

# **PEROXIREDOXINS**

## **Novel mediators of cellular timekeeping**

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**M. Sc. Prince Saforo Amponsah**

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### **Prüfungskommission**

- 1. Gutachter: Prof. Dr. Bruce Morgan
- 2. Gutachter: Prof. Dr. Zuzana Storchová
- Vorsitzender: Prof. Dr. Johannes M. Herrmann

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D386

*...To my family*

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# LIST OF PUBLICATIONS

**Amponsah PS**, Metwally G, Mergel S, Storchova Z and Morgan B (2019). Peroxiredoxins couple metabolism and cell division in an ultradian clock. *Nat Chem Biol* (under revision)

Calabrese G, Peker E, **Amponsah PS**, Hoehne MN, Riemer T, Mai M, Deponte M, Morgan B, Riemer J (2019). Hyperoxidation of mitochondrial peroxiredoxin limits H<sub>2</sub>O<sub>2</sub>-induced cell death in yeast. *EMBO J* 38(18):e101552

**Amponsah PS** (2016). Cellular redox – living chemistry. *Science in School* 36: 15-17

Morgan B, Van Laer K, Owusu TN, Ezeriņa D, Pastor-Flores D, **Amponsah PS**, Tursch A, Dick TP (2016). Real-time monitoring of basal H<sub>2</sub>O<sub>2</sub> levels with peroxiredoxin-based probes. *Nat Chem Biol* 12(6): 437-43

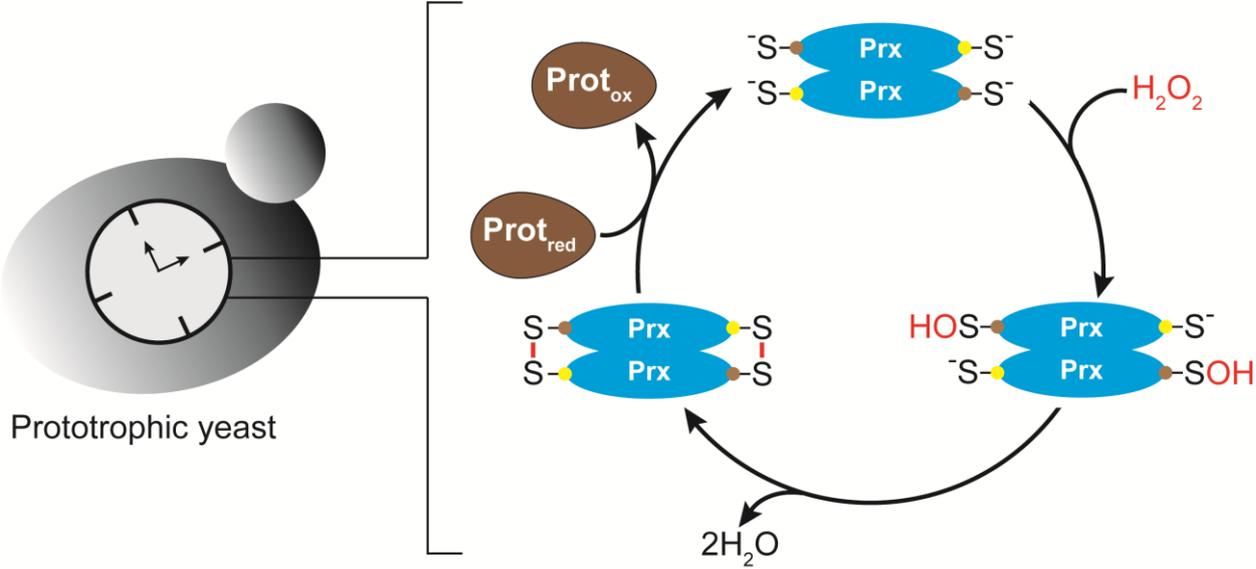
# SUMMARY

Biological clocks exist across all life forms and serve to coordinate organismal physiology with periodic environmental changes. The underlying mechanism of these clocks is predominantly based on cellular transcription-translation feedback loops in which clock proteins mediate the periodic expression of numerous genes. However, recent studies point to the existence of a conserved timekeeping mechanism independent of cellular transcription and translation, but based on cellular metabolism. These metabolic clocks were concluded based upon the observation of circadian and ultradian oscillations in the level of hyperoxidized peroxiredoxin proteins. Peroxiredoxins are enzymes found almost ubiquitously throughout life. Originally identified as  $H_2O_2$  scavengers, recent studies show that peroxiredoxins can transfer oxidation to, and thereby regulate, a wide range of cellular proteins. Thus, it is conceivable that peroxiredoxins, using  $H_2O_2$  as the primary signaling molecule, have the potential to integrate and coordinate much of cellular physiology and behavior with metabolic changes. Nonetheless, it remained unclear if peroxiredoxins are passive reporters of metabolic clock activity or active determinants of cellular timekeeping. Budding yeast possess an ultradian metabolic clock termed the Yeast Metabolic Cycle (YMC). The most obvious feature of the YMC is a high amplitude oscillation in oxygen consumption. Like circadian clocks, the YMC temporally compartmentalizes cellular processes (e.g. metabolism) and coordinates cellular programs such as gene expression and cell division. The YMC also exhibits oscillations in the level of hyperoxidized peroxiredoxin proteins.

In this study, I used the YMC clock model to investigate the role of peroxiredoxins in cellular timekeeping, as well as the coordination of cell division with the metabolic clock. I observed that cytosolic 2-Cys peroxiredoxins are essential for robust metabolic clock function. I provide direct evidence for oscillations in cytosolic  $H_2O_2$  levels, as well as cyclical changes in oxidation state of a peroxiredoxin and a model peroxiredoxin target protein during the YMC. I noted two distinct metabolic states during the YMC: low oxygen consumption (LOC) and high oxygen consumption (HOC). I demonstrate that thiol-disulfide oxidation and reduction are necessary for switching between LOC and HOC. Specifically, a thiol reductant promotes switching to HOC, whilst a thiol oxidant prevents switching to HOC, forcing cells to remain in LOC. Transient peroxiredoxin inactivation triggered rapid and premature switching from LOC to HOC. Furthermore, I show that cell division is normally synchronized with the YMC and that deletion of typical 2-Cys peroxiredoxins leads to complete uncoupling of cell division from metabolic cycling. Moreover, metabolic oscillations are crucial for regulating cell cycle entry and exit. Intriguingly, switching to HOC is crucial for initiating cell cycle entry whilst switching to LOC is crucial for cell cycle completion and exit. Consequently, forcing cells to remain in HOC by application of a thiol reductant leads to multiple rounds of cell cycle entry despite failure to complete the preceding cell cycle. On the other hand, forcing cells to remain in LOC by treating with a thiol oxidant prevents initiation of cell cycle entry.

In conclusion, I propose that peroxiredoxins – by controlling metabolic cycles, which are in turn crucial for regulating the progression through cell cycle – play a central role in the coordination of cellular

metabolism with cell division. This proposition, thus, positions peroxiredoxins as active players in the cellular timekeeping mechanism.



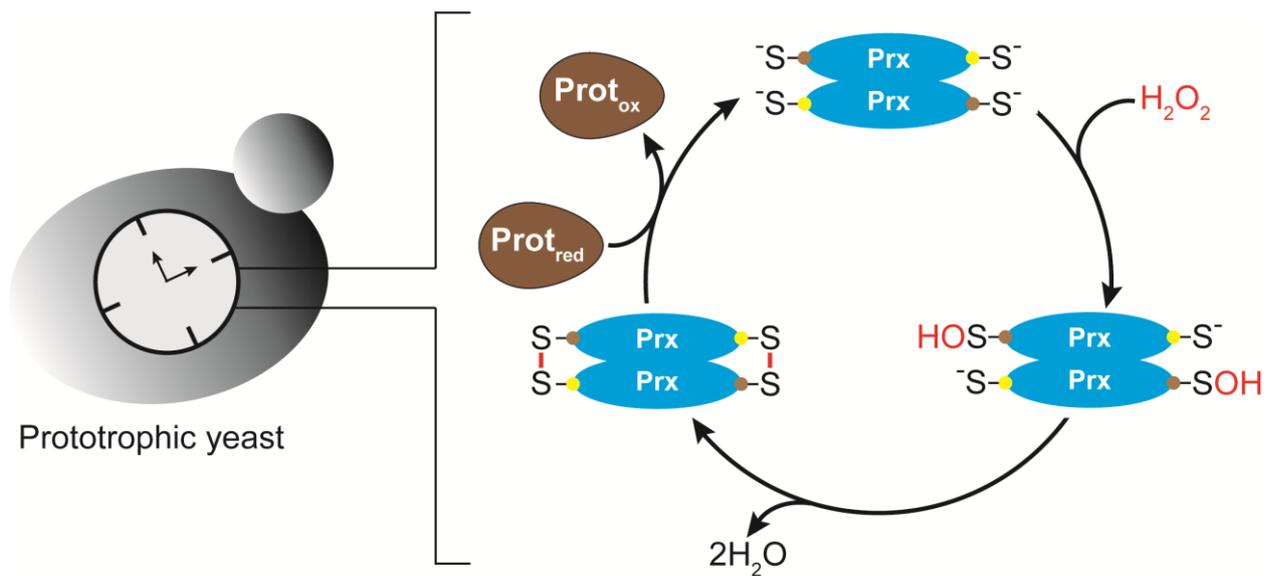
# ZUSAMMENFASSUNG

In sämtlichen Lebensformen gibt es biologische Uhren, die die Physiologie des jeweiligen Organismus mit sich periodisch verändernden Umweltbedingungen koordinieren. Der molekulare Mechanismus dieser Uhren besteht zumeist aus zellulären Transkriptions-Translations-Feedback-Loops, in denen Zeitgeber-Proteine die periodische Expression zahlreicher Gene steuern. Jüngere Studien legen jedoch nahe, dass eine weitere konservierte Zeitgeberfunktion existiert, die unabhängig von Transkription und Translation ist, sondern auf dem zellulären Metabolismus beruht. Die Existenz solcher metabolischen Uhren wurde postuliert basierend auf der Beobachtung, dass die Level von hyperoxidierten Peroxiredoxin-Proteinen circadianen und ultradianen Schwankungen unterliegen. Peroxiredoxine sind Enzyme, die es fast in sämtlichen lebenden Zellen gibt. Ursprünglich als Radikalfänger zur Entgiftung von Wasserstoffperoxid identifiziert, ist heute bekannt, dass Peroxiredoxine die Oxidation – und damit Regulation – einer großen Bandbreite von Proteinen bewerkstelligen. Es liegt daher nahe, dass Peroxiredoxine Wasserstoffperoxid als primäres Signalmolekül nutzen können, um einen Großteil der zellulären Physiologie und Verhalten mit metabolischen Veränderungen zu integrieren und koordinieren. Unklar blieb jedoch bislang, ob Peroxiredoxine lediglich passive Reporter für die Aktivität metabolischer Uhren sind, oder vielmehr aktive Zeitgeber darstellen. Auch die Bäckerhefe besitzt eine ultradiane metabolische Uhr, den Yeast Metabolic Cycle (YMC). Dessen offensichtlichste Eigenschaft ist die starke periodische Schwankung im zellulären Sauerstoff-Bedarf. Ähnlich den circadianen Uhren trennt der YMC zelluläre Prozesse (z.B. metabolische) zeitlich voneinander und koordiniert zelluläre Programme wie etwa Genexpression und Zellteilung. Auch im YMC zeigen sich Oszillationen in der Menge an hyperoxidierten Peroxiredoxinen.

In der vorliegenden Arbeit habe ich den YMC als Modell genutzt, um die Rolle der Peroxiredoxine in der zellulären Zeitgebung und der Koordination der Zellteilung mit der metabolischen Uhr zu untersuchen. Ich habe cytosolische 2-Cys Peroxiredoxine als essentiell für eine robuste Funktion der metabolischen Uhr identifiziert. Auch konnte ich zyklische Schwankungen sowohl in der zellulären  $H_2O_2$ -Menge als auch im oxidativen Status eines Peroxiredoxins und eines Modellsubstrates während des YMC messen. Dieser besitzt zwei verschiedene metabolische Phasen, eine mit geringem Sauerstoffverbrauch (LOC, low oxygen consumption) und eine mit hohem Sauerstoffverbrauch (HOX, high oxygen consumption). Ich konnte zeigen, dass Thiol-Disulfid-Oxidation und -Reduktion notwendig für das Umschalten zwischen LOC und HOC sind. Insbesondere triggert ein Thiol-Oxidans das Umschalten in die HOC-Phase, während umgekehrt Thiol-Reduktion dieses Umschalten verhindert und Zellen in der LOC-Phase hält. Eine vorübergehende Inaktivierung von Peroxiredoxinen führte zu einem schnellen und vorzeitigen Switch von der LOC- in die HOC-Phase. Darüber hinaus zeigte ich, dass Zellteilung normalerweise mit dem YMC synchronisiert ist und die Deletion eines typischen 2-Cys Peroxiredoxins zu einer kompletten Entkopplung von Zellzyklus und metabolischer Uhr führt. Metabolische Oszillationen sind wichtig für die Regulation von Eintritt und Ende des Zellzyklus. Dabei ist das Umschalten in die HOC-Phase entscheidend für die Initiation der Zellteilung, während Eintritt in die LOC-Phase wichtig ist für die

Vervollständigung und den Austritt aus dem Zellzyklus ist. Folgerichtig führt das Verweilen von Zellen in der HOC-Phase, erzwungen durch Zugabe von Thiol-Reduktantien, zu mehreren Runden von Zellzyklus-Eintritt, obwohl der vorangegangene Zellzyklus nicht vollendet werden konnte. Umgekehrt verhindert das Arretieren von Zellen in der LOC-Phase den Eintritt in die Zellteilung.

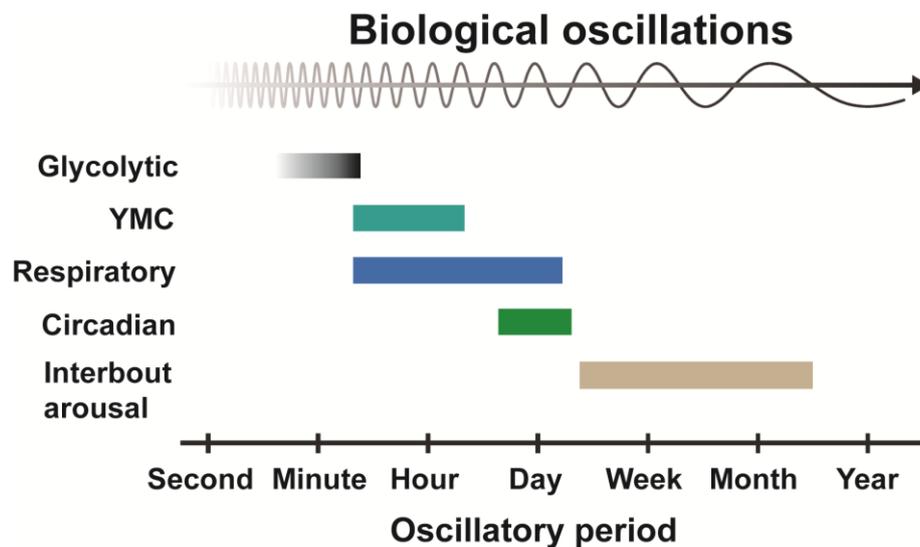
Basierend auf diesen Beobachtungen schlage ich vor, dass Peroxiredoxine eine zentrale Rolle in der Koordination von zellulärem Metabolismus und Zellzyklus spielen, indem sie metabolische Zyklen kontrollieren, die wiederum ausschlaggebend für die Progression durch den Zellzyklus sind. Peroxiredoxine spielen somit eine aktive Rolle in der zellulären Zeitgebung..



# 1 INTRODUCTION

All living species order their biological processes - at the molecular, cellular, tissue or organ levels. The timing of these biological processes is intrinsic and essential for coordination of periodic physiological and behavioral responses. This intrinsic timekeeping mechanism is called the biological clock, which enables living species to anticipate oscillatory environmental changes – day/night cycles, temperature rhythms, time of feeding etc. Underlying biological clocks are biological rhythms with periods of approximately 24 hours (termed Circadian) or less (termed Ultradian) or periods ranging from weeks to months and years (Fig. 1.0). Essential features of circadian clocks include:

- Free-running or self-sustained in nature – this means that the rhythms continue to exist even under constant conditions, such as constant darkness, without the influence of external signals. This distinguishes circadian rhythms from diurnal rhythms, which are influenced by external cues such as light.
- Rhythms are entrainable - this means that the rhythm is reset or synchronized by external time cues called *Zeitgebers* (German word meaning ‘time giver’).
- Rhythms are temperature-compensated – this means that the circadian period is not dictated by the prevailing environmental temperature [1].

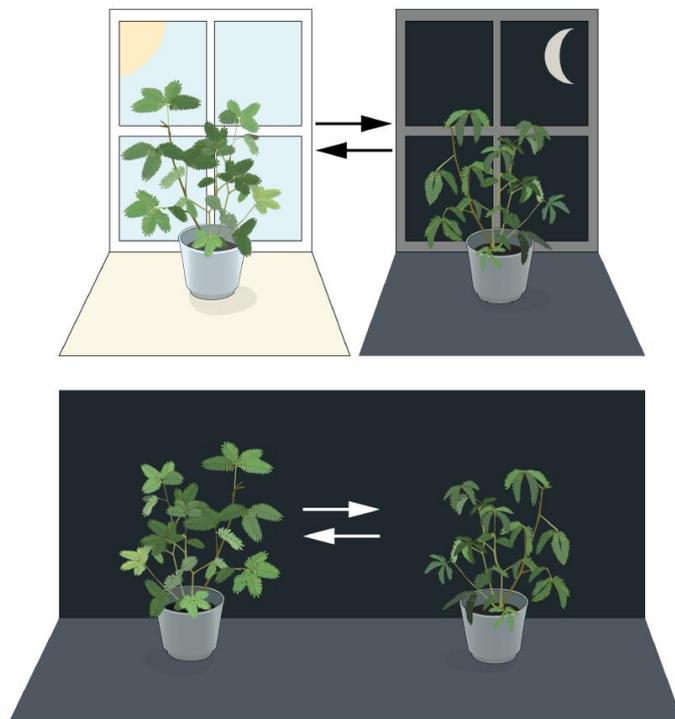


**Figure 1.0: Biological oscillations in living systems.** Diverse species exhibit biological rhythms that span periods ranging from several seconds to hours and months. These oscillations optimize the species' adaptability to its environment. Image modified from [2].

## 1.1 Historical perspectives of cellular timekeeping

The term Circadian originates from the Latin words, *circa* – meaning ‘around’ and *dies* – meaning ‘day’, coined by Franz Halberg (a Romanian chronobiologist) in 1959 [3]. This implied that physiological rhythms occur within periods of about 24 hours. The first documented description of an intrinsic circadian rhythm was the observation that leaves of *Mimosa pudica* plant opened and closed with a 24-hour period

when kept under constant darkness conditions (Fig. 1.1). This experiment was performed by the French Geophysicist Jean-Jacques d'Ortous de Mairan in 1729, and showed for the first time that circadian clocks were self-sustaining or free-running in nature, without the influence of external stimuli [4].



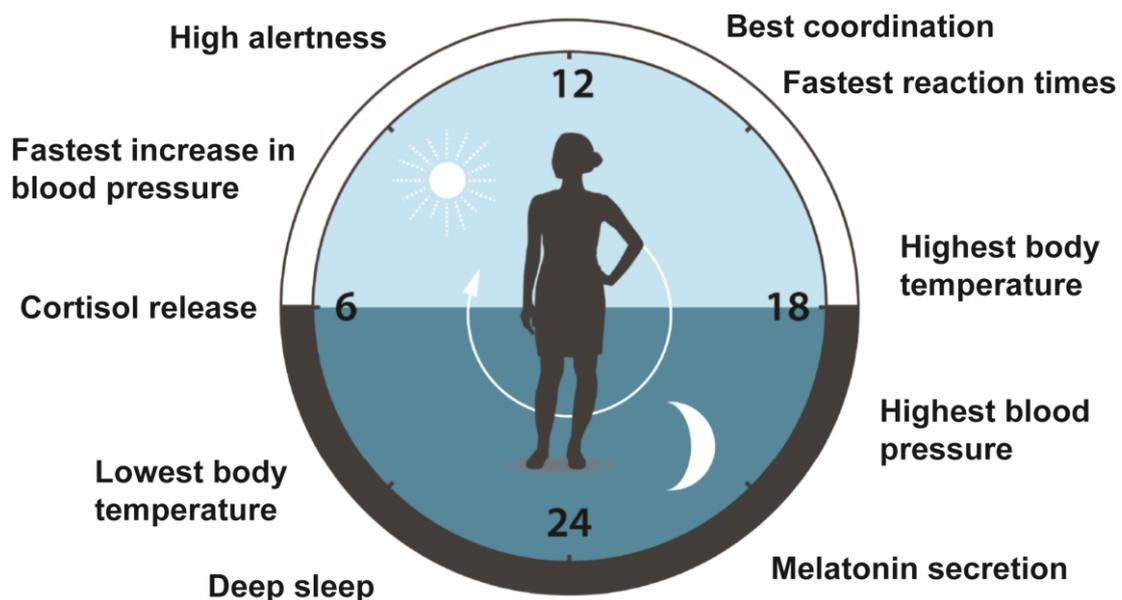
**Figure 1.1: The plant, *M. pudica*, possesses an internal biological clock.** Leaves of mimosa plants open towards the sun during daytime and close at dusk. Jean Jacques d'Ortous de Mairan placed a mimosa plant in constant dark and found that the leaves continued to follow their daily rhythm for several days. This suggested that mimosa plants have a cell autonomous clock that can maintain the biological rhythm even under constant conditions. Image and legend adapted from [@nobelprize.org](https://www.nobelprize.org).

Michel Siffre, the French cave explorer, lived isolated in a cave in the French Alps for two months to investigate the body's clock. His initial plan was to undertake a geological expedition to study underground glaciers for about fifteen days. However, he extended his stay in the cave to two months, with the idea to investigate how the natural rhythms of human life would be affected by living "beyond time". To achieve this, he lived in the cave without access to clock, calendar or sun and slept and ate only when his body told him to. Additionally, he had a team at the entrance of the cave who took notice of his sleep-wake activity without him personally knowing what time it was on the outside. He called them only when he woke up, when he ate, and just before he went to sleep. During his wake times, he performed a psychological test by counting from 1 to 120, at the rate of one digit per second. He also took record of his pulse and measured his body temperature, which got as low as 34 °C. After the experiment, Siffre noted that he had completely lost conscious perception of the passing of time. It took him five minutes to count to 120. He went into the cave on July 16 and planned to finish the experiment on September 14. When he was notified that the date was due, he thought it was August 20, believing he still had another month to spend in the cave. Thus, his psychological time had compressed by a factor of two. Siffre's experiment showed that humans, like lower mammals such as rat, possess an internal biological clock [5, 6]. In line with Siffre's reports, the German physician, Jürgen Aschoff, observed in laboratory settings that the circadian clock controlled daily physiological and psychological parameters such as blood pressure, body temperature, plasma hormone levels and cognitive performance [7] (Fig. 1.2).

These descriptions of biological timekeeping ignited interests in previously observed, but unexplained, rhythmic behavior. For instance, Antle and Silver described in their article, circadian rhythms in feeding times of bees as observed by Hugo Berthold von Buttel-Reepen and Auguste Forel in the early 20<sup>th</sup> Century [8]. Observations by Hugo Berthold von Buttel-Reepen, a German zoologist, indicated that

bees exhibited a time-sense for feeding, thus, visited a buckwheat field only in the morning while the flowers were open and secreting nectar. On the other hand, Auguste Forel, during his vacation in the Swiss Alps observed that bees came to feed on his marmalade while he ate breakfast outdoor. As the days went by, the number of bees which visited him increased until he was unable to eat outdoors anymore. He noted that the bees persistently arrived during breakfast time, although he and his marmalade were safely indoors, suggesting these insects appeared to have a time-memory for when breakfast was served [8]. Although these observations did not necessarily constitute evidence of clocks, they demonstrate how organismal behavior is timed with environmental changes.

Subsequent studies have demonstrated that insects such as *Drosophila sp.* possessed an endogenous circadian clock [9, 10]. The discovery and functional annotation of the *period* (“*per*”) gene as a key determinant of *Drosophila sp.* circadian rhythmicity, birthed the transcription-translation feedback loop (TTFL) model of circadian timekeeping, for which the Noble Prize in Physiology and Medicine was awarded to Jeffrey Hall, Michael Rosbash and Michael Young in 2017.



**Figure 1.2: The circadian clock has an impact on many aspects of human physiology.** This clock helps to regulate sleep patterns, feeding behavior, hormone release, blood pressure and body temperature. Image modified and legend adapted from [@nobelprize.org](https://www.nobelprize.org).

## 1.2 Circadian clocks: Molecular players and mechanisms

Until the late 20<sup>th</sup> century, the prevailing evidence for biological timekeeping were based on organismal behavioral rhythmicity, such as ‘time-sense’ feeding of bees and closure and opening of plant leaves in response to day-night cycles. However, the basic mechanistic underpinnings for these rhythms were largely not understood. Subsequent discovery and characterization of several ‘clock genes’ in diverse organisms led to the hypothesis that the cellular clockwork must consist of a feedback system that is able to generate approximately 24-hour cycles in various cellular parameters (i.e. TTFL) [11].

Although the TTFL model of the clockwork is the prominent mechanistic basis for circadian behavior, ‘clock genes’ are not conserved across different species. Moreover, some ‘clock genes’ are non-essential, without which circadian rhythms persist [12, 13]. These apparent ‘anomalies’ to the TTFL-based model therefore ignited interests in the search for alternative cellular timekeeping models that function independent of cellular transcription (ie. non-transcriptional clocks). I summarize below some key findings that contributed to postulations on transcription and non-transcription based clock models.

### 1.2.1 Transcriptional clocks

As recounted earlier, the identification of the *per* gene as a genetic determinant of circadian rhythmicity in *Drosophila* made way for the postulation of the TTFL-model as the underlying mechanism of circadian clocks [14]. To arrive at this hypothesis, Konopka and Benzer performed a genetic screen for abnormal eclosion rhythms in *Drosophila* flies. Eclosion is the process of transition from pupa into adult *Drosophila*. Before the genetic screen by Konopka and Benzer, Colin Pittendrigh had earlier observed that the eclosion process exhibited a circadian behavior and showed that temperature played an essential role in determining the period of eclosion rhythm, but not its rhythmicity [15]. Konopka and Benzer’s genetic screen revealed three mutations that traced to the same genomic locus. Of the three mutant alleles, one was arrhythmic; another had short-period rhythms of 19 hours, and the third had long-period rhythms of 28 hours [14]. They called this genomic locus *period* (“*per*”). Subsequently, the labs of Ronald Konopka, Jeffrey Hall, Michael Roshbash and Michael Young isolated and characterized *per* and showed that both *per* mRNA and its protein were expressed in a circadian manner [11, 14, 16-18]. They further demonstrated that the *per* locus was the center of the circadian rhythm and that loss of *per* terminates circadian activity. They therefore proposed a model where PER protein, in cooperation with another protein called TIMELESS (TIM), auto-regulated its own expression [11, 19, 20] (Fig. 1.3A). Further studies led to the discovery of two transcriptional factors, CLOCK (CLK) and CYCLE (CYC), which were proposed to control the expression of *tim* and *per* to provide a closure to the feedback loop [21-23]. The expression of *clock* (“*clk*”) and *cycle* (“*cyc*”) were also shown to be auto-regulated by their own protein products (Fig. 1.3B).

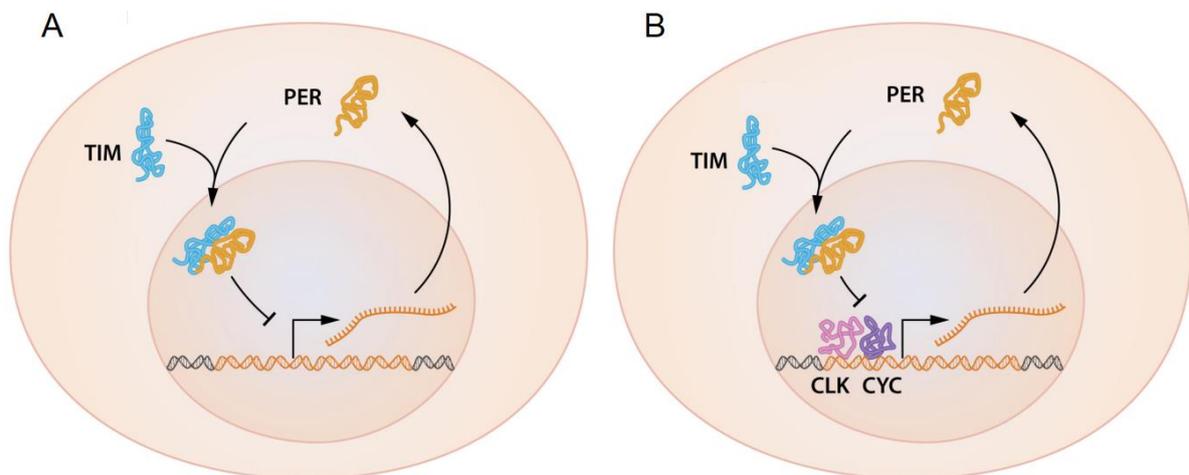
The basic mechanism of the TTFL-model is dependent on a transcriptional activator that induce the transcription of a repressor. The repressor accumulate over time until it reaches levels enough to inhibit its own activation [2] (Fig. 1.4). This clock model has been described in a number of species that demonstrate circadian activity. The core clock components are not conserved across species, although the mechanisms remain similar [24].

In mammals, CLOCK (NPAS2) and BMAL1 are the central transcriptional machinery that activate expression of *per* and *cryptochrome* (“*cry*”) gene families. PER and CRY protein products accumulate over time in the cytoplasm and form complexes that repress CLOCK and BMAL1 transactivation upon translocation into the nucleus [25]. A second feedback loop also involves upregulation of REV-ERB $\alpha$  and

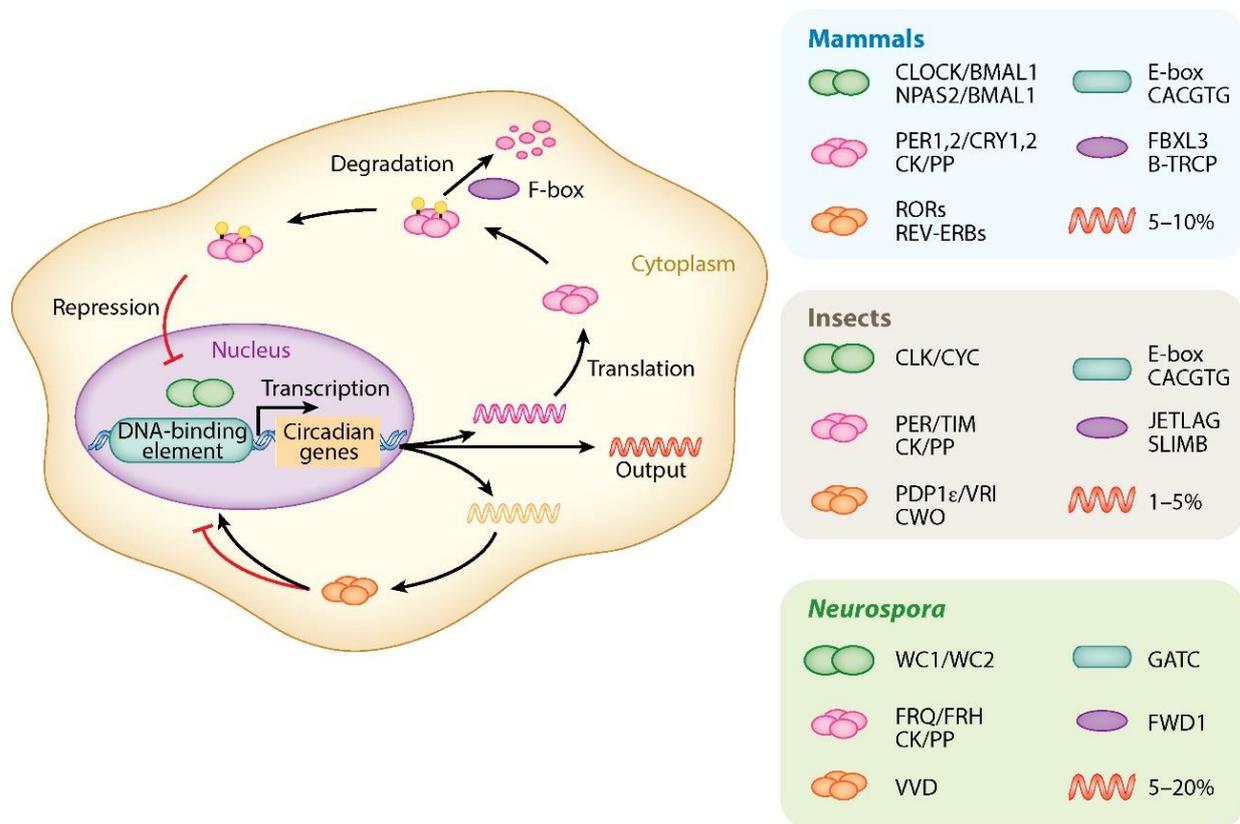
REV-ERB $\beta$  transcription by CLOCK and BMAL1, which in turn accumulate to repress *Bmal1* transcription [26] (Fig. 1.4).

In *Drosophila*, the central clock proteins, CLK and CYC, form the positive elements of the transcriptional loop whilst PER and TIM mediate the negative feedback. CLK and CYC form heterodimers that positively regulate transcription of *per* and *tim* by binding directly to E-box elements found in the *per* and *tim* promoters. PER and TIM self-antagonize their own expression by binding to the CLK/CYC complex to prevent binding to DNA. A second transcriptional feedback loop consists of the transcription factor VRILLE (VRI) whose repression alters *tim* expression as well as the rhythm [24, 27] (Fig. 1.4).

In *Neurospora crassa*, the protein product of *frequency* (“*frq*”), FRQ, represses its own transcription. Protein products of the core clock oscillator, *white collar* (“*wc*”), WC1 and WC2, form heterodimers to activate expression of *frq*. FRQ accumulates and translocate into the nucleus to repress its own mRNA levels by binding to and interfering with WC1 and WC2 activity [28, 29] (Fig 1.4).



**Figure 1.3: A simplified illustration of the feedback regulation of the *period* gene. (A)** Both *period* mRNA and PER protein oscillate, with PER protein accumulating several hours after the peak in *period* mRNA. PER protein localizes in the nucleus, and the *period* gene activity oscillates because of PER protein feedback inhibition of its own gene. TIM protein, encoded by the *timeless* gene also oscillates and interacts with PER protein. This interaction is critical for PER protein nuclear accumulation and repression of the *period* gene. **(B)** CLK and CYC, encoded by the clock and cycle genes, are two transcription factors that activate the *period* gene. Image and legend modified from [@nobelprize.org](http://nobelprize.org).



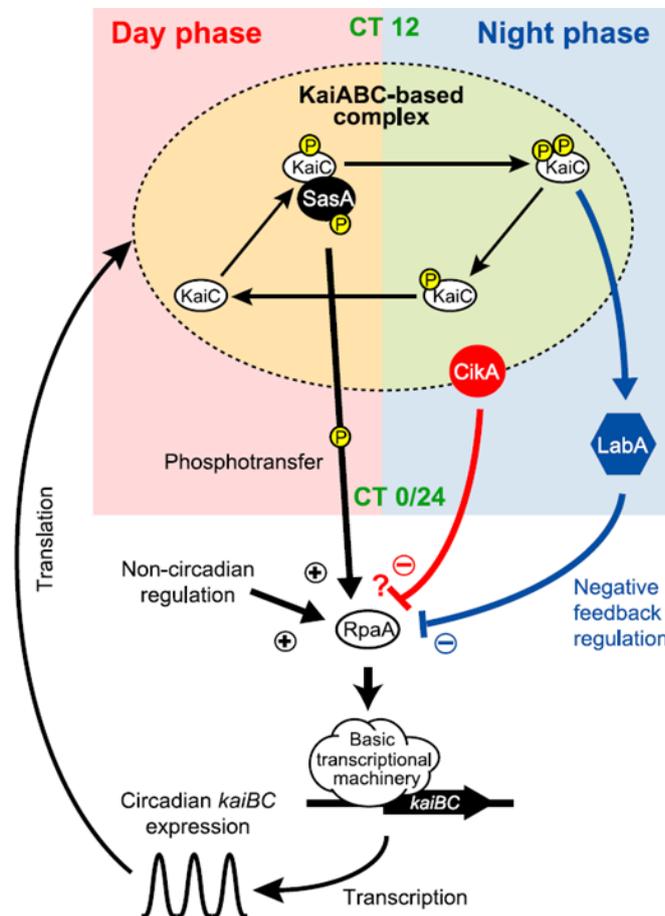
**Figure 1.4: The TTFL model of cellular timekeeping.** The architecture and basic principle (left) of transcription-based clocks seems to be conserved among species, although the core clock components differ. The core clock components for three different model organisms are shown (right). Image adapted from [2].

## 1.2.2 Non-transcriptional clocks

The notion that non-transcriptional processes might drive circadian clocks dates back to the year 1960. Observations in the single nucleus unicellular alga *Acetabularia sp.* showed that the enucleated plant cell could retain viability over several weeks and exhibited intrinsic rhythms in photosynthesis and chloroplast shape in constant conditions [30, 31]. Furthermore, pharmacologically inhibiting cellular transcription in this plant did not affect the observed rhythms in the first fortnight of treatment, suggesting that transcription is dispensable for generating rhythmicity. Nonetheless, transcription was required to maintain the levels of key oscillator components [32].

The first intriguing evidence for an autonomous non-transcriptional oscillator came from studies in *S. elongates* by Kondo and colleagues. The core oscillator in this organism consists of three genes – *kaiA*, *kaiB* and *kaiC* – which, together with their protein products control the periodic transcription of other genes [33-35]. In this system, KaiC is the main oscillator with both auto-kinase and -phosphatase activities; KaiA activates the kinase and inhibits the phosphatase activities of KaiC, whilst KaiB antagonizes the activity of KaiA. The interactions between these proteins lead to 24-hour oscillations in KaiC phosphorylation state, which successively drives rhythmic changes in transcription of genes, including that of the *kaiBC* operon (Fig. 1.5). Subsequent experiments showed that the oscillations in KaiC phosphorylation state was not dictated by the rhythmic transcription of the *kaiBC* operon. A simple biochemical experiment reconstituting

the three Kai proteins in a test tube, supplemented with ATP as a source of phosphates showed that the 24-hour rhythms in phosphorylation and de-phosphorylation of the KaiC protein could persist. This observation demonstrated the existence of an oscillator that functioned independent of transcription. Nonetheless, transcription was required to maintain the levels of *Kai* proteins *in vivo*.



**Figure 1.5: The cyanobacterial circadian clock.** The cyanobacterial clock demonstrated that phosphorylation of a substrate can exhibit 24-hour rhythms *in vivo* (even upon inhibition of transcription) and *in vitro*, providing the first mechanistic explanation for non-transcriptional rhythms. The core oscillator consists of three proteins (KaiA, KaiB, and KaiC), which via a feedback loop, regulate the expression of numerous genes including the *kaiBC* promoter. Image adapted from [36].

John O’Neil and colleagues showed evidence for the existence of a non-transcription based clock mechanism that functions together with the TTFL model in *Ostreococcus tauri* [37]. They demonstrated that after complete termination of transcription in constant darkness, *O. tauri* cultures could restore transcriptional rhythms once transferred to constant light. They concluded that a non-transcriptional mechanism ran in parallel to preserve the phase of the clock, since the transfer to light did not alter the phase of the oscillations [37]. The subsequent

discovery of redox oscillations in the levels of hyperoxidized (or sulfynylated) peroxiredoxin proteins in human RBCs (which lack nucleus) and *O. tauri* (even during transcriptional arrest) showed that non-transcriptional oscillators might not only be restricted to small organisms like cyanobacteria [37, 38]. Later on, peroxiredoxin hyperoxidation rhythms have been shown to be conserved across all domains of life [39]. These peroxiredoxin hyperoxidation rhythms, together with oscillations in cellular metabolites such as  $\text{NAD}^+/\text{NADH}$ ,  $\text{NADP}^+/\text{NADH}$  and  $\text{ADP}/\text{ATP}$  underlie cellular metabolic clocks [37, 38, 40]. Metabolic clocks can function independent of cellular transcription.

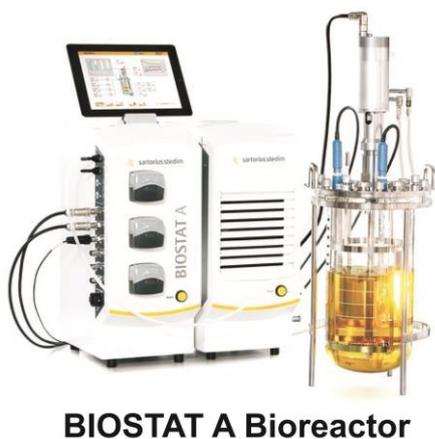
Furthermore, oscillations in cellular biochemical parameters were described and proposed to complement the TTFL to explain circadian behavior. For instance, Cornelius and Rensing reported that  $\text{Mg}^{2+}$ -dependent ATPase activity in the membranes of RBCs cultured *in vitro* was rhythmic [41]. Radha *et al.* showed that *in vitro* cultures of human platelets exhibited oscillations in the levels of glutathione [42]. Edmunds and colleagues described a non-transcriptional feedback loop model for observed rhythms in nicotine adenine dinucleotides,  $\text{NAD}^+$  and  $\text{NADP}^+$ , in *Euglena sp.* [43]. More recently, circadian oscillations in magnesium ( $\text{Mg}^{2+}$ ) and potassium ( $\text{K}^+$ ) levels were also observed in cultured human cells and *O. tauri* (even during transcriptional arrest) under constant conditions [44].

### 1.3 The Yeast Metabolic Cycle as a model metabolic clock

In budding yeast, *Saccharomyces cerevisiae*, long period oscillations in oxygen consumption – termed Yeast Metabolic Cycle (YMC) – occur over several hours during continuous, glucose-limited growth [45-48]. *S. cerevisiae*, is a unicellular eukaryote that shares many protein homologs with other multicellular organisms.

The most obvious feature of the YMC is high amplitude changes in oxygen consumption in a continuous culture system (Fig. 1.6). There exists two main phases of the YMC: a high oxygen consumption (HOC) phase where cells rapidly consume oxygen and a low oxygen consumption (LOC) phase where there is highest dissolved oxygen levels during the continuous culture (Fig. 1.7). Cyclical changes in the levels of more than 50 % of cellular genes also occur during the YMC. Additionally, the YMC appears to regulate entry into and exit from the cell division cycle. Strikingly, DNA replication and cell division are precisely gated to temporal windows when oxygen consumption decreases, in ways reminiscent of the circadian gating of cell division observed in cyanobacteria, mouse liver, and cultured fibroblasts [46, 49-55]. Moreover, genetic or chemical perturbation of cellular redox processes with H<sub>2</sub>O<sub>2</sub> can strongly disrupt the YMC [47, 49, 56].

It is not entirely clear how the oscillations in the YMC are generated and sustained. However, it has been proposed that cells build and accumulate storage carbohydrates, such as trehalose and glycogen during LOC [51]. Upon accumulating enough of these carbohydrates, a proportion of the yeast population are thought to commit to HOC and liquidate their storage carbohydrates in the process, to generate energy for cellular biosynthetic processes via aerobic metabolism [57]. Through a mechanism termed, YMC-to-YMC coupling, it is believed that this metabolically committed yeast sub-population produce and secrete metabolites such as ethanol, acetaldehyde and dihydrogen sulfide that serve to shift the YMC phase of other cells in order to achieve synchronous oxygen consumption [58-60]. It has also been shown that during the YMC, cyclical changes in cell metabolism is coordinated with cellular processes such as gene expression, respiration, mitochondria biogenesis, ribosome biogenesis, DNA replication, cell division, fatty acid oxidation, glycolysis and vacuole-mediated catabolism [47, 49, 50].

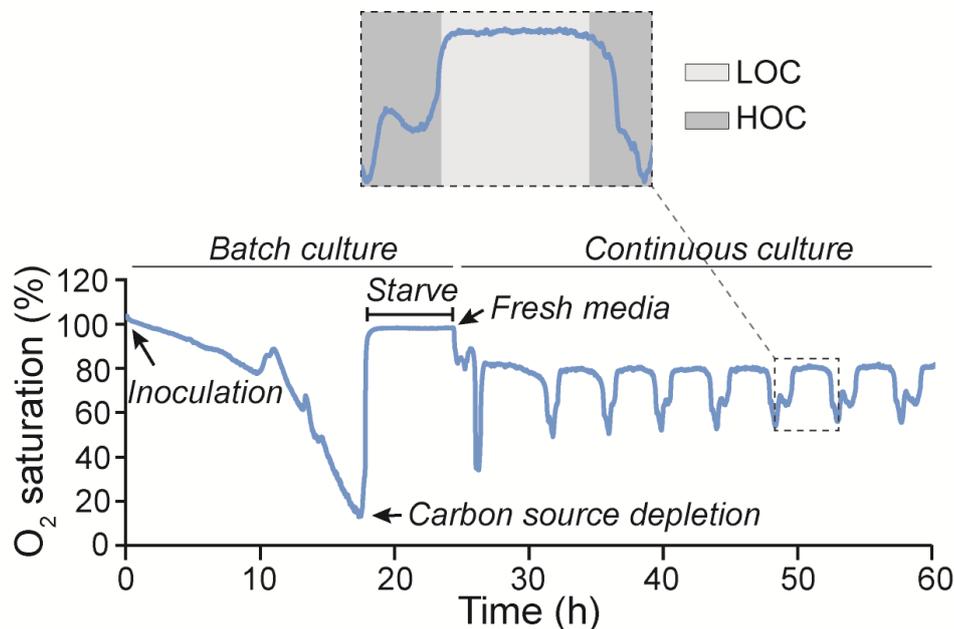


**BIOSTAT A Bioreactor**

**Figure 1.6: The Chemostat system for generation of the YMC.** The bioreactor consists of a culture vessel connected via tubes to a system unit. The system unit is equipped with probes to measure pH and dissolved oxygen in the culture vessel. Peristaltic pumps are affixed to control media in-flow and out-flow. An additional unit serves as a cooling chamber to control the operating temperature. Image sourced from @Satorious GmbH.

The YMC represents the yeast metabolic clock and shares several features that are conserved with circadian clocks in other organisms [40, 61]. These include (1) generation of temperature-compensated rhythms, (2) exhibition of oscillations that are coupled with the cell division cycle, (3) oscillatory period that is determined by post-translational mechanisms, such as phosphorylation of clock proteins by casein kinase

1 (CK1) and glycogen synthase kinase  $\beta$  (GSK $\beta$ ), and (4) rhythmic oscillations in the cellular redox state (eg. hyperoxidation of peroxiredoxin proteins) [40, 47]. On this basis, the YMC serves as a convenient model to investigate metabolic clock activity.



**Figure 1.7: The Yeast Metabolic Cycle.** Representative oxygen trace showing the procedure for establishing YMC-synchronized continuous cultures. Cells inoculated into the fermenter are allowed to grow in a batch phase to maximum density until dissolved oxygen is almost depleted. Following starvation for nearly 6 hours, fresh media is pumped into the culture vessel at a constant dilution rate of 0.05 h<sup>-1</sup>, which results in the generation of synchronized self-sustained oscillations between fermentative and respiratory metabolism with a period of nearly 5 hours. **LOC** – low oxygen consumption, **HOC** – high oxygen consumption.

## 1.4 Hydrogen peroxide in redox signaling

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is a redox metabolite that functions as a secondary messenger in a variety of cell signaling events [62]. These include, but not limited to, antimicrobial defense, inflammation, cell migration, cell proliferation, angiogenesis and regulation of gene expression [63]. Within the cell, H<sub>2</sub>O<sub>2</sub> production is regulated, and mediated by enzymes such as superoxide dismutases (SODs), NADPH oxidases, as well as the mitochondrial electron transport chain (ETC). Under physiological conditions, steady-state cellular H<sub>2</sub>O<sub>2</sub> concentration ranges between approximately 1 - 10 nM, and can rise to approximately 0.5 - 0.7  $\mu$ M during oxidative events such as aerobic respiration and fatty acid  $\beta$ -oxidation [64, 65].

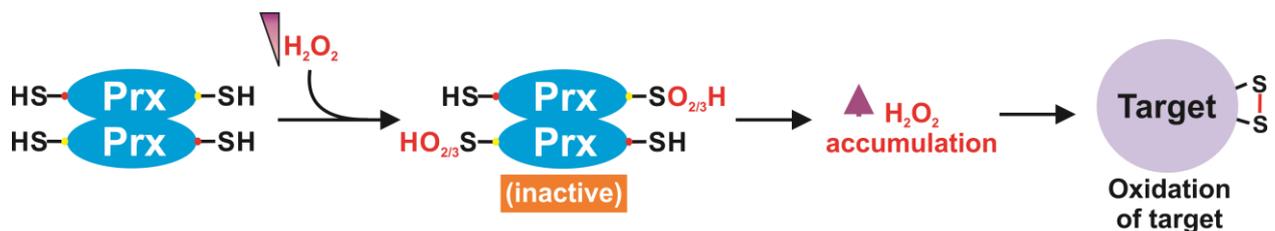
It is not entirely clear how H<sub>2</sub>O<sub>2</sub> oxidizes protein thiols within cells. This is because most redox-regulated proteins exist in low abundance within cells. Moreover, most thiol-containing redox-regulated proteins generally exhibit poor direct reactivity towards H<sub>2</sub>O<sub>2</sub>, with  $k \approx 10^0$ – $10^2$  M<sup>-1</sup>s<sup>-1</sup> [66]. Furthermore, peroxiredoxins – the most prominent group of thiol peroxidases – exhibit the highest intrinsic reactivity of any protein towards H<sub>2</sub>O<sub>2</sub>, and are predicted to capture nearly all of the H<sub>2</sub>O<sub>2</sub> generated inside cells [67]. In addition, peroxiredoxins are highly expressed proteins with overall cytosolic concentration 2 - 3 orders of magnitude higher than that of most redox-regulated proteins. Owing to their high abundance and high

reactivity, peroxiredoxins are effective competitors for  $H_2O_2$  compared to all other protein thiols [68]. Glutathione peroxidases are also a group of thiol peroxidases that exhibit high reactivity towards  $H_2O_2$ .

Although the mechanisms that ensure target specificity of  $H_2O_2$ -induced thiol oxidation events are not fully resolved, two contrasting but mutually non-exclusive schools of thought explain  $H_2O_2$  signaling within cells: direct oxidation or ‘*floodgate*’ and facilitated oxidation or ‘*relay*’ hypotheses.

### 1.4.1 Direct oxidation or ‘*floodgate*’ hypothesis

The ‘*floodgate*’ hypothesis suggests that at sources of generation,  $H_2O_2$  accumulates to high levels and directly mediates oxidation of protein thiols. Here, peroxiredoxins and other thiol peroxidases are viewed as competitors or scavengers of  $H_2O_2$ , thereby thwarting direct  $H_2O_2$ -mediated oxidation of protein thiols [63]. Therefore for direct thiol oxidation to occur, peroxiredoxins must be temporarily rendered inactive by posttranslational modifications such as hyperoxidation or phosphorylation. Under such conditions,  $H_2O_2$  locally accumulates to levels that allow for selective and direct oxidation of protein thiols with modest intrinsic  $H_2O_2$  reactivity (Fig. 1.8). This hypothesis makes two predictions: (1) upon  $H_2O_2$ , cysteine sulfenic acid (Cys-SOH) should form directly on target protein thiols, and (2) upon deletion of peroxiredoxins,  $H_2O_2$ -induced protein thiol oxidation should increase. Evidence for direct  $H_2O_2$ -mediated thiol oxidation was described in A431 human epidermoid carcinoma cells. The Rhee lab showed that transient increase in the intracellular concentration of  $H_2O_2$  either by exogenous addition or via epidermal growth factor (EGF) signaling caused inactivation of recombinant protein-tyrosine phosphatase 1B (PTP1B) *in vitro* by oxidizing its catalytic site cysteine, most likely to sulfenic acid [69]. Although the thiol state of peroxiredoxins was not analyzed in their study, it is plausible that peroxiredoxins were hyperoxidized by the amounts of  $H_2O_2$  used. In a related study, they also showed that localized inactivation of membrane-associated PrxI by phosphorylation in response to EGF leads to local accumulation of  $H_2O_2$  for cell signaling [70].

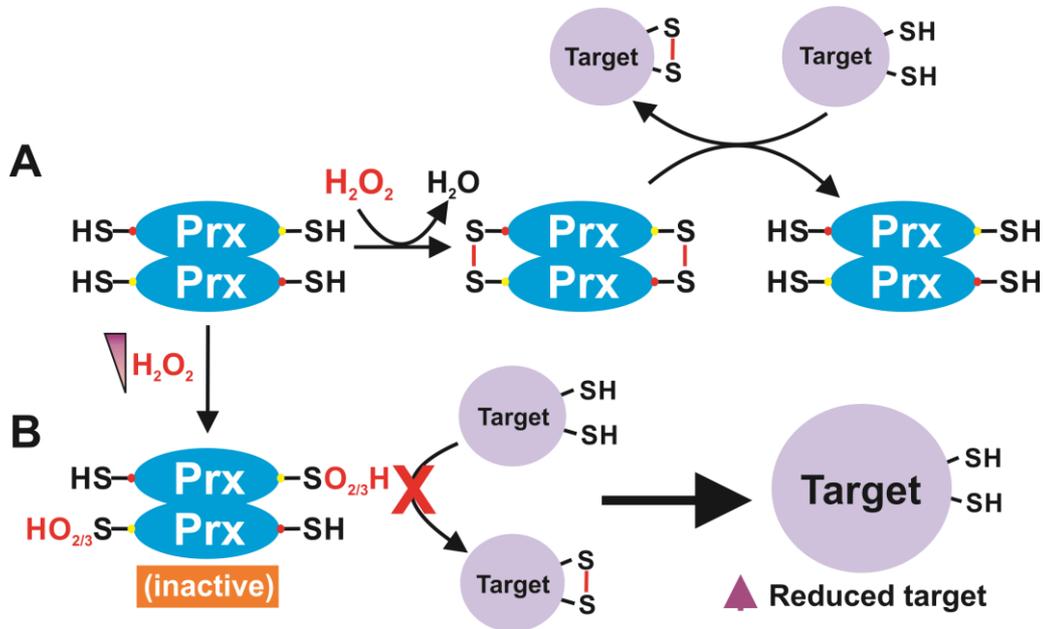


**Figure 1.8: Floodgate hypothesis of  $H_2O_2$  signaling.** This model of signaling positions peroxiredoxins as scavengers of  $H_2O_2$ , which frustrate thiol protein oxidation within cells.

### 1.4.2 Facilitated oxidation or ‘*relay*’ hypothesis

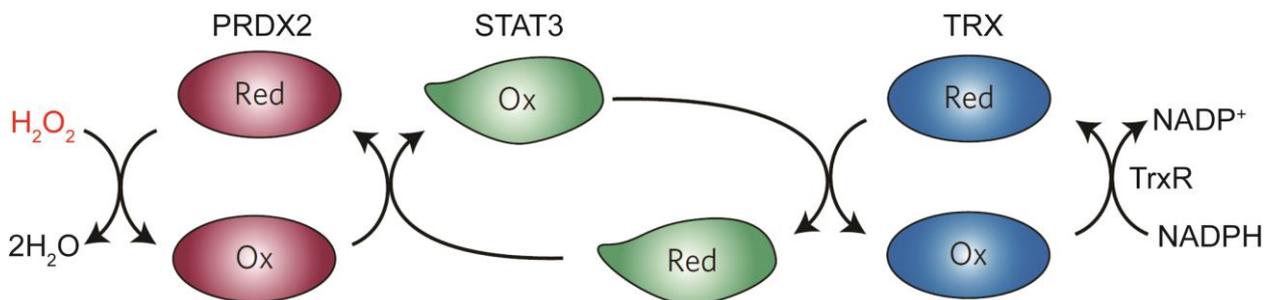
The ‘*relay*’ hypothesis suggests that the levels and exceptional intrinsic reactivity of thiol peroxidases to  $H_2O_2$  position them as effective recipients and competitors for  $H_2O_2$ , above all other potential protein thiols [71]. These proteins must therefore receive oxidizing equivalents from  $H_2O_2$  via the action of thiol peroxidases – which act as transmitters –, in order to become oxidized. Here, thiol peroxidases are

viewed as enablers of protein thiol oxidation, not as competitors. As such, inactivation by hyperoxidation or phosphorylation inhibits this ‘relay’ function and renders target protein thiols reduced (Fig. 1.9). In contrast to the ‘floodgate’ model, this hypothesis predicts that: (1) upon  $H_2O_2$ , Cys-SOH should not form directly on target protein thiols, as only thiol peroxidases are prone to react directly with  $H_2O_2$ , and (2) upon deletion of thiol peroxidases,  $H_2O_2$ -induced protein thiol oxidation should decrease.



**Figure 1.9: Relay hypothesis of  $H_2O_2$  signaling.** This model of signaling positions peroxiredoxins as facilitators or enablers of protein thiol oxidations, without which thiol protein oxidations barely occur within cells. (A) Peroxiredoxin-mediated oxidation of protein thiol. (B) Protein thiol oxidation is inhibited upon peroxiredoxin hyperoxidation.

Evidence for the ‘relay’ hypothesis has been demonstrated in yeast and mammalian cells. Recent studies by Stöcker and colleagues suggest that mammalian proteins such as ANXA2, ASK1 and CBS mostly depend on the relay function of PRDX1 and PRDX2 in order to facilitate their thiol oxidation [72]. It has also been shown that PRDX2 transfers oxidizing equivalents to the STAT3 transcription factor via a redox relay [73] (Fig 1.10). In *S. cerevisiae*, the thiol peroxidase Orp1 forms mixed disulfides with the Yap1 transcription factor, leading to the activation of Yap1 via an intramolecular disulfide [74]. In *S. pombe*, the 2-Cys peroxiredoxin, Tpx1, activates the p38/JNK homolog, Sty1, upon  $H_2O_2$  [75].



**Figure 1.10: Example of endogenous peroxiredoxin redox relay.** Redox-regulated protein, STAT3, receive oxidizing equivalents from a peroxiredoxin, PRDX2. The oxidized STAT3 translocate into the nucleus to mediate transcription of genes involved in cell growth and apoptosis. The oxidized STAT3 can be reduced by thioredoxin. Image modified from [73]

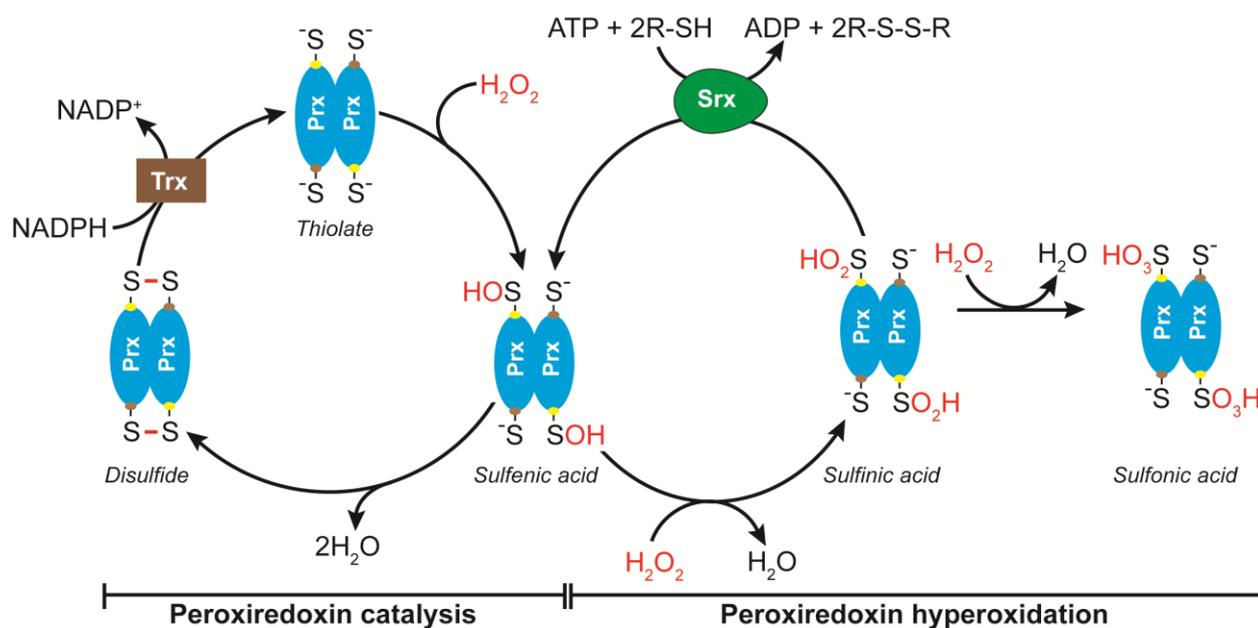
## 1.5 Peroxiredoxins: structure, catalytic mechanism and function

Budding yeast possesses eight thiol peroxidases, consisting of three cysteine-dependent glutathione peroxidases (Gpx1, Gpx2, Gpx3/Orp1) and five peroxiredoxins. Peroxiredoxins (Prxs) are a very large and highly conserved family of thiol peroxidases that are distributed across cellular compartments: three cytosolic (i.e. Tsa1, Tsa2 and Ahp1) one nuclear (i.e. Dot5) and one mitochondrial (i.e. Prx1). Of all thiol peroxidases, peroxiredoxins are the most reactive towards  $\text{H}_2\text{O}_2$  with rate constants,  $k \approx 10^7\text{--}10^9 \text{ M}^{-1} \text{ s}^{-1}$  [76-79].

A highly conserved cysteine (Cys) residue called the ‘peroxidatic’ cysteine ( $\text{C}_\text{P}$ ) acts as the site of oxidation by peroxides and mediates catalytic activity of peroxiredoxins [80, 81]. During catalysis, the  $\text{C}_\text{P}$  performs a nucleophilic attack on one of the oxygens in  $\text{H}_2\text{O}_2$ , which leads to the oxidation of the  $\text{C}_\text{P}$  sulfhydryl ( $\text{C}_\text{P}\text{-SH}$ ) to a sulfenic acid ( $\text{C}_\text{P}\text{-SOH}$ ) intermediate. Depending on the number of Cys residues involved in the catalysis, peroxiredoxins can be classified into either 1-Cys or 2-Cys. In 2-Cys peroxiredoxins, the  $\text{C}_\text{P}\text{-SOH}$  reacts with another Cys residue, called the ‘resolving’ cysteine ( $\text{C}_\text{R}$ ) to form a disulfide that is subsequently reduced by an appropriate electron donor such as thioredoxins (Trxs) to complete the catalytic cycle. One-Cys peroxiredoxins (e.g. Prx1) lack a  $\text{C}_\text{R}$  and therefore the  $\text{C}_\text{P}\text{-SOH}$  is reduced by small molecule antioxidants such as glutathione [82].

The location of the  $\text{C}_\text{R}$  residue in 2-Cys peroxiredoxins further results in two subclasses: typical 2-Cys and atypical 2-Cys peroxiredoxins [83, 84]. Typical 2-Cys peroxiredoxins (e.g. Tsa1, Tsa2) are catalytically active as homodimers with a  $\text{C}_\text{P}$  and  $\text{C}_\text{R}$  per monomer. The  $\text{C}_\text{P}\text{-SOH}$  intermediate on one monomer is resolved by the  $\text{C}_\text{R}$  from the other monomer to form an intermolecular disulfide (S-S) bond. In atypical 2-Cys Prxs (e.g. Ahp1), a  $\text{C}_\text{R}$  from the same subunit resolves the  $\text{C}_\text{P}\text{-SOH}$  intermediate to form an intramolecular disulfide bond.

The  $\text{C}_\text{P}\text{-SOH}$  intermediate of 2-Cys peroxiredoxins can undergo further oxidation to generate reversible sulfinic ( $\text{C}_\text{P}\text{-SO}_2\text{H}$ ) and irreversible sulfonic ( $\text{C}_\text{P}\text{-SO}_3\text{H}$ ) acid forms [85-87]. This phenomenon results in catalytic peroxiredoxin inactivation and is termed hyperoxidation. The  $\text{C}_\text{P}\text{-SO}_2\text{H}$  form can be reactivated by sulfiredoxin (Srx) through an ATP-dependent reduction reaction [88] (Fig. 1.11).



**Figure 1.11: Catalytic mechanism of typical 2-Cys Peroxiredoxins.** Upon encountering  $\text{H}_2\text{O}_2$ , the peroxidatic cysteine of peroxiredoxins undergo peroxidation to form sulfenic acid ( $\text{C}_\text{P}\text{-SOH}$ ), followed by disulfide bond ( $\text{S-S}$ ) formation mediated by the resolving cysteine ( $\text{C}_\text{R}$ ). The peroxiredoxin is recycled via a step catalyzed by thioredoxin (Trx). However, upon further exposure to  $\text{H}_2\text{O}_2$ , the sulfenic acid intermediate can be oxidized to sulfinic and sulfonic acid forms ( $\text{C}_\text{P}\text{-SO}_2\text{H}$ ). The overoxidized  $\text{C}_\text{P}\text{-SO}_3\text{H}$  can be recycled through ATP-dependent reduction by sulfiredoxin (Srx). The hyperoxidized  $\text{C}_\text{P}\text{-SO}_3\text{H}$  form is irreversible.

## 1.6 Genetically encoded thiol peroxidase-based biosensors

Taking advantage of naturally occurring redox relays and the extreme sensitivity of the  $\text{C}_\text{P}\text{-SH}$  of thiol peroxidases towards  $\text{H}_2\text{O}_2$ , genetically encoded  $\text{H}_2\text{O}_2$  fluorescent sensors have been developed by fusing a thiol peroxidase to redox sensitive green fluorescent protein (e.g. roGFP2) [89-91]. Typical examples of such biosensors include the roGFP2-Orp1, roGFP2-Tsa1, roGFP2-Tsa2, roGFP2-PRDX2 [90-92]. RoGFP2 is based upon an enhanced GFP, modified to contain two cysteine residues capable of forming a disulfide bond. These biosensors work on the principle that upon encountering oxidizing equivalents, the thiol peroxidase gets oxidized and transfers its oxidation state via a multi-step process to the cysteine residues in roGFP2 (Fig. 1.12). RoGFP2 exhibits two excitation maxima at 405 nm and 488 nm when fluorescence emission is monitored at 510 nm [93]. The two excitation wavelengths permit ratiometric measurements, which renders the sensor readout independent of changes in sensor concentration.

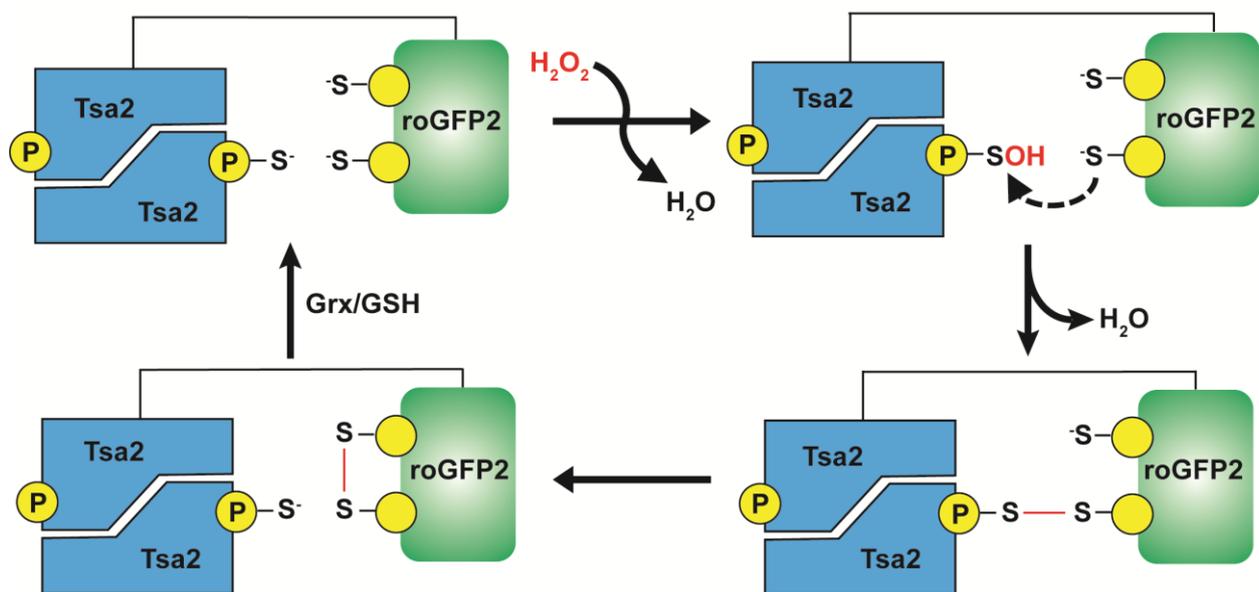
Together with my colleagues, we developed and applied a more sensitive (approximately 20-fold) variant of the roGFP2-Tsa2 sensor, roGFP2-Tsa2 $\Delta\text{C}_\text{R}$ , in the measurement of ‘basal’ endogenous  $\text{H}_2\text{O}_2$  levels in yeast [90]. We fused the thiol specific antioxidant Tsa2, a typical 2-Cys peroxiredoxin from *S. cerevisiae*, deleted for its resolving cysteine (i.e. Tsa2 $\Delta\text{C}_\text{R}$ ), to roGFP2. We deleted the resolving cysteine of the Tsa2 moiety in order to limit the formation of Tsa2 disulfide and subsequent reduction by thioredoxins. This ensures direct transfer of oxidation from the peroxidatic cysteine of Tsa2 $\Delta\text{C}_\text{R}$  to roGFP2 and enhances the fluorescence signal (i.e. sensitivity). The roGFP2-Tsa2 $\Delta\text{C}_\text{R}$  biosensor specifically

measures  $\text{H}_2\text{O}_2$  levels and does not significantly contribute to cellular  $\text{H}_2\text{O}_2$  scavenging. RoGFP2 itself exhibits poor direct reactivity towards  $\text{H}_2\text{O}_2$ ; hence, any  $\text{H}_2\text{O}_2$ -driven oxidation of roGFP2 is mediated by the Tsa2 $\Delta\text{C}_R$  moiety. However, roGFP2 reduction is mediated by cellular glutaredoxins (Grx) using glutathione (GSH).

Functionally, roGFP2-thiol peroxidase fusion biosensors represent an artificial reconstitution of an entire thiol peroxidase redox relay. However, instead of a transcriptional response upon oxidation, as is the case with natural relays, a fluorescence response is elicited in the context of the biosensor. Excess or non-physiological levels of  $\text{H}_2\text{O}_2$  can hyperoxidize Tsa2 $\Delta\text{C}_R$ . When the  $\text{C}_P\text{-SH}$  of the Tsa2 $\Delta\text{C}_R$  becomes hyperoxidized to the sulfinic or sulfonic acid, transfer of oxidizing equivalents to roGFP2 is prevented and the sensor ceases to function as an  $\text{H}_2\text{O}_2$  sensor. Upon Tsa2 $\Delta\text{C}_R$  inactivation, roGFP2 equilibrates with GSH/GSSG via the action of glutaredoxins. A reduced roGFP2 in this scenario does not represent an absence of, or low,  $\text{H}_2\text{O}_2$  levels.

I have relied on the capacity of the roGFP2-Tsa2 $\Delta\text{C}_R$  sensor to make long-term, non-disruptive, real-time, fully dynamic  $\text{H}_2\text{O}_2$  measurements in yeast, to answer the outstanding question of whether the peroxiredoxin hyperoxidation rhythms that underlie circadian and ultradian metabolic clocks have any functional relevance. I have re-engineered the sensor and expressed it from the genome of prototrophic yeast to measure cyclical  $\text{H}_2\text{O}_2$  changes and evaluate peroxiredoxin activity during the YMC.

### roGFP2-Tsa2 $\Delta\text{C}_R$ : a reconstituted thiol peroxidase redox relay



**Figure 1.12: Mechanism of the roGFP2-Tsa2 $\Delta\text{C}_R$  sensor.** Diagram to illustrate the mechanism of the roGFP2-Tsa2 $\Delta\text{C}_R$  sensor, which is based on the principle of a thiol peroxidase-based redox relay. All cysteine residues of the sensor remain in a reduced thiolate state in the absence of  $\text{H}_2\text{O}_2$ . Upon encountering  $\text{H}_2\text{O}_2$ , the peroxidatic cysteine ( $\text{C}_P\text{-SH}$ ) of the Tsa2 $\Delta\text{C}_R$  performs a nucleophilic attack to extract oxidizing equivalents from  $\text{H}_2\text{O}_2$ . The oxidizing equivalents are transferred via a two-step process resulting in the formation of an intramolecular roGFP2 disulfide bond. The oxidized roGFP2 can be reduced by glutaredoxins (Grx) using glutathione (GSH).

## 1.7 Crosstalk between the cellular redox state, metabolism, cell cycle and circadian/ultradian clocks

### 1.7.1 Reciprocal regulation of clock mechanism by redox homeostasis

Preliminary evidence for the existence of crosstalk between metabolism, redox homeostasis and circadian clocks was demonstrated using a non-physiological *in vitro* biochemical assay. In their studies, Rutter *et al.* showed that nuclear NAD<sup>+</sup>/NADH and NADP<sup>+</sup>/NADPH ratios influence the transcriptional activity of the CLOCK (NPAS2)/BMAL1 heterodimer [94]. It has also been shown that oscillations in NAD(P)<sup>+</sup> and NAD(P)H cofactors accompany peroxiredoxin-sulfinylation rhythms that underlie metabolic clocks [37, 38].

Redox changes regulate expression of clock-related genes in multiple systems. In *S. cerevisiae*, the glutathione peroxidase, Orp1, relays signals from H<sub>2</sub>O<sub>2</sub> to the Yap1 transcription factor to elevate antioxidant response systems [74]. Yap1 shuttles in and out of the nucleus during the YMC [47]. In zebrafish, H<sub>2</sub>O<sub>2</sub> generated from light regulates the expression of clock genes, *cry1* and *per2*, via the action of catalase [95]. In *N. crassa* and cyanobacteria, H<sub>2</sub>O<sub>2</sub> influences the daily expression pattern of clock and clock-controlled genes involved in coordinating photosynthesis [96-98]. Furthermore, the redox sensor, light-dependent period A (LdpA) modulates cyanobacterial circadian clock period length by controlling the levels of *CikA* and *KaiA*, in response to light [99, 100]. Oscillations in overexpressed mammalian PRDX2 nuclear levels dampen oscillations in BMAL1 in HaCaT keratinocytes [101, 102]. Hyperoxidation of mitochondrial PRDX3 has also been shown to be essential for circadian rhythms in adrenal steroidogenesis [103].

Alternatively, clock genes can regulate the expression of multiple redox systems. For instance, wildtype Canton S flies exhibit oscillations in glutamate-cysteine ligase and glutathione S transferase D1 transcript levels, which is abolished in mutants lacking the *per* and *cyc* clock genes [104]. *D. melanogaster* flies exhibit diurnal rhythms in susceptibility to H<sub>2</sub>O<sub>2</sub>, which are abolished in *per* gene mutants [105]. *A. thaliana* exhibits diurnal rhythms in the generation and scavenging of H<sub>2</sub>O<sub>2</sub>, which is perturbed in mutants of the core clock regulator, circadian clock-associated 1 (CCA1) [106]. Finally, it was shown that *Bmal1*<sup>-/-</sup> mice accumulate higher ROS levels compared to their wildtype counterparts, which impinges on aging pathologies [107].

Although abundance of evidence for reciprocal crosstalk between cellular metabolism, redox homeostasis and circadian/ultradian systems exist, the underlying mechanisms by which redox changes may govern circadian/ultradian rhythms are not fully understood.

## 1.7.2 Redox/metabolic state versus cell division cycle

In order to grow, living organisms undergo cell division. During this highly ordered and regulated process, living cells give rise to either two (ie. somatic or mitotic division) or four (i.e. gametic or meiotic division) daughter cells. *S. cerevisiae* undergoes mitotic cell division, which is ordered in four phases: G<sub>1</sub> (Gap1), S (Synthesis), G<sub>2</sub> (Gap2) and M (Mitosis). In G<sub>1</sub> the cell increases in size until it reaches the checkpoint at which critical decisions to undergo division, or not, is taken. This ‘committed step’ is termed ‘restriction point’ in mammals and ‘Start’ in budding yeast [108-111]. During the S phase, cellular DNA is duplicated and cells proceed to segregate this DNA into two daughter cells during the M phase. In S phase, the fidelity of DNA replication is controlled by checkpoint mechanisms that become activated to repair damaged DNA if replication forks stall [112]. Between the transition from S to M phase is a G<sub>2</sub> that is less distinct in *S. cerevisiae* [113].

It has been suggested that the decision to undergo cell division is determined by the cellular metabolic state (or metabolic oscillator), and governed by the availability of macromolecules and energy to power cells through the process [114]. For instance, budding yeast cell cycle ‘Start’ is coordinated with the liquidation of storage carbohydrates and their daughter cells spend more time in G<sub>1</sub>, accumulating biomass until they have reached a critical size before committing to cell division [57, 115].

Several reports indicate that the cellular redox state is a key regulator of the cell cycle, i.e. transient oxidative mechanisms control key cell cycle regulatory proteins possibly via thiol-disulfide exchanges at critical cysteine residues [116-119]. It is believed that low levels of cellular oxidation, possibly from superoxide (O<sub>2</sub><sup>-</sup>) and H<sub>2</sub>O<sub>2</sub>, are required for proper mitogenic signaling [117, 120, 121]. In mammalian cells, this oxidative mechanism is thought to control the activities of cyclin-dependent kinases (Cdks) and the retinoblastoma protein at G<sub>1</sub>, to permit entry into S phase for DNA replication [122-124]. Thereafter, a more general reduction of the cellular environment is necessary to enable cells progress to the G<sub>2</sub> and M phases [125]. Moreover, it has been suggested that the G<sub>1</sub> phase is characterized by low cellular GSH, which levels must necessarily increase to facilitate cell cycle progression from G<sub>1</sub> to S [126].

Although redox metabolism cannot be divorced from cell cycle regulation, the identification of redox-regulated proteins or thiol peroxidase relays relevant to each cell cycle phase remains an enormous challenge.

## 1.8 Aims of this thesis

Circadian and ultradian peroxiredoxin hyperoxidation rhythms exist across the three domains of life, including during the yeast metabolic cycle (YMC), and have been proposed as the mechanistic basis for cellular metabolic clocks [37-40]. However, to date it has remained largely unclear if peroxiredoxins are involved in cellular timekeeping or are merely convenient reporters of metabolic oscillations. The aims of this thesis were to investigate (1) whether peroxiredoxins are active determinants of cellular timekeeping, using the yeast metabolic clock as a model, and (2) whether peroxiredoxins are important for the coordination between metabolic clocks and the cell division cycle. My specific objectives included the following:

- a) Establishment and characterization of YMC-synchronized cultures.
- b) Engineering prototrophic yeast strain capable of expressing the roGFP2-Tsa2 $\Delta$ C<sub>R</sub> biosensor from its genome.
- c) Interrogating whether H<sub>2</sub>O<sub>2</sub> levels, peroxiredoxin oxidation, and the oxidation of peroxiredoxin target protein(s) oscillate during the YMC.
- d) Determining whether thiol disulfide changes influence or regulate metabolic clock function.
- e) Investigating if peroxiredoxins are crucial for YMC oscillation.
- f) Verifying whether cell division is synchronized with the YMC, and if metabolic oscillations are crucial for regulating cell cycle entry and exit.
- g) Examining whether loss or inactivation of peroxiredoxins affects coupling of cell metabolism to the cell division cycle.

## 2 RESULTS

Circadian and ultradian oscillations in the level of hyperoxidized peroxiredoxin proteins underlie cellular metabolic clocks. However, whether peroxiredoxins are important components of the clock mechanism or just convenient markers of oscillatory redox metabolism remained enigmatic. In this study, I sought to investigate whether peroxiredoxins are active participants in cellular timekeeping, using the YMC clock model. For this purpose, I established YMC-synchronized cultures and interrogated the role of peroxiredoxins using genetic and biochemical approaches.

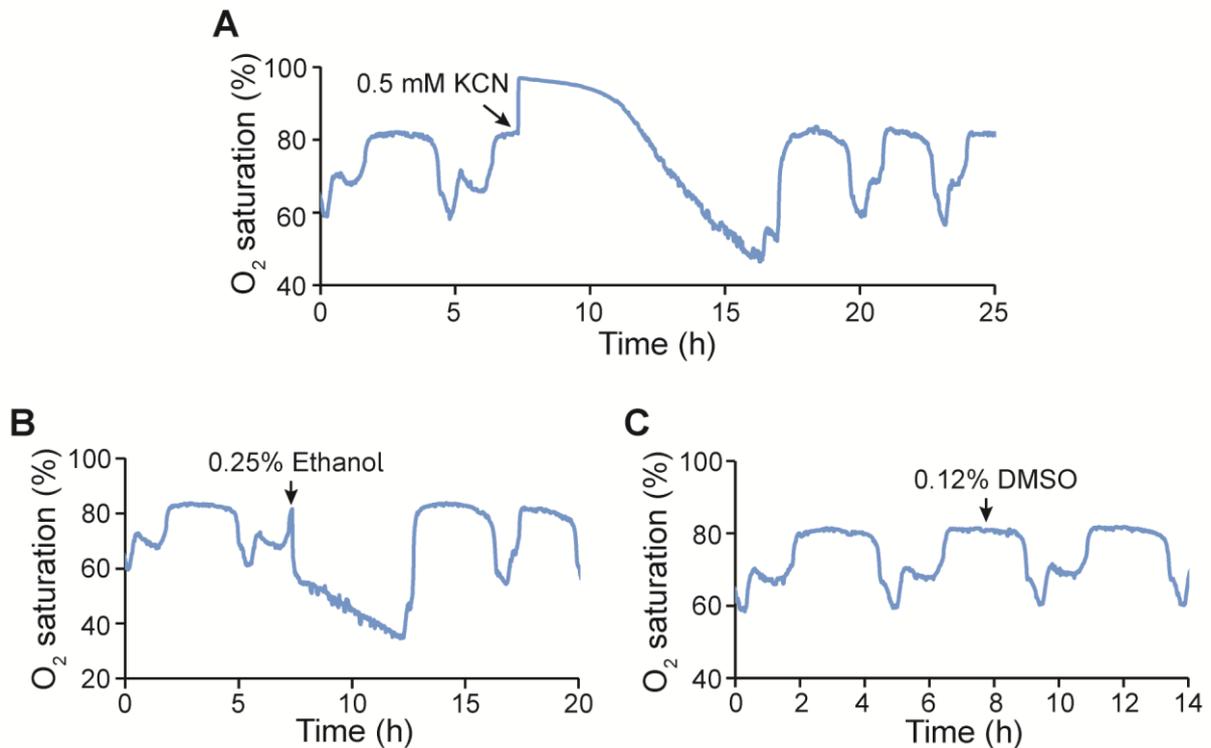
### 2.1 Establishment of YMC-synchronized cultures

When prototrophic yeast cells are grown under nutrient-limited conditions in continuous culture using chemostat, they exhibit high amplitude periodic changes in oxygen consumption. These self-sustained oscillations are termed the Yeast Metabolic Cycle (YMC) and display periods ranging from approximately 40 minutes to over 10 hours, depending on the type of strain as well as growth conditions [46, 47, 52, 127, 128]. The YMC is the yeast ultradian metabolic clock, which consists of oscillations in cell metabolism, levels of cellular transcripts and the cell division cycle [47, 49, 56]. I used the YMC model because it shares features that are conserved with circadian rhythms in mammalian cells. The YMC exhibits oscillations that are temperature-compensated and are coupled with the cell division cycle. It also displays rhythmic oscillations in the cellular redox state (e.g. hyperoxidation of peroxiredoxin proteins) [40, 47].

#### 2.1.1 The YMC consists of synchronized respiratory oscillations

To use the YMC as the model metabolic clock for my study, I cultured yeast cells under the chemostat conditions described by Tu *et al.* [47] with slight modifications, as described in Materials and Methods. After growth in a batch phase, starvation for at least 6 hours and continuous feed-in of fresh media into the culture vessel at a dilution rate of  $0.05 \text{ h}^{-1}$ , I observed self-synchronized oscillations in oxygen consumption by the yeast population. These oscillations consisted of long phases of low oxygen consumption (LOC) and short phases of high oxygen consumption (HOC), which persisted as long as media was available. The amplitude of each oscillation ranged between 60 – 80% of dissolved oxygen and the period of each wildtype YMC lasted approximately 5 hours (Fig. 1.7). Blocking respiratory activity with potassium cyanide (KCN) immediately halted oxygen consumption and returned YMC-synchronized cells to a ‘starvation mode’ that lasted until KCN was diluted out of the culture vessel (Fig. 2.1A). KCN inhibits complex IV of the mitochondrial electron transport chain. Addition of a bolus of respiratory substrate, ethanol, at the start of a LOC phase immediately triggered oxygen consumption that lasted for a longer period until the substrate was depleted (Fig. 2.1B). However, an inert solvent such as dimethyl sulfoxide (DMSO) had no observable effect on the YMC (Fig. 2.1C). Taken together, I have successfully established

the YMC for my study and shown that the oscillations in oxygen consumption observed during the YMC are largely respiratory in nature.



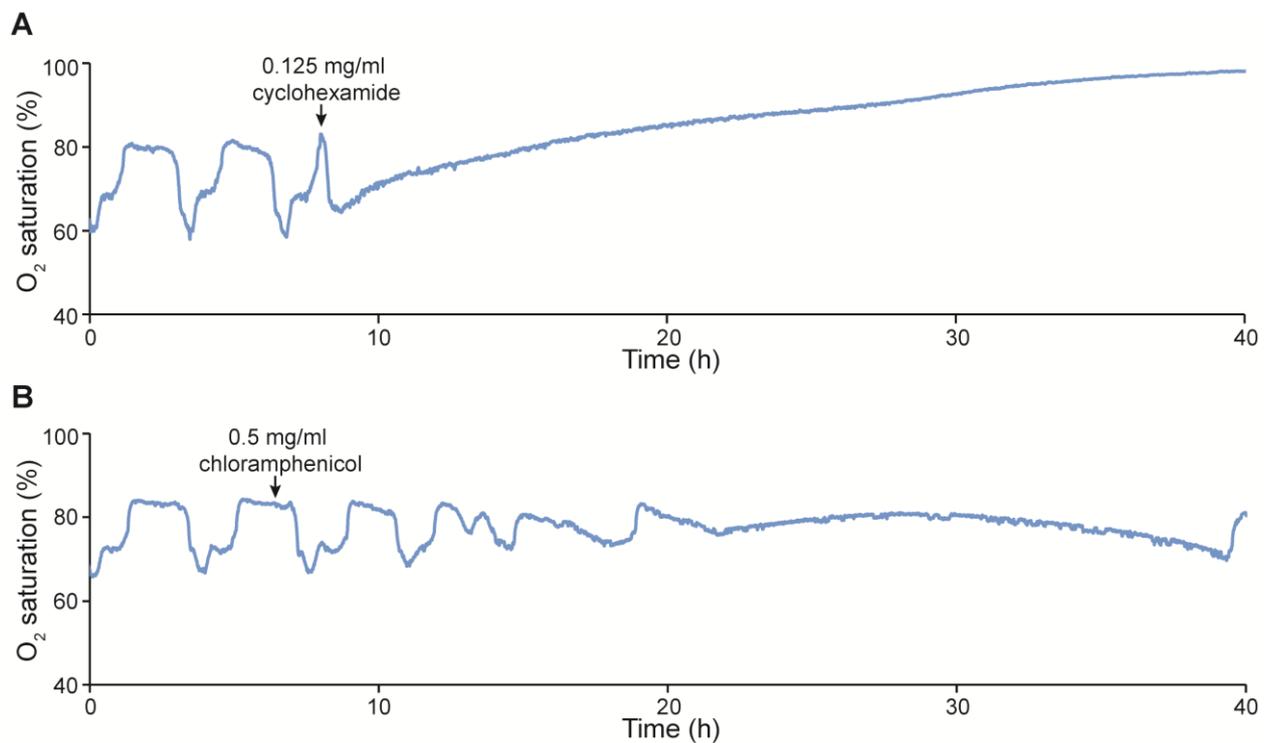
**Figure 2.1: The yeast metabolic cycle consists of respiratory oscillations. (A)** Representative oxygen trace showing how inhibition of respiration with 0.5 mM KCN immediately stops dissolved oxygen consumption and terminates oscillatory YMC for a prolonged period. Oscillatory behavior returns only after KCN is diluted out of the vessel. **(B)** Representative oxygen trace showing how ethanol, a respiratory substrate, forces synchronized cells to switch from fermentative to respiratory metabolism thereby inducing rapid dissolved oxygen consumption. **(C)** Representative oxygen trace showing that the organic solvent, DMSO, has no observable effect on yeast respiratory oscillations. All experiments were repeated at least twice with completely independent YMC-synchronized cultures.

### 2.1.2 Chemical inhibition of gene translation perturbs the YMC

It has been suggested that during the YMC, metabolic processes are temporally compartmentalized into phases that are tightly coordinated with gene expression and cell division [47, 50]. Gene expression analysis by microarray showed that periodic changes in the levels of more than 50% of cellular transcripts occur during the YMC. These periodic genes were grouped into three clusters. Based on these gene clusters, the YMC was classified into three phases: reductive building (RB), reductive charging (RC) and oxidative (Ox) [47].

I asked whether interfering with gene translation would have any impact upon the YMC. To answer this question, I performed chemical inhibition of translation using cyclohexamide and chloramphenicol. Cyclohexamide interferes with whole cell protein synthesis by blocking translation elongation whilst chloramphenicol specifically inhibits mitochondrial protein synthesis. Addition of cyclohexamide at the start of LOC initially triggered transient oxygen consumption and steadily slowed down oxygen consumption until the cells assumed a 'starvation mode' (Fig. 2.2A). Similarly, addition of chloramphenicol at the start of LOC destabilized the YMC and led to loss of oscillations in oxygen consumption (Fig. 2.2B).

In summary, these observations indicate that interfering with gene translation affects the YMC. Thus, cellular transcription/translation cycles are essential components of the YMC.

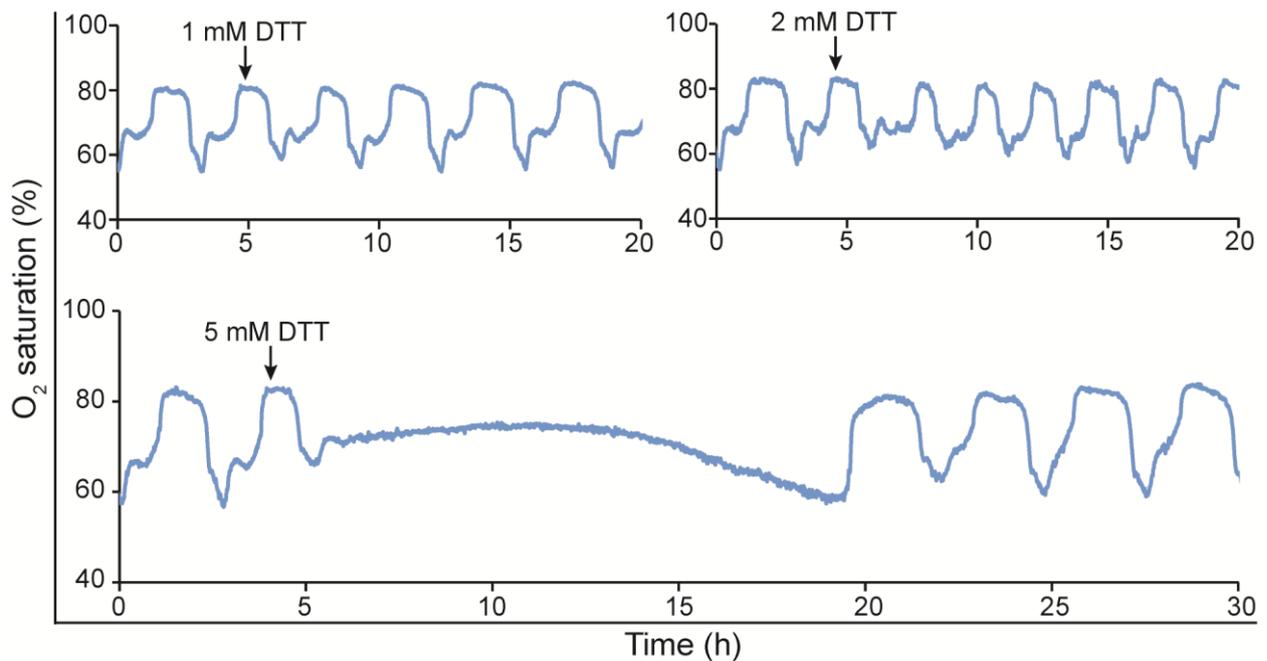


**Figure 2.2: The YMC is coupled to cellular transcription/translation programs. (A)** Representative oxygen trace showing that blocking cytosolic translation with 0.125 mg/ml cyclohexamide (dissolved in sterile milliQ-H<sub>2</sub>O) has a prolonged effect of inhibiting dissolved oxygen consumption and halting oscillatory YMC. **(B)** Representative oxygen trace showing that inhibition of mitochondrial translation with 0.5 mg/ml chloramphenicol (dissolved in 0.12% DMSO) destabilizes the YMC. All experiments were repeated at least twice with completely independent YMC-synchronized cultures.

## 2.2 The YMC is responsive to and modulated by chemical redox changes

The YMC primarily exhibits rhythms in consumption of oxygen, an important redox molecule. The levels of several redox enzymes and metabolites also oscillate during the YMC. Moreover, genetic or chemical disruption of redox processes affects the YMC [49, 56]. These observations therefore position the YMC as a “redox clock”. Hence, I sought to ascertain how chemical perturbation of cellular redox processes would influence the YMC that I have established. In their study, Chen *et al.* showed that H<sub>2</sub>O<sub>2</sub> and methionine advance the phase of the YMC of wildtype cells from RC to the Ox phase [49]. In this study, I applied the thiol reductant, dithiothreitol (DTT) and the thiol oxidant, N,N,N',N'-tetramethylazodicarboxamide (diamide) to wildtype YMC-synchronized cultures.

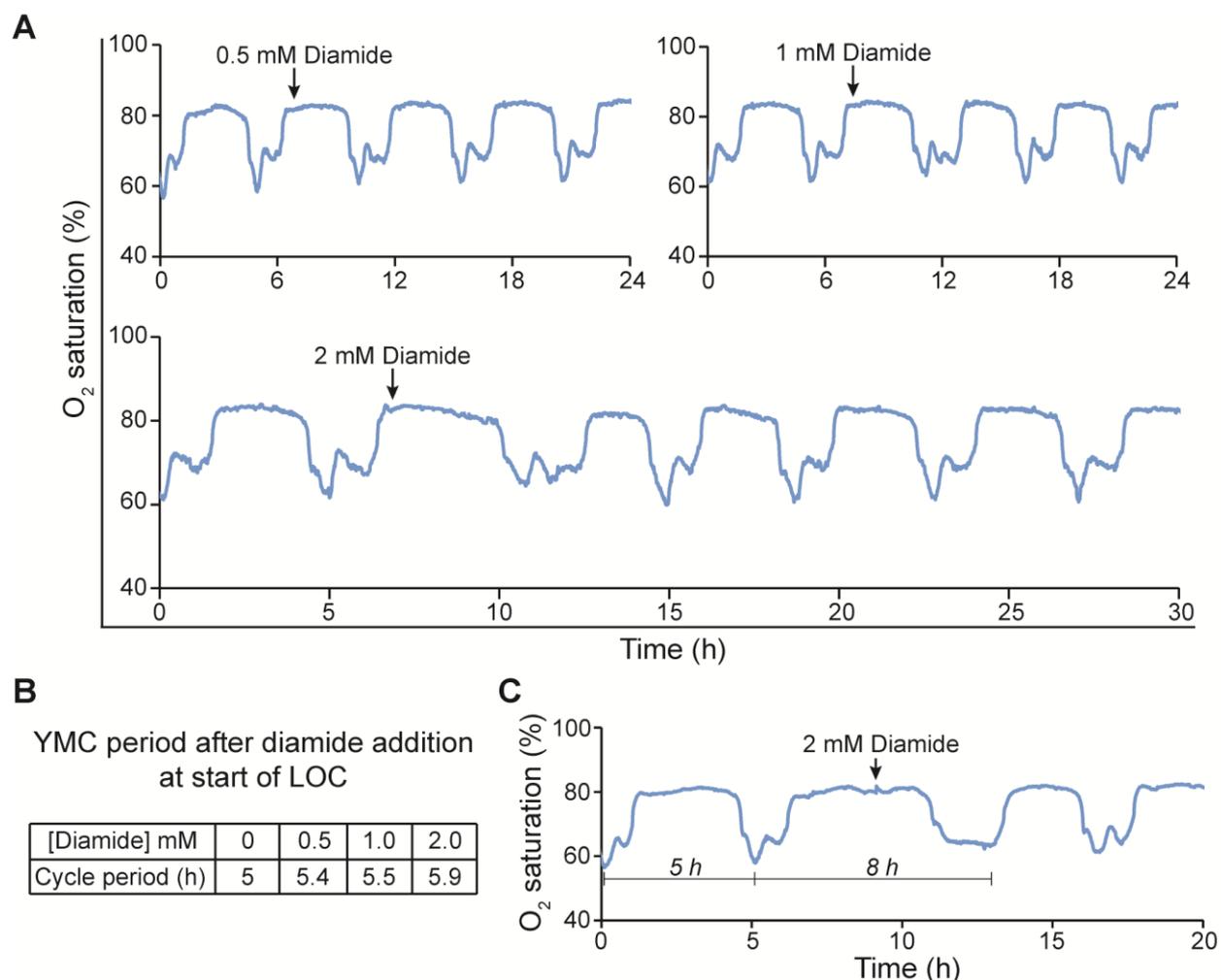
Addition of DTT at the start of LOC shortened time spent in LOC and facilitated switch to HOC in a concentration dependent manner. With 5 mM DTT, cells were forced to stay in a HOC phase for more than 10 hours, suggesting that a thiol reductant favors high oxygen consumption (Fig. 2.3).



**Figure 2.3: Reduction facilitates switch to high oxygen consumption.** Representative oxygen trace showing the response of the YMC to the addition of DTT at the start of LOC, in a concentration dependent manner. With 5 mM DTT, oxygen consumption was sustained for a prolonged period. All experiments were repeated at least twice with completely independent YMC-synchronized cultures.

In contrast, addition of diamide at the start of LOC extended the duration of the LOC phase and delayed transition to HOC, in a concentration dependent manner (Fig. 2.4A-B). This suggests that a thiol oxidant promotes low oxygen consumption. Intriguingly, cells just about to initiate HOC could be forced to further stay longer in LOC for approximately 2 hours by addition of 2 mM diamide towards the end of

LOC (Fig. 2.4C). Taken together, these observations show that cellular respiratory rate, and thus the YMC, is responsive to and perhaps regulated by cellular redox changes.



**Figure 2.4: Oxidation delays switch to high oxygen consumption. (A)** Representative oxygen trace showing the response of the YMC to the addition of diamide at the start of LOC, in a concentration dependent manner. **(B)** Table showing the period of a cycle of the YMC before and after addition of diamide at the indicated concentrations in (A). **(C)** Representative oxygen trace showing the response of the YMC to the addition of 2 mM diamide towards the end of LOC. Diamide delays switch to the HOC phase. All experiments were repeated at least twice with completely independent YMC-synchronized cultures.

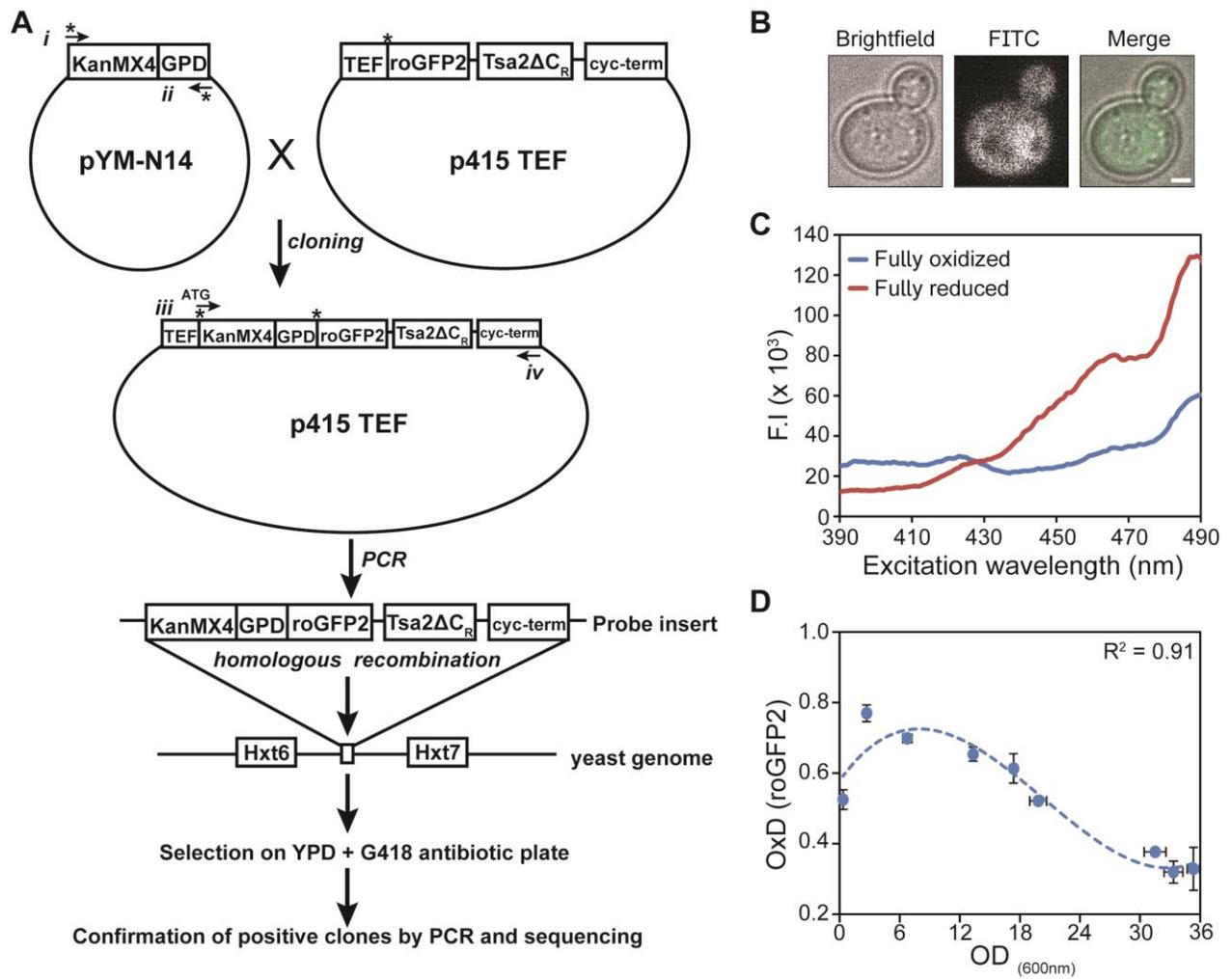
## 2.3 Cyclical changes in cellular H<sub>2</sub>O<sub>2</sub> occur during the YMC

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is an important cellular redox metabolite that functions as a second messenger in a variety of cell signaling pathways and can elicit large-scale transcriptional responses [129]. By exogenous addition to YMC-synchronized cultures, H<sub>2</sub>O<sub>2</sub> has been suggested as a signal that advances YMC phase and controls the gate for cell cycle entry [49]. However, until recently, no study had reported whether oscillations in H<sub>2</sub>O<sub>2</sub> existed in circadian and ultradian metabolic clocks, under physiological conditions. I hypothesized that as a “redox clock”, oscillations in cellular H<sub>2</sub>O<sub>2</sub> levels could occur during the YMC.

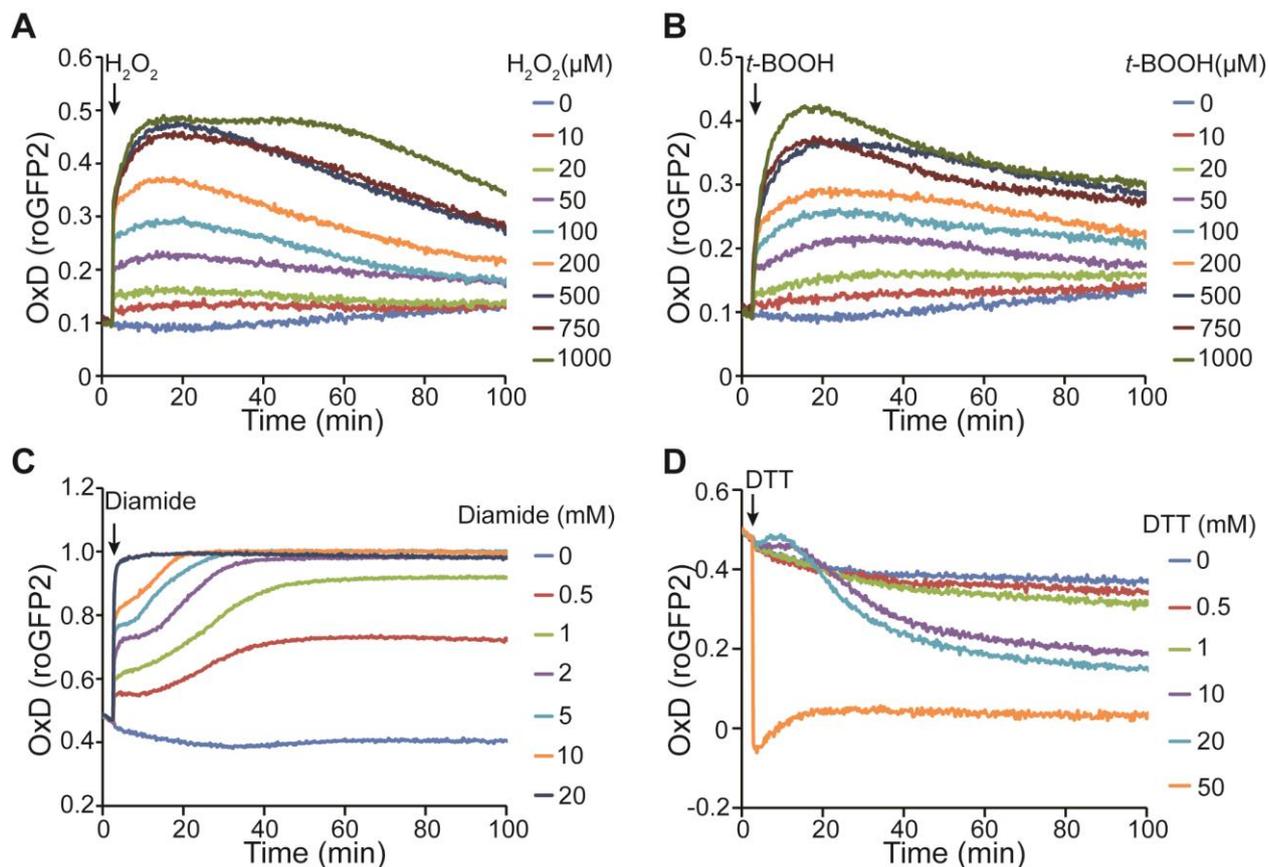
### 2.3.1 Integration of roGFP2-Tsa2ΔC<sub>R</sub> biosensor into yeast genome

To test the above hypothesis, I integrated the roGFP2-Tsa2ΔC<sub>R</sub> biosensor into yeast genome (Fig. 2.5A). This was done in part to circumvent the non-suitability of amino acid-based plasmid selection in the prototrophic CEN.PK yeast background. I specifically replaced the constitutive TEF promoter with a stronger expressing GPD promoter, coupled to a Kanamycin resistance marker gene (i.e. *KanMX4*) at its N-terminus for selection. This construct was re-constituted in a p415 plasmid and amplified by PCR using primer sequences that allowed integration into the intergenic region between hexose transporters *HXT6* and *HXT7*, just upstream of the conserved promoter region for *HXT7* [130]. This unusually long (~3kb) region was chosen in part because there are several other hexose transporters in the yeast genome, which render them functionally redundant in case of damage [131]. Moreover, this is the only region of the yeast genome that has been suggested to have no known function [47]. Successful construction and integration of the genomic sensor was confirmed by PCR and sequencing (Appendix A1). I could also confirm sensor expression and cytosolic localization by fluorescence microscopy (Fig. 2.5B). Additionally, the sensor exhibited an excitation spectra characteristic of roGFP2 with maxima at 405 (fully oxidized) and 488 nm (fully reduced), at an emission wavelength of 510 nm (Fig. 2.5C). The genomic sensor was also sensitive to changes in the cellular redox environment due to changing nutrient and oxygen availability (Fig. 2.5D).

Furthermore, the sensor could respond to a wide range of redox chemicals including H<sub>2</sub>O<sub>2</sub>, the organic *tert-butyl* hydroperoxide (*t*-BOOH), the thiol oxidant diamide and thiol reductant DTT, in a concentration dependent manner (Fig. 2.6). To ascertain whether the sensor was functional during the YMC, I collected cells from different phases of a YMC-synchronized culture of the biosensor expressing cells and treated them with different concentrations of H<sub>2</sub>O<sub>2</sub> in a plate reader format. The biosensor was oxidized upon H<sub>2</sub>O<sub>2</sub> in a concentration dependent manner, suggesting that the biosensor was active under continuous culture conditions in the fermenter (Appendix A2). Taken together, these results show that the genomic expression of the roGFP2-Tsa2ΔC<sub>R</sub> biosensor was successful. This sensor also afforded me the opportunity to study peroxiredoxin function *in vivo* since roGFP2 redox state was dependent upon a functional Tsa2ΔC<sub>R</sub>. More so, changes in roGFP2 redox state could reflect changes in the redox state of endogenous peroxiredoxin relay(s).



**Figure 2.5: Construction and characterization of a genomically integrated roGFP2-Tsa2 $\Delta$ C<sub>R</sub> sensor-expressing yeast strain.** (A) Scheme illustrating the cloning strategy for generating CEN.PK113-1A strain with a genomically integrated construct for roGFP2-Tsa2 $\Delta$ C<sub>R</sub> biosensor expression. (B) Fluorescence microscopy to show cytosolic localization of roGFP2-Tsa2 $\Delta$ C<sub>R</sub> biosensor. (C) Fluorescence excitation spectra of CEN.PK cells expressing roGFP2-Tsa2 $\Delta$ C<sub>R</sub>. Fully oxidized and fully reduced spectra were obtained by treating the cells with 20 mM diamide and 100 mM DTT respectively. (D) Sensor oxidation changes with cell density in glucose media. The degree of roGFP2 oxidation (OxD) was measured from YPD culture of the sensor-expressing strain every 2 hours. Data represent mean of three independent repeats, whilst error bars represent standard deviation. Note: (i) to (iv) in (A) represent primers P1 to P4 in Materials and Methods.



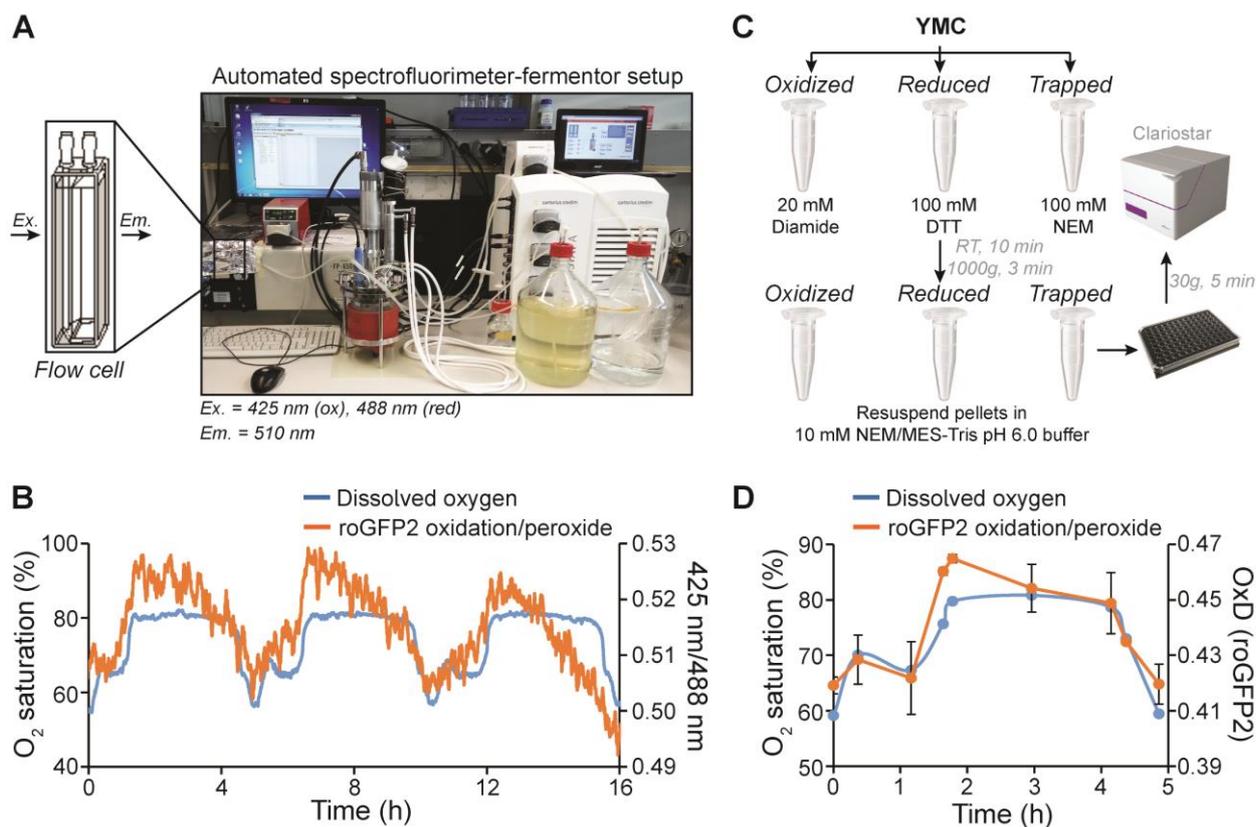
**Figure 2.6: Genomically integrated roGFP2-Tsa2 $\Delta$ C<sub>R</sub> sensor is sensitive to a variety of redox chemicals.** Response of the roGFP2-Tsa2 $\Delta$ C<sub>R</sub> biosensor, expressed in CEN.PK cells, to the addition of (A) H<sub>2</sub>O<sub>2</sub>, (B) *t*-BOOH, (C) diamide, and (D) DTT, at the indicated concentrations. All experiments were performed in triplicates.

### 2.3.2 Cyclical H<sub>2</sub>O<sub>2</sub> changes are synchronized to YMC phase

To ascertain whether basal levels of H<sub>2</sub>O<sub>2</sub> changed during the YMC, I cultured the sensor-expressing yeast strain under conditions for metabolic cycling. To monitor sensor fluorescence in real-time, I developed a system in-house, consisting of a flow cell placed in the chamber of a spectrofluorimeter and connected via tubes to the fermenter culture vessel (Fig. 2.7A). With the aid of a peristaltic pump, I drove cells from the culture vessel through the flow cell and back into the vessel. I measured the fluorescent intensity of cells at 425 nm and 488 nm excitation and 510 nm emission at 30 s intervals. By computing the ratio between fluorescent intensities at 425 nm and 488 nm, I could qualitatively monitor oxidation of the biosensor during the YMC. By overlaying biosensor fluorescence ratios and oxygen saturation curves from three metabolic cycles, I observed that oscillations in roGFP2 oxidation state accompanied the YMC. RoGFP2 oxidation peaked at the entry into LOC, whilst a reduction of roGFP2 correlated with the switch from LOC to HOC (Fig. 2.7B).

However, the signal to noise ratio from the real-time fluorescence measurements was high. As such, it was plausible that the observed oscillations in roGFP2 redox state was an artifact. To verify these oscillations in roGFP2 oxidation state, I collected samples from specific points of the YMC and analyzed their roGFP2 redox state via an N-Ethylmaleimide (NEM)-based trapping technique (Fig. 2.7C), as

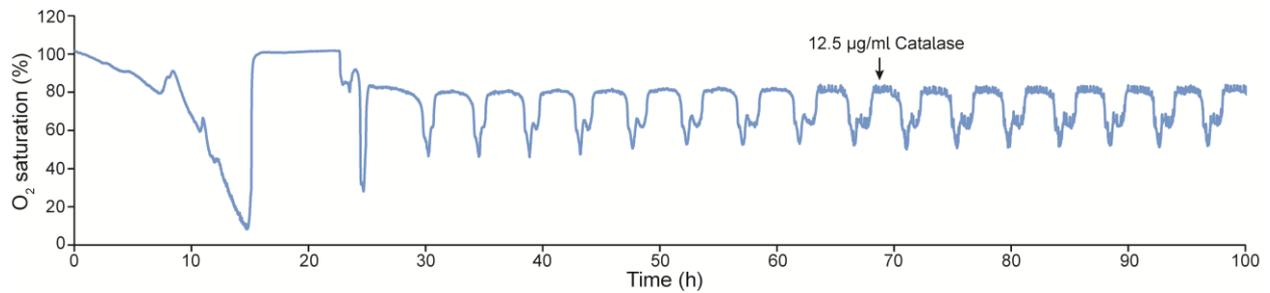
described in Materials and Methods. NEM irreversibly alkylates cysteine residues to protect the redox state of protein thiols. By repeating this experiment for three independent metabolic cycles, calculating degree of sensor oxidation (OxD) and superimposing the results over one representative YMC, I could recapitulate the observations made with the flow-cell based real-time measurements (Fig. 2.7D). Taken together, these data demonstrate that oscillations in basal  $H_2O_2$  levels occur during the YMC. Moreover, the redox state of peroxiredoxins as well as peroxiredoxin target protein(s) during the YMC is rhythmic.



**Figure 2.7: Oscillations in basal  $H_2O_2$  levels during the YMC.** (A) Setup of an automated system to monitor fluorescence of sensor-expressing cells during the YMC. (B) Representative trace of dissolved oxygen levels and roGFP2 fluorescence excitation ratio over three complete YMC cycles. (C) Experimental workflow for measuring steady-state roGFP2 oxidation by NEM-trapping, in a plate reader format. (D) The degree of roGFP2-Tsa2ΔC<sub>R</sub> oxidation measured from YMC-synchronized culture samples at the indicated time points, based on the NEM-trapping technique in (C). Data points represent average of three independent experiments while error bars represent standard deviation. Data points of percent oxygen saturation represent phase of YMC at which cell samples were taken.

### 2.3.3 $H_2O_2$ is not important for population synchrony

A model for signaling events during the YMC posits that liquidation of storage carbohydrates by a fraction of cells in HOC leads to the secretion of metabolites that trigger other cells to synchronously consume oxygen. These metabolites include ethanol, acetaldehyde and dihydrogen sulfide [58, 59, 115]. Having observed that  $H_2O_2$  oscillates during the YMC, I asked whether it was secreted by cells into the medium to serve as a trigger for other cells to synchronously consume oxygen. To do this, I injected 12.5μg/ml of catalase into the culture vessel. Surprisingly, the YMC remained unperturbed for the entire course of fermenter run (Fig. 2.8). This suggests that  $H_2O_2$ , even if exogenously secreted by cells into the surrounding medium, is not important for YMC synchrony.



**Figure 2.8: Effect of exogenous catalase on the YMC.** Catalase solution at a final concentration of 12.5 µg/ml was added into the culture vessel at the start of a LOC phase as indicated, for exogenous H<sub>2</sub>O<sub>2</sub> scavenging.

## 2.4 A signaling role for H<sub>2</sub>O<sub>2</sub> and peroxiredoxins during the YMC

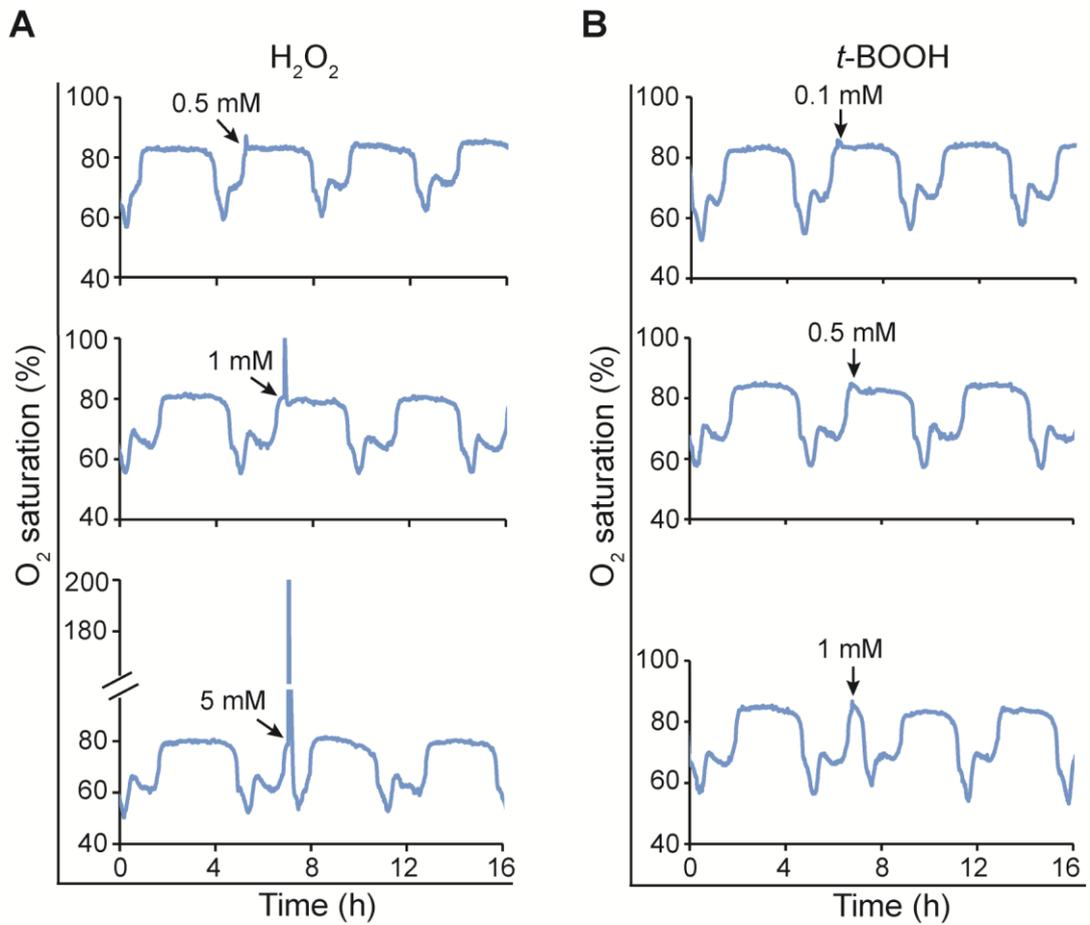
Having ruled out an inter-cellular communication function for H<sub>2</sub>O<sub>2</sub>, I asked whether H<sub>2</sub>O<sub>2</sub> served an endogenous function during the YMC. If so, could peroxiredoxins act as mediators in such signaling event?

### 2.4.1 High exogenous H<sub>2</sub>O<sub>2</sub> induces metabolic switch to HOC

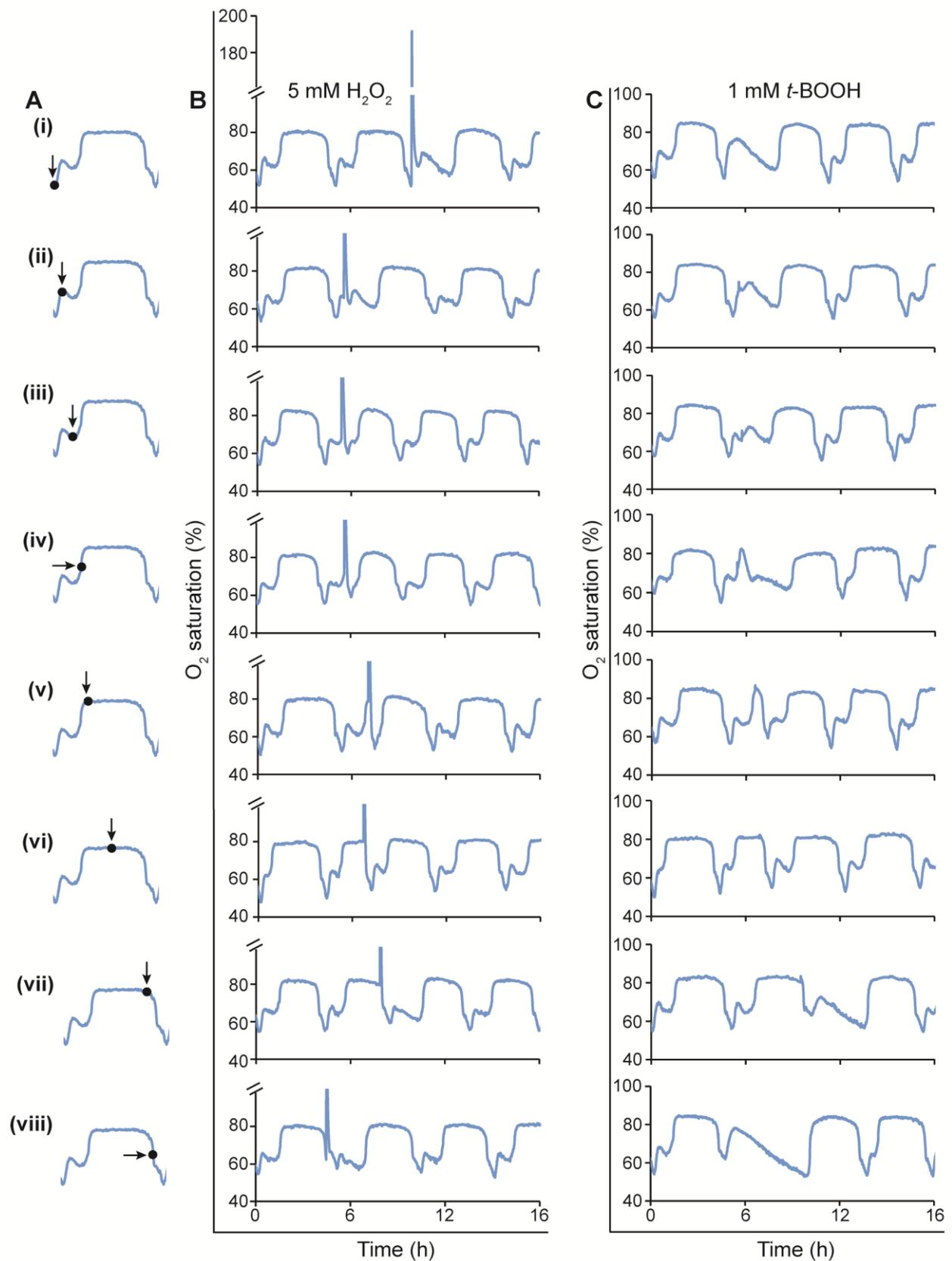
To ascertain an endogenous role during the YMC, I added boli of H<sub>2</sub>O<sub>2</sub> at different concentrations to the start of LOC of wildtype YMC-synchronized cultures. While 0.5 and 1 mM had no significant effect on the YMC, 5 mM H<sub>2</sub>O<sub>2</sub> rapidly and transiently induced HOC, which lasted nearly 2 h before return to the LOC phase (Fig. 2.9A). However, addition of H<sub>2</sub>O<sub>2</sub> to the culture vessel resulted in molecular oxygen production due to endogenous catalase activity, which could be detected by the dissolved oxygen (DO) probe, and thus interfered with my measurements. To circumvent this “side-effect”, I applied the organic hydroperoxide *t*-BOOH. Unlike H<sub>2</sub>O<sub>2</sub>, *t*-BOOH cannot be catabolized by catalase. Interestingly, I observed that 0.1 and 0.5 mM *t*-BOOH had no significant effect on the YMC, whilst 1 mM *t*-BOOH rapidly induced premature switch to HOC similar to 5 mM H<sub>2</sub>O<sub>2</sub> (Fig. 2.9B). In sum, these observations indicate that peroxides can induce premature LOC-to-HOC transition when applied to YMC-synchronized cultures at high non-physiological concentrations.

### 2.4.2 High peroxide-induced switch to HOC during YMC is phase-independent

Having observed the induction of LOC-to-HOC transition upon high peroxide addition at the start of LOC of the YMC, I asked whether this response was phase-specific or could occur at any point of the YMC. To address this, I added either 5 mM H<sub>2</sub>O<sub>2</sub> or 1 mM *t*-BOOH to different points of the YMC. Surprisingly, HOC could be induced independent of the phase of peroxide addition (Fig. 2.10).



**Figure 2.9: Effect of peroxides on the YMC. (A)** Representative responses of YMC-synchronized culture of wildtype cells to the addition of  $\text{H}_2\text{O}_2$  at the indicated concentrations. **(B)** Representative responses of YMC-synchronized cultures of wild-type cells to the addition of *t*-BOOH at the indicated concentrations.

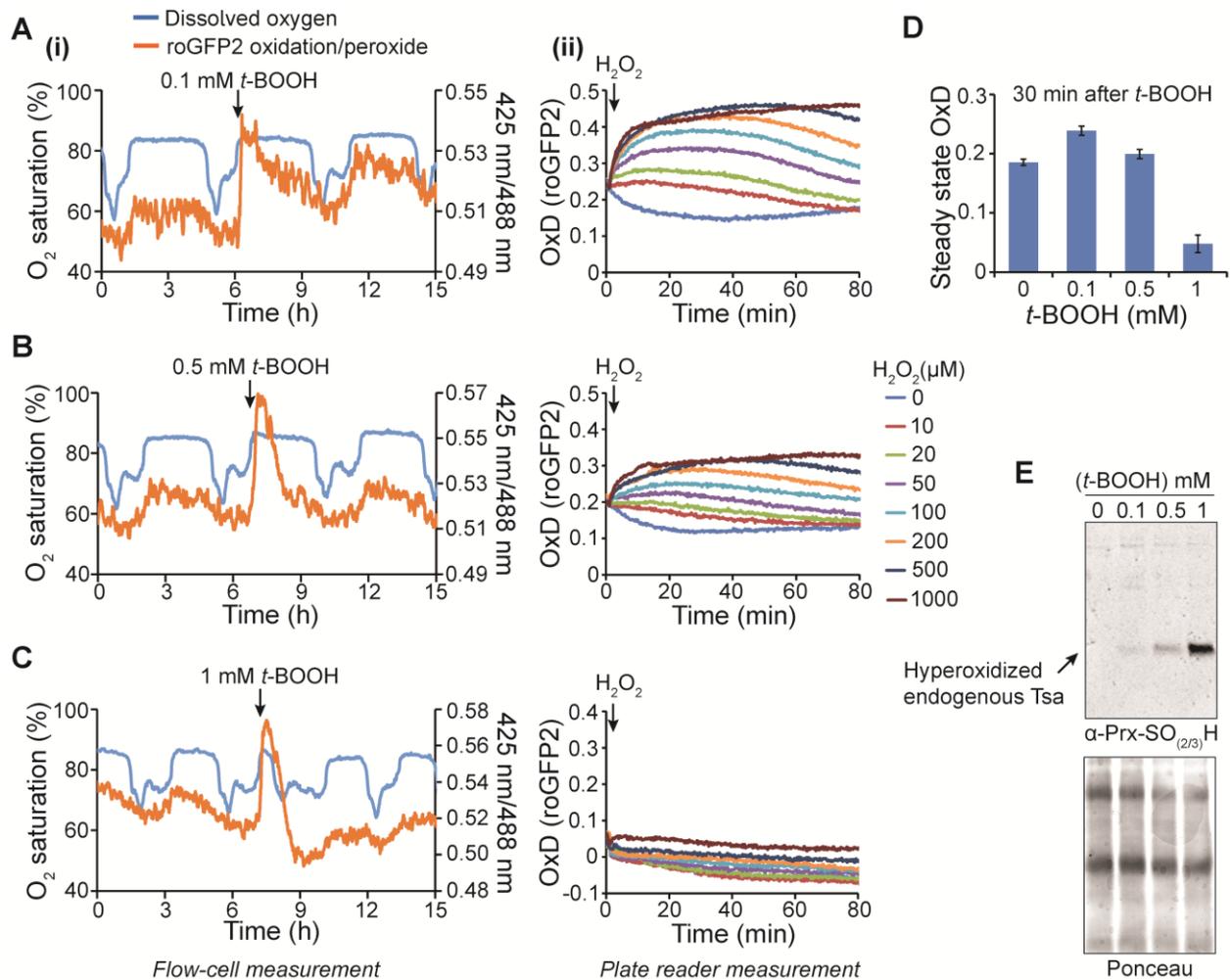


**Figure 2.10: High amounts of peroxide can induce switch to HOC at any phase of the YMC. (A)** Points on the YMC at which treatments were carried out. **(B)** Representative responses of YMC-synchronized cultures of wildtype cells to the addition of 5 mM H<sub>2</sub>O<sub>2</sub>. **(C)** Representative responses of YMC-synchronized cultures of wildtype cells to the addition of 1 mM *t*-BOOH.

### 2.4.3 Peroxiredoxin inactivation upon high peroxide mediates metabolic switch to HOC

Living cells respond to  $\text{H}_2\text{O}_2$  via regulation of protein function leading to increased production of antioxidant enzymes and cofactors. The regulated proteins generally include kinases, phosphatases and transcription factors that mediate a variety of cellular processes such as transcription, growth, nutrient sensing and mitochondria biogenesis [132]. It has been suggested that the oxidation of most redox-regulated proteins by  $\text{H}_2\text{O}_2$  is almost completely dependent on peroxiredoxins [72]. Accordingly, I sought to ascertain whether the premature LOC-to-HOC transition upon high peroxide could be mediated by peroxiredoxins. To do this, I cultured the sensor-expressing cells under YMC conditions and monitored sensor fluorescence in real-time before and upon addition of the different concentrations of *t*-BOOH previously used. At bolus concentrations of 0.1 and 0.5 mM *t*-BOOH, I observed roGFP2 oxidation, which gradually reduced to 'normal' levels before the switch to HOC. Counter-intuitively, upon 1 mM *t*-BOOH, roGFP2 oxidized transiently and reduced in a manner that correlated with increased oxygen consumption (i.e. LOC-to-HOC transition) (Fig. 2.11A-C(i)). Interestingly, roGFP2 appeared to have reduced to levels below the initial oxidation state (Fig. 2.11C(i)). This observation was indicative of hyperoxidation-based inactivation of the Tsa2 $\Delta\text{C}_\text{R}$  moiety of the roGFP2-Tsa2 $\Delta\text{C}_\text{R}$  sensor. This phenomenon is conceivable because upon excess peroxide, the  $\text{C}_\text{P}$  of the Tsa2 $\Delta\text{C}_\text{R}$  moiety becomes hyperoxidized and inactivated. Consequently, the transduction of oxidizing equivalents from peroxide to roGFP2 is inhibited. The redox state of roGFP2 thus depends on equilibration with the cellular glutathione pool (Fig. 2.12).

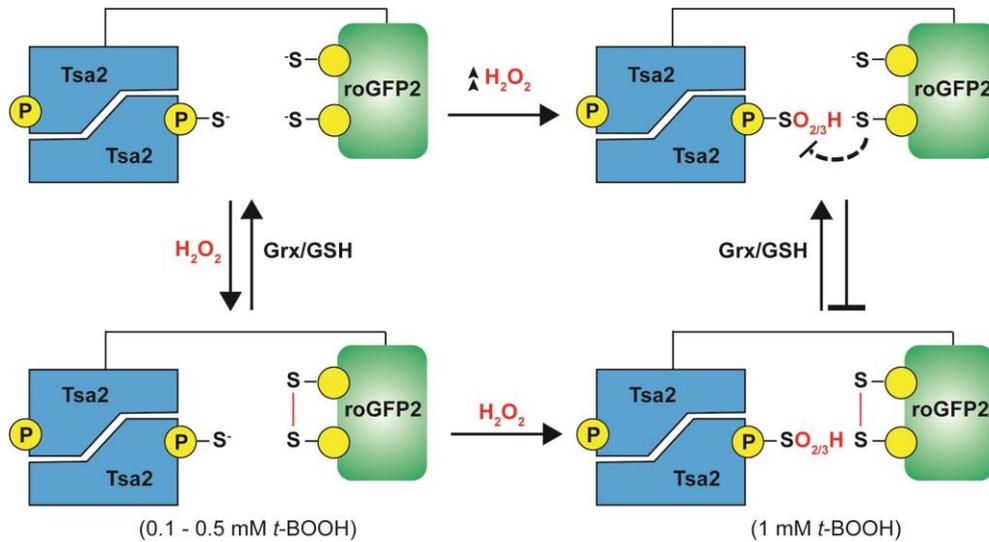
To verify whether the observed reduction of roGFP2 redox state upon high peroxide was due to hyperoxidation and inactivation of Tsa2 $\Delta\text{C}_\text{R}$ , I collected YMC-synchronized cells from the fermenter at the start of LOC, before and 30 mins after *t*-BOOH treatment. I then assessed roGFP2 fluorescence via the plate-reader method. This assay works on the principle that if the Tsa2 $\Delta\text{C}_\text{R}$  moiety of the sensor was still functional after *t*-BOOH addition to cells during the YMC, further oxidation of roGFP2 should be achieved upon exogenous addition of  $\text{H}_2\text{O}_2$  to cells in a 96-well plate, in a concentration dependent manner. On the contrary, if the Tsa2 $\Delta\text{C}_\text{R}$  moiety of the sensor becomes inactivated upon *t*-BOOH addition to cells during the YMC, roGFP2 becomes unresponsive independent of the amount of exogenous  $\text{H}_2\text{O}_2$  added to cells in the plate. In line with this reasoning, sensor-expressing cells collected after 30 mins of 0.1 and 0.5 mM *t*-BOOH addition to YMC-synchronized cultures were responsive to external  $\text{H}_2\text{O}_2$  in a concentration dependent manner (Fig. 2.11A-B(ii)). On the other hand, sensor-expressing cells collected after 30 mins of 1 mM *t*-BOOH addition to YMC-synchronized cultures were unreactive to any concentration of external  $\text{H}_2\text{O}_2$  (Fig. 2.11C(ii)). These observations confirmed that the peroxiredoxin of the biosensor was inactivated upon 1 mM *t*-BOOH addition to YMC-synchronized cells. Furthermore, the steady-state OxD of roGFP2 was rendered more reduced in 1 mM *t*-BOOH samples (Fig. 2.11D). Since the roGFP2-Tsa2 $\Delta\text{C}_\text{R}$  biosensor represented a reconstituted thiol peroxidase redox relay, I imagined that endogenous peroxiredoxin inactivation and subsequent reduction of the redox state of endogenous peroxiredoxin substrate(s) could be responsible for premature LOC-to-HOC transition upon high peroxide.



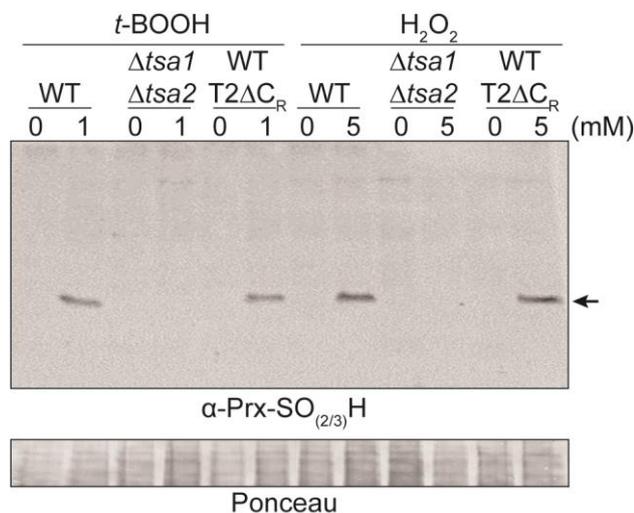
**Figure 2.11: LOC-to-HOC transition upon high peroxide correlates with peroxiredoxin hyperoxidation. (A-C)** Representative responses of a roGFP2-Tsa2 $\Delta$ C<sub>R</sub> probe in YMC-synchronized cultures treated with either 0.1 mM, 0.5 mM or 1 mM *t*-BOOH. **(i)** Left panels represent traces of dissolved oxygen and roGFP2 fluorescence before and after treatment with *t*-BOOH in the fermenter at the indicated concentrations. **(ii)** Right panels represent sensor response to exogenous addition of the indicated concentrations of H<sub>2</sub>O<sub>2</sub> in a plate-reader format, 30 mins after *t*-BOOH bolus in the fermenter. **(D)** The steady-state oxidation of roGFP2 (substrate) was measured in a plate reader format. Data represent average of three independent experiments, while error bars represent standard deviation. **(E)** Western blot for hyperoxidized endogenous Tsa1 and Tsa2 following application of *t*-BOOH to continuous cultures at the indicated concentrations.

To test whether endogenous peroxiredoxins were hyperoxidized upon *t*-BOOH addition to YMC-synchronized cultures, the cell samples collected before and 30 mins after *t*-BOOH addition were assayed for endogenous peroxiredoxin hyperoxidation by Western blot. The antibody I used for this assay was raised against a Keyhole limpet hemocyanin (KLH)-coupled sulfonlated peptide corresponding to the active site sequence of human Prdx I to IV (AbFrontier, LF-PA004). This antibody was also shown to be specifically reactive towards yeast typical 2-Cys peroxiredoxins, Tsa1 and Tsa2 (Fig. 2.13, [90]). Intuitively, after assaying cells collected before and after addition of 0.1, 0.5 and 1 mM *t*-BOOH to YMC-synchronized cultures, I observed that the hyperoxidation of endogenous yeast typical 2-Cys peroxiredoxins, Tsa1 and Tsa2, occurred slightly in 0.5 mM and strongly in 1 mM *t*-BOOH treated samples (Fig. 2.11E). In addition, the pattern of endogenous peroxiredoxin hyperoxidation, as visualized on the Western blot, precisely mimicked the biosensor peroxiredoxin response observed with the plate-reader (compare Fig. 2.11C(ii) and

Fig. 2.11E). In sum, I have demonstrated that peroxide prematurely triggers LOC-to-HOC transition only when added at concentrations high enough to induce hyperoxidation of typical 2-Cys peroxiredoxins, a phenomenon that leads to reduction of the redox state of peroxiredoxin target protein(s). Although this concentration of peroxide is non-physiological, these observations suggest that peroxiredoxins have an important role in regulating LOC-to-HOC transition. Consequently, peroxiredoxins and peroxiredoxin target protein(s) constitute a molecular switch that regulate LOC-to-HOC transition and thus the YMC.

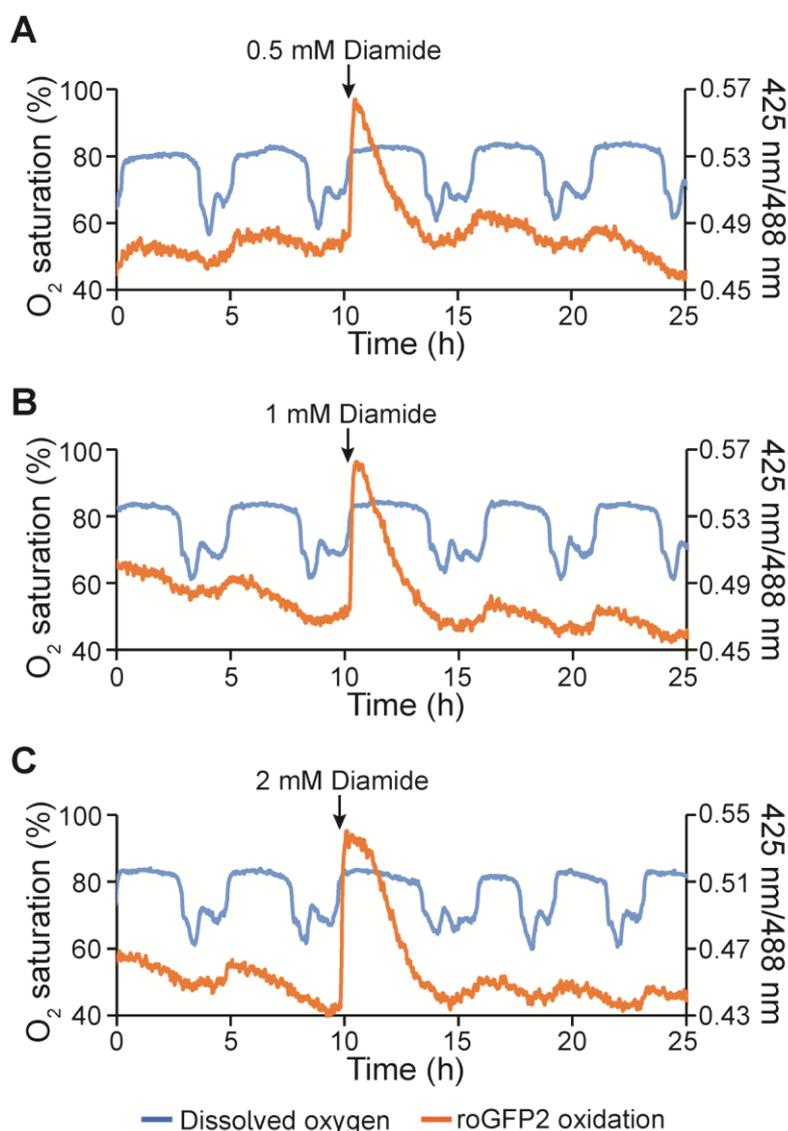


**Figure 2.12: Scheme explaining sensor behavior after *t*-BOOH bolus in fermenter.** At steady state, the sensor remains mostly in the reduced thiolate state. Upon encountering basal  $H_2O_2$ , the ratio of oxidized to reduced probe levels change depending on the amount of  $H_2O_2$ , as depicted by the cyclical changes during the YMC. RoGFP2 acts as substrate to receive oxidizing equivalents from  $H_2O_2$  with the  $C_P$  of the  $Tsa2\Delta C_R$  acting as the transducer. In this state,  $Tsa2\Delta C_R$  remains very active in this role. Upon exogenous addition of 0.1 or 0.5 mM *t*-BOOH, the oxidized levels of the sensor increase and more oxidizing equivalents passed onto roGFP2 by an active  $Tsa2\Delta C_R$ . RoGFP2 stays more oxidized until peroxide is diluted out of the culture to basal levels, and thereafter reversed to a reduced thiolate state by Grxs using GSH. Upon excess peroxide (e.g. 1 mM *t*-BOOH), the sensor becomes only transiently oxidized and remains reduced thereafter. This is because the  $C_P$  of the  $Tsa2\Delta C_R$  becomes overwhelmed by the amount of peroxide and therefore is inactivated by hyperoxidation. Subsequently, the transduction of oxidizing equivalents to roGFP2 is hindered, resulting immediately in the return to a more reduced thiolate roGFP2 via the action of Grxs using GSH.



**Figure 2.13: Antibody detection of peroxiredoxin hyperoxidation is Tsa-specific.** Western blot showing specificity of Prx-SO<sub>(2/3)</sub>H antibody towards the yeast typical 2-Cys peroxiredoxins, Tsa1 and Tsa2. Key: WT - wildtype CEN.PK cells, WT T2 $\Delta C_R$  - genomic sensor-expressing cells,  $\Delta tsa1\Delta tsa2$  - cells deleted for *TSA1* and *TSA2*.

In fact, it was instructive to observe an apparent reduction of roGFP2 redox state upon 1 mM *t*-BOOH since peroxides function as oxidants. Therefore, I ascertained whether the thiol oxidant diamide caused a reduction in roGFP2 redox state under similar conditions. In contrast, by monitoring sensor response in real-time, diamide could only induce roGFP2 oxidation when added at the start of LOC. LOC-to-HOC transition was only achieved after diamide was removed from the culture vessel by dilution and roGFP2 redox state reduced to ‘normal’ levels (Fig. 2.14). Thus, unlike high levels of peroxides, diamide only induced roGFP2 oxidation, which was not required for LOC-to-HOC transition, suggesting that LOC-to-HOC transition is regulated by thiol reduction. This thiol reduction is achieved either via low signaling H<sub>2</sub>O<sub>2</sub> levels under physiological conditions or upon high non-physiological peroxide levels that hyperoxidize and inactivate peroxiredxins, a phenomenon explainable by the ‘relay’ hypothesis.



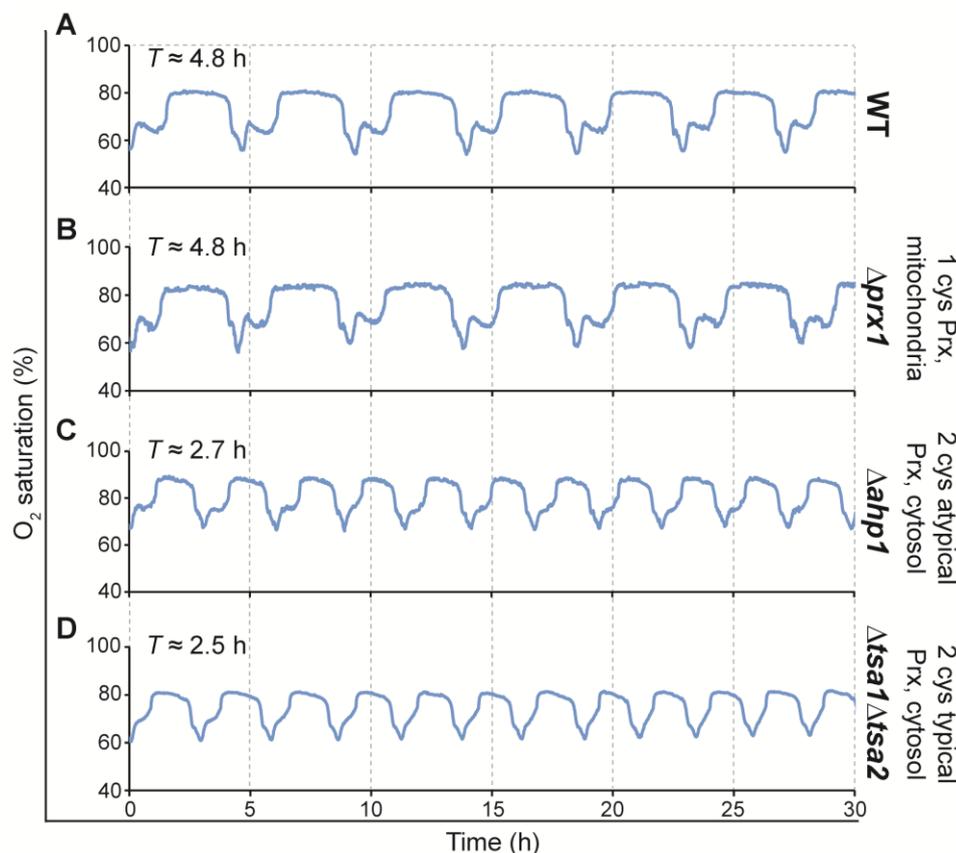
**Figure 2.14: The oxidant diamide does not induce roGFP2 reduction as well as rapid LOC-to-HOC transition.** Representative graphs showing YMC response to (A) 0.5 mM (B) 1 mM and (C) 2 mM diamide addition at start of LOC together with the response of the genomically integrated roGFP2-Tsa2ΔC<sub>R</sub> probe. All experiments were repeated at least twice with completely independent YMC-synchronized cultures.

## 2.5 Cytosolic peroxiredoxins are essential for YMC regulation

Given that peroxiredoxin hyperoxidation correlated with the switch to HOC from LOC, I asked whether loss of peroxiredoxins would affect the YMC *per se*, as well as the response of YMC-synchronized cells to the levels of *t*-BOOH needed to induce LOC-to-HOC switching.

### 2.5.1 Loss of cytosolic 2-Cys peroxiredoxins is associated with decreased YMC period

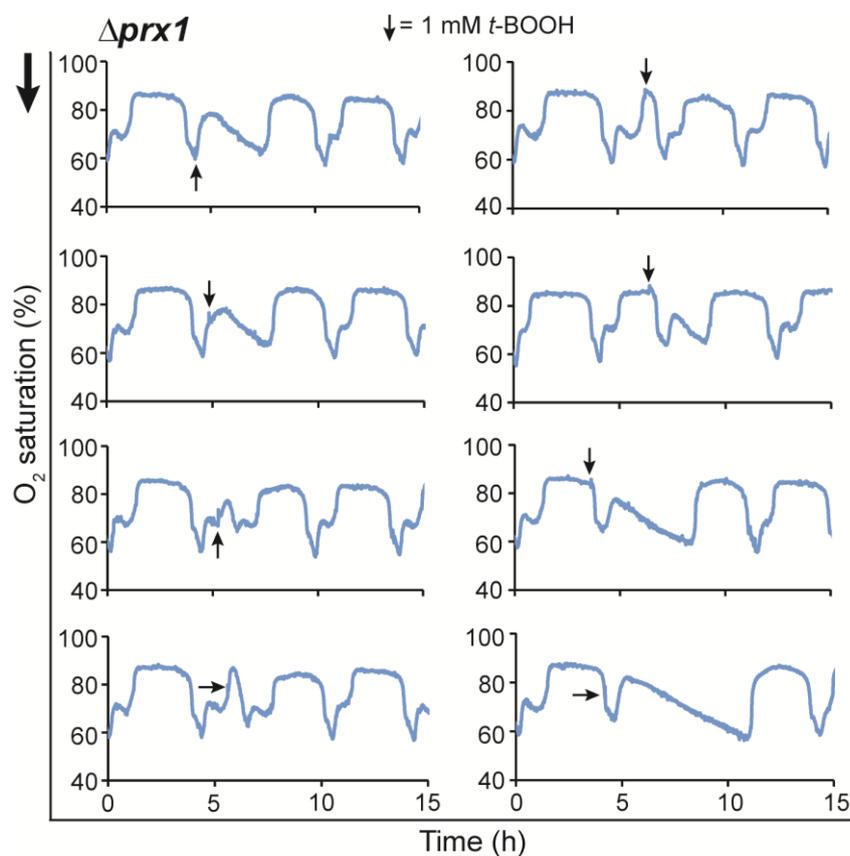
To address the above question, I performed single and double deletions of peroxiredoxins in yeast cytosol or mitochondria. Loss of the mitochondria 1-Cys peroxiredoxin, *PRX1*, had no observable effect on YMC shape and period. However, a single deletion of the cytosolic atypical 2-Cys peroxiredoxin and alkylhydroperoxidase, *AHP1*, significantly affected YMC shape and decreased YMC period to approximately 2.7 hours. Similarly, double deletion of the cytosolic typical 2-Cys peroxiredoxins, *TSA1* and *TSA2* significantly affected YMC shape and decreased YMC period to approximately 2.5 hours (Fig. 2.15). Taken together, these observations suggest that cytosolic 2-Cys peroxiredoxins may be essential for YMC regulation.



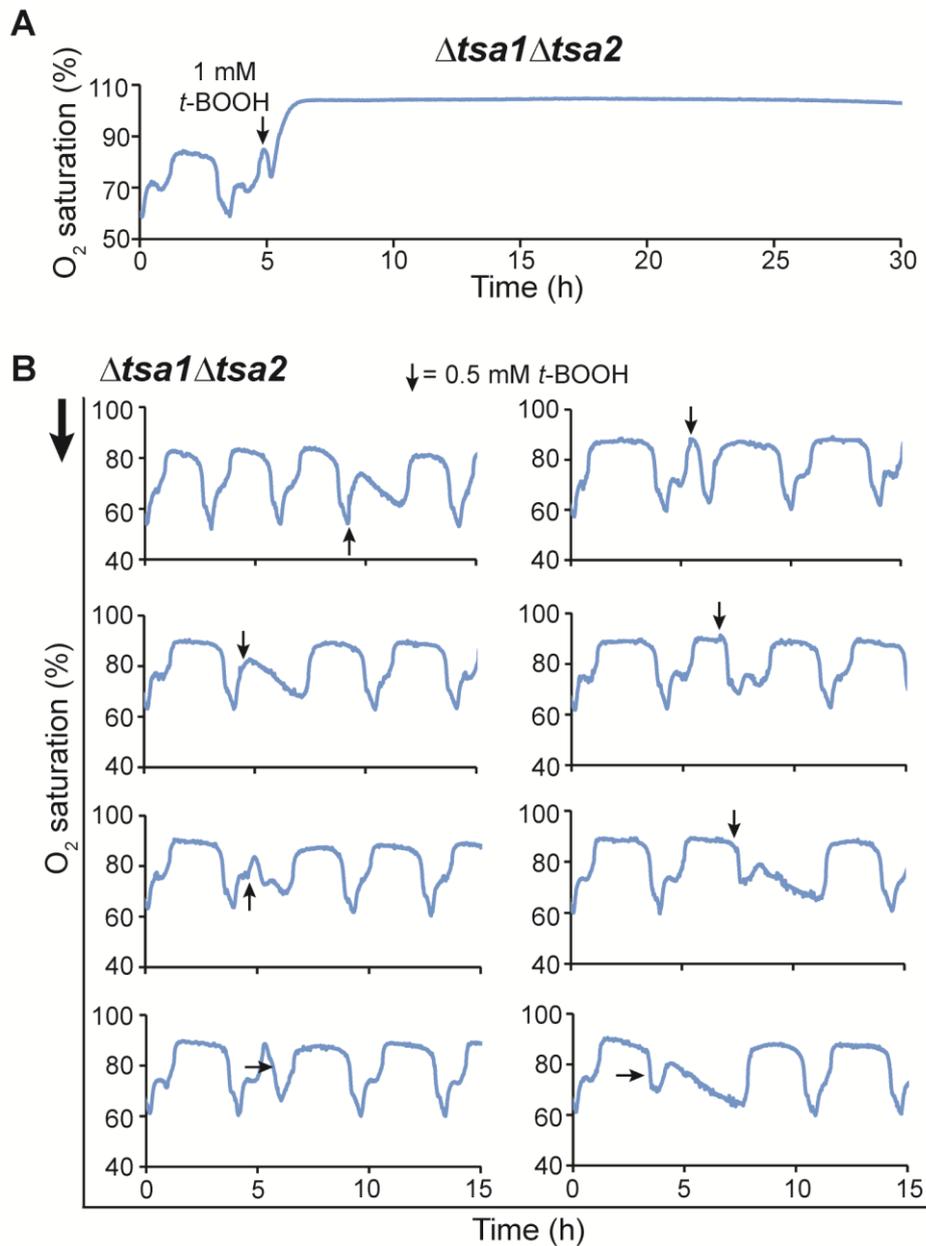
**Figure 2.15: Effect of peroxiredoxin deletion on the YMC.** Representative oxygen traces to show the YMC for (A) Wildtype CEN.PK113-1A, (B) Cells deleted for the mitochondrial 1-Cys peroxiredoxin *PRX1*, (C) Cells deleted for the cytosolic atypical 2-Cys peroxiredoxin *AHP1* and (D) Cells deleted for the cytosolic typical 2-Cys peroxiredoxins *TSA1* and *TSA2*. All experiments were repeated at least twice with completely independent YMC-synchronized cultures.

## 2.5.2 Multiple peroxiredoxins function to mediate peroxide-induced metabolic switch to HOC

Next, I sought to understand how loss of peroxiredoxins might modulate response to the peroxide levels needed to induce switch to HOC in wildtype cells, if any. To do this, I added 1 mM *t*-BOOH at points of the YMC described in Fig. 2.10A. At this concentration of *t*-BOOH, I observed induction of HOC in  $\Delta prx1$  cells in a manner synonymous to wildtype cells (Fig. 2.16). However, this concentration was lethal to  $\Delta tsa1\Delta tsa2$  cells and resulted in loss of oxygen consumption and metabolic cycles, probably due to the strongly diminished antioxidant capacity of these cells (Fig. 2.17A). Nonetheless, with 0.5 mM *t*-BOOH, HOC could still be induced similar to wildtype and  $\Delta prx1$  cells (Fig. 2.17B).



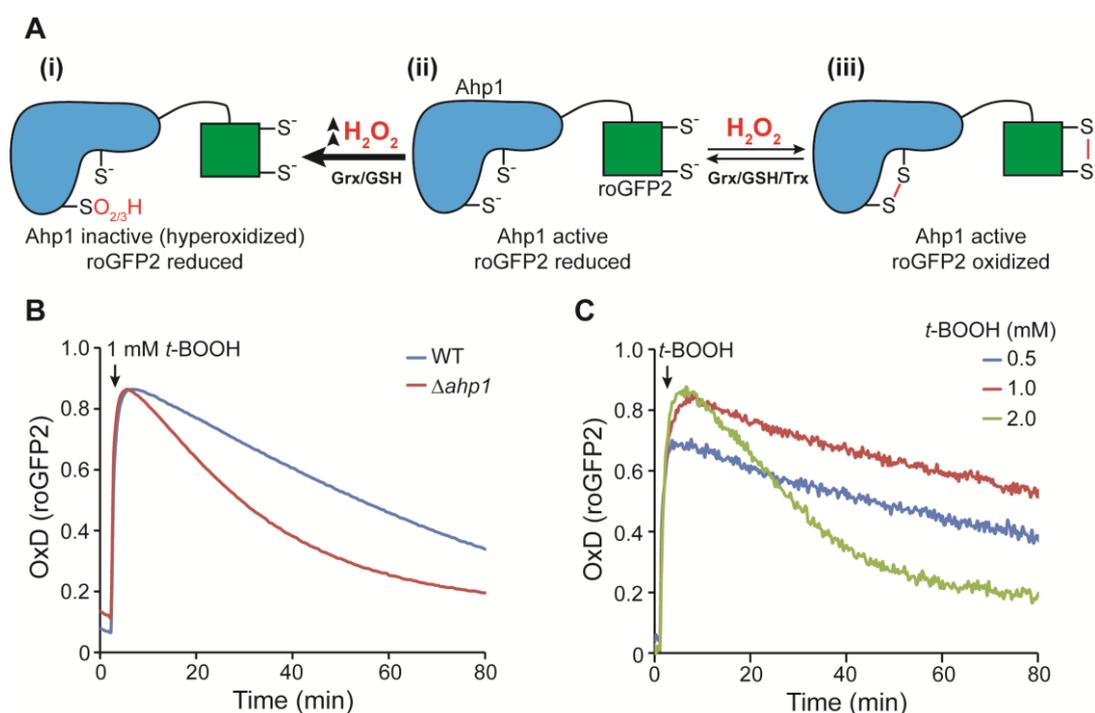
**Figure 2.16: High peroxide induces switch to HOC in *PRX1*-deleted cells.** Representative oxygen traces showing YMC response of  $\Delta prx1$  cells to 1 mM *t*-BOOH. Thick black arrow indicates sequence of YMC phase of *t*-BOOH addition as depicted in Fig. 2.10A.



**Figure 2.17: High peroxide induces switch to HOC in  $\Delta tsa1\Delta tsa2$  cells.** Representative oxygen traces showing YMC response of  $\Delta tsa1\Delta tsa2$  cells to **(A)** 1 mM and **(B)** 0.5 mM *t*-BOOH. Thick black arrow indicates sequence of YMC phase of *t*-BOOH addition as depicted in Fig 2.10A.

It was instructive to note that YMC-synchronized cultures of  $\Delta tsa1\Delta tsa2$  cells could exhibit LOC-to-HOC switching upon high amounts of *t*-BOOH, suggesting that peroxiredoxin hyperoxidation might have still occurred. This reasoning is not far-fetched since thiol peroxidases exhibit functional redundancy in their response to peroxides [129]. Given that  $\Delta ahp1$  cells displayed YMC with shape and period similar to  $\Delta tsa1\Delta tsa2$  cells, I asked whether endogenous Ahp1 might have been hyperoxidized and inactivated in wildtype as well as  $\Delta tsa1\Delta tsa2$  cells to mediate peroxide-induced switch to HOC during the YMC. However, there was no easy and direct way to answer this question since there was no known antibody for detection of hyperoxidized Ahp1 levels by Western blot. Meanwhile, we had developed a sensor similar to roGFP2-Tsa2 $\Delta$ C<sub>R</sub>, by fusing Ahp1 to the C-terminus of roGFP2 [90]. I therefore tested whether it was

possible to hyperoxidize the Ahp1 moiety of this biosensor in the BY4741 or BY4742 yeast backgrounds, using the plate reader assay. The principle behind this assay is that if Ahp1 becomes hyperoxidized upon peroxide, roGFP2 would be reduced. On the other hand, roGFP2 would stay oxidized in the absence of Ahp1 hyperoxidation upon peroxide (Fig. 2.18A). Interestingly in the BY4741 background, the roGFP2-Ahp1 sensor was rapidly reduced in a  $\Delta ahp1$  compared to a wildtype strain, upon 1 mM *t*-BOOH (Fig. 2.18B). In a BY4742  $\Delta tsa1\Delta tsa2$  strain, the sensor could increasingly be oxidized with 0.5 and 1 mM *t*-BOOH. However, it was briefly oxidized and quickly reduced with 2 mM *t*-BOOH, an indication of rapid roGFP2 reduction upon Ahp1 hyperoxidation and inactivation (Fig. 2.18C). In a separate experiment, hyperoxidation of the roGFP2-Tsa $\Delta C_R$  sensor could be achieved with 0.75 mM *t*-BOOH in the wildtype background of these strains (data not shown). Summarily, these observations suggest that during the YMC, Tsa1 and Tsa2 are preferentially inactivated upon high peroxide, after which Ahp1 could take over such role. Interestingly, contributions of Ahp1 toward YMC regulation had not been considered in previous studies.



**Figure 2.18: RoGFP2 fluorescence measurements reveal possible Ahp1 hyperoxidation upon high peroxide.** (A) Scheme showing mechanism of an Ahp1 coupled roGFP2 sensor. Ahp1 acts as the transducer of oxidizing equivalents from peroxide to roGFP2. (ii) All cysteine residues of the roGFP2-Ahp1 sensor remain in a reduced thiolate state in the absence of peroxide. (iii) Upon encountering peroxide, oxidizing equivalents are transferred via the C<sub>P</sub> of Ahp1, resulting ultimately in the formation of an intramolecular Ahp1 and roGFP2 disulfide bonds. (i) In the presence of excess peroxide, the C<sub>P</sub> of Ahp1 becomes hyperoxidized and inactivated, rendering the transfer of oxidizing equivalents non-functional. Thus, Grxs return the cysteine residues of roGFP2 to a more reduced thiolate state using GSH. The presence of the C<sub>R</sub> of Ahp1 in this sensor, unlike in the roGFP2-Tsa2 $\Delta C_R$  sensor, leads to competition between roGFP2 oxidation and intramolecular Ahp1 disulfide bond reduction by thioredoxins. (B) Degree of sensor oxidation in a BY4741 wildtype and  $\Delta ahp1$  backgrounds upon 1 mM *t*-BOOH. (C) Degree of sensor oxidation in a BY4742  $\Delta tsa1\Delta tsa2$  background upon 0.5, 1 and 2 mM *t*-BOOH. Data represent mean of at least three independent experiments.

### 2.5.3 Combined loss of *TSA1* and *AHP1* in prototrophic yeast leads to non-viability

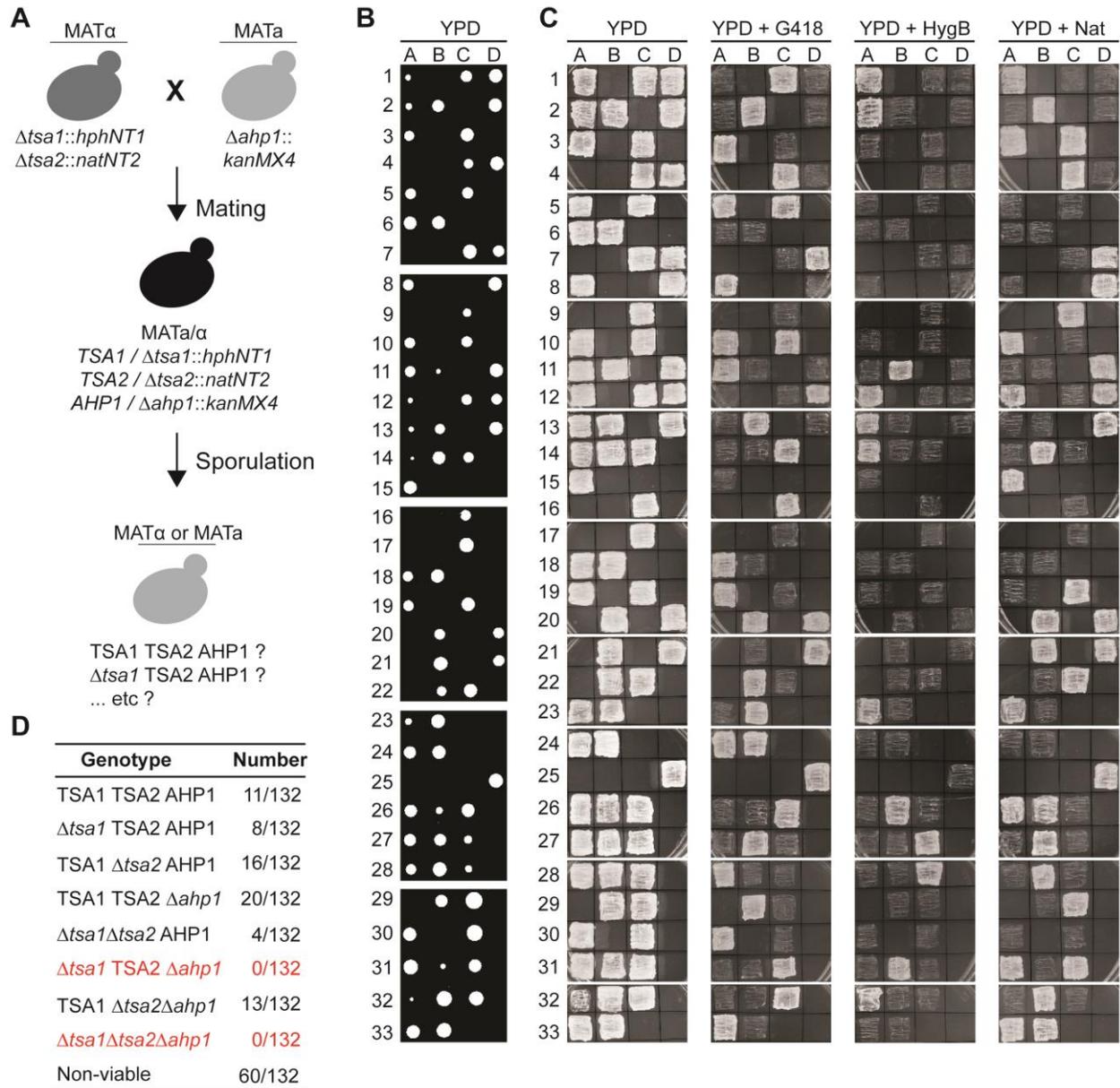
Previous works have demonstrated that yeast thiol peroxidases are functionally redundant; hence, it was possible to delete all eight thiol peroxidases in the BY4742 background and still have a viable yeast strain [129]. I therefore sought to ascertain the consequence on the YMC of deleting all three cytosolic 2-Cys peroxiredoxins together (i.e. *TSA1*, *TSA2* and *AHP1*). To do this, I intended to delete *AHP1* from the  $\Delta tsa1\Delta tsa2$  background by the same homologous recombination-based gene deletion approach. Surprisingly, I was unable to generate either  $\Delta tsa1\Delta ahp1$  or  $\Delta tsa1\Delta tsa2\Delta ahp1$  mutants in the CEN.PK 113-1A background upon several attempts (data not shown). Similarly, I could not obtain a  $\Delta tsa1\Delta ahp1$  strain by deleting *AHP1* in a  $\Delta tsa1$  background using the same approach. Meanwhile, I could obtain the  $\Delta tsa1\Delta ahp1$  and  $\Delta tsa1\Delta tsa2\Delta ahp1$  strains in the BY4742 background by homologous recombination (data not shown).

To ensure that the supposed failure was not due to technical challenges, but perhaps, a biological phenomenon peculiar to the CEN.PK background, I sought to obtain the desired strains via mating, sporulation and tetrad dissection experiments as described in Materials and Methods. I crossed  $\Delta tsa1\Delta tsa2$  from the CEN.PK113-1A (Mat- $\alpha$ ) background with  $\Delta ahp1$  from the CEN.PK113-7D (Mat-a) background (Fig. 2.19A). Interestingly, I could not obtain any viable  $\Delta tsa1\Delta ahp1$  or  $\Delta tsa1\Delta tsa2\Delta ahp1$  spores from any of the 33 tetrads dissected (Fig 2.19B-D). These observations suggest that combined loss of *TSA1* and *AHP1* is lethal in the CEN.PK background, an observation that is in sharp contrast with the common lab yeast strain BY4742.

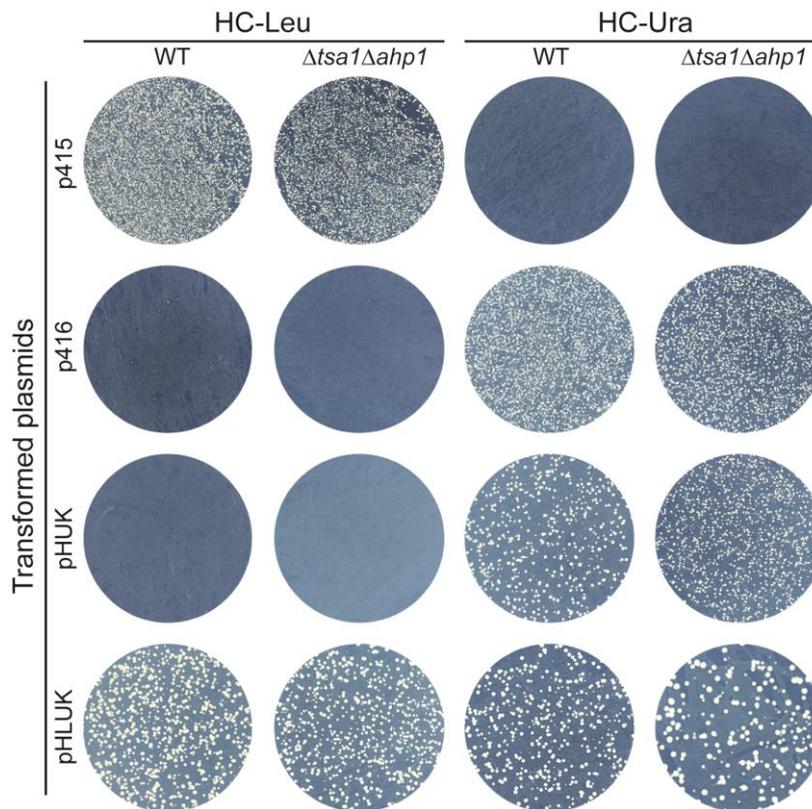
The main difference between the CEN.PK and BY4742 is that the former is a prototrophic strain capable of synthesizing all its amino acids, whilst the latter is an auxotrophic strain that is incapable of synthesizing all of its amino acids and thus must be supplied in the media for growth. I therefore asked whether the difference between the two strains, with respect to the deletion of 2-Cys peroxiredoxins, could be explained by the differences in their auxotrophic markers. To answer this question, I transformed BY4742  $\Delta tsa1\Delta ahp1$  cells, as well as their wildtype counterparts with a pHLUK plasmid to replace all auxotrophic markers in order to assume a 'CEN.PK-like' state. I also independently transformed these cells with pHUK, p415 and p416 plasmids as controls. The pHUK plasmid replaced all auxotrophic markers except leucine, whilst the p415 and p416 plasmids replaced leucine and uracil, respectively. I then grew the transformed cells on Hartwell Complete (HC) agar plates lacking either leucine (L) or uracil (U) for selection. Interestingly, cells transformed with pHLUK were fully viable; suggesting that absence of viable  $\Delta tsa1\Delta ahp1$  or  $\Delta tsa1\Delta tsa2\Delta ahp1$  spores in the CEN.PK background could not be due to their amino acid prototrophy (Fig 2.20).

It is plausible that in the CEN.PK background, *TSA1* and *AHP1* may be essential for spore formation or viability although I do not have direct evidence for this. It is also possible that peroxiredoxins will have a much broader role for the survival of CEN.PK yeast, independent of antioxidant defense, although this is yet to be proven. Furthermore, another difference between CEN.PK and most laboratory

strains, such as BY4742 and W303, is that the latter are known to be unable to establish synchronized metabolic oscillations [47, 56]. Based on the above reasoning, it is tempting to speculate that the differential requirement for peroxiredoxins in typical laboratory strains versus ‘less domesticated’, prototrophic yeast strains, such as CEN.PK is related to the ability to establish synchronized metabolic cycles.



**Figure 2.19: Double deletions of *TSA1* and *AHP1* leads to loss of viability in prototrophic yeast. (A)** Scheme illustrating the mating, sporulation and tetrad dissection procedure. **(B)** Images of tetrad dissection plates for all 33 tetrads dissected. **(C)** Images showing growth of cells from all recovered viable spores on media containing the indicated antibiotics to assess for the presence of the antibiotic resistance cassettes used for gene deletion. **(D)** Table showing the eight possible genotypes and the number of spores recovered with each genotype.

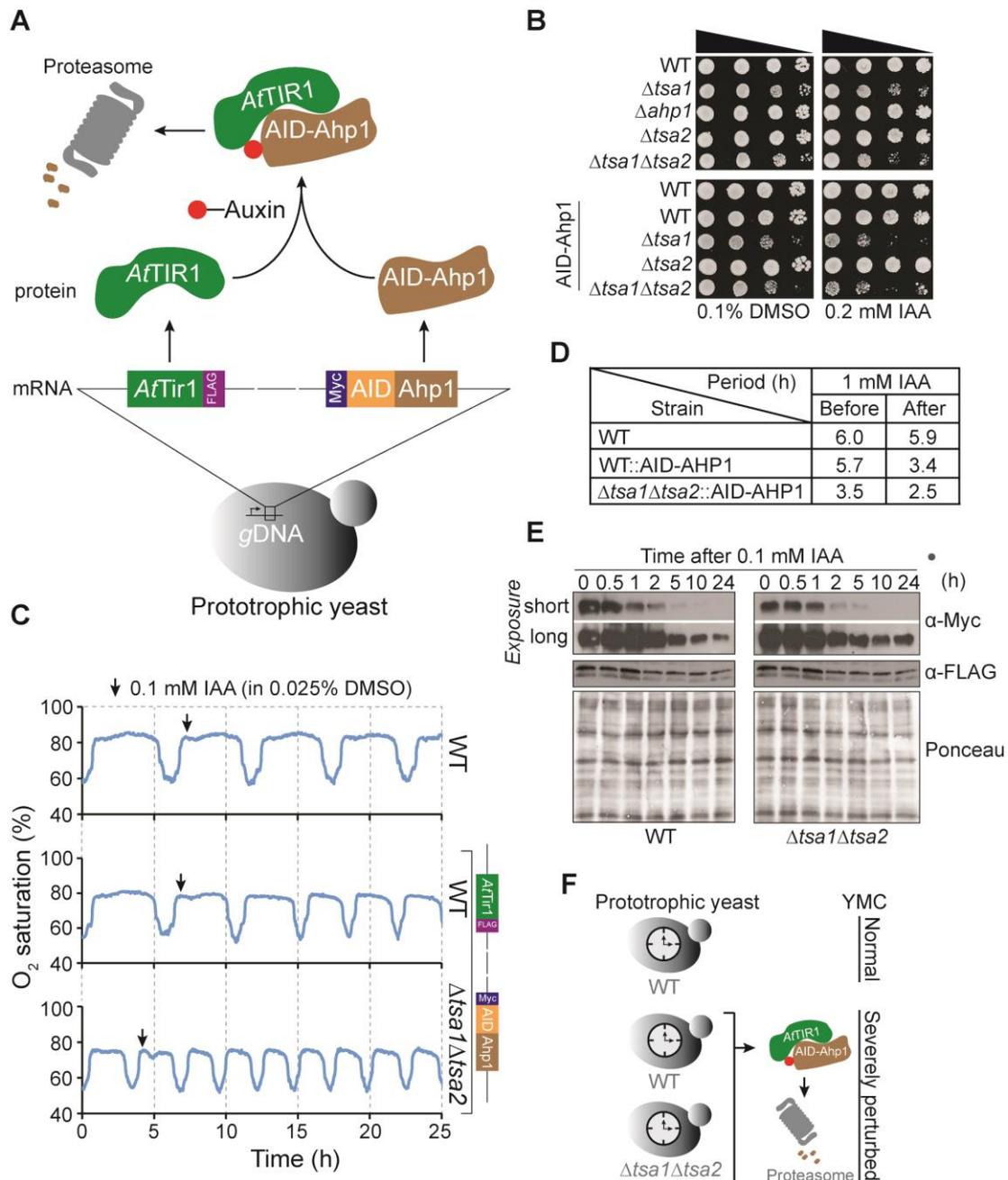


**Figure 2.20: Non-viability of  $\Delta tsa1\Delta ahp1$  cells in a CEN.PK background is not due to prototrophy.** Images of colonies of BY4742 wildtype and  $\Delta tsa1\Delta ahp1$  cells formed on indicated selective plates following transformation with the indicated plasmids. BY4742 cells are uracil and leucine auxotrophs. Therefore, cells transformed with p416 and pHUK plasmids, which both harbor URA3 as a selective marker, would not be expected to grow on media lacking leucine. Likewise, cells transformed with p415 (LEU2 as selective marker) should not grow on plates lacking uracil. These conditions therefore serve as controls. Cells transformed with a pHLUK plasmid to replace all four auxotrophic markers grow well on media lacking either leucine or uracil. Key: H – histidine, L – leucine, U – uracil, K – lysine.

## 2.5.4 An auxin-inducible degron system reveals toxicity of Ahp1 degradation in a *Atsa1* background

Since I could obtain neither a viable *Atsa1Δahp1* nor *Atsa1Atsa2Δahp1* strain by both homologous recombination and tetrad dissection experiments, I genomically fused *AHP1* with an auxin regulable degron (AID) in a *Atsa1Atsa2* background. This approach was used in order to first, verify the viability phenotype due to the combined loss of *TSA1* and *AHP1* in the CEN.PK background, and second, to assess the effect on the YMC of the combined loss of *TSA1* and Ahp1. This technique allows for rapid degradation of AID-fusion proteins upon supplementation with the auxin hormone indole-3-acetic acid (IAA) [133, 134]. The *Arabidopsis thaliana* F-box protein, *AtTIR1*, which is coupled to the AID construct functions as part of the ubiquitin ligase system to ubiquitinate and deliver AID-fusion proteins to the proteasome for degradation (Fig. 2.21A). I obtained the AID/*AtTIR1* construct on a plasmid from the lab of Prof. Blanche Schwappach (Göttingen, Germany). I amplified this construct by PCR and transformed it into yeast for genomic integration and fusion to the N-terminus of *AHP1* (Appendix A3). After successful strain construction, I tested the effect of this technique in a drop dilution growth assay, performed on YPD plates supplemented with either 0.2 mM IAA or 0.1% DMSO as vehicle control. Interestingly, *Atsa1* and *Atsa1Atsa2* strains

harboring the Ahp1 degron exhibited decreased viability on plates supplemented with IAA, as compared with their wildtype or *Atsa2* counterparts (Fig. 2.21B). This result demonstrates that depletion of Ahp1 in a CEN.PK *Atsa1* or *Atsa1Atsa2* background is detrimental for growth.



**Figure 2.21: Conditional depletion of Ahp1 is lethal in  $\Delta tsa1$  cells and severely perturbs the YMC. (A)** Scheme illustrating the mechanism of Ahp1 depletion by the AID system. Both AfTIR1 and AID-Ahp1 are expressed from the genome of prototrophic yeast. Auxin binding to AfTIR1 promotes the interaction between AfTIR1 and AID-Ahp1. Subsequently, AfTIR1 acts as an E3 ubiquitin ligase to recruit an E2 ligase resulting in polyubiquitylation of AID-Ahp1 (not shown). Ahp1 is then delivered to the proteasome for degradation. **(B)** Drop dilution assay showing the growth of the indicated yeast strains on YPD plates containing either 0.1% DMSO as a vehicle control or 0.2 mM indole-3-acetic acid (IAA). Growth inhibition was monitored by incubation at 30 °C for 48 h. **(C)** Representative oxygen traces showing the effect of treating YMC-synchronized cultures of wildtype cells or wildtype and  $\Delta tsa1\Delta tsa2$  cells expressing Ahp1 genetically fused to an auxin inducible degron, with 0.1 mM indole-3-acetic acid (auxin). **(D)** Table illustrating effect of IAA on YMC period. **(E)** Western blot of Ahp1 degradation after IAA treatment. Samples were taken at 0, 0.5, 1, 2, 5, 10 and 24 h after treatment to test for Ahp1 degradation by Western blot. **(F)** Schematic explaining effect of IAA on the YMC of the cell types used. WT YMC remains unaffected whilst YMC of AID-Ahp1/AfTIR1 expressing cells are severely perturbed upon IAA, due to gradual Ahp1 degradation. All experiments were repeated at least twice with completely independent YMC-synchronized cultures.

## 2.5.5 Repression of Ahp1 in a $\Delta tsa1\Delta tsa2$ background perturbs the YMC

Further, I tested the effect of auxin on the YMC of wildtype- and  $\Delta tsa1\Delta tsa2$ - AID-*AHP1*/*AtTIR1* expressing cells in comparison with their wildtype non-construct expressing counterpart. I cultured these cells under metabolic cycling conditions and added IAA at a final concentration of 0.1 mM at the start of LOC. The cycles of wildtype and  $\Delta tsa1\Delta tsa2$  cells expressing the AID construct had periods remarkably shortened from 5.7 to 3.4 hours and 3.5 to 2.5 hours respectively, upon 0.1 mM IAA. The YMC of non-construct expressing wildtype cells was barely affected, maintaining periods of about 6 hours before and after 0.1 mM IAA (Fig. 2.21C-D). The reduction of YMC period in the AID-construct expressing cells was consistent with Ahp1 protein degradation as visualized by Western blot (Fig. 2.21E). The cycles could not be completely abolished as I anticipated. This was explainable by the incomplete removal of Ahp1 upon film visualization at long exposure. In short, these observations taken together demonstrate that cytosolic 2-Cys peroxiredoxins, especially Tsa1 and Ahp1, regulate the YMC and are important for the yeast timekeeping mechanism (Fig. 2.21F).

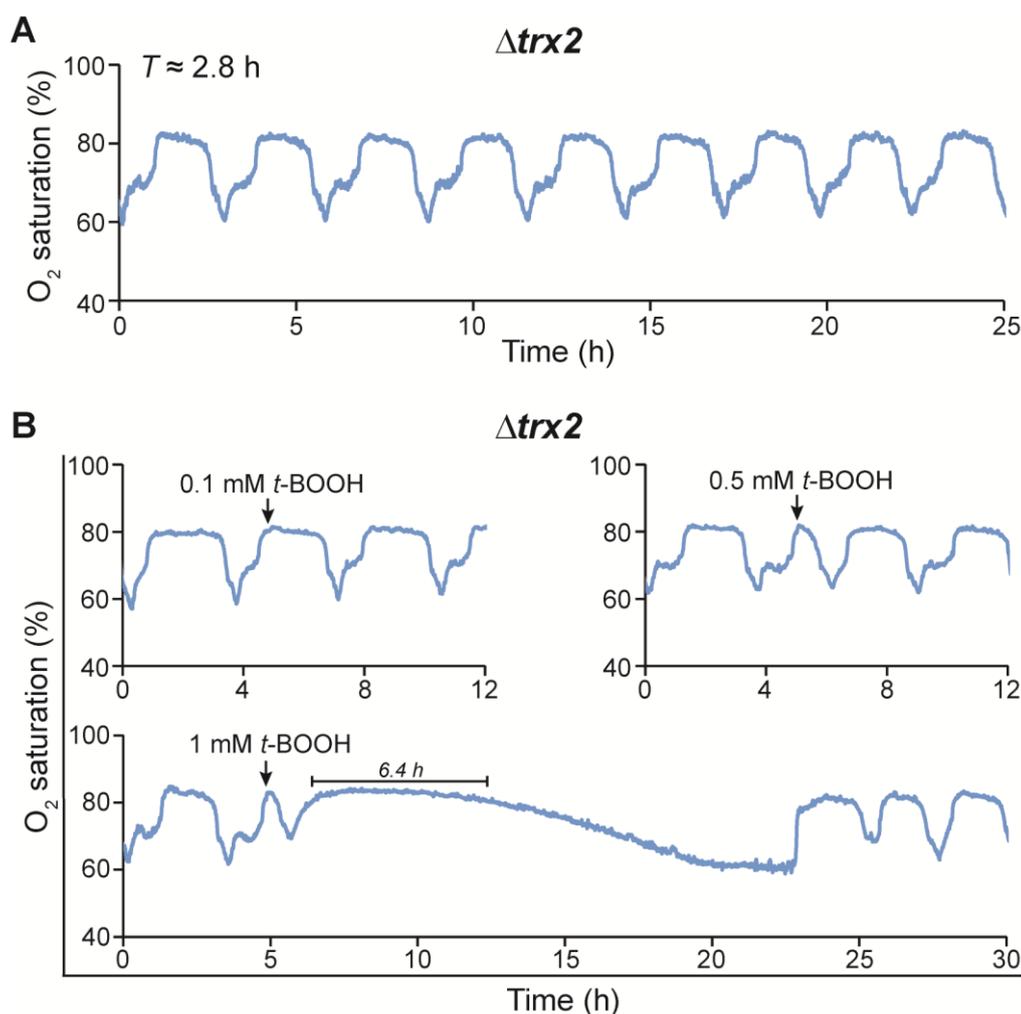
## 2.6 Peroxiredoxin-mediated YMC regulation is thioredoxin dependent

Thioredoxins (Trxs) are evolutionarily conserved proteins that facilitate protein thiol-disulfide reduction on specific target proteins, including peroxiredoxins [135]. Budding yeast harbors three thioredoxins; two in the cytosol, Trx1 and Trx2, and one in the mitochondria, Trx3. Thioredoxins and the NADPH-dependent thioredoxin reductase (TrxR) form a protein reductive system that mediate maintenance of cellular redox homeostasis and repair of oxidatively modified proteins, such as PTPs and STAT3 [69, 73]. Active peroxiredoxin catalysis requires disulfide reduction by thioredoxins. Similarly, active recycling of peroxiredoxins via the Trx/TrxR system is essential for hyperoxidation since the peroxiredoxin disulfide is protected from further oxidation [136].

I imagined two mechanisms by which thioredoxins could be important during the YMC: first, thioredoxins may reduce/repair/recycle target protein(s) oxidatively modified by peroxiredoxins, and second, they might directly reduce oxidized peroxiredoxins to limit oxidation of target protein(s). These plausible mechanisms could be affected in the absence of thioredoxins and possibly perturb the YMC, especially if peroxiredoxin relay(s) that regulate YMC oscillation become modulated. Therefore, I asked whether loss of thioredoxins (especially Trx1 and Trx2) could affect the YMC. I tested the impact of loss of *TRX2* on the YMC *per se*, and in response of the YMC to *t*-BOOH. Remarkably, *TRX2* deletion shortens YMC period similar to combined loss of *TSA1* and *TSA2* or loss of *AHP1* alone (Fig. 2.22A). Moreover, 0.5 mM *t*-BOOH was enough to trigger “hyperoxidation-based” LOC-to-HOC transition (Fig. 2.22B). In

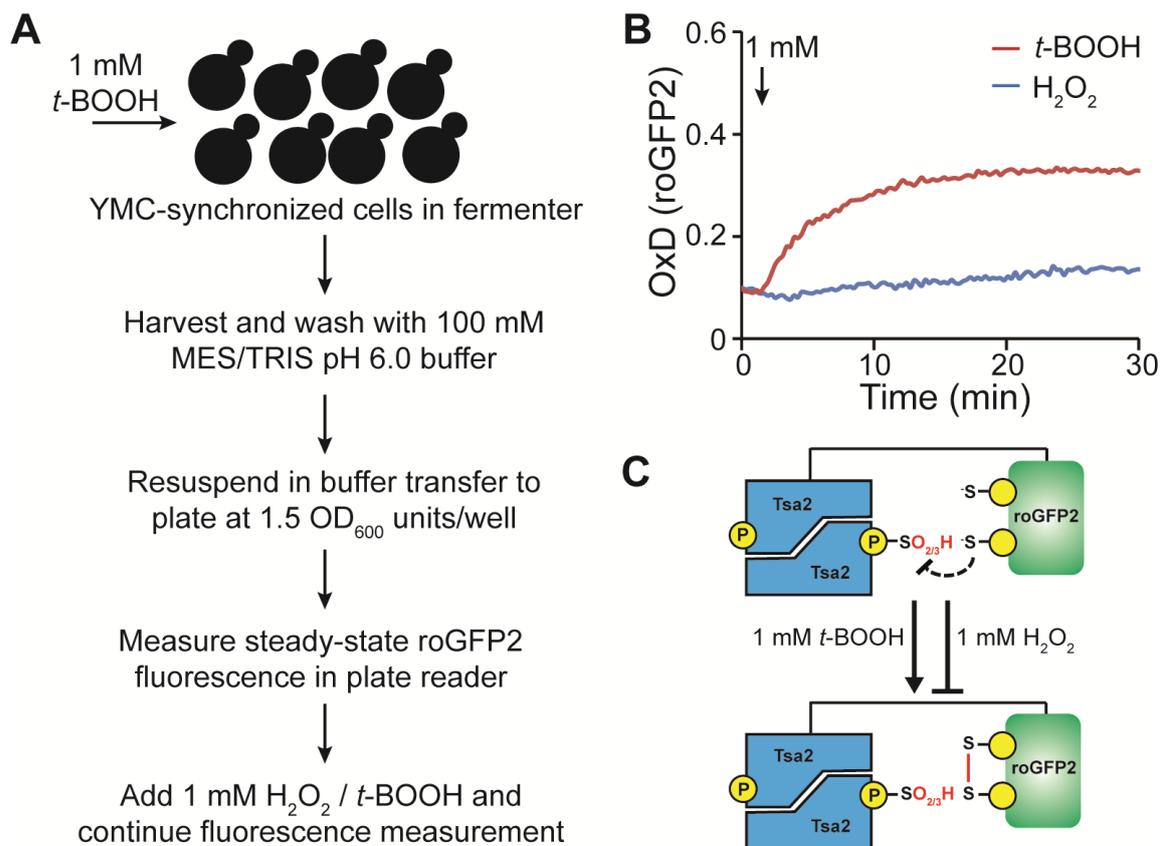
contrast to wildtype and  $\Delta tsa1\Delta tsa2$  cells, 1 mM *t*-BOOH only triggered transient HOC and an immediate return of the YMC to a prolonged LOC phase that lasted more than 6 hours (Fig. 2.22B).

Having earlier observed that an oxidative process (e.g. diamide) was required to keep cells in the LOC phase, I hypothesized that the prolonged LOC in  $\Delta trx2$  cells upon 1 mM *t*-BOOH could be due to direct oxidation of protein thiols by *t*-BOOH. This situation is plausible because  $\Delta trx2$  cells may have a diminished ‘reductive capacity’ occasioned by the loss of *TRX2*, thus, frustrating their ability to facilitate effective removal of *t*-BOOH. Furthermore, unlike  $H_2O_2$  that is largely dependent on peroxiredoxins to effectively oxidize protein thiols [72]; *t*-BOOH on the other hand can directly oxidize protein thiols in the absence of peroxiredoxins. To test the latter claim, I collected samples from the YMC of wildtype cells expressing the genomic roGFP2-Tsa $\Delta 2C_R$  sensor, 30 mins after 1 mM *t*-BOOH-mediated hyperoxidation-based LOC-to-HOC transition. Samples were processed for fluorescence measurements in a plate-reader format, and further treated with either 1 mM  $H_2O_2$  or 1 mM *t*-BOOH (Fig. 2.23A). Interestingly, 1 mM *t*-BOOH could directly oxidize roGFP2 despite Tsa $\Delta 2C_R$  inactivation, whilst 1 mM  $H_2O_2$  could not (Fig. 2.23B-C).

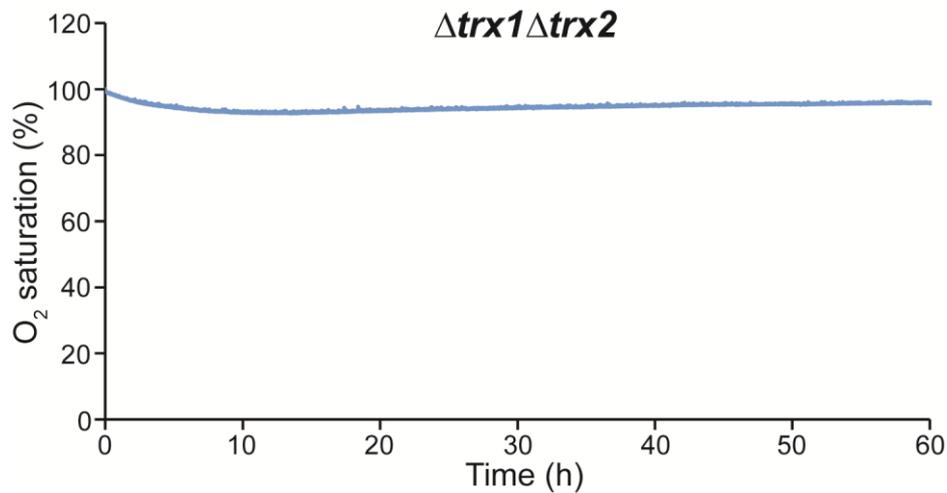


**Figure 2.22: Loss of *TRX2* affects the YMC *per se*, as well as YMC response to *t*-BOOH upon peroxiredoxin hyperoxidation during the YMC. (A) Representative oxygen traces to show the YMC for  $\Delta trx2$  cells. (B) Representative oxygen traces showing the effect of *t*-BOOH on YMC-synchronized cultures of a  $\Delta trx2$  strain at the indicated concentrations.**

To test the consequence of loss of both cytosolic *TRXs* on the YMC, I sought to delete *TRX2* in a  $\Delta trx1$  background or *vice versa*, by the homologous recombination-based gene deletion approach. This attempt was unsuccessful; however, the  $\Delta trx1\Delta trx2$  strain could be obtained by subsequent mating, sporulation and tetrad dissection (Shamala Riemann, data not shown). That notwithstanding, the  $\Delta trx1\Delta trx2$  culture could only grow in a batch phase without the ability to consume the levels of dissolved oxygen in the culture vessel, hence, could not establish synchronized metabolic cycles (Fig. 2.24). Taken together, these observations indicate that thioredoxins are important for YMC regulation. These results further speak in favor of function(s) for the peroxiredoxin and thioredoxin systems in the yeast metabolic clock.



**Figure 2.23: Unlike H<sub>2</sub>O<sub>2</sub>, *t*-BOOH mediates direct protein thiol oxidation upon peroxiredoxin hyperoxidation.** (A) Schematic describing procedure by which sensor-expressing cells treated with *t*-BOOH during the YMC were processed to test for further peroxide response by the plate reader method. (B) Sensor expressing cells respond differently to *t*-BOOH and H<sub>2</sub>O<sub>2</sub> after peroxiredoxin hyperoxidation in the fermenter. (C) Schematic explaining hyperoxidized probe response upon further *t*-BOOH or H<sub>2</sub>O<sub>2</sub>. Note: Unlike H<sub>2</sub>O<sub>2</sub>, *t*-BOOH can bypass the inactivated Tsa2 $\Delta$ C<sub>R</sub> and react directly with roGFP2 to keep it oxidized. Therefore, in the absence or decreased levels of reducing equivalents from cellular thioredoxins, *t*-BOOH mediates hyperoxidation of peroxiredoxins during the YMC to induce switch to HOC, which is only short-lived, and immediately returned to a prolonged LOC phase possibly via direct oxidation of endogenous peroxiredoxin target protein(s).



**Figure 2.24:  $\Delta\text{trx1}\Delta\text{trx2}$  cells do not generate synchronized metabolic cycles.** Representative oxygen trace showing that  $\Delta\text{trx2}\Delta\text{trx2}$  cells only grow in a batch phase and could not effectively consume dissolved oxygen to generate synchronized metabolic cycles.

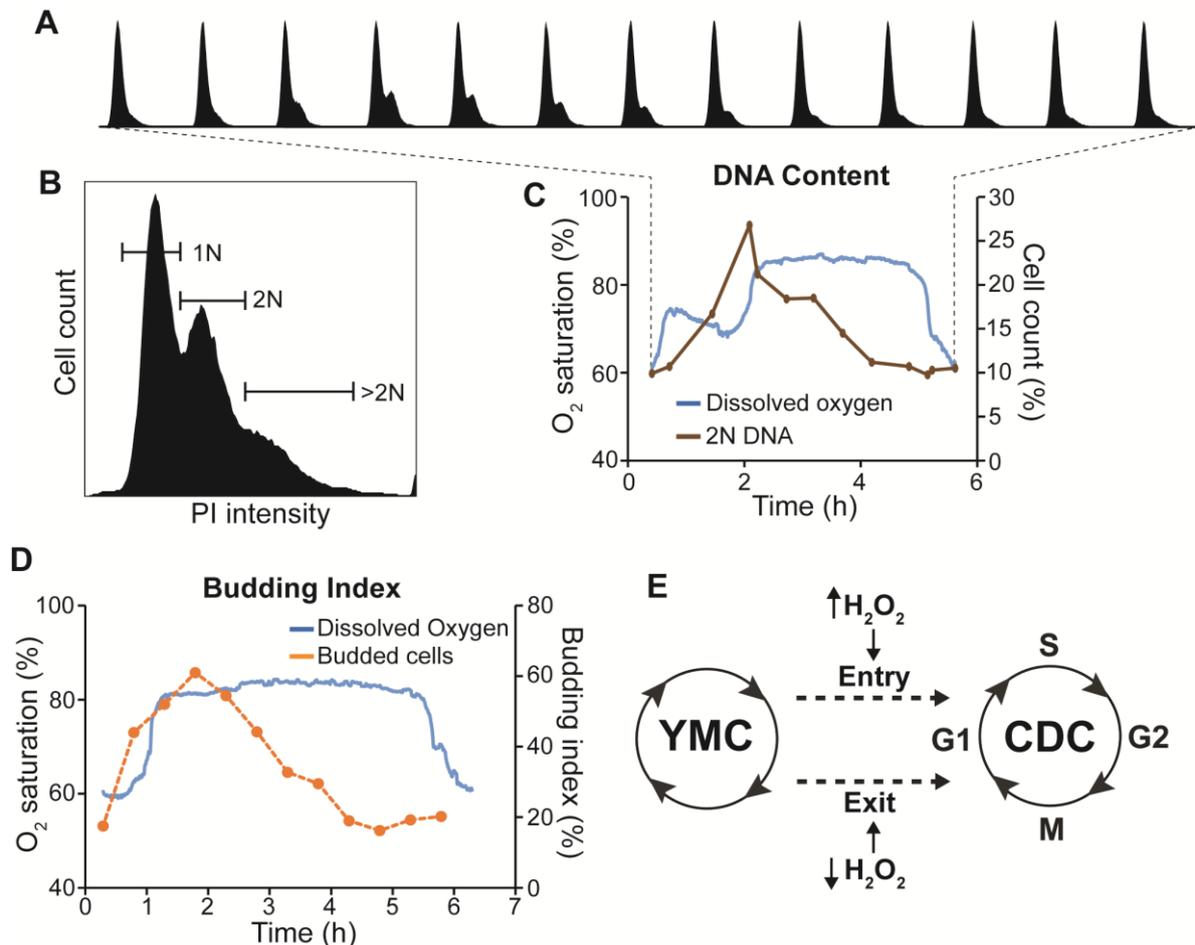
## 2.7 Peroxiredoxins couple metabolic oscillations in yeast to the cell division cycle (CDC)

Circadian and ultradian clocks are coupled to the cell division cycle (CDC) in a variety of species [50, 53-55]. In cyanobacteria, algae, fungi and mammals, the circadian clock gates the CDC such that cell cycle events are arrested at checkpoints during prohibitive circadian phases [53, 55, 137-139]. It has also been suggested that DNA replication and cell division are synchronized to, and temporally regulated by the YMC [49]. In other words, metabolism gates the decision to undergo division, as such; cells lacking essential metabolites will not bypass the ‘committed step’ to cell division [111, 140-142].

However, the mechanistic details on how metabolic changes may temporally be coordinated with the CDC remains unresolved. Moreover, some evidence point to the existence of a regulatory redox cycle within the cell cycle [143, 144]. This proposition is strengthened by the presence of redox-sensitive motifs in a variety of cell cycle regulatory proteins, suggesting that periodic oscillations in intracellular redox state could play a significant role in regulating cell cycle progression [119, 123]. However, it is not completely clear whether peroxiredoxins are involved in cell cycle regulation. I hypothesized that oscillations in the intracellular redox state (e.g.  $\text{H}_2\text{O}_2$  levels) during the YMC could represent a fundamental mechanism linking cell metabolism to cell cycle regulation. If so, this mechanism might rely on peroxiredoxins as transducers of  $\text{H}_2\text{O}_2$ .

## 2.7.1 Oscillatory metabolism is coordinated with cell division

To explore the relationship between the YMC and the CDC, I monitored the progression of DNA replication throughout the YMC, by performing propidium iodide (PI) staining of ethanol-fixed cells collected from YMC-synchronized cultures. I then subjected these samples to flow cytometry analysis. By sampling cells at specific phases of the YMC of wildtype cells, I observed that nuclear DNA replication was initiated during HOC, with increasing proportion of cells acquiring twice (2N) their DNA content before the LOC phase. The proportion of cells with 2N DNA content then decreased gradually towards the end of LOC and into the next HOC (Fig. 2.25A-C). This increase and decrease in DNA replication during the YMC correlated precisely with the increase and decrease in cellular H<sub>2</sub>O<sub>2</sub> levels as well as roGFP2 oxidation demonstrated earlier (see Fig. 2.7B,D), suggesting a communication between the redox and cell division cycles. Thus, it appears that increasing cellular H<sub>2</sub>O<sub>2</sub> levels may be required for DNA synthesis and cell cycle entry, whilst decreasing H<sub>2</sub>O<sub>2</sub> levels permit cell division and cell cycle exit. This reasoning aligns with the suggestion by Chen *et al.* that H<sub>2</sub>O<sub>2</sub> might regulate cell cycle entry [49].



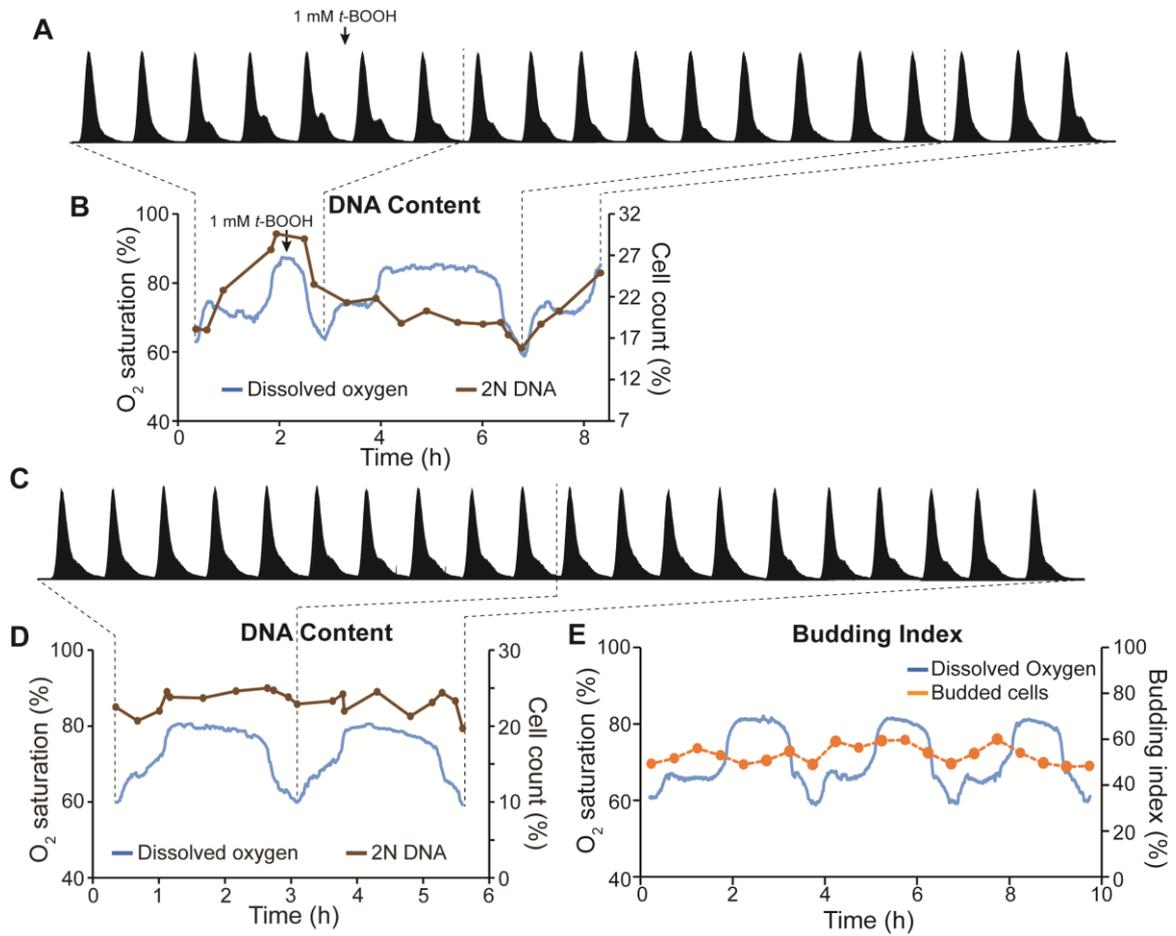
**Figure 2.25: Coupling of YMC and CDC during the YMC.** (A) Representative flow cytometry histograms showing DNA content in samples harvested from a culture of YMC-synchronized wildtype cells. (B) Illustration showing how flow cytometry histograms were assessed for DNA content. (C) Change in DNA content during the YMC for wildtype cells, determined based on the histograms in (A). (D) Representative graph showing the budding index determined in samples of wildtype yeast cells collected at the indicated time points from YMC-synchronized cultures. (E) Scheme summarizing mode of coupling between the YMC and CDC.

To verify this supposed YMC-CDC coordination, I collected samples from an independent YMC-synchronized culture of wildtype cells, fixed them in 70% ethanol and determined cell budding index by microscopy, in collaboration with Dr. Galal Metwally (Molecular Genetics, TU Kaiserslautern). Interestingly, I observed that the proportion of budding cells increased and decreased in correlation with the proportion of cells acquiring 2N DNA content (Fig. 2.25D). I therefore opined that redox/H<sub>2</sub>O<sub>2</sub> changes during the YMC might in part be coupled to modulate target-protein activity to regulate the cell division cycle (Fig. 2.25E). In other words, under physiological conditions DNA replication might not necessarily be restricted to the non-respiratory phase of the YMC in order to prevent oxidative DNA damage as previously suggested [47, 49], rather, increasing H<sub>2</sub>O<sub>2</sub> levels may be necessary to trigger DNA synthesis and entry into the CDC. Alternatively, redox/H<sub>2</sub>O<sub>2</sub> changes might control the metabolic state of cells, which in turn gates the CDC.

### **2.7.2 Temporary inactivation or loss of peroxiredoxins leads to decoupling of CDC from YMC**

Next, I sought to ascertain whether peroxiredoxins might be essential to the synchrony between the YMC and CDC. To do this, I collected samples from YMC-synchronized cultures of wildtype cells before and after treatment with 1 mM *t*-BOOH at the start of LOC. I then analyzed the DNA content by flow cytometry as described earlier. I observed once again that the proportion of cells with 2N DNA content peaked before entry to LOC; however, upon 1 mM *t*-BOOH and peroxiredoxin hyperoxidation, the proportion of cells with 2N DNA content began to decrease and correlated with switch to HOC. The proportion of cells with 2N DNA content remained low on the next immediate cycle, suggesting a temporary loss of coupling between the YMC and CDC. Synchrony between the YMC and CDC returned upon the second next cycle, presumably when active peroxiredoxin catalysis was restored (Fig. 2.26A,B).

To ascertain what the combined loss of *TSA1* and *TSA2* could mean for YMC-CDC synchrony, I further collected samples from YMC-synchronized cultures of  $\Delta tsa1\Delta tsa2$  cells and analyzed their DNA content by flow cytometry. Interestingly, nuclear DNA replication stayed constant over the two metabolic cycles sampled, suggesting a decoupling of CDC from the YMC (Fig. 2.26C,D). Subsequently, I collected samples from an independent YMC-synchronized culture and fixed them in 70% ethanol for budding index determination. Here, I also observed equal number of budding cells at every stage of the YMC from which samples were taken (Fig. 2.26E). Taken together, these data suggest that peroxiredoxins may be crucial in coupling metabolic/redox changes during the YMC to regulate DNA replication and cell division



**Figure 2.26: Coupling of YMC to CDC is mediated by peroxiredoxins.** (A) Representative flow cytometry histograms showing DNA content in samples harvested from a culture of YMC-synchronized wildtype cells before and after 1 mM  $t$ -BOOH. (B) Change in DNA content during the YMC for wildtype cells treated with 1 mM  $t$ -BOOH, determined based on the histograms in (A). (C) Representative flow cytometry histograms showing DNA content in samples collected from a culture of YMC-synchronized  $\Delta tsa1\Delta tsa2$  cells at the indicated time points. (D) Change in DNA content during the YMC for  $\Delta tsa1\Delta tsa2$  cells, as determined based on the data in (C). (E) Graph showing the budding index determined in samples of  $\Delta tsa1\Delta tsa2$  yeast cells collected at the indicated time points from YMC-synchronized cultures. All experiments were repeated at least twice with completely independent YMC-synchronized cultures.

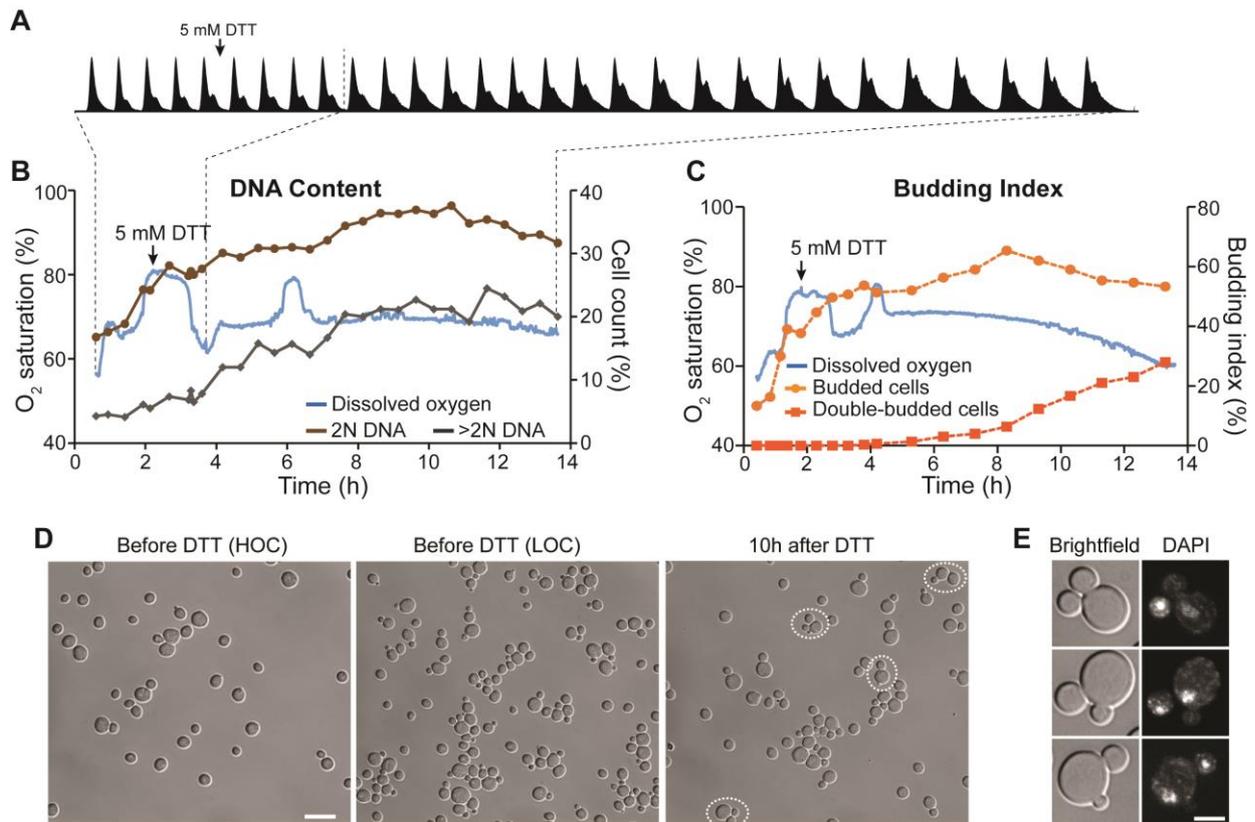
## **2.8 Chemical redox perturbations of the YMC modulate entry into and exit from the cell division cycle**

Given that, I could predictably manipulate switching between LOC and HOC of the YMC by applying thiol redox compounds such as diamide and DTT: i.e. diamide delays switch to HOC, whilst DTT promotes switch to HOC, I asked what the consequence would be of these molecules, on the coordination of the YMC to the CDC.

### **2.8.1 Induction of HOC upon thiol disulfide reduction promotes cell cycle entry**

To address this question, I first treated YMC-synchronized cultures of wildtype cells with 5 mM DTT at the start of LOC to induce switch to HOC. Subsequently, I collected cell samples at defined time points before and after, and analyzed their DNA content by flow cytometry (Fig. 2.27A,B). Strikingly, upon DTT addition to induce LOC-to-HOC transition and a prolonged HOC phase for more than 10 hours, I observed a consistent increase in the proportion of cells with 2N DNA content. Interestingly, nearly 4 hours after DTT treatment, I also observed the presence of cells that appear to contain more than 2N DNA content (Fig. 2.27A,B).

To confirm the above observation, I setup an independent YMC-synchronized wildtype culture, collected samples in similar fashion as before and fixed them in 70% ethanol for microscopic analysis and budding index determination. Upon determination of budding index, I observed a consistent increase in the proportion of cells with one bud after DTT treatment. Surprisingly, I also saw cells with more than one bud appearing nearly 4 hours after DTT addition (Fig. 2.27C-D). Upon DAPI staining of nuclear DNA and microscopic analysis, I could show that cells with more than one bud possessed more than 2N DNA content (Fig. 2.27E). Importantly, in budding yeast the occurrence of a new bud is timed with initiation of DNA replication [145]. Thus, the above observations suggest that artificially inducing YMC-synchronized cells to remain in a prolonged HOC phase compels them to trigger DNA synthesis and start a new cell cycle irrespective of whether the previous cell division had been fully accomplished. This therefore results in the accumulation of cells with increasing DNA content and more than one bud. Consequently, switching to HOC appears to be a prerequisite for initiation of cell division.



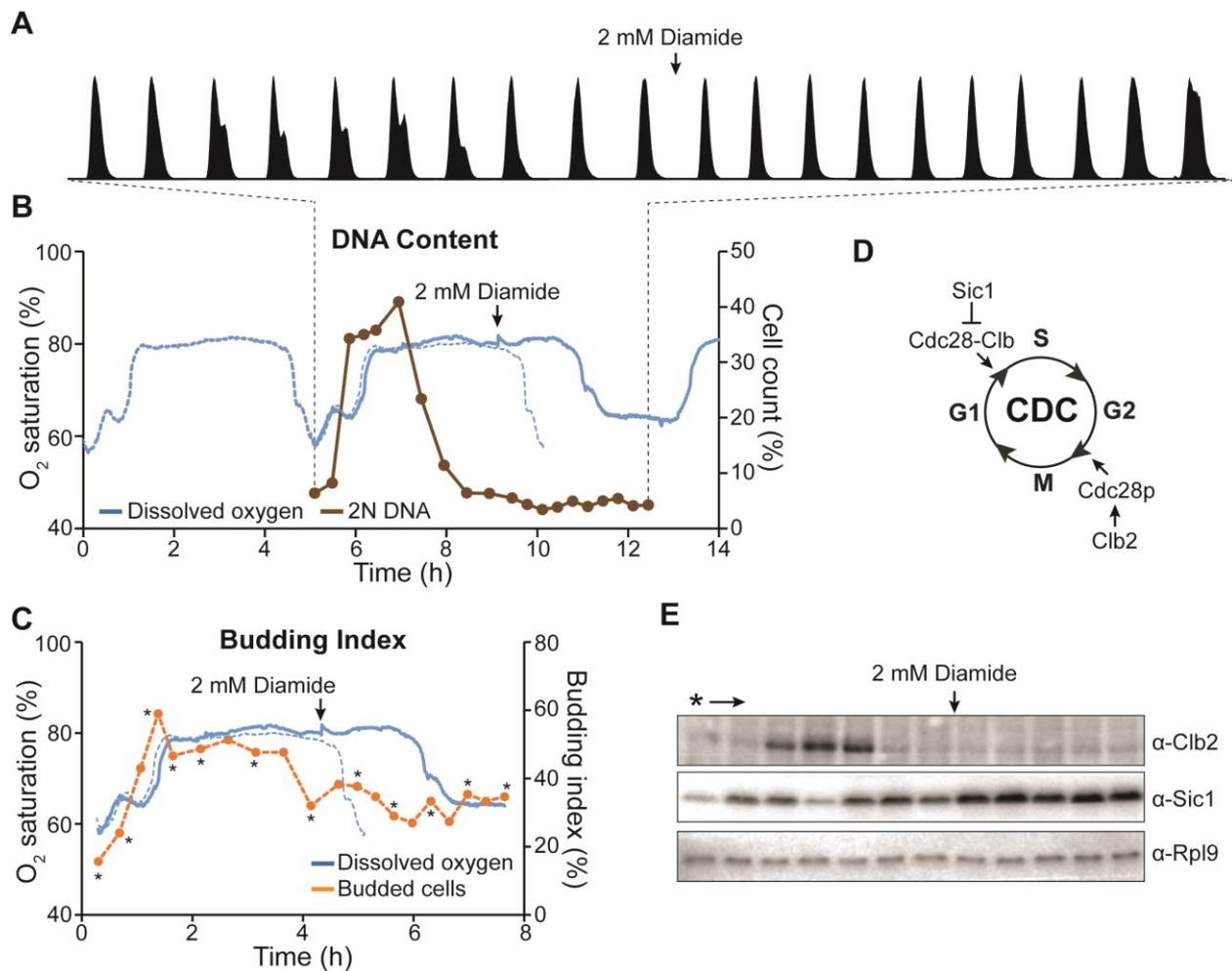
**Figure 2.27: Effect of thiol reduction on DNA replication and cell budding.** (A) Representative flow cytometry histograms showing change in cellular DNA content before and after addition of 5 mM DTT to YMC-synchronized cultures of wildtype cells. (B) Graph showing the impact of 5 mM DTT treatment on the YMC as assessed by monitoring of oxygen consumption as well as the impact on cell cycle based upon cellular DNA content determined from the histograms in (A). (C) Graph showing the change in budding index of YMC-synchronized wildtype cells before and after the addition of 5 mM DTT. (D) Representative microscopy images of yeast cells before and ~10 h after 5 mM DTT addition to YMC-synchronized wildtype cells. (E) Representative microscopy images of DAPI stained cells with 2 buds, isolated from YMC-synchronized cultures ~10 h after addition of 5 mM DTT. Scale bar is 2  $\mu$ m.

## 2.8.2 Prolongation of LOC upon thiol disulfide oxidation delays cell cycle entry

Similarly, I treated YMC-synchronized wildtype cells with 2 mM diamide towards the end of LOC and collected samples at defined time points for DNA content analysis by flow cytometry (Fig. 2.28A,B). In contrast to DTT, addition of 2 mM diamide towards the end of LOC of YMC-synchronized wildtype cells delayed LOC-to-HOC transition as well as start of DNA replication by nearly 2 hours (Fig. 2.28A,B). I then subjected these samples to microscopic analysis to determine their budding index. Strikingly, the proportion of budding cells stayed low during the period of LOC extension and into the next HOC (Fig. 2.28C).

During cell division, proteins that control the activity of cyclin-dependent kinases (Cdks) regulate cell cycle entry and exit. For example, Sic1 is a Cdk inhibitor that blocks initiation of S-phase and must be degraded at the G<sub>1</sub>-S transition to facilitate cell cycle entry [146, 147]. Likewise, Clb2 is a B-type cyclin that accumulates during G<sub>2</sub> and M phases of the cell cycle and repressed by the end of mitosis; it activates Cdc28p to promote the transition from G<sub>2</sub> to M phase [148, 149] (Fig. 2.28D).

To ascertain what was happening to these regulatory protein levels during the YMC and upon diamide treatment, I performed Western blot analysis against Sic1 and Clb2 in collaboration with Galal, using the above cells. I observed that upon diamide treatment, Sic1 levels increased whilst Clb2 levels that were diminished shortly before treatment did not reappear over the duration of the experiment (Fig. 2.28E). Taken together, these observations suggest that switching to LOC seems to be a pre-requisite for completion of cell division, as such; a prolonged LOC phase upon diamide treatment appears to trap cells that have exited mitosis in the G<sub>1</sub> phase, unable to initiate a new cell cycle.



**Figure 2.28: Effect of thiol oxidation on DNA replication and cell budding.** (A) Representative flow cytometry histograms showing change in cellular DNA content before and after addition of 2 mM diamide to YMC-synchronized cultures of wildtype cells. (B) Graph showing the impact of 2 mM diamide treatment on the YMC as assessed by monitoring of oxygen consumption, as well as the impact on cell cycle based upon cellular DNA content determined from the histograms in (A). (C) Graph showing the change in budding index of YMC-synchronized wildtype cells before and after the addition of 2 mM diamide. Dotted blue lines in (B) and (C) represent an overlay of the YMC cycle immediately prior to the one indicated with the continuous blue line. (D) Scheme showing some proteins involved in regulating cell cycle progression. (E) Western blot using anti-Sic1 and anti-Clb2 antibodies. Rpl9 was used as a loading control. Samples were collected as indicated by (\*) in (C).

## 3 DISCUSSION

The aim of this study was to investigate whether peroxiredoxins are active players in the cellular timekeeping mechanism, using the yeast metabolic clock as a model. I utilized the reconstituted peroxiredoxin redox relay, roGFP2-Tsa2 $\Delta$ C<sub>R</sub>, and performed genetic manipulations and biochemical assays to show that yeast 2-Cys peroxiredoxins are crucial for regulating the yeast metabolic clock. This, I propose they do by coupling cyclical changes in H<sub>2</sub>O<sub>2</sub> during the yeast metabolic cycle (YMC) to regulate the redox state of redox-regulated proteins. I have also shown that metabolic oscillations are coupled with the cell division cycle, an important feature of circadian and ultradian metabolic clocks. As such, perturbation of cell metabolism with thiol redox modifiers influences entry into and exit from the cell division cycle. More importantly, I demonstrate that 2-Cys peroxiredoxins are essential for coordinating metabolic changes to the cell division cycle. In this section, I discuss the above findings and summarize this novel role for peroxiredoxins in the cellular clockwork.

### 3.1 The YMC is a redox clock that is regulated by thiol switch(es)

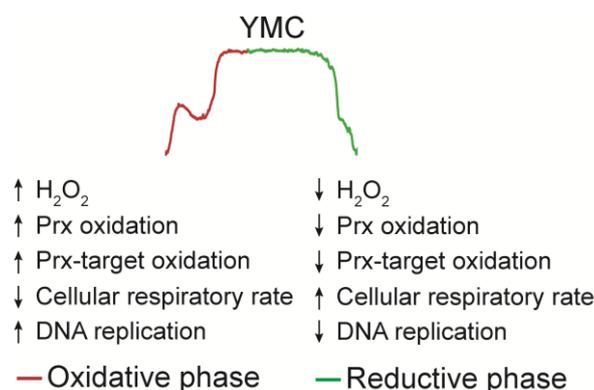
I have engineered prototrophic yeast to genomically harbor a genetically encoded biosensor that allows real-time, dynamic monitoring of the flux of oxidation through a peroxiredoxin redox relay during the YMC. My observations principally reveal that: (1) oscillations in basal H<sub>2</sub>O<sub>2</sub> levels accompany oscillatory metabolism, (2) there exist in the yeast metabolic clock, changes in the flux of oxidation through a peroxiredoxin redox relay, (3) these flux changes are large enough to induce changes in the oxidation state of peroxiredoxin target protein(s). The yeast metabolic clock is characterized by two phases dependent upon the rate of oxygen consumption, namely; a high oxygen consumption (HOC) phase in which dissolved oxygen levels decrease and a low oxygen consumption (LOC) phase where the levels of dissolved oxygen are high (Fig 1.7). My observations indicate that H<sub>2</sub>O<sub>2</sub> levels rise during ‘decreasing’ HOC and peak before HOC-to-LOC transition thereby inducing peroxiredoxin and peroxiredoxin target protein(s) oxidation. H<sub>2</sub>O<sub>2</sub> then decreases gradually during LOC to levels that render peroxiredoxin and peroxiredoxin target(s) more reduced to trigger LOC-to-HOC switching. A decrease in HOC is triggered once again by accumulating H<sub>2</sub>O<sub>2</sub> levels and subsequent peroxiredoxin and peroxiredoxin target protein(s) oxidation (Fig. 2.7 and 3.1).

In *N. crassa* and cyanobacteria, the daily expression pattern of clock and clock-controlled genes involved in coordinating photosynthesis is influenced by H<sub>2</sub>O<sub>2</sub> [96-98]. It has also been shown that visible light alters the YMC by inhibiting respiration [150]. In cultured mouse, monkey and humans cells, light stimulates H<sub>2</sub>O<sub>2</sub> production via photoreduction of flavin-containing enzymes such as peroxisomal acyl-coenzyme A (CoA) oxidase [151]. Recent evidence suggests that yeast peroxisomal flavin-containing fatty acyl CoA oxidase, Pox1, converts visible light into H<sub>2</sub>O<sub>2</sub> signal that is sensed by the peroxiredoxin Tsa1 and transduced to thioredoxin, to counteract PKA-dependent Msn2 phosphorylation [152]. Additionally,

Tu *et al.* demonstrated that the levels of *POX1* transcripts oscillate during the YMC [47]. Moreover, zebrafish circadian clock is coupled to light signals via  $H_2O_2$  [95]. Thus, it is tempting to speculate that by oscillating during the YMC,  $H_2O_2$  acts as a signal to entrain the yeast metabolic clock. The oscillations in  $H_2O_2$  levels that underlie the yeast metabolic clock as I have demonstrated, may not only be unique to this clock model, but rather, represent an important feature of all circadian and ultradian metabolic clocks. In support of this reasoning, recent studies by Liu and colleagues suggest that oscillations in  $H_2O_2$  levels occur in single N2a murine neuroblastoma and human U2OS cells in a circadian manner [153]. They also observed diurnal oscillations in  $H_2O_2$  levels in the liver of mice [153]. I envisage that these observations would be replicated in several metabolic clock models in the near future.

Furthermore, I note that oscillations in  $H_2O_2$  levels during the YMC serve a causative role, rather than being just a by-product of cell metabolism. I propose that this causative function may be intrinsic to each yeast cell, but the outcome may be orchestrated on the population level to achieve metabolic synchrony. Although it has been suggested that population synchrony of budding yeast in low-glucose bioreactors could arise from signaling between cells due to secreted metabolites such as ethanol, acetaldehyde and dihydrogen sulfide [58, 59], secreted  $H_2O_2$  molecules do not play a role in such intercellular communications. This reasoning was confirmed by catalase-mediated  $H_2O_2$  scavenging activity in the culture vessel, which had no observable effect on the YMC (Fig 2.8). Like  $H_2O_2$ , oscillations in other redox metabolites such as GSH/GSSG,  $NAD^+/NADH$  and  $NADP^+/NADPH$  accompany the YMC [56, 154, 155]. Moreover, transcript levels of redox proteins and enzymes oscillate during the YMC [47]. These observations, taken together, affirm that the YMC is indeed a redox clock.

Although I could report oscillations in  $H_2O_2$  levels during the YMC using two different approaches, i.e. thiol-based NEM-trapping technique and real-time measurements with a flow cell (Fig 2.7), the amplitude of change was small. Nonetheless, an  $\approx 5\%$  oscillation in basal  $H_2O_2$  levels or physiological oxidations during the YMC, as I have demonstrated herein, is not trivial. Sobotta *et al.* showed that in the PRDX2-STAT3 redox relay in mammalian cells, only a very small subpopulation of STAT3 is oxidatively modified to compromise STAT3 transcriptional activity, in response to cytokines [73]. Therefore, it is conceivable that an  $\approx 5\%$  change in the redox state of yet to be identified peroxiredoxin redox relay(s) may be enough to regulate the YMC.

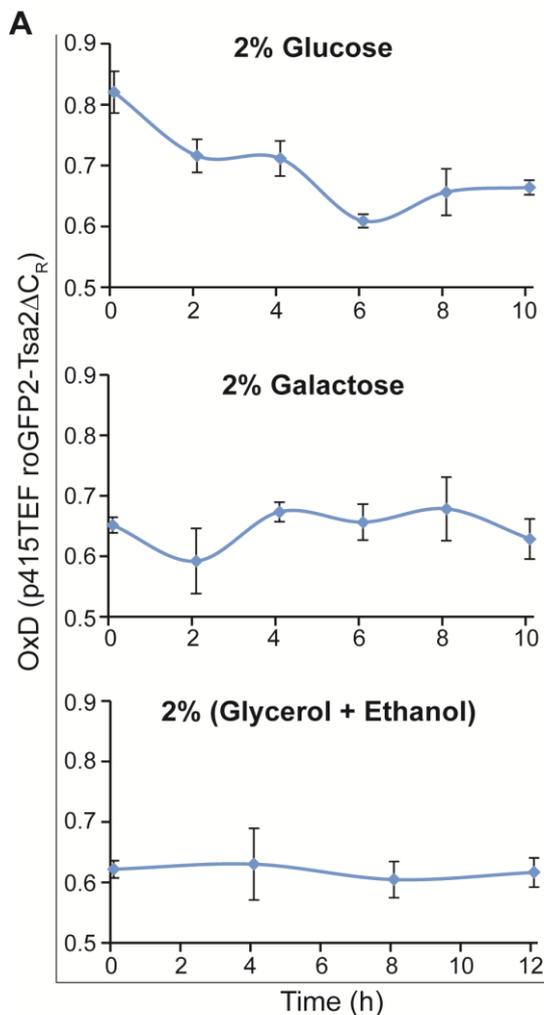


**Figure 3.1: The YMC is a redox clock.** Scheme describing events occurring at each phase of the yeast metabolic cycle as observed in this study.

On the other hand, the observed “small” amplitude of change in physiological oxidations could be explained with respect to cellular physiology and technical/experimental conditions. First, the biosensor used to monitor cellular  $H_2O_2$  was localized to the cell’s cytoplasm. Oscillations in cytoplasm-localized  $H_2O_2$  levels could most likely be counteracted by the high reducing capacity of the cytoplasm. Reducing molecules such as GSH and NADPH are highly abundant within the cytoplasm of cells to mitigate any significant rise in physiological oxidations. Nonetheless, this reductive capacity does not completely eliminate signaling  $H_2O_2$  levels that could be detected and transduced by peroxiredoxins to regulate protein thiols, as observed with the biosensor. Secondly, during real-time biosensor fluorescence measurements, experimental conditions such as the distance or time it takes for cells to travel from the culture vessel into the spectrofluorimeter, as well as air bubbles arising from culture aeration and stirring could frustrate ‘actual’ fluorescence signals. Thirdly, although I tried to immediately ‘trap’ the redox state of the biosensor after collecting cell samples from the fermenter, it is plausible that the NEM-trapping technique might not have been effective as envisaged, to monitor fluctuations in basal  $H_2O_2$  levels on such a huge time-scale. It is also plausible that some thiol alkylation might have been lost during sample processing. Since this is the first study utilizing the flow cell technique to measure oscillations in basal  $H_2O_2$  levels in real-time, it could be improved upon and standardized for future studies. These notwithstanding, the  $\approx 50\%$  oxidation state of roGFP2 as observed with the NEM-trapping technique accurately reflects the steady-state degree of oxidation of the roGFP2-Tsa2 $\Delta C_R$  biosensor under physiological conditions in wildtype cells, as have been previously reported [90]. The real-time flow cell measurements also qualitatively reveal  $\approx 50\%$  roGFP2 oxidation ratio.

An outstanding question that remains to be fully answered is how these  $H_2O_2$  oscillations are generated and sustained. In other words, how is  $H_2O_2$  produced during the YMC? Are the observed oscillations due to  $H_2O_2$  production or consumption? While these questions could be the subject of future investigations, some explanations can be attempted. First, I note that when the sensor redox state of genomically engineered yeast cells grown in glucose batch culture was trapped with NEM and roGFP2 fluorescence measured, the oxidation state of roGFP2 decreased with culture density (Fig. 2.5D). This suggests that once glucose became limiting,  $H_2O_2$  production gradually decreased and roGFP2 consistently became reduced. Secondly, in a comparative measurement of roGFP2 fluorescence in wildtype BY4742 yeast cells transformed with a plasmid expressing the roGFP2-Tsa2 $\Delta C_R$  sensor and grown in media supplemented separately with three different carbon sources (i.e. glucose, galactose and glycerol/ethanol); I could observe only in glucose media, change in roGFP2 oxidation state with respect to culture duration. The sensor was almost 80% oxidized in fresh glucose and gradually reduced to about 60% oxidation over time, probably when glucose was depleted. This nearly 60% oxidation state was what was consistently achieved in galactose and glycerol/ethanol media over the duration of the experiment (Fig 3.2A). Similarly, by growing the plasmid expressing cells in glucose to mid-log phase, harvesting, diluting and further growing them separately in the three media described above, for NEM trapping experiments over a period

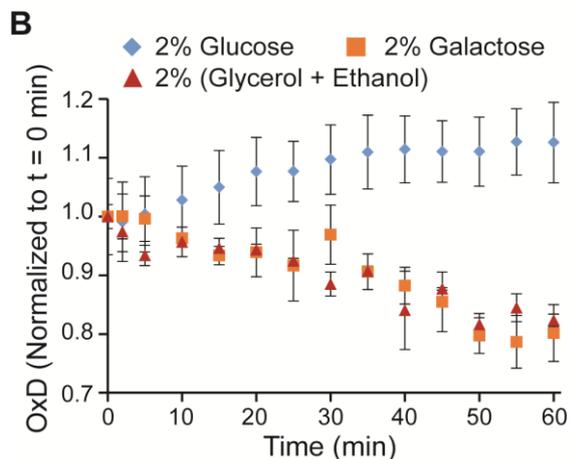
of 1 hour, I observed a consistent increase in the degree of roGFP2 oxidation only in glucose media (Fig.



3.2B). Based on these observations, I concluded that glucose stimulated  $H_2O_2$  production.

Consequently, the controlled but consistent supply of glucose during continuous culture could be responsible for the generation of  $H_2O_2$  that underlie the YMC. This reasoning is also supported by the fact that interference with cellular  $H_2O_2$  generation systems inhibits the generation of, or perturbs the YMC. For instance, deletion of yeast cytosolic Cu-Zn superoxide dismutase 1 (*SOD1*) – an enzyme that catalyzes the breakdown of superoxide radical to molecular oxygen and  $H_2O_2$  – inhibits respiratory growth and YMC generation (Fig. 3.3A). Moreover, deletion of yeast superoxide-generating NADPH oxidase, *YNO1*, strongly perturbs the YMC (Fig. 3.3B).

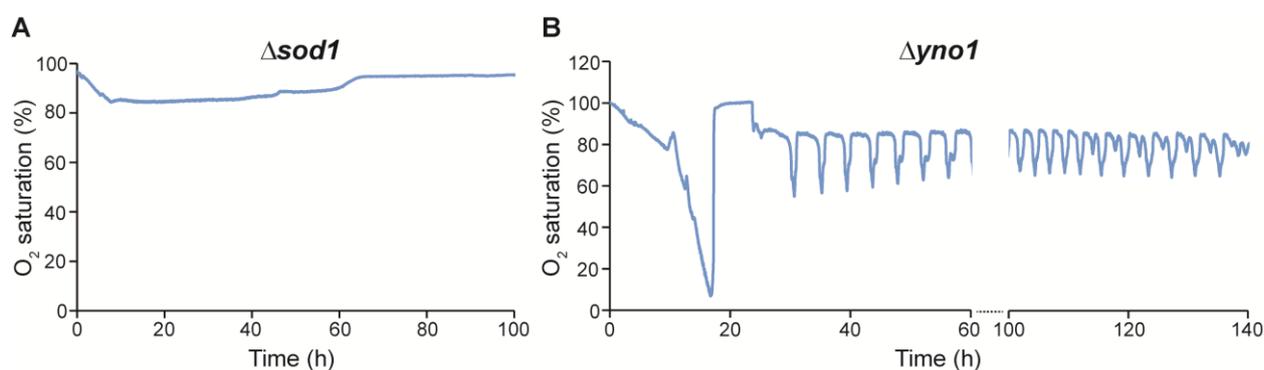
**Figure 3.2: Glucose stimulates  $H_2O_2$  production.** WT BY4742 cells expressing the roGFP2-Tsa2 $\Delta$ C<sub>R</sub> plasmid were (A) cultured in the respective media at OD<sub>600</sub> = 0.25 and trapped with NEM to monitor steady-state roGFP2 oxidation over the stated duration. (B) grown in 2% glucose media to OD<sub>600</sub> = 3.5, diluted in the respective media and trapped in NEM at specific time intervals for 1 h. Data represent at least three independent replicates and error bars represent standard deviation.



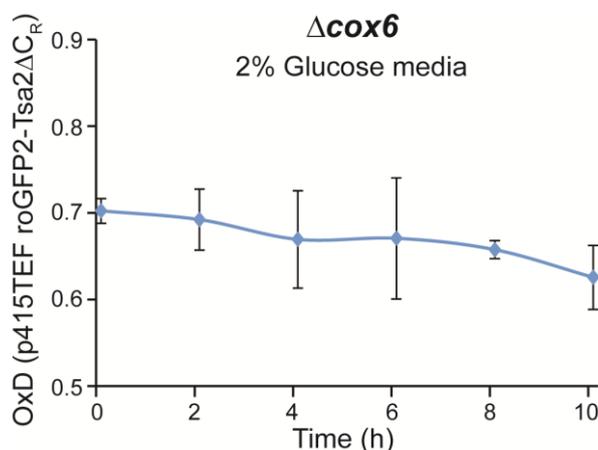
Another important source of  $H_2O_2$  within the cell is the mitochondria. Mitochondrial ROS are mainly generated at complexes I and III of the ETC in the form of superoxide, which is then converted to  $H_2O_2$  by the superoxide dismutase Sod2 [156]. In mammalian cells, the protein product of an mRNA isoform of the mitochondrial *SHC1* gene, p66<sup>Shc</sup>, has been implicated in the generation of  $H_2O_2$  that oxidizes the circadian clock protein, CLOCK [153]. It is believed that p66<sup>Shc</sup> functions

as an oxidoreductase to transfer electrons from cytochrome c to molecular oxygen, leading to the generation of mitochondrial  $H_2O_2$  [157]. Liu and colleagues showed that both p66<sup>Shc</sup> mRNA and protein products are expressed in a circadian manner, and that knockout of p66<sup>Shc</sup> compromises circadian  $H_2O_2$  oscillations in hepatocytes and SCN neurons [153]. The significance of a functional ETC in the generation of  $H_2O_2$  is also underscored by the fact that loss of a subunit of yeast cytochrome c oxidase (complex IV) of the ETC, *COX6*, decreases the starting OxD of roGFP2 and eliminates the  $H_2O_2$  dynamics observed in 2% glucose batch culture of wildtype BY4742 cells (Fig 3.4).

Conversely, culture aeration and glucose supply remained constant during the YMC, although dissolved oxygen levels changed. It is plausible that  $H_2O_2$  is constantly produced during the YMC. It is also conceivable that the oscillations in  $H_2O_2$  during the YMC is underscored by  $H_2O_2$  consumption or removal, rather than production. One major enzyme involved in  $H_2O_2$  removal or transduction in budding yeast is the peroxiredoxin Tsa1. Strikingly, I observed that the levels of endogenous Tsa1 protein peaked during phases of decreasing  $H_2O_2$  levels (Fig. 3.5A). In line with this observation, Tu *et al.* reported changes in *TSA1* transcript levels during the YMC, which levels peaked during the ‘reductive charging’ phase [47] (Fig. 3.5B). The ‘reductive charging’ phase described by Tu *et al.* is the phase of decreasing  $H_2O_2$  levels in this study. Furthermore, the study by Tu *et al.* also showed that the transcription factor Yap1 translocated into and accumulated in the nucleus during the ‘oxidative’ phase of the YMC [47]. Nuclear translocation of Yap1 leads to elevated expression of genes encoding most antioxidants and components of the cellular thiol-reducing pathways [158, 159]. These elevated antioxidant defense mechanisms during the so-called ‘oxidative’ phase, I propose, was responsible for the decreased  $H_2O_2$  and roGFP2 oxidation levels I observed in this study during this phase of the YMC. Therefore, in contrast to their characterization, I propose a YMC consisting of two phases – oxidative and reductive – based on the mode of  $H_2O_2$  changes (Fig. 3.1).



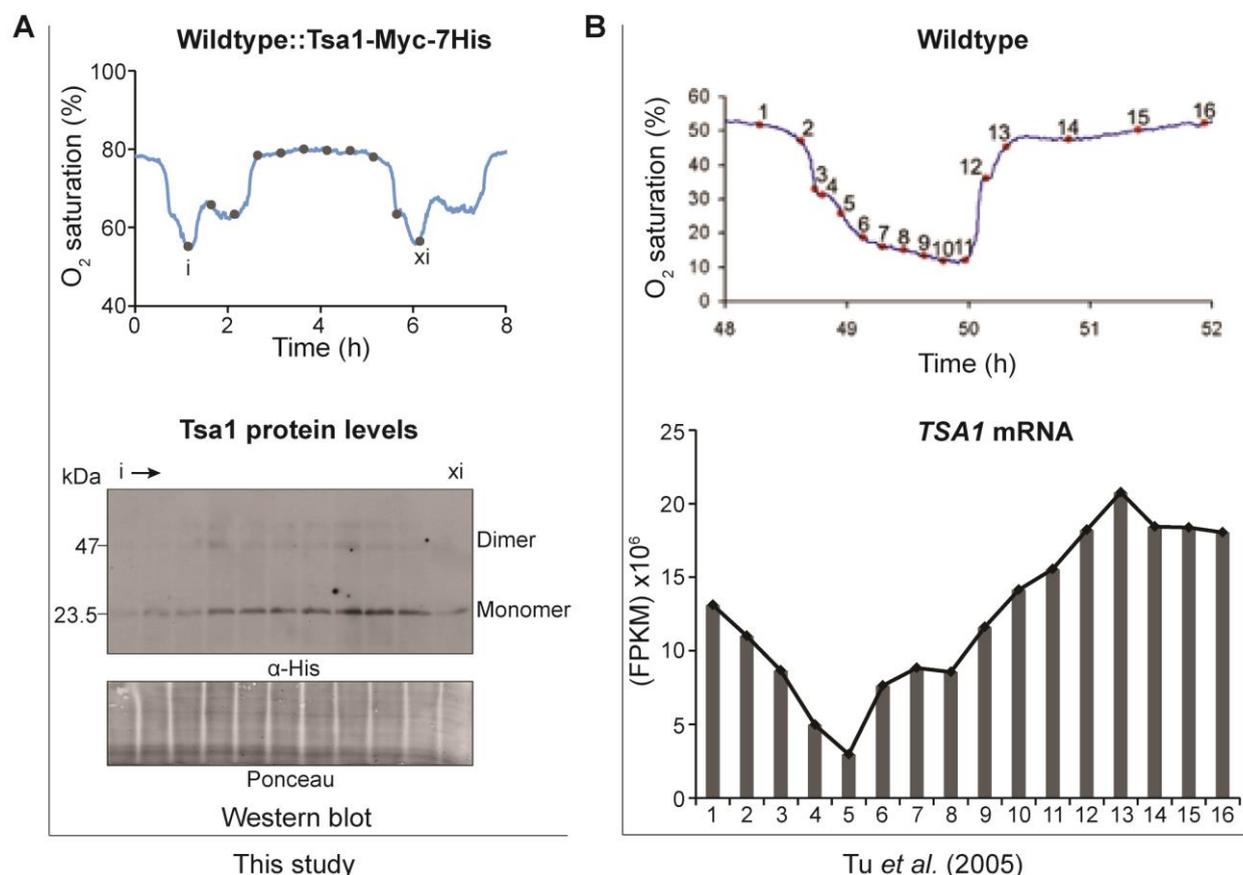
**Figure 3.3: Interference with  $H_2O_2$  generating pathways affect the YMC.** (A) Representative oxygen trace showing that  $\Delta sod1$  cells only grow in a batch phase and could not effectively consume dissolved oxygen to generate synchronized metabolic cycles. (B) Representative oxygen trace showing that loss of *YNO1* perturbs the YMC.



**Figure 3.4: Glucose-stimulated  $H_2O_2$  dynamics is eliminated in  $\Delta cox6$  cells.** BY4742  $\Delta cox6$  cells expressing the roGFP2-Tsa2 $\Delta C_R$  plasmid were cultured in glucose media at  $OD_{600} = 0.25$  and trapped with NEM to monitor steady-state roGFP2 oxidation over the stated duration. Data represent at least three independent replicates and error bars represent standard deviation.

The YMC has historically been studied in low-glucose chemostats. Burnett *et al.* propose that this is so because the budding yeast population self-synchronizes in low-glucose conditions [115]. I propose that such self-synchronization in low-glucose chemostats may be achieved in part via glucose stimulated  $H_2O_2$  generation. On the other hand, it has also been proven that yeast metabolic oscillations can occur on the population level, outside chemostat conditions and in non-glucose media. For instance, metabolic oscillations occurred in a batch culture upon diauxic shift to pure respiration on ethanol from aerobic fermentation [160]. Moreover, batch-grown and phosphate-starved yeast cells with ethanol as a sole carbon source exhibited metabolic oscillations [161].

The causative role for  $H_2O_2$  during the YMC was reinforced by exogenous addition of non-physiological peroxide levels that was enough to trigger phase shifting of the YMC from LOC to HOC (Fig 2.9). Interestingly, this observation was independent of the phase of peroxide addition (Fig 2.10). More importantly, hyperoxidation and inactivation of endogenous typical 2-Cys peroxiredoxins as well as the 2-Cys peroxiredoxin of the  $H_2O_2$  biosensor accompanied the switch from LOC to HOC, upon high peroxide (Fig 2.11). This peroxide-induced peroxiredoxin hyperoxidation was also reflected in the reduction of the redox state of the peroxiredoxin target reporter, roGFP2, and in extension, endogenous peroxiredoxin protein target(s), although I did not provide direct evidence for the latter.

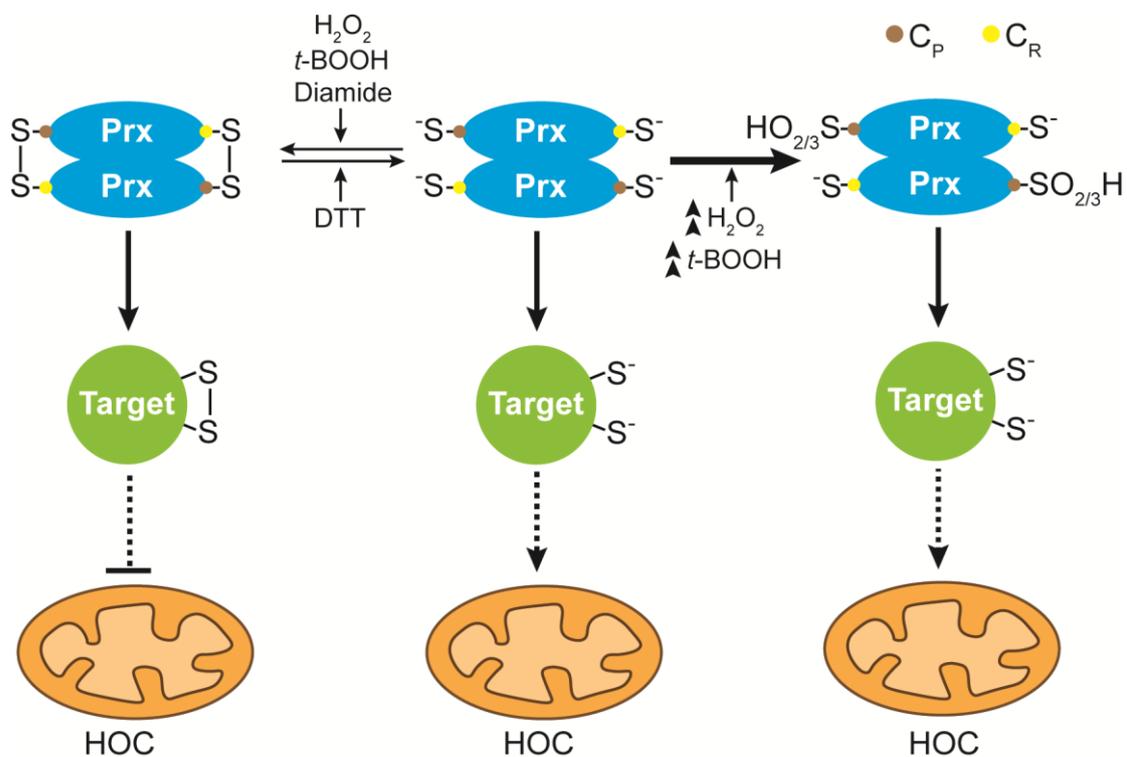


**Figure 3.5: Tsa1 protein and transcript levels peak during the LOC phase, before entry to HOC.** (A) Representative oxygen trace (top) and Western blot (bottom) indicating points of sampling and Tsa1 protein levels, respectively, as observed in this study. (B) Representative oxygen trace (top) and TSA1 mRNA levels (bottom) as reported by Tu *et al.* [47].

Based on the above observations, I concluded that the YMC is regulated by thiol redox switch(es) that are controlled by peroxiredoxins. In other words, cyclical  $H_2O_2$  changes are transduced by peroxiredoxins to regulate the redox state of protein target(s) that control oscillatory metabolism. I opined that if this hypothesis held true, then two predictions could easily be tested: Firstly, the addition of thiol oxidants and reductants should predictably perturb the YMC. Meaning, a thiol oxidant should directly oxidize protein thiols and thus prolong the LOC phase, whilst, a thiol reductant should directly reduce protein thiols and trigger HOC. This prediction works independent of peroxiredoxin activity. Secondly, peroxiredoxin deletion should strongly affect the YMC.

The first prediction was tested by separate additions of 0.5, 1 and 2 mM diamide to independent wildtype YMC-synchronized cultures at the start of LOC, which extended the duration of LOC in a concentration dependent manner. Strikingly, addition of 2 mM diamide just before the switch from LOC to HOC extended the duration of LOC and profoundly delayed switch to HOC (Fig 2.4). Furthermore, DTT at 1, 2 and 5 mM shortened time in LOC and mediated rapid switch to HOC in a concentration dependent manner when added at the start of LOC in independent wildtype YMC-synchronized cultures. Importantly, 5 mM DTT profoundly extended the duration of HOC beyond 10 hours – the time needed to complete two metabolic cycles (Fig 2.3). Hence, by applying thiol redox modifiers, the YMC could be switched between LOC and HOC metabolic states.

In conclusion, I could demonstrate that redox processes that include thiol disulfide exchanges or switches regulate the yeast metabolic clock (Fig. 3.6). Identification of the specific thiol switch(es) involved could be an interesting subject for future investigations.



**Figure 3.6: Peroxiredoxin-mediated thiol-disulfide exchanges regulate the yeast metabolic clock.** Scheme describing the effect of chemical redox perturbations on the yeast metabolic cycle.

## 3.2 Peroxiredoxins and thioredoxins are crucial for stable metabolic clock function

The second prediction stated above was tested by deleting one or more peroxiredoxins from the yeast genome by homologous recombination of an antibiotic resistance cassette. By this method, I successfully generated cells lacking the mitochondrial matrix 1-Cys peroxiredoxin – i.e.  $\Delta prx1$ , cells lacking the cytosolic atypical 2-Cys peroxiredoxin – i.e.  $\Delta ahp1$ , and cells lacking both cytosolic typical 2-Cys peroxiredoxins – i.e.  $\Delta tsa1\Delta tsa2$ . In comparison to wildtype YMC-synchronized cultures, cells lacking the cytosolic 2-Cys peroxiredoxins displayed YMCs with shortened periods and diminished shapes, whilst  $\Delta prx1$  YMC remained virtually unaffected (Fig 2.15). In other words, loss of one or more cytosolic 2-Cys peroxiredoxin(s) profoundly affects the stability of the yeast metabolic clock.

The importance of peroxiredoxins for yeast metabolic clock function could further be deduced from the observation that combined loss of *TSA1* and *AHP1* leads to non-viability of the CEN.PK strain (Fig. 2.19). In fact, conditional depletion of Ahp1 in a  $\Delta tsa1\Delta tsa2$  strain further affects YMC stability (Fig. 2.21). Peroxiredoxin-mediated YMC regulation was mirrored in the behavior of the YMC upon temporary inactivation of peroxiredoxins with excess peroxide. Of note, is the correlation between peroxiredoxin hyperoxidation and immediate switch from LOC to HOC upon 1 mM *t*-BOOH or 5 mM  $H_2O_2$  (Fig. 2.9 and Fig. 2.11). Meanwhile, this peroxide-induced HOC could also occur in a  $\Delta prx1$  or  $\Delta tsa1\Delta tsa2$  strain, although at different peroxide concentrations (Fig. 2.16 and Fig. 2.17). Thus, it is possible that multiple peroxiredoxins were hyperoxidized simultaneously during the peroxide-induced HOC in wildtype cells. To buttress this point, I have shown that the endogenous cytosolic typical 2-Cys peroxiredoxins, Tsa1 and Tsa2, were hyperoxidized via Western blots (Fig. 2.11D). Additionally, the hyperoxidation of the Tsa2 $\Delta C_R$  and Ahp1 moieties of the biosensors upon excess peroxide, could be demonstrated (Fig. 2.11C(ii) and Fig. 2.18). Therefore, it was not surprising that similar peroxide-induced HOC could be achieved in  $\Delta prx1$  and  $\Delta tsa1\Delta tsa2$  cells. It would be interesting to test in future studies, the contribution of the nuclear peroxiredoxin, Dot5, to YMC regulation. Additionally, raising antibodies against the hyperoxidized forms of other peroxiredoxins could augment our understanding of the contributions of each enzyme to the observed phenomenon. Taken together, these results confirm the functional redundancy between the different peroxiredoxins with respect to YMC control, and support the conclusion that peroxiredoxins and peroxiredoxin redox relays are crucial for YMC regulation.

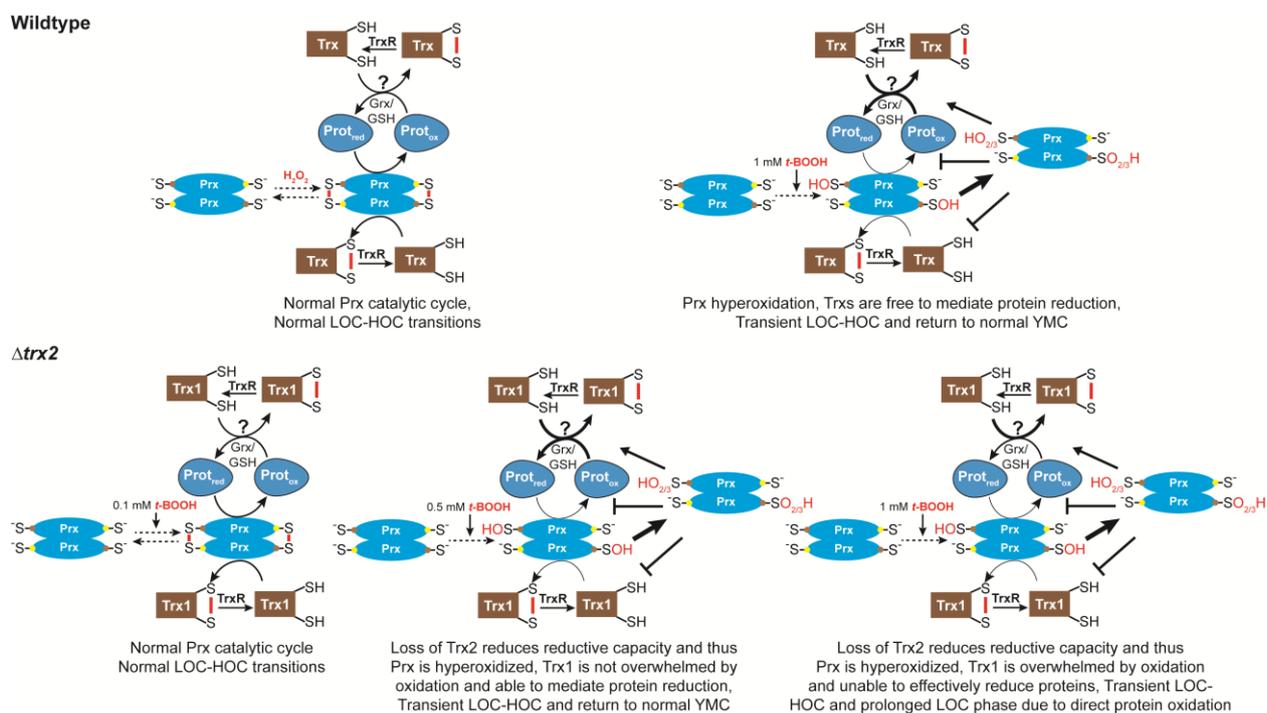
I have proposed that peroxiredoxins control thiol switch(es) that mediate YMC regulation. By using roGFP2 as a substrate, I have demonstrated that: (1) roGFP2 reduction in a ‘normal’ metabolic cycle correlates with switch to HOC, and (2) hyperoxidation of peroxiredoxins leads to reduced roGFP2, which correlates with switch from LOC to HOC. Next, I asked if indeed peroxiredoxin redox relays are crucial for YMC regulation, what might the target proteins of these relays be? Although numerous targets of peroxiredoxin relays have been identified in other organisms, only a few have so far been described in yeast. These include the transcription factor Yap1, which is oxidized by the glutathione peroxidase homolog

Orp1 [74], and the transcription factor Cad1, which was suggested to be oxidized by Ahp1 [162]. Indirect evidence suggests that there are many more unidentified targets of peroxiredoxin redox relays. For instance, a study from the lab of Vadim Gladyshev showed that about 50% of all yeast transcripts respond to H<sub>2</sub>O<sub>2</sub> in a manner that is almost completely dependent on the presence of peroxiredoxins [129]. Interestingly, it was recently reported that Tsa1 can regulate redox modifications on the yeast protein kinase A (PKA) subunit, Tpk1 [163]. The general stress responsive transcription factor, Msn2, is a target of PKA, and Msn2 deletion has been shown to strongly disrupt the YMC, particularly the switching from LOC to HOC [164]. Other targets of PKA include the general heat shock response transcription factor, Hsf1, and metabolic enzymes, such as Pfk2, encoding phosphofructokinase. Thus, it is possible that peroxiredoxins, via redox regulation of PKA, enable cells to regulate a wide range of transcriptional and metabolic processes in response to changing H<sub>2</sub>O<sub>2</sub> levels. Elucidating the mechanistic underpinnings and their relevance, for example, in coupling metabolic changes with the cell cycle, would be an interesting subject of future investigations.

Active peroxiredoxin catalysis endogenously require thioredoxins – enzymes that facilitate the reduction of proteins by catalyzing cysteine thiol-disulfide exchange reactions [165]. Thus, in place of the artificial roGFP2, thioredoxins represent endogenous substrates of peroxiredoxins. Although I did not directly identify in this study, the specific thiol switch(es) involved in regulating the yeast metabolic clock, I opined that thioredoxins could be of interest if indeed peroxiredoxin relay(s) was/were crucial for YMC regulation. Mechanistically, thioredoxins contain two conserved cysteines that either exist in a reduced (-SH) or an oxidized (S-S) form. They donate electrons from their active site dithiol to protein disulfide bonds, which are then reduced to dithiols. The resulting oxidized thioredoxin disulfide is reduced by thioredoxin reductase with electrons donated by NADPH [165]. I imagined that this catalytic mechanism could be important for regenerating oxidized peroxiredoxins to mediate the oscillatory H<sub>2</sub>O<sub>2</sub> signaling phenomenon observed during the YMC. Consequently, loss of either cytosolic thioredoxins, ie. *TRX1* or *TRX2* should have an effect on the YMC. Intuitively, a *Δtrx2* strain exhibited cycles similar to that of *Δtsa1Δtsa2* and *Δahp1* strains, suggesting that loss of *TRX2* affects YMC regulation (Fig. 2.22A). More importantly, a *Δtrx1Δtrx2* strain could not generate metabolic cycles (Fig. 2.24).

Furthermore, loss of *TRX2* affected hyperoxidation-based LOC-to-HOC switching in a significant way. I noted that unlike wildtype YMC-synchronized cultures, 0.5 mM *t*-BOOH was enough to induce rapid LOC-to-HOC transition in *Δtrx2* cultures. Strikingly, 1 mM *t*-BOOH induced a short LOC-to-HOC and immediately returned the YMC to a prolonged LOC phase spanning more than 6 hours (Fig. 2.22B). This observation is explainable as follows: unlike wildtype cells, loss of *TRX2* diminishes the cellular ‘reductive capacity’; hence, 0.5 mM *t*-BOOH was enough to achieve peroxiredoxin hyperoxidation. This concentration of *t*-BOOH however does not overwhelm Trx1-mediated reduction of protein thiols, hence HOC induction occurred ‘normally’ and the YMC was returned to ‘normal’ wildtype behavior. On the other hand, 1 mM *t*-BOOH overpowers rapid Trx1-mediated thiol reductions, as such, LOC-to-HOC transition is short-lived and cells are returned to a prolonged LOC phase, possibly due to direct oxidation of

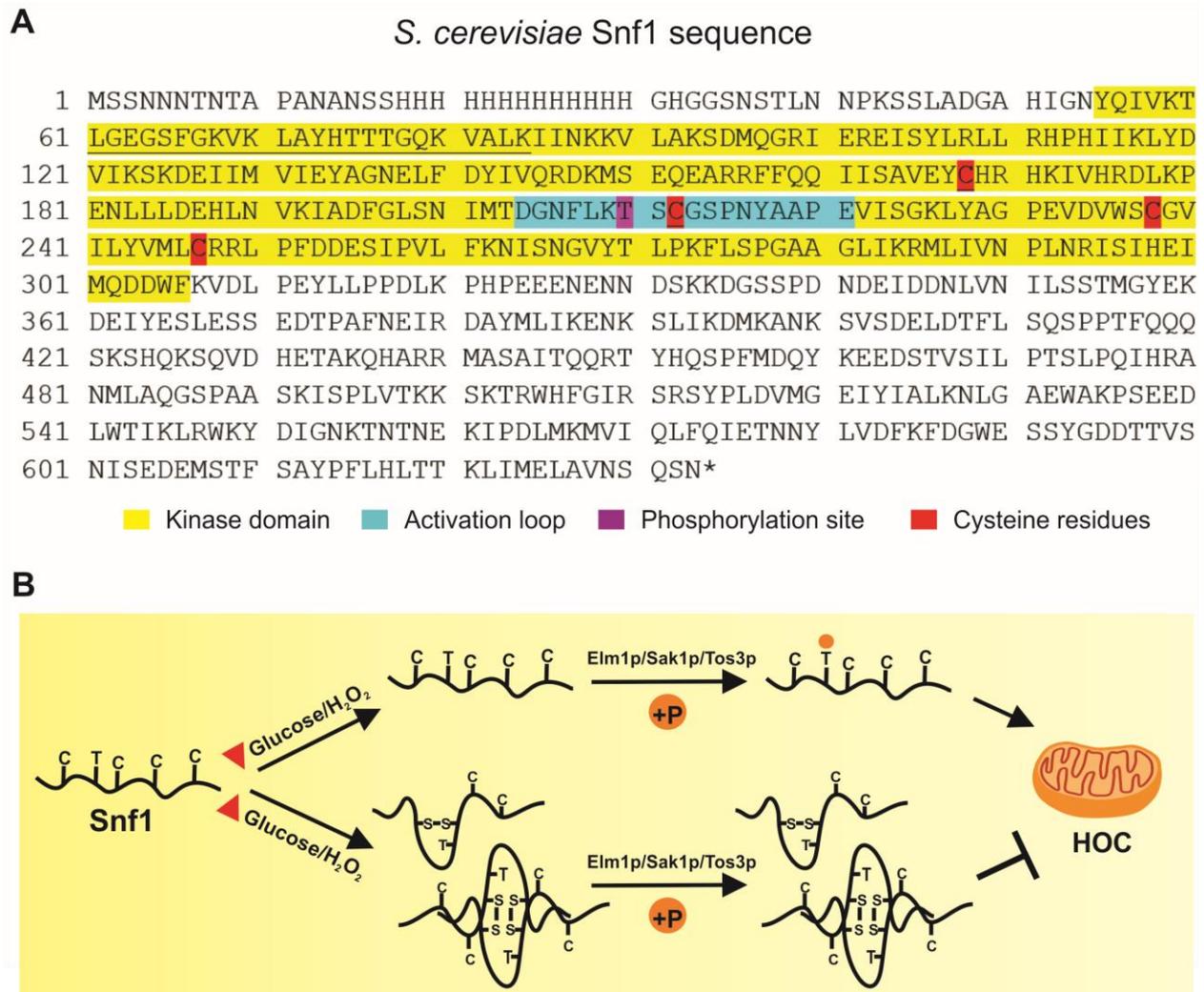
peroxiredoxin-regulated thiol switch(es) by *t*-BOOH (Fig. 3.7), in ways similar to diamide. Indeed, unlike  $H_2O_2$ , which essentially depends on peroxiredoxins to mediate protein thiol oxidations [72, 166]; *t*-BOOH can directly react with protein thiols with or without peroxiredoxins. This was revealed in the response of roGFP2 to further addition of either 1 mM  $H_2O_2$  or *t*-BOOH upon hyperoxidation of the Tsa2 $\Delta$ C<sub>R</sub> moiety of the biosensor (Fig. 2.23).



**Figure 3.7: Scheme explaining YMC behavior in wildtype and  $\Delta$ trx2 cells upon peroxide.** Loss of *TRX2* decreases cellular reductive capacity and facilitates a prolonged LOC phase upon peroxiredoxin hyperoxidation due to direct target protein oxidation by excess *t*-BOOH.

A conceivable role for thioredoxins in circadian clock function was occasioned by the observation that mammalian AMP-activated protein kinase (AMPK), the master regulator of metabolism, is a direct target of Trx1 – i.e. Trx1 regulates the redox state and activation of AMPK during energy metabolism [167]. AMPK has been shown to regulate the circadian metabolic clock in mammals via cryptochrome (Cry) phosphorylation and degradation [168]. Snf1 is the yeast homolog of mammalian AMPK. In budding yeast, Snf1 is important for increased transcription of genes required for metabolic adaptation in low-glucose or non-glucose media [169]. Snf1 activation requires phosphorylation by Sak1/Elm1/Tos3 kinases, of a threonine residue (Thr210) in the activation loop of the kinase domain [170, 171]. Inside the kinase domain are four cysteine residues; Cys168, Cys212, Cys238 and Cys247, two of which directly flank Thr210 (Fig. 3.8A). Shao *et al.* showed that AMPK forms oxidative aggregates in response to energy stress, via intermolecular disulfide bonds at the conserved Cys130 (Cys168 in *S. cerevisiae*) and Cys174 (Cys212 in *S. cerevisiae*) residues, which inhibit activating phosphorylation of AMPK. Consequently, AMPK activation by phosphorylation is restored by Trx1-mediated reduction of these cysteine residues [167]. Crystal structure analysis shows that formation of Snf1 dimer involves segments around Thr210 and

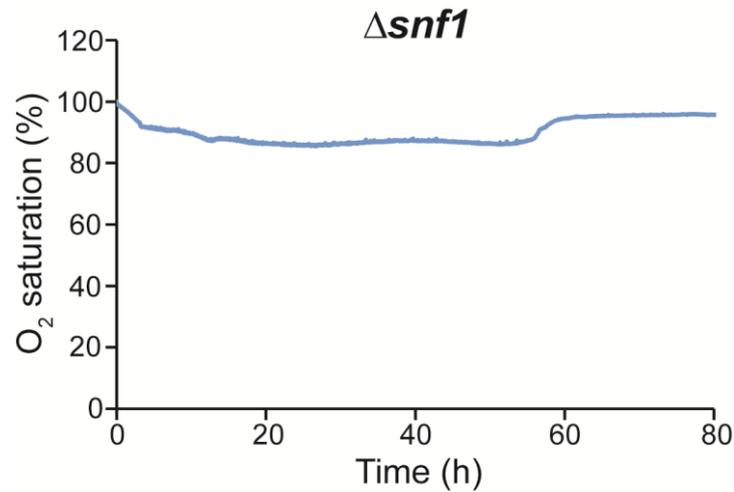
Cys212, predominantly via hydrophobic interactions [172]. Based on their results, Shao *et al.* suggested that disulfide bond formation might be involved in both Snf1 and AMPK dimerization [167]. Upon dimerization, the activation loop and substrate-binding site of Snf1 are buried inside the dimer, resulting in an inactive protein [172]. I imagined that such a disulfide bond could modulate Snf1 function as shown (Fig. 3.8B).



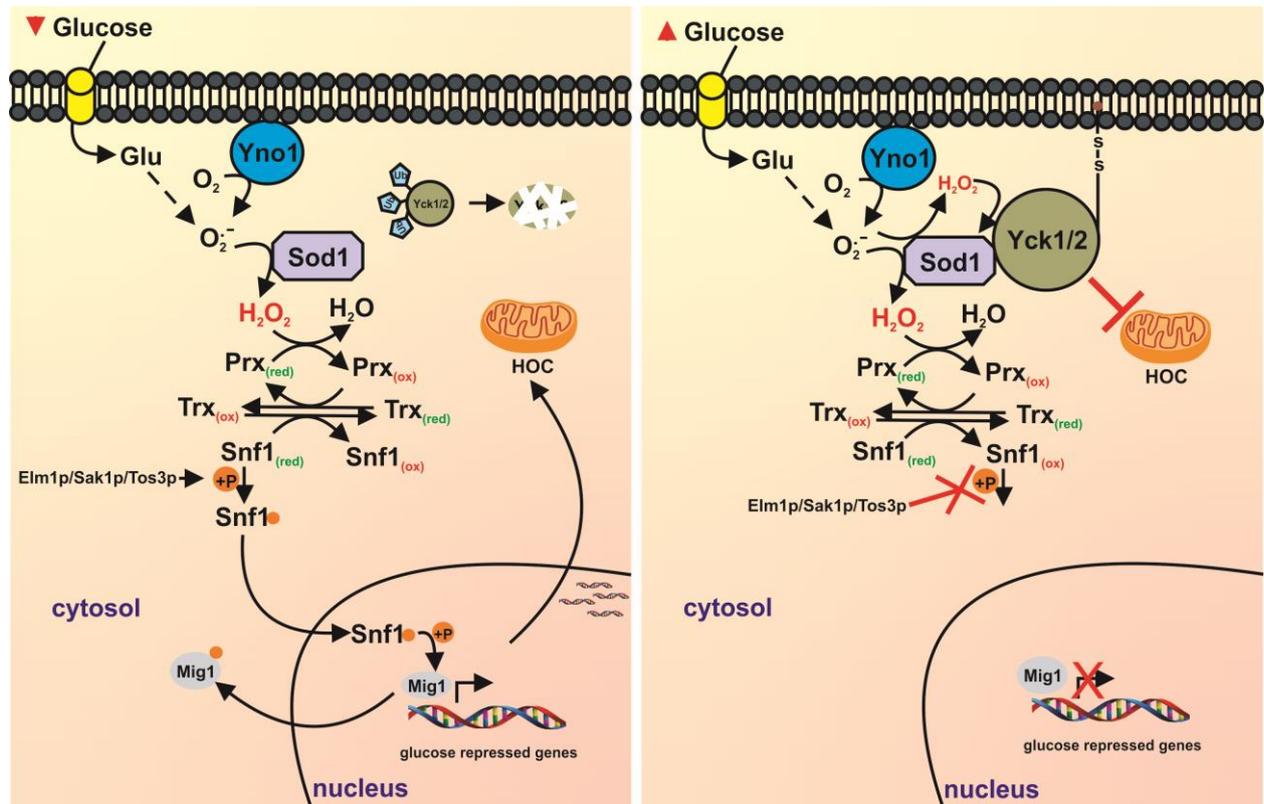
**Figure 3.8: Snf1 could potentially be redox regulated. (A)** Annotated sequence of Snf1. Sequence was obtained from the Yeast Genome Database. **(B)** Inter- or intra-molecular disulfide bond formation at Cys168 and/or Cys212 under high physiological  $H_2O_2$  or high glucose could inhibit Snf1 activation by phosphorylation. Consequently, expression of genes required for respiration and HOC could be suppressed.

It is plausible that Snf1 function may be required for metabolic cycling in low-glucose chemostats, since a  $\Delta snf1$  strain could not grow in batch culture to establish the YMC (Fig. 3.9). Such a function could probably be mediated via thiol redox modifications at Cys168 and/or Cys212, in response to changing metabolic states, which ultimately affect activating phosphorylation at Thr210. During the YMC, these Snf1 thiol disulfide modifications might occur in a cyclical manner in response to oscillations in  $H_2O_2$ , and possibly be regulated by the peroxiredoxin and thioredoxin systems. Consequently, shuttling of Snf1 in and out of the nucleus would influence changes in expression of genes that are needed for respiration, as well

as to mediate LOC and HOC metabolic states of the YMC (Fig 3.10). It would be interesting to pursue this hypothesis in the future and test the impact of Snf1 cysteine mutants on YMC oscillation.



**Figure 3.9: Loss of *SNF1* affects generation of the YMC.** Representative oxygen trace showing that  $\Delta snf1$  cells only grow in a batch phase and could not effectively consume dissolved oxygen to generate synchronized metabolic cycles.



**Figure 3.10: Redox regulation of Snf1 could be essential for YMC oscillation.** Scheme illustrating a possible mode of Snf1 regulation in low-glucose or low signaling  $H_2O_2$  conditions (left), as well as in high-glucose or high signaling  $H_2O_2$  conditions (right). Under low-glucose, Snf1 stays reduced due to low signaling levels of  $H_2O_2$ . Consequently, Snf1 could be activated by phosphorylation to translocate into the nucleus to induce expression of genes required for respiration and HOC. Under high-glucose, Snf1 becomes oxidized due to high signaling levels of  $H_2O_2$ . Consequently, phosphorylation of Snf1 is inhibited and Snf1 stays in the cytoplasm. Sod1-mediated stabilization of Yck1p1/Yck2p under high signaling levels of  $H_2O_2$  leads to repression of respiration [173].

### 3.3 The cellular redox/metabolic state regulates the cell division cycle

Herein, I have shown that during the YMC, levels of  $H_2O_2$  begin to rise at the height of oxygen consumption (i.e. trough of the YMC) and peak just before the entry into LOC. During LOC,  $H_2O_2$  levels begin to decrease and is lowest at LOC-to-HOC transition. Simultaneously, accumulation of 2N DNA content was observed to begin in correlation with rising  $H_2O_2$  levels and peaked just before the entry into LOC. Thereafter, the proportion of cells with 2N DNA content decreased and was lowest during LOC-to-HOC transition. Furthermore, cell budding was highest before entry to LOC and cell division was completed before entry into HOC (Fig. 2.25 and Fig. 3.1). These observations suggest that changes in  $H_2O_2$  signals during oscillatory metabolism is required to synchronously drive cells through the cell division cycle. I therefore hypothesized that initiation of DNA synthesis might require input signals from  $H_2O_2$ . For a successful completion of cell division however, signaling levels of  $H_2O_2$  must decrease.

In line with this reasoning, it was suggested that oscillations in the intracellular redox state during cell cycle progression represents a fundamental mechanism that may link oxidative metabolism to cell cycle regulation [119]. The idea that redox changes may regulate the cell division cycle is supported by evidence from Tu *et al.* who demonstrated that several DNA replication and cell cycle regulatory genes are expressed during a reductive non-respiratory phase of the YMC (i.e. LOC) whilst cell cycle initiation occurs very late during a respiratory oxidative phase (i.e. peak of HOC or trough of the YMC) [47]. Although it is not entirely clear which redox half-reactions control either cell cycle entry or exit, it has been suggested that transient increase in oxidation early in  $G_1$  is necessary for  $G_1$ -to-S phase transition and thus inhibiting this transient increase in oxidation causes cell cycle arrests in the  $G_1$  phase [122]. Similarly, GSH levels were reported to be significantly higher in the  $G_2$  and M phases of the cell cycle compared to  $G_1$ , suggesting that cells in the  $G_2$  and M phases are at a more reduced redox state compared to  $G_1$  phase cells [125]

The plausibility of periodic oscillations in the intracellular redox state playing a crucial role in regulating cell cycle progression is not far-fetched since several cell cycle regulatory proteins harbor redox-sensitive motifs [125]. These proteins include but not limited to cyclins, Cdks and Cdk inhibitors. For instance, the activity of the 20S proteasome in *S. cerevisiae* is inhibited by S-glutathionylation following  $H_2O_2$  treatment, meanwhile, proteasomal degradation of cyclins is central to cell cycle regulation [174]. In line with this, it has been shown in Her14 fibroblasts that exposure to  $H_2O_2$  leads to accumulation of cyclin D1 at  $G_1$  phase, due to the inhibition of cyclin D1 protein degradation [175]. Moreover, direct redox modification of cyclin D1 itself has been proposed as an alternative mechanism for its regulation. Cyclin D1 contains two phosphorylation sites – Thr286 and Thr288 – that can be phosphorylated by glycogen synthase kinase (GSK-3 $\beta$ ) and Mirk/dyrk kinase, for proteasomal degradation [176, 177]. It is suggested that redox thiol modification of cyclin D1 at Cys285 could induce a conformational change that might influence degradation phosphorylation at Thr286 and/or Thr288 [119]. Furthermore, Cdc25, an activator of cyclin–Cdk complex kinase activity could be inhibited either by the thiol-alkylating agent NEM or via

mutation of a single conserved cysteine residue [178, 179]. In addition, H<sub>2</sub>O<sub>2</sub> induced degradation of Cdc25c proteins via formation of intramolecular disulfide bond at Cys377 and Cys330, whilst double mutants of Cys377 and Cys330 were resistant to H<sub>2</sub>O<sub>2</sub>-induced degradation [180].

Recent observations suggest that metabolic cycles are crucial for regulating cell cycle entry and exit [114, 115]. I imagined that the redox state *per se* might not only be necessary to modulate cell cycle regulatory proteins and thereby controlling the cell cycle, rather, the redox state may influence the metabolic state that eventually determines whether cells are ready to initiate the cell cycle or not. In other words, cell cycle initiation is dictated by the metabolic state, which in itself is controlled by the cellular redox environment (Fig 3.11). I have shown that HOC is a trigger for DNA synthesis, and LOC-to-HOC transition is characterized by low H<sub>2</sub>O<sub>2</sub> (or oxidation) levels under physiological conditions. Intriguingly, inducing a reducing environment with DTT is enough to artificially maintain a prolonged HOC phase and cause multiple rounds of DNA synthesis and cell cycle initiation even though a previous cell division cycle remains uncompleted (Fig. 2.27). Conversely, a LOC phase was critical for cell cycle completion and exit, and entry into LOC was triggered by high H<sub>2</sub>O<sub>2</sub> (or oxidation) levels under physiological conditions. Accordingly, temporarily prolonging the LOC phase and delaying LOC-to-HOC transition with diamide delays initiation of DNA synthesis and start of a new cell cycle (Fig. 2.28).

It has been shown that more than 50% of yeast metabolome changes during cell cycle progression downstream of *Start*, causally linking global metabolism changes to cell division [181]. The mechanistic details on how metabolic changes might regulate cell division or *vice versa* remains to be fully explored. Cell division in itself is suggested to involve several coupled, but nonetheless independent oscillators, in addition to the classic and well understood cyclin-Cdk system. Recent observations suggest that Cdk1 directly modulates metabolism of storage carbohydrates via activation of trehalase, Nth1, and glycogen phosphorylase, Gph1, thus coordinating carbohydrate metabolism and the cell cycle in yeast [181, 182]. I have proposed earlier that peroxiredoxins might regulate PKA to trigger a wide range of transcriptional and metabolic processes in response to changing H<sub>2</sub>O<sub>2</sub> levels. Interestingly, it has been shown that Gph1 and Nth1 are not only targets of Cdk1, but also of PKA [182, 183]. Moreover, some studies have identified Bcy1, the regulatory subunit of PKA, as a Cdk substrate [184, 185]. More importantly, PKA has been shown to regulate the activity of Cln-Cdc28 at G<sub>1</sub> and multiple G<sub>1</sub> cyclins (i.e. Cln1, Cln2, and Cln3) harbor consensus PKA phosphorylation sites [182, 186]. It is plausible that Cdk and PKA not only interact with similar substrates, but also regulate each other. This believe stems from the observation that PKA and Cdk activity peak at the same time during cell cycle *Start* [187]. Thus, peroxiredoxins, PKA, Cdk and their substrates might form a regulatory circuit that mediate the coordination of metabolism with cell division in the yeast metabolic cycle.

### 3.4 Peroxiredoxins couple changes in cell metabolism to cell division

My own observations, as well as previous reports show that metabolic clocks are coupled to cellular programs such as transcription/translation and cell division [47, 49, 56]. Intriguingly, I find that temporary inactivation or loss of the cytosolic typical 2-Cys peroxiredoxins, Tsa1 and Tsa2, do not only affect the yeast metabolic cycle, but also profoundly upsets coordination of the metabolic cycle with the cell division cycle (Fig. 2.26). Consequently, the mechanism by which the metabolic state controls cell division might be governed by peroxiredoxins.

I propose that under physiological conditions in the yeast metabolic cycle, peroxiredoxins sense oscillations in H<sub>2</sub>O<sub>2</sub> levels to regulate cellular metabolism [188] and possibly the redox state of cell cycle modulators. During periods of low signaling H<sub>2</sub>O<sub>2</sub> levels, peroxiredoxins become less oxidized and their target(s) stay(s) reduced. Reduced peroxiredoxin target(s) induce(s) a HOC state, which is a pre-requisite for DNA synthesis and cell cycle entry. Upon high signaling H<sub>2</sub>O<sub>2</sub> levels, peroxiredoxins become oxidized and transfer oxidizing equivalents to their target(s). Oxidized peroxiredoxin target(s) induce(s) a LOC state, which is a pre-requisite for cell division and cell cycle exit. By artificially modulating the redox state of peroxiredoxin target(s) directly or indirectly either via peroxiredoxin hyperoxidation with excess *t*-BOOH, oxidation with diamide or reduction with DTT, a HOC or LOC metabolic state can be achieved to trigger entry into or exit from the cell division cycle (Fig. 3.11). Importantly, a prolonged LOC phase prolongs duration of cell cycle exit and inhibits entry, whilst a prolonged HOC phase favors DNA replication and cell cycle entry and inhibits cell cycle exit.

The involvement of peroxiredoxins in regulation of cell cycle processes may represent a novel and exciting area of scientific research. Recent observations suggest this ‘new’ role for peroxiredoxins may either be achieved indirectly via endogenous substrates such as thioredoxins or through direct redox modulation of cell cycle regulators. For instance, the rate-limiting enzyme of deoxyribonucleotide triphosphate (dNTP) biogenesis – ribonucleotide reductase (RNR) – relies on electrons from thioredoxins or glutaredoxins for recycling during DNA replication and repair in the cell cycle [189, 190]. Boronat and colleagues demonstrated that the cytosolic thioredoxin, Trx1, is the primary electron donor for the RNR large subunit, Cdc22, in *S. pombe*. Genetic depletion of *TRX1* and *TRX3* leads to severe replication stress that is partially overcome by activation of the Rad3-Cds1 DNA replication checkpoint to induce transcription of Cdc22. However, loss of the peroxiredoxin Tpx1, a major substrate of Trx1, in a *Δtrr1Δgrx1* strain favored the reduction and functionality of RNR to allow DNA synthesis, cell cycle progression and cell growth [191].

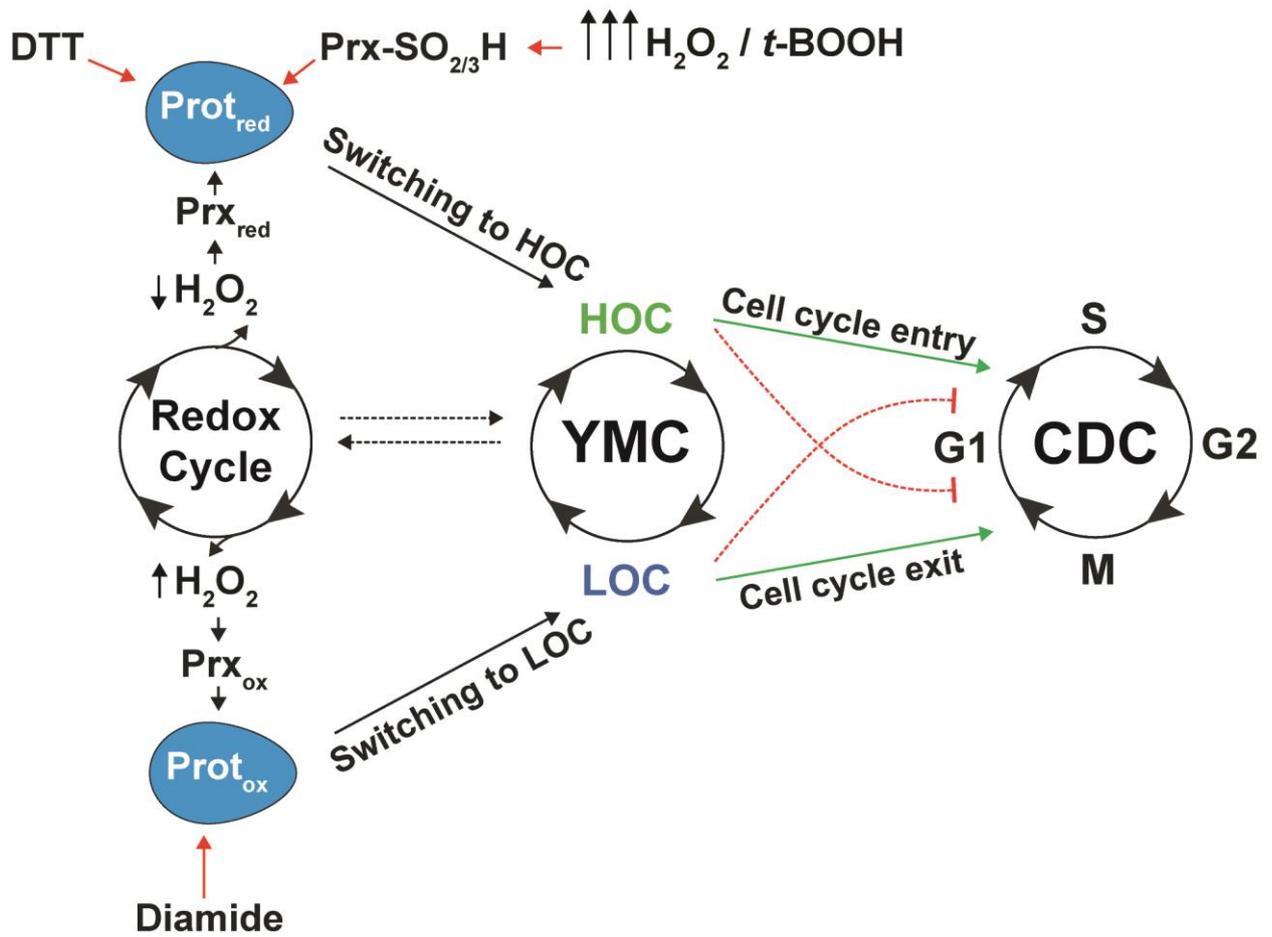
Furthermore, it has been proposed that the replication of DNA requires coordination between replication fork progression and metabolic pathways involved in dNTP biogenesis. In the absence of such coordination or during adverse metabolic conditions such as elevated ROS production, replication fork integrity might be compromised, thereby undermining the fidelity of genome duplication [49, 192].

Perturbation of RNR leads to elevated ROS levels and an imbalance of dNTPs in human cells, which can affect genome integrity by inducing replication fork stalling, DNA breaks and impairment of polymerase proofreading activity [193]. Somyajit *et al.* proposed a mechanism by which redox signaling couples fluctuations of dNTP biogenesis with replisome activity to reduce stress during genome duplication. This mechanism is mediated by the interaction between a component of the replisome, TIMELESS and peroxiredoxin 2 (PRDX2). In low ROS levels, PRDX2 binds TIMELESS to accelerate the replication fork and enable timely genome duplication. In elevated ROS levels, PRDX2 oxidization disrupts this binding and compels the displacement of TIMELESS from the replisome leading to replication fork slowdown [193, 194].

Cell cycle progression through mitosis depends upon the activation of the Cdk1-cyclin B complex in early mitosis [195]. This activation occurs first at the centrosome during prophase and amplified via multiple feedback loops involving kinases such as cyclin B, Cdc25 and Aurora A [196-198]. The activity of these kinases are in turn regulated by anaphase-promoting complex/cyclosome (APC/C)-mediated ubiquitination and subsequent degradation in the proteasome. Meanwhile, APC/C activation is dependent upon Cdh1, whose phosphorylation by Cdk1 inhibits this interaction [199, 200]. Cdk-opposing phosphatases such as Cdc14B, PP1 and PP2A restore Cdh1 activation via de-phosphorylation [201-203].

It is suggested that the intracellular concentration of H<sub>2</sub>O<sub>2</sub>, especially at the centrosome, increases as the cell cycle progresses. Recent reports show that this local accumulation of H<sub>2</sub>O<sub>2</sub> results from Cdk1-mediated inhibition of PrxI oxidase activity via phosphorylation [204, 205]. At the centrosome, H<sub>2</sub>O<sub>2</sub> is believed to oxidatively inhibit Cdk1-opposing phosphatases such as Cdc14B at Cys228 and Cys314, leading to the accumulation of phosphorylated Cdh1 [205]. Accordingly, by Cdk1-mediated phosphorylation and inhibition of PrxI at the centrosome, H<sub>2</sub>O<sub>2</sub> locally accumulates to control the activity of Cdk1 regulators to promote mitotic entry. My observation that H<sub>2</sub>O<sub>2</sub> levels peak just before entry into LOC, a phase characterized by cell division, underscores the above reports. It will be interesting to elucidate the exact mechanisms involved during the YMC.

My observations also show that the YMC persisted, albeit in a perturbed manner, upon deletion of the genes encoding the two cytosolic typical 2-Cys peroxiredoxins, Tsa1 and Tsa2. The mutant cells divide completely asynchronously from the metabolic cycles and from each other. Although the consequence of the loss of YMC-CDC coupling in 2-Cys peroxiredoxin-deleted cells remains to be fully elucidated, this loss of synchrony could explain why for example, thiol peroxidase-deficient (e.g. *Δtsa1*) cells exhibit decreased growth fitness and are prone to spontaneous genetic mutations and gross chromosomal rearrangements, compared to their wildtype counterparts [206-208]. More so, deletion of peroxiredoxins is associated with an increased incidence of cancer in higher organisms [207, 209]. These phenotypes are typically attributed to supposedly higher levels of ROS in peroxiredoxin deficient cells. However, an attractive alternative hypothesis arising from this study is that in the absence of peroxiredoxins, the uncoupling of cell division from metabolism allows cells to divide at 'inappropriate' times, with respect for example to metabolite availability and prevailing metabolic conditions.



**Figure 3.11: Model illustrating the role of peroxiredoxins in the YMC.** A proposed role for peroxiredoxins in regulation of metabolic cycling and the coordination of metabolism with cell division. Red arrows indicate non-physiological treatments that have been used in this study to test the model.

## 4 OUTLOOK

In this study, I have demonstrated that peroxiredoxins and peroxiredoxin redox relays are important for metabolic clock function, and in coupling of metabolism to the cell division cycle. I was able to demonstrate that 2-Cys peroxiredoxins, especially Tsa1 and Ahp1, are crucial for this function, loss of both being detrimental for prototrophic yeast viability.

I could not in this study identify specific redox relays important for YMC control and CDC coupling, although previous reports provide some insights into this [74, 162, 164]. It would therefore be interesting for future studies to use high throughput approaches to identify some of these relays. Protein-protein interaction techniques such as proximity biotinylation could be helpful in this regard. Fusion and coexpression of Tsa1 with the biotin affinity tag and biotin-ligase BirA\*, for instance, and treating a YMC-synchronized culture of the tagged yeast strain with appropriate amounts of commercially available biotin would lead to promiscuous biotinylation of Tsa1 interacting partners [210-212]. By phase-specific sampling and proteomic analysis, it would be possible to analyze in an unbiased manner, phase-specific expression changes of such relays or interactions. An alternative strategy would be to trap the thiol redox state of samples collected at specific phases of the YMC and subject them to redox proteomic analysis techniques such as OxICAT [213, 214] or SICyLIA [215].

Changes to cellular transcript levels during the YMC have been investigated [47]. One subject that would be interesting to explore in the future would be to compare transcriptomic profiles to proteomic profiles during the same YMC. It is plausible that oscillatory changes in transcript levels are reflected at the proteomic level, or not. However, this is yet to be demonstrated. It would also be interesting to know how these transcriptome and proteome profiles are altered in response to the concentrations of peroxides used in this study. A proper experimental protocol would be to extract both mRNAs and proteins simultaneously from same cell aliquots in an assay and subject them to transcriptomics and non-targeted proteomic analysis. The GenElute™ RNA/DNA/Protein Purification Plus Kit (RDP300) from Sigma-Aldrich would be helpful for this purpose.

The requirement of Tsa1 and Ahp1 for prototrophic yeast viability is a novel observation that warrants further interrogations in the future. It would be interesting to know if the differential requirement for Tsa1 and Ahp1 is idiosyncratic of all prototrophic yeast strains that undergo metabolic cycling. It is also plausible that the more domesticated lab strains, such as BY4742 and W303, might have lost other important biological features that are essential for understanding cellular mechanisms on the systems biology level (e.g. Proteostasis). The challenge for yeast biologists, which is worthy of debate, is whether before drawing conclusions from experiments performed in auxotrophic yeast strains, it would be necessary to confirm same in their prototrophic counterparts, in similar fashion as cell culture experiments are verified in primary cells or animal models.

The importance of thioredoxins in redox processes and ribonucleotide biosynthesis position these enzymes at the interface of YMC-CDC coupling. My 'biased' selection of *TRX2* for scrutiny of its impact

in YMC regulation revealed an important finding that is worth pursuing. The requirement of thioredoxins for peroxiredoxin recycling was reflected in the effect on the YMC, of the loss of *TRX2*. It is possible that  $\Delta trx2$  cells will exhibit CDCs decoupled from the YMC, although yet to be proven. In their review, Chen and McKnight argued as follows: “Based on the redox nature of cell cycle gating by the YMC, the regulatory factor(s) might possess the following attributes: (1) redox sensitivity; (2) expression and/or activity that is switched on or off at the oxidative-reductive transition point; and (3) direct or indirect interaction with key factors controlling DNA replication” [216]. It is my view that thioredoxins meet these criteria. A careful dissection of the contributions of thioredoxins and thioredoxin reductases to YMC regulation is worth pursuing. Moreover, the mechanisms that underlie YMC-CDC coupling as well as impacts of the loss of such coupling on cell physiology could become clearer upon identification of the redox relays that govern such process using the techniques aforementioned.

Although other redox metabolites such as NADP(H) and GSH/GSSG have been shown to oscillate during the YMC [56], the approach used to interrogate  $H_2O_2$  dynamics in this study is novel and less prone to direct human manipulations. Real-time measurements provide high resolution, second to second interval snapshots of redox metabolite dynamics. During the course of this study, I have also engineered CEN.PK yeast strain expressing a genomically integrated roGFP2-Grx1 biosensor, capable of monitoring in real-time, oscillations in the GSH/GSSG couple during the YMC. It would be good to use this strain in the future to monitor GSH/GSSG dynamics during the YMC. While expression of plasmids in the CEN.PK background was assumed impossible due to the inability for plasmid selection, I devised a technique, by deleting the *HIS3* gene from the CEN.PK genome and supplementing this with a *HIS3* plasmid. By so doing, it was possible to develop a  $\Delta his3$  CEN.PK strain expressing a *HIS3* plasmid and capable of generating synchronized YMCs just like a wildtype strain (data not shown). I am aware that plans are advanced by the Morgan lab to develop plasmid-based genetically encoded biosensors to monitor cellular NADP(H) in yeast. Such plasmids, when ready, can be expressed in the  $\Delta his3$  CEN.PK strain to monitor cycling changes in this metabolite during the YMC, if any. Such NADPH oscillations are expected to run anti-phasic to  $H_2O_2$  changes, owing to the antioxidant role of NADPH. It would be interesting to see how this turns out in the future. The source of  $H_2O_2$  during the YMC, as well as the mechanisms by which their oscillations are generated and maintained is worth following up on.

# 5 MATERIALS AND METHODS

This section contains methods that are standard protocols in the Morgan lab. Methods that were developed by me as part of this project are described in details. Methods modified from other publications are duly cited and referenced.

## 5.1 Molecular Biology Methods

### 5.1.1 Plasmid DNA isolation from *Escherichia coli* (*E. coli*)

I isolated plasmid DNA from *E. coli* cells after cloning of vectors. To do this, I inoculated 5 ml of selective LB-media with a single bacteria colony and incubated at 37 °C with shaking at 140 rpm overnight. I harvested and extracted DNA from 2 ml of *E. coli* culture using the NucleoSpin Plasmid-Kit (Macherey-Nagel, #740609.250) according to the manufacturer's instructions.

### 5.1.2 Determination of DNA concentration

I used the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific) to evaluate DNA concentration and purity. This was done with 1 µl of DNA after cleaning and blanking of instrument with milliQ-H<sub>2</sub>O. I measured absorbance at 280, 260 or 230 nm. The ratios of 280/230 and 260/230 were used to determine contamination by proteins, RNA and organic compounds. For low contamination, a ratio of around 2 was sufficient.

### 5.1.3 Polymerase chain reaction for DNA amplification

I performed polymerase chain reaction (PCR) to amplify DNA for homologous recombination, plasmid construction and successful clone verification. For homologous recombination and plasmid construction, the PCR reaction volume was 50 µl, consisting of 100 ng template DNA, 40 pmol each of forward and reverse primers, 0.2 mM dNTPs, 1 U polymerase and 1x reaction buffer, and made up with milliQ-H<sub>2</sub>O. For verifications after cloning, the PCR reaction volume was scaled down to 25 µl.

Successful integration of antibiotic resistance cassette by homologous recombination was confirmed by colony PCR. To do this, one colony of yeast was dissolved in 30 µl of 0.2 % SDS and boiled for 10 min at 95 °C. Afterwards, cell debris and intact cells were pelleted using a small tabletop centrifuge at 17,000g for 1 min. Thereafter, 1 µl of the supernatant was used as template DNA for PCR.

All reactions were performed with the Phusion High-Fidelity DNA Polymerase kit (New England BioLabs or NEB, #M0530L). The PCR was run under the following conditions: initial denaturation at 98°C for 3 min, 30 cycles of denaturation at 98 °C for 30 s, followed by annealing at 60 °C for 20 s and extension

at 72 °C for 1 min/kb. The final extension of PCR product was carried out at 72 °C for 10 min and cooling to 4 °C.

#### **5.1.4 Restriction digestion of DNA**

Restriction digestion was used to confirm plasmid construct before and after cloning. PCR products or plasmids were digested in a 50 µl reaction volume containing 15 U of restriction enzyme and 1x reaction buffer (CutSmart, NEB, #B7204S) as recommended by the manufacturer. To avoid self-ligation, 1 U of calf intestinal phosphatase (CIP, NEB, #M0290S) was added to the reaction where necessary. The reaction mixture was incubated at 37 °C for 2 h and analyzed in agarose gel electrophoresis. The respective DNA fragment was purified after electrophoresis or directly after the digestion reaction using the NucleoSpin® PCR Clean-up kit (Macherey-Nagel) according to the manufacturer's instructions. For analytic purposes, 500 ng of plasmid DNA was digested for 30 min at 37 °C in a 20 µl reaction volume and analyzed by agarose gel electrophoresis.

#### **5.1.5 Ligation of DNA fragments with vectors**

Insert fragments were ligated into vector plasmid DNA in a 3:1 ratio in a reaction volume of 20 µl. This consisted of 100 ng of vector, 2 µl of *T4 DNA ligase* (NEB, #M0202S), 2 µl of 10x ligase reaction buffer (NEB, #E6289), and made up with milliQ-H<sub>2</sub>O. The reaction was performed at 16 °C overnight and 2 µl of the ligation reaction was transformed into *E. coli* cells.

#### **5.1.6 Agarose gel electrophoresis**

Agarose was used for verification of DNA fragments after restriction digest and cloning. The gel was prepared at 1% (w/v) in 1x TAE buffer (40 mM Tris, 1.14% acetic acid, 10 mM EDTA pH 8.0) by heating in a microwave. The gel was casted into a slide and 0.5 µg/ml ethidium bromide was added to visualize DNA under ultra violet (UV) light. Samples were mixed with 6x loading dye (60 mM Tris/HCl pH 7.5, 30 mM sodium acetate, 12 mM EDTA, 60% (v/v) glycerol, 0.36% (w/v) orange G) and loaded onto wells in the gel. The electrophoresis was performed in 1x TAE buffer at 10 V/cm. The gels were analyzed under UV light.

### **5.1.7 Chemical transformation of *E. coli* cells**

Chemo competent *E. coli* cells were transformed for the amplification of plasmid DNA. Cells were thawed slowly on ice and mixed with either 2 µl of a ligation reaction or 20 ng of plasmid DNA. The mixture was further incubated on ice for 30 min followed by a 45 s heat shock at 42 °C and incubation on ice for another 1 min. Afterwards, 100 µl LB-media was added and the suspension incubated further at 37°C for 1 h on 750 rpm shaking. The cell suspension was immediately plated onto LB agar plates supplemented with 100 µg/ml Ampicillin and incubated at 37 °C overnight until colonies appeared. The *E. coli* cells used in this study are described in Table 5.1.

### **5.1.8 One-step transformation of *S. cerevisiae* cells**

One milliliter of log phase yeast cells were harvested at 900g for 3 min. For homologous recombination to genomically integrate a DNA cassette, cell pellet was washed with 1 ml sterile milliQ-H<sub>2</sub>O and re-suspended in 200 µl of one-step buffer (0.2 M Li-Acetate, 40 % PEG 3350, 100 mM DTT), 10µl of 100 mg/ml single strand DNA from Salmon sperm (denatured at 95 °C for 5 min) and 500 ng of PCR product. In case of transformation with plasmid DNA for sensor expression, cell pellet was washed with 1 ml sterile milliQ-H<sub>2</sub>O and re-suspended in 100 µl one-step buffer, 5 µl Salmon sperm DNA and 100ng of plasmid DNA. The mixture was vortexed briefly and incubated at 45 °C for 30 min on 750 rpm shaking.

With plasmid transformations, the suspension was immediately spread onto HC agar plates lacking the respective amino acid for selection. For homologous recombination, cells were pelleted at 900g for 3min, re-suspended in 1 ml of fresh YPD media and transferred into flasks containing 4 ml of fresh YPD media. The cell suspension was then incubated overnight in a shaking incubator at 30 °C. Thereafter, cells were harvested at 900g for 3 min and re-suspended in 200 µl of fresh YPD media. The cells were then plated onto the appropriate selective media as follows: 180 µl of the immediate suspension was spread on a YPD agar plate (90% plate), and the remaining 20 µl suspension was further diluted with 180 µl of fresh YPD media and plated onto another agar plate (10% plate). All plates were incubated at 30 °C for 2-3 days until colonies appeared.

### **5.1.9 Plasmids and primers**

Plasmids used in this thesis are described in Table 5.2, whilst primers used in plasmid and strain construction are listed in Table 5.3. Plasmids were designed for expression in yeast. In some instances, primer names are denoted by the yeast strain they were used to construct and confirm.

## 5.2 Cell Biology Methods

### 5.2.1 Yeast strains

The CEN.PK 113-1A strain background was used to perform all experiments using the fermenter. Most deletion mutants were created in this background, unless otherwise stated. The BY4741 and BY4742 backgrounds were also used for some experiments. Gene deletion strains were created using a standard, homologous recombination-based gene deletion approach (Table 5.4). All deletion strains were confirmed by PCR on isolated genomic DNA using primers designed to anneal 100–200 base pairs up- and downstream of the gene of interest (Table 5.3).

### 5.2.2 Construction of a genomically integrated roGFP2-Tsa2 $\Delta$ C<sub>R</sub> expressing yeast strain

The peroxiredoxin-based H<sub>2</sub>O<sub>2</sub> sensor, roGFP2-Tsa2 $\Delta$ C<sub>R</sub>, was modified for integration and expression from the yeast genome. Briefly, the coding sequence for roGFP2-Tsa2 $\Delta$ C<sub>R</sub> was genetically fused to the KanMX4 resistance cassette and a strong constitutive promoter, GPD, both derived from a pYM-N14 plasmid [217]. The KanMX4-GFP fragment was amplified by PCR using primers P1 and P2, which possess a *NheI* restriction site to enable annealing to the *XbaI* restriction site of the p415TEF roGFP2-Tsa2 $\Delta$ C<sub>R</sub> plasmid. The complete construct was assembled in a p415TEF plasmid and confirmed by sequencing. The entire KanMX4-GPD-roGFP2-Tsa2 $\Delta$ C<sub>R</sub> construct was then amplified by PCR using primers P3 and P4, which were designed to have overhangs complementary to a non-coding genome region between the *HXT6* and *HXT7* genes. The PCR product was transformed into yeast for homologous recombination, positive colonies were selected on YPD agar plates supplemented with G-418 antibiotic, and confirmed by PCR of the genomic DNA using primers P5 and P6 (see Appendix A1).

### 5.2.3 Construction of yeast strains capable of conditional genomic Ahp1 depletion via an auxin-inducible degron (AID) system

A plasmid construct (AP2099) of both the auxin-inducible degron and *Arabidopsis thaliana* F-box protein, transport inhibitor response 1 (*AtTir1*), was a generous gift from Prof. Dr. Blanche Schwappach (Göttingen, Germany). The construct was modified and amplified by PCR for genomic integration and fusion to the N-terminus of *AHP1*, using primers P7 and P8. Positive clones were selected on YPD agar plates supplemented with G-418 antibiotic and verified by PCR using primers P9 and P10 or P9 and KanMX4 REV (see Appendix A3). Sensitivity to auxin was determined by spotting serial dilutions of exponentially growing yeast cultures on YPD plates containing 0.2 mM of auxin.

## 5.2.4 Yeast mating, sporulation and tetrad dissection

A CEN.PK113-1A  $\Delta tsa1\Delta tsa2$  strain was crossed with CEN.PK113-7D  $\Delta ahp1$  strain on YPD agar plate. The resultant diploid cells were selected and enriched by growth on YPD plates supplemented with G418, nourseothricin and hygromycin antibiotics. These cells were subsequently inoculated into 10 ml of fresh YPD media and incubated overnight with shaking at 140 rpm, 30 °C. Next, 500  $\mu$ l of YPD culture was harvested by centrifugation at 900g for 3 min and re-suspended in 10 ml of sporulation media (10 g/l Potassium acetate, 1 g/l yeast extract, 0.5 g/l glucose). The cell suspension was further incubated at 30 °C for 4 days to allow for sporulation. About 500  $\mu$ l of sporulated cell suspension was harvested by centrifugation at 900g for 3 min and re-suspended in 100  $\mu$ l of 1.2 M sorbitol buffer supplemented with zymolyase and incubated for 15 min at room temperature (RT). Zymolyase-treated cells were further diluted 1:50 in 1.2 M sorbitol buffer and a few microliters of the suspension transferred onto the side of a dried YPD plate under sterile conditions. The plate was dried for a further 10 min and tetrads dissected using a tetrad dissection microscope (Nikon ECLIPSE 50i). Dissected tetrads were incubated at 30 °C for 48 h and characterized by growth on appropriate antibiotic containing media.

## 5.2.5 Media for *E. coli* cultivation

Bacteria was cultivated in liquid culture or on agar plates. LB medium (1% bacto-tryptone, 0.5% yeast extract, 1% sodium chloride, pH was adjusted to 7.5 with NaOH) supplemented with 100  $\mu$ g/ml ampicillin was used for liquid culture and plasmid selection. For agar plates, 2% bacto-agar (w/v) was dissolved in LB medium and autoclaved. After cooling of the agar solution, 100  $\mu$ g/ml of ampicillin was added prior to pouring in plates.

## 5.2.6 Media for *S. cerevisiae* cultivation

Yeast cells were cultured in full YP-media (1% yeast extract, 2% peptone, pH was adjusted to 5.5 with HCl) supplemented with 2% glucose as carbon source. YPD agar plates were prepared by mixing 2% agar, 1% yeast extract and 2% peptone, adjusted to pH 5.5 with HCl and autoclaved. Autoclaved media was supplemented with 2% of glucose and 100  $\mu$ g/ml of G418, Nourseothricin or Hygromycin B solution for selection where necessary. The mixture was poured into petri dishes.

Yeast strains were streaked onto YPD agar plates for growth before culture in liquid media. Cells streaked on agar plates were grown in a stationary incubator, whilst cells cultured in liquid media were grown in a shaking incubator (140 rpm) at 30 °C. Yeast transformed for gene deletion by homologous recombination was grown on YPD agar plates supplemented with the respective antibiotic for selection. Backgrounds transformed with plasmids were grown in Hartwell's complete (HC) media lacking the appropriate amino acids for plasmid selection, with 2% glucose as carbon source. The composition of HC media is described in Table 5.5. All media components were sterile filtered before use.

## 5.2.7 Establishment of continuous culture

A Biostat® A fermenter (Module BB-8822002, Serial # 00337/16, Sartorius Stedim Systems, Guxhagen, Germany) was used for all continuous culture experiments. Fermenter runs were initiated by the addition of a 20 ml starter culture, which had been grown to stationary phase in YPD media at 30 °C. The culture working volume was 800 ml. The fermenter was run at a constant temperature of 30 °C, with a constant pH of 3.4 maintained by automated addition of 10% (w/v) NaOH. The culture was continuously aerated at 1 L/min with stirring at 530 rpm. After growth to stationary phase as a batch culture, continuous culture was initiated by the addition of growth media at a constant dilution rate of 0.05 h<sup>-1</sup>.

Growth media consisted of 10 g/l glucose, 1 g/l yeast extract (SERVA, #24540.03), 5 g/l (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 g/l KH<sub>2</sub>PO<sub>4</sub>, 0.5 g/l MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g/l CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.02 g/l FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g/l ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.005 g/l CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.001 g/l MnCl<sub>2</sub>·4H<sub>2</sub>O, 2.5 ml 70% H<sub>2</sub>SO<sub>4</sub> and 0.5 ml/l Antifoam 204 (Sigma). Media components and trace metals were separately prepared, sterilized and re-constituted to a total volume of 5L. Briefly, a solution consisting of 1 g/l yeast extract (SERVA), 5 g/l (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 g/l KH<sub>2</sub>PO<sub>4</sub>, 0.5 g/l MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g/l CaCl<sub>2</sub>·2H<sub>2</sub>O was prepared to the appropriate volume and autoclaved. To this solution were added the appropriate amounts of separately autoclaved 100 g/l glucose solution and 70 % H<sub>2</sub>SO<sub>4</sub> solution. ZnSO<sub>4</sub>·7H<sub>2</sub>O, CuSO<sub>4</sub>·5H<sub>2</sub>O and MnCl<sub>2</sub>·4H<sub>2</sub>O were prepared as a 1000x trace metal stock and separately autoclaved. Finally, FeSO<sub>4</sub>·7H<sub>2</sub>O was dissolved to 10 mg/ml in water, sterile filtered, and the appropriate amount added to the media mix, together with the appropriate volume of antifoam 204.

## 5.2.8 Online monitoring of roGFP2-Tsa2ΔC<sub>R</sub>

To enable the continuous ‘on-line’ monitoring of roGFP2-Tsa2ΔC<sub>R</sub> fluorescence, I developed an in-house system. Briefly, a peristaltic pump was used to continuously pump culture from the fermenter, through a flow-cell (Type 71-F, Starna GmbH, Göttingen) inserted into a JASCO FP-6500 spectrofluorimeter. Fluorescence was measured at fixed excitation wavelengths of 425 nm and 488 nm with emission monitored at 510 nm. The excitation and emission bandwidths were set to 5 nm. A measurement was recorded every 30 s. RoGFP2 oxidation was qualitatively determined by computing the ratio between fluorescence intensities at 425 nm and 488 nm

## 5.2.9 NEM-based alkylation for degree of oxidation of roGFP2-Tsa2ΔC<sub>R</sub> determination

To monitor changes in roGFP2-Tsa2ΔC<sub>R</sub> oxidation during the YMC an NEM-based probe oxidation ‘trapping’ method was used. Briefly, cells were removed from the culture vessel at the indicated time points. Aliquots of 450 μl were immediately added to separate Eppendorf tubes containing either 50 μl 1 M NEM (i.e. 100 mM final), 50 μl 1 M DTT (i.e. 100 mM final) or 50 μl 0.2 M diamide (i.e. 20 mM final). The samples were incubated at RT for 10 mins. Cells were subsequently harvested by centrifugation

at 900g for 3 min and re-suspended in 100 mM MES-Tris buffer (pH 6.0) containing 10 mM NEM. Cells were then transferred to a 96-well plate and probe oxidation was measured using a BMG Labtech CLARIOstar fluorescence plate reader. The degree of sensor oxidation was determined according to Equation 1, as previously described, based on the fluorescence emission intensity at 510 nm with excitation at both 405 nm and 488 nm for the fully oxidized, fully reduced and control samples respectively [90, 93, 218].

Equation 1

$$\text{OxD}_{\text{roGFP2}} = \frac{(I_{405_{\text{sample}}} * I_{488_{\text{red}}}) - (I_{405_{\text{red}}} * I_{488_{\text{sample}}})}{(I_{405_{\text{sample}}} * I_{488_{\text{red}}} - I_{405_{\text{sample}}} * I_{488_{\text{ox}}}) + (I_{405_{\text{ox}}} * I_{488_{\text{sample}}} - I_{405_{\text{red}}} * I_{488_{\text{sample}}})}$$

### 5.2.10 Flow cytometry analysis of DNA content

Aliquots of 1.0 OD<sub>600</sub> units of fermenter cultures (~1 x 10<sup>7</sup> cells) were harvested at 6,000g for 1min. Cells were re-suspended and fixed in 1 ml 70 % (v/v) ethanol at 4 °C overnight. Fixed cells were then pelleted at 6,000g for 1 min and washed with milliQ-H<sub>2</sub>O. Cells were subsequently re-suspended in 250 µl FxCycle™ PI/RNase staining solution (Life Technologies, #F10797), incubated at RT in the dark for 30min and then stored at 4 °C for 72 h. Samples were sonicated at 30 % amplitude for 20 s and run on an Attune™ Flow Cytometer. Data analyses was performed using the FlowJo™ software (v.10). Samples were gated for single cells and a histogram of cell count against PI intensity plotted. The percentage of cells with 1N DNA and 2N DNA content were determined as follows: 1N; area under the histogram from 2.7 to 4.5 x 10<sup>5</sup> PI intensity and 2N; area under the histogram from 4.5 to 7 x 10<sup>5</sup> PI intensity. Cell populations with PI intensity greater than 7 x 10<sup>5</sup> were considered to have more than 2N DNA content.

### 5.2.11 Budding index determination and DNA visualization by DAPI staining and microscopy

Fermenter culture samples were harvested at the indicated times and fixed with 70% (v/v) ethanol at 4 °C for 30 min. Cells were washed twice with 1x phosphate-buffered saline (PBS). Budded and non-budded cells in several random fields were scored to calculate budding index. To visualize DNA 4,6-diamidino-2-phenylindole (DAPI) was added to the harvested cells at a final concentration of 1 µg/ml in PBS and incubated in the dark for 10 mins. Cells were then washed and re-suspended in PBS for visualization. Cells were visualized with a fully automated Zeiss inverted microscope (AxioObserver Z1) equipped with the CSU-X1 spinning disk confocal head (Yokogawa). Image acquisition was performed using a CoolSnap HQ camera (Roper Scientific) and a 40x air or 63x oil objective under the control of the Slidebook software (Intelligent Imaging Innovations, Denver, CO).

### 5.2.12 Fluorescence Microscopy

Genomically integrated sensor-expressing strains were grown to mid-log phase in YPD. About 1  $\mu$ l of the culture was placed on a microscopic slide and covered with a cover slip. Cells were viewed using a Nikon ECLIPSE E600 fluorescent microscope. Images were acquired with a 100 $\times$  oil immersion lens and Nikon equipped camera using NIS-Elements D 4.50.00 software. Images were then transferred to PhotoScape (version 3.7) or ImageJ (version 1.52a) for processing.

## 5.3 Protein biochemistry methods

### 5.3.1 Protein extraction

For detection of hyperoxidized peroxiredoxin proteins, 2.0 OD<sub>600</sub> units of cells were collected from the fermenter 30 min after *t*-BOOH treatment and immediately treated with 100 mM NEM for 10 min at RT. Subsequently, cells were isolated by centrifugation at 900g for 3 min, re-suspended in 100  $\mu$ l of 100 mM NaOH and incubated for 10 min at RT. Cells were then centrifuged at 900g for 3 mins, re-suspended in 15  $\mu$ l of lysis buffer (50 mM HEPES, pH 8.0, 50 mM NaCl, 1% SDS, 10  $\mu$ M EDTA, 20 mM NEM) and mixed with 15  $\mu$ l of 2 $\times$  non-reducing SDS-PAGE sample loading buffer (50 mM Tris-HCl pH 6.8, 10% glycerine, 2% SDS, 0.01% bromophenol blue).

For western blots against Myc-, FLAG- and His- tagged proteins, 1.0 OD<sub>600</sub> units of cells were harvested at the indicated time points, re-suspended in lysis buffer and mixed with an equal volume of 2 x SDS-PAGE sample loading buffer.

To control for specificity of anti-Prx-SO<sub>2</sub>3H antibody towards Tsa, CEN.PK113-1A wildtype,  $\Delta$ *tsa1* $\Delta$ *tsa2* and sensor-expressing cells were grown to mid log phase (OD<sub>600</sub> of 3-3.5) in fermenter media at 30 °C in a shaking incubator. About 2.0 OD<sub>600</sub> units of cells were aliquoted into eppis containing a final of 0 and 1 mM *t*-BOOH or 0 and 5 mM H<sub>2</sub>O<sub>2</sub>.

### 5.3.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins by their size. In this method, proteins are denatured and assume a negative charge due to the SDS detergent. This allows protein migration through an electric field towards the anode of the gel chamber. The network of acrylamide fibers leads to a slow migration of unfolded and large proteins whereas small or partially folded proteins run faster through the gel. Gels were casted in-house using the components shown in Table 5.6. Samples were mixed with appropriate sample buffer prior to loading in gels. Protein sizes were determined with the aid of an unstained marker from peQLab or PageRuler™ Prestained Protein Ladder from ThermoFisher Scientific. Each gel was run at 25 mA for 2 h in SDS running buffer (25 mM Tris-HCl pH 8.3, 190 mM glycine, 0.1% SDS).

### 5.3.3 Western blot transfer and detection

Proteins separated in SDS-PAGE were transferred onto a cellulose membrane using the semi-dry method. Two whatman papers (sized 17 cm x 12 cm) were briefly incubated in blotting buffer (20 mM Tris, 150 mM glycine, 0.08% SDS, 20% methanol) and placed in the blotting chamber. Nitrocellulose membrane (sized 15 cm x 10 cm) and the SDS-gel were also incubated in the blotting buffer. The cellulose membrane was placed on top of the whatman paper followed by the gel. An additional Whatman paper was placed on top of the gel and the chamber was tightly closed. All processes were performed to avoid air bubbles. Proteins were transferred onto the membrane at 200 mA for 1.5 h. Membrane-transferred proteins were visualized by staining in Ponceau S solution (0.2% (w/v) Ponceau S, 3% (w/v) acetic acid) for 2 – 5 mins.

Next, membranes were incubated reeling for 30 min in 5 % milk or BSA in 1x TBS buffer (10 mM Tris/HCl pH 7.5, 150 mM NaCl) to prevent unspecific binding of antibodies. Afterwards, the membranes were incubated with the primary antibody (as indicated below) overnight reeling at 4°C. The membranes were washed three times for 10 mins with TBS buffer. Afterwards, the membranes were incubated for at least 30 min, reeling at RT with horse reddish peroxidase-coupled secondary antibodies (anti-mouse or anti-rabbit, 1:10,000 in 5 % milk or BSA in TBS). Membranes were thereafter washed again thrice for 10 mins. Membranes were then visualized by chemiluminescence using a 1:1 ratio of ECL 1 (100 mM Tris/HCl pH 8.5, 0.044% (w/v) luminol, 0.0066% p-coumaric acid) and ECL 2 (100 mM Tris/HCl pH 8.5, 0.03% H<sub>2</sub>O<sub>2</sub>) solutions. The primary antibodies used include rabbit polyclonal  $\alpha$ -Prx-SO3 (LF-PA0004, AbFRONTIER, 1:1000 in 5 % Milk), mouse monoclonal  $\alpha$ -FLAG M2 (F3165, SIGMA, 1:500, 5 % Milk), mouse monoclonal  $\alpha$ -Myc Tag (9E11) (MAI-16637, Invitrogen, 1:500 in 5% Milk) and rabbit polyclonal  $\alpha$ -His (Herrmann lab, 1.000 in 5% BSA).

### 5.3.4 Autoradiography

Luminescent membranes were detected by autoradiography, by placing radiosensitive films (Fuji Medical X-Ray Film Super RX or Kodak BioMax MR Film) on top of the dried cellulose membrane. After the desired exposure time, the films were developed using the Optimax TR (MS Laborgeräte) machine.

**Table 5.1: Genotypes of the different *E. coli* strains used in this study**

Strain	Genotype	Use	Reference
MH1	MC1061 derivative; araD139, lacX74, <i>galU</i> , <i>galK</i> , <i>hsr</i> , <i>hsm+</i> , <i>strA</i>	Plasmid amplification	[219]
DH5 $\alpha$	K12 derivative; F <sup>-</sup> $\phi$ 80 <i>dlacZ</i> $\Delta$ M15, $\Delta$ ( <i>lacZYA-argF</i> )U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (rk <sup>-</sup> mk <sup>+</sup> ), <i>phoA</i> , <i>supE44</i> , $\lambda^-$ , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	Plasmid amplification	[220]

**Table 5.2: List of plasmids used in this study**

Name	Resistance/ Selection marker	Backbone	Insert	Reference
pYM-N14	<i>Amp<sup>R</sup>/kanMX4</i>	p413-GPD	KanMX4-GPD	[217]
pYM46	<i>Amp<sup>R</sup>/kanMX4</i>	pYM1	MYC-7His	[217]
p415GPD roGFP2-Tsa2 $\Delta$ C <sub>R</sub>	<i>Amp<sup>R</sup>/LEU2</i>	p415-GPD	roGFP2-Tsa2 $\Delta$ C <sub>R</sub>	This study
p415TEF roGFP2-Tsa2 $\Delta$ C <sub>R</sub>	<i>Amp<sup>R</sup>/LEU2</i>	p415-TEF	roGFP2-Tsa2 $\Delta$ C <sub>R</sub>	[90]
p415TEF roGFP2-Ahp1	<i>Amp<sup>R</sup>/LEU2</i>	p415-TEF	roGFP2-Ahp1	[90]
AP2099	<i>Amp<sup>R</sup>/kanMX4</i>	pCEV-Nop1	mycAID-A/Tir1-Flag	Blanche, Göttingen
pFA6 $\alpha$ - <i>natNT2</i>	<i>Amp<sup>R</sup>/natNT2</i>	pEG202	natNT2	[217]
pFA6 $\alpha$ - <i>hphNTI</i>	<i>Amp<sup>R</sup>/hphNTI</i>	p425-GalI	hphNTI	[217]
pFA6 $\alpha$ - <i>kanMX4</i>	<i>Amp<sup>R</sup>/kanMX4</i>	pYM3	kanMX4	[221, 222]
PHUK	<i>Amp<sup>R</sup></i>	N/A	HUK	Addgene
PHLUK	<i>Amp<sup>R</sup></i>	N/A	HLUK	Addgene

**Table 5.3: List of primers used in this study**

Primer name	5' → 3' Sequence
P1	GGTCCC GCGGCTAGCCGTACGCTGCAGGTCG
P2	GGCAAAGGGCTAGCCATCGATGAATTCTCTGTCG
P3	CCATTTAATTCCACCTTCGGATTTTTTTGCATAAACTCTCAATTT CCCCGCGATCCATCTGCCGAATGCGTACGCTGCAGGTCGAC
P4	CGTTCCTGTCAATCTAAGACTTGAAGATTACAGGACTTTTTTTTT TCTTACTGTATTTTTCCGTAGAGTGGTACCGGCCGCAAATTAAG G
P5	CGATTTGTTGATTCTATCCCAAGATTGAG
P6	CTCTTTCAGCCTTGTTTGATGGTGTAGATAAAACC
P7	GAAATTTCAACAAACCAGAACAACAAGTACTACCAATAACC ACAACAAAACATGCTTCGAGCGTCCCAAAACC
P8	GCAATGTATTGGAATTTGTAGTCGCCAGCTGGGAATTTCTTGTT AACTAAGTCAGAACCTGATACCTTCACGAACGC
P9	TTTCTGATTTGTAATTATACGGGGA
P10	ATTTTGTTCGAAACGCATATAATGT
KanMX4 confirm FWD	TGATTTTGATGACGAGCGTAAT
KanMX4 confirm REV	CTGCAGCGAGGAGCCGTAAT
HphNTI confirm FWD	GGCTTGTATGGAGCAGCAGAC
HphNTI confirm REV	CAAAGCATCAGCTCATCGAGAG
natNT2 confirm FWD	GCGCTCTACATGAGCATGCC
natNT2 confirm REV	CATCCAGTGCCTCGATG
Tsa1-S1	CTCGTTCAATTGCTCACAACCAACCACAACACTACATACACATACA TACACAATGcgtacgctgcaggtcgac
Tsa1-S2	CGTAAAGAGTGAATTTTAAATAAGTAGTCATTTAGACAACCTCTG CAAGCGTCTTAATCGATGAATTCGAGCTCG
Tsa1-S3	CCATCAAGCCAACCGTTGAAGACTCCAAGGAATACTTCGAAGC TGCCAACAAACGTACGCTGCAGGTCGAC
Tsa1 confirm FWD	TGCGTTTAAGGTGTACGAAAACCC
Tsa1 confirm REV	GGTTTACGCGTTTTAGAGCCAGAC
Tsa2-S1	CACTATTACTGTTTTTTGCTCAAGAATATATTAGCCTTACAAGA ACGTAAAAAACCAATCATG cgtacgctgcaggtcgac
Tsa2-S2	CATATATAGGGTGTATTTTTTAATTATTTAATAGGGCCTAGC GTTATCGTGCGAAGATTAatcgatgaattcgagctcg
Tsa2 confirm FWD	GTTACCCGAGTAATCAAGGATCAACTATGG

Tsa2 confirm REV	GGTCATTTGCGTCTTCTGGATATAAAGATG
Ahp1-S1	CGAAATTTCAACAAACCAGAACAACACAAGTACTACCAATAAC CACAACAAAACATGcgtacgctgcaggtcgac
Ahp1-S2	CCTTGTACAGAATCGTTTTCTATTTTGAATTTTTTTATATAAAC ATGGTTTTATTGTCTATTACATAGCATCTAatcgatgaattcgagctcg
Ahp1 confirm FWD	TTTCTGATTTGTAATTATACGGGGA
Ahp1 confirm REV	ATTTTGTTCGAAACGCATATAATGT
Prx1-S1	GTGCTTCTAGATTCTCGCAGTAGGATGAGATAAATTTCAAAGA AGCAGGAAGCAAAGGATGCGTACGCTGCAGGTCGAC
Prx1-S2	GATAAAAGTTTTAGTTAGAGATACTTCATATACCTGTATATAGT AAAGTCGTTTTATTCAAAGCTTAATCGATGAATTCGAGCTCG
Prx1 confirm FWD	GTTTATCTTTATACAATATACAAAAGGTCACCCAG
Prx1 confirm REV	GGTCTTGGTCAGAATCTCGATTATC
Sod1-S1	GGAAAAACAGGCAAGAAAGCAATCGCGCAAACAAATAAAACA TAATTAATTTATAATGCGTACGCTGCAGGTCGAC
Sod1-S2	GCGCTTACTACTTACTTACATACGGTTTTTATTCAAGTATATTAT CATTAAACATTAATCGATGAATTCGAGCTCG
Sod1 confirm FWD	GTAAACCGGTGTGTCCGAATTAGTAAG
Sod1 confirm REV	CTGGAACCATCAAGACCGTTTTG
Yno1-S1	ATATCTCGTCGCCAGAACTTCTAATTTGGTAAGCCTTCCAATA AATATGCGTACGCTGCAGGTCGAC
Yno1-S2	ATTTCCGAGCATATTGCGTAAGACATTATTATTCTTTTCTTTTCC CCTCAATCGATGAATTCGAGCTCG
Yno1 confirm FWD	GATCGCTGCTCCGTAATACCGATATAC
Yno1 confirm REV	CCGATTAATAATGCGTACAACCTGTCAAG
Trx1-S1	CCCTGAAACTGCATTAGTGTAATAGAAGACTAGACACCTCGAT ACAAATAATGCGTACGCTGCAGGTCGAC
Trx1-S2	TATAACAAACACAGTATAGAAACACAATATATCGGTCATTGGG TGAGTTAATCGATGAATTCGAGCTCG
Trx1 confirm FWD	CGATATGTATATTCTTTTCGTTGGAAAAGATGTC
Trx1 confirm REV	CCTCTTGTGTGAAAAATTAATTGTTTCCTCC
Trx2-S1	GAATTATACACGCACACATACACGAGAGTCTACGATATCTTTA AATAACACATCAATAATGcgtacgctgcaggtcgac
Trx2-S2	GTTTATTTAAACTGGTAAACATGATGTACTTTACGTAGCGTTAA TATACCGGCAACTAatcgatgaattcgagctcg
Trx2 confirm FWD	CTCCCTACAAGGTGGCTCTTTTCTTACTAAGC

Trx2 confirm REV	CAAAGGTGCAGAAAGCTGCACCTTGTAAG
Cox6-S1	CGAACAATTGTATTTGACACATAAACTAATAAATATACAACAA TGCGTACGCTGCAGGTCGAC
Cox6-S2	ACAACAAATTACAGACGTTGTGTGGTAGCTTTTTCCTTATTATT AATCGATGAATTCGAGCTCG
Cox6 confirm FWD	GCCAGATCTCAAGGTTACCTCATTTTC
Cox6 confirm REV	TTCTGAGTGGATGAATATCCATAAAGGG

**Table 5.4: Genetic composition of yeast strains used in this study**

Strain	Genotype	References
CEN.PK113-1A	<i>MAT<math>\alpha</math></i>	P. Kötter, Frankfurt
CEN.PK113-7D	<i>MAT<math>\alpha</math></i>	P. Kötter, Frankfurt
BY4741	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math>0 met15<math>\Delta</math>0 ura3<math>\Delta</math>0</i>	Euroscarf
BY4742	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0</i>	Euroscarf
$\Delta$ <i>prx1</i>	CEN.PK113-1A <i><math>\Delta</math>prx1::hphNT1</i>	This study
$\Delta$ <i>tsa1</i>	CEN.PK113-1A <i><math>\Delta</math>tsa1::hphNT1</i>	This study
$\Delta$ <i>tsa2</i>	CEN.PK113-1A <i><math>\Delta</math>tsa2::natNT2</i>	This study
$\Delta$ <i>tsa1<math>\Delta</math><i>tsa2</i></i>	CEN.PK113-1A <i><math>\Delta</math>tsa1::hphNT1 <math>\Delta</math>tsa2::natNT2</i>	This study
$\Delta$ <i>tsa1<math>\Delta</math><i>tsa2</i></i>	BY4742 <i><math>\Delta</math>tsa1::natNT2 <math>\Delta</math>tsa2::kanMX4</i>	[90]
$\Delta$ <i>ahp1</i>	CEN.PK113-1A <i><math>\Delta</math>ahp1::hphNT1</i>	This study
$\Delta$ <i>ahp1</i>	CEN.PK113-7D <i><math>\Delta</math>ahp1::kanMX4</i>	This study
$\Delta$ <i>ahp1</i>	BY4741 <i><math>\Delta</math>ahp1::kanMX4</i>	Euroscarf
CEN.PK T2- $\Delta$ C <sub>R</sub>	CEN.PK113-1A <i>kanMX4-P<sub>GPD</sub>-roGFP2-Tsa2<math>\Delta</math>C<sub>R</sub></i>	This study
WT::AID-AHP1	CEN.PK113-1A <i>Af-Tir1-FLAG-Myc-kanMX4-AID-AHP1</i>	This study
$\Delta$ <i>tsa1</i> ::AID-AHP1	CEN.PK113-1A <i><math>\Delta</math>tsa1::hphNT1 Af-Tir1-FLAG-Myc-kanMX4-AID-AHP1</i>	This study
$\Delta$ <i>tsa2</i> ::AID-AHP1	CEN.PK113-1A <i><math>\Delta</math>tsa2::natNT2 Af-Tir1-FLAG-Myc-kanMX4-AID-AHP1</i>	This study
$\Delta$ <i>tsa1<math>\Delta</math><i>tsa2</i>::AID-AHP1</i>	CEN.PK113-1A <i><math>\Delta</math>tsa1::hphNT1 <math>\Delta</math>tsa2::natNT2 Af-Tir1-FLAG-Myc-kanMX4-AID-AHP1</i>	This study
$\Delta$ <i>sod1</i>	CEN.PK113-1A <i><math>\Delta</math>sod1::hphNT1</i>	This study
$\Delta$ <i>yno1</i>	CEN.PK113-1A <i><math>\Delta</math>yno1::natNT2</i>	This study
$\Delta$ <i>cox6</i>	BY4742 <i><math>\Delta</math>cox6::kanMX4</i>	[90]
$\Delta$ <i>snf1</i>	CEN.PK113-1A <i><math>\Delta</math>snf1::kanMX4</i>	This study

**Table 5.5: Composition of Hartwell's Complete (HC) media for growth of plasmid transformed yeast strains**

HC Media		Dropout mix (DOM)	
Component	Amount/L (ml)	Amino acids	10x (g/l)
10 x DOM	100	Methionine	0.2
10 x YNB	100	Tyrosine	0.6
ml 40% Glucose	50	Isoleucine	0.8
1g/l Uracil	35	Phenylalanine	0.5
1g/l Adenine	20	Glutamic acid	1.0
10g/l Lysine	12	Threonine	2.0
10g/L Tryptophan	8	Aspartic acid	1.0
20g/l Leucine	4	Valine	1.5
10g/l Histidine	2	Serine	4.0
milliQ-H <sub>2</sub> O	669	Arginine	0.2

**Table 5.6: Composition of SDS-PAGE gels**

Gel	Composition
Running gel	16% acrylamide/14% acrylamide 0.11% bisacrylamide 375 mM Tris-HCl pH 8.8 0.1% SDS 0.1% ammonium persulfate (APS) 0.03% N,N,N',N'-Tetramethylethylenediamine (TEMED)
Stacking gel	5% acrylamide 0.03% bisacrylamide 60 mM Tris-HCl pH 6.8 0.1% SDS 0.05% APS 0.1% TEMED
Base gel	20% acrylamide 0.13% bisacrylamide 375 mM Tris-HCl pH 8.8 0.1% SDS 0.05% APS 0.1% TEMED

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# ABBREVIATIONS

°C	Grade Celsius
µg	Microgram
µl	Microliter
µM	Micromolar
AID	Auxin inducible degron
BSA	Bovine serum albumin
CDC	Cell division cycle
Cdk	Cyclin dependent kinase
CIP	Calf intestinal phosphatase
C <sub>P</sub>	Peroxidactic cysteine
C <sub>R</sub>	Resolving cysteine
Cry	Cryptochrome
Cyc	Cytochrome
Cys	Cysteine
DAPI	4,6-diamidino-2-phenylindole
Diamide	N,N,N',N'-tetramethylazodicarboxamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetate
ETC	Electron transport chain
Grx	Glutaredoxin
GSH	Glutathione
h	Hours
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HC	Hartwell's Complete
HOC	High oxygen consumption
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid
IAA	Indole-3-acetic acid
kb	Kilobase
kDa	Kilodalton
L or l	liter

LB	Lysogeny broth media
LOC	Low oxygen consumption
M	Molarity
mg	Milligram
milliQ-H <sub>2</sub> O	Double distilled water
min	Minute
ml	Milliliter
mM	Millimolar
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NEM	N-Ethylmaleimide
nm	Nanometer
OD <sub>600</sub>	Optical density at 600 nm
OxD	Degree of oxidation
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Per	Period
PKA	Protein kinase A
Prx	Peroxiredoxin
RNA	Ribonucleic acid
RNR	Ribonucleotide reductase
roGFP2	Redox sensitive green fluorescent protein 2
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
s	Seconds
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>S. pombe</i>	<i>Saccharomyces pombe</i>
SDS	Sodium dodecyl sulfate
SOD (or Sod)	Superoxide dismutase
<i>t</i> -BOOH	<i>tert</i> -butyl hydroperoxide
TAE	Tris acetate EDTA
TBS	Tris buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
Tim	Timeless

Tpx	Thioredoxin peroxidase
Tris	Tris-(hydroxymethyl)-aminomethane
Trx	Thioredoxin
TrxR	Thioredoxin reductase
Tsa	Thiol specific antioxidant
TTFL	Transcription-translation feedback loop
U	Units
UV	Ultraviolet
w/v	Weight per volume
YMC	Yeast Metabolic Cycle
Yno1	Yeast NADPH oxidase 1

# ACKNOWLEDGEMENTS

The journey of a thousand miles, it is said, begins with one step. This is how far the steps have brought me. I am most grateful to God Almighty, without whom it would have been impossible for me to pursue my PhD. I am grateful to my wife, Joyce Amponsah, for the moral support to carry me through the course of this study. Joyce, you understood and cooperated with me during my late-night stays in the lab, considering the strain it would have had on our relationship, for this, I am most grateful. Now to every member of the Morgan and Herrmann labs who have contributed immensely to this feat, I am indebted in gratitude to you. I appreciate all your assistance in diverse ways, and apologize in advance if I am unable to mention here your individual direct contributions.

Dear **Bruce**, you are an amazing supervisor, and I speak plainly, when I say so. I am very grateful for your mentorship and guidance right from our time in Heidelberg to Kaiserslautern and to Saarbrücken. When you asked me to join you in Kaiserslautern for PhD, though I was initially hesitant due to the influence around Heidelberg, I defied the odds to pursue this mission. I am grateful for the opportunity because it paid off. You gave me the flexibility to build my knowledge in the field by attending conferences and presenting my data at any opportunity. The topic was challenging but you helped me through it. The success story is evidenced in the many awards and recognitions I have received over the period. I owe you tones of gratitude. On the other hand, I hope I have fulfilled my part of the bargain, when I promised to join you to form an unbeatable team ☺. I can boldly say your “second place is the first loser” catch phrase is ingrained in my character and propels me to aim higher. Thank you very much Bruce.

Dear **Hannes**, I am very grateful to you for your support throughout my PhD. You challenged me to aim higher. You made me perform experiments that were “near impossible” and I was always ready to rise to the occasion. These acts have made me a better scientist, for which I am very grateful. You were technically my second supervisor ☺ and your inputs into this project are enormous. Thank you for also allowing me stay around to share the oxygen in your lab when the Morgan group moved to Saarbrücken. I am also grateful to you for accepting to be the ‘Vorsitz’ for my thesis examination.

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Thank you **Vera Nehr** for providing me with technical assistance on this project. Thank you **Andrea Trinkhaus** for being my mum in the lab. I acknowledge your help in getting an apartment to stay in when I was almost stranded. Your efforts helped my wife and I have a home to live in. I am happy to make you an Oma in the near future ☺.

Many thanks to **Simone Adkins**, the best Secretary in the world! You were readily available to solve all my problems ☺. Thank you for help with all my documentations and German translations. When it became difficult for my wife to join me in Kaiserslautern, you made all the calls and wrote all the necessary letters. God bless you!

I am especially grateful to **Felix Boos** for helping to translate the summary of this thesis into the German language. Thank you very much Felix!

To the rest of the Hannes lab, past and present, I am very grateful to you all for your diverse support throughout this project and my stay in Kaiserslautern. Thank you for your criticisms and contributions. Thank you for the cakes ☺ and times we shared together. Thank you **Connie** for making the lab wares ready for experiment and giving me the opportunity to interrupt your work whenever I needed to use the autoclave. Thank you **Sabine** for being an amazing Technician. Thank you **Katja** for learning to be a 'good wife' and showing me what a 'good husband' should do ☺. Thank you **Sree** for being a great friend and colleague. Together with **Gurleen**, you helped prepare my apartment for habitation, for which I owe you two much gratitude. Thank you **Anna** for allowing me to 'de-stress' on you. It was a pleasure to scare you every day, 'Schlagoushi'! Thank you **Sandra** for making me not see you in... 2 years? Thank you **Janina** for being a trustworthy friend and colleague. You will forever remain my 'schatzi' ☺. Thank you **Eva** and **Katharina** for being great colleagues. Thank you **Lena** and **Carina** for being great companions during the final phase of my studies. It was a pleasure to share lab space with you two amazing ladies. You shared my responsibilities in the lab, without me asking, a gesture I really appreciate. Thank you **Vale**, **Michael** and **Ajay** for crossing paths with me in this scientific journey.

To my family and friends in Germany and Ghana, thank you for your kind support. Your prayers and encouragements have made this feat attainable. God bless you all. I also acknowledge financial support from the DFG Priority Program SPP1710, EMBO, TU Nachwuchsring, GlaxoSmithKline and the DAAD for my participation at International conferences.

# CURRICULUM VITAE

**Prince Saforo Amponsah**

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## Education

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01/2016 – 01/2020	<b>Ph.D.</b> , Technische Universität Kaiserslautern Thesis lab: Cellular Biochemistry, Group of Prof. Dr. Bruce Morgan Thesis title: “ <i>PEROXIREDOXINS: Novel mediators of cellular timekeeping</i> ”
10/2013 – 11/2015	<b>Master of Science</b> , Ruprecht-Karls-Universität Heidelberg Course: Molecular Biosciences, Major: Cancer Biology Thesis lab: Molecular OncoSurgery, Universitätsklinikum Heidelberg and German Cancer Research Center (DKFZ) Heidelberg, Group of Prof. Dr. Ingrid Herr Thesis title: “ <i>microRNA regulation of gemcitabine resistance in pancreatic cancer</i> ”
08/2008 – 05/2012	<b>Bachelor of Science</b> , University of Ghana, Legon Specialization: Biochemistry Thesis supervisor: Dr. Kwadwo Asamoah Kusi (Immunology Department, Noguchi Memorial Institute for Medical Research) Thesis title: “ <i>Relationship between avidity and functionality of anti-malarial antibodies from Plasmodium falciparum-exposed individuals</i> ”
2004 – 2007	<b>High School Education/West Africa Senior School Examination</b> , Keta Secondary School

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## Further Education

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09/2011 – 12/2011	<b>CUSAC Semester abroad program</b> at Carleton University, Ottawa (ON), Canada
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## Scholarships, Awards and Prizes

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15/09/2019	<b>SPP1710 Young Researchers Travel Grant</b> , Thiol-based switches and redox regulation - from microbes to men, St. Feliu de Guixols, Spain
29/05/2019	“ <b>Kongressreisen Stipendien</b> ” of the DAAD, Bonn, Germany
11/04/2019	<b>Best Poster Prize</b> , Nature Conference on Cellular Metabolism, Xiamen, China
27/02/2019	“ <b>Mobilitätsförderung</b> ” (Travel Grant) of the TU Nachwuchsring, TU Kaiserslautern, Germany
28/06/2018	“ <b>Mobilitätsförderung</b> ” (Travel Grant) of the TU Nachwuchsring, TU Kaiserslautern, Germany
22/06/2018	<b>Best Talk Prize</b> , Young Researchers Symposium 2018, Fraunhofer-Center Kaiserslautern, Germany
08/02/2018	<b>Best Poster Prize</b> , Emerging concepts in Mitochondrial Biology Conference, Weizmann Institute of Science, Rehovot, Israel

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20/12/2017	<b>Travel Grant</b> , GlaxoSmithKline, München, Germany
21/09/2017	<b>Best Elevator Talk Prize</b> , EMBO Workshop on Thiol oxidation in toxicity and signaling, Sant Feliu de Guixols, Spain
30/06/2017	<b>EMBO Travel Grant</b> (Registration Fee Waiver)
05/07/2016	<b>Best Poster Prize</b> , 3rd Meeting of the study group Redox Biology of the “Gesellschaft für Biochemie und Molekularbiologie” (GBM), Düsseldorf, Germany
08/2015 – 02/2016	<b>Study grant</b> for Master’s thesis, “Gesellschaft der Freunde Universität Heidelberg eV” (GdF), Heidelberg, Germany
28/05/2014	<b>Public Prize for Best Poster Presentation</b> , Young Researchers in Life Sciences Conference, Paris, France
09/2011 – 12/2011	<b>Bursary for Academic Exchange</b> in Canada, Commonwealth Universities Study Abroad Consortium (CUSAC), London, United Kingdom
06/05/2011	<b>Alumni and Shell prizes</b> for Best Level 200 Science Student, University of Ghana, Legon
08/2009 – 05/2011	<b>Academic Sponsorship</b> , Kapadia Education Foundation (for Bachelor’s degree in University of Ghana, Legon)
09/2004 – 06/2007	<b>Government of Ghana Academic Merit Award</b> , Keta Secondary School, Ghana

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## List of Publications

- Amponsah PS, Metwally G, Mergel S, Storchova Z and Morgan B (2019). Peroxiredoxins couple metabolism and cell division in an ultradian clock. *Nat Chem Biol* (under revision)
- Calabrese G, Peker E, Amponsah PS, Hoehne MN, Riemer T, Mai M, Deponte M, Morgan B, Riemer J (2019). Hyperoxidation of mitochondrial peroxiredoxin limits H<sub>2</sub>O<sub>2</sub>-induced cell death in yeast. *EMBO J* 38(18):e101552
- Amponsah PS, Fan P, Bauer N, Zhao Z, Gladkich J, Fellenberg J, Herr I (2017). microRNA-210 overexpression inhibits tumor growth and potentially reverses gemcitabine resistance in pancreatic cancer. *Cancer Lett* 388: 107-117
- Amponsah PS (2016). Cellular redox – living chemistry. *Science in School* 36: 15-17
- Morgan B, Van Laer K, Owusu TN, Ezeriņa D, Pastor-Flores D, Amponsah PS, Tursch A, Dick TP (2016). Real-time monitoring of basal H<sub>2</sub>O<sub>2</sub> levels with peroxiredoxin-based probes. *Nat Chem Biol* 12(6): 437-43
- Fan P, Liu L, Yin Y, Zhao Z, Zhang Y, Amponsah PS, Xiao X, Bauer N, Abukiwan A, Nwaeburu CC, Gladkich J, Gao C, Schemmer P, Gross W, Herr I (2016). MicroRNA-101-3p reverses gemcitabine resistance by inhibition of ribonucleotide reductase M1 in pancreatic cancer. *Cancer Lett* 373(1):130-7

# DECLARATION

I hereby declare that this thesis is a record of bonafide work carried out by me, under the supervision of Prof. Dr. Bruce Morgan, for the award of a Doctorate degree at the Technische Universität Kaiserslautern. No other sources or aids for assistance, other than those specified, were used in the writing of this thesis.

I further declare that the work reported in this thesis has not been submitted and will not be submitted, either in part or full, for the award of any other degree or diploma in this institute or any other institute or University.

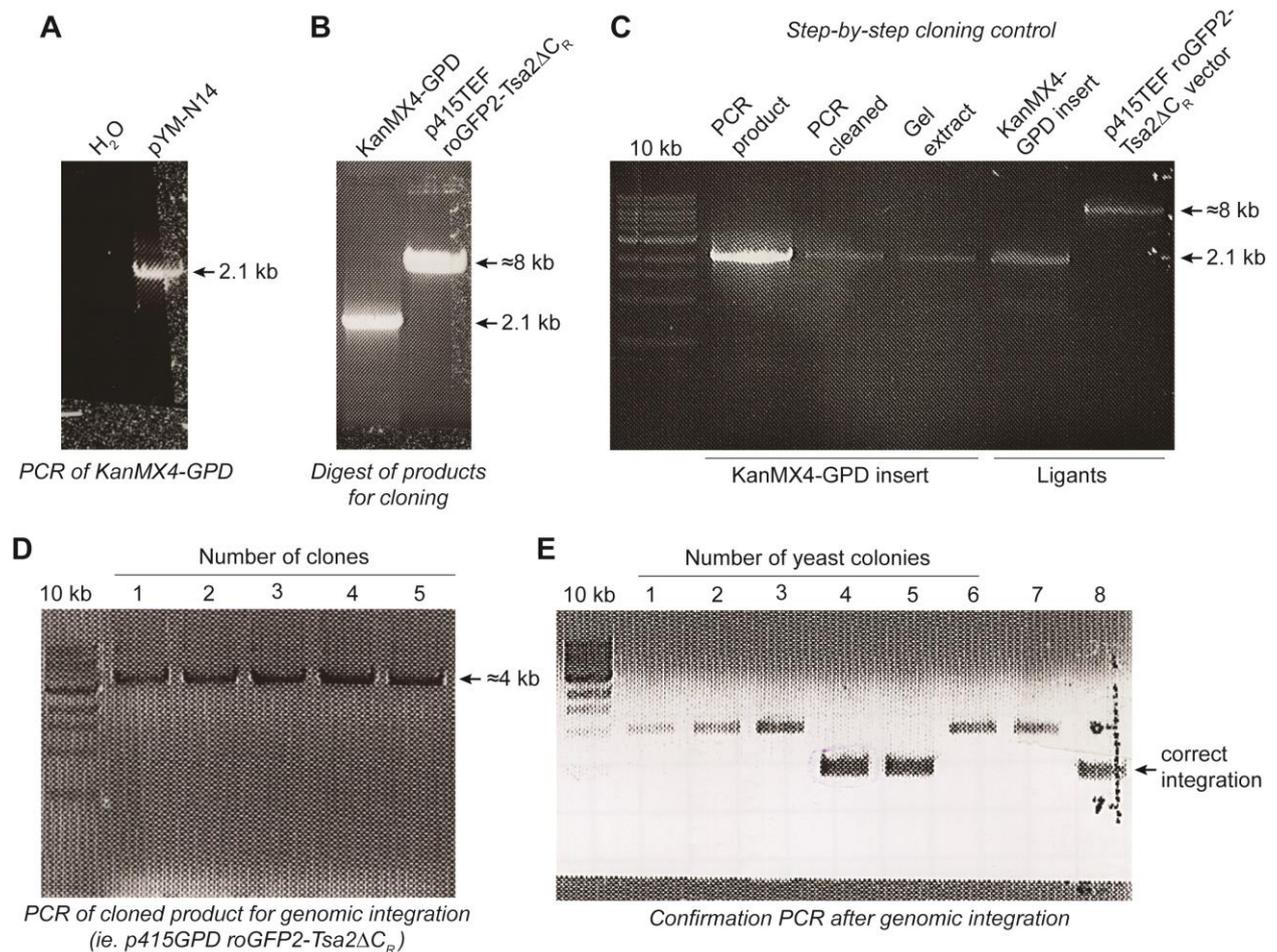
**Prince Saforo Amponsah**

Kaiserslautern, 28.11.2019

# APPENDIX

## A1: Construction of a genomically integrated roGFP2-Tsa2 $\Delta$ C<sub>R</sub> expressing yeast strain

### A1.1: PCR amplifications, cloning and confirmation of engineered yeast



Agarose gel showing:

- KanMX4-GPD PCR product amplified from the pYMN-14 plasmid.
- Digest of KanMX4-GPD PCR product and p415TEF roGFP2-Tsa2 $\Delta$ C<sub>R</sub> plasmid with appropriate restriction enzymes, for subsequent ligation.
- Step-by-step control of KanMX4-GPD roGFP2-Tsa2 $\Delta$ C<sub>R</sub> plasmid construction process.
- Confirmation of constructed KanMX4-GPD roGFP2-Tsa2 $\Delta$ C<sub>R</sub> plasmid by PCR.
- Confirmation by PCR, of yeast cells showing successful genomic integration of the KanMX4-GPD roGFP2-Tsa2 $\Delta$ C<sub>R</sub> biosensor construct.

**A1.2: Sequence map of re-constituted p415 roGFP2-Tsa2ΔCr plasmid for PCR and genomic integration (5' → 3')**

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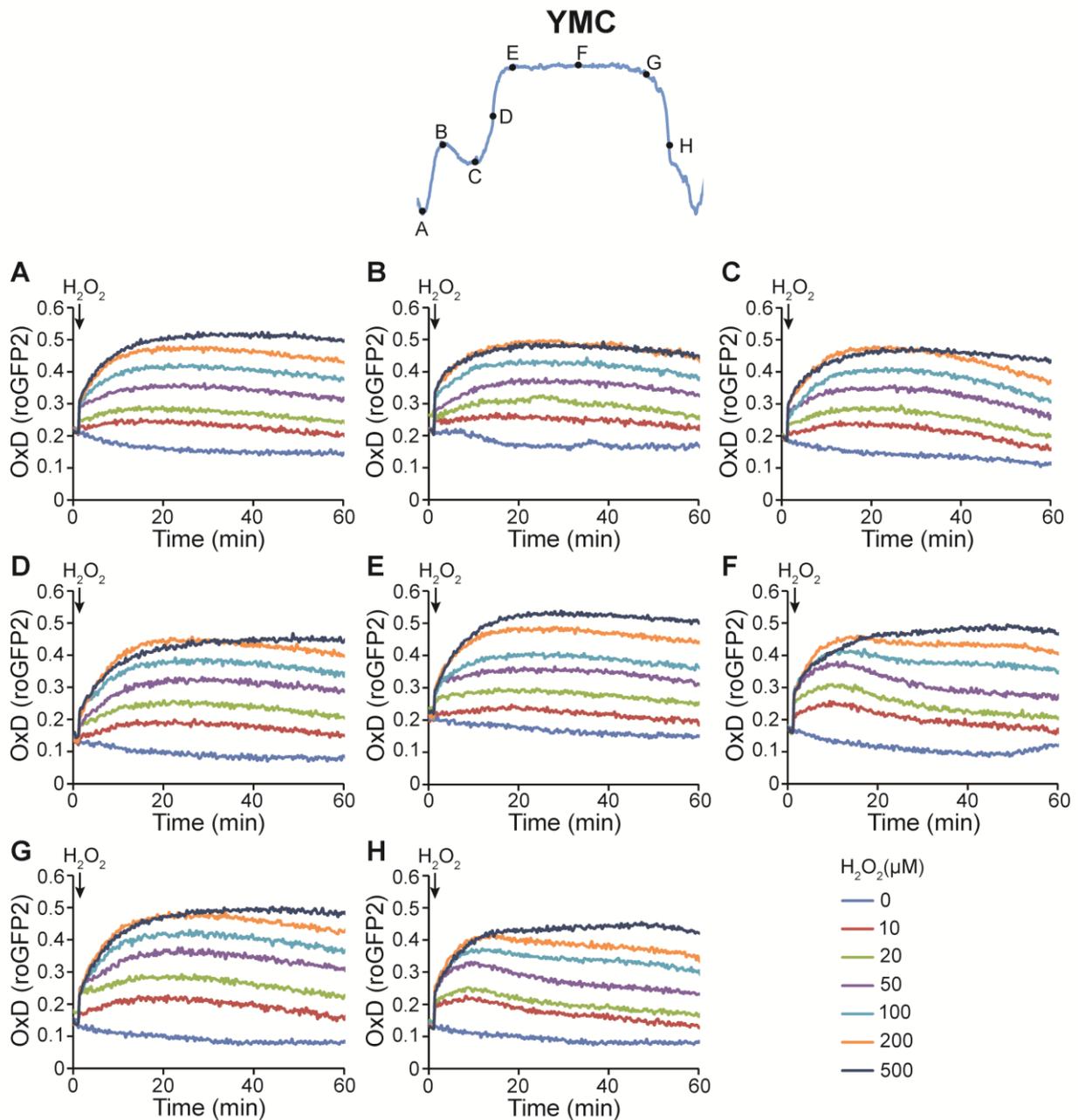
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CAGATTTGCCAAAGAATAAGGTTGACCCTATCGCCACTATCTTGTCTGCTGCAATGATGTTG  
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GAGGATAGTAAAGGAATACAGGTAAGCAAATTGATACTAATGGCTCAACGTGATAAGGAAA  
AGAATTGCACTTTAACATTAATATTGACAAGGAGGAGGGCACCACACAAAAAGTTAGGTG  
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AAAAATACAACAAATAAAAAACACTCAATGACCTGACCATTTGATGGAGTTTAAGTCAATA  
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ATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTACCCGT  
CATCACCGAAACGCGCGA

**Colour codes:**

-  KanMX4 marker
-  GPD promoter
-  roGFP2 domain
-  Linker domain
-  Tsa2 $\Delta$ C<sub>R</sub> domain
- Binding sites for primers P3 and P4
-  Restriction sites

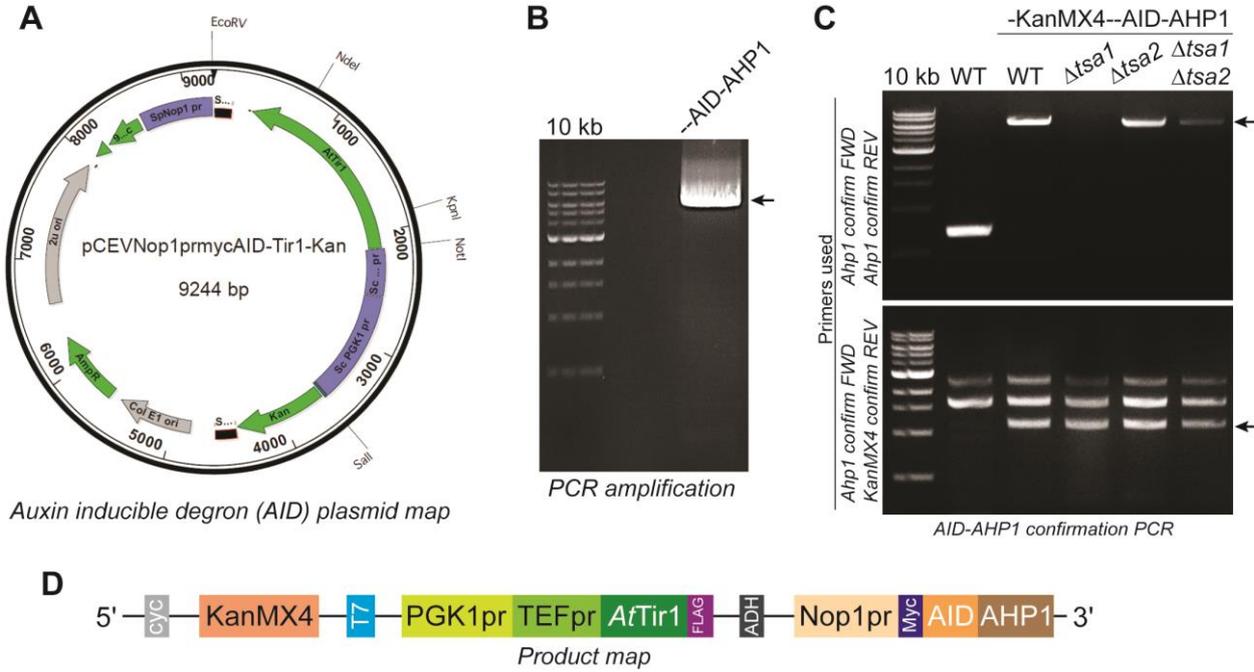
## A2: Confirmation of functional roGFP2-Tsa2ΔC<sub>R</sub> biosensor under continuous culture conditions in the fermenter



RoGFP2-Tsa2ΔC<sub>R</sub> biosensor expressing cells sampled from various phases of the YMC respond to H<sub>2</sub>O<sub>2</sub> in a concentration dependent manner.

### A3: Construction of yeast strains capable of conditional genomic Ahp1 depletion via an auxin-inducible degron (AID) system

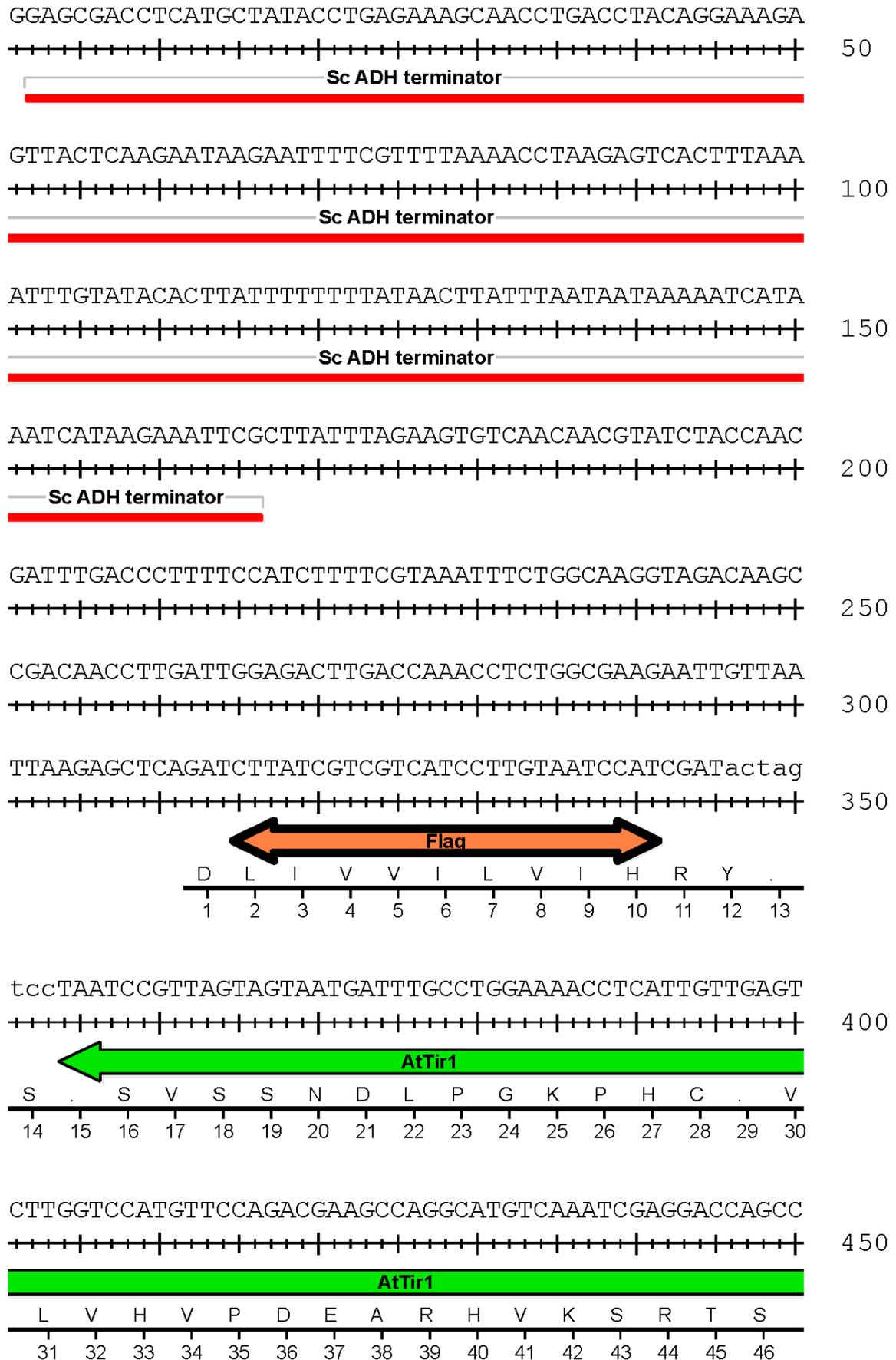
#### A3.1: PCR amplifications, cloning and confirmation of engineered yeast



- A. Map of AID/A/TIR1 plasmid.
- B. Agarose gel showing PCR product from amplification of the AID/A/TIR1 plasmid for genomic integration.
- C. Agarose gel showing confirmation by PCR, of various yeast strains showing successful genomic integration of the Ahp1-AID/A/TIR1 construct.
- D. Product map of the genomically integrated construct

**A3.2: Sequence map of AP2099 (pCEVNop1prmycAID-Tir1-Kan) as provided by Prof. Dr.**

**Blanche Schwappach (5' → 3')**

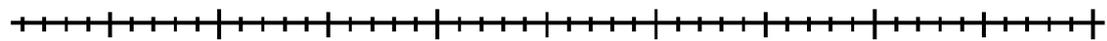








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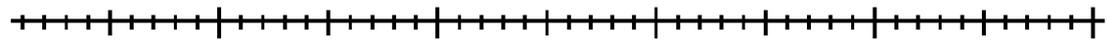
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**AtTir1**

R E R Y R . T R V N I W S H F C S

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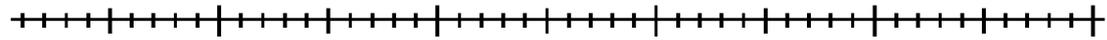
1450

**AtTir1**

V P T S A Q F L Q L R C S L K . S

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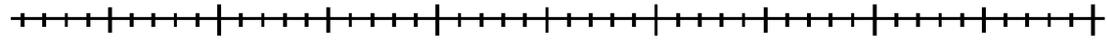
1500

**AtTir1**

S Q F F K W N S S V K L E R L E

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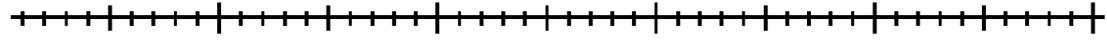
1550

**AtTir1**

I G T P S H Q P F Q S R E T D L R

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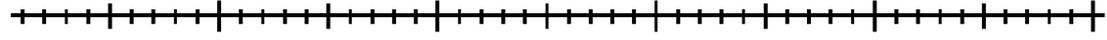
1600

**AtTir1**

C . A R Y I E . Y Q R S V C I W E

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1650

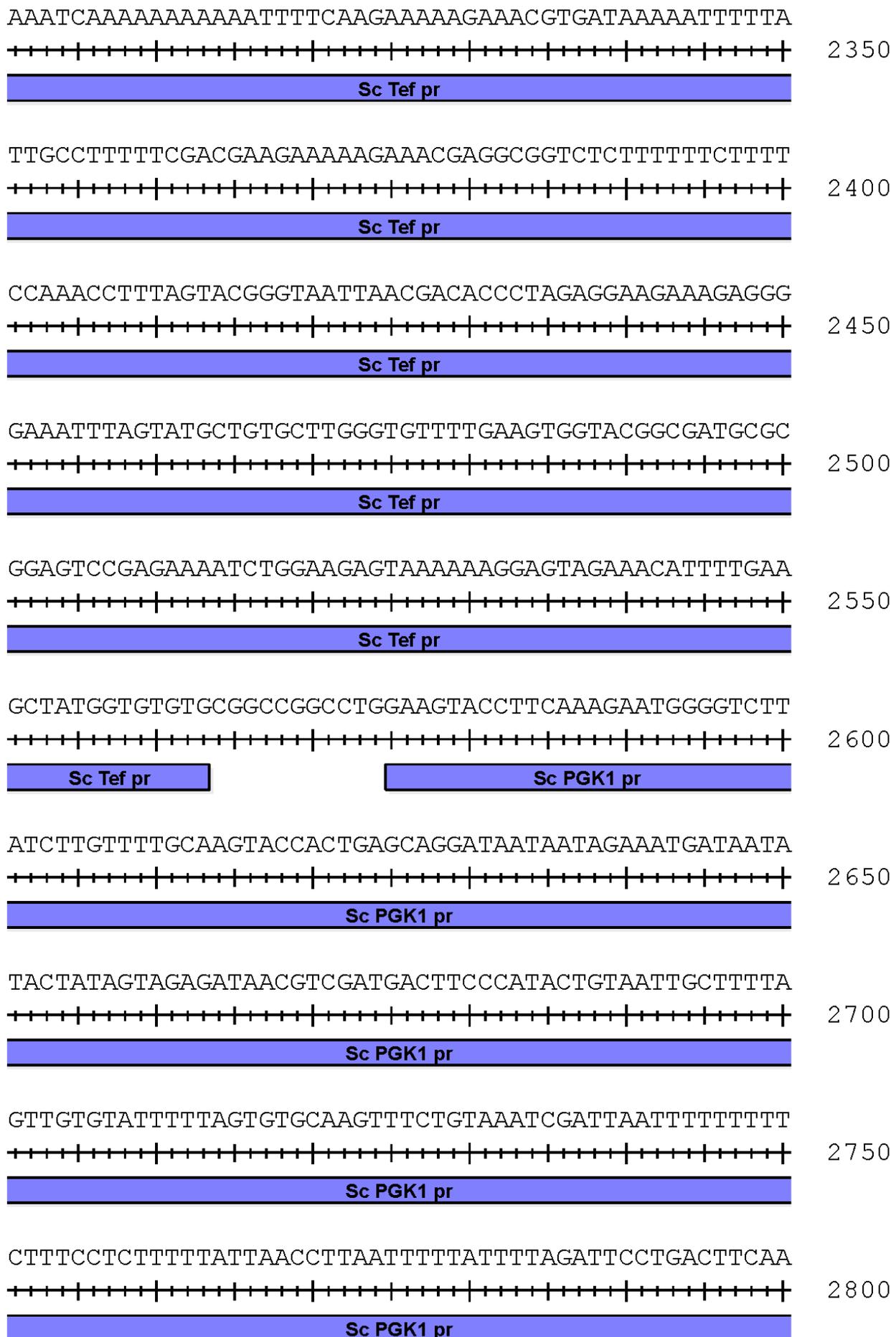
**AtTir1**

M A K P V A T N V V N I T L S .

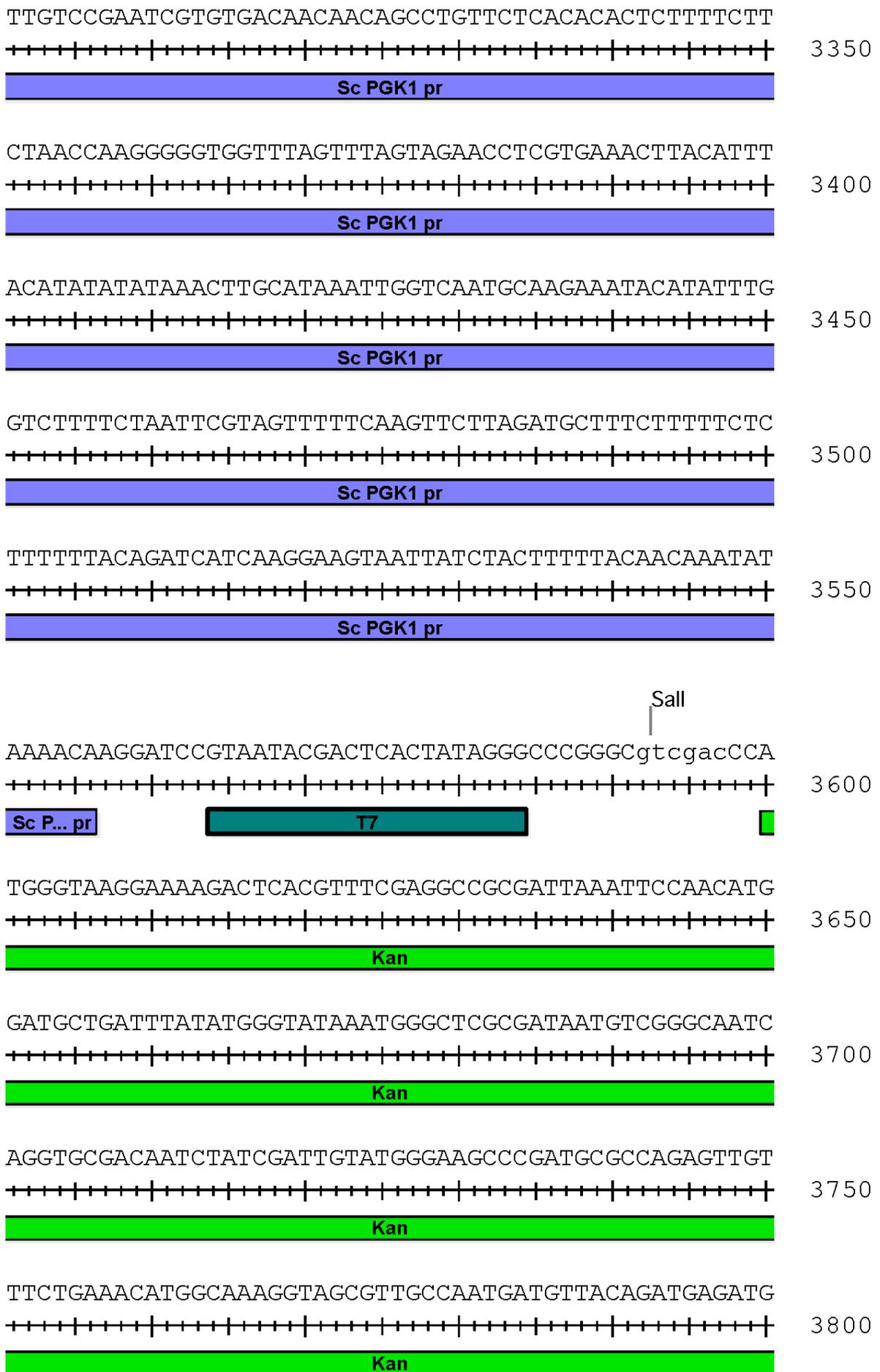
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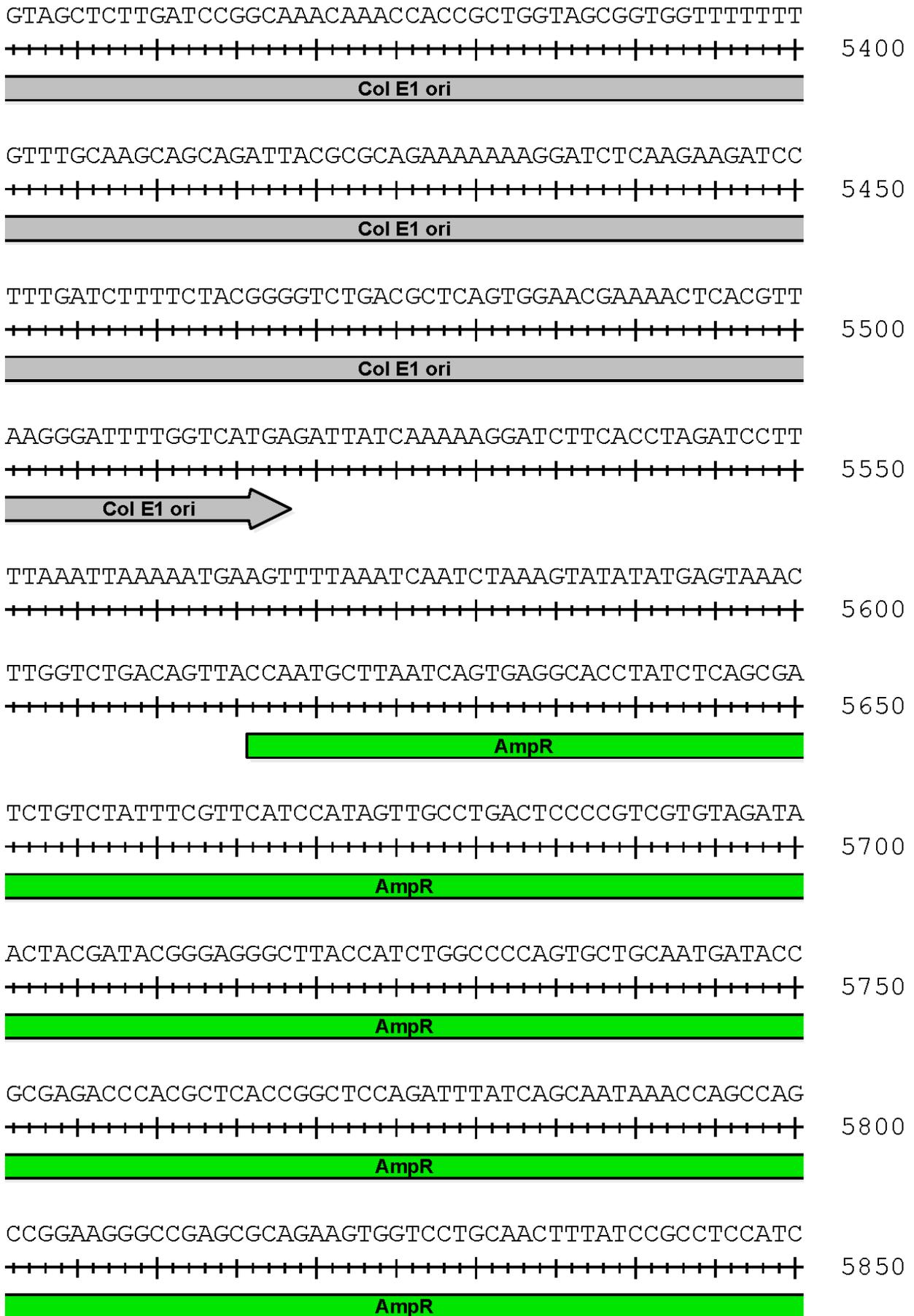
















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2u ori

AAGGTTAGAAGAAGGCTACTTTGGTGTCTATTTTCTCTTCCATAAAAAAA 7050

2u ori

GCCTGACTCCACTTCCCGCGTTTACTGATTACTAGCGAAGCTGCGGGTGC 7100

2u ori

ATTTTTTCAAGATAAAGGCATCCCCGATTATATTCTATACCGATGTGGAT 7150

2u ori

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2u ori

TCAGAAAATTATGAACGGTTTCTTCTATTTTGTCTCTATATACTACGTAT 7250

2u ori

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2u ori

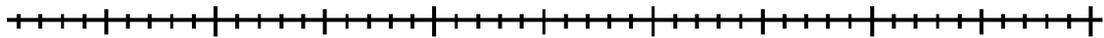
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2u ori

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2u ori

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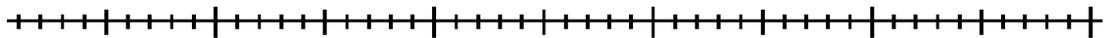


7500



2u ori

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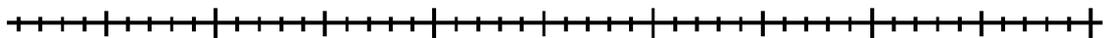


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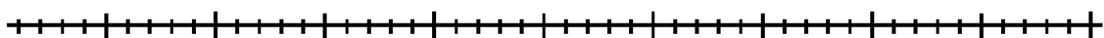


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2u ori

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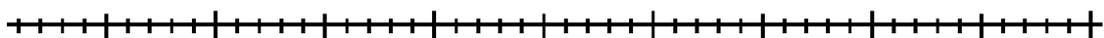


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2u ori

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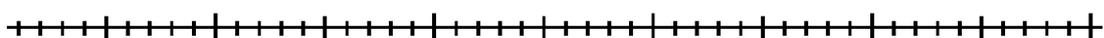


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2u ori

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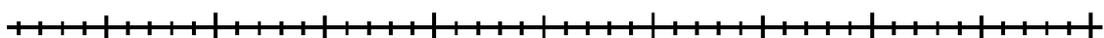


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2u ori

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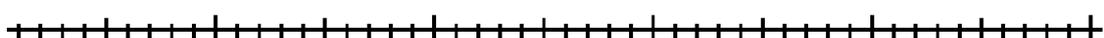


7800



2u ori

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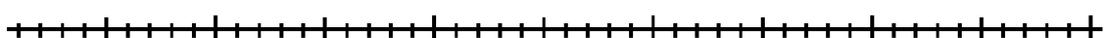


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2u ori

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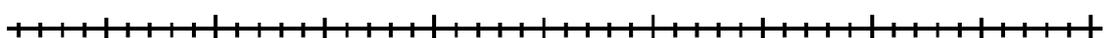


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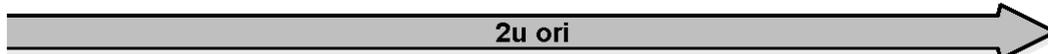


2u ori

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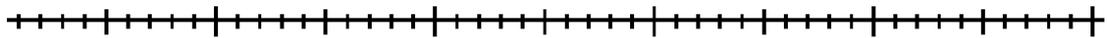


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2u ori

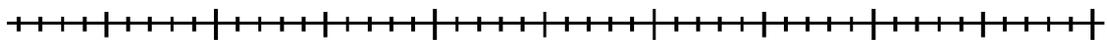
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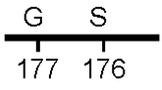
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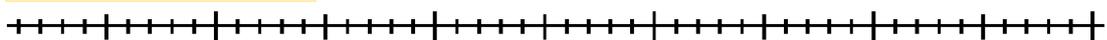
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8050



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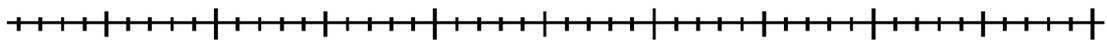


8100



V K V F A A A E P G G S S K Q C S  
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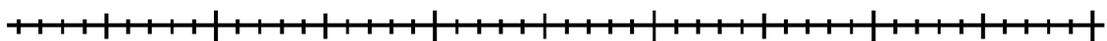


8150



V M V N K R Y S R V P P W G V V Q  
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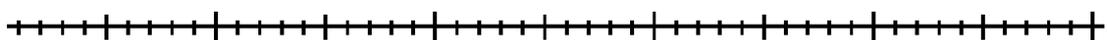


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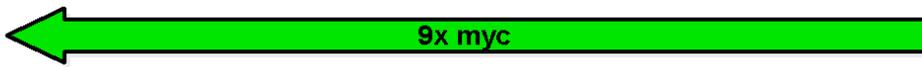


A K A P P K A P D K P M F E R T  
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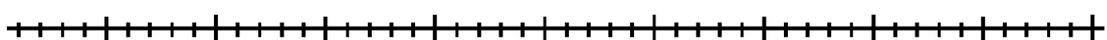


8250



S G N L D E E S I L K Q E G N L D  
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8300



E E S I L K Q E G N L D E E S I L  
108 107 106 105 104 103 102 101 100 99 98 97 96 95 94 93 92







„Nature gave men two ends – one to sit on and one to think with. Ever since then man’s success or failure has been dependent on the one he used most.“

*George R. Kirkpatrick*