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Dose dependent gene expression is dynamically modulated by the history, physiology and age of yeast cells

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Abstract

Cells respond to external stimuli with transient gene expression changes in order to adapt to environmental alterations. However, the dose response profile of gene induction upon a given stress depends on many intrinsic and extrinsic factors. Here we show that the accurate quantification of dose dependent gene expression by live cell luciferase reporters reveals fundamental insights into stress signaling. We make the following discoveries applying this non-invasive reporter technology. (1) Signal transduction sensitivities can be compared and we apply this here to salt, oxidative and xenobiotic stress responsive transcription factors. (2) Stress signaling depends on where and how the damage is generated within the cell. Specifically we show that two ROS-generating agents, menadione and hydrogen peroxide, differ in their dependence on mitochondrial respiration. (3) Stress signaling is conditioned by the cells history. We demonstrate here that positive memory or an acquired resistance towards oxidative stress is induced dependent on the nature of the previous stress experience. (4) The metabolic state of the cell impinges on the sensitivity of stress signaling. This is shown here for the shift towards higher stress doses of the response profile for yeast cells moved from complex to synthetic medium. (5) The age of the cell conditions its transcriptional response capacity, which is demonstrated by the changes of the dose response to oxidative stress during both replicative and chronological aging. We conclude that capturing dose dependent gene expression in real time will be of invaluable help to understand stress signaling and its dynamic modulation.

1. Introduction

All living cells can experience adverse environmental conditions, which threaten their homeostasis and require a proper stress defense in order to avoid damage or death. Transient activation of gene expression is a universal mechanism to combat the stress and recover cellular homeostasis [1,2,3]. These transcriptional defense programs often involve the activation of hundreds of genes in unicellular models such as yeast, which however will not respond in a uniform manner [4,5]. Gene expression changes upon stress are furthermore highly divergent between closely related species indicating that the stress response allows great variability without affecting the fitness of the organism [6,7,8]. Even in one species, the same stress or environmental condition can have very different transcriptional readouts at different up- or down-regulated genes. Additionally, very often the stress dose is intimately linked to the specific adaptive response of the cell. Here, it is crucial how the stress is converted into a signal at the beginning of intracellular signaling pathways. In yeast, we know that dose dependent signaling of essential nutrients (for example sugars or iron) or osmotic stress triggers diverse transcriptional programs at different subsets of target genes strictly dependent on the stimulus concentration [9,10,11,12,13,14,15]. Additionally, stress-generated signals are brought to the genome very often by more than one specific transcription factor (TF) [16]. The yeast response to salt, nutrient or xenobiotic stress includes the simultaneous activation of multiple, often structurally unrelated TFs [17,18,19]. Thus the use of different transcriptional activators could create different gene expression patterns at specific sets of target genes. Additionally, different TFs can form hierarchical networks by regulatory connections between them [20,21,22], which makes it necessary to determine the sensitivities of individual TFs.

Stress-activated TFs convert signals into a defined gene expression output by allowing RNAPII to engage in active transcription. Here, yet other regulatory mechanisms exist to define the strength and timing of transcriptional activation. Active chromatin remodeling is crucial for efficient stimulus-activated transcription. The nucleosome structure of the inducible upstream region can determine the dynamics of the gene expression at a given genomic locus, which has been reported for different stress and developmental adaptations in yeast [23,24,25]. As a consequence, the response to different stress doses might imply the contribution of distinct chromatin remodeling complexes [26]. Finally the distribution of promoter binding sites and their affinity to

the TF ultimately define the quantitative performance of a genomic locus upon environmental activation [27,28].

Multiple steps from signal generation to the first wave of gene transcription can change dynamically during the induction of stress genes. Apart from this, also extrinsic factors can change the way a cell triggers gene expression upon environmental changes. Previous exposure to stress can change the dynamics of the transcriptional response in experienced cells as compared to naïve cells. Epigenetic mechanisms have been revealed in yeast, which cause a faster re-activation of the second round of transcription in pre-treated cells in several cases of nutrient regulated genes [29,30]. This epigenetic memory can be produced by an altered chromatin structure at the previously induced locus or its transient translocation to the nuclear envelope [31,32,33,34]. However, other mechanisms have been described such as the inheritance of reinforced signal transduction to make experienced cells faster and more sensitive in their environmental response [35,36]. Additionally, the physiological robustness is decisive for the dose dependent induction of stress gene expression. It has been shown that specifically stress sensitive mutants tend to mount maximal gene induction at lower stress concentrations as compared to the more resistant wild type [37]. Oppositely, the accumulation of defense proteins in experienced cells can contribute to changes in the stress response during the process of acquired resistance [38]. Finally, the age of the cell might be of importance for the dynamic transcriptional adaptation to changing environments. This is suggested by the fact that genomic expression generally changes during the aging process [39,40,41,42], however, it remains to be determined if and how aging impacts on the sensitivity of stress induced gene expression.

As environmental stress triggers very dynamic transcriptional responses, it is essential to monitor gene expression at more than one stress dose. Only determining the transcriptional outputs over large ranges of stressor concentrations allows to compare dose dependent gene expression profiles and how they change by genetic, physiological and environmental alterations. An adequate experimental setup for this purpose is the application of destabilized luciferase reporters, which allow the parallel and time-elapsed determination of gene expression changes in living yeast cultures [37,43]. In this system, a modified firefly luciferase gene is used, which contains degradation motifs at the protein and mRNA levels. As a result, the expression of this very short-lived reporter can be measured continuously by the light emission from small yeast culture aliquots in a microplate luminometer. The application of stress gradients in this

system allows to assay inducible gene expression in a dose-dependent manner. Here we apply this technology to gain insights into dynamic stress signaling and how it is modulated by intracellular signal transduction, upon physiological changes, previous stress encounters and during aging.

2. Materials and Methods

2.1. Yeast strains and growth conditions

The *Saccharomyces cerevisiae* strains used in this study were wild type BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and isogenic strains carrying the mutant alleles *rpo41::KanMX4*, *yap1::KanMX4*, and *skn7::KanMX4* [44]. Yeast strains containing the indicated luciferase fusion genes on plasmids were grown at 28°C over night from fresh precultures to exponential growth phase in synthetic dextrose (SD) medium lacking histidine (0.67% yeast nitrogen base, 2% glucose, 50 mM succinic acid [pH5.5], 0.1 g/l leucine, 0.1 g/l methionine, 0.025 g/l uracil). Yeast strains carrying integrative luciferase reporter fusions were grown at 28°C over night from fresh precultures to exponential growth phase in yeast extract peptone containing 2% glucose (YPD) medium. For the aging experiments, the mother enrichment program strain UCC4925 (*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 lys2Δ0/+ trp1Δ63/+ hoΔ::SCW11pr-Cre-EBD78-NATMX/hoΔ::SCW11pr-Cre-EBD78-NATMX loxP-CDC20-Intron-loxP-HPHMX/loxP-CDC20-Intron-loxP-HPHMX loxP-UBC9-loxP-LEU2/loxP-UBC9-loxP-LEU2*) was used [45].

2.2. Plasmid constructions

For the construction of centromeric luciferase reporter fusions using natural promoters we employed pAG413-lucCP$^+$ (HIS3, CEN) described in [43]. The stress activated GRE2-luciferase reporter pAG413-pGRE2-lucCP$^+$ was used according to [36]. For the expression of destabilized luciferase under the control of specific *cis* regulatory elements, we employed pAG413-CYC1Δ-lucCP$^+$ [43]. Synthetic double stranded oligonucleotides with BspEI compatible ends were used to generate AP-1-, OSRE-, STRE-, CRE-, Hot1$_{UAS}$-, and PDRE-dependent luciferase reporters. The following sequences were used (relevant TF binding motifs are underlined). AP-1: CCGGCATCGATCTTACTAAGCGCGAAATTAGTAACCGGCTAATTACTAAGT;

OSRE: CCGGCGATATCGGCTGGCTAGAATACAGCCGGCCTATTCTAAGGCTGGCT; STRE: CCGGCGATATCAGCCCCTGGAAAAAGCCCCTGCGCAAAGCCCCT; CRE: CCGGCGATATCATTACGTAATAGAATACATTACGTAATCGCGATCATTACGTAAT; UAS_{Hot1}: CCGGCGATATCTGGGACAATGTAGAATACATTGTCCCTCGCGATCTGGGACAAT; PDRE: CCGGCGATATCTCCGCGGATAGAATACATCCGCGGATCGCGATCATCCGCGGAT. All constructions were verified by sequencing. In order to allow the genomic replacement of ORF sequences with the destabilized luciferase reporter, we amplified the $lucCP^+$ gene by PCR and cloned it upstream of the dominant KanMX marker of plasmid pUG6 [46]. The resulting construction, pUG6-$lucCP^+$-Kan, allows the amplification of $lucCP^+$-KanMX cassettes with gene specific primers (see Fig 1 for primer sequences) to create genomic promoter fusions with destabilized luciferase. Integrative *GRE2-* and *SOD2-*$lucCP^+$ fusions were created in this way in the present work.

2.3. Serial stress treatments

Yeast strains grown to exponential phase were adjusted to the same number of cells and exposed to the indicated stress gradients in white 96-well plates (Costar). 135 µl of cell culture was mixed with 15 µl of 10 fold concentrated stock solutions of H_2O_2, NaCl or menadione prepared in growth medium to minimize solvent effects. In the case of menadione, 100 fold concentrated stocks were first prepared in DMSO, which were further 10 fold diluted in growth medium. Mock incubations contained the same amount of solvent in each case. Cell viability was determined by plating appropriate dilutions of the cultures at the indicated times onto YPD agar plates and counting the colony forming units.

2.4. Time-elapsed luciferase assays

Yeast strains containing the indicated luciferase fusion genes were grown to exponential phase in SD lacking histidine or YPD medium adjusted to pH 3.0 with 50 mM succinic acid. Cultures were adjusted to the same cell density and incubated on a roller for 60 min at 28°C with 0.5 mM luciferin (free acid; Synchem, Felsberg, Germany) from a 10

mM stock prepared in DMSO. The cells were then transferred in 135 µl aliquots to white 96-well plates (Costar), which contained the indicated stressor concentrations. The light emission was immediately measured in a GloMax microplate luminometer (Promega) in three biological replicates. The light emission was continuously recorded over the indicated time and raw data processed with Microsoft Excel software. The light units were corrected for the absolute cell number at time point 0 and the relative light units were representedfor each stress treatment. The maximal luciferase activity (A_{max}) and maximal luciferase induction (IF_{max}) was calculated as described elsewhere [37].

2.5. Memory experiments

Cells containing the plasmid encoded GRE2-lucCP$^+$ live cell reporter were grown overnight in SD-his medium adjusted to pH 3.0. The cultures were divided and one half was treated with 0.5 M NaCl or 0.2 mM H_2O_2 while the other half was mock treated with the same amount of solvent. Cells were then briefly collected by centrifugation, washed once with growth medium and finally resuspended to identical density in fresh SD-his medium for 90 min with luciferin treatment, as described above, in the last 60 min. The indicated H_2O_2 concentrations were then applied and the continuous dose response recorded comparing experienced and naïve cells. The live cell titer was determined for each treatment by plating the appropriate dilutions onto YPD plates. We confirmed that none of the memory regimes affected cell viability.

2.6. Aging experiments

For chronological and replicative aging experiments, yeast strain UCC4925 was modified by the integration of the pSOD2-lucCP$^+$::KanMX fusion to allow the time elapsed determination of gene expression in response to oxidative stress. The cells were allowed to grow to early exponential growth phase on YPD medium adjusted to pH 3.0. A first dose response profile was determined as indicated with a hydrogen peroxide gradient. Cell cultures were then split and the different aging regimes applied. For chronological aging, the cells were maintained in stationary phase and the DR profile was daily recorded as described above. For replicative aging, the cell culture was supplemented with 1 µM estradiol (from a 10 mM stock in DMSO) to induce the selective inactivation of daughter cells and the DR profile of luciferase expression in response to H_2O_2 was recorded in the following 5 days. Before each luciferase assay the titer of living cells in the cultures was determined by washing cell aliquots with fresh

YPD medium and plating appropriate dilutions onto YPD agar plates. The raw data obtained in each DR experiment were normalized for the number of living cells in the assay.

2.7. Statistical analyses

All live cell gene expression studies were performed on three independent culture aliquots for each stress dose, which were adjusted to the same cell number. Data were represented as the mean value with the corresponding standard deviation. The significance of the variations in DR profiles was estimated with the unpaired Student´s t-test.

3. Results

We have previously established the use of destabilized luciferase reporters (lucCP$^+$) for the time-elapsed quantification of gene expression in yeast [43]. The lucCP$^+$ system has been then applied to quantitatively compare transcriptional stress responses [37] and to decipher the mechanisms of gradual control of gene expression [36]. The major advantage of determining gene expression outputs in parallel upon exhaustive stress gradients is obtaining the dose response (DR) profile of a cell. The DR profile is the collection of all gene expression changes, which occur at a specific gene from lowest to highest stress concentrations and contains quantitative parameters related to its stress defense. We reasoned that the way a given stress signal is converted into a gene expression output is highly variable and in general might depend on where and how the signal is created and transduced inside the cell and on specific parameters of the cell itself, such as its age, metabolic state or previous stress encounters. Therefore we wanted to prove whether dynamic DR modulation and its detection by lucCP$^+$ reporters can be applied in a much more general manner to decipher changes in cellular signaling. We started with generating a complete set of lucCP$^+$ reporters to adjust the technology for diverse approaches.

3.1. A versatile toolbox of live cell luciferase reporters for dynamic gene expression studies

241 We wanted to create a complete set of lucCP$^+$ applications expressing extremely short
242 lived luciferase. Full length or truncated natural promoters can be fused to lucCP$^+$ on
243 single copy plasmids (pAG413-lucCP$^+$, Fig 1). More specific reporters, only containing
244 one (or few) type(s) of transcription factor (TF) binding sites, can be generated by
245 inserting artificial DNA segments into an unregulated *CYC1* core promoter (pAG413-
246 pCYC1Δ-lucCP$^+$). Finally we combined lucCP$^+$ with the KanMX marker for targeted
247 PCR amplification, which allows genomic replacement of any non essential gene of
248 interest with destabilized luciferase (pUG6-lucCP$^+$-KAN). All autonomous or
249 integrative constructions can be used to capture complete DR profiles in real time upon
250 the adequate stimulation and will be applied here to discover DR variability and
251 dynamics in diverse stress signaling processes.
252
253 *3.2. The dose dependent transcriptional response to stress is modulated by growth*
254 *conditions*
255
256 Stress resistance in yeast is determined by the metabolic activity of the cell [47]. We
257 asked whether the choice of the growth medium, rich amino acid supplemented medium
258 (YPD) versus synthetic medium (SD), had an influence of the DR upon cell stress and
259 whether possible DR variations could be faithfully determined with the lucCP$^+$
260 technology. We investigated the expression of the *GRE2* gene, which responds with
261 high activation folds upon different stresses such as osmotic or oxidative stress [48]. We
262 employed yeast strains with integrated GRE2-lucCP$^+$ reporters and quantified their DR
263 upon NaCl exposure after just 1 hour of pre-growth in either medium. Importantly, the
264 GRE2-luciferase fusion reproduces the previously reported mRNA induction profiles
265 for increasing salt stress [49]. As shown in Fig 2A and B, the short incubation in either
266 synthetic or rich medium changed the DR profile to salt stress significantly. Cells in
267 rich medium induced the expression of the salt responsive reporter with the highest
268 amplitude at significantly lower stress conditions as compared to cells coming from
269 synthetic medium mainly because they do not sustain efficient expression levels at
270 higher stress doses. As a consequence, the shift from rich to synthetic medium moves
271 the DR profile towards higher stress doses by at least 100 mM of NaCl. One possible
272 explanation for this effect is a general pre-disposition towards stress conditions of cells
273 adapted to minimal medium as opposed to cells with completely repressed stress
274 responses on rich medium. In line with this interpretation we observed that NaCl

induced gene expression occurred faster in minimal media cells upon higher stress doses (Fig 2 C). Thus yeast cells experience fast changes in their stress activated DR according to their environmental conditions and reflecting their robustness towards stress defense in general. We confirmed (Fig. 2D) that all stress conditions did not cause a loss of viability in this experiment. Fig 2 also demonstrates that DR shifts can be experimentally determined with live cell luciferase reporters. Therefore we were encouraged to exploit the dynamics within DR profiles and apply this approach to different fundamental aspects of cell signaling.

3.3. Deciphering intracellular stress targets by differential transcriptional dose responses

Stress does not affect normally the whole cell but is originated at specific sites within the cell. The nature of these stress targets is important for signal generation and transduction in order to efficiently induce gene expression. Oxidative stress is an important example as it can occur at many different cellular locations in response to different intrinsic and extrinsic stressors. We wanted to explore the possibility to use dynamic DR outputs to trace the oxidative damage produced by two different oxidants and their relation to mitochondrial respiration. Mitochondria have been previously shown to have differential pro- and anti-oxidant potential depending on the nature of ROS production [50,51]. We applied the hydrophilic and membrane permeable molecule H_2O_2 and the lipophilic molecule menadione, causing ROS production at soluble cell compartments or at intracellular membranes, respectively [52,53,54]. The DR profiles upon both oxidants were determined in yeast cells with the internal generic stress reporter GRE2-lucCP$^+$ (Fig 3A). Three different energy sources were applied to gradually induce mitochondrial respiration: Glucose (full repression), galactose (derepression) and glycerol/ethanol (full mitochondrial induction). In the case of hydrogen peroxide, we observed similar DR profiles along the induction of respiratory metabolism with a slight shift of the DR towards lower doses indicating a modest increase in H_2O_2 susceptibility upon mitochondrial respiration (Fig 3B). For menadione treatment, however, we observed a pronounced shift of the DR towards very low doses upon full respiration (Fig 3B) because the cells responded much less at higher stress doses. The different oxidative stress treatments did not cause a significant loss of

viability in the cell cultures (Fig. 3C). Taken together, this suggested that menadione action could be intimately linked to mitochondrial performance.

We next examined how the loss of mitochondrial function would impact on the DR profiles activated by the two oxidants. We employed a plasmid-encoded oxidative stress reporter, which drives destabilized luciferase expression from a repeat of AP-1 binding sites [37]. We compared wild type cells with a mutant in the mitochondrial RNA polymerase *rpo41*, which exhibits a greatly diminished mitochondrial activity. We observed (Fig 4) that the DR profiles upon both oxidants were differentially affected by the loss of mitochondrial function. Hydrogen peroxide seemed to elicit a more sensitive response in the mitochondria defective strain, while menadione caused a less sensitive DR in the absence of mitochondrial function (Fig 4B). These data are in agreement with a ROS scavenging function of mitochondria upon H_2O_2 stress and a pro-oxidant function of mitochondria upon menadione stress. We suggest that dynamic DR profiles are sensitive indicators of intracellular stress targets.

We next asked whether the different oxidative signals generated by H_2O_2 and menadione might be transduced differentially by transcription factors. We focused at the two main oxidative stress transcriptional activators Yap1 and Skn7, which recognize distinct DNA sequences, AP-1 and OSRE respectively [55,56]. As shown in Fig 5A, we constructed specific live cell reporters for both TFs by inserting artificial repeats of their recognition sequences according to Fig 1. The DR of wild type cells upon exposure to the two oxidants was quantified and the two TFs compared. While the Yap1 reporter gave consistently higher response amplitudes as opposed to Skn7, it discriminated much more between the two oxidants, with menadione causing only 20% of the H_2O_2 response (Fig 5B). Signaling through Skn7 seemed to be less restrictive as both peroxide and menadione signals were converted into transcriptional activation in a much more comparable manner. These data suggested that external stressors elicit gene expression profiles dependent on their intracellular targets and that different TFs participate to various degrees in the signal transduction process.

3.4. Determining TF sensitivities by their DR profiles

The same cellular stress is very often recognized by different signal transduction pathways involving several specific TFs. Although it is biologically relevant, it remains challenging to determine whether the different TFs have distinguishable sensitivities for

342 specific stressors. Thus we aimed at applying dynamic DR profiling to determine
343 sensitivities among different stress activated yeast TFs. We first examined three
344 different specific TFs, which are commonly activated by hyperosmotic stress. In the
345 yeast osmostress response, the Hog1 MAP kinase is the master regulator, which directly
346 targets several downstream TFs, such as Sko1, Hot1 or Msn2 [18,57]. It remains
347 unclear whether the different transcriptional activators respond to high osmolarity with
348 distinct sensitivities. Multiple binding sites for each TF were placed to drive lucCP^{+}
349 expression from centromeric plasmids in order to generate TF specific live cell reporters
350 (Fig 6A). All reporters were suitable for the determination of the NaCl elicited DR
351 profiles (Fig 6B). The comparison of the three transcriptional activators revealed that
352 Sko1 was the factor, which responded with the highest sensitivity to salt stress (Fig 6C).
353 The Sko1 DR profile showed a shift by >100 mM towards lower NaCl doses, while
354 Hot1 and Msn2 had similar response profiles.
355 We next applied DR profiling to other stress responsive TFs. Organic oxidants can be
356 recognized in yeast cells by different transcriptional activators. The above mentioned
357 Yap1 is activated by a conformational change induced by the direct oxidation of the
358 protein [58], while Pdr1 is activated by binding of the xenobiotic molecule within the
359 multidrug response [59]. Specific live cell reporters for both pathways (Fig 7A) were
360 able to determine the DR profiles for menadione exposure (Fig 7B). We found that the
361 multidrug response had a significantly higher sensitivity towards menadione as
362 compared to the stress activated Yap1 (Fig 7C). These data commonly suggested that
363 indeed hierarchies exist among yeast stress responsive TFs and that DR profiling is a
364 sensitive and easy experimental approach for its determination.
365
366 *3.5. Capturing different DR dynamics upon repeated stress treatments*
367
368 The transcriptional response upon stress changes in cells that have been previously
369 exposed to the same or different stresses. Yeast has been an instructive model in the
370 investigation of transcriptional memory. Different genetic and physiological
371 mechanisms have been identified to contribute to an alteration in the way experienced
372 cells transcriptionally respond to stress as compared to naïve cells [60]. It is not easy to
373 predict these alterations as for example positive memory would generally facilitate
374 while acquired resistance could actually reduce the second round of gene activation. We
375 reasoned that it would be critical to determine dynamic DRs during repeated stress

exposure in order to better understand the changes occurring upon different stress pre treatments. We investigated how different stress pre-exposures modulated the DR upon oxidative stress for two specific genes, *SOD2* and *GRE2* (Fig 8). The *SOD2* gene was chosen because its expression is highly inducible specifically upon oxidative stress [37]. In a "crossed stress" treatment with NaCl followed by H_2O_2 we observed that the experienced cells performed better in their transcriptional response along all oxidant concentrations (Fig 8A and 8B). This involved a faster and more efficient gene activation in the second stress encounter and clearly demonstrates a general positive memory effect. Alternatively we applied a "same stress" treatment were experienced cells passed through two H_2O_2 exposures (Fig 8C). In this case we observed that the stress response of the pre-treated cells was reduced for lower stressor concentrations and significantly higher for harsher H_2O_2 treatments (Fig 8D). Thus the "same stress" regime caused a shift of the DR profile towards higher stressor concentrations. In summary, DR profile analyses are important and efficient for the determination of different memory effects during previous stress encounters. In the light of the results obtained for short-term memory, we wanted to extend our studies to longer effects and studied the dynamic DR changes during the process of aging.

3.6. Modulation of the oxidative stress DR during aging

ROS accumulation and oxidative stress susceptibility are hallmarks of aging cells. We wanted to investigate whether the capacity to induce gene expression upon oxidative stress suffers alterations during the aging process. We applied integrative *pSOD2* driven live cell luciferase fusions as sensitive oxidative stress reporters along chronological and replicative aging (Fig 9). In exponentially growing cells, SOD2-lucCP$^+$ yields a dynamic DR upon H_2O_2 stress with the most dynamic response at low (300 μM) doses (Fig 9A). During survival in stationary phase, the same cells show a dramatic loss of DR dynamics within a few days, which is exacerbated over time without an obvious loss in cell viability (Fig 9A and 9B, right panel). This effect is accompanied by a continuous delay in the gene expression response (Fig 9B).

The same luciferase reporter was used in a replicative life span experiment using the "mother enrichment program", which permits the analysis of yeast cultures with an increasing percentage of old mother cells by selectively killing newborn daughter cells [45]. Induction of this process removes initially about half of the number of viable cells,

410 which remains constant for 3 days while the surviving mother cells age. In this time we
411 observed a continuous decline in the DR profile, which in this case, however, was not
412 accompanied by a response delay (Fig 9A and 9B, left panel). Taken together, our
413 results indicate that aging yeast cells display DR profiles with reduced dynamics and
414 efficiencies upon oxidative stress.
415
416 **4. Discussion**
417 The motivation of this work was to improve our understanding of transcriptional
418 regulation by investigating it as a dose dependent biological function. Many studies
419 have addressed dynamic changes of gene expression during environmental stress in
420 individual cells [61], isogenic cell populations [1] or even evolutionarily related species
421 [6]. However, the fact that gene expression is regulated over large stress gradients has
422 not been fully considered. This is important because the biological information of a
423 gene expression profile at just one stimulus concentration is limited and does not allow
424 straightforward comparisons. A decreased response to oxidative stress for example can
425 have fundamentally opposite explanations and might be due to an inability to respond
426 but also due to an acquired resistance. Here we show that stress induced DR profiles
427 contain very useful information and that they can be readily determined in yeast cells.
428 Furthermore, dose dependent transcription can change by the use of different
429 intracellular signaling pathways, upon previous experience of the cell or during aging.
430
431 *4.1. Understanding transcriptional regulation as a dose dependent dynamic process*
432
433 Most studies on environmental gene induction are done at single stress conditions to
434 cause a robust transcriptional activation. These conditions, however, are often far
435 beyond the stimulation which cells encounter in natural environments. Additionally the
436 response to increasing stress doses is finely tuned by the cell, probably in order to spend
437 only the necessary resources to efficiently reestablish cellular homeostasis after the
438 insult. In yeast, constantly changing and transient gene expression bursts can be
439 detected over considerable concentration ranges of harmful compounds [36,37,43]. A
440 typical DR profile will start with weak induction at low threshold concentrations and
441 then produce continuously growing expression until a characteristic high threshold
442 concentration is reached [43]. Beyond this point a decline in the transcriptional response
443 might be observed because at these stress conditions the gene induction process in

general is negatively affected by additional damage. DR profiles are not static and have been previously found to be influenced by chromatin remodeling or the particular stress resistance of a cell [36,37,62]. Here we demonstrate that precise DR profiling in vivo serves as a diagnostic tool to quantitatively distinguish intracellular signaling as well as to characterize external modulation of stress resistance.

4.2. Intracellular signaling sensitivities can be revealed by DR profiles

In many occasions, stress is signaled via several TFs to regulate groups of distinguishable or overlapping target genes. We show here that Sko1 is a more sensitive transcriptional activator when compared to other osmostress responsive TFs. These results validate our DR profiling strategy as a measure of the signal sensitivity of redundant TFs. Sko1, Hot1 and Msn2 are all activated by direct phosphorylation via the Hog1 MAP kinase [63,64,65]. However, at least Sko1 and Msn2 are additionally PKA phosphorylation targets, which might modulate their sensitivities [64,66]. More importantly, the three TFs differ in their mode of activation. Msn2 is mostly cytosolic upon favorable conditions and accumulates in the nucleus dependent on PKA phosphorylation [66], where it activates genes of the general environmental stress program. In turn, Sko1 and Hot1 activate defense genes, which are specific for osmostress adaptation. The main mechanism of Hot1 activation is the phosphorylation dependent stimulation of DNA binding in the nucleus [63]. Sko1 is activated by inactivating Tup1-mediated repression and allowing the recruitment of additional co-activator complexes [67]. This switch most likely occurs at Sko1 while it is bound at its target promoters and might rapidly unmask its activation domain [68]. In general, the kinase-mediated counteraction of Tup1 repression at chromatin might be a more sensitive and rapid way of gene induction. Additionally to differential upstream signaling, also differences in the binding affinities of the TFs could contribute to DR changes.

It is important to note that the Sko1 and Hot1 targets differ substantially. Sko1 upregulates genes encoding stress related transporters, enzymes and regulators as well as other TFs [69], while Hot1 targets seem to be specific for glycerol production upon salt stress [70]. Osmolyte production might be more relevant at higher stress doses as opposed to adaptation to ionic imbalance, reflected here by the distinguishable sensitivities of Hot1 and Sko1. The special sensitivity of Sko1 in the osmostress

network might be the reason why this TF has been found to directly control the expression of many other specific TFs [22,69]. Sko1 might act as a sensitive signaling hub controlling the hierarchical osmostress response. In general, DR profiling might help to understand the function of transcriptional networks in yeast.

Here we show that the transcriptional activators Pdr1 and Yap1 have different sensitivities towards the oxidant menadione. In this case, the different mode of sensing might be the key to explain this distinction. Pdr1 binds xenobiotic molecules directly via a specialized protein domain, and substrate recognition then triggers RNA pol II recruitment and rapid gene expression [59]. This activation at the chromatin of the target genes, similar to Sko1, might make these regulations especially sensitive. Yap1 is instead indirectly activated via the redox sensing peroxidase Gpx3 upon hydrogen peroxide [71]. However, alternative direct activation of Yap1 has been reported for other oxidants [72,73], which might occur with distinct sensibilities. Oxidation of Yap1 at specific cysteine residues triggers then nuclear accumulation and subsequent gene activation [74]. This indirect activation mechanism likely contributes to its lesser sensitivity and here we find that it is optimized for the hydrophilic hydrogen peroxide as opposed to the lipophilic oxidant menadione. However, other oxidative stress signal transducers such as Skn7 are less discriminative for different oxidants as shown in this work. Skn7 is activated by phosphorylation through stress signaling cascades such as the osmostress two component system [75], which might have yet a different substrate specificity. The DR profiling methods described here will be a powerful tool to determine the selectivities and sensitivities of signaling pathways responding to many environmental stresses.

Looking at the sensitivity of a transcriptional response can also shed light on the physiologically important targets of a particular stress. This was first described generally for mutants lacking antioxidant defenses. These mutants show large shifts in their DR profiles towards very low oxidant concentrations [37]. Here we further extend this connection and demonstrate that two different classes of oxidants trigger distinct cellular signaling dependent on mitochondrial respiration. Menadione activates stress responses more sensitively in actively respiring cells and less sensitively in cells with a general mitochondrial defect. Mitochondria are thus a physiologically relevant target of this oxidant. Oppositely, hydrogen peroxide acts independently on the rate of respiration and instead activates a more sensitive stress response upon mitochondrial dysfunction, which identifies the mitochondria as important for peroxide defense but not for its

toxicity. This is in agreement with large scale phenotypic screenings where mitochondrial mutants were identified by a hydrogen peroxide growth defect [50]. However, we predict that DR profiling will be a more sensitive and versatile method to decipher stress targets because these experiments are performed at low stressor concentrations where the cells are actively responding to the particular stress and do not involve the assessment of cell death. As an example, live cell DR profiling has contributed to decipher toxicity targets of different mycotoxins [76].

4.3. Gene expression dynamics change in experienced and aged cells

The way a stress signal is transformed into a gene expression pulse is highly dynamic according to the DR profiling results presented in this work. It is sufficient to expose yeast cells to a medium with limited nutrient content for just one hour to provoke a considerable shift of their stress responsiveness towards higher stress conditions. Nutrient limitation is known to partially activate stress responses not only in yeast [77], and nutrient sensing pathways such as TOR or protein kinase A repress stress resistance in general and stress-activated gene expression [78]. Therefore a yeast cell in a nutrient rich environment has low defense resources and responds to stress insults in a highly sensitive manner. DR profiling in vivo is an elegant and straightforward tool to quantify these adaptations. One application is the visualization of changes in the stress response, which occur dependent on the recent history of the cell. Transcriptional memory in the case of the repeated exposure to a certain nutrient, for example galactose, consists in a much faster and more sensitive gene activation in experienced cells [36], to which both epigenetic mechanisms and the induction of signaling molecules can contribute [35,79]. However, in other environmental stress scenarios this picture might get more complicated and it is not clear whether an experienced cell will respond with a higher or lower intensity. Salinity induced gene expression, for example, is generally attenuated in experienced cells and oxidative stress resistance is conditioned by both the inheritance of antioxidant enzymes and facilitating the second round of transcription [36,38]. In any case, it is essential to compare DR profiles applying stress gradients instead of single dose responses to determine how stress adaptation is modulated in changing environments. Here we show that treatment with a related but not identical stress prepares cells generally in a second stress encounter. In this case, the induction of the general environmental stress response might make cells respond faster and more efficiently to any dose of subsequent stress [80]. However, upon repeated treatment with the exact same stress, we can expect that the experienced cell will be equipped

with the optimal defense protein combination in the pre-treatment, which actually causes less transcriptional activation upon mild stress and a general shift of the DR towards higher stress doses.

The serial luciferase reporter strategy employed here is able to distinguish different patterns of stress defense acquired in different environments, but more importantly can be applied to monitor stress responses in developmental processes. As reported here, the dose response to oxidative stress generally declines in chronologically and replicatively aging yeast. This adds to the growing number of biological processes, which deteriorate in older cells [81]. However, interesting differences exist between the two aging regimes. Early stationary phase cells show first an acquired resistance phenotype with the characteristic shift of their DR profile towards high stress. In later stages, the transcriptional responsiveness generally declines accompanied by an important delay in the onset of transcriptional activation, which might reflect a general signaling defect. Replicative aging instead does not delay the cellular response, thus the signaling events leading to oxidative stress induced gene expression seem intact. However, the dynamic response to growing oxidative stress conditions is completely lost in old mother cells. This behavior is very similar to the truncated DR profiles observed for induced gene expression in chromatin remodeling mutants [36] and might indicate that aging cells lose the ability to efficiently remodel highly inducible stress loci. Future genetic approaches combined with high resolution DR profiling will further discover the mechanisms of environmentally and developmentally changing stress responses.

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Figure Legends

Fig. 1. Different destabilized luciferase reporters for the determination of dynamic transcriptional dose response profiles in yeast. The workflow shows the introduction of plasmid encoded or integrative lucCP^{+} live cell reporters, the obtaining of complete DR profiles in real time and how these profiles could change dynamically. Large promoter sequences or artificial TF binding sites can be placed upstream of lucCP^{+} with

the indicated restriction sites in centromeric plasmids (upper panel right). For genomic integration, the lucCP$^+$ gene together with the KanMX marker can be amplified by PCR using gene specific primers with the indicated plasmid sequences (upper panel left). The start ATG of the luciferase gene is highlighted. The complete DR profile is determined by the application of serial dilutions of the stress treatment of interest in parallel and time elapsed light recording in a luminometer (middle panel, see Materials and Methods). DR profiles can dynamically change upon the indicated intrinsic and extrinsic factors (lower panel). lucCP$^+$ = gene encoding destabilized firefly luciferase; CL1 = yeast protein degradation motif; PEST = protein degradation domain from mouse ornithin decarboxylase; ARE = mRNA degradation motif; Cyc1T = transcriptional terminator from *CYC1*; TF = transcription factor; *pCYC1Δ* = *CYC1* core promoter without regulatory sequences.

Fig. 2. The stress induced dose response changes quickly dependent on the culture medium. (A) Left panel: Schematic overview of the transcriptional regulation at the *GRE2* gene in response to oxidative and osmotic stress. Right panel: Yeast cells harboring the integrated GRE2-lucCP$^+$ reporter were pre-incubated in YPD (rich medium) or SD (minimal medium) and the DR profiles were captured upon NaCl stress. (B) The maximal reporter activity (A_{max}) was plotted against the stress dose to visualize the DR shift upon the different media. (C) The time to reach maximal reporter activity upon the different NaCl concentrations was compared for SD and YPD grown cells. (D) Cell viability upon the different salt treatments in SD and YPD medium. The number of live cells was arbitrarily set to 100 for time point 0. Three independent biological replicas were analyzed, data are mean ± SD. * marks significant differences according to the Student´s t-test ($p < 0.05$).

Fig. 3. DR modulation by two oxidants dependent on respiratory metabolism. Hydrogen peroxide and menadione were used as oxidative stressors in yeast wild type cells with the integrated GRE2-lucCP$^+$ reporter. (A) The DR profiles were captured for cells grown in YPD (glucose), YPGal (galactose) and YPGE (glycerol/ethanol). (B) The maximal reporter activity (A_{max}) was plotted against the stress dose to visualize the DR shifts upon the different energy sources. (C) Cell viability upon representative treatments with the two oxidants comparing glucose and glycerol/ethanol media. The

number of live cells was arbitrarily set to 100 for time point 0. Three independent biological replicas were analyzed, data are mean ± SD.

Fig. 4. Mitochondrial function differentially modulates the DR elicited by H_2O_2 and menadione. (A) DR profiles upon the two oxidants were determined in SD medium in wild type and *rpo41* mutant cells harboring the oxidative stress specific live cell reporter 3xAP1-lucCP$^+$ on a centromeric plasmid. (B) The maximal reporter activity (A_{max}) was plotted against the stress dose to visualize the DR shifts upon loss of mitochondrial function in the case of the two oxidants. Three independent biological replicas were analyzed, data are mean ± SD.

Fig. 5. Differential signaling through Yap1 and Skn7 upon H_2O_2 and menadione stress. (A) Yap1 and Skn7 transduce oxidative stress signals via distinct promoter elements. The indicated *cis* elements (AP-1 and OSRE) were introduced into lucCP$^+$ expression vectors (left panel) and the induction profiles were recorded upon H_2O_2 treatment (0.2mM) in the indicated yeast strains (right panel). (B) Maximal fold induction (IF_{max}) levels for both reporter genes were plotted against the concentration of both oxidants. The percentage of inducibility was calculated for Yap1 and Skn7 (right panel). IF_{max} for H_2O_2 was arbitrarily set to 100%. Three independent biological replicas were analyzed, data are mean ± SD.

Fig. 6. Different sensitivities among osmostress responsive TFs determined by dynamic DR profiles. (A) The Hog1 MAP kinase targets several specific transcriptional activators upon osmotic stress. The indicated artificial binding sites for Msn2, Hot1 and Sko1 were introduced into lucCP$^+$ expression vectors. (B) The DR profile for each TF was determined upon NaCl shock. (C) The maximal reporter inducibility (IF_{max}) was plotted against the stress dose to visualize differences in the DR profile of the three TFs. Three independent biological replicas were analyzed, data are mean ± SD. Significant differences according to the Student´s t-test are marked (* $p < 0.05$; ** $p < 0.01$).

Fig. 7. Differential signaling through oxidative and xenobiotic stress response pathways. (A) Menadione activates both the oxidative (Yap1) and the xenobiotic (Pdr1) stress response. TF specific lucCP$^+$ reporters were constructed as described in

Fig 5 and 6. (B) DR profiles obtained for Yap1 and Pdr1 in response to menadione exposure. (C) The maximal reporter activity (A_{max}) was plotted against the stress dose to visualize differences in the DR profiles of the two pathways. Three independent biological replicas were analyzed, data are mean ± SD. Significant differences according to the Student´s t-test are marked (* $p < 0.05$; ** $p < 0.01$).

Fig. 8. Dynamic DR profiles reveal different memory patterns upon repeated stress treatment. Yeast wild type cells harboring SOD2- or GRE2-lucCP$^+$ plasmidic live cell reporters were used. (A) Cross stress regime: NaCl followed by H_2O_2 exposure. (B) DR profiles of *SOD2* and *GRE2* comparing experienced (red) and naïve (black) cells. (C) Plotting the maximal reporter activity against the stressor concentrations reveals a general positive memory pattern for the two genes. (D) Same stress regime: Repeated H_2O_2 exposure. (E) DR profiles of *SOD2* and *GRE2* comparing experienced (red) and naïve (black) cells. (F) Plotting the maximal reporter activity against the stressor concentrations reveals a DR shift towards higher stress (right panel). Three independent biological replicas were analyzed, data are mean ± SD. Significant differences according to the Student´s t-test are marked (* $p < 0.05$; ** $p < 0.01$).

Fig. 9. Oxidative stress DR dynamics during chronological and replicative aging. The MEP strain UCC4925 was employed with an integrated SOD2-lucCP$^+$ live cell reporter. (A) Top panel: DR profile upon H_2O_2 exposure of exponentially growing cells. Right panel: Chronological aging in stationary phase, left panel: Replicative aging by the induction of MEP with estradiol. The H_2O_2 activated DR profiles were determined at the indicated time points. Data are normalized for the number of viable cells. (B) Upper panel: The maximal reporter activity (A_{max}) was plotted against the stress dose to visualize differences in the DR profiles during survival in stationary phase (right panel) and during replicative aging (left panel). Lower panel: The time to reach maximal reporter activity is shown for two H_2O_2 concentrations for the two types of aging. The corresponding numbers of viable cells during the different aging regimes are shown. Three independent biological replicas were analyzed, data are mean ± SD.

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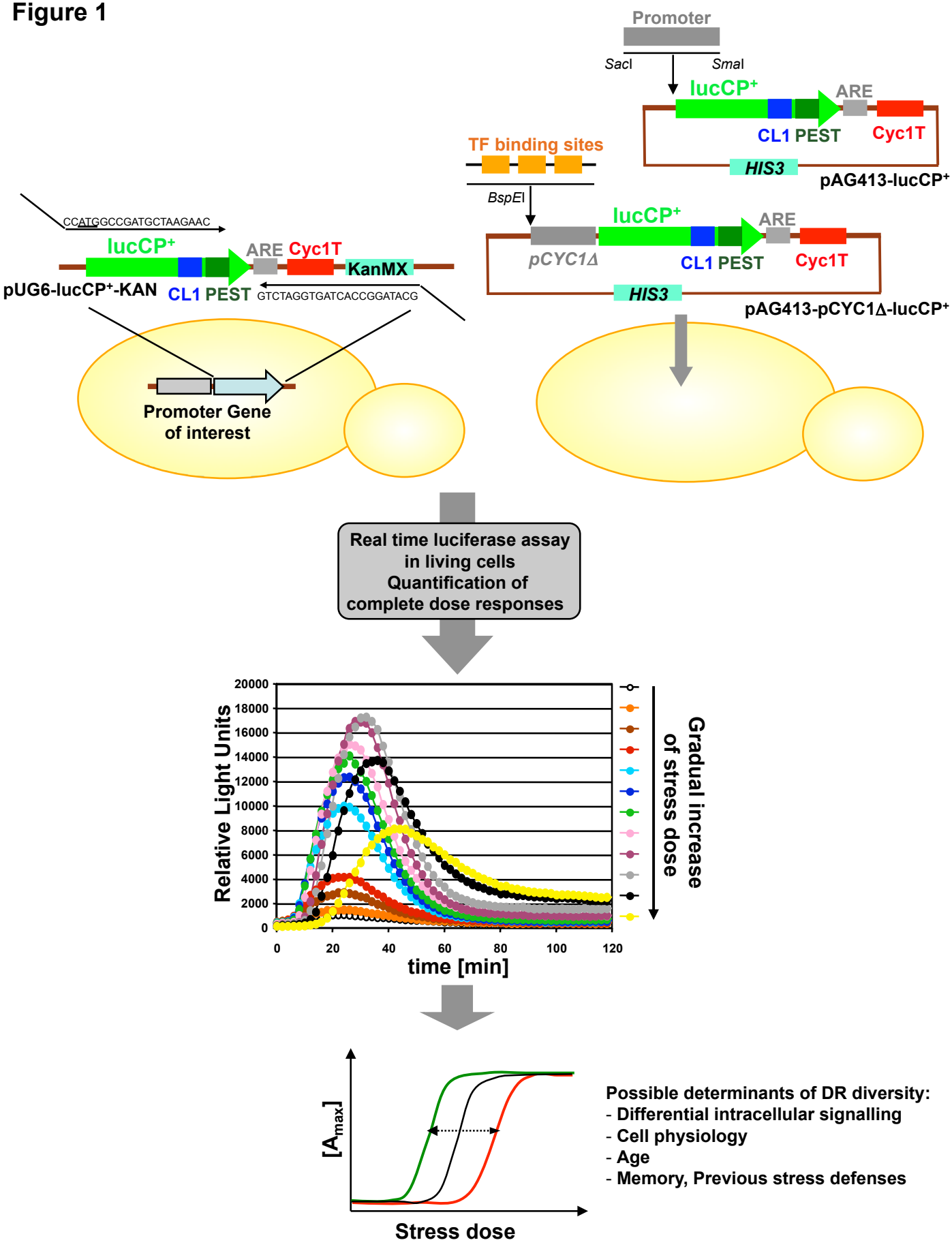
Figure 1


Promoter
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Cyc1T
HIS3
pAG413-lucCP+
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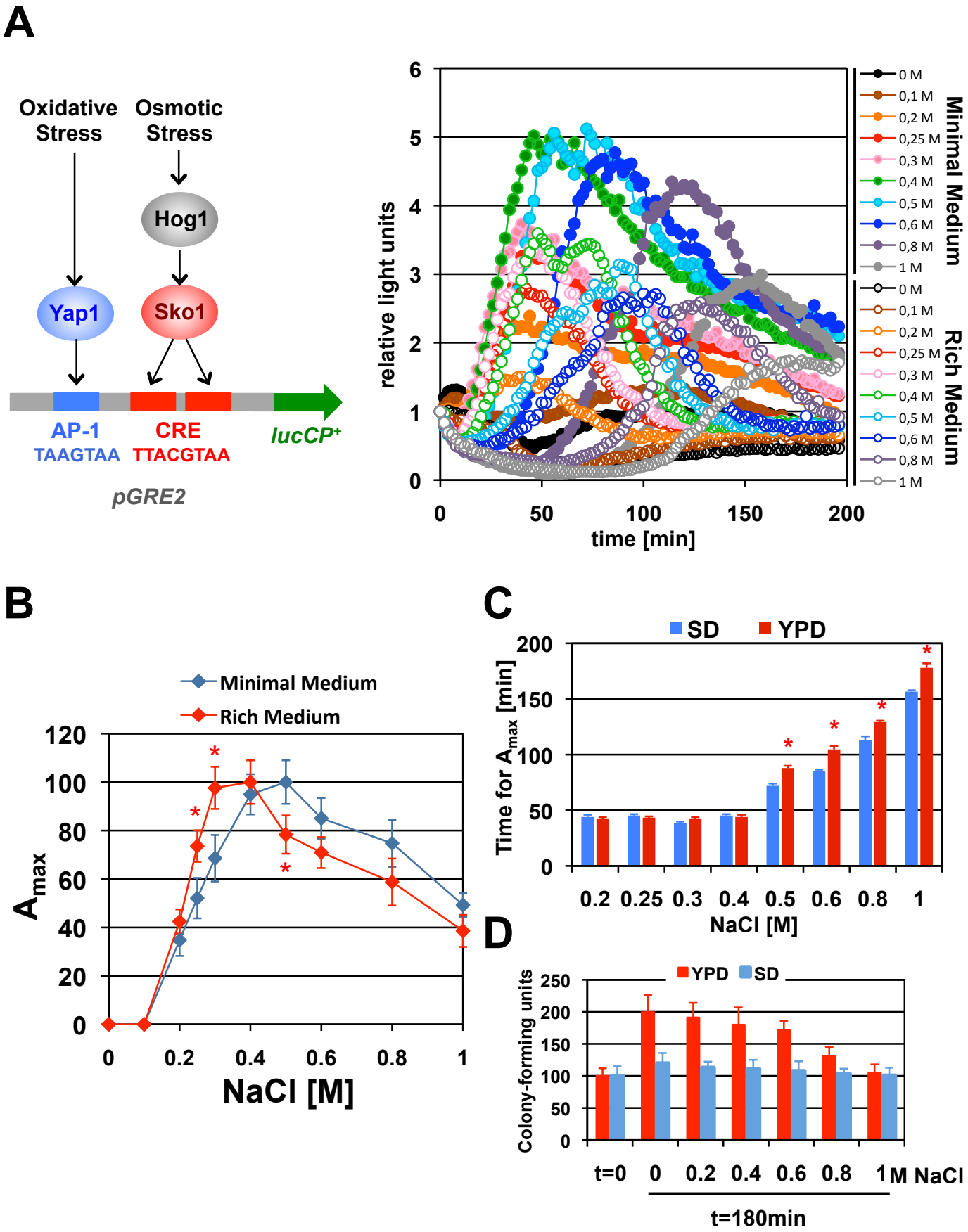
BspEI
pCYC1Δ
pAG413-pCYC1Δ-lucCP+
CCATGGCCGATGCTAAGAAC
pUG6-lucCP+-KAN
KanMX
GTCTAGGTGATCACCGGATACG
Promoter Gene of interest
Real time luciferase assay in living cells Quantification of complete dose responses
Relative Light Units
time [min]
Gradual increase of stress dose
[Amax]
Stress dose
Possible determinants of DR diversity:
- Differential intracellular signalling
- Cell physiology
- Age
- Memory, Previous stress defenses

Figure 2


A
Oxidative Stress
Osmotic Stress
Hog1
Yap1
Sko1
AP-1
TAAGTAA
CRE
TTACGTAA
lucCP+
pGRE2
relative light units
time [min]
Minimal Medium
Rich Medium
0 M
0,1 M
0,2 M
0,25 M
0,3 M
0,4 M
0,5 M
0,6 M
0,8 M
1 M
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Minimal Medium
Rich Medium
Amax
NaCl [M]
C
SD
YPD
Time for Amax [min]
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D
YPD
SD
Colony-forming units
t=0
M NaCl
t=180min

Figure 3

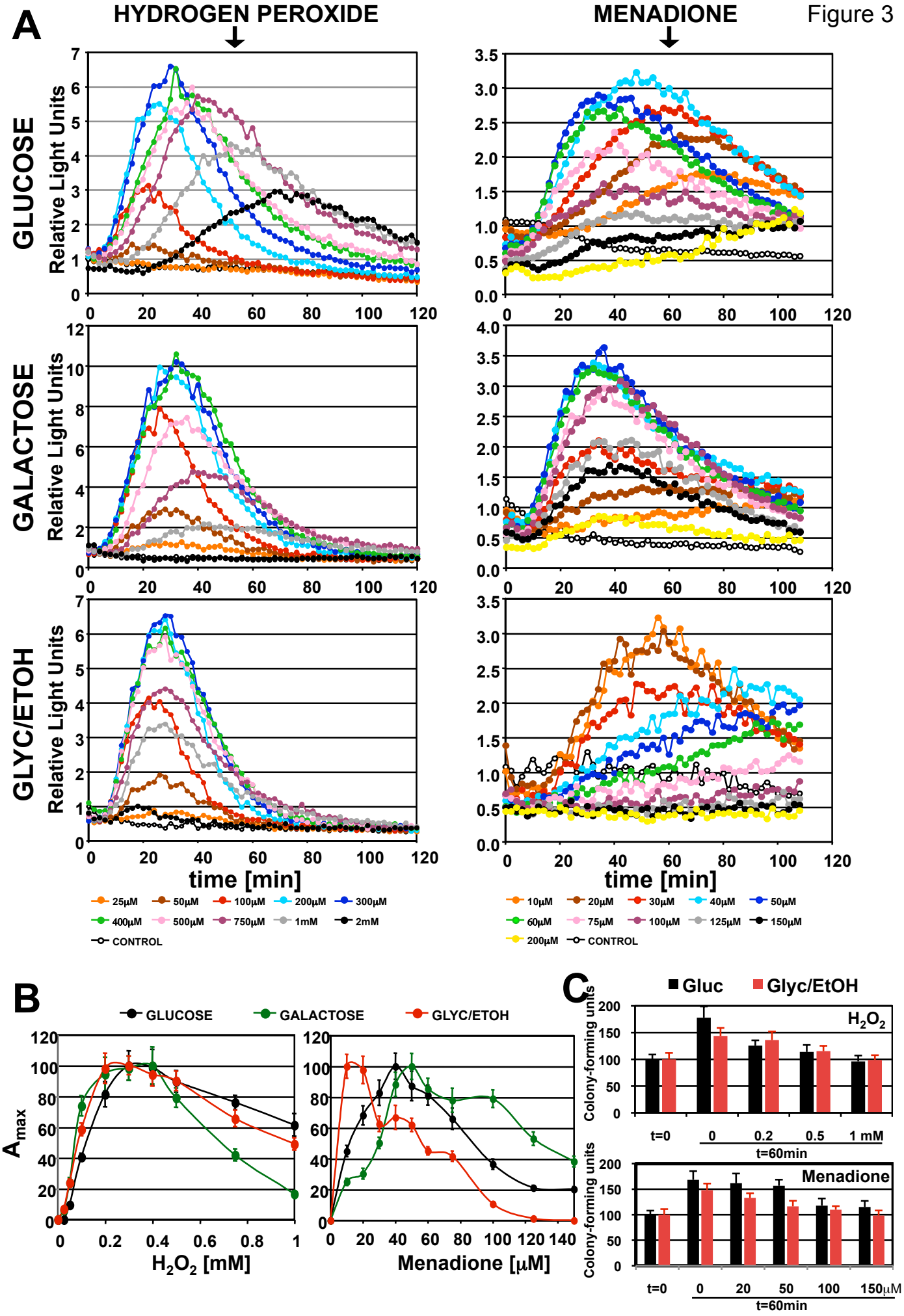

A
HYDROGEN PEROXIDE
MENADIONE
GLUCOSE
GALACTOSE
GLYC/ETOH
Relative Light Units
time [min]
25µM 50µM 100µM 200µM 300µM
400µM 500µM 750µM 1mM 2mM
CONTROL
10µM 20µM 30µM 40µM 50µM
60µM 75µM 100µM 125µM 150µM
200µM CONTROL
B
GLUCOSE GALACTOSE GLYC/ETOH
Amax
H2O2 [mM]
Menadione [µM]
C
Gluc Glyc/EtOH
H2O2
Menadione
Colony-forming units
t=0 0 0.2 0.5 1 mM
t=60min
t=0 0 20 50 100 150µM
t=60min

Figure 4

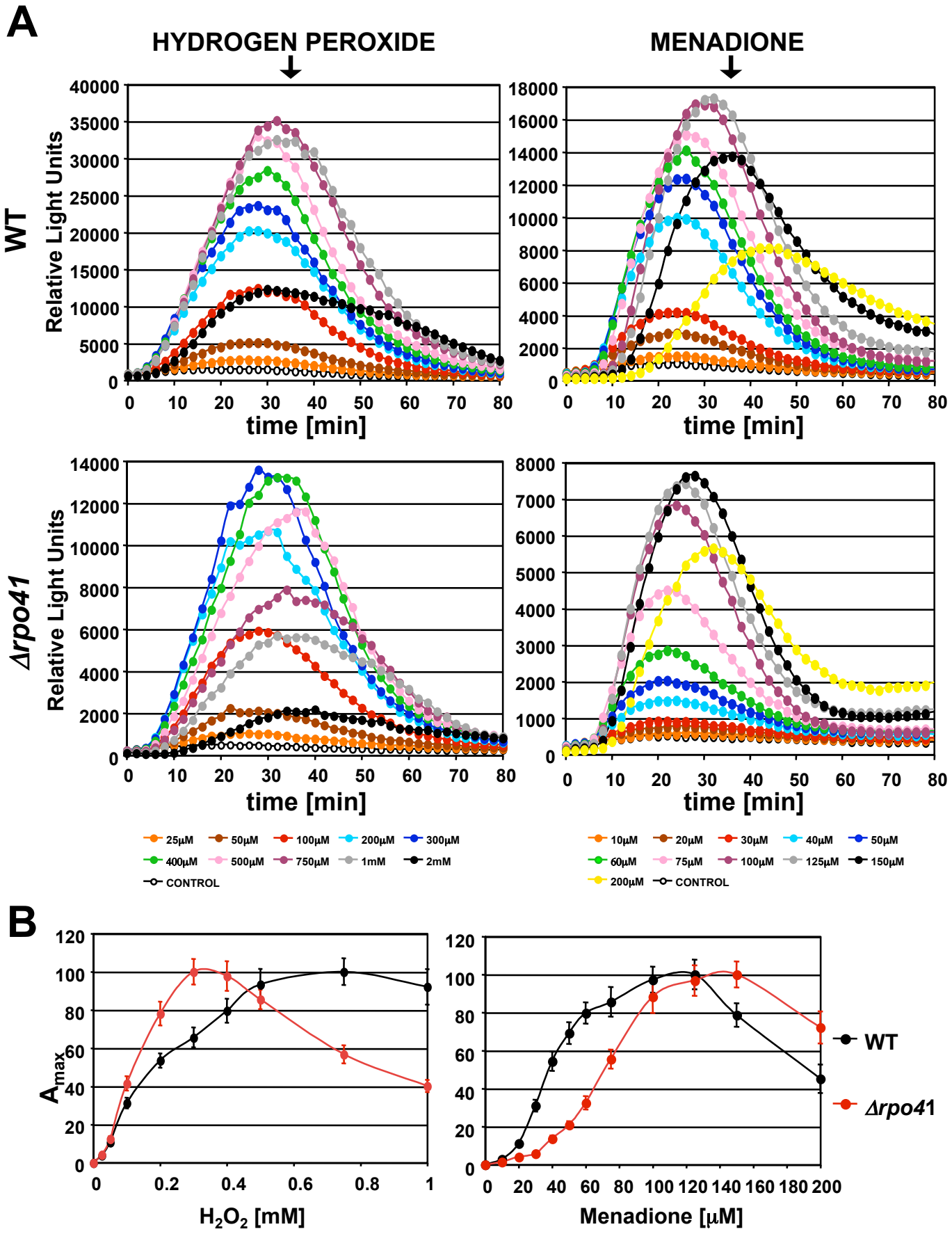

A
HYDROGEN PEROXIDE
MENADIONE
WT
Δrpo41
Relative Light Units
time [min]
25µM 50µM 100µM 200µM 300µM
400µM 500µM 750µM 1mM 2mM
CONTROL
10µM 20µM 30µM 40µM 50µM
60µM 75µM 100µM 125µM 150µM
200µM CONTROL
B
Amax
H2O2 [mM]
Menadione [µM]
WT
Δrpo41

Figure 5

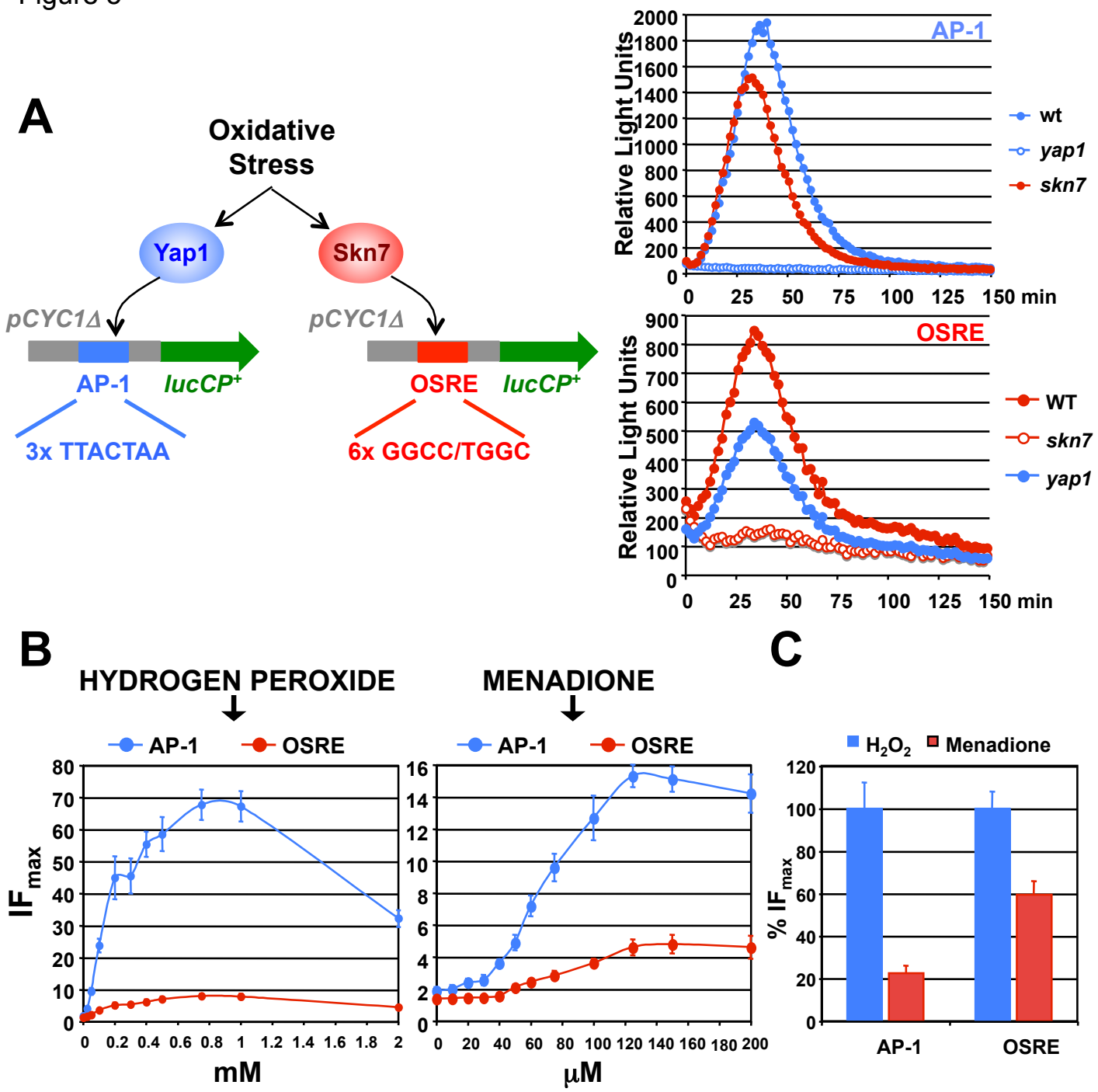

A
Oxidative Stress
Yap1
Skn7
pCYC1Δ
pCYC1Δ
AP-1
lucCP+
3x TTACTAA
OSRE
lucCP+
6x GGCC/TGGC
AP-1
Relative Light Units
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0 25 50 75 100 125 150 min
wt
yap1
skn7
OSRE
Relative Light Units
900
800
700
600
500
400
300
200
100
0
0 25 50 75 100 125 150 min
WT
skn7
yap1
B
HYDROGEN PEROXIDE
MENADIONE
AP-1
OSRE
AP-1
OSRE
IFmax
80
70
60
50
40
30
20
10
0
0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8 2
mM
16
14
12
10
8
6
4
2
0
0 20 40 60 80 100 120 140 160 180 200
μM
C
H2O2
Menadione
% IFmax
120
100
80
60
40
20
0
AP-1
OSRE

Figure 6

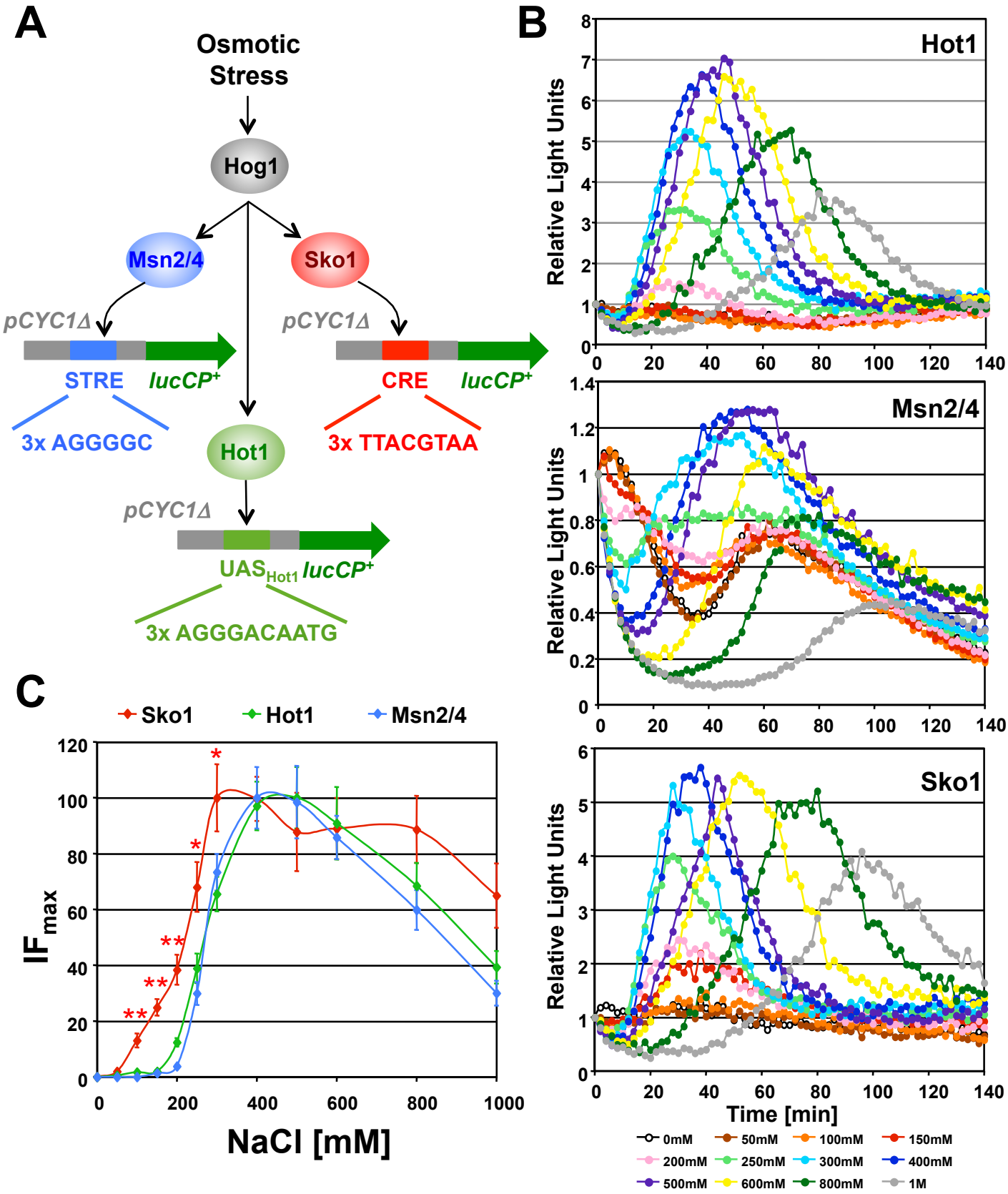

A
Osmotic Stress
Hog1
Msn2/4
Sko1
pCYC1Δ
STRE
lucCP+
3x AGGGGC
pCYC1Δ
CRE
lucCP+
3x TTACGTAA
Hot1
pCYC1Δ
UAS Hot1
lucCP+
3x AGGGACAATG
B
Hot1
Msn2/4
Sko1
Relative Light Units
Time [min]
0mM
50mM
100mM
150mM
200mM
250mM
300mM
400mM
500mM
600mM
800mM
1M
C
Sko1
Hot1
Msn2/4
IFmax
NaCl [mM]

Figure 7

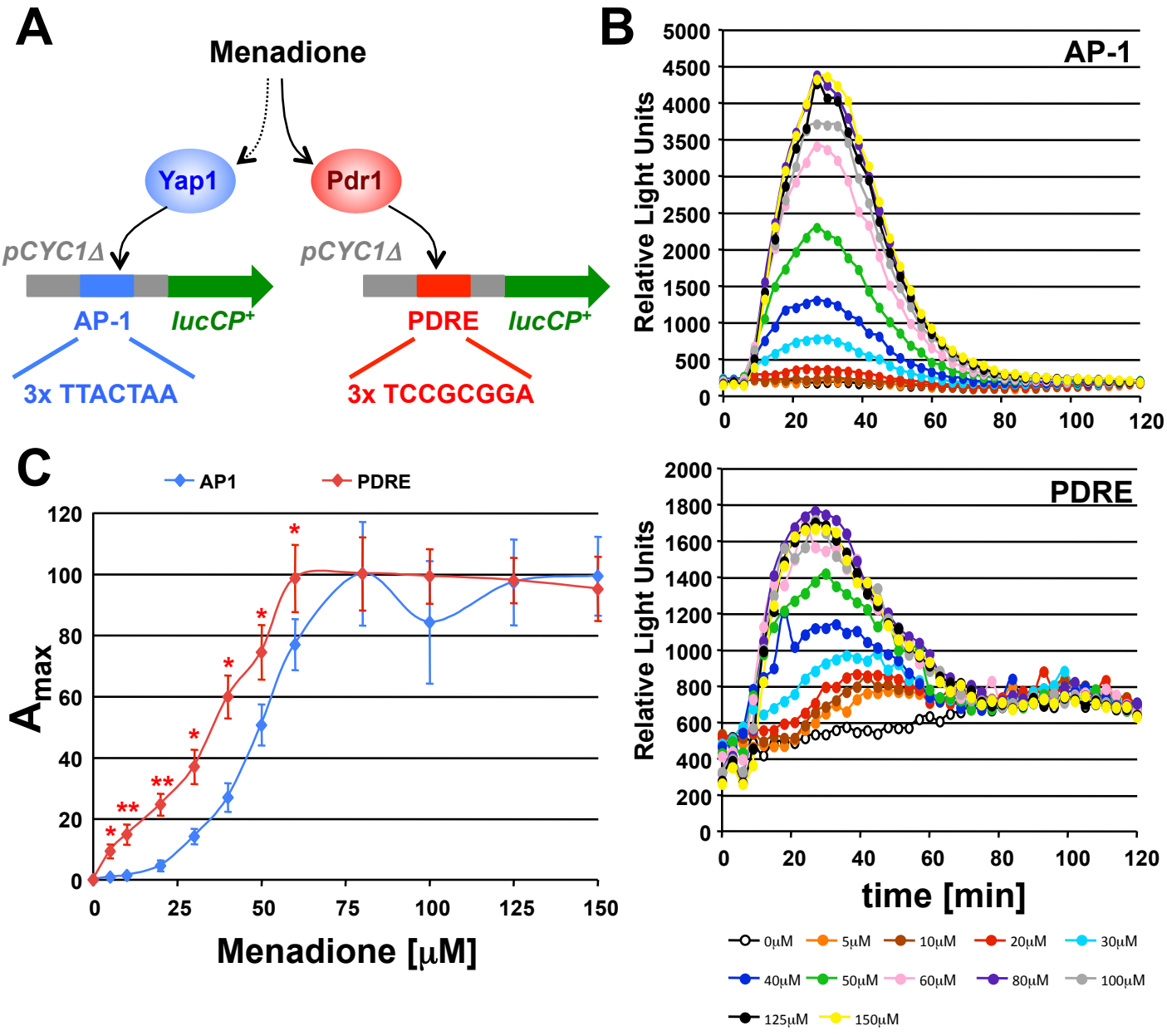
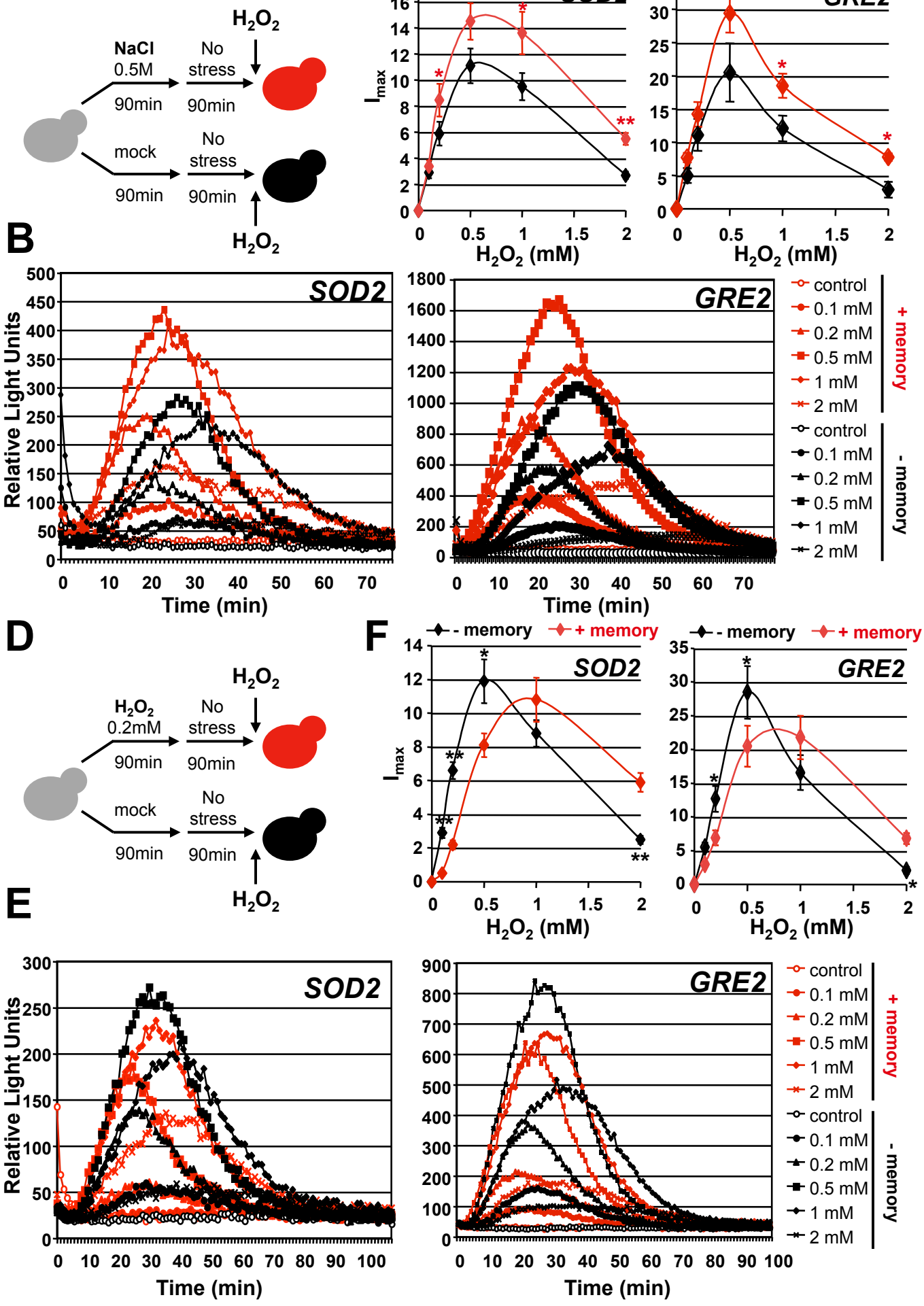
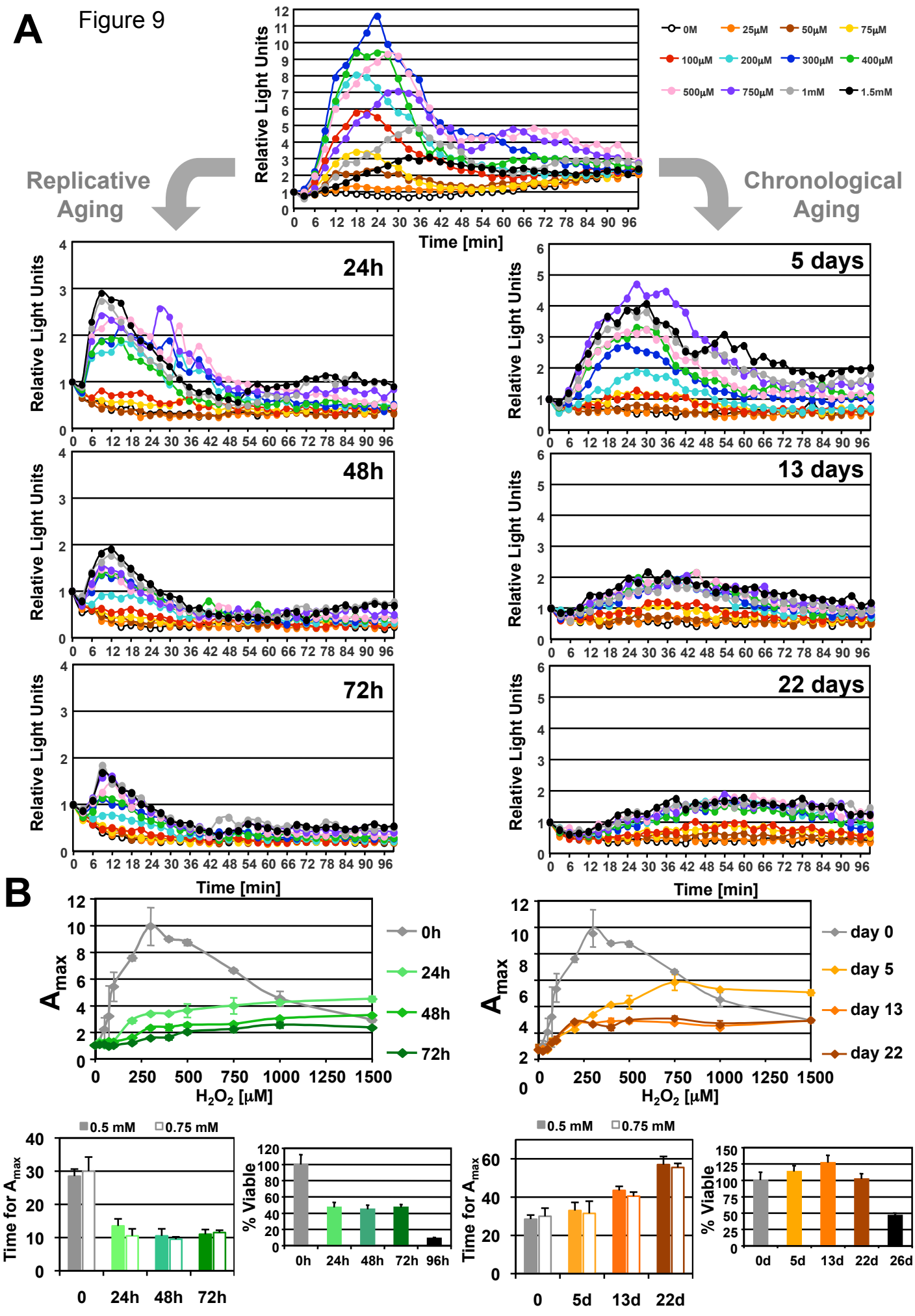

A
Menadione
Yap1
Pdr1
pCYC1Δ
pCYC1Δ
AP-1
lucCP+
PDRE
lucCP+
3x TTACTAA
3x TCCGCGGA
B
AP-1
Relative Light Units
PDRE
Relative Light Units
time [min]
0μM
5μM
10μM
20μM
30μM
40μM
50μM
60μM
80μM
100μM
125μM
150μM
C
AP1
PDRE
Amax
Menadione [μM]

Figure 8


A
NaCl
0.5M
90min
No
stress
90min
H_2O_2
mock
90min
No
stress
90min
H_2O_2
B
SOD2
Relative Light Units
Time (min)
GRE2
Time (min)
control
0.1 mM
0.2 mM
0.5 mM
1 mM
2 mM
+ memory
- memory
C
- memory
+ memory
SOD2
GRE2
I_{max}
H_2O_2 (mM)
D
H_2O_2
0.2mM
90min
No
stress
90min
H_2O_2
mock
90min
No
stress
90min
H_2O_2
E
SOD2
Relative Light Units
Time (min)
GRE2
Time (min)
control
0.1 mM
0.2 mM
0.5 mM
1 mM
2 mM
+ memory
- memory
F
- memory
+ memory
SOD2
GRE2
I_{max}
H_2O_2 (mM)


A
Figure 9
Relative Light Units
Time [min]
0M
25μM
50μM
75μM
100μM
200μM
300μM
400μM
500μM
750μM
1mM
1.5mM
Replicative Aging
Chronological Aging
24h
48h
72h
5 days
13 days
22 days
B
Time [min]
A_{max}
H_2O_2 [μM]
0h
24h
48h
72h
day 0
day 5
day 13
day 22
0.5 mM
0.75 mM
Time for A_{max}
% Viable
0h 24h 48h 72h 96h
0 24h 48h 72h
0d 5d 13d 22d 26d
0 5d 13d 22d