



Proteome-wide tagging with an H₂O₂ biosensor reveals highly localized and dynamic redox microenvironments

Paraskevi Kritsiligkou^{a,1}, Katharina Bosch^{a,b,1}, Tzu Keng Shen^{a,b}, Matthias Meurer^{c,d}, Michael Knop^{c,d}, and Tobias P. Dick^{a,b,2}

Edited by Rafael Radi, Universidad de la Republica Facultad de Medicina, Montevideo, Uruguay; received August 15, 2023; accepted September 20, 2023

Hydrogen peroxide (H₂O₂) sensing and signaling involves the reversible oxidation of particular thiols on particular proteins to modulate protein function in a dynamic manner. H₂O₂ can be generated from various intracellular sources, but their identities and relative contributions are often unknown. To identify endogenous “hotspots” of H₂O₂ generation on the scale of individual proteins and protein complexes, we generated a yeast library in which the H₂O₂ sensor HyPer7 was fused to the C-terminus of all protein-coding open reading frames (ORFs). We also generated a control library in which a redox-insensitive mutant of HyPer7 (SypHer7) was fused to all ORFs. Both libraries were screened side-by-side to identify proteins located within H₂O₂-generating environments. Screening under a variety of different metabolic conditions revealed dynamic changes in H₂O₂ availability highly specific to individual proteins and protein complexes. These findings suggest that intracellular H₂O₂ generation is much more localized and functionally differentiated than previously recognized.

hydrogen peroxide | genetically encoded probes | redox signaling | redox regulation

Hydrogen peroxide (H₂O₂) is a molecule highly relevant to aerobes. It is formed by the partial reduction of O₂ and is a product of aerobic respiration and oxidases. H₂O₂ leads to protein thiol oxidation, either directly or through the mediation of enzymes (1). It is mainly through reversible protein thiol oxidation, a regulatory posttranslational modification, that cells respond and adapt to changes in H₂O₂ availability (2).

Genetically encoded H₂O₂ probes have greatly advanced our understanding of H₂O₂ in biological systems. There are two types of such probes, one coupling the bacterial H₂O₂-sensing protein OxyR to a circularly permuted fluorescent protein (HyPer probes) (3–5) and the other coupling a thiol peroxidase to a redox-sensitive fluorescent protein (roGFP2-Orp1, roGFP2-Tsa2ΔC_R, and others) (6–9). Until now, these probes were typically expressed as freely diffusible proteins with a targeting peptide and predominantly used to monitor average H₂O₂ within membrane-bounded compartments (the cytosol, nucleus, peroxisomes, chloroplasts, mitochondria, and the mitochondrial intermembrane space). In a few cases, H₂O₂ probes have also been targeted and anchored to membranes (10). Overall, these studies revealed differences and crosstalk between the classical subcellular compartments and helped to clarify the role of compartment-specific redox enzymes in H₂O₂ homeostasis (11, 12).

However, one important question remains largely unexplored. How fine-grained are the differences and fluctuations in H₂O₂ concentration within a membrane-bounded compartment? This question cannot be addressed with probes that sample a whole compartment by free diffusion. If nanometer-scale “hotspots” of elevated H₂O₂ concentration do exist, their influence on the probe will be “washed out” in the overall signal as probe molecules quickly diffuse in and out of nanoscale regions. It is important to keep in mind that the existing probes are dynamic, i.e., subject to reduction by oxidoreductases, which means that an H₂O₂ probe leaving a local H₂O₂ hotspot will not maintain its oxidized state.

It is becoming increasingly clear that cells are not only structured by membrane-bounded compartments. Instead, there seems to be an abundance of membraneless “condensates”, which recruit or exclude select components. This implies that H₂O₂ generating and scavenging enzymes may also be recruited to or excluded from certain nanometer scale condensates or aggregates. Therefore, it seems increasingly likely that measuring an average H₂O₂ signal across a whole compartment is missing out on the most interesting information.

In this paper, we asked whether the intracellular H₂O₂ distribution is fine-grained on the nanoscale, whether it is variable and dynamic on the level of individual proteins and protein complexes within the same compartment. To this end, we fused the genetically encoded H₂O₂ biosensor HyPer7 to the C-terminus of each individual yeast protein to create a library of yeast strains in which every protein is equipped with its own proximal H₂O₂ probe. We chose HyPer7 because it is believed to function as a monomer, unlike the even more sensitive roGFP2 peroxidoredoxin fusion proteins, which strictly require

Significance

So far, genetically encoded H₂O₂ probes have been mostly expressed as freely diffusible proteins within individual membrane-bounded compartments. However, it is becoming increasingly clear that cells are also compartmentalized on a much smaller scale, by membraneless condensates. This study addresses the question to which extent differences and changes in H₂O₂ concentration exist on the nanoscale. Using a yeast library in which every protein is equipped with its own proximal H₂O₂ probe, this study reveals redox differences and changes on the scale of individual protein complexes, invisible to H₂O₂ probes that sample a whole membrane-bounded compartment by random diffusion. These observations indicate that redox control by H₂O₂ is much more local, differentiated, and dynamic than previously appreciated.

Author contributions: P.K., K.B., M.M., M.K., and T.P.D. designed research; P.K., K.B., and T.K.S. performed research; P.K., K.B., T.K.S., and T.P.D. analyzed data; and P.K., K.B., M.K., and T.P.D. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

Copyright © 2023 the Author(s). Published by PNAS. This open access article is distributed under [Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 \(CC BY-NC-ND\)](#).

¹P.K. and K.B. contributed equally to this work.

²To whom correspondence may be addressed. Email: T.Dick@dkfz-heidelberg.de.

This article contains supporting information online at <https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2314043120/-/DCSupplemental>.

Published November 22, 2023.

assembly into at least dimers. We made use of the H₂O₂-insensitive HyPer7 mutant SypHer7 to recognize and exclude artifacts. Tethered to a protein, HyPer7 reports on the immediate redox environment of that particular protein. Thus, increased HyPer7 oxidation within the context of a fusion protein indicates the protein's proximity to an H₂O₂ source. It does not necessarily indicate that the attached protein is itself a generator of H₂O₂. Nor does it allow conclusions about the redox state of the attached protein.

Using this approach, we found the following: First, we confirmed that the redox state of tethered HyPer7 differs substantially between individual proteins in the same subcellular compartment. As expected, these protein context-dependent redox differences cannot be observed with freely diffusible H₂O₂ probes. Second, we identified both known and unknown intracellular hotspots of H₂O₂ generation. Third, we observed that changes in metabolism (nutrient availability) cause changes in the tethered HyPer7 redox state for highly distinct sets of proteins, which are often metabolic enzymes. We also observed that opposing redox changes occur in parallel within the same subcellular compartment. Fourth, by crossing the HyPer7 fusion protein library with a deletion library, we identified H₂O₂-generating and consuming enzymes controlling context-specific HyPer7 oxidation and reduction. Taken together, these findings strongly support the notion that nanoscale redox domains exist on the level of individual protein complexes/aggregates, which potentially are part of condensates. These observations show that redox control by H₂O₂ and oxidoreductases is much more local, differentiated, and dynamic than previously appreciated.

Results

Construction and Screening of a HyPer7/SypHer7 Fusion Library.

To create a library of yeast strains in which HyPer7 is fused to each individual gene product, the SWAp tag (SWAT) approach was used (13, 14). Briefly, the coding sequence for HyPer7 was inserted at the C-terminus of each individual open reading frame (ORF) to create a gene locus encoding a fusion protein with a 15-amino acid linker, ORF-RTLQVDGGSGGGGS-HyPer7 (Fig. 1A). The resulting HyPer7 fusion protein library contains 5366 individual strains. In addition, a matching control library in which each protein is fused to the oxidation-insensitive HyPer7 mutant HyPer7(C121S), named SypHer7, was generated. Both libraries were verified at random by PCR and immunoblotting (*SI Appendix, Fig. S1 A and B*).

To demonstrate that fused HyPer7 is functional in principle, we treated selected library strains with antimycin A, an inhibitor of the electron transport chain (ETC) known to induce mitochondrial H₂O₂ generation. HyPer7 fused to ETC subunits was oxidized almost immediately, whereas cytosolic or nuclear fusion proteins responded after a lag phase, showing that protein-specific HyPer7 tagging provides spatiotemporal resolution (*SI Appendix, Fig. S1C*). For whole proteome measurements, the combined HyPer7/SypHer7 fusion protein library was arrayed and grown on agar plates containing synthetic complete defined (SCD) medium with 2% glucose (Glc) for 24 h (*SI Appendix, Fig. S1D*).

We previously reported that the fluorescence ratio of SypHer7 is higher than that of reduced HyPer7 (15). This was an unexpected observation because SypHer7 cannot form the disulfide bridge in the OxyR domain and therefore should mimic the fully reduced state of HyPer7. The biophysical reason for the higher-than-expected SypHer7 baseline ratio is unclear, but it can be assumed that the C121S mutation alters the conformational dynamics in such a way that the fluorophore protonation equilibrium is shifted toward

deprotonation (i.e., a higher fluorescence ratio). We therefore asked whether the SypHer7 baseline shift applies to our libraries. An analysis of reference strains expressing the probes as free (unfused) proteins in the cytosol confirmed that SypHer7 exhibits a higher ratio than HyPer7 (Fig. 1B). The same phenomenon is seen when the ratio distributions of HyPer7 and SypHer7 fusion proteins are compared (Fig. 1C). As a consequence, direct ratio comparisons between HyPer7 and SypHer7 fusion proteins are not meaningful. We therefore chose a different approach to compare HyPer7 and SypHer7 responses in a stringent manner.

Unfused cytosolic HyPer7 is almost completely reduced (15). Thus, we first identified HyPer7 fusion proteins exhibiting ratios above the 99% percentile of the unfused HyPer7 ratio distribution (Fig. 1D, *Top*). These fusion proteins are most likely to contain (partially) oxidized HyPer7. However, there is also the theoretical possibility that a fusion-protein-specific structural perturbation increases the ratio without oxidation. To identify potential oxidation-independent ratio effects, we identified SypHer7 fusion proteins with ratios above the 99% percentile of the unfused SypHer7 ratio distribution (Fig. 1D, *Bottom*). We then excluded all proteins from analysis that showed an elevated ratio in both HyPer7 and SypHer7 fusions. Interestingly, these proteins mostly function in ubiquitination and proteolysis, suggesting that proximity of HyPer7/SypHer7 to these machineries can perturb the fluorophore in a redox-independent manner (*SI Appendix, Fig. S2A and Dataset S1*). The remaining set of HyPer7 fusion proteins we considered to be (at least partially) oxidized, as it strictly conformed to our two criteria, namely an increased ratio relative to unfused HyPer7 (Fig. 1E, *Upper*) without an increase of the corresponding SypHer7 fusion protein relative to unfused SypHer7 (Fig. 1E, *Lower*).

Applying the above criteria, growth on glucose-containing SCD medium for 24 h led to the identification of 43 oxidized HyPer7 fusion proteins (Fig. 1F and *Dataset S2*). Not unexpectedly, the majority of these proteins is mitochondrial. Numerous proteins are part of the ETC, including complex II (Sdh1, Sdh4), complex III (Qcr2, Qcr9), complex IV (Cyt1), and complex V (Atp18, Atp7). We also identified both subunits of the E1 component (Pda1, Pdb1) of the pyruvate dehydrogenase complex, a known source of H₂O₂ (16). Other proteins are associated with sites or processes that have been linked to H₂O₂ generation, for example, components of the peroxisome (Pex11, Pex25) and the heme biosynthetic pathway (Hem15, Mcx1) (17).

Redox Changes Caused by Carbon Source Switching. Having identified H₂O₂-sensing HyPer7 fusion proteins in cells growing on glucose for 24 h, we then evaluated the influence of changing metabolic conditions in a metabolic switch experiment. Cells were first grown on SCD for 24 h, measured, and then transferred (i.e., repinned) to plates containing raffinose (Raf) (SCR). After 24 h, SCR plates were measured and cells again transferred to SCD plates, to be measured after another 24 h (Fig. 2A).

Using the procedure and criteria defined above, we identified oxidized fusion proteins for the three conditions (Fig. 2B and *Dataset S2*): Upon shifting from Glc to Raf, more fusion proteins were found to be oxidized ($n = 43$ vs. $n = 117$). Following the back-shift to Glc, again fewer fusion proteins were found to be oxidized ($n = 48$). The same trend was seen across different cellular compartments (Fig. 2C). The oxidation of 7, 77, and 6 fusion proteins was specific to primary Glc growth, Raf growth, and secondary Glc growth, respectively (Fig. 2B). Numerous fusion proteins ($n = 24$), mainly mitochondrial, were found to be oxidized under all conditions (Fig. 2D). Other fusion proteins ($n = 27$), including many cytosolic proteins, were only seen to be oxidized under Raf

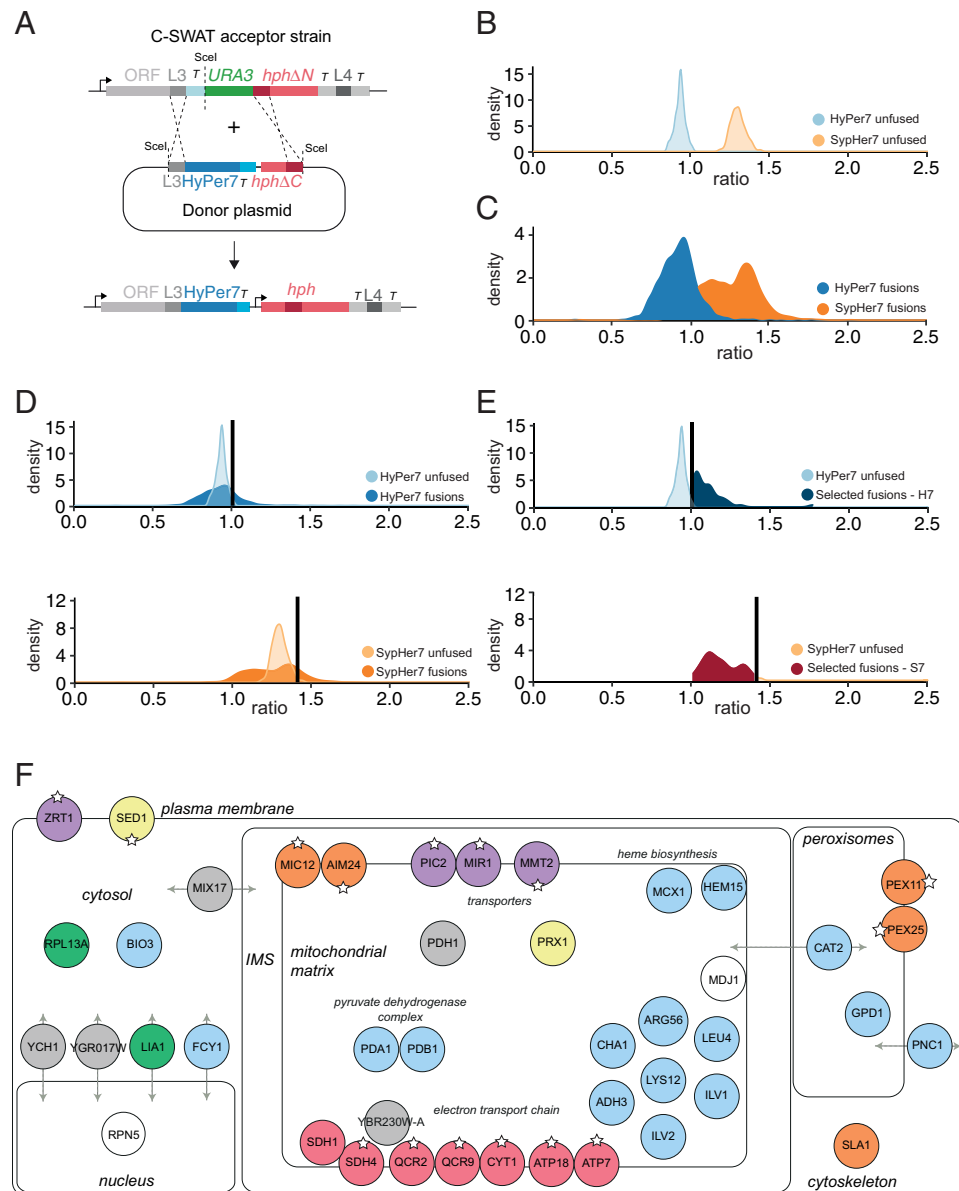


Fig. 1. Construction and screening of a HyPer7/SypHer7 fusion library. (A) Library generation using the C-SWAT approach. HyPer7 is fused to the C-terminus of all ORFs. Successful recombination leads to hygromycin resistance. (B) Ratio distributions for free (unfused) HyPer7 (light blue) and SypHer7 (light orange), as measured on SCD plates after 24 h. (C) Ratio distributions for HyPer7 (blue) and SypHer7 (orange) fusion proteins, as measured on SCD plates after 24 h. (D) Ratio distribution overlay for fused and unfused probes. Same data as in B and C. The 99th percentile of the unfused probe is indicated by a black vertical line. *Top*: HyPer7 (blue hues). *Bottom*: SypHer7 (orange hues). (E) Ratio distributions of fusion proteins classified as oxidized. *Top*: HyPer7 fusion proteins above the 99th percentile of unfused HyPer7 (dark blue). *Bottom*: SypHer7 fusion proteins below the 99th percentile of unfused SypHer7 (dark red). (F) Intracellular location of 43 proteins classified as oxidized after growth on SCD for 24 h. Dashed gray arrows indicate alternative locations. Stars indicate the predicted or annotated location of the C-terminus in transmembrane proteins. Color code: ETC (pink), metabolic enzymes (blue), stress-related proteins (yellow), structural proteins (orange), transporters (purple), translation (green), uncharacterized (gray), and others (white).

(Fig. 2E). However, these fusion proteins were not measurable under Glc. This is not surprising because the expression of many proteins depends on the carbon source. Therefore, not all fusion proteins can be measured across all conditions. Thus, for further analysis, we specifically focused on those proteins for which we could obtain HyPer7 and SypHer7 ratios across all three conditions ($n = 846$). We only considered HyPer7 ratio changes if there was no concurrent change in the corresponding SypHer7 ratio. The transition from Glc to Raf decreased the HyPer7 ratio of 104 fusion proteins while increasing the ratio of just two (Fig. 2 F, *Left*). The return from Raf to Glc increased the HyPer7 ratio of 85 fusion proteins while decreasing the ratio of just two (Fig. 2 F, *Right*). Comparing primary and secondary Glc growth, only 6 out of 838 fusion proteins differed in their ratio (SI Appendix, Fig. S2B). Ratio

changes of example fusion proteins are shown in Fig. 2G. In conclusion, while growth on Raf led to the detection of additional pro-oxidative protein environments (Fig. 2E), many fusion proteins shifted towards a more reductive environment under Raf (Fig. 2F).

Redox Changes Caused by Nutrient Depletion. We then asked how nutrient depletion influences the redox state of individual HyPer7 fusion proteins. We compared strains grown on SCD for 24 h and 48 h (SCD_24h vs. SCD_48h). Colony growth on minimal medium (SD) was slower and therefore only allowed measurements after 48 h. We therefore compared strains grown on SCD (24 h and 48 h) with those grown on SD at 48 h. When grown on SCD, fewer fusion proteins were oxidized after 48 h ($n = 25$) than after 24 h ($n = 48$) (Fig. 3A and Dataset S2). Eight

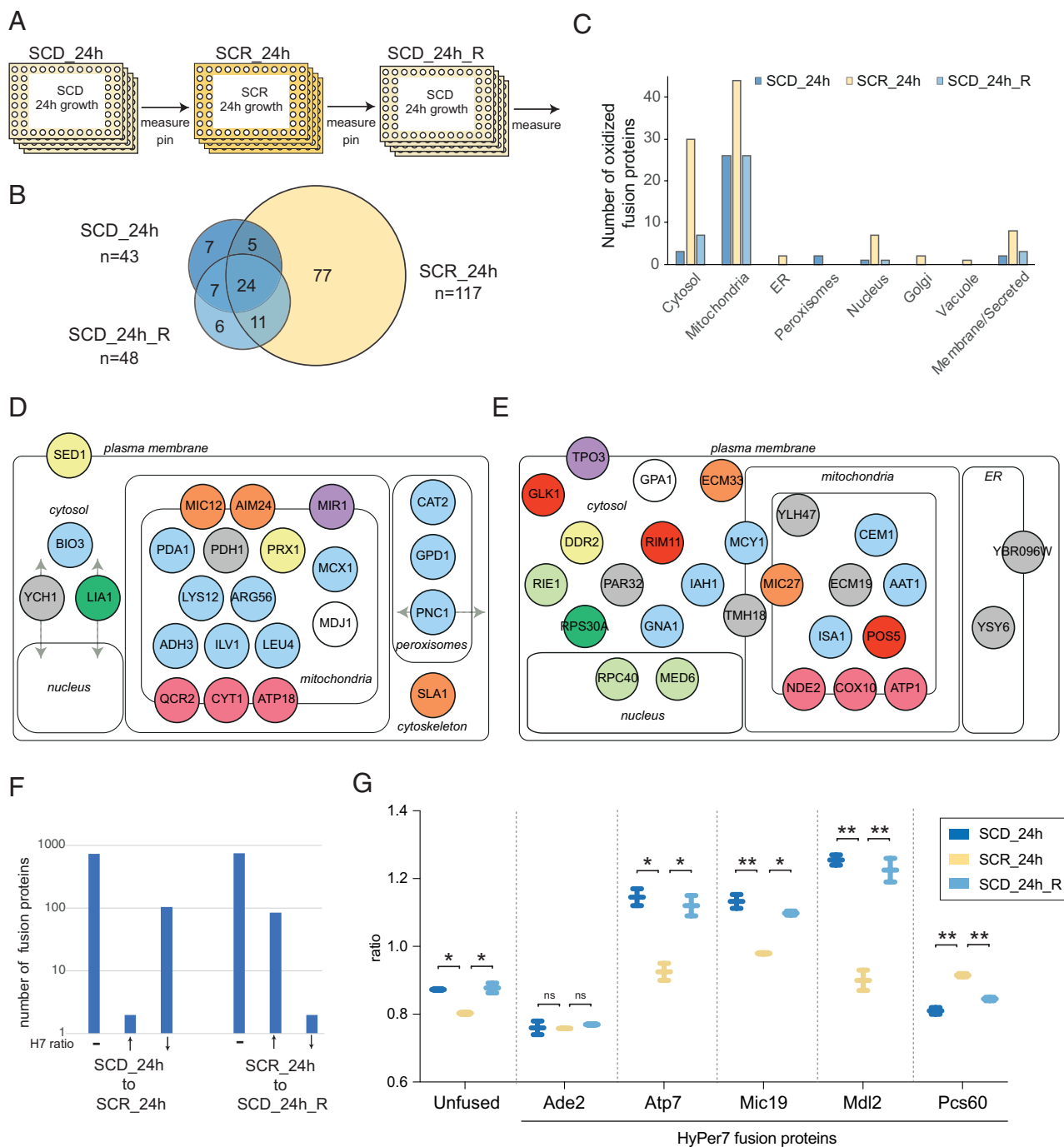


Fig. 2. Redox changes caused by carbon source switching. (A) Experimental design of the metabolic switch experiment: Cells were grown on SCD for 24 h (SCD_24h), measured, pinned to SCR plates, grown for 24 h (SCR_24h), measured, pinned again onto SCD plates (SCD_24h_R) and measured. (B) Venn diagram depicting overlapping sets of oxidized fusion proteins in three metabolic conditions: SCD_24h (blue), SCR_24h (yellow), and SCD_24h_R (light blue). (C) Classification of oxidized fusion proteins by subcellular localization. Same color code as in B. (D) Intracellular location of 24 proteins associated with oxidized HyPer7 across all three metabolic conditions. Color code: ETC (pink), metabolic enzymes (blue), stress-related proteins (yellow), structural proteins (orange), transporters (purple), translation (green), uncharacterized (gray), and others (white). (E) Intracellular location of 27 fusion proteins associated with oxidized HyPer7 and only detectable on Raf (SCR_24h). Color code: ETC (pink), metabolic enzymes (blue), stress-related proteins (yellow), structural proteins (orange), transporters (purple), translation (green), RNA-associated (light green), kinases (red), uncharacterized (gray), and others (white). (F) Response of HyPer7 ratios to carbon source switching. Only fusion proteins measurable across all three conditions are included. All fusion proteins with changing SypHer7 ratios were excluded from analysis. Left: Change from Glc to Raf. Right: Change from Raf back to Glc. (G) Examples of HyPer7 fusion proteins exhibiting ratio changes during carbon source switching: SCD_24h (blue), SCR_24h (yellow), and SCD_24h_R (light blue).

proteins were oxidized at both time points, and 17 proteins were uniquely oxidized after 48 h. Comparing SCD to SD, many more fusion proteins were found to be oxidized on SD (Fig. 3A). At 48 h, there was no overlap between SCD and SD. SCD at 24 h and SD at 48 h shared only 7 oxidized fusion proteins. Interestingly, many more cytosolic fusion proteins are oxidized on SD relative to

SCD (Fig. 3B). The comparison of HyPer7 ratios across the three conditions revealed dynamic behavior in both directions (Fig. 3C). Comparing ratios between SCD at 24 h and SCD at 48 h, 720 were unchanged, 15 increased, and 26 decreased. Comparing ratios between SCD at 24 h and SD at 48 h, 300 were unchanged, 31 increased, and 8 decreased. Comparing ratios between SCD

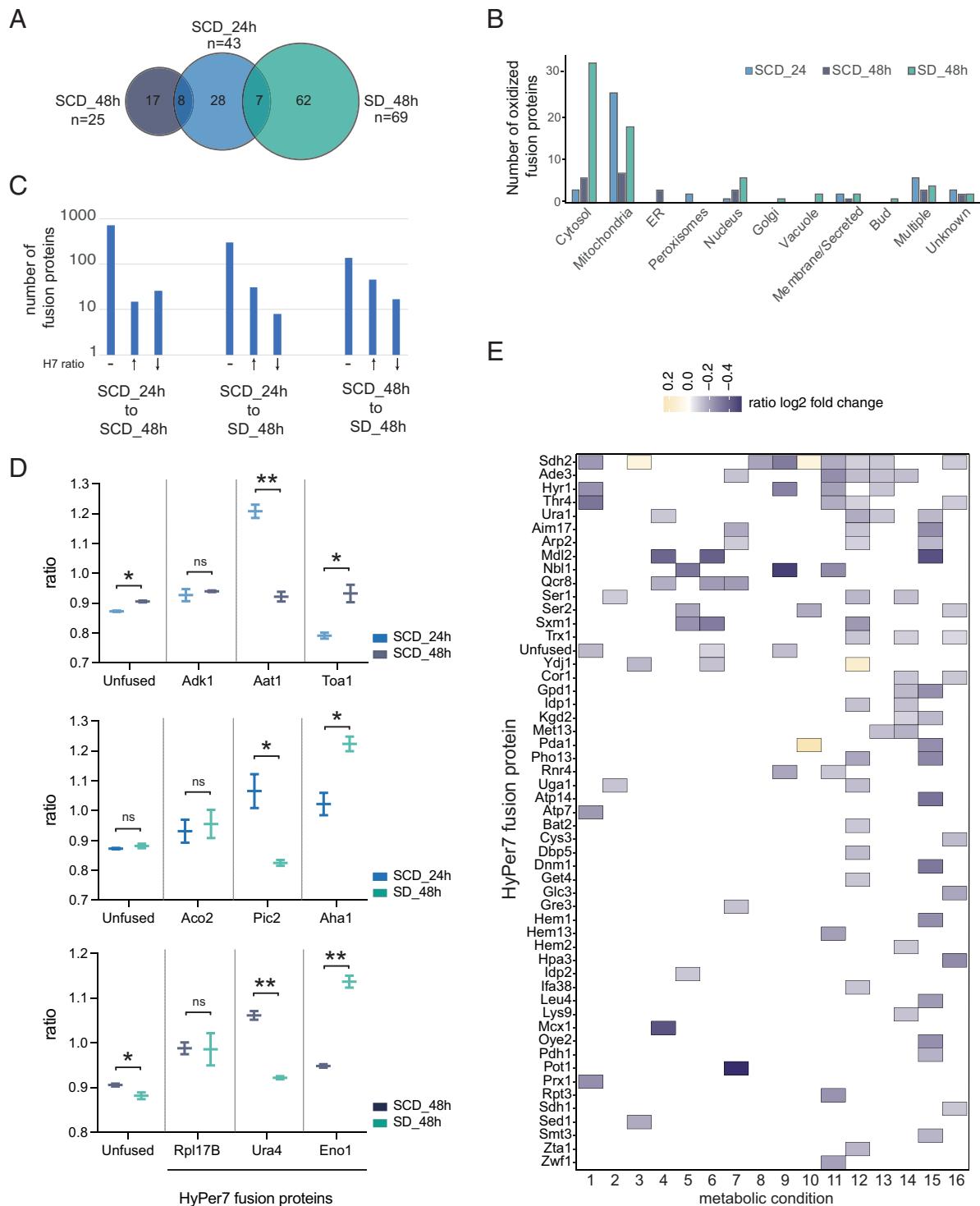


Fig. 3. Redox changes caused by nutrient depletion and medium composition. (A) Venn diagram depicting overlapping sets of oxidized fusion proteins in three metabolic conditions: SCD_24h (blue), SCD_48h (dark blue), and SD_48h (green). (B) Classification of oxidized fusion proteins by subcellular localization. Same color code as in A. (C) Response of HyPer7 ratios to changing conditions. Only fusion proteins measurable across all three conditions are included. All fusion proteins with changing SypHer7 ratios were excluded from analysis. *Left:* Change from SCD_24h to SCD_48h. *Middle:* Change from SCD_24h to SD_48h. *Right:* Change from SCD_48h to SD_48h. (D) Examples of HyPer7 fusion proteins showing different kinds of responses to metabolic change. *Top:* SCD_24h vs. SCD_48h; *Middle:* SCD_24h vs. SD_48h; *Bottom:* SCD_48h vs. SD_48h. Same color code as in A. (E) Heat map depicting significant ($P > 0.05$) changes in the HyPer7 ratio relative to growth on SCD, in the absence of SypHer7 ratio changes. Media: 1. SCD-QE; 2. SCD-FWY; 3. SCD-IV; 4. SD; 5. SD+P; 6. SD+Q; 7. SD+E; 8. SD+QE; 9. SC+0.2%Glc; 10. SC+4%Glc; 11. SC+2%Raf; 12. SC+1%Raf+1%Gal; 13. SC+2%Suc; 14. SC+2%Frc; 15. SCD with MSG replacing ammonium sulfate; 16. SCD with proline replacing ammonium sulfate.

and SD, both at 48 h, 139 were unchanged, 46 increased, and 17 decreased. Individual examples of fusion proteins exhibiting HyPer7 redox differences are shown in Fig. 3D. Additionally, we investigated the impact of low glucose (0.2%). Relative to growth on 2% glucose, unfused cytosolic HyPer7 was more oxidized

(SI Appendix, Fig. S3A) and 417 out of 677 measured fusion proteins showed a shift toward increased oxidation (SI Appendix, Fig. S3B). This observation is in line with the notion that the regeneration of reducing equivalents is limited under low glucose, thus affecting probe redox states more globally.

Pre2-HyPer7 fusion protein was significantly more reduced in the absence of Sod1, under all three metabolic conditions. Among all tested deletions, the lack of Sod1 had the most significant effect on Pre2-HyPer7 (*SI Appendix, Fig. S4C*). Likewise, the lack of Sod2 decreased HyPer7 oxidation in several fusion proteins (9 and 10, in Glc and Raf, respectively) (*SI Appendix, Fig. S4D and E*), suggesting that Sod2 may act as a localized H₂O₂ generator, as previously proposed (20). However, the observed effects could also be caused more indirectly, and compensation effects cannot be excluded.

We then looked at enzymes known to scavenge H₂O₂. Prx1 is the principal mitochondrial thiol peroxidase, and its deletion is expected to increase H₂O₂ availability within the mitochondrial matrix. Indeed, the lack of Prx1 led to a significant increase in oxidation of seven fusion proteins, all of them localized to the mitochondrial matrix (Fig. 4C). IMS-localized fusion proteins, e.g., Ccp1-HyPer7, did not respond to the absence of Prx1. The lack of the cytosolic peroxiredoxin Tsa1 led to increased oxidation of several fusion proteins, while some others were more reduced (*SI Appendix, Fig. S5A*). The pro-oxidative effect of Tsa1 deficiency was more pronounced on Raf, mostly affecting cytosolic fusion proteins (including Ola1, Arc1, Gis2, Rps7b, Mdh2, Tma17, Pho13, Cub1, and Get4) (*SI Appendix, Fig. S5B*). For Rps7b, the lack of Tsa1 led to oxidation across all three conditions, more than any other deletion strain that was tested (*SI Appendix, Fig. S5C*).

In sum, these results reconfirmed that HyPer7 fusion proteins reflect local H₂O₂ availability. We then asked about the role of less-characterized enzymes. The yeast NADPH oxidase homologue (Yno1/Aim14) has been proposed to contribute to H₂O₂ generation (21, 22). On SCD medium, four proteins were less oxidized in the absence of Yno1 (*SI Appendix, Fig. S6A*). However, the influence of Yno1 was more obvious on SD medium, where 10 fusion proteins were less oxidized (*SI Appendix, Fig. S6B*). The lack of D-arabinono-1,4-lactone oxidase (Alo1) led to increased oxidation of numerous fusion proteins (*SI Appendix, Fig. S6C*), suggesting that Alo1 may have a previously unrecognized role in limiting H₂O₂ availability. Alo1 generates both H₂O₂ and D-erythroascorbic acid. The latter is acting as an antioxidant that may counteract H₂O₂ generation by scavenging superoxide (23). A graphical overview of the tested combinations of fusion proteins and deletions, across three different growth conditions, is shown in *SI Appendix, Fig. S7*.

Discussion

The purpose of this work was to create a genetically tractable platform for identifying highly localized “H₂O₂ hotspots” inside yeast cells. The fusion of HyPer7 to each individual protein allowed us to measure the HyPer7 redox state in the spatial context of that protein. There are several ways of how protein context-specific HyPer7 oxidation may reflect an actual H₂O₂ hotspot. For instance, HyPer7 may be directly fused to an enzyme that produces H₂O₂, or it may be fused to a protein localized in proximity to an enzyme producing H₂O₂.

Although HyPer7 is usually described as a H₂O₂ probe, it is rather a probe reporting on the balance of H₂O₂-dependent OxyR oxidation and thioredoxin-dependent OxyR reduction (5, 15). This means that changes in the HyPer7 redox state can be caused not only by increased or decreased H₂O₂ availability but also by increased or decreased reductase activity. Therefore, the accessibility of the HyPer7 OxyR domain to oxidoreductases may also play a role in causing redox differences in specific locations. Even though HyPer7 is not a pure H₂O₂ probe, its redox state can be taken to indicate the thiol oxidation–reduction balance in a particular location.

The implementation of the approach confronted us with a fundamental technical challenge. In HyPer7, the extrinsic sensitivity toward thiol oxidation–reduction (OxyR domain) is conformationally coupled to the intrinsic sensitivity toward fluorophore protonation–deprotonation (cpYFP domain), on which the excitation-ratiometric fluorescence readout is based. The key advantage of HyPer7 vs. its predecessors (HyPer 1 to 3) is its insensitivity toward ambient pH (5). Nevertheless, we considered it important to generate SypHer7 controls to make sure that an observed difference or change depends on the presence of the H₂O₂-reactive (i.e., peroxidatic) cysteine in the OxyR domain.

However, as reported here and noted by us previously (15), the fluorescence ratio of SypHer7 does not mimic fully reduced HyPer7, unlike the situation with earlier SypHer-HyPer versions. Our interpretation is that the C121S mutation alters the conformational dynamics in such a way that the fluorophore protonation equilibrium is shifted toward deprotonation. This observation had two implications. First, it necessitated an alternative approach to comparing HyPer7 and SypHer7 responses, as described above. Second, it suggested the possibility that the HyPer7 fluorophore protonation state, while being much less sensitive to ambient pH, is potentially more sensitive to redox-independent conformational effects. Indeed, in screening our libraries, we identified numerous fusion proteins in which an HyPer7 ratio increase was accompanied by a SypHer7 ratio increase, suggesting that redox-independent fluorophore protonation shifts do indeed occur, probably caused by conformational influences acting on the cpYFP domain in a particular environment. Interestingly, in many such cases, HyPer7 is attached to proteins involved in protein degradation, which may expose HyPer7 to the drag and pull of chaperones and ubiquitination-conjugating enzymes. Importantly, the protein-specific HyPer7/SypHer7 comparisons allowed us to exclude problematic fusion proteins from further analysis.

Overall, our study led to five major insights:

First, we find that the redox state of tethered HyPer7 differs substantially between individual proteins. Proteins located within the same subcellular compartment show very different HyPer7 redox states. These protein-context-dependent redox differences are not resolved with freely diffusible H₂O₂ probes. This means that the conventional approach of expressing freely diffusible redox biosensors is missing a lot of information that exists on the nanoscale. This observation is in line with the notion that there is no overall representative subcompartmental (e.g., cytosolic) redox state but rather a patchwork of highly differentiated nanoscale regions. These nanoscale “redox domains” seem to exist at the level of individual protein complexes and may also correspond to condensates.

Second, the approach reveals intracellular hotspots of H₂O₂ generation. Several of them correspond to protein complexes long known to release O₂^{•−}/H₂O₂. This includes the succinate dehydrogenase (SDH) complex, complex III of the respiratory chain, and the PDH complex. In these cases, we obtained the redox state of HyPer7 as directly tethered to subunits of these complexes. Other hotspots were revealed by proteins in the larger periphery of an H₂O₂ source. For example, HyPer7 oxidation associated with peroxisomal proteins (Pex11, Pex25, and Gpd1/Pnc1) appears to reflect acyl-CoA oxidase (Pox1) activity. The approach also revealed novel hotspot candidates. For example, HyPer7 tethered to deoxyhypusine hydroxylase (Lia1) is consistently oxidized. Lia1 uses a nonheme diiron center to reduce O₂ and to form a peroxo intermediate. Although this enzyme has not been described to oxidize substrates other than eIF5A (24), it may be a previously unrecognized source of H₂O₂.

Third, changes in metabolism cause changes in the HyPer7 redox state for highly distinct sets of proteins, in both directions, meaning that HyPer7 becomes more oxidized in the periphery of

some proteins and more reduced in the periphery of others. This implies that opposing redox changes (HyPer7 oxidation/reduction) occur in parallel, within the same subcellular compartment. Moreover, different nutrients affect different sets of proteins. For example, the availability or scarcity of a single amino acid shifts the HyPer7 redox state in association with numerous proteins. These observations show that redox control by H₂O₂ and thiol reductases is much more local, differentiated, and dynamic than previously appreciated. Again, these metabolism-dependent differences and changes were not resolvable with the conventional approach of expressing a biosensor as a freely diffusible protein.

Fourth, the crossing of HyPer7/SypHer7 fusion libraries with deletion libraries is a methodological avenue to identify proteins influencing context-specific HyPer7 oxidation and reduction. Our so-far limited subset of crosses (59 × 24 = 1,416) revealed expected factors, but also additional ones. As expected, deletion of *Pox1* affected peroxisomal HyPer7 fusion proteins. The deletion of superoxide dismutases and peroxiredoxins had relatively broad but also some specific effects. Some fusion proteins were influenced by the NADPH oxidase homologue *Yno1*. The oxidase *Alo1* was identified as a potential player in H₂O₂ homeostasis. *Alo1* generates H₂O₂ together with erythroascorbic acid, which is believed to act as an antioxidant (23). Further analysis, beyond the scope of the current study, is needed as some effects may be indirect and/or may involve compensatory changes.

Fifth, the HyPer7/SypHer7 fusion libraries provide a resource of protein-specific H₂O₂ biosensors for the community. While our current study aimed to look at the overall picture of highly context-dependent redox changes, individual fusion proteins can now be used as tools to address specific questions. Moreover, the use of HyPer7/SypHer7 fusion protein libraries can now be expanded to include additional measurement modalities (flow cytometry and microscopy) and to study the impact of external stresses and drugs.

Methods

Growth Conditions. Yeast strains to be transformed were grown on YEPD (yeast extract peptone dextrose) [1% w/v yeast extract (Gerbu), 2% w/v peptone (Formedium), and 2% w/v glucose (Sigma)]. Selection was performed on YEPD plates solidified with 2% agar (Millipore) containing either G418 (Sigma) or hygromycin (Invitrogen). Screening was performed using the following media: 1. SCD (synthetic complete dextrose) [0.17% w/v yeast nitrogen base without amino acids, with 0.5% w/v ammonium sulfate (Difco, BD), 2% w/v glucose (Sigma), Kaiser complete amino acid mix (Formedium)]. 2. SCR (synthetic complete Raf) (glucose replaced by 2% w/v Raf; Formedium). 3. SD (synthetic defined) [0.17% w/v yeast nitrogen base without amino acids, with 0.5% w/v ammonium sulfate (Difco, BD), 2% w/v glucose (Sigma), supplemented with essential amino acids (Leu, His, Met, Lys; Sigma) and Ura (Sigma)]. 4. SC with low glucose: 0.2% w/v glucose. Colonies were grown at 30 °C for either 24 or 48 h as indicated. Additional metabolic conditions tested: SC with high glucose: 4% w/v glucose; SCD with custom drop-out Kaiser amino acid mixtures (–QE, –FWY, –IV; Formedium); SD with amino acid drop-in media (+P, +Q, +E, +QE; Sigma). Alternative carbon sources: raffinose (Formedium), galactose (Sigma), sucrose (Sigma), and fructose (Sigma) were used at the indicated concentrations. Alternative nitrogen sources: YNB without amino acids and without ammonium sulfate (Formedium) was supplemented with either monosodium glutamate (MSG; Sigma) or proline (Sigma).

Library Generation. The C-SWAT acceptor library and the donor strain (YMaM639), containing the *Scel* expression module and the haploid selection cassette, were used as described (13). The plasmid pMAM484 was used to generate the donor vectors: Plasmids pMAM_HyPer7 and pMAM_SypHer7 containing the HyPer7 and SypHer7 coding sequences, respectively, were generated using the *Bam*HI and *Spe*I cloning sites. The plasmids were verified by sequencing (Euroscarf-GATC) and subsequently transformed into the donor strain using the lithium acetate protocol. The C-SWAT library was generated as described previously, using Singer Rotor technology (13, 25). Briefly, the C-SWAT acceptor

library (arrayed in 384 colony format) was mated with the donor strain containing either the HyPer7 or SypHer7 donor plasmid. Following 2 d of mating, the library was plated on selection plates for diploid selection, and this step was repeated twice. Strains were left to sporulate for 7 d at 23 °C and subsequently plated on haploid selection media. Mat strains were selected using the *HIS* cassette (26). The strains were then plated on galactose-containing medium, to induce *Scel* endonuclease expression and promote recombination. The strains were then selected for hygromycin resistance, indicative of correct recombination between the acceptor library and the donor plasmid. Selection was repeated twice. Finally, colonies were grown on SCD media plates. The strains were stored in 96-well plates at –80 °C (95 plates per library). Given the well-characterized efficiency of library generation and the antibiotic selection steps, we performed random PCR analysis on a subset of strains. For this, we used a reverse primer within the HyPer7/SypHer7 sequence and a forward primer within the ORF (27).

Control and Reference Strains. Nontransformed BY4741 and BY4742 strains were used as autofluorescence background controls. To generate strains expressing genetically integrated unfused HyPer7 or SypHer7, the respective coding sequences were cloned from p415 (15) into pRS303H (28), using Gibson assembly. Resulting plasmids were validated by sequencing (Euroscarf-GATC). Following digestion, the linearized plasmid was transformed into BY4741 and plated on hygromycin-containing plates for selection. Strains were validated by PCR.

Screening Procedures and Conditions. For the primary screen, strains were arrayed in 1536 colony format. Each plate contains 60 pairs of matching HyPer7/SypHer7 strains, representing 60 ORFs, each in triplicate colonies. In addition, each plate includes a fixed set of 59 pairs of HyPer7/SypHer7 reference reporter strains (expressing fusion proteins chosen to represent different subcellular locations, redox environments, and expression levels), and one pair expressing unfused cytosolic HyPer7/SypHer7. Furthermore, each plate contains a total of 240 nonfluorescent parental strain colonies, evenly distributed over the whole plate, to serve as autofluorescence background controls, and to allow for the correction of position-related variations (spatial normalization), as potentially caused by gradients in agar thickness, temperature, humidity, and oxygen availability (SI Appendix, Fig. S1D). Plate borders are filled with strains expressing unfused HyPer7 and SypHer7 but excluded from the measurements due to variation in nutrient availability and thus colony size. In total, the library consists of 95 plates with 1536 colonies each. The plates are freshly repinned to SCD plates, and after 24 h of growth in a static incubator (30 °C), they are repinned to the measurement plates and allowed to grow for 24 h. Colonies were then repinned to plates containing SCR medium, grown for 24 h and measured (“SCR_24h”). Colonies were then again repinned to SCD medium plates, grown for 24 h, and measured (“SCD_24h_R”). In addition, the library was measured after growth on SCD plates for 48 h (“SCD_48h”) or on SD medium for 48 h (“SD_48h”). Measurements were performed in an excitation ratiometric manner using a fluorescence plate reader (see below). All measurements were independently repeated, yielding two complete data sets for each metabolic condition. For the extended metabolic switch experiment, a similar approach was used. Of note, 119 fusion proteins were selected and arranged in a similar format as described above, with 3 colonies representing one HyPer7 fusion protein and 3 colonies representing its SypHer7 counterpart, arranged next to each other together with nonfluorescent strains. Strains expressing unfused cytosolic HyPer7/SypHer7 were also included. The arrayed plate was repinned several times on SCD for 24 h. Each plate was measured in triplicate for each condition.

Synthetic Genetic Array Analysis. To combine the expression of HyPer7 fusion proteins with gene deletions, 59 fusion proteins were selected on hygromycin resistance plates and then expanded to ensure they lost KanMX resistance. Gene deletions of 24 proteins (*ahp1*, *aim14*, *alo1*, *cta1*, *ctt1*, *dot5*, *erv2*, *fms1*, *gpx1*, *gpx2*, *grx1*, *hyr1*, *jhd2*, *lpd1*, *pox1*, *prx1*, *sod1*, *sod2*, *trx1*, *tsa1*, *tsa2*, *yhb1*, *ylr456w*, *ypr172w*) marked with KanMX resistance in the BY4742 background were taken from the deletion collection (Euroscarf). The 59 HyPer7 fusion protein strains were arrayed in the middle of a 96-well plate. Each deletion strain was arrayed in a separate 96-well plate. The plates were then mated with the HyPer7 fusion protein plate. Diploids were selected using KanMX and hygromycin and then sporulated in a static incubator for 7 d at 23 °C. Haploid selection was performed as described above, followed by selection for the correct antibiotic resistance markers. The plates were then repinned and arrayed onto a 1536 plate. The 1536

plate was divided into 60 squares of 16 colonies each. Each square of 16 colonies contained four colonies of a wild-type fusion protein strain, four colonies of the fusion protein strain in the deletion background, four colonies of BY4742 autofluorescence background control, and four colonies of the respective deletion in BY4742. Each 1536 colony plate had a total of 59 such squares. As we assessed 24 different deletions, this led to 24 different 1536 plates. These plates were grown several times in SCD for 24 h at 30 °C, before they were pinned on plates containing SCD. SCD plates were measured after 24 h and then repinned to SCR plates or SD plates. As before, SCR plates were grown for 24 h at 30 °C, and SD plates for 48 h at 30 °C. Each was done in duplicates. The plates were measured as described. Like before, plate border areas were filled with HyPer7 expressing strains but excluded from measurement.

Plate Reader Measurements. All plates were measured with a PHERAstar FSX plate reader (BMG Labtech) at 30 °C. Excitation/emission wavelengths were 405/520 nm and 488/520 nm, with a bandwidth of 10 nm.

Data Processing and Analysis. Data processing, analysis, plotting, and reporting were done using R within RStudio. Data handling, processing, and cleaning were done using R packages of the tidyverse collection (29): janitor, here, writexl, and readxl. Base R and the rstatix package were used for statistical testing. Following import, data were checked for completeness. Invalid measurements such as overflow or extreme blank values, i.e., any values above or below 6 x IQR (interquartile range) of all nonfluorescent measurements, were removed. A spatial normalization procedure was performed for each plate, and the predicted autofluorescence background values were used for blank correction. The algorithm for spatial normalization was developed by Silvia Calderazzo and Nicholas Schreck (DKFZ Biostatistics Core Facility). Reference strains were used to exclude obvious differences between plates. To obtain a reliable signal to noise ratio, a threshold was applied in addition to the removal of values below blank. A per-plate threshold defined by the 99th percentile blank value of each plate was used for both channels. Only strains (i.e., fusion proteins) fulfilling this criterion for at least 2 out of 3 technical replicates in both HyPer7 and SypHer7 libraries, and in both independent screens, were considered for further analysis. Having excluded all strains not meeting these quality criteria, we calculated the conventional fluorescence emission ratio (485 nm/400 nm) for each colony. Statistical tests were performed as indicated. Values were log2 transformed for the *t* test and ANOVA. Packages used for reading, tidying, and analysis of data included ggplot2 from

the tidyverse (29). Packages viridis, ggrepel, ggpubr, and cowplot were used for plotting. Preprocessed data are included with this manuscript (Dataset S3). Dataset S1 lists all fusion proteins that showed elevated ratios in both HyPer7 and SypHer7, in both repeats. Similarly, Dataset S2 lists fusion proteins matching the criteria defined for oxidized HyPer7 in both repeats.

Cell Lysis, Gel Electrophoresis, and Immunoblotting. Yeast cells were lysed by bead beating in PBS containing complete Protease Inhibitor Cocktail (Roche) and N-ethylmaleimide. Lysates were run on 4 to 12% gradient NuPage gels (ThermoFischer). Probe expression was visualized by immunoblotting with a polyclonal anti-GFP antibody (PABG1, Chromotek). Detection of phosphoglycerate kinase with an anti-Pgk1 antibody (Life Technologies) served as a loading control.

Antimycin A Treatment. Selected yeast strains from the HyPer7 library and a strain expressing unfused cytosolic HyPer7 were grown in SCD until mid exponential phase ($OD_{600} = 0.6$ to 1). Cells were harvested, resuspended in MES-TRIS buffer (pH 6), and dispensed into a 96-well black plate with a clear flat bottom (Falcon). Antimycin A diluted in ethanol was added to a final concentration of 50 μ M (0.5% final ethanol concentration). The HyPer7 response was measured at 30 °C using a PHERAstar FSX plate reader (BMG Labtech). Excitation/emission wavelengths were 405/520 nm and 488/520 nm, with a bandwidth of 10 nm.

Data, Materials, and Software Availability. All study data are included in the article and/or supporting information. Yeast library strains are available from the first author upon request.

ACKNOWLEDGMENTS. We gratefully acknowledge Dr. Silvia Calderazzo and Dr. Nicholas Schreck (German Cancer Research Center, Biostatistics core facility) for help with the implementation of the spatial normalization algorithm. We thank Virginie Malak for technical assistance. P.K. acknowledges support from the Christiane Nüsslein-Volhard Foundation and the German Stem Cell Network. This work was funded by the European Commission (742039, to T.P.D.).

Author affiliations: ^aDivision of Redox Regulation, German Cancer Research Center (DKFZ), DKFZ-ZMBH Alliance 69120 Heidelberg, Germany; ^bFaculty of Biosciences, Heidelberg University, 69120 Heidelberg, Germany; ^cCenter for Molecular Biology of Heidelberg University (ZMBH), DKFZ-ZMBH Alliance, 69120 Heidelberg, Germany; and ^dResearch Group Cell Morphogenesis and Signal Transduction, German Cancer Research Center (DKFZ), DKFZ-ZMBH Alliance, 69120 Heidelberg, Germany

1. S. Stöcker, M. Maurer, T. Ruppert, T. P. Dick, A role for 2-Cys peroxiredoxins in facilitating cytosolic protein thiol oxidation. *Nat. Chem. Biol.* **14**, 148–155 (2018).
2. A. Delaunay, D. Pflieger, M. B. Barrault, J. Vinh, M. B. Toledano, A thiol peroxidase is an H₂O₂ receptor and redox-transducer in gene activation. *Cell* **111**, 471–481 (2002).
3. V. V. Belousov *et al.*, Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. *Nat. Methods* **3**, 281–286 (2006).
4. D. S. Bilan *et al.*, HyPer-3: A genetically encoded H₂O₂ probe with improved performance for ratiometric and fluorescence lifetime imaging. *ACS Chem. Biol.* **8**, 535–542 (2013).
5. V. V. Pak *et al.*, Ultrasensitive genetically encoded indicator for hydrogen peroxide identifies roles for the oxidant in cell migration and mitochondrial function. *Cell Metab.* **31**, 642–653.e6 (2020).
6. M. Gutscher *et al.*, Proximity-based protein thiol oxidation by H₂O₂-scavenging peroxidases. *J. Biol. Chem.* **284**, 31532–31540 (2009).
7. B. Morgan, Reassessing cellular glutathione homeostasis: Novel insights revealed by genetically encoded redox probes. *Biochem. Soc. Transact.* **42**, 979–984 (2014).
8. G. Calabrese *et al.*, Hyperoxidation of mitochondrial peroxiredoxin limits H₂O₂-induced cell death in yeast. *EMBO J.* **38**, e101552 (2019), 10.1525/embj.2019101552.
9. M. Carmona *et al.*, Monitoring cytosolic H₂O₂ fluctuations arising from altered plasma membrane gradients or from mitochondrial activity. *Nat. Commun.* **10**, 4526 (2019).
10. S. De Henau, M. Pagès-Gallego, W.-J. Pannekoek, T. B. Dansen, Mitochondria-derived H₂O₂ promotes symmetry breaking of the *C. elegans* zygote. *Dev. Cell* **53**, 263–271.e6 (2020).
11. M. N. Hoehne *et al.*, Spatial and temporal control of mitochondrial H₂O₂ release in intact human cells. *EMBO J.* **41**, e109169 (2022).
12. M. Secilmis *et al.*, A co-culture-based multiparametric imaging technique to dissect local H₂O₂ signals with targeted HyPer7. *Biosensors* **11**, 338 (2021).
13. M. Meurer *et al.*, Genome-wide C-SWAT library for high-throughput yeast genome tagging. *Nat. Methods* **15**, 598–600 (2018).
14. I. Yofe *et al.*, One library to make them all: Streamlining the creation of yeast libraries via a SWAp-Tag strategy. *Nat. Methods* **13**, 371–378 (2016).
15. P. Kritsiligkou, T. K. Shen, T. P. Dick, A comparison of Prx- and OxyR-based H₂O₂ probes expressed in *S. cerevisiae*. *J. Biol. Chem.* **297**, 100866 (2021).
16. K. H. Fisher-Wellman *et al.*, Mitochondrial glutathione depletion reveals a novel role for the pyruvate dehydrogenase complex as a key H₂O₂-emitting source under conditions of nutrient overload. *Free Radical Biol. Med.* **65**, 1201–1208 (2013).
17. T. A. Dailey, P. Meissner, H. A. Dailey, Expression of a cloned protoporphyrinogen oxidase. *J. Biol. Chem.* **269**, 813–815 (1994).
18. V. I. Bunik, Redox-driven signaling: 2-Oxo acid dehydrogenase complexes as sensors and transmitters of metabolic imbalance. *Antioxid. Redox Signal.* **30**, 1911–1947 (2019).
19. A. Kawaguchi *et al.*, Stereochemistry of dehydrogenation catalyzed by acyl-CoA oxidase1. *J. Biochem.* **88**, 1481–1486 (1980).
20. F. R. Palma *et al.*, Mitochondrial superoxide dismutase: What the established, the intriguing, and the novel reveal about a key cellular redox switch. *Antioxid. Redox Signal.* **32**, 701–714 (2020).
21. M. Rinnerthaler *et al.*, Yno1p/Aim14p, a NADPH-oxidase ortholog, controls extramitochondrial reactive oxygen species generation, apoptosis, and actin cable formation in yeast. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 8658–8663 (2012).
22. M. Weber *et al.*, Actin cytoskeleton regulation by the yeast NADPH oxidase Yno1p impacts processes controlled by MAPK pathways. *Antioxidants* **10**, 322 (2021).
23. W.-K. Huh *et al.*, D-Erythroascorbic acid is an important antioxidant molecule in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **30**, 895–903 (1998).
24. V. V. Vu *et al.*, Human deoxyhypusine hydroxylase, an enzyme involved in regulating cell growth, activates O₂ with a nonheme diiron center. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 14814–14819 (2009).
25. U. Weill *et al.*, Genome-wide SWAp-Tag yeast libraries for proteome exploration. *Nat. Methods* **15**, 617–622 (2018).
26. A. H. Y. Tong, C. Boone, "Synthetic genetic array analysis in *Saccharomyces cerevisiae*" in *Yeast Protocols* (Humana Press, 2005), pp. 171–192.
27. I. Yofe, M. Schuldiner, Primers-4-Yeast: A comprehensive web tool for planning primers for *Saccharomyces cerevisiae*: Primers-4-Yeast. *Yeast* **31**, 77–80 (2014).
28. C. Taxis, M. Knop, System of centromeric, episomal, and integrative vectors based on drug resistance markers for *Saccharomyces cerevisiae*. *Biotechniques* **40**, 73–78 (2006).
29. H. Wickham *et al.*, Welcome to the tidyverse. *J. Open Source Softw.* **4**, 1686 (2019).