

# Methodologies for the Mechanistic Study of Compartment-Specific Hydrogen Peroxide Perturbations

by

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Reactive oxygen species (ROS) are an interesting class of molecules because of their ability to promote contradictory phenotypes depending on their intracellular concentration. Most significantly, their elevation has been linked with several pathologies, including cancer. The selective cancer killing hypothesis hinges on the idea that certain cancers will be more susceptible to toxicity via a redox-based mechanism than their surrounding healthy counterparts, and provides an attractive target for those studying redox biology. In order to effectively leverage this strategy, quantitative knowledge of intracellular ROS, specifically hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and its associated pathway proteins, is necessary. The mitochondria are a putative major source of intracellular  $\text{H}_2\text{O}_2$ , as well as a site for  $\text{H}_2\text{O}_2$ -mediated signaling. However, quantitative relationships between mitochondrial  $\text{H}_2\text{O}_2$  and phenotypic response are currently poorly characterized, despite the importance of this organelle in  $\text{H}_2\text{O}_2$ -mediated signaling.

This thesis developed tools and methodology for quantitative and mechanistic studies of  $\text{H}_2\text{O}_2$  in the mitochondria. Both experimental and computational tools were developed and implemented to analyze mitochondrial  $\text{H}_2\text{O}_2$ , peroxiredoxin (Prx) proteins, and primary patient tumor cells. A genetically-encoded  $\text{H}_2\text{O}_2$  generator, D-amino acid oxidase (DAAO), was targeted to the mitochondria of human cells, and its utility in investigating cellular response to a range of  $\text{H}_2\text{O}_2$  doses over time was assessed. Organelle-specific Prx dimerization and protein S-glutathionylation were measured as indicators of increased  $\text{H}_2\text{O}_2$  flux due to the activity of DAAO. Cell death was observed in a concentration- and time-dependent manner, and protein oxidation shifted in localization as the dose increased. This work presents the first systematic study of  $\text{H}_2\text{O}_2$ -specific perturbation of mitochondria in human cells, and it reveals a marked sensitivity of this organelle to increases in  $\text{H}_2\text{O}_2$  in comparison with prior studies that targeted the cytosol.

To improve our dynamic and mechanistic understanding of the mitochondrial  $\text{H}_2\text{O}_2$  reaction network in HeLa cells, a kinetic model of the system was built. This model used the most current quantitative proteomics and kinetic data available to model species abundances and rate constants, respectively. This model also considered responses on different time scales. It was predicted that basal mitochondrial  $\text{H}_2\text{O}_2$  was in the low nM range ( $\sim 3$  nM) and was inversely dependent on the total pool of peroxiredoxin-3 (Prx3). Additionally, the mitochondrial reaction network was expected to control perturbations well, up to  $\text{H}_2\text{O}_2$  generation rates  $\sim 50$   $\mu\text{M/s}$  (0.25 nmol/mg-protein/s), above which point the (Prx3) system collapses. Prx3 was found to control the observed  $\text{H}_2\text{O}_2$  dynamics. Experimental validation of these results demonstrated good trend agreement at short times ( $\leq 15$  min) but at longer times suggested a greater impact of transport of  $\text{H}_2\text{O}_2$  out of the mitochondria and into the cytosol, as evidenced by peroxiredoxin-2 (Prx2) oxidation. These results have applications for understanding the physiologically relevant concentration ranges of  $\text{H}_2\text{O}_2$  in mammalian epithelial cells, as well as which thiol modifications are possible under these conditions via direct, bimolecular reaction mechanisms.

Pheochromocytoma and paraganglioma are rare but life-threatening cancers. Certain sub-types of these cancers carry succinate dehydrogenase b (SDHB) mutations in Complex II of the mitochondrial electron transport chain, which drive the disease. This mutation may make these tumors susceptible to treatment via a redox-based chemotherapeutic, but the lack of a model system currently makes it difficult to test this hypothesis. A method for using primary patient tumor samples and testing their susceptibility to treatment with a redox-based drug, piperlongumine, while maintaining rigorous statistical power was developed. This method leveraged high-throughput imaging systems and automated image analysis software. Preliminary data indicates that these tumors may be sensitive to piperlongumine.

In addition to controlling  $\text{H}_2\text{O}_2$  levels by reducing  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$ , Prxs have recently been discovered to participate in signaling reactions by oxidizing transcription factors via thiol-disulfide redox relays. The factors that control the specificity of these reactions are currently completely unknown. Prxs are remarkably conserved across all kingdoms of life and through evolutionary time. Their catalytic mechanism involves a conformational change across the length of the protein, from the peroxidatic to the resolving cysteine, in order for a disulfide bond to form. To better understand the structural mechanisms involved in protein-protein interactions and dimer formation, statistical coupling analysis (SCA) was performed to elucidate evolutionarily conserved clusters of residues within the peroxiredoxin-2 (Prx2) and peroxiredoxin-1 (Prx1) sub-families of proteins. Eigenvalue decomposition of the correlation matrices was used to analyze protein sectors. Prx2 was found to have four evolutionarily conserved clusters, and Prx1 was found to have three, with each protein demonstrating evidence of distinct protein sectors. These data can be used to better understand the structure-function relationships in Prx2 and Prx1.

All in all, these tools provide a basis for understanding the quantitative relationship between changes in  $\text{H}_2\text{O}_2$  and phenotypic response. They also establish the importance of the Prx proteins as controlling enzymes in the  $\text{H}_2\text{O}_2$  reaction network, as well as potential biomarkers. Finally, these tools can be used in other cancer cell systems to better understand the quantitative  $\text{H}_2\text{O}_2$  signaling mechanisms and to suggest chemotherapeutic targets within those pathways.

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