Peroxiredoxin 6 (Prdx6) supports NADPH oxidase 1 (Nox1)-based reactive oxygen species generation and cell migration: collaboration of oxidant generating and scavenging systems

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Abstract

Nox1 is an abundant source of reactive oxygen species (ROS) in colon epithelium recently shown to function in wound healing and epithelial homeostasis. We identified Peroxiredoxin 6 (Prdx6) as a novel binding partner of Nox activator 1 (Noxa1) in yeast two-hybrid screening experiments using the Noxa1 SH3 domain as bait. Prdx6 is a unique member of the Prdx antioxidant enzyme family that exhibits both glutathione peroxidase and phospholipase A_2 activities. We confirmed this interaction in cells overexpressing both proteins, showing Prdx6 binds to and stabilizes wild-type Noxa1, but not the SH3 domain mutant form, Noxa1 W436R. We demonstrated in several cell models that Prdx6 knockdown suppresses Nox1 activity, whereas enhanced Prdx6 expression supports higher Nox1-based ROS release. Prdx6-dependent enhanced ROS production by Nox1 depends on both the peroxidase and phospholipase A_2 activities of Prdx6, since peroxidase and lipase-deficient mutant forms failed to bind to or stabilize Nox1 components or support Nox1-mediated ROS generation. Furthermore, Wild-type Prdx6, but not lipase or peroxidase mutant forms, supports Nox1-mediated cell migration in the HCT-116 colon epithelial cell model of wound closure. These findings highlight a novel pathway in which an antioxidant enzyme positively regulates an oxidant-generating system to support cell migration and wound healing.

Introduction

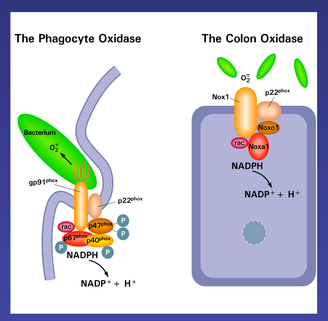
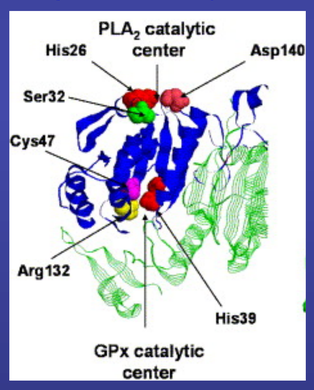


Figure 1. Comparison of multicomponent gp91phox (Nox2)- and Nox1-based NADPH oxidases [2]
Like other Nox enzymes, Nox1 and Nox2 contain all the conserved structural features involved in electron transport and superoxide generation. Both Nox core components associate with p22phox in the membrane and are activated by GTP-Rac. The Nox activator (Noxa1) and Nox organizer (Noxo1) proteins are homologous to p67phox and p47phox adaptor proteins of the prototypical phagocyte oxidase.

Reactive oxygen species (ROS) are now regarded as important signaling molecules in biological systems and have diverse roles in health and disease. Along with mitochondria, a family of NADPH oxidase (Nox) enzymes has been identified as a major source of cellular ROS in many cell types. These enzymes are membrane-integrated protein complexes that utilize molecular oxygen and NADPH to deliberately produce superoxide or H_2O_2. Nox1, the first of the non-phagocytic Nox family members to be identified, is most abundant in colon epithelial cells [1] and is suggested to participate in mucosal innate immunity, wound healing, and epithelial homeostasis [2-4]. Nox1 is structurally most closely related to the phagocytic prototype, Nox2 (a.k.a., gp91phox). As inappropriate or excessive ROS production can damage surrounding tissues and promote inflammation, the activity of NADPH oxidases needs to be tightly regulated. The physiological agonists and molecular mechanisms that modulate the activity of Nox1 complex are still poorly understood.

In recent studies, Prdx6 was found to participate in the activation of Nox2 in human neutrophils, alveolar macrophages, and endothelium (5, 6). Prdx 6 is one member of the ubiquitous antioxidant family of Prdxs, which have peroxidase activity that catalyzes removal of H_2O_2 and other hydroperoxides. Prdx6 differs from other mammalian Prdxs both in its ability to reduce phospholipid hydroperoxides and by exhibiting phospholipase A_2 (PLA_2) activity [7, 8].

Two enzyme activities in one protein Prdx6



Fisher AB, Antioxid Redox Signal 15:831-844, 2011

Recently, several non-phagocytic Nox enzymes were shown to promote cell migration or wound healing processes in epithelial and vascular tissues (Sc). The Nox1 enzyme complex was shown to promote colon epithelial cell migration and wound healing through formyl-peptide receptor pathways [3, 4] that were previously well described in phagocytic cells. Here we identify Prdx6 as an antioxidant enzyme that promotes Nox1-based ROS generation and cell migration through both its peroxidase and PLA_2 activities. Prdx6 deficiency results in destabilization of the Nox1-supportive cofactors, whereas Prx6 overexpression supports higher levels of oxidase activity. These results highlight a novel pathway in which an oxidant-scavenging enzyme positively regulates an oxidant generator to promote cellular activities involving higher oxidative output. Based on these observations, we propose a model in which Prdx6 recruitment into the Nox1 NADPH oxidase complex leads to increased stabilization and increased superoxide production.

Materials & Methods

ROS Detection
Extracellular superoxide production was measured as previously described (Ueyama). Briefly, superoxide dismutase-inhibitable chemiluminescence was detected using Diogenes reagent (National Diagnostics, Atlanta, GA, USA). Cells were collected by trypsinzation and washed twice with Hank's Balanced Salt Solution (HBSS; Invitrogen). Kinetic ROS measurements were performed by chemiluminescence in 96-well plates at 37°C over a 45 min time course using a Luminoskan luminometer (Thermo, Waltham, MA, USA).

Transfections and Plasmids
All cell lines were obtained ffrom ATTC. Adherent cells were transfected with Fugene 6 (Roche) or Lipofectamine-2000 (Life Technologies) at ~40-70 % confluence according to manufacturer's protocol. Most functional assays were performed 24 hours later. The cloning and construction of human Nox1, Noxa1-myc, Noxo1-V5 cDNA-encoding plasmids were described previously (Ueyama). Prdx6 constructs were by PRC. Mutated constructs were generated by PCR using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Western Blot Analysis
Total cell lysates were processed for Western blotting as previously described (). The following antibodies were purchased as indicated: rabbit polyclonal anti-GADPH Ab, rabbit polyclonal anti-myc from Sigma-Aldrich; mouse monoclonal anti-V5 Ab from Invitrogen; rabbit polyclonal anti-Nox1 Ab (NBP1-31546) from Novus Biologicals (Novus Biologicals, Littleton, CO); rabbit polyclonal anti-Nox1 Ab-Agarose conjugate (sc-25545 AC), rabbit polyclonal anti-p22phox Ab-agarose conjugate (sc-20781 AC), rabbit polyclonal anti-Cbl, rabbit polyclonal anti-Sos1, and rabbit polyclonal Prdx6 from Santa Cruz; Mouse monoclonal anti-HA Ab from Covance (Covance, Princeton, NJ, USA). Rabbit polyclonal Ab against Noxa1 and Mouse monoclonal Ab against p22phox (no. 449) were described previously (MCB 2006, Ueyama T. et al).

Migration Assays
Six hours after transfection with Prdx6 and Nox1 expression vectors, HCT-116 cells were collected by trypsinization and reseeded into silcone culture inserts (Ibidi LLC, Verona, Wisconsoin, USA) that created dual chambers, which were mounted within collagen1-coated 12-well culture plates. The cells were allowed to adhere over 24 hours, and then the insert frames were removed to allow the cells to migrate into the 0.5 mm gaps created between chambers. Cells were fixed in methanol and stained with Eosin Y and Azure B (Diff Stain Kit, IMEB Inc, San Marcos, CA, USA) 20 hours later. Cell migration data were processed from three independent transfection experiments, which included replicate transfections for assays of superoxide production and Western blot analysis performed in parallel.

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Results

I. Wild Prdx6 associates with the SH3 domain of Noxa1 and stabilizes its expression

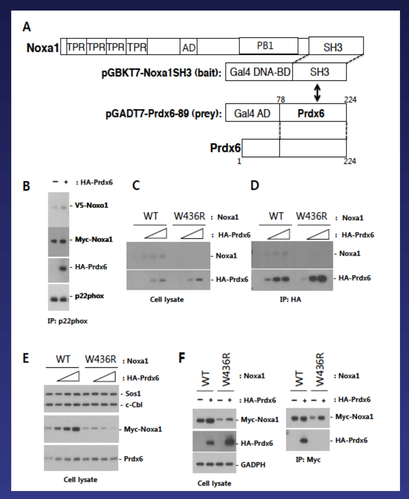

Figure 1. (A). Interaction of the Noxa1 C-terminal SH3 domain with Prdx6 detected by yeast two-hybrid screening of a human kidney cDNAs identified Prdx6 as a potential binding partner of the SH3 domain of Noxa1 (res. 468-526). Cotransfectants exhibited Gal4-based transcriptional activation enabling survival on His/Leu/Ade-deficient selective medium. (B). Full-length Prdx6 associates with the Nox1 NADPH oxidase complex. Nox1, Noxo1, and Noxa1 plasmids (0.2 ug each) were cotransfected with or without HA-Prdx6 (0.6 ug) in HEK-293 cells. Cleared lysates were incubated with anti-$p22^{phox}$ to pull down Nox1 complexes and associated HA-Prdx6 was analyzed by Western blotting. (C). Overexpressed Prdx6 enhances WT Noxa1 production but not the SH3 domain mutant form, Noxa1 W436R. CHO-K1 cells were cotransfected with Noxa1 plasmid (0.3 ug) and Prdx6 plasmid (0, 0.3, 0.6, 0.9 ug) for 48 hrs. Production of HA-Prdx6 and Noxa1 was analyzed in cell lysates by Western blotting with anti-HA or anti-Noxa1 antibody.

(D). Prdx6 associates with WT Noxa1 but not Noxa1 W436R. CHO-K1 cells were transfected as in C. Cleared lysates were harvested 48 hours later and incubated with anti-HA antibody to pull down Prdx6. Associated proteins were analyzed by Western blotting with anti-HA or anti-Noxa1 antibody. (E). Overexpression of HA-Prdx6 enhances detection of Noxa1 but not Noxa1 W436R in HEK-293 cells. Nox1, Noxo1, and Noxa1 (0.05 ug each) were cotransfected with HA-Prdx6 (0, 0.05, 0.1, or 0.4 ug) and the cells were processed as described in C. (F). Prdx6 associates with WT Noxa1 but not with Noxa1 W436R in HEK-293 cells. Noxa1 (0.1 ug) was cotransfected with or without Prdx6 (0.8 ug) in HEK-293 cells. Products of HA-Prdx6 and Myc-Noxa1 were analyzed in cell lysates harvested 48 hours later by Western blotting with anti-HA or anti-Myc antibody (left). Cleared lysates were incubated with anti-Myc to pull down Noxa1 and bound Prdx6 detected by Western blotting (right).

II. Prdx6 supports Nox1 NADPH oxidase activity

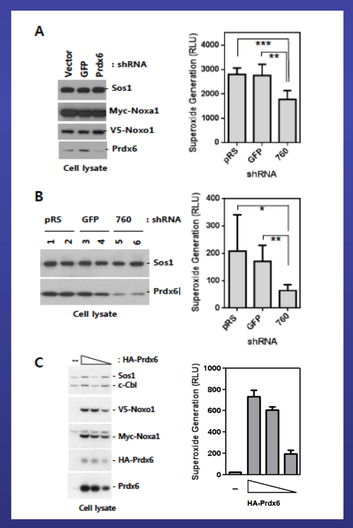

Figure 2. (A). Transient knockdown of Prdx6 suppresses ROS generation by Nox1 reconstituted in HEK-293 cells. Cells were co-transfected with vector (pRS), shRNA-GFP (GFP), or shRNA-Prdx6 plasmid (760) (0.6 ug), along with Nox1, Noxo1, and Noxa1 plasmids (0.1 ug each). After 76 hrs, cells were harvested for Western blotting (left) and extracellular superoxide generation (right). Unpaired t-test p value of 760 vs pRS = 0.0002. P value of 760 vs GFP = 0.0031. Error bars reflect mean ± S.D (n=3). (B). Stable knockdown of Prdx6 suppresses superoxide generation by HT-29 colon epithelial cells. HT-29 cells were transfected with vector (pRS), shRNA-GFP (GFP), or shRNA-Prdx6 (760) plasmids. Left panels: Western blotting of expressed proteins in two representative clones of each shRNA transfectant. Right: extracellular superoxide generation from trypsinized cells. Unpaired t-test p value of 760 vs pRS = 0.0245. P value of 760 vs GFP = 0.002. Error bars reflect mean ± S.D (n=4). (C) Overexpressed Prdx6 enhances production of transfected Nox1 components, Noxo1 and Noxa1, and extracellular superoxide generation. CHO-K1 cells were cotransfected with various amounts of Prdx6 plasmid (0, 2, 1.5, 1 ug) along with 0.05 ug each of Nox1, Noxo1, and Noxa1 plasmids. After 48 hr, extracellular superoxide generation from trypsinized cells was measured (right). Left panel: cell lysates were prepared and protein expression was analyzed by Western blot analysis. .

III. Both phospholipase A_2 and peroxidase activities of Prdx6 support Nox1 activity

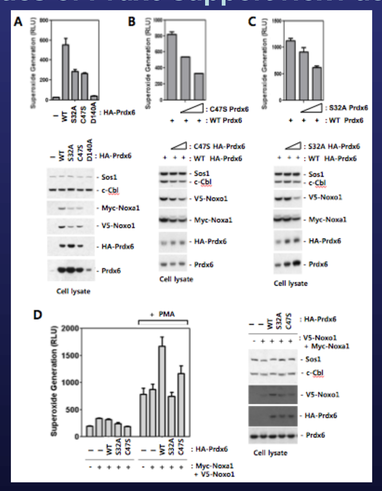

Figure 3.. (A). CHO-K1 cells were co-transfected with WT, S32A (PLA_2 mutant), D140A (PLA_2 mutant), or C47S (peroxidase mutant) Prdx6 (1.5 ug), along with Nox1, Noxo1, and Noxa1 plasmids (0.05 ug each). After 48 hrs, ROS generation from trypsinized cells was measured by Diogenes luminescence assay. Cell lysates were prepared and Noxo1, Noxa1, and Prdx6 levels were analyzed by Western blot analysis (lower panels). (B). Nox1-supportive effects of WT Prdx6 are suppressed by peroxidase mutant, C47S. CHO-K1 cells were cotransfected with various amount of C47S (inactive peroxidase) Prdx6 plasmid (0, 0.5, 1.5 ug), along with the same amounts of Nox1, Noxo1, Noxa1 (0.05 ug each) and WT Prdx6 (1 ug) plasmids. Upper panel: after 48 hrs, extracellular superoxide generation from trypsinized cells was measured by Diogenes luminescence assay. Lower Panel: cell lysates were prepared and levels of Noxo1, Noxa1, and Prdx6 were analyzed by Western blot analysis. (C). Nox1-supportive effects of WT Prdx6 are suppressed by PLA_2 mutant, Prdx6 S32A. CHO-K1 cells were cotransfected with increasing amounts of Prdx6 S32A, along with Nox1, Noxo1, Noxa1, and WT Prdx6 plasmids as in B. Upper panel: after 48 hrs, extracellular ROS generation from trypsinized cells was measured. Lower panels: cell lysates were prepared and Noxo1, Noxa1, and Prdx6 levels were analyzed by Western blotting. Error bars reflect mean ± S.D; data show a representative triplicate assay of 2 independent experiments. (D). Endogenous Nox1 activity in HT-29 cells is supported by WT Prdx6 but not by PLA_2 or peroxidase mutants. Cells were transfected with 2 ug of Prdx6 along with Noxo1 and Noxa1 (0.5 ug each). Left: after 48 hrs, extracellular ROS generation from trypsinized cells was measured in the absence or presence of PMA (1 uM). Right: corresponding Western blotting of the lysates from the same transfected cells.

IV. TNFα stimulation induces Prdx6, Noxo1, and Noxa1 and recruitment of Prdx6 into the Nox1 complex

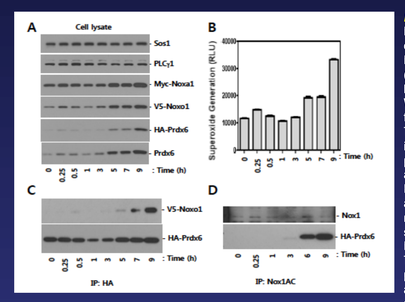

Figure 4. (A). TNFα enhances stabilization of transfected Nox1 components and Prdx6. A stable Prdx6 knockdown clone of HEK-293 cells was cotransfected with Nox1, Noxa1, Noxo1 (0.1 ug each), and Prdx6 (0.3 ug) plasmids (48 hrs) and then stimulated with TNFα (50 ng/ml). Cell lysates at each time point were prepared and analyzed by Western blotting. (B). Extracellular superoxide generation from trypsinized cells collected at each time point. (C). TNFα stimulation induced Noxo1 recruitment into immunoprecipitated Prdx6 complexes. Cell lysates prepared as in B were incubated with agarose-conjugated anti-HA antibody. Coimmunoprecipitated V5-Noxo1 and HA Prdx6 in each immune complex were detected with anti-V5 and anti-HA, respectively. (D) TNFα induces Prdx6 recruitment to the Nox1 complex at later phases of stimulation. HEK-293 cells transfected with Nox1, Noxa1, Noxo1, and Prdx6 plasmids, as in B, were stimulated with TNFα, and then cell lysates were prepared at each time point and incubated with agarose-conjugated anti-Nox1 antibody. Coimmunoprecipitated Prdx6 in each immune complex was analyzed by anti-HA Western blotting.

V. Both PLA_2 and peroxidase activities of Prdx6 stabilize Nox1 components and support ROS generation in TNFα-treated HEK-293 cells

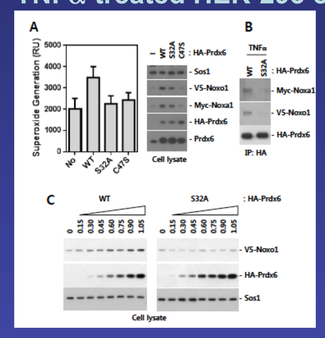

Figure 5. (A). Overexpressed WT Prdx6 enhanced extracellular superoxide generation in TNFα-treated HEK-293 cells, whereas PLA_2 mutant (S32A) and peroxidase mutant (C47S) forms did not. A stable Prdx6-knockdown clone of HEK-293 cells was cotransfected with or without various forms of Prdx6 (0.2 ug), along with Nox1, Noxo1, and Noxa1 (0.1 ug each). At 48 hrs post-transfection cells were treated with TNFα for 6 hours and extracellular superoxide generation from trypsinized cells was measured by the Diogenes superoxide assays. Unpaired t-test p value of WT Prdx6 vs None (No) = 0.001. P value of WT Prdx6 vs S32A = 0.0042. P value of WT Prdx6 vs C47S = 0.0037. Error bars reflect mean ± S.D (n=3). Right panel: corresponding Western blot analysis of expressed proteins. (B). The PLA_2 activity mutant Prdx6 S32A showed a markedly reduced association with the Nox1 system in TNFα-treated HEK-293 cells. A stable Prdx6 knockdown clone of HEK-293 cells was cotransfected with Nox1, Noxa1, Noxo1, and Prdx6 plasmids WT or S32A as in A (48 hrs); cells were then stimulated with TNFα (50 ng/ml) for 9 hours. Cell lysates were incubated with ant-HA antibody and associated Noxo1 and Noxa1 were detected with anti-V5 or anti-Myc antibody, respectively. Data show a representative blot of 3 independent experiments. (C). WT Prdx6 dose-dependently enhanced Noxo1 protein production, whereas the PLA_2 mutant Prdx6 S32A did not.

VI. Stability of the Nox1-supportive components is reduced by expression of a low activity mutant of Nox1

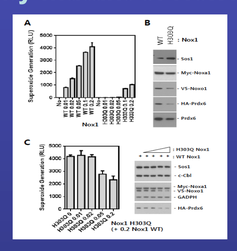

Figure 6 (A). Nox1 H303Q showed almost 10-fold lower superoxide generation in comparison with WT Nox1. Various amounts of WT or H303Q Nox1 plasmids were transfected into HEK-293 cells along with fixed amounts of Noxo1 (0.2 ug), Noxa1 (0.2 ug), and Prdx6 (0.4 ug). Trypsinized cells were measured for extracellular superoxide generation using the Diogenes luminescence assay. (B). Expression of a low activity mutant of Nox1 (H303Q) (0.2 ug) along with Noxa1 (0.2 ug), Noxo1 (0.2 ug), and Prdx6 (0.4 ug) plasmids induced lower production of Noxa1, Noxo1, and Prdx6 in comparision with WT Nox1. (C). Coexpression of H303Q Nox1 with WT Nox1 leads to decreased Noxo1, Noxa1, and Prdx6 protein production and extracellular superoxide generation in a dose-dependent manner. Various concentrations of H303Q mutant Nox1 plasmids were transfected into HEK-293 cells along with fixed amounts of Nox1 (0.2 ug), Noxo1 (0.2 ug), Noxa1 (0.2 ug), and Prdx6 (0.4 ug). Trypsinized cells were measured for extracellular superoxide generation by Diogenes assays. Error bars reflect mean ± S.D (n=3). Right panel: corresponding Western blot analysis of expressed proteins. All data show a representative of 2 independent experiments.

VII. Prdx6 modulates Nox1-derived ROS generation and migration of HCT-116 colon epithelial cells

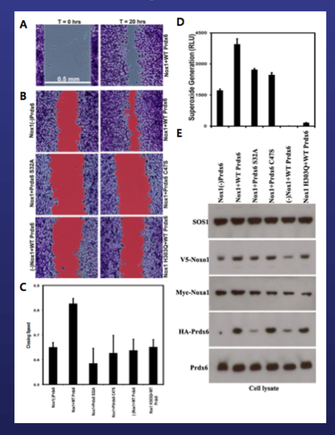

Figure 7 (A). Representative cell migration images of HCT-116 cells transfected with Nox1, Noxo1, Noxa1, and WT Prdx6, as described methods; shown are fixed and stained cell migration images detected at T= 0 (left) and 20 hrs (right). (B). Overexpressed WT Prdx6 augments Nox1-mediated colon epithelial cell migration. Cells were transfected with Noxo1, and Noxa1, along with plasmids encoding various forms of Nox1, HA-Prdx6, or GFP, as controls. Representative fixed and stained cell images after 20 hrs of migration, showing fully reconstituted Nox1 with overexpressed WT Prdx6 (upper right) supports maximum migration. Image analysis of 20-hr migration endpoints highlight (in red) the gap area calculated between cell boundaries, as described in Materials and Methods. (C) Calculated mean gap closure between migrating cell boundaries detected at 20-hr endpoints from 3 independent experiments. In each experiment, 3-4 replicate Ibidi dual chambers were seeded and gap widths were calculated from multiple micrograph images (n), as shown in A and B. (D) WT Prdx6 supports maximum Nox1-derived superoxide generation by HCT-116 cells. Shown are results of assays (48 hrs post-transfection) from one of three representative experiments, here using replicate transfected cell cultures from the same experiment shown in B. (E) Western blotting of Nox- and Prdx6- transfected HCT-116 cell lysates (48 hrs) from the same experiment shown in B and D.

Summary

- **We identified Peroxiredoxin (Prdx) 6 as a novel positive regulator of the Nox1-based reactive oxygen generating enzyme complex. Earlier work showed that Prdx 6 also regulates the Nox2-based oxidase, the closest relative of Nox1.**
- **Prdx6 was initially identified as an interacting partner of the SH3 domain of Noxa1. Interaction of the full-length proteins was confirmed in several transfected cell models. Furthermore, Prdx6 assembled with other components of the Nox1 complex.**
- **Overexpressed Prdx6 was shown to stabilize higher levels of the Nox1-supportive cofactors, resulting in higher levels of ROS generation. In contrast, lower Nox1-derived ROS was observed in cells in which Prdx6 expression was suppressed.**
- **The Nox1-supportive activity of Prdx 6 was shown to require both its peroxidase and phospholipase A_2 activities. Prdx 6 also supported Nox1-mediated colon epithelial cell migration, requiring both its peroxidase and lipase activities.**
- **ROS generated by Nox1 appeared to exert positive feedback effects in stabilizing higher levels of Prdx6 as well as other Nox1-supportive cofactors, Noxo1 and Noxa1. These results highlight a novel pathway in which an oxidant-scavenging enzyme positively regulates an oxidant generator to promote cellular activities involving higher oxidative output.**