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Proximity-dependent biotin labeling in yeast using the engineered ascorbate peroxidase APEX2

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Abstract

The engineered ascorbate peroxidase (APEX2) has been effectively employed in mammalian cells to identify protein-protein interactions. APEX2 fused to a protein of interest covalently tags nearby proteins with biotin-phenol (BP) when H₂O₂ is added to the cell culture medium. Subsequent affinity purification of biotinylated proteins allows for identification by mass spectrometry. BP labeling occurs in 1 min, providing temporal control of labeling. The APEX2 tool enables proteomic mapping of subcellular compartments as well as identification of dynamic protein complexes, and has emerged as a new methodology for proteomic analysis. Despite these advantages, a related APEX2 approach has not been developed for yeast. Here we report methods to enable APEX2-mediated biotin labeling in yeast. Our work demonstrated that high osmolarity and disruption of cell wall integrity permits live-cell biotin labeling in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, respectively. Under these conditions, APEX2 permitted targeted and proximity-dependent labeling of proteins. The methods described herein set the stage for large-scale proteomic studies in yeast. With modifications, the method is also expected to be effective in other organisms with cell walls, such as bacteria and plants.

Keywords

APEX2; proximity-dependent biotinylation; yeast; protein-protein interaction; sorbitol; ascorbate peroxidase

INTRODUCTION

Proximity-dependent labeling techniques that detect protein-protein interactions have been developed in recent years. For example, the proximity-dependent biotin identification (BioID) method has been successfully used in biochemical screens for interaction partners in a native environment (1–3). This approach is based on fusion of bacterial biotin ligase mutant BirA to a targeting protein. After an overnight incubation with biotin, proteins biotinylated by BirA can be recovered by affinity purification using Streptavidin beads and identified by mass spectrometry (MS). This simple technique has been used to identify

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proteins that interact weakly or transiently with the protein of interest. However, a long half-life (>minutes) of the BirA product, a biotin-adenylate ester, could be a drawback since it leads to a large labeling radius, making it hard to distinguish true and false-positives. Moreover, the slow reaction of BirA (requiring 18–24 hours) makes it difficult to investigate highly dynamic cellular events or acute cellular responses upon environmental changes.

To improve the spatial and temporal specificity, a method that uses an engineered variant of ascorbate peroxidase (APEX2) was developed (4–6). In the presence of H₂O₂, APEX2 catalyzes the conversion of biotin-phenol (BP) to biotin-phenoxyl radicals that covalently link biotin to electron-rich amino acid side chains within a short radius. As in BioID-based analysis, the biotinylated proteins are purified using Streptavidin beads and then analyzed by MS. Biotin labeling in this case is achieved after only 1 min as opposed to a longer incubation in the BioID approach. The biotin-phenoxyl radical is short-lived (<1 ms), generating a small labeling radius (<20 nm). Given its small labeling radius and short labeling time, APEX2-based proximity labeling has the potential to provide higher spatial and temporal specificity. Indeed, the APEX2 tool has emerged as a powerful system to provide proteomic maps of proteins present in challenging compartments such as mitochondrial intermembrane space, ER-PM junctions, primary cilia, and the synaptic cleft (5,7–10). Besides spatial information, the APEX2 technique is also expected to offer temporal proteomic maps as well, uncovering signaling dynamics in living biological systems. A combined approach of APEX2 with stable isotope labeling by amino acids in cell culture-based quantitative proteomics allowed for higher spatial and temporal resolution (9).

Yeast is a powerful model organism for studying protein function and cellular processes (11). With short generation times and well-characterized genomes, a major advantage of yeast systems is the ability to perform high-throughput genetic screens to identify genes and pathways. Given the ease of the genetic manipulation of yeast, application of APEX2 to yeast proteomics studies would provide another powerful tool for addressing complex biological questions. Although well established in mammalian system, APEX2 has not been adapted for yeast to date.

In this paper, we describe methods to permit APEX2-based live-cell biotin labeling in yeast. We observed that applying a high osmotic solution such as 1.2 M sorbitol to fission yeast *S. pombe* dramatically increased APEX2-dependent live-cell biotinylation. We used the APEX2 system to confirm known protein-protein interactions. In cells expressing a Dsc5-APEX2 fusion protein, we observed specific biotinylation of its known direct binding partner Cdc48 (12). A similar labeling protocol using Zymolyase to remove the cell wall allowed APEX2-mediated biotinylation in the budding yeast *S. cerevisiae*, demonstrating that the APEX2 approach can also be extended to this organism. Our results open the door to application of the APEX2 system to proteomic studies in the two most common yeast model organisms.

EXPERIMENTAL PROCEDURES

Materials

We obtained Edinburgh minimal medium (EMM) and Complete Supplement Mixture (CSM) from MP Biomedicals; yeast extract, peptone, agar from Becton Dickinson; oligonucleotides from Integrated DNA Technologies; complete EDTA-free protease inhibitor from Roche; amino acids, D-sorbitol, D-Galactose, yeast nitrogen base without amino acids, sodium ascorbate, (S)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) from Sigma-Aldrich; Prestained Protein Standards from Bio-Rad; Biotin-phenol from Iris Biotech; Streptavidin magnetic beads from Promega; Gibson Assembly Master Mix from NEB BioLabs; Zymolyase from Seikagaku distributed by MP Biomedicals.

Strains and media

Wild-type haploid *S. pombe* (*h- leu1-32 ura4-D18 ade6-M210 his3-D1*) and derived strains were grown to exponential phase at 30°C in EMM medium (32.2 g/L EMM plus 20 g/L glucose supplemented with 225 mg/L each of adenine, uracil, leucine, histidine, and lysine) (13). *S. pombe dsc5* strain (*h- leu1-32 ura4-D18 ade6-M210 his3-D1 dsc5-D1::kanMX6*) was previously described (12). Wild-type *S. cerevisiae* cells (*MATa his3 1 leu2 0 met15 0 ura3 0*) were grown in synthetic complete (SC) medium (20 g/L glucose, 6.7 g/L yeast nitrogen base without amino acids, 0.89 g/L CSM) with 2% sucrose at 30°C (14). Galactose (2% w/v) was used to induce APEX2 protein expression. Standard yeast transformations were performed as previously described (14,15).

Antibodies

We obtained anti-FLAG M2 from Sigma (F1804). Antiserum to Cdc48 was a kind gift of R. Hartmann-Petersen (University of Copenhagen). IRDye800CW Goat anti-Mouse and Rabbit IgG, IRDye680RD Goat anti-Mouse and Rabbit IgG, and IRDye800CW Streptavidin were from LI-COR.

Plasmids

All oligonucleotides used in PCR analyses are listed in Supplemental Table 1. A plasmid containing *Flag-APEX2* under control of *S. pombe adh1*⁺ promoter (16) was generated via Gibson Assembly protocol by assembling two PCR-amplified fragments of *Flag-APEX2* from pcDNA3 APEX2-NES (Plasmid #49386, Addgene) (by primers oJH39/40) and *adh1*⁺ promoter-driven vector pJB114 (by primers oJH41/42) following manufacturer's protocol (NEB). The *dsc5-Flag-APEX2* and *dsc5- UBX-Flag-APEX2* were generated via Gibson Assembly by assembling two PCR-amplified fragments of *Flag-APEX2* from pcDNA3 APEX2-NES (using oJH116/117 and oJH113/117, respectively) and *adh1*⁺ promoter-driven *dsc5* vector pREB30 (oJH118/119 and oJH118/114, respectively). A plasmid containing *Flag-APEX2* under control of *S. cerevisiae GAL1* promoter was generated via Gibson Assembly by assembling two PCR-amplified fragments of *GAL1*-driven vector pRS425 (a gift from Susan Michaelis Lab, Johns Hopkins University) (by oJH124/125) and *Flag-APEX2* (oJH126/127) from pcDNA3 APEX2-NES.

Western blotting

Equal amounts of protein (30–50 µg) from each sample were loaded on an SDS-PAGE gel. Gels were transferred to nitrocellulose using Trans-Blot Turbo Transfer system (Bio-Rad), blocked using a solution of 5% milk-PBST (PBS with 0.05% Tween 20), and probed with primary antibody followed by mouse or rabbit IgG (IRDye800CW or IRDye680RD) or IRDye800CW Streptavidin. Blots were scanned through channel 700 or 800 using an Odyssey CLx imager from LI-COR and quantified using LI-COR Image Studio 4.0 Software.

Biotin-phenol labeling

The original biotin-phenol labeling protocol for mammalian cells was modified for yeast (4,9). Exponentially growing cells ($5 - 10 \times 10^8$) were harvested and washed with water. Cell pellets were resuspended in 1 ml of 1.2 M sorbitol dissolved in H₂O. Biotin-phenol (2.5 mM) was added and cells were incubated for 1 hr at room temperature. H₂O₂ (1 mM) was added for 1 min to initiate biotin-phenol labeling and cells were quickly spun down. To stop the reaction, the solution was aspirated off and cell pellets were then washed four-five times with a quenching solution consisting of 5 mM Trolox, 10 mM sodium azide and 10 mM sodium ascorbate in 1.2 M sorbitol dissolved in H₂O. Cell pellets were washed with 1 ml of 1.2 M sorbitol once more, and lysed by addition of 27 mM NaOH, 1% (v/v) 2-mercaptoethanol for 10 min on ice. Total protein was precipitated with trichloroacetic acid (20% w/v) by incubating on ice for 10 min and centrifuging at 20,000 g for 10 min at 4°C. The protein pellet was washed with 0.5 ml ice-cold acetone once. The protein pellet was then solubilized by sonication at 30% of max power for 10 seconds (Fisher Scientific Model 500 Sonic Dismembrator) in 200 µl lysis buffer (25 mM Tris pH 7.4, 150 mM NaCl, 2% SDS) containing Complete Protease Inhibitor EDTA free. After denaturation by heating at 75°C for 15 min, protein samples were either directly analyzed by western blotting or subjected to enrichment of biotinylated proteins. For enrichment, protein samples (1–2 mg in 0.5–1 ml) were first dialyzed in dialysis buffer (25 mM Tris pH 7.4, 150 mM NaCl, 0.2% SDS) using 3.5K MWCO dialysis tubing (Thermoscientific) for 2–3 hr at room temperature, and then incubated with 50–100 µl Streptavidin-coated magnetic beads for 1–2 hr at room temperature. Dialysis was performed to remove intracellular unreacted free BP, which may compete with biotinylated proteins for binding to Streptavidin beads. Streptavidin beads were then washed 6X with Wash buffer (25 mM Tris pH 7.4, 150 mM NaCl, 0.3% SDS) followed by 1X wash with 2 M urea/50 mM Tris pH7.4, 1X wash with 1 M KCl, and 1X wash with Wash buffer. Biotinylated proteins were eluted by incubating the beads with 30 µl 2X SDS sample loading buffer (4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 100 mM Tris pH6.8, 10% (v/v) 2-mercaptoethanol) and heating to 75°C for 15 min. For Zymolyase incubation, cell pellets from exponentially growing cells ($5 - 10 \times 10^8$) were resuspended in 500 µl of Zymolyase-100T solution (1000 U/ml of Zymolyase in 1.2 M sorbitol dissolved in H₂O). After 10 min or at the same time as Zymolyase treatment, biotin-phenol (2.5 mM) was added and the cells were further incubated for 20–60 min at room temperature rotating before addition of H₂O₂.

RESULTS and DISCUSSION

Optimization of APEX2-mediated biotin labeling in fission yeast

To apply the APEX2 system to yeast, we first asked whether APEX2 is expressed and active in fission yeast. To express APEX2 in *S. pombe*, a plasmid carrying *Flag-APEX2* gene under *adh1⁺* promoter was transformed into leucine-auxotrophic wild-type strain. Four transformants were tested for Flag-APEX2 expression by western blotting. All isolates tested expressed *Flag-APEX2* with a predicted molecular weight of ~28 kDa (Figure 1A). A single isolate was used for subsequent analysis. To examine whether APEX2 is active and mediates biotinylation in yeast, we initiated labeling by adding 2.5 mM BP to the culture medium of *Flag-APEX2* expressing cells. After 1 hr incubation at room temperature, H₂O₂ was added for 1 min and then the labeling was terminated. Streptavidin blot of whole cell lysates revealed endogenous biotinylated proteins in untreated cells, and H₂O₂ treatment for 1 min had no effect despite using 5-fold more BP than used in mammalian cells (Figure 1B, lanes 1–2). Because the BP probe is thought not to have high membrane permeability, we suspected that BP might not be entering the cell. To improve uptake, we disrupted cell wall structure using Zymolyase treatment. *Flag-APEX2* expressing cells were pre-incubated with Zymolyase-100T for 10 min at room temperature, and then 2.5 mM BP was added for 1 hr. Streptavidin blot of cell lysates showed specific H₂O₂-dependent biotinylation of proteins (Figure 1B, lanes 3–4). Thus, the BP probe is delivered to cells when the yeast cell wall architecture is compromised. In this experiment, APEX2 biotinylation is presumably non-specific, occurring on cytosolic proteins in proximity to soluble APEX2.

Because Zymolyase treatment was performed in 1.2 M sorbitol, we next tested whether BP delivery required Zymolyase, sorbitol or both. The APEX2 labeling reaction was performed using control or *Flag-APEX2* cells. Streptavidin blot analysis of cell lysates showed that numerous endogenous proteins were biotinylated when cells were pre-treated with Zymolyase in 1.2 M sorbitol in a reaction that required APEX2, BP, and H₂O₂ (Figure 1C). Surprisingly, we observed identical results when cells were pre-incubated with 1.2 M sorbitol alone (Figure 1C, lanes 7–9), indicating that the high osmotic sorbitol solution may permit BP labeling. Indeed, significant rearrangement in cell wall architecture occurs upon osmotic shock (17). Also, it has been previously reported that transient pre-incubation of intact fission yeast cells with a high osmotic solution (e.g. 2 M sorbitol) before electrical application dramatically improved DNA transformation efficiency of *S. pombe* (15). Thus, remodeled cell wall structure in response to sudden change in osmolarity might improve BP uptake in a similar way as for nucleic acid uptake. In support of this hypothesis, we observed little to no biotinylation above the background from endogenous biotinylated proteins when cells were incubated with the lower concentrations of sorbitol solution (0–600 mM) (Figure 1D, lanes 1–5). In contrast, when cells were incubated in a high osmolar solution of 1 M KCl we observed labeling equal to 1.2 M sorbitol (Figure 1D, lanes 5–6), suggesting that an increase in osmolarity promotes BP uptake. Alternatively but not exclusively, high osmolarity may prevent BP export out of the cell. Sorbitol incubation and Zymolyase treatment are routinely used for yeast experiments and have been demonstrated not to affect yeast cell viability (18–20). Thus, this condition for BP delivery condition is expected to be non-toxic.

To our surprise, incubation of cells in EMM minimal medium containing 1.2 M sorbitol did not result in APEX2-dependent protein biotinylation (Figure 1E), indicating that the EMM culture medium may contain an inhibitory factor(s). To ask whether glucose in the EMM medium interferes with uptake of BP, we examined the effect of 1.2 M sorbitol solution on biotin labeling in the absence or presence of glucose. Streptavidin blot analysis of cell lysates showed that the presence of 2% glucose (w/v) in 1.2 M sorbitol solution completely inhibited biotin labeling compared to 1.2 M sorbitol alone (Figure 1F, lane 2 and 4). Also, we observed no APEX2-specific biotinylated proteins when cells were incubated in 2% glucose solution. Thus, glucose blocked BP accumulation in the cell. Development of structural variants of the BP probe that bypass glucose inhibition may improve BP delivery into fission yeast.

We performed additional experiments to optimize BP labeling, varying BP concentration (Figure 1G) and H₂O₂ labeling time (Figure 1H). Streptavidin blot analysis of lysates from cells incubated with different concentrations of BP showed that protein biotinylation was maximal at 2.5 mM BP (Figure 1G). A requirement for a higher concentration of BP in fission yeast compared to mammalian cells (2.5 mM versus 0.5 mM) may be due to the different physical barriers (5). We observed that 1 min of H₂O₂ labeling was optimal, and that longer H₂O₂ treatment did not enhance the protein biotinylation level (Figure 1H). From these experiments, we established a labeling protocol for APEX2 in fission yeast.

APEX2 proximity-dependent biotin labeling detects specific protein-protein interactions in fission yeast

APEX2-catalyzed biotin-phenoxyl radicals are short lived, thus acting within a small radius from APEX2 (4). This property permits the detection of specific and direct protein-protein interactions (7). To examine whether APEX2 allows detection of specific protein interactions in fission yeast, we fused APEX2 to C-terminus of the Golgi protein Dsc5 (Dsc5-Flag-APEX2). Dsc5 is a subunit of the Golgi Dsc E3 ligase complex and contains a UBX domain, which interacts with the AAA-ATPase Cdc48 (12). Our previous *in vitro* and *in vivo* binding assays demonstrated that the Dsc5 UBX domain directly binds Cdc48, and that Dsc5 lacking the UBX domain (Dsc5^{UBX}) failed to recruit Cdc48 to the Dsc E3 ligase (12). Based on these data, we predicted that Dsc5-APEX2 would label Cdc48 with biotin, but that Dsc5^{UBX}-APEX2 would not label Cdc48 (Figure 2A).

To test the utility of the APEX2 system, yeast cells expressing Dsc5-Flag-APEX2, Dsc5^{UBX}-Flag-APEX2 or Dsc5 were generated, and expression of APEX2 fusion proteins was verified by western blotting (Figure 2B). We further developed a protocol to purify biotinylated proteins from cells (Figure 2C). Briefly, following a 1 hr pre-incubation of cells with BP in 1.2 M sorbitol labeling was initiated by the addition of H₂O₂ for 1 min. After quenching with anti-oxidant reagents, cells were lysed in a denaturing buffer. To remove unreacted free BP, we dialyzed the cell lysate, and biotinylated proteins were then enriched using Streptavidin beads. After stringent washing with different buffers, biotinylated proteins were eluted from beads by heating samples in SDS at 75°C. Whole cell lysates and bead-bound fractions were then analyzed by western blot to detect proteins of interest. Streptavidin and anti-Flag blot analysis showed that both Dsc5-APEX2 and Dsc5^{UBX}-

APEX2 induced biotinylation of multiple endogenous proteins, but that Dsc5 alone without APEX2 fusion did not (Figure 2D). Importantly, Cdc48 was specifically recovered from cells expressing Dsc5-APEX2, but not Dsc5^{UBX}-APEX2. Quantification of western blot band intensities showed that ~2% of total Cdc48 (average from two biological replicates) was recovered from cells expressing Dsc5-APEX2. In contrast, only ~0.1% and ~0.01% of Cdc48 was recovered from Dsc5^{UBX}-APEX2 and control cells, respectively, demonstrating that APEX2 detects specific protein-protein interactions in fission yeast.

Cell wall removal enables APEX2-mediated biotin labeling in budding yeast

Next, we sought to develop a similar approach for the budding yeast *S. cerevisiae*. To express APEX2 in *S. cerevisiae*, we generated cells carrying a plasmid expressing *Flag-APEX2* gene under the control of the galactose-inducible *GAL1* promoter. Yeast cells carrying an empty vector under the same promoter served as a negative control. Western blotting of whole cell lysates revealed that expression of Flag-APEX2 protein was induced in a galactose-dependent manner (Figure 3A). We then subjected cells to the same sorbitol labeling procedure optimized for *S. pombe*. To our surprise, this same method did not result in protein biotinylation above the background from endogenous biotinylated proteins (Figure 3B). The general composition of the cell wall in *S. cerevisiae* and *S. pombe* yeast cells is similar, but substantial differences exist (21,22). Thus, osmotic shift may have different effects on cell permeability in two organisms.

Next, we tested whether cell wall removal using Zymolyase would enable labeling. After 10 min pre-incubation of cells with Zymolyase in 1.2 M sorbitol solution, we added the BP probe, incubated cells further for an additional 1 hr, and treated cells with H₂O₂ for 1 min. Under these conditions, we observed APEX2-dependent protein biotinylation (Figure 3C), demonstrating that cell wall removal permitted BP labeling in *S. cerevisiae*. BP uptake after Zymolyase treatment occurs rapidly, because 20 min BP incubation following 10 min pre-treatment with Zymolyase allowed protein biotinylation to a similar level that was obtained from 60 min incubation (Figure 3D, lanes 3 and 5). In this experiment, we pretreated with Zymolyase for 10 min prior to the addition of BP to allow spheroplast formation, but BP may be also added at the same time as Zymolyase.

In this report, we describe a method for the application of APEX2-dependent proximity biotinylation in yeast. To date, the APEX2 system has only been used in mammalian cells, but our results demonstrate that the APEX2 technique can also identify protein-protein interactions in yeast. This new technique expands the toolkit for detection of protein-protein interactions in these model organisms. Importantly, this technique opens the door for detailed studies examining whether APEX2 methodology can be extended to large-scale protein-protein interaction screens. In addition to specific binding partners, APEX2 fusion proteins will also label non-binding, protein neighbors due to effects of molecular crowding. However, specific binding partners should be labeled with higher efficiency, and these will differ among APEX2 fusion proteins. Indeed, this methodology may be most powerful when applied on a large scale since non-specifically labeled proteins will be common among samples, but specific binding partners will not. Application of software like that developed to analyze data from affinity purification-based approaches should identify high confidence

interacting proteins for subsequent validation (23). Should the APEX2 technique extend to large-scale proteomic studies, this methodology will allow construction of spatial and temporal molecular maps of biological pathways and networks that can be paired with existing datasets from these well-studied model organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations list

The abbreviations used are:

Dsc	Defective for SREBP cleavage
BP	biotin-phenol
BioID	proximity-dependent biotin identification
MS	mass spectrometry

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Summary statement

Protein fusions to the peroxidase APEX2 allow identification of protein-protein interactions and organellar proteomes in mammalian cells. We developed protocols for APEX2-dependent proximity biotinylation in two yeast models. Our methods open the door to application of APEX2 to yeast proteomics.

Figure 1. APEX2-based biotin labeling of yeast proteome in *S. pombe*

(A) Western blot was probed with anti-Flag IgG of whole cell lysates. Wild-type *S. pombe* cells carrying either empty vector (EV) or a *Flag-APEX2* plasmid were grown in minimal medium lacking leucine for 16 hr. Four independent isolates (A–D) of transformants were tested for protein expression. (B) Western blot analysis using IRDye Streptavidin of biotinylated yeast proteins from whole cell lysates. Yeast cells carrying a *Flag-APEX* plasmid were labeled by addition of 2.5 mM biotin-phenol (BP) for 1 hr and subsequent addition of 1 mM H₂O₂ for 1 min in either minimal medium (EMM) or Zymolyase-containing 1.2 M sorbitol solution (Zymo). *S. pombe* has two main endogenously biotinylated proteins, acetyl-CoA carboxylase and pyruvate carboxylase, which were observed in negative controls (-H₂O₂) marked as red asterisks (24). (C) Yeast cells carrying either empty vector (EV) or a *Flag-APEX2* plasmid were labeled with BP and H₂O₂ as in (B) in 1.2 M sorbitol solution with or without Zymolyase, and then lysed. Western blot of biotinylated proteins was visualized by IRDye Streptavidin. (D–F) *Flag-APEX2* expressing yeast cells were labeled in different solutions with BP for 1 hr followed by treatment with 1 mM H₂O₂ for 1 min: (D) increasing concentrations of sorbitol buffer or 1 M KCl; (E) EMM, EMM containing 1.2 M sorbitol; (F) 2% glucose, or 2% glucose in 1.2 M sorbitol. Western blot was probed using IRDye Streptavidin of whole cell lysates. (G) *Flag-APEX2* expressing yeast cells were labeled in 1.2 M sorbitol buffer with different concentrations of

BP for 1 hr followed by treatment with 1 mM H₂O₂ for 1 min. Western blot was probed using IRDye Streptavidin of whole cell lysates. **(H)** Flag-APEX2 expressing yeast cells were labeled by addition of 2.5 mM BP in 1.2 M sorbitol buffer for 1 hr followed by addition of H₂O₂ for indicated times. Western blot was probed using IRDye Streptavidin of whole cell lysates. Experiments in Figures A and D were performed once. More than three biological replicates were performed for Figures B, C, and E–H.

Figure 2. Specificity of APEX2-mediated proximity-dependent biotin labeling in *S. pombe*

(A) Model showing expected results from labeling experiment. Dsc5-Flag-APEX2 generates biotin-phenoxy radicals that covalently conjugate to nearby proteins such as Cdc48 and Dsc5-APEX2 itself. In contrast, APEX2 fused to Dsc5 lacking UBX domain does not label Cdc48 with biotin, but does label Dsc5-UBX-APEX2 itself. (B) Western blot was probed with anti-Flag IgG of whole cell lysates from *dsc5*⁻ cells expressing *dsc5-Flag-APEX2*, *dsc5-UBX-Flag-APEX2*, or *dsc5*⁺. Two to four independent isolates were tested. (C) Schematic showing labeling protocol in fission yeast. Yeast cells are pre-incubated in 1.2 M sorbitol with 2.5 mM biotin-phenol for 1 hr. Labeling is initiated by the addition of 1 mM H₂O₂ to cells. After quenching, cells are subjected to lysis using NaOH and 2-mercaptoethanol followed by precipitation of the extracted protein with trichloroacetic acid, dissolution in 2% SDS lysis buffer, and heating for denaturation (25). To remove unconjugated BP, dialysis is performed in dialysis buffer (25 mM Tris pH 7.4, 150 mM

NaCl, 0.2% SDS) for 2–3 hr at room temperature. Biotinylated proteins are recovered with Streptavidin-coated magnetic beads, sequentially washed with stringent buffers to remove non-biotinylated proteins, and eluted for protein analysis. See “Experimental procedures” for more details. **(D)** Indicated yeast strains were labeled and lysed as described in (C). Western blot of whole cell lysates and biotinylated proteins recovered using Streptavidin beads were probed with IRDye Streptavidin, anti-Flag IgG, or anti-Cdc48 serum. Experiment in Figure B was performed once. Two biological replicates were performed for Figure D.

Figure 3. APEX2-based biotin labeling of yeast proteome in *S. cerevisiae*

(A) *S. cerevisiae* wild-type cells were transformed with the empty vector (EV) or a plasmid encoding Flag-tagged APEX2 under the control of a galactose-inducible promoter (*GAL*). Cells were grown to exponential phase in SC medium with 2% sucrose, and then 2% galactose was added to the medium for 2 or 5 hr to induce protein expression. Equal amounts of whole cell lysates were subjected to western blot analysis using anti-Flag IgG.

(B) *S. cerevisiae* wild-type cells expressing empty vector (EV) or *Flag-APEX2* (AX) were grown in the presence of galactose for 3 hr to induce APEX2 expression and were labeled by addition of 2.5 mM BP for 1 hr and subsequent addition of 1 mM H₂O₂ for 1 min to either culture medium or 1.2 M sorbitol solution. Western blot of whole cell lysates was probed using IRDye Streptavidin and anti-Flag IgG. Endogenously biotinylated proteins are marked with red asterisks.

(C) Indicated *S. cerevisiae* strains were subjected to labeling as in (B) in Zymolyase-containing 1.2 M sorbitol buffer, then lysed. Western blot of whole cell lysates was probed using IRDye Streptavidin and anti-Flag IgG.

(D) Indicated *S. cerevisiae* strains were grown in the absence or presence of galactose for 3 hr to induce APEX2 expression and were pre-treated with Zymolyase for 10 min. The BP probe was then added to cells with Zymolyase and further incubated for indicated times. Western blot of whole cell lysates was probed using IRDye Streptavidin and anti-Flag IgG. Two biological replicates were performed for Figures A–D.