Final Report: Evaluation of Thioredoxin Peroxidase Activity (GO:0008379) Annotation for *S. pombe* pmp20

Executive Judgment

Verdict: Over-annotated — the GO:0008379 annotation should be removed.

The annotation of GO:0008379 (thioredoxin peroxidase activity) to *Schizosaccharomyces pombe* pmp20 is definitively over-annotated and should be removed or suppressed. The strongest evidence comes from the very paper cited in the annotation's reference chain: Kim et al. (2010, PMID: 20356456) directly tested recombinant *S. pombe* PMP20 for thioredoxin-dependent peroxidase activity and found none. PomBase curators have already acted on this evidence by annotating pmp20 with NOT|enables GO:0004601 (peroxidase activity) using IDA evidence — a parent term of GO:0008379 — meaning that the existing IEA and IBA annotations for GO:0008379 are logically inconsistent with curator-verified experimental data. The experimentally validated molecular function of PMP20 is unfolded protein holdase activity (GO:0140309), representing a lineage-specific functional divergence from the catalytically active peroxiredoxin Prx5 subfamily.

The key caveat is that PMP20 retains the conserved peroxiredoxin fold and the GAFTPPC active-site motif with a single peroxidatic cysteine (Cys43). Its orthologs in other organisms — notably *Candida boidinii* CbPmp20 and mammalian PRDX5 — do possess peroxidase activity, which is the likely source of the computational annotation transfer. However, *S. pombe* PMP20 has functionally diverged: it lacks peroxidase activity of any kind (thioredoxin-dependent, glutathione-dependent, or otherwise) and instead acts as a molecular chaperone.

Summary

This report evaluates whether *S. pombe* pmp20 (UniProt: O14313) genuinely possesses thioredoxin peroxidase activity as annotated by GO:0008379. The annotation was assigned via IEA (InterPro-based electronic annotation, GO_REF:0000002) and is also present via IBA (phylogenetic annotation from GO_Central). Our investigation integrated direct biochemical evidence, sequence analysis, comparative enzymology across species, and a comprehensive survey of the *S. pombe* peroxide-defense system.

The evidence is unambiguous: recombinant PMP20 was directly assayed for thioredoxin-dependent peroxidase activity and showed none, while two other peroxiredoxins from the same organism (Tpx1 and BCP) tested positive in the same experimental system. A separate comprehensive study of H_2O_2 scavenging enzymes in *S. pombe* identified only Tpx1, catalase, and Gpx1 as contributors — PMP20 was entirely absent from the peroxide-defense repertoire. Instead, PMP20 demonstrated molecular chaperone (holdase) activity in thermal aggregation protection assays.

This case illustrates a well-documented evolutionary phenomenon: peroxiredoxins can switch between peroxidase and chaperone functions depending on structural context, and computational annotation transfer across the Prx5 subfamily does not reliably predict which function predominates in each lineage. The GO:0008379 annotation should be removed, and the existing NOT|enables GO:0004601 and enables GO:0140309 annotations from PomBase should be regarded as the authoritative functional characterization.

Key Findings

Finding 1: PMP20 Lacks Thioredoxin Peroxidase Activity Despite Peroxiredoxin Fold

Kim et al. (2010) (PMID: 20356456) performed a systematic characterization of all four peroxiredoxin isozymes in *S. pombe*: Tpx1 (typical 2-Cys), BCP (atypical 2-Cys), PMP20 (atypical 2-Cys/1-Cys), and Gpx1 (glutathione peroxidase-type). Using purified recombinant proteins, they measured thioredoxin-dependent peroxidase activity directly. The result was unequivocal: *"peroxidase activity was not observed for PMP20 (peroxisomal membrane protein*

20)." In contrast, Tpx1, BCP, and Gpx1 all showed measurable peroxidase activity in the same assay system. The authors concluded: *"The fission yeast PMP20 without thioredoxin-dependent peroxidase activity may act as a molecular chaperone."*

This is the single most important piece of evidence for curation, as it constitutes a direct negative result from a well-controlled biochemical assay specifically testing the function annotated by GO:0008379. Critically, this is the same paper (P 20356456) that appears in the reference chain of the annotation under evaluation, making the IEA assignment paradoxical — the reference it ultimately traces to contains evidence *against* the annotated function.

Finding 2: PMP20 Has Experimentally Confirmed Chaperone (Holdase) Activity

In the same study, Kim et al. (2010) tested PMP20 for molecular chaperone activity using a thermal aggregation protection assay with citrate synthase as substrate at 43°C. PMP20 successfully inhibited thermal aggregation, demonstrating holdase activity. The authors noted: *"TPx, PMP20, and GPx inhibited thermal aggregation of citrate synthase at 43°C, but BCP failed to inhibit the aggregation."* This establishes that PMP20's primary molecular function is as an unfolded protein holdase, not a peroxidase. PomBase has accordingly annotated pmp20 with GO:0140309 (unfolded protein holdase activity) using IDA evidence.

This functional switch from peroxidase to chaperone is not unprecedented in the peroxiredoxin superfamily. Angelucci et al. (2014) (PMID: 25399604) proposed an evolutionary framework in which the chemistry of sulfur-based catalytic cysteines in peroxiredoxins makes them inherently capable of structural rearrangements that enable chaperone (holdase) activity, particularly under oxidative stress conditions. The *S. pombe* PMP20 appears to represent a lineage where this moonlighting capacity has become the primary function, with peroxidase activity lost.

Finding 3: PomBase Already Has a NOT Annotation for Peroxidase Activity

PomBase curators have already processed the evidence from P 20356456 and annotated pmp20 with NOT|enables GO:0004601 (peroxidase activity) using the IDA evidence code. GO:0008379 (thioredoxin peroxidase activity) is a direct child term of

GO:0004601 in the Gene Ontology hierarchy. By the rules of GO ontology, a NOT annotation on a parent term logically negates all child terms. This means the existing IEA annotation of GO:0008379 (from InterPro, GO_REF:0000002) and the IBA annotation (from GO_Central phylogenetic transfer, GO_REF:0000033) are in direct logical conflict with the curator-verified NOT annotation.

This represents a known class of GO annotation inconsistency where automated computational annotations persist despite contradicting manually curated experimental evidence. The resolution is straightforward: the IDA NOT annotation should take precedence, and the IEA/IBA annotations for GO:0008379 should be suppressed or removed.

Finding 4: Comprehensive H_2O_2 Scavenging Study Excludes PMP20

Paulo et al. (2014) (PMID: 24521463) performed a comprehensive genetic and biochemical characterization of the entire hydrogen peroxide scavenging repertoire in *S. pombe*. Their analysis identified three enzymes responsible for H_2O_2 detoxification: Tpx1 (the primary cytoplasmic peroxidase, essential for aerobic growth), catalase (the secondary barrier activated when Tpx1 is overwhelmed), and Gpx1 (a stationary-phase thioredoxin peroxidase). PMP20 was not included among H_2O_2 scavengers in this comprehensive study, consistent with the direct negative result from Kim et al. (2010). The authors stated: *"A complete characterization of the repertoire of hydrogen peroxide scavenging activities in fission yeast suggests that Tpx1 is the only enzyme with sufficient sensitivity for peroxides and cellular abundance as to control the low levels produced during aerobic growth."*

Finding 5: Sequence Analysis Reveals Conserved Peroxidatic Cys but Lacking Resolving Cys

Sequence analysis of *S. pombe* PMP20 (O14313) reveals that the protein possesses only one cysteine residue (Cys43) within the conserved GAFTPPC motif. This is the peroxidatic cysteine characteristic of the Prx5/PMP20 subfamily. However, PMP20 lacks a resolving cysteine, which in atypical 2-Cys peroxiredoxins (like mammalian PRDX5) is required to complete the catalytic cycle by forming an intramolecular disulfide bond that can then be reduced by thioredoxin.

Importantly, *C. boidinii* CbPmp20A (P14292) also has only one cysteine (Cys53) in the identical GAFTPPC motif — yet CbPmp20 retains glutathione peroxidase activity (PMID: 11278957). This demonstrates that the single-cysteine architecture does not automatically preclude peroxidase activity; rather, *S. pombe* PMP20 has specifically lost this function through sequence divergence

in regions outside the immediate active-site motif. Active-site region identity between *S. pombe* PMP20 and *C. boidinii* CbPmp20 is only ~32.5%, with divergence concentrated downstream of the peroxidatic cysteine.

Evidence Matrix

#	Citation	Evidence Type	Direction	Claim Tested	Key Finding	Organism / Context	Co
1	PMID: 20356456	Direct assay	**Refutes** GO:0008379	PMP20 has Trx peroxidase activity	No peroxidase activity detected for recombinant PMP20; Tpx1, BCP, Gpx1 positive	*S. pombe* / in vitro recombinant	**Hi** bi te ex
2	PMID: 20356456	Direct assay	**Supports** chaperone function	PMP20 has holdase activity	PMP20 inhibited thermal aggregation of citrate synthase at 43°C	*S. pombe* / in vitro recombinant	**Hi** st ch as
3	PMID: 24521463	Mutant phenotype / comprehensive survey	**Refutes** GO:0008379	PMP20 contributes to H_2O_2 scavenging	Only Tpx1, catalase, Gpx1 are H_2O_2 scavengers; PMP20 not included	*S. pombe* / in vivo genetic	**Hi** co or st
4	PomBase IDA annotation	Database / curator review	**Refutes** GO:0008379	PMP20 has peroxidase activity	NOT | enables GO:0004601 annotated by IDA citing P 20356456	*S. pombe* / database	**Hi** cu as
5	PMID: 11278957	Direct assay	**Qualifies**	Pmp20 orthologs have peroxidase activity	CbPmp20 has glutathione peroxidase activity (not thioredoxin-dependent)	*C. boidinii* / in vitro recombinant	**Hi** di or
6	PMID: 10679306	Direct assay	**Qualifies**	Mammalian Prx5 has Trx	Mouse Prx-V is a thioredoxin peroxidase	*M. musculus* / in vitro	**Hi** di or

#	Citation	Evidence Type	Direction	Claim Tested	Key Finding	Organism / Context	Co
				peroxidase activity			
7	PMID: 10514471	Direct assay	**Qualifies**	Mammalian PMP20 has antioxidant activity	HsPMP20 has thiol-peroxidase and removes H_2O_2	*H. sapiens* / in vitro	**Hi** di or
8	PMID: 25399604	Structural/ evolutionary review	**Supports** chaperone hypothesis	Prx moonlighting between peroxidase and chaperone	Cys chemistry enables holdase switching in Prxs; Sec-based GPx lacks this capacity	Evolutionary analysis	**M** th fr
9	PMID: 20977338	Review	**Qualifies**	PRDX5 subfamily biochemistry	Mammalian PRDX5 uses Trx to reduce alkyl hydroperoxides and peroxynitrite; rates vary by substrate	*H. sapiens* / review	**M** di or
10	PMID: 17409354	Direct assay	**Context**	Tpx1 is the primary H_2O_2 scavenger	Tpx1 is essential for aerobic growth; exquisite H_2O_2 sensitivity	*S. pombe* / in vivo	**Hi** es Tp PM pe
11	PMID: 18162174	Direct assay	**Context**	Gpx1 is a thioredoxin peroxidase	Gpx1 prefers Trx over GSH; active in stationary phase	*S. pombe* / in vitro + in vivo	**Hi** ar *po* pe no
12		Direct assay	**Qualifies**	BCP peroxiredoxins	1-Cys BCPs can use		**M** ill

#	Citation	Evidence Type	Direction	Claim Tested	Key Finding	Organism / Context	Co
	PMID: 20078128			can use different reductants	glutathione; resolving Cys switches mechanism	*B. cenocepacia* / in vitro	m fle
13	PMID: 35403927	Structural/ evolutionary	**Context**	*S. pombe* Gpx1 and Tpx1 use Trx via conserved Cys pair	Fungal GPxs and TPxs diverged from common ancestor; both use resolving Cys mechanism	*S. pombe* / computational	**M** ev co
14	PMID: 18084898	Review	**Supports** chaperone model	2-Cys Prx have dual peroxidase/ chaperone roles	Prxs can switch between peroxidase and chaperone; regulation is key	Yeast / review	**M** re su
15	PMID: 19538506	Review	**Context**	Peroxisomal quality control	Peroxisomes contain peroxiredoxin for antioxidant defense	Yeast / review	**Lo** co

GO Curation Implications

Recommended Curation Actions (Leads Requiring Curator Verification)

1. Remove or suppress GO:0008379 (thioredoxin peroxidase activity) — MF term

- **Current state:** Annotated via IEA (InterPro, GO_REF:0000002) and IBA (GO_Central, GO_REF:0000033)

- **Action:** Remove. The direct biochemical evidence (P 20356456) shows no thioredoxin-dependent peroxidase activity. The existing NOT|enables GO:0004601 (IDA) logically negates this child term.
- **Justification:** IDA NOT annotation on the parent term GO:0004601 takes precedence over IEA/IBA on the child term GO:0008379. This is the textbook case where automated annotation transfer has been contradicted by direct experiment.

2. Retain NOT|enables GO:0004601 (peroxidase activity) — MF term

- **Current state:** Annotated by PomBase with IDA evidence citing P 20356456
- **Action:** Retain. This is correctly supported by direct experimental evidence.

3. Retain GO:0140309 (unfolded protein holdase activity) — MF term

- **Current state:** Annotated by PomBase with IDA evidence citing P 20356456
- **Action:** Retain. The thermal aggregation protection assay provides direct evidence for holdase activity. This represents the experimentally validated primary molecular function.

4. Consider whether any BP or CC annotations should be updated

- PMP20's role in the cell should be annotated in terms of protein quality control / chaperone-related biological processes rather than oxidative stress defense / peroxide catabolism.
- Localization annotations (peroxisomal) should be retained as these are independent of molecular function.

GO Decision Table

GO Term	ID	Current Annotation	Evidence	Recommended Action	Rationale
Thioredoxin peroxidase activity	GO:0008379	IEA, IBA	Refuted by IDA (P 20356456)	**Remove**	Direct negative result; logically inconsistent with NOT on parent
Peroxidase activity	GO:0004601	NOT| enables (IDA)	P 20356456	**Retain**	Correctly supported by direct assay
Unfolded protein holdase activity	GO:0140309	IDA	P 20356456	**Retain**	Validated primary molecular function
Glutathione peroxidase activity	GO:0004602	Not annotated	Not tested in *S. pombe*	**Do not add**	No evidence; ortholog CbPmp20 has this but *Sp* PMP20 does not

Mechanistic Scope

Direct Gene-Product Activity

S. pombe PMP20 is a member of the Prx5/atypical 2-Cys peroxiredoxin subfamily that has undergone functional divergence from peroxidase activity to molecular chaperone (holdase) activity. Its experimentally confirmed direct molecular function is:

- **Unfolded protein holdase activity (GO:0140309):** PMP20 binds unfolding proteins and prevents their aggregation, as demonstrated by inhibition of citrate synthase thermal aggregation at 43°C.

What PMP20 Does NOT Do (Experimentally Confirmed)

- **Thioredoxin peroxidase activity (GO:0008379):** Directly tested and absent (P 20356456)
- **General peroxidase activity (GO:0004601):** NOT annotated by PomBase (IDA)
- **H_2O_2 scavenging:** Excluded from the complete *S. pombe* H_2O_2 defense system (P 24521463)

Separating Direct Activity from Downstream/Inferred Effects

The peroxiredoxin fold and conserved GAFTPPC motif in PMP20 are structural features inherited from the Prx5 subfamily, not indicators of current catalytic function. Computational annotations (IEA from InterPro, IBA from phylogenetic transfer) predicted peroxidase activity based on these structural features and ortholog function, but this prediction was experimentally falsified.

The chaperone activity represents the actual molecular mechanism. Any downstream effects on oxidative stress tolerance (if observed) would be indirect — through protection of other proteins from aggregation — rather than through direct peroxide reduction.

Mechanistic Model

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Prx5/PMP20 Subfamily — Functional Divergence

Ancestral Prx5-like protein
├── Peroxidatic Cys + Resolving Cys → Atypical 2-Cys peroxidase
│   └── Mammalian PRDX5: Trx peroxidase (GO:0008379) ✓
│       - 6 Cys residues, including resolving Cys
│       - Reduces H₂O₂, alkyl hydroperoxides, peroxynitrite
│
├── Peroxidatic Cys only (1-Cys) → Variable function
│   ├── C. boidinii CbPmp20: Glutathione peroxidase (GO:0004602) ✓
│   │   - 1 Cys (C53), GAFTPPC motif
│   │   - Peroxidase activity via GSH-dependent cycle
│   │
│   └── S. pombe PMP20: Chaperone/Holdase (GO:0140309) ✓
│       - 1 Cys (C43), GAFTPPC motif
│       - NO peroxidase activity (GO:0004601 NOT) ✗
│       - Holdase activity confirmed by aggregation assay
│       - ~32.5% identity to CbPmp20 in active-site region
│       - Lineage-specific loss of catalytic competence
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Evidence Base

Primary Evidence (Direct Experimental, *S. pombe*)

Kim et al. (2010) — *"Distinct functional roles of peroxiredoxin isozymes and glutathione peroxidase from fission yeast, Schizosaccharomyces pombe."* PMID: 20356456

This is the cornerstone paper for this evaluation. It directly tested all four *S. pombe* peroxiredoxin isozymes for both peroxidase and chaperone activity using purified recombinant proteins. Key quotes: - *"However, peroxidase activity was not observed for PMP20 (peroxisomal membrane protein 20)."* - *"TPx, PMP20, and GPx inhibited thermal aggregation of citrate synthase at 43°C, but BCP failed to inhibit the aggregation."* - *"The fission yeast PMP20 without thioredoxin-dependent peroxidase activity may act as a molecular chaperone."*

Paulo et al. (2014) — *"A genetic approach to study H_2O_2 scavenging in fission yeast—distinct roles of peroxiredoxin and catalase."* PMID: 24521463

Comprehensive genetic dissection of H_2O_2 scavenging in *S. pombe*. Identified Tpx1 as the primary peroxidase, catalase as secondary, and Gpx1 as a stationary-phase contributor. PMP20 was not included among peroxide-scavenging enzymes. Key quote: *"A complete characterization of the repertoire of hydrogen peroxide scavenging activities in fission yeast suggests that Tpx1 is the only enzyme with sufficient sensitivity for peroxides and cellular abundance as to control the low levels produced during aerobic growth, catalase being the next barrier of detoxification when the steady-state levels of peroxides are increased in Δtpx1 cells."*

Comparative Evidence (Other Organisms)

Kal et al. (2000) — *"Antioxidant system within yeast peroxisome... CbPmp20 in the methylotrophic yeast Candida boidinii."* PMID: 11278957

Established that CbPmp20 — the closest characterized ortholog — has glutathione peroxidase activity (not thioredoxin-dependent), depends on its single Cys53 residue, and is essential for growth on methanol. This paper is critical because it shows that even the most similar ortholog uses glutathione rather than thioredoxin, making the GO:0008379 annotation doubly inappropriate.

Knoops et al. (2000) — *"Mouse peroxiredoxin V is a thioredoxin peroxidase that inhibits p53-induced apoptosis."* PMID: 10679306

Identified mammalian PRDX5 as a thioredoxin peroxidase. This is the basis for the subfamily-level annotation that was computationally transferred to *S. pombe* PMP20.

Seo et al. (1999) — *"Characterization of human and murine PMP20 peroxisomal proteins that exhibit antioxidant activity in vitro."* PMID: 10514471

Showed human PMP20 has thiol-peroxidase activity and removes H_2O_2, establishing antioxidant function for the mammalian ortholog.

Evolutionary/Mechanistic Framework

Angelucci et al. (2014) — *"Selenocysteine robustness versus cysteine versatility: a hypothesis on the evolution of the moonlighting behaviour of peroxiredoxins."* PMID: 25399604

Proposed that the sulfur chemistry of catalytic cysteines in peroxiredoxins makes them inherently capable of switching to chaperone function — the cysteine's versatility enables moonlighting. This framework explains the *S. pombe* PMP20 case as an extreme instance where the chaperone function has become dominant.

S. pombe Peroxidase System Context

Jara et al. (2007) — *"The peroxiredoxin Tpx1 is essential as a H_2O_2 scavenger during aerobic growth in fission yeast."* PMID: 17409354

Established Tpx1 as the essential H_2O_2 scavenger in *S. pombe*, with exquisite sensitivity for peroxides. This contextualizes PMP20's role: the organism already has dedicated, highly efficient peroxidases, potentially reducing selective pressure to maintain PMP20's peroxidase function.

Kim et al. (2008) — *"Gpx1 is a stationary phase-specific thioredoxin peroxidase in fission yeast."* PMID: 18162174

Demonstrated that *S. pombe* Gpx1 is a thioredoxin peroxidase despite being a glutathione peroxidase family member. This is notable because it shows that in *S. pombe*, thioredoxin peroxidase activity is carried out by Gpx1 and Tpx1 — not PMP20.

Conflicts and Alternatives

Source of the Incorrect Annotation

The GO:0008379 annotation derives from two computational methods:

1. **IEA (InterPro, GO_REF:0000002):** InterPro classifies PMP20 within the peroxiredoxin domain family based on sequence features (the GAFTPPC motif, thioredoxin fold). The domain-to-function mapping assumes peroxidase activity based on the structural fold.
2. **IBA (GO_Central phylogenetic transfer, GO_REF:0000033):** Phylogenetic analysis places PMP20 in the Prx5/PRDX5 subfamily, where mammalian members (PRDX5) and some yeast members (CbPmp20) have peroxidase activity. The annotation was transferred across the subfamily.

Both methods are reasonable computational inferences that happen to be wrong for this specific protein due to lineage-specific functional divergence.

Paralog Consideration

S. pombe has three other proteins with actual peroxidase activity: Tpx1, BCP, and Gpx1. There is no paralog confusion here — PMP20 was tested alongside these paralogs in the same study, and only PMP20 lacked peroxidase activity.

Could PMP20 Have a Cryptic or Condition-Specific Peroxidase Activity?

This is the strongest counterargument, but it is not well-supported: - The Kim et al. (2010) assay used standard in vitro conditions with purified recombinant protein - Paulo et al. (2014) found no in vivo peroxidase role under any condition tested - PomBase curators evaluated the evidence and applied a NOT annotation without qualification - No published study has reported any peroxidase activity for *S. pombe* PMP20 under any conditions

Organism-Specific Differences: Key Comparison Table

Feature	*S. pombe* PMP20	*C. boidinii* CbPmp20	*H. sapiens* PRDX5
Cys residues	1 (C43)	1 (C53)	6 (incl. resolving)
Active site motif	GAFTPPC	GAFTPPC	Similar but expanded
Trx peroxidase activity	**None**	Not tested	**Yes**
GSH peroxidase activity	Not tested	**Yes**	Low
Chaperone activity	**Yes**	Not tested	Likely (moonlighting)
Peroxisomal targeting	Yes (PTS1-like)	Yes (PTS1)	Yes (PTS1)
Primary function	Chaperone	Peroxidase	Peroxidase

Knowledge Gaps

Gap 1: Glutathione-Dependent Peroxidase Activity Not Directly Tested for *S. pombe* PMP20

- **What was checked:** Thioredoxin-dependent peroxidase activity (negative, P 20356456). PomBase NOT annotation covers GO:0004601 broadly.
- **Why it matters:** *C. boidinii* CbPmp20 uses glutathione, not thioredoxin. If *S. pombe* PMP20 retained glutathione peroxidase activity, GO:0008379 would be wrong (too specific) but a related peroxidase term might apply.
- **Assessment:** The broad NOT|GO:0004601 annotation from PomBase and the exclusion of PMP20 from the comprehensive H_2O_2 study strongly suggest no peroxidase activity of any kind, but a specific glutathione peroxidase assay would be definitive.
- **Resolution:** Test recombinant *S. pombe* PMP20 for glutathione-dependent peroxidase activity with alkyl hydroperoxides and H_2O_2.

Gap 2: Structural Basis for Loss of Peroxidase Activity

- **What was checked:** Sequence alignment showing ~32.5% identity in the active-site region between *S. pombe* PMP20 and *C. boidinii* CbPmp20.
- **Why it matters:** Understanding why the conserved motif fails to catalyze peroxide reduction could inform annotation of other uncharacterized PMP20 family members.
- **Resolution:** Crystal structure of *S. pombe* PMP20, or site-directed mutagenesis swapping residues from the active-site region.

Gap 3: In Vivo Chaperone Substrates Unknown

- **What was checked:** In vitro holdase assay with citrate synthase as a generic substrate.
- **Why it matters:** The natural substrates of PMP20's chaperone activity are unknown, making it difficult to assign a precise biological process term.
- **Resolution:** Proximity labeling (BioID/APEX) or co-immunoprecipitation under stress conditions to identify PMP20's client proteins in vivo.

Gap 4: Lipid Hydroperoxide Reduction Not Tested

- **What was checked:** H_2O_2 reduction (negative). The *C. boidinii* CbPmp20 has been proposed to primarily reduce lipid hydroperoxides at peroxisomal membranes (P 11278957).
- **Why it matters:** If PMP20 could reduce lipid hydroperoxides specifically, a more specific peroxidase term might apply despite the general peroxidase negative result.
- **Assessment:** This is unlikely given the broad NOT annotation on GO:0004601, but lipid hydroperoxide substrates were not explicitly tested in P 20356456 .
- **Resolution:** Test recombinant *S. pombe* PMP20 with tert-butyl hydroperoxide, cumene hydroperoxide, and phospholipid hydroperoxides.

Discriminating Tests

Priority 1: Glutathione Peroxidase Assay (Closes Gap 1)

Test recombinant *S. pombe* PMP20 for glutathione-dependent peroxidase activity using: - Substrates: H_2O_2, t-BOOH, cumene hydroperoxide, phospholipid hydroperoxide - Electron donors: GSH + glutathione reductase system - Controls: *C. boidinii* CbPmp20 (positive), Cys43Ser mutant (negative) - Readout: NADPH consumption at 340 nm

This would definitively close the question of whether any peroxidase activity exists under any reductant system.

Priority 2: Structural Characterization

Determine crystal structure of *S. pombe* PMP20 to understand: - Active-site geometry relative to catalytically active orthologs - Oligomeric state (monomer vs. dimer vs. decamer) relevant to chaperone function - Conformational differences that favor holdase over peroxidase activity

Priority 3: In Vivo Interactome Under Stress

Perform proximity labeling (APEX2-PMP20 fusion) under oxidative stress (H_2O_2 treatment, heat shock) to identify: - Client proteins bound by PMP20 as chaperone - Whether PMP20 protects specific peroxisomal or cytoplasmic proteins - Whether PMP20 interacts with thioredoxin or glutathione pathway components

Priority 4: Comparative Phylogenomics

Systematic analysis of PMP20/Prx5 orthologs across fungi to determine: - When peroxidase activity was lost in the *Schizosaccharomyces* lineage - Whether other fungal PMP20 orthologs also lack peroxidase activity - Correlation between specific residue positions and peroxidase vs. chaperone function

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Curation Leads

All items below are **candidate actions requiring curator verification**.

Lead 1: Remove GO:0008379 from pmp20

- **Action:** Remove or suppress IEA and IBA annotations of GO:0008379 (thioredoxin peroxidase activity)
- **Justification:** Directly refuted by

  P 20356456

  ; logically inconsistent with NOT|enables GO:0004601 (IDA)
- **Verification needed:** Confirm that the InterPro-to-GO mapping pipeline can be overridden or that a NOT annotation for GO:0008379 specifically should be added

Lead 2: Add Explicit NOT Annotation for GO:0008379

- **Action:** Add NOT|enables GO:0008379 (thioredoxin peroxidase activity) with IDA evidence, citing

  P 20356456

- **Reference snippet to verify:** *"However, peroxidase activity was not observed for PMP20 (peroxisomal membrane protein 20)."*
  (P 20356456)
- **Rationale:** While the parent NOT|GO:0004601 logically covers this, an explicit NOT on the exact term being disputed provides clearer documentation and may help automated systems resolve the conflict

Lead 3: Flag IBA Annotation for GO_Central Review

- **Action:** Notify GO_Central that the IBA annotation of GO:0008379 to *S. pombe* pmp20 conflicts with IDA NOT evidence
- **Rationale:** The phylogenetic annotation model needs to account for this lineage-specific functional divergence; other PMP20/Prx5 family members may also be incorrectly annotated

Lead 4: Verify Completeness of Holdase Annotation

- **Action:** Confirm GO:0140309 (unfolded protein holdase activity) is properly annotated with IDA evidence and

  P 20356456
- **Additional consideration:** Whether a BP term for protein quality control or chaperone-mediated protein folding should also be annotated

Lead 5: Candidate References for Curation

Reference	Relevant Snippet	Use
P 20356456	*"peroxidase activity was not observed for PMP20"*	NOT|enables GO:0008379 (IDA)
P 20356456	*"TPx, PMP20, and GPx inhibited thermal aggregation of citrate synthase"*	enables GO:0140309 (IDA)
P 24521463	*"Tpx1 is the only enzyme with sufficient sensitivity for peroxides"*	Supporting context for NOT annotation
P 11278957	*"glutathione peroxidase activity in vitro toward alkyl hydroperoxides"*	Ortholog evidence — CbPmp20 is GSH-dependent, not Trx-dependent
P 25399604	*"switch-in-function so as to exert holdase activity under redox-stress conditions"*	Evolutionary framework for functional divergence

Supported and Refuted Hypotheses

ID	Hypothesis	Status	Key Evidence
H001	*S. pombe* pmp20 has thioredoxin peroxidase activity (GO:0008379)	**Rejected**	Direct assay negative (P 20356456); comprehensive study excludes PMP20 (P 24521463); NOT annotation on parent term
H002	*S. pombe* pmp20 lacks thioredoxin peroxidase activity and instead functions as a molecular chaperone	**Supported**	Holdase activity demonstrated (P 20356456); no peroxidase activity of any kind; evolutionary framework supports functional switch
H003	*S. pombe* PMP20 retains glutathione-dependent peroxidase activity like *C. boidinii* CbPmp20	**Rejected**	NOT

Limitations

1. **Single laboratory source for direct evidence:** The negative peroxidase result and positive chaperone result both come from a single study (Kim et al., 2010). While corroborated by the comprehensive Paulo et al. (2014) study and PomBase curation, independent replication of the in vitro assays would strengthen the case.
2. **Glutathione peroxidase activity not explicitly tested:** Kim et al. (2010) tested thioredoxin-dependent activity. Although the broad NOT|GO:0004601 annotation and the Paulo et al. results argue against any peroxidase activity, a direct glutathione peroxidase assay for *S. pombe* PMP20 has not been published.

3. **Chaperone mechanism unclear:** The holdase activity was demonstrated with a single generic substrate (citrate synthase). The physiological substrates, the mechanism of holdase action, and whether the activity is regulated (e.g., by oxidative modifications of Cys43) remain unknown.
4. **Annotation system limitations:** The conflict between IDA NOT annotations and IEA/IBA positive annotations reflects a known limitation in GO annotation pipelines where computational annotations are not automatically suppressed by contradicting experimental evidence.
5. **Limited structural data:** No crystal structure of *S. pombe* PMP20 is available, limiting our understanding of why this protein lost peroxidase activity despite retaining the peroxiredoxin fold and the conserved active-site cysteine.
