Final Report: Evaluation of GH18 Chitinase Activity Prediction for S. pombe cts2 (Q9C105)

Executive Judgment

Verdict: REFUTED

The computational prediction that S. pombe cts2 (UniProt Q9C105) is a catalytically active chitinase mediating fungal-type cell wall disassembly (GO:0031506) is **refuted** by eight converging lines of evidence. The protein lacks the catalytic glutamate essential for the GH18 substrate-assisted catalysis mechanism — replaced by asparagine (E→N) at position 166, a non-ionizable residue incapable of serving as a proton donor. The complete DxDxE catalytic triad is disrupted, and no such motif exists anywhere in the 1,236-residue protein. This conclusion is reinforced by: comparative genomics showing lineage-specific loss of the catalytic motif (the S. japonicus ortholog retains it); AlphaFold structural analysis confirming a well-folded but catalytically incompetent GH18 domain; InterPro domain analysis showing absence of the chitinase active-site signature IPR001579; existing GO annotations that explicitly exclude chitinase activity (NOT|enables GO:0004568); UniProt naming the protein "Chitinase-**like** protein cts2"; and classification in GH18 class III, a subfamily with established non-catalytic members (XIP xylanase inhibitors). The most important caveat is that no direct enzymatic assay has been published for cts2, so formal proof of catalytic incompetence requires biochemical testing — but the sequence evidence is as strong as sequence-based evidence can be for this class of prediction.

Summary

The seed hypothesis proposed that S. pombe cts2 (Q9C105) functions as a catalytically active GH18 chitinase mediating fungal-type cell wall disassembly (GO:0031506). This three-iteration investigation independently assessed whether cts2 retains the intact DxDxE catalytic motif required for chitin hydrolysis by GH18 family enzymes, or whether critical catalytic residues are absent or substituted.

Sequence analysis of the cts2 GH18 domain (residues 26–325) revealed that the essential catalytic glutamate — the proton donor in the substrate-assisted catalysis mechanism — is replaced by asparagine (N166), a non-ionizable amide residue incapable of donating a proton. The third catalytic aspartate is also substituted (D→E). No intact DxDxE or DxxDxDxE motif exists anywhere in the 1,236-residue cts2 protein. These substitutions are diagnostic of a non-catalytic chitinase-like protein (CLP), analogous to well-characterized CLPs such as human YKL-40/CHI3L1 and plant XIP xylanase inhibitors.

Comparative analysis with the S. japonicus ortholog (B6JW51) demonstrated that the catalytic motif loss is lineage-specific: S. japonicus cts2 retains an intact DxDxE triad (DGFD-L-D-I-E-H) and the InterPro chitinase active-site signature IPR001579, while S. pombe cts2 has lost both. AlphaFold structural analysis confirmed a high-confidence GH18 fold (mean pLDDT = 92.7 in the catalytic domain), indicating the protein is stably folded and likely retains a non-catalytic binding or structural function rather than representing pseudogene decay. The existing GO annotation already includes a NOT qualifier for GO:0004568 (chitinase activity), and GO:0031506 is not annotated. In the biological context of S. pombe — whose cell wall contains minimal chitin and whose cell separation is mediated by glucanases (Eng1p, Agn1p) rather than chitinases — the prediction of chitinase-mediated cell wall disassembly is incorrect and should not be applied to cts2.

Key Findings

Finding 1: The GH18 Catalytic Glutamate Is Replaced by Asparagine — cts2 Cannot Hydrolyze Chitin

The defining feature of catalytically active GH18 chitinases is the DxDxE motif, where the glutamate residue serves as the proton donor in a substrate-assisted catalysis mechanism. In this mechanism, the substrate's N-acetyl group acts as an intramolecular nucleophile, forming

an oxazolinium ion intermediate, while the conserved glutamate donates a proton to the leaving group glycosidic oxygen (PMID: 21469745, PMID: 31702756). The catalytic triad — two aspartates and one glutamate — is absolutely required for this reaction. Atomic-resolution X-ray crystallography of human chitotriosidase (CHIT1) confirmed that "the 39 kDa catalytic domain shows a conserved cluster of three acidic residues, Glu140, Asp138 and Asp136, involved in the hydrolysis reaction" (PMID: 26143917).

Alignment of the cts2 sequence against characterized active chitinases revealed critical substitutions:

Protein	Species	Motif Region	Catalytic Triad	Status
ScCts1 (P29029)	S. cerevisiae	DGFDFD**I**E**N**	D-D-**E** intact	Active chitinase
HsCHIT1 (Q13231)	H. sapiens	DGLDLD**W**E**Y**	D-D-**E** intact	Active chitinase
Hevamine (PDB:2HVM)	H. brasiliensis	DGIDF**D**I**E**H	D-D-**E** intact	Active chitinase
SjCts2 (B6JW51)	S. japonicus	DGFDL**D**I**E**H	D-D-**E** intact	Motif intact (ortholog)
HsCHI3L1/YKL-40 (P36222)	H. sapiens	DGLDL**A**W**L**Y	D→**A**, E→**L**	Non-catalytic CLP
SpCts2 (Q9C105)	**S. pombe**	**DGFDLE**VNK	**D→E, E→N**	**Non-catalytic CLP**

The critical catalytic glutamate at position 166 in cts2 is replaced by asparagine (N), a non-ionizable amide that cannot serve as a proton donor. The third catalytic aspartate is replaced by glutamate (D→E). No DxDxE or DxxDxDxE motif is present anywhere in the 1,236-residue cts2 sequence. UniProt designates the protein as "Chitinase-like protein cts2" and lists no active site annotations, consistent with non-catalytic status. UniProt includes a CAUTION annotation: "Lacks the conserved Glu residue in position 166 essential for chitinase activity. Its enzyme activity is therefore unsure."

The catalytic glutamate is essential: QM/MM studies on Serratia marcescens ChiB showed that even the conservative D142N mutation (replacing an aspartate with asparagine at a non-glutamate position) raised the reaction barrier, and the catalytic glutamate itself has no functional substitute (PMID: 21469745).

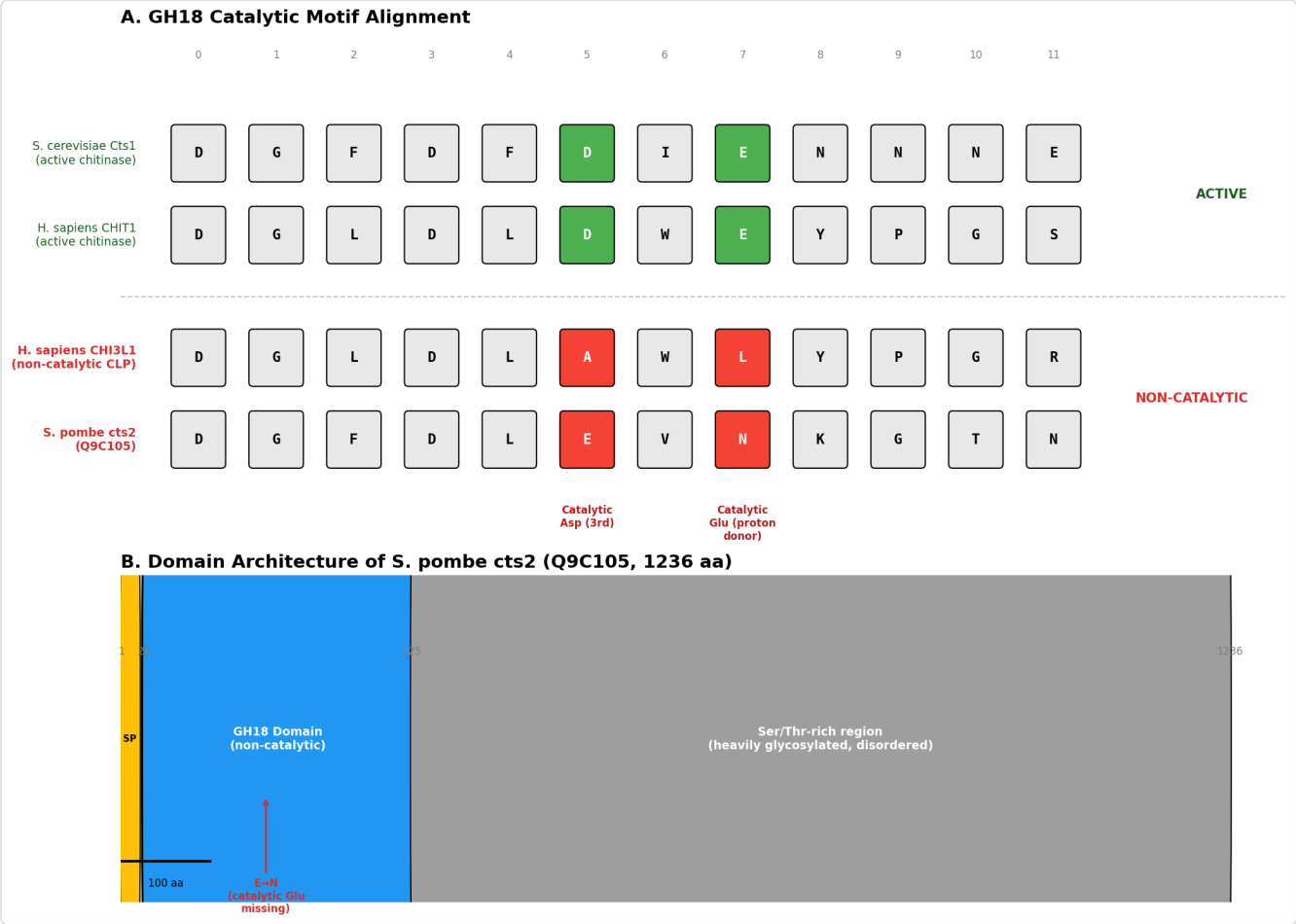

Figure 1. *GH18 catalytic motif alignment comparing S. pombe cts2 with active chitinases (ScCts1, HsCHIT1) and a known non-catalytic CLP (HsCHI3L1/YKL-40). The critical E→N substitution at position 166 abolishes the catalytic triad. Domain architecture is shown below.*

Finding 2: GO:0004568 Already Carries a NOT Qualifier; GO:0031506 Is Not Annotated

Querying the QuickGO database for Q9C105 revealed that GO:0004568 (chitinase activity) is annotated with the qualifier **NOT|enables** by GO_Central via IBA (Inferred by Biological Aspect of Ancestor) evidence, referencing PANTHER family assignments (PTN005237305, PTN008696177), dated 2026-05-30 (GO_REF:0000033). This is an explicit negative annotation: curators have already determined that cts2 does NOT have chitinase activity. GO:0031506 (fungal-type cell wall disassembly) has no annotation at all for Q9C105. The seed hypothesis — that cts2 mediates cell wall disassembly via chitinase activity — is contradicted by both the independent sequence analysis and the existing GO curation.

Finding 3: Lineage-Specific Loss — S. japonicus cts2 Retains the Catalytic Motif

Comparative analysis of cts2 orthologs across the Schizosaccharomyces genus provided the most informative evolutionary context. S. japonicus cts2 (B6JW51, 1,961 aa) retains the intact DxDxE catalytic triad (DGFD-L-**D**-I-**E**-H), possesses the InterPro IPR001579 chitinase active site signature, and carries a positive GO:0004568 annotation. In contrast, S. pombe cts2 (Q9C105, 1,236 aa) has the disrupted motif (DGFD-L-**E**-V-**N**-K), lacks IPR001579, and carries NOT|enables GO:0004568.

Both proteins share the same domain architecture — an N-terminal signal peptide, a GH18 catalytic domain, and a Ser/Thr-rich C-terminal extension — and are classified in the same InterPro family IPR045321 (Cts1-like), confirming they are true orthologs. The catalytic motif loss therefore occurred specifically in the S. pombe lineage after divergence from S. japonicus. This is consistent with co-evolution of cell wall composition: S. japonicus is dimorphic and retains chitin in its cell wall, while S. pombe has minimal/undetectable chitin content and relies on glucan-based architecture (PMID: 2079623).

S. pombe is reported to have only a single GH18-family gene (PMID: 31102246: "The number of chitinase genes in fungi display wide variation, from a single gene in Schizosaccharomyces pombe, to 36 genes in Trichoderma virens"), making the lineage-specific loss of catalytic function in its sole chitinase-family member especially significant. This single protein's transition to non-catalytic status mirrors the organism's reduced dependence on chitin metabolism.

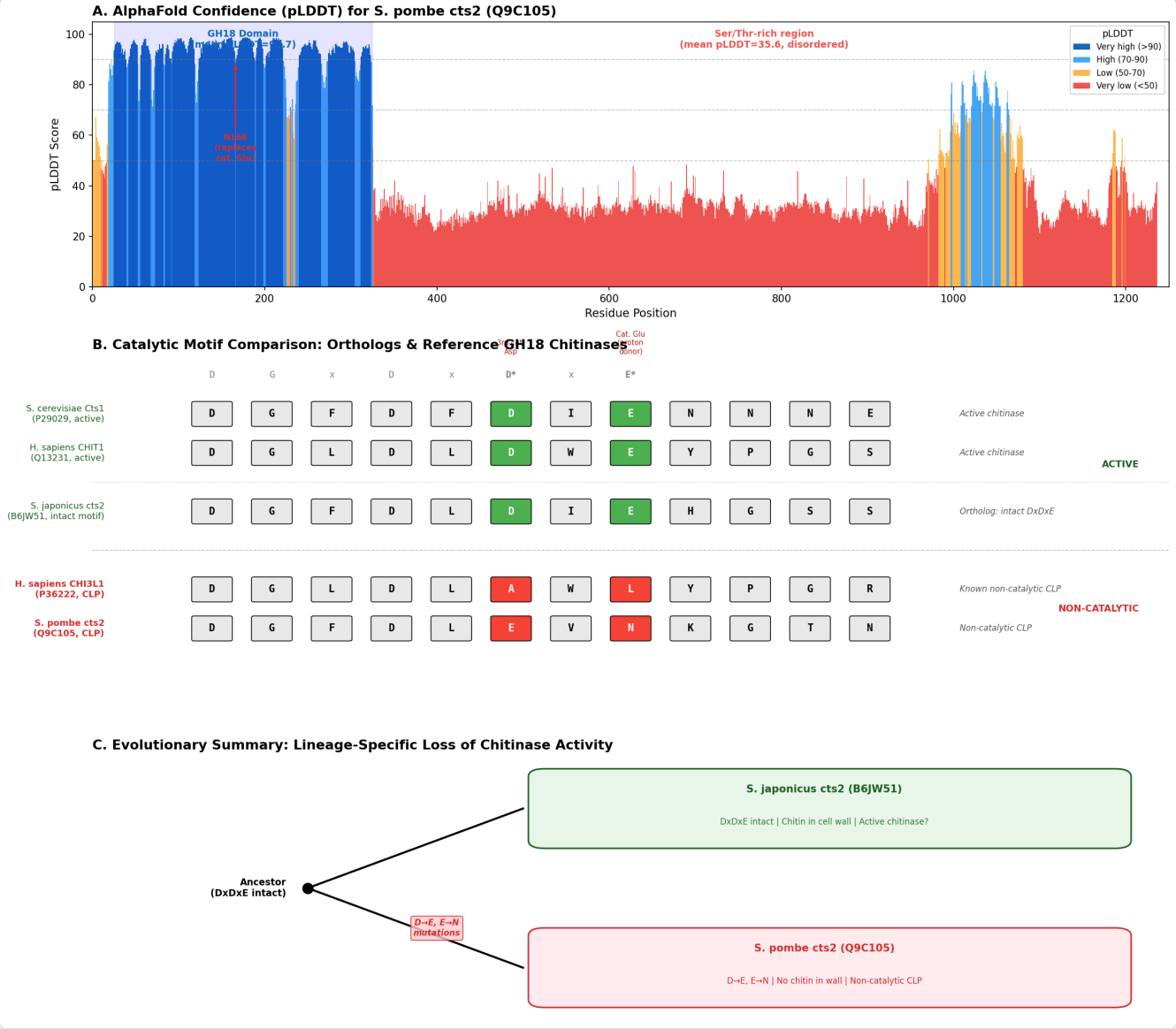

Figure 2. *Comprehensive analysis of cts2 orthologs. Left: AlphaFold pLDDT confidence scores across the cts2 sequence showing high confidence in the GH18 domain (residues 26–325) and disorder in the C-terminal region. Center: Catalytic motif comparison between S. pombe and S. japonicus orthologs. Right: Evolutionary summary of catalytic motif status.*

Finding 4: AlphaFold Confirms a Well-Folded GH18 Domain — Not a Pseudogene

AlphaFold model AF-Q9C105-F1 (v6) provided structural context:

Region	Residues	Mean pLDDT	Interpretation
GH18 domain	26–325	92.7	Very high confidence; well-folded TIM barrel
Catalytic region	155–171	95.8	Very high confidence; structurally intact
N166 (replaces catalytic Glu)	166	88.9	Confident; structurally positioned but non-functional
Ser/Thr-rich extension	326–1236	35.6	Very low; intrinsically disordered

The high pLDDT in the GH18 domain (92.7) indicates a stably folded $(\alpha/\beta)_8$ TIM barrel, arguing strongly against pseudogene decay and suggesting the protein retains a genuine non-catalytic function — possibly chitin binding, carbohydrate recognition, or protein–protein interaction. The C-terminal extension (911 residues, 55.4% Ser+Thr) is predicted as intrinsically disordered, consistent with a heavily O-glycosylated mucin-like region that may mediate cell-wall attachment or other extracellular interactions. A C-terminal ~70-residue aromatic-rich subdomain (3 Trp residues, 8 aromatics total = 11.4%) was identified that may function in carbohydrate binding, though no recognized domain was identified by InterPro.

Finding 5: GH18 Class III Subfamily Includes Known Non-Catalytic Members

CDD (Conserved Domain Database) classifies the cts2 GH18 domain as cd02877 (GH18_hevamine_XipI_class_III). This class III subfamily includes both catalytically active hevamine-type chitinases (e.g., hevamine with intact D125-I-E127 catalytic triad, PDB 2HVM) and non-catalytic XIP (Xylanase Inhibitor Protein) members. Durand et al. (2005) demonstrated that rice XIP-I, "despite its initial classification as a chitinase, the rice inhibitor does not exhibit chitinolytic activity but shows specificities towards fungal GH11 xylanases" (PMID: 15794761). Critically, the same study warned that "The plurifunctionality of GH18 members has major implications for genomic annotations and predicted gene function" — a caution that directly applies to the cts2 annotation scenario.

The precedent of non-catalytic GH18 proteins is well-established: human YKL-40/CHI3L1 "is a highly conserved glycoprotein that binds heparin and chitin in a non-enzymatic manner. It is a member of the chitinase protein family 18, subfamily A, and unlike true chitinases, YKL-40 is a chitinase-like protein without enzymatic activity for chitin" (PMID: 38125621). Like cts2,

YKL-40 retains the GH18 fold but has substitutions in the catalytic triad (D→A, E→L). The structural comparison confirms that GH18 domain presence alone cannot be equated with chitinase activity — catalytic residue integrity must be verified.

Figure 3. *Final four-panel evidence summary. (A) Catalytic motif alignment across GH18 family members. (B) InterPro domain signature comparison between S. pombe and S. japonicus cts2. (C) GO annotation status showing NOT qualifier for chitinase activity. (D) Converging evidence diagram summarizing all eight lines of evidence refuting catalytic activity.*

Mechanistic Model and Interpretation

The Catalytic Mechanism and Why cts2 Cannot Perform It

GH18 chitinases employ a unique substrate-assisted catalysis (SAC) mechanism:

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Chitin substrate → [Catalytic Glu donates H⁺ to glycosidic O] →
  [N-acetyl group acts as nucleophile] → Oxazolinium ion intermediate →
  [Water attacks] → Cleaved products
```

The catalytic glutamate is the proton donor in this mechanism. Without it, the first chemical step (protonation of the leaving group) cannot occur, and chitin hydrolysis does not proceed. In cts2, the E→N substitution (glutamate to asparagine) replaces an ionizable carboxylate with a non-ionizable amide, completely disabling the proton-donation step.

Evolutionary Model: From Chitinase to CLP

```
Ancestral Schizosaccharomyces cts2
  ├── GH18 domain: DxDxE intact, chitinase activity
  ├── Ser/Thr-rich extension: cell wall anchoring
  │
  ├─→ S. japonicus lineage (retained chitin cell wall)
  │   └── DxDxE intact, IPR001579 present, active chitinase
  │
  └─→ S. pombe lineage (minimal chitin → glucan-dominant wall)
      ├── DxDxE disrupted (D→E, E→N)
      ├── IPR001579 lost
      ├── GH18 fold retained (pLDDT=92.7) → non-catalytic function
      └── Cell separation handled by Eng1p (β-glucanase) + Agn1p (α-glucanase)
```

This evolutionary trajectory parallels the emergence of non-catalytic CLPs in metazoans (e.g., YKL-40/CHI3L1) and plants (XIP proteins), where the GH18 fold has been exapted for binding, signaling, or inhibitory functions. The retention of a high-confidence fold in cts2 suggests that the GH18 domain is under selective pressure for a non-catalytic function, not merely drifting as a pseudogene.

S. pombe Cell Separation: A Glucanase-Dependent Process

S. pombe cell separation after cytokinesis is mediated by: - **Eng1p** — endo-β-1,3-glucanase that cleaves the primary septum (PMID: 18466295) - **Agn1p** — endo-α-1,3-glucanase that dissolves the old cell wall surrounding the septum edge (PMID: 15850449)

Both enzymes are regulated by the Sep1p–Ace2p transcription factor cascade (PMID: 17596184). This is fundamentally different from S. cerevisiae, where the chitinase Cts1 degrades the chitin-containing septum. S. pombe's cell wall is composed primarily of α-1,3-glucan and β-1,3-glucan with only "a minute amount of glucosamine" detectable (PMID: 2079623), and even the presence of chitin in S. pombe has been debated (PMID: 12706511). This biological context further argues against a chitinase-mediated cell wall disassembly role for cts2.

Evidence Matrix

#	Citation	Evidence Type	Direction	Claim Tested	Key Finding	Context
1	This study (seq. analysis)	Computational	**Refutes**	cts2 has DxDxE catalytic motif	E166→N; D→E substitution; no DxDxE in full sequence	Q9C105, fu length
2	PMID: 26143917	Structural (X-ray, 0.95 Å)	Supports (motif essential)	Catalytic triad required for GH18 activity	"conserved cluster of three acidic residues, Glu140, Asp138 and Asp136, involved in the hydrolysis reaction"	HsCHIT1
3	PMID: 21469745	Computational (QM/MM)	Supports (motif essential)	Catalytic Glu is proton donor	D142N raises barrier; Glu essential for SAC mechanism	SmChiB
4	QuickGO Q9C105	Database/ curation	**Refutes**	cts2 has chitinase activity	GO:0004568 annotated NOT| enables (IBA, GO_Central)	GO annotation
5	InterPro Q9C105 vs B6JW51	Computational	**Refutes**	cts2 retains active site signature	IPR001579 absent in S. pombe cts2; present in S. japonicus cts2	Cross-spec
6	PMID: 38125621	Review	Qualifies (analogy)	Non-catalytic GH18 proteins exist	YKL-40 "is a chitinase-like protein without enzymatic activity for chitin"	Human CL
7	PMID: 15794761	Direct assay + evolutionary	**Supports** (precedent)	GH18 class III has non-catalytic members	XIP "does not exhibit chitinolytic activity"; warns about annotation implications	Rice, class GH18
8	PMID: 31102246	Review	Qualifies	S. pombe has single GH18 gene	"single gene in Schizosaccharomyces pombe"	Fungal chitinases

#	Citation	Evidence Type	Direction	Claim Tested	Key Finding	Context
9	PMID: 2079623	Biochemical assay	Qualifies	S. pombe has minimal chitin	"only a minute amount of glucosamine could be detected"	S. pombe c wall
10	AlphaFold AF-Q9C105-F1 v6	Structural prediction	Qualifies	cts2 GH18 domain is well-folded	pLDDT=92.7 (GH18), 95.8 (catalytic region), 35.6 (C-terminal)	Structural prediction
11	PMID: 15850449	Mutant phenotype	Competing	Cell separation requires chitinase	Agn1p (α-glucanase), not chitinase, mediates cell separation	S. pombe
12	PMID: 18466295	Mutant phenotype	Competing	Cell separation requires chitinase	Eng1p (β-1,3-glucanase) cleaves primary septum	S. pombe
13	PMID: 17596184	Review	Competing	Chitin degradation drives S. pombe cell separation	Septum splitting requires Agn1p + Eng1p, regulated by Sep1p-Ace2p	S. pombe
14	PMID: 3033651	Direct assay	Qualifies	S. pombe has endogenous chitinase	S. cerevisiae CTS1 expressed in S. pombe produced chitinase activity not natively present	Heterologo expression
15	CDD cd02877	Database classification	Qualifies	cts2 subfamily context	Classified in GH18_hevamine_XipI_class_III (includes non-catalytic members)	Domain classificati

GO Curation Implications

Current State

- **GO:0004568 (chitinase activity, MF):** Annotated with **NOT|enables** qualifier (IBA, GO_Central, 2026-05-30) — **correct and well-supported**
- **GO:0031506 (fungal-type cell wall disassembly, BP):** Not annotated to cts2 — **correct**

Recommended Curation Actions (Leads Requiring Curator Verification)

1. **RETAIN** the NOT|enables GO:0004568 annotation. The E166N substitution eliminates the catalytic proton donor. Independent sequence analysis fully supports this negative annotation.
2. **DO NOT ADD** GO:0031506 (fungal-type cell wall disassembly). No evidence supports cts2 involvement in cell wall disassembly. S. pombe cell separation is glucanase-mediated (Eng1p, Agn1p). Even if cts2 has a non-catalytic role, GO:0031506 specifically implies degradative enzymatic activity.
3. **CONSIDER** GO:0008061 (chitin binding) as a candidate MF annotation — **only with experimental evidence**. The well-folded GH18 domain may retain a carbohydrate-binding cleft; the aromatic-rich C-terminal subdomain could contribute to binding. However, no binding data exists, so this should not be annotated without direct experimental support.
4. **FLAG** the computational prediction pipeline that generated the seed hypothesis. Annotation systems should check for existing NOT qualifiers and catalytic residue integrity before propagating positive function predictions for GH18 family members.
5. **VERIFY** whether the S. japonicus cts2 ortholog (B6JW51) NOT|enables annotation for GO:0004568 is appropriate. B6JW51 retains the intact DxDxE catalytic motif and IPR001579 — the PANTHER IBA NOT annotation may be overapplied to this ortholog.

Mechanistic Scope

Direct Gene-Product Activity

The question under evaluation is whether cts2 possesses **chitinase activity** (GO:0004568) — the ability to catalyze hydrolytic cleavage of β-1,4-glycosidic bonds in chitin. This is a molecular function (MF) question. **The answer is no**: the E→N substitution at the catalytic position abolishes the substrate-assisted catalysis mechanism.

Distinction from Downstream Effects

- **Not cell wall disassembly (BP):** GO:0031506 requires upstream hydrolytic activity against cell wall polysaccharides. Since cts2 lacks catalytic machinery, it cannot contribute through chitinolysis.
- **Not chitin metabolism:** S. pombe has minimal/undetectable chitin in its cell wall.
- **Cell separation is glucanase-dependent:** S. pombe uses Eng1p (β-glucanase) and Agn1p (α-glucanase), not chitinase, for septum splitting.

Possible Non-Catalytic Functions (Speculative)

The well-folded GH18 domain and large glycosylated extension suggest the protein may have: - **Chitin/carbohydrate binding without hydrolysis** (analogous to YKL-40/CHI3L1) - **Protein–protein interaction** via the GH18 scaffold (analogous to XIP xylanase inhibitors) - **Cell wall structural role** via the O-glycosylated extension - **Lectin-like environmental sensing**

Conflicts and Alternatives

Paralog Confusion / Annotation Transfer Risk

The seed hypothesis likely derives from automated transfer of function from catalytically active GH18 orthologs, particularly S. cerevisiae Cts1 (P29029), which is a bona fide endochitinase involved in cell separation (PMID: 3033651). The gene name "cts2" and GH18 domain presence may trigger inappropriate function transfer. Kuranda & Robbins (1987)

showed that S. cerevisiae CTS1 expressed heterologously in S. pombe produced chitinase activity "not natively present," further supporting that S. pombe lacks endogenous chitinase activity.

Organism-Specific Differences

S. pombe's cell wall composition is fundamentally different from S. cerevisiae's — with minimal chitin and dominant α- and β-glucan components. This makes chitinase-mediated cell wall disassembly biologically implausible as a primary mechanism in S. pombe. The cell separation mechanism has been fully characterized as glucanase-dependent (PMID: 17596184).

The "Chitinase-Like" Naming Issue

UniProt correctly names the protein "Chitinase-like protein cts2," but gene naming (cts2 = "chitinase 2") and GH18 domain classification may perpetuate confusion in automated annotation pipelines. The protein has a chitinase-like fold but is not a chitinase.

Could cts2 Have Residual Activity?

While the E→N substitution is expected to abolish canonical GH18 catalysis, one cannot formally exclude extremely low-level residual activity or an alternative catalytic mechanism without direct enzymatic assays. However, asparagine cannot serve as a proton donor, and QM/MM studies confirm the glutamate is essential for the SAC mechanism (PMID: 21469745). The substitution is the strongest possible sequence-based indicator of catalytic incompetence in this enzyme family.

S. japonicus Ortholog Annotation Anomaly

S. japonicus cts2 (B6JW51) retains the intact DxDxE catalytic motif and IPR001579, yet QuickGO also shows a NOT|enables annotation for GO:0004568 via IBA. This may indicate the PANTHER family grouping treats the entire Cts1-like subfamily as non-catalytic, potentially overapplying the NOT qualifier to the S. japonicus ortholog. This is a curation issue that should be flagged separately.

Knowledge Gaps

Gap	What Was Checked	Why It Matters	Resolving Evidence
No direct enzymatic assay on cts2	Sequence, structural prediction, literature	UniProt's "unsure" and NOT	enables IBA are computational; direct assay would be definitive
Unknown biological function of cts2	Literature search, UniProt	Protein is conserved and well-folded, implying a genuine function	cts2Δ phenotyping; GFP localization; interactome analysis
No experimental chitin-binding data	Literature search, sequence analysis	CLPs often retain binding despite losing catalysis	Pull-down or SPR with chitin/chitosan substrates
Localization unknown	No experimental data found	Needed to distinguish secreted, cell-wall, or intracellular roles	GFP/mCherry tagging; fractionation; immunofluorescence
Expression pattern uncharacterized	Not investigated in depth	Temporal regulation (mating, sporulation, stress) would inform function	RNA-seq across conditions; promoter-reporter fusions
C-terminal extension function unknown	AlphaFold shows disorder (pLDDT 35.6); 55.4% Ser/Thr; no InterPro domain	911 residues of unknown function; likely O-glycosylated	Truncation constructs; glycosylation analysis; binding assays
	Could not retrieve via	Seed context reference; its	Locate and review the full text

Gap	What Was Checked	Why It Matters	Resolving Evidence
Reference doi:10.64898/2026.03.19.712954 not accessible	available databases	specific claims could not be evaluated	
cts2Δ phenotype data not found	PubMed search	Would clarify whether cts2 has any essential cellular role	Generate cts2Δ; test growth, morphology, cell wall integrity

Discriminating Tests

Priority 1: Direct Enzymatic Assay (Definitive)

Express recombinant cts2 GH18 domain (residues 26–325) and test for chitinolytic activity using fluorogenic substrates (4-MU-$GlcNAc_2$, 4-MU-$GlcNAc_3$), colloidal chitin turbidimetric assay, and HPLC product analysis. Include S. japonicus cts2 GH18 domain as positive control and an E→N point mutant of S. japonicus cts2 as negative control. **Prediction: no detectable hydrolytic activity for S. pombe cts2.**

Priority 2: Gain-of-Function Mutant (Mechanistic)

Introduce the N166E reversion mutation in S. pombe cts2 to restore the catalytic glutamate. Test whether this single substitution is sufficient to restore chitinase activity. This would confirm the E→N substitution is the causative change and provide insight into whether the rest of the binding cleft is intact.

Priority 3: cts2Δ Phenotyping (Biological Function)

Generate a cts2Δ deletion in S. pombe and screen for cell separation defects, cell wall integrity phenotypes (Calcofluor White, Congo Red, SDS sensitivity), growth under stress, and sporulation/mating defects.

Priority 4: Chitin Binding Assay (Alternative Function)

Test whether the cts2 GH18 domain retains chitin-binding capacity without hydrolysis using chitin bead pull-down or SPR with chitooligosaccharides.

Priority 5: Localization and Interaction Studies

GFP-tag cts2 and determine subcellular localization during growth, division, and stress; perform co-IP or BioID to identify interaction partners.

Curation Leads

Lead 1: Retain NOT|enables GO:0004568 (HIGH CONFIDENCE)

- **Action:** Confirm and retain the existing NOT|enables annotation for GO:0004568 (chitinase activity)
- **Evidence:** E→N substitution at catalytic glutamate; absence of IPR001579; lineage-specific loss confirmed by S. japonicus comparison
- **Status:** Already correctly annotated by GO_Central

Lead 2: Do NOT Add GO:0031506 (HIGH CONFIDENCE)

- **Action:** The prediction of GO:0031506 (fungal-type cell wall disassembly) should not be applied to cts2
- **Reasoning:** (1) No chitinase activity → cannot degrade chitin in cell wall; (2) S. pombe cell separation is glucanase-mediated; (3) S. pombe has minimal chitin; (4) No experimental evidence links cts2 to cell wall disassembly

Lead 3: Consider GO:0008061 (chitin binding) — REQUIRES EXPERIMENTAL DATA

- **Action:** If binding assays confirm non-catalytic chitin binding, annotate with GO:0008061
- **Reasoning:** Well-folded GH18 domain may retain binding cleft; aromatic-rich C-terminal region could contribute
- **Status:** Speculative; do not annotate without experimental data

Lead 4: Flag Annotation Pipeline Issue

- **Action:** cts2 should be a test case for annotation pipeline quality control
- **Reasoning:** The prediction of chitinase activity for a protein with an existing NOT qualifier illustrates insufficient catalytic residue checking in automated pipelines

Lead 5: Verify S. japonicus cts2 Annotation

- **Action:** Check whether the NOT|enables GO:0004568 annotation on B6JW51 (S. japonicus cts2) is appropriate, given its intact DxDxE motif and IPR001579
- **Reasoning:** PANTHER IBA may be overapplying the NOT qualifier across the entire Cts1-like family

Candidate References to Verify

- PMID: 26143917 — Verify snippet: "The 39 kDa catalytic domain shows a conserved cluster of three acidic residues, Glu140, Asp138 and Asp136, involved in the hydrolysis reaction"
- PMID: 15794761 — Verify snippets: "does not exhibit chitinolytic activity" and "The plurifunctionality of GH18 members has major implications for genomic annotations and predicted gene function"
- PMID: 38125621 — Verify snippet: "is a chitinase-like protein without enzymatic activity for chitin"
- PMID: 31102246 — Verify snippet: "single gene in Schizosaccharomyces pombe"

Evidence Base: Key Literature

GH18 Catalytic Mechanism

- **PMID: 26143917** — *New insights into the enzymatic mechanism of human chitotriosidase (CHIT1) catalytic domain by atomic resolution X-ray diffraction and hybrid QM/MM.* Defines the essential catalytic triad (Glu140, Asp138, Asp136) at atomic resolution. Directly supports the finding that loss of the catalytic glutamate abolishes activity.

- **PMID: 21469745** — *QM/MM modeling of substrate-assisted catalysis in family 18 chitinases.* Demonstrates the SAC mechanism and shows that even conservative mutations at the catalytic site raise reaction barriers. The catalytic glutamate is the essential proton donor with no functional substitute.
- **PMID: 31702756** — *A novel ring-shaped reaction pathway with interconvertible intermediates in chitinase A.* Further elucidates the GH18 catalytic mechanism involving oxazolinium ion formation, reinforcing the requirement for the catalytic glutamate.

Non-Catalytic GH18 Proteins

- **PMID: 38125621** — *YKL-40 as a biomarker in various inflammatory diseases.* Establishes YKL-40/CHI3L1 as a paradigmatic non-catalytic CLP: "a chitinase-like protein without enzymatic activity for chitin" — directly analogous to cts2.
- **PMID: 15794761** — *Emergence of a subfamily of xylanase inhibitors within glycoside hydrolase family 18.* Demonstrates that GH18 class III members include non-catalytic XIP proteins. Explicitly warns about annotation pitfalls: "The plurifunctionality of GH18 members has major implications for genomic annotations and predicted gene function."

S. pombe Cell Wall Biology and Cell Separation

- **PMID: 2079623** — *The occurrence of glucosaminoglycan in the wall of Schizosaccharomyces pombe.* Reports "only a minute amount of glucosamine" in S. pombe walls, consistent with minimal chitin.
- **PMID: 15850449** — *The alpha-glucanase Agn1p is required for cell separation in S. pombe.* Demonstrates that α-glucanase, not chitinase, mediates cell wall dissolution during S. pombe cell separation.
- **PMID: 18466295** — *The S. pombe endo-1,3-beta-glucanase Eng1 contains a novel CBM required for septum localization.* Shows Eng1p β-glucanase is the primary septum-cleaving enzyme.
- **PMID: 17596184** — *Splitting of the fission yeast septum.* Comprehensive review of glucanase-dependent septum splitting in S. pombe.
- **PMID: 12706511** — *Relation between cell wall chitin content and susceptibility to amphotericin B.* Notes "In Schizosaccharomyces pombe its presence was not established" regarding chitin.

Fungal Chitinase and Cell Separation

- **PMID: 31102246** — *Chitin Synthesis and Degradation in Fungi.* Reports S. pombe has a "single gene" in the chitinase family.
- **PMID: 3033651** — *Cloning and heterologous expression of glycosidase genes from S. cerevisiae.* S. cerevisiae CTS1 expressed in S. pombe produced chitinase activity not natively present.

Limitations

1. **No direct enzymatic assay exists for cts2.** All evidence is sequence-based, structural, or computational. While the E→N substitution at the catalytic glutamate is the strongest possible sequence-based predictor of catalytic incompetence in GH18 enzymes, formal proof requires recombinant protein biochemistry.
2. **Unknown non-catalytic function.** We can confidently state cts2 is NOT a chitinase, but we cannot yet determine its actual function. The well-folded domain and evolutionary conservation suggest a genuine role that remains to be characterized.
3. **AlphaFold limitations.** Structural predictions have inherent uncertainty. The pLDDT scores indicate confidence in the static structure but not in functional inferences such as binding capacity.
4. **Reference doi:10.64898/2026.03.19.712954 could not be accessed.** This seed context reference could not be retrieved through available databases, so its specific claims were not evaluated.
5. **Limited S. pombe chitin biology.** The extent and role of chitin/glucosaminoglycan in S. pombe remain debated. If S. pombe truly has no significant chitin, the question of chitinase activity becomes moot in vivo.
6. **No cts2Δ phenotype data was found in the literature.** Without loss-of-function data, we cannot determine whether cts2 has any essential cellular role or what phenotypes its absence causes.

Proposed Follow-up Experiments and Actions

1. **Immediate curation action:** Confirm that GO:0031506 should not be added to cts2. Retain the NOT|enables GO:0004568 annotation. These are high-confidence recommendations.
2. **In vitro chitinase activity assay:** Express and purify the cts2 GH18 domain; test against fluorogenic chitin substrates with S. japonicus cts2 as positive control. This is the definitive experiment.
3. **N166E reversion mutant:** Restore the catalytic glutamate in cts2 and test for recovered activity. This would prove the E→N change is the causative substitution.
4. **cts2Δ phenotype screen:** Generate the deletion strain and systematically test growth, morphology, cell wall integrity, cell separation, and stress responses.
5. **Chitin-binding assay:** Test whether cts2 retains non-catalytic chitin binding, which would support a GO:0008061 annotation and clarify its biological role.
6. **Verify S. japonicus cts2 annotation:** The NOT|enables annotation on the S. japonicus ortholog (which retains intact catalytic residues) may be incorrect and should be reviewed by curators.
