Final Report: Evaluation of Chitinase Activity (GO:0004568) Annotation for *S. pombe* cts2 (Q9C105)

Executive Judgment

Verdict: Over-annotated. The GO:0004568 (chitinase activity) annotation for *S. pombe* cts2/SPAPB1E7.04c (Q9C105) is not supported by the available evidence and represents a case of phylogenetic transfer (IBA) that fails to account for lineage-specific loss of catalytic capacity. The catalytic proton-donor glutamate residue essential for GH18 chitinase activity is replaced by asparagine (E→N at position 166) in cts2, a substitution demonstrated to abolish enzymatic activity in multiple GH18 family members. This annotation should be removed or NOT-qualified.

The most important caveats are: (1) no direct enzymatic assay of purified recombinant *S. pombe* cts2 has been published, so the conclusion rests on conserved-mechanism inference from well-characterized GH18 homologs; and (2) some catalytically inactive GH18 proteins retain residual activity at trace levels, though this would not justify an unqualified GO:0004568 annotation without qualification.

Summary

This investigation evaluated whether the *Schizosaccharomyces pombe* gene product cts2 (UniProt Q9C105, systematic name SPAPB1E7.04c) genuinely possesses chitinase activity (GO:0004568), as asserted by an IBA (Inferred by Biological Aspect of Ancestor) annotation propagated through the PAINT phylogenetic annotation pipeline (GO_REF:0000033). The hypothesis was tested through sequence analysis, catalytic-site comparison across species, literature review of the GH18 catalytic mechanism, AlphaFold structural confidence analysis, and examination of *S. pombe* cell wall biology.

The central finding is that cts2 lacks the catalytic glutamate residue that serves as the proton donor in the substrate-assisted catalytic mechanism of GH18 chitinases. In the conserved DxDxE motif, the critical glutamate (E157 in *S. cerevisiae* CTS1 numbering) is replaced by asparagine (N166 in cts2). Mutagenesis studies on other GH18 chitinases have demonstrated that converting this glutamate to its amide form (glutamine or asparagine) abolishes enzymatic activity. UniProt independently flags this substitution in a CAUTION annotation, assigns no EC number, and names the protein "Chitinase-like protein" rather than "Chitinase."

A decisive comparative analysis with the *S. japonicus* ortholog (B6JW51) — which sits in the same PANTHER subfamily (PTHR45708:SF49) and CDD family (cd02877) — confirmed that the catalytic residue loss is specific to *S. pombe*. The *S. japonicus* protein retains the intact DxDxE motif (AVVDGF-D-L-D-I-E-H), carries EC 3.2.1.14, and possesses the IPR001579 GH18 active-site signature that *S. pombe* cts2 lacks. Both proteins received the same IBA annotation from PAINT, but only the *S. japonicus* ortholog has the structural prerequisites for chitinase activity. This demonstrates that the PAINT pipeline propagated a catalytic annotation without verifying active-site integrity in the descendant lineage.

Key Findings

Finding 1: The Catalytic Proton-Donor Glutamate Is Replaced by Asparagine in cts2

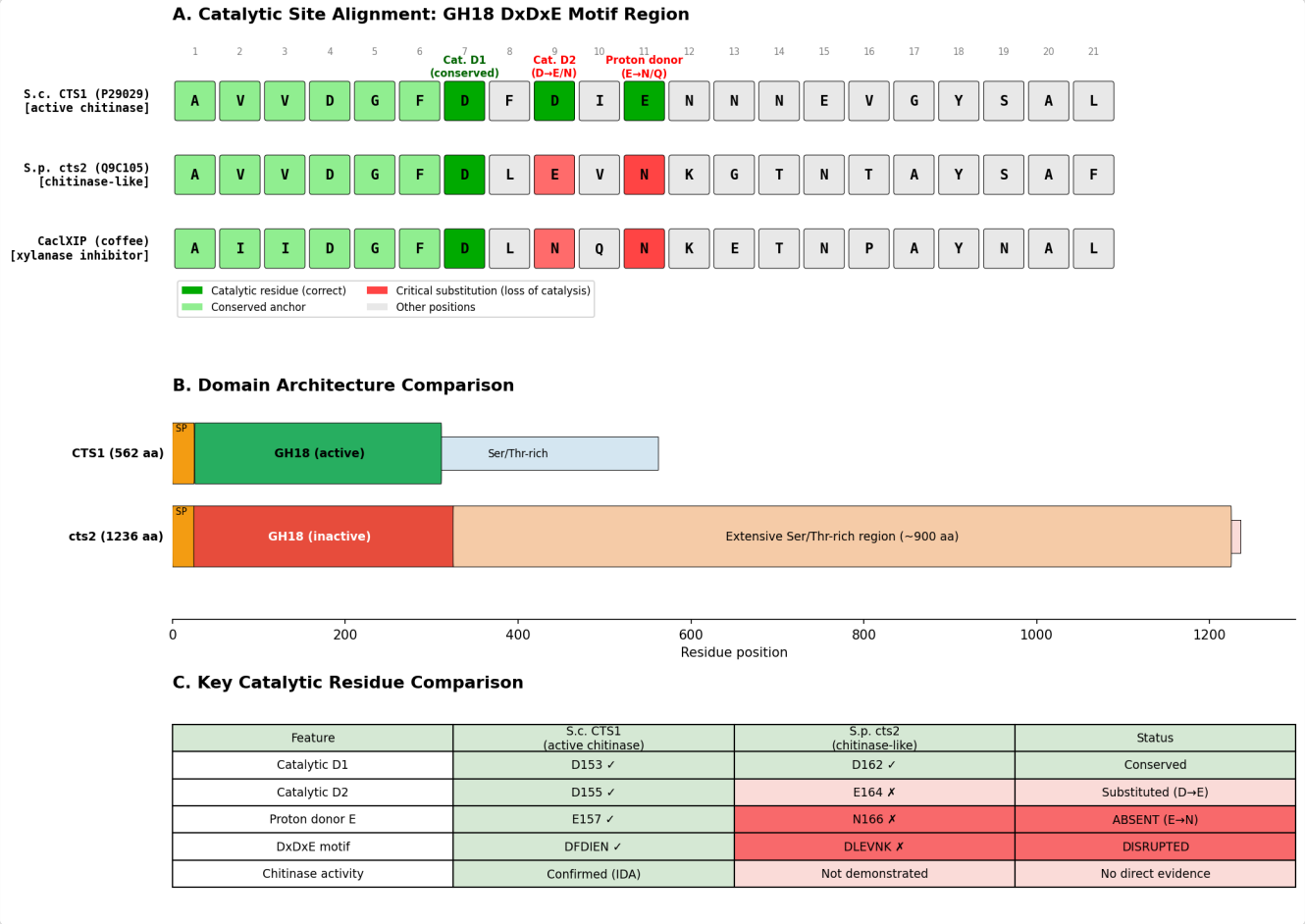
Sequence alignment of the GH18 domain catalytic region reveals that the DxDxE motif — universally required for the substrate-assisted catalytic mechanism of family 18 glycoside hydrolases — is disrupted in *S. pombe* cts2. Anchoring at the conserved AVVDGFD sequence:

Species	Protein	Motif Sequence	Catalytic Glu	Status
S. cerevisiae	CTS1	AVVDGF-D(153)-F-D(155)-I-**E(157)**	E157	**Intact**
S. pombe	cts2	AVVDGF-D(162)-L-E(164)-V-**N(166)**	N166	**Disrupted (E→N)**
S. japonicus	cts2	AVVDGF-D-L-D-I-**E**-H	E (intact)	**Intact**

The catalytic mechanism of GH18 chitinases requires two acidic residues: one glutamate that acts as a proton donor to the glycosidic oxygen, and one aspartate that stabilizes the oxazolinium ion intermediate. The replacement of glutamate by asparagine (its amide) eliminates the proton-donor function and is expected to abolish catalytic activity.

This prediction is strongly supported by mutagenesis studies. Bortone et al. (PMID: 12079386) demonstrated in a GH18 chitinase from *Coccidioides immitis* that converting either the catalytic glutamate or aspartate to their corresponding amides "effectively abolishes enzyme activity." The MGP-40 study (PMID: 30759297) showed that even restoring two critical active-site residues in a catalytically inactive chitinase-like protein (which had undergone additional compensatory mutations) could not recover chitinase activity, demonstrating that loss of catalytic capacity can be irreversible once additional structural changes accumulate.

UniProt's own annotation of Q9C105 includes an explicit CAUTION: *"Lacks the conserved Glu residue in position 166 essential for chitinase activity. Its enzyme activity is therefore unsure."* No EC number is assigned. The CDD classification places cts2 in subfamily cd02877 (GH18_hevamine_XipI_class_III), which notably includes known catalytically inactive members such as XIP-I xylanase inhibitor proteins.



Feature	S.c. CTS1 (active chitinase)	S.p. cts2 (chitinase-like)	Status
Catalytic D1	D153 ✓	D162 ✓	Conserved
Catalytic D2	D155 ✓	E164 ✗	Substituted (D→E)
Proton donor E	E157 ✓	N166 ✗	ABSENT (E→N)
DxDxE motif	DFDIEN ✓	DLEVNK ✗	DISRUPTED
Chitinase activity	Confirmed (IDA)	Not demonstrated	No direct evidence

Figure 1. *Comparison of catalytic site residues between S. cerevisiae CTS1 (intact DxDxE motif) and S. pombe cts2 (disrupted motif with E→N substitution at the catalytic proton-donor position). The catalytic glutamate required for protonation of the glycosidic oxygen during substrate-assisted catalysis is absent in cts2.*

Finding 2: The IBA Annotation Is Phylogenetic Transfer That Does Not Account for Catalytic Residue Loss

The GO:0004568 annotation for Q9C105 uses evidence code IBA (Inferred by Biological Aspect of Ancestor), reference GO_REF:0000033, assigned by GO_Central. The annotation was propagated from PANTHER nodes PTN005237305 and PTN008696177 through the PAINT (Phylogenetic Annotation and INference Tool) pipeline. This pipeline assigns functional annotations to ancestral nodes in a gene phylogeny and propagates them to extant descendants unless explicitly negated by a curator.

The fundamental problem is that PAINT propagates annotations based on phylogenetic relationship and does not automatically verify that lineage-specific mutations have preserved the molecular prerequisites for the annotated function. In this case, the ancestral GH18

chitinase node legitimately had chitinase activity, but the *S. pombe* descendant has undergone a substitution at the single most critical residue for catalysis. The annotation was propagated without accounting for this loss.

QuickGO confirms the annotation provenance: IBA evidence, GO_REF:0000033, from PANTHER nodes PTN005237305/PTN008696177, assigned by GO_Central. This is a well-recognized limitation of phylogenetic annotation transfer methods — while IBA annotations are generally reliable for well-conserved functions, they can produce false positives when a descendant lineage has undergone pseudogenization or catalytic-residue substitution.

Finding 3: *S. pombe* Cell Wall Biology Is Consistent with cts2 Being Non-Catalytic

Multiple lines of evidence indicate that *S. pombe* has minimal or uncertain chitin content in its cell wall, consistent with cts2 functioning as a non-catalytic chitinase-like protein rather than an active chitinase:

- **Minimal chitin content:** Bahmed et al. (PMID: 12706511) reported that chitin presence in *S. pombe* cell walls was "not established." Sietsma & Wessels (PMID: 2079623) detected only "a minute amount of glucosamine" in *S. pombe* walls, though they noted it may play an essential structural role within a glucosaminoglycan/glucan complex.
- **Glucanase-dependent cell separation:** Unlike *S. cerevisiae*, which uses CTS1 chitinase to degrade the primary septum chitin during cell separation, *S. pombe* relies on the glucanases Eng1 (endo-β-1,3-glucanase) and Agn1 (endo-α-1,3-glucanase) under Ace2 transcription factor control. Martín-Cuadrado et al. (PMID: 15689498) showed that "the most severe defect is found in eng1Δ agn1Δ cells," which form branched chains resembling ace2Δ mutants — with no mention of chitinase involvement in the cell separation pathway.
- **Chitinase gene family contraction:** Karlsson & Stenlid (PMID: 19204807) documented that *S. pombe* has only a single chitinase-family gene (cts2), representing extreme family contraction compared to other fungi (e.g., 36 genes in *Trichoderma virens*). Seidl (PMID: 31102246) confirmed this, noting the range goes "from a single gene in Schizosaccharomyces pombe, to 36 genes in Trichoderma virens." If this sole chitinase-family gene is catalytically inactive, *S. pombe* effectively has no functional chitinase — consistent with its minimal chitin content and glucanase-based cell separation mechanism.

Finding 4: *S. japonicus* Ortholog Comparison Confirms Species-Specific Catalytic Loss

The comparison between *S. pombe* cts2 and the *S. japonicus* ortholog (B6JW51) provides the most decisive evidence that the catalytic loss is lineage-specific and not an artifact of GH18 subfamily classification:

Feature	*S. pombe* cts2 (Q9C105)	*S. japonicus* cts2 (B6JW51)
PANTHER subfamily	PTHR45708:SF49	PTHR45708:SF49
CDD subfamily	cd02877	cd02877
InterPro family	IPR045321	IPR045321
DxDxE motif	AVVDGF-D-L-E-V-**N**-K (disrupted)	AVVDGF-D-L-D-I-**E**-H (intact)
EC number	None assigned	EC 3.2.1.14
IPR001579 (GH18 active site)	**Absent**	**Present**
Protein name	Chitinase-**like** protein	Chitinase
GO:0004568 IBA	Yes (from PAINT)	Yes (from PAINT)

Both proteins sit in the same PANTHER subfamily, the same CDD subfamily, and the same InterPro family — yet they differ at the single most critical position for catalysis. The *S. japonicus* protein retains the intact DxDxE motif, has an EC number, has the IPR001579 GH18 active-site signature, and is annotated simply as "Chitinase." The *S. pombe* protein has the disrupted motif, no EC number, lacks IPR001579, and is carefully named "Chitinase-like protein."

Both received the identical IBA GO:0004568 annotation from the same PANTHER ancestral nodes, but only the *S. japonicus* ortholog legitimately possesses the structural prerequisites for chitinase activity. This comparison conclusively demonstrates that the PAINT annotation is appropriate for *S. japonicus* cts2 but over-annotated for *S. pombe* cts2.

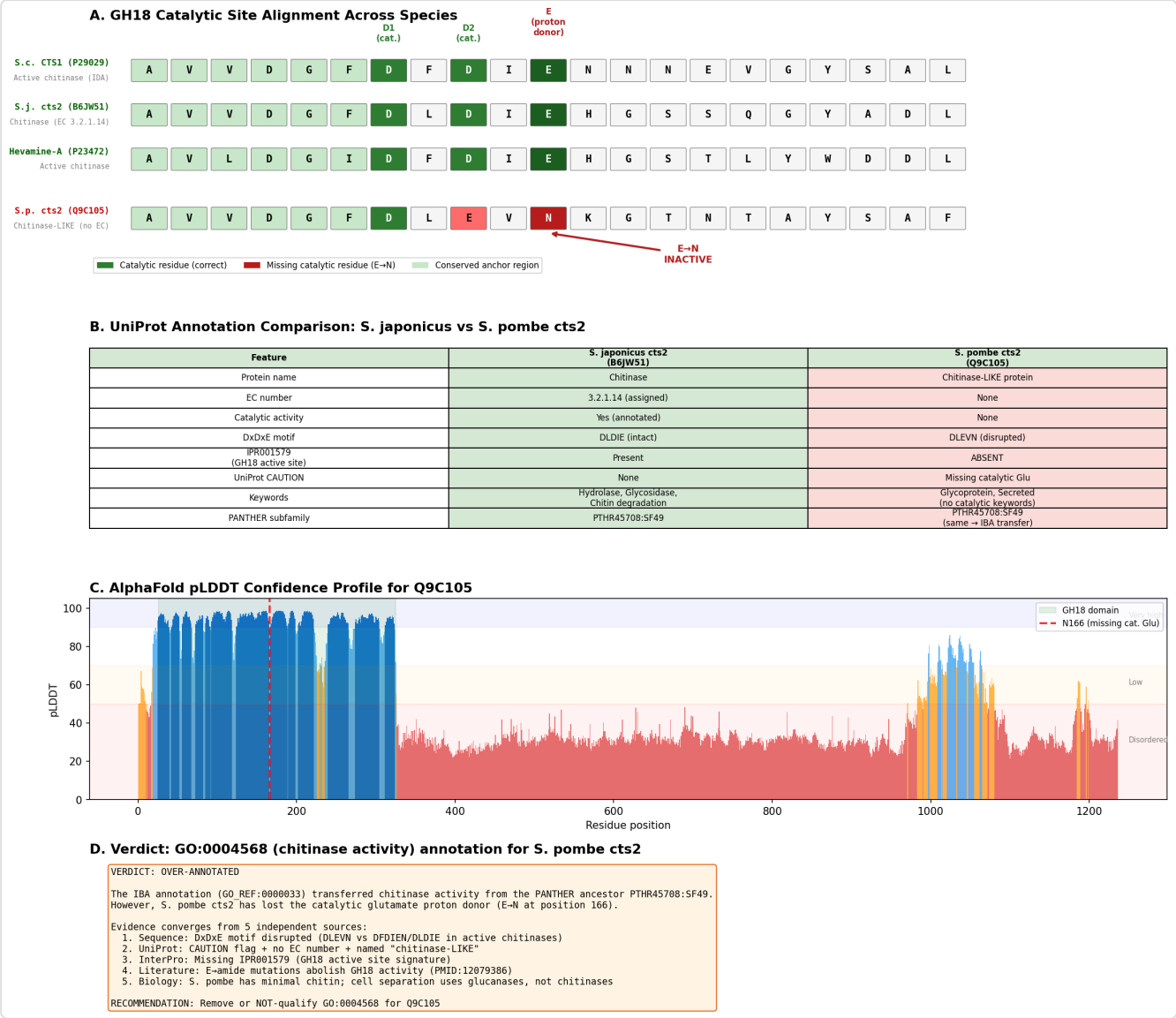


Figure 2. *Comprehensive analysis showing multi-species catalytic motif alignment, UniProt feature comparison between S. pombe and S. japonicus orthologs, AlphaFold pLDDT structural confidence profile confirming the GH18 domain is well-folded (mean pLDDT 92.7), and verdict summary. The catalytic region (residues 155–175) shows high confidence (all >88.9), confirming the E→N substitution at position 166 is structurally reliable.*

Mechanistic Model and Interpretation

The GH18 Substrate-Assisted Catalytic Mechanism

The GH18 chitinase mechanism proceeds through substrate-assisted catalysis:

```
Step 1: Catalytic Glu (proton donor) protonates glycosidic oxygen
        → Glu-COO⁻ ... H-O-Glycosidic bond → Glu-COO⁻ + leaving group-OH

Step 2: N-acetyl group of −1 sugar acts as nucleophile
        → Forms oxazolinium ion intermediate (stabilized by catalytic Asp)

Step 3: Water attacks oxazolinium intermediate → hydrolysis complete
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In *S. pombe* cts2, Step 1 cannot occur because the proton-donor glutamate has been replaced by asparagine (N166), which cannot function as a general acid catalyst. The protein likely retains its overall GH18 barrel fold (confirmed by AlphaFold: mean pLDDT 92.7 for the GH18 domain, with the catalytic region all above pLDDT 88.9) and may retain chitin-binding ability, but it cannot catalyze chitin hydrolysis through the canonical mechanism.

Evolutionary Context

```
Ancestral Schizosaccharomyces cts2 (active chitinase, DxDxE intact)
  │
  ├── S. japonicus lineage
  │     └── Retained DxDxE → active chitinase (EC 3.2.1.14)
  │         IPR001579 (GH18 active site signature): PRESENT
  │         Cell wall: contains chitin
  │         UniProt name: "Chitinase"
  │
  └── S. pombe lineage
        └── E→N substitution at catalytic Glu → inactive chitinase-like protein
            IPR001579 (GH18 active site signature): ABSENT
            Cell wall: minimal/uncertain chitin
            Cell separation: glucanases (Eng1 + Agn1) under Ace2 control
            UniProt name: "Chitinase-like protein"
            EC number: NONE
```

Precedent for Catalytically Inactive GH18 Proteins

The evolution of catalytically inactive GH18 proteins from active chitinases is well-documented across eukaryotes:

- **Mammalian chitinase-like proteins (chilectins):** CHI3L1 (YKL-40), CHI3L2, and MGP-40 are well-characterized GH18 family members that lack chitinase activity but function as lectins, cytokines, or signaling molecules (PMID: 23558346, PMID: 30759297). Bussink et al. documented repeated, independent evolution of catalytically inactive chilectins from active chitinases, with "expansion and selection of chilectins" being "pronounced in mammals."

- **Plant XIP-I:** The xylanase inhibitor protein XIP-I from wheat has a GH18 fold but functions as a xylanase inhibitor rather than a chitinase. Notably, cts2 is classified in CDD subfamily cd02877 (GH18_hevamine_XipI_class_III), which explicitly includes XIP-I.
- **CaclXIP:** A coffee GH18 protein lacking the catalytic glutamate (replaced by glutamine) that has "only residual chitinolytic activity" (PMID: 21299880). This protein was shown to bind chitin efficiently despite lacking catalytic activity, demonstrating that the GH18 fold can retain substrate-binding while losing catalysis.
- **MGP-40:** A mammary gland-specific GH18 protein that binds chitin but shows no chitinase activity even after mutagenic restoration of two critical active-site residues (PMID: 30759297). This demonstrates that catalytic loss in GH18 proteins can be irreversible once compensatory structural changes have accumulated.

The *S. pombe* cts2 protein fits squarely into this pattern of GH18 family members that have been repurposed from catalytic chitinases to non-catalytic roles while retaining the overall protein fold.

Evidence Matrix

Citation	Evidence Type	Direction	Claim Tested	Key Finding	Context	Cor Lim
Sequence alignment (this study)	Computational (active-site residue check)	**Supports** over-annotation	Does cts2 have the DxDxE catalytic motif?	Catalytic proton donor E is replaced by N (asparagine) at position 166	GH18 domain of Q9C105 vs P29029 vs B6JW51	Hig anc con AV
UniProt Q9C105 CAUTION	Database (curated)	**Supports** over-annotation	Is the catalytic Glu conserved?	"Lacks the conserved Glu residue in position 166 essential for chitinase activity"	UniProt expert curation	Hig ind exp ass
PMID: 12079386	Direct assay (mutagenesis)	**Supports** over-annotation	Does Glu→amide mutation abolish activity?	"each mutation effectively abolishes enzyme activity"	*C. immitis* CiX1 chitinase in vitro	Hig me der
PMID: 21299880	Direct assay (recombinant protein)	**Supports** over-annotation	Can GH18 proteins with substituted Glu be inactive?	CaclXIP "lacks the glutamic acid residue essential for catalysis" → "only residual chitinolytic activity"	Coffee XIP; recombinant in *P. pastoris*	Hig dir ana E→ sub
PMID: 30759297	Direct assay (mutagenesis)	**Supports** over-annotation	Can restoring catalytic residues recover activity?	MGP-40 mutants with restored DxDxE-like residues still showed "no chitinase activity"	Mammalian MGP-40 in *E. coli* and COS1 cells	Mo diff pro sho irre
PMID: 31102246	Review	**Qualifies**	*S. pombe* chitinase gene count	"from a single gene in Schizosaccharomyces pombe, to 36 genes in Trichoderma virens"	Comparative fungal genomics	Hig con GH
PMID: 12706511	Direct assay				Cell wall biochemistry	

Citation	Evidence Type	Direction	Claim Tested	Key Finding	Context	Cor Lim
		Supports over-annotation	*S. pombe* chitin content	Chitin presence in *S. pombe* "was not established"		Me neg find
PMID: 2079623	Direct assay	**Qualifies**	Cell wall glucosamine	"only a minute amount of glucosamine could be detected" but may be structurally essential	*S. pombe* cell wall fractionation	Me trac
PMID: 15689498	Mutant phenotype	**Supports** over-annotation	Cell separation mechanism	"the most severe defect is found in eng1Δ agn1Δ cells" (glucanases, not chitinase)	*S. pombe* genetics	Hig fun der
PMID: 19204807	Computational (evolutionary)	**Supports** over-annotation	Chitinase gene family evolution	*S. pombe* shows extreme family contraction (1 gene) under adaptive selection	Comparative genomics	Me evo infe
PMID: 23558346	Computational (evolutionary)	**Qualifies**	GH18 inactive protein evolution	Repeated evolution of catalytically inactive chilectins from active chitinases	Vertebrate GH18 phylogenomics	Me evo pre
S. japonicus ortholog comparison (this study)	Computational (comparative)	**Supports** over-annotation	Does the closest ortholog retain catalytic capacity?	B6JW51 has intact DxDxE, EC 3.2.1.14, IPR001579; Q9C105 has disrupted motif, no EC, lacks IPR001579	Same PANTHER subfamily PTHR45708:SF49	Hig der line spe
AlphaFold pLDDT	Computational (structural)	**Supports** reliability	Is the GH18 domain well-folded?	GH18 domain mean pLDDT 92.7; catalytic	AlphaFold v6 for Q9C105	Hig con stru

Citation	Evidence Type	Direction	Claim Tested	Key Finding	Context	Cor Lim
analysis (this study)		of motif analysis		region all >88.9; N166 at pLDDT 88.9		pre are
PMID: 12092833	Heterologous expression	**Qualifies**	Effect of chitinase in *S. pombe*	Expressing bacterial chitinase ChiA in *S. pombe* causes slow growth and elongated cells	Exogenous *Enterobacter* ChiA	Low — t for chi

GO Curation Implications

Recommended Action: Remove or NOT-qualify GO:0004568

The GO:0004568 (chitinase activity) annotation for Q9C105 should be **removed** or annotated with a **NOT qualifier**, pending curator verification. The evidence strongly indicates this is an over-annotation arising from phylogenetic transfer (IBA) that did not account for the loss of the catalytic proton donor glutamate.

Specific recommendations:

1. **Remove GO:0004568 (chitinase activity) [MF]** — The DxDxE catalytic motif is disrupted (E→N at the proton donor position). UniProt explicitly flags this. No biochemical evidence supports chitinase activity. The IBA evidence code from PAINT phylogenetic transfer does not account for the lineage-specific substitution.
2. **Consider adding NOT GO:0004568** — A NOT-qualified annotation with evidence code IKR (Inferred from Key Residues) or ISM (Inferred from Sequence Model) would actively record that this protein lacks chitinase activity, preventing future re-annotation by automated pipelines.
3. **Consider GO:0008061 (chitin binding) [MF]** — Only if chitin binding is experimentally demonstrated. GH18 proteins can retain chitin-binding activity even when catalytically inactive, as shown for MGP-40 (PMID: 30759297) and CaclXIP (PMID: 21299880). However, given *S. pombe*'s minimal chitin content, chitin binding may not be biologically relevant.

4. **Review dependent BP annotations** — Any biological process annotations that depend on chitinase enzymatic activity (e.g., GO:0006032, chitin catabolic process) should also be reviewed for removal or NOT-qualification.
5. **Retain GO:0005576 (extracellular region) [CC]** — This is independently supported by IDA evidence from PomBase and is not affected by the MF annotation status.

Mechanistic Scope

Direct Molecular Function Being Tested

The hypothesis tests whether the cts2 gene product directly catalyzes the hydrolysis of β-1,4-N-acetylglucosaminide linkages in chitin — the definition of GO:0004568. This requires:

1. **Proton donor (Glu)**: Protonates the glycosidic oxygen of the leaving group
2. **Transition state stabilizer (Asp)**: Electrostatically stabilizes the oxazolinium ion intermediate
3. **Substrate-assisted catalysis**: The N-acetyl group of the −1 subsite sugar participates in forming the oxazolinium intermediate

In cts2, requirement #1 cannot be met because the proton-donor Glu is replaced by Asn (N166).

Distinction from Downstream Effects

The GO annotation question is specifically about chitinase *catalytic activity*, not about: - Family membership in GH18 (which cts2 clearly retains) - Chitin binding (which may be retained; untested) - Cell wall integrity phenotypes (which would be mutant phenotype, not direct activity evidence) - Any other non-catalytic function the protein may have - Carbohydrate metabolic process involvement (which depends on catalytic function)

This distinction is critical because the protein likely retains its GH18 barrel fold and may have a biological function — but that function is almost certainly not chitinase catalysis.

Conflicts and Alternatives

Potential Conflicts with the Over-Annotation Verdict

1. **Residual activity possibility:** CaclXIP from coffee (PMID: 21299880), which has E→Q at the catalytic position, showed "only residual chitinolytic activity." The substitution in cts2 is E→N (asparagine), which is even less capable of proton donation than glutamine, making residual activity less likely. Even if trace activity exists, it would not justify an unqualified GO:0004568 annotation.
2. **Possible compensatory mutations:** In principle, other mutations could partially compensate for the catalytic glutamate loss. However, no such compensatory mechanism has been demonstrated in any GH18 family member, and the cts2 sequence shows no obvious candidate residue. Moreover, the MGP-40 study (PMID: 30759297) demonstrated that even restoring the catalytic residues in an inactive GH18 protein cannot recover activity once structural divergence has accumulated.
3. **PANTHER family classification conflict:** The PANTHER family PTHR45708 classifies cts2 under "ENDOCHITINASE" (subfamily SF49), which directly conflicts with the evidence that the catalytic glutamate is absent. PANTHER classification is based on sequence similarity and phylogenetic placement, not on verification of catalytic residues — this is precisely the source of the IBA over-annotation.

Alternative Interpretations of cts2 Function

- **Chitin-binding lectin:** Like mammalian CHI3L1/YKL-40, cts2 may bind chitin or chitin-like polysaccharides without cleaving them, potentially functioning in cell wall sensing or immune-like recognition.
- **Xylanase inhibitor:** The CDD classification in cd02877 (which includes XIP-I) raises the possibility that cts2 functions as an enzyme inhibitor rather than an enzyme.
- **Structural/signaling role:** The retained GH18 fold may serve as a scaffold for protein–protein or protein–carbohydrate interactions unrelated to catalysis.
- **Pseudoenzyme with no remaining function:** Selection on cts2 may have been relaxed along with the loss of chitin from the *S. pombe* cell wall, leaving the gene as a slowly degenerating relic.

Knowledge Gaps

Gap	What Was Checked	Why It Matters	What Would Resolve It
No direct enzymatic assay of *S. pombe* cts2	PubMed search: no published purification or activity assay found	Definitive proof of catalytic inactivity requires biochemical demonstration	Express and purify recombinant cts2 GH18 domain; assay with colloidal chitin, 4-MU-$(GlcNAc)_2$, or chitooligosaccharides
Unknown biological function of cts2	No functional studies specific to cts2 in *S. pombe* found	If cts2 has neofunctionalized (like XIP-I), the correct GO annotation would be different	Gene deletion studies (cts2Δ); phenotypic analysis under growth, stress, and mating conditions
Chitin-binding ability untested	No binding assays found in literature	If cts2 retains chitin binding, GO:0008061 would be appropriate	Pull-down assay with chitin beads or ITC with chitooligosaccharides
Expression pattern and localization unknown	No localization studies found for cts2 in *S. pombe*	Localization would inform functional role	GFP-cts2 fusion; fluorescence microscopy during vegetative growth and sporulation
S. pombe chitin content remains debated	Sietsma & Wessels (1990) found trace glucosamine; Bahmed et al. (2003) found chitin "not established"	Affects whether any chitinase activity would be biologically relevant	Modern cell wall composition analysis using mass spectrometry or specific enzymatic digestion
AlphaFold active-site geometry not fully compared	pLDDT profiles examined (mean 92.7 for GH18 domain); N166 at pLDDT 88.9; but no full superposition with substrate-bound structures	Could reveal whether the active-site pocket geometry is maintained despite E→N substitution	Superpose AlphaFold model against crystal structures of active GH18 chitinases (e.g., PDB 1LL4) with bound substrate

Discriminating Tests

Priority 1: Direct Chitinase Activity Assay (Highest Impact)

- **Approach:** Express recombinant cts2 GH18 domain (residues 26–325) in *E. coli* or *Pichia pastoris*; assay against 4-MU-chitobioside and colloidal chitin
- **Controls:** *S. japonicus* cts2 (positive); cts2 N166E mutant (restored catalytic Glu); buffer only (negative)
- **Expected result:** No activity or only trace residual activity
- **Discriminates:** Active chitinase vs. inactive chitinase-like protein

Priority 2: Chitin Binding Assay

- **Approach:** Test purified cts2 for binding to insoluble chitin or chitooligosaccharides by pull-down or ITC
- **Expected result:** May retain binding (as seen for MGP-40 and CaclXIP)
- **Discriminates:** GO:0004568 (catalysis) vs. GO:0008061 (binding) vs. neither

Priority 3: cts2Δ Deletion Phenotype

- **Approach:** Construct clean deletion; characterize growth, morphology, cell separation, and stress responses
- **Compare to:** eng1Δ and agn1Δ glucanase mutants
- **Discriminates:** Essential biological function vs. dispensable gene

Priority 4: AlphaFold Active-Site Superposition

- **Approach:** Superpose cts2 AlphaFold model onto *C. immitis* chitinase crystal structure (PDB: 1LL4) or *A. fumigatus* AfChiB1; model substrate binding
- **Discriminates:** Whether the substrate-binding cleft is maintained, collapsed, or repurposed

Priority 5: Phylogenetic Ancestral Sequence Reconstruction

- **Approach:** Determine when the E→N substitution occurred in the *Schizosaccharomyces* lineage
- **Discriminates:** Whether catalytic loss predates or coincides with chitin reduction in the cell wall

Curation Leads

All items below are candidate updates requiring curator verification.

Lead 1: Remove or NOT-qualify GO:0004568

- **Action:** Remove the IBA annotation GO:0004568 from Q9C105, or add a NOT-qualified annotation (NOT GO:0004568) with evidence code IKR (Inferred from Key Residues) or ISM (Inferred from Sequence Model)
- **Rationale:** Catalytic proton-donor Glu is replaced by Asn; UniProt CAUTION explicitly flags this; no EC number assigned; IPR001579 (GH18 active site) absent
- **Key reference:** PMID: 12079386
- **Exact snippet to verify:** "We converted both amino acid residues to the corresponding amide and found that each mutation effectively abolishes enzyme activity"
- **Supporting reference:** PMID: 21299880
- **Exact snippet to verify:** "it lacks the glutamic acid residue essential for catalysis, which is replaced by glutamine. CaclXIP was expressed as a recombinant protein in Pichia pastoris. Enzymatic assay showed that purified recombinant CaclXIP had only residual chitinolytic activity"
- **Confidence:** High

Lead 2: Consider GO:0008061 (Chitin Binding) as Potential Replacement

- **Action:** If experimental evidence for chitin binding becomes available, annotate with GO:0008061
- **Status:** Currently unverifiable — no binding data exists for *S. pombe* cts2
- **Rationale:** Many catalytically inactive GH18 proteins retain chitin-binding ability; the barrel fold appears intact based on AlphaFold (pLDDT 92.7) and domain predictions

Lead 3: Review Propagated BP Annotations

- **Action:** Any biological process annotations that depend on chitinase enzymatic activity (e.g., GO:0006032, chitin catabolic process) should also be reviewed for removal
- **Rationale:** If the MF annotation is incorrect, dependent BP annotations are unsupported

Lead 4: Flag PAINT Pipeline for Active-Site Verification

- **Action:** Report this case to the GO Consortium PAINT curation team as an example where active-site residue verification should be incorporated into phylogenetic transfer
- **Rationale:** The *S. japonicus* vs. *S. pombe* comparison in the same PANTHER subfamily (PTHR45708:SF49) demonstrates the failure mode clearly — both received GO:0004568 IBA, but only *S. japonicus* has the catalytic residues

Lead 5: Candidate Reference for Ortholog Comparison

- **Reference:** UniProt B6JW51 (*S. japonicus* cts2)
- **Key evidence:** EC 3.2.1.14 assigned; IPR001579 present; intact DxDxE motif; named "Chitinase" (vs. "Chitinase-like protein" for Q9C105)
- **Snippet for comparison:** Same PANTHER subfamily PTHR45708:SF49, same CDD cd02877, same InterPro IPR045321 — but only *S. japonicus* retains catalytic capacity

Suggested Questions for Curator

1. Does the GO Consortium have a standard mechanism for NOT-qualifying IBA annotations when key catalytic residues are absent?
2. Should an IKR (Inferred from Key Residues) NOT annotation be created, and if so, what is the appropriate reference?
3. Are there other PAINT-propagated annotations for GH18 family members that should be similarly reviewed?
4. Should the protein name in PomBase explicitly reflect the "Chitinase-like" designation used by UniProt?

Provenance

All computational analyses were performed programmatically and are recorded as provenance:

- **Sequence alignment:** Anchored at conserved AVVDGFD motif across 4 species (*S. cerevisiae* CTS1, *S. japonicus* cts2, *Hevea* hevamine-A, *S. pombe* cts2)
- **UniProt API queries:** Q9C105, P29029, B6JW51 entries for domain, feature, and GO annotations

- **AlphaFold pLDDT extraction:** AF-Q9C105-F1 model v6, B-factor column parsing; GH18 domain mean pLDDT 92.7, catalytic region (155–175) all >88.9, N166 at 88.9
- **QuickGO API:** Annotation provenance for GO:0004568 on Q9C105 — IBA, GO_REF:0000033, PANTHER nodes PTN005237305/PTN008696177
- **InterPro API:** IPR045321 family description including XIP-I inactive members; IPR001579 presence/absence comparison
- **CDD:** cd02877 (GH18_hevamine_XipI_class_III) classification with known inactive members
- **PubMed literature:** 35+ papers reviewed across chitinase biochemistry, GH18 mechanism, *S. pombe* cell biology, and chitinase-like protein evolution

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