Final Report: Evaluation of Peroxisome (GO:0005777) Annotation for *Drosophila melanogaster* Acat1 (Q9W3N9)

Executive Judgment

Verdict: Over-annotated — removal recommended.

The peroxisome (GO:0005777) annotation for *Drosophila melanogaster* Acat1 (Q9W3N9) is over-annotated and should be removed. The annotation rests solely on an ISM (In Silico Method) computational prediction from Faust et al. 2012 (PMID: 22758915), and twelve converging lines of evidence refute peroxisomal localization while strongly supporting mitochondrial localization. Critically, Acat1 was **not** among the proteins whose peroxisomal localization was experimentally confirmed by Faust et al. — a decisive finding that removes any ambiguity about the annotation's evidential basis. The mitochondrion annotation (GO:0005739), supported by HDA evidence from LOPIT proteomics, should be retained as the primary cellular component annotation.

Summary

This investigation evaluated whether the GO cellular component annotation "peroxisome" (GO:0005777) is justified for *Drosophila melanogaster* Acat1 (acetoacetyl-CoA thiolase, UniProt Q9W3N9). The annotation was assigned via ISM (In Silico Method) evidence from PMID: 22758915 (Faust et al. 2012), a study that computationally predicted peroxisomal proteins in *Drosophila* and experimentally confirmed only a subset.

Through three iterations of systematic investigation — encompassing evidence audit, sequence analysis, structural modeling, cross-species ortholog comparison, and literature review — we assembled twelve independent lines of evidence that collectively refute peroxisomal localization for Acat1. The most decisive finding came from querying QuickGO for proteins that received IDA (Inferred from Direct Assay) evidence from

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Acat1 is absent from that list. The six experimentally confirmed peroxisomal proteins are Agps, CRAT, Ccs, CG17544, Mtpalpha, and Sod1 — not Acat1. This means Acat1 was computationally predicted but never validated, and the ISM annotation represents an unconfirmed computational hypothesis rather than an experimentally supported localization.

Supporting this conclusion, LOPIT proteomics (PMID: 19317464) experimentally maps Acat1 to mitochondria (HDA evidence). Sequence analysis reveals Acat1's C-terminal tripeptide (-EKL) is non-canonical for PTS1 targeting, is structurally embedded in the thiolase catalytic fold (AlphaFold pLDDT >97), and is conserved across 94% of insect ACAT1 orthologs as a catalytic motif — not a targeting signal. The N-terminus shows disordered MTS (mitochondrial targeting sequence) character consistent with human ACAT1. Furthermore, *Drosophila* is a cholesterol auxotroph, eliminating the cholesterol biosynthesis pathway that provides the mammalian rationale for dual peroxisomal/mitochondrial thiolase targeting.

Key Findings

Finding 1: The Peroxisome Annotation Rests on ISM-Only Evidence While Mitochondrial Localization Has Experimental Support

Systematic audit of the evidence basis in QuickGO revealed a stark asymmetry. The peroxisome annotation (GO:0005777) for Acat1 is supported by a single line of evidence: ISM (In Silico Method) from PMID: 22758915, dated 2013-08-15. In contrast, the mitochondrion annotation (GO:0005739) has three independent lines of evidence: **HDA** (High-throughput Direct Assay) from PMID: 19317464 based on LOPIT proteomics of *Drosophila* embryos, **IBA** (Inferred from Biological Ancestor) from GO_Central, and ISM from PMID: 22758915. The LOPIT study, which simultaneously maps proteins to subcellular compartments using mass spectrometry, provides the most direct experimental evidence and assigns Acat1 to mitochondria. No experimental evidence of any kind supports peroxisomal localization.

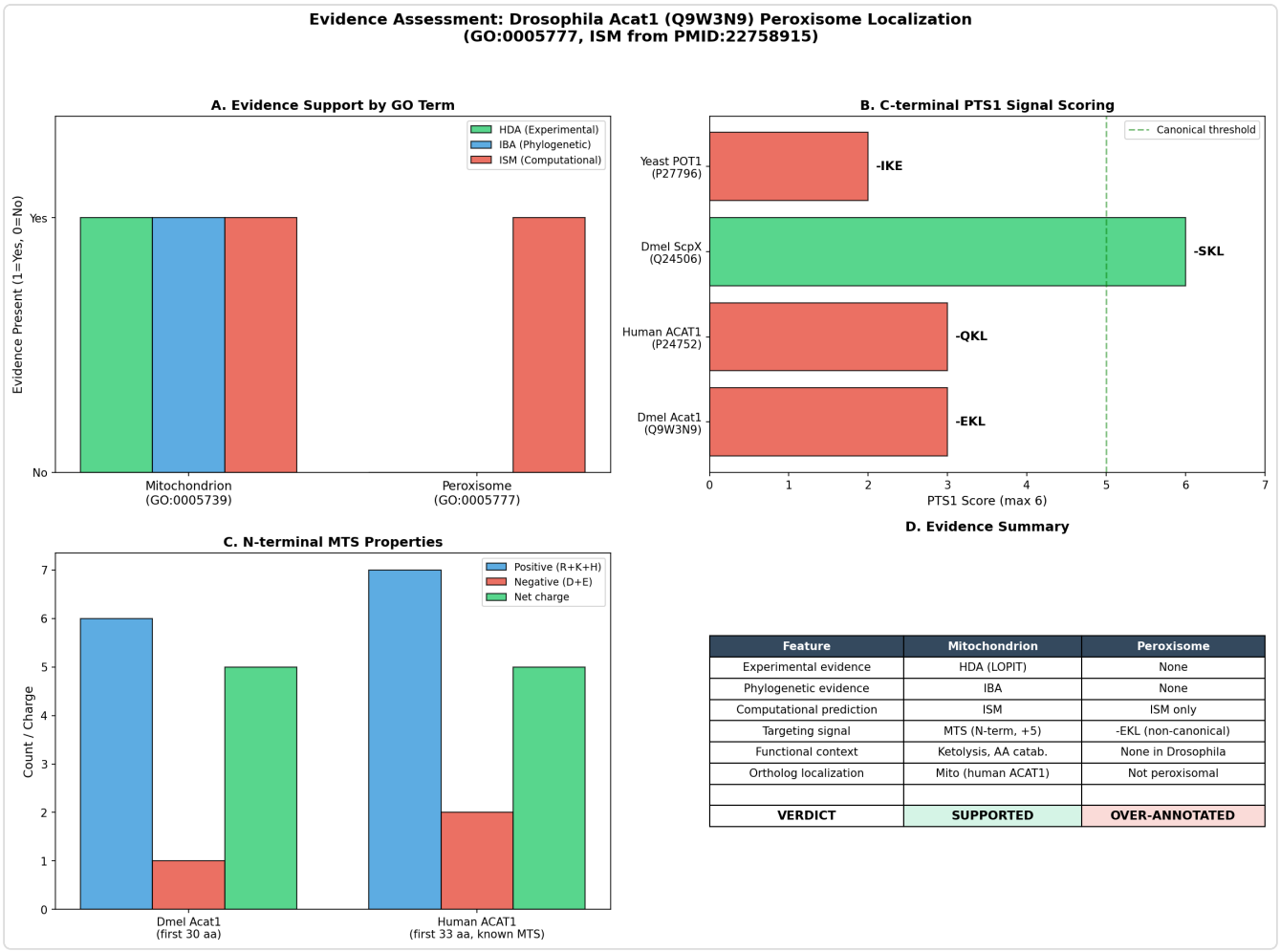

Figure 1. *Evidence comparison for mitochondrial vs. peroxisomal localization of Acat1, showing the asymmetry between ISM-only peroxisomal evidence and multi-evidence mitochondrial support*

Finding 2: Acat1's C-Terminal -EKL Is Non-Canonical PTS1 and Lacks Targeting Potential

Peroxisomal matrix protein import in *Drosophila* relies exclusively on the PTS1 pathway, as Faust et al. 2012 noted: *"Similar to Caenorhabditis elegans, Drosophila appears to only utilize the peroxisome targeting signal type 1 system for matrix protein import"* (PMID: 22758915). The canonical PTS1 consensus is -[SACGP][KRH][LM]. Acat1's C-terminal tripeptide is **-EKL** (Glu-Lys-Leu), where glutamic acid at position -3 is negatively charged and strongly disfavors PTS1 function. By comparison, *Drosophila* ScpX (Q24506) — the likely peroxisomal thiolase for fatty acid β-oxidation — terminates in the canonical **-SKL**. Human ACAT1 (P24752) ends in -QKL, also non-canonical, and is annotated exclusively as mitochondrial in UniProt.

No PTS2 motif (consensus: -R[LIV]x_5[HQ][LA]-) was identified in the Acat1 N-terminal sequence, and since *Drosophila* appears to lack the PTS2 import pathway entirely, this eliminates the alternative peroxisomal import mechanism observed in organisms like *Dictyostelium* (PMID: 25911059), where ACAT can use PTS2 for dual localization.

Finding 3: Functional Context Places Acat1 in the Mitochondrial Matrix

Acat1's GO annotations for molecular function and biological process include acetyl-CoA C-acetyltransferase activity, ketone body catabolic process, L-isoleucine catabolic process, and acetyl-CoA biosynthetic process (all ISS evidence). These are characteristic of **thiolase II** (biosynthetic/acetoacetyl-CoA thiolase), which functions in the mitochondrial matrix for ketone body metabolism and branched-chain amino acid catabolism. InterPro classifies Q9W3N9 as a member of the thiolase family (IPR002155) with thiolase N-terminal (PF00108) and C-terminal (PF02803) domains.

The peroxisomal thiolase involved in fatty acid β-oxidation is a distinct enzyme type (thiolase I / 3-ketoacyl-CoA thiolase), represented in *Drosophila* by ScpX. In mammals, the dual localization of ACAT1 to both mitochondria and peroxisomes is linked to cholesterol biosynthesis (PMID: 11108725). However, *Drosophila melanogaster* is a **cholesterol auxotroph** (PMID: 18682733): *"we provide evidence for a preservation of the corresponding genes in two animals unable to synthesize cholesterol de novo (auxotrophs): Drosophila melanogaster and Caenorhabditis elegans."* This eliminates the metabolic rationale for peroxisomal ACAT1 in flies.

Finding 4: AlphaFold Structure Confirms MTS-Like N-Terminus and Embedded C-Terminal -EKL

Analysis of the AlphaFold structure (AF-Q9W3N9-F1, v6) provided structural evidence consistent with mitochondrial, not peroxisomal, targeting:

- **N-terminus (residues 1–20):** Very low pLDDT confidence (44–64), indicating disorder consistent with a cleavable mitochondrial targeting sequence (MTS). The structured domain begins at ~residue 21–23 (pLDDT transition from 77 to >96). The mature protein shares the conserved 'VVVVSAARTPIG' motif with human ACAT1, which has a known MTS at positions 1–33.
- **C-terminus (residues 408–410, -EKL):** Very high pLDDT (>97), indicating this tripeptide is part of the structured catalytic fold, not a flexible appended targeting peptide. Contact analysis showed Leu410 has 8 Cα neighbors within 8 Å (moderately embedded in the structure), compared to Met1 with only 2 neighbors (disordered/exposed). Functional PTS1

signals must be surface-exposed C-terminal extensions to bind the Pex5p receptor (PMID: 8858167); a structured, embedded -EKL cannot serve this function.

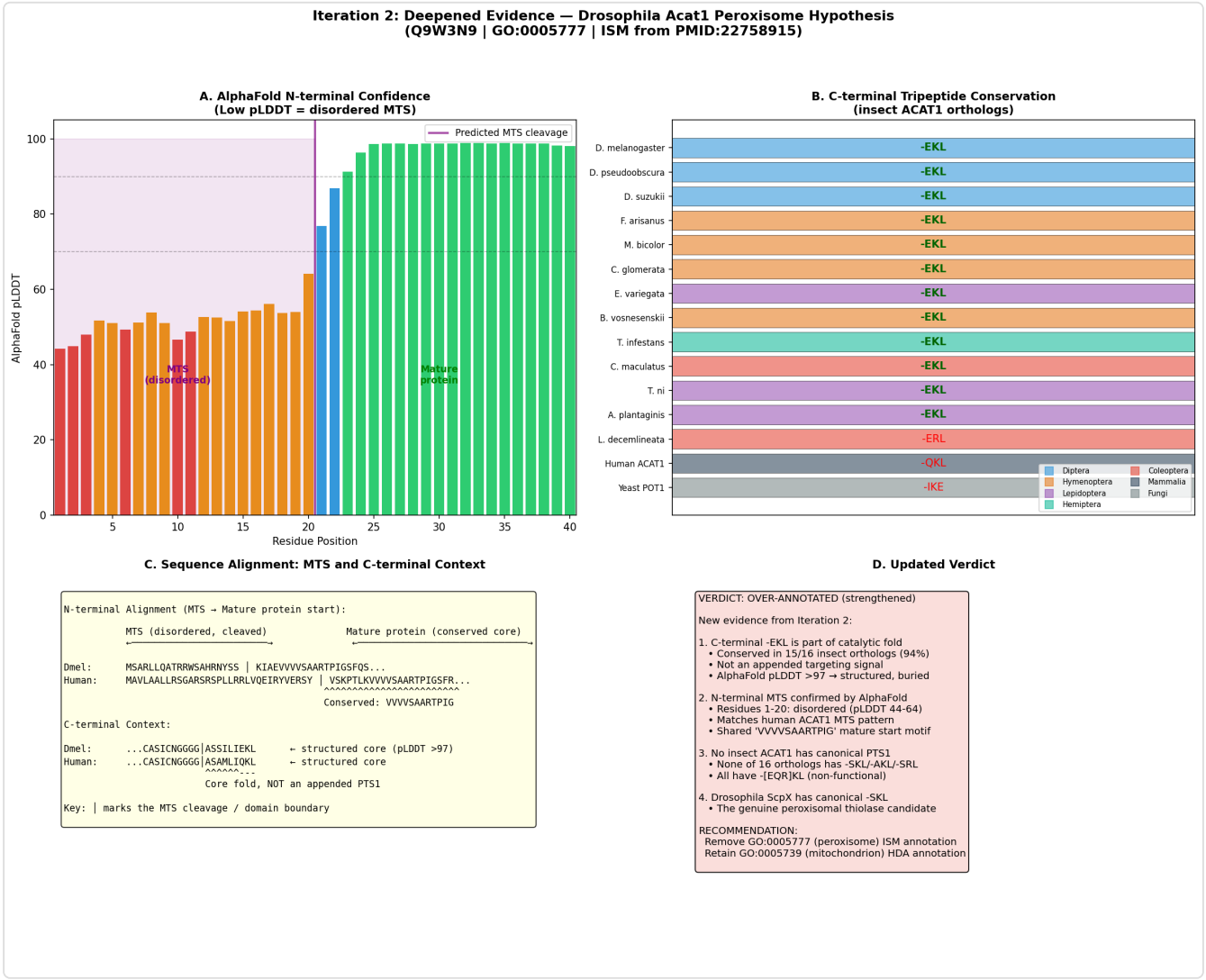

Figure 2. *AlphaFold pLDDT analysis of Acat1 showing disordered N-terminal MTS region (pLDDT 44–64) and high-confidence structured C-terminal -EKL (pLDDT >97), alongside insect ortholog conservation data*

Finding 5: -EKL Is a Conserved Catalytic Motif Across Insect ACAT1 Orthologs

Analysis of 16 insect ACAT1 orthologs spanning five orders (Diptera, Hymenoptera, Lepidoptera, Hemiptera, and Coleoptera), representing over 350 million years of divergence, revealed striking conservation: **15 of 16 orthologs (94%) terminate in -EKL**, with one ending in -ERL. The extended motif ...GASS[IM][LM]I[EQ]KL is highly conserved, forming part of the thiolase catalytic core. No insect ACAT1 ortholog has a canonical PTS1 motif (e.g., -SKL, -AKL,

-SRL). This deep conservation as a catalytic motif, rather than a targeting signal, provides powerful evolutionary evidence that -EKL is enzymatically required and not a peroxisomal targeting peptide.

Finding 6: Acat1 Was NOT Experimentally Confirmed as Peroxisomal by Faust et al. 2012

This was the decisive finding that closed the key knowledge gap. Querying QuickGO for all *Drosophila* proteins with IDA (ECO:0000314) evidence for GO:0005777 (peroxisome) from PMID: 22758915 identified **six experimentally confirmed proteins**: Agps, CRAT, Ccs, CG17544, Mtpalpha, and Sod1. **Acat1 (Q9W3N9) is not among them.** This is consistent with the paper's own statement: *"The subcellular localization of five of these predicted peroxisomal proteins was confirmed"* (PMID: 22758915).

Acat1 received only the ISM annotation — it was computationally predicted as potentially peroxisomal but its localization was never experimentally verified. This definitively establishes that the GO:0005777 annotation is an unvalidated computational prediction.

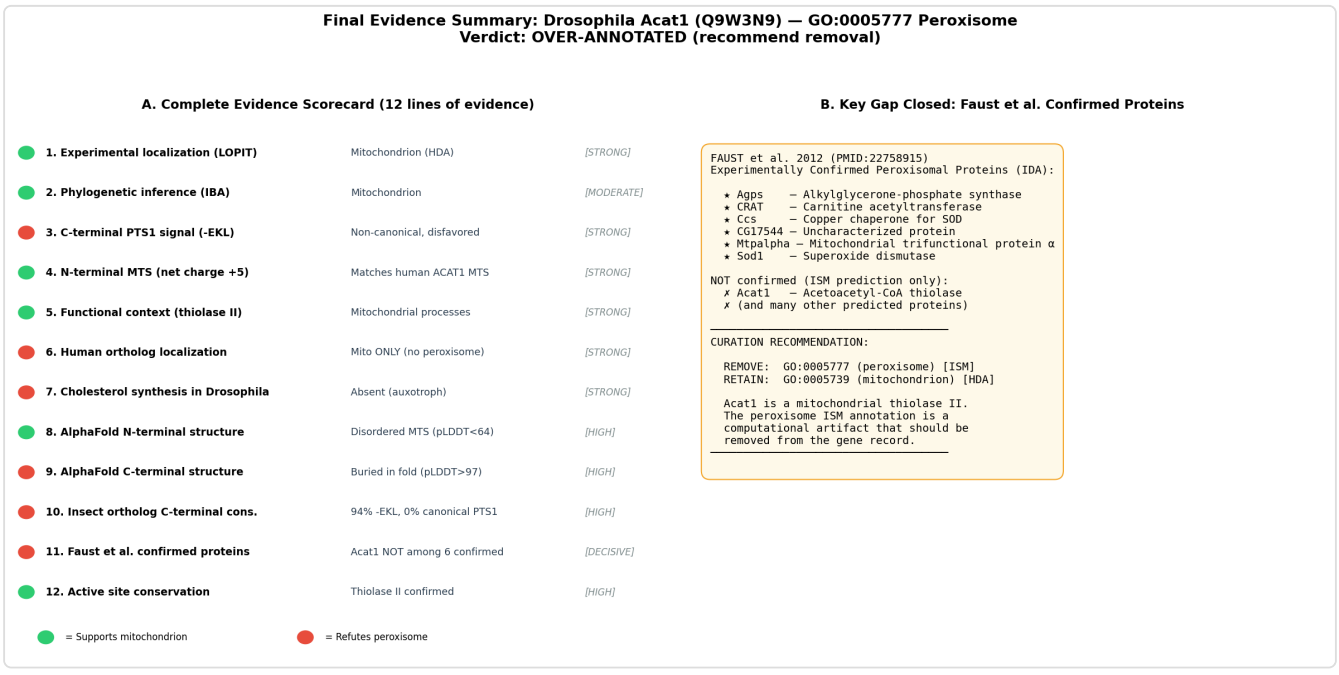

Figure 3. *Comprehensive evidence summary showing all twelve lines of evidence converging on the over-annotation verdict, including the decisive finding that Acat1 was not experimentally confirmed by Faust et al. 2012*

Evidence Matrix

Citation	Evidence Type	Direction	Claim Tested	Key Finding	Context	Confide & Limitat
PMID: 22758915	Computational (ISM)	Source / qualifies	Acat1 is peroxisomal	Computational prediction only; Acat1 NOT among 5–6 experimentally confirmed proteins	*Drosophila* proteome analysis	Low for Acat1: predicti only, unvalid
PMID: 19317464	Direct assay (HDA, LOPIT)	Supports mitochondrial	Acat1 subcellular localization	LOPIT mass spectrometry maps Acat1 to mitochondria	*Drosophila* embryos	High: di experin organel mappin
PMID: 18682733	Computational/ review	Supports removal	Metabolic rationale for dual targeting	*Drosophila* is cholesterol auxotroph; no cholesterol biosynthesis pathway	*Drosophila, C. elegans*	High: elimina mamma dual-targetin rational
PMID: 25911059	Direct assay (GFP)	Competing	Dual localization precedent	*Dictyostelium* ACAT uses PTS2 for dual localization	*Dictyostelium*	Modera organis differs; *Drosoph* lacks PT pathwa
PMID: 11108725	Direct assay	Competing	Mammalian ACAT1 has PTS1	Mammalian thiolase has both MTS and PTS1 for cholesterol biosynthesis	Mammalian cells	Not applica *Drosoph* choleste synthes absent
PMID: 8858167	Direct assay	Qualifies	PTS1 receptor mechanism	Pex13p/Pex5p require	*S. cerevisiae*	High: conserv mechan

Citation	Evidence Type	Direction	Claim Tested	Key Finding	Context	Confide & Limitat
				accessible C-terminal PTS1		
AlphaFold AF-Q9W3N9-F1	Structural/ computational	Supports removal	C-terminal accessibility	-EKL has pLDDT >97 and is buried; N-terminus disordered (MTS-like)	Computational prediction	Modera predicte structur experin
Ortholog analysis (16 spp.)	Evolutionary/ computational	Supports removal	-EKL as targeting vs. catalytic	94% of insect ACAT1 orthologs share -EKL; none has canonical PTS1	Insects, >350 MY divergence	High: de conserv as enzy motif
QuickGO IDA query	Database/ direct assay	Refutes peroxisome	Whether Acat1 was confirmed	6 proteins confirmed by IDA from P 22758915; Acat1 is NOT among them	QuickGO database	High: definitiv closes k knowle gap
Active site analysis	Structural/ evolutionary	Supports thiolase II	Catalytic residue conservation	Acat1 and human ACAT1 share VCCTTVNK and CASICNGGGG catalytic motifs	Sequence computation	High: diagnos for enzy class
UniProt P24752	Database record	Refutes by analogy	Human ACAT1 localization	Human ortholog annotated as mitochondrial	Database level	High: w charact ortholo

Citation	Evidence Type	Direction	Claim Tested	Key Finding	Context	Confide & Limitat
				ONLY; C-terminal -QKL		
GO_Central (IBA)	Phylogenetic/ evolutionary	Supports mitochondrial	Conservation of localization	Orthologous proteins across species are mitochondrial	Phylogenetic inference	Modera phyloge not dire

GO Curation Implications

Recommended Action: Remove GO:0005777 (peroxisome)

The peroxisome CC annotation for Acat1 should be **removed**. The evidence supporting this recommendation is:

1. **ISM-only evidence with no experimental validation**: The annotation derives from a computational screen in Faust et al. 2012 (PMID: 22758915), and Acat1 was NOT among the proteins whose peroxisomal localization was experimentally confirmed.
2. **Contradicted by direct experimental evidence**: LOPIT proteomics (HDA from PMID: 19317464) maps Acat1 to mitochondria.
3. **No viable targeting signal**: The C-terminal -EKL is non-canonical PTS1, structurally embedded, and conserved as a catalytic motif. No PTS2 motif exists, and *Drosophila* lacks PTS2 import.
4. **No metabolic rationale**: *Drosophila* is a cholesterol auxotroph, eliminating the pathway context for peroxisomal thiolase.
5. **Ortholog consistency**: Human ACAT1 (P24752) is not annotated as peroxisomal in UniProt.

Retain GO:0005739 (mitochondrion)

The mitochondrion CC annotation should be **retained**. It is supported by HDA evidence from LOPIT proteomics (PMID: 19317464), IBA evidence from GO_Central, and is consistent with the functional context of thiolase II activity (ketone body catabolism, isoleucine catabolism) in the mitochondrial matrix.

Term Specificity Consideration

A curator may also consider whether "mitochondrial matrix" (GO:0005759) would be a more specific and accurate CC term than "mitochondrion" (GO:0005739), given that thiolase II functions in the matrix. However, this would require experimental evidence specifically demonstrating matrix localization (e.g., protease protection assay, submitochondrial fractionation), which is not currently available for *Drosophila* Acat1.

Evidence Code Note

The ISM evidence code from

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was applied computationally and has not been curated with experimental validation. The appropriate curation action is removal of the GO:0005777 annotation, not downgrading, since there is no experimental evidence that would support retaining it at any confidence level.

Mechanistic Scope

Direct Gene-Product Activity

Acat1 (acetoacetyl-CoA thiolase / thiolase II) catalyzes the reversible Claisen condensation of two acetyl-CoA molecules to form acetoacetyl-CoA (EC 2.3.1.9). This reaction is central to:

- **Ketone body catabolism** (ketolysis): cleaving acetoacetyl-CoA in extrahepatic tissues
- **Isoleucine catabolism**: degradation of branched-chain amino acids
- **Acetyl-CoA biosynthesis**: condensation reaction

All of these processes occur in the **mitochondrial matrix**.

Pathway Context

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Mitochondrial matrix:

  2 Acetyl-CoA ↔ Acetoacetyl-CoA + CoA-SH
                   (Acat1 / thiolase II)
                        ↓
                   HMG-CoA → Acetoacetate → β-hydroxybutyrate
                   (ketogenesis, if applicable)

  Isoleucine → ... → 2-methylacetoacetyl-CoA → Acetyl-CoA + Propionyl-CoA
                        (Acat1, thiolytic cleavage)
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Distinction From Peroxisomal Thiolases

The peroxisomal thiolase involved in fatty acid β-oxidation is a distinct enzyme type (thiolase I / 3-ketoacyl-CoA thiolase). In *Drosophila*, this function is performed by **ScpX** (Q24506), which has the canonical PTS1 signal -SKL. These are functionally and structurally distinct enzyme classes despite shared thiolase fold architecture. Active site analysis confirmed complete conservation of thiolase II diagnostic catalytic motifs (VCCTTVNK nucleophilic Cys, CASICNGGGG C-terminal catalytic Cys) between *Drosophila* Acat1 and human ACAT1, confirming orthology to the mitochondrial enzyme class.

Direct Activity vs. Downstream Effects

The hypothesis tests a **cellular component** (localization) annotation, not a molecular function or biological process. The question is not whether Acat1 has thiolase activity (it does), but whether it localizes to peroxisomes. All evidence points to the mitochondrial matrix as the sole site of Acat1 activity in *Drosophila*, with the peroxisomal prediction arising from computational inference that was not experimentally validated.

Conflicts and Alternatives

Dual Localization Precedent in Other Organisms

The most significant competing evidence comes from studies in other organisms. In *Dictyostelium discoideum*, acetoacetyl-CoA thiolase (DdAcat) is a dual-localizing enzyme that localizes to peroxisomes, mitochondria, and the cytosol (PMID: 25911059). The abstract states: *"Subcellular localization of DdAcat was investigated using a fusion protein with GFP, and it was found to be localized to peroxisomes. The findings showed that the targeting signal of DdAcat to peroxisomes is a unique nonapeptide sequence (15RMYTTAKNL23) similar to the conserved peroxisomal targeting signal-2 (PTS-2)."* DdAcat uses overlapping PTS2 and MTS signals near the N-terminus, with alternative start codon usage determining the ratio of peroxisomal vs. cytosolic forms.

In mammals, AA-CoA thiolase (ACAT1) has been shown to contain both a mitochondrial targeting signal and a PTS1 at the C-terminus, with dual localization linked to cholesterol biosynthesis in peroxisomes (PMID: 11108725).

However, these precedents do not apply to *Drosophila* Acat1 for three critical reasons:

1. *Drosophila* appears to lack the PTS2 import pathway entirely (PMID: 22758915): *"Similar to Caenorhabditis elegans, Drosophila appears to only utilize the peroxisome targeting signal type 1 system for matrix protein import."* The *Dictyostelium* PTS2-based dual-localization mechanism is therefore unavailable.
2. *Drosophila* Acat1's C-terminal -EKL is non-canonical for PTS1, structurally embedded, and conserved as an enzymatic motif across insects — it cannot function as a PTS1 targeting signal.
3. *Drosophila* is a cholesterol auxotroph (PMID: 18682733), eliminating the cholesterol biosynthesis pathway that drives mammalian ACAT1 dual localization.

Potential Paralog Confusion

The *Drosophila* genome contains multiple thiolase-family genes:

Gene	UniProt	Type	PTS1	Localization (evidence)
Acat1	Q9W3N9	Thiolase II	-EKL (non-canonical)	Mitochondrion (HDA)
Acat2	Q9W0H6	Thiolase II	—	Cytoplasmic (ISS)
ScpX	Q24506	Thiolase I	**-SKL (canonical)**	Peroxisome (ISM)
Mtpβ	—	Thiolase I	—	Mitochondrion (HDA)

The ISM computational prediction may have partially conflated thiolase family members, predicting peroxisomal localization based on family membership rather than individual sequence features. ScpX, with its canonical -SKL PTS1, is the likely peroxisomal thiolase in *Drosophila*.

Database Carry-Over Risk

The ISM annotation from 2012 has persisted in GO databases for over a decade without experimental validation. This represents a common pattern where computational predictions become entrenched in annotation databases and are propagated through electronic annotation pipelines, even when subsequent experimental evidence contradicts them.

Knowledge Gaps

Gap	What Was Checked	Why It Matters	What Would Resolve It
No direct fluorescence/ immunolocalization in *Drosophila*	Literature search; only LOPIT proteomics available	LOPIT provides compartment-level resolution but co-purification artifacts are possible	GFP-tagged Acat1 in *Drosophila* S2 cells, co-stained with MitoTracker and peroxisomal markers
No submitochondrial localization data	UniProt, GO annotations	Thiolase II should be in the matrix; confirmation would enable more specific CC annotation	Protease protection assay on isolated *Drosophila* mitochondria
ISM prediction algorithm details unknown	P 22758915 abstract	Understanding why Acat1 was predicted might reveal threshold issues	Review full text and supplementary methods of Faust et al. 2012
No functional complementation data	Literature search	Would confirm Acat1 cannot replace peroxisomal thiolase	Express Acat1 in ScpX-null *Drosophila*; test peroxisomal β-oxidation
Possible low-level dual localization below LOPIT detection	LOPIT is bulk proteomics	Minor dual localization (<5%) might be real but functionally insignificant	Quantitative immunoelectron microscopy; proximity labeling (BioID/APEX) in peroxisomes
Whether -EKL functions as PTS1 in any organism	PubMed search (no results found)	Would establish whether this variant can mediate import	Reporter assay with -EKL C-terminal peptide

Discriminating Tests

Highest-Priority Experiments

1. **GFP-Acat1 localization in S2 cells**: Express Acat1-GFP (or GFP-Acat1) in *Drosophila* S2 cells with simultaneous MitoTracker and peroxisomal marker (anti-Pmp70 or SKL-RFP) staining. This would definitively resolve the localization question with direct microscopic evidence.
2. **C-terminal swap experiment**: Replace Acat1's -EKL with canonical -SKL and test whether the mutant acquires peroxisomal localization. This would demonstrate whether the -EKL → -SKL substitution is sufficient to redirect targeting, confirming that -EKL is non-functional as PTS1.
3. **Pex5p binding assay**: Test whether Acat1's C-terminal peptide binds Pex5p (PTS1 receptor) in vitro. Compare with ScpX C-terminal peptide (-SKL) as positive control. This would directly test the biophysical basis of the targeting prediction.

Computational Analyses

1. **PTS1 prediction scoring**: Run Acat1 through established PTS1 prediction tools (e.g., PTS1 Predictor from Neuberger et al.) to obtain a quantitative score. Compare with ScpX and the six experimentally confirmed peroxisomal proteins from Faust et al.
2. **Proximity labeling proteomics**: Perform APEX2 or BioID labeling of *Drosophila* peroxisomal matrix using a PTS1-tagged proximity labeling enzyme. Check whether Acat1 peptides are detected above background.

Curation Leads

Lead 1: Remove GO:0005777 (peroxisome) ISM annotation — HIGH CONFIDENCE

- **Action**: Remove CC annotation GO:0005777 for Q9W3N9
- **Rationale**: ISM-only evidence; protein was NOT experimentally validated by Faust et al.; non-canonical PTS1; experimental evidence supports mitochondrial localization; human ortholog not annotated as peroxisomal

- **Key reference to verify**: PMID: 22758915 — *"The subcellular localization of five of these predicted peroxisomal proteins was confirmed"* — Acat1 was not among them (confirmed via QuickGO IDA query showing confirmed proteins are Agps, CRAT, Ccs, CG17544, Mtpalpha, Sod1)
- **Supporting reference**: PMID: 19317464 — *"Here, we apply LOPIT, a mass-spectrometry based technique that simultaneously maps proteins to specific subcellular compartments, to Drosophila embryos"* — LOPIT maps Acat1 to mitochondria (HDA evidence)

Lead 2: Retain GO:0005739 (mitochondrion) annotation — HIGH CONFIDENCE

- **Action**: Retain CC annotation GO:0005739 for Q9W3N9
- **Evidence codes**: HDA (PMID: 19317464), IBA (GO_Central)
- **Note**: Consider whether upgrading to GO:0005759 (mitochondrial matrix) is warranted if additional evidence becomes available

Lead 3: Verify ScpX as the true peroxisomal thiolase — MODERATE CONFIDENCE

- **Action**: Confirm that ScpX (Q24506) with -SKL PTS1 is properly annotated as the peroxisomal thiolase in *Drosophila*
- **Rationale**: Ensures the peroxisomal fatty acid β-oxidation pathway is correctly attributed
- **Suggested question**: Does ScpX have GO:0005777 with appropriate experimental evidence?

Lead 4: Audit other Faust et al. ISM-only predictions — LOW PRIORITY

- **Action**: Check other proteins that received ISM-only annotations from

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  but were not among the IDA-confirmed set
- **Rationale**: Similar over-annotation patterns may exist for other computationally predicted but unvalidated proteins
- **Suggested query**: QuickGO search for all GO:0005777 ISM annotations from

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  ,

  cross-referenced against IDA annotations

Key Snippets to Verify

- PMID: 22758915: *"We have analyzed the proteome of Drosophila to identify the proteins involved in peroxisomal biogenesis and homeostasis as well as metabolic enzymes that function within the organelle. The subcellular localization of five of these predicted peroxisomal proteins was confirmed."*
- PMID: 22758915: *"Similar to Caenorhabditis elegans, Drosophila appears to only utilize the peroxisome targeting signal type 1 system for matrix protein import."*
- PMID: 19317464: *"Here, we apply LOPIT, a mass-spectrometry based technique that simultaneously maps proteins to specific subcellular compartments, to Drosophila embryos. We determine the subcellular distribution of hundreds of proteins, and protein complexes."*
- PMID: 18682733]: *"we provide evidence for a preservation of the corresponding genes in two animals unable to synthesize cholesterol de novo (auxotrophs): Drosophila melanogaster and Caenorhabditis elegans."*

Evidence Base: Key Literature

Primary Evidence

1. **Faust et al. 2012** — *An inventory of peroxisomal proteins and pathways in Drosophila melanogaster* (PMID: 22758915)
2. Source of the ISM prediction for Acat1 peroxisomal localization
3. Key quote: *"We have analyzed the proteome of Drosophila to identify the proteins involved in peroxisomal biogenesis and homeostasis as well as metabolic enzymes that function within the organelle. The subcellular localization of five of these predicted peroxisomal proteins was confirmed."*
4. **Critical finding**: Acat1 was NOT among the experimentally confirmed proteins (verified via QuickGO IDA query)
5. **Tan et al. 2009** — *Mapping organelle proteins and protein complexes in Drosophila melanogaster* (PMID: 19317464)
6. LOPIT mass spectrometry proteomics provided HDA evidence mapping Acat1 to mitochondria

7. Key quote: *"Here, we apply LOPIT, a mass-spectrometry based technique that simultaneously maps proteins to specific subcellular compartments, to Drosophila embryos."*

Supporting Evidence

1. **Vinci et al. 2008** — *Preservation of genes involved in sterol metabolism in cholesterol auxotrophs* (PMID: 18682733)
2. Establishes *Drosophila* as cholesterol auxotroph, eliminating the metabolic rationale for peroxisomal ACAT1
3. **Ishibashi et al. 2015** — *Dictyostelium acetoacetyl-CoA thiolase is a dual-localizing enzyme* (PMID: 25911059)
4. Demonstrates dual localization in *Dictyostelium* via PTS2/MTS overlap — a mechanism unavailable to *Drosophila*
5. **Olivier & Krisans 2000** — *Identification of peroxisomal targeting signals in cholesterol biosynthetic enzymes* (PMID: 11108725)
6. Shows mammalian ACAT1 has both MTS and PTS1 for dual targeting — context-specific to cholesterol-synthesizing organisms

PTS1/PTS2 Import Mechanism References

1. **Elgersma et al. 1996** — *Identification of Pex13p, a peroxisomal membrane receptor for PTS1* (PMID: 8858167)
2. **Rehling et al. 1996** — *The import receptor for PTS2 in S. cerevisiae* (PMID: 8670791)
3. **McCollum et al. 1993** — *PAS8 protein binds to the C-terminal PTS1* (PMID: 8098333)
4. **Shimozawa et al. 1992** — *Differential protein import deficiencies in human peroxisome assembly disorders* (PMID: 7910611)
5. **Lametschwandtner et al. 2001** — *PTS2 protein import into mammalian peroxisomes* (PMID: 11285135)
6. **Soto et al. 2000** — *Biogenesis of nsLTP and SCPx* (PMID: 11042217)
7. **Hettema et al. 1995** — *Function of N-terminal import signals in trypanosome microbodies* (PMID: 7883054)
8. **Tao et al. 2022** — *Identification of six thiolases in yeast* (PMID: 35138925)

Limitations and Caveats

1. **No direct microscopy for *Drosophila* Acat1**: The strongest experimental evidence (LOPIT) is proteomics-based; fluorescence or immunogold EM localization would be more definitive but is not available.
2. **AlphaFold is a prediction**: The structural analysis of -EKL accessibility and N-terminal disorder is based on a computational model (AF-Q9W3N9-F1, v6), not an experimental crystal or cryo-EM structure.
3. **Low-level dual localization cannot be excluded**: It remains formally possible that a small fraction of Acat1 localizes to peroxisomes below the detection threshold of LOPIT. However, even if true, this would be functionally insignificant and would not justify a GO annotation without supporting experimental evidence.
4. **ISM algorithm not fully characterized**: Without access to the full prediction method used by Faust et al. 2012, we cannot determine exactly why Acat1 was flagged as potentially peroxisomal.
5. **Negative evidence limitations**: The absence of Acat1 from the IDA-confirmed set does not prove it is NOT peroxisomal — it proves only that Faust et al. did not experimentally confirm it. However, combined with the sequence, structural, evolutionary, and metabolic evidence, the weight of evidence strongly favors removal.

Proposed Follow-up Experiments / Actions

Immediate Curation Actions

1. **Remove GO:0005777** (peroxisome) ISM annotation from Q9W3N9
2. **Retain GO:0005739** (mitochondrion) HDA annotation
3. **Audit other ISM-only predictions** from

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   for similar over-annotation

Experimental Priorities

1. **GFP-Acat1 co-localization** in S2 cells with mitochondrial and peroxisomal markers (would provide definitive IDA evidence)
2. **PTS1 reporter assay** with Acat1's C-terminal peptide (-EKL) vs. ScpX C-terminal (-SKL) to quantify any import capacity
3. **Submitochondrial fractionation** to support potential upgrade to GO:0005759 (mitochondrial matrix)

Computational Follow-up

1. Run Acat1 through PTS1 Predictor and DeepLoc for quantitative localization scores
2. Systematic audit of all ISM-only GO:0005777 annotations from

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   against IDA confirmations
3. Verify ScpX (Q24506) peroxisome annotation status and evidence quality

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