

Spatiotemporal proteomic remodeling of lipid droplets drives astaxanthin accumulation in *H. pluvialis*

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Research Article

Keywords: Haematococcus pluvialis, Lipid droplets, Spatiotemporal proteomics, Metabolic remodeling, Astaxanthin accumulation, Organellar interaction

Posted Date: July 28th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9698299/v1>

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Additional Declarations: No competing interests reported.

Abstract The green alga *Haematococcus pluvialis* is of great economic importance in astaxanthin hyperaccumulation, yet the dynamic remodeling of lipid droplet proteomes during encystment remains poorly understood, hindering bioprocess optimization. Here, we constructed the first integrated spatiotemporal proteomic atlas of *H. pluvialis* lipid droplets (HPLDs) across five critical encystment stages, quantifying 3,396 high-confidence proteins. We reveal that HPLDs undergo profound stage-specific proteomic remodeling, redefine them as dynamic metabolic and communication hubs. Bioinformatic analysis revealed distinct functional shifts in HPLDs, including lipid metabolism, β -carotene biosynthesis, vesicular trafficking, antioxidant defense, and inter-organellar communication. Time-resolved clustering and pathway enrichment uncover four distinct expression modules, delineating a precise functional succession: from precursor mobilization and transient biosynthetic bursts (e.g., phospholipid:diacylglycerol acyltransferase surges 13.3-fold at HPLDs-3), through sustained ABC transporter-mediated trafficking, to dramatic structural reinforcement (Caleosin rises 11.5-fold at HPLDs-5) and the assembly of a compartmentalized antioxidant shield (Catalase surges 20-fold). Notably, terminal astaxanthin synthesis enzymes (BKT/CHYb) are absent from HPLDs, indicating spatial uncoupling of synthesis from storage and leading us to propose a conserved two-step sequestration and stabilization model for ketocarotenoids. These findings establish HPLDs as protein-governed platforms that integrate chloroplast, endoplasmic reticulum, and mitochondrial functions to drive carbon repartitioning, redox homeostasis, and astaxanthin ester deposition. Beyond providing a mechanistic blueprint for engineering astaxanthin hyperaccumulation, this spatiotemporal proteome resource illuminates fundamental principles of LD functionalization in photosynthetic organisms—principles that are broadly shared with land plants and offer new targets for improving stress tolerance and lipid-based metabolite storage in green lineages.

Keywords: *Haematococcus pluvialis*, Lipid droplets, Spatiotemporal proteomics, Metabolic remodeling, Astaxanthin accumulation, Organellar interaction

Introduction

Lipid droplets (LDs), once regarded as inert cytoplasmic inclusions for neutral lipid storage, are now recognized as dynamic, functionally versatile organelles that orchestrate cellular lipid flux, membrane homeostasis, and stress adaptation across

eukaryotes (Blot et al., 2025; Farese and Walther, 2025; Mathiowetz and Olzmann, 2024). In photosynthetic organisms—from green microalgae to vascular plants—LDs exhibit unique features, they establish intimate physical contacts with chloroplasts, serve as transient sinks for photoassimilate-derived carbon, and sequester cytotoxic hydrophobic metabolites such as carotenoids and phylloquinone (Blot et al., 2025; Zhao et al., 2025). It was also reported that LDs exhibit unique characteristics and engage in specialized interactions with plastids, underscoring their integrated role in photoassimilate partitioning and stress resilience (Xu et al., 2023; Zhao et al., 2025). In microalgae, LDs play particularly pivotal roles, not only in buffering excess carbon under nutrient limitation but also in the sequestration and storage of high-value secondary metabolites, such as the potent antioxidant astaxanthin (Blot et al., 2025; Wang et al., 2025a; Xu et al., 2023; Zhang et al., 2025). Critically, the functional diversity and adaptability of LDs are governed by their specific and dynamically changing proteome—the array of proteins embedded within or associated with their surface monolayer (Cai and Horn, 2025; Farese and Walther, 2025; Olzmann and Carvalho, 2019; Xu et al., 2023). This protein coat dictates LD biogenesis, growth, stability, inter-organelle contacts, and metabolic channeling, transforming LDs from passive lipid reservoirs into active, protein-driven metabolic platforms (Cai and Horn, 2025; Xu et al., 2023; Zhang et al., 2025).

The carotenogenic microalga *Haematococcus pluvialis* is the most prolific natural producer of astaxanthin, a high-value ketocarotenoid with potent antioxidant properties and wide applications in nutraceuticals, cosmetics, and aquaculture (Yang et al., 2025a). In *H. pluvialis*, astaxanthin accumulation is intrinsically linked to a profound morphological transition from motile vegetative cells to non-motile cysts, triggered by environmental stressors such as high light or nutrient deprivation (Cui et al., 2025; Pick et al., 2019; Yang et al., 2025a). During this encystment process, triacylglycerols (TAGs) and astaxanthin esters massively accumulate within cytoplasmic LDs, which expand dramatically to serve as the primary storage compartment (Pick et al., 2019; Wang et al., 2025a; Yang et al., 2025a). Increasing evidence suggests that the sequestration of astaxanthin into LDs is not a passive filling process but hypothesized to be an active, protein-mediated event crucial for pigment stabilization, cytotoxicity mitigation, and final yield determination (Cui et al., 2025; Pick et al., 2019; Wang et al., 2025a; Yang et al., 2025a).

Recent multi-omics studies have begun to illuminate the complex regulatory networks underlying astaxanthin biosynthesis in *H. pluvialis* (Cui et al., 2025; Gu et al., 2024; Wang et al., 2025a; Yan et al., 2025). For instance, quantitative proteomics under weak light stress identified key enzymes like farnesyl pyrophosphotransferase (FDFT1) and isocitrate dehydrogenase (IDH) that significantly enhance astaxanthin production in heterologous systems, highlighting the power of proteomics in uncovering regulatory nodes within the biosynthetic pathway (Wang et al., 2025a). Nevertheless, most existing studies provide only snapshot views, often focusing on individual pathway enzymes or terminal cyst stages, thereby missing the dynamic proteomic remodeling that orchestrates LD biogenesis, expansion, and functional maturation throughout the encystment cycle (Cui et al., 2025; Gu et al., 2024). A critical gap remains in our understanding of the organelle-specific machinery directly responsible for astaxanthin storage and LD dynamics (Cui et al., 2025).

While previous studies focused on snapshot views or pathway enzymes, a stage-resolved, organelle-specific proteome of LDs across the entire encystment cycle is missing. A comprehensive, spatiotemporally resolved analysis of the LD proteome across the complete life cycle is essential for deciphering the stage-specific composition and function of the *H. pluvialis* lipid droplets (HPLDs). Such an atlas would not only elucidate how HPLDs serve as metabolic and communication hubs integrating multiple organellar functions but also provide a protein-centric blueprint for targeted metabolic engineering and bioprocess optimization. In this study, we construct the first integrated spatiotemporal proteomic atlas of HPLDs across five critical encystment stages. By mapping stage-specific changes in protein composition and abundance, we aimed to elucidate how HPLDs serve as metabolic and communication hubs that integrate multiple organellar functions to drive carbon reallocation, antioxidant protection, and astaxanthin accumulation in *H. pluvialis*, thereby providing a mechanistic framework and candidate targets for engineering lipid-based metabolite storage in higher plants.

Results

Morphological and physiological changes during encystment

Astaxanthin biosynthesis in *H. pluvialis* is tightly coupled with triacylglycerol (TAG) accumulation, a process highly induced under adverse conditions such as high light or nutrient deprivation, rather than during vegetative growth (Cui et al., 2025; Shah et al., 2016; Wilawan et al., 2024). To delineate the morphological and physiological context

for lipid droplet (LD) dynamics, we first monitored cellular growth, pigmentation, and the carotenoid-to-chlorophyll (Car/Chl) ratio across five defined life cycle stages (HPLDs-1 to HPLDs-5).

Light microscopy revealed a distinct and progressive morphological transition, characterized by a color shift from green to bright red (Fig. 1A). This visual metamorphosis signifies a substantial increase in intracellular orange-red pigmentation, indicative of the gradual formation and enrichment of cytoplasmic LDs with carotenoids, primarily astaxanthin esters, under combined nutrient starvation and high-light stress—a phenomenon consistent with prior reports (Li et al., 2019; Pick et al., 2019; Shah et al., 2016). Cell density analysis over the 25-day cultivation period showed a characteristic pattern (Fig. 1B). Starting from an initial density of $6.14 \pm 0.72 \times 10^5 \text{ cells} \cdot \text{mL}^{-1}$, cultures underwent rapid growth during the first 3 days. Subsequently, from day 3 to day 15, the cell density stabilized at approximately $1.0 \times 10^6 \text{ cells} \cdot \text{mL}^{-1}$, marking the transition from logarithmic proliferation to a sustained encystment phase under continuous stress. During the final period (days 15-25), the particle count plateaued, reflecting the maturation of red non-motile cysts (HPLDs-5) rather than net vegetative growth.

Concurrently, we observed a dramatic reprogramming of cellular pigment composition, as evidenced by the Car/Chl ratio (Fig. 1B). During the exponential growth phase (days 0-3, corresponding to HPLDs-1 and HPLDs-2), the ratio remained low (~ 0.3), aligning with a photosynthetic, vegetative state. A sharp increase ensued from day 3 to day 7, with the ratio stabilizing around 0.65 through days 7-15 (HPLDs-3 and HPLDs-4), signaling active carotenoid accumulation. The ratio peaked at 1.34 ± 0.04 in the mature red cyst stage (HPLDs-5), representing a greater than five-fold increase from the initial vegetative stage. This substantial shift underscores a massive metabolic reallocation: the aggressive accumulation of secondary carotenoids (astaxanthin esters) within cytosolic LDs, coupled with the active degradation of chlorophylls. Together, these coordinated changes drive the hallmark color transformation and encapsulate the core physiological signature of stress-triggered encystment in *H. pluvialis*.

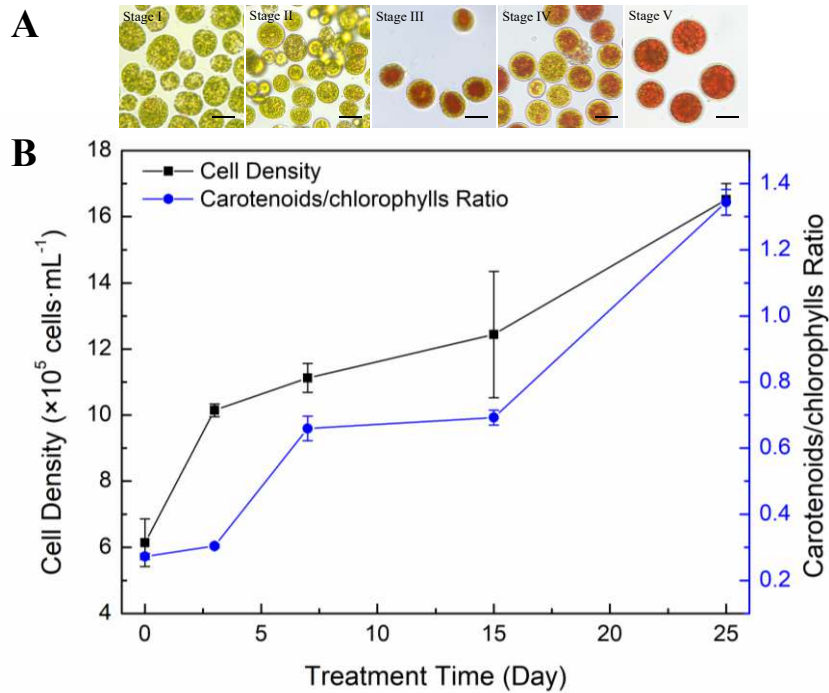


Figure 1. Morphological transition and physiological changes in *H. pluvialis* during its life cycle under inductive conditions. (A) Light microscopy images of cells at five life cycle stages. Scale bar = 10 µm. (B) Time course of cell density and cellular Car/Chl ratio during cultivation. Data points represent mean $\pm$ SD (n=3).

Alt text: Light microscopy images showing the progressive color transition of *H. pluvialis* cells from green (HPLDs-1) to bright red (HPLDs-5) over five encystment stages. The accompanying line graph depicts cell density stabilizing after an initial increase, while the carotenoid-to-chlorophyll ratio increases steadily, peaking in the mature cyst stage (HPLDs-5).

Isolation and purity assessment of HPLDs

To enable a definitive analysis of the LD proteome, high-purity HPLDs were essential. We therefore isolated LDs from all five life cycle stages using an optimized differential ultracentrifugation protocol based on established methods (Yoneda et al., 2016), which effectively separates intact LDs from cellular debris and organelles (Fig. 2A).

The integrity and morphology of the isolated HPLDs were confirmed by confocal laser scanning microscopy. Representative images of HPLDs-1 and HPLDs-5, selected as polar stages representing LD initiation and mature astaxanthin-enriched cysts, respectively, revealed populations of spherical structures (Fig. 2B). The purified LDs exhibited a size distribution ranging from approximately 50 nm to 10 µm in diameter, with notable stage-dependent variations in size.

Given that mechanical cell disruption can release soluble proteins that contaminate LD fractions (Wang et al., 2016), we rigorously assessed the purity of our preparations by

immunoblotting. We probed for canonical markers of major organelles: the RuBisCO large subunit (rbcL) for chloroplasts, alternative oxidase (AOX) for mitochondria, and binding immunoglobulin protein (BIP) for the endoplasmic reticulum (ER). While these markers were readily detected in whole-cell (WC) lysate controls from corresponding stages, they were undetectable in the purified HPLD fractions from all five stages (Fig. 2C). This result demonstrates that the isolated HPLDs had minimal contamination from chloroplasts, mitochondria, and the endoplasmic reticulum (ER), ensuring the subsequent proteomic analysis specifically reflects the bona fide LD-associated proteome.

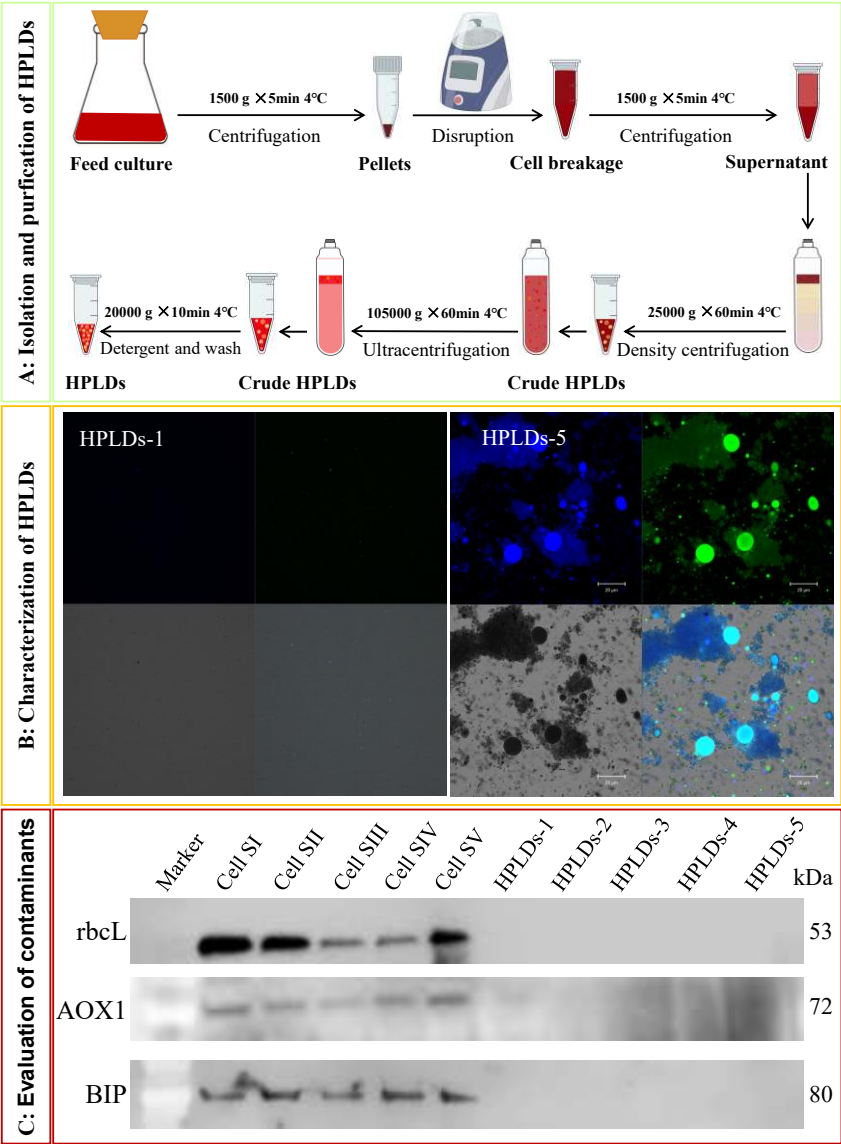


Figure 2. Purification, visualization, and purity assessment of HPLDs from five life cycle stages. (A) Schematic of the HPLD extraction and purification workflow. (B) Representative confocal laser scanning microscopy images of purified intact HPLDs

(HPLDs-1 and HPLDs-5). (C) Western blot analysis assessing contamination of purified HPLD fractions by specific organelle markers.

Alt text: Schematic workflow illustrating the isolation and purification of HPLDs via differential ultracentrifugation. Confocal microscopy images show purified HPLDs as spherical structures ranging from 50 nm to 10 µm in diameter. Western blot analysis detects organelle marker proteins (RBCL for chloroplasts, AOX for mitochondria, BIP for endoplasmic reticulum) in whole-cell lysates but not in purified HPLD fractions, confirming high purity.

HPLDs undergo profound proteomic remodeling during encystment

To decipher the protein machinery of HPLDs, we first assessed the quality and consistency of LD-isolated proteins. SDS-PAGE analysis followed by silver staining revealed distinct and reproducible protein banding patterns across the five encystment stages (Fig. 3A). While replicates within each stage showed high consistency, clear visual differences in band intensity and distribution were evident among stages, providing an initial indication of stage-specific proteomic composition.

We then performed TMT-based quantitative proteomics on HPLDs from all stages. After rigorous data filtering, a total of 15,797 peptides were identified, corresponding to 3,396 protein groups quantified with high and medium confidence (Supplementary Table S1; ProteomeXchange identifier PXD069513). This extensive dataset enabled a comprehensive functional annotation of the HPLD proteome. Gene Ontology (GO) analysis classified these proteins primarily into “cellular process” and “metabolic process” within the biological process category, “cellular anatomical entity” and “protein-containing complex” within the cellular component category, and “catalytic activity” and “binding” within the molecular function category (Fig. 3B).

KEGG pathway enrichment further delineated the functional landscape (Fig. 3C). Proteins involved in core metabolic processes dominated the profile. “Carbohydrate metabolism” constituted the largest group (246 proteins), followed by “Energy metabolism” (209 proteins), “Amino acid metabolism” (172 proteins), and “Lipid metabolism” (30 proteins). Beyond metabolism, a significant enrichment of 222 proteins in “Translation”-related pathways was notable. This includes ribosomal proteins and translation factors, which, while historically considered potential contaminants, have emerging roles in localized translation on LDs in other organisms. In *H. pluvialis*, their presence may support rapid LD biogenesis or astaxanthin esterification during encystment. Additionally, substantial numbers of proteins were assigned to “Folding, sorting and degradation” (165 proteins), “Transport

and catabolism” (99 proteins), “Transcription” (83 proteins), “Signal transduction”, and “Environmental adaptation”. Five proteins were annotated as membrane transporters, including lipid transporters, suggesting regulated metabolite exchange. Collectively, this functional profile positions HPLDs not merely as passive lipid reservoirs but as dynamic, multifunctional organelles engaged in core metabolism, stress adaptation, and inter-organellar communication crucial for astaxanthin accumulation and cyst formation.

Proteomic analysis also predicted diverse subcellular origins for the HPLD-associated proteins (Fig. 3D). Cytoplasmic proteins formed the largest contingent (1,107), followed by chloroplast-localized (732) and mitochondrial proteins (615). Notable contributions also came from the nucleus (255 proteins), endoplasmic reticulum (ER, 150 proteins), and peroxisomes (124 proteins). Fewer proteins were annotated to the Golgi apparatus (61), plasma membrane (45), and vacuole (14). This compartmentalization implies multifaceted functional integration: the strong chloroplast association aligns with precursor supply and photooxidative stress mitigation; the enrichment of ER and cytoplasmic proteins supports roles in lipid biosynthesis and trafficking; the unexpected presence of nuclear proteins may hint at regulatory functions during encystment. Interestingly, 262 proteins were annotated to the extracellular compartment, suggesting potential, albeit requiring validation, roles in secretory pathways related to LD dynamics. Overall, the HPLD proteome reflects its identity as a dynamic metabolic hub interfacing with multiple organelles to drive encystment. This multi-origin proteome composition suggests that HPLDs are not passive containers but rather dynamic organelles whose functions must shift dramatically during encystment. To deconstruct these functional dynamics, we next analyzed how the HPLD proteome changes across the five life cycle stages.

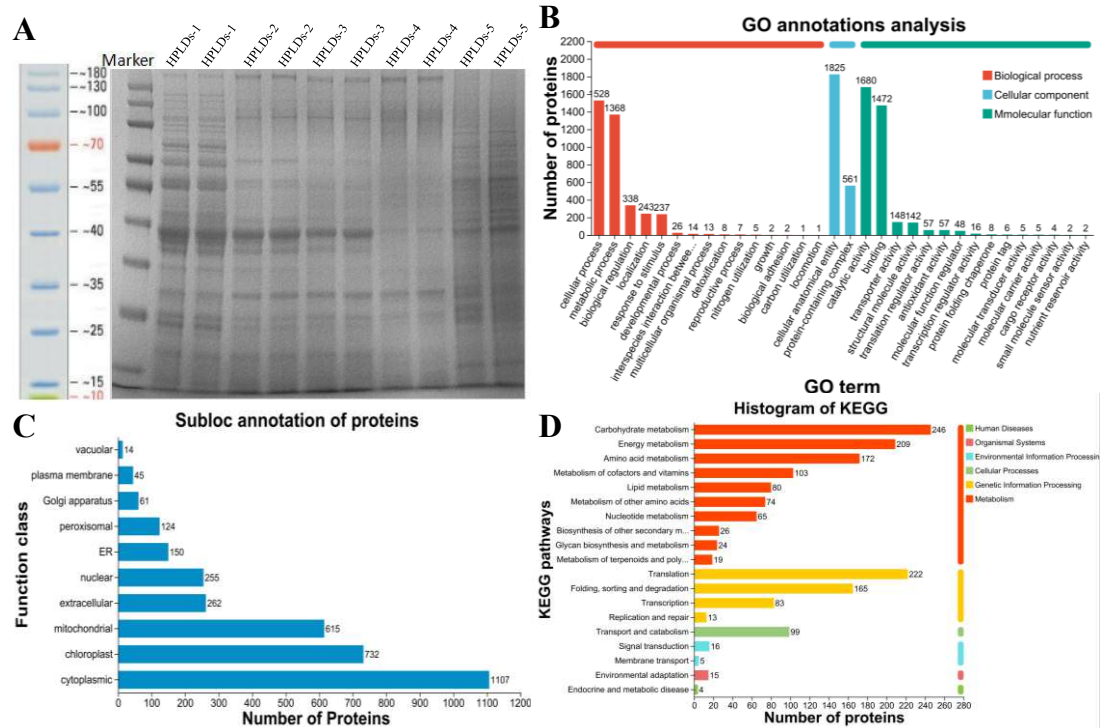


Figure 3. Proteomic profiling and functional annotation of HPLDs. (A) SDS-PAGE analysis of total HPLD proteins visualized by silver-staining. (B) Gene Ontology (GO) functional annotation of the identified HPLD proteins. (C) Predicted subcellular localization of the identified HPLD proteins. (D) KEGG pathway enrichment analysis of the identified HPLD proteins.

Alt text: (A) Silver-stained SDS-PAGE gel showing distinct protein banding patterns across the five HPLD stages. (B) Gene Ontology classification showing HPLD proteins are primarily involved in cellular and metabolic processes, with catalytic and binding functions. (C) Predicted subcellular localization indicates proteins originate mainly from the cytoplasm, chloroplast, and mitochondria. (D) KEGG pathway enrichment reveals HPLD proteins are associated with carbohydrate, energy, amino acid, and lipid metabolism, as well as translation and transport pathways.

Comparative proteomics reveals stage-specific functional shifts

To visualize and quantify the global proteomic shifts suggested by the stage-specific banding patterns (Fig. 3A), we first performed a principal component analysis (PCA) on the entire HPLD proteomic dataset. The PCA plot (Fig. 4A) revealed clear stage-dependent segregation. PC1 (40.00% of variance) effectively separated the early, nutrient-replete green stages (HPLDs-1, HPLDs-2) from the later, stress-induced reddening stages (HPLDs-3, HPLDs-4, HPLDs-5). PC2 (29.60% of variance) further distinguished the motile green stage (HPLDs-1) from the non-motile stages. Notably, the HPLDs-1, HPLDs-3, and HPLDs-5 groups displayed the most pronounced separation from each other. The tight clustering of biological replicates within each stage confirmed high experimental reproducibility.

Pairwise comparative analysis quantified the proteomic remodeling between consecutive life cycle stages (Fig. 4B). The most dramatic shift occurred during the initial transition from the motile vegetative stage to the early encystment stage (HPLDs-1 vs. HPLDs-2), with 1,580 proteins significantly upregulated and 639 downregulated. Subsequent transitions involved more modulated changes: HPLDs-3 versus HPLDs-2 showed 378 upregulated and 298 downregulated proteins. A prominent trend toward downregulation emerged from HPLDs-3 to HPLDs-4 (488 up vs. 812 down), which persisted, though slightly attenuated, in the final transition to the mature cyst (HPLDs-5 vs. HPLDs-4: 771 up vs. 884 down). The sustained high number of differentially abundant proteins (DAPs) throughout the encystment process underscores the dynamic and continuous adaptation of the HPLD proteome.

In this study, DAPs were defined as proteins with an abundance change >1.2 -fold or <0.83 -fold ($p < 0.05$). In total, 2,771 DAPs were identified across all five stages. Hierarchical clustering of these DAPs generated a heatmap (Fig. 4C) that revealed distinct, stage-specific protein expression profiles with high reproducibility among replicates. The clustering pattern grouped the samples into three major clusters corresponding to key physiological states: the flagellated motile stage (HPLDs-1), the stress-induced encystment stages (HPLDs-2, HPLDs-3, HPLDs-4), and the mature red cyst stage (HPLDs-5). This clear separation confirms the substantial proteome remodeling associated with each major phase transition.

Collectively, these comparative proteomic analyses demonstrate that the HPLD proteome undergoes profound and stage-specific reprogramming. The sequential waves of protein up- and down-regulation align with the shifting functional demands of HPLDs, from supporting motility and photosynthesis in green cells to facilitating active biosynthesis and expansion during mid-encystment, and finally to ensuring stable storage and protection in the astaxanthin-rich red cyst.

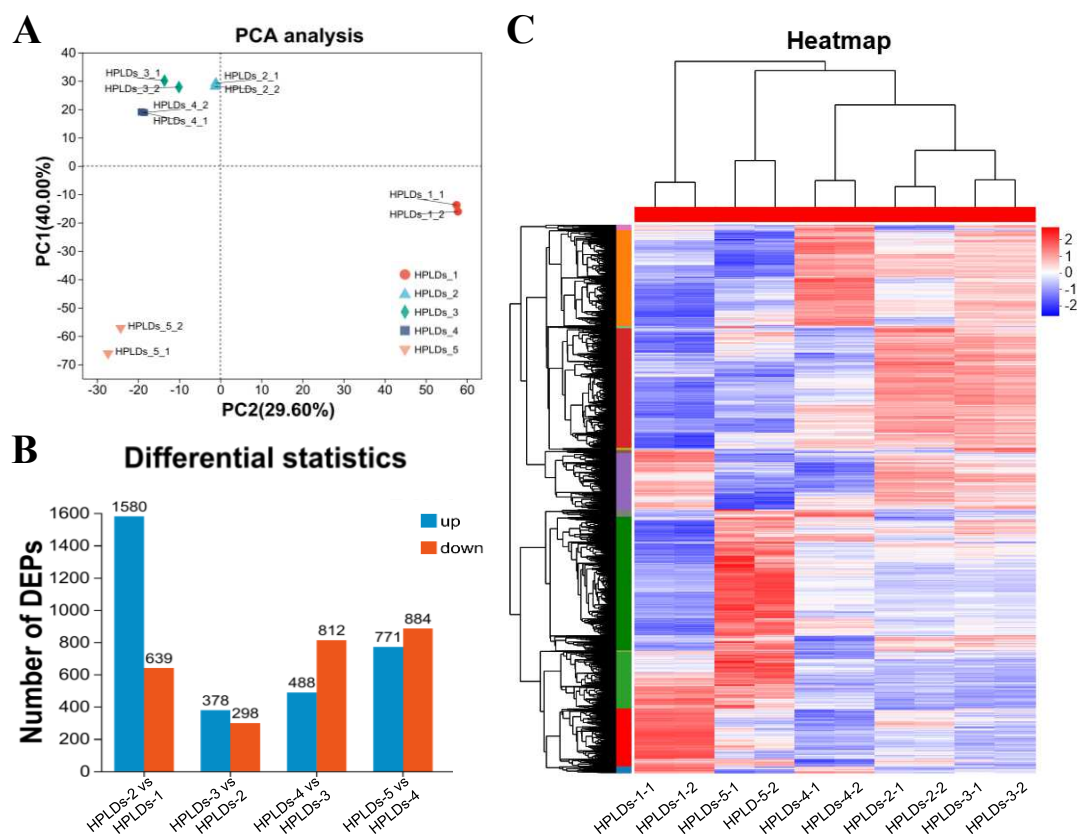


Figure 4. Comparative proteomic analysis of HPLDs across distinct life cycle stages. (A) Principal Component Analysis (PCA) of HPLD proteomes. (B) Quantitative analysis of differentially abundant proteins (DAPs) between stage groups (blue: upregulated; red: downregulated). (C) Hierarchical clustering heatmap of DAPs across all stages (red: upregulated; blue: downregulated relative to overall mean).

Alt text: (A) Principal component analysis shows clear separation of the HPLD proteomes, with early green stages (HPLDs-1,2) distinct from later reddening stages (HPLDs-3,4,5). (B) Bar graph displaying the number of upregulated and downregulated differentially abundant proteins between consecutive stages, with the most dramatic change occurring from HPLDs-1 to HPLDs-2. (C) Hierarchical clustering heatmap reveals three major sample clusters corresponding to motile, encysting, and mature cyst stages, demonstrating stage-specific protein expression profiles.

Time-resolved proteomic analysis reveals stage-specific functional modules

While pairwise comparisons identified stage-specific proteomic shifts, deciphering the continuous functional remodeling of HPLDs throughout encystment required an analysis of expression dynamics across the entire life cycle. To uncover the underlying temporal expression programs, we performed time-series clustering (Mfuzz) on the 2,771 DAPs that met the criteria ($\max \{ \log_2(\text{Abundance} + 1) \} > 1$; $\text{SD} > 1$). Soft clustering revealed 15 distinct temporal expression profiles (Supplementary Figure S1). Based on their expression trends, these clusters were consolidated into four major subgroups: Group 1 (Clusters 3, 7, 15; 881 DAPs) exhibiting a continuous increase;

Group 2 (Clusters 2, 8, 9; 645 DAPs) showing a continuous decrease; Group 3 (Clusters 1, 4, 5, 10, 11, 12, 13, 14; 1,461 DAPs) displaying transient upregulation peaking at intermediate stages (HPLDs-2 to HPLDs-4); and Group 4 (Cluster 6; 167 DAPs) demonstrating a U-shaped pattern with high abundance in both early (HPLDs-1) and late (HPLDs-5) stages (Supplementary Table S2).

KEGG enrichment analysis of these subgroups unveiled stage-specific functional partitioning intimately linked to LD dynamics and astaxanthin sequestration (Fig. 5, Supplementary Table S2). Proteins with continuously increasing abundance (**Group 1**) were significantly enriched in metabolic pathways that supply energy and carbon skeletons for extensive lipid deposition. Key enriched pathways included the pentose phosphate pathway (PPP), crucial for generating NADPH for fatty acid biosynthesis, and carbon fixation in photosynthetic organisms, which enhances the flux of carbon toward glycerol-3-phosphate, the backbone of triacylglycerols (TAGs). The concomitant upregulation of pathways like amino sugar and nucleotide sugar metabolism, cysteine and methionine metabolism, and glutathione metabolism likely supports LD membrane stabilization and provides redox homeostasis against oxidative stress induced by massive astaxanthin esterification. Conversely, proteins decreasing continuously (**Group 2**) were predominantly associated with translation (e.g., Ribosome pathway), indicating a systematic downregulation of the protein synthesis machinery as cells transition from active growth (HPLDs-1) to a dedicated storage phase (HPLDs-5), presumably to reallocate cellular resources toward LD biogenesis. Proteins showing transient upregulation (**Group 3**), which peaked during the phases of most active LD expansion (HPLDs-2 to HPLDs-4), were enriched in pathways fueling high biosynthetic demand. This included central energy-generating pathways such as the citrate cycle (TCA cycle) and oxidative phosphorylation, highlighting a surge in mitochondrial ATP production. A pronounced but temporary enrichment of the Ribosome pathway further underscores a transient boost in protein synthesis capacity specifically to support the assembly of the HPLD proteome and related metabolic enzymes. The U-shaped expression pattern (**Group 4**) featured proteins abundant in both motile vegetative cells and mature cysts but suppressed during intermediate restructuring. This group showed enrichment in fructose and mannose metabolism, whose early depression may indicate a redirecting of carbon flux from glycolysis toward acetyl-CoA production for lipid synthesis. Enrichment in RNA

degradation and glutathione metabolism suggests a finely tuned balance between resource recycling, stress adaptation, and the rapid lipid/astaxanthin sequestration characteristic of the encystment process.

In summary, time-resolved proteomic profiling delineates a precise functional succession: from an initial shift toward precursor and energy supply (Group 1), coupled with a burst of biosynthetic capacity (Group 3), to a subsequent decline in general protein synthesis (Group 2), all coordinated with stress-responsive mechanisms (Group 4) to facilitate the spatiotemporal remodeling of LDs for astaxanthin accumulation.

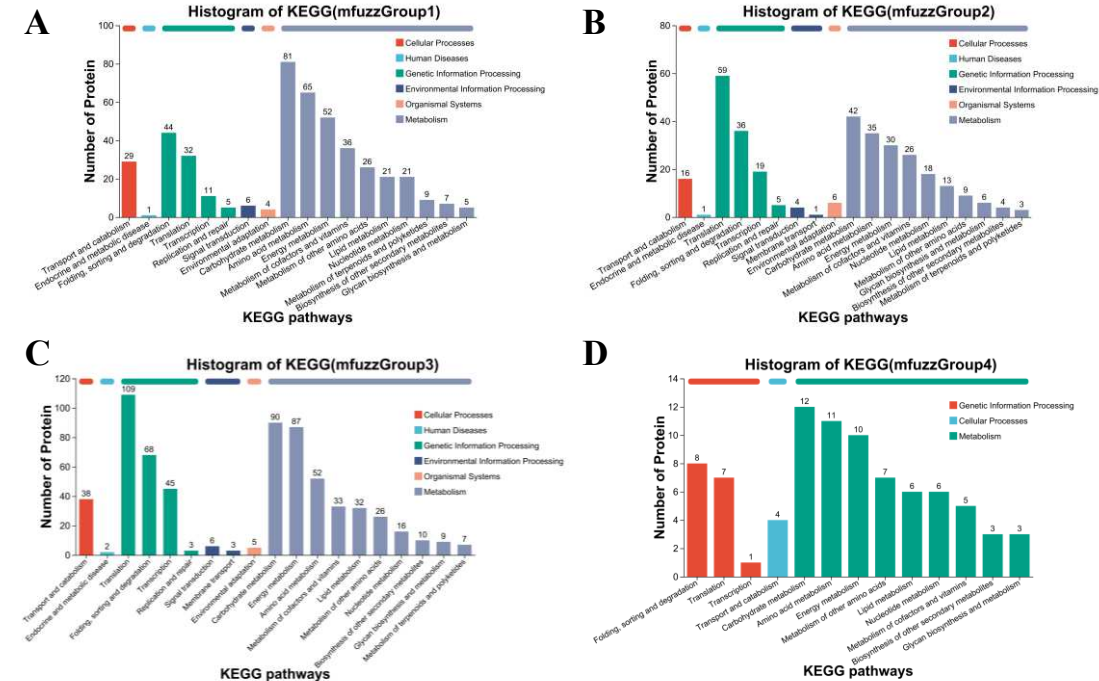


Figure 5. KEGG pathway enrichment analysis of the four major Mfuzz expression groups. (A) Group 1: Proteins with continuously increasing abundance, enriched in pathways for precursor and energy supply (e.g., pentose phosphate pathway, carbon fixation). (B) Group 2: Proteins with continuously decreasing abundance, primarily enriched for the ribosome, indicating a shutdown of general protein synthesis. (C) Group 3: Proteins with a transient peak during mid-encystment, enriched in energy metabolism (TCA cycle, oxidative phosphorylation) and the ribosome, supporting active LD expansion. (D) Group 4: Proteins with a U-shaped pattern, enriched in stress-related pathways (glutathione metabolism, RNA degradation), suggesting a role in fine-tuning redox balance during the transition.

Alt text: KEGG enrichment dot plots for four temporal expression groups: Group 1 (continuously increasing) is enriched in pentose phosphate pathway, carbon fixation, and glutathione metabolism; Group 2 (continuously decreasing) is primarily enriched for ribosome pathway; Group 3 (transient mid-stage peak) is enriched in TCA cycle, oxidative phosphorylation, and ribosome; Group 4 (U-shaped pattern) is enriched in fructose and mannose metabolism, RNA degradation, and glutathione metabolism.

DAPs associate with the specific pathways in HPLDs

The four temporal expression modules identified above (Fig. 5) point to broad shifts in metabolic priorities. To obtain a more granular view of the specific machinery driving lipid and astaxanthin accumulation, we next analyzed the stage-specific expression patterns of DAPs associated with key metabolic and cellular processes (Figs. 6-8, Table 1).

Firstly, key enzymes in the “fatty acid biosynthesis” pathway (KEGG map00061) exhibited starkly differential expression (Fig. 6). Enzymes involved in initiation and elongation showed contrasting trends. ACC1, catalyzing the committed step for malonyl-CoA production, showed negligible expression in early stages (HPLDs-1/2) but surged more than 19-fold in HPLDs-5. Early elongation proteins (FabD, FabF, FabH) were predominantly active in motile/green stages (HPLDs-1/2) but declined sharply (up to 85% reduction) during early encystment (HPLDs-3/4), with partial recovery in HPLDs-5. FabG (β -ketoacyl reductase) displayed a unique biphasic pattern, with moderate upregulation in HPLDs-2/3 (2–3-fold) and a dramatic 19.9-fold increase in HPLDs-5. Terminal modification and activation enzymes were strongly induced in cysts, including FabI/MECR (enoyl-ACP reductase) and FAB2 (stearoyl-ACP desaturase), which increased more than 12-fold and 5-fold, respectively, in HPLDs-5 compared with HPLDs-1. ACSL (acyl-CoA synthetase) also rose 12.35-fold in red cysts (HPLDs-5). These patterns suggest a metabolic transition from membrane lipid biosynthesis in vegetative stages (HPLDs-1/2) to energy-rich neutral lipid storage in cysts (HPLDs-4/5).

Consistent with this shift toward neutral lipid storage, we investigated the expression of phospholipid:diacylglycerol acyltransferase (PDAT), a key enzyme catalyzing TAG formation during early encystment (Sah et al., 2024; Zhang et al., 2021; Zhao et al., 2020). A single PDAT homolog was identified in the HPLD proteome, exhibiting a striking stage-specific expression pattern (Table 1). Its expression was minimal in the green motile stage (HPLDs-1) but increased 9.2-fold in the green nonmotile stage (HPLDs-2). The expression peaked dramatically in the brown nonmotile stage (HPLDs-3), representing a 13.3-fold increase relative to HPLDs-1, followed by a precipitous decline in the orange and red cyst stages (HPLDs-4/5). This transient peak coincides with the initial phase of TAG and lipid droplet accumulation.

Next, we examined DAPs annotated to the carotenoid biosynthesis pathway (Fig. 7).

Phytoene desaturase (PDS) was rapidly upregulated (6 to 8-fold compared to HPLDs-1) during the transition to nonmotile stages (HPLDs-2/3), then displayed moderate downregulation in cysts (HPLDs-4/5). Zeta-carotene desaturase (ZDS) showed moderate upregulation throughout, peaking at 2.6-fold in HPLDs-5. Cytochrome P450 97A3 (LUT5) exhibited dramatic induction in the red cyst stage (HPLDs-5), increasing up to 6.8-fold. Carotenoid cleavage dioxygenase 8 (CCD8) displayed isoform-specific patterns; one isoform increased continuously to ~4-fold in HPLDs-5, while another was transiently induced 9-fold in HPLDs-2. Notably, enzymes directly responsible for astaxanthin synthesis from β -carotene, such as Beta-carotene ketolase (BKT) and β -carotene hydroxylase (CHYb), were not significantly detected in HPLDs, suggesting their primary localization outside LDs or extremely low abundance within them.

Given the apparent spatial separation of terminal astaxanthin synthesis from its storage compartment, we investigated potential transport machinery. Analysis of the HPLD proteome revealed five ABC transporter homologs identified with high or medium confidence (Supplementary Table S1), four of which were DAPs. Their distinct stage-specific expression patterns (Table 1) suggest specialized roles in trafficking lipid and carotenoid precursors to developing HPLDs. Notably, two transporters, predicted to localize to peroxisomes and the Golgi apparatus respectively, showed sustained upregulation from the early encystment stage (HPLDs-2) through to the mature cyst, peaking in HPLDs-5 with a >4-fold and >10-fold induction, compared to the vegetative stage (HPLDs-1). In contrast, two cytoplasmic-localized transporters were transiently induced during early-to-mid encystment (HPLDs-2 to HPLDs-4) before declining, peaking in HPLDs-2/3 with a >4-fold and >6-fold induction in comparison with HPLDs-1. The presence and dynamic regulation of these transporters imply specialized, stage-dependent roles in mobilizing hydrophobic precursors (e.g., fatty acids, carotenoids, or their esters) to the developing LDs.

To understand how these metabolites are trafficked to and within LDs, we then analyzed DAPs associated with “Interactions in vesicular transport” (Fig. 8). ER-associated proteins (Sec20, Sec22, Bet1) peaked in abundance in early nonmotile stages (HPLDs-2/3), suggesting active ER-derived lipid transfer during LD initiation. The R-SNARE protein Ykt6 was rapidly upregulated (6-fold) in HPLDs-2 and remained highly elevated thereafter, indicating sustained Golgi-mediated vesicular trafficking to LDs throughout encystment. Other regulators (Gos1, STX6) displayed transient surges in

mid-stages (HPLDs-2/3), implicating a role in early endosomal sorting. Syntaxin proteins STX8 and Vti1 exhibited a sharp, transient peak specifically in HPLDs-2, likely involved in initiating LD biogenesis, as their abundance diminished once LD nucleation was established. SNAP23 expression increased sharply, peaking in HPLDs-3, and remained elevated, suggesting a constitutive role in membrane fusion for LD growth and maintenance.

Further analysis highlighted Caleosin, a key structural protein of LDs (Fu et al., 2025), which exhibited a biphasic expression pattern (Table 1). It was initially upregulated in HPLDs-2, then showed a profound and significant upregulation (11.5-fold vs. HPLDs-1) specifically in the red cyst stage (HPLDs-5). This pronounced overexpression in mature cysts strongly suggests a defining role in stabilizing the LD monolayer during massive astaxanthin ester sequestration, potentially facilitating the docking or embedding of astaxanthin esters within the LD core.

Beyond their metabolic roles, HPLDs function as dynamic hubs for oxidative stress mitigation by stage-specifically recruiting antioxidant machinery. We identified compartmentalized and stage-specific enrichment of key antioxidant enzymes within HPLDs (Table 1). Superoxide dismutase (SOD) isoforms displayed distinct dynamics: chloroplastic SODs peaked in HPLDs-1 (early photosynthetic protection) and rebounded in HPLDs-5; mitochondrial SODs surged 3–4-fold during mid-encystment (HPLDs-2 to HPLDs-4) to meet heightened energy demands; and an ER-localized SOD increased >27-fold by HPLDs-4, likely responding to ER stress during active lipid synthesis. Catalase (CAT) displayed a biphasic induction, rising moderately to HPLDs-4 before surging 20-fold in HPLDs-5, poised to counter cyst-stage peroxide accumulation. Glutathione reductase (GSR) isoforms operated in compartment-specialized modules, with chloroplastic forms dominating early and late stages, cytoplasmic forms sustaining mid-encystment, and an ER-targeted isoform recovering in HPLDs-5. A chloroplastic glutathione peroxidase (GPX) also rebounded 4.4-fold in HPLDs-5, synergizing with CAT. Together, these findings indicate that HPLDs serve as dynamic platforms that spatiotemporally coordinate a multi-layered antioxidant defense to ensure redox homeostasis throughout the encystment process.

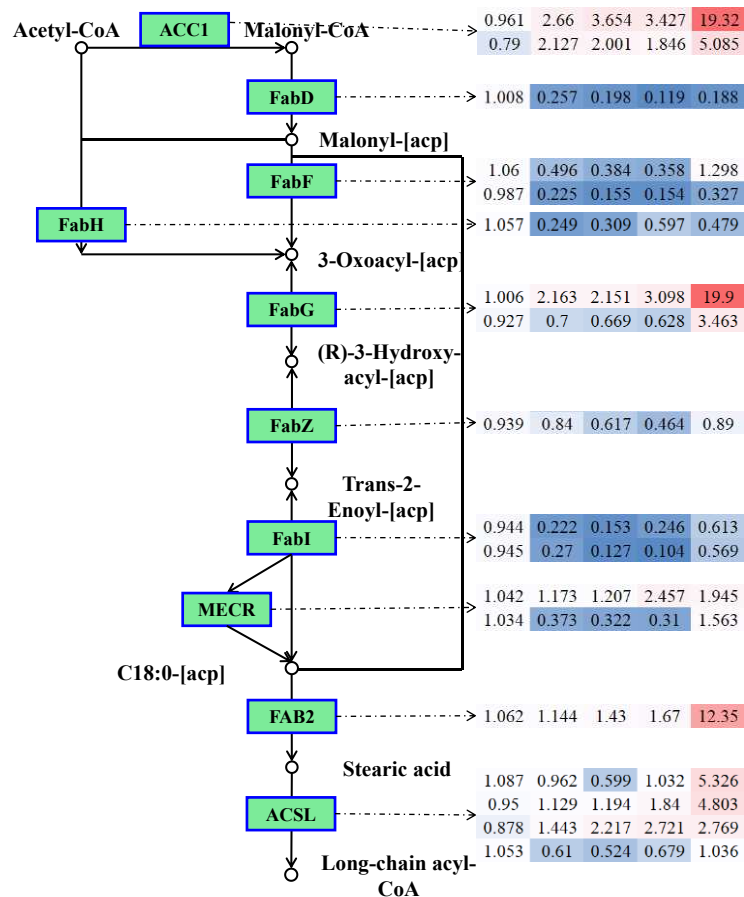


Figure 6. Expression patterns of DAPs enriched in the "Fatty acid biosynthesis" pathway. Heatmap visualization shows relative protein abundance (red: high; blue: low). **Alt text:** Heatmap showing relative abundance of fatty acid biosynthesis enzymes across HPLD stages. Initiation enzymes (FabD, FabF, FabH) are highly expressed in early stages, while late-stage enzymes (ACC1, FabG, FabI, ACSL) show dramatic upregulation in mature cysts (HPLDs-5), indicating a metabolic shift from membrane lipid synthesis to neutral lipid storage.

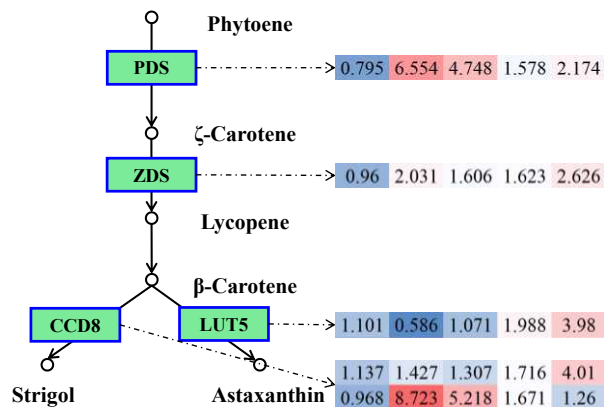


Figure 7. Expression patterns of DAPs enriched in the "Carotenoid biosynthesis" pathway. Heatmap visualization shows relative protein abundance (red: high; blue: low).

Alt text: Heatmap displaying expression patterns of carotenoid biosynthesis enzymes. Phytoene desaturase (PDS) peaks during early encystment, while zeaxanthin epoxidase and other late enzymes increase in mature cysts. Notably, the terminal astaxanthin synthesis enzymes BKT and CHYb are not detected in the HPLD proteome, suggesting their localization outside lipid droplets.

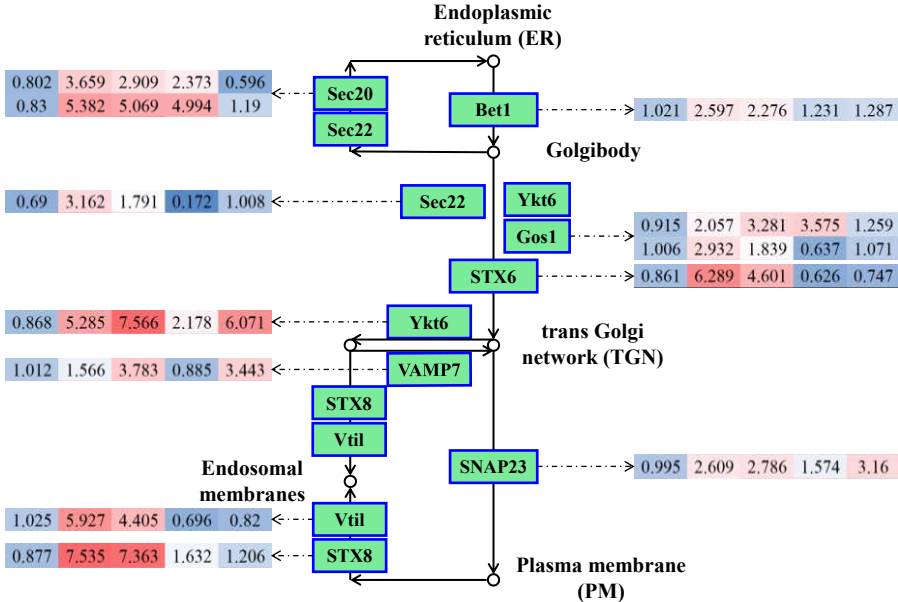


Figure 8. Expression patterns of DAPs enriched in the "Interactions in vesicular transport" pathway. Heatmap visualization shows relative protein abundance (red: high; blue: low).

Alt text: Heatmap illustrating dynamic expression of vesicular transport proteins. ER-resident SNAREs (Sec20, Sec22) peak during early encystment, while Ykt6 and SNAP23 remain elevated throughout. Syntaxins STX8 and Vtil show a sharp, transient peak specifically at HPLDs-2, implicating them in lipid droplet initiation. This pattern supports ER- and Golgi-mediated vesicular trafficking for lipid droplet biogenesis and expansion.

Discussion

Our integrated spatiotemporal proteomic atlas of HPLDs across five defined life cycle stages unveils extensive and stage-specific reprogramming of the LD proteome. This dynamic remodeling demonstrates how HPLDs successively recruit proteins from multiple organelles to orchestrate carbon repartitioning, vesicular trafficking, energy supply, and antioxidant defense, thereby driving astaxanthin hyperaccumulation. Building on these findings, we discuss how the compositional and functional plasticity of HPLDs underpins their role as integrated metabolic hubs during encystment in *H. pluvialis*.

HPLDs as Integrative Metabolic Hubs: A Multi-Origin Proteome Redefines Organellar Function

Our study substantially revises the functional understanding of HPLDs. Far from being inert storage inclusions, they are emerged as dynamic and integrative metabolic hubs that actively orchestrate carbon repartitioning, membrane remodeling, redox homeostasis, and astaxanthin sequestration during stress-induced encystment. This aligns with the paradigm shift recognizing LDs as protein-governed platforms for metabolic channeling and stress adaptation in plants and algae (Blot et al., 2025; Farese and Walther, 2025; Mathiowetz and Olzmann, 2024; Xu et al., 2023; Zhang et al., 2025; Zhao et al., 2025).

The foundation of this hub function is their proteome of diverse origins, assembled dynamically. The identification of 3,396 high-confidence proteins with diverse subcellular origins (Fig. 3D) positions HPLDs at the crossroads of inter-organelle communication, mirroring the role of LDs as central regulators of lipid flux (Blot et al., 2025; Mathiowetz and Olzmann, 2024; Wang et al., 2025a). This multi-organelle signature likely reflects functional recruitment rather than contamination, as confirmed by our purity checks (Fig. 2C). For instance, the substantial enrichment of chloroplast-derived proteins underscores a critical chloroplast-HPLD axis. This axis likely channels photosynthetic carbon, isoprenoid precursors, and possibly galactolipids from thylakoid disassembly for LD monolayer reinforcement and astaxanthin backbone supply (Cai and Horn, 2025; Cui et al., 2025; Li et al., 2019; Shah et al., 2016; Zhao et al., 2025). Concurrently, the temporal dynamics of ER-associated machinery, exemplified by the sharp, transient peak of PDAT in HPLDs-3 (Table 1), pinpoints the ER as a primary site for TAG synthesis fueling initial LD expansion (Arlt et al., 2022; Farese and Walther, 2025; Mathiowetz and Olzmann, 2024; Zhang et al., 2021). This echoes and extends recent findings in *Arabidopsis*, where LDs establish direct membrane contacts with chloroplasts and peroxisomes for metabolite channeling (Brocard et al., 2017; Fan et al., 2017), and where PDAT1 plays a pivotal role in membrane lipid turnover during seed development and senescence (Fan et al., 2014; Sah et al., 2024).

Notably, our atlas places mitochondrial proteins (peaking at HPLDs-3/HPLDs-4, Fig. 3D) and the induction of TCA cycle and oxidative phosphorylation components (Fig. 5C) directly at the LD interface. This suggests HPLDs may help establish a localized metabolic microenvironment to meet the high energy (ATP) and reducing power (NADPH) demands for massive lipid and astaxanthin biosynthesis. The significant

enrichment of ribosomal proteins, often considered potential contaminants, is intriguing (Moellering and Benning, 2010). While their definitive role here requires validation, it provides circumstantial support for emerging roles of LDs as platforms for localized translation or protein quality control in other systems, which could coordinate rapid synthesis of specific proteins with LD biogenesis and expansion (Roberts and Olzmann, 2020; Vijayan et al., 2024).

Furthermore, the presence and dynamic regulation of multiple ABC transporters within the HPLD proteome (Table 1, Supplementary Table S1) provides correlative evidence supporting the model that HPLDs actively recruit transport systems to facilitate directed metabolite flux (Cui et al., 2025; Salvador Lopez and Van Bogaert, 2021; Wang et al., 2025b; Zhang et al., 2025). Their distinct stage-specific expression patterns suggest specialized roles: transporters induced during early-to-mid encystment (HPLDs-2 to HPLDs-4) may facilitate rapid LD nucleation and initial loading, while those sustained into the mature cyst stage (HPLDs-5) could mediate long-range precursor import or inter-organellar lipid/astaxanthin exchange and sequestration (Chen et al., 2022; Wang et al., 2025b; Yang et al., 2025a).

A key novel insight is the suggested role of HPLDs in astaxanthin ester sequestration and stabilization. The extraordinary overexpression of the structural protein Caleosin specifically in red cysts (HPLDs-5, 11.5-fold increase; Table 1) is pivotal. As studies represent that Caleosins are crucial for LD stability and inter-organellar tethering in plants (Blot et al., 2025; Fu et al., 2025; Mathiowetz and Olzmann, 2024; Roberts and Olzmann, 2020; Vijayan et al., 2024). Its massive upregulation here strongly implies a specialized function in stabilizing the expanded LD monolayer during massive astaxanthin ester deposition, potentially facilitating the embedding of these hydrophobic pigments into the LD core or mediating contacts for precursor import. This is reminiscent of the role of *Arabidopsis* Caleosin3 in stabilizing LDs under abiotic stress and mediating ER-LD contacts (Fu et al., 2025; Kim et al., 2016).

In conclusion, the spatiotemporal remodeling of the HPLD proteome drives a functional metamorphosis—from precursor-importing and assembly platforms in early encystment, to energized biosynthetic hubs during expansion, and finally into highly fortified storage and protection organelles in the mature cyst. This dynamic, protein-mediated transformation positions HPLDs as central executors of the encystment

program.

Stage-Specific Proteomic Reprogramming Mirrors Conserved LD Lifecycle Transitions

The HPLD proteome undergoes a tightly regulated, stage-specific transformation, reflecting the inherent dynamism of LDs (Du et al., 2025; Farese and Walther, 2025; Listenberger et al., 2025; Mathiowetz and Olzmann, 2024; McLelland et al., 2023; Olarte et al., 2022; Olzmann and Carvalho, 2019; Yang et al., 2025b). Our time-resolved analysis allows us to propose that HPLDs evolve through three consecutive functional phases, each defined by distinct proteomic signatures (Fig.s 4–6, Table 1).

Phase I: Initiation and Precursor Mobilization (HPLDs-1 to HPLDs-2). This transition is marked by the most dramatic proteomic upheaval (1,580 proteins upregulated; Fig. 4B), signifying a global shift in cellular priorities. Key events include the induction of vesicular trafficking machinery (e.g., STX8, Vti1; Fig. 8), suggesting active ER-derived lipid transfer for LD nucleation (Arlt et al., 2022; Farese and Walther, 2025; Wang et al., 2016; Zhang et al., 2021). The concomitant transient upregulation of specific cytoplasmic ABC transporters (Table 1) coincides with this peak, suggesting a coordinated mechanism for initial lipid loading (Chen et al., 2022; Farese et al., 2023; Yang et al., 2025b; Zhang et al., 2025). Upregulation of the pentose phosphate pathway and carbon fixation (Group 1, Fig. 5A) ensures a supply of NADPH and carbon skeletons for biosynthesis. Notably, PDAT exhibits a striking 9.2-fold induction in HPLDs-2 (Table 1), highlighting its potential key role in initial TAG synthesis, possibly utilizing phospholipids as acyl donors during this rapid stress response (Ikeda et al., 2020; Li et al., 2024; Sah et al., 2024; Zhang et al., 2021). The initial upregulation of carotenoid pathway components (e.g., PDS; Fig. 7) links LD formation with precursor flux toward astaxanthin biosynthesis (Bai et al., 2022; Ota et al., 2018).

Phase II: Active Expansion and Biosynthetic Integration (HPLDs-3 to HPLDs-4). This phase represents the peak of LD biogenesis and expansion. The proteome is dominated by proteins showing transient upregulation (Group 3, Fig. 5C), including mitochondrial components (TCA cycle, oxidative phosphorylation) and ribosomal proteins. This underscores a surge in energy production and biosynthetic capacity to support rapid expansion (Fan et al., 2014, 2017; Farese, and Walther, 2023; Mathiowetz and Olzmann, 2024). The dramatic peak of PDAT in HPLDs-3 (13.3-fold; Table 1) coincides with maximal TAG accumulation. Enrichment of chloroplast-derived proteins suggests

intensive precursor channeling (Blot et al., 2025; Li et al., 2019), while vesicular transport shifts toward sustained machinery (e.g., Ykt6, SNAP23; Fig. 8) for continued LD growth and maintenance (Blot et al., 2025; Olarte et al., 2022).

Phase III: Maturation, Storage, and Stabilization (HPLDs-5). This phase is characterized by structural consolidation and long-term protection. Key classes are sharply induced: the sharp induction of *de novo* fatty acid synthesis enzymes (e.g., ACC1, FabG; Fig. 6), potentially modifying the lipid core composition for stability (Gupta and Mandal, 2025; Xu et al., 2023); the extraordinary induction of the structural protein Caleosin (11.5-fold, Table 1), suggesting a critical role in monolayer stabilization and potentially astaxanthin ester embedding (Blot et al., 2025; Fu et al., 2025; Roberts and Olzmann, 2020; Zhang et al., 2025); and a reconfiguration of antioxidant defenses (e.g., CAT surges 20-fold, Table 1), creating a fortified redox shield to protect stored lipids and carotenoids (Gupta and Mandal, 2025; Olzmann and Carvalho, 2019; Ota et al., 2018; Zhang et al., 2025). Conversely, the decline in general ribosomal machinery (Group 2, Fig. 5B) reflects a shift away from active growth to a storage-optimized state. The role of lipophagy (LD degradation via autophagy) in *H. phuvialis* cyst metabolism remains an open question (Amari et al., 2024; Ge et al., 2025). While our data show induction of general "Transport and catabolism" pathways, future studies should investigate whether specific lipases or autophagy-related proteins are recruited to HPLDs for potential turnover or quality control.

The three-phase functional succession we delineate in HPLDs—initiation, expansion, stabilization—is strikingly reminiscent of the LD lifecycle in *Arabidopsis* mesophyll cells during dark-induced senescence and in oilseed cotyledons during germination (Arlt et al., 2022; Brocard et al., 2017; Fan et al., 2017; Gidda et al., 2016; Roberts and Olzmann, 2020). This parallel suggests that the fundamental principles governing LD biogenesis, maturation, and turnover are deeply conserved across the green lineage, from unicellular algae to complex vascular plants, while the specific protein machinery recruited at each phase has been adapted to meet lineage-specific metabolic demands, such as astaxanthin hyperaccumulation in *H. phuvialis*.

HPLDs during encystment

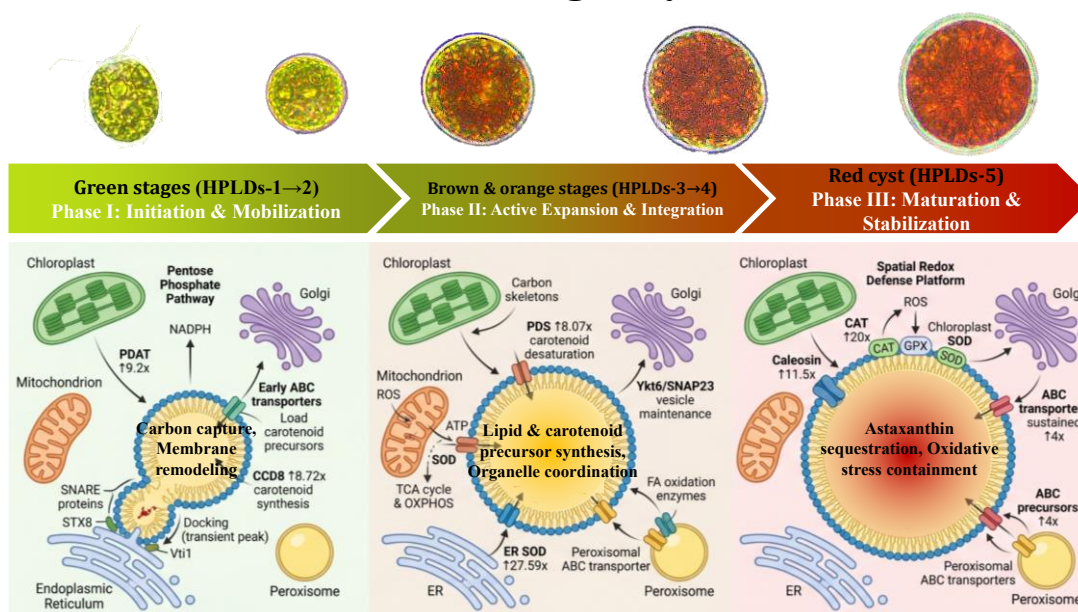


Figure 9. A comprehensive model illustrating the functional evolution of HPLDs driven by spatiotemporal proteomic remodeling: from passive storage depot to active metabolic and communication hub.

Alt text: Schematic model depicting the three-phase functional evolution of HPLDs during encystment. Phase I (Initiation) involves ER-derived vesicular trafficking and precursor mobilization. Phase II (Expansion) shows integration with mitochondria for energy and chloroplasts for carbon skeletons, with PDAT driving triacylglycerol synthesis. Phase III (Maturation) features structural reinforcement by Caleosin and assembly of an antioxidant shield (CAT, SOD) to stabilize stored astaxanthin esters, positioning HPLDs as a central hub integrating chloroplast, mitochondrial, and ER functions.

A Two-Step Sequestration and Stabilization Model for Astaxanthin

A central finding is the spatial uncoupling between terminal astaxanthin biosynthesis and its storage. While HPLDs are the definitive reservoir, key enzymes (BKT, CHYb) were not detected in the HPLD proteome (Fig. 7), suggesting that in *H. pluvialis*, these terminal reactions are spatially uncoupled from the LD, occurring primarily in plastids or at the ER, as previously hypothesized (Cui et al., 2025). This, coupled with the dramatic induction of Caleosin in HPLDs-5, prompts us to propose a refined Two-Step Sequestration and Stabilization Model.

Step 1: Synthesis and Modification External to the LD Core. The core pathway up to β -carotene occurs in plastids (Cui et al., 2025; Salvador Lopez and Van Bogaert, 2021; Yang et al., 2025a). Subsequent ketolation/hydroxylation by BKT/CHYb is associated with plastid membranes or ER (Cui et al., 2025; Wang et al., 2025; Yang et al., 2025a). The absence of BKT/CHYb from our HPLD proteome suggests these final steps likely

occur externally to the LD core, possibly at ER or plastid-LD contact sites. This spatial separation may allow for independent regulation of synthesis and storage fluxes (Blot et al., 2025; Mathiowetz and Olzmann, 2024). Efficient transport of astaxanthin or its precursors to the LD surface is also implied by the presence of dynamically regulated ABC transporters in the HPLD proteome (Table 1, Supplementary Table S1) (Chen et al., 2022; Salvador Lopez and Van Bogaert, 2021; Wang et al., 2025; Yang et al., 2025b; Zhang et al., 2025).

Step 2: Esterification and Active Sequestration at the LD Interface. Free astaxanthin is esterified for stabilization and sequestration into the neutral lipid core (Pick et al., 2019; Yang et al., 2025a). We posit this esterification occurs at or near the HPLD surface, potentially facilitated by as yet unidentified acyltransferases recruited to the LD monolayer. The extraordinary induction of Caleosin in mature cysts suggests it stabilizes the LD monolayer during massive astaxanthin ester deposition, providing a mechanistic clue for the final stabilization step (Blot et al., 2025; Fu et al., 2025; Ikeda et al., 2020; Zhao et al., 2020). We hypothesize Caleosin plays a critical role in: (i) reinforcing the phospholipid monolayer to accommodate massive ester deposition; (ii) facilitating ordered embedding of astaxanthin esters into the core; and/or (iii) mediating inter-organellar contacts for precursor import. The presence of diverse ABC transporters supports active, stage-dependent precursor (e.g., fatty acids for esterification, carotenoids) influx (Table 1) (Chen et al., 2022).

This model frames HPLDs not merely as passive sinks but as the central organizing compartments that actively manage the sequestration, stabilization, and protection of astaxanthin, analogous to protective roles of LDs in sequestering other hydrophobic or potentially disruptive molecules (Ge et al., 2025; Mathiowetz and Olzmann, 2024; Olzmann and Carvalho, 2019; Zhao et al., 2025). The two-step model we propose—synthesis outside the LD, followed by esterification and protein-facilitated embedding—thus offers a conceptual framework applicable to diverse processes in plants, such as the sequestration of lycopene in tomato fruit chromoplasts or the accumulation of protective carotenoids in stress-acclimated leaves, where LDs are increasingly recognized as dynamic and transient reservoirs for hydrophobic metabolites (Blot et al., 2025; Du et al., 2025; Zhao et al., 2025).

HPLDs as Dynamic Platforms for Spatially Organized Redox Homeostasis

Our proteomic analysis reveals that HPLDs function as dynamic platforms for redox

regulation, actively recruiting a compartmentalized suite of antioxidant enzymes—including superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSR), and glutathione peroxidase (GPX)—in a tightly stage-specific manner (Table 1). This spatially organized recruitment underscores the active role of HPLDs in maintaining cellular redox homeostasis during the oxidative stress-prone process of astaxanthin hyperaccumulation (Cui et al., 2025; Yang et al., 2025a), exemplifying a conserved function of LDs in mitigating lipid peroxidation and associated cellular damage in plants (Doner et al., 2025; Zhao et al., 2025).

The recruitment strategy is compartmentalized and temporally orchestrated. During active expansion and high metabolic activity (HPLDs-2 to HPLDs-4), induction of mitochondrial SOD isoforms coincides with peak energy metabolism and oxidative phosphorylation activity (Fig. 5C), likely scavenging superoxide generated from heightened respiration at potential LD-mitochondria contact sites (Du et al., 2025; Farese and Walther, 2023; Listenberger et al., 2025; McLelland et al., 2023; Olarte et al., 2022; Wang et al., 2025; Yang et al., 2025b; Zhang et al., 2021). Simultaneously, a surge in an ER-localized SOD likely counters ROS produced during intense lipid assembly at the ER-LD interface (Farese and Walther, 2025; Mathiowetz and Olzmann, 2024; Roberts and Olzmann, 2020; Vijayan et al., 2024; Zhang et al., 2021).

In the mature red cyst stage (HPLDs-5), the dramatic induction of CAT and a chloroplastic GPX provides a robust, localized defense system on the LD surface against H₂O₂ accumulation. This compartmentalized antioxidant recruitment aligns with the understanding that LDs can serve as sinks for ROS and hubs for antioxidant defense, protecting both the LD contents and surrounding cellular structures (Gu et al., 2024; Fan et al., 2017; Salvador Lopez and Van Bogaert, 2021; Wang et al., 2025; Xu et al., 2023; Yang et al., 2025a; Zhang et al., 2025). The extraordinary 20-fold surge of catalase in mature HPLDs-5 is particularly striking and suggests that LD-associated CAT may represent a common adaptation for protecting stored lipids and carotenoids from photooxidative damage—a function likely relevant to evergreen leaves and developing oilseeds exposed to high light. This finding resonates with recent reports in *Arabidopsis*, where LDs in stressed leaves recruit CAT to mitigate lipid peroxidation, suggesting that LD-associated catalase represents a conserved adaptation for protecting storage compounds from photooxidative damage in the green lineage (Bouchnak et al.,

2023; Doner et al., 2025; Du et al., 2025).

The compartmentalized and stage-specific recruitment of SOD, CAT, GSR, and GPX to HPLDs reveals that LDs actively manage their own redox environment rather than passively enduring oxidative stress. This finding resonates with recent reports in *Arabidopsis* where LDs in drought-stressed leaves recruit CAT and GPX to mitigate lipid peroxidation, suggesting that the function of LDs as platforms for spatially organized antioxidant defense is a conserved feature in the plant kingdom (Xu et al., 2023; Zhao et al., 2025). The spatially organized antioxidant shield model here represents a sophisticated adaptation, explaining how *H. pluvialis* sustains massive astaxanthin accumulation without oxidative collapse. It identifies the stage-specific recruitment of LD-associated antioxidant enzymes as a potential engineering target for enhancing cellular stress tolerance and final astaxanthin yield.

Implications for Inter-Organellar Communication and LD Motility

Our proteomic data provide compelling evidence that HPLDs are hubs for inter-organellar communication, consistent with recent advances in LD biology (Blot et al., 2025; Zhang et al., 2025). The enrichment of vesicular transport proteins (Fig. 8) implies dynamic interactions, potentially involving contact sites with other organelles. Future studies using live-cell imaging are needed to directly assess LD motility in *H. pluvialis*. Furthermore, the diverse subcellular origins of the HPLD proteome imply the existence of physical contact sites with chloroplasts, mitochondria, and the ER, analogous to the ER-LD contact machinery described in mammals and plants. These contacts, potentially mediated by proteins like Caleosin, may enable direct metabolite transfer, enhancing the efficiency of precursor channeling and energy coupling (Blot et al., 2025; Olzmann and Carvalho, 2019; Zhang et al., 2025). The motility of LDs, often linked to the cytoskeleton, likely facilitates these dynamic interactions and their role as communication hubs, though this aspect warrants future investigation in *H. pluvialis*. The stage-specific expression of trafficking components aligns with phased needs: early induction correlates with LD nucleation from the ER; mid-phase enrichment supports sustained expansion via Golgi and endosomal systems; and late-phase profiles may reflect stabilized contacts. Integrating this perspective, we propose that HPLDs are not only metabolic hubs but also communication hubs that physically and functionally link multiple organelles. This dynamic interactome likely underpins the high efficiency of astaxanthin compartmentalization and stress adaptation in *H. pluvialis*, offering a new

dimension for understanding and engineering microalgal metabolite storage.

While our proteomic atlas provides a comprehensive resource and suggests extensive inter-organellar communication, direct physical evidence, such as visualization of contact sites between HPLDs and other organelles by transmission electron microscopy, is still needed to validate these interactions. Furthermore, the proposed functions of key proteins, such as the stage-specific ABC transporters and Caleosin, are inferred from their abundance patterns. Future studies should focus on their functional characterization using targeted gene knockout or overexpression approaches to definitively establish their causal roles in astaxanthin sequestration and LD stabilization.

Conclusion

In summary, this study constructs the first comprehensive, spatiotemporally resolved proteomic atlas of lipid droplets across five critical encystment stages in *Haematococcus pluvialis*. By quantifying 3,396 high-confidence proteins, we demonstrate that dynamic remodeling of the HPLD proteome drives its functional evolution from a passive lipid reservoir into an active metabolic and communication hub, thereby orchestrating astaxanthin hyperaccumulation.

Our findings reveal that HPLDs govern encystment progression through a tightly regulated functional succession: from initial precursor mobilization and transient biosynthetic bursts during early expansion, to sustained metabolite trafficking, and finally, dramatic structural reinforcement coupled with the assembly of a compartmentalized antioxidant shield in the mature cyst. Integrating these insights, we propose a refined two-step sequestration and stabilization model for astaxanthin, wherein its terminal synthesis is spatially uncoupled from, yet coordinated with, protein-facilitated esterification and embedding into the lipid droplet core.

This study fundamentally advances our understanding by positioning HPLDs as central, protein-governed organizers that integrate metabolic, structural, and defensive functions through dynamic protein recruitment. Beyond elucidating the mechanistic basis of astaxanthin biogenesis, this stage-resolved proteomic resource delivers a blueprint for metabolic engineering. The key regulatory nodes identified herein—such as the transient PDAT surge, stage-specific ABC transporters, and the dramatic induction of Caleosin and CAT—represent promising targets for strain improvement to enhance astaxanthin yield. More broadly, the conserved principles of lipid droplet functionalization revealed here offer new perspectives for engineering lipid metabolism

and stress tolerance in diverse plant systems.

Materials and methods

Algal strain and culture conditions

H. pluvialis 192.80 were obtained from Sammlung von Algenkulturen Culture Collection of Algae at Gottingen University. The microalgal cells were inoculated from the agar plate into a 50-ml Erlenmeyer flask containing 30 ml of BBM medium and cultured for six days at 22 °C, light intensity of 25 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with a 16:8 h light/dark cycle, and around 50% RH relative humidity. The culture was then transferred to 300 ml of medium in a 500-ml flask for and incubated for an additional six days under the same conditions. Afterwards, the cells were inoculated at 10% (v/v) into 900-ml columns and cultured for six days (logarithmic phase, 5×10^5 cells $\cdot\text{mL}^{-1}$) to prepare seed cultures under the following conditions: 22 °C, light intensity, 25 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, continuous illumination, around 50% relative humidity, and continuous aeration through a 0.22- μm Millex Syringe driven filter unit (Millipore, Ireland). Subsequently, the algal cells were collected by centrifuging the culture fluid at 1500g for 5 min under sterile conditions at 22 °C. Three biological replicates of these *H. pluvialis* cells were gathered and defined as green vegetative motile stage (stage I, HPLDs-1), while the remaining cells were pooled, and washed with 0N0P BBM medium for three times. The cells were resuspended in the 0N0P BBM medium to achieve a cell density of approximately 5×10^5 cells $\cdot\text{mL}^{-1}$. These suspended cells were evenly divided into 12 aliquots of 1000 mL each in 2000-mL Erlenmeyer flasks and incubated for three days under standard conditions (temperature, 22 °C; light intensity, 25 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; continuous illumination; relative humidity around 50%). Then, 3 of the 12 *H. pluvialis* cultures were randomly selected and collected by centrifugation at 1500g for 5 min, these cells were defined as green nonmotile stage (stage II, HPLDs-2). The remaining 9 *H. pluvialis* cultures were transferred to another high light incubator (light intensity, 350 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; continuous illumination; temperature 22°C; relative humidity 50%) to induce the formation of lipid droplets and astaxanthin accumulation in the cells. The cultures were incubated for 4 days, 12 days, and 22 days to achieve the other three stages of *H. pluvialis*, that is, brown nonmotile stage (stage III, HPLDs-3), orange nonmotile stage (stage IV, HPLDs-4), and red nonmotile cyst stage (stage V, HPLDs-5), respectively. For each stage of *H. pluvialis*, three cultures were randomly selected and the microalgae cells were harvested by centrifugation, each treatment was performed in triplicates. Harvested

cells were immediately frozen with liquid N₂ and kept at 80 °C until further analysis. For proteomic analysis, two out of the three biological replicates per stage were randomly selected for TMT labeling and LC-MS/MS analysis.

Growth parameter measurements and pigment analysis

At each stage of LDs isolation, *H. pluvialis* cells were examined with an Olympus BX61 microscope equipped with an Olympus DP10 digital camera to monitor cell morphology and color changes. Growth, lipid and pigments analyses were quantified according to a previously established method (Hu et al., 2021).

Lipid droplet isolation and purification

LDs from *H. pluvialis* (HPLDs) were isolated and purified as previously described with some modifications based on density gradient centrifugation (Fig. 2A) (Hu et al., 2021; Wang et al., 2019; Yan et al., 2025). Briefly, the harvested microalgal cells were washed three times using Tris buffer (10mM Tris–HCl, pH 7.6) by centrifugation at 3,000 × g for 10min at room temperature, resuspended in the sterile lysis buffer (0.2 M sucrose, 10 mM MOPs, 10 mM KCl, 5 mM NaEDTA, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, and protease inhibitor cocktail (cOmplete, Roche Diagnostics), pH 7.4 adjusted with KOH), and disrupted in Lysing Matrix E tubes (MP biomedical, Irvine, CA, USA) using a semi-automatic homogenizer (FastPrep[®] instrument). Cell breakage was verified based on light optical microscopy measurements (Olympus BX61, Olympus, Hamburg, Germany). Unbroken cells and large pieces of cell debris were removed as a pellet by centrifugation at 1,500 × g for 10 min at 4 °C, and the supernatant containing the LDs was collected. A discontinuous sucrose gradient (0.8M, 0.6M, 0.4M, and 0.2M sucrose in lysis buffer) was prepared in 10 mL SW40Ti tubes by layering 2 mL each of lysis buffer from bottom to top. Approximately 1.0 mL of the post-1500 × g supernatant was carefully loaded on top of the gradient. The tubes were centrifuged again at 25,000 × g for 60 min at 4 °C (SW40Ti rotor, Beckman Coulter, Brea, CA, USA), the floating pad of crude HPLDs was recovered from the surface of the sucrose cushion (approximately 500 µL per tube). And transferred to a new SW40Ti tube. The crude HPLDs fraction was diluted in 8 mL sucrose lysis buffer and re-centrifuged at 100,500 × g for 60 min at 4°C. Harvested aliquots of the purified HPLDs was collected (500 µL per tube). The collected LDs were then centrifuged at 20,000 × g for 10 min at 4 °C to remove any remaining buffer at the bottom using a manual syringe. The concentrated HPLDs were then incubated with 1 mL weak detergent buffer (0.2%

Triton X-100/Tris-HCl buffer, (v/v)) on ice for 10 min, followed by $20,000 \times g$ centrifugation for 10 min at 4 °C to remove the debris. The washing was repeated three times. Finally, the HPLDs were washed twice with the Tris buffer (Tris-HCl buffer, 10 mM, pH 7.6) at $20,000 \times g$ for 10 min at 4 °C, collected and stored at -80 °C until further analysis. All procedures were performed on ice unless specified and were carried out in three independent biological replicates.

Observation of microalgal morphology and HPLDs

Light microscopy of our preparations showed a mixed-sized population of yellow–red globules ranging from about 200 nm to 5 µm in diameter (Fig. 2). Isolated HPLDs were imaged under differential interference contrast (DIC) and fluorescence modes according a previously described method (Wang et al., 2019). For fluorescence imaging, HPLDs were stained with the fluorescence BODIPYTM 505/515 (Catalog No. D3921, Invitrogen) at a working concentration of 1 mg/mL, and observed under a Leica TCS SP5II DMI6000CS Fluorescence Microscope (Leica, Wetzlar, Germany). BODIPYTM 505/515 fluorescence was detected with excitation at 488 nm and emission between 579 and 641 nm, whereas chlorophyll fluorescence was monitored by excitation at 488 nm and capture zone ranging from 650 and 700 nm. Bright field acquisitions were also performed. Acquisition was saved under the Zeiss. czi format and imported with ImageJ 1.48v using the BIO-FORMATS plugins 5.7.2 for analysis.

Contamination evaluation of purified HPLDs

Proteins derived from the purified HPLDs were extracted and removed residual lipids by incubation in cold acetone and ethyl acetate at -20 °C, and then dissolved in the lysis buffer as previously described (Lupette et al., 2019; Wang et al., 2019). The proteins were stored at -80 °C until further analysis. To assess possible contamination of LDs by other organelles, Western blot analysis was independently performed as previously described using three markers, RBCL, AOX, and BIP, which target to chloroplast, mitochondria, and endoplasmic reticulum (ER), respectively (Wang et al., 2019). Total HPLD-derived proteins were obtained from three biological replicates in equal amounts (10 µg per replicate) and pooled together to form mixed HPLD protein samples across all five life stages (HPLDs-1 to HPLDs-5). Stage-matched control pools were similarly prepared from total cellular proteins. Antibodies for these markers were obtained from Agrisera AB (Catalog Nos. AS03037, AS06152, and AS09481, Vännäs, Sweden).

Proteomic analyses

For proteomic analyses, HPLDs-derived proteins of all triplicate biological replicates were extracted and purified as described in the preceding section. Two replicates (R1 and R2) were randomly selected for independent proteome analysis. The concentration of the purified HPLDs proteins was determined using a BCA Protein assay kit (ThermoFisher scientific, California, USA). The quantity of purified HPLD proteins was evaluated on proper aliquots, loaded onto SDS-PAGE and stained using the SilverQuest staining kit (Invitrogen, Carlsbad, CA, USA). The remaining proteins were concentrated and eluted for TMT-based quantitative proteomics analysis (Wang et al., 2024). The determination of HPLDs' lipids and proteins were performed as follows. Although three biological replicates were harvested per stage, two replicates per stage were randomly selected for proteomic analysis to balance analytical depth and cost-effectiveness. The high reproducibility between replicates was confirmed by principal component analysis and hierarchical clustering (Fig. 4A, C). This approach is consistent with previously published large-scale proteomic studies of lipid droplets (e.g., Brocard et al., 2017; Wang et al., 2019).

Protein determination

RAW proteomic data were processed using Proteome Discoverer (Thermo Scientific, Version 2.4) against the UniProt database (UP000485058, 28,444 entries). The search parameters included a precursor mass tolerance of 20 ppm and fragment mass tolerance of 0.02Da, and a false discovery rate (FDR) ≤ 0.01 for peptide identification. Differently abundant proteins (DAPs) were identified using a threshold of $|\log_2FC| \geq 0.585$, p value < 0.05 for pairwise comparisons.

Protein functions and subcellular localizations were annotated and predicted using EggNOG (Evolutionary genealogy of genes: Non-supervised Orthologous Groups), Gene ontology (GO), KEGG, Non-Redundant Protein Sequence Database (NR), Pfam, String, Universal Protein (Uniprot), Wolfpsort, and CELLO. Protein-protein interaction (PPI) networks were analyzed using String v11.5. GO and KEGG enrichment significance was defined as p value < 0.05 using a two-tailed Fisher's exact test. Functional annotation and enrichment analysis focused primarily on pathways and processes related to lipid metabolism, carotenoid metabolism, lipid droplet biogenesis and dynamics, membrane trafficking, and stress response. Proteomic data analysis was performed using ISanger eCloud Platform (Majorbio), and the Majorbio cloud platform (<https://cloud.majorbio.com>) (Ren et al., 2022).

Statistical analysis

All numerical data were analyzed using IBM SPSS Statistics 25 and GraphPad Prism 8.0.1. Results are presented as mean $\pm$ standard error and visualized with bar graphs or dot graphs. Differences between two groups were conducted by unpaired two-tailed Student t-test, p -value < 0.05 was regarded as statistically significant. For comparison among multiple groups, one-way ANOVA followed by Tukey's post hoc test was performed, and differences were considered statistically significant at $p < 0.05$. When assumptions normality and homogeneity of variance was violated, Kruskal-Wallis test was used. For lipidomic data, false discovery rate (FDR) correction was performed to adjust p -value. Differentially abundant proteins (DAPs) were identified using thresholds of $|\log_2FC| \geq 0.585$ and an adjusted p -value < 0.05 .

Accession numbers

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD069513.

Acknowledgments

We apologize for not being able to discuss or cite many studies due to limited space. We thank the Sammlung von Algenkulturen (Göttingen University, Germany) for providing the *Haematococcus pluvialis* strain 192.80. We also acknowledge the support of the Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) for providing the bioinformatics analysis platform. And, we would like to thank the editors, and the anonymous readers who provided me with such helpful comments and advice during the revision process.

During the preparation of this manuscript, the authors used DeepSeek, a large language model-based AI assistant, for language polishing and improving the clarity and readability of the text. The authors have thoroughly reviewed and edited all AI-assisted content and take full responsibility for the final version of the manuscript.

Author contributions

Q.-J. H. designed research, performed experiments, and wrote the paper; T.-W.Y. and Y.-Y.W. analyzed data; Q.-J. L, Y.-H. T and Q.-S. X provided resources; C.-G.W. and X.J.Y. supervised the project and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Supplementary Material

The data generated in this study are included in this article and the online supplementary

material:

Supplementary Figure S1. Time-series clustering of differentially abundant proteins in HPLDs across five life cycle stages of *H. pluvialis*. Fifteen distinct protein clusters (Cluster 1–15) were identified by Mfuzz analysis, representing temporal expression patterns of 2771 differentially abundant proteins (DAPs) across HPLDs-1 to HPLDs-5. Each line represents the standardized abundance trend of proteins within a cluster throughout encystment. Clusters were subsequently grouped into four major expression trends: continuously increasing (Group 1), continuously decreasing (Group 2), transiently peaking (Group 3), and U-shaped (Group 4), as summarized in Table S2.

Supplementary Table S1. Proteomic annotation and stage-specific expression profiles of 3,396 proteins across five life cycle stages of HPLDs during encystment.

Supplementary Table S2. KEGG pathway enrichment of the identified DAPs in four groups based on Mfuzz analysis.

Funding

This research was supported by the Scientific Research Foundation for Talents of Zhejiang Ocean University (JX6311103223), the National Key R&D Program of China (2021YFA0910800), and the General Research Project of Zhejiang Provincial Education Department (Y202353950).

Data availability

The data underlying this article are available in the article and the online supplementary material.

981 Table 1. Abundance analysis of PDAT, Caleosin and antioxidant enzymes within DAPs of HPLDs across *H. phuvialis* life cycle stages.

Protein	Subcellular		HPLDs-1	HPLDs-2	HPLDs-3	HPLDs-4	HPLDs-5
	Accession Number	Location					
PDAT	MSTRG.25016.1_pep1	nuclear	0.94±0.09 ^c	8.58±0.85 ^b	12.50±1.88 ^a	1.56±0.34 ^c	1.33±0.23 ^c
Caleosin	MSTRG.13498.4_pep1	cytoplasmic	0.94±0.09 ^c	1.81±0.20 ^b	2.08±0.08 ^b	1.88±0.01 ^b	10.76±6.92 ^a
	MSTRG.49609.4_pep1	peroxisomal	1.04±0.06 ^b	1.26±0.12 ^b	1.54±0.08 ^b	4.01±0.16 ^a	4.03±3.59 ^{ab}
ABC	MSTRG.49609.16_pep4	Golgi apparatus	1.08±0.12 ^c	2.89±0.42 ^b	4.18±0.68 ^b	10.37±0.99 ^a	9.27±7.76 ^{abc}
Transporters	MSTRG.43734.1_pep1	cytoplasmic	0.99±0.01 ^c	3.95±0.12 ^a	3.85±0.84 ^{ab}	2.32±0.02 ^b	1.67±0.10 ^b
	MSTRG.33214.1_pep1	cytoplasmic	0.89±0.15 ^d	5.14±0.26 ^{ab}	5.96±0.99 ^a	3.83±0.33 ^b	1.95±0.03 ^c
SOD	Isoform000473	chloroplast	1.01±0.02 ^a	0.51±0.00 ^b	0.24±0.10 ^c	0.27±0.01 ^c	0.49±0.05 ^b
	MSTRG.68495.1_pep1	mitochondrial	0.98±0.02 ^c	3.00±0.10 ^b	2.79±0.04 ^b	3.72±0.18 ^a	0.56±0.05 ^d
	MSTRG.47782.1_pep1	cytoplasmic	1.00±0.00 ^a	0.71±0.02 ^b	0.73±0.09 ^b	0.38±0.04 ^c	0.32±0.02 ^c
	MSTRG.27083.1_pep1	chloroplast	1.00±0.01 ^a	0.13±0.00 ^c	0.10±0.03 ^c	0.11±0.03 ^c	0.28±0.00 ^b
	MSTRG.58476.3_pep4	ER	1.02±0.02 ^b	6.38±0.09 ^b	11.17±0.98 ^b	27.59±7.90 ^a	2.94±1.01 ^b
	MSTRG.45957.1_pep3	mitochondrial	0.99±0.01 ^c	1.92±0.00 ^b	1.88±0.01 ^b	2.63±0.02 ^a	0.70±0.05 ^d
	MSTRG.7898.3_pep1	chloroplast	1.02±0.02 ^a	0.13±0.02 ^c	0.12±0.04 ^c	0.12±0.01 ^c	0.30±0.01 ^b
	MSTRG.66665.1_pep1	cytoplasmic	0.99±0.01 ^a	0.16±0.02 ^c	0.10±0.02 ^c	0.09±0.02 ^c	0.48±0.03 ^b
CAT	MSTRG.64600.2_pep1	chloroplast	1.08±0.11 ^b	2.04±0.03 ^b	2.50±0.21 ^b	2.99±0.09 ^b	20.33±1.53 ^a

GSR	Isoform008838	chloroplast	0.99±0.02 ^a	0.34±0.01 ^b	0.19±0.00 ^c	0.12±0.01 ^c	0.37±0.01 ^b
	MSTRG.24197.1_pep1	cytoplasmic	0.97±0.05 ^c	1.91±0.01 ^a	1.67±0.05 ^b	0.41±0.05 ^d	0.51±0.01 ^d
	MSTRG.16622.2_pep1	cytoplasmic	1.07±0.10 ^{bc}	1.48±0.09 ^{ab}	1.75±0.02 ^a	0.76±0.29 ^{cd}	0.54±0.07 ^{cd}
	MSTRG.13447.1_pep1	chloroplast	0.99±0.02 ^b	2.07±0.03 ^a	1.76±0.25 ^a	0.28±0.05 ^c	0.30±0.05 ^c
	MSTRG.3939.2_pep1	chloroplast	0.97±0.04 ^a	0.37±0.03 ^b	0.22±0.03 ^c	0.24±0.00 ^c	0.40±0.03 ^b
	MSTRG.13447.3_pep1	cytoplasmic	1.00±0.00 ^b	1.71±0.07 ^a	1.68±0.13 ^a	0.90±0.19 ^b	0.45±0.02 ^c
	MSTRG.53322.2_pep1	ER	0.98±0.03 ^a	0.77±0.00 ^b	0.52±0.00 ^c	0.35±0.01 ^c	0.78±0.09 ^b
GPX	MSTRG.63848.7_pep1	chloroplast	1.03±0.04	1.16±0.08	2.02±0.36	0.98±0.04	2.11±1.38
	MSTRG.59575.1_pep1	chloroplast	0.98±0.03 ^a	0.59±0.01 ^b	0.74±0.09 ^b	0.25±0.01 ^c	1.07±0.08 ^a

Note: Values within a column followed by different lowercase letters are significantly different at $p < 0.05$ according to unpaired two-tailed Student t-test.

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