FRONT MATTER

Title

- Full title: **HSPB8 regulates CTP synthase filaments to couple nucleotide metabolism and autophagy in tumors**

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Abstract

Metabolic enzyme compartmentalization is increasingly recognized as a regulatory strategy, yet its functional relevance in cancer remains incompletely understood. Here, we demonstrate that polymerization of cytidine triphosphate synthase (CTPS) into filaments is essential for tumor growth in vivo and for three-dimensional proliferation. Glutamine deprivation, a hallmark of the nutrient-poor core of solid tumors, promotes CTPS filament assembly, suppressing enzymatic activity and limiting phospholipid synthesis and autophagic flux. Using proximity labeling, we identify the chaperone HSPB8 as a filament-associated

regulator. HSPB8 antagonizes CTPS filament assembly by facilitating clearance of misfolded proteins, thereby restoring CTPS activity. Filament disassembly enhances CTP production, elevates autophagy-related phosphatidylinositol and phosphatidylethanolamine synthesis, and accelerates autophagic flux. Disruption of CTPS filament formation impairs xenograft tumor growth. Consistently, analysis of patient datasets reveals that high CTPS and low HSPB8 expression correlate with poor survival across multiple cancers. These findings identify CTPS filament dynamics as a metabolic vulnerability in tumors.

MAIN TEXT

Introduction

Adapting to chronic nutrient scarcity is a fundamental challenge for malignant progression. In solid tumors, poor vascularization leads to profound glutamine deprivation, forcing cancer cells to develop sophisticated survival strategies (*1*). To maintain amino acid homeostasis, cells utilize a coordinated network of membrane transport, de novo biosynthesis, and proteostasis—the balance between protein synthesis and autophagic breakdown. Among these adaptive responses, cells increasingly rely on asparagine (Asn) to maintain proliferation under glutamine-depleted conditions (*2-4*). Consequently, asparaginase treatment—which depletes extracellular and intracellular asparagine—is a key therapeutic strategy (*5, 6*). However, the efficacy of this treatment is often limited by adaptive cellular responses, including the induction of autophagy (*7-9*).

Emerging evidence suggests that cellular metabolism is regulated through the dynamic compartmentalization of enzymes into membrane-less organelles or biomolecular condensates. Cytidine triphosphate synthase (CTPS), the rate-limiting enzyme in de novo pyrimidine synthesis, is a prominent example of this phenomenon. CTPS catalyzes the ATP-dependent conversion of UTP to CTP using glutamine as a nitrogen donor. Under glutamine-deprived conditions, mammalian CTPS undergoes a phase transition to form higher-order polymers and filament bundles, facilitated by specific residues in the linker (R294) and glutaminase (H355) domains (*10, 11*). Furthermore, we have previously shown that the amino acid, histidine can trigger these filamentous structures to assemble along with cytokeratin networks and allow rapid cell growth after stress relief (*12, 13*). While these filaments are known to respond to nutrient cues, the molecular machinery that coordinates their assembly with broader cellular survival programs remains poorly defined.

The maintenance of proteostasis is primarily overseen by molecular chaperones, among which the small heat shock protein B8 (HSPB8) plays a specialized role. Unlike general chaperones, HSPB8 specifically recognizes unfolded peptides and defective ribosomal products (DRiPs), targeting them for degradation via the chaperone-assisted selective autophagy (CASA) complex (*14-16*). By forming a functional unit with the co-chaperone BAG3 and the scaffold protein HSP70, HSPB8 has been shown to actively promote autophagic flux to clear proteotoxic aggregates (*17-19*). While HSPB8 is well-characterized in the context of neurodegenerative protein aggregation, its involvement in regulating the

structural dynamics of metabolic enzymes has not been explored. Given that metabolic stress often coincides with proteostatic imbalance, HSPB8 emerges as a potential link between the cellular protein quality control machinery and the adaptive compartmentalization of enzymes like CTPS.

In this study, we identify the availability of asparagine as a key environmental factor governing CTPS filament dynamics. Through proteomic screening, we discovered that HSPB8 serves as a critical metabolic rheostat that antagonizes CTPS polymerization. We demonstrate that filament assembly is driven by the accumulation of unfolded peptides, which act as structural scaffolds for filament bundling. Our results reveal that the HSPB8-mediated disassembly of CTPS filaments is a requisite step for activating autophagic flux under nutrient stress. By releasing soluble, active CTPS, cells increase CTP production to fuel the phospholipid biosynthesis required for autophagosome membrane expansion. Crucially, we show that disrupting this balance to reduce CTPS filament formation leads to significantly impaired tumor growth, identifying the HSPB8-CTPS-autophagy axis as a vital survival response. These findings define a therapeutic vulnerability in cancers with high CTPS expression and provide a new perspective on how cells coordinate their physical and chemical states to survive starvation.

Results

CTPS filament assembly is essential for xenograft tumor progression

Given that CTPS filaments have been identified in clinical tumor specimens (*20*), we hypothesized that these macromolecular structures serve a functional role in oncogenesis. To investigate whether CTPS polymerization regulates tumor growth *in vivo*, we performed xenograft experiments using HCT116 cells expressing either wild-type (WT) CTPS or the filament-deficient H355A mutant (Fig. 1A-B). The filament-deficient H355A mutant cells exhibited a significant reduction in tumor volume compared to WT controls (Fig. 1C-E). Notably, these phenotypic differences occurred in the absence of systemic toxicity, as indicated by stable murine body weights across cohorts (Fig. 1F).

To further elucidate the mechanisms driving this growth disparity, we characterized the proliferative capacity of these cells under varying environmental conditions. Under glutamine deprivation in three-dimensional (3D) culture (Gln(-)S(+)), HCT116 cells showed negligible growth. However, in nutrient-replete conditions (Gln(+)S(+)), the H355A mutant displayed significantly impaired proliferation specifically in suspension sphere assays (Fig. S1B). This contrast is striking given that the mutant and WT cells exhibited indistinguishable growth rates in standard two-dimensional (2D) monolayer cultures (Fig. S1A). Collectively, these data suggest that CTPS filament assembly is not merely a cellular feature but a critical regulator of 3D proliferative capacity and *in vivo* tumor formation.

Asparaginase inhibits CTP synthase polymerization by depleting intracellular asparagine pools

Asparagine (Asn) is a known survival factor during glutamine deprivation (*3*). Consistent with this role, we observed that CTP synthase (CTPS) filament formation under glutamine-

124 deprived conditions was inhibited by asparaginase (ASNase) treatment (Fig. 2, A to F) without
125 altering total CTPS protein levels (Fig. 2G). Conversely, exogenous Asn supplementation
126 promoted CTPS polymerization (Fig. 2, C to F). To further define the specificity of this effect,
127 we screened the remaining non-essential amino acids—alanine, asparagine, aspartate, and
128 proline—for their capacity to induce filamentation. Our results confirm that Asn induces
129 CTPS filaments, comparable to the histidine positive control (Fig. S2A). Supporting this, the
130 combination of Asn and ASNase treatment nearly abolished CTPS filamentation in EBSS, a
131 conditional medium without any amino acid supplementation (Fig. S2B).
132 Given that Asn is absent from DMEM, we hypothesized that serum serves as the primary
133 source of Asn for filament assembly. Quantitative metabolic analysis revealed that Asn levels
134 increase during glutamine deprivation relative to standard DMEM but are depleted upon
135 serum withdrawal or ASNase treatment (Fig. S2C). Although extracellular nutrients can be
136 scavenged via macropinocytosis to support proliferation under nutrient stress (*21*), Asn can
137 also be synthesized de novo by asparagine synthetase (ASNS). However, ASNS knockdown
138 did not affect CTPS filamentation (Fig. S2, D and E), likely due to substrate limitations of
139 glutamine required for ASNS enzymatic activity (*22*). Collectively, these data demonstrate that
140 intracellular Asn levels regulate CTPS filament dynamics, suggesting a metabolic adaptation
141 where cells modulate amino acid homeostasis to confer nutrition stress during glutamine
142 deprivation.

143 **Identification of CTPS-associated proteins via APEX2 proximity labeling**

144 To elucidate the mechanisms governing CTP synthase (CTPS) filament dynamics, we
145 employed APEX2-based proximity labeling to identify protein interactors in situ (*23*).
146 Although CTPS filaments assemble during nutrient starvation, their structural integrity is
147 highly sensitive to cell lysis, which typically precludes traditional biochemical purification.
148 We therefore generated HEp-2 cell lines stably expressing a CTPS-APEX2 fusion protein to
149 biotinylate proximal proteins in living cells (*13*). To ensure high specificity for filament-
150 associated partners, we utilized two negative controls representing a non-filamentous state:
151 glutamine-induced disassembly and a polymerization-deficient R294D mutant (*12, 24*) (Fig.
152 3A). Western blot analysis confirmed that both endogenous CTPS and exogenous CTPS-
153 APEX2 levels were markedly elevated under glutamine deprivation, consistent with reports
154 that filamentation protects CTPS from proteasomal degradation (*12*). Furthermore, the
155 enrichment of the known filament-associated protein IMPDH2 (*25*) in the CTPS-APEX2 pull-
156 down validated the spatial accuracy and efficacy of our proximity labeling system (Fig. 3B).

157 **Discovery of HSPB8 as a CTPS filament-associated protein regulated by asparaginase**

158 To define the CTPS filament proteome with high stringency, we integrated data from two
159 independent proteomics experiments and applied a fold-change cutoff based on IMPDH2
160 enrichment (Fig. S3, A to C). This filtering strategy yielded 184 overlapping proteins
161 significantly enriched in pathways involving de novo purine biosynthesis and protein
162 ubiquitination (Fig. 3C). IMPDH2 and PFAS in the purine de novo biosynthesis category have
163 been demonstrated to form distinct compartments, filament, and purinosome, respectively (*26,*

27). Notably, the identification of multiple heat shock proteins (e.g., HSPA4, HSPB8, HSPH1) and deubiquitinating enzymes in the protein ubiquitination category suggests that protein quality control (PQC) machinery localizes proximally to CTPS filaments during metabolic stress. Given that asparagine (Asn) is a key effector of ATF4-mediated amino acid homeostasis, we hypothesized that Asn availability modulates this PQC-filament association (*4, 28*). Quantitative proteomics and subsequent western blotting validation revealed that the small heat shock protein HSPB8 is downregulated during glutamine deprivation but specifically upregulated upon ASNase treatment (Fig. 3, D and E). We further confirmed the physical proximity between CTPS and HSPB8 through a proximity ligation assay (PLA) (Fig. S3D), establishing HSPB8 as a putative regulator of CTPS filament dynamics under glutamine-deprived stress.

HSPB8 regulates the compartmentalization of CTPS filaments

HSPB8, a member of the small heat shock protein (sHSP) family, acts in concert with BAG3—and often within the chaperone-assisted selective autophagy (CASA) complex alongside HSP70 and E3 ligases—to facilitate the clearance of misfolded or aggregation-prone protein. Given its established role in maintaining proteostasis, we hypothesized that the ASNase-induced upregulation of HSPB8 promotes the disassembly of CTPS filaments. To test this, we performed loss- and gain-of-function assays to evaluate the requirement of HSPB8 for CTPS filament dynamics. Under glutamine-deprived conditions, HSPB8 knockdown significantly enhanced CTPS filament assembly compared to control groups (Fig. 4, A and B). Conversely, we observed that the restoration of glutamine, which typically triggers filament disassembly, was less effective in the absence of HSPB8, resulting in persistent filament structures (Fig. 4, A and B).

In contrast to the knockdown phenotypes, overexpression of HSPB8 markedly reduced the prevalence of CTPS filaments assembly, suggesting that HSPB8 facilitates the release of CTPS from filamentous compartments into the cytosol (Fig. 4, C and D). Crucially, we found that HSPB8 depletion was sufficient to restore CTPS filament assembly even in the presence of ASNase treatment, which normally prevents filament assembly (Fig. 4, E and F). Collectively, these results demonstrate that ASNase disassembles CTPS filaments by upregulating HSPB8, identifying this chaperone as a key molecular rheostat for CTPS assembly during glutamine-deprived stress.

Clinical significance of the HSPB8-CTPS axis in cancer prognosis

Although CTPS expression and enzymatic activity have been established as drivers of malignant progression (*29*), the clinical relevance of its regulatory partnership with HSPB8 remains poorly defined. To investigate this, we analyzed expression profiles across several cancer types using The Cancer Genome Atlas (TCGA) database. Our analysis revealed that CTPS expression is significantly elevated, while HSPB8 expression is markedly reduced, in multiple malignancies compared to adjacent normal tissues, including breast invasive carcinoma (BRCA), colon adenocarcinoma (COAD), head and neck squamous cell carcinoma (HNSCC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), rectum

adenocarcinoma (READ), stomach adenocarcinoma (STAD), and thyroid carcinoma (THCA) (Fig. S4, A and B).

To evaluate the prognostic impact of this inverse correlation, we performed Kaplan-Meier survival analysis. We found that patients with high CTPS expression combined with low HSPB8 expression exhibited significantly poorer survival outcomes in adrenocortical carcinoma (ACC), pancreatic adenocarcinoma (PAAD), sarcoma (SARC), and pheochromocytoma and paraganglioma (PCPG) (Fig. S4C). These clinical data suggest that an environment characterized by low HSPB8 levels—which we have shown promotes CTPS filament assembly—may drive tumor progression and serves as a negative prognostic signature in several human cancers.

Glutamine deprivation-induced misfolded protein accumulation drive CTPS filamentation

Since HSPB8 targets misfolded proteins for autophagic clearance and our proteomic data identified protein quality control (PQC) machinery in the CTPS interactome (Fig. 3C), we hypothesized that the accumulation of unfolded proteins modulates CTPS filament dynamics. Proximity ligation assays (PLA) confirmed that the CTPS-HSPB8 association is spatially restricted to CTPS filaments (Fig. S5A). Furthermore, we observed an increased association between HSPB8 and its co-chaperone BAG3 under glutamine deprivation (Fig. S5B), suggesting an active role for the HSPB8-BAG3 complex in managing protein misfolding during metabolic stress.

To evaluate the direct impact of proteotoxic stress on CTPS polymerization, we treated cells with puromycin to induce the premature termination of translation and the subsequent generation of defective ribosomal products (DRiPs) (*30*). Under glutamine-deprived conditions, puromycin treatment resulted in the formation of significantly thicker CTPS filaments compared to untreated controls (Fig. 5, A and B). The proximity biotin-labeling with streptavidin pull-down experiment revealed that puromycylated polypeptides are enriched in the immediate vicinity of CTPS filaments (Fig. 5C), implying that local proteotoxic stress exerts a stabilizing effect on filament dynamics.

Consistent with its role in protein clearance, the knockdown of HSPB8 led to a marked accumulation of puromycylated polypeptides (Fig. 5D and Fig. S5C). Conversely, the intensity of these misfolded protein signals significantly declined upon ASNase treatment (Fig. 5E). Taken together, these findings demonstrate that HSPB8 regulates CTPS filament dynamics by modulating the local concentration of unfolded proteins, positioning the filament as a metabolic structure sensitive to cellular proteostasis.

CTPS filament disassembly promotes autophagic flux under glutamine-deprived stress

Autophagy is a critical survival mechanism during asparaginase-induced cytotoxicity. Given that CTP is a requisite substrate for the de novo synthesis of choline phospholipids—catalyzed by PCYT1A (phosphate cytidylyltransferase 1, choline, alpha) to replenish autophagosome membranes (*31*)—we investigated whether the disassembly of CTPS filaments enhances autophagic capacity. We previously established that non-filamentous CTPS

244 possesses higher enzymatic activity than its polymerized counterpart. Consistent with this, our
245 in vivo enzymatic assays, measuring the $^{13}C^{15}N$-CTP to $^{13}C^{15}N$-UTP ratio, demonstrated that
246 filament-null mutants (R294D and H355A) exhibit significantly higher catalytic activity
247 compared to wild-type CTPS (Fig. S6A).
248 To determine if increased CTPS activity promotes autophagy, we utilized mRFP-GFP-
249 LC3 reporter cell lines to monitor autophagic flux (*32*). Under glutamine deprivation,
250 $CTPS^{R294D}$ or $CTPS^{H355A}$ cells displayed a higher density of mRFP-positive/GFP-negative
251 puncta per cell than $CTPS^{WT}$ cells, indicating an acceleration of autolysosome formation (Fig.
252 6, A and B; Fig. S6, B and C). Transmission electron microscopy (TEM) further confirmed a
253 marked accumulation of both early (AVi) and late (AVd) autophagic vacuoles in filament-null
254 mutants, whereas wild-type cells exhibited significantly fewer structures (Fig. 6, C and D; Fig.
255 S6, D and E). To distinguish between increased induction and blocked degradation, we treated
256 cells with Bafilomycin A1 (BafA1) to inhibit lysosomal fusion. Under BafA1 treatment,
257 filament-null cells accumulated significantly higher levels of LC3-II compared to wild-type
258 controls, confirming that CTPS disassembly actively enhances autophagic flux (Fig. 6, E and
259 F). Collectively, these findings suggest that the metabolic regulation of CTPS filament
260 dynamics modulates autophagic activity.

261 **HSPB8 promotes autophagy by antagonizing CTPS filament assembly**

262 Building on findings identifying the chaperone HSPB8 as a negative regulator of CTP
263 synthase (CTPS) polymerization, we investigated the role of CTPS filament dynamics within
264 the HSPB8-autophagy axis during glutamine-deprived nutrient stress. HSPB8, a member of
265 the small heat shock protein family, specifically binds unfolded peptides and has been
266 previously implicated in the induction of autophagy (*33*). To determine if CTPS filament
267 disassembly is a requisite step in this process, we evaluated autophagic flux under glutamine-
268 and serum-deprived conditions (Gln(-)S(-))In cells expressing wild-type CTPS ($CTPS^{WT}$),
269 overexpression of HSPB8 significantly increased the number of autolysosomes (mRFP-
270 positive/GFP-negative) and total autophagic vesicles compared to vehicle-treated controls
271 (Fig. 7, A, C, and E). In contrast, HSPB8 overexpression failed to further enhance autophagic
272 vesicle accumulation in filament-deficient $CTPS^{H355A}$ cells (Fig. 7, B, D, and E). These results
273 suggest that the pro-autophagic effect of HSPB8 under glutamine deprivation is mediated
274 through the inhibition of CTPS filament assembly, identifying the transition from polymerized
275 to dimeric or tetrameric CTPS as a critical metabolic switch for autophagic activation.

276 **Reduced volume of $CTPS^{H355A}$ tumors correlates with excessive autophagy activity**
277 Given that $CTPS^{H355A}$ cells exhibit both impaired filament assembly and elevated
278 autophagy in vitro, we sought to determine if this relationship holds true in a physiological
279 tumor model. We analyzed the widely-used autophagy marker LC3B in $CTPS^{WT}$ - and
280 $CTPS^{H355A}$ -derived tumors (from Fig. 1) to understand how defective filament assembly
281 interferes with tumor progression (Fig. S7A). Consistently, the LC3B-II/LC3B-I ratio, a
282 hallmark of autophagic flux, was elevated in $CTPS^{H355A}$ tumors (Fig. S7B). These findings
283 suggest that the anti-tumor effect observed when CTPS filament assembly is disrupted is

284 associated with an induction of excessive autophagic activity.

285 **Phospholipid biosynthesis is upregulated in filament-deficient CTPS mutants**

286 Because CTPS facilitates phospholipid biosynthesis through CTP production, we
287 hypothesized that filament-deficient mutants exhibit elevated autophagy due to an increased
288 supply of membrane building blocks. Given that lipids and lipid-associated proteins are
289 indispensable for autophagosome formation and maturation, we performed liquid
290 chromatography-mass spectrometry (LC-MS) to compare the lipidomic profiles of $CTPS^{WT}$
291 and $CTPS^{R294D}$ HEp-2 cells. We identified 75 annotated lipids that met the significance criteria
292 of $P < 0.05$, fold change > 1.3, and a Variable Importance in Projection (VIP) score > 1 (Fig.
293 8A and Fig. S8, A to C). A heatmap of 43 high-confidence lipids which was verified by
294 MS/MS identification revealed a consistent elevation in $CTPS^{R294D}$ cells, correlating with the
295 increased enzymatic activity of the non-filamentous mutant (Fig. 8B). LIPEA pathway
296 analysis indicated a significant enrichment of autophagy-related lipids, specifically
297 phosphatidylethanolamines (PE) and phosphatidylinositols (PI), which were significantly
298 higher in $CTPS^{R294D}$ cells (Fig. 8, C and D). Since PI and PE are essential for autophagosome
299 membrane initiation and expansion, these findings suggest that the metabolic output of
300 disassembled CTPS directly supports the lipid requirements for enhanced autophagic flux.
301

302 **Discussion**

303 Metabolic enzymes increasingly are recognized for their ability to undergo higher-order
304 compartmentalization in response to physiological stress, yet the functional consequences of
305 these transitions remain poorly understood. Our findings demonstrate that the polymerization
306 of CTP synthase (CTPS) into filamentous structures is a critical regulatory event that dictates
307 tumor growth and metabolic adaptation. By integrating biochemical analysis, 3D cell culture,
308 and clinical datasets, we show that CTPS filaments serve as a metabolic switch that balances
309 enzyme activity with proteostatic demands (**Fig. 1 and Fig. S1**). Specifically, under glutamine
310 deprivation—a hallmark of the nutrient-poor core of solid tumors (*1*)—the assembly of CTPS
311 filaments suppresses enzymatic activity, thereby modulating phospholipid availability and
312 autophagic flux to influence tumor progression.

313 A key revelation of this study is the identification of the HSPB8-CTPS filament axis as a
314 novel branch of the cellular protein quality control (PQC) system. Building on our previous
315 evidence that ubiquitination regulates CTPS polymerization (*34, 35*), we identified a suite of
316 PQC-related proteins associated with the filament interactome (**Fig. 3**). The chaperone
317 HSPB8, acting within the HSPB8-BAG3 complex, emerged as a master regulator of these
318 dynamics. Our data suggest that misfolded proteins, or defective ribosomal products (DRiPs),
319 localize to and stabilize CTPS filaments. By modulating the clearance of these proteotoxic
320 species, HSPB8 dictates the architectural state of CTPS. The knockdown of HSPB8 promotes
321 filament bundling, while its overexpression triggers disassembly (**Fig. 4**). This mirrors the role
322 of the HSPB8-BAG3-HSP70 complex in regulating the phase transition of stress granules,
323 suggesting a universal role for HSPB8 in maintaining the fluidity and dynamics of
324 biomolecular condensates and membrane-less organelles.

The biological significance of this regulation is underscored by our analysis of TCGA datasets, where we observed a pervasive inverse relationship between CTPS and HSPB8 expression across 75% of cancer types (**Fig. S4, A and B**). The "high-CTPS/low-HSPB8" signature—which our model predicts would favor stable filament formation—correlates significantly with reduced patient survival (**Fig. S4C**). This suggests that the stabilization of CTPS filaments may provide a selective advantage in aggressive malignancies, likely by sequestering enzymatic activity until metabolic conditions require its mobilization. Consequently, the molecular machinery governing CTPS assembly represents a promising, yet unexplored, therapeutic vulnerability in cancer.

Mechanistically, we establish that the transition from filamentous to soluble CTPS is a prerequisite for autophagic activation (**Fig. 6 and Fig. S6**). While it is well known that autophagy is essential for surviving starvation, we found that the pro-autophagic effects of HSPB8 are strictly dependent on the filamentous state of CTPS (**Fig. 7**). The disassembly of these structures facilitates CTP production, providing the necessary energy and substrates for phosphatidylcholine biosynthesis, which is required for autophagosome membrane expansion. Our lipidomic profiles confirm that autophagy-essential lipids, such as PI and PE, are upregulated upon filament disassembly (**Fig. 8**). In the context of the tumor microenvironment, the mobilization of CTPS and the resulting surge in autophagy may interfere with tumor growth (**Fig. S7**), as excessive autophagic flux can trigger apoptosis or metabolic exhaustion (*36, 37*).

Finally, our study identifies the structural dynamics of CTPS as a direct downstream target of the cellular PQC response. We propose that the accumulation of unfolded peptides serves as a molecular scaffold that promotes CTPS filament bundling. This process is highly sensitive to the cellular methylation status (*12*), as evidenced by the disassembly of CTPS filaments upon treatment with the methylation inhibitor Adox (**Fig. S2F**). Under metabolic stress and asparaginase treatment, the upregulation of HSPB8 facilitates the clearance of these filament-associated misfolded proteins (**Fig. 2 and Fig. 3E**). This HSPB8-mediated transition, potentially modulated by methylation-dependent signaling (*38*), ensures that cells can reallocate metabolic resources from a sequestered state into active CTP synthesis to meet the demands of autophagic flux. Collectively, these findings position the CTPS filament as a structural bridge between protein quality control and metabolic homeostasis, revealing that targeting the unfolded protein-HSPB8 axis could disrupt the safeguards enabling cancer cell survival (**Fig. 8E**).

Materials and methods

Cells

Human HEp-2 and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% fetal bovine serum. Human HCT116 cells were culture in McCoy's 5A Medium (Sigma). All of cells were maintained at 37°C under 5% CO^2, and passage was done every 3~4 days. Cells were wash with 1X PBS twice before any medium change.

366 **3D spheroid culture**
367 HCT116 cells were seeded in the R^3CE platform (Acrocyte Therapeutics Inc., New Taipei
368 City, Taiwan) and incubated in McCoy's 5A medium (with 10% FBS), and appropriate
369 amount of fresh medium is replenished every 3-4 days. Spheroid images were captured by
370 Nikon Eclipse-TS100 microscope with LeadView-2000AIO camera and the spheroid sizes
371 were quantified by imageJ. The spheroids at day 21 were fixed by 4% formaldehyde for 1 h 30
372 mins and immunostained with CTPS, followed with image capturing by Leica Stellaris SP8
373 confocal microscope.
374
375 **Xenograft mouse**
376 Fresh HCT116 WT-CTPS and H355A-CTPS are suspended in McCoy's 5A (without FBS)
377 with Matrigel (M5A: Matrigel=1:1) and subcutaneously injected into right dorsal flank of
378 NUDE mice (BALB/cAnN.Cg-Foxn1nu/CrlNarl). From tumor start to be seen, tumor volume
379 is calculated by length and width (tumor volume V = ½ (Length × Width2)) twice a week.
380 Until 28 days after injection, mice are sacrificed and tumors are collected for further
381 experiments.
382
383 **Statistical Analysis**
384 Experiment data are analyzed by Student's t test or Kaplan-Meier analysis. Results includes at
385 least 3 biological repeats show means and standard deviation (SD) values with P-values: *<0.05,
386 **<0.01, ***<0.001, ****<0.0001. Other statistical details are included in figure legends.
387
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Writing – review & editing: Y.S.C., C.K.L., and J.G.

Competing interests:

All other authors declare they have no competing interests.

Data and materials availability:

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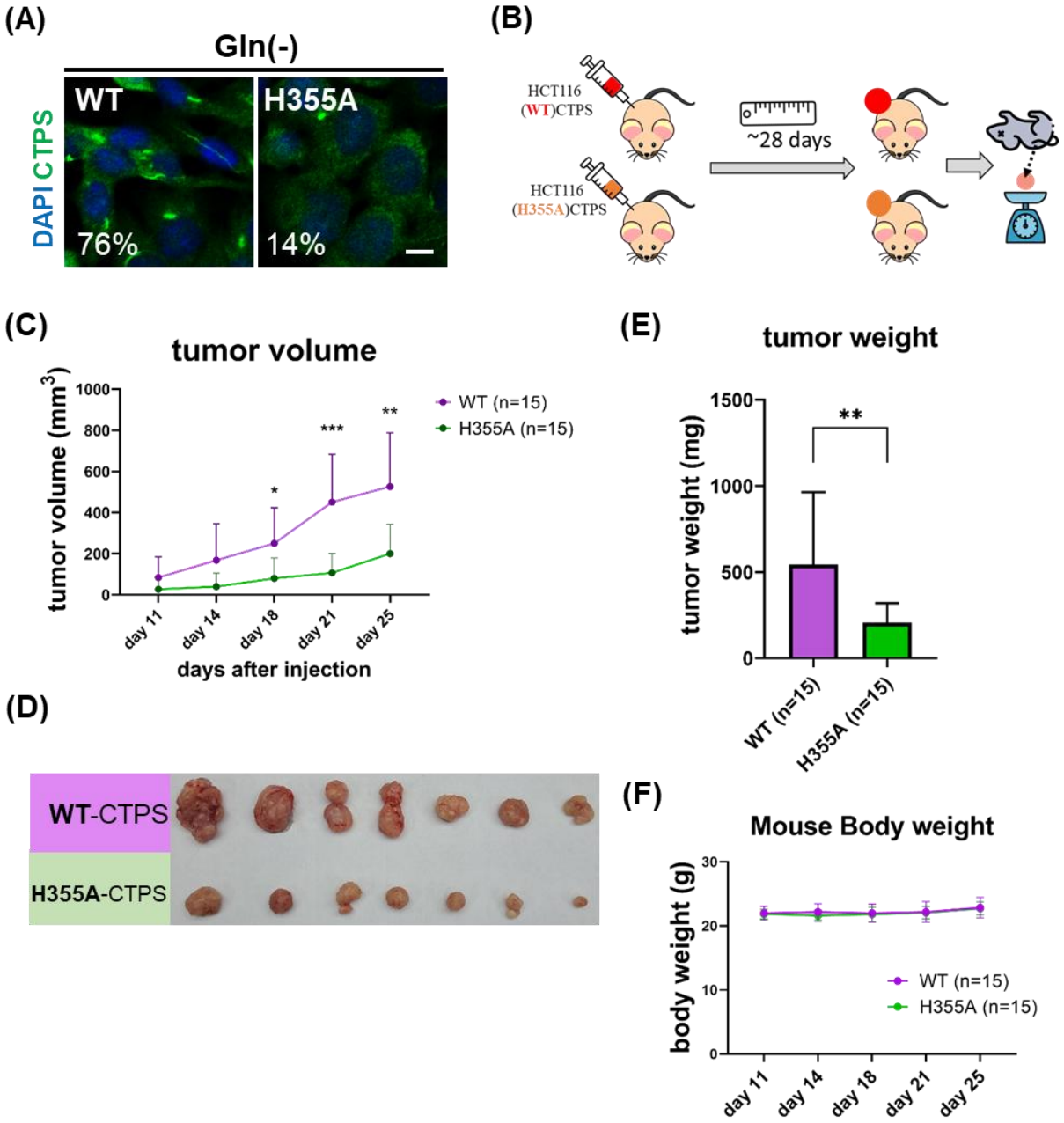
520 **Figures**



521 **Figure 1. Disrupting CTPS filament assembly impairs HCT116 tumor growth in vivo**
522 **(A)** HCT116 cells stably expressing $CTPS^{WT}$ or $CTPS^{H355A}$ were cultured in Gln-free DMEM
523 for 24 h, and immunostained for CTPS (green); nuclei were counterstained with DAPI (blue).
524 **(B)** Schematic of the xenograft mouse experiment. Thirty mice were used, with 15 injected with
525 HCT116 $CTPS^{WT}$ cells and 15 injected with HCT116 $CTPS^{H355A}$ cells. The experiment was
526 terminated day 28 after injection. **(C)** Tumor volume measured by external caliper throughout
527 the experimental period. **(D)** Representative images of tumors derived from $CTPS^{WT}$ or
528 $CTPS^{H355A}$ cells excised at day 28 after injection; tumor weights are quantified in **(E)**. **(F)** Mouse
529 body weight recorded by scale over the course of the experiment.
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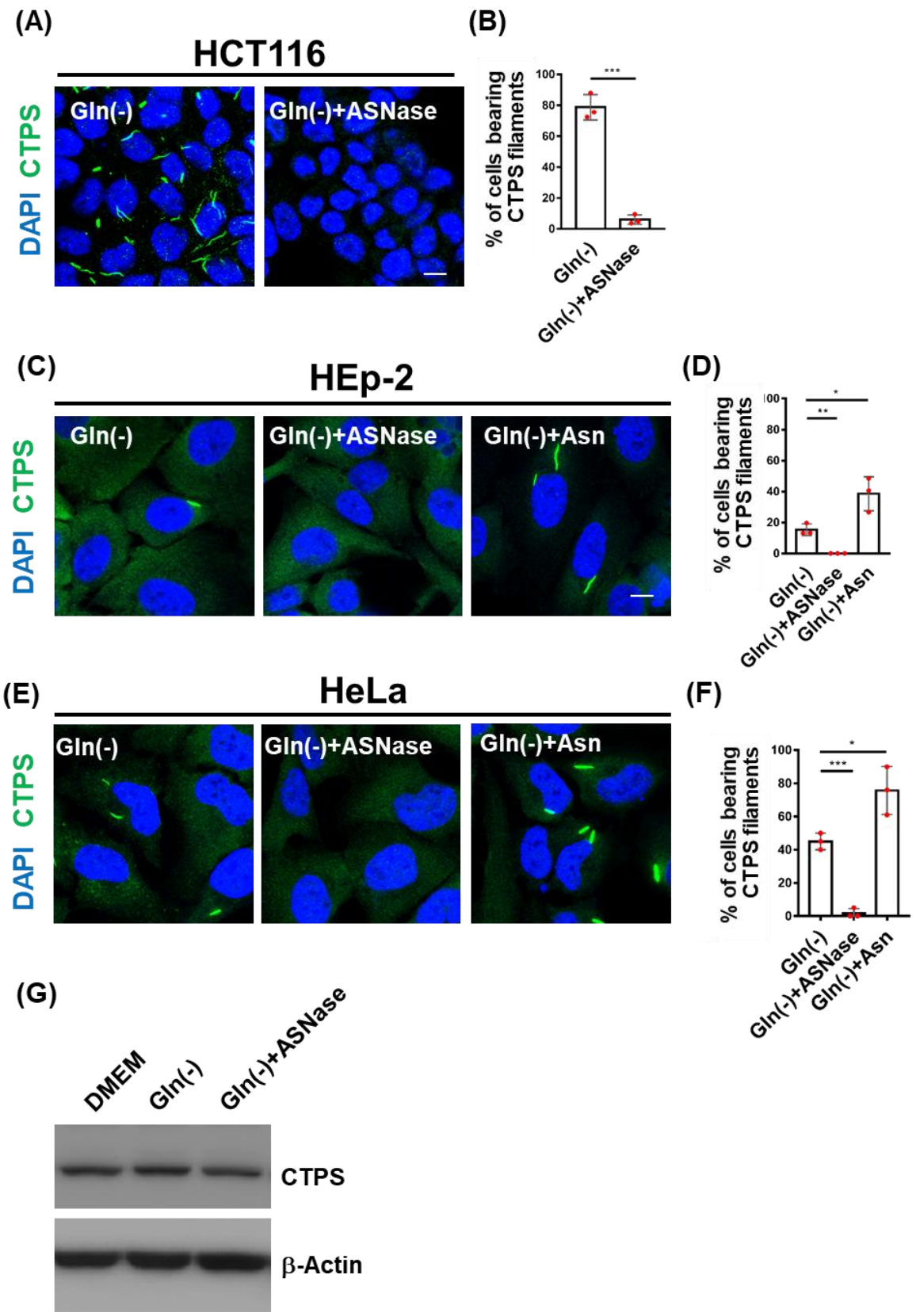

Figure 2. Asparagine availability is essential for CTPS filament assembly under glutamine deprivation

(A, B) HCT116 cells were incubated in Gln-free DMEM or Gln-free DMEM supplemented with ASNase (2U/ml) for 24 h, followed by immunostaining for CTPS (green) and DAPI (blue). **(C, D)** HEp-2 cells were incubated in Gln-free DMEM supplemented with ASNase or Asn (0.34mM) for 24 h, followed by immunostaining for CTPS (green) and DAPI (blue). **(E,**

537 **F)** HeLa cells were incubated with Gln-free DMEM supplemented with ASNase or Asn
538 (0.34mM) for 24 h, followed by immunostaining against CTPS (green) and DAPI (blue).
539 **(G)** HEp-2 cells were incubated in indicated conditions for 24 h, harvested and lysed for
540 Western blotting analysis. Scale bars: 10 mm.
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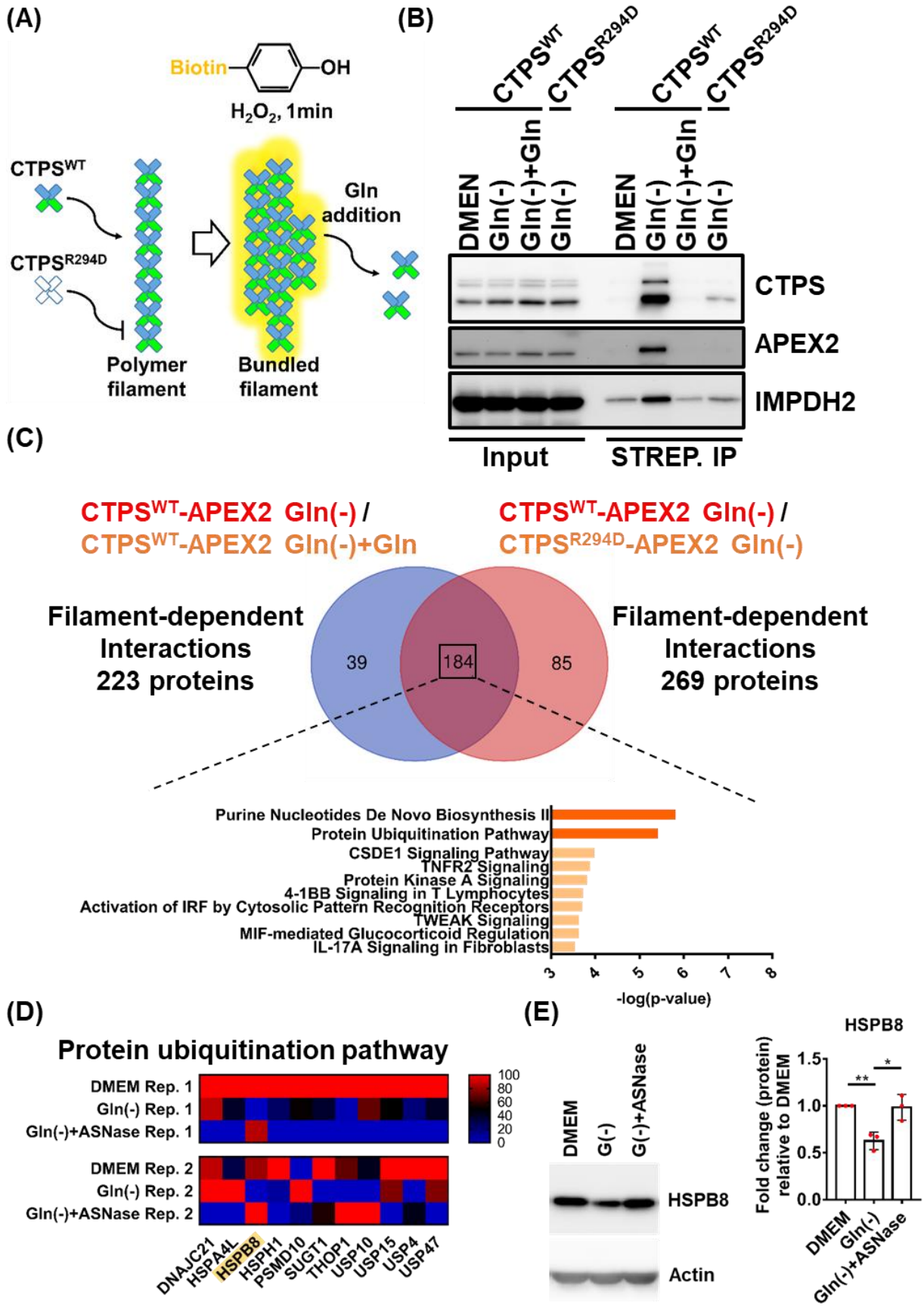

543 **Figure 3. Proximity labeling identifies the protein quality control factor HSPB8 as a**
544 **condition-specific interactor of CTPS**
545 **(A)** Schematic of the CTPS-APEX2 proximity labeling strategy. **(B)** HEp-2 cells expressing
546 CTPS^{WT}-APEX2 were cultured in DMEM, Gln-free DMEM, or Gln-free DMEM supplemented

547 with Gln for 24 h. HEp-2 cells expressing CTPS^{R294D}-APEX2 were cultured in Gln-free DMEM
548 for 24 h. Biotinylated proteins were affinity purified using streptavidin-conjugated magnetic
549 beads and analyzed by Western blotting. **(C)** Venn diagram showing 184 proteins common to
550 the comparisons of CTPS^{WT}-APEX2 cells cultured in Gln-free DMEM versus Gln-free DMEM
551 supplemented with Gln, and CTPS^{WT}-APEX2 versus CTPS^{R294D}-APEX2 cells cultured in Gln-
552 free DMEM. Enriched pathways associated with CTPS-interacting proteins under Gln
553 deprivation were identified by Ingenuity Pathway Analysis (IPA). **(D)** Heatmap showing
554 proteins involved in the unbiquitination pathway in cells cultured in DMEM, Gln-free DMEM,
555 or Gln-free DMEM supplemented with ASNase. **(E)** HSPB8 protein levels in cells cultured in
556 DMEM, Gln-free DMEM, or Gln-free DMEM supplemented with ASNase.
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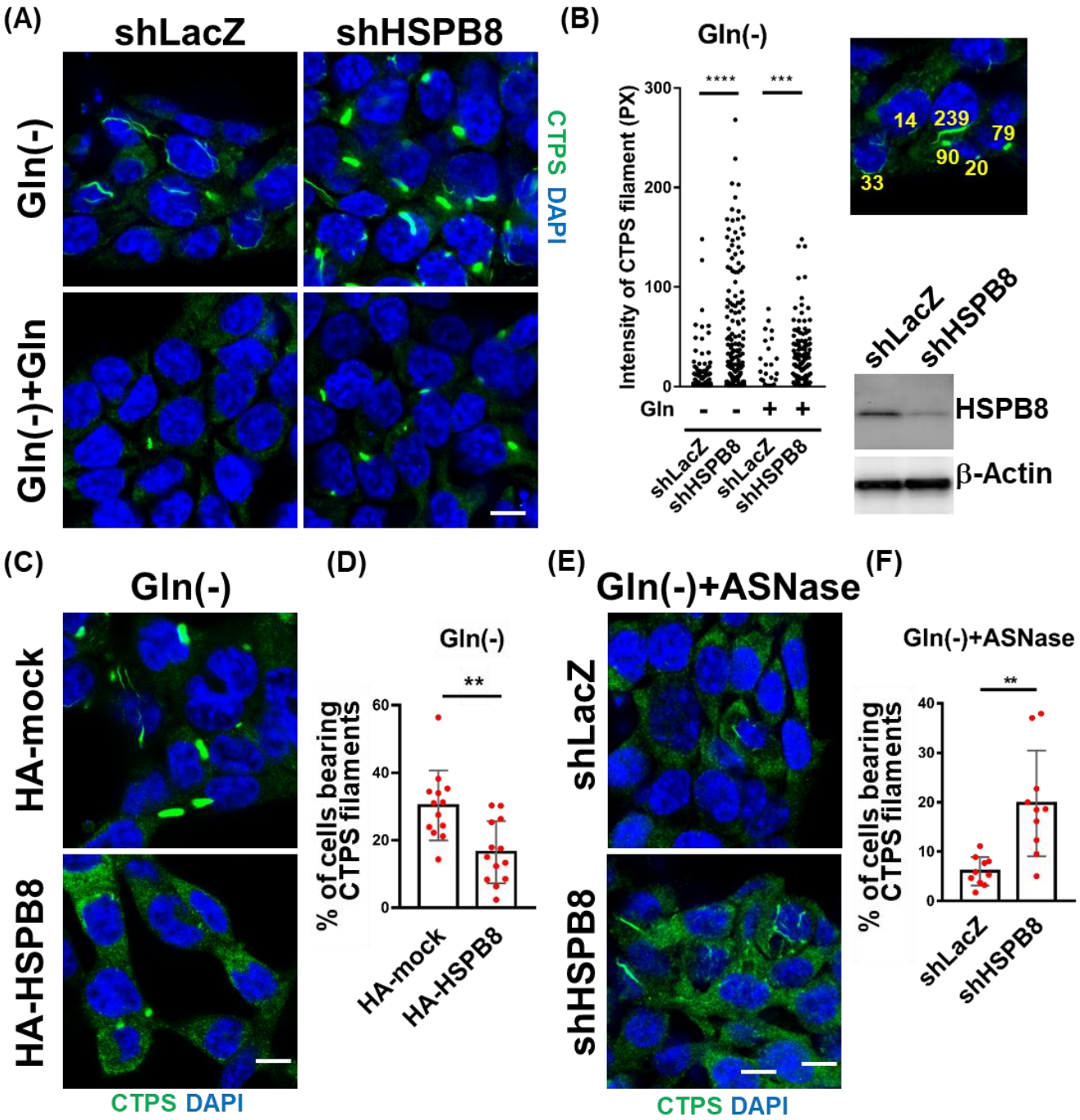

Figure 4. HSPB8 is required for the disassembly of CTPS filaments during nutrient stress

(A and B) HCT116 cells expressing shLacZ or shHSPB8 were cultured in glutamine-free DMEM for 24 h, with glutamine added for the final 30 min where indicated. Cells were immunostained for CTPS (green) and DAPI (blue). CTPS filament intensity was quantified using ImageJ, with representative filament lengths indicated by pixel values (yellow). HSPB8 protein levels were assessed by Western blotting.

(C and D) HCT116 cells transfected with HA-mock or HA-HSPB8 were cultured in glutamine-free DMEM for 24 h and immunostained for CTPS (green) and DAPI (blue).

(E and F) shLacZ- and shHSPB8-expressing HCT116 cells were cultured in glutamine-free DMEM supplemented with asparaginase (ASNase) for 24 h and immunostained for CTPS (green) and DAPI (blue).

570 Scale bars, 10 μm.

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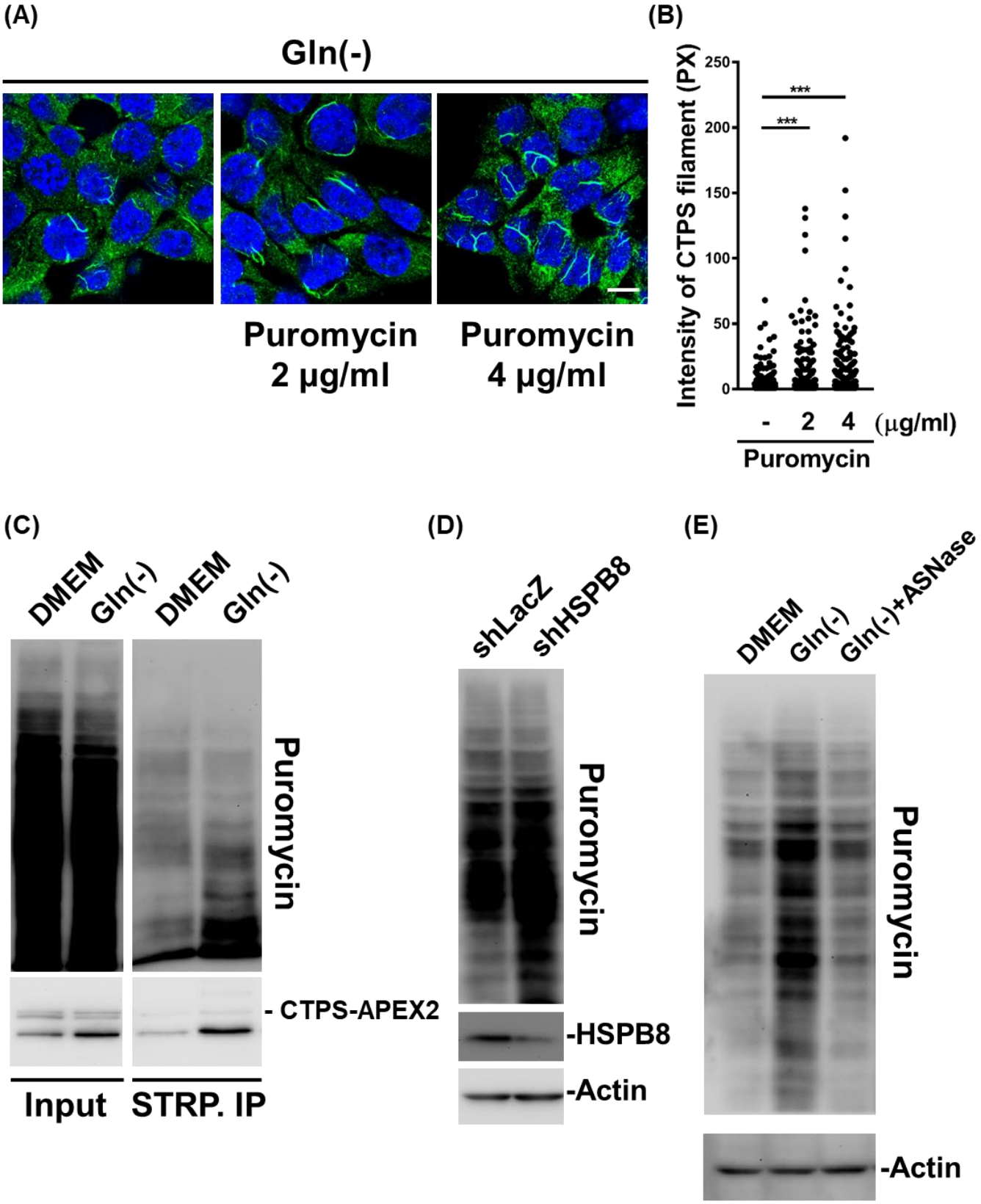

572 **Figure 5. HSPB8-mediated protein quality control is associated with CTPS filament**
573 **formation**
574 **(A and B)** HCT116 cells were cultured in Gln-free DMEM for 24 h, with puromycin added
575 during the final 3 h, followed by immunostaining for CTPS (green) and DAPI (blue). CTPS
576 filament intensity was quantified using ImageJ.
577 **(C)** HCT116 cells expressing CTPS^{WT}-APEX2 were cultured in Gln-free DMEM for 24 h,
578 with puromycin (10 μg/ml) added during the final 3 h. Proximity labeling was performed, and

biotinylated proteins were analyzed by Western blotting.
(D) shLacZ- and shHSPB8-expressing HEp-2 cells and **(E)** HEp-2 cells were cultured in Gln-free DMEM for 24 h, with puromycin (20 μg/ml) added during the final 10 min. Cells were harvested and analyzed by Western blotting for puromycin incorporation, HSPB8, and actin. Scale bars, 10 μm.

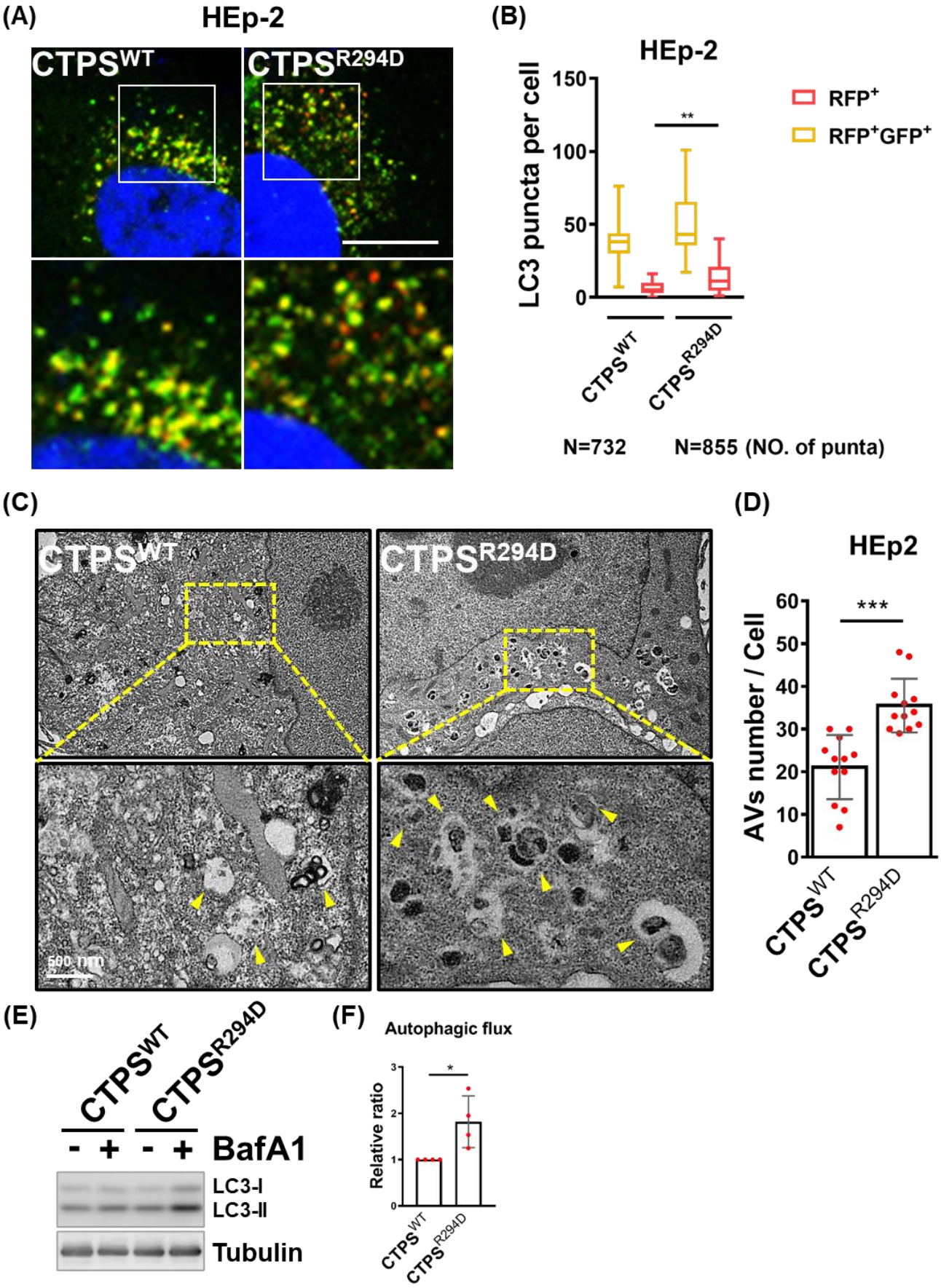

Figure 6. CTPS filament modulates autophagic flux under glutamine deprivation

(A and B) HEp-2 cells stably expressing $CTPS^{WT}$ or $CTPS^{R294D}$ and the tandem GFP–RFP–LC3 reporter were cultured in Gln-free DMEM for 24 h. GFP- and RFP-positive puncta were quantified using ImageJ.

589 **(C and D)** Transmission electron microscopy images of $CTPS^{WT}$ and $CTPS^{R294D}$ HEp-2 cells
590 cultured in Gln-free DMEM for 24 h. Autophagic vesicles are indicated by yellow arrowheads.
591 **(E and F)** Quantification of LC3-II levels in $CTPS^{WT}$ and $CTPS^{R294D}$ HEp-2 cells cultured in
592 Gln-free DMEM for 24 h, with or without bafilomycin A1 (BafA1; 100 nM) added during the
593 final 16 h.
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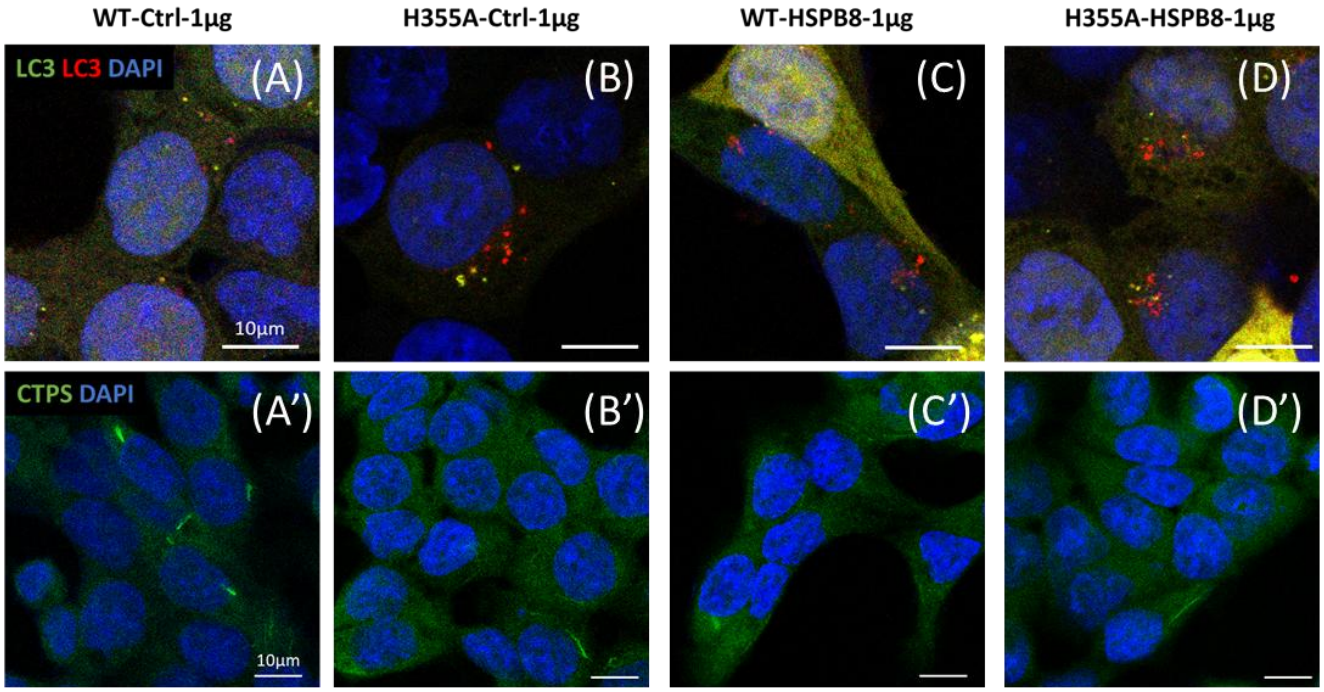


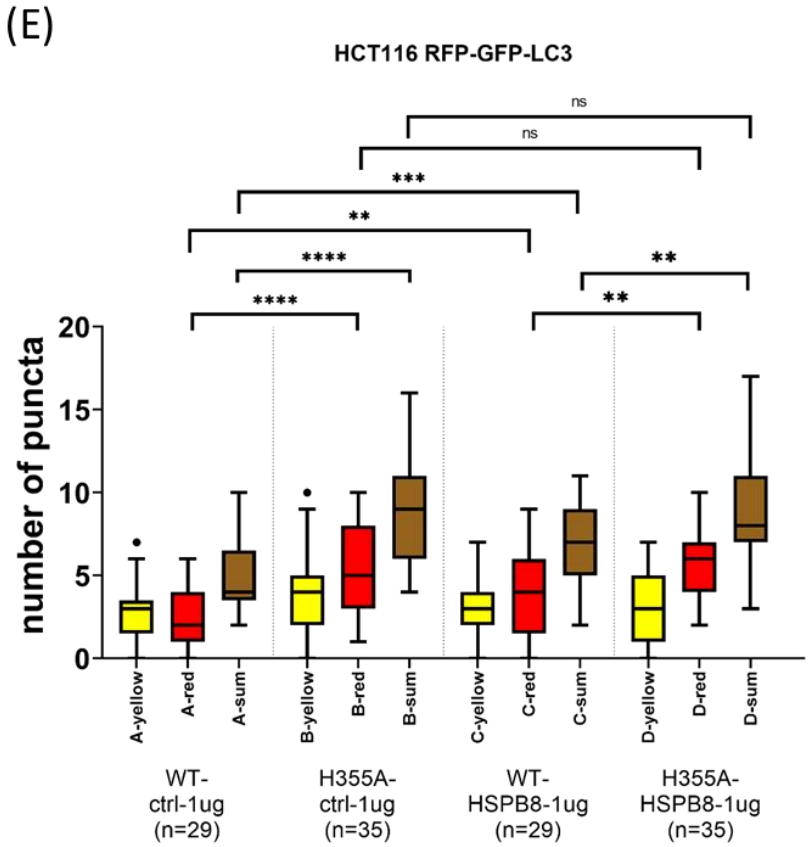
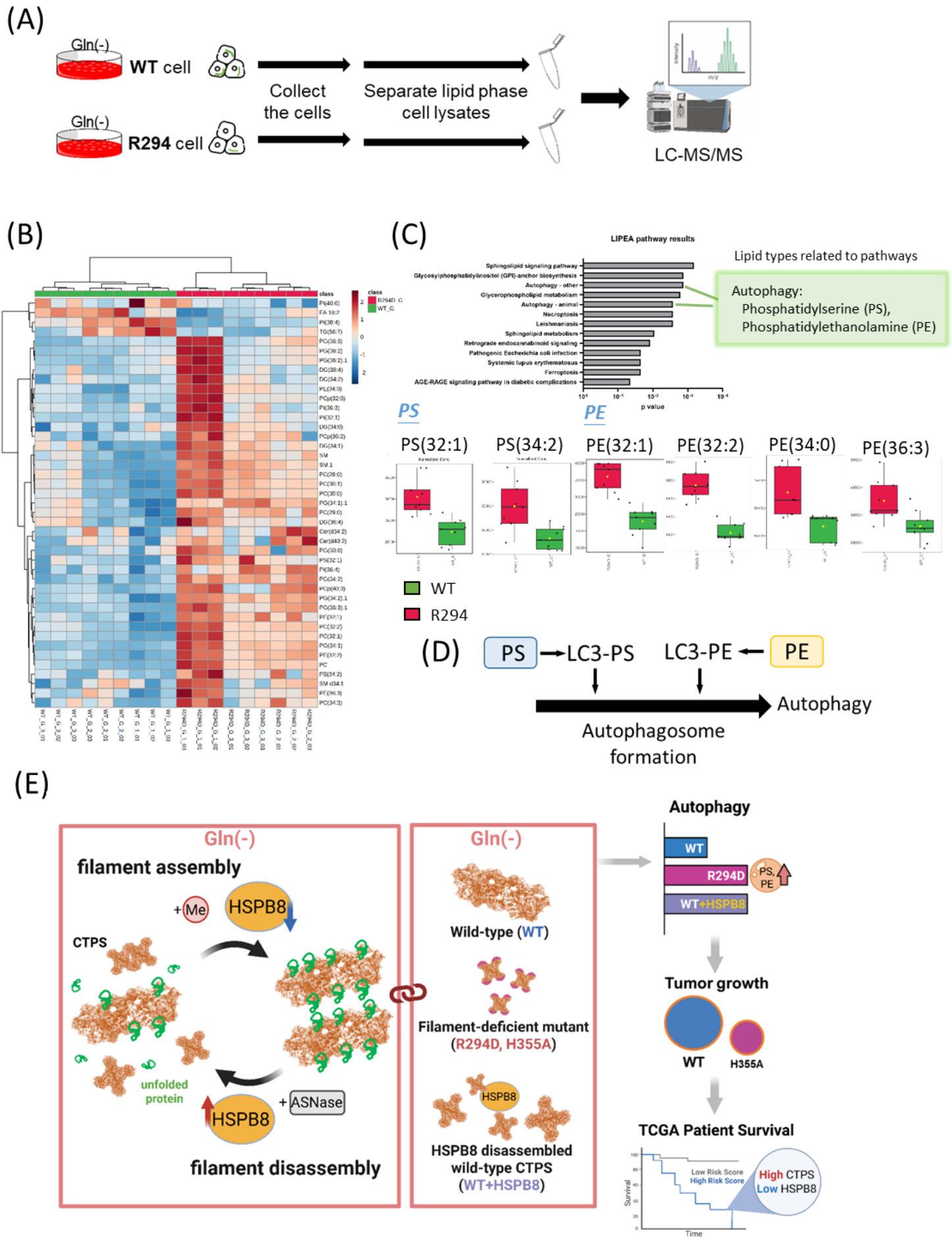


Figure 7. HSPB8 overexpression enhances autophagy inversely correlated with CTPS filament formation

(A–D) Representative images of autophagic vesicles in HCT116 cells expressing the tandem RFP–GFP–LC3 reporter. Red-only puncta indicate autolysosomes, while yellow puncta (RFP/GFP colocalized) mark autophagic structures; nuclei were counterstained with DAPI (blue).

(A′–D′) Corresponding images showing CTPS filament formation by immunostaining for CTPS (green) with DAPI (blue).

604 **(E)** Quantification of autophagic puncta per cell, including red-only, yellow, and total puncta.
605 Numbers below each condition (n) indicate the number of cells analyzed per group.
606 Scale bars, 10 μm.
607
608



609 **Figure 8. Metabolomic profiling of phospholipids in cells expressing CTPS^{WT} versus**

610 **filament-null CTPS mutant**
611 **(A)** Scheme of the experiment design. HEp-2 cells stably expressing $CTPS^{WT}$ and $CTPS^{H355A}$
612 were cultured in Gln-free DMEM for 24 h, and subjected to lipid extraction, followed by
613 LC/MS-MS analysis. Three independent experiments were performed and included in the
614 analysis. **(B)** Heatmap showing the relative abundance of 43 identified metabolites across
615 conditions. **(C)** Pathway annotation analysis of the 43 identified metabolites using LIPEA. **(D)**
616 Phosphatidylserine (PS) and Phosphatidylethanolamine (PE) contribution to autophagy
617 pathway. **(E)** Schematic illustrating the HSPB8–CTPS axis reveals a metabolic vulnerability
618 in glutamine-stressed tumors. CTPS filament dynamics is suggested as a metabolic switch.
619 Accumulation of unfolded peptides promotes CTPS filament bundling, a process modulated
620 by cellular methylation. Under glutamine deprivation, reduced HSPB8 levels lead to the
621 accumulation of misfolded peptides, thereby facilitating CTPS filament assembly. In contrast,
622 increased HSPB8 expression, induced by asparaginase (ASNase), promotes CTPS filament
623 disassembly and enhances CTPS enzymatic activity. Elevated CTPS activity supports CTP-
624 dependent phospholipid synthesis, including phosphatidylinositol (PI) and
625 phosphatidylethanolamine (PE), thereby driving autophagosome expansion and suppressing
626 tumor growth. This regulatory axis reveals a metabolic vulnerability in tumors characterized
627 by high CTPS and low HSPB8 expression.
628 Abbreviations: Me, methylation; ASNase, asparaginase.
629