Loss of dihydroceramide desaturase drives neurodegeneration by disrupting endoplasmic reticulum and lipid droplet homeostasis in glial cells

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ABSTRACT

Dihydroceramide desaturases convert dihydroceramides to ceramides, the precursors of all complex sphingolipids. Reduction of DEGS1 dihydroceramide desaturase function causes pediatric neurodegenerative disorder hypomyelinating leukodystrophy-18 (HLD-18). We discovered that *infertile crescent (ifc)*, the *Drosophila DEGS1* homolog, is expressed primarily in glial cells to promote CNS development by guarding against neurodegeneration. Loss of *ifc* causes massive dihydroceramide accumulation and severe morphological defects in cortex glia, including endoplasmic reticulum (ER) expansion, failure of neuronal ensheathment, and lipid droplet depletion. RNAi knockdown of the upstream ceramide synthase *schlank* in glia of *ifc* mutants rescues ER expansion, suggesting dihydroceramide accumulation in the ER drives this phenotype. RNAi knockdown of *ifc* in glia but not neurons drives neuronal cell death, suggesting that *ifc* function in glia promotes neuronal survival. Our work identifies glia as the primary site of disease progression in HLD-18 and may inform on juvenile forms of ALS, which also feature elevated dihydroceramide levels.

INTRODUCTION

Sphingolipids are key structural and functional components of the cell membrane in all eukaryotic cells and are enriched in glial cells, such as oligodendrocytes, where they comprise up to 30% of all membrane lipids.[1] All complex sphingolipids, like glycosylsphingolipids and sphingomyelin, derive from ceramide.[2,3,4] In the *de novo* ceramide biosynthesis pathway, dihydroceramide desaturases, such as DEGS1, produce ceramide from dihydroceramide by catalyzing the formation of a *trans* double bond between carbons 4 and 5 of the sphingoid backbone, which enhances conformational plasticity.[5,6] In humans, bi-allelic mutations in *DEGS1* cause hypomyelinating leukodystrophy-18 (HLD-18), a progressive, often fatal pediatric neurodegenerative disease marked by cerebral atrophy, white matter reduction, and hypomyelination.[7,8,9] The primary neural cell type impacted by loss of *DEGS1* function and the cell biology of how disruption of ceramide synthesis leads to neurodegeneration remain unknown.

The *de novo* ceramide biosynthetic pathway is well conserved among higher metazoans.[4,10,11,12,13] *De novo* ceramide synthesis occurs in the endoplasmic reticulum (ER) and starts with the rate-limiting activity of the serine palmitoyltransferase (SPT) complex, which condenses serine and palmitoyl-CoA (lauoryl-CoA in flies) to form 3-Ketosphinganine, which is converted to sphinganine by 3-Ketosphinganine reductase. Ceramide synthases condense sphinganine with acyl-CoA to generate dihydroceramide, which is converted to ceramide by dihydroceramide desaturases. Ceramide is then efficiently transported by the specific ceramide transporter CERT from the ER to the Golgi,[14] where it undergoes headgroup modifications to produce complex sphingolipids that eventually translocate to the plasma membrane.[15] Mutations in most members of the SPT complex, ceramide synthases, and DEGS1 lead to neurodegeneration,[4,12,13] identifying the *de novo* ceramide biosynthesis pathway as a hotspot for neurodegenerative disease mutations.

Consistent with its central role in ceramide biogenesis, reduction or loss of *DEGS1* function in human patients or cell lines, mice, zebrafish, and flies drive dihydroceramide accumulation and ceramide depletion.[8,9,16,17] HLD-18 patients display reduced myelin sheath thickness in peripheral nerves, and knockdown of *DEGS1* function in zebrafish reduces the number of myelin basic protein-positive oligodendrocytes,[8,9] suggesting *DEGS1* regulates Schwann cell and oligodendrocyte development. In *Drosophila*, genetic ablation of *infertile crescent* (*ifc*), the fly *DEGS1* ortholog, drives activity-dependent photoreceptor degeneration,[17] suggesting that *ifc* function is crucial for neuronal homeostasis. In addition, forced expression of a wildtype *ifc* transgene in neurons, glia, or muscles was shown to rescue the *ifc* mutant phenotype.[17] Whether *ifc/DEGS1* acts primarily in glia, neurons, or other cells to regulate nervous system development then remains to be determined.

Research on *DEGS1* points to dihydroceramide accumulation as the driver of nervous system defects caused by *DEGS1* deficiency. Pharmacological or genetic inhibition of ceramide synthase function, which should reduce dihydroceramide levels, suppresses the observed reduction of MBP-positive oligodendrocytes in zebrafish and the activity-dependent photoreceptor degeneration in flies triggered by reduction of *DEGS1/ifc* function.[9,17] Dihydroceramide accumulation may also contribute to other neurodegenerative diseases, as gain of function mutations in components of the SPT complex cause juvenile amyotrophic lateral sclerosis due to elevated sphingolipid biosynthesis, with dihydroceramides showing the greatest relative increase of all sphingolipids.[18,19,20,21] How dihydroceramide accumulation alters the cell biology of neurons, glia, or both to trigger myelination defects and neurodegeneration is unclear.

With its vast genetic toolkit, *Drosophila* is a powerful system in which to dissect the genes and pathways that regulate glial development and function.[22,23] In flies, six morphologically and functionally distinct glial subtypes regulate nervous system development and homeostasis.[24,25] The surface perineurial and subperineural glia act as physiochemical barriers to protect the

nervous system and control metabolite exchange with the hemolymph, carrying out a similar function as the human blood-brain-barrier.[26,27] Residing between the surface glia and the neuropil, cortex glia ensheath the cell bodies of neuroblasts and neurons in the CNS in protective, nutritive, honeycomb-like membrane sheaths. Ensheathing glia define the boundary of the neuropil and insulate axons, dendrites, and synapses from neuronal cell bodies in the CNS.[28] Astrocyte-like glia extend fine membrane protrusions that infiltrate the neuropil and form a meshwork of cellular processes that ensheath synapses and regulate synaptic homeostasis in the CNS.[28] In the PNS, wrapping glia reside internally to the surface glia and insulate axons in the PNS to enhance neuronal signaling and provide energy support.

Using the *Drosophila* model, we found that *ifc* acts primarily in glia to regulate CNS development with its loss disrupting glial morphology. Our work supports a model in which inappropriate accumulation and retention of dihydroceramide in the ER drives ER expansion, glial swelling, and the failure of glia to enwrap neurons, ultimately leading to neuronal degeneration as a secondary consequence of glial dysfunction. Given the conserved nature of *de novo* ceramide biosynthesis, our findings likely illuminate the exact mechanism through which elevated dihydroceramide levels drive neuronal degeneration and cell death in flies and humans.

RESULTS

ifc contributes to the regulation developmental timing and CNS structure

In an EMS-based genetic screen, we uncovered three non-complementing mutations that when homozygous or trans-heterozygous to each other resulted in identical phenotypes, including a 3-day or greater delay in reaching the late-third larval instar stage, reduced brain size, progressive ventral nerve cord elongation, axonal swelling, and lethality at the late larval or early pupal stage

(Figure 1A; data not shown). Whole genome sequencing revealed that *ifc* was independently mutated in each line: *ifc*^{js1} and *ifc*^{js2} encode V276D and G257S missense mutations, respectively, and *ifc*^{js3} encodes a W162* nonsense mutation (Figures 1A and 1B). Sanger sequencing also uncovered a missense mutation, E241K, in the molecularly uncharacterized *ifc*^{1} allele (Figure 1B). All four mutations reside in the fatty acid desaturase domain, the hotspot for mutations in human *DEGS1* that cause HLD-18 (Figures 1B-B').

As a prior study reported that a CRISPR-generated, gene-specific deletion of *ifc, ifc-KO,* resulted in early larval lethality[17], we first confirmed the genetic nature of our *ifc* alleles because they caused late larval-early pupal lethality. Complementation crosses of each *ifc* allele against a deficiency of the region (Df(2L)BSC184) and *ifc-KO* revealed that all combinations, including larvae trans-heterozygous for *ifc-KO* over Df(2L)BSC184, survived to the late larval-early pupal stage and yielded phenotypes identical to those detailed above for the newly uncovered *ifc* alleles. Flies homozygous for *ifc-KO*, however, died as early larvae. Further analysis uncovered second site mutation(s) in the 21E2 chromosomal region responsible for the early lethal phenotype of the *ifc-KO* chromosome (see Methods). When uncoupled from these mutation(s), larvae homozygous for the “clean” *ifc-KO* chromosome developed to the late larval-early pupal stage and manifested phenotypes identical to the other *ifc* alleles. This analysis defined the correct lethal phase for *ifc* and identified our *ifc* alleles as strong loss of function mutations.

Loss of *ifc* function drives ceramide depletion and dihydroceramide accumulation

As *ifc/DEGS1* converts dihydroceramide to ceramide, we used untargeted lipidomics on whole larvae and the isolated CNS from wild-type and *ifc-KO/ifc*^{JS3} larvae (hereafter termed *ifc*$^{-/-}$ larvae) to assess the effect of loss of *ifc* function on metabolites in the ceramide pathway (Figure 1C). Loss of *ifc* function resulted in a near complete loss of ceramides and a commensurate increase

in dihydroceramides in the CNS and whole larvae (Figures 1E, S1). Sphinganine, the metabolite directly upstream of dihydroceramide, also exhibited a significant increase in its levels in the absence of *ifc* function, while metabolites further upstream were unchanged in abundance or undetectable (Figures 1E, S1). Ceramide derivatives like sphingosine, CPE, and Glucosyl-Ceramide (Glc-Cer), were reduced in levels and replaced by their cognate dihydroceramide forms (e.g., Glc-DiCer) (Figures 1E, S1). Loss of the enzymatic function of Ifc then drives dihydroceramide accumulation and ceramide loss.

ifc governs glial morphology and survival

To connect this metabolic profile to a cellular phenotype, we assayed *ifc* function in the CNS. We leveraged the expression of fatty acid binding protein (FABP) as a marker of cortex glia[29] and that of the *M{3xP3-RFP.attP}* phi-C31 "landing pad" transgene, which resided in the isogenic target chromosome of our screen, as a general glial marker (Figure S2). In addition, we labeled neuroblasts with Deadpan (Dpn), neurons with ELAV, and axons with N-Cadherin (NCad). In *ifc*$^{-/-}$ larvae, we observed a clear reduction in Dpn-positive neuroblasts in the optic lobe, swelling of glia in peripheral nerves, enhanced RFP expression in the CNS, and the presence of large swollen, cortex glia identified by RFP labeling and fatty acid binding protein (FABP) expression (Figure 1F). In wild-type larvae, cortex glia display compact cell bodies and fully enwrap individual neuronal cell bodies with their membrane sheaths (Figures 1F and S3).[29] In *ifc*$^{-/-}$ larvae, cortex glia display swollen cell bodies, fail to fully enwrap neuronal cell bodies, displace neurons from their regular arrangement, and appear to contain brightly fluorescent RFP-positive aggregates (Figure 1F, S4-S5). *ifc* is then necessary for glial development and function in the larval nervous system.

To track the impact of *ifc* on glial morphology, we combined GAL4 lines specific for each glial subtype with a UAS-linked membrane-tagged GFP transgene (Myr-GFP) and the MultiColor FlpOut system.[30,31,32] Using this approach, we determined that loss of *ifc* function affects all CNS glial subtypes except perineurial glia (Figures 2E-E' and 2J-J'). Cortex glia appeared swollen, failed to enwrap neurons, and accumulated large amounts of Myr-GFP$^+$ internal membranes (Figures 2A-A' and 2F-F'). Ensheathing glia (Figures 2B-B' and 2G-G') and subperineurial glia (Figures 2D-D' and 2I-I') also displayed swollen, disorganized cell bodies and accumulated Myr-GFP$^+$ internal membranes. Astrocyte-like glia displayed smaller cell bodies, reduced membrane extensions, and disrupted organization along the dorsal ventral nerve cord (Figures 2C-C'). We conclude that *ifc* regulates the morphology of most glial subtypes in the larval CNS.

Next, we asked if loss of *ifc* function alters the number of each glial subtype. Using the same glial subtype-specific GAL4 lines to drive a nuclear-localized GFP transgene, we counted the total number of all CNS glial subtypes, except perineurial glia, in wild type and *ifc*$^{-/-}$ larvae. To remove the small size of the brain in *ifc*$^{-/-}$ larvae as a confounding factor, we focused our analysis on the ventral nerve cord. The number of subperineurial glia was unchanged between the two genotypes, but we observed a 12%, 40%, and 72% reduction in the number of astrocyte-like, ensheathing, and cortex glia, respectively, in *ifc*$^{-/-}$ larvae relative to wild-type (Figures 2K-N). Our data reveal a broad role for *ifc* in regulating glial cell morphology and number in the *Drosophila* larval CNS.

ifc acts in glia to regulate glial and CNS development

Prior work in zebrafish showed that *DEGS1* knockdown reduced the number of myelin basic protein-positive oligodendrocytes;[9] in flies, loss of *ifc* function in the eye drove photoreceptor degeneration.[17] Neither study uncovered the cell type in which *ifc/DEGS1* acts to regulate neural

development. To address this question, we used the GAL4/UAS system, RNAi-mediated gene depletion, and gene rescue approaches to see if *ifc* acts in neurons or glia to control glial development and CNS morphology. First, we used a UAS-linked *ifc*-RNAi transgene to deplete *ifc* function in all neurons (*elav-GAL4*; *repo-GAL80*) or all glia (*repo-GAL4*). Focusing on FABP-positive cortex glia due to their easily scorable phenotype, we found that pan-glial, but not pan-neuronal, knockdown of *ifc* recapitulated the swollen cortex glia phenotype observed in *ifc* mutant larvae (Figures 3C-D and 3J).

To complement our RNAi approach, we asked if GAL4-driven expression of a wild-type *Drosophila ifc* or human *DEGS1* transgene rescued the $ifc^{-/-}$ CNS phenotype. In the absence of a GAL4 driver, the *ifc* transgene drove weak rescue of the cortex glia phenotype (Figures 3E and 3K), consistent with modest GAL4-independent transgene expression reported for UAS-linked transgenes.[33] Pan-neuronal expression of *ifc* drove modest rescue of the *ifc* CNS phenotype beyond that observed for the *ifc* transgene alone (Figures 3F and 3K), but pan-glial expression of *ifc* fully rescued the *ifc* mutant cortex glia phenotype and other CNS phenotypes (Figures 3H, 3K, and S6). Identical experiments using the human *DEGS1* transgene revealed that only pan-glial *DEGS1* expression provided rescuing activity (Figures 3G, 3I, and 3K). We infer that *ifc* acts primarily in glia to govern CNS development and that human *DEGS1* can partially substitute for *ifc* function in flies despite a difference in the preferred length of the sphingoid backbone in flies versus mammals.[10]

***ifc* is predominately expressed in glia and localizes to the ER**

Next, we tracked *ifc* expression in the CNS via RNA in-situ hybridization and an *ifc-T2A-GAL4* transcriptional reporter. RNA in-situ hybridization revealed that *ifc* is widely expressed in the CNS (Figure 4A), most obviously in the distinctive star-shaped astrocyte-like glia (Figure 4B), which are

marked by Ebony expression.[34] RNA in-situ hybridization was not ideal for tracing *ifc* expression in other cells, likely due to signal diffusion. Thus, we paired *ifc-T2A-GAL4* with a nuclear RFP (nRFP) transgene[35] and confirmed authenticity of the *ifc-T2A-GAL4* line by its strong expression in astrocyte-like glia (Figure 4C). Using this approach, we found that *ifc* is strongly expressed in all glial cells (Figures 4D and S7A) and more weakly neurons in the larval CNS (Figures 4E and S7B).

Using a fosmid transgene that harbors a GFP-tagged version of *ifc* flanked by ~36-kb of its endogenous genomic region and molecular markers for ER, cis-Golgi, and trans-Golgi,[36,37,38,39] we found that the Ifc-GFP colocalized strongly with the ER markers Calnexin 99A (CNX99A) and ESYT (Figures 4F-F' and 4G-G') and weakly with the cis-Golgi marker GOLGIN84 (Figure S7C) and the trans-Golgi marker GOLGIN245 (Figure S7D). Our results indicate that Ifc localizes primarily to the ER, aligning with the presumed site of *de novo* ceramide biosynthesis and prior work on *DEGS1* localization in cell lines.[8,40]

Loss of *ifc* drives ER expansion and lipid droplet loss in cortex glia

We next asked if loss of *ifc* function altered ER, Golgi, or lysosome morphology. Focusing on cortex glia, we observed a clear expansion of the ER marker CNX99A (Figures 4H-H' and K-K'), a mild enrichment of the Golgi markers, GOLGIN84 and GOLGIN245, in diffuse "clouds" (Figures 4I-I', 4L-L', S7E-F), and a reduction in expression of the lysosome marker LAMP (Figures 4J-J' and M-M') in *ifc*$^{-/-}$ larvae. The expansion of ER markers in *ifc* mutant larvae compelled us to obtain high-resolution views of organelle structure in cortex glia via transmission electron microscopy (TEM). In wild-type, cortex glia display a compact cytoplasm that surrounds a large nucleus (Figures 5A-B) and extend glial sheaths that fully enwrap adjacent neuronal cell bodies (black arrows; Figures 5C-D). In *ifc*$^{-/-}$ larvae, cortex glia display enlarged cell bodies with a maze-like

pattern of internal membranes (solid white arrows; Figures 5A'-B' and 5E-E') and fail enwrap neurons (hollow white arrows; Figures 5C'-D'). The internal membrane structures appear to assume an ER-like identity, as we observed significant overlap between the ER marker CNX99A and the membrane marker Myr-GFP when Myr-GFP was driven by a cortex glia-specific GAL4 line (Figure 5F). We observed similar yet milder effects on cell swelling and internal membrane accumulation in subperineurial and wrapping glia in abdominal nerves (purple and pink shading, respectively; Figures 5G-G' and 5H-H'), indicating that loss of *ifc* drives internal membrane accumulation in and swelling of multiple glial subtypes.

TEM analysis also revealed a near complete depletion of lipid droplets in the CNS of *ifc*$^{-/-}$ larvae (compare Figures 5A, 5C, and 5D to 5A', 5C' and 5D'; lipid droplets marked by asterisk; Figure 5M), which we confirmed using BODIPY to mark neutral lipids in FABP-positive cortex glia (Figure 5I-L). In the CNS, lipid droplets form primarily in cortex glia[29] and are thought to act as building blocks of membrane lipids versus an energy source in the brain.[41] Consistent with lipid droplet depletion indicating increased membrane lipid biogenesis, RNA-seq assays of dissected nerve cords revealed that loss of *ifc* drove transcriptional upregulation of genes that promote membrane lipid biogenesis, such as *SREBP*, the conserved master regulator of lipid biosynthesis, *SCAP*, an activator of SREBP, and *Pcyt1/Pcyt2,* which promote phosphatidylcholine and phosphatidylethanolamine synthesis.[42,43,44] The spliced form of *Xbp-1* mRNA (*Xbp-1s*) (Figure 5J), which promotes membrane lipid synthesis required for ER biogenesis and is activated by the unfolded protein response (UPR),[45] is also upregulated in the CNS of *ifc* mutant larvae. Most ER chaperones, which are typically transcriptionally upregulated upon UPR activation,[46] were however downregulated (Figure S8A), suggesting that in this case misfolded protein is not the factor that triggers UPR activation and increased ER membrane biogenesis upon loss of *ifc*.

Loss of *ifc* increases the saturation levels of triacylglycerols and membrane phospholipids

Lipid droplets are composed largely of triacylglycerols (TGs),[44] and we observed a 5-fold and 3-fold drop in TG levels in the CNS and whole larvae of *ifc* mutant larvae relative to wild-type (Figure 6A). Our lipidomics analysis also revealed a shift of TGs toward higher saturation levels in the absence of *ifc* function (Figures 6A-C). Consistent with this, we observed transcriptional upregulation of most genes in the Lands cycle, which remodels phospholipids by replacing existing fatty acyl groups with new fatty acyl groups (Figures S8B-C).[47,48,49,50] As TGs function as building blocks of membrane phospholipids, we asked if changes in TG levels and saturation were reflected in the levels or saturation of the membrane phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). In the absence of *ifc* function, PC and PE exhibited little change in quantity (Figures 6D, 6G), but the levels of the less abundant PS were increased three-fold increase in the CNS of *ifc* mutant larvae relative to wild-type (compare Figure 6J to Figures 6D and 6G). All three phospholipids, however, displayed increased saturation levels: The relative levels of all PC, PE, and PS species were reduced, except for the most saturated form of each phospholipid – 18:1/18:1 – which is increased (Figures 6E-F and 6H-L). This increase was more pronounced in the CNS than whole larvae (Figures 6F, 6I, and 6L), implying that loss of *ifc* function creates greater demand for lipid remodeling in the CNS.

Reduction of dihydroceramide synthesis suppresses the *ifc* CNS phenotype

Following its synthesis, CERT transports ceramide from the ER to the Golgi, but CERT is less efficient at transporting dihydroceramide than ceramide.[13] The expanded nature of the ER in *ifc* mutant larvae supports a model in which loss of *ifc* triggers excessive accumulation and retention of dihydroceramide in the ER, driving ER expansion and glial swelling. If this model is correct, reduction of dihydroceramide levels should suppress the *ifc* CNS phenotype. In agreement with this model, we found that the *schlank*^{G0365} loss of function allele dominantly suppressed the enhanced RFP expression (Figure 7M) and CNS elongation phenotypes of *ifc*

(Figure 7N). We also found that glial-specific depletion of *schlank* suppressed the internal membrane accumulation (Figures 7A-B), reduced lipid droplet size (Figures 7C-D, and 7O), glial swelling (Figures 7E-F, and S10), enhanced RFP expression (Figure 7M), CNS elongation (Figure 7N), and reduced optic lobe (Figure S4) phenotypes observed in otherwise *ifc* mutant larvae. Our data support the model that inappropriate retention of dihydroceramide in the ER drives ER expansion and glial swelling and dysfunction.

Glial-specific depletion of *ifc* function triggers neuronal cell death

Loss of *DEGS1/ifc* in human and flies has been shown to promote neurodegeneration and neuronal cell death,[8,9,17] but whether neuronal death results from an intrinsic defect in neurons or is induced by glial dysfunction remains unclear. To see if loss of *ifc* causes neuronal cell death, we used Cleaved Caspase-3, a marker of cell death,[51,52] and ELAV to track neuronal cell death in the brain and ventral nerve cord of wild-type and $ifc^{-/-}$ mutant late-third instar larvae. In wildtype, little to no cell death was evident in the brain or nerve cord and neurons appear smoothly packed next to each other; in contrast, in $ifc^{-/-}$ larvae, significant cell death was apparent in the brain and to a lesser degree in the nerve cord, with Caspase-3 staining often highlighting small perforations in the neuronal cell layer (Figures 7G-H, and 7P). This perforated pattern was associated with and more expansive than Caspase-3 staining, indicating the perforations mark Caspase-positive dying neurons and Caspase-negative dead neurons. Glial-specific depletion of *schlank* function in otherwise $ifc^{-/-}$ larvae suppressed the neuronal cell death phenotype (Figures 7I and 7P), supporting the model that loss of *ifc* function specifically in glia, rather than a specific requirement for *ifc* function in neurons, drives neuronal cell death. To directly test whether *ifc* function in glia is required to guard against neuronal cell death, we used the GAL4-UAS system and RNAi mediated gene interference to deplete *ifc* function specifically in glia (*repo-GAL4*) or in neurons (*elav-GAL4; repo-GAL80*) and found that glial-specific, but not neuronal-specific, depletion of *ifc*

function drove significant neuronal cell death in the brain and to a greater extent the nerve cord, a phenotype that was enhanced upon removal of one copy of *ifc* (Figures 7J-L, 7Q, and S11). Loss of *ifc* function then triggers glial dysfunction, whin in turn drives neuronal cell death.

DISCUSSION

Our work on *ifc* supports a model in which loss of *ifc/DEGS1* function drives glial dysfunction through the accumulation and inappropriate retention of dihydroceramide in the ER of glia with this proximal defect driving ER expansion, glial swelling, and subsequent neuronal cell death and neurodegeneration. A large increase in dihydroceramide would also impact ER membrane structure: ER membranes are typically loosely packed, thin, semi-fluid structures, with sphingolipids comprising just 3% of ER phospholipids.[44] Sphingolipids in general and dihydroceramide in specific are highly saturated lipids, promote lipid order, tighter lipid packing, membrane rigidity, and thicker membranes.[53,54] Our observation of thick ER membranes in cortex glia in *ifc*$^{-/-}$ larvae (Figure 5) aligns with increased dihydroceramide levels in the ER. In this context, we note that of many clinical observations made on an HLD-18 patient, one was widening of the ER in Schwann cells,[8] a finding that when combined with our work suggests that dihydroceramide accumulation in the ER is the proximal cause of HLD-18.

Although we think ER expansion represents the most proximal effect of loss of *ifc/DEGS1* function and dihydroceramide accumulation on cortex glia, other organelles and their functions are likely also disrupted, minimally as a consequence of ER disruption. For example, the apparent reduction of the lysosome marker, LAMP, in cortex glia of *ifc* mutant larvae correlates with increased RFP levels and the presence of bright RFP puncta or aggregates in this cell type, suggesting impaired lysosome function contributes to increased RFP perdurance and aggregation. Defects in the activity of multiple organelles may collaborate to elicit the full

phenotype manifested by loss of *ifc/DEGS1* function, resulting in glial dysfunction and subsequent neuronal cell death.

Increased dihydroceramide levels may contribute to a broader spectrum of neurodegenerative diseases than simply HLD-18. Recent work reveals that gain of function mutations in *SPTLC1* and *SPTLC2*, which encode components of the SPT complex that catalyzes the initial, rate limiting step of *de novo* ceramide and sphingolipid biosynthesis,[55] cause juvenile ALS via increased sphingolipid biosynthesis.[18,19,20,21] Of all sphingolipids, the relative levels of dihydroceramide were increased the most in patient plasma samples, suggesting that DEGS1 activity becomes limiting in the presence of enhanced SPT activity and that dihydroceramide accumulation contributes to juvenile ALS. Any mutations that increase metabolite flux through the ceramide pathway upstream of DEGS1 may then increase dihydroceramide levels, drive ER expansion and cell swelling, and lead to neurodegeneration, with disease severity predicated on the extent of excessive dihydroceramide accumulation. Model systems, like flies, can harness the power of genetic modifier screens to identify genes and pathways (potential therapeutic targets), that can be tweaked to ameliorate the effect of elevated dihydroceramide levels on neurodegeneration.

Our work appears to pinpoint the cell type impacted by loss of *ifc/DEGS1* function: Glia, specifically glia that exhibit great demand for membrane biogenesis like cortex glia in the fly and oligodendrocytes or Schwann cells in mammals. In larvae, *ifc* is expressed at higher levels in glial cells than in neurons, and its genetic function is required at a greater level in glia than neurons to govern CNS development. Our unpublished work on other genes in the ceramide metabolic pathway reveals similar glial-centric expression patterns in the larval CNS to that observed for *ifc*, suggesting they too function primarily in glia rather than neurons at this stage. In support of this idea, a study on CPES, which converts ceramide into CPE, the fly analog of sphingomyelin, revealed that CPES is required in cortex glia to promote their morphology and homeostasis and

to protect flies from photosensitive epilepsy.[56] Similarly, ORMDL, a dedicated negative regulator of the SPT complex, is required in oligodendrocytes to maintain proper myelination in mice.[57] Given that many glial cell types, are enriched in sphingolipids and exhibit a great demand for new membrane biogenesis during phases of rapid neurogenesis and axonogenesis, we speculate that glial cells, such as cortex glia, oligodendrocytes, and Schwann cells, rather than neurons manifest a greater need for *ifc/DEGS1* function and ceramide synthesis during developmental stages marked by significant nervous system growth.

Will this glial-centric model for *ifc/DEGS1* function, and more generally ceramide synthesis, hold true in the adult when neurogenesis is largely complete and the demand for new membrane synthesis in glia dissipates? Recent work in the adult fly eye suggests it may not. Wang et al.[58] argued that the GlcT enzyme, which converts ceramide to glucosylceramide, is expressed at much higher levels in neurons than glia, and that glucosylceramide is then transported from neurons to glial cells for its degradation, suggesting cell-type specific compartmentalization of sphingolipid synthesis in neurons and its degradation in glia in the adult. In the future, it will be exciting to uncover whether genes of the sphingolipid metabolic pathway alter their cell-type specific requirements as a function of developmental stage.

Altered glial function may derive not just from dihydroceramide buildup in the ER, but also from altered cell membrane structure due to the replacement of ceramide and its derivates, such as GlcCer and CPE, with the cognate forms of dihydroceramide. Relative to dihydroceramide species, the 4-5 *trans* carbon-carbon double bond in the sphingoid backbone of ceramide-containing sphingolipids enables them to form more stable hydrogen bonds with water molecules and facilitates their ability to associate with lipids of different saturation levels.[5] A high dihydroceramide to ceramide ratio has been shown to form rigid gel-like domains within model membranes and to destabilize biological membranes by promoting their permeabilization.[59,60] As even minor alterations to membrane properties can disrupt glial morphology,[61] such alterations in

membrane rigidity and stability may underlie the failure of cortex glia to enwrap adjacent neurons. The observed increase in saturation of PE, PC, and PS in the CNS of *ifc* mutant larvae may reflect a compensatory response employed by cells to stabilize cell membranes when challenged with elevated levels of dihydroceramide.

The observed loss of lipid droplets in cortex glia may also contribute to their inability to fully enwrap neuronal cell bodies. Under wild-type conditions, many lipid droplets are present in cortex glia during the rapid phase of neurogenesis that occurs in larvae. During this phase, lipid droplets likely support the ability of cortex glia to generate large quantities of membrane lipids to drive membrane growth needed to ensheath newly born neurons. Supporting this idea, lipid droplets disappear in the adult *Drosophila* CNS when neurogenesis is complete and cortex glia remodeling stops.[29] We speculate that lipid droplet loss in *ifc* mutant larvae contributes to the inability of cortex glia to enwrap neuronal cell bodies. Prior work on lipid droplets in flies has focused on stress-induced lipid droplets generated in glia and their protective or deleterious roles in the nervous system.[62,63,64] Work in mice and humans has found that more lipid droplets are often associated with the pathogenesis of neurodegenerative diseases,[65,66,67] but our work correlates lipid droplet loss with CNS defects. In the future, it will be important to determine how lipid droplets impact nervous system development and disease.

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Author Contributions

BAW, HL, Yi Zhu, and JBS completed the genetic screen that identified the *ifc* alleles and completed the initial genetic characterization of these alleles. Yuqing Zhu, JD, and JBS completed all other experiments except for the lipidomics analyses, which were completed by KC and GJP. Yuqing Zhu and JBS wrote the manuscript and compiled figures with help from JD with figures. All authors read, commented on, and edited the manuscript.

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Declaration of interests: G.J.P. has collaborative research agreements with Agilent Technologies and Thermo Scientific. G.J.P. is the chief scientific officer of Panome Bio. The remaining authors declare no competing interests.

STAR Methods

Key resources table:

Fly stocks used

Genotype	Source	Notes
y[1] M{RFP[3xP3.PB] GFP[E.3xP3]=vas-int.Dm}ZH-2A w[*]; M{3xP3-RFP.attP}ZH-51D	BDSC #24483	Wildtype. Control for 3xP3 background.
Gpdh1[nSP4] ifc[1] wg[spd-fg] pr[1]/CyO, cl[4]	BDSC #4963	The line carrying *ifc*^{1} mutant allele
w[1118]; Df(2L)ED334, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED334/SM6a	BDSC #9343	A collection of deficiency lines that deletes the *ifc* locus
w[1118]; Df(2L)BSC184/CyO	BDSC #9612	
w[1118]; Df(2L)BSC353/CyO	BDSC #24377	
y[1] w[*];TI{GFP[3xP3.cLa]=CRIMIC.TG4.2}ifc[CR70115-TG4.2]/SM6a	BDSC #92710	
v[1]; Kr[If-1]/CyO; P{y[+t7.7] v[+t1.8]=UAS-TransTimer.v+}attP2	BDSC #93411	
y[1] w[*]; PBac{y[+mDint2] w[+mC]=UAS-hDEGS1.HA}VK00033	BDSC #79200	
w[1118] P{y[+t7.7] w[+mC]=hs-FLPG5.PEST}attP3; PBac{y[+mDint2] w[+mC]=10xUAS(FRT.stop)myr::smGdP-HA}VK00005 P{y[+t7.7] w[+mC]=10xUAS(FRT.stop)myr::smGdP-V5-THS-10xUAS(FRT.stop)myr::smGdP-FLAG}su(Hw)attP1	BDSC #64085	Referred to as “MCFO1”
w[1118] P{y[+t7.7] w[+mC]=hs-FLPG5.PEST}attP3; *ifc*^{JS3}/CyOTb; PBac{y[+mDint2] w[+mC]=10xUAS(FRT.stop)myr::smGdP-HA}VK00005 P{y[+t7.7] w[+mC]=10xUAS(FRT.stop)myr::smGdP-V5-THS-10xUAS(FRT.stop)myr::smGdP-FLAG}su(Hw)attP1	Skeath lab	MCFO1 with one copy of *ifc*^{JS3}
w[*]; P{y[+t7.7] w[+mC]=10XUAS-IVS-myr::GFP}attP2	BDSC #32197	
w[1118]; P{y[+t7.7] w[+mC]=GMR54H02-GAL4}attP2	BDSC #45784	Cortex glia GMR-GAL4
ifc-KO/CyO, P{2xTb[1]-RFP}; P{y[+t7.7] w[+mC]=GMR54H02-GAL4}attP2	Skeath lab	Cortex glia GMR-GAL4 with one copy of *ifc-KO*
w[1118]; P{y[+t7.7] w[+mC]=GMR86E01-GAL4}attP2	BDSC #45914	Astrocyte-like glia GMR-GAL4
ifc-KO/CyO, P{2xTb[1]-RFP}; P{y[+t7.7] w[+mC]=GMR86E01-GAL4}attP2	Skeath lab	Astrocyte-like glia GMR-GAL4 with one copy of *ifc-KO*
w[1118]; P{y[+t7.7] w[+mC]=GMR56F03-GAL4}attP2	BDSC #39157	Ensheathing glia GMR-GAL4

ifc-KO/CyO, P{2xTb[1]-RFP}; P{y[+t7.7] w[+mC]=GMR56F03-GAL4}attP2	Skeath lab	Ensheathing glia GMR-GAL4 with one copy of *ifc-KO*
w[1118]; P{y[+t7.7] w[+mC]=GMR54C07-GAL4}attP2	BDSC #50472	Subperineurial glia GMR-GAL4
ifc-KO/CyO, P{2xTb[1]-RFP}; P{y[+t7.7] w[+mC]=GMR54C07-GAL4}attP2	Skeath lab	Subperineurial glia GMR-GAL4 with one copy of *ifc-KO*
w[1118]; P{y[+t7.7] w[+mC]=GMR85G01-GAL4}attP2	BDSC #40436	Perineurial glia GMR-GAL4
ifc-KO/CyO, P{2xTb[1]-RFP}; P{y[+t7.7] w[+mC]=GMR85G01-GAL4}attP2	Skeath lab	Perineurial glia GMR-GAL4 with one copy of *ifc-KO*
P{w[+mW.hs]=GawB}elav[C155]	BDSC #458	
FlyFos019206(pRedFlp-Hgr)(ifc[27951]::2XTY1-SGFP-V5-preTEV-BLRP-3XFLAG)dFRT	VDRC #318826	
P{VSH330794}	VDRC # 330794	UAS-*ifc* RNAi
M{UAS-ifc-ORF-3xHA.attP}86Fb	F003887 (FlyORF)	
ifc^{JS1} M{3xP3-RFP.attP}ZH-51D/CyoTb	Skeath Lab	V276D loss-of-function allele
ifc^{JS2} M{3xP3-RFP.attP}ZH-51D/CyoTb	Skeath Lab	G257S loss-of-function allele
ifc^{JS3} M{3xP3-RFP.attP}ZH-51D/CyoTb	Skeath Lab	W162* loss-of-function allele
P{w[+mC]=GAL4-elav.L}2/CyO ; Repo-GAL80/Tb	Skeath Lab	
ifc-KO/CyO	Dr. Chih-Chiang Chan	Jung et al., 2017[17]

Antibodies and lipid marker used

Antibody/marker	Dilution	Source	RRID
Rabbit anti-FABP	1:500	Skeath lab	
Rabbit anti-EBONY	1:500	Gift from Dr. Haluk Lacin	AB_2314354
Mouse anti-REPO	1:100	DSHB	AB_528448
Rat anti-ELAV	1:100	DSHB	AB_528218
Mouse anti-CXN99A	1:20	DSHB	AB_2722011
Mouse anti-GOLGIN84	1:20	DSHB	AB_2722113
Goat anti-GOLGIN245	1:500	DSHB	AB_2618260
Rabbit anti-ESYT	1:300	Gift from Dr. Dion Dickman	
GFP Antibody Dylight™ 488 Conjugated Pre-Adsorbed, Goat Polyclonal	1:500	Rockland (600-141-215)	AB_1961516
Anti-LAMP1 antibody - Lysosome Marker	1:500	Abcam (ab 30687)	AB_775973
Cleaved Caspase-3 (Asp175) Antibody	1:400	Cell Signaling Technology® (#9661)	AB_2341188
Anti-HA antibody produced in rabbit	1:500	Millipore Sigma (H6908)	AB_260070
ANTI-FLAG® antibody, Rat monoclonal	1:500	Millipore Sigma (SAB4200071)	AB_10603396
Chicken V5 Tag Polyclonal Antibody	1:500	Bethyl laboratories (A190-118A)	AB_66741
BODIPY™ 493/503 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-*s*-Indacene)	1:200	Invitrogen™ (D3922)	
Goat anti-Chicken IgY (H+L) Secondary Antibody, Alexa Fluor™ 488	1:1000	Invitrogen™ (A-11039)	AB_2534096
Donkey anti-Rat IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 555	1:1000	Invitrogen™ (A48270)	AB_2896336
Cy™5 AffiniPure Donkey Anti-Rat IgG (H+L)	1:1000	Jackson ImmunoResearch (712-175-153)	AB_2340672
Cy™5 AffiniPure Donkey Anti-Goat IgG (H+L)	1:1000	Jackson ImmunoResearch (705-175-147)	AB_2340415
Cy™5 AffiniPure Donkey Anti-Mouse IgG (H+L)	1:1000	Jackson ImmunoResearch (715-175-151)	AB_2340820
Cy™5 AffiniPure Donkey Anti-Rabbit IgG (H+L)	1:1000	Jackson ImmunoResearch (711-175-152)	AB_2340607

Deposited data

Raw and processed RNA sequencing data	GEO: GSE263308	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE263308
Untargeted lipidomics	PR001967: ST003162	https://dx.doi.org/10.21228/M8G72D

Software used

Software	Source	Website
ImageJ2 2.3.0/1.53q	NIH; Schindelin et al., 2012	https://imagej.net/ij/
ZEN Microscopy Software	Carl Zeiss AG; Jena, Germany	https://www.zeiss.com/microscopy/en/home.html
Photoshop 23.5.5	Adobe; San Jose, California, USA	https://www.adobe.com/products/photoshop.html
GraphPad Prism 10.2.2	GraphPad; Boston, MA, USA	https://www.graphpad.com/features
BioRender	BioRender; Toronto, CAN	https://www.biorender.com
Microsoft Office 365 16.83 (24031120)	Microsoft; Redmond, USA	https://www.microsoft.com/en-us/microsoft-365

Method details

Fly husbandry

Flies were raised on standard molasses-based food at 25°C. Unless otherwise noted, wild type is an otherwise wild-type stock harboring the *M{3xP3-RFP.attP}ZH-51D* insert in an isogenic second chromosome.

Mutagenesis

A standard autosomal recessive forward genetic screen was carried out using 25-30 µm EMS to mutagenize a *M{3xP3-RFP.attP}ZH-51D* isogenic second chromosome. Homozygous mutant third instar larvae were visually screened under a standard fluorescent microscope for defects in CNS morphology. A detailed description of the screen and all other identified genes will be described elsewhere.

Creation of recombinant lines and identification of second site mutations in *ifc-KO* chromsome

Standard genetic methods were used to generate fly strains that contained specific combinations of GAL4 and UAS-linked transgenes in the *ifc*^{js3} or *ifc-KO* background. During this process, we uncovered that the *ifc-KO* deletion could be unlinked from at least one second chromosomal mutation that caused early larval lethality, resulting in homozygous *ifc-KO* flies that survived to late L3 to early pupa. A subsequent EMS-based F2 lethal non-complementation screen using the *M{3xP3-RFP.attP}ZH-51D* isogenic second chromosome as target chromosome and screening against the *ifc-KO* chromosome identified multiple mutations in four complementation groups that led to an early larval lethal phenotype. Whole genome sequencing identified these genes as *Med15*, *lwr*, *Nle*, and *Sf3b1*; all four genes reside within ~90 Kb of each other in chromosomal bands 21D1-21E2 near the telomere in chromosome 2L, identifying the site of the associated lethal mutations and suggesting the actual lesion may be a small deletion that removes these genes. The following alleles of these genes are available at Bloomington Stock Center: *Med15*^{js1} (Q175*), *Med15*^{js2} (Q398*), *Med15*^{js3} (C655Y), *lwr*^{js1} (I4N), *lwr*^{js2}(E12K), *Nle*^{js1} (W146R), *Nle*^{js2} (L125P), *Nle*^{js3} (Q242*), *Sf3b1*^{js1} (R1160*), *Sf3b1*^{js2} (Q1264*), *Sf3b1*^{js3} (570 bp deletion at the following coordinates chr2L571720-572290; this deletion removes amino acids 1031 through 1222 and introduces a frameshift into the reading frame).

Gene rescue and in vivo RNAi phenocopy assays

To restrict UAS-linked transgene expression specifically to glia, we used the *repoGAL4* driver line. To restrict UAS-linked transgene expression specifically to neurons, we paired the *elavGAL4* driver lines, which activates transgene expression strongly in all neurons and moderately in glia, with *repoGAL80*, which blocks GAL4-dependent activation in glia. We used *P{VSH330794}* (VDRC 330794) for the RNAi experiments and *UAS-ifc*^{68} and *UAS-DEGS1*

(BDSC 79200) for the gene rescue assays. All gene rescue experiments were performed in the *ifc*^{js3}/*ifc-KO* background with the UAS-transgene placed into the *ifc*^{js3} background and the GAL4 drivers into the *ifc-KO* background.

The GAL4-UAS method was also used to assess the phenotype of each glial subtype. In combination with the *UAS-Myr-GFP* transgene, which labels cell membranes, we used the following GAL4 lines to trace the morphology of each glial subtype in wild type and *ifc*$^{-/-}$ larvae: *GMR85G01-GAL4* (perineurial glia; BDSC 40436), *GMR54C07-GAL4* (subperineurial glia; BDSC 50472), *GMR54H02-GAL4* (cortex glia; BDSC 45784), *GMR56F03-GAL4* (ensheathing glia; BDSC 77469 and 39157), and *GMR86E01-GAL4* (astrocyte-like glia; BDSC45914). The *UAS-Myr-GFP* transgene was placed into the *ifc*^{js3} background; each glial GAL4 line was placed into the *ifc-KO* background.

DNA sequencing

Genomic DNA was obtained from wild type larvae or larvae homozygous for each relevant mutant line and provided to GTAC (Washington University) or GENEWIZ for next-generation or Sanger sequencing.

RNA in-situ hybridization

ifc RNA probes for in-situ hybridization chain reaction (HCR) were designed and made by Molecular Instruments (HCR™ RNA-FISH v3.0).[69] Wild type CNS was harvested and fixed in 2% paraformaldehyde at late L3. The fixed CNS underwent gradual dehydration and rehydration, followed by standard hybridization and amplification steps of the HCR protocol.[69] For double labeling with antibody, the post-HCR labeled CNS was briefly fixed for 30 minutes prior to standard antibody labeling protocol to identify specific CNS cell type(s) with highly localized *ifc* RNAs.[70,71]

MultiColor FlpOut labeling of glial subtypes

For glial labeling in the control background, the *MCFO1* line was crossed to *GMR-GAL4* driver lines for each glial subtype (Supplemental Table 1).[32] For glial labeling in *ifc*$^{-/-}$ background, the *MCFO1* line was placed in the *ifc*^{JS3} background, each of the five glial-specific GMR-GAL4 lines were placed individually into the *ifc-KO* background, and then the MCFO, *ifc*^{JS3} line was crossed to each glial specific GAL4, *ifc-KO* line. Flies were allowed to lay eggs for 24 hr at 25°C, and progeny were raised at 25°C for 4 days prior to heat-activated labeling. On day 4 after egg-laying, F1 larvae were incubated in a 37°C water bath for 5-minutes. When wild-type or *ifc*$^{-/-}$ mutant larvae reached late L3, which was day 5-6 for control and day 9-10 for *ifc*$^{-/-}$ mutants, the CNS was dissected, fixed, stained, and then analyzed under a Zeiss LSM 700 Confocal Microscope for the presence of clones, using Zen software.

Antibody generation

YenZym (CA, USA) was used as a commercial source to generate affinity purified antibodies against a synthetic peptide that corresponded to amino acids 85-100 (TLDGNKLTQEQKGDKP) of FABP isoform B. Briefly, the peptide was conjugated to KLH, used as an immunogen in rabbits to generate a peptide-specific antibody response, and antibodies specific to the peptide were affinity purified. The affinity-purified antibodies were confirmed to label cortex glia specifically based on comparison of antibody staining relative to Myr-GFP when a UAS-Myr-GFP was driven under control of the cortex specific glial GAL4 driver GMR-54H02 (Figure S3). The FABP antibodies are used at 1:500-1:1000.

Immunofluorescence and lipid droplet staining

Gene expression analysis was performed essentially as described in Patel.[72] Briefly, the larval CNS was dissected in PBS, fixed in 2.5% paraformaldehyde for 55 minutes, and washed in PTx

(1xPBS, 0.1% TritonX-100. The fixed CNS was incubated in primary antibody with gentle rocking overnight at 4°C. Secondary antibody staining was conducted for at least two hours to overnight at room temperature. All samples were washed in PTx at least five times and rocked for an hour before and after secondary antibody staining. A detailed list of the primary and secondary antibodies is available in Supplemental Table 2. Dissected CNS were mounted either in PTx or dehydrated through an ethanol series and cleared in xylenes prior to mounting in DPX mountant.[73] All imaging was performed on a Zeiss LSM-700 Confocal Microscope, using Zen software.

For lipid droplet staining, the fixed CNS was incubated for 30 minutes at room temperature at 1:200 dilution of 1mg/mL BODIPY™ 493/503 (Invitrogen: D3922). It was then rinsed thoroughly in PBS and immediately mounted for imaging on a Zeiss LSM-700 Confocal Microscope, using Zen software.

Transmission electron microscopy (TEM) imaging

For transmission electron microscopy (TEM), samples were immersion fixed overnight at 4°C in a solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.15 M cacodylate buffer with 2 mM CaCl2, pH 7.4. Samples were then rinsed in cacodylate buffer 3 times for 10 minutes each and subjected to a secondary fixation for one hour in 2% osmium tetroxide/1.5% potassium ferrocyanide in cacodylate buffer. Following this, samples were rinsed in ultrapure water 3 times for 10 minutes each and stained overnight in an aqueous solution of 1% uranyl acetate at 4˚C. After staining was complete, samples were washed in ultrapure water 3 times for 10 minutes each, dehydrated in a graded acetone series (50%, 70%, 90%, 100% x4) for 15 minutes in each step, and infiltrated with microwave assistance (Pelco BioWave Pro, Redding, CA) into Spurr's resin. Samples were then cured in an oven at 60˚C for 72 hours and post-curing, 70 nm thin sections were cut from the resin block, post-stained with uranyl acetate and Sato's lead and

imaged on a Transmission Electron Microscope (JEOL JEM-1400 Plus, Tokyo, Japan) operating at 120 KeV.

Lipidomics

Untargeted lipidomics analysis was conducted on whole larva and dissected CNS of wild type and *ifc*$^{-/-}$ mutants at the late-third instar stage. Five replicates were prepared for each set of experiments. For whole larvae, at least 15 larvae of each genotype were used for each replicate. For the dissected CNS, at least 50 wild type and 60 *ifc*$^{-/-}$ CNS were used per replicate. Immediately following collection or dissection, larvae and the dissected CNS were flash frozen in liquid nitrogen and placed at -80°C.

Lipids were extracted from frozen whole larvae and dissected larval CNS samples by using an Omni Bead Ruptor Elite Homogenizer using acetonitrile:methanol:water (2:2:1; 40 μL/mg tissue). Two Ultrahigh performance LC-(UHPLC)/MS systems were used in this work: a Thermo Vanquish Flex UHPLC system with a Thermo Scientific Orbitrap ID-X and an Agilent 1290 Infinity II UPLC system with an Agilent 6545 QTOF as described previously.[74] Lipids were separated on a Waters Acquity HSS T3 column (150 x 2.1 mm, 1.8 mm). The mobile-phase solvents were composed of A: 0.1% formic acid, 10 mM ammonium formate, 2.5 μM medronic acid in 60:40 acetonitrile:water; and B = 0.1% formic acid, 10 mM ammonium formate in 90:10 2-propanol:acetonitrile. The column compartment was maintained at 60 °C. The following linear gradient was applied at a flow rate of 0.25 mL min-1: 0 – 2 min, 30% B; 17 min, 75% B; 20 min, 85% B; 23 – 26 min, 100% B. The injection volume 4 μL for all lipids analysis. Data was acquired in positive ion.

LC/MS data were processed and analyzed with the open-source Skyline software.[75] Lipid MS/MS data were annotated with Agilent Lipid Annotator software.

RNA sequencing and analysis

To determine the CNS-specific transcriptional changes upon loss of *ifc*, RNA-seq was conducted on five replicates of dissected CNS tissue derived from wild type and *ifc*$^{-/-}$ mutant late-third instar larvae. For each replicate, roughly 30-35 dissected CNS of wild type or *ifc-/-* larvae were used. Invitrogen™ RNAqueous™-Micro Total RNA Isolation Kit (AM1931) was used to extract RNA. Agilent 4200 TapeStation system was used for RNA quality control.

Samples were prepared according to library kit manufacturer's protocol, indexed, pooled, and sequenced on an Illumina NovoSeq. Basecalls and demultiplexing were performed with Illumina's bcl2fastq software and a custom python demultiplexing program with a maximum of one mismatch in the indexing read. RNA-seq reads were then aligned to the Ensembl release 76 primary assembly with STAR version 2.5.1a.[76] Gene counts were derived from the number of uniquely aligned unambiguous reads by Subread:featureCount version 1.4.6-p5.[77] Isoform expression of known Ensembl transcripts were estimated with Salmon version 0.8.2.[78] To find the most critical genes, the raw counts were variance stabilized with the R/Bioconductor package DESeq2[79] and was then analyzed via weighted gene correlation network analysis with the R/Bioconductor package WGCNA.[80]

Statistics

All data are presented as mean ± SEM. Statistical significance between groups was determined using Student's *t*-test or one-way ANOVA with multiple comparisons, and with varying levels of significance assessed as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, and ns, not significant.

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Figure legends

Figure 1. *ifc* regulates CNS and glial morphology. A) Ventral views of late-third instar larvae of indicated genotype showing 3xP3 RFP labeling of CNS and nerves. Arrowheads indicate nerve bulges; scale bar is 200μm. B-B') Schematic of Ifc (B) and human DEGS1 (B') proteins indicating location and nature of *ifc* mutations and 15 HLD-18-causing *DEGS1* mutations.[7,8,9] C) Schematic of *de novo* ceramide biosynthesis pathway indicating the subcellular location of ceramide synthesis and ceramide modifications. D) Chemical structure of dihydroceramide and ceramide; arrow indicates *trans* carbon-carbon double bond between C4 and C5 in the sphingoid backbone created by the enzymatic action of Ifc/DEGS1. E) Normalized quantification of the relative levels of dihydroceramide, ceramide, and six related sphingolipid species in the dissected CNS of wild-type and $ifc^{-/-}$ late-third instar larvae. F) Ventral views of *Drosophila* CNS and peripheral nerves in wild-type and $ifc^{-/-}$ mutant late-third instar larvae labeled for NCAD to mark the neuropil, HRP to label axons, RFP to label glia, Dpn to label neuroblasts, ELAV to label neurons, and FABP to label cortex glia. Anterior is up; scale bar is 100μm for whole CNS images and 20μm for peripheral nerve image. Statistics: * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, **** *denotes* $p < 0.0001$.

Figure 2. Loss of *ifc* disrupts glial morphology. A-E') High magnification ventral views and X-Z and Y-Z projections of the nerve cord of wildtype and *ifc*$^{-/-}$ late third instar larvae labeled for ELAV (magenta) for neurons and Myr-GFP (green) for cell membranes of indicated glial subtype. Anterior is up; scale bar is 40µm. F-J') High magnification views of individual glial cells of indicated glial subtype in the nerve cord of wildtype and *ifc*$^{-/-}$ larvae created by the MultiColor-FlpOut method.[32] Anterior is up; scale bar is 20µm. K-N) Quantification of total number of indicated glial subtype in the nerve cord of wildtype and *ifc*$^{-/-}$ late third instar larvae (n = 7 for K, L, N; n = 6 for M). Statistics: * denotes $p < 0.05$, **** denotes $p < 0.0001$, and ns, not significant.

Figure 3. *ifc* acts in glia to regulate CNS structure and glial morphology. A-I) Ventral views of photomontages of the CNS of late third instar larvae labeled for FABP (greyscale) to mark cortex glia in late third instar larvae of indicated genotype. Neuronal-specific transgene expression was achieved by using *elav-GAL4* combined with *repo-GAL80*; glial-specific transgene expression was achieved by using *repo-GAL4*. J-K) Quantification of the number of swollen cortex glia in the abdominal segments of the CNS of late-third instar larvae of the indicated genotype for the RNAi (J) and gene rescue assays (K). Statistics: * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, **** denotes $p < 0.0001$, and ns, not significant.

Figure 4. Loss of ifc drives ER expansion in cortex glia. A-E) Dorsal (A, B-B'', and C-C'') and ventral (D-D'' and E-E'') views of the CNS of late-third instar wild-type larvae labeled for *ifc* RNA (grey in A; magenta in B'), *ifc-GAL4>nRFP* (magenta; C'-E'), EBONY to mark astrocytes (green; B and C), REPO to mark glia (green; D), and ELAV to mark neurons (green; E). Panels D-D'' and E-E'' show surface and interior views, respectively, along the Z-axis on the ventral side of the nerve cord. Arrowheads in E-E'' identify neurons with low level *ifc-GAL4* expression. F-G) High

magnification ventral views of thoracic segments in the CNS of wild-type late third instar larvae labeled for GFP (green; F and G), CNX99A (magenta; F'), ESYT (magenta; G'). H-M) Late third instar larvae of indicated genotype labeled for 3xP3-RFP (green; H'-M'), CNX99A (magenta; H and K), GOLGIN84 (magenta; I and L), and LAMP (magenta; J and M). Anterior is up; scale bar is 100μm for panel A and 30μm for panels B-M.

Figure 5. Loss of *ifc* leads to internal membrane accumulation and lipid droplet loss in cortex glia. A-E') TEM images of cortex glia cell body (A-A' and B-B') and neuronal cell bodies (C-C' and D-D') at low (A-A') and high (B-B', C-C', and D-D') magnification in the nerve cord of wildtype (A-D) and *ifc*$^{-/-}$ (A'-D' and E-E') late third instar larvae. (A-A') Dotted lines demarcate cell boundary of cortex glia; yellow squares highlight regions magnified in B, B', and E'. Scale bar is 3μm for A and A' and 1μm for B-B'. (B-B') Cy denotes cytoplasm; Nu denotes nucleus. Solid white arrows highlight the layered internal membranes that occupy the cytoplasm of *ifc*$^{-/-}$ cortex glia. (C-C' and D-D') Black arrows highlight cortex glia membrane extensions that enwrap neuronal cell bodies; hollow white arrows denote the absence of cortex glia membrane extensions; white asterisk denotes lipid droplets. Scale bar is 2μm. E-E') An additional example of membrane-filled cortex glia cell body in *ifc*$^{-/-}$ larvae. Scale bar is 2μm for E and 1μm for E'. F) Cortex glia in *ifc* mutant larvae labeled for Myr-GFP (green) to label membranes and CNX99A to label ER membranes. Scale bar is 30μm. G-H) Black and white and colored TEM cross-sections of peripheral nerves in wild type and *ifc*$^{-/-}$ late-third instar larvae. Blue marks perineurial glia; purple marks subperineurial glia; pink marks wrapping glia. Scale bar: 2μm. I-I') High magnification ventral views of abdominal segments in the ventral nerve cord of wild-type (I) and *ifc* mutant (I') third instar larvae labeled for BODIPY (green) to mark lipid droplets and FABP (magenta) to label cortex glia. Anterior is up; scale bar is 30μm. J) Graph of log-fold change of transcription of five genes that promote membrane lipid synthesis in *ifc*$^{-/-}$ larvae relative to wildtype. K-M) Quantification of the number (G)

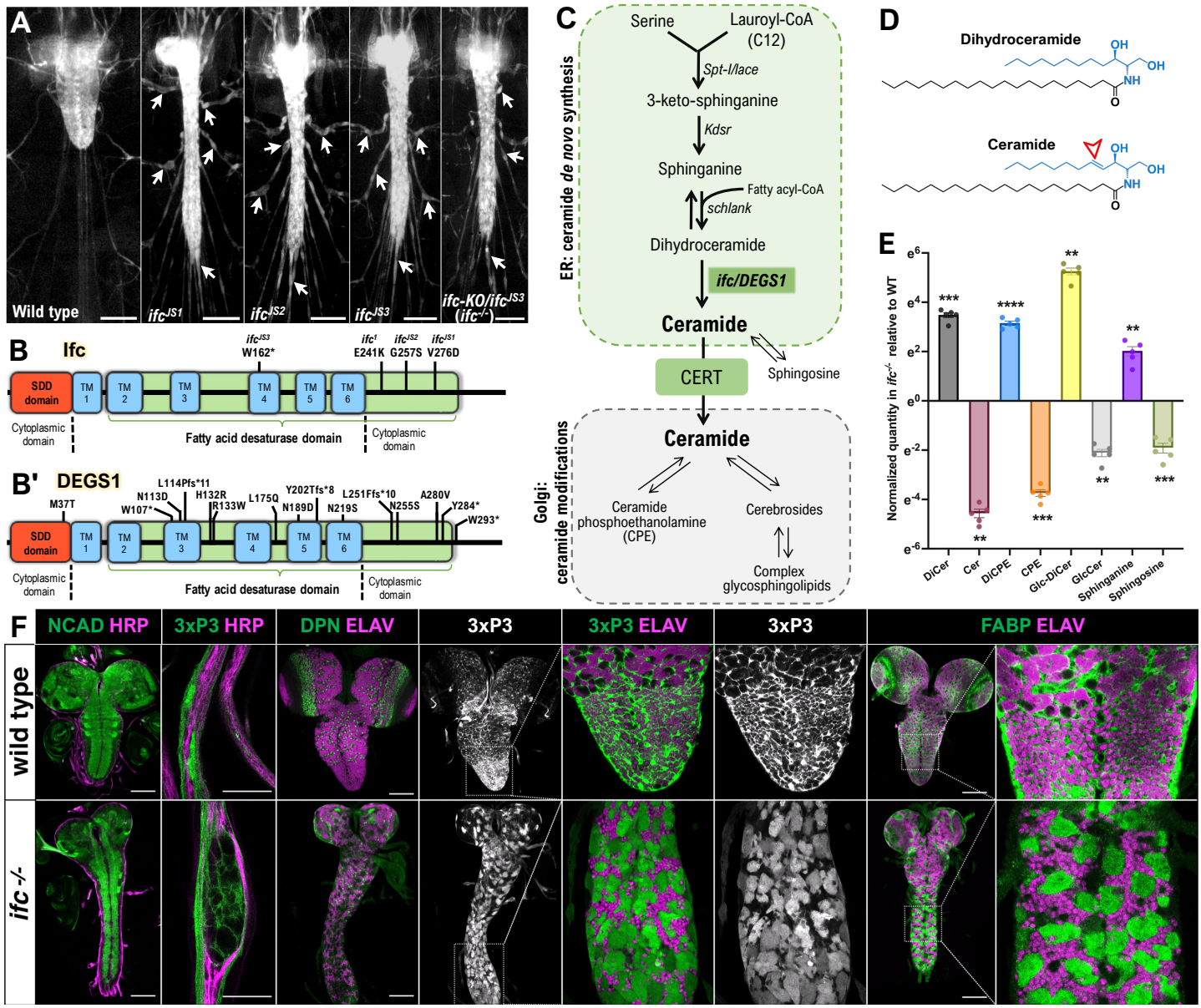
and area of lipid droplets (H and I) in the dissected CNS of wildtype and *ifc*$^{-/-}$ larvae. Statistics: * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, **** denotes $p < 0.0001$, and ns, not significant.

Figure 6. PC, PE, PS, and TG exhibit higher saturation levels in a CNS-specific manner in *ifc* mutant late third instar larvae. A-C) Quantification of total (A) and species-specific (B and C) TGs in whole larvae (A and C) and dissected CNS (A, B, and C) of wildtype and *ifc*$^{-/-}$ larvae. D-F). Quantification of total (D) and species-specific (E and F) PCs in whole larvae (D and F) and dissected CNS (D, E, and F) of wildtype and *ifc*$^{-/-}$ larvae. G-I) Quantification of total (G) and species-specific (H and I) PEs in whole larvae (G and H) and dissected CNS (G, H, and I) of wild-type and *ifc*$^{-/-}$ larvae. J-L) Quantification of total (J) and species-specific (K and L) PSs in the whole larvae (J and L) and dissected CNS (J, K, and L) of wild-type and *ifc*$^{-/-}$ larvae. Statistics: * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, **** denotes $p < 0.0001$, and ns, not significant.

Figure 7. Glial-specific knockdown of *ifc* triggers neuronal cell death. A-D) TEM images of cortex glia cell body (A and B) and neuronal cell bodies (C and D) at low (A) and high (B, C, and D) magnifications in the nerve cord of *ifc*$^{-/-}$; *repo>schlank RNAi* late third instar larvae. Dotted lines demarcate cell boundary of cortex glia; yellow squares highlight regions magnified in A. Scale bar: 3μm for A, 1μm for B, and 2μm for C and D. (B) Cy denotes cytoplasm; Nu denotes nucleus. (C-D) Black asterisk denotes lipid droplets. E-F) Ventral views of abdominal sections of CNS of *ifc*$^{-/-}$; *UAS-schlank RNAi/+* larvae (E) and *ifc*$^{-/-}$; repoGAL4/*UAS-schlank RNAi* larvae (F) labeled for neurons (ELAV, Green) and cortex glia (FABP, magenta/grey). Scale bar is 30μm for E-F. G-L) Low (G-L) and high (G'-L' and G''-L'') magnification views of the brain (G-I) and nerve cord (J-L) of late-third instar larvae of the indicated genotypes labeled for ELAV (magenta or greyscale)

and Caspase-3 (green). Arrows indicate regions of high Caspase-3 signal and/or apparent neuronal cell death identified by perforations in the neuronal cell layer. Scale bar is 50μm for panels G-L and 10μm for panels G'-L''. M-N) Quantification of CNS elongation (M) and 3xP3 RFP intensity (N) in *ifc* mutants alone, *ifc* mutants with one copy of *schlank[G0365]* loss-of-function allele, or *ifc* mutants in which *schlank* function is reduced via RNAi in glial cells. O) Quantification of the area of lipid droplets in dissected CNS of *ifc* mutants and *ifc* mutants in which *schlank* function is reduced via RNAi in glial cells. Anterior is up in all panels. P-Q) Quantification of Cleaved Caspase-3 neurons for panels G-I (P) and J-L (Q). Statistics: * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, **** denotes $p < 0.0001$, and ns, not significant.

Figure 1.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ifc^JS2
ifc^JS3
ifc-KO/ifc^JS3 (ifc^-/-)
B
Ifc
ifc^JS3 W162*
ifc^1 E241K
ifc^JS2 G257S
ifc^JS1 V276D
SDD domain
TM 1
TM 2
TM 3
TM 4
TM 5
TM 6
Cytoplasmic domain
Fatty acid desaturase domain
Cytoplasmic domain
B'
DEGS1
M37T
W107*
N113D
L114Pfs*11
H132R
R133W
L175Q
Y202Tfs*8
N189D
N219S
L251Ffs*10
N255S
A280V
Y284*
W293*
C
ER: ceramide de novo synthesis
Serine
Lauroyl-CoA (C12)
Spt-I/lace
3-keto-sphinganine
Kdsr
Sphinganine
Fatty acyl-CoA
schlank
Dihydroceramide
ifc/DEGS1
Ceramide
CERT
Sphingosine
Golgi: ceramide modifications
Ceramide
Ceramide phosphoethanolamine (CPE)
Cerebrosides
Complex glycosphingolipids
D
Dihydroceramide
Ceramide
OH
NH
O
E
Normalized quantity in ifc^-/- relative to WT
DiCer
Cer
DiCPE
CPE
Glc-DiCer
GlcCer
Sphinganine
Sphingosine
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FABP ELAV
wild type
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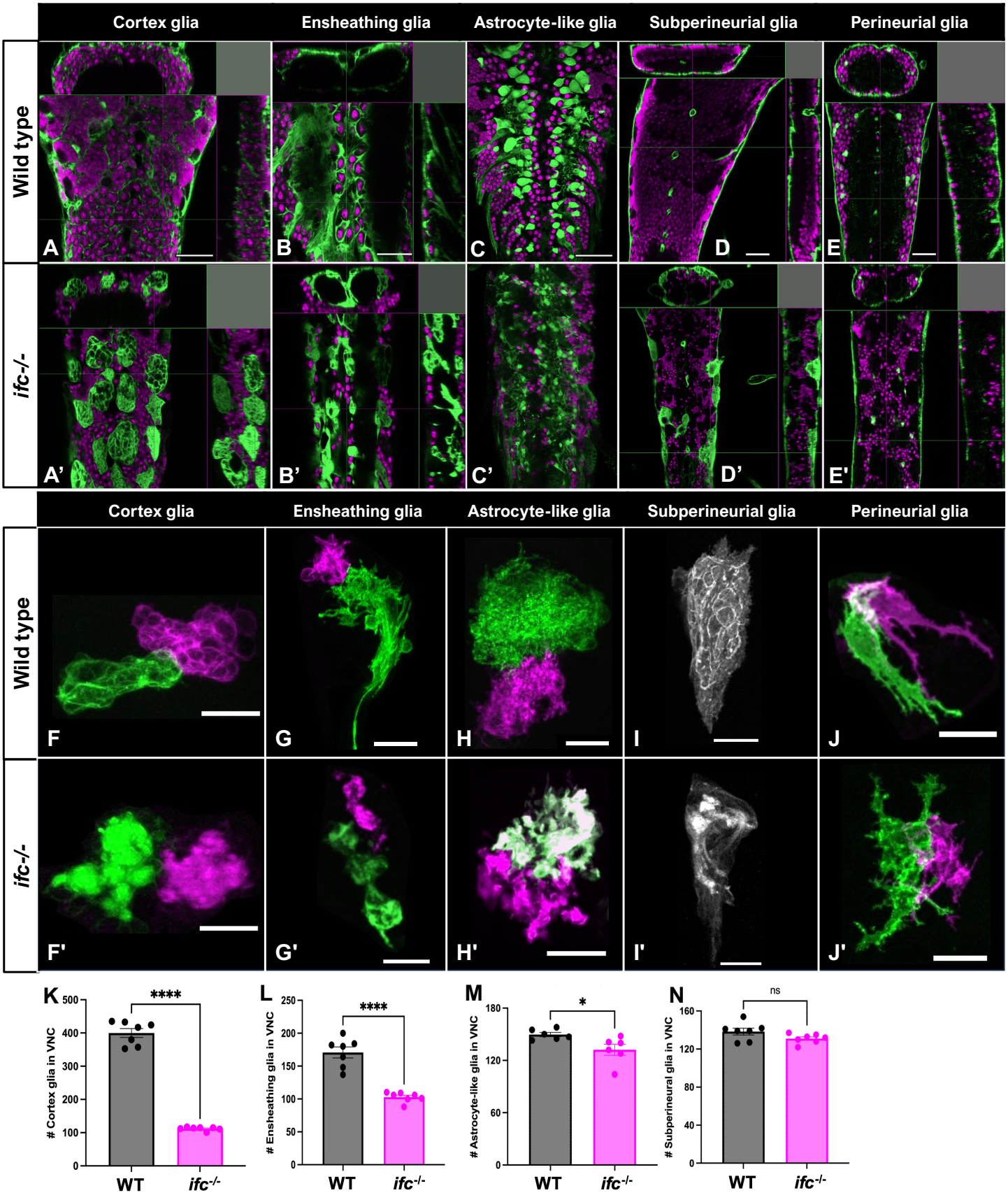
Figure 2.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Ensheathing glia
Astrocyte-like glia
Subperineurial glia
Perineurial glia
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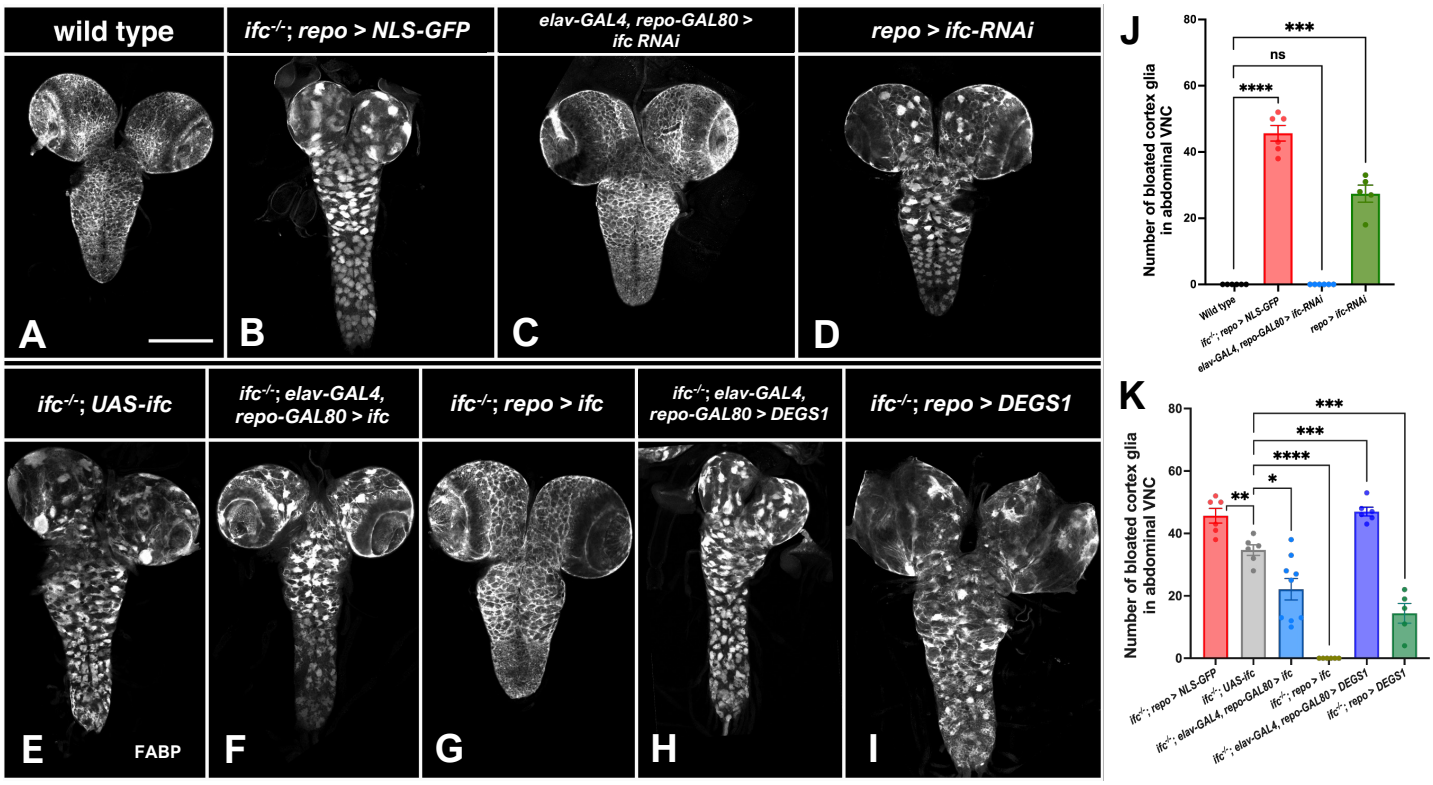
Figure 3.



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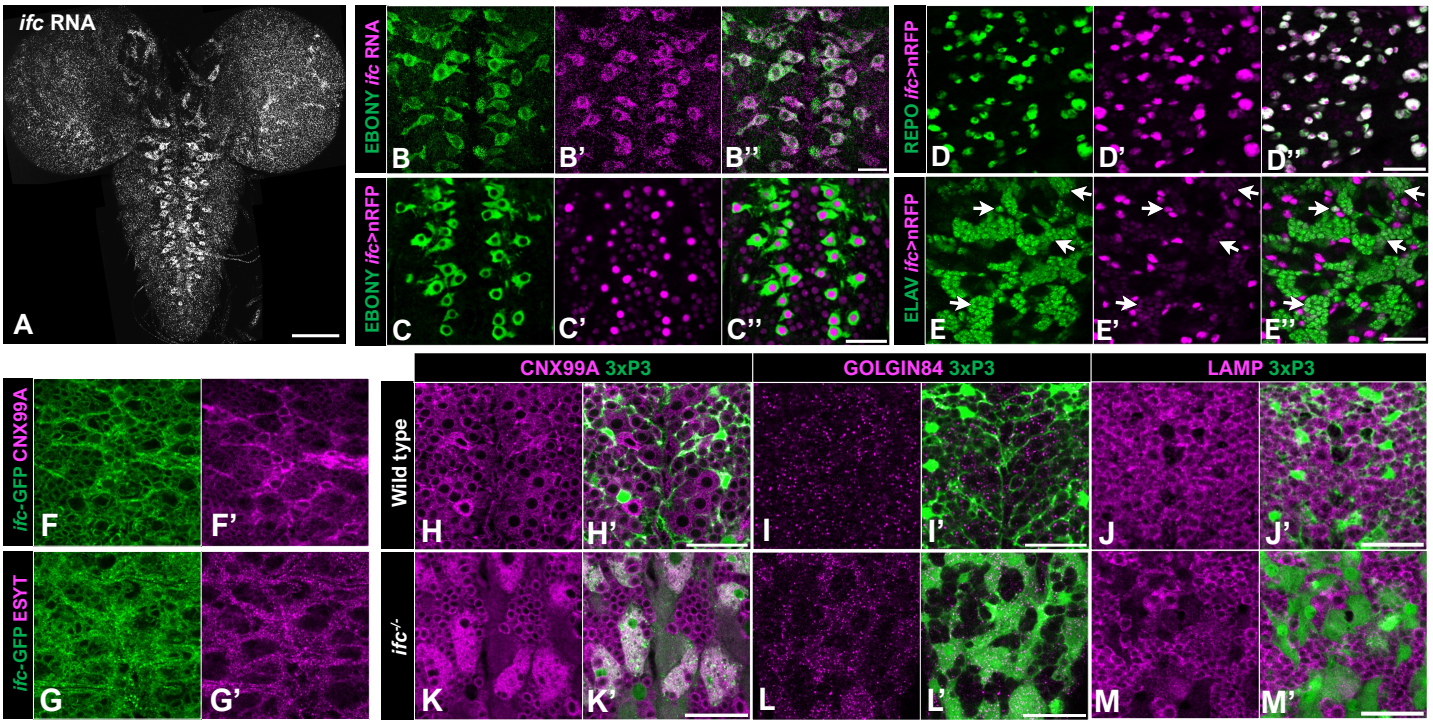
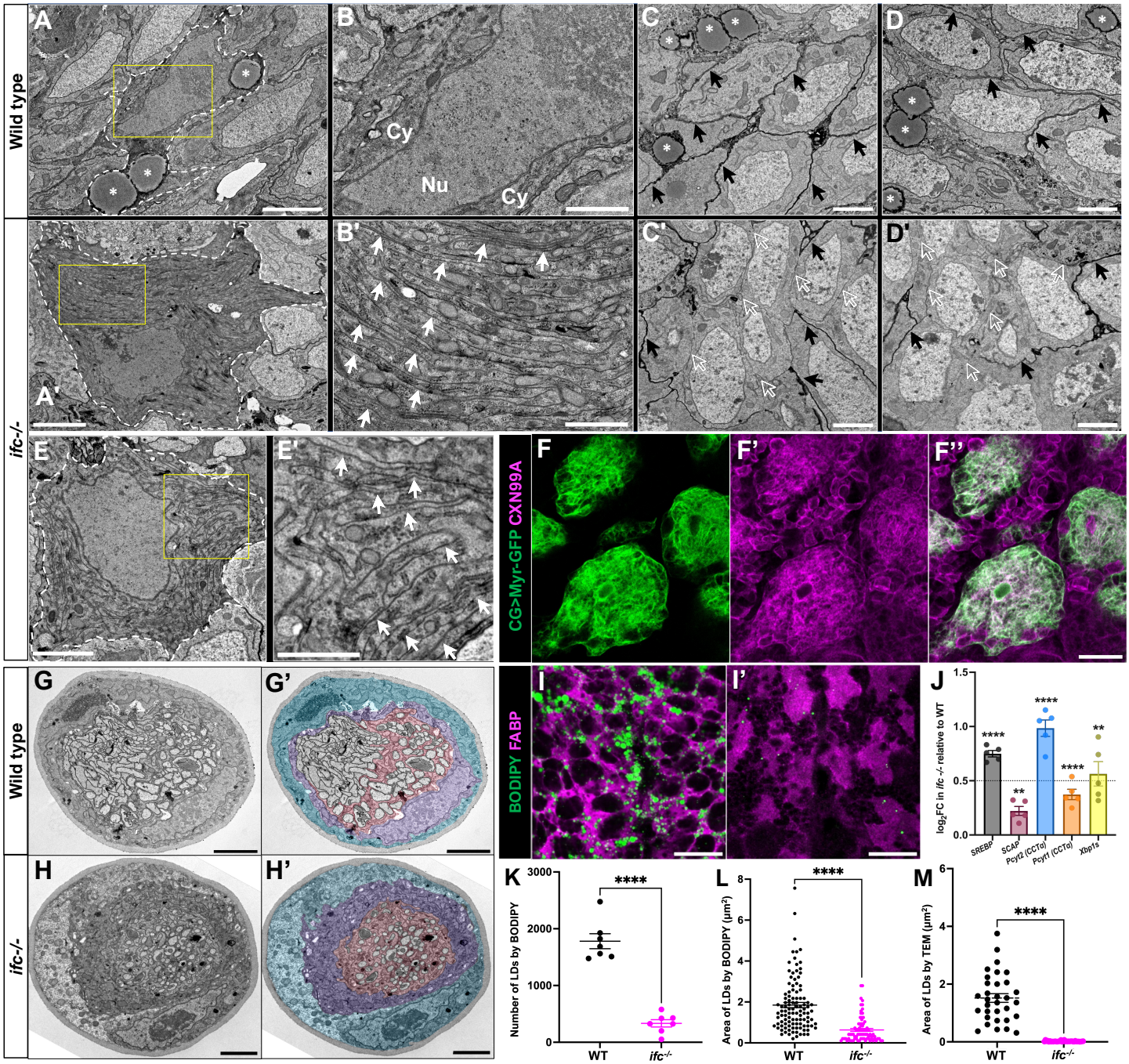


Figure 5.


Wild type
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CG>Myr-GFP CXN99A
G
G'
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I'
BODIPY FABP
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log2FC in ifc -/- relative to WT
SREBP
SCAP
Pcyt2 (CCTα)
Pcyt1 (CCTα)
Xbp1s
K
Number of LDs by BODIPY
L
Area of LDs by BODIPY (μm²)
M
Area of LDs by TEM (μm²)
WT
ifc-/-
****

Figure 6.

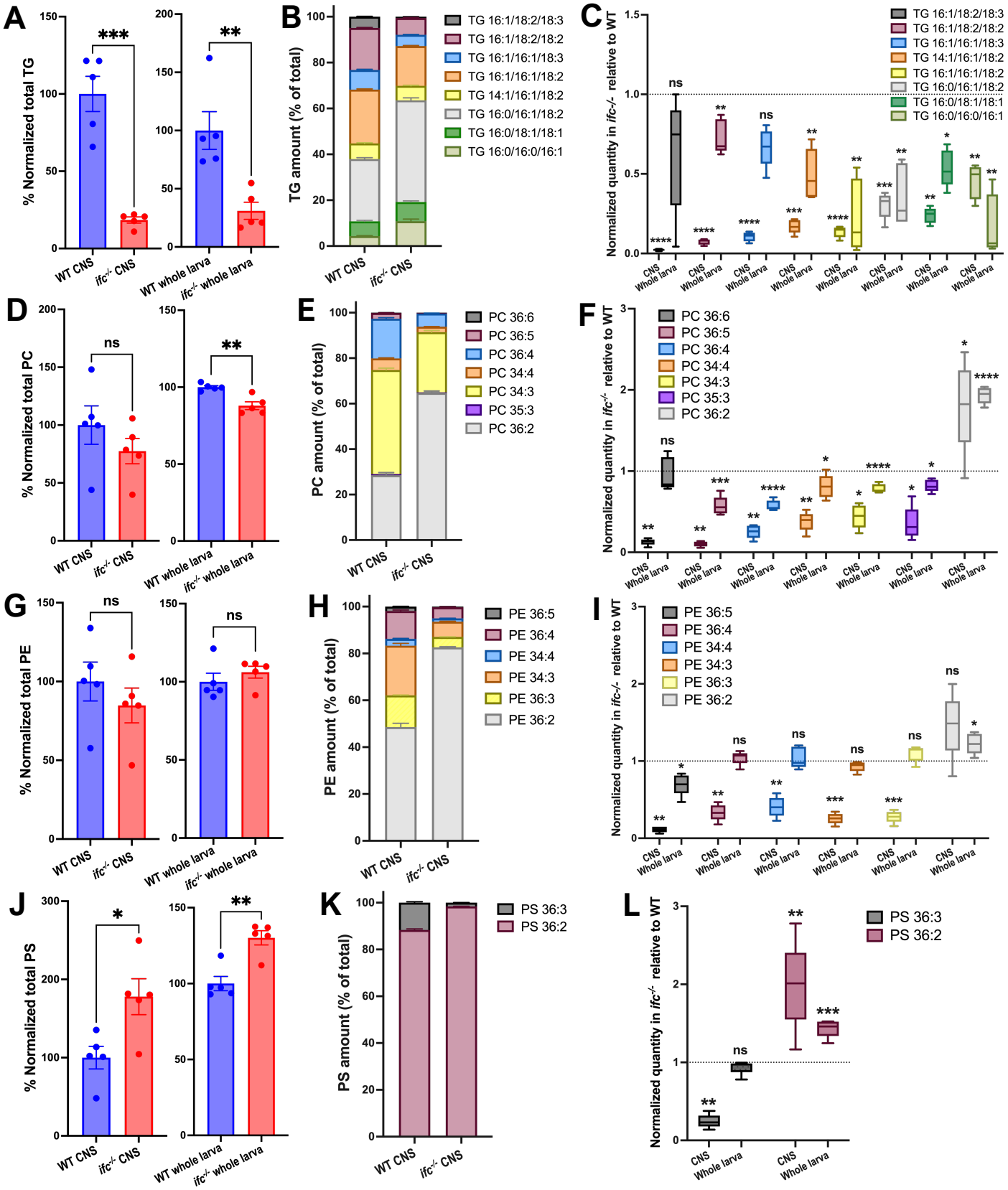
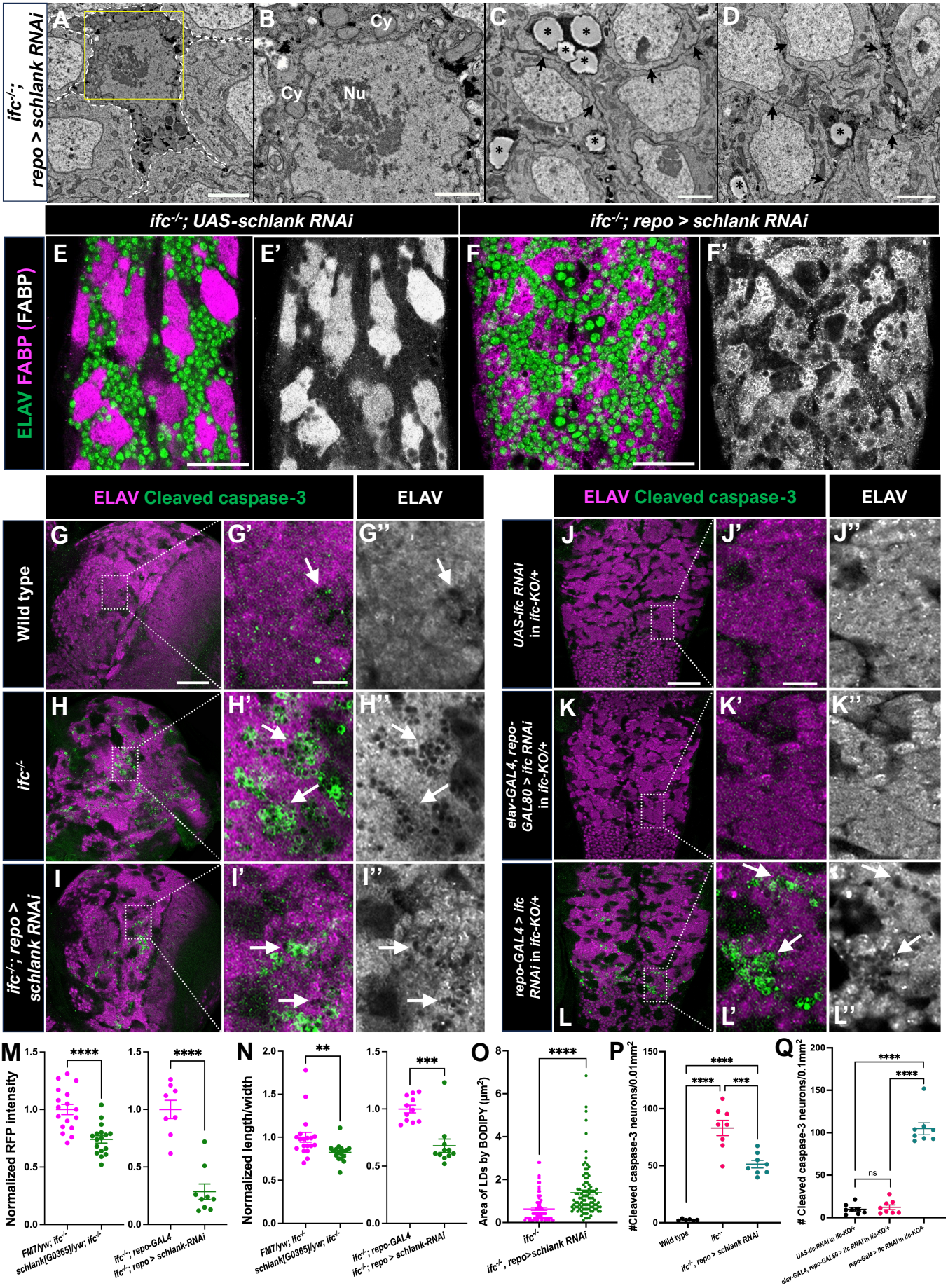

A
% Normalized total TG
WT CNS
ifc-/- CNS
WT whole larva
ifc-/- whole larva
B
TG amount (% of total)
TG 16:1/18:2/18:3
TG 16:1/18:2/18:2
TG 16:1/16:1/18:3
TG 16:1/16:1/18:2
TG 14:1/16:1/18:2
TG 16:0/16:1/18:2
TG 16:0/18:1/18:1
TG 16:0/16:0/16:1
C
Normalized quantity in ifc-/- relative to WT
CNS
Whole larva
D
% Normalized total PC
E
PC amount (% of total)
PC 36:6
PC 36:5
PC 36:4
PC 34:4
PC 34:3
PC 35:3
PC 36:2
F
Normalized quantity in ifc-/- relative to WT
G
% Normalized total PE
H
PE amount (% of total)
PE 36:5
PE 36:4
PE 34:4
PE 34:3
PE 36:3
PE 36:2
I
Normalized quantity in ifc-/- relative to WT
J
% Normalized total PS
K
PS amount (% of total)
PS 36:3
PS 36:2
L
Normalized quantity in ifc-/- relative to WT

Figure 7.


ifc-/-; repo > schlank RNAi
A
B
Cy
Cy
Nu
C
D
ifc-/-; UAS-schlank RNAi
ifc-/-; repo > schlank RNAi
ELAV FABP (FABP)
E
E'
F
F'
ELAV Cleaved caspase-3
ELAV
Wild type
G
G'
G''
UAS-ifc RNAi in ifc-KO/+
J
J'
J''
ifc-/-
H
H'
H''
elav-GAL4, repo-GAL80 > ifc RNAi in ifc-KO/+
K
K'
K''
ifc-/-; repo > schlank RNAi
I
I'
I''
repo-GAL4 > ifc RNAi in ifc-KO/+
L
L'
L''
M
Normalized RFP intensity
****
****
FM7/yw; ifc-/- schlank[G0365]/yw; ifc-/-
ifc-/-; repo-GAL4 ifc-/-; repo > schlank-RNAi
N
Normalized length/width
**
***
O
Area of LDs by BODIPY (μm2)
****
ifc-/-
ifc-/-, repo>schlank RNAi
P
#Cleaved caspase-3 neurons/0.01mm2
****
****
***
Wild type
ifc-/-
ifc-/-, repo > schlank RNAi
Q
Cleaved caspase-3 neurons/0.1mm2
****
****
ns
UAS-ifc-RNAi in ifc-KO/+
elav-GAL4, repo-GAL80 > ifc RNAi in ifc-KO/+
repo-Gal4 > ifc RNAi in ifc-KO/+