Dihydroceramide desaturase directs the switch between exosome production and autophagy for neuronal maintenance

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ABSTRACT

Exosomes play important roles in the nervous system. Mutations in the human

dihydroceramide desaturase gene, *DEGS1*, are recently linked to severe neurological

disorders, but the cause remains unknown. Here, we show that Ifc is required for the

morphology and function of *Drosophila* photoreceptor neurons and not in the

surrounding glia, but the degeneration of *ifc-KO* eyes can be rescued by glial expression

of *ifc*, possibly mediated by exosomes. We develop an *in vivo* assay using Drosophila eye

imaginal discs and show that the level and activity of Ifc correlates with the detection of

exosome-like vesicles. While *ifc* overexpression and autophagy inhibition both enhances

exosome production, combining the two had no additive effect. Moreover, *ifc-KO* reduces

the density of the exosome precursor intraluminal vesicles (ILVs) in vivo, and DEGS1

promotes ILV formation *in vitro*. In conclusion, dihydroceramide desaturase promotes

exosome formation and prevents its autophagic degradation in the nervous system.

Introduction

Exosomes are secreted vesicles of approximately 100 nm in diameter that mediate the transport of proteins, RNAs, or lipid cargos. In the central nervous system, exosomes serve as a form of intercellular communication and participate in neurodevelopment, synaptic function, and neuronal maintenance (Budnik et al., 2016). In neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease, exosomes affect disease progression by facilitating the clearance of protein aggregates or the spread of toxic signals (Budnik et al., 2016). Exosomes originate from the invagination of the endosomal membrane in multivesicular endosomes (MVEs) to form intra-luminal vesicles (ILVs) (Raposo and Stoorvogel, 2013). ILVs are formed by a mechanism involving the Endosomal Sorting Complexes Required for Transport (ESCRT) complex of more than 20 proteins that recruit ubiquitinated protein cargos (Katzmann et al., 2001) and facilitate the budding and scission of the membrane to form ILVs into the MVE lumen (Adell et al., 2014). Alternatively, ILVs can also form via an ESCRT-independent, ceramide (Cer)-dependent mechanism. Trajkovic et al. (2008) show that the inhibition of neutral sphingomyelinase blocks the conversion of sphingomyelin to Cer and reduces exosome release from oligodendrocytes, likely because low Cer disfavors membrane budding.

Once formed, ILVs inside MVEs may be targeted for autophagic or lysosomal degradation, or be released to the extracellular space as exosomes when the MVE membrane fuses with the plasma membrane. Although a ubiquitin-like post-translational modification called ISGylation has been described to promote MVE degradation and thus block exosome secretion (Villarroya-Beltri et al., 2016), the control of the switch between secretion and degradation remains largely unknown.

Sphingolipids such as sphingomyelin and Cer are major components of cellular

membranes in eukaryotic cells, typically represent ~10-20% of cellular lipids (van Meer

et al., 2008). In addition to sphingomyelin hydrolysis, as much as one-third of cellular

Cer is produced via the *de novo* synthesis pathway (Lee et al., 2012). The final step of

Cer *de novo* synthesis is catalyzed by the enzyme dihydroceramide desaturase (DEGS1 in

human and Ifc in *Drosophila*) which inserts a 4,5-*trans*-double bond into the sphingolipid

backbone of dihydroceramide (dhCer) to produce Cer. This structural difference has a

profound effect on the biophysical properties of Cer; therefore, the relative abundance of

Cer versus dhCer in cellular membranes may have a profound impact on microdomain

segregation, signal transduction, membrane permeability, and membrane bending

curvature (Li et al., 2002). Indeed, genetic or drug inhibition of DEGS1/Ifc has been

shown to cause significant accumulation of dhCer which increases reactive oxygen

species, induces autophagy and ER stress, causes cell cycle arrest, and even results in cell

death (Barbarroja et al., 2015, Flores et al., 1998, Kraveka et al., 2007, Munoz-Guardiola

et al., 2020, Siddique et al., 2013). Although ceramide is known to promote exosome

biogenesis, the role of dhCer in exosome has never been explored beyond reports of its

detection in exosomes (Brzozowski et al., 2018, Podbielska et al., 2016, Vallabhaneni et

al., 2015). Considering that autophagy is involved in the degradation of MVE and

DEGS1/Ifc inhibition is a potent inducer of autophagy both *in vitro* and *in vivo*

(Gagliostro et al., 2012, Hernandez-Tiedra et al., 2016, Jung et al., 2017, Siddique et al.,

2013), it is possible that dhCer metabolism may affect the autophagic degradation of

MVEs to regulate exosome production and release.

Three recent reports linked variants of the human dhCer desaturase gene, *DEGS1*,
to leukodystrophy and systemic neuropathy (Dolgin et al., 2019, Karsai et al., 2019, Pant
et al., 2019). These reports not only confirm an essential role for *DEGS1* in the human
nervous system, but they also showed that loss-of-function mutations of *DEGS1* cause
significant accumulation of dhCer and a shift of cellular sphingolipid pool towards the
dihydro forms. However, the underlying cause of neuropathy remains unknown. Our
previous study shows that systemic knock-out of *Drosophila ifc* results in larval lethality,
which can be partially rescued tissue-specific expression of *ifc* only in neurons or muscle,
suggesting that Ifc can function cell non-autonomously (Jung et al., 2017). We also show
that, unlike the conventional belief that the conversion of dhCer to Cer occurs in the ER,
Ifc predominantly localizes to the endosomes in *Drosophila* photoreceptors. Furthermore,
Ifc colocalizes with an endosomal protein Rab4, which is also known to be enriched in
exosomes (Vidal and Stahl, 1993). Therefore, we hypothesized that *ifc* influences
exosome biogenesis to exert its cell non-autonomous function. In the present study, we
explored the necessity and sufficiency of *ifc* in neurons versus glia for the maintenance of
photoreceptor function in the *Drosophila*. We also developed an assay to observe whether
ifc plays a role in exosome biogenesis *in vivo* using *Drosophila* eye imaginal discs and
evaluated the effect of *ifc* inhibition on autophagy versus exosome release. Lastly, we
employed the giant unilamellar vesicle (GUV) system to examine the ability of human
DEGS1 to promote the formation of ILVs *in vitro*. Our findings demonstrate an important
role for DEGS1/Ifc in regulating the switch between autophagy and exosome biogenesis,
providing a possible mechanism of its cell non-autonomous function in the nervous

system.

Results

Drosophila ifc is differentially required in photoreceptor neurons versus glia

We have previously reported that _ifc-KO_ whole eye clones in _Drosophila_ show light-

dependent degeneration of photoreceptors as evidenced by the disorganized ommatidial

pattern and the decreased depolarizing amplitudes measured by electroretinogram (ERG)

(Jung et al., 2017). However, the whole-eye _ifc-KO_ clones take out _ifc_ in both

photoreceptor neurons and the surrounding glia. To further decipher whether _ifc_ is

required in neurons, glia, or both, we created cell-type-specific _ifc_ knock-out clones using

two different FLP/FRT site-directed recombination systems. We flanked the _ifc_ coding

sequence with FRT sites and depleted _ifc_ either strictly in the photoreceptor cells using

the _ey3.5-FLP_ (Mehta et al., 2005) or in the neighboring glial cells by _repo-FLP_ (Xiong

and Montell, 1995). We found that clonal depletion of _ifc_ only in the photoreceptor

neurons was sufficient to cause significant neurodegeneration after 10 days of light

exposure as evidenced by both the morphological degradation of ommatidial organization

(Figure 1A,B, quantified in Figure 1C) and the functional declined in depolarization

amplitude measured by ERG (Figure 1D, quantified in Figure 1,E). In contrast, knocking-

out _ifc_ in the neighboring glial cells caused no significant difference in the morphology of

photoreceptors (Figure 1F-H) and only resulted in a slight reduction in the depolarizing

amplitudes as detected by ERG (Figure 1I,J). These results indicate that the photoreceptor

neurons had a strict requirement for _ifc_, whereas the function of _ifc_ in glial cells was

likely to be less essential.

<u>Glial expression of *ifc* rescues the requirement for *ifc* in neurons</u>

Our previous report finds that *ifc* expressed in different tissues under different Gal4

drivers can rescue the larval lethality phenotype of ubiquitous *ifc-KO*, suggesting cell

non-autonomous rescue. Here, we further tested if *ifc* expression in glial cells alone was

sufficient to rescue the degenerative phenotype of *ifc-KO* whole-eye clones by comparing

the effect of expressing the UAS transgene of wild-type *ifc(WT)-mCherry* in glial cells

only (*repo-GAL4*) versus in the whole eye (*GMR-GAL4*). We found that overexpression

of *GMR>ifc(WT)-mCherry* and *repo>ifc(WT)-mCherry* both significantly rescued the *ifc-*

KO whole-eye clones, and the rescue effect was at comparable levels as determined by

ommatidial morphology (Figure 2A-E) and functional electroretinogram (Figure 2F,G).

Because *ifc* is required in the photoreceptor neurons, the results here show that cell non-

autonomous supply of *ifc* from glia to photoreceptor neurons is sufficient to rescue the

eye degenerative phenotype of *ifc-KO*.

<u>Knocking-out *ifc* reduces the density of ILVs in the MVEs</u>

One possible mechanism underlying the cell non-autonomous rescue is via exosome, we

thus examined the morphology of MVE, the first cellular structure related to exosome

production, by analyzing the ultrastructural images of wild-type (WT) and *ifc-KO*

photoreceptors by transmission electron microscopy. The MVEs in the WT

photoreceptors typically contained densely packed ILVs (Figure 3A). In comparison, the

MVEs in the *ifc-KO* photoreceptors appeared bloated (Figure 3B). Indeed, while the

numbers of MVE per view field were similar between WT and mutant photoreceptors

(Figure 3C, p = 0.3213), the size of the MVEs was significantly larger in *ifc-KO*

photoreceptors (Figure 3D, p = 0.0226). Moreover, the average number of ILVs in each

MVE was slightly reduced in *ifc-KO*, although the difference from WT was not

statistically significant (Figure 3E, p = 0.1153). However, when the size of MVEs was

taken into consideration, we found that the density of ILVs was significantly reduced in

the mutant MVEs comparing with that of WT (Figure 3F, p < 0.0001). This phenotype

was reminiscent of some ESCRT complex mutants (Adell et al., 2014, Buono et al., 2017,

Teis et al., 2010), suggesting that Ifc may function in the MVEs and participate in the

production of ILVs.

<u>Ifc activity positively correlates with the production of exosome-like vesicles</u>

Drosophila imaginal discs are capable of secreting exosomes (Gross et al., 2012). We

collected particles released by *Drosophila* eye imaginal discs and determined their sizes

by nanoparticle tracking analysis. The average diameter of the secreted vesicles was

113.8 ± 14.3 nm (Figure S1), consistent with the size range of exosomes. To evaluate the

role of *ifc* in exosome biogenesis, we established an *in vivo* system with the eye imaginal

discs utilizing the Dorsal-Eye Gal4 (*DE-GAL4*) system (Morrison and Halder, 2010) to

express the exosome marker of CD63 fused to GFP (Panakova et al., 2005). In this setup,

the expression of UAS-GFP-*CD63* is restricted to the dorsal half of the disc, and the

release of GFP-CD63+ extracellular vesicles can be observed on the ventral side,

especially in the acellular space above the morphogenetic furrow as evidenced by the

absence of DAPI staining (illustrated in Figure 4A). We quantified the GFP-CD63+

vesicles in the ventral morphogenetic furrow. In the *ifc-KO* eye disc clones, the number

of GFP puncta detected away from the dorsal compartment was significantly reduced in

comparison with the control eye discs (Figure 4B-D), suggesting that *ifc* promotes the

production of CD63+ extracellular vesicles. We tested another exosome marker, the

flotillin protein Flo2-RFP (Bischoff et al., 2013) to further characterize the extracellular

vesicles in eye discs. Consistent with the results observed with GFP-CD63, *ifc-KO*

reduced the number of Flo2-RFP puncta in the ventral compartment (Figure 4E-G). Since

Cer has been shown to promote exosome formation by favoring membrane budding, our

data suggest that *ifc-KO* hinders exosome production by affecting membrane properties

due to the accumulation of dhCer over Cer.

Conversely, using another exosome marker TSG101 (Thery et al., 2001), we

found that overexpression of *ifc(WT)-mCherry* increased the detection of extracellular

puncta labeled with TSG101-HA (Figure 5A-C). We further showed that *ifc* may have a

dose-dependent effect on the number of vesicles detected since overexpression of

ifc(WT)-mCherry increased the number of GFP-CD63+ puncta comparing with the

mCherry-CAAX as a UAS control (Figure 5D,E,G). To investigate whether the catalytic

activity of Ifc was required, we mutated one of the highly conserved histidine boxes that

are required for the enzyme activity of Ifc (Figure S2A) (Ternes et al., 2002). Expression

of *ifc(C3^{mut})-mCherry* failed to rescue the degeneration phenotype of *ifc-KO* eye clones

(Figure S2B-G). The lack of rescue was not due to a difference in the protein level as our

Western blot analysis showed that the level of Ifc(C3^{mut})-mCherry was similar to that of

Ifc(WT)-mCherry (Figure S2H,I), suggesting a functional deficiency. Consistently,

overexpression of *ifc(C3^{mut})-mCherry* did not increase the number of GFP-CD63+ puncta

released in comparison with the *mCherry-CAAX* control (Figure 5D,F,G), suggesting that

the desaturase activity of Ifc is required to promote the production of GFP-CD63+

exosome-like vesicles (ELVs). Together, these findings show that these extracellular

vesicles exhibit several main characteristics of exosomes based on exosome marker

proteins and the size distribution (Thery et al., 2018), thus support the use of GFP-CD63

as a surrogate to monitor the extracellular ELVs produced in the system of *Drosophila*

eye imaginal discs. Also, the results demonstrated a correlation between the level and

activity of Ifc with the number of ELVs detected.

Ifc directs the switch between the release of ELVs and their degradation via autophagy

186   We and others have reported autophagy induction as a common outcome of

DEGS1/ifc inhibition not only in *Drosophila* but also in mammalian cell lines and animal

models (Barbarroja et al., 2015, Holland et al., 2007, Jung et al., 2017, Kraveka et al.,

2007). Autophagy and exosome pathways are known to be interconnected: inhibition of

autophagy has been shown to increase exosome release (Poehler et al., 2014), whereas

autophagy induction shifts the fate of ILV-containing MVE for autophagy-mediated

degradation thus reduces exosome release (Fader et al., 2008). We examined the

localization of CD63 to MVEs and autophagosomes in *Drosophila* eye imaginal discs. In

the wild-type controls, very little CD63 colocalized with the autophagosomal marker

Atg8a. The colocalization of CD63 and Atg8 dramatically increased in *ifc-KO* eye

imaginal discs with a concomitant induction in autophagy indicated by increased Atg8a

(Figure 6A-C). On the contrary, the colocalization between CD63 and the MVE marker

Rab5 was significantly reduced upon *ifc-KO* (Figure 6D-F). We further examined the

colocalization between CD63 and the constitutively active form of Rab5 (Rab5^{CA}) in

adult eyes. Rab5^{CA} is known to block endosome maturation and has been widely used for

analyzing MVE formation (Stenmark et al., 1994, Wegner et al., 2010). Consistently, the

level of colocalization between GFP-CD63 and Rab5 also significantly decreased in the

enlarged endosomal vesicles in the photoreceptor neurons (Figure S3). The results

support that Ifc controls the fate of MVE likely by controlling the targeting of MVEs for

autophagy-mediated degradation.

To further determine the interaction between *ifc* and autophagy, we inhibited

autophagy by feeding the *Drosophila* larvae with 3-methyladenine (3-MA) and

chloroquine that block the formation of autophagosome and prevent autophagosomes-

lysosome fusion, respectively (Tang et al., 2017, Jung et al., 2017). Inhibition of

autophagy by 3-MA and chloroquine both increased the numbers of GFP-CD63+ vesicles

released in the wild-type background (Figure 7A-D). Consistent with the results shown in

Figure 5, over-expression of *ifc(WT)-mCherry* increased the number of ELVs.

Interestingly, feeding 3-MA or chloroquine to larvae overexpressing *ifc* did not further

increase the number of GFP-CD63 puncta detected in the ventral morphogenetic furrow

(Figure 7E-H, compared with 7D). These data suggest that Ifc promotes the production of

ELVs by inhibiting autophagy.

<u>Ifc participates in the early stage of ILV formation</u>

Considering the effect of Cer on membrane budding, we hypothesize that Ifc functions in

the early step of membrane invagination during ILV formation. Since the role of ESCRT

proteins in regulating the process of ILV formation is well studied, we investigated the

genetic interactions between *ifc* and the ESCRT-0 gene *hrs* and the ESCRT-II gene *vps-*

25. Because homozygous mutations of *hrs^{D28}* and *vps25^{A3}* are both lethal (Lloyd et al.,

2002, Vaccari and Bilder, 2005), we analyzed whether the heterozygous mutation affects

exosome production in the eye discs with overexpressing *ifc*. In eye imaginal discs where

we overexpressed *ifc(WT)-mCherry* using the *DE-GAL4* system, we found that *hrs$^{D28/+}$*

greatly reduced the number of GFP-CD63 puncta, whereas *vps25$^{A3/+}$* did not have a

significant effect (Figure 8A-D). The result indicates that, for exosome formation,

overexpression of *ifc* can overcome the requirement for *vps25* but not *hrs*. Because the

ESCRT-II complex is involved in the initiation of membrane invagination and the

recruitment of the ESCRT-III complex [ref], this finding supports a role for Ifc in the

early stage of membrane inward bending during ILV biogenesis.

DEGS1 promotes the formation of ILVs *in vitro*

To further evaluate if dhCer desaturase is sufficient to promote ILV formation, we

resorted to the *in vitro* model membrane system of GUV. After the generation of GUVs

using a mixture of dioleoylphophatidylcholine (DOPC) and cholesterol, we observed

whether there was spontaneous formation of ILVs over time. No ILV formation occurred

either with or without the inclusion of dhCer in the lipid mixture (Figure 9A,B-B',D-D').

Interestingly, when we added human DEGS1 recombinant protein to the GUVs with the

indicated lipid composition, we were able to observe the formation of ILVs in the GUVs

that containing dhCer but not without within 5 minutes (Figure 9A,C-C',E-E'). When we

added the DEGS1 inhibitor fenretinide to the system, the formation of ILVs induced by

DEGS1 was suppressed by fenretinide (Figure 9F,J-J'). The ethanol solvent control did

not inhibit ILV formation in the GUVs (Figure 9F,H-H'). The effect required the presence

of dhCer (Figure 9G-G', I-I'). These results show that the activity of DEGS1 was

required to promote the formation of ILV in the GUVs, likely by converting dhCer to Cer.

These data further support that the catalytic activity of DEGS1 promotes membrane

bending for the initiation of exosome biogenesis.

Discussion

In the present study, we developed an *in vivo* system for the analysis of exosome

production using *Drosophila* eye imaginal discs with the expression of GFP-CD63. We

showed that knocking out *ifc* in *Drosophila* eye imaginal discs reduced the production

and release of ELVs. In *ifc-KO*, the ELV marker CD63 became less associated with

endosomes but colocalized more with autophagosomes. On the contrary, transgenic

overexpression of *ifc(WT)-mCherry* promotes exosome biogenesis. In addition, our

genetic analysis and GUV assay support a role for Ifc in the early stage of ILV formation,

likely by regulating the invagination of the MVE membrane. We also showed that the

enzyme activity of DEGS1/Ifc is required to drive exosome formation since the

expression of a catalytic mutant form of *ifc* failed to promote exosome production and

inhibiting DEGS1 activity blocked ILV formation *in vitro*. Together, our data show that

DEGS1/Ifc promotes membrane invagination of ILV formation during exosome

biogenesis and controls the switch between exosome release and the autophagic

degradation of MVE, providing a mechanism for the cell non-autonomous function of

DEGS1/Ifc in the nervous system.

Between the ESCRT-dependent and Cer-mediated mechanisms of exosome

biogenesis, it is generally believed that the ESCRT-dependent mechanism is involved in

the selective sorting of cargos, whereas the Cer-dependent mechanisms are important to

control the initial membrane budding (Larios et al., 2020, Teis et al., 2010, Trajkovic et

al., 2008). However, *Kajimoto et al. (2013)* show that the continuous Cer catabolism

produces sphingosine 1-phosphate that is required for cargo sorting into ILVs in human

cells. Consistently, we found that *ifc-KO* reduced the localization of CD63-GFP to Rab5

in endosomes. While *in vitro* experiments demonstrated the ability of Cer to promote ILV

formation, the ESCRT-mediated and Cer-induced mechanisms are likely to be not

mutually exclusive of each other *in vivo*. Exosome membranes share similar lipid

composition with lipid rafts, both highly enriched for cholesterol and sphingolipids

(Hebbar et al., 2008). Lipid rafts are rigid membrane microdomains that serve as a

platform for protein-protein interaction and are essential for protein activation and signal

transduction (Lingwood and Simons, 2010). Cer-rich domains have been recognized to

affect raft properties by competing and displacing cholesterol (Megha and London,

2004). The Cer-rich membrane domains may not only serve as a permissive environment

to promote membrane invagination for ILV formation, but may also behave as a platform

to facilitate the segregation of exosome cargos. Our previous finding that Ifc resides in

endosomes also suggests that the on-site conversion of dhCer into Cer could regulate the

dynamic change of membrane property (Jung et al., 2017). Cer enrichment promotes the

formation of highly ordered gel phases and may induce the congregation of lipid rafts

into forming larger domains (Bieberich, 2018). The effects of dhCer accumulation on

cellular membranes need to be investigated to better elucidate the mechanism underlying

the systemic and peripheral neuropathies caused by human *DEGS1* deficiency.

Several previous studies have shown that inhibition of autophagosome-lysosome

degradation enhances exosome secretion (Danzer et al., 2012, Fader et al., 2008, Iguchi et

al., 2016). Interestingly, inhibition of autophagy by Bafilomycin A1 (BafA1) not only

increases the secretion of CD63+ exosomes but also increases the detection of autophagic

proteins p62 and LC3 in exosomes (Minakaki et al., 2018). However, autophagy proteins

ATG12–ATG3 have been shown to interact with the ESCRT-associated protein Alix to

promote late endosome to lysosome trafficking as well as exosome biogenesis (Murrow

et al., 2015). Also, knockout of ATG5 but not ATG7 significantly reduces the number of

exosomes, suggesting that not all of the autophagy core components participate in the

regulation of exosome release (Guo et al., 2017). In addition, exosomes purified from

cultured cortical neurons treated with BafA1 show a ~2-fold increase in certain

sphingolipid species, including Cer and dhCer, indicating BafA1 affects the lipid

composition of exosomes (Miranda et al., 2018). Leidal et al. (2020) report that LC3 and

LC3-conjugation machinery recruit specific cargos to pack into LC3-positive EVs for

secretion, a process which does not require the ESCRT complex but is dependent on the

activity of neutral sphingomyelinase 2, an enzyme which hydrolyzes sphingomyelin to

produced Cer. In the present study, we showed that exosome production increased with

the inhibition of autophagy by either 3-MA or chloroquine. However, combining

autophagy inhibition and *ifc* overexpression did not further increase exosome production,

suggesting *ifc* overexpression may inhibit autophagy. This is the opposite effect of *ifc-KO*

which induces autophagy. Together, these results suggest that the balance of dhCer and

Cer regulates the switch between autophagy and exosomes; further study is warranted to

elucidate the mechanism in detail.

While the effects of Cer have been extensively studied in various contexts, our

findings draw attention to the long-ignored sphingolipid species, dhCer. Although dhCer

is structurally different from Cer only in the lack of the 4,5-double bond, the two

sphingolipid species have widely different biophysical properties and bioactivities.

Several previous studies have hinted at a role for dhCer in the nervous system. Podbielska

et al. (2016) showed that treating human oligodendroglioma cells with inflammatory

cytokines greatly enhances the secretion of exosomes and significantly increases the

exosome and cellular levels of several sphingolipid species including dhCer.

Sphingolipidomics data from the Baltimore Longitudinal Study of Aging by following

992 cognitively normal individuals aged 55 years and older for an average of 15 years

concluded that plasma dhCer increase with age, but the trend was much steeper in women

than men and suggest dhCer may underlie the gender difference for degenerative diseases

such as Alzheimer's disease (Mielke et al., 2015). Fonteh et al. (2015) show that the level

of dhCer level in the cerebrospinal fluid increased significantly in patients with clinically

probable AD than it is in people with mild cognitive impairment, suggesting dhCer may

be used as an early marker for the progression to AD. Future studies are needed to

investigate whether the level and distribution of dhCer contribute to age-related

deterioration of the nervous system.

Materials and Methods

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-Na$^+$/K$^+$ ATPase	Developmental Studies Hybridoma Bank (DSHB)	Cat# a5; RRID: AB_2166869
Mouse anti-CD63		Cat# H5C6; RRID: AB_528158
Anti-Atg8a	Abcam	Cat# ab109364; RRID: AB_10861928
Anti-Rab5		Cat# ab31261; RRID: AB_882240
Alexa Fluor^{TM} 647 Phalloidin	ThermoFisher	Cat# A22287; RRID: AB_2620155
DAPI		Cat# D1306; RRID: AB_2629482
Anti-RFP	Rockland	Cat# 600-401-379; RRID: AB_2209751
Anti-HA	Cell Signaling Technology	Cat# 3724; RRID:

		AB_1549585
Anti-mCherry	GeneTex	Cat# GTX630189
Anti-Actin	Millipore	Cat# MAB1501; RRID: AB_2223041
anti-mouse-HRP	Jackson ImmunoResearch Laboratories, Inc.	Cat# 115-005-003; RRID: AB_2338447
Drosophila **Strains**		
ey3.5-FLP	Bloomington stock center (BDSC)	35542
cl, FRT40A		5622
FRT40A		5615
GMR>*w* RNAi		43344
DE-Gal4		29650
UAS-mCD8-GFP		5137
UAS-mCherry-CAAX		59021
UAS-hTSG101-HA		77977
rh1-Gal4, *ey*FLP		43347
UAS-Rab5CA		43335
nsyb-Gal4		5138
hrs^{D28}		54574
vps25^{A3}		39633
ifc-KO, FRT40A	Jung et al. (2017)	
UAS-*ifc*(WT)-mCherry		
UAS-EGFP-CD63	Gift from Dr. Suzanne Eaton	

	(Panakova et al., 2005)	
UAS-*ifc*(C1,2,3^{mut})-mCherry	This study	
UAS-Flo2-RFP	Bischoff et al. (2013)	
repo-FLP	Silies et al. (2007)	
repo-ifc-mCherry	This study	
Chemicals and other reagents		
DEGS1	Abcam	ab160100
Sucrose	Sigma	S7903
NaCl		31434
Glucose		G8270
NADPH		1010782400
ATP		A2383
MgCl$_2$	Calbiochem	442611

Lead Contact

343 Further information and requests for resources and reagents should be directed to and will

344 be fulfilled by the Lead Contact, Shu-Yi Huang (shuyih@ntuh.gov.tw).

Materials Availability

347 All unique reagents generated in this study are available from the Lead Contact and may

348 require completion of a Materials Transfer Agreement.

Experimental Model and Subject Details

Drosophila **strains and genetics**

Drosophila stocks were maintained at 25°C on standard medium and genetic crosses were

performed following standard fly husbandry procedures. Information on individual fly

strains can be found on FlyBase (flybase.org) unless otherwise noted in the above Key

Resources Table.

Eye-specific mosaic and whole-eye clones of *ifc-KO* were generated using the *ey-FLP* system as previously described (Jung et al., 2017). The photoreceptor-specific *ey3.5-FLP* was used to selectively create the homozygous *ifc* mutant clones in photoreceptors

(Mehta et al., 2005), whereas *repo-FLP* was used to generate glial-specific *ifc-KO* clones

(Silies et al., 2007). For light-stimulation experiments, a constant, 600 Lux LED light

source was provided for the indicated duration before the flies were used for

immunohistochemistry or electroretinogram

Immunohistochemistry and confocal microscopy

For immunofluorescence staining, larval eye discs and adult retina tissues were dissected,

fixed, washed, and immunostained with primary and secondary antibodies as previously

described (Jung et al., 2017). The antibodies used were listed in the above Key Resources

Table. All samples were mounted in Vectashield (Vector Laboratories) and analyzed on a

Carl Zeiss LSM880 confocal microscope with LAS AF software. Imaging data were

processed and quantified using Photoshop (CS6; Adobe), Illustrator (CS6; Adobe), and

ImageJ (National Institutes of Health).

Electroretinogram

Electroretinograms were performed, and the electric signals were amplified, filtered, and

recorded as previously described (Jung et al., 2017). To quantify the depolarization, all

experiments were carried out in triplicates with minimally 10 recordings per replicate for

each genotype and experimental condition.

Site-directed mutagenesis of *UAS-ifc* mutants

To generate point mutations in the catalytic domain of *UAS-ifc* transgene, the pUASt-ifc

was used as a backbone and site-directed mutagenesis was done with QuikChange Kit

(Agilent 200523) following the manufacturer's instruction. All constructs were verified

by sequencing. Transgenic flies were generated by embryo injection according to

standard protocols of integrase-mediated insertion (Bischof et al., 2007) followed by a

germline transmission screen.

Primer sequences:

ifc-C1[mut]-FWD	GCCGAGATCTCGGCCAACCTGGCC
ifc-C1[mut]-REV	GGCCGAGATCTCGGCAACGGCCAG
ifc-C2[mut]-FWD	GCCCTGGAAGCCGCCCGTTACCAA
ifc-C2[mut]-REV	GGCGGCTTCCAGGGCGTACTTTTT
ifc-C3[mut]-FWD	GCCAACGAGGCCGCCGACTTTCCG
ifc-C3[mut]-REV	GGCGGCCTCGTTGGCGTAGCCCAC
repo-ifc-mCherry-FWD	ATATGCGCGGCCGCCAAAATGGGACAGAAAGTTTCGCGCA
repo-ifc-mCherry-REV	CTAGTCTAGATTACTTGTACAGCTCGTCCATGCCGC

	CGGT

Western blotting

The protein extracts were prepared with dissected fly tissues that were homogenized for

10 min at 4°C in 1x RIPA buffer containing protease inhibitors (ThermoFisher A32955),

followed by centrifugation at 16000g for 15min at 4°C to remove the debris. The

supernatant of the homogenates was mixed with 4X sample buffer (20% glycerol, 4%

SDS, 100 mM Tris pH 6.8, 0.002% Bromophenol blue), boiled for 10 min, separated by

SDS-PAGE, and transferred in a wet-transfer tank to PVDF membranes (Millipore,

IPVH00010) following manufacturer's instructions (Bio-Rad). PVDF membranes were

incubated with 5% non-fat milk in TBST [10 mM Tris (pH 8.0), 150 mM NaCl, 0.1%

Tween 20] for 1 h at room temperature and then incubated with primary antibodies at 4°C

overnight. Membranes were then washed three times with TBST for 10 min each before

incubating with secondary antibodies in TBST for 1 h at room temperature, washed with

TBST three times, and developed with ECL reagents (Millipore, WBKLS0500). The

signals were captured by the BioSpectrum^{TM} 600 Imaging System (UVP Ltd), and the

relative intensities of bands were quantified by densitometry using the Image J software

(Schneider et al., 2012).

Transmission electron microscopy and image analysis of MVE morphology

Transmission electron microscopy was performed as previously described (Jung et al.,

2017). To characterize the MVE morphology, the fields of imaging for the

characterization within the eye of each genotype were selected randomly, and the image

files were blinded for the analyses of both the area of MVE and the number of ILV. The

area and number of MVE were quantified using Photoshop.

Nanoparticle tracking analysis

Fifty to 100 eye imaginal discs were dissected and incubated in sterile-filtered Schneider

medium (Gibco 21720024) with low-speed agitation (7 rpm) at room temperature for 2.5

h. The supernatant was then separated by low-speed centrifugation at 3,000 x g for 15

min to remove large debris, followed by another centrifugation at 12,000 x g for 15 min.

The samples were then passed through a Smart SEC single column (System Biosciences,

USA) for EV isolation. The size distribution was determined using NanoSight NS300

(Malvern Panalytical Ltd, United Kingdom) under constant flow condition (flow rate =

70) at 25°C. The recordings were analyzed by NTA 3.4 software Build 3.4.003 with the

detection threshold set to 6.

Giant Unilamellar Vesicle (GUV) assay

GUV was prepared by the electroformation method (Lee et al., 2018). Briefly, the lipid

mixture of Dioleoylphosphatidylcholine (DOPC) and cholesterol was dissolved in

chloroform at 1:0.67 molar ratio to the final concentration of 15mM. For lipid mixture

with dhCer, the lipid mixture included DOPC:dhCer:cholesterol at 1:1:0.67 molar ratio to

the total concentration of 15mM. The lipid mixture was then deposited into a chamber

made with a rubber O-ring on an electrically conductive ITO glass. The setup was

vacuum dried at 50°C for 1 h, and the solvent was evaporated thereby producing a thin

lipid film. The chamber was then re-filled with 2ml of 400mM sucrose. Depending on the

experimental conditions, 0.2 μM of the DEGS1 inhibitor fenretinide might be added to

the sucrose solution. After sealing the chamber with another piece of ITO glass, the setup

was supplied with a sinusoidal voltage waveform of 3.5 V amplitude at 10 Hz frequency

and kept at 65°C overnight. The unilamellar vesicles were cooled down and resuspended

in 200 μl of 400mM glucose, and confocal images were acquired using Zeiss Cell

Observer SD to show the morphology of GUV at time = 0. The recombinant DEGS1

protein at the concentration of 9.62 nM and co-factors (ATP 4 mM, NADPH 2 mM,

MgCl2 2.4 mM, NaCl 50 mM, and sucrose 50 mM) were then supplemented to the

GUV/sucrose mixture. The post-treatment images were acquired after 5 min of

incubation at 37°C.

Quantification and statistical analysis

Quantitative data were analyzed using a two-tailed unpaired Student's *t*-test or one-way

ANOVA with Tukey's multiple comparison test. Data analysis was performed using

GraphPad Prism 5. All data in bar graphs are shown as mean ± SEM. The quantified

GFP-CD63 puncta in the experimental groups were normalized to the average of the

controls which was set as 1. *P* values of less than 0.05 were regarded as statistically

significant. All quantifications included at least three experimental replicates.

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Declaration of interests

469 The authors declare no competing financial interests.

Abbreviations

Cer, ceramide;

dhCer, dihydroceramide;

ELVs, exosome-like vesicles;

ESCRT, Endosomal Sorting Complexes Required for Transport;

ERG, electroretinogram;

GUV, giant unilamellar vesicles;

ILVs, intraluminal vesicles;

MVEs, multivesicular endosomes.

References

Adell, M. A., Vogel, G. F., Pakdel, M., Muller, M., Lindner, H., Hess, M. W. & Teis, D.
484   2014. Coordinated binding of Vps4 to ESCRT-III drives membrane neck
485   constriction during MVB vesicle formation. *J Cell Biol,* 205**,** 33-49. DOI:
486   https://doi.org/10.1083/jcb.201310114.
487   Barbarroja, N., Rodriguez-Cuenca, S., Nygren, H., Camargo, A., Pirraco, A., Relat, J.,
488   Cuadrado, I., Pellegrinelli, V., Medina-Gomez, G., Lopez-Pedrera, C., Tinahones,
489   F. J., Symons, J. D., Summers, S. A., Oresic, M. & Vidal-Puig, A. 2015. Increased
490   dihydroceramide/ceramide ratio mediated by defective expression of degs1
491   impairs adipocyte differentiation and function. *Diabetes,* 64**,** 1180-92. DOI:
492   https://doi.org/10.2337/db14-0359.
493   Bieberich, E. 2018. Sphingolipids and lipid rafts: Novel concepts and methods of
494   analysis. *Chem Phys Lipids,* 216**,** 114-131. DOI:
495   https://doi.org/10.1016/j.chemphyslip.2018.08.003.
496   Bischof, J., Maeda, R. K., Hediger, M., Karch, F. & Basler, K. 2007. An optimized
497   transgenesis system for Drosophila using germ-line-specific phiC31 integrases.
498   *Proc Natl Acad Sci U S A,* 104**,** 3312-7. DOI:
499   https://doi.org/10.1073/pnas.0611511104.
500   Bischoff, M., Gradilla, A. C., Seijo, I., Andres, G., Rodriguez-Navas, C., Gonzalez-
501   Mendez, L. & Guerrero, I. 2013. Cytonemes are required for the establishment of
502   a normal Hedgehog morphogen gradient in Drosophila epithelia. *Nat Cell Biol,*
503   15**,** 1269-81. DOI: https://doi.org/10.1038/ncb2856.
504   Brzozowski, J. S., Jankowski, H., Bond, D. R., Mccague, S. B., Munro, B. R., Predebon,
505   M. J., Scarlett, C. J., Skelding, K. A. & Weidenhofer, J. 2018. Lipidomic profiling
506   of extracellular vesicles derived from prostate and prostate cancer cell lines.
507   *Lipids Health Dis,* 17**,** 211. DOI: https://doi.org/10.1186/s12944-018-0854-x.
508   Budnik, V., Ruiz-Canada, C. & Wendler, F. 2016. Extracellular vesicles round off
509   communication in the nervous system. *Nat Rev Neurosci,* 17**,** 160-72. DOI:
510   https://doi.org/10.1038/nrn.2015.29.
511   Buono, R. A., Leier, A., Paez-Valencia, J., Pennington, J., Goodman, K., Miller, N.,
512   Ahlquist, P., Marquez-Lago, T. T. & Otegui, M. S. 2017. ESCRT-mediated vesicle
513   concatenation in plant endosomes. *J Cell Biol,* 216**,** 2167-2177. DOI:
514   https://doi.org/10.1083/jcb.201612040.
515   Danzer, K. M., Kranich, L. R., Ruf, W. P., Cagsal-Getkin, O., Winslow, A. R., Zhu, L.,
516   Vanderburg, C. R. & Mclean, P. J. 2012. Exosomal cell-to-cell transmission of
517   alpha synuclein oligomers. *Mol Neurodegener,* 7**,** 42. DOI:
518   https://doi.org/10.1186/1750-1326-7-42.
519   Dolgin, V., Straussberg, R., Xu, R., Mileva, I., Yogev, Y., Khoury, R., Konen, O., Barhum,
520   Y., Zvulunov, A., Mao, C. & Birk, O. S. 2019. DEGS1 variant causes neurological

disorder. *Eur J Hum Genet,* 27**,** 1668-1676. DOI: https://doi.org/10.1038/s41431-019-0444-z.

Fader, C. M., Sanchez, D., Furlan, M. & Colombo, M. I. 2008. Induction of autophagy promotes fusion of multivesicular bodies with autophagic vacuoles in k562 cells. *Traffic,* 9**,** 230-50. DOI: https://doi.org/10.1111/j.1600-0854.2007.00677.x.

Flores, I., Martinez, A. C., Hannun, Y. A. & Merida, I. 1998. Dual role of ceramide in the control of apoptosis following IL-2 withdrawal. *J Immunol,* 160**,** 3528-33.

Fonteh, A. N., Ormseth, C., Chiang, J., Cipolla, M., Arakaki, X. & Harrington, M. G. 2015. Sphingolipid metabolism correlates with cerebrospinal fluid Beta amyloid levels in Alzheimer's disease. *PLoS One,* 10**,** e0125597. DOI: https://doi.org/10.1371/journal.pone.0125597.

Gagliostro, V., Casas, J., Caretti, A., Abad, J. L., Tagliavacca, L., Ghidoni, R., Fabrias, G. & Signorelli, P. 2012. Dihydroceramide delays cell cycle G1/S transition via activation of ER stress and induction of autophagy. *Int J Biochem Cell Biol,* 44**,** 2135-43. DOI: https://doi.org/10.1016/j.biocel.2012.08.025.

Gross, J. C., Chaudhary, V., Bartscherer, K. & Boutros, M. 2012. Active Wnt proteins are secreted on exosomes. *Nat Cell Biol,* 14**,** 1036-45. DOI: https://doi.org/10.1038/ncb2574.

Guo, H., Chitiprolu, M., Roncevic, L., Javalet, C., Hemming, F. J., Trung, M. T., Meng, L., Latreille, E., Tanese De Souza, C., Mcculloch, D., Baldwin, R. M., Auer, R., Cote, J., Russell, R. C., Sadoul, R. & Gibbings, D. 2017. Atg5 Disassociates the V1V0-ATPase to Promote Exosome Production and Tumor Metastasis Independent of Canonical Macroautophagy. *Dev Cell,* 43**,** 716-730 e7. DOI: https://doi.org/10.1016/j.devcel.2017.11.018.

Hebbar, S., Lee, E., Manna, M., Steinert, S., Kumar, G. S., Wenk, M., Wohland, T. & Kraut, R. 2008. A fluorescent sphingolipid binding domain peptide probe interacts with sphingolipids and cholesterol-dependent raft domains. *J Lipid Res,* 49**,** 1077-89. DOI: https://doi.org/10.1194/jlr.M700543-JLR200.

Hernandez-Tiedra, S., Fabrias, G., Davila, D., Salanueva, I. J., Casas, J., Montes, L. R., Anton, Z., Garcia-Taboada, E., Salazar-Roa, M., Lorente, M., Nylandsted, J., Armstrong, J., Lopez-Valero, I., Mckee, C. S., Serrano-Puebla, A., Garcia-Lopez, R., Gonzalez-Martinez, J., Abad, J. L., Hanada, K., Boya, P., Goni, F., Guzman, M., Lovat, P., Jaattela, M., Alonso, A. & Velasco, G. 2016. Dihydroceramide accumulation mediates cytotoxic autophagy of cancer cells via autolysosome destabilization. *Autophagy,* 12**,** 2213-2229. DOI: https://doi.org/10.1080/15548627.2016.1213927.

Holland, W. L., Brozinick, J. T., Wang, L. P., Hawkins, E. D., Sargent, K. M., Liu, Y., Narra, K., Hoehn, K. L., Knotts, T. A., Siesky, A., Nelson, D. H., Karathanasis, S. K., Fontenot, G. K., Birnbaum, M. J. & Summers, S. A. 2007. Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. *Cell Metab,* 5**,** 167-79. DOI: https://doi.org/10.1016/j.cmet.2007.01.002.

Iguchi, Y., Eid, L., Parent, M., Soucy, G., Bareil, C., Riku, Y., Kawai, K., Takagi, S., Yoshida, M., Katsuno, M., Sobue, G. & Julien, J. P. 2016. Exosome secretion is a key pathway for clearance of pathological TDP-43. *Brain,* 139**,** 3187-3201. DOI: https://doi.org/10.1093/brain/aww237.

Jung, W. H., Liu, C. C., Yu, Y. L., Chang, Y. C., Lien, W. Y., Chao, H. C., Huang, S. Y., Kuo, C. H., Ho, H. C. & Chan, C. C. 2017. Lipophagy prevents activity-dependent neurodegeneration due to dihydroceramide accumulation in vivo. *EMBO Rep,* 18**,** 1150-1165. DOI: https://doi.org/10.15252/embr.201643480.

Kajimoto, T., Okada, T., Miya, S., Zhang, L. & Nakamura, S. 2013. Ongoing activation of sphingosine 1-phosphate receptors mediates maturation of exosomal multivesicular endosomes. *Nat Commun,* 4**,** 2712. DOI: https://doi.org/10.1038/ncomms3712.

Karsai, G., Kraft, F., Haag, N., Korenke, G. C., Hanisch, B., Othman, A., Suriyanarayanan, S., Steiner, R., Knopp, C., Mull, M., Bergmann, M., Schroder, J. M., Weis, J., Elbracht, M., Begemann, M., Hornemann, T. & Kurth, I. 2019. DEGS1-associated aberrant sphingolipid metabolism impairs nervous system function in humans. *J Clin Invest,* 129**,** 1229-1239. DOI: https://doi.org/10.1172/JCI124159.

Katzmann, D. J., Babst, M. & Emr, S. D. 2001. Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. *Cell,* 106**,** 145-55. DOI: https://doi.org/10.1016/s0092-8674(01)00434-2.

Kraveka, J. M., Li, L., Szulc, Z. M., Bielawski, J., Ogretmen, B., Hannun, Y. A., Obeid, L. M. & Bielawska, A. 2007. Involvement of dihydroceramide desaturase in cell cycle progression in human neuroblastoma cells. *J Biol Chem,* 282**,** 16718-28. DOI: https://doi.org/10.1074/jbc.M700647200.

Larios, J., Mercier, V., Roux, A. & Gruenberg, J. 2020. ALIX- and ESCRT-III-dependent sorting of tetraspanins to exosomes. *J Cell Biol,* 219. DOI: https://doi.org/10.1083/jcb.201904113.

Lee, C. W., Chiang, Y. L., Liu, J. T., Chen, Y. X., Lee, C. H., Chen, Y. L. & Hwang, I. S. 2018. Emerging Roles of Air Gases in Lipid Bilayers. *Small,* 14**,** e1802133. DOI: https://doi.org/10.1002/smll.201802133.

Lee, S. Y., Kim, J. R., Hu, Y., Khan, R., Kim, S. J., Bharadwaj, K. G., Davidson, M. M., Choi, C. S., Shin, K. O., Lee, Y. M., Park, W. J., Park, I. S., Jiang, X. C., Goldberg, I. J. & Park, T. S. 2012. Cardiomyocyte specific deficiency of serine palmitoyltransferase subunit 2 reduces ceramide but leads to cardiac dysfunction. *J Biol Chem,* 287**,** 18429-39. DOI: https://doi.org/10.1074/jbc.M111.296947.

Leidal, A. M., Huang, H. H., Marsh, T., Solvik, T., Zhang, D., Ye, J., Kai, F., Goldsmith, J., Liu, J. Y., Huang, Y. H., Monkkonen, T., Vlahakis, A., Huang, E. J., Goodarzi, H., Yu, L., Wiita, A. P. & Debnath, J. 2020. The LC3-conjugation machinery specifies the loading of RNA-binding proteins into extracellular vesicles. *Nat Cell Biol,* 22**,** 187-199. DOI: https://doi.org/10.1038/s41556-019-0450-y.

Li, L., Tang, X., Taylor, K. G., Dupre, D. B. & Yappert, M. C. 2002. Conformational characterization of ceramides by nuclear magnetic resonance spectroscopy. *Biophys J,* 82**,** 2067-80. DOI: https://doi.org/10.1016/S0006-3495(02)75554-9.

Lingwood, D. & Simons, K. 2010. Lipid rafts as a membrane-organizing principle. *Science,* 327**,** 46-50. DOI: https://doi.org/10.1126/science.1174621.

Lloyd, T. E., Atkinson, R., Wu, M. N., Zhou, Y., Pennetta, G. & Bellen, H. J. 2002. Hrs regulates endosome membrane invagination and tyrosine kinase receptor signaling in Drosophila. *Cell,* 108**,** 261-9. DOI: https://doi.org/10.1016/s0092-

8674(02)00611-6.

Megha & London, E. 2004. Ceramide selectively displaces cholesterol from ordered lipid domains (rafts): implications for lipid raft structure and function. *J Biol Chem,* 279**,** 9997-10004. DOI: https://doi.org/10.1074/jbc.M309992200.

Mehta, S. Q., Hiesinger, P. R., Beronja, S., Zhai, R. G., Schulze, K. L., Verstreken, P., Cao, Y., Zhou, Y., Tepass, U., Crair, M. C. & Bellen, H. J. 2005. Mutations in Drosophila sec15 reveal a function in neuronal targeting for a subset of exocyst components. *Neuron,* 46**,** 219-32. DOI: https://doi.org/10.1016/j.neuron.2005.02.029.

Mielke, M. M., Bandaru, V. V., Han, D., An, Y., Resnick, S. M., Ferrucci, L. & Haughey, N. J. 2015. Demographic and clinical variables affecting mid- to late-life trajectories of plasma ceramide and dihydroceramide species. *Aging Cell,* 14**,** 1014-23. DOI: https://doi.org/10.1111/acel.12369.

Minakaki, G., Menges, S., Kittel, A., Emmanouilidou, E., Schaeffner, I., Barkovits, K., Bergmann, A., Rockenstein, E., Adame, A., Marxreiter, F., Mollenhauer, B., Galasko, D., Buzas, E. I., Schlotzer-Schrehardt, U., Marcus, K., Xiang, W., Lie, D. C., Vekrellis, K., Masliah, E., Winkler, J. & Klucken, J. 2018. Autophagy inhibition promotes SNCA/alpha-synuclein release and transfer via extracellular vesicles with a hybrid autophagosome-exosome-like phenotype. *Autophagy,* 14**,** 98-119. DOI: https://doi.org/10.1080/15548627.2017.1395992.

Miranda, A. M., Lasiecka, Z. M., Xu, Y., Neufeld, J., Shahriar, S., Simoes, S., Chan, R. B., Oliveira, T. G., Small, S. A. & Di Paolo, G. 2018. Neuronal lysosomal dysfunction releases exosomes harboring APP C-terminal fragments and unique lipid signatures. *Nat Commun,* 9**,** 291. DOI: https://doi.org/10.1038/s41467-017-02533-w.

Morrison, C. M. & Halder, G. 2010. Characterization of a dorsal-eye Gal4 Line in Drosophila. *Genesis,* 48**,** 3-7. DOI: https://doi.org/10.1002/dvg.20571.

Munoz-Guardiola, P., Casas, J., Megias-Roda, E., Sole, S., Perez-Montoyo, H., Yeste-Velasco, M., Erazo, T., Dieguez-Martinez, N., Espinosa-Gil, S., Munoz-Pinedo, C., Yoldi, G., Abad, J. L., Segura, M. F., Moran, T., Romeo, M., Bosch-Barrera, J., Oaknin, A., Alfon, J., Domenech, C., Fabrias, G., Velasco, G. & Lizcano, J. M. 2020. The anti-cancer drug ABTL0812 induces ER stress-mediated cytotoxic autophagy by increasing dihydroceramide levels in cancer cells. *Autophagy***,** 1-18. DOI: https://doi.org/10.1080/15548627.2020.1761651.

Murrow, L., Malhotra, R. & Debnath, J. 2015. ATG12-ATG3 interacts with Alix to promote basal autophagic flux and late endosome function. *Nat Cell Biol,* 17**,** 300-10. DOI: https://doi.org/10.1038/ncb3112.

Panakova, D., Sprong, H., Marois, E., Thiele, C. & Eaton, S. 2005. Lipoprotein particles are required for Hedgehog and Wingless signalling. *Nature,* 435**,** 58-65. DOI: https://doi.org/10.1038/nature03504.

Pant, D. C., Dorboz, I., Schluter, A., Fourcade, S., Launay, N., Joya, J., Aguilera-Albesa, S., Yoldi, M. E., Casasnovas, C., Willis, M. J., Ruiz, M., Ville, D., Lesca, G., Siquier-Pernet, K., Desguerre, I., Yan, H., Wang, J., Burmeister, M., Brady, L., Tarnopolsky, M., Cornet, C., Rubbini, D., Terriente, J., James, K. N., Musaev, D., Zaki, M. S., Patterson, M. C., Lanpher, B. C., Klee, E. W., Pinto, E. V. F., Wohler, E., Sobreira, N. L. M., Cohen, J. S., Maroofian, R., Galehdari, H., Mazaheri, N.,

Shariati, G., Colleaux, L., Rodriguez, D., Gleeson, J. G., Pujades, C., Fatemi, A., Boespflug-Tanguy, O. & Pujol, A. 2019. Loss of the sphingolipid desaturase DEGS1 causes hypomyelinating leukodystrophy. *J Clin Invest,* 129**,** 1240-1256. DOI: https://doi.org/10.1172/JCI123959.

Podbielska, M., Szulc, Z. M., Kurowska, E., Hogan, E. L., Bielawski, J., Bielawska, A. & Bhat, N. R. 2016. Cytokine-induced release of ceramide-enriched exosomes as a mediator of cell death signaling in an oligodendroglioma cell line. *J Lipid Res,* 57**,** 2028-2039. DOI: https://doi.org/10.1194/jlr.M070664.

Poehler, A. M., Xiang, W., Spitzer, P., May, V. E., Meixner, H., Rockenstein, E., Chutna, O., Outeiro, T. F., Winkler, J., Masliah, E. & Klucken, J. 2014. Autophagy modulates SNCA/alpha-synuclein release, thereby generating a hostile microenvironment. *Autophagy,* 10**,** 2171-92. DOI: https://doi.org/10.4161/auto.36436.

Raposo, G. & Stoorvogel, W. 2013. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol,* 200**,** 373-83. DOI: https://doi.org/10.1083/jcb.201211138.

Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods,* 9**,** 671-5. DOI: https://doi.org/10.1038/nmeth.2089.

Siddique, M. M., Li, Y., Wang, L., Ching, J., Mal, M., Ilkayeva, O., Wu, Y. J., Bay, B. H. & Summers, S. A. 2013. Ablation of dihydroceramide desaturase 1, a therapeutic target for the treatment of metabolic diseases, simultaneously stimulates anabolic and catabolic signaling. *Mol Cell Biol,* 33**,** 2353-69. DOI: https://doi.org/10.1128/MCB.00226-13.

Silies, M., Yuva, Y., Engelen, D., Aho, A., Stork, T. & Klambt, C. 2007. Glial cell migration in the eye disc. *J Neurosci,* 27**,** 13130-9. DOI: https://doi.org/10.1523/JNEUROSCI.3583-07.2007.

Stenmark, H., Parton, R. G., Steele-Mortimer, O., Lutcke, A., Gruenberg, J. & Zerial, M. 1994. Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis. *EMBO J,* 13**,** 1287-96.

Tang, Y., Cai, Q. H., Wang, Y. J., Fan, S. H., Zhang, Z. F., Xiao, M. Q., Zhu, J. Y., Wu, D. M., Lu, J. & Zheng, Y. L. 2017. Protective effect of autophagy on endoplasmic reticulum stress induced apoptosis of alveolar epithelial cells in rat models of COPD. *Biosci Rep,* 37. DOI: https://doi.org/10.1042/BSR20170803.

Teis, D., Saksena, S., Judson, B. L. & Emr, S. D. 2010. ESCRT-II coordinates the assembly of ESCRT-III filaments for cargo sorting and multivesicular body vesicle formation. *EMBO J,* 29**,** 871-83. DOI: https://doi.org/10.1038/emboj.2009.408.

Ternes, P., Franke, S., Zahringer, U., Sperling, P. & Heinz, E. 2002. Identification and characterization of a sphingolipid delta 4-desaturase family. *J Biol Chem,* 277**,** 25512-8. DOI: https://doi.org/10.1074/jbc.M202947200.

Thery, C., Boussac, M., Veron, P., Ricciardi-Castagnoli, P., Raposo, G., Garin, J. & Amigorena, S. 2001. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J Immunol,* 166**,** 7309-18. DOI: https://doi.org/10.4049/jimmunol.166.12.7309.

Thery, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., Antoniou, A., Arab, T., Archer, F., Atkin-Smith, G. K., Ayre, D. C., Bach, J.

M., Bachurski, D., Baharvand, H., Balaj, L., Baldacchino, S., Bauer, N. N., Baxter, A. A., Bebawy, M., Beckham, C., Bedina Zavec, A., Benmoussa, A., Berardi, A. C., Bergese, P., Bielska, E., Blenkiron, C., Bobis-Wozowicz, S., Boilard, E., Boireau, W., Bongiovanni, A., Borras, F. E., Bosch, S., Boulanger, C. M., Breakefield, X., Breglio, A. M., Brennan, M. A., Brigstock, D. R., Brisson, A., Broekman, M. L., Bromberg, J. F., Bryl-Gorecka, P., Buch, S., Buck, A. H., Burger, D., Busatto, S., Buschmann, D., Bussolati, B., Buzas, E. I., Byrd, J. B., Camussi, G., Carter, D. R., Caruso, S., Chamley, L. W., Chang, Y. T., Chen, C., Chen, S., Cheng, L., Chin, A. R., Clayton, A., Clerici, S. P., Cocks, A., Cocucci, E., Coffey, R. J., Cordeiro-Da-Silva, A., Couch, Y., Coumans, F. A., Coyle, B., Crescitelli, R., Criado, M. F., D'souza-Schorey, C., Das, S., Datta Chaudhuri, A., De Candia, P., De Santana, E. F., De Wever, O., Del Portillo, H. A., Demaret, T., Deville, S., Devitt, A., Dhondt, B., Di Vizio, D., Dieterich, L. C., Dolo, V., Dominguez Rubio, A. P., Dominici, M., Dourado, M. R., Driedonks, T. A., Duarte, F. V., Duncan, H. M., Eichenberger, R. M., Ekstrom, K., El Andaloussi, S., Elie-Caille, C., Erdbrugger, U., Falcon-Perez, J. M., Fatima, F., Fish, J. E., Flores-Bellver, M., Forsonits, A., Frelet-Barrand, A., et al. 2018. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles,* 7**,** 1535750. DOI: https://doi.org/10.1080/20013078.2018.1535750.

Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., Schwille, P., Brugger, B. & Simons, M. 2008. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science,* 319**,** 1244-7. DOI: https://doi.org/10.1126/science.1153124.

Vaccari, T. & Bilder, D. 2005. The Drosophila tumor suppressor vps25 prevents nonautonomous overproliferation by regulating notch trafficking. *Dev Cell,* 9**,** 687-98. DOI: https://doi.org/10.1016/j.devcel.2005.09.019.

Vallabhaneni, K. C., Penfornis, P., Dhule, S., Guillonneau, F., Adams, K. V., Mo, Y. Y., Xu, R., Liu, Y., Watabe, K., Vemuri, M. C. & Pochampally, R. 2015. Extracellular vesicles from bone marrow mesenchymal stem/stromal cells transport tumor regulatory microRNA, proteins, and metabolites. *Oncotarget,* 6**,** 4953-67. DOI: https://doi.org/10.18632/oncotarget.3211.

Van Meer, G., Voelker, D. R. & Feigenson, G. W. 2008. Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol,* 9**,** 112-24. DOI: https://doi.org/10.1038/nrm2330.

Vidal, M. J. & Stahl, P. D. 1993. The small GTP-binding proteins Rab4 and ARF are associated with released exosomes during reticulocyte maturation. *Eur J Cell Biol,* 60**,** 261-7.

Villarroya-Beltri, C., Baixauli, F., Mittelbrunn, M., Fernandez-Delgado, I., Torralba, D., Moreno-Gonzalo, O., Baldanta, S., Enrich, C., Guerra, S. & Sanchez-Madrid, F. 2016. ISGylation controls exosome secretion by promoting lysosomal degradation of MVB proteins. *Nat Commun,* 7**,** 13588. DOI: https://doi.org/10.1038/ncomms13588.

Wegner, C. S., Malerod, L., Pedersen, N. M., Progida, C., Bakke, O., Stenmark, H. & Brech, A. 2010. Ultrastructural characterization of giant endosomes induced by

751   GTPase-deficient Rab5. *Histochem Cell Biol,* 133**,** 41-55. DOI:
752   https://doi.org/10.1007/s00418-009-0643-8.
753 Xiong, W. C. & Montell, C. 1995. Defective glia induce neuronal apoptosis in the repo
754   visual system of Drosophila. *Neuron,* 14**,** 581-90. DOI:
755   https://doi.org/10.1016/0896-6273(95)90314-3.
756

Figure 1.Tissue-specific requirements for *ifc-KO* in *Drosophila* photoreceptors and the surrounding glia. (A and B) Representative confocal images showing ommatidial morphology after 10 days of light stimulation in the control (A) and *ey3.5-FLP*-induced *ifc-KO* (B). Scale bar = 10 μm. **(C)** Quantification of rhabdomeres per ommatidium. **(D)** Representative electroretinogram (ERG) traces. **(E)** Quantification of the depolarization amplitudes of ERG. **(F and G)** Representative confocal images of the control (F) and *repo-FLP*-induced *ifc-KO* in glia (G). **(H)** Quantification of rhabdomeres per ommatidium. **(I)** Representative ERG traces. **(J)** Quantification of ERG depolarization amplitudes after 10 days of light stimulation. Phalloidin shows rhabdomeres in green; anti-Na+/K+-ATPase shows neuronal membrane in magenta. Bar charts show mean ± SEM of at least three independent experiments. *** p < 0.001, ns: Not significant (Student's *t*-test).

Figure 2. Cell non-autonomous rescue of *ifc-KO* eye clones. (A-C) Representative confocal images of ommatidial morphology of *ifc-KO* whole eye clones created by *ey-FLP* (A-A'') and *ifc-KO* with eye expression of *ifc(WT)-mCherry* driven by *GMR-Gal4* (B-B'') or *ifc(WT)-mCherry* in the glia under *repo-Gal4* (C-C'') after 10 days of light stimulation. Phalloidin staining shows rhabdomere in green and anti-Na+/K+-ATPase shows neuronal membrane in magenta. Scale bars: 10 μm. **(D)** Quantification of rhabdomeres per ommatidium. **(E)** Representative electroretinogram (ERG) traces after 10 days of light stimulation. **(F)** Quantification of the depolarization amplitudes of ERG. Bar charts show mean ± SEM of at least three independent experiments. ***P < 0.001 (Student's *t*-test).

Figure 3. Knocking-out ifc reduces the density of intraluminal vesicles (ILVs) inside of multivesicular endosomes (MVEs) in *Drosophila* photoreceptors. (A and B) Representative transmission electron microscopy (TEM) images of the control (A) and *ifc-KO* eyes (B) after 3 days of light stimulation. Scale bars: 250 nm. **(C)** Quantification of the number of MVEs per field. **(D)** Quantification of the area of MVEs. **(E)** Quantification of the number of ILVs per MVE. **(F)** Quantification of the number of ILVs per unit area inside MVEs. Bar charts show mean ± SEM of at least three independent experiments. *** $p < 0.001$, * $p < 0.05$, ns: Not significant (Student's *t*-test).

Figure 4. Knocking out *ifc* reduces the number of exosome-like vesicles (ELVs) detected in *Drosophila* eye imaginal discs. (A) A schematic illustration of the *Dorsal-Eye Gal4* (*DE-Gal4*) system for observing the release of ELVs. *DE-Gal4* drives the expression of *UAS-GFP-CD63* in the dorsal half of the eye imaginal disc (mint green). The secreted ELVs that move towards the ventral side alone the morphogenetic furrow (MF) into the area of observation (red box) were counted. **(B-C)** Representative confocal images of *Drosophila* eye imaginal discs with the expression of GFP-CD63 in the dorsal compartment of control (B-B") and *ifc-KO* (C-C") showing signals merged with DAPI in blue (B,C), GFP-CD63 enhanced by anti-CD63 staining in green with the ROIs marked in white boxes (B',C'), and enlarged images of the ROIs with enhanced brightness and contrast to show the GFP-CD63 puncta in the MF (B",C"). **(D)** Quantification of GFP-CD63 puncta. **(E-F)** Representative confocal images of *Drosophila* eye imaginal discs expressing Flo2-RFP in the dorsal compartment in the control (E-E") and *ifc-KO* eyes (F-F") showing merged images with DAPI in blue (E,F), Flo2-RFP fluorescence in red (E',F'), Flo2-RPF signal enhanced by anti-RFP staining pseudocolored in green (E",F"), and enlarged and enhanced images of the ROI to reveal Flo2-RFP puncta in the MF in the ROIs (E'"-F'"). **(F)** Quantification of Flo2-RFP puncta. Scale bars: 5 μm. Bar charts show mean ± SEM of at least three independent experiments. *** p < 0.001 (Student's *t*-test).

Figure 5. Overexpression of functional Ifc increased the detection of extracellular exosome-like vesicles (ELVs) in *Drosophila* eye imaginal discs. (A-B) Representative confocal images of *Drosophila* eye imaginal discs expressing *TSG101-HA* in the dorsal compartment with *mCherry-CAAX* (control, A-A''') and *ifc(WT)-mCherry* (B-B''') showing merged images with DAPI in blue (A,B), mCherry fluorescence in red (A',B'), anti-HA staining in green (A'',B''), and enlarged images of the ROI with enhanced brightness and contrast to reveal TSG101-HA puncta in the MF (A''',B'''). **(C)** Quantification of TSG101-HA puncta. **(D-F)** Representative confocal images of *Drosophila* eye imaginal discs expressing *GFP-CD63* with the control *mCherry-CAAX* (D-D'''), *ifc(WT)-mCherry* (E-E'''), and *ifc(C3^{mut})-mCherry* (F-F''') showing merged images with DAPI in blue (D,E,F), mCherry fluorescence in red (D',E',F'), anti-CD63 staining in green (D'',E'',F''), and enlarged images of the ROI with enhanced brightness and contrast to reveal GFP-CD63 puncta in the MF (D''',E''',F'''). **(G)** Quantification of GFP-CD63 puncta. Scale bars: 5 μm. Bar charts show mean ± SEM of at least three independent experiments. *** p < 0.001; ns: Not significant (Student's *t*-test).

Figure 6. GFP-CD63 becomes more associated with autophagosomes and less with the endosomes in *ifc-KO* eye discs. (A-B) Representative confocal images of *Drosophila* eye imaginal discs expressing *GFP-CD63* in the dorsal compartment in control (A-A''') and *ifc-KO* (B-B''') showing anti-CD63 staining in green (A,B), anti-Atg8a staining in red (A',B'), merged images of CD63 and Atg8a immunostaining signals (A'',B''), and merged images with DAPI in blue (A''',B'''). **(C)** Quantification of the colocalization between CD63 and Atg8a. **(D-F)** Representative confocal images of *Drosophila* eye imaginal discs expressing *GFP-CD63* in control (D-D''') and *ifc-KO* (E-E''') showing anti-CD63 staining in green (D,E), anti-Rab5 staining in red (D',E'), merged images of CD63 and Rab5 immunostaining signals (D'',E''), and merged images with DAPI in blue (D''',E'''). **(G)** Quantification of the colocalization between CD63 and Rab5. Scale bars: 5 µm. Bar charts show mean ± SEM of at least three independent experiments. *** $p < 0.001$; ns: Not significant (Student's *t*-test).

Figure 7. Overexpression of Ifc increases the production of exosome-like vesicles via autophagy inhibition. (A-C) Representative confocal images of *Drosophila* eye imaginal discs expressing *GFP-CD63* in the dorsal compartment treated with ddH$_2$O solvent (A-A'''), 3-methyladenine (3-MA, B-B'''), and chloroquine (C-C''') showing merged images with DAPI in blue (A,B,C), mCherry control in red (A',B',C'), anti-CD63 staining in green (A'',B'',C''), and enlarged images of the ROI with enhanced brightness and contrast to show GFP-CD63 puncta in the MF (A''',B''',C'''). **(D)** Quantification of GFP-CD63 puncta. **(E-G)** Representative confocal images of *Drosophila* eye imaginal discs expressing GFP-CD63 and *ifc(WT)-mCherry* in the dorsal compartment treated with ddH$_2$O solvent (E-E'''), 3-methyladenine (3-MA, F-F'''), and chloroquine (G-G''') showing merged images with DAPI in blue (E,F,G), Ifc(WT)-mCherry in red (E',F',G'), anti-CD63 staining in green (E'',F'',G''), and enlarged images of the ROI with enhanced brightness and contrast to show GFP-CD63 puncta in the MF (E''',F''',G'''). **(H)** Quantification of GFP-CD63 puncta. Scale bars: 5 μm. Bar charts show mean ± SEM of at least three independent experiments. *** $p < 0.001$; ns: Not significant (Student's *t*-test).

Figure 8. Ifc participates in early exosome biogenesis. (A-C) Confocal images of *Drosophila* eye imaginal discs expressing *GFP-CD63* and *ifc(WT)-mCherry* in the control (A), *Hrs*[D28/+] (B), and *Vps25*[A3/+] (C) showing merged images with DAPI in blue (A,B,C), mCherry fluorescence in red (A',B',C'), anti-CD63 staining in green (A",B",C"), and enlarged images of the ROI with enhanced brightness and contrast to reveal GFP-CD63 puncta in the MF in the ROIs (A'",B'",C'"). **(D)** Quantification of GFP-CD63 puncta. Scale bars: 5 μm. Bar charts show mean ± SEM of at least three independent experiments. *** p < 0.001, ns: Not significant (one-way ANOVA).

Figure 9. DEGS1 promotes the formation of ILVs in the giant unilamellar vesicles (GUVs) *in vitro*.
(A) The percentages of GUVs that formed ILVs within 5 min. The GUVs were composed of DOPC:cholesterol at the molar ratio of 1:0.67 with or without dhCer and recombinant DEGS1 protein. **(B-E)** Representative images of GUVs in (A). **(F)** The percentages of GUVs that showed ILV formation within 5 min. The GUVs consisted of DOPC:cholesterol:dhCer at the molar ratio of 1:1:0.67 with and without DEGS1 protein in the presence of ethanol (EtOH) solvent or 0.2 µM fenretinide, a DEGS1 inhibitor. **(G-J)** Representative images of GUVs in (F). Scale bar: 5 µm.