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**The cellular and molecular origins of extracellular vesicles released by the
helminth pathogen, *Fasciola hepatica***

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25   **Abstract**

26   Parasitic helminths secrete extracellular vesicles (EVs) which have potent immunomodulatory
27   effects. Whilst the cargo of EVs has been characterised for many species, we know little about the
28   mechanisms that govern their biogenesis and release. Using antibodies raised against a panel of
29   *Fasciola hepatica* EV (FhEV) marker proteins, we have identified multiple sites of EV production
30   in the parasite. Discrete immunofluorescence patterns were observed within the gastrodermal cells
31   and tegumental syncytium for different marker proteins whilst the protonephridial (excretory)
32   system and parenchymal-type 2 cells were identified as additional sites of production (or transit) of
33   FhEVs. Ligation was used to mechanically block the oral sucker, excretory pore, or both, to
34   determine the effect on FhEV release from live adult flukes *in vitro*. This revealed that FhEVs are
35   predominately derived from the gut, whilst the tegument releases EVs to a lesser extent. The data
36   also suggest that the protonephridial system contributes to the small (120K) EV sub-population.
37   Sphingomyelinase (SMase) activity is a key driver of EV biogenesis in mammalian cells and we
38   have previously identified SMases in FhEVs by mass spectrometry. SMase activity associated with
39   isolated FhEVs was susceptible to the chemical inhibitor GW4869 and treatment of adult flukes
40   with GW4869 led to a significant reduction in 120K EV release *in vitro* suggesting that a ceramide-
41   dependent mechanism could drive 120K EV formation. In contrast, the release of the larger 15K
42   EVs was only moderately impacted, indicating that they form independently of SMase activity.
43   Ultrastructural observation of GW4869-treated *F. hepatica* tissue showed severe disruption to the
44   parenchyma and vacuolation of the tegument, gastrodermal cells and epithelial lining of the
45   excretory ducts. This work establishes that targeted disruption of EV biogenesis and release in
46   helminths is possible and provides proof-of-concept for future studies investigating EV secretion as
47   a target for parasite control.

48

49   **Keywords:** *Fasciola hepatica*, trematode, helminth, extracellular vesicle, secretion, excretion

50   **Introduction**

51   The liver fluke, *Fasciola hepatica*, is a pathogen of medical and veterinary significance and can
52   maintain chronic infections within its mammalian hosts for many years. Like other helminths, it
53   does this by releasing excretory-secretory (E/S) products to modulate the host immune response.
54   Using mass spectrometry-based proteomics techniques, several groups have built up a
55   comprehensive profile of the E/S material (Cwiklinski et al., 2015; Di Maggio et al., 2016;
56   Mophew et al., 2007; Robinson et al., 2009; Wilson et al., 2011) and identified individual
57   immunomodulatory components (Dalton et al., 2013). However, the discovery of extracellular
58   vesicles (EVs) within the *F. hepatica* secretome has shown that the composition of the E/S products
59   is more complex than originally thought (Marcilla et al., 2012). At least two distinct EV
60   populations, which differ in size and resident protein markers, have been characterised from *F.*
61   *hepatica* secretions and proteomics analysis has identified a range of known immunomodulators
62   amongst the *F. hepatica* EV (FhEV) cargo proteins (Cwiklinski et al., 2015; Marcilla et al., 2012;
63   de la Torre-Escudero et al., 2019). Indeed, recent work has shown that liver fluke EVs protected
64   against inflammation (independently of T and B cells) in a mouse model of colitis (Roig et al.,
65   2018), thus confirming their immunomodulatory capacity.

66   Given the important immunomodulatory roles of *Fasciola* EVs (and those of other
67   helminths; reviewed by Zakeri et al., 2018), impairing their release could tip the balance of the host-
68   parasite interaction in favour of the host and enable immune-mediated elimination of the parasite.
69   Although technically challenging, blocking EV release has shown promise in cancer therapy (El
70   Andaloussi et al., 2013), and it was recently demonstrated that blocking *Brugia malayi* EV release
71   is an action of the anthelmintic ivermectin (Harischandra et al., 2018). As the pharmacologically-
72   relevant concentrations of ivermectin used had no direct toxic effect on *B. malayi in vitro*, it was
73   suggested that preventing EV secretion contributes to the immune-mediated killing of the parasite
74   (Harischandra et al., 2018). Based on this precedent, developing therapeutics to inhibit the release

of FhEVs could have anthelmintic potential. However, this approach requires a better understanding of the mechanism(s) driving FhEV biogenesis and release.

Some insight into EV biogenesis in helminths has been provided by proteomics studies. Orthologues of sphingomyelinases (SMases) and endosomal sorting complex required for transport (ESCRT) proteins, the major players in multivesicular body-driven exosome formation in mammalian cells (Kowal et al., 2014; Trajkovic et al., 2008), were identified in FhEVs, suggesting that similar mechanisms may operate in the parasite (Cwiklinski et al., 2015; de la Torre-Escudero et al., 2019, 2016). However, the molecular pathways driving FhEV formation, their mechanisms of release, and their sites of secretion from the parasite remain largely unknown.

In this study, we have used a panel of antibodies, raised against proteins enriched in FhEVs, to determine sites of EV production in adult fluke tissue sections. Our data reveal discrete localisation patterns in the tegument, gut and protonephridial system, and further immunostaining of vesicular structures within parenchymal cells suggests that EVs may also be produced in this tissue before being trafficked to other sites for extra-corporeal release. Ligation of adult flukes *in vitro* showed that, whilst EVs are predominately derived from the gastrointestinal tract, some are released from the protonephridial system, via the excretory pore, whilst others may be shed directly from the tegument. We also demonstrate that SMase activity associated with FhEVs is susceptible to the chemical inhibitor GW4869. Treatment of flukes with GW4869 suppressed secretion of 120K EVs *in vitro* and led to major disruption of parenchymal cell ultrastructure. Collectively, our data show that targeted disruption of helminth EV biogenesis and release is possible which provides a framework for future studies using *in vivo* infection models.

Materials and Methods

Immunolocalisation

Adult *F. hepatica* were collected from the bile ducts of sheep immediately after slaughter at a local abattoir (Dungannon, Northern Ireland). Worms were fixed for 4 h in 4% paraformaldehyde in 0.1

101 M PBS (Sigma-Aldrich) and subsequently embedded in JB-4 resin (Sigma-Aldrich) according to
102 the manufacturer's instructions. Semi-thin (2 μ m) sections were cut on a pyramitome and mounted
103 on clean glass slides. For immunofluorescence, tissue sections were washed in PBS then incubated
104 overnight in primary antibody in antibody diluent (AbD: PBS containing 0.2% (v/v) Triton X-100)
105 at room temperature (18-21°C). For details of the primary antibodies, including their target
106 peptides, working concentrations and source, see Supplementary Table 1. As a negative control,
107 tissue sections were also incubated with pre-immune serum in AbD. The sections were then washed
108 three times in AbD before incubation in an appropriate secondary antibody-fluorophore conjugate
109 (goat anti-rabbit IgG-FITC or goat anti-mouse IgG-TRITC, Sigma-Aldrich) at a 1:200 dilution in
110 AbD for 1 h at room temperature. Following four washes in PBS, the sections were mounted in
111 glycerol containing 10% (v/v) PBS and 0.1 M propyl gallate (Sigma-Aldrich) then viewed under a
112 Leica DM2500 fluorescent microscope.

113

114 *Ligation and in vitro culture of adult F. hepatica*

115 Adult flukes were obtained from the bile ducts of sheep immediately after slaughter. After
116 thoroughly washing the flukes in PBS containing 0.1% (w/v) glucose at 37°C, those that had
117 regurgitated their gut contents were selected for ligation using the method of Rohrbacher (1957).
118 Briefly, flukes were ligated around the oral sucker or the excretory pore, or both using sterile
119 surgical thread (5-0 Prolene® Polypropylene Suture, Ethicon). Any flukes that were damaged by
120 the ligation process were discarded. Flukes were ligated in groups of 30, in biological triplicate
121 (with non-ligated flukes acting as control) and maintained at 1 worm/ml in RPMI-1640 culture
122 medium containing 0.1% (w/v) glucose, 100 U/ml penicillin and 100 μ g/ml streptomycin (Sigma-
123 Aldrich) for 3 h at 37°C to collect EVs. During culture, the integrity and survival of the flukes was
124 monitored visually. The wet weight of each group was used to normalise downstream protein
125 concentration. After 3 h incubation, EVs were isolated from the culture media by differential
126 centrifugation as described by de la Torre-Escudero et al. (2019) to determine the effect of ligation

127 on the release of the 15K EVs (post-15,000 x g pellet) and 120K EVs (post-120,000 x g pellet)
128 characterised by Cwiklinski et al. (2015). Both EV pellets were resuspended in filter-sterilised PBS,
129 snap-frozen and stored at -80°C until further analysis.

130

131 *Determining particle and protein concentrations*

132 The protein concentration of lysed EVs was determined using the Pierce^{TM} BCA Protein Assay Kit
133 (ThermoFisher Scientific). Nanoparticle tracking analysis (NTA) was carried out using the
134 NanoSight NS300 system (Malvern Instruments Ltd, United Kingdom) equipped with a 638 nm
135 laser and NanoSight NTA software version 3.2. EVs that had undergone a single freeze-thaw cycle
136 were diluted in filter-sterilised PBS and five 1 min videos of each sample were recorded with
137 optimised set parameters and a 10 s delay between recordings. Data for each sample are presented
138 as the mean of the five recordings. All measurements for each experimental group were performed
139 in biological triplicate and the dilution factor for each sample was set to account for the wet weight
140 of flukes in each replicate.

141

142 *Immunoblot analysis of F. hepatica extracellular vesicles*

143 Antibodies raised against FhEV proteins (de la Torre-Escudero et al., 2019) were used as markers to
144 determine the effect of ligation on the release of EVs from adult worms during *in vitro* culture. For
145 immunoblotting, EVs normalised by wet weight of the cultured flukes (2 µl 15K EVs/g fluke or 4
146 µl 120K EVs/g fluke) were run on reducing NuPAGE Novex 4-12% Bis-Tris gels (ThermoFisher
147 Scientific) and transferred to nitrocellulose membranes (GE Healthcare) at 30 V for 40 minutes.
148 Following transfer, the membrane was incubated in blocking solution (TBST: 20 mM Tris-HCl, 150
149 mM NaCl, and 1% Tween-20, pH 7.6) containing 5% skimmed milk for 1 h at room temperature.
150 Blots were then probed with primary antibodies (for details see Supplementary Table 1) diluted in
151 TBST containing 1% skimmed milk for 1 h at room temperature. After washing in TBST (3 x 10
152 minutes), blots were incubated with an appropriate alkaline phosphatase-conjugated IgG secondary

153 antibody (Sigma-Aldrich) for 1 h at room temperature before detection using the BCIP/NBT
154 substrate (Sigma-Aldrich). Blots were imaged using the Syngene G:BOX F3 system (Synoptics Ltd,
155 UK) and densitometric quantification was performed with ImageJ software version 1.48.

156

157 *Statistical analysis*

158 Data are presented as the mean of three biological replicates, $\pm$ standard error. Significance values
159 were calculated using a two-tailed t-test with two-sample unequal variance. Statistical analysis was
160 performed using Excel version 16.0.9126.2295 (Microsoft). A p value < 0.05 was considered
161 significant.

162

163 *Bioinformatics analysis of F. hepatica sphingomyelinases*

164 The evolutionary relationship of *Homo sapiens* (Hs) SMases (HsSMPD1, P17405; HsSMPD2,
165 O60906; HsSMPD3, Q9NY59; HsSMPDL3a, Q92484; HsSMPDL3b, Q92485) and *F. hepatica*
166 orthologues (FhSMPD2, BN1106_s7135B000046; FhSMPDL3¹⁵⁹, BN1106_S1285B000159;
167 FhSMPDL3¹³⁸, BN1106_S3568B000138) was inferred using the neighbour-joining method (Saitou
168 and Nei, 1987). The superscript number in FhSMPDL3^{159/138} refers to the last three digits of the
169 protein's identifier to distinguish it from the other isoform. The bootstrap consensus tree inferred
170 from 1000 replicates was taken to represent the evolutionary history of the taxa analysed
171 (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap
172 replicates were collapsed. The evolutionary distances were computed using the Poisson correction
173 method (Zuckerandl and Pauling, 1965) and are in the units of the number of amino acid
174 substitutions per site. All positions containing gaps and missing data were eliminated. There were a
175 total of 284 positions in the final dataset. Evolutionary analyses were conducted in MEGA6
176 (Tamura et al., 2013). The domain organisation of *F. hepatica* (Fh)SMases was determined using
177 InterPro (<https://www.ebi.ac.uk/interpro/>).

178

179 *GW4869 inhibition assay*

180 The susceptibility of native SMase activity associated with adult FhEVs to GW4869 was assayed
181 using the Amplex Red Sphingomyelinase Assay Kit (ThermoFisher Scientific) according to the
182 manufacturer's instructions. Four µg of 15K EVs or 2 µg 120K EVs were incubated with 0-50 µM
183 GW4869 (dissolved in DMSO). DMSO alone was added to some EVs as a vehicle control. Assays,
184 performed in biological triplicate, were incubated for 15 min (120K EVs) or 1 h (15K EVs) in the
185 dark at 37°C before fluorescence measurements were made using a POLARstar Omega
186 Spectrophotometer (BMG LABTECH, Germany) (excitation 544 nm/emission 590 nm).

187

188 *In vitro treatment of F. hepatica with GW4869*

189 Adult flukes were obtained from sheep, as described above, and washed thoroughly in PBS
190 containing 0.1% (w/v) glucose at 37°C. Those that had regurgitated their gut contents were
191 incubated in triplicate groups of 30 at 2 worms/ml in RPMI-1640 culture medium containing 0.1%
192 (w/v) glucose, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) and either 25 µM
193 GW4869 (Sigma-Aldrich) or 0.5% DMSO (vehicle control) for 3 h at 37°C. The flukes were then
194 placed in fresh media and incubated for a further 3 h to collect parasite secretions. The wet weight
195 of each group was determined and used to normalise downstream protein concentrations. During
196 culture the integrity and survival of the flukes was monitored visually. Following culture the EVs
197 were collected and quantified by protein concentration, NTA and immunoblotting as described
198 above.

199

200 *Processing of adult F. hepatica for microscopy*

201 Following *in vitro* culture, control and GW4869-treated flukes were fixed and processed for
202 transmission electron microscopy as previously described (Robinson et al., 2002). Briefly, whole
203 worms were fixed in 4% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4)
204 containing (w/v) 3% sucrose for 30 min. Worms were then dissected, and mid-body sections were

205 placed in fresh fixative for 4 h. Post-fixation, the tissue was washed in 0.1 M sodium cacodylate
206 buffer (pH 7.4) containing 3% (w/v) sucrose for 24 h. Fixed tissue was then placed in 1% osmium
207 tetroxide for 1 h and washed again in 0.1 M sodium cacodylate buffer (pH 7.4) containing 3% (w/v)
208 sucrose for 24 h. The tissue was dehydrated in ethanol before infiltration and embedding in Agar
209 100 epoxy resin (Agar Scientific, UK). Ultrathin sections (80 nm) were mounted on uncoated
210 copper grids then stained with uranyl acetate and aqueous lead citrate at the Electron Microscopy
211 facility at the Conway Institute, University College Dublin. The grids were viewed using a Jeol
212 1400 TEM operating at 80 kV at the Agri-Food and Biosciences Institute (Stormont, Northern
213 Ireland).

214

215 **Results**

216 *F. hepatica* extracellular vesicle markers localise to the gut, tegument, protonephridial ducts and
217 parenchymal tissue

218 To determine the sites of FhEV production, trafficking and release, a panel of antibodies
219 raised against proteins found to be enriched in FhEVs by mass spectrometry (Cwiklinski et al.,
220 2015; de la Torre-Escudero et al., 2019) were used to probe adult *F. hepatica* tissue sections. These
221 targets included orthologues of known EV biogenesis proteins such as TSG101 and ALIX which
222 are involved in the ESCRT-dependent pathway (Christ et al., 2017); Ral-A which is associated with
223 a non-canonical form of the ESCRT pathway that utilises only a limited selection of ESCRT
224 components to form exosomes (Hyenne et al., 2016); and SMases which operate independently of
225 ESCRT in EV biogenesis (van Niel et al., 2018). Other antibodies targeted the fluke-specific
226 proteins tegumental antigen 1 (FhTeg1), T cell immunomodulatory protein (FhTIP) and DM9
227 domain-containing protein (FhDM9) (Cwiklinski et al., 2015), whilst α -tubulin was used as a
228 marker of EVs that pellet at low centrifugal forces (Menck et al., 2017).

229 Antibodies against several FhEV markers labelled the gastrodermal cells, albeit with
230 discrete localisation patterns (Fig. 1). *F. hepatica* gastrodermal cells line the gut caeca and have a

pyramidal shape (Fig. 1A) and showed no specific immunofluorescence when sections were probed with pre-immune control serum (Fig. 1B). However, immunostaining with the anti-FhALIX, FhDM9 and FhTIP antibodies revealed foci of immunofluorescence concentrated towards the gastrodermal cell apices that become more dispersed towards the basolateral membrane. Points of immunofluorescence were also visible in rear-cell extensions that penetrated backwards into the parenchymal tissue (Fig. 1C, F-G). Similar immunofluorescent puncta were observed clustered in and around parenchymal cells underlying the gastrodermis for FhALIX, FhDM9, FhTIP and FhSMPDL3¹⁵⁹ (Fig. 1C, E, F-G). Only FhDM9 showed immunopositivity in the gut lumen where larger globular structures were labelled (Fig. 1F). Faint FhSMPD2 and FhSMPDL3¹⁵⁹-positive immunofluorescence was seen in the gastrodermal cell lamellae (Fig. 1D-E), and discrete puncta of immunofluorescence were also observed in this region for tyrosinated α -tubulin (Fig. 1H). These proteins showed contrasting immunostaining in the cell bodies, however, with FhSMPD2 exhibiting diffuse immunofluorescence, whilst labelling was absent for FhSMPDL3¹⁵⁹ and tyrosinated α -tubulin (Fig. 1D-E, H).

The tegumental syncytium of adult *F. hepatica* is the major barrier between the parasite and the host environment and encloses the parenchymal tissue and internal organs (Threadgold, 1963; Fig. 2A). No specific immunofluorescence was observed in the tegument in sections probed with pre-immune serum (Fig. 2B), but the tegumental syncytium was labelled by antibodies targeting FhSMPDL3¹⁵⁹, FhDM9 and FhTeg1 (Fig. 2C-E). This immunofluorescence was concentrated at the base of the syncytium, but FhDM9 and FhTeg1 were also localised to the apical plasma membrane. Additionally, FhDM9 and FhTeg1 displayed a more granular immunofluorescence pattern than FhSMPDL3¹⁵⁹, with FhTeg1 present in discrete puncta that were similarly visible in the cytoplasmic connections that join the syncytium to the underlying tegumental cell bodies. FhSMPDL3¹⁵⁹ also showed intense immunostaining in the cytoplasmic connections, along with fainter immunofluorescence around the periphery of tegumental cell bodies (Fig. 2C). The tegumental cell bodies themselves displayed diffuse FhDM9 and FhTeg1 immunoreactivity

257 punctured by larger foci of immunofluorescence within the cytoplasm (Fig. 2D-E). This was more
258 intense for FhTeg1 and consistent with pockets of T1 secretory bodies arising from Golgi
259 complexes (Hanna, 1980). The anti-FhDM9 antibody also labelled vesicular structures situated in
260 large clusters within parenchymal cells or dispersed below the tegumental syncytium (Fig. 2D).

261 The protonephridial (excretory) system is a major source of lipid-expulsion by the parasite
262 (Burren et al., 1967) and consists of a network of ducts lined by a cellular epithelium (Wilson and
263 Webster, 1974; Fig. 3A). Whilst no specific immunofluorescence was seen in these areas in sections
264 probed with pre-immune control serum (Fig. 3B), both the protonephridial duct epithelium and
265 pockets of vesicular structures in sub-protonephridial parenchymal cells were labelled by antibodies
266 targeting FhTSG101, FhALIX, FhRal-A, FhSMPD2, and FhDM9 (Fig. 3C-G). The
267 immunolocalisation pattern within the protonephridial duct epithelium, however, varied with
268 FhTSG101 and FhSMPD2 showing diffuse immunofluorescence (Fig. 3C and F), FhALIX and
269 FhRal-A displaying a granular immunofluorescence pattern (Fig. 3D and E) (with FhALIX
270 resembling its gastrodermal cell localisation pattern seen in Fig. 1C), whilst FhDM9-positive
271 immunofluorescence was situated mainly in perinuclear regions of the duct epithelial cells (Fig.
272 3G).

273 To investigate the labelling of parenchymal cells by antibodies targeting FhEV markers,
274 sections that were first used for immunolocalisation were subsequently stained with toluidine blue
275 to help classify the labelled cells. The immunopositive vesicular structures, seen clustered within
276 parenchymal cells, stained dark blue which indicates that they are basophilic (Fig. 4). The
277 cytoplasm of some of these cells displayed slight basophilia and the vesicular structures could
278 occupy the majority of the cytoplasm (Fig. 4). This cell type was commonly located in sub-
279 gastrodermal and sub-tegumental regions and resembled the PC2-type parenchymal cells in *F.*
280 *gigantica* described by Pankao et al. (Pankao et al., 2006).

281
282 *Ligation of the oral sucker prevents the release of F. hepatica extracellular vesicles in vitro*

283 To determine the principal route(s) of export of FhEVs, adult *F. hepatica* were ligated at the oral
284 sucker (OSL), excretory pore (EPL), or both (double ligated, DL), and then maintained for 3 h *in*
285 *vitro*. The flukes remained motile following incubation and representative images of the ligated
286 flukes are shown in Fig. 5. In comparison to the prominent 15K EV pellet recovered from non-
287 ligated control fluke culture media, the OSL and DL 15K EV pellets were almost invisible (Fig. 5).
288 In contrast, the 15K EV pellets recovered from the EPL flukes looked similar to the controls (Fig.
289 5). The 120K EV pellets are typically too faint to be compared visually.

290 Varied analyses were performed to determine the protein concentration, particle
291 concentration and particle size of 15K and 120K EVs collected from control and ligated flukes.
292 OSL resulted in a 31% reduction in 15K EV protein concentration and a 25% reduction in 15K EV
293 particle concentration compared to control 15K EVs, although these reductions were not
294 statistically significant (Supplementary Fig. S1A-B). Additionally, it was estimated that OSL flukes
295 released protein associated with 15K EVs at a rate of approximately 790 ng/fluke/h and 15K EV
296 particles at $\sim 4.27 \times 10^8$ particles/fluke/h, as opposed to the control flukes which released
297 approximately 1150 ng/fluke/h and $\sim 5.69 \times 10^8$ particles/fluke/h. The quantity of protein per
298 particle was similar, averaging around 2.0 femtograms (fg)/particle in both the control and OSL
299 15K EV pellets, and there was no significant difference in the average size of 15K EVs collected
300 from OSL flukes (224 nm) compared to the control 15K EVs (219 nm). Western blotting was used
301 to determine whether ligation affected the release of EVs bearing different protein markers.
302 Consistent with their association with the gut, FhSMPDL3¹⁵⁹, tyrosinated α -tubulin, cathepsin L1
303 (FhCL1) and CD63 receptor (FhCD63rec) showed 65 – 93% reduction within the 15K EV
304 population after OSL, with all but FhCL1 being statistically significant (Fig. 6A). Collectively,
305 these experiments demonstrate that OSL had a marked impact on the release of 15K EVs from adult
306 flukes *in vitro*.

307 OSL had an even greater effect on the release of 120K EVs, with > 50% reduction ($p <$
308 0.01) in protein concentration and > 65% reduction in particle concentration (Supplementary Fig.

309 S1A-B) compared to the control 120K EVs. Additionally, it was estimated that OSL flukes released
310 protein associated with 120K EVs at a rate of 70 ng/fluke/h and 120K EV particles at 2.58×10^7
311 particles/fluke/h, as opposed to the control flukes which released approximately 140 ng/fluke/h and
312 7.90×10^7 particles/fluke/h. Again, the protein content of control and OSL 120K EVs was ~ 2.0
313 fg/particle and there was no significant difference in the particle sizes or size distribution between
314 them. The reductions in protein and particle concentration were similarly reflected in Western blots
315 (Fig. 6B) with the release of FhSMPDL3¹⁵⁹-positive and FhCD63rec-positive 120K EVs
316 significantly reduced by OSL ($p < 0.01$ and $p < 0.05$, respectively). These findings demonstrate that
317 OSL substantially reduced the release of 120K EVs by *F. hepatica in vitro*.

318

319 *Excretory pore ligation of F. hepatica has differing effects on the release of EV populations in vitro*
320 EPL showed an increase (though not significant) of 28% and 25% in 15K EV protein and particle
321 concentrations, respectively, in comparison to control 15K EVs (Supplementary Fig. S1C-D).
322 Accordingly, it was estimated that EPL flukes released protein associated with 15K EVs at a rate of
323 650 ng/fluke/h and 15K EV particles at $\sim 4.23 \times 10^9$ particles/fluke/h, compared to the control
324 flukes which released approximately 590 ng/fluke/h and $\sim 3.73 \times 10^9$ particles/fluke/h. There were
325 no differences in the sizes of control and EPL 15K EVs (both averaging approximately 235 nm), the
326 protein content (0.16 fg/particle on average) and they also displayed similar size distributions. In
327 accordance with the protein and particle concentrations, increases in the levels of the FhEV marker
328 proteins tyrosinated α -tubulin, FhCL1, and FhCD63rec were observed within the EPL 15K EV
329 preparation relative to the control 15K EVs (Fig. 6C), indicating that EPL increases the number of
330 15K EVs derived from the gut.

331 In contrast to the 15K EVs, EPL caused a decrease in 120K EV protein and particle
332 concentrations by approximately 40 – 50% (Supplementary Fig. S1C-D). The EPL flukes released
333 protein associated with 120K EVs at a rate of approximately 90 ng/fluke/h and 120K EV particles at
334 $\sim 1.55 \times 10^9$ particles/fluke/h, as opposed to the control flukes which released approximately 170

335 ng/fluke/h and $\sim 3.09 \times 10^9$ particles/fluke/h. There were no differences in the sizes of control and
336 EPL 120K EVs (both averaged between 163 – 164 nm), the protein content (both 0.05 – 0.06
337 fg/particle), and both EPL and control 120K EVs displayed similar size distributions. Although
338 there was some variation between replicates, densitometric analysis of Western blots showed that
339 the average band intensities for FhTSG101, FhSMPDL3¹⁵⁹ and FhCD63rec were reduced in the
340 EPL 120K EV population relative to control 120K EVs (Fig. 6D), and a significant reduction ($p <$
341 0.05) in 120K EVs positive for FhRal-A was observed.

342

343 *Ligating both the oral sucker and the excretory pore of F. hepatica (double ligation) blocks 120K*
344 *EV release in vitro and significantly alters 15K EV composition*

345 Remarkably, DL had no effect on protein concentration, particle concentration or size distribution
346 of 15K EVs compared to the controls (Supplementary Fig. S1E-F). It was also estimated that
347 control and DL flukes released 15K EV-associated protein and particles at approximately the same
348 rate: 590 ng/fluke/h and $3.58 - 3.73 \times 10^9$ particles/fluke/h. Equally, there was no difference in 15K
349 EV protein content between the control and DL flukes (both ~ 0.16 fg/particle). In contrast, Western
350 blotting showed that the EV markers tyrosinated α -tubulin, FhCL1, FhCD63rec and FhSMPDL3¹⁵⁹
351 displayed a 40 – 75% reduction in band intensity (Fig. 6E), indicating that (similarly to OSL) DL
352 markedly reduced the release of 15K EVs carrying these proteins from the gut.

353 In contrast to the effect on 15K EVs, DL reduced 120K EV protein concentration by $> 70\%$
354 (Supplementary Fig. S1E), and the particle concentration was reduced by $\sim 90\%$ compared to the
355 control 120K EVs (Supplementary Fig. S1F). Additionally, it was estimated that control flukes
356 released protein associated with 120K EVs at a rate of 170 ng/fluke/h and 120K EV particles at 3.09
357 $\times 10^9$ particles/fluke/h, whilst the rates of 120K EV release by DL flukes were ~ 50 ng/fluke/h and $\sim$
358 3.35×10^8 particles/fluke/h. The estimated protein content of DL 120K EVs was almost three times
359 greater than control 120K EVs (0.14 fg/particle compared to 0.05 fg/particle), although no
360 significant difference in average size of the EVs was observed (both 161 – 163 nm). Western

361 blotting similarly demonstrated a decrease of over 50% in the levels of FhTSG101, FhSMPDL3¹⁵⁹,
362 FhCD63rec, and FhRal-A in the DL 120K EV population (Fig. 6F). These findings indicate that DL
363 significantly inhibited the release of 120K EVs by *F. hepatica* *in vitro*.

364
365 *Phylogenetic and sequence analysis of F. hepatica sphingomyelinases*

366 The conversion of sphingomyelin to ceramide by SMases is a key driver of ESCRT-independent
367 EV biogenesis (Verderio et al., 2018) and acid and neutral SMase family members (characterised by
368 their pH optima) have both been implicated this process (Gradilla et al., 2014; Menck et al., 2017;
369 Trajkovic et al., 2008; Truman et al., 2012). BLAST searches indicated that only two acid-like
370 SMases and one neutral SMase are conserved in *F. hepatica*. Phylogenetic analysis revealed that
371 human SMases and *F. hepatica* orthologues group by functional similarity, rather than into species-
372 specific clades (Supplementary Fig. S2A). Whilst FhSMPD2 grouped with its neutral SMase family
373 orthologue (HsSMPD2), FhSMPDL3¹⁵⁹ and FhSMPDL3¹³⁸ formed their own discrete cluster within
374 the acid-like SMase family, set apart from HsSMPD1 and HsSMPDL3a/b. Despite their
375 segregation, the predicted domain architectures of FhSMPDL3¹⁵⁹ and FhSMPDL3¹³⁸ showed that
376 they were more similar to HsSMPDL3a/b (Supplementary Fig. S2B): both have a predicted N-
377 terminal signal peptide and metallo-phosphatase catalytic domain, but lack the N-terminal saposin
378 domain of HsSMPD1 (Gorelik et al., 2016b). FhSMPDL3¹³⁸ also had an additional predicted
379 transmembrane region but neither protein was predicted to have a GPI anchor which is a feature of
380 SMPDL3b (Heinz et al., 2015). FhSMPD2 displayed the same domain architecture as HsSMPD2
381 (Supplementary Fig. S2B), including two transmembrane regions which are essential for activity
382 (Tomiuk et al., 2000). Catalytic residues were conserved in FhSMPD2, including Asp¹¹¹ and Lys¹¹⁶
383 of the DK switch, a motif involved in both catalysis and interactions with the neutral SMase
384 inhibitor GW4869 (Airola et al., 2017). In contrast, fewer functionally-important residues were
385 conserved in the fluke acid-like SMases, particularly in FhSMPDL3¹⁵⁹ which lacked residues such
386 as His⁹⁷ and Lys¹⁴⁰ which are important in substrate recognition and binding (Gorelik et al., 2016a).

387 FhSMPDL3¹⁵⁹ and FhSMPDL3¹³⁸ also lacked key *N*-linked glycosylation sites required for
388 HsSMPDL3a function and, like in HsSMPDL3a/b, only the terminal Asn residues of the acid
389 SMase (SMPD1) consensus motif NXXXCXXXN (where X is any amino acid) were conserved.

390

391 *GW4869 inhibits F. hepatica sphingomyelinase activity and suppresses release of extracellular*
392 *vesicles in vitro*

393 To determine whether native FhSMase activity associated with EVs was sensitive to GW4869, an
394 inhibitor of mammalian neutral SMases (Trajkovic et al., 2008), FhEVs were treated with
395 increasing concentrations of the inhibitor (Fig. 7A and Supplementary Fig. S3A). Whilst 5 μ M
396 GW4869 had no significant effect on SMase activity, 25 μ M GW4869 decreased 15K and 120K-
397 associated SMase activity by 27% and 35%, respectively. Treatment with 50 μ M GW4869
398 decreased 120K-associated SMase activity by 64% (Fig. 7A), but this increased concentration had
399 no additional inhibitory effect on 15K EV-associated activity (Supplementary Fig. S3A).

400 As FhSMase activity was sensitive to GW4869, adult *F. hepatica* were maintained for 6 h *in*
401 *vitro* in the presence of 25 μ M GW4869 (or 0.5% DMSO as a vehicle control) to determine the
402 effect on FhEV release. At the end of the incubation period, the flukes remained motile, but
403 GW4869 treatment resulted in a significant ($p < 0.05$) 25% reduction in 15K EV protein
404 concentration and a 24% reduction in 15K EV particle concentration compared to control 15K EVs
405 (Supplementary Fig. S3B-C). Additionally, GW4869-treated flukes released protein associated with
406 15K EVs at a rate of 470 ng/fluke/h and 15K EV particles at $\sim 2.04 \times 10^9$ particles/fluke/h, as
407 opposed to the control flukes which released 630 ng/fluke/h and $\sim 2.69 \times 10^9$ particles/fluke/h.
408 GW4869 treatment had no effect on the quantity of protein per particle (0.23 fg/particle for 15K
409 EVs derived from both treated and untreated flukes) or on the average size of 15K EVs (~ 255 nm)
410 which both had similar size distributions. Western blotting was also used to assess whether
411 GW4869 treatment could affect the release of FhEVs bearing particular protein markers. As shown
412 in Supplementary Fig. S3D, the levels of EV markers tyrosinated α -tubulin, FhCD63rec, FhCL1,

413 and FhSMPDL3¹⁵⁹ were reduced by 16 – 31%, although this was only significant ($p < 0.05$) for
414 FhSMPDL3¹⁵⁹. Collectively, these results demonstrate that GW4869 treatment had a measurable,
415 but modest, impact on 15K EV release from adult flukes *in vitro*.

416 In contrast, GW4869 substantially impacted the release of 120K EVs, with a 55% reduction
417 ($p < 0.01$) in protein concentration (Fig. 7B) and an 80% ($p < 0.05$) reduction in particle
418 concentration (Fig. 7C) compared to 120K EVs recovered from vehicle-treated flukes. Furthermore,
419 GW4869-treated flukes released protein associated with 120K EVs at a rate of approximately 17
420 ng/fluke/h and 120K EV particles at $\sim 3.24 \times 10^7$ particles/fluke/h, compared to the control flukes
421 which released approximately 38 ng/fluke/h and $\sim 1.59 \times 10^8$ particles/fluke/h. The protein content
422 of the 120K EVs was 0.24 fg/particle for control 120K EVs compared with 0.53 fg/particle for the
423 120K EVs from GW4869-treated flukes which could indicate the presence of more protein
424 aggregates in the 120K EV pellet derived from GW4869-treated flukes. There was no significant
425 difference in 120K EV particle size or size distribution. Western blotting showed an 86% ($p < 0.05$)
426 and 89% ($p < 0.001$) reduction in the release of FhSMPDL3¹⁵⁹-positive and FhCD63rec-positive
427 120K EVs (Fig. 7D), respectively, reflecting the change in particle concentration rather than total
428 protein concentration. These findings show that GW4869 treatment significantly reduced the release
429 of 120K EVs by adult *F. hepatica in vitro*.

430 431 *GW4869 causes ultrastructural disruption of adult fluke tissues in vitro*

432 Fluke tissue sections were viewed by TEM to observe the effects of GW4869 treatment at the
433 ultrastructural level. Whilst the tegument of control *F. hepatica* appeared normal (Fig. 8A), the
434 tegumental syncytium of GW4869-treated flukes was severely vacuolated, although no blebbing of
435 the apical plasma membrane (a common feature of drug-induced damage in trematodes) was
436 apparent (Fig. 8B). Furthermore, whilst fewer T2 secretory bodies were visible, the distribution and
437 number of T1 secretory bodies appeared similar to the controls and the spines appeared normal (Fig.
438 8B). In comparison to controls (Fig. 8C), the gastrodermal cells of GW4869-treated flukes appeared

439 to be undergoing higher levels of autophagy, as evidenced by the abundance of autophagosomes
440 (Fig. 8D). Some vacuolation of the gastrodermal cell cytoplasm was visible following GW4869
441 treatment (Fig. 8D), whilst the lamellae appeared normal (Fig. 8D). TEM also revealed that,
442 compared to the controls (Fig. 8E), the parenchymal tissue of GW4869-treated *F. hepatica* appeared
443 highly disorganised with numerous large electron-lucent vacuoles dominating the tissue (Fig. 8F).
444 Additionally, compared to the controls (Fig. 8G), more vacuolation was evident in the
445 protonephridial duct epithelia of GW4869-treated *F. hepatica* (Fig. 8H), although the lipid droplets
446 within the ducts appeared normal.

447

448 **Discussion**

449 Helminth EVs (hEVs) are emerging as important mediators of the host-parasite interaction and are
450 consequently being investigated as novel targets for parasite control (Wu et al., 2018). These studies
451 have so far focussed on preventing the internalisation of hEVs by host cells (Chaiyadet et al., 2015;
452 de la Torre-Escudero et al., 2019) and on the potential of hEVs as vaccine candidates (Kifle et al.,
453 2017). However, the effect of blocking hEV release on parasite survival within the host has yet to
454 be investigated due to our limited understanding of the molecular machinery that drives hEV
455 biogenesis and secretion. Accordingly, this work aimed to develop our knowledge of the
456 mechanisms underlying hEV formation by firstly investigating the sites of EV secretion by *F.*
457 *hepatica* and secondly attempting to disrupt FhEV release by targeting putative EV biogenesis
458 molecules.

459 FhEVs can be separated into two distinct (albeit crude) populations by differential
460 centrifugation at 15,000 x g (15K EVs) and 120,000 x g (120K EVs) which were originally
461 proposed to originate from the gut and the tegument, respectively (Cwiklinski et al., 2015; de la
462 Torre-Escudero et al., 2019; Marcilla et al., 2012). However, our immunolocalisation data suggest
463 that FhEVs are derived from multiple cell types. The localisation of several FhEV marker proteins
464 to vesicular structures within the parenchyma indicates that these cells play a previously unknown

465 role in EV biogenesis. Immunolabelling of parenchymal cells resembling the PC2-type described in
466 *F. gigantica* (Pankao et al., 2006) suggested that these cells have a secretory function in addition to
467 their proposed roles in lipid storage and metabolism. For the parenchymal cell-derived vesicles to
468 be secreted extra-corporeally, they would have to be trafficked to suitable sites within the fluke that
469 are permissive to their release. In this regard, vesicles bearing the same markers were also seen
470 outside the parenchymal cells (around their periphery or in the interstitial material) and in close
471 proximity to the gut, tegument and protonephridial ducts, indicating that they are trafficked within
472 the parenchyma to these potential sites of release. Moreover, similar structures were visible in rear
473 extensions of gastrodermal cells that protruded into the parenchymal tissue, suggesting that there
474 may be a transition of these vesicles between the parenchyma and the basolateral membrane of
475 gastrodermal cells. A possible mechanism for EV transfer between cells is adsorptive-mediated
476 transcytosis which enables EVs released by erythrocytes to cross the blood-brain barrier
477 (Matsumoto et al., 2017). Such a process in gastrodermal cells could be mediated by Rab11
478 (enriched on the surface of 120K EVs; de la Torre-Escudero et al., 2019) which mediates
479 transcytosis of vesicles across canine kidney cells (Casanova et al., 1999; Wang et al., 2000),
480 although this mechanism requires experimental investigation.

481 As had been proposed by Cwiklinski et al. (2015), the gut was found to be the dominant site
482 of 15K EV release. In particular, those EVs immunopositive for tyrosinated α -tubulin,
483 FhSMPDL3¹⁵⁹, FhCL1 and FhCD63rec were shown by oral sucker ligation to originate from the
484 gastrointestinal tract. Unexpectedly, oral sucker ligation also demonstrated that the majority of
485 120K EVs originate from the gut. Components of both the ESCRT-dependent (FhALIX) and
486 ESCRT-independent (SMases) EV biogenesis pathways were identified in the gastrodermal cells
487 and the varied localisation of FhEV marker proteins indicates that the gut is capable of secreting
488 multiple sub-populations of EVs. Whilst the gastrodermal cells may just act as a conduit for release
489 of vesicles trafficked from the parenchyma, we have previously detected FhRab-A in *F. hepatica*
490 gastrodermal cells (de la Torre-Escudero et al., 2019) which is thought to initiate a pathway

491 involving ALIX and syntenin that results in the budding of ILVs (Hyenne et al., 2016). However,
492 co-localisation and functional studies would be required to verify this mechanism.

493 The immunolocalisation studies and *in vitro* ligation experiments showed that the tegument
494 could also contribute to 15K and 120K EV release, although to a lesser extent than the gut.
495 Surprisingly, DL did not affect 15K EV total protein or particle concentration. However, the DL
496 15K EV population did have a markedly different protein profile (with a significant reduction in
497 gut-derived proteins), indicating that some of the material was shed from the tegument as a result of
498 ligation which masked the true effect of DL on 15K EV total protein and particle concentration.
499 Notably, there was no increase in the protein content of the DL 15K EVs, suggesting the shedding
500 of membranous material (which pelleted at 15,000 x g) rather than protein aggregates. Therefore,
501 DL may have induced the release of blebs which pinch directly from the apical plasma membrane
502 (APM) of the tegumental syncytium (de la Torre-Escudero et al., 2016). This supports the proposal
503 that external stress (in this case, DL) to helminths causes the release of blebs as a mechanism of
504 tegument repair. A small number of 120K EVs were still released by *F. hepatica* following DL.
505 Whilst these could be leakage from the gut and the protonephridial system due to incomplete
506 ligation, it could also indicate that some 120K EVs originate from the tegument. TEM studies have
507 observed MVB-like structures releasing small (< 50 nm) EVs at the tegument APM (de la Torre-
508 Escudero et al., 2016; Wilson et al., 2011) and FhTeg1, which was identified in 120 K EVs by mass
509 spectrometry (Cwiklinski et al. 2015), showed this distribution pattern. As FhTeg1
510 immunolabelling was not observed at any other secretory interface, the tegument is likely to be the
511 source of FhTeg1-positive 120K EVs.

512 Compared to the gut and the tegument, the role of the protonephridial system in the host-
513 parasite interaction has been somewhat overlooked. During infection, the protonephridial system
514 has been associated with the excretion of lipids and anthelmintics (Burren et al., 1967; Kusel et al.,
515 2009; Wilson and Webster, 1974), but our current data suggests an additional role in the release of
516 FhEVs. The *in vitro* ligation experiment indicated that a FhRal-A-positive sub-population of 120K

EVs are released from the excretory pore and the localisation of several EV biogenesis proteins (FhALIX, FhTSG101, FhRal-A and FhSMPD2) to the protonephridial duct epithelium suggests that 120K EVs may also be generated (or at least transported) by this organ system. Recently, the excretory system has also been suggested as a source of EVs from the nematodes *Ascaris suum* and *Brugia malayi* (Hansen et al., 2019; Harischandra et al., 2018). Interestingly, ALIX was localised to the excretory duct of *B. malayi* (Harischandra et al., 2018) which could indicate a conserved role of this protein in the formation of EVs in the excretory ducts of helminths. Notably, EV biogenesis proteins did not localise to lipid droplets in the protonephridia of *F. hepatica*, nor did SMase inhibition impact lipid droplet formation (see below). Whilst the molecular mechanism responsible for lipid droplet formation in helminths remains unresolved, the initial characterisation of the lipid droplets released from the excretory pore of *F. hepatica* suggested that they have a complex composition and function in the removal of lipid (or lipid-soluble) metabolic waste products from parasite tissues (Burren et al., 1967; Moss, 1970). The effects of excreting such vast quantities of lipid (10% of the fluke's total lipid content during 4 h *in vitro* culture; Burren et al., 1967) on host cell physiology are unknown. However, the recent description of the lipase-independent release of lipid-filled vesicles from mouse adipocytes and their effects on macrophage differentiation and function (Flaherty et al., 2019) sets an interesting precedent for the possible roles of such bioactive lipids, or proteins embedded in the lipid droplets' lamellar coat (Bennett, 1977), excreted during helminth infection.

BLAST surveys indicated that *F. hepatica* has a distinct SMase repertoire to humans and phylogenetic analysis showed that fluke acid-like SMases clustered separately to their human counterparts. Furthermore, key functional residues were not conserved, including *N*-glycosylation sites essential for HsSMPDL3a nucleotide phosphodiesterase activity (Traini et al., 2017) which indicates that the fluke acid-like enzymes have distinct functions or catalytic mechanisms. Whilst HsSMPDL3a does not have SMase activity (Traini et al., 2014), HsSMPDL3b is thought to have SMase activity when tethered to a lipid bilayer (Gorelik et al., 2016a). Conceivably, FhSMPDL3¹³⁸

could be tethered to lipid bilayers by its predicted transmembrane region and proteomic analysis has shown that FhSMPDL3¹⁵⁹ and FhSMPDL3¹³⁸ are associated with FhEV membranes (Cwiklinski et al., 2015; de la Torre-Escudero et al., 2019), whilst FhSMPDL3¹⁵⁹ was localised to the periphery of cells. This localisation pattern of FhSMPDL3¹⁵⁹ and FhSMPDL3¹³⁸ could therefore permit SMase activity, but direct biochemical characterisation of these proteins would be required to confirm this.

Functionally important residues and motifs were, however, conserved in FhSMPD2, including the DK switch which acts in both catalysis and interactions with the neutral SMase inhibitor GW4869 (Airola et al., 2017). Indeed, treating FhEVs with GW4869 inhibited FhEV-associated neutral SMase activity in a dose-dependent manner. However, residual activity following GW4869 treatment suggests that FhSMPD2 may be refractory to this inhibitor (compared to human enzymes) as has been reported for insect SMase (Zhou et al. 2017).

Treating adult *F. hepatica* with GW4869 *in vitro* had a minor impact on 15K EV release which points to a SMase-independent mode of biogenesis for these vesicles in *F. hepatica*. In contrast, GW4869 caused a major reduction in the release of 120K EVs which reflects the impact of this molecule on exosome/small EV release reported in the literature (Essandoh et al., 2015; Li et al., 2013; Menck et al., 2017; Willms et al., 2016) and suggests that 120K EVs form in a ceramide-dependent manner. Some 120K EVs, however, were still released following GW4869 treatment which indicates either that GW4869 does not completely inhibit FhSMase activity (as was demonstrated in the assays using isolated EVs) or that hybrid/multiple EV biogenesis pathways exist in *F. hepatica*; a proposition supported by the enrichment of ESCRT proteins in the 120K EVs in particular (Cwiklinski et al., 2015; de la Torre-Escudero et al., 2019).

GW4869-treated fluke tissue was examined by microscopy to provide insight into its mechanism of action. Whilst increased autophagy indicated that there was some disruption to the gut, more severe disruption was observed in the parenchymal tissue of GW4869-treated flukes, including in sub-gastrodermal and sub-protonephridial regions. This further supports the differential origins of 15K EVs and 120K EVs bearing the same markers (Cwiklinski et al., 2015; de la Torre-

Escudero et al., 2019). For example, GW4869 treatment had no effect on the release of FhCD63rec-positive 15K EVs (secreted directly by gastrodermal cells) but had a significant impact on the release of FhCD63rec-positive 120K EVs (which may be synthesised in the parenchymal cells before being trafficked to the gastrodermal cells for secretion). The extensive vacuolation of fluke tissue may be attributed to the inhibition of sphingomyelin hydrolysis by GW4869. Indeed, the ultrastructural effects of GW4869 were broadly similar to those seen in sphingomyelin storage diseases such as Niemann-Pick disease where sphingomyelin accumulates in the cell (Elleder, 1989). Whilst GW4869 was non-lethal *in vitro*, it remains to be determined whether the inhibition of FhEV release seen here would be sufficient to impair parasite survival *in vivo*. However, ivermectin-induced suppression of EV release from *B. malayi* has been proposed to enable immune-mediated parasite killing *in vivo* (Harischandra et al., 2018).

This study has shown that EV biogenesis in *F. hepatica* is more complex than first thought, with the parasite capable of secreting a range of FhEV subtypes from a variety of tissues/organ systems and via multiple biogenesis pathways. However, despite the complexities of FhEV biogenesis identified in this study, it was revealed for the first time that targeted disruption of EV biogenesis and release in helminths is possible. In doing so, we provide proof-of-concept for future *in vivo* studies investigating EV secretion as a target for parasite control.

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594   **References**

- 595   Airola, M. V, Shanbhogue, P., Shamseddine, A.A., Guja, K.E., Senkal, C.E., Maini, R., Bartke, N.,
596   Wu, B.X., Obeid, L.M., Garcia-Diaz, M., Hannun, Y.A., 2017. Structure of human nSMase2
597   reveals an interdomain allosteric activation mechanism for ceramide generation. Proc. Natl.
598   Acad. Sci. U. S. A. 114, E5549–E5558.
- 599   Bennett, C.E., 1977. *Fasciola hepatica*: development of excretory and parenchymal systems during
600   migration in the mouse. Exp. Parasitol. 41, 43–53.
- 601   Burren, C.H., Ehrlich, I., Johnson, P., 1967. Excretion of lipids by the liver fluke (*Fasciola hepatica*
602   L). Lipids 2, 353–356.
- 603   Casanova, J.E., Wang, X., Kumar, R., Bhartur, S.G., Navarre, J., Woodrum, J.E., Altschuler, Y.,
604   Ray, G.S., Goldenring, J.R., 1999. Association of Rab25 and Rab11a with the apical recycling
605   system of polarized Madin-Darby canine kidney cells. Mol. Biol. Cell 10, 47–61.
- 606   Chaiyadet, S., Sotillo, J., Smout, M., Cantacessi, C., Jones, M.K., Johnson, M.S., Turnbull, L.,
607   Whitchurch, C.B., Potriquet, J., Laohaviroj, M., Mulvenna, J., Brindley, P.J., Bethony, J.M.,
608   Laha, T., Sripa, B., Loukas, A., 2015. Carcinogenic liver fluke secretes extracellular vesicles
609   that promote cholangiocytes to adopt a tumorigenic phenotype. J. Infect. Dis. 212, 1636–45.
- 610   Christ, L., Raiborg, C., Wenzel, E.M., Campsteijn, C., Stenmark, H., 2017. Cellular functions and
611   molecular mechanisms of the ESCRT membrane-scission machinery. Trends Biochem. Sci.
612   42, 42–56.
- 613   Cwiklinski, K., de la Torre-Escudero, E., Trelis, M., Bernal, D., Dufresne, P.J., Brennan, G.P.,
614   O'Neill, S., Tort, J., Paterson, S., Marcilla, A., Dalton, J.P., Robinson, M.W., 2015. The
615   extracellular vesicles of the helminth pathogen, *Fasciola hepatica*: biogenesis pathways and
616   cargo molecules involved in parasite pathogenesis. Mol. Cell. Proteomics 14, 3258–3273.
- 617   Dalton, J.P., Robinson, M.W., Mulcahy, G., O'Neill, S.M., Donnelly, S., 2013. Immunomodulatory

618 molecules of *Fasciola hepatica*: candidates for both vaccine and immunotherapeutic
619 development. Vet. Parasitol. 195, 272–85.

620 de la Torre-Escudero, E., Bennett, A.P.S., Clarke, A., Brennan, G.P., Robinson, M.W., 2016.
621 Extracellular vesicle biogenesis in helminths: more than one route to the surface? Trends
622 Parasitol. 32, 921–929.

623 de la Torre-Escudero, E., Gerlach, J.Q., Bennett, A.P.S., Cwiklinski, K., Jewhurst, H.L., Huson,
624 K.M., Joshi, L., Kilcoyne, M., O'Neill, S., Dalton, J.P., Robinson, M.W., 2019. Surface
625 molecules of extracellular vesicles secreted by the helminth pathogen *Fasciola hepatica* direct
626 their internalisation by host cells. PLoS Negl. Trop. Dis. 13, e0007087.

627 Di Maggio, L.S., Tirloni, L., Pinto, A.F.M., Diedrich, J.K., Yates Iii, J.R., Benavides, U., Carmona,
628 C., da Silva Vaz, I., Berasain, P., 2016. Across intra-mammalian stages of the liver fluke
629 *Fasciola hepatica*: a proteomic study. Sci. Rep. 6, 32796.

630 El Andaloussi, S., Mäger, I., Breakefield, X.O., Wood, M.J.A., 2013. Extracellular vesicles: biology
631 and emerging therapeutic opportunities. Nat. Rev. Drug Discov. 12, 347–57.

632 Elleder, M., 1989. Niemann-Pick disease. Pathol. Res. Pract. 185, 293–328.

633 Essandoh, K., Yang, L., Wang, X., Huang, W., Qin, D., Hao, J., Wang, Y., Zingarelli, B., Peng, T.,
634 Fan, G.C., 2015. Blockade of exosome generation with GW4869 dampens the sepsis-induced
635 inflammation and cardiac dysfunction. Biochim. Biophys. Acta - Mol. Basis Dis. 1852, 2362–
636 2371.

637 Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution
638 (N. Y). 39, 783–791.

639 Flaherty, S.E., Grijalva, A., Xu, X., Ables, E., Nomani, A., Ferrante, A.W., 2019. A lipase-
640 independent pathway of lipid release and immune modulation by adipocytes. Science 363,
641 989–993.

642 Gorelik, A., Heinz, L.X., Illes, K., Superti-Furga, G., Nagar, B., 2016a. Crystal structure of the acid
643 sphingomyelinase-like phosphodiesterase SMPDL3B provides insights into determinants of
644 substrate specificity. *J. Biol. Chem.* 291, 24054–24064.

645 Gorelik, A., Illes, K., Heinz, L.X., Superti-Furga, G., Nagar, B., 2016b. Crystal structure of
646 mammalian acid sphingomyelinase. *Nat. Commun.* 7, 1–9.

647 Gradilla, A.C., González, E., Seijo, I., Andrés, G., Bischoff, M., González-Mendez, L., Sánchez, V.,
648 Callejo, A., Ibáñez, C., Guerra, M., Ortigão-Farias, J.R., Sutherland, J.D., González, M.,
649 Barrio, R., Falcón-Pérez, J.M., Guerrero, I., 2014. Exosomes as Hedgehog carriers in
650 cytoneme-mediated transport and secretion. *Nat. Commun.* 5, 5649.

651 Hanna, R.E.B., 1980. *Fasciola hepatica*: autoradiography of protein synthesis, transport, and
652 secretion by the tegument. *Exp. Parasitol.* 50, 297–304.

653 Hansen, E.P., Fromm, B., Andersen, S.D., Marcilla, A., Andersen, K.L., Borup, A., Williams, A.R.,
654 Jex, A.R., Gasser, R.B., Young, N.D., Hall, R.S., Stensballe, A., Ovchinnikov, V., Yan, Y.,
655 Fredholm, M., Thamsborg, S.M., Nejsum, P., 2019. Exploration of extracellular vesicles from
656 *Ascaris suum* provides evidence of parasite-host cross talk. *J. Extracell. vesicles* 8, 1578116.

657 Harischandra, H., Yuan, W., Loghry, H.J., Zamanian, M., Kimber, M.J., 2018. Profiling
658 extracellular vesicle release by the filarial nematode *Brugia malayi* reveals sex-specific
659 differences in cargo and a sensitivity to ivermectin. *PLoS Negl. Trop. Dis.* 12, e0006438.

660 Heinz, L.X., Baumann, C.L., Köberlin, M.S., Snijder, B., Gawish, R., Shui, G., Sharif, O., Aspalter,
661 I.M., Müller, A.C., Kandasamy, R.K., Breitwieser, F.P., Pichlmair, A., Bruckner, M.,
662 Rebsamen, M., Blüml, S., Karonitsch, T., Fauster, A., Colinge, J., Bennett, K.L., Knapp, S.,
663 Wenk, M.R., Superti-Furga, G., 2015. The lipid-modifying enzyme SMPDL3B negatively
664 regulates innate immunity. *Cell Rep.* 11, 1919–28.

665 Hyenne, V., Labouesse, M., Goetz, J.G., 2016. The Small GTPase Ral orchestrates MVB

666 biogenesis and exosome secretion. *Small GTPases* 9, 445–451.

667 Kifle, D.W., Sotillo, J., Pearson, M.S., Loukas, A., 2017. Extracellular vesicles as a target for the
668 development of anti-helminth vaccines. *Emerg. Top. Life Sci.* 1, 659–665.

669 Kowal, J., Tkach, M., Théry, C., 2014. Biogenesis and secretion of exosomes. *Curr. Opin. Cell*
670 *Biol.* 29, 116–125.

671 Kusel, J.R., McVeigh, P., Thornhill, J.A., 2009. The schistosome excretory system: a key to
672 regulation of metabolism, drug excretion and host interaction. *Trends Parasitol.* 25, 353–358.

673 Li, J., Liu, K., Liu, Y., Xu, Y., Zhang, F., Yang, H., Liu, J., Pan, T., Chen, J., Wu, M., Zhou, X.,
674 Yuan, Z., 2013. Exosomes mediate the cell-to-cell transmission of IFN- α -induced antiviral
675 activity. *Nat. Immunol.* 14, 793–803.

676 Marcilla, A., Trelis, M., Cortés, A., Sotillo, J., Cantalapiedra, F., Minguez, M.T., Valero, M.L.,
677 Sánchez del Pino, M.M., Muñoz-Antoli, C., Toledo, R., Bernal, D., 2012. Extracellular
678 vesicles from parasitic helminths contain specific excretory/secretory proteins and are
679 internalized in intestinal host cells. *PLoS One* 7, e45974.

680 Matsumoto, J., Stewart, T., Sheng, L., Li, N., Bullock, K., Song, N., Shi, M., Banks, W.A., Zhang,
681 J., 2017. Transmission of α -synuclein-containing erythrocyte-derived extracellular vesicles
682 across the blood-brain barrier via adsorptive mediated transcytosis: another mechanism for
683 initiation and progression of Parkinson's disease? *Acta Neuropathol. Commun.* 5, 71.

684 Menck, K., Sönmezer, C., Worst, T.S., Schulz, M., Dihazi, G.H., Streit, F., Erdmann, G., Kling, S.,
685 Boutros, M., Binder, C., Gross, J.C., 2017. Neutral sphingomyelinases control extracellular
686 vesicles budding from the plasma membrane. *J. Extracell. vesicles* 6, 1378056.

687 Morpew, R.M., Wright, H.A., LaCourse, E.J., Woods, D.J., Brophy, P.M., 2007. Comparative
688 proteomics of excretory-secretory proteins released by the liver fluke *Fasciola hepatica* in
689 sheep host bile and during *in vitro* culture ex host. *Mol. Cell. Proteomics* 6, 963–72.

690 Moss, G.D., 1970. The excretory metabolism of the endoparasitic digenean *Fasciola hepatica* and
691 its relationship to its respiratory metabolism. *Parasitology* 60, 1–19.

692 Pankao, V., Sirisriro, A., Grams, R., Vichasri-Grams, S., Meepool, A., Kangwanrangsan, N.,
693 Wanichanon, C., Ardseungneon, P., Viyanant, V., Upatham, E.S., Sobhon, P., 2006.
694 Classification of the parenchymal cells in *Fasciola gigantica* based on ultrastructure and their
695 expression of fatty acid binding proteins (FABPs). *Vet. Parasitol.* 142, 281–292.

696 Robinson, M.W., Menon, R., Donnelly, S.M., Dalton, J.P., Ranganathan, S., 2009. An integrated
697 transcriptomics and proteomics analysis of the secretome of the helminth pathogen *Fasciola*
698 *hepatica*: proteins associated with invasion and infection of the mammalian host. *Mol. Cell.*
699 *Proteomics* 8, 1891–907.

700 Robinson, M.W., Trudgett, A., Hoey, E.M., Fairweather, I., 2002. Triclabendazole-resistant
701 *Fasciola hepatica*: β -tubulin and response to *in vitro* treatment with triclabendazole.
702 *Parasitology* 124, 325–38.

703 Rohrbacher, G.H., 1957. Observations of the survival *in vitro* of bacteria-free adult common liver
704 flukes, *Fasciola hepatica* Linn., 1758. *J. Parasitol.* 43, 9–18.

705 Roig, J., Saiz, M.L., Galiano, A., Trelis, M., Cantalapiedra, F., Monteagudo, C., Giner, E., Giner,
706 R.M., Recio, M.C., Bernal, D., Sánchez-Madrid, F., Marcilla, A., 2018. Extracellular vesicles
707 from the helminth *Fasciola hepatica* prevent DSS-induced acute ulcerative colitis in a T-
708 lymphocyte independent mode. *Front. Microbiol.* 9, 1036.

709 Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing
710 phylogenetic trees. *Mol. Biol. Evol.* 4, 406–25.

711 Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular
712 Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–9.

713 Threadgold, L.T., 1963. The tegument and associated structures of *Fasciola hepatica*. *Quart. J.*

714 micr. Sci 104, 505–512.

715 Tomiuk, S., Zumbansen, M., Stoffel, W., 2000. Characterization and subcellular localization of
 716 murine and human magnesium-dependent neutral sphingomyelinase. J. Biol. Chem. 275,
 717 5710–5717.

718 Traini, M., Kumaran, R., Thaysen-Andersen, M., Kockx, M., Jessup, W., Kritharides, L., 2017. *N*-
 719 glycosylation of human sphingomyelin phosphodiesterase acid-like 3A (SMPDL3A) is
 720 essential for stability, secretion and activity. Biochem. J. 474, 1071–1092.

721 Traini, M., Quinn, C.M., Sandoval, C., Johansson, E., Schroder, K., Kockx, M., Meikle, P.J.,
 722 Jessup, W., Kritharides, L., 2014. Sphingomyelin phosphodiesterase acid-like 3A (SMPDL3A)
 723 is a novel nucleotide phosphodiesterase regulated by cholesterol in human macrophages. J.
 724 Biol. Chem. 289, 32895–32913.

725 Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., Schwille, P., Brügger,
 726 B., Simons, M., 2008. Ceramide triggers budding of exosome vesicles into multivesicular
 727 endosomes. Science 319, 1244–1247.

728 Truman, J.P., Al Gadban, M.M., Smith, K.J., Jenkins, R.W., Mayroo, N., Virella, G., Lopes-Virella,
 729 M.F., Bielawska, A., Hannun, Y.A., Hammad, S.M., 2012. Differential regulation of acid
 730 sphingomyelinase in macrophages stimulated with oxidized low-density lipoprotein (LDL) and
 731 oxidized LDL immune complexes: role in phagocytosis and cytokine release. Immunology
 732 136, 30–45.

733 van Niel, G., D’Angelo, G., Raposo, G., 2018. Shedding light on the cell biology of extracellular
 734 vesicles. Nat. Rev. Mol. Cell Biol. 19, 213–228.

735 Verderio, C., Gabrielli, M., Giussani, P., 2018. Role of sphingolipids in the biogenesis and
 736 biological activity of extracellular vesicles. J. Lipid Res. 59, 1325–1340.

737 Wang, X., Kumar, R., Navarre, J., Casanova, J.E., Goldenring, J.R., 2000. Regulation of vesicle

738 trafficking in Madin-Darby canine kidney cells by Rab11a and Rab25. J. Biol. Chem. 275,
739 29138–46.

740 Willms, E., Johansson, H.J., Mäger, I., Lee, Y., Blomberg, K.E.M., Sadik, M., Alaarg, A., Smith,
741 C.I.E., Lehtiö, J., EL Andaloussi, S., Wood, M.J.A., Vader, P., 2016. Cells release
742 subpopulations of exosomes with distinct molecular and biological properties. Sci. Rep. 6,
743 22519.

744 Wilson, R.A., Webster, L.A., 1974. Protonephridia. Biol. Rev. 49, 127–160.

745 Wilson, R.A., Wright, J.M., de Castro-Borges, W., Parker-Manuel, S.J., Dowle, A.A., Ashton, P.D.,
746 Young, N.D., Gasser, R.B., Spithill, T.W., 2011. Exploring the *Fasciola hepatica* tegument
747 proteome. Int. J. Parasitol. 41, 1347–1359.

748 Wu, Zhenyu, Wang, Lingling, Li, J., Wang, Lifu, Wu, Zhongdao, Sun, X., 2018. Extracellular
749 vesicle-mediated communication within host-parasite interactions. Front. Immunol. 9, 3066.

750 Zakeri, A., Hansen, E.P., Andersen, S.D., Williams, A.R., Nejsun, P., 2018. Immunomodulation by
751 helminths: intracellular pathways and extracellular vesicles. Front. Immunol. 9.

752 Zhou, Y., Lin, X.-W., Begum, M.-A., Zhang, C.-H., Shi, X.-X., Jiao, W.-J., Zhang, Y.-R., Yuan, J.-
753 Q., Li, H.-Y., Yang, Q., Mao, C., Zhu, Z.-R., 2017. Identification and characterization of
754 *Laodelphax striatellus* (Insecta: Hemiptera: Delphacidae) neutral sphingomyelinase. Insect
755 Mol. Biol. 26, 392–402.

756 Zuckerandl, E., Pauling, L., 1965. Evolutionary divergence and convergence in proteins, in: Bryson,
757 V., Vogel, H.J. (Eds.), *Evolving Genes and Proteins*. Academic Press, New York, pp. 97–166.

758

759 **Legends to Figures**

760 Fig. 1. Proteins enriched in *F. hepatica* EVs localise to the gastrodermal cells in adult *F. hepatica*
761 tissue sections. (A) Toluidine blue staining showing the architecture of the gastrodermal tract. (B)
762 Pre-immune serum showing no specific immunofluorescence in the gut. (C-H) Immunostaining
763 (green, or red in H) patterns in the gut show puncta of fluorescence concentrated at the apices of the
764 gastrodermal cells (arrowheads in C, FhALIX; F, FhDM9; G, FhTIP; and H, tyrosinated α -tubulin);
765 diffuse FhSMPD2-positive immunofluorescence (D); low intensity staining of the lamellae (D,
766 FhSMPD2; E, FhSMPDL3¹⁵⁹); and puncta of fluorescence in the sub-gastrodermal region (E,
767 FhSMPDL3¹⁵⁹; F, FhDM9; G, FhTIP). FhDM9 is also present in large vesicular structures in the
768 gut lumen (arrow, F). Panels B, D, and E display DAPI nuclear staining (blue). G, gut; L, lumen.
769 Scale bars: 25 μ m (A), 7.5 μ m (B-H).

770

771 Fig. 2. Immunostaining (green) of proteins enriched in *F. hepatica* EVs in the tegument and sub-
772 tegumental regions of adult *F. hepatica* tissue sections. (A) Toluidine blue staining showing the
773 architecture of the tegumental region. (B) Pre-immune serum showing no specific
774 immunofluorescence. (C) The anti-FhSMPDL3¹⁵⁹ antibody labels the cytoplasmic connections
775 (arrows) and basal part of the tegumental syncytium. (D-E) Immunoreactivity against FhDM9 (D)
776 and FhTeg1 (E) is present in the tegumental apical plasma membrane, syncytium, and sub-
777 tegumental areas (arrowheads). APM, apical plasma membrane; PN, protonephridial duct; S, spine;
778 T, tegumental syncytium. Panels B, C, and E display DAPI nuclear staining (blue). Scale bars: 25
779 μ m (A), 7.5 μ m (B-E).

780

781 Fig. 3. Immunolocalisation of proteins enriched in *F. hepatica* EVs (green) to the protonephridial
782 ducts (PN) and associated parenchymal cells in adult *F. hepatica* tissue sections. (A) Toluidine blue
783 staining showing a protonephridial duct embedded in the parenchymal tissue. (B) Pre-immune
784 serum showing no specific immunofluorescence. (C-G) The lining of the protonephridial ducts

785 display immunofluorescence positive for FhTSG101 (arrows, C), FhALIX (D), FhRal-A (E) and
786 FhSMPD2 (F), whilst FhDM9 immunoreactivity is localised to the perinuclear region (arrow, G).
787 Clusters of vesicular structures (arrowheads) are also visible in parenchymal cells, including those
788 adjacent to the protonephridial ducts. Panels B, C, E, and F display DAPI nuclear staining (blue).
789 Scale bars: 7.5 μm (A-B, D-G), 25 μm (C).

790

791 Fig. 4. Parenchymal cells containing vesicular structures immunopositive for proteins enriched in *F.*
792 *hepatica* EVs (green, top row) and the same cells stained with toluidine blue (bottom row). Panel
793 (B) has DAPI nuclear staining (blue). Arrowheads indicate immunopositive clusters of vesicular
794 structures within parenchymal cells. (A) FhTSG101. (B) FhSMPD2. (C) FhDM9. (D-F) The sub-
795 gastrodermal parenchymal cells labelled by these antibodies have a slightly basophilic cytoplasm
796 and contain many vesicular structures which are highly basophilic. This cell type resembles the PC2
797 parenchymal cells characterised by Pankao et al. (2006). G, gut; L, gut lumen; PN, protonephridia.
798 Scale bars, 7.5 μm .

799

800 Fig. 5. Representative images of ligated adult *F. hepatica* after 3 h *in vitro* culture (left-hand
801 panels), and the resulting 15K EV pellet recovered from the culture media (right-hand panels). The
802 dashed circle highlights the location of the pellet. (A) Non-ligated control. (B) Oral sucker ligated.
803 (C) Excretory pore ligated. (D) Oral sucker and excretory pore ligated.

804

805 Fig. 6. The effect of oral sucker ligation (OSL; A-B), excretory pore ligation (EPL; C-D), and oral
806 sucker and excretory pore ligation (double ligation, DL; E-F) on the release of *F. hepatica* 15K (A,
807 C, F) and 120K (B, D, F) EVs as determined by Western blot against selected EV markers.
808 Experiments were carried out in biological triplicate. Representative blots are shown in the left-
809 hand panel and mean densitometric values expressed as a percentage of the controls ($\pm$ standard

810 error) in the right-hand panel. *, $p < 0.05$; **, $p < 0.01$, *** $p < 0.001$, ****, $p < 0.0001$; ***** p
811 < 0.000001 (Student's t-test). Ctrl, control.

812

813 Fig. 7. (A) The effect of GW4869 on sphingomyelinase activity associated with 120K EVs. Bars
814 represent percentage sphingomyelinase activity of EVs treated with either the vehicle control or
815 increasing concentrations of GW4869. (B-D) The effect of GW4869 treatment on 120K EV release
816 as determined by protein (B) and particle (C) concentration (expressed as a percentage of the
817 controls) and Western blots against selected EV markers (D). Representative blots are shown in the
818 left-hand panel and the right-hand panel displays mean densitometric values expressed as a
819 percentage of the controls. Experiments were carried out in biological triplicate and mean values $\pm$
820 standard error are shown. Ctrl, control. *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$; ****, $p < 0.0001$
821 (Student's t-test).

822

823 Fig. 8. Transmission electron micrographs showing the effect of GW4869 treatment on the
824 ultrastructure of adult *F. hepatica*. (A) Control tegumental syncytium. (B) GW4869-treated
825 tegumental syncytium displaying extensive vacuolation (asterisks). (C) Control gastrodermal cell.
826 (D) GW4869-treated gastrodermal cell showing an accumulation of autophagosomes (arrows) and
827 some vacuolation (asterisk). (E) Control parenchymal tissue. (F) The parenchymal tissue of
828 GW4869-treated *F. hepatica* displays large, electron lucent vacuoles (asterisks). (G) Control
829 protonephridial duct with lipid droplets (arrows). (H) Areas of vacuolation (arrowheads) are more
830 evident in the protonephridial duct of GW4869-treated *F. hepatica*, whilst lipid droplets (arrows) are
831 still present. PN, protonephridial duct; S, spine; V, vitelline follicle. Scale bars: 2 μm (A, C), 5 μm
832 (B, E, H), 1 μm (D), 10 μm (F, G).

833

834 **Legends to Supplementary Figures**

835 Supplementary Fig. S1. The effect of oral sucker ligation (OSL; A-B), excretory pore ligation (EPL;
836 C-D), and oral sucker and excretory pore ligation (double ligation, DL; E-F) on 15K and 120K EV
837 protein (A, C, E) and particle (B, D, F) concentration (expressed as a percentage of the controls).
838 Experiments were carried out in biological triplicate and mean values $\pm$ standard error are shown. *,
839 $p < 0.05$; **, $p < 0.01$ (Student's t-test).

840

841 Supplementary Fig. S2. (A) A bootstrapped (1000 trials) neighbour-joining phylogenetic tree of
842 human neutral (HsSMPD2 and HsSMPD3) and acid-like sphingomyelinase (SMase) family
843 members (HsSMPD1, HsSMPDL3a and HsSMPDL3b) with *F. hepatica* orthologues (FhSMPD2,
844 FhSMPDL3¹⁵⁹ and FhSMPDL3¹³⁸). Numbers next to nodes represent bootstrap values (given as
845 percentages). Values over 65% are displayed. (B) Cartoon representation of domains in human
846 neutral and acid-like sphingomyelinases in comparison to *F. hepatica* orthologues. Catalytic
847 domains (EEP, endonuclease/exonuclease/phosphatase; MPP, metallo-phosphodiesterase) are in
848 black, signal peptides (SP) in dark grey, and transmembrane regions (TM) in blue. Protein domain
849 architectures were generated using InterPro (www.ebi.ac.uk/interpro; Mitchell et al., 2015).

850

851 Supplementary Fig. S3. (A) The effect of GW4869 on sphingomyelinase activity associated with
852 15K EVs. Bars represent percentage sphingomyelinase activity of EVs treated with either the
853 vehicle control or increasing concentrations of GW4869. (B-D) The effect of GW4869 treatment on
854 15K EV release as determined by protein (B) and particle (C) concentration (expressed as a
855 percentage of the controls) and Western blots against selected EV markers (D). Representative blots
856 are shown in the left-hand panel and the right-hand panel displays mean densitometric values
857 expressed as a percentage of the controls. Experiments were carried out in biological triplicate and
858 mean values $\pm$ standard error are shown. Ctrl, control. *, $p < 0.05$; **, $p < 0.01$ (Student's t-test).

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862   Supplementary Table 1: List of primary antibodies used for immunolocalisation and immunoblot analysis.

Target protein	Target identifier	Antibody	Target peptide	Host	Working concentration/dilution		Source
					Immunolocalisation	Immunoblotting	
<i>Polyclonal peptide antibodies</i>							
FhTSG101	BN1106_s410B000432	TSG101_18	KSYKYARDVTNDVKC	Rabbit	10 µg/ml	1 µg/ml	GenScript
FhALIX	BN1106_s1871B000313	ALIX_2	CTSSKKRPAPDRPPP	Rabbit	10 µg/ml	-	GenScript
FhRal-A	BN1106_s637B000246	RAL_28	CNKIDLTQERTVPFE	Rabbit	10 µg/ml	1 µg/ml	GenScript
FhSMPD2	BN1106_s7135B000046	NSM_65	CMDESPSDPDTKASG	Rabbit	10 µg/ml	-	GenScript
FhSMPDL3 ¹⁵⁹	BN1106_s1285B000159	ASM_1_41	IEKGNDEGYAENKPC	Rabbit	5 µg/ml	1 µg/ml	GenScript
FhCD63 receptor	BN1106_s1657B000161	Cd63_9	CGAKFTHKDSAAGRT	Rabbit	-	0.125 µg/ml	GenScript
FhDM9-containing protein	BN1106_s5689B000026	DM9_26	CPSGRGDVPHNAVQG	Rabbit	7.5 µg/ml	-	GenScript
FhTiP	BN1106_s92B000559	TiP_5	CYGASDSKRGTFLLKP	Rabbit	7.5 µg/ml	-	GenScript
FhTeg1	Q9Y0H2	Teg1_44	CPKGDGDPKPEPKGD	Rabbit	10 µg/ml	-	GenScript
<i>Commercial monoclonal</i>							
Tyrosinated α-tubulin	-	TUB-1A2	-	Mouse	1:100	1:2500	Sigma-Aldrich
<i>Anti-serum</i>							
FhCL1	U62288	FhCL1	Recombinant <i>F. hepatica</i> cathepsin L1	Sheep	-	1:15,000	de la Torre-Escudero et al. (2019)

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880 Figure 4

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