

Extracellular Vesicle Biogenesis in Helminths: More than One Route to the Surface?

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1	Extracellular vesicle biogenesis in helminths: more than one route to the surface?
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13 Abstract

14 The quite recent discovery that parasites release extracellular vesicles (EVs) that can transfer a range of effector molecules to host cells has made us re-think our understanding of the host-15 parasite interface. In this opinion article we will consider how recent proteomics and 16 transcriptomics studies, together with ultrastructural observations, suggest that more than one 17 18 mechanism of EV biogenesis can occur in helminths. We propose that distinct EV sub-types 19 have roles in immune-modulation and repair of drug-induced damage, and put forward the case for targeting EV biogenesis pathways to achieve parasite control. In doing so we raise a 20 21 number of outstanding research questions that must be addressed before this can happen.

23 Molecular communication at the host-parasite interface

The persistence of helminth parasites in their mammalian hosts has been ascribed to their 24 striking ability to manipulate host immune responses. Many helminths are obligate blood 25 feeders and can deliver secreted molecules into the bloodstream where they exert an 26 immunosuppressive activity on the host immune system [1]. Other molecules are secreted 27 from the gut, excretory pores and surface cuticle/tegument into the local microenvironment, 28 often the host intestine [2-4]. For many years the paucity of material made these secretions 29 extremely difficult to analyse but the application of mass spectrometry-based proteomics 30 31 techniques has allowed a detailed understanding of the type and abundance of the various 32 proteins present in soluble helminth secretions (reviewed in [5]). However, until quite recently the contribution of extracellular vesicles (EVs; see glossary) to helminth secretomes 33 34 has been overlooked.

EVs are small membrane-bound structures that are shed by most cell types. Although 35 once considered to act solely as a cellular waste disposal system [6], EVs are now recognised 36 as important mediators of cell-cell communication by transferring a range of effector 37 molecules including proteins, lipids, mRNA, microRNA and other non-coding RNA species. 38 39 Variously described as exosomes or microvesicles (MVs) depending on their composition, size and mode of biogenesis (see below), EVs not only perform a variety of roles in the 40 41 maintenance of normal physiology but also participate in pathological settings, notably in 42 tumour progression [7-9]. A growing number of studies have also shown that parasite-derived EVs play an important role during infection and pathogenesis [10]. Although most of these 43 44 studies have focused on profiling the molecular cargo packaged into parasite EVs, they have 45 provided a valuable first insight into the putative mechanism(s) used by parasites for EV biogenesis and release. Here, we will briefly summarise the current understanding of EV 46

biogenesis in mammalian cells and examine the ultrastructural and biochemical evidence for
EV biogenesis and export mechanisms used by helminth parasites.

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50 EV biogenesis pathways in mammalian cells

There are two major subtypes of EVs that are actively released by viable cells: exosomes and 51 52 microvesicles (MVs). Exosomes are typically 30-100 nm in diameter and originate from the endosomal pathway of eukaryotic cells. The process starts with inward budding of the late 53 endosome membrane which forms multivesicular bodies (MVBs) containing a number of 54 intraluminal vesicles (ILVs). The ILVs are then secreted from the cell surface (as exosomes) 55 following fusion of the MVB with the plasma membrane. The principal machinery that drives 56 57 this process is the endosomal sorting complex required for transport (ESCRT). ESCRT-0, -I, and -II complexes cluster ubiquitinated proteins to the endosomal membrane, whilst 58 ESCRT-III forms polymeric filaments that result in the invagination of the membrane, 59 60 forming the ILV [11-14]. The final abscission of the budding ILVs into the MVB lumen requires the AAA-ATPase VPS4 [15]. However, ESCRT-independent pathways have been 61 reported that centre around several members of the tetraspanin family [16] or that require the 62 63 generation of ceramide by neutral sphingomyelinase [17]. In this respect, Colombo et al. [18] proposed four molecular machineries involved in ILV formation: (1) the ESCRT pathway; 64 (2) the lipid pathway primarily involving the lipid hydrolases neutral sphingomyelinase and 65 phospholipase D2; (3) the tetraspanins route where CD63 plays a principal role; and (4) a 66 hybrid mechanism combining elements of all the former machineries together. The 67 subsequent fusion of MVBs with the plasma membrane is dependent on small GTPases 68 including Ral-1, Rab8b, Rab11, Rab27a and Rab27b and the Rab effectors otoferlin and 69 70 synaptotagmin-like protein [19-22].

71 Microvesicles (MVs) are larger than exosomes (diameter 100-1000 nm) and originate by direct budding from the plasma membrane in response to a variety of external stimuli 72 which generally lead to elevated intracellular Ca²⁺ levels and cytoskeleton-driven membrane 73 remodelling [23,24]. This Ca^{2+} signalling cascade activates scramblase that is responsible for 74 the translocation of phosphatidylserine to the outer leaflet of the plasma membrane. The 75 76 resulting membrane asymmetry is thought to drive localised curvature of the plasma membrane. MV budding is driven further by the rearrangement of the actin cytoskeleton 77 conducted by calpain [25]. Many studies suggest that small GTPases, such as ARF6, play a 78 79 key role in MV formation by indirectly activating proteases in response to the activation of the extracellular signal-regulated kinase (ERK) [24]. Although the final abscission 80 81 mechanism of the MV is not fully understood, the late ESCRT component VPS4 is likely to 82 be involved in the same way that it drives abscission of the budding ILVs into the MVB 83 lumen during exosome biogenesis [26]. Whilst both exosomes and MVs can deliver cargo to target cells, and are released by viable cells in an energy-dependent manner, most cells 84 85 constitutively produce exosomes whilst MVs are generally released in response to specific signals [27]. 86

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88 EVs as potential targets for anti-parasite therapy

Several studies have described the modulatory effects of parasite-derived EVs on the host immune system [28-30] or the pathological effects on host cells [31]. Indeed, the role of helminth EVs as vehicles for transfer of small RNAs (especially miRNA) to host cells has been extensively documented in the last few years [28,32-35] suggesting a new mechanism used by parasites to influence host cell function at a molecular level. Targeting key regulators of EV biogenesis (including sphingomyelinase, ESCRT components and Rab GTPases) using chemical inhibitors or RNAi has been shown to significantly reduce the number of EVs

96 released by mammalian cells (Table 1). Indeed, suppressing EV production to inhibit their 97 effect in disease is an emerging therapeutic strategy that has yielded impressive results, notably in cancer therapy (reviewed in [8]). In the same vein, parasite EV biogenesis 98 99 pathways might be an attractive therapeutic target if selective inhibitors could be identified. Disrupting the packaging, biogenesis and/or release of parasite EVs would prevent the 100 101 delivery of a plethora of protein or small RNA immunomodulators to host cells; thus, 102 allowing the host to mount an effective immune response against the parasite. Silencing of a range of orthologues of molecules that function at different stages of EV biogenesis pathways 103 104 in mammalians cells led to varied aberrant phenotypes in *Caenorhabditis elegans* (Table 1). Whilst the direct impact on EV production was not assessed for all of these targets, MVBs 105 106 accumulated below the cuticle layer (and did not fuse with the plasma membrane) when the 107 vATPase V0 subunit and GTPase Ral-1 were mutated or silenced [22,45]. The challenge now 108 is to determine how silencing EV biogenesis components in parasitic helminths impacts their ability to establish infection and avoid the host immune response. For instance, silencing 109 syntenin (involved in ILV formation and cargo sorting) had no effect on the ability of 110 schistosomulae and adult schistosomes to survive in vitro culture however their ability to 111 persist in mammalian hosts was not investigated [46]. 112

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114 Exosome biogenesis in helminths – insights from Fasciola hepatica

To date, much of our understanding of EV biogenesis in helminths comes from studies on the liver fluke, *Fasciola hepatica*. A detailed proteomics analysis of its secretome showed that *F*. *hepatica* releases at least two major sub-populations of EVs that differ according to size, cargo molecules and probable site of origin [47]. The sub-population of smaller EVs (30-100 nm diameter) contain a number of well-known exosomal markers, also identified in other helminth parasites, such as Hsp70, ALIX, tetraspanin CD63 and several Rab GTPases suggesting that *Fasciola* secretes *bona fide* exosomes. In support of this, mass spectrometry data strongly suggests an endosomal origin of the smaller secreted vesicles; up to ten proteins from the ESCRT pathway were found associated with the vesicles. These included members of the ESCRT-III complex and the Vps4 ATPase responsible for ILV budding and abscission respectively as well as the ESCRT-associated proteins ALIX and syntenin [47].

Compared with the plasma membrane, exosomal membranes are enriched in certain 126 phospholipids as well as cholesterol and ceramide. They also contain numerous lipid-binding 127 proteins such as annexins that are organized in specialised lipid rafts called tetraspanin-128 129 enriched microdomains (TEMs). Lipid hydrolases (typically sphingomyelinases and phospholipase B) and cholesterol transporters are also characteristic features of exosome 130 membranes [9]. TEMs are believed to have roles in both exosome biogenesis and the sorting 131 132 of cargo by promoting specific compartmentalisation of proteins and receptor molecules into the budding ILVs [16]. According to the mass spectrometry data, the F. hepatica exosomes 133 contain all the necessary proteins to organise the membrane into TEMs (including several 134 members of the tetraspanin and annexin families, an acid sphingomyelinase, a phospholipase 135 B2-like enzyme as well as the cholesterol transporters Niemann Pick C1 and C2 proteins; 136 [47]). Due to their biophysical properties, TEMs have also been defined as detergent-resistant 137 microdomains [48]. Such organization would confer a high rigidity to F. hepatica EV 138 membranes (and presumably to those from other helminths) at neutral pH that would help to 139 140 preserve their structural integrity, and persistence, in host bile and other biological fluids. Indeed, exosome-like vesicles from the nematode parasite Brugia malayi have been detected 141 in host circulatory blood, suggesting that they may act at effector sites well beyond that of the 142 143 local host microenvironment [30]. Recently it was shown that exosome membranes from the nematode parasite Heligmosomoides polygyrus are enriched with plasmalogens and other 144 specialised ether phospholipids that confer greater vesicle rigidity compared with host-145

derived exosomes [49]. Thus, is it likely that the unique lipid and protein composition of
parasite EV membranes has evolved to confer the greatest protection possible to the cargo
packaged within the vesicle lumen.

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150 Ultrastructural evidence for EV biogenesis in helminths

Whilst much of the biochemical and proteomics data to date supports an endosomal origin for helminth exosomes, further ultrastructural studies are required to confirm this. Exosome-like vesicles have been described associated with the lining of the gut in *Schistosoma mansoni* and in *H. polygyrus* [28,50]. Secretion of exosomes from the cuticle surface in parasitic nematodes has yet to be documented although exosomes released by *C. elegans* epithelial cells are more likely to contribute to formation of the overlaying cuticle rather than be secreted into the extracellular environment from the worm surface [22].

Transmission electron microscopy has revealed the presence of structures within the 158 tegumental syncytium of parasitic trematodes that resemble the MVBs observed in 159 mammalian cells (Figure 1) [4,32,51]. These structures have been shown to fuse with the 160 apical plasma membrane at the tegumental surface from where vesicles, compatible in size 161 with exosomes, are discharged [51]. These observations, and the fact that trematode 162 exosomes contain a considerable number of tegumental-associated proteins [47], suggest a 163 role for the tegument in exosome secretion. It is noteworthy, given the multitude of 164 165 ultrastructural studies performed on F. hepatica and other trematodes, that these putative MVBs have not been described more widely in the literature. Wilson et al. [4] reported the 166 appearance of MVB-like structures within the F. hepatica tegumental syncytium in response 167 168 to culturing the flukes in vitro which could suggest that some helminth EVs are released in response to stress. Indeed, the transient nature of MVB formation and exosome release makes 169

the phenomenon difficult to observe (even in mammalian cells) [18], but it could alsoindicate that alternative mechanisms of EV release are in operation in the parasite.

172

173 Are there novel EV biogenesis pathways in helminths?

Several studies have suggested the existence of atypical EV biogenesis pathways that 174 combine elements of the ESCRT, lipid and tetraspanin pathways in mammalian cells [18]. 175 Whilst the proteomics data largely supports an ESCRT-dependent origin for the exosome-like 176 EVs released by F. hepatica, we also identified some components of ESCRT-independent 177 pathways, such as sphingomyelinase and various members of the tetraspanin family, in its 178 secretome [47]. Whilst this may be due to the presence of several sub-populations of EVs 179 (either produced by distinct mechanisms or released from different parasite tissues), it could 180 also indicate the coordinated participation of proteins from ESCRT-dependent and -181 182 independent pathways in the production of exosome-like vesicles in F. hepatica, as has been described in other cell types [52]. In contrast, the larger Fasciola EVs were shown to be 183 184 specifically enriched with the inactive 37 kDa cathepsin L zymogen and may originate from the specialised secretory cells that line the parasite gastrodermus [47]. Our preliminary 185 biochemical characterisation of these EVs suggest they are distinct from the smaller 186 exosome-like vesicles with respect to their cargo molecules. Data pertaining to their 187 mechanism of biogenesis is currently lacking, although MVBs have not been described in the 188 parasite gastrodermal cells. One possibility is the breakdown of the long finger-like 189 membrane-bound lamellae, which extend from the gastrodermal cells into the gut lumen, to 190 release free EVs. A similar mechanism has been described in the protozoan parasite, 191 Trypanosoma brucei rhodesiense. These single-celled parasites produce membrane-bound 192 nanotubes that extend up to 20 µm from the cell that disassociate into free EVs of around 80 193 nm in diameter [53]. Further proteomics analysis of the gastrodermal cell-derived EVs and 194

subsequent immunolocalisation studies are required to investigate this possibility in fluke andother helminths.

197

198 Are drug-induced "blebs" functionally equivalent to microvesicles?

The appearance of membrane-bound "blebs" on the parasite surface is a common initial 199 response to drug-induced stress that has been observed in various flatworm and roundworm 200 parasites [54-56]. Blebs appear as vesicular structures that eventually pinch off from the 201 plasma membrane bounding the parasite surface and have been best described by scanning 202 203 electron microscopy studies of the trematode tegument. The released blebs are variable in size, usually ranging between 200 nm and a few micrometres, but can be more than 20 µm in 204 205 diameter. Complementary transmission electron microscopy studies have confirmed that the 206 blebs pinch directly from the tegumental surface and are released into the extracellular environment (Figure 2) [55,57-59]. It has been suggested that blebbing is an attempt by 207 helminths to replenish tegument that has been lost or damaged due to drug action [60] or 208 209 humoral immune challenge [61]. Given the abundance of pumps such as P-glycoprotein 1 in the parasite EV membrane [47] it is possible that blebs sequester anthelmintics before their 210 release; thus, reducing their effective local concentration in the parasite tissues. Indeed, P-211 glycoprotein reversal agents have been shown to increase the efficacy of triclabendazole 212 213 (TCBZ) against TCBZ-resistant F. hepatica [62].

The mechanism of bleb formation in helminths has yet to be determined, but it is thought to be a calcium-dependent process [63,64]. MVs also bud directly from the plasma membrane in response to raised intracellular Ca^{2+} levels [65]. Given these biochemical and structural similarities, it is conceivable that parasite blebs are formed using the same molecular machinery as the MVs released by mammalian cells; i.e. Ca^{2+} -dependent cytoskeleton-driven remodelling of the plasma membrane. Transcriptomics and proteomics analysis indicates that the major players in this process (including ARF6 GTPase, pivotal in
MV biogenesis in mammalian cells) are conserved in *F. hepatica* although functional studies,
such as RNAi, are needed to confirm their role in MV/bleb formation [47].

223

224 Concluding remarks

The discovery of parasite-derived EVs has changed our view of the host-parasite interface. 225 The formation of EVs enables the parasite to release a range of effector molecules (including 226 degradation-susceptible protein and RNA species) in a protected manner to ensure their safe 227 delivery to host cells. The cargo of helminth exosome-like EVs contain numerous 228 immunomodulatory proteins and microRNAs that alter host immune cell phenotype to 229 support parasite survival and reproduction [28,33,47]. Additionally, helminth blebs/MVs 230 likely represent a vital membrane repair mechanism in response to drug-induced damage or 231 host antibody attack. If the molecular machinery responsible for either of these EV biogenesis 232 pathways could be disrupted using selective inhibitors/blocking antibodies, it might just be 233 234 possible to tip the host immune balance in favour of parasite elimination; by preventing 235 immune suppression by EV-delivered parasite immunomodulators or to enhance the potency of existing anthelmintic drugs by impairing parasite membrane repair mechanisms. Whilst 236 recent proteomics and functional studies have considerably advanced our understanding of 237 parasite EVs, many basic research questions remain (see Outstanding Questions). By 238 addressing these areas, we may have a realistic chance of targeting EVs to achieve parasite 239 control in the future. 240

241

242 Glossary

243

• **Bleb:** a blister-like protrusion of the plasma membrane on the cell surface.

Endosomal sorting complex required for transport (ESCRT): a series of up to 30
 membrane-associated proteins that interact directly to form distinct molecular
 machineries that drive recruitment of cargo molecules, inward budding and abscission
 of the endosomal membrane during ILV or MVB formation.

• Extracellular vesicle: small membrane-bound vesicles shed from most viable cell types. Includes exosomes (30-100 nm in diameter) that are formed from the endosomal pathway and microvesicles (100-1000 nm in diameter) that pinch directly from the plasma membrane.

• Multivesicular body (MVB): a mature endosome that contains numerous vesicles (termed intra-luminal vesicles; ILVs) within their interior. They are formed by inward budding of the endosomal membrane followed by abscission of the ILV into the endosome lumen.

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260 **References**

266

- Molina-Hernández, V. *et al.* (2015) *Fasciola hepatica* vaccine: we may not be there yet but we're on the right road. *Vet. Parasitol.* 208, 101–111
- 264
 2. Fairweather, I. *et al.* (1999) Development of *Fasciola hepatica* in the mammalian host. In *Fasciolosis* (Dalton, J.P., ed) pp. 1–29, CABI
- 267 3. Collins, P.R. *et al.* (2004) Cathepsin L1, the major protease involved in liver fluke
 268 (*Fasciola hepatica*) virulence: propeptide cleavage sites and autoactivation of the
 269 zymogen secreted from gastrodermal cells. *J. Biol. Chem.* 279, 17038–17046
- 4. Wilson, R.A. *et al.* (2011) Exploring the *Fasciola hepatica* tegument proteome. *Int. J. Parasitol.* 41, 1347–1359

273		
274	5.	van der Ree, A.M. and Mutapi, F. (2015) The helminth parasite proteome at the host-
275		parasite interface - informing diagnosis and control. Exp. Parasitol. 157, 48–58
276		
277	6.	Thébaud, B. and Stewart, D.J. (2012) Exosomes: cell garbage can, therapeutic carrier,
278		or trojan horse? Circulation 126, 2553-2555
279		
280	7.	Yáñez-Mó, M. et al. (2015) Biological properties of extracellular vesicles and their
281		physiological functions. J Extracell Vesicles 4:27066.
282		
283	8.	EL Andaloussi, S. et al. (2013) Extracellular vesicles: biology and emerging
284		therapeutic opportunities. Nat. Rev. Drug. Discov. 12, 347–357
285		
286	9	Record M. et al. (2014) Exosomes as new vesicular lipid transporters involved in
287	<i>.</i>	cell-cell communication and various pathophysiologies <i>Biochim Biophys Acta</i>
288		1841 108–120
200		1041, 100-120
200	10	Coakley G at al. (2015) Exosomes and other extracellular vesicles: the new
201	10.	communicators in parasite infections. Trands Parasital 31, 177, 80
291		communicators in parasite infections. Trends Tarasitol. 51, 477–69
292	11	Scherey IS at al. (2014) Excomes and other extracellular variables in best pathogen
295	11.	international EMBO Bar 16 24 42
294		Interactions. EMBO Rep. 10, 24–45
295	10	Altern LC at d (2012) Biogeneratic of extracellular variables (EV), exceeding
296	12.	Akers, J.C. <i>et al.</i> (2015) Biogenesis of extracentular vesicies (EV): exosonies,
297		microvesicies, retrovirus-like vesicies, and apoptopic bodies. J. Neurooncol. 113, 1–
298		
299	10	
300	13.	Kowal, J. <i>et al.</i> (2014) Biogenesis and secretion of exosomes. <i>Curr. Opin. Cell Biol.</i>
301		29, 116–125
302		
303	14.	Simons, M. and Raposo, G. (2009) Exosomes – vesicular carriers for intercellular
304		communication. Curr. Opin. Cell Biol. 21, 575–581
305		
306	15.	Hasegawa, T. et al. (2011) The AAA-ATPase VPS4 regulates extracellular secretion
307		and lysosomal targeting of α -synuclein. <i>PLoS ONE</i> 6, e29460
308		
309	16.	Andreu, Z. and Yáñez-Mó, M. (2014) Tetraspanins in extracellular vesicle formation
310		and function. Front. Immunol. 4, 442
311		
312	17.	Trajkovic, K. et al. (2008) Ceramide triggers budding of exosome vesicles into
313		multivesicular endosomes. Science 319, 1244–1247
314		
315	18.	Colombo, M. et al. (2014) Biogenesis, secretion, and intercellular interactions of
316		exosomes and other extracellular vesicles. Annu. Rev. Cell Dev. Biol. 30, 255-89
317		
318	19.	Chen. S. et al. (2001) Rab8b and its interacting partner TRIP8b are involved in
319		regulated secretion in AtT20 cells. J. Biol. Chem. 276. 13209-13216
320		
321	20.	Savina, A. et al. (2005) Rab11 promotes docking and fusion of multivesicular bodies
322		in a calcium-dependent manner. Traffic 6, 131–143
		· · · · · · · · · · · · · · · · · · ·

323	01	Ordered $(1, 2010)$ D-h27, and D-h27h and different dama of the
324 325	21.	exosome secretion pathway. <i>Nat. Cell. Biol.</i> 12, 19–30
326	\mathbf{r}	Hyperpart $V_{\rm res}$ of d (2015) DAL 1 controls multivacioular body biogenesis and evecome
327	<i>LL</i> .	socration <i>L Cell Biol</i> 211, 27, 27
520 220		secretion. J. Cell Blot. 211, 27–37
229	22	Hugel P at al. (2005) Membrane microperticles: two sides of the coin <i>Physiclem</i>
221	23.	(Rethesda) 20, 22–7
333		(Demesau) 20, 22 T
332	24	Muralidharan-Chari V et al. (2009) ARE6-regulated shedding of tumor cell-derived
334	21.	nlasma membrane microvesicles <i>Curr Biol</i> 19 1875–85
335		plushia memorane mierovesieles. Curr. Diot. 19, 1075-05
336	25	Fox, J.E. <i>et al.</i> (1990) Role of the membrane skeleton in preventing the shedding of
337	201	procoagulant-rich microvesicles from the platelet plasma membrane. J. Cell Biol. 111.
338		483–93
339		
340	26.	Booth, A.M. et al. (2006) Exosomes and HIV Gag bud from endosome-like domains
341		of the T cell plasma membrane. J. Cell Biol. 172, 923–35
342		
343	27.	Angelot, F. et al. (2009) Endothelial cell-derived microparticles induce plasmacytoid
344		dendritic cell maturation: potential implications in inflammatory diseases.
345		Haematologica 94, 1502–12
346		
347	28.	Buck, A.H. et al. (2014) Exosomes secreted by nematode parasites transfer small
348		RNAs to mammalian cells and modulate innate immunity. Nat. Commun. 5, 5488
349		
350	29.	Wang, L. et al. (2015) Exosome-like vesicles derived by Schistosoma japonicum adult
351		worms mediates M1 type immune- activity of macrophage. Parasitol. Res. 114,
352		1865–1873
353		
354	30.	Zamanian, M. et al. (2015) Release of small RNA-containing exosome-like vesicles
355		from the human filarial parasite Brugia malayi. PLoS Negl. Trop. Dis. 9, e0004069
356		
357	31.	Chaiyadet, S. et al. (2015) Carcinogenic liver fluke secretes extracellular vesicles that
358		promote cholangiocytes to adopt a tumorigenic phenotype. J. Infect. Dis. 212, 1636-
359		45
360		
361	32.	Bernal, D. et al. (2014) Surface analysis of Dicrocoelium dendriticum. The molecular
362		characterization of exosomes reveals the presence of miRNAs. J. Proteomics 105,
363		232–41
364	22	
365	33.	Hansen, E.P. <i>et al.</i> (2015) Secretion of RNA-containing extracellular vesicles by the
366		porcine wnipworm, Trichuris suis. J. Parasitol. 101, 336–40
367	24	Ensure D (1 (2015) The second
308 200	54.	Fromin, B. et al. (2015) The revised microKINA complement of <i>Fasciola hepatica</i>
309 270		immuno regulatory microPNAs in extracellular variable. Let I Devented 45,607
37U 271		minumo-regulatory microxinas in extracemular vesicles. <i>Int. J. Parasitol.</i> 45, 697–
271 271		102
512		

373 374 375 376	35.	Nowacki, F.C. <i>et al.</i> (2015) Protein and small non-coding RNA-enriched extracellular vesicles are released by the pathogenic blood fluke <i>Schistosoma mansoni</i> . <i>J. Extracell. Vesicles</i> 4, 28665
377 378 379 380	36.	Colombo, M. <i>et al.</i> (2013) Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. <i>J. Cell Sci.</i> 126, 5553–65
381 382 383	37.	Baietti, M.F. et al. (2012) Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. Nat. Cell Biol. 4, 677-85
384 385 386	38.	Bianco, F. <i>et al.</i> (2009) Acid sphingomyelinase activity triggers microparticle release from glial cells. <i>EMBO J.</i> 28, 1043–54
387 388 389	39.	Hsu, C. <i>et al.</i> (2010) Regulation of exosome secretion by Rab35 and its GTPase- activating proteins TBC1D10A-C. <i>J. Cell Biol.</i> 189, 223–32
390 391 392 393	40.	Beckett, K. <i>et al.</i> (2013) Drosophila S2 cells secrete wingless on exosome-like vesicles but the wingless gradient forms independently of exosomes. <i>Traffic</i> 14, 82–96
394 395 396	41.	Abrami, L. <i>et al.</i> (2013) Hijacking multivesicular bodies enables long-term and exosome-mediated long-distance action of anthrax toxin. <i>Cell Rep.</i> 5, 986–96
397 398 399	42.	Koles, K. <i>et al.</i> (2012) Mechanism of evenness interrupted (Evi)-exosome release at synaptic boutons. <i>J. Biol. Chem.</i> 287, 16820–34
400 401 402	43.	Gross, J.C. et al. (2012) Active Wnt proteins are secreted on exosomes. Nat. Cell Biol. 14, 1036–45
403 404 405	44.	Ghossoub, R. <i>et al.</i> (2014) Syntenin-ALIX exosome biogenesis and budding into multivesicular bodies are controlled by ARF6 and PLD2. <i>Nat. Commun.</i> 5, 3477
406 407 408 409	45.	Liégeois, S. et al. (2006) The V0-ATPase mediates apical secretion of exosomes containing Hedgehog-related proteins in <i>Caenorhabditis elegans</i> . J. Cell Biol. 173, 949-61
410 411 412	46.	Figueiredo, B.C. <i>et al.</i> (2014) Schistosome syntenin partially protects vaccinated mice against <i>Schistosoma mansoni</i> infection. <i>PLoS Negl. Trop. Dis.</i> 8, e3107
413 414 415 416	47.	Cwiklinski, K. <i>et al.</i> (2015) The extracellular vesicles of the helminth pathogen, <i>Fasciola hepatica</i> : biogenesis pathways and cargo molecules involved in parasite pathogenesis. <i>Mol. Cell. Proteomics</i> 14, 3258–73
417 418 419	48.	de Gassart, A. et al. (2003) Lipid raft-associated protein sorting in exosomes. Blood 102, 4336-44
420 421 422	49.	Simbari, F. <i>et al.</i> (2016) Plasmalogen enrichment in exosomes secreted by a nematode parasite versus those derived from its mouse host: implications for exosome stability and biology. <i>J Extracell Vesicles</i> 5, 30741

423		
424	50.	Wilson, R.A. (2012) Proteomics at the schistosome-mammalian host interface: any
425		prospects for diagnostics or vaccines? <i>Parasitology</i> 139, 1178–94
426		
427	51.	Marcilla, A. et al. (2012) Extracellular vesicles from parasitic helminths contain
428		specific excretory/secretory proteins and are internalized in intestinal host cells. <i>PLoS</i>
429		ONE 7. e45974
430		
431	52	Vilette D et al. (2015) Efficient inhibition of infectious prions multiplication and
432	02.	release by targeting the exosomal pathway <i>Cell Mol Life Sci</i> 72, 4409–27
433		Tolease by aligeting the exosonial painway. Cell. Mol. Life Sel. 12, 1109 27
131	53	Szempruch A I et al. (2016) Extracellular vesicles from Trypanosoma brucei mediate
434	55.	virulence factor transfer and cause host anemia. Call 164, 246-57
435		virulence ractor transfer and cause nost anemia. Cett 104, 240–37
450	51	Aninhagmit W and Sabhan D (1006) Onigtherabic viverning affect of preziguental
437	54.	Aphiliasinit, w. and Sobion, P. (1990) Opismorchis viverrini. effect of praziquanter
438		on the adult legument. Southeast Asian J. Trop. Mea. Public Health 27, 304–11
439	~ ~	
440	55.	Robinson, M.W. et al. (2003) The effect of the microtubule inhibitor tubulozole-C on
441		the tegument of triclabendazole-susceptible and triclabendazole-resistant Fasciola
442		hepatica. Parasitol. Res. 91, 117–29
443		
444	56.	Zeng, X. et al. (2013) Angiostrongylus cantonensis: tegumental and hypodermic
445		alterations of the fourth-stage larvae following administration of tribendimidine in
446		vivo and in vitro. Parasitol. Res. 112, 3035–3040
447		
448	57.	Halferty, L. et al. (2009) Electron microscopical study to assess the in vitro effects of
449		the synthetic trioxolane OZ78 against the liver fluke, Fasciola hepatica. Parasitology
450		136, 1325–37
451		
452	58.	Toner, E. et al. (2010) Tegumental surface changes in adult Fasciola hepatica in
453		response to treatment <i>in vivo</i> with triclabendazole in the sheep host. Vet. Parasitol.
454		172, 238–48
455		
456	59.	Tansatit, T. et al. (2012) Fasciola gigantica: the in vitro effects of artesunate as
457		compared to triclabendazole on the 3-weeks-old invenile. <i>Exp. Parasitol</i> 131, 8–19
458		
459	60	Bennett C F et al (1980) Fasciola henatica: changes in tegument during killing of
460	00.	adult flukes surgically transferred to sensitized rats. <i>Parasite Immunol</i> 2, 39–55
460		addit fluxes surgrouny transferred to sensitized fais. I drastic flutation. 2, 37-35
462	61	Abdeen SH at al (2012) Illtrastructural changes of adult Schistosoma mansoni
402	01.	worms recovered from C57BL/6 mice passively immunized with normal and
403		vaccinated rabbit sera in vivo Parasital Pas 110 37 47
404		
405	62	Mattian I at al. (2006) Desistance induced shances in trialshandered transport in
466	02.	Mottler, L. <i>et al.</i> (2006) Resistance-induced changes in triclabelidazole transport in $E_{\rm res}$ is the second standard from the probability of the second standard standa
467		Fasciola nepatica: ivermecun reversal effect. J. Parasitol. 6, 1555–1560
468	<i>(</i> 2)	$D_{n} = \frac{1}{2} \left(\frac{1}{2} + \frac{1}{2} \right) \left(\frac{1}{2} + \frac$
469	63.	Bricker, U.S. <i>et al.</i> (1983) The relationship between tegumental disruption and muscle
470		contraction in <i>Schistosoma mansoni</i> exposed to various compounds. Z. Parasitenkd.
471		09, 01-/1
472		

- 473 64. Xiao, S. *et al.* (1984) Praziquantel-induced vesicle formation in the tegument of male
 474 *Schistosoma mansoni* is calcium-dependent. *J. Parasitol.* 70, 177–179
- 65. Cocucci, E. *et al.* (2009) Shedding microvesicles: artefacts no more. *Trends Cell Biol*. 19, 43–51

481 Figure 1. Biogenesis of exosome-like extracellular vesicles in the tegument of
482 trematodes.

(A) Schematic representation of the proposed exosome biogenesis pathway in the trematode 483 484 tegument and the major components likely involved based upon mass spectrometry analysis of secreted exosome-like extracellular vesicles (EVs). Endosomal sorting complexes required 485 for transport (ESCRT)-dependent (TSG101, ALIX and VPS4) and -independent (aSMase 486 and CD63 tetraspanin) components may contribute to the initial formation of multivesicular 487 bodies (MVBs), which are directed towards the apical plasma membrane (APM) of the 488 489 tegumental syncytium. Here, soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNAREs) (such as syntaxin, SNAP-25 and VAMP7) together with small GTPases 490 491 (including Rab11, Rab27 and Ral-A) may facilitate the tethering and fusion of the MVB to 492 the plasma membrane allowing the release of parasite-derived exosomes into the host 493 microenvironment. S, spine. (B) Transmission electron micrograph of the tegumental syncytium of adult Fasciola hepatica containing structures that resemble MVBs. Inset, 494 magnification of a MVB-like structure containing several intraluminal vesicles (ILVs). 495 aSMase, acid sphingomyelinase. 496

497

Figure 2. Microvesicle/bleb release from the apical plasma membrane of the trematode tegument.

500 (A) Schematic representation of the proposed mechanism of bleb/microvesicle (MV) 501 formation and release from the apical plasma membrane (APM) of the trematode tegument. 502 An increase in intracellular Ca^{2+} concentration triggers a signalling cascade that promotes the 503 excision of MVs/blebs directly from the tegmental surface. This "budding" process requires a 504 state of membrane asymmetry, conducted by phospholipid translocases (e.g. scramblase), 505 which is driven further by calpain-dependent cleavage and rearrangement of the actin

cytoskeleton. The actin-binding proteins ezrin, radixin and moesin (ERM) are also involved in the reassembly of the cytoskeleton following MV/bleb formation. S, spine. (B) Scanning electron micrograph of the surface of the Fasciola hepatica tegument showing profuse blebbing. (C) Transmission electron micrograph of F. hepatica tegumental syncytium showing the pinching of blebs/MVs from the apical plasma membrane and their release from the tegumental surface. Arrowheads; MV/blebs.

Protein	Function	EV subtype	RNAi phenotype in	Present in	RNAi phenotypes of	Refs
			mammalian cells	Helminth EVs	C. elegans orthologues	
HRS/HGS	ESCRT-0 component	Exo	EV secretion ↓	-	Gro, Dpy, Unc	[36]
STAM	ESCRT-0 component	Exo	EV secretion ↓	-	Emb, Lva, Let, Ste, Gro, Stp	[36]
TSG101	ESCRT-I component	Exo & MV	EV secretion ↓	Fh ^d	Emb, Lva, Let, Ste, Gro, Stp	[36,37]
VPS22/SNF8	ESCRT-II component	Exo	EV secretion ↓	-	Emb, Lva, Let, Ste, Gro, Stp	[37]
CHMP2A	ESCRT-III component	Exo	EV secretion \downarrow	Fh	Emb, Lva, Let, Ste, Gro, Stp	[37]
CHMP4	ESCRT-III component	Exo	EV secretion ↑↓	-	Emb, Let, Bmd, Prl	[36,37]
VPS4	EV abscission	Exo & MV	EV secretion ↑↓	Fh, Sm	Emb, Let, Unc, Prl, Gro	[36,37]
ALIX/PDCD6IP	ILV formation/cargo sorting	Exo	EV secretion ↓	Fh, Sm, Dd, Hp	Emb, Lva, Let, Ste, Gro, Stp	[36,37]
Syndecan	ILV formation/cargo sorting	Exo	EV secretion \downarrow	-	Let	[37]
Syntenin	ILV formation/cargo sorting	Exo	EV secretion ↓	Fh, Ov, Sm	-	[37]
Sphingomyelinase	Ceramide-dependent ILV	Exo & MV	EV secretion ↓ ^b	Fh	Emb, Lva, Let, Ste, Gro, Stp	[17,38]
	formation					
CD63 antigen	ILV formation/cargo sorting	Exo	No effect	Fh, Ov, Sm, Sj	-	[37]
Rab27	Fusion of MVB with the PM	Exo	EV secretion \downarrow	Fh	Emb, Lva, Let, Ste, Gro, Stp	[21]
Rab35	Fusion of MVB with the PM	Exo	EV secretion ↓	Fh	Let	[39]
Rab11	Fusion of MVB with the PM	Exo	EV secretion ↓	Fh, Sm, Sj, Hp	Emb, Lva, Let, Ste, Gro, Stp	[40,41]
Ral-1/Ral-A	Fusion of MVB with the PM	Exo	EV secretion ↓	Fh, Hp	Let	[22]
Syntaxin	Q-SNARE	Exo	EV secretion ↓	Fh, Sm	Let	[42]
SNAP-25	T-SNARE	Exo	ND	Fh, Sm	Emb, Lva, Let, Ste, Gro, Stp	[8]
YKT6	R-SNARE	Exo	EV secretion \downarrow	-	Clr, Emb, Sck, Gro, Stp	[43]
Phospholipid	PM curvature	MV	ND	Fh	Emb, Lva, Let, Ste, Gro, Stp,	[25]
translocases ^a					Spn	

Table 1. Key regulators of EV biogenesis in mammalian cells and their orthologues in helminths

Calpain	Cytoskeletal remodelling	MV	ND	Fh, Ov, Sm	Emb, Lva, Let, Ste, Gro, Stp	[25]
Phospholipases	Signal-induced cytoskeletal	Exo & MV	EV secretion \downarrow	Fh	Emb, Lva, Let, Ste, Gro, Stp	[44]
	regulation					
ARF6	Reorganization of the actin	Exo & MV	EV secretion ↓	Fh, Sj	Emb, Lva, Let, Ste, Gro, Stp	[44]
	cytoskeleton					
vATPase (V0)	Fusion of MVB with the PM	Exo	MVB accumulation ^c	Fh, Sj, Hp, Bm	Clr, Emb, Sck, Gro, Stp	[45]

^a flippases, floppases and scramblases; ^b phenotype observed using chemical inhibitors; ^c in *C. elegans.* ^dAbbreviations: Fh, *Fasciola hepatica*; Ov, *Opisthorchis viverrini*; Sm, *Schistosoma mansoni*; Sj, *Schistosoma japonicum*; Dd, *Dicrocelium dendriticum*; Hp, *Heligsomoides polygyrus*; Bm, *Brugia malayi*; PM, plasma membrane; Exo, exosome, MV, microvesicle. *Ste*, sterile; *Lva*, larval arrest; *Emb*, embryonic lethal; *Gro*, slow growth; *Unc*, locomotion abnormal; *Dpy*, dumpy; *Clr*, clear; *Stp*, sterile progeny; *Bmd*, organism morphology abnormal; *Let*, larval lethal; *Prl*, paralysed; *Sck*, sick; *Spn*, Abnormal spindle orientation; ND, not determined.

Trends

- Parasite-derived EVs are now recognised as important mediators of molecular communication between host and parasite.
- Transcriptomics and proteomics profiling has identified a range of immunomodulatory cargo molecules packaged into these but has also begun to shed light on the mechanisms used by helminths to generate and release EVs into the host microenvironment.
- Although technically challenging, selective inhibition of parasite EV biogenesis pathways would prevent the delivery of a range of immunomodulators to host cells and tip the immune balance in favour of parasite elimination.

Outstanding questions

- What mechanisms/pathways are used by helminths to produce and export EVs?
- Are these pathways novel or distinct from those used by mammalian cells?
- Are helminth EVs secreted constitutively or in response to stress?
- Do drug-induced MVs/blebs contribute to mechanisms of drug resistance in parasites?
- Are parasite-derived EVs essential for survival in the mammalian host?
- Can selective inhibitors of key "checkpoints" in parasite EV biogenesis pathways be developed?





Figure 2

