

1 **Tracking the uptake of labelled host-derived extracellular vesicles by the human fungal pathogen *Aspergillus***  
2 ***fumigatus*.**

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16  
17 **Running Title:** Genetic-based reporter system for labelling host EVs

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25 **ABSTRACT**

26 Extracellular vesicles (EVs) have gained attention as facilitators of intercellular as well as interkingdom  
27 communication during host-microbe interactions. Recently we showed that upon infection, host  
28 polymorphonuclear leukocytes produce antifungal EVs targeting the clinically important fungal pathogen  
29 *Aspergillus fumigatus*; however, the small size of EVs (< 1  $\mu\text{m}$ ) complicates their functional analysis. Here, we  
30 employed a more tractable, reporter-based system to label host alveolar epithelial cell-derived EVs and enabled  
31 their visualisation during *in vitro* *A. fumigatus* interaction. Fusion of EV marker proteins (CD63, CD9, and CD81)  
32 with a Nanoluciferase (NLuc) and a green fluorescent protein (GFP) facilitated their relative quantification by  
33 luminescence and visualization by a fluorescence signal. The use of an NLuc fused with a GFP is advantageous as  
34 it allows for quantification and visualisation of EVs simultaneously without additional external manipulation and  
35 to distinguish subpopulations of EVs. Using this system, visualisation and tracking of EVs was possible using  
36 confocal laser scanning microscopy and advanced imaging analysis. These experiments revealed the propensity  
37 of host cell-derived EVs to associate with the fungal cell wall and ultimately colocalize with the cell membrane of  
38 *A. fumigatus* hyphae in large numbers. In conclusion, we have created a series of tools to better define the  
39 complex interplay of host-derived EVs with microbial pathogens.

ORIGINAL UNEDITED MANUSCRIPT

## 40 INTRODUCTION

41 Human pathogenic fungi continue to pose a global threat, especially to immunocompromised individuals and  
42 those with underlying diseases such as leukemia, chronic obstructive pulmonary disease, or severe influenza,  
43 (Köhler *et al.* 2017; WHO 2022; Denning 2024). In the recent World Health Organization fungal priority pathogen  
44 list, the ubiquitously distributed, filamentous saprobe *Aspergillus fumigatus* was added to the critical priority  
45 group due to its ability to cause disease ranging from allergic reactions to life-threatening invasive aspergillosis  
46 in immunocompromised hosts (Brakhage 2005; van de Veerdonk *et al.* 2017; Latgé *et al.* 2019; WHO 2022).  
47 *A. fumigatus* typically undergoes propagation through the asexual production of spores called conidia (Latgé *et al.*  
48 *et al.* 2019). With their small size of only 2-3  $\mu\text{m}$  in diameter and their hydrophobic surface structure, conidia are  
49 dispersed through the air and can be easily inhaled. In the lungs, they can reach the alveoli, where they are  
50 rapidly cleared by innate immune cells in immunocompetent hosts, but able to germinate, form hyphae, and  
51 cause invasive fungal growth in immunocompromised patients (Heinekamp *et al.* 2015).

52 During infection, both professional phagocytic immune cells (Lionakis *et al.* 2023) and lung epithelial  
53 cells (Amin *et al.* 2014; Ewald *et al.* 2021; Jia *et al.* 2023) are required for the coordinated clearance of  
54 *A. fumigatus* conidia from the lungs. In recent years, extracellular vesicles (EVs) have gained attention for  
55 facilitating communication between human cells or between hosts and microorganisms; however, the potential  
56 role of host-derived EVs during *A. fumigatus* infection remains largely unexplored. Recently it was shown that  
57 upon infection, host polymorphonuclear leukocytes (PMNs) produce EVs distinct from those released during a  
58 healthy state and that are able to inhibit *A. fumigatus* conidia and hyphae (Shopova *et al.* 2020). EVs are a  
59 heterogenous group of membrane-delimited vesicles secreted by almost all cell types that play important roles  
60 in cell-to-cell communication during physiological and pathological states (Brakhage *et al.* 2021). Classification of  
61 EVs into subtypes is difficult and currently distinction is mostly based on their type of biogenesis, resulting in  
62 three primary subgroups named exosomes, ectosomes/microvesicles, and apoptotic bodies (Abels *et al.* 2016;  
63 van Niel *et al.* 2018; Brakhage *et al.* 2021). Apoptotic bodies are a heterogenous group of vesicles associated  
64 with cell death and are frequently considered a confounding factor in studies of EVs in otherwise healthy cells  
65 (Kakarla *et al.* 2020), and even more challenging to assess during infection situations. Exosomes are produced in  
66 the endosomal pathway by invagination and subsequent inward budding and fission of the limiting membrane  
67 of endosomes to form intraluminal vesicles (ILVs). During the maturation process from early to late endosomes,  
68 these structures accumulate several ILVs, leading to the generation of multivesicular bodies (MVBs). MVBs are  
69 then either directed to lysosomes for degradation of their contents or fused with the cell cytoplasmic membrane  
70 releasing the ILVs as exosomes into the extracellular space. Ectosomes are formed through direct outward  
71 budding and fission of the cytoplasmic membrane (Abels *et al.* 2016; van Niel *et al.* 2018; Brakhage *et al.* 2021).

72 EVs carry a wide variety of cargo molecules that can elicit specific cellular functions in the recipient cell,  
73 including proteins, lipids, polysaccharides, and nucleic acids (Yáñez-Mó *et al.* 2015). In comparison to  
74 spontaneously produced EVs, EVs produced by *A. fumigatus*-infected PMNs were shown to be larger in size and  
75 exhibit a higher enrichment of the tetraspanin CD63 in their membranes, as well as a more diverse protein cargo  
76 and specifically, larger amounts of proteins and peptides displaying antimicrobial activity. Challenging  
77 *A. fumigatus* hyphae with such infection-derived EVs resulted in the localization of the EVs to or within the cell  
78 wall as well as infrequently to the cytoplasm of the hyphae, which ultimately resulted in the damage and  
79 apparent death of the fungus (Shopova *et al.* 2020).

80 There are numerous studies on labeling EVs in the literature, either using lipophilic dyes such as DiI  
81 (Lázaro-Ibáñez *et al.* 2021), radionucleotide labelling (Lázaro-Ibáñez *et al.* 2021), or tagging of proteins  
82 commonly associated with EVs (Hikita *et al.* 2018; Cashikar *et al.* 2019; Gupta *et al.* 2020; Hikita *et al.* 2020; Levy  
83 *et al.* 2020; Lázaro-Ibáñez *et al.* 2021). Many of these methods either enabled the detection and quantification  
84 of EVs *in vitro* as well as *in vivo*, or their visualization, whereas others combined different fusion proteins to  
85 allow both detection and visualization simultaneously (Shpigelman *et al.* 2021). In this study we sought to build  
86 on these works by creating a system that would allow for easy detection and visualization of EVs independent of  
87 external labelling and thus potential alteration of their behavior. Therefore, we labelled host cell-derived EVs by  
88 genetically fusing the commonly accepted EV marker proteins CD63, CD9, and CD81 with a Nanoluciferase  
89 (NLuc) luminescence reporter as well as an appropriate fluorescent protein tag. The resulting constructs were  
90 transiently transfected into the human lung epithelial cell line A549. EVs isolated from these cells can be  
91 detected and visualized through measurement of the luminescence signal in cell culture supernatants and by  
92 confocal laser scanning microscopy (CLSM), respectively. We used this system to track host-derived EVs upon  
93 coinubation with *A. fumigatus* and revealed robust association of these labeled EVs with fungal hyphae,  
94 establishing a system for future dissections of the role of EVs in cross-kingdom delivery of molecules from host  
95 to pathogen.

## 96 97 **RESULTS**

### 98 ***A. fumigatus*-infected A549 cells secrete EVs associated with well-characterized tetraspanins.**

99 We selected an *in vitro* cellular system fulfilling two requirements, *i.e.*, it was relevant to fungal infection and  
100 readily transfectable, to establish a toolkit for the further study of host-derived EVs. A549 lung epithelial cells  
101 met both demands, as they have a history as a cell culture infection model for *A. fumigatus* and are routinely  
102 transfected with high efficiency (Jia *et al.* 2023). First, we examined the secretion of EVs by A549 epithelial cells  
103 under normal culture conditions and after infection with opsonized *A. fumigatus* conidia, a condition known to

104 elicit antifungal EVs in other systems (Shopova *et al.* 2020; Rafiq *et al.* 2022). Spontaneously released EVs (EVs)  
105 from uninfected cells and *A. fumigatus* infection-derived EVs (idEVs) produced by cells challenged with conidia  
106 were isolated from conditioned cell culture media using a combination of low-speed centrifugation,  
107 ultrafiltration, and size-exclusion chromatography. While unconditioned media prepared with EV-free FCS did  
108 not contain any measurable EV-sized particles (**Sup. Fig. 1a**), isolation of EVs using size-exclusion  
109 chromatography resulted in most of the particles being released in fractions 7-9 (**Sup. Fig. 1b**), which were also  
110 positive for common EV markers (**Sup. Fig. 1c**). Transmission electron microscopy confirmed the presence of  
111 round, cup-shaped structures ranging in size from 50 to 100 nm in sample preparations from both infected and  
112 non-infected A549 cells (**Fig. 1a**). Next, the concentration and size of EVs and idEVs isolated at different time  
113 points were determined using nanoparticle tracking analysis. We observed a significant increase in the particle  
114 concentration in the samples after eight hours of incubation for EVs and idEVs but no differences in the  
115 concentration between the two culture conditions (**Fig. 1b**). Similarly, size distributions did not greatly differ  
116 between EVs and idEVs after shorter periods of incubation and ranged between about 70-300 nm, with a peak at  
117 approximately 110 nm. After eight hours, nanoparticle tracking analysis measurements of idEVs revealed a  
118 broader size range of isolated particles in the idEV samples with major peaks at around 120 nm and 140 nm, and  
119 a smaller peak at 215 nm that was not observed in the EV group, however still resides within the expected size  
120 range for EVs (van Niel *et al.* 2018) (**Fig. 1c**). Cell viability was confirmed to be unaffected by infection using LDH  
121 release assays (**Sup. Fig. 1d**).

122 EVs have gained attention with respect to their roles during infections; however, visualization of EVs is  
123 limited due to their small sizes. We previously suggested the association and apparent internalization of human  
124 primary neutrophil-derived EVs into *A. fumigatus* hyphae using a lipophilic dye (Shopova *et al.* 2020). Here, we  
125 aimed to improve on our previous efforts by tagging several previously characterized EV marker proteins with a  
126 combination of luciferase and fluorescent protein tags. To aid in selection of suitable EV marker proteins, EVs  
127 were isolated from conditioned A549 cell culture medium after a 24-hour incubation period from both infected  
128 and uninfected cells by size-exclusion chromatography. The isolated EVs were subjected to LC-MS/MS-based  
129 proteomic analysis, revealing 884 unique proteins with 30 and 55 proteins solely found in the EV and idEV  
130 sample, respectively (**Dataset S1**). Principal component analysis revealed that these samples were not grossly  
131 different, but EV sample 2 was a slight outlier driven by principle component 2 at 5.54% (**Fig. 1d, Sup. Fig. 2a**).  
132 We did observe significant differences for a limited number of proteins (**Sup. Fig. 2b**), but in general the  
133 proteome of EVs appeared quite similar between the EVs and idEVs, with the most obvious changes occurring as  
134 serum proteins (*e.g.*, IGHM, APOA1 increased in the idEVs) likely introduced by the opsonization process of *A.*  
135 *fumigatus* conidia prior to infection of the host cells. EVs and idEVs were both tested for their antifungal

136 capacity using an *A. fumigatus* mitochondrial GFP reporter strain (Ruf *et al.* 2018) in a host cell-free system as  
137 previously described for PMNs (Shopova *et al.* 2020) and PLB-985 cells (Rafiq *et al.* 2022), in which *A. fumigatus*  
138 germlings are challenged with isolated EVs for 16 hours, followed by the evaluation of the mitochondrial  
139 network integrity. In our assay, in both cases the mitochondrial network appeared largely intact and similar to  
140 the untreated growth control, suggesting that EVs isolated from naïve or *A. fumigatus*-infected A549 cells are  
141 not overtly antifungal against *A. fumigatus* (**Sup. Fig. 3**). This finding might not be surprising as A549 EVs lacked  
142 most of the antimicrobial proteins previously detected in PMN EVs (Shopova *et al.* 2020). We were able to  
143 confirm the presence of the three tetraspanins CD9, CD81, and CD63 that are generally accepted EV marker  
144 proteins. Although the nuclear lamina protein, lamin A (LMNA), was generally found in EVs from A549 cells, both  
145 EV samples were negative for the endoplasmic reticulum-associated protein calnexin (CANX), indicative of good  
146 purity of the isolated EV fractions using size-exclusion chromatography (Welsh *et al.* 2024). Due to the similarity  
147 between EVs and idEVs in terms of protein content and the lack of apparent antifungal activity, the remainder of  
148 the study was performed using spontaneously released EVs to exclude potential artifacts arising from opsonins  
149 that are introduced by the opsonization of conidia during the infection process.

150

#### 151 **Transfected A549 epithelial cells properly express and localize tetraspanin-fusion proteins.**

152 After identifying appropriate EV marker proteins, we created plasmids encoding fusion proteins of one of these  
153 EV marker proteins, a Nanoluciferase (NLuc), and the fluorescent protein GFP-Spark (green fluorescent protein;  
154 GFP). Despite the presence of LMNA in the EVs samples as detected in our proteomic analysis, plasmids  
155 encoding for NLuc-GFP-labelled CANX and LMNA fusion proteins were generated as potential controls intended  
156 to serve as indicators of the level of introduction of cellular contaminants during EV isolation (**Fig. 2a**). Fusion  
157 proteins were each driven by the CMV promoter and constructed to encode an N-terminal NLuc followed by the  
158 sequence for GFP and the respective tetraspanin or LMNA at the C-terminal end to not mask the nuclear  
159 localization sequence (Anderson *et al.* 2021). Accordingly, since CANX has a signal peptide localized at its N-  
160 terminus (Paskevicius *et al.* 2023), the fusion protein was generated to encode N-terminal CANX followed by  
161 NLuc and a C-terminal GFP. Purified plasmids were used for the transient transfection of A549 alveolar epithelial  
162 cells using a liposome-based approach. Successful transfection and expression of the fusion proteins were  
163 verified after 24-hour incubation by fluorescence microscopy and measurement of the luminescence signal in  
164 the cell culture supernatant. Generally, GFP fluorescence was detected in cells transfected with all the plasmids.  
165 Lactate dehydrogenase (LDH) assays were performed on wild-type and transfected cells to test for cytotoxicity  
166 caused by the transfection itself or the specific plasmid preparations. All transfected cells showed slight  
167 decreases in viability compared to the non-transfected control cells; however, this was not influenced by the



199 Following EV isolation, NLuc activity was assessed in the resuspended EV pellets as well as in the EV-  
200 depleted supernatants. EV isolation using this method resulted in a high luminescence signal deriving from the  
201 resuspended EV pellets compared to the EV-depleted supernatants of cells expressing the CD63 and CD9 fusion  
202 proteins, indicative of the fusion proteins being associated with EVs (**Fig. 3a**). In the case of the CD81-fusion  
203 proteins the enrichment of the signal in the EV fraction was not as prominent and a comparably high signal was  
204 also derived from the supposedly EV-depleted supernatant. As expected, little NLuc activity was detected in the  
205 EV pellets of cells expressing the CANX and LMNA fusion proteins, but high signals were measurable in the EV-  
206 depleted supernatants, suggesting that the respective protein fusions were not associated with EVs.

207 To further confirm the association of the labelled tetraspanins with EVs, conditioned cell culture media  
208 were treated with the non-denaturing detergent Triton X-100 prior to EV isolation. Addition of Triton X-100  
209 leads to the lysis of EVs in the sample (**Sup. Fig. 5c**), releasing EV-associated proteins and molecules into the  
210 media and preventing their subsequent precipitation during polymer-assisted EV isolation. Thus, assuming the  
211 association of the labelled tetraspanins with EVs, in this setting successful lysis of the EV membrane will result in  
212 the luminescence signal deriving from the EV-depleted supernatants instead of the pellets as seen before.  
213 Indeed, Triton X-100 treatment led to a clear shift of the luminescence signal from the EV pellet to the EV-  
214 depleted supernatant for samples of cells expressing either of the labelled tetraspanins (**Fig. 3b**). This finding  
215 further strengthened our hypothesis that these fusion proteins are associated with EVs.

216 Lastly, transfected cells were treated with the neutral sphingomyelinase inhibitor GW4869 prior to  
217 collection of conditioned cell culture media. GW4869 is known to partially block exosome biogenesis (Trajkovic  
218 *et al.* 2008; Willms *et al.* 2016; Catalano *et al.* 2020). Assuming that the tetraspanin fusion proteins are  
219 associated with EVs, treatment of transfected cells with the inhibitor was expected to decrease the EV-derived  
220 luminescence signal in our assays. Cell culture supernatants were collected 6 hours post treatment either with  
221 the inhibitor or DMSO as a control and subjected to EV isolation by polymer-assisted precipitation.  
222 Subsequently, the resulting EV-enriched pellets were resuspended, and the NLuc signal measured (**Fig. 3c**). Due  
223 to high variability of the luminescence signal values between different biological replicates, values were  
224 normalised to the untreated controls. Indeed, cell culture media obtained from cells treated with GW4869  
225 revealed a significantly decreased NLuc signal in the EV-pellet (**Fig. 3c**). The decrease in measurable signal  
226 correlated positively with increasing concentrations of the inhibitor. In contrast, cells treated with DMSO did not  
227 exhibit significantly different NLuc signals compared to the non-treated controls, indicating that the effect is not  
228 correlated to DMSO-induced cell toxicity. In addition, LDH assays were performed to verify that the dose-  
229 dependent decrease in EV release is not caused by cell death due to the inhibitor itself. A significant decrease of  
230 viability was only seen for cells expressing the CD63-fusion proteins when treated with 20  $\mu$ M GW4869 (**Sup.**



231 **Fig. 5d**). Thus, the LDH assay indicates that cell death is not solely responsible for the significantly reduced NLuc  
232 signal in EV-pellets from transfected cells and supports our previous findings that the fusion proteins are  
233 associated with EVs.

234 Collectively, these results suggest that first, the tetraspanin fusion proteins are associated with EVs of  
235 A549 epithelial cells, and second, that transfected A549 cells can be used as reporters for the detection and  
236 quantification of secreted EVs after their isolation by measurement of luminescence signal.

237

#### 238 **GFP-labelled EVs can be visualized using imaging flow cytometry and CLSM.**

239 We next wanted to assess whether the isolated EVs from cells expressing the GFP fusion proteins can be  
240 visualized. We subjected isolated EVs from transfected and non-transfected cells to imaging flow cytometry. All  
241 events that exhibited either a brightfield, side scatter, or GFP signal were captured, including the device internal  
242 beads that are important for adjusting the focus. Measurement of the samples revealed a distinct population of  
243 events that was only detected in samples expressing the tetraspanin fusion proteins and mostly absent in the  
244 sample isolated from non-transfected control cells (**Sup. Fig. 5e**). Further investigation of this population  
245 revealed a high abundance of particles with no brightfield or side scatter signal but a visible GFP signal, likely  
246 corresponding to single EVs.

247 Next, to visualise EVs using CLSM, we set up a cell-free system in which EVs isolated from transfected  
248 cells were added to *A. fumigatus* germlings. The fungus was cultivated in RPMI in glass-bottom microscopy  
249 channel slides that offer limited space for vertical growth and instead force horizontal, planar growth of the  
250 hyphae. EV pellets resuspended in small volumes of culture media were added to the fungus and co-incubated  
251 for 16 hours in a humidified incubator. After the co-incubation time, samples were directly observed by CLSM.  
252 To increase the contrast and resolution, we used the *A. fumigatus* strain AfS150 (Lothar *et al.* 2014), a  
253 genetically modified strain expressing the fluorescent protein dTomato within its cytoplasm. Using this strain,  
254 overlapping of emission signals was limited and additional washing steps required for most staining protocols  
255 that could lead to the loss of EVs were eliminated. CLSM revealed visible green-fluorescent signals in samples  
256 treated with EVs isolated from transfected cells expressing any of the labelled tetraspanins compared to  
257 untreated control samples (**Fig. 4**). The visible signal derived from small dot-like to roundish structures, most of  
258 which appeared to consist of clusters of EVs. These clusters exhibited varying intensities and sizes with most  
259 being far below or around 1000 nm based on the green fluorescence signal, similar to the findings of other  
260 groups (Levy *et al.* 2020). Our nanoparticle tracking analysis data of A549 EVs isolated *via* polymer-assisted  
261 precipitation with sizes of A549 EVs ranging from approximately 70 – 450 nm (**Sup. Fig. 5b**) supports our  
262 hypothesis that the visible signals partly derive from clumps of EVs. In all cases, labelled EVs were found to co-

263 localise with the fungus, indicating that A549 EVs are indeed able to attach to or associate with *A. fumigatus*  
264 hyphae and confirming the value of our system in tracking these interactions.

265 To further verify our findings, we also applied EVs isolated from non-transfected, wild-type A549 cells.  
266 To visualise these EVs, we used a novel fluorogenic membrane dye called MemGlow 560 that exhibits minimal  
267 fluorescence in its free form. Only the integration into a lipid bilayer leads to high fluorescence signals, which  
268 harbours the advantage that isolated EVs can be stained without the need for subsequent washing steps. Since  
269 the dye exhibited red fluorescence when integrated into membranes, stained EVs were added to the genetically  
270 modified *A. fumigatus* strain AfS35 pJW103 expressing a green-fluorescent mitochondrial tag (Ruf *et al.* 2018)  
271 that was also used to assess the antifungal capacity. Conidia were seeded in 8-well microscopy slides and  
272 allowed to germinate for 8 hours prior to addition of the EVs. EVs in turn were isolated from cell culture  
273 supernatants of A549 cells *via* size-exclusion chromatography, concentrated, and subsequently stained and  
274 added to the germlings. After 16 hours of co-incubation, hyphal cell walls were visualised using calcofluor white  
275 and the samples were subjected to CLSM. Negative control samples of hyphae treated with the dye diluted in  
276 non-EV containing medium displayed no or only minimal red fluorescence signal, showing that the dye is not  
277 able to penetrate the fungal cell wall and therefore does not stain the hyphal membrane. In samples treated  
278 with stained EVs, several dots of red fluorescence in varying size and intensity accumulating at fungal hyphae  
279 were visible (**Fig. 5**). In general, based on the sizes of the red fluorescence signal, EVs stained with the  
280 membrane dye appeared to be smaller in size than the genetically labelled EVs, potentially due to reduced  
281 clumping and cluster formation after isolation using size-exclusion chromatography.

282 The CLSM images were subsequently subjected to imaging analysis and 3D reconstruction to further  
283 investigate the localisation of EVs regarding *A. fumigatus* hyphae (**Fig. 6**). Since labelled EVs were added to the  
284 *A. fumigatus* strain AfS150 expressing a cytoplasmic dTomato protein, our 3D models depict the limiting  
285 boundaries of the cytoplasm, equivalent to the hyphal membrane. Reconstruction of the EV signal revealed the  
286 association of labelled EVs with the hyphal membrane and their internalisation into the hyphal lumen (**Fig. 6 a,b**;  
287 **Sup. Video 1**). In the case of MemGlow-stained EVs, experiments were performed using the calcofluor white-  
288 stained *A. fumigatus* AfS35 pJW103 strain, with the hyphal cell wall depicted in blue (**Fig. 6c**). Most of the  
289 MemGlow signal was localised within the hyphae. Interestingly, the signal appeared to accumulate as a tube-like  
290 structure underneath the cell wall, which became even more obvious upon reconstruction of the signal from the  
291 GFP-labelled mitochondria within the hyphal lumen. These finding indicate an accumulation of the signal within  
292 or associated with the hyphal membrane, consistent with what was observed using the genetically labelled EVs.

293 Finally, we wanted to test whether the genetically labelled EVs can be used to track EVs in our  
294 experimental set-up using live cell imaging. As a proof-of-concept we tested EVs isolated from NLuc-GFP-CD9

295 expressing cells using polymer-assisted precipitation. Western blots confirmed the presence of CD9 in EVs  
296 isolated from cells expressing either of the labelled tetraspanins or non-transfected control cells. In addition, a  
297 clear band corresponding to the NLuc-GFP-CD9 fusion protein was visible for EVs isolated from cells that were  
298 transfected with the respective plasmid (**Sup. Fig. 5f**). Isolated EVs were then added to 8-hour-old germlings of  
299 the *A. fumigatus* strain AfS150 and imaging was performed over night with a z-stack image being taken every 30  
300 minutes (**Sup. Video 2-4**). In the beginning, EVs appeared as small green fluorescent dots that over time started  
301 to accumulate at the growing hyphae. Once attached, these EVs still displayed some mobility, but remained  
302 attached to the hyphae.

303

## 304 DISCUSSION

305 PMN-derived EVs were previously found to display antibacterial (Timár *et al.* 2013; Lőrincz Á *et al.* 2015) and  
306 antifungal activities (Shopova *et al.* 2020; Rafiq *et al.* 2022) under specific conditions. Here, we attempt to  
307 investigate the first steps in the mechanism by which host EVs exert their antifungal functions by exploring the  
308 interaction between host cell-derived EVs and *A. fumigatus* hyphae. Our main goal was the visualisation and  
309 tracking of these EVs in a live-cell setting; however, EVs are difficult to visualise based on their small size (<1 µm)  
310 and heterogeneity. Various methods have been employed in the past such as electron microscopy, staining with  
311 dyes, immunofluorescence, and genetic labelling of abundant EV cargo molecules, each with their own  
312 advantages and disadvantages (Chuo *et al.* 2018; Verweij *et al.* 2021). In this study, we used two different  
313 approaches, lipophilic dyes to stain EV membranes and genetic labelling of abundant EV proteins, which allowed  
314 us to detect, visualise, and track genetically labelled EVs in association with *A. fumigatus* hyphae.

315 A549 alveolar epithelial cells served as a tractable model as they are easy to transfect compared to  
316 immune cells and have been well-studied during infection with *A. fumigatus* conidia (Paris *et al.* 1997; Zhang *et al.*  
317 *et al.* 2005; Amin *et al.* 2014; Jia *et al.* 2014; Zhang *et al.* 2020; Jia *et al.* 2023). We confirmed the successful  
318 isolation of EVs and little induction of cell death by LDH release assay consistent with the literature, followed by  
319 analysis of their proteomic cargo and antifungal capacity. Interestingly, and in contrast to PMN idEVs (Shopova  
320 *et al.* 2020) and PLB-985 idEVs (Rafiq *et al.* 2022), none of the EV populations isolated exhibited antifungal  
321 activity in our host cell-free system based on an *A. fumigatus* mitochondrial integrity reporter strain.  
322 Consistently, analysis of the proteomic cargo confirmed the absence of any obvious antimicrobial proteins,  
323 opposite that of PMN idEVs (Shopova *et al.* 2020). Although the literature reports both activation and repression  
324 of inflammatory phenotypes in A549 cells upon co-incubation with *A. fumigatus* conidia (Paris *et al.* 1997; Zhang  
325 *et al.* 2005; Amin *et al.* 2014; Jia *et al.* 2014; Escobar *et al.* 2016; Escobar *et al.* 2018; Jia *et al.* 2023), the  
326 auxotrophic *A. fumigatus pyrG*-deficient strain used here appeared to result in a limited proinflammatory

327 response and may contribute to our observations. This strain provides a fungal stimulus to the cells, while  
328 allowing for collection of larger amounts of EVs necessary for proteomics over longer incubations. It remains a  
329 potential complication of these experiments that longer infection time points may result in increased cell stress,  
330 low level cell death, and/or alterations to the proteomic cargo of EVs. Fungal proteins were detected  
331 inconsistently in the proteomic samples and likely derive from co-isolation of fungal proteins during EV isolation  
332 as “contaminants” (Shopova *et al.* 2020; Rafiq *et al.* 2022).

333 Proteomic analysis of EVs confirmed several commonly associated proteins, including the tetraspanins  
334 CD63, CD9, and CD81, which are routinely used as EV marker proteins (Lötvall *et al.* 2014; Kowal *et al.* 2016;  
335 Lischnig *et al.* 2022; Tognoli *et al.* 2023) and to label EVs (Hikita *et al.* 2018; Cashikar *et al.* 2019; Görgens *et al.*  
336 2019; Gupta *et al.* 2020). CANX and LMNA were included as negative controls to assess EV purity and the  
337 association of the fusion proteins with EVs (Tognoli *et al.* 2023). According to the minimal information for  
338 studies of extracellular vesicles (MISEV) guidelines, these proteins are typically absent or underrepresented in  
339 EVs; however, they are found in some subtypes of EVs (Théry *et al.* 2018; Welsh *et al.* 2024). Proteomic analysis  
340 of wild-type A549 cells indicated that CANX was indeed absent; however, LMNA could be detected in our EV  
341 samples. Nonetheless, we created reporters for both control markers and the tetraspanin proteins to have a full  
342 repertoire of options for expansion into multiple cell systems in the future. Successful transient overexpression  
343 of the panel of NLuc-GFP fusion proteins in A549 cells was confirmed by green fluorescence and luminescence  
344 signals in the cell culture media from transfected cells, including those encoding the CANX and LMNA fusion  
345 proteins. It is worth noting that overexpression of tetraspanins can impact EV biogenesis and cargo loading, a  
346 caveat that must always be considered with any reporter (Strohmeier *et al.* 2021), and subsequently future  
347 studies will be required to more finely localize our new reporters in particular EV subpopulations. Nonetheless,  
348 we observed release of all the fusion proteins into the cell culture supernatant in accordance with previous  
349 observations (Cashikar *et al.* 2019; Gupta *et al.* 2020); however, isolation of EVs from cell culture supernatants  
350 revealed that the CANX and LMNA fusion proteins were not associated with the EV fraction, as initially expected.  
351 In addition, lysis of EVs in cell culture supernatants prior to their isolation demonstrated the association of the  
352 fusion proteins with A549 EVs. As an orthogonal approach, we confirmed a dose-dependent decrease of signal in  
353 the EV-containing fraction upon treatment of transfected cells with the neutral sphingomyelinase inhibitor  
354 GW4869 known to reduce EV biogenesis via the ESCRT-independent exosome biogenesis pathway (Trajkovic *et al.*  
355 2008; Kulshreshtha *et al.* 2013; Essandoh *et al.* 2015). We used high levels of GW4869 as described in the  
356 literature and did observe some LDH release indicative of cell death in this assay, yet overall the result appears  
357 to imply that the reporters decreased along with EV numbers. The successful generation of the reporter panel  
358 allowed for detection and relative quantification of EVs by luminescence signal, while simultaneously enabling

359 their visualisation within the cells for subcellular localisation and transfection efficiency, simplifying the overall  
360 workflow associated with such a reporter assay.

361 Imaging flow cytometry on the tetraspanin reporters from isolated EVs detected events of green  
362 fluorescent particles with minimal to no visible brightfield or side scatter signal. The absence of the brightfield  
363 signal is indicative of particles with sizes below the optical resolution limit of the device of 200 nm, consistent  
364 with sizes corresponding to small EVs (Headland *et al.* 2014; Abels *et al.* 2016) and in accordance with previous  
365 studies investigating GFP-labelled EVs from THP-1 monocytes (Görgens *et al.* 2019) and HEK293 cells  
366 (Jurgielewicz *et al.* 2020), as well as immuno-labelled EVs (Ricklefs *et al.* 2019). Using CLSM, EVs appeared as  
367 small, green fluorescent dots of varying size and intensity similar to the findings of previous studies using  
368 genetically labelled fluorescent EVs (Corso *et al.* 2019) and immunolabelled or stained EVs (Mondal *et al.* 2019).  
369 Importantly, part of the visible signal was clearly associated with fungal hyphae, indicating that A549 EVs can  
370 interact with *A. fumigatus*. This co-localisation was seen for all EVs regardless of the labelled tetraspanin  
371 expressed, hinting towards a nonspecific interaction. We verified these findings using MemGlow stained A549  
372 EVs from non-transfected cells. While the dye alone was almost completely unable to penetrate the cell wall of  
373 *A. fumigatus* hyphae, addition of stained EVs led to accumulation of co-localised signal with *A. fumigatus* hyphae  
374 as dot-like structures, similar to the EVs labelled with reporter proteins. One major difference observed between  
375 the two experiments was the amount of EVs binding to the hyphae, which is likely caused by the higher  
376 proportion of labelled EVs using the MemGlow approach. These EVs were isolated via size-exclusion  
377 chromatography from a large number of cells. Even though this isolation method led to a dilution of the sample  
378 (Welsh *et al.* 2024), the total amount of labelled EVs in the final sample was likely higher than from the low-  
379 efficiency transfection experiments. While the lipophilic dye can stain many subsets of EVs, it was previously  
380 shown that the tetraspanins CD63, CD81, and CD9 distribute heterogeneously across EV subsets (Ricklefs *et al.*  
381 2019; Han *et al.* 2021), resulting in even fewer labelled versus stained EVs. Different tagging approaches can  
382 alter the properties and fate of EVs, so it is recommended to use different labelling methods when studying EVs  
383 (Loconte *et al.* 2023). Here, the similar outcomes of the two strategies employed provides confirmatory  
384 evidence for the use of either our tetraspanin fusion proteins or MemGlow dye as the situation necessitates, but  
385 certainly orthogonal support is required for each specific experiment.

386 Lastly, imaging analysis and 3D-reconstruction of the microscopy images indicated that both labelled  
387 and stained A549 EVs are attached and partly internalised by *A. fumigatus* hyphae. This was an interesting  
388 finding that brings us one step further into understanding how EVs can exert effects on the fungus. First, it is  
389 now clear that the interaction of EVs with fungal hyphae is not limited to EVs produced by infected cells, but  
390 instead a more widespread phenomena, potentially even serving as a food source for the fungus in some cases.

391 It is also likely that the interaction of EVs alone cannot explain the antifungal activity previously observed for  
392 PMN (Shopova *et al.* 2020) and PLB-985 idEVs (Rafiq *et al.* 2022). The mechanisms behind the attachment of EVs  
393 to hyphae and their subsequent internalisation remain unclear and will be objectives of future studies, but  
394 based on their ability to fuse with membranes (Morandi *et al.* 2022), one hypothesis could be that EVs are able  
395 to pass the cell wall, either actively or passively, followed by fusion of the EV membrane with the hyphal  
396 membrane to release cargo. A more active form of fungal cell-induced endocytosis also remains possible. In  
397 mammalian cells, evidence suggests that EVs can be taken up into the endosomal/lysosomal pathway via  
398 endocytosis and that the release of EV cargo within recipient cells is often pH-dependent (Mulcahy *et al.* 2014;  
399 Bonsergent *et al.* 2021). More generally, successful uptake and cargo delivery of host cell EVs by fungi was  
400 previously demonstrated in plant-fungal-interactions (Cai *et al.* 2018; Wang *et al.* 2024).

401 In conclusion, our knowledge of host cell-derived EVs in immunity to *A. fumigatus* is still very limited.  
402 With these findings, we can confirm that EVs produced by naive human alveolar epithelial cells robustly interact  
403 with *A. fumigatus* hyphae, but fail to trigger an antifungal response. In the future, this methodological proof-of-  
404 principle using genetic labelling of A549 cell-derived EVs will serve as a platform to be expanded to other cell  
405 types as well as other target proteins.

## 407 MATERIALS AND METHODS

### 408 Culture conditions of microorganisms.

409 *A. fumigatus* conidia (Sup. Table 1) were plated on malt agar (Sigma Aldrich) and incubated at 37°C. On day five,  
410 conidia were collected from plates by adding 10 mL of sterile ultrapure water (dH<sub>2</sub>O) to the plate and scraping  
411 the conidia off the agar using a disposable T-shaped scraper. The conidia-water suspension was filtered through  
412 a 30 µm cell strainer (MACS, Miltenyi Biotec GmbH) for the removal of mycelium. Conidia were washed by  
413 centrifugation at 1,800 × *g* and 4°C for 5 min followed by removal of the supernatant and resuspension in sterile  
414 dH<sub>2</sub>O. Conidia were stored at 4°C for no longer than a week before use.

### 416 Cell culture.

417 A549 epithelial cells were cultivated in Kaighn's Modification of Ham's F-12 (F-12K) medium (Gibco)  
418 supplemented with 10% (v/v) artificial FCS (FetalClone III; Cytiva) and penicillin/streptomycin to a final  
419 concentration of 1%. Cells were seeded into T25 or T75 cell culture flasks at concentrations of 2×10<sup>5</sup> and 3×10<sup>5</sup>,  
420 or 6×10<sup>5</sup> and 1×10<sup>6</sup> cells per flask and passaged after four and three days, respectively. Spent media was  
421 removed by aspiration and cells were washed using prewarmed Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS (Gibco). Cells were detached  
422 from the cell culture flasks through the addition of trypsin (Thermo Fisher Scientific) and were subsequently

423 incubated at 37°C with 5% (v/v) CO<sub>2</sub> for 5 minutes followed by gentle tapping of the culture flask to assure the  
424 detachment of all cells. Trypsinisation was stopped by the addition of FCS-containing F-12K medium and cells  
425 were transferred to a fresh tube. Cell concentrations were determined using the Luna cell counter and cells  
426 were seeded into a new culture flask containing pre-warmed media for culturing or into plates as needed for the  
427 respective experiments. Seeded cells were incubated at 37°C and 5% (v/v) CO<sub>2</sub>. If required, cells were seeded in  
428 EV-depleted medium consisting of F-12K medium supplemented with 1% (v/v) EV-depleted FCS (“exosome-free  
429 serum”, Life Technologies GmbH).

430

#### 431 **Infection of cells with *A. fumigatus*.**

432 A549 epithelial cells were seeded into 15 cm culture dishes at concentrations of  $2 \times 10^7$  cells/dish in EV-depleted  
433 medium on the day prior to infection and incubated at 37°C and 5% (v/v) CO<sub>2</sub>. On the day of infection, fungal  
434 conidia suspensions were washed by centrifugation with  $1,800 \times g$  at 4°C for 5 minutes and the pellet was  
435 resuspended in 900  $\mu$ L sterile PBS. Conidia were opsonised by the addition of 100  $\mu$ L normal human serum  
436 followed by incubation at 37°C and centrifugation with 350 rpm for 30 minutes. Subsequently, the conidia were  
437 washed three times with sterile PBS and the concentration determined using a Thoma haemocytometer. Cells  
438 were washed using PBS and fresh pre-warmed EV-depleted medium in addition to opsonised fungal conidia to a  
439 multiplicity of infection (MOI) of 5, was added to the cells. Control experiments were treated with medium  
440 without fungal conidia. The plates were incubated at 37°C and 5% (v/v) CO<sub>2</sub> for 8 or 24 hours before EV isolation  
441 by size-exclusion chromatography.

442

#### 443 **EV isolation by size-exclusion chromatography (SEC).**

444 Conditioned cell culture medium was collected from cells and centrifuged at  $3000 \times g$  and 4°C for 15 minutes,  
445 filtered through a 0.22  $\mu$ m syringe filter (Carl Roth) and concentrated using Amicon Ultra-15 centrifugal filters  
446 (molecular weight cut-off: 100 kDa; Merck) by centrifugation at 4°C and  $3,220 \times g$ . First, as a pre-experiment to  
447 test for successful isolation of EVs samples were loaded onto pre-washed qEV 70 nm size-exclusion  
448 chromatography columns (Izon). Samples were isolated using Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS (Gibco). The first 2 mL of flow  
449 through corresponding to fractions 1-4 were collected followed by the collection of single fractions of 0.5 mL  
450 each until fraction 12. All of these samples were subjected to NTA analysis to check for particle size and  
451 concentration in the different fractions. Finally, throughout the remaining study, concentrated samples were  
452 loaded onto pre-washed qEV 70 nm size-exclusion chromatography columns (Izon) and isolated using Ca<sup>2+</sup>/Mg<sup>2+</sup>-  
453 free PBS (Gibco). Based on the results from the pre-experiment and in accordance with the manufacturer’s  
454 protocol, the first 3 mL corresponding to fractions 1-6 was considered as void volume and discarded, followed by

455 collection of the 1.5 mL EV-containing fractions 7-9. EVs were used for nanoparticle tracking analysis (NTA) or in  
456 case of sample preparation for proteomic analysis, western blots, or treatment of fungal hyphae, further  
457 concentrated using 10 kDa cut-off Amicon Ultra-0.5 ml filters (Merck) and stored at -20°C until usage.

458

#### 459 **Nanoparticle tracking analysis (NTA).**

460 Particle concentrations and size distributions of purified EV samples were assessed by NTA using a  
461 NanoSight 300 device (Malvern Instruments Ltd.). Samples were measured at ambient room temperature at a  
462 constant flow rate of 20 with the camera level set to 14 and the detection threshold to 4. For each sample,  
463 either three videos of 45 second each or five videos of 60 seconds each were obtained as indicated and  
464 subsequently analyzed with the NTA 3.2.16 software.

465

#### 466 **Proteomic analysis of isolated EVs.**

467 EVs were isolated by size-exclusion chromatography from conditioned medium of uninfected A549 cells and  
468 cells infected with the *A. fumigatus pyrG* strain after 24 hours of incubation at 37°C and 5% (v/v) CO<sub>2</sub> in EV-  
469 depleted medium. Preparation of protein samples from SEC isolated EV samples, LC-MS/MS analysis, and  
470 database search and analysis were performed as described before (Rafiq et al. 2022), except for the following  
471 changes: LC-MS/MS analysis was performed on a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific).  
472 Gradient elution was as follows: 0-5 min at 4% B, 30 min at 7% B, 60 min at 10% B, 100 min at 15% B, 140 min at  
473 25% B, 180 min at 45% B, 200 min at 65% B, 210-215 min at 96% B, and 215.1-240 min at 4% B. The instrument  
474 was operated in full MS/data-dependent MS2 (Top10) mode. Precursor ions were monitored at a resolution of  
475 140,000 FWHM (full width at half maximum). After higher-energy collisional dissociation (HCD) fragmentation at  
476 30% normalized collision energy (NCE), MS2 ions were scanned at 17,500 FWHM and a maximum injection time  
477 of 120 ms. Dynamic exclusion of precursor ions was set to 30 s. Tandem mass spectra were searched against the  
478 UniProt database of *Homo sapiens* (<https://www.uniprot.org/proteomes/UP000005640>; 21 March 2019) and  
479 *Aspergillus fumigatus* (<https://www.uniprot.org/proteomes/UP000002530>; 21 March 2019) using Proteome  
480 Discoverer (PD) 2.2 (Thermo) and the algorithms of Mascot 2.4.1 (Matrix Science), Sequest HT (version of  
481 PD2.2), and MS Amanda 2.0.

482

#### 483 **Assessment of the antifungal capacity of EVs.**

484 To assess the antifungal capacity of EVs,  $1 \times 10^4$  conidia of the *A. fumigatus* AfS35/pJW103 strain (Ruf et al. 2018)  
485 were seeded into an 8-well microscopy slide ( $\mu$ -Slide 8 Well Polymer Coverslip, Ibidi) in 200  $\mu$ L Roswell Park  
486 Memorial Institute (RPMI) medium (Gibco) and allowed to germinate for 8 hours at 37°C and 5% CO<sub>2</sub>. EVs were



487 isolated from uninfected or infected A549 cells after 8 hours via SEC and added to the germlings followed by  
488 incubation over night at the above-mentioned conditions. On the following day fungal hyphae were stained with  
489 Calcofluor white, subjected to CLSM imaging using a Zeiss LSM 780 confocal microscope, and evaluated with the  
490 Zen software (Carl Zeiss).

491

#### 492 **Generation of the plasmids encoding fusion proteins and transformation of *Escherichia coli*.**

493 DNA fragments for Gibson cloning were prepared as follows: DNA fragments were amplified from an  
494 appropriate template by PCR using a primer pair (**Sup. Table 2, 3**), and PCR fragments were purified using the  
495 GeneJet PCR Purification kit (Thermo Fisher Scientific). The plasmid backbone was prepared by restriction  
496 digestion of the circular plasmid pNLF1-N (Promega) with *EcoRV* or *EcoRI* for C- or N-terminal cloning,  
497 respectively. Gibson cloning was performed using the NEBuilder HiFi DNA Assembly Master Mix (New England  
498 Biolabs) according to the manufacturer's protocol using the primers described in **Sup. Table 3**, and the reaction  
499 was subsequently used for the transformation of chemically competent *E. coli* cells (NEB® Turbo Competent *E.*  
500 *coli*, New England Biolabs). The final constructs are shown in **Fig. 2**, where each reporter construct is driven by a  
501 CMV promoter.

502

#### 503 **Transient transfection of A549 epithelial cells using Lipofectamine 3000®.**

504 Transfection using Lipofectamine® 3000 (Invitrogen) was performed in 24-well plates according to the  
505 manufacturer's instructions. Briefly, on the day before transfection, A549 epithelial cells were seeded into a 24-  
506 well plate at densities of  $1 \times 10^5$  cells/well and incubated at 37°C and 5% (v/v) CO<sub>2</sub> for 20 hours to allow the cells  
507 to attach. The transfection solution per well was prepared as follows: In two separate reactions, 1.5 µL  
508 Lipofectamine® and 1 µL P3000® reagent, together with 500 ng of the desired plasmid DNA for transfection,  
509 were diluted in 25 µL F12-K medium (Gibco). The contents of both tubes were combined to yield the  
510 transfection reaction, which was incubated at room temperature for 10-15 minutes. In the meantime, the cells  
511 were washed using pre-warmed PBS and 450 µL pure F-12K medium was added to each well. 50 µL of the  
512 transfection reaction was added to the wells in a dropwise manner, followed by gentle swirling of the plate to  
513 ensure an equal distribution. The plates were incubated at 37°C and 5% (v/v) CO<sub>2</sub>. 22 hours post transfection,  
514 cells were washed with pre-warmed PBS and incubated in 1 mL EV-depleted medium. The cells were incubated  
515 under the above-mentioned conditions for the appropriate times depending on the following experiments. For  
516 microscopy, cells were seeded on glass cover slips prior to transfection. On the day of microscopy, cell  
517 membranes were stained with Cell Mask Deep Red (Invitrogen) for 10 minutes at 37°C and nuclei with DAPI-  
518 containing mounting medium (Roti-Mount FluorCare DAPI; Carl Roth).

519

520 **Assessment of cell viability.**

521 To assess cell viability, lactate dehydrogenase (LDH) assays were performed using a commercially available  
522 fluorometric LDH assay kit (Abcam) according to the manufacturer's protocol. Briefly, culture medium was  
523 collected from transfected and non-transfected control cells after 24 hours, centrifuged at  $10,000 \times g$  at  $4^{\circ}\text{C}$  for  
524 5 min, and supernatants were transferred to fresh microcentrifuge tubes. Cell lysates were also prepared from  
525 untreated cells by scraping and resuspension in assay buffer to assess the maximum LDH amount required for  
526 the calculation of cell viability. Cell supernatants and lysates were kept on ice and either directly used for  
527 measurement or snap frozen and stored at  $-20^{\circ}\text{C}$  until the following day. Standards and reagents were prepared  
528 freshly for every measurement. Samples and standards were diluted in assay buffer and after addition of the  
529 assay reagent, fluorescence signal was measured in kinetic mode at  $37^{\circ}\text{C}$  at excitation/emission  $535 \pm 15$   
530  $\text{nm}/587 \pm 20 \text{ nm}$  every 2 min for a total of 30 min using a plate reader M200 PRO plate reader (Tecan group Ltd.).  
531 Viability was calculated according to the method in the protocol.

532

533 **GW4869 treatment of cells.**

534 GW4869 (Sigma Aldrich) was resuspended in DMSO to a final concentration of 10 mM. This oversaturated stock  
535 solution was used for further dilution in cell culture media as previously described in the literature (Józefowski  
536 *et al.* 2010; Essandoh *et al.* 2015; Slivinski *et al.* 2022). Transfected A549 cells were washed with PBS 22 hours  
537 post transfection and incubated in 1 mL F-12K medium containing 1% (v/v) EV-depleted FCS and the appropriate  
538 amount of GW4869 to obtain final concentrations of 20  $\mu\text{M}$  and 40  $\mu\text{M}$ . Negative controls were incubated in  
539 culture media without the addition of GW4869 while DMSO controls were incubated in culture media containing  
540 the identical volume of DMSO as 40  $\mu\text{M}$  GW4869 stock solution in the treated samples. Since DMSO is itself  
541 toxic, the highest volume of DMSO without the inhibitor was used as a control to ensure potential outcomes  
542 were not due to DMSO toxicity. Cells were incubated at  $37^{\circ}\text{C}$  and 5% (v/v)  $\text{CO}_2$  for 6 hours. All conditions were  
543 carried out as 3 technical replicates per biological replicate.

544

545 **EV isolation using polymer-assisted precipitation.**

546 Conditioned cell culture medium was collected from transfected cells after 6 or 24 hours, depending on the  
547 experiment, and centrifuged at  $3,000 \times g$  at  $4^{\circ}\text{C}$  for 15 minutes to pellet dead cells and debris. For EV isolation  
548 500  $\mu\text{L}$  of the supernatants were transferred to fresh microcentrifuge tubes and the miRCURY<sup>®</sup> Exosome  
549 Cell/Urine/CSF kit (Qiagen) was used according to the manufacturer's protocol. Briefly, 200  $\mu\text{L}$  of precipitation  
550 buffer B was added to the supernatant and mixed thoroughly by vortexing. The samples were incubated at  $4^{\circ}\text{C}$

551 for one hour followed by centrifugation with  $10,000 \times g$  at room temperature for 30 minutes to pellet EVs. The  
552 supernatants were completely removed by gentle pipetting and stored for NLuc measurement when required.  
553 The remaining EV pellet was resuspended in filtered PBS. Samples were used directly for NTA analysis,  
554 measurement of the NLuc signal, or snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until usage.

555

#### 556 **Western blot analysis.**

557 Cell lysates of transfected and non-transfected control cells were prepared by scraping and resuspension in  
558 radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific) supplemented with 1X protease  
559 inhibitor (Roche). EV lysates were prepared by addition of RIPA buffer and 1X protease inhibitor to isolated EVs.  
560 Protein concentrations were determined using the Qubit 4 Fluorometer with the Qubit Protein Broad Range  
561 Assay Kit (Thermo Fisher). 15 or 40  $\mu\text{g}$  of protein were mixed with 1x NuPAGE LDS sample buffer (Thermo Fisher)  
562 and incubated at  $95^{\circ}\text{C}$  for 10 minutes. Protein preparations were loaded onto NuPAGE 4-12% Bis Tris gels  
563 (Invitrogen) for separation followed by blotting onto a 0.2  $\mu\text{m}$  pore size PVDF membrane (Invitrogen) in an iBlot  
564 3 Western Blot Transfer System device (Thermo Fisher Scientific) using the pre-set settings for broad-range  
565 protein transfer. Membranes were blocked with 5% (w/v) milk powder in PBS with 0.5% (v/v) Tween20 for 1  
566 hour at room temperature and subsequently incubated with the respective primary antibodies diluted in 5%  
567 (w/v) milk powder in PBS with 0.5% (v/v) Tween20 at  $4^{\circ}\text{C}$  overnight. The following day, membranes were  
568 washed three times with PBS with 0.5% (v/v) Tween20 for 10 minutes before incubation with the secondary  
569 antibody for 1 hour at room temperature. Washing steps were repeated as described above and membranes  
570 were developed by addition of 1-Step Ultra TMB Blotting Solution (Pierce, Thermo Fisher Scientific). Anti-CD9  
571 (Abcam, AB236630), Anti-CANX (Abcam, AB22595), Anti-CD81 (Abcam, AB109201), Anti-CD63 (Abcam,  
572 AB271286 and AB134045) Anti-Lamin A (Cell Signaling Technology, 133A2), and Anti-ALIX (Cell Signaling  
573 Technology, 3A9) were used as primary antibodies. The secondary antibodies used were goat Anti-Rabbit IgG  
574 H&L (HRP) (Abcam, AB6721) and horse Anti-mouse IgG (HRP) (Cell Signaling Technology, 7076S).

575

#### 576 **Lysis of EVs.**

577 Conditioned cell culture media was collected from transfected cells after 24 hours and centrifuged with  $3,000 \times$   
578  $g$  at  $4^{\circ}\text{C}$  for 15 minutes to pellet dead cells and debris. 400  $\mu\text{L}$  of the supernatants were treated with 10% (v/v)  
579 Triton-X 100 at a final concentration of 0.2% (v/v) for 15 minutes at room temperature. During this time samples  
580 were inverted and vortexed regularly. After incubation, 300  $\mu\text{L}$  of the sample was transferred to a new  
581 microcentrifuge tube for EV isolation using the miRCURY<sup>®</sup> Exosome Cell/Urine/CSF kit (Qiagen) as described  
582 earlier.

583

584 **Measurement of the NLuc activity.**

585 NLuc activity was measured from cell culture media and isolated EVs from transfected cells using the NanoGlo®  
586 Luciferase Assay System (Promega GmbH, Germany) according to the manufacturer's protocol with slight  
587 modifications. Briefly, 10 µL of sample and 15 µL of dH<sub>2</sub>O were pipetted into the wells of a white, flat-bottom 96  
588 well plate. The assay reagent was prepared by mixing 2 µL of NanoGlo® Luciferase Assay Substrate with 100 µL  
589 of NanoGlo® Luciferase Assay Buffer. 25 µL of the reagent was added to each well and mixed on a plate shaker.  
590 After 3 minutes of incubation at room temperature, the NLuc activity was examined by measurement of the  
591 luminescence signal using an Infinite M200 PRO plate reader (Tecan group Ltd.).

592

593 **Imaging flow cytometry.**

594 Imaging flow cytometry of isolated EVs from transfected cells was performed using an Amnis® ImageStream<sup>x</sup>  
595 MKII device. Briefly, cells were transfected and washed as described above. Conditioned cell culture media from  
596  $2.4 \times 10^6$  cells per condition and construct was collected after 24 hours and EVs were isolated via polymer-  
597 assisted precipitation. Isolated EVs were resuspended in 100 µl 0.22 µm filtered Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS (Gibco) and  
598 stored at -20°C. EVs from non-transfected cells served as negative control. The device was calibrated before  
599 each use. The magnification was set to 60x, flow speed to low, and sensitivity to high. Brightfield images were  
600 obtained in channel 4 and 10, green fluorescence (488 nm) in channel 2, and the side scatter (SSC, 785 nm) in  
601 channel 6. Laser intensities were adjusted to 120 mW for the green laser and 5.16 mW for the SSC. All events  
602 including the device-internal speed beads were acquired, and the number of events was set to 20,000 per  
603 sample. Analysis of the data was performed using the Image Data Exploration and Analysis Software (IDEAS®,  
604 Amnis), version 6.2. Briefly, EVs were considered to appear as particles without a visible brightfield signal, hence  
605 the area of the brightfield signal was plotted against the intensity of the brightfield signal. This allowed us to  
606 exclude all events with a brightfield signal from the sample population and included the device-internal  
607 SpeedBeads as well as clumps of cellular material. Subsequently, to visualise GFP-labelled EVs, the signal  
608 intensity of the GFP channel was plotted against the normalized frequency of the occurring events. Within these  
609 plots a cut-off was set at  $1 \times 10^3$  for particles to be assessed as GFP-positive while particles with GFP intensity  
610 signals below this threshold were considered to be GFP-negative.

611

612 **Treatment of fungal hyphae with reporter gene-expressing EVs.**

613 *A. fumigatus* conidia were washed by centrifugation at  $1800 \times g$  and 4°C for 5 minutes, resuspended in sterile  
614 dH<sub>2</sub>O and the concentration was determined using a Thoma haemocytometer.  $2 \times 10^4$  spores in 300 µL RPMI

615 were pipetted into a glass bottom channel slide ( $\mu$ -Slide Luer, 0.6, Glass Bottom, Ibidi) and incubated at 37°C and  
616 5% (v/v) CO<sub>2</sub> for 8 hours or overnight to allow for germination or the formation of hyphae, respectively. For EV  
617 treatment, 150  $\mu$ L of the spent media was removed from the channel slide and isolated EVs from  $2.4 \times 10^6$   
618 transfected cells resuspended in 150  $\mu$ L fresh RPMI were added to the hyphae. Due to challenges in quantifying  
619 the exact number of tagged extracellular vesicles, we can only approximate the number of particles delivered to  
620 *A. fumigatus* based on our transfection efficiency and quantification of total particles by NTA. We used a ratio of  
621 roughly 250 to 1 for cells to fungus, but as we expect only 10-15% of cells to be transfected and many EVs to be  
622 lost during isolation, we expect to have only a minor excess of EVs to fungal hyphae in the actual experiment. To  
623 achieve a more homogenous distribution of the EVs within the channel slide, 150  $\mu$ L media was removed from  
624 the channel opening on the right-side and re-added into the left-side opening of the channel. This step was  
625 repeated 5 times for every sample. Negative controls were treated similarly but with cell culture media lacking  
626 labelled EVs. Channel slides were then incubated at 37°C and 5% (v/v) CO<sub>2</sub> for a total of 24 hours prior to being  
627 subjected to CLSM imaging using a Zeiss LSM 780 confocal microscope with a 63x oil immersion objective lens  
628 with a numerical aperture of 1.40, a refractive index of  $n=1.518$ , and voxel size of  $188 \times 188 \times 850$  nm. Evaluation  
629 of the images was performed using the Zen software (Carl Zeiss). Live cell imaging was performed by addition of  
630 labelled EVs to *A. fumigatus* germlings as described above. Samples were incubated in the incubation chamber  
631 of the Zeiss LSM 780 confocal microscope at 37°C with 5% (v/v) CO<sub>2</sub>. A pre-set z-stack of images was captured  
632 every 30 minutes using the 63x oil immersion objective lens with a numerical aperture of 1.40 and a refractive  
633 index of  $n=1.518$ .

634

#### 635 **Treatment of fungal hyphae with stained EVs.**

636  $1 \times 10^4$  conidia in 200  $\mu$ L RPMI were seeded into the chambers of an 8-well microscopy slide ( $\mu$ -Slide 8 Well  
637 Polymer Coverslip, Ibidi) and incubated at 37°C and 5% (v/v) CO<sub>2</sub> for 8 hours to allow for germination. After the  
638 incubation time, MemGlow 560 (Cytoskeleton) was used to stain concentrated EV samples isolated from  $1 \times 10^7$   
639 non-transfected A549 cells per sample by SEC. More precisely, MemGlow 560 was added to the sample to reach  
640 a final concentration of 200 nM and the sample was directly mixed thoroughly by vortexing and immediately  
641 thereafter added into the media of the respective wells of the microscopy slide. The samples were then mixed  
642 by carefully pipetting up and down and the microscopy slide was returned to 37°C and 5% (v/v) CO<sub>2</sub> for 16 hours.  
643 Control samples were treated with MemGlow 560 diluted in unconditioned media to a final concentration of  
644 200 nM. The following day hyphae were stained with Calcofluor white, subjected to CLSM imaging using a Zeiss  
645 LSM 780 confocal microscope with a 63x oil immersion objective lens with a numerical aperture of 1.40, a

646 refractive index of  $n=1.518$ , and a voxel size of  $132 \times 132 \times 400$  nm. Images were evaluated with the Zen software  
647 (Carl Zeiss).

648

#### 649 **Imaging Analysis.**

650 Three-dimensional images were provided in the CZI (Carl Zeiss Image) native microscopy format. The main steps  
651 of the image processing and quantification are summarized in **Sup. Fig 6**. At first, the raw 3D images were  
652 deconvolved using the Huygens Professional software (SVI, Hilversum, The Netherlands, <https://www.svi.nl>),  
653 applying a measured point spread function detected from the actual samples using 170 nm fluorescent plastic  
654 beads with excitation and emission wavelengths that matched those of the biological samples. The deconvolved  
655 images were saved in TIFF format and proceeded to Imaris (Bitplane, Zürich, Switzerland,  
656 <https://www.bitplane.com>) for further analysis. Here Imaris 10.2 was applied, where we used batch processing  
657 scripts to segment all components, including the hyphal wall, EVs, and the mitochondria. Each processed image  
658 was manually checked for possible segmentation errors. The hyphal segmentation revealed the actual wall  
659 boundaries, allowing the precise measurement and visualisation of the EVs' spatial position relative to the  
660 hyphal cell wall. Furthermore, EVs were quantified after segmentation as Spots objects in Imaris, where they  
661 were allowed to have various radii, in order to account for the possible presence of unresolvable small EV  
662 clusters (**Fig. 6**).

663

#### 664 **Data Availability.**

665 The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the  
666 PRIDE (Perez-Riverol *et al.* 2022) partner repository with the dataset identifier PXD050398.

667

#### 668 **Statistical Analysis.**

669 GraphPad Prism 10.1.0 software was used for data plotting and statistical analysis. The Student's t test was used  
670 for the comparison of two groups and one-way or two-way ANOVA for the comparison of multiple groups. All  
671 bar graphs are depicted with standard deviation of the mean (SEM). Significance was defined as follows: \*,  
672  $p = <0.05$ ; \*\*,  $p = <0.01$ ; \*\*\*,  $p = <0.001$ ; ns, not significant.

673

#### 674 **ACKNOWLEDGEMENTS**

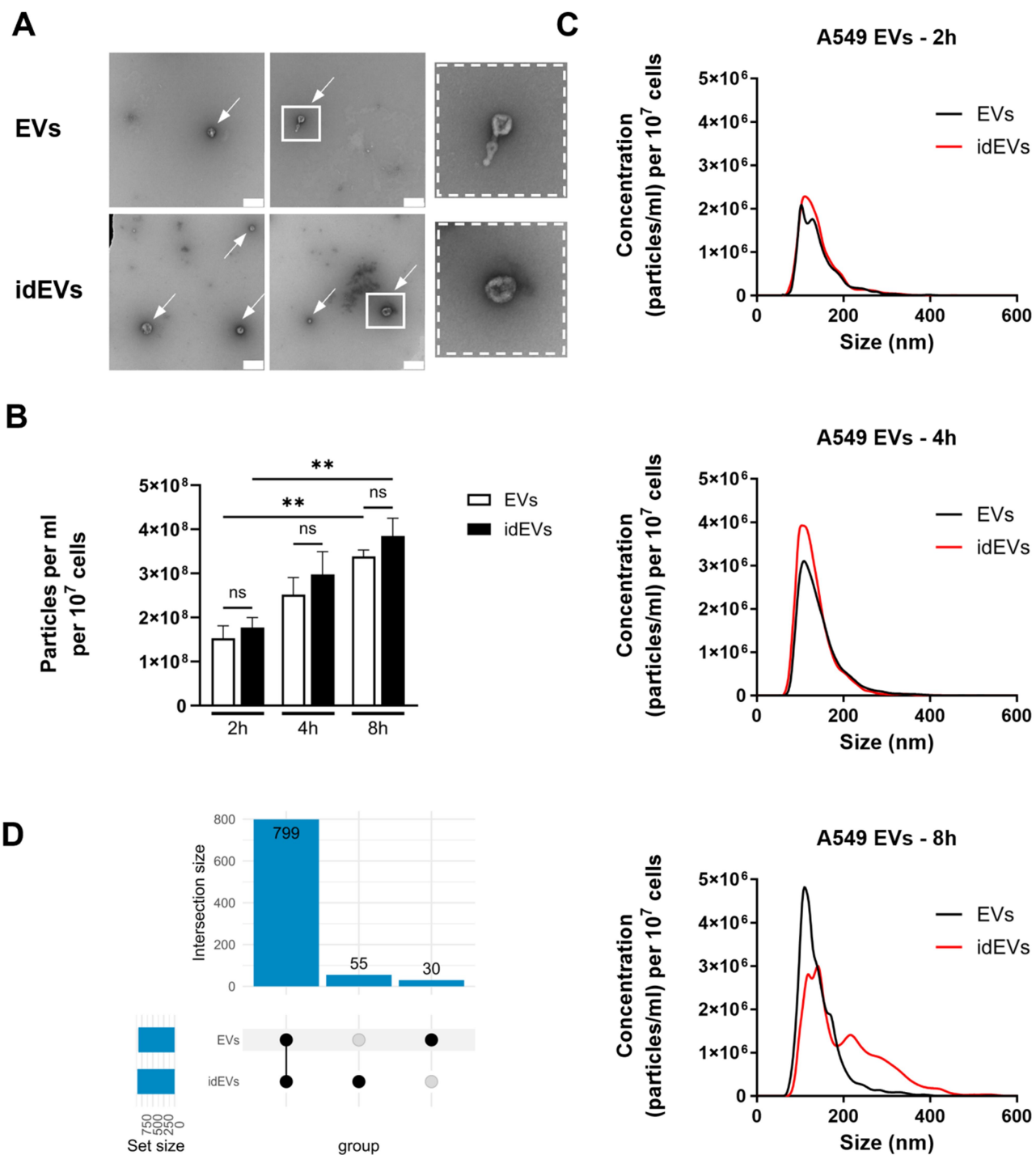
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683 manuscript. The authors declare no conflicts of interest.

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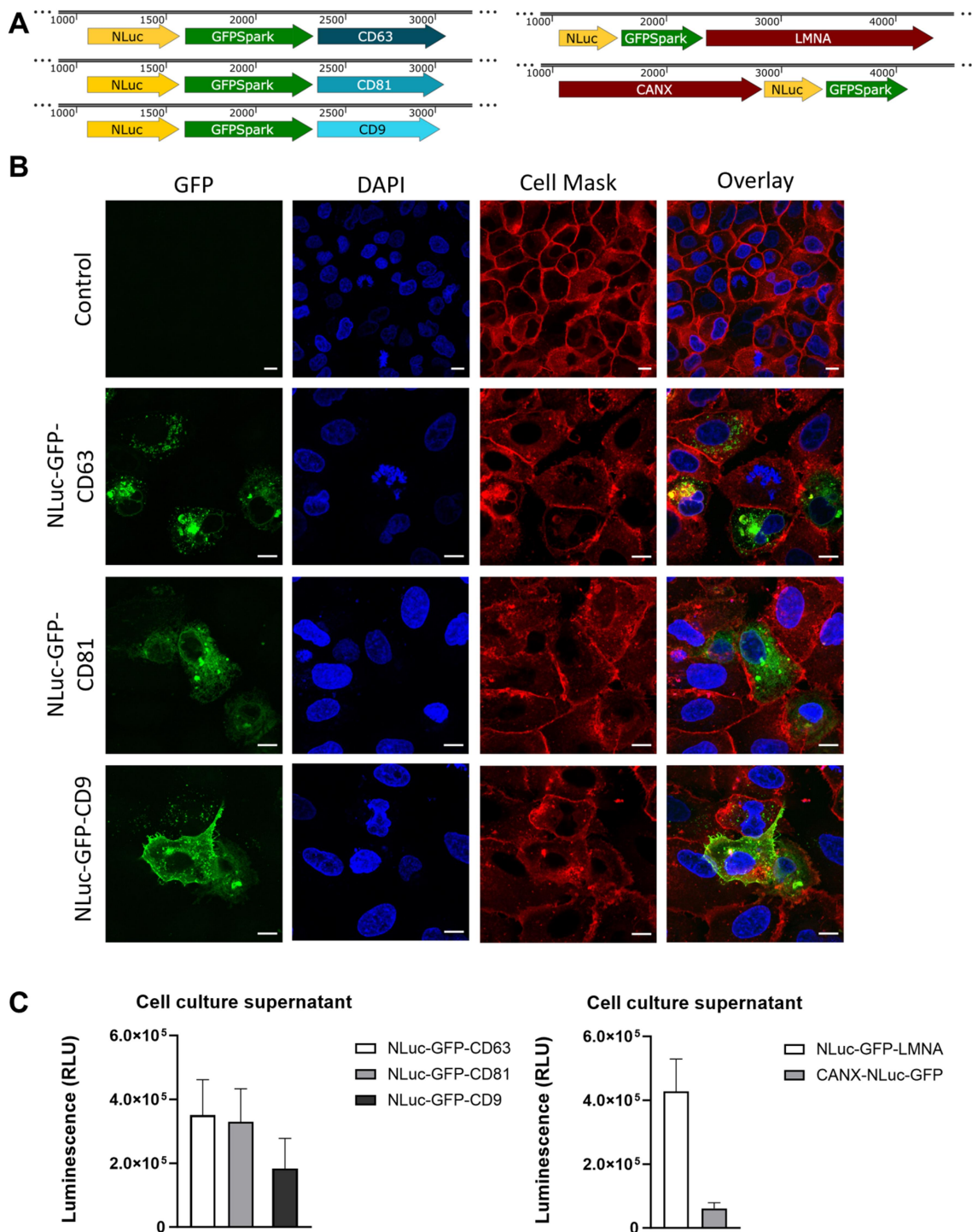
688 **Fig 1.**

689 **A)** Representative transmission electron microscopy images of A549 EVs (arrows). Upper lane: EVs were isolated  
 690 from cell culture supernatants of untreated cells (spontaneously produced EVs; EVs). Lower lane: EVs were  
 691 isolated from cell culture supernatants of cells previously infected with *A. fumigatus* conidia (*A. fumigatus*-



692 induced EVs; idEVs). Scale bar: 200 nm. **B)** Concentration of particles isolated from cell culture supernatants of  
693 untreated or infected A549 cells at different time points. **C)** Size distribution of the isolated particles.  
694 Concentration and sizes of isolated EVs were measured using NTA. **D)** Upset R plot showing the intersection of  
695 proteins detected by LC-MS/MS proteomic analysis of EVs isolated from A549 cells either left untreated (EVs) or  
696 infected with *A. fumigatus* (idEVs). Data obtained from 3 biological replicates. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; ns, not  
697 significant.  
698

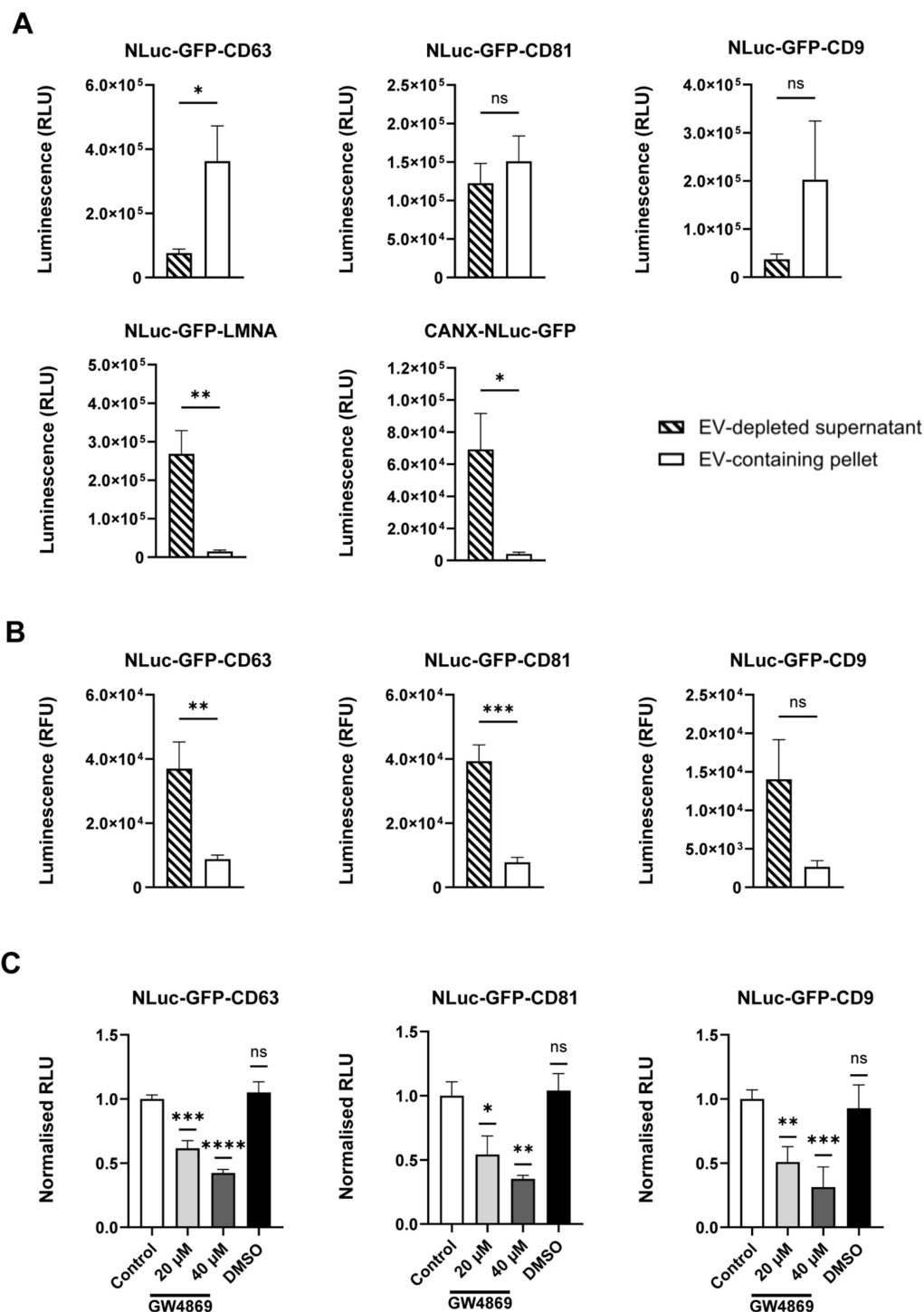
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700  
701 **Fig 2.**

702 **A)** Scheme of the fusion proteins. The tetraspanins CD63, CD81, and CD9, and LMNA were N-terminally tagged  
703 while CANX was C-terminally tagged with a Nanoluciferase (NLuc) and a GFPSpark (short: GFP). **B)** CLSM analysis

704 revealed the expression of the different tetraspanin fusion proteins within transfected A549 epithelial cells  
705 based on the GFP signal. Non-transfected cells imaged with the same settings for GFP showed no background  
706 signal or autofluorescence. Nuclei of the cells were stained with DAPI and the cell membrane was stained using  
707 Cell Mask deep red. Scale bar: 10  $\mu\text{m}$ . **C)** Measurement of the luminescence signal in the cell culture supernatant  
708 of transfected cells. Positive signals were obtained for cells transfected with either of the plasmids encoding the  
709 tetraspanin as well as control fusion proteins. Results were obtained from at least 4 biological replicates.  
710

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712

713

Fig 3.

714

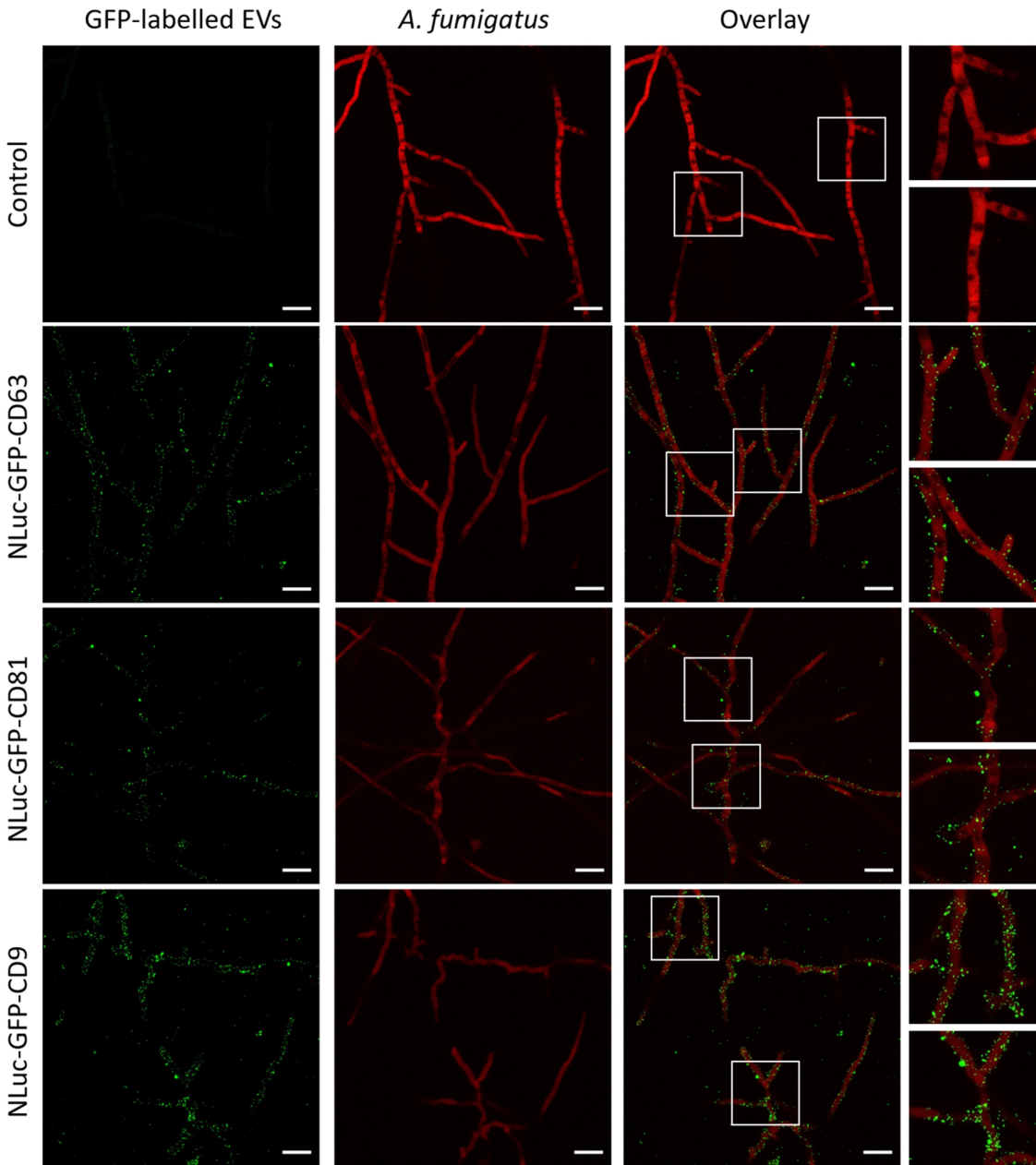
**A)** Measurement of the luminescence signal in the EV-depleted supernatant compared to the EV-enriched pellet after EV isolation from transfected A549 epithelial cells using a polymer-precipitation based method. EV

715

716 isolation led to an increased luminescence signal deriving from the EV-containing pellet compared to the EV-  
717 depleted supernatant in cases of cells expressing the tetraspanin fusion proteins. EV isolation from cell culture  
718 media of cells expressing the LMNA and CANX control fusion proteins resulted in most of the signal deriving  
719 from the EV-depleted supernatant. **B)** Measurement of the luminescence signal in the EV-depleted supernatant  
720 and EV-containing pellet of A549 EVs treated with Triton X-100 prior to EV isolation. Lysis of EVs resulted in a  
721 shift of the luminescence signal from the pellet to the supernatant. **C)** Measurement of the luminescence signal  
722 in the EV-containing pellet after EV isolation from transfected A549 cells treated with GW4869 or DMSO as a  
723 control. Treatment of cells with GW4869 resulted in a dose-dependent decrease of the luminescence signal in  
724 the pellet. Results of isolated and lysed EVs were obtained from at least 4 biological replicates. Inhibitor assays  
725 using GW4869 were performed as three biological replicates \*,  $p = <0.05$ ; \*\*,  $p = <0.01$ ; \*\*\*,  $p = <0.001$ , \*\*\*\*,  $p$   
726 =  $<0.0001$ ; ns, not significant.

727

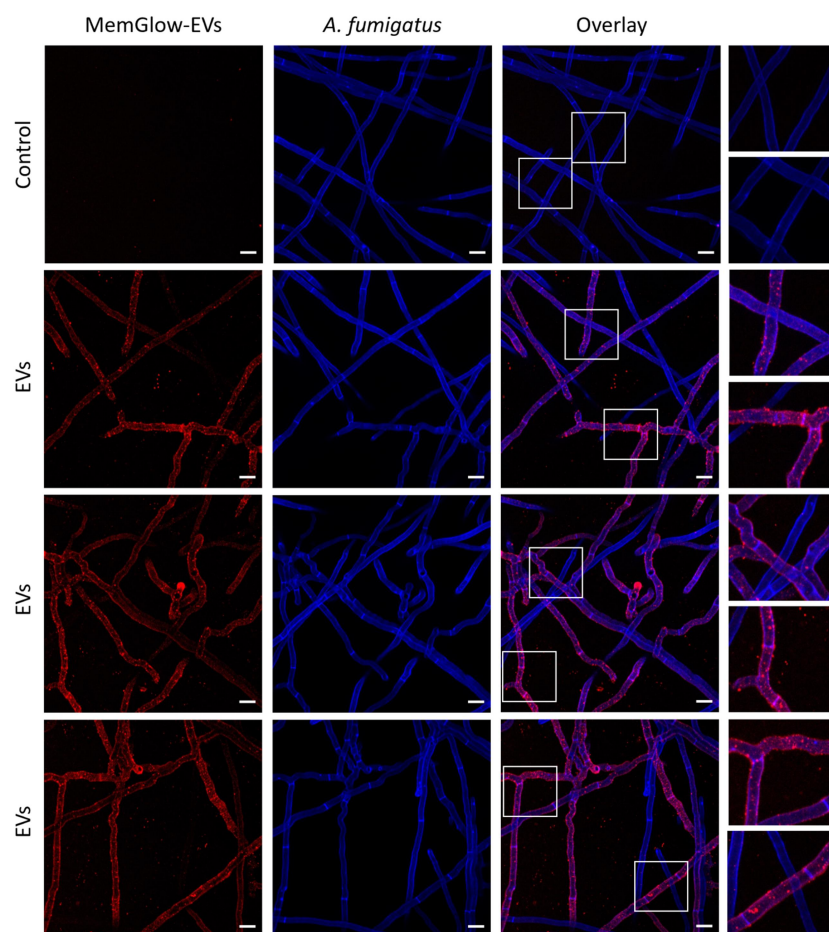
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729 **Fig 4.**

730 CLSM images revealing that labelled A549 EVs can be visualised and are able to associate with *A. fumigatus*  
 731 hyphae. Resuspended EVs isolated from transfected cells expressing the NLuc-GFP-labelled tetraspanins were  
 732 added to 7 hour-old *A. fumigatus* dTomato germlings in a host cell-free system and incubated overnight. EVs  
 733 appeared as green-fluorescent dots with varying sizes. *A. fumigatus* treated with EVs isolated from non-  
 734 transfected cells were used a control. Images were taken using a Zeiss LSM 780 confocal microscope. Scale bar:  
 735 20  $\mu$ m. Representative images from >3 biological replicates per construct.



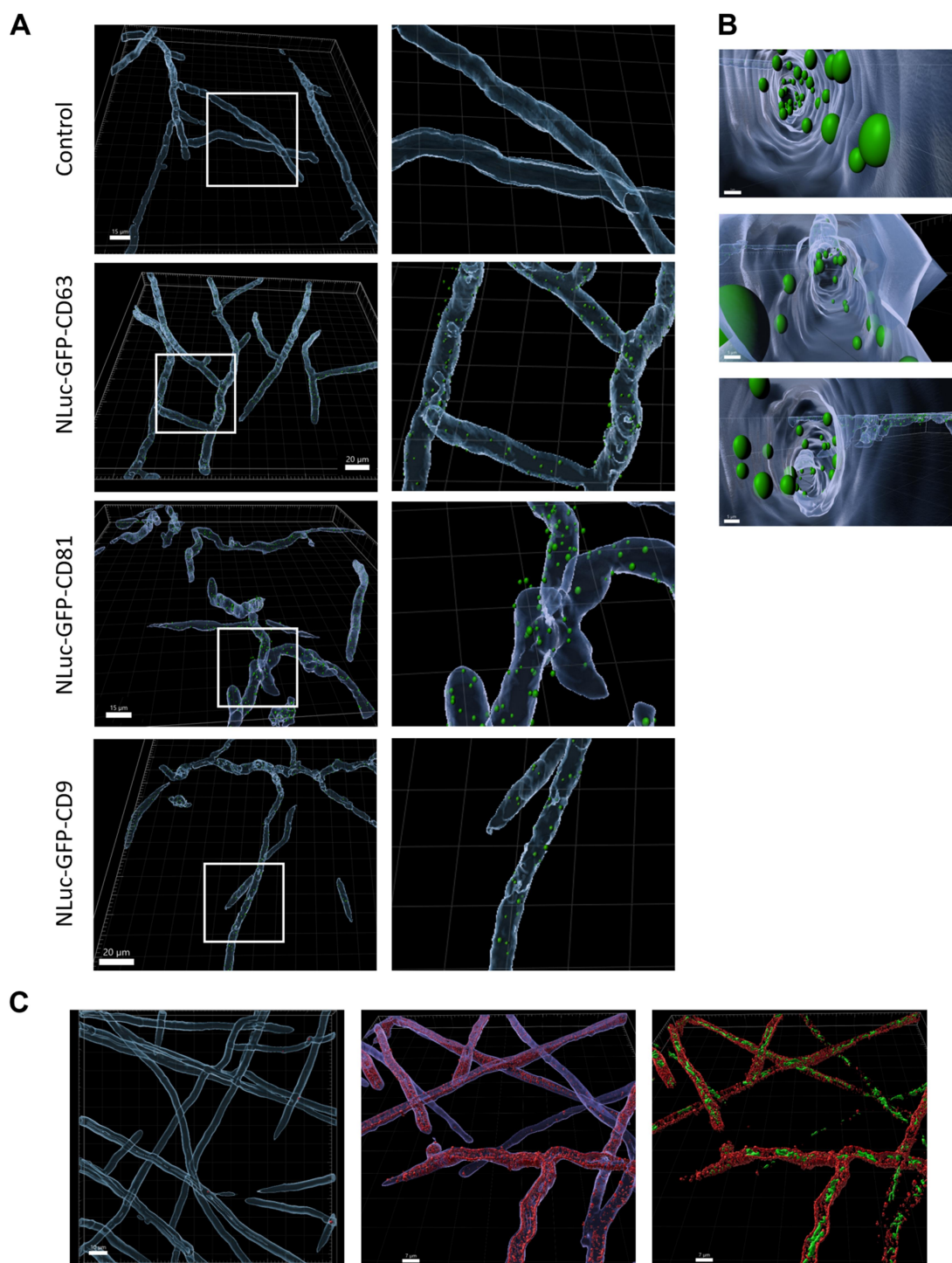
737

738 **Fig 5.**

739 Representative CLSM images of stained EVs co-localising with the *A. fumigatus* strain Afs35. EVs were isolated  
 740 from non-transfected cells using size exclusion chromatography and stained with MemGlow 560 prior to  
 741 addition to 7 hour-old *A. fumigatus* germlings in a host cell-free system. After overnight incubation hyphal cell  
 742 walls were stained with calcofluor white (CFW) and subjected to CLSM. EVs appeared as red fluorescent dot-like  
 743 signals with varying sizes. Control hyphae treated with MemGlow 560 in PBS exhibit no staining. Images were  
 744 taken using a Zeiss LSM 780 confocal microscope. Scale bar: 20  $\mu$ m. Representative images from three biological  
 745 replicates.

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749

**Fig 6.**

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**A)** 3D reconstruction of *A. fumigatus* hyphae CLSM images reveals the association of labelled EVs depicted as green dots with hyphae. Since the *A. fumigatus* AfS150/ pSK537 strain expressing a cytoplasmic dTomato protein was used here, the blue signal is equivalent with the limiting border of the hyphal lumen and thus the



753 hyphal membrane. **B)** Magnification of the 3D reconstructed hyphae confirms the association and partly  
754 internalization of NLuc-GFP-tetraspanin labelled EVs into the hyphal lumen of *A. fumigatus*. **C)** MemGlow  
755 stained EVs (in red) accumulate within *A. fumigatus* hyphae as a tube-like structure underneath the cell wall  
756 (depicted in blue), likely accumulating at the hyphal cell membrane. The finding is supported upon the  
757 reconstruction of the GFP-tagged mitochondria (in green).  
758

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- 760 Abels, E. R., and X. O. Breakefield. 2016. 'Introduction to extracellular vesicles: biogenesis, RNA cargo selection,  
761 content, release, and uptake', *Cell Mol Neurobiol*, 36: 301-312.
- 762 Amin, S., A. Thywissen, T. Heinekamp, H. P. Saluz, and A. A. Brakhage. 2014. 'Melanin dependent survival of  
763 *Aspergillus fumigatus* conidia in lung epithelial cells', *Int J Med Microbiol*, 304: 626-636.
- 764 Anderson, Corey L., Emma R. Langer, Timothy C. Routes, Seamus F. McWilliams, Igor Bereslavskyy, Timothy J.  
765 Kamp, and Lee L. Eckhardt. 2021. 'Most myopathic lamin variants aggregate: a functional genomics  
766 approach for assessing variants of uncertain significance', *npj Genomic Medicine*, 6: 103.
- 767 Bonsergent, Emeline, Eleonora Grisard, Julian Buchrieser, Olivier Schwartz, Clotilde Théry, and Grégory Lavieu.  
768 2021. 'Quantitative characterization of extracellular vesicle uptake and content delivery within  
769 mammalian cells', *Nat Commun*, 12: 1864.
- 770 Brakhage, A. A. 2005. 'Systemic fungal infections caused by *Aspergillus* species: epidemiology, infection process  
771 and virulence determinants', *Curr Drug Targets*, 6: 875-886.
- 772 Brakhage, A. A., A. K. Zimmermann, F. Riviuccio, C. Visser, and M. G. Blango. 2021. 'Host-derived extracellular  
773 vesicles for antimicrobial defense', *Microlife*, 2: uqab003.
- 774 Cai, Qiang, Lulu Qiao, Ming Wang, Baoye He, Feng-Mao Lin, Jared Palmquist, Sienna-Da Huang, and Hailing Jin.  
775 2018. 'Plants send small RNAs in extracellular vesicles to fungal pathogen to silence virulence genes',  
776 360: 1126-1129.
- 777 Cashikar, A. G., and P. I. Hanson. 2019. 'A cell-based assay for CD63-containing extracellular vesicles', *PLoS One*,  
778 14: e0220007.
- 779 Catalano, M., and L. O'Driscoll. 2020. 'Inhibiting extracellular vesicles formation and release: a review of EV  
780 inhibitors', *J Extracell Vesicles*, 9: 1703244.
- 781 Chuo, Steven Ting-Yu, Jasper Che-Yung Chien, and Charles Pin-Kuang Lai. 2018. 'Imaging extracellular vesicles:  
782 current and emerging methods', *Journal of Biomedical Science*, 25: 91.
- 783 Corso, G., W. Heusermann, D. Trojer, A. Görgens, E. Steib, J. Voshol, A. Graff, C. Genoud, Y. Lee, J. Hean, J. Z.  
784 Nordin, O. P. B. Wiklander, S. El Andaloussi, and N. Meisner-Kober. 2019. 'Systematic characterization of  
785 extracellular vesicle sorting domains and quantification at the single molecule - single vesicle level by  
786 fluorescence correlation spectroscopy and single particle imaging', *J Extracell Vesicles*, 8: 1663043.
- 787 Denning, D. W. 2024. 'Global incidence and mortality of severe fungal disease', *Lancet Infect Dis*.
- 788 Eppler, Felix, Verena Semmling, Cora Schild, Yahya Homsy, Shoshana Levy, Thorsten Lang, Christian Kurts, and  
789 Waldemar Kolanus. 2011. 'CD81 is essential for the formation of membrane protrusions and regulates  
790 Rac1-activation in adhesion-dependent immune cell migration', *Blood*, 118: 1818-1827.
- 791 Escobar, N., S. R. Ordonez, H. A. Wösten, P. J. Haas, H. de Cock, and H. P. Haagsman. 2016. 'Hide, keep quiet, and  
792 keep low: Properties that make *Aspergillus fumigatus* a successful lung pathogen', *Front Microbiol*, 7:  
793 438.
- 794 Escobar, Natalia, Ivan D. Valdes, Esther M. Keizer, Soledad R. Ordonez, Robin A. Ohm, Han A. B. Wösten, and  
795 Hans de Cock. 2018. 'Expression profile analysis reveals that *Aspergillus fumigatus* but not *Aspergillus*  
796 *niger* makes type II epithelial lung cells less immunological alert', *BMC Genomics*, 19: 534.
- 797 Essandoh, K., L. Yang, X. Wang, W. Huang, D. Qin, J. Hao, Y. Wang, B. Zingarelli, T. Peng, and G. C. Fan. 2015.  
798 'Blockade of exosome generation with GW4869 dampens the sepsis-induced inflammation and cardiac  
799 dysfunction', *Biochim Biophys Acta*, 1852: 2362-2371.
- 800 Ewald, J., F. Riviuccio, L. Radosa, S. Schuster, A. A. Brakhage, and C. Kaleta. 2021. 'Dynamic optimization reveals  
801 alveolar epithelial cells as key mediators of host defense in invasive aspergillosis', *PLoS Comput Biol*, 17:  
802 e1009645.
- 803 Fan, Yé, Cédric Pionneau, Federico Cocozza, Pierre-Yves Boëlle, Solenne Chardonnet, Stéphanie Charrin, Clotilde  
804 Théry, Pascale Zimmermann, and Eric Rubinstein. 2023. 'Differential proteomics argues against a general  
805 role for CD9, CD81 or CD63 in the sorting of proteins into extracellular vesicles', 12: 12352.

806 Görgens, A., M. Bremer, R. Ferrer-Tur, F. Murke, T. Tertel, P. A. Horn, S. Thalmann, J. A. Welsh, C. Probst, C.  
807 Guerin, C. M. Boulanger, J. C. Jones, H. Hanenberg, U. Erdbrügger, J. Lannigan, F. L. Ricklefs, S. El-  
808 Andaloussi, and B. Giebel. 2019. 'Optimisation of imaging flow cytometry for the analysis of single  
809 extracellular vesicles by using fluorescence-tagged vesicles as biological reference material', *J Extracell*  
810 *Vesicles*, 8: 1587567.

811 Gupta, D., X. Liang, S. Pavlova, *et al.* 2020. 'Quantification of extracellular vesicles in vitro and in vivo using  
812 sensitive bioluminescence imaging', *J Extracell Vesicles*, 9: 1800222.

813 Hall, M. P., J. Unch, B. F. Binkowski, M. P. Valley, B. L. Butler, M. G. Wood, P. Otto, K. Zimmerman, G. Vidugiris, T.  
814 Machleidt, M. B. Robers, H. A. Benink, C. T. Eggers, M. R. Slater, P. L. Meisenheimer, D. H. Klaubert, F.  
815 Fan, L. P. Encell, and K. V. Wood. 2012. 'Engineered luciferase reporter from a deep sea shrimp utilizing a  
816 novel imidazopyrazinone substrate', *ACS Chem Biol*, 7: 1848-1857.

817 Han, C., H. Kang, J. Yi, M. Kang, H. Lee, Y. Kwon, J. Jung, J. Lee, and J. Park. 2021. 'Single-vesicle imaging and co-  
818 localization analysis for tetraspanin profiling of individual extracellular vesicles', *J Extracell Vesicles*, 10:  
819 e12047.

820 Headland, Sarah E., Hefin R. Jones, Adelina S. V. D'Sa, Mauro Perretti, and Lucy V. Norling. 2014. 'Cutting-edge  
821 analysis of extracellular microparticles using ImageStreamX imaging flow cytometry', *Sci Rep*, 4: 5237.

822 Heinekamp, T., H. Schmidt, K. Lapp, V. Pätz, I. Shopova, N. Köster-Eiserfunke, T. Krüger, O. Kniemeyer, and A. A.  
823 Brakhage. 2015. 'Interference of *Aspergillus fumigatus* with the immune response', *Semin*  
824 *Immunopathol*, 37: 141-152.

825 Hikita, T., M. Miyata, R. Watanabe, and C. Oneyama. 2018. 'Sensitive and rapid quantification of exosomes by  
826 fusing luciferase to exosome marker proteins', *Sci Rep*, 8: 14035.

827 Hikita, T., M. Miyata, R. Watanabe, and C. Oneyama. 2020. 'In vivo imaging of long-term accumulation of cancer-  
828 derived exosomes using a BRET-based reporter', *Sci Rep*, 10: 16616.

829 Jia, L. J., M. Rafiq, L. Radosa, P. Hortschansky, C. Cunha, Z. Cseresnyés, T. Krüger, F. Schmidt, T. Heinekamp, M.  
830 Straßburger, B. Löffler, T. Doenst, J. F. Lacerda, A. Campos, Jr., M. T. Figge, A. Carvalho, O. Kniemeyer,  
831 and A. A. Brakhage. 2023. '*Aspergillus fumigatus* hijacks human p11 to redirect fungal-containing  
832 phagosomes to non-degradative pathway', *Cell Host Microbe*, 31: 373-388.e310.

833 Jia, Xiaodong, Fangyan Chen, Weihua Pan, Rentao Yu, Shuguang Tian, Gaige Han, Haiqin Fang, Shuo Wang, Jingya  
834 Zhao, Xianping Li, Dongyu Zheng, Sha Tao, Wanqing Liao, Xuelin Han, and Li Han. 2014. 'Gliotoxin  
835 promotes *Aspergillus fumigatus* internalization into type II human pneumocyte A549 cells by inducing  
836 host phospholipase D activation', *Microbes and Infection*, 16: 491-501.

837 Józefowski, Szczepan, Maciej Czerkies, Anna Łukasik, Alicja Bielawska, Jacek Bielawski, Katarzyna Kwiatkowska,  
838 and Andrzej Sobota. 2010. 'Ceramide and ceramide 1-phosphate are negative regulators of TNF- $\alpha$   
839 production induced by lipopolysaccharide', *The Journal of Immunology*, 185: 6960-6973.

840 Jun, Mi-Hee, Young-Wu Jun, Kun-Hyung Kim, Jin- A. Lee, and Deok-Jin Jang. 2014. 'Characterization of the  
841 cellular localization of C4orf34 as a novel endoplasmic reticulum resident protein', *BMB reports*, 47.

842 Jurgielewicz, B. J., Y. Yao, and S. L. Stice. 2020. 'Kinetics and Specificity of HEK293T Extracellular Vesicle Uptake  
843 using Imaging Flow Cytometry', *Nanoscale Res Lett*, 15: 170.

844 Kakarla, Ramesh, Jaehark Hur, Yeon Ji Kim, Jaeyoung Kim, and Yong-Joon Chwae. 2020. 'Apoptotic cell-derived  
845 exosomes: messages from dying cells', *Experimental & Molecular Medicine*, 52: 1-6.

846 Köhler, J. R., B. Hube, R. Puccia, A. Casadevall, and J. R. Perfect. 2017. 'Fungi that infect humans', *Microbiol*  
847 *Spectr*, 5.

848 Kowal, J., G. Arras, M. Colombo, M. Jouve, J. P. Morath, B. Primdal-Bengtson, F. Dingli, D. Loew, M. Tkach, and C.  
849 Théry. 2016. 'Proteomic comparison defines novel markers to characterize heterogeneous populations  
850 of extracellular vesicle subtypes', *Proc Natl Acad Sci U S A*, 113: E968-977.

851 Kulshreshtha, A., T. Ahmad, A. Agrawal, and B. Ghosh. 2013. 'Proinflammatory role of epithelial cell-derived  
852 exosomes in allergic airway inflammation', *J Allergy Clin Immunol*, 131: 1194-1203, 1203.e1191-1114.

853 Latgé, J. P., and G. Chamilos. 2019. '*Aspergillus fumigatus* and aspergillosis in 2019', *Clin Microbiol Rev*, 33.

854 Lázaro-Ibáñez, E., F. N. Faruqu, A. F. Saleh, A. M. Silva, J. Tzu-Wen Wang, J. Rak, K. T. Al-Jamal, and N. Dekker.  
855 2021. 'Selection of fluorescent, bioluminescent, and radioactive tracers to accurately reflect extracellular  
856 vesicle biodistribution *in vivo*', *ACS Nano*.

857 Levy, Daniel, Mai Anh Do, Annie Brown, Kyle Asano, David Diebold, Hanzhe Chen, Jiayi Zhang, Brendan Lawler,  
858 and Biao Lu. 2020. 'Chapter One - Genetic labeling of extracellular vesicles for studying biogenesis and  
859 uptake in living mammalian cells.' in Sheila Spada and Lorenzo Galluzzi (eds.), *Methods in Enzymology*  
860 (Academic Press).

861 Lionakis, M. S., R. A. Drummond, and T. M. Hohl. 2023. 'Immune responses to human fungal pathogens  
862 and therapeutic prospects', *Nat Rev Immunol*, 23: 433-452.

863 Lischnig, A., M. Bergqvist, T. Ochiya, and C. Lässer. 2022. 'Quantitative proteomics identifies proteins enriched in  
864 large and small extracellular vesicles', *Mol Cell Proteomics*, 21: 100273.

865 Loconte, L., D. Arguedas, R. El, A. Zhou, A. Chipont, L. Guyonnet, C. Guerin, E. Piovesana, J. L. Vázquez-Ibar, A.  
866 Joliot, C. Théry, and L. Martín-Jaular. 2023. 'Detection of the interactions of tumour derived extracellular  
867 vesicles with immune cells is dependent on EV-labelling methods', *J Extracell Vesicles*, 12: e12384.

868 Lórinicz Á, M., M. Schütte, C. I. Timár, D. S. Veres, Á Kittel, K. R. McLeish, M. L. Merchant, and E. Ligeti. 2015.  
869 'Functionally and morphologically distinct populations of extracellular vesicles produced by human  
870 neutrophilic granulocytes', *J Leukoc Biol*, 98: 583-589.

871 Lothar, Jasmin, Tanja Breitschopf, Sven Krappmann, C. Oliver Morton, Maria Bouzani, Oliver Kurzai, Matthias  
872 Gunzer, Mike Hasenberg, Hermann Einsele, and Juergen Loeffler. 2014. 'Human dendritic cell subsets  
873 display distinct interactions with the pathogenic mould *Aspergillus fumigatus*', *International Journal of*  
874 *Medical Microbiology*, 304: 1160-1168.

875 Lötvall, J., A. F. Hill, F. Hochberg, E. I. Buzás, D. Di Vizio, C. Gardiner, Y. S. Gho, I. V. Kurochkin, S. Mathivanan, P.  
876 Quesenberry, S. Sahoo, H. Tahara, M. H. Wauben, K. W. Witwer, and C. Théry. 2014. 'Minimal  
877 experimental requirements for definition of extracellular vesicles and their functions: a position  
878 statement from the International Society for Extracellular Vesicles', *J Extracell Vesicles*, 3: 26913.

879 Mathieu, Mathilde, Nathalie Névo, Mabel Jouve, José Ignacio Valenzuela, Mathieu Maurin, Frederik J. Verweij,  
880 Roberta Palmulli, Danielle Lankar, Florent Dingli, Damaris Loew, Eric Rubinstein, Gaëlle Boncompain,  
881 Franck Perez, and Clotilde Théry. 2021. 'Specificities of exosome versus small ectosome secretion  
882 revealed by live intracellular tracking of CD63 and CD9', *Nat Commun*, 12: 4389.

883 Mondal, A., K. A. Ashiq, P. Phulpagar, D. K. Singh, and A. Shiras. 2019. 'Effective visualization and easy tracking of  
884 extracellular vesicles in glioma cells', *Biol Proced Online*, 21: 4.

885 Morandi, Mattia I., Petro Busko, Efrat Ozer-Partuk, Suman Khan, Giulia Zarfati, Yael Elbaz-Alon, Paula  
886 Abou Karam, Tina Napso Shogan, Lana Ginini, Ziv Gil, Neta Regev-Rudzki, and Ori Avinoam. 2022.  
887 'Extracellular vesicle fusion visualized by cryo-electron microscopy', *PNAS Nexus*, 1: pgac156.

888 Mulcahy, L. A., R. C. Pink, and D. R. Carter. 2014. 'Routes and mechanisms of extracellular vesicle uptake', *J*  
889 *Extracell Vesicles*, 3.

890 Myhill, Nathan, Emily M. Lynes, Jalal A. Nanji, Anastassia D. Blagoveshchenskaya, Hao Fei, Katia Carmine  
891 Simmen, Timothy J. Cooper, Gary Thomas, and Thomas Simmen. 2008. 'The subcellular distribution of  
892 calnexin is mediated by PACS-2', *Molecular Biology of the Cell*, 19: 2777-2788.

893 Paris, S., E. Boisvieux-Ulrich, B. Crestani, O. Houcine, D. Taramelli, L. Lombardi, and J. P. Latgé. 1997.  
894 'Internalization of *Aspergillus fumigatus* conidia by epithelial and endothelial cells', *Infect Immun*, 65:  
895 1510-1514.

896 Paskevicius, Tautvydas, Rabih Abou Farraj, Marek Michalak, and Luis B. Agellon. 2023. 'Calnexin, more than just  
897 a molecular chaperone', 12: 403.

898 Perez-Riverol, Yasset, Jingwen Bai, Chakradhar Bandla, David García-Seisdedos, Suresh Hewapathirana,  
899 Selvakumar Kamatchinathan, Deepti J Kundu, Ananth Prakash, Anika Frericks-Zipper, Martin Eisenacher,  
900 Mathias Walzer, Shengbo Wang, Alvis Brazma, and Juan Antonio Vizcaíno. 2022. 'The PRIDE database

resources in 2022: a hub for mass spectrometry-based proteomics evidences', *Nucleic Acids Research*, 50: D543-D552.

Rafiq, M., F. Riviuccio, A. K. Zimmermann, C. Visser, A. Bruch, T. Krüger, K. González Rojas, O. Kniemeyer, M. G. Blango, and A. A. Brakhage. 2022. 'PLB-985 neutrophil-like cells as a model to study *Aspergillus fumigatus* pathogenesis', *mSphere*, 7: e0094021.

Ricklefs, F. L., C. L. Maire, R. Reimer, *et al.* 2019. 'Imaging flow cytometry facilitates multiparametric characterization of extracellular vesicles in malignant brain tumours', *J Extracell Vesicles*, 8: 1588555.

Ruf, D., V. Brantl, and J. Wagener. 2018. 'Mitochondrial fragmentation in *Aspergillus fumigatus* as early marker of granulocyte killing activity', *Front Cell Infect Microbiol*, 8: 128.

Shopova, I. A., I. Belyaev, P. Dasari, *et al.* 2020. 'Human neutrophils produce antifungal extracellular vesicles against *Aspergillus fumigatus*', *mBio*, 11.

Shpigelman, Jonathan, Fitzgerald S. Lao, Shiyin Yao, Chenyang Li, Tetsuya Saito, Fumi Sato-Kaneko, John P. Nolan, Nikunj M. Shukla, Minya Pu, Karen Messer, Howard B. Cottam, Dennis A. Carson, Maripat Corr, and Tomoko Hayashi. 2021. 'Generation and application of a reporter cell line for the quantitative screen of extracellular vesicle release', 12.

Slivinschi, Bianca, Federico Manai, Carolina Martinelli, Francesca Carriero, Camilla D'Amato, Martina Massarotti, Giorgia Bresciani, Claudio Casali, Gloria Milanese, Laura Artal, Lisa Zanoletti, Federica Milélla, Davide Arfini, Alberto Azzalin, Sara Demartis, Elisabetta Gavini, and Sergio Comincini. 2022. 'Enhanced delivery of rose bengal by amino acids starvation and exosomes inhibition in human astrocytoma cells to potentiate anticancer photodynamic therapy effects', *Cells*, 11: 2502.

Strohmeier, K., M. Hofmann, F. Hauser, D. Sivun, S. Puthukodan, A. Karner, G. Sandner, P. E. Le Renard, J. Jacak, and M. Mairhofer. 2021. 'CRISPR/Cas9 genome editing vs. over-expression for fluorescent extracellular vesicle-labeling: a quantitative analysis', *Int J Mol Sci*, 23.

Théry, C., K. W. Witwer, E. Aikawa, *et al.* 2018. 'Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines', *J Extracell Vesicles*, 7: 1535750.

Timár, C. I., A. M. Lorincz, R. Csépanyi-Kömi, A. Vályi-Nagy, G. Nagy, E. I. Buzás, Z. Iványi, A. Kittel, D. W. Powell, K. R. McLeish, and E. Ligeti. 2013. 'Antibacterial effect of microvesicles released from human neutrophilic granulocytes', *Blood*, 121: 510-518.

Tognoli, M. L., J. Dancourt, E. Bonsergent, R. Palmullij, O. G. de Jong, G. Van Niel, E. Rubinstein, P. Vader, and G. Lavieu. 2023. 'Lack of involvement of CD63 and CD9 tetraspanins in the extracellular vesicle content delivery process', *Commun Biol*, 6: 532.

Trajkovic, K., C. Hsu, S. Chiantia, L. Rajendran, D. Wenzel, F. Wieland, P. Schwillé, B. Brügger, and M. Simons. 2008. 'Ceramide triggers budding of exosome vesicles into multivesicular endosomes', *Science*, 319: 1244-1247.

van de Veerdonk, F. L., M. S. Greshigt, L. Romani, M. G. Netea, and J. P. Latgé. 2017. '*Aspergillus fumigatus* morphology and dynamic host interactions', *Nat Rev Microbiol*, 15: 661-674.

van Niel, G., G. D'Angelo, and G. Raposo. 2018. 'Shedding light on the cell biology of extracellular vesicles', *Nat Rev Mol Cell Biol*, 19: 213-228.

Verweij, F. J., L. Balaj, C. M. Boulanger, *et al.* 2021. 'The power of imaging to understand extracellular vesicle biology *in vivo*', *Nat Methods*, 18: 1013-1026.

Wang, S., B. He, H. Wu, Q. Cai, O. Ramírez-Sánchez, C. Abreu-Goodger, P. R. J. Birch, and H. Jin. 2024. 'Plant mRNAs move into a fungal pathogen via extracellular vesicles to reduce infection', *Cell Host Microbe*, 32: 93-105.e106.

Welsh, Joshua A., Deborah C. I. Goberdhan, Lorraine O'Driscoll, *et al.* 2024. 'Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches', *J Extracell Vesicles*, 13: e12404.

WHO. 2022. "WHO fungal priority pathogens list to guide research, development and public health action." In, 48.

949 Willms, E., H. J. Johansson, I. Mäger, Y. Lee, K. E. Blomberg, M. Sadik, A. Alaarg, C. I. Smith, J. Lehtiö, S. El  
950 Andaloussi, M. J. Wood, and P. Vader. 2016. 'Cells release subpopulations of exosomes with distinct  
951 molecular and biological properties', *Sci Rep*, 6: 22519.  
952 Yáñez-Mó, M., P. R. Siljander, Z. Andreu, *et al.* 2015. 'Biological properties of extracellular vesicles and their  
953 physiological functions', *J Extracell Vesicles*, 4: 27066.  
954 Zhang, X., D. He, S. Gao, Y. Wei, and L. Wang. 2020. 'iTRAQ-based proteomic analysis of the interaction of A549  
955 human lung epithelial cells with *Aspergillus fumigatus* conidia', *Mol Med Rep*, 22: 4601-4610.  
956 Zhang, Zhihong, Rongyu Liu, Jacobien A. Noordhoek, and Henk F. Kauffman. 2005. 'Interaction of airway  
957 epithelial cells (A549) with spores and mycelium of *Aspergillus fumigatus*', *Journal of Infection*, 51: 375-  
958 382.  
959

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