

1 ***Ehrlichia ruminantium* uses its transmembrane protein Ape to adhere to**  
2 **host bovine aortic endothelial cells**

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20

21 **Abstract**

22 *Ehrlichia ruminantium* is an obligate intracellular bacterium, transmitted by ticks of the genus  
23 *Amblyomma* and responsible for heartwater, a disease of domestic and wild ruminants. High  
24 genetic diversity of *E. ruminantium* strains hampers the development of an effective vaccine  
25 against all strains present in the field. In order to develop strategies for the control of  
26 heartwater through both vaccine and alternative therapeutic approaches, it is important to  
27 first gain a better understanding of the early interaction of *E. ruminantium* and its host cell.  
28 Particularly, the mechanisms associated with bacterial adhesion remain to [be elucidated](#).  
29 Herein, we studied the role of *E. ruminantium* membrane protein ERGA\_CDS\_01230  
30 (UniProt Q5FFA9), a probable iron transporter, in the adhesion process to host bovine aortic  
31 endothelial cells (BAEC). The recombinant version of the protein ERGA\_CDS\_01230,  
32 successfully produced in the *Leishmania tarentolae* system, is O-glycosylated. Following *in*  
33 *vitro* culture of *E. ruminantium* in BAEC, the expression of CDS ERGA\_CDS\_01230 peaks  
34 at the extracellular infectious elementary body stages. This result suggest the likely  
35 involvement of ERGA\_CDS\_01230, named hereafter Ape for Adhesion protein of *Ehrlichia*,  
36 in the early interaction of *E. ruminantium* with its host cells. We showed using flow cytometry  
37 and scanning electron microscopy that beads coated with recombinant ERGA\_CDS\_01230  
38 (rApe) adheres to BAEC. In addition, we also observed that rApe interacts with proteins of  
39 the cell lysate, membrane and organelle fractions. Additionally, enzymatic treatment  
40 degrading dermatan and chondroitin sulfates on the surface of BAEC is associated with a  
41 50% reduction in the number of bacteria in the host cell after a development cycle, indicating  
42 that glycosaminoglycans seem to play a role in the adhesion of *E. ruminantium* to the host  
43 cell. Finally, Ape induces a humoral response in vaccinated animals. Globally, our work  
44 identifying the role of Ape in *E. ruminantium* adhesion to host cells makes it a gold vaccine  
45 candidate and represents a first step toward the understanding of the mechanisms of cell  
46 invasion by *E. ruminantium*.

47

48 **Introduction**

49 *Ehrlichia ruminantium* is an obligate intracellular Gram-negative bacterium responsible for  
50 the fatal and neglected heartwater disease of domestic and wild ruminants (Allsopp, 2010).

51 This bacterium belongs to the Anaplasmataceae family in the order Rickettsiales that  
52 includes many pathogens and symbionts of veterinary and public health importance  
53 (Moumene and Meyer, 2015). *E. ruminantium* is transmitted by ticks of the genus

54 *Amblyomma* in the tropical and sub-Saharan areas, as well as in the Caribbean islands. It

55 constitutes a major threat for the American livestock industries since a suitable tick vector is  
56 already present in the American mainland and potential introduction of infected *A.*

57 *variegatum* through migratory birds or uncontrolled movement of animals from Caribbean  
58 could occur (Deem, 1998). Kasari et al., 2010). The disease is also a major obstacle to the

59 introduction of animals from heartwater-free to heartwater-infected areas into sub-Saharan  
60 Africa and thus restrains the breeding to upgrade local stocks (Allsopp, 2010). The small

61 genome of *E. ruminantium* (1.5 Mb) shows an unique process of contraction/expansion in  
62 non-coding regions and targeted at tandem repeats (Frutos et al., 2006). This genome

63 plasticity, associated with a high genetic diversity, suggests a capacity of adaptation upon  
64 exposure to a novel environment and could also explain the low field efficacy of available

65 vaccines (Cangi et al., 2016).

66 After adhesion and entry of infectious elementary bodies into host cells, *E. ruminantium*  
67 replicates by binary fission of reticulated bodies into an intracellular vacuole bounded by a

68 lipid bilayer membrane derived from the eukaryotic host endothelial cell membrane (Dumler  
69 et al., 2001). *Ehrlichia* spp. have evolved sophisticated mechanisms to invade and multiply

70 in host tissues by hijacking/subverting host cell processes ranging from host signaling,  
71 modulation of vesicular traffic, protection from oxidative burst, acquisition of nutrients, and

72 control of innate immune activation (Moumene and Meyer, 2015). Notably, *E. chaffeensis*  
73 secretes the type IV effector Etf-1 to induce autophagy and capture nutrients, whereas it

74 uses Etf-2 to delay endosome maturation to avoid phagolysosomal fusion for the benefit of  
75 bacterial replication (Lin et al., 2016; Yan et al., 2018). Moreover, recent work identified that

76 *E. chaffeensis* uses EtpE invasin to enter mammalian cells via the binding to its receptor  
77 DNaseX, a glycosylphosphatidylinositol-anchored cell surface receptor (Mohan Kumar et

78 al., 2013). That receptor-triggered entry simultaneously blocks the generation of reactive  
79 oxygen species (ROS) by host monocytes and macrophages (Teymournejad et al., 2017).

80 Due to the lack of some key metabolic genes that are required for host-free living and  
81 similarly to what is observed in other intracellular bacteria, entry into the eukaryotic host

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86 cells is crucial for *E. ruminantium* to sustain its life and disseminate (Pizarro-Cerda and  
87 Cossart, 2006) ; (Frutos et al., 2007). Computational studies have predicted type IV effectors  
88 in *E. ruminantium* (Noroy et al., 2019) which, however, remain to be characterized; the  
89 mechanisms of adhesion and entry are still unknown and may be active or passive  
90 depending on the pathogen. *E. ruminantium* could either inject a type IV effector across  
91 the host cell membrane to trigger actin rearrangement and pathogen phagocytosis such as  
92 *Bartonella henselae* (Truttmann et al., 2011). An another option is the use of homologous  
93 system of *E. chaffeensis* invasion/receptor pair (Mohan Kumar et al., 2013) or an outer  
94 membrane protein of the OmpA family that actively trigger internalization as was observed  
95 in *Coxiella burnetii* (Martinez et al., 2014).

96 Bacterial pathogens are capable of exploiting and diverting host components such as  
97 proteoglycans for their pathogenesis (Aquino et al., 2018). These assemblies of  
98 glycosaminoglycans (GAGs) chains fixed around a protein nucleus (Bartlett and Park, 2010)  
99 are expressed constitutively on the cell surface, in intracellular compartments as well as in  
100 the extracellular matrix. They are known to act as receptors for pathogens in many cases of  
101 infection (Aquino et al., 2018); (Rajas et al., 2017); (Gagoski et al., 2015). Many pathogens  
102 – e.g. *Chlamydia trachomatis* with a biphasic life cycle like *E. ruminantium* – use GAGs as  
103 an initial anchor site of low affinity; this facilitates interaction with their respective secondary  
104 receptor allowing internalization but GAGs are sometimes also used as bridging molecules  
105 (Aquino et al., 2010). Thus, whether *E. ruminantium* uses GAGs as a portal of entry or any  
106 specific bacterial surface protein is unknown but essential for developing any anti-infective  
107 measures.

108 The outer membrane proteome study of *E. ruminantium* Gardel strain revealed that a  
109 hypothetical protein, the possible major ferric iron binding protein precursor the putative iron  
110 transporter ERGA\_CDS\_01230, is uniquely expressed in the outer-membrane fraction  
111 (Moumene et al., 2015). This protein was also shown to be O-glycosylated only in *E.*  
112 *ruminantium* (Marcelino et al. 2019). Moreover, homologous counterparts of this protein in  
113 other pathogenic species play a key role in bacterial survival within the host by scavenging  
114 iron from mammalian serum iron transport proteins (Brown et al., 2010). Interestingly, we  
115 previously showed that iron starvation induces expression of virulence factors such as type  
116 IV secretion genes (Moumene et al., 2017).

117 In this study, we show that ERGA\_CDS\_01230 (UniProt Q5FFA9, named herein Ape for  
118 Adhesion protein of *Ehrlichia ruminantium*,) is involved in the binding of *E. ruminantium* to  
119 bovine aortic endothelial cells (BAEC). In order to study whether Ape alone can mediate the

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**a supprimé:** Computational studies allowed the prediction of type IV effectors for *E. ruminantium*

**a supprimé:** that remain to be fully characterized but detailed mechanisms of adhesion and entry are still unknown. These processes can be active or passive depending on the pathogen

126 invasion of host cells by adhering to endothelial cells membrane, we used latex beads  
127 coated with recombinant Ape. These beads covered with the protein adhered and seemed  
128 to enter endothelial cells similarly to what is observed with *E. ruminantium in vitro* (Moumene  
129 and Meyer, 2015). Subsequent investigation uncovered that rApe is a glycosylated protein  
130 that induces a strong humoral immune response in vaccinated goats making it a possible  
131 vaccine candidate.

132

## 133 **Methods**

### 134 **Synchronous culture**

135 *E. ruminantium* (strain Gardel) was propagated in Bovine Aortic endothelial cells (BAEC  
136 isolated at CIRAD, Guadeloupe, France) in Baby Hamster Kidney (BHK-21) cell medium  
137 supplemented with 1% L-glutamine 200mM (Eurobio), 10% heat-inactivated fetal bovine  
138 serum (FBS, Thermofisher), 1% penicillin 10000UI/ streptomycin 10000 µg (Eurobio), 1x  
139 amphotericin B (Sigma), 5% NaHCO<sub>3</sub> 5.5%, under 5% CO<sub>2</sub> at 37°C modified from  
140 (Marcelino et al., 2005). The cell trypsinization (Trypsin-Versen, Eurobio) was carried out  
141 with a splitting ratio of ½ when monolayer reached 100% confluence. Synchronous infection  
142 of a new BAEC monolayer was obtained using a bacterial suspension previously harvested  
143 at 120 hpi by scraping the TC flask (TCF) and passing infected lysed cells through 18G and  
144 26G needles before reinfection at a ratio of 1/20 (Marcelino et al., 2005).

145

### 146 **Quantitative reverse transcription RT-PCR along bacterial cycle**

147 *E. ruminantium*-infected BAE cells were harvested for RNA extraction by trypsinization at  
148 24, 48, 72 and 96 hours post infection (hpi), centrifuged 1700 x g, 5 min at 4°C and by cell  
149 lysate scrapping at 120 hpi, centrifuged 20000 x g, 15 min at 4°C. All pellets were dissolved  
150 in 1 mL TRIZOL (Thermofisher), RNA was extracted following manufacturer's  
151 recommendations and eluted in 100 µL H<sub>2</sub>O RNase/DNase free. RNA was treated by Turbo  
152 DNase (Ambion) according to supplier's instructions and precipitated overnight at -20°C in  
153 2.5 volume (v/v) cold absolute ethanol (Normapur), 1/10 volume of 3 M sodium acetate and  
154 1 µL glycogen 10 mg/mL (Thermofisher). Pellet obtained by centrifugation 15000 x g, 10  
155 min, 4°C was washed with 1 mL 75% ethanol, air dried after centrifugation 9000 x g, 7 min,  
156 4°C and dissolved in 20 µL H<sub>2</sub>O RNase/ DNase free. Two µg ARN were reverse transcribed  
157 using "SuperScript™ VILO™ cDNA Synthesis Kit" (Invitrogen), according to the supplier's  
158 specifications.

159 **Pre amplification of *ape* gene**

160 Quantification started by 15 cycles of pre-amplification (same reaction mix and cycling  
161 conditions as below). ERGA\_CDS\_01230 (Ape) was amplified in 25 µL reaction mix  
162 containing 250 nM of the forward ERGA\_CDS\_01230F AATGGAGAATGAGGGGGAAG  
163 and reverse ERGA\_CDS\_01230R ACCCAAACCAAATCCATCA primers, 12.5 µL of  
164 Power SYBR® Green PCR Master Mix (Thermofisher), 12.5 µL H<sub>2</sub>O RNase/ DNase free  
165 and 20 µL pre-amplified DNA. Reactions were performed in a Quantstudio 5 (Thermofisher)  
166 as follow: 50°C, 2 min for Uracil-N-Glycosylase activation, 95°C, 10 min for Uracil-N-  
167 Glycosylase inactivation and polymerase activation; 40 cycles 95°C, 15 sec denaturation  
168 and 59°C, 60 sec hybridization and elongation. The specificity of the PCR product was  
169 confirmed by the dissociation curves.

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170 **Quantification of *E. ruminantium* along the bacterial cycle**

171 *E. ruminantium* quantification for normalization as described by (Pruneau et al., 2012), was  
172 performed by DNA extraction according to manufacturer's specifications (QiaAmp DNA  
173 minikit, QIAGEN, Courtaboeuf) on 1/10 of the harvested volume, after 20000 x g, 15 min  
174 centrifugation and dissolution in 200 µL PBS 1x, followed by a [quantitative polymerase chain  
175 reaction \(qPCR\)](#) *So1* targeting the pCS20 region (Cangi et al., 2017).

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176 **Normalization of *ape* gene expression**

177 Normalization by *E. ruminantium* quantification was calculated:  $R_{x\ hpi} = [cDNA\ copy\ number$   
178  $(ERGA\_CDS\_01230)] / [E.\ ruminantium\ pCS20\ DNA\ copy\ number]$ , allowing fold change (FC)  
179 determination, compared to expression at 96 hpi (corresponding to the stationary phase of  
180 the bacterial growth):  $FC = R_{x\ hpi} / R_{96\ hpi}$ . Results were represented in log<sub>2</sub>, according to  
181 Pruneau et al. (2012) and confirmed by 2 others [biological replicates](#) (data not shown).

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183 **Recombinant protein production**

184 pLEXY\_I-blecherry3 plasmid (Bioscience, 2011) and 400 ng of amplified  
185 CDS\_ERGA\_01230 were digested 10 min at 37°C by BamHI and Sall (Thermo Scientific,  
186 USA) and column purified (Macherey Nagel, Germany). The digested product was ligated  
187 in pLEXY\_I-blecherry3 plasmid using T4 DNA Ligase (Thermo Scientific) for 1 h at 22°C.  
188 pLEXY\_I-blecherry3 plasmid was modified with the addition of a sequence coding GFP at  
189 C-terminus of the insert and 6X His tags at N-terminus of insert. It is to note that, GFP and  
190 the coding sequence of CDS\_ERGA\_01230 were linked with the sequence encoding  
191 cleavage site for Xa factor. Ligated plasmid was column purified and 50 ng of the ligation  
192 mix was electro-transferred to competent bacteria *E. coli* XL10 (Miller and Nickoloff, 1995).

198 100 µL of transformed culture were spread on LB medium Petri dish supplemented with  
199 carbenicillin (50µg/ml). To confirm the presence of the plasmid and the insert, a PCR was  
200 performed on a colony using vector specific primers (Forward,  
201 CGCATCACCATCACCATCAGC; Reverse, ACCAAAATTGGGACAACACCAGTG). PCR  
202 product was then sequenced. Transformed E. coli clone was grown 16 h at 37°C under 200  
203 rpm stirring until optical density (OD) reached 3. The plasmid was isolated  
204 using "GenElute™ Plasmid Midiprep" kit (Sigma Aldrich) and digested with *SmaI* (Thermo  
205 Scientific)

206 *Leishmania tarentolae* preculture was grown in 10 mL LEXSY BHI medium with TC flask at  
207 26°C. 3 days after, preculture was diluted 10 fold in 10 mL LEXSY BHI medium and  
208 incubated overnight at 26°C (flat position). Next day, *Leishmania tarentolae* were  
209 centrifuged 5 min ( 2000 x g) and half of the medium was removed. Cells were resuspended  
210 in remaining medium to get 10<sup>8</sup> cells/ mL and incubated 10 min on ice. 350 µL cells were  
211 electroporated at 450 V, 450 µF, 5 - 6 msec impulses with 5 µg linearized plasmid (smal  
212 digested). Cells were immediately incubated for 10 min on ice, transfer to 10 mL LEXSY BHI  
213 medium (Bioscience) and incubated overnight. 10 µL bleomycin (100 mg/mL, (Bioscience)  
214 was then added to nonclonal selection for 3 more days. 5 mL of culture supernatant was  
215 centrifuged for 3 min at 1000 x g. Pellet was resuspended in 10 mL LEXSY BHI medium  
216 containing bleomycin and incubated at 26°C for 5 days. After nonclonal selection,  
217 expression of protein was induced in 45 mL BHI medium supplemented with bleomycin and  
218 tetracyclin 10 mg/mL (Bioscience). 5 ml of culture from nonclonal selection was added  
219 incubated for 72 h at 26°C with shaking at 100 rpm. Supernatant was harvested and  
220 concentrated in dialyses bag (3.5 kDa membrane, Serva) in a polyethylene glycol solution  
221 20000 overnight at 4°C. Proteins were concentrated and purified by Sephadex gel filtration.

### 222 [Mass spectrometry analysis](#)

223 Protein size was verified by [MALDI-TOF](#) (Microflex, Bruker). The presence of GFP tag and  
224 associated fluorescence was checked respectively by immunoblotting and aggregation on  
225 Probond beads followed by fluorescence microscopy. Mass spectrometry allowed to check  
226 rApe size [and Orbitrap allowed to confirm identity of the protein.](#)

227

### 228 **3D Structure and localization prediction**

229 Protein structure prediction was accomplished using I-TASSER-MTD (Xiaogen Zhou,  
230 2022) and view was generated by MacPyMol (DeLano, 2009). The subcellular localization  
231 was predicted by "CELLO 2.5: subCELLular LOcalization predictor" (Yu et al., 2006), from

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236 the protein sequence, accession number CAI27575.1. Ape structural homology was  
237 determined using "Swiss model, Expasy" (Waterhouse et al., 2018).

238

#### 239 **Western Blot for O-GlcNAc Glycoprotein detection**

240 rApe was migrated on an acrylamide gel "SDS page" (NuPAGE bis-tris, Novex) for 2 h 30  
241 min at 100V and 400mA in MOPS buffer (Novex), according to the supplier's instructions  
242 (Nu PAGE Technical Guide). Approximately 10 µg protein [and BSA for the negative control](#)  
243 were denatured at 70°C for 10 min with LDS buffer and reducing agent (Novex) and migrated  
244 with a [3.1 to 198 kDa](#) molecular weight marker (SeeBlue plus 2, Invitrogen). A gel-sized PVDF  
245 membrane (Amersham, Hybond-P) was soaked for 30 sec in methanol (Normapur) and  
246 incubated in transfer buffer (20x, NuPAGE) for at least 15 min with the same size filter  
247 papers (Whatman) and 4 sponges. The transfer assembly was performed according to the  
248 NuPAGE technical guide and the transfer was run 1 h 15 min at 30 V, 170 mA. The  
249 membrane was then immersed in a solution of Culvert Red (AMRESCO) ~1 min and rinsed  
250 with water prior to picture. The protocol for western blot detection was modified from  
251 (Marcelino et al., 2019). The membrane was blocked for 3 h at room temperature (RT) with  
252 stirring in PBS (pH 7.4), 0.05% Tween20 (PBS-T) and 5% milk. Then, membrane was  
253 incubated overnight with anti-O-GlcNAc antibody (Santa Cruz), [monoclonal IgM](#), diluted 200  
254 fold in blocking solution. After three washes with PBS-T, membrane was incubated with anti-  
255 mouse antibody (IgM-HRP, Molecular probes) diluted 1000 fold in PBS-T for one hour. The  
256 membrane was washed 3 times 10 min with PBS-T before the addition of the TMB substrate  
257 (Pierce) and gel reader picture once the color was developed.

258

#### 259 **Glycosaminoglycans degradation assays**

260 10, 5, 1 and 0.3 µg/ mL heparan sulfate (Jonquieres et al., 2001; Kobayashi et al., 2010) and  
261 9 µg/ mL rApe (positive control) were adsorbed in 50 µL of carbonate/bicarbonate buffer pH  
262 9.6 (Martinez et al., 1993), distributed in Nunc Maxisorp wells, 1 h at 37°C with gentle stirring  
263 then overnight at 4°C. The next day, 3 washes were carried out with 200 µL of PBS-T per  
264 well. Blocking was done 1 h at 37°C under agitation with 100 µL of blocking buffer PBS  
265 tween 20 0.05% milk 3%, followed by 3 washes with 200 µL of PBS-T per well. 50 µL/ well  
266 of rApe at 14.3 µg/mL diluted in PBS tween 20 0.05% milk 3% was incubated 1 h at 37°C  
267 with stirring. Three washes of 200 µL PBS-T per well were performed, followed by addition  
268 of 50 µL anti-GFP antibody diluted to 4,000 in PBS-T with milk 3% and incubation 1 h at  
269 37°C with stirring. Washings were repeated as above. Addition of 200 µL TMB substrate

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271 allowed revelation within 30 min at 37°C before to stop the reaction with 100 µL of H2SO4  
272 2N. The reading was done at 450 nm (Multiskan, Thermofisher). 2 cm<sup>2</sup> wells were inoculated  
273 with ~1.1x10<sup>4</sup> BAEC in 500 µL BHK21 medium. When confluence was reached, several  
274 concentrations of [chondroitinase \(Sigma Aldrich\)](#) were tested as follows: 0.2U, 0.4U and  
275 0.9U/ mL [chondroitinase](#) was incubated 2 h before infection with the BAEC in 1X PBS and  
276 bovine fetal serum (SBF)-free BHK21 medium (Sava et al., 2009;Rajas et al., 2017). The  
277 medium was renewed with standard BKH21 prior infection at a ratio 1/20 and 2 h after  
278 infection. At lysis stage, all the wells were scraped, centrifuged for 15 min at 20,000 x g.  
279 DNA was extracted using the QiaAmp DNA minikit (Qiagen) and quantified using qPCR™  
280 Sol1, targeting the pCS20 region (Cangi et al., 2017). The results were treated using the  
281  $\Delta\Delta C_t$  method and represented in  $2^{-\Delta\Delta C_t}$ .

282

#### 283 **Flow cytometry for attachment quantification**

284 We used the cytometer to quantify the fluorescence-labelled cells following incubation with  
285 rApe. Six-well Nunc plates (9.6 cm<sup>2</sup>/ well) with confluent BAEC were incubated for 2 h at  
286 37°C, 5% CO<sub>2</sub> with rApe concentrations ranging from 6.4 to 102.4 µg/ mL following the  
287 principle described in (Lundberg et al., 2003). The negative control consisted of confluent  
288 BAE cells. After incubation with recombinant protein, each well was rinsed twice with 1 mL  
289 of 1X PBS and 1.5 mL of 1X PBS was used to scrape the well. After centrifugation during  
290 10 min at 200 x g, at 4°C cells were resuspended in Isoflow (Beckman) for further reading  
291 the percentage of fluorescent labelled cells on the cytometer (FC500, Beckman Coulter).

292

#### 293 **Far Western Blot**

294 The BHK21 culture medium of a 175 cm<sup>2</sup> TCF was removed, the TCF was washed with 5mL  
295 of PBS 1x containing anti-protease (Roche). ~10mL of cold PBS 1x was added to gently  
296 scrap the cell mat. Centrifugation 10 min at 200 x g and 4°C was performed to remove the  
297 supernatant. Lysis of the pellet was performed by addition of 3 mL native lysis buffer (150  
298 mM NaCl, 50 mM Tris-HCl, pH 7.4, NP-40 1%, anti-proteases 1%), followed by 4  
299 "freeze/thaw" cycles by first immersing the lysate 2 min in an ethanol/ ice bath, then 2 min  
300 in a 37°C water bath. The lysate was vortexed between each bath. The cells were broken  
301 by passing the lysate 4 times through an 18G needle with a syringe. The cell debris were  
302 pelleted at 10000 x g for 30 min prior to supernatant transfer into a new tube.

303 Four different fractions (F1, cytosol components; F2, membrane and organelle components;  
304 F3, nucleus components; F4, the cytoskeleton) were extracted using the "ProteoExtract

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307 Subcellular Proteome Extraction kit" (Calbiochem/ MERCK) according to the supplier's  
308 instructions. Each fraction was placed in acetone (at least 5x the volume of the extracted  
309 fraction) for overnight precipitation at -20°C. The day after, each pellet was re-suspended in  
310 500µL of native lysis buffer after 20 min centrifugation at 15000 x g and 4°C.  
311 18 µL of each cell fraction and 35 µL of lysate were migrated onto acrylamide gel, with  
312 addition of LDS, under the same conditions as for the glycosylation tests (but without  
313 addition of reducing agent or heating), as well as for the transfer onto PVDF membrane.  
314 The membrane was stored in TTBS 1x (10mM TRIS, 150mM NaCl, 0.05% Tween 20, pH  
315 8,3), then blocked for 1 h at RT under gentle agitation in 2% Bovine Serum Albumine (BSA)  
316 in TTBS and finally rinsed 3 times for 5 min with TTBS. The membrane was incubated  
317 overnight at 4°C under slow rotation with 0.5 mg rApe in 1% BSA in TTBS. The negative  
318 control (without recombinant protein) was incubated in the same conditions. The next day,  
319 the membrane was washed 3x for 5 min with TTBS and incubated 1 h at RT under agitation  
320 with anti-GFP-HRP (ThermoFisher) diluted 2,500 fold in 1% BSA in TTBS. The membrane  
321 was washed again 3 x for 5 min with TTBS. The binding of anti- GFP-HRP was revealed by  
322 the addition of 4.5 mL peroxide substrate (Pierce ThermoFisher) + 0.5mL chromogen DAB  
323 (ThermoFisher) and incubation 10 min at RT. Picture was taken by colorimetry reading.

324

#### 325 **Scanning electronic microscopy for binding assays**

326 Two kinds of fluorescent latex beads (with a sulfate group) were adsorbed with rApe through  
327 electrostatic interactions based on (Martinez et al., 2014). A total of  $10^{10}$  beads (0.1 µm in  
328 diameter) and  $7 \times 10^9$  beads (0.5 µm in diameter) were adsorbed with 100 µg/mL rApe in 1  
329 mL of 25 mM MES, pH 6.1 (Sigma Aldrich) during 4h at RT under slow rotation. Then, three  
330 washes were performed using the same buffer before to be resuspended in 1 mL of 1% BSA  
331 MES. 24-well plates containing 13 mm diameter lamellae (VWR) were inoculated with  
332 BAEC. A deposit of 0.1 or 0.5 µm-diameter beads ( $1.82 \times 10^8$  per well) was made in BHK21  
333 medium. The negative control consisted of recombinant protein-free beads incubated with  
334 BAEC. The plate was centrifuged 5 min at 200 x g at RT before incubation 30-120 min at  
335 37°C under 5% CO<sub>2</sub> atmosphere. Three washes with PBS 1X removed the excess of  
336 unbound beads before overnight fixation with 2% paraformaldehyde. Lamellae were then  
337 removed from each well and dehydrated in series of acetone solutions of increasing  
338 concentration, dried to critical point in CO<sub>2</sub> and sputter-coated with gold before observation  
339 with a FEI Quanta 250 electron microscope at 20 kV.

340

### 341 ELISA-based binding assays

342 The antibody response to Ape during vaccination of goats was tested by ELISA. Sera for  
343 vaccinated goats obtained from previous studies (Marcelino et al. 2015 a, b) were incubated  
344 in wells coated with rApe, followed by incubation with anti-goat [IqG](#) antibody coupled to  
345 HRP.

346 The adsorption of [4 µg/ mL](#) rApe diluted in 100 µL of carbonate bicarbonate buffer pH 9.6  
347 was performed in a Nunc plate (Maxisorp). One hour incubation was carried out at 37°C with  
348 stirring at 150 rpm then overnight at 4°C. The plate was washed with 300 µL/ well of wash  
349 buffer (PBS 1x pH 7.2, Tween 20 0.1%). Blocking was carried out at 37°C with stirring in  
350 300 µL blocking buffer (PBS1x, tween 20 0.1%, casein 2%) for 1 h. Washings were repeated  
351 as described in the previous step. 100 µL of each goat serum diluted 100-fold in blocking  
352 buffer was incubated 1 h at 37°C with agitation (Perez et al., 1998); 2 blank wells were  
353 incubated with 100 µL blocking buffer. Five washes with Wash Buffer preceded the deposit  
354 of 100 µL of anti-goat [IqG](#) antibody (Rockland) diluted 20,000-fold in blocking buffer and 1  
355 h incubation at 37°C with agitation. Five washes were performed. The revelation was  
356 performed by addition of 100 µL of TMB (Neogen) and stopping of the reaction after 5 min  
357 development by the addition of 50 µL of 0.5 M H<sub>2</sub>SO<sub>4</sub>. Antibody response was detected by  
358 ELISA titers and optical density (OD) was read with a spectrophotometer at 450nm. The OD  
359 of the wells without serum were valid when < 0.1 ; OD of negative samples were valid when  
360 < 0.2.

361

### 362 Results

363

#### 364 ERGA\_CDS\_01230 (*ape*) is highly expressed at infectious elementary body stages of 365 *E. ruminantium* development inside mammalian cells.

366 In order to measure the expression of the *ape* gene, normalization was carried out in relation  
367 to the number of bacteria present at each stage of development since no reference gene  
368 with a sufficiently stable expression is available for *E. ruminantium*. The development cycle  
369 of *E. ruminantium* is synchronized when the lysis occurs 5 days after BAEC infection.  
370 Quantification of the number of bacteria present in the BAEC every 24 hpi by qPCR Sol1  
371 showed a sigmoidal curve represented in log<sub>10</sub> (Figure 1A). The bacterium had a slow  
372 growth phase between 24 and 48 hpi then an exponential development with a slowing down  
373 of the growth, a stationary phase after 96 hpi and a maximum of copies reached at 120 h  
374 (release of elementary bodies). The number of transcripts was determined by qPCR and a

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377 ratio calculated at each time as follow: (*E. ruminantium* cDNA number) / (*E. ruminantium* pCS20  
378 DNA copy number). The fold change (FC) was determined in relation to bacterial expression at 96  
379 hpi (stationary phase) and represented graphically in Figure 1B. The expression of *ape*  
380 gene peaks at the elementary body infectious stages of *E. ruminantium* life cycle which  
381 correspond to 120 hpi (host cell lysis) and 24 hpi (lag phase). These data indicate that *ape*  
382 is expressed when the bacterium is released and ready to infect new cells.

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### 384 **Ape protein presents a C-clamp structure and is predicted to be an outer-membrane** 385 **protein**

386 The 3D structure proposed by I-TASSER-[MTD](#) software (Figure 2A) showed that Ape  
387 harbours a succession of helices  $\alpha$  linked by loops distributed around a  $\beta$  sheet and showed  
388 a "c-clamp" three-dimensional structure. By homology with other known I<sup>ry</sup>, II<sup>ry</sup> or III<sup>ry</sup>  
389 structures, "Swiss model" showed a strong analogy of Ape with a "C-clamp" structure,  
390 capable of binding iron. According to the "CELLO 2.5" software, Ape has a dominant  
391 cytoplasmic localization (score of 2.418) but is also present at the outer membrane (1.652)  
392 (Figure 2B). This canonical sub-cellular localization of a transmembrane protein is in  
393 accordance with previous results finding this protein in the outer membrane proteome  
394 (Moumene et al., 2015).

### 396 **rApe protein shows O-glycosylated post-traductional modifications**

397 Western blot analysis showed that rApe was detected with an anti-O-GlcNAc antibody at the  
398 expected size of 66kDa (Figure 3). This size confirmed the data obtained by mass  
399 spectrometry and is 30% larger than the one estimated by the amino acid sequence  
400 encoded by *ape* gene (41 kDa for 365 amino acids). [The identity of rApe protein sequence](#)  
401 [blasted with the possible major ferric iron binding protein precursor \(Q5FFA9\)](#). Altogether  
402 these results demonstrate the O-glycosylation of rApe.

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### 404 **Enzymatic treatment of BAEC with chondroitinase decreases invasion by *E.*** 405 ***ruminantium***

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406 To investigate whether GAGs have a role in *E. ruminantium* adhesion process, BAEC were  
407 treated with [chondroitinase](#) and then infected with *E. ruminantium*. The number of bacteria  
408 was calculated at the end of growth, during the lysis of the BAEC, by qPCR Sol1 for each  
409 treatment. After treatment with [chondroitinase](#) at 0.2U/mL, the FC corresponding to the  
410 bacterial amount differential was higher than for the condition without treatment but this may

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415 be explained by inter-well variability (Figure 4). Increasing [chondroitinase](#) concentrations to  
416 0.4 and 0.9U/mL resulted in FC of 0.52 and 0.58, respectively, corresponding to a 50%  
417 reduction of the number of bacteria compared to the untreated condition, highlighting the  
418 role of [chondroitin](#) sulfate and dermatan sulfate in adhesion of *E. ruminantium* to the cell.  
419 No affinity of rApe for heparan sulfate could be revealed using HRP anti-GFP antibody (data  
420 not shown).

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421

#### 422 **rApe binds to BAEC**

423 To go further in the understanding of the role of Ape in *E. ruminantium* invasion of host cells,  
424 we analyzed the interaction of rApe with the surface of endothelial cells using flow cytometry.  
425 Adhesion of recombinant proteins to BAEC was measured using flow cytometry to detect  
426 the fluorescence of rApe harboring GFP tag. The dot plot profile clearly showed that the  
427 percentage of labelled BAEC with rApe increases with the amount of rApe protein (Figure  
428 5). As a negative control, the auto-fluorescence of the cells was measured on cells without  
429 recombinant protein incubation. The percentage of labelled cells increased with protein  
430 concentration up to 36 µg, showing a dose-effect relationship. Above these concentrations,  
431 [bending of the curve starts](#) with 50% of rApe labelled BAE.

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432

#### 433 **Ape interacts with BAEC lysate, membrane and organelles cell fractions**

434 The Far Western blot shows the interaction between rApe and the cell fractions as well as  
435 the lysate of the BAEC. rApe interacted with proteins of the cell lysate and more specifically  
436 with those of the membrane and organelle fraction (Figure 6).

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437

#### 438 **rApe coated beads adhere to BAEC and start internalization mechanism**

439 The visualization of interaction between rApe and the host cell was possible through usage  
440 of beads adsorbed with rApe (mimicking *E. ruminantium*) and incubated with BAEC.  
441 Adhesion was evaluated by scanning electron microscopy. The negative control showed  
442 that non-coated beads did not adhere to the surface of the BAEC, that harbor their classical  
443 fried egg shape (Figure 7A). In contrast, Figure 7B showed swollen BAEC, dotted with rApe  
444 adsorbed beads after 30 minutes incubation, revealing an interaction between rApe and the  
445 BAEC. Figure 7C displayed that rApe-coated beads also adhere (black arrow) and begin to  
446 be invaginated (white arrow) by the endothelial cell membrane. Beads diameter did not  
447 affect interaction of rApe and the BAEC. The images shown are representative of the  
448 observations made in other fields. These data reinforcing the results obtained by

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454 immunoblotting and flow cytometry prove that Ape interacts with the host cell membrane  
455 and is involved in the adhesion of *E. ruminantium* to the host cell.

456

#### 457 **Ape induces an antibody response in vaccinated goats**

458 In order to verify if *E. ruminantium* Ape protein induces a humoral response following  
459 vaccination in goats, we tested sera from *in vivo* experiments on animals vaccinated with an  
460 inactivated or attenuated bacterial vaccine (Marcelino et al. 2015 a, b). Goats #614 and #915  
461 were both naïve prior to vaccination, characterized by the absence of antibodies against  
462 Ape at 3 and 5 weeks post-vaccination, respectively (Figure 8). For #915, the ELISA test  
463 showed an increase in humoral response against rApe over time. In fact, the antibody  
464 response was developing between 5 and 7 weeks post-vaccination, the latter corresponding  
465 to the vaccine boost. For goat #614, inoculation of an attenuated bacterial vaccine also  
466 conduced to a humoral response, including response against rApe. These results suggest  
467 that rApe could be a relevant target for further studies to see whether it could be a protective  
468 immunogen in *E. ruminantium* infection *in vivo*.

469

#### 470 **Discussion**

471 Controlled and determined *Ehrlichia* entry into host cell is a fundamental first step for an  
472 effective infectious developmental cycle, particularly for an obligate intracellular pathogen  
473 that strictly relies on its host to grow. In the last decade, comparative genomics and cellular  
474 microbiology allowed major discoveries in the molecular pathogenesis of Anaplasmataceae.  
475 These integrated approaches led to the effective identification of several bacterial virulence  
476 determinants (e.g. effectors and regulators) and their diverse mechanisms of action  
477 (Martinez et al., 2005; Cheng et al., 2008); (Rikihisa, 2017); Marcelino et al., 2015b ;  
478 (Moumene et al., 2017). Notably, Rikihisa *et al.* identified that the EtpE protein governs the  
479 binding and entry of *E. chaffeensis* into its host cells (Mohan Kumar et al., 2013). The binding  
480 of EtpE to DNaseX elicits a signaling cascade that results in cytoskeleton modification,  
481 filopodial induction and finally endocytosis into the host cell (Green et al., 2020). Previous  
482 studies demonstrated that functional conservations of molecular pathogenicity determinants  
483 can occur between of *E. chaffeensis* and *E. ruminantium* (Moumene et al., 2017) but such  
484 bacterial ligand was still unknown in *E. ruminantium* at the beginning of this study. Among  
485 other Rickettsiales, a receptor-mediated endocytosis was only reported for *Rickettsia conorii*  
486 (Moumene et al., 2017) ; (Martinez et al., 2005).

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488 Our aim being to determine some major pathogenicity determinants of *E. ruminantium*, we  
489 chose to analyze ERGA\_CDS\_01230 (Ape, UniProt Q5FFA9), a putative iron-transporter  
490 previously identified in the outer-membrane proteome of *E. ruminantium* (Moumene et al.,  
491 2015). Indeed, we postulated that some key bacterial proteins involved in the early  
492 interaction with the eukaryotic host cell should be overexpressed at early stages of the  
493 developmental cycle and at the bacterial-host interface. Moreover, we previously showed  
494 that iron was a triggering environmental cue for several *E. ruminantium* molecular virulence  
495 determinants. Indeed, iron depletion induced a master regulatory gene, genes encoding  
496 outer-membrane proteins of the Map1 family and genes of the Type IV Secretion System, a  
497 major bacterial pathogenicity determinant (Moumene et al., 2017). Therefore, taking into  
498 account that iron is a virulence triggering signal of *Ehrlichia*, Ape protein being a putative  
499 iron transporter made it an excellent candidate for further characterization. In the present  
500 study, we focused on the role of Ape protein in the interaction between *E. ruminantium* and  
501 its mammalian host cell, notably during adhesion. As depicted in our working model for *E.*  
502 *ruminantium* binding and invasion of its host endothelial cell (Figure 9), we showed that Ape  
503 epitopes are recognized by the immune system of goats vaccinated with live attenuated  
504 strain or killed strain or *E. ruminantium*. Indeed, after vaccination, we detected Ape  
505 antibodies in sera of vaccinated goats indicating a global humoral response. Following this  
506 model, the initial binding of *E. ruminantium* onto the host cell surface seems to involve  
507 glycosaminoglycans (GAGs) as [chondroitinase](#) treatment of BAEC resulted in a significant  
508 decrease of the number of bacteria present at the end of development cycle. The *ape* gene  
509 is highly expressed at the elementary body developmental stages of *E. ruminantium*,  
510 particularly during host cell lysis which precedes *E. ruminantium* release from host cells to  
511 initiate a new cycle of infection. Interestingly, recombinant protein rApe is an O-glycosylated  
512 protein that interacts with cell membrane and latex beads coated with rApe were able to  
513 adhere to the BAEC surface to initiate internalization and follows a similar pattern of entry  
514 like that of *E. ruminantium* (Moumene and Meyer, 2015).

515 Our results showed that Ape protein, an *Ehrlichia* ligand different from previously identified  
516 *E. chaffeensis* EtpE (Mohan Kumar et al., 2013), is important for *E. ruminantium* adhesion  
517 to the mammalian host cells. The mechanisms used by *E. ruminantium* to invade its host  
518 are still not elucidated compared to other pathogens of the order *Rickettsiales*. Indeed  
519 *Rickettsia conorii* uses OmpA (Hillman et al., 2013) to adhere to the host cell whereas two  
520 different receptors were described for *Anaplasma marginale* and *A. phagocytophilum*  
521 mobilizing Msp1a and OmpA (de la Fuente et al., 2003), (Hebert et al., 2017), Asp14 and

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524 OmpA (Kahlon et al., 2013), (Ojogun et al., 2012), respectively to attach cell membrane.  
525 Adhesins and invasins were largely described in other bacteria as surface located structures  
526 for specific interaction with host cell receptors (Niemann et al., 2004). *Yersinia* outer  
527 membrane invasin interacts with  $\beta$ 1 integrin receptors, inducing several reactions including  
528 actin rearrangements at the site of bacterial entry, promoting invasion. *Salmonella*  
529 translocates several effectors into target cells, some of them allowing the initial uptake of  
530 the bacterium, whereas *Listeria* uses InIA and InIB-dependent molecular pathways, (Pizarro-  
531 Cerda and Cossart, 2006). Ligands often show elongated molecules containing domains  
532 commonly found in eukaryotic proteins (Niemann et al., 2004).

533 We showed that rApe is O-glycosylated. Post-translational modifications are one of the most  
534 important mechanisms for activating, changing, or suppressing protein functions, being  
535 widely used by pathogens to interact with their hosts. *E. ruminantium* glycoproteomics  
536 showed a high percentage of glycoproteins, many of them being O-glycosylated (Marcelino  
537 et al., 2019). *E. ruminantium* "mucin", which is also glycosylated, was presented as an  
538 adhesin for tick cells, reinforcing a role of glycans in *Ehrlichia* adhesin molecules (de la  
539 Fuente et al., 2004). The strength of ligand-receptor bacterial interactions is optimized  
540 depending on their environment but weak enough to allow a bacterium to detach regularly  
541 and migrate to other locations (Formosa-Dague et al., 2018). Glycan-glycan interactions in  
542 bacterial-mammalian cells systems were characterized as low-affinity weak interactions  
543 preceding high-affinity protein-glycan or protein-protein interactions but recent studies have  
544 documented the importance of such interactions in bacterial adhesion (Formosa-Dague et  
545 al., 2018). Indeed, we determined that the presence GAG on the surface of BAE plays a key  
546 role in the attachment of *Ehrlichia* to bovine endothelial cells *in vitro*, reinforcing the  
547 hypothesis that several receptors are probably required in *E. ruminantium* adhesion and  
548 subsequent infection of host cells. [Chondroitinase](#) treatment significantly affected *Ehrlichia*  
549 entry compared to the untreated condition. The enzymatic digestion of [chondroitin](#) and  
550 dermatan from BAEC reduced the rate of infection of the BAEC, as *E. ruminantium* can no  
551 longer adhere to the surface of the cells. Indeed, in other models like Chlamydia, GAGs  
552 were shown to be used for initial attachment to host cells (Tiwari et al., 2012); Lyme disease  
553 *Borreliae* requires glycosaminoglycan binding activity to colonize and disseminate to tissues  
554 (Lin et al., 2017). Even though heparan sulfate appears to be the most important GAG  
555 species involved in bacterial binding, both heparan sulfate and [chondroitin](#) sulfate were able  
556 to influence the attachment of mucoid *P. aeruginosa*, *H. influenza* and *B. cepacia* in specific  
557 ways that were dependent on the cell line involved (Martin et al., 2019). *Borrelia burgdorferi*

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562 has multiple surface proteins with different binding specificities to GAGs depending on the  
563 tissue affected (Leong et al., 1998). Different GAGs act as receptors for *B. burgdorferi*  
564 depending on the host cells; both heparin sulfate and heparan sulfate are essential in  
565 adherence to primary endothelium and adult kidney Vero cells, but only dermatan sulfate is  
566 involved in attachment to human embryonic kidney cells (Garcia et al., 2016).

567 Although we did not establish the interaction of rApe adhesion with a cellular receptor, we  
568 can still hypothesize that Ape actively triggers *Ehrlichia* internalization by the mean of a  
569 ligand/receptor interaction. Kumar *et al.* already suggested the existence of additional  
570 mammalian receptors for *Ehrlichia* infection (Mohan Kumar et al., 2013). The present study  
571 identified the bacterial of a putative second *Ehrlichia* invasion-receptor pair and highlights  
572 the importance of this molecular control of invasion for the *Anaplasmataceae* intracellular  
573 bacteria. The probable existence of several ligand-receptor systems could indeed serve the  
574 bacteria to infect a broader host range of animal reservoirs and vector ticks. Moreover, GAG  
575 degradation following [chondroitinase](#) treatment severely impaired *Ehrlichia* infection. This  
576 suggests that Ape could interact with a glycosylphosphatidylinositol (GPI)-anchored protein  
577 as previously shown for PSGL-1 that is required for the binding and infection of human HL-  
578 60 cells by *A. phagocytophylum* (Herron et al., 2000), This remains to be further studied as  
579 well as its role in iron uptake of *Ehrlichia ruminantium* (Reneer et al., 2008).

580 In summary, with the identification of Ape (ERGA\_CDS\_01230), we found the first *Ehrlichia*  
581 *ruminantium* protein that is involved in host cell invasion. Whether the *E. chaffeensis* EtpE  
582 homolog (ERGA\_CDS\_08340) is functional in *E. ruminantium* remain to be explored, but  
583 these outer-membrane proteins can now be considered as immune-dominant pathogen-  
584 associated molecular patterns (Budachetri et al., 2020). Our next step is now [to](#) investigate  
585 the use of rApe as a new vaccine candidate against Heartwater. In light of the lack of  
586 prophylactic measures against *Ehrlichia* spp. and the rising appearance of antibiotic  
587 resistances, deep understanding of invasion mechanisms is of prime importance and will  
588 help to propose efficient alternative therapeutics blocking the early interaction between  
589 these obligate intracellular bacteria and their host cells.

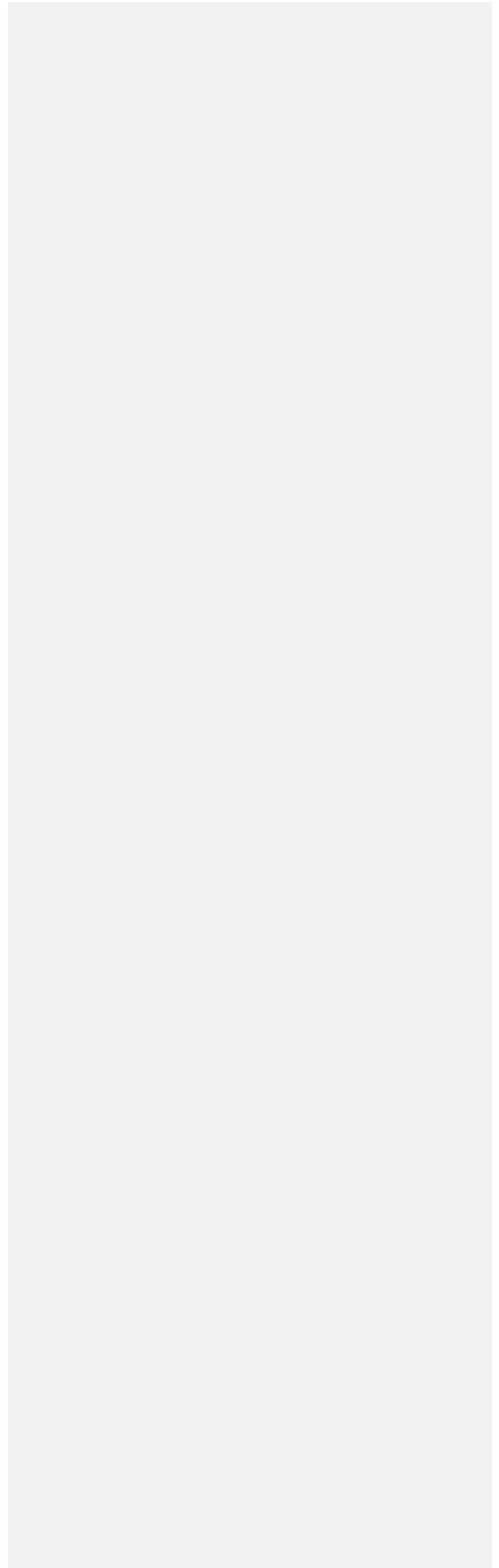
590

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750

751 **Figure captions**

752 **Figure 1. Temporal expression of the (ERGA\_CDS\_01230) ape gene in *E. ruminantium***  
753 **determined by qRTPCR.**

754 (A) *E. ruminantium* sigmoidal growth curve was determined by qPCR targeting *pCS20*  
755 region and represented as relative bacterial number along the cycle of development

a supprimé: log 10

756 (B) Transcript levels were determined from 24, 48, 72 and 120 hpi by qRTPCR targeting  
757 ERGA\_CDS\_01230 gene. Levels were normalized by the quantity of bacteria measured by  
758 qPCR Sol1 and the ratio was compared to 96 hpi, allowing fold-change (FC) determination,  
759 expressed as log 2. Values at each time point are the means +/- standard deviations for 2  
760 biological replicates. Gp38 is for *E. ruminantium* Gardel strain, passage #38 (virulent strain).

761

762 **Figure 2. Structural characterization of Ape protein.**

763 (A-H) I-TASSER-MTD derived 3D-prediction of the ERGA\_CDS\_01230 gene product (Ape  
764 protein in green) interacting with oxo-iron molecule (red). Front (A-B), back (C-D) and side  
765 (E-F) views of Ape showing a succession of  $\alpha$ -helices (turquoise) linked by loops (pink)  
766 distributed around  $\beta$ -sheets (purple), organized as a three-dimensional "c-clamp" structure.  
767 The enlarged panels (G-H) of the binding site allow the visualization of the residues involved  
768 in polar (yellow) and non polar (light blue) interactions. The oxo-iron molecule (CIN1)  
769 constituted by 3 atoms of iron (orange) and 12 atoms of oxygen (red) interacts with Ape  
770 protein (green) via polar interaction (hydrogen bond with T99 in yellow) and non-polar  
771 interactions (hydrophobic interactions in light blue involving L120 and Q122) bonds.

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772 (J) Reliability score prediction by CELLO 2.5 software of the subcellular localization of the  
773 native *E. ruminantium* Ape protein. Ape presents a typical subcellular localization of an  
774 active transporter, with a dominant cytoplasmic localization and is also present at the outer  
775 membrane.

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776

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777 **Figure 3. rApe is an O-glycosylated recombinant protein.**

778 Composite picture of a Western blot detecting O-glycosylation of recombinant Ape protein  
779 (lane 2). Recombinant proteins were separated by SDS-PAGE, then transferred to PVDF  
780 and incubated with anti-O-GlcNAc antibody. The Western blot was probed with anti-mouse  
781 IgM-HRP antibody and revealed by TMB substrate. Lane 1: negative control : BSA; lane 2:  
782 rApe. Numbers and black arrowheads indicate molecular masses in kilodaltons (kDa). The  
783 recognized rApe is significantly larger (66 kDa) than the one predicted by the amino acid  
784 sequences encoded by ERGA\_CDS\_01230 gene (41 kDa).

793

794 **Figure 4. Chondroitinase impairs BAEC infection by *E. ruminantium*.** Using 0.4 and  
795 0.9U/mL [chondroitinase](#) resulted in a halving of the number of bacteria compared to the  
796 untreated condition. Bacterial quantification was performed at lysis stage by qPCR sol1 after  
797 [chondroitinase](#) treatment at three different concentration. Fold-change (FC) was obtained  
798 by calculating the bacterial amount differential for each condition compared to the condition  
799 without treatment and represented as  $2^{-\Delta\Delta Ct}$ .

800

801 **Figure 5. rApe attaches to the host cells.**

802 Different concentrations of rApe tagged with GFP were incubated with BAEC. Adherence of  
803 GFP-rApe to prefixed BAEC was evaluated by flow cytometry and showed a dose-effect  
804 relationship up to 36  $\mu$ g of rApe. Fluorescent-labelled cells quantified by flow cytometry are  
805 represented in % for each concentration point. Auto-fluorescence was evaluated with cells  
806 without recombinant protein.

807

808 **Figure 6. rApe interacts with cell lysate and membrane fractions.**

809 Composite picture of a Far-Western blot detecting Ape protein. Lysate and cell fractions  
810 were separated by SDS-PAGE, transferred to PVDF and incubated either with rApe (left  
811 panel) or PBS as a negative control (right panel). The Western blot was probed with rabbit  
812 anti-GFP-HRP antibody, and revealed by peroxide substrate mixed with chromogen DAB.  
813 1: protein ladder, 2 and 5: cell lysate, 3: cytoskeleton fraction, 4 and 6: membrane and  
814 organelles fraction.

815

816 **Figure 7. rApe is sufficient for adhesion of latex beads to bovine endothelial cells.**

817 Representative images of BAEC incubated 30 minutes with fluorescent latex beads  
818 ( $1.82 \times 10^8$ ) coated with rApe and processed for scanning electron microscopy.

819 (A) Endothelial cells did not retain non-adsorbed beads (0.1 $\mu$ m diameter) on their cell  
820 surface. (B) On the opposite, the whole surface of the same type of cells are covered with  
821 adherent beads (0.5 $\mu$ m diameter) absorbed with rApe. (C) Enlarged view of adherent beads.  
822 Such beads present two kinds of localizations. Some of them are already internalized (black  
823 arrow heads) while others are still located outside the cell remaining in contact with the  
824 cytoplasmic membrane (white arrow heads). Scale bars are indicated.

825

826 **Figure 8. Ape induces an antibody response in sera of vaccinated goats.**

a supprimé: Chondroitinase

a supprimé: chondroitinase

a supprimé: chondroitinase

830 The antibody response to Ape during vaccination kinetic was tested by ELISA. Sera of  
831 vaccinated goats were incubated in wells coated with rApe, followed by incubation with anti-  
832 goat antibody coupled to HRP. Antibody response was detected by ELISA titers (optical  
833 density at 450 nm). Shown are representative results from five vaccinated goats. #614: goat  
834 vaccinated with an attenuated vaccine. #915: goat vaccinated with an inactivated vaccine.  
835 The time (in weeks) post-vaccination is indicated. Goats vaccinated with inactivated vaccine  
836 were also challenged for resistance to *E. ruminantium* Gardel strain seven weeks post  
837 vaccination.

838

839 **Figure 9. Schematic representation of *Ehrlichia ruminantium* binding to mammalian  
840 cells and rApe interaction with the host cell surface.**

841 Ape is located at *E. ruminantium* outer membrane and is recognized by antibody from sera  
842 of vaccinated animals. *E. ruminantium* can adhere and enter into BAEC but infection is  
843 reduced when GAG like [chondroitin](#) and dermatan sulfate are degraded. The recombinant  
844 version of *E. ruminantium*, rApe, is glycosylated and latex beads coated with rApe bind to  
845 BAE cell surface and start to enter in BAEC, in a similar manner that of *E. ruminantium*.  
846 Whether Ape binds to a cellular receptor and the following triggered signaling cascade  
847 remain to be determined.

a supprimé: chondroïtine





!

<b>BACTERIAL LOCALIZATION</b>	<b>ERGA_CDS_01230</b>
Cytoplasmic	<b>2,418</b>
Outer membrane	<b>1,652</b>
Periplasmic	0,424
Inner membrane	0,404
Extracellular	0,102

Figure 2

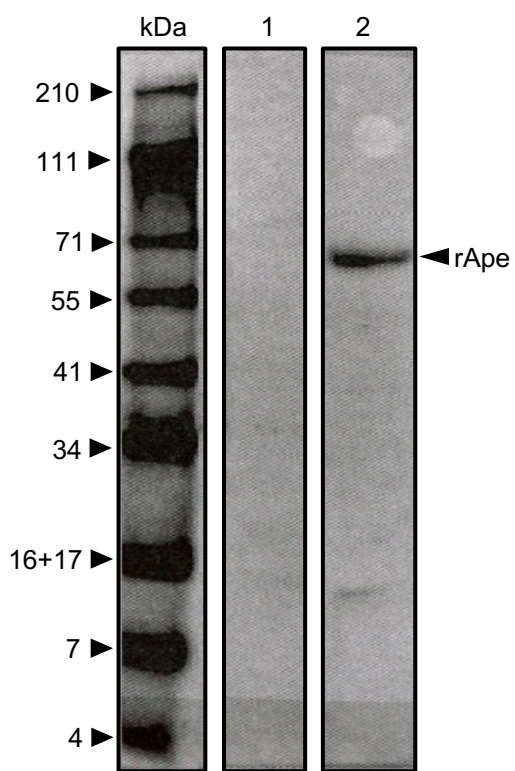


Figure 3

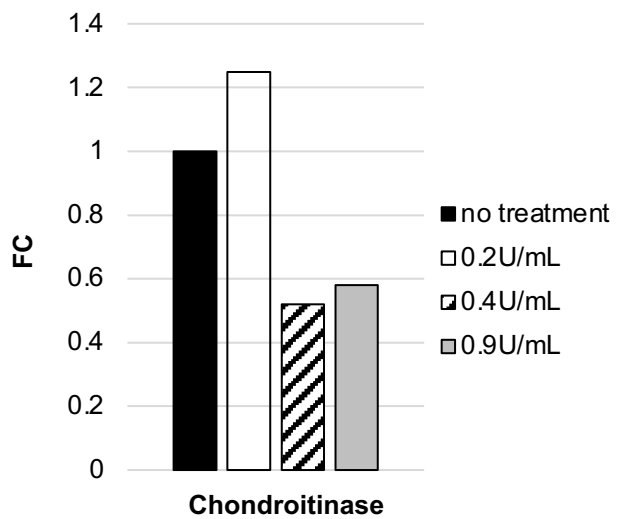


Figure 4

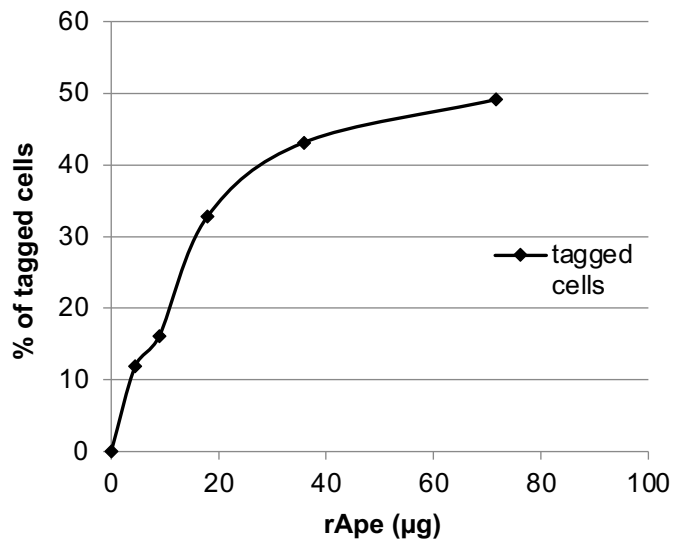


Figure 5

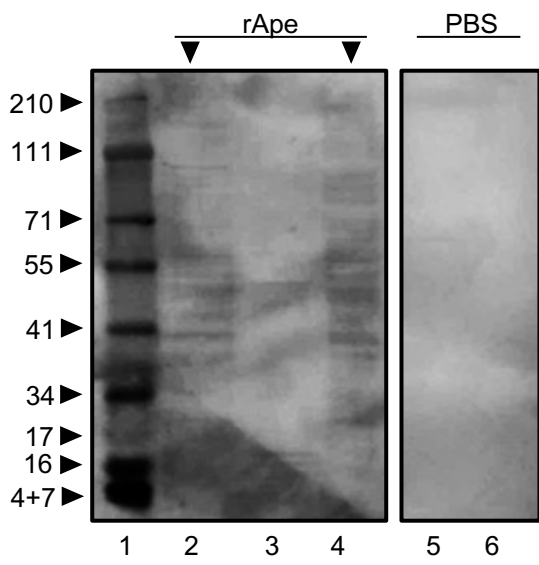


Figure 6

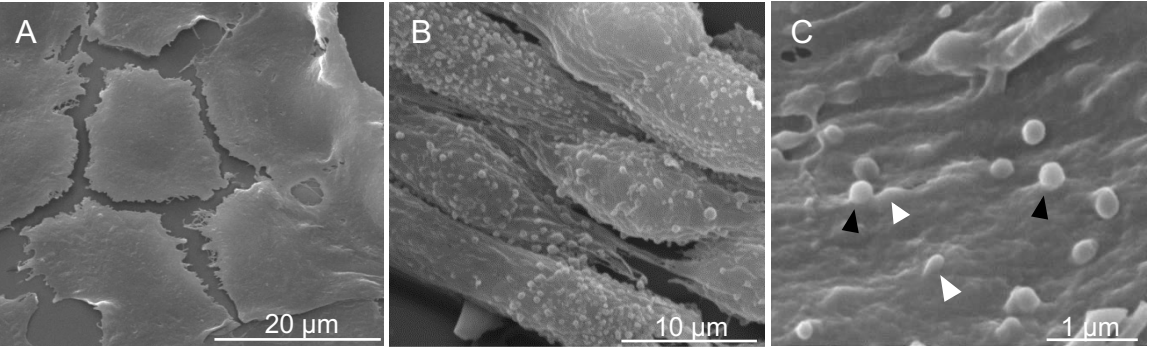


Figure 7

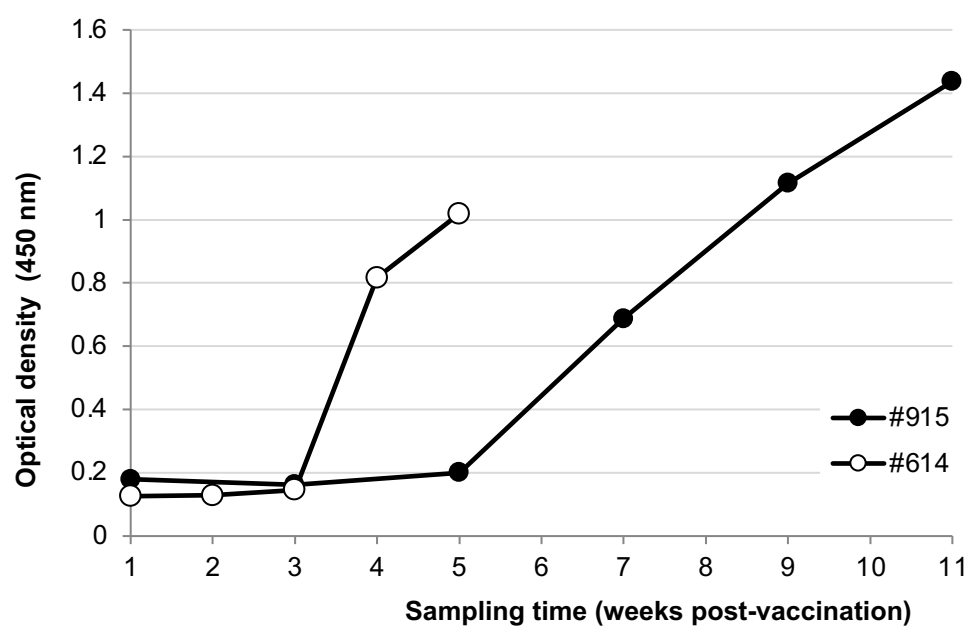


Figure 8



