**Introduction**

Eukaryotic flagella present a remarkable evolutionary conservation of their structure and constituents [1]. They have been mostly studied in *Chlamydomonas reinhardtii* [2,3] and *Trypanosoma brucei* [4,5], two flagellated organisms. Thus, intrflagellar transport was first described in *C. reinhardtii* before being generalized to all eukaryotic flagella and cilia [6,7]. Stressing their practical importance, protozoan studies allowed the identification of human orthologue genes whose mutations are responsible for pleiotropic, severe genetic diseases, such as polycystic kidney disease or Bardet-Biedl syndrome [8].

*Leishmania* are flagellated protozoan kinetoplastid parasites [9]. They exist alternatively as amastigotes (intracellular mammalian forms) and promastigotes (extracellular insect forms) [10]. While the amastigote flagellum barely sticks out of the cell body, its promastigote counterpart may be twice as long as the cell body. The flagellum is essential for promastigote motility within the insect digestive tract, allowing the migration of the parasites to the mouth parts and hence their transmission to a mammalian host through biting [11]. Recent elegant experiments have also shown that the flagellum is indispensable for infectious promastigotes to infect mammalian host macrophages where they transform into amastigotes [12]. Moreover, the flagellum is also suspected to bear other essential functions like cellular organization and sensory perception [13]. The differentiation from amastigotes to promastigotes and reciprocally requires the successive assembly and disassembly of hundreds of proteins. The dynamics of these processes is tightly regulated and their understanding necessitates the characterization of individual elements.

Bin/Amphiphysin/Rvs (BAR) domains are 200-amino-acid modular elements found in many eukaryotic multi-domain proteins [14]. Although their amino acid sequence may be poorly conserved and therefore difficult to identify, their structure is well conserved throughout evolution [15]. The archetype BAR domain consists of a monomer of three α-helices folded onto each other. Dimers form banana-shaped molecules with a positively charged concave face that interacts with the negatively charged lipid membranes [16]. BAR domains recognize or generate membrane curvature by inserting into the lipid bilayer. The specific membrane to which they bind to depends on adjacent domains, e.g., PH domains [17]. Since their discovery [18], BAR domains revealed diverse and have been categorized into several families and sub-families; some associate with concave, others with convex membranes [19]. BAR domain proteins are involved in membrane shaping, in the formation of endocytosis vesicles, tubules, endosomes, T-tubules, podosomes, filopodia, mitochondria and autophagosomes [20].

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**Abstract**

During the *Leishmania* life cycle, the flagellum undergoes successive assembly and disassembly of hundreds of proteins. Understanding these processes necessitates the study of individual components. Here, we investigated *Ld*Flabarin, an uncharacterized *L. donovani* flagellar protein. The gene is conserved within the *Leishmania* genus and orthologous genes only exist in the *Trypanosoma* genus. *Ld*Flabarin associates with the flagellar plasma membrane, extending from the base to the tip of the flagellum as a helicoidal structure. Site-directed mutagenesis, deletions and chimera constructs showed that *Ld*Flabarin flagellar addressing necessitates three determinants: an N-terminal potential acylation site and a central BAR domain for membrane targeting and the C-terminal domain for flagellar specificity. In vitro, the protein spontaneously associates with liposomes, triggering tubule formation, which suggests a structural/morphogenetic function. *Ld*Flabarin is the first characterized *Leishmania* BAR domain protein, and the first flagellum-specific BAR domain protein.
Here we report the characterization of *LdFlabarin* (*L. donovani* FLAgellar BAR domain proteIN). To our knowledge, it is the first BAR domain protein associated with a eukaryotic flagellum and the first BAR domain protein found in *Leishmania*. We show that its flagellar addressing depends on several determinants and presents some originality within the genus *Leishmania* and the BAR domain superfamily. It arranges into a helicoidal structure around the flagellum and provokes the tubulation of artificial membranes in vitro. This suggests a role in flagellar morphogenesis or structural stability.

**Results**

**Identification of the *Leishmania* Flabarin**

We previously discovered the involvement of small G proteins in the biogenesis of the *Leishmania* flagellum [21–23], which led us to try and identify effectors. One of the approaches used was bioinformatics: the *L. major* genome [24], the only available *Leishmania* genome sequence at the time of the experiments, was searched for homologues of known partners of the human ARF/ARL small G proteins [25–27].

We found *LmjF.27.1730*, an unannotated 340-aa protein, as potential homologue of human *HsArfaptin-1* (Genbank U52521), a partner of several ARF/ARL family members [25,28]. The N-terminal region of *LmjF.27.1730* (amino acids 10–231) showed some identity (Fig. S1A) with the C-terminal region of *HsArfaptin-1* (amino acids 61–339 for a total length of 341). The presence of a polypyrimidine tract 59–212 nt upstream of the start codon, followed by an AG dinucleotide (7–6 nt upstream of the start codon), a potential spliced leader attachment site [29], validated the predicted functionality of the *LmjF.27.1730* ORF.

For consistency with our previous work, we chose to focus on *L. donovani*. The orthologue was PCR amplified from *L. donovani* LV9 genomic DNA, using oligonucleotides designed from the *LmjF.27.1730* sequence. The *L. donovani* protein comprised 339 amino acids, with a predicted molecular mass of 37 827 Da and a pI of 5.17. Its amino acid sequence was 90% identical to *LmjF.27.1730* and differed by one amino acid with the now available *L. infantum* orthologue sequence (*LinJ.27.1630*) (Fig. S1B).

For reasons becoming obvious below, these new proteins were named Flabarins for FLAgellar BAR domain proteINS.

**Phylogenetic analysis of Flabarins**

Blast searches and available data [24] revealed the existence of *LdFlabarin* orthologues in trypanosomatids (Fig. S1B). Flabarins amino acid sequences and lengths were well conserved within the genus *Leishmania* (*L. infantum*, *L. major*, *L. mexicana*, and *L. braziliensis*): 339–340 amino acids, 99.7–100% identity with *LdFlabarin*. By contrast, *Trypanosoma* (*T. cruzi*, *T. vivax*, *T. congolense*, and *T. brucei*) Flabarins were shorter (222–269 amino acids) and more divergent (18–20% identity). Synteny was observed within these species [24] except for *T. vivax* (though *TvFlabarin* sequence did not differ much from the other *Trypanosoma* Flabarin sequences). The *T. brucei* and *T. gambiensis* Flabarin sequences were identical and the most divergent (18% identity). To our knowledge, the only previous report of trypanosomatid Flabarin is that of *T. brucei*, identified as a flagellar protein (*Tb927.11.2410*, formerly *Tb11.22.0001*) in the flagellome [4,5] and not further studied. There is no apparent homologue in any other sequenced genome (Group OGI _140786_ [30]), which makes the study of Flabarins particularly interesting.

**LdFlabarin is a flagellar protein**

*LdFlabarin* intracellular localization was investigated in *L. amazonensis* BA125 cells co-expressing mRed-*LdFlabarin* (with a free *LdFlabarin* C-terminus) and *LdFlabarin*-GFP (with a free

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**Figure 1. Intracellular localization of *LdFlabarin***. *L. amazonensis* BA125 cells were co-transfected with pNUS mRedN-D-*LdFlabarin* and pNUS *LdFlabarin*-GFPcH. (A–B) Fluorescence images from a cell expressing both mRed-*LdFlabarin* (red) and *LdFlabarin*-GFP (green). (C) Overlay of A and B. (D) Overlay of DAPI staining (blue) and phase contrast. (E) A cell expressing *LdCentrin*-GFP (green) and *LdFlabarin*-mRed.

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LaFlabarin N-terminus). Fluorescence microscopy showed that mRed-LaFlabarin was diffusely distributed throughout the cell (Fig. 1A,C,D), while LaFlabarin-GFP localized exclusively to the flagellum (Fig. 1B,C,D). About 2% of LaFlabarin-GFP-expressing cells displayed a stronger fluorescence signal at the base of the flagellum (Fig. 1B), an area different from the basal body, as revealed by the marker LdCentrin-GFP [31] (Fig. 1E) and much larger.

Since the LaFlabarins tagged at the N- or C-terminus did not co-localize, the localization of native LaFlabarin was unclear. To elucidate this point, we produced a specific anti-LaFlabarin rabbit antiserum. First, C-terminus His6-tagged recombinant LaFlabarin (LaFlabarin-His6) was synthesized in E. coli using the pET29b expression vector. After IPTG induction, a 55 kDa band (for a predicted 41.3 kDa, including the His6-tag) was visible in extracts submitted to SDS-PAGE (Fig 2A, SI) while no band was visible without induction (Fig 2A, SNI). The difference between the predicted and the observed molecular masses may be due to intrinsic migration properties. Surprisingly, under non-denaturing conditions, partially purified LaFlabarin-His6 (Fig 2A, F16) presented several high molecular mass bands (~240, ~360, and ~570 kDa, Fig. 2B). When submitted to a denaturing second dimension electrophoresis, these high molecular mass bands all dissociated to 55 kDa spots (Fig 2C1), all of them being reactive to anti-His6 antiserum (Fig 2C2); thus, the complexes comprised mostly LaFlabarin-His6 proteins devoid of major E. coli contaminants, since no other spot was visible by Coomassie staining, although the presence of minor components, eventually responsible for the molecules aggregation, cannot be excluded.

For the rabbit immunization, the recombinant LaFlabarin-His6 was partially purified (Fig 2A, F16). The obtained anti-LaFlabarin antiserum was used to probe western-blotts of L. amazonensis cell extracts. Native LaFlabarin was detected as a 50 kDa band (for a predicted 37.8 kDa; Fig 2D, left panel, lane 1); no band was seen with the preimmune serum (not shown) or an anti-mRed antiserum (Fig 2D, right panel, lane 2). Using LaFlabarin-mRed-expressing cell extracts, the anti-LaFlabarin recognized a main additional band of 90 kDa (Fig. 2D, left panel, lane 2) (for a predicted 63.3 kDa), also detected by the anti-mRed antiserum (Fig 2D, right panel, lane 2). The difference between the predicted and the observed molecular masses is not clear at the moment, and may be due to post-translational modifications or intrinsic migration properties. An additional 75 kDa band was also detected by the anti-LaFlabarin (Fig 2D, left panel, lane 2); however, the intensity of this extra-band, feebly detected by the anti-mRed antiserum (Fig 2D, right panel, lane 2), was variable from an experiment to another, suggesting the existence of a preferential cleavage site within the LaFlabarin-mRed sequence; the protease(s) involved could be partially active in spite of the presence of an anti-protease cocktail during the cell lysis, although it remains possible that this partial proteolysis occurred before lysis.

Probing untransformed L. amazonensis cells by indirect immunofluorescence, the anti-LaFlabarin revealed a punctuated labeling along the flagellum (Fig. 2E1 and E2). As with the GFP/mRed fusion proteins, a spot was sometimes observed at the flagellum base (Fig. 2E, lower cell). The flagellum most distal part remained unlabelled (Fig. 2E, both cells); whether the endogenous LaFlabarin was absent or in too low amount to be detected by the antiserum remains unknown; conversely, the more abundant tag LaFlabarin-GFP was visible from bottom to tip of the flagellum (Fig. 1B). In conclusion, our data confirmed the flagellar localization of native LaFlabarin and validated the observations made with the C-terminally tagged LaFlabarin-GFP (Fig. 1B): a

Figure 2. Production of recombinant LaFlabarin-His6 and localization of LaFlabarin in Leishmania. (A) 5 μg supernatant of non-induced (SN), IPTG-induced (SI) and purified LaFlabarin-His6 (F16) were separated by SDS-PAGE (denaturing polyacrylamide gel electrophoresis). (B) The purified fraction (F16) was submitted to a blue-native gel (BN-PAGE) allowing separation of complex. (C) The complex were separated by electrophoresis in first non-denaturing conditions (BN-PAGE), and then the track was subjected to a second electrophoresis under denaturing conditions (SDS-PAGE) to separate the components of the different complex. The gel was stained with Coomassie (C1) or transferred to membranes and revealed with 1:10000 anti-His6 antiserum.
anti-His (C2). (D) 3.10^6 L. amazonensis BA125 untransfected control cells (lanes 1) and LdFlabarin-mRed-expressing cells (lanes 2) were submitted to SDS-PAGE, transferred to membranes and revealed with 1:2000 anti-LdFlabarin (left panel) or 1:10000 anti-mRed (right panel) and 1:2500 anti-rabbit IgG conjugate as in [59]. (E) Localization of native LdFlabarin in L. amazonensis BA125 by indirect immunofluorescence. Cells were fixed and incubated with anti-LdFlabarin (1:1000) and Alexa-labelled anti-rabbit IgG (8 μg/ml). Panel 1, LdFlabarin green fluorescence image of two cells in the same field; panel 2, overlay of panel 1 with DAPI staining (blue) and phase contrast.

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free N-terminus is indispensable for LdFlabarin flagellar addressing.

Ultrastructural localization of LdFlabarin

When observed by indirect immunofluorescence (anti-LdFlabarin plus Alexa-labelled anti-rabbit IgG), the green fluorescence signal of LdFlabarin-mRed (Fig. 3A1) was usually confined to the periphery of the flagellum, while the inside region remained darker (Fig. 3A1 and A3), as expected for a plasma membrane labelling; this pattern was less obvious with LdFlabarin-GFP (Fig. 1B) or the direct visualisation of LdFlabarin-mRed fluorescence (Fig. 3A2), which could be attributed to different diffusion characteristics of the fluorophores. For comparison, we used a paragangellar rod (PFR) marker. PFR is a cytoskeletal structure of trypanosomatid, euglenoid and dinoflagellate flagella [32] that extends inside the flagellum, along the axoneme, from the exit of the flagellar pocket to the tip of the flagellum [33]. Its main constituents are two structurally related proteins, PFR1 and PFR2 [33,34], which, as other cytoskeleton-associated proteins, remain insoluble after treatment with non-ionic detergents [35,36]. When expressed as fusion proteins with GFP at their N-terminus, PFR proteins are addressed to the flagellum [37]. Here the L. amazonensis PFR2C (one of the tandemly repeated isoforms fused to GFP) was co-expressed with LdFlabarin-mRed in L. amazonensis. GFP-LaPFR2C was visible as a thin flagellar filament (Fig. 3B2), whereas LdFlabarin-mRed extended further inside the flagellar pocket and, in that particular case, was visible as two external red filaments on each side of the green GFP-LaPFR2C signal (Fig. 3B1, B3 and B4); clearly, LdFlabarin-mRed did not co-localize with GFP-LaPFR2C.

To investigate the possible association of LdFlabarin with the flagellar cytoskeleton, cells co-expressing GFP-LaPFR2C and LdFlabarin-mRed were treated with increasing concentrations (0.0005 to 1%) of the non-ionic detergent NP-40 before paraformaldehyde (PFA) fixation (Fig. 3B). From 0.0005% (Fig. 3B5–8) to 0.001% (Fig. 3B9–12) up to 1% NP-40 (not shown), GFP-LaPFR2C remained associated to the cell ghosts (Fig. 3B6 and B10), while LdFlabarin-mRed was destabilized with as little as 0.0005% NP-40 (Fig. 3B5) and completely solubilized with 0.001% NP-40 (Fig. 3B9), indicating that LdFlabarin is not associated with the flagellar cytoskeleton.

Then we performed immuno-electron microscopy on non-transfected and LdFlabarin-mRed-expressing L. amazonensis cells using anti-LdFlabarin or a rabbit anti-mRed antibody plus an anti-rabbit IgG-gold particles conjugate. The gold particles (black dots) localized to the flagellar plasma membrane (Fig. 4A and B upper panel) in longitudinal cross-sections of L. amazonensis cells. The specificity of the labelling was ascertained by the absence of any black dot when the anti-LdFlabarin primary antiserum was omitted (Fig 4D). A cumulative transverse cross-sectional image was generated (Fig. 4B, lower panel; artificial color: white dots), and LdFlabarin-mRed appeared both randomly distributed on the surface of the flagellum and randomly oriented with respect to the axoneme or the PFR. Conversely, in longitudinal sections, the black dots appeared clustered in small bundles, obliquely aligned and separated by a mean distance of 178±31 nm (Fig. 4C and F), compatible with a helicoidal arrangement around the flagellum.

Figure 3. LdFlabarin is not associated with the flagellar cytoskeleton. (A) Comparison of LdFlabarin/LdFlabarin-mRed localization by immunodetection with rabbit anti-LdFlabarin and mRed fluorescence. L. amazonensis BA125 cells expressing LdFlabarin-mRed were treated with anti-LdFlabarin plus Alexa-labelled anti-rabbit IgG. A1, native LdFlabarin and LdFlabarin-mRed revealed by anti-LdFlabarin plus Alexa-labelled anti-rabbit IgG (green); A2, same cell, LdFlabarin-mRed revealed by mRed fluorescence (red); A3, overlay of A1 and A2; A4, overlay of A1, A2, DAPI staining (blue) and phase contrast images; A5, magnification of the framed area of A1. (B) Co-expression of LdFlabarin-mRed and GFP-LaPFR2C in L. amazonensis BA125. Cells were co-transfected with pNUS LdFlabarin-mRedcD (red, B1) and pNUS GFPnP-H-LaPFR2C (green, B2; B3 is an overlay of B1 and B2), stained with DAPI (blue, B4), B5–12, NP-40 treatment. Prior to PFA fixation, cells co-expressing LdFlabarin-mRed and GFP-LaPFR2C were treated for 5 min at room temperature with 0.0005% (B5–8) and 0.001% (B9–12) NP-40. B1/B5/B9, LdFlabarin-mRed (red); B2/B6/B10, GFP-LaPFR2C (green); B3/B7/B11, overlay image of B1–2/B5–6/B9–10; and B4/B8/B12, overlay image of B1–2/B5–6/B9–10 plus DAPI staining (blue) and phase contrast.

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Localization and point to a structural function for the occasional protein accumulation observed at the base of the result of a limited proteolysis. The presence of a small amount of additional 75 kDa band, which behaved like the 90 kDa band in the presence of detergent (Fig 5A lane SN versus PN). The fraction (Fig 5A lane S) while it translocated to the soluble fraction (100 000 g pellet) (Fig 5A lane P) compared to the soluble Ld membrane or lumen (Fig. 4E).

Consistently, after cell fractionation, the 90 kDa band of LdFlabarin-mRed was mainly associated with the membrane fraction (100 000 g pellet) (Fig 5A lane P) compared to the soluble fraction (Fig 5A lane S) while it translocated to the soluble fraction in the presence of detergent (Fig 5A lane SN versus PN). The additional 75 kDa band, which behaved like the 90 kDa band in fractionation experiments, was present in variable amounts, depending of the lysate (Fig 5A) which confirmed it may be the result of a limited proteolysis. The presence of a small amount of LdFlabarin-mRed in the 100 000 g supernatants maybe reflected the occasional protein accumulation observed at the base of the flagellum, which did not seem to be associated to membranes according to the electron microscopy images (Fig. 4E).

Taken together, these data demonstrate a plasma membrane localization and point to a structural function for LdFlabarin.

Predicted consensus motifs of LdFlabarin

Trypanosomatid Flabarin sequences were analyzed for the presence of consensus motifs and potential post-translational modification sites, and their conservation among orthologues (Table S1).

Many Leishmania membrane proteins are heavily and variably glycosylated [38,39]. LdFlabarin has a potential N-glycosylation site at asparagine 267 (N267), conserved in all Leishmania Flabarins but absent in Trypanosoma Flabarins (their sequence being shorter; Fig. S1B). Inactivation of this site by replacement of the asparagine (Fig 5C) by a glutamate (LdFlabarin/N267Q-mRed) did not affect flagellar localization (Fig. 5B) (nor cell viability).

Some membrane proteins require acylation (palmitoylation) of N- or C-terminal cysteine residues for membrane anchoring [40]. Using the CSS-Palm software [41], we found a potential palmitoylation site in the LdFlabarin N-terminus (amino acids 1–7, MPLCASL) with a palmitoylatable cysteine at position 4. This site is conserved in Leishmania and Trypanosoma Flabarins, with the exception of T. vivax (Table S1). The cysteine 4 was replaced by a serine (LdFlabarin/C4S-mRed); as a consequence, the potential palmitoylation site was destroyed. Compared to the flagellar wild-type LdFlabarin-mRed (Fig 5C), the fluorescence was diffuse throughout the cell body (Fig. 5D). Cell fractionation showed that the mutant protein LdFlabarin/C4S-mRed was not associated with the membrane fraction (Fig 5A; lane P) but remained soluble in the 100 000 g supernatant (Fig 5A, lane S). Thus, the cysteine 4 was indispensable for the membrane localization as for the flagellar addressing of LdFlabarin.

Structural domains of LdFlabarin

The amino acid sequence of LdFlabarin was analyzed by several online programs for structure predictions. As summarized in Fig. S2, LdFlabarin comprise: (i) a short N-terminal β-strand (S1, aa 1–15), (ii) a putative BAR domain (aa 16/22 to 199/218), limits depending on the prediction program) with 6 α-helices (H1–6), a β-strand (S2) and 5 loops (L1–5), (iii) a “linker” consisting of one loop (L6) and one α-helix (H7), and (iv) a C-terminal disordered domain (D, aa 256–339). The D domain may overlap with loop L6/α-helix H7 and may comprise an additional α-helix (H8) (not shown). Three amphiphilic helix-rich regions (AHRs) were detected within the BAR domain (AHR-1, aa 19–123; AHR-2, aa 202–221) and the C-terminus with a lower probability (AHR-3, aa 291–335). Three potential dimerization regions were predicted on each side of the BAR domain. Although this model will require validation by the 3D-structure determination of LdFlabarin, it provides useful insights on the properties of the protein.

The BAR domain. Proteins of the BAR domain superfamily bind and tubulate liposomes in vitro [42,43] and anchor proteins to membranes in vivo. To test the prediction of the presence of a functional BAR domain within LdFlabarin, we produced liposomes and examined their morphology by electron microscopy in the presence and absence of LdFlabarin. Incubation with purified LdFlabarin-His6 resulted in deformations of the liposomes that exhibited tubules of similar diameter (15.9±1.7 nm, mean of 92 measurements) but various length (Fig. 6A–C and F). Tubule length varied with protein concentration and incubation time but diameter was constant and there was no branching. By contrast, incubation without LdFlabarin-His6 showed no lipidome deformation/tubulation (Fig. 6D). To rule out the possibility that the His6-tag was responsible for the tubulation, it was removed by thrombin digestion, the protein was further purified and similar tubulation occurred (Fig 6E). Thus, like other BAR domain–containing proteins, LdFlabarin was able to bind lipids and induce membrane curvature in vitro.

Concerned with its role in vivo, we replaced LdFlabarin BAR domain by the mRed protein and expressed the chimera F(1–125)-mRed-F(256–339) in L. amazonensis. The protein localized diffusely to the entire cell body (Fig. 5E); cell fractionation revealed that it was soluble, absent from the 100 000 g pellet (Fig 5A, lane P) but present in the supernatant (Fig 5A, lane S) (same with detergent, Fig 5A, lanes SN and PN). Thus the putative BAR domain was necessary for LdFlabarin flagellar addressing.

However, the domain was not sufficient for LdFlabarin flagellar addressing, as the protein F(1–255)-mRed, obtained after deletion the tail (aa 256–339), localized mainly to membranes (Fig 5F) and could be found in the 100 000 g pellet (Fig 5A, lane P) but not in the supernatant (Fig 5A, lane S) except in the presence of detergent (Fig 5A lane SN).

In conclusion, LdFlabarin is a flagellar protein; its flagellar addressing depends on three determinants: an N-terminal potential acylation (palmitoylation) site and a BAR domain, which direct the protein to plasma membranes, and a C-terminal domain which directs the membrane protein to the flagellum.
Discussion

Flabarins are not Arfaptin homologues and are unique to eukaryotes

We report the characterization of Flabarin, a novel *Leishmania* flagellar protein. It was identified based on its sequence homology to *Hs* Arfaptin-1. *Hs* Arfaptin-1/-2 localize to the trans-Golgi network (TGN) [25]; they interact with the small G proteins *Hs* Rac-1 [28], *Hs* ARF-1, -3, -6 [25,44] and participate in the assembly of trafficking vesicles together with *Hs* ARF-1 [45] or *Hs* ARL-1 [46,47]. On the other hand, *Leishmania* Flabarins are flagellar proteins; their ultrastructural localization and arrangement make it unlikely that they participate in intracellular trafficking. Besides, a close look at the sequence alignments (Fig. S1A) reveals that Flabarins and Arfaptins share identity only in the BAR domains which are located in the C-terminus of *Hs* Arfaptin-1 and in the N-terminus of *Lm* Flabarin. Flabarins belong to only one orthologous group, the Group OG5_148786 [30], which exists only in trypanosomatids. Thus, these proteins are certainly not functional homologues.

Flabarins are flagellar proteins

The flagellum is a complex structure, comprising notably a lumen, a plasma membrane, an axoneme, and in the case of trypanosomatids, an additional structure called the PFR (paraflagellar rod). Flagellar targeting consists probably in several different mechanisms, for every protein must be targeted to its specific location and there are several hundreds of them. These mechanisms are being progressively deciphered [48] and reveal complex. Sequence motifs have been found to be involved in a specific flagellar targeting; for example, a simple HLA C-terminal motif is necessary, but not sufficient, for the...
Proteins PFRA and ARP to reach their location in the T. brucei PFR structure [37,49], while another N-terminal motif is necessary and sufficient to address the protein ADK-A to the same structure [50]. Concerning flagellar membrane proteins, two main mechanisms have been documented. The first one concerns the Leishmania glucose transporter, where particular internal epitopes and amino-acids are essential [51,52]. The second category of proteins must be myristoylated and palmitoylated at their N-termini for reaching the flagellar membrane; it is the case of the T. cruzi FCaBP [53,54], of the T. brucei Caflagnins [55] and of the Leishmania HASPB [56] and SMP-1 [57,58].

For LdFlabarin, flagellar membrane targeting should involve another mechanism: there is no myristoylation site, only a potentially palmitoylatable site at the N-terminus, which raises questions about the trafficking pathway used by the flagellar membrane-anchoring role as the N-terminal amphipathic α-helix of the N-BAR domains; to our knowledge, it is the first BAR domain determined; crystallization attempts are actively pursued. LdFlabarin binds to, deforms and induces the tubulation of lipid membranes in vitro (Fig. 6) like other BAR domains proteins do. Moreover, our deletion studies uncovered two distinctive features of the LdFlabarin BAR domain. First, the BAR domain is necessary, but not sufficient, for membrane binding in vivo, which requires the presence of cysteine 4, a potentially palmitoylated amino acid, and suggests that palmitate could play the same membrane-anchoring role as the N-terminal amphipathic α-helix of N-BAR domains; to our knowledge, it is the first BAR domain of the sort. Second, the flagellar addressing, which depends on the C-terminal region, makes LdFlabarin the first BAR domain protein localized to a flagellum.

BAR domains are dimerization domains. Dimerization regions have been predicted for LdFlabarin (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml [69]). Although our data need confirmation, they suggest that native LdFlabarin auto-associates to form oligomers of discrete sizes (Fig. 2B–C); the ultrastructural helicoidal arrangement around the flagellum would be consistent with such a controlled oligomerization (Fig. 4). In this perspective, we are planning to perform in vitro and in vivo interaction studies with recombinant truncated proteins.

Potential role of Flabarins

Many BAR domain proteins are involved in the biogenesis of membrane vesicles, by protrusion (intracellular traffic) and invagination (endocytosis), depending on the concave or convex curvature they induce or recognize. It is difficult to envision such processes at the flagellum surface because in Leishmania, vesicular endocytosis/exocytosis occur exclusively inside the flagellar pocket [70]. Given its helicoidal arrangement along the flagellum, LdFlabarin could have a
morphogenetic function. The nature of the BAR domain (i.e., the banana-shaped dimer) is known to determine the curvature of the membrane to which it binds. Thus, “F-BAR domains typically induce wider membrane tubules compared with the ones induced by BAR/ N-BAR domains” [71]; however, there is a large difference in diameter between the LdFlabarin-induced tubules in vitro (15.9±1.7 nm) and the flagellum (30±±38 nm). LdFlabarin alone could not generate the flagellum as a large tubule but may help stabilizing its structural complex.

Gene disruption/replacement experiments are in progress, which will hopefully provide some understanding of LdFlabarin role and its involvement in Leishmania flagellar assembly, structure or functioning.

Materials and Methods

Cell culture and transfection

Leishmania amazonensis (MHOM/BR/1987/BA125; MHOM/BR/1987/BA276) and L. donovani (MHOM/ET/1967/Hu3/LV9) promastigotes were cultured at 24°C in AM medium with 7.5% FCS [21,72]. Electroporations were done in duplicate with 50 μg plasmid, and stable transfecteds selected by addition of 50 μg/ml hygromycin (Euromedex) or 10 μg/ml blasticidin (InvivoGen) 24 h later [21].

DNA technology

Conventional methods were used for DNA extractions [73]. Restriction and modification enzymes were from New England Biolabs, oligonucleotides from Eurofins MWG Operon. DNA fragments were amplified with Phusion DNA polymerase (Finnzymes) and cloned into the pUC-18 or pMOS vectors [451].

Plasmid constructions

The ORF was PCR amplified from L. donovani LV9 genomic DNA with oligonucleotides (designed from the L. mexicana sequence) [34]. The LaPFR2C ORF (submitted to GenBank, accession number JN874564) comprised 1797 bp/591 amino acids (98%/98.7% identity to LmPFR2C, respectively) [34] with 36 nucleotides/8 amino acids differences. The ORF was cloned into the Acc65 I site of the plasmid pNUS-GFPnH vector (pNUS-GFPnH-LaPFR2C).

LaCentrin (GenBank AF106767) was amplified from L. donovani LV9 genomic DNA with oligonucleotides G446 (gatcatatatGGGCGGCGTTGGAATGCGCCAGAAGGCC). Recombinant LdFlabarin-His6, anti-LdFlabarin rabbit antiserum

The LdFlabarin ORF was excised from the pNUS-LdBFlabarin-mRedD and transferred to the bacterial expression vector pET-29b (Novagen) (pET29b-LdFlabarin-His6) for expression in the E. coli strain BL21(DE3). The transformed bacteria were grown at 36°C in LB broth containing 100 μg/ml kanamycin. When cultures reached an OD at 600 nm of 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a concentration of 0.4 mM to induce expression of LdFlabarin-His6. Bacteria were harvested 3–4 h later.

Recombinant LdFlabarin-His6 was purified by fast protein liquid chromatography (AKTA Purifier system) at 4°C. After IPTG induction, bacteria were resuspended in 10 ml binding buffer (5 mM imidazole in 10 mM Tris pH 8, 300 mM NaCl) and sonicated (3×90 s pulses interrupted with cooling on ice). After elimination of cell debris by centrifugation at 25000 g, the supernatant was applied to a nickel-chelated agarose affinity column (Qiagen). After extensive washes with binding buffer, the protein was eluted with an imidazole gradient (3–1000 mM) and 2 ml fractions were collected. The protein was eluted with about catATGCCGCTCTcGGCCAGCATC (with a c mutation at position 11) to replace C4 with S4/G391 and cloned into pNUS-mRedD (generating pNUS-LdBFlabarin/C4S-mRedD).

The F1–12–mRedD vector between Nde I and Kpn I. The chimera F1–12–mRedD (F256–339) was constructed in three steps as follows. First, the C-terminal fragment F256–339 was amplified from the LdFlabarin ORF with the oligonucleotides G381/G441 (gatctgcgatcatTCACATCAGTTTGGTCGACAC) and cloned between Nde I and Kpn I sites of the pNUS-mRedD vector (thus replacing the mRedD ORF), to generate the vector pNUS-F256–339. Second, for the N-terminal fragment F1–12 (nt 1–36), 5 μg complementary oligonucleotides G344B (tagcgcgctctgcgccagcagctggatgcagctggatgcagctggatgcagcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgcagctggatgc4
150 mM imidazole (Fraction 16: F16). Fractions were analyzed by SDS-PAGE. The recombinant protein was concentrated to 1 mg/ml in Hapes 30 mM pH 7.5, 150 mM KCl, glycerol 10% using Vivasin ultrafiltration device (Vivascience Sartorius, cutoff 10000 Da) and stored at 4°C. Protein concentration was determined by UV spectra and by the Bradford method [79].

Four 5-μl aliquots of LdFlabarin-His6 were injected every other week to a female NZW rabbit (Charles River Laboratories) at the IRD animal facility. The first injection was done with 50% complete Freund adjuvant, the last 3 with incomplete Freund adjuvant). Each time, ten (0.1 μl) aliquots of the homogenate were injected into the right side of the rabbit. The reactivity of the antiserum was tested 10 days after the third injection by Western blot. The final anti-LdFlabarin antiserum was collected 10 days after the fourth injection.

Cell lysis, cell fractionation and Western blotting
Leishmania total protein extraction was done as described [21]. For cell fractionation, 100 ml log phase cells (1.5 \times 10^7 cells/ml) incubated for 2 h with anti-Mowiol. For indirect immunofluorescence, coverslips were spread onto poly-L-lysine-treated coverslips and fixed with 4% PFA for 20 min at room temperature. Coverslips were washed twice with PBS, resuspended in 1 ml hypotonic Buffer I [10 mM Hapes pH 7.4, 1 mM DTT, 2 mM EDTA, 1/100 dilution of Sigma P8215 protease inhibitors cocktail] and incubated for 10 min in ice. Cells were mechanically broken by 20 passages through a 26GX1/2“ gauge. After addition of cold 3.5 ml Buffer II (same as Buffer I except 50 mM Hapes pH 7.4 instead of 10 mM) +0.5% NP-40, the lysates were cleared by 1500 g 15 min centrifugation and the supernatants further centrifuged at 100 000 g for 1 h at 4°C (SW55Ti rotor, Beckman Coulter Optima LE-80K centrifuge). The last supernatants represented the soluble fractions and the pellets, resuspended in 4.5 ml Buffer II +0.5% NP-40, the membrane fractions.

Western blotting was done as described [21] and revelation as in [59], anti-LdFlabarin (1:2000 dilution) was additioned with BL-21 E. coli homogenate for eliminating an eventual reactivity of the rabbit serum with bacterial contaminants of the purified recombinant LdFlabarin.

Fluorescence microscopy
Fluorescence microscopy was done as described [21]. Cells were spread onto poly-L-lysine-treated coverslips and fixed with 4% PFA for 20 min at room temperature. Coverslips were washed 3 x 5 min with PBS and DNA was stained with DAPI (10 μg/ml) during the last 5-min wash before mounting on microscope slides with Mowiol. For indirect immunofluorescence, coverslips were incubated for 2 h with anti-LdFlabarin (1:1000 dilution), and for 2 h with 8 μg/ml goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular Probes). Observations were done with an Axioscop 2 Zeiss fluorescence microscope and a 100x oil lens. Images were acquired with a Princeton Instruments or Photometrics CoolSnap HQ camera and analyzed with Metaview (Universal Imaging) and Adobe Photoshop. Acquisition times: phase contrast, 100 ms; DAPI, 50 ms; red and green channels, 50–500 ms, depending on the fluorescence level to avoid saturation.

Freezing and freeze substitution for ultrastructural studies
Leishmania pellets (0.5–1 \times 10^9 cells) were washed three times in PBS buffer, incubated with 2% dextran in PBS buffer for 1 h and placed on the surface of a Formvar-coated copper electron microscopy grid (400 mesh). Each loop was quickly submersed in precooled liquid propane and kept at −180°C in liquid nitrogen. The loops were incubated in 4% osmium tetroxide in dry acetone at −62°C for 48 h (substitution fixation), gradually warmed to room temperature, and washed three times in dry acetone. Specimens were stained for 1 h with 1% uranyl acetate in acetone at 4°C in the dark. After another rinse in dry acetone, the loops were infiltrated progressively with araldite (epoxy resin; Fluka). Ultrathin sections were contrasted with lead citrate.

ImmunoGold electron microscopy
Leishmania cells were cryofixed in 2% dextran as above and freeze substituted with acetone plus 0.1% glutaraldehyde for 3 days at −82°C. Samples were rinsed with acetone at −20°C and embedded progressively at −20°C in LR Gold resin (EMS). Resin polymerization was carried out at −20°C for 3 days under UV illumination, after which ultrathin LR Gold sections were collected on Formvar-coated nickel grids. Sections were incubated at room temperature with 1 mg/ml glycine for 5 min, with FCS (1:20) for 5 min, with anti-Red antiserum (1:250) (MOBIO ML75489) or anti-LdFlabarin (1:2000) for 45 min and with an anti-rabbit/10 nm gold particles conjugate (1:500) (BioCell) for 45 min. The sections were rinsed with distilled water and contrasted for 5 min with 2% uranyl acetate in water followed by 1% lead citrate for 1 min. Specimens were observed with a HITACHI 7650 electron microscope (Electronic Imaging Pole of Bordeaux Imaging Center).

Liposome preparation and in vitro tubulation assays
Phosphatidylcholine (DOPC) and phosphatidylethanolamine (DOPE) (ratio: 70/30; 10 mg/ml in chloroform; Avanti Polar Lipids) were dried under a stream of argon and kept under vacuum for at least 2 h. The lipids were then redissolved in Hapes 50 mM pH 7.5, KCl 150 mM, glycerol 10% to a final concentration of 1 mg/ml, gently vortexed, subjected to seven freeze–thaw cycles, and immediately extracted 21 times through a 50- or 100-nm polycarbonate membrane (Avanti Polar Lipids). The homogeneity of the liposome preparations was tested using dynamic light scattering (DLS). For tubulation assays, 10 μl of liposomes were incubated with 10 μl of recombinant LdFlabarin-His6 (1 mg/ml = 24 μM) or LdFlabarin without Histidine-Tag (repurified after thrombin digestion) for 2 h at room temperature. Samples were applied for 15 min to Formvar/carbon-coated copper grids, washed with distilled water and stained with 2% uranyl acetate in water for 1 min. Specimens were examined on a HITACHI 7650 electron microscope as above.

Supporting Information
Figure S1 A: Sequence alignment between H. sapiens Arfaptin-1 and L. major Flabarin. Alignment of HsArfaptin-1 (Genbank U52521) and LmFlabarin (LmjF.27.1730) obtained from GeneDB [GeneDB-Blast-Lmajor, 2015 #13595]. Score =84 (34.6 bits), Expect =0.033, P = 0.032. Identities =54/213 (25%), Positives =88/213 (41%). Identical amino acids are highlighted in black, similar amino acids (apolar, polar) in grey.

B: Sequence alignment of LdFlabarin and its orthologues
Clustal W (1.83) [http://www.ch.embnet.org/software/ClustalW.html] alignment of LdFlabarin and its orthologues (Group O6G_148786, http://orthomcl.org; L. infantum [LmjF.27.1630], 99.7% id.; L. major (LmjF.27.1730), 90.3% id.; L. mexicana (LncM.27.1730), 88.2% id.; L. braziliensis (LbM.27.1860), 73.5% id.; Trypanosoma cruzi (TcCLB.506125.20 indicated by #, and TcCLB.304153.30), 20.3 and 19.8% id., respectively; T. vivax (TvA486_0013090), 19.2% id.; T. congolense (TcHL3000.11.2210.1, 18.3% id.; T. brucei (Tb927.11.2410, formerly Tb11.22000.1), 18% id. Two other orthologues, T4272/mp.22.0001 (from another T. brucei strain) and Tbg572.11.2660 (T. gambiense), were not included.
because their amino acid sequences are identical to \( T^8927.11.2410 \). Identical amino acids are highlighted in black; *, and correspond to "identity"; "semi-conservative substitution" and "conservative substitution" respectively.

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Figure S2 Analysis of structural domains and motifs of \( Ld\)Flabarin. Schematic representation of \( Ld\)Flabarin structural domain predictions by NCBI-Blast (http://www.ncbi.nlm.nih.gov/blast/blast2/Blast.cgi), secondary structure predictions by PredictProtein (http://www.predictprotein.org/) and Disopred (http://bioinf.cs.ucl.ac.uk/disopred/), and potential amphiphilic helices by Helquest (http://helquest.ipmc.cnrs.fr). Amino acids are numbered starting with the first methionine. (i) the \( \alpha \)-helix H7 (227–256); PredictProtein) is considered as belonging to the disordered region (D) by Disopred and (ii) there may be a \( \alpha \)-helix in the middle of the disordered region (PredictProtein). PredictProtein/Disopred: red is for \( \alpha \)-helix (H), blue for \( \beta \)-strand (S), green for loop (L), and yellow for disordered (D); solid lines limited by solid triangles correspond to potential dimerization domains. Heliques: AHR, amphiphilic \( \alpha \)-helix region. The four selected 18-aa windows show \( \alpha \)-helices as viewed from above: for a comparison, the first on the left (M1-R18) is not amphiphilic, the other three are; non-polar amino acids are yellow, grey, and green; polar amino acids are blue for cationic, red for anionic, and pink and purple for neutral.

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Table S1 Potential modification sites of Flabarins and conservation between species.

(DOC)

Acknowledgments

The authors thank P. P. Bastien for his support and encouragements; Dr. A. Barral for the \( L. amazonensis \) strains BA125 and BA276; Y. Balard, N. Biteau, L. Crobu, C. Giroud, L. Taligan and Drs. S. Besteiro, C. Blainve, M. Pagès, D. Robinson, Y. Sterkers and D. Tareste for their help; C. Hubert (Sequencing Facility of Bordeaux, France) for some DNA sequencing; P. Boutinaud and N. Barouguier (Animal Facility, IRD, Montpellier, France) for the rabbit immunization and the anti-\( Ld\)Flabarin antiserum collection; the Electronic Imaging Pole of Bordeaux Imaging Center. This work was initiated at the UMR CNRS 5234 (Bordeaux) and continued at both the UMR CNRS 5290 (formerly 2724, Montpellier) and the UMR CNRS 5095 (Bordeaux).

Author Contributions

Conceived and designed the experiments: ET GM. Performed the experiments: ML ET MT BS FB CB GS CM BE GM. Analyzed the data: ML ET MT BS FB CB GS CM BE GM. Wrote the paper: ET GM.

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(ROC)

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Author Contributions

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