Biochemical and Biophysical Research Communications 505 (2018) 290–294

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Detecting phospholipase activity with the amphipathic lipid packing sensor motif of ArfGAP1

Pablo Yunes Quartino [a, b], Gerardo Daniel Fidelio [a, b], Jean-Baptiste Manneville [c], Bruno Goud [c], Ernesto Esteban Ambroggio [a, b, *]

[a] Departamento de Química Biológica-Ranwel Caputto, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende S/n, Córdoba, X5000HUA, Argentina
[b] CONICET, Universidad Nacional de Córdoba, Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC), Haya de la Torre y Medina Allende S/n, Córdoba, X5000HUA, Argentina
[c] Institut Curie, PSL Research University, CNRS, UMR 144, 26 Rue d'Ulm, 75248, Paris Cedex 05, France

ARTICLE INFO

Article history:
Received 3 September 2018
Accepted 18 September 2018
Available online 22 September 2018

Keywords:
Phospholipases
ALPS
COPI
Arf1
Intracellular trafficking
Golgi apparatus
Lipid packing defects
Liposome
Lipid spontaneous curvature
Membrane curvature

ABSTRACT

The amphipathic lipid packing sensor (ALPS) motif of ArfGAP1 brings this GTPase activating protein to membranes of high curvature. Phospholipases are phospholipid-hydrolyzing enzymes that generate different lipid products that alter the lateral organization of membranes. Here, we evaluate by fluorescence microscopy how *in-situ* changes of membrane lipid composition driven by the activity of different phospholipases promotes the binding of ALPS. We show that the activity of phospholipase A2, phospholipase C and phospholipase D drastically enhances the binding of ALPS to the weakly-curved membrane of giant liposomes. Our results suggest that the enzymatic activity of phospholipases can modulate the ArfGAP1-mediated intracellular traffic and that amphiphilic peptides such as the ALPS motif can be used to study lipolytic activities at lipid membranes.

1. Introduction

During COPI-mediated intracellular trafficking, specific lipids and membrane curvature regulate the activity of the ADP-ribosylation factor GTPase-activating protein 1 (ArfGAP1) which induces the detachment of Arf1 from Golgi membranes [1–3]. The sensitivity of ArfGAP1 to such membrane physicochemical parameters is given by its amphipathic lipid packing sensor (ALPS) motif [3,4]. ALPS motifs are generally soluble peptides which, when absorbed onto lipid interfaces that display a high density of lipid packing defects, adopt an amphipathic and flexible α-helix structure with an hydrophilic region containing a very low number of charged residues [4]. ALPS motifs bind liposomes of high curvature but can also interact with large liposomes of low curvature if their bilayer contains lipids that generate lipid-packing defects [5]. Diacyl-glycerol (DAG), phosphatidic-acid and lyso-phospholipids are examples of these types of lipids with high spontaneous curvature (Fig. 1A), molecules that cause lateral stress when present in lipid membranes [6]. Interestingly, in the cell, these lipid molecules can be produced *in-situ* by lipolytic enzymes whose substrates are phospholipids. Specifically, phospholipases (PL) hydrolyze phospholipids and, depending on the type of phospholipase (PLA, PLB, PLC or PLD), the product is either a fatty-acid (in the case of PLA and PLB) or a lipophilic substance such as DAG (in the case of PLC) or phosphatidic-acid lipids (in the case of PLD) [7]. The change in the lipid geometrical properties (i.e. the spontaneous curvature of lipids [8,9]) generates lipid packing defects [6].

Here we tested whether the ALPS motif could detect such

Abbreviations: ALPS, amphipathic lipid packing sensor; DAG, diacyl-glycerol lipids; PA, phosphatidic-acid lipids; PL, phospholipase; PLA, PLC or PLD, phospholipase A, C or D; DOG, 1-2-dioleoyl-*sn*-glycerol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid; BodTRCer, BODIPY® TR ceramide.

* Corresponding author. Departamento de Química Biológica-Ranwel Caputto, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende S/n, Córdoba, X5000HUA, Argentina.
E-mail address: ernesto@mail.fcq.unc.edu.ar (E.E. Ambroggio).

https://doi.org/10.1016/j.bbrc.2018.09.116
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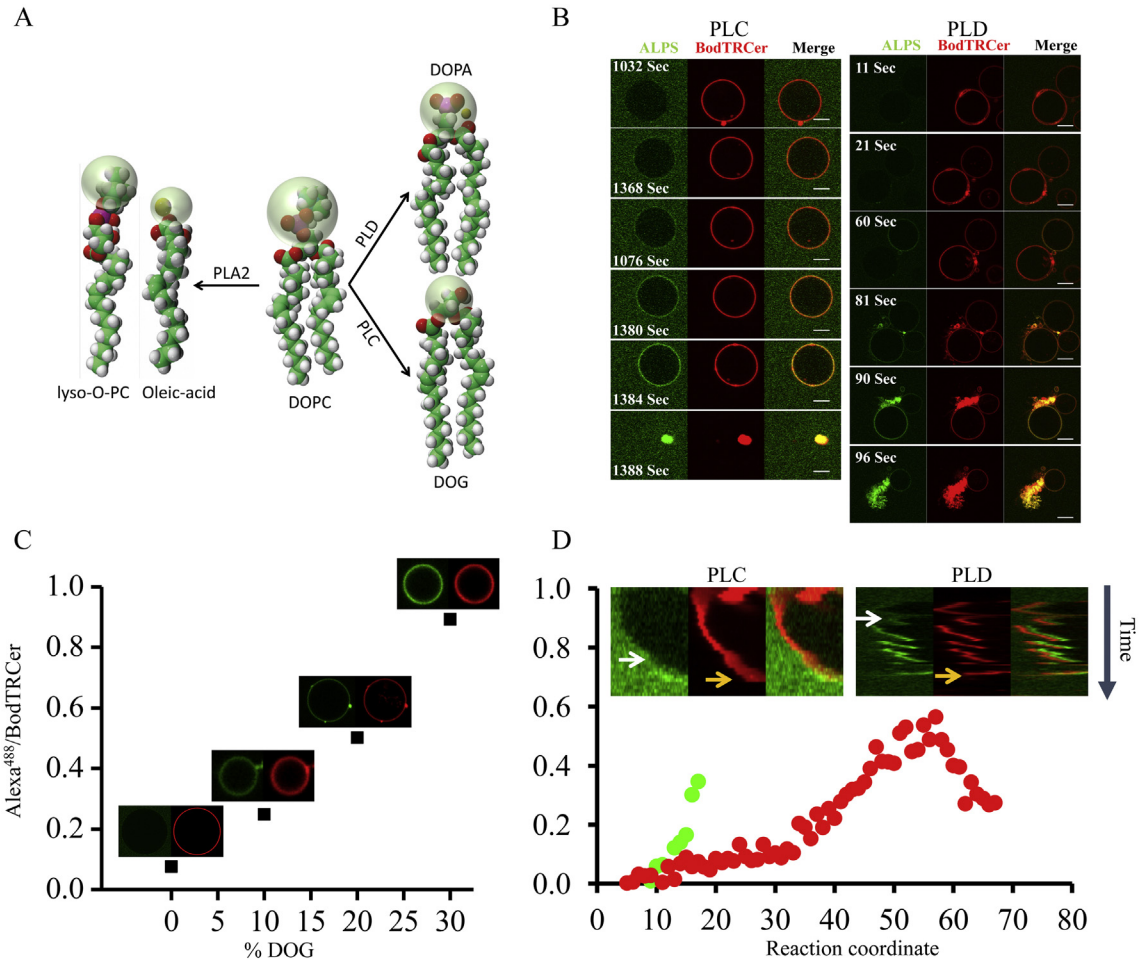

Fig. 1. Binding of ALPS to low-curvature GUVs is controlled by the activity of PLC and PLD. A) PLA2, PLC and PLD products when DOPC lipids are the enzyme substrate. B) Time-lapse images of DOPC GUVs labelled with BodipyTR-ceramide (red channel) in the presence of 1 μM ALPS-Alexa^{488} (green channel) and 10 units of PLC (left panel) or PLD (right panel). C) DOPC:DOG GUVs containing increasing amounts of DOG (0, 10, 20 and 30% DOG mol/mol) labelled with BodipyTR-ceramide (inserted images, red channel) in the presence of 1 μM ALPS-Alexa^{488} (inserted images, green channel). Increasing the DOG content in the GUV membrane enhances ALPS binding. D) Time-course analysis of ALPS binding upon PL activity. Quantification of ALPS binding to DOPC GUVs in the presence of PLC (green dots and kymograph on the left) or PLD (red dots and kymograph on the right). The white arrows in the kymographs signpost the starting point for ALPS binding. The orange arrows in the kymographs indicate membrane collapse. Scale bars, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

membrane physicochemical changes when phospholipases act on the lipid bilayer of giant unilamellar vesicles (GUVs). Our work shows how the ALPS region of ArfGAP1 is able to bind low-curvature membranes when DOPC GUVs are in the presence of PLA, PLC and PLD enzymes. In the context of membrane trafficking, our results provide direct evidence that phospholipase activity induces lipid packing defects as monitored by ALPS binding to membranes. In addition, we propose that ALPS motifs could be used as sensors of the protein lipolytic activity.

2. Materials and methods

2.1. Reagents

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1-2-dioleoyl-sn-glycerol (DOG) were purchased from Avanti Polar Lipids. N-((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl)phenoxy)acetyl)sphingosine (BODIPY® TR ceramide; BodTRCer) and Alexa 488 C5-maleimide were obtained from Life technologies. PLC (Clostridium Perfingens), PLD (Streptomyces chromofuscus) and PLA2 (Naja-Naja and porcine pancreas) and all other chemicals were purchased from Sigma Aldrich.

2.2. Peptide and protein purification

The purification and fluorescent labeling with Alexa 488 of the ALPS motif from ArfGAP1 (ALPS-Alexa^{488}) was performed as described previously [10]. Briefly, transformed bacteria with the GST-fused 192–304 (with the mutation K297C) fragment of ArfGAP1 inserted in a pGEX-2T expression vector (Pharmacia), were resuspended in 50 mM Tris (pH 7.4) and 150 mM NaCl (TN buffer), supplemented with protease inhibitors (1 mM PMSF, 1 mM pepstatin, 10 mM bestatin, 10 mM phosphoramidon) and 1 mM DTT. After bacterial lysis (french press) and cell lysate centrifugation (160000*g* for 30–60 min), the supernatant was then incubated for 1 h with glutathione-Sepharose 4B beads (Amersham). The beads were washed three times with TN buffer. To release ALPS from GST, the beads were incubated overnight with thrombin (1U). After cleavage, DTT was added to 1 mM final concentration and the supernatant was passed through a C18 phase-reverse column (Chromolite Performance RP-18 100–4.6, Merck). The separation was achieved with an acetonitrile gradient. The fractions were analyzed by SDS-PAGE. Those corresponding to the peptide molecular weight were pooled, lyophilized and stored at −20 °C. The punctual K297C mutation in the peptide sequence allows labeling

with the Alexa^{488} C5-maleimide fluorophore. The labeling reaction was performed by resuspending the desired amount of lyophilized peptide in 2 mL of HK (HEPES 50 mM, KAcetate 120 mM)-DMF (5%) buffer and then adding this volume to lyophilized maleimide reactive fluorophore (protein/dye ratio: 0.1 to 0.2). After 1–2 h, the labeling reaction was stopped with 10 mM Cys and the fluorescently-labelled peptide was separated from non-labeling dye by reverse phase chromatography using an acetonitrile gradient for the elution. The ratio of dye to total protein was always around 1. Peptide integrity and labelling was finally checked by mass spectrometry (not shown).

2.3. Preparation of giant unilamellar vesicles

GUVs were grown at room temperature on ITO slides using the electroformation technique described previously (Angelova et al., 1992). Briefly, a 0.5 mg/mL solution of DOPC or DOPC:DOG (up to a maximum of 30% mol/mol DOG above which GUVs electroformation was inhibited) doped with red fluorescent BodipyTR-ceramide (0.5% mol/mol) in chloroform:methanol (2:1 vol/vol) was spread onto two ITO slides and dried under N_2 stream. The slides were further dried for 2 h into a high-vacuum chamber to remove traces of organic solvent. The ITO slides were assembled to form a chamber into which sucrose was added at an osmotic pressure matching that of the buffer in which the enzymatic reaction was performed (see below). The ITO slides were connected to a function generator allowing to apply a 1 V voltage at a 10 Hz frequency to the chamber. After 4–6 h of electroformation the GUVs were pipetted out of the chamber by aspiration with a gel loading tip.

2.4. Enzymatic reaction

A suspension of DOPC GUVs was injected into a 5–10 μL microchamber (previously incubated with casein 10 mg/mL in HKM buffer, see Ref. [11]) containing 0.5 μM ALPS-Alexa^{488} in HKM buffer. After 10 min of incubation, PLC (1 U/mL), PLD (80 U/mL; $CaCl_2$ 10 mM) or PLA2 (1U/mL) was injected into the top of the chamber and GUVs were imaged by time-lapsed confocal microscopy on a Zeiss LSM-Pascal microscope equipped with a 63× objective. The 488 and 546 nm laser lines were used to excite Alexa^{488} and BodipyTR dyes, respectively. Control experiments were performed by inhibiting enzyme activity with EGTA 3 mM.

3. Results

3.1. In-situ production of DOG and DOPA promotes ALPS binding to GUVs

ArfGAP1 can be activated when phosphatidylcholine-derived diacylglycerols are present in liposomes extruded through a 0.1 or 0.4 μm filter [12]. Although the liposome size was not reported in this work, the vesicle radius of liposomes extruded through the mentioned filter size should be above 90 nm diameter [2,13]. Thus, the size of such liposomes corresponds to a curvature much smaller than the threshold curvature for ArfGAP1 and ALPS binding to membranes [2,3,10,14,15]. Because of the spontaneous curvature of DOG (small polar region, Fig. 1A; see Ref. [16]), lateral lipid packing is expected to be altered in the presence of DOG [6,9,17]. Similarly, phosphatidic acid displays a similar geometry compared to DOG [16,17] and has been described as an intermediate for DAGs production in cells [12]. Our hypothesis is that ALPS, facing an enhancement of lipid-packing defects produced by the appearance of such lipids with high spontaneous curvature upon PL activity, would be able to bind the low-curvature membrane of GUVs. To test this hypothesis, we incubated DOPC GUVs of 5–25 μm diameter with PLC (DOG production) or PLD (DOPA production) in the presence of the fluorescently labelled version of ALPS, ALPS-Alexa^{488}. Fig. 1 B and D (see movies MS1 and MS2 in the supporting information section) show that the binding of ALPS to the GUV membrane increases in both conditions reaching a maximum until the membrane collapses. Membrane collapse occurs when changes in lipid packing due to the production of DOG or DOPA in the outer leaflet can no longer be accommodated by the lipid bilayer [18]. Consistently, modulating the amount of DOG in GUVs (Fig. 1C) also shows a marked increase in ALPS binding.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.09.116

Time-lapse movies showing the increase of ALPS-Alexa^{488} binding to GUVs (initially composed of DOPC) in the presence of PLC (MS1), PLD (MS2) and PLA2 phospholipases from porcine pancreas (MS3) or from Naja-Naja (MS4).

3.2. Generation of lyso-phospholipids also enhances ALPS binding to GUV membranes

To further test how membrane packing changes is a signal for ALPS binding to the lipid interface, we took advantage of the activity of two PLA2 phospholipases that generate oleoyl-lyso PC and oleic acid from DOPC GUVs [7]. We hypothesized that the appearance of such products (detergent-like molecules) may also create a membrane lateral packing stress, because of drastic changes in lipid spontaneous curvature [6,16], that could be detected by ALPS which would therefore bind to the stressed GUV hemi-layer. Indeed, when DOPC GUVs are exposed to either PLA2 from porcine pancreas or from Naja-Naja cobra (movies MS3 and MS4, respectively, in the supporting information section), the fluorescent version of ALPS starts binding to the low-curvature membrane until the GUV membrane breakdowns because of high amounts of lyso-lipids [19] (Fig. 2). From these data, we confirm that changes in membrane packing are a strong signal for the modulation of protein binding through ALPS domains.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.09.116.

4. Discussion

Arf1-GTP can activate PLD activity generating PA at the membrane [20,21]. PA can be hydrolyzed by a phosphodiesterase producing a local enrichment of DAGs at the cell membrane [12,22]. Moreover, it was reported that DAG is required for the formation of COPI vesicles in the Golgi-to-ER transport pathway and of tubulovesicular structures at the Golgi apparatus, and for ArfGAP1 recruitment at the Golgi apparatus [23,24]. Phosphatidic acid is thought to play a key role for the complete achievement of COPI vesicles [25]. In this context, ArfGAP1 is markedly active when Arf1-GTP is bound to liposomes containing DAGs [12]. From our data, it is clear that the *in-situ* production either of DOG or DOPA (by PLC or PLD respectively) (Fig. 1) or of lyso-phospholipids (by PLA2) at the membrane of GUVs strongly promotes the binding of ALPS to the lipid interface. PLA2 activity produces oleoyl lyso-phospholipids and oleic acid from DOPC. Both products exhibit a spontaneous curvature different from their substrate. Such drastic changes in lipid spontaneous curvature in one layer of the membrane induce an elastic lateral stress that can be sensed by ALPS [6]. From our data, the GUV radius of curvature is typically 5–10 μm and is considerably larger than the radius of curvature of the 40 nm-diameter liposomes used in previous works [3,14]. However, the production of lipids with high spontaneous curvature generates a density of lipid-packing defects large enough to induce the binding

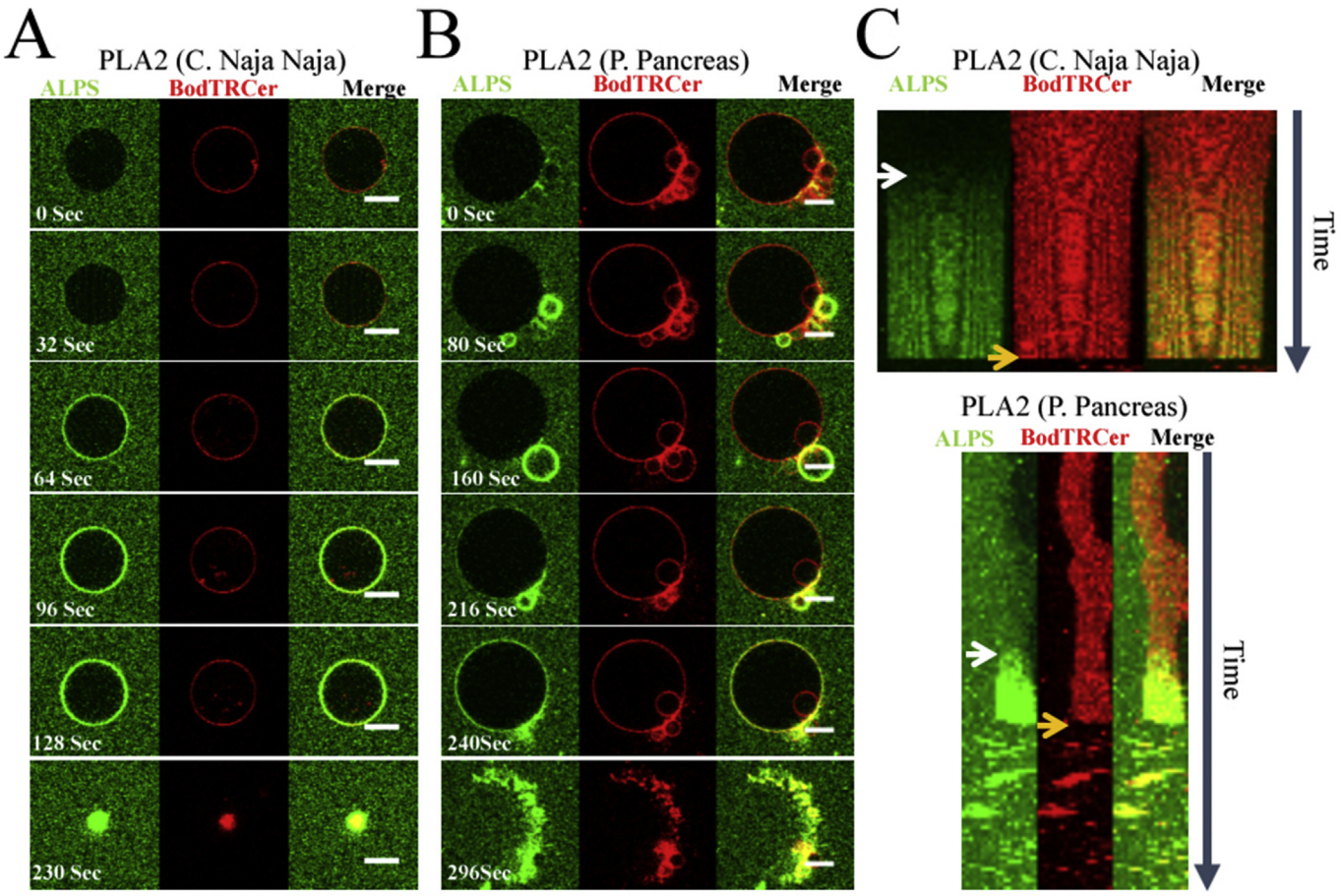

Fig. 2. Binding of ALPS to low-curvature GUVs is controlled by the activity of PLA2. Time-lapse images of DOPC GUVs labelled with BodipyTR-ceramide (red channel) in the presence of 1 μM ALPS-Alexa^{488} (green channel) and 10 units of Cobra *Naja Naja* PLA2 (A) or Porcine pancreatic PLA2 (B). C) Kymograph analysis of ALPS binding to DOPC GUVs in the presence of Cobra *Naja Naja* PLA2 (upper panel) or Porcine pancreatic PLA2 (bottom panel). The white arrows signpost the starting point for ALPS binding. The orange arrows indicate membrane collapse. Scale bars, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of the ALPS motif. In cells, the local production of these lipids may drive a localized enrichment of ArfGAP1, promoting the cycling of Arf1 and the COPI coat. Our findings are thus relevant to membrane trafficking as they show that ArfGAP1 activity may be modulated not only by the classical pathway of DAG production at the Golgi membrane [12] but also by PL activity which alters lipid packing.

Several intracellular pathogens are responsible for hijacking the COPI transport machinery [26]. For example, cytosolic PLA2 (cPLA2) was shown to translocate to the nuclear membrane upon swelling of the nucleus [27]. The stretching of the nuclear envelope could induce lipid packing defects, which may facilitate the binding of cPLA2 [28]. According to our results, the local production of lysolipids by cPLA2 should generate lateral elastic stresses and lipid packing defects in the nuclear membrane and thus amplify the binding of cPLA2 by a feedback mechanism. Consistently, it was shown that alterations in these cellular pathways correlate with changes in the activity of lipolytic enzymes [29].

Our experiments also provide a new way to evaluate how enzyme activity could modulate membrane composition and how soluble proteins respond to changes in the chemistry of the membrane. Developing new sensors is crucial to monitor the dynamics of enzymatic activity. Here we propose that fluorescently labelled ALPS could be used as a PL sensor to simultaneously follow PL activity in real-time and to evaluate the integrity of the membrane in terms of lateral lipid packing. To conclude, our work suggests that ALPS motifs allow to precisely regulate the binding of ALPS domain proteins to biomembranes in cells not only by sensing local changes in membrane curvature but also through a specific control of membrane biochemistry.

Acknowledgments

E.E.A. and G.D.F. are Career Members of CONICET (Argentina). E.E.A. thanks to CONICET for the financial support from (PIP 2013–2015) and Agencia Foncyt (PICT 2015-2575). We are grateful to the “Centro de micro y nanoscopia de Córdoba (CEMINCO)”, integrated to the “Sistema Nacional de Microscopía (SNM-MINCyT)”. J-B.M was funded by a grant from the French Ministry of Research. Other fundings are from Institut Curie, CNRS (Program ‘Prise de risque à l'interface Physique-Biologie’) and ANR (grant number ANR09-JCJC-0020-01).

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.09.116.

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