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Vesicular PtdIns(3,4,5)P_3 and Rab 7 as key effectors of nuclear membrane assembly

[1,4,5]Marta G. Lete, [2]Richard D. Byrne, [1]Alicia Alonso, [3]Dominic Poccia, [1,4]Banafshé Larijani

[1]Instituto Biofisika (UPV/EHU, CSIC) and Departamento de Bioquímica, Universidad del País Vasco, Barrio Sarriena s/n, 48940 Leioa, Spain.

[2]The Francis Crick Institute, Mill Hill Laboratory The Ridgeway London, NW7 1AA, UK.

[3]Department of Biology, Amherst College, Amherst, MA 01002, USA.

[4]Cell Biophysics Laboratory, Ikerbasque Basque Foundation for Science, Research Centre for Experimental Marine Biology and Biotechnology (PiE), Instituto Biofisika (CSIC, UPV/EHU) and, University of the Basque Country (UPV/EHU), Leioa 48940, Spain.

[5]Actual address: Department of Molecular and Cellular Medicine, College of Medicine, Texas A&M Health Sciences Center, College Station, Texas 77843-1114, USA.

In higher eukaryotes, the nuclear envelope (NE) is assembled and disassembled in every mitotic cycle. This reassembly is also observed during fertilisation around the male pronucleus (MPN). But the mechanisms by which NEs are formed are not totally understood, even though it is agreed that the bulk of the NE is derived from endoplasmic reticulum (ER) membranes that undergo fusion to enclose the chromatin (Burke and Ellenberg, 2002; Poccia and Larijani, 2009; Prunuske and Ullman, 2006).

Previous work has characterised the precursor membrane vesicles (MVs) implicated in this process and shown two distinct populations. Most of the MVs correspond to a population that derives from the ER, termed MV2. The minor component, MV1, has strikingly different features. It has an unusual lipid composition, up to a 60 mole % of phosphoinositides (PIPns), and it is enriched in PLC γ and its regulator SFK1 (Byrne et al., 2012; Byrne et al., 2007). Moreover, PIPns are key molecules in fusion events (Poccia and Larijani, 2009). For instance, a key regulator of endosomal fusion events is phosphoinositide 3-kinase (PI3K) (Fili et al., 2006), which was also seen to be indispensable for the NE assembly. Previous work (Larijani et al., 2001) had demonstrated, with the cell-free assay from sea urchin extracts, that PI3K is required for the NE assembly. In this work we have shown, by specifically inhibiting class I PI3K, that this specific isoform is required for the complete NE formation (Figure 1). This particular isoform phosphorylates preferentially PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3. So this highly phosphorylated derivative has a role in the assembly.

Since PtdIns(3,4,5)P_3 is the product of the essential class I PI3K, we investigated its localisation, both *in vitro* and *in situ* during NE assembly. In the first approach, we purified the PH domain contained in Grp1 known to recognise PtdIns(3,4,5)P_3 with a high affinity tagged with GST. We used the cell-free assay and added the purified recombinant protein to track PtdIns(3,4,5)P_3. We observed by immunofluorescence that PtdIns(3,4,5)P_3 was localised in patches around the NE. We suggest that these clusters can enhance bulging of the region, which could promote protein recruitment required for membrane fusion. To investigate *in situ* the localisation and role of PtdIns(3,4,5)P_3 during male and female pronuclear fusion, we used fertilised sea urchin eggs. We observed that PtdIns(3,4,5)P_3 was located in vesicles within the cortex and upon fertilisation such vesicles concentrated near the male and female pronucleus around the time of fusion (Figure 2) suggesting a. role during this process.

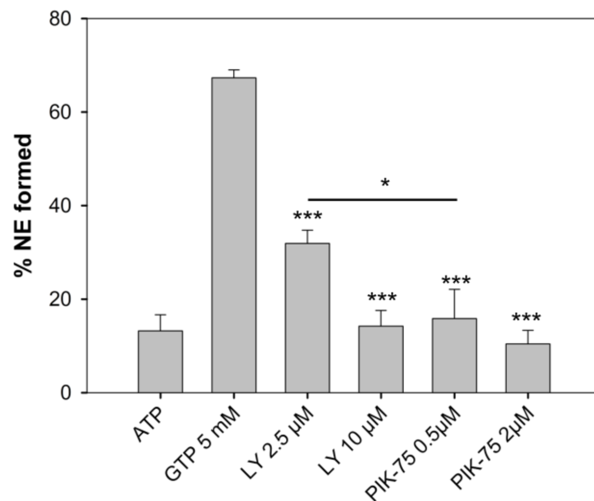

Figure 1. NE formation is blocked by class I PI3K inhibition *in vitro.* Cell-free assay was used for testing sensitivity to PI3K inhibitors. Percentage of NE formed for different conditions. First bar represents the NE formation when only an ATP generation system is added. The second bar corresponds to the NE formation after the addition of 5 mM GTP. Next, general PI3K inhibitor (LY 294002) and class I PI3K inhibitor (PIK-75) were added at the indicated concentrations. The NE formation is blocked with both inhibitors, but more effectively by the class I PI3K inhibitor. Three independent set of experiments were done, in each experiment at least 100 nuclei were scored. Error bars indicate S.D. (Lete *et. al*)

The family of Rab GTPases are often linked with membrane fusion in different cell compartments (Stenmark, 2009) many times in conjunction with PIPns. In particular Rab 7 has been connected to SFK activity. So we explored the localisation of the enzymes and lipids involved in NE membrane fusion in the sea urchin eggs. Using immunofluorescence we observed that PtdIns(3,4,5)P_3 vesicles surrounding the pronuclei of fertilised eggs co-localised significantly more with Rab7 than unfertilized eggs suggesting a recruitment of the GTPase. Also, post-fertilization the co-localisation

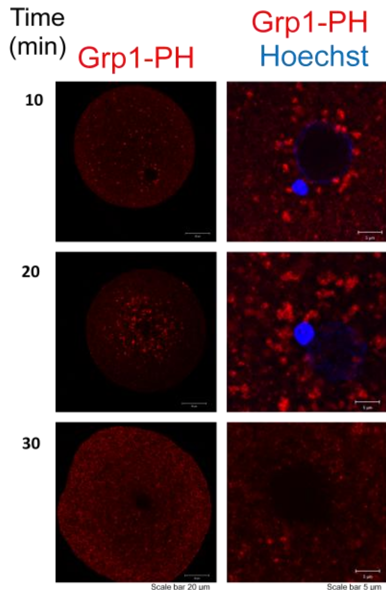

Figure 2. PtdIns(3,4,5)P_3 is localised around the MPN and the FPN during fertilisation *in situ.* Nuclei were labelled with Hoechst (blue), and PtdIns(3,4,5)P_3 with the purified GST-Grp1-PH probe tagged with conjugated anti-GST (red), and imaged by confocal microscopy. Fertilised P.*lividus* eggs were fixed at different times (indicated on the left) post fertilisation. Vesicles containing PtdIns(3,4,5)P_3 were observed around the eggs upon fertilisation and close to the MPN and FPN at the moment of fusion. Scale bar 20 μm (whole egg) or 5 μm (zoom).

of Rab7 and SFK in the egg cortex was significantly increased which could lead to activation of SFK.

As co-localisation is not enough to determine protein-lipid interaction between Rab7 PtdIns(3,4,5)P_3 we took advantage of time-resolved coincidence amplified FRET monitored by FLIM. Our data indicates a direct interaction between Rab7 and PtdIns(3,4,5)P_3 during zygote nuclear membrane fusion.

Our working model is shown in Figure 3, vesicles enriched in PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 assemble with inactive Rab7, SFK1, and PLCγ. PtdIns(3,4,5)P_3 is maintained by a class I PI3-K and is recognized by both Rab7. Initiation of membrane fusion by GTP activates Rab7 and removes the block to SFK1 activity, possibly caused by Csk inhibition. Phosphorylation of PLCγ by SFK1 activates it, catalysing the hydrolysis of PtdIns(4,5)P_2 to the fusogenic lipid diacylglycerol.

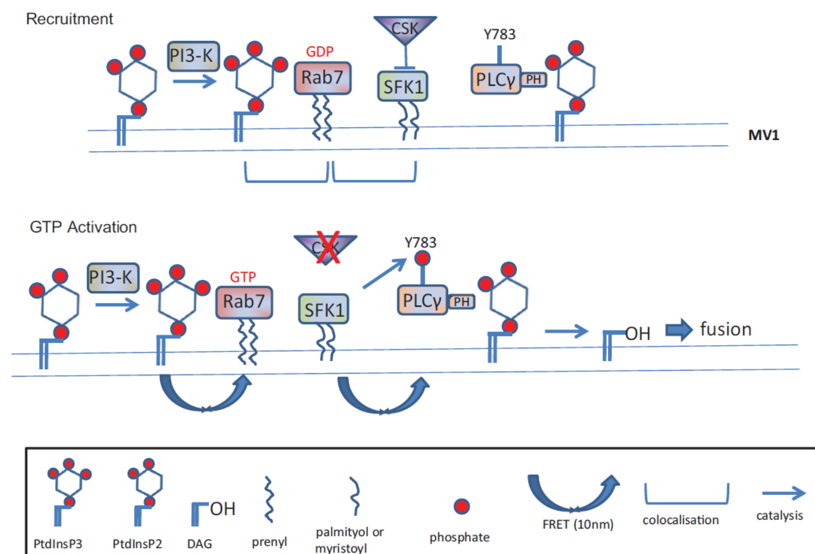

Figure 3. Working model for NE fusion platform (Lete *et. al*)

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