Conservation of proteo-lipid nuclear membrane fusion machinery during early embryogenesis

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Abbreviations: DAG, diacylglycerol; FLIM, fluorescence lifetime imaging microscopy; FPN, female pronucleus; FRET, Förster resonance energy transfer; MPN, male pronucleus; MV, membrane vesicle; NE, nuclear envelope; PLCγ, phospholipase Cγ; PtdIns(4,5)P2, phosphatidylinositol (4,5) bis phosphate; SFK1, src-family kinase 1; TSA, tyramide signal amplification

The fusogenic lipid diacylglycerol is essential for remodeling gamete and zygote nuclear envelopes (NE) during early embryogenesis. It is unclear whether upstream signaling molecules are likewise conserved. Here we demonstrate PLCγ and its activator SFK1, which co-operate during male pronuclear envelope formation, also promote the subsequent male and female pronuclear fusion. PLCγ and SFK1 interact directly at the fusion site leading to PLCγ activation. This is accompanied by a spatially restricted reduction of PtdIns(4,5)P2. Consequently, pronuclear fusion is blocked by PLCγ or SFK1 inhibition. These findings identify new regulators of events in the early embryo and suggest a conserved “toolkit” of fusion machinery drives successive NE fusion events during embryogenesis.

Introduction

Shortly after fertilization the sperm nuclear envelope (NE) is broken down and reformed with NE precursor membranes derived from the egg cytoplasm. The sperm nucleus, now referred to as the male pronucleus (MPN), subsequently congresses toward, and fuses with the female pronucleus (FPN) to form a diploid zygote nucleus. In select marine invertebrates, including the sea urchin, whose eggs are arrested after the completion of meiosis, this initiates the re-entry into the cell cycle and embryogenesis proceeds with a series of rapid mitotic divisions. These are sometimes followed at late anaphase and/or telophase by the formation of specialized structures, called karyomeres, chromosomes with individual NEs that subsequently fuse to form a single nucleus. Thus, in these organisms development is punctuated by distinct dynamic events at the center of which is the nuclear membrane.

All of these events have in common a reliance on membrane fusion of juxtaposed membranes into a continuous double bilayer enclosing genetic material. Membrane fusion is ubiquitous throughout membrane trafficking events in eukaryotes, and several components of the fusion machinery such as SNAREs and Rab GTPases are highly conserved. While the endoplasmic reticulum provides the bulk of material to form the male pronuclear envelope, its formation is also dependent on the localized generation of the fusogenic lipid diacylglycerol (DAG), the product of PtdIns(4,5)P2 hydrolysis by PLCγ. PLCγ itself is activated by a src family kinase, SFK1. SFK1, PLCγ and PtdIns(4,5)P2 are all enriched on a NE precursor membrane population, termed MV1, which is the site of fusion initiation. Karyomere resolution and formation of the NE after somatic mitoses are dependent on DAG production, while DAG homeostasis is also important for NE expansion and shape. Thus, the membrane fusion events underlying NE assembly studied to date are driven by the localized generation of DAG. However, male and female pronuclear fusion has not been examined, and few regulators of this process have been identified in higher eukaryotes. Yeast screens have identified several proteins that regulate nuclear fusion during mating, including Prm3, Kar2, Kar5, and Kar8. The localization of some of these proteins is known, as is the point during karyogamy at which they act. However, their molecular mechanisms remain unclear, and it seems unlikely they will involve lipid metabolism.

Thus, we investigated whether the signaling events culminating in DAG generation also regulated pronuclear fusion. This would identify novel regulators of zygote formation and demonstrate the proteo-lipid signaling pathways underlying nuclear membrane fusion are conserved through embryogenesis.

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We demonstrated the necessity for PLCγ, SFK1, and PtdIns(4,5)P2 during the fusion of the MPN and FPN, which represent elements of a conserved pathway in NE assembly.

Results and Discussion

PLCγ and SFK1 are localized on the male pronucleus until pronuclear fusion

We have previously demonstrated a role for PLCγ and SFK1 in the formation of the male pronuclear envelope via the production of the fusogenic lipid DAG.6,8,15 PLCγ and SFK1, are retained on the MPN after its formation.8 We hypothesized that the retention of PLCγ and SFK1 serves to facilitate a subsequent fusion event, specifically male and female pronuclear fusion. We thus examined the localization of PLCγ in eggs prior to, and undergoing pronuclear fusion. As previously described, PLCγ was localized to the MPN surface after its formation and persisted until the male and female pronuclei began to fuse (Fig. 1A). Strikingly, PLCγ was frequently localized to the point where the MPN and FPN came into contact, and thus would fuse (Fig. 1A, solid arrow). Interestingly, we also observed PLCγ on vesicles located between the congressing pronuclei (Fig. 1A, open arrows), raising the possibility that a further population of PLCγ-enriched membrane vesicles may be recruited for pronuclear fusion. A complete z-series showing PLCγ localization on fusing pronuclei is shown in Figure S1. Where we observed PLCγ positive structures, SFK1 was also present (Fig. 1B; see Fig. S2 for a complete z-series of fusing pronuclei), including vesicles where pronuclear fusion was predicted as (open arrows), or was taking place (solid arrows). Together, these data indicate that core fusion machinery from MV1 is localized on the MPN prior to fusion with the FPN.

PLCγ and SFK1 directly interact during male and female pronuclear fusion

While PLCγ and SFK1 co-localized on the MPN, this did not mean they were necessarily interacting. Indeed, our previous work has shown that while PLCγ and SFK1 co-localize for the duration of MPN formation, they only directly interact for a portion of this time.8 To examine the interaction kinetics of PLCγ and SFK1 we utilized FRET, detected by two-photon FLIM. This technique has previously been demonstrated to be suitable for such studies.8 Samples in which the pronuclei were either congressing (Fig. 2A) or undergoing fusion (Fig. 2C) were labeled with PLCγ-Atto 488 (donor chromophore), PLCγ-Atto 488, and SFK1-HRP-Cy5 (donor plus acceptor chromophore), or PLCγ-Atto 488 and HRP-Cy5 (donor plus “false acceptor” chromophore). Samples were subsequently subjected to a tyramide signal amplification (TSA) step to enhance the acceptor signal (see Fig. S3A). This amplification methodology has been robustly tested in our laboratory and is suitable for FRET studies.16 The lifetime of Atto 488 (donor) in each of these three conditions was determined in a region of interest that (1) surrounded the MPN or (2) encompassed the whole egg. In eggs where the pronuclei were congressing (Fig. 2B), compared with the donor alone, we did not see a significant decrease in the...
lifetime of Atto 488 in the presence of acceptor (Cy5), either around the MPN (1.72 ± 0.24 ns vs 1.28 ± 0.27 ns; \( P = 0.1376 \)) or in the entire egg (1.52 ± 0.18 ns vs 1.33 ± 0.13 ns; \( P = 0.4297 \)). Thus, while the pronuclei congress PLCγ and SFK1 are not directly interacting. However, upon pronuclei contact and fusion (Fig. 2D), the donor lifetime decreased specifically around the MPN (1.62 ± 0.25 ns vs 1.00 ± 0.12 ns; \( P = 0.0309 \)), but not in the egg cytoplasm (1.33 ± 0.23 ns vs 1.44 ± 0.27 ns; \( P = 0.3813 \)). Thus a direct interaction between PLCγ and SFK1 was detected, but only in the vicinity of the MPN. The specificity of this interaction at the MPN, and its dependency on the presence of anti-SFK1 was demonstrated by a lack of FRET in samples where anti-SFK1 was omitted (1.62 ± 0.25 ns vs 1.55 ± 0.27 ns; \( P = 0.8614 \)); thus the HRP-Cy5 signal alone was insufficient to act as an acceptor (see also Fig. S3B). Together, these data indicate that PLCγ and SFK1 are co-localized on the surface of

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**Figure 2.** PLCγ and SFK1 directly interact at pronuclear fusion, as determined by FRET measured by 2-photon FLIM. Sea urchin eggs were fertilized, fixed, and labeled with anti-PLCγ-Atto 488 (donor), anti-PLCγ and SFK1-HRP-Cy5 (donor plus acceptor) or PLCγ-Atto 488 and HRP-Cy5 (donor plus false acceptor). The lifetime of Atto 488 under these three conditions was acquired by 2-photon FLIM in eggs where the pronuclei were congressing (A, B), or undergoing fusion (C, D). Qualitative images of congressing (A) and fusing nuclei (C) are shown. (B, D) Multiple eggs were analyzed, with each egg being divided into regions of interest (red shading) surrounding the MPN (left hand data points), or encompassing the whole egg (right hand data points). The resulting Atto 488 lifetimes were expressed as Box and Whisker plots, indicating minima, maxima, and median values. Individual data points are shown as red dots. * denotes a significant decrease in the donor (Atto 488) lifetime, indicative of FRET (1-tailed \( t \) test, \( P < 0.05 \)).
the MPN, but crucially, only at the point of pronuclear fusion do they directly interact.

Depletion of PtdIns(4,5)P₂ corresponds to the site of active PLCγ during pronuclear fusion

Given the co-localization and interaction kinetics of PLCγ and SFK1, we predicted the outcome of this interaction would be the phosphorylation and subsequent activation of PLCγ, as judged by the phosphorylation status of Tyr783.¹ Consistent with the localization of total PLCγ reported above (Fig. 1A), we observed active PLCγ localized between congressing pronuclei <5 μm apart (Fig. 3A, solid arrows) and at the point of pronuclear fusion (open arrows; see Fig. S4 for a complete z-series). To quantify any changes in the pool of active PLCγ over time, z-series of nuclei were reconstructed (Fig. 3B), and the number of pTyr783 positive structures in contact with the MPN were scored. Our results show that while the MPN and FPN are congressing, the quantity of active PLCγ on the MPN remains unchanged (P = 0.7219). However, upon pronuclear contact and the initiation of fusion, the number of active PLCγ molecules increased significantly, approximately doubling (P = 0.0175).

Figure 3. The selective accumulation of active PLCγ coincides with PtdIns(4,5)P₂ depletion during pronuclear fusion. (A) Sea urchin eggs were fertilized and fixed with the male (M) pronucleus >5 μm (top row), <5 μm (middle row) or in contact and/or fusing (bottom row) with the female (F) pronucleus. Eggs were stained for PLCγ phospho-Tyr783 (red), total membranes (green) or DNA (blue) and imaged by confocal microscopy. Female DNA is denoted with a dashed line. Active PLCγ localized in between the pronuclei (solid arrows) and at the point of pronuclear fusion (open arrows) is indicated. (B) Multiple z-series for each time point were re-constructed in Imaris and the pTyr783 signal on the MPN surface quantified. Scale bar is 2 μm. Quantitative data are expressed as mean ± s.e.m (n = 5–9), * denotes a significant change compared with the preceding data point (2-tailed t test, P < 0.05). (C-D) As (A-B), with eggs labeled with GST-PLCδ1-PH to detect PtdIns(4,5)P₂ (red), total membranes (green) and DNA (blue). PtdIns(4,5)P₂ localized at the predicted pronuclear fusion point (solid arrows, middle), and around the MPN periphery (open arrows, bottom) is indicated. Quantitative data are expressed as mean ± s.e.m (n = 8–13).
The localization of active PLCγ at the point of pronuclear fusion indicated that at such sites phosphoinositide metabolism would also be taking place; specifically the hydrolysis of PtdIns(4,5)P_2 to DAG and Ins(1,4,5)P_3. We have previously demonstrated that the localized generation of DAG (but not Ins(1,4,5)P_3) is the trigger for the formation of NE precursor membranes during MPN assembly, 5,6,17 and thus predicted the hydrolysis of PtdIns(4,5)P_2 during male and female pronuclear fusion also. Using the GST-PLCδ1-PH recombinant fusion protein, which preferentially binds to PtdIns(4,5)P_2 (ref. 18; Fig. S5A), we detected PtdIns(4,5)P_2 in vesicles throughout the egg cytoplasm, consistent with the localization of PLCγ and SFK1 (Fig. S5E). While PtdIns(4,5)P_2 has been previously detected at the plasma membrane, 19 its localization to vesicles in echinoderm eggs has not previously been reported, nor has its metabolism during zygote formation. We observed PtdIns(4,5)P_2 on the surface of the MPN when distant (>5 μm) from the FPN (Fig. 3C, top row), until the point of pronuclear contact and fusion initiation. In a manner similar to that of pTyr783, the number of PtdIns(4,5)P_2 containing structures on the MPN surface were quantified during pronuclear approach until contact (Fig. 3D). We observed a significant increase in PtdIns(4,5)P_2 structures on the MPN surface during congression (P = 0.0332). Consistent with the activation of PLCγ on the MPN, upon contact and the initiation of fusion between the pronuclei, this PtdIns(4,5)P_2 pool was depleted, and levels significantly decreased (P = 0.0242) to their pre-contact levels. In addition to the quantitative changes in PtdIns(4,5)P_2 levels, we also observed a qualitative depletion of PtdIns(4,5)P_2 from the surface of the MPN undergoing fusion with the FPN. PtdIns(4,5)P_2 on the MPN surface away from this fusion region remained unchanged (Fig. 3C, open arrows, see Fig. S5B for a complete z-series). This reduction in PtdIns(4,5)P_2 was specific to the MPN, as PtdIns(4,5)P_2 levels were unchanged in the egg cortex during pronuclear congression and fusion (Fig. S5D). Given the pivotal role of PtdIns(4,5)P_2 hydrolysis to the fusogen DAG during MPN formation, we interpret the rise in PtdIns(4,5)P_2 during congression as a replenishment of the depleted PtdIns(4,5)P_2 levels on the MPN. Thus upon contact of the male and female pronuclei, the MPN has a sufficient reservoir of PtdIns(4,5)P_2 from which DAG can again be generated to drive the fusion of the male and female pronuclear membranes. While the observed decrease in PtdIns(4,5)P_2 during pronuclear fusion could in principle occur because of a conversion to other phosphoinositides aside from DAG, it should be noted that these pathways are by no means mutually exclusive. However, given the localization of active PLCγ at the point of pronuclear fusion it is likely that some of this PtdIns(4,5)P_2 reduction is due to

**Figure 4.** (A) PLC and SFK catalytic activities are required for pronuclear fusion. Sea urchin eggs were fertilized and after 10 min treated with DMSO vehicle or the inhibitor indicated. At 25 min post fertilization eggs were fixed, Hoechst stained and analyzed by confocal microscopy. Eggs were scored on the basis of the MPN position relative to that of the FPN: >5 μm apart, <5 μm apart, in contact or having undergone fusion. Example images (top) and quantified eggs (bottom) are shown. * denotes a significant reduction of fused pronuclei in inhibitor treated samples compared with untreated eggs (2-tailed t test, P < 0.05). (B) Model of pronuclear fusion. As the pronuclei congress, PLCγ and SFK1 are present on the MPN surface (gray) though they do not interact (red dashed box). Upon pronuclear contact and the initiation of fusion, PLCγ is phosphorylated by SFK1 on its Tyr783 residue (yellow circle), leading to PLCγ activation. Active PLCγ subsequently hydrolyses PtdIns(4,5)P_2 to DAG (black squares), which promotes the fusion of the male and female pronuclear membranes. Consistent with this model pronuclear fusion is blocked by the pharmacological inhibition of PLC and SFK. F is the female pronucleus.
PLCy activity. Interestingly, we also observed a distinct pool of PtdIns(4,5)P$_2$ on the NE of the FPN, close to the predicted point of MPN and FPN fusion (Fig. 3C, closed arrows). Like the PLCy enriched vesicles localized between congressing pronuclei, this raises the possibility that fusion machinery may not just be localized to the MPN, but there may also be a contribution from cytoplasmic or FPN localized molecules. Regardless of the relative contributions of these sources, it appears that in sea urchins pronuclear fusion is nonetheless initiated at a single point on the surface of the pronuclear envelopes.20

Pronuclear fusion is blocked by pharmacological inhibitors of PLC and SFK

The localization of active PLCy, SFK1 and selective PtdIns(4,5)P$_2$ metabolism at the point of MPN and FPN fusion strongly suggested that this "toolkit" of machinery, capable of generating fusogenic lipids, was involved in driving the formation of the zygote. To test this hypothesis we treated developing embryos with inhibitors of PLC (U73122) and SFK (PP2), or the inhibitor inactive analogs (U73343 and PP3 respectively). Inhibitors were added at 10 min, once MPN formation was complete so as not to interfere with this process, and eggs were subsequently left to develop and analyzed at 25 min (Fig. 4). Between 10 and 25 min, most eggs had developed to the point at which the MPN and FPN were either in contact or had fused. This progression was not affected by DMSO. However, eggs treated with either U73122 (P = 0.00234) or PP2 (P = 0.0472) frequently failed to form the zygote nucleus, and significantly, this process was blocked at the point of pronuclear contact, indicating that the fusion step was blocked specifically (congression was unaffected by the inhibitors). These data strongly support our observation above that some of the observed PtdIns(4,5)P$_2$ depletion must result from PLCy activity on the MPN generating DAG. Consistent with the inhibition of pronuclear fusion by PP2, we observed a significant reduction (60%) in the pTyr783 MPN signal (P = 0.0323), and in the global egg pTyr783 signal (approximately 50%, P = 0.0081), confirming the role of SFK in PLCy phosphorylation and activation (Fig. S6A and B). The inactive analogs U73343 (P = 0.1994) and PP3 (P = 0.9584) had no effect on pronuclear fusion. Given the conserved requirement of PtdIns(4,5)P$_2$ hydrolysis by PLCy for MPN formation and its subsequent fusion with the FPN, this also raises the question of the calcium requirement for pronuclear fusion. We have previously demonstrated that MPN formation requires basal calcium (<1 nM free Ca$^{2+}$),21,22 but not elevated levels; it is sensitive to BAPTA but not EGTA.15 As such we predict basal calcium levels would also be necessary for the pronuclear fusion described here.

Taken together, our data suggest that specific proteo-lipid signaling molecules are retained on the MPN to facilitate the formation of the zygote nucleus (Fig. 4B). We suggest that PLCy, SFK1 and PtdIns(4,5)P$_2$ represent conserved components of a molecular “toolkit” whose aim is to promote membrane fusion events at the heart of developmentally distinct NE assemblies. Moreover these signaling molecules are the first regulators of phosphoinositide metabolism shown to be involved in zygote formation.

Methods

Sea urchin gamete handling and preparation

Lytechinus pictus and Paracentrotus lividus sea urchins were procured from Marinus Scientific and Macrae and Company respectively. Gametes were obtained as previously described.21 Eggs were fertilized, the excess sperm washed away, and fixed at the appropriate time point to allow visualization of MPN and FPN congression or fusion.

Preparation of fixed egg samples and antibody labeling for confocal analysis

Fertilized eggs were gently shaken at 20 °C, and at the required time resuspended in 1 vol 7.4% paraformaldehyde in ice-cold isolation buffer (80 mM PIPES, pH 7.2, 5 mM EGTA, 5 mM MgCl$_2$, 1 M glycerol), fixed for 1 h at 20 °C with shaking. Eggs were washed twice in PBS (150 g, 1 min, 4 °C) and finally resuspended in 0.05% azide in PBS and stored at 4 °C.

For confocal analysis, an aliquot of eggs was blocked and/or permeabilized in 3% fatty acid free BSA/0.5% saponin in PBS for 15 min. Eggs were quenched in 1 mg/ml sodium borohydride for 2 min. Slides were labeled with anti-PLCy, anti-SFK1, anti-pTyr783 (Santa Cruz), or GST-PLCδ1-PH (2 μg/ml; Echelon). Primary antibodies were illuminated with commercial secondary antibodies conjugated to either DyLight 488, or DyLight 649 (secondary antibodies alone did not produce a signal, Fig. S7, panel C). GST-PLCδ1-PH was detected with an anti-GST-DyLight 650 antibody (abcam). Antibody incubations were for 1 h in 3% fatty acid free BSA/0.5% saponin in PBS. Eggs were additionally labeled with 2 μg/ml Hoechst 33342 and 2 μM DiOC$_6$ (as appropriate), settled onto freshly prepared 0.005% poly-L-lysine coverslips for 15 min, and mounted in ProLong Gold.

Where eggs were labeled with anti-PLCy and anti-SFK1 together (Fig. 1B and 2), which are both raised in chickens, a novel “capping” method was applied to prevent false co-localization from occurring, for example if both anti-chick DyLight 488 and anti-chick 649 were both to bind to an anti-PLCy primary antibody. This entailed labeling eggs with anti-PLCy, followed by anti-chicken DyLight 649. Any remaining exposed antigen sites on anti-PLCy were subsequently saturated with a goat anti-chicken IgG applied in excess (25 μg/ml), before the subsequent labeling of the samples with anti-SFK1 and anti-chicken DyLight 488. The data in Figure S7 show this to be an effective method.

Acquisition parameters for confocal microscopy and subsequent data treatment

Images were acquired on a Zeiss 710 upright confocal microscope run by Zen software (2009), with a 63/1.4NA oil-immersion objective. The chromophores were excited (Ex) at the wavelengths indicated, with emitted light (Em) collected between the bandwidths indicated: Hoechst (Ex 405 nm, Em 410–470 nm), DiOC$_6$, (Ex 458 nm, Em 496–592), DyLight 488 (Ex 488nm, Em 506–727), DyLight 647 (Ex 633 nm, Em 638–759), DyLight 650 (Ex 633 nm, Em 639–759). The images shown represent a field of view taken at 4’ zoom (33.7 μm by
were excited with the 920 nm laser line of a 6W Mira (Coherent) immersion objective and a Hamamatsu ORCA camera. Samples were acquired with 0.4 μm intervals between sections. The Hoechst channel was used to create an artificial “surface” over the nucleus, while the pY787/83 or PtdIns(4,5)P2 signal was labeled as “spots” of 0.5 μm diameter, the size of NE precursor membrane vesicles as previously reported.6 Only labeled vesicles that were in contact with the surface of the nucleus were scored. This process was repeated for each MPN analyzed (n = 5–13).

Conjugation of Atto 488 to anti-chicken F(ab’)_2 fragments

100 μg of antibody was conjugated to 25 μg of chromophore in 100 μl adjusted to pH 8.5 with 50 mM sodium borate buffer. Atto 488 (donor chromophore) was conjugated to an anti-chick F(ab’) fragment for 1 h in the dark at 20 °C with shaking. Free chromophore was separated from antibody-dye conjugate on a PD Minintrap™ G-25 column (GE Healthcare). The dye:protein ratio of the chromophore-antibody conjugate was calculated as per the manufacturer’s instructions. Conjugates had a dye:protein ratio of <3:1.

Data acquisition for the measurement of FRET by 2-photon FLIM

Fixed eggs were blocked/permeabilised and quenched as described above. Protein labeling took place with anti-PLCy followed by anti-chicken F(ab’),-Atto 488 and anti-SFK1, followed by anti-chicken HRP. The anti-chicken F(ab’),-Atto 488 and anti-SFK1 incubations were separated by a goat anti-chicken IgG incubation as described above. All antibody incubations took place in 3% fatty acid free BSA/0.5% saponin in PBS for 1 h. To create an enhanced acceptor signal, eggs were treated with TSA-Cy5 for 10 min at room temperature, as per the manufacturer’s instructions, with the enhancement reaction being terminated by PBS washing the eggs (150 g, 1 min). Eggs were additionally labeled with 2 μg/ml Hoechst 33342 and mounted in ProLong Gold. Images were acquired on a Nikon TE2000-E inverted microscope with a 60´/1.49NA oil immersion objective and a Hamamatsu ORCA camera. Samples were excited with the 920 nm laser line of a 6W Mira (Coherent) laser at a power of 27 mW and were subjected to a maximum of 800 scans to obtain a sufficient number of photons (>800) for a bi-exponential Marquardt curve fit. The scanning number was controlled to ensure no photobleaching occurred. The 920 nm excitation does not directly excite Cy5.8 Calculation of the donor chromophore (Atto 488) lifetime was performed in TRI2 custom software (Paul Barber, Dept. of Oncology, University of Oxford).

Lipid overlay assay

GST-PLCδ1-PH protein (0.5μg/ml) was incubated with a pre-blocked Membrane Lipid Strip™ (P-6002, Echelon) in 3% Fatty acid free BSA/PBS/0.2% Tween 20 (PBST) for 1 h at RT. Excess protein was removed by five washes in PBST. Recombinant protein binding was detected with an anti-GST primary antibody (Millipore), followed by an anti-mouse HRP conjugated secondary (GE Healthcare).

Inhibitor study

Eggs were fertilized and at T+10 and were left untreated, or treated with DMSO vehicle (0.1%), U73122/U73343 (30 μM), or PP2/PP3 (10 μM). At T+25 samples were fixed, stained with Hoechst 33342 and examined by confocal microscopy. Between 16 and 74 eggs (mean = 39) were scored per condition.

Statistical analysis

Data was analyzed in Graphpad Prism v6.0a. Data were subjected to unpaired 1- or 2-tailed t tests with Welch’s Correction and 95% confidence intervals.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/nucleus/article/34422

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