Untangling Par complex dynamics during embryogenesis using single-molecule TIRF microscopy

Presented by Lars Nico Jan Deutz

In partial fulfillment of the requirements for graduation with the
Dean's Scholars Honors Degree in Neuroscience

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ABSTRACT

Proper neuronal development requires localizing cellular components in asymmetric ways to enable differences in cell behavior. This process, called cell polarity, is regulated by proteins in the Par complex. The kinase aPKC (along with its cofactor Par6) is a key member of this network and can be recruited to the plasma membrane by either the small GTPase Cdc42 or the scaffolding protein Par3. Although interactions among these proteins are well established in test tubes, much is still unknown about the complexes they form in life during development. In addition, at later stages of development, much is still unknown about how Cdc42 or Par3 interact with aPKC, especially at the 8-cell stage. Here, to enable the study of membrane-associated complexes, we used a synthetic chemistry tool to rapidly isolate membrane proteins from single C. elegans zygotes into lipid nanodiscs. We show that this tool allows us to detect native complexes involving Cdc42, which are undetectable when cells are lysed in detergent. We found that Cdc42 interacts more strongly with aPKC during polarity maintenance than polarity establishment, two developmental stages that are separated by only a few minutes. Further, we show that Par3 rapidly assembles during zygotic polarity with aPKC but not during apical-basal polarity. Overall, our findings establish a new tool for studying membrane-associated signaling complexes and reveal an unexpected mode of polarity regulation via Cdc42.

INTRODUCTION

Proper development requires localizing cellular components asymmetrically, a phenomenon termed cell polarity. This process is essential for a wide range of biological processes including the proliferation of stem cells, specification of cell fate during embryogenesis, and neurite outgrowth (Dhonukshe, 2013). A loss of cell polarity is a hallmark of epithelial tumor progression; conversely, cancer cells may upregulate polarity machinery to aid in tissue invasion and metastasis.

In the early embryo, polarity is regulated by partitioning defective (Par) complex proteins. The kinase aPKC is an essential, strongly conserved component in this system, whereby it enables asymmetry by phosphorylating cytoskeletal proteins, cell fate determinants, and other targets that enable polarity (Hong, 2018). aPKC forms a tight complex with its partner protein Par6. The Par6/aPKC complex is thought to be localized to the membrane through either the scaffolding protein Par3 or the small GTPase Cdc42. The localization of aPKC onto the proper domains on the cell cortex is one of the key mechanisms by which polarity is regulated (Hong, 2018).

Cell polarity is widely studied in the one-cell *C. elegans* embryo. In this system, polarization proceeds through distinct establishment and maintenance stages which correspond to interphase and mitosis, respectively (Lang & Munro, 2017). Before polarity is established, cell determinants are found to be distributed throughout the cell. During polarity establishment, membrane flows transports Par3-bound aPKC clusters to the anterior membrane (Lang & Munro, 2017). Once those Par3-aPKC clusters are on the cortex, Par3 is thought to disassociate and aPKC instead becomes stabilized to the cortex through Cdc42, proceeding the cell into maintenance.

These interactions are disrupted in disease. In models of breast cancer, disrupting the interaction between Par3 and aPKC induces tumor invasion and migration (Hu et al., 2022). In addition, increased expression of Par3 and aPKC are tied to lower survival of breast cancer patients in a national cohort. Although Cdc42 is infrequently mutated in cancer, its expression is significantly downregulated in several carcinomas including those of the central nervous system, the adrenal gland, and the pancreas (Xiao et al., 2018). Ultimately, studying thermodynamic properties can give us insight into protein complex stability. Aberrant interference with complex stability (e.g. via post-translational modifications like phosphorylation) has been implicated in a wide array of disease processes (Singh et al., 2022; Zhong et al., 2023).

Given that cell polarity is a biological process difficult to recapitulate *in vitro* and there is a disease relevance to studying these interactions, the Dickinson lab has developed a technique to study protein interactions ex vivo (Sarıkaya & Dickinson, 2021a). In brief, we start by isolating a *C elegans* zygote in a microfluidic device. This embryo has been genetically modified to express key proteins attached to various fluorescent tags (for instance, Cdc42 and aPKC). Then, we use a pulsed infrared laser to lyse the embryo and visualize the proteins as they go into solution and bind to antibodies on the coverslip using TIRF microscopy (Sarıkaya & Dickinson, 2021a). We can measure the strength of protein interaction by observing the time it takes for the prey protein to unbind from the complex. From this technique, we can measure certain thermodynamic properties (such as k_{off}) of certain protein complexes.

This assay is termed single-cell Single-Molecule Pulldown (sc-SiMPull), first introduced in 2017 (Dickinson et al., 2017).

sc-SiMPull requires the proper solubilization of cellular components and thus readily detects cytoplasmic protein interactions. However, studying membrane-associated protein interactions is more difficult because traditional detergent solubilization usually disrupts the underlying lipid membrane environment (Krishnarjuna & Ramamoorthy, 2022). To overcome this hurdle, chemists have developed a wide array of synthetic polymers that form native lipid nanodiscs, however, their use in these types of assays is largely unknown.

By including maleic-acid copolymers which form lipid nanodiscs in our sc-SiMPull experiments, we showed that we were able to readily detect Cdc42/Par6 complexes during development. This revealed that the interaction between Cdc42 and Par6 is four-fold stronger in polarity maintenance than in polarity establishment (Deutz et al., 2025). In addition, we showed how phosphorylation of Cdc42 is sufficient to explain this discrepancy in development (Packer et al., 2024). Finally, by looking at later stages of polarity, we showed how Par3 is cooperative at polarity establishment, but not the 8-cell stage. Our results broadly demonstrate a new method of studying membrane protein interactions and refine our understanding of protein interactions essential to life.

MATERIALS AND METHODS

C. elegans strain construction and maintenance

C. elegans were maintained on NGM medium and fed E. coli according to standard procedures. Strains with fluorescent tags were generated using CRISPR/Cas9-triggered homologous recombination and/or genetic crosses, as previously established (Dickinson et al., 2013).

Imaging of C. elegans zygotes with RNA interference

RNA interference (RNAi) against cdc-42 was performed by feeding on plates. Cultures containing RNAi feeding clones were grown overnight in 4 mL of LB broth with ampicillin. After being concentrated 5X, the cultures were spotted onto plates with 25 μg/mL carbenicillin and 1 mM IPTG and left to dry overnight. L4 worms were picked onto these plates and embryos were dissected 24h afterward.

Embryos were mounted on glass slides with 22.8-μm beads (Whitehouse Scientific, Chester, UK) as spacers in egg buffer (5 mM HEPES (pH 7.4), 118 mM NaCl, 40 mM KCl, 3.4 mM MgCl2, 3.4 mM CaCl2). Images were acquired using a Nikon Ti2 inverted microscope with a 60x objective lens.

Fabrication of microfluidic devices with antibodies

Following established protocols, microfluidic devices were fabricated using SU-8 photolithography (Deutz et al., 2025; Sarıkaya & Dickinson, 2021b). A 10:1 mixture of PDMS and curing agent was prepared and poured onto moles. After degassing the molds, the devices are transferred to a spin-cotor set for 300 rpm for 3s and then left to bake in a 85°C incubator for 20 min. After baking, the devices were peeled off the molds and the inlet and outlet wells are cut out with a 2-mm biopsy punch. PDMS devices were plasma treated and placed on 24 x 60 mm glass coverslips that have been cleaned and UV-ozone treated. Upon placement, the devices make a permanent bond with the glass coverslip. To passivate the devices, a mixture containing 0.02% biotin-PEG-silane and 2% water in mPEG-silane was prepared. 2 μL of this mixture was placed in each inlet wel and 0.5 μL into each outlet wel. After incubating in RT for 30 min, devices were aspirated and rinsed 2x with water.

Immediately before an experiment, devices were functionalized with mNG nanobodies. A 0.2% mg/mL solution of Neutravidin in Tween buffer was prepared and 1.5 uL was placed in each inlet well. After 10 minute incubation, devices were rinsed 4x in Tween buffer. Subsequently, 1.5uL of 1 μM solution of biotinylated anti-mNG nanobodies in Tween buffer was placed in each inlet and left to incubate for 10 more minutes. Devices were again rinsed 4x with Tween buffer and devices were sealed with clear tape. Right before use, tape was cut with a scalpel and peeled off.

Lysis buffers

For each day of experiments, a 50 mg/mL stock of DIBMA 12 Tris was prepared. For sc-SiMPull experiments, lysis buffers for DIBMA (10 mM Tris (pH 8.0), 50–150 mM NaCl, 1% DIBMA, and 0.1 mg/mL bovine serum albumin) and detergent samples (10 mM Tris (pH 8.0), 50 mM NaCl, 0.1–1.0% Triton X-100, and 0.1 mg/mL bovine serum albumin) were prepared.

sc-SiMPull with TIRF microscopy

Adults with embryos were dissected in egg buffer and rinsed in lysis buffer. Separately, the microfluidic device channel was rinsed 4x with lysis buffer. Embryos were transferred to inlet well and pushed into the channel with a clean needle and/or pulled in with a vacuum. The channel inlet and outlet was sealed with clear tape. A microfluidic device with live zygote was then transferred to the microfluidic device.

TIRF microscopy was performed using a custom-built microscope, as previously described (Sarıkaya & Dickinson, 2021b). The instrument is equipped with excitation lasers for 488-, 561- and 638-nm and a custom four-color image splitter that allows for simultaneous imaging of four wavelengths. After transfer to the microscope, embryos were located with transmitted light and a snapshot of the embryo's developmental stage was taken. The embryo is then lysed with a single shot of a 1068-nm pulse laser which generates a cavitation bubble which mechanically lyses the cell. After lysis, the stage was moved 60–120 μm and TIRF images were acquired for 8000-10,000 frames at 50-ms exposure.

Electron microscopy

Preceding an experiment, electron microscopy grids were plasma treated. Wild-type embryos were prepared and lysed in channels that have not been functionalization with antibodies and with different buffer conditions (see: *Lysis buffers*). After optical lysis, cell lysate was transferred to the EM grid with a mouth pipette and clean glass needle (approx: 50nL). The EM grid was subsequently negative stained with 35 μL of 2% (w/v) uranyl acetate solution and blotted to remove excess stain. The grid was imaged with a FEI Technai TEM at 80 kV at the biomedical core facility at the University of Texas.

Fluorescence intensity measurement in live embryos

To quantify enrichment of Par proteins at the cortex, cortical and cytoplasmic F.I.s were measured from line scans perpendicular to the cortex in the anterior part of the cell. Measurements were made using FIJI and scatterplots prepared using Graphpad Prism.

Processing of dynamic sc-SiMPull data

SiMPull data analysis was performed using open-sourced MATLAB package (https://github.com/dickinson-lab/SiMPull-Analysis-Software/). The process of analyzing this data has been described extensively (Deutz et al., 2025; Dickinson et al., 2017; Sarıkaya & Dickinson, 2021b). Co-appearance versus time plots were generated by a plotting tool within the SiMPull analysis software package. Data were fit with a single-exponential decay function to obtain koff and confidence intervals. Dwell time analyses were analyzed by subtracting kobserved from paired mNG::HaloTag control samples; kobserved = kbleach + koff.

RESULTS

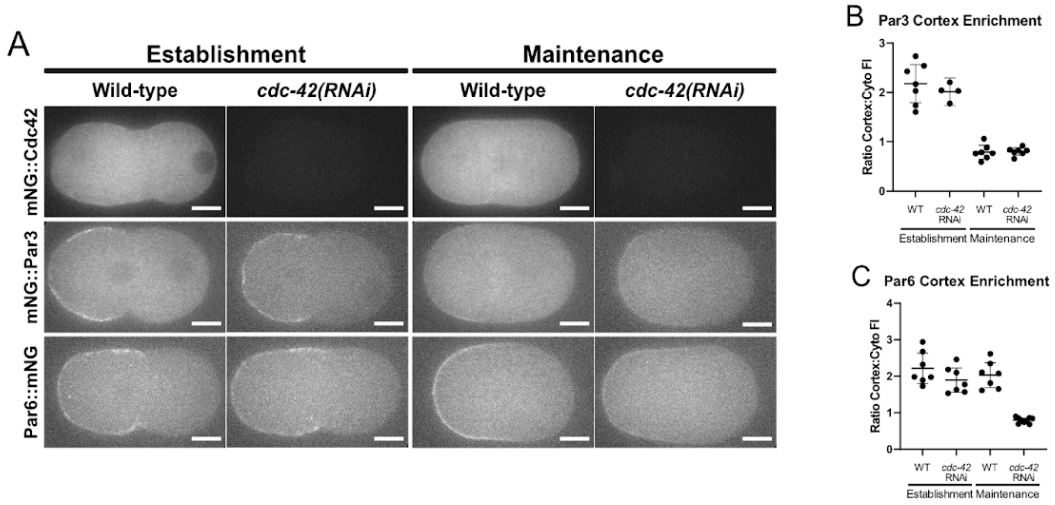

Figure 1: Figure and legend from *Deutz et al 2025*. Cdc42 is necessary for Par6 recruitment to the plasma membrane during polarity maintenance, but not establishment. (A) Confocal images of C. elegans zygotes carrying endogenous tags for Cdc42, Par3 or Par6 during polarity establishment or polarity maintenance, with or without cdc-42. Scale bars represent 10 µm. (B) Ratio of cortex to cytoplasm fluorescence intensity of Par3, from line scans taken during establishment or maintenance in either control or cdc-42(RNAi) conditions. Each data point is a separate embryo. Error bars indicate mean ± 95% confidence interval. (D) Ratio of cortex to cytoplasm fluorescence for Par6::mNG as described in (C).

Motivated by recent results that have clarified the relationship between Par3, aPKC/Par6, and Cdc42 in vitro (Vargas & Prehoda, 2023), we set out to measure the complexes these proteins form in vivo. To this end, we chose the C. elegans zygote as a model system due to its stereotyped development and establishment (interphase) and maintenance (mitosis) where polarity is thought to rely on either Par3 or Cdc42, respectively. We wanted to first confirm the consensus that Par3 and Cdc42 have different effects on the localization of Par6/aPKC during development (Aceto et al., 2006). In control embryos, Par6 is found on the anterior cell membrane throughout these two stages of development. In embryos without Cdc42, Par6 properly localizes to the cell membrane during establishment but loses this localization during maintenance (Fig 1A, 1C). Cdc42 reduction does not affect Par3, which is found on the cortex during establishment but not maintenance (Fig 1A, 1B).

Interested in studying this complex biochemically, we turn to a technique that the Dickinson lab pioneered called single-cell Single-Molecule Pulldown (sc-SiMPull). Briefly, sc-SiMPull involves isolating a *C. elegans* zygote in a microfluidic chamber. This zygote has been endogenously tagged against proteins of interest (e.g. mNeonGreen::Cdc42). A pulsed laser then generates a cavitation bubble, mechanically lysing the embryo. We can use microscopy to detect these complexes as they spill out of the embryo and bind to antibodies on the coverslip.

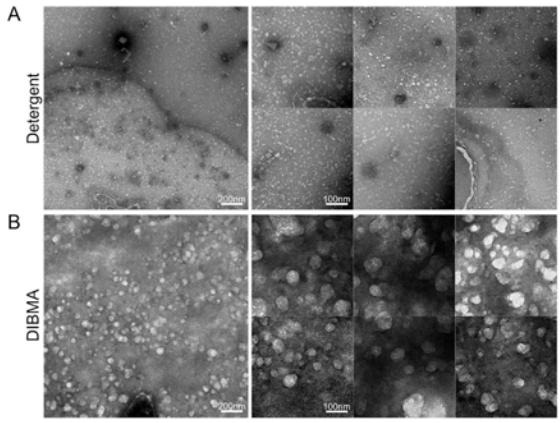

Figure 2: Figure and legend from *Deutz et al 2025*. DIBMA polymers rapidly form lipid nanodiscs from C. elegans embryos. (A–B) Electron micrographs of cell lysate extracted from C. elegans embryos. Embryos were lysed in either (A) detergent buffer, (B) 1% DIBMA with 7.5mM CaCl2 and then extracted from the microfluidic channel, applied to an EM grid, and negative-stained.

Typically, these experiments are performed in detergent backgrounds. Despite biochemical evidence for a Cdc42-Par6 interaction, these complexes were undetectable in detergent backgrounds (Deutz et al., 2025). Then, we turned to synthetic polymers which have shown to preserve the stability of membrane associated complexes by forming native lipid nanodiscs. To verify the formation of nanodiscs in our experiments we extracted lysate from single embryos in either Detergent or DIBMA, a polymer which forms lipid nanodiscs. This lysate was transferred to an electron microscopy grid and negative-stained to enhance contrast, a technique previously reported (Yi et al., 2019). In detergent conditions, the distribution of lipids is amorphous and heterogeneous (Fig 2A). In contrast, in the presence of DIBMA, we see the formation of stereotyped size of lipid nanodiscs between 20-50nm in diameter (Fig 2B).

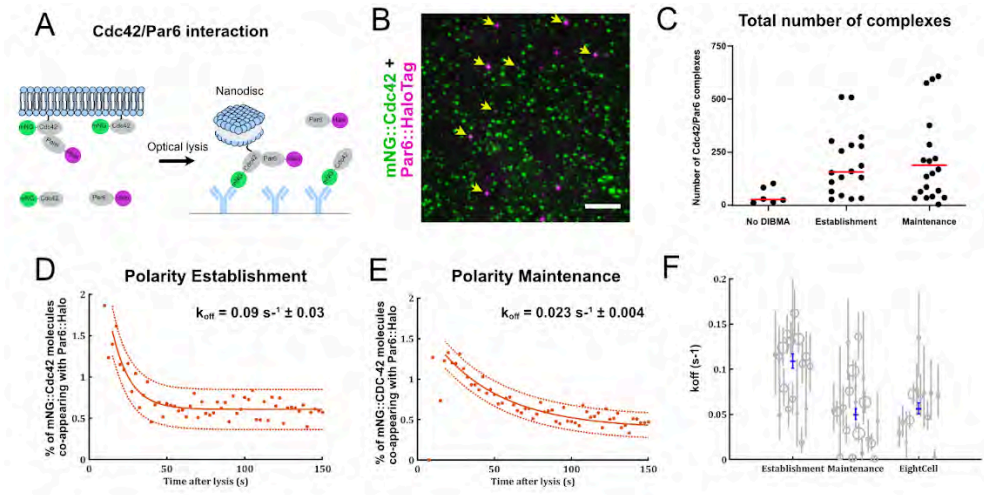

Figure 3: Figure and legend from *Deutz et al 2025*. The interaction of Cdc42 and Par6 is developmentally regulated. (A) Schematic of the sc-SiMPull experiment with embryos being lysed in DIBMA buffer. (B) Image showing mNG::Cdc42/Par6::HaloTag complexes detected after lysis in DIBMA buffer. Arrows indicate single mNG::Cdc42/Par6::HaloTag complexes. Scale bar represents 5 µm. (C) Number of Cdc42/Par6 complexes detected per experiment in the indicated conditions. Each data point represents one embryo, and red lines indicate the medians. (D and E) Fraction of mNG::Cdc42 molecules co-appearing with Par6::HaloTag as a function of time. Curves were fit to single-exponential decay functions, resulting in the indicated estimates for koff. (F) Calculated koff values for the Cdc42/Par6 interaction based on the distribution of single-molecule dwell times subtracted by bleaching control.

With the validation experiments complete, we turned to our original goal of biochemically studying the Cdc42-Par6 complex interaction using sc-SiMPull. In DIBMA, we detected a robust number of Cdc42-Par6 complexes (Fig 3A-3C). Protein complexes can be treated as ligand-binding assays where the complexes relax to equilibrium. When performing this analysis, we find (shockingly) a $k_{off} = 0.09 \pm 0.03$ for embryos in establishment and $k_{off} = 0.023 \pm 0.004$ for embryos in maintenance (Fig 4D-4E), a nearly four-fold different in disassociation rates. Another way to analyze the same data is to look at the time a prey molecule (e.g. aPKC) is found in a complex, known as a dwell time. The $k_{observed}$ is a product of the natural unbinding between two proteins k_{off} and the bleaching rate k_{bleach}. After correcting for bleaching, we determine a k_{off} of 0.109 ± 0.008 s−1 during polarity establishment and $k_{off} = 0.050 \pm 0.006$ s−1 during polarity maintenance and $k_{off} = 0.056 \pm 0.006$ during the 8-cell stage. Both of these analyses show that the Cdc42/Par6 interaction is downregulated during polarity establishment compared to later stages of development.

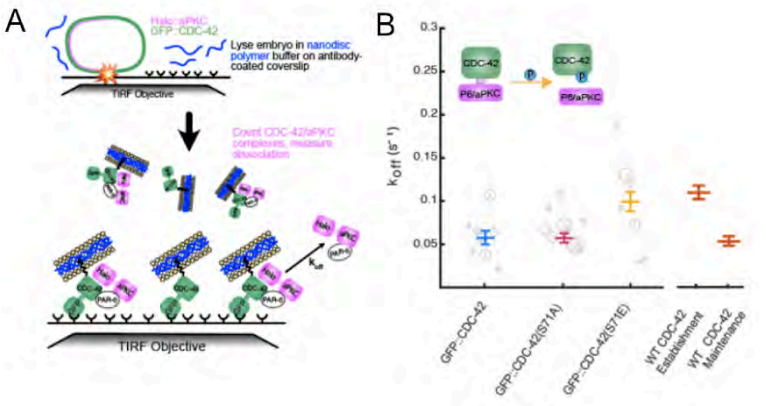

Figure 4: Figure and legend from *Packer et al 2023*. Cdc42 phosphorylation on Serine-71 promotes its aPKC dissociation. (A) Graphical representation of the lipid nanodisc single-cell pull-down experiment. (B). Dissociation rate constants measured for Cdc42/aPKC, Cdc42(S71A)/aPKC and Cdc42(S71E)/aPKC complexes extracted from mixed-stage single C. elegans zygotes. Gray circles represent individual experiments, with the size of the circle indicating the number of complexes captured from each zygote and the error bars representing the Bayesian 95% credible interval for the estimated koff. Orange bars are measurements of koff for the complex of wild-type Cdc42 and Par6 during polarity establishment or polarity maintenance (Deutz et al., 2023), shown here for comparison.

Given the fact that only a few minutes separate polarity establishment and maintenance, it is likely that the mechanism for this regulation is post-translational. To this end, we collaborated with the Rodriguez lab at Newcastle which identified a possible post-translational modification: phosphorylation. Cdc42's Serine-71 site can be phosphorylated by aPKC in vitro (Packer et al., 2024). To investigate whether phosphorylation can explain the discrepancy in koff observed during development we perform sc-SiMPull experiments with Cdc42 mutated to be non phosphorylatable (S71A) or phosphomimetic (S71E) (Fig 5A). We find that the k_{off} observed in the phosphomimetic and non phosphorylatable mutant resembles almost exactly the k_{off} observed during establishment and maintenance, respectively (Fig 5B). Thus, phosphorylation at Cdc42's Serine-71 site is sufficient to explain its dynamic binding to Par6 during development.

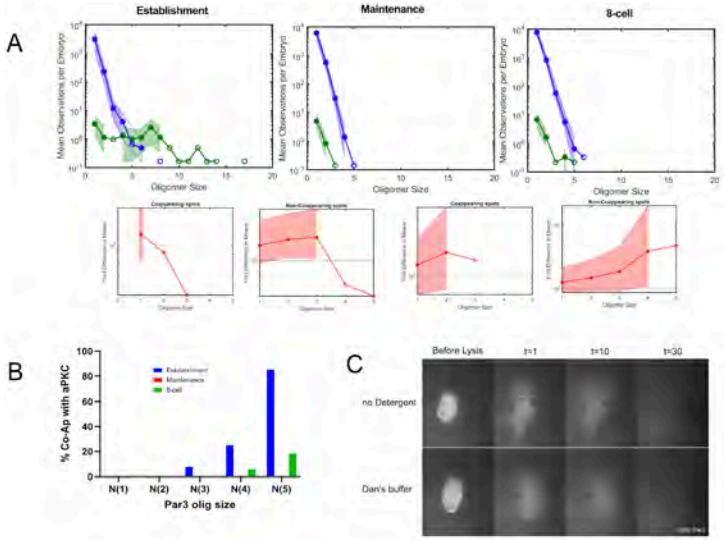

Figure 5: Par3 clusters are smaller in later stages of development and associate less with aPKC. (A) Mean number of Par3 clusters separated by oligomer size and their coAppearance with aPKC at different stages. Statistical analysis comparing Estab/Maint and Maint/8-cell are below each graph; the two conditions are significantly different if the confidence interval does not include zero. (B) Percent co-appearance with aPKC for different Par3 oligomer sizes in different stages of development. (C) Snapshots of embryos endogenously tagged with mNG::Par3 after laser lysis in different buffer conditions.

Switching gears, we were next interested in understanding how Par3 regulates the transition between establishment and maintenance. During establishment, Par3 forms large oligomers which almost always bind aPKC (Dickinson et al., 2017). After maintenance and the 8-cell stage, these large Par3 clusters are no longer present, and their association with aPKC is thus reduced (Fig 5A-5B). In addition to the number of large Par3 clusters being reduced after establishment, it also turns out that the same clusters bind aPKC less effectively during maintenance and the 8-cell stage (Fig 5B). The reduction in large Par3 clustering can likely not be explained to improper dissolution because Par3 is fully soluble after laser lysis (Fig 5C).

The result that Par3 does not form large clusters during the 8-cell stage is surprising because recent work has shown that Par3 is playing an essential role in cap formation and is essential for proper inside-out polarization (Stolpner et al., 2023).

DISCUSSION

In this study, we begin with a straightforward observation: when levels of the signaling protein Cdc42 are reduced, the Par complex is properly localized to the membrane during polarity establishment but improperly detaches a few minutes later during maintenance. To understand this phenomenon, we use a tool (developed by the Dickinson lab) with a technical enhancement to study Cdc42-Par complex interactions. Our findings reveal that the interaction between Cdc42 and the Par complex is highly dynamic throughout the cell cycle. Additionally, we uncover a novel, yet unexpected role for Par3, a key protein that stabilizes aPKC at the cortex, in regulating these dynamics.

Cdc42 is a member of a class of enzymes termed small GTPases. Small GTPases, such as Cdc42, act as crucial “on-off” switches in cell biology, cycling between active GTP-bound and inactive GDP-bound states. In our study, we demonstrate that the phosphorylation of Cdc42 at its Serine-71 site serves as an critical regulatory mechanism for its dynamic binding to the Par complex. This work builds upon previous studies showing that phosphorylation can influence the GTP/GDP cycling of other Rho-family GTPases in different model systems (Kwon et al., 2000; Waschbüsch & Khan, 2020).

The finding that phosphorylation regulates Cdc42 activity has significant therapeutic potential. Despite decades of effort, small GTPases like Cdc42 or Ras are considered “undruggable” due to a lack of deep pockets for ligand binding (Yin et al., 2023). In contrast, kinases are classic examples of druggable targets and are the second most common small molecule target (Xie et al., 2023). Therefore, targeting small GTPases through their kinase regulators may offer significant promise for developing new therapeutics.

The application of nanodisc-forming polymers to study membrane-associated complexes also represents a significant technical advancement, in addition to its biological relevance. The study of membrane protein complexes has long been challenging due to the limited availability of assays, particularly those that preserve native complexes (Carpenter et al., 2008). Single-molecule assays, fortunately, have emerged as powerful tools for studying signaling complexes, providing exceptional resolution and insights into key biological processes (Deniz et al., 2008). By leveraging synthetic chemistry to retain the native membrane environment, we can now make feasible the assessment of a wide range of protein complexes.

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