1. Applicant

Dr. Sophie Tschirpke, PostDoc at TU Delft (group of L. Laan)

Achievement 1: During my PhD in the group of L. Laan I reconstituted central parts of yeast polarity establishment as a model system to unravel how different types of molecular interactions in large regulatory networks contribute to robust cellular function – a fundamental question in biology. My approach enables a high level of control to advance mechanistic studies, deepening our understanding biological robustness. I successfully addressed the main challenges: (1) purifying large and partially unstructured proteins and (2) reliably controlling multi-protein experiments. I achieved this through establishing a number of protocols and biochemical assays [1, 2]. By publishing a detailed guide about our methodology we aim to benefit the community and make future studies more efficient [1]. In addition, I trained a new lab technician in protein purification to reproducibly purify existing and new proteins, resulting in a library of 20+ purified proteins to date. My work thus built a solid fundament for all future *in vitro* work in the Laan group, lowers the entry barrier for other researchers, and enables controllable quantitative studies of this important system.

Achievement 2: Enabled by achievement 1, I investigated the regulation of the central enzyme in yeast polarity establishment (Cdc42). I showed that two kinds of regulators individually ***and*** synergistically boost the activity of Cdc42, and that proteins that are not traditionally thought part of the yeast polarity network also enhance Cdc42's activity. My findings challenge the current view that regulation is only achieved through a small number of regulatory proteins that interact with specific partners. Instead, it proposes a novel view where Cdc42 regulation is defined through a larger network of specific and non-specific interactions. The role of non-specific interactions in regulatory networks is so-far unexplored, and could play a crucial role in establishing robustness in cellular functionality [3].

2. Title of the project

Disentangling molecular mechanisms of the Cdc42 GTPase activity regulation

3. Kavli host groups

Group 1: Dr. ir. Liedwij Laan (TU Delft), www.tudelft.nl/laanlab **Group 2:** Dr. Martin Depken (TU Delft), www.tudelft.nl/tnw/over-faculteit/afdelingen/bionanoscience/about-the-department/dr-martin-depken

4. Abstract

Robust functioning is essential for cells, and can be achieved through expansive regulatory networks. How do these networks facilitate robustness at the molecular level? I will answer this question in the context of polarity establishment in budding yeast. Here a large regulatory protein network drives the accumulation of the enzyme Cdc42 to one spot on the cell membrane to initiate bud formation. Key to this process is the regulation of Cdc42's GTPase activity. Building on my discovery of novel regulatory interactions within the network (**achievement 2**), I will use the seed funding to unravel the precise effect of these molecular interactions. To test various hypothesis I will use quantitative modelling to make predictions and compare them to data from *in vitro* reconstituted networks. My combined experimental and theoretical approach will provide a new mechanistic lens through which robust Cdc42 regulation can be viewed, impacting research domains beyond yeast.

5. Description of the proposed research

Background: Cell polarity - the directional organization of cellular components and functions within a cell - is vital for processes such as cell division, directed cellular growth, and motility. How the interplay of components within the complexity of a cell leads to robust establishment of cell polarity is still heavily debated. I explore this problem using polarity establishment in budding yeast. To initiate budding, the protein Cdc42 exits the cytoplasm and accumulates in a spot on the cell membrane (Fig. 1a). This step is vital, as it initiates all down-stream processes of cell division. Although all key polarity proteins (approx. 35) have been identified, the molecular mechanism driving Cdc42 accumulation is still debated [4, 5]. A key element is the GTPase activity regulation of Cdc42. GTPase activity involves 3 steps (Fig. 1b): (1) binding of GTP to Cdc42, (2) hydrolysis of GTP, and (3) release of GDP from Cdc42. The regulatory network of Cdc42 contains two classes of GTPase activity regulators: GTPase activating proteins (GAPs) boost step 2 and GDP/GTP exchange factors (GEFs) boost step 3 [6]. My recent work found a synergistic effect between GAPs and GEFs, and that even proteins that interact non-specifically with Cdc42 boost its GTPase activity [3]. The molecular mechanisms through which these effects arise remain elusive.

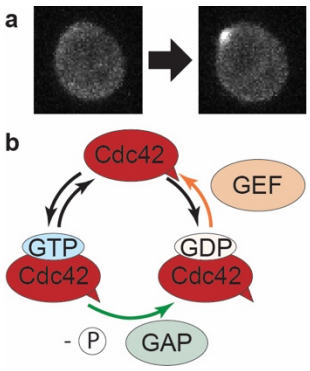


Figure 1: a) Life cell microscopy showing the localization of labelled Cdc42 (white) during polarity establishment. **b)** GTPase cycle steps.

Aim of the project: To dissect the molecular mechanism through which synergies arise and through which non-specifically interacting proteins affect Cdc42 activity. To achieve this aim I will focus on the sub-steps of the GTPase cycle, answering two key questions:

Q1: Which steps of the GTPase cycle are affected by non-specifically interacting proteins, and what is the magnitude of the effect they induce?
Q2: To what extent does each step of the GTPase cycle give rise to the synergy between GEFs and GAPs?
I will integrate the answers of **Q1** and **Q2** to contextualize how polarity proteins regulate Cdc42. The prevailing view is that only GEFs and GAPs, classified as such by their GEF/GAP domains, regulate Cdc42's GTPase activity. However, through which chemical interactions GTPases hydrolyze GTP and how GAPs catalyze the same reaction remains controversial [7, 8]. This allows for the possibility that more polarity proteins exhibit a GEF- or GAP-like function, or enhance GTPase activity via a third pathway. Answering **Q1** will show if our current definition of a GEF or GAP is too restrictive and needs to be expanded, or if a third regulatory class exists. Answering **Q2** focusses on the interplay of components and will indicate where further synergies within the network may arise.

Approach: We have purified the following polarity proteins: Cdc42 (GTPase), Cdc24 (GEF), Bem3 and Rga2 (GAPs), Bem1 and Rdi1 (other polarity proteins). Using a bulk GTPase assay I have shown that (1) Cdc24 and Rga2 individually boost the GTPase cycle of Cdc42, (2) together they show synergy, and (3) that proteins that are not part of the yeast polarity protein network (BSA, Casein) can boost the Cdc42 GTPase cycle. With my input a former PostDoc in our group (W. Daalman) developed a model which I used to quantitatively analyze the data.
The GTPase assay, which I used so-far, studies the effect of a protein on the entire GTPase cycle of Cdc42 (step 1-3). In this project I will set up another bulk assay (mant-assay) with which I will examine sub-steps of the GTPase cycle (step 2 and step 2+3). I will use the same proteins (Cdc24, Rga2, BSA, Casein) in the mant-assay to determine if the overall effects seen in the GTPase assay can be traced back to a single reaction step. I will develop and test reaction-kinetic models based on how well they quantitatively predict the mant-assay data. These models will be analyzed in steady state to describe how GTPase activity is affected by regulatory proteins, the enzyme (Cdc42), nucleotides, and through the reaction network with which they are connected. These findings will assist me in postulating so-far unexplored mechanisms through which Cdc42 GTPase activity is regulated. I will use other (already purified) polarity proteins, in combination with the proteins used so-far, in additional GTPase and mant-assays, to test to which extent my findings can be generalized and to conjure how this contributes to robustness in Cdc42 regulation.

6. Host environment

The experimental group of L. Laan will provide the environment in which the assays will be performed and measurements can be conducted on departmental machines. M. Depken will provide his molecular-modelling expertise to guide and support me in developing models for hypothesis testing and quantitatively analyzing the data.

Costs:

PostDoc (8 month):	Costs for consumables and chemicals:	**Total**
~ 40'000 EUR	~ 15'000 EUR	**55'000 EUR**

8. Benefit to career of the fellow

This project will enable me to add an intricate mechanistic understanding to my findings of **achievement 2**, elevating their significance and impact. We aim to submit a research paper on this topic before the project end (1.4.2024). My findings can have a high impact on the polarity community, as they challenge the current view and provide a new lens through which Cdc42 regulation can be viewed. As both Cdc42 and its regulation through GEFs and GAPs is highly conserved within eukaryotes, my findings will inform multiple Cdc42-related research areas incl. those on mammalian systems. Working with M. Depken on developing models will expose me to theoretical biophysics and programming – skills that highly complement my experimental expertise. I will be starting a PostDoc in a bioengineering group at UC Berkeley in Spring 2024, working on protein-mediated cellular uptake strategies for novel DNA-origami based carriers. Modelling expertise on dynamic protein interactions, as gained here, will translate to this new project and will position me to successfully work on protein systems that necessitate experimental and theoretical expertise.

9. Benefit to the synergy of KIND

The project will start the first collaboration between the experimental group of L. Laan and the theory group of M. Depken. The group of L. Laan will continue to investigate the mechanisms governing the regulation and accumulation of Cdc42 and its multi-component network through *in vitro* reconstitutions. The Depken group has a general interest in robustness and specificity in biological function, and this work will allow the group to enter the field of systems level modelling. With both groups offering complementary expertise, the collaboration initiated here has the potential for long-term success. Both group having a track record in the field will help attract external funding for future collaborations.

References:
[1] Tschirpke *et al.* Heliyon 2023 (submitted).
[2] Tschirpke *et al.* 2023 (in preparation).
[3] Tschirpke *et al.* eLife 2023 (in preparation).
[4] Vendel *et al.* J Cell Sci 2019.
[5] Goryachev & Leda, Mol Biol Cell 2017.
[6] Martin, BioEssays 2015.
[7] Calixto *et al.* ACS Omega 2020.
[8] Patel *et al.* Comput Biol Chem 2023.