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Research Article

Sortase A-Mediated Farnesylation of Cdc42 *In Vitro*

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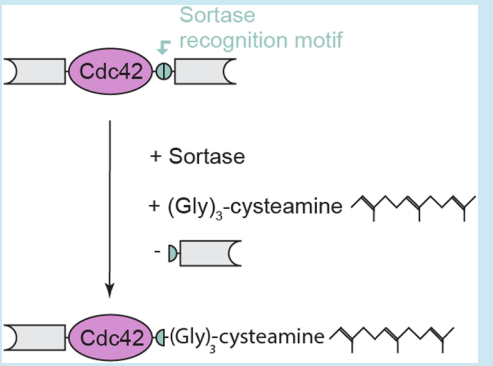
ABSTRACT: Cdc42, a Rho-family GTPase, plays a pivotal role in establishing polarity in *Saccharomyces cerevisiae* by accumulating on the membrane at the site of bud emergence. Cdc42's ability to bind to membranes, mediated by prenylation, is essential for its function. Prenylation involves either the post-translational addition of a 15-carbon farnesyl group or a 20-carbon geranylgeranyl group to Cdc42's C-terminus. One of the major challenges in studying the physical and chemical interactions of Cdc42 at the polarity spot *in vitro* is obtaining prenylated Cdc42. Here, we present a streamlined, sortase A-based approach to farnesylate Cdc42 *in vitro*. This method uses *Escherichia coli*-expressed Cdc42 with a sortase A recognition motif, facilitating efficient farnesylation and purification using a size exclusion-based strategy. The farnesylated Cdc42 retains functionality, as evidenced by membrane binding and by its GEF-regulatory GTPase activity, making it suitable for further biophysical and biochemical investigations.



KEYWORDS: *farnesylation, prenylation, post-translational modification, Rho GTPases, Cdc42, protein engineering*

INTRODUCTION

Rho small GTPases are important signaling proteins in the cell and are highly evolutionary conserved. They are involved in many essential cellular processes including cell polarity, actin and microtubule assembly, gene transcription, and vesicle formation.[1,2] Many of these cellular processes take place at cellular membranes, making the ability of Rho GTPases to dynamically associate with membranes critical for their function. This membrane association is typically mediated by prenylation, a C-terminal post-translational lipid modification. While small GTPases have been extensively studied in both *in vitro* and *in vivo* cytosolic contexts, it has become increasingly clear that their activity is tightly coupled to membrane interactions. Membranes not only serve as platforms for GTPase signaling but also actively regulate protein function: lipid composition can influence protein localization and activity, proteins can induce lipid domain formation, and reciprocal feedback between proteins and membranes can shape both structural and functional properties.[3−8] These insights highlight the importance of studying prenylated proteins in controlled *in vitro* membrane systems. Further insights into how prenylation affects cellular signaling can also contribute to the development of targeted therapies for major diseases, such as cancer.[9]

The majority of Rho small GTPases undergo prenylation, a C-terminal post-translational modification in which either a farnesyl or geranylgeranyl isoprenoid group is covalently attached.[10−12] This modification occurs at a conserved C-terminal CAAX motif, which consists of a cysteine (C), two aliphatic amino acids (AA), and a terminal residue (X).[13] The identity of the terminal amino acid determines whether the protein is recognized by farnesyltransferase (FTase) or geranylgeranyltransferase type I (GGTase-I), both of which catalyze lipid attachment at the cysteine residue. Upstream of the CAAX motif, most Rho GTPases contain a polybasic region (PBR); together, these elements form the hypervariable region (HVR). The HVR, in combination with the attached lipid group, governs membrane association and subcellular targeting of Rho GTPases.[13−15] Importantly, the identity of the isoprenoid group influences membrane affinity and orientation dynamics, which can lead to functional differences in signaling behavior.[16,17]

Here, we present a convenient and robust method for the *in vitro* prenylation of purified proteins, enabling their use in reconstituted membrane systems to both dissect the molecular mechanisms of cell polarization and build modules that can spatially organize a synthetic cell.

As a model system, we focus on the farnesylation of Rho GTPase Cdc42 from the budding yeast *Saccharomyces cerevisiae*. In *S. cerevisiae*, Cdc42 is a central regulator of polarity establishment. Polarization is initiated by the accumulation of Cdc42 at a specific site on the plasma

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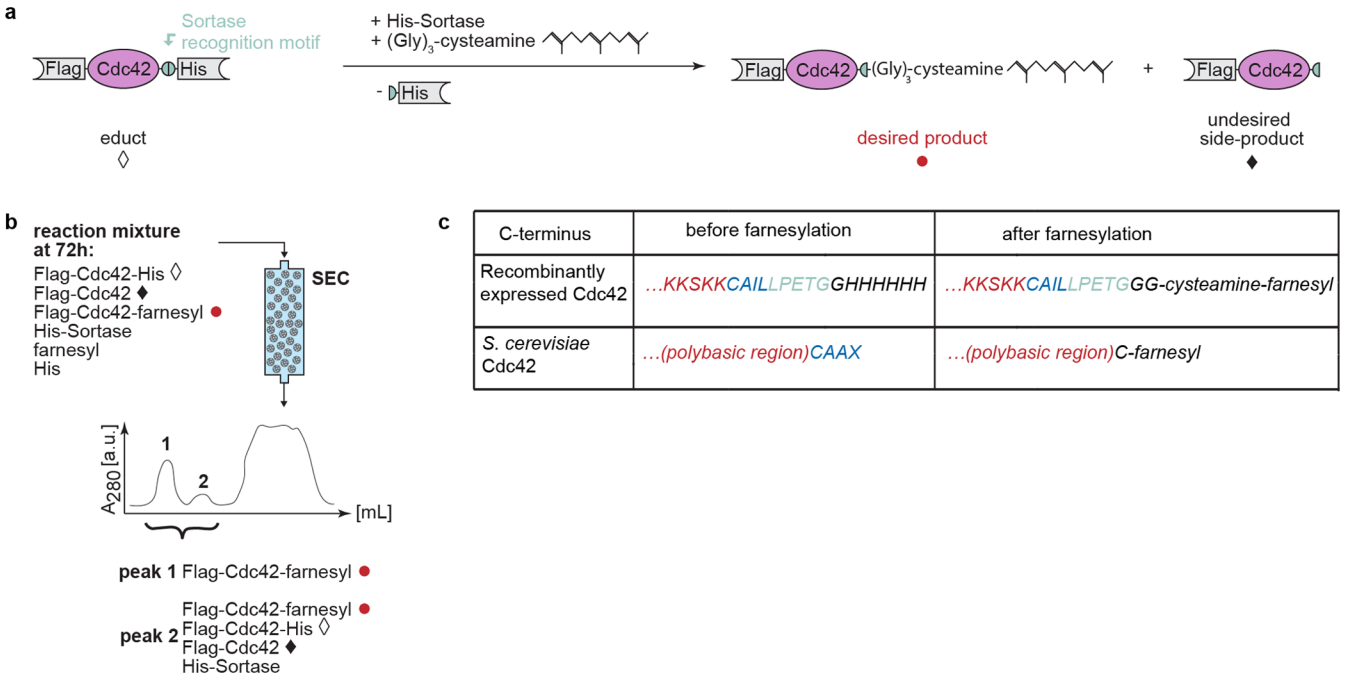

c

C-terminus	before farnesylation	after farnesylation
Recombinantly expressed Cdc42	…KKSKKCAILLPETGGHHHHHH	…KKSKKCAILLPETGGG-cysteamine-farnesyl
S. cerevisiae Cdc42	…(polybasic region)CAAX	…(polybasic region)C-farnesyl

Figure 1. Schematic illustration of the pipeline for sortase-mediated farnesylation of recombinantly expressed Cdc42. (a) Modified Cdc42 construct with the sortase recognition motif flanked with two purification tags. Sortase cleaves at the recognition motif. Sortase ligates the available $(Gly)_3$-cysteamine to the C-terminus. (b) Clean-up procedure for the labeling reaction: after an incubation of 72 h, size exclusion chromatography (SEC) is used to separate unreacted farnesylated triglycine and cleaved purification His-tags from proteins. (c) C-terminal Cdc42 features before and after farnesylation compared between sortase-mediated farnesylation of recombinantly expressed Cdc42 and Cdc42 as occurring in vivo. The C-terminal polybasic region is highlighted in red, CAAX box is highlighted in blue, and the sortase recognition motif is highlighted in green.

membrane, driven by interconnected positive feedback loops.[18,19] Membrane association of Cdc42 depends on prenylation, including both farnesylation and geranylgeranylation.[20,21] Although extensive *in vivo* and *in vitro* studies have elucidated the molecular regulation of Cdc42 activity and its role in polarity establishment,[19,22−25] these have predominantly focused on cytosolic aspects. A major limitation for studying membrane-bound Cdc42 *in vitro* is the availability of prenylated protein as well as precise control over its lipidation state. These constraints hinder the development of minimal synthetic polarity systems and reconstitution approaches, which require chemically well-defined prenylated species, high sample purity, and controlled membrane binding dynamics. Reliable amounts of active protein can be obtained using standard *Escherichia coli*-based recombinant expression systems, but *E. coli* lacks the required enzymatic machinery for prenylation; thus, purification of prenylated Cdc42 cannot be achieved using this method. Other methods that have been used to obtain prenylated Cdc42 are effective but can be difficult to implement for various reasons. The most commonly used method is through insect cell expression systems.[26−32] Insect cell expression systems require cell culturing facilities that are not available at every research location, and purification would result in a mixture of geranylated and farnesylated Cdc42. A second method would be to purify membrane-bound Cdc42 directly from yeast cells.[33,34] The limitation here is that this does not allow for overexpression and results in the purification of a mixture of geranylated and farnesylated Cdc42. For *in vitro*-based prenylation systems, there are two main strategies for obtaining prenylated Cdc42. The first one involves adding purified prenyltransferases[35] to a reaction mixture with purified recombinantly expressed Cdc42. This method does require additional purification of prenyltransferase FTase and/or GGTase-I, which poses their own challenges and can have varying activities across different batches. Lastly, *E. coli* cell-free prenylated protein synthesis systems can be used.[36] Setting up *E. coli* cell-free prenylated protein synthesis systems demands considerable time, especially for establishing efficient purification techniques for prenylated Cdc42. Furthermore, these methods are not always as accessible and reproducible for researchers from a nonbiochemistry group as needed.

To complement these strategies and provide an accessible method for protein prenylation based on commercially available components, we utilize a protein labeling method called "sortagging" or sortase-mediated transpeptidation[37] to prenylate Cdc42. Transpeptidase sortase A, derived from *Staphylococcus aureus*, can label proteins in a precise site-specific manner due to its ability to perform both cleavage and ligation in one step. Sortase A recognizes a LPXTG motif (where L is leucine, P is proline, X is a variable amino acid, T is threonine, and G is glycine) in a substrate, cleaves the bond between threonine and glycine in the motif, and subsequently catalyzes the formation of an amide bond between the threonine of the substrate and the N-terminal glycine of a compatible probe.[38,39] Sortase has almost no requirements for the structure or sequence of the probe as long as it contains an oligoglycine at the N-terminus. Sortagging has been used for many different labeling purposes ranging from labeling with fluorescent dyes,[40] nucleic acids,[41] or lipid chains[42] as well as protein−protein[43] and protein−membrane[44] conjugations both *in vivo* and *in vitro*. Although several studies with lipidation through sortagging have been conducted, this technique has not been applied to the conjugation of farnesyl to proteins thus far.

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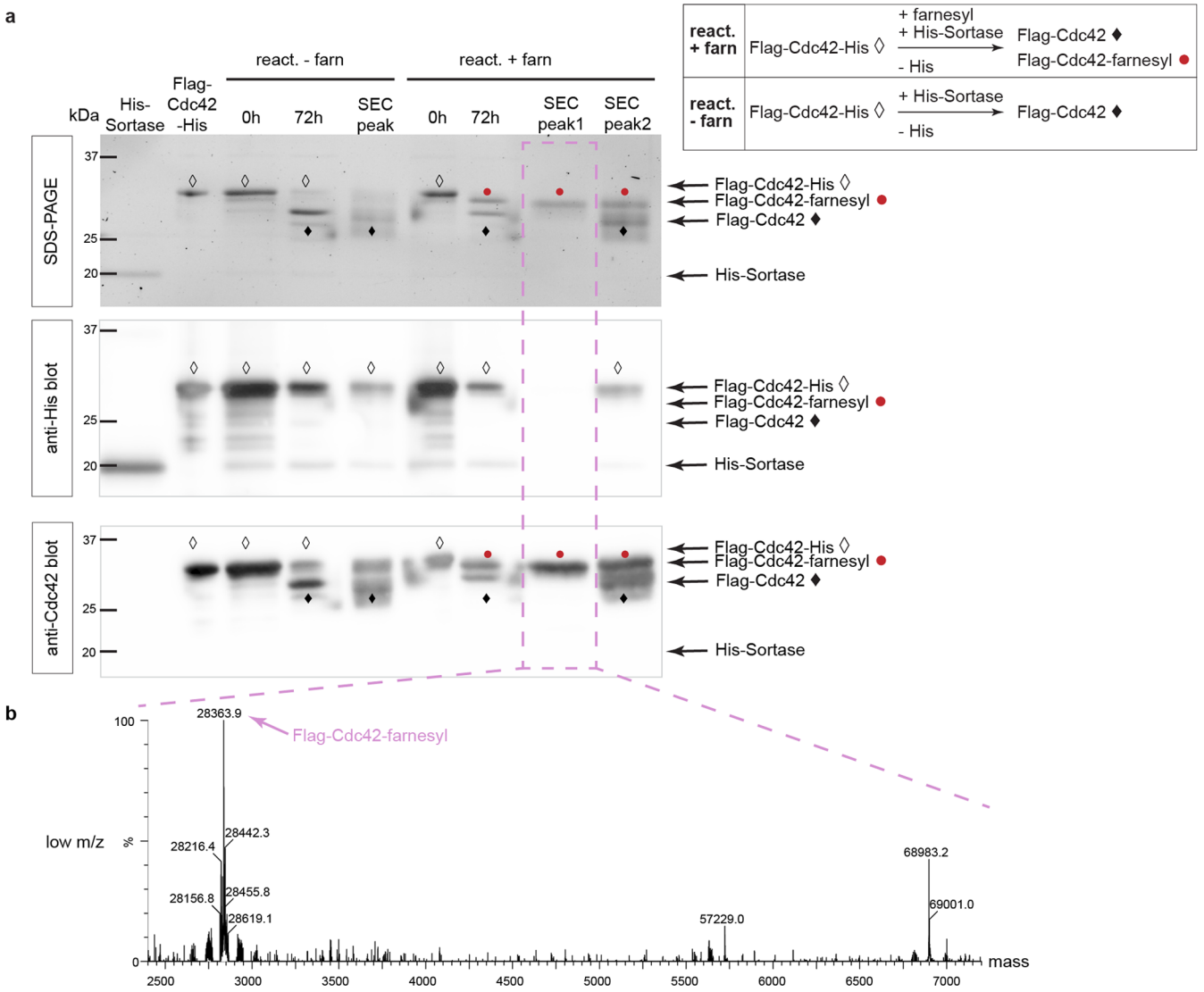

Figure 2. Cdc42 can be farnesylated in a sortase-mediated reaction. (a) SDS-PAGE and Western blot analysis of the separate components of the reaction mixture, the reaction mixture, prereaction (0h), postreaction (72h), and SEC peaks for both a reaction mixture with and without farnesylated triglycine. Western blots performed with anti-His-tag antibodies and anti-Cdc42 antibodies. (b) Deconvoluted mass analysis spectrum of SEC peak 1 after the sortase-mediated farnesylation reaction with Flag-Cdc42-His. Mass analysis was conducted by Max Perutz Laboratories Vienna.

Here, we describe the synthesis of a single-type prenylated protein, farnesylated Cdc42, through a versatile *in vitro* sortase A-mediated reaction and a single-step purification through a size exclusion approach. Our data show that sortase A-mediated reactions are an effective method for farnesylating Cdc42, resulting in functional and active membrane-binding proteins for *in vitro* biophysical and biochemical studies, enabling synthetic biology applications that rely on controllable polarity components.

Pipeline for Sortase-Mediated Farnesylation of Cdc42

To explore approaches to obtain Cdc42 that can bind membranes, we reviewed the literature for existing methods. We describe our experience using these methodologies in the Supporting Information, with which we ultimately did not yield reproducible or interpretable outcomes. Given the constraints of the explored methods, we employed an alternative approach using a sortase-mediated labeling reaction. For the sortase-mediated method, a Cdc42 protein construct compatible with sortase-mediated labeling was designed and purified in *E. coli* by introducing a LTPEG sortase A recognition motif at the C-terminus of Flag-Cdc42-His through recombinant expression. The C-terminus is flanked by an additional glycine and a His_6-purification tag. During the labeling reaction, the sortase enzyme cleaves Cdc42's C-terminal His-tag and ligates farnesylated triglycine to the protein (Figure 1a). Our design incorporates a small linker into the final educt compared to naturally occurring farnesylated Cdc42 from *S. cerevisiae*, composed of parts of the sortase recognition motif and the oligoglycine from the probe between Cdc42's C-terminus and the farnesyl group (Figure 1c).

In this study, H_2N-Gly_3-cysteamine-farnesyl ("farnesylated triglycine") was synthesized and the purity was confirmed through NMR (Figure S6) and the molar mass (452 g/mol) was confirmed through mass analysis (Figure S6) (Max Perutz Laboratories, Vienna). At the starting point of this study, peptides with farnesyl modifications were not yet commercially available, although several companies offer them now, adding

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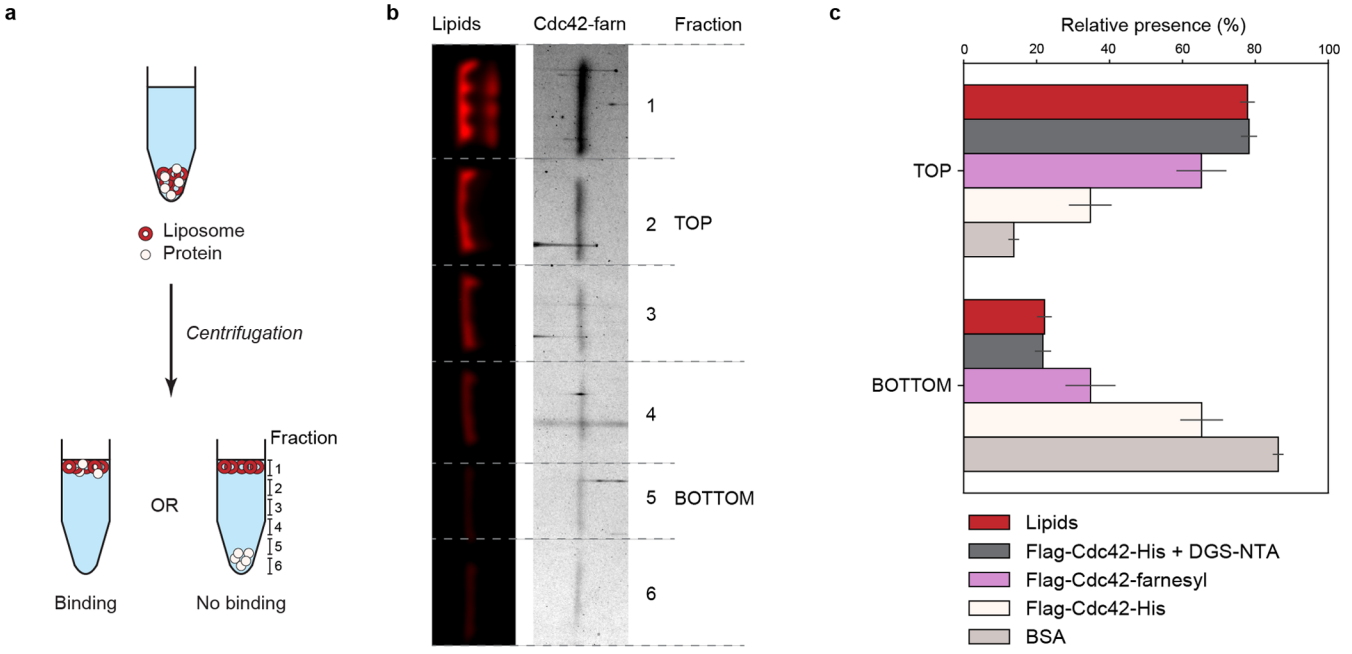

Figure 3. Farnesylated Cdc42 can bind membranes in liposome cofloatation assay. (a) Schematic illustration of the liposome cofloatation assay. (b) Representative example of Flag-Cdc42-farnesyl and liposomes per fraction after ultracentrifugation in cofloatation assay. (Left) SDS-PAGE imaged to visualize fluorescently labeled lipids per fraction. (Right) Silver-stained SDS-PAGE of six fractions to visualize the presence of protein. Full SDS-PAGE images shown in Figure S7. (c) Relative presence of lipids and proteins in the pooled TOP and BOTTOM fractions after the liposome cofloatation assay. Error bars represent the standard error of the mean (n = 6).

to the accessibility and convenience of the sortase-mediated protocol. The protocol further utilizes a commercially available His-tagged sortase mutant with improved catalytic properties,[40] available from BPS Bioscience, for the labeling reactions.

The reaction is carried out at 4 °C for 72 h to balance protein and farnesyl peptide stability and sortagging efficiency, and an excess of farnesyl peptide is added to boost product formation. The final reaction mixture can contain the following three Cdc42 species: the educt of Flag-Cdc42-His (29 kDa); the desired product of Flag-Cdc42-farnesyl (28.5 kDa); and the undesired side product of Flag-Cdc42 (28 kDa). Additionally, sortase (22 kDa) and nonligated farnesylated triglycine can be part of the mixture. The desired product, Flag-Cdc42-farnesyl, can be sorted from the other reaction mixture components through size exclusion chromatography (SEC) (Figure 1b). Further cleanup steps could include His-affinity purification (His-AC), depending on the purity requirements for follow-up experiments.

This pipeline thus utilizes recombinant Cdc42 expressed in *E. coli*, eliminating the need for specialized cell culturing skills and facilities, and leverages commercially available peptides and enzymes combined with straightforward SEC cleanup into an effective method for farnesylating Cdc42.

Cdc42 Can Be Farnesylated in a Sortase-Mediated Reaction

To qualify and quantify the labeling success, we analyzed all reaction and purification steps by SDS-PAGE and anti-His and anti-Cdc42 Western blotting (Figures 2a and S7), providing an accessible method for indirect evidence of farnesylation. Bands that are visible on both anti-His and anti-Cdc42 blots correspond to the educt (Flag-Cdc42-His, diamond); the educt runs at 31 kDa on SDS-PAGE. Bands that are visible on the anti-Cdc42 blot but not on the anti-His blot correspond to farnesylated Cdc42 (red dot) or the undesired side product (Flag-Cdc42, filled diamond). The cleaving of the His-tag and ligation of a farnesyl group can lead to a small distinctive mobility shift[45] on SDS-PAGE despite the small differences in molecular size, enabling a preliminary distinction between the educt, side product, and farnesylated Cdc42.

For a reaction mixture without farnesyl peptide, the 28 kDa band after a 72 h reaction suggests that most protein has been cleaved at the sortase recognition site (Figure 2a). A small fraction of roughly 15% remains uncleaved, based on the 31 and 28 kDa band intensity measurements.

After 72 h of a reaction with all components including the farnesyl peptide, the SDS-PAGE shows three distinct bands: one low-intensity band at 31 kDa (best visible on the anti-His blot) and two bands of similar intensities running at 30 and 28 kDa. The top band is visible on the SDS-PAGE, anti-His blot, and anti-Cdc42, suggesting that this 31 kDa band is Flag-Cdc42-His, meaning that a small fraction of the educt does not get cleaved by sortase. Based on the band intensities, we estimate that only 5% of the educt remains uncleaved. The lowest band running at 28 kDa is visible on the SDS-PAGE and anti-Cdc42 blot and corresponds to the same band as the cleaved product of the control reaction without farnesyl peptide. The middle band, running at 30 kDa, is visible on the SDS-PAGE and anti-Cdc42 blot, but not on the anti-His blot, shows a mobility shift compared to the educt, and is not present in the control reaction without farnesyl peptide, suggesting that the 30 kDa band is the desired Flag-Cdc42-farnesyl (Figure 2a). Based on the band intensities, we estimate a labeling efficiency of roughly 40%.

With SEC, the reaction mixture of the full reaction with farnesyl peptide is separated into three main peaks (Figure S8). Considering the proteins' sizes, SEC separates a mixture of the three Cdc42 species and sortase enzyme in the first two peaks from remaining farnesylated triglycine and cleaved His-tag

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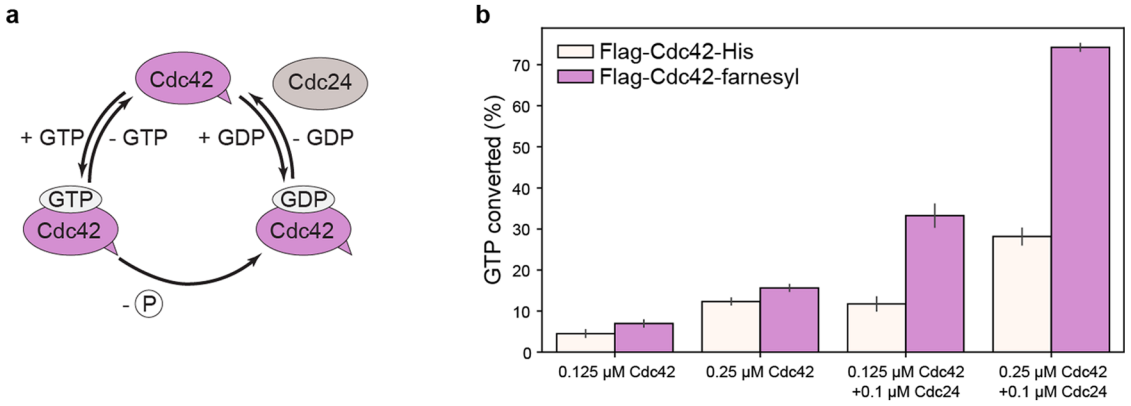

Figure 4. Farnesylated Cdc42 shows tunable GTPase activity. (a) Schematic overview of Cdc42's GTPase activity cycle, adapted from Tschirpke et al.[46] (b) Percentage of converted GTP after 5 h for different protein concentrations with and without Cdc24. Error bars represent the standard error of the mean ($n_{0.125\ \mu M\ Flag\text{-}Cdc42\text{-}His}$ = 9, $n_{0.125\ \mu M\ Flag\text{-}Cdc42\text{-}farnesyl}$ = 18, $n_{0.25\ \mu M\ Flag\text{-}Cdc42\text{-}His}$ = 9, $n_{0.25\ \mu M\ Flag\text{-}Cdc42\text{-}farnesyl}$ = 13, $n_{0.125\ \mu M\ Flag\text{-}Cdc42\text{-}His+Cdc24}$ = 17, $n_{0.125\ \mu M\ Flag\text{-}Cdc42\text{-}farnesyl+Cdc24}$ = 14, $n_{0.25\ \mu M\ Flag\text{-}Cdc42\text{-}His+Cdc24}$ = 25, $n_{0.25\ \mu M\ Flag\text{-}Cdc42\text{-}farnesyl+Cdc24}$ = 10).

peptides in the third peak (Figure 1b). The first peak is visible as a single band on the SDS-PAGE and anti-Cdc42 and corresponds to the previously observed middle band (30 kDa), suggesting that this peak fraction contains only the desired product Flag-Cdc42-farnesyl. The first peak is absent in the control reaction without farnesyl. The second peak fraction contains three bands corresponding to both the bands for Flag-Cdc42-farnesyl and Flag-Cdc42 as well as a third, likely breakdown, product (Figure 2a). Interestingly, the signal on SDS-PAGE for SEC peak 1 was not stronger than that of peak 2 on SDS-PAGE, despite peak 1 having a higher absorbance during SEC. The decrease in the signal implies that the actual labeling efficiency before cleanup steps is higher than 40%. The weaker SDS-PAGE signal for SEC peak 1 may be due to farnesylated Cdc42, adhering to the surfaces of tubes and pipettes, leading to loss during handling. The addition of a detergent can aid the reaction yield, increase protein solubility, and prevent adherence to surfaces after the highly hydrophobic farnesyl peptide has been added. In agreement with the literature, showing that the detergent Brij58 specifically is effective in increasing the solubility of prenylated protein,[36] our method utilizes Brij58 throughout the labeling reaction.

To provide direct evidence of successful labeling, mass analysis was performed: We further cleaned SEC peak 1 using His-AC to further remove all His-tagged components (His-Sortase, Flag-Cdc42-His) from the sample. The His-AC flow-through samples was prepared for mass analysis (Max Perutz Laboratories, Vienna), which confirmed Flag-Cdc42-farnesyl (molar mass: 28364.95 Da) to be the main component in the first SEC peak (Figure 2b) and its weaker presence in the second SEC peak (Figure S9).

Taken together, our data demonstrate that the presented pipeline provides an efficient method for successful sortase-mediated farnesyl labeling of Cdc42.

Farnesylated Cdc42 Can Bind Membranes

To check whether the prenylated Cdc42 had functional membrane binding, we carried out a liposome cofloatation assay. Here, the liposome-bound protein is distinguished from the unbound protein by ultracentrifugation through a sucrose gradient (Figure 3a). Protein was mixed with fluorescently labeled neutrally charged liposomes (99.99% DOPC, 0.01% Liss Rhod PE unless otherwise stated) into the first layer of high sucrose density. After ultracentrifugation, six fractions per sample were analyzed for the presence of both liposomes and protein by imaging the fluorescence signal of the lipids and the protein band intensity of silver-stained SDS-PAGE, respectively (Figures 3b and S7). The top and bottom three fractions were pooled into a TOP and BOTTOM fraction after the quantification of the band intensity of the six separate fractions.

As a positive control for membrane binding, liposomes were supplemented with 20% DGS-NTA, ensuring a strong interaction between the liposomes and the His-tagged protein. Flag-Cdc42-His comigrated with the DGS-NTA liposomes in the cofloatation assay, confirming that if it is membrane-bound, Cdc42 will also show abundantly in the top fractions on SDS-PAGE (Figure 3c). Control experiments with BSA result in a comigration of only 14% to the top fractions, suggesting that unbound Cdc42 will not show abundantly in the top fractions on SDS-PAGE but that a small percentage of protein will comigrate even in the absence of membrane binding (Figure 3c). Flag-Cdc42-His samples have an average comigration of 35% to the top fractions (Figure 3c). The increased comigration compared to nonbinding protein BSA samples shows that even without a farnesyl modification, Cdc42 can weakly bind membranes. For Flag-Cdc42-farnesyl, we observe a comigration of 60% of the protein to the top fractions (Figure 3c); thus, the farnesylated protein has a strong increase in membrane binding compared to its unfarnesylated educt. Flag-Cdc42-farnesyl shows a lower presence in the top fractions compared to the positive control with DGS-NTA liposomes with Flag-Cdc42-His. Although the liposome cofloatation assay cannot be conclusive about the exact membrane binding dynamics of the tested proteins, it is expected that farnesylated Cdc42 will have a lower binding strength than the strong interaction between Nickel and His, given that *in vivo* Cdc42 is transiently bound to the plasma membrane.[46] The sortase-mediated farnesylated Cdc42 binds membranes, suggesting that the linker region between the protein's C-terminus and the farnesyl group does not impede membrane interaction.

GTPase Activity of Farnesylated Cdc42 Is Enhanced by Cdc24

Next, we investigated the effects of our farnesylation method on the GTPase activity of Cdc42 to confirm that the regulatory potential of Cdc42 was not impaired. Cdc42 regulates the hydrolysis of GTP in a cyclic three-step process, of which

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hydrolysis of GTP to GDP can be boosted by the guanine nucleotide exchange factor Cdc24[47] (Figure 4a). We used the GTPase Glo assay (Promega), an assay to quantify the *in vitro* bulk properties of the entire GTPase cycle to measure the GTPase activity of our farnesylated Cdc42 and its educt in the absence of vesicles.[47] We measured the end-point amount of remaining GTP after the incubation of different protein concentrations in the presence of GTP with and without the effector Cdc24. After a 5 h incubation of proteins and GTP, the reaction is quenched and the remaining GTP is converted into luminescent ATP. The measured luminescence per sample is normalized and transformed into the percentage of converted GTP.

Our earlier work shows that the Flag-Cdc42-His protein shows reasonable activity *in vitro* and that its GTPase activity can be enhanced by Cdc24.[47] After 5 h, Flag-Cdc42-farnesyl shows comparable amounts of converted GTP as Flag-Cdc42-His for both tested concentrations with a roughly twofold decrease in activity for a twofold decrease in protein concentration (Figure 4b). This indicates that both the farnesylated protein and its educt show an expected concentration dependence in GTPase activity.

The similarities in the measured intrinsic Cdc42 GTPase activity further suggest that the protein stability of farnesylated Cdc42 was not impaired by the addition of the farnesyl peptide compared to the unfarnesylated Cdc42 for time scales up to 5 h.

The addition of purified Cdc24 increased the activity of both Flag-Cdc42-farnesyl and Flag-Cdc42-His. For the educt Flag-Cdc42-His, addition of the Cdc24 increases the activity of the high- and low-concentration Cdc42 by roughly 2.5 times for both tested concentrations (Figure 4b). For Flag-Cdc42-farnesyl, the activity is boosted by a factor of 4.8 for both concentrations (Figure 4b). The increase in GTP-to-GDP conversion shows that the farnesylated Cdc42 is sensitive to the enhancement of activity through the Cdc24. It is surprising that farnesylated Cdc42 seems more susceptible to Cdc24 activity than the unfarnesylated protein. This could point to a novel regulatory interaction between Cdc24 and the farnesyl moiety, but it may also arise from an assay artifact as the great acceleration of nucleotide cycling in the presence of Cdc24[48] makes the assay sensitive to small variations in concentration.

Although the presented GTPase assay cannot fully explain the surprising difference in the enhancement of activity by Cdc24 for the farnesylated protein compared to its educt, it does shows that the farnesylated Cdc42 has tunable GTPase activity.

DISCUSSION

In this study, we utilized a sortase-mediated reaction to farnesylate Cdc42. We show effective C-terminal labeling of recombinantly expressed Cdc42 utilizing versatile, commercially available components.

We adopted an indirect approach for detection of the farnesylated product by blotting for all Cdc42 species, including the educt, and used a control reaction to identify the undesired side product. Visualization on SDS-PAGE and blots allowed us to exclude bands corresponding to non-farnesylated Cdc42 species and accurately identify the farnesylated product (Figure 2). A more straightforward approach could be to implement an antifarnesyl blotting protocol, which, if reliably developed, could significantly benefit the wider scientific community. However, previously used Western blotting protocols proved ineffective.[49,50] Alternatively, using a fluorescently labeled farnesylated triglycine, as described by Kai et al.,[36] would allow us to directly detect and measure the formation of the farnesylated Cdc42. To adopt a more direct approach of protein identification, we verified the presence of farnesylated Cdc42 by using mass spectrometry (Figure 2).

In addition to the challenges of detecting farnesylated Cdc42, working with it without significant loss is difficult due to the characteristics of the strongly hydrophobic farnesyl group. We suspect that a substantial portion of the farnesylated Cdc42 is lost during the labeling and purification processes as is suggested by the observation that despite high absorbance during SEC, the gel of SEC peak 1 (containing the majority of farnesylated Cdc42) shows only a faint signal (Figure 2). We found that using low-binding consumables and the detergent Bij58 helped reduce the overall loss of farnesylated Cdc42. Given these challenges, we believe that an overall farnesylation yield of ∼40% after all purification steps is high.

We opted for the sortase enzyme to mediate the farnesylation, which is versatile, commercially available, and easily purified (a process supported by well-established purification protocols).[38,41,42,51] Given the versatility of sortase-mediated labeling reactions, our method could be adapted to mediate prenylation of other Rho small GTPases with little effort. Other GTPases can be prepared for the sortase-mediated labeling method by recombinantly expressing a protein construct with an appended sortase recognition site at the C-terminus. Another interesting modification to our protocol would be to use a triglycine-geranylgeranyl group to produce geranylgeranylated protein through sortase-mediated labeling.

Our approach appends a sortase recognition motif downstream of the CAAX box, resulting in a 10-amino acid linker (Figure 1). We demonstrate that this linker does not impair membrane binding or GTPase activity, indicating that Cdc42 remains functionally intact despite the modification. Consistent with this, our previous work showed that neither C-terminal tags nor extended unstructured C-terminal regions affect Cdc42 GTPase activity or its interaction with Cdc24.[52] Here, we confirm these findings for farnesylated Cdc42 using a GTPase activity assay, which reveals robust intrinsic activity and stimulation by Cdc24 for both farnesylated and unmodified protein.

However, while overall functionality is preserved, subtle quantitative changes in protein−protein and protein−substrate interactions cannot be excluded. Consequently, this farnesylation strategy may not be ideally suited for directly translating *in vitro* rate measurements to *in vivo* conditions, for example, due to the potential effects of the linker on the protein's distance from the membrane. These effects could be minimized by shortening the linker to seven amino acids, for instance, by reducing the CAAX box to the cysteine residue and appending the sortase motif immediately downstream.

Our farnesylated Cdc42 shows strong membrane recruitment with only a small fraction of spontaneous unbinding in the liposome cofloatation assay. This is consistent with the strong binding observed on supported lipid bilayers in two recent *in vitro* studies, in which Cdc42 was prenylated either via the addition of GGTase[6] or with an *E. coli* cell-free protein synthesis system.[36] The exact binding kinetics of the differently produced prenylated proteins cannot be compared directly as the cofloatation assay captures binding at a single time point.

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Unbinding of prenylated Cdc42 is known to be strongly enhanced by the guanine nucleotide dissociation inhibitor (GDI).[31] Direct observation of the GDI-mediated unbinding, as shown in the two studies mentioned above,[6,36] could provide further insights into how different prenylation methods affect the full cycle of binding and unbinding behavior, as well as associated protein−protein interactions. We show in this study that a different protein−protein interaction, between Cdc42 and its guanine nucleotide exchange factor Cdc24, continues to result in an enhancement of GTPase activity after farnesylation. The observed difference in enhancement by Cdc24 between farnesylated and unfarnesylated proteins shows the relevance of studying the biophysical and chemical properties of Rho small GTPases that have been prenylated. Systematic comparison of these protein properties across different prenylation techniques, given their advantages and disadvantages as discussed in the Introduction, could help further explore how *in vitro* prenylation shapes membrane association and regulatory protein interactions.

Membrane composition can have a large influence on binding dynamics and was, in this study, chosen to be very minimalistic. Although other reports[31,33] show the importance of other negatively charged lipids for reliable Cdc42 membrane binding, we have observed strong binding with a simple two-component artificial membrane.

This study presents a sortase-mediated method for the *in vitro* prenylation of Cdc42, yielding a functional protein that retains membrane binding and GTPase activity. The method is readily adaptable to produce geranylgeranylated Cdc42 and to prenylate other Rho small GTPases. The ability to generate purified Rho GTPases with a controlled prenylation state expands the experimental toolkit for bottom-up *in vitro* studies, enabling a systematic investigation of how bidirectional regulation between Rho GTPases and lipids gives rise to self-organized spatial signals. In addition, these well-defined components provide a foundation for modules that spatially organize synthetic cells.

METHODS

Plasmid Construction

The gene of interest (Cdc42) was obtained from the genome of *S. cerevisiae* W303 and was amplified through PCR. The target vector was also amplified through PCR. Additionally, each PCR incorporated a small homologous sequences needed for Gibson assembly.[53] After Gibson assembly, the resulting mixture was used to transform chemically competent Dh5α and BL21 DE::3 pLysS cells and plated onto a Petri dish containing lysogeny broth agar and the correct antibiotic marker. A list of all protein constructs and plasmids used throughout this publication is stated in Table 1. Construction routes of plasmids and primers used for each PCR, as well as the amino acid sequences of the proteins used in this publication, are listed in Figure S10 and table S1. The plasmid for Cdc24-His was received from D. McCusker (University of Bordeaux) (published in Rapali et al.[34]).

Table 1. List of Protein Constructs/Plasmids Used throughout This Publication

plasmid	description	source
pET28a-His-mcm10-Sortase-Flag	template for pRV073	received from N. Dekker (TU Delft), based on pBP6 in Douglas and Diffley[54]
pRV073	flag-Cdc42-His	this work
pDM272	Cdc24-His	received from D. McCusker (University of Bordeaux) (published in Rapali et al.[34])

Buffer Composition

If not mentioned otherwise, buffers are of the composition stated in Table 2.

Table 2. Buffer Composition

Buffer	Composition
lysis buffer	50 mM Tris-HCl (pH = 8.0), 1 M NaCl, 5 mM imidazole, 1 mM 2-mercaptoethanol, supplemented with an EDTA-free protease inhibitor cocktail (Roche), and 1 mM freshly prepared PMSF
His-AC washing buffer	50 mM Tris-HCl (pH = 8.0), 1 M NaCl, 5 mM imidazole, and 1 mM 2-mercaptoethanol
His-AC elution buffer	50 mM Tris-HCl (pH = 8.0), 100 mM NaCl, 500 mM imidazole, and 1 mM 2-mercaptoethanol
SEC buffer	50 mM Tris-HCl (pH = 7.5), 100 mM NaCl, 10 mM MgCl2, and 1 mM 2-mercaptoethanol

Protein Expression and Purification

Flag-Cdc42-His (pRV073) was expressed in the Bl21:DE3 pLysS cells. Cells were grown in lysogeny broth at 37 °C until an OD600 of 0.7. The expression was induced through the addition of 1.0 mM IPTG, after which cells were grown for 3 h at 37 °C. Cells were harvested through centrifugation. Cell pellets were resuspended in lysis buffer and lysed with a high-pressure homogenizer (French press cell disruptor, CF1 series Constant Systems) at 4 °C using 5−10 rounds of exposing the sample to pressurization. The cell lysate was centrifuged at 37,000*g* for 30 min and the supernatant was loaded onto a HisTrap excel column (Cytiva). After several rounds of washing with His-AC washing buffer, the protein was eluted in a gradient of His-AC washing buffer and His-AC elution buffer. The protein was dialyzed twice in SEC buffer. After the addition of 10% glycerol, samples were flash frozen in liquid nitrogen and kept at −80 °C for storage.

Cdc24 (pDM272) was expressed and purified following the same steps as for Cdc42 with modification mentioned hereafter based on the protocol in Rapali et al.[34] (1) The expression was induced through the addition of 0.2 mM IPTG, after which cells were grown for 18 h at 18 °C. (2) After His-AC elution, the sample was further purified by size exclusion chromatography using SEC buffer and a HiPrep 16/60 Sephacryl S-300 HR (Cytiva) column. Fractions containing full-size protein were concentrated using AmiconUltra 4 mL centrifugal filters (Merck). After the addition of 10% glycerol, samples were flash-frozen in liquid nitrogen and kept at −80 °C for storage.

Note on His-AC: HisTrap excel (Cytiva) columns bought by 2020 or later required a higher amount of imidazole in the lysis and washing buffer as stated in Table 2, as indicated by the recommendation "use 20−40 mM imidazole in the sample and binding buffer for the highest purity" on the column package. For these columns, the amount of imidazole in lysis and His-AC washing buffer was increased to 50 mM.

Synthesis of H_2N-Gly_3-Cysteamine-Farnesyl ("Farnesylated Triglycine")

General Information. Boc-Gly_3−OH was purchased from Bachem. DCM, DMF, PyBOP, DiPEA, cysteamine hydrochloride, farnesyl bromide, TFA, 7 M NH_3 in MeOH, and MeOH were purchased from Sigma-Aldrich. DMSO-d_6 was purchased from Eurisotop. Deionized (Milli-Q) water was made in our laboratory. Unless stated otherwise, all chemicals were used as received. For all synthetical steps, anhydrous solvents were used. NMR spectra were recorded on an Agilent-400 MR DD2 (399.67 MHz) instrument. Measurements were taken at 298 K.

Synthesis. An overview of the synthesis steps is given in Figure 5.

Boc-Gly_3-Cysteamine. Synthesis proceeded according to a modified procedure of Agarwal et al.[55] Boc-Gly_3OH (579 mg, 2.0

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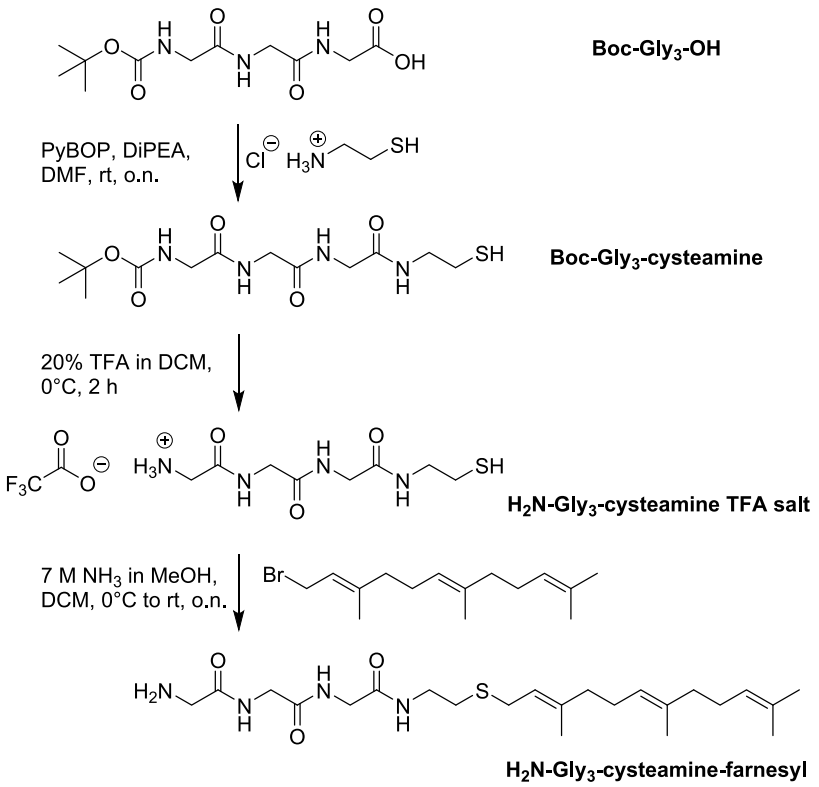

Figure 5. General synthetic procedure going from Boc-triglycine to the C-terminally farnesylated triglycine derivative ("farnesylated triglycine"). Abbreviations: rt: room temperature and o.: overnight.

mmol, 1.0 equiv) was dissolved in anhydrous DMF (7.0 mL) under argon. PyBOP (1.56 g, 3.0 mmol, 1.5 equiv) and anhydrous DiPEA (1.75 mL, 10.0 mmol, 5.0 equiv) were added, and the clear solution was stirred for 10 min at room temperature upon which it turned yellow. Cysteamine hydrochloride (455 mg, 2.0 mmol, 2.0 equiv) was added upon which the solution turned colorless again. The reaction mixture was stirred overnight under argon at room temperature. The crude reaction mixture was concentrated *in vacuo*, coevaporated with heptane, and crystallized from MeCN to obtain Boc-Gly_3-cysteamine (210 mg, 0.60 mmol, 30%) as a colorless solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.10 (dt, J = 12.5, 5.9 Hz, 2NH), 7.90 (d, J = 5.9 Hz, 1NH), 7.01 (t, J = 5.6 Hz, 1NH), 3.73 (d, J = 5.5 Hz, 2H), 3.67 (d, J = 5.8 Hz, 2H), 3.58 (d, J = 5.8 Hz, 2H), 3.21 (q, J = 6.5 Hz, 2H), 2.53 (m, 2H, note: partly overlapping with DMSO signal), 2.38 (t, J = 8.2 Hz, 1SH), 1.38 (s, 9H).

H_2N-Gly_3-Cysteamine TFA Salt. Boc-Gly_3-cysteamine (50.0 mg, 143.5 μmol, 1.0 equiv) was placed under argon (Schlenk tube) and cooled to 0 °C (ice bath). A degassed solution of TFA (1 mL, 20% v/v in anhydrous DCM) was separately prepared under argon, cooled to 0 °C (ice bath), and added to the Boc-protected starting material. The reaction mixture was stirred for 2 h at 0 °C (ice bath). The solvent was removed via cold distillation by subjecting the mixture to a fine vacuum at 0 °C (ice bath), while stirring to obtain the crude NH_2-Gly_3-cysteamine TFA salt, which was directly used without further purification. Note: We found the use of degassed solvents and removal under reduced pressure at lower temperatures to be essential to avoid oxidation of the free thiol group. Furthermore, it is crucial to remove the Boc group before introducing the farnesyl residue due to its high lability toward acids.[56]

H_2N-Gly_3-Cysteamine-Farnesyl. Synthesis proceeded according to a modified procedure of Cini et al.[57] Upon removal of DCM and TFA by vacuum distillation, the crude NH_2-Gly_3-cysteamine TFA salt (see above; 143.5 μmol, 1.0 equiv) was directly placed under argon (Schlenk tube) at 0 °C (ice bath) and subsequently dissolved in a mixture of anhydrous DCM (400 μL) and ammonia (7 M in MeOH, 513 μL, 3.59 mmol, 25.0 equiv). Farnesyl bromide (58.4 μL, 215 μmol, 1.5 equiv) was added, and the reaction mixture was stirred overnight and allowed to warm to room temperature under argon. The reaction mixture was concentrated *in vacuo* (room temperature), before suspending the crude product in a mixture of 1-butanol (15 mL) and H_2O (15 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated *in vacuo* (room temperature) to obtain a crude orange oil. The residue was redissolved in anhydrous MeOH (15 mL) and washed with hexane (20 mL). The methanol phase was concentrated *in vacuo* (room temperature), redissolved in anhydrous dioxane (10 mL), and lyophilized to obtain H_2N-Gly_3-cysteamine-farnesyl as a thick orange solid. Note: The compound was directly subjected to analysis and no further purification was attempted due to the labile nature of the farnesyl residue, which is well studied.[56] ^{1}H NMR (400 MHz, DMSO) δ 8.63 (t, J = 5.7 Hz, 1NH), 8.25 (t, J = 5.9 Hz, 1NH), 8.08 − 7.96 (m, 2NH), 7.79 (s, 1NH), 5.28 − 5.14 (m, 1H), 5.13 − 5.01 (m, 2H), 3.84 (d, J = 5.7 Hz, 1H), 3.69 (d, J = 5.8 Hz, 1H), 3.61 (s, 1H), 3.35 (m, 4H, note: partly overlapping with the H_2O signal), 3.27 − 3.19 (m, 1H), 3.15 (d, J = 7.7 Hz, 1H), 3.03 (s, 1H), 2.50 (m, 2H, note: partly overlapping with the DMSO signal), 2.13 − 1.87 (m, 8H), 1.63 (s, 6H), 1.56 (s, 6H).

Sortase-Mediated Farnesylation

A step-by-step description of the sortase A-mediated farnesylation reaction and cleanup by SEC is described in Tschirpke et al.[58,60] In brief, sortase A (Octamutant, BPS Bioscience) was incubated with Cdc42 and farnesylated triglycine (dissolved in DMSO) at 2:100:10,000 molar ratio in labeling buffer (204 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 20 mM $CaCl_2$, 1 mM 2-mercaptoethanol, 1% Brij58 (Surfact-Amps 58, Thermo Scientific), supplemented with 2 mM freshly prepared dithiothreitol, with a final DMSO content of 22%) for 72 h at 4 °C. The sample was spun for 5 min at 13,000 × g, after which precipitated protein was removed. Reaction mixtures were gel-filtrated on a HiPrep 16/60 Sephacryl S-300 HR column (Cytiva) equilibrated with SEC buffer that was supplemented with 0.1% Brij58 (Surfact-Amps 58, Thermo Scientific). Peak fractions were pooled, and after the addition of

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Table 3. Antibodies Used for Western Blotting

blot	primary antibody	secondary antibody
anti-His	his tag antibody, mouse (OAEA00010, Aviva Systems Biology) (dilution: 1:4000)	IgG2b Antibody HRP-conjugated (Goat Anti-Mouse) (OASA06620, Aviva Systems Biology) (dilution: 1:1000)
anti-Cdc42	anti-Cdc42 antibody, Rabbit (ab64533, ab- cam) (dilution: 1:2000)	polyclonal goat anti-rabbit immunoglobulins/HRP (goat anti-rabbit) (P0448, Agilent Dako) (dilution: 1:500)

10% glycerol, samples were flash-frozen in liquid nitrogen and kept at −80 °C for storage.

Mass Analysis of Farnesylated Cdc42

To further purify the samples for mass analysis, SEC peak fractions were loaded onto a HisTrap Excel column (Cytiva). The flow-through was loaded again and this cycle was repeated for a total of three times. After the last cycle, the flow-through was concentrated (Amicon Ultra Centrifugal Filter, 10 kDa MWCO, Millipore) and flash-frozen in liquid nitrogen for storage.

For mass analysis, samples were dialyzed in an SEC buffer. Samples were analyzed using intact protein mass spectrometry analysis, carried out by Max Perutz Labs Vienna: Protein samples were diluted directly to 10 ng/μL with H_2O or in the case of reduction-diluted 1:10 with 1 mM TCEP, incubated for 30 min at RT, and then diluted to 10 ng/μL. Up to 2 μL (20 ng) was loaded on an XBridge Protein BEH C4 column (2.5 μm particle size, dimensions: 2.1 × 150 mm; Waters) using a Vanquish Horizon UHPLC system (Thermo Fisher Scientific) with a working temperature of 50 °C, 0.1% formic acid as solvent A, 99.92% acetonitrile, and 0.08% formic acid as solvent B. The gradient started with 12% solvent B and ramped in 5 min to 40% solvent B, in 1 min from 40 to 64% solvent B, and in 4 min from 64 to 72% solvent B at a flow rate of 250 μL/min. The eluting proteins were analyzed on a Synapt G2-Si instrument coupled via a ZSpray ESI source (Waters). MS1 spectra were recorded with MassLynx V 4.1 software (Waters) in positive polarity and resolution mode in an m/z range of 600−2000. Every 30 s, the peptide GluFib was recorded over the LockSpray and used for internal calibration of the spectra. Data were analyzed using the MaxEnt 1 process to reconstruct the uncharged average protein mass. Mass spectrometry analysis as described here was also performed on samples dialyzed in SEC buffer supplemented with 0.1% DDM and SEC buffer supplemented with 0.1% CHAPS, which led to the same results.

SDS-PAGE

Proteins were analyzed using freshly prepared SDS-PAGE gels (12% acrylamide). In brief, a solution of 375 mM Tris-HCl (pH 8.8), 30−37.5 v/v% 40% acrylamide solution (Bio-Rad), 0.2 w/v% sodium dodecyl sulfate (Sigma-Aldrich), 0.5 v/v% 2,2,2-trichloroethanol (Sigma-Aldrich), 0.1 w/v% ammonium persulfate (Sigma-Aldrich), and 0.1 v/v% *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine (Sigma-Aldrich) was prepared and cast into 1.00 mm mini-protean glass plates (Bio-Rad), filling them up to 80%. To protect the gel surface from drying, a layer of isopropyl alcohol (Sigma-Aldrich) was added. The gel was allowed to solidify for 20 min, after which the isopropanol layer was removed. A solution of 155 mM Tris-HCl (pH 6.5), 10 v/v% 40% acrylamide solution (Bio-Rad), 0.2 w/v% sodium dodecyl sulfate (Sigma-Aldrich), 0.1 w/v% ammonium persulfate (Sigma-Aldrich), and 0.1 v/v% *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine (Sigma-Aldrich) was prepared and added to the existing gel layer, after which a well comb (Bio-Rad) was added. The gel was let to solidify for 20 min. Cell and protein samples were mixed with the SDS loading buffer (Laemmli buffer[59]). Before loading onto the SDS-PAGE gels, they were kept for 5 min at 95 °C. Gels were run for 5 min at 130 V followed by 55 min at 180 V (PowerPac Basic Power Supply (Bio-Rad)). Imaging was done on a ChemiDoc MP (Bio-Rad) using the "Stain-Free Gels" feature and automatic exposure time determination. Precision Plus Protein Unstained (Bio-Rad) was used as the protein standard.

Western Blotting

After SDS-PAGE, the sample was transferred from the SDS-PAGE to a blotting membrane (Trans-Blot Turbo Mini 0.2 μm PVDF Transfer Pack, Bio-Rad) using the "Mixed MW" program of the Trans-Blot Turbo Transfer System (Bio-Rad). The blotting membrane was incubated with an Immobilon signal enhancer (Millipore) at 4 °C for 18 h. The blotting membrane was incubated with the primary antibody, diluted in the Immobilon signal enhancer (Table 3), at room temperature for 1 h. It was washed three times with TBS-T (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1 v/v% Tween-20 (Sigma-Aldrich)). For each washing step, the blotting membrane was incubated with TBS-T at room temperature for 20 min. The blotting membrane was incubated with the secondary antibody, diluted in the Immobilon signal enhancer (Table 3), at room temperature for 1 h, after which it was again washed thrice with TBS-T. The SuperSignal West Pico Mouse IgG Detection Kit (Thermo Scientific) was used for activation. Imaging was done on a ChemiDoc MP (Bio-Rad) using the "Chemi Sensitive" feature and automatic exposure time determination. Precision Plus Protein Unstained Standard (Bio-Rad) and Precision Plus Protein All Blue Standard (Bio-Rad) were used as protein standards.

Silver Staining

The cofloatation gels were silver-stained for better visualization using the basic protocol of the SilverQuest Silver Staining Kit (Thermo Scientific). Volumes used for all solutions were 100 mL per gel unless mentioned otherwise. After SDS-PAGE, the sample was washed with ultrapure water and left in a fixative solution (40% v/v ethanol, 10% v/v acetic acid) overnight. The sample was then washed in 30% v/v ethanol for 10 min and incubated with sensitizing solution for 10 min followed by two washes of 10 min with first 30% v/v ethanol and next ultrapure water. Then, the sample is incubated for 15 min with the staining solution and briefly washed with ultrapure water followed by an incubation of 10 min in the developing solution until bands started to appear. The staining was then stopped by the addition of 10 mL of stopper per gel and incubated on a shaker for 10 min. The sample is then washed in ultrapure water and imaged on a ChemiDoc MP (Bio-Rad) using the "Silver Stain Gel" feature and "optimal autoexposure" time determination.

Liposome Cofloatation Assay

Based on the protocol described by Meca et al.,[22] lipid stocks of 10 mg/mL in chloroform were mixed into 200 μL of chloroform in a molar ratio of 99.99 DOPC:0.01 Liss Rhod PE (DOPC: 1,2-dioleoyl-sn-glycero-3-phosphocholine, Liss Rhod PE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl), Avanti Polar Lipids) to a final concentration of 5 mg/mL in 300 μL of 0% sucrose buffer (20 mM Tris (pH = 7.5), 150 mM NaCl). The lipid solution was then gently dried under a N_2 air stream and left in a desiccated form for 1 h. Lipids were then rehydrated with 300 μL of liposome buffer (20 mM Tris (pH = 7.5), 150 mM NaCl) by vortexing three times for 30 s followed by a 30 resting period. Five cycles of freeze−thaw were applied in liquid nitrogen alternated with a 45 °C water bath after which the lipids were bath-sonicated for 15 min. After liposome formation, 0.1% Brij58(Surfact-Amps 58, Thermo Scientific) was added to the mixture to match the detergent concentration of the protein samples. GTP is then added to the protein to a final concentration of 300 mM GTP, which is about 10,000-fold excess with the protein. All protein was for this assay was handled with Protein LoBind tubes (Eppendorf) and low-binding TripleA pipet tips (Westburg) to prevent protein loss due to the hydrophobic prenylation sticking to the plastics. 17 μL of the protein−GTP mixture was then added to 38 μL of liposomes. A small scoop, about one-third of the total sample volume, was placed in the tubes and left overnight on a turntable at 4 °C to take up the detergent. 40 μL of the mixture was carefully pipetted out, without

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taking the biobeads, and incubated for 30 min at 30 °C to promote protein−liposome binding. Then, a 60% sucrose buffer (20 mM Tris (pH = 7.5), 150 mM NaCl, 60% w/v sucrose) was mixed into a final sucrose content of 30% w/v. This was overlaid by 80 μL of 25% sucrose buffer (20 mM Tris (pH = 7.5) and 150 mM NaCl, 25% w/v sucrose) and finally overlaid by 80 μL of 0% sucrose buffer (20 mM Tris (pH = 7.5), 150 mM NaCl), resulting in a sucrose density gradient with three layers. The samples were then centrifuged for 30 min at 42,000 rpm at 4 °C (Optima L-90K, Beckman Coulter). After centrifugation, the three layers were separated through carefully pipetting six 40 μL fractions from the top of the sample and mixing with SDS loading buffer (Laemmli buffer[59]) for further analysis on SDS-PAGE. The lipids were visualized by imaging the SDS-PAGE with a GE Amersham Typhoon gel imager, and the protein presence per fraction was determined by measuring band intensity on silver-stained SDS-PAGE. Band intensity was normalized to "relative presence(%)" as follows

$$\text{relative presence (\%)} = \sum_{i=1-3 \text{ or } 4-6} \frac{\text{band intensity}_i}{\sum_{j=1-6} \text{band intensity}_j} \quad (1)$$

GTPase Assay

Based on the protocol described by Tschirpke et al.,[47] the GTPase Glo assay (Promega) was used. All proteins are dialyzed and diluted in SEC buffer (Table 2) supplemented with 0.1% Brij58 (Surfact-Amps 58, Thermo Scientific). 5 μL of protein was mixed in with 5 μL of GTP solution (10 μM GTP, 50 mM Tris-HCl (pH = 7.5), 100 mM NaCl, 10 mM MgCl2, 1 mM 2-mercaptoethanol (Sigma-Aldrich), 1 mM dithiothreitol) and incubated for 5 h at 30 °C on an Innova 2300 platform shaker (New Brunswick Scientific) (120 rpm). Then, the reaction is quenched by adding 10 μL of Glo buffer to convert the remaining GTP to ATP during a 30 min incubation. Then, 20 μL of detection reagent is added, making the ATP luminescent, and the resulting luminescence is measured on a plate reader (Synergy HTX reader, BioTek). Wells without protein containing the used buffer were then used to calculate the percentage of converted GTP as follows

$$\text{GTP}_{\text{converted}} = \left(1 - \frac{\text{luminescence protein}}{\text{luminescence buffer}}\right) \times 100 \quad (2)$$

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this publication are openly available at https://data.4tu.nl at doi.org/10.4121/b044bbb9-edf3-476a-b729-e3252bd7ad6d (CC BY-SA 4.0).[60]

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.6c00136.

Overview of methods for obtaining membrane-binding Cdc42, ^{1}H NMR spectra of farnesylated triglycine synthesis steps and mass conformation of farnesylated triglycine, overview of SDS-PAGE, blots, silver-stained SDS-PAGE and fluorescence images used in this publication, gel filtration spectra of the labeling reaction with and without farnesylated triglycine, mass analysis spectrum of SEC peak 2, and plasmid construction and protein sequences Flag-Cdc42-His (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Boc	*tert*-butoxycarbonyl protecting group
Gly	glycine
DCM	dichloromethane
DMF	*N,N*-dimethylformamide
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
DiPEA	*N,N*-diisopropylethylamine
TFA	trifluoroacetic acid
MeOH	methanol
NMR	nuclear magnetic resonance
DMSO	dimethylsulfoxide

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