Research article

Distinct Bin/Amphiphysin/Rvs (BAR) family proteins may assemble on the same tubule to regulate membrane organization *in vivo*

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ABSTRACT

Intracellular membrane tubules play a crucial role in diverse cellular processes, and their regulation is facilitated by Bin-Amphiphysin-Rvs (BAR) domain-containing proteins. This study investigates the roles of *Drosophila* ICA69 (dICA69) (an N-BAR protein) and *Drosophila* CIP4 (dCIP4) (an F-BAR protein), focusing on their impact on *in vivo* membrane tubule organization. In contrast to the prevailing models of BAR-domain protein function, we observed colocalization of endogenous dICA69 with dCIP4-induced tubules, indicating their potential recruitment for tubule formation and maintenance. Moreover, actin-regulatory proteins such as Wasp, SCAR, and Arp2/3 were recruited at the site of CIP4-induced tubule formation. An earlier study indicated that F-BAR proteins spontaneously segregate from the N-BAR domain proteins during membrane tubule formation. In contrast, our observation supports a model in which different BAR-domain family members can associate with the same tubule and cooperate to fine-tune the tubule width, possibly by recruiting actin modulators during the generation of tubules. Our data suggests that cooperative activities of distinct BAR-domain family proteins may determine the length and width of the membrane tubule *in vivo*.

1. Introduction

BAR domain-containing proteins are crucial for membrane organization, fission, dynamics, and tubule generation within the cells [1–6]. Structural analysis reveals that the BAR domain possesses a characteristic crescent-like shape, enabling it to bind and induce curvature in cellular membranes [7–10]. BAR domain proteins actively participate in numerous cellular processes by sculpting the membrane, including endocytosis, membrane trafficking, filopodia, and tubule formation [11–14]. Through their ability to sense and shape membrane curvature, BAR domain proteins can facilitate the generation of tubular structures from flat membranes [15]. These tubules are dynamic, essential for cellular compartmentalization, and serve as conduits for efficiently transporting various cargo, such as proteins and lipids.

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The BAR domain proteins act as versatile membrane organizers and dynamic regulators, pivotal in maintaining cellular homeostasis and ensuring proper intracellular communication and function [16,17]. Among various BAR domain-containing proteins, F-BAR (Formin-Binding Protein 1 Homology-Bin-Amphiphysin-Rvs) and N-BAR domains have emerged as potential membrane remodelers for inducing membrane dynamics and remodeling [12,18,19]. F-BAR domain-containing proteins are typically involved in the early stages of membrane deformation, leading to the generation of wider, tubular structures [7]. However, N-BAR domains primarily function in the later stages of membrane remodeling, stabilizing the curved membrane and facilitating the formation of narrower, more rounded tubules [20].

Despite several structural and biochemical studies, the *in vivo* interaction of N-BAR and F-BAR proteins remains elusive at the cellular level. Earlier biochemical and genetic studies have revealed that dCIP4 interacts with Wasp and SCAR/WAVE and recruits actin-nucleation-promoting factors to the tubulating membranes [21]. Moreover, cell culture studies have shown that dCIP4 and dICA69 are potential molecules for tubule formation and relocalize actin regulators during filopodia formation in cultured cells [2,21]. Hence, we endeavored to characterize the interplay between dCIP4 (an F-BAR domain-containing protein) and dICA69 (an N-BAR domain-containing protein) in regulating membrane dynamics in S2R + cells. Our finding revealed that: a) the N-BAR and F-BAR domain-containing proteins may associate with the same tubules; b) the F-BAR domain proteins can alter the distribution of N-BAR proteins; and c) actin regulatory proteins get recruited and induce actin-polymerization at the site of membrane tubule formation. Thus, we suggest that different classes of BAR proteins may assemble on the same tubule to regulate the membrane organization *in vivo*.
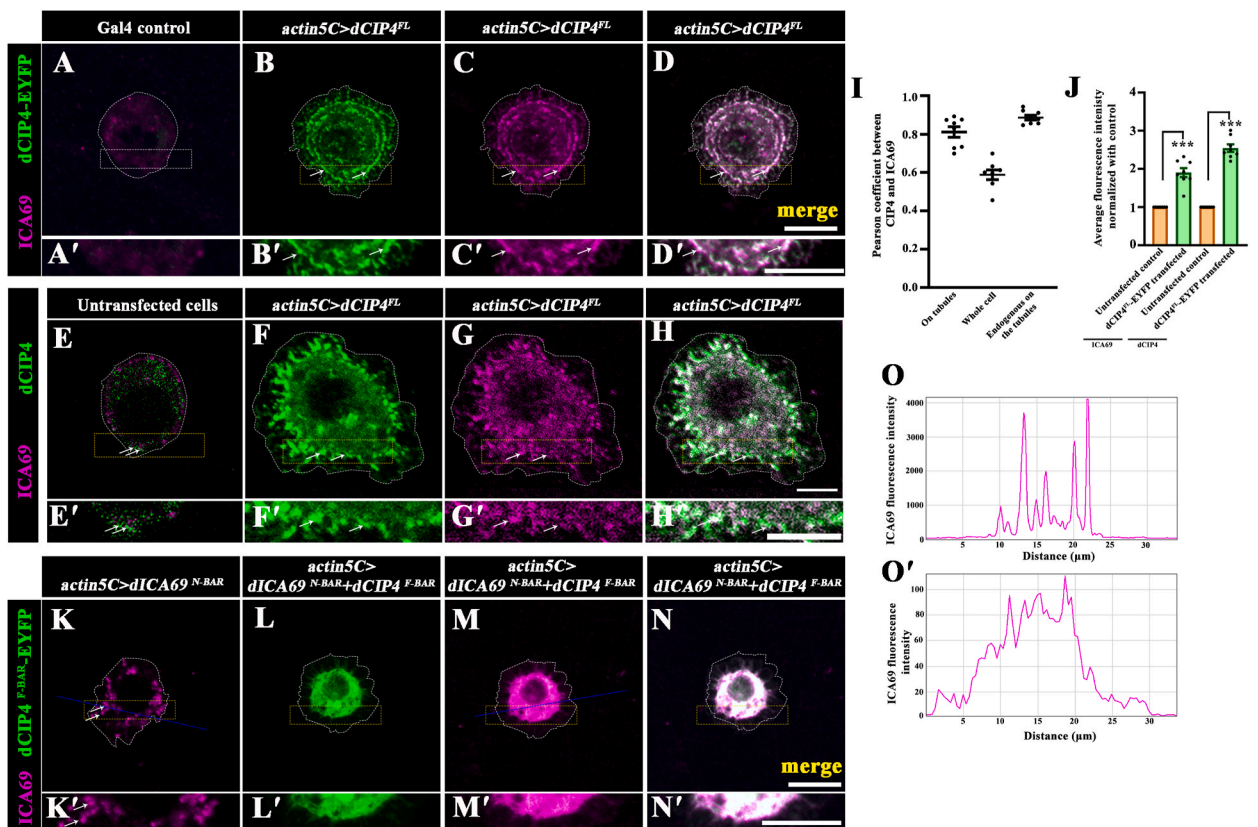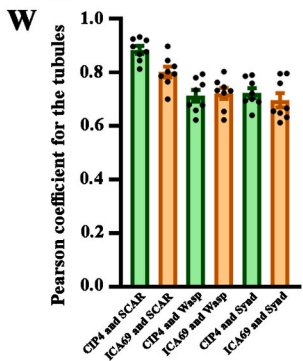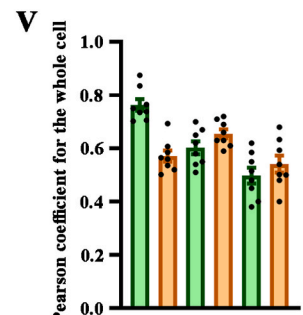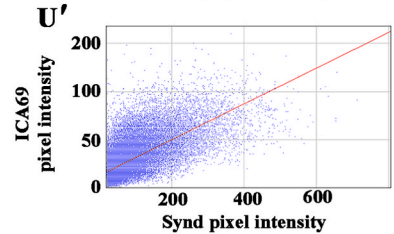

Fig. 1. dCIP4 overexpression relocalizes endogenous dICA69 to the same membrane tubules. (A-H') Confocal images of control S2R + cells transfected with actin5C-Gal4 with transfection reagent (Mirus TransIT; A) or cells transfected with EYFP-tagged full-length dCIP4, UAS-dCIP4^{FL} co-labeled with dICA69 and anti-GFP antibodies (A-D') and dICA69 and dCIP4 antibodies (E-H'). Scale bar: 10 μm (A–D); 4 μm (A'-D'); 5 μm (E-E'). The dCIP4 and dICA69 positive tubules are marked with an arrow in the figure. Note that overexpression of dCIP4^{FL} leads to relocalization of endogenous dICA69 to dCIP4-induced tubules. (I) Quantification of colocalization of the dCIP4 protein with ICA69 by determination of Pearson coefficient on the tubules, of whole cell and of endogenous proteins (using antibodies for both dCIP4 and dICA69) in cells transfected with the dCIP4^{FL}. (J) Histogram showing the average fluorescence intensity of the dICA69 (1.904 + 0.11) and dCIP4 (2.543 + 0.09) in the untransfected and dCIP4^{FL} transfected cells. The statistical analysis was done using one-way ANOVA followed by post-hoc Tukey's test. The error bar represents the standard error of the mean (SEM). ***p < 0.001. (K–N') Confocal images of S2R + cells co-transfected with actin5C-Gal4 and N-BAR domain of dICA69 (UAS-dICA69^{N-BAR}) (K–K') or actin5C-Gal4, UAS-dICA69^{N-BAR}, and dCIP4^{F-BAR} (L-N') and co-labeled with anti-dICA69 and anti-GFP antibodies. Scale bar: 10 μm (K–N); 4 μm (K'-N'). The overexpression of the dICA69^{N-BAR} domain alone resulted in the perinuclear distribution of dICA69 punctae (marked with the arrow). Note that co-expression of the dCIP4^{F-BAR} domain redistributes dICA69^{N-BAR} punctae in S2R + cells. (O–O') Plot profiles showing the distribution of the dICA69 in the control cells and in the cells cotransfected with the dCIP4^{F-BAR} and dICA69^{N-BAR}.
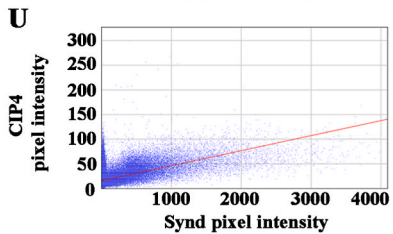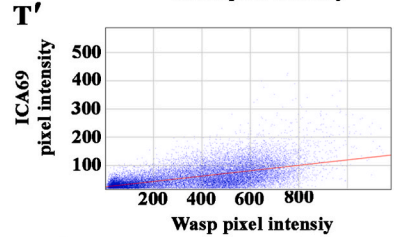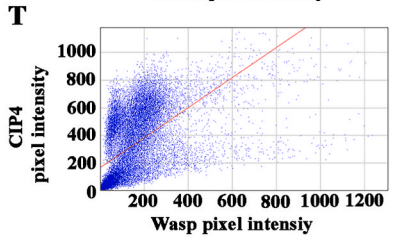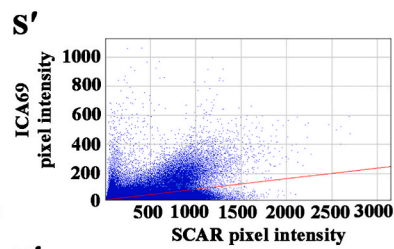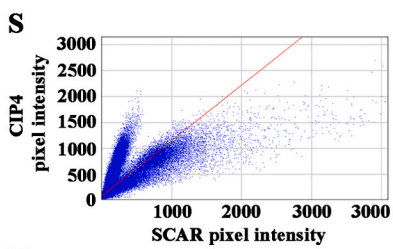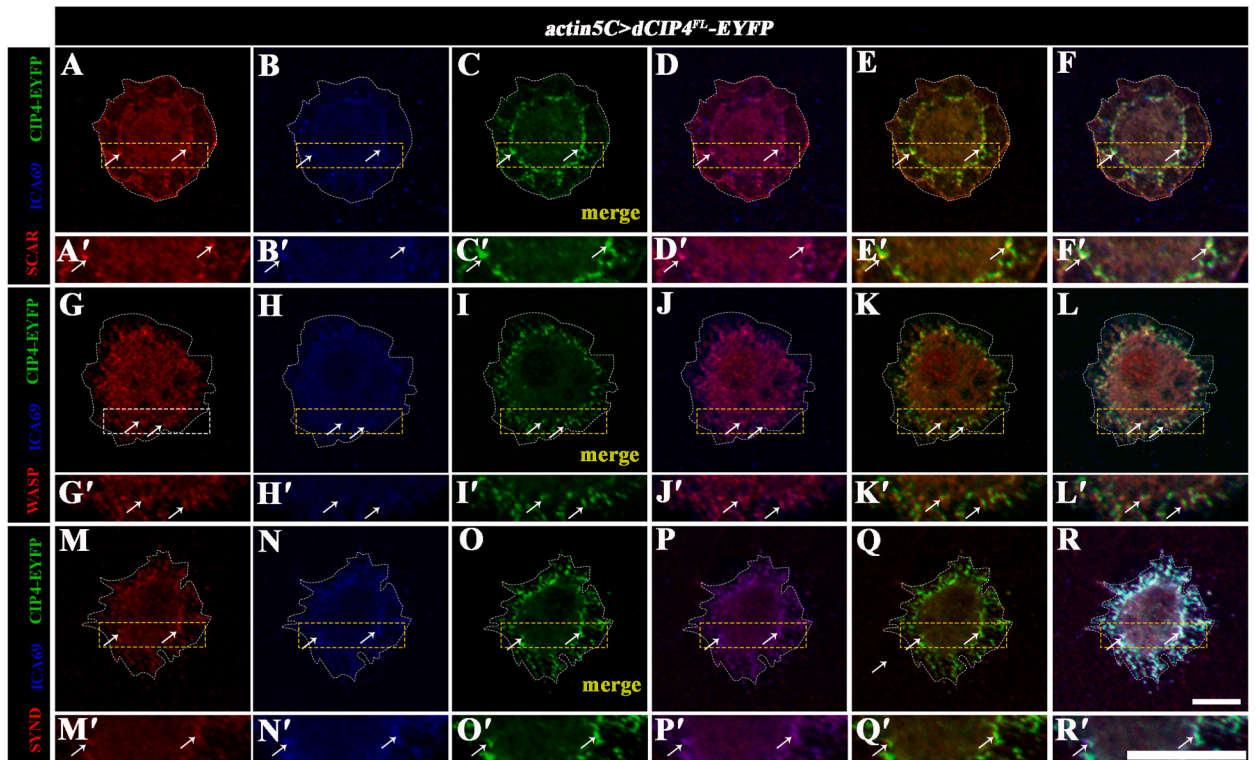
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Fig. 2. dCIP4 overexpression relocalizes Syndapin, Wasp, and SCAR at the site of membrane tubules. (A-R') Confocal images of S2R + cells co-transfected with full-length dCIP4 (dCIP4^{FL}) and immunolabelled with dCIP4 (green), SCAR/Wasp/Synd (red), and dICA69 (blue) antibodies. Note that SCAR, Wasp, and Syndapin are highly enriched at the dCIP4-positive membrane tubules (marked with arrows). The scale bar represents 10 μm for A-R, and 3 μm for A'-R'. (S–U') Images representing the cytoflorograms of dCIP4 (S, T, U) and dICA69 (S', T', U') with the SCAR, Wasp, and Synd. (V) Histogram showing the quantifications of colocalization of the dCIP4 and dICA69 proteins with SCAR, Wasp, and Synd of whole cells by determination of Pearson's coefficients. (W) Histogram showing the quantifications of colocalization of the dCIP4 and dICA69 proteins with SCAR, Wasp, and Synd on membrane tubules by determination of Pearson's coefficients. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2. Results

2.1. dCIP4 and dICA69 localize to the same membrane tubules

To understand the nature of dCIP4^{FL}-induced membrane tubules, we immunostained transiently expressing S2R + cells positive for dCIP4^{FL}-EYFP with dICA69 antibodies. Our immunostaining results revealed that endogenous dICA69 colocalizes with the CIP4-positive membrane tubules (Fig. 1A-D'). Furthermore, colocalization analysis between the dICA69 and dCIP4 suggests a strong association with the membrane tubules (Fig. 1A–I). We confirmed the colocalization of ICA69 and dCIP4 on the same membrane tubules by using antibodies against these proteins. Indeed, we found a tight colocalization of endogenous proteins on the same tubules (Fig. 1E–I). Next, we assessed the extent of CIP4 and dICA69 protein levels on the membrane tubules. We found that the level of CIP4 was about 2.5-fold more than the untransfected controls. Similarly, levels of ICA69 were found to be about 2.0-fold more on the tubules compared to untransfected controls (Fig. 1J). Consistent with the previous finding, overexpression of dICA69^{N-BAR} did not induce any detectable tubules but formed puncta-like structures at the perinuclear region of the cells (Fig. 1K and K') [2]. Next, to understand the *in vivo* interaction of F-BAR and N-BAR domains, we co-express dICA69^{N-BAR} and dCIP4^{F-BAR} domains in S2R + cells. Interestingly, the co-expression of the dCIP4^{F-BAR} domain results in the redistribution of dICA69^{N-BAR} punctae to a cytoplasmic distribution in S2R + cells (Fig. 1L-N'). The intensity plot profiles further confirm the redistribution of ICA69 punctae by coexpressing dICA69^{N-BAR} and dCIP4^{F-BAR} domains in the S2R + cells (Fig. 1O-O').

Thus, in contrast to the previous findings, this data revealed a novel observation elucidating the *in vivo* regulation of N-BAR (dICA69) and F-BAR (dCIP4) proteins that might contribute towards governing the dynamics and organization of membrane tubules [21,22]. While our observation does not discriminate direct N-BAR: F-BAR heterodimerization on the same tubules, it does raise the possibility that such colocalization on the same tubule could expand the scope of modulatory activities by different BAR-domain proteins *in vivo*.

2.2. dCIP4 overexpression relocalizes actin regulators at the site of tubule formation

Mammalian Cip4/Toca has been shown to coordinate actin dynamics through a pathway that involves Cdc42 and Wasp family protein-dependent activation of Arp2/3 [23]. Moreover, the conjunctional interaction of BAR proteins is likely to expand the functional aspects regulated by BAR proteins by controlling the range of membrane tubule generation and recruitment of binding partners or signaling molecules at the site of membrane deformation [16]. Hence, we next assessed if actin-regulators or other BAR domain-containing proteins were recruited at the site of membrane tubules induced by CIP4 expression. Using antibodies against actin regulatory proteins, we found that dCIP4^{FL}-induced membrane tubules show enhancement of Wasp and SCAR, two of the positive regulators of actin polymerization (Fig. 2 A-L'). Analysis of the extent of colocalization between CIP4 and SCAR or Wasp revealed a very strong colocalization of these proteins on the membrane tubules (Fig. 2S-T' and Fig. 2V-W). Additional immunostaining and colocalization analysis by Pearson's correlation coefficient (PCC) revealed that CIP4-positive tubules also contained Syndapin, an F-BAR-containing protein (Fig. 2 M-R', U–U' and Fig. 2V-W). These data suggest that dCIP4 relocalizes actin-regulatory proteins at the site of tubule formation in S2R + cells and that membrane tubules may contain the same or different families of BAR-domain-containing proteins. Since we observed the recruitment of actin-regulatory proteins at the location of Cip4-induced membrane tubules, we next asked if there is also relocalization of Arp2/3 proteins and actin polymerization at the membrane tubules.

Interestingly, we found that Arp2 and Arp3 were enriched at the site of membrane tubules compared to the whole cell, as revealed by the PCC analysis (Fig. 3A-L', S-T' and Fig. 3V-W). Moreover, we observed increased F-Actin at the site of membrane tubules induced by CIP4 (Fig. 3M-R', U–U' and Fig. 3V-W). Thus, we propose that actin regulation at the site of membrane tubule remodeling, combined with the recruitment of other BAR-domain proteins, forming heterodimers with other BAR-domain family members, could be a critical event in membrane remodeling.

3. Discussion

This study shows that the N-BAR and F-BAR domain-containing proteins can localize on the same tubules. Furthermore, we defined the role of the dCIP4^{F-BAR} domain in regulating the distribution of dICA69^{N-BAR} perinuclear punctae, suggesting the *in vivo* interaction of N-BAR and F-BAR proteins in the cells. Finally, we showed that CIP4-induced membrane tubules relocalize actin regulatory proteins, possibly to stabilize nascent membrane tubules.

The N-BAR and F-BAR proteins play a crucial role in regulating actin dynamics at the plasma membrane through the interaction of actin-regulatory proteins such as Wasp (Wiskott-Aldrich Syndrome Protein) and NPFs (Nucleation promoting factors) [19,21,24,25].

actin5C>dCIP4^{FL}-EYFP
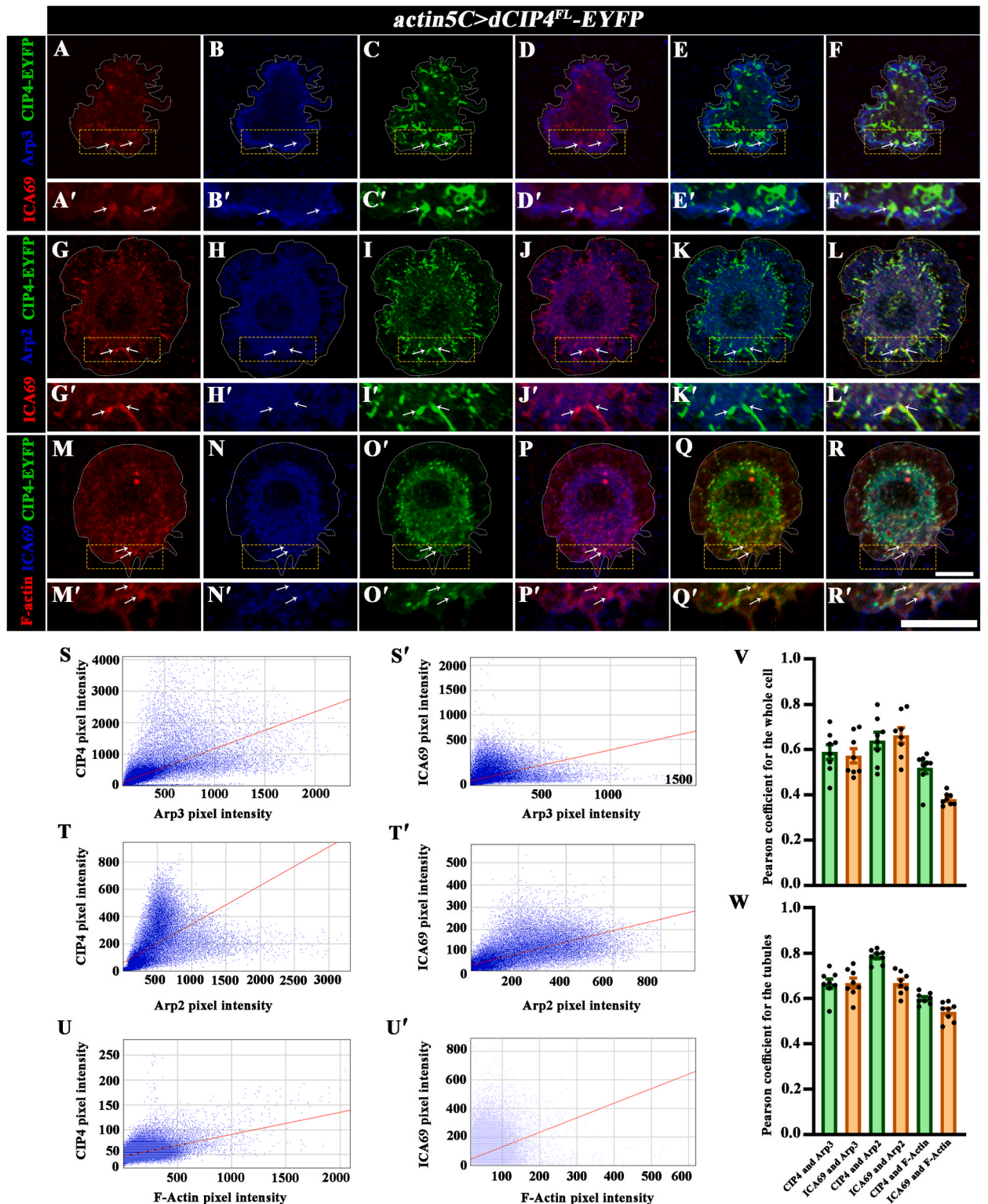
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Fig. 3. The actin nucleator Arp2/3 is recruited to the dCIP4-induced membrane tubules.

(A-L′) Confocal images of cells transfected with full-length dCIP4 and immunolabelled for dCIP4 (green), Arp2/Arp3 (blue), and dICA69 (red). Confocal images show that dCIP4-positive tubules are highly enriched with Arp2/3 actin regulatory proteins (marked with arrows). The scale bar represents 10 μm for A-L and 3 μm for A′-L′.

(M-R′) Confocal images of cells transfected with full-length dCIP4 and immunolabelled with dCIP4 (green), dICA69 (blue), and F-actin (red) dyes, respectively. F-actin is highly enriched at the dCIP4-positive tubules (marked with arrows). The scale bar represents 10 μm for M-R and 3 μm for M′-R′. (S–U′) Images representing the cytoflorograms of dCIP4 (S, T, U) and dICA69 (S′, T′, U′) with the Arp3, Arp2, and F-Actin. (V) Histogram showing the quantifications of colocalization of the dCIP4 and dICA69 proteins with Arp3, Arp2, and F-Actin of whole cell by determination of Pearson's coefficients. (W) Histogram showing the quantifications of colocalization of the CIP4 and ICA69 proteins with Arp3, Arp2, and F-Actin on tubules by determination of Pearson's coefficients. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Recruitment of actin regulatory proteins induces actin polymerization, generating forces that help further deform the membrane and elongate tubules. Additionally, our previous report suggests that overexpression of dICA69 localizes actin regulatory proteins at the site of filopodia [2]. Consistent with this idea, our data indicate that endogenous dICA69 might relocalize Wasp, SCAR, at the site of dCIP4-induced membrane tubules, possibly through interaction with the actin regulators in S2R + cells. Therefore, this actin-based pushing force, in coordination with the curvature-sensing ability of N-BAR and F-BAR proteins, enables efficient tubule extension and membrane remodeling during various cellular processes.

Additionally, earlier studies have indicated that N-BAR and F-BAR domain-containing proteins are segregated and localized to distinct tubules within the cells; more specifically, N-BAR proteins are localized to shorter, rounded, tighter tubules, whereas F-BAR protein induces longer, elongated, and wider tubules [7,22]. In contrast, we observed three distinct characteristics in the context of dCIP4^{BAR} and ICA69^{N-BAR} proteins: a) Overexpression of dCIP4^{F-BAR} redistributes dICA69^{N-BAR} perinuclear punctae resulting in an altered pattern within the cell, suggesting possible interaction of N-BAR and F-BAR proteins *in vivo*; b) dICA69 levels were enhanced in dCIP4-positive tubules when compared to the untransfected control cells; and c) dCIP4 and dICA69/Syndapin are recruited to the same membrane tubules, further suggesting that a distinct set of N-BAR and F-BAR proteins can cooperate and form mixed protein scaffolds at the membrane surface, thus generating diversity in the width of membrane tubules. The formation of these heteromeric protein complexes possibly allows for enhanced curvature sensing and membrane tubulation. However, despite several structural and biochemical analyses, the interaction of N-BAR and F-BAR proteins still needs to be understood at the cellular level.

Overall, the interplay between N-BAR and F-BAR proteins and their interaction with other cellular components form an intricate regulatory network that governs membrane dynamics, tubule formation, and actin regulation. These processes are essential for various cellular functions, including endocytosis, cell migration, and vesicular trafficking [26–28]. Previous findings suggest that N-BAR and F-BAR domain-containing proteins are segregated and localized to distinct tubules within the cells [22]. However, our finding extends the previous model, indicating the localization of N-BAR and F-BAR proteins to the same membrane tubules, at least in *Drosophila* S2R + cells. Whether dCIP4 and dICA69 directly interact within the cells or through indirect mechanisms to regulate tubule generation *in vivo* is a matter of further investigation. We suggest that dICA69 and dCIP4 proteins might interact directly or indirectly, localize on the same membrane tubules, and regulate tubule width through a mechanism involving the participation of actin-regulatory proteins. Such mechanisms may generate diversity in the width and length of membrane tubules necessary for *in vivo* functions within cells to regulate tubule dynamics in S2R + cells (Fig. 4A–C). Prior reports have indicated that the high expression of dCIP4 levels is linked to renal dysfunction in a rat model of 5/6 nephrectomy [29]. Hence, further studies on the molecular mechanisms underlying N-BAR and F-BAR protein functions and their interactions will strengthen our understanding of fundamental cellular processes. They may offer potential therapeutic targets for diseases associated with membrane trafficking and cell migration dysregulation.
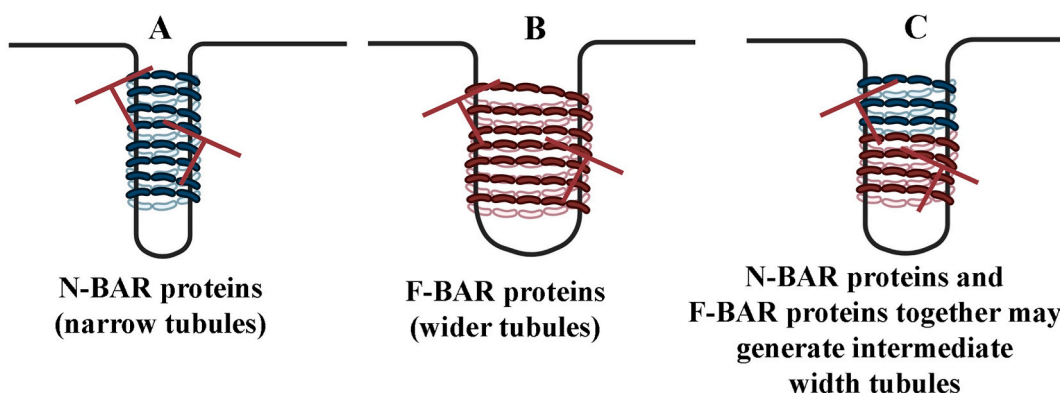

Fig. 4. Model depicting regulation of dCIP4 and dICA69 during tubule formation in S2R + cell. (A) The N-BAR domain with intrinsic curvature of ~22 nm forms narrow tubules. (B) The F-BAR with intrinsic curvature of ~60 nm forms wider tubules. (C) Recruitment of N-BAR and F-BAR proteins on the same tubules may generate intermediate-width tubules in the cells. We suggest that cooperative activities of distinct N-BAR and F-BAR domain proteins may regulate tubule width through a mechanism that also involves actin regulation.

4. Material and methods

4.1. Drosophila S2R + cell culture

Drosophila S2R + cells were cultured in 1x Schneider's *Drosophila* media (Thermo Fisher Scientific, MA USA) supplemented with 10 % fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin in 25-cm^2 T-flasks (Corning) at room temperature. The cells ($\sim$3x10^3) were seeded in a six well-culture plate and transiently co-transfected with pUAST-ICA69^{N-BAR} (1–228 aa), pUAST-CIP4^{F-BAR} (1–300 aa), pUAST-CIP4^{FL}, pUAST-ICA69^{FL}, and *actin5C*-Gal4 (1 μg each) using Mirus *TransIT* transfection reagent as described previously [2,30].

4.2. Antibodies and immunocytochemistry

For microscopic analysis, S2R + cells co-expressing pUAST-ICA69^{N-BAR}, pUAST-CIP4^{F-BAR}, pUAST-CIP4^{FL}, and *actin5C*-Gal4 were adhered onto Concanavalin A (Sigma-Aldrich, St. Louis, MO, USA) coated coverslips and fixed with 4 % formaldehyde solution for 15 min. The first 361 amino acids were cloned to generate dICA69 antibodies, which contain the N-BAR domain of dICA69 [2]. Anti-ICA69 antibodies [2] and dCIP4 antibodies [21] were used at 1:1000 dilutions. The polyclonal antibodies anti-GFP (Roche, Basel, Switzerland), anti-Arp2, and anti-Arp3 were used at 1:200 dilutions. Other antibodies and dyes used were anti-syndapin [3,31], anti-SCAR [32], anti-Wasp [33], and Rhodamine conjugated phalloidin (Thermo Fisher Scientific, MA, USA) (1:200 dilutions). Anti-mouse and anti-rabbit Alexa fluor 488/568 conjugated secondary antibodies were incubated at 1:800 dilutions (Thermo Fisher Scientific, MA, USA). The cells were mounted in Vectashield (Vector Laboratory, Newark, California, USA) and imaged with 63x/1.4NA objective in Zeiss LSM780 confocal microscope (Carl-Zeiss, Germany) as described previously [34–37].

4.3. Quantification and statistical analysis

All the colocalization analyses were performed using the JACoP ImageJ plugin as described previously [38], and the thresholding was done manually to analyze the colocalization of proteins on tubules and the whole cell. For the fluorescence intensity quantification, the confocal images were captured under the same conditions, and ROI was drawn across each cell to determine the quantification section to be used in our analysis. The images were thresholded for the fluorescence intensity on the membrane tubules, and the mean intensity was calculated manually using ImageJ/Fiji software (National Institutes of Health, Bethesda). The intensity was normalized with the average fluorescence intensity of the respective proteins in the untransfected control cells. A line was drawn using the line tool in ImageJ, and plots were generated using the Plot Profile function in ImageJ. For multiple comparisons, one-way ANOVA followed by post hoc Tukey's test was used. GraphPad Prism 8 was used to plot all the graphs. Error bars in all the histograms represent SEM. ***$p < 0.001$.

Data availability statement

All data presented in this article have been included or referenced.

CRediT authorship contribution statement

Bhagaban Mallik: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Srikanth Pippadpally:** Writing – review & editing, Visualization, Methodology, Investigation. **Anjali Bisht:** Validation, Investigation. **Sajad Bhat:** Writing – original draft, Investigation, Formal analysis. **Surabhi Mukherjee:** Visualization, Investigation. **Vimlesh Kumar:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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