

Reviewed Preprint

v1 • April 15, 2025

Not revised

Reviewed Preprint

v2 • July 17, 2026

Revised by authors

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Competing interests:

No competing interests declared

Funding:

See [page 37](#)

Reviewing editor:

Alphee Michelot, Mechanobiology Institute, Singapore

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Nim1-related kinases regulate septin organization and cytokinesis by modulating Hof1 at the cell division site

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eLife Assessment

In this **valuable** study, Bhojappa et al. investigate the function of four septin-associated kinases (Elm1p, Gin4p, Hsl1p and Kcc4p) in controlling septin ring stability, actomyosin ring organization and constriction, and cytokinesis in budding yeast. The evidence supporting the conclusions is **convincing**. The results are a meaningful addition to previously published work and provide a clearer picture of a complex mechanism involving proteins of partially redundant functions.

<https://doi.org/10.7554/eLife.106366.2.sa4>

Abstract

The septin scaffold recruits and organizes actomyosin ring (AMR) components, thus, ensuring faithful cytokinesis. The septin-associated kinases - Elm1, Gin4, Hsl1, and Kcc4 are thought to stabilize and regulate the septin architecture at the bud neck, but the underlying mechanisms remain largely unknown. Here, we present a comprehensive, quantitative analysis of these four septin-associated kinases and reveal major roles for Elm1 and Gin4 in septin stability and architectural transitions during the cell cycle. We find that Elm1 and Gin4 play a previously overlooked role in AMR organization and constriction during cytokinesis. We report that the Gin4 kinase interacts directly with the AMR component and F-BAR protein Hof1 via its C-terminal membrane-binding kinase associated-1 (KA1) domain, and is likely involved in the proper organization and anchoring of Hof1 at the bud neck, representing an unappreciated mode of regulation during cytokinesis. We further show that Gin4 controls septin organization and AMR constriction in a kinase-independent manner, similar to Elm1. Using an extensive GFP-GBP-based tethering assay in *elm1Δ* and *gin4Δ* cells, we identify an important role for Hsl1 in maintaining septin organization and cell shape in coordination with Elm1, Gin4, and Kcc4, independent of its role in the morphogenetic checkpoint. Furthermore, our data indicate that Hsl1 acts downstream of Elm1, with its membrane-binding KA1 domain being critical for its function. Together, these findings reveal new insights into the modes by which the kinases Gin4 and Elm1 regulate cytokinesis, highlight a redundant role for Hsl1 in controlling septin organization and cytokinesis, and uncover the inherent redundancy and adaptability of the septin kinase network in *Saccharomyces cerevisiae*.

Introduction

Septins, the fourth cytoskeletal element¹, are an evolutionarily conserved family of GTP-binding proteins that assemble into filamentous structures on cell membranes^{2,3}. Although septins belong to the P-loop GTPases⁴, recent studies have revealed that some septin proteins have lost the ability to hydrolyze bound GTP during evolution^{5,6}. They play critical roles in various fundamental processes such as cytokinesis, ciliogenesis, spermatogenesis, and phagocytosis^{7–10}. Septins

polymerize into hetero-oligomeric complexes that further assemble into higher-order structures, such as filaments, rings, bundles, and gauzes^{3,11–15}. These structures function as scaffolds that recruit and organize proteins required for cytokinesis and cell division across eukaryotes^{16,17}. Septins act as a diffusion barrier at the cell division site in fungi and animal cells, contributing to the assembly, maturation, and constriction of the contractile AMR machinery during cytokinesis^{18–22}. However, studies have suggested that redundant mechanisms can compensate for the septin diffusion barrier, rendering it dispensable for AMR constriction and cytokinesis, indicating that septins may not be strictly essential for AMR function in all contexts^{15,17}. The budding yeast *S. cerevisiae* is an excellent model system for studying the relationship between septin organization and cytokinesis. In this organism, five mitotic septins Cdc3, Cdc12, Cdc10, Cdc11, and Shs1^{7,23–26} assemble into an octameric complex that subsequently polymerizes into septin filaments or rods capable of organizing diverse higher-order architectures at the plasma membrane^{2,3,27}.

The terminal subunits of the octameric complex influence the architecture of the septin structure: Cdc11 promotes septin filament bundling, whereas Shs1 favours the formation of ring-like structures^{28,29}. Septin filaments assemble at the mother-bud neck to form a stable "hourglass" structure that acts as a scaffold for proteins involved in cytokinesis, including components of the AMR complex^{14,17,18}. At mitotic exit, this hourglass structure undergoes dynamic remodeling into a double-ring structure, marking the onset of cytokinesis^{30–32}. This transition allows proper anchoring of the AMR to the plasma membrane and leads to efficient ring constriction and centripetal septum deposition between the dividing cells^{18,33–36}. The mitotic exit network (MEN) is thought to play a crucial role in initiating this transition^{30,31}. Despite its importance, the precise molecular mechanisms regulating the hourglass to double-ring (HDR) transition remain incompletely understood.

Several kinases associate with septin structures and regulate septin dynamics during different phases of the cell cycle. These septin-associated kinases localize to the septin hourglass at the beginning of the G1 phase and dissociate from the bud neck at the onset of cytokinesis, coincident with the start of the HDR transition^{37–40}. In *S. cerevisiae*, four conserved septin-associated kinases - Elm1^{38,39,41–43}, Gin4^{38,40,44–50}, Hsl1^{51–56}, and Kcc4^{57,58} are thought to exert distinct and overlapping regulatory effects on septin stability and organization^{38,39,50,59,60}. Elm1 and Gin4 deletion significantly destabilizes the septin architecture^{38,39,41,59}, while loss of Hsl1 and Kcc4 does not have any effect⁵⁹. Several hypotheses have been proposed to describe the functions of these kinases during cytokinesis. Previous studies suggested that the LKB1-like kinase Elm1 and the Nim1-related kinases (Gin4, Hsl1, Kcc4) function in parallel pathways to regulate septin architecture at the bud neck^{59,61}. More recently, it was shown that Elm1 acts as the most upstream kinase in this septin kinase network by regulating the activation and phosphorylation of the Nim1-related kinases^{39,62}. In addition, Elm1 is known to regulate septin organization by binding to its substrate Bni5 independently of its kinase activity³⁹. Gin4, on the other hand, is thought to regulate septin architecture by phosphorylating Shs1⁴⁹. Hsl1 is best known for its role in negatively regulating Swe1 in the morphogenetic checkpoint pathway by forming a complex with Hsl7^{52,55,59}. How Hsl1 and Kcc4 regulate septin organization remains unknown, and the mechanisms by which these kinases coordinate septin organization and cytokinesis remain incompletely understood.

Despite significant progress in understanding septin architecture and its regulation, the coordinated effects of septin-associated proteins on this process are only beginning to be understood, and several important questions remain open in the field of septin biology. In particular, it is unclear how septin-associated kinases contribute to the organization and remodeling of septin structures, and how these regulatory pathways are coordinated with AMR assembly and cytokinesis. Furthermore, whether these kinases influence cytokinesis through direct interactions with components of the cytokinetic machinery or indirectly through effects on septin organization remains largely unexplored. In this study, we investigate the roles of the septin-associated kinases Elm1, Gin4, Hsl1, and Kcc4 in regulating septin organization and cytokinesis in *S. cerevisiae*, with the goal of understanding how this kinase network coordinates septin dynamics with AMR function during cell division.

Results

Dynamics of septin-associated kinases during the cell cycle and defects associated with their deletions

S. cerevisiae contains four major septin-regulatory kinases Elm1, Gin4, Hsl1, and Kcc4 that are known to localize to the mother-bud neck and contribute to septin stability and the morphogenesis checkpoint during the cell cycle^{39,59}. Three of these kinases, commonly referred to as Nim1-related kinases⁵⁹: Gin4, Hsl1, and Kcc4 contain a membrane-binding Kinase associated-1 (KA1) domain at the C-terminus (Fig. 1A [↗](#)). The KA1 domain shows affinity for acidic phospholipids (e.g., phosphatidylserine) on the plasma membrane and is required for the bud neck targeting^{47,48,51,57}. To characterize their temporal kinetics and localized accumulation at the bud neck septin scaffold, we performed time-lapse imaging of endogenously GFP-tagged septin kinases in cells co-expressing the core septin Cdc3 tagged with mCherry. All four kinases showed simultaneous, gradual recruitment to the septin hourglass during bud emergence and exhibited a sequential pattern of disappearance during the onset of cytokinesis, consistent with previous observations^{38,39} (Fig. S1A-S1G [↗](#), Movie-S1 [↗](#)). Elm1 and Kcc4 exhibited lower fluorescence intensities at the bud neck prior to septin remodeling, suggesting a lower abundance at this site at the defined time period (Fig. S1E-S1G [↗](#)), compared with the higher abundance of Gin4 and Hsl1, which exhibited increased fluorescence intensities prior to septin remodeling (Fig. S1E-S1G [↗](#)). At the onset of cytokinesis, when the septin scaffold undergoes remodeling from the hourglass structure to a double-ring structure (HDR transition), all four kinases left the bud neck just before or during the HDR transition in a sequential manner (Fig. S1E-S1G [↗](#), Movie-S1 [↗](#)). Interestingly, fluorescence intensities of Elm1 and Kcc4 began to decrease ~4-6 minutes before the HDR transition (Fig. S1E-S1G [↗](#)). In contrast, Gin4 and Hsl1 fluorescence signal intensities began to decrease just before or during the HDR transition, suggesting that they may serve as a trigger or key molecular cue for septin remodeling that subsequently crosstalks with downstream events of cytokinesis (Fig. S1E-S1G [↗](#)). These observations suggest a defined temporal order of removal of these kinases from the bud neck during cytokinesis, which may directly rely on the interdependency of their localizations on one another. Overall, these data show that the four septin kinases are associated with the septin hourglass structure with similar patterns of recruitment but distinct removal kinetics, suggesting that they may play both overlapping and non-overlapping roles in septin hourglass stability and the HDR transition.

Septin-associated kinases Elm1 and Gin4 majorly regulate septin stability, HDR transition, and cellular morphology

Septin rearrangement during cytokinesis is believed to be partly governed by post-translational modifications (PTMs), including phosphorylation, ubiquitination, acetylation, and SUMOylation^{37,45,63–68}. Previously, different studies have highlighted the role of kinase-mediated modifications in septin remodeling, but a comprehensive quantitative analysis for all the four septin-associated kinases under similar conditions is not present^{39,49,50,69,70}. Thus, to understand the extent to which different septin kinases affect cell physiology and septin organization, we characterized cell morphology, cell growth, and septin organization in wild-type cells and in cells harbouring deletions of the septin-regulatory kinases - Elm1, Gin4, Hsl1, and Kcc4. We observed that cells exhibited elongated and clumped morphologies in the *elm1Δ* and *gin4Δ* backgrounds, respectively, at all three temperatures (23°C, 30°C, and 37°C) tested, consistent with previous observations^{38,41,59,71} (Fig. S2A, S2B [↗](#)). Quantitative analysis revealed *elm1Δ* cells displayed a more severe defect, with nearly 100% of cells showing elongated morphology that failed to undergo isometric growth, whereas *gin4Δ* cells exhibited clumped morphology in over 73.33% of the cellular population (Fig. S2A, S2B [↗](#)). While *hsl1Δ* cells largely showed round morphology similar to wild-type at 23°C and 30°C, a fraction of cells exhibited mild morphological defects at 37°C (Fig. S2A, S2B [↗](#)). In contrast, no alterations in morphology were observed in *kcc4Δ* cells (Fig. S2A, S2B [↗](#)). Growth assays of these kinase deletions showed a similar trend: *elm1Δ* and *gin4Δ* cells

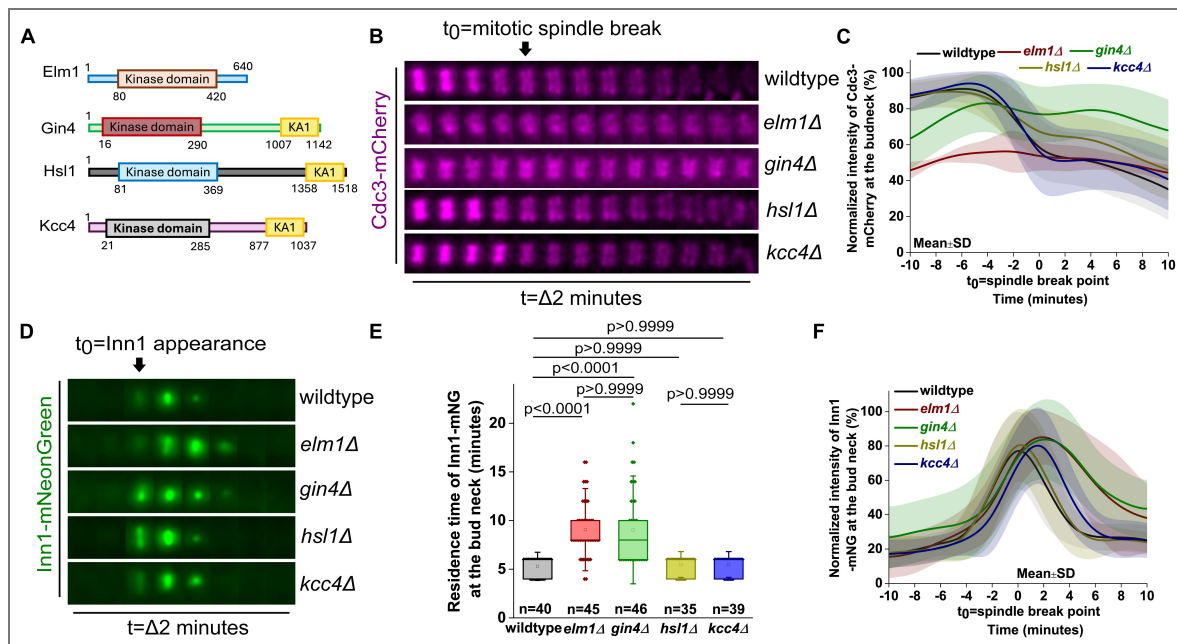


Figure 1. Septin organization and actomyosin ring (AMR) dynamics are defective in *elm1* Δ and *gin4* Δ cells.

(A) Domain-level architecture of Elm1, Gin4, Kcc4 and Hsl1 kinases. The KA1 domain represents the membrane-binding domain. (B) Representative time-lapse images showing Cdc3-mCherry dynamics in septin-kinase deletion strains, captured at two-minute time intervals (t_0 =spindle breakpoint). (C) Normalized fluorescence intensity profile showing the temporal kinetics of Cdc3-mCherry in the indicated strains shown in (B) (wild-type: $n=41$, $elm1\Delta$: $n=29$, $gin4\Delta$: $n=33$, $hsl1\Delta$: $n=34$, $kcc4\Delta$: $n=36$ cells). (D) Representative time-lapse images of Inn1-mNG constriction captured at two-minute time intervals in septin-kinase deletion strains (t_0 = appearance of Inn1-mNG at the bud neck). (E) Quantification of the residence time of Inn1-mNG during the onset of cytokinesis in the indicated strains of (D), Kruskal-Wallis nonparametric test (****: $p<0.0001$, ns: $p>0.05$). (F) Normalized fluorescence intensity graph showing the kinetics of Inn1-mNG in the indicated strains of (D). A population of $elm1\Delta$ and $gin4\Delta$ cells exhibiting asymmetric constriction is included in the plot (wild-type: $n=21$, $elm1\Delta$: $n=13$, $gin4\Delta$: $n=23$, $hsl1\Delta$: $n=21$ and $kcc4\Delta$: $n=18$ cells).

showed growth sensitivity at both 23°C and 30°C, which was heightened at 37°C for *elm1Δ*, while *hsl1Δ* exhibited growth sensitivity only at the higher temperature 37°C, in contrast to *kcc4Δ* cells, which grew identical to the wild-type strain (Fig. S2C [↗](#)).

Next, we sought to systematically assess the effects of the absence of these kinases on septin architecture and dynamics. The core septin Cdc3 fused to mCherry was used to visualize the septin structures during the cell cycle⁷². Cdc3-mCherry localized to the emerging bud site in wild-type, *elm1Δ*, *gin4Δ*, *hsl1Δ*, and *kcc4Δ* cells but was subsequently mislocalized to the bud cortex, consistent with previous findings^{39,41,50,61} in ~100% of *elm1Δ* cells and 80.48% of *gin4Δ* cells (Fig. S2E, S2G [↗](#), Movie-S2 [↗](#)). In wild-type cells, septin formed an stable hourglass structure at the bud neck, which subsequently split into double rings coinciding with spindle breakage and mitotic exit (Fig. S2D, S2F [↗](#)). The septin hourglass architecture appeared abnormal in *elm1Δ* and *gin4Δ* cells and exhibited a defective HDR transition (Fig. 1B, 1C [↗](#), Movie-S2). The *elm1Δ* cells showed a stronger defect in septin organization during bud emergence and the HDR transition compared with *gin4Δ* cells (Fig. 1B, 1C [↗](#), S2E, S2G [↗](#)). This suggests a more prominent role for Elm1 in the maintenance of septin filament architecture, consistent with previous literature reporting a role for Elm1 in stabilizing the daughter-side of the septin hourglass structure by regulating septin filament pairing³⁹. Cells lacking the Hsl1 and Kcc4 kinases showed normal septin organization during bud emergence and HDR transition at the onset of cytokinesis, similar to wild-type cells (Fig. 1B, 1C [↗](#), S2E, S2G [↗](#)). However, approximately 7.14% of *hsl1Δ* cells displayed septin mislocalization to the bud cortex during bud emergence (Fig. S2E, S2G [↗](#)), indicating that Hsl1 may also play a minor or supporting role in septin dynamics through a compensatory mode of regulation. These results therefore demonstrate a major requirement for Elm1 and Gin4 in proper septin arrangement and dynamics at the cell division site, while also hinting at a parallel minor role for Hsl1.

Elm1 and Gin4 regulate AMR organization and constriction

Given that our data suggest major defects in septin regulation occur in the absence of Elm1 and Gin4, we asked whether these defects also translate into effects on AMR-mediated cytokinesis. We therefore investigated the roles of Elm1 and Gin4 in AMR organization at the cell division site and in constriction dynamics during cytokinesis. The AMR machinery generates the force required to drive plasma membrane ingression for successful cell separation^{18,73}. In budding yeast, the AMR machinery consists primarily of actin filaments, tropomyosin, formins, Myo1, Hof1, Iqg1, Cyk3, and Inn1, along with many other regulatory proteins^{18,21,74–76}. We used endogenously labelled Myo1-mNeonGreen (mNG) the sole non-muscle myosin-II in *S. cerevisiae* to determine AMR organization during bud emergence. Previous studies have shown that Myo1 recruits to the bud neck in a bi-phasic manner - via Bni5 prior to cytokinesis and via Iqg1 during cytokinesis^{72,77}. Consistent with its interaction with septin-binding protein Bni5⁷², we observed that, similar to Cdc3-mCherry, Myo1-mNG was mislocalized to the bud cortex during bud emergence in 75.86% and 30% of *elm1Δ* and *gin4Δ* cells, respectively (Fig. S3A, S3B [↗](#)). We then used Inn1 which arrives two-minutes after the start of AMR constriction and aids in plasma membrane ingression by coupling AMR constriction with primary septum (PS) formation as a marker to determine AMR constriction dynamics during cytokinesis^{75,76}. In wild-type cells, Inn1-mNG arrived at the bud neck just prior to spindle breakage and constricted symmetrically as part of the AMR complex with a residence time of ~5-6 minutes, and eventually disappeared from the bud neck (Fig. 1D-1F [↗](#)). In *elm1Δ* and *gin4Δ* cells, Inn1-mNG was recruited normally to the cell division site but displayed an abnormal constriction pattern with an increased duration of constriction (~8-10 minutes) (Fig. 1D-1F [↗](#), Movie-S3 [↗](#)). By contrast, in *hsl1Δ* and *kcc4Δ* cells, Inn1-mNG was recruited normally and constricted similarly to the wild-type strain (Fig. 1D-1F [↗](#), Movie-S3 [↗](#)). These results agree with the regulation of these kinases on septin dynamics, suggesting that Elm1 and Gin4 may directly or indirectly regulate AMR constriction and cytokinesis.

Inn1 interacts with Hof1, an F-BAR protein known to associate with the septin hourglass, transit to the AMR during the split-ring trigger^{73,74,78–82}, and sense membrane curvature^{74,76,82}. Inn1 also functions in parallel with Cyk3; Hof1, Inn1 and Cyk3 form a tripartite complex referred to as the Ingression Progression Complex (IPC)^{76,83–85}. This complex then activates the primary component

of the PS, Chitin Synthase II (Chs2)^{76,83–85}. Thus, we monitored the constriction pattern and dynamics of Chs2-mNG and observed an increased residence time for Chs2 at the bud neck in both *elm1Δ* and *gin4Δ* cells (Fig. S3C–S3E [↗](#)). Our observations suggest that loss of the septin kinases Elm1 and Gin4 may result in improper organization and constriction dynamics of the AMR, and possibly impaired IPC formation, resulting in abnormal cytokinesis.

To test the organization of the AMR/IPC at the bud neck, we assessed the organization of the F-BAR protein Hof1. Deletion of Hof1 has been shown to result in asymmetric AMR constriction and improper chitin deposition during PS synthesis⁷⁶. We then analyzed the residence time of Hof1-mNG at the bud neck after the spindle breakpoint during cytokinesis. Hof1-mNG exhibited a residence time of 10–12 minutes after the mitotic spindle break in wild-type cells, which increased to >15 minutes in both *elm1Δ* and *gin4Δ* cells (Fig. 2A, 2B [↗](#), Movie-S4 [↗](#)). By contrast, we did not observe any significant change in the recruitment and temporal kinetics of Hof1-mNG in *elm1Δ* and *gin4Δ* cells compared with wild-type during the mitotic spindle break (Fig. 2C [↗](#)). Next, we analyzed the organization of Hof1-mNG at the cell division site. In wild-type cells, Hof1-mNG appeared as a continuous, uniform ring at the bud neck in 93.05% of the cellular population, whereas discontinuous or fragmented Hof1-mNG rings were observed in 82.69% and 71.07% of *elm1Δ* and *gin4Δ* cells, respectively (Fig. 2D, 2E [↗](#)). These observations suggest that Elm1 and Gin4 regulate the proper spatial assembly of various AMR components to ensure robust cytokinesis. The effect on AMR dynamics observed in *elm1Δ* and *gin4Δ* cells may be an indirect consequence mediated through septins, because septin organization is significantly defective in these backgrounds. However, a previous study has shown that disruption of the septin scaffold does not affect cytokinesis, suggesting that Elm1 and Gin4 could regulate AMR organization and constriction independently of their effects on septin organization¹⁷. Together, these results reveal an unappreciated role for Elm1 and Gin4 in AMR assembly, organization, and constriction, opening avenues for future investigation.

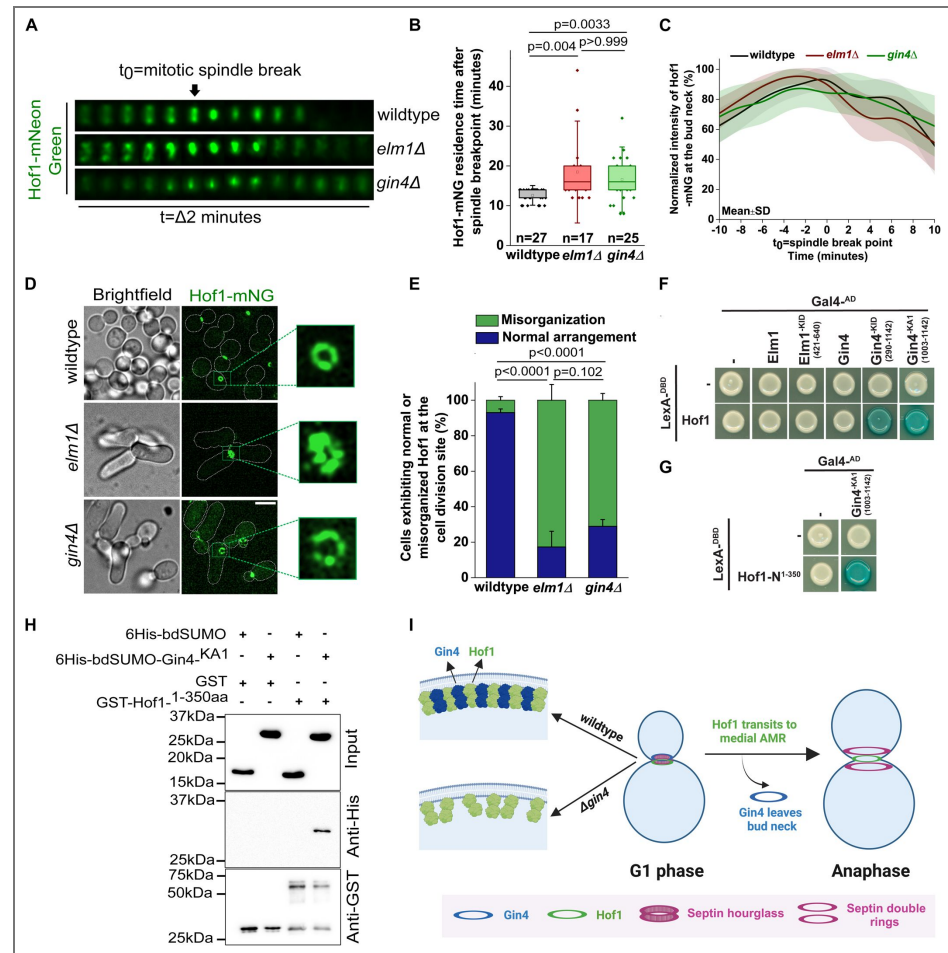


Figure 2. Gin4 regulates Hof1 organization and dynamics through interaction with the N-terminal membrane-binding F-BAR domain.

(A) Representative time-lapse images of Hof1-mNG in wild-type, *elm1Δ* and *gin4Δ* cells imaged at two-minute time intervals (t_0 =spindle breakpoint). (B) Quantification of the residence time of Hof1-mNG after spindle breakpoint in the indicated strains shown in (A), Kruskal-Wallis nonparametric test (**: $p < 0.01$, ns: $p > 0.05$). (C) Normalized fluorescence intensity profile for the temporal kinetics of Hof1-mNG during cytokinesis in the indicated strains shown in (A), (wild-type: $n = 33$, *elm1Δ*: $n = 21$, *gin4Δ*: $n = 23$ cells). (D) Representative images showing the organization of Hof1-mNG at the bud neck in the indicated strains shown in (A). Scale bar-5 μ m. (E) Bar graph showing the percentage of cells exhibiting misorganization of Hof1 at the cell division site in the indicated strains shown in (D), one-way ANOVA with Tukey's multiple-comparison test (****: $p < 0.0001$, ns: $p > 0.05$). p-values correspond to the population of cells exhibiting disorganized Hof1-mNG at the bud neck. (N=3, wild-type: $n = 518$, *elm1Δ*: $n = 225$, *gin4Δ*: $n = 384$ cells). (F) Representative images from the Yeast Two-Hybrid assay depicting the interaction of full-length Gin4, Gin4-KID and Gin4-KA1 with full-length Hof1. (G) Representative Yeast Two-Hybrid images showing the interaction between Gin4-KA1 (1003-1142aa) and N-terminal F-BAR containing domain of Hof1 (1-350aa). (H) Immunoblot showing the in vitro binding assay for the interaction between 6xHis-bdSUMO-Gin4^{KA1} and GST-Hof1^{F-BAR} fragments. GST-tagged Hof1 fragment is immobilized on glutathione resin, and bound 6xHis-bdSUMO-tagged Gin4 fragments were analyzed by SDS PAGE. Input (top) and pull-down fractions (middle) were probed with anti-His antibody, while GST-Hof1 fragments (bottom) were detected using anti-GST antibody, (+) present (-) absent. (I) Proposed model depicting the possible interplay between Gin4 and Hof1 at the plasma membrane interface. Created with [BioRender.com](https://www.biorender.com).

Gin4 directly interacts with the N-terminal F-BAR domain of Hof1 to regulate proper cytokinesis

To understand how the Elm1 and Gin4 kinases affect AMR dynamics, we performed a Yeast Two-Hybrid (Y2H) assay to identify novel interacting partners of these proteins. We found that two Gin4 fragments - Kinase Independent Domain (KID; 290-1142 aa) and Kinase associated-1 (KA1) (1003-1142 aa) but not the full-length protein, interacted with the full-length Hof1 protein (Fig. 2F). Further, the N-terminal Hof1 fragment (1-350 aa), which contains its F-BAR domain, but not the other fragments, interacted with the Gin4-KA1 domain (Fig. 2G, S3H). By contrast, neither full-length Elm1 nor its C-terminal fragment (421-640 aa) showed any interaction with Hof1 in our Y2H assay (Fig. 2F). However, the Y2H assay for detecting protein-protein interactions has inherent limitations, including a bias toward high-affinity interactions and susceptibility to false-positive detection⁸⁶. As a result, biologically relevant interactions may be missed, particularly when one or both interacting partners are membrane-associated, self-activating, require post-translational modifications, or interact indirectly through adaptor proteins⁸⁶. Given these limitations of the Y2H approach, we performed an in-vitro binding assay between the Gin4^{KA1} domain and the N-terminal region of Hof1^{N (1-350 aa)} to independently validate the direct interaction between these proteins.

We purified 6His-bdSUMO-Gin4-KA1 (1003-1142 aa) and GST-Hof1-N-terminus (1-350 aa) fragments from *E. coli*. Strikingly, our results revealed that the N-terminal, membrane-binding, F-BAR-containing domain of Hof1 (1-350 aa) interacts strongly with the C-terminal membrane-binding KA1 domain of Gin4 (1003-1142 aa) (Fig. 2H). The Hof1 N-terminal F-BAR fragment did not pull down the control 6His-bdSUMO fragment but specifically pulled down 6His-bdSUMO-Gin4^{KA1} under identical experimental conditions (Fig. 2H), confirming the specificity of the interaction between Hof1 F-BAR domain and Gin4-KA1.

Overall, these results reveal a direct physical link between the Gin4 and the AMR component Hof1, suggesting a novel and direct mode of regulation of cytokinesis by the septin kinase network in *S. cerevisiae*. The involvement of the membrane-binding interfaces of these proteins also suggests a possible coordinative mechanism in which Gin4 may regulate Hof1 to control its proper assembly and anchoring to the membrane prior to cytokinesis (Fig. 2I). This is supported by the disorganized and irregular Hof1 ring at the cell division site observed in *gin4Δ* cells (Fig. 2D, 2E). Moreover, *gin4Δ* cells also show asymmetric AMR constriction (Fig. 2A), which is a known characteristic phenotype of *hof1Δ* cells⁷⁶. Thus, our data, combined with these line of evidences, strongly suggest that Gin4 exerts direct control over AMR dynamics by physically interacting with Hof1.

To further understand the direct role of Gin4's membrane-binding KA1 domain in cytokinesis and Hof1 dynamics, we generated an endogenously tagged *Gin4-ka1Δ*-GFP strain and compared its localization kinetics with those of *Gin4*-GFP during mitotic spindle breakdown. We observed a marked reduction in *Gin4-ka1Δ*-GFP localization at the bud neck during cytokinesis, suggesting that the KA1 domain contributes to the recruitment of Gin4 (Fig. S4A-S4C). In addition, we analyzed the temporal kinetics of Hof1-mNG at the bud neck in both *gin4Δ* and *Gin4-ka1Δ* cells. Consistent with previous findings by Moravcevic et al. (2010), the *Gin4-ka1Δ* mutant phenocopied the *gin4Δ* deletion, exhibiting a clumped morphology⁵⁷. In both *gin4Δ* and *Gin4-ka1Δ* cells, Hof1-mNG exhibited a comparable constriction pattern after mitotic spindle breakdown, extending beyond 20 minutes (Fig. S4D, S4E). Additionally, Hof1-mNG exhibited similar localization kinetics during cytokinesis in *gin4Δ* and *Gin4-ka1Δ* cells (Fig. S4D, S4F). Taken together, these results strongly suggest that the Gin4-KA1 domain is indispensable for Gin4 function and may directly regulate AMR maturation and constriction by influencing Hof1 dynamics at the cell division site.

Gin4 exerts its control over septin organization and AMR components in a kinase-independent manner

Elm1 kinase activity is essential for its localization to the bud neck but is dispensable for its role in septin stability³⁹. Bni5 is a myosin-recruiting protein that stabilizes and crosslinks septin filaments by binding to the terminal septin Cdc11^{72,87–89}. Elm1 regulates the septin hourglass structure and stability through its binding partner Bni5, independent of its kinase activity³⁹. We therefore asked whether Gin4 kinase activity is required for its functions in the regulation of septin organization and cytokinesis. To address this, we complemented *gin4Δ* cells with the kinase-dead *gin4-K48A* allele^{38,44,90} (henceforth referred to as Gin4^{KD}), integrated into the *leu2* locus. Interestingly, Gin4^{KD}-GFP localized to the bud neck, similar to endogenously tagged Gin4 and Gin4^{FL}-GFP, and complemented the septin mislocalization defects during bud emergence in 78% of total *gin4Δ* cells (Fig. 3A, 3B [↗](#)). Additionally, expression of Gin4^{KD}-GFP in *gin4Δ* cells restored the residence time of Inn1-mNG at the bud neck to ~5-7 minutes and the temporal kinetics during cytokinesis, similar to the wild-type strain (Fig. 3C-3E [↗](#)). Furthermore, Gin4^{KD} restored Hof1 misorganization defects at the cell division site when expressed in *gin4Δ* cells in 77.14% of the cellular population (Fig. S3F, S3G [↗](#)). Gin4 kinase is known to stabilize septins by phosphorylating its substrate, Shs1⁴⁹. Hof1 has been shown to exhibit synthetic lethality with Gin4, possibly by regulating septins in a parallel pathway⁷⁴. To test whether this synthetic lethality depends on the kinase activity of Gin4, we expressed Gin4^{FL} and Gin4^{KD} in cells carrying the *gin4Δ hof1Δ* double deletion (Fig. S3I [↗](#)). Interestingly, Gin4^{KD} rescued the synthetic lethality, similar to Gin4^{FL}, suggesting that the kinase activity of Gin4 is not required to restore viability in the absence of Hof1 (Fig. S3I [↗](#)). These findings provide a strong evidence that Gin4 kinase activity is largely dispensable for its localization and for its functions in septin organization and cytokinesis, addressing an important aspect of how Gin4 may perform these functions in the absence of catalysis while raising several new questions.

Hsl1 kinase can partially compensate for Elm1 function upon forced tethering to the bud neck

Marquardt et al. (2024) showed that Gin4 and Elm1 exhibit reciprocal regulation during different cell cycle stages, and that their localization to the bud neck is interdependent³⁸. The study also reported that Gin4 recruitment and localization to the bud neck is hampered as the cell cycle progresses in *elm1Δ* cells³⁸. We independently corroborated these results and observed that Gin4 was mislocalized to the bud cortex during bud emergence (Fig. S5A [↗](#)) and exhibited defective localization to the bud neck during later cell cycle stages in *elm1Δ* cells (Fig. S5B-S5D [↗](#)). Next, we asked whether restoring Gin4 localization to the bud neck could rescue the elongated morphology in cells lacking Elm1. To test this hypothesis, we performed an artificial tethering assay using the GFP-GBP strategy (Fig. 4A [↗](#)). The GFP-Binding Protein (GBP) is a ~13 kDa soluble protein derived from the heavy chain of camelid (*llama*) antibodies⁹¹. This single-domain antibody binds GFP-tagged proteins with very high affinity, in the picomolar range. GBP also binds other GFP variants, such as Yellow Fluorescent Protein (YFP), with a strict 1:1 stoichiometric ratio⁹². By contrast, GBP does not bind to Cyan Fluorescent Protein (CFP) or to derivatives of DsRed, including monomeric Red Fluorescent Protein (mRFP) and mOrange⁹². Owing to its high binding affinity and specificity, the GFP-GBP system was initially developed for the purification of GFP-tagged proteins^{91,92}. More recently, this strategy has been adapted to artificially redirect proteins to non-canonical subcellular locations^{38,91,92}. Based on this approach, we tagged the terminal septin Shs1^{49,93} and the bud neck-localizing septin-associated proteins (SAPs) the anillin-like protein - Bud4⁹⁴, Bni5³⁹, and Hsl1 with GBP at their C-termini. These GBP-tagged proteins were then used to redirect mislocalized Gin4-GFP to the bud neck, allowing us to assess whether forced relocalization of Gin4 could rescue the elongated morphology exhibited by *elm1Δ* cells.

Forced tethering of Gin4-GFP to the bud neck via its related kinase Hsl1-GBP restored normal cellular morphology in 63.75% of the population in *elm1Δ* cells (Fig. 4B, 4C [↗](#), Table 1 [↗](#)). To quantitatively assess this rescue, we also analyzed the aspect ratio, measured as the ratio of the

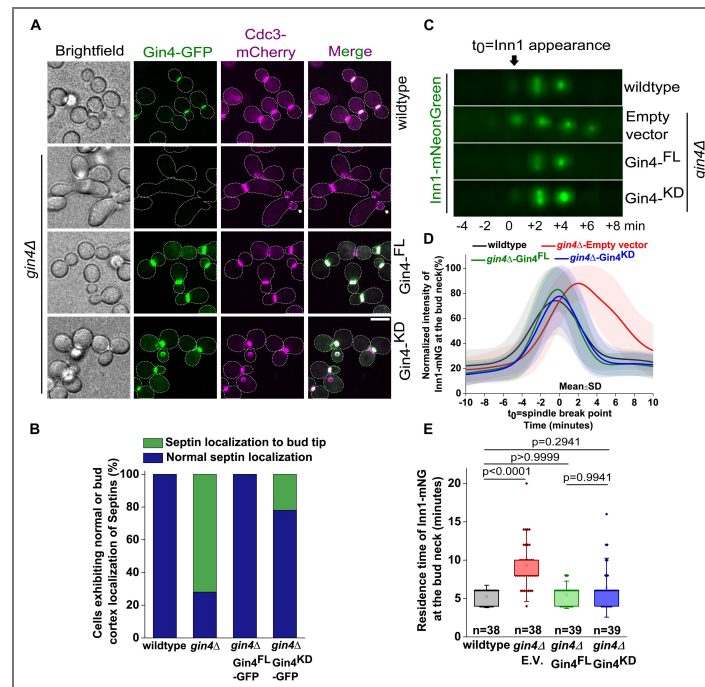


Figure 3. Gin4 controls septin organization and AMR dynamics independently of its kinase activity

(A) Representative images showing septin defects or mislocalization during bud emergence in *gin4Δ* cells and rescue of the mislocalization by *Gin4^{FL}-GFP* and *Gin4^{KD}-GFP* constructs expressed under pTEF promoter. White arrows indicate mislocalized Cdc3-mcherry in the represented strains. Scale bar=5μm. (B) Quantitation of septin mislocalization and its rescue in the indicated strains shown in (A) (wild-type: n=38, *gin4Δ*: n=32, *gin4Δ-Gin4^{FL}-GFP*: n=32, *gin4Δ-Gin4^{KD}-GFP*: n=36 cells). (C) Representative time-lapse images showing Inn1-mNG dynamics acquired at two-minute time intervals in *gin4Δ* cells expressing *Gin4^{FL}* and *Gin4^{KD}* constructs cloned under endogenous promoter (t_0 =appearance of Inn1-mNG at the bud neck). (D) Plot of normalized fluorescence intensity of Inn1-mNG in indicated strains shown in (C). A population of *gin4Δ*-Empty vector cells exhibiting asymmetric constriction is plotted in the graph (t_0 =spindle breakpoint) (wild-type: n=38, *gin4Δ*-Empty vector: n=21, *gin4Δ-Gin4^{FL}*: n=39, *gin4Δ-Gin4^{KD}*: n=31 cells). (E) Quantification of the residence time of Inn1-mNG during cytokinesis in indicated strains of (C), Kruskal-Wallis nonparametric test (****: $p<0.0001$, ns: $p>0.05$).

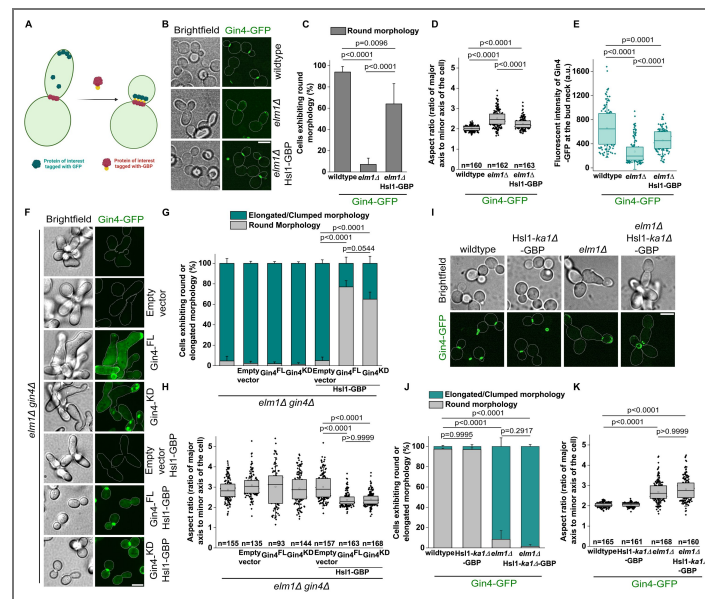


Figure 4. Artificial tethering of Gin4-GFP to the bud neck via Hsl1-GBP restores functionality in *elm1Δ* cells in a Gin4 kinase-independent manner.

(A) Schematic representation of GFP-GBP artificial tethering strategy, in which the protein of interest is targeted the bud neck by C-terminal GFP tagging, while the binding partner is tagged with GBP. Created with *BioRender.com*. **(B)** Representative images showing artificial tethering of Gin4-GFP with Hsl1-GBP in *elm1Δ* cells. Scale bar-5μm. **(C)** Bar graph showing the percentage of cells exhibiting round morphology in the indicated strains shown in (B), one-way ANOVA with Tukey's multiple-comparison test (**: $p < 0.01$, ****: $p < 0.0001$), (N=3, wild-type: n=407, *elm1Δ*: n=508, *elm1Δ*-Hsl1-GBP: n=400 cells). **(D)** Quantification of aspect ratios in the indicated strains shown in (B), Kruskal-Wallis nonparametric statistical test (****: $p < 0.0001$), (N=3, n>150 cells/strain). **(E)** Graph depicting the raw fluorescence intensity of Gin4-GFP at the large bud in the indicated strains shown in (B), Kruskal-Wallis nonparametric statistical test (****: $p < 0.001$, ****: $p < 0.0001$) (N=3, wild-type: n=125, *elm1Δ*: n=125, *elm1Δ*-Hsl1-GBP: n=118 cells). **(F)** Representative images showing the localization of Gin4^{FL}-GFP and Gin4^{KD}-GFP constructs expressed under the pTEF promoter and their tethering to bud neck with Hsl1-GBP in *elm1Δ gin4Δ* double-deletion strains. Scale bar-5μm. **(G)** Stacked column graph showing the percentage of cells exhibiting round and elongated/clumped morphologies in the indicated strains shown in (F), one-way ANOVA with Tukey's multiple-comparison test (****: $p < 0.0001$, ns: $p > 0.05$). p-values correspond to population of cells exhibiting round morphology (N=3, *elm1Δ gin4Δ*: n=339, *elm1Δ gin4Δ*-Empty vector: n=327, *elm1Δ gin4Δ*-Gin4^{FL}: n=173, *elm1Δ gin4Δ*-Gin4^{KD}: n=236, *elm1Δ gin4Δ*-Empty vector-Hsl1-GBP: n=356, *elm1Δ gin4Δ*-Gin4^{FL}-Hsl1-GBP: n=365, *elm1Δ gin4Δ*-Gin4^{KD}-Hsl1-GBP: n=459 cells). **(H)** Quantification of aspect ratios in the indicated strains shown in (F), Kruskal-Wallis nonparametric statistical test (****: $p < 0.0001$, ns: $p > 0.05$) (N=3, n>90 cells/strain). **(I)** Representative images showing artificial tethering of Gin4-GFP with Hsl1-*ka1Δ*-GBP in *elm1Δ* cells. Scale bar-5μm. **(J)** Stacked column graph showing the percentage of cells exhibiting round and elongated/clumped morphologies in the indicated strains shown in (I), one-way ANOVA with Tukey's multiple comparison test (****: $p < 0.0001$, ns: $p > 0.05$) p-values correspond to the population of cells exhibiting round morphology (N=3, wild-type: n=674, Hsl1-*ka1Δ*-GBP: n=419, *elm1Δ*: n=572, *elm1Δ* Hsl1-*ka1Δ*-GBP: n=337 cells). **(K)** Quantification of aspect ratios in the indicated strains shown in (I), Kruskal-Wallis nonparametric statistical test (****: $p < 0.0001$, ns: $p > 0.05$), (N=3, n>150 cells/strain).

major axis to the minor axis of the cell. As expected, the mean aspect ratio was higher in *elm1Δ* cells, than in wild-type cells, reflecting their elongated morphology (Fig. 4D [↗](#)), and decreased in cells where Gin4-GFP was tethered to the bud neck with Hsl1-GBP, corroborating the rescue of cell morphology observed after restoring Gin4-GFP localization to the bud neck (Fig. 4D [↗](#)). We measured fluorescence intensities of Gin4-GFP with and without tethering to the bud neck via Hsl1-GBP in large-budded *elm1Δ* cells, which validated that Gin4-GFP was indeed tethered to the bud neck (Fig. 4E [↗](#)). In contrast to the rescue observed after tethering with Hsl1-GBP, artificial tethering of Gin4-GFP via other bud neck proteins Shs1, Bni5, or Bud4 did not rescue the elongated phenotype of *elm1Δ* cells, despite restoration of bud neck localization (Fig. S5E-S5G [↗](#)). These results suggest that the observed rescue may be due to two underlying causes: (i) restoration of Gin4 protein at the bud neck, and (ii) tethering of Gin4 to Hsl1.

To test whether Gin4 kinase activity is required for the observed rescue in cellular morphology upon artificial tethering of Gin4-GFP to the bud neck with Hsl1-GBP. We tethered Gin4^{FL}-GFP and Gin4^{KD}-GFP, expressed under the pTEF promoter, via Hsl1-GBP in *gin4Δ elm1Δ* cells, and observed that both constructs restored cellular morphology and aspect ratio to wild-type levels in 77.26% and 65.57% of cells, respectively (Fig. 4F-4H [↗](#), Table 1 [↗](#)). These results suggest that Gin4 tethering to the bud neck via Hsl1 may compensate for Elm1 function in a kinase-independent manner, further reinforcing the kinase-independent nature of Gin4 function. Because Hsl1 also contains a membrane-binding C-terminal KA1 domain^{51,57}, we tested its requirement for the observed functional rescue in *elm1Δ* cells and found that artificial tethering of Gin4-GFP via Hsl1-*ka1Δ*-GBP did not rescue the cellular morphology of *elm1Δ* cells (Fig. 4I-4K [↗](#)). Together, these observations suggest that (i) restoring Gin4 localization to the bud neck via Hsl1 is necessary to partially rescue the morphological defects and cell shape exhibited by *elm1Δ* cells (ii) Gin4 might form a complex with Hsl1, which becomes essential in the absence of Elm1, indicating that Elm1 might play a significant role in regulating the Gin4-Hsl1 complex and (iii) proper membrane tethering of Hsl1 via its KA1 domain may be essential for its function in *elm1Δ* cells.

Septin and cytokinesis defects are rescued by artificial tethering of Gin4-GFP to the bud neck via Hsl1-GBP in *elm1Δ* cells

Rescue of cell shape alone does not necessarily imply restoration of underlying physiological processes such as septin organization, AMR dynamics, or cytokinesis. To address this, we analyzed septin dynamics using Cdc3-mCherry in *elm1Δ* cells with and without artificial tethering of Gin4-GFP to the bud neck via Hsl1-GBP. Specifically, we examined the initial recruitment of septins during bud emergence. Consistent with previous results^{38,39} and our observations, 100% of *elm1Δ* cells exhibited mislocalization of Cdc3-mCherry to the bud cortex during bud emergence (Fig. 5A, 5B [↗](#)). By contrast, only 35.71% of cells showed septin mislocalization when Gin4-GFP was artificially tethered to the bud neck via Hsl1-GBP (Fig. 5A, 5B [↗](#)). We also analyzed the localization kinetics of Gin4-GFP in these strains and found that nearly 100% of *elm1Δ* cells exhibited Gin4-GFP mislocalization to the bud cortex in the absence of tethering, whereas this was reduced to 42.85% upon tethering via Hsl1-GBP (Fig. 5A, 5C [↗](#)). The extent of rescue in Cdc3-mCherry mislocalization (64.28%) and Gin4-GFP mislocalization (57.14%) in *elm1Δ* cells closely correlated with the fraction of cells that also exhibited rescue of cellular morphology upon tethering with Hsl1-GBP (Fig. 4B-4C [↗](#), 5A-5C [↗](#)).

Protein tagged with GFP	Protein tagged with GBP	Genetic background	Number of cells exhibiting round morphology	Number of cells exhibiting elongated/clumped morphology	Total number of cells	Percentage of cellular population exhibiting round morphology	Rescue of elongated or clumped morphology
Gin4	Shs1	<i>elm1</i> Δ	24	464	488	4.91	No
Gin4	Bud4	<i>elm1</i> Δ	20	451	471	4.24	No
Gin4	Hsl1	<i>elm1</i> Δ	255	145	400	63.75	Yes
Gin4	Hsl1- <i>ka1</i> Δ	<i>elm1</i> Δ	6	331	337	1.78	No
Gin4	Bni5	<i>elm1</i> Δ	17	431	448	3.79	No
Gin4-Full length	Hsl1	<i>elm1</i> Δ <i>gin4</i> Δ	282	83	365	77.26	Yes
Gin4-Kinase dead	Hsl1	<i>elm1</i> Δ <i>gin4</i> Δ	301	158	459	65.57	Yes
Hsl1	Shs1	<i>elm1</i> Δ	264	462	726	36.36	Yes
Hsl1	Bud4	<i>elm1</i> Δ	110	607	717	15.34	No
Hsl1	Bni5	<i>elm1</i> Δ	4	354	358	1.11	No
Hsl1	Gin4	<i>elm1</i> Δ	521	233	754	69.09	Yes
Hsl1	Kcc4	<i>elm1</i> Δ	498	364	862	57.77	Yes
Kcc4	Shs1	<i>elm1</i> Δ	21	566	587	3.57	No
Kcc4	Bud4	<i>elm1</i> Δ	60	603	663	9.04	No
Kcc4	Hsl1	<i>elm1</i> Δ	516	192	708	72.88	Yes
Kcc4	Bni5	<i>elm1</i> Δ	6	563	569	1.05	No
Kcc4	Gin4	<i>elm1</i> Δ	119	364	483	24.63	No
Elm1	Bud4	<i>gin4</i> Δ	616	270	886	69.52	Yes
Elm1	Hsl1	<i>gin4</i> Δ	399	289	688	57.99	Yes
Elm1	Hsl1- <i>ka1</i> Δ	<i>gin4</i> Δ	139	470	609	22.82	No
Elm1	Shs1	<i>gin4</i> Δ	499	301	800	62.375	Yes

Table 1. Table summarizing the rescue of elongated or clumped morphology upon redirecting the Nim1-related kinases to the bud neck in *elm1*Δ cells and Elm1-GFP in *gin4*Δ cells using GFP-GBP artificial tethering strategy.

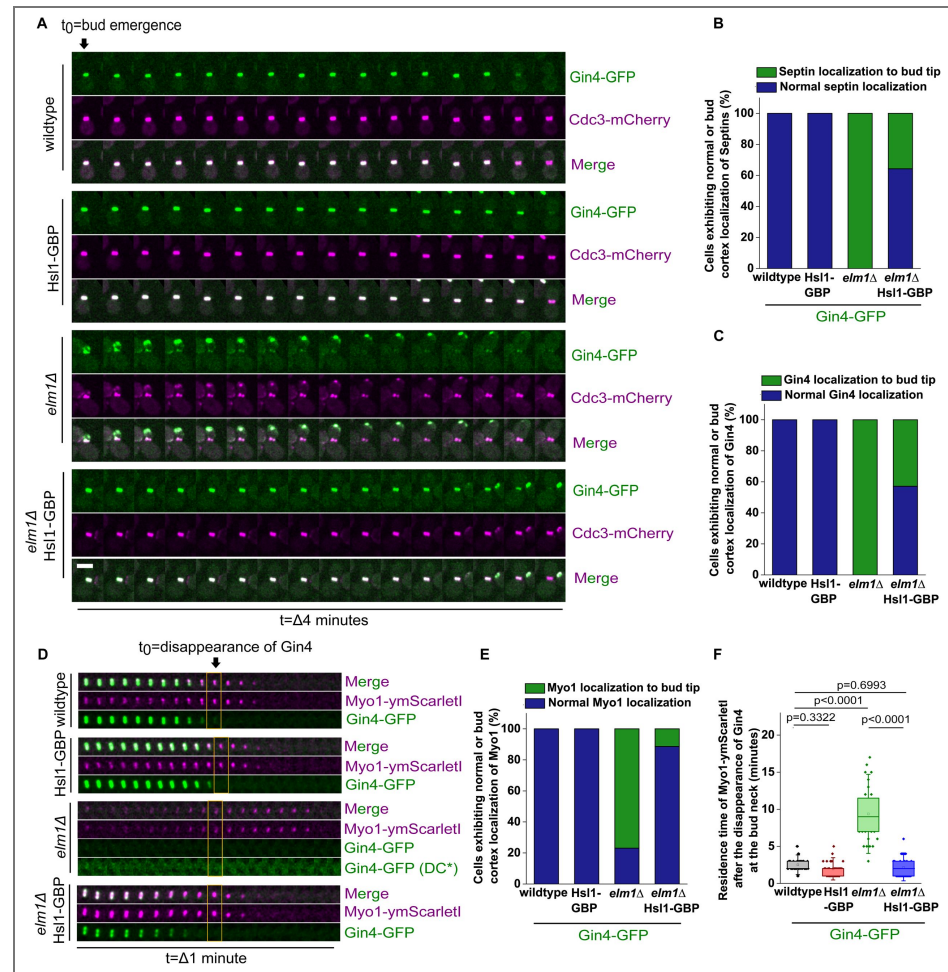


Figure 5. Redirecting Gin4 to the bud neck via Hsl1 in *elm1* Δ cells rescues septin organization and AMR dynamics.

(A) Representative time-lapse montages showing the rescue of mislocalization of both Gin4-GFP and Cdc3-mCherry upon artificial tethering with Hsl1-GBP in *elm1* Δ cells. Scale bar=5 μ m. (B) Stacked column graph showing the percentage of cells exhibiting normal septin localization or septin mislocalization to bud cortex during the bud emergence in the indicated strains shown in (A) (wild-type: n=39, Hsl1-GBP: n=39, *elm1* Δ : n=39, *elm1* Δ -Hsl1-GBP: n=42 cells). (C) Quantification of Gin4-GFP localization rescue in the indicated strains shown in (A) (wild-type: n=39, Hsl1-GBP: n=39, *elm1* Δ : n=39, *elm1* Δ -Hsl1-GBP: n=42 cells). (D) Representative montages of the bud neck showing the constriction kinetics of Myo1-ymScarletI in *elm1* Δ strains in which Gin4-GFP is artificially tethered to bud neck via Hsl1-GBP. (E) Quantitative analysis for normal localization and mislocalization of Myo1-ymScarletI in the indicated strains shown in (D) (wild-type: n=41, Hsl1-GBP: n=37, *elm1* Δ : n=39, *elm1* Δ -Hsl1-GBP: n=44 cells). (F) Graph showing the constriction dynamics of Myo1-ymScarletI in the indicated strains shown in (D). Disappearance of Gin4 from the bud neck is used to normalize the initial time point for Myo1 constriction. Kruskal-Wallis nonparametric statistical test (****: $p<0.0001$, ns: $p>0.05$) (wild-type: n=39, Hsl1-GBP: n=30, *elm1* Δ : n=32, *elm1* Δ -Hsl1-GBP: n=39 cells).

To determine whether AMR defects were similarly rescued, we analyzed the dynamics of Myo1 C-terminally tagged with ymScarletI during cytokinesis. In *elm1* Δ cells, 76.92% of cells displayed Myo1 mislocalization to the bud cortex during its recruitment, consistent with previous reports³⁹. Upon artificial tethering of Gin4-GFP to the bud neck via Hsl1-GBP, the proportion of cells exhibiting Myo1 mislocalization was reduced to 11.36% (Fig. 5D, 5E). Additionally, we quantified the residence time of Myo1 at the bud neck by defining the disappearance of Gin4 as t_0 , corresponding to the onset of constriction, and measuring the time intervals until complete loss of the Myo1 signal from the bud neck. In wild-type cells, Myo1 constriction was completed within

approximately 4 minutes, whereas in *elm1Δ* cells this process was prolonged to nearly 10 minutes (Fig. 5D, 5F). The residence time was rescued to ~4 minutes upon tethering Gin4-GFP to the bud neck with Hsl1-GBP in *elm1Δ* cells (Fig. 5D, 5F).

Taken together, these results indicate that (i) rescue of cellular morphology correlates with restoration of both Gin4 and septin localization dynamics as the cell cycle progresses, suggesting a direct mode of regulation of Gin4 and Hsl1 downstream of Elm1 and (ii) restoration of AMR dynamics in *elm1Δ* cells upon artificial tethering of Gin4 to the bud neck via Hsl1 suggests an unconventional role for Hsl1 in the absence of Elm1.

Rescue of septin localization in *elm1Δ* cells upon redirecting Gin4-GFP to the bud neck via Hsl1-GBP is largely independent of the morphogenetic checkpoint

Swe1 is a checkpoint kinase that regulates the morphogenetic signaling pathway and plays a key role in controlling cell shape by regulating isometric cell growth^{41,59,61}. Previous studies have shown that Elm1, Gin4, Hsl1, and Kcc4 negatively regulate Swe1 through inhibitory phosphorylation, thereby promoting isometric growth and maintaining a round cell morphology^{41,59}. It has also been reported that deletion of Swe1 in combination with septin kinase deletions (*elm1Δ swe1Δ*, *gin4Δ swe1Δ*, or *hsl1Δ swe1Δ*) rescues the elongated and clumped morphologies associated with the corresponding individual kinase deletions^{41,61}. However, this rescue is specific to cell shape and does not restore septin localization or cytokinesis defects^{41,61}. Based on these observations, we asked whether the rescue of cellular morphology and aspect ratio observed in *elm1Δ* cells upon artificial redirection of Gin4-GFP to the bud neck via Hsl1-GBP is mediated through negative regulation of Swe1 during mitotic spindle breakdown, or instead represents a direct consequence of restoring Gin4 and Hsl1 localization.

To address this, we deleted Swe1 in *elm1Δ* cells in which Gin4-GFP was either untethered or artificially tethered to the bud neck using Hsl1-GBP. Consistent with Bouquin et al., (2000)⁶¹, deletion of Swe1 alone rescued the elongated morphology in 60.66% of the *elm1Δ* population when Gin4-GFP was not tethered to the bud neck (Fig. S6A-S6C). Strikingly, deletion of Swe1 did not alter the proportion of round cells in *elm1Δ* cells in which Gin4-GFP was redirected to the bud neck via Hsl1-GBP (Fig. S6A-S6C).

We further analyzed septin organization using Cdc3-mCherry in *elm1Δ* and *elm1Δ swe1Δ* cells, with and without artificial tethering of Gin4-GFP to the bud neck via Hsl1-GBP. Although cell shape was rescued in both *elm1Δ swe1Δ* control cells and *elm1Δ swe1Δ* cells with Gin4-GFP tethered via Hsl1-GBP, only 23.63% of cells in the *elm1Δ swe1Δ* control strain exhibited rescue of septin mislocalization during bud emergence (Fig. 6A, 6B). This partial rescue by Swe1 deletion also correlated with restoration of mislocalized Gin4 during bud emergence in *elm1Δ* cells (Fig. 6C, 6D). Notably, more than 70% of cells exhibited rescue of both Gin4 and Cdc3 mislocalization in *elm1Δ swe1Δ* cells when Gin4-GFP was artificially tethered to the bud neck via Hsl1-GBP, in contrast to the rescue exhibited by only ~23% of cells in which Gin4-GFP was not tethered (Fig. 6A-6D).

Collectively, these findings suggest that (i) the rescue of elongated cell morphology to normal round cell morphology in *elm1Δ* cells upon artificial tethering of Gin4-GFP to the bud neck via Hsl1-GBP may depend on Hsl1-mediated regulation of the morphogenesis checkpoint; however, (ii) the observed rescue in septin and Gin4 localization clearly does not depend on the morphogenesis checkpoint and reflects direct regulation of septin organization by Gin4 and Hsl1.

Intrigued by the rescue in cellular morphology observed only after tethering Gin4-GFP to Hsl1-GBP and not to other bud neck proteins, we extended our assay to its related kinases and tethered Hsl1-GFP and Kcc4-GFP via various bud-neck proteins in *elm1Δ* cells (Fig. 7A-7C and S7A-S7C). Our results revealed that Hsl1-GFP restored normal cell morphology in 36.36% of cells in *elm1Δ* when tethered with Shs1-GBP, while tethering with Gin4-GBP or Kcc4-GBP resulted in significantly higher rescue of 69.09% and 57.77%, respectively, suggesting possible coordination among these kinases (Fig. 7A-7C, Table 1). Strikingly, like Gin4-GFP, Kcc4-GFP tethering also restored cell

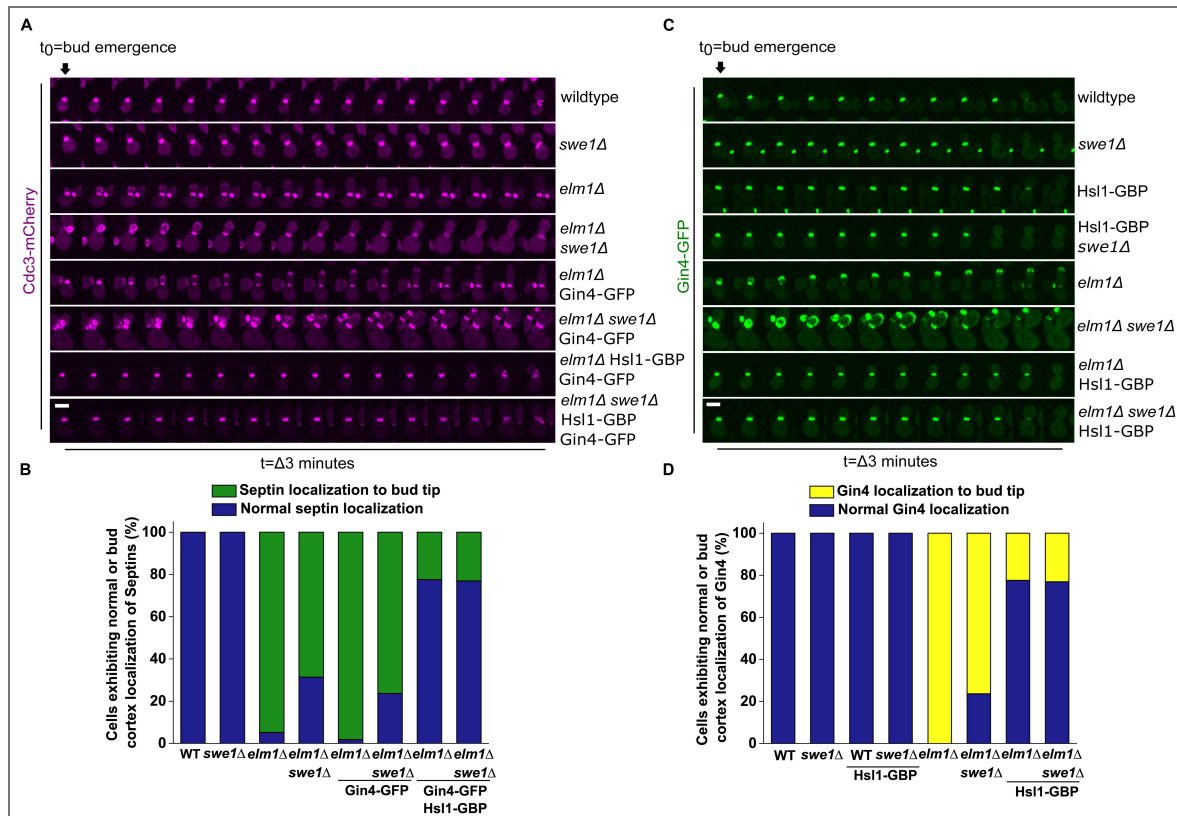


Figure 6. Gin4 tethering to the bud neck via Hsl1 bypasses the requirement for Elm1 independently of the morphogenetic checkpoint kinase Swe1.

(A) Time-lapse montages representing the dynamics of Cdc3-mCherry in both *elm1Δ* and *elm1Δ swe1Δ* strains upon artificial tethering of Gin4-GFP to bud neck with Hsl1-GBP (t_0 = bud emergence). Scale bar=5μm. (B) Stacked column graph showing the percentage of cells exhibiting normal localization and mislocalization of Cdc3-mCherry in the indicated strains shown in (A) (wild-type: n=55, *swe1Δ*: n=49, *elm1Δ*: n=58, *elm1Δ swe1Δ*: n=51, *elm1Δ*-Gin4-GFP: n=55, *elm1Δ swe1Δ*-Gin4-GFP: n=55, *elm1Δ*-Gin4-GFP-Hsl1-GBP: n=58, *elm1Δ swe1Δ*-Gin4-GFP-Hsl1-GBP: n=52 cells). (C) Representative time-series montages showing the localization of Gin4-GFP in both *elm1Δ* and *elm1Δ swe1Δ* strains upon artificial tethering of Gin4-GFP to the bud neck via Hsl1-GBP (t_0 = bud emergence). Scale bar=5μm. (D) Quantification graphs depicting the normal localization and mislocalization of Gin4-GFP at the bud cortex during bud emergence in the indicated strains shown in (C) (wild-type: n=57, *swe1Δ*: n=52, Hsl1-GBP: n=54, Hsl1-GBP *swe1Δ*: n=51, *elm1Δ*-Gin4-GFP: n=55, *elm1Δ swe1Δ*-Gin4-GFP: n=55, *elm1Δ*-Gin4-GFP-Hsl1-GBP: n=58, *elm1Δ swe1Δ*-Gin4-GFP-Hsl1-GBP: n=52 cells).

morphology similar to wild-type in 72.88% of the population, only when tethered to the bud neck with Hsl1-GBP (Fig. S7A-S7C [↗](#), Table 1 [↗](#)). However, Kcc4-GFP tethering via different bud neck proteins did not rescue the phenotype, although a minor rescue in 24.63% of cells was observed upon tethering with Gin4-GBP, suggesting possible crosstalk between Kcc4 and Gin4 (Fig. S7A-S7C [↗](#), Table 1 [↗](#)).

To support these results, we analyzed the localization of Hsl1 and Kcc4 in *elm1Δ* and *gin4Δ* cells. As reported previously³⁹, Kcc4 exhibited mislocalization to the bud cortex during the initial phases of the cell cycle and defective localization at the onset of cytokinesis (Fig. S8B-S8E [↗](#)). Recruitment and localization of Hsl1 to the bud neck were also severely impaired in *elm1Δ* cells (Fig. 7D-7F [↗](#), S8A [↗](#)), suggesting a role for Elm1 in maintaining Hsl1 localization at the bud neck. By contrast, recruitment and localization of Hsl1 and Kcc4 were similar to wild-type in *gin4Δ* cells (Fig. 7D-7F [↗](#), S8A-S8E [↗](#)). These data suggest that loss of Hsl1 at the bud neck may contribute significantly to the elongated phenotype observed in *elm1Δ* cells, and, consistent with this idea, restoration of Hsl1 localization rescues the observed morphological defects in our experiments. The degree of phenotypic rescue appears to depend on the presence of Hsl1 at the bud neck, and higher rescue percentages are observed when Hsl1 is tethered with another kinase (Gin4 or Kcc4), suggesting coordinated effects among these kinases and that Hsl1 may act as a downstream effector in the septin kinase network.

Our results reveal that Hsl1 regulates normal cell morphology, possibly through downstream signaling events triggered by the presence of Elm1 in wild-type cells, in coordination with either Gin4 or Kcc4. Thus, Elm1 may act upstream to control the localization and activation of the Nim1-related kinases (Hsl1, Gin4, and Kcc4) at the septin scaffold in a precise, spatiotemporal manner, within a network with multiple feedback regulatory links. Overall, the ability to restore normal function by simply rewiring the septin kinase network demonstrates the inherent redundancy and robustness of this network in *S. cerevisiae*.

Hsl1 and its membrane-binding ability are required for the rescue of *gin4Δ* cells by artificially tethered Elm1

Elm1 does not localize to the bud neck in the absence of Gin4 and can rescue the elongated/clumped phenotype exhibited by *gin4Δ* cells upon artificial tethering to the bud neck septin scaffold³⁸. We corroborated these findings independently and found that Elm1 is not recruited to the bud neck in *gin4Δ* cells (Fig. S9A-S9D [↗](#)). We found that Elm1-GFP tethering via Shs1-GBP, Bud4-GBP, or Hsl1-GBP could restore normal cell morphology and aspect ratio in *gin4Δ* cells (Fig. S9E-S9G [↗](#)). We therefore asked whether the presence of Hsl1 is necessary for the observed rescue upon Elm1 tethering to the bud neck in *gin4Δ* cells. To test this, we artificially tethered Elm1-GFP to the bud neck via Shs1-GBP in *gin4Δ* and *gin4Δ hsl1Δ* cells. While 62.37% of *gin4Δ* cells showed restoration of cell morphology and aspect ratio, no rescue was observed in the *gin4Δ hsl1Δ* double deletion strain upon tethering Elm1 to the bud neck, indicating that Hsl1 is required downstream of Elm1 tethering in *gin4Δ* cells (Fig. 8A [↗](#), 8C, 8D [↗](#), Table 1 [↗](#)). Additionally, we observed rescue of septin misorganization upon tethering Elm1-GFP to the bud neck with Shs1-GBP in *gin4Δ* cells in 82.92% of the cellular population, which was lost in *gin4Δ hsl1Δ* cells in >75% of the population (Fig. 8A, 8E [↗](#)). Redirected Elm1-GFP also showed mislocalization to the bud cortex during bud emergence in *gin4Δ hsl1Δ* cells (Fig. 8F [↗](#)). A loss of rescue was observed when Elm1-GFP was tethered to the bud neck in *gin4Δ* cells via Hsl1-*ka1Δ*-GBP, suggesting that Hsl1 acts downstream of Elm1 and that its membrane-binding KA1 domain is required for its function (Fig. S9H-S9J [↗](#)). These data support the idea that Hsl1 acts downstream of Elm1 and Gin4 in the septin kinase network to control septin stability.

Next, we asked whether Elm1-GFP tethering to the bud neck in *gin4Δ* cells could rescue other observed cellular defects related to AMR dynamics during cytokinesis. To test this, we first assessed the recruitment and localization of the Myo1 during bud emergence. We observed that Myo1-3xmCherry exhibited mislocalization to the bud tip in 12.82% *gin4Δ* cells during bud emergence (Fig. S10A, S10B [↗](#)). However, Myo1-3xmCherry localization was restored to the bud neck upon artificial tethering of Elm1-GFP in *gin4Δ* cells. The cellular population with mislocalized

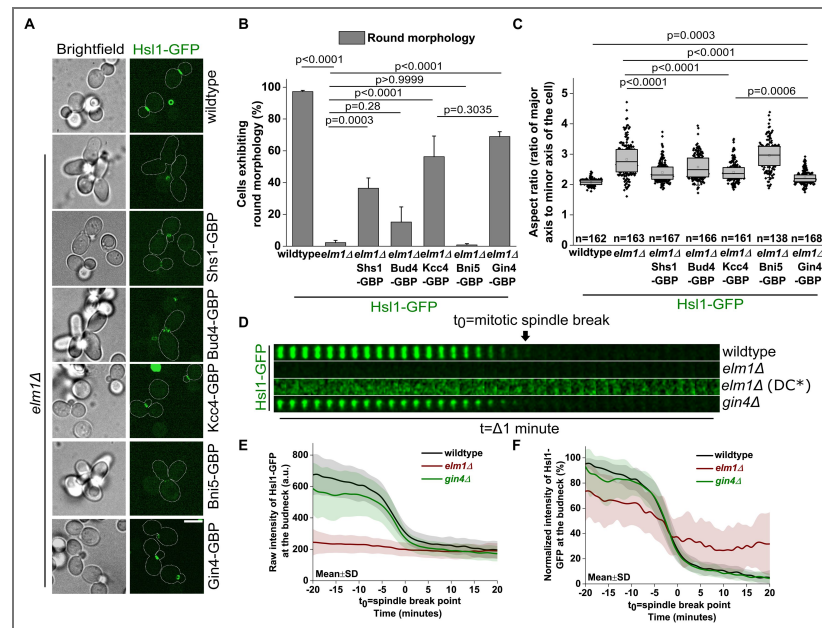


Figure 7. Restoring Hsl1-GFP localization to the bud neck via septins or its related kinases can bypass the requirement of Elm1.

(A) Representative images showing artificial tethering of Hsl1-GFP to the bud neck via Shs1-GBP, Bud4-GBP, Kcc4-GBP, Bni5-GBP and Gin4-GBP in *elm1Δ* cells. Scale bar-5μm. (B) Bar graph showing the percentage of cells exhibiting round morphology in the indicated strains shown in (A), one-way ANOVA with Tukey's multiple comparison test (****: $p < 0.0001$, ns: $p > 0.05$) (N=3, wild-type: n=724, *elm1Δ*: n=458, *elm1Δ*-Shs1-GBP: n=726, *elm1Δ*-Bud4-GBP: n=717, *elm1Δ*-Kcc4-GBP: n=862, *elm1Δ*-Bni5-GBP: n=358 and *elm1Δ*-Gin4-GBP: n=754 cells). (C) Quantification of aspect ratios in the indicated strains shown in (A), Kruskal-Wallis nonparametric statistical test (***: $p < 0.001$, ****: $p < 0.0001$) (N=3, n>135 cells/strain). (D) Representative montages of the bud neck depicting the localization of Hsl1-GFP in *elm1Δ* and *gin4Δ* cells during cytokinesis. DC*=Differential contrast. (E) Plot of raw fluorescence intensity of Hsl1-GFP in the indicated strains shown in (D) (t_0 =spindle breakpoint) (wild-type: n=29, *elm1Δ*: n=24, *gin4Δ*: n=38 cells). (F) Plot of normalized fluorescence intensity of Hsl1-GFP in the indicated strains shown in (D) (t_0 =spindle breakpoint).

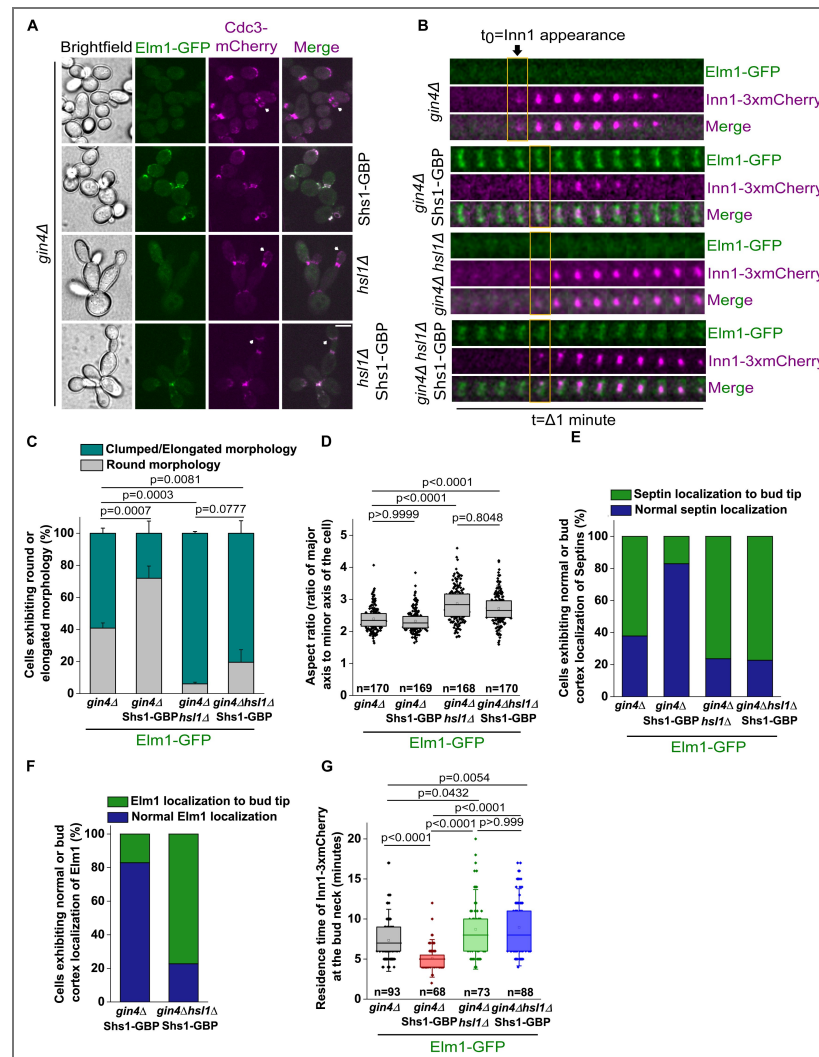


Figure 8. A non-canonical role for Hsl1-Kinase in regulating septin organization and AMR dynamics.

(A) Representative images showing Cdc3-mCherry mislocalization during bud emergence in *gin4Δ* and *gin4Δ hsl1Δ* cells in which Elm1-GFP is artificially tethered to bud neck via Shs1-GBP. White arrows indicate mislocalized Cdc3-mCherry in the represented strains. Scale bar=5μm. (B) Representative time-lapse montages of the bud neck showing the constriction profile of Inn1-3xmCherry in *gin4Δ* and *gin4Δ hsl1Δ* cells in which Elm1-GFP is artificially tethered to bud neck via Shs1-GBP. (C) Bar graph showing the percentage of cells exhibiting round and elongated morphologies in the indicated strains shown in (A), one-way ANOVA with Tukey's multiple-comparison test (**: $p < 0.01$, ***: $p < 0.001$, ns: $p > 0.05$). p-values correspond to population of cells exhibiting round morphology, (N=3, *gin4Δ*: n=689, *gin4Δ*-Shs1-GBP: n=639, *gin4Δ hsl1Δ*: n=492, *gin4Δ hsl1Δ*-Shs1-GBP: n=590 cells). (D) Quantification of aspect ratios in the indicated strains shown in (A), Kruskal-Wallis nonparametric statistical test (****: $p < 0.0001$, ns: $p > 0.05$), (N=3, n=165 cells/strain). (E) Stacked column graph representing the percentage of cells exhibiting normal septin localization and septin mislocalization in the indicated strains of (A) (*gin4Δ*: n=44, *gin4Δ*-Shs1-GBP: n=41, *gin4Δ hsl1Δ*: n=38, *gin4Δ hsl1Δ*-Shs1-GBP: n=44 cells). (F) Quantification of normal localization and mislocalization of Elm1-GFP in the indicated strains shown in (A) (*gin4Δ*-Shs1-GBP: n=37, *gin4Δ hsl1Δ*-Shs1-GBP: n=44 cells) (G) Quantification of the residence time of Inn1-3xmCherry during cytokinesis in the indicated strains shown in (B), Kruskal-Wallis nonparametric statistical test (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.0001$, ns: $p > 0.05$) (N=2, n>60 cells/strain).

Myo1-3xmCherry increased further upon deletion of Hsl1 in *gin4Δ* cells, even when Elm1-GFP was artificially tethered to the bud neck with Shs1-GBP, reaching up to 87.17% of the cellular population (Fig. S10A, S10B). We also assessed the residence time of Inn1-3xmCherry in these cells to measure constriction dynamics of the AMR^{75,76}. We found that the Inn1-3xmCherry residence time was similar to that of wild-type cells when Elm1-GFP was tethered via Shs1-GBP in *gin4Δ* cells, but was significantly increased when Elm1-GFP was tethered via Shs1-GBP in *gin4Δ hsl1Δ* cells (Fig. 8B, 8G). These results suggest that (i) Elm1 tethering to the bud neck restores normal Myo1 localization and AMR constriction dynamics in the absence of Gin4, (ii) Hsl1 is required for Elm1 to exert its influence over AMR organization and dynamics after bud neck tethering in *gin4Δ* cells, and (iii) the membrane-binding KA1 domain of Hsl1 is required for this activity downstream of Gin4.

Taken together, our results suggest that Hsl1 functions both downstream of and in coordination with Elm1 and Gin4 to maintain septin stability and ensure proper cytokinesis (Fig. 9). In wild-type cells, Hsl1 likely acts downstream of Elm1 and Gin4 and localizes normally to the bud neck, where it may help coordinate septin architecture with AMR constriction during cytokinesis (Fig. 9). In the absence of Elm1, localization of both Gin4 and Hsl1 becomes defective during later cell cycle stages, disrupting the potential crosstalk between Gin4 and Hsl1. This disruption may lead to septin mislocalization and disorganization of the AMR machinery (Fig. 9). Notably, restoring Hsl1 localization using a GFP-GBP tethering strategy strengthens this link, suggesting that Elm1 plays an important role in recruiting or stabilizing Hsl1 at the bud neck. These observations indicate that Hsl1 acts downstream of Elm1 in this pathway, with partially overlapping functions. Furthermore, the requirement for Hsl1 in the rescue observed upon Elm1 tethering in *gin4Δ* cells supports the idea that Hsl1 mediates downstream effects of Elm1 in regulating septin stability and cytokinesis. Therefore, we propose that Hsl1, through a compensatory pathway, facilitates functional crosstalk between the septin kinase network and septin/AMR components, which is essential for proper septin organization and symmetric AMR constriction, ultimately ensuring faithful cytokinesis.

Discussion

How septin architecture remodeling crosstalk with the AMR machinery and cytokinesis remains a dynamic area of investigation, as these processes are spatiotemporally controlled by septin-interacting proteins and PTMs^{37,46,63–68}. Yet the regulatory mechanisms governing septin remodeling and AMR constriction during the cell cycle in yeast and higher eukaryotes remain incompletely understood. In *S. cerevisiae*, four septin-associated kinases Elm1, Gin4, Hsl1, and Kcc4 have been reported to primarily regulate septin-dependent processes^{38,39,59,61}. However, the precise mechanisms by which these kinases influence septin architecture and coordinate cytokinesis remain poorly understood. In this study, we identify critical roles for Elm1 and Gin4 in maintaining proper septin organization and AMR dynamics during cytokinesis. Our results further reveal that Gin4 exerts its functions in septin stability and AMR dynamics in a kinase-independent manner, challenging the prior view of kinase-dependent functions. We also identify a direct physical interaction between Gin4 and the AMR protein Hof1, providing new insights into how septin regulatory pathways directly crosstalk with the cytokinetic machinery.

Septin remodeling marks the onset of AMR constriction and cytokinesis^{17,18,30,82}, but the molecular mechanisms coordinating these processes remain largely unclear. We observed that septins are severely mislocalized to the bud cortex in *elm1Δ* and *gin4Δ* cells, supporting previous reports that these kinases are required for proper septin organization at the bud neck^{38,39,59}. Consistent with these defects, the dynamics of the AMR complex proteins Inn1 and Hof1 were significantly perturbed, leading to delayed constriction in the absence of Elm1 and Gin4. These observations suggest that septin-associated kinases regulate intermediate signaling events that coordinate septin architecture with the assembly and stability of the cytokinetic machinery, and they highlight an underappreciated mode of regulation of AMR organization and dynamics (Fig. 1D-1F, 2A-2E).

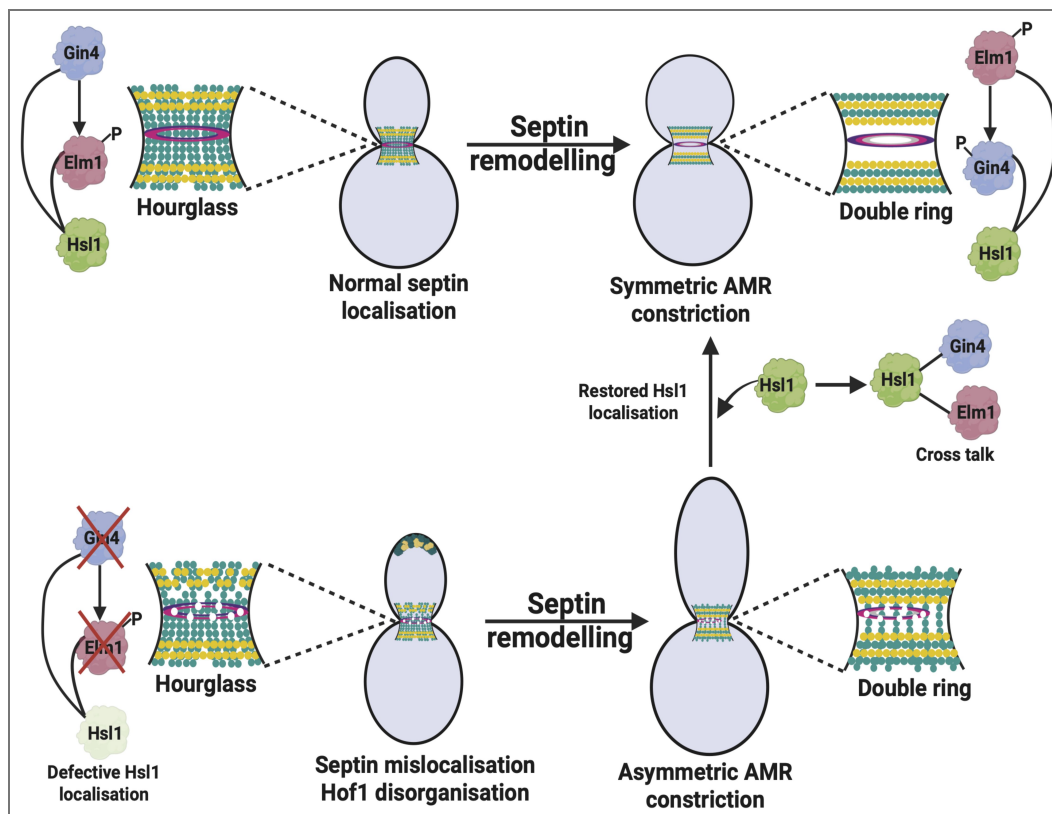


Figure 9. Representative model for the role of Hsl1 kinase in septin organization and AMR constriction downstream of Gin4 and Elm1.

Created with [BioRender.com](https://www.biorender.com).

A key question arising from these observations is whether the crosstalk between the septin kinase network and AMR dynamics is direct and septin-independent, or indirect and septin-dependent. Previous studies have shown that AMR constriction can proceed in certain septin mutants that disrupt septin organization, suggesting that septin kinases may also exert direct regulatory effects on the cytokinetic machinery¹⁷, further supporting a direct contribution of Elm1 and Gin4 to the dysregulated cytokinetic events observed in this study. Supporting this idea, we found that Gin4 interacts with the F-BAR protein Hof1, as demonstrated by yeast two-hybrid and in-vitro binding assays (Fig. 2F-2H [↗](#)). Notably, the membrane-binding domains of Gin4 and Hof1 were sufficient for this interaction, suggesting that Gin4 activation at the membrane^{48,57} may directly influence Hof1 organization and function during AMR maturation (Fig. 2H [↗](#)). These findings suggest a potential mechanism for anchoring Hof1 to the plasma membrane prior to cytokinesis (Fig. 2I [↗](#)). Interestingly, previous work has shown that *gin4Δ* and *hof1Δ* are synthetic lethal and that Hof1 exhibits mislocalization in *gin4Δ* cells⁷⁴. These results also point to a coordinative mechanism in which Gin4 directly crosstalks with an AMR component, Hof1, to regulate AMR organization and constriction. To our knowledge, this represents the first evidence physically linking the septin kinase network to AMR proteins and highlights a novel regulatory input on AMR assembly in *S. cerevisiae*. Future work is required to delineate the molecular mechanisms underlying this regulation.

Interestingly, our results further demonstrate that the kinase activity of Gin4 is not required for its role in septin organization, AMR constriction (Fig. 3A-3E [↗](#)), or cell viability in the absence of Hof1 (Fig. S3I [↗](#)). This highlights an important aspect of Gin4 function in cells and is similar to the kinase-independent function reported for Elm1³⁹. These observations raise an important question regarding the functional significance of kinase activity within the septin kinase network. Our results, together with previous studies^{38,59}, establish that built-in functional redundancies exist among the septin kinases (Elm1, Gin4, Hsl1 and Kcc4), and that their recruitment to the bud neck is also interdependent. Kinase activities may play an important role in regulating the signaling that coordinates the precise arrival and spatiotemporal activation of these kinases at the bud neck. These results also raise important questions about post-translational regulatory mechanisms of septin filament organization, as a number of studies have shown that these kinases directly phosphorylate septins⁴⁹. Thus, the role of post-translational regulation of septins will be better understood with future work in this direction.

Although these kinases exhibit distinct functions, they also display mutual interdependence^{38,62}. Recent studies have proposed a new perspective regarding this crosstalk, revealing reciprocal regulation between Gin4 and Elm1 through direct binding and phosphorylation at different stages of the cell cycle³⁸. The existence of redundancy in the septin kinase network explains the longstanding differences in the phenotypes observed in *elm1Δ* and *gin4Δ* cells. The *elm1Δ* cells show more pronounced abnormalities in cell shape and septin organization than *gin4Δ* cells, even though Elm1 is known to be absent from the bud neck in *gin4Δ* cells^{38,41,59}. Our analysis of the temporal localization dynamics of Hsl1 and Kcc4 suggests a potential explanation for this discrepancy. Specifically, the recruitment of Hsl1 and Kcc4 to the bud neck was strongly perturbed in *elm1Δ* cells but remained largely intact in *gin4Δ* cells (Fig. 7D-7F [↗](#), S8A-S8E [↗](#)). These findings suggest that the localization of Hsl1 and Kcc4 to the bud neck is crucial for their normal function and may explain the less prominent phenotype observed following loss of Gin4, emphasizing the redundant roles of Hsl1 and Kcc4 in septin organization.

Consistent with this interpretation, our GFP-GBP tethering experiments demonstrated that restoring localization of Hsl1 in *elm1Δ* cells, together with Gin4 and Kcc4, can bypass the requirement for Elm1 during the cell cycle, independent of its function in the morphogenetic checkpoint pathway (Fig. 4B-4E [↗](#), 6A-6D [↗](#), 7A-7C [↗](#) and S7A-S7C [↗](#)). These findings highlight a previously underappreciated level of functional redundancy among the Nim1-related kinases. Our results also suggest that Hsl1 can play a non-canonical role in regulating septin organization and AMR dynamics downstream of Elm1 and Gin4 (Fig. 5A-5F [↗](#), 8A-8G [↗](#), S10A, S10B [↗](#)). We therefore speculate that synergy exists among Elm1, Gin4, and Hsl1 to regulate downstream signaling pathways that control septin organization and cytokinesis. Investigating the requirement for Hsl1

kinase activity in the absence of Elm1 or Gin4 may provide further insight into the molecular mechanisms underlying Hsl1 regulation. Additionally, elucidating the phosphorylation-dependent modifications involved, along with identifying the specific regulatory residues in future studies, will help clarify the molecular mechanisms by which these kinases coordinate with one another to ensure the precise execution of cytokinesis.

Strikingly, deletion of the membrane-binding KA1 domain of Hsl1 abolished its ability to rescue the elongated defect exhibited in the absence of Elm1 (Fig. 4I–4K [↗](#) and S9H–S9J [↗](#)), emphasizing the importance of membrane association for the proper function of these kinases⁵⁷. Membrane binding may facilitate kinase activation or enable interactions with septin filaments and other regulatory proteins at the bud neck. However, the precise mechanisms by which membrane association contributes to Hsl1 function remain unclear and will require further investigation. The underlying molecular mechanisms and actual cause-and-effect relationships by which Hsl1 and its related kinases regulate septin organization at the filament-level remain to be understood. Approaches such as platinum-replica electron microscopy (PREM) and in-vitro reconstitution assays may prove instrumental in dissecting how these kinases influence septin filament organization and higher-order assembly in future studies^{27,39,82}. Such studies will provide critical insights into the modes of regulation of septins through the coordinated action and signaling inputs from multi-septin kinase modules across eukaryotes.

In summary, our study provides a comprehensive quantitative analysis of septin-regulatory kinases in controlling septin organization and cytokinesis. We identify Elm1 and Gin4 as key regulators of septin architecture and uncover previously uncharacterized roles for these kinases in AMR organization and constriction. These findings pave the way for future investigations into the intricate molecular mechanisms and cross-regulatory interactions within the septin-kinase network and with AMR proteins during the cell cycle in *S. cerevisiae* and other related organisms, where septins and their associated proteins play key roles in processes such as cell division.

Materials and Methods

Strain construction

All yeast strains in this study were constructed using the *S. cerevisiae* wild-type strains ESM356 and YPH499, derived from the S288C genetic background. The strain number and the genotype are listed in [supplementary table S1 \[↗\]\(#\)](#). C-terminal epitope tagging and endogenous gene deletions were carried out based on the PCR-based integration strategy^{95,96}. All strains were cultured at 23°C with shaking at 180 rpm. Synthetic complete (S.C.) media lacking tryptophan, uracil, leucine, and histidine were used to select auxotrophic recombinants. Antibiotic selections for endogenous gene manipulation were done on YPD agar plates supplemented with 200 µg/ml G418 (Sisco Research Laboratories Pvt. Ltd.; catalogue no. 58327), 100 µg/ml hygromycin-B (Sisco Research Laboratories Pvt. Ltd.; catalogue no. 67317), and 100 µg/ml nourseothricin (Jena Biosciences; catalogue no. AB-102). To visualize septin dynamics, Cdc3-mCherry, YIP211-cdc3-mCherry^{72,97}, and YIP204-cdc3-mCherry⁷⁷ plasmids were cut with BglII (catalogue no. R0144S; New England Biolabs) and integrated into the CDC3 locus. Cell cycle dynamics were visualized using a yeast expression vector with mRuby2-Tub1 under the His3 promoter (pHIS3p:mRuby2-Tub1+3"UTR::Ura3)⁹⁸ and pAFS125-GFP-TUB1⁹⁹, which were cut with ApaI (Catalogue no. R0114S; New England Biolabs) and StuI (catalogue no. R0187S; New England Biolabs), respectively, and integrated into the ura3 loci. Yeast transformation for epitope tagging and endogenous gene deletions was performed using a lithium acetate (LiOAc)-based protocol as described¹⁰⁰.

Oligonucleotides and Plasmids

All oligonucleotides used for endogenous gene tagging and deletion in this study are listed in [supplementary table S2 \[↗\]\(#\)](#). The plasmids used and generated for this study are listed in [supplementary table S3 \[↗\]\(#\)](#). For the generation of clones used in Fig. 3A and 3B [↗](#), Gin4^{FL} was amplified from the endogenous locus and integrated into pRS305 under the pTEF promoter. The ligation reaction was performed using NEB Hifi-Builder (New England Biolabs; cat. no.: E2621L).

Catalytically dead Gin4^{KD}(K48A) was constructed based on the site-directed mutagenesis strategy using the oligos (P855, P856, P857, and P1024). The kinase-dead mutant was confirmed correct by sequencing. To construct Gin4^{FL} and Gin4^{KD} used in Fig. 3C-3E, S3F, S3G, and S3I, the Gin4 gene fragment was amplified from the genomic loci with the endogenous promoter using PCR and was ligated into a pRS305 integration vector cut with BamHI-HF (Catalogue no. R3136S; New England Biolabs).

Live-cell imaging

Cells were inoculated in S.C. media and incubated overnight at 23°C. The secondary culture was diluted to log phase (optical density-0.4 to 0.6) from the overnight saturated culture. 6% Concanavalin A type 6 (Sigma-Aldrich; catalogue no. C2010) was coated onto a confocal dish (ibidi GmbH-catalogue no. 81218-200; Cellvis-catalogue no. D35C4-20-1.5-N) and was incubated at 23°C. The secondary culture cells were pelleted at 3200 rpm for 3 minutes. The cells were washed in S.C. media to remove non-adherent cells and subjected to live-cell imaging.

The laser point scanning confocal microscope Olympus FV-3000, equipped with high-sensitivity GaAsP photomultiplier tube (PMT) detectors and solid-state lasers (488 nm and 561 nm), was used to acquire time-lapse imaging for Fig. S1A-S1D, S2A. Images were acquired at a one-minute time interval with 0.5 µm step size and seven z-slices for 2-3 hours. The raw images were three-dimensionally (3D) deconvolved in Olympus CellSens Dimension (3.1) software. Maximum-intensity projection images were used for representation.

The Olympus IX83 widefield microscope equipped with a CoolLED PE-4000 LED light source and Prime-BSI sCMOS camera was used for acquiring images shown in Fig. 1B, 1D, 2A, 3C, and S3C. All images were acquired with a 100x oil-immersion objective with 1.45 N.A., 1 µm step-size, and three z-slices at a 2-minute time interval for 60-150 minutes. The raw images were processed for 3D deconvolution using Olympus CellSens Dimension (3.1) software. Maximum-intensity projection images were used for representation.

The images for Hof1 mis-organization and artificial tethering experiments in Fig. 2D, S3A, S3F, 4B, 4I, 7A, S5E, S7A, S9E, and S9H were acquired using the Oxford Andor Dragonfly 502 spinning disc confocal system equipped with a fully motorized Dmi8 inverted setup. The raw images were captured with a 100x oil immersion objective with 0.5 µm step-size, 11 z-slices, in an Andor Sona sCMOS camera, and deconvolved using Andor Fusion software. The 3D-deconvolved and max-projected images were used for representation.

The Olympus IX-83 Laser scanning spinning disc Yokogawa-W1 microscope was used to capture Fig. 3A, 4F, S6A, 8A, and S10A images. Raw images were acquired with a 100x oil-immersion objective with 1.45 N.A., using 1 µm step-size and five z-slices. The time-lapse imaging for Fig. S1E, S2D, S4A, S4D, 5A, 5D, S5A, S5B, 6A, 6C, 7D, 8B, S8A-S8C, S9A, and S9B was acquired at a one-minute time interval with 60x objective with 1.42 N.A. The images and time-lapse montages were 3D-deconvolved in Olympus CellSens Dimension (3.1) software. Maximum-intensity projection images were used for representation.

Protein accumulation kinetics quantification and analysis

The live-cell imaging data were opened in Fiji (ImageJ2) image analysis software¹⁰¹ using the protocol established¹⁰². In brief, a region of interest (ROI) was drawn around the bud neck of the sum-intensity projected image. The extracted fluorescent intensity values were corrected using background values. All the individual fluorescent intensity values obtained in an excel sheet at each time point were normalized using the minimum and maximum values according to the formula below:

$$\text{Normalized value} = (\text{Raw value} - \text{minimum value}) / (\text{maximum value} - \text{minimum value})$$

The data points ranging between the -20 and +20 time points were separately extracted for the 'n' number of cells indicated with each graph, respectively. The mean and standard deviation were calculated. A line plot was generated using Origin-Pro (2015, Sr2, 69.2.272; Origin Lab, USA) image

analysis software to represent the temporal accumulation kinetics of the protein of interest. The small bud is considered as t_0 for quantifications involving bud emergence. The complete drop in Cdc3-mCherry intensity is considered as the HDR transition in Figure S1E. The spindle breakpoint, where the spindle splits during the cytokinesis, is considered as t_0 or the reference time-point for measuring Hof1, Inn1 and Chs2 temporal dynamics during cytokinesis.

Quantitative analysis of septin and Hof1 misorganization was performed manually by counting the number of cells exhibiting septin mislocalization to the bud cortex during bud emergence and the defective/discontinuous Hof1 ring at the bud neck. The residence time of AMR components at the bud neck during cytokinesis was calculated from the time-lapse movies as the time interval between the first appearance of the signal and its complete disappearance from the bud neck.

Quantitative analysis of cellular morphology

Brightfield images were acquired using a 100x oil-immersion objective. Cells containing a large bud were selected for analysis. Cell boundaries were manually drawn by selecting a polygonal ROI in Fiji. The marked ROIs were analyzed to obtain measurements of cell area, major axis length, minor axis length, and aspect ratio. The aspect ratio, defined as the ratio of the cell's major axis to the minor axis, was used as a quantitative parameter to distinguish between round and elliptical cell shapes.

Yeast Two-Hybrid Assay

Genes or gene fragments of interest were amplified and cloned into NotI restriction enzyme (NEB; Catalogue No. R3189S)-cut pMM5S and pMM6S plasmids consisting of the DNA binding domain of LexA (Lex^{DBD}) and activation domain of Gal4 (Gal4^{AD}), respectively. The clones were transformed into the yeast strains SGY37 (MATa) and YPH500 (MATalpha). The transformants were selected on S.C. media lacking histidine and leucine, respectively. Mating was performed on YPD plates and was incubated at 30°C for 2 days, followed by replica plating on SC-His-Leu double selection plates. The plates were incubated for 2 days at 30°C, then overlaid with freshly prepared X-Gal mix (500 mM sodium phosphate buffer (pH 7.0), 10% SDS, 1 M KCl, 1 M MgCl₂, 0.04% X-gal, and 0.4% low-melting agarose) for detection of β -galactosidase activity^{103,104}. Interaction of LexA^{DBD}-Protein of interest A with Gal4^{AD}-Protein of interest B resulted in expression of the lacZ gene encoding β -galactosidase, which converted X-gal to a product with blue color¹⁰⁵. The plates were scanned and documented after 14-16 hours of incubation with overlay mix.

Protein expression, purification, and in vitro binding assay

The N-terminal fragment of Hof1 containing the F-BAR domain (1-350 aa) and KA1 fragment of Gin4 (1003-1142 aa) were PCR-amplified from *S. cerevisiae* genomic DNA and cloned into pGEX-5X1 cut with EcoRI restriction enzyme (Catalogue No. R3101S) and pET28a-6His-bdSUMO vector cut with NdeI restriction enzyme (Catalogue No. R0111S), respectively, using NEB Hifi Builder. Both plasmids were transformed into BL21-DE3 E. coli competent cells and were selected on Luria Bertani (LB) plates containing Kanamycin (50 mg/ml) for pGEX-5X1-Hof1^{N(1-350)} and Ampicillin (100 mg/ml) for pET28a-6His-bdSUMO-Gin4^{KA1}. LB broth with Kanamycin or Ampicillin was used to grow primary cultures overnight at 37°C with constant shaking at 200 rpm. The primary culture was diluted to 0.1 O.D. in 500 ml LB broth with Kanamycin or Ampicillin. Protein expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) once the O.D. reached log phase (0.4-0.5). The culture was incubated at 30°C with shaking at 200 rpm for 3-4 hrs. The cells were pelleted at 4°C, 7000 rpm, for 20 min. Media were aspirated and the pellets were washed with cold phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and were stored at -80°C.

Purification of 6His-bdSUMO-Gin4^{KA1}

Pellets were kept on ice for 10 min. and were subjected to lysis with the lysis buffer (50 mM Tris pH 8, 200 mM NaCl, 10% glycerol). The lysed pellets were clarified at 4°C and 15,000xg. The clarified lysate was mixed with Ni-NTA agarose resin (cat. no. 786-940, G-Biosciences), washed with

wash buffer (50 mM Tris pH 8, 200 mM NaCl, 10 mM imidazole), and was incubated at 4°C for 2 hrs. The lysate with Ni-NTA resins was then transferred to poly-prep chromatography columns (cat. no. 731-1550, BIO-RAD laboratories Inc.), and was subjected to gradient elution with increasing concentrations of imidazole. The eluted protein fractions were buffer-exchanged with storage buffer (50 mM Tris-HCl pH 7.4, 2000 mM NaCl, 1 mM DTT, and 10% glycerol) using PD Midi Trap G-10 Sephadex columns (GE Healthcare, cat. No. GE28-9180-11). The purified proteins were flash-frozen in liquid nitrogen and were stored at -80°C. The protein concentration was estimated using a spectrophotometer at 280 nm and was analyzed, along with known concentrations of Bovine Serum Albumin (BSA) standards, on Sodium Dodecyl-Sulfate PolyAcrylamide Gel electrophoresis (SDS-PAGE).

Purification of GST-Hof1^N (1-350)

Pellets were kept on ice for 10 min. and were subjected to lysis with the lysis buffer (PBS, 0.5 mM EDTA, 1 mM DTT, 1 mg/ml lysozyme, and complete mini-EDTA-free protease inhibitor cocktail tablets). The clarified lysate was incubated with 1% Triton-X-100 for 20 min. on ice and centrifuged at 4°C, 22,000xg, for 30 min. The lysate was then transferred to a tube containing glutathione sepharose-4B resin (cat. no. GE17-0756-01, GE), washed with wash buffer (5x) (PBS, 0.5 mM EDTA, and 1 mM DTT), and was incubated at 4°C for 2-3 hrs. The lysate with glutathione Sepharose resins was then transferred to poly-prep chromatography columns after incubation, washed with wash buffer, and was stored at 4°C.

In vitro binding assay

GST or GST-Hof1^N (1-350) (~3 µg) bound to glutathione beads were mixed with ~1 µg 6His-bdSUMO-Gin4^{KA1} and incubated in binding buffer (PBS, 0.2% NP-40) for 1 hour at 4°C. The beads were washed 3-5 times with wash buffer (PBS, 1% NP-40) and were analyzed by western blot using Anti-His (H-3) mouse monoclonal IgG1 (Santa Cruz/#sc-8036/Lot. No. C0421), Anti-GST (B-14) mouse monoclonal IgG1 (Santa Cruz/#sc-138/Lot. No. K1020), and Anti-mouse IgG-HRP (Cell Signalling/#7076S/Lot. No. 36).

Spot assay

The yeast strains were grown in liquid YPD broth overnight at 23°C. The cultures were diluted to 1.0 O.D. and were serially diluted in the order 10⁰, 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴. These dilutions were spotted on three YPD plates and incubated at 23°C, 30°C, and 37°C. Growth was monitored, and plates were scanned after 48 hrs of incubation.

The strains used for rescue of *gin4Δ hof1Δ* synthetic lethality (Fig. S3I [↗](#)) were grown overnight in S.C. media lacking Uracil and Leucine. The culture was diluted to an O.D. of 1.0 and was serially diluted in the order 10⁰, 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴. These dilutions were spotted on three SC-Ura-Leu plates and were incubated at 23°C for two days. Subsequently, the colonies were patched onto 5-FOA plates (1 mg/ml) to select for Ura-cells.

Statistical analysis

All experiments were performed independently three times. Statistical significance for bar graphs and stacked column plots was determined using one-way ANOVA with Tukey's multiple-comparison test, whereas residence-time plots were analyzed using the Kruskal-Wallis nonparametric test. All statistical analyses were performed using in-built functions of GraphPad Prism (v.6.04). A p-value of p<0.05 was considered statistically significant. (*: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001, ns: p>0.05).

Supplementary tables

Strain number	Genotype	Source
YSP002	<i>MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200 (ESM356)</i>	¹⁰⁶
YSP120	<i>ESM356-Elm1-GFP-Hph Cdc3-mCherry-Trp</i>	This study
YSP121	<i>ESM356-Gin4-GFP-Hph Cdc3-mCherry-Trp</i>	This study
YSP122	<i>ESM356-Kcc4-GFP-Hph Cdc3-mCherry-Trp</i>	This study
YSP123	<i>ESM356-Hsl1-GFP-Hph Cdc3-mCherry-Trp</i>	This study
YSP524	<i>ESM356-Cdc3-mCherry-Trp, Tub1-GFP-ura</i>	This study
YSP520	<i>ESM356-Cdc3-mCherry-Trp, Tub1-GFP-Ura, Δelm1::His3mX6</i>	This study
YSP521	<i>ESM356-Cdc3-mCherry-Trp, Tub1-GFP-Ura, Δgin4::His3mX6</i>	This study
YSP1104	<i>ESM356-Cdc3-mCherry-Trp, Tub1-GFP-Ura, Δkcc4::His3mX6</i>	This study
YSP518	<i>ESM356-Δhsl1-His3mX6, Cdc3-mCherry-Trp, Tub1-GFP-Ura</i>	This study
YSP058	<i>ESM356-Δelm1::His3MX6</i>	This study
YSP062	<i>ESM356-Δgin4::His3MX6</i>	This study
YSP050	<i>ESM356-Δhsl1::His3MX6</i>	This study
YSP115	<i>ESM356-Δkcc4::KanMX6</i>	This study
YSP950	<i>ESM356-Inn1-mNG-KanMX6, pHis3-mRuby2-Tub1-Ura3</i>	This study
YSP1017	<i>ESM356-Inn1-mNG-KanMX6, pHis3-mRuby2-Tub1-Ura3, Δelm1::3HA-Trp</i>	This study
YSP1004	<i>ESM356-Inn1-mNG-KanMX6, pHis3-mRuby2-Tub1-Ura3, Δgin4::3HA-Trp</i>	This study
YSP1014	<i>ESM356-Inn1-mNG-KanMX6, pHis3-mRuby2-Tub1-Ura3, Δhsl1::3HA-Trp</i>	This study
YSP1037	<i>ESM356-Inn1-mNG-Kanmx6, pHis3-mRuby2-Tub1-Ura3, Δkcc4::3HA-Trp</i>	This study
YSP948	<i>ESM356-Hof1-mNG-KanMX6, pHis3-mRuby2-Tub1-Ura3</i>	This study
YSP1024	<i>ESM356-Hof1-mNG-KanMX6, pHis3-mRuby2-Tub1-Ura3, Δelm1::3HA-Trp</i>	This study
YSP1095	<i>ESM356-Hof1-mNG-Kanmx6, pHis3-mRuby2-Tub1-Ura3, Δgin4::3HA-Trp</i>	This study
YSP947	<i>ESM356-Chs2-mNG-KanMX6, pHis3-mRuby2-Tub1-Ura3</i>	This study

YSP1019	<i>ESM356-Chs2-mNG-KanMX6, pHis3-mRuby2-Tub1-Ura3, Δelm1::3HA-Trp</i>	This study
YSP1002	<i>ESM356-Chs2-mNG-KanMX6, pHis3-mRuby2-Tub1-Ura3, Δgin4::3HA-Trp</i>	This study
YSP003	<i>MATα ura3-52 lys2-801amber ade2-101ochre trp1Δ63 his3Δ200 leu2Δ1 (YPH500)</i>	100
YSP004	<i>MATα leu2 ADE2 ura3-52::URA3-lexA-op-LacZ his3 trp (SGY37)</i>	100
YSP997	<i>ESM356-Cdc3-mCherry-Ura, Δgin4::3HA-Trp</i>	This study
YSP1581	<i>ESM356-Cdc3-mCherry-Ura Δgin4::3HA-Trp pRS305-(Vector alone) cut with Kas I and integrated into Leu locus</i>	This study
YSP1582	<i>ESM356-Cdc3-mCherry-Ura Δgin4::3HA-Trp pRS305-pTEF-Gin4(FL)-GFP-tCyc1 cut with Kas I and integrated into Leu locus</i>	This study
YSP1583	<i>ESM356-Cdc3-mCherry-Ura Δgin4::3HA-Trp pRS305-pTEF-Gin4(K48A)-GFP-tCyc1 cut with Kas I and integrated into Leu locus</i>	This study
YSP1236	<i>ESM356-Inn1-mNG-KanMX6, pHis3-mRuby2-Tub1-Ura3, Δgin4::3HA-Trp, pRS305-(Empty vector)-cut with Kas I and integrated into leu locus</i>	This study
YSP1237	<i>ESM356-Inn1-mNG-KanMX6, pHis3-mRuby2-Tub1-Ura3, Δgin4::3HA-Trp, pRS305-pGin4-Gin4(FL)-tGin4-cut with Kas I and integrated into leu locus</i>	This study
YSP1238	<i>ESM356-Inn1-mNG-KanMX6, pHis3-mRuby2-Tub1-Ura3, Δgin4::3HA-Trp, pRS305-pGin4-Gin4(Kinase dead)-tGin4-cut with Kas I and integrated into leu locus</i>	This study
YSP1312	<i>ESM356-Hof1-mNG-Kanmx6, pHis3-mRuby2-Tub1-Ura3, Δgin4::3HA-Trp, pRS305-(Empty vector)-cut with Kas1 and integrated into leu locus</i>	This study
YSP1313	<i>ESM356-Hof1-mNG-Kanmx6, pHis3-mRuby2-Tub1-Ura3, Δgin4::3HA-Trp, pRS305-pGin4-Gin4(FL)-tGin4-cut with Kas1 and integrated into leu locus</i>	This study
YSP1314	<i>ESM356-Hof1-mNG-Kanmx6, pHis3-mRuby2-Tub1-Ura3, Δgin4::3HA-Trp, pRS305-pGin4-Gin4(Kinase dead)-tGin4-cut with Kas1 and integrated into leu locus</i>	This study
YSP001	<i>MATα ura3-52 lys2-801amber ade2-101ochre trp1Δ63 his3Δ200 leu2Δ1 (YPH499)</i>	100

YSP1086	YPH499- Δ hof1::TRP1 pRS316-HOF1 Δ gin4::HIS3MX6 (MFY1474)	⁷⁴
YSP1357	YPH499 Δ hof1::TRP1 pRS316-HOF1 Δ gin4::HIS3MX6, pRS305-Empty Vector-cut with Kas1 and integrated into Leu locus	This study
YSP1358	YPH499 Δ hof1::TRP1 pRS316-HOF1 Δ gin4::HIS3MX6, pRS305-pGin4-Gin4(FL)-tGin4-cut with Kas1 and integrated into Leu locus	This study
YSP1359	YPH499 Δ hof1::TRP1 pRS316-HOF1 Δ gin4::HIS3MX6, pRS305-pGin4-Gin4(K48A)-tGin4- cut with Kas1 and integrated into Leu locus	This study
YSP034	ESM356-Gin4-GFP-Hph	This study
YSP1345	ESM356-GFP-Gin4-GFP-Hph Δ elm1::3HA-Trp	This study
YSP1419	ESM356-Gin4-GFP-Hph, Δ elm1::3HA-Trp, Hsl1- GBP-KanMX6	This study
YSP1651	ESM356- Δ elm1::3HA-Trp Δ gin4::6HA NatNT2	This study
YSP1665	ESM356- Δ elm1::3HA-Trp Δ gin4::6HA NatNT2 pRS305 cut with Kas I and integrated into Leu locus	This study
YSP1666	ESM356- Δ elm1::3HA-Trp Δ gin4::6HA NatNT2 pRS305-pTEF-Gin4(FL)-GFP-tCyc1 cut with Kas I and integrated into Leu locus	This study
YSP1667	ESM356- Δ elm1::3HA-Trp Δ gin4::6HA NatNT2 pRS305-pTEF-Gin4(K48A)-GFP-tCyc1 cut with Kas I and integrated into Leu locus	This study
YSP1658	ESM356- Δ elm1::3HA-Trp Δ gin4::6HA NatNT2 pRS305 cut with Kas I and integrated into Leu locus Hsl1-GBP-KanMX6	This study
YSP1652	ESM356- Δ elm1::3HA-Trp Δ gin4::6HA NatNT2 pRS305-pTEF-Gin4(FL)-GFP-tCyc1 cut with Kas I and integrated into Leu locus Hsl1-GBP-KanMX6	This study
YSP1653	ESM356- Δ elm1::3HA-Trp Δ gin4::6HA NatNT2 pRS305-pTEF-Gin4(K48A)-GFP-tCyc1 cut with Kas I and integrated into Leu locus Hsl1-GBP-KanMX6	This study
YSP1737	ESM356-Gin4-GFP-Hph, Hsl1- Δ ka1-GBP-KanMX6	This study
YSP1738	ESM356-Gin4-GFP-Hph Δ elm1::3HA-Trp Hsl1- Δ ka1- GBP-KanMX6	This study
YSP1590	ESM356-Gin4-GFP-Hph mRuby-Tub1-Ura	This study
YSP1594	ESM356-Gin4-GFP-Hph Δ elm1::3HA-Trp, pHis3- mRuby2-Tub1-Ura3	This study

YSP1411	<i>ESM356-Gin4-GFP-Hph, Δelm1::3HA-Trp Shs1-GBP-KanMX6</i>	This study
YSP1415	<i>ESM356-Gin4-GFP-Hph, Δelm1::3HA-Trp, Bud4-GBP-KanMX6</i>	This study
YSP1423	<i>ESM356-Gin4-GFP-Hph, Δelm1::3HA-Trp, Bni5-GBP-KanMX6</i>	This study
YSP119	<i>ESM356-Hsl1-GFP-Hph</i>	This study
YSP1485	<i>ESM356-Hsl1-GFP-Hph Δelm1::3HA-Trp</i>	This study
YSP1605	<i>ESM356-Hsl1-GFP-Hph Δelm1::3HA-Trp Shs1-GBP-KanMX6</i>	This study
YSP1606	<i>ESM356-Hsl1-GFP-Hph Δelm1::3HA-Trp Bud4-GBP-KanMX6</i>	This study
YSP1740	<i>ESM356-Hsl1-GFP-Hph, Δelm1::3HA-Trp Kcc4-GBP-KanMX6</i>	This study
YSP1745	<i>ESM356-Hsl1-GFP-Hph, Δelm1::3HA-Trp Bni5-GBP-KanMX6</i>	This study
YSP1560	<i>ESM356-Hsl1-GFP-Hph, Δelm1::3HA-Trp Gin4-GBP-KanMX6</i>	This study
YSP036	<i>ESM356-Kcc4-GFP-Hph</i>	This study
YSP1482	<i>ESM356-Kcc4-GFP-Hph Δelm1::3HA-Trp</i>	This study
YSP1734	<i>ESM356-Kcc4-GFP-Hph, Δelm1::3HA-Trp Hsl1-GBP-KanMX6</i>	This study
YSP1730	<i>ESM356-Kcc4-GFP-Hph, Δelm1::3HA-Trp Shs1-GBP-KanMX6</i>	This study
YSP1732	<i>ESM356-Kcc4-GFP-Hph, Δelm1::3HA-Trp Bud4-GBP-KanMX6</i>	This study
YSP1747	<i>ESM356-Kcc4-GFP-Hph, Δelm1::3HA-Trp Bni5-GBP-KanMX6</i>	This study
YSP1518	<i>ESM356-Kcc4-GFP-Hph Δelm1::3HA-Trp Gin4-GBP-KanMX6</i>	This study
YSP1906	<i>ESM356-Kcc4-GFP-Hph pHis3-mRuby2-Tub1-Ura3</i>	This study
YSP1908	<i>ESM356-Hsl1-GFP-Hph pHis3-mRuby2-Tub1-Ura3</i>	This study
YSP1910	<i>ESM356-Kcc4-GFP-Hph Δelm1::3HA-Trp pHis3-mRuby2-Tub1-Ura3</i>	This study
YSP1912	<i>ESM356-Kcc4-GFP-Hph Δgin4::3HA-Trp pHis3-mRuby2-Tub1-Ura3</i>	This study
YSP1914	<i>ESM356-Hsl1-GFP-Hph Δelm1::3HA-Trp pHis3-mRuby2-Tub1-Ura3</i>	This study

YSP1916	ESM356-Hsl1-GFP-Hph Δ gin4::3HA-Trp <i>pHis3-mRuby2-Tub1-Ura3</i>	This study
YSP1588	ESM356-Elm1-GFP-Hph <i>pHis3-mRuby2-Tub1-Ura3</i>	This study
YSP1592	ESM356-Elm1-GFP-Hph Δ gin4::3HA-Trp <i>pHis3-mRuby2-Tub1-Ura3</i>	This study
YSP032	ESM356-Elm1-GFP-Hph	This study
YSP1346	ESM356-Elm1-GFP-Hph Δ gin4::3HA-Trp	This study
YSP1410	ESM356-Elm1-GFP-Hph, Δ gin4::3HA-Trp <i>Shs1-GBP-KanMX6</i>	This study
YSP1414	ESM356-Elm1-GFP-Hph, Δ gin4::3HA-Trp, <i>Bud4-GBP-KanMX6</i>	This study
YSP1418	ESM356-Elm1-GFP-Hph, Δ gin4::3HA-Trp, <i>Hsl1-GBP-KanMX6</i>	This study
YSP1422	ESM356-Elm1-GFP-Hph, Δ gin4::3HA-Trp, <i>Bni5-GBP-KanMX6</i>	This study
YSP1735	ESM356-Elm1-GFP-Hph, <i>Hsl1-Δka1-GBP-KanMX6</i>	This study
YSP1736	ESM356-Elm1-GFP-Hph Δ gin4::3HA-Trp <i>Hsl1-Δka1-GBP-KanMX6</i>	This study
YSP1591	ESM356-Elm1-GFP-Hph Δ gin4::3HA-Trp <i>Cdc3-mCherry-Ura</i>	This study
YSP1453	ESM356-Elm1-GFP-Hph Δ gin4::3HA-Trp, <i>Shs1-GBP-KanMX6, Cdc3-mCherry-Ura</i>	This study
YSP1715	ESM356-Elm1-GFP-Hph Δ gin4::3HA-trp <i>Δhsl1::natNT1 Cdc3-mCherry-Ura</i>	This study
YSP1717	ESM356-Elm1-GFP-Hph Δ gin4::3HA-trp <i>Shs1-GBP-KanMX6 Δhsl1::natNT1 Cdc3-mCherry-Ura</i>	This study
YSP1757	ESM356-Elm1-GFP-Hph Δ gin4::3HA-trp <i>Inn1-3xmCherry-His3MX6</i>	This study
YSP1759	ESM356-Elm1-GFP-Hph, Δ gin4::3HA-Trp <i>Shs1-GBP-KanMX6 Inn1-3xmCherry-His3MX6</i>	This study
YSP1755	ESM356-Elm1-GFP-Hph Δ gin4::3HA-trp <i>Δhsl1::natNT1 Inn1-3xmCherry-His3MX6</i>	This study
YSP1754	ESM356-Elm1-GFP-Hph Δ gin4::3HA-trp <i>Shs1-GBP-KanMX6 Δhsl1::natNT1 Inn1-3xmCherry-His3MX6</i>	This study
YSP1724	ESM356-Elm1-GFP-Hph Δ gin4::3HA-trp <i>Myo1-3xmCherry-His3MX6</i>	This study
YSP1726	ESM356-Elm1-GFP-Hph, Δ gin4::3HA-Trp <i>Shs1-GBP-KanMX6 Myo1-3xmCherry-His3MX6</i>	This study

YSP1722	ESM356-Elm1-GFP-Hph Δ gin4::3HA-trp Δ hsl1::natNT1 Myo1-3xmCherry-His3MX6	This study
YSP1721	ESM356-Elm1-GFP-Hph Δ gin4::3HA-trp Shs1-GBP-KanMX6 Δ hsl1::natNT1 Myo1-3xmCherry-His3MX6	This study
YSP1417	ESM356-Gin4-GFP-Hph, Hsl1-GBP-KanMX6	This study
YSP1589	ESM356-Gin4-GFP-Hph Cdc3-mCherry-Ura	This study
YSP1593	ESM356-Gin4-GFP-Hph Δ elm1::3HA-Trp Cdc3-mCherry-Ura	This study
YSP1595	ESM356-Gin4-GFP-Hph Δ elm1::3HA-Trp Hsl1-GBP-KanMX6 Cdc3-mCherry-Ura	This study
YSP1599	ESM356-Gin4-GFP-Hph Hsl1-GBP-KanMX6 Cdc3-mCherry-Ura	This study
YSP1489	ESM356-Gin4-GFP-Hph Hsl1-GBP-KanMX6 Myo1-ymScarlet-I	This study
YSP1491	ESM356-Gin4-GFP-Hph Δ elm1::3HA-Trp Hsl1-GBP-KanMX6 Myo1-ymScarlet-I	This study
YSP1627	ESM356-Gin4-GFP-Hph Myo1-ymScarlet-I-Nat	This study
YSP1629	ESM356-Gin4-GFP-Hph Δ elm1::3HA-Trp Myo1-ymScarlet-I-Nat	This study
YSP951	ESM356-Cdc3-mCherry-Ura	This study
YSP1033	ESM356-Cdc3-mCherry-Ura, Δ elm1::3HA-Trp	This study
YSP2374	ESM356-Cdc3-mCherry-Ura Δ swe1::His3MX6	This study
YSP2376	ESM356-Gin4-GFP-Hph Δ elm1::3HA-Trp Cdc3-mCherry-Ura Δ swe1::His3MX6	This study
YSP2377	ESM356-Gin4-GFP-Hph Δ elm1::3HA-Trp Hsl1-GBP-KanMX6 Cdc3-mCherry-Ura Δ swe1::His3MX6	This study
YSP2380	ESM356-Cdc3-mCherry-Ura, Δ elm1::3HA-Trp Δ swe1::His3MX6	This study
YSP1348	ESM356-Gin4- Δ ka1-GFP-mRuby-Tub1-Ura	
YSP1590	ESM356-Gin4-GFP-Hph mRuby-Tub1-Ura	This study
YSP1207	ESM356-Hof1-mNG, mRuby-Tub1, Gin4- Δ ka1::3HA-Trp	This study

Supplemental Table 1. Yeast strains used in this study

Supplemental Table 2. Oligonucleotides used in this study

Primer number	Primer name	Sequence
P86	Elm1-S1	GATATTATTTTTGAACGCCAGTTAACAATAA TTACTTAGCATGAAATGCGTACGCTGCAGGTC GAC
P87	Elm1-S2	GATATTATTTTTGAACGCCAGTTAACAATAA TTACTTAGCATGAAATGCGTACGCTGCAGGTC GACTCCGACAGATATCATCCTGTAGTTTCATC TAATCGATGAATTCGAGCTCG
P88	Elm1-S3	CAGTAGAAGATGTAAAAACGTATCTGAACCTT GCAGATAATGGTCAAATACGTACGCTGCAGG TCGAC
P110	Gin4-S1	TTGATCAAGAAAGATATTCGCAGCACAAATACA ATAATAACATTCAAATGCGTACGCTGCAGGT CGAC
P111	Gin4-S2	GGCAGAACAAAACGAAGGAGACAAAACATGA TTGCATTACATTAGCACTAATCGATGAATTCG AGCTCG
P112	Gin4-S3	TGGTCAATGAAGTTGAGAACGCTCTGAATAAG GAAGGCGTTCTACAAAACGTACGCTGCAGG TCGAC
P113	Hsl1-S1	ACTCCTTTATTATTTGTTATTAATATTTTTATT TTTACCACACGACATGCGTACGCTGCAGGTC GAC
P114	Hsl1-S2	AATTTTTCAAATTATTGTTGATAATTATATAAC ATCTATATAGAATTTAATCGATGAATTCGAGCT CG
P115	Hsl1-S3	AAAAGTTTAAATGATGATGTGGAGAGAGTAATT CGAAATGCCGACGTTACGTACGCTGCAGG TCGAC
P107	Kcc4-S1	ATAGGCTTGATAATAGTGCTGACGTAAATTAC CAGAACTACTGCAGTATGCGTACGCTGCAGG TCGAC
P108	Kcc4-S2	GTTACATCGTATTGTCCATTTGGGATCGATT ATCCCTCCCTTTTTTTAATCGATGAATTCGAG CTCG
P109	Kcc4-S3	TTGCTACAGAAATCCAAATTATTTACAAAAG AAGGTGTTTTGGACAAACGTACGCTGCAGGT CGAC
P90	Hof1-S2	TTTCTTTTATCAGAAAACAGTAAATTGATAT ACATCGAGATCAatcgatgaattcgagctcg
P91	Hof1-S3	GGATTAATCCCTATAATTTCAATTCAGCTACTG CATCAAGGTCTTcgtacgctgcaggtcgac

Supplemental Table 2. (continued)

P698	Inn1-S2	TTCTATTTAAATATTAGTATTACAATAACAGCA TGTTTCTCTTGTCATTAAATCGATGAATTCGAG CTCG
P699	Inn1-S3	GGAAGAATTCGATGTCTCCTACAAGAAAAAGA CCACCTCCAAGGCTCAGCCGTACGCTGCAGG TCGAC
P742	Chs2-S2	TGAGAAAAAGAGGGAATGACGAGAAATTAG CTGAAAAATACTGGCATTAAATCGATGAATTCG AGCTCG
P741	Chs2-S3	CATGGGAAGTCTCTAAATTAGACTTACCAAAT GTTTTCCACAAAAAGGCCGTACGCTGCAGG TCGAC
P855	pRS305-Gin4- Fwd	GGCGGCCGCTCTAGAAGTAGTCTGGTGTTAA AGCAGAAATCACG
P1021	pRS305-Gin4- Rvs	atcgaattctgcagcccggttTATACAAATAGTA ATCAACACG
P856	Gin4-K48A- Rvs	TGCTTTTGATATTACAGCAACTGCCGCTCTT GT
P857	Gin4-K48A- Fwd	CAAGAGGCGGCAGTTGCTGTAATCAAAAGC AG
P60	Shs1-S2	TATTTATTTATTTATTTGCTCAGCTTTGGATTTT GTACAGATACAACCTCAATCGATGAATTCGAGC TCG
P61	Shs1-S3	AAAAAATGACACGTATACTGATTTAGCCTCT ATTGCATCGGGTAGAGATCGTACGCTGCAGG TCGAC
P81	Bud4-S2	CTCTCAGTCATCAGATTATATGCTGTTTTTCAAT CCATTAATCACCTTCTAATCGATGAATTCGAG CTCG
P82	Bud4-S3	AGGAGGAAAAGACTCGAACAACCGGTCATAA TTTGAAAGCAAGATTTTAACTGACGCTGCAGG TCGAC
P84	Bni5-S2	TCTGTCTTAATTTATAAATATTATAACAACCC TTGGCGTAATGTAATCAATCGATGAATTCGAG CTCG
P85	Bni5-S3	GAAAGCAAATTGATCGTGGCGAAAATGGCCA ATTTTGATTGGAACCTAACGTACGCTGCAGG TCGAC
P906	Hof1-(1-350 aa)-Fwd	ATCTGATCGAAGGTCGTGGGATCCCCATGAG CTACAGTTATGAAGCTTGT
P908	Hof1-350-Rvs	TGCGGCCGCTCGAGTCGACCCGGTGTTGAA TTTCTAAATTGTGTAGCCATG
P1251	pET28a- bdSUMO- Gin4-FL-Rvs	CGAGTGCGGCCGCAAGCTTGCTATTTTGTGA GAACGCCTTCCTTATTGAG
P1254	pET28a- bdSUMO- PIP2BD/KA1- Fwd	gcccaTGCTGCACCAAAGTGGAGCGGCTCGCA GGCAAAGGAT
P340	Gin4-Fwd-Y2H	ggttctgccccggtcagggaATGGCAATCAATGGTAA CAGTA
P341	Gin4-Rvs-Y2H	gatatcgaattctgcagcccTATTTTGTAGAACGCC TTCC
P613	Gin4-KID-290- Fwd	ggttctgccccggtcagggaGGATCCCAAAATATCC AAGTATAAGAG
P1291	Gin4- PIP2BD/KA1(1 003)-Fwd	ggttctgccccggtcagggaGGATCCACCTGTGTCAA GGCTTGATC
P338	Elm1-Fwd	ggttctgccccggtcagggaATGTCACCTCGACAGCT TATAC
P339	Elm1-Rvs	gatatcgaattctgcagcccTATATTTGACCATTATC TGCA
P611	Elm1-KID- 420Fwd	ggttctgccccggtcagggaGGATCCTCATCTGCCAG TGTGAACCC
P132	Hof1-Y2H-Fwd	ggttctgccccggtcagggaATGAGCTACAGTTATGA AGCT
P133	Hof1-Y2H-Rvs	gatatcgaattctgcagcccTCAAAGACCTTGATGCA GTAG
P618	Hof1-261-Rvs	gatatcgaattctgcagcccTTCATCCGCTGCCGTGA ATG
P619	Hof1-350-Rvs	gatatcgaattctgcagcccTGTGGAATTTCTAAATTG TGTA
P620	Hof1-334-Fwd	ggttctgccccggtcagggaGGATCCCCATTGCCATC TCCAGAAGT
P621	Hof1-600-Rvs	gatatcgaattctgcagcccGACTGGAAAACCTTCAC TGGTG

P622	Hof1-600-Fwd	ggttctgccccggtcagggaGGATCCGTCATTGAATA TGCCAAGGC
P1179	Gin4-KA1-S1	GAGCCACCATTGAACAAATCTGTGCAAAAAAT TAGGGAAAAAATGCTGGCTAAcgtacgtgcaggt cgac
P1180	Gin4-KA1-S3	GAGCCACCATTGAACAAATCTGTGCAAAAAAT TAGGGAAAAAATGCTGGCcggtacgtgcaggtcgac
P1184	Hsl1-KA1-S3	AATAATACAGAAAAATACTACTCTAAAAATACCA AAACCCTCAACGATTAAAcgtacgtgcaggtcgac
P63	Myo1-S2	TTTAAATAAAGGATATAAAGTCTTCCAAATTTT TAAAAAAAAGTTCTTAATCGATGAATTCGAG CTCG
P64	Myo1-S3	TAGGCTCGAAAAATATTGATAGTAACAATGCA CAGAGTAAAAATTTTCAGTCGTACGCTGCAGGT CGAC
P1766	Swe1-S1	CGTAGTGCTGGGGAAAAAGTAAACACACACAG GCGCACACGAGAACAGATGcgtacgtgcaggtcga c
P1767	Swe1-S2	GTGTGGGAAAAAGTATGTAAATAAAACAAGG TTTTTTGTTCCATTTATCAatgatgaattcgagctcg
P1764	Swe1-Col1	CAACAACACAGTTGAACATTG
P1765	Swe1-Col2	CCATCCTTCCTTATTCTCAA
P21	Elm1-Col1	GATGCCGCTCAACAGAGGTTATG
P22	Elm1-Col2	CGCAATAGAAGACCACTAAACTC
P48	Gin4-Col1	GTTTTGCGCCAAGTTTCGTCT
P49	Gin4-Col2	TAAGGGTAAATATAATAGTAC
P50	Hsl1-Col1	CTCCCTTTGGATCGTAAGTTC
P51	Hsl1-Col2	ACCCCTCCAGGTTGTATTTA
P46	Kcc4-Col1	ATGTTTGCCATGGTAGGTTGA
P47	Kcc4-Col2	TTTCTGCTATTACAATTAAAG
P334	His3MX6-Int- Fwd	gcgcgggtactagtcttact
P335	His3MX6-Int- Rvs	tgtccaaagcgatggcaac
P1175	Trp-Int-Fwd	TACCTAAGTAATCAGCACCAT
P1176	Trp-Int-Rvs	GCCATTAAATATGTTACCAAAT
P185	Nat-Int-Fwd	ggctggaggtcaccaacgtca
P1523	Nat-Int-Rvs	cctcccgagggtgaagccgt

Supplemental Table 2. (continued)

Supplemental Table 3. Plasmids used in this study

Plasmid number	Description	Source
piSP23	pFA6a-HIS3-MX6	96
piSP14	pYM25 (yeGFP-hphNT1)	95
piSP20	pYM22 (3HA-kiTRP1)	95
piSP185	pYM17 (6HA-natNT2)	96
piSP659	pDK88 (mNeogreen cassette. mNG-kanmx6)	Gift from Prof. Gislene Pereira
piSP1075	pHA29-GBP-KAN	107
piSP31	YIP211-cdc3-mCherry (URA3)	72
piSP34	YIP204-cdc3-mCherry (TRP1)	77
piSP678	pAFS125-GFP-TUB1	99
piSP1224	pHIS3p:mRuby2-Tub1+3"UTR::Ura3	98
piSP1	pRS305	100
piSP1341	pRS305-pGin4-Gin4(FL)-tGin4	This Study ^a
piSP1739	pRS305-pGin4-Gin4(K48A)-tGin4	This Study ^a
piSP1614	pRS305-pTEF-Gin4-GFP-tCYC1	This Study ^b
piSP1704	pRS305-pTEF-Gin4 (K48A)-GFP-tCYC1	This Study ^b
piSP656	pBPE39-1(3xmCherry-His3MX6 cassette)	Gift from Prof. Gislene Pereira
piSP112	pMM5S-Y2H (Empty vector)	108
piSP113	pMM6S-Y2H (Empty vector)	108
piSP635	pMM5S-Gin4-FL (1-1142aa)	This Study
piSP645	pMM6S-Gin4-FL (1-1142aa)	This Study
piSP632	pMM5S-Elm1-FL (1-640aa)	This Study
piSP642	pMM6S-Elm1-FL (1-640aa)	This Study
piSP685	pMM5S-Gin4-KID (290-1142aa)	This Study
piSP691	pMM6S-Gin4-KID (290-1142aa)	This Study
piSP683	pMM5S-Elm1-KID (421-640aa)	This Study
piSP689	pMM6S-Elm1-KID (421-640aa)	This Study
piSP568	pMM5S-Hof1-FL (1-669aa)	This Study
piSP580	pMM6S-Hof1-FL (1-669aa)	This Study
piSP1275	pMM5S-Hof1(1-350aa)	This Study
piSP1277	pMM6S-Hof1(1-350aa)	This Study
piSP1279	pMM5S-Hof1(334-600aa)	This Study
piSP1281	pMM6S-Hof1(334-600aa)	This Study
piSP1283	pMM5S-Hof1(600-669aa)	This Study
piSP1285	pMM6S-Hof1(600-669aa)	This Study
piSP1839	pMM5S-Gin4-KA1(1003-1142aa)	This Study
piSP1844	pMM6S-Gin4-KA1(1003-1142aa)	This Study
piSP65	pET28a-6HIS-bdSUMO	109
piSP1094	pGEX-5X1	GE Healthcare, ¹¹⁰
piSP1320	pGEX-5X1-Hof1(1-350)	This Study
piSP1712	pET28a-6HIS-bdSUMO-Gin4(KA1)	This Study

^{a & b} plasmids piSP1341, piSP1739, piSP1614 and piSP1704 were cut with KsaI restriction enzyme and integrated into *Leu* locus.

Data availability

All relevant data are within the paper and its supporting information files.

Acknowledgements

We thank the DST-FIST microscope facility of the Department of Biochemistry, Indian Institute of Science. We also thank the Bioimaging facility, Division of Biological Sciences. We thank Prof. Erfei Bi (University of Pennsylvania) and Prof. Gislene Periera (Centre for Organismal Studies (COS) Heidelberg) for the plasmids. We are grateful for the help of Ms. Spoorthi Hiremath in image acquisition at the DST-FIST microscope facility. We thank Prof. P. N. Rangarajan, Prof. Franz Meitinger, and Prof. Ramanujam Srinivasan for their valuable suggestions on the manuscript. Schematic diagrams were created in BioRender. B.B. acknowledges the DST-INSPIRE Fellowship. A.D. and J.K. acknowledge the IISc Ph.D. fellowship from IISc. F.C. acknowledges the ICMR fellowship.

Additional information

Author contributions

Conceptualization: S.P.; Funding acquisition: S.P.; Supervision: S.P.; Project administration: S.P.; Methodology: S.P., B.B.; Investigation: B.B., A.D., B.V.T., J.K., F.C., D.G.; Formal analysis: B.B., A.D., V.R.; Resources: S.P., B.B., B.V.T., J.K.; Data curation: B.B.; Visualization: B.B.; J.K.; F.C. B.V.T; Writing-original draft: S.P., A.D., B.B.; Writing-review and editing: All authors

Funding

This work was financially supported by a Department of Biotechnology-Wellcome Trust India Alliance intermediate fellowship (IA/I/21/1/505633), SERB SRG grant (SRG/2021/001600), and an Indian Institute of Science (IISc) start-up grant awarded to S.P.

Funding

Funder	Grant reference number	Author
Wellcome Trust DBT India Alliance (India Alliance)	IA/I/21/1/505633	Saravanan Palani
SERB SRG	SRG/2021/001600	Saravanan Palani

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Additional files

Supplementary figures 

Supplementary video 1  Representative movies showing the recruitment of Elm1, Gin4, Hsl1, and Kcc4 during bud emergence and cytokinesis.

Supplementary video 2  Representative movies showing the localization and dynamics of Cdc3-mCherry in wild-type, *elm1Δ*, *gin4Δ*, *kcc4Δ*, and *hsl1Δ* backgrounds.

Supplementary video 3  Representative movies showing Inn1-mNG constriction in wild-type, *elm1Δ*, *gin4Δ*, *kcc4Δ*, and *hsl1Δ* backgrounds.

Supplementary video 4  Representative movies showing the localization and constriction of Hof1-mNG in wild-type, *elm1Δ*, and *gin4Δ* cells during the cell cycle.

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Peer reviews

Reviewer #1 (Public review):

Summary:

The authors wanted to better understand how the various septin-associated kinases contribute to septin organization and function in budding yeast. This question has been recently addressed by similar kinds of studies but there are still some open questions, particularly as regards to what extent the kinases may interact with and/or modify components of the contractile ring that drives cytokinesis.

Strengths:

This study uses sensitive imaging with good temporal and spatial resolution to monitor the localization of various proteins in living cells. Particularly informative is the use of a GFP/GFP-binding-protein "tethering" approach to ask if the requirement for one protein can be bypassed by physically tethering another protein to a third protein. Results from a yeast two-hybrid assay for measuring protein-protein interactions in vivo are buttressed by direct in vitro binding assays using purified proteins, which is important given the likelihood of "bridging" interactions between yeast proteins in the two-hybrid approach. The authors' conclusions are quite well supported by the data.

Weakness:

Ultimately, while the study provides some interesting and novel insights, we still don't understand which phosphorylation events on which proteins are important for the events occurring at the molecular level, so the advance in knowledge is somewhat incremental.

<https://doi.org/10.7554/eLife.106366.2.sa3>

Reviewer #2 (Public review):

Summary and strengths:

In this study, Bhojappa et al. investigate the roles of the septin-associated kinases Elm1, Gin4, Hsl1, and Kcc4 in septin organization and cytokinesis in budding yeast. Through quantitative analyses of kinase localization dynamics, septin organization, actomyosin ring (AMR) constriction, and cell morphology, the authors demonstrate that Elm1 and Gin4 play particularly important roles in maintaining proper septin architecture and cytokinetic progression. The work further identifies an interaction between the Gin4 KA1 domain and the Hof1 F-BAR domain and provides evidence that several cytokinesis-related functions of Gin4 are independent of its kinase activity. Artificial tethering approaches further supports that spatial organization at the bud neck is critical for the execution of septin-dependent cytokinetic processes. The authors combine live-cell imaging, quantitative analyses, biochemical interaction assays, and genetic perturbations to build a comprehensive framework for understanding how these kinases contribute to cytokinesis.

Comments on revised version:

The revised manuscript has been substantially improved. The authors have carefully addressed the concerns raised during review by providing additional experiments, analyses, quantifications, clarifications, and improved presentation of the data. The conclusions are now well supported by the experimental evidence. The study advances our understanding of the mechanisms linking septin organization to cytokinesis and will be of interest to researchers studying septins, cell division, and cytoskeletal regulation.

Overall Assessment:

This work provides valuable mechanistic insight into the coordination of septin organization and cytokinesis by septin-associated kinases. The experiments are carefully executed, the analyses are thorough, and the conclusions are supported by the data presented. The manuscript represents a useful contribution to the fields of cytokinesis and septin biology.

<https://doi.org/10.7554/eLife.106366.2.sa2>

Reviewer #3 (Public review):

Summary:

The study by Bhojappa et al. brings new and interesting elements about the stability of the septin ring and the crosstalk between septin and actomyosin ring assemblies. The study focuses on the four kinases associated with the septin ring, Elm1p, Gin4p, Hsl1p and Kcc4p. Elm1 and Gin4 show the strong knock-out phenotypes, whereas Hsl1p and Kcc4p show the weak knock-out phenotypes. The Elm1p/Kccp1p and Gin4p/Hsl1p pairs show similar timing at the bud neck. While these kinases share redundant functions, Gin4 appears to have a unique interaction with the BAR domain protein Hof1, revealing a novel direct interaction between the septin and actomyosin rings. Interestingly, the kinase activity of Gin4 is not required for its role in septin organisation and AMR constriction. The last part of the manuscript shows an original protein tethering protocol used to show that Hsl1 and its membrane binding ability are required for phenotype rescue of gin4null cells.

Comments on revised version:

I thank the authors for their thorough and thoughtful response to my review. The revised manuscript clearly reflects their efforts to provide rigorous and high-quality science. Addressing the concerns raised in the review required significant effort, but the improvements in the manuscript make it clear that the work was well worth it.

<https://doi.org/10.7554/eLife.106366.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public review):

Summary:

The authors wanted to better understand how the various septin-associated kinases contribute to septin organization and function in budding yeast. This question has been recently addressed by similar kinds of studies but there are still some open questions, particularly as regards to what extent the kinases may interact with and/or modify components of the contractile ring that drives cytokinesis.

Strengths:

This study uses sensitive imaging with good temporal and spatial resolution to monitor the localization of various proteins in living cells. Particularly informative is the use of a GFP/GFP-binding-protein "tethering" approach to ask if the requirement for one protein can be bypassed by physically tethering another protein to a third protein. Results from a yeast two-hybrid assay for measuring protein-protein interactions in vivo are buttressed by direct in vitro binding assays using purified proteins, which is important given the likelihood of "bridging" interactions between yeast proteins in the two-hybrid approach. The authors' conclusions are quite well supported by the data.

Weaknesses:

A control for non-specific binding is missing from the in vitro binding assay. The figures suffer sometimes from the very small text in the labels, which obscures understanding. Ultimately, while the study provides some interesting and novel insights, we still don't understand which phosphorylation events on which proteins are important for the events occurring at the molecular level, so the advance in knowledge is somewhat incremental.

We thank the reviewer for highlighting the strengths of our imaging pipelines and protein-protein interaction data. We have now included appropriate controls for the *in vitro* binding assays, which demonstrate that the observed interactions are specific (Fig. 2H). We have also revised all figures to improve clarity, including increasing font sizes to enhance visibility across panels. We agree that mapping the specific phosphorylation sites regulated by these septin kinases would provide valuable mechanistic insights. However, only a few studies have addressed this direction so far (Mortensen et al., 2002; Asano et al., 2006 and Marquardt et al., 2024) [1-3]. The current study focuses on the interplay among septin-associated kinases and their role in regulation of the actomyosin machinery (AMR). In this context, we highlight several key findings:

(i) a molecular link between the septin kinase network and AMR through physical interaction between the KA1 domain of Gin4 and F-BAR domain of Hof1 (Fig. 2F-2H and S3H);

- (ii) a kinase-independent role for Gin4 in coordinating septin organization and AMR dynamics (Fig. 3A-3E, S3F & S3G, S3I and 4F-4H);
- (iii) a novel role for Hsl1 in regulating septins and the AMR downstream of Gin4 and Elm1, potentially through plasma-membrane binding (Fig. 4I-4K, 5A-5F, 8A-8G and S9H-S9J); and
- (iv) crosstalk between Gin4 and Hsl1 that is independent of their role in the morphogenetic checkpoint (Fig. 6A-6D and S6A-S6C).

We have clarified this scope in the Discussion section and explicitly stated that mapping these phosphorylation sites will be an important direction for future work (Lines 623-626).

Reviewer #2 (Public review):

Summary:

In this paper, Bhojappa et al. provide insights into the function of septin-related kinases Elm1, Gin4, Hsl1, and Kcc4 in septin organization and actomyosin ring (AMR) structure and constriction. Their findings are both corroborative of and complementary to previous related studies.

*First, the authors provide a comparative analysis of the dynamic localization of these kinases at the bud neck, as well as a comparative analysis of defects in septin localization, splitting dynamics, AMR constriction rates, and cell morphology in kinase deficient cells. They find that septin localization and splitting kinetics, as well as AMR constriction rates, are significantly perturbed in *elm1Δ* and *gin4Δ* mutants but remain largely unaffected in *hsl1Δ* and *kcc4Δ*. A similar trend is observed in terms of cell morphology and viability.*

*Next, the authors focus on *elm1Δ* and *gin4Δ* cells, demonstrating that the residence time of the F-BAR protein Hof1 is significantly increased and defective in these mutants. Using yeast two-hybrid (Y2H) and in vitro binding assays, they show that the KA1 domain of Gin4 interacts with the F-BAR domain of Hof1, which may explain the cytokinesis-related functions of Elm1 and Gin4. Supporting this, they find that Gin4's role in septin localization, AMR constriction kinetics, and Hof1 bud neck localization is kinase-independent.*

*The authors then conduct a series of artificial tethering experiments given their bud neck localization is mostly interdependent. They first demonstrate that artificially tethering Gin4 to the bud neck rescues the morphology defects of *elm1Δ* cells, with the strongest rescue observed when Gin4 was forced to interact with Hsl1—an effect that was also kinase-independent. Additionally, artificial tethering of Hsl1 to the bud neck restores the morphology of *elm1Δ* cells in a KA1 domain-dependent manner, suggesting that Hsl1 functions downstream of Elm1 to maintain normal cell morphology. Consistently, artificial tethering of Elm1 to the bud neck in *gin4Δ* cells rescues morphology defects, as well as defects in Myo1 localization and AMR constriction, but only in the presence of full-length Hsl1. The rescue fails in the absence of Hsl1 or when using a version of Hsl1 lacking the KA1 domain, which supports the role of Hsl1 downstream to Elm1 in cytokinesis.*

Strengths:

Altogether, this study offers valuable insights into the mode of cytokinesis regulation mediated by the septin-related kinases, mainly Elm1, Gin4, and Hsl1, and would be an important contribution to the field of septins and cytokinesis after addressing current weaknesses.

We thank the reviewer for the detailed summary and for highlighting the novel findings of our study.

Weaknesses:

(1) When assessing rescue of the elm1Δ phenotype, it needs to become clearer whether only morphology or also cytokinesis and septin organization are rescued.

To clarify the extent of rescue observed in *elm1Δ* cells, we extended our analysis beyond morphological parameters by quantifying septin organization and AMR constriction dynamics. These analyses now show that artificial tethering partially restores septin organization and AMR constriction kinetics in *elm1Δ* cells in addition to improving cell morphology. These results are now described in detail in Fig. 5 and lines 373-406.

(2) The quantification of the microscopy data does not always match up with the example images, and it's not always clear how the authors quantitatively analyzed their data.

We revised the manuscript to clearly outline the quantification methods used for microscopy data analysis and specified the statistical tests, number of cells analyzed, and number of experimental replicates in the figure legends. We also clarified the criteria used for phenotype scoring and quantification in the Materials and Methods section. In addition, We replaced representative images where necessary to accurately reflect the quantified data throughout the revised manuscript.

(3) The forced tethering data are key to the paper, but the lack of a summarizing table makes it difficult to grasp the full picture.

We agree with the reviewer and have now included a new summary table (Table 1) that compiles the results of all artificial tethering experiments presented in this study, including the percentage of rescue in cellular morphology observed upon forced tethering of these kinases to the bud neck, thereby providing a clearer overview of these experiments.

(4) Novel results and those confirming earlier results could be better distinguished.

We have improved the overall clarity of the manuscript to distinguish novel findings from the results that corroborate previous studies, and have cited the appropriate literature throughout the revised manuscript.

Reviewer #3 (Public review):

Summary:

The study by Bhojappa et al. brings new and interesting elements about the stability of the septin ring and the crosstalk between septin and actomyosin ring assemblies. The study focuses on the four kinases associated with the septin ring, Elm1p, Gin4p, Hsl1p, and Kcc4p. Elm1 and Gin4 show strong knock-out phenotypes, whereas Hsl1p and Kcc4p show weak knock-out phenotypes. The Elm1p/Kccp1p and Gin4p/Hsl1p pairs show similar timing at the bud neck. While these kinases share redundant functions, Gin4 appears to have a unique interaction with the BAR domain protein Hof1, revealing a novel direct interaction between the septin and actomyosin rings. Interestingly, the kinase activity of Gin4 is not required for its role in septin organisation and AMR constriction. The last part of the manuscript shows an original protein tethering protocol used to show that Hsl1 and its membrane binding ability are required for phenotype rescue of gin4null cells.

Strengths:

The combination of genetics, cell imaging, and biochemical characterization of proteinprotein interactions is attractive.

We thank the reviewer for recognizing the significance of our findings and for the helpful suggestions.

Weaknesses:

(1) Imaging and data analysis is the main weakness of this manuscript. The authors must avoid manual counting and selection when easy analysis software can be used to limit bias. Instead of presenting unclear statistics of "percentage phenotypes", they need to define clear metrics to offer meaningful phenotype analysis.

We agree that improving the quantitative rigour of the image analysis is essential for this study. Accordingly, we implemented a semi-automated image analysis workflow in the revised manuscript that defines reproducible metrics, such as aspect ratio, and reduces reliance on subjective phenotypic scoring. The inclusion of these parametric measurements enables clearer and more objective comparison of the rescued phenotypes.

(2) This manuscript examines a very complex mechanism with four kinases of overlapping function using new data and existing literature. A clearer picture/model at the end of the manuscript that synthesizes the current knowledge would be beneficial:

We incorporated a new representative model (Fig. 9) that integrates current knowledge in the field with our findings. This model highlights crosstalk among Elm1, Gin4, and Hsl1 as a key mechanism coordinating septin architectural transitions with AMR constriction during cytokinesis and is discussed in lines 520-536 of the revised manuscript.

We sincerely thank all the reviewers for their insightful comments. We incorporated new results in Fig. S3A, S3B, S4A-S4F, 5A-5F, 6A-6D, S6A-S6C, 8E, 8F, S10B and 9, along with Table 1 summarizing the artificial tethering experiments in the revised manuscript. We believe that these revisions have improved the rigor of our analyses and enhanced the overall clarity of the manuscript.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

(1) Line 70: " abnormal cytokinesis defects": this language is redundant, either "abnormal" or "defects" would suffice:

We thank the reviewer for identifying the redundant wording. We have rephrased the final paragraph of the Introduction section.

Please refer to line numbers 86-97.

(2) Currently, the final paragraph of the Introduction is an extensive, detailed summary of the results. This is unnecessary, as the Abstract and the Results sections summarize the results. Better would be a short statement of the questions addressed in the manuscript:

We thank the reviewer for this suggestion. We have revised the final paragraph of the Introduction to remove the detailed summary of results and instead outline the key questions addressed in this study. The revised paragraph now emphasizes the knowledge gap regarding how septin-associated kinases regulate septin organization and coordinate cytokinesis. Please refer to line numbers 86-97.

(3) In the Results, the wording of the heading associated with the first section is confusing. "and their defects during the cell cycle": it is not expected that the kinases

themselves will have defects; defects may be observed in cells upon mutation of the kinases, but that is not clear from this wording:

We thank the reviewer for this comment. The heading has been revised from “and their defects during the cell cycle” to “and defects associated with their deletions” for improved clarity.

Please refer to line numbers 99-100.

(4) Lines 107-109 (" they may act as molecular signals for the transition and a trigger for crosstalk of cytokinesis") are redundant with earlier lines 104-105 (" suggesting them to be a possible trigger for septin remodelling"):

We have rephrased the text to improve clarity and avoid redundancy.

Please refer to line number 123-125.

(5) Lines 121-122: "Septin-associated kinases are believed to play an essential regulatory role": is the role essential, or is it regulatory? Since these kinases are not individually essential for cytokinesis, "essential" doesn't seem appropriate here:

This has been corrected in the revised manuscript.

(6) Figure 1 panels C and F: the font size is exceedingly small for the labels and should be greatly increased. This is true for multiple panels in Figures 2-5 and the Supplemental Figures as well:

We thank the reviewer for highlighting this issue. We have significantly increased the font sizes of the text and the x- and y-axis labels across all figures in the manuscript.

(7) The timing of mitotic spindle breakdown was used as a timepoint for comparison but it is not made clear in the manuscript how this was determined. Presumably, the mRuby2-Tub1 marker was visualized and the timepoint when the mitotic spindle separated into two discrete entities was called the "breakpoint" timepoint, but it would be important to better describe (and ideally show an example) of how that timepoint was determined. The only images I find with labelled tubulin are Tub1-GFP and these do not show spindle breakdown:

We thank the reviewer for raising this point. We used GFP-Tub1 (pAFS125-GFP-TUB1) and mRuby2-Tub1 (pHIS3p:mRuby2-Tub1+3'UTR::URA3) plasmids to visualize spindle dynamics across the cell cycle, and in both cases defined the spindle breakpoint as time zero. As suggested, we have now included time-lapse images of Cdc3-mCherry and GFP-Tub1 in wild-type cells in Fig. S2D to illustrate the spindle breakpoint event used for temporal alignment. Corresponding changes have also been made in the Materials and Methods section to explicitly describe this analysis.

Please refer to line numbers 760-761.

(8) Line 165: "F-BAR protein Hof1, which senses and induces membrane curvature" and lines 170-172 "the F-BAR protein Hof1, which is known to be associated with septin hourglass and transit to AMR during split ring trigger". It is awkward to introduce the same protein twice, in two different ways, within a few lines of each:

We thank the reviewer for noting the redundant description. We have rephrased the text to introduce the F-BAR protein Hof1 in a more concise and streamlined manner while retaining the relevant functional information.

Please refer to line numbers 206-208.

(9) Throughout, it would be helpful to introduce more line breaks and organize the Results into smaller paragraphs:

We thank the reviewer for this suggestion. We have revised the layout of the Results section and introduced additional line breaks to improve readability.

(10) This is somewhat of a personal preference, but in the interest of transparency (and with the understanding that the 0.05 value is entirely arbitrary), would authors be willing to show actual *P* values in the figure panels rather than "***" or "ns", for example? Some readers may wish to apply a different standard of significance than 0.05, and not showing the *P* values makes this impossible. Furthermore, some readers (like this one) may interpret differently a *P* value of 0.044 versus "*" or 0.051 vs "ns":

We agree with the reviewer's suggestion. To improve the transparency of the quantitative analyses, we have modified the graphs across all main and supplementary figures to display the "actual p-values" and specified the corresponding statistical tests in the figure legends, with significance indicated by asterisks. An example is provided in the attached image showing the residence time of Inn1-mNG in wild-type and *gin4Δ* cells complemented with kinase-active and kinase-dead Gin4 constructs (Fig. 3E in the revised manuscript). For this analysis, significance was assessed using the nonparametric Kruskal-Wallis statistical test.

(11) It is written that the authors "performed a Yeast Two Hybrid screen to find novel interacting partners at the bud neck" but I do not find evidence anywhere of a "screen" being performed, i.e., an unbiased search of many proteins to find a few interactors. Instead, it appears that the authors performed a two-hybrid assay to visualize interactions between a small, specific set of proteins. "Screen" should be replaced with "assay", as is currently the case in the Methods section. It is probably also valuable to point out in the text that using a yeast two-hybrid assay to assess interactions between yeast proteins has the caveat that any interactions observed could be indirect, as they may be "bridged" by endogenous yeast proteins.

We thank the reviewer for raising this point. Our Yeast Two-Hybrid experiments were performed using a small subset of septin-associated proteins rather than as an unbiased screen. Accordingly, we have replaced the term "screen" with "assay" throughout the revised manuscript and in the Materials and Methods section.

Please refer to line 241.

We also agree with the limitations inherent to this assay and have now explicitly stated it in the revised manuscript, please see lines 248-255.

(12) Panel 2H: I do not understand what the middle (as opposed to the top and bottom) blot segment represents. It is labelled "anti-HIS", like the one above it, but it is not associated with any molecular weight/ladder marker and I do not know what other species in the binding reaction in that lane would be recognized by the anti-HIS antibody. Perhaps the top segment is an "input" sample, and below it (in the middle segment) is what was bound to the beads. The figure legend is uninformative in this regard. Also, panel I in this figure is unnecessary to show, assuming that the bands shown in H are what I think they are. The blot makes the point without the need for quantification.

As suggested by the reviewer, we have added molecular weight markers for each blot panel showing the input and bead-bound fractions. The figure legend has also been updated accordingly to clearly describe the different blot segments and experimental conditions.

Please refer to figure legend 2H.

In addition, as suggested by the reviewer, we have removed the quantification graph corresponding to the in-vitro binding assay from the revised manuscript.

(13) *The results in Figure 2H demonstrate that the Gin4-KA1 fragment is not non-specifically "sticky", because it does not bind GST alone, but there is no demonstration that binding by the Hof1 fragment is specific because there is no equivalent negative control for binding:*

We thank the reviewer for this suggestion. We repeated the in-vitro binding assay using 6His-bdSUMO as a negative control alongside 6His-bdSUMO-Gin4^{KA1} to demonstrate binding specificity of the Hof1 fragment. The Hof1 N-terminal F-BAR fragment did not pull down the control 6His-bdSUMO fragment but specifically pulled down 6His-bdSUMO-Gin4^{KA1} under identical experimental conditions (Fig. 2H), confirming the specificity of the interaction between Hof1 F-BAR domain and Gin4KA1.

The corresponding text and results have been updated in the revised manuscript.

Please refer to Fig. 2H and lines 259-263.

(14) *Line 257-258: "Localisation via Hsl1 is necessary to rescue the morphological defects exhibited by $\Delta elm1$ cells partially": what does "partially" refer to here? To the rescue, or the defects?*

The term “partially” refers to the extent of rescue. Specifically, elongated cell morphology was rescued in 63.75% of the *elm1Δ* cell population, rather than in all cells, upon artificial tethering of Gin4-GFP to the bud neck via Hsl1-GBP.

(15) *Lines 292-293: "can restore the morphological defects": this wording is unclear. "Restore" means "return to a former condition", which in this case would be normal cellular morphology, not defective cellular morphology. Similarly, see line 304: "While Myo1-3xmCherry mislocalisation was restored upon Elm1-GFP tethering": presumably the proper localization was restored, not the mislocalization:*

In Lines 292-293, by phrase “can restore the morphological defects” was intended to indicate rescue of the elongated/clumped morphology associated with *gin4Δ* cells upon artificial tethering of Elm1-GFP to the bud neck via Shs1-GBP. We have now rephrased this sentence as: “can rescue the elongated/clumped phenotype exhibited by *gin4Δ* cells”.

Please refer to line numbers 481-482.

Similarly, in Line 304, the statement “While Myo1-3xmCherry mislocalisation was restored upon Elm1-GFP tethering” referred to rescue of the Myo1 mislocalization phenotype observed in *gin4Δ* cells. We have rephrased this sentence as: “However, Myo1-3xmCherry localization was restored to the bud neck upon artificial tethering of Elm1-GFP in *gin4Δ* cells”.

Please refer to lines 506-508 in the revised manuscript.

(16) *Lines 295-296: "We find that Elm1-GFP tethering via Shs1-GBP, Bud4-GBP, and Hsl1-GBP" this should be "or", not "and":*

Thank you for pointing this out. We have now corrected the text accordingly.

Please refer to line number 485.

(17) *Lines 311 and 312 refer to "Inn1-3xmCherry lifetime" but previously Inn1 residence time was measured. Since fluorescence lifetime is a distinct kind of measurement/ assay, it seems important to clarify here what kind of experimental data are being referred to:*

The term “Inn1-3xmCherry lifetime” was intended to describe the residence time of Inn1 at the cell division site, defined as the interval between the initial appearance of the Inn1 fluorescence signal and its complete disappearance during cytokinesis. For clarity and consistency, we have replaced the term “lifetime” with “residence time” in the revised manuscript.

Please refer to the line numbers 511 and 512.

(18) Discussion: "Septins are considered as the fourth cytoskeletal elements due to their extensive structural and functional diversity." This sentence is confusing, as it seems to imply that what defines a protein as being "cytoskeletal" is structural and functional diversity rather than anything to do with forming filaments, etc. The rest of this first paragraph of the Discussion also sounds like a summary of the background, is quite redundant with the Introduction, and should be shortened:

We thank the reviewer for this suggestion. We have rewritten the first paragraph of the Discussion to improve clarity, reduce redundancy with the Introduction, and better emphasize the main findings of the study.

Please refer to the line numbers 538-552 in the Discussion section.

(19) Lines 365-366: "Gin4 and Hof1 are synthetic lethal": this should be revised to "gin4Δ and hof1Δ are synthetic lethal". This is a good place to point out that standard yeast nomenclature inserts the Δ symbol after the gene name, not before (as is done in E. coli genetics, for example):

We thank the reviewer for this suggestion. We have replaced “Gin4 and Hof1 are synthetic lethal” with “gin4Δ and hof1Δ are synthetic lethal” in the revised manuscript.

Please refer to line number 576.

In addition, we have now consistently placed the Δ symbol after the gene throughout the manuscript in accordance with standard yeast nomenclature.

(20) Line 399: "We also performed an extensive GFP-GBP screens": again, here "screen" implies that a large collection of genes/proteins were assayed, perhaps in an unbiased way, which does not accurately portray what was actually done, which was an extensive tethering study using GFP-GBP:

We thank the reviewer for this suggestion. We have replaced the term “GFP-GBP tethering screen” with “GFP-GBP tethering assay” throughout the revised manuscript. In addition, we have included a brief description of the specificity and functionality of GBP nanobody and its application in the GFP-GBP tethering strategy, extensively used in this study.

Please refer to line numbers 326-336.

Reviewer #2 (Recommendations for the authors):

Major points:

(1) Analysis of morphological defects of elm1Δ does not directly reflect the defects in cytokinesis and septin organization. For example, the deletion of SWE1 rescues the morphological defects of elm1Δ cells but not the cytokinesis or septin mislocalization (Bouquin et al., 2000). Considering this, the authors should address whether artificial tethering of Hsl1 to Gin4 in elm1Δ cells rescues the septin and cytokinesis defects or just the morphology. Is the role of Hsl1 in cytokinesis dependent on its role in the morphogenesis checkpoint? Can the authors comment on how much the defects

observed by Hsl1 tethering to the bud neck may be a result of bypassing the morphogenesis checkpoint?:

We thank the reviewer for this important point. We performed time-lapse imaging of Cdc3-mCherry in strains where Gin4-GFP partially rescued the elongated phenotype of *elm1Δ* cells (63.75%) when tethered to the bud neck via Hsl1-GBP. Under these conditions, 64.29% of cells showed rescue of Cdc3-mCherry mislocalization, and Gin4 localization itself was restored to the bud neck in 57.85% of cells. We also examined Myo1-ymScarletI dynamics, while 76.92% of untethered *elm1Δ* cells displayed Myo1 mislocalization to the bud cortex, this was reduced to 11.36% upon Gin4-GFP tethering via Hsl1-GBP. Together, these results indicate that the morphological rescue observed in *elm1Δ* cells is accompanied by restoration of normal septin organization and AMR dynamics.

Previous work (Bouquin et al., 2000) [4] showed that Swe1 deletion rescues cell elongation in *elm1Δ* cells without restoring septin organization. Consistent with this, we found that 60.66% of *elm1Δ swe1Δ* cells exhibited a round morphology, but tethering of Gin4-GFP to the bud neck via Hsl1-GBP in *elm1Δ swe1Δ* background did not further enhance morphological rescue. These results suggest that the rescue of cellular morphology observed in our tethering experiments may, at least in part, depend on Hsl1-mediated regulation of the morphogenesis checkpoint.

Importantly, despite the lack of additional morphological rescue, a clear restoration of septin localization was observed when Gin4-GFP was tethered to the bud neck via Hsl1-GBP in *elm1Δ swe1Δ* cells. Overall, these results suggest that while Hsl1-dependent morphogenesis checkpoint regulation may contribute to cell shape rescue, the restoration of septin organization is independent of Hsl1's function in morphogenesis checkpoint and instead reflects a direct requirement for Gin4 and Hsl1 at the bud neck.

Please refer to Figures 5, 6, and S6 of the revised manuscript for these additional data.

(2) As suggested by the authors, the interaction of the Gin4-KA1 domain with the FBAR domain of Hof1 may explain the cytokinesis-related functions of Gin4. As an orthogonal approach, how does KA1 domain deletion of Gin4 affect cytokinesis and Hof1 bud neck localization?

We thank the reviewer for this suggestion. We first examined the bud neck localization of Gin4-*ka1Δ*-GFP in comparison with full-length Gin4-GFP. We observed that the localization kinetics of Gin4-*ka1Δ*-GFP were significantly altered relative to the full-length protein, with reduced recruitment and earlier removal from the bud neck. We also analyzed the localization kinetics of Hof1-mNG in both *gin4-ka1Δ* and *gin4Δ* cells. Our results show that Hof1-mNG displays increased residence time and altered accumulation kinetics during cytokinesis in both genetic backgrounds. Thus, loss of the KA1 domain phenocopies loss of the full-length Gin4 and is consistent with disruption of the physical interaction between Gin4 and Hof1.

Please refer to Figure S4 for these results in the revised manuscript.

(3) The authors state that "Elm1 and Kcc4 were present at lower abundance at the bud neck (Fig S1A-D) compared to the higher abundance of Gin4 and Hsl1, as observed in their fluorescence intensities (Figures S1B-C)". This is not evident in the figures. The authors should show a quantification of how they judged abundance at the bud neck:

We thank the reviewer for this question. Quantification of septin kinase fluorescence intensity at the bud neck was performed using the established protocol for measuring protein accumulation kinetics described by Okada et. al. 2020 [5]. Time-lapse imaging for kinetic analysis of septin-associated kinases during bud emergence shown in Fig. S1A-S1D was carried out using a point-scanning confocal microscope with a 100×oilimmersion objective.

Different laser intensities were required because the fluorescence signals of Kcc4 and Elm1 were comparatively weak and not readily detectable above cellular background under the imaging conditions used for Gin4 and Hsl1. The images shown in Fig. S1A-S1D are therefore displayed using differential contrast settings to facilitate visualization. We have now explicitly clarified this in the figure legend.

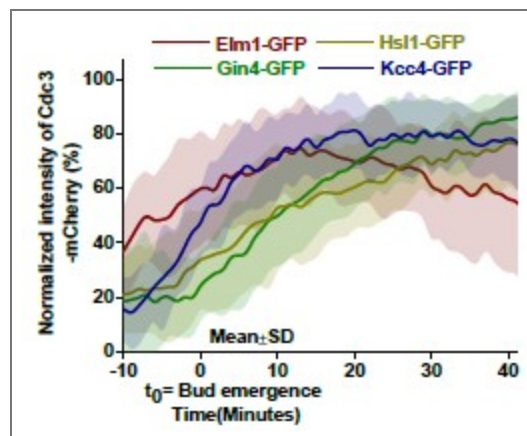
A more direct comparison of septin kinase abundance at the bud neck is now provided in Fig. S1E-F, where localization kinetics during the HDR transition/septin remodelling stage were captured using a laser-scanning spinning-disk microscope under similar imaging conditions. We have additionally included raw fluorescence intensity profiles during the HDR transition to better illustrate the relative abundance of these kinases at the bud neck during cytokinesis.

Please refer to Figures S1E and S1F in the revised manuscript for the updated images and quantitative analyses.

(4) How did the authors determine G1 and M-phase in the experiments shown in Figures S1A-D? Can the authors mark these phases on the timelapse images? Also, How do the authors explain the different behaviour of Cdc3 in graphs S1A-D among different strains?

We thank the reviewer for this comment. Cell cycle stages were initially inferred based on bud size, where kinase accumulation at the bud neck correspond to bud emergence (small bud, G1), and kinase disappearance coincided with septin splitting (large bud, M phase). However, we agree that accurate assignment of cell cycle stages would require specific cell cycle markers. To avoid confusion, we have removed the cellcycle-specific stage assignments from the Results section and describe the kinetics relative to $t=0$ (bud emergence).

The differential dynamics observed in the Cdc3-mCherry kinetic profiles likely reflect heterogeneity within the cellular population. To address this, we combined the normalized fluorescence intensity profiles of Cdc3-mCherry from the strains expressing GFP-tagged septin kinases during bud emergence and have included this data as reference (Author response image 1).



Author response image 1. Plot showing spatiotemporal kinetics of Cdc3-mCherry in strains expressing either Elm1-GFP, or Gin4-GFP, or Hsl1-GFP, or Kcc4-GFP.

(5) Forced tethering based experiments are one of the key sets of experiments for this work, but it is difficult to have a comprehensive understanding of all the data considering how large the data set is and how dispersed it is in the supplemental and main figures (Figures 3-4-5 and Figures S4-S5-S6). It would be helpful to provide a table summarizing the tested forced tethering's and the phenotypic outcome in the tested yeast strains (Wt/mutant):

We thank the reviewer for recognising the extensive dataset generated from the artificial tethering experiments and for suggesting the inclusion of a summary table. We have now added Table 1, which summarizes the proteins used in the GFP-GBP artificial tethering experiments, their genetic backgrounds, the total number of cells quantified across three independent replicates, and the phenotypic outcomes associated with bud neck tethering under each condition.

Please refer to Table 1 and lines 342, 361, 451, 453, 456 and 492 in the revised manuscript.

(6) In the introduction section, it would help the reader to provide more information on already known molecular roles of septin-associated kinases in septin organization and AMR. Later in the results section (i.e. Figure S1, S2, and S6), the authors extensively explain and show data that independently corroborate some earlier findings, which makes it difficult for the reader to distinguish novel findings from the repeated findings. I suggest shortening the text for the corroborative results, which will help to put more emphasis on their novel findings:

We thank the reviewer for this suggestion. We have now included the canonical roles of these four septin-associated kinases in the Introduction section of the revised manuscript.

Please refer to line numbers 79-85.

We have also revised sections describing corroborative findings and explicitly cited previous studies wherever relevant in the Results section to better distinguish previously established observations from the novel findings presented in this work.

Other minor comments:

(1) In Figure 1D-1F, also show the data for hsl1Δ and kcc4Δ - which are shown in S3AC in the current version:

We have now included the Inn1-mNG residence time in *hsl1Δ* and *kcc4Δ* cells, alongside *elm1Δ* and *gin4Δ* in Fig. 1E.

Please refer to Fig. 1E in the revised manuscript.

(2) The authors should be more careful in interpreting their negative Y2H data in Figure 2F.

We have now explicitly discussed the caveats and inherent limitations of the Yeast Two-Hybrid assay in the manuscript.

Please refer to line numbers 248-255.

(3) Please provide quantification for Figure 5A, Figure S6I:

We have added quantitative analyses showing rescue of Cdc3-mCherry and Myo13xmCherry mislocalization in *gin4Δ* and *gin4Δ hsl1Δ* strains upon artificial tethering of Elm1-GFP to the bud neck via Shs1-GBP.

Please refer to Figures 8E, 8F, and S10B in the revised manuscript.

(4) In Figure S6: label is missing "Δ" in front of hsl1:

We thank the reviewer for pointing out this error. We have corrected the labels accordingly.

(5) As common consensus on yeast gene nomenclature, I suggest the use of "geneΔ" instead of "Δgene":

We thank the reviewer for this suggestion. We have now consistently placed the Δ symbol after deleted gene names throughout the manuscript in accordance with standard yeast nomenclature.

(6) Lines (535-536): *min(distribution) and max(distribution) in the formula is confusing. Clarify it or if possible use "minimum value", "maximum value" instead:*

We thank the reviewer for this suggestion. We have replaced the term “distribution” with “value” in the formula for protein accumulation kinetics analysis.

Please refer to the updated formula in the Materials and Methods section (Lines 752-753).

(7) In line 86, *"Dynamics of Septin-associated kinases and their defects during the cell cycle": Change the title as it is not clear what is meant by "their defects" given the discussed results under this title:*

We thank the reviewer for this suggestion. We have revised the section heading from “and their defects during the cell cycle” to “and defects associated with their deletions”.

Please refer to line numbers 99-100.

(8) On the Hof1-mNG image (Fig2C), show the line used for the line scan profile. Additionally, a similar line-scan profile could be useful in Figure S3G:

As suggested by Reviewer 3, we removed the line-scan analysis from Figure 2 in the revised manuscript because Hof1 ring organization showed substantial heterogeneity across cells, making line-scan analysis difficult to interpret reliably.

Reviewer #3 (Recommendations for the authors):

Major points:

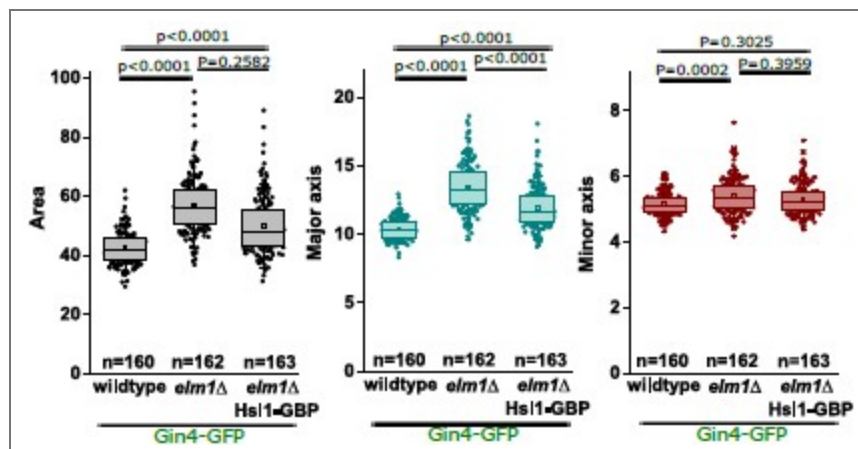
(1) *The % phenotype units are terrible. With no explanation, we do not really know whether they represent the percentage of cells that have a particular phenotype, or whether they correspond to a metric that measures some deviation between normal and extreme phenotypes. I would strongly recommend using precise quantitative metrics systematically (i.e. intensities, aspect ratios, division times, etc.) to properly quantify phenotypes:*

We thank the reviewer for suggesting the inclusion of precise quantitative metrics to assess phenotypic differences in the GFP-GBP tethering experiments. In the revised manuscript, we adopted quantification workflows that have been extensively validated and widely used in the literature, including those reported by Marquardt et al., 2024 (Fig. 7B and 7D) [2] from the Bi Lab. In response to the reviewer’s suggestion, we have now incorporated additional quantitative measurements, including cell area and aspect ratio (defined as the ratio of the cell’s major axis to the minor axis), for the experimental datasets presented in the manuscript.

In the main figures, we now include aspect ratio quantification, while additional parameters are provided for the reviewer’s reference. We also quantified the fluorescence intensity of tethered proteins at the large bud neck and present these data together with the aspect ratio analysis in Figure 4 for *elm1 Δ* cells in which Gin4GFP is artificially tethered to the bud neck via Hsl1-GBP. These quantitative analysis corroborates our qualitative observations and further strengthens our conclusions. Please refer to Figures 4D and 4E as representative examples.

We have also changed the y-axis labels throughout the revised manuscript. For example, the y-axis in Fig. 7B is now labelled as “Cells exhibiting round morphology (%)”. Please refer to

Fig. 4H, 4K, 7C, 8D, S5G, S6C, S7C, S9G and S9J for inclusion of aspect ratio quantification. Statistical analyses for the represented graphs were performed using Kruskal-Wallis nonparametric test, (N=3, n>150 cells/strain) (*: $p<0.05$, **: $p<0.01$, ****: $p<0.0001$, ns: $p>0.05$).



Author response image 2.

(2) Some quantitative analyses were performed manually where simple automated analysis should be performed to provide unbiased, accurate quantification:

We fully agree with the reviewer that automated image analysis approaches, such as segmentation-based methods, are generally preferred for minimizing bias in morphological quantification. However, *elm1*Δ and *gin4*Δ cells exhibit severe phenotypes, including pronounced elongation and clumping, which makes reliable automated segmentation technically challenging for accurate quantification of parameters such as aspect ratio and cell area. For this reason, we used manual annotation for these analyses, as this approach enabled accurate delineation of individual cell and reliable measurements of morphological parameters such as cell area and size across the datasets despite being more time-consuming.

Please refer lines 769-775 in the Materials and Methods section.

(3) The "tethering" data also lack clear quantification. The authors should properly quantify the average intensity of Hsl1-GFP at the bud neck in each condition and correlate the results with cell aspect ratios or any other relevant parameters. For example, when comparing *elm1*null and *elm1*null Bud4-GBP with *elm1*null Kcc4-GBP, the visual impression is that as much Hsl1-GFP protein is recruited to the bud neck, whereas the phenotypes are dramatically different:

We thank the reviewer for pointing this out. We have revised the image representation to facilitate clearer interpretation of the tethering experiments. In addition, we performed the key GFP-GBP tethering experiments using GBP-ymScarletI constructs, allowing direct visualisation of both the GFP-tagged protein and the GBP-tagged partner at the bud neck following tethering. We have included quantitative analyses of cellular morphology, raw fluorescence intensities of GFP-tagged proteins at the large bud neck, and the corresponding aspect ratio measurements for these updated datasets (Author response images 3, 4, 5). We have also included a summary table (Author response table 1) compiling these quantitative results for easier comparison.

(4) I am very confused by Figure 3 which shows normal localization of Gin4-GFP in *elm1*null cells and seems to contradict other claims in the manuscript. This is very problematic for the interpretation of most of the "tethering" data:

We understand the reviewer's concern and have replaced the representative images of Gin4-GFP in *elm1Δ* cells in Figure 4B. Although Gin4-GFP is initially recruited to the presumptive bud neck during bud emergence in *elm1Δ* cells, it subsequently becomes mislocalized to the bud cortex during early cell cycle stages, resulting in reduced bud neck localization. As the cell cycle progresses, the Gin4-GFP signal at the bud neck decreases substantially in *elm1Δ* cells while remaining stable in wild-type cells until its departure prior to septin HDR remodelling (Fig. S5A-S5D).

(5) Figure 6 is neither explained in the text nor in its legend. Could the authors explain the model and offer a comprehensive picture of the current knowledge?

We have simplified the representative model to more clearly distinguish previously established knowledge from the findings presented in this study. Based on our results, We propose that Hsl1 functions both downstream of and in coordination with Elm1 and Gin4 to regulate septin stability and the timely execution of cytokinesis. Deletion of Elm1 disrupts the normal localization and crosstalk between Gin4 and Hsl1 at the bud neck, leading to septin mislocalization and misregulation of AMR dynamics, thereby revealing a previously uncharacterized role for Hsl1 in cytokinesis. The Results section has also been updated to reflect the revised model.

Please refer to Figure 9 and lines 520-536.

(6) Could the authors provide information about the double/triple mutant kinase phenotypes to clarify the overlap of functions among them?:

Barral et al., 1999 [6] reported that individual deletions of Hsl1 and Gin4 result in mild cytokinetic defects, whereas deletion of Kcc4 does not produce any striking phenotype compared to wild-type cells. In contrast, the *hsl1Δ gin4Δ kcc4Δ* triple mutant remains viable but exhibits severe morphological abnormalities, including branched chains of elongated cells with defective cell separation. These mutants also display aberrant septin organization at the bud neck, characterized by irregular patch-like structures. Analysis of double mutants (*hsl1Δ gin4Δ*, *gin4Δ kcc4Δ*, and *hsl1Δ kcc4Δ*) revealed intermediate phenotypes between the corresponding single and triple mutants, with the *hsl1Δ gin4Δ* combination showing the strongest defects. Together, these findings suggest that the Nim1-related kinases function redundantly to regulate Swe1 activity and maintain septin architecture at the bud neck.

Further supporting this model, Bouquin et al., 2000 [4] demonstrated that Elm1 operates independently of the Nim1-related kinases in controlling septin organization. The *hsl1Δ gin4Δ kcc4Δ elm1Δ* quadruple mutant exhibits severe septin localization defects and strong growth defects, in contrast to the *elm1Δ* single mutant, which primarily displays septin mislocalization from the bud neck to the bud cortex. These findings indicate that the combined activity of these kinases is essential for proper septin anchorage at the division plane and for assembly of the septin ring.

Importantly, the progressively stronger phenotypes observed in double, triple and quadruple mutants also suggest that these kinases retain partially specialized functions at the bud neck. Consistent with this framework, our results support a model in which Nim1-related kinases function redundantly to regulate septin architecture and cytokinesis, likely through modulation of the AMR machinery. Because the localization of these kinases appears interdependent, as reported previously (Marquardt et al., 2020; Marquardt et al., 2024) [2,7] and corroborated by our findings, interpretation of mutant phenotypes remains complex and future studies will be required to delineate their individual contributions more precisely.

Minor points:

(1) Abbreviations are not defined in the manuscript.

We have now expanded and defined all abbreviations throughout the manuscript.

| *(2) Some of the writing in the figures is too small. Please make sure that a minimal size of letters/numbers is respected:*

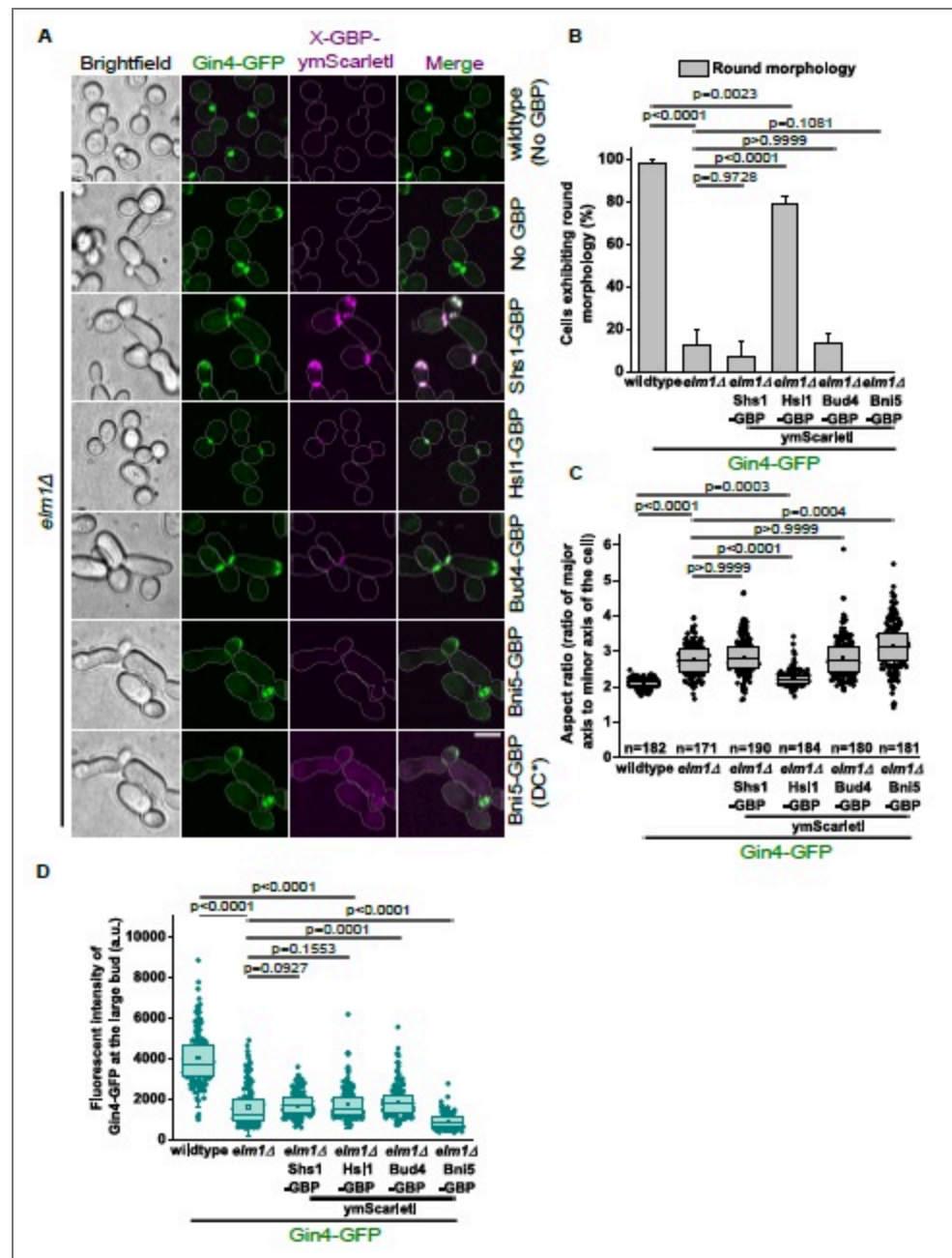
We thank the reviewer for raising this issue. We have enlarged the figure labels and axis labels throughout the revised manuscript to improve readability.

| *(3) Figure 2D. I am not sure that the line scans bring any useful information as the rings in mutant cells are quite heterogenous. This panel is also not cited in the text. Please make sure that every panel is cited at least once:*

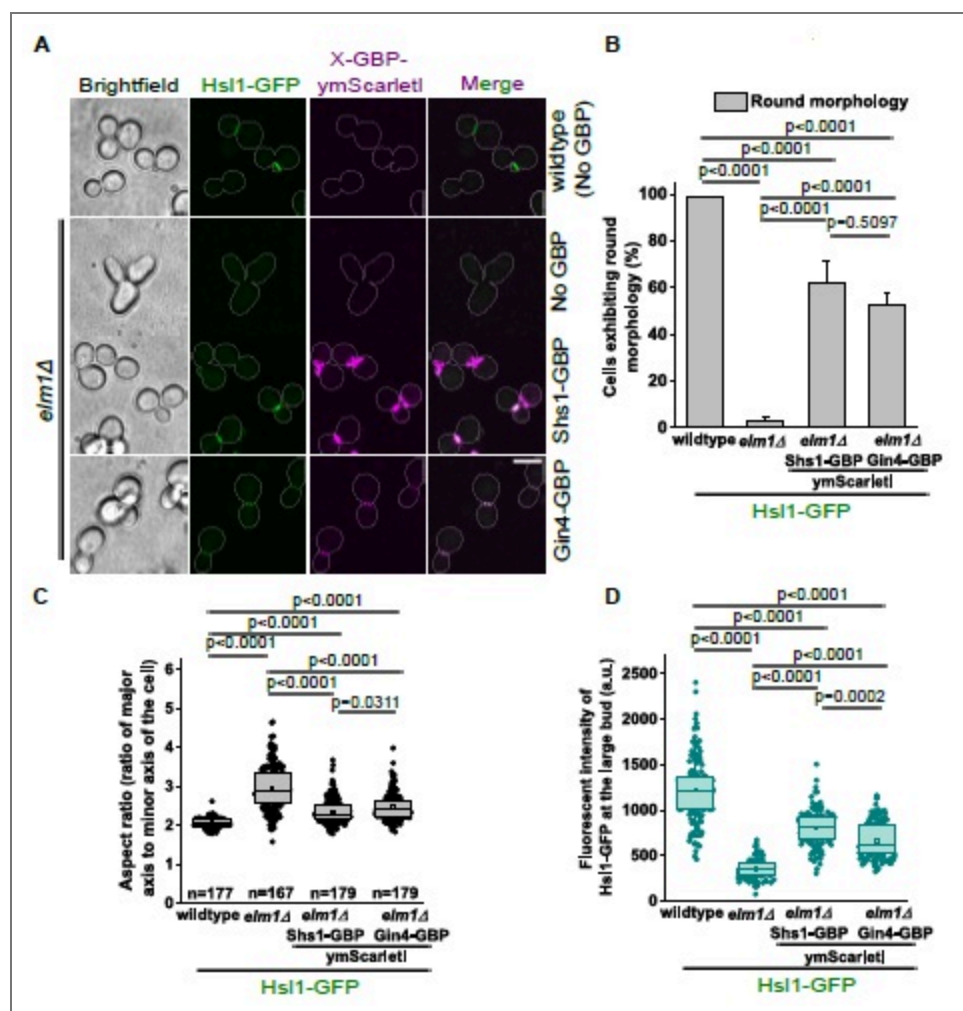
We agree with the reviewer regarding the heterogeneity observed in the Hof1 ring organization in mutant cells and have therefore removed the line-scan analysis from the revised manuscript.

| *(4) Knocked-out genes are written incorrectly. Please use the usual yeast nomenclature:*

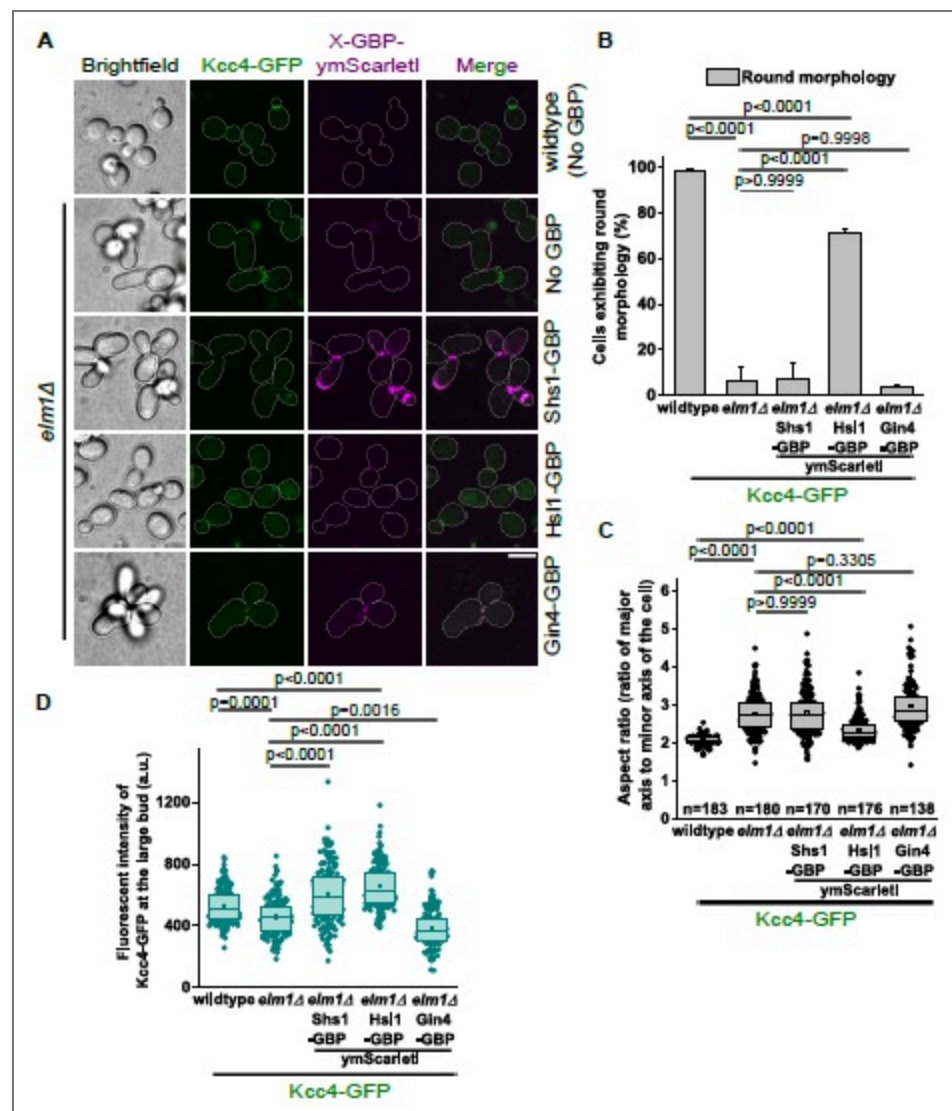
We thank the reviewer for this suggestion. We have corrected the nomenclature for all deleted genes throughout the manuscript in accordance with standard yeast nomenclature.



Author response image 3. Artificial tethering of Gin4-GFP to the bud neck via Hsl1-GBP-ymScarletI rescues cellular morphology in *elm1Δ* cells. (A) Representative images showing artificial tethering of Gin4-GFP via Shs1-GBP-ymScarletI, Hsl1-GBP-ymScarletI, Bud4-GBP-ymScarletI and Bni5-GBP-ymScarletI in *elm1Δ* cells. DC*=Differential contrast. Scale bar 5μm. (B) Bar graph representing the percentage of cells exhibiting round morphology in the indicated strains shown in (A), one-way ANOVA Tukey's multiple-comparison test (**: $p < 0.01$, ***: $p < 0.0001$, ns: $p > 0.05$), (N=3, wildtype: n=397, *elm1Δ*: n=408, *elm1Δ*-Shs1-GBP-ymScarletI: n=467, *elm1Δ*-Hsl1-GBP-ymScarletI: n=462, *elm1Δ*-Bud4-GBP-ymScarletI: n=401 and *elm1Δ*-Bni5-GBP-ymScarletI: n=317 cells). (C) Quantification of aspect ratios in the indicated strains shown in (A), Kruskal-Wallis nonparametric statistical test (***: $p < 0.001$, ****: $p < 0.0001$, ns: $p > 0.05$), (N=3, n>170 cells/strain). (D) Graph depicting the raw fluorescence intensity of Gin4-GFP at the large bud in the indicated strains shown in (A), Kruskal-Wallis nonparametric statistical test (***: $p < 0.001$, ****: $p < 0.0001$, ns: $p > 0.05$), (N=3, wildtype: n=166, *elm1Δ*: n=177, *elm1Δ*-Shs1-GBP-ymScarletI: n=176, *elm1Δ*-Hsl1-GBP-ymScarletI: n=188, *elm1Δ*-Bud4-GBP-ymScarletI: n=185 and *elm1Δ*-Bni5-GBP-ymScarletI: n=151 cells).



Author response image 4. Artificial tethering of Hsl1-GFP to the bud neck via septins or Nim1-related kinases rescues cellular morphology in *elm1Δ* cells. (A) Representative images showing the relocalization of Hsl1-GFP to the bud neck in *elm1Δ* cells via Shs1-GBP-ymScarletI and Gin4GBP-ymScarletI. Scale bar-5μm. (B) Bar graph representing the percentage of cells exhibiting round morphology in the indicated strains shown in (A), one-way ANOVA Tukey's multiple comparison test (****: $p < 0.0001$, ns: $p > 0.05$), (N=3, wildtype: n=508, *elm1Δ*: n=418, *elm1Δ*Shs1-GBP-ymScarletI: n=535 and *elm1Δ*-Gin4-GBP-ymScarletI: n=482 cells). (C) Quantification of the aspect ratios in the indicated strains shown in (A), Kruskal-Wallis nonparametric statistical test (*: $p < 0.05$, ****: $p < 0.0001$), (N=3, n>165 cells/strain). (D) Quantification of raw fluorescence intensity of Hsl1-GFP at the large bud in the indicated strains shown in (A), Kruskal-Wallis nonparametric statistical test (***: $p < 0.001$, ****: $p < 0.0001$), (N=3, wildtype: n=156, *elm1Δ*: n=166, *elm1Δ*-Shs1-GBP-ymScarletI: n=169 and *elm1Δ*-Gin4-GBP-ymScarletI: n=165 cells).



Author response image 5. Targeted localization of Kcc4-GFP to the bud neck via Hsl1-GBP-ymScarletI rescues cellular morphology in *elm1Δ* cells. (A) Representative images showing artificial tethering of Kcc4-GFP to the bud neck in *elm1Δ* cells via Shs1-GBP-ymScarletI, Hsl1-GBP-ymScarletI and Gin4-GBP-ymScarletI. Scale bar = 5 μm. (B) Quantitative analysis representing the percentage of cells exhibiting round morphology in the indicated strains shown in (A), oneway ANOVA Tukey's multiple-comparison test (****: $p < 0.0001$, ns: $p > 0.05$), (N=3, wildtype: n=443, *elm1Δ*: n=489, *elm1Δ*-Shs1-GBP-ymScarletI: n=312, *elm1Δ*-Hsl1-GBP-ymScarletI: n=563 and *elm1Δ*-Gin4-GBP-ymScarletI: n=337 cells). (C) Quantification of the aspect ratios in the indicated strains shown in (A), Kruskal-Wallis nonparametric statistical test (****: $p < 0.0001$, ns: $p > 0.05$), (N=3, n>165 cells/strain). (D) Quantification for the raw fluorescence intensity of Kcc4-GFP at the large bud in the indicated strains shown in (A), Kruskal-Wallis nonparametric statistical test (**: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$), (N=3, wildtype: n=163, *elm1Δ*: n=168, *elm1Δ*-Shs1-GBP-ymScarletI: n=161, *elm1Δ*-Hsl1-GBP-ymScarletI: n=172 and *elm1Δ*-Gin4-GBP-ymScarletI: n=150 cells).

Protein tagged with GFP	Protein tagged with GBP	Genetic background	Number of cells exhibiting round morphology	Number of cells exhibiting elongated/clumped morphology	Total number of cells	Percentage of cellular population exhibiting round morphology	Rescue of elongated or clumped morphology
Gln4	Shs1	<i>elm1Δ</i>	37	430	467	7.92	No
Gln4	Bud4	<i>elm1Δ</i>	52	349	401	12.96	No
Gln4	Hsl1	<i>elm1Δ</i>	364	98	462	78.78	Yes
Gln4	Bni5	<i>elm1Δ</i>	0	317	317	0	No
Hsl1	Shs1	<i>elm1Δ</i>	333	202	535	62.24	Yes
Hsl1	Gln4	<i>elm1Δ</i>	258	224	482	53.52	Yes
Kcc4	Shs1	<i>elm1Δ</i>	24	288	312	7.69	No
Kcc4	Hsl1	<i>elm1Δ</i>	402	161	563	71.40	Yes
Kcc4	Gln4	<i>elm1Δ</i>	11	326	337	3.26	No

Author response table 1. Summary table showing rescue of elongated morphology in *elm1Δ* cells upon forced recruitment of Nim1-related kinases via septins or its related kinases tagged with GBPyScarletI.

Additional changes:

The graph in Fig. S3D (revised preprint) has been updated to reflect a slight increase in the Chs2-mNG residence time in both the *elm1Δ* and *gin4Δ* strains, whereas our previous version indicated a delay only in the *gin4Δ* strain. Because the *elm1Δ* strain exhibited a more pronounced phenotype than the *gin4Δ* strain, we re-examined the analysis. The revised results show that the residence time of Chs2 during cytokinesis is modestly prolonged by approximately 2 minutes in both backgrounds. Accordingly, the graph and statistical analyses have been updated.

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<https://doi.org/10.7554/eLife.106366.2.sa0>