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1 **Septin filament compaction into rings requires the anillin Mid2**
2 **and contractile ring constriction**

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11 **Running title: Mid2 promotes fission yeast septin ring assembly**

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1 **SUMMARY**

2 Septin filaments assemble into high-order molecular structures that associate with
3 membranes, acting as diffusion barriers and scaffold proteins crucial for many cellular
4 processes. However, how septin filaments organize in such structures is still not well
5 understood. In this study, we used fission yeast to explore septin filament organization
6 during cell division and decipher key factors responsible for their regulation. Live-
7 imaging and polarization microscopy analysis uncovered that septin filaments are
8 initially recruited as a diffuse meshwork surrounding the acto-myosin contractile ring
9 (CR) in anaphase, which undergoes compaction into two rings when CR constriction is
10 initiated. We found evidence that the anillin-like protein Mid2 is necessary to promote
11 this novel compaction step, possibly acting as a bundler for septin filaments. We also
12 found that Mid2-driven septin compaction requires inputs from the Septation Initiation
13 Network (SIN) as well as CR constriction or the $\beta(1,3)$ -glucan synthase Bgs1. This work
14 highlights the complex regulations that allow the coordination between septin ring
15 assembly and cell cycle progression.

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1 INTRODUCTION

2 Cytokinesis is an irreversible process which must precisely partition an equal set of the
3 replicated chromosomes and organelles into each daughter cell. Defects in this process
4 can lead to chromosome mis-segregation or aneuploidy, a hallmark of cancer (Storchova
5 and Pellman, 2004; Fujiwara et al., 2005; Lacroix and Maddox, 2012). Cytokinesis
6 completion requires the participation of different components of the cytoskeleton,
7 namely, the actin cytoskeleton at the basis of the contractile ring (CR) that pinches in
8 the plasma membrane to create the cleavage furrow, microtubules involved in division
9 plane signaling in animal cells, as well as septins which are present at the division site
10 from yeast to mammals.

11 Septins are unique cytoskeletal components capable of self-assembling to form high-
12 order molecular structures of varied shapes such as filaments and rings. Their ability to
13 interact with cellular membranes through interaction with negatively charged
14 phospholipids, such as phosphatidylinositol 4,5-bisphosphate (PIP2) as well as with actin
15 filaments and microtubules allows them to participate in many biological processes,
16 working as diffusion barriers for protein compartmentalization and/or scaffolds for
17 protein-protein interactions (Bertin et al., 2010; Saarikangas and Barral, 2011; Mostowy
18 and Cossart, 2012; Bridges et al., 2014; Bridges and Gladfelter, 2015; Bridges et al., 2016;
19 Marquardt et al., 2019). These conserved GTP-binding proteins were initially discovered
20 in budding yeast, based on their role in cytokinesis, a function which was found to be
21 conserved in animals (Hartwell, 1971; Neufeld and Rubin, 1994; Gladfelter et al., 2001;
22 Surka et al., 2002; Kozubowski et al., 2005; Versele and Thorner, 2005; McMurray and
23 Thorner, 2009; Estey et al., 2010; Wu et al., 2010; Kim et al., 2011; Kechad et al., 2012;
24 El Amine et al., 2013; Founounou et al., 2013; Cauvin and Echard, 2015). Importantly,
25 septins in budding yeast serve as a scaffold for the sequential and ordered assembly of
26 the acto-myosin based CR (Juanes and Piatti, 2016; Meitinger and Palani, 2016; Bhavsar-
27 Jog and Bi, 2017). Later on, when CR constriction occurs, septins act as cortical barriers
28 to create specialized confined zones at the cleavage furrow where membrane
29 reorganization and septum formation happen during cell division (Dobbelaere and
30 Barral, 2004).

1 In budding yeast septin organization undergoes multiple transitions during the cell cycle.
2 In early G1, septins initially assemble as a patch-like structure at the presumptive bud
3 site. Upon bud emergence, this patch narrows to form a single ring marking the site of
4 bud growth. Once the bud has formed, the septin ring extends into an hourglass-shaped
5 collar present at the mother-bud neck until mitotic entry. At cytokinesis onset, the
6 septin hourglass splits into two distinct rings that sandwich the CR (Gladfelter et al.,
7 2001). Finally, after cell division, the septin rings disassemble and the septin subunits
8 are recycled for a new cycle to begin (McMurray and Thorner, 2009).

9 One important factor in the organization of septins is the conserved protein anillin,
10 whose disruption leads to defects in animal cytokinesis (Oegema et al., 2000; Somma et
11 al., 2002; Echard et al., 2004; Field et al., 2005; Straight et al., 2005; Zhao and Fang, 2005;
12 Hickson and O'Farrell, 2008; Piekny and Glotzer, 2008). Indeed, anillin bridges septins
13 and the CR during cell division (Oegema et al., 2000; Kinoshita et al., 2002; Field et al.,
14 2005; Kechad et al., 2012; Liu et al., 2012). *In vitro* studies have shown that anillin can
15 bind septin and actin filaments. Animal anillin interacts with septins and PIP2 through a
16 C-terminal PH domain located after the anillin homology domain (Piekny and Glotzer,
17 2008; Piekny and Maddox, 2010; Kechad et al., 2012; Liu et al., 2012). The C-terminal
18 region of Bud4, the anillin-like protein in budding yeast, is important to direct septin
19 organization during bud site selection and bud growth and to preserve the integrity of
20 the septin ring during cytokinesis (Wloka et al., 2011; Eluere et al., 2012; Kang et al.,
21 2013; Wu et al., 2015). Thus, in *bud4Δ* mutants the septin double ring disassembles
22 during cytokinesis (Kang et al., 2013), whereas the overexpression of Bud4 leads to the
23 formation of extra septin structures. More recently, polarized fluorescence microscopy
24 studies have described a key role of Bud4 in the reorientation of septin filaments in the
25 hourglass to double ring transition (McQuilken et al., 2017).

26 The fission yeast *Schizosaccharomyces pombe*, a recognized model system for
27 cytokinesis studies, is a rod-shaped organism that divides by the assembly and
28 constriction of a medially placed actomyosin-based CR. *S. pombe* has two anillin-like
29 proteins, Mid1 and Mid2, with non-overlapping functions (Berlin et al., 2003; Tasto et
30 al., 2003). Mid1 accumulates at the medial cortex at mitosis onset, where it recruits
31 components involved in CR assembly (Pollard and Wu, 2010; Lee et al., 2012; Rincon and

1 Paoletti, 2012; Rincon and Paoletti, 2016). CR constriction and synthesis of the septum
2 (the extracellular cell wall separating the two daughter cells) are triggered upon mitotic
3 exit by the SIN pathway, related to the budding yeast Mitotic Exit Network and the
4 metazoan Hippo pathway (Hergovich et al., 2006; Cortés et al., 2016). *S. pombe* septins
5 were identified by sequence homology with the *S. cerevisiae* counterparts (Wood et al.,
6 2002). Spn1-4 are expressed in vegetatively growing cells and localize to the division site.
7 However, differently from *S. cerevisiae*, where septins are necessary for CR formation,
8 fission yeast septins are recruited after the CR has fully assembled (Wu et al., 2003).
9 Septins form first a single ring in late mitosis which splits in two during CR constriction
10 and septum formation. This double ring does not constrict together with the CR, but
11 remains at the base of the septum to delineate the boundaries of the cleavage furrow
12 and dissociates after cell separation (Berlin et al., 2003; Tasto et al., 2003; Wu et al.,
13 2003; An et al., 2004; Juanes and Piatti, 2016; Cortés et al., 2016). Septin mutants have
14 defects in cell-cell separation since septins have a key role, in concert with the exocyst
15 complex, in the delivery of the two septum hydrolytic enzymes (Agn1 and Eng1) to the
16 area around the septum, regulating thereby the step that finishes cytokinesis (Alonso-
17 Nunez et al., 2005; Martin-Cuadrado et al., 2005). Recently, it has also been shown that
18 septins play a role in driving the proper recruitment and maintenance in the septum
19 region of the SIN effector kinase Sid2 and of the glucan synthase enzymes Bgs1 and Ags1,
20 to guarantee a successful cytokinesis (Zheng et al., 2018). Mid2 localizes at the division
21 site after septin recruitment in a septin-dependent manner (Berlin et al., 2003; Ramos
22 et al., 2019). FRAP analysis has revealed that, in the absence of Mid2, septins are more
23 dynamic, indicating a role of Mid2 in septin organization (Berlin et al., 2003).
24 Overexpression of a non-degradable Mid2 mutant let septin filaments persist through
25 the next cell cycle, indicating that Mid2 is also involved in septin ring maintenance (Tasto
26 et al., 2003; An et al., 2004).

27 How septins and Mid2 are precisely regulated in space and time to properly perform
28 their function and how Mid2 contributes to septin ring organization remains elusive.
29 Here, by using live cell imaging and precise timers for mitotic progression we describe
30 in detail septin and Mid2 behavior in fission yeast. Our approach identified a new step
31 in septin ring assembly: septin filament compaction from a loose meshwork surrounding

1 the CR into a tight ring when CR constriction is initiated. We show that this step requires
2 Mid2, which has the properties of a bundler for septin filaments promoting their
3 compaction. Polarization microscopy in living dividing cells show that septin filaments
4 transition from a disorganized state to a highly ordered state where they become
5 parallel to the CR actin filaments during CR constriction, in a mechanism dependent on
6 Mid2. Finally, we show that the process of septin compaction requires the SIN pathway
7 signaling and CR constriction. In summary, we show that septin filaments organization
8 depends on the anillin-like protein Mid2 and on signaling inputs that coordinate
9 cytokinesis events in fission yeast.

10

11 **RESULTS**

12 **Septins form a diffuse network in the vicinity of the CR before compacting into a ring** 13 **structure**

14 Fission yeast septins localize to the division site where they form non-contractile rings
15 defining the edge of the cleavage furrow while the CR constricts and the septum is built
16 (Berlin et al., 2003; Tasto et al., 2003). However, relatively little is known about how
17 septin rings assemble. We therefore decided to characterize by time-lapse imaging the
18 dynamic organization of septins with respect to spindle assembly and elongation, used
19 as a proxy for mitosis progression, and CR assembly and constriction.

20 To do so, we created a strain producing the septin Spn1 C-terminally fused to GFP, as
21 well as the regulatory light chain of myosin II Rlc1 fused to mCherry to monitor the CR,
22 and the spindle pole body (SPB) component Sid4 tagged with mCherry to monitor the
23 mitotic spindle. Indeed, since septins function strictly as heteromeric complexes,
24 imaging Spn1 is sufficient to have an overview of the whole septin network (An et al.,
25 2004). In these movies, SPB separation was defined as the time for mitotic entry ($t=0$ in
26 Fig 1).

27 Septins appeared on the cortex as a band surrounding the CR about 18 minutes after
28 SPB separation, when the SPBs were the furthest apart, corresponding to maximum
29 spindle elongation in anaphase (Fig 1A and 1D, $t=18.6 \pm 2.3$ min). 7 minutes later, septins
30 compacted into a tight ring structure ($t=25.6 \pm 2.8$ min), concomitant with the initiation
31 of CR constriction ($t=26.1 \pm 2.9$ min). This compaction phenomenon is best visualized on

1 enlargements of the medial region of the cell recorded at 1-minute intervals (Fig 1C left,
2 dark green asterisk). About 20 minutes later, by the end of CR constriction ($t=48.5 \pm 3.6$
3 min), septins were visualized as a double ring ($t=43.4 \pm 6.5$ min, red asterisk). Eventually,
4 septins re-spread to the new cell tip generated when the two sister-cells separated from
5 one-another by cleavage of the septum (yellow asterisk).

6 Quantitative analysis of the septin signal further revealed that septin intensity increased
7 for a period of about 15 minutes after they appeared on the medial cortex. The intensity
8 then stayed roughly constant for 30 minutes before decreasing rapidly over 10 minutes
9 (Fig 1E). Furthermore, analyzing the width of the cortical region on which septins were
10 distributed revealed a two-fold reduction in width, from $1.4 \pm 0.1 \mu\text{m}$ at their
11 appearance to $0.75 \pm 0.1 \mu\text{m}$ after compaction when CR constriction starts ($n=30$, Fig 1F
12 light green dots).

13 To conclude, this detailed live analysis of septins establishes the timing of the major
14 transitions in septin organization and reveals a new step in the process of septin ring
15 assembly that we have named compaction.

16

17 **The anillin Mid2 is necessary for septin ring compaction**

18 A similar imaging strategy was taken to define the dynamic organization of the anillin-
19 like protein Mid2, known to co-localize with septins and modulate their dynamics (Berlin
20 et al., 2003; Tasto et al., 2003). Using a strain expressing Mid2 C-terminally fused to the
21 green fluorescent protein ENVY, as well as Rlc1-Cherry and Sid4-mCherry, we found that
22 Mid2 appeared as a narrow band 8 minutes later than septins ($t=26.9 \pm 2.5$ min),
23 coincident with the time of septin compaction (Fig 1C right, light green asterisk in the
24 Mid2 kymograph and Fig 1C left, dark green asterisk in the Spn1 kymograph, Fig 1D) and
25 CR constriction ($t=25.2 \pm 2.4$ min, Fig 1B-E). The Mid2 domain transformed into two rings
26 concomitantly to the double septin rings (Fig 1C, red asterisk) and spread out on the
27 newly formed cell tips at the end of cytokinesis (Fig 1C right, yellow asterisk).

28 Quantitative analysis of Mid2 signal confirmed the late recruitment of Mid2 compared
29 to septins and showed that its intensity increased for about 30 minutes, stayed constant
30 for 10 minutes and decreased rapidly, similarly to septins (Fig 1E)

31 The coincidence between CR constriction, septin compaction and Mid2 recruitment
32 suggested that these events may be coupled. To determine if this was the case, we first

1 tested whether Mid2 was involved in septin compaction. To do so, we analyzed septin
2 ring assembly in a *mid2Δ* mutant (Berlin et al., 2003), using the same markers for mitosis
3 and cytokinesis progression as before. Time-lapse movies showed that in *mid2Δ* cells,
4 initial septin recruitment occurred normally (Fig 2A-D), but septin intensity stopped
5 increasing prematurely, about 8 minutes after septins appeared, i.e. at the time when
6 Mid2 normally appears (Fig 2F). As a consequence, septin maximum intensity was
7 decreased by 3-fold in *mid2Δ* cells compared to control cells (n=6 for each strain; Fig 2E).
8 Furthermore, septin compaction was completely abolished (Fig 2C and 2F). Instead,
9 septins retained a diffuse distribution around the CR and the septum-associated
10 membrane (Fig 2C and S1A). As a consequence, splitting into two rings did not occur.
11 Finally, whereas the CR constriction took place normally (Fig 2D), cell separation was
12 delayed and the diffuse septin network persisted during the next cell cycle, as reported
13 in a *mid2Δ* mutant (Fig 2A-E; Berlin et al., 2003; Tasto et al., 2003).

14

15 **Mid2 may function as bundler for septin filaments**

16 The fact that Mid2 is necessary for septin compaction, suggested that Mid2 may function
17 as a bundler for septin filaments promoting their alignment and assembly into a ring
18 structure. To perform this activity, Mid2 should be able not only to interact with septins,
19 but also to dimerize or multimerize in order to bridge together two or more septin
20 filaments.

21 In line with this hypothesis, Mid2 co-immunoprecipitated with Spn1 (see Fig S1B). To
22 test if Mid2 self-associates, we designed co-immunoprecipitation assays between
23 differentially tagged versions of Mid2 co-expressed in the same cells. These experiments
24 were performed in the presence or in the absence of Spn1, to exclude the detection of
25 differentially tagged molecules of Mid2 bridged by septin filaments. Our experiments
26 show that Mid2-mEGFP co-immunoprecipitates with Mid2-13Myc regardless of the
27 presence or absence of Spn1 (Fig 3A). This interaction was also detected even in the
28 absence of Spn1-5 (Fig S2A).

29 In order to map the Mid2-Mid2 interaction site, we also performed co-
30 immunoprecipitation assays with a truncated version of Mid2 lacking the PH domain
31 (Mid2 Δ PH) or with the isolated PH domain (see Fig 3C for Mid2 domains organization).
32 Interestingly, Mid2 -mEGFP co-immunoprecipitated with Mid2- Δ PH-13Myc and Mid2 PH

1 domain also showed the ability to self-interact, even in the absence of Spn1 (Fig 3A and
2 3B). These interactions were also detected in *spn1-5Δ* cells (Fig S2A and B), indicating
3 that Mid2-Mid2 binding is septin-independent. To strengthen this notion, a 2-hybrid
4 assay using Mid2 PH domain as a prey and as a bait was performed; the result suggests
5 that Mid2 PH domain directly interacts with itself (Fig S2C). Furthermore, recombinant
6 GST-Mid2-PH interacted with MBP-Mid2-PH in an *in vitro* binding assay (Fig 3D) showing
7 that the interaction is direct.

8 To better define the contribution of Mid2 domains to septin ring compaction, we
9 analyzed the function of Mid2-ΔPH lacking the PH domain. This protein has been
10 previously shown not to localize to the division site and therefore it does not contribute
11 to septin function (Tasto et al., 2003). To overcome this situation, Mid2-ΔPH was fused
12 to the GFP-binding protein (GBP) and expressed as a Mid2 unique copy in a strain
13 expressing Spn1-GFP to target it to septins by interaction between the GBP and GFP
14 tags. The analysis of Spn1-GFP indicated that it appeared as a broad band that eventually
15 compacted, in spite of the absence of Mid2 PH domain (Fig 3E and 3F). However, the
16 septin rings collapsed as the CR constricts, suggesting that the PH domain contributes to
17 septin ring stability. It should be noted though that, in this situation, Spn1-GFP tends to
18 form clumps that follow the CR during its constriction and remain longer at the division
19 site. Nonetheless, expression of Spn1-GFP in the *mid2-ΔPH-GBP* background partially
20 rescued the cell separation defect showed by cells lacking Mid2 (Fig 3G).

21 This altogether indicates that Mid2 has the ability to form structures of higher molecular
22 order and that Mid2 lacking the PH domain domain is sufficient to drive septin
23 compaction, if it is artificially bound to septins. Mid2 PH domain on the other hand may
24 contribute to Mid2 association with septins and to septin ring stability at later stages.
25 Since Mid2 arrival at the cortex also coincides with septin compaction and Mid2 is
26 necessary for this step, our biochemical data support the hypothesis that Mid2 may
27 promote septin compaction by bundling septin filaments.

28

29 **Septins and Mid2 form wide bands surrounding the CR when cells are blocked in** 30 **mitosis**

31 Our work shows that septin filaments appear at a very precise time in mitosis, when the
32 anaphase spindle length is maximum, and compact into rings when Mid2 appears and

1 the CR starts constricting. This provides evidence for a tight coupling between septin
2 ring assembly and cell cycle progression. To get insight into how this coupling is
3 achieved, we first analyzed the localization of Spn1 and Mid2 in mutants blocked in
4 mitosis, with a high cyclin-dependent kinase (CDK) activity. We first used the cold
5 sensitive β -tubulin mutant *nda3-KM311* producing Spn1-GFP or Mid2-ENVY together
6 with Rlc1-mCherry and Sid4-mCherry. Upon cold treatment, cells were blocked in
7 mitosis carrying a CR, and formed a large band of septins and Mid2 surrounding the CR
8 instead of the septin or Mid2 ring detected in control cells (Fig 4A left). Interestingly,
9 Spn1-GFP and Rlc1-mCherry intensity measurements by linescans along the cell axis
10 showed a decrease in Spn1-GFP signal at the exact position of the CR (Fig 4A right and
11 Fig S3A and B). Furthermore, imaging these cells at 1-hour intervals after the
12 temperature shift showed that the septin domain gradually spread along the cortex in
13 *nda3-KM311* cells (from $1.2 \mu\text{m} \pm 0.03$ after 1 hour at 18°C to $2.4 \mu\text{m} \pm 0.2$ after 7 hours
14 at 18°C), while narrow rings were always present in control cells ($0.9 \mu\text{m} \pm 0.03$, Fig S3C).
15 A similar situation was observed for the Mid2 domain, its width increasing from $1.0 \mu\text{m}$
16 ± 0.1 after 1 hour at 18°C to $2.0 \mu\text{m} \pm 0.1$ after 7 hours in *nda3-KM311* cells compared
17 to narrow rings in control cells ($0.9 \mu\text{m} \pm 0.4$, Fig S3C). Release from the mitotic block by
18 transferring the cells back to 25°C for 1 hour resulted in a compaction of both septin and
19 Mid2 domains with the domain widths decreasing to $1.2 \mu\text{m} \pm 0.1$ and $1.3 \mu\text{m} \pm 0.2$,
20 respectively (Fig S3C).

21 We also used the thermo-sensitive kinesin-5 *cut7-24* mutant, which forms a monopolar
22 mitotic spindle at 36°C , producing a temporary cell cycle arrest in metaphase with a high
23 CDK activity (Fu et al., 2009; Hagan and Yanagida, 1990; Hagan and Yanagida, 1992). Live
24 cell imaging of the Spn1-GFP Rlc1-mCherry Sid4-mCherry strain in the wild type cells at
25 36°C slightly accelerated mitosis progression compared to 25°C (Fig 4B and C compared
26 to Fig 1A and D), but septin appearance occurred again at the time of maximum spindle
27 elongation and septin compaction coincided with the initiation of CR constriction, like
28 at 25°C . In the *cut7-24* mutant, since no SPB separation could be observed, the time of
29 transfer to 36°C was defined as time 0 for this experiment. The timing measured in this
30 background can therefore not be compared to those measured in the wild type
31 background. In the *cut7-24* mutant, septins appeared 9 minutes after the temperature

1 shift in a large band ($1.7 \pm 0.2 \mu\text{m}$ compared to $1.45 \pm 0.1 \mu\text{m}$ in control cells, Fig 4F) and
2 were excluded from the CR as seen in *nda3-KM311* cells. Unlike in control cells, where
3 septins compacted to $0.9 \pm 0.05 \mu\text{m}$, these bands spread along the membrane over time
4 in *cut7-24* cells ($2.0 \pm 0.1 \mu\text{m}$ Fig 4D-F), until they escaped the cell cycle arrest 29.6 ± 4.4
5 min in mean after transfer to 36°C , as visualized by the initiation of CR constriction,
6 which was again coincident with septin band compaction ($t=29.3 \pm 4.4$ min). Later on,
7 septins were observed as two rings at $t=41.9 \pm 7.3$ min until cell separation at $t=55.6 \pm$
8 5.1 min (Fig 4D and E).

9 Live cell imaging of the Mid2-ENVY Rlc1-mCherry Sid4-mCherry *cut7-24* mutant strain at
10 36°C showed that Mid2-ENVY formed a faint band surrounding the CR that enlarged
11 slightly over time (Fig S3D-H), until cells escaped mitosis and CR constriction initiated
12 ($t=33.6 \pm 2.3$ min, Fig S3F-H). Ring splitting and re-spreading were strongly delayed
13 ($t=45.7 \pm 1.2$ min and $t=66.4 \pm 5.2$ min, respectively; Fig S3G).

14 Overall, these data suggest that high CDK activity does not interfere with initial septin
15 and Mid2 recruitment. However, later events, such as further septin accumulation at
16 the division site and septin compaction and re-spreading may require a reduction in CDK
17 activity. This in turn suggests that Mid2 bundling activity may be impaired upon high
18 CDK activity.

19 This prompted us to investigate if Mid2 had still the ability to self-interact in this context.
20 To do so, we performed co-immunoprecipitations between Mid2-13Myc and Mid2-GFP,
21 Mid2 ΔPH -GFP or the single PH domain of Mid2 tagged with GFP in the control or *nda3-*
22 *KM311* mutant cells, after 8 hours of incubation at 18°C to block cells into mitosis (Fig
23 S2D). These experiments did not reveal any modification in the ability of Mid2 to self-
24 interact.

25 We then tested if high CDK activity could reduce the avidity of Mid2 for septins. To test
26 this hypothesis, co-immunoprecipitation assays between Spn1-GFP and Mid2-13Myc
27 were performed in control and *nda3-KM311* cells after 8 hours of incubation at 18°C or
28 after 8 hours at 18°C followed by 1 hour at 25°C to release cells from the mitotic block
29 (Fig S1B). Although the amount of Mid2 in mitotic cells is higher because its expression

1 peaks at this stage of the cell cycle (Alonso-Nuñez et al., 2005), these experiments did
2 not reveal significant differences in the ability of Mid2 to interact with septins.

3 Altogether, these data suggest that, although Mid2 is necessary for septin compaction,
4 it is not proficient for this function when CDK activity is high, independently of its ability
5 to self-interact or to interact with septins. Since Mid2 is hyperphosphorylated during
6 mitosis (Tasto et al., 2003), one hypothesis is that Mid2 phosphorylation prevents septin
7 compaction. Or else, CDK activity regulates septin compaction independently of Mid2.

8

9 **Mid2 controls the orientation of septin filaments**

10 In order to test further our hypothesis that Mid2 functions as a septin filament bundler
11 and to get insights into the role of Mid2 in septin filament organization, we decided to
12 measure septin filament organization using polarization-resolved fluorescence
13 microscopy (Vrabioiu and Mitchison, 2006; DeMay et al., 2011b; DeMay et al., 2011a;
14 Kress et al., 2013; Wang et al., 2013). Polarimetry measurements with the regular Spn1-
15 GFP fusion, which includes a flexible linker between the septin and the GFP (RIPGLI),
16 revealed randomly oriented GFP dipoles at all stages of cytokinesis (septin recruitment,
17 compaction, splitting and re-spreading) consistent with the flexibly-linked GFP exploring
18 orientations in all directions relative to Spn1 orientation (Fig S4A).

19 Thus, to use dipole orientation measurements of the GFP as a readout of septin filament
20 orientation, the mobility of GFP relative to Spn1 had to be minimized. We therefore
21 screened for Spn1-GFP fusions in which several amino acids between the C-terminus of
22 Spn1 and the N-terminus of GFP were deleted without impairing Spn1 localization and
23 function nor GFP fluorescence, and identified a fusion of Spn1 to rotationally constrain
24 GFP (conGFP), Spn1-conGFP (see methods for details).

25 The Spn1-conGFP construct was produced from the genomic *spn1 locus*, under the
26 control of its own promoter, in a strain containing Rlc1-mCherry Sid4-mCherry. This
27 strain had a wild type phenotype indicating that the fusion was functional. Accordingly,
28 Spn1-conGFP localized and redistributed similarly to Spn1-GFP (Fig S4B and C).

1 We measured GFP dipole orientation at four different stages during cytokinesis, namely
2 septin recruitment (Appearance), septin compaction (Early), CR constriction (Late), and
3 CR disassembly (Post-constriction) in this strain. We found that GFP dipoles were highly
4 disordered when septins are first recruited to the division site, as evidenced by the
5 random distribution of angles between the GFP dipoles and the axis of the CR (Fig 5A).
6 However, at later stages when septins compact into tight rings and throughout CR
7 constriction, the distribution of angles between GFP dipoles and the CR axis became
8 narrow, with GFP dipoles mostly oriented perpendicular to the CR axis (Fig 5A). Their
9 distribution eventually started becoming broader again after CR disassembly, when the
10 septin rings start to collapse. The narrowing of the GFP dipole orientation distributions
11 upon and throughout CR constriction, which also persists post-constriction, indicates
12 that the associated septin filaments are highly organized during these stages.

13 To determine the orientation of the GFP dipoles relative to septin filaments and be able
14 to deduce septin filament orientation with respect to the CR axis, we took advantage of
15 a Spn4-conGFP fusion, (Fig S4D and E; see methods for details), which, in addition to its
16 normal localization to the septin ring (white asterisks in Fig S4D), also assembled into
17 ectopic elongated cytoplasmic bars (arrows in Fig S4D). These ectopic bars most likely
18 represent bundles of tightly packed parallel septin filaments, similar to elongated bar-
19 like septin structures induced in *Ashbya gossypii* upon treatment with the septin
20 filament stabilizing drug forchlorfenuron (DeMay et al., 2011a) and very similar to the
21 ectopic bar-like structures detected in *spn2* Δ cells containing both Spn1 and Spn4 (An et
22 al., 2004). GFP dipoles were perpendicular to these septin bars (Fig S4D) suggesting that
23 the septin filaments are oriented perpendicular to the GFP dipole in the Spn4-conGFP
24 fusion. Importantly, GFP dipoles were also perpendicular to the CR axis during CR
25 constriction and disassembly in these cells (Fig S4E), suggesting that septin filaments run
26 parallel to the CR axis of constriction. Given that Spn4 is in a complex with Spn1 (An et
27 al., 2004) we deduce that the septin filaments in the Spn1-conGFP construct are also
28 oriented perpendicular to the GFP dipoles, and propose that initially randomly organized
29 septin filaments orient parallel to the plane of CR constriction upon compaction and
30 keep this orientation throughout CR constriction, progressively losing their organization
31 after CR constriction as septin rings progressively collapse (Fig 5D).

1 Septin filament orientation was then analyzed in *mid2Δ* cells. Similar to the wild type,
2 septin filaments appeared in a very disorganized manner in *mid2Δ* cells (Fig 5B).
3 However, as CR constriction took place, GFP dipoles failed to reorient perpendicularly
4 to the CR (Fig 5B). Septin filaments also failed to align in *nda3-KM311* cells where CDK
5 activity is high (Fig 5C).

6 Overall, this data indicates that Mid2 contributes to the orientation of septin filaments
7 parallel to the CR axis of constriction, supporting the hypothesis that Mid2 functions as
8 a septin bundler (Fig 5D) during the septin compaction phase. Moreover, Mid2 bundling
9 activity is negatively regulated by CDK activity in a direct or indirect manner, preventing
10 septin orientation and compaction in early mitosis.

11

12 **SIN activity and CR constriction are required for normal septin accumulation and** 13 **compaction at the division site**

14 Since septin compaction is coincident with the initiation of CR constriction, an event
15 known to be triggered by the SIN pathway (McCollum and Gould, 2001), we next
16 wondered if septins and Mid2 were also under the control of this pathway.

17 To test this hypothesis, we analyzed the behavior of septins in the temperature sensitive
18 hypo-active SIN mutant *sid2-250* at the non-permissive temperature of 36°C
19 (Balasubramanian et al., 1998; Sparks et al., 1999). Live cell imaging of *sid2-250* cells
20 producing Spn1-GFP Rlc1-mCherry Sid4-mCherry revealed that in the majority of cells
21 septins appeared as a diffuse cortical band at the normal timing compared to control
22 cells (Fig 6A). However, the intensity of the septin signal was diminished by ~ 4.5-fold
23 (Fig 6B) and compaction failed in 75% cells, while a loose compaction was observed in
24 the remaining 25%. Indeed, quantification of the extent of compaction showed that,
25 while in control cells the width of the septin domain decreased from $1.5 \pm 0.1 \mu\text{m}$ to 0.9
26 $\pm 0.1 \mu\text{m}$ between appearance and compaction state, in the majority of *sid2-250* mutant
27 cells, the initial width of the septin domain increased instead from $1.6 \pm 0.1 \mu\text{m}$ to $1.9 \pm$
28 $0.1 \mu\text{m}$ in a similar period of time, although it decreased in a minority of cells (from 1.6
29 $\pm 0.1 \mu\text{m}$ to $1 \pm 0.1 \mu\text{m}$ (Fig 6C).

1 The lack of septin compaction suggested a similar defect in the function of Mid2.
2 Therefore, the behavior of Mid2 was analyzed in *sid2-250* cells producing Mid2-ENVY
3 Rlc1-mCherry Sid4-mCherry (Fig S5A). These cells displayed a strong defect (3.5 fold) in
4 the accumulation of Mid2 (Fig S5B), similar to the one observed in Spn1 accumulation.

5 Altogether, these data suggest that the SIN pathway is not responsible for the initial
6 recruitment of septins or Mid2, but is required for their accumulation. The defects
7 observed in septin compaction points towards an important role of the SIN in this event.

8 Since the SIN pathway triggers CR constriction, $\beta(1,3)$ -glucan synthase Bgs1 localization
9 and septum synthesis initiation (Liu et al., 2000; Jin et al., 2006; Cortes et al., 2018), we
10 next wondered if the SIN acts directly on septin compaction or indirectly by inducing CR
11 constriction.

12 To test this, we decided to use the $\beta(1,3)$ -glucan synthase mutant *cps1-191* which halts
13 cytokinesis progression due to the inability to construct a septum, with an assembled
14 CR that cannot constrict. Interestingly, it had been shown that the SIN is active in this
15 mutant and required for the stability of the CR (Liu et al., 2000), which provides the best
16 scenario to answer this question. Spn1-GFP and Mid2-ENVY behavior were analyzed in
17 a *cps1-191* mutant producing Rlc1-mCherry and Sid4-mCherry at restrictive temperature
18 (Fig 6D and S4C). Both proteins showed a very defective accumulation (Fig 6E and S4D),
19 septins did not compact (Fig 6F) and eventually dissociated from the division site, even
20 when the CR remained stable (Fig 6D and Fig S5C).

21 Altogether, these data strongly suggest that the CR itself is not sufficient to promote
22 septin accumulation, and that the SIN pathway, by inducing CR constriction and/or
23 septum synthesis initiation allows septin filaments accumulation. Following Mid2
24 recruitment, septin filament further accumulate, compact and align parallel to the axis
25 of CR constriction to assemble the septin rings in a process strictly regulated by CR
26 constriction and septum ingression.

27

28 **DISCUSSION**

1 The assembly of macromolecular structures is a distinctive feature of cytoskeletal
2 elements. Septins are GTP-binding proteins with the ability to form non-polar filaments,
3 which can assemble into a variety of membrane-associated structures such as filament
4 networks or rings (Ong et al., 2014). A number of factors influence the ability of septins
5 to get assembled into those structures, such as the septin monomers themselves, the
6 lipid bilayer composition, post-translational modifications or regulatory proteins.

7 Here, we investigated the role of the septin-binding protein, Mid2 in the assembly of
8 supramolecular septin filament structures, using fission yeast as a model system. Mid2
9 is an anillin ortholog, previously involved in the stability of the septin ring assembled
10 during cytokinesis (Berlin et al., 2003; Tasto et al., 2003). Similar to other anillin
11 orthologs, such as *Saccharomyces cerevisiae* Bud4, Mid2 is only recruited at the end of
12 cytokinesis. In contrast to budding yeast, in which septins are first recruited to the
13 presumptive bud site at the onset of G1 (reviewed in (Juanes and Piatti, 2016)), in fission
14 yeast septins only localize to the cortex during cell division. Indeed, septin recruitment
15 requires the assembly of the CR prior to their localization.

16 Our data indicate that septins localize to the division site as a diffuse band that compacts
17 into a tight ring by the time of CR constriction. Our detailed analysis of septin
18 recruitment shows that the faint band where septins are recruited becomes very intense
19 as septin filament compaction takes place. This might explain the differences found with
20 Wu and collaborators, who established that septins appeared around 22 minutes after
21 SPB separation (Wu et al., 2003). This compaction phenomenon is a novel step in the
22 behavior of septins, coincident with the appearance of Mid2, suggesting a cause-effect
23 relationship. Accordingly, the lack of Mid2 results in the inability of septins to compact.

24 How might Mid2 trigger septin band compaction? One possibility is that Mid2 serves as
25 a bridge connecting septin filaments. In fact, anillin is a scaffolding protein, able to
26 interact with many other proteins (Field et al., 2005; Piekny and Glotzer, 2008). Anillin
27 (and Mid2) contains a C-terminal septin-interacting domain. If Mid2 were to bundle
28 septin filaments, it should be able to interact with itself so that each molecule could bind
29 to a different septin filament. Our biochemical analysis of Mid2 self-association shows
30 that Mid2 can directly interact with other Mid2 molecules via its C-terminal PH domain.
31 The Mid2 fragment lacking the PH domain can also self-associate independently of

1 septins (although we cannot rule out that other proteins may bridge this interaction)
2 and is sufficient to trigger septin compaction if an interaction with septins is artificially
3 triggered using the GBP system. This suggests that at least two molecules of Mid2 could
4 bridge two septin filaments together. Increasing levels of Mid2 would contribute to
5 septin filaments compaction into a tight ring. Importantly, in the absence of Mid2, septin
6 accumulation is strongly affected; since Mid2 recruitment to the division site is strongly
7 dependent on septins (Berlin et al., 2003; Tasto et al., 2003), we propose that septins
8 and Mid2 establish a positive feedback loop to get properly accumulated at the division
9 site.

10 Septin filaments show a dynamic organization pattern during cell cycle progression. Our
11 data based on polarization microscopy let us conclude that septin filaments are not
12 organized when they first appear at initial stages of cytokinesis. Coincident with the
13 arrival of Mid2, septin filaments become aligned parallel to the CR axis of constriction, a
14 pattern that is absent upon deletion of *mid2*. This strongly supports the notion that Mid2
15 functions to bridge septin filaments and organize them. In budding yeast, after the initial
16 recruitment to the bud site and the early stages of ring formation septins show a “fluid”
17 state, followed by “frozen” state once the ring transforms into a collar (Caviston et al.,
18 2003; Dobbelaere et al., 2003); after ring duplication septin dynamics increases again.
19 At the same time, during the collar stage, septin filaments are parallel to the mother-
20 bud axis (Vrabioiu and Mitchison, 2006), while they reorganize to become parallel to the
21 plane of CR constriction in later stages (DeMay et al., 2011a) in a process that might
22 implicate disassembly and reassembly of septin filaments (Ong et al., 2014). This process
23 might be different in fission yeast, where septin filaments only transition from a
24 disordered to an ordered organization, parallel to the plane of CR constriction. In any
25 case, anillin-like proteins, seem to play the conserved role of septin filament
26 reorganization and septin ring stabilization, as it is also the case for budding yeast anillin
27 Bud4 (McQuilken et al., 2017).

28 What signals trigger the dynamic behavior of septins? SUMOylation, acetylation and
29 phosphorylation are the best characterized post-translational modifications involved in
30 septin regulation. Our data is consistent with a tight control from the cell cycle
31 machinery. In wild type cells, septin recruitment to the division site takes place during

1 anaphase, when mitotic spindle reaches its maximum length. Mid2 appearance happens
2 8 minutes later, by the time of detectable CR constriction and septum synthesis
3 initiation. Analysis of Spn1 and Mid2 behavior in the beta-tubulin mutant *nda3-KM311*
4 or the kinesin-5 mutant *cut7-24*, blocked at early stages of mitosis demonstrate that
5 both, septins and Mid2, strongly accumulate at the division site when CDK activity is
6 high. Although we have no proof of a direct phosphorylation by CDK, Mid2 is highly
7 phosphorylated during septation (Tasto et al., 2003) and our non-published data show
8 a number of CDK consensus sites phosphorylated in *nda3-KM311*-blocked cells. This
9 would not be surprising since both septins and anillin orthologs have been shown to be
10 direct targets of CDK in other model systems (Sinha et al., 2007; Eluere et al., 2012).

11 Detailed observation of septins and Mid2 in mitotic blocked cells opens two questions.
12 First, septin and Mid2 initial meshworks are physically excluded from the precise CR
13 position on the cortex, raising the question on the molecular mechanisms at work to
14 allow such an exclusion and whether steric hindrance on the plasma membrane surface
15 might be sufficient. Whatever the mechanisms, it can be inferred from this observation
16 that the compaction of the two septin domains on each side of the CR, might
17 immediately produce a double septin ring, although it may not be detectable until the
18 septum has grown enough to push them apart during the maturation stage of the
19 complete septum (Muñoz et al., 2013), allowing the observation of the septin double
20 ring. Initial septin recruitment observation by super-resolution microscopy will
21 contribute to clarify this point.

22 Second, even though septins and Mid2 co-localize and interact in this context, this is not
23 sufficient to trigger septin ring compaction. This suggests that another mechanism
24 contributes to septin ring compaction. It might involve post-translational modifications
25 of Mid2, additional proteins working together with Mid2 to promote septin filament
26 bundling or yet another mechanism to be identified.

27 Importantly, compaction of the septin filaments is coincident with the onset of the CR
28 constriction triggered by the SIN pathway and we found the SIN plays a critical role in
29 septin ring assembly. Non-exclusive possibilities may explain this observation. First, it
30 has been shown that septin accumulation is inhibited in latrunculin A-treated cells, in
31 which CR assembly is abolished (Tasto et al., 2003); indeed, although the CR is assembled

1 is SIN mutants, this ring is not homogeneous and eventually disassembles (Hachet et al.,
2 2008). Second, the poor accumulation of Mid2 in the absence of SIN activity, may arise
3 from the poor accumulation of septin filaments and could also affect their
4 reorganization. This suggests that the SIN, directly or indirectly, regulates Mid2 to
5 control septin accumulation and compaction.

6 However, we also found that the $\beta(1,3)$ -glucan synthase Bgs1 mutant allele *cps1-191*, in
7 which the CR assembles and is stable and the SIN pathway is active but CR constriction
8 and initial septum synthesis are defective (Liu et al., 2000), phenocopies the SIN mutant
9 *sid2-250* in terms of septin defects. This suggests a role for the septum structure to
10 trigger septin and Mid2 accumulation and compaction. Although we cannot rule out the
11 hypothesis that the SIN may target Mid2, this demonstrates that SIN activity on its own
12 is not directly responsible for triggering the full accumulation of septins and Mid2 and
13 the compaction of septin filaments. Rather, CR constriction and septum formation by
14 themselves might cooperate with Mid2 to promote septin filament accumulation and
15 reorientation. Indeed, Bgs1 is the only glucan synthase localized to the division site at
16 the time of CR constriction onset (Cortes et al., 2018). One possibility to explore in the
17 future is that CR constriction or the initial deposition of septum material by the $\beta(1,3)$ -
18 glucan synthase Bgs1 may modify the plasma membrane locally at the ingression furrow,
19 favoring Mid2 and septin accumulation on the cortex and septin filament reorientation.

20 In summary, we have shown that the crosslinking activity of the anillin-like protein Mid2
21 promotes the compaction of an initial broad band of septin filaments into tight rings at
22 the onset of CR constriction and septum formation. The activity of the SIN is necessary
23 but not sufficient to trigger this effect and CR constriction and septation themselves, or
24 the $\beta(1,3)$ -glucan synthase Bgs1 downstream of the SIN may be the real trigger. It will
25 be interesting to explore whether the cleavage furrow acts on the properties of other
26 anillin-like proteins in a similar manner in other systems.

27

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15

16 **AUTHOR CONTRIBUTIONS**

17 Federica Arbizzani performed the experiments, analyzed the data and wrote the
18 manuscript. Manos Mavrikis and Sophie Brasselet performed the polarization
19 microscopy experiments and edited the manuscript. Marta Hoya performed the Mid2
20 self-interaction experiments. Juan Carlos Ribas checked the functionality of the Mid2-
21 Δ PH-GBP fusion and edited the manuscript. Anne Paoletti and Sergio Rincon directed
22 the study and edited the manuscript.

23

24 **AUTHOR CONTRIBUTIONS**

25 The authors declare no competing financial interests.

26

27 **FIGURE LEGENDS**

28 **Figure 1: Dynamics of septins and Mid2 during cell division**

1 Time lapse imaging of wild type cells producing Spn1-GFP, Rlc1-mCherry and Sid4-
2 mCherry (A) or Mid2-ENVY, Rlc1-mCherry and Sid4-mCherry (B) growing at 25°C.
3 Maximum intensity projections of confocal images are shown. Time 0 corresponds to
4 mitotic entry. Scale bars: 4 μm. C: Kymographs showing the medial region of the cell
5 producing Spn1-GFP Rlc1-mCherry (left) or Mid2-ENVY Rlc1-mCherry (right). There is a
6 1-minute interval between successive images. The major transitions in the behavior of
7 Spn1 and Mid2 are indicated with the elapsed time shown in minutes and highlighted at
8 the far right, in magnified panels with an asterisk (color code matches that of the graph
9 in D; green for Spn1 and Mid2 appearance, dark green for Spn1 compaction, magenta
10 for Spn1 and Mid2 splitting and yellow for Spn1 and Mid2 re-spreading). A scheme of a
11 cell and the selected area are shown at the bottom. Scale bars: 2 μm. D: Plot depicting
12 the timing of Spn1-GFP (solid bars) and Mid2-ENVY (striped bars) transitions throughout
13 cell division. The indicated times correspond to the elapsed time of each transition, using
14 SPB separation as a timer for mitotic entry (t=0 in A). Mean ± sd are shown. n=51 cells
15 for each strain. E: Analysis of Spn1-GFP (light green) and Mid2-ENVY (dark green)
16 fluorescence intensities in the cell middle from mitotic onset throughout cell division.
17 The average line graphics ± sd are displayed. n=6 cells in each case. F: Quantification of
18 the extent of Spn1-GFP (light green) width compaction over time. n=30 cells

19

20 **Figure 2: Defects in septin behavior in the absence of Mid2**

21 Time lapse imaging of wild type (A) and *mid2Δ* cells (B) producing Spn1-GFP, Rlc1-
22 mCherry and Sid4-mCherry growing at 25°C. Maximum intensity projections of confocal
23 images are shown. Time 0 corresponds to mitotic entry. Scale bars: 2 μm. C: Kymographs
24 showing the medial region of wild type (left) or *mid2Δ* cells (right) producing Spn1-GFP,
25 Rlc1-mCherry and Sid4-mCherry, as displayed in the scheme of a cell at the bottom.
26 Frames are spaced by 1-minute intervals. The major transitions in Spn1-GFP dynamics
27 and the defects observed in absence of *mid2* are indicated with the elapsed time shown
28 in minutes and highlighted at the far right, in magnified panels with an asterisk (color
29 code matches that of the graph in D). A scheme of a cell and the selected area are shown
30 at the bottom. Scale bars: 2 μm. D: Plot displaying the dynamics of Spn1-GFP in wild type
31 (solid bars) and *mid2Δ* cells (striped bars) throughout cell division. Mean ± sd are shown.

1 n=52 cells for each strain. **E:** Analysis of Spn1-GFP fluorescence intensity in the cell
2 middle in wild type (green) and in *mid2Δ* cells (magenta) from mitotic onset throughout
3 cell division. The average line graphics ± sd are displayed. n=6 cells in each case. **F:**
4 Quantification of the extent of Spn1-GFP width compaction over time in wild type
5 (green) and in *mid2Δ* cells (magenta). n=20 cells.

6

7 **Figure 3: Mid2 interacts with other Mid2 molecules through different regions of the**
8 **protein, contributing to septin compaction**

9 **A:** Co-immunoprecipitation assay between full length Mid2-mEGFP and Mid2-13Myc or
10 Mid2 ΔPH-13Myc either in the presence (*spn1⁺*) or in the absence of Spn1 (*spn1Δ*).
11 Magenta arrowheads show the expected Mid2 bands; black arrowheads show
12 degradation products; green arrowheads show the immunoglobulin bands. **B:** Co-
13 immunoprecipitation assay between the PH domain of Mid2 tagged with 13XMyc and
14 the PH domain of Mid2 tagged with GFP either in the presence (*spn1⁺*) or in the absence
15 of Spn1 (*spn1Δ*). Magenta arrowheads show the expected Mid2 bands; black
16 arrowheads show degradation products; green arrowheads show the immunoglobulin
17 bands. **C:** Diagram showing the structure of the anillin-like protein Mid2 with the main
18 domains highlighted. **D:** *In vitro* binding assay of recombinant MBP-Mid2-PH with GST-
19 Mid2-PH or GST alone. Left panels show the inputs. Right panels show the GST
20 pulldowns. **E:** Kymographs showing the medial region of wild type (left) or *mid2ΔPH-*
21 *GBP* cells (right) producing Spn1-GFP, Rlc1-mCherry and Sid4-mCherry. There is a 2-
22 minutes interval between successive images. Scale bar: 2 μm. **F:** Quantification of the
23 extent of Spn1-GFP width compaction over time in wild type (green) and in *mid2ΔPH-*
24 *GBP* cells (magenta). n=20 for wt cells and 25 for *mid2ΔPH-GBP* cells. **G:** Percentage of
25 septating cells in the indicated strains at log phase analyzed by calcofluor staining.
26 Values correspond to the average of 3 experiments, with over 500 cells analyzed per
27 strain each time.

28

29 **Figure 4: Septins accumulate as a broad band at the cell middle cortex under high CDK**
30 **activity**

1 **A:** Epifluorescence images of cells producing Spn1-GFP Rlc1-mCherry Sid4-mCherry (left
2 panels) or Mid2-ENVY Rlc1-mCherry Sid4-mCherry (right panels) in wild type or *nda3-*
3 *KM311* cells growing at 18°C during 4 hours. Fluorescence intensity analysis of Spn1-GFP,
4 Rlc1-mCherry and Sid4-mCherry signals along the entire cell length of *nda3-KM311* cells
5 incubated during 7 hours at 18°C. Individual lines are shown in light green (wild type
6 cells) and magenta (*nda3-KM311* cells), while average lines are shown in dark green
7 (wild type cells) and purple (*nda3-KM311* cells). n=10 cells (far right panel). A scheme of
8 a cell displaying the linescan used for quantification of fluorescence intensity is shown.
9 Time lapse imaging of wild type (**B**) or *cut7-24* cells (**D**) producing Spn1-GFP, Rlc1-
10 mCherry and Sid4-mCherry growing at 36°C. Maximum intensity projections of confocal
11 images are shown. Time 0 corresponds to mitotic entry. Scale bars: 2 μm. Plot showing
12 Spn1-GFP transitions throughout cell division in the wild type (**C**) or the *cut7-24* mutant
13 (**E**). Mean ± sd are shown. n=45 cells in each case. The graph showing the behavior of
14 the *cut7-24* cells was obtained by the analysis of the cytokinetic events of those cells
15 that escaped the cytokinetic arrest (termed “Escape”). The backward analysis of
16 previous cytokinetic events in the same cells is shown in the “Block” portion of the plot.
17 **F:** Quantification of the extent of Spn1-GFP width compaction over time in wild type
18 (green) and *cut7-24* cells (magenta). n=20 cells in each case.

19

20 **Figure 5: Septin filament organization changes during CR constriction and septum**
21 **synthesis depends on Mid2**

22 **A:** Polar-plot histograms of GFP dipole orientations of Spn1-conGFP filaments from wild
23 type cells at different stages of cytokinesis (top panels). The number of analyzed cells
24 per stage is indicated below each polar-plot histogram (typically 120-200 analyzed pixels
25 per cell). Confocal images of Spn1-conGFP from selected wild type cells and an overlaid
26 color-coded stick representation of the measured orientations (angles ρ) per analyzed
27 pixel at each cytokinetic stage (bottom panels). Scale bar: 1 μm. **B:** Polar plots of GFP
28 dipole orientations of Spn1-conGFP filaments from *mid2Δ* cells at different stages of
29 cytokinesis (top panels). The number of analyzed cells per stage is indicated below each
30 polar-plot histogram. Confocal images of Spn1-conGFP from selected *mid2Δ* cells and an
31 overlaid color-coded stick representation of the measured orientations per pixel, as in

1 A, at each cytokinetic stage (bottom panels). Scale bar: 1 μm . **C:** Polar plot of GFP dipole
2 orientations of Spn1-conGFP filaments from 57 *nda3-KM311* cells incubated 7 hours at
3 18°C (top panel). Confocal images of Spn1-conGFP from *nda3-KM311* cells and an
4 overlaid color-coded stick representation of the measured orientations per pixel, as in A
5 (bottom panels). Scale bar: 1 μm . **D)** Schematic representation of septin localization
6 related to major CR and septum transitions (top) and detail of septin filament
7 orientation at each stage (bottom). The cartoon on the bottom right depicts the
8 orientation of a septin filament with respect to the orientation of GFP dipoles (double
9 arrowheads), as deduced from Spn4-conGFP fusions (see Fig S3D and E). The GFP barrels
10 orient with their long axes parallel to the septin filament axis, i.e. with the GFP dipoles
11 perpendicular to the septin filament axis. The numbers correspond to the septin
12 subunits Spn1-4 and the dashed lines delimit a putative octameric septin complex within
13 the filament. The septin subunit order is based on An et al (2004) and their homology
14 with budding yeast septins.

15

16 **Figure 6: Septin recruitment and compaction are defective in the SIN mutant *sid2-250***
17 **and the $\beta(1-3)$ -glucan synthase *Bgs1* mutant *cps1-191***

18 **A:** Time lapse imaging of wild type (left series) and *sid2-250* cells (right series), producing
19 Spn1-GFP, Rlc1-mCherry and Sid4-mCherry incubated at 36°C. Maximum intensity
20 projections of confocal images are shown. Time 0 corresponds to mitotic entry. Scale
21 bars: 2 μm . **B:** Analysis of Spn1-GFP fluorescence intensity from mitotic onset
22 throughout cell division in control (green) and *sid2-250* cells (magenta). The average line
23 graphics \pm sd are displayed. n=6 cells in each case. **C:** Quantification of the extent of
24 Spn1-GFP width compaction over time in wild type (green) and *sid2-250* cells (magenta).
25 Note that some *sid2-250* cells escape SIN phenotype. n=20 cells. **D:** Time lapse imaging
26 of wild type (left series) and *cps1-191* cells (right series), producing Spn1-GFP, Rlc1-
27 mCherry and Sid4-mCherry incubated at 36°C. Maximum intensity projections of
28 confocal images are shown. Time 0 corresponds to mitotic entry. Scale bars: 2 μm . **E:**
29 Analysis of Spn1-GFP fluorescence intensity from mitotic onset throughout cell division
30 in control (green) and *cps1-191* cells (magenta). The average line graphics \pm sd are

1 displayed. n=6 cells. **F:** Quantification of the extent of Spn1-GFP width compaction over
2 time in wild type (green) and *cps1-191* cells (magenta). n=20 cells in each case.

3

4 **EXPERIMENTAL PROCEDURES**

5 **Yeast genetics and culture conditions**

6 Standard *S. pombe* media and genetic manipulations were used as described in (Moreno
7 et al., 1991). All strains used in the study were isogenic to wild type h⁻ 972 and are
8 described in Supplemental Table S1. Strains from genetic crosses were selected by
9 random spore germination and replica in plates with the appropriate supplements or
10 drugs.

11 Plasmids and DNA transformations were performed by using the lithium acetate-DTT
12 method. Briefly, 20 ml of exponentially growing cells (optical density 0.5-0.8 at 600 nm)
13 were harvested by centrifugation and washed with 10 mM Tris HCl pH 7.4. Then, these
14 cells were re-suspended in 100 mM lithium acetate with 10 mM DTT and incubated on
15 an orbital wheel at room temperature for 40 minutes. 100 µl of these cells were mixed
16 with 80 µl of 100 mM lithium acetate, 10 µl of single stranded DNA from salmon testes
17 (D9156-5ML, Sigma) and 2 µg of the desired plasmid or the purified PCR product. After
18 10 minutes of incubation at room temperature on an orbital wheel, 300 µl of PEG 4000,
19 previously diluted 1:1 in 100 mM lithium acetate, were added. After a second round of
20 10 minutes at room temperature on the wheel, 15 µl of DMSO were added and the cells
21 were subjected to heat shock at 42°C for 20 minutes in a water bath. Cells were then
22 washed, resuspended in 100 µl of sterile water and plated on the appropriate selection
23 plates.

24

25 **Production of mutant and tagged strains**

26 Wild type *mid2* was fused to the DNA sequence coding for the green fluorescent tag
27 ENVY (Slubowski et al., 2015) or for the 13Xmyc tag at its 3' end by PCR amplification of
28 a cassette with the corresponding fusion from the plasmid pFA6a-ENVY-kanMX6 or
29 pFA6a-13Xmyc-natMX6, respectively, as described in (Bahler et al., 1998).

30

31

1 To create the strains used for the co-immunoprecipitation experiments shown in Figures
2 3A, S1B and S2D, constructs containing *mid2*⁺, or the *mid2* fragments coding for Mid2-
3 ΔPH (aminoacids 1-581) or for the PH domain of Mid2 (aminoacids 582-685) were cloned
4 into a pJK148-derived plasmid (Keeney and Boeke, 1994) containing *mEGFP* followed by
5 the terminator of *nmt1*⁺, to be expressed under the control of the *mid2*⁺ promoter. The
6 resulting plasmids were digested with NruI or Tth111 for plasmid linearization to allow
7 integration in the endogenous *leu1-32 locus* upon yeast transformation.

8 To create the strains used for the co-immunoprecipitation experiments shown in Figures
9 S2A and B, an integration plasmid for Mid2 constructs was made by insertion of 1 kb of
10 the *mid2*⁺ promoter and terminator into pFA6a-*mEGFP-kanMX6* (Bahler et al., 1998)
11 between Sall and BamHI sites and PmeI and SacII sites, respectively. The *mid2*⁺ open
12 reading frame was then inserted upstream of *mEGFP* between BamHI and XmaI. The
13 *mid2* fragments coding for Mid2-ΔPH (aminoacids 1-569) or for the PH domain of Mid2
14 (aminoacids 569-706) were cloned into this plasmid, replacing full length *mid2*⁺, to
15 express the Mid2 truncations used for co-immunoprecipitations in figure 1. pFA6a-*GFP-*
16 *kanMX6*-derived plasmid was digested with NotI to release the linear DNA fragment that
17 directs homologous recombination upon yeast transformation.

18 For production of Mid2-13Myc truncations in Figures S2A and B, 1 kb of the *mid2*⁺
19 promoter was cloned into pBP-*ade6* (Beaudoin et al., 2006) using the sites Sall and XmaI.
20 The resulting plasmid was used to clone a fragment coding for *13xmyc* and 1 kb of *nmt1*⁺
21 terminator obtained from pSR98 (Rincon et al., 2014) using the sites NotI and SacI.
22 Finally, the *mid2* truncations were cloned in this plasmid using the sites XmaI and NotI.
23 The resulting plasmids were digested with AatII for plasmid linearization to allow
24 integration in the endogenous *ade6*⁺ *locus* upon yeast transformation.

25 To create the strains used for the co-immunoprecipitation experiment shown in Figure
26 3B, a construct containing the *mid2* fragment coding for the PH domain of Mid2
27 (aminoacids 582-685) was cloned into a pJK148-derived plasmid containing *13xMyc*
28 followed by the terminator of *nmt1*⁺, to be expressed under the control of the *mid2*⁺
29 promoter. The resulting plasmids were digested with NruI for plasmid linearization and
30 integration in the endogenous *leu1-32 locus* of a strain containing the PH domain of
31 Mid2 (aminoacids 582-685) followed by *mEGFP* expressed from *mid2*⁺ endogenous
32 *locus*.

1 DNA constructs expressing Mid2-mEGFP full length, Mid2 Δ PH-mEGFP (1-581aa) and the
2 C-terminal PH domain of Mid2 (aa 582-685) tagged with mEGFP, used in figure S2D were
3 integrated at the *leu1-32 locus* under the control of *mid2⁺* promoter. Briefly, a fragment
4 containing 1 kb of *mid2⁺* promoter and the different *mid2* fragments were amplified
5 from genomic DNA purified from a wild type strain and cloned between KpnI and NotI
6 sites of pSR2 (pJK148 - *Pcdr2⁺-cdr2⁺-GFP-Tnmt1⁺*) (Keeney and Boeke, 1994). The
7 plasmids obtained were circularized by NruI or Tth111 digestion to allow integration into
8 the *leu1-32 locus* of a wild type strain.

9 A DNA construct expressing the PH domain of Mid2 (582-685 aa) was integrated into the
10 *leu1-32 locus* under the control of *mid2⁺* promoter. Briefly, a fragment containing 1 kb
11 of *mid2⁺* promoter and the *mid2* fragment coding for the PH domain of Mid2 (582-685aa)
12 were amplified from genomic DNA purified from a wild type strain and cloned between
13 KpnI and NotI sites of pSR98 (pJK148 *Pcdr2⁺-cdr2⁺-13xMyc-Tnmt1⁺*) (Rincon et al., 2014).
14 The obtained plasmid was linearized by NruI digestion to allow integration into the *leu1-*
15 *32 locus* of a wild type strain.

16 To create the strains producing Spn1 fused to monomeric superfolder GFP lacking the
17 12 N-terminal aminoacids (*Spn1-conGFP*, *spn1⁺-msfGFP Δ N12*), the *msfGFP* sequence
18 was first amplified from plasmid pEX-A128-*msfGFP* and cloned into the pFA6a-EGFP-
19 *kanMX6* plasmid (Bahler et al., 1998), replacing the original EGFP for *msfGFP* using the
20 Gibson Assembly cloning strategy (Gibson et al., 2009). *msfGFP Δ N12* and *spn1⁺* were
21 amplified and fused in a two-round PCR and the PCR product was used to replace
22 *msfGFP* in the pFA6a-*msfGFP-kanMX6* plasmid. This construct was amplified with long
23 oligos containing 80 bases from *spn1⁺* promoter and terminator to direct the
24 homologous recombination, producing the integration module which was used to
25 transform the wild type, *mid2 Δ* and *nda3-KM-311* strains and replace *spn1⁺* with *spn1⁺-*
26 *msfGFP Δ N12*. A similar strategy was used to create the strains producing Spn4 lacking
27 the 6 C-terminal aminoacids fused to GFP lacking the 7 N-terminal aminoacids (*Spn4-*
28 *conGFP*, *spn4- Δ C6-mEGFP- Δ N7*).

29 To create the strains producing Mid2- Δ PH-GBP, a DNA fragment coding for the GFP
30 binding protein was obtained from pFA6a-*GBP-kanMX6* (Chen et al., 2017) by digestion
31 with PacI-Ascl, and cloned into the plasmid pFA6a-*mid2⁺-mEGFP-kanMX6*, replacing the

1 mEGFP for GBP. The obtained plasmid was linearized by digestion with NotI before yeast
2 transformation.

3 All plasmids were confirmed by PCR and restriction enzyme digestion, and the DNA
4 fragments amplified by PCR were confirmed by DNA sequencing.

5

6 **Microscopy and image analysis**

7 Cells were incubated at 25°C in YE5S except in the case of cell cycle temperature
8 sensitive mutants (*cut7-24*, *sid2-250*, *cps1-191*), which were incubated for 3 hours at
9 36°C or *nda3-KM311* cold sensitive mutant, which was incubated up to 7h at 18°C.

10 Epifluorescence images were taken on a DMRXA2 upright microscope (Leica
11 Microsystems), equipped with a 100×/1.4NA oil immersion PlanApo objective and a
12 Coolsnap HQ CCD camera (Photometrics). The exposure time was 2 s for GFP, 1 s for
13 mCherry. Quantification in the *nda3-KM311* mutant of Spn1-GFP fluorescence intensity
14 with respect to the red fluorescence signal was performed by analysis along a line drawn
15 throughout the cell length (dashed lines in Fig 4A and Fig S3A and B). The values obtained
16 were background-subtracted using an equivalent region outside the cell. The line
17 graphics obtained were then centered with respect to the position of the ring.

18 For time-lapse imaging, 1 ml of exponentially growing cells were harvested by
19 centrifugation for 60 s at 800 g in a Minispin Eppendorf centrifuge equipped with a F-
20 45-12-11 rotor, most of the supernatant was discarded and 1 µl of the cells was
21 deposited in a 2% YE5S agar pad at the center of polydimethylsiloxane slide chamber
22 prepared as described in (Costa et al., 2013).

23 Time-lapse movies were performed on an inverted spinning disk confocal microscope
24 (Roper/Nikon Instruments), equipped with a Plan Apochromat 100×/1.4 NA objective
25 (Nikon Instruments), a PIFOC (perfect image focus) objective stepper, and a charge-
26 coupled device camera (EMCCD 512x512 QuantEM; Photometrics). The microscope was
27 equipped with a thermostatic methacrylate chamber to perform imaging at 25°C (or
28 36°C when analyzing thermosensitive mutants). To analyze Spn1-GFP Rlc1-mCherry
29 Sid4-mCherry wild type as well as Mid2-ENVY Rlc1-mCherry Sid4-mCherry in the
30 respective cell cycle thermosensitive mutants, z-stacks of 7 planes spaced by 1 µm
31 between planes were acquired at 1 min interval for 3h (binning 1, 300 EM gain; 200 ms
32 exposure for each channel).

1 Fluorescent images for each channel were scaled equivalently to their respective control
2 and analyzed with MetaMorph software 7.7.8.

3 Analysis of Spn1-GFP and Mid2-ENVY fluorescent signals were quantified in cells at
4 mitotic onset (SPBs separation) by measuring the average fluorescence intensity of the
5 medial region with the linescan tool (MetaMorph software 7.7.8) of 5 μm in length and
6 13 μm in width perpendicularly oriented to the long axis of the cell on maximum
7 intensity projection images. Background values, measured as the average intensity of an
8 equivalent region outside the cell, were then subtracted.

9 To compare the width of Spn1-GFP and Mid2-ENVY at the two different time points
10 indicated in the text in control and mutant cells, the linescan tool (MetaMorph software
11 7.7.8) was oriented parallel to the long axis of the cell. The values obtained are the
12 average of 3 measurements over the cell width within the bands corresponding to Spn1-
13 GFP or Mid2-ENVY.

14

15 **Co-immunoprecipitation experiments**

16 Co-immunoprecipitation experiments were performed as described in (Guzman-Vendrell
17 et al., 2013). For the experiments shown in figures 3A and B and S1B and S2D, 200 ml of
18 early log phase cells were incubated to an optical density of 1.0 at 600 nm at 25°C in
19 YE5S medium concentrated twice compared to the regular YE5S medium (YE5S2X),
20 harvested in cold STOP buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM Na₃N)
21 and resuspended in 300 μl Lysis buffer (50 mM Hepes, 100 mM NaCl, 1 mM EDTA, 1 %
22 NP-40, 50 mM NaF, 20 mM β -glycerophosphate) with 1 mM PMSF and a tablette of
23 protease inhibitor cocktail (Thermo, 11697498001). Extracts were incubated with anti-
24 mouse IgG magnetic beads (M-280 DYNAL, Invitrogen), previously coupled to 6 μg of
25 anti-GFP mAb (Roche). For the experiments shown in figure S2A and B, 200 ml of early
26 log phase cells were incubated to an optical density of 0.7 at 600 nm at 25°C in YE5S
27 medium harvested in cold STOP buffer, and finally resuspended in 300 μl Lysis buffer
28 with proteases inhibitors (1 mM PMSF, 0.5 μM aprotinin, 0.5 μM leupeptin, 0.5 μM
29 pepstatin A, 0.1 μM NaVO₃). Extracts were incubated with protein A-magnetic beads
30 (Thermo, 10006D) previously conjugated with 5 μl of polyclonal anti-GFP antibody
31 (Thermo A-6455).

1 Western blots were probed with mouse anti-GFP mAb (1/500 dilution, Roche Cat No.
2 11814460001) or mouse anti-myc mAb 9E10 (1/666 dilution, Roche Cat No.
3 11667149001). Secondary goat anti-mouse antibodies were coupled to horse radish
4 peroxidase (Jackson Immunoresearch).

5 For *nda3-KM311* synchronization experiments, 1 liter of cell culture was incubated
6 overnight to an optical density of 0.6 at 600 nm. Then, the cells were synchronized by
7 an 8 hours incubation at 18°C before being collected and processed.

8

9 ***In vitro* binding assay**

10 DNA fragments encoding Mid2 PH domain (aminoacids 569-706) were PCR-amplified
11 and inserted into the GST-producing vector pJC20 or the MBP-producing vector pMAL-
12 p2 (New England Biolabs Inc.) between the sites NdeI - BamHI or BamHI – Sall,
13 respectively. The resulting plasmids were transformed into the E. coli strain BL21. 200
14 ml of LB cultures supplemented with ampicillin at an O.D. of 2.0 at 600 nm after
15 induction of protein production with 0.1 mM IPTG for 3 h at 28 °C were harvested. For
16 GST fusions, cells were resuspended and incubated 30 min at room temperature in 10
17 ml of CellLytic B Cell Lysis Reagent (Sigma-Aldrich, C8740) containing 50 u/ml Benzonase
18 (Sigma-Aldrich, E1014), 1 mg/ml lysozyme and protease inhibitors (1 mM PMSF, 0.5 μM
19 aprotinin, 0.5 μM leupeptin, 0.5 μM pepstatin A). After a centrifugation at 10.000 x g for
20 30 min at 4 °C, the lysate was incubated with 100 μl glutathione-conjugated magnetic
21 beads (Cube Biotech) in rotation overnight at 4 °C. The beads were then PBS-washed
22 three times and resuspended in 200 μl of binding buffer (20 mM Tris-HCl pH 7.5, 0.2 M
23 NaCl, 1 mM EDTA, 0.5 % NP-40). For MBP fusions, cells were resuspended and incubated
24 10 min in rotation at room temperature in mM Tris-HCl pH 8.0, 20 % sucrose, 1 mM
25 EDTA (80 ml of buffer per gram of cells). After centrifugation, the pellet was incubated
26 for 10 minutes in ice in 80 ml of 5 mM MgSO₄ buffer per gram of cells, frequently shaking
27 gently. After a centrifugation at 8.000 x g for 20 min at 4 °C, 800 μl of 50 % of amylose
28 resin (NEB, E8021) per 40 ml of lysate and Tris-HCl pH 7.5 to a final concentration of 20
29 mM were added and incubated in rotation at 4 °C overnight. The resin was then washed
30 three times in washing buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA) and
31 the MBP fusions were eluted by adding a total of 6 ml 10 mM maltose. Samples were
32 concentrated using a Y-30 centricon after washing five times with binding buffer. 30 μl

1 of GST or GST-Mid2-PH were mixed with 30 μ l MBP-Mid2-PH in a total volume of 100 μ l
2 and incubated at 4 $^{\circ}$ C for 2 h. Magnetic beads were washed three times with binding
3 buffer, resuspended in 35 μ l of laemli buffer and analyzed by SDS-PAGE and western
4 blotting.

5

6 **Septin-GFP fusions for orientation measurements**

7 The Spn1-GFP fusion used throughout this study includes a flexible linker (RIPGLI)
8 between the Spn1 and the GFP sequences rendering this fusion not appropriate for
9 orientation measurements (Fig S3A). To generate fusions of septins with rotationally
10 constrained GFP (conGFP) we generated and screened C-terminal GFP fusions of Spn1
11 and Spn4 with no linker sequence between the septin and the GFP, and with the C-
12 termini of septins and the N-terminus of GFP truncated to different extents, indicated
13 as δ C and Δ N, respectively. The coiled-coil prediction algorithm COILS was used to
14 identify the ends of the predicted coiled-coils in Spn1 and Spn4 to guide the C-terminal
15 truncations. We started with Spn1 fusions to monomeric EGFP (mEGFP) deleting C-
16 terminal Spn1 and N-terminal GFP amino acids, generating Spn1 Δ C5-mEGFP Δ N5 (Spn1
17 1-464; mEGFP starting at EELF), Spn1 Δ C5-mEGFP Δ N6, Spn1 Δ C5-mEGFP Δ N7 and
18 Spn1 Δ C5-mEGFP Δ N11; the first three fusions behaved normally localization- and
19 septation-wise, but the GFP was still flexible, whereas the last one was not fluorescent.
20 Further trimming of Spn1 C-terminus to generate Spn1 Δ C12-mEGFP Δ N5, Spn1 Δ C12-
21 mEGFP Δ N6 and Spn1 Δ C12-mEGFP Δ N7 led to ectopic cytoplasmic bar-like structures
22 (like the ones shown in FigS3 D). We thus turned to monomeric superfolder GFP
23 (msfGFP) which tolerates more extensive N-terminal truncations (Pédélec et al., 2006),
24 and generated a fusion of full-length Spn1 to msfGFP without using a linker and
25 additionally removing residues before the first stave of the GFP β -barrel to obtain Spn1-
26 msfGFP- Δ N12 (Spn1 1-469; msfGFP starting at VPILV). Spn1-msfGFP Δ N12, designated as
27 Spn1-conGFP in this study, was rotationally constrained and was used for orientation
28 measurements. We also generated Spn4 fusions to mEGFP deleting C-terminal Spn4 and
29 N-terminal GFP amino acids to obtain Spn4 Δ C6-mEGFP Δ N5 (Spn4 1-374; EGFP starting
30 at EELF), Spn4 Δ C6-mEGFP Δ N6, Spn4 Δ C6-mEGFP Δ N7 and Spn4 Δ C6-mEGFP Δ N11. The
31 first two constructs behaved normally, the third one formed ectopic cytoplasmic bar-

1 like structures in addition to its localization in septin rings (Fig S3D), whereas the last
2 one was not fluorescent. The GFP in Spn4 Δ C6-mEGFP Δ N7 was rotationally constrained
3 and this fusion, designated as Spn4-conGFP in this study, was used to infer the
4 orientation of septins with respect to the GFP dipole.

5 6 **Spinning-disk polarization-resolved fluorescence microscopy**

7 Yeast cells were prepared for live polarimetry measurements as described above for live
8 microscopy. Polarization-resolved fluorescence images were acquired on a custom-built
9 optical setup employing a confocal spinning disk unit (CSU-X1-M1 from Yokogawa)
10 connected to the side-port of a Perfect Focus System-equipped inverted microscope
11 (Eclipse Ti2-E, Nikon Instruments), using a Nikon Plan Apo \times 100/1.45 NA oil immersion
12 objective lens, 488- and 561-nm Sapphire laser lines (Coherent) and an iXon Ultra 888
13 EMCCD camera (1024 \times 1024 pixels, 13 \times 13 μ m pixel size, Andor, Oxford Instruments),
14 resulting in an image size of 1 pixel = 65 nm. Polarimetry images were obtained by
15 varying the incident linear polarization with a Pockels Cell electro-optic modulator (No
16 28-NP Quantum Technology) synchronized to the EMCCD camera, followed by a quarter
17 waveplate (WPQ05M-488, Thorlabs) (Wang et al., 2013). The quality of the polarization
18 state at the sample plane was ensured by using a compensating dichroic beamsplitter
19 similar to the one present in the spinning disk unit (405/488/561/635 nm Quad Dichroic
20 mirror) but placed at 90 $^\circ$ reflection to exchange s and p polarization state phase-shifts.
21 A polarization stack is made of 18 polarized images using an incident linear polarization
22 varying from 0 $^\circ$ (corresponding to the horizontal direction in the image) to 170 $^\circ$ with steps
23 of 10 $^\circ$. The recorded stack is used to obtain the polarization response of the sample at
24 each pixel, from which GFP orientation information can be deduced. Three to five large
25 field-of-view images (66 \times 66 μ m) typically containing 10-20 cells during cytokinesis per
26 image, were collected for wild-type and *nda3-KM311* cells; seven to eight such images
27 were collected for *mid2* Δ cells to account for the smaller number of *mid2* Δ dividing cells
28 per field of view. An exposure time of 0.5 s was used per image. Before each polarimetry
29 measurement, a two-color z-stack was acquired ($\Delta z = 1.0 \mu$ m) to image both septin-GFP
30 fusions and myosin/spindle bodies; these images were used for staging the measured
31 cells and correlating the measured orientation distributions with the different stages of
32 cytokinesis. A polarization stack was then recorded for each position within a z-stack (Δz

1 = 1.0 μm) for the septin-GFP channel, and thus allowed to obtain polarimetry images
2 throughout the septin rings, containing both tangential-most views where the entirety
3 of the ring can be seen parallel to the xy plane, and more equatorial views showing cross-
4 sections that appear as spots on either side of the ring. To minimize bias in the measured
5 orientations due to the contribution of off-plane orientations we focused on the
6 tangential-most views, as shown in Fig 5 and Fig S3.

7 Polarization stack images were first processed with the open-source image processing
8 software ImageJ/Fiji. Images within each polarization stack were systematically
9 registered using the StackReg plugin to correct for drift during the acquisition. The
10 proper z planes, i.e. the ones containing the tangential-most views of the ring, were then
11 identified for each cell, and polarimetry data were analyzed according to the framework
12 defined by (Kress et al., 2013) to obtain the mean GFP dipole orientation (angle ρ) per
13 pixel. Analysis and data representation, including color-coded stick representations of
14 the measured orientations per pixel and polar-plot histograms, were done by using the
15 Polarimetry Analysis software which is a Matlab App Designer standalone application.
16 The source code is available at [gitHub.com/cchandre/Polarimetry](https://github.com/cchandre/Polarimetry), and the desktop app
17 can be freely obtained at <https://www.fresnel.fr/polarimetry> under a BSD license.

18 The regions of interest for analysis were selected by a combination of intensity
19 thresholding and manual selection of the features to analyze. Each region of interest
20 contained typically 120-200 analyzed pixels i.e. 120-200 orientation color-coded sticks
21 in the tangential-most view of the septin ring per cell (Fig 5 and Fig S3). The mean
22 orientation per pixel is represented as a stick whose orientation and color are the
23 measured angle ρ within the resolution limit of the microscope (~ 200 nm). The
24 distribution of mean orientations was represented in 0-180° polar-plot histograms. To
25 assess the extent to which the measured orientations were more parallel or more
26 perpendicular with respect to the CR axis, the ring axis in each cell was used to normalize
27 the angle distributions from 0-180° to 0-90° and generate 0-90° polar plots (Fig 5 and Fig
28 S3), with 0° and 90° defining orientations parallel and perpendicular to the ring axis,
29 respectively. To facilitate the visual comparison of orientation distributions between
30 cells and also relate directly to the 0-90° polar plots, representative cells in Fig 5 and Fig
31 S3 have been rotated using the Polarimetry Analysis software so that their CR axes
32 correspond to the horizontal orientations (0°). 0-90° normalized polar-plot histograms

1 from all cells in a given condition were concatenated to represent all analyzed data in
2 each condition, then they were rescaled to their respective maximum value to facilitate
3 comparisons, and orientations were sorted in bins of 6°. The normalization of
4 orientation distributions with respect to the CR ring axis, the normalization of 0-180° to
5 0-90° polar plots and the rescaling of histograms were done with custom-generated
6 Matlab codes, the source codes of which are available at
7 github.com/cchandre/Polarimetry.

8

9 **Statistical analysis**

10 The number of samples analyzed (n) is defined in each figure and were derived from 3
11 independent experiments, except for the co-immunoprecipitations assays in Figure S1B
12 and S2D, which were repeated twice. The error bars correspond to standard deviation
13 (sd) between experiments and are specifically indicated in each figure.

14

15 **SUPPLEMENTAL INFORMATION**

16 Supplemental information includes five figures and one table.

17

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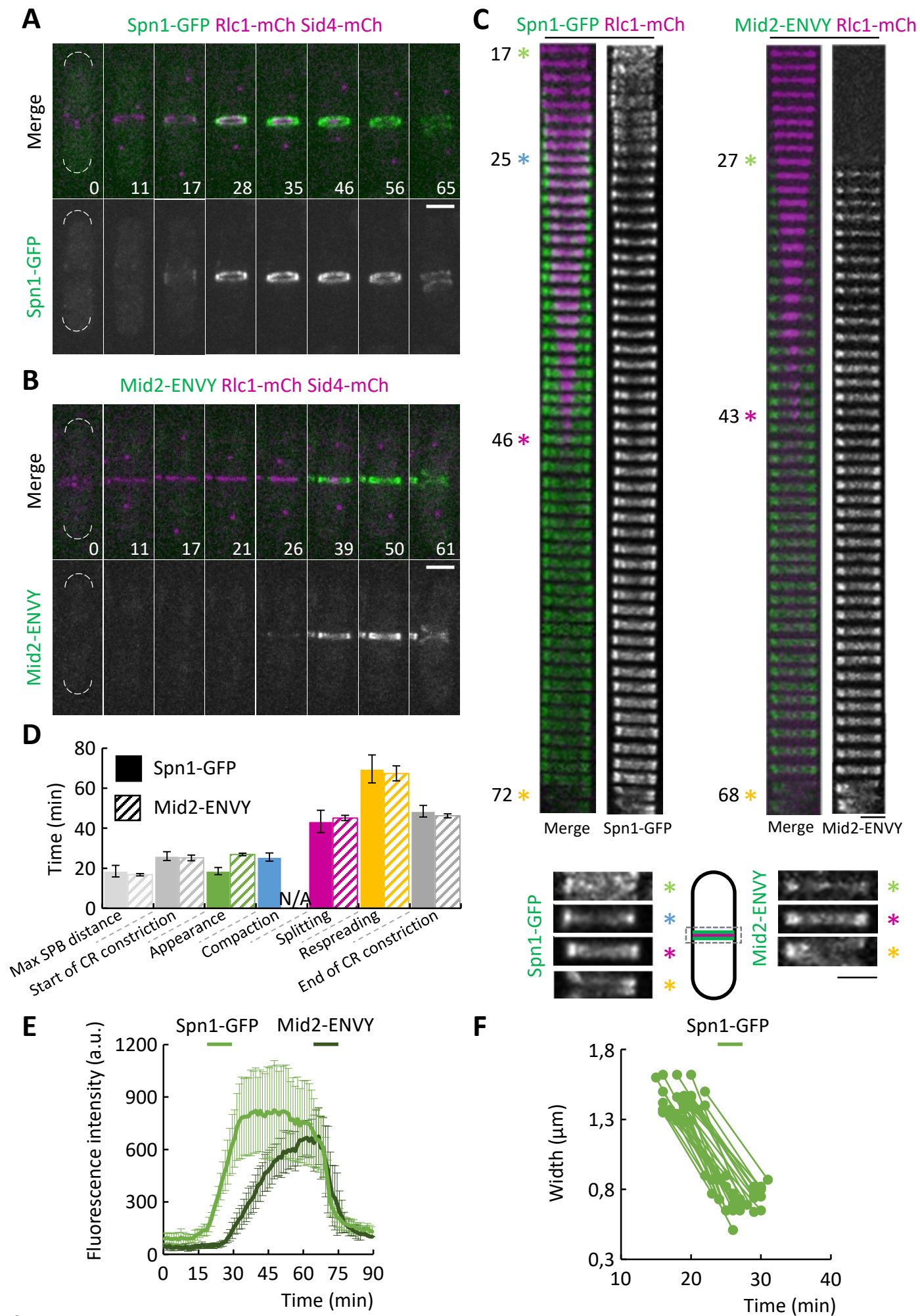
44

45 **ABBREVIATIONS**

46 CR: Contractile Ring

47 SIN: Septation Initiation Network

- 1 CDK: Cyclin-Dependent Kinase
- 2 Sd: standard deviation



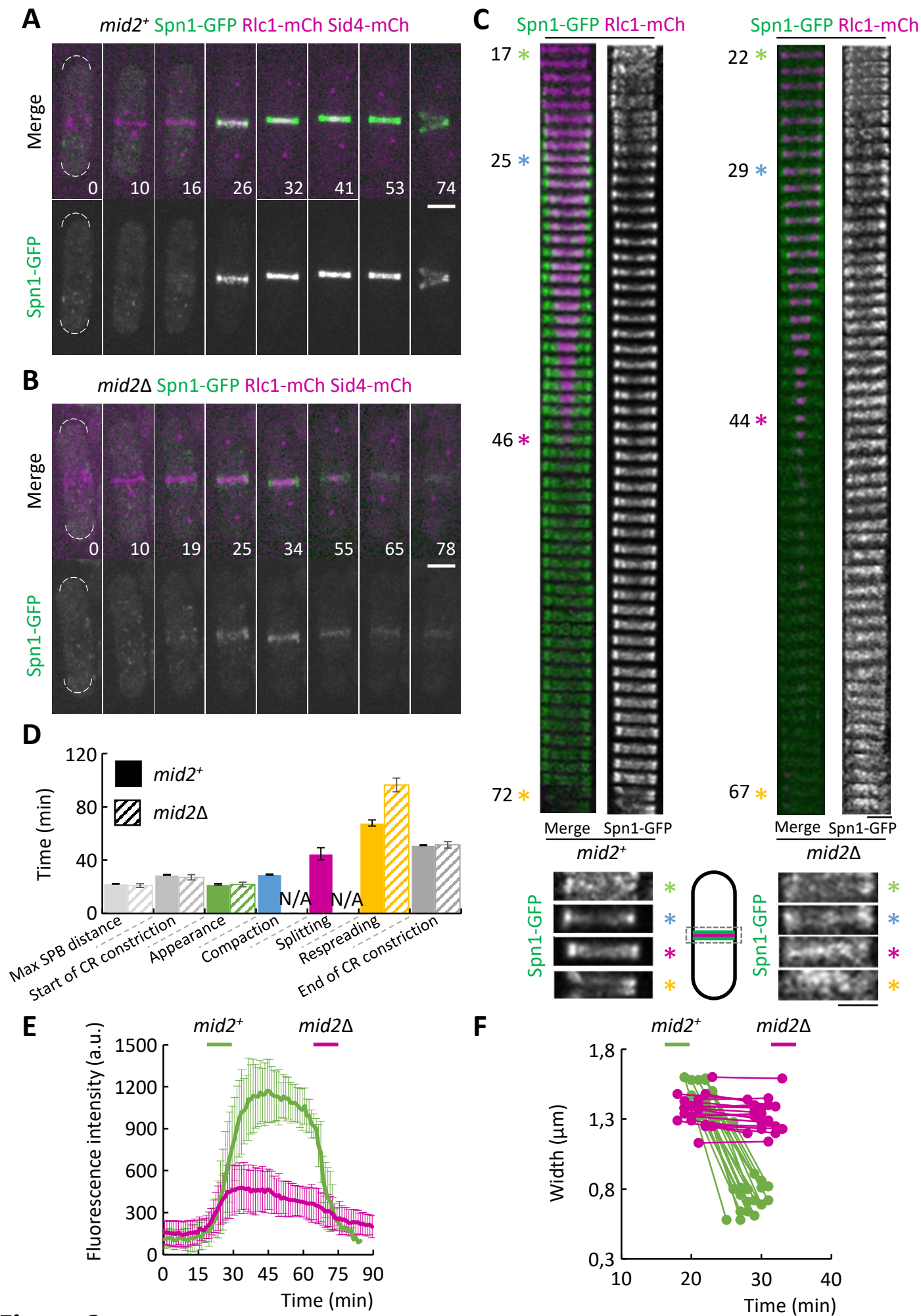
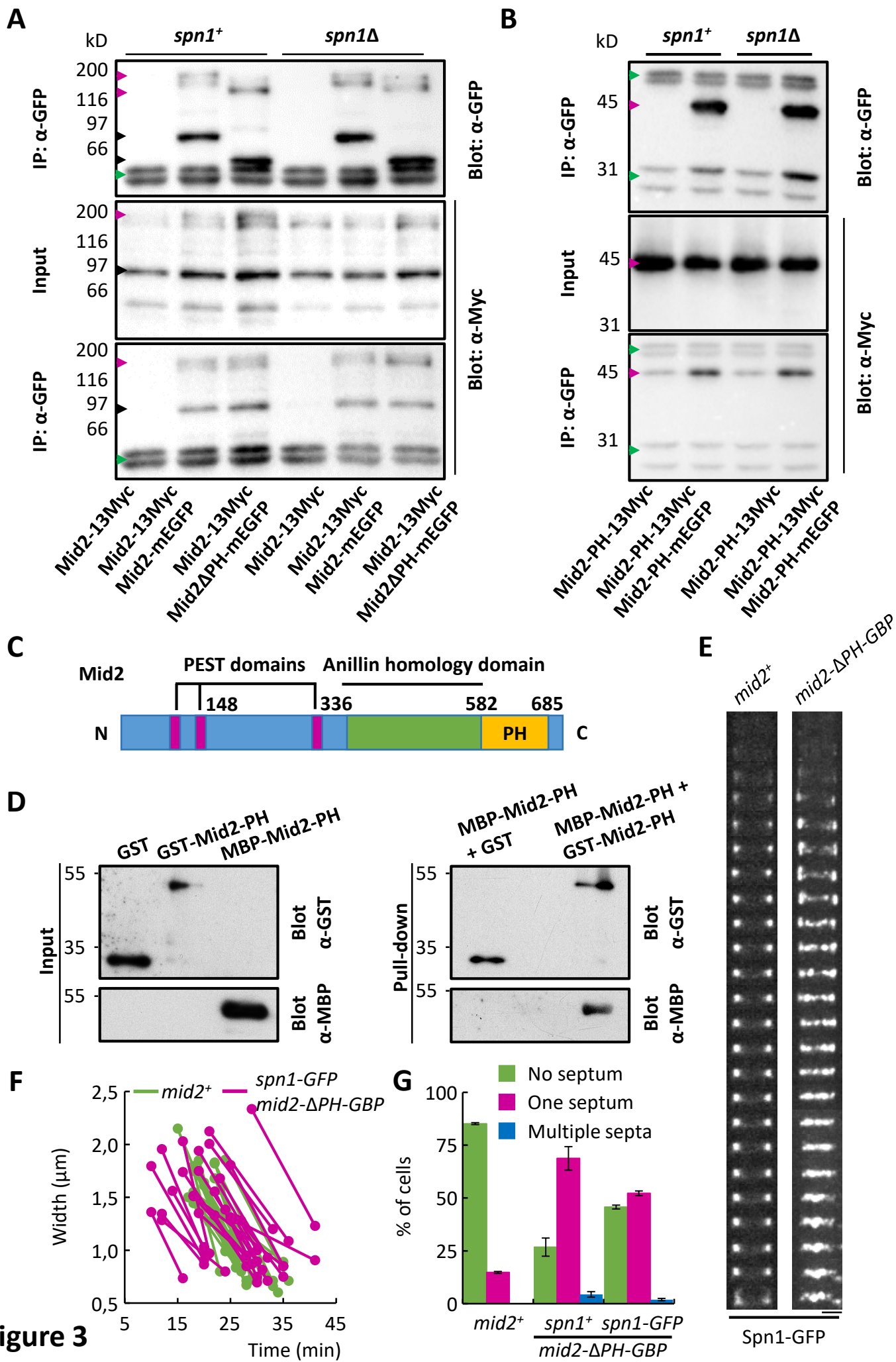


Figure 2



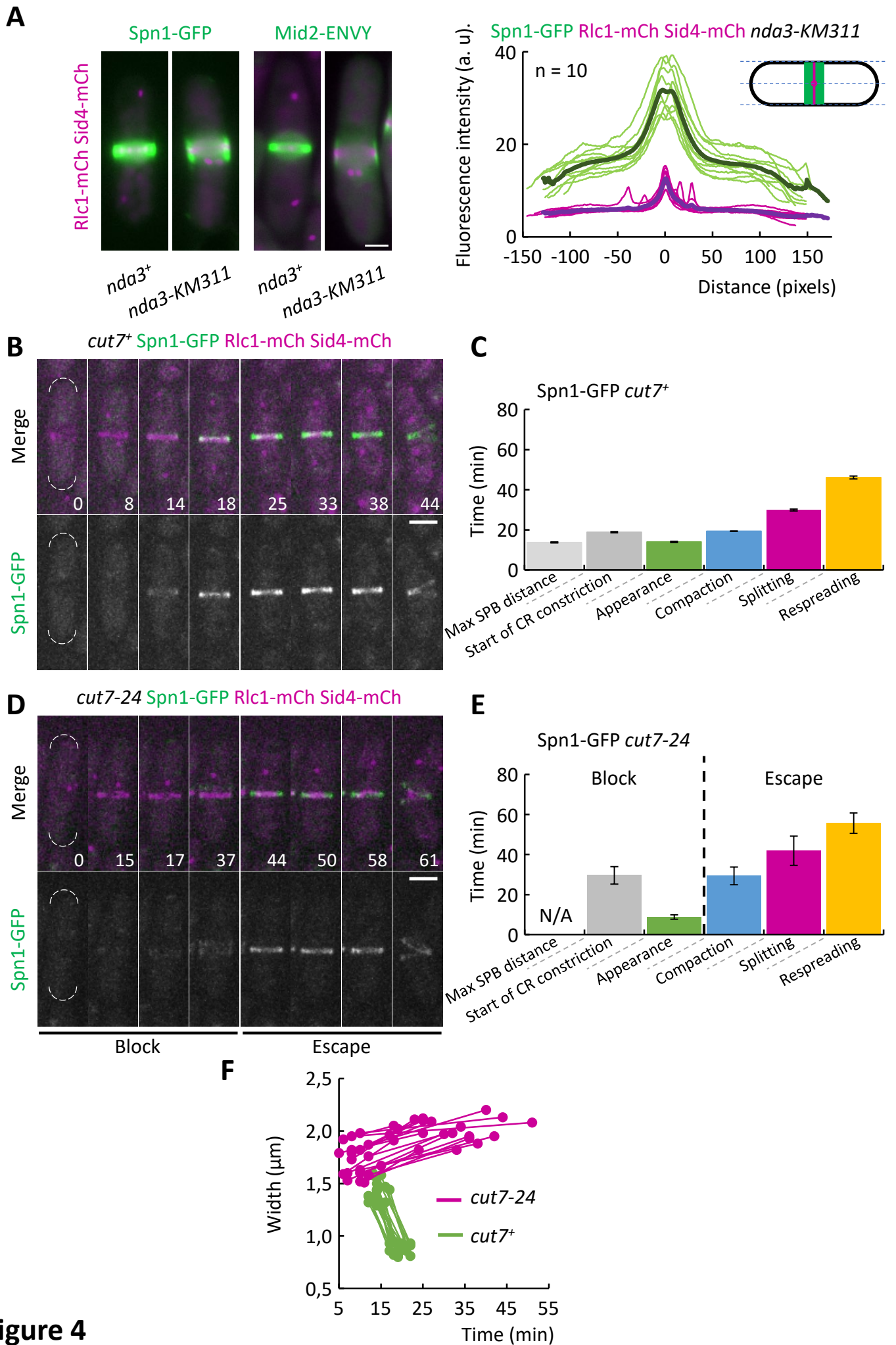
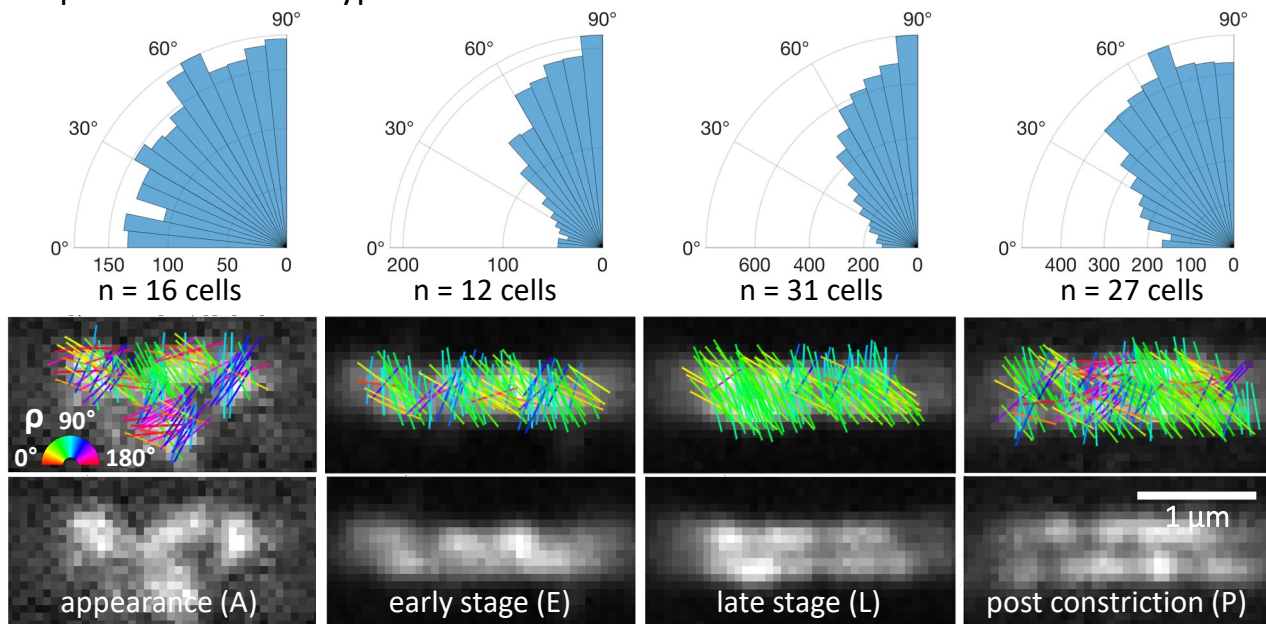
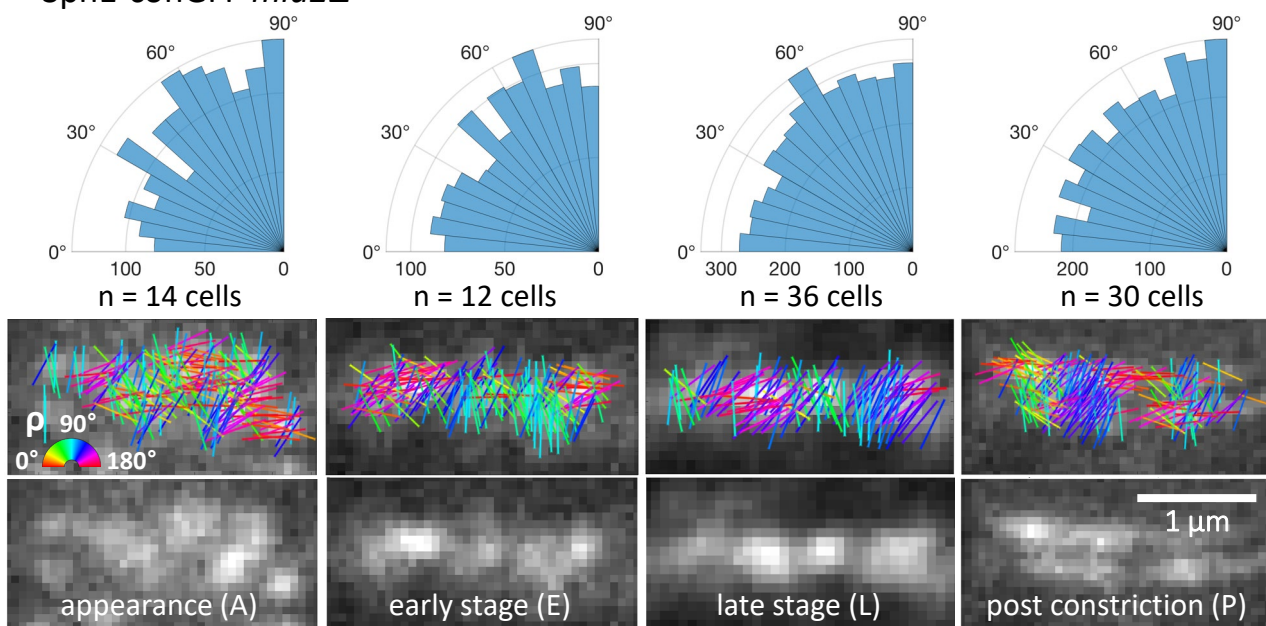


Figure 4

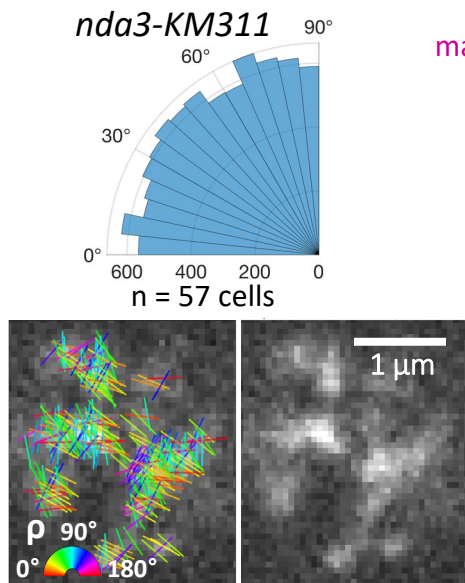
A Spn1-conGFP wild-type



B Spn1-conGFP *mid2Δ*



C Spn1-conGFP *nda3-KM311*



D septin appearance septin compaction starting ring septin splitting advanced ring after ring constriction

max SPB elongation constriction constriction constriction

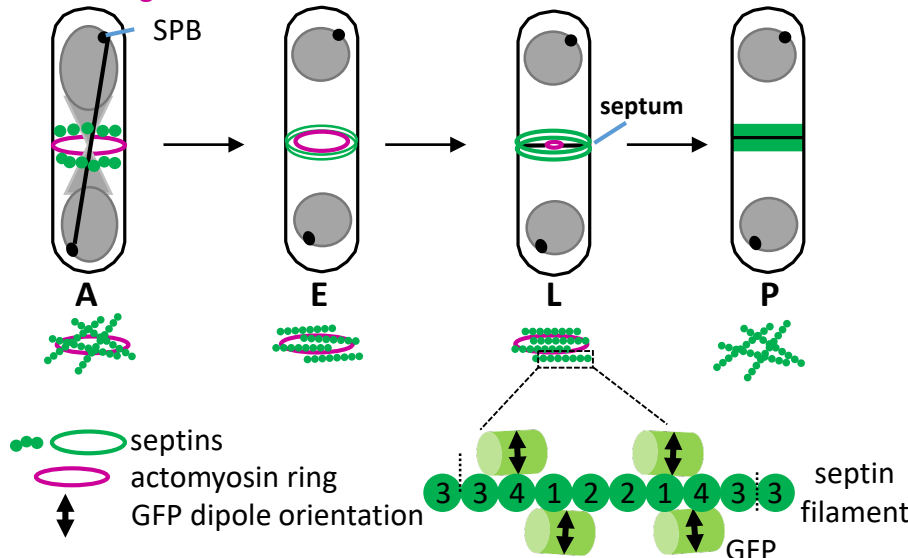


Figure 5

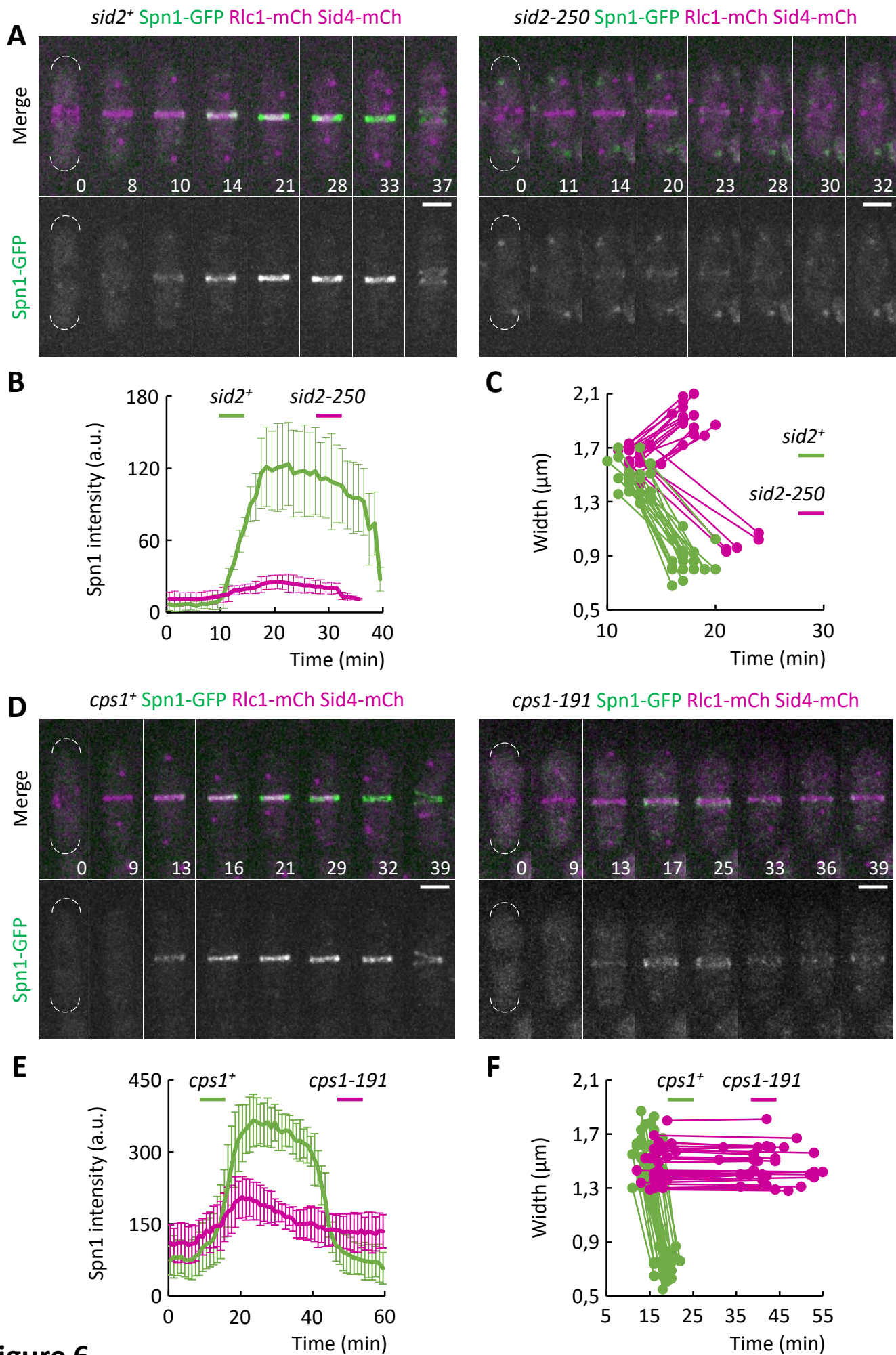


Figure 6

SUPPLEMENTAL INFORMATION

Table S1. Table of strains used in this study.

Strain	Genotype	Source
Figure 1		
AP5501	<i>spn1-GFP:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
AP5678	<i>mid2-ENVY:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
Figure 2		
AP5501	<i>spn1-GFP:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
AP5582	<i>spn1-GFP:kanMX6 mid2Δ::kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
Figure 3		
AP5823	<i>mid2-13xMyc:Nat ade6-M210 ura4-D18 leu1-32</i>	This study
AP5831	<i>leu1+:Pmid2-mid2-mEGFP mid2-13xMyc:Nat ade6- ura4-D18 leu1-32</i>	This study
AP5829	<i>leu1+:Pmid2-mid2ΔPH-mEGFP mid2-13xMyc:Nat ade6- ura4-D18 leu1-32</i>	This study
AP5918	<i>spn1Δ::ura4+ mid2-13xMyc:Nat ade6- ura4-D18 leu1-32</i>	This study
AP5920	<i>spn1Δ::ura4+ leu1+:Pmid2-mid2-mEGFP mid2-13xMyc:natMX6 ade6-</i>	This study
AP5927	<i>spn1Δ::ura4+ leu1+:Pmid2-mid2ΔPH-mEGFP mid2-13xMyc:natMX6 ade6- ura4-D18 leu1-32</i>	This study
AP5814	<i>leu1+:Pmid2-mid2-PH-13xMyc ade6- ura4-D18 leu1-32</i>	This study
AP5932	<i>mid2-PH-mEGFP:kanMX6 leu1+:Pmid2-mid2-PH-13xMyc ade6- ura4-D18 leu1-32</i>	This study
AP5930	<i>spn1Δ::ura4+ leu1+:Pmid2-mid2-PH-13xMyc ade6- ura4-D18 leu1-32</i>	This study
AP5943	<i>spn1Δ::ura4+ mid2-PH-mEGFP:kanMX6 leu1+:Pmid2-mid2-PH-13xMyc ade6- ura4-D18 leu1-32</i>	This study
SR1370	<i>mid2Δ::mEGFP:kanMX6 ade6: :Pmid2-mid2-PH-13xMyc ade6- ura4-D18 leu1-32</i>	This study
AP5501	<i>spn1-GFP:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study

SR1216	<i>mid2-ΔPH-GBP:kanMX6 spn1-GFP:hphMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
SR1210	<i>mid2-ΔPH-GBP:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
Figure 4		
AP5501	<i>spn1-GFP:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
AP5515	<i>spn1-GFP:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 nda3-KM311 ade6- ura4-D18 leu1-32</i>	This study
AP6268	<i>spn1-GFP:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 cut7-24 ade6- ura4-D18 leu1-32</i>	This study
AP5678	<i>mid2-ENVY:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
AP5514	<i>mid2-ENVY:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 nda3-KM311 ade6- ura4-D18 leu1-32</i>	This study
Figure 5		
AP6313	<i>spn1-msfGFPΔN12:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
AP6334	<i>spn1-msfGFPΔN12:kanMX6 mid2::hphMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
AP6324	<i>spn1-msfGFPΔN12:kanMX6 nda3-KM311 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
Figure 6		
AP5501	<i>spn1-GFP:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
AP5585	<i>spn1-GFP:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 sid2-250 ade6- ura4-D18 leu1-32</i>	This study
AP5633	<i>spn1-GFP:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 cps1-191 ade6- ura4-D18 leu1-32</i>	This study
Figure S1		
AP5501	<i>spn1-GFP:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
AP5582	<i>spn1-GFP:kanMX6 mid2Δ::kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
AP5823	<i>mid2-13xMyc:Nat ade6-M210 ura4-D18 leu1-32</i>	This study

AP6274	<i>mid2-13xMyc:natMX6 nda3-KM311 ade6- ura4-D18 leu1-32</i>	This study
AP6276	<i>spn1-GFP:KanMX6 mid2-13xMyc:natMX6 ura4-D18 leu1-32</i>	This study
AP6280	<i>spn1-GFP:KanMX6 mid2-13xMyc:natMX6 nda3-KM311 ura4-D18 leu1-32</i>	This study
Figure S2		
AP6274	<i>mid2-13xMyc:natMX6 nda3-KM311 ade6- ura4-D18 leu1-32</i>	This study
AP6069	<i>leu1+:Pmid2-mid2-mEGFP mid2-13xMyc:natMX6 nda3-KM311 ade6- ura4-D18 leu1-32</i>	This study
AP6073	<i>leu1+:Pmid2-mid2ΔPH-mEGFP mid2-13xMyc:natMX6 nda3-KM311 ade6- ura4-D18 leu1-32</i>	This study
AP6074	<i>leu1+:Pmid2-mid2-PH-mEGFP mid2-13xMyc:natMX6 nda3-KM311 ade6- ura4-D18 leu1-32</i>	This study
SR1344	<i>mid2-mEGFP:kanMX6 ade6:mid2-13xMyc ade6- ura4-D18 leu1-32</i>	This study
SR1234	<i>spn1-5Δ::ura4+ mid2-mEGFP:kanMX6 ade6:mid2-13xMyc ade6- ura4-D18</i>	This study
SR1346	<i>mid2-ΔPH-mEGFP:kanMX6 ade6:mid2-13xMyc ade6- ura4-D18 leu1-32</i>	This study
SR1236	<i>spn1-5Δ::ura4+ mid2-ΔPH-mEGFP:kanMX6 ade6:mid2-13xMyc ade6- ura4-D18</i>	This study
SR1348	<i>PH-mid2-mEGFP:kanMX6 ade6: PH-mid2-13xMyc ade6- ura4-D18 leu1-32</i>	This study
SR1237	<i>spn1-5Δ::ura4+ PH-mid2-mEGFP:kanMX6 ade6: PH-mid2-13xMyc ade6- ura4-D18</i>	This study
Figure S3		
AP5678	<i>mid2-ENVY:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
AP6271	<i>mid2-ENVY:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 cut7-24 ade6- ura4-D18 leu1-32</i>	This study
AP5501	<i>spn1-GFP:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
AP5515	<i>spn1-GFP:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 nda3-KM311 ade6- ura4-D18 leu1-32</i>	This study
AP5678	<i>mid2-ENVY:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study

AP5514	<i>mid2-ENVY:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 nda3-KM311 ade6- ura4-D18 leu1-32</i>	This study
Figure S4		
AP5501	<i>spn1-GFP:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
AP6313	<i>spn1-msfGFPΔN12:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
AP6334	<i>spn1-msfGFPΔN12:kanMX6 mid2::hphMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
AP6324	<i>spn1-msfGFPΔN12:kanMX6 nda3-KM311 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
AP6234	<i>spn4ΔC6-msfGFPΔN7:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
Figure S5		
AP5678	<i>mid2-ENVY:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 ade6- ura4-D18 leu1-32</i>	This study
AP5564	<i>mid2-ENVY:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 sid2-250 ade6- ura4-D18 leu1-32</i>	This study
AP5855	<i>mid2-ENVY:kanMX6 rlc1-mCherry:natMX6 sid4-mCherry:hphMX6 cps1-191 ade6- ura4-D18 leu1-32</i>	This study

SUPPLEMENTAL FIGURE LEGENDS

Figure S1: Septin compaction is defective in *mid2Δ* cells, and Mid2 and septins interact in *nda3-KM311*-blocked cells and Mid2 self-interaction is septin independent

A: Kymographs of the lateral region of the medial cortex (indicated in the scheme at the bottom) of wild type (left) or *mid2Δ* cells (right) producing Spn1-GFP, Rlc1-mCherry and Sid4-mCherry. Elapsed time is shown in minutes. The major transitions in the behavior of Spn1 from mitosis onset and the defects observed in the absence of Mid2 are highlighted in magnified panels at the far right and indicated with a color-coded asterisk that matches that of the kymograph in figure 1C. **B:** Co-immunoprecipitation assay between Mid2-13XMyc and Spn1-GFP in control and *nda3-KM311* cells incubated at 18°C for 7 hours (lanes 1, 2, 3 and 4) and released for 1 hour at 25°C (lanes 5, 6, 7 and 8). Magenta arrowheads show the expected Spn1 or Mid2 bands; black arrowheads show degradation products; green arrowheads depict the immunoglobulin bands.

Figure S2: The PH domain of Mid2 self-interacts in two hybrid assays and Mid2 co-immunoprecipitates with itself in *nda3-KM311*-blocked cells

A: Co-immunoprecipitation assay between full length Mid2-13Myc and Mid2-mEGFP or Mid2 ΔPH -13Myc and Mid2ΔPH-GFP either in the presence (wt) or in the absence of septins (*spn1-5Δ*). Magenta arrowheads show the expected Mid2 bands; black arrowheads show degradation products. **B:** Co-immunoprecipitation assay between the PH domain of Mid2 tagged with 13XMyc and the PH domain of Mid2 tagged with GFP either in the presence (wt) or in the absence of septins (*spn1-5Δ*). **C:** Two-hybrid assay showing the interaction of the PH domain of Mid2 with itself. The serially diluted growth of the indicated transformants is shown under non-selective (left panel) or selective conditions (right panel). **D:** Co-immunoprecipitation assay between Mid2-13XMyc and full length Mid2-GFP, Mid2ΔPH-GFP or the PH domain of Mid2 tagged with GFP in *nda3-KM311* cells incubated at 18°C for 8 hours. Magenta arrowheads show the expected Mid2 bands; black arrowheads show degradation products; green arrowheads depict the immunoglobulin bands.

Figure S3: Under high CDK activity, septins and Mid2 appear as a broad band at both sides of the CR

Fluorescence intensity analysis of Spn1-GFP, Rlc1-mcherry and Sid4-mcherry signal measured along the entire cell in epifluorescence images of the *nda3-KM311* mutant incubated at 18°C for 8 hours in a representative cell where the SPBs are aligned (A) or not (B) with respect to the ring position. C: Quantification of Spn1-GFP and Mid2-ENVY width over time in wild type (light green for Spn1-GFP and dark green for Mid2-ENVY) and *nda3-KM311* mutant cells (magenta for Spn1-GFP and purple for Mid2-ENVY) incubated at 18°C during 8 hours. The last data point has been taken after switching the cells to 25°C for 1 hour. The average curves \pm sd are displayed. $n > 500$ cells. Time lapse imaging of wild type (D) or *cut7-24* cells (F) expressing Mid2-ENVY, Rlc1-mcherry and Sid4-mcherry incubated at 36°C for 3 hours. Maximum intensity projections of confocal images are shown. Time 0 corresponds to mitotic entry. Scale bars: 2 μ m. Plot displaying Mid2-ENVY dynamics throughout cell division in control (E) and *cut7-24* cells (G). Mean \pm sd are shown. $n = 45$ cells in each strain. H: Quantification of the extent of Mid2-ENVY width compaction over time in wild type (dark green) and *cut7-24* cells (purple). $n = 20$ cells in each case.

Figure S4: Localization and orientation measurements of Spn1-conGFP and Spn4-conGFP in wild type cells

A: Polar-plot histograms of GFP dipole orientations of Spn1-GFP filaments from wild type cells at different stages of cytokinesis (top panels). The number of analyzed cells per stage is indicated below each polar-plot histogram. Confocal grayscale images of Spn1-GFP from selected wild type cells and an overlaid color-coded stick representation of the measured orientations (angles ρ) per analyzed pixel at each cytokinetic stage (bottom panels). The mean orientation of GFP dipoles per pixel is represented as a stick whose orientation and color are the measured angle ρ . Scale bar: 1 μ m. B: Maximum intensity projection images of Spn1-GFP, Rlc1-mCherry and Sid4-mCherry in wild type cells (left) and Spn1-conGFP, Rlc1-mCherry and Sid4-

mCherry (right) in wild type, *mid2Δ* and *nda3-KM311* cells at each stage of cytokinesis. Representative large field-of-view maximum intensity projection images of wild type *rlc1-mCherry sid4-mCherry* cells producing Spn1-conGFP (C) or Spn4-conGFP (D) used for binning cells in each cytokinetic stage before polarimetry measurements. White asterisks in the insets in (D) point to septin rings in selected cells (outlined rectangles), while white arrows point to ectopic cytoplasmic bar-like structures upon Spn4-conGFP production. Scale bars: 10 μm. An example of a polar plot of dipole orientations in a Spn4-conGFP bar-like structure is shown on the right. A confocal grayscale image of the bar-like structure and an overlaid color-coded stick representation of the measured orientations in this structure are shown below the polar-plot histogram. Scale bar: 1 μm. E: Examples of cells with Spn4-conGFP in septin rings during CR constriction (left) and during early stages of post constriction (right). Confocal grayscale images of selected cells are shown together with the respective overlaid color-coded stick representation of the measured orientations and the corresponding polar-plot histograms.

Figure S5: Mid2 recruitment to the division region is compromised under low SIN activity or in the β(1,3)-glucan synthase Bgs1 mutant *cps1-191*

A: Time lapse imaging of wild type (left series) and *sid2-250* cells (right series) producing Mid2-ENVY, Rlc1-mCherry and Sid4-mCherry incubated at 36°C for 3 hours. Maximum intensity projections of confocal images are shown. Time 0 corresponds to mitotic entry. Scale bars: 2 μm. **B:** Analysis of Mid2-ENVY fluorescence intensity from mitotic onset throughout cell division in control (dark green) and *sid2-250* cells (purple). The average line graphics ± sd are displayed. n=6 cells in each case. **C:** Time lapse imaging of wild type (left series) and *cps1-191* cells (right series) producing Mid2-ENVY, Rlc1-mCherry and Sid4-mCherry incubated at 36°C for 3 hours. Maximum intensity projections of confocal images are shown. Time 0 corresponds to mitotic entry. Scale bars: 2 μm. **D:** Analysis of Mid2-ENVY fluorescence intensity from mitotic onset throughout cell division in control (dark green) and *cps1-191* cells (purple). The average line graphics ± sd are displayed. n=6 cells in each case.

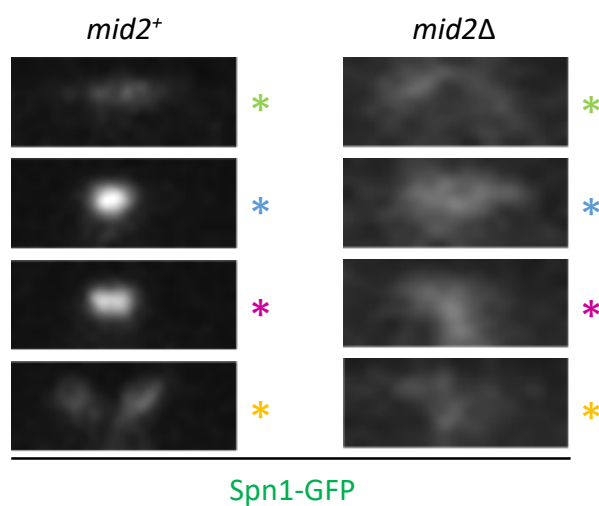
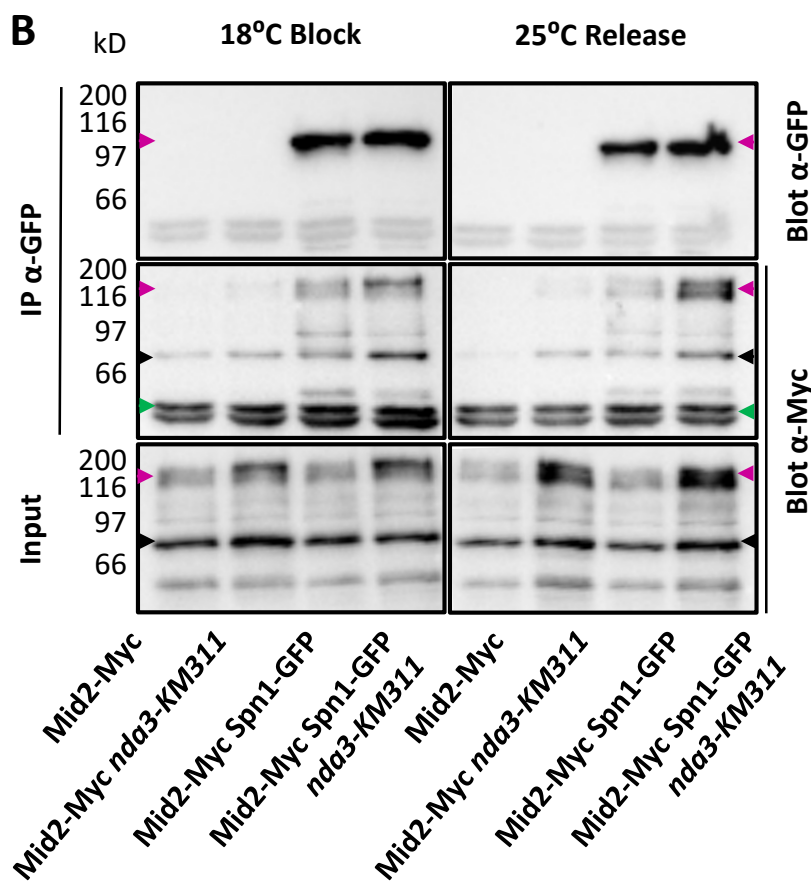
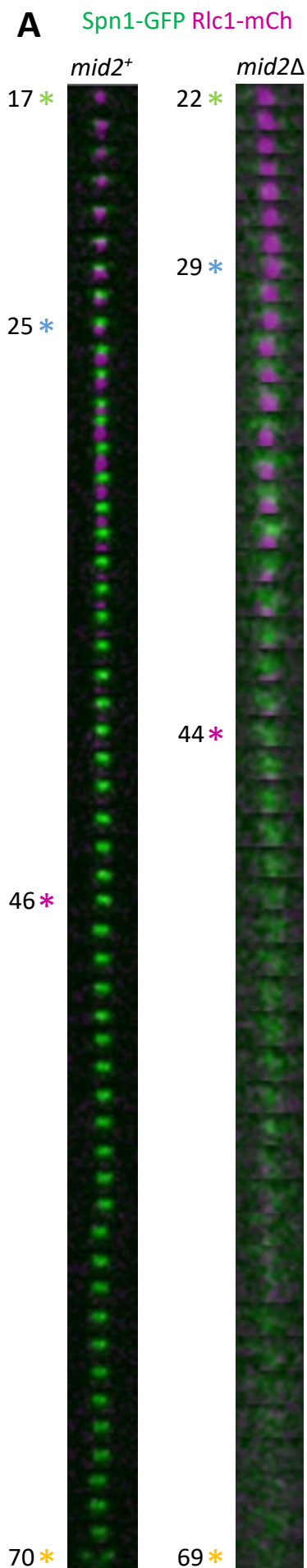


Figure S1

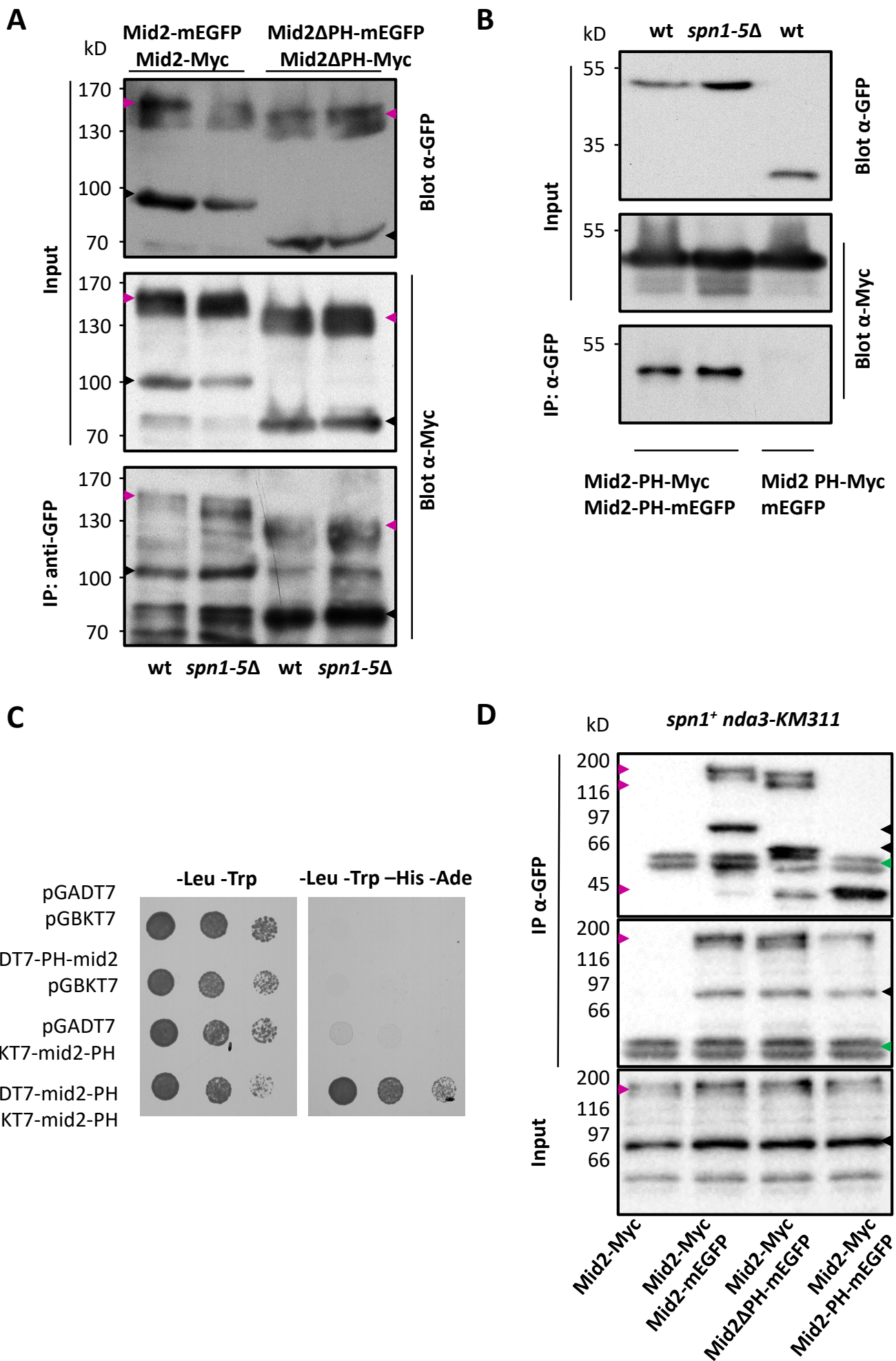


Figure S2

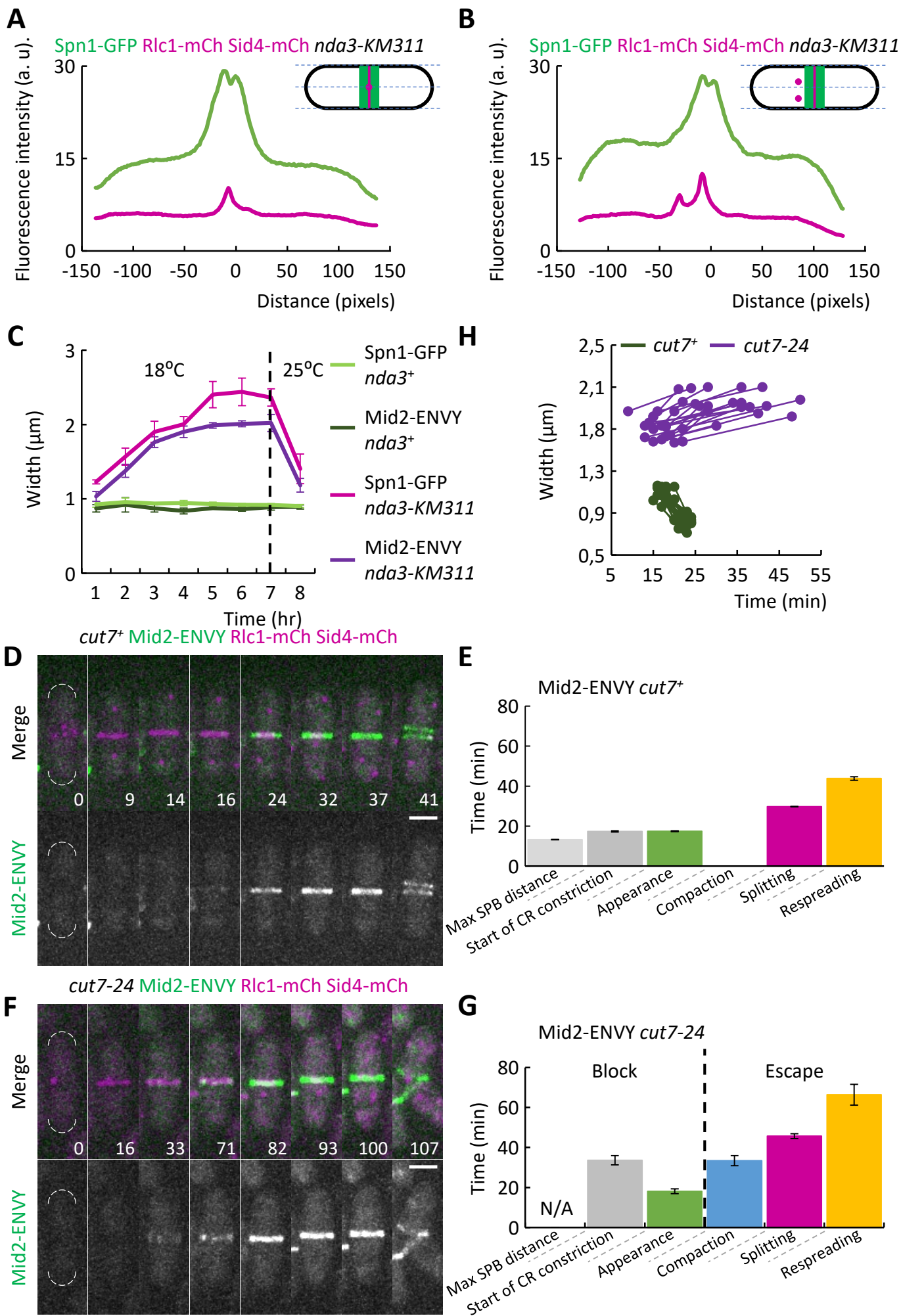
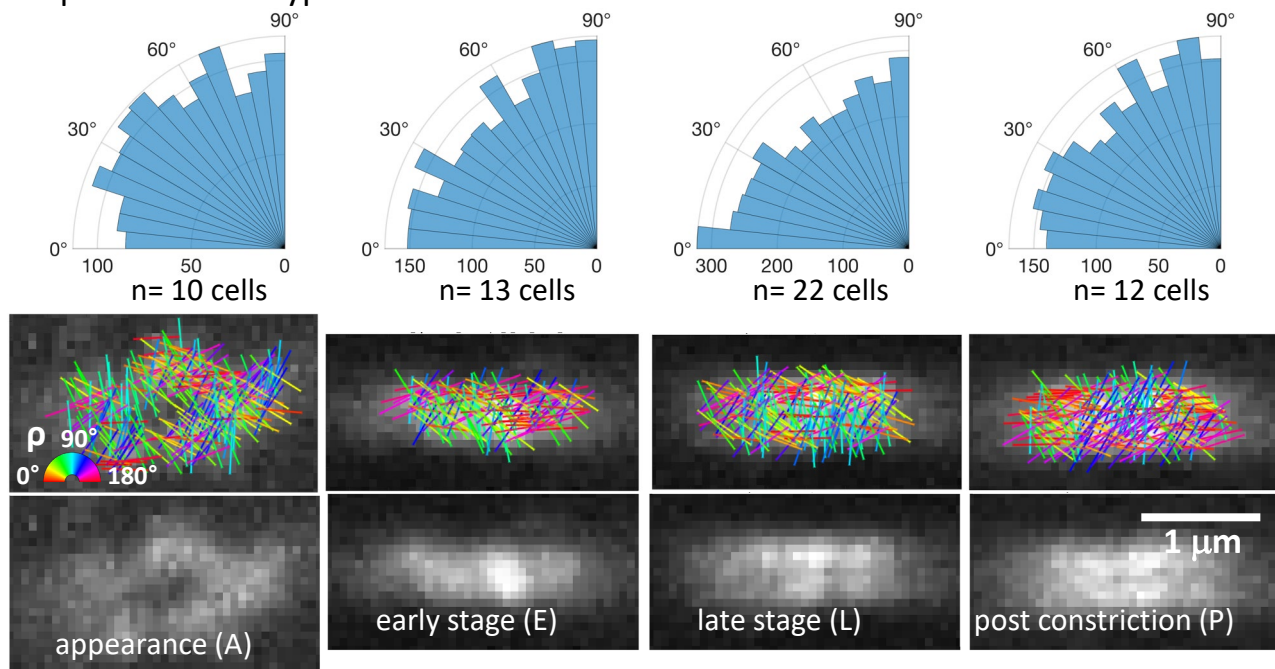


Figure S3 Block Escape

A

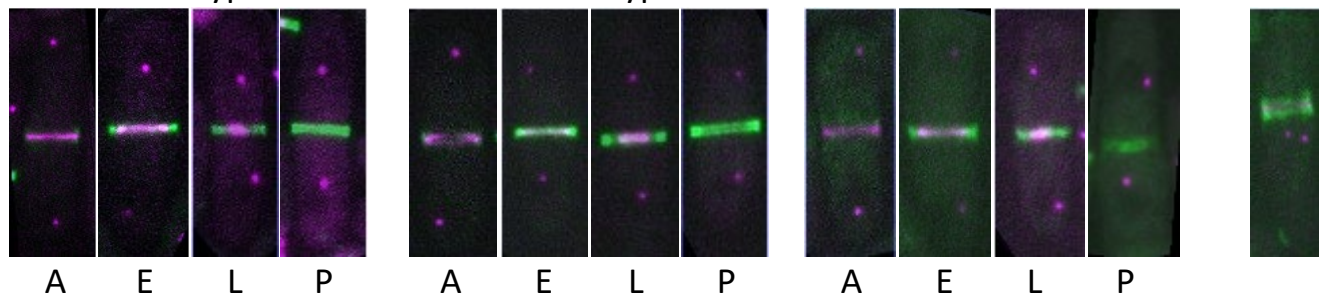
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**B**

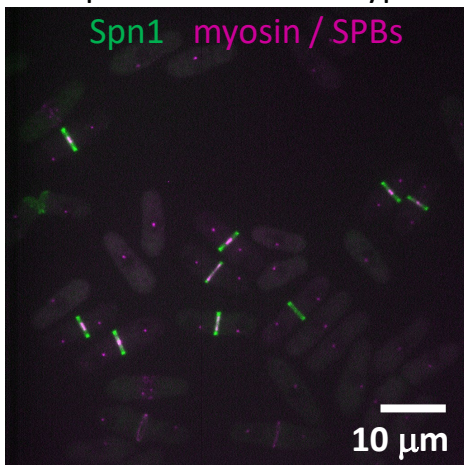
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wild-type

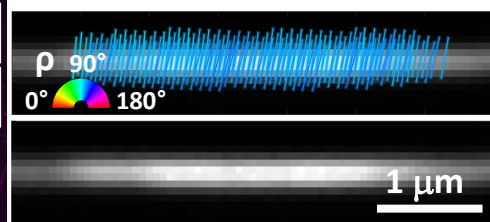
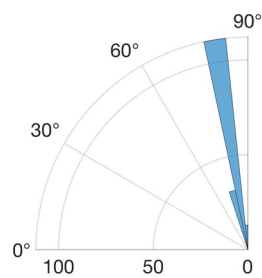
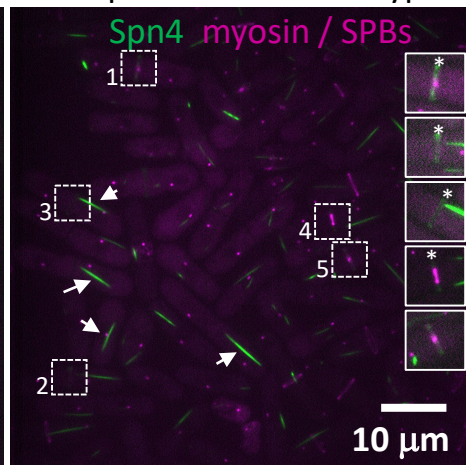
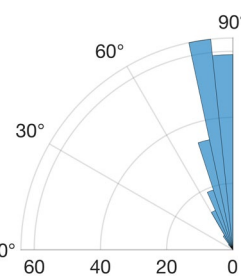
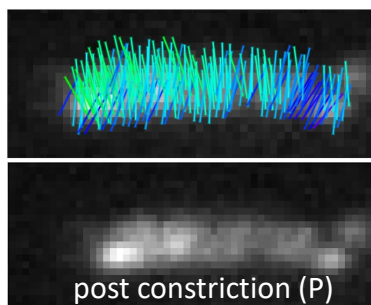
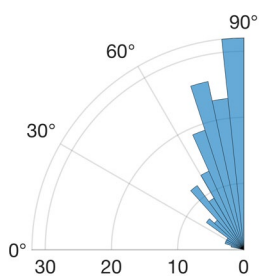
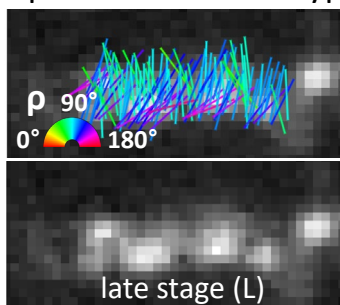
Spn1-conGFP

mid2 Δ *nda3-KM311***C** Spn1-conGFP wild-type

Spn1 myosin / SPBs

**D** Spn4-conGFP wild-type

Spn4 myosin / SPBs

**E** Spn4-conGFP wild-type**Figure S4**

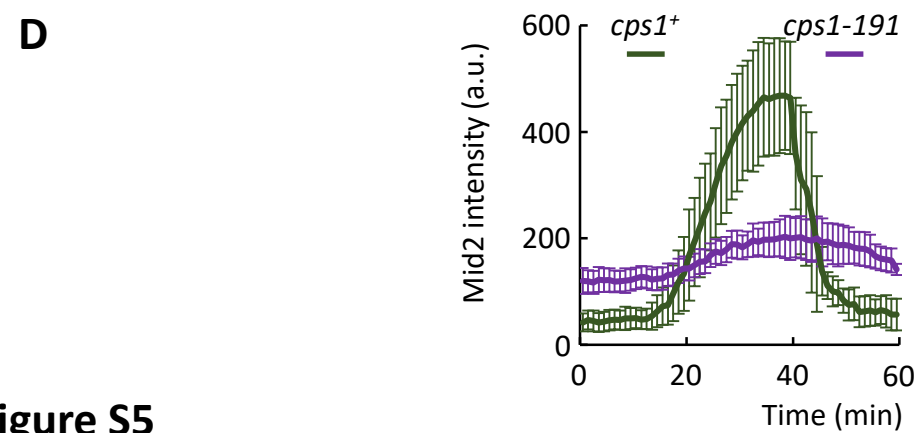
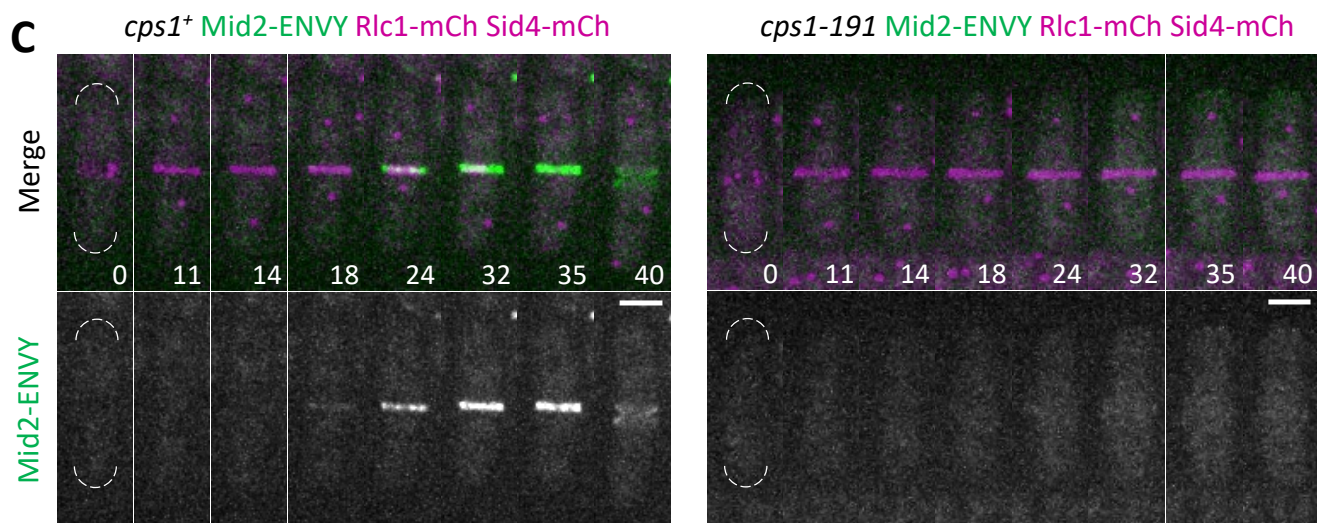
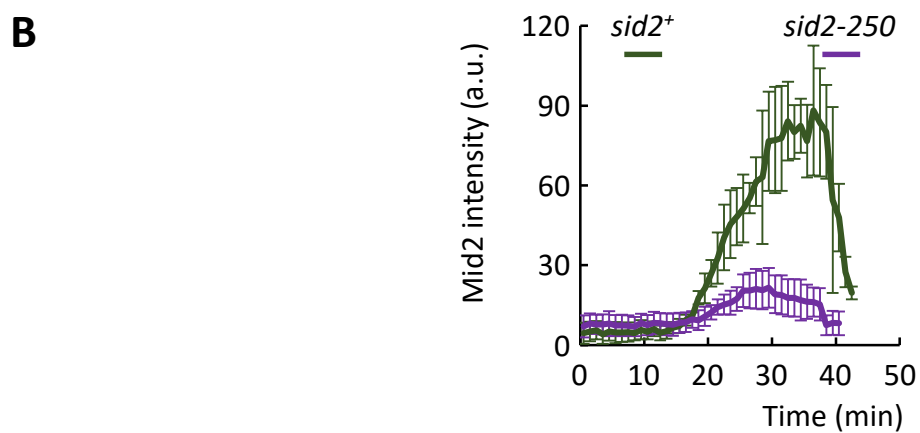
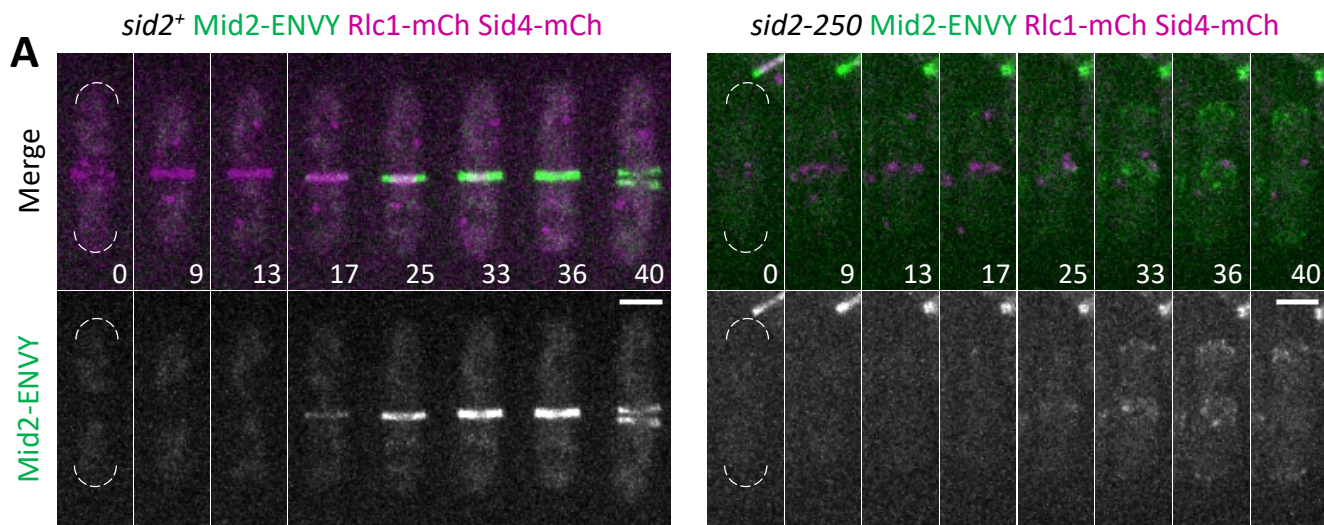


Figure S5