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Report

Identification of SIN Pathway Targets Reveals Mechanisms of Crosstalk between NDR Kinase Pathways

Sneha Gupta,[1] Sebastian Mana-Capelli,[1] Janel R. McLean,[2] Chun-Ti Chen,[1,3] Samriddha Ray,[1,4] Kathleen L. Gould,[2] and Dannel McCollum[1,*]
[1]Department of Microbiology and Physiological Systems and Program in Cell Dynamics, University of Massachusetts Medical School, Worcester, MA 01605, USA
[2]Howard Hughes Medical Institute and Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

Summary

The septum initiation network (SIN) regulates multiple functions during late mitosis to ensure successful completion of cytokinesis in *Schizosaccharomyces pombe*. One mechanism by which the SIN promotes cytokinesis is by inhibiting a competing polarity pathway called the MOR [1], which is required for initiation of polarized growth following completion of cytokinesis [2]. Mutual antagonism between the two NDR kinase pathways, SIN and MOR, is required to coordinate cytoskeletal rearrangements during the mitosis-interphase transition. To determine how the SIN regulates the MOR pathway, we developed a proteomics approach that allowed us to identify multiple substrates of the SIN effector kinase Sid2, including the MOR pathway components Nak1 kinase and an associated protein, Sog2. We show that Sid2 phosphorylation of Nak1 causes removal of Nak1 from the spindle pole bodies, which may both relieve Nak1 inhibition of the SIN and block MOR signaling by preventing interaction of Nak1 with the scaffold protein Mor2. Because the SIN and MOR are conserved in mammalian cells (Hippo and Ndr1/2 pathways, respectively), this work may provide important insight into how the activities of these essential pathways are coordinated.

Results and Discussion

Identification of Sid2 Substrates

To determine how the septum initiation network (SIN) performs its various functions in late mitosis, including MOR inhibition, we developed an approach to identify new Sid2 substrates. Sid2-family kinases preferentially phosphorylate the consensus sequence RXXS (where S becomes phosphorylated) [3, 4], which in its phosphorylated form is also the core consensus binding site for 14-3-3 proteins [5, 6]. Because Sid2 phosphorylation of Clp1 at RXXS sites is required for the 14-3-3 protein Rad24 to bind Clp1 [3], we hypothesized that other Sid2 substrates might be identified through SIN-dependent interaction with Rad24. Because many kinases besides Sid2 create 14-3-3 binding sites, Rad24-TAP protein complexes were purified from cells with constitutively active SIN (*cdc16-116*) and cells with inactive SIN (*sid1-239*, *cdc11-123*) for comparison. Protein samples were digested and analyzed by two-dimensional liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify Rad24 binding partners. The abundance (spectral counts) of each Rad24 interactor was normalized to Rad24 abundance for each experiment and then averaged over two biological replicates. The ratio of individual protein abundance in SIN "ON" (*cdc16-116)* versus SIN "OFF" (*sid1-239*, *cdc11-123*) cells was calculated, revealing many proteins significantly enriched in SIN "ON" conditions, with the top 13 proteins enriched at least 8-fold (Figure 1A; see also Table S1 available online). Validating our methodology, the two known Sid2 substrates Clp1 and Cdc11 were among our top hits [3, 4]. Most other top hits were plausible Sid2 targets with annotated roles in contractile ring and septum assembly, the spindle checkpoint, and/or mitosis. In addition to the identification of peptides, LC-MS/MS analysis also revealed phosphorylation sites on predicted Sid2 motifs (RXXS) in most of the putative SIN targets (Figures 1A and S1; Table S2).

We next tested whether the SIN "ON" enriched proteins could be directly phosphorylated by the Sid2 kinase in vitro. Seven of the top candidate Sid2 substrates could be purified as soluble recombinant proteins, and all but one of these was phosphorylated by Sid2 kinase purified from yeast (Figure 1B). The exception was Pos1, which was likely copurified with its binding partner and Sid2 substrate Spa2 (K.L.G., unpublished data).

Nak1 and Sog2 Are Part of a Complex Whose SPB Localization Is Regulated by Sid2

We next sought to use the information acquired about Sid2 substrates to understand how the SIN inhibits the MOR pathway. Our previous studies indicated that the SIN inhibits the MOR by blocking the ability of Nak1 to phosphorylate and activate the Orb6 kinase [2]. Thus it was striking that we identified Nak1 and a predicted Nak1 binding partner (Sog2) (based on the known function of its homolog in budding yeast [7]) as top hits in our screen for Sid2 substrates. Further analysis showed that *sog2*, like *nak1*, is an essential gene and that germinating *sog2Δ* spores have a round cell morphology like other MOR pathway mutants (Figure 2A) [1, 8]. Nak1 and Sog2 are in a complex (Figure 2B) and show very similar localization patterns; they are localized to spindle pole bodies (SPBs) in early mitosis, then leave the SPBs in anaphase when the SIN gets activated and localize to the division site late in cytokinesis [9] (Figure 2C).

To test whether the SIN regulates Nak1 and Sog2 localizations, we observed their green fluorescent protein (GFP) fusions in a *sid2-250* mutant after incubation at the semipermissive temperature of 33°C (incubation at full restrictive temperature [36°C] impaired GFP fluorescence). Interestingly, both proteins persisted at the SPBs longer in anaphase in *sid2-250* mutants compared to wild-type cells, and the distance between the SPBs when either SPB contained either Nak1 or Sog2 was significantly longer in *sid2-250* mutants (Figure 2D). These results raise two questions: (1) What does Nak1 do at

[3]Present address: Department of Biology, Boston College, Chestnut Hill, MA 02467, USA
[4]Present address: Department of Cell Biology, Duke University, Durham, NC 27710, USA
*Correspondence: dannel.mccollum@umassmed.edu

A

Protein Pombe (Human homolog)	Fold enriched when SIN ON	% coverage	RXXS phosphorylation identified by MS
Sog2 (LRRC57)	59	65	S421, S449, S665
Nak1 (MST4)	27	52	S491, S501
Spa2 (?)	22	88	S167
Clp1 (CDC14)	21	66	S396, S467/S468
Rgf1 (ARHGEF8)	21	48	S35, S68, S342
Ase1 (PRC1)	17	64	S537
Pos1 (?)	14	80	
Ppk25 (MARK2)	11	61	S38, S404
Mph1 (MPS1)	11	64	S259, S277
Ipp1 (PPA1)	9	68	
Scw1 (?)	8	79	S191, S232, S325, S361
Cdc11 (Centriolin)	8	34	
Mug72 (?)	8	75	S379, S564

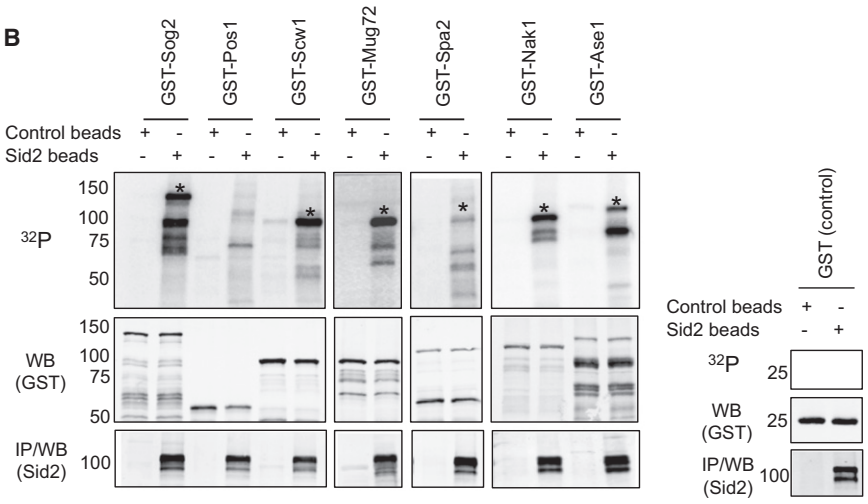

Figure 1. Identification and Validation of Sid2 Kinase Candidate Substrates

(A) Proteins most enriched in Rad24-TAP purifications when the SIN is activated and any RXXS phosphorylation sites identified by LC-MS/MS are shown. See also Tables S1 and S2.

(B) Sid2 phosphorylates candidate substrates in vitro. Sid2 kinase assays were performed using the indicated substrates purified from bacteria as glutathione S-transferase (GST) fusions. Substrate-GST fusions or GST alone was incubated with (Sid2 beads) or without (control beads) Sid2. Half of the kinase reaction was used to detect phosphorylation by autoradiography (^{32}P), and half was used in western blots to determine the levels of substrate (GST) and kinase (Sid2). Asterisks mark phosphorylation of the indicated full-length substrate.

See also Table S3.

SPBs in early mitosis? and (2) Why is it important for the SIN to remove Nak1 from the SPBs in anaphase?

The SIN is normally kept inactive in early mitosis to keep cells from initiating cytokinesis before chromosome segregation has initiated [4, 10]. We therefore reasoned that SPB-localized Nak1 might inhibit the SIN (which also localizes to SPBs) in early mitosis and that SIN-dependent removal of Nak1 from the SPBs in anaphase may enhance SIN activity. To test whether Nak1 inhibits SIN activity in early mitosis, we inactivated Nak1 using the *nak1-167* mutation in cells that were first arrested in metaphase by overexpression of the spindle checkpoint protein Mad2 [11]. Although some wild-type cells eventually began to septate despite the metaphase arrest, premature septation was significantly increased in *nak1-167* mutant cells (Figure 2E). The ectopic septation in *nak1-167* cells was blocked by the *sid2-250* mutation (Figure 2E), indicating that increased septation in *nak1-167* mutants can be attributed to premature SIN activation. Together, these experiments are consistent with a model where Nak1 helps prevent premature SIN activation in metaphase and SIN-dependent removal of Nak1 from the SPBs in anaphase promotes full SIN activation.

Phosphorylation of Nak1 by the Sid2 Kinase Is Required for SIN-Mediated Inhibition of Polarized Growth

Because the SIN interferes with the ability of Nak1 to activate its downstream kinase Orb6 [2], we sought to understand how Sid2 phosphorylation affects Nak1. Nak1 contains seven Sid2 consensus phosphorylation motifs (RXXS), two of which (S491 and S501) we identified as phosphorylated in vivo by LC-MS/MS (Figure S1). Three other Sid2 consensus sites (S479, S533, and S561) were identified in phosphoproteomic mass spectrometry studies [12–14]. These five sites, which cluster in an 82 aa region of the Nak1 C-terminal noncatalytic domain (Figure 3A), were mutated to either alanine (*nak1-5A*) or glutamic acid (*nak1-5E*) to create nonphosphorylatable or phosphomimetic mutants, respectively. Because we expected Sid2 phosphorylation to inhibit Nak1, it was not surprising that the Nak1-5A protein was functional, as judged by its ability to rescue the viability and shape defects of the *nak1Δ* deletion mutant, whereas the *nak1Δ* cells expressing Nak1-5E were viable but had defects in cell shape (Figure 3B). Unlike Nak1, recombinant Nak1-5A could not be phosphorylated by Sid2 in vitro (Figure 3C). We also examined the in vivo phosphorylation status of Nak1-5A compared to Nak1 using phosphospecific antibodies that recognize the RXXS motif. Western blot analysis of Nak1 immunoprecipitates from asynchronously growing wild-type cells and cells in which the SIN pathway was activated using the *cdc16-116* mutation showed that wild-type Nak1, but not Nak1-5A, was phosphorylated on RXXS sites specifically in cells with activated SIN signaling (Figure 3D).

SIN activation causes cessation of polarized cell growth by inhibiting the MOR pathway [2]. To determine whether Sid2 phosphorylation of Nak1 mediates its inhibition of the MOR, we tested whether Nak1-5A could bypass SIN-mediated inhibition of polarized cell growth. Wild-type Nak1, Nak1-5E, or Nak1-5A was expressed in *cdc16-116* mutant cells where the SIN is constitutively active (note that for all experiments where the SIN was activated, the *cdc3-124* mutation, which blocks septum formation [15], was also present to block nonspecific effects on cell growth triggered by ectopic septation upon SIN activation). Nak1-5A, but not wild-type Nak1 or Nak1-5E, bypassed SIN-mediated inhibition of cell elongation (Figures 4A and 4B). Cells expressing the nonphosphorylatable form of Nak1 were approximately twice as long as control cells (Figure 4A). Furthermore, unlike Nak1 or Nak1-5E, Nak1-5A expression in cells with active SIN was able to partially restore Orb6 kinase activity (Figure 4D), which is normally inhibited by

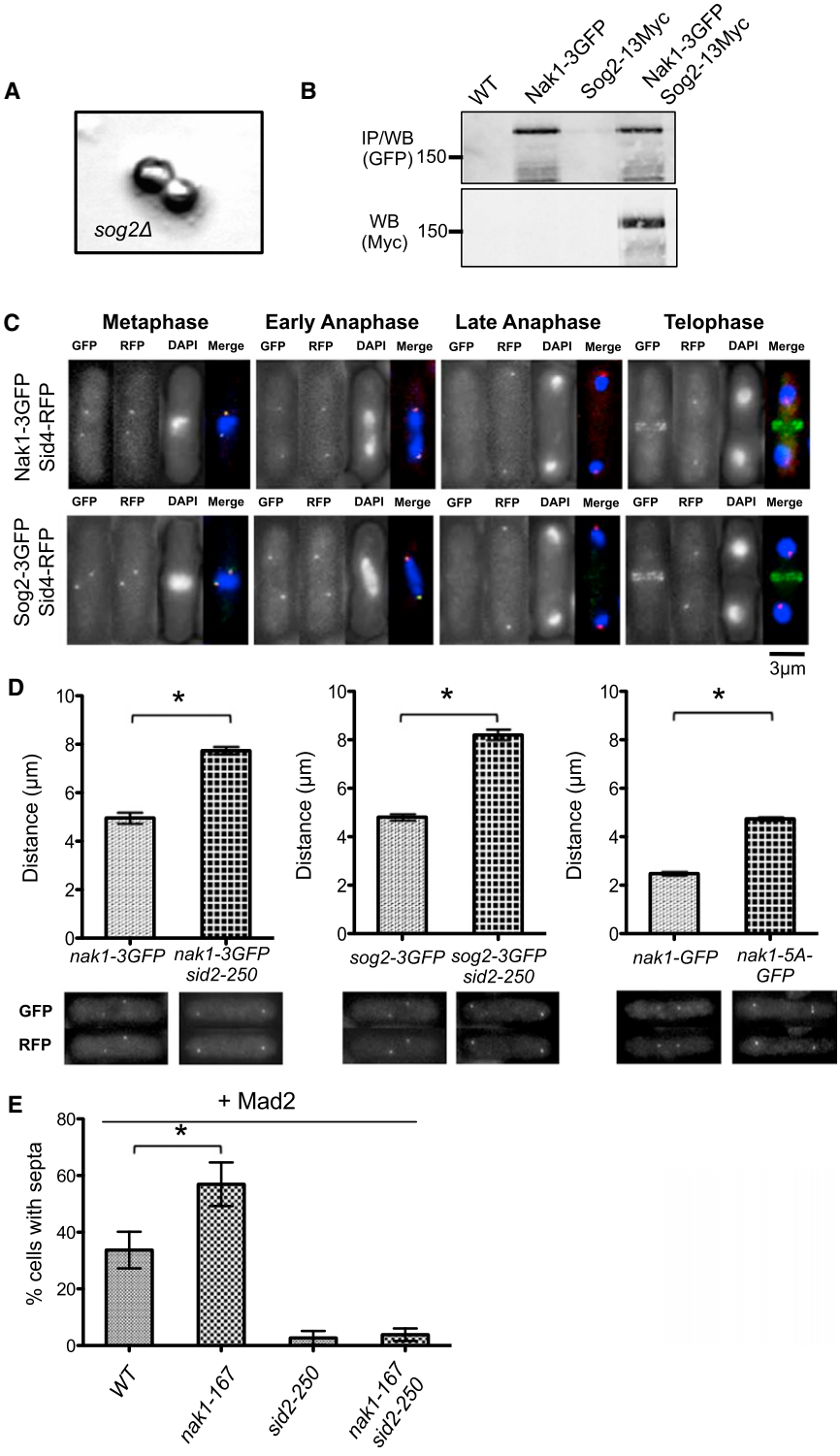

Figure 2. Mitotic Localization of Nak1 and Sog2 to the Spindle Pole Body Is Inhibited by the SIN

(A) Phenotype of *sog2*Δ cells. Diploid cells heterozygous for the *sog2Δ::ura4+* mutation were sporulated, and the tetrads were dissected and allowed to germinate. Haploid cells carrying *sog2Δ::ura4+* divided once or twice then stopped dividing and died with a round phenotype. An example of the *sog2Δ::ura4+* phenotype is shown.

(B) Sog2-13Myc coimmunoprecipitates with Nak1-3GFP. Extracts were prepared from cells of the indicated genotypes following overnight growth in yeast extract (YE) at 25°C. Nak1-3GFP was immunoprecipitated using an anti-GFP antibody, and the immunoprecipitates were analyzed by immunoblotting for GFP and Myc.

(C) Localization of Nak1-3GFP and Sog2-3GFP during mitosis. Sid4-RFP was used as a marker for SPBs. Cells were grown at 25°C in YE, fixed in methanol, and then imaged. Representative images are shown. GFP (green), RFP (red), and DAPI (blue) are shown in merged images.

(D) Sid2 promotes loss of Nak1 and Sog2 from anaphase SPBs. Upper panels depict the average distance between SPBs, showing localization of the indicated proteins. Lower panels show representative images of the cells examined above. Sid4-RFP marked SPBs. Cells were grown at 25°C in YE, followed by a shift to 33°C for 2 hr, fixed in methanol, and then imaged (left and middle panels). In the right panel, *nak1-GFP* and *nak1-5A-GFP* cells were grown overnight in YE at 25°C and then fixed and imaged. Error bars denote SD values obtained from the average distance measured from three separate experiments. Statistical analysis using paired t tests (brackets) indicates that the difference in SPB separation between the control and the experimental values is statistically significant (all $p < 0.007$; a minimum of 25 cells were scored for every experiment).

(E) Nak1 inhibits SIN-dependent ectopic metaphase septation. Cells of the indicated genotypes were grown at 25°C in the absence of thiamine for 16 hr to induce expression of the *mad2* gene under control of the full-strength thiamine-inducible promoter (*nmt1*) and then shifted to 36°C for 3 hr. Cells were then fixed in methanol and imaged, and a minimum of 100 cells were scored for the presence of ectopic septa. Error bars denote SD values obtained from percentages measured from three separate experiments. Statistical analysis using paired t tests (brackets) indicates that the difference in ectopic septation between the wild-type (WT) control and the *nak1-167* cells is statistically significant ($p = 0.0182$).

See also Tables S2 and S3.

the SIN [2]. These results indicate that the SIN inhibits Orb6 kinase activity and MOR-mediated polarized growth at least in part through phosphorylation of the Nak1 kinase.

We also examined the localization of Nak1-5A to determine whether SIN-dependent removal of Nak1 from the SPBs in anaphase depended on direct phosphorylation of Nak1. This analysis showed that Nak1-5A persisted on the SPB longer in anaphase than the wild-type protein, consistent with Sid2 phosphorylation displacing Nak1 from the SPB (Figure 2D).

Sid2 Phosphorylation of Nak1 Inhibits Its Interaction with the Scaffold Protein Mor2

We next tested further whether Sid2 phosphorylation of Nak1 could explain our previous observation that Sid2 inhibits the ability of Nak1 to activate Orb6 [2]. Both Nak1 and Orb6 kinase

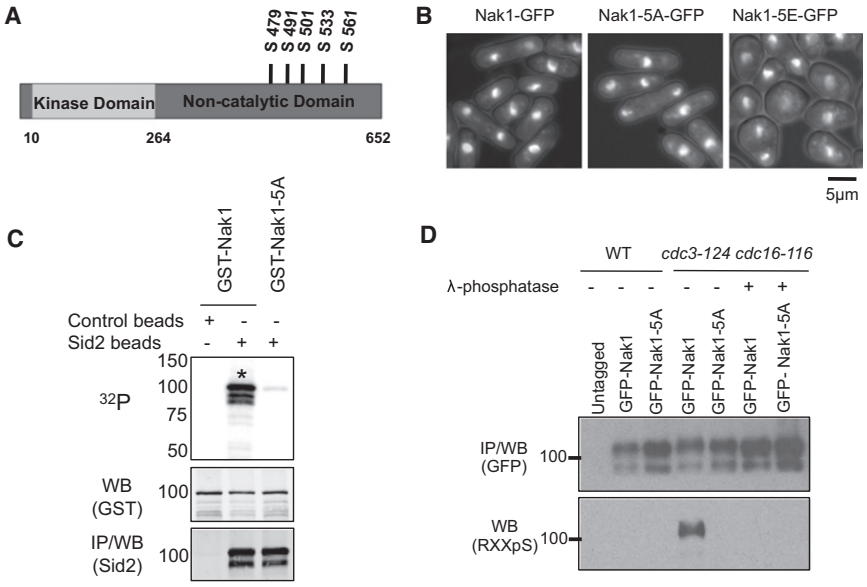

Figure 3. The Nak1-5A Mutant Cannot Be Phosphorylated by Sid2

(A) Diagram of Nak1. The diagram indicates the five Sid2 phosphorylation sites (RXXS) mutated to either alanine or glutamic acid to create Nak1-5A and Nak1-5E, respectively (see Table S2 and Figure S1).

(B) Rescue of the *nak1Δ* deletion mutant by the Nak1 phosphorylation site mutants. Plasmids for the Nak1 phosphorylation site mutants were integrated into *nak1Δ* heterozygous diploids, sporulated, and clones carrying the Nak1 phosphorylation site mutants and the *nak1Δ* deletion were isolated and grown at 36°C for 4 hr.

(C) Sid2 phosphorylates GST-Nak1 in vitro, but not GST-Nak1-5A. Kinase assays were performed on GST-Nak1 and GST-Nak1-5A as described in Figure 1B.

(D) GFP-Nak1, but not GFP-Nak1-5A, is phosphorylated in vivo. GFP-Nak1 and GFP-Nak1-5A were immunoprecipitated from extracts prepared from cells of the indicated genotypes expressing GFP-Nak1 or GFP-Nak1-5A from a thiamine-repressible promoter. Cells were grown in the absence of thiamine for 16 hr, and the SIN pathway was then activated by shifting *cdc3-124 cdc16-116* cells to 36°C for 3 hr. In addition, extracts were prepared from cells with activated SIN for treatment with λ-phosphatase following immunoprecipitation. Levels of Nak1 RXXpS phosphorylation and GFP-Nak1 were detected by western blotting with anti-GFP antibody and a phosphospecific antibody against the RXXS motif, respectively.

See also Table S3.

interact with a *Drosophila* furry-like protein, called Mor2 [16, 17]. Mor2 functions as a scaffold that allows Nak1 to activate Orb6 [18]. We hypothesized that Sid2 phosphorylation of Nak1 might block the Nak1-Mor2 interaction. If this were the case, then fusing Nak1 to Mor2 would bypass SIN inhibition of cell elongation. Therefore we constructed a Nak1-Mor2 fusion (Figure S2A) that was expressed on a plasmid using a thiamine-repressible promoter. This fusion rescued both *nak1-167* and *mor2-286* mutants (under repressed conditions), indicating that both proteins in the fusion were functional (data not shown). Next, its expression was induced in cells with activated SIN (*cdc3-124 cdc16-116*) (Figures 4D, S2B, and S2C). Expression of the fusion caused a huge increase in Orb6 kinase activity, even when the SIN was active (Figure 4D). Although SIN activation blocked cell elongation in cells with vector control, Nak1 or Mor2 alone, or coexpression of unfused Nak1 and Mor2, expression of the Nak1-Mor2 fusion caused a remarkable increase in cell length under both induced and repressed conditions (Figure S2B and S2C; data not shown). Cells expressing the Nak1-Mor2 fusion grew to more than three times the length of control cells after 9 hr of SIN activation, supporting the model that the SIN inhibits cell elongation by interfering with Nak1-Mor2 interaction.

To test more directly whether Sid2-mediated Nak1 phosphorylation disrupted the Nak1-Mor2 interaction, we utilized the phosphomimetic mutant Nak1-5E. Although Nak1-5E could not support polarized growth or bypass SIN-mediated inhibition of cell elongation (Figures 3B, 4A, and 4B), fusion of Nak1-5E to Mor2 (Nak1-5E-Mor2) did bypass SIN inhibition of polarized cell growth similar to the Nak1-Mor2 fusion (Figures S2B and S2C). These results are consistent with the idea that phosphorylation of Nak1 by Sid2 inhibits its ability to interact with Mor2. To directly test this hypothesis in another manner, we examined whether phosphomimetic mutations in Nak1 could disrupt the interaction between Nak1 and Mor2. Although we could not observe interaction between endogenous Nak1 and Mor2 by coimmunoprecipitation, as with a previous study, we could observe an interaction between the two proteins by two-hybrid analysis [17]. Whereas we observed an interaction between Nak1 (or Nak1-5A) and the Mor2 amino terminus, as previously reported, this interaction was greatly reduced in the Nak1-5E mutant (Figure 4C). Together, these results support the model that Sid2-mediated phosphorylation of Nak1 blocks its interaction with the Mor2 scaffold.

The SIN has multiple essential functions in late mitosis mediated by its effector kinase Sid2, but our understanding of these events has been hampered by our lack of knowledge of Sid2 substrates. Our proteomics screen for Sid2 targets yielded many promising candidate mediators of SIN signaling. Although some of the candidate substrates may be indirectly regulated by Sid2, we expect that many are direct targets. Two previously identified Sid2 substrates (Clp1 and Cdc11) [3, 4] were hits in our screen, validating our methodology, and many of the other candidates, including the MOR component Nak1, are phosphorylated on Sid2 consensus sites in vivo and can be phosphorylated by Sid2 in vitro. We expect that characterizing other hits from our screen will illuminate how the SIN regulates additional mitotic events. Particularly compelling candidates for follow-up studies include Rgf1, Scw1, and Mph1. Rgf1 is a guanine exchange factor for Rho1, a GTPase essential for cytokinesis that regulates actomyosin ring assembly and septum deposition [19, 20], and a multicopy suppressor of SIN mutants [21]. Mutants of the RNA-binding protein Scw1 suppress SIN mutants, suggesting that it could be inhibited by the SIN [22, 23]. And finally, Mph1 (Mps1 in budding yeast and humans) is a spindle checkpoint kinase, and the SIN inhibits the spindle checkpoint through an unknown mechanism [24, 25].

This work also clarifies another mechanism of crosstalk between the SIN and MOR pathways. We found first that

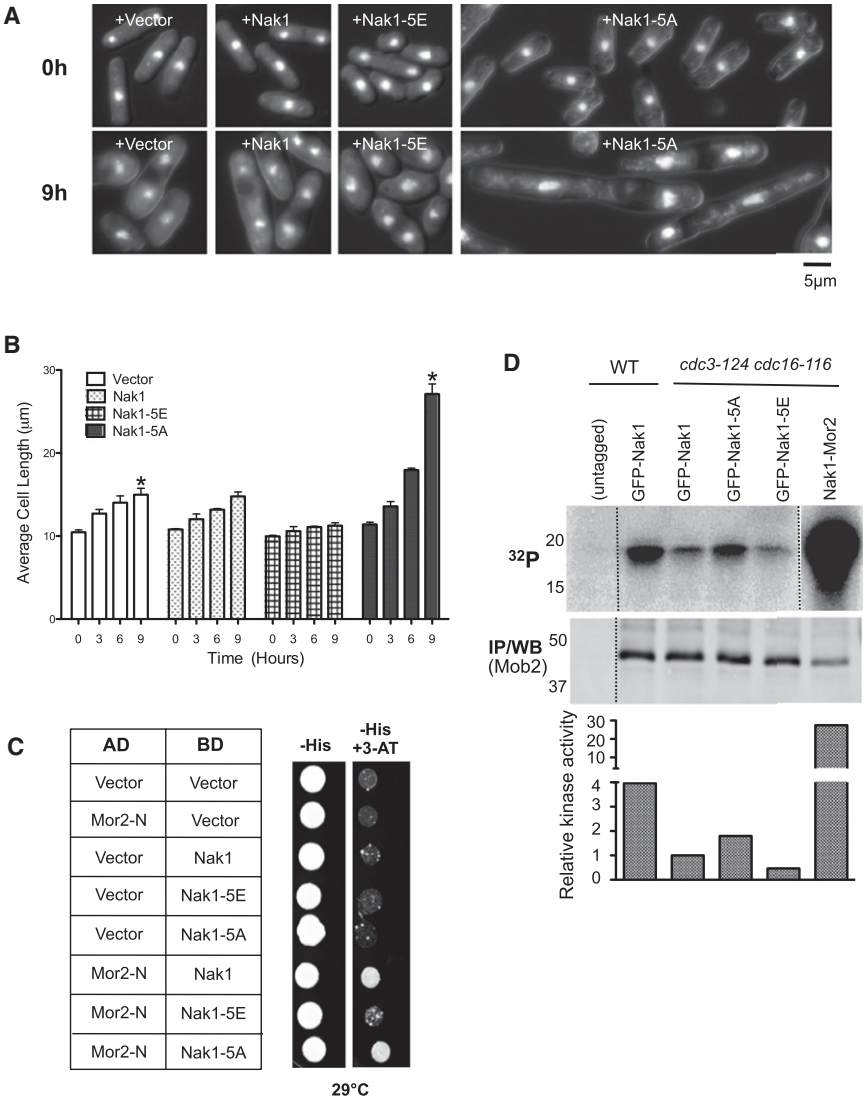

Figure 4. Elimination of Sid2 Phosphorylation of Nak1 Bypasses SIN-Mediated Inhibition of Polarized Growth

(A and B) Expression of Nak1-5A bypasses SIN inhibition of cell elongation. *cdc16-116 cdc3-124* cells with the indicated plasmids were grown at 25°C in the absence of thiamine for 16 hr (to induce expression of the indicated proteins) and then shifted to 36°C to activate the SIN. Samples were collected every 3 hr.

(A) Images representing the indicated cells at 0 hr and 9 hr time points are shown. Cells were stained with DAPI and visualized by a combination of fluorescence and differential interference contrast microscopy.

(B) Cell lengths of 50 cells were measured for each time point. Error bars denote SD values obtained from the average cell lengths measured from three separate experiments. Statistical analysis using paired t tests (as indicated by asterisks) shows that the difference in cell lengths between cells expressing the vector control and the Nak1-5A construct is statistically significant ($p < 0.001$).

(C) Nak1, but not the Nak1-5E mutant, shows a physical interaction with the Mor2-N terminus by yeast two-hybrid analysis. *Saccharomyces cerevisiae* Y190 cells expressing the Mor2-N terminus (aa 1–1095) fused to the Gal4 activation domain (AD) along with either Nak1, Nak1-5E, or Nak1-5A fused to the Gal4 DNA-binding domain (BD) were spotted on SD agar plates lacking histidine ± 3-aminotriazole (3-AT). Cell growth was observed after culturing at 29°C for 3 days.

(D) Nak1-5A and the Nak1-Mor2 fusion cause Orb6 kinase activation, even when the SIN is active. All strains except the control (untagged) express the indicated Nak1 proteins and Mob2-13Myc, which was used to pull down its associated kinase Orb6. The temperature-sensitive *cdc3-124 cdc16-116* background was used to activate the SIN. Cells were grown at 25°C and then shifted to 36°C for 3 hr. Orb6 immunocomplex kinase assays were performed by first pulling down the Orb6 regulatory subunit Mob2. Myelin basic protein (MBP) was used as substrate. Half of the kinase reaction was used to detect phosphorylation by autoradiography (^{32}P; upper panel), and half was used in western blots to determine the levels of Mob2 (lower panel). Orb6 kinase activity for each strain was normalized to Mob2 levels and relative to the activity of GFP-Nak1-expressing *cdc3-124 cdc16-116* cells.

See also Figure S2 and Table S3.

Nak1, perhaps through its SPB localization in early mitosis, helps prevent premature activation of the SIN. Further investigation will be required to determine how Nak1 inhibits the SIN. Second, at anaphase onset, Sid2 phosphorylation of Nak1 removes Nak1 from the SPBs, promotes SIN activation, and inhibits MOR signaling by blocking interaction of Nak1 with the Mor2 scaffold protein. The SIN may inhibit the MOR through additional mechanisms. An obvious possibility is via phosphorylation of the Nak1 binding partner Sog2, given that we identified it as another probable Sid2 substrate. Because SIN and MOR pathway components are conserved in mammalian cells, we expect that these studies will be informative for understanding coordination of the activities of the counterpart Hippo and Ndr1/2 pathways.

Supplemental Information

Supplemental Information includes three tables, two figures, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.01.014.

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