

Growth-Dependent Activation of Protein Kinases Suggests a Mechanism for Measuring Cell Growth

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ABSTRACT In all cells, progression through the cell cycle occurs only when sufficient growth has occurred. Thus, cells must translate growth into a proportional signal that can be used to measure and transmit information about growth. Previous genetic studies in budding yeast suggested that related kinases called *Gin4* and *Hsl1* could function in mechanisms that measure bud growth; however, interpretation of the data was complicated by the use of gene deletions that cause complex terminal phenotypes. Here, we used the first conditional alleles of *Gin4* and *Hsl1* to more precisely define their functions. We show that excessive bud growth during a prolonged mitotic delay is an immediate consequence of inactivating *Gin4* and *Hsl1*. Thus, acute loss of *Gin4* and *Hsl1* causes cells to behave as though they cannot detect that bud growth has occurred. We further show that *Gin4* and *Hsl1* undergo gradual hyperphosphorylation during bud growth that is dependent upon growth and correlated with the extent of growth. Moreover, gradual hyperphosphorylation of *Gin4* during bud growth requires binding to anionic phospholipids that are delivered to the growing bud. While alternative models are possible, the data suggest that signaling lipids delivered to the growing bud generate a growth-dependent signal that could be used to measure bud growth.

KEYWORDS cell cycle; cell growth; cell size; *Gin4*; *Hsl1*

KEY cell cycle transitions occur only when sufficient growth has occurred. To enforce this dependency relationship, cells must convert growth into a proportional signal that triggers cell cycle progression when it reaches a threshold. However, the molecular mechanisms by which cells generate proportional signals to measure and limit cell growth have remained deeply mysterious.

In budding yeast, growth occurs in three distinct intervals that are characterized by different rates and patterns of growth (McCusker *et al.* 2007; Goranov *et al.* 2009; Ferrezuelo *et al.* 2012; Leitao and Kellogg 2017). The first interval occurs during G1 phase and is characterized by slow growth over the entire cell surface. The second interval is initiated at the end of G1 phase when a new daughter cell emerges and undergoes polar growth. The third interval is

initiated at entry into mitosis and is marked by a switch from polar bud growth to growth that occurs more widely over the bud surface. Bud growth then continues throughout mitosis. The distinct size and shape of a yeast cell is ultimately defined by the extent of growth during each of these intervals.

Several observations suggest that mechanisms that control the duration and extent of bud growth play a major role in the control of cell size (Leitao and Kellogg 2017). First, little growth occurs during G1 phase. For example, cells growing in rich nutrients increase in size by only 20% during G1 phase. Rather, most growth occurs during bud growth in mitosis, and the rate of growth in mitosis is around threefold faster than growth during the other intervals. Therefore, failure to tightly control the duration and extent of bud growth, particularly during mitosis, would have large consequences for cell size.

Additional evidence that bud growth is tightly controlled comes from analysis of the effects of nutrient availability on cell growth and size. Growth of cells in a poor nutrient source causes a large reduction in average cell size (Johnston *et al.* 1977, 1979). However, growth in poor nutrients has little effect on cell size at completion of G1 phase (Bean *et al.* 2006; Leitao and Kellogg 2017). Furthermore, mutant cells

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that lack all known regulators of cell size in G1 phase show robust nutrient modulation of cell size (Jorgensen and Tyers 2004a). In contrast, poor nutrients cause a large decrease in the extent of growth in mitosis, which causes daughter cells to complete cytokinesis at a substantially reduced size (Hartwell and Unger 1977; Leitao and Kellogg 2017). Furthermore, the duration of growth in mitosis is increased in poor nutrients, which suggests that cells compensate for slow bud growth by increasing the duration of growth (Leitao and Kellogg 2017). These observations point to the existence of mechanisms that measure and modulate both the duration and extent of bud growth to ensure that mitotic progression is linked to cell growth.

A model in which the extent of bud growth is tightly controlled requires a molecular mechanism for measuring growth. Here, we searched for proteins that could play a role in measuring bud growth during mitosis. A candidate-based approach identified three related kinases: *Gin4*, *Hsl1*, and *Kcc4*, which we refer to as *Gin4*-related kinases. Similar kinases are found in all eukaryotes. Genetic analysis reaching back over 30 years has suggested that *Gin4*-related kinases could play roles in the control of cell growth and size (Young and Fantes 1987; Ma *et al.* 1996; Altman and Kellogg 1997; Okuzaki *et al.* 1997; Barral *et al.* 1999). For example, loss of *Gin4*-related kinases in both budding yeast and fission yeast causes excessive growth during a prolonged delay in mitotic progression (Young and Fantes 1987; Ma *et al.* 1996; Okuzaki *et al.* 1997; Barral *et al.* 1999). A model that could explain this phenotype is that *Gin4*-related kinases play roles in measuring bud growth. In this case, cells that lack *Gin4*-related kinases would behave as though they cannot detect that bud growth has occurred. However, an alternative model is that aberrant growth caused by loss of *Gin4*-related kinases is an indirect consequence of mitotic delays. For example, there is evidence that loss of *Gin4*-related kinases causes defects in positioning the mitotic spindle, which could cause chromosome segregation defects and associated mitotic delays that lead to abnormally prolonged growth (Fraschini *et al.* 2006; Grava *et al.* 2006; Gihana *et al.* 2018). In this case, growth defects would be a secondary consequence of mitotic spindle defects.

A major problem with distinguishing models is that previous genetic analysis of *Gin4*-related kinases utilized gene deletions that cause severe terminal phenotypes. This has made it difficult to discern which aspects of the phenotype are an immediate and direct consequence of loss-of-function, vs. aspects of the phenotype that are the result of secondary defects accumulated over multiple generations. An additional major limitation of previous studies is that they provided limited information on the physiological signals that control *Gin4*-related kinases. If *Gin4*-related kinases are involved in measuring growth, then their activity must in some way be mechanistically linked to growth. Previous studies found that *Gin4* is gradually hyperphosphorylated and activated during mitosis (Altman and Kellogg 1997). Since bud growth occurs gradually throughout mitosis, this observation

suggests that *Gin4* activity could be a readout of growth; however, the data are correlative and activation of *Gin4* or *Hsl1* has not been analyzed in the context of cell growth.

Here, we created the first conditional alleles of *Gin4*-related kinases and used them to show that defects in the control of bud growth are an immediate and direct consequence of inactivating *Gin4*-related kinases. We further show that *Gin4*-related kinases influence the duration and extent of growth during metaphase, and that they do so partly via regulation of *Cdk1* inhibitory phosphorylation. Finally, we show that *Gin4*-related kinases undergo gradual phosphorylation during bud growth that is dependent upon bud growth and correlated with the extent of bud growth. While alternative models remain possible, the data suggest a model in which *Gin4*-related kinases generate and/or relay growth-dependent signals that could be used to measure the extent of bud growth.

Materials and Methods

Yeast strain construction, media, and reagents

All strains were in the W303 background (*leu2-3,112 ura3-1 can1-100 ade2-1 his3-11,15 trp1-1 GAL+ ssd1-d2*). The additional genetic features of strains are listed in Table 1. Cells were grown in YP medium (1% yeast extract, 2% peptone, and 40 mg/liter adenine) supplemented with 2% dextrose (YPD), or 2% glycerol and 2% ethanol (YPG/E). For live cell imaging, cells were grown in complete synthetic medium (CSM) supplemented with 2% dextrose and 40 mg/ml adenine.

Gene deletions and C-terminal epitope tagging were performed by standard PCR amplification and homologous recombination (Longtine *et al.* 1998; Janke *et al.* 2004; Lee *et al.* 2013). *gin4-ΔKA1-LactC2* (Lactadherin C2) constructs integrated at the *GIN4* locus were created by gene splicing with overlap extension (Horton *et al.* 1990). Briefly, LactC2 fragments were PCR amplified from plasmids pKT2100 or pKT1995 (Takeda *et al.* 2014) with 40 bp of flanking sequence at the 5' end that was homologous to the *GIN4* ORF just upstream of the kinase-associated 1 (KA1) domain (amino acids 1007–1142) using oligos *Gin4-39* and *Gin4-40* (Table 2). The 3xHA::His3MX6 fragment was amplified from pFA6a-3HA-His3MX6 (Longtine *et al.* 1998) with 5' homology to the terminal sequence of LactC2 and 3' homology to the DNA sequence just downstream of the *Gin4* ORF using primers *Gin4-38* and *Gin4-41*. The two fragments were then gel purified, annealed to each other, and elongated for 15 PCR cycles in the absence of primers, followed by PCR amplification using primers *Gin4-38* and *Gin4-39*. The resulting fragments were then transformed into wild-type cells and correct integrants were identified by western blotting with anti-HA antibody. To create the GFP-tagged versions of the *gin4-ΔKA1-LactC2* constructs, GFP-His3MX6 was amplified from pFA6a-GFP-His3MX6 (Longtine *et al.* 1998) and spliced to LactC2 as described above.

Table 1 Strains used in this study

Strain	Mating type	Genotype	Source
DK186	a	<i>bar1</i> Δ	Altman and Kellogg (1997)
DK3418	a	<i>bar1</i> Δ <i>HSL1-6XHA::His3MX6</i>	This study
SH24	a	<i>bar1</i> Δ <i>swe1</i> Δ:: <i>URA3</i>	Harvey and Kellogg (2003)
DK1600	a	<i>bar1</i> Δ <i>swe1</i> Δ:: <i>His3MX6 sec6-4::KanMX6</i>	Anastasia et al. (2012)
DK3510	a	<i>bar1</i> Δ <i>his3::His3MX6+TIR1 leu2::LEU2+TIR1 SPC42-yomRuby2::KanMX6</i>	This study
DK3307	a	<i>bar1</i> Δ <i>his3::His3MX6+TIR1 leu2::LEU2+TIR1 SPC42-GFP::HphNTI gin4-AID::KanMX6</i>	This study
DK3308	a	<i>bar1</i> Δ <i>his3::His3MX6+TIR1 leu2::LEU2+TIR1 SPC42-GFP::HphNTI hsl1-AID::TRP</i>	This study
DK3327	a	<i>bar1</i> Δ <i>his3::His3MX6+TIR1 leu2::LEU2+TIR1 SPC42-GFP::HphNTI gin4-AID::KanMX6 hsl1-AID::TRP1</i>	This study
DK3330	a	<i>bar1</i> Δ <i>his3::His3MX6+TIR1 leu2::LEU2+TIR1 SPC42-GFP::HphNTI gin4-AID::KanMX6 hsl1-AID::TRP1 swe1</i> Δ:: <i>URA3</i>	This study
DK3350	a	<i>bar1</i> Δ <i>GIN4-GFP::His3MX6 SPC42-yomRuby2::KanMX</i>	This study
DK3790	a	<i>bar1</i> Δ <i>gin4-ΔKA1-GFP::His3MX6 SPC42-yomRuby2::KanMX</i>	This study
DK3351	a	<i>bar1</i> Δ <i>gin4-ΔKA1-LactC2-GFP::His3MX6 SPC42-yomRuby2::KanMX</i>	This study
DK3823	a	<i>bar1</i> Δ <i>gin4-ΔKA1-LactC2^{AAA}-GFP::His3MX6 SPC42-yomRuby2::KanMX</i>	This study
DK888	a	<i>bar1</i> Δ <i>gin4</i> Δ:: <i>LEU2</i>	Mortensen et al. (2002)
HT159	a	<i>bar1</i> Δ <i>hsl1</i> Δ:: <i>His3MX6</i>	This study
DK3784	a	<i>bar1</i> Δ <i>gin4</i> Δ:: <i>LEU2 hsl1</i> Δ:: <i>His3MX6</i>	This study
DK2158	a	<i>bar1</i> Δ <i>gin4</i> Δ:: <i>LEU2 hsl1</i> Δ:: <i>HphNTI swe1</i> Δ:: <i>URA3</i>	This study
DK373	a	<i>bar1</i> Δ <i>GIN4-3xHA::TRP1</i>	This study
DK2822	a	<i>bar1</i> Δ <i>gin4-ΔKA1-3xHA::His3MX6</i>	This study
DK3286	a	<i>bar1</i> Δ <i>gin4-ΔKA1-LactC2-3xHA::His3MX6</i>	This study
DK3295	a	<i>bar1</i> Δ <i>gin4-ΔKA1-LactC2^{AAA}-3xHA::His3MX6</i>	This study
DK3621	a	<i>bar1</i> Δ <i>GIN4-3xHA::TRP1 hsl1</i> Δ:: <i>HphNTI</i>	This study
DK3624	a	<i>bar1</i> Δ <i>gin4-ΔKA1-3xHA::His3MX6 hsl1</i> Δ:: <i>HphNTI</i>	This study
DK3627	a	<i>bar1</i> Δ <i>gin4-ΔKA1-LactC2-3xHA::His3MX6 hsl1</i> Δ:: <i>HphNTI</i>	This study
DK3630	a	<i>bar1</i> Δ <i>gin4-ΔKA1-LactC2^{AAA}-3xHA::His3MX6 hsl1</i> Δ:: <i>HphNTI</i>	This study
DK1440	a	<i>bar1</i> Δ <i>sec6-4::KanMX6</i>	Anastasia et al. (2012)

To generate strains with an auxin-inducible degron (AID) tag on *GIN4* and/or *HSL1*, the *HSL1* gene was tagged at the C-terminus with an AID tag marked with KanMX6 in a parent strain that has two copies of the *TIR1* gene. The KanMX6 marker was then replaced by a *TRP1* marker. Next, a second AID tag marked with KanMX6 was incorporated at the *GIN4* locus. The *SPC42* gene in all four AID-tagged strains was fused to GFP at the C-terminus using standard PCR and homologous recombination. The parent strain that contains *2xTIR1* was used as the control strain and was modified to express endogenous *SPC42* fused with yeast-optimized mRuby2 (*yomRuby2*). Auxin was dissolved in 100% ethanol to make a 50 mM stock solution.

Cell cycle time courses and western blotting

Cell cycle time courses were carried out as previously described (Harvey et al. 2011). Briefly, cells were grown to log phase at room temperature overnight in YPD or YPG/E to an OD (OD₆₀₀) of 0.5–0.7. Cultures were adjusted to the same OD and were then arrested in G1 phase by incubation in the presence of 0.5 μg/ml α factor at room temperature for 3 hr. Cells were released from the arrest by washing three times with fresh YPD or YPG/E. All time courses were carried out at

25° unless otherwise noted, and α factor was added back at 70 min to prevent initiation of a second cell cycle. For experiments involving auxin-mediated destruction of proteins, a single culture synchronized in G1 phase was split into two culture flasks and 0.5 mM auxin was added to one flask 20 min after release from the G1-phase arrest. An equivalent volume of ethanol was added to the control flask.

For western blotting, 1.6-ml sample volumes were collected in screw-cap tubes and centrifuged at 13,000 rpm for 30 sec. After discarding the supernatant, 200 μl acid-washed glass beads were added to the tubes and the samples were frozen in liquid nitrogen. Cells were lysed in 140 μl sample buffer (65 mM Tris HCl pH 6.8, 3% SDS, 10% glycerol, 50 mM sodium fluoride, 100 mM β-glycerophosphate, 5% β-mercaptoethanol, and bromophenol blue) supplemented with 2 mM PMSF immediately before use. For experiments involving immunoblotting for *Gin4-AID/Hsl1-AID* proteins, the sample buffer also included a protease inhibitor cocktail (1mg/ml leupeptin, 1 mg/ml pepstatin, 1 mg/ml chymostatin, used at 1/500 dilution). Sample buffer was added to cells immediately after they were removed from liquid nitrogen and the cells were then lysed in a mini-beadbeater 16 (Bio-Spec) at top speed for 2 min. After brief centrifugation, the

Table 2 Primers used in this study

Primer name	Primer sequence (5' to 3')
Gin4-39	TGTGCAAAAAATTAGGGAAAAAAATGCTGGCTCGCAGGCATGCACTGAACCCCTAGGCCT
Gin4-40	ACAGCCCAGCAGCTCCACT
Gin4-41	GCACAACCGTATCACCTGCGAGTGGAGCTGCTGGGCTGTCGGATCCCCGGGTTAATTAA
Gin4-38	AACGAAGGAGACAAAACATGATTGCATTACATTAGCACTAGAATTCGAGCTCGTTTAAAC

samples were placed in a boiling water bath for 5 min and were then centrifuged again at 13,000 rpm for 3 min before loading onto SDS-PAGE gels. SDS-PAGE was carried out as previously described (Harvey *et al.* 2011). 10% polyacrylamide gels with 0.13% bis-acrylamide were used for analysis of *Gin4*, *Clb2*, and *Nap1* (loading control); 9% polyacrylamide gels with 0.14% bis-acrylamide were used for *Hsl1* and *Swe1* blots; and 7.5% polyacrylamide gels with 0.17% bis-acrylamide were used for *Hsl1*-AID blots. Proteins were immobilized onto nitrocellulose membranes using wet transfers for 1 hr 45 min. Blots were probed with the primary antibody at 1–2 $\mu\text{g/ml}$ at room temperature overnight in 5% milk in PBST (1 \times phosphate-buffered saline, 250 mM NaCl, and 0.1% Tween-20) containing 5% nonfat dry milk and 0.02% azide. Primary antibodies used to detect *Clb2*, *Gin4*, *Nap1*, and *Swe1* were rabbit polyclonal antibodies generated as described previously (Kellogg and Murray 1995; Sreenivasan and Kellogg 1999; Mortensen *et al.* 2002). *Hsl1*-AID was detected by a mouse monoclonal antibody (#MA515253; Thermo Fisher Scientific; 0.2 $\mu\text{g/ml}$) that recognizes a V5-epitope present on the AID tag. Primary antibodies were detected by an HRP-conjugated donkey anti-rabbit secondary antibody (#NA934V; GE Healthcare) or HRP-conjugated donkey anti-mouse secondary antibody (#NXA931; GE Healthcare) incubated in PBST containing 5% nonfat dry milk for 1 hr at room temperature. Blots were rinsed in PBS before detection via chemiluminescence using ECL reagents (#K-12045-D50; Advansta) with a Bio-Rad (Hercules, CA) ChemiDoc imaging system.

Quantification of *Gin4* protein in Figure S1C was performed using Image Lab (Bio-Rad). The ratio of total *Gin4* signal to a corresponding loading control band (anti-*Nap1*) was calculated for every time point. The 20-min time point in each condition (ethanol/auxin) was given a value of 1 and the relative abundance of the normalized *Gin4* protein was plotted for each time point. Two biological replicates were analyzed and averaged to obtain the relevant information.

Coulter counter analysis

Cell cultures were grown in 10 ml YPD medium to an OD_{600} between 0.4 and 0.6. Cells were fixed by addition of a 1/10 volume of 37% formaldehyde to the culture medium followed by incubation at room temperature for 1 hr. Cells were then pelleted and resuspended in 0.5 ml PBS containing 0.02% sodium azide and 0.1% Tween-20, and analyzed on the same day. Cell size was measured using a Coulter counter (Channelizer Z2; Beckman, Fullerton, CA) as previously

described (Jorgensen *et al.* 2002; Artiles *et al.* 2009). Briefly, 40 μl of fixed cells were diluted in 10 ml diluent (Isoton II; Beckman) and sonicated for five pulses of ~ 0.5 sec each at low power. The Coulter counter data shown in the figures represent the average of three biological replicates, which are each the average of three technical replicates. For Figure 7B, the strains were grown to log phase overnight at room temperature, diluted to OD_{600} 0.1 in 5 ml fresh YPD, and then incubated for 4–5 hr at 30° to observe temperature-dependent phenotypes of the mutants.

Microscopy

For DIC imaging, cells were grown to log phase in YPD and fixed in 3.7% formaldehyde for 30 min, and then resuspended in PBS with 0.1% Tween-20 and 0.02% sodium azide. Images were obtained using a Zeiss-Axioskop 2 Plus microscope fitted with a 63x Plan-Apochromat 1.4 n.a. objective and an Axio-Cam HR camera ([Carl Zeiss], Thornwood, NY). Images were acquired using AxioVision software and processed on Fiji (Schindelin *et al.* 2012).

For live-cell time-lapse imaging, the control strain (DK3510) and AID-tagged strains (DK3307, DK3308, DK3327, or DK3330) were grown in CSM overnight to an OD_{600} of 0.1–0.2 and then arrested in G1 phase with α factor. The control and the AID-tagged strains were mixed in a 1.6-ml tube and then washed 3 \times in CSM prewarmed to 30° to release the cells from the G1-phase arrest. After resuspending the cells in CSM, ~ 200 μl of cells were immobilized onto a concanavalin A-treated chambered #1.5 Coverglass system (Nunc catalog number 155409; Labtek-II) for 5 min. Unbound cells were washed away by repeated washes with CSM. The cells were then incubated in 500 μl CSM at 27° for the duration of the imaging. Auxin was added to the cells to a final concentration of 0.5 mM 20 min after the first wash used to release the cells from the α factor arrest.

Scanning confocal images were acquired on a Zeiss 880 confocal microscope running ZEN Black software using a 63 \times /1.4 n.a. Plan Apo objective. The microscope was equipped with a heat-block stage insert with a closed lid and exterior chamber for temperature control. The microscope was allowed to equilibrate at the set temperature of 27° for ≥ 1 hr to ensure temperature stability prior to imaging. Definite Focus was used to keep the sample in focus during the duration of the experiment. The 1 \times 2 tiled z-stack images were acquired every 3 min. Zoom and frame size were set to 0.8 \times magnification to achieve a consistent pixel area of 1024 \times 1024 pixels in XY, and the pixel dwell time was 0.5 μs . Optical sections were taken for a total of 14 z-planes

Table 3 Plasmids used in this study

Plasmid name	Details	Source
pKT1995	pRS306-GFP-LactC2 ^{AAA} -URA3	Takeda <i>et al.</i> (2014)
pKT2100	pRS306-GFP-LactC2-URA3	Takeda <i>et al.</i> (2014)
pAID1	Auxin-inducible degron-tagging genes at the C-terminus; kanamycin resistance	Nishimura <i>et al.</i> (2009)
pTIR2	Plasmid containing the TIR1 gene under the control of the <i>GPD1</i> promoter. After PmeI digestion, recombines at the <i>HIS3</i> locus	Nishimura <i>et al.</i> (2009)
pTIR4	Plasmid containing the TIR1 gene under the control of the <i>GPD1</i> promoter. After PmeI digestion, recombines at the <i>LEU2</i> locus	Nishimura <i>et al.</i> (2009)
pFA6a-yomRuby2::KanMx	Yeast-optimized mRuby2	Lee <i>et al.</i> (2013)

every 0.37 μm with frame averaging set to 2 to reduce noise. The 488-nm laser power was set to 0.2% and the 561-nm laser power was set to 1% to minimize cell damage. The gains for GFP, red fluorescent protein (RFP), and brightfield were set to 550, 750, and 325, respectively. The same settings were used for each experiment. GFP signal was acquired on a GaAsP (Gallium arsenide phosphide) detector and collected between 498 and 548 nm. Brightfield images were collected simultaneously. RFP signal was acquired on a GaAsP detector and collected between 577 and 629 nm.

To visualize the localization of *Gin4* constructs fused to GFP, cells were grown in CSM overnight, fixed in 3.7% formaldehyde for 15 min, and then resuspended in 500 μl 1 \times PBST. Images were acquired on a spinning disk confocal microscope with a Solamere system running MicroManager (Schindelin *et al.* 2012). The microscope was based on a Nikon (Garden City, NY) TE2000 stand and Coherent OBIS lasers. We used a 100 \times /1.4 n.a. Plan Apo objective for Figure 7A and a 63 \times /1.4 Plan Apo objective for data collection in Figure S6. Pixel sizes were 0.11 μm in X,Y and z-stack spacing was set to 0.5 μm with a total of 17 z-slices. GFP was excited at 488 nm and collected through a 525/50-nm band pass filter (Chroma) onto a Hamamatsu ImageEMX2 EMCCD (Electron Multiplying Charge Coupled Device) camera. Gain levels were set to 200 to maximize signal without hitting saturation. GFP and brightfield images were collected sequentially.

Image analysis

All images were analyzed on Fiji (Schindelin *et al.* 2012). For visualization of GFP-tagged *Gin4* constructs, a sum projection of z-slices was used. Movies for the time-lapse were processed as previously described (Leitao and Kellogg 2017). The brightfield images were processed using the “Find Focused Slices” plugin available on Fiji to create a stack with the focused slice \pm one slice for each time point. A z-projection with sum of slices was performed on this stack and then bud volumes were determined using the plugin BudJ (Ferrezuelo *et al.* 2012).

The timings of cell cycle events were determined as previously reported (Leitao and Kellogg 2017). Briefly, bud initiation was manually determined by the appearance of a protrusion on the surface of the mother cell. The initiation of metaphase was marked by the appearance of separation of

spindle poles to 2–3 μm apart. Initiation of anaphase was marked by further separation of the spindle poles and segregation of one of the poles into the daughter cell. We defined the completion of anaphase as the point at which the spindle poles reached their maximal distance apart.

For a quantitative comparison of the localization of GFP-tagged *Gin4* constructs in Figure S6, a z-projection with sum of slices was performed on the images and an elliptical region of interest was drawn around the bud neck. The maximum pixel intensity was determined for each cell after subtracting the background pixel intensity.

Statistical analysis

Data acquired from the image analysis were plotted as scatter dot plots using GraphPad Prism. The scatter dot plots show the data distribution along with the mean and SD for each strain. For all scatter dot plots, the unpaired Student's *t*-test was calculated using the Mann–Whitney *U* test for non-Gaussian distributions and the two-tailed *P*-values were mentioned.

Data availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Figure S1 relates to Figure 1 and provides a characterization of the *gin4-AID* and *hsl1-AID* alleles. Figure S2 relates to Figure 2 and shows further evidence for the role of *Gin4* and *Hsl1* in the control of bud growth. Figure S3 relates to Figure 3 and shows a time-dependent increase in the *gin4-AID hsl1-AID* phenotype. Figure S4 relates to Figure 4 and shows the rapid response of *Swe1* dephosphorylation upon the inactivation of *Gin4* and *Hsl1*. Figure S5 relates to Figure 6 and shows the effect of growth inactivation at different times during bud growth on *Gin4* phosphorylation. Figure S6 relates to Figure 7 and shows peak pixel intensity of the various *Gin4*-GFP constructs localized to the bud neck. Supplemental Videos 1 and 2 show time-lapse videos of 2x*TIR1* and *gin4-AID hsl1-AID* cells as they progress through the cell cycle. The tables show yeast strains (Table 1), primers (Table 2), and plasmids (Table 3) used in this study. Supplemental material available at figshare: <https://doi.org/10.25386/genetics.12014943>.

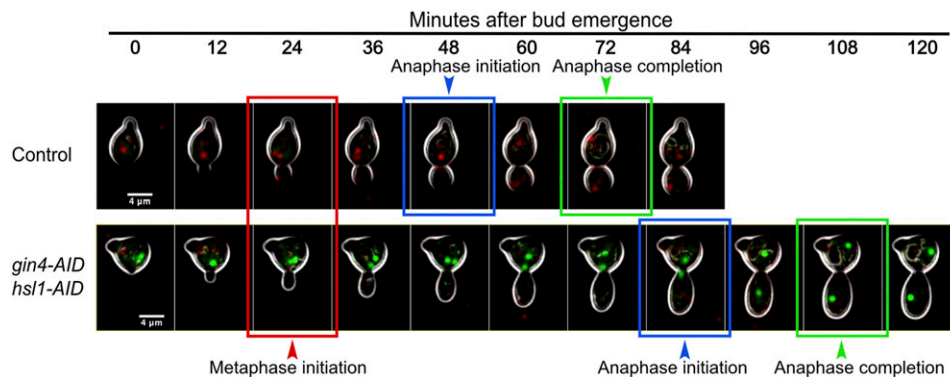


Figure 1 *Gin4* and *Hsl1* are required for normal control of cell growth and size in mitosis. Control cells and *gin4-AID hsl1-AID* cells were differentially marked with fluorescently tagged mitotic spindle poles. Thus, control cells express *SPC42-mRuby2*, while the *gin4-AID hsl1-AID* cells express *SPC42-GFP*. Both strains include two copies of the *TIR1* gene. Cells growing in CSM were arrested with α factor and then mixed together before releasing from the arrest. Auxin was added to 0.5 mM at 20 min after release from arrest, which corresponds to \sim 30 min before bud emergence. Cells were then

imaged at 3-min intervals by confocal microscopy at 27°. Bud emergence was used to set the zero time point. Key mitotic transitions are highlighted for each strain. AID, auxin-inducible degron; CSM, complete synthetic medium.

Results

Gin4-related kinases are required for normal control of bud growth during mitosis

Gin4 and *Hsl1* are the most important *Gin4*-related kinases in budding yeast. Loss of either kinase alone causes defects in the control of bud growth, whereas loss of both causes severe defects (Barral *et al.* 1999; Longtine *et al.* 2000). Loss of *Kcc4*, a paralog of *Gin4*, has little effect. We therefore focused on *Gin4* and *Hsl1*. To avoid the complications associated with long-term inactivation of the *Gin4*-related kinases, we created AID-tagged versions of *Gin4* and *Hsl1*, which allowed us to define the immediate effects of inactivation (Nishimura *et al.* 2009). A strain carrying AID-tagged versions of both *GIN4* and *HSL1* had no size defects in the absence of auxin (Figure S1A). Addition of auxin before mitosis in synchronized cells caused reduced levels of both *Gin4-AID* and *Hsl1-AID* (Figure S1, B–D). In both cases, small amounts of protein persisted in the presence of auxin, which indicated that auxin may cause a partial loss-of-function. Addition of auxin to *gin4-AID hsl1-AID* cells also caused a delay in mitotic progression (Figure S1E).

We first tested how conditional inactivation of *Gin4* and *Hsl1* influences bud growth and mitotic progression via live analysis of single cells. We included a fluorescently tagged spindle pole protein to monitor the duration of metaphase and anaphase [see *Materials and Methods* and Leitao and Kellogg (2017)]. The spindle poles in wild-type and *gin4-AID hsl1-AID* cells were marked with different fluorescent tags, which allowed simultaneous imaging of both strains under identical conditions.

We analyzed the effects of *gin4-AID* or *hsl1-AID* alone, as well as the combined effects of *gin4-AID hsl1-AID*. Cells were released from a G1 arrest and auxin was added immediately before initiation of bud emergence, which ensured that *Gin4* and *Hsl1* were depleted by the time of mitotic entry. Bud sizes and mitotic spindle dynamics were then analyzed at 3-min intervals to determine how loss of *Gin4* and *Hsl1* influenced

bud growth and the duration of mitosis. Examples of wild-type and *gin4-AID hsl1-AID* cells are shown in Figure 1 and Supplemental Video 1. Both cells initiated bud emergence at nearly the same time, but the wild-type cell completed bud growth and exited mitosis while the *gin4-AID hsl1-AID* cell remained delayed in metaphase as the bud continued to grow. The *gin4-AID hsl1-AID* cell eventually completed mitosis, but at a substantially larger bud size than the wild-type control cell. The daughter bud was more elongated in the *gin4-AID hsl1-AID* cell, which indicated a defect in control of polar growth.

Quantitative analysis of multiple cells showed that destruction of *Gin4* and/or *Hsl1* caused an increase in the duration of metaphase, but had little effect on the duration of anaphase (Figure 2A). Destruction of *Gin4* and *Hsl1* also caused an increase in bud size at the completion of each mitotic interval (Figure 2B). *gin4-AID hsl1-AID* did not cause significant effects on the growth rate of the daughter cell (Figure S2A). The effects of *gin4-AID* and *hsl1-AID* were not additive (Figure 2, A and B), which was surprising because *hsl1* Δ and *gin4* Δ cause strong additive effects on cell size and shape [Figure S2B and Barral *et al.* (1999)]. This issue is addressed below. Inactivation of *gin4-AID* caused polar bud growth to continue in mitosis, whereas inactivation of *hsl1-AID* did not. This effect was quantified by measuring axial ratios of daughter buds at completion of anaphase (Figure 2C).

Previous studies found that *gin4* Δ and *hsl1* Δ cause defects in cytokinesis that lead to the formation of clumps of interconnected cells (Ma *et al.* 1996; Okuzaki *et al.* 1997; Barral *et al.* 1999). Consistent with this, we observed that *gin4-AID hsl1-AID* caused a failure in cell separation in nearly all cells at the end of the first cell cycle following addition of auxin (Supplemental Video 2). Previous studies also found that *gin4* Δ and *hsl1* Δ cause defects in spindle positioning (Fraschini *et al.* 2006; Grava *et al.* 2006; Gihana *et al.* 2018). We observed only a few defects in spindle positioning during the first cell division after the

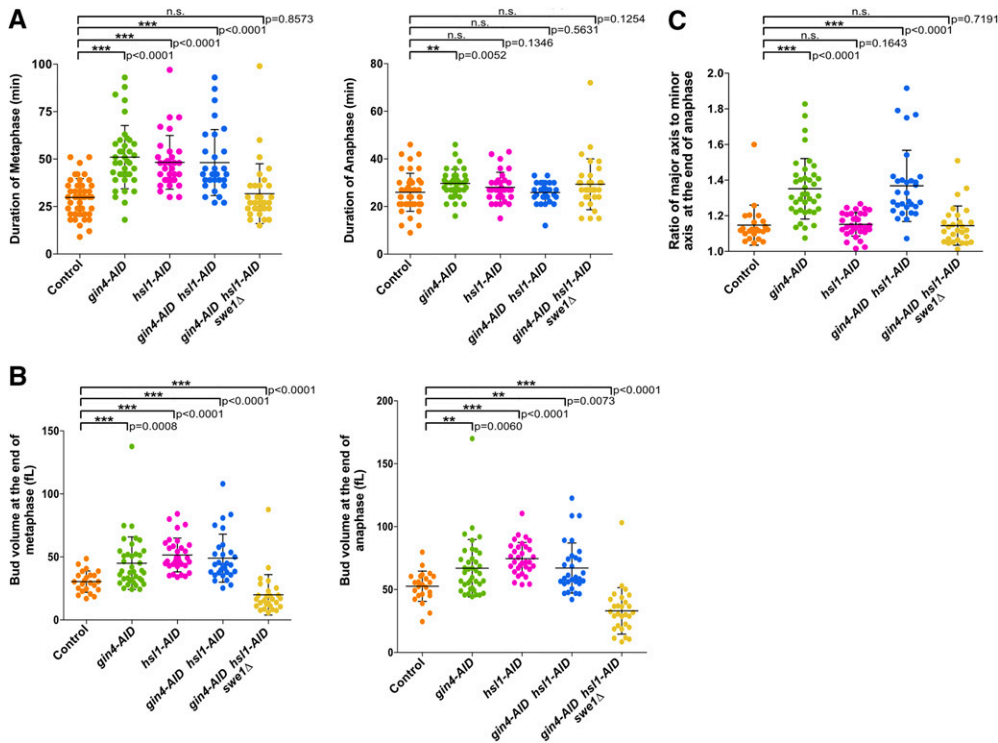


Figure 2 *Gin4* and *Hsl1* are required for normal control of cell growth and size in mitosis. Cells of the indicated genotypes were released from a G1 arrest and analyzed by confocal microscopy as described for Figure 1. All strains included two copies of the *TIR1* gene. (A) Scatter plots showing the duration of metaphase and anaphase. (B) Scatter plots showing bud size at completion of metaphase and anaphase. (C) Scatter plots showing the ratio of major axis to minor axis of the bud at completion of anaphase. For panels (A–C) the mean and SD for each strain are shown. AID, auxin-inducible degen; n.s., not significant.

addition of auxin to *gin4-AID hsl1-AID* cells. However, in the second cell division, many cells showed aberrant movement of the metaphase spindle into the daughter cell before anaphase (Supplemental Video 2). Thus, defects in spindle orientation appear to be a secondary consequence of inactivating *Gin4* and *Hsl1*.

***Gin4*-related kinases are required for normal control of mother cell growth**

In wild-type cells, little growth of the mother cell occurs after bud emergence (McCusker *et al.* 2007; Ferrezuelo *et al.* 2012). However, in *gin4-AID hsl1-AID* cells, we found that mother cell growth often continued throughout the interval of daughter cell growth. Example plots of mother and daughter cell size as a function of time for wild-type and *gin4-AID hsl1-AID* cells are shown in Figure 3A. Quantitative analysis revealed that *gin4-AID* and *hsl1-AID* had additive effects upon mother cell growth, so that most *gin4-AID hsl1-AID* cells underwent abnormal mother cell growth (Figure 3B).

Previous work has shown that daughter cell size is correlated with mother cell size (Schmoller *et al.* 2015; Leitao and Kellogg 2017). Thus, large mother cells give birth to large daughter cells. As a result, the increased size of mothers in *gin4-AID hsl1-AID* cells would be expected to drive increased daughter cell size in subsequent cell divisions, which could lead to gradually increasing defects in cell growth and size. Therefore, the role of *Gin4* and *Hsl1* in the control of mother cell growth could help explain why prolonged loss of *Gin4* and *Hsl1* causes strong additive effects on cell growth and size. We found that the effects caused by *gin4-AID hsl1-AID*

increased substantially with prolonged incubation in the presence of auxin, consistent with a model in which the terminal phenotype caused by *gin4Δ hsl1Δ* is partially the result of defects that accumulate over multiple cell cycles (Figure S3).

Gin4*-related kinases influence the duration of metaphase via inhibitory phosphorylation of *Cdk1

Genetic analysis has shown that *Gin4*-related kinases are negative regulators of the *Wee1* kinase, which phosphorylates and inhibits mitotic *Cdk1* (Ma *et al.* 1996; Longtine *et al.* 2000). The budding yeast homolog of *Wee1* is referred to as *Swe1*. Previous studies have shown that *Swe1* influences the timing of mitotic entry as well as the duration of metaphase (Harvey and Kellogg 2003; Lianga *et al.* 2013; Leitao *et al.* 2019). Therefore, we tested whether *Gin4* and *Hsl1* influence the duration of metaphase and cell size via *Swe1*. To do this, we analyzed the effects of *swe1Δ* on bud growth and mitotic duration in *gin4-AID hsl1-AID* cells. This revealed that *swe1Δ* eliminated the prolonged metaphase delay caused by loss of *Gin4* and *Hsl1* (Figure 2A). Furthermore, *swe1Δ* caused daughter buds in *gin4-AID hsl1-AID* cells to complete metaphase and anaphase at sizes smaller than the wild-type control cells (Figure 2B), and it eliminated the bud elongation caused by *gin4-AID* (Figure 2C). As reported previously, *swe1Δ* caused a reduced growth rate, which is thought to be due to the decreased size of mother cells (Figure S2A) (Leitao *et al.* 2019).

Several observations demonstrated that *Gin4* and *Hsl1* do not work solely via *Swe1*. Previous studies found that

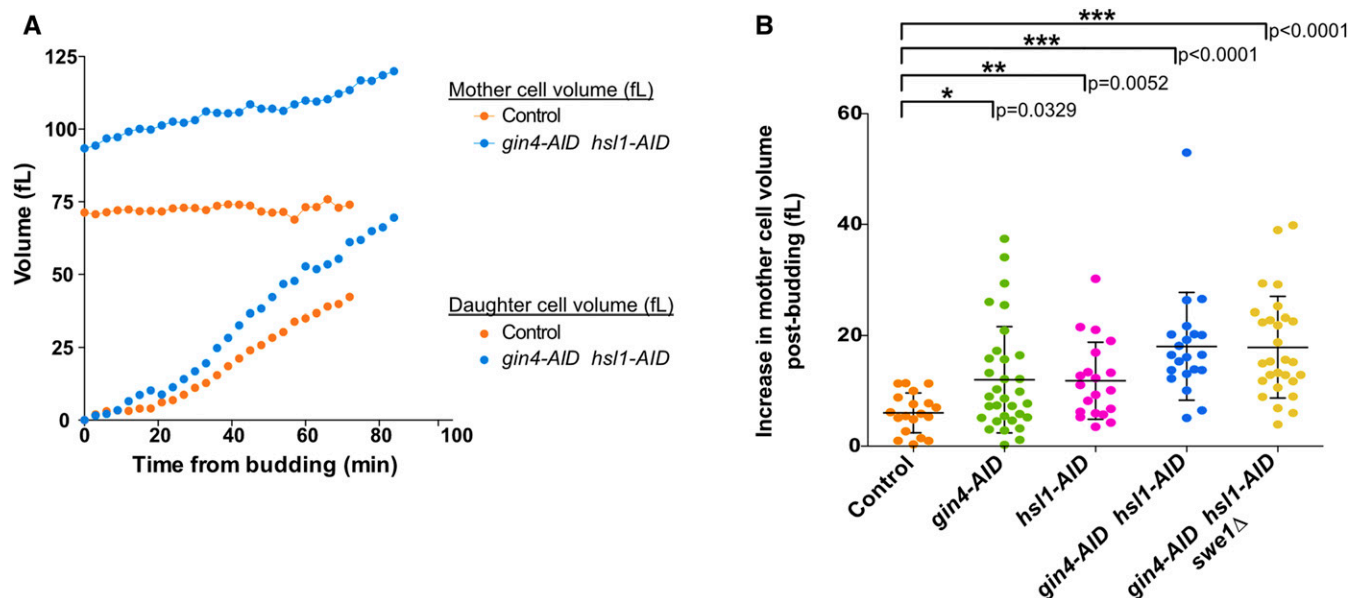


Figure 3 *Gin4* and *Hsl1* are required for normal control of mother cell growth. Cells of the indicated genotypes were released from a G1 arrest and analyzed by confocal microscopy as described for Figure 1. (A) A representative plot of mother and daughter cell size as a function of time. In each case, the daughter cell is the daughter of the mother cell shown in the same plot. (B) Scatter plots showing the net increases in mother cell volume from the time of bud emergence to completion of anaphase. The plot shows the mean and SD for each strain. AID, auxin-inducible degenon.

swe1Δ cells have a shorter metaphase than wild-type cells (Liang *et al.* 2013; Leitao *et al.* 2019). Here, we found that *swe1Δ* reduced the duration of metaphase in *gin4-AID hsl1-AID* cells, but it did not make the duration of metaphase in these cells shorter than metaphase in wild-type cells, indicating that acute loss of *Gin4* and *Hsl1* still influenced the duration of metaphase in *swe1Δ* cells. In addition, *swe1Δ* did not fully rescue growth defects caused by *gin4Δ hsl1Δ* (Figure S2B). Finally, *swe1Δ* did not eliminate inappropriate growth of mother cells in *gin4-AID hsl1-AID* cells (Figure 3B). Together, these observations demonstrate that *Gin4* and *Hsl1* have *Swe1*-dependent and *Swe1*-independent functions.

Gin4* and *Hsl1* are required for full hyperphosphorylation of *Swe1

We next investigated how *Gin4* and *Hsl1* control *Swe1*. In previous work, we showed that *Swe1* undergoes complex regulation in mitosis (Sreenivasan and Kellogg 1999; Harvey *et al.* 2005, 2011). In early mitosis, *Cdk1* phosphorylates *Swe1* on *Cdk1* consensus sites, which activates *Swe1* to bind and inhibit *Cdk1*. This form of *Swe1*, which we refer to as partially hyperphosphorylated *Swe1*, works in a systems-level mechanism that maintains a low level of *Cdk1* activity during metaphase. Further phosphorylation events drive full hyperphosphorylation of *Swe1*, leading to inactivation of *Swe1* and release of fully active *Cdk1*. *Swe1* is proteolytically destroyed at the end of mitosis; however, mutants that block *Swe1* have no effect on mitotic progression, so the function

of *Swe1* destruction remains unknown (Thornton and Toczyski 2003; Raspelli *et al.* 2011).

A previous study suggested that *hsl1Δ* causes defects in the phosphorylation of *Swe1* but did not provide sufficient resolution of differently phosphorylated forms of *Swe1* to determine which events were affected (Shulewitz *et al.* 1999). Here, we found that conditional inactivation of *gin4-AID* and *hsl1-AID* before mitosis caused a failure in full hyperphosphorylation of *Swe1* (Figure 4). Similarly, addition of auxin to asynchronous *gin4-AID hsl1-AID* cells caused loss of fully hyperphosphorylated *Swe1* within 60 min (Figure S4). These data show that *Gin4* and *Hsl1* are required for generation of the fully hyperphosphorylated inactive form of *Swe1*, consistent with genetic data showing that *Gin4*-related kinases are negative regulators of *Swe1*.

Hyperphosphorylation of *Gin4* and *Hsl1* is correlated with the extent of bud growth

We previously found that *Gin4* undergoes gradual hyperphosphorylation and activation during bud growth, reaching peak hyperphosphorylation and peak kinase activity in late mitosis as growth ends (Altman and Kellogg 1997). Thus, gradual phosphorylation and activation of *Gin4* is correlated with gradual bud growth, which suggests that *Gin4* could be activated by growth-dependent signals that provide a molecular readout of the extent of bud growth. To begin to test this hypothesis, we first carried out additional experiments to test whether hyperphosphorylation of *Gin4* and *Hsl1* is correlated with bud growth. To do this, we took advantage of the fact that the durations of both metaphase and anaphase are increased in cells growing slowly on a poor carbon source, even

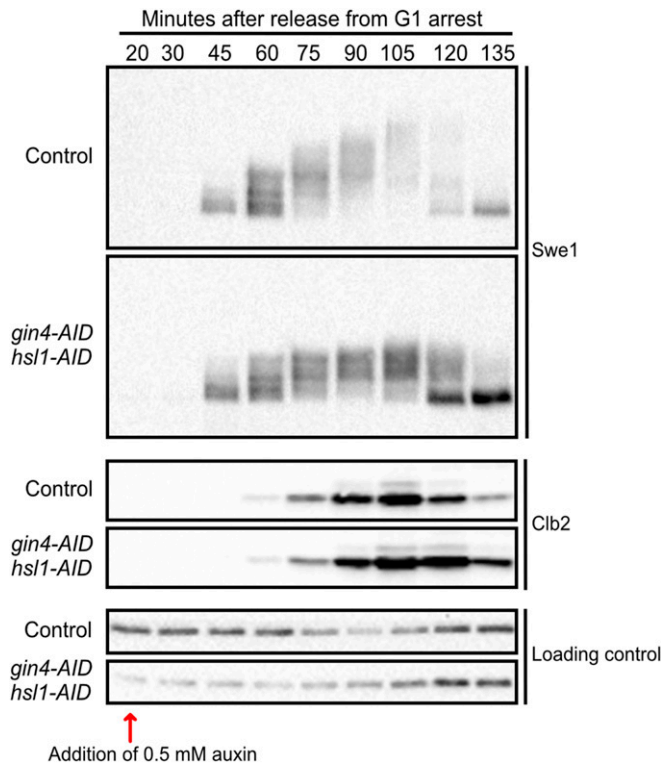


Figure 4 *Gin4* and *Hsl1* are required for full hyperphosphorylation of *Swe1*. Control cells and *gin4-AID hsl1-AID* cells growing in YPD were released from a G1 arrest at 25°, and 0.5 mM auxin was added to both strains 20 min after release. Both strains included two copies of the *TIR1* gene. Samples were taken at the indicated intervals and the behaviors of *Swe1* and *Clb2* were analyzed by western blot. A background band from the *Clb2* blot is provided as a loading control. AID, auxin-inducible degron.

as the extent of growth in mitosis is reduced (Leitao and Kellogg 2017; Leitao *et al.* 2019). In other words, cells in poor carbon spend more time growing in both metaphase and anaphase, but complete mitosis at a smaller daughter cell size. Therefore, we reasoned that if hyperphosphorylation of *Gin4* and *Hsl1* is correlated with the extent of bud growth, the duration of hyperphosphorylation should be increased in cells growing in poor carbon, while the extent of hyperphosphorylation should be reduced. Alternatively, if phosphorylation of *Gin4* and *Hsl1* is more closely linked to a mitotic event unrelated to growth, such as the positioning of the mitotic spindle, one might expect that the timing and/or extent of hyperphosphorylation would not be influenced by the carbon source.

Wild-type cells growing in rich (2% glucose) or poor carbon (2% glycerol and 2% ethanol) medium were released from a G1 arrest, and phosphorylation of *Gin4* and *Hsl1* was assayed by western blot to detect phosphorylation events that cause electrophoretic mobility shifts. The same samples were also probed for the mitotic cyclin *Clb2* as a marker for mitotic duration. Cells growing in poor carbon showed delayed mitotic entry and a prolonged mitosis compared to cells growing in rich carbon, as previously described

(Figure 5, A and B) (Leitao and Kellogg 2017; Leitao *et al.* 2019). *Gin4* is present throughout the cell cycle, while *Hsl1* is synthesized anew before mitosis and destroyed at the end of mitosis (Altman and Kellogg 1997; Burton and Solomon 2000). In both carbon sources, hyperphosphorylation of *Gin4* and *Hsl1* increased gradually during mitosis, with peak phosphorylation occurring near peak *Clb2* levels. In addition, the interval during which hyperphosphorylation occurred was prolonged in poor carbon. Previous studies have shown that the increased duration of mitosis in poor carbon is not an artifact caused by poor synchrony (Leitao and Kellogg 2017; Leitao *et al.* 2019). Importantly, we also observed that growth in poor carbon caused a reduction in the maximal extent of hyperphosphorylation reached during mitosis (Figure 5, A–C).

These observations are consistent with the hypothesis that hyperphosphorylation of *Gin4* and *Hsl1* is correlated with the extent of bud growth rather than a mitotic event that is unrelated to growth. One interpretation of the results is that the extent of hyperphosphorylation of *Gin4* and *Hsl1* required for mitotic exit is reduced in poor carbon, which allows cells to complete mitosis at a smaller bud size.

Hyperphosphorylation of *Gin4* is dependent upon bud growth

We next tested whether hyperphosphorylation of *Gin4* is dependent upon bud growth. To do this, we used a temperature-sensitive allele of *SEC6* (*sec6-4*) to block bud growth. *Sec6* is a component of the exocyst complex, which is required at the plasma membrane for the docking and fusion of vesicles that drive bud growth. In previous work, we showed that inactivation of *Sec6* blocks bud growth and triggers an arrest in early mitosis (Anastasia *et al.* 2012). The arrest is enforced by *Swe1*. Thus, *sec6-4 swe1Δ* cells fail to undergo bud growth, yet enter mitosis and complete chromosome segregation before eventually arresting in late mitosis. Therefore, we analyzed *Gin4* phosphorylation in *sec6-4 swe1Δ* cells, which allowed us to distinguish whether effects of *sec6-4* could be a consequence of a failure to undergo bud growth or a failure in mitotic progression. As controls, we also analyzed *Gin4* hyperphosphorylation in wild-type and *swe1Δ* cells.

Cells were released from a G1 arrest and shifted to the restrictive temperature for the *sec6-4* allele before bud emergence. *Gin4* phosphorylation was assayed by western blot (Figure 6A). The same samples were probed for *Clb2* as a marker for mitotic progression (Figure 6B). The *sec6-4 swe1Δ* cells entered mitosis but arrested in late mitosis with high levels of mitotic cyclin, as previously reported (Anastasia *et al.* 2012). Hyperphosphorylation of *Gin4* failed to occur in both *sec6-4* and *sec6-4 swe1Δ* cells (Figure 6, A and C and Figure S5A). Direct comparison of the extent of *Gin4* hyperphosphorylation in mitosis showed a complete loss of *Gin4* phosphorylation (Figure 6C). Thus, hyperphosphorylation of *Gin4* is dependent upon membrane trafficking events that drive bud growth. Moreover, since mitotic spindle assembly and chromosome segregation occur normally in *sec6-4 swe1Δ* cells (Anastasia *et al.*

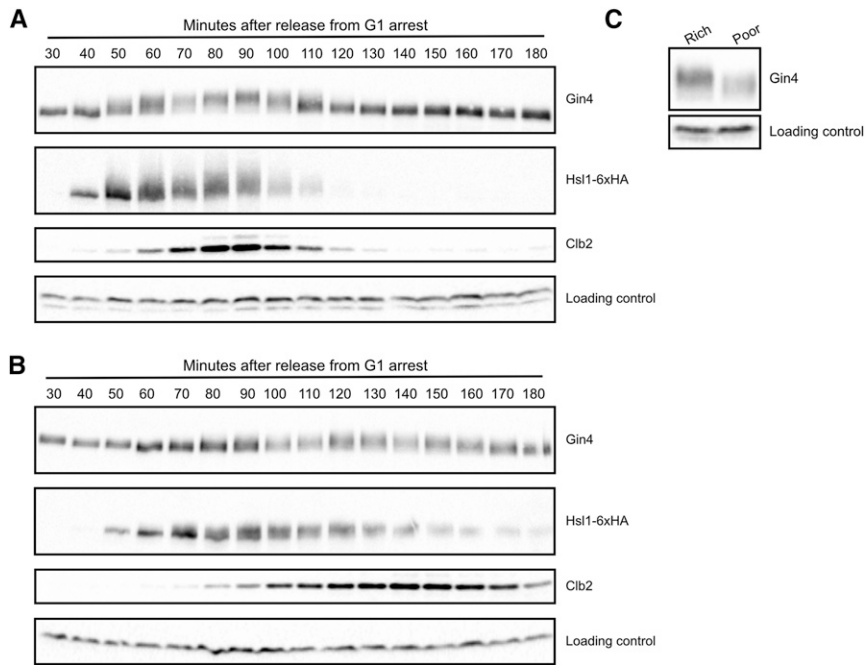


Figure 5 Hyperphosphorylation of *Gin4* and *Hsl1* is correlated with the extent of bud growth. Wild-type cells grown overnight in YPD (A) or YPG/E (B) were arrested with α factor. The cells were then released from the arrest at 25° and samples were taken at 10 min intervals. The behaviors of *Gin4*, *Hsl1*-6XHA, and *Clb2* were assayed by western blot. (C) A direct comparison of the maximal extent of *Gin4* phosphorylation in rich or poor carbon was made by comparing samples taken at peak *Clb2* expression in each condition (90 min in rich carbon and 140 min in poor carbon). An anti-Nap1 antibody was used as a loading control. YPG/E, YP medium containing 2% glycerol and 2% ethanol.

2012), the data further suggest that *Gin4* phosphorylation is independent of the signals that drive chromosome segregation.

There is some evidence that growth rate increases with cell size (Jorgensen and Tyers 2004b; Tzur *et al.* 2009; Sung *et al.* 2013). Therefore, we considered the possibility that *Gin4* phosphorylation provides a readout of growth rate rather than a readout of how much growth has occurred. In this case, *Gin4* phosphorylation could be driven by dynamic signals generated in association with ongoing events of membrane growth. To investigate, we tested the effects of inactivating *Sec6* during bud growth. If *Gin4* phosphorylation is a readout of growth rate, an acute block to bud growth should cause rapid loss of *Gin4* phosphorylation. In contrast, if *Gin4* hyperphosphorylation reports on the extent of bud growth, acute inactivation of *Sec6* should stop progression of *Gin4* phosphorylation but should not cause a loss of *Gin4* phosphorylation. Wild-type and *sec6-4* cells were released from a G1 arrest and shifted to the restrictive temperature in early mitosis, as mitotic cyclin levels were rising and *Gin4* phosphorylation was beginning to occur. *Gin4* phosphorylation persisted in the *sec6-4* cells but did not disappear or increase further after the shift to the restrictive temperature (Figure S5B). Inactivation of *Sec6* late in mitosis when bud growth was largely complete had no effect on *Gin4* phosphorylation (Figure S5C). Thus, *Gin4* phosphorylation appears to be correlated with the extent of bud growth, rather than the rate of bud growth.

Phosphorylation of *Gin4* during bud growth requires binding to anionic phospholipids

We next investigated the mechanisms that drive hyperphosphorylation of *Gin4*-related kinases, since these could

provide clues to how growth-dependent signals are generated and relayed. Both *Gin4* and *Hsl1* have well-defined C-terminal KA1 domains that bind phosphatidylserine and other anionic phospholipids (Moravcevic *et al.* 2010). Phosphatidylserine is preferentially localized to the growing bud and appears to be the most important effector for KA1 domains (Ejsing *et al.* 2009; Moravcevic *et al.* 2010; Fairn *et al.* 2011b; Klose *et al.* 2012). The KA1 domain of *Hsl1* is required for efficient localization to the bud neck, but the mechanism by which the domain promotes bud neck localization remains unknown. More generally, lipid-binding domains have been shown to play diverse roles in biochemical mechanisms that generate or relay signals at the plasma membrane, which indicates that their functions reach beyond simply localizing proteins. For example, binding of phosphatidylserine to KA1 domains can promote an open and active conformation that could lead to autophosphorylation or phosphorylation by another kinase (Wu *et al.* 2015; Emptage *et al.* 2017, 2018). Gradual phosphorylation of *Gin4* during bud growth is dependent upon *Gin4* kinase activity, consistent with a model in which binding of phosphatidylserine drives autophosphorylation (Altman and Kellogg 1997). Together, these observations led us to hypothesize that phosphatidylserine delivered to the plasma membrane during bud growth could drive hyperphosphorylation of *Gin4*-related kinases, thereby generating a growth-dependent molecular signal that is correlated with the extent of growth. Alternatively, binding to phosphatidylserine could help localize *Gin4*-related kinases to a location where they can receive and relay growth-dependent signals.

To investigate further, we focused on *Gin4*. We found that *gin4- Δ KA1-GFP* failed to localize to the bud neck normally

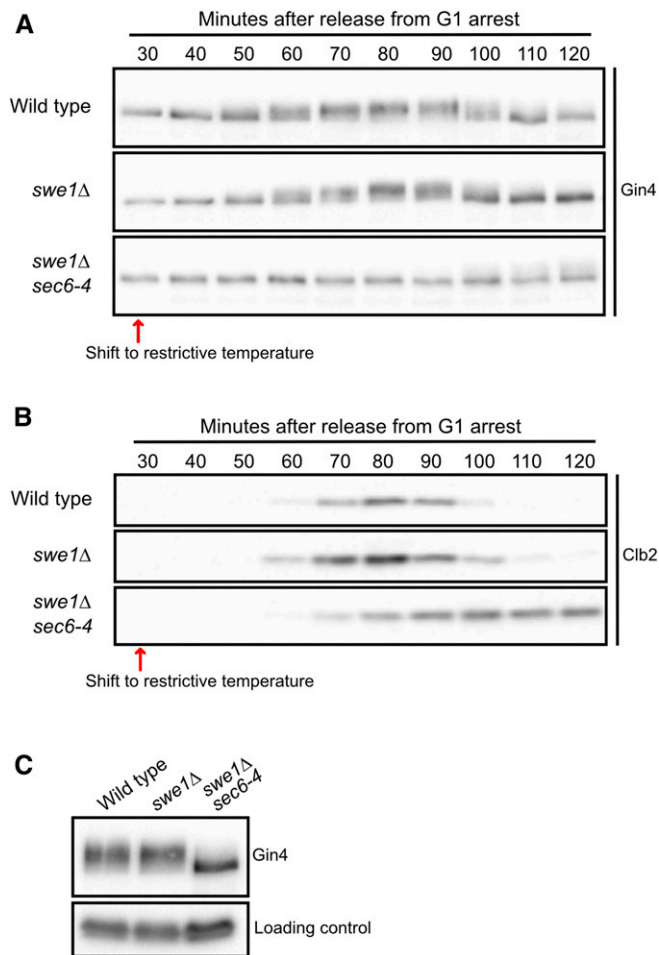


Figure 6 Hyperphosphorylation of *Gin4* is dependent upon bud growth. Wild-type, *swe1Δ*, and *swe1Δ sec6-4* cells were released from a G1 arrest in YPD at room temperature, and shifted to the restrictive temperature (34°) 30 min after release from arrest. Samples were taken at the indicated intervals and the behaviors of *Gin4* (A) and *Clb2* (B) were analyzed by western blot. (C) A direct comparison of the extent of *Gin4* phosphorylation was made by loading samples from all three strains taken at 90 min (wild-type), 80 min (*swe1Δ*), and 100 min (*sec6-4 swe1Δ*).

and was observed primarily in the cytoplasm (Figure 7A). Weak localization to the bud neck could be detected in a fraction of cells, indicating that determinants outside the KA1 domain contribute to *Gin4* localization, as previously seen for *hsl1-ΔKA1* (arrowheads, Figure 7A) (Finnigan *et al.* 2016). We further found that *gin4-ΔKA1* cells showed increased cell size and an elongated bud phenotype similar to *gin4Δ* cells (Figure 7, B and C). *gin4-ΔKA1* in an *hsl1Δ* background caused a phenotype similar to *gin4Δ hsl1Δ* (Figure 7C). These findings extend results from a previous analysis that utilized an overexpressed version of *gin4-ΔKA1-GFP* (Moravcevic *et al.* 2010).

Previous work found that the KA1 domain of *Hsl1* could be functionally replaced by a heterologous bovine LactC2 domain that binds phosphatidylserine (Finnigan *et al.* 2016). Similarly, we found that replacing the KA1 domain of *Gin4* with the LactC2 domain rescued the defects in localization, cell size, and cell growth caused by *gin4-ΔKA1* (Figure 7,

A–C). We also found that mutation of three amino acids in the LactC2 domain that are required for efficient binding to phosphatidylserine (*gin4-ΔKA1-LactC2^{AAA}*) (Yeung *et al.* 2008) caused a phenotype similar to *gin4Δ* and *gin4-ΔKA1* (Figure 7, A–C). The mutant *LactC2^{AAA}* domain partially restored *Gin4* localization to the bud neck; however, the amount of *gin4-ΔKA1-LactC2^{AAA}-GFP* at the bud neck was reduced relative to both *GIN4-GFP* and *gin4-ΔKA1-LactC2-GFP* (Figure 7A and Figure S6). Together, these observations show that the ability of the KA1 domain to bind to anionic phospholipids is essential for the function of *Gin4*, as seen previously for *Hsl1* and *Kcc4* (Crutchley *et al.* 2009; Moravcevic *et al.* 2010; Finnigan *et al.* 2016).

No previous studies analyzed whether the KA1 domain influences mitotic duration or the phosphorylation of *Gin4*-related kinases. We found that *gin4-ΔKA1* completely failed to undergo hyperphosphorylation during bud growth (Figure 7D). Analysis of *Clb2* levels in synchronized cells showed that *gin4-ΔKA1* cells exhibited an increased duration of mitosis (Figure 7E). The LactC2 domain restored gradual hyperphosphorylation of *Gin4* during bud growth in *gin4-ΔKA1-LactC2* cells, as well as normal mitotic duration (Figure 7, D and E). The mutant version of the LactC2 domain that cannot bind efficiently to phosphatidylserine failed to restore *Gin4* hyperphosphorylation (Figure 7D). Together, these data show that binding to anionic phospholipids is required for gradual hyperphosphorylation and activation of *Gin4* during bud growth.

Discussion

Gin4-related kinases influence the duration and extent of growth during metaphase

Previous analysis of the functions of *Gin4*-related kinases used gene deletions. However, phenotypes caused by gene deletions can be the outcome of cumulative defects gained over multiple generations. Therefore, it has not been possible to discern the immediate and direct consequences of loss-of-function of *Gin4*-related kinases. Here, conditional alleles allowed us to rigorously define the functions of *Gin4*-related kinases. We show for the first time that a prolonged delay in metaphase is an immediate consequence of inactivating *Gin4* and *Hsl1*. Growth continues during the delay, leading to aberrant cell size. We also discovered that inactivation of *Gin4*-related kinases leads to aberrant growth of the mother cell, which indicates that *Gin4*-related kinases are required for mechanisms that restrict growth to the daughter bud. Since *Gin4*-related kinases are localized to the bud neck, they are ideally positioned to define a domain of growth in the daughter bud. Defects in cytokinesis are another primary consequence of inactivating *Gin4*-related kinases. Severe defects in mitotic spindle positioning were only observed in the second cell cycle after inactivation of *Gin4*-related kinases, which suggests that they are an indirect consequence.

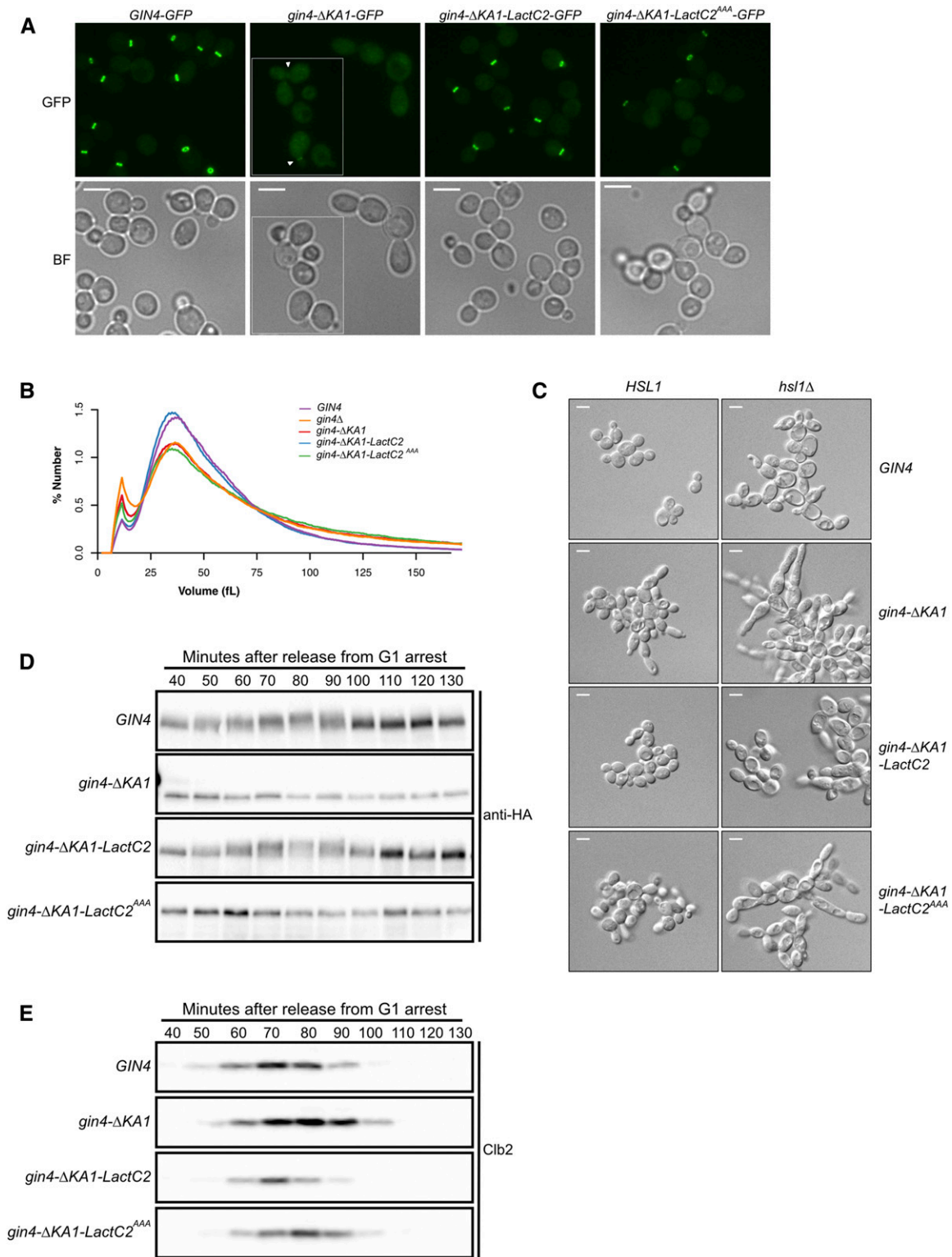


Figure 7 Phosphorylation of *Gin4* during bud growth requires binding to anionic phospholipids. (A) Cellular localization of *Gin4*, *Gin4-ΔKA1*, *Gin4-ΔKA1-LactC2*, and *Gin4-ΔKA1-LactC2^{AAA}* fused to GFP at the C-terminus. All four strains were excited by the GFP-laser with identical settings at 100× magnification and displayed with the same brightness levels to compare relative levels of *Gin4* localized to the bud neck. *Gin4-ΔKA1-GFP* shows mostly cytoplasmic localization with a small level of bud neck localization (white arrowheads). The BF images for each field are shown below. (B) Cells of the indicated genotypes were grown in YPD overnight, diluted in fresh YPD, and then incubated for 5 hr at 30°. The size distribution for each strain was analyzed using a Coulter counter. (C) Cells of the indicated genotypes were grown to log phase in YPD at 25° and imaged by DIC optics. (D and E) Cells of the indicated genotypes were released from a G1 arrest in YPD at 30°. The behaviors of *Gin4* (D) and *Clb2* (E) were analyzed by western blot. In each strain, *Gin4* constructs were marked with a 3xHA tag and detected with anti-HA antibody. The signals for the *gin4-ΔKA1* and the *LactC2* constructs were weaker, so these blots were exposed for longer. *Gin4-ΔKA1-3xHA* was ~16 kDa smaller than the other proteins. Bar, 5 μm. BF, brightfield.

A key question concerns the relationship between the mitotic delay and the excessive cell growth caused by inactivation of *Gin4*-related kinases. One can imagine two opposing models. In one model, excessive growth is an indirect consequence of the mitotic delay. In the other model, increased mitotic duration is a consequence of a failure to properly detect growth. Current data do not fully distinguish these models. The former model has dominated interpretation of the phenotypes caused by cell division control mutants for decades. Yet the fact that cell cycle progression is dependent upon cell growth suggests that it should be possible to identify mutants that cause cell cycle delays due to a failure to properly detect that growth has occurred. We favor a model in which the mitotic delay caused by inactivation of *Gin4*-related kinases is a direct consequence of a failure in growth-dependent signals that are used to detect and measure cell growth. However, the data do not yet rule out the possibility that a more proximal cause of the delay is failure of an event that is unrelated to cell growth. Since the mitotic delay is imposed by *Swe1*, a key step toward distinguishing models will be to gain a better understanding of the signaling mechanisms that control the activity of *Swe1*.

The effects of inactivating *Gin4* and *Hsl1* on the duration and extent of growth in metaphase were not additive. This was surprising because *gin4* Δ and *hsl1* Δ show strong additive effects on cell size and shape (Barral *et al.* 1999). The discovery that loss of *Gin4*-related kinases causes inappropriate mother cell growth during mitosis suggests an explanation. Previous studies have shown that large mother cells drive increased growth of daughter cells (Schmoller *et al.* 2015; Leitao and Kellogg 2017). Thus, we hypothesize that the increased size of mother cells caused by loss of *Gin4* and *Hsl1* amplifies aberrant growth in subsequent divisions. However, other factors could contribute to the additive effects of *gin4* Δ and *hsl1* Δ . For example, the severe spindle positioning defects that appear in the second cell division after inactivation of *Gin4* and *Hsl1* could cause prolonged mitotic delays that lead to further aberrant growth. Defects in growth control could also be amplified by failures in cytokinesis that create chains of conjoined cells in which the signals that control cell growth and size are no longer effectively compartmentalized.

Our analysis also showed that *Gin4* and *Hsl1* play different roles in controlling polar bud growth. Destruction of *Gin4* caused excessive polar growth in the first cell cycle following destruction, whereas destruction of *Hsl1* did not. Previous work suggested that *Gin4* binds and regulates *Bnr1*, a formin protein that controls the location of actin cables that deliver vesicles to sites of membrane growth (Buttery *et al.* 2012). *Bnr1* is localized to the bud neck, and loss of *Bnr1* is thought to cause inappropriate polar growth because the actin cables that direct isotropic growth are lost (Pruyne *et al.* 2004; Gao *et al.* 2010). Thus, polar growth caused by loss of *Gin4* could be due, at least in part, to misregulation of *Bnr1*.

Inactivation of *Gin4* and *Hsl1* had little effect on the duration of anaphase or the extent of growth in anaphase. In a previous study, we found that extensive growth occurs during anaphase, and that the duration of anaphase and the extent of growth in anaphase are both modulated by nutrient-dependent signals (Leitao and Kellogg 2017). Our results suggest that *Gin4*-related kinases are unlikely to control the anaphase growth interval. The anaphase growth interval is also unlikely to be controlled by *Cdk1*-inhibitory phosphorylation (Leitao *et al.* 2019). The mechanisms that control anaphase duration in response to nutrient-dependent signals therefore remain mysterious.

***Gin4*-related kinases influence the duration of metaphase via *Cdk1* inhibitory phosphorylation**

The use of conditional alleles allowed us to show that *Gin4*-related kinases influence metaphase duration, and that they do so primarily via *Swe1*-dependent *Cdk1* inhibitory phosphorylation. Early work suggested that *Wee1*-related kinases work at mitotic entry. However, more recent work in both vertebrates and yeast found that *Wee1* also controls events after mitotic entry (Deibler and Kirschner 2010; Harvey *et al.* 2011; Lianga *et al.* 2013; Vassilopoulos *et al.* 2014; Toledo *et al.* 2015; Leitao *et al.* 2019). Several previous studies implied that *Gin4*-related kinases could be responsible for controlling *Cdk1* inhibitory phosphorylation during metaphase; however, interpretation of the results was complicated by the use of gene deletions, and the experiments did not directly test whether *Gin4*-related kinases control metaphase via *Cdk1* inhibitory phosphorylation (Carroll *et al.* 1998; Barral *et al.* 1999; Sreenivasan and Kellogg 1999; Sreenivasan *et al.* 2003). Here, acute conditional inactivation of *Gin4* and *Hsl1* provided definitive evidence that *Gin4*-related kinases influence the duration of metaphase via *Cdk1* inhibitory phosphorylation. We also showed that loss of *Gin4*-related kinases causes a failure in full hyperphosphorylation of *Swe1*, which is thought to be required for inactivation of *Swe1*. Previous studies have shown that *Gin4*-related kinases from fission yeast can directly phosphorylate *Wee1*; however, there is no evidence yet that this is true in budding yeast (Coleman *et al.* 1993; Kano and Russell 1998; Opalko *et al.* 2019).

Analysis of the effects of conditional inactivation of *Gin4*-related kinases suggested that they do not influence the duration of metaphase solely via *Swe1*. Previous studies showed that the duration of metaphase in *swe1* Δ cells is shorter than wild-type cells (Lianga *et al.* 2013; Leitao *et al.* 2019). Here, we found that *swe1* Δ substantially reduces the metaphase delay caused by inactivation of *Gin4*-related kinases but did not cause metaphase duration to be shorter than wild-type cells. Moreover, *swe1* Δ did not fully rescue the elongated buds, cell separation defects, and inappropriate mother cell growth caused by loss of *Gin4*-related kinases. Together, these observations suggest that *Gin4*-related kinases influence growth in metaphase partly via a mechanism that works downstream or independently of *Cdk1* inhibitory

phosphorylation. The fission yeast homolog of *Gin4* is also known to execute functions that are independent of *Wee1* (Breeding *et al.* 1998).

Hyperphosphorylation of *Gin4*-related kinases is dependent upon bud growth

Gin4 and *Hsl1* undergo gradual hyperphosphorylation and activation during mitosis. Growth of the bud also occurs gradually during mitosis, and loss of *Gin4*-related kinases causes inappropriate growth during a metaphase delay. These and other data led us to hypothesize that hyperphosphorylation of *Gin4*-related kinases reflects the activity of signaling mechanisms that measure bud growth during mitosis. To begin to test this hypothesis, we further investigated the correlation between bud growth and hyperphosphorylation of *Gin4* and *Hsl1*. To do this, we analyzed hyperphosphorylation of *Gin4* and *Hsl1* in cells growing in poor carbon, which increases the duration of bud growth in mitosis, while also decreasing the extent of growth. Poor carbon prolonged the interval during which hyperphosphorylation of *Gin4* and *Hsl1* took place in mitosis. Importantly, poor carbon also reduced the maximal extent of *Gin4* and *Hsl1* hyperphosphorylation achieved in mitosis. Thus, hyperphosphorylation of *Gin4* and *Hsl1* is correlated with both the duration and extent of growth in mitosis.

The increased duration of *Gin4* and *Hsl1* hyperphosphorylation in poor carbon is unlikely to be due to poor cell cycle synchrony because analysis of mitotic spindle dynamics, in both live single cells and synchronized populations of cells, has shown that the durations of both metaphase and anaphase are substantially increased in poor carbon (Leitao and Kellogg 2017; Leitao *et al.* 2019). Moreover, poor synchrony cannot explain why the maximal extent of *Gin4* and *Hsl1* hyperphosphorylation is dramatically reduced in poor carbon. If poor synchrony were the cause, one would expect to see a lower fraction of *Gin4* reaching the maximally hyperphosphorylated state in poor carbon. Rather, we observe a complete loss of the maximally hyperphosphorylated forms of *Gin4* and *Hsl1* in poor carbon.

To further investigate the relationship between bud growth and phosphorylation of *Gin4*, we tested whether hyperphosphorylation of *Gin4* is dependent upon growth. We found that blocking membrane trafficking events that drive plasma membrane growth causes a complete failure in *Gin4* phosphorylation. Furthermore, blocking membrane growth after initiation of bud growth halted the progression of *Gin4* phosphorylation, but did not cause a loss of *Gin4* phosphorylation, consistent with the idea that the *Gin4* phosphorylation is correlated with the extent of bud growth, rather than growth rate or time spent in mitosis.

Blocking membrane growth causes a prolonged metaphase delay that is dependent on *Cdk1* inhibitory phosphorylation (Anastasia *et al.* 2012). Elimination of *Swe1*-mediated *Cdk1* inhibitory phosphorylation abrogates the delay but does not restore *Gin4* hyperphosphorylation, suggesting

that *Gin4* hyperphosphorylation is upstream of *Cdk1* inhibitory phosphorylation. Moreover, in previous work, we found that *Gin4* hyperphosphorylation occurs normally in cells treated with drugs that block the formation of a mitotic spindle (Mortensen *et al.* 2002). Thus, *Gin4* hyperphosphorylation is dependent upon membrane trafficking events that drive bud growth, and appears to be independent of the events of mitotic spindle assembly and chromosome segregation.

Hyperphosphorylation of *Gin4* requires binding to anionic phospholipids

We found that growth-dependent hyperphosphorylation of *Gin4* is dependent upon the KA1 domain, which binds anionic phospholipids. Moreover, the functions of the KA1 domain that are required for *Gin4* phosphorylation could be fully replaced by a heterologous bovine LactC2 domain that binds phosphatidylserine. Mutations in the LactC2 domain that reduce binding to phosphatidylserine block hyperphosphorylation of *Gin4* and cause a phenotype similar to *gin4* Δ . Together, these observations demonstrate that gradual hyperphosphorylation of *Gin4* during bud growth is dependent upon binding to anionic phospholipids.

The KA1 domain binds preferentially to phosphatidylserine but can also bind other anionic phospholipids, such as phosphatidylinositol (Moravcevic *et al.* 2010; Wu *et al.* 2015). In contrast, the LactC2 domain appears to bind only to phosphatidylserine (Shao *et al.* 2008). The fact that the KA1 domain can be functionally replaced by the LactC2 domain therefore suggests that phosphatidylserine may be the predominant anionic phospholipid that drives gradual hyperphosphorylation of *Gin4* (Moravcevic *et al.* 2010; Fairn *et al.* 2011a). Phosphatidylserine is preferentially localized to the growing bud and is enriched at the bud neck (Moravcevic *et al.* 2010; Fairn *et al.* 2011a). Phosphatidylserine is also preferentially localized to sites of membrane growth in fission yeast (Haupt and Minc 2017).

Hyperphosphorylation of both *Gin4* and *Hsl1* is dependent upon their kinase activity, which suggests that gradual hyperphosphorylation of these kinases during bud growth is due to autophosphorylation (Altman and Kellogg 1997; Barral *et al.* 1999). Moreover, previous studies suggested that binding of anionic phospholipids to KA1 domains in protein kinases that are related to *Gin4* can drive formation of an open, active conformation (Wu *et al.* 2015; Emptage *et al.* 2017, 2018). Together, these observations suggest that anionic phospholipids delivered to the growing bud could drive autophosphorylation by directly binding and activating *Gin4*-related kinases. This model could explain why *gin4*- Δ KA1-*LactC2*^{AAA} causes a complete loss of *Gin4* phosphorylation, despite allowing a fraction of *Gin4* to be localized to the bud neck. Alternatively, binding of *Gin4*-related kinases to anionic phospholipids could help recruit them to a location where they receive other signals that are required for hyperphosphorylation. Both scenarios could

help generate or relay a molecular readout of the extent of bud growth.

Several studies suggested that mammalian kinases related to *Gin4* are autoinhibited by determinants within or near the KA1 domain, which raise the question of how the heterologous LactC2 domain can replace the KA1 domain. In one example, autoinhibition is mediated by a cluster of basic amino acids in the KA1 domain that also mediate binding to anionic phospholipids (Emptage *et al.* 2017). If this mechanism holds for *Gin4*, one could imagine that basic amino acids found in LactC2 could be sufficient to mediate autoinhibition. In another example, autoinhibition is mediated by a short sequence immediately adjacent to the KA1 domain (Wu *et al.* 2015). In this case, autoinhibition would occur even when the KA1 domain is replaced by the LactC2 domain.

A growth-dependent signaling hypothesis for the functions of *Gin4*-related kinases

Together, the data suggest a model in which anionic phospholipids delivered to the growing bud bind and activate *Gin4*-related kinases, thereby generating a signal that is correlated with the extent of growth during metaphase. We further propose that once the signal reaches a threshold, *Gin4*-related kinases drive full hyperphosphorylation and inactivation of *Swe1*, thereby triggering mitotic progression and termination of the metaphase growth interval. We refer to this model as the growth-dependent signaling hypothesis.

The data do not yet rule out alternative models. Nevertheless, the growth-dependent signaling hypothesis is consistent with the available data. It would explain why loss of *Gin4*-related kinases causes an abnormally prolonged interval of growth during metaphase, leading to aberrant cell size. In this case, the hypothesis suggests that loss of *Gin4*-related kinases causes cells to behave as though they cannot measure how much bud growth has occurred. The hypothesis also explains why *Gin4*-related kinases undergo gradual hyperphosphorylation and activation during bud growth, and why hyperphosphorylation is dependent upon membrane trafficking events that drive cell growth, but is seemingly independent of the events of mitotic spindle assembly and chromosome segregation. In this model, the *Gin4*-related kinases would be direct molecular sensors of a critical event that drives cell growth (*i.e.*, delivery of anionic phospholipids to the plasma membrane). The data are also consistent with a model in which the *Gin4*-related kinases relay a growth-dependent signal generated by another mechanism.

The growth-dependent signaling hypothesis could also explain why the maximal extent of *Gin4* and *Hsl1* hyperphosphorylation achieved in mitosis appears to be reduced in poor nutrients. We hypothesize that the reduction in cell size at completion of metaphase in poor nutrients is driven by nutrient-dependent signals that reduce the threshold activity of *Gin4*-related kinases required for mitotic progression.

Measuring cell growth cannot be the sole function of *Gin4*-related kinases. *Gin4*-related kinases are required for the organization of septins at the bud neck, and for controlling the pattern and location of growth. *Gin4*-related kinases also appear to be embedded in the Target of Rapamycin Complex 2 (TORC2) network, which influences cell size and growth rate, although the functional relationships between the TORC2 network and *Gin4*-related kinases are poorly understood (Alcaide-Gavilan *et al.* 2018). In each case, *Gin4*-related kinases appear to be closely associated with signals that influence various aspects of cell growth. Thus, it is possible that the mechanisms that measure growth are embedded in the mechanisms that drive and coordinate the events of growth.

Theoretical analysis has shown that cell size control can be achieved by an “adder” mechanism, in which a constant increment of growth is added during each cell cycle (Campos *et al.* 2014). In the adder model, cells measure growth rather than size. Adder behavior has been reported in cells ranging from bacteria to vertebrates, yet a mechanistic explanation for how growth could be measured has remained elusive (Campos *et al.* 2014; Cadart *et al.* 2018). Here, we propose that growth-dependent activation of *Gin4*-related kinase events could be part of an adder mechanism that measures bud growth. Thus, delivery of signaling lipids to sites of growth could be the critical event that is monitored to measure bud growth. Growth-dependent signaling could be broadly relevant, as it would be readily adaptable to cells of diverse size and shape. It could also influence cell shape by controlling the extent of growth at specific locations on the cell surface. Further analysis of the mechanisms that drive growth-dependent signaling should yield new insights into control of cell growth and size.

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