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Cell growth and nutrient availability influence the mitotic exit signaling network in budding yeast

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MOLECULAR, CELL, AND DEVELOPMENTAL BIOLOGY

By

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

CELL GROWTH AND NUTRIENT AVAILABILITY INFLUENCE THE MITOTIC EXIT SIGNALING NETWORK IN BUDDING YEAST

The adult human body is made up of trillions of cells and yet began as just one. This could not be possible without careful coordination of cell growth and cell division. The molecular mechanisms that drive and control cell growth as well as its coordination with the cell cycle remain poorly understood.

Cell size is the outcome of cell growth. Thus, a decoupling of growth and division would result in cells of increasingly heterogenous sizes. Interestingly, the severity of a cancer is often correlated with increasingly heterogenous size of cells within a tissue. Furthermore, genes that are thought to play important roles in cell growth are often dysregulated in a variety of cancers. This suggests that the misregulation of mechanisms that control cell growth and cell size plays a role in cancer cell development.

How cell growth influences cell cycle progression to control cell size is perhaps best understood in budding yeast where key cell cycle transitions are delayed until sufficient growth has occurred. It had been long believed that the predominant cell size checkpoint in budding yeast occurred at cell cycle entry. Recent work, however, suggests that bud growth strongly influences cell cycle progression in mitosis. In this thesis, I present evidence to support a model in which cells influence the extent and duration of bud growth by controlling mitotic exit, a cell cycle transition that seizes bud growth and finalizes daughter cell size.

Dedications and acknowledgements

I dedicate this dissertation to my family, without whom this would not have been possible. My parents have provided unwavering love, support, and patience during this nearly decade-long journey that has tested me both professionally and personally. To my sister and brother-in-law, Carolina and Sam, who are in so many ways what I strive to be. With their seemingly endless supply love and guidance they have been both therapists and friends. With them I have grown in ways I could not have imagined. And to my extended family, there are too many to name, but they all have played important roles in who am I today, thank you.

To my advisor, Dr. Doug Kellogg, whose mentorship has been instrumental. If a coach were to take someone from spectating a sport to understanding it and being able to practice it, then that's what you have done for me with science. Whether it be troubleshooting a protocol or preparing for qualification exams, I 'll miss learning from such a great scientific mind. I am grateful to have trained under your guidance and honored to say I've come from the Kellogg Lab.

And a message to any fellow graduate student reading this: "leave the beach cleaner than you left it" (*My Dad et al.*). After having been consumed by competitive soccer my whole life, research appealed to me in part because science is advertised as being uncompetitive. Competition, like in sport, implies a zero-sum game. So, if science in its purest form is a collective and collaborative effort towards

the pursuit of knowledge, then it is inherently not a zero-sum game. Thus, the toxic, self-imposed culture of measuring our self-worth in the number of hours spent in lab or the journal our work is published in is at odds with the notion that, in the pursuit of knowledge, one's gain is not at the expense of another. We as graduate students are a special cog in the academia machine with the power to change its rhythm. I don't pretend to have torn up trees. In fact, I often it difficult to find where I could "do my part". I eventually found my pocket in TAing and mentoring at the bench but my message is simply this: pay it forward.

In the time I have been in graduate school I have lived through two graduate student strikes, a pandemic, the George Floyd riots, apocalyptic fires, global and national sociopolitical unrest, and unrest within the department, to name a few. At times it has been difficult to feel optimistic of the future of the planet and society. So it was interesting that I came across this quote from Mahatma Ghandi on the eve of submitting this dissertation: "friendship that insists upon agreement on all things is not worthy of the name".

Chapter I: Introduction

Part I: On being the right size

How do cells control their size? What determines cell size? Even though these questions are largely absent from modern textbooks they have puzzled scientists for at least a century. In 1926 J.B.S Haldane mused in his essay, *On Being the Right Size*, over the relationship between the size of an animal and its life strategy (Haldane, 1926). Why does a mouse require larger eyes relative to its body size compared to a whale? In the same year, a German scientist showed that cell division of *Amoeba proteus* cells could be indefinitely inhibited by repeated amputation of cytoplasmic extensions (Hartmann, 1926). Amputated cells rapidly reset their nuclear to cytoplasmic ratio and resumed cell growth, yet cell division failed to occur so long as the dissection was repeated once the cell regained its preoperative size. Once the dissections were stopped cell division resumed normally, suggesting that the failure in cell division was not due to defects in basic cellular functions. Rather, it suggested that cell growth and/or cell size influenced cell cycle progression. His seminal work was corroborated in 1956 (Prescott, 1956).

In the late 70's groundbreaking work in yeast pushed the pursuit to understand cell size from musings and observations to a focused endeavor. Using the famous cell division cycle (cdc) mutants it was observed that budding yeast cells that were arrested at various points in the cell cycle continued cell growth (Johnston

et al., 1977b, 1977a). The finding that cell growth could be decoupled from cell cycle progression suggested that cell growth was a cellular process independent of cell cycle progression. They also reported that not only are daughter cells born at a smaller size than the mother but also that the daughter cell had a prolonged period of growth before budding compared to the mother (Hartwell and Unger, 1977). These observations became the basis of further studies that together pointed to the existence of a cell size checkpoint in G1 phase that inhibits cell cycle entry until sufficient growth has occurred (Johnston *et al.*, 1977b, 1977a; Nash *et al.*, 1988; Cross, 1990; Sommer *et al.*, 2021). Work done around the same time in fission yeast suggested the existence of a analogous cell size threshold at mitotic entry (Fantes and Nurse, 1977). The suggestion of a conserved link between cell growth or size and cell cycle transitions suggested the possibility that the signaling mechanisms which regulate this connection may also be conserved.

These early bodies of work laid the foundation for a field seeking to understand a fundamental aspect of cell biology: what are the molecular mechanisms that control cell growth and cell size and how are they coordinated with cell cycle progression?

Part II: A different view of the cell

The basic unit of life is the cell. Thus, cell growth is a fundamental requirement of life. For single cells, the extent of growth during a cell cycle defines cell size and must be controlled to maintain a size that is in balance with the nutrient conditions in the environment. Insufficient cell growth prior to division would result in increasingly small cells that would be outcompeted. Excessive cell growth during the cell cycle would result in large cells that exceed the biosynthetic capacity of its environment. No control over the extent of cell growth would result in a population of cells heterogenous in size. Thus, the earliest cells would have had to evolve mechanisms to control the duration and extent of growth during the cell cycle to ensure that division occurs at an appropriate cell size. This suggests that there are universal mechanisms for control of cell growth and cell size that are as conserved as the mechanisms that control DNA replication and the cell cycle.

Part III: Cell size control in human health and disease

A discussion of the mechanisms that control cell growth and size in budding yeast is found in *Part IV.* This section highlights insightful observations that have been made in multicellular organisms. For instance, a survey of the different cell tissues of the human body shows that cell size ranges over several orders of magnitude between different cell types. Yet, cells of a particular type are

homogenous around an average cell size. This highlights that cells of different cell types can and must undergo varying amounts of growth, while the extent of cell growth within a specific type is tightly controlled.

Defects in cell size are strongly correlated with human disease (Brimo *et al.*, 2013; Hoda *et al.*, 2018; Asa, 2019; Asadullah *et al.*, 2021; Gothwal *et al.*, 2021; Sandlin *et al.*, 2022). In cancer, for example, defects in cell size, shape, and nuclear-to-cytoplasmic ratio are strongly correlated with malignancy and have formed the basis of cancer pathology for almost a century. A system based on the standardization of cytological atypia (abnormal cell shape and size) as a means of grading the severity of prostate adenocarcinomas was first introduced in 1966 by Dr. Donald Gleason and is referred to as the Gleason Score. It is still used today despite advances in medical diagnostics (Brimo *et al.*, 2013; Chen and Zhou, 2016). Cell size defects are not limited to prostatic cancers as similar tests have been adopted for cancers of different etiologies, suggesting that dysregulation of mechanisms that regulate cell size and/or growth may be a key step in tumorigenesis (Epstein *et al.*, 2005; Trpkov, 2015; Chen and Zhou, 2016). Notably, one study found that only cells with high cytological atypia were capable of tumorigenesis when transplanted into mouse cheek, suggesting that cellular atypia may not be a simple consequence of general cellular dysfunction (Caspersson *et al.*, 1963) Nearly a century later the molecular basis of cytological atypia and cellular

dysplasia in cancer pathology remains unknown. Thus, novel strategies to target cancer cells will likely be found from discovering the mechanisms that regulate cell growth and size in normal cells and the roles their dysfunction plays in mammalian diseases.

Insights into how cell growth and cell size are regulated in mammalian cells can be found outside of the context of cancer as well. For instance, when the liver of a canine is transplanted into a similarly sized dog the size of the liver goes unchanged post-operatively. However, a liver transplanted into a larger dog will quickly grow to a size which is characteristic of the larger animal (Kam *et al.*, 1987; Francavilla *et al.*, 1988). This phenomenon has also been reported in rat kidney, suggesting that organ size homeostasis may be conserved in mammals (Silber, 1976).

Additionally, changes in kidney cell size and organ size occurs during normal adaptive responses and diabetes (Chen *et al.*, 2005, 2009; Sakaguchi *et al.*, 2006; Boehlke *et al.*, 2010; Lieberthal and Levine, 2012). For example, when one kidney is surgical removed from a mouse, the remaining kidney enlarges by approximately 40% within 20 days as a result of increases in cell size rather than cell number (Chen *et al.*, 2005, 2009). While the mechanisms behind this adaptive response are unknown, it nonetheless requires careful control of cell growth, cell division, and cell number.

Additional observations regarding cell size, cell growth, and organ size have been made in other organs; however, suffice it to say that these data suggest that control of cell growth and cell size likely play an important role in mammalian physiology and disease.

Part IV: Cell growth, cell size, and the budding yeast cell cycle

Early studies in yeast and humans cells observed that cell size within a population is smallest at cell cycle entry and highest at mitotic exit (Killander and Zetterberg, 1965; Johnston *et al.*, 1977a). And, as noted in Chapter I, budding yeast cells divide at a point where the daughter cell is smaller than the mother. The daughter cell will then undergo a prolonged period of growth in G1 phase relative to the larger mother cell before initiating bud formation. This difference in the period of growth prior to cell cycle entry between mother and daughter cell is exacerbated when cells are grown under poor nutrient conditions, where daughter cells are born smaller (Hartwell and Unger, 1977; Johnston *et al.*, 1977b; Leitao and Kellogg, 2017; Sommer *et al.*, 2021). Finally, newborn daughter cells that are switched from poor to rich carbon conditions in G1 phase rapidly reset their growth rate and initiate budding at a larger size relative to unshifted cells (Sommer *et al.*, 2021). Early studies found that fission yeast cells that are rapidly shifted to a richer carbon source rapidly reset the entry to mitosis, indicating conserved mechanistic

links between growth, nutrients, and cell cycle progression (Fantes and Nurse, 1977). Together, these data suggested that cell cycle entry, a critical commitment point, is delayed until cells achieve a critical threshold amount of growth and/or cell size.

Based on their observations, Hartwell et al. proposed a model for cell size control in yeast. It was suggested that modulation of cell cycle progression in response to growth and nutrient dependent signals occurred predominantly in G1 phase while the period of bud growth was invariant with respect to nutrient condition. Thus, this proposed model predicted that daughter cells are born at a smaller size in poor carbon due simply to a reduced growth rate.

However, recent evidence indicates that cell growth and size is regulated beyond G1 phase. Key components of the cell cycle machinery in G1 phase are influenced by growth and nutrient-dependent signals, such as the early G1 phase cyclin Cln3. However, cells lacking Cln3 and other upstream regulators of the late G1 cyclins, Cln1/2, remain capable of mitotic entry and nutrient modulation of cell size (Jorgensen and Tyers, 2004). The break though came over a decade later when novel evidence demonstrated that most cell growth during the cell cycle occurs during the interval of bud growth. It further showed that bud growth occurs predominantly during in mitosis (Leitao and Kellogg, 2017). Moreover, both the rate of bud growth and mitotic progression were modulated by nutrient availability.

Thus, while the rate of bud growth in poor carbon is reduced the duration of bud growth was extended. One interpretation of this result is that cells prolong the duration of bud growth in poor carbon to compensate for the reduced growth rate to ensure that sufficient bud growth occurs before division.

Lastly, the conserved Protein Phosphatase 2A in association with its regulatory subunit Rts1 ($PP2A^{Rts1}$) has been identified as being required for nutrient modulation of cell size (Artiles *et al.*, 2009). And proteome-wide mass spectrometry analysis of $PP2A^{Rts1}$ targets corroborated evidence indicating that it controls key regulators of mitotic progression as well as cell cycle entry (Zapata *et al.*, 2014; Leitao *et al.*, 2019).

Together, these data demonstrate that the interval of bud growth during mitosis is regulated by mechanisms that coordinate nutrient-dependent signals and cell growth with mitotic progression to strongly influence cell size. The evidence presented in this thesis suggests that cells modulate the duration and extent of bud growth by regulating a conserved signaling network that is required for mitotic exit. In doing so, this body of work challenges canonical views of the physiological signals that regulate this key cell cycle transition.

Chapter II (Published results): Cell growth and nutrient availability control the mitotic exit signaling network in budding yeast

Introduction

In all orders of life, cell cycle progression is dependent upon cell growth, which ensures that dividing cells attain sufficient volume and mass so that cell division produces viable cells of an appropriate size (Jorgensen and Tyers, 2004; Turner et al., 2012; Ginzberg et al., 2015). The threshold amount of growth required for cell cycle progression is reduced in poor nutrients, which leads to a large reduction in cell size (Kellogg and Levin, 2022). The reduced growth threshold in poor nutrients likely confers a competitive advantage, as it allows cells to divide and proliferate more rapidly when nutrients become limiting. The mechanisms that set and modulate the threshold amount of growth required for cell cycle progression are largely unknown.

In budding yeast, most cell growth takes place during growth of the daughter bud, which occurs almost entirely during mitosis (Leitao and Kellogg, 2017). Bud growth is required for progression through mitosis, and nutrient availability strongly influences the rate, duration and extent of bud growth (Anastasia et al., 2012; Leitao and Kellogg, 2017). Thus, in poor nutrients the rate of bud growth is reduced, yet the duration of bud growth is extended, presumably to

allow more time for bud growth. In effect, the threshold amount of bud growth required for completion of mitosis is reduced, leading to birth of very small daughter cells. These observations suggest the existence of nutrient and growth-sensing mechanisms that modulate the duration and extent of bud growth to ensure that daughter cells are born at an appropriate size.

As the bud grows during mitosis, approximately equal volumes are added in metaphase and anaphase (Leitao and Kellogg, 2017). Nutrient availability modulates the duration and extent of bud growth in both intervals, and recent work suggests that the two intervals are controlled by different mechanisms (Leitao et al., 2019; Jasani et al., 2020). The metaphase interval is influenced by a pair of related kinases called Gin4 and Hsl1. Both kinases are required for normal control of bud growth and undergo gradual hyperphosphorylation and activation that appear to be dependent upon bud growth and proportional to the extent bud growth, which suggests that they play roles in measuring growth (Jasani et al., 2020)7/19/23 2:00:00 PM. Once they have been fully activated, Gin4 and Hsl1 promote mitotic progression by inhibiting Swe1, the budding yeast homolog of the conserved Wee1 kinase that directly phosphorylates and inhibits mitotic Cdk1/cyclin complexes (Ma et al., 1996; Longtine et al., 2000). In both mammalian cells and budding yeast, it is thought that Wee1 keeps mitotic Cdk1 activity low during metaphase and that inhibition of Wee1 leads to a full activation of Cdk1 that drives the metaphase to

anaphase transition (Deibler and Kirschner, 2010; Harvey et al., 2011). Gin4 and Hsl1 are required for normal control of the duration and extent of bud growth in metaphase but are not required during anaphase/telophase (Leitao et al., 2019; Jasani et al., 2020). These data suggest that growth in anaphase/telophase is regulated by mechanisms that are distinct from those that work in metaphase. The mechanisms by which nutrients modulate the duration and extent of bud growth in anaphase are unknown.

Here, we used proteome-wide mass spectrometry to search for proteins that influence the duration and extent of bud growth in late mitosis. The results point to the Mitotic Exit Network (MEN), an essential signaling network that includes highly conserved proteins, as a likely target of signals that link mitotic exit to cell growth and nutrient availability. Previous studies suggested that the MEN links mitotic exit to mitotic spindle orientation and elongation (Bardin et al., 2000; Pereira et al., 2000; Campbell et al., 2020). Our studies establish for the first time that key components of the MEN also respond to signals associated with bud growth and nutrient availability.

Results

Multiple components of the mitotic exit network are controlled by nutrient-dependent signals

Previous studies found that nutrients modulate the duration and extent of bud growth in both metaphase and anaphase, and that changes in nutrient availability immediately influence cell cycle progression and the threshold amount of growth required for cell cycle progression (Fantes and Nurse, 1977; Leitao and Kellogg, 2017). The data suggest that a reduced growth rate caused by a shift to poor nutrients triggers an immediate mitotic delay to allow more time for bud growth (Leitao and Kellogg, 2017). Therefore, to identify signals that could play a role in modulating the duration and extent of growth in mitosis, we used proteome-wide mass spectrometry to search for proteins that undergo large changes in phosphorylation in response to a rapid shift from rich to poor carbon during mitosis. We reasoned that these could include proteins that play roles in setting the threshold amount of bud growth required for cell cycle progression.

Cells growing in medium containing a rich carbon source (YP + 2% dextrose) were released from a G1 arrest and rapidly shifted to poor carbon medium (YP + 2% glycerol/2% ethanol) at 90 minutes, when the mitotic cyclin Clb2 reached peak levels (Figure 1). Proteins were isolated from the shifted and unshifted control cells 10 minutes after the shift and proteolytic peptides were analyzed by quantitative

proteome-wide mass spectrometry to search for changes in phosphorylation. The ratio of phosphorylated to unphosphorylated peptides in shifted versus unshifted control cells was log2 transformed. Thus, a negative log2 ratio indicates a loss of phosphorylation in response to a shift to poor carbon, whereas a positive log2 ratio indicates a gain of phosphorylation. A total of three biological replicates were analyzed, which allowed calculation of average log2 ratios and standard deviation. The complete data set is shown in Table S1.

We searched the mass spectrometry data for proteins involved in mitotic progression and cell growth, which are summarized in Table 1. A log2 ratio of +/- 1.32, corresponding to a 2.5-fold change in phosphorylation occupancy, was used as an arbitrary cutoff to define substantial changes in phosphorylation. Numerous components of a signaling network that regulates exit from mitosis, referred to as the "mitotic exit network" (MEN), were amongst the proteins that showed the largest loss of phosphorylation when cells were shifted from rich to poor nutrients in mitosis. These proteins include Net1, Cdc14, Lte1, Cdc15, Bfa1, Nud1, and Zds1. Previous work suggested that the MEN plays a central role in triggering mitotic exit by initiating destruction of the mitotic cyclin Clb2 and inhibition of mitotic Cdk activity (Visintin et al., 1998, 1999; Jaspersen et al., 1999; Shou et al., 1999; Stegmeier et al., 2002). It has been proposed that the MEN initiates mitotic exit in response to signals generated by proper orientation of the mitotic spindle in the

daughter bud (Bardin et al., 2000; Pereira et al., 2000). Here, the discovery that numerous components of the mitotic exit network show large changes in phosphorylation in response to a shift to poor nutrients indicates that the MEN is strongly regulated by nutrient-dependent signals. Since the MEN can influence the duration of late mitosis, the data further suggest that the MEN could be the target of nutrient-dependent signals that influence the duration and extent of bud growth in late mitosis.

The mass spectrometry analysis also identified multiple components of TOR Complex 2 (TORC2) and its surrounding signaling network (Table 1). These proteins include Avo2, Tsc11, Ypk1, Mss4, Pkh2, Gin4, Hsl1, Rts1, and Prk1. The identification of components of the TORC2 network is consistent with previous studies that found that TORC2 signaling is required for nutrient modulation of cell size and growth rate (Lucena et al., 2018). As an example, loss of Rts1, a regulatory subunit for PP2A, causes misregulation of TORC2 signaling, as well as a complete loss of the proportional relationship between cell size and growth rate during bud growth (Lucena et al., 2018; Leitao et al., 2019).

Finally, the analysis identified several core components of the COPII complex that is required for ER to Golgi transport (Sec16 and Sec31) as well as a key regulator of ribosome biogenesis (Sch9) (Table 1). Since a shift to poor nutrients leads to a rapid reduction in growth rate, these findings suggest that poor nutrients

trigger signals that rapidly reduce the rates of both ribosome biogenesis and membrane growth to match reduced biosynthetic rates in poor nutrients.

Cellular Process	***Site Position***	***Sequence***	***A-score***	***# of peptides***	***Log2(Poor/Rich)***
Mitotic Exit Network					
NET1					
	212	VST#PLAR	0	3	-4.497
	259	ISS#GIDAGK	24.43	6	-4.409
	280	S#ATVDPDK	30.83	2	-4.343
	362	ITS#GM*LK	24.43	3	-4.259
	388	EGPSSPAS#ILPAK	5.08	3	-3.419
	270	SS#IVEEDIVSR	9.34	1	-3.180
	447	S#QSSIADNNGS#PVK	100.02	3	-3.059
	301;304	LLSGT#PIM*STMT#PNR	4.08	3	-2.740
	166	LNNGS#PQSVQPQQQIPSSSGVLR	15.01	2	-1.640
	1164	S#ASAALGK	30.83	2	-1.331
	48	TNM*AQSAGDAS#LQYANLR	33.66	2	-1.315
CDC14					
	537	RTTS#AAGGIR	6.59	1	-2.903
	494;497	KNDISS#ASS#SR	17.01;9.34	1	-1.590
	429	SSAVPQTS#PGQPR	30.83	5	-1.533
LTE1					
	789;793	VQS#IAIT#PTK	110.63	3	-2.413
	718	ST#IDGLEK	24.44	1	-2.220
	779	QAVVRPAS#GR	1000	1	-1.857
	799	ELS#IVDPEQNK	1000	2	-1.481
CDC15					
	940	VSSVT#AAIGSS#PTK	2.81	1	-1.668
BFA1					
	317	HCS#NQNVQLNGPAK	1000	1	-1.614
NUD1					
	352	APS#ILDK	1000	1	-1.469
TORC2 Signaling					
AVO2					
	305	VNS#INVK	1000	1	-3.872
	273	SS#ITNPVFNPR	17.01	2	-2.293
	330;333	HSAT#PTS#PHNNIALINR	4.08;6.59	1	-2.061
	310	T#PVGVS#PK	1000	1	-1.432
	310;315	T#PVGVS#PK	1000;1000	2	-1.387
TAP42					
	251;260	ESNDDDS#TGFT#DK	85.26;13.38	1	-3.872
GIN4					
	471	RAS#VINVEK	1000	3	-3.100
	382;389	RQS#ISSVS#VSPSK	20.17	1	-2.569

Cellular Process	***Site Position***	***Sequence***	***A-score***	***# of peptides***	***Log2(Poor/Rich)***
	805	HFS#ESNK	30.83	2	-2.338
	639	SIS#APM*ENEEK	0	1	-1.648
	384	RQS#IS#SVSVSPSK	13.21	1	-1.594
YPK1					
	57	GT#INPSNSSVVPVR	59.30	5	-2.807
	57;64	GT#INPS#NSSVVPVR	59.30;17.01	4	-2.017
HSL1					
	899	NIS#QPVNSK	9.30	1	-2.790
	1210	ETT#EEILSK	9.34	2	-2.082
	641	AIHAS#PSTK	33.66	1	-1.893
	629;631;632	SPS#RY#S#LSR	30.83;24.24;17.01	1	-1.848
BIT61					
	164	AS#GFFNR	1000	1	-2.721
TSC11					
	19	S#VTNTTPLLTPR	48.87	1	-2.372
	24;28	SVTNT#TPLLT#PR	9.34;65.44	1	-2.176
	50;52	NITSSS#PS#TITNESSK	24.43;9.34	1	-1.936
	251	NSQQNLNRNS#TVNSR	6.59	1	-1.742
	19;28;32	S#VTNTTPLLT#PRHS#R	48.87;65.44;63.08	1	-1.586
AVO1					
	537	DTVIS#GK	33.66	1	-2.332
	543	EPTS#LTSSNR	9.34	2	-1.872
	588	VSDS#VLHR	6.59	3	-1.704
	326	SHFPTS#QK	0	1	-1.632
MSS4					
	341	RSES#ATAEIK	20.17	3	-2.039
	238	HS#QILPM*DDSDVIK	88.82	2	-2.025
PKH2					
	1005	THSQS#PSIS#K	11.01	4	-1.585
PRK1					
	488	LS#PTIT#SK	30.83	1	-1.506
SLM2					
	7;11	NS#ARAS#LDLR	1000;1000	1	-1.466
Secretory Pathway					
SEC16					
	1961	AS#TNQYR	24.44	2	-3.849
	759	GVS#NAS#VGSSASFGAR	62.66	3	-3.163
	2144	TSPSPTGPNPNNSPSPS#S#PISR	13.38	3	-2.022
	759;762	GVS#NAS#VGSSASFGAR	62.66; 87.26	5	-1.992
	806	YAPVS#PTVQQK	6.59	5	-1.882
	766;768	GVSNASVGSS#AS#FGAR	4.08; 17.01	1	-1.407
SEC31					
	980	APSS#VSM*VS#PPPLHK	71.34	1	-1.611
	988;992	NS#RVPS#LVATSESPR	113.18; 99.28	6	-1.514
	988	NS#RVPS#LVAT#SESPR	113.18	3	-1.475
Ribosome Biogenesis					
DOT6					
	322;326;331	RSS#FNVS#SNNT#SR	24.44; 0; 9.34	1	-4.104

Cellular Process	Site Position	Sequence	A-score	# of peptides	Log2(Poor/Rich)
	322	RSS#FNVSSNNTSR	24.44	2	-3.133
	247	SNS#HSFTNSLNQDPIVR	11.01	2	-1.687
	322;331	RSS#FNVS#SNNTSR	24.44; 9.34	4	-1.424
TOD6					
	300;304	NSHS#VISS#R	37.54; 9.34	1	-3.938
	298;304	NS#HSVIS#SR	13.38; 9.34	1	-3.484
	318;329	RSS#FNSHAPTEPIS#R	0; 12.43	1	-2.103
	298	NS#HSVIS#SR	13.38	1	-1.956
	298;300	NS#HSVISS#R	13.40; 37.54	1	-1.382
STB3					
	337;341	LTATS#EPTS#R	13.38; 17.25	4	-3.382
	341	LTATS#EPTS#R	17.25	2	-3.144
	336;341	LTAT#SEPT#SRR	24.44; 17.25	6	-2.754
	336	LTAT#SEPT#SRR	24.44	1	-1.767
IFH1					
	1041	RQS#MVEAAAENLR	1000		-2.340
ENP1					
	5;6	M*ARAS#S#TK	4.08; 6.59	1	-1.504
SCH9					
	288	S#S#SQLDQLNSCSSVTDPSK	5.75	4	-1.397

Table 2.1. A summary of key peptides that undergo substantial changes in phosphorylation in response to a shift to poor carbon during mitosis. The A-score indicates the likelihood a phosphorylation site is correctly localized within a peptide. A given phosphorylation site is correctly assigned with >95% confidence when the A-score is >13. An A-score of 0 indicates that the phosphorylated residue within the peptide sequence cannot be precisely determined. An asterisk (*) represents oxidation of the preceding methionine, an artifact of the sample preparation. A hashtag indicates that the preceding amino acid is likely the relevant phosphorylation site.

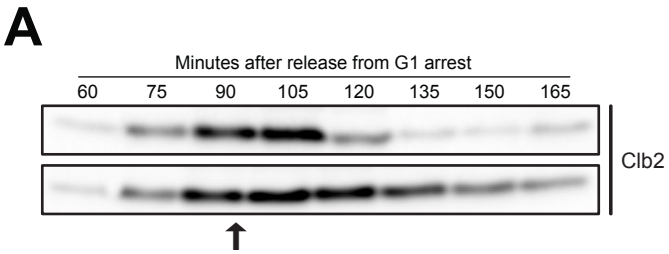


Figure 2.1. A shift to poor carbon in metaphase causes a mitotic delay. Cells were grown overnight in YPD and arrested in G1 phase with alpha factor. The cells were

the released from arrest into fresh YPD at 25°C. At the point indicated by the arrow, half of the culture was shifted to poor carbon (bottom panel). Samples were collected at the indicated time points to analyze Clb2 levels by western blot. Samples for mass spectrometry analysis were collected ten minutes after the shift to poor carbon.

Net1 hyperphosphorylation occurs before mitotic exit

The discovery that multiple components of the MEN are regulated in response to changes in carbon source suggested that the MEN could play a role in linking mitotic exit to cell growth and nutrient availability. In this context, the discovery that Net1 phosphorylation is strongly modulated by carbon source was particularly interesting. Net1 showed large decreases in phosphorylation at 11 sites, including one site that has not been detected in any previous studies. Net1 was also identified as a top hit in a mass spectrometry screen for proteins that undergo large changes in phosphorylation in response to an arrest of plasma membrane growth during early mitosis (Clarke *et al.*, 2017). Net1 plays a critical role in initiation of mitotic exit and also controls ribosome biogenesis, which is a major facet of cell growth (Shou *et al.*, 1999, 2001; Straight *et al.*, 1999; Visintin *et al.*, 1999; Shou and Deshaies, 2002; Hannig *et al.*, 2019). Net1 is localized in the nucleolus early in the cell cycle, where it binds and inhibits Cdc14 (Shou *et al.*, 1999; Visintin *et al.*, 1999; Traverso *et al.*, 2001). Hyperphosphorylation of Net1 is thought to be a critical step that helps release Cdc14 from Net1 so that it can leave the nucleolus to initiate mitotic exit (Shou *et al.*, 1999; Yoshida and Toh-e, 2002; Visintin

et al., 2003). In previous work we found evidence that the events of bud growth generate signals that are dependent upon growth and proportional to growth (Anastasia *et al.*, 2012; Jasani *et al.*, 2020). We therefore considered a working hypothesis in which the events of bud growth generate a signal that contributes to Net1 phosphorylation and helps trigger activation of the MEN, while nutrient-dependent signals modulate the strength of the growth-dependent signal needed to initiate mitotic exit.

Since hyperphosphorylation of Net1 is thought to be a key early step in the initiation of mitotic exit, we carried out new experiments to determine whether Net1 phosphorylation is influenced by nutrients or growth, which would begin to test the hypothesis that the MEN links mitotic exit to cell growth. Previous studies reported that hyperphosphorylation of Net1 can be detected via electrophoretic mobility shifts; however, we found that the large size of Net1 made reproducible detection of these shifts difficult. We therefore developed an additional tool for detection of Net1 phosphorylation. We reasoned that expression of a truncated version of Net1 could provide a readout of phosphorylation while also allowing easier detection due to the reduced size of the truncated Net1. We analyzed proteome-wide mass spectrometry data from multiple studies that have been collated on BioGrid, which indicated that most phosphorylation of Net1 occurs in the first two thirds of the protein. We therefore constructed a plasmid that expresses a version of Net1 that

lacks the last 328 amino acids and is tagged with 3xHA (Net1$^{\Delta 328}$-3xHA). The truncated Net1 is expressed from the endogenous promoter and can be integrated at URA3 so that cells express both full-length Net1 as well as the truncated reporter. The reporter includes an N-terminal Cdc14 binding site that is thought to undergo phosphorylation that helps release Cdc14 (Traverso *et al.*, 2001).

Current models suggest that phosphorylation of Net1 is a critical event that helps initiate mitotic exit, which would suggest that Net1 phosphorylation is initiated late in mitosis during mitotic exit. However, in a previous study it appears that phosphorylation of Net1 is initiated early in mitosis as mitotic cyclin levels are rising, which could suggest that Net1 phosphorylation is initiated before mitotic exit (Visintin *et al.*, 2003). To further investigate, we tested whether arresting cells at metaphase, before mitotic exit, blocked hyperphosphorylation of Net1. To do this, we released cells from a G1 phase arrest into YPD medium containing benomyl, which depolymerizes microtubules and activates a mitotic spindle checkpoint that arrests cells before metaphase. Depolymerization of microtubules will also eliminate any signals generated by proper orientation of the mitotic spindle, which is thought to be a critical trigger for mitotic exit. As a control, we also released cells from the arrest into YPD medium that does not contain benomyl. Phosphorylation of both full length Net1 and Net1$^{\Delta 328}$ was analyzed by western blot to detect electrophoretic mobility shifts, and levels of the mitotic cyclin Clb2 were analyzed as

a molecular marker for mitotic progression. In the control cells without benomyl, both Net1 and Net1$^{\Delta 328}$ underwent hyperphosphorylation as cells progressed through mitosis, with peak phosphorylation appearing to occur shortly after peak Clb2 levels, which likely corresponds to anaphase/telophase (Figure 2.2A,B). Both Net1 and Net1$^{\Delta 328}$ underwent extensive hyperphosphorylation when cells were arrested before mitotic exit with benomyl. The extent of Net1 hyperphosphorylation was greater in benomyl-arrested cells compared to the control cells, which will be addressed further below.

To further test whether Net1 phosphorylation occurs before mitotic exit we used depletion of Cdc20 as an alternative means of arresting cells in metaphase (Figure 2.2C). Again, Net1 underwent full hyperphosphorylation in metaphase-arrested cells.

These results show that hyperphosphorylation of Net1 that can be detected via electrophoretic mobility shifts does not depend on the events of mitotic exit or any functions of the mitotic spindle.

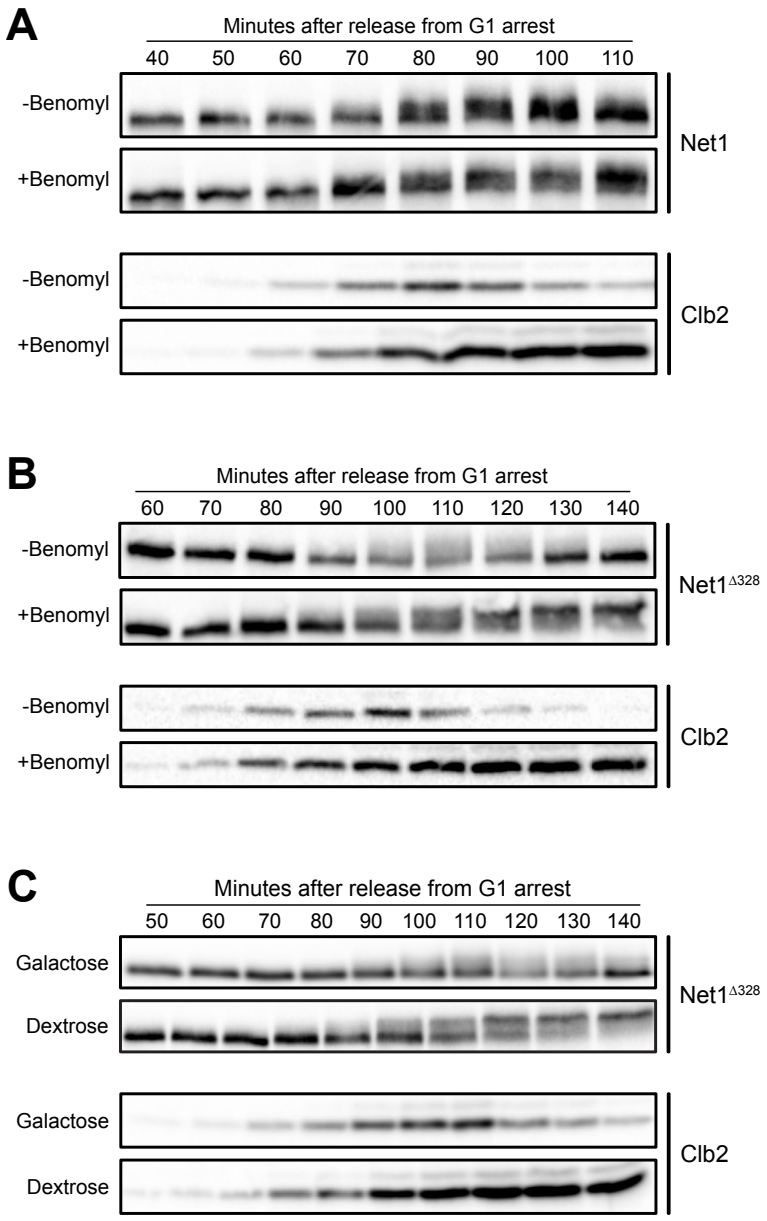


Figure 2.2. Net1 hyperphosphorylation occurs before mitotic exit. *net1-6xHA* (A,B) or *net1$^{\Delta 328}$-3xHA* (C,D) cells growing in YPD were released from a G1 arrest at 25°C into YPD or YPD containing benomyl. Samples were collected at the indicated time points to assay Net1 phosphorylation and Clb2 protein levels by western blot. Alpha factor was added back to control cells at 60 minutes after release to prevent a second cell cycle. (E) Untagged control cells (lanes 1, 2) or *net1-6xHA* cells (lanes 3, 4) were grown overnight in YPD to log phase. Cells from each culture were then transferred to YPD containing benomyl (Lane 2, 4). Samples were collected after three hours for western blot analysis.

Phosphorylation of Net1 is correlated with daughter bud growth

We next analyzed whether Net1 phosphorylation is influenced by carbon source. In previous studies we showed that the durations of both metaphase and anaphase are increased in cells growing in poor carbon, while the extent of bud growth during both intervals is reduced (Leitao and Kellogg, 2017). Cells growing in rich or poor carbon were released from a G1 arrest and Net1 phosphorylation was assayed by western blot (**Figure 2.3**). Clb2 levels were analyzed in the same samples to provide a marker for mitotic progression. In rich carbon, both full length Net1 and Net1$^{\Delta 328}$ underwent hyperphosphorylation during mitosis. In poor carbon, the extent of Net1 phosphorylation was reduced and the duration of Net1 phosphorylation was extended. Since the rate and extent of bud growth are strongly reduced in poor carbon, these observations suggest that extent of Net1 phosphorylation is correlated with the rate and extent of bud growth. Previous work found that Gin4 and Hsl1, which are thought to relay growth-dependent signals before mitotic exit, also undergo hyperphosphorylation that is correlated with the rate and extent of bud growth in early in mitosis (Jasani *et al.*, 2020).

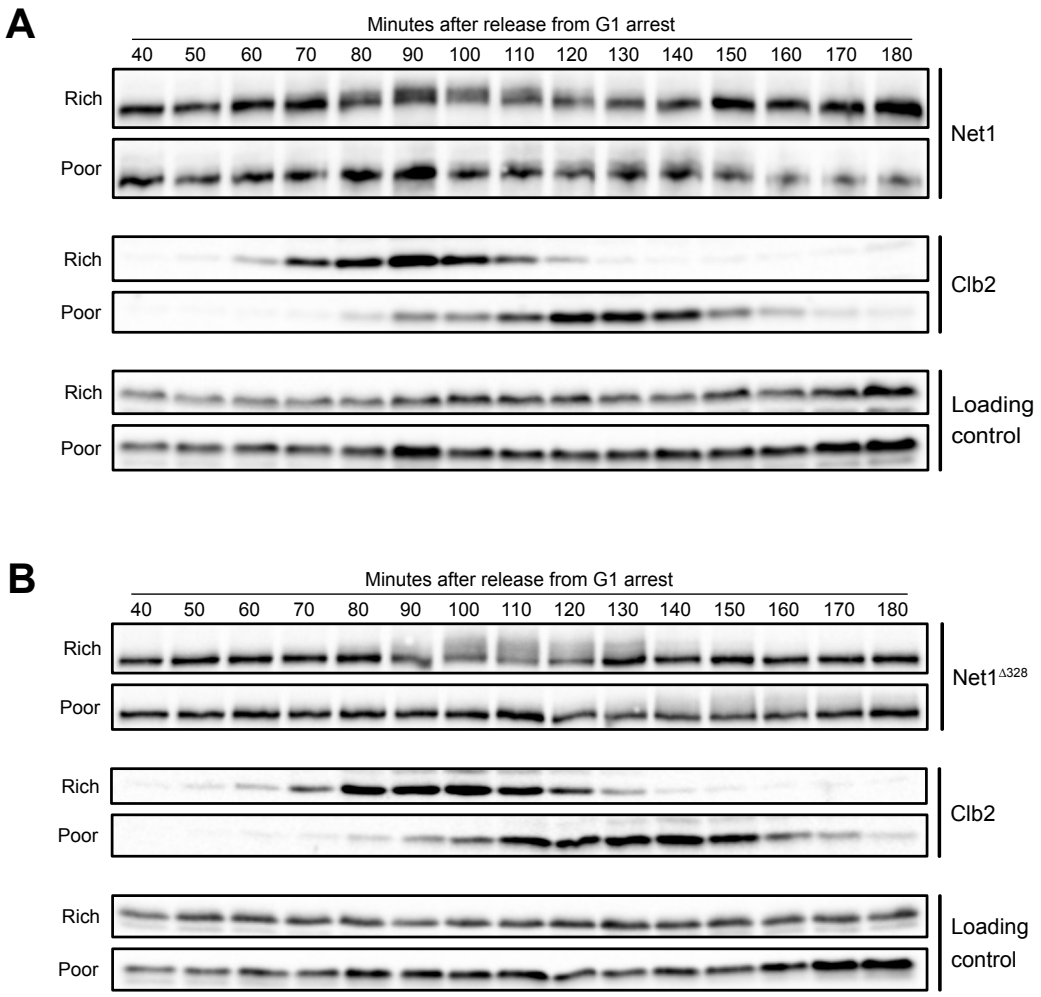


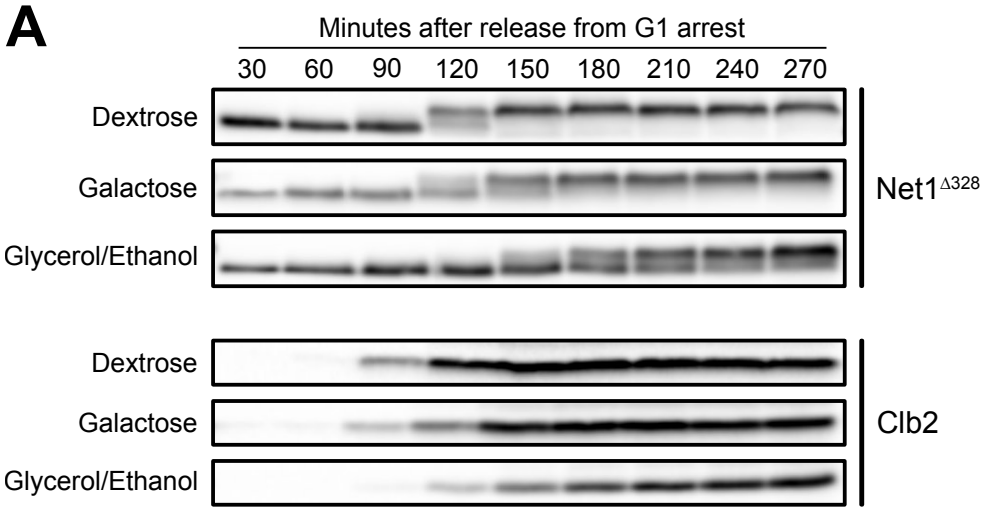
Figure 2.3. Net1 phosphorylation is reduced in poor carbon. *net1-6xHA* (A) or *net1$^{\Delta 328}$-3xHA* (B) cells grown overnight in YPD or YPG/E were released from a G1 phase arrest into fresh medium at 25°C Samples were collected at the indicated time points to assay for Net1 and Clb2 by western blot. An anti-Nap1 antibody was used for a loading control. Alpha factor was added back to the cultures 60 minutes after release to prevent a second cell cycle.

Hyperphosphorylation of Net1 is correlated with the rate and extent of bud growth

To further investigate the relationship between Net1 phosphorylation and cell growth, we took advantage of the fact that bud growth continues during a prolonged mitotic arrest, which leads to growth of unusually large buds as well as an unusually long interval of bud growth (Gihana *et al.*, 2021). We also took advantage of the fact that the rate and extent of bud growth in mitosis can be modulated by the quality of the carbon source present in the culture medium (Leitao and Kellogg, 2017). Thus, we tested whether the extent of Net1 hyperphosphorylation was correlated with growth when bud growth was prolonged and increased by a benomyl arrest, and when the rate of growth during the arrest was modulated by carbon source.

Cells were grown in carbon sources of varying quality and released from a G1 phase arrest into media containing benomyl to arrest the cell cycle before metaphase. The cells were then maintained at the arrest point for a prolonged interval of time. We utilized dextrose as a rich carbon source, galactose as an intermediate quality carbon source, and glycerol/ethanol as a poor carbon source. Samples were taken at regular intervals during bud growth and phosphorylation of the Net1$^{\Delta 328}$ reporter was measured by western blot (**Figure 2.4A**) and median cell size was measured with a Coulter Channelyzer. Cell size was plotted as a function of time to provide a measure of the rate and extent of bud growth (**Figure 2.4B**).

In rich carbon, buds underwent rapid and extensive growth. Net1$^{\Delta 328}$ phosphorylation appeared to go to completion during the prolonged arrest, and the maximal extent of Net1$^{\Delta 328}$ phosphorylation achieved during the prolonged benomyl arrest was greater than in cells going through a normal cell cycle (compare top panels in Figures 2.4A and 2.2B). Since daughter buds grow to be much larger in benomyl-arrested cells, this observation suggests that the extent of Net1 phosphorylation is correlated with the extent of bud growth. In galactose, the rate of bud growth was reduced and it took longer for Net1$^{\Delta 328}$ phosphorylation to go to completion. In glycerol/ethanol, the poorest carbon source, the rate and extent of bud growth were strongly reduced and Net1 failed to reach full hyperphosphorylation. Together, these results provide further evidence that Net1 phosphorylation is strongly correlated with the rate and extent of bud growth.



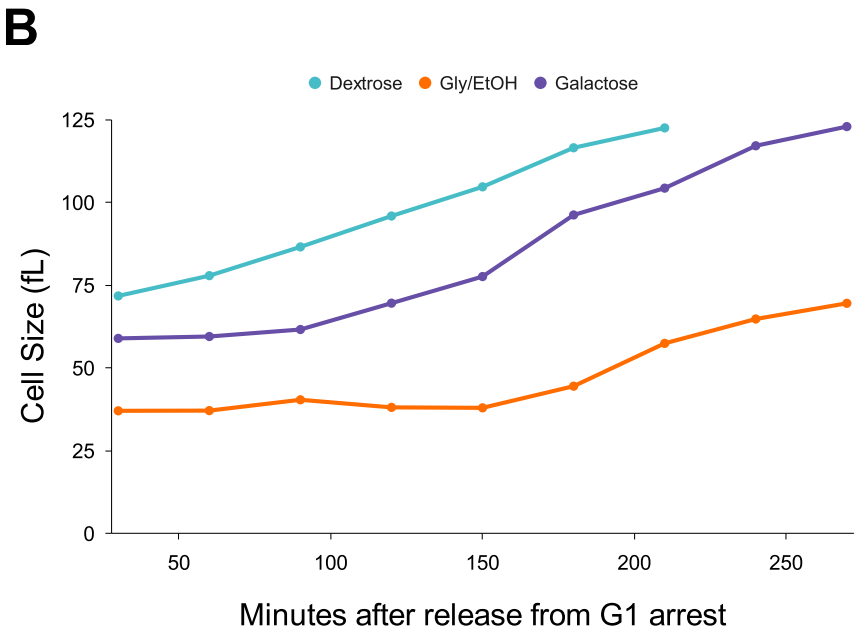


Figure 2.4. The extent and rate of Net1 phosphorylation is correlated with the quality of carbon source. *net1$^{\Delta 328}$-3xHA* cells grown overnight to log phase in YPD, YPGal, or YPG/E were released from a G1 phase arrest at 25°C into fresh media supplemented with benomyl. Samples were taken at the indicated times to assay for Net1$^{\Delta 328}$-3xHA and Clb2. An anti-Nap1 antibody was used for a loading control. (A). Samples were also fixed at the indicated time intervals to measure median cell size using a Coulter Channelizer (B).

Hyperphosphorylation of Net1 is dependent upon membrane trafficking events that drive bud growth

To further investigate the relationship between Net1 phosphorylation and bud growth, we tested whether hyperphosphorylation of Net1 is dependent upon bud growth. In previous work, we tested for dependency of signaling events on bud growth by blocking plasma membrane growth in the daughter bud, which can

be achieved by inactivating Sec6, a component of the exocyst complex that is required for fusion of vesicles with the plasma membrane during bud growth (Anastasia *et al.*, 2012). A temperature sensitive allele of *SEC6* (*sec6-4*) can be used to achieve rapid conditional inactivation of Sec6.

Inactivation of Sec6 before bud emergence causes cells to arrest at metaphase, due most likely to a failure in bud growth during early mitosis (Anastasia *et al.*, 2012). Swe1, the budding yeast homolog of Wee1, is required for the arrest and is thought to respond to signals that measure bud growth before metaphase. Loss of Swe1 allows cells that lack Sec6 function to proceed through metaphase to late anaphase/telophase, and the cells show normal mitotic spindle assembly and chromosome segregation within the mother cell (Anastasia *et al.*, 2012). Therefore, we analyzed Net1 phosphorylation in wild type and *sec6-4 swe1*Δ cells, which ensured that any effects on Net1 phosphorylation were not due to a metaphase arrest. Cells were released from a G1 phase arrest and shifted to the restrictive temperature at 20 minutes after release from the arrest, before bud emergence. Hyperphosphorylation of full length Net1 was assayed by western blot and levels of Clb2 were assayed as a marker for mitotic progression. Hyperphosphorylation of Net1 failed to occur when *sec6-4* was inactivated (Figure 2.5). Controls showed that *sec6-4* alone also caused a failure in Net1 phosphorylation, while *swe1*Δ alone had no effect on Net1 phosphorylation (not shown). Thus, hyperphosphorylation of

Net1 is independent of mitotic exit and mitotic spindle function but is completely dependent upon membrane trafficking events that are required for bud growth. Furthermore, the mitotic cyclin Clb2 accumulates to high levels in *sec6-4 swe1*Δ cells and is capable of driving mitotic spindle assembly and segregation of chromosomes in anaphase (Anastasia *et al.*, 2012), which indicates that Clb2/Cdk1 activity is not sufficient to drive Net1 phosphorylation. This finding is consistent with a previous study that found that Clb2/Cdk1 activity likely plays a minor role in regulation of Net1, but only after MEN signaling has been initiated (Azzam *et al.*, 2004).

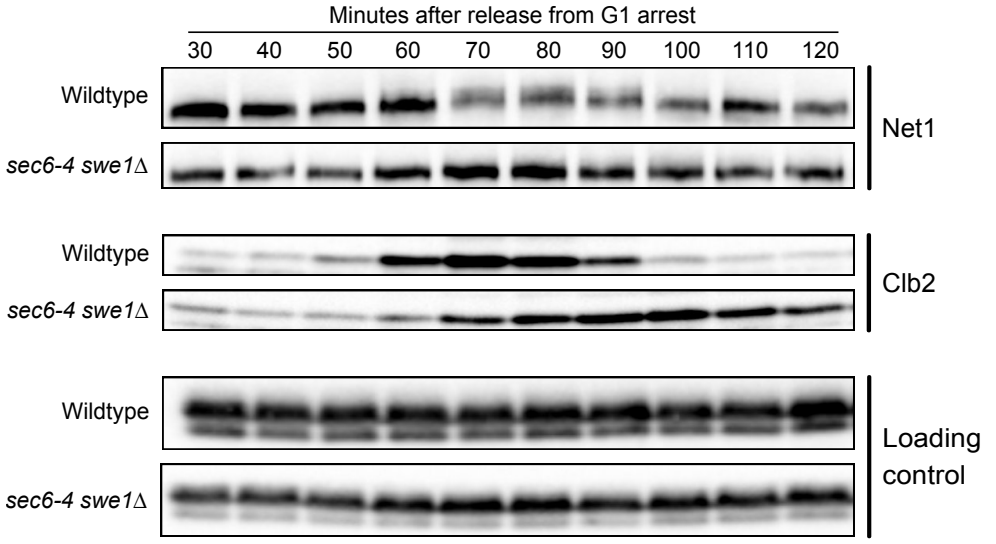


Figure 2.5. Net1 hyperphosphorylation is dependent upon membrane trafficking events that are required for bud growth. *net1*$^{\Delta 328}$*-3xHA* and *net1*$^{\Delta 328}$*-3xHA sec6-4 swe1*Δ cells grown overnight to log phase in YPD were released from a G1 phase arrest at room temperature and were then shifted to the restrictive temperature (34°C) 20 minutes after release. Samples were taken at the indicated time intervals

to assay for Net1$^{\Delta 328}$-3xHA, Clb2 by western blot. An anti-Nap1 antibody was used for a loading control.

An allele of *CDC14* that is not inhibited by Net1 bypasses the anaphase arrest caused by a failure in bud growth

The data thus far suggest that hyperphosphorylation of Net1 is dependent upon bud growth and correlated with the extent of bud growth. To explain the data, we hypothesized that hyperphosphorylation of Net1 is driven by growth-dependent signals. We further hypothesized that growth-dependent hyperphosphorylation of Net1 plays a role in activation of the MEN, which would help ensure that mitotic exit occurs only when sufficient growth has occurred. This model suggests that the late anaphase arrest in *sec6-4 swe1*Δ cells is caused by a failure in the release of Cdc14 from Net1. To test this model, we determined whether an allele of Cdc14 that shows reduced binding to Net1 (*cdc14^{TAB6}*) is sufficient to bypass the late mitotic arrest in *sec6-4 swe1*Δ cells. The *cdc14^{TAB6}* allele was identified in a screen for mutants that bypass a telophase arrest caused by loss of Cdc15 function, which indicates that the allele can bypass a failure to activate the MEN (Shou *et al.*, 2001; Shou and Deshaies, 2002). We released *sec6-4 swe1*Δ *cdc14^{TAB6}* cells and control cells from a G1 phase arrest at the permissive temperature and then shifted to the restrictive temperature 20 minutes later and assayed Clb2 protein levels by western blot to assess mitotic progression (**Figure**

2.6A). The *cdc14*^{TAB6} allele fully abrogated the late anaphase arrest caused by *sec6-4*.

To further test the model, we analyzed the effects of deleting the *BFA1* gene, which encodes a GAP thought to inhibit the MEN via inhibition of Tem1 (Pereira *et al.*, 2000; Wang *et al.*, 2000; Ro *et al.*, 2002). Loss of *BFA1* partially abrogated the late mitotic arrest in *sec6-4 swe1*Δ cells (Figure 2.6B). These results suggest that the late mitotic arrest caused by *sec6-4* is due to a failure to activate the MEN, consistent with a model in which the events of bud growth generate signals that promote Net1 phosphorylation and are required for full activation of the MEN.

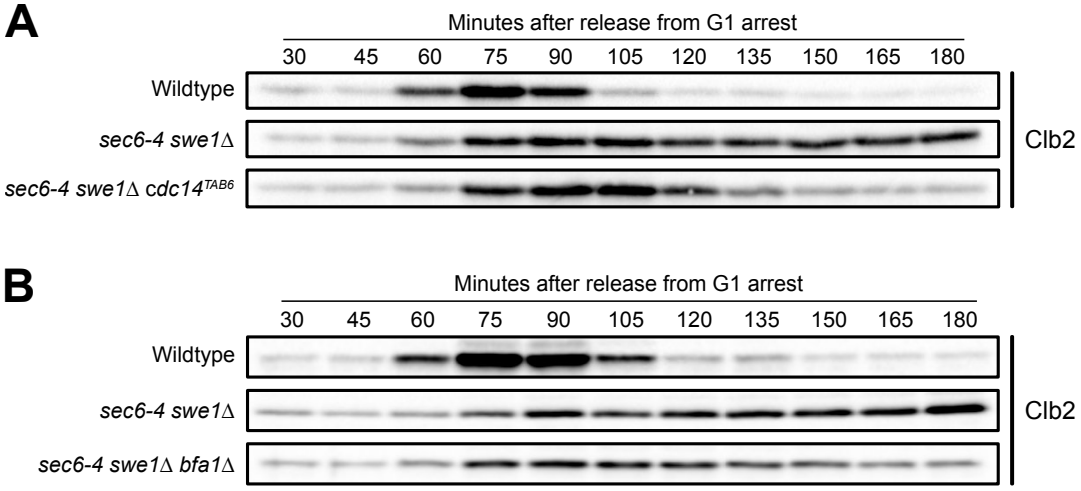


Figure 2.6. Net1-dependent inhibition of Cdc14 is required for the late anaphase arrest caused by inhibition of membrane trafficking. *net1-6xHA*, *net1-6xHA sec6-4 swe1*Δ, and *net1-6xHA sec6-4 swe1*Δ *cdc14*^{TAB6} cells grown overnight in YPD to log

phase were released from a G1 phase arrest into fresh media at 25°C. 20 minutes after release the cultures were shifted to 34°C to inactivate sec6-4 and samples were collected at the indicated time points to assay levels of Clb2.

Components of the TORC2 signaling network are required for hyperphosphorylation of Net1

The discovery that inactivation of Sec6 causes a failure in hyperphosphorylation of Net1, as well as a late mitotic arrest that is dependent upon binding of Net1 to Cdc14, is consistent with a model in which activation of the MEN requires growth-dependent signals that report on the extent of bud growth. However, the data do not rule out alternative models. A key question concerns the nature of the signals that drive Net1 hyperphosphorylation. However, these signals are poorly understood. Over 90 phosphorylation sites have been detected on Net1 and multiple kinases have been implicated (Shou and Deshaies, 2002; Azzam *et al.*, 2004; Mah *et al.*, 2005; Stark *et al.*, 2010; Zhou *et al.*, 2021). Mitotic Cdk1 activity is thought to contribute to Net1 phosphorylation; however, only 10 of the many sites that have been detected on Net1 correspond to the minimal Cdk1 consensus site (S/TP). Furthermore, a previous study showed that mitotic spindle assembly, chromosome segregation, and mitotic spindle elongation occur normally in *sec6-4 swe1*Δ cells, despite the failure in bud growth (Anastasia *et al.*, 2012). Since all these key events of mitosis require mitotic Cdk1 activity, the fact that they occur normally in *sec6-4 swe1*Δ cells that show a complete failure in Net1

hyperphosphorylation suggests that hyperphosphorylation of Net1 is not a consequence of mitotic Cdk1 activity. Another kinase that has been proposed to phosphorylate Net1 is the Cdc5/Polo kinase; however, mutation of sites thought to be phosphorylated by Cdc5 has no effect on release of Cdc14 or mitotic progression in vivo (Shou and Deshaies, 2002; Zhou *et al.*, 2021). Additionally, Net1 hyperphosphorylation induced by Cdc5 overexpression is dependent upon Tem1, which suggests that Cdc5 could influence Net1 phosphorylation via Tem1-dependent activation of other kinases (Visintin *et al.*, 2003). No kinase has been shown to be required for the extensive hyperphosphorylation of Net1 seen in vivo. Although kinases have been identified that are capable of disrupting the Net1-Cdc14 complex in vitro it remains unclear whether they work directly to disrupt the complex in vivo (Shou and Deshaies, 2002; Azzam *et al.*, 2004).

To investigate further we first tested whether kinases previously proposed to phosphorylate Net1 are required for Net1 phosphorylation in vivo. Cells harboring an analog-sensitive allele of *CDC5* (*cdc5-as1*) were released from a G1 phase arrest and analog inhibitor was added 30 minutes after release. Inhibition of cdc5-as1 caused cells to arrest with hyperphosphorylated Net1$^{\Delta 328}$, which indicates that Cdc5 is not required for Net1 hyperphosphorylation (**Figure S2.2A**). Similarly, inhibition an analog-sensitive allele of the *CDC15* kinase (*cdc15-as1*), a key component of the MEN signaling cascade, had no effect on Net1$^{\Delta 328}$ phosphorylation in cells released

from a G1 phase arrest into medium containing benomyl (Figure S2.2B). Since Cdc15 is required for activation of Dbf2, these results indicate that none of the key kinases in the MEN are required for hyperphosphorylation of Net1, which suggests that the hyperphosphorylation of Net1 that can be detected via electrophoretic mobility shifts is not due to MEN activity. This is consistent with previous studies that found that the MEN is not active in metaphase-arrested cells (Fesquet *et al.*, 1999; Jaspersen and Morgan, 2000; Hu *et al.*, 2001; Visintin and Amon, 2001; Hu and Elledge, 2002). The results do not rule out the possibility that MEN kinases phosphorylate a small number of sites that do not influence the electrophoretic mobility of Net1.

The evidence for links between Net1 phosphorylation and bud growth suggest that signals associated with cell growth could play a role in hyperphosphorylation of Net1. We therefore tested whether signals known to be associated with cell growth are required for hyperphosphorylation of Net1. Previous work has shown that TOR kinases play important roles in control of cell growth. The TOR kinases are assembled into two distinct multiprotein complexes, referred to as Target of Rapamycin Complexes 1 and 2 (TORC1 and TORC2) (Loewith and Hall, 2011). TORC1 is best understood because it can be inhibited with rapamycin and plays roles in control of ribosome biogenesis, amino acid biosynthesis, and autophagy. A key target of TORC1 is the conserved Sch9 kinase, which controls

transcription of ribosome biogenesis genes. To test whether TORC1 is required for hyperphosphorylation of Net1 we synchronized cells and added rapamycin shortly after bud emergence. Rapamycin had no effect on hyperphosphorylation of Net1$^{\Delta 328}$ in cells released from a G1 phase arrest into medium containing benomyl (**Figure S2.2C**). Similarly, inhibition of an analog-sensitive version of Sch9 (*sch9-as*) had no effect on hyperphosphorylation of Net1$^{\Delta 328}$ (**Figure S2.2D**).

Recent studies have shown that TORC2 activity is correlated with growth rate and the extent of growth. Moreover, a signaling network that surrounds TORC2 is required for normal control of cell size and for nutrient modulation of cell size (Alcaide-Gavilán *et al.*, 2018; Lucena *et al.*, 2018; Leitao *et al.*, 2019). A key downstream target of TORC2 that is required for normal control of cell growth and size is a pair of redundant kinase paralogs called Ypk1 and Ypk2, which are the budding yeast homologs of mammalian SGK kinases (Niles *et al.*, 2012). Inhibition of an analog-sensitive allele of *YPK1* in a *ypk2*Δ background (*ypk1-as ypk2*Δ) caused a substantial failure in hyperphosphorylation of Net1$^{\Delta 328}$ in cells released from a G1 phase arrest into medium containing benomyl (**Figure 2.7)**. A previous study found that inhibition of Ypk1/2 does not block bud growth (Clarke *et al.*, 2017). Together, these observations suggest that the TORC2 signaling network could play a role in the signals that drive hyperphosphorylation of Net1, consistent with the mass spectrometry results showing that multiple components of the TORC2 signaling

network are rapidly regulated in response to a shift from rich to poor carbon during mitosis (Table 1).

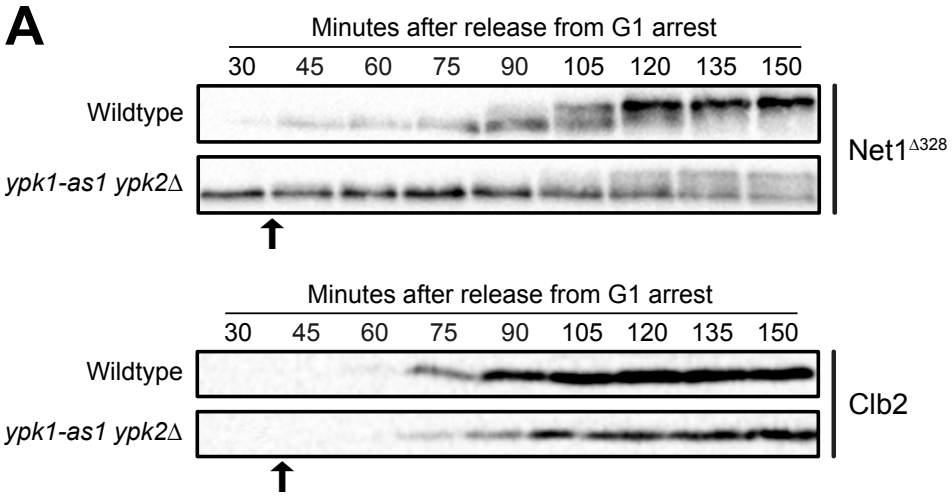


Figure 2.7. Ypk1/2 and Yck1/2 are required for normal hyperphosphorylation of Net1 *in vivo*. *net1$^{\Delta 328}$-3xHA* and *net1$^{\Delta 328}$-3xHA ypk2Δ ypk1-as1* (A) or *net1$^{\Delta 328}$-3xHA yck2Δ yck1-as1* (B) grown overnight in YPD were released from a G1 phase arrest at 25°C. Adenine analog inhibitors, 3MB-PP1 (A) or 3BrB-PP1 (B), were added to a final concentration of 25 uM at 40 minutes after release. Samples were taken at the indicated time intervals to assay for Net1$^{\Delta 328}$-3xHA and Clb2 by western blot.

Discussion

Numerous components of the MEN are controlled by nutrient-dependent signals

In budding yeast, most cell growth occurs during mitosis as the daughter bud undergoes rapid expansion (Leitao and Kellogg, 2017). Furthermore, bud growth is required for progression through mitosis (Anastasia *et al.*, 2012; Jasani *et al.*, 2020). Previous studies led us to hypothesize that the extent of bud growth is measured to ensure that an appropriate amount of growth occurs, and that the threshold amount of growth required for mitotic progression is reduced in poor nutrients, which would explain why daughter cells are born at a very small size in poor nutrients (Anastasia *et al.*, 2012; Jasani *et al.*, 2020). Testing this hypothesis will require identification of molecular mechanisms that link mitotic progression to bud growth, as well as mechanisms that set the threshold amount of bud growth required for cell cycle progression. Here, we searched for molecular mechanisms that link completion of mitosis to bud growth. To do this, we took advantage of the fact that changes in nutrient availability rapidly re-set the threshold amount of growth required for cell cycle progression (Fantes and Nurse, 1977; Leitao and Kellogg, 2017). We reasoned that proteins involved in measuring cell growth and setting the growth threshold should show rapid changes in phosphorylation in response to a shift to poor nutrients. Thus, we used proteome-wide mass spectrometry to identify proteins that undergo rapid changes in phosphorylation when cells in mitosis are shifted from rich to poor

carbon. This revealed that numerous components of the MEN are regulated by nutrient availability. Since activation of MEN signaling initiates exit from mitosis, the MEN is well-positioned to receive physiological signals related to nutrients and cell growth to ensure that mitotic exit is initiated only when an appropriate amount of growth has occurred. A role for the MEN in controlling mitotic exit in response to nutrient and growth cues would be compatible with previously proposed models in which migration of the daughter nucleus into the daughter bud promotes mitotic exit. For example, signals associated with cell growth and nutrient availability could influence initiation of mitotic exit, while migration of the nucleus into the daughter bud could initiate positive feedback that fully activates the MEN to complete mitotic exit.

An alternative hypothesis could be that a shift to poor carbon reduces the availability of ATP, leading to a general slowdown in cellular events and a decrease in the rate of phosphorylation of proteins. Several observations argue against this interpretation. First, a shift to poor carbon late in mitosis does not cause a delay in mitotic exit, which suggests that the shift does not cause a non-specific slowdown of mitotic events (Leitao and Kellogg, 2017). Rather, a shift to poor carbon in late mitosis may not cause a delay because sufficient bud growth has already occurred. A further argument against a general reduction in ATP is that the top proteins that underwent dephosphorylation are enriched in proteins involved in the MEN, TORC2 signaling,

ribosome biogenesis and membrane trafficking, all of which are involved in growth or cell cycle progression and would be expected to change when growth rate is slowed by poor nutrients. In contrast, few proteins involved in mitotic spindle dynamics, which are known to undergo phosphorylation during mitosis, were identified.

Hyperphosphorylation of Net1 occurs before mitotic exit

Among the MEN components identified by mass spectrometry, Net1 was amongst the most prominent with 11 phosphorylation sites that showed substantial loss of phosphorylation. Net1 prevents mitotic exit by binding and inhibiting the Cdc14 phosphatase in the nucleolus. The dissociation of Cdc14 from Net1 initiates mitotic exit as well as a positive feedback loop that promotes mitotic exit. It is thought that hyperphosphorylation of Net1 promotes dissociation of Cdc14, thereby initiating mitotic exit, although there is evidence that hyperphosphorylation of Net1 may not be sufficient to release Cdc14 (Traverso *et al.*, 2001; Visintin *et al.*, 2003; Azzam *et al.*, 2004; Zhou *et al.*, 2021). A previous study found that Cdc14 is not released from the nucleus in benomyl-arrested cells, consistent with the idea that phosphorylation of Net1 is not sufficient to drive release of Cdc14 from the nucleus (Stegmeier *et al.*, 2002). Here, we found that Net1 undergoes full hyperphosphorylation in metaphase-arrested cells, which indicates that Net1 hyperphosphorylation is not strictly correlated with mitotic exit and does not depend upon the events of mitotic exit. The

finding that Net1 is fully hyperphosphorylated in metaphase arrested cells is consistent with previously proposed models in which hyperphosphorylation of Net1 is necessary but not sufficient to initiate exit from mitosis.

Phosphorylation of Net1 is correlated with daughter bud growth

What are the physiological signals that drive hyperphosphorylation of Net1 before mitotic exit? Several observations suggest that signals associated with cell growth influence Net1 phosphorylation. First, hyperphosphorylation of Net1 occurs gradually during bud growth, which indicates that Net1 phosphorylation is correlated with bud growth. Furthermore, we found that metaphase-arrested cells undergo unusually extensive bud growth, which leads to abnormally large daughter buds as well as unusually extensive hyperphosphorylation of Net1. Similarly, we used growth in poor carbon sources to show that reducing the rate and extent of bud growth leads to a corresponding reduction in the rate at which hyperphosphorylated forms of Net1 accumulate as well as a reduction in the maximal extent of Net1 phosphorylation. Thus, hyperphosphorylation of Net1 is strongly correlated with the rate and extent of bud growth, which suggests that the events of growth generate signals that help drive hyperphosphorylation of Net1.

Hyperphosphorylation of Net1 is dependent upon membrane trafficking events that drive bud growth

Further evidence for a link between Net1 phosphorylation and cell growth came from our finding that hyperphosphorylation of Net1 is dependent upon membrane trafficking events that drive bud growth. In *sec6-4 swe1Δ* cells the initial steps of mitotic exit occur normally as the spindle elongates during anaphase (Anastasia *et al.*, 2012), yet hyperphosphorylation of Net1 fails to occur. These observations provide further evidence that hyperphosphorylation of Net1 is closely associated with bud growth, rather than with early steps of anaphase and mitotic exit. Previous studies have shown that an arrest of membrane trafficking events that are required for plasma membrane growth leads to inhibition of ribosome biogenesis (Mizuta and Warner, 1994; Li *et al.*, 2000). Therefore, the failure in Net1 phosphorylation in *sec6-4 swe1Δ* cells could be a more direct consequence of an arrest of ribosome biogenesis, although it is unclear from previous studies whether arresting membrane growth causes an arrest of ribosome biogenesis on a sufficiently rapid time scale to account for the observed effects on Net1 phosphorylation. It is also possible that the inhibition of ribosome biogenesis caused by blocking membrane trafficking events is due to a failure in Net1 phosphorylation, as previous studies have shown that Net1 is required for normal transcription of rRNA genes (Shou *et al.*, 2001; Hannig *et al.*, 2019).

The fact that Net1 is intimately connected to ribosome biogenesis, a major facet of cell growth, further suggests a potential connection between Net1 hyper-phosphorylation and cell growth (Straight *et al.*, 1999; Shou *et al.*, 2001; Hannig *et al.*, 2019). An interesting possibility is that gradual phosphorylation of Net1 during bud growth ensures that the rate of rDNA gene transcription increases during growth to meet the increased protein synthesis requirements of larger cells. Consistent with this, a previous study found that phosphorylation of Net1 promotes binding and activation of RNA polymerase PolI (Hannig *et al.*, 2019). This would suggest a model in which the events of growth generate feedback signals that promote further growth and also provide a measure of the extent of growth.

Bypassing the MEN relieves a late mitotic arrest caused by a failure in bud growth

In previous work, we found that *sec6-4 swe1Δ* cells arrest in late anaphase with long mitotic spindles, separated nuclei, and high levels of mitotic cyclin, which indicates that they fail to complete mitotic exit (Anastasia *et al.*, 2012). The cause of this late mitotic arrest was unknown. Here, we found that the late anaphase arrest in *sec6-4 swe1Δ* cells can be bypassed by preventing Net1-dependent inhibition of Cdc14. Loss of Bfa1, a Tem1 GAP that inhibits the MEN, also partially bypasses the late mitotic *sec6-4 swe1Δ* cells. Together, these observations suggest that the late mitotic arrest caused by blocking bud growth is due to a failure to activate the MEN,

which is consistent with the hypothesis that activation of the MEN is dependent upon bud growth.

Net1 undergoes extensive hyperphosphorylation that is not dependent upon key components of the MEN

Net1 is one of the most highly phosphorylated proteins in budding yeast. Various mass spectrometry analyses have identified 91 phosphosites. Of the 11 sites identified in our analysis, 10 were identified in previous analyses, while 1 was not previously identified.

The functions of Net1 phosphorylation remain poorly understood. Many of the 91 sites were identified in non-quantitative proteome-wide analyses, which makes it unclear when they are phosphorylated in a cell cycle-dependent manner or whether they are phosphorylated at significant stoichiometries that are likely to be biologically relevant. A Net1 mutant in which all 91 sites were mutated (*net1-91A*) is viable and causes only a slight delay in mitotic exit (Zhou *et al.*, 2021). However, whether the *net1-91A* mutant eliminates phosphorylation-induced electrophoretic mobility shifts in vivo was not tested, so it is unclear whether relevant sites were mutated. Furthermore, mutating 91 phosphorylation sites almost certainly eliminates regulation by multiple kinases, which makes interpretation of the results difficult. For example, if Net1 plays both positive and negative roles in controlling ribosome biogenesis and

mitotic exit, as suggested by previous studies, elimination of 91 phosphorylation sites could cause both gain- and loss-of-function effects that cancel each other out (Straight *et al.*, 1999; Shou *et al.*, 2001; Hannig *et al.*, 2019). The fact that mutating subsets of sites appears to cause stronger effects is consistent with the possibility (Zhou *et al.*, 2021).

Previous studies suggested that Cdc5 and Dbf2, two key components of the MEN, could phosphorylate Net1 (Shou and Deshaies, 2002; Yoshida and Toh-e, 2002; Visintin *et al.*, 2003; Mah *et al.*, 2005; Zhou *et al.*, 2021). Previous studies mapped phosphorylation sites on Net1 that are dependent upon Cdc5 or Cdc15. In some cases, mutation of these sites causes mitotic delays, especially in sensitized genetic backgrounds (Loughrey Chen *et al.*, 2002; Shou and Deshaies, 2002; Shou *et al.*, 2002; Zhou *et al.*, 2021). However, the phosphorylation site mutants do not cause a failure in mitotic exit or a loss of viability, and they appear to have only mild effects in an otherwise wildtype background, which indicates that they are not essential for mitotic exit. No previous studies have tested whether Cdc5, Cdc15 or Dbf2 are required for hyperphosphorylation of Net1 that can be detected via electrophoretic mobility shifts.

Here, we found that Cdc5 and Cdc15 are not required for hyperphosphorylation of Net1 in vivo. Since Cdc15 is required for activation of Dbf2, these findings suggest that none of the protein kinases in the MEN are required for

the extensive hyperphosphorylation of Net1 that can be detected via electrophoretic mobility shifts. Previous studies found that key components of the MEN are inactive during a metaphase arrest, which provides further evidence that hyperphosphorylation of Net1 in metaphase-arrested cells is not due to signaling from components of the MEN (Fesquet *et al.*, 1999; Jaspersen and Morgan, 2000; Hu *et al.*, 2001; Visintin and Amon, 2001; Hu and Elledge, 2002). Furthermore, the fact that full hyperphosphorylation of Net1 occurs even when mitotic spindle assembly is blocked shows that there is extensive hyperphosphorylation of Net1 that is not dependent upon any events associated with the function or orientation of the mitotic spindle. A potential explanation for these observations is that hyperphosphorylation of Net1 is the consequence of early signals that are necessary but not sufficient for initiating full activation of the MEN signaling network.

TORC2 signaling is required for growth-dependent hyperphosphorylation of Net1

Since hyperphosphorylation of Net1 appears to be correlated with growth and dependent upon growth, we tested whether it is influenced by the activity of TORC1 or TORC2, which play essential roles in growth control. Inactivation of TORC1 with rapamycin had no effect on Net1 hyperphosphorylation. Similarly, inactivation of Sch9, a key target of TORC1 that controls ribosome biogenesis, also had no effect on the extent of Net1 phosphorylation. In contrast, inactivation of Ypk1 and Ypk2, key

targets of TORC2 that are homologs of mammalian SGK kinases, caused a nearly complete failure in Net1 hyperphosphorylation.

The discovery that components of the TORC2 network are required for Net1 phosphorylation is particularly interesting. Multiple components of the TORC2 network were identified as top hits in our mass spectrometry analysis of the effects of a shift from rich to poor carbon during mitosis. These include core components of the TORC2 complex (Avo2, Avo1 and Tsc11) as well as a key downstream effector, Ypk1. Furthermore, previous studies have shown that Ypk1/2 and their surrounding signaling network are required for normal control of cell size and for nutrient modulation of cell size (Lucena *et al.*, 2018). For example, decreased activity of Ypk1/2 causes a large decrease in cell size. Ypk1/2 activate ceramide synthase, which produces signaling lipids required for normal control of cell size. An inhibitor of the ceramide synthesis pathway causes a dose-dependent decrease in cell size, and loss of ceramide synthase causes a complete failure of nutrient modulation of TORC2 signaling and cell size. TORC2 signaling, as measured via phosphorylation of Ypk1/2, is modulated by nutrient availability and increases throughout the interval of bud growth (Alcaide-Gavilán *et al.*, 2018; Lucena *et al.*, 2018). Finally, loss of Rts1, a conserved PP2A regulatory subunit that influences TORC2 signaling, causes a complete breakdown in the relationship between cell size and growth rate during bud growth in mitosis (Leitao *et al.*, 2019). Together, these observations suggest that

gradually rising TORC2 signaling during bud growth could drive growth-dependent phosphorylation of Net1, and that nutrient modulation of TORC2 signaling could explain the effects of nutrient availability on Net1 phosphorylation and cell size at completion of mitosis.

What are the signals that drive mitotic exit?

A puzzling aspect of the MEN has been that Cdc14, which plays perhaps the most critical role in initiation of mitotic exit, is conserved across eukaryotic cells, yet the role of Cdc14 in promoting mitotic exit is not conserved, as budding yeast is the only known organism in which Cdc14 functions in mitotic exit. For example, the fission yeast homolog of Cdc14 appears to function in a pathway that controls mitotic entry (Trautmann *et al.*, 2001). A potential explanation could come from the fact that budding yeast is the only known organism in which most cell growth occurs during mitosis. Thus, it seems possible that Cdc14 has been co-opted in budding yeast to function in an unusual pathway that links mitotic exit to bud growth. Our results support this model, as they suggest that the MEN responds to growth-dependent signals that define the duration and extent of bud growth in late mitosis. The data do not rule out previously suggested models in which the MEN ensures that mitotic exit does not occur until the daughter nucleus has entered the bud. Indeed, the existence of a mechanism to ensure that nuclear segregation occurs only when the

bud has become sufficiently large to accommodate the daughter nucleus would appear to be essential for yeast cells that rely on a highly unusual growth mode (i.e. bud growth in mitosis) to reproduce.

Supplemental Figures

Figure 2.7 – figure supplement 1

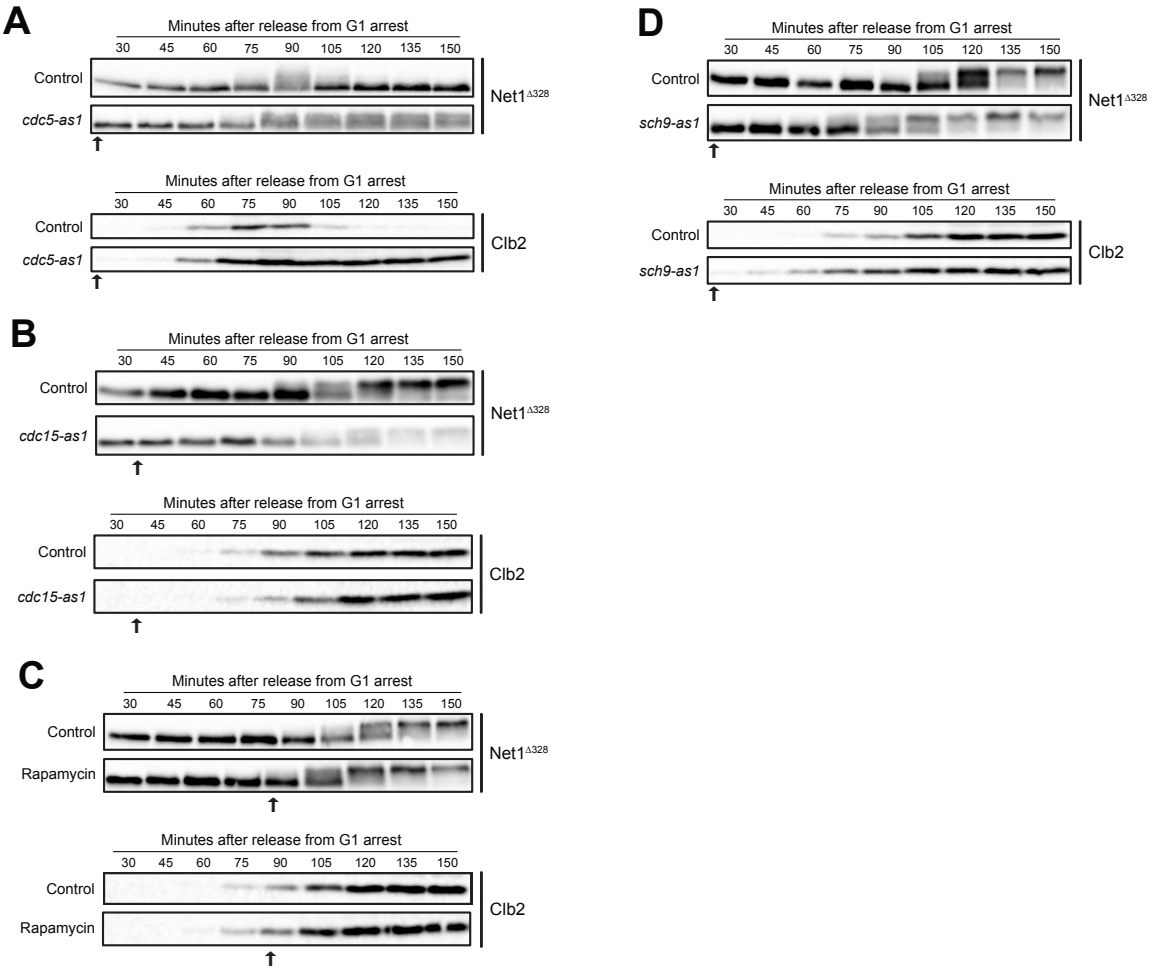


Figure S2 – Kinases found not to be required for hyperphosphorylation of Net1 *in vivo*. For all experiments, cells were released from a G1 phase arrest and inhibitor was added at the times indicated by the arrow. Samples were then taken at the indicated time points to assay for Net1 and Clb2 by western blot. (A) *net1$^{\Delta 328}$-3xHA* and *net1$^{\Delta 328}$-3xHA cdc5-as1* cells released from G1 phase into fresh media, 10 uM CMK was added 30 minutes after release. (B) *net1$^{\Delta 328}$-3xHA* cells released from G1 phase arrest into fresh media containing benomyl, vehicle or 0.219 uM rapamycin was added to one half of the culture 90 minutes after release. (C) *net1$^{\Delta 328}$-3xHA* and *net1$^{\Delta 328}$-3xHA cdc15-as1* cells released from G1 phase into fresh media containing

benomyl. 10 uM 1-NA-PP1 was then added 40 minutes after release. (D) *net1*$^{\Delta 328}$*-3xHA* and *net1*$^{\Delta 328}$*-3xHA sch9-as1* cells released from G1 phase into fresh media containing benomyl. 5 uM 1-NM-PP1 was added 30 minutes after release.

Chapter III (unpublished results):

Phosphorylation and abundance of Net1 are reduced in poor carbon

The detection of phosphorylation-induced mobility shifts in Net1 by standard SDS-PAGE proved to be difficult. This is likely due to a combination of Net1's high molecular weight and that it is one of the most highly phosphorylated proteins in budding yeast. As an alternative method to detect mobility shifts in Net1, we used polyacrylamide gels supplemented with Phos-Tag. Phos-Tag is a small molecule that coordinates phosphorylated residues during electrophoresis, slowing the mobility of phosphorylated proteins through the gel matrix. Thus, Phos-Tag can substantially improve the resolution between protein phosphoforms (Kinoshita *et al.*, 2006; Kinoshita and Kinoshita-Kikuta, 2011).

By this method we show that the extent Net1 hyperphosphorylation is greatly reduced in poor carbon, suggesting the signals which drive Net1 phosphorylation are correlated with the extent of growth in mitosis (Figure 2.3). The result is highlighted well in Figure 3.1C, obtained from a PhosTag SDS-PAGE, where the resolution between upper and lower phosphoforms of Net1 is especially pronounced in the poor carbon condition whereas Net1 completely shifts into the upper form in rich carbon.

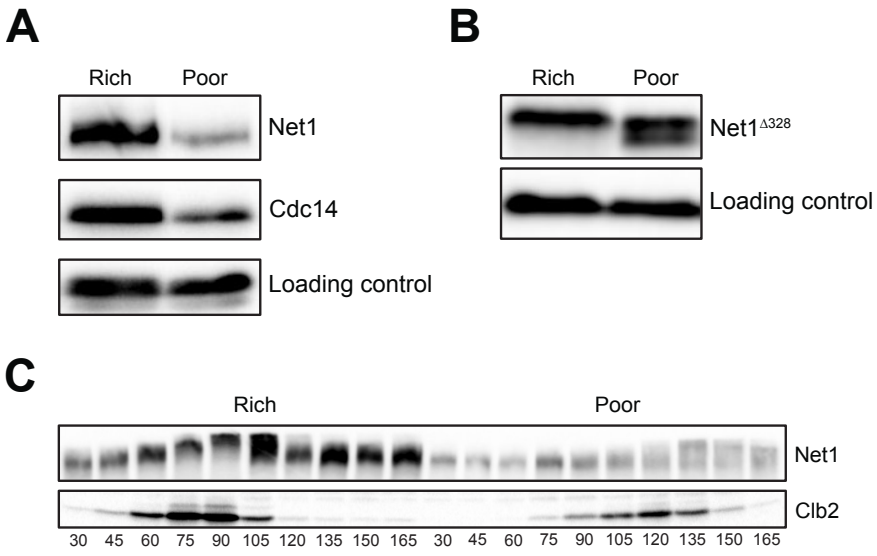


Figure 3.1. Nutrient-dependent regulation of Net1 protein abundance and phosphorylation. (A) Samples were taken from Net1-6xHA cells that were grown overnight in YPD or YPG/E to log phase at room temperature. Net1 and Cdc14 protein were assayed by western blot (B) Wildtype cells containing Net1$^{\Delta 328}$ were grown to log phase overnight in YPD or YPG/E then shifted to benomyl-supplemented media. Samples were collected three hours after the shift to benomyl-supplemented media. (C) Cells grown overnight in YPD or YPG/E were released from a G1 arrest into fresh media and samples were taken at the indicated time points to assay for Net1 and Clb2 by western blot. Where applicable, Nap1 serves as the loading control.

Nutrient modulation of cell size requires modulation of the threshold amount of growth necessary for cell cycle progression. This requires a mechanism capable of promoting the same cell cycle transition despite undergoing varying amounts of growth due to differences in nutrient availability. For example, in budding yeast cells must achieve a critical size threshold before initiating cell cycle entry (Hartwell and Pringle '77). Current models propose cell entry is triggered by the early G1

phase cyclin Cln3 that promotes sufficient accumulation of late G1 cyclins Cln1/2 via inactivation of Whi5. However, in poor carbon Cln3 protein levels are reduced by 75% relative to total protein while Whi5 abundance remains approximately constant (Sommer *et al.*, 2021). Thus, how does a cell cycle promote cell cycle entry despite accumulating substantially less Cln3? The mechanisms by which this occurs remains poorly understood.

The data presented in Chapter II proposes a similar problem with respect to bud growth in mitosis. Namely, how is mitotic exit triggered despite significantly less bud growth occurring in poor carbon? Mitotic exit is driven by activation of Cdc14 phosphatase, which results in diffusion from the nucleolus to cytoplasm. A critical step that is thought to drive this process is Net1 phosphorylation, the extent of which is reduced in poor carbon. Thus, how is mitotic exit initiated despite Net1 undergoing less phosphorylation in poor carbon? Interestingly, we found during the studies described in **Figure 3.1B** and **Figure 2.2** that the abundance of Net1 is reduced in poor carbon (**Figure 3.1A**). One hypothesis to explain these data is that the threshold amount of growth required for mitotic exit in poor carbon is reduced by reducing the amount of Cdc14 that must be released from the nucleolus. In this model, a reduction in the extent of growth-dependent phosphorylation of Net1 can be compensated for by reducing the pool of Net1 that requires phosphorylation to relieve inhibition of Cdc14. To test this model, it will be important to quantify both

the amount of Cdc14 as well as the extent of Cdc14 released from the nucleolus as a function of carbon source.

The effects of a C-terminally truncated form of Net1 on mitotic progression and protein abundance

The technical challenges associated with Phos-Tag supplemented SDS-PAGE made its continued use impractical. The C-terminally truncated form of Net1, Net1$^{\Delta 328}$, used in Chapter II on the other hand consistently produced detectable mobility shifts by standard western blotting techniques. Importantly, the mobility shifts in Net1$^{\Delta 328}$ were similar in extent and timing to that of full length Net1, validating the use of Net1$^{\Delta 328}$ as a viable reporter of Net1 phosphorylation (Figure 3.2A).

A recent study reported that expressing a C-terminally truncated form of Net1, *net1Δctr*, as the sole copy causes significant growth defects, including abnormal bud growth and increased doubling time (Hannig). While *net1Δctr* retains its ability to form the RENT complex, the authors show that the growth defects are likely due to Net1's functions in ribosome biogenesis. To avoid any of these defects we integrated Net1$^{\Delta 328}$ in cells expressing endogenous Net1. In doing so, it presented interesting questions about the potential effects of increasing Net1 protein abundance on cell growth and cell cycle progression.

One potential hypothesis is that an additional copy of Net1, assuming normal localization, would increase the pool of Net1-bound Cdc14. Thus, the expected outcome would be a delay in mitotic exit due to delays in Cdc14 release. To test this, cells that contained either full length (*FL*) Net1 or both endogenous and truncated (*trunc.*) forms of Net1 were released from a G1 arrest and their progression through mitosis monitored by western blot. Peak Clb2 levels in cells containing the truncated reporter were advanced relative to wildtype, indicating only minor defects in mitotic progression caused by the second copy of Net1 (**Figure 3.2A**). Additionally, the slow decline in Clb2 levels in Net1-6xHA Net1$^{\Delta 328}$-3xHA cells indicates possible defects in mitotic exit. These results suggests that increased abundance of Net1 cause only minor defects on mitotic progression. However, it may be informative to compare the timing of mitotic exit in diploids cells which are heterozygous or homozygous for Net1. Additionally, it would be important to test if Net1$^{\Delta 328}$ causes cell size defects and is properly localized and bound to Cdc14.

The abundance of Net1 protein is modulated by carbon source (**Figure 3.1A**). Insights into the cause of this effect can be found in the Net1$^{\Delta 328}$ allele used in this study. Figure 3.1B reveals that the abundance of the Net1$^{\Delta 328}$ allele does not change with carbon source like that of full length Net1. This allele of Net1 is missing 328 amino acids from the C-terminal end and it is integrated at the URA3

locus, suggesting that the C-terminal region of the protein may be required for post-translation modification which regulate protein turnover. Alternatively, while Net1$^{\Delta 328}$ transcription is driven by its native promoter there may be additional cis elements near its native locus that regulate its transcription in response to nutrient conditions.

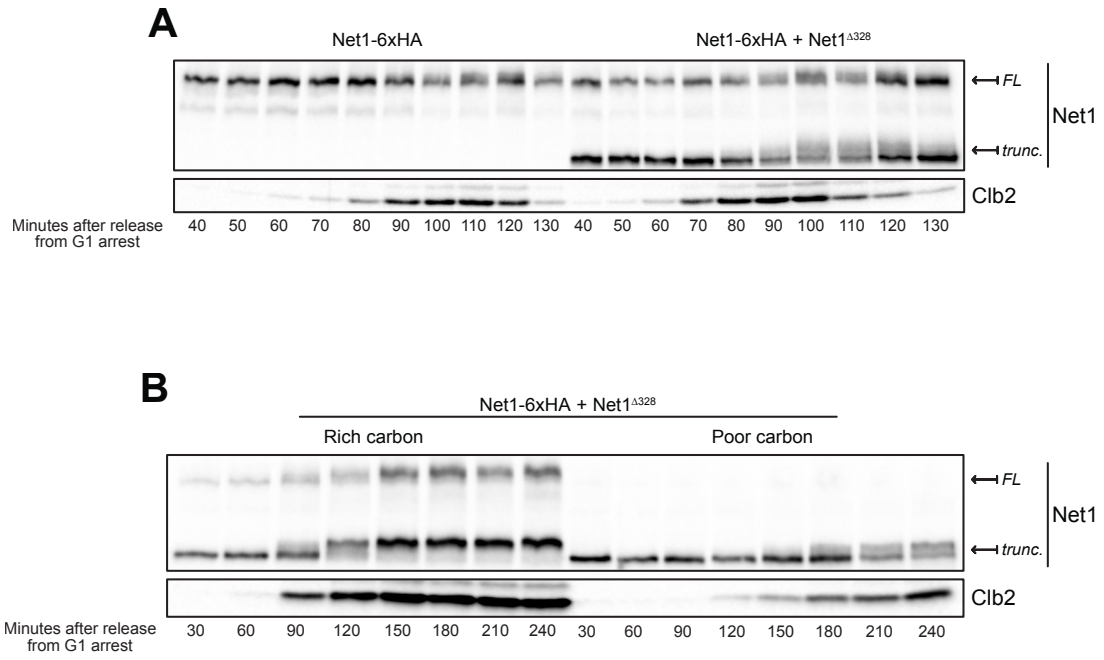


Figure 3.2. Validity of Net1$^{\Delta 328}$ as a phosphorylation sensor and its effects on cell cycle progression and Net1. (A) Cells grown overnight in YPD to log phase were released from a G1 arrest into fresh media. Samples were taken at the indicated time points to assay by western blot for both forms of Net1 using an anti-HA monoclonal antibody. Clb2 was assayed as a marker of mitotic progression. (B) Net1-6xHA Net1$^{\Delta 328}$-3xHA cells grown in either YPD or YPG/E to log phase were released from a G1 arrest into benomyl-supplemented media.

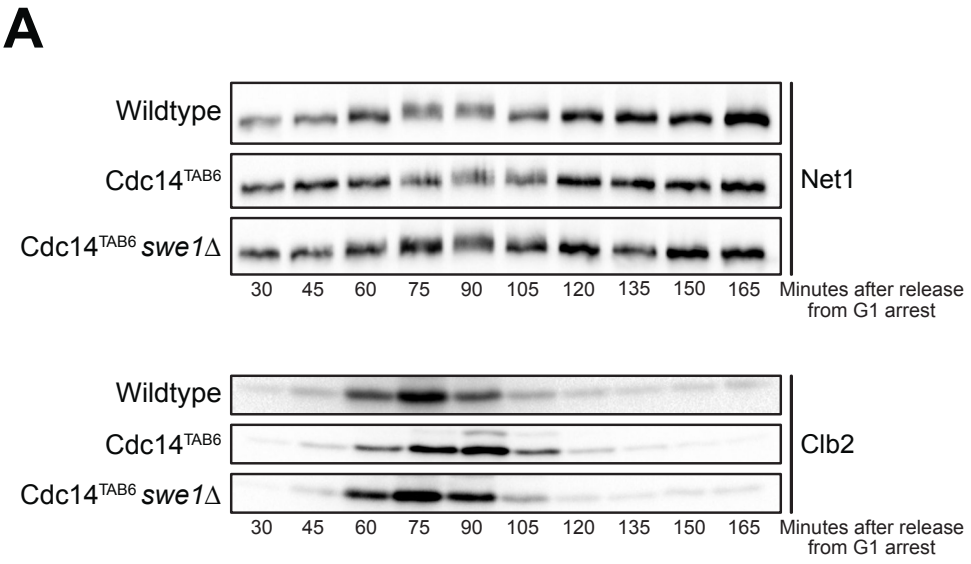
Cdc14^{TAB6} causes Swe1-dependent defects on mitotic progression and Net1 phosphorylation

In Figure 2.6 of Chapter II, we report that phosphorylation of Net1 fails upon inhibition of membrane trafficking by inactivation of Sec6. We further show that *cdc14^{TAB6}* rescues the late anaphase arrest in sec6-4 swe1Δ cells. In this context, Net1 phosphorylation still fails (data not shown). Possible explanations for the failure in Net1 phosphorylation include Swe1-dependent effects or uncontrolled dephosphorylation by Cdc14^{TAB6}. Figure 3.3A rules out these possibilities as Net1 phosphorylation occurs normally in *cdc14^{TAB6}* and *cdc14^{TAB6}* swe1Δ cells, suggesting the failure in Net1 phosphorylation is most likely due to inhibition of membrane growth.

Interestingly, Cdc14^{TAB6} alone causes perturbations in mitotic entry and exit. Possible explanations include effects of the *cdc14^{TAB6}* allele or uncharacterized functions of Cdc14 in early mitosis. The former is a likely explanation as previous studies have found that the *cdc14^{TAB6}* allele has compromised phosphatase activity and potential defects in nucleolar release (Shou and Deshaies, 2002; Yoshida and Toh-e, 2002). Loss of Swe1 completely rescued the defects in mitotic progression caused by *cdc14^{TAB6}*. This result may suggest the delay in mitotic entry in *cdc14^{TAB6}* cells could be due to Cdc14-dependent regulation of Swe1 but does not rule a model in which Cdc14 and Swe1 function in parallel. Interestingly, loss of Swe1

rescues the defects in mitotic progression of Cdc14^{TAB6} cells but does not rescue the delay in peak Net1 hyperphosphorylation, effectively decoupling Net1 phosphorylation and mitotic progression. This is consistent with Figure 2.2 where we find that Net1 phosphorylation does not depend on mitotic exit.

Finally, *cdc14^{TAB6}* causes a Swe1-dependent increase in cell size. Thus, *cdc14^{TAB6}* swe1Δ are comparable in size to wildtype. One hypothesis to explain this result is that *swe1*Δ rescues the increased cell size effects of *cdc14^{TAB6}* by reducing the duration of the interval of bud growth.



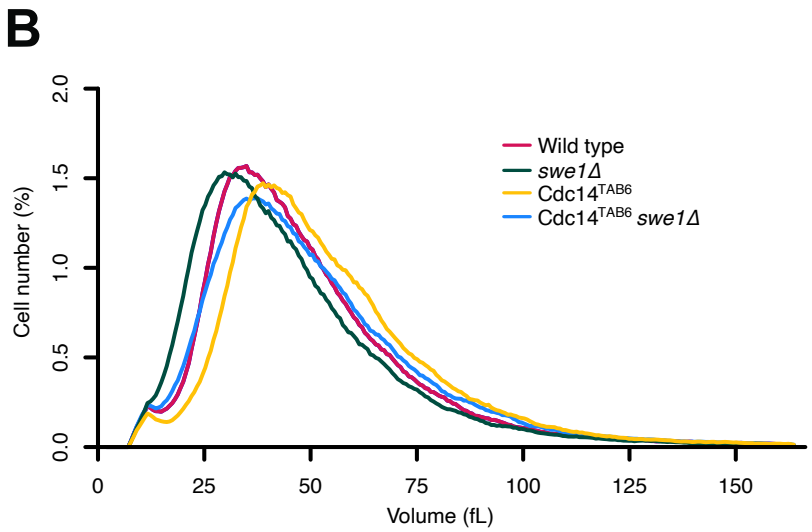


Figure 3.3. The Swe1-dependent effects of Cdc14^{TAB6} on cell size, cell cycle progression, and Net1 phosphorylation. (A) Cells of the indicated genotypes grown overnight in YPD were released from a G1 arrest into fresh media. Samples were collected at the indicated time points to assay for full length Net1 (top panels) and Clb2 (bottom panels). (B) Samples were collected from log phase cell cultures of the indicated genotypes grown in YPD for cell size analysis using a Coulter Channelyzer. Data from (A) and (B) were collected from different experiments.

Spindle Orientation Checkpoint proteins are not required for a late anaphase arrest caused by a failure in bud growth

Previously published work from our lab showed that inhibition of membrane trafficking events required for bud growth causes a cell cycle arrest at mitotic entry ((Anastasia *et al.*, 2012). It was further shown that the G2/M phase arrest is caused by a Swe1-dependent mechanism. Thus, *sec6-4 swe1*Δ cells at the restrictive temperature progress through mitosis but then arrest again in late anaphase with

high levels of mitotic cyclin and anaphase spindles. Until now, the cause of the late anaphase arrest had been unresolved.

In Figure 2.6 we show that *sec6-4 swe1*Δ cells fail to exit mitosis due to Net1-dependent inhibition of Cdc14. This suggested that the late anaphase arrest in these cells is due to a failure to activate the MEN. To test this, we hypothesized that loss of Bub2, Bfa1, or both may lead to sufficient hyperactivation of the MEN to drive mitotic exit in *sec6-4 swe1*Δ cells at restrictive temperature. Bub2 and Bfa1 are GAPs of Tem1 that are thought to inhibit Tem1, and therefore the MEN (Pereira *et al.*, 2000; Geymonat *et al.*, 2002; Ro *et al.*, 2002). Loss of Bub2 caused only a weak decrease in mitotic cyclin levels, while loss of Bfa1 and Bub2 had no effect. The lack of an effect of *bub2*Δ could be explained by previous work showing overexpression Bfa1 has a stronger inhibitory effect on mitotic exit than Bub2 (Ro *et al.*, 2002). However, why *bfa1*Δ caused a pronounced decrease in Clb2 levels while *bub2*Δ *bfa1*Δ failed to cause any effect is more difficult to explain. One hypothesis is that the failure in bud formation upon Sec6 inactivation causes a failure to establish the bud-specific signal that is thought to activate the MEN. Thus, *bub2*Δ *bfa1*Δ would be insufficient to activate the MEN in this context despite relieving Tem1 of inhibition. However, this hypothesis cannot explain why *bfa1*Δ alone caused a partial rescue of Clb2 levels. One study found that both Bub2 and Bfa1 are required for normal localization of Tem1 to the SPBs (Pereira *et al.*, 2000). Thus,

loss of both Bub2 and Bfa1 may fail to prematurely activate the MEN due to Tem1 mislocalization. One study found that Tem1 can weakly associate to the dSPBs in either *bub2*Δ or *bfa1*Δ, although it appears only weakly in late anaphase. Thus, an alternative hypothesis is that loss of Bfa1 but not Bub2 may be sufficient to induce Clb2 degradation in *sec6-4 swe1*Δ cells due to sufficient Tem1 localization to SPBs, albeit weakly and delayed. Interpretations of these data are made difficult by previous experiments showing differential effects of Bub2 and Bfa1 on Tem1 activity (Geymonat *et al.*, 2002).

The finding that loss of neither Bub2 nor Bfa1 can promote mitotic exit to the same extent as *cdc14*^{TAB6} in *sec6-4 swe1*Δ cells indicates that a late anaphase arrest caused by inhibition of membrane growth is not solely due to inactivation of the MEN. One model to explain these data is that full release of Cdc14 requires growth-dependent phosphorylation of Net1. Consistent with this model, *bfa1*Δ may fail to fully rescue the cell cycle arrest in *sec6-4 swe1*Δ cells because key growth-dependent signals had failed to target key phosphorylation sites of Net1.

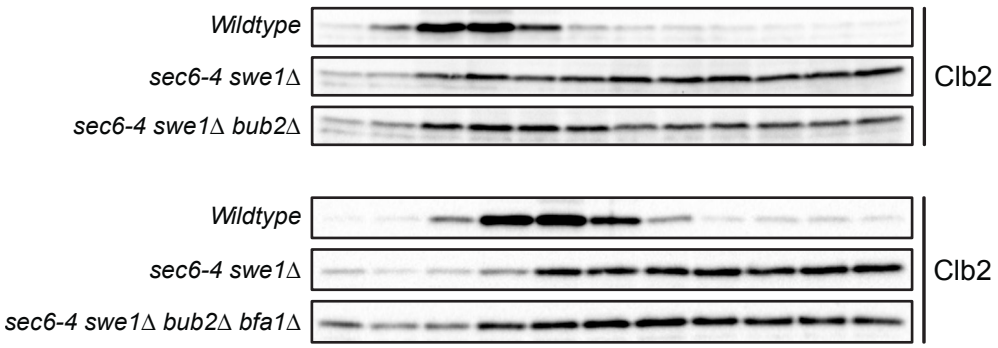


Figure 3.4. Bub2 and Bfa1 are not required for a late anaphase arrest caused by a failure in membrane growth. (A-B) Cells grown to log phase were released from a G1 arrested into fresh media and shifted to the restrictive temperature 20 minutes afterwards. Samples were taken at the indicated time points to assay for Clb2 by western blot to monitor mitotic progression.

The search for kinases that are required for growth-dependent hyperphosphorylation of Net1

In Chapter II we show that Net1 undergoes hyperphosphorylation in a manner that is inconsistent with current models of Net1 and the MEN. The data strongly suggest that Net1 hyperphosphorylation is not dependent upon events of mitotic exit and that it is driven by MEN-independent mechanisms. Rather, our evidence suggests that Net1 phosphorylation in vivo is correlated with and dependent upon growth. We further show that Net1 hyperphosphorylation in vivo is partially dependent upon the yeast homolog of SGK, Ypk1. To test whether Ypk1 directly phosphorylates Net1, we purified Net1 and Ypk1 from wildtype cells and asked whether Ypk1 alone is sufficient to induce detectable mobility shifts in Net1 by western blot. Ypk1 alone fails to phosphorylate full length Net1 in vitro, suggesting that Ypk1-dependent phosphorylation of Net1 in vivo occurs indirectly. However, it cannot be ruled out that Ypk1 requires additional cofactors or that it phosphorylates Net1 in vitro in a manner that does not induce a mobility shift detectable by western blot.

Plasma membrane growth is required for cell growth and failure to add new membrane to the site of bud growth triggers a cell cycle arrest at the entry to mitosis (Anastasia *et al.*, 2012). The data suggests that delivery of vesicles to the site of bud growth generates signals that are proportional to the extent of growth, which are then read by downstream components to determine when sufficient bud growth has occurred. Our observation that plasma membrane growth is also required for Net1 phosphorylation led us to hypothesize that Net1 may be one of those targets. Yck1/2 are palmitoylated kinases that are transported to the plasma membrane of the growing bud by vesicle traffic (ref). Moreover, loss of function of Yck1/2, or failure to localized Ypk1/2 to the plasma membrane, leads to severe defects in the control of bud growth (ref). They are therefore well positioned to relay signals generated by bud growth to Net1. We found that inhibition of an analog-sensitive allele of *YCK1* (*yck1-as1*) in a *yck2*Δ background caused a substantial failure in Net1 hyperphosphorylation. These data are consistent with a hypothesis in which Net1 is the target of signals related to bud growth in mitosis.

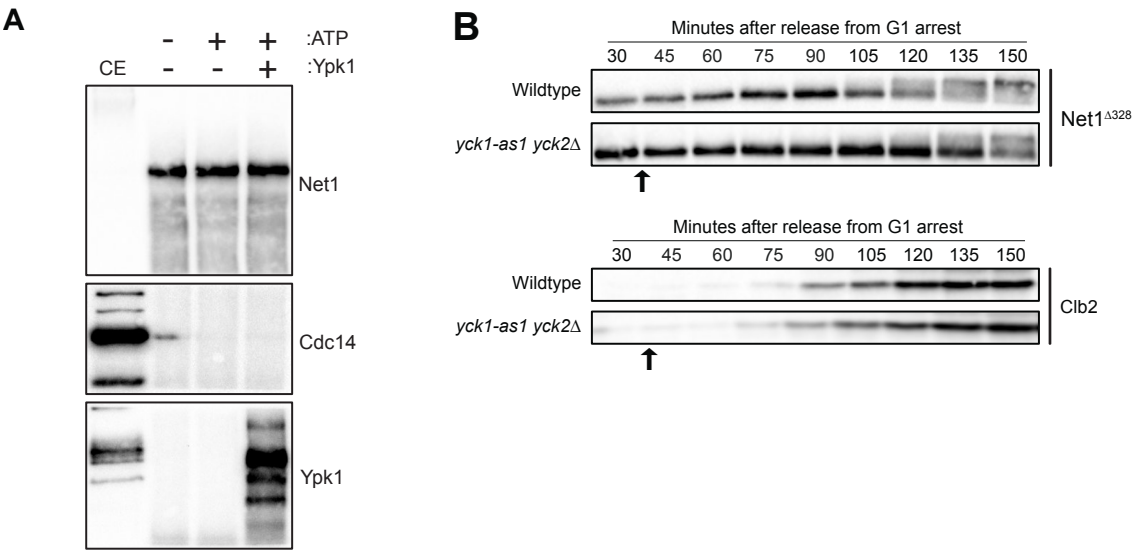
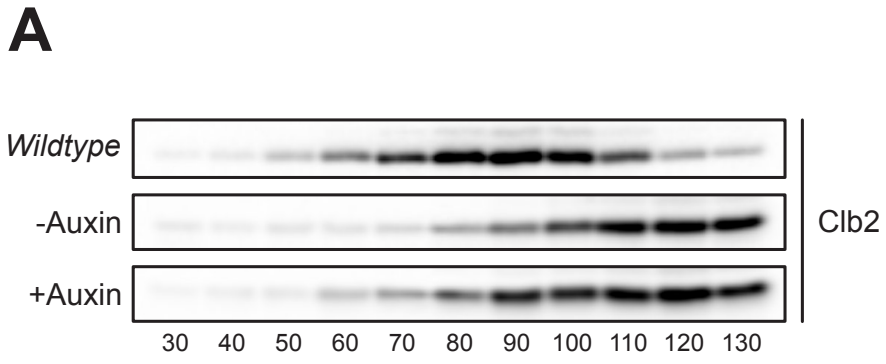


Figure 3.5. Investigating kinases that are required for Net1 phosphorylation. (A) Net1-6xHA was immunoprecipitated from wildtype cells incubated with purified Ypk1 in the presence of ATP and assayed by western blot. (B) yck2Δ cells harboring an analog-sensitive allele of yck1 were released from a G1 arrest into benomyl-supplemented media. The analog XXX was added YYY minutes after release samples taken for western blot analysis.

Inhibition of ribosome biogenesis has strong effects on TORC2 signaling

Evidence suggesting that Net1 plays a role in ribosome biogenesis and nucleolar functions date back to the late 1990s (Straight *et al.*, 1999; Shou *et al.*, 2001; Shou and Deshaies, 2002). In Chapter II we show that Net1 undergoes phosphorylation that is correlated with and dependent upon bud growth. We therefore hypothesized that signals related to cell growth are relayed via a kinase associated with ribosome biogenesis to influence mitotic exit via phosphorylation of Net1. We identified the atypical kinase Rio1 as a potential candidate due to its similar roles to Net1 in controlling rRNA and ribosome biogenesis, including

regulation of Pol I activity, Sir2 localization and 35s rRNA transcription (Angermayr *et al.*, 2002; Iacovella *et al.*, 2018; Berto *et al.*, 2019). To test this, we utilized an auxin-inducible degron version of Rio1 that allowed for conditional degradation due to Rio1 being essential for viability. We found that *rio1-AID* cells in the absence of auxin have a substantial delay in mitotic entry that is rescued upon addition of auxin (**Figure 3.6A**). The cause of the delay in cell cycle progression is unknown however **Figure 3.6B** shows that Rio1-AID cells in the presence of auxin have dramatic reductions in T662 phosphorylation of Ypk1 relative to wildtype. Hyperphosphorylation of full length Net1 appeared to largely fail in the presence of auxin (**Figure 3.6B,C**). However, Net1$^{\Delta 328}$ does accumulates into the upper phosphorylated form although it appears to be delayed and not to the same extent as wildtype (**Figure 3.6D**).



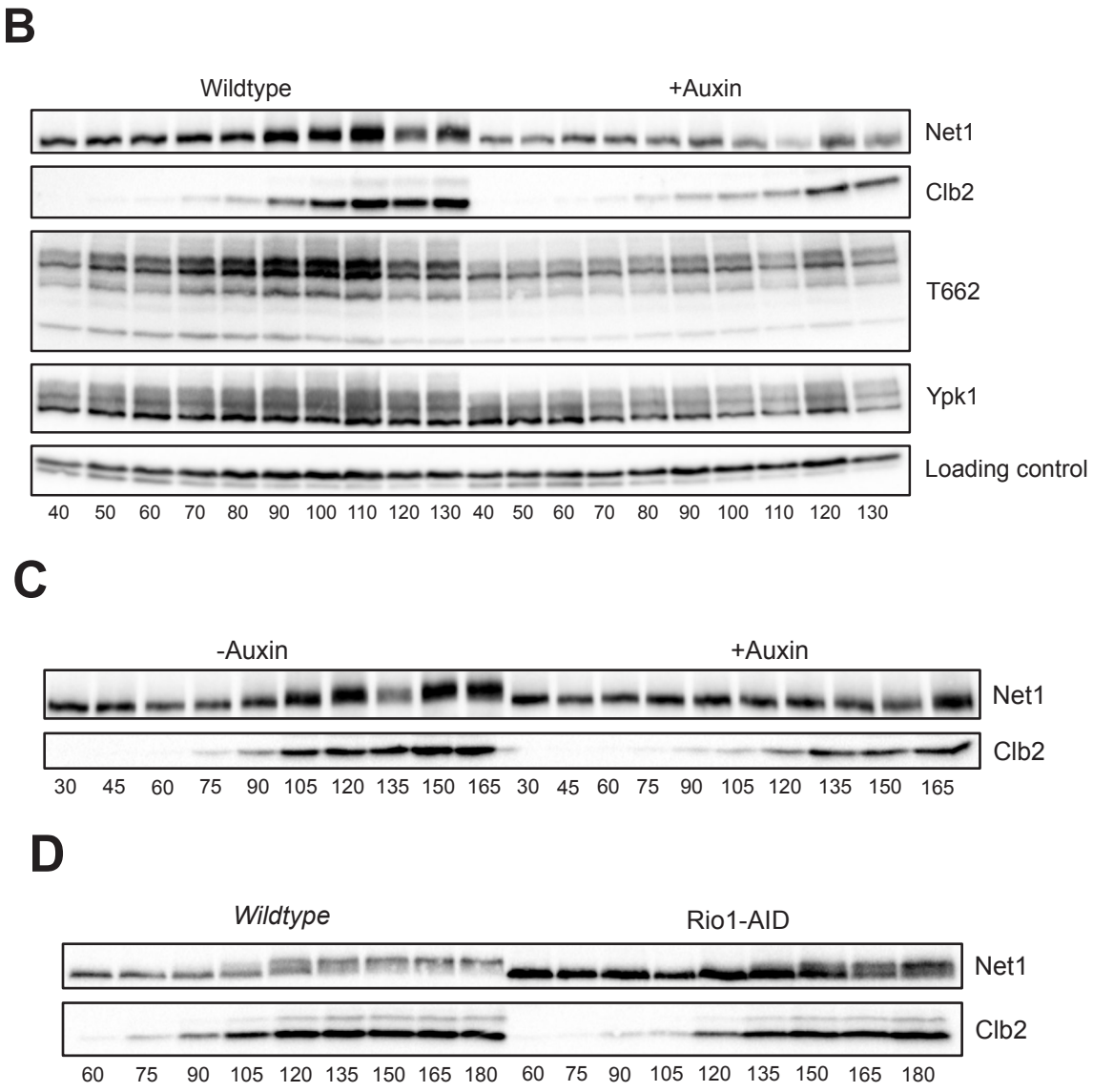


Figure 3.6. Effects of Rio1 depletion on TORC2 activity and Net1 phosphorylation. (A) Cells were released from a G1 arrest into fresh media. 30 minutes after release 1 mM auxin or DMSO was to Rio1-AID cells. Samples were taken at the indicated time points to assay for Clb2. (B) Wildtype and Rio1-AID cells were released from a G1 arrest into benomyl supplemented media. 26 minutes after release 1 mM auxin was added to Rio1-AID cells. (C) Rio1-AID cells were released from a G1 arrest into benomyl supplemented media and 1 mM auxin or DMSO was added 26 minutes after release. (D) Wildtype and Rio1-AID cells containing Net1$^{\Delta 328}$ were released from a G1 arrest into media containing benomyl. 1 mM auxin was added to Rio1-AID cells 30 minutes after release.

While addition of auxin inducible degrons to proteins often results in compromised function, the delay in mitotic entry of Rio1-AID cells in absence of auxin is striking. One possible explanation comes from Figure 3.6B where it was found that cell cycle dependent increases in T662 phosphorylation of Ypk1 fails. Previous work from our lab has shown that growth rate increases approximately 3-fold when cells enter mitosis in rich carbon (Leitao and Kellogg, 2017). Additionally, TORC2-dependent phosphorylation of Ypk1 is correlated with cell growth rate (Lucena *et al.*, 2018). Thus, it could suggest that delays in cell cycle progression of Rio1-AID cells could be due to a reduced growth rate caused by reduced TORC2 activity. Additional analysis, including measurements of the rate of growth and budding will be required to test this hypothesis. Our data from Figure 2.7 suggests that growth-dependent phosphorylation of Net1 requires Ypk1/2. Consistent with this there is a partial loss of Net1 hyperphosphorylation in Rio1-AID cells, suggesting it may be due to defects in TORC2-depedent regulation of Ypk1/2.

Net1 plays a critical role in regulating mitotic exit in budding yeast, a key cell cycle transition that influences the extent of bud growth and size. Net1's role in regulating rRNA and ribosome biogenesis is poorly understood. Moreover, our data suggests the possibility that the TORC2 system plays a role in regulating Net1 phosphorylation in response to growth and nutrient dependent signals. Thus, our

data provides possible novel insight into the physiological signals that regulate Net1's role sequestration of Cdc14. The work in thesis suggests that Net1 may represent a mechanistic integration of signals related to ribosome biogenesis and cell growth, which together regulate cell cycle machinery to influence mitotic exit.

Table 2.2: Strains used in this study

Strain	Genotype	Figure
DK2017	*CLN3-6XHA::His3MX6, bar1*	Figure 2.1
DK3822	*NET1-6XHA::kanMX4, bar1*	Figure 2.2, 2.3, 2.6, 3.1, 3.2, 3.4. 3.5
DK4616	*net1Δ328-3XHA::URA, bar1*	Figure 2.2, 2.4, 2.7, 3.1
DK4661	*net1Δ328-3XHA::URA, pGAL-CDC20::kanMX5, bar1*	Figure 2.2
DK4563	*NET1-6XHA::kanMX4, net1Δ328-3XHA::URA, bar1*	Figure 2.3, 3.2,
DK4066	*NET1-6XHA:: hphNT1*	Figure 2.5, 2.6, 3.3
DK4180	*NET1-6XHA:: hphNT1, sec6-4::kanMX6, swe1Δ::His5*	Figure 2.5, 2.6
DK4005	*NET1-6XHA::hphNT1, sec6-4::kanMX6, swe1Δ::His5, cdc14TAB6-1*	Figure 2.6
DK3838	*NET1-6XHA::hphNT1, sec6-4::kanMX6, swe1Δ::His5, bar1*	Figure 2.6, 3.4
DK4442	*NET1-6XHA::hphNT1, sec6-4::kanMX6, swe1Δ::His5, bfa1::caURA, bar1*	Figure 2.6
DK4708	*net1Δ328-3XHA::URA, ypk2::His3, ypk1-as1, bar1*	Figure 2.7
DK4624	*net1Δ328-3XHA::URA, cdc5-as1, bar1*	Figure 2.7S1
DK4706	*net1Δ328-3XHA::URA, cdc15-as1, bar1*	Figure 2.7S1
DK4654	*net1Δ328-3XHA::URA, sch9-as1*	Figure 2.7S1
DK4669	*Net1-6xHA::hphNT1, rio1-AID::KanMX4, TIR1-9MYC::Ura3*	Figure 3.6
DK4247	*NET1-6XHA::hphNT1, Cdc14^{TAB6-1}*	Figure 3.3
DK4162	*NET1-6XHA::hphNT1, cdc14^{TAB6-1}, swe1Δ::His5*	Figure 3.3
DK177	*BAR1*	Figure 3.3
SH24	*swe1::His5*	Figure 3.3
SH803	*cdc14^{TAB6-1}*	Figure 3.3
DK3860	*cdc14^{TAB6-1}, swe1::His5*	Figure 3.3
DK4343	*Sec6-4::kanMX6, swe1Δ::His5, NET1-6XHA::hphNT1, bub2Δ::CloNat*	Figure 3.4
DK4432	*Sec6-4::kanMX6, swe1Δ::His5, NET1-6XHA::hphNT1, bub2Δ::CloNat bfa1Δ::caURA*	Figure 3.4
DK4488	*NET1-6XHA::hphNT1, yck1Δ::KanMX6, yck2-as1*	Figure 3.5

Material and Methods

Strain construction, media, and reagents

All strains are in the W303 background (leu2-3,112 ura3-1 can1-100 ade2-1 his3-11,15 trp1-1 GAL+ ssd1-d2). Additional genetic features are listed in Table 2. Gene deletions and epitope tagging were performed by standard PCR amplification and homologous recombination (Longtine et al. 1998; Janke et al. 2004; Lee et al. 2013).

Strains were grown in YEP medium (1% yeast extract, 2% peptone, and 40 mg/mL adenine) containing 2% dextrose (YPD), 2% galactose (YPGal), or 2% glycerol/2% ethanol (YPG/E), as indicated in the text. In experiments involving analog-sensitive alleles of kinases, cells were grown in media that was not supplemented with adenine.

The Net1$^{\Delta 328}$ phosphorylation reporter was created by amplifying a region of the *NET1* gene that includes the promoter and the first 806 amino acids (oligos: ATAcc-cgggCGTAGGGAGCGATATGTGCATTATG and ATAggtaccCTTCTTGTTTGAa-ttcggataaaagcttttcgcc). The resulting PCR fragment was cloned into the KpnI and XmaI sites of pDK51, which expresses proteins with a C-terminal 3XHA tag and carries the *URA3* marker, to create pDK131. The plasmid is cut with StuI to target integration at the *URA3* gene.

Benomyl was solubilized in 100% DMSO to prepare a 20 mg/mL stock solution. Media containing benomyl was prepared by first heating the media to 100°C before adding the drug to a final concentration of 30 ug/mL. The media was then allowed to cool at room temperature while stirring.

Cell cycle time courses and western blotting

For cell cycle time courses, cultures were grown overnight at room temperature to log phase (OD_{600} = 0.4 - 0.7). The cells were then arrested in G1

phase by the addition of 0.5 ug/mL or 15 ug/mL alpha factor for *bar1* or BAR1 strains, respectively. Cultures were arrested for 3 to 3.5 hours and then released from the arrest by three washes in fresh media not containing mating pheromone. All time courses were performed at 25°C, except for experiments involving *sec6-4* strains, which were carried out at 34°C. Alpha factor was added back to cultures 60 minutes after release to prevent initiation of a second cell cycle. At each time point, 1.6 mL of sample were collected in screw cap tubes and centrifuged at 15,000 rpm for 15 seconds. The supernatant was then removed and 200 uL of acid-washed glass beads were added before freezing samples in liquid nitrogen. Cell pellets were lysed in 140 uL 1x SDS-PAGE sample buffer (65 mM Tris-HCl pH 6.8, 3% SDS, 10% glycerol, 100 mM β-glycerophosphate, 50 mM NaF, 5% β-mercaptoethanol, 3 mM PMSF, and bromophenol blue) by bead beating in a Biospec Mini-Beadbeater-16 at 4°C for 2 minutes. Lysed samples were spun down in a microcentrifuge for 15 seconds at 15,000 rpm before incubating in a 100°C water bath for 6 minutes followed by centrifugation at 15,000 rpm for 10 minutes.

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 Net1, Clb2, and Nap1 (loading control). Proteins were immobilized onto nitrocellulose membranes using wet transfers for 2 hr 45 min. Blots were probed with the primary antibody at 1–2 mg/ml at 4°C overnight in 4.5% milk in PBST (13 phosphate-buffered saline, 250 mM NaCl, and 0.1% Tween-20) containing 4.5% nonfat dry milk. Primary antibodies used to detect Clb2 and Nap1 were rabbit polyclonal antibodies generated as described previously (Kellogg and Murray, 1995; Sreenivasan and Kellogg, 1999; Mortensen *et al.*, 2002). Net1-6xHA and Net1$^{\Delta 328}$-3xHA were detected by a mouse monoclonal antibody (12CA5). 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 4.5% 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.

Coulter Counter Analysis

For Figure 4B, cell cultures were grown overnight at room temperature in YPD, YPGal, or YPG/E to an OD_{600} between 0.4 and 0.6. During the time course, 450 ul of culture was collected at each time point and fixed by addition of 50 uL of 37% formaldehyde to the culture medium followed by incubation at room temperature for 1 hour. Cells were then pelleted and resuspended in 0.25 ml PBS containing 0.02% sodium azide and 0.1% Tween-20. Cell size was measured on the same day as the sample collection using a Coulter counter (Channelizer Z2; Beckman, Fullerton, CA) as previously described.

Tandem Mass Spectrometry analysis

Cells were grown overnight to log phase in YPD medium and were then arrested in G1 phase with alpha factor. The cells were released from the arrest into fresh YPD medium and at 90 minutes after release one half of the culture was shifted to poor carbon (YPG/E) by pelleting the cells and resuspending in YPG/E three times. At ten minutes after the first wash into poor carbon, the cells from 50 mLs of both cultures (O.D. 600 of 0.5) were pelleted in a 50 ml conical tube, resuspended in 1 ml of media, transferred to a new 1.6 ml screw top, and then pelleted again for 15 sec in a microfuge. After removing the supernatant, 200 uL of acid-washed beads were added and the samples were frozen on liquid nitrogen. Parallel samples were taken for western blotting to ensure that the shift to poor carbon occurred at peak Clb2 levels. A total of 2 biological replicates were carried

out and analyzed by mass spectrometry. A strain carrying Cln3-6XHA (DK2017) was used for these experiments because we wanted to test whether the Cln3 protein present in mitosis responds rapidly to a shift to poor carbon. Western blotting showed that Cln3 rapidly disappeared when the cells were shifted to poor carbon within 5 minutes, as observed previously for Cln3 in G1 phase.

Cell pellets were lysed in a Biospec Mini-Beadbeater-16 at 4°C in 500 uL of Lysis Buffer (8M urea, 75 mM NaCl, 50 mM Tris, pH 8.0, 50 mM B-glycerolphosphate, 1 mM $NaVO_3$, 10 mM sodium pyrophosphate, 1 mM PMSF) by two 1-minute rounds of bead-beating at 4°C, with 1 minute of chilling in an ice water bath in between. The lysate was then centrifuged at 14,000 rpm at 4°C for 10 minutes and the supernatant was transferred to a new tube and frozen on liquid nitrogen. The samples were prepared for quantitative proteome and phosphoproteome profiling following the SL-TMT protocol that includes the "mini-phos" phosphopeptide enrichment strategy (Navarrete-Perea, Yu et al. 2018).

Additional contributions to published work

During my time as a graduate student in the Kellogg lab, I had the opportunity to work on a fellow graduate student's project. My contributions earned a second authorship for the following publication: Ricardo M Leitao, Akshi Jasani, Rafael A. Talavera, Annie Pham, Quincy J. Okobi, and Douglas R Kellogg (2019) A conserved PP2A Regulatory Subunit Enforces Proportional Relationships Between Cell Size and Growth Rate. Published in Genetics: DOI: 10.1534/genetics.119.301012. My contributions to this work can be observed in Figure 4A.

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