Mitotic Exit and Separation of Mother and Daughter Cells

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ABSTRACT Productive cell proliferation involves efficient and accurate splitting of the dividing cell into two separate entities. This orderly process reflects coordination of diverse cytological events by regulatory systems that drive the cell from mitosis into G1. In the budding yeast *Saccharomyces cerevisiae*, separation of mother and daughter cells involves coordinated actomyosin ring contraction and septum synthesis, followed by septum destruction. These events occur in precise and rapid sequence once chromosomes are segregated and are linked with spindle organization and mitotic progress by intricate cell cycle control machinery. Additionally, critical parts of the mother/daughter separation process are asymmetric, reflecting a form of fate specification that occurs in every cell division. This chapter describes central events of budding yeast cell separation, as well as the control pathways that integrate them and link them with the cell cycle.

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Overview

Proliferation of most eukaryotic cells requires productive partitioning of intracellular components and the physical separation of this material into two distinct and independent living entities. This involves coordination of diverse processes and mechanical systems in time and space. In some cases these oppose each other, as exemplified by the construction, constriction, and disassembly of the actomyosin ring. General understanding of the molecular machines and regulatory networks underlying eukaryotic cell division and separation has been significantly advanced by studies in *S. cerevisiae*, which uses mechanisms that are conserved in fundamental organization, and often their precise components, across vast evolutionary distances.

Like other hemiascomycetes, *Saccharomyces cerevisiae* is an asymmetric organism that proliferates by forming a daughter bud that grows rapidly while the mother cell remains essentially the same size. This asymmetry persists through the process of division and cell separation, producing two individual cells with different properties. In mitosis, the mitotic spindle is positioned to ensure proper segregation of chromosomes, and separation proceeds in temporally distinct stages. First, beginning in late G1, the cytokinesis site is organized at the narrow mother/bud neck. Second, the cytoplasm is roughly halved by cytokinesis, via two interrelated but partly independent mechanisms: actomyosin ring contraction and formation of a specialized wall between the two cells called the septum. Finally, the septum is degraded and the mother and daughter cells physically dissociate. The process of cytokinesis and cell separation is closely linked to the positioning and division of the nucleus, which occurs without breakdown of the nuclear envelope. Nuclear processes are organized by the mitotic spindle, which is disassembled at about the same time as cytokinesis.

In three related parts, this chapter describes physical events and regulatory pathways that underlie separation of mother and daughter budding yeast cells into two distinct entities as they pass from mitosis into G1. Part 1 provides an overview of critical mechanical events of cell separation. This is followed in part 2 by a summary of the regulatory systems that control mitotic exit and link the mechanical processes of division to the cell cycle. These include the FEAR (Cdc **f**ourteen **e**arly **a**naphase **r**elease) and MEN (**m**itotic **e**xit **n**etwork) pathways, which drive passage from the metaphase/anaphase transition in late mitosis to early G1, as well as the RAM network (**r**egulation of **A**ce2 and **m**orphogenesis), which controls septum destruction and thus the final events of cell separation. Part 3 discusses the regulatory connections that link these control pathways to the processes that drive cell division.

This chapter emphasizes mechanisms that orchestrate the execution and timing of the late events of cell division, in particular processes that happen after the actomyosin ring contracts. I mention some critical subjects largely in overview that are described extensively elsewhere. These include the late mitotic spindle and contraction of the cytokinetic apparatus, which are covered in other YeastBook chapters by Bi and Park (2012) and Winey and Bloom (2012), respectively, and are also reviewed extensively elsewhere (Tolliday *et al.* 2001; Walther and Wendland 2003; Balasubramanian *et al.* 2004; Moseley and Goode 2006; Moseley and Nurse 2009; Roncero and Sanchez 2010).

Mechanics of Mother/Daughter Separation

Two major things happen when budding yeast cells divide: partitioning and separation of the cytoplasm and division of the nucleus. These processes are closely interlinked to ensure that genetic material is properly segregated to the mother and daughter cells. Division of the cytoplasm comprises (A) construction and function of a contractile actomyosin ring and (B) deposition of a multilayered septum, followed by (C) destruction of the septum to allow final separation of the divided cells. Coinciding with these processes, (D) division of the nucleus and disassembly of the spindle occurs concurrently with cytokinesis.

Assembly and contraction of the actomyosin ring

Assembly of the *S. cerevisiae* cytokinesis site begins at the earliest stages of budding, with the formation of a narrow bud neck as cells pass from G1 into S phase. During the first stages of bud formation the septin proteins, which form filaments without intrinsic polarity (Frazier *et al.* 1998), are recruited from a soluble cytoplasmic pool to a polymeric form at the border between the mother cell and emerging daughter bud (reviewed in Weirich *et al.* 2008; Caudron and Barral 2009; McMurray and Thorner 2009; Oh and Bi 2011). As diagrammed in Figure 1, this septin filament system eventually forms a focused band that extends around the bud neck, close to the plasma membrane (Longtine and Bi 2003; Kinoshita 2006; Oh and Bi 2011). The septin lattice is initially highly dynamic, but reorganizes into a more stable structure as bud growth proceeds (Dobbelaere *et al.* 2003; Dobbelaere and Barral 2004; Vrabioiu and Mitchison 2006; Demay *et al.* 2011). The septin ring at the bud neck functions as a barrier that prevents diffusion of membrane proteins and other cell cortex material (Barral *et al.* 2000; Dobbelaere and Barral 2004; Vrabioiu and Mitchison 2006; Caudron and Barral 2009).

Other proteins involved in bud neck morphogenesis and cytokinesis are recruited to the septin collar as bud emergence occurs. These include Myo1, a type II myosin directly involved in actomyosin ring contraction, which localizes to the bud neck almost as soon as it forms but has no apparent function there until cytokinesis at the M/G1 transition. The septin band extends continuously through the bud neck until cytokinesis, when it splits in two, leaving rings on both the mother and daughter sides of the bud neck. This has been proposed to create a restricted membrane domain at the bud neck between the two remaining septin rings, allowing concentration of phosphoinositides and membrane-associated

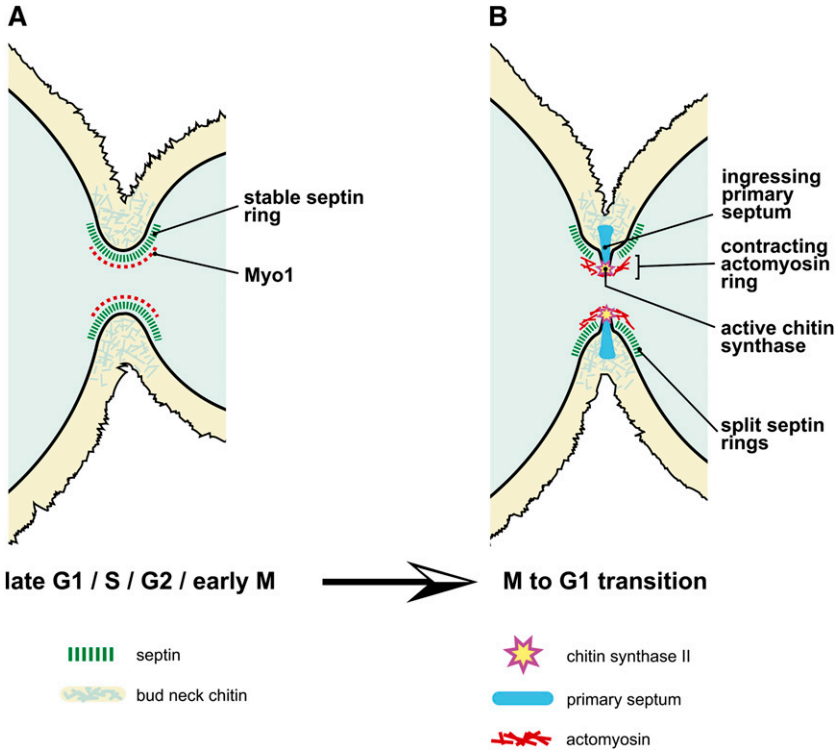

Figure 1 Early organization of the cytokinesis site and initiation of cytokinesis. (A) When the bud is formed in G1/S, septin filaments (green dashes) help organize the bud neck and promote recruitment of Myo1 (red dashes); filamentous actin is not markedly present. Chitin (blue lines) is present in the bud neck from early stages in its formation. The septin lattice also serves as a barrier that prevents diffusion of material associated with the daughter cell cortex into the mother cell. (B) During early cytokinesis, a contractile actomyosin ring (red lines) is assembled that helps guide deposition of a primary septum (blue) through the localized action of Chs2 (yellow star).

proteins involved in actomyosin ring contraction (Dobbelaere and Barral 2004).

Septation

Overview: Actomyosin ring contraction is accompanied by synthesis of a specialized multilayered septum between mother and daughter cells (Figure 1), which is built in distinct but nearly coincident phases. First, a thin primary septum composed primarily of chitin, a polymer of *N*-acetylglucosamine, forms behind the ingressing cytokinetic furrow. Primary septum synthesis and actomyosin ring contraction are intimately linked, with the ingressing furrow guiding an active zone of chitin synthesis. Closely following primary septum formation, the structure is thickened on both mother and daughter sides by localized deposition of additional chitin and other wall polymers (Figure 2), forming the secondary septum. This occurs as the actomyosin ring disassembles and the chitin synthase II machinery (see below) is endocytosed. Once the primary and secondary septa are complete, the enzymes responsible for chitin export are internalized, and the synthesis of other septum components is also downregulated.

Septation is a form of cell wall synthesis, and so in addition to dedicated enzymes it uses many of the same synthetic machines that assemble the dynamic cell wall covering the rest of the cell. Indeed, defects in protein mannosylation affect septation; this process occurs as proteins that span the plasma membrane or are secreted move through the endomembrane system, and is broadly important for the function of cell wall components (Schmidt *et al.* 2005). A forthcoming YeastBook chapter by Orlean and Strahl will discuss cell wall organization mechanisms in detail; additionally, the process by which this septum is built is the subject of a number of excellent reviews (Cabib *et al.* 2001; Roh *et al.* 2002; Roncero and Sanchez 2010).

Synthesis of the primary septum: The primary septum is made of chitin deposited as long fibers that are not extensively cross-linked to other cell wall components (Cabib and Duran 2005). Chitin in the primary septum is synthesized *in situ* by chitin synthase II, of which the protein Chs2 is the catalytic subunit. This ~110-kDa enzyme spans the plasma membrane and likely forms a channel through which the chitin polymer is extruded (Sburlati and Cabib 1986). Chs2 is exclusively involved in septation (Shaw *et al.* 1991). Consistent with this role, it is largely produced in mitosis, but is retained in the endoplasmic reticulum (ER) until mitotic exit (Chuang and Schekman 1996; Zhang *et al.* 2006; Teh *et al.* 2009). As discussed in part 3, when Chs2 is dephosphorylated as cells progress from M to G1, it proceeds through the secretory pathway to the site of cytokinesis and concentrates at the actomyosin ring (Schmidt *et al.* 2002; VerPlank and Li 2005; Teh *et al.* 2009). Chitin synthase II function is intimately connected with actomyosin ring contraction and polarization of membrane trafficking to the cytokinesis site and is also directly controlled by a mitotic exit regulatory system (Bi 2001; Schmidt *et al.* 2002; Oh *et al.* 2012).

While the primary septum's main structure is provided by chitin fibrils extruded by Chs2, additional proteins concentrate at the structure to promote its integration with the rest of the septum. A fraction of the cell wall reorganizing

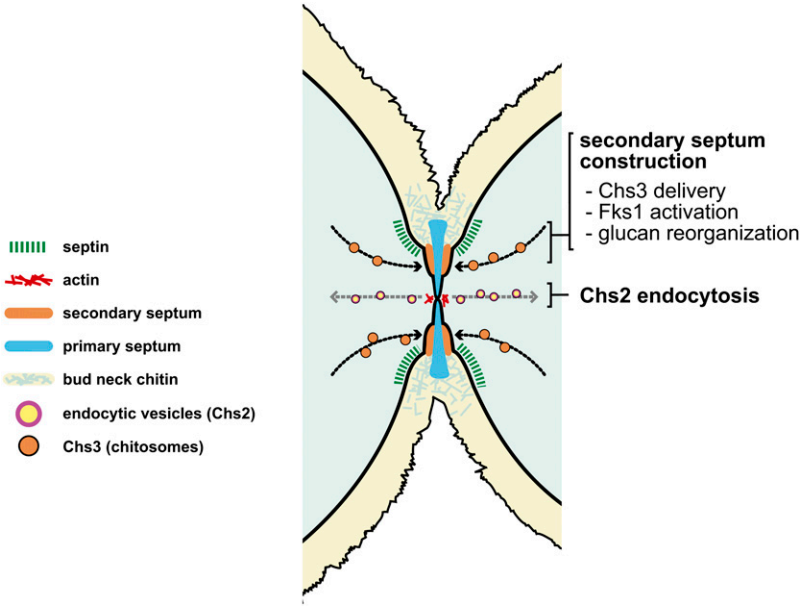

Figure 2 Completion of the primary septum and secondary septum synthesis. Coincident with completion of the primary septum (blue), the actomyosin ring (red lines) is disassembled and Chs2 is internalized through endocytosis (yellow circles). Deposition of the secondary septum (orange) occurs on both mother and daughter sides of the primary septum. This involves synthesis of glucan and chitin by Fks1 (which is resident in the plasma membrane) and Chs3, which is delivered in by exocytic vesicles (orange circles) along with other material that helps build up the structure.

enzyme Gas1 localizes to the primary septum upon cleavage of a GPI-linked portion of the protein that anchors it to the plasma membrane (Rolli *et al.* 2009). Similarly, Cwp1 is also trafficked to the site depending on when it is produced (Smits *et al.* 2006). These proteins help cross-link the outer layers of the chitinous primary septum to the adjacent glucan-rich secondary septum, ensuring that the multilayered structure does not delaminate.

The role of Rho GTPases in primary septum formation: Rho family GTPases are critical for eukaryotic cytokinesis, and concentration of active Rho at the cell division site with proper timing is important for normal progression (Nishimura *et al.* 1998; Bement *et al.* 2005; Piekny *et al.* 2005). In budding yeast the GTPase Rho1, which plays important roles in spatial organization of cell growth and membrane trafficking, localizes to the cytokinesis site and performs key functions in multiple steps of septum biogenesis (Kohno *et al.* 1996; Qadota *et al.* 1996). Assembly of the actomyosin ring, which guides deposition of the primary septum, requires Rho1. This in part reflects its role in cytokinetic activation and targeting of the formin Bni1, which nucleates assembly of actin cables (Tolliday *et al.* 2002). Upon phosphorylation by the *polo*-like kinase Cdc5 in late M phase, the Rho guanine nucleotide exchange factor (GEF) Tus1 accumulates at the cytokinesis site and recruits Rho1 (Yoshida *et al.* 2006) by an unclear mechanism. The GEF then generates active Rho1–GTP at that site, which triggers formin-mediated actin cable assembly (Yoshida *et al.* 2009). Rho1 may also directly regulate other aspects of actomyosin ring contraction and primary septum deposition.

Synthesis of the secondary septum and remedial septation: The secondary septum is a region of new cell wall material deposited next to the ingressing primary septum. Synthesis of β1–3 glucan by Fks1/glucan synthase is critical for this process (Cabib *et al.* 2001; Lesage *et al.* 2004, 2005; Lesage and Bussey 2006). The chitin synthase Chs3 also localizes to the site of secondary septum formation, providing additional structural reinforcement with chitin (Ziman *et al.* 1998; Cabib *et al.* 2001; Schmidt *et al.* 2002; Cabib and Schmidt 2003; Ortiz and Novick 2006). Delivery of other wall components by secretion is probably important, as there is a strong concentration of exocytic machinery to the site (Dobbelaere and Barral 2004; Zhang *et al.* 2006). For example, the "exocyst" component Sec3 (reviewed in Heider and Munson 2012; Liu and Guo 2012) acts in septation parallel to actomyosin ring contraction, suggesting that delivery of material through the secretory system promotes secondary septum formation (Dobbelaere and Barral 2004).

Budding yeast septation is robust and can occur in the absence of either primary septum synthesis or actomyosin ring contraction. This occurs via synthesis of a "remedial septum," which is essentially a secondary septum deposited at the bud neck in a way that is disorganized yet sufficient to separate mother and daughter cells (Cabib and Schmidt 2003; Tolliday *et al.* 2003). In fact, complete absence of chitin in both the primary and secondary septa can be compensated by deposition of other polymers in this remedial structure, albeit poorly (Schmidt 2004). Intriguingly, elimination of the primary septum synthesis machinery selects for whole chromosome aneuploidies that enhance remedial septum formation (Rancati *et al.* 2008).

Rho1's role in secondary septum formation: In addition to its GEF-mediated recruitment, Rho1 has a short plasma membrane localization domain at its C terminus, containing a CAAX prenylation motif and a polybasic sequence (PBS) that mediates phospholipid association (Heo *et al.* 2006). This likely helps concentrate Rho1 at membrane regions

enriched in phosphatidylinositol 4,5-bisphosphate, (PIP2), a phospholipid generated by the phosphoinositide kinase Mss4 (Homma *et al.* 1998; Yoshida *et al.* 2009). Intriguingly, a Rho1 C-terminal fragment concentrates at the bud neck during late division, consistent with localized PIP2 generation in a restricted region of membrane at the bud neck bounded by septin cortical barriers (Dobbelaere and Barral 2004). This mechanism for Rho1 recruitment may be crucial for secondary septum formation: when primary septum deposition is abrogated, increasing either Rho1 or PIP2 levels helps alleviate the resulting division defect (Yoshida *et al.* 2006). Rho1 activates Fks1 glucan synthase and initiates recruitment of Chs3, likely promoting secondary septum construction by locally activating these polymer synthesis machines (Mazur and Baginsky 1996; Qadota *et al.* 1996; Inoue *et al.* 1999). Accordingly, loss of the Rho–GTPase activating protein (GAP) Lrg1 results in much thicker secondary septa (Svarovsky and Palecek 2005). Rho1 is also involved in activation and recruitment of Sec3 and the exocytic apparatus, which delivers chitin synthases for construction of both primary and secondary septa (Guo *et al.* 2001; Roumanie *et al.* 2005; Baek *et al.* 2010; Wu and Brennwald 2010; Yamashita *et al.* 2010). Overall, these findings suggest that Rho1 directly coordinates multiple distinct processes to ensure that cytokinesis and septation proceed rapidly and robustly (Figure 3).

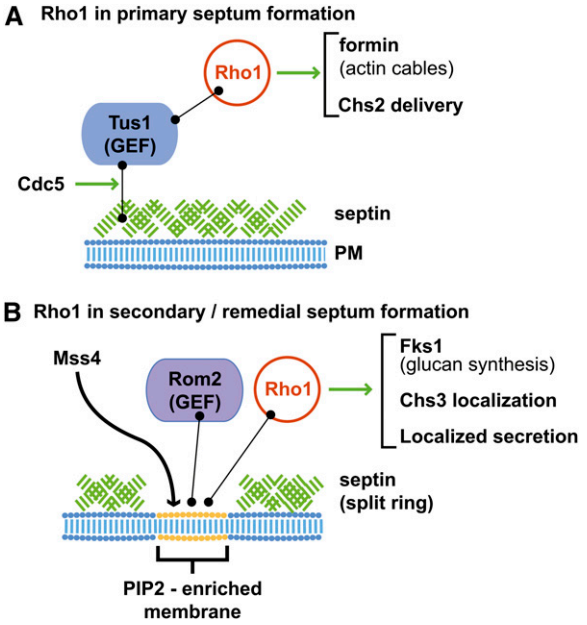

Figure 3 Model for Rho1 action in coordination of primary and secondary septum synthesis. (A) Early in cytokinesis, Cdc5-mediated GEF recruitment localizes Rho1–GTP, which promotes assembly of actin cables. It is not clear if direct association with septins (green) mediates this. (B) Splitting of the septin ring permits formation of a spatially constrained PIP2-rich membrane domain generated by Mss4. This concentrates Rho1 to the site via binding to phospholipids and the Rom2 GEF. Rho1 promotes multiple events, indicated at right.

Septum destruction

Overview: Once the cytokinetic furrow resolves and the primary and secondary septa are completed, mother and daughter cells remain linked by the septum. This connection is then cut, allowing the two cells to dissociate (Figure 4). Destruction of the septum requires enzymatic digestion of chitin in the inner layer, and to a more subtle extent, the digestion and/or remodeling of the secondary septum (Cabib *et al.* 2001; Walther and Wendland 2003; Roncero and Sanchez 2010).

Unlike the transmembrane machinery that synthesizes chitin and glucan polymers, cell separation enzymes are secreted and hence are beyond the reach of intracellular regulatory mechanisms. Yet, because of the critical importance of wall integrity, septum degradation must proceed via carefully targeted dissolution and reorganization of cell wall materials, coordinated with wall reinforcement. Septum destruction occurs nearly immediately after cytokinesis, displaying a temporal coordination with the completion of membrane abscission and septum synthesis that remains poorly understood.

Remarkably, the septum is degraded exclusively from the daughter cell side. This explains a long-noted observation: mother cells are left with a chitin-rich remnant of the septum called the "bud scar" after separation, while daughter cells have a region of new cell wall material at the corresponding site referred to as the "birth scar" (Belin 1972; Powell *et al.* 2003). This asymmetry reflects exclusive expression of septum destruction proteins in the newly born daughter cell, via mechanisms discussed in part 3.

Enzymes of septum destruction: chitinase: Degradation of chitin in the primary septum is the key event in the final separation of mother and daughter cells. This is carried out by the chitinase Cts1, an enzyme with an N-terminal glycoside hydrolase domain plus a C-terminal carbohydrate-binding module that binds specifically to chitin, which may foster localized activity by anchoring it to specific regions of the septum and surrounding wall (Kuranda and Robbins 1991). Deletion of *CTS1* causes dramatic defects in cell separation. The crystal structure of Cts1's enzymatic domain (Hurtado-Guerrero and van Aalten 2007) yields insight into its specificity for the chitin oligosaccharide. The protein localizes to the region of the septum of a cell undergoing separation, in a distribution biased toward the daughter cell side (Colman-Lerner *et al.* 2001), consistent with its daughter-specific expression pattern (see part 3). Cts1 is highly glycosylated, but the details of its secretory trafficking, and whether any steps are regulated, are unknown.

Enzymes of septum destruction: endoglucanases/glucanosyltransferases: In addition to Cts1, efficient septum removal requires a suite of known and putative hydrolytic enzymes that target wall components other than chitin. Some of these probably remove cell wall polymers in the secondary septum, while others locally reorganize the wall by transferring glucan chains from one macromolecule to another. These enzymes may function primarily to open up the secondary septum to facilitate delivery of chitinase to

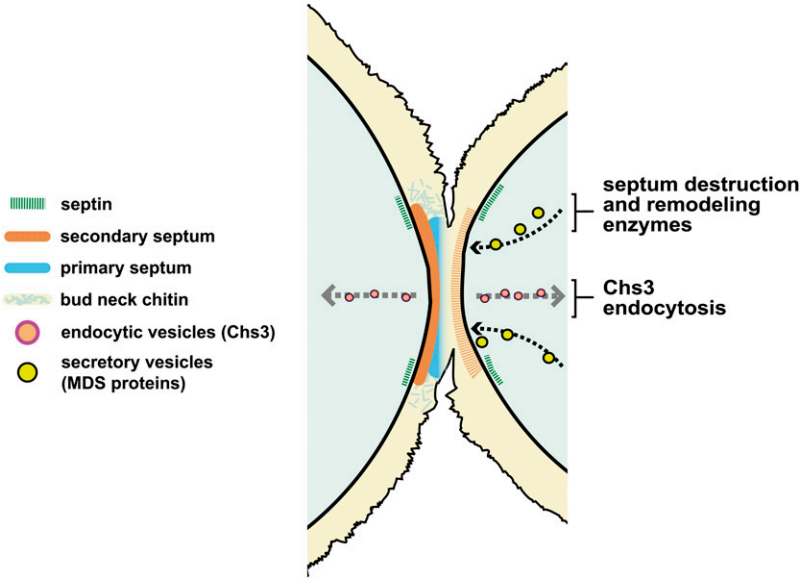

Figure 4 Septum destruction. After the secondary septum is complete, Chs3 is endocytosed (orange circles)and presumably Rho1–Fks1 is partially inactivated (not shown). Coincident with this, mother/daughter separation enzymes (MDS proteins) that reorganize and degrade the septum are produced in the daughter cell and delivered to the division site. The chitin synthase Chs1 is also delivered to the site (not shown), where it repairs damage caused by degradative enzymes.

the primary septum. The proteins Dse2, Dse4, Egt2, and Scw11 are known or likely hydrolytic enzymes that function exclusively in mother/daughter separation. Additionally, optimal cell separation requires glucan remodeling proteins that function more broadly in wall organization during bud growth. These include the glucanosyl transferase Gas1 and members of a closely related group of putative glucanases collectively referred to as SUN proteins (Sim1, Uth1, Nca3; also Sun4 and possibly Tos1). These proteins probably do not function in primary septum degradation, and loss of any one causes relatively subtle separation defects. In the few cases tested, combined deletion of multiple genes enhances the separation defect (Mouassite *et al.* 2000a,b). Thus, these proteins probably play partially overlapping or redundant roles in secondary septum destruction and remodeling, in addition to functioning in cell wall organization during bud growth.

Dse4/Eng1 endoglucanase: The best understood cell separation endoglucanase is Dse4, which was extensively characterized in studies that refer to it as Eng1 (Baladron *et al.* 2002). It is highly glycosylated, with a C-terminal catalytic domain that endohydrolytically cleaves 1,3-β-glycosidic bonds *in vitro*. Like Cts1, Dse4 localizes specifically to the daughter cell side of the septum in large budded cells that have not yet completed separation. Intriguingly, Dse4 may function as a transglucosidase, rearranging the linkages in glucan chains at high substrate concentrations rather than digesting and eliminating the polymer. Dse4 may contain a GPI linkage that attaches it to the plasma membrane and/or wall matrix (De Groot *et al.* 2003).

Other hydrolases specific to septum destruction: Egt2, Dse2, and Scw11 are also likely hydrolases that function primarily in septum degradation (Adams 2004). All contain glucanase domains, but their enzymatic activities have not been directly assessed. Dse2 is required for efficient cell separation (Doolin *et al.* 2001), and like Dse4 may be GPI linked (De Groot *et al.* 2003). Scw11 is closely related to two other wall glucanases, Scw4 and Scw10, that are soluble proteins released from the cell wall upon treatment with reducing agent (Cappellaro *et al.* 1998). Genetic analysis indicates that Scw11 is involved in septum destruction while Scw4 and Scw10 have different functions in wall morphogenesis. Egt2 is a GPI-anchored protein, but it is not clear if its function requires cleavage of this moiety (Hamada *et al.* 1999). While most glucanases solely involved in septum destruction are expressed only in daughter cells, the *EGT2* gene is regulated by Swi5 and thus expressed in both mother and daughter cells (Kovacech *et al.* 1996). Thus, Egt2 may have a special role in remodeling the septum on the mother cell side.

SUN-family glucanases: The role of the SUN family glucanases in cell separation is subtle, and complicated by their additional function in cell wall organization during budding (Mouassite *et al.* 2000a,b). All contain related domains with strong similarity to known glycosidases. Sun4 was identified by release from cell walls, where it is abundant. The *UTH1* gene was initially identified in screens for mutations that increased cell lifespan. Uth1 has been proposed to act directly in both wall organization and autophagic destruction of mitochondria (Camougrand *et al.* 2003, 2004; Kissova *et al.* 2004), but this is controversial (Kanki *et al.* 2009). Recent evidence strongly suggests that Uth1 is primarily a cell wall hydrolase and many phenotypes seen when it is deleted are the result of strengthened cell walls (Ritch *et al.* 2010).

Gas1: Gas1 belongs to a group of closely related GPI-linked proteins that contain glucanosyl transferase domains (Gas1–5) (Popolo *et al.* 2001, 2008; Ragni *et al.* 2007a,b; Rolli *et al.* 2011). These proteins transfer glucan chains extruded from β1–3 glucan synthase to other cell wall components, a critical role in wall organization. There are two extracellular pools of Gas1. Prior to cleavage of a GPI

linkage from its tail, the protein remains associated with the plasma membrane (PM) and is present broadly. This membrane-anchored fraction functions in wall organization, in concert with Fks1/2, to build a lattice of cross-linked glucan. A significant fraction of Gas1 associates with chitin in the bud neck and close to the primary septum, and this anchorage probably happens after directed cleavage of the GPI anchor from its tail (Rolli *et al.* 2011). This fraction is involved in both bud neck morphogenesis and cell separation: loss of septum-localized Gas1 causes cell separation defects, suggesting that transglucosidase prepares the structure for the action of exoglucanases and chitinase. Intriguingly, another fraction of Gas1 plays an intracellular role in chromatin silencing (Koch and Pillus 2009), apparently unlinked to its role in cell wall organization.

An integrated model for the process of septum destruction: Figure 5 illustrates two plausible models for septum destruction. In one view (Figure 4, A–C), chitinase and other cell wall remodeling enzymes are secreted into the periplasmic space beneath the secondary septum in the daughter cell. Additionally (and not shown in this model), some glucan-remodeling enzymes are present at the cytokinesis site because they were deposited before septation, most notably when the mother/bud neck formed early in the budding process. In this model, the glucan network of the secondary septum is then opened and remodeled to allow rapid diffusion of Cts1 to the primary septum. Once this payload of chitinase is delivered, it both binds and digests the lattice of chitin filaments. Simultaneous with or just behind this wave of hydrolytic and glucan-remodeling enzymes, β1–3 glucan (from Rho1-activated Fks1) and a small amount of chitin (from Chs1) are locally synthesized and transferred into a cross-linked mesh. Thus, the extracellular lattice's integrity would be maintained as the secondary septum is dynamically remodeled.

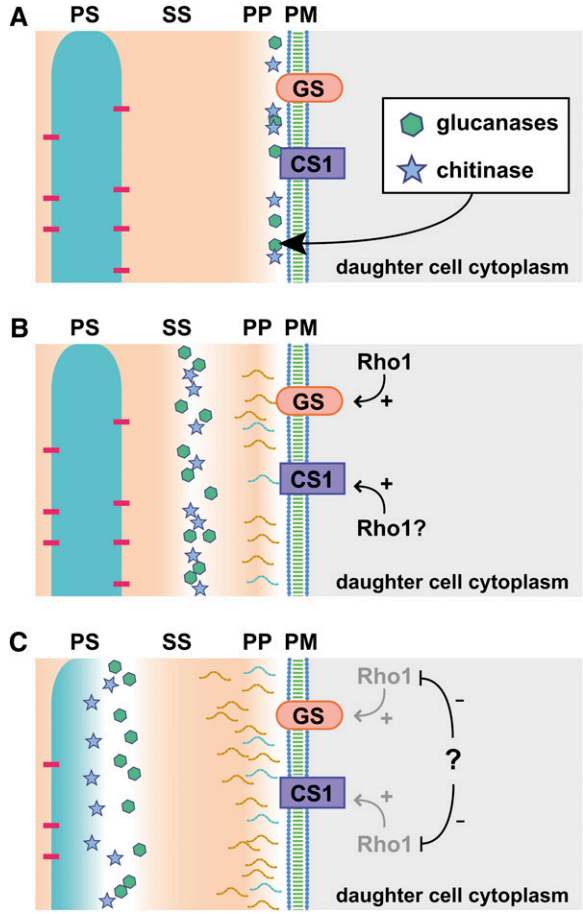

Figure 5 A model for enzymatic destruction of the septum. (A) Upon completion of septation, in response to an unknown signal, chitinase and a suite of glucanases are exocytosed into the small periplasmic space (PP) between the secondary septum (SS) and the outer leaflet of the plasma membrane (PM). Coincident with this, glucan and chitin are synthesized by transmembrane enzymes Fks1 (GS) and Chs1 (CS1), respectively. These oligosaccharide chains are transferred enzymatically to other wall components. (B) Exocytosis of septum destruction enzymes ceases. Digestion and chain rearrangement by glucanases and glucanosyltransferases creates a zone into which chitinase can diffuse. This is pushed forward by new wall synthesis. (C) New wall synthesis is attenuated, and chitinase reaches the primary septum (PS). Digestion breaks the link between mother and daughter.

Resolving the nucleus: spindle breakdown and karyofission

In addition to partitioning of mother and daughter cell cytoplasms, productive cell separation requires proper resolution of the divided nucleus. In budding yeast, as in other ascomycetes, nuclear division occurs without breakdown of the nuclear envelope. Once anaphase chromosome movement and spindle elongation are complete, a thin thread of nucleoplasm and central spindle microtubules encased by nuclear membrane remains (Winey *et al.* 1995). Final resolution of this closed mitosis requires dissolution of the central mitotic spindle (Maddox *et al.* 2000). Spindle disassembly happens just prior to cytokinesis and is linked to mitotic exit by a recently defined regulatory network (Woodruff *et al.* 2010). Additionally, the strand of doubly membrane-encased nucleoplasm that stretches between the mother and daughter cells must be cut. This process is here termed "karyofission," and it is not well understood. This homotypic membrane fusion event appears to be linked to cytokinesis, but it remains unclear if machinery specifically dedicated to karyofission exists (Latterich *et al.* 1995; Lippincott and Li 2000). Intriguingly, nuclear division in filamentous fungi takes place in a common cytoplasm without the aid of a cytokinetic contractile apparatus or septation, suggesting the existence of an independent karyofission apparatus.

Mitotic Exit: Regulatory Systems That Control Mother/Daughter Separation

The mechanical events of late cell division and mother/daughter separation happen in rapid succession and precise order, reflecting a remarkable coordination of diverse and sometimes directly opposing processes in time and space. This is achieved by a regulatory system that links the cell

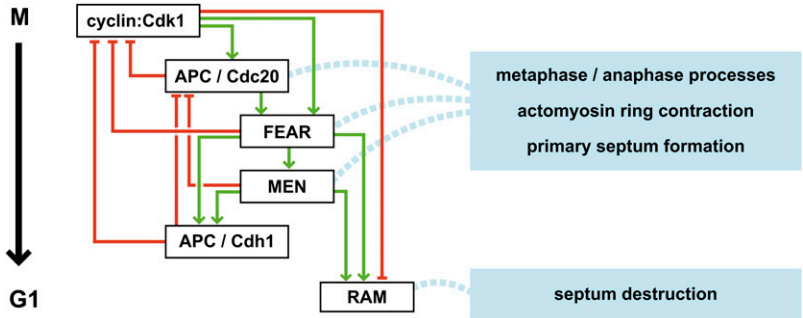

Figure 6 Overall organization of mitotic exit control. Mitotic exit is controlled by a series of systems that both activate (green pointed arrows) and inhibit (red block arrows) one another as cells pass from M into G1 phase. The temporal sequence in which the systems function during mitotic exit is ordered from top to bottom. A recurring theme is that a regulator will activate a downstream process that ultimately antagonizes the activator. For example, mitotic cyclin–Cdk1 promotes APC^{Cdc20} function and the FEAR pathway, which both then oppose M-phase CDK function. The large-scale processes occurring sequentially during mitotic exit are shown in blue boxes on the right, connected by dashed lines to the regulatory systems that promote them.

cycle's core cyclin–CDK oscillator to the assembly and function of molecular machines that divide the cell in two. The part of cell division that begins with anaphase chromosome movement and ends after resolution of cell separation is referred to as "mitotic exit." Studies in yeast are achieving an unprecedented understanding of regulatory systems that control this phase of the cell cycle and drive the ordered events of late cell division and separation.

Unsurprisingly, mitotic exit control pathways are integrated with numerous other aspects of the cell division cycle. For example, checkpoints monitoring spindle assembly, DNA damage, completion of replication, and even bud neck morphogenesis, impinge on the M/G1 transition. While this chapter necessarily focuses on pathways that underlie mitotic exit and cell separation, I urge the reader to avoid drawing artificially rigid boundaries between the major cell cycle regulation systems. Indeed, understanding how these pathways are dynamically integrated is an important frontier in contemporary molecular biology whose exploration will certainly be led by ongoing studies in yeast.

Overview of mitotic exit pathways: APC/C, FEAR, MEN, and RAM

As cells make the transition from M to G1 the effects of mitotic cyclin–CDK are reversed and the regulatory pathways that promote late cell cycle events are activated. A key early insight into the control of mitosis was the determination that progression from metaphase to anaphase involves ubiquitin-mediated proteolytic destruction of mitotic cyclins (reviewed in Morgan 2007; Sullivan and Morgan 2007; Enserink and Kolodner 2010). It is now well established that cyclin destruction in mitosis is a key regulated step in the cell cycle, and failure to degrade mitotic cyclin blocks division at metaphase arrest (reviewed in Pines 2011). Additionally, reversing the effects of mitotic cyclin–CDK requires mitotic phosphorylations to be removed. In budding yeast this is largely carried out by the proline-directed phosphatase Cdc14 (Stegmeier and Amon 2004; Amon 2008).

As diagrammed in Figure 6, mitotic exit control can be roughly broken into four interlaced regulatory systems that are sequentially activated as cells pass from metaphase into G1. The first to act is the **a**naphase **p**romoting **c**omplex/**c**yclosome (APC/C) (Pines 2011; Song and Rape 2011) in complex with the protein Cdc20, which initiates degradation of the mitotic cyclins as well as the material that keeps replicated metaphase chromosomes from separating. Mitotic cyclin–CDK promotes assembly of the APC/C, which is held inactive until checkpoints that monitor chromosome attachment to the mitotic spindle are satisfied (Peters 2002; Elia *et al.* 2003; Silva *et al.* 2011). The second and third mechanisms to act are the Cdc **f**ourteen **e**arly **a**naphase **r**elease (FEAR) pathway and the **m**itotic **e**xit **n**etwork (MEN) (Dumitrescu and Saunders 2002; de Bettignies and Johnston 2003; Stegmeier and Amon 2004; Sullivan and Morgan 2007; Queralt and Uhlmann 2008a). The MEN is essential for mitotic exit, while the FEAR pathway is not strictly required. These pathways control Cdc14, which dephosphorylates mitotic CDK substrates, and also directly regulate processes important for productive cell division. Activation of the FEAR and MEN systems helps convert the APC/C to a different form, a complex with the protein Cdh1. A fourth pathway acts later by driving localization and activation of the transcription factor Ace2, which turns on expression of separation genes, as well as other mechanisms that promote septum destruction. This system also functions in cell morphogenesis control and is thus referred to as the **r**egulation of **A**ce2 and **m**orphogenesis (RAM) network (Nelson *et al.* 2003; Maerz and Seiler 2010).

"Hippo" pathways: the MEN and the RAM networks have similar core organization: Before discussing the MEN and the RAM networks in detail as separate systems, it is worth noting their similar components and functional organization. As diagrammed in Figure 7, both comprise core elements of ancient pathways that contribute to the control of cell growth, proliferation, and morphogenesis in diverse eukaryotes. These pathways have been labeled Mst/*hippo* or Ndr/LATS signaling systems, after mammalian and *Drosophila* kinases involved (Edgar 2006; Harvey and Tapon 2007; Hergovich 2011, 2012; Hergovich and Hemmings 2009; Varelas and Wrana 2012). These networks appear deep in the tree of eukaryotic life, with related pathways present from humans to *Protista* (Manning *et al.* 2011; Tavares *et al.* 2012).

In these pathways, GCK group "Mst/*hippo*" kinases directly control AGC group "Ndr/LATS" kinases, which form

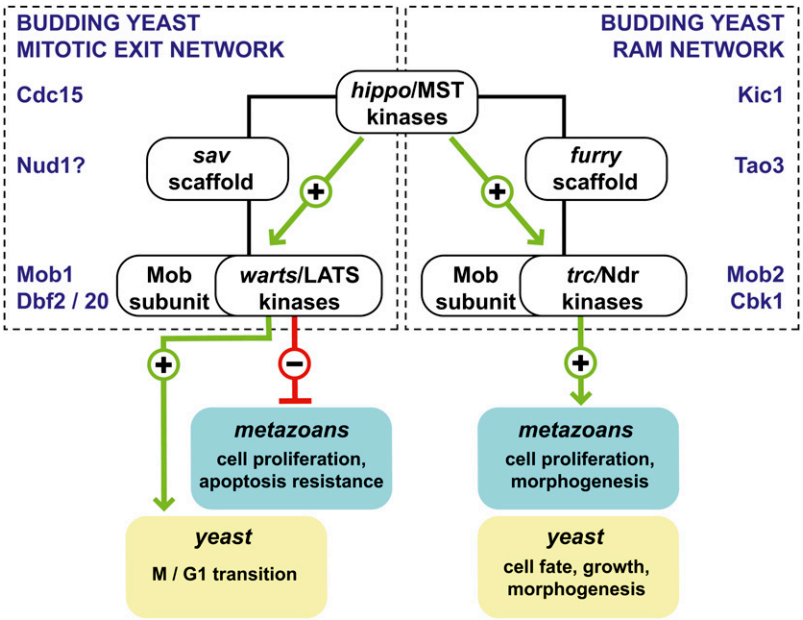

Figure 7 Conserved structure of Mst/hippo signaling pathways. Pathways similar to the MEN and the RAM network are broadly conserved in eukaryotes. Activating phosphorylations are shown as green arrows, while inhibitory phosphorylation is shown as a red block arrow. The MEN is most similar to the pathway in the left box, although no *sav* ortholog is present. The RAM is most similar to the pathway in the right box.

a crucial association with highly conserved co-activating "Mob" subunits. The Ndr/LATS kinases function as the downstream-most components that control cell proliferation, gene expression, and morphogenesis. Flow of information in these systems involves Mst/*hippo* kinase phosphorylation of the Ndr/LATS kinase at a hydrophobic motif C-terminal to the kinase catalytic core, referred to as the hydrophobic motif (HM) site. This regulatory phosphorylation is broadly conserved in AGC group kinases and is thought to play regulatory roles that include enhancement of kinase activity and recruitment to substrate proteins *in vivo* (Biondi *et al.* 2002; Yang *et al.* 2002a,b; Sarbassov *et al.* 2005). It is not clear if HM site modification of Ndr/LATS kinases works the same way. The Ndr/LATS kinases have a conserved activation loop regulatory phosphorylation site, which in Ndr kinases gets modified by an intramolecular reaction *in vitro* and *in vivo* (Bichsel *et al.* 2004; Jansen *et al.* 2006). Ndr/LATS family kinase activations loops also generally contain an insert of unknown functional significance, which is not as highly conserved as the surrounding kinase domain (Bichsel *et al.* 2004; Maerz and Seiler 2010).

There appear to be two distinct kinds of Mst/*hippo* signaling pathways that are conserved as separate systems in a wide range of eukaryotes. In one, the downstream-most component is a LATS kinase bound to a Mob1 co-activator; in another it is an Ndr kinase bound to a Mob2 co-activator. As diagrammed in Figure 7, the MEN is a "LATS type" pathway, and the RAM network is an "Ndr type" pathway. As in yeast, metazoan pathways have distinct functions: the "*salvador/warts/hippo*" system is a LATS-type pathway that restrains cell proliferation and promotes apoptosis (Harvey and Tapon 2007; Pan 2010; Sudol and Harvey 2010; Zhao *et al.* 2011), whereas the Ndr-type "*furry/tricornered/hippo*" pathways drive cell division and control cell polarization (Emoto *et al.* 2006; Seiler *et al.* 2006; Gao 2007; Cameron and Rao 2010; Cornils *et al.* 2011a,b; Staley and Irvine 2012). The mechanistic implications of the similarity between Ndr and LATS branches (and more specifically the MEN and the RAM network) are unclear.

The APC/C

The APC/C is an E3 ubiquitin ligase that recognizes specific mitotic proteins and targets them for destruction by the proteasome, precipitating anaphase onset. It is important for mitotic exit and mother/daughter separation, and thus is discussed here in overview; recent reviews provide a more in-depth treatment (Pines 2011; Song and Rape 2011). In metaphase cells, APC/C is present but cannot trigger degradation of target proteins because its function requires association with one of two sequentially acting co-activating subunits, Cdc20 and Cdh1 (Sullivan and Morgan 2007). Cdc20 acts first, associating with APC/C at the end of metaphase to form a complex denoted APC^{Cdc20}.

Activated APC^{Cdc20} initiates ubiquitination and degradation of mitotic cyclins, and also completely destroys securin, an inhibitor of the protease known as separase (in yeast, Esp1); separase destroys proteins that link replicated sister chromosomes at metaphase and promotes release of the phosphatase Cdc14 from inhibitory sequestration (Yeong *et al.* 2000; Sullivan *et al.* 2001; Yeong *et al.* 2002; Buonomo *et al.* 2003; Sullivan *et al.* 2004b). As discussed further below, APC^{Cdc20}-mediated destruction of spindle-associated proteins is important for the mechanical progress of anaphase onset. Formation of APC^{Cdc20} is blocked by a checkpoint signal generated by kinetochores that have not formed a bipolar attachment to the mitotic spindle, linking the initiation of anaphase to completion of spindle assembly (Nezi and Musacchio 2009).

While APC^{Cdc20} begins the destruction of mitotic cyclins, their complete degradation and persistent instability in the subsequent G1 phase requires the APC/C to associate with the Cdh1 subunit. This complex, known at APC^{Cdh1}, completes the inactivation of mitotic cyclin–CDK and also promotes destruction of other key mitotic proteins that are not targeted by APC^{Cdc20}; in fact, Cdc20 itself is a target of APC^{Cdh1} (Prinz *et al.* 1998). One mechanistic reason for the transition between APC^{Cdc20} and APC^{Cdh1} is that activity of APC^{Cdc20} is optimally maintained when cyclin–CDK levels are high, while APC^{Cdh1} function is actually antagonized by mitotic CDK phosphorylation (Zachariae *et al.* 1998; Yeong *et al.* 2000; Toth *et al.* 2007; Holt *et al.* 2008; Pines 2011). Once mitosis is complete, APC^{Cdh1} helps define the G1 state: it turns off mitotic exit by targeting the *polo*-family protein kinase Cdc5 for destruction in late M or early G1 and destabilizes mitotic cyclins throughout G1 (Wasch and Cross 2002; Visintin *et al.* 2008).

Cdc14: core regulator of mitotic exit events

Overview: Two major molecular processes must occur for cells to pass from M phase into G1. First, the mitotic cyclin–CDK complex has to be inactivated. Second, phosphorylation of key CDK substrates needs to be reversed. APC^{Cdc20} is critical for initiating cyclin destruction, chromosome separation, and anaphase spindle elongation, but it is not sufficient for total destruction of mitotic cyclin or for completion of the M-to-G1 transition (Jaspersen *et al.* 1998; Morgan 1999; Sullivan and Morgan 2007; Holt *et al.* 2008). In budding yeast, completion of these tasks requires the phosphatase Cdc14 (Visintin *et al.* 1998; Jaspersen *et al.* 1999; Shou *et al.* 1999; Jaspersen and Morgan 2000; D'Amours and Amon 2004; Stegmeier and Amon 2004; Amon 2008). This CDK-counteracting phosphatase is inactive for most of the cell cycle because it is trapped in the nucleolus (Stegmeier and Amon 2004; Amon 2008). As cells pass from metaphase to anaphase, this entrapment is weakened and Cdc14 eventually floods into the cytoplasm to reverse CDK phosphorylations.

Notably, Cdc14 orthologs are not essential for mitotic exit in many organisms (Mocciaro and Schiebel 2010), including other hemiascomycetes such as *Candida albicans* (Clemente-Blanco *et al.* 2006). This likely reflects functional redundancy, with other phosphatases taking on the task of reversing mitotic CDK phosphorylations (Uhlmann *et al.* 2011). Nevertheless, Cdc14 is highly conserved, and in many organisms it may control specific mitotic subprocesses rather than mitotic exit as a whole. In both *C. albicans* and the distantly related yeast *Schizosaccharomyces pombe*, for example, Cdc14 orthologs are important for postcytokinetic processes such as septum destruction (Oliferenko and Balasubramanian 2001; Papadopoulou *et al.* 2010).

Cdc14 acts in direct opposition to mitotic CDK and controls diverse processes: Cdc14 is a "dual-specificity" phosphatase (DSP) that strongly prefers dephosphorylate phosphoserines or phosphothreonines that are immediately followed by proline, a motif that corresponds to a minimal CDK phosphorylation site. Thus, the enzyme can reverse the phosphorylation state of mitotic CDK substrates. The structure of a human Cdc14 catalytic domain has provided insight into the enzyme's evolution and site preference (Gray *et al.* 2003; Wang *et al.* 2004). Cdc14's enzyme core consists of tandem DSP domains, in which the C-terminal "B domain" provides catalytic activity while a pocket formed between the two domains enforces specific site recognition.

Cells lacking Cdc14 function arrest division in telophase, with chromosome masses segregated between mother and daughter cells by a fully elongated mitotic spindle. Such cells can be driven into G1 and made to initiate new budding cycles by overproduction of the CDK inhibitor Sic1, but this occurs without completion of cytokinesis (Luca *et al.* 2001). Thus, while Cdc14 is not strictly indispensible for cell cycle progress if mitotic CDK can be inactivated, it is essential for mother/daughter separation. Accordingly, Cdc14 localizes to the bud neck during cytokinesis (Bembenek *et al.* 2005), suggesting that it functions at this site during cell separation and that control of its cytoplasmic localization might contribute to its regulation.

How does Cdc14 control division? It has numerous roles in mitotic exit, as has been extensively reviewed (Stegmeier and Amon 2004; Amon 2008; Clifford *et al.* 2008; Queralt and Uhlmann 2008a; Mocciaro and Schiebel 2010; Meitinger *et al.* 2012). One of its important functions is to promote the transition from APC^{Cdc20} to APC^{Cdh1}, by dephosphorylating CDK sites on Cdh1 that block its association with the APC/C (Visintin *et al.* 1997; Zachariae *et al.* 1998; Jaspersen *et al.* 1999). However, Cdc14 has many more functions, and its reversal of a large number of CDK phosphorylations clearly influences the progress of late mitotic events. For example, it controls organization of the rDNA repeats, which form a highly distinct chromatin structure whose condensation and resolution is dependent on Cdc14 (D'Amours *et al.* 2004; Sullivan *et al.* 2004a). It also suppresses microtubule dynamics at anaphase onset by dephosphorylating the kinetochore component Ask1, thereby stabilizing the mitotic spindle as it begins the mechanical process of chromosome segregation (Li and Elledge 2003; Higuchi and Uhlmann 2005). Additionally, Cdc14 stabilizes spindles by dephosphorylating the microtubule binding protein Fin1, and shuts off the ability of anaphase-separated chromosomes to trigger a checkpoint signal by dephosphorylating the kinetochore component Sli15. Some key Cdc14 targets whose dephosphorylation is important for mother/daughter separation are discussed in the next section, but the current list is certainly incomplete.

How does a single phosphatase drive events that happen at different times in the transition from M to G1? Cdc14 might interact with different substrate targeting or localization subunits that direct its specificity and help define the sequential order of substrate dephosphorylation. Alternatively, Cdc14 appears to dephosphorylate different target

proteins with different efficiency, with good Cdc14 substrates dephosphorylated faster and more completely than poor substrates (Bouchoux and Uhlmann 2011). Hence, as CDK activity levels drop, good Cdc14 substrates would become dephosphorylated earlier than poor substrates (Bouchoux and Uhlmann 2011; Uhlmann *et al.* 2011).

Cdc14 is controlled by regulated sequestration to the nucleolus: Ectopic activation of Cdc14 can drive mitotic exit, and so an intricate regulatory system ensures that its activity is tightly controlled. During mitotic division Cdc14 localizes to the nucleolus and is essentially inactive until the end of metaphase. This reversible sequestration involves binding to a protein complex present on rDNA repeats in the nucleolus (Petes 1979). Specifically, Cdc14 binds the nucleolar protein Net1, also known as Cfi1, which is part of a system that transcriptionally silences rDNA repeats (Shou *et al.* 1999; Straight *et al.* 1999; Visintin *et al.* 1999). As cells begin anaphase, Cdc14's anchoring to the nucleolus is weakened due to increased phosphorylation of key components of the anchoring system. The release of Cdc14 proceeds in two distinct stages. First, under the control of the FEAR pathway, Cdc14 is released from the nucleolus into the nucleoplasm, but only a very small amount enters the cytoplasm. Then, coincident with cytokinesis and resolution of the mitotic spindle, sustained release of Cdc14 into the cytoplasm is triggered by the MEN.

The polo-like kinase Cdc5: a regulator of both FEAR and MEN

The highly conserved *polo*-like kinase Cdc5 is critical for mitotic exit, and thus for the initiation of mother/daughter separation. The amount of Cdc5 present in cells is strongly linked with mitotic progress: it accumulates in S phase, reaches maximum levels in late M phase, and is rapidly degraded in G1. Like other kinases of this family, Cdc5 has an essential C-terminal domain known as the polo box domain (PBD), which mediates association with S-(pS/pT)-(P/X) motifs in which the central residue is phosphorylated (Elia *et al.* 2003). Indeed, a crystal structure of the PBD from a human polo-like kinase reveals that phosphopeptide binding is likely a highly conserved function of this domain (Cheng *et al.* 2003), and mutation of PBD residues needed for phosphopeptide recognition inactivates yeast Cdc5 (Song *et al.* 2000). The PBD recognition motif can correspond to a minimal CDK site, and thus an early view of Cdc5's intimate relationship with mitotic CDK has been that CDK phosphorylation "primes" proteins for Cdc5 regulation. This is clearly the case for some Cdc5 substrates (Yoshida *et al.* 2006). Intriguingly, however, recent analyses indicate that phosphopeptide binding by the Cdc5 PBD is not truly essential, and instead is required for only a subset of Cdc5-dependent processes (Chen and Weinreich 2010; Ratsima *et al.* 2011).

Cdc5 controls both the FEAR pathway and the MEN. As discussed in detail below, in essence Cdc5 turns on both pathways and thereby increases the activity of Cdc14 (Figure 8). This is probably triggered by changes in Cdc5 kinase activity rather than in PBD-mediated docking to substrates (Ratsima *et al.* 2011). Intriguingly, since Cdc5 is a robust target of APC^{Cdh1} in G1 phase, the kinase effectively plants the seeds of its own destruction by upregulating Cdc14 (Visintin *et al.* 2008).

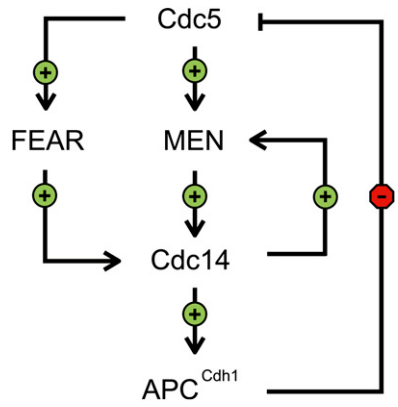

Figure 8 Cdc5 turns on its own destruction. The overall signaling interactions downstream of Cdc5 activation converge to activate Cdc14. This activates the APC^{Cdh1} form of the APC, which promotes degradation of Cdc5 and thereby closes a negative feedback loop.

FEAR pathway

As cells transition from metaphase to anaphase, the release of Cdc14 from its inhibited localization in the nucleolus begins. The FEAR pathway (Figure 9) controls early stages of this release. While this pathway is not strictly essential, it helps coordinate the timing of Cdc14-driven anaphase events.

Nucleolar Cdc14 sequestration: NET1 and the RENT complex: Nucleolar sequestration of Cdc14 requires association with Net1. An N-terminal region of Net1 binds Cdc14 and strongly inhibits its ability to dephosphorylate substrates, so that Net1 both anchors and inactivates the phosphatase (Traverso *et al.* 2001). In addition to Net1, Cdc14's sequestration requires a suite of other proteins. Most notably, Fob1 and Spo12 concentrate in the nucleolus and control the Net1–Cdc14 association. Fob1 blocks the progress of replication forks at specific locations in the rDNA repeats that form the bulk of the nucleolar region (Kobayashi and Horiuchi 1996; Mohanty and Bastia 2004). Spo12 is a binding partner of Fob1, and together they help keep the Cdc14 sequestration machinery in the nucleolus (Toyn and Johnston 1993; Stegmeier *et al.* 2002, 2004; Buonomo *et al.* 2003; Tomson *et al.* 2009; Bairwa *et al.* 2010). Fob1 anchors a Cdc14–Net1 complex in association with Sir2, a silencing protein, to the rDNA repeats. This complex is called the **re**gulator of **n**ucleolar silencing and **t**elophase (RENT), and it suppresses transcription and recombination in the rDNA repeats in addition to localizing Cdc14 (Shou *et al.* 1999; Straight *et al.* 1999; Visintin *et al.* 1999; Huang and Moazed 2003; Kobayashi *et al.* 2004; Huang *et al.* 2006).

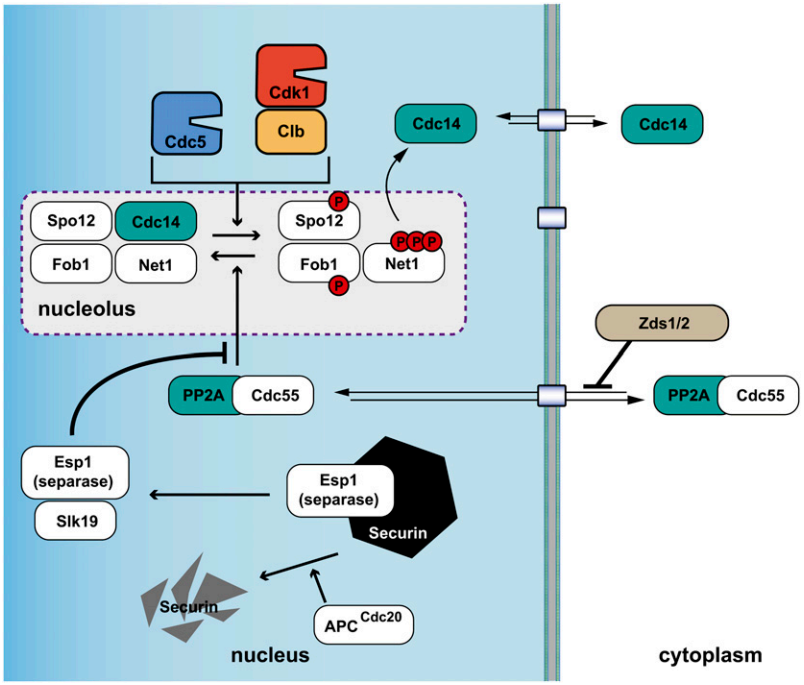

Figure 9 The FEAR pathway. The balance of phosphorylation and dephosphorylation of RENT components, most notably Net1, determines whether Cdc14 is sequestered or released from the nucleolus. Nucleocytoplasmic trafficking determines how much Cdc14 gets out of the nucleus and how much $PP2A^{Cdc55}$ gets in. Esp1 inhibits $PP2A^{Cdc55}$ dephosphorylation of RENT components.

Triggering the first pulse of Cdc14 release: Release from Net1 is a key part of Cdc14 activation, and thus the M/G1 transition. How is this interaction controlled? The phosphorylation state of Net1 and other RENT complex components is critically important. As diagrammed in Figure 9, mitotic CDK can phosphorylate Net1 at six key sites. Cdc5 is also required for Cdc14's early anaphase release, but this role remains mysterious (Sullivan *et al.* 2001; Sullivan and Uhlmann 2003; Visintin *et al.* 2003; Azzam *et al.* 2004; Rahal and Amon 2008; Manzoni *et al.* 2010). CDK phosphorylation of Spo12 helps potentiate disengagement of the Cdc14–Net1 interaction (Tomson *et al.* 2009).

Although CDK phosphorylation of Net1 and other RENT components causes release of Cdc14 (Azzam *et al.* 2004), this is counteracted by the protein phosphatase PP2A. This phosphatase is a multiprotein complex with different regulatory subunits that mediate substrate association; the regulatory subunit for Cdc14 release is Cdc55 (Queralt *et al.* 2006). Thus, a dynamic balance of kinase and phosphatase activities controls Cdc14 release from the nucleolus. $PP2A^{Cdc55}$ dominates this balance prior to anaphase, keeping Net1 dephosphorylated (Wang and Burke 1997; Queralt *et al.* 2006; Wang and Ng 2006; Yellman and Burke 2006). Suppression of $PP2A^{Cdc55}$ activity is thus a key trigger of mitotic exit.

When the FEAR pathway is activated, $PP2A^{Cdc55}$ activity toward Net1 is inhibited, and the balance of activities on the RENT complex tips toward CDK-mediated phosphorylation (Queralt and Uhlmann 2008a; Rossio and Yoshida 2011). The budding yeast separase ortholog Esp1 plays a critical role in this trigger, distinct from its essential role as the protease that dissolves the physical connection between replicated metaphase chromosomes. After APC^{Cdc20} degrades an Esp1 inhibitor, securin, Esp1 triggers release of Cdc14 by promoting CDK phosphorylation of Net1 (Sullivan *et al.* 2001; Sullivan and Uhlmann 2003; Azzam *et al.* 2004). Intriguingly, this function is independent of Esp1 protease activity (Sullivan and Uhlmann 2003). The Esp1-associated protein Slk19 is necessary for optimal Cdc14 release: while Slk19 is actually cleaved by the protease, this does not seem to be functionally important for the FEAR pathway (Stegmeier *et al.* 2002).

The proteins Zds1 and Zds2, which associate with and regulate $PP2A^{Cdc55}$ (Yasutis *et al.* 2010; Wicky *et al.* 2011) help tip the kinase–phosphatase balance in favor of Cdk1 (Queralt and Uhlmann 2008b). They probably accomplish this by binding and retaining $PP2A^{Cdc55}$ in the cytoplasm, thereby lowering its nuclear concentration (Rossio and Yoshida 2011), but the precise mechanism remains unclear. Analysis of their localization in isolated nucleoli suggests that Zds proteins may regulate a nucleolar pool of the phosphatase (Calabria *et al.* 2012).

FEAR pathway is an oscillator: FEAR-mediated release of Cdc14 can cycle with a defined periodicity even when mitotic cyclin–CDK activity levels are locked at discrete levels using nondegradable cyclins (Lu and Cross 2010; Manzoni *et al.* 2010). These pulses of Cdc14 release are entrained by Cdk1 to produce a single early burst of Cdc14 release. Normally, when mitotic CDK is only partially inactivated the FEAR system quenches itself by reducing the amount of CDK-phosphorylated Net1 in the cell, leading to resequestration in the nucleolus. Setting CDK activity at different levels reveals distinct oscillatory mechanisms. When CDK activity is high, cells stay arrested in mitosis and Cdk1 and Cdc5 drive Cdc14 release. Consistent with the negative

feedback loop shown in Figure 8, Cdc5 is then degraded allowing resequestration of Cdc14 (Visintin *et al.* 2008). Resynthesis and reactivation of Cdc5 then reset the cycle. At lower CDK levels, Cdc5 likely does not reset the cycle, and the MEN (described below) plays an essential role in driving repeated bursts of Cdc14 release.

MEN

Overview: Consistent with its nonessential nature, FEAR-driven Cdc14 release cannot drive full exit from mitosis: it is incomplete and results in very little accumulation of the phosphatase in the cytoplasm. Complete mitotic exit requires the MEN, which promotes robust relocalization of Cdc14, leading to full dephosphorylation of cytoplasmic CDK substrates and numerous downstream events (Charles *et al.* 1998; Bosl and Li 2005; Toth *et al.* 2007). The core MEN pathway consists of three functional modules: the G protein Tem1, the protein kinase Cdc15, and the protein kinases Dbf2 and Dbf20 in complex with their co-activating protein Mob1 (Figure 10) (Visintin *et al.* 1998; Shou *et al.* 1999; Tinker-Kulberg and Morgan 1999; Bardin *et al.* 2000; Jaspersen and Morgan 2000; Pereira and Schiebel 2001; Visintin and Amon 2001).

MEN components concentrate dramatically at the spindle pole body (SPB), the budding yeast centrosome equivalent, with a strong initial asymmetric bias toward the SPB that enters the daughter cell cytoplasm (Cenamor *et al.* 1999; Menssen *et al.* 2001; Visintin and Amon 2001; Yoshida *et al.* 2002). This localization of Tem1, Cdc15, and Mob1–Dbf2/20 to the SPB is critical for the system's activation in response to proper spindle position (Figure 11) and is largely accomplished through their association with the centriolin-related SPB protein Nud1 (Luca *et al.* 2001; Yoshida *et al.* 2002; Rock and Amon 2011). As discussed further in part 3 of this chapter, the MEN proteins also concentrate at the cytokinesis site just prior to actomyosin ring contraction and septation and probably function directly in those processes.

Core MEN components: the Dbf2–Mob1 module: The paralogous protein LATS-family kinases Dbf2 and Dbf20 are the MEN's downstream-most "LATS kinase" components (Figure 7) (Toyn *et al.* 1991; Toyn and Johnston 1994; Jaspersen *et al.* 1998; Lee *et al.* 2001a; Luca *et al.* 2001). Most analysis has focused on Dbf2, which appears to be the more important paralog (Toyn *et al.* 1991), using temperature-sensitive *dbf2* alleles in *dbf20Δ* cells. Only Dbf2 is discussed here, though Dbf20 is assumed to have largely similar properties. Dbf2, like all other characterized LATS kinases, binds to a Mob family co-activating subunit, in this case Mob1 (Figure 10) (Komarnitsky *et al.* 1998; Luca and Winey 1998; Luca *et al.* 2001). Mob1 is essential for mitotic exit. Three-dimensional structures have been obtained for Mob1 orthologs from budding yeast, *Xenopus laevis*, and humans (Stavridi *et al.* 2003; Ponchon *et al.* 2004; Mrkobrada *et al.* 2006). The Mob1 core structure

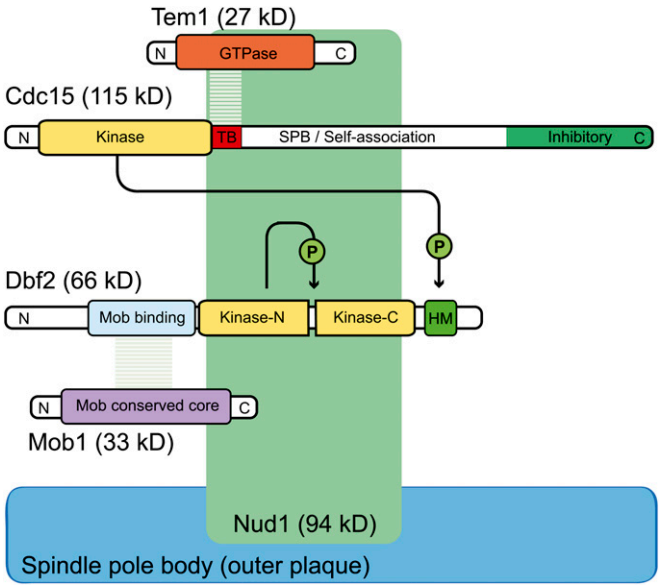

Figure 10 Molecular organization of the core MEN pathway. Activating phosphorylations are noted, and the domain organization of the Cdc15–Tem1 module and the Mob1–Dbf2 module are shown. Cdc15 is an Mst/*hippo*-related kinase that binds the Ras-family GTPase Tem1 through a region C-terminal to its kinase domain. Other parts of Cdc15 may act to promote self-association and MEN inhibition. Dbf2 is an Ndr/LATS family protein kinase that binds to Mob1 via a region N-terminal to its kinase domain. Nud1 acts as a scaffold that brings elements of the pathway together at the outer plaque of the SPB.

includes an electronegative surface that may create a binding region for the conserved positively charged Mob-binding peptide found N-terminal to the kinase domain in Dbf2 (Stavridi *et al.* 2003; Ponchon *et al.* 2004; Mrkobrada *et al.* 2006). *In vitro* studies showed that Dbf2 is basophilic, preferring to phosphorylate serine or threonine residues that are three residues C-terminal to arginine (R-X-X-[S/T]) (Mah *et al.* 2005). This short consensus motif, which is shared by other basophilic kinases, has been borne out for several *in vivo* substrates (Mohl *et al.* 2009; Oh *et al.* 2012).

Core MEN components: the Cdc15–Tem1 module: Cdc15, which functions upstream of Dbf2/Mob1 in the MEN (Figure 10), is a ~117-kDa protein with an Mst/*hippo*-related kinase domain near its N terminus. While Cdc15's enzymatic activity does not seem to fluctuate over the cell cycle, its function is blocked prior to the metaphase/anaphase transition by CDK phosphorylation (Jaspersen and Morgan 2000). Cdc15's C-terminal domain is crucial for its regulation and contains an important short segment next to the kinase domain that binds the Ras-related GTPase protein Tem1 (Asakawa *et al.* 2001; Bardin *et al.* 2003). Like other small GTPases, Tem1 probably activates its effectors only when loaded with GTP, but this has not been directly established. As discussed further below, Tem1 provides a key regulatory linkage that connects Cdc15's ability to activate Mob1–Dbf2 to the status of mitotic processes like spindle orientation (Shirayama *et al.* 1994; Jaspersen *et al.* 1998; Jaspersen

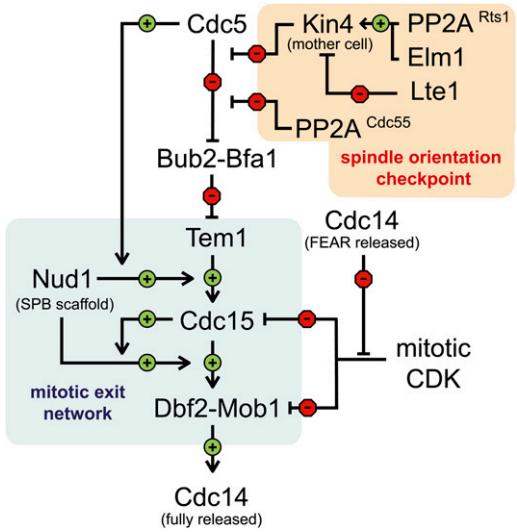

Figure 11 The logic of the MEN. Positive interactions in the broader MEN are indicated as pointed arrows, and negative ones as block arrows. The core MEN (Tem1–Cdc15–Dbf2 complexed with Nud1) is negatively regulated by both mitotic CDK and the Tem1 GTPase activating protein (GAP) Bub2–Bfa1. Cdc14 reverses the effects of CDK phosphorylation. Cdc5 turns Bub2–Bfa1 off, and Kin4 and PP2ACdc55 negatively regulate this. Cdc5 also activates the MEN more directly.

and Morgan 2000; Lee *et al.* 2001a,b; Menssen *et al.* 2001; Rock and Amon 2011). While specific amino acids in Cdc15's Tem1-binding region are important for the association, the biochemical basis of the interaction is incompletely understood. In addition to this short Tem1-interacting segment, a C-terminal portion of Cdc15 appears to function as an inhibitory region. Finally, Cdc15's central region mediates self-association, but it is not clear if this is direct or requires higher-order complex formation (Bardin *et al.* 2003).

Core MEN components: Nud1, a platform for SPB localization: The SPB protein Nud1 is an essential MEN component (Figure 10). Loss of its function causes arrest at the M/G1 transition, and it is required for cytokinesis even when this block to G1 entry is overridden by overexpression of a CDK inhibitor (Gruneberg *et al.* 2000; Luca *et al.* 2001; Yoshida *et al.* 2002). It associates with the cytoplasmic face of the SPB, which is embedded in the nuclear envelope (Winey and Bloom 2012). Nud1 recruits other components of the MEN to the SPB, and this localization is critical for MEN activation (Jaspersen *et al.* 1998; Cenamor *et al.* 1999; Shou *et al.* 1999; Visintin and Amon 2001; Molk *et al.* 2004; Rock and Amon 2011; Valerio-Santiago and Monje-Casas 2011). In fact, Cdc15 regulation of the Mob1–Dbf2 complex is probably mediated by co-association on Nud1 at the SPB. Nud1 contains an ~150-amino-acid region related to the metazoan protein centriolin, a cytokinesis factor that associates with centrioles and the site of cytokinetic abscission (Gromley *et al.* 2003). This conserved domain probably mediates association of membrane trafficking machinery (Gromley *et al.* 2005), but the importance of this association in yeast is unknown. Budding yeast Nud1 connects the γ-tubulin binding protein Spc72 to the SPB outer plaque and is thus an important anchor for cytoplasmic microtubules (Pereira *et al.* 1999; Gruneberg *et al.* 2000). It is not known if Nud1's role in microtubule organization is related to its mitotic exit function.

MEN's functional organization: *Controlling MEN: the core pathway:* As diagrammed in Figure 10, Cdc15's direct phosphorylation of Dbf2's C-terminal HM site is a key part of MEN activation, similar to homologous metazoan pathways (Hergovich and Hemmings 2009; Emoto 2011). *In vitro* studies show that Cdc15 phosphorylation of this HM site dramatically increases Dbf2 enzymatic activity (Mah *et al.* 2001, 2005). Furthermore, Mob1 greatly increases the efficiency of Cdc15's *in vitro* phosphorylation of Dbf2. Corroborating these *in vitro* studies, genetic analysis indicates that Cdc15's phosphorylation of Dbf2 is critical for its *in vivo* function (Mah *et al.* 2001). Mutation of Cdc15 phosphorylation sites on Dbf2 to alanine significantly abrogates MEN function, while mutation to glutamic acid (to mimic phosphorylation) renders Cdc15 dispensable.

Controlling MEN: modulating Tem1's nucleotide state and SPB localization: A possible logic diagram for MEN organization is diagrammed in Figure 11, which summarizes models and results from numerous investigators. As noted above, the core pathway comprising the Tem1–Cdc15–Dbf2/Mob1 cascade is activated by recruitment to the SPB by Nud1. This recruitment probably promotes Cdc15's phosphorylation of Dbf2's HM site, although the exact mechanism for this is not known. Additionally, Cdc15 is required to bring Dbf2/Mob1 to the SPB, suggesting that the upstream kinase controls the ability of Nud1 to bind the downstream kinase complex. The likely negative regulation of Cdc15 and/or Dbf2/Mob1 by mitotic CDK (Jaspersen and Morgan 2000) may be relieved by dephosphorylation of these proteins by Cdc14, after its release by the FEAR pathway prior to MEN activation (Konig *et al.* 2010).

Tem1 appears to be the first MEN component loaded onto Nud1 at the SBP, and photobleaching analysis shows that it is initially highly dynamic in early anaphase (Cenamor *et al.* 1999; Bardin *et al.* 2000; Menssen *et al.* 2001; Visintin and Amon 2001; Molk *et al.* 2004; Rock and Amon 2011; Valerio-Santiago and Monje-Casas 2011). Tethering Tem1 at the SPB by fusing it to another outer plaque component increases recruitment of Cdc15 (Valerio-Santiago and Monje-Casas 2011). However, this does not cause a corresponding increase in localization of the Dbf2/Mob1 complex, further suggesting that Cdc15 must be activated and modify components of the local protein environment before Dbf2/Mob1 can stably associate.

Tem1's localization and nucleotide state are important in MEN control. The proteins Bub2 and Bfa1 were proposed to form a bipartite GAP that converts Tem1 to an inactive GDP-bound form (Bardin *et al.* 2000; Krishnan *et al.* 2000; Pereira *et al.* 2000; Wang *et al.* 2000; Lee *et al.* 2001b). However,

other studies suggest that Tem1 by itself efficiently hydrolyzes GTP and exchanges GDP, and Bfa1 may in fact act as an inhibitor of GDP exchange (Geymonat *et al.* 2002). Regardless, assembly and function of the Bub2–Bfa1 complex is clearly a key aspect of MEN regulation (Fraschini *et al.* 2006; Geymonat *et al.* 2009). Like other MEN components, Bub2–Bfa1 localizes to the SPB, with a notable bias to the pole that migrates into the daughter cell cytoplasm; in fact, the complex strongly stabilizes SBP localization of Tem1 (Bardin *et al.* 2000; Pereira *et al.* 2000; Monje-Casas and Amon 2009), but not its initial recruitment (Valerio-Santiago and Monje-Casas 2011).

At least two distinct models have been proposed for Bub2–Bfa1's control of mitotic exit. In one view, the Bub2–Bfa1 complex diffuses from the SPB to inhibit cytoplasmic pools of Tem1–Cdc15, with SPBs located in the mother cell generating a diffusible MEN-suppressing signal by producing active Bub2–Bfa1 (Fraschini *et al.* 2006; Caydasi and Pereira 2009). In a different view, Tem1 must turn on the MEN at the SPB, and rapid flux of Bub2–Bfa1 quickly removes the GTPase from this location. The fact that tethering Tem1 to the SPB triggers mitotic exit supports this hypothesis, which essentially posits that rapid loss of the Bub2–Bfa1 complex from mother-localized SPBs sweeps away Tem1 (Valerio-Santiago and Monje-Casas 2011).

Cdc5 inactivates Bub2–Bfa1, probably by directly phosphorylating Bfa1, and thereby positively regulates the MEN (Figure 11) (Hu *et al.* 2001; Hu and Elledge 2002; Pereira *et al.* 2002; Geymonat *et al.* 2003; Park *et al.* 2003). Separately, Cdc5 may directly promote recruitment of the Cdc15–Tem1 module to Nud1, although by unclear means (Luca *et al.* 2001; Yoshida *et al.* 2002; Park *et al.* 2008). This forms a feed-forward loop (FFL) in which Cdc5 both turns off inhibition of the Cdc15–Tem1 module and separately promotes this module's Nud1 association and activation of Dbf2.

How is Tem1 activated? The Lte1 protein turns on MEN signaling and resembles a GEF (Bardin *et al.* 2000; Pereira *et al.* 2000; Jensen *et al.* 2002). Lte1 localizes to the bud cortex (Figure 12), leading to early models in which SPB migration into the daughter cell directly promotes GTP loading on Tem1. Recent studies suggest that Lte1 does not serve this purpose; rather, it appears to block the function of the protein kinase Kin4, which as discussed further below is an inhibitor of the MEN (Geymonat *et al.* 2009; Chan and Amon 2010; Bertazzi *et al.* 2011). Overall, the mechanism by which Lte1 activates the MEN remains uncertain.

Controlling MEN: spindle orientation and the "zone model": The MEN is the target of a signaling mechanism that blocks M/G1 progress when mitotic spindle is not properly oriented to segregate chromosomes to mother and daughter cells (Figure 12). This system, termed the "spindle orientation checkpoint," ensures that mitotic exit does not occur until one of the two SPBs enters the daughter cell cytoplasm (Adames and Cooper 2000; Bardin *et al.* 2000; Bloecher *et al.* 2000; Pereira *et al.* 2000 and reviewed in Lew and

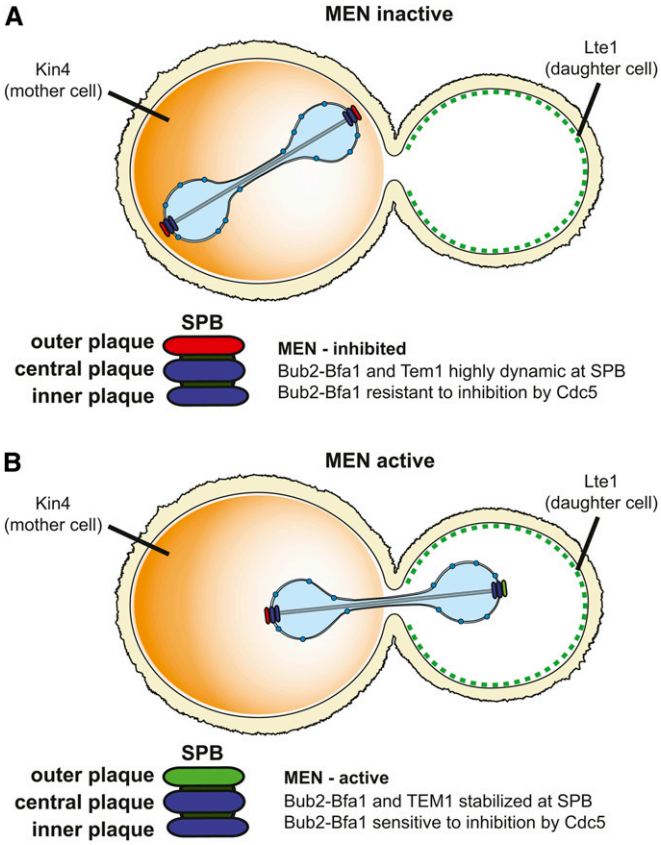

Figure 12 MEN activation is closely tied to the mitotic spindle. Activation of the MEN is closely tied to the location of the mitotic spindle. Kin4, which antagonizes Bub2–Bfa1 inactivation, is localized to the mother cell; Lte1, which acts positively on the pathway, is located in the daughter cell. (A) When SPBs are present in the mother cell cytoplasm, the MEN is inhibited at the outer plaque (red). (B) Movement of one of the SPBs into the daughter is permissive for MEN activation (green SPB outer plaque), as long as Cdc5 is active.

Burke 2003; Fraschini *et al.* 2008; Burke 2009). Asymmetric regulation of Bub2–Bfa1 by the MEN-inactivating kinase Kin4 is the basis of a zone model for this checkpoint (Figure 12). As diagrammed in Figure 11, Kin4 phosphorylates Bfa1, and this prevents Cdc5 from turning off Bub2–Bfa1 (Hu *et al.* 2001; Hu and Elledge 2002; Maekawa *et al.* 2007). Kin4 concentrates at the mother cell cortex, the bud neck, and SPBs located in the mother cell cytoplasm (D'Aquino *et al.* 2005; Pereira and Schiebel 2005). Thus, it maintains Bub2–Bfa1 in a highly active state at SPBs present in the mother cell cytoplasm and appears to increase the rate of Bub2–Bfa1 turnover there (Caydasi and Pereira 2009; Chan and Amon 2010). In contrast, Kin4 disappears from SPBs that have entered the daughter cell, presumably allowing activation of SPB-associated Tem1 and downstream MEN components. This normally occurs only when the spindle is correctly aligned, when one of the SPBs moves into the daughter cell cytoplasm and enters a region of low Kin4 concentration (Figure 12).

If this zone model is correct, how do relatively small yeast cells set up and maintain such sharply defined subcellular

regulatory regions? Kin4's localization and activity are clearly important, and the mechanism that spatially restricts its function remains a subject of active investigation. The protein phosphatase PP2A, in complex with its regulatory subunit Rts1, is required both for restriction of Kin4's localization to the mother cell cortex and for its recruitment to SPBs (Chan and Amon 2010). Additionally, the bud neck localized protein kinase Elm1 activates Kin4 by directly phosphorylating a critical activation loop site. Lte1, which is localized in the daughter cell, blocks Kin4's ability to phosphorylate Bfa1 (Bertazzi *et al.* 2011); this requires the p21-activated kinase Cla4 (Seshan *et al.* 2002; Chiroli *et al.* 2003). Intriguingly, cells lacking cytoplasmic SPB-anchored microtubules exhibit a partial checkpoint defect, and the interaction of these microtubules with the bud neck has been proposed to activate the spindle orientation checkpoint (Adames *et al.* 2001; Moore *et al.* 2009).

MEN may function in metaphase spindle positioning and mRNA stabilization: The prevailing view of MEN function is that its activity is closely coupled with the final stages of mitosis, leading into the initiation and completion of cytokinesis. Intriguingly, however, Mob1–Dbf2 and the MEN may have important functions significantly earlier. These include a possible role in orientation of the mitotic spindle in metaphase (Hotz *et al.* 2012) and stabilization of the early mitotic transcripts *CLB2* and *SWI5* (Trcek *et al.* 2011). In the latter case, a fraction of Dbf2 and Dbf20 associate with the mRNAs and suppress their degradation. Surprisingly, this appears to be independent of their kinase activity. For Dbf2, its loading onto mRNAs depends entirely on the promoter, not mRNA sequence context. Anaphase MEN activation appears to antagonize Dbf2/20's mRNA stabilization role, and this may help close the window of the cell cycle in which the *SWI5* and *CLB*2 mRNAs can produce protein.

Mob1–Dbf2/Cdc14 release positive feedback loop: *In vitro* and *in vivo* studies indicate that Mob1–Dbf2 directly drives cytoplasmic accumulation of Cdc14 by phosphorylating it at sites near its C-terminal nuclear localization sequence (NLS) (Mohl *et al.* 2009). This inhibits the NLS's function, shifting Cdc14's distribution from the nucleus to the cytoplasm and thus allowing it to remove CDK phosphorylations on cytoplasmic proteins. The Mob1–Dbf2 complex appears to enter the nucleus prior to final release of Cdc14 to the cytoplasm (Stoepel *et al.* 2005), suggesting that it regulates Cdc14 in the nucleus. Additionally, Mob1–Dbf2 must phosphorylate at least one other nuclear protein to promote dissociation of Net1 and Cdc14, and this may be Net1 itself (Mah *et al.* 2005; Mohl *et al.* 2009). Intriguingly, the Net1-related protein Tof2 also sequesters a fraction of Cdc14 to the nucleolus, but this is refractory to the FEAR and is only released upon full MEN activation (Waples *et al.* 2009).

RAM network

Overview: As cells complete synthesis of the primary and secondary septa, the RAM network turns on processes needed for septum destruction. This pathway is regulated by mitotic exit but is distinct from FEAR and MEN (and less extensively studied). By activating the transcription factor Ace2, it drives a sharp increase in transcription of mother/daughter separation genes. In addition to a mother/daughter separation function during the M-to-G1 transition, the RAM network functions during other parts of the cell cycle to promote sustained polarized growth and localized cell wall expansion. The RAM name (**r**egulation of **A**ce2 and **m**orphogenesis) reflects these dual roles (Nelson *et al.* 2003).

The RAM network thus far comprises six proteins that function in at least two distinct modules (Figures 13 and 14) (Racki *et al.* 2000; Bidlingmaier *et al.* 2001; Colman-Lerner *et al.* 2001; Weiss *et al.* 2002; Nelson *et al.* 2003). Cells lacking any of these six components have indistinguishable cell separation defects, and loss of multiple components does not further exacerbate this phenotype (Bidlingmaier *et al.* 2001; Nelson *et al.* 2003). The downstream-most module of the system is the Ndr family protein kinase Cbk1, which functions in complex with its co-activator Mob2 (Weiss *et al.* 2002). Cbk1 is regulated by the Mst/*hippo* protein kinase Kic1, which associates with the activating subunit Hym1 (Nelson *et al.* 2003; Brace *et al.* 2011). The very large protein Tao3 is critical for this regulatory interaction, but its role is not well defined (Du and Novick 2002; Jorgensen *et al.* 2002; Nelson *et al.* 2003). Additionally, the leucine-rich protein Sog2 is important for RAM network function, and most likely directly interacts with the Kic1–Hym1 complex (Nelson *et al.* 2003). All components except Sog2 are conserved, with putative paralogs present from fungi to metazoans (reviewed in Hergovich *et al.* 2006; Gao 2007; Maerz and Seiler 2010; Emoto 2011; Hergovich 2011; and see also Emoto *et al.* 2004; Chiba *et al.* 2009; Fang and Adler 2010; Cornils *et al.* 2011a,b).

Current knowledge about the RAM network's control of septum destruction is discussed in part 3, in particular its control of the transcription factor Ace2. However, for discussions immediately below it is important to note that Ace2 is a direct *in vivo* phosphorylation target of Cbk1 (Mazanka *et al.* 2008; Mazanka and Weiss 2010). The RAM network's role in defining cell architecture is less well understood and is largely beyond the scope of this chapter.

RAM components: the Mob2–Cbk1 module: Cbk1, which stably associates with the Mob2 protein, is the sole Ndr family kinase in budding yeast (Racki *et al.* 2000; Bidlingmaier *et al.* 2001; Colman-Lerner *et al.* 2001) (Figure 13). Both Cbk1 and Mob2 are present throughout the cell cycle and appear to bind each other constitutively (Weiss *et al.* 2002); they have been identified as a complex in large-scale protein interaction analyses (Reguly *et al.* 2006; Stark *et al.* 2006, 2011). Cbk1's activity fluctuates modestly over the cell

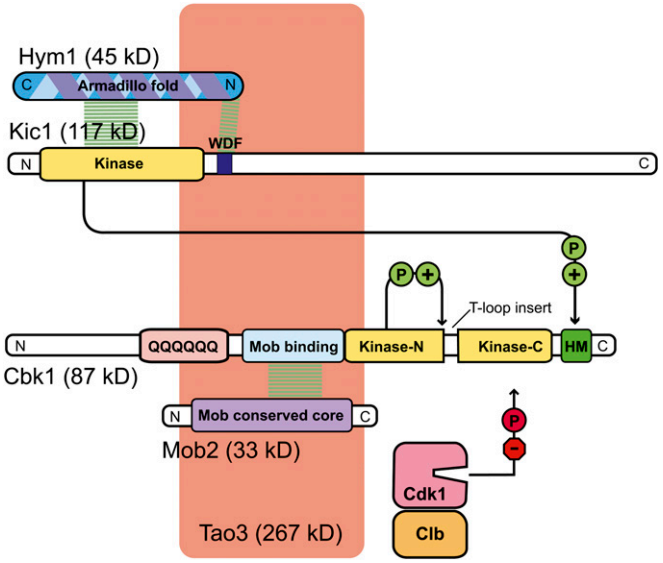

Figure 13 The RAM network. This figure represents the overall organization of the core components of the RAM network. Activating phosphorylations are noted, and the domain organization of the Hym1–Kic1 module and the Mob2–Cbk1 module are shown. Kic1 is an Mst/*hippo*-related kinase that likely binds the MO25 ortholog Hym1 through its kinase domain and a C-terminal peptide motif. Cbk1 is an Ndr/LATS family protein kinase that binds to Mob2 via a region N-terminal to its kinase domain. Cbk1's N-terminal region contains a polyglutamine tract, but is not highly conserved. Mitotic CDK phosphorylation likely inhibits Cbk1. Tao3 may act as a scaffold that brings elements of the pathway together, but there is little evidence for or against this notion.

cycle, with peaks occurring during bud formation and cell separation (Weiss *et al.* 2002), but the importance of this change is not clear. Indeed, alleles that drastically compromise Cbk1's kinase activity have modest effects on the *in vivo* function of the Mob2–Cbk1 complex, suggesting that control of its specific activity is a relatively unimportant regulatory input (Racki *et al.* 2000; Colman-Lerner *et al.* 2001; Weiss *et al.* 2002; Jansen *et al.* 2006; Bourens *et al.* 2009).

Cbk1 has a distinctive phosphorylation consensus site preference that has proven extremely helpful in identifying its *in vivo* phosphorylation targets. Unbiased *in vitro* analysis of its specificity showed that Cbk1 strongly favors phosphorylation of serine or threonine in the motif H-X-R-R-X-(S/T), where X represents any amino acid (Mazanka *et al.* 2008). This sequence closely matches sites phosphorylated by metazoan LATS/*warts* family protein kinases in the *yorkie*/YAP/TAZ transcriptional co-activators (Zhao *et al.* 2007; Zhang *et al.* 2008; Wang *et al.* 2009), indicating that this consensus motif preference is conserved in Ndr/LATS family kinases. Cbk1's preference for histidine at position −5 is very strong *in vivo* and *in vitro*; lysines can be substituted for arginines at positions −2 or −3, but, as for Dbf2, they cannot be eliminated (Mah *et al.* 2005; Mazanka *et al.* 2008).

Mob2, like the MEN component Mob1, is a member of the Mob-family of proteins that appear to function in complexes with Ndr/LATS protein kinases in all eukaryotes (Hergovich 2011). As with Mob1 and Dbf2/20, Mob2 association with Cbk1 is critical for normal protein kinase function (Weiss *et al.* 2002), suggesting that it acts as an essential co-activator. Additionally, binding of Mob2 may stabilize Cbk1 *in vivo* (Weiss *et al.* 2002; Nelson *et al.* 2003). The three-dimensional structure of Mob2 has not been determined, but its similarity to Mob1 suggests that it adopts a broadly similar conformation in its core (Luca and Winey 1998; Stavridi *et al.* 2003; Mrkobrada *et al.* 2006). A notable difference is that Mob1 contains a motif that binds zinc, while Mob2 does not. Unlike Mob1, there is no indication that Mob2 dimerizes, but *in vitro* and *in vivo* analyses suggest that Mob1 forms heterodimers with Mob2, potentially linking the Mob1–Dbf2 module with Mob2–Cbk1 (Mrkobrada *et al.* 2006).

The localization of Cbk1 and Mob2 is consistent with their dual roles in control of cell morphogenesis and mother/daughter separation gene transcription. They concentrate prominently at the mother/bud neck of large budded cells in telophase, prior to actomyosin ring contraction, and localize somewhat more faintly to the cortex of the growing daughter cell and the tip of the mating projection formed by cells responding to mating pheromone (Racki *et al.* 2000; Bidlingmaier *et al.* 2001; Colman-Lerner *et al.* 2001; Weiss *et al.* 2002). The proteins depend on one another for robust recruitment to cortical sites. Intriguingly, their cortical localization does not require other RAM network components

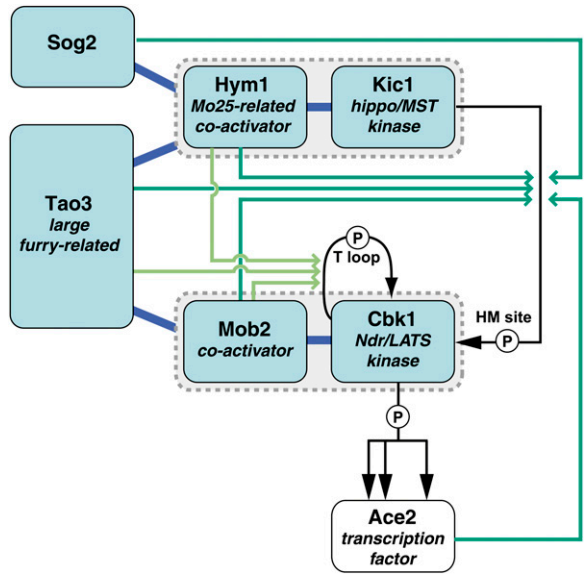

Figure 14 Signaling structure of the RAM network. Protein–protein interactions, (shown as thick blue lines) and importance for *in vivo* phosphorylation levels of individual sites (shown as green lines) suggest how modules in the RAM network relate to Cbk1 phosphoregulation. Kic1 probably directly phosphorylates Cbk1's C-terminal HM site, and Cbk1 autophosphorylates its own activation loop in *cis*. All RAM network components are required for normal HM site phosphorylation, as is Ace2. Tao3, Mob2, and Hym1 are required for normal activation loop autophosphorylation.

(Weiss *et al.* 2002; Nelson *et al.* 2003). The proteins also accumulate in the daughter cell nucleus as cells pass from M to G1 in an Ace2-dependent manner, suggesting that they enter the nucleus as a ternary complex with the transcription factor (Colman-Lerner *et al.* 2001; Weiss *et al.* 2002).

RAM components: the Kic1–Hym1 module: As noted above, Kic1 is an Mst/*hippo*-related kinase with an N-terminal catalytic domain highly similar to Cdc15's (Sullivan *et al.* 1998). One distinctive feature is a conserved three amino acid motif C-terminal to the Kic1 kinase domain termed "site D" (Zeqiraj *et al.* 2009a; Filippi *et al.* 2011); in Kic1 the sequence is WDF. Kic1 was initially identified as a two-hybrid interactor with the calmodulin-related protein Cdc31 (Sullivan *et al.* 1998), which is essential for SPB duplication and aspects of mRNA export from the nucleus (Baum *et al.* 1986; Jani *et al.* 2009). While this interaction may be functionally important (Sullivan *et al.* 1998; Ivanovska and Rose 2001), its *in vivo* significance remains unclear.

Kic1 associates with the helical repeat protein Hym1, which is closely related to metazoan MO25 proteins (Sullivan *et al.* 1998; Bidlingmaier *et al.* 2001; Jorgensen *et al.* 2002; Nelson *et al.* 2003; Reguly *et al.* 2006; Stark *et al.* 2006, 2011) (Figure 13). Like the Cbk1/Mob2 module, both Kic1 and Hym1 localize to sites of cell growth and wall remodeling, including the daughter bud cortex, mating projection tip, and bud neck, with Hym1 recruitment to these sites dependent on Kic1 (Bidlingmaier *et al.* 2001; Nelson *et al.* 2003). Neither protein has been detected in nuclei. Overexpression studies suggest that Hym1 might have a role outside of the RAM network in G1 progression, possibly indicating that the protein interacts with kinases other than Kic1 (Bogomolnaya *et al.* 2004, 2006).

Hym1 is highly conserved, and analysis of mammalian MO25 reveals biochemical mechanisms that are probably critical for RAM network function. Mammalian MO25 binds the inactive Kic1-related pseudokinase STRAD with high affinity and associates more weakly with at least five different catalytically active mammalian Mst/*hippo*-related kinases (Hawley *et al.* 2003; Boudeau *et al.* 2004; Milburn *et al.* 2004; Zeqiraj *et al.* 2009a; Zeqiraj *et al.* 2009b; Filippi *et al.* 2011). The three-dimensional structure of mammalian MO25 has been solved alone and in complex with STRAD (Milburn *et al.* 2004; Zeqiraj *et al.* 2009a). It is broadly similar to armadillo-repeat proteins, with three-helix elements stacked atop one another to form a slightly concave solenoid. The STRAD:MO25 complex provides a plausible model for MO25's binding and activation of Mst/*hippo* kinases. One set of interactions between MO25's concave surface and the kinase N-terminal lobe helps position the kinase activation loop; this resembles the mechanism of CDK activation by cyclin binding (Jeffrey *et al.* 1995; Zeqiraj *et al.* 2009a,b; Filippi *et al.* 2011). Additionally, the conserved site D motif on the Mst/*hippo* kinases associates with an N-terminal pocket on MO25 (Zeqiraj *et al.* 2009a). Overall, it is possible that Hym1–Kic1 interaction is regulated *in vivo* to control the RAM network (and, more broadly, other Mst/*hippo* pathways), but this has not yet been addressed.

RAM components: Tao3 and Sog2: Tao3 (also known as Pag1) is a large (~350 kDa) protein essential for RAM network function (Du and Novick 2002; Jorgensen *et al.* 2002; Nelson *et al.* 2003). Tao3 physically interacts with both Kic1 and Cbk1, and thus may facilitate communication between these two modules (Figure 13) (Nelson *et al.* 2003; Reguly *et al.* 2006; Stark *et al.* 2006, 2011). Like other RAM network components, Tao3 localizes to the bud cortex as well as the mother/bud neck in large budded cells, but it has not been detected in nuclei. Its localization does not require other RAM network components, and vice versa (Nelson *et al.* 2003). Thus, there are likely multiple independent mechanisms for recruitment of RAM network proteins to the cell cortex.

Tao3 is conserved from yeast to metazoans, and its likely orthologs function along with Ndr family kinases in control of cell proliferation and morphogenesis. These are broadly defined as the "*furry*" family of proteins, after a *Drosophila melanogaster* ortholog involved in the organization of cellular extensions such as bristles, wing hairs, and aristal laterals (Cong *et al.* 2001; He *et al.* 2005; Fang *et al.* 2010). Like the Ndr kinases, *furry*-related proteins are also involved in neuron morphogenesis (Zallen *et al.* 1999; Emoto *et al.* 2004; Gallegos and Bargmann 2004; Jia and Emmons 2006; Gao 2007). Phylogenetic analysis of these putative Tao3 orthologs indicates that the broad family has five sequence blocks that exhibit significant conservation from metazoans to yeast, and that a significant portion of the protein has HEAT repeats predicted to adopt an extended β-catenin–like structure (Gallegos and Bargmann 2004; Chiba *et al.* 2009; Goto *et al.* 2010)

Sog2 is a leucine-rich protein that interacts with Kic1 and most likely with Tao3 and localizes to the cell cortex (Nelson *et al.* 2003). The protein is otherwise not well understood, and is not as well conserved as other components of the RAM network.

Functional organization of the RAM network: How does information flow within the RAM network to allow its control of cell separation and morphogenesis? While some important aspects of this system are known (Figure 13), far less is understood about it than either the MEN or the FEAR pathway. Since loss of any of the system's components produces indistinguishable nonadditive defects in cell separation and morphogenesis, it is not possible to order the pathway through epistasis analysis of known phenotypes. *In vitro* protein kinase assays using immunoprecipitated Cbk1 suggest that it is the most downstream component: all other RAM network proteins of the system are essential for full *in vitro* enzymatic activity of the Cbk1 (Weiss *et al.* 2002; Nelson *et al.* 2003). Most effort toward understanding

information flow within the RAM network and its coordination with mitotic exit has focused on regulation of the Mob2–Cbk1 module.

Regulatory phosphorylation of Cbk1's hydrophobic motif site and activation loop: While the protein kinase activity of immunoprecipitated Mob2–Cbk1 may be a useful proxy for RAM network function, there are several caveats to this analysis. Until recently, most such kinase assays used histone H1 as the *in vitro* test substrate, which is probably not ideal (Jansen *et al.* 2006; Mazanka *et al.* 2008). More direct assessment of Cbk1's HM site and activation loop phosphorylation using phosphospecific antibodies has provided additional insights (Jansen *et al.* 2006; Brace *et al.* 2011). Both sites are phosphorylated *in vivo* (Jansen *et al.* 2006), an observation corroborated by large-scale mass spectrometry (Bodenmiller and Aebersold 2011). Phosphorylation of the HM site is dynamic over the cell cycle, peaking during cell separation and polarized growth of the daughter bud (Jansen *et al.* 2009). At peak times, only a small fraction of the kinase, between 3 and 5%, carries this phosphorylation (Brace *et al.* 2011). Phosphorylation of the activation loop site occurs through an intramolecular autophosphorylation reaction (Jansen *et al.* 2006), as with mammalian Ndr kinases (Tamaskovic *et al.* 2003), and remains relatively constant throughout the cell cycle.

Genetic analysis indicates that phosphorylation of both the HM site and activation loop site is important for Cbk1 function, although to different degrees (Jansen *et al.* 2006; Bourens *et al.* 2009; Panozzo *et al.* 2010). Neither modification is required for recruitment of Cbk1 to the daughter cell cortex and bud neck. Substitution of alanine at the activation loop phosphoacceptor site severely compromises Cbk1's catalytic activity, making it nearly undetectable; yet this allele has a notably mild phenotype, exhibiting moderate defects in polarized growth, mating, and cell separation (Jansen *et al.* 2006; Bourens *et al.* 2009). In contrast, replacing the phosphoacceptor threonine at Cbk1's HM site position completely abolishes its *in vivo* function, but not the *in vitro* kinase activity of Mob2–Cbk1 immunoprecipitated from asynchronous cells. It is pertinent here that HM site phosphorylation does increase catalytic activity of related kinases (Millward *et al.* 1998; Stegert *et al.* 2005), and substitution with glutamic acid at this site significantly increases Cbk1 kinase activity (Brace *et al.* 2011). Thus, since only a small fraction of Cbk1 is phosphorylated at the HM site, it is likely that analysis of immunoprecipitated Mob2–Cbk1 cannot accurately measure the degree of enzyme activation by this modification. Overall, the precise function of this modification remains unknown.

The RAM network and Ace2 in Cbk1 phosphoregulation: Cbk1's HM site phosphorylation requires all other RAM network components, again consistent with the Mob2–Cbk1 complex acting as the downstream-most part of the system (Jansen *et al.* 2006) (Figure 14). However, phosphorylation of Cbk1's HM site is also greatly reduced in cells that lack Ace2, which as discussed in part 3 is one of the kinase's *in vivo* substrates. This suggests a possible feedback loop in which the kinase's substrate synergistically enhances the activation of its upstream regulator. The mechanism by which Ace2 enhances Cbk1 HM site phosphorylation is unknown; it might promote binding of Mob2–Cbk1 to Kic1–Hym1 or it might shield a fraction of Mob2–Cbk1 from a phosphatase that normally reverses HM site phosphorylation, perhaps by partitioning Mob2–Cbk1 to the nucleus. Finally, it is possible that one of Ace2's transcriptional targets, rather than Ace2 itself, is responsible for modulating phosphorylation of the HM site. Regardless, it is unlikely that Cbk1 HM site phosphorylation fully depends on Ace2, as the phosphorylation site is essential for Cbk1's role in polarized growth, whereas Ace2 is not (Weiss *et al.* 2002; Voth *et al.* 2005; Jansen *et al.* 2006).

Autophosphorylation of Cbk1's activation loop site exhibits a more nuanced set of genetic dependencies (Figure 14) (Jansen *et al.* 2006). As expected, Mob2 is required. Tao3 is also required, suggesting that it either helps Cbk1 adopt a conformation suitable for autophosphorylation or shields it from a phosphatase activity. Intriguingly, this phosphorylation is reduced (though not absent) in cells lacking Hym1, but is unaffected in cells lacking Kic1 and Sog2.

It is likely that the Kic1–Hym1 module functions upstream of the Mob2–Cbk1 module by directly phosphorylating Cbk1's HM site. Bacterially expressed Kic1 phosphorylates this site *in vitro* (Brace *et al.* 2011). Kic1 and Mob2–Cbk1 are reminiscent of Cdc15 and Mob1–Dbf2, where this regulatory interaction is well established (Mah *et al.* 2001; Visintin and Amon 2001). Similarly, in metazoans Mst/*hippo* family kinases like Kic1 phosphorylate the HM site of Ndr/LATS family kinases closely related to Cbk1 (Stegert *et al.* 2005; Hergovich and Hemmings 2009). Also, substitution with the phosphomimetic amino acid glutamic acid at the phosphoacceptor residue in Cbk1's HM site renders upstream RAM components dispensable, at least for some functions, arguing that control of this regulatory site on Cbk1 is a major focus of the system's upstream components (Panozzo *et al.* 2010).

Regulation of the RAM network by FEAR and MEN: Consistent with mitotic exit control, Cbk1's HM site is briefly phosphorylated during cytokinesis and cell separation, just prior to Ace2's relocalization from the cytoplasm to the daughter cell nucleus (Jansen *et al.* 2006; Brace *et al.* 2011). As diagrammed in Figure 15, both the FEAR pathway and the MEN appear to coordinate this regulatory event with mitotic progress. Phosphorylation of the Cbk1 HM site is undetectable in both *cdc5-1* and *cdc14-2* cells arrested at restrictive temperature, consistent with FEAR regulation of the process or synergistic control by both FEAR and Cdc5. In contrast, phosphorylation of Cbk1's HM site occurs in *cdc15-2* cells arrested at restrictive temperature. These findings suggest that the FEAR pathway promotes the activation of upstream components of the RAM network, possibly by permitting interaction of Kic1–Hym1 with Mob2–Cbk1. Since

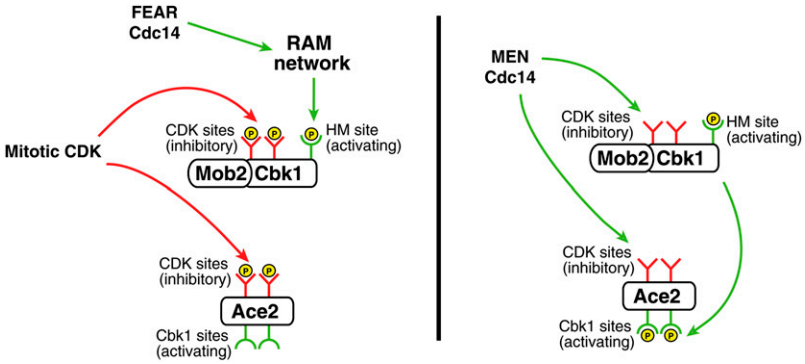

Figure 15 MEN and FEAR control of the RAM network. The FEAR pathway activates phosphorylation of Cbk1's HM site in early anaphase, while direct phosphorylation by mitotic CDK inhibits Ace2 nuclear import as well as Cbk1/RAM network function. Activation of the MEN then releases large amounts of Cdc14, resulting in removal of inhibitory CDK phosphorylations on Cbk1 (and possibly other RAM network components) and Ace2. MEN activation also likely sustains RAM-network mediated phosphorylation of Cbk1's HM site.

cells lacking FEAR activity do not have dramatic problems with cell separation, activation of the RAM network is likely a role played by Cdc14, and any mechanism that releases it from the nucleolus (but not necessarily from the nucleus) is sufficient.

While necessary, phosphorylation of Cbk1's HM site is not sufficient for the kinase to phosphorylate Ace2 and drive it into the daughter nucleus (Brace *et al.* 2011). Cbk1 (and possibly other components of the RAM network) appear to be held inactive by CDK phosphorylation that blocks Cbk1's ability to interact with Ace2 until MEN activation. Upon sustained release of Cdc14, these modifications are reversed, likely permitting Cbk1 regulation of Ace2. Overall, the apparent dual control of Cbk1/RAM function at mitotic exit (Figure 15) emphasizes the importance of Cdc14 as a coordinator of late cell cycle events.

Control of Cell Separation Processes

The transition from M to G1 is marked by massive reorganization of numerous cellular components, coordinated with the mechanical action of the mitotic spindle and cytokinetic apparatus. Mitotic control systems therefore clearly drive a wide variety of processes, and these regulatory linkages remain incompletely understood. A few direct targets of mitotic exit pathways have been identified that are relevant to the separation of mother and daughter cells, and a few common regulatory themes emerge. Some target processes are controlled by APC-mediated destruction of key protein components. Other important proteins are held inactive in metaphase by mitotic CDK and activated upon dephosphorylation by Cdc14. Finally, in some cases the FEAR pathway, the MEN, or the RAM network directly regulate downstream events.

This section focuses mainly on control of septum destruction. The paralogous transcription factors Swi5 and Ace2 and their control are at the heart of this event and are important links between the regulatory systems that drive mitotic exit and the processes of cell separation and G1 onset. Thus, these important transcription factors are discussed in some depth.

Mitotic spindle stability

The mitotic spindle goes through major structural transitions as cells pass from M phase to early G1. It is highly stable only during early mitosis, and is destabilized upon mitotic exit. The protein Fin1 stabilizes the mitotic spindle, and the precise window in which it can act is under CDK control, so that it does not associate with the spindle when phosphorylated in S phase by the Clb5–CDK1 complex (Woodbury and Morgan 2007a). Upon dephosphorylation by Cdc14, Fin1 is released from inhibition and can strengthen the bipolar spindle (van Hemert *et al.* 2003; Woodbury and Morgan 2007b). As cells exit mitosis, coincident with spindle disassembly, APC^{Cdh1} targets Fin1 for degradation.

Other spindle-stabilizing systems are controlled by mitotic exit. For example, CDK phosphorylation blocks spindle association of the midzone protein Ase1, and dephosphorylation by Cdc14 reverses this (Schuyler *et al.* 2003; Crasta *et al.* 2006; Khmelinskii *et al.* 2007; Khmelinskii and Schiebel 2008). Additionally, the Sli15–Ipl1 complex localizes from kinetochores (where it regulates their spindle attachment) to the spindle itself when Cdc14 reverses CDK phosphorylation of Sli15 (Pereira and Schiebel 2003). When localized to the central spindle, the Sli15–Ipl1 complex appears to help stabilize the structure.

Regulation of septation machinery and the cytokinetic apparatus

In addition to its role in spindle function, Cdc14 directly promotes cytokinetic events, including the formation of the primary septum. Like components of the MEN and RAM network, Cdc14 localizes to the site of cell division at the mother/bud neck, but it remains unclear if it functions there. In addition to Cdc14, the MEN plays important roles in separation and cytokinesis. Aspects of the cell cycle regulation of this part of mother/daughter separation are reviewed in other YeastBook chapters (Bi and Park 2012; Howell and Lew 2012) and are discussed only briefly here.

Mitotic control of chitin synthase trafficking: The chitin synthase II catalytic subunit Chs2 is subject to multiple levels of cell cycle control that likely ensure that it is only available to form the primary septum. The *CHS2* gene is subject to cell cycle regulation, and its expression peaks in early M phase (Pammer *et al.* 1992; Spellman *et al.* 1998). The Chs2 protein is then retained in the endoplasmic reticulum until

cytokinesis, whereupon it moves through the secretory system to the bud neck, coincident with recruitment of exocytosis and endocytosis machinery to the site of cell division (Chuang and Schekman 1996; VerPlank and Li 2005; Zhang *et al.* 2006; McMurray *et al.* 2011). Chs2's entry into the secretory system is antagonized by phosphorylation of CDK sites in its N terminus (Teh *et al.* 2009), and Cdc14 reverses this modification to allow trafficking of Chs2, the site of primary septum formation (Chin *et al.* 2012). Intriguingly, while CDK phosphorylation likely blocks Chs2's secretion, it also greatly stabilizes the protein. This might reflect inhibition of proteolytic processing of a Chs2 zymogen. Moreover, the instability of dephosphorylated Chs2 might protect cells from inappropriate activation of this specialized chitin synthase (Martinez-Rucobo *et al.* 2009). The MEN has recently been demonstrated to directly regulate Chs2, through Dbf2 phosphorylation of at least one critical site (Oh *et al.* 2012). Dbf2 localizes to the division site as primary septum synthesis proceeds and appears to enhance Chs2's synthesis of chitin. Intriguingly, this modification is also important for efficient dissociation of Chs2 from the division site, suggesting that Dbf2's enhancement of primary septum synthesis ultimately helps turn the process off.

Regulation of the Hof1–Cyk3–Inn1 complex: As discussed more fully by Bi and Park (2012) the assembly of a trimeric complex composed of the SH3-domain containing proteins Cyk3 and Hof1 and the proline-rich protein Inn1 is critical for the initiation of a properly placed primary septum (Jendretzki *et al.* 2009; Nishihama *et al.* 2009; Meitinger *et al.* 2010, 2011). This complex localizes to the actomyosin ring before it contracts, a process that involves coordinated phosphorylation of Hof1 by Mob1–Dbf2, Cdc5, and mitotic CDK. The overall effect of this regulation is to shift Hof1 from a septin-bound pool to an actomyosin association (Korinek *et al.* 2000; Vallen *et al.* 2000; Sanchez-Diaz *et al.* 2008; Meitinger *et al.* 2010, 2011). As a result, Inn1 and Cyk3 promote the localized activity of Chs2 and thus growth of the primary septum in concert with actomyosin ring contraction. This interaction requires Cdc14, which removes mitotic CDK phosphorylations on Inn1 that block its interaction with Cyk3′s SH3 domain (Palani *et al.* 2012).

Disassembly of the contractile apparatus: In cytokinesis, the final separation of cytoplasms involves removal of the actomyosin ring components and resolution of the membrane junction that the contractile apparatus has brought together. In metazoans, this process is known as "abscission" (Barr and Gruneberg 2007); it is not clear if budding yeast undergo an identical event, although some similar processes are clearly needed (Dobbelaere and Barral 2004; Yoshida *et al.* 2009). One key event in yeast is the disassembly of the actomyosin ring. APC^{Cdh1} plays an important role in this process, both during and after ring contraction (Tully *et al.* 2009). Cells lacking APC^{Cdh1} show delayed disappearance of the contractile ring myosin Myo1, the IQGAP protein Iqg1, and the myosin light chains Mlc1 and Mlc2. Aberrant actomyosin ring disassembly leads to pronounced defects in the completion of the septum, with many cells failing to complete closure of a small region in the septum's center. Part of this APC^{Cdh1} function involves destruction of Iqg1, and Mlc1 likely plays a second reinforcing role (Ko *et al.* 2007; Tully *et al.* 2009).

Control of septum destruction and G1 entry: Ace2 and Swi5

Overview: The septum is rapidly destroyed minutes after it is built, starkly exemplifying precise coordination of opposing processes by the cell cycle regulatory system. Obviously, premature action of enzymes that efficiently degrade chitin and destroy or remodel other septum components would greatly complicate the structure's construction during contraction of the cytokinetic furrow. Furthermore, septum destruction is spatially regulated, proceeding from the daughter cell side.

The control of this process in time and space is largely accomplished by RAM network regulation of both transcription and translation, making septum destruction an event occurring only once in a cell's life, when it is a newly born daughter. This section describes current understanding of the control mechanisms underlying Ace2-driven transcription of genes involved in mother/daughter separation and daughter cell identity and then discusses recent insights into the RAM-mediated translational regulation of some of these genes by the mRNA binding protein Ssd1.

Ace2 works at the M/G1 transition along with the paralogous but functionally distinct transcription factor Swi5, which controls the expression of a distinct set of genes that close mitotic exit and function in G1 processes. Figure 16 shows the proteins' cycle of nuclear localization, plus regulatory events important for their control. A discussion of their similarities and differences helps describe mechanisms of the mother/daughter separation process. However, since Swi5 is not critical for mother/daughter separation, I discuss Ace2 more extensively.

Functional organization of Ace2 and Swi5: As diagrammed in Figure 17, Ace2 has C2H2 binuclear zinc finger motifs proximal to its C terminus, which likely recognizes the DNA motif GCTGG[G/T/C] (Dohrmann *et al.* 1992, 1996; Harbison *et al.* 2004; Badis *et al.* 2008). Separate motifs mediate nuclear import and export: a NLS and a nuclear export sequence (NES). Both have been experimentally verified, and the NES binds exportin Crm1/Xpo1 *in vitro* and *in vivo* (Jensen *et al.* 2000; Mazanka *et al.* 2008). A region in the middle of Ace2 mediates association with Cbk1 in two-hybrid studies (Racki *et al.* 2000) and appears to be functionally important in domain swap experiments with Swi5 (McBride *et al.* 1999; Sbia *et al.* 2008).

Ace2 and Swi5 are closely related paralogs, likely resulting from an ancient genome duplication event (Byrne and

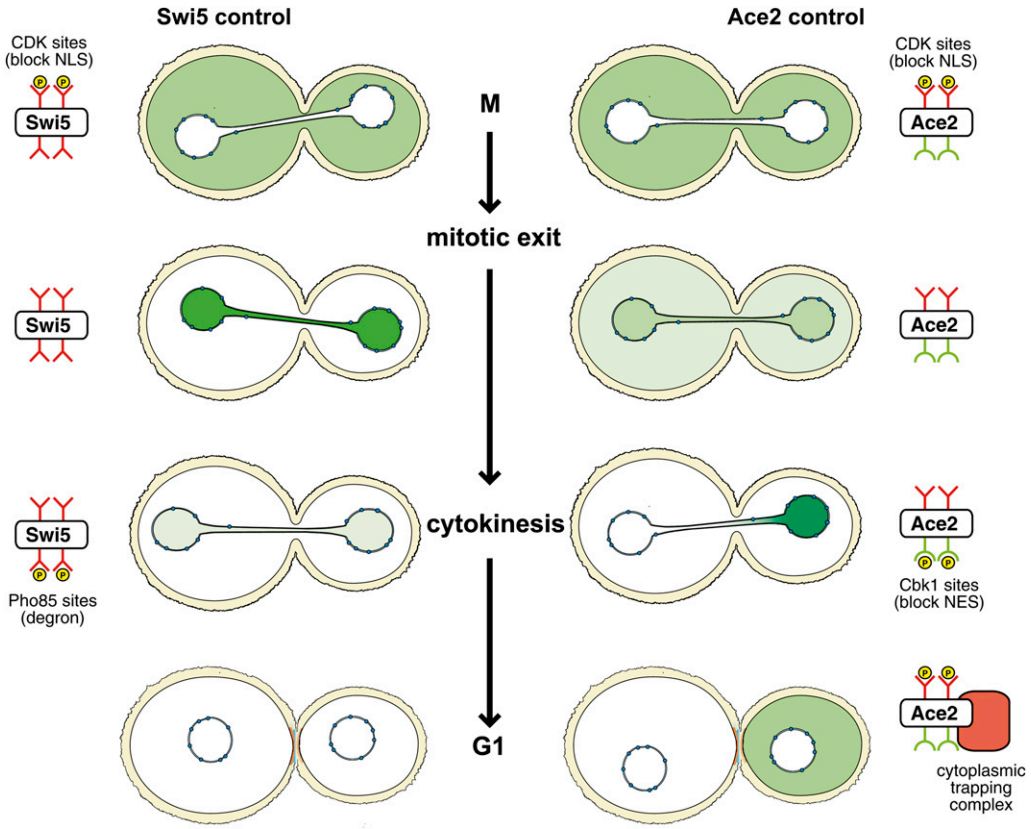

Figure 16 Comparison of Ace2 and Swi5 localization. Swi5 and Ace2 are represented separately as green color in the left and right columns of cell diagrams, and cartoons of the proteins with relevant modifications or proposed protein–protein interactions are shown next to the corresponding cells. In anaphase and very early telophase neither protein enters the nucleus; both proteins' NLS's are inactive due to phosphorylation by mitotic CDK. Later in telophase, both proteins are dephosphorylated by Cdc14 and can enter both mother and daughter nuclei. This is sufficient for accumulation of Swi5, which lacks an NES, to high concentrations. Ace2, in contrast, does not accumulate significantly in nuclei. Prior to actomyosin ring contraction, Ace2's NES is phosphorylated, inhibiting its nuclear export. This probably occurs specifically in the daughter cell. Ace2 then accumulates specifically in the daughter cell nucleus and is depleted in the still-contiguous cytoplasm of mother and daughter cells. In mid-G1, Ace2's NES is dephosphorylated and the protein is exported from the nucleus. Due to cytoplasmic trapping, Ace2 cannot reenter the nucleus of the newly born daughter cell once it exits.

Wolfe 2005, 2006). While their N termini have diverged, their Zn-finger domains are highly similar, and they can bind the same DNA sequence motifs (Dohrmann *et al.* 1992, 1996). Although Swi5 appears to lack an NES, it has a related C-terminal NLS (Butler and Thiele 1991; Moll *et al.* 1991; Dohrmann *et al.* 1992). Despite their similarities, Ace2 and Swi5 behave differently *in vivo* and with a few exceptions do not regulate the same genes (Doolin *et al.* 2001; Voth *et al.* 2005). Paradoxically, Swi5 associates *in vivo* with binding sites at the promoters of genes that are exclusively Ace2 driven, but does not turn on their transcription. This failure of Swi5 to activate promoters to which it binds is due to local "antiactivation" mediated by the Rpd3 acetyltransferase complex recruited by the Forkhead-related DNA binding proteins Fkh1 and Fkh2 (Voth *et al.* 2007).

Ace2 and Swi5 at the dawn of G1: Ace2 controls cell separation genes; Swi5 closes mitotic exit: Ace2 regulates genes encoding the most important septum destruction proteins, in a coherent pulse of transcription that coincides precisely with mother/daughter separation and is among the sharpest in the yeast cell cycle (Spellman *et al.* 1998; Pramila *et al.* 2002; Lu *et al.* 2007). These genes include *CTS1*, *SCW11*, *DSE2*, *DSE3*, and *DSE4*, which as discussed above encode known or putative septum hydrolases (Dohrmann *et al.* 1992; O'Conallain *et al.* 1998, 1999; McBride *et al.*

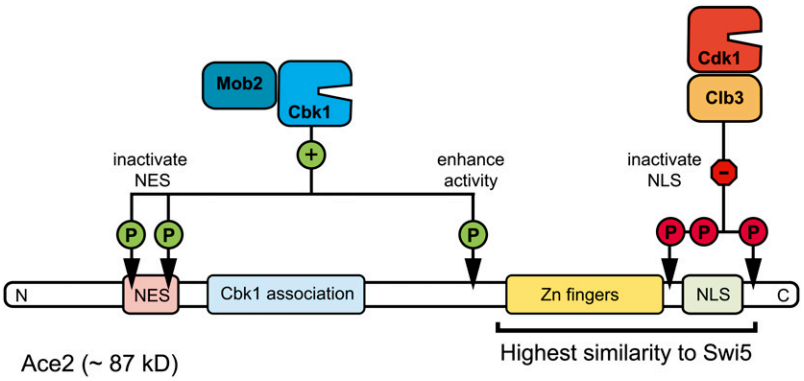

Figure 17 Domain organization of Ace2. The Ace2 protein contains a C-terminal Zn-finger domain that, in addition to immediately surrounding sequence, is related to Swi5. A C-terminal NLS is regulated by CDK phosphorylation. Ace2's NES is medially located and is negatively regulated by Cbk1 phosphorylation. An additional site of Cbk1 phosphorylation activates Ace2. A region in the middle of Ace2 mediates association with Cbk1.

1999; Bidlingmaier *et al.* 2001; Colman-Lerner *et al.* 2001; Doolin *et al.* 2001; Di Talia *et al.* 2009). Ace2 also clearly drives expression of the bud site selection gene *BUD9* as well as *DSE1*, which may be involved in bud scar organization and bud site selection (Bidlingmaier *et al.* 2001; Voth *et al.* 2005; Draper *et al.* 2009; Frydlova *et al.* 2009). Additionally, Ace2 may promote transcription of the *SUN4* glucanase (Bidlingmaier *et al.* 2001; Di Talia *et al.* 2009). For brevity, this group of Ace2-regulated genes are henceforward referred to as mother–daughter separation (MDS) genes. In a fascinating mechanism of cell cycle control that is beyond the scope of this chapter, Ace2 also lengthens G1 in daughter cells by inhibiting expression of the early G1 cyclin *CLN3*, helping them to attain a size suitable for budding (Laabs *et al.* 2003; Di Talia *et al.* 2009).

The targets of Swi5 notably include the protein Sic1 (Toyn *et al.* 1997), a potent CDK inhibitor. Its expression and activation during the M-to-G1 transition creates a negative feedback loop that makes mitotic exit an irreversible process (Luca *et al.* 2001; Lopez-Aviles *et al.* 2009; He *et al.* 2011). Swi5 also drives the expression of the cell separation gene *EGT2* and the transcriptional repressor *ASH1*, an mRNA that is transported to the daughter cell and ultimately translated there to suppress mating type switching. Since the *ASH1* mRNA must be transported to the daughter cell prior to cytokinesis, this further emphasizes the fact that Swi5′s regulation of transcription happens just prior to division.

Ace2 and Swi5 are produced in mitosis but sequestered in the cytoplasm: Ace2 and Swi5 are initially regulated at the level of their own transcription. Both genes are activated in early M phase under the control of a transcription factor complex known as SFF, which includes the Forkhead-related transcription factor Fkh2, the general transcription factor Mcm1, and the co-activator Ndd1 (Hollenhorst *et al.* 2000, 2001; Koranda *et al.* 2000; Zhu *et al.* 2000). SFF drives coherent expression of the "*CLB2* cluster" of genes, which in addition to *ACE2* and *SWI5* includes other genes involved in mitotic control and cytokinesis (Spellman *et al.* 1998).

Unsurprisingly given their shared origin, some of the same mechanisms control the nuclear accumulation of both Ace2 and Swi5. Nuclear entry of each protein is inhibited by mitotic CDK activity, with phosphorylation directly inactivating their NLS and hence trapping them in the cytoplasm until mitotic exit (Figure 16, see diagrams next to cell outlines). Appending Swi5′s NLS to a heterologous protein confers cell-cycle–dependent nuclear localization, involving CDK phosphorylation of sites near the NLS; this was one of the earliest identifications of a physiologically important Cdk1 substrate (Moll *et al.* 1991). Similarly, Ace2 is present in early mitotic cells but cannot enter the nucleus because its NLS is not functional until mitotic exit has commenced (Dohrmann *et al.* 1992; O'Conallain *et al.* 1999). As in Swi5, the Ace2 NLS is flanked by minimal CDK phosphorylation motifs (Figure 17); and alanine replacement at these sites allows nuclear entry of Ace2 (O'Conallain *et al.* 1999; Sbia *et al.* 2008; Mazanka and Weiss 2010; Brace *et al.* 2011).

In Ace2's case, the Clb3:Cdk1 complex likely phosphorylates the key NLS-inactivating CDK sites, and large-scale mass spectrometry confirms that Ace2 is an *in vivo* substrate of mitotic Cdk1 (Archambault *et al.* 2004; Holt *et al.* 2009). In addition to sites adjacent to its NLS, Ace2 is phosphorylated on numerous other minimal CDK motifs, suggesting extensive regulation by cyclin–CDK (Archambault *et al.* 2004). These sites appear to participate in the Ace2's inactivation in G1 (Mazanka and Weiss 2010), but their function in mitosis remains unclear.

Cdc14 relieves inhibition of Swi5 and Ace2 nuclear import: Cdc14 efficiently removes CDK phosphorylations that inhibit Swi5′s nuclear import, making nuclear entry of Swi5 strongly dependent on mitotic exit (Visintin *et al.* 1998; Traverso *et al.* 2001; Stegmeier and Amon 2004). Similarly, Ace2 was found to bind Cdc14 in a mass spectrometric analysis (Breitkreutz *et al.* 2010). Furthermore, Ace2 is heavily phosphorylated in mitotically arrested cells, Cdc14 removes most of this phosphorylation *in vitro*, and dephosphorylation of Ace2's CDK consensus motifs occurs abruptly upon release from metaphase arrest in a manner dependent on Cdc14 (Archambault *et al.* 2004; Sbia *et al.* 2008; Mazanka and Weiss 2010). As discussed further below, dephosphorylation by Cdc14 is both necessary and sufficient for Swi5 to induce transcription of its target genes, while for Ace2 this is necessary but not sufficient.

Ace2 localizes to the daughter cell nucleus just prior to cytokinesis: The timing and daughter-specific expression of MDS genes arises from M/G1 activation and asymmetric partitioning of Ace2, which is diagrammed and compared with Swi5 in Figure 16. To summarize work of multiple groups, Ace2 is initially isotropically distributed in mitotic cells, but is excluded from nuclei until well into anaphase. The protein becomes weakly visible in nuclei of mother and daughter cells in early telophase. If nuclear export is blocked at this stage by either genetic or pharmacological inhibition of the exportin Crm1/Xpo1, Ace2 accumulates strongly in both nuclei. Thus, at this time of the cell cycle Ace2 undergoes active import and export from nuclei, and there is no evident asymmetry between mother and daughter in these rates. A few minutes prior to actomyosin ring contraction, Ace2 rapidly shifts to nearly exclusive accumulation in the daughter cell's nucleus, where it remains until shortly after mother/daughter separation is complete. Ace2's localization to the daughter cell nucleus is accompanied by a corresponding reduction in its cytoplasmic concentration in both mother and daughter cells and occurs independently of the protein's ability to bind DNA (Dohrmann *et al.* 1992; O'Conallain *et al.* 1999; Racki *et al.* 2000; Bidlingmaier *et al.* 2001; Colman-Lerner *et al.* 2001; Doolin *et al.* 2001; Weiss *et al.* 2002; Nelson *et al.* 2003; Voth *et al.* 2005;

Bourens *et al.* 2008; Mazanka *et al.* 2008; Sbia *et al.* 2008; Di Talia *et al.* 2009; Mazanka and Weiss 2010; Brace *et al.* 2011).

Soon after separation is completed in early G1, and coincident with disappearance of MDS transcripts, Ace2 disappears from daughter cell nuclei; this is not due to degradation but rather to its relocalization to the cytoplasm where it is retained by a complex cytoplasmic trapping mechanism (Sbia *et al.* 2008; Mazanka and Weiss 2010; Brace *et al.* 2011). The Ace2-driven asymmetry of MDS gene expression has been visualized as daughter-specific accumulation *CTS1* mRNA and daughter cell fluorescence in strains carrying a GFP variant under the control of the *DSE1* promoter (Colman-Lerner *et al.* 2001; Bourens *et al.* 2008). This partitioning is quite stringent, and the DSE1 promoter has been exploited to construct a genetically encoded system called the "mother enrichment program" that conditionally kills only daughter cells, to assist studies of aging and replicative lifespan (Lindstrom and Gottschling 2009; Lindstrom *et al.* 2011).

Ace2 partitioning to the daughter cell nucleus resembles segregation of Ash1, a transcriptional repressor that blocks mating type switching in daughter cells. However, these asymmetries are generated by different mechanisms. Ash1 accumulates mainly in the nuclei of daughter cells because its mRNA is first moved as a translationally inactive ribonucleoprotein particle (RNP) to the daughter cell by the type V myosin Myo4 traveling on cytoplasmic actin cables and then translated as the cells divide. (Darzacq *et al.* 2003; Paquin *et al.* 2007; Hasegawa *et al.* 2008; Paquin and Chartrand 2008). Ace2 localization does not require cytoskeletal components needed for Ash1 asymmetry (Weiss *et al.* 2002). Rather, the segregation of Ace2 is most likely diffusion mediated and involves selective inactivation of its nuclear export in daughter cells by the RAM network; by contrast, Ash1 segregation does not require RAM network function (Weiss *et al.* 2002). A significant fraction of Ash1 is present in the nuclei of mother cells (McBride *et al.* 1999), suggesting that *ASH1* mRNA segregation is not as stringent a mechanism for protein localization as the partitioning of Ace2.

RAM network activates Ace2 and traps it in the daughter cell nucleus: Removal of CDK phosphorylation near Ace2's NLS permits the protein to enter nuclei, and is necessary but not sufficient for its localization and transcriptional activation of MDS genes (O'Conallain *et al.* 1999; Mazanka *et al.* 2008; Sbia *et al.* 2008; Mazanka and Weiss 2010; Brace *et al.* 2011). This contrasts with Swi5: NLS unmasking during mitotic exit is both necessary and sufficient for Swi5 to accumulate to high levels in nuclei and activate transcription of its target genes (Moll *et al.* 1991; Jans *et al.* 1995; Doolin *et al.* 2001; Sbia *et al.* 2008). The reason for this difference is that Ace2 has a highly active NES, and its equilibrium concentration in nuclei is not high enough to drive expression of its target genes when both its import and export are unrestrained. In contrast, Swi5 lacks an NES and thus rapidly accumulates in nuclei when its NLS is uninhibited (Sbia *et al.* 2008).

Cbk1 directly regulates Ace2's nucleocytoplasmic shuttling by phosphorylating and inactivating its NES. Two Cbk1 consensus motifs are present within Ace2's NES (Figure 17). Cbk1 phosphorylates these *in vitro*, and mass spectrometry revealed *in vivo* phosphorylation of both residues (Mazanka *et al.* 2008; Gnad *et al.* 2009; Bodenmiller and Aebersold 2011). A phosphospecific antibody raised against one of these sites indicates that the modification occurs shortly after release from mitotic block, coincident with the transcription factor's nuclear localization, and that it requires Cbk1 function (Mazanka and Weiss 2010; Brace *et al.* 2011). Mutations that inactivate Ace2's NES, including phosphomimetic substitutions at either Cbk1 site, cause it to localize strongly to both mother and daughter nuclei and make MDS gene expression and septum destruction independent of the RAM network (Racki *et al.* 2000; Mazanka *et al.* 2008; Sbia *et al.* 2008). Binding of the exportin Crm1/Xpo1 to Ace2's NES has been reconstituted *in vitro*, and phosphorylation of either Cbk1 site disrupts the association (Mazanka and Weiss 2010). Importantly, eliminating these Cbk1 sites prevents Ace2's concentration in the daughter nucleus, resulting instead in a faint mother–daughter distribution like that seen just after mitotic exit.

Cbk1 also regulates Ace2 by phosphorylating a more C-terminal site (S436). This is not required for Ace2's localization to the daughter nucleus, but rather enhances its ability to activate target genes (Mazanka *et al.* 2008). Thus, Cbk1 controls Ace2 in two synergistic ways. NES inactivation drives accumulation in the daughter cell nucleus, but is not fully necessary for induction of Ace2-responsive genes. Likewise, S436 phosphorylation is not absolutely necessary for Ace2 function when the NES phosphorylation sites are present.

Turning Ace2 and Swi5 off: The transcription of Swi5- and Ace2-driven genes occurs in an exceptionally sharp burst in early G1 daughter cells. (Spellman *et al.* 1998; Pramila *et al.* 2006; Li *et al.* 2009). Such a pulse requires both an "on" and "off" switch. As shown in Figure 16, the bursts of Ace2 and Swi5 localization follow subtly different courses that reflect important differences in their regulation (Sbia *et al.* 2008; Mazanka and Weiss 2010). In many cases, inactivation phases of biochemical pulse-generating systems involve destruction of key components (*e.g.*, Pomerening *et al.* 2003). In fact, Swi5′s inactivation follows this route: the protein is degraded in early G1, likely triggered by the G1 CDK Pho85 (Tebb *et al.* 1993; Measday *et al.* 2000; Sbia *et al.* 2008). In vivid contrast, Ace2 remains stable throughput the cell cycle and is turned off by a complex system that results in its nuclear export and stable cytoplasmic sequestration (Sbia *et al.* 2008; Mazanka and Weiss 2010). This includes periods in G1 in which Mob2–Cbk1 and the RAM network is active in the control of cell morphogenesis (Racki *et al.* 2000;

Bidlingmaier *et al.* 2001; Colman-Lerner *et al.* 2001; Weiss *et al.* 2002; Nelson *et al.* 2003; Jansen *et al.* 2006, 2009).

The first phase of Ace2 inactivation involves dynamic nuclear retention that determines the length of the pulse of Ace2-driven transcription. In early G1, Cbk1 continuously rephosphorylates and thus inactivates Ace2's NES in the nucleus (Mazanka and Weiss 2010). Inhibition of Cbk1 causes rapid loss of Ace2 NES phosphorylation and immediate export of the protein from the nucleus, indicating that Cbk1 acts in the presence of an unknown countervailing phosphatase and that a dynamic balance of competing kinase and phosphatase activities determines how long Ace2 stays in the nucleus. During normal G1 transit Cbk1 eventually loses this competition, and Ace2's NES is dephosphorylated and thus activated (Mazanka *et al.* 2008; Sbia *et al.* 2008; Mazanka and Weiss 2010).

Ace2 cannot reenter the nucleus once it is exported in G1 (Bourens *et al.* 2008; Mazanka *et al.* 2008; Sbia *et al.* 2008; Mazanka and Weiss 2010), due to a multistage mechanism that traps it in the cytoplasm. This sequestration requires either of two G1 CDKs: Pho85 or Cdk1 (Mazanka and Weiss 2010). In principle, these G1 CDKs could trap Ace2 in the cytoplasm entirely by phosphorylating sites that inactivate Ace2's NLS, recapitulating control by M phase Cdk1. However, Ace2 becomes extensively phosphorylated at CDK sites prior to its export from the nucleus, and a mutant form of Ace2 (*ace2-AP*) lacking all 21 minimal CDK sites is only partially defective in G1 cytoplasmic sequestration (Mazanka and Weiss 2010). Moreover, in cells arrested at G1/S by Cdk1 inhibition, both Ace2-AP and wild-type Ace2 proteins are identically blocked from entering the nucleus. How is Ace2 kept in the cytoplasm without direct phosphorylation of its CDK sites? Importantly, G1 cytoplasmic trapping of Ace2-AP is essentially absent in *pho85*Δ cells, but loss of Pho85 kinase activity does not cause this effect (Mazanka and Weiss 2010). Thus, Pho85's role here is independent of its kinase function, and perhaps a Pho85–cyclin complex binds Ace2 in G1 and blocks its nuclear import, with CDK phosphorylation promoting either Pho85–cyclin association or its binding to Ace2.

Thus, Ace2 inactivation may occur in multiple stages, by contrast with the relatively simple degradation of Swi5 (Figure 18; also see Figure 16). Early G1 CDK phosphorylation of Ace2 (by Cdk1, Pho85, or a transcription-associated CDK) may inactivate its NLS while it is still in the nucleus, ensuring that unmasking of Ace2's NES rapidly turns off expression of its target genes. Later in G1, Pho85 and/or Cdc28 activate Ace2 cytoplasmic trapping, which involves a kinase-independent function of Pho85. Such a multistage system might allow both a fast block to Ace2 nuclear reentry that sharpens the off phase of MDS gene expression, as well as a slower-maturing retention mechanism that is more stable and resistant to protein phosphatases. These models remain speculative, however. It is also not clear if there is any benefit to retaining stable Ace2 in the cytoplasm; conceivably, it could be rapidly released under certain growth conditions (Butler and Thiele 1991).

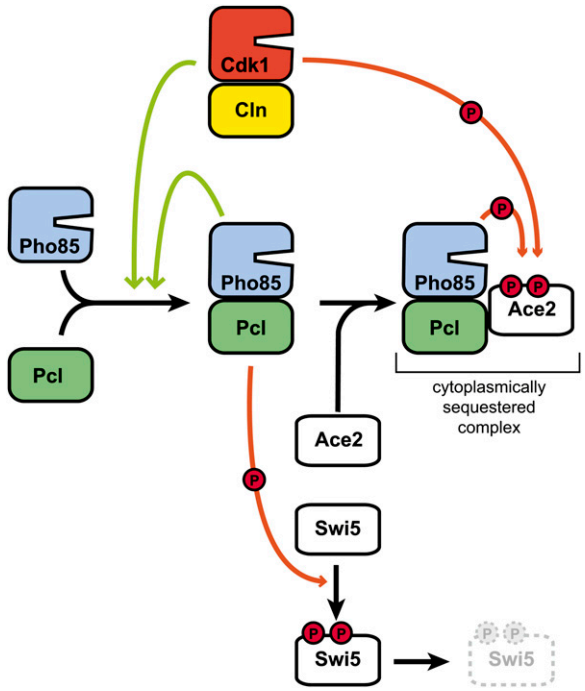

Figure 18 Inactivation of Swi5 and Ace2 in G1. In this model, red arrows indicate direct phosphorylation that has an inhibitory effect, and green arrows indicate a positive regulatory interaction. Swi5 is degraded following direct phosphorylation by the G1 CDK Pho85 in complex with an unknown "Pcl" cyclin. For Ace2, following dephosphorylation of its NES it is exported to cytoplasm and sequestered there. This sequestration appears to occur through two mechanisms, one involving direct phosphorylation of CDK sites in Ace2 and another involving a kinase-independent function of Pho85. See text for details.

An unknown mechanism restricts the RAM network's control of Ace2 to the daughter cytoplasm: Ace2's rapid concentration in the daughter cell nucleus prior to actomyosin ring contraction is a compelling example of the partitioning of a transcription factor that is initially distributed evenly in a contiguous cytoplasm. This process appears to be entirely mediated by diffusion, with the daughter nucleus acting as a trap for Ace2. Intriguingly, Ace2's nuclear accumulation does not occur if nuclear division occurs entirely in the mother cell, suggesting that the daughter cell cytoplasm has a special ability to promote inactivation of the Ace2 NES (Mazanka *et al.* 2008). Since Mob2–Cbk1 (and other RAM network proteins) concentrate at the bud cortex, it is tempting to speculate that their localization is causal. However, while Ace2 physically interacts with the Mob2–Cbk1 module, it has never been seen to colocalize with them at the cell cortex, and it is not clear why Ace2 activated in the cytoplasm would not diffuse into the mother cell. Phosphatase activities localized to the mother or bud neck could maintain a steep gradient in Cbk1 activation. Indeed, a point mutation in Cbk1's C-terminal tail that probably hyperactivates the kinase increases localization of Ace2 in the mother cell nucleus (Panozzo *et al.* 2010). However, Ace2 asymmetry cannot exclusively involve spatial control of Cbk1's HM site, as acidic substitutions at Cbk1's HM

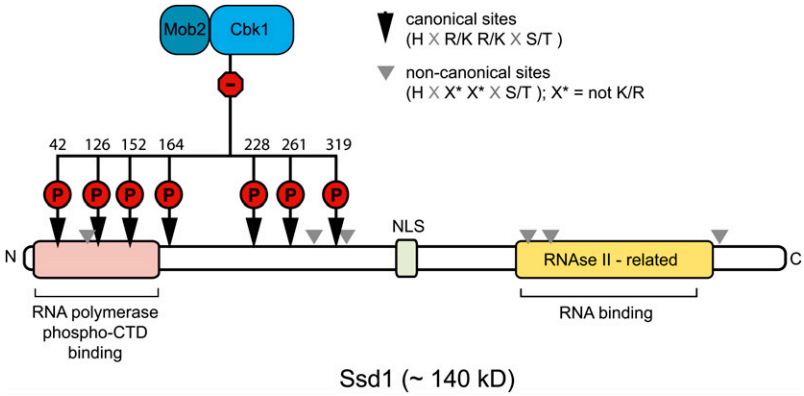

Figure 19 Domain organization of Ssd1. Ssd1 is an RNAse II-related protein that probably lacks catalytic activity. Canonical Cbk1 consensus motifs (black arrows) are concentrated in the protein's N-terminal region, including a portion that mediates interaction with phosphorylated RNA polymerase II C-terminal domain (PCTD). A sequence that can act as an NLS is present in the middle part of the protein.

phosphorylation site create a gain-of-function allele but do not cause symmetrical Ace2 distribution (Panozzo *et al.* 2010; Brace *et al.* 2011). Therefore, the mechanism responsible for the diffusion-mediated partitioning of Ace2 remains obscure.

RAM network controls translation of cell separation proteins

Recent analysis has shown that Cbk1, and by extension the RAM network, controls the translation of specific proteins by directly regulating the mRNA binding protein Ssd1. In brief, Ssd1 is an RNAseII-related protein broadly conserved in fungi that appears to lack catalytic activity; it has been implicated in numerous processes. It binds specific mRNAs and suppresses their translation, and Cbk1 efficiently directly negatively regulates this Ssd1 function *in vivo* (Hogan *et al.* 2008; Jansen *et al.* 2009; Kurischko *et al.* 2011a). This is critical for cell wall remodeling during bud growth; however, the Ssd1 translational control system's important role in cell morphogenesis outside of the process of mother/daughter separation is not discussed further in this chapter. As diagrammed in Figure 19, Cbk1 regulates Ssd1 through direct *in vivo* phosphorylation of sites in Ssd1's N-terminal region (Jansen *et al.* 2009).

Ssd1 associates with mRNAs that encode some of the most important MDS proteins, such as Cts1, Dse2, and most of the SUN-family glucanases (Hogan *et al.* 2008; Jansen *et al.* 2009); the *CTS1* and *SUN4* mRNAs are particularly strongly enriched in Ssd1 pull-down experiments. Translation rate measurements show that Ssd1-associated messages are significantly suppressed when Cbk1 activity is acutely blocked (Jansen *et al.* 2009). Ssd1 can localize to cytoplasmic granules known as P-bodies, which are associated with translational suppression (Balagopal and Parker 2009; Jansen *et al.* 2009; Kurischko *et al.* 2011a), but Ssd1 can clearly carry out its translational repression function in cells lacking these structures. Ssd1 likely assembles a translationally silent mRNP in the nucleus: it associates with unspliced and centromeric mRNAs, interacts with the phosphorylated C-terminal tail of RNA polymerase II, and requires a sequence that can function as an NLS (Phatnani *et al.* 2004; Jansen *et al.* 2006; Hogan *et al.* 2008; Kurischko *et al.* 2011b). The mechanisms and precise function of Ssd1's control of translation remain poorly understood, but since RAM network proteins concentrate at the bud neck during cell separation, the Ssd1 circuit could allow the cytokinesis site to exert post-transcriptional control over the expression of proteins needed at different stages of the process (Nelson *et al.* 2003; Jansen *et al.* 2006; Kurischko *et al.* 2011a).

Model for septum destruction control: cascading feed-forward loops

The RAM network's control of cell separation is a remarkable multilayered system. By regulating both Ace2 and Ssd1, the pathway activates both the transcription and translation of a specific set of genes. As diagrammed in Figure 20, Mob2–Cbk1 phosphorylation of these effectors creates a FFL network motif. More specifically, this configuration forms a coherent feed-forward loop, in which the system's two branches exert an effect of a similar sign on the downstream component or process (Mangan and Alon 2003). Such network motifs are significantly enriched in biology and can create systems that turn on slowly but off quickly (Mangan and Alon 2003; Dekel *et al.* 2005; Kalisky *et al.* 2007). Intriguingly, upstream control of Ace2 and Mob2–Cbk1 during the M/G1 transition may constitute a similar network motif, with Cdc14 activating both Mob2–Cbk1 and Ace2. This

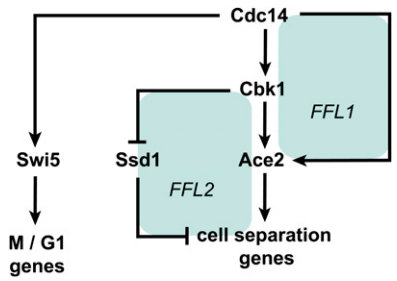

Figure 20 Model for septum destruction control. A simplified structure of Ace2 and RAM network mitotic control shows that Cdc14 activates both systems, in a coherent feed-forward loop (FFL1). Cbk1 control of both Ace2 and Ssd1 constitutes a second feed-forward loop (FFL2). In contrast, Cdc14 regulation of Swi5 appears to involve simple activation, consistent with relative kinetics of the two transcription factors' activities.

produces a pathway in which two such loops are linked in a cascading fashion, consistent with the construction of complex biological regulatory systems from recurring simple network motifs (Milo *et al.* 2002; Yeger-Lotem *et al.* 2004; Kashtan and Alon 2005; Alon 2007). While much remains to be learned about the septum destruction control system, it is possible that this network organization helps ensure that the process does not start before cytokinesis is complete.

Concluding Remarks

Studies of the asymmetric division of budding yeast cells have greatly illuminated molecular machines and regulatory systems that allow the spectacular feat of cell division and has identified systems that are broadly important for eukaryotic cell proliferation. In particular it has become clear that mitotic exit control pathways coordinate multiple independent processes, which act in rapid sequence to split the dividing cell into two unequal progeny. However, while the molecular components involved in both the mechanics and control of this event are better known, much remains mysterious. It seems clear that we are only beginning to identify targets of the APC/C, Cdc14, the MEN kinases Cdc15 and Dbf2, and the RAM network that are relevant to the processes that productively cut the dividing cell in two.

It is still not clear how the relative timing of cytokinetic events is enforced: for example, why do cells not start destroying the septum before they have finished building it? Similarly, we do not fully understand how mitotic exit control pathways are integrated such that they act sequentially. The events leading to RAM network activation are not well understood, nor is it known how these proteins get to the cell cortex and bud neck or if this is even important for their function. On the cytological side, mechanisms for cutting the divided nucleus, forming the secondary septum, and halting septum synthesis remain unknown. While the activation and nuclear localization of Ace2 is well worked out, the mechanism responsible for its asymmetric partitioning is not. Finally, the RAM network's role in cell morphogenesis and translational control is only dimly understood: it is not clear how Ssd1 suppresses translation of associated mRNAs and if it promotes their localized translation. Future research in these areas will define how diverse systems are integrated to achieve effective cell division.

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