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Review

Cell biology of yeast zygotes, from genesis to budding

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ABSTRACT

The zygote is the essential intermediate that allows interchange of nuclear, mitochondrial and cytosolic determinants between cells. Zygote formation in *Saccharomyces cerevisiae* is accomplished by mechanisms that are not characteristic of mitotic cells. These include shifting the axis of growth away from classical cortical landmarks, dramatically reorganizing the cell cortex, remodeling the cell wall in preparation for cell fusion, fusing with an adjacent partner, accomplishing nuclear fusion, orchestrating two steps of septin morphogenesis that account for a delay in fusion of mitochondria, and implementing new norms for bud site selection. This essay emphasizes the sequence of dependent relationships that account for this progression from cell encounters through zygote budding. It briefly summarizes classical studies of signal transduction and polarity specification and then focuses on downstream events.

1. Introduction

Zygotes are essential intermediates in the life cycle of organisms that reproduce sexually and undergo a haploid-to-diploid transition (Fig. 1). In many species, preparation for fertilization entails major molecular and spatial reorganization of parental genomes and cytoplasmic structures to generate germ cells that are sexually dimorphic. By contrast, zygote formation in *Saccharomyces cerevisiae* is not known to involve molecular reorganization of the genome, and cells of the two mating types are indistinguishable upon microscopic examination. Partly because zygote formation is a facultative function, multiple aspects of the process have been studied in depth. Zygote formation in budding yeast has defined paradigms of broad cell biological, evolutionary and genetic interest.

To form zygotes, parental cells of *S. cerevisiae* must be able to recognize and signal to cells of the opposite mating type, to interrupt their cell cycles, and to generate or recruit essential molecular equipment that makes possible "chemotropic" polarization toward a mating partner. These preliminary events are followed by establishment of a zone of contact (ZOC) and lead to formation of sonication-resistant "prezygotes," in which the two polarized haploid cells adhere to each other. Once the intervening cell wall has been remodeled, as we discuss below, it seems reasonable to speak of the enclosed "ZOC compartment" that lies between the two cells. Upon cell fusion, the nuclear envelope (NE) remains intact (as during the yeast mitotic cell cycle), quite unlike fertilization in many higher eukaryotes, for which the NE breaks down [1,2].

After nuclear fusion (karyogamy), early zygotes reenter the cell cycle and then bud repeatedly [3–5]. During this period, the mitochondrial genomes replicate and parental mitochondria fuse with each other after a delay, allowing recombination to occur [6–8]. At least during the first several hours, parental vacuoles do not fuse together and mature peroxisomes, although they intermix, also do not fuse with each other [9,10]. Moreover, many proteins of the parental plasma membrane domains do not intermix rapidly, reflecting the low diffusional mobility of many cortical proteins in yeast [11,12].

Yeast zygotes in which karyogamy is inhibited have often been used as an intermediate for cytoduction, in which a cytoplasmic element (mitochondria, prions, virus) is transferred from one haploid parent to a distinct haploid recipient [13,14]. Related strategies have been used to transfer chromosomes or plasmids, thereby providing an unusual opportunity to investigate the origins and consequences of aneuploidy [15–18]. A further point of interest in studying zygotes pertains to transgenerational inheritance: In zygotes that result from fusion of genetically distinct parents, if mitosis occurs before thorough mixing of parental organelles, distinct parental characteristics can be passed to subsets of progeny.

Abbreviations: bud scar, the chitin-rich remnant of the cell wall that demarcates the site of cytokinesis; chemotropic polarization, cell polarization that results from exposure to a gradient of pheromone, as opposed to isotropic pheromone; GEF, guanine nucleotide exchange factor; karyogamy, nuclear fusion; NE, nuclear envelope; prezygote, the intermediate in which two polarized haploid cells adhere to each other but have not fused; SPB, spindle pole body; ZOC, the zone of contact at which haploid cells establish contact in forming prezygotes; ZOC compartment, the extracellular compartment delimited by the apices of haploid cells in prezyogtes, along with the lateral portion of the cell wall that spans the gap between the two cells

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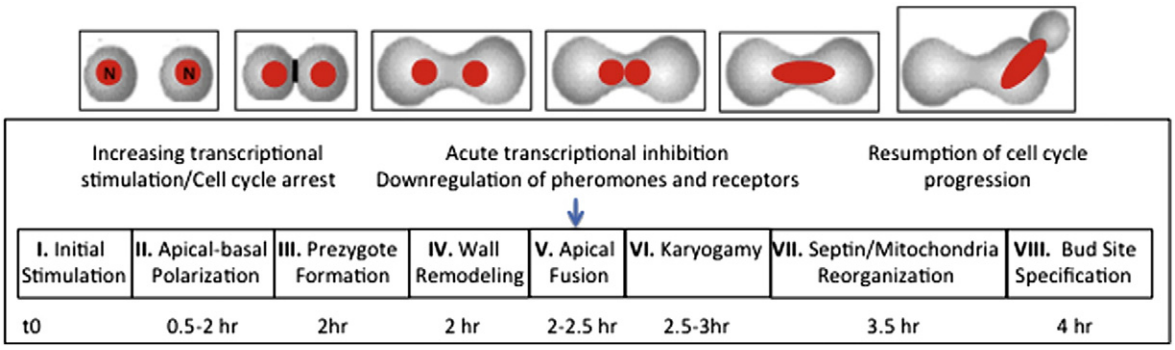

Fig. 1. Schematic of zygote genesis showing the approximate timing at 23 °C. The diagram indicates the successive steps: I: initial stimulation due to pheromone-receptor interactions, II: establishment of cell contact at a specialized interface (vertical black line), along with apical–basal polarization of the cortex, III: formation of sonication-resistant pairs (prezygotes), IV: cell wall remodeling, V: cell fusion, VI: nuclear congression and fusion (karyogamy), VII: redistribution of septins, allowing contacts between parental mitochondria, and VIII: bud site specification. These events constitute a series of dependent steps that are set in motion when mating pheromones stimulate cells of opposite mating type that are adjacent to each other. The transcriptional program elicited by pheromones is terminated (arrow) when cell fusion allows the a1–α2 heterodimeric transcriptional inhibitor to form. Especially because this ends transcription of Far1, cell cycle progression can then resume (see text). The indicated timing is approximate, both because of intrinsic cell-to-cell variability and because in standard mating protocols with unsynchronized cells both partners must arrive at the beginning of the cell cycle in order to advance through the indicated steps. Nuclei are red.

2. Initial cell stimulation; transcriptional response — Fig. 2

The classical pathway for protein secretion involves synthesis in the ER, transport through the Golgi complex into secretory vesicles, and exocytosis. A typical cargo for this pathway is the pheromone, alpha factor, that is synthesized by MAT α cells. By contrast, a limited number of proteins synthesized on free ribosomes are released from cells via ABC transporters in the plasma membrane. The best-characterized prototype – and the only example in *S. cerevisiae* – is the pheromone produced by MAT a cells (a-factor) which undergoes proteolytic cleavage as well as post-translational prenylation and carboxymethylation. Homologs of some of the enzymes responsible for these post-translational modifications contribute to equivalent modifications of lamins in higher eukaryotes. The lamin subfamily of intermediate filament proteins is however not found in *S. cerevisiae*. Curiously, the Axl1 protease that normally participates in cleavage of the a-factor precursor also functions as an axial landmark for bud site selection and is required for efficient cell fusion [19–25] — see below.

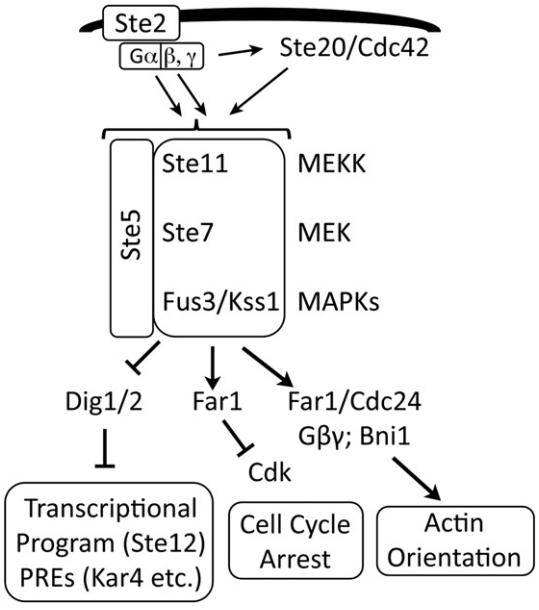

Fig. 2. Signaling pathways downstream of pheromone receptors. The alpha factor receptor, Ste2, is indicated, as are downstream elements, all of which are identical for both mating types. Receptor stimulation has profound transcriptional consequences, arrests cell cycle progression, and reorients actin toward the receptor. Additional critical interactions contribute to these events, some of which are mentioned in the text. PREs: Genes with pheromone-response elements in their promoter sequences. Ste12 drives most of the transcriptional program upon stimulation. As indicated in the text, both the kinase, Fus3 and Far1 serve multiple roles during zygote formation. Much of the information on signaling has been obtained by treating MAT a cells with synthetic α-factor. Now that a facile synthesis of a-factor is available, it should become possible to compare the downstream consequences of each pheromone [28].

The limited solubility of prenylated pheromones may endow their concentration gradients with properties that optimize partner selection. Furthermore, there could be some yet-unappreciated value to having the two pheromones secreted by distinct mechanisms, judging from the observation that this dichotomy is characteristic of other *Ascomycetes*. Nevertheless, the many pheromones of *Basidiomycetes* are all prenylated and presumably undergo ABC cassette-mediated export [26]. Moreover, when pairs of *S. cerevisiae* strains are engineered to produce pheromones, both of which or neither of which is prenylated, they are able to mate with each other [27]. Although the biosynthesis of mating factors in *S. cerevisiae* involves multiple covalent modifications (proteolysis, prenylation, carboxymethylation, glycosylation), there is no evidence that these modifications are differentially regulated.

The pheromone receptors expressed by the two mating types (Ste2, Ste3) are not closely homologous to each other, but each has seven membrane-spanning domains and is coupled to identical heterotrimeric G-proteins. Strains carrying mutations of these receptors and mutants that carry lesions in downstream effectors were discovered using selections and screens to recover cells that are deficient in mating or deficient in growth arrest when exposed to pheromone. Given the conservation of the basic paradigms of G-protein-coupled receptors, yeast has been engineered to express mammalian receptors that can function in conjunction with the yeast G proteins. This has made possible aggressive programs to identify ligands and drugs that interact with the mammalian receptors, e.g. [29].

There is a wealth of information concerning the multiple consequences of receptor stimulation. Fig. 2 calls attention to the resulting transcriptional impact, cell cycle arrest, and repolarization of the actin cytoskeleton. In brief, the receptor-coupled $G_{\beta\gamma}$ subunits signal via the PAK kinase, Ste20, to a MAP kinase cascade. The scaffold protein, Ste5, plays a pivotal role since it binds all members of the cascade and also mediates the activation of Fus3 via Ste7 kinase. Fus3 kinase then activates the transcription factor, Ste12 by inhibiting Dig1/2. Fus3 also targets Far1 to cause cell cycle G1 arrest, and the formin, Bni1, to redirect actin. The MAP kinase cascade itself is not required for formation of prezygotes, so long as Ste12 is expressed. The cascade is however required to yield zygotes and diploid progeny [30].

More than 100 transcripts show significant induction when cells are exposed to isotropic pheromone. This group encodes proteins that function in agglutination, the pheromones, their receptors and coupled G protein subunits, as well as proteins that function in a-factor release, cell fusion, in karyogamy, and in cell cycle arrest. The products of these transcripts therefore result in major intensification of signaling. The logic behind the increase of many other transcripts that are upregulated is however intriguingly obscure. Upregulation of a subset of transcripts by Ste12 also requires Kar4, which is among the targets of Ste12. Many Ste12 targets have "pheromone-response elements" in their promoters [24,31,32] {Valtz, 1995 #16396; Valtz, 1997 #16395}. Since zygotes can be purified, it should become possible to learn whether the zygote transcriptome has additional distinct characteristics [33,34].

3. Reorientation of the axis of growth — Fig. 3

When cells of opposite mating type are close enough to each other to sense pheromone gradients, mutual stimulation of their pheromone receptors initiates "courtship." The result is pairing with a single partner, with strong preference given to the partner that produces the most pheromone [35–38] (Fig. 3).

Chemotropic polarization results from exposure to a gradient of pheromone, e.g. produced by a partner of opposite mating type or by pheromone released from a pipette tip. In this situation, the receptors and their canonical G protein subunits cluster along the proximal segment of the cortex and actin filaments then reorient toward the receptor. This reorientation relies on release of the Far1/Cdc24 complex from the nucleus, which bridges between receptor $G_{\beta\gamma}$ subunits and actin. In this process, Ste20 and the entire MAP kinase cascade, as well as Bem1 (which serves as a scaffold for Cdc42, Cdc24 and Ste20) are also recruited to the cell apex. Additionally, G_{α} stabilizes the Far1–$G_{\beta\gamma}$ complex and promotes Fus3 synthesis at the cell apex via the RNA-binding protein, Scp160 [39–49]. Since chemotropic growth can occur in the absence of a mating partner, it does not depend on cell contact per se. It nevertheless seems likely that it does require the high intensity of focused receptor stimulation.

When haploid cells are exposed to isotropic pheromone – and perhaps when they are exposed simultaneously to multiple identical partners – they produce a mating projection that orients toward the cortical landmark proteins that govern cell polarity during mitotic growth. The actin guidance protein, Spa2/Pea1, is required in this situation, but Far1 is not. Such "default" behavior is also seen in selected *far1* and *cdc24* mutants and when cells of opposite mating type, although near to each other and able to polarize, become "confused" by excessive amounts of soluble pheromone [41,50–54]. The inability of such cells to reorient their axis of growth away from landmarks presumably reflects the absence of spatial unanimity of receptor signaling, coupled with the cooperative impact of the preexisting landmark determinants. As is explained further below, landmark-dependent bud site specification in haploid and diploid cells depends on two GTPases, Bud1/Rsr1 and Cdc42.

The common practice of studying responses to isotropic pheromone is thus an imperfect model for the polarization that leads to zygote formation.

4. Prezygote formation – surface polarization – Fig. 4

Both when haploid cells are exposed to isotropic pheromone and when they form prezygotes, the cortex of each cell starts to become polarized within the first hour, forming an apical domain and a basal domain. These events are concurrent with – and in at least some cases result from – the reorientation of actin polarity discussed above. The apical and basal domains are separated from each other by a circumferential belt of septins. Curiously, some apical proteins are tightly concentrated at the tip, while others appear composite or define a broader cap [11,55–59] — Fig. 4. Features of this polarization remain conspicuous in mature zygotes.

The apical domain of prezygotes is surely a site of intense signaling: It is highly enriched in the ABC cassette a-factor exporter, the corresponding pheromone receptors, and components of the downstream signaling cascade, as well as proteins that function in exocytosis and endocytosis. It will be valuable to learn whether the apical localization of

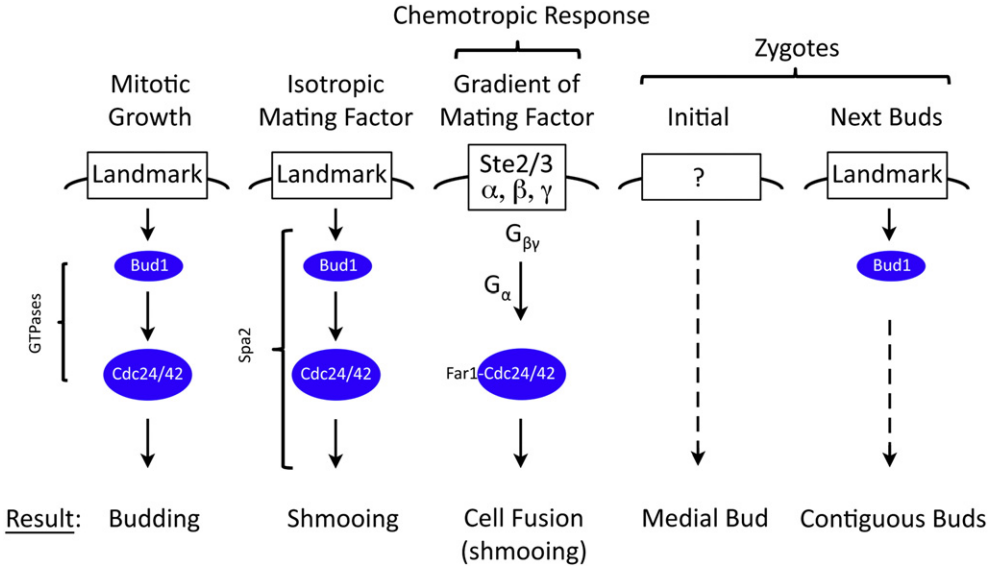

Fig. 3. Reorientation of actin. This simplified scheme highlights the differences between the mechanisms of actin guidance in mitotic cells (left), cells stimulated with isotropic pheromone or a gradient of pheromone, and in budding by zygotes. In cycling cells, axial or bipolar landmarks interact with the Bud1/Rsr1 GTPase module that serves as an essential intermediate. The Bud1 module signals to Cdc42 which leads to actin guidance. In the presence of isotropic pheromone, the mating projection/shmoo – as in typical haploid cells – forms adjacent to axial landmarks/bud scars that mark the site of the preceding bud. When a pheromone gradient is present, cells elongate along the gradient, with the receptor in the lead (chemotropic growth). In this response, Far1 functions as part of a complex with Cdc24. In zygotes, as is described below, most initial buds are medial. Bud1 is not required for their positioning. Later buds are contiguous to the initial bud and require axial landmarks and Bud1 for this contiguity. The dotted arrows in the two schemes for zygotes signify that unidentified intermediates may be present.

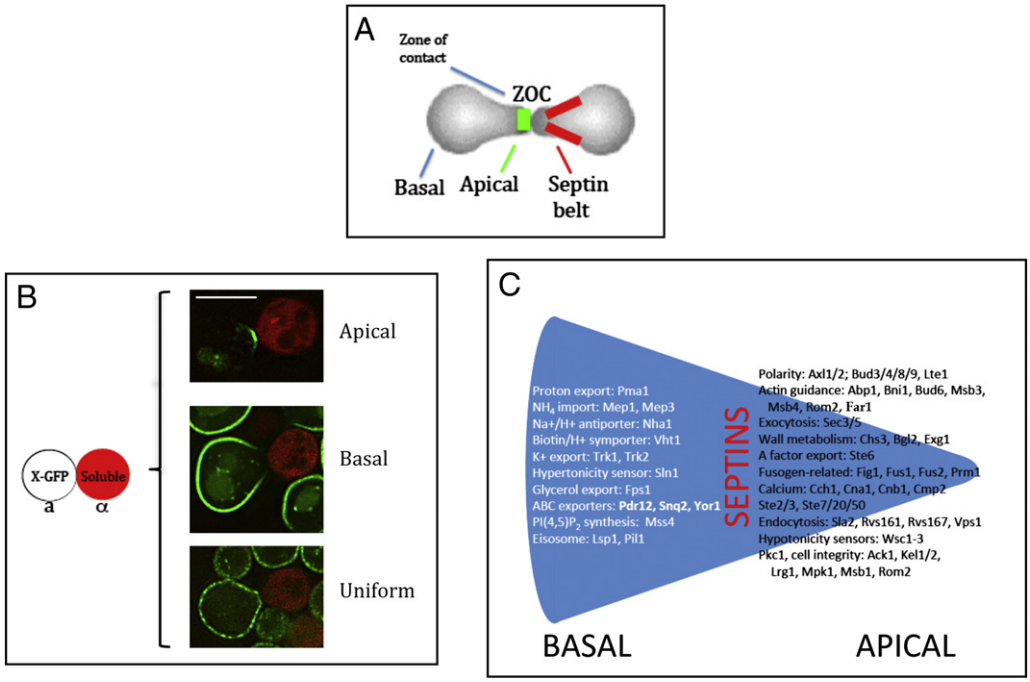

Fig. 4. Polarized distribution of cortical proteins in prezygotes. A: Diagram of the symmetric organization of prezygotes, showing the circumferential septin belt (red), apical and basal domains and the zone of contact (ZOC). B: Examples of apical (Fus1-GFP), basal (Pma1-GFP) and uniformly distributed (Can1-GFP) cortical proteins. In each case, a strain expressing the tagged protein (X-GFP) was crossed with a strain expressing cytoplasmic DsRed. Since the DsRed is freely diffusible in the cytoplasm, this arrangement makes it possible to know whether cell fusion has occurred. The images are either included in or described in reference [11]. Bar = 5 μm. C: Diagram summarizing polarized distributions of cortical proteins in prezygotes. Additionally, many nutrient transporters are uniformly distributed around the cortex [11].

each of these proteins is in fact needed for cell fusion and zygote formation. Judging from surveys and studies of individual proteins, the apical group also includes adhesion proteins, proteins important for actin orientation (e.g. Far1 and Cdc24), wall metabolism, calcium metabolism, the "cell integrity path," and proteins that contribute to cell–cell fusion, as is discussed below [11,24,55].

Moreover, many small vesicles – presumably functioning in endo- and exocytosis – are adjacent to the ZOC, although there is no direct evidence that endocytosis occurs [60–62]. Some proteins that have been localized to the apical domain may in fact be confined to adjacent vesicles, making it possible for them to be recruited to the surface. Taken together, these observations support the notion that the prezygote performs functions similar to those of a symmetric synapse, e.g. [63].

For proteins that are delivered to the surface in conjunction with secretory vesicles, apical localization could be a simple consequence of exocytosis at the sites where actin filaments contact the cortex, with the proviso that they do not diffuse to the basal surface, e.g. because of the belt of septins [64]. If they are endocytosed from the apical domain, subsequent exocytosis would be expected to return them to the apex. In fact, localization of several proteins to the tip of the mating projection does depend on endocytosis (and actin polarization), e.g. as judged from studies of their distribution in ts endocytic (*end4*) mutants [12]. Apical localization has also been thought to be linked to association with lipid rafts [65–67]. At least for the cell fusion factor, Fus1, specific cytosolic sequences and glycosylation promote apical localization [68–70]. Additionally, apical localization of a subset of proteins depends on Cdc24 [71]. For others, apical localization depends on localization of their mRNAs, which requires that their 3′-UTRs bind to the apical RNA-binding protein, Scp160, in association with cortical ER. In the absence of Scp160, apical localization of these proteins and zygote formation are strongly inhibited [72].

As summarized in Fig. 4, the group of proteins at the basal domain that are absent from the apical domain contributes to proton and glycerol efflux and to phosphoinositide synthesis. In addition, many ABC cassette nutrient transporters are found around the entire perimeter of prezygotes [11]. There have been no investigations of the determinants that cause proteins to assume a basal distribution or to be uniformly distributed around the cortex.

The environment within the ZOC compartment (that is bounded by the apex of each of the two cells and the lateral wall) is unknown; however – as described above – several transporters and channels are not detected at this site. This environment is therefore likely to be poor in glycerol and potassium and not to be acidic. This environment places limits on the biochemistry that accomplishes wall remodeling and cell fusion.

5. Wall remodeling and the osmotic challenge — Fig. 5

Bud growth during the mitotic cell cycle requires ongoing remodeling of the wall, as well as insertion of new wall material [73–75]. An equivalent danger exists during zygote formation since intervening wall must be removed before cell fusion can occur. It remains unclear how these concurrent events are accomplished without risking osmotic lysis, although the "cell integrity pathway" is considered to play a protective role — Fig. 5. This pathway is activated by exposure to hypotonicity and circumstances thought to stretch the plasma membrane. Moreover, mere application of a pressure differential to the surface of spheroplasts will activate ion flux, including calcium entry [76–81].

Indeed, crosses of certain mutants that cannot fuse efficiently, and even crosses of wt cells, are accompanied by varying degrees of lysis [75,82–84]. Collateral damage might be minimized by focusing any exocytosis of lytic enzymes precisely at the apex, so that they attack the intervening wall rather than the rest of the wall. Specificity could also be ensured by a) delaying release of lytic enzymes until the ZOC compartment is sufficiently mature to restrict lateral diffusion of such enzymes, or b) releasing enzymes whose specificity corresponds to that of the intervening wall. Consistent with this latter possibility, the wall at the tip of mating projections and at the ZOC is newly-synthesized, and at least the wall at the tip of the projection has a distinct appearance and lectin-binding properties [11,85–90]. There has, however, been no report of

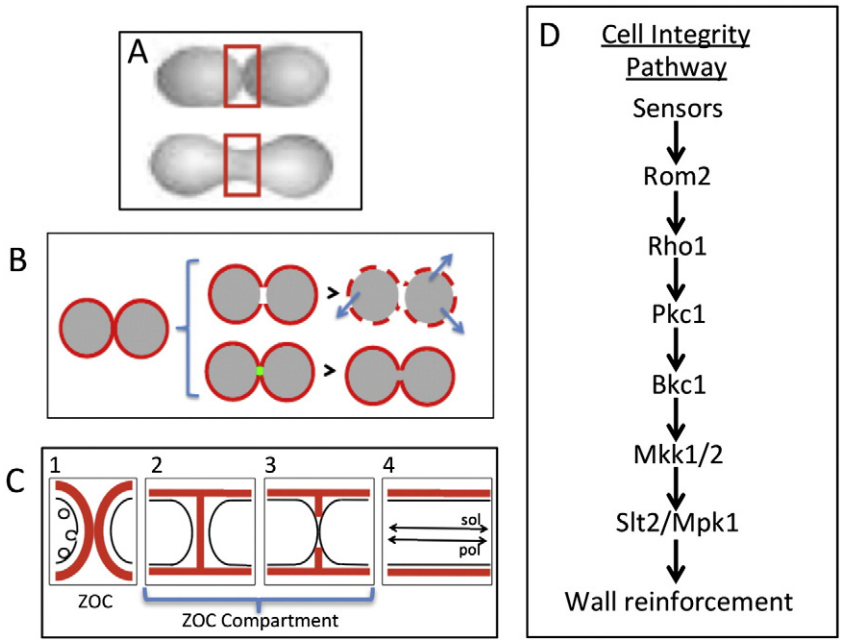

Fig. 5. The osmotic challenge of cell fusion. A: Overview of the prezygote (upper) and early zygote (lower). The ZOC compartment/midzone is boxed. B: The challenge. When the two cells contact each other, if the intervening wall (red) is dissolved using enzymes that can also attack the rest of the wall, it is likely that lysis will occur (upper). On the other hand, if a chemically distinct type of wall lies between the two cells (green) it could be selectively dissolved (lower). C: Model. The two cells surrounded by a wall (red) establish contact, forming a prezygote (1). Both are actively performing exocytosis/endocytosis (indicated in the cell at the left). The prezygote adds new wall to seal off the ZOC (upper and lower horizontal red lines in panel (2)). This generates the enclosed "ZOC compartment." An intervening wall segment is present (vertical red line). Once the intervening wall has been selectively perforated (3), the plasma membranes can contact each other in trans. Upon fusion, soluble proteins (sol) and polysomes (pol) will diffuse between the parental domains (horizontal arrows in (4)). The continuity of the wall between the parental cells and continuity with the intervening wall segment are detected by electron microscopy [60,91]. D: Cell integrity pathway. Transmembrane sensor(s) for this pathway (e.g. Wsc1–3) are perturbed by mechanical stretch and/or hypotonicity. This triggers a cascade of signals that culminate in wall reinforcement. Other sensors include the transmembrane proteins, Mid2 and Mtl1.

wall isolation and compositional analysis after exposure to mating pheromone.

Exposure of cells to isotropic pheromone produces a mating projection without frequent lysis. Any concomitant release of degradative enzymes is thus not catastrophic under these circumstances [92]. A minimalist model to explain the timing of wall dissolution could be based on the hypothesis that degradative enzymes are continually released, and that their local impact at the apex becomes significant only when the presence of a partner en face restricts their free permeation and dissipation [93].

Alternatively, a step-wise model can be proposed, according to which the balance of wall synthesis vs degradation at the ZOC is under precise control, with a synthetic phase being followed by a degradative phase — Fig. 6. This view is supported by the observation that pheromone stimulates the cell integrity pathway, leading to synthesis of wall β-glucans, organization of the actin cytoskeleton, and delivery of at least chitin synthase (Chs3) from the *trans*-Golgi to the cell surface [94–98]. Wall synthesis therefore is expected to predominate before formation of the ZOC.

Sensors of the integrity pathway (the transmembrane proteins, Wsc1–3) and especially protein kinase C (Pkc1) [99] seem well-suited to play a central role in regulating wall integrity. For example, the transmembrane Wsc proteins have a domain embedded in the wall and therefore could be affected by changes in wall mechanics and tension.

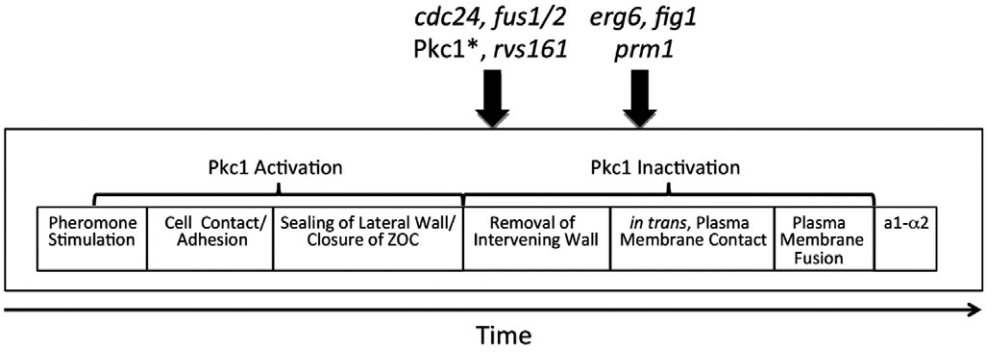

Fig. 6. Cell wall remodeling. According to this model, pheromone stimulates the cell integrity pathway, activating Pkc1 and therefore promoting wall synthesis, that occurs primarily at the apex. Upon cell–cell contact, the lateral wall becomes stabilized ("sealed") across the midzone, forming the ZOC compartment (Fig. 5C). Once formed, either the internal environment of the ZOC compartment or surface tension/stretch of its surface reduces stimulation of the adjacent sensors of the cell integrity pathway. Pkc1 therefore becomes less active and apical cell wall synthetic activity is reduced. Moreover, exocytosis of degradative enzymes (glucanases ?) could be stimulated. Perforation of the intervening wall allows plasma membrane contact and fusion. a1–α2 designates the formation of this heterodimeric transcriptional inhibitor. Its presence and the endocytosis of the pheromone receptors terminate cell stimulation. At the top, the bold arrows designate the approximate sites of interruption of prezygote maturation in the indicated mutants. The central point is that some prezygotes arrest with the intervening cell wall still present, while in other mutants the two plasma membranes that face the ZOC compartment are in contact with each other. Pkc1* is a hyperactive pseudosubstrate mutant form of Pkc1. This mutant causes accumulation of prezygotes in which the intervening wall is present [102].

Moreover, activation of Pkc1 is known to promote wall synthesis, to cause accumulation of prezygotes and to inhibit zygote formation [99]. Furthermore, consistent with the well-established stimulation of Pkc1 and the cell integrity pathway by hypotonicity, cell fusion can also be inhibited by making the cytosol relatively hypertonic, e.g. in glycerol channel mutants (*fps1Δ*) that overaccumulate glycerol [99].

It recently has become possible to interpret some of these observations in a spatial context since, while elements of the cell integrity pathway localize at the apex, the critical glycerol channel (Fps1) has a basal distribution [11]. The ZOC compartment therefore could be expected to be differentially sensitive to local change in osmotic pressure or stretch. Thus, damage to the ZOC compartment would trigger a "cell fusion checkpoint" by stimulating Pkc1 [99], whose local corrective action would strengthen the wall, as in the healing of focal lesions at the cortex of haploid cells [100].

In the normal course of events, however, as previously suggested [99], changes of surface tension at the ZOC could diminish apical wall synthesis, e.g. due to sealing of the lateral walls that border this region. These events could stimulate release or activation of degradative enzymes, perhaps via a regulated secretory pathway. The identity of these enzymes is not known, however, the putative glucanases/transferases, Scw4 and Scw10, that are needed for efficient mating, could play a major role [101]. Interestingly, in mitotic cells, vesicles that convey Chs3 to the surface also transport the fusion factor, Fus1 [70,103]. Moreover, in cycling cells, at least two vesicle populations carry distinct cargoes from the Golgi to the cell surface [104].

When wall remodeling has been completed, each cell must indicate that it is in a "state of readiness" before proceeding to fusion, per se. This could be ensured if the two surfaces of the ZOC compartment were focally modified in some mutually complementary fashion. If the internal condition of each cell transmitted a corresponding change to the external face of the membrane at this site, removal of intervening wall could allow the resulting *cis–trans* spatial coincidence and contact to signal mutual readiness across the gap.

Upon cell–cell fusion, the transcription factors a1 (made by MAT a cells) and α2 (made by MAT α cells) associate with each other. This heterodimer binds and inhibits the transcription of 23 genes in diploid cells, and presumably targets the same genes in zygotes [105]. These genes include *FAR1* (which therefore allows resumption of cell cycle progression), subunits of the receptor-coupled G protein, pheromones, and elements of pheromone receptor signaling (*FUS3*, *GPA1*, *STE5*). Since the average half-life of yeast mRNAs is ~15 min, the transcriptional program of early zygotes is expected quickly to lose most signs of ongoing pheromone responses. Normalization is also achieved through turnover of the key regulators, e.g. [106].

Further potentially relevant proteins are also among the targets of the a1–α2 transcriptional repressor. These are the central kinase of the hypertonicity response pathway, Hog1, and a cell wall mannoprotein, Ccw12 [107]. One polarity landmark protein, Axl1, is also in this group, as is discussed below.

6. Apical fusion — Fig. 6

Much of what is known about the mechanisms of membrane fusion concerns events in which the cytosolic faces of membranes initiate contact. Interactions between the surfaces that are the topological equivalent of the extracellular space must face equivalent thermodynamic hurdles, but use distinct equipment, as for budding from the ER. Cell surface fusion and karyogamy in yeast provide genetically tractable models to investigate both of these topologies.

There has been no direct biochemical evaluation of the activity of proteins that contribute to yeast cell fusion, e.g. in reconstituted systems. The involvement of specific proteins has instead been inferred from the performance of the corresponding mutant cells. These mutants were first isolated because they do not form diploids or do not arrest in the presence of pheromone, because they exacerbate other mating defects, because the corresponding transcripts are upregulated by mating pheromone, or because of structural characteristics of the corresponding proteins [84,91,99,108–116]. Significant limitations for mutant identification have been the necessity of introducing the same mutation into both partners and the necessity to avoid interrupting mitotic growth. Most mutations that do inhibit fusion have only partial effects — perhaps because of underlying redundancies. For this reason, several studies have sought to identify mutants that show a major deficiency when crossed with an already "enfeebled" partner. Several proteins that localize to the apex have been strongly implicated in fusion. Among the transmembrane proteins are:

- *Fus1* — detected serendipitously and by a screen. Exacerbation of the *fus1Δ* fusion defect led to identification of the associated protein, Fus2 (which is not a transmembrane protein). Overexpression of either protein suppresses mutation of the other, as well as other fusion mutants. Fus2 is released from the nucleus upon pheromone stimulation, associates with Cdc42 and Rvs161 – which binds lipids in conjunction with Rvs167 – and forms a circle at the cell apex either before or shortly after cell fusion. Both *fus2Δ* and *rvs161Δ* prezygotes accumulate vesicles adjacent to the ZOC compartment, although *fus1Δ* prezygotes appear not to do so [60,103,117–122].
- *Fig1* — detected because of its major upregulation upon exposure of haploid cells to pheromone. The cell fusion defect that is evident in its absence can be overcome by increasing extracellular calcium levels. Moreover, *fig1Δ* cells are deficient in low-affinity calcium uptake — see below [112,123,124].
- *Prm1* — a multispanning transmembrane protein that is upregulated by pheromone. The endpoint reached in *prm1Δ* and *fig1Δ* zygotes has the peculiarity that the plasma membrane abutting on the ZOC compartment intrudes ("bubbles") from one cell across the midpoint into the cytoplasm of the partner — possibly reflecting an osmotic differential. Zygote formation in *prm1Δ* crosses is further inhibited by deletion of the proteases Kex1 or Kex2 or by addition of the styryl dye, FM4-64, that remains in the outer leaflet of the lipid bilayer. Especially *prm1Δ* crosses are accompanied by significant cell lysis [62,83,91,123,125].
  Additionally, β-hydroxy sterols are critical for fusion. Thus, when certain atypical sterols are substituted for ergosterol – although cells can divide and form prezygotes – cell fusion is inhibited [126–129]. There is controversy as to whether it is meaningful to use filipin binding to report on the sterol content of distinct regions of membranes [12,65]. It is therefore not known whether the membrane surfaces that face the ZOC are rich in sterols.
  Experiments with ts secretion mutants show that ongoing exocytosis is required for zygote formation [125]. By contrast, although proteins implicated in endocytosis (Sla2, Rvs161, Rvs167) localize to the cell apex, endocytosis has been reported not to be required, as judged from studies of endocytic ts mutants [60,130,131].

Given the frequent importance of extracellular calcium for exocytosis, one might expect calcium influx to be required for local exocytosis at the ZOC and for cell fusion. Nevertheless – except in the absence of Prm1 – extracellular calcium is not required for zygote formation [123,132]. It is therefore of interest that Cch1, which concentrates at the apex, constitutes a high affinity calcium influx system in conjunction with Mid1. Fig1 contributes to low affinity calcium uptake upon treatment with pheromone [123,124,133].

Several further mutants implicate additional proteins in cell fusion. These include proteins that affect actin orientation (Spa2/Pea1 [50,109,134,135], and Kel1/2 [136]), the regulator of the cell integrity pathway, Lrg1 [137], and Ste6 [138].

Intervening cell wall persists in prezygotes generated from cells with activated Pkc1 [99,102], in *fus1Δ*, *fus2Δ* and *rvs161Δ* prezygotes and also in certain *cdc24* prezygotes. In other types of prezygotes (*prm1Δ*, *fig1Δ*

and *erg6Δ*) wall is absent [60]. As indicated in Fig. 6, the former group of mutants therefore appears to interrupt the progress of fusion at an earlier step than the latter group.

7. Karyogamy — Figs. 7/8

Apart from skeletal and cardiac muscles in vertebrates, and for various protozoa and fungi, it is unusual to have more than a single nucleus in a cell. Moreover, there are few instances of nuclear fusion, other than during fertilization in organisms for which the nuclear envelope remains intact, e.g. [139–144]. In *S. cerevisiae*, pheromone stimulation is a prerequisite for karyogamy, judging from studies of yeast dikaryons created without pheromone stimulation [145,146].

In prezygotes and in cells exposed to isotropic pheromone, the plus end of the microtubule cable that is anchored to the spindle pole body (SPB) extends toward the cell apex. Upon cell fusion, the microtubule cables extend toward each other, the nuclei congress and their SPBs contact each other. It has been known for decades that the point of contact is at the lateral "half-bridge" of each SPB [61]. Early evidence showed that microtubule function is required for congression [147,148]. Although cable shortening and lateral sliding had been thought to accomplish congression, investigations based on EM tomography show that congression results from the cables establishing lateral contact with the opposite SPB, where the minus-end directed motor, Kar3, exerts a pulling force [149]. It remains unclear whether the primary role of the cable is to bring the nuclei together or rather to cause the SPBs to contact each other. Once contact has been established, the two parental SPBs remain apposed to each other without immediately merging [150]. By contrast, in many organisms, the centrioles of one mating partner are lost during fertilization [151]. This could minimize any functional imbalance that might lead to aneuploidy.

When nuclear contact has been established, the order of fusion of the outer and inner membranes can be inferred by following the relative timing of flux of mobile fluorescent proteins that are characteristic of each membrane. Rather than this being a concerted reaction, the outer membrane fuses minutes before the inner membrane [150,152]. Interestingly, inner membrane fusion is not accompanied by immediate dilation of the point of internuclear contact. It is only several minutes later that this nexus widens to generate an oblong unified nucleus [150] — Fig. 8. This delay is reminiscent of the gradual opening of the fusion pore of secretory granules at the cell surface [153].

The delay between fusion of the outer and inner membrane suggests that some preparation for inner membrane fusion is set in motion after outer membrane fusion. Coordination between the two membranes could depend on the composite SPB that forms in early zygotes, in which the two parental SPBs lie adjacent to each other [150]. Since SUN and KASH proteins link the inner and outer nuclear membranes in other cell types, the yeast SUN protein, Mps3, that localizes to the SPB, could play a critical role [154].

Identification of molecular participants in karyogamy has depended on mutant isolation and on serendipitous observations [114–116,152,155–159]. The initial genetic selections were designed to make better beer by learning how to transfer mitochondria between strains. This effort yielded a mutant (*kar1-1*) that upon fusion with wildtype cells produced haploid progeny including a mitochondrial marker from the *trans* parent [13]. In a later genetic screen, strains were recovered that – after a change of mating type and self-fusion – did not yield diploids with normal efficiency, as judged by the low incidence of particular recombinants [115].

Karyogamy mutants are often classified as being unilateral or bilateral (meaning that one or both parents must carry the mutation) and according to whether congression itself is inhibited. In the case of at least some unilateral mutants, the proteins in question likely needs to be built into the SPB before cell fusion, thereby explaining why the presence of a wt copy cannot complement functional deficiency [160]. Most mutants that inhibit rapid congression of nuclei affect microtubule functions or the SPB (e.g. *bik1*, *cik1* [121,161]). *KAR4* encodes a transcription factor and *kar4* mutants also inhibit congression due to its being required to induce the kinesins, Cik1 and Kar3 [31,162]. Intriguingly, studies of the first of the congression mutants to be described, *kar1-1*, suggest that single parental nuclei can somehow disappear from the resulting dikaryons [163,164].

A minimal model for fusion invokes a positioning function of the SPBs followed by participation of fusion factors that also operate along the secretory and endocytic pathways. NE fusion itself depends on a surprisingly large number of snare proteins (Bos1, Sec20, Ufe1, Use1) – judging from investigation of crosses conducted at semi-permissive temperature – and the snare disassembly factor, Sec18 — judging from "2-step crosses" in which cells are first allowed to fuse while congression is reversibly inhibited [150,165]. Earlier studies have also provided evidence for participation of the ATPase, Cdc48 [166]. Considering the topology of membrane fusion, in which cytoplasmic leaflets of outer membranes establish contact first (Fig. 7), it seems plausible that snare involvement is for this initial step. Mutations in a group of ER/NE transmembrane proteins (Kar5/Fig3, Kar7/Sec71, Sec63, Sec72) also inhibit karyogamy. Their involvement could signify that their cytosolic domains must interact in trans after congression [167]. The well-documented importance of the lumenal Hsp70 family member, Kar2, along with the lumenal DnaJ family member, Kar8/Jem1, could reflect their association with the lumenal domains of the same group of transmembrane proteins. Deletion of the NE tail-anchored membrane protein, Prm3, also blocks fusion. This outer membrane protein somehow associates with Kar5 [168–171]. Fusion can be conveniently inhibited in the second step of 2-step crosses by addition of the reducing agent, DTT, likely due to sequestration of Kar2 and other folding factors within the ER lumen. Protein translocation into the ER itself is however not required [150,167,172]. Additional mutations also inhibit karyogamy. These include *cdc4* [173], *kar9* [169,170], *kem1* [174] and *nep98* [175].

Electron microscopic studies conclude that the arrest in *prm3Δ* precedes *kar2*, *kar5* and *kar8* arrest [176]. Nevertheless, there is no evidence that any of these proteins function in inner membrane fusion. In fact, little is known of inner membrane fusion. Given its topology, factors involved in budding from the ER could be required.

In wt crosses, contact of the two SPBs is quickly followed by an explosive nucleation of cytoplasmic microtubules that reach into both parental domains [148,177]. Possibly because the yeast genome remains tethered to the SPB during almost the entirety of the cell cycle [178], first studies of the fate of tagged genetic loci upon fusion suggest that the two parental contributions remain spatially separate from each

Fig. 7. Model of nuclear envelope fusion. The outer membrane (o.m.) fuses before the inner membrane (i.m.). We suggest that snare involvement is for outer membrane fusion. Proteins that function in ER budding could contribute to inner membrane fusion. Red circles: SPBs.

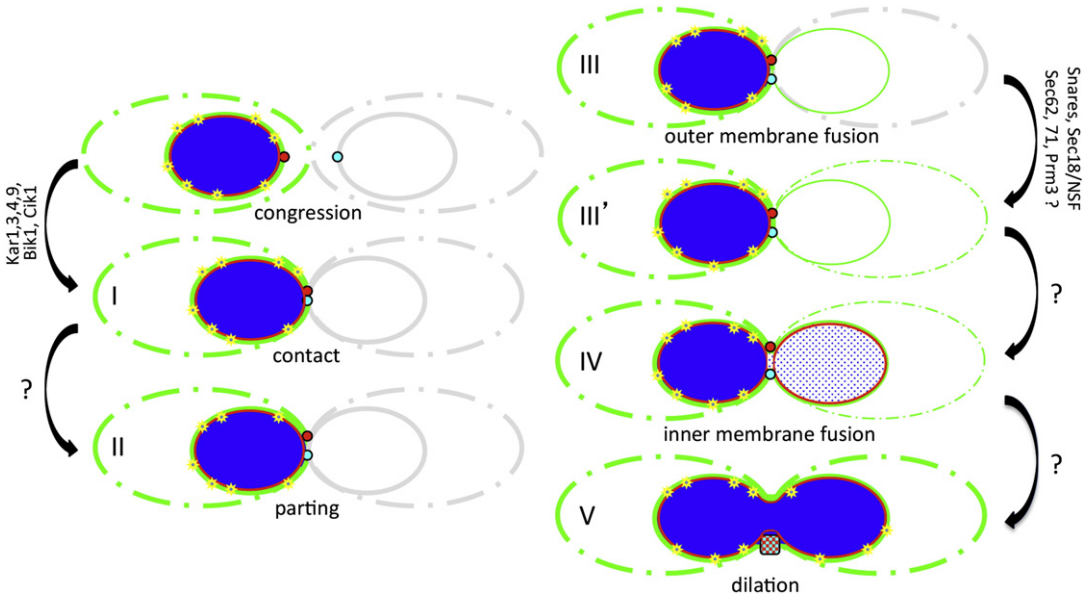

Fig. 8. Sequential events of nuclear fusion. The steps are (I) contact of the two parental SPBs (red, blue), (II) appearance of a discontinuity of the nuclear envelope — NE ("parting") adjacent to the SPBs, (III) initial transfer of tagged outer membrane proteins (green) — which is first detected in the *trans*-NE — followed (III′) by continued transfer and spreading of the *trans*-NE signal to the cortical ER, (IV) transfer of tagged inner membrane proteins (red) and the lamina equivalent, as well as initial nucleoplasmic continuity (stippling throughout the nucleoplasm), and (V) visible dilation of the point of contact, transfer of NPCs (yellow) and disengagement of the SPB (designated as a checkered square) from one face of the NE.
Modified from [150].

other prior to the initial diploid anaphase. The two contributing parental nucleoli generally remain separate during this period [150].

8. Mitochondrial unification – septin morphogenesis – parental zones

When early zygotes form terminal buds, mitochondrial genomes contributed by a single parent are preferentially inherited by early buds that emerge at the corresponding end [7,179–184]. These longstanding observations are surprising since soluble proteins exchange rapidly throughout early zygotes. It is therefore of interest that an annular partition occupies the midzone of early zygotes, separating the flanking zones of distinct parental origin—Fig. 9. The annulus includes at least four septins and Myo1 and appears, as for the bud neck septin annulus during the mitotic cell cycle, to serve a "fence" function [57,64]. Thus, when the visibility of the annulus diminishes, coincident with relocalization of septins to form an hourglass at the neck of the emerging bud, parental mitochondria fuse together. Reorganization of actin filaments that extend from bud neck formins into the body of the zygote appears to be required, judging from the impact of latrunculin on fusion [57]. ER discontinuity between parental domains is also conspicuous at the midzone of the early zygote [150].

When parental mitochondria do fuse, their nucleoids intermix much later than their matrix proteins, presumably because of their large size and linkage to the mitochondrial membranes [179,185,186]. Moreover– by contrast to the sequential fusion of outer and inner membranes of the NE – the timing of interchange of markers of the outer mitochondrial membrane coincides with confluence of matrix markers [57]. The ultimate fusion of mitochondria in zygotes is presumably accomplished by the same mechanisms that have been characterized in mitotic cells [185,187].

In parallel with the delay of encounter of parental mitochondria, *cis–trans* diffusion of polysomes and the [*PSI* +] form of Sup35 prion are also restricted in the middle of the zygote [57]. If indeed the medial impasse discriminates according to size, one therefore can conclude that its mesh size is much finer than the size of mitochondria themselves. These considerations provide a point of reference for understanding the transmission of supramolecular complexes during the mitotic cell cycle [188–193].

For unknown reasons, although both parental mitochondrial genomes are initially present in zygotes, the zygotes and any heteroplasmic diploid progeny become homoplasmic within a few generations [8,194–196]. Since dozens of nucleoids are present in single haploid cells, this observation might signify that there is only a small

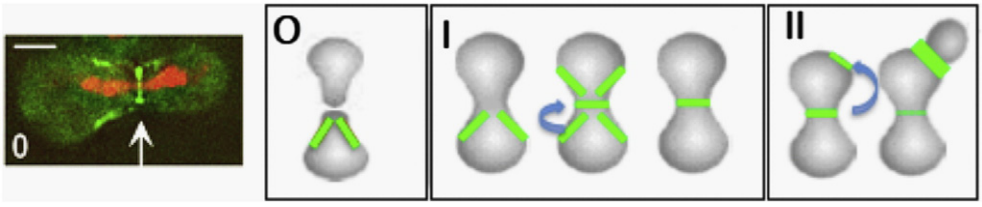

Fig. 9. Steps of septin morphogenesis. Left: An early zygote expressing the septin, Cdc3, as a GFP fusion, and a red fluorescent marker of the nucleoplasm (Htb2-mRFP). The nuclei have fused and extend across the midzone. The green septin annulus encircles the middle of the zygote (arrow). Right: Stages of septin morphogenesis. 0 represents the distribution of a tagged septin prior to cell fusion. I indicates its redistribution to the *trans* domain upon cell fusion and the appearance of the annulus. With time, a patch of septins appears at the site of imminent bud emergence (II). As it matures into an hourglass at the bud neck, the annulus vanishes.
Modified from [57].

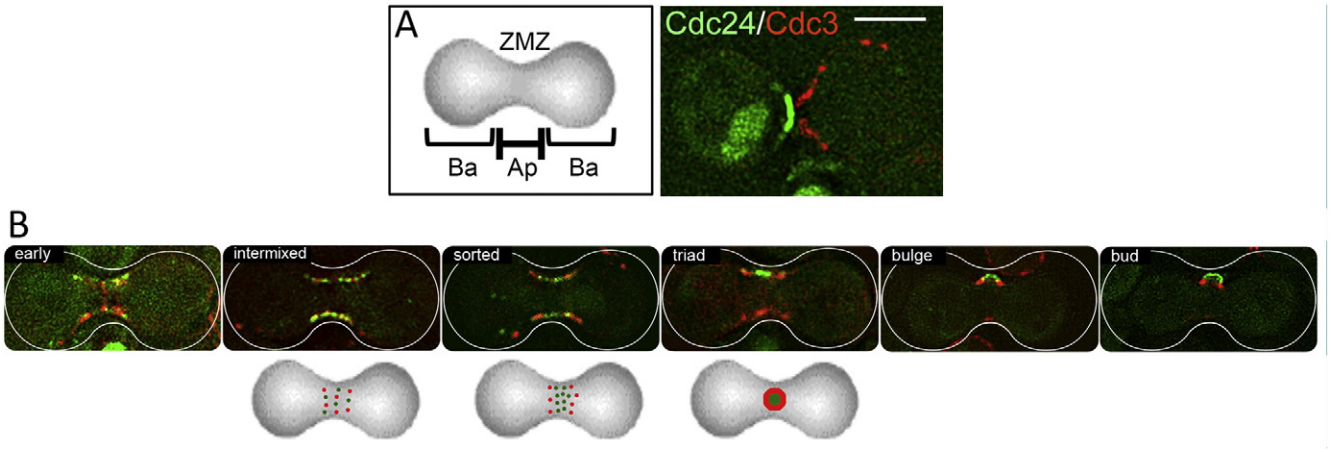

Fig. 10. Stages of growth of an initial medial bud. (A) In the uppermost row, the diagram at the left indicates the retention of domain identity that is seen after cell fusion. Early zygotes have one shared "apical" domain (Ap) and a pair of lateral "basal" domains (Ba). The midzone of the zygote is indicated as ZMZ. Adjacent to this model is an image of a prezygote in which one parental cell expresses Cdc24-GFP and the other expresses the tagged septin, Cdc3-mCherry. Note the apical concentration of Cdc24-GFP and the cortical septins that avoid the cell apex. A nuclear pool of Cdc24 is also seen. (B) The six images in the next row illustrate the progressive redistribution of the tagged proteins that occurs after cell fusion. During and after formation of the medial annulus (first image), both proteins are detected as cortical foci, scattered throughout the midzone. With time, they reorganize to generate a cortical triad in which the septin foci flank foci of Cdc24-GFP. As the septin foci coalesce, they encircle Cdc24-GFP. The cortex subsequently bulges and then becomes increasingly enlarged distal to the septin belt, during bud growth. The reorganization of the foci is diagrammed in the lower set of panels.

number of binding sites (for nucleoids ?) in buds. Alternatively, if frequently-occurring variant mitochondrial genomes confer a metabolic advantage upon their host or replicate at an especially high rate, homoplasmy could result from selection.

9. Bud emergence/bud site specification — Figs. 10/11

When the titer of Far1 is reduced due to formation of the a1–α2 transcriptional inhibitor in early zygotes, the cell cycle can resume. Zygotes then produce medial, lateral or terminal buds. The causes of this spatial variability represent a long-standing puzzle. In agreement with earlier suggestions, recent studies show that most initial buds are medial [3,4,11,197,198]. This positioning is reminiscent of mammalian fertilization in which the arbitrary site of sperm entry into the oocyte guides the polarity of initial cleavage [199]. The medial preference in yeast could simply be the direct result of the convergence of actin guidance proteins at the ZOC prior to fusion and their persistence at the zygote midzone. Indeed, when cell–cell fusion occurs, cortical markers retain their relative distributions between apical and basal domains. For example, a number of proteins that had been at the apex before fusion, e.g. Cdc24, Bni1 and Sec5, remain in the midzone of the zygote, while proteins that had a basal distribution in pre-zygotes remain toward the extremities of the parental zones. Since a collar of septins no longer separates these extremities from the zygote midzone, it is unclear why intermixing does not occur. Within the midzone, apical proteins scatter to form multiple foci that then intermix with septin foci of comparable size. As a preliminary to assembly of the initial bud, the foci then sort out along the cortex so that the septins take up lateral positions, whereupon they form a belt that encircles the nascent bud. Thus, many proteins that became polarized to the cell apex upon exposure to pheromone ultimately end up lining the cortex of initial zygotic buds — Fig. 10 [11].

In the "axial" budding that is usually characteristic of haploid cells, buds initiate adjacent to cortical landmark proteins and successive buds are contiguous to each other. The history of budding is therefore stably marked by a chain of scars in which each scar is contiguous to the scar at the site of the preceding and following buds. By contrast, diploid cells usually exhibit "bipolar" budding, with each bud initiating at a patch of distinct landmark proteins, and successive bud scars clustering without contacting each other or defining linear patterns. Daughter cells first bud at the pole that is distal to the mother and then alternate between poles, while the mother cell rebuds at either pole. Regardless of the budding pattern, the cortical landmarks orient actin filaments. The GTPases, Bud1/Rsr1 and Cdc42 (and their GEFs, Bud5 and Cdc24) are essential intermediaries for this guidance, as is the formin, Bni1. Orientation of actin toward landmarks results in polarized transport of secretory vesicles containing new surface proteins and cell wall components. Deletion of either GTPase randomizes successive budding in cycling cells [40,200–207].

Despite the apparently distinct roles of the two groups of landmarks, seven of the classical landmarks that guide axial and bipolar budding are present in both haploid and diploid cells. The exception is the transmembrane protein, Axl1, that is absent from diploid cells, due to repression of its transcription by the a1–α2 heterodimeric transcriptional inhibitor. Expression of Axl1 in diploid cells allows these cells to bud axially [20]. Along with the other axial landmark proteins, Axl1 therefore appears to be required for contiguous bud site specification.

Quite unlike bud site specification in haploid and diploid cells, the Bud1/Rsr1 GTPase is not required for positioning of the initial medial zygotic bud. Moreover, at least three "axial" landmarks and three "bipolar" landmarks also are not required. This is unprecedented [11] — Fig. 3.

After formation of the initial bud, the next zygotic buds are generally contiguous to the initial medial bud and – as in haploid cells – this contiguity requires Bud1 and each of the axial landmarks. Subsequent buds are progressively less often contiguous to previous buds and become enriched at terminal sites, in part due to bipolar landmarks. As expected from studies of diploid bud site selection, deliberate expression of Axl1 through this period markedly increases the extent and duration of contiguous budding [11] — Fig. 11.

Although there are three phases of zygotic bud site specification (medial, contiguous, further dispersed), adherence to this order is imprecise, reminiscent of the extensive but imperfect conformity of haploid and diploid bud site selection to the axial and bipolar norms described above. Cell/developmental morphogenesis could benefit from such variability.

10. Concluding thoughts

Zygotes are also of interest in other contexts:

- *Speciation*: Inter-species incompatibility can have either pre-zygotic or post-zygotic causes. In the former case, zygotes do not form. One intriguing example of such incompatibility depends

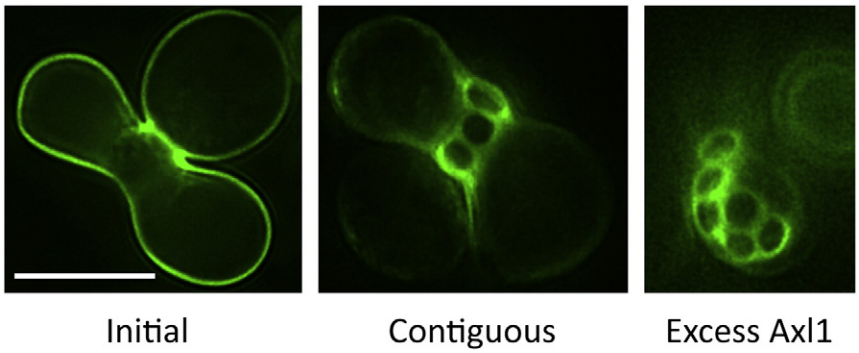

Fig. 11. Zygotes and bud scars stained with Calcofluor White [11]. Left: Zygote with an initial medial bud. Middle: An encircling necklace of contiguous scars. Right: Exaggerated contiguity of scars as a result of excess Axl1. Note that one scar is at the middle, that a chain of scars emanates from this point, and that the scars contact each other. One parental lobe of the zygote is illustrated.

on prion variants that are characteristic of fungi that exhibit "heterokaryon incompatibility" [208]. In post-zygotic incompatibility, sporulation cannot occur [209–211]. Some such incompatibilities can result from the need for an accurate match between mitochondrial and nuclear genomes, as detected in studies of chromosome substitution strains [212]. Since even single mutations can have widespread secondary genetic consequences, the interspecies barriers could be far-removed from the phenotypes that are directly caused by single known mutations [213].

- *Fertilization*: Practical dividends of investigations of yeast zygotes surely will materialize in relation to fertilization. In addition to the need for precise molecular compatibility between partners, the coordination of timing between two contributing cell cycles and coordination of their preparedness for unification seem likely to be critical for zygote formation in all organisms. Several further transkingdom themes have indeed already emerged. For example, in the green alga, *Chlamydomonas*, key membrane proteins localize to the sites of contact of gametes of distinct mating types, with Fus1 being required for gamete adhesion and Hap2 functioning in plasma membrane fusion. At least Hap2 is related to proteins implicated in fertilization in *Arabidopsis* and in *Plasmodium* [214]. In *Chlamydomonas*, both proteins are quickly degraded after cell fusion [214,215]. In mice, the surface proteins Izumo and Juno interact directly and are required for fertilization [216]. There are also likely to be meaningful parallels between the roles of the yeast cell wall and the extracellular coats that surround gametes in other organisms [217–220]. Although the intervening wall at the ZOC compartment is normally thought of as a barrier, this wall, or products derived from it, might also play a positive role in promoting cell fusion.
- *Functional complementation*: Zygote formation provides the opportunity to investigate functional complementation on a rapid time scale, when distinct genomes and cytoplasms are unified. Indeed, many studies of functional complementation and inter-organellar relations – as well as identification of mutations that affect zygote formation – have made use of *Chlamydomonas* [221,222]. Related powerful strategies could be based on experimental use the a1–α2 heterodimeric transcriptional inhibitor to investigate the consequences of sudden elimination of single RNAs at the moment of cell fusion. Equivalent protocols could be based on strains that allow sudden induction of selected transcripts at the moment of fusion. Rapid complementation studies might also be performed with zygotes of *S. pombe*. Nevertheless, unusual conditions would be needed since *S. pombe* zygotes are short-lived and diploids are normally unstable [48,223,224].

The sequence of dependent relationships that account for zygote formation provides an elegant prototype for subdividing and analyzing this classical developmental itinerary.

Conflict of interest

I am not aware that there is any conflict of interest.

Acknowledgements

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