PERSPECTIVE

What better measure than ribosome synthesis?

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Cells grow; cells divide. Some cells grow without dividing: neurons, or oocytes. Some cells divide without growing: developing zygotes. For most cells, however, growth and division are coupled, thereby maintaining cell size within narrow limits. The basis of this coupling has been elusive.

Cell growth was the subject of some of the earliest molecular biological investigations when it was shown that, on a variety of media, the ribosome content of *Escherichia coli* was proportional to the growth rate (Maaloe and Kjeldgaard 1966). Soon thereafter, the study of growth was overshadowed by the study of gene expression with its emphasis on the regulated transcription of individual genes.

More recently, the recognition that growth and cell division are so closely intertwined has led to new interest in the study of growth per se, as suggested by the recent publication of two reviews (Kozma and Thomas 2002; Saucedo and Edgar 2002) and a monograph, *Cell Growth: Control of Cell Size* (Hall et al. 2004). In considering cell growth and division, two points are paramount: (1) Cell growth requires the synthesis of proteins; the synthesis of proteins requires ribosomes. Thus, ultimately the control of cell growth must involve the control of ribosome synthesis. (2) A cell must somehow match the replication of its genome, with the implicit decision to divide, to its size, or perhaps to its predicted size at the time division will occur.

These two aspects have often been considered in parallel, the studies of macromolecular synthesis, transcription, and translation proceeding with little care for their implications for cell division, and the studies of the intricate dance of cyclins that powers the cell cycle relatively unconcerned with the macromolecular events within the cell that ultimately drive the decision to divide. Those involved in the cell cycle, however, have been more attentive, if no more successful at understanding the mechanisms controlling the relationship between growth and division. Thus, the dean of the cell cycle field recently wrote, "The existence of a 'size con-

trol' is well known ... but it has been remarkably resistant to molecular analysis. ... It is a vital link coordinating cell growth with periodic events of the cycle." (Mitchison 2003).

Genetic approaches to cell size control

It has long been known for *Saccharomyces cerevisiae* that cell size is proportional to ploidy, and also depends on nutritional state. Cells growing slowly in poor medium are small; shifting cells from rich to poor medium decreases the critical size, and vice versa, an effect that is mediated in part by the Ras/PKA pathway (for review, see Jorgensen et al. 2004b). But what are the signals that designate size?

An early insight into this problem derived from the analysis by Hartwell and coworkers (Johnston et al. 1977) of the growth of *S. cerevisiae* cells blocked in the division cycle, and, conversely, the cycling of cells inhibited in growth. They concluded that growth did not depend on a functioning cycle, but that some event in G1 "...cannot be completed until a critical size is attained." In short, "growth, rather than progress through the DNA-division cycle, is normally limiting for cell proliferation." The identification of *wee* mutants in *Schizosaccharomyces pombe* (Thuriaux et al. 1978) and *whi* mutants in *S. cerevisiae* (Carter and Sudbery 1980; Nash et al. 1988), with significantly smaller cells than wild type, provided a genetic approach to the problem. In *S. cerevisiae*, in which the variable part of the cell cycle is G1, such mutants were considered to be making a premature decision to replicate their DNA, and thus to "Start": to enter the next cell cycle. It is noteworthy that many such mutants had the same doubling time as wild-type cells, demonstrating that cell size is not necessarily connected to cell growth rate.

Basically similar relationships have been reported for *Drosophila*. Genetic manipulation to increase or decrease the rate of cell cycling alters the cell number but not the size of the imaginal disc involved (Neufeld and Edgar 1998). Similarly, deletion of the S6 kinase, a potential regulator of ribosome synthesis, leads to slowly developing, small flies with small but not fewer cells. (Montagne et al. 1999).

Yet the molecular basis of size control has remained

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obscure. A substantial contribution to this problem is reported in this issue of *Genes & Development*, where Tyers and coworkers have followed up their earlier genome-wide screen for mutants affecting cell size (Jorgensen et al. 2002) with experiments that lead them to conclude that the coupling between cell growth and cell division is mediated by two genes involved in the regulation of the rate of ribosome biosynthesis (Jorgensen et al. 2004a). In short, "...nutrient control of the critical cell size threshold ... is communicated to the Start machinery via proximal events in ribosome biosynthesis, rather than by downstream changes in protein synthetic rate."

Cell size

Briefly, the key player in cell size homeostasis in *S. cerevisiae* appears to be a G1 cyclin, Cln3 (Cross 1988), that functions to coordinate Start with cell growth and with the supply of nutrients. A critical concentration of Cln3 in the nucleus results in the activation of the Cln3-Cdc28 kinase. This kinase phosphorylates and releases Whi5, a negative regulator and interacting partner of the SBF/MBF transcription factor complexes (Costanzo et al. 2004; de Bruin et al. 2004). The dissociation of Whi5 activates the SBF/MBF transcriptional machinery and leads to the activation of a set of some ~120 genes including two other G1 cyclins, *CLN1* and *CLN2*, and the B-type cyclins *CLB5* and *CLB6*. The Clb5 and Clb6 proteins thus formed are complexed with the negative regulator Sic1. The phosphorylation and subsequent degradation of Sic1 by the Cln1 and Cln2-Cdc28 complexes, respectively, activates Clb5/6-Cdc28 complexes to trigger "Start" and thus initiate DNA replication (for review, see Rupes 2002). Overexpression of Cln3 leads to an accelerated Start and a decrease in cell size, but with little effect on the doubling time. Conversely, deletion of *CLN3* leads to a delayed start, and thus a considerable increase in cell size.

How then does the cell make the decision that sufficient Cln3 has accumulated to initiate S? There have been invocations of cytoplasmic volume, ratio of cytoplasmic to nuclear volume, rate of translation, activity of translation initiation factors, and many others (for re-

view, see Rupes 2002). An intriguing observation in *S. cerevisiae* is that the 5'-leader of the *CLN3* transcript has a small open reading frame (ORF), analogous to that of *GCN4*, suggesting that regulation of translation is at work. Indeed, disruption of the upstream ORF, presumably permitting far more efficient translation of Cln3, also leads to small cells (Polymenis and Schmidt 1997).

Ribosome biosynthesis

If cell size depends on some aspect of ribosome synthesis, it is important to consider what features distinguish the control of ribosome synthesis from most other control pathways.

(1) Ribosome synthesis requires the coordinated activities of all three RNA polymerases: Pol I for rRNA, Pol II for the ribosomal protein (RP) genes, and Pol III for 5S RNA.

(2) Quantity matters. The cell needs precisely equimolar amounts of rRNA and each of the 79 RPs. Excess rRNA will not be properly assembled into a ribosome with insufficient RPs. Insufficiency of any one of the 79 RPs will, in most cases, lead to aberrant processing and insufficient ribosomal subunits.

(3) The processing of rRNA and the assembly of ribosomes requires an army of nearly 200 proteins (Fatica and Tollervey 2002), whose synthesis is regulated in tandem with, but not identically to, that of the RPs (the *ribi* regulon) (Gasch et al. 2000; Jorgensen et al. 2004a).

(4) Ribosome synthesis is a massive consumer within the economy of the yeast cell, where rRNA transcription represents ~60% of total transcription and RP mRNA transcription represents ~50% of the total Pol II transcription initiation events (Warner 1999). Thus, up- or down-regulation of ribosome synthesis can have a substantial ripple effect on the expression of other genes.

(5) Perhaps reflecting its central role in the regulation of growth, ribosome synthesis requires positive inputs from several sources, and can be repressed by negative inputs from several sources (Fig. 1).

New factors in the control of cell size

In their previous screen of the yeast gene deletion set for mutants affecting size, Jorgensen et al. (2002) identified

Figure 1. Regulation of ribosome biosynthesis in *S. cerevisiae*. Ribosome biosynthesis requires several elements working in tandem: an active TOR pathway; an active PKA pathway; sufficient amino acids; and the factors Rap1, Fhl1, Ifh1, Sfp1, Sch9, Rrn3 (for rRNA), and perhaps others (see text for details). Repression of ribosome synthesis can come through inactivation of the TOR pathway, reduction of PKA activity, signaling via PKC, deprivation of an amino acid, stress of many kinds including heat shock, free radicals, radiation, and so on (for review, see Jorgensen et al. 2004b).

several small (*whi*) cell phenotypes with deletions in potential repressors of Start. The strongest *whi* mutants resulted from the deletion of either of two nonessential genes, *SFP1* or *SCH9*. Sfp1 is a zinc-finger protein that has been implicated in ribosome biogenesis (Fingerman et al. 2003). Deletion of *SFP1* leads not only to exceedingly small cells, but also to slow growth and to reduced levels of mRNA both for the RP genes and for the *ribi* regulon (Jorgensen et al. 2002). Expression of Sfp1 from the Gal promoter in such cells resulted in the prompt induction of many genes, initially those of the *ribi* regulon, and somewhat later the RP genes. Thus Sfp1 is implicated in the regulation of ribosome synthesis at two levels, because the *ribi* regulon is mediated by conserved promoter elements called RRPE and PAC (Wade et al. 2001), whereas the RP regulon is regulated by Rap1p-binding sites (and others, see below).

SCH9 encodes a putative protein kinase, whose C-terminal kinase domain is 49% identical to that of human protein kinase B (PKB). Intriguingly, in both *Drosophila* and mouse, PKB is involved in cell size control by regulating the rate at which ribosomal components are produced (for reviews, see Kozma and Thomas 2002; Saucedo and Edgar 2002). In *S. cerevisiae*, however, Sch9 appears to be related to the ras/PKA pathway (Toda et al. 1988), which has been shown to be a key regulator of ribosome biosynthesis (Neuman-Silberberg et al. 1995). Should Sch9 be considered a PKA or a PKB? Thus, Sfp1 and Sch9 are strong candidates for the elusive proteins that couple ribosome synthesis with cell size. Indeed, overexpression of either Sfp1 or Sch9 leads to large cells. In contrast, in the absence of Sfp1 and Sch9, SBF- and MBF-dependent transcription, as well as G1-to-S transition, are accelerated after synchronous release of G1-phase daughter cells obtained by centrifugal elutriation. These results further confirm the negative regulatory effect of Sfp1 and Sch9 on Start, and suggest that they act upstream to the G1/S transcriptional network. By the criterion of cell size, deletion of *SFP1* is not fully epistatic to mutants that basically inactivate the Cln3 regulatory elements of cell cycle control (Jorgensen et al. 2004a). A reasonable conclusion is that Sfp1 or Sch9 influences cell size by sensing nutrient availability and matching that with the rate of ribosome synthesis. It remains to be seen whether ribosome synthesis itself is a negative regulator of Start, or whether these two proteins have dual functions.

These proteins have an interesting geographical response to stress, either from inactivation of the TOR pathway or from nutritional signals through the PKA pathway. Both Sch9, usually found largely at the periphery of the vacuole, and Sfp1, usually confined to the nucleus, disperse throughout the cell. An independent genomic screen has also identified Sfp1 as being unusually responsive to stress in this way, and has provided evidence suggesting that Sfp1 is a direct regulator of RP gene expression (Marion et al. 2004). Indeed, they find that Sfp1 is present at RP genes, by ChIP analysis, but only under conditions in which transcription is occurring, although others report that enrichment of Sfp1 at

RP genes or *ribi* genes is barely detectable (Lee et al. 2002; Fingerman et al. 2003; Jorgensen et al. 2004a). Furthermore, the absence of Sfp1 clearly reduces the residence at RP genes of two transcription factors, Fhl1 and Ifh1 (Jorgensen et al. 2004a; Marion et al. 2004), that are implicated in RP gene transcription (Hermann-Le et al. 1994; Cherel and Thuriaux 1995; S. Schawalter and D. Shore, pers. comm.; D. Rudra, Y. Zhao, and J.R. Warner, in prep.). It remains to be seen how four proteins—Rap1, Fhl1, Ifh1, and Sfp1—all interact at the same site on each of the RP genes! A most interesting observation is that whenever RP transcription is repressed, and Sfp1 is dispersed from the nucleus, both Fhl1 and Ifh1 appear to concentrate in the nucleolus (Jorgensen et al. 2004a). This could be an effective way for the cell to maintain these scarce proteins readily available for use once RP transcription resumes.

Sfp1 and Sch9 appear to operate through different pathways. There is lack of genetic epistasis. Deletion of both, or simultaneously reducing their activity in a dose-dependent manner, leads to synthetic lethality. Unlike $\Delta SFP1$, $\Delta SCH9$ does not affect the nuclear localization of Fhl1 or Ifh1. Yet, ultimately these pathways must converge on ribosome biosynthesis and on cell size determination; it seems unlikely that they have no influence on each other.

It is quite remarkable that two groups have identified Sfp1p as a key regulator using quite different criteria, small cell size (Jorgensen et al. 2002) or movement from nucleus to cytoplasm in response to stress (Marion et al. 2004). What remains to be seen is the role actually played by Sfp1p. Deletion of *SFP1* leads to small cells that have fewer ribosomes and make protein at a slower rate (Fingerman et al. 2003). But that is true of many conditions of slow growth. Another issue is the directness of the effect. Recovery from depletion of Sfp1 initiates with increased transcription of the *ribi* regulon, the genes responsible for ribosome processing/assembly, with the RP genes lagging significantly behind (Jorgensen et al. 2004a). Results of over- or underexpression of Sfp1p on transcription of reporter constructs carrying the signature RRPE and PAC elements of the *ribi* regulon confirm a relatively direct effect (Fingerman et al. 2003). Does that mean that the Sfp1 effect on transcription of the *ribi* regulon is primary, with the effects on RP transcription being a secondary response to that? A very recent report finds that cells deprived of phosphorous or uracil specifically down-regulate the RP but not the *ribi* regulon (Saldanha et al. 2004). Where will we find Sfp1 under these conditions?

The notion of Sfp1, and to a lesser extent Sch9, linking cell size determination with the regulation of ribosome biosynthesis is intriguing, as far as it goes. Indeed, for a cell undertaking the perilous steps of replicating its genome and subsequently dividing into two daughter cells, what better measure of its own health than vigorous and efficient ribosome synthesis? Yet, in the case of Sfp1, we see the opposite story: poor and inefficient ribosome synthesis is coupled to premature passage through Start. In contrast, other mutations that slow ribosome biosynthe-

sis and slow growth, for example, deletions of a RP gene or of the RP gene transcription factor Fhl1p, have little effect on cell size (Jorgensen et al. 2004a).

This suggests that Sfp1 is part of a more subtle strategy: under positive growth conditions, the cell has an interest in vigorous ribosome biosynthesis to enable rapid growth and at the same time a delay in cell division to permit the cell to grow to an optimal size. One can postulate that (in response to signals from Sch9?) Sfp1 does both, through interacting with the RP (and rRNA?) genes and through delaying Start, by means we do not yet know. Then when conditions deteriorate, in response to stress, or as cells approach late log phase, Sfp1 retreats from the nucleus, and ribosome synthesis ceases in order to provide more resources for the cell to respond to sub-optimal conditions and to approach stationary phase (Ju and Warner 1994; Gray et al. 2004).

Interesting results raise interesting questions

Sfp1 is a putative transcription factor. How does it activate both the *ribi* and the RP regulons, which have different regulatory sequences? Does Sfp1 itself have transcriptional activity, or does it facilitate the activity of other factors, such as Fhl1 and Ifh1? How is the relocation of Sfp1 brought about? Is it the cause or the consequence of the stress-related repression of RP gene transcription?

Sch9 is a putative kinase. What are its substrates? Is Sfp1 a substrate? What does the activity of Sch9 depend on? How is it regulated by stress? What is the implication of Sch9 being localized at the vacuolar surface? Although overexpression of Sch9 partially suppresses mutations in the *ras*/PKA pathway (Toda et al. 1988), their pathways seem to be parallel rather than redundant (Dennis and Audino 1991). What is their relationship? The deletion of *SCH9* leads to extended longevity of cells in minimal medium (Fabrizio et al. 2001). Is this related to their reduced investment in ribosome biosynthesis?

The arguments developed above are derived from data about the levels of mRNA for the *ribi* regulon and the RP regulon. However, one must also consider the role of the regulation of rRNA transcription by RNA polymerase I (for review, see Grummt 2003; Moss 2004). Indeed, we do not know if the regulation of rRNA and RP synthesis occurs in parallel or if one is the primary target and, in turn, regulates the other. It is clear, however, that in almost all physiological conditions, the two are closely coupled. Regulation of transcription of Pol I in *S. cerevisiae* is largely through the transcription factor Rn3p (Peyroche et al. 2000; Claypool et al. 2004). Do Sfp1 and Sch9 play a role in the regulation of Rn3p and/or rRNA transcription? What role does rRNA transcription play in cell size determination?

A number of proteins involved in ribosome synthesis and assembly appear to be associated with the replication apparatus. The ORC complex interacts with several, including Nog1, Nop5, and Nop7 (Du and Stillman 2002). Noc3, which is required for rRNA processing, serves as a key connection between the ORC and other

replication initiation proteins (Zhang et al. 2002). Does the vigorous ribosome synthesis of rapidly growing cells starve the replication apparatus of these essential proteins? On the other hand, one might expect that if the absence of Sfp1 leads to a very low level of synthesis of these *ribi* proteins, replication would be delayed, leading to larger cells. Paradoxes abound.

Growth control and ribosome synthesis in metazoa

Do these observations coupling ribosome biosynthesis to cell size and the cell cycle have implications for higher organisms? Indeed, there has been an increasing appreciation of the role of ribosome biosynthesis in cell growth and its regulation, cell cycle control, and apoptosis in higher organisms. Perhaps the most telling experiment was carried out by Thomas and coworkers (Volarevic et al. 2000). They generated a mouse in which selective induction of *cre* led to the deletion of both genes for ribosomal protein S6 specifically in the liver. In spite of the lack of 40S ribosome synthesis in their livers, 60S ribosome synthesis continued and the mice survived for several weeks. Their livers could respond to fasting and refeeding cycles, in which the liver changes in mass nearly twofold. However, whereas partial hepatectomy in normal animals leads to rapid regrowth and cell division, the livers of mice defective in the production of 40S ribosomal subunits did not regrow and showed no signs of cell division. Thus, in this case functional ribosome synthesis appears to be essential for initiation of the cell division cycle.

Ribosome synthesis and cancer/apoptosis

It has been known for some time that key regulatory molecules of the cell influenced and were influenced by ribosome synthesis. Thus, the interaction of Rb with UBF, a key factor in the transcription of rRNA, was shown to down-regulate the transcription by RNA polymerase I (Cavanaugh et al. 1995). MDM2, a key regulator of p53 turnover in mouse cells, is bound by ribosomal protein L5 (Marechal et al. 1994).

Yet only recently have specific observations linked ribosome synthesis or ribosomal components to apoptosis or to cancer. Bop1 is a mouse protein that is essential for rRNA processing. A dominant-negative allele of Bop1p leads to a strong cell cycle arrest at the G1/S transition, dependent on p53 (Pestov et al. 2001). Ribosomal protein L11 has an affinity for the HDM2 protein (the human version of MDM2), whose usual function is to facilitate the degradation of p53. When overexpressed, or when it has no rRNA to bind to, L11 binds HDM2, leading to accumulation of p53, and the cells enter the apoptotic pathway (Lohrum et al. 2003; Jin et al. 2004). In this way, the cells can respond to aberrant nucleolar activity by undergoing apoptosis. Indeed, a recent analysis of damage to the nucleolus has led to the suggestion that “a p53 response, rather than being induced, has to be constantly prevented by a fully functioning nucleolus” (Rubbi and

Milner 2003). Strikingly, the nucleolar protein p19^{Arf}, another partner that binds MDM2 to initiate apoptosis, interacts also with the nucleolar ribosome assembly factor B23 and seems to inhibit rRNA processing (Sugimoto et al. 2003; Bertwistle et al. 2004).

Surprisingly, in the zebrafish haploinsufficiency for any of several ribosomal proteins led to unusual malignant tumors of the peripheral nerve sheath (Amsterdam et al. 2004). This is a distinctly different situation from that of *Drosophila*, in which haploinsufficiency of RP genes generally leads to “minutes,” relatively normal flies with minute bristles (Lambertsson 1998), although haploinsufficiency for S6 can lead to hematopoietic overgrowth (Watson et al. 1992; Stewart and Denell 1993).

Aside from the work on S6 described above, there is a surprising dearth of reports either of natural conditions of RP haploinsufficiency in the human population, or of the effects of the deletion of RP genes in mice. The single classical case is that of Diamond-Blackfan anemia, in which a variety of mutations in the gene for ribosomal protein S19 have been identified in 25% of the cases (Draptchinskaia et al. 1999). These mutations lead to an anemia with variable penetrance and to an increased incidence of leukemia. A recent report characterizes the spontaneous, semidominant, homozygous lethal *Bst* mutation in mouse as a deletion within the gene encoding ribosomal protein L24. The resulting phenotype includes a white ventral midline spot, a kinked tail, and other skeletal abnormalities, hardly what one might predict from the mutation of such a critical gene (Oliver et al. 2004). Although there is a clear inhibition of rRNA processing in mutant cells, there is little evidence of apoptosis, as one might expect based on the L11 results discussed above. It should be noted, however, that in yeast, L24 is one of the few RPs that is not essential for growth (Baronas Lowell and Warner 1990).

Is the lack of RP haploinsufficiency in mammals a case of “the dog that didn’t bark”? Perhaps the haploinsufficiency of an RP leads to such serious defects that a mammalian organism is inviable, either because of fundamental defects in development, or because the cells are programmed to apoptose if ribosome biosynthesis becomes too aberrant.

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