Regulation of ribosomal DNA amplification by the TOR pathway

Carmen V. Jack[a,1], Cristina Cruz[a,1], Ryan M. Hull[a], Markus A. Keller[b], Markus Ralser[b,c], and Jonathan Houseley[a,2]

[a]Epigenetics Programme, The Babraham Institute, Cambridge CB22 3AT, United Kingdom; [b]Cambridge Systems Biology Centre and Department of

conditions, so rapid rDNA amplifications cannot be explained by such a mechanism (10, 22). This implies the existence of a mechanism that can monitor rDNA copy number and instigate

intermediates in the 18S rRNA synthesis pathway. As expected, based on growth rates, rapamycin treatment decreased ribosome synthesis, but nicotinamide had no effect on 35S levels in the presence or absence of rapamycin, nor did it lead to any change in the pattern of ribosome synthesis intermediates that would indicate increased transcription (SI Appendix, Fig. S2C). This shows that the repression of rDNA amplification by rapamycin does not stem from reduced RNA pol I transcription.

These experiments demonstrate that the repression of rDNA amplification by rapamycin cannot be attributed to defects in growth or RNA pol I transcription and is instead mediated through a nicotinamide-sensitive pathway.

Rapamycin Acts Through Sir2 but Not Hst3 and Hst4 HDACs. Nicotinamide inhibits Sir2 (30) as well as the Sir2 homologs Hst3 and Hst4 (31), all of which affect rDNA recombination rate (11, 21). To determine which of these HDACs controls rDNA amplification, we tested whether rapamycin represses rDNA amplification in $rDNA_{35}$ cells lacking Hst3 and Hst4, which have a largely degenerate activity, or lacking Sir2.

As before, rDNA amplification was strongly repressed in $rDNA_{35}$ cells treated with rapamycin (Fig. 2, lanes 1–7 and upper distribution plot). However, significant rDNA amplification occurred in $rDNA_{35}$ sir2Δ cells in the presence of rapamycin, although this was very limited compared with nontreated cells (Fig. 2, lanes 8–14, middle distribution plot and histogram). Therefore, rapamycin acts partially but not completely through Sir2 to repress rDNA amplification. In $rDNA_{35}$ hst3Δ hst4Δ cells, the ability of rapamycin to block amplification was also compromised, and more heterogeneous rDNA expansions were observed, in-

SI Appendix, Fig. S5B). This demonstrates that rDNA amplification in normal cells can occur in the absence of Rad52.

This does not, however, demonstrate that the non-HR-dependent pathway is active when Rad52 is present; to assess the contribution of the non-HR-dependent pathway in cells expressing Rad52, we deleted the replicative kinase Dun1. Loss of Dun1 has little effect on Rad52-dependent HR but completely inhibits the non-HR-dependent amplification pathway (20, 36, 37). $rDNA_{35}$ dun1Δ cells underwent rDNA amplification after the introduction of pFOB1

high copy plasmid at the same time as the FOB1 plasmid. Comparing rDNA amplification in cells overexpressing PNC1 to empty vector controls showed that decreasing nicotinamide levels strongly repressed rDNA amplification (Fig. 4C). This demonstrates that the rate of rDNA amplification can be modulated by changes in PNC1 expression.

Caloric restriction induces PNC1 overexpression, which represses the BIR pathway (24), suggesting that rDNA amplification rate may be modulated by caloric restriction. To confirm this, we transformed pFOB1 into $rDNA_{35}$ cells and grew them in normal glucose [2% (wt/vol)] or under caloric restriction (0.05% glucose), leading to PNC1 overexpression as expected (SI Appendix, Fig. S6A). After 60 generations of growth, we observed that caloric restriction significantly repressed rDNA amplification (Fig. 4D), showing that the rate of rDNA copy number change is directly linked to environmental nutrient availability. As with rapamycin treatment, caloric restriction impairs growth and reduces RNA pol I transcription; however, the repression of rDNA amplification is clearly separable from growth, as overexpression of PNC1 reduces rDNA amplification, but not growth rate (SI Appendix, Fig. S6 B and C and Fig. S2C).

The effect of PNC1 expression on rDNA amplification led us to question whether PNC1 levels are altered in cells with low rDNA copy number, which would be an important indicator of an active mechanism responding to low rDNA copy number. Indeed, PNC1 mRNA is significantly reduced in $rDNA_{35}$ cells compared with isogenic $rDNA_{180}$ controls (Fig. 4E). This does not fully explain the rDNA amplification phenotype of $rDNA_{35}$ cells, as amplification is not completely suppressed by caloric restriction, whereas the PNC1 mRNA level is fully restored (compare Figs. 4D and SI Appendix, Fig. S6A), but clearly shows that the activity of Sir2 and Hst3/4 is selectively reduced in these cells through an increase in nicotinamide concentration.

Taken together, our results show that rDNA amplification is a tightly controlled process that is modulated in response to nutrient availability. rDNA amplification requires TOR signaling, which simultaneously controls the activity of multiple HDACs. These HDACs in turn regulate HR-dependent and non-HR-dependent rDNA recombination pathways that are both required for efficient rDNA amplification (Fig. 5).

D r n

C ntr f rDNA Am f at n n R n t t Env r nm nt. It has long been known that some organisms can amplify rDNA copy number, indicating the existence of controlled mechanisms for copy number change (18, 19). Here we have demonstrated that rDNA amplification in budding yeast is regulated by the TOR pathway and is performed by at least two recombination pathways under the control of multiple HDACs.

The rapamycin-sensitive Target of Rapamycin Complex 1 (TORC1) orchestrates budding yeast cell growth in response to nutrient levels (reviewed in ref. 39), and therefore the repression of rDNA amplification by rapamycin or caloric restriction firmly links rDNA copy number to nutrient availability. TOR inhibition can alter the rate of marker loss from the rDNA (23, 24); however, this occurs through the BIR pathway, which acts primarily to homogenize rDNA sequences, and it is not clear why rDNA homogenization should respond to the environment. In contrast, cells with suboptimal rDNA copy number are forced to up-regulate RNA pol I transcription to maintain ribosome synthesis, and rDNA amplification is a logical response in this situation; although ribosome synthesis can be enhanced temporarily by increasing RNA pol I transcription, this strategy is harmful in the long term (22, 40). Controlled rDNA amplification is therefore a response to available nutrients being in excess compared with ribosome synthesis capacity. Caloric restriction has been extensively investigated in yeast (41), but conversely, the effects of nutrient or caloric excess are largely unexplored because of complications from the osmolarity of high-glucose solutions (42). We observe that cells with low rDNA copy number in normal glucose media show reduced expression of PNC1, a gene that is overexpressed on caloric restriction and is required for lifespan extension (24, 29). Interestingly,PNC1

Although Sir2, Hst3, and Hst4 are structurally related, they have very different effects on recombination. Sir2 regulates expression of ncRNAs in the rDNA spacer, removing cohesin and allowing a broken replication fork to undergo BIR with unmatched repeats (10). In contrast, Hst3 and Hst4 control recombination pathway choice at stalled replication forks; disturbance of the H3 K56 acetylation cycle prevents HR with a sister chromatid (34), and at the rDNA instigates non-HR-dependent recombination, leading to amplification. Because the non-HR-dependent pathway causes constitutive gain of rDNA copies, cells could regulate rDNA amplification by modulating H3 K56 acetylation (see model Fig. 5). This method of regulation may seem unlikely, as loss of Hst3 and Hst4 leads to general genome instability (31, 34); however, the loss of HDAC activity need not be complete. HR proteins are excluded from the nucleolus (48), and the highly repetitive rDNA is an excellent substrate for non-HR-dependent recombination (20).