Compositional reorganization of the nucleolus in budding yeast mitosis

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ABSTRACT The nucleolus is a membraneless organelle of the nucleus and the site of rRNA synthesis, maturation, and assembly into preribosomal particles. The nucleolus, organized around arrays of rRNA genes (rDNA), dissolves during prophase of mitosis in metazoans, when rDNA transcription ceases, and reforms in telophase, when rDNA transcription resumes. No such dissolution and reformation cycle exists in budding yeast, and the precise course of nucleolar segregation remains unclear. By quantitative live-cell imaging, we observed that the yeast nucleolus is reorganized in its protein composition during mitosis. Daughter cells received equal shares of preinitiation factors, which bind the RNA polymerase I promoter and the rDNA binding barrier protein Fob1, but only about one-third of RNA polymerase I and the processing factors Nop56 and Nsr1. The distribution bias was diminished in nonpolar chromosome segregation events observable in dyn1 mutants. Unequal distribution, however, was enhanced by defects in RNA polymerase I, suggesting that rDNA transcription supports nucleolar segregation. Indeed, quantification of pre-rRNA levels indicated ongoing rDNA transcription in yeast mitosis. These data, together with photobleaching experiments to measure nucleolar protein dynamics in anaphase, consolidate a model that explains the differential partitioning of nucleolar components in budding yeast mitosis.

INTRODUCTION
The nucleolus, a prominent subcompartment of the nucleus, forms around arrays of rRNA genes (rDNA), which are therefore called nucleolar organizer regions (NORs) (Boisvert et al., 2007; Taddei et al., 2010; McStay, 2016). In humans, such rDNA arrays are found on five acrocentric chromosomes, while there is a single rDNA array of around 150 gene repeats on chromosome XII in the budding yeast Saccharomyces cerevisiae. The main function of the nucleolus is to supply cells with the large number of ribosomes needed for growth and proliferation. To this end, the rDNA locus is transcribed by RNA polymerase I (pol I) into a rRNA precursor molecule, which is processed, modified, and assembled with ribosomal proteins to form small and large preribosome particles, which are further matured on their way from the nucleolus to the cytoplasm (Kressler et al., 2010; Woolford and Baserga, 2013).

In addition to providing cells with ribosomes, pol I activity is of major relevance to the architecture of the nucleolus. Studies in yeast and also synthetic biology in higher eukaryotes suggest that rRNA synthesis is the essential process triggering assembly of the nucleolus by self-organization (Oakes, Johzuka, et al., 2006; Grob et al., 2014). Preinitiation factors bind to different promoter elements upstream of the budding yeast 35S rRNA gene, and under favorable nutrient conditions, the pol I holoenzyme is recruited to the promoter in an Rrn3-dependent manner and transcription of the 35S rRNA gene is initiated (Schneider, 2012; Claypool, French, et al., 2004). Synthesized pre-rRNA likely acts as a seed recruiting numerous processing factors (Grob and McStay, 2014) and many processing steps occur cotranscriptionally (Tschochner and Hurt, 2003; Koš and Tollervey, 2010; Albert et al., 2012). This can be seen in electron and cryo-electron microscopy as so-called Miller trees representing transcribed rDNA with emerging transcripts that contain processing complexes as terminal knobs (Miller and Beatty, 1969;
Barandun, Hunziker, and Klinge, 2018). Therefore, pol I activity provides a transient physical link between rDNA and rRNA processing factors.

During cell division in metazoans, the nucleolus dissolves temporarily (Hernandez-Verdun, 2011; Mangan et al., 2017). At the beginning of mitosis, transcription by pol I ceases and the dense fibrillar and granular components of nucleoli disassemble as they relocate to the chromosome periphery. Following chromosome segregation in anaphase, transcription by pol I resumes and nucleoli reassemble at NORs. However, there are also factors that stay associated with rDNA during anaphase, such as the high-mobility group protein UBF, which establishes a bookmark for the recruitment of the pol I transcription machinery and the reassembly of nucleoli in telophase (Grob and McStay, 2014).

In contrast to higher eukaryotes, the budding yeast nucleolus is thought to stay assembled during the course of mitosis (Fuchs and Loidl, 2004; Sirri et al., 2008). The single budding yeast rDNA array is segregated equally between the mother and daughter cell in mitosis. However, like cell and nuclear division, the geometry of chromosome segregation in budding yeast is highly polar (Jorgensen et al., 2007; Renshaw et al., 2010). At the onset of anaphase, cohesin is not completely inactivated across chromosome arms and sister chromatids remain linked also by catenation (Titos et al., 2014). This leads to a zipperlike segregation of chromosomes, in which the chromatids transmitted from the mother to the daughter cell undergo pronounced stretching and subsequent condensin-dependent recoiling events that progress from centromere to telomere. In addition to residual cohesin and catenation applying to chromosome segregation in general, pol I activity is known to establish linkages between sister rDNA-arrays in budding yeast mitosis (Machin et al., 2006; Tomson et al., 2006). Under particular conditions, where rDNA segregation is impeded, switching off pol I activity can abort segregation defects. In more detail, cleavage of cohesin by separase is sufficient for segregation of most chromosomes (Uhlmann et al., 2000), but in the case of the rDNA locus activity of the phosphatase, Cdc14 is an additional requirement for segregation (Granot and Snyder, 1991; Sullivan et al., 2004; Torres-Rosell, Machin, et al., 2004). Cdc14 is released from the nucleolus during anaphase (Shou et al., 1999; Visintin et al., 1999) and may contribute to rDNA segregation via multiple pathways. Recruitment of condensin to rDNA depends on Cdc14 activity (D’Amours et al., 2004), and the phosphatase also enhances the integrity of the mitotic spindle by dephosphorylation of the microtubule-bundling protein Ase1 (Khmelinski, Lawrence, et al., 2007). The process of rDNA segregation in budding yeast has been the subject of several studies. However, it is still unknown how the proteinaceous components of the nucleolus segregate during mitosis.

RESULTS

To study how the nucleolus divides during mitosis in budding yeast, we followed various green fluorescent protein (GFP)-fused nucleolar proteins by spinning-disk confocal fluorescence microscopy. The GFP fusions were made by tagging endogenous gene copies to maintain physiological expression levels. All GFP-fused nucleolar proteins produced the characteristic crescent-shaped subnuclear fluorescence signal in interphase and supported wild-type rates of cell growth (Supplemental Figure 1, A and B), indicating functionality of the constructs. Integrity of the fusion proteins was confirmed by Western blot analysis (Supplemental Figure 1C). For live-cell imaging, cells were overlaid with an agar pad of synthetic-complete dextrose (SD) medium to provide a constant supply of nutrients, and z-stacks were taken at 2 min intervals. Under microscopy conditions, cells continued to divide at rates comparable to that of unperturbed growth in liquid medium (Supplemental Figure 2). In addition to visualizing nucleolar proteins during mitosis, we also determined the amount passed on to the nucleolus of the daughter cell. To this end, we quantified signals in the mother and daughter cell nuclei for three time points after complete signal segregation in typically 10 or more cells. The median and interquartile range (IQR) were used as measures of center and spread, respectively.

To validate the experimental setup, we analyzed a yeast strain that carried a GFP-fused gene copy of histone H2A (HTA2-GFP) and coexpressed a nucleolar localization sequence–fused version of mCherry (mCherry-NLS; Arnold, Höckner, and Seufert, 2015). This allowed us to follow the mitotic segregation of chromatin and nucleoloplasm in parallel. Previous work had indicated that the yeast cell nucleus divides asymmetrically (Jorgensen et al., 2007). Consistent with this, we observed that the nucleoloplasmic marker protein segregated asymmetrically, with daughter cells receiving 37.7% (IQR = 3.3) of the total mCherry-NLS signal (Figure 1). A similar value was obtained in measurements of the nuclear volume (Supplemental Figure 3A). In contrast, H2A-GFP segregated almost equally, with daughter cells receiving 46.3% (IQR = 1.3) of the signal (Figure 1). The minor deviation from the expected equal distribution might be caused by a small fraction of H2A-GFP not being incorporated into chromatin. Unlike the uniform signal concentration of mCherry-NLS, the H2A-GFP signal was significantly more concentrated in daughter cells (Supplemental Figure 3B), suggesting that total chromatin is hypercondensed in yeast daughter cells in telophase. Consistent with this observation, previous work reported hypercondensation of chromosome arms segregated to daughter cells in yeast mitosis (Neurohr, Naegeli, et al., 2011). Together, these results indicate that the experimental setup is suitable for quantitative measurements under physiological conditions.

Differential segregation of nucleolar proteins

Transcription of the rRNA genes by RNA polymerase I (pol I) is a major biosynthetic activity in the nucleolus (Warner, 1999; Woolford and Baserga, 2013). To follow pol I during mitosis, we fused GFP to Rpa190 and Rpa135, the two largest pol I subunits which together form the catalytic center in the core of the holoenzyme (Neyer, Kunz, et al., 2016; Tafur et al., 2016). GFP was also fused to the Rpa43 subunit, which localizes to the polymerase stalk and serves to facilitate promoter recruitment of pol I (Peyroche et al., 2000; Kuhn et al., 2007). In addition to the characteristic crescent-shaped structures in preanaphase cells, Rpa190-GFP, Rpa135-GFP, and Rpa43-GFP gave rise to various elongated signals during chromosome segregation, which is known to involve stretching and recoiling of chromosomes (Harrison et al., 2009; Renshaw et al., 2010). Covisualization of the nucleoloplasmic marker protein mCherry-NLS indicated that pol I remained localized to distinct subnuclear structures throughout anaphase (Figure 2, A–C). Nucleoloplasmic pools of GFP-tagged pol I subunits were comparatively low in interphase and anaphase cells, as indicated by line intensity plots (Supplemental Figure 4). Together, these data suggest that pol I remains associated with the rDNA locus during mitosis. Interestingly, quantification of the GFP signals revealed that pol I split unequally between the mother and daughter cell nucleoli, as the daughter cell nucleolus contained on average only 35% of the total signal (Rpa190-GFP: 37.2% IQR = 4.1; Rpa135-GFP: 33.3% IQR = 4.1; Rpa43-GFP: 35.5% IQR = 5.5; Figure 2).

During its synthesis, the nascent pre-rRNA is bound at its 5′-portion by the small subunit (SSU) processome, a large ribonucleoprotein complex needed for pre-rRNA maturation (Dragon et al., 2002; Barandun, Chaker-Margot, Hunziker, et al., 2017;
Barandun, Hunziker, and Klinge, 2018). To follow this particle, we fused GFP to the Nop56 subunit of the U3 small nucleolar ribonucleoprotein particle (snORNP), a subcomplex of the SSU processome (Lafontaine and Tollervey, 2000). GFP was also fused to the yeast orthologue of human nucleolin, Nsr1, a ribosome biogenesis factor that is not part of the SSU processome (Kondo and Inouye, 1992; Chaker-Margot et al., 2017). Like the pol I subunits, Nop56-GFP and Nsr1-GFP localized to elongated subnuclear structures during chromosome segregation indicating persistent rDNA association in mitosis (Figure 2, D and E). And again, there was an obvious segregation bias with daughter nucleoli obtaining 36.1% (IQR = 5.6) and 31.0% (IQR = 5.9) of the total Nop56-GFP and Nsr1-GFP signal, respectively (Figure 2). Consistent with these data, unequal mitotic segregation of Nop56 has been observed before (Menendez-Benito et al., 2013). Thus, in addition to pol I, components of the pre-rRNA processing machinery partition unequally during mitosis.

Various initiation factors associated with the rDNA promoter region are required for the onset of transcription by pol I. These include the core factor (CF), subunits of which interact with Rrn3, the TATA-binding protein (TBP), and the upstream activating factor (UAF; Knutson et al., 2014). To visualize the core factor, the CF-subunits Rrn6 and Rrn7 were fused to GFP. Consistent with their low abundance (Ghaemmaghami et al., 2003), Rrn6-GFP and Rrn7-GFP generated weak fluorescence signals, but characteristic rDNA structures were observed throughout anaphase (Figure 2, F and G). In contrast to pol I and rRNA biogenesis factors, however, these CF subunits segregated equally between the nucleoli of mother and daughter cells (Figure 2). Signal quantification in time-lapse series revealed that Rrn1-GFP segregated symmetrically or almost symmetrically in the case of Rrn10-GFP (45.5%; IQR = 6.4; Figure 2). Thus, unlike components of the pol I holoenzyme and rRNA processing machineries, the promoter-associated CF and UAF components partitioned equally between the mother and daughter cell nucleoli.

Finally, we checked the rDNA binding protein Fob1, which has no direct role in rDNA transcription. Fob1 binds to replication fork barrier sites present in a nontranscribed spacer region of the rDNA locus. There, Fob1 blocks replication in a polar manner to prevent collisions of the replication and transcription machinery and thereby supports integrity of the rDNA locus (Kobayashi, 2003; Mohanty and Bastia, 2004). During anaphase, Fob1-GFP localized to typical nucleolar structures and visualized the stretching and recoiling of the rDNA locus (Figure 2). Nucleoli of daughter cells contained 49.8% (IQR = 6.2) of the total Fob1-GFP signal. Thus, Fob1-GFP resembles the rDNA promoter-associated CF and UAF components in its equal partitioning between the nucleoli of mother and daughter cells.

Together, these data suggest that during chromosome segregation in budding yeast mitosis the nucleolus persists, but its composition is rearranged with the daughter cell nucleolus receiving a smaller portion of the rDNA transcription and processing machineries, but an equal share of rDNA-associated factors.

Low mobility of nucleolar proteins in anaphase
Because pol I subunits and rRNA processing factors showed a mitotic segregation bias quantitatively similar to the unequal segregation of the nucleoplasm marked by mCherry-NLS, we asked whether these nucleolar proteins are mobile and exchange rapidly with the nucleoplasm in anaphase. To investigate their intranuclear dynamics, we performed fluorescence loss in photobleaching (FLIP) experiments (Figure 3). To this end, we chose anaphase cells with completely separated nucleoli and bleached the GFP-fused
Asymmetric nucleolar inheritance caused by the polarity of chromosome segregation

Chromosome segregation in budding yeast is inherently polar. At the onset of anaphase, the spindle rapidly elongates into the daughter cell (Straight et al., 1997; Shaw et al., 1998). Spindle forces are opposed by residual cohesion and catenation of chromosome arms, so that sister chromatids are segregated in a zipper-like manner from centromeres to telomeres (Renshaw et al., 2010; Titos et al., 2014). The daughter cell–directed chromatids undergo pronounced decompactions followed by condensin-mediated recondensation. To ask whether the specific geometry of chromosome segregation is relevant to the observed unequal partitioning of nucleolar components, we studied division of the nucleolus in dyn1 mutant strains. Dyn1, the cytoplasmic microtubule motor protein dynein, helps to position the nucleolus at the bud neck and orient the spindle along the mother-bud axis. In its absence, chromosome segregation may occur in the nucleus at the bud neck and orient the spindle along the mother-bud axis. In its absence, chromosome segregation may occur in the mother cell nucleus (reduction to 6.6%; SD = 5.9; n = 5) had little effect on the daughter cell signal (88.1%; SD = 11.3; n = 5), while the mCherry-NLS signal was lost in both portions of the nucleus. Some exchange of the Nop56-GFP signal was detectable thereafter, but it occurred at a very low rate. Within 4 min after the bleach, the Nop56-GFP signal increased to 18.1% (SD = 7.7) in the mother cell nucleolus and decreased to 75.7% (SD = 18.8) in the daughter cell nucleolus. This suggests that, similarly to pol I, the SSU processome remains localized to the nucleolus in anaphase.

With this approach, nucleolar retention was observed also for the GFP-fused nucleolin Nsr1 (signal reduction to 5.3% [SD = 6.4] and 72.9% [SD = 17.1] in the mother and daughter cell nucleoli, respectively; Figure 3C). Signal exchange in the 4-min postbleach interval (19.2% [SD = 10.0] and 47.5% [SD = 15.0] in the mother and daughter cell nucleoli, respectively) indicated that relative to Rpa135-GFP and Nop56-GFP, the nucleolin Nsr1 is somewhat more mobile.

Together, these data suggest that RNA pol I, the SSU processome, and nucleolin do not exchange rapidly with the nucleoplasm, but are retained efficiently in the nucleolus during anaphase. Thus, a direct link between the unequal segregation of these nucleolar proteins and the nucleolus seems unlikely.
Nop56-3mCherry signal (Figure 4A) and therefore serves as a measure for the completion of rDNA segregation. In our further analysis, we used a dyn1 mutant rDNA dot strain coexpressing a 3mCherry fusion of Spc42 to visualize the spindle pole body (SPB) (Donaldson and Kilmartin, 1996). This allowed us to monitor spindle elongation as well as stretching and recoiling of the chromosome arm carrying the rDNA locus by tracking SPB–SPB and SPB–dot distances (Figure 4H).

Consistent with previous studies, the majority of dyn1 mutant cells managed to elongate their mitotic spindles through the bud neck (Figure 4B). In these regular mitotic divisions, spindle elongation showed the typical biphasic pattern (Yeh et al., 1997) and SPBs reached an average distance of 7.2 µm at 20 min after onset of elongation (Figure 4, B and D). In addition, chromosomes that partitioned into the daughter cell underwent significant stretching, as indicated by the increase of the rDNA dot–SPB distance (Figure 4F). In contrast, when chromosome segregation was confined to the mother cell body, spindles elongated somewhat more slowly and reached a typical length of 5 µm (Figure 4D) and, importantly, chromosome stretching did not occur (Figure 4G). Moreover, it took much longer to complete rDNA segregation (Figure 4E). Thus, inside the mother cell bodies of dyn1 mutants, chromosomes segregate somewhat more slowly and in a symmetric, nonpolar manner.

To ask how the polarity of chromosome segregation influences the partitioning of the nucleolus, we followed GFP-fused nucleolar proteins in dyn1 mutants. Subunits of pol I, the SSU processome, and nucleolin partitioned asymmetrically when chromosomes segregated through the bud neck. Under these conditions, daughter cells received 30.7% (IQR = 6.7) of the Rpa135-GFP signal, 33.9% (IQR = 12.0) of Nop56-GFP, and 32.7% of Nsr1-GFP (IQR = 7.2; Figure 5). In contrast, when chromosomes segregated inside the mother cell body, these nucleolar components partitioned much more symmetrically, with the nuclear portion closer to the daughter cell receiving 45.6% (IQR = 5.8) of the Rpa135-GFP signal, 41.3% (IQR = 5.5) of Nop56-GFP, and 43.8% (IQR = 5.0) of Nsr1-GFP. These observations suggest that the specific geometry of nuclear division in budding yeast, where one set of chromosomes is rapidly transported out of the mother into the daughter cell, is a major cause for the asymmetric segregation of the rRNA transcription and biogenesis machineries of the nucleolus.

Consistent with earlier work (Yeh et al., 1995), we observed that some cells, which had segregated their nucleoli inside the mother cell body, continued mitosis by moving one portion of the two-lobed nucleus into the daughter cell. No significant change in the daughter directed portion of the signal of Rpa135-GFP was observed after passage of the bud neck (Supplemental Figure 5). Thus, passage of chromosomes through the bud neck does not delocalize pol I from the nucleolus, arguing against a possible view that a bud neck barrier is responsible for the asymmetric segregation of the rRNA transcription machinery.

RNA polymerase I promotes nucleolar inheritance
rRNA is a key organizer of the nucleolar structure (Grob and McStays, 2014; Shin and Brangwynne, 2017). To study how transcription by pol I affects the mitotic segregation of the nucleolus, we used cells carrying an rrn3 ts allele. This conditional mutation hinders recruitment of pol I to the promoter and thereby inhibits pol I transcriptional activity (Claypool, French, et al., 2004). Consistent with this work, we observed that a 30-min shift of rrn3 ts cells from 25°C to 37°C decreased pre-rRNA levels to ~5% of the wild-type value (see Figure 9 later in this article). Time-lapse microscopy and signal quantification of the nucleoplasm marker mCherry-NLS and the histone H2A-GFP indicated that, overall, nuclear division and segregation of chromatin occurred normally upon inactivation of pol I (Supplemental Figure 6, A and B). Inviability of rrn3 ts cells at the restrictive temperature was confirmed by a growth assay (Supplemental Figure 8).

We next analyzed the mitotic segregation of pol I by following Rpa135-GFP. Incubation of rrn3 ts cells at the restrictive temperature for 30 min caused some delocalization of Rpa135-GFP to the nucleoplasm during interphase and mitosis (Figure 6, A and B). Measurement of the Rpa135-GFP signal in the mother and daughter cell portions of the nucleus during anaphase revealed that the
FIGURE 4: Nonpolar rDNA segregation in a dyn1 mutant. A tetO array was introduced at the telomere-proximal flank of the rDNA locus and visualized by tetR GFP. (A) Mitotic segregation of the rDNA dot with Nop56-3mCherry as comarker. Scale bar, 5 µm. t = 0 is set arbitrarily. (B, C) Mitotic segregation of the rDNA-dot and Spc42-3mCherry in dyn1 cells elongating the mitotic spindle through the bud neck, B, or inside the mother cell body, C. t = 0 is the last point in time with SPB-SPB distance < 3 µm. Scale bars, 5 µm. (D) SPB–SPB distance in RRN3 and rrn3 ts cells over time. Mean values and SDs (n = 10). (E) Percentage of dyn1 cells (spindle elongation through the bud neck or inside the mother, respectively) showing segregated rDNA dots (distance permanently > 1 µm) over time. (F) Distance of rDNA dot and SPB over time for mother- and daughter-directed chromatids when segregation proceeds through the bud neck. Mean values and SDs are shown. (H) Scheme to illustrate the distances measured in D–G.
mother cell bias of pol I segregation was further increased in \textit{rm3}\textsubscript{ts} cells (Figure 6C). Daughter cells received 35.1\% (IQR = 7.1) and 28.4\% (IQR = 6.2) of the \textit{Rpa135-GFP} signal in wild-type and \textit{rm3}\textsubscript{ts} cells, respectively. Thus, after acute inactivation of pol I transcription, a sizeable fraction of pol I remains localized to the nucleolus, and reduced amounts of pol I are associated with the rDNA copy transmitted to the daughter cell. These data suggest that ongoing transcription helps to supply the daughter cell with a regular amount of nucleolar-localized pol I.

The GFP-fused rRNA biogenesis factors Nop56 and Nsr1 behaved similarly. The presence of extranucleolar speckles, in addition to the major nucleolar signal (Supplemental Figure 6C), point to...
FIGURE 6: Increased asymmetry of pol I and processing factors in an \textit{rrn3} ts mutant. (A, B) Line intensity profiles depicting the maximum-intensity distribution of mCherry-NLS and Rpa135-GFP in interphase and anaphase (last time point showing unsegregated signal) for wild-type and \textit{rrn3} ts cells at 37°C, respectively. (C–F) Mitotic segregation of (C) Rpa135-GFP, (D) Nop56-GFP, (E) Nsr1-GFP, and (F) Fob1-GFP in \textit{RRN3} and \textit{rrn3} ts cells at 37°C. Rrn3 was inactivated in growing, nonsynchronized cells by a shift to 37°C for 30 min before time series of 28 min at 37°C were acquired. \( t = 0 \) is the first point in time with completely segregated mother and daughter signals in the GFP-channel. Scale bars, 5 µm. Box plots depict the percentage of total nuclear signal inherited by daughter cells (from top to bottom: \( n = 12, 12, 10, 10; 11, 11, 10, 10; *p < 0.05; **p < 0.01; ***p < 0.001 \)).

partial delocalization of these proteins in \textit{rrn3} ts mutant cells. Relative to wild-type cells, reduced amounts of Nop56-GFP were inherited by daughter cells (\textit{RRN3} cells: 36.7% [IQR = 5.1]; \textit{rrn3} ts cells: 31.9% [IQR = 10.1]; Figure 6D). In the case of Nsr1-GFP, the daughter cell signal was unusually small and dot-shaped, and the bias was even more pronounced (\textit{RRN3} cells: 37.7% [IQR = 7.1]; \textit{rrn3} ts cells: 23.7% [IQR = 13.6]; Figure 6E). Thus, the inhibition of pol I transcription compromised the passage of the SSU processome and nucleolin to daughter cells during mitosis.

Unlike the above-described rRNA transcription and biogenesis factors, the GFP-fused rDNA binding protein Fob1 did not detectably delocalize from the nucleolus in the \textit{rrn3} ts mutant (Figure 6F). Well-defined subnuclear structures were visible throughout the mitotic segregation process. Fob1-GFP signals were more compact in the mutant, but the equal segregation pattern was maintained (\textit{RRN3} cells: 50.8% [IQR = 6.6]; \textit{rrn3} ts cells: 47.5% [IQR = 10.4]).

Together, these observations indicate that transcription by pol I supports inheritance of the rRNA transcription and processing machinery by the daughter cell nucleus, but is dispensable for the transmission of other nucleolar components, as exemplified by the rDNA-binding protein Fob1.

To characterize in more detail how the rDNA locus segregates after inhibiting pol I transcription, we tracked the SPB labeled by Spc42-3mCherry and a chromosome dot inserted into the telomere-proximal flank of the rDNA locus. Following \textit{rrn3} ts-mediated inactivation of pol I, spindles elongated at normal rates (Figure 7, A–C). Segregation of the rDNA locus, however, was noticeably accelerated in the \textit{rrn3} ts mutant (Figure 7D). This suggests that rDNA transcription opposes the mitotic segregation of the rDNA locus and accounts for its late completion. To analyze the stretching and recoiling of chromosome arms, we measured the distance between the SPB and the respective rDNA dot (Figure 7, E and F). In the chromatid, which remained in the mother cell body, the rDNA dot–SPB distance did not change much. In contrast, chromosome stretching was pronounced in the case of the chromatid segregating into the daughter cell, as average distances larger than 2.5 µm were observed for ~8 min. Interestingly, this stretched state of the daughter cell–directed rDNA locus was reduced in the \textit{rrn3} ts mutant, as it lasted only ~2 min on average. Together, these data indicate that transcription by pol I sets the timing of rDNA segregation and intensifies chromosome stretching. These observations confirm earlier work that showed that pol I transcriptional activity can interfere with rDNA segregation (Machin et al., 2006; Tomson et al., 2006).

To further evaluate the effect of pol I transcription on the mitotic segregation of nucleolar components, we tested cells lacking the nonessential Rpa12 subunit of pol I. Synthesis of pre-rRNA and cell growth are reduced in the \textit{rpa12} deletion mutant (Nogi et al., 1991, 1993). Nuclear division and chromosome segregation proceeded normally, as indicated by measurements of mCherry-NLS and
H2A-GFP (Supplemental Figure 7, A and B). The absence of Rpa12 had some influence on pol I levels and nucleolar integrity, as the Rpa135-GFP signal was reduced in rpa12 cells and a minor portion of Nop56-GFP, and Nsr1-GFP localized to speckles in the nucleus (Supplemental Figure 7, C and D). Still, the major part of these proteins localized to characteristic nucleolar structures during interphase and mitosis. Signal quantification revealed that in rpa12 mutant cells the fractions of Rpa135-GFP, Nop56-GFP, and Nsr1-GFP passed on to the daughter cell nucleolus were lower than with wild-type cells, indicating an increased mother cell segregation bias (Figure 8, A–C). This contrasted with the behavior of Fob1-GFP, whose nucleolar localization and equal segregation pattern remained essentially unchanged (Figure 8D). These data substantiate the notion that transcription by pol I facilitates transmission of the pol I and pre-rRNA processing machineries to the daughter cell nucleolus in mitosis.

Figure 7: Altered timing of rDNA segregation in an rrn3 ts mutant. A tetO array was introduced at the telomere-proximal flank of the rDNA locus and visualized by tetR-GFP. (A, B) Mitotic segregation of the rDNA dot and Spc42-3mCherry in (A) RRN3 and (B) rrn3 ts cells at 37°C. Rrn3 was inactivated in growing, nonsynchronized cells by a shift to 37°C for 30 min before time series of 28 min at 37°C were acquired. t = 0 is the last point in time with SPB–SPB distance <3 µm. Scale bars, 5 µm. (C) SPB–SPB distance in RRN3 and rrn3 ts cells over time. Mean values and SDs (n = 10). (D) Percentage of RRN3 and rrn3 ts cells showing segregated rDNA dots (distance permanently >1 µm) over time. (E) Distance of rDNA dot and SPB over time for mother-directed chromatids in RRN3 and rrn3 ts cells. Mean values and SDs are given. (F) Distance of rDNA dot and SPB over time for daughter-directed chromatids in RRN3 and rrn3 ts cells. Mean values and SDs are given. (G) Scheme to illustrate the distances measured in C–F.
Transcription by RNA polymerase I during mitosis

Because our studies had indicated that defects in pol I compromised the mitotic inheritance of nucleolar components, we were interested in determining the transcriptional activity of pol I during mitosis in budding yeast. To this end, we synchronized yeast cells by release from a pheromone-induced arrest in G1 and took samples of cells that had completed S-phase at 8-min intervals to adequately resolve mitotic progression. To measure transcription by pol I, we quantified the levels of the newly synthesized pre-rRNA by reverse transcription–quantitative real-time PCR (RT-qPCR), using primers that span the A0 and A1 cleavage sites in the 5′ external transcribed spacer region (Supplemental Figure 9). Owing to their rapid cleavage, the corresponding regions are present only in the newly synthesized rRNA precursor and their level can therefore be used as a measure of pol I activity. This type of assay has been used before by others (Clemente-Blanco et al., 2009; Iacovella, Golfieri, et al., 2015). As a positive control, we also used this RT-qPCR assay to measure pol I transcription in rm3 ts mutant cells. Following a shift to 37°C for 30 min, levels of the A0 and A1 cleavage site-spanning fragments decreased by a factor of 25 and 32, respectively (Figure 9, A and E), confirming that this assay is suitable to measuring pol I transcription. To factor of 25 and 32, respectively (Figure 9, A and E), confirming that this assay is suitable to measuring pol I transcription. To

FIGURE 8: Increased asymmetry of pol I and processing factors in a rpa12 mutant. (A–D) Mitotic segregation of (A) Rpa135-GFP, (B) Nop56-GFP, (C) Nsr1-GFP, and (D) Fob1-GFP in RPA12 and rpa12 cells. t = 0 is the first point in time with completely segregated mother and daughter signals in the GFP channel. Scale bars, 5 µm. Box plots depict the percentage of total nuclear signal inherited by daughter cells (from top to bottom: n = 13, 12; 17, 15; 12, 14; 10, 10; *p < 0.05; **p < 0.01; ***p < 0.001).

In this study we analyzed by live-cell imaging how the single nucleolus of budding yeast is partitioned between the mother and daughter cells during nuclear division. We observed that the nucleolus is not passed on as a uniform entity, but is reorganized in its protein composition upon segregation of the rDNA locus in mitosis. While several rDNA-bound proteins were distributed equally, daughter cells received only about one-third of pol I and certain ribosome biogenesis factors. To explain this differential segregation of nucleolar proteins, we propose a model illustrated in Figure 10. On the basis of the available data, we consider the nucleolus a highly coherent network formed by multivalent interactions among various nucleolar proteins and nucleic acids. A previous study suggested that nucleolar proteins are highly mobile in the nuclei of mammalian cells (Dundr et al., 2002). However, extrachromosomal nucleoli from Xenopus laevis oocytes behave like liquid droplets (Brangwynne et al., 2011), suggesting a highly coherent, yet dynamic, architecture of the nucleolus. In the case of budding yeast, live-cell imaging and computational modelling indicated that phase separation of the rDNA from the rest of the genome might play a major role in organizing the nucleolus (Albert, Mathon, et al., 2013; Hult et al., 2017). The view of the budding yeast nucleolus as a coherent network is further supported by our photobleaching experiments. On a time scale of minutes, pol I subunits and rRNA processing factors failed to equilibrate between the separated nucleoli of anaphase cells, providing evidence for their stable residence in the nucleolus during mitosis (Figure 3). Thus, the budding yeast nucleolus might be a complex multiphase “droplet” even though it does not assume a spherical
FIGURE 9: Pre-rRNA levels throughout the cell cycle. (A–E) bar1 cells were synchronized by a pheromone induced G1 arrest and release into fresh medium. (A) Maximum change of RNAs in the synchronized culture over time compared with the change of RNAs in RRN3 wild-type and rm3 ts mutant cells following shift from 25 to 37°C for 30 min. (B, C) Synchrony of the culture was tested by flow cytometry providing DNA-profiles (1C, G1 cells; 2C, G2 and M cells) (B) and counting of DAPI stained cells (n = 100) to follow nuclear division (C). (D) Levels of CLB2, SIC1, and CTS1 mRNAs in the synchronized culture shown as fold actin mRNA. (E) Levels of the pre-rRNA portion spanning the cleavage sites A0 and A1 and an 18S rRNA segment are shown as fold actin mRNA. Fold actin mRNA values for cycling cells at 25°C and for RRN3 wild-type and rm3 ts mutant cells after 30 min at 37°C are shown for comparison. Means and SDs for three qPCR replicas are shown.
A chromosome XII
ribosomal RNA
cohesin
centromere
Fob1, stable rDNA-binding
Pol I engaged/unengaged
rRNA processing factors engaged/unengaged
rDNA independent
coherence forces

pre-anaphase

B

bud-neck
daughter cell

mid-anaphase

C

post-anaphase

FIGURE 10: Model for the mitotic inheritance of nucleolar proteins in budding yeast. (A) The nucleolus is a dynamic multicomponent assembly showing a high degree of coherence. Multiple protein–protein, protein–nucleic acid, and nucleic acid–nucleic acid interactions contribute to this behavior. Active and inactive factors can reside in the nucleolus. (B) During anaphase, the chromatin transmitted from the mother to the daughter cell body undergoes stretching and recoiling. Transcription by pol I in anaphase causes efficient association of pol I and processing factors with rDNA. This results in cohesive forces between rDNA sister arrays and facilitates efficient transport of the engaged rRNA transcription and processing machineries into the daughter nucleus. (C) Following anaphase, mother and daughter nuclei contain equal numbers of proteins stably binding to rDNA. The noncollectively and nonpermanently associated transcription and processing components are inherited asymmetrically.

shape. Spatial constraints such as anchorage of the rDNA to the nuclear envelope (Mekhail et al., 2008) might counteract surface tension–driven sphericity.

During anaphase, one of the sister rDNA arrays is transported out of the nucleolar interaction network located in the mother cell body in a rapid and highly polar manner. In line with previous reports on residual cohesion of chromosomes, catenation, and pol I–dependent rDNA linkages (Machin et al., 2006; Tomson et al., 2006; Renshaw et al., 2010; Titos et al., 2014), we observed more intense stretching and recoiling of the daughter cell–directed rDNA array (Figures 4 and 7), as well as completion of rDNA segregation ahead of schedule following inhibition of rDNA transcription by pol I through the rm3 ts mutation (Figure 7). We envisage that the rDNA array pulled out of the coherent nucleolar network serves as a carrier for the transmission of nucleolar proteins to the daughter cell, so that components tightly associated with the rDNA locus or with emerging pre-rRNA transcripts will be passed on efficiently to the daughter cell. This is expected to be the case for the actively engaged pool of pol I and processing factors. For the nonengaged fraction, however, rDNA-independent nucleolar coherence forces may prevail, hindering daughter-directed transport and supporting preferential retention in the mother cell nucleolus. Thus, a pool of nonengaged molecules, together with the polarity of chromosome segregation, is suggested to be the source of the observed asymmetry in the partitioning of pol I and processing factors in yeast mitosis (Figures 2 and 10).

Indeed, a substantial fraction of pol I and processing factors still localized to the nucleolus after inactivation of pol I transcription (Figures 6 and 9; Supplemental Figures 6 and 10) and a smaller portion was passed on to the daughter cell nucleolus under these conditions (Figures 6 and 8). In addition, the existence of a pool of unengaged pol I has been reported for mammalian cells (Dundr et al., 2002; Mais, Wright, et al., 2005). Further support for our model comes from the observation that the asymmetry of nucleolar protein segregation was reduced in the mother cell–confined nonpolar chromosome segregation events of dyn1 mutant cells (Figures 4 and 5).

Unlike pol I, the SSU subunit Nop56, and the nucleolin Nsr1, which segregated asymmetrically, GFP-fused subunits of the CF and UAF, as well as the RFB protein Fob1, partitioned equally between the mother and daughter cell nucleoli (Figure 2). According to our model, we expect these factors to bind rDNA tightly and lack any substantial pool of unbound protein in the nucleolus. This view is supported by the low abundance of these proteins (Ghaemmaghami et al., 2003) and by biochemical work that indicated stable association of the CF subunits Rrn6 and Rrn7, as well as UAF, with the rDNA promoter (Keys et al., 1994; Keys, Lee, et al., 1996).

In addition, a recent study reported that Fob1, unlike other nucleolar proteins, stayed associated with the rDNA during starvation-induced nucleophagy (Mostofa et al., 2018), supporting the view of persistent rDNA binding by Fob1.

Another important aspect of our model concerns the role of rDNA transcription in mitosis. We observed that acute inactivation or chronic reduction of transcription by pol I reduced the fraction of pol I and processing factors partitioned to the daughter cell (Figures 6 and 8). This supports that rDNA transcription supports nucleolar inheritance, likely through providing a sufficiently stable link to the rDNA array transmitted to the daughter cell. Indeed, quantitative analysis of pre-rRNA levels indicated that rDNA transcription persists during mitosis. Essentially constant levels, with fluctuations by
no more than 1.7-fold, were found by RT-qPCR analysis of samples taken from synchronized cells at short intervals (Figure 9; Supplemental Figures 10 and 11). This analysis, done in two biological and several technical replicates, recovered the known cell cycle fluctuation of the CLB2, SIC1, andCTS1 mRNAs and a decrease of pre-rRNA levels by more than 25-fold following acute pol I inactivation in the rm3 ts mutant (Figure 9). Consistent with our findings and interpretation, stable levels of pre-rRNA during mitosis (lacovella, Golferi, et al., 2015) and rDNA transcription in anaphase (de los Santos-Velazquez, de Oya, Manzano-Lopez, and Monje-Casas, 2017) were reported by others. Moreover, forced inhibition of pol I resulted in premature rDNA segregation (Figure 7D; Machin et al., 2006; Tomson et al., 2006), indicating that no such global inhibition of pol I occurs during normal, unperturbed mitosis. We therefore conclude that rDNA transcription is active during yeast mitosis and facilitates nucleolar inheritance.

A previous study reported on the inhibition of rDNA transcription in yeast mitosis serving as a prerequisite for the condensation and segregation of the rDNA locus proposed (Clemente-Blanco et al., 2009). To reconcile these observations, we assume that rDNA transcription in mitosis may be down-regulated only partially, so that transcription by pol I remains sufficiently active to support the segregation of nucleolar components to daughter cells. Indeed, rDNA transcription by pol I in anaphase and condensin-mediated rDNA compaction in anaphase need not necessarily hinder each other. The rDNA repeats of budding yeast are known to occur in distinct chromatin states: transcriptionally active repeats bound by the HMG-like protein Hmo1, and transcriptionally silent repeats with nucleosomal organization (Conconi et al., 1989; Merz et al., 2008). rDNA repeats organized in these distinct chromatin states have been shown to coexist in mitosis (Wittner et al., 2011). The presence of inactive repeats may provide adequate condensin binding capacity and render at least global pol I inactivation superfluous. Thus, rDNA compaction and segregation may come along with ongoing rDNA transcription in anaphase to ensure a continuous supply of ribosome building blocks and to support inheritance of the pol I transcription and ribosome biogenesis machineries.

The chromosomal rDNA locus is partitioned equally between mother and daughter cells and according to our model serves as a carrier for nucleolar proteins. However, rDNA does not occur exclusively in the chromosomal context in budding yeast. Owing to its repetitive nature, rDNA is prone to intrachromosomal recombination, resulting in formation of extrachromosomal rDNA circles (ERCs) (Sinclair and Guarente, 1997). ERCs are a cause for aging in yeast and are retained in mother cells, so that the newborn daughters are rejuvenated. Moreover, activity of the longevity factor and protein deacetylationSir2 suppresses intrachromosomal recombination at the rDNA locus and hence the generation of ERCs (Gottlieb and Esposito, 1989; Kaeberlein, McVey, and Guarente, 1999). To assess whether ERCs might contribute to the asymmetric distribution of nucleolar proteins, we investigated the mitotic segregation of Rpa135-GFP in sir2 mutant cells that are known to carry increased levels of ERCs (Kaeberlein, McVey, and Guarente, 1999). No significant change in the segregation bias of Rpa135-GFP was observed in the sir2 mutant (Supplemental Figure 12). We therefore conclude that ERCs do not make a major contribution to the nucleolar asymmetry observed in this study.

The asymmetric segregation of nucleolar components might serve relevant biological functions. Owing to the differential inheritance of pol I and processing factors, initial rates of rDNA transcription are expected to be lower in newborn daughter cells. This might contribute to the special characteristics of daughter cells, such as their lengthened G1 cell cycle period, and might balance rRNA with ribosomal protein production, presumably reduced as well in the small daughter cells. In addition, the preferential inheritance of the actively engaged fraction of pol I and processing factors might serve as a built-in quality control system for retaining damaged or otherwise nonfunctional molecules in the nucleolar network of the mother cell and for supplying specifically active factors to the daughter cell. Thus, the unequal transmission of nucleolar proteins might add to a growing list of quality control mechanisms associated with the asymmetric cell division of budding yeast (Higuchi-Sanabria et al., 2014). To test for possible contributions of the differential nucleolar protein inheritance to the fitness and rejuvenation of daughter cells is an important objective for the future.

**MATERIALS AND METHODS**

**Yeast methods and strains**

For growth, transformation, mating and sporulation of budding yeast cells, standard protocols were used (Ausubel et al., 2005). Strains used in this study are listed in Supplemental Table 1 and are isogenic derivatives of W303 or S288c as indicated. Deletion strains rpa12, dyn1, and sir2 were purchased from EUROSCARF, Frankfurt. In the W303 background, dyn1 was deleted by transformation of a PCR based deletion construct (Longtine et al., 1998). The thermo-sensitive rm3 (S213P) mutant originates from the study of Claypool, French, et al. (2004; strain NOY1075). YPD+ (10 g/l yeast extract, 20 g/l peptone, 2% glucose, 0.2 g/l tryptophan, 0.1 g/l adenine, 10 mM KH2PO4) was used as a standard growth medium.

**DNA constructs and genetic manipulations**

For endogenous tagging with fluorescent protein genes, different strategies were used. The PCR-based pFA6a (Longtine et al., 1998) and pFA6a-link (Sheff and Thorn, 2004), as well as a plasmid-based approach, were used. For the plasmid-based approach, C-terminal fragments of the genes of interest were PCR-amplified, sequenced, and finally cloned into pRS-based (Sikorski and Hieter, 1989) GFP or mCherry plasmids. Plasmids were linearized within the C-terminal fragment using a single cutter enzyme for integration at the respective endogenous locus. The mCherry-NLS construct (pTEF2-mCherry-Sw15(codons 569–709)/S646A/S664A-MYC13-RCYC1) has been described (Arnold, Höckner, and Seufert, 2015). It consists of the constitutive TEF2 promoter, mCherry, a SW15-derived NLS fragment, and a myc13 epitope. Serine residues S646 and S664 within the Swi5-NLS were mutated to alanine to confer permanent nuclear localization (Moll et al., 1991; Arnold, Höckner, and Seufert, 2015). The mCherry-NLS construct was integrated at the HIS3, URA3, or TRP1 marker locus as indicated (Supplemental Table 1). In the case of Spc42, no endogenous tagging was done but a plasmid carrying a pSPC42-SPC42-3mCherry construct was integrated into the genome at the TRP1 locus. For chromosome dots at the telomere proximal flank of the DNA-array, the 3' UTR of YLR163C was cloned into a plasmid containing 11x tetO-repeat. The plasmid was linearized for integration at the YLR163C locus. Visualization of tetO-arrays was done by a pURA3-NLS-TetR-GFP construct. The original tetR-GFP and 112x tetO plasmids were provided by Kim Nasmyth (Oxford University, UK; plasmids 3524 and 3525, respectively; Michaelis et al., 1997). Disruption of BAR1 was done by insertion of a functional LEU2-cassette into the BAR1-coding sequence (Xbal-digested plasmid pZV77).

**Fluorescence microscopy, image processing, and quantification**

To follow budding and chromosome segregation in synchronized cells, ethanol-fixed samples were resuspended in sodium citrate...
For live-cell microscopy, cultures were grown overnight at 25°C in YPD+ containing 2% glucose. The next day, cells in the exponential growth phase were harvested, resuspended in water to remove YPD+, pipetted on cover slides, and covered with an agarose block containing synthetic complete medium (2% glucose, yeast nitrogen base, amino acids, nucleobases) for analysis in an inverted microscope. Wild-type and mutant strains used for comparison of fluorescence signals were generated by mating and sporulation to ensure equivalence of marker-integrated gene constructs. Spinning-disk microscopy was done with an Axio Observer.Z1 (Carl Zeiss) in combination with a CSU-X1 spinning disk unit (Yokogawa), an AxioCam MRm (Carl Zeiss) for detection, and AxioVision or ZEN software (Carl Zeiss). Fourteen z-slices with a distance of 400 nm were acquired. FLIP experiments were done using a TCS-SP8 confocal laser scanning microscope (Leica) with photomultiplier tubes (Leica) and Leica Application Suite software (Leica). Fourteen z-slices with a distance of 430 nm were acquired. The appointed areas were bleached 15 times at high laser intensity to effectively erase the fluorescence signal (one bleaching event taking 1.736 s). Plan Apochromat 63×/1.40 oil objectives were used for all microscopy, mCherry and GFP were excited using 561- and 488-nm laser lines, respectively. Most experiments were performed at room temperature (20–23°C). For the rrn3 ts experiments, cells were pipetted in eight-well μ-slides (ibidi), covered with an agarose block, and preincubated at the microscope for 30 min in a heating chamber (ibidi) with lid and plate set to 37°C. Heating continued during time-lapse microscopy. All movies were of duration 28 min. The ibidi system was also used for growth analysis under microscopy conditions at 25°C (Supplemental Figure 2).

Image processing was done using the software ImageJ (National Institutes of Health, Bethesda, MD; Schneider et al., 2012). For quantification of mCherry-NLS, H2A-GFP, Rpa190-GFP, Rpa135-GFP, Rpa43-GFP, and Nop56-GFP, z-stacks were summed up, the mean background value calculated from regions outside cells was subtracted, and the Raw Integrated Density of the nuclear/nucleolar population was measured. Owing to prominent cytoplasmic portions and/or weak signal intensities close to background levels, Nsr1-GFP, Rrn6-GFP, Rrn7-GFP, Rrn5-GFP, Rrn10-GFP, and Fob1-GFP were quantified from unprojected z-stacks using the 3D object counter plugin for ImageJ (Bolte and Cordelières, 2006; http://imagejdocu.tudor.lu/doku.php?id=plugin:analysis:3d_object_counter:start; Schneider et al., 2012). The mean background value was calculated from regions outside cells for single z-planes and subtracted. To quantify the segregation of fluorescence signals, total signals in mother- and daughter-located parts of anaphase nuclei/nucleoli were averaged for three time points after complete segregation, as defined by threshold setting. The daughter signal, as a percentage of the total signal, was then determined by normalization of the daughter portion to the added mother and daughter signal. In the case of concentration, volume, and distance measurements, the 3D object counter plug-in was used to determine integrated signal values, volumes, or center-of-mass coordinates in xyz, respectively. Distances in xyz were calculated in Excel (Microsoft) using the center-of-mass coordinates and the Euclidean distance formula. Threshold settings were used for all image evaluation mentioned above. For line intensity plots, maximum projections of z-stacks were used and the mean background values calculated from regions outside cells were subtracted.

Cell synchronization, sample collection, RNA extraction, and RT-qPCR

For cell synchronization, cultures of bar1 cells were grown in YPD+ at 25°C and treated with 50 ng/ml α-factor for 2 h. Cells were washed twice with YPD+ and released into fresh medium. Cell samples for flow cytometry analysis and DAPI staining were centrifuged for 2 min at 2000 rpm, resuspended in water, and fixed using ethanol. Cells for RNA extraction were centrifuged for 2 min at 2000 rpm, resuspended in ice-cold water, and centrifuged for 15 s at 13,200 rpm and 4°C. The supernatant was removed, and cell pellets were immediately frozen on dry ice. For extraction of RNA, a hot acid phenol/chloroform procedure was used (modified from Collart and Oliwiero, 1993). Frozen cell pellets were resuspended in 400 µl TES solution (10 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.5% SDS) and 400 µl acid phenol preheated to 65°C was added. After brief vortexing, samples were incubated at 65°C for 1 h and vortexed every 10 min. Samples were placed on ice for 5 min and centrifuged for 5 min at 13,200 rpm and 4°C, and the aqueous phase was transferred to a clean tube. Hot acid phenol (400 µl) was added to the aqueous phase and samples were placed on ice for 5 min and centrifuged for 5 min at 13,200 rpm and 4°C. The aqueous phase was transferred to a clean tube, 400 µl chloroform was added, and samples were vortexed and centrifuged for 5 min at 13,200 rpm and 4°C. The aqueous phase was transferred to a clean tube and 40 µl of 3 M sodium acetate (pH 5.3) was added, as well as 1 ml ice-cold ethanol. RNA was precipitated at –80°C for at least 20 min, pelleted by centrifugation at 13,200 rpm and 4°C (original experiment: 5 min; biological replicate: 30 min), washed with 70% ice-cold ethanol, and dissolved in water. RNA concentrations were determined photometrically (BioPhotometer; Eppendorf), and quality assessment was done by agarose gel electrophoresis and ethidium bromide staining. For cDNA-synthesis, the QuantiT Nova Reverse Transcription Kit (Qiagen) was used according to the manufacturer’s instructions. To test for genomic DNA contamination, controls using water instead of the reverse transcriptase enzyme were tested. The QuantiT Nova SYBR Green PCR Kit (Qiagen) was used for RT-qPCR and reactions were run on a Rotor-Gene Q machine (Qiagen) using an identical gain setting for all runs. Reactions for samples and controls were run in triplicate, and identical threshold settings were used for all quantifications executed with the Rotor-Gene Q Series Software (Qiagen). Primer sequences are given in Supplemental Table 2.

Flow cytometry

Flow cytometry was done as described in Gei et al., 2008. A CyFlow Space cytometer (Partec, Münster, Germany) was used, and 20,000 cells were analyzed for each sample to obtain DNA profiles.

Protein analysis

Growing cultures were harvested and cells were resuspended in 1 ml ice-cold H2O. A lysis buffer (150 µl: 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 50 mM NaF, 5 mM EDTA, 0.1% IGEPAL CA-630) was added and cells were lysed by shaking with an equal volume of glass beads in a mixer mill (Retsch, Haan, Germany) at 4°C. Cell debris was pelleted by centrifugation for 3 min at 13,200 rpm and 4°C. Equal volumes of protein lysate and 2× Laemmli sample buffer were mixed and incubated for 10 min at 100°C. SDS–PAGE and Western blot analysis were performed as described in Schwab et al. (1997, 2001). Mouse monoclonal anti-GFP antibody (Roche, Basel, Switzerland) and goat anti-mouse IRDye 800 anti-body (Li-COR Biosciences) were used for detection with an Odyssey Infrared Imaging System (Li-COR Biosciences, Bad Homburg, Germany).
Graphs and statistical analyses

Box plots and statistical analyses were done with the software Origin (OriginLab Corporation). Box plots are defined as follows: the central box spans the quartiles Q1 and Q3, the line inside the box represents the median, and a square represents the mean. Whiskers extending from the quartiles have a maximum length of 1.5 × interquartile range (IQR). Values <Q1 – 1.5 × IQR or >Q3 + 1.5 × IQR are considered outliers. For statistical analyses, a two-sample, two-sided, unpaired Student's t test and the unequal-variance t-procedure were used. Outliers were excluded from statistical analyses. Graphs other than box plots were done in Excel (Microsoft).

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