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Reverse Genetic Analysis of the Yeast RSC Chromatin Remodeler Reveals a Role for RSC3 and SNF5 Homolog 1 in Ploidy Maintenance

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The yeast “remodels the structure of chromatin” (RSC) complex is a multi-subunit “switching deficient/sucrose non-fermenting” type ATP-dependent nucleosome remodeler, with human counterparts that are well-established tumor suppressors. Using temperature-inducible degron fusions of all the essential RSC subunits, we set out to map RSC requirement as a function of the mitotic cell cycle. We found that RSC executes essential functions during G1, G2, and mitosis. Remarkably, we observed a doubling of chromosome complements when degron alleles of the RSC subunit SFH1, the yeast hSNF5 tumor suppressor ortholog, and RSC3 were combined. The requirement for simultaneous deregulation of SFH1 and RSC3 to induce these ploidy shifts was eliminated by knockout of the S-phase cyclin CLB5 and by transient depletion of replication origin licensing factor Cdc6p. Further, combination of the degron alleles of SFH1 and RSC3, with deletion alleles of each of the nine Cdc28/Cdk1-associated cyclins, revealed a strong and specific genetic interaction between the S-phase cyclin genes CLB5 and RSC3, indicating a role for Rsc3p in proper S-phase regulation. Taken together, our results implicate RSC in regulation of the G1/S-phase transition and establish a hitherto unanticipated role for RSC-mediated chromatin remodeling in ploidy maintenance.

Introduction

Maintenance of ploidy is crucial for sexual reproduction in eukaryotes because the ploidy changes that take place during gametogenesis require two identical chromosome complements. Polyploid plant, insect, amphibian, and mammalian species have been documented, and various forms of somatic polyploidy have been described, including mammalian hepatocytes, megakaryocytes, and trophoblasts, insect oocyte nurse cells, and plant endosperm [1–3]. At the cellular level, polyploidy usually represents a highly differentiated state, with increased cell size and elevated metabolic activity. To become polyploid, cells enter a process called endocycling. This usually commences by aborting the mitotic cycle anywhere between G2 (endoreduplication) and cytokinesis (endomitosis), followed by replication [2–4]. Depending on the timing of mitotic exit, cells have multiple chromosome sets contained within a single nucleus or they become multinucleate.

Factors known to drive the switch between mitotic cycling and endocycling include S-phase cyclin-Cdk complexes and their regulators [3,5], as well as the replication origin licensing factors Cdc6, Cdt1, and geminin [6–9]. Such specialized cell-cycle transitions can involve switching between expression of protein isoforms, as reported for cyclin D variants in mammalian trophoblasts [10], or they can be restricted to a variation in oscillation of gene expression, as observed for cyclin E in Drosophila nurse nuclei [3]. Finally, mutations in multiple components of the yeast spindle pole body (Msp1p, Msp2p, Mob1p, Cdc31p, Ndc1p, and Kar1p), the fungal centrosome, have been reported to result in numerical chromosome doubling events in yeast [11–15].

In order to remodel chromosomes, eukaryotes have evolved multi-subunit protein complexes that can alter chromatin structure covalently, by modifying nucleosomes [16,17], or mechanically, via ATP-dependent chromatin remodeling (SNF2-type ATPases) [18,19]. Within the latter class, the SWI2/SNF2 enzymes are represented in yeast by the Sth1p and Swi2p/Snf2p ATPases that reside in the related multi-subunit complexes “remodels the structure of chromatin” (RSC) [20] and mating type “switching deficient/sucrose non-fermenting” (SWISNF) [21,22], respectively. RSC and SWI/SNF complexes are structurally related, sharing three subunits and harboring five paralogs [23,24]. Despite their extensive structural homology, dysfunction of various essential RSC components cannot be compensated for by over-expression of SWISNF paralogs, arguing that protein motifs that mediate complex assembly and function differ [20,25]. Furthermore, genetic evidence indicates that SWISNF and RSC differ fundamentally with respect to interaction with chromatin since histone and SPT6 mutations that suppress snf2A mutants actually enhance conditional sht1SS06L.T881M mutant phenotypes [26].

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Abbreviations: CFU, colony-forming unit; HU, hydroxyurea; MBF, MluI cell cycle box-binding factor; ORF, open reading frame; RSC, remodels the structure of chromatin; SWI/SNF, mating type switching deficient/sucrose non-fermenting

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Author Summary

Some molecules responsible for altering the 3-D organization of chromatin are RSC subunits. Two such complexes are called “remodels the structure of chromatin” (RSC) in yeast and “switching deficient/sucrose non-fermenting” (SWI/SNF) in man. SWI/SNF is known to inhibit the advent of multiple types of human cancers. Since cancer is a disease whereby cells unduly divide, we sought to define when in the yeast cell division cycle RSC executes essential functions. Using a generic method to induce inactivation of essential proteins in otherwise healthy yeast cells, we found that the RSC complex is important before chromosome replication as well as before chromosome segregation. Interestingly, combining two of the mutations we had generated caused doubling of the entire chromosome complement of yeast. As it is known that such multiplication of the cellular chromosome complements results in an increased malleability of the genetic patrimony, which itself is known to underlie some of the aggressive traits of human cancers, our discovery suggests new models as to why SWI/SNF is such a potent tumor suppressor, and this may in turn provide valuable new inroads for cancer treatment.

To date, genetic and molecular analyses have implicated RSC in a variety of cellular processes including chromosome cohesion and transmission, DNA repair, and transcriptional regulation [27–35]. In addition, RSC interacts with a PKC pathway that impinges on cell polarity through Bim1p, a microtubule-associating protein that ensures spindle pole body asymmetry through Kar9p [36,37].

To address fundamental questions with respect to RSC function, we analyzed generic degron alleles of essential RSC subunits. Here, we report that RSC executes essential functions in G1, G2, and mitosis. Strikingly, integral ploidy shifts occurred when degron alleles of the yeast hSNF5 tumor suppressor ortholog SFH1 [38,39] and the cell cycle-regulated RSC3 subunits were combined. Combination of the sfh1td and rsc5td alleles with cyclin deletion alleles revealed a strong genetic interaction between the S-phase cyclin gene, CLB5, and RSC3, indicating a role for Rsc3p in proper S-phase regulation. Furthermore, impairing rereplication control mediated by Clb5p and the replication origin-licensing factor Cdc6p eliminated the requirement for concomitant deregulation of SFH1 and of RSC3 to induce ploidy doubling events. Our data implicate RSC in regulation of the G1/S-phase transition and establish an unanticipated role for RSC-mediated chromatin remodeling in ploidy maintenance.

Results

Generation of Conditional Alleles of All Essential RSC Complex–Specific Subunits

In order to investigate the role of RSC in cellular physiology, we utilized an inducible protein degradation system based on fusion of an N-terminal heat-inducible ubiquitin ligase-target peptide (“degron”) [40] to the open reading frames (ORFs) of all essential RSC-specific subunits. This included replacement of the endogenous promoters by the Pgal::UBR1 promoter, resulting in Cu2+ driven transcription of the rsc3td alleles. The system also included integration of the Pgal::UBR1 promoter at the UBR1 locus, which encodes the N-end rule E3 ligase Ubr1p, that recognizes the N-terminal arginine residue of the degron fusions [41]. This permits suppression of degron fusion degradation by growing cells in glucose media, which represses the Pgal promoter, and allows priming of degron-fusion degradation by pre-growing cells in galactose media at 25 °C, which represses the Poai1-10 promoter, and allows priming of degron-fusion degradation by pre-growing cells in galactose media at 25 °C. Thus, this system permits heat shock–induced, polyubiquitin-mediated degradation of existing cellular protein fusions [41].

Cells expressing degron alleles of the essential RSC subunits Pgal::UBR1 expression at 25 °C by galactose, rsc3td strains (but none of the other RSC degron strains) arrested growth, and colony formation was strongly diminished (Figure 1A and 1C). This indicates that Rsc3p is exquisitely sensitive amongst RSC subunits to the presence of the N-terminal degron.

Following incubation of rsc3td strains in galactose at 37 °C, growth arrest ensued for all subunits within 3–4 h (Figure 1B). Western blot analysis indicated that degron fusions were depleted to nondetectable levels within 2 h (Figure S1 and
unpublished data) and Sth1pTAP level also decreased (Figure S1), indicating impaired complex integrity. Flow cytometry analysis of cellular DNA content revealed G2/M cell-cycle arrests in rsc6Δ, rsc8Δ, and sfh1Δdiploid strains (Figures 1A and S2). sfh1Δdiploid strains gave variable results, usually yielding almost exclusively G2/M cells, though occasionally significant levels of G1-blocked cells were observed. In contrast, both G1- and G2/M-arrested cells were invariably observed in rsc8Δdiploid and rsc3Δdiploid strains. Importantly, every combination of RSC degrons that was tested induced both G1 and G2/M arrests (Figures 1A and S2).

Irreversible lethal effects, observed as a decrease in colony-forming units (CFUs) upon seedling-out onto 2% glucose plates and incubation at 25 °C, were more pronounced and occurred earlier in the 37 °C time course in rsc8Δdiploid and rsc3Δdiploid strains, as well as in strains harboring multiple RSC degron fusions (Figure 1A). In the extreme case of the rsc6Δ, rsc8Δ, and sfh1Δdiploid strain, less than 1% CFUs remained after 3 h of heat shock (Figure 1A), while equivalent fractions of pre- (Figure 1C) and post-replicative (Figure 2C) cells were observed.

Altogether, these data suggest that the cell-cycle phase of arrest correlates with the kinetics of RSC complex inactivation, with G2/M cells being more sensitive to RSC inactivation than G1 cells since G2/M cells accumulate when RSC function is least impaired, as assayed by cell survival.

RSC Is Essential in the G1 Phase of the Cell Cycle

As no essential role has previously been described for RSC during G1, we wished to determine whether the G1 arrest we observed upon RSC depletion (Figures 1A and S2) resulted from functional failure in the course of the preceding cell cycle or whether this reflected a genuine essential function for RSC during G1. To this end, the triple rsc6Δ, rsc8Δ, sfh1Δdiploid degron combination, which conveyed >99% lethality within 3 h of heat shock (Figure 1A), was employed to deplete RSC from synchronously cycling cells (Figure 2). Cells were grown overnight in galactose at 25 °C and then blocked in G1 by exposure to α-pheromone (5 h; boost at 2.5 h). Release into the cell cycle was achieved by removal of pheromone (0 h). Aliquots of synchronized cells were taken at 30-min intervals and incubated for 3-h periods at 37 °C so as to deplete RSC from cells traversing consecutive stages of the cell cycle synchronously. After 3 h at 37 °C, cellular DNA content was determined and cells were seeded-out onto permissive plates to determine viability levels by colony formation (Figure 2). RSC inactivation in synchronized cells proved lethal for >95% of the cells in every case (Figure 2), and RSC inactivation resulted in homogeneous G1 and G2/M arrests, depending on the time when heat shock was applied (Figure 2).

We conclude that RSC executes essential functions in G1 in addition to its essential roles in G2/M. As we did not observe cell arrest in the process of DNA replication (corresponding to the 0.5 h to 3.5 h time point), our experiments suggest that RSC activity is not required per se for chromosome replication. However, we cannot exclude the possibility that a small portion of the genome failed to be replicated upon RSC depletion (Figure 2, 0.5 h to 3.5 h time point).

sfh1Δdiploid and rsc3Δdiploid Cells Together Induce Single Rounds of Ploidy Doublings

The above analyses indicated that RSC performs crucial functions during mitosis, G1, and G2, and they implicate RSC in proper cell-cycle progression. This perception was further strengthened in the process of generating yeast strains harboring combinations of degron alleles of essential RSC subunits. Whereas most diploid strains heterozygous for two or three degron alleles produced >80% viable spores, diploid strains heterozygous for sfh1Δdiploid and rsc6Δdiploid and homozygous for Pgal::UBR1 yielded less than 10% viable spores (Table S1). This dominant meiotic-lethal phenotype was not due to aberrant ploidy of the parental strains, as both haploid rsc6Δdiploid and sfh1Δdiploid strains displayed the expected haploid DNA contents (Figure 1A). Furthermore, the sfh1Δdiploid and rsc6Δdiploid strains were able to individually mate with other haploid rsc6Δdiploid strains to produce diploids that produced >80% viable spores with the expected segregation frequencies of heterozygous markers (Table S1).

We tested the involvement of the Pgal::UBR1 allele by mating a UBR1, sfh1Δdiploid strain to a UBR1, rsc3Δdiploid strain. These diploids were fertile (64% viable progeny); however, none of the surviving progeny harbored both the rsc3Δdiploid and the sfh1Δdiploid alleles (Table 1). This demonstrates that the dominant meiotic-lethal phenotype displayed by double heterozygous rsc3Δdiploid/rsc3Δ/UBR1, sfh1Δdiploid/UBR1 diploids was due to repression of UBR1 expression. This suggests that RSC and
Ub1p, or physiological Ub1p substrates [42], are part of genetic pathways that are redundant to some extent or that form one large pathway in meiosis.

Interestingly, the dominant meiotic-lethal phenotype of diploids homozygous for Pgal::UBR1 and heterozygous for rsc3<sup>td</sup> and sfh1<sup>td</sup> could be rescued by inclusion of a single copy of rsc9<sup>td</sup>, but not by inclusion of sth1<sup>td</sup>, rsc6<sup>td</sup>, rsc8<sup>td</sup>, or rsc58<sup>td</sup> alleles (unpublished data). Remarkably, we observed that every single descendant spore of the triple heterozygous diploids bore both the sfh1<sup>td</sup> and the rsc3<sup>td</sup> alleles gave rise to large, mono-nucleated cells that had a diploid DNA content, regardless of the presence of the rsc58<sup>td</sup> allele (>50 tetrads analyzed). The DNA profile of one sfh1<sup>td</sup>, rsc3<sup>td</sup> nonparental di-type tetrad is shown in Figure 3A. Both progeny that inherited the rsc3<sup>td</sup> and the sfh1<sup>td</sup> alleles have 2C + 4C DNA contents, while the two other spores display the 1C + 2C DNA content expected for haploid yeast. The endodiploid sfh1<sup>td</sup>, rsc3<sup>td</sup> strains responded to mating pheromone (unpublished data) and could mate to produce tetraploid strains (4C8C, Figure 3B). The ploidy shift took place after meiotic segregation of the chromosomes because inheritance of all the heterozygous chromosomal loci obeyed the Mendelian 2:2 frequency.

In order to test whether endodiploid strains could be generated independently of passage through meiosis, we performed endogenous locus replacement experiments in haploid cells. When UB1, rsc3<sup>td</sup> strains were transformed with vectors to convert the wild-type SFH1 allele to sfh1<sup>td</sup>, 10% of the resulting colonies were haploid, 80% were diploid, and 10% also harbored tetraploid cells (n = 48, Figure 3C and Table 2). Thus, the endodyce induced by the rsc3<sup>td</sup> and sfh1<sup>td</sup> alleles could also occur independently of meiosis. Transformation of UB1, sfh1<sup>td</sup> haploid strains with the rsc3<sup>td</sup> locus conversion construct yielded fewer endodiploid clones (4%; n = 48, Table 2). This suggests that the presence of rsc3<sup>td</sup> primed cells to undergo a ploidy shift, a fact that may well relate to the sensitivity of rsc3<sup>td</sup> strains (but no other rsc<sup>td</sup>-containing strains) to overexpression of the E3 ligase Ub1p at 25 °C (Figure 1C).

To assess the role of UB1 in rsc3<sup>td</sup> + sfh1<sup>td</sup> mediated ploidy shifts, the above endogenous locus replacement experiment was also performed in Pgal::UBR1 cells grown in glucose. Inhibition of UB1 significantly reduced the frequency of observed ploidy shifts (Table 2), consistent with an ancillary role for Ub1p in this phenomenon.

We conclude that together, degenon alleles of RSC3 and SFH1 disrupt a facultative cell-cycle process that is crucial to maintain ploidy levels in yeast. Furthermore, the fact that ploidy shifts only took place once or twice strongly suggests that a third biological parameter is involved, and that this parameter was triggered in both the endogenous locus conversion and the meiotic segregation experiments.

**RSC Genetically Interacts with the Cyclin-Dependent Kinase Cdc28p/Cdk1p**

In budding yeast, cell-cycle progression is orchestrated by a single cyclin-dependent kinase, Cdc28p/Cdk1p [43]. As we found RSC to be crucial for passage through multiple stages of the cell cycle, we wished to assess functional interactions between RSC and Cdc28p/Cdk1p. To this end, we employed a cdc28<sup>td</sup> degenon allele [40]. Upon Ub1p overexpression, the cdc28<sup>td</sup> allele led to a severe decrease in CFUs. This phenotype was exacerbated by inclusion of the sth1<sup>td</sup> allele (Figure 4), arguing that hypomorphic alleles of RSC and Cdc28p/Cdk1p genetically interact. This notion was further substantiated by the observation that sth1<sup>td</sup> cells overexpressing Saccharomyces cerevisiae WEE1 (SWE1), a tyrosine kinase that controls mitosis entry by inhibition of Cdc28p/Cdk1p activity [43,44], were

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**Table 1: Synthetic Lethality between rsc3<sup>td</sup> and sfh1<sup>td</sup>**

<table>
<thead>
<tr>
<th>Frequency</th>
<th>SFH1, RSC3</th>
<th>sfh1&lt;sup&gt;td&lt;/sup&gt;, RSC3</th>
<th>SFH1, rsc3&lt;sup&gt;td&lt;/sup&gt;</th>
<th>sfh1&lt;sup&gt;td&lt;/sup&gt;, rsc3&lt;sup&gt;td&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>37</td>
<td>30</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>Expected if not SL</td>
<td>38</td>
<td>38</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>Expected if SL</td>
<td>38</td>
<td>38</td>
<td>38</td>
<td>38</td>
</tr>
</tbody>
</table>

UB1, sfh1<sup>td</sup>, and UB1, rsc3<sup>td</sup> strains of opposing mating type were mated, and resulting diploid strains were induced to sporulate. Spores from 38 ascii were analyzed for colony formation and segregation of sfh1<sup>td</sup> and rsc3<sup>td</sup> alleles, as well as several auxotrophic markers. SL, synthetic lethal.

**Figure 3. Post-Meiotic and Mitotic Ploidy Doubling of sfh1<sup>td</sup>, rsc3<sup>td</sup> Cells**

(A) FACS analysis of the DNA content of the four spores of a single representative tetrad derived from a SFH1/sfh1<sup>td</sup>, RSC3/rsc3<sup>td</sup>, RSC9/rsc9<sup>td</sup>, Pgal::UBR1/Pgal::UBR1 diploid. The genotypes of the spores are SFH1, RSC3, RSC9, Pgal::UBR1 (blue), SFH1, RSC3, rsc9<sup>td</sup>, Pgal::UBR1 (red), sfh1<sup>td</sup>, rsc3<sup>td</sup>, RSC9, Pgal::UBR1 (green), sfh1<sup>td</sup>, rsc3<sup>td</sup>, rsc9<sup>td</sup>, Pgal::UBR1 (black). Inset: Light scatter plots indicating cell sizes.

(B) Tetraploid strain (blue) derived from mating of two endodiploids (black). For comparison, a haploid was included (red).

(C) FACS analysis of clones generated by transformation of a rsc3<sup>td</sup> strain with a sfh1<sup>td</sup> allele. Haploid clones are indicated in red, endodiploids in gray, and tetraploids in blue.

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Table 2: Ploidy Alterations by Endogenous Locus Conversion

<table>
<thead>
<tr>
<th>Strain</th>
<th>Integrate</th>
<th>UBR1</th>
<th>Pgal:UBR1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IC 2C</td>
<td>4C</td>
</tr>
<tr>
<td>WT</td>
<td>sfh1td</td>
<td>100 0 0</td>
<td>100 0 0</td>
</tr>
<tr>
<td>rsc3td</td>
<td>sfh1td</td>
<td>10 80 10</td>
<td>48 52 0</td>
</tr>
<tr>
<td>WT</td>
<td>rsc3td</td>
<td>100 0 0</td>
<td>100 0 0</td>
</tr>
<tr>
<td>sfh1td</td>
<td>rsc3td</td>
<td>96 4 0</td>
<td>100 0 0</td>
</tr>
</tbody>
</table>

Wild-type (WT), sfh1td, or rsc3td strains were transformed with endogenous locus conversion constructs for sfhfd or rsc3td and selected for integration of the respective alleles. Resulting transformants were analyzed for DNA content by flow cytometry (see Figure 3) and results are shown as percentage of colonies analyzed (n = 48). Experiments were performed using cells expressing endogenous levels of UBR1 (UBR1), or cells repressing UBR1 expression (Pgal:UBR1) as indicated.

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also exquisitely sensitive to overexpression of Ubr1p at 25 °C (Figure 4). Together, these synthetic lethal effects demonstrate that the RSC catalytic ATPase subunit Sth1p genetically interacts with the cyclin-dependent kinase pathway.

Specific Genetic Interaction between the rsc3td Allele and the CLB5 S-Phase Cyclin

Our results suggest that a specific cell-cycle process is impaired in cells that harbor both the sfh1td and rsc3td degron alleles, and that this could relate to a specific cyclin-dependent kinase pathway. In order to map this process, we mated Pgal:UBR1, rsc3td and Pgal:UBR1, sfh1td strains to a panel of deletion strains that lacked any one of the nine Cdk1p/Cdc28p associated cyclins and assessed spore viability on glucose plates. This analysis did not reveal significant genetic interactions between sfh1td and any of the cyclin deletions (Figure 5A). In the case of the rsc3td allele, however, a significant loss of spore viability was observed upon combination with the clb5Δ allele. As a matter of fact, we did not recover a single UBR1 clone that harbored both the clb5Δ and the rsc3td alleles, indicating that the latter alleles form a lethal combination, and that lethality was suppressed by repression of Ubr1p levels (using the Pgal:UBR1 allele; Figure 5B). Other UBR1, cyclin deletion, rsc3td double mutants were recovered with the expected frequency, demonstrating a specific interaction between clb5Δ and rsc3td. We conclude that the rsc3td allele impairs a cell-cycle process that also relies on Clb5p. As this cyclin is known to control late S-phase progression [43,45,46], this suggests that an important S-phase event is disrupted by the rsc3td allele.

The Rereplication Control Machinery Antagonizes rsc3td-Mediated Ploidy Shifts

Endocycling of eukaryotic cells (e.g., mammalian hepatocytes and megalakaryocytes) commonly relies on alternative regulation of genes essential for replication control, such as G1/S cyclins, Cdc6, geminin, and Cdt1 [3,6,7,9]. We therefore assessed the role of the yeast origin licensing factor Cdc6p [47,48] in ploidy shifts induced by rsc3td and sfh1td. As Cdc6p is an essential protein, we attenuated its cellular levels using a strain expressing CDC6 solely from a methionine repressible promoter [49]. Cells were incubated in the presence of 2 mM methionine for 45 min to repress CDC6 transcription, and then they were made competent for transformation. These cells were transformed with control constructs, or with endogenous locus conversion constructs for the sfh1td or rsc3td alleles. Clones were then selected at 25 °C on glucose plates expressing either Pgal:UBR1 or Pgal:SWE1. This analysis did not reveal significant genetic interactions between sfh1td and any of the cyclin deletions (Figure 5A). In the case of the rsc3td allele, however, a significant loss of spore viability was observed upon combination with the clb5Δ allele. As a matter of fact, we did not recover a single UBR1 clone that harbored both the clb5Δ and the rsc3td alleles, indicating that the latter alleles form a lethal combination, and that lethality was suppressed by repression of Ubr1p levels (using the Pgal:UBR1 allele; Figure 5B). Other UBR1, cyclin deletion, rsc3td double mutants were recovered with the expected frequency, demonstrating a specific interaction between clb5Δ and rsc3td. We conclude that the rsc3td allele impairs a cell-cycle process that also relies on Clb5p. As this cyclin is known to control late S-phase progression [43,45,46], this suggests that an important S-phase event is disrupted by the rsc3td allele.

Figures

Figure 4. Synthetic Sickness Phenotype of sfh1td, cdc28Δ, and sth1td, Pgal:SWE1 Double Mutants

Haploid yeast strains harboring Pgal:UBR1 and combinations of sfh1td and cdc28Δ (cdk1td) or Pgal:SWE1 were analyzed for their ability to form colonies after growth in galactose for the indicated times at the indicated temperatures, by seeding-out 10-fold serial dilutions of an equal number of cells onto solid glucose medium containing 0.1 mM CuSO4. Note the dramatically increased severity of the lethal phenotypes of cdc28Δ and Pgal:SWE1 alleles when they were combined with sth1td. The data shown are from one experiment and are representative of at least three independent experiments.

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plates lacking methionine so as to restore CDC6 transcription. Control cells that had not been depleted of Cdc6p yielded exclusively haploid clones upon conversion of the RSC3 or SFH1 loci to the corresponding degron alleles (Figure 6). In contrast, ploidy shifts were efficiently induced in cells depleted of Cdc6p upon conversion of the RSC3 locus to rsc3 td, but not upon conversion of the SFH1 locus to sfh1 td (n = 60; 60% and 0%, respectively, Figure 6). Thus, temporary depletion of Cdc6p appears to phenocopy the sfh1 td allele but not the rsc3 td allele.

Next, we turned to the cyclin Clb5p. Besides a role in spindle pole body maturation and duplication [50,51], Clb5p plays a dual role in replication regulation as it is required for proper timing of S-phase initiation, as well as to prevent re-initiation of replication forks that have already fired [52]. Furthermore, deregulation of CLB5 levels has been associated with the occurrence of endoreplication [52]. Wild type and clb5Δ strains were transformed with the same constructs as above. In this experimental setup, and contrary to meiotic segregation, UBR1, rsc3 td, clb5Δ mutants could be recovered. Analysis of the resultant rsc3 td clones (n = 72) showed efficient ploidy doubling in the clb5Δ background (74%; Figure 6) in contrast to control constructs. Conversion of SFH1 to sfh1 td in the clb5Δ background could also produce endodiploid clones, though at a much lower frequency (1%, Figure 6). Taken together, this indicates that the cellular rereplication inhibition pathway that depends on CLB5 and CDC6 [18,52] antagonizes the effects of the degron alleles of RSC3 and SFH1.

RSC and Transcriptional Activity of the CLB5 Locus

Previous observations indicate that RSC is recruited to the CLB5 promoter [27], and CLB5 induction was observed in microarray experiments using a rsc3 allele [53]. To further assess the role for RSC in regulation of CLB5 expression, impaired S-phase progression by exposure to hydroxyurea (HU), an inhibitor of deoxyribonucleotide synthesis. HU treatment activates the S-phase checkpoint that signals through Rad53p and phosphorylation of various targets, including Sth1p, thus culminating in inhibition of S-phase progression [54-56]. Following exposure to HU for 3 h we monitored association of RSC with a number of loci by Sth1p TAP chromatin immunoprecipitation (Figure 7A), and we assessed expression of CLB5 and TPS3 (Figure 7B). HU treatment resulted in up to 3-fold increased association of Sth1p with the CLB5 promoter (Figure 7A), concomitant with repression of CLB5 expression (Figure 7B), much as reported for HTA1 (Figure 7A, [27]). The increased association of RSC complexes with the CLB5 and HTA1 promoters upon HU treatment was specific, as no such effects were observed at TPS3, FUR4, CEN4, at an ORF-free chromosomal element on Chromosome I (ORF-FREE) or in the CLB5 ORF (CLB5-ORF, Figure 7A and 7B). Taken together, these results correlate increased binding of RSC to the CLB5 promoter with inactivation of this locus upon HU treatment (Figure 7A and 7B) and further implicate RSC in transcriptional control of CLB5 expression.

Discussion

The RSC ATP-dependent nucleosome remodeling complex [20] encompasses 17 subunits, and the mutually exclusive paralogs Rsc1p and Rsc2p define two RSC isoforms [57]. The Rsc3p/Rsc30p heterodimer [20,53] preferentially associates with the Rsc1p-bearing RSC isoform (Campsteijn et al., unpublished data). Here, we analyzed RSC requirement...
during the course of the cell cycle using conditional degradation alleles (N-degrons) of all essential RSC-specific subunits. We find that RSC controls cell-cycle progression at multiple stages of the cell cycle and uncovered a strong genetic interaction between RSC and cyclin-dependent kinase 1 (Figure 4).

**RSC Functions in G2/M**

We temporally dissected the mitotic requirement for RSC by depleting RSC subunits from cells harboring G2 or mitosis checkpoint mutations (Figure S3). In keeping with a role for RSC in G2 and mitotic prophase, RSC degron alleles synergized with overexpression of the G2/M transition regulator **SWE1** [43,44], and the same RSC alleles were partially epistatic to a degron allele of the spindle checkpoint factor **CDC20** [58] (Figure S3). On the other hand, a degron allele of the mitotic exit network kinase **CDC15** [59] weakly suppressed the lethal effects of those same RSC subunit degron alleles (Figure S3). Collectively, these results indicate that RSC activity is central to achieving a proper mitosis and that RSC appears to be somewhat more important before the metaphase/anaphase transition than afterward (Figure S3).

While these results are consistent with published reports, it remains to be seen whether the essential role of RSC in G2 and in mitosis relates to a role for RSC in gene expression [27,28,35,53], in higher order chromatin structure [32–34,60,61], or both.

**RSC Functions in G1**

Several lines of evidence provided here argue that RSC functionally intersects with regulation of the G1/S-phase transition. First, cells deprived of RSC arrest in G1 (Figures 1 and 2). Second, we and others [27] find that RSC associates with several **MluI** cell cycle box-binding factor (MBF) targets including the **HTA1/HTB1** and **CLB5** promoters (Figure 7, unpublished data). Both **HTA1/HTB1** and **CLB5** are expressed during the G1/S transition and association of RSC correlates with transcriptional inactivity of these loci (Figure 7, [27]).

Third, we discovered that the **rsc3Δ** allele is synthetic lethal with a deletion allele of the cyclin **CLB5** when combined via meiotic segregation (Figure 5). When these two alleles were combined by endogenous locus conversion through DNA transformation, surviving clones could be recovered, however, and the resulting **clb5Δ, rsc3Δ** strains underwent integral ploidy increases (Figure 6). This was also the case when the replication origin licensing factor Cdc6p was transiently depleted (Figure 6). As these genes are crucial for G1/S-phase transition, this very strongly suggests that RSC plays an important role in ploidy maintenance when this stage of the cell cycle is perturbed.

Consistent with this notion, RSC has been reported to interact physically with Swi6p [62], a component of the central heterodimeric G1/S transcription regulators MBF (with Mbp1p) and SBF (with Swi4p), which are considered to be the functional analogs of mammalian E2Fs [63]. Finally,
rsc1 cells were shown to display a large cell phenotype that is indicative of impaired cell-cycle entry as has been observed in cln3, beh2, swi4, and swi6 strains [64].

RSC Has a Facultative Role in Ploidy Maintenance

The endocycle phenotype we observe in sfh1 td, rsc3 td double mutants underscores the important role of RSC in proper cell-cycle progression. The endocycles occur under conditions when the degron fusions were least affected since the levels of the degron-activating ubiquitin ligase Ubr1p were repressed by glucose and since the yeast were kept at 25 °C to keep the DHFR ts degron fragment folded (Figure 3A) [40,41]. The ploidy shifts must therefore arise from rather subtle changes that keep the DHFRts degron fragment folded (Figure 3A) [40,41]. Further, we found that Rsc3p is actively degraded in late S-phase upon Ubr1p overexpression (Figure S4). Furthermore, we note that Sfh1p is phosphorylated during G1 and this is thought to compromise necessary oscillations in expression pattern of multiple MBF target genes, resulting in reduced cell-cycle phase identity, and, under specific environmental conditions, in ploidy shifts.

Our experiments suggest that both CLB5 deletion (Figure 6) and CLB5 derepression (Figure 7) could aid in the induction of ploidy shifts. These opposing observations can be reconciled by the requirement for simultaneous deregulation of multiple MBF-target genes to observe ploidy doublings, as well as by the fact that Clb5p is required for both activation and inactivation of pre-replication complexes [48,52,67–69]. As such, diminished or untimely oscillation in expression level rather than over- or underexpression would result in ploidy shifts, a phenomenon that has been reported for the S-phase cyclin E in Drosophila nurse nuclei [3]. This hypothesis is consistent with the observation that hyperstabilization of CLB3 mRNA suffices to induce ploidy shifts [52].

Finally, we note that CDC6 expression, which normally peaks during late mitosis, has been reported to peak in a MBF-dependent fashion at the G1/S transition only in cells that have not undergone a recent mitosis [49,70]. As this would be the case following spore germination or cell transformation by the lithium procedure, this may therefore account for the single round of ploidy shifts observed here and for the observed lack of RSC-association with the CDC6 promoter in cycling cells (unpublished data).

Role of the P gal:UBR1 Allele in Ploidy Shifts

It is known that Ubr1p participates in cohesin degradation in mitosis [42]. However, our results indicate that the effects of the P gal:UBR1 allele in our experiments were largely mediated through Ubr1p’s role in polyubiquitylation of the N-terminal degron fusions we studied. For instance, repressing Ubr1p levels suppressed rather than enhanced the occurrence of ploidy shifts in rsc3 td, sfh1 td strains (Table 2). Furthermore, repressing Ubr1p expression through P gal:UBR1 suppressed rather than enhanced the synthetic lethal interaction between rsc3 td and clb5 A (Figure 5B). However, since the dominant meiotic-lethal phenotype displayed by double heterozygous rsc3 td, sfh1 td sfh1, P gal:UBR1/Lp gal:UBR1 diploids was due to repression of UBR1 expression (Table 1), our results do suggest that RSC and Ubr1p are part of genetic pathways that are redundant to some extent or that form one large pathway in meiosis.

Conclusion

Our experiments indicate that the rsc3 td and sfh1 td degron alleles interfere in synergistic ways with cell-cycle progression resulting in environmentally conditioned ploidy shifts. These results formally implicate RSC in ploidy maintenance. The RSC complex has previously been implicated in multiple
molecular processes, including regulation of chromatin cohesion [32], DNA damage response [29-31], nucleocytoplasmic transport [28,71], and transcription control [27,28,55,57,72]. Furthermore, and underscoring the complexity of RSC function, viable RSC subunit deletion strains have been identified that display long (npl6A, hfl1A, and ldb7A) or short (rsc2A) telomeres, hinting toward ambivalent roles for RSC in maintenance of telomere length [73,74], a process that occurs in late S-phase [75]. In light of the pleiotropic physiological functions of RSC, further dissecting RSC-mediated ploidy control will require a detailed understanding of the roles and modes of regulation of individual RSC subunits, as well as understanding the functional interplay of the various processes that rely on RSC.

The functions we ascribe here to RSC, namely ploidy maintenance and control of G1/S-phase transition, appear conserved for human RSC-like complexes [76-81]. Interestingly, it has been shown that mutant forms of the human ortholog of Sfh1, the tumor suppressor INI1/hSNF5 [38,39,82,83], can induce the appearance of tetraploid cells [76,81]. Thus, an ancient RSC-dependent ploidy doubling inhibition mechanism may have been recruited in the course of animal evolution to avert cancer.

Materials and Methods

Yeast strains, plasmids, and culturing. With the exception of the S288c mbp1A strain (Figure S5) and the S288c cyclin deletion strains (Figure 5), all the yeast used here were descendants of W303 strains. Deegrons were introduced in diploid yeast (YN106) by ends-in homologous recombination of plasmids at the endogenous loci (Table S1; details are available on request). Verification of the integration events was based on PCR analysis and western blot detection of the modified gene products. For sporulation, diploids were grown overnight on YEP-10% glucose, with or without 0.1 mM CuSO4, and incubated at 25 °C or 37 °C. At an indicated times, 5 µl of cells and 5- or 10-fold serial dilutions were spotted onto YEP plates supplemented with 2% glucose, 40 µg/ml adenine, and 0.1 mM CuSO4 at 25 °C. The cells were then seeded into a second overnight culture in YEP supplemented with 2% galactose. For deletion, cells were diluted to 2×10^6 cells/ml into YEP 2% galactose, with or without 0.1 mM CuSO4, and incubated at 25 °C or 37 °C. At an indicated times, 5 µl of cells and 5- or 10-fold serial dilutions were spotted onto YEP plates supplemented with 2% glucose, 40 µg/ml adenine, and 0.1 mM CuSO4. The plates were incubated at 25 °C and pictures were taken after 2-4 d. For DNA damage experiments (Figure 7), cells were cultured in nonselective medium at 30 °C (to an optical density of 0.6, followed by exposure to 150 mM HU (Sigma-Aldrich, http://www.sigmaldrich.com) for 3 h.

Flow cytometry analysis. Cells were grown in nonselective medium overnight, pelleted, and collected into 70% ethanol and kept at -20 °C. Subsequently, cells were suspended into 50 mM sodium citrate, sonicated briefly, treated for 2 h with 0.2 mg/ml RNase A at 37 °C, and DNA was stained with 1 µg/ml Sytox dye (Molecular Probes, http://www.probes.invitrogen.com). DNA content was quantified at FL1 on a Becton-Dickinson (http://www.bd.com) Calibur fluorescence activated cell sorter.

Chromatin immunoprecipitation, RNA extraction, and quantitative PCR. Chromatin was prepared as described [84] with several modifications. Cells (20-40 ml) were treated with 1% formaldehyde for 15-20 min at room temperature under constant rotation. Glycine was added to a final concentration of 330 mM and incubation continued for an additional 5-10 min. Cells were gently washed three times with 0.15 M NaCl and 1% SDS. The remaining cell pellet was re-suspended in lysis buffer (FA-lysis buffer complemented with 1% Triton X-100 and 0.5% SDS) and crosslinking was done for 4-5 h at 65 °C, and DNA was purified by phenol extraction followed by ethanol precipitation in the presence of 20 µg glycogen.

RNA was extracted with hot acid-phenol: chloroform and cDNA synthesis was carried out using 2 µg of total RNA. Quantitative PCR was performed in an E-Free Radial (http://www.bio-rad.com) MxIQ Single Color Real-Time PCR Detection System using a 2X iQ SYBR Green Supermix. For ChIP, 1/50 of the immunoprecipitated material was used and abundance of immunoprecipitated fragments was compared to 1% input. For cDNA, 1/25 of total cDNA was used and values were normalized as indicated.

Supporting Information

Figure S1. Depletion of Rsc8pΔ Compromises RSC Integrity

A rsc8Δ sth1Δ strain (YN438) was grown overnight in YP-Gal medium containing 0.1 mM CuSO4 at 25 °C and was subsequently shifted to YP-Gal medium at 37 °C to induce degradation of Rsc8pΔ. Aliquots were harvested at the indicated time points and equal amounts of whole cell extracts were analyzed by western blot. Rsc8pΔ was visualized using anti-HA mouse antibody and Sth1pTAP using peroxidase-conjugated anti-peroxidase rabbit antibody (Sigma-Aldrich).

Found at doi:10.1371/journal.pgen.0030092.sg001 (786 KB PDF).

Figure S2. Simultaneous Depletion of Multiple RSC Degrons Invariably Yields G1 and G2/M Arrests

Indicated strains were incubated under nonpermissive conditions for 4 h after which DNA content was determined by FACS analysis. To assess colony-forming potential, strains were incubated for 2, 6, 9, or 12 h under nonpermissive conditions, after which 5-fold serial dilutions of the cultures were spotted on YP-glucose plates containing 0.1 mM CuSO4 followed by incubation at 25 °C and photography (inset).

Found at doi:10.1371/journal.pgen.0030092.sg002 (355 KB PDF).

Figure S3. RSC Requirement after Replication

(A-C) Strains harboring the indicated rscΔ alleles and/or the conditional cell division cycle alleles for cdc15, cdc20, or swe1 were cultured as described (Materials and Methods), followed by shift to 37 °C in galactose medium lacking CuSO4. Aliquots of these cultures were seeded under permissive conditions at the indicated time points.

(D-F) Indicated strains were incubated for 9 h under nonpermissive conditions, after which cellular DNA content was assessed by FACs analysis.

Found at doi:10.1371/journal.pgen.0030092.sg003 (3.7 MB PDF).

Figure S4. Sfh1pΔ Association with RSC Depends on Ubr1p Levels

RSC was purified using an STH1TAP allele from wild-type (YN400) or sfh1Δ (YN435) strains following overnight culturing at 25 °C in the presence of CuSO4 in glucose (Glu) or galactose (Gal) media to repress or overexpress Ubr1p, respectively. Equal amounts of RSC were loaded in each lane. The position of Sfh1pΔ (empty arrowhead) is indicated. All strains used in this figure contain the Pgal::UBR1 allele. Note that loss of Sfh1Δ did not perceptibly affect complex integrity.

Found at doi:10.1371/journal.pgen.0030092.sg004 (532 KB PDF).

Figure S5. Implication of Mbp1p in RSC-Mediated Ploidy Shifts

S288c mbp1Δ cells were transformed with the rscΔ strain endogenous locus conversion construct. The DNA content of cells from one clone is shown. Note the presence of 4C and 8C cells, indicative of continuous endopolyploidization.

Found at doi:10.1371/journal.pgen.0030092.sg005 (69 KB PDF).

Table S1. Fertility of Diploids Generated by Mating of Various rscΔ Strains

Diploids generated by mating the indicated haploids were considered fertile (+) when over 80% of spores were able to form haploid colonies. Less than 10% of spores from a sfh1Δ, rsc3Δ diploid were able to form colonies (−). Not all combinations were generated, as indicated by NT (not tested).
Ploidy Control by RSC

Found at doi:10.1371/journal.pgen.0030092.s001 (30 KB DOC).

Table S2. Yeast Strains Employed by Campsteijn et al.

Notes: (i) All the yeast strains we generated are descendents of the W303-derived strain YN2; (ii) The deletion alleles were first introduced into the YN16 diploid strain and haploid strains were obtained by sporulation; (iii) Lysine auxotrophy was not systematically verified. When a strain is ‘lys–’ it harbors the Δlys2::KanMX50 allele [85].

Found at doi:10.1371/journal.pgen.0030092.s002 (96 KB DOC).

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