Characterization of the molecular crosstalk within the essential Grc3/Las1 pre-rRNA processing complex

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ABSTRACT
Grc3 is an essential well-conserved eukaryotic polynucleotide kinase (PNK) that cooperates with the endoribonuclease Las1 to process the preribosomal RNA (rRNA). Aside from being dependent upon Las1 for coordinated kinase and nuclease function, little is known about Grc3 substrate specificity and the molecular mechanisms governing kinase activity. Here we characterize the kinase activity of Grc3 and identify key similarities and differences between Grc3 and other polynucleotide kinase family members. In contrast to other PNK family members, Grc3 has distinct substrate preference for RNA substrates in vitro. By disrupting conserved residues found at the Grc3 kinase active site, we identified specific residues required to support Grc3-directed Las1-mediated pre-rRNA cleavage in vitro and in vivo. The crosstalk between Grc3 and Las1 ensures the direct coupling of cleavage and phosphorylation during pre-rRNA processing. Taken together, our studies provide key insight into the polynucleotide kinase activity of the essential enzyme Grc3 and its molecular crosstalk with the endoribonuclease Las1.

Keywords: polynucleotide kinase; Grc3; Las1; RNA processing; crosstalk

INTRODUCTION
Polynucleotide kinases (PNKs) are enzymes that play fundamental roles in numerous cellular processes including the repair of DNA, as well as the processing, maturation, repair, and degradation of RNA (Weinfeld et al. 2011; Das et al. 2014; Dikfidan et al. 2014; Remus et al. 2016). PNKs target the 5′-terminus of DNA and RNA substrates and catalyze the transfer of the γ-phosphate from nucleoside triphosphate (NTP) to the 5′-hydroxyl terminus of the polynucleotide substrate. The 5′-phosphorylation status of DNA and RNA is critically important in the cell, thus underscoring the need for the PNK family of enzymes (Peach et al. 2015). For example, phosphorylation of 5′-hydroxyl termini following DNA damage is required for subsequent DNA repair (Weinfeld et al. 2011); whereas RNA turnover by the eukaryotic Xrn 5′-exonuclease family requires a 5′-phosphate for RNA degradation (Heindl and Martinez 2010; Jinek et al. 2011).

All known PNKs are members of the P-loop NTPase superfamily, yet each member has distinct substrate specificity. Bacteriophage T4 PNK is the founding member of the PNK family and is a bifunctional enzyme that harbors both PNK and phosphatase domains for repairing the 5′ and 3′-ends of damaged tRNA (Richardson 1965; Cameron and Uhlenbeck 1977). Furthermore, T4 PNK can efficiently phosphorylate both DNA and RNA substrates in vitro; a feature that has turned T4 PNK into a molecular biology workhorse for labeling DNA/RNA substrates (Richardson 1965; Wang and Shuman 2001; Galburt et al. 2002; Wang et al. 2002). Mammalian polynucleotide kinase/phosphatase (mPNKP) is another well-studied PNK family member which is required for DNA repair and is specific for damaged DNA substrates (Bernstein et al. 2005, 2009). There are also several PNK family members which target RNA in vivo including the bacterial Pnkp/Hen1 complex, which is required for bacterial RNA repair (Wang et al. 2012, 2015); Trl1, which is involved in tRNA maturation (Sawaya et al. 2003); and the Clp1/Grc3 subfamily, which is involved in multiple RNA processing pathways (Dikfidan et al. 2014; Weitzer et al. 2015).

The Clp1/Grc3 subfamily includes homologs of two distinct PNK enzymes, Clp1 and Grc3. In contrast to other well-characterized PNK enzymes, Grc3 and Clp1 are not bifunctional and they lack a phosphatase domain (Galburt et al. 2002). Clp1 is evolutionarily well-conserved across eukaryotes and homologs have been identified in archaea (Shuman and Hurwitz 1979; Jain and Shuman 2009; Weitzer et al. 2015). Clp1 has been implicated in at least three RNA pathways including mRNA, tRNA, and siRNA processing (Weitzer and Martinez 2007; Holbein et al. 2011; Haddad et al. 2012; Hanada et al. 2013; Karaca et al. 2014; Schaffer...
et al. 2014; Weitzer et al. 2015; Salzman et al. 2016). In contrast to Clp1, much less is known about Grc3 (No19 in mammals), which is required for marking preribosomal RNA (rRNA) through 5′-phosphorylation for degradation by the Rat1 (Xrn2 in mammals) 5′-exonuclease (Braglia et al. 2010; Heindl and Martinez 2010; Castle et al. 2012, 2013; Gasse et al. 2015; Pillon et al. 2017).

One major distinguishing feature between Clp1 and Grc3 is that Grc3 is reliant on its binding partner, the endoribonuclease Las1, for kinase activity (Pillon et al. 2017). Las1 is the endoribonuclease responsible for cleavage at the C2 site during pre-rRNA processing (Gasse et al. 2015). C2 cleavage is a critical step during the middle stages of ribosome assembly that triggers the removal of the internal transcribed spacer 2 (ITS2) that lies between the 5.8S and 25S rRNA (Konikkat and Woolford 2017; Konikkat et al. 2017). Failure to remove the ITS2 results in the generation of aberrant 60S particles with translational defects (Sarkar 2017). Failure to remove the ITS2 results in the generation of aberrant 60S particles with translational defects (Sarkar 2017). Failure to remove the ITS2 results in the generation of aberrant 60S particles with translational defects (Sarkar 2017). Failure to remove the ITS2 results in the generation of aberrant 60S particles with translational defects (Sarkar 2017).

To confirm that Grc3/Las1 uses the γ-phosphate of ATP for its PNK activity, we asked whether ADP or the non-hydrolyzable ATP analog, ADP•P, could support RNA phosphorylation in the absence of ATP. Similar to other members of the P-loop NTPase superfamily (Wang et al. 2012; Das et al. 2014; Dikfidan et al. 2014). To assess the requirements for metal ions, we set up RNA phosphorylation reactions with 2 μM Grc3/Las1, 15 μM of a fluorescently labeled single-stranded RNA substrate and 1 mM ATP in the presence of 15 mM divalent metal or 5 mM EDTA. RNA phosphorylation was measured with a labeled 5′-hydroxyl 21-mer single-stranded RNA substrate that we previously determined was refractory to Las1 cleavage and has been previously used to study human Clp1 (Weitzer and Martinez 2007; Pillon et al. 2017). These reactions were incubated for 60 min at 37°C, quenched with loading dye and urea, and resolved on sequencing gels (Fig. 1A). Grc3/Las1-mediated RNA phosphorylation was supported in the presence of magnesium, but was significantly hindered with excess EDTA (Fig. 1A). We explored additional divalent metals to determine the metal selectivity of Grc3/Las1. Metals with typical octahedral coordination such as Mg2+, Mn2+, Ca2+, and Ni2+ supported Grc3/Las1 PNK activity in vitro, whereas Zn2+, which is often bound through tetrahedral geometry, could not support catalysis under the experimental conditions examined (Fig. 1A).

Next, we determined the NTP/dNTP specificity of Grc3. P-loop motifs are a hallmark for NTP binding, often responsible for engaging ATP or GTP (Saraste et al. 1990), and PNK family members have a wide variety of NTP donor specificities (Munir and Shuman 2017). To determine the NTP specificity of the Grc3/Las1 complex, we carried out RNA phosphorylation reactions in the presence of 1 mM ATP, GTP, CTP, UTP, dATP, dGTP, dCTP, and dTTP. Reactions were incubated for 60 min at 37°C with 2 μM Grc3/Las1 and 15 μM of a fluorescently labeled single-stranded RNA substrate. The reactions were quenched with loading dye supplemented with urea and resolved on sequencing gels (Fig. 1B). We did not detect phosphorylation in the absence of nucleotide and observed that Grc3/Las1 can use any NTP or dNTP as the phosphate donor (Fig. 1B). Next, we analyzed the nucleotide specificity of Grc3/Las1 by repeating our phosphorylation reactions with titrations of ATP, GTP, CTP, UTP, dATP, dGTP, dCTP, and dTTP. Reactions were incubated for 60 min at 37°C with 2 μM Grc3/Las1 and 15 μM of a fluorescently labeled single-stranded RNA substrate. The reactions were quenched with loading dye supplemented with urea and resolved on sequencing gels (Fig. 1C). The trend for nucleotide preference corresponds to the variability in binding affinity as measured by the Michaelis constant (Km) while the catalytic constant (Kcat) remained largely unchanged (Fig. 1D).

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We investigated RNA phosphorylation activity of Grc3/Las1 on a mimic of the in vivo substrate. The pre-rRNA sequence harboring the C2 site forms a predicted stem–loop structure (van Nues et al. 1995a,b; Pillon et al. 2017). Las1 cleaves the C2 site following a bulged adenine residue, therefore we created a substrate with the local sequence and structure to mimic the Las1 cleavage product (Fig. 3A; Grc3 primed). To avoid multiple phosphorylation sites, we engineered a loop at the top of the stem to remove the free 5′ end. In the presence of the cleaved C2 RNA mimic, Grc3/Las1 displayed RNA phosphorylation at the C2 site with a stronger binding affinity ($K_m$) than with the nonspecific single-stranded RNA substrate (Fig. 1C, D versus Fig. 3B). Finally, we mixed equimolar amounts of the nonspecific 21-mer with the cleaved C2 mimic and measured RNA phosphorylation over a titration of Grc3/Las1. Grc3/Las1 shows a clear preference for the C2 mimic over the nonspecific 21-mer. The specific activity of Grc3/Las1 was calculated from the linear range of the protein titration to show a 3.5-fold higher specific activity in phosphotransferase activity toward the C2 mimic (Grc3 primed RNA: 1.1 min$^{-1}$) relative to the nonspecific 21-mer (ssRNA: 0.31 min$^{-1}$), suggesting that Grc3/Las1 has selectivity for the cleaved C2 substrate over the nonspecific substrate (Fig. 3C).

**Identification of conserved motifs within the PNK domains from the Clp1/Grc3 family**

After establishing the requirements for Grc3 phosphotransferase activity, we next sought to understand the importance of conserved features within the PNK domain of Grc3, which shares ~20% sequence identity with Clp1 and is flanked by distinct N- and C-terminal domains (Fig. 4A). Recent high-resolution structures of Clp1 from *Caenorhabditis elegans* in complex with ATP analogs and dinucleotide RNA substrates provide snapshots of this enzyme in several steps along the kinase reaction pathway revealing the presence of four key structural motifs important for catalysis and RNA specificity (Dikfidan et al. 2014). Multiple sequence alignments across Grc3 and Clp1 homologs revealed that these four motifs, including the P-loop, Walker B, Clasp, and Lid, are mostly well-conserved within the Grc3 and Clp1 homologs discovered (Fig. 4B; Dikfidan 2013). The P (phosphate binding) motif (also known as the Walker A motif), contains the consensus sequence, GxxxxGK[T/S] (where x is any residue), and is characteristic of proteins that bind and hydrolyze ATP or GTP (Saraste et al. 1990). The lysine residue within this motif is traditionally thought to be important for nucleotide binding, however crystal structures of Clp1 suggest that within Clp1 this lysine residue (K127 in Clp1) serves as a molecular switch that regulates ATP turnover (Dikfidan et al. 2014). The Walker B motif (D/E hhQ, where h is a hydrophobic residue) follows the P-loop and contains a well-conserved D/E residue essential for hydrolysis that is often referred to as the catalytic base (Koonin 1993). Within the Walker B motif from the Clp1/ Grc3 family there is also a well-conserved glutamine residue which
interacts with the 5′–3′ bridging phosphate group of the ultimate base and is important for hydrolysis (Dikfidan et al. 2014). Downstream lies the Clasp motif [T/S/L]xGW important for RNA specificity and unique to the Clp1/Grc3 family (Fig. 4B; Dikfidan et al. 2014). The final feature of the PNK domain is the Lid module (Fig. 4B), encoding arginine residues that in Clp1 stabilize the transition state (Dikfidan et al. 2014). Unlike the P-loop, Walker B, and Clasp motifs; the Lid module is not as well-conserved across Clp1 and Grc3 homologs and its functional significance within Grc3 is unknown. To visualize the S. cerevisiae Grc3 PNK active site, we constructed a homology model using the PNK domain from the S. cerevisiae Clp1 crystal structure (PDB ID 2NPI) (Noble et al. 2007). All four conserved Grc3 PNK motifs cluster in space to form a continuous and distinct active site (Fig. 4C) suggesting that these residues are important for Grc3’s PNK activity.

Grc3 PNK motifs are essential in S. cerevisiae

To investigate the roles of the P-loop, Walker B, Clasp, and Lid motifs for cell viability in S. cerevisiae, we generated a series of single and double Grc3 PNK domain mutants to well-conserved residues within the: P-loop (K252, S253), Walker B (D283, Q286), Clasp (L361, W364), and Lid (R433, R440) (Table 1; Fig. 4C). We then performed genetic complementation assays using a S. cerevisiae strain encoding the tetracycline-inducible promoter (tetO7) upstream of endogenous GRC3. Prior to carrying out complementation assays, we modified the Grc3 tetO7 strain by the addition of a 5X-Flag tag to the C terminus of endogenous Las1 for downstream detection purposes. Addition of doxycycline (DOX) represses expression of endogenous Grc3 leading to the suppression of cell growth (Fig. 5A; Castle et al. 2013). We first tested the complementation of Grc3 WT, with and without an N-
terminal 3X-Myc tag in the host strains by repressing endogenous GRC3 expression with DOX at 30°C. Yeast expressing Grc3 WT from the ARS1-CEN4 YCplac vector (Gietz and Sugino 1988) grew well in the presence of doxycycline while the empty YCplac vector could not restore growth in any of the conditions tested (Fig. 5B). Addition of a 3X-Myc tag to WT Grc3 (Grc3 versus Myc-Grc3) or a 5X Flag tag to endogenous Las1 (tet-Grc3 versus tet-Grc3/Las1-Flag strains) did not alter growth (Fig. 5B). Thus, all subsequent variants of Grc3 included a 3X-Myc tag (Table 1), unless specified otherwise.

Variants of GRC3 harboring missense mutations at conserved residues within the P-loop (K252A/S253A, K252A, K252R, S253A), Walker B (D283A/Q286A, D283A, D283N, D283E, Q286A), Clasp (L361A/W364A, L361A, L361I, W364A), and Lid (R433A/R440A, R433A, R433K, R440A, R440K) were expressed from the yeast ARS1-CEN4 YCplac vector transformed into tetO7-GRC3-Las1-5XFlag (Table 2).

We tested the complementation of these Grc3 PNK variants in the host strain by repressing endogenous GRC3 expression with DOX followed by serial dilution plating and growth at 30°C (Fig. 5B). Thus, all subsequent variants of Grc3 included a 3X-Myc tag (Table 1), unless specified otherwise.

Disruption of the conserved K[T/S] residues within the P-loop had a severe growth defect that could not be restored even with the conservative single K252R mutation (Fig. 5C), highlighting the importance of these residues within the P-loop. Mutation of the aspartic acid residue (D283) within the Walker B motif to either an alanine, glutamic acid, or asparagine residue had a significant growth defect, while mutation of the Walker B glutamine residue (Q286) to alanine, only resulted in a minor growth defect (Fig. 5C,D). Disruption of the Clasp motif also resulted in a severe growth defect, as yeast expressing the Clasp mutants (L361A/W364A, L361A) could not restore growth in the presence of DOX (Fig. 5C,D). Replacement of L361 with the conservative isoleucine mutation could restore growth to almost wild-type levels, while a single mutation of W364A had a moderate effect on cell growth (Fig. 5C,D). In contrast to the P-loop, Walker B, and Clasp motifs, expression of Grc3 with mutations to the Lid motif restored cell proliferation under the experimental conditions tested by serial dilution plating, suggesting that the Lid motif plays a less significant role in Grc3 function in vivo (Fig. 5C). To further assess the role of the Lid motif,
we measured growth curves on the single arginine mutants (Fig. 5D). Mutation of the first arginine residue (R433A) within in the Lid motif had no effect on cell growth while mutation of the second arginine residue within the Lid motif (R440A) had a mild effect on rates of cell proliferation (Fig. 5D). This corresponds well with sequence alignment of the Lid motif highlighting the lack of conservation for R433 and semi-conservation of R440 (Fig. 4B). The doubling time for the Lid double mutant (R433A, R440A: 170 min) in YPD supplemented with DOX was slightly longer than the WT strain (140 min) emphasizing the subtlety of this growth defect.

The mild growth phenotype seen by mutagenesis of the Walker B motif at conserved residue Q286 and arginine residues had no effect on cell growth and proliferation, confirming the subtlety of this growth defect.

Grc3 PNK motifs do not disrupt Las1 interaction

To confirm that our Grc3 PNK variants did not compromise Grc3 stability or disrupt Las1 binding, we carried out a series of coimmunoprecipitation experiments. Grc3 PNK variants were grown in the tetO7-GRC3-Las1-5XFlag strain (Table 2) to mid-log phase in the absence and presence of DOX at 30°C. Cells were harvested, lysed, run on SDS-PAGE gels, and analyzed by western blot. Grc3 was detected using an anti-Myc antibody, while endogenous Las1 was detected with an anti-Flag antibody and tubulin was used as a loading control. The addition of DOX to the medium to repress endogenous Grc3 did not significantly impact the expression of endogenous Las1 with the exception of the vector only control (Fig. 7A versus 7B). This confirms earlier reports that Grc3 and Las1 are dependent upon one another for protein stability (Castle et al. 2013). Moreover, the presence or absence of DOX did not affect the protein levels of the Grc3 PNK variants, indicating that the PNK mutations do not severely impact the stability of Grc3 in S. cerevisiae. Finally, cells grown in the presence of DOX were coimmunoprecipitated with Myc-tagged Grc3. All Grc3 PNK variants were able to pull down endogenous Las1, indicating that these mutations do not hinder Las1 binding in S. cerevisiae (Fig. 7B). Taken together, these data suggest the growth defects observed upon disrupting the Grc3 P-loop, Walker B, Clasp, and Lid motifs are not due to instability or Las1 dissociation, but a defect in Grc3 function.

**Grc3 PNK motifs are essential for RNA phosphorylation in vitro**

After establishing the significance for the Grc3 P-loop, Walker B, Clasp, and Lid motifs for cell viability in *S. cerevisiae*, we wanted to determine their role in RNA phosphorylation in vitro. First, we generated a series of double mutations to the PNK motifs in an *Escherichia coli* expression vector (Table 3). The Grc3 PNK variants were coexpressed with full-length poly-histidine tagged Las1 in *E. coli* and purified

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### TABLE 1. Yeast plasmids used in this study

| Plasmid | GRC3 | Vector | Source |
|---------|------|--------|--------|
| pMP 003 | WT; residues 1–632 | YCplac111 | This study |
| pMP 483 | WT; residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 484 | P-loop (K252A, S253A); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 486 | P-loop (K252A); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 487 | P-loop (K252R); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 488 | P-loop (S253A); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 485 | Walker B (D283A, Q286A); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 489 | Walker B (D283A); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 490 | Walker B (D283N); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 491 | Walker B (D283E); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 492 | Walker B (Q286A); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 512 | Clasp (L361A, W364A); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 493 | Clasp (L361A); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 494 | Clasp (L361I); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 507 | Lid (R433A, R440A); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 496 | Lid (R433A); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 497 | Lid (R433A, R433K); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 498 | Lid (R440A); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |
| pMP 499 | Lid (R440K); residues 1–632, 3X N-terminal Myc Tag | YCplac111 | This study |

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**FIGURE 5.** Grc3 PNK active site motifs are critical in *S. cerevisiae.* (A) Schematic of tetracycline-off system. In the presence of doxycycline (DOX), the tetracycline-transcription activator (tTA) cannot bind the tetracycline response element (TRE); thus repressing transcription of endogenous *GRC3.*

(B) *S. cerevisiae tetO7-GRC3* and *S. cerevisiae tetO7-GRC3–Las1 5X Flag* strains were transformed with plasmids encoding wild-type Grc3 with and without an N-terminal Myc-tag and the ARS1-CEN4 YCplac vector. Serial dilutions were spotted on YPD agar plates in the absence and presence of DOX (20 µg/mL) and incubated at 30°C for 2–3 d.

(C) *S. cerevisiae tetO7-GRC3–Las1 5X Flag* were transformed with plasmids encoding variants of the Grc3 PNK active site motifs (P-loop [red], Walker B [blue], Clasp [purple], Lid [orange]). Serial dilutions were spotted on YPD agar plates in the absence and presence of doxycycline (20 µg/mL) and incubated at 30°C for 2–3 d.

(D) Growth curves from selected Grc3 PNK variants grown in the absence or presence of DOX (20 µg/mL) at 30°C. The absorbance at 595 nm was recorded over a 25-h time period. Each curve is the average of three independent replicates and error bars mark the standard deviation.
using Ni²⁺-affinity chromatography followed by gel filtration (Fig. 8A). All four double Grc3 PNK variants bound by Las1 were stably expressed and eluted similarly by gel filtration when compared to wild-type (WT) Grc3/Las1, confirming our in vivo results which showed that these mutations do not disrupt or destabilize the interaction between Grc3 and Las1 (Fig. 7B). Moreover, we measured the stability of the Grc3/Las1 PNK variants by thermal shift, and did not

| Strain | Genotype | Source |
|--------|----------|--------|
| tetO₇-Grc3 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA MATa his3-1 leu2-0 met15-0 | Tet-promoter Hughes Collection (GE Dharmacon) |
| tetO₇-Grc3 + pMP 003 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA MATa his3-1 leu2-0 met15-0; pMP003 (Grc3 WT) | This study |
| tetO₇-Grc3 + pMP 483 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA MATa his3-1 leu2-0 met15-0; pMP 483 (3Myc-Grc3; WT) | This study |
| tetO₇-Grc3 + pMP 484 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA MATa his3-1 leu2-0 met15-0; pMP 484 (3Myc-Grc3; P-loop [K252A/S253A]) | This study |
| tetO₇-Grc3 + pMP 485 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA MATa his3-1 leu2-0 met15-0; pMP 485 (3Myc-Grc3; Walker B [D283A/Q286]) | This study |
| tetO₇-Grc3 + pMP 512 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA MATa his3-1 leu2-0 met15-0; pMP 512 (3Myc-Grc3; Clasp [L361A/W364A]) | This study |
| tetO₇-Grc3 + pMP 507 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA MATa his3-1 leu2-0 met15-0; pMP 507 (3Myc-Grc3; Lid [R433A/R440A]) | This study |
| tetO₇-Grc3 Las1-5Flag | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0 | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 483 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 483 (3Myc-Grc3 WT) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 484 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 484 (3Myc-Grc3; P-loop [K252A, S253A]) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 486 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 486 (3Myc-Grc3; P-loop [K252A]) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 487 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 487 (3Myc-Grc3; Walker B [D283A]) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 488 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 488 (3Myc-Grc3 WT; P-loop [S253A]) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 489 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 489 (3Myc-Grc3; Walker B' [D283N]) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 490 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 490 (3Myc-Grc3; Walker B [D283E]) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 491 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 491 (3Myc-Grc3; Walker B [D283N]) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 492 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 492 (3Myc-Grc3; Walker B [Q286E]) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 512 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 512 (3Myc-Grc3; Clasp [L361A, W364A]) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 493 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 493 (3Myc-Grc3; Clasp [L361A]) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 494 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 494 (3Myc-Grc3; Clasp [L361K]) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 495 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 495 (3Myc-Grc3; Clasp [W364K]) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 507 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 507 (3Myc-Grc3; Lid [R433A, R440A]) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 496 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 496 (3Myc-Grc3; Lid [R433A]) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 497 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 497 (3Myc-Grc3; Lid [R433K]) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 498 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 498 (3Myc-Grc3; Lid [R440A]) | This study |
| tetO₇-Grc3 Las1-5Flag + pMP 499 | URA::CMV-tTA Grc3::kanR-tetO₇-TATA Las1-5XFlag MATa his3-1 leu2-0 met15-0; pMP 499 (3Myc-Grc3; Lid [R440K]) | This study |
observed any significant changes to the stability of the variants in comparison to the Grc3/Las1 WT complex (Fig. 8B). We performed a protein titration of Grc3 variants bound by Las1 with constant 5'-hydroxyl single-stranded RNA in the presence of ATP (Fig. 8C). The Grc3/Las1 WT complex displayed a 17-fold higher specific activity in phosphotransferase activity (0.87 min⁻¹) when compared to the Lid variant (0.05 min⁻¹) and 40-fold higher specific activity relative to the Clasp mutant (0.02 min⁻¹). Intriguingly, despite causing marked defects in phosphotransferase activity in vitro, the Lid double mutant only caused a mild growth phenotype while the Clasp double mutant was inviable (Fig. 5C,D). These data suggest that the Lid mutant presents a lower limit in PNK activity which can still support survival. The P-loop and Walker B variants had almost no detectable RNA phosphorylation activity under the experimental conditions examined preventing the measurement of their specific activity. This is in agreement with our cell viability assays, which revealed that the P-loop and Walker B variants could not support growth.

To determine the individual roles of specific residues within the four PNK motifs in RNA phosphotransferase activity, we generated 13 single mutants (Table 3). The single PNK mutants were expressed and purified as described above for the double Grc3 PNK variants. All 13 single mutants were stably expressed, copurified with Las1, and eluted similarly by gel filtration as seen with the WT and double PNK variants (Fig. 8D). We measured RNA phosphorylation at a fixed protein concentration of the single point mutants (Fig. 8E). Individual point mutants to the P-loop (K252A, K252R, and S253A), Walker B (D283A, D283N, D283E), and Clasp (W364A) motifs had almost no detectable kinase activity, while other single mutants from the Walker B (Q286A) and Clasp (L361A) motifs had a four- to fivefold reduction in total phosphorylated RNA compared to WT (Fig. 8E). Within the Lid motif, we could not detect a significant defect in activity with the R433A/K mutants, but observed a decrease in activity for the R440A/K mutants (approximately four- to fivefold reduction). With the exception of L361A, the in vitro kinase assays nicely complement the in vivo growth assays. Thus, mutations that abolish kinase activity cause severe growth defects, while mutants that only impair kinase activity have a more modest effect on cell growth. Residue L361 presents an exception to this trend since mutation of L361 to an alanine shows a moderate reduction in PNK activity, but a severe growth defect. These results suggest that aside from supporting kinase activity L361 may have an additional role in vivo.

**Grc3 PNK motifs are critical for assembly of the large ribosomal subunit**

Grc3 and its mammalian counterpart, Nol9, are essential for production of the large ribosomal subunit (60S) (Heindl and Martinez 2010; Castle et al. 2013; Pillon et al. 2017). To assess whether the reduction in cell proliferation and the defects in RNA phosphorylation in vitro cause deficiencies in ribosome production, we performed sucrose gradient fractionation from tetO₇-GRC3-Las1-5XFlag strains expressing WT Grc3 and select Grc3 PNK variants (K252A, S253A, D283A, L361A/W364A, R433A/R440A) in the presence of DOX. Ribosomes purified from yeast expressing WT Grc3 produced three distinct peaks representing the small subunit (40S), large subunit (60S), and intact 80S ribosomes, as well as a series of peaks indicating the presence of polyribosomes (polysomes) (Fig. 9; WT). In comparison to WT Grc3, the five Grc3 PNK mutants (K252A, S253A, D283A, L361A/W364A, R433A/R440A) that we analyzed marked a reduction in the 60S ribosomal subunit and a corresponding decrease in the 80S monosome and polysomes (Fig. 9). Moreover, the accumulation of ribosome halfmers further emphasizes the importance for Grc3 phosphotransferase activity in ribosome assembly. Interestingly, mutations to the Lid motif caused a mild-phenotype in 60S production, in agreement with our growth assays which revealed that mutations to this motif had a mild effect on cell proliferation (Fig. 9;
LidR433A/R440A). We interpret this to mean the Grc3 Lid motif plays an important role in enhancing the efficiency of ribosome production, but is not strictly required for cell viability like the P-loop, Walker B and Clasp motifs. To determine whether Grc3 variants and endogenous Las1 still associate with preribosome particles, we analyzed the sedimentation pattern of Grc3 variants and Las1 in our sucrose gradient fractions by western blot. Myc-Grc3 variants and Las1-Flag co-sediment with fractions corresponding to the 60S ribosomal particle (Fig. 9). This confirms that mutations to the Grc3 PNK motifs do not hinder Grc3/Las1 binding to pre-60S particles.

Las1 crosstalk is dependent on the integrity of the Grc3 PNK domain

The higher-order assembly of the Grc3/Las1 super-dimer is critical for supporting crosstalk between the Las1 nuclease and the Grc3 polynucleotide kinase (Pillon et al. 2017). Disrupting the Grc3 Walker B motif causes a deficiency in crosstalk leading to a reduction in Las1-mediated C2 cleavage of the S. cerevisiae ITS2 in vitro (Pillon et al. 2017). To assay whether the Grc3 P-loop, Clasp, and Lid motifs are also required for Grc3 to direct efficient Las1-mediated C2 cleavage, we performed in vitro C2 cleavage assays using an ITS2 RNA mimic (Fig. 10A) and Grc3/Las1 complexes with the double PNK variants. As shown previously, mutations to the Walker B motif cause a significant reduction in C2 cleavage. Changes to the Lid motif resulted in a similar RNA cleavage deficiency while mutations to the Clasp motif significantly impaired C2 cleavage in vitro (Fig. 10B). Conversely, the Grc3 P-loop was dispensable for Grc3/Las1 crosstalk, since C2 cleavage was similar to WT (Fig. 10B).

To assess the individual contribution of the residues within the PNK motifs, we also carried out nuclease assays at a fixed protein concentration with single point mutants (Fig. 10C). From these individual mutants, we identified three residues (D283, L361, R440) which are important for Grc3-directed C2 cleavage activity. These cleavage assays highlight the contributions of the Grc3 PNK motifs in supporting the coordinated crosstalk between these two essential RNA processing enzymes.

To assess whether the observed Grc3/Las1 crosstalk also occurs in S. cerevisiae, we looked at the accumulation of pre-rRNA intermediates by northern blot analysis. We extracted RNA from tetO7-GRC3 strains expressing Grc3 PNK variants grown in the presence of DOX. Defects to the Grc3 P-loop, Walker B, and Clasp motifs, lead to a reduction in the mature 25S rRNA and an accumulation in RNA intermediates, including the 35S, 27S, and 7S precursor rRNA, highlighting their importance in pre-rRNA processing (Fig. 10D–F). Disruption of the Lid motif also lead to a pronounced abundance of the 27S, but unlike the P-loop, Walker B, or Clasp motifs, there was no stark reduction in the mature 25S or a severe accumulation of either the 35S or 7S (Fig. 10F). This is in good agreement with our in vitro biochemical assays and in vivo yeast viability and sucrose gradient assays suggesting the Lid motif plays a minor role in pre-rRNA processing. Interestingly, the accumulation of the 27S intermediate indicates a defect in C2 cleavage (Fig. 10D). The most dramatic C2 cleavage defect is observed with the Clasp variant...
and 7S pre-rRNA maturation in port varying roles for pre-rRNA processing at the C2 site crosstalk occurs in vivo and that the Grc3 PNK motifs sup-

for Grc3/Las1 in earlier steps of pre-rRNA processing.

phosphorylate the 26S or it may highlight an unknown role in the 35S pre-rRNA upon disrupting the P-loop, Walker B, and Clasp motifs (Fig. 10F). This may either reflect a regula-
tory feedback loop that is activated due to the inability to interpret this to mean the Lid motif is important for efficient activity toward the Las1 cleaved C2 substrate. Similarly, mammalian PNKP has a substrate preference for DNA oligo-
nucleotides with recessed 5′-OH groups, which is consistent with its role in certain DNA repair pathways (Bernstein et al. 2005). Taken together, our results show that the Grc3 poly-
nucleotide kinase shares similar metal- and nucleotide-selectivity to known members of the P-loop NTPase superfamily, but exhibits critical differences in its substrate selection in comparison with other PNK enzymes. These differences in substrate selection likely influence Grc3′s biological target(s) in vivo.

We further enhanced our understanding of the Grc3 PNK domain by generating missense mutations within four key motifs that are shared with the ancestral Clp1, including the P-loop, Walker B, Clasp, and Lid (Fig. 11). The P-loop, Walker B, and Clasp motifs are all essential for cell viability, kinase activity, and ribosome production (Fig. 11). In contrast, the Lid motif is not essential for cell viability, but disruption of the Lid motif results in minor impairments to cell growth, ribosome production and a 17-fold reduction in specific activity of the phosphotransferase (Fig. 11). We inter-

pret this to mean the Lid motif is important for efficient rRNA processing, but is not essential for survival. This is in agreement with the report that pre-rRNA transcription is the rate limiting step in ribosome assembly (Nomura et al. 1984), and not pre-rRNA processing. Similar to the Lid muta-
tant, mutagenesis of other ribosome assembly factors, such as Fap7 and Has1, displayed defects in enzymatic function in vitro, while maintaining wild-type cell growth and proliferation (Granneman et al. 2005; Rocak et al. 2005; Dembowski et al. 2013).

Through the generation of individual missense mutations we were able to discern differences in activity within individu-
al motifs. One of the most significant differences was (L361A/W364A); whereas, the P-loop mutant (K252A/ S253A) has comparable levels to WT (Fig. 10E,F). This observation corre-

sponds well with our in vitro C2 cleavage assay suggesting the P-loop is dispensable for Las1 nuclease activ-

ation. Moreover, yeast expressing the double Grc3 P-loop vari-
rual motifs. One of the most significant differences was con-
sidering the P-loop, Walker B, Clasp, and Lid (Fig. 11). The P-loop, Walker B, and Clasp motifs are all essential for cell viability, kinase activity, and ribosome production (Fig. 11). In contrast, the Lid motif is not essential for cell viability, but disruption of the Lid motif results in minor impairments to cell growth, ribosome production and a 17-fold reduction in specific activity of the phosphotransferase (Fig. 11). We inter-

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tant, mutagenesis of other ribosome assembly factors, such as Fap7 and Has1, displayed defects in enzymatic function in vitro, while maintaining wild-type cell growth and proliferation (Granneman et al. 2005; Rocak et al. 2005; Dembowski et al. 2013).

Through the generation of individual missense mutations we were able to discern differences in activity within individu-
al motifs. One of the most significant differences was

| TABLE 3. E. coli expression plasmids used and constructed in this study |
|-------------------------|-------------------------|-------------------------|
| Plasmid | Description | Vector | Source |
| pMP001 | Grc3 WT: 1-632 aa; 6XHis-Las1 WT: 1-502 aa | pST39 | (Pillon et al. 2017) |
| pMP338 | Grc3 P-loop (K252A, S253A): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |
| pMP335 | Grc3 Walker B (D283A, Q286A): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | (Pillon et al. 2017) |
| pMP337 | Grc3 Clasp (L361A, W364A): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |
| pMP339 | Grc3 Lid (R433A, R440A): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |
| pMP500 | Grc3 P-loop (K252R): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |
| pMP511 | Grc3 P-loop (K252A): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |
| pMP519 | Grc3 Walker B (D283E): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |
| pMP520 | Grc3 Walker B (D283E): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |
| pMP501 | Grc3 Walker B (D283A): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |
| pMP502 | Grc3 Walker B (D283N): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |
| pMP503 | Grc3 Walker B (D283E): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |
| pMP523 | Grc3 Walker B (Q286A): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |
| pMP511 | Grc3 Clasp (L361A): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |
| pMP504 | Grc3 Clasp (W364A): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |
| pMP519 | Grc3 Lid (R433K): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |
| pMP520 | Grc3 Lid (R433A): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |
| pMP505 | Grc3 Lid (R440A): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |
| pMP506 | Grc3 Lid (R440K): 1-632 aa; 6XHis-WT Las1 1-502 aa | pST39 | This study |

DISCUSSION

Prior to this study little was known about the activity of the PNK domain of the essential polynucleotide kinase Grc3, therefore we characterized the Grc3 PNK domain through a series of in vivo and in vitro assays. Here we establish that the PNK domain has a requirement for a divalent metal ion for catalysis and has a nucleotide preference for ATP. Similar to the Trl1 and Pnkp/Hen1 phosphotransferases, the NTP donor selectivity of Grc3/Las1 includes all four nu-

cleotides, but like Clp1, Grc3/Las1 shows a preference for ATP (Sawaya et al. 2003; Noble et al. 2007; Das et al. 2013). We also determined that Grc3/Las1 has enhanced activity toward the Las1 cleaved C2 substrate. Similarly, mammalian PNKP has a substrate preference for DNA oligo-
nucleotides with recessed 5′-OH groups, which is consistent with its role in certain DNA repair pathways (Bernstein et al. 2005). Taken together, our results show that the Grc3 poly-
nucleotide kinase shares similar metal- and nucleotide-selectivity to known members of the P-loop NTPase superfamily, but exhibits critical differences in its substrate selection in comparison with other PNK enzymes. These differences in substrate selection likely influence Grc3′s biological target(s) in vivo.

We further enhanced our understanding of the Grc3 PNK domain by generating missense mutations within four key motifs that are shared with the ancestral Clp1, including the P-loop, Walker B, Clasp, and Lid (Fig. 11). The P-loop, Walker B, and Clasp motifs are all essential for cell viability, kinase activity, and ribosome production (Fig. 11). In contrast, the Lid motif is not essential for cell viability, but disruption of the Lid motif results in minor impairments to cell growth, ribosome production and a 17-fold reduction in specific activity of the phosphotransferase (Fig. 11). We inter-

pret this to mean the Lid motif is important for efficient rRNA processing, but is not essential for survival. This is in agreement with the report that pre-rRNA transcription is the rate limiting step in ribosome assembly (Nomura et al. 1984), and not pre-rRNA processing. Similar to the Lid muta-
tant, mutagenesis of other ribosome assembly factors, such as Fap7 and Has1, displayed defects in enzymatic function in vitro, while maintaining wild-type cell growth and proliferation (Granneman et al. 2005; Rocak et al. 2005; Dembowski et al. 2013).

Through the generation of individual missense mutations we were able to discern differences in activity within individu-
al motifs. One of the most significant differences was
observed for two key residues within the Clasp motif ([T/S/L]xGW). The L361A mutation resulted in a lethal phenotype while the W364A mutation only impaired growth. However when we carried out in vitro kinase assays we found that the W364A mutation had a more significant impact on phosphotransferase activity than the L361 mutation. These results suggested that aside from supporting kinase activity, L361 must have another critical role in the cell and could be important for supporting Las1 nuclease activity.

To delineate the role of the PNK motifs in molecular crosstalk between the Las1 nuclease and the Grc3 kinase active sites, we looked at Las1 nuclease activity in vitro and in vivo. Previously established that Grc3 and Las1 are dependent upon one another for higher ordered assembly and enzyme activation (Pillon et al. 2017). Mutation of the key active site residues within Las1 (R129/H134) disrupts Grc3 kinase activity (Fig. 11). Likewise, mutation of the Walker B motif within the Grc3 PNK active site and truncation of the Grc3 C terminus (residues 623–632) disrupts Las1 nuclease function (Fig. 11; Pillon et al. 2017). Here we establish that in addition to the Walker B motif and C-terminal tail, the Clasp motif and, to a lesser extent, the Lid motif are important for Grc3-directed Las1 nuclease activity in vivo and in vitro. Through the generation of single point mutants we were able to identify two residues critical for directing crosstalk including the Walker B catalytic base, D283, and L361 from the Clasp motif, which lie in close proximity to one another in our homology model (Fig. 4C). Thus, molecular crosstalk between Grc3 and Las1 relies on a series of motifs within the nuclease active site (R129/H134 from Las1), the kinase active site (D283/L361 from Grc3), and the C-terminal tail of Grc3 (residues 623–632) (Fig. 11). Together, Grc3/Las1 generate cleavage and phosphorylation products with RNA ends that cannot be resealed by any known ligase. Phosphorylation of the 26S pre-rRNA by Grc3/Las1 also primes the 26S pre-rRNA for processing by Rat1/Rai1 (Gasse et al. 2015). Therefore, the unique combination of enzymatic activities of the Grc3/Las1 complex commits the cell to processing the ITS2 following C2 cleavage.

In contrast to the Walker B, Lid, and Clasp motifs, the P-loop motif is dispensable for Las1 nuclease activity. This marks a striking difference between Grc3/Las1 and the related endoribonucleases Ire1 and RNase L, which are both dependent upon their kinase domains for high-ordered assembly and activation of their nuclease domains (Pillon and Stanley 2017). In contrast to Grc3/Las1, both Ire1 and
RNase L require ATP binding to their respective kinase/pseudo kinase domains for nuclease activation (Wreschner et al. 1982; Dong et al. 1994; Sidrauski and Walter 1997; Dong and Silverman 1999; Huang et al. 2014). We previously established that ATP binding is not a requirement for Las1 nuclease function and this is further supported by mutation of the P-loop residues, critical for ATP binding (Castle et al. 2013; Pillon et al. 2017). One of the major differences between Grc3/Las1 and Ire1 and RNase L, is that Grc3 phosphotransferase activity is vital in vivo as Grc3 directly phosphorylates the Las1 cleavage product. From a functional standpoint, coupling C2 cleavage with RNA phosphotransferase activity would be beneficial as this ensures the Grc3/Las1 RNA processing machinery strictly commits to C2 cleavage if both catalytic sites of the Grc3/Las1 complex are functionally competent. This is achieved through a molecular crosstalk network supported by the catalytic base from the Walker B motif as opposed to the P-loop. Thus, the alternative wiring of the crosstalk network seen for Grc3/Las1 compared to its related RNA processing machinery, Ire1 and RNase L, may reflect a structural feature or unknown function of the distinct Grc3 N-terminal and C-terminal domains.

Together, this work reveals the checks and balances necessary for cells to commit to ITS2 processing through the Grc3/Las1 RNA processing machinery. We hypothesize that the Grc3/Las1 active sites share key residues, including Grc3 Walker B and Clasp motif residues, to form a composite active site to properly position the RNA substrate for efficient and accurate cleavage and phosphorylation. While this hypothesis awaits high resolution structural determination for validation, our data provides additional insight into the intricate molecular crosstalk between the essential enzymes Grc3 and Las1 that ensures C2 cleavage and phosphorylation of the 26S pre-rRNA is directly coupled.

**MATERIALS AND METHODS**

**Cloning of Grc3/Las1 variants**

Generation of bacterial expression plasmids of *S. cerevisiae* (Sc) Grc3/Las1 (pMP 001; Grc3 residues 1–632 and Las1 residues 1–502) and the Walker B variant Grc3<sup>283A</sup>, Clasp<sup>L361A/W364A</sup>, Lid<sup>R433A/R440A</sup> of Grc3. Black arrows mark ribosome halfmers and brackets highlight the polysomes. Sucrose gradient fractions were precipitated, run on SDS-PAGE gels, and analyzed by western blot with anti-Myc (Grc3), anti-Flag (Las1), and anti-RPL8 (60S ribosomal protein) antibodies.

**FIGURE 9.** Grc3 PNK active site motifs are critical for ribosome production in *S. cerevisiae*. Polysome profile analysis of transformed *tetO7-GRC3-Las1 5X Flag* expressing WT and PNK variants (P-loop<sup>K252A</sup>, P-loop<sup>S253A</sup>, Walker B<sup>283A</sup>, Clasp<sup>L361A/W364A</sup>, Lid<sup>R433A/R440A</sup>) of Grc3. Black arrows mark ribosome halfmers and brackets highlight the polysomes. Sucrose gradient fractions were precipitated, run on SDS-PAGE gels, and analyzed by western blot with anti-Myc (Grc3), anti-Flag (Las1), and anti-RPL8 (60S ribosomal protein) antibodies.
The Grc3 (pMP 003; residues 1–632) plasmid used for proliferation assays was modified from the original by the inclusion of three additional Myc tags (pMP 484) onto the N terminus for detection purposes. Kinase-deficient Grc3 variants were generated by Q5 site-directed mutagenesis (NEB) using pMP 484 as a template and are listed in Table 1. All plasmids were verified by DNA sequencing (GeneWiz).

Expression and purification of Grc3 and Las1 constructs

Grc3/Las1 variant proteins were produced as described previously (Pillon et al. 2017) with minor modifications. Briefly, Grc3/Las1 variants were overexpressed in *E. coli* LOBTSR (Kerafast) cells. Cells were harvested by centrifugation and resuspended in 50 mM Tris pH 8.0, 500 mM NaCl, 5 mM MgCl₂, 1% (v/v) Triton X-100, 10% (v/v) glycerol and disrupted by sonication. Lysate was clarified at 26,916g for 50 min at 4°C and the supernatant was applied to a gravity flow column loaded with His60 Ni Superflow Resin (Clontech). The column was washed with 200 mL of wash buffer (50 mM Tris pH 8.0, 500 mM NaCl, 5 mM MgCl₂, 15 mM imidazole, 10% glycerol) and eluted with imidazole (50 mM Tris pH 8.0, 500 mM NaCl, 5 mM MgCl₂, 200 mM imidazole, 10% glycerol). Samples were resolved by a HiLoad 16/600 Superdex-200 Prep Grade (GE Healthcare) gel filtration column equilibrated with storage buffer (20 mM Tris pH 8.0, 200 mM NaCl, 5 mM MgCl₂, 5% glycerol).

Thermal shift assay

The melting temperature (Tm) was measured for purified Sc Grc3/Las1 variants (1.0 µM) in storage buffer supplemented with 5× SYPRO Orange Protein Gel Stain (Sigma-Aldrich). Mixtures were
heated from 25°C–95°C at a rate of 0.05°C/sec. Relative fluorescence was measured using excitation and emission wavelengths 490 nm and 580 nm, respectively, using a QuantStudio 7 Flex (Applied Biosystems). The mean and standard deviation were calculated from three independent replicates.

**Phosphorylation assay**

RNA phosphorylation was measured with 2 µM Grc3/Las1 variants, unless specified otherwise, in kinase reaction buffer (50 mM Tris pH 8.0, 100 mM NaCl, 15 mM MgCl₂, and 1 mM ATP). Fluorescently labeled RNA (10–15 µM) encoding a 5’-hydroxyl included: single-stranded 21-mer RNA (5’-ACGUACGGCGAACAUCUGAA-TAMRA-3’) and Grc3 primed RNA (5’-GGUUUACCAACUGCGGCUAUCGUACGGCGUUUUA-TAMRA-3’). To ascertain NTP and dNTP concentration dependence, ATP, UTP, CTP, GTP, and dATP titrations (0–250 µM) were used where indicated. To test metal selectivity, we purified Grc3/Las1 variants in storage buffer lacking metal and supplemented reactions with 5 mM EDTA or 15 mM divalent metal ion, where indicated. Nucleotide selectivity was measured using modified kinase reaction buffer with 1 mM nucleotide, as indicated. ATP/ADP and ATP/ADPnP competition mixtures included constant ATP (30 µM) with a titration of ADP or ADPnP (0–5 mM). Phosphorylation reactions were incubated for 60 min at 37°C and quenched by adding equal volume urea loading dye (20 mM Tris pH 8.0, 8 M urea, 0.05% bromophenol blue, 1 mM EDTA). Reaction mixtures were boiled and loaded onto a 15% polyacrylamide (8 M urea) gel in 0.5× tris–borate–EDTA buffer. Gels were visualized on a Typhoon FLA 9500 (GE Healthcare). All

![Diagram of Grc3/Las1 molecular crosstalk](image)

**FIGURE 11.** Model of Grc3/Las1 molecular crosstalk. Grc3/Las1 assembles into a super dimer driven by homodimerization of the Las1 nuclease domain and Grc3 PNK domain (Pillon et al. 2017). Each Grc3 PNK domain encodes four motifs, including the P-loop (red), Walker B (blue), Clasp (purple), and Lid (orange), important for kinase activity, nuclease activity, and cell viability. Approximate positions of the motifs relative to one another are based upon the homology model from Figure 4C. PNK residues identified to be critical for molecular crosstalk are enclosed (Grc3 D283 and L361). Yellow asterisks highlight the Grc3 C-terminal tail and the Las1 HEPN active site, which were previously established to also mediate molecular crosstalk (Pillon et al. 2017). The X marks residues that are dispensable, whereas the checkmark identifies critical residues.
phosphorylation reactions were done in triplicate and representative gels are shown in the figures. Gels were analyzes using ImageJ software (Abramoff et al. 2004).

**Yeast growth conditions and proliferation assays**

The *S. cerevisiae* strain encoding a tetracycline-titratable promoter (tetO2) upstream of the endogenous GRC3 gene was obtained from Open Biosystems (GE Dharmacon). A 5XFlag tag was added onto the C terminus of endogenous Las1 in the tetO2–GRC3 strain for detection purposes with the pFA6a-5FLAG-natMX6 vector (Noguchi et al. 2008). The tetO2–GRC3–Las1–5XFlag strain was transformed with plasmids encoding Grc3 variants (see Table 1) and expression of endogenous GRC3 was repressed by supplementing YPD media with doxycycline (20 µg/mL). Proliferation assays spotted transformed tetO2–GRC3–Las1–5XFlag strains (Table 2) on YPD in the absence and presence of doxycycline (20 µg/mL) and incubated at 30°C for 2–3 d, unless specified otherwise. Growth curves were generated from cultures (100 µL) with and without doxycycline (20 µg/mL) inoculated at an OD600 of 0.05 and incubated at 30°C for 25 h. Saturation of the cultures were monitored by measuring the absorbance at 595 nm every 15 min using an Infinicy 200 Pro (Tecan). The mean and standard deviation were calculated from three independent replicates.

**Western blot and coimmunoprecipitation**

* TetO2–GRC3–Las1–5XFlag strain was transformed with plasmids listed in Table 1. Cultures were grown in YPD in the absence and presence of doxycycline (20 µg/mL) and harvested at mid-log phase. Whole-cell lysate was prepared by lysing the cells using glass beads followed by protein precipitation using trichloroacetic acid. Coimmunoprecipitation was carried out by resuspending cells in 50 mM Tris pH 8.0, 300 mM NaCl, 5 mM MgCl2, 0.5% NP-40 prior to cell lysis using glass beads. Whole-cell lysate was incubated with anti-myc resin (Pierce) for 30 min at 4°C and washed using 50 mM Tris pH 8.0, 300 mM NaCl, 5 mM MgCl2, 5% Glyceral. Samples were resolved by SDS-PAGE and analyzed by western blot using anti-Myc (Grc3: EMD Millipore), anti-Flag (Las1; Sigma-Aldrich), and anti-a-tubulin (Abcam) antibodies.

**C2 RNA cleavage assays**

Las1 endoribonuclease activity was measured as previously described (Pillon et al. 2017) with minor modifications. Briefly, Grc3/Las1 variants (0.8–0.8 µM) were incubated with fluorescently labeled *S. cerevisiae* rRNA mimic (0.1 µM) encoding the C2 site (5′-GUGGUUUAGGUUUCACACUGCGGC-FAM-3′) for 60 min at 37°C. Samples were resolved on 15% polyacrylamide (8 M urea) gels in 1× tris–borate–EDTA buffer and visualized using the Typhoon FCA 9500. The mean and standard deviations were calculated from three independent replicates.

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