

# The Homologous Putative GTPases Grn1p from Fission Yeast and the Human GNL3L Are Required for Growth and Play a Role in Processing of Nucleolar Pre-rRNA<sup>□</sup>

Xianming Du,\* Malireddi R.K. Subba Rao,<sup>†‡</sup> Xue Qin Chen,<sup>\*‡</sup> Wei Wu,\*  
Sundarasamy Mahalingam,<sup>†</sup> and David Balasundaram\*

\*Laboratory of Nucleopore Biology, Institute of Molecular and Cell Biology, National University of Singapore, Singapore 117609, Singapore; and <sup>†</sup>Laboratory of Molecular Virology, Centre for DNA Fingerprinting and Diagnostics, Hyderabad 500076, India

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**Grn1p from fission yeast and GNL3L from human cells, two putative GTPases from the novel HSR1\_MMR1 GTP-binding protein subfamily with circularly permuted G-motifs play a critical role in maintaining normal cell growth. Deletion of *Grn1* resulted in a severe growth defect, a marked reduction in mature rRNA species with a concomitant accumulation of the 35S pre-rRNA transcript, and failure to export the ribosomal protein Rpl25a from the nucleolus. Deleting any of the Grn1p G-domain motifs resulted in a null phenotype and nuclear/nucleolar localization consistent with the lack of nucleolar export of preribosomes accompanied by a distortion of nucleolar structure. Heterologous expression of GNL3L in a  $\Delta$ *grn1* mutant restored processing of 35S pre-rRNA, nuclear export of Rpl25a and cell growth to wild-type levels. Genetic complementation in yeast and siRNA knockdown in HeLa cells confirmed the homologous proteins Grn1p and GNL3L are required for growth. Failure of two similar HSR1\_MMR1 putative nucleolar GTPases, Nucleostemin (NS), or the dose-dependent response of breast tumor autoantigen NGP-1, to rescue  $\Delta$ *grn1* implied the highly specific roles of Grn1p or GNL3L in nucleolar events. Our analysis uncovers an important role for Grn1p/GNL3L within this unique group of nucleolar GTPases.**

## INTRODUCTION

The nucleolus is the principal site for the generation of rRNA as also for ribosome assembly and maturation (see Venema and Tollervy, 1999; Tschochner and Hurt, 2003). The initial rRNA processing is concomitant with the formation of the 90S ribosomal precursor particle that separates into 40S and 60S subunits. The pre-60S ribosomes undergo a series of RNA processing reactions that begin within the nucleolus followed by export of the ribosomes through the nuclear pore complex (NPC; Venema and Tollervy, 1999; Milkereit *et al.*, 2001; Fatica and Tollervy, 2002; Nissan *et al.*, 2002; Tschochner and Hurt, 2003). Nucleotide-binding proteins comprising several putative GTPases are known to be associated with pre-60S ribosomes on their journey from the nucleolus to the cytoplasm (Nissan *et al.*, 2002; Tschochner and Hurt, 2003). However, their precise function at the molecular level is unclear. All studies to date point to their role(s) in rRNA processing/maturation and/or 60S ribosomal subunit export from the nucleolus/nucleus (Tschochner and Hurt, 2003). Although most of the nucleotide-bind-

ing GTPases possess both GDP/GTP-binding and GTP-hydrolyzing potential from the structural point of view, some members lack either one or both abilities in vivo (Takai *et al.*, 2001). The YAWG/YlqF/HSR1\_MMR1 GTP-binding protein subfamily of GTPases belongs to the TRAFAC (for Translation factor-related) subclass of GTPases characterized by a circular permutation of their GTPase signature motifs (G1–5) so that the G4, -5, and -6 subdomains are relocated from the C-terminal side of the protein to the N-terminus (Daigle *et al.*, 2002; Leipe *et al.*, 2002). Several members of this family from human (NGP-1 and Nucleostemin; Racevskis *et al.*, 1996; Tsai and McKay, 2002, 2005), and yeast (Nug1p, Nug2p/Nog2p; Bassler *et al.*, 2001; Saveanu *et al.*, 2001) have been shown to localize to the nucleolus including some closely associated with ribosomal assembly and nucleolar/nuclear export. One member of this group, Nucleostemin (NS), was preferentially expressed in nuclei of CNS stem cells, embryonic stem cells and several cancer cell lines and may control cell cycle progression in those cell lines (Tsai and McKay, 2002; Liu *et al.*, 2004; Sijin *et al.*, 2004).

There are at least four predicted HSR1\_MMR1 nucleolar/nuclear GTP-binding proteins in the fission yeast. Fission yeast has served as an excellent genetically tractable model organism for the study of the molecular aspects of growth and regulation (Lee and Nurse, 1987; Lee and Nurse, 1988; Sheldrick and Carr, 1993; Hermeking *et al.*, 1997; Nurse, 1997; Rustici *et al.*, 2004). Given the growing awareness regarding the importance of nucleolar GTPases such as NS as modulators of cell growth and proliferation in cancer (Normile, 2002; Tsai and McKay, 2002; Bernardi and Pandolfi, 2003; Misteli, 2005; Tsai and McKay, 2005), we wanted

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<sup>‡</sup> These authors contributed equally to this work.

Address correspondence to: David Balasundaram ([davidb@imcb.a-star.edu.sg](mailto:davidb@imcb.a-star.edu.sg)).

**Table 1.** Yeast strains

Strains (YNB)	Parent strain	Plasmid/Character/Genotype	Marker	Reference
	YNB483	<i>leu1-32 ura4-D18 his3-Δ1</i> (Wild type)		This study
	YNB484	<i>leu1-32 ΔSPBC26H8.08c::ura4<sup>+</sup> his3-Δ1</i> (Mutant)		This study
YNB544	YNB484	pBNB190	<i>Leu2</i>	This study
YNB545	YNB484	pBNB189	<i>Leu2</i>	This study
YNB546	YNB484	pCDL280	<i>Leu2</i>	This study
YNB566	YNB484	pBNB203	<i>Leu2</i>	This study
YNB567	YNB484	pBNB202	<i>Leu2</i>	This study
YNB568	YNB484	pBNB204	<i>Leu2</i>	This study
YNB611	YNB484	pBNB217	<i>Leu2</i>	This study
YNB631	YNB484	pBNB221	<i>Leu2</i>	This study
YNB795	YNB484	pBNB284	<i>Leu2</i>	This study
YNB805	YNB484	pBNB316	<i>Leu2</i>	This study
YNB858		<i>leu1-32 ura4-D18 his3-Δ1, ΔSPBC26H8.08c::GNL3L-FLAG KanMX6 h<sup>-</sup></i>	<i>G418<sup>R</sup></i>	This study
YNB859		<i>leu1-32 ura4-D18 his3-Δ1, SPBC26H8.08c::FLAG KanMX6 h<sup>-</sup></i>	<i>G418<sup>R</sup></i>	This study
YNB860	YNB484	pBNB395	<i>Leu2</i>	This study
YNB956	YNB484	pBNB412	<i>Leu2</i>	This study
YNB961	YNB484	pBNB494	<i>Leu2</i>	This study
YNB1003	YNB484	pBNB561	<i>Leu2</i>	This study
YNB1075	YNB858	pBNB221	<i>G418<sup>R</sup>, Leu2</i>	This study
YNB1076	YNB859	pBNB221	<i>G418<sup>R</sup>, Leu2</i>	This study

*G418<sup>R</sup>* is the geneticin (*G418*) resistance gene.

to identify and characterize similar nucleolar/nuclear GTPases in the fission yeast *S. pombe* in order to understand their role in cell growth and regulation.

## MATERIALS AND METHODS

### Yeast Strains and Plasmids

*Schizosaccharomyces pombe* strains used in this study are listed in Table 1. Yeast strains were maintained in YES (yeast extract plus supplements) or EMM (Edinburgh minimal media) medium supplemented with appropriate amino acids and  $\pm 15 \mu\text{M}$  thiamine routinely to repress (it must be noted that repression is not 100%) or induce, respectively, the *nmt1* promoter (Moreno *et al.*, 1991). Unless otherwise specified, yeast cultures were maintained or grown at 32°C and harvested at 0.4–1.0 OD<sub>600</sub> for all experiments. YNB483 (*leu1-32 ura4-D18 his3-Δ1*) and YNB484 (*leu1-32 ΔSPBC26H8.08c::ura4<sup>+</sup> his3-Δ1*) were the principal yeast strains used in this study and are referred to in the text as either wild type and null mutant or  $\Delta\text{grn1}$ , respectively.

### Biochemical Methods

Standard laboratory techniques were used for extraction of DNA, total RNA, or protein.

### Plasmid Constructions

Plasmids generated for this study are described in Table 2. DNA fragments used to create plasmids for this study were generated by PCR using high-fidelity enzyme Turbo Pfu (Stratagene, La Jolla, CA). All constructs were confirmed by DNA sequencing. Oligonucleotide primers are listed in Table 3.

### Deletion of *Grn1*

PCR-based gene deletion of *Grn1* was performed using previously described procedures (Bahler *et al.*, 1998; Chen *et al.*, 2004). The open reading frame (ORF) *SPBC26H8.08c* encodes the gene *Grn1* (GTPase in Ribosomal export from the Nucleolus) expressing a putative GTPase. Briefly, the *Ura4*-marker cassette with *SPBC26H8.08c* flanking (5' and 3') homology regions was generated by PCR using the primers NB110 and NB111. This 2.2-kb fragment was directly used to transform the homozygous diploid YNB400 (*ade6-M210/ade6-M216 leu1-32 ura4-D18 his3-Δ1, h+/h*). Diploid transformants were selected on EMM-ura-ade plates. Sporulation of the heterozygous diploid and dissection of tetrads from at least 24 independent asci yielded four haploid spores/tetrad. On germination, two of the four spores from each tetrad grew extremely slowly on rich medium. Each one of these slow-growing colonies was confirmed as having the *SPBC26H8.08c* deletion by colony PCR using 5' and 3' primers flanking the gene.

### Construction of Strains YNB858 and YNB859 by Gene Integration

PCR-based gene integration of *GNL3L* into the *Grn1* locus using the *KanMX6* marker was performed using previously described procedures (Bahler *et al.*, 1998; Chen *et al.*, 2004). Oligonucleotides representing the FLAG sequence (AGATGGACTACAAGGATGACGATGACAAATAA) were annealed and inserted into pBNB341 to replace the *GFP* ORF generating pBNB373. Next, the gene encoding *KanMX6* (BNB343) was inserted into pBNB373 downstream of *GNL3L-FLAG* fusion in the reverse orientation (pBNB396). The *GNL3L-FLAG KanMX6* cassette was amplified by PCR using pBNB396 as the template with the primers NB1004 (5' end) + NB1125 (3' end). *FLAG KanMX6* cassette was amplified using the same template and 3' end primer as for the *GNL3L* cassette, whereas the 5' end primer (NB1002) corresponded to 76 nts of *Grn1* ORF sequence immediately upstream of its stop codon. The integrations resulted in the replacement of wild-type *Grn1* ORF with *GNL3L-FLAG KanMX6* (YNB858) and the fusion of *Grn1* with FLAG epitope (YNB859).

### Fluorescence Microscopy

Fission yeast cells were prepared for DAPI, GFP fluorescence or indirect immunofluorescence as previously described (Balasundaram *et al.*, 1999; Chen *et al.*, 2004; Varadarajan *et al.*, 2005). All epi-fluorescence microscopy were performed at 1000 $\times$  magnification using a Leica DMLB microscope (Deerfield, IL) equipped with an Optronics DEI-750T coded CCD camera (Goleta, CA) with Leica Qwin proprietary software. For some experiments, samples were viewed with an upright Nikon E800 confocal microscope (Melville, NY), and images were acquired using a Nikon DXM1200 camera with Image Proplus 4.5 software (Media Cybernetics, Silver Spring, MD). Cos-7 cells in chamber culture slides (BD Biosciences, San Diego, CA) were infected with vaccinia virus vTF7-3 and transfected with *GNL3L-GFP* (BNB341) and *Grn1-GFP* (BNB338) expression plasmids using Lipofectin (Invitrogen, Carlsbad, CA). After 12 h, cells were fixed with 3% paraformaldehyde and mounted in mounting medium (Vector Laboratories, Burlingame, CA). Localization of *GNL3L* and *Grn1p* was determined by confocal microscopy. Nucleoli were revealed by immunostaining with monoclonal anti-nucleolin (Upstate Biotechnology, Charlottesville, VA). Adobe Photoshop software was used to process all image presentations (San Jose, CA).

### *Rpl25a* Localization for $\Delta\text{grn1}$ , *Grn1-FLAG*, and $\Delta\text{grn1}::\text{GNL3L-FLAG}$ Strains

Strains expressing *nmt1::Rpl25a::GFP* were cultured in EMM medium supplemented with appropriate amino acids and 15  $\mu\text{M}$  thiamine were grown to log-phase, after which the cells were washed in medium without thiamine and diluted to an OD<sub>595</sub> 0.1 in fresh medium with (*nmt1* OFF) or without thiamine (*nmt1* ON). Samples of cells were harvested at early log-phase (OD<sub>595</sub> 0.5–0.8), stained with DAPI, and examined for DAPI and GFP fluorescence.

**Table 2.** Plasmids/Constructs

Construct	Description	Marker <sup>a</sup>	Reference
pCDL280	pREP1 with GFP (S65T)	Amp <sup>R</sup>	Varadarajan <i>et al.</i> (2005)
pBNB168	pBlueScript KS II-Ura4	Amp <sup>R</sup>	Chen <i>et al.</i> (2004)
pBNB189	ΔG1 (AA <sup>276-283</sup> ): Nucleotides encoding AA <sup>1-275</sup> and AA <sup>284-470</sup> were generated by PCR using pBNB190 as template with NB380+NB379 and NB378+NB377 sets of respective primers. The two fragments were used in fusion PCR using primers NB380 and NB377. The PCR product was inserted into pCDL280 as in pBNB190.	Amp	This study
pBNB190	The full-length <i>Grn1</i> gene without its intron was amplified by PCR using <i>S. pombe</i> genomic DNA as template and primers NB380 and NB377. The resulting <i>Grn1</i> was digested with <i>SalI</i> and <i>NotI</i> and inserted immediately upstream of the GFP gene in vector pCDL280.	Amp <sup>R</sup>	This study
pBNB202	ΔCC (AA <sup>70-90</sup> ): Nucleotides encoding AA <sup>1-69</sup> and AA <sup>91-470</sup> were generated by PCR using BNB190 as template with NB380+NB454 and NB453+NB377 sets of respective primers. The resulting fragments were used in fusion PCR with NB380 and NB377 as the primers. The PCR product was inserted into pCDL280 as in pBNB190.	Amp <sup>R</sup>	This study
pBNB203	ΔG5 (AA <sup>164-175</sup> ): Nucleotides corresponding to AA <sup>1-163</sup> and AA <sup>176-470</sup> were amplified by PCR using BNB190 as template with NB380+NB458 and NB457+NB377 sets of respective primers. The products were used for fusion PCR with primers NB380 and NB377. The resulting fragment was inserted in pCDL280 as in pBNB190.	Amp <sup>R</sup>	This study
pBNB204	ΔRG (AA <sup>405-415</sup> ): Nucleotides corresponding to AA <sup>1-404</sup> and AA <sup>416-470</sup> were amplified by PCR using BNB190 as template with NB380+NB456 and NB455+NB377 sets of respective primers. The PCR products were used for fusion PCR with primers NB380 and NB377. The resulting product was inserted in pCDL280 as in pBNB190.	Amp <sup>R</sup>	This study
pBNB217	ΔG4 (AA <sup>195-208</sup> ): Nucleotides corresponding to AA <sup>1-194</sup> and AA <sup>209-470</sup> were amplified by PCR using BNB190 as template with NB380+NB525 and NB524+NB377 sets of respective primers. The PCR products were used for fusion PCR with primers NB380 and NB377. The resulting product was inserted in pCDL280 as in pBNB190.	Amp <sup>R</sup>	This study
pBNB221	The <i>Rpl25a</i> gene ( <i>SPBC106.18</i> ) was generated without its intron. The N- and C-terminal fragments were amplified by PCR using the genomic DNA as template with NB535+NB541 and NB540+NB536 sets of respective primers. The fusion product was obtained by PCR using N- and C-terminal products as template with NB535 and NB536 as primers. The resulting product was inserted into pCDL280 using <i>SalI</i> and <i>NotI</i> .	Amp <sup>R</sup>	This study
pBNB284	The full-length human <i>NGP-1</i> gene was amplified by PCR using a HeLa cDNA library as template, with primers NB712 and NB713. The PCR product was digested with <i>SalI</i> and <i>NotI</i> and inserted in pCDL280 similarly as in pBNB190.	Amp <sup>R</sup>	This study
pBNB316	The full-length <i>GNL3L</i> gene was amplified by PCR using a HeLa cDNA library as template and primers NB762 and NB763. The PCR product was cloned into pCDL280 immediately upstream of GFP gene with <i>SalI</i> and <i>NotI</i> .	Amp <sup>R</sup>	This study
pCDNA3.1		Amp <sup>R</sup>	Novagen
pBNB335	ΔG5-GFP fusion was amplified using pBNB203 as template and NB864+NB865 as primers. The PCR was cloned into pCDNA3.1 with <i>KpnI</i> and <i>XhoI</i> .	Amp <sup>R</sup>	This study
pBNB336	ΔG4-GFP fusion gene was cloned into pCDNA3.1 following the same strategy as in pBNB335 except using pBNB217 as template for PCR.	Amp <sup>R</sup>	This study
pBNB337	ΔG1-GFP fusion gene was cloned in pCDNA3.1 following the same strategy as in pBNB335 using pBNB189 as template.	Amp <sup>R</sup>	This study
pBNB338	The <i>Grn1</i> -GFP fusion gene was cloned into pCDNA3.1 following the same strategy as in pBNB335 using pBNB190.	Amp <sup>R</sup>	This study
pBNB339	ΔRG-GFP fusion gene was cloned into pCDNA3.1 following the same strategy as in pBNB335 using pBNB204.	Amp <sup>R</sup>	This study
pBNB340	GFP was released from pBNB8 by <i>BamHI</i> / <i>XhoI</i> and inserted in pCDNA3.1 containing the same unique sites.	Amp <sup>R</sup>	This study
pBNB341	GNL3L-GFP fusion gene was cloned into pCDNA3.1 following the same strategy as pBNB335 cloning using pBNB316 as template and NB883+NB865 as primers.	Amp <sup>R</sup>	This study
pBNB343	pBlueScript KS II-KanMX6	Amp <sup>R</sup>	Chen <i>et al.</i> (2004)
pBNB373	Nucleotides encoding the FLAG epitope were annealed and cloned into pBNB341 with <i>NotI</i> and <i>XhoI</i> . FLAG replaces GFP in this vector.	Amp <sup>R</sup>	This study
pBNB376	Primers NB907+NB908 were annealed to form a siRNA fragment specific for <i>GNL3L</i> at nt 1047-1065 and inserted in the pSIREN shuttle vector (BD Biosciences).	Kan <sup>R</sup>	This study
pBNB377	Primers NB909+NB910 were annealed to form a scrambled version of <i>GNL3L</i> siRNA (pBNB376) and inserted in pSIREN shuttle vector.	Kan <sup>R</sup>	This study
pBNB378	Oligonucleotides specific for the Luciferase gene were annealed to form a siRNA fragment and inserted in pSIREN shuttle vector.	Kan <sup>R</sup>	This study
pBNB395	Nucleotides encoding the FLAG epitope were annealed and cloned into pBNB316 with <i>NotI</i> and <i>XhoI</i> . FLAG replaces GFP in this vector.	Amp <sup>R</sup>	This study
pBNB396	The KanMX6 cassette (BNB343) was inserted into pBNB373 using <i>ApaI</i> and <i>XbaI</i> .	Amp <sup>R</sup>	This study
pBNB412	ΔG3 (AA <sup>326-329</sup> ): Nucleotides encoding AA <sup>1-325</sup> and AA <sup>330-470</sup> were generated by PCR using BNB190 as template with NB380+NB1007 and NB1006+NB377 sets of respective primers. The two fragments were used in fusion PCR using primers NB380 and NB377. The resulting product was inserted in pCDL280 as in pBNB190.	Amp <sup>R</sup>	This study
pBNB417	ΔG3-GFP fusion was cloned into pCDNA3.1 following the same strategy as in pBNB335 using pBNB412.	Amp <sup>R</sup>	This study
pBNB494	The human nucleostemin (NS) gene was amplified by PCR using a HeLa cDNA library as template, with primers NB1160 and NB1161. The PCR product was cloned into pCDL280 following the same strategy as in pBNB190.	Amp <sup>R</sup>	This study
pBNB561	The <i>S.cerevisiae</i> <i>NUG1</i> gene was amplified by PCR using yeast genomic DNA as template, with primers NB1309 and NB1310. The PCR product was cloned into pCDL280 following the same strategy as in pBNB190.	Amp <sup>R</sup>	This study

<sup>a</sup> R, resistance.

**Table 3.** Oligonucleotides

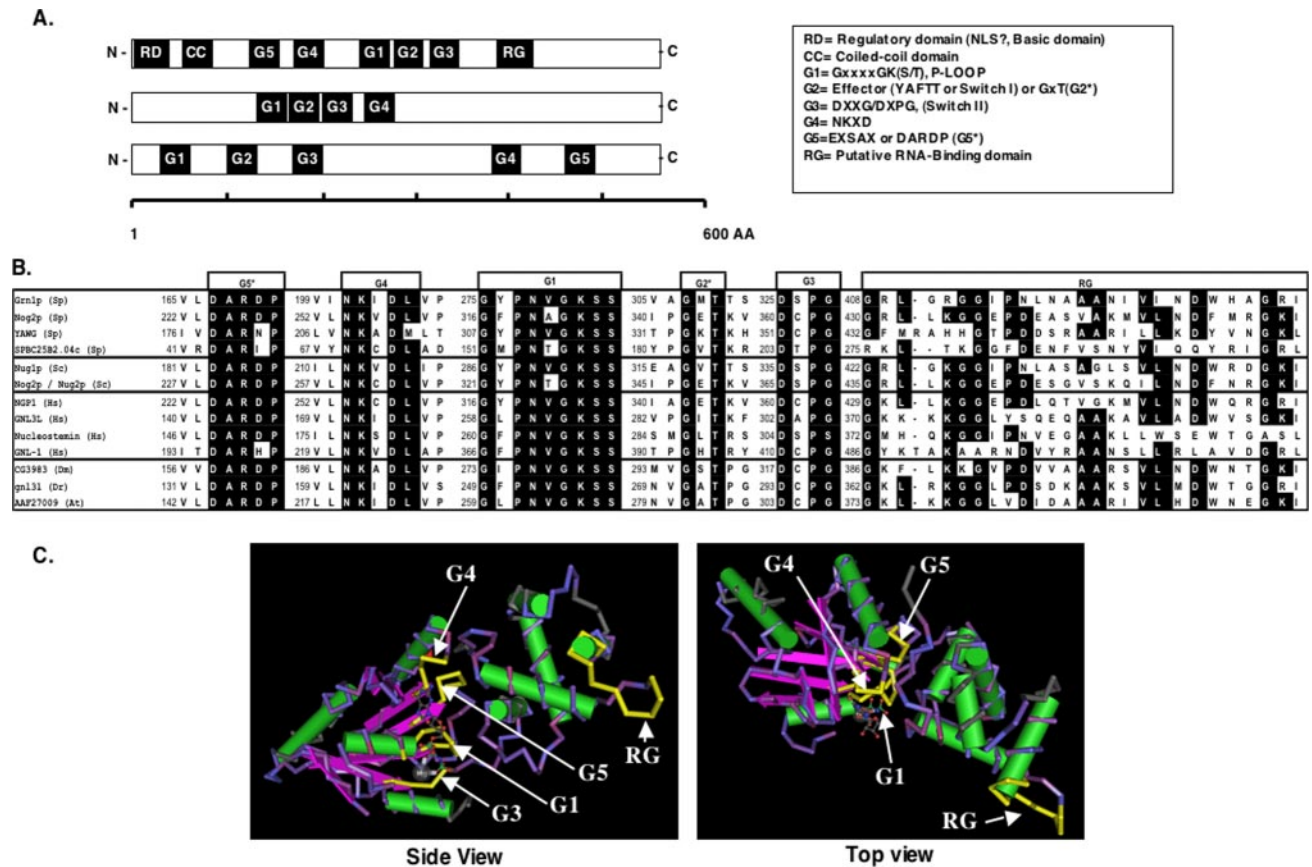
Number <sup>a</sup>	Sequence (5'-3')
NB110 <sup>F</sup>	GGTTAAAAAAGAATAATCGGTAATGTTTTTCTCTAGACAACCAACTGTAAAATTTGTAACACAGCATTTTTTACAATGCAACAGCTAT GACCGGCTACCAATTCACCCGCTCAACCCCTCACTAAAGGGAAC
NB111 <sup>R</sup>	GAAAAACCGCAACCGAAAACCAATCCCAAAATATAAGCTCTAAGCAACAATAGCTTTTTTTCGTAAGTTGAAAACCTCATTTGTAACG ACGGCCCGTTCTGCGGAGCATGACGACACTATAGGGCGAATTGG
NB373 <sup>F</sup>	GCATTGCTAACTAAGGAAATCTTTCATAATGTGAATATAAATTACTAATTAGCTTCAACTTTAAAAATAACGAGGGAATTCGAGCTCGT TTAAAC
NB375 <sup>R</sup>	GTTCTTCTTTTACTCTTTTTTCTTAAAGAAATAAGTTAGAAATAGTTACGCGTGCATATACTTACTTAAAGGAACTTTGTATAGTTTCATCC
NB377 <sup>R</sup>	CATCTGCGGCCGCGGAAAATCATTAAGGTCAA
NB378 <sup>F</sup>	CTTACAGTCGGTGTAATTTCTGTTATTAACGCTCTT
NB379 <sup>R</sup>	AAGAGCGTTAATAACAGAAATACACCGACTGTAAG
NB380 <sup>F</sup>	CATATGTCGACTATGGTTTCCCTTAAAAAAAAGAGTAAAAGAAG
NB453 <sup>F</sup>	GATCGAAGAACAGAAGCGCGAAGACGCTGTTGATGAA
NB454 <sup>R</sup>	TTCATCAACAGCGCTTCGCGCTTCTGTTCTTCGATC
NB455 <sup>F</sup>	GCTACTGATTTTTTAGTCAATATTAATCCAAATCTTAACGCTGC
NB456 <sup>R</sup>	GCAGCGTTAAGATTTGGAATAATATTGACTAAAAATCAGTAGC
NB457 <sup>F</sup>	AAGTTGTTGAAGCGTCAGAAGGGACTCGTTCAAAAAG
NB458 <sup>R</sup>	CTTTTGAACGAGTCCCTTCTGACGCTTCAACAACTT
NB524 <sup>F</sup>	GCATCTTCTGCTGAAGAATCAGAAGTACTCAACAAG
NB525 <sup>R</sup>	CTTGTTGAGTACTTCTGATTTCTCAGCAGAAGATGC
NB629 <sup>R</sup>	CCCAAAAAGTTAAAAGATGG
NB631 <sup>R</sup>	TCGTTCAACACCTCATC
NB700 <sup>R</sup>	TCGTTAGAGGTGAGACAA
NB702 <sup>F</sup>	AGAAGTGAAAAAGGAGAC
NB712 <sup>F</sup>	CATATGTCGACTATGGTGAAGCCCAAGTACAAAAG
NB713 <sup>R</sup>	CATCTGCGGCCGCGGCTGCTTTTGTCTGAATTTT
NB762 <sup>F</sup>	CATATGTCGACTATGATGAACTTAGACACAA
NB763 <sup>R</sup>	CATCTGCGGCCGCGGGTACCAACACCATCATCAGC
NB864 <sup>F</sup>	GACTCAGGTACCATGGTTTCCCTTAAAAAAAAG
NB865 <sup>R</sup>	GACTCACTCGAGCTATTTGTATAGTTTCAT
NB883 <sup>F</sup>	GACTCAGGTACCATGATGAACTTAGACACAA
NB907 <sup>F</sup>	GATCCGCTATATGGCGTCTCTGGGTTCAAGAGACCCAGAGACGCCATAATAGCTTTTTTGGTACCG
NB908 <sup>R</sup>	AATTCGGTACCAAAAAGCTATTTATGGCGTCTCTGGGTCCTTGAACCCAGAGACGCCATAATAGCG
NB909 <sup>F</sup>	GATCCGCTATATGGCGTCTGGTCTGCAAGAGACGACCAGACCGCAATATAGCTTTTTTGGTACCG
NB910 <sup>R</sup>	AATTCGGTACCAAAAAGCTATTTATGGCGTCTGGTCTGCTTGAACGACCAGACCGCAATATAGCG
NB963 <sup>F</sup>	GGCCGAGATGGACTACAAGGATGACGATGACAAATAAC
NB964 <sup>R</sup>	TCGAGTTATTTGTCTATCGTCAATCCTTGTAGTCCATCTGC
NB1002 <sup>F</sup>	GTTAAAAAAGAATAATCGGTAATGTTTTTCTCTAGACAACCAACTGTAAAATTTGTAACACAGCATTTTTTACAATGATGAAACTTAGAC ACAA
NB1004 <sup>F</sup>	CGAAAAAGAACTCATCTGAAGTTCAGGATACTCAAATCGTTACTGAGTGGGCCAAAGAATTTGACCTTAATGATTTTCCGCGGCCGAGATG GACTAC
NB1006 <sup>F</sup>	AACAAATTACGTTTGGTCAATGTTTTTCCTTCTAGT
NB1007 <sup>R</sup>	ACTAGAAGGAAAAACAATGACCAACGTAATTTGTT
NB1102 <sup>F</sup>	GATATAATTAATTCAGAC
NB1125 <sup>R</sup>	AACCGCAACCGAAAACCAATCCCAAAATATAAGCTCTAAGCAACAATAGCTTTTTTTCGTAAGTTGAAAACCTCTCTAGAACTAGTGGATCTG
NB1149 <sup>F</sup>	GACGCAGGTACCGTACGACGAGCTCCTTTATATTAATAAATTAATTAATTGC
NB1150 <sup>R</sup>	GCTATCTTTGAAATCATTAGGGATCCAT
NB1151 <sup>F</sup>	ACTACAGCATTTTTTACAATGGTTTTCCTTAAAAAGCTGC
NB1152 <sup>R</sup>	GCAGCTTTTAAAGGAAACCATGTAATAAATGCTGTAGT
NB1153 <sup>F</sup>	ACTACAGCATTTTTTACAATGGTTTTCCTTAAAAAATCCGC
NB1154 <sup>R</sup>	GCGGATTTTTTAAAGGAAACCATGTAATAAATGCTGTAGT
NB1160 <sup>F</sup>	CATATGTCGACTATGAAAAGGCTAAGTTAAAG
NB1161 <sup>R</sup>	CATCTGCGGCCGCGGCACATAATCTGTAAGTGC
NB1478 <sup>F</sup>	TTTCGCTGCGTCTTC
NB1160 <sup>F</sup>	CATATGTCGACTATGAAAAGGCTAAGTTAAAG
NB1161 <sup>R</sup>	CATCTGCGGCCGCGGCACATAATCTGTAAGTGC
NB1309 <sup>F</sup>	CATATGTCGACTATGAGAGTCAGAAAAGCGCC
NB1310 <sup>R</sup>	CATCTGCGGCCGCGGTTCCATCATTTGTATCTTGATC

<sup>a</sup> F, forward; R, reverse.**Western Analysis**

To analyze the expression of GFP- or FLAG-tagged Grn1p or GNL3L in yeast, 5–10 ml cultures were grown in appropriate medium and harvested at an OD<sub>595</sub> of 0.5–1.0. Because wild-type and mutant strains grew at different rates, great care was taken to harvest strains at the same OD<sub>595</sub>. Unless otherwise specified, equal numbers of cells as judged by OD<sub>595</sub> measurements were processed for Western blots. Proteins were extracted by resuspending cell pellets in 1% SDS phosphate-saline buffer (pH 8.0) and lysing them with an equal

volume of glass beads in a bead-beater four times for 30 s at 4°C. In some instances, to concentrate protein, TCA was added to the above yeast lysate at 4°C to a final concentration of 25%. Precipitated proteins were resuspended in SDS/sample buffer containing 8 M urea and pH adjusted with 1.0 M Tris-base buffer. After separation on 12% SDS-PAGE gels, proteins were transferred to PVDF membranes (Millipore, Bedford, MA). Specific epitope-tagged proteins were visualized by their reaction either with polyclonal anti-GFP (Molecular Probes, Eugene, OR) or polyclonal anti-FLAG (Sigma, St. Louis, MO).





**Figure 1.** Grn1p is member of a unique family of HSR1\_MMR1-nucleolar GTPases with a highly conserved circularly permuted “G”-domain. (A) Schematic representation of three types of G-proteins illustrating the relative positions of the motifs that make up the G-domain. Top bar: GTPases with a circular permutation of the classic G-domain; middle bar: a small group of GTPases that belong to the Nog-subfamily; bottom bar represents the “classic” G-proteins as exemplified by the Ras, EF-2, and heterotrimeric G-protein families. (B) Alignment of the above circularly permuted G-domain showing individual motifs G5\*, G4, G1, G2\*, G3, and the putative RNA-binding domain (RG). Motifs G5\* and G2\* correspond to G5-like and G2-like, respectively (see text). Representatives were chosen from a broad selection of eukaryotes: *S. pombe* (Sp), *S. cerevisiae* (Sc), human (Hs), *Drosophila melanogaster* (Dm), *Danio rerio* (Dm), and *Arabidopsis thaliana* (At). Identical residues are shaded black (conservative substitutions are not indicated). Numbers to the left of each motif indicate the beginning of the amino acid motif. (C) 3D representation of the highly conserved circularly permuted GTPases based on the structure of *Bacillus subtilis* Y1qf GTPase as the consensus for the HSR1\_MMR1 GTP-binding domain from the 3D-structure Entrez database, *MMDB* (<http://www.ncbi.nlm.nih.gov/Structure/MMDB/mmdb.shtml>). Images were visualized and recorded using NCBI’s Cn3D4.1 software. G-motifs and the RG-domain are indicated with arrows. Flat arrows represent  $\beta$ -sheets, whereas cylinders represent  $\alpha$ -helices.

### RNA Extraction and Northern Analysis

Total RNA was isolated by phenol:chloroform method following standard methods and was analyzed on a 1.2% agarose-acrylamide gel. After electrophoresis, ethidium bromide-stained-RNA bands were imaged to record 25S and 18S mature rRNA species and then transferred onto Hybond N+ membrane (Amersham Biociences, Bucks, United Kingdom). All oligonucleotide probes were more-or-less based on Good *et al.* (1997) and are shown in Figure 3. To identify 35S pre-rRNA species, a DIG-labeled PCR probe specific for 5' externally transcribed spacers (ETS) was synthesized using *S. pombe* genomic DNA as template, DIG-DNA labeling Mix as substrate and primer sets of NB700 + NB702 (all commercial reagents for Northern analysis were from Roche, Mannheim, Germany). The resulting PCR product corresponded to the sequence -900 nucleotides upstream of the 18S rRNA ORF. The 5.8S probe corresponds to a sequence within the 5.8S ORF (NB1478), whereas the ITS1 oligonucleotide probes (NB629 and NB1102) corresponded to the D→A2 and A3→B1 cleavage sites, respectively, and the ITS2 oligonucleotide probe (NB631) corresponding to the sequence within E→C1 cleavage sites. All four were generated by 3' end labeling using DIG-11-ddUTP according to manufacturer's instructions. Hybridization of RNA with the above probes was made with commercially available buffers. For 5' ETS northern, hybridization was performed at 55°C and washes at 65°C, whereas for 5.8S, ITS1 and ITS2, hybridization and washes were performed at 30°C. The membrane containing RNA was hybridized with above probes and subsequently detected with anti-DIG AP fragment and developed using CSPD according to the manufacturer's instructions. A commercial actin RNA DIG-labeled probe was used to detect yeast actin mRNA levels as an internal control.

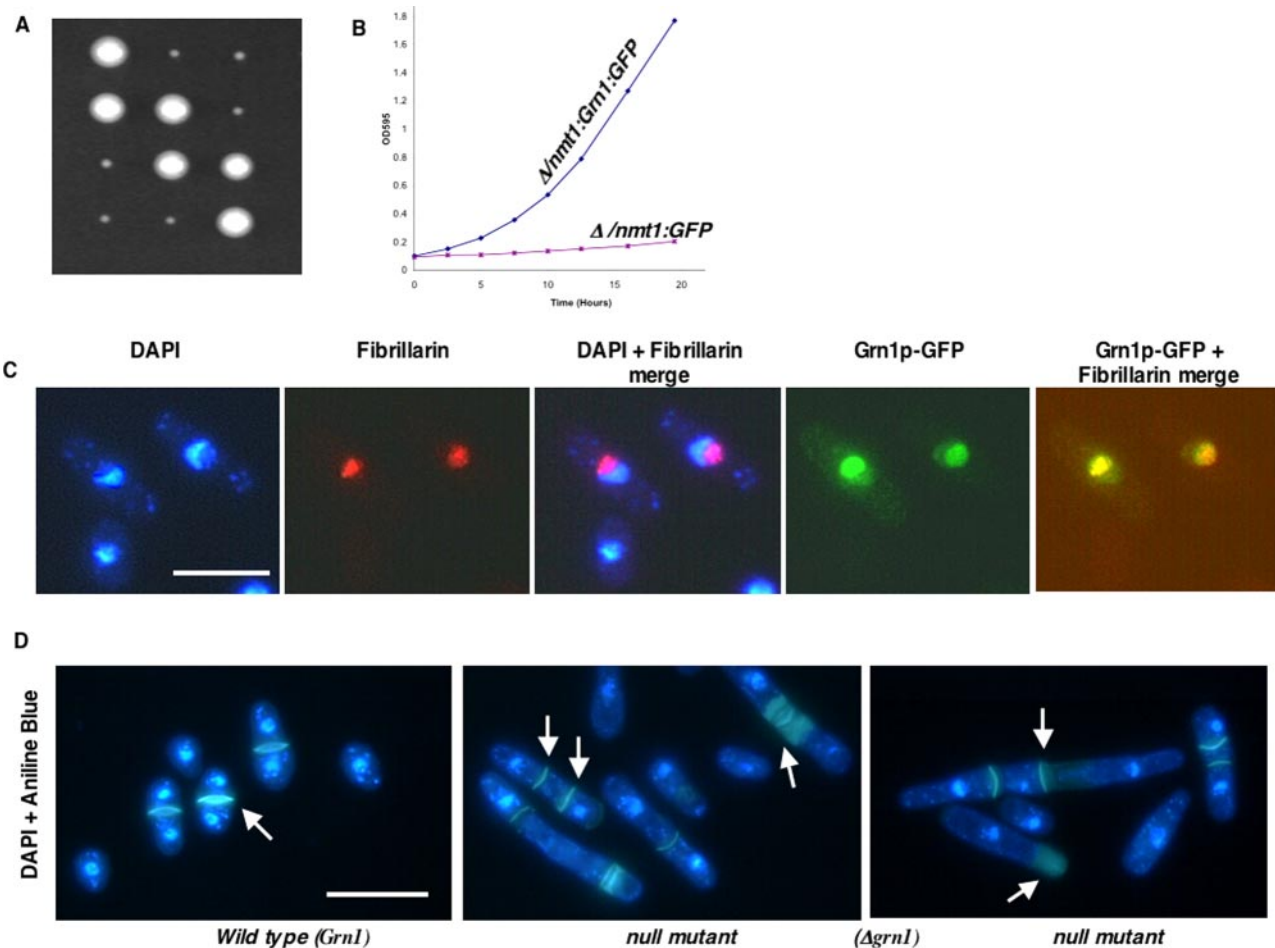
### Small Inhibitory RNA Knockdown

A unique sequence of *GNL3L* (nt1047–1065) was chosen as the target sequence for RNA interference. NB907/908 containing the target sequence, 5'-CTAT-TATGGCGTCTCTGGG-3' and a scrambled version, 5'-CTATATTGCGGTCT-GGTCCG-3' (NB909/910; used as a negative control) were cloned into the pSIREN vector (BD Biosciences) under the control of the human U6 promoter. A small inhibitory RNA (siRNA) targeted to the Luciferase gene was used as an additional negative control. All siRNA expressing constructs (9  $\mu$ g of each) were cotransfected with pcDNA3 vector (0.9  $\mu$ g) into HeLa cells. After 120 h selection in G418 (500 mg/ml), the cells were photographed and total RNA was isolated using spin minicolumns according to manufacturer's instructions. RT-PCR was performed by a standard protocol and the *GNL3L*-specific signal was amplified using the following primers: *GNL3L* forward: 5'ATGT-GCGAATTCATGTGAAACTTAGACACAAAAATAAAAAGCC and *GNL3L* reverse: 5'CACCATGATATCCCGGATGAACCTGTCCAGGTAGAC.  $\beta$ -actin was amplified with the following primers: forward: 5'GGCGACGAG-GCCAGA and reverse: 5'CGATTTCCTCCGCGC as an internal control to normalize the equal quantity of RT products used in PCR.

## RESULTS

### *Grn1p* Is a Member of a Novel G-Protein Family

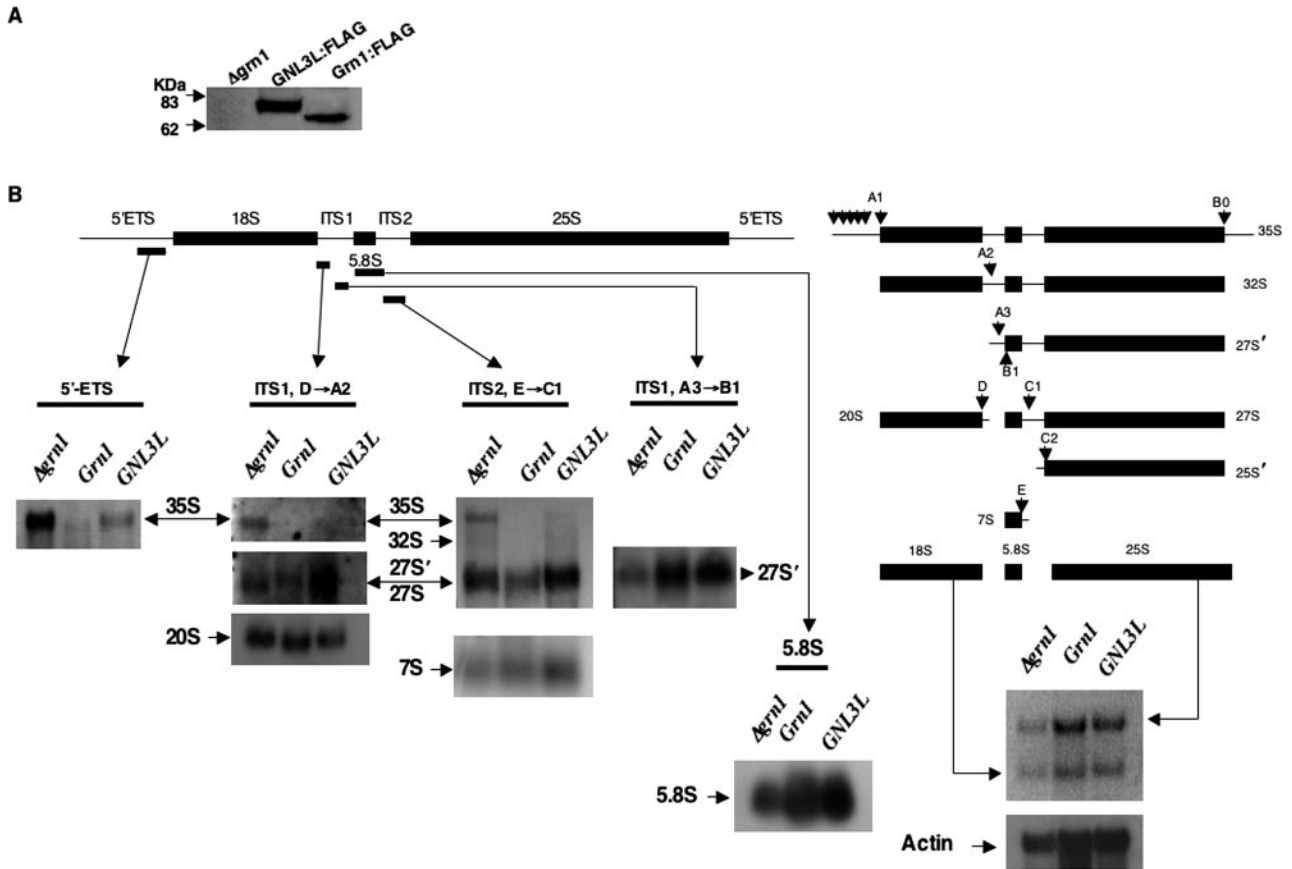
*Grn1* encodes a predicted protein of 470 residues. PSORT analysis (Nakai and Horton, 1999) identifies a predicted



**Figure 2.** *Grn1* is required for wild-type growth and encodes a nucleolar protein. (A) *Grn1* (*SPBC26H8.08c*) was deleted as described in the text. Tetrad dissection yielded two fast-growing (Wild type) and two slow-growing (null mutant) colonies. (B) A null mutant expressing full-length Grn1p:GFP (YNB544) or an empty vector (YNB546) were in EMM-leu medium. Optical density ( $OD_{595}$ ) was determined at the indicated time points. (C) YNB544 (see above) was used to show the localization of Grn1p. Nuclear DNA was stained with DAPI. Nucleoli were revealed by indirect immunostaining with anti-Fibrillarin (AbCam, Cambridge, United Kingdom). Independent or merged images are indicated. (D) Wild-type (YNB483) and null mutant (YNB484) strains were stained with DAPI and Aniline blue for visualizing the nucleus and septum, respectively. Arrows indicate the septum. Bar, 10  $\mu$ m.

coiled-coil domain and at least four GTPase-consensus motifs designated here as G1, G3, G4, and G5\* that define a G protein (Takai *et al.*, 2001; Leipe *et al.*, 2002; Figure 1A). In addition, there is a putative RNA-binding domain at the C-terminus (RG-stretch). A BLASTp search (Altschul *et al.*, 1997) of the predicted protein data banks showed that highly related sequences are found in yeast as well as in diverse eukaryotes with the "G"-domain displaying an extremely high degree of sequence homology (Figure 1B). A CD-search of conserved domain databases (CDD; Marchler-Bauer *et al.*, 2005), Pfam (Bateman *et al.*, 2004) and clusters of orthologous groups (COGS; Tatusov *et al.*, 2000) revealed some very interesting aspects of this GTPase. All the diagnostic motifs of the putative GTPases described above were present albeit in altered juxtaposition to each other. Rather than the usual G1-G2-G3-G4-G5, found in the superfamily of regulatory GTP hydrolases (Takai *et al.*, 2001; Leipe *et al.*, 2002), the G1 motif or P-loop (GXXXXGK(S/T)) is situated between the G4 (KXDL) and G3 (DXXG/DXPG) motifs as G5\*-G4-G1-G2\*-G3 (Figure 1A) in what has been described as a circularly permuted G-motif (Daigle *et al.*, 2002; Leipe *et al.*, 2002). Thus, the domain structure of the putative GTPase like other

previously studied nucleolar GTPases, Nug1p (Bassler *et al.*, 2001), Nog2p (Nug2p; Saveanu *et al.*, 2001), NGP-1 (Racvskis *et al.*, 1996), and NS (Tsai and McKay, 2002, 2005) conforms to that observed for the HSR1\_MMR1 GTP-binding protein GNL-1, (Vernet *et al.*, 1994) and members of the Ylqf/YawG family of GTPases (Leipe *et al.*, 2002). The so-called G2\* (YAFTT or Effector or Switch I) is a less conserved motif and is not present in all GTPases and G5\* (EXSAX; Takai *et al.*, 2001) appear to be ill defined in this group. However, in keeping with a previous report (Saveanu *et al.*, 2001), the amino acid residues DARDP and GxT will be referred to as G5\* and G2\*, respectively. The predicted structure of the putative GTPase based on consensus motifs from the Ylqf/YawG family show the six-stranded  $\beta$ -sheet of the G-domain wherein the conserved sequence elements, G5\*-G4-G1-G3 stack almost perfectly over each other (Figure 1C). Interestingly, except for a bacterial ancestor of this group of GTPases, YjeQ (Daigle *et al.*, 2002), none of the eukaryotic members shown in Figure 1, A and B, have proven GTPase activity.



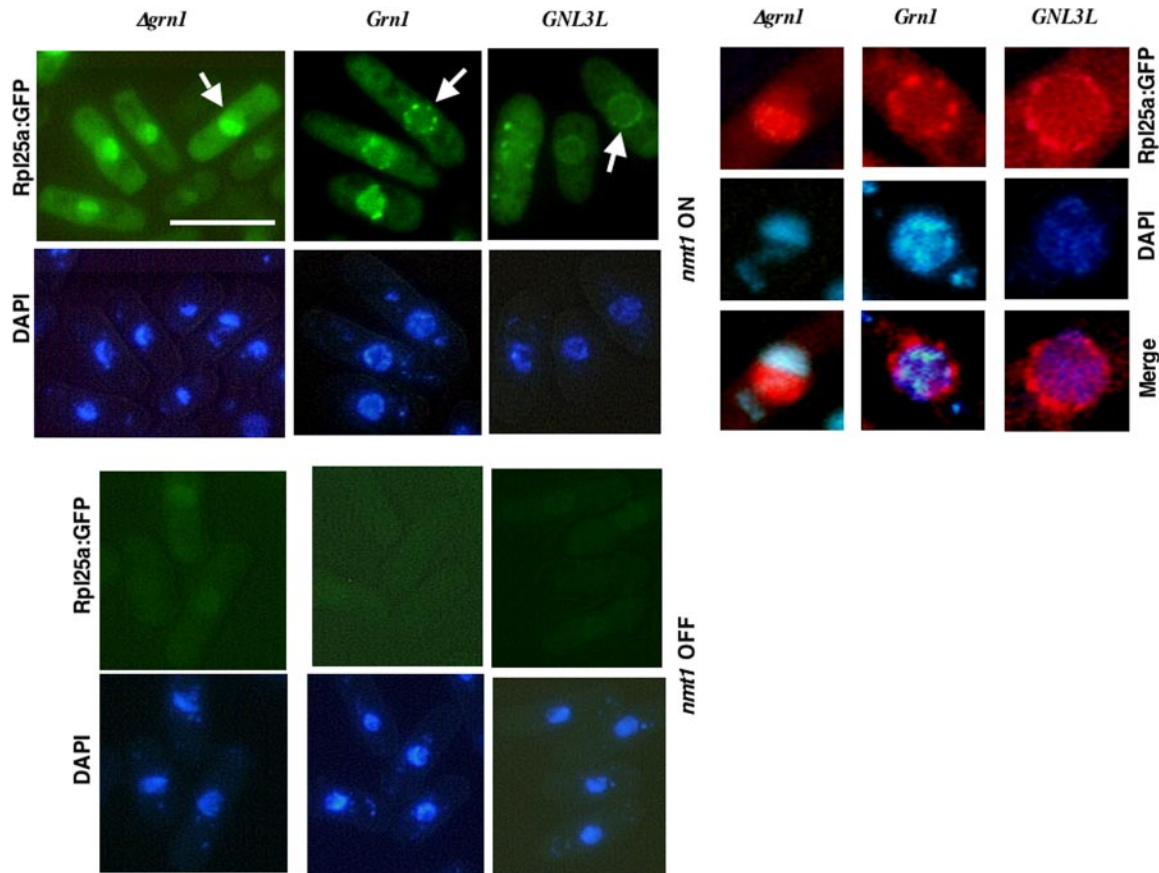
**Figure 3.** Effect of Grn1p and GNL3L on processing of 35S pre-rRNA species. (A) *Grn1:FLAG* (YNB859) and *GNL3L:FLAG* (YNB858) were tested for genomically expressed FLAG-tagged Grn1p and GNL3L by Western analysis and probing with anti-FLAG. The null mutant (YNB484) was used as control. (B) Pre-rRNA and mature rRNA species were detected in the above strains by Northern hybridizational analysis. DNA probes specific for 5' ETS, 5.8S, ITS1 or ITS2 are indicated by bars under the respective flanks. The rRNA processing pathway was adapted from Good *et al.* (1997). Downward pointed arrows indicate relative positions of processing sites.

### *Grn1* Is Required for Optimal Growth of *S. pombe* and Localizes to the Nucleolus

To study the function of this putative GTPase, the ORF *SPBC26H8.08c* was deleted and replaced with the *Ura4* gene by homologous recombination using a previously described PCR-based deletion strategy (Bahler *et al.*, 1998; Chen *et al.*, 2004). Dissection of tetrads from at least 24 independent asci yielded two of the four spores from each tetrad that grew extremely slow on YES medium when compared with the wild-type strain at 32°C (Figure 2A) and similarly at 24 or 37°C (results unpublished data). Each one of these slow-growing colonies was confirmed as having the *SPBC26H8.08c* deletion. In contrast, genes encoding similar putative GTPases from yeast, *Nug1* (*YER006W*) and *Nug2/Nog2* (*YNR053C*; Bassler *et al.*, 2001; Saveanu *et al.*, 2001) were essential for viability. To investigate the subcellular localization of the Grn1p, its ORF, *Grn1*, was cloned as a C-terminal fusion to the green fluorescent protein (GFP) downstream from an inducible promoter, *nmf1*, and transformed into the null mutant. Figure 2B shows that the growth phenotype of the null mutant was rescued. To establish that Grn1p localizes to the nucleolus, we used as a nucleolar reference marker, Fibrillarin/Nop1p (Aris and Blobel, 1988; Henriquez *et al.*, 1990). Monoclonal antibody staining detected Fibrillarin/Nop1p in a discrete region (Figure 2C, image showing red staining) that selectively excluded the DAPI-stained area of the nucleus establishing

the nucleolar region (Figure 2C, DAPI + fibrillarin merge). The bulk of the Grn1p:GFP (green) signal was observed within the area colocalized by the Fibrillarin/Nop1p (red), resulting in the yellow merged signal shown in the last image of the panel in Figure 2C. However some signal was also evident in the nucleoplasm area. Our results therefore suggest that Grn1p accumulates primarily in the nucleolus in *S. pombe*. Because the episomally expressed gene is able to complement the growth defect in a null mutant, we confirm the involvement of this nucleolar protein in growth. In addition to *Grn1*, *S. pombe* has at least three other ORFs predicted to generate putative nuclear/nucleolar GTPases with an HSR1\_MMR1-type domain (Figure 1B). We conclude the function of the protein encoded by *Grn1* does not overlap directly with those of the other three putative GTPases. Our genetic data implies that the gene is not essential for viability. We did, however, observe that 20–40% of cells exhibited morphogenic aberrations represented by irregular, uneven, or overdeposition of septum material as well as septation and cell separation defects, often resulting in pseudofilamentous or multiseptated cells (Figure 2D), suggesting a failure of cytokinesis in those cells. Cultures of null mutant cells did not appear to exhibit a growth arrest. Because GTPases are known to be involved in nuclear-cytoplasmic transport (Saitoh *et al.*, 1996; Moy and Silver, 1999; Seedorf *et al.*, 1999; Bassler *et al.*, 2001), we asked whether the *Grn1* deletion would lead to defects in the nuclear pore complexes





**Figure 4.** *Rpl25a* localization in  $\Delta grn1$ , *Grn1*-FLAG, and  $\Delta grn1::GNL3L$ -FLAG strains. The null mutant (YNB484), *Grn1*:FLAG (YNB859), and *GNL3L*:FLAG (YNB858) were transformed with *nmt1::Rpl25a::GFP* (BNB221) to give YNB631, YNB1076, and YNB1075, respectively. GFP fluorescence was visually inspected in >100 cells for each of the indicated strains. Bar, 10  $\mu$ m. In > 90% null mutant cells (YNB631) *Rpl25a::GFP* appeared inside nucleus with a significant accumulation within the nucleolus. For YNB1075 and YNB1076, >90% showed localization to the nuclear rim, with no accumulation within the nucleolus. A representative image of each strain is depicted. The top right panel depicts the GFP and DAPI images of a single nucleus (indicated by arrowhead) that were enlarged and digitally manipulated to convert the GFP-green fluorescence to red.

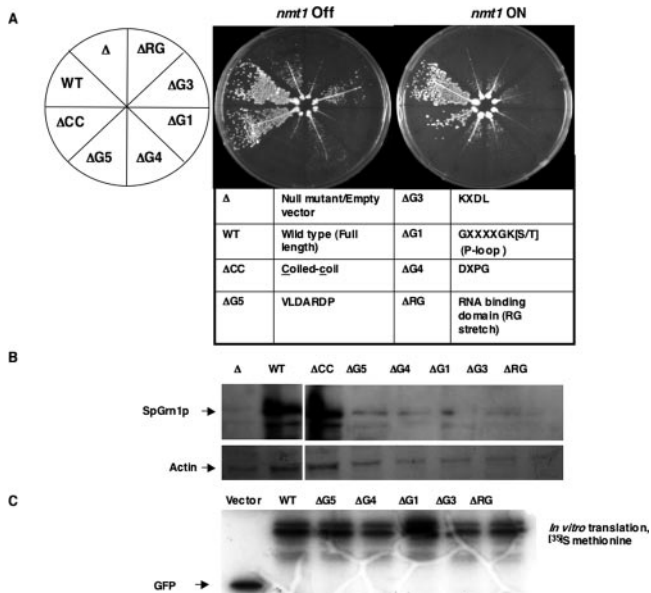
(NPC), nuclear import, and mRNA export. However, cultures of null mutant cells did not appear to have defects in either the NPC or nuclear-cytoplasmic transport (protein import or mRNA export; unpublished data).

#### *Pre-rRNA Processing Is Impaired in Grn1-depleted Cells*

Because the *Saccharomyces cerevisiae* GTPases Nug1p and Nug2p were linked closely with pre-rRNA processing (Bassler *et al.*, 2001; Saveanu *et al.*, 2001), we asked if the  $\Delta grn1$  mutant was defective in the processing of 35S pre-rRNA precursor to mature rRNA species by performing a Northern blot analysis. The yeast rDNA unit is made up of the 35S pre-rRNA operon and two nontranscribed spacers (NTS) interrupted by the 5S rRNA gene. The 35S pre-rRNA operon, flanked on either end by ETS, 5' ETS and 3' ETS, eventually gives rise to the mature 18S, 5.8S, and 25S rRNA species (Venema and Tollervey, 1999). The *S. pombe* rRNA processing pathway (Figure 3B) is similar to that of *S. cerevisiae* and several other eukaryotes although it may depart from the same in specific processing steps (Good *et al.*, 1997). Probes corresponding to the 5' ETS, 5.8S, ITS1 and ITS2 regions are indicated in Figure 3B. Our 5' ETS probe beginning at -900 bp upstream of the 5' end of 18S rRNA covers all the putative 5' ETS processing sites and would thus

identify all intermediates from 35S to 32S pre-rRNA (it will not detect 32S) species, whereas the ITS1 probes (D $\rightarrow$ A2) and (A3 $\rightarrow$ B1) identify 35S, 32S, and 20S; and 35S, 32S, and 27S', respectively. The ITS2 probe (E $\rightarrow$ C1) detects 35S, 32S, 27S', 27S, and 7S species. The 27S', 27S rRNA precursors are indistinguishable (Good *et al.*, 1997). To detect 5.8S mature rRNA, the probe was based on a sequence within the 5.8S operon. To investigate rRNA processing and other experiments (see below) in wild-type and mutant strains, we constructed the strain YNB859 (Table 1), wherein the *FLAG KanMX6* sequence was integrated at the 3' end of *Grn1* as described in *Materials and Methods*. YNB859 expressed a stable C-terminus tagged Grn1p:FLAG fusion protein when tested for protein expression along with the null mutant, YNB484 (Figure 3A). The results depicted in Figure 3B show the accumulation of the 35S pre-rRNA species in the null mutant (YNB484) when compared with the wild type (YNB859) with a concomitant decrease in the 25S, 18S, and 5.8S mature rRNA species. Under wild-type conditions it may not be possible to see the 35S pre-rRNA species because it is processed very rapidly (Good *et al.*, 1997; Venema and Tollervey, 1999). The observations that mature rRNA species in the null mutant are significantly lower than that of the wild type, coupled with the increase in levels of the 35S





**Figure 5.** The G-domain and RG-domain of Grn1p are required for growth. (A) The growth of all the indicated strains: WT (YNB544),  $\Delta grn1$  (YNB546),  $\Delta RG$  (YNB568),  $\Delta G3$  (YNB956),  $\Delta G1$  (YNB545),  $\Delta G4$  (YNB611),  $\Delta G5$  (YNB566), and  $\Delta CC$  (YNB567) was determined. Strains were struck for single colonies on EMM-leu plates with (*nmt1* OFF) or without 15  $\mu$ M thiamine (*nmt1* ON). (B) The total proteins of all the strains were isolated and processed by Western analysis using anti-GFP antibody. (C) GFP, Grn1:GFP,  $\Delta G5$ -Grn1:GFP,  $\Delta G4$ -Grn1:GFP,  $\Delta G1$ -Grn1:GFP,  $\Delta G3$ -Grn1:GFP,  $\Delta RG$ -Grn1:GFP, from pBNB340, pBNB338, pBNB335, pBNB336, pBNB337, pBNB417, and pBNB339, respectively (Table 2), were transcribed and translated in the presence of L-[<sup>35</sup>S]methionine using a TNT-coupled Reticulocyte Lysate System (Promega, Madison, WI) according to the manufacturer's instructions. One microliter of each of product was analyzed on a 12% SDS-PAGE gel and exposed to x-ray film.

pre-rRNA species, is suggestive of a significant inhibition or slowing down of the early pre-rRNA processing steps.

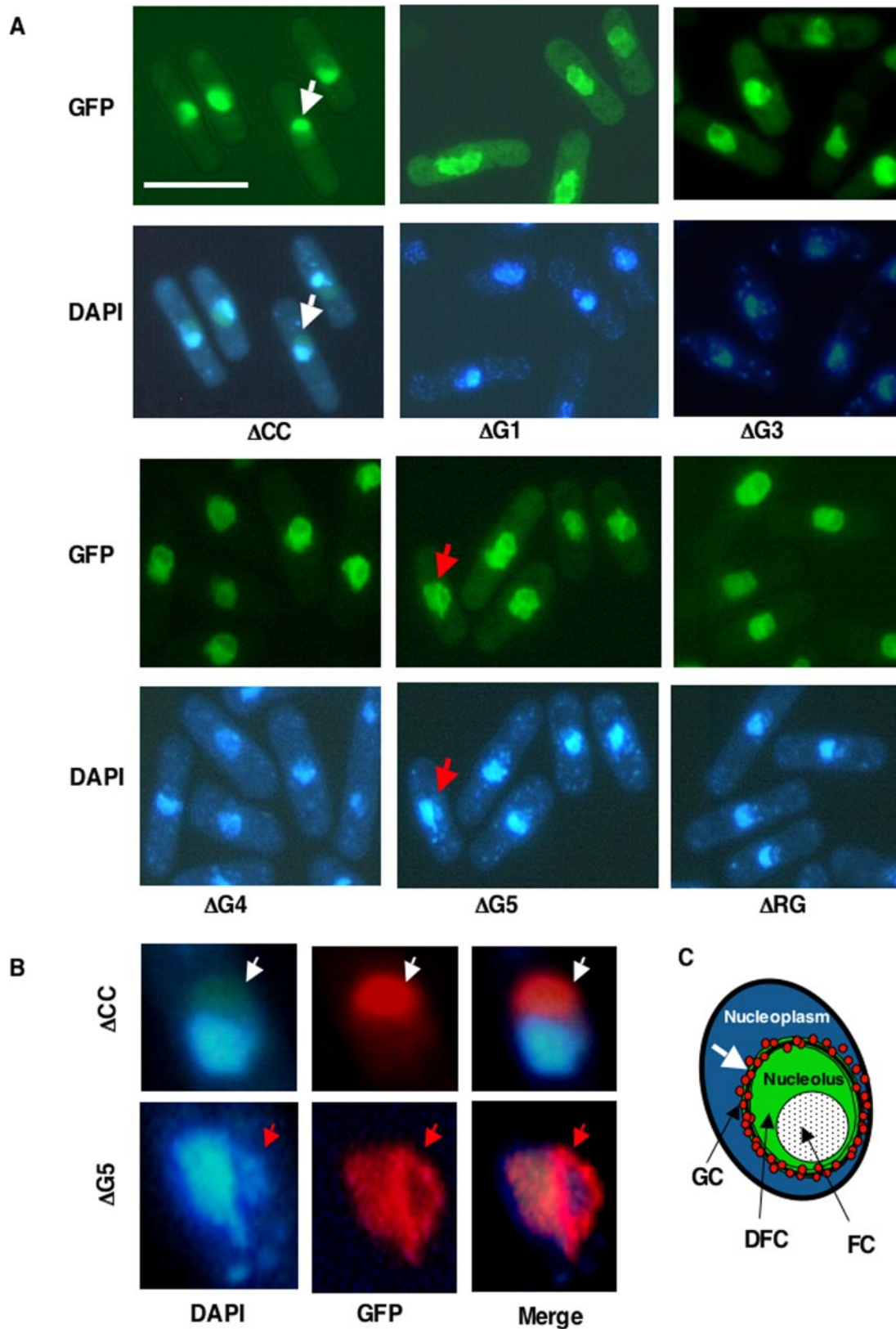
#### Grn1p Is Required for Nuclear Export of the Putative Ribosomal Protein Rpl25a

RNA export from the nucleus is linked to its proper processing and packaging into ribonucleoprotein complexes within the nucleus (Strasser and Hurt, 1999; Tschochner and Hurt, 2003). The use of functional GFP-tagged ribosomal protein reporters has greatly facilitated the elucidation of the large-subunit (Hurt *et al.*, 1999; Stage-Zimmermann *et al.*, 2000; Gadai *et al.*, 2001) and small-subunit (Grandi *et al.*, 2002; Milkereit *et al.*, 2003) ribosome assembly and nucleolar/nuclear export pathway. L25 (Rpl25 in yeast and L23 in plant/mammal/human) is perhaps the most extensively studied and highly conserved eukaryotic ribosomal protein (Supplemental Figure S1). The *S. cerevisiae* Rpl25:GFP, binds to pre-rRNA, assembles with 60S ribosomal subunits after its import into the nucleolus and is subsequently exported into the cytoplasm, thus allowing for monitoring of the localization of pre-60S and 60S particles by fluorescence microscopy (Hurt *et al.*, 1999). We therefore used an in vivo assay (Hurt *et al.*, 1999) exploiting the GFP-tagged version of the *S. pombe* putative ribosomal protein Rpl25a encoded by the ORF SPBC106.18 that is homologous to ORF YOL127W of *S. cerevisiae* encoding Rpl25 (Supplemental Figure S1). We cloned the *S. pombe* putative Rpl25a into a vector and expressed *nmt1*:Rpl25a:GFP in wild-type (*Grn1*) and null mu-

tant ( $\Delta grn1$ ) strains. When induced (*nmt1* ON), Rpl25a:GFP was detected as punctate foci primarily outside the nucleolus at what appears to be the nuclear membrane of wild-type cells (Figure 4). However, in stark contrast, the null mutant consistently revealed a nucleolar accumulation of Rpl25a:GFP (Figure 4, panels showing enlarged nucleus). The combination of our results regarding the impaired release of Rpl25a:GFP from the nucleolus in the  $\Delta grn1$  mutant coupled with its inability to efficiently process the 35S pre-rRNA transcript is suggestive of its involvement in early 5'-end pre-rRNA processing step(s) and in the assembly and export of Rpl25a/pre-ribosomal complexes from the nucleolus.

#### The Canonical G Domain and a Putative RNA-binding Domain (RG) Are Required for Grn1p Function

To dissect the molecular mechanism underlying Grn1p function and to assess the functional significance of the signature GTP-binding motifs and the RG domain, we tested the ability of Grn1p with deletions of those motifs/domains to complement the growth defect of the null mutant. Constructs were engineered so that C-terminal GFP fusion proteins were generated with deletions of the putative coiled-coil domain  $\Delta CC$  (AA70–AA90),  $\Delta G5^*$  (AA164–AA175),  $\Delta G4$  (AA195–AA208),  $\Delta G1$  (AA276–AA283),  $\Delta G3$  (AA326–AA329), and the RNA binding domain  $\Delta RG$  (AA405–AA415; Figure 5A). The  $\Delta grn1$  strain was transformed with plasmids bearing the above constructs (Tables 1 and 2). An *nmt1* promoter drove transcription of wild-type and mutant versions of Grn1 in the absence of thiamine as described below. Independent transformants were struck for single colonies onto selective growth medium allowing for induction of gene expression. Figure 5A depicts growth of the various mutants in comparison to the wild-type and null mutant. The WT and the  $\Delta CC$  mutant fully complement the null mutant. However, the  $\Delta G5$ ,  $\Delta G4$ ,  $\Delta G1$ ,  $\Delta G3$ , and  $\Delta RG$  mutants were unable to rescue the null growth defect, indicating that those domains or motifs were required for its function. We noted that even in the presence of thiamine (*nmt1* turned off), WT and  $\Delta CC$  grew very well. The *nmt1* promoter is known to be "leaky," and as a result, even a low expression is sufficient to rescue growth. Expression of WT and mutant GFP-tagged proteins was verified by Western analysis using anti-GFP (Figure 5B), after which the membrane was stripped and reprobed with actin antibody to visualize actin levels. Figure 5B shows that levels of  $\Delta G5$ ,  $\Delta G4$ ,  $\Delta G1$ ,  $\Delta G3$ , and  $\Delta RG$  deletion proteins are extremely low when compared with the WT or  $\Delta CC$  levels of expression. Because equal amounts of cells were sampled for Western blots (*Materials and Methods*), the protein expression levels may in fact reflect the growth of the above strains. Actin levels are consistent with the above results. Our growth results show that deletion of any one of the  $\Delta G$ - or  $\Delta RG$  motifs results in a null phenotype and that the G5, G4, G1, G3, and RG motifs are equally critical for Grn1p function. Actin, here an example of a "house-keeping" protein, was used as an index of the protein levels of the cell. Actin levels in the above mutants appear to be similar to that of the null mutant. To make sure that the low levels of protein observed in these mutants was not because of defective constructs used, we detected the quantity and size of each protein by in vitro transcription-translation from a rabbit reticulocyte system. Figure 5C shows that an equivalent amount of each protein of the expected size shown was realized. We noted that in each case doublet bands were evident (including wild type) that were not present in the null mutant. Similar doublets were also observed in the Western blots in Figure 5B.



**Figure 6.** Effect of deletions in the G-domain and RG-domain on the localization of Grn1p. (A) Strains indicated in Figure 5A with plasmids containing GFP-tagged mutant versions of Grn1p expressed from an *nmt1* inducible promoter were grown in EMM-leu medium with and without 15  $\mu M$  thiamine. Only cells that were induced (*nmt1* ON) are shown. Bar, 10  $\mu m$ . (B) The GFP and DAPI images of a single nucleus from  $\Delta G5$  (YNB566) and  $\Delta CC$  (YNB567), marked with colored arrowheads, were enlarged and digitally manipulated to convert the GFP-green fluorescence to red in order to render a sharper contrast against the blue DAPI fluorescence and thus render and delineate more

### Mutations in the Functional Domains of *Grn1p* Alter Its Localization within the Nucleus

In Figure 2C, we established that full-length *Grn1p*:GFP localized to the nucleolus. We therefore wanted to know if the growth function of *Grn1p* and mutants observed in Figure 5A was correlated with their ability to localize to the nucleolus. GFP localization data are depicted in Figure 6A. The  $\Delta$ CC mutant localized to the nucleolus like the wild type (Figure 2C), whereas  $\Delta$ G1,  $\Delta$ G3,  $\Delta$ G4,  $\Delta$ G5, and  $\Delta$ RG exhibited an aggregation of GFP signal at the border of the nuclear and nucleolar regions (Figure 6, A and B). The evidence is clear from the enlarged images of the nucleus (Figure 6B) showing  $\Delta$ CC (an example of wild-type nucleolar localization) and  $\Delta$ G5 (a representative of the  $\Delta$ G-domain and  $\Delta$ RG-mutants). The  $\Delta$ CC mutant exhibits GFP fluorescence evenly distributed throughout the nucleolus (area defined by DAPI exclusion), whereas in the  $\Delta$ G5 mutant, the  $\Delta$ G5:GFP fluorescence appears to be concentrated in a specific area rather than be evenly distributed (Figure 6B, bottom panel). This area may be consistent with the granular component (GC) region of the nucleolus (Figure 6C). We also noted significant distortion of nucleolar/nuclear morphology with the  $\Delta$ G-motifs and  $\Delta$ RG mutants when compared with the WT (Figure 2C) or  $\Delta$ CC (Figure 6A).

### A Human Homolog FLJ10613 (*GNL3L*) Encoding a Hypothetical Protein Rescues the $\Delta$ *grn1* Growth Defect in *S. pombe*

As mentioned earlier, BLASTp and CD-search searches of available databases identified several HSR1\_MMR1 GTP-binding proteins similar to *Grn1p* (Figure 1, A–C). However, our attention was drawn to the association of these human proteins with cancer. NGP-1 (*GNL2*) was identified as a nucleolar breast tumor-associated autoantigen (Racevskis *et al.*, 1996). NS, a nucleolar GTPase controlling stem cell proliferation, was found in several cancer cell lines (Tsai and McKay, 2002; Liu *et al.*, 2004; Sijin *et al.*, 2004; Tsai and McKay, 2005). *GNL3L* (also referred to as FLJ10613; Ota *et al.*, 2004), on the other hand, is essentially an uncharacterized and hypothetical protein predicted to be a GTPase. To determine whether *GNL3L*, NGP-1, or NS could complement the growth defect in the *S. pombe*  $\Delta$ *grn1* mutant, we cloned their ORFs from a HeLa cell cDNA library into an inducible (*nmt1*) yeast expression vector and assayed growth of the yeast transformants (Figure 7A). *GNL3L* rescued the  $\Delta$ *grn1* deletion in a manner similar to that of *Grn1*. Similarly, the null growth phenotype is rescued when the *GNL3L* is expressed from the endogenous *Grn1* promoter instead. For these experiments, the wild-type *Grn1* ORF was replaced with *GNL3L*-FLAG *KanMX6* (YNB858, Table 1) so the latter would be transcribed from the *S. pombe* *Grn1* native promoter (see *Materials and Methods*). Therefore, strains with *GNL3L*-FLAG (YNB858) and *Grn1*::FLAG (YNB859) are isogenic. Figure 7B shows that though *GNL3L*-FLAG exhibited a longer lag, its growth rate became almost equal to that of *Grn1*. Thus, under identical endogenous promoter activities, *GNL3L* expression complements the *Grn1* deletion. We noted that although all these proteins were expressed in fission yeast, NGP-1, NS, or the yeast *ScNug1* were unable to complement

*SpGrn1* activity when induced. Surprisingly however, we observed that NGP-1 was able to fully complement *SpGrn1* when induced very weakly (*nmt1* OFF, Figure 7A) implying that *nmt1*-dependent overexpression of NGP-1 was toxic to cell growth. Our results suggest that these HSR1\_MMR1 GTPases could have unique activities within the nucleolus/nucleus.

To test whether *GNL3L* was also a nucleolar protein and that fission yeast *Grn1p*:GFP would similarly be targeted to the nucleolus in human cells, Cos-7 cells were transfected with *GNL3L*:GFP or *Grn1p*:GFP. The human nucleolar protein nucleolin was used as a marker for the nucleolus. Figure 7C shows that both putative GTPases are targeted to the mammalian nucleolus. Our genetic complementation thus identifies *GNL3L* as a homolog of *Grn1p*. Figure 7D shows the localization of *GNL3L*:FLAG in *S. pombe*. Comparing the localization of fibrillar and *GNL3L* (Figure 7D, see arrow), we noted the latter was not concentrated in the nucleolus to the same degree as *Grn1p*:GFP (compared with Figure 2C), suggesting that *GNL3L* and *Grn1p* may differ in their ability to either be targeted to, or retained by, the nucleolus.

### *GNL3L* Functionally Complements *Grn1p*

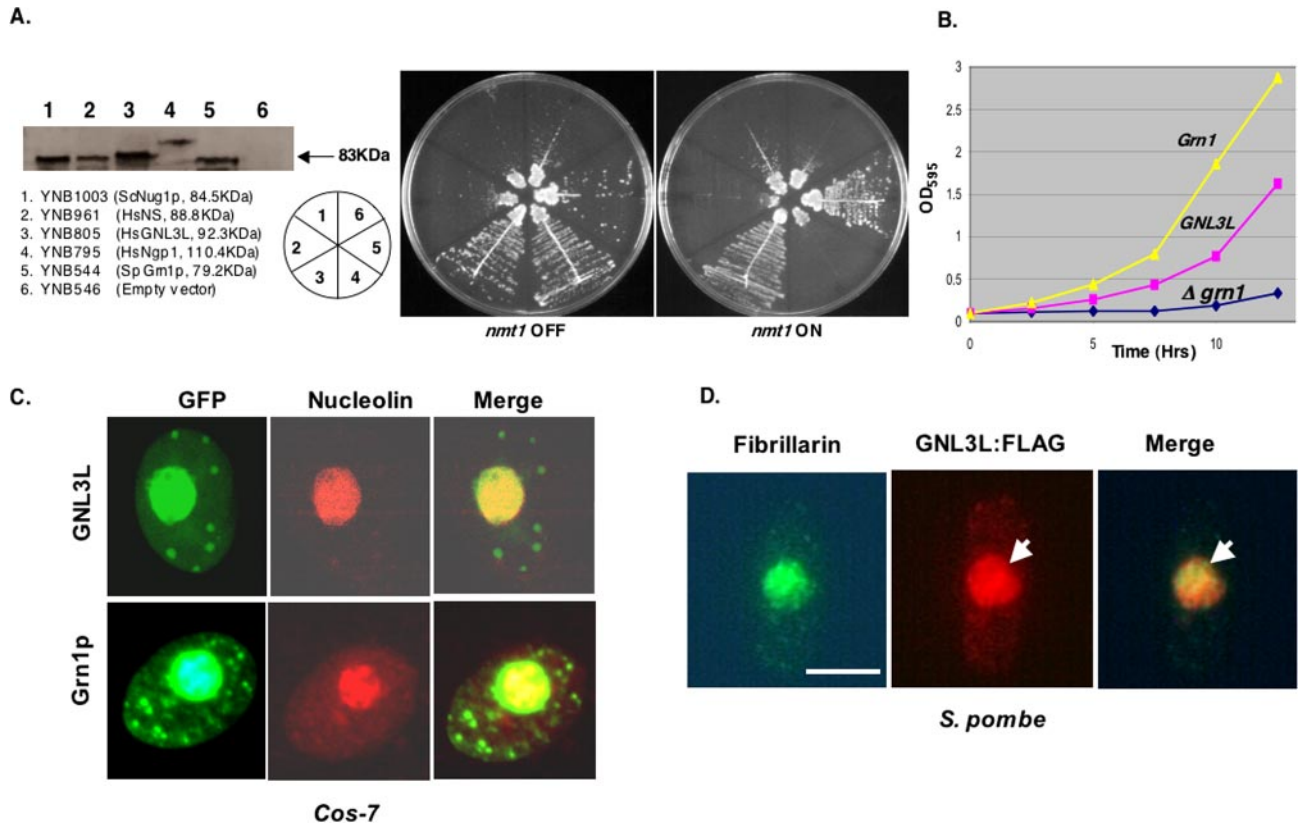
Because *GNL3L* rescued the  $\Delta$ *grn1* growth defect and the GFP-tagged protein localized to the nucleolus, we wanted to know if its expression in fission yeast would 1) rescue the rRNA processing defect and prevent the accumulation of the 35S pre-rRNA and 2) rescue the Rpl25a:GFP nuclear export defect. Strains expressing *GNL3L*-FLAG or *Grn1*::FLAG from the endogenous *Grn1* promoter and the null mutant were investigated for rRNA processing using 5' ETS, 5.8S, ITS1 and ITS2 probes. Figure 3 shows that in the *GNL3L*-FLAG strain there is a marked reduction in accumulation of 35S pre-rRNA accompanied by a significant increase in the amounts of 5.8S, 18S, and 25S mature rRNA species when compared with the null mutant. 5' ETS, ITS1 and ITS2 probing confirmed the reduction in accumulation of 35S pre-rRNA when *GNL3L* was expressed. Thus, expression of *GNL3L* rescues the pre-rRNA processing defect in the null mutant although it was not equivalent to the wild type. Because nuclear export of Rpl25a:GFP was blocked in the null mutant (Figure 4), we asked whether *GNL3L* could rescue that ribosome export defect. As shown in Figure 4, Rpl25a:GFP accumulated within the nucleoli of the *grn1* null but was exported to the nuclear periphery and cytoplasm with equal efficiency in both *GNL3L* and *Grn1p* strains. Thus, *GNL3L* functionally complements *Grn1p*.

### *GNL3L* Is Required for Proliferation of Mammalian Cells

Our results imply an important and unique role for the putative GTPase *Grn1p* in fission yeast because it is required for wild-type growth despite the presence of three other HSR1\_MMR1 putative nucleolar GTPases (see Figure 1B). In human cells, depletion or overexpression of NS causes a reduction in cell proliferation in CNS stem cells and transformed cells (Tsai and McKay, 2002). In another study, HeLa cells wherein NS expression was knocked down with siRNA could not complete DNA synthesis to pass through S phase and resulted in an increase of cells in the G0/G1 phase (Sijin *et al.*, 2004). We therefore investigated if decreasing expression of *GNL3L* would result in adversely affecting cellular proliferation in HeLa cells. HeLa cells were transfected with *GNL3L*-siRNA, a scrambled version of the *GNL3L*-siRNA, Luciferase-specific siRNA, and an empty vector, pCDNA3. Cultures transfected with *GNL3L*-siRNA showed consistently a 30–40% decrease in the number of cells when compared with Luciferase siRNA or *GNL3L* nonspecific (scrambled sequence) siRNA-transfected cells used as negative

**Figure 6 (cont).** vividly the nucleolar region from the extranucleolar region. (C) Cartoon shows a single nucleus with morphological subcompartments of the nucleolus. FC, fibrillar center; DFC, dense fibrillar component; GC, the granular component. White arrow indicates the accumulation of GFP-signal at the granular component on the nucleolus.





**Figure 7.** Expression of a human gene *GNL3L* rescues the growth defect of the null mutant. (A) Yeast strains expressing the indicated proteins transcribed from an *nmt1* promoter were tested for growth in EMM-leu medium in the presence (*nmt1* OFF) and absence (*nmt1* ON) of thiamine. (B) Growth of *GNL3L:FLAG* (YNB858) and *Grn1:FLAG* (YNB859) is compared with a null mutant (YNB484) in YES medium. (C) *GNL3L* and *Grn1p* colocalize with nucleolin in Cos-7 cells. Localization of *GNL3L* and *Grn1p* was determined by confocal microscopy. Nucleoli were revealed by immunostaining with anti-nucleolin. (D) *S. pombe* cells showing localization of *GNL3L:GFP* in *S. pombe*. Wild-type *S. pombe* was transformed with an expression vector containing *GNL3L:FLAG* (BNB395). Nucleoli were revealed by immunostaining with anti-fibrillarilin and *GNL3L* with anti-FLAG. Independent or merged images are indicated. Bar, 4  $\mu$ m.

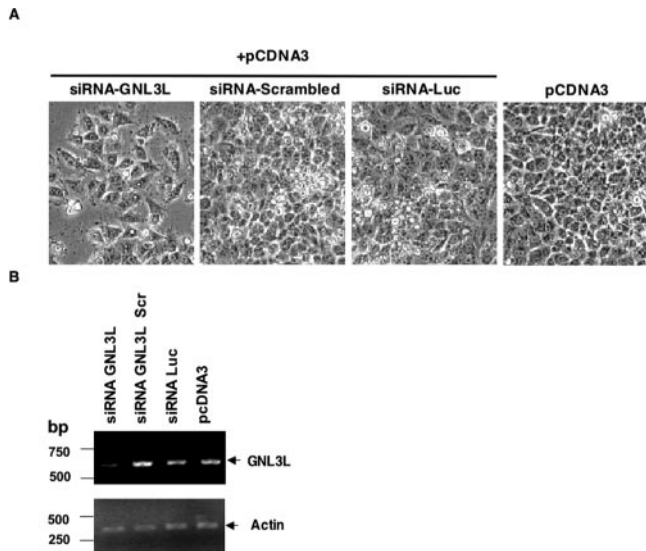
controls (Figure 8A). RT-PCR analysis was performed using primers for a 600-bp *GNL3L*-specific product or 460-bp actin-specific product. Our analysis confirmed a reduction in *GNL3L* RNA in cultures transfected with *GNL3L*-specific siRNA when compared with cultures transfected with control siRNA (Figure 8B). Similar levels of a house-keeping gene, actin-specific PCR product from all above siRNA treatments ensured there was no bias for either RNA or the RT-PCR reaction. We demonstrate that the specific knock-down of *GNL3L* expression is consistent with a decrease in HeLa cell proliferation and is also consistent with the growth function of its homolog *Grn1p* in *S. pombe*.

## DISCUSSION

### Role of *Grn1p* and *GNL3L* in Nucleolar/Nuclear Function

Because many aspects of ribosome biogenesis and nuclear export are conserved to a significant extent between yeast and humans (Venema and Tollervey, 1999; Tschochner and Hurt, 2003), we reasoned that should expression of *GNL3L* rescue the *grn1* null mutant, it would complement rRNA processing and the Rpl25a export defect too. We have demonstrated in this article that was indeed the case. Our rRNA processing and Rpl25a export data suggest that *Grn1p*/*GNL3L* acts to reinstate efficient processing of the 35S pre-rRNA transcript to wild-type levels of mature RNA and the

nucleolar export of the ribosomal protein Rpl25a in a *grn1* null mutant. Our conclusions are in general, consistent with those of the *S. cerevisiae* paralogues, Nug1 and Nug2/Nog2p (Bassler *et al.*, 2001; Saveanu *et al.*, 2001) with one notable difference. Although *Grn1p*/*GNL3L* is required for processing of the 35S preribosomal rRNA, Nug1p's involvement in pre-rRNA cleavage is not apparent (Bassler *et al.*, 2001), whereas Nog2p is required for processing of the 27S precursor to 25S and 5.8S rRNA (Saveanu *et al.*, 2001). There is, however, a close and almost inseparable relationship within the nucleolus between the two processes: rRNA processing and preribosome/Rpl25 export (Venema and Tollervey, 1999; Tschochner and Hurt, 2003). Such a relationship is exemplified by other studies (also in *S. cerevisiae*) as well, where depletion of the ribosomal protein L25 (Rpl25) led to a severe reduction in the levels of the large subunit rRNAs with a concomitant accumulation of the 35S pre-rRNA (van Beekvelt *et al.*, 2001). Interestingly, the same study also established a similar phenotype if binding of Rpl25 to the preribosome was abolished reiterating the notion that the assembly of Rpl25 with the 60S preribosome is required for rRNA processing (van Beekvelt *et al.*, 2001). Furthermore, Rpl25 is incorporated into nascent pre-60S ribosomes and is known to bind both, 35S pre-rRNA as well as the 25S rRNA in yeast (el-Baradi *et al.*, 1987; van Beekvelt *et al.*, 2000; van Beekvelt *et al.*, 2001). Therefore, because nuclear or nucleolar



**Figure 8.** siRNA knockdown of *GNL3L* in HeLa cells. (A) Cultures of HeLa cells were transfected with the indicated siRNA sequences. After a 24-h transfection, the siRNA expression cells were selected in the presence of neomycin (500  $\mu\text{g}/\text{ml}$ ) for 120 h and photographed. (B) RT-PCR analysis of *GNL3L* transcript. Total RNA was isolated from the cells transfected with the indicated siRNA. RT-PCR analysis was performed as described in *Materials and Methods*.  $\beta$ -actin was used as internal control.

retention of Rpl25 has been observed for mutants defective in 60S ribosome biogenesis and/or nucleocytoplasmic transport, it is predicted that such a phenotype may stem from an inability to process rRNA properly resulting in ribosome maturation and transport defects (Tschochner and Hurt, 2003). As depicted in Figure 6C, mammalian nucleoli contain fibrillar centers (FC) known to house rDNA genes, surrounded by a layer called the dense fibrillar component (DFC) in which the maturation of pre-rRNA transcripts is said to take place, which in-turn is surrounded by a granular component (GC) wherein the assembly of preribosomes takes place (Carmo-Fonseca *et al.*, 2000). Similar morphological nucleolar subcompartments are found in *S. pombe* (Leger-Silvestre *et al.*, 1997) and *S. cerevisiae* (Trumtel *et al.*, 2000). Though the presence of FC and DFC in yeast is currently a debated issue, the existence of a granular zone (GC?) is accepted as comprising preribosomes/ribosomes (Thiry and Lafontaine, 2005). Thus, accumulation of GFP signal at what appears to be the GC region and compared with nucleolar accumulation in a  $\Delta\text{CC}$  mutant as shown in Figure 6B (here,  $\Delta\text{G5}$  is used as a representative because all of them have a similar phenotype) suggests that the absence of G- or RG-motifs may block release from the preribosome assembly from the nucleolus, thereby restricting cycling of the GTPase in and out of the nucleolar compartment or that the movement of preribosomes from the nucleolus-nucleus interface to the NPC is compromised. Because wild-type and the  $\Delta\text{CC}$  mutant Grn1p proteins are nucleolar and those strains do not have any growth defect, we must conclude from Figures 5 and 6 that failure of Grn1p to either localize or relocate to the nucleolus is responsible for the null phenotype and that nucleolar localization was indeed essential for Grn1p function. We also noted a significant distortion of nucleolar structure in all our mutants except for the  $\Delta\text{CC}$  and our observations are consistent with those for NS (Tsai and McKay, 2002), wherein mutations that affected its nor-

mal localization disrupted nucleolar stability and integrity. Such disruptions in nucleolar integrity may be regarded as signs of cellular stress (Olson, 2004). Studies with NS suggest that there exists a dynamic partitioning of the protein between the nucleolus and nucleoplasm (Tsai and McKay, 2002, 2005), possibly the driving force behind signal-mediated activities within nuclear subcompartments (Misteli, 2005). It would be difficult to reconcile our rRNA processing and Rpl25 data with the concept of "nucleolar retention rather than release," as being the cornerstone of Grn1p/*GNL3L* activity as has been suggested for NS (Misteli, 2005) because it would be necessary for the putative GTPase to be physically present within the nucleolus to execute such functions. However, despite their similarities, the modus operandi of NS and Grn1p/*GNL3L* may be quite different (see below).

#### *Grn1p and GNL3L as Members of a Novel Group of Putative Nucleolar GTPases with a Circularly Permuted G-Domain*

The circularly permuted G-domain is conserved in evolution and is present in GTPases from prokaryotes (bacteria) to eukaryotes (mammals; Mans *et al.*, 2004). Interestingly, bacterial permuted GTPases were probably involved in translation regulation and in ribosome assembly, whereas the presence of duplicated or multiple forms of permuted GTPases in eukaryotes correlates with the emergence of a nucleus/nucleolus (Mans *et al.*, 2004). A recurrent theme emerging from recent proteomic analyses is that besides its traditional role(s) in ribosome biogenesis, the nucleolus serves as a repository for molecules involved in "nonribosomal" activities as well (Olson *et al.*, 2000, 2002; Andersen *et al.*, 2002; Dundr and Misteli, 2002; Scherl *et al.*, 2002; Leung *et al.*, 2003; Staub *et al.*, 2004; Olson and Dundr, 2005). Could the existence of multiple nucleolar/nuclear GTPases as depicted in Figure 1B (including Grn1p or *GNL3L*) with a unique circularly permuted G-domain serve as a paradigm for understanding nucleolar ribosomal/nonribosomal-mediated regulation of cell growth? Of the 700-or-so human nucleolar proteins in the nucleolar protein database (<http://lamondlab.com/nopdb/>), four putative GTPases-NGP-1 (*GNL2*), *GNL3L*, NS, and *GNL-1* possess a circularly permuted G-domain containing the so-called G5\* motif [DARDP] (Figure 1B). According to AceView (<http://www.ncbi.nlm.nih.gov/IEB/Research/AceView>), these genes are expressed at 4.1 or very high (*NGP-1*), 3.2 or high (*GNL3L*), 0.8 or well expressed (*NS*) times the average gene (*GNL-1* expression is moderate to low). We have examined three of them, *NGP-1*, *NS*, and *GNL3L* for their ability to complement *Grn1*. Despite the fact that all three were expressed in *S. pombe*, only *GNL3L* rescued the  $\Delta\text{grn1}$  mutant. The *S. cerevisiae Nug1* did so only very weakly. *NGP-1* complemented *Grn1* at low levels of induction (*nmt1* OFF) but inhibited growth at high levels of expression (*nmt1* ON). Because *NGP-1* localizes exclusively to the nucleolus and nucleolar organizer regions (NORs; Racevskis *et al.*, 1996), it is possible that excess *NGP-1* in fission yeast disrupts nucleolar function. However, applying the same analogy, it is intriguing that excess *GNL3L* is not toxic. It would be interesting to find the basis of such a dosage-dependent nucleolar response to *GNL3L* and *NGP-1*. Our work clearly demonstrates that *NS* does not complement *Grn1* implying that the former does not play a role in rRNA processing and/or ribosomal export. Besides *GNL3L* (this work) and *NGP-1* (Racevskis *et al.*, 1996), only *NS* appears to have been studied in some detail (Tsai and McKay, 2002; Baddoo *et al.*, 2003; Schwartz *et al.*, 2003; Cai *et al.*, 2004; Liu *et al.*, 2004; Sijin *et al.*, 2004; Xu *et al.*, 2004; Grasberger and Bell, 2005; Politz *et al.*,

2005; Tsai and McKay, 2005; Yang *et al.*, 2005). Although no well-defined molecular function has been attributed to NS, its ability to shuttle between the nuclear-nucleolar compartments in a GTP-dependent manner is a key factor in modulating growth of stem and cancer cells (Tsai and McKay, 2002; Misteli, 2005; Tsai and McKay, 2005). Additional support of our conclusion that GNL3L and NS may be acting via noninclusive or independent pathways comes from the high-resolution electron spectroscopic imaging analysis of NS localizing to the GC regions of the nucleolus having little or no rRNA, thus leading to the prediction that NS is *not* associated with ribosome biogenesis/rRNA processing (Politz *et al.*, 2005). Furthermore, our finding that *Grn1* is nonessential in fission yeast implies that its activity is redundant. Yet, because the null mutant has an acute growth defect despite the presence of at least three other circularly permuted putative GTPases (in the fission yeast), it is appropriate to conclude that Grn1p/GNL3L has specialized functions or responds to molecular signals that are distinct from those of other similar G-domain GTPases.

Finally, although the circularly permuted and highly conserved G-domain of NS or Grn1p/GNL3L may be critical for function as Tsai and McKay (2002, 2005) and we (this report) have established, it is likely that the key to differences in their localization vis-à-vis activities lies in domain(s) with least homology (See Supplementary Figure S2). Because nuclear subcompartments are in a state of constant flux, it is anticipated that molecular components would likewise move from one subcompartment to another (Dundr and Misteli, 2001; Olson and Dundr, 2005). It is known that the nucleolar localization and nuclear shuttling of NS is dependent on its N-terminal basic domain (targeting sequence?) and regulation of the latter by its G1/GTP-binding state (Tsai and McKay, 2002, 2005). Though we have not investigated the phenomenon ourselves, GNL3L appears in the Nucleolar Proteome Database as a dynamic component of the nucleolus based on SILAC analysis (stable isotope labeling by amino acids in cell culture; <http://lamondlab.com/nopdb/>). Our preliminary analysis of the N-terminus of Grn1p identified a putative nuclear/nucleolar sequence similar but not identical to either GNL3L or NS, which when deleted, failed to concentrate Grn1p:GFP in the nucleolus (Supplementary Figure S3). Quite surprisingly, these mutants did not exhibit any growth defect compared with either the null mutant or G-motif/RG mutants tested. Absence of nucleolar sequestration may thus allow these cells to “override” the wild-type requirement for this particular pathway. Conversely, as envisaged for NS by Tsai and McKay (2005) and Misteli (2005), the function(s) of Grn1p are realized in shuttling between the nucleolus/nucleoplasm interface (GC) and the nucleoplasm. In the absence of any one of the G-motifs or RG, Grn1p activity was impeded at the GC/nucleolus/nuclear border as shown in Figure 6B, whereas deletion of the putative targeting sequence allowed some of the protein to be retained in the nucleoplasm where it could continue to function. Thus, should it be that differences in circularly permuted GTPase activities are related to subnucleolar/nuclear compartmentalization as exemplified by NS, their locales transient or static, must define specific metabolic areas (or activities) within the nucleus. In this context, such GTPases may help to shed light on key sites of nonribosomal or ribosomal activity in the nucleolus/nucleus and their respective roles in growth.

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