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# Subcellular Distribution of the Human Putative Nucleolar GTPase GNL1 is Regulated by a Novel Arginine/Lysine-Rich Domain and a GTP Binding Domain in a Cell Cycle-Dependent Manner

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GNL1, a putative nucleolar GTPase, belongs to the MMR1-HSR1 family of large GTPases that are emerging as crucial coordinators of signaling cascades in different cellular compartments. Members of this family share very closely related G-domains, but the signals and pathways regulating their subcellular localization with respect to cell growth remain unknown. To understand the nuclear transport mechanism of GNL1, we have identified a novel arginine/lysine-rich nucleolar localization signal in the $NH_2$-terminus that is shown to translocate GNL1 and a heterologous protein to the nucleus/nucleolus in a pathway that is independent of importin-α and importin-β. In addition, the present investigation provided evidence that GNL1 localized to the nucleus and the nucleolus only in G2 stage, in contrast to its cytoplasmic localization in the G1 and S phases of the cell cycle. Using heterokaryon assay, we have demonstrated that GNL1 shuttles between the nucleus and the cytoplasm and that the motif between amino acids 201 and 225 is essential for its export from the nucleus by a signal-mediated CRM1-independent pathway. Alanine-scanning mutagenesis of conserved residues within G-domains suggests that the G2 motif is critical for guanine nucleotide triphosphate (GTP) binding of GNL1 and further showed that nucleolar retention of GNL1 is regulated by a GTP-gating-mediated mechanism. Expression of wild-type GNL1 promotes G2/M transition, in contrast to the G-domain mutant (G2m), which fails to localize to the nucleolus. These data suggest that nucleolar translocation during G2 phase may be critical for faster M-phase transition during cell proliferation. Replacement of conserved residues within the G5 motif alters the stability of GNL1 without changing GTP binding activity. Finally, our data suggest that ongoing transcription is essential for the efficient localization of GNL1 to the nucleolus. Overall, the results reported here demonstrate that multiple mechanisms are involved in the translocation of

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Abbreviations used: GTP, guanine nucleotide triphosphate; NLS, nuclear localization signal; HIV, human immunodeficiency virus; NoLS, nucleolar localization signal; GFP, green fluorescent protein; Act-D, actinomycin D; GST, glutathione *S*-transferase; LMB, leptomycin B; CHX, cycloheximide; MPA, mycophenolic acid; NES, nuclear export signal; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole.

GNL1 to the nucleolus in a cell cycle-dependent manner to regulate cell growth and proliferation.



## Introduction

The nucleolus is a subnuclear membrane-free organelle that was shown to be the site of ribosomal DNA transcription and ribosome assembly.[1–5] The process of ribosome assembly involves more than 70 ribosomal proteins and 200 nonribosomal factors.[6] However, recently, many other proteins that have no known function in ribosome assembly have been identified to localize to the nucleolus. Some of the well-known examples are p53, MDM2, ARF, Cdc14, and Pch2, which are known to be involved in cell cycle regulation.[7–14] This new knowledge has brought the nucleolus into the limelight as a "cell cycle regulatory center." Currently, it is estimated that the human nucleolar proteome contains approximately 720 proteins.[15,16] Of these, some proteins are in a dynamic equilibrium with the nucleoplasm, while others are known to localize to the nucleolus based on the stage of the cell cycle. The nucleolus also acts as a sequestration center for cell cycle regulatory proteins. This might be a mechanism for regulating the intranuclear/nucleoplasmic concentration of these proteins. For instance, Cdc14 and PP1γ are sequestered in the nucleolus throughout the interphase and released at the onset of mitosis.[17–25] It is also known that p14ARF physically sequesters MDM2 into the nucleolus in response to stress signals and defective ribosome biogenesis.[26] Knowledge of proteins that localize to the nucleolus in response to a specific signal/cascade of events might be very valuable in further understanding the regulation of the cell cycle.

Compartmentalization is a well-documented form of predicting and defining the functions of proteins. Constant exchange of molecules between the nucleus and the cytoplasm occurs through aqueous channels spanned across the nuclear envelope formed by larger multiprotein assemblies called nuclear pore complexes (NPC). Molecules greater than 40–45 kDa are transported across the nuclear envelope via signal-mediated energy-dependent pathways by interacting with transport receptors such as importins and exportins. In general, the proteins destined to be imported into the nucleus carry defined signal sequences known as nuclear localization signals (NLSs), which are rich in positively charged amino acids. The proteins containing these signal sequences are recognized directly by the transport receptor importin-β or through an adapter (usually importin-α) and facilitate nuclear translocation. Although many NLSs have been identified, the following types of NLS have so far been characterized in detail: classical NLS, encompassing a stretch of lysine residues, was originally identified in SV40 T antigen and interacts with importin-α;[27,28] arginine-rich NLS, identified in human immunodeficiency virus (HIV) Rev, directly binds to importin-β;[29,30] glycine-rich NLS (M9 NLS), initially identified in proteins involved in mRNA transport, interacts with transportin, which is closely related to importin-β.[31–33] Once the cargo binds to its respective receptor, it will then be imported into the nucleus in a Ran-GTP-dependent manner.[27–34] Many arginine-rich motifs are implicated in protein nuclear/nucleolar transport, and a consensus sequence for nucleolar targeting is yet to emerge. Since the nucleolus is not separated from the rest of the nucleus by a membrane, it is likely that mechanisms of protein localization to the nucleolus may follow a paradigm different from those operating to sequester proteins to membrane-bound organelles.

Several nucleotide binding proteins are known to be involved in ribosome biogenesis. Of these, the YawG/YIqF subfamily of GTPases is conserved across evolution from prokaryotes to highly evolved mammals and humans.[35] This family of GTPases is characterized by the circular permutation of their guanine nucleotide triphosphate (GTP) binding motifs. GTP binding motifs G1–G5 are arranged in a G5–G4–G1–G2–G3 order in cpGTPases, in contrast to the G1–G2–G3–G4–G5 arrangement in classical GTPases.[36] In humans, there are at least four well-known members in this subfamily: GNL1, GNL2 (NGP-1), GNL3 (nucleostemin), and GNL3L. Studies on the members of this family in yeast and humans have shown them to be involved in ribosome assembly and ribosomal RNA processing.[5] Depletion of GNL3 and GNL3L has abrogated G1/S and G2/M transition, respectively, indicative of their role in cell cycle progression.[37–40] Furthermore, both GNL3 and GNL3L were shown to regulate the activities of p53, MDM2, and TRF1.[41–44] The gene encoding *GNL1* is located less than 2 kb centromeric to HLA-E on chromosome 6.[45] GNL1 is a putative nucleolar GTPase, but its function remains unknown.

To understand the function of GNL1, we first identified the signals and mechanism regulating the subcellular distribution of GNL1. To this end, we have cloned and expressed the novel putative nucleolar GTPase GNL1. Immunofluorescence analysis indicates that GNL1 exhibits three different patterns of localization: cytoplasmic, nuclear/cytoplasmic, and nucleolar. A combination of mutagenesis and subcellular localization analyses suggests that R3K4-5 acts as the nucleolar localization signal (NoLS) and that nucleolar retention of GNL1 is dependent on the G2 phase of the cell cycle. In addition, we have shown that GNL1 is a nucleocytoplasmic shuttling protein

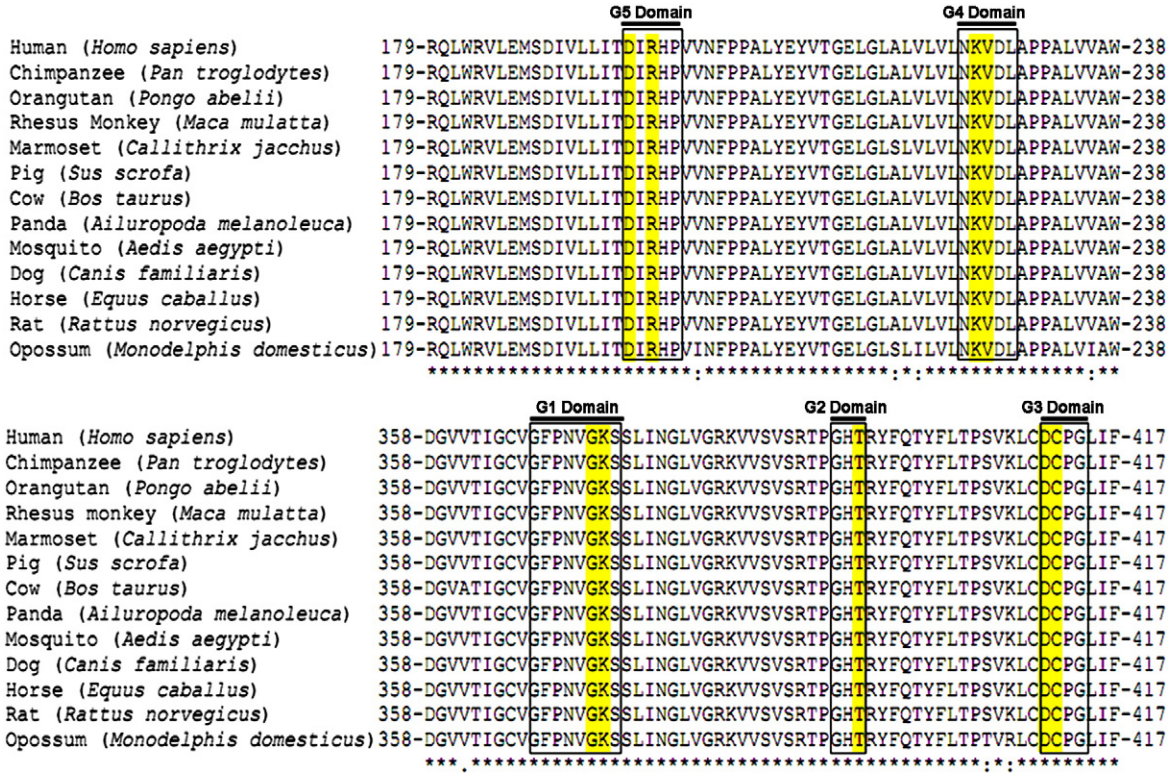


**Fig. 1.** Multiple alignments of deduced amino acid sequences encompassing the GTP binding domains (G-domains) of proteins related to human GNL1. The amino acid sequences were aligned using ClustalW, version 1.83 (www.genome.jp/tools/clustalw). Amino acids forming G-domains are boxed, and the residues selected for mutagenesis (to understand their role in GNL1 functions) are shaded in yellow. The organisms and GenBank accession numbers are as follows: human (*Homo sapiens*; gene ID: 2794), chimpanzee (*Pan troglodytes*; gene ID: 462541), orangutan (*Pongo abelii*; gene ID: 100172755), rhesus monkey (*Macaca mulatta*; gene ID: 713303), marmoset (*Callithrix jacchus*; gene ID: 100409463), pig (*Sus scrofa*; gene ID: 100151741), cow (*Bos taurus*; gene ID: 532528), panda (*Ailuropoda melanoleuca*; gene ID: 100475181), mosquito (*Aedis aegypti*; gene ID: 5577121), dog (*Canis familiaris*; gene ID: 474825), horse (*Equus caballus*; gene ID: 100051374), rat (*Rattus norvegicus*; gene ID: 309593), and opossum (*Monodelphis domestica*; gene ID: 100022283). The G-domains are found to be conserved across species.

and that the signal between amino acids 201 and 225 is responsible for its export from the nucleus. Furthermore, our data demonstrate that the nucleolar retention of GNL1 is dependent on ongoing transcription and its ability to bind GTP. Finally, our data provided evidence that nucleolar localization of GNL1 is critical for mitotic phase transition. Together, the present investigation provides evidence that multiple mechanisms are involved in the translocation of GNL1 to the nucleolus in a G2-phase-dependent manner to regulate cell growth control.

## Results

### GNL1 is an evolutionarily conserved nucleolar GTP binding protein

GNL1 was initially identified as a putative nucleolar GTPase that encodes a polypeptide of 607 amino acids with a predicted molecular mass of 65 kDa. Phylogenetic analysis of GNL1 sequences showed that it is evolutionarily conserved from bacteria to humans (Fig. S1), suggesting an important role for this gene. Multiple alignments of GNL1 amino acid sequences (amino acids 179–417) identified five GTP binding motifs (G1–G5; boxed) that were conserved in various organisms (Fig. 1). The amino acid residues highlighted in yellow within the G1–G5 domains were selected for mutagenesis to understand their role in GNL1 function. The presence of G-domains defines GNL1 as a GTP binding protein, and nearly all regulatory GTPases have five conserved polypeptide loops designated G1 through G5, which form contact sites for the guanine nucleotide. Rather than the usual G1–G2–G3–G4–G5 found in the superfamily of regulatory GTPases, the G1 motif ($Gx_4GK(S/T)$) in GNL1 is located between G4 (NKxD) and G2 (GxT) as G5–G4–G1–G2–G3, which has been described as a circularly permuted G-motif. A conserved domain

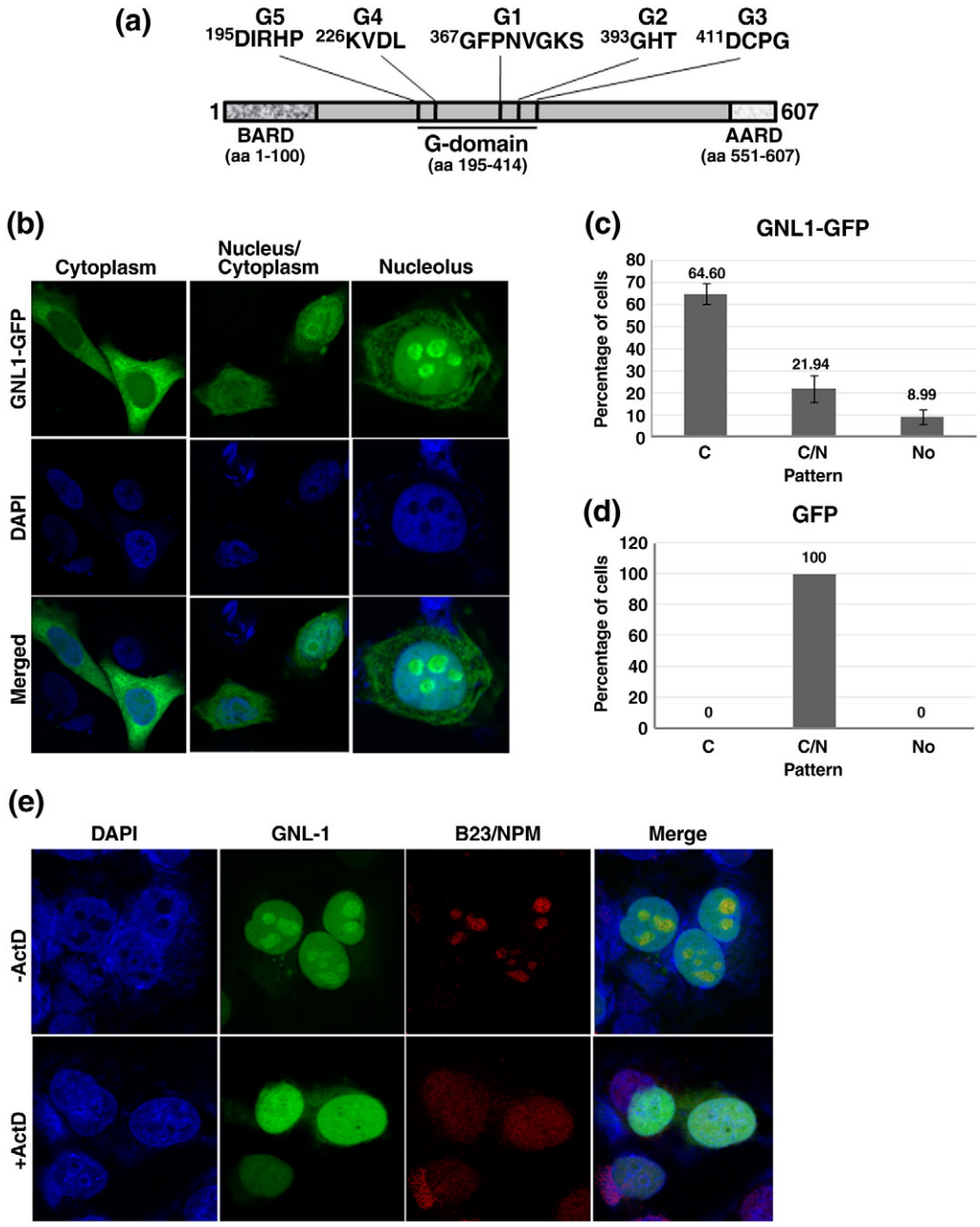


**Fig. 2.** GNL1 localizes in different cellular compartments in cycling cells. (a) Diagrammatic representation of the different functional domains of GNL1. (b) Subcellular distribution of GNL1. A mammalian expression vector carrying GNL1/GFP was transfected in HeLa cells. After 16–24 h, the cells were fixed with 3% paraformaldehyde in 1× PBS, the nucleus was stained with DAPI, and the images were captured by a Carl Zeiss laser scanning confocal microscope using Zen2009 software. Green fluorescence indicates GNL1/GFP localization. GNL1 localized in different cellular compartments. Percent fluorescent cells representing the cytoplasmic (C), nucleolar (No), and nucleocytoplasmic (C/N) distribution patterns of GNL1/GFP (c) and GFP (d) were counted in three different independent experiments. (e) HeLa cells were transfected with an expression vector encoding GNL1/GFP. After 16 h, the medium was replaced with either fresh medium or medium containing 5 μg/ml Act-D and incubated for 3 h. Subcellular distribution of GNL1/GFP (green) was determined directly by a fluorescence microscope. The nucleolar protein B23/NPM was visualized by probing the cells with anti-B23 antibodies, followed by Alexa 594 staining (red). (f) Schematic representation of GNL1 variants. All the indicated GNL1 deletion constructs expressed the correctly sized polypeptides (left). (g) HeLa cells were transiently transfected with the expression vectors encoding GFP-tagged wild type and various deletion mutants of GNL1. The numbers on the top of each panel corresponding to the amino acids within GNL1 were expressed. Sixteen hours posttransfection, the cells were fixed and subcellular distribution of GNL1 was determined directly by a fluorescence microscope.

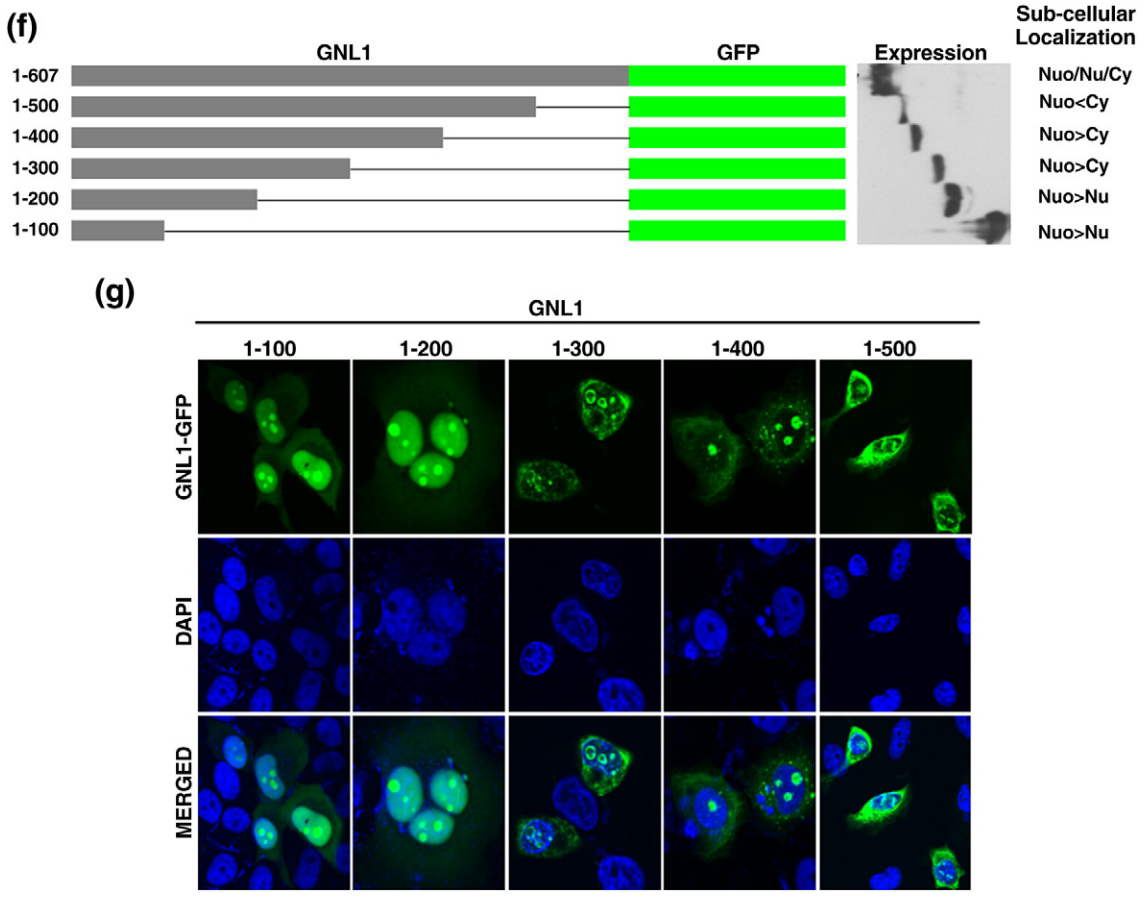


**Fig. 2** (*legend on previous page*)

search analysis indicated that GNL1 encompasses the basic amino-acid-rich region in the amino-terminus, proline-rich domain (possible SH3 binding motifs), and acidic amino-acid-rich domain in the carboxy-terminus in addition to the G-domains (G1–G5) in the middle region (Fig. 2a). The spacing between individual G-domains and the domain structure in GNL1 is similar to known nucleolar GTPases Nug1p, Nug2p, and Ngp1p; nucleostemin and GNL3L; and members of the Ylqf/YawG family of GTPases.[46–50] The presence of five GTP binding motifs in GNL1 suggests that it may act as a molecular switch in different cellular processes such as control of cell growth and differentiation, protein trafficking, and signal transduction.[36,51–54]

### GNL1 localizes to different cellular compartments

The existence of several closely related proteins in the large MMR1-HSR1 GTPase family suggests the possibility that they may have shared or independent roles in regulating different cellular events. Full-length GNL1 was amplified from a HeLa cDNA library and cloned as carboxyl-terminal fusion with enhanced green fluorescent protein (GFP), as described in Materials and Methods, in order to understand the mechanisms of cellular localization. The subcellular localization of GNL1/GFP was determined by direct fluorescence microscopy upon transient expression in HeLa cells. We selected GFP as a partner for generating fusion protein, since it allows the visualization of fusion proteins without antibody staining and is known to localize to the nucleus when attached to a functional NLS.[55,56] The predicted size of the GNL1/GFP fusion protein is around 94 kDa, which exceeds the passive diffusion limit of the nuclear pore complex. Fluorescence analysis in HeLa cells showed that GNL1/GFP is distributed in different cellular locations that are nuclear/cytoplasmic, completely cytoplasmic, and predominantly nucleolar with a faint cytoplasmic distribution (Fig. 2b). Quantitative analysis of GNL1/GFP revealed 64% cytoplasmic staining, 22% nuclear/cytoplasmic staining, and 8.99% nucleolar staining (Fig. 2c) of GNL1 among fluorescence-positive cells. GFP alone, in comparison, was diffusely distributed both in the nucleus and in the cytoplasm (Fig. 2d). We counted 200 fluorescing cells

from three independent experiments and observed similar patterns of GNL1 subcellular distribution. Although GNL1 belongs to nucleolar GTPases, we noticed cytoplasmic localization in the majority of GNL1-positive cells. These data suggest the following possibilities: (i) it may be shuttling between the nucleus and the cytoplasm continuously, and (ii) since we used unsynchronized HeLa cells, the observed differential subcellular distribution may be dependent on the stage of the cell cycle. Treatment of GNL1/GFP-expressing HeLa cells with the RNA polymerase inhibitor actinomycin D (Act-D) [Act-D blocks transcription by intercalating between nucleotide base pairs and induces nucleolar reorganization[57]] resulted in nuclear accumulation with a faint cytoplasmic distribution of GNL1. A similar pattern of cellular localization was also observed for B23/NPM (a well-known nonribosomal nucleolar protein) upon Act-D treatment (Fig. 2e). These results suggest that ongoing transcription may be critical for the retention of GNL1 in the nucleolus.

### Amino-terminal domain regulates the nuclear/nucleolar targeting of GNL1

In order to understand the mechanisms of GNL1 nucleolar transport, we first analyzed the predicted amino acid sequence of GNL1. Such an analysis indicated the presence of positively charged residues in the $NH_2$-terminal region GNL1. Since the domains containing clusters of positively charged amino acids act as nuclear/nucleolar targeting signals for many cellular and viral proteins,[27–30] we generated variants of GNL1 harboring truncations in the COOH-terminus (Fig. 2f). Wild-type or truncation mutants of GNL1 were transfected in HeLa cells, and their expression was determined by Western blot analysis using anti-GFP antibodies. Results in Fig. 2f showed that all the mutants expressed correct size polypeptides, suggesting that truncations from the C-terminus did not alter the expression of GNL1 variants. Subcellular localization analysis by fluorescence microscopy indicated that mutant proteins containing amino acids 1–100 and 1–200 of GNL1 efficiently localized exclusively to the nucleolus and the nucleoplasm (Fig. 2g). Surprisingly, GNL1 mutant proteins $GNL1^{1–300}$ and $GNL1^{1–400}$ were retained in the nucleolar compartments with faint cytoplasmic staining (Fig. 2g). However, we observed a predominantly cytoplasmic staining for the mutant protein $GNL1^{1–500}$, with some nucleolar staining (Fig. 2g). These results suggest that the amino-terminal domain (amino acids 1–100) contained necessary and sufficient information to translocate and retain GNL1 in the nucleolus by a signal-mediated process. In addition, the observed cytoplasmic localization of GNL1 variants containing amino acids 201–500 leads to the hypothesis that GNL1 may be a nucleocytoplasmic shutting protein and that the signal responsible for export from the nucleus may reside within amino acids 201–500 of GNL1.

To identify the minimal signal essential for efficient nucleolar targeting of GNL1, we generated truncation mutants within the $NH_2$-terminal domain containing amino acids 1–100 as GFP fusion (Fig. 3a). All the constructs were transfected into HeLa cells as described in Materials and Methods, and Western blot analysis of the transfected cell lysates using anti-GFP antibodies indicated that all the constructs expressed correct size polypeptides (Fig. 3b). Subcellular localization of GNL1 variants was determined by transfecting the indicated clones in chamber slides containing HeLa cells. Immunofluorescence analysis suggested that the GFP fusion protein containing 1–25 amino acids of GNL1 efficiently targeted to the nucleolus (Fig. 3c). In contrast, fusion proteins containing amino acids 26–607 and 51–607 of GNL1 localized both in the nucleus and in the cytoplasm but were excluded from the nucleolar compartment (Fig. 3c, arrows). These results provided evidence that the domain containing amino acids 1–25 contains necessary and sufficient information for efficient targeting and retention of GNL1 in the nucleolus. A detailed analysis of the amino acid sequences of this domain ($GNL1^{1–25}$) showed clusters of positively charged lysine and arginine residues. In order to identify the critical residues that are important for the nucleolar retention of GNL1, we used a site-directed mutagenesis approach to generate variants of GNL1 in which the basic amino acid residues were exchanged with alanines individually or in combination within the amino-terminal domain 1–25 as well as in the full-length protein (Fig. 3d and g). Western blot analysis of the transfected cell lysates showed that all the indicated variants of GNL1/GFP expressed correct size polypeptides (Fig. 3e and h), suggesting that mutations did not alter the expression profile of mutant proteins.

GFP-tagged GNL1 variants were transiently expressed in HeLa cells as described in Materials and Methods, and their subcellular distribution was determined by fluorescence microscopy. Results in Fig. 3f indicated that replacement of R3,K4-5A within $GNL1^{1–25}$ impaired the wild-type nucleolar localization. In contrast, we observed nucleolar and cytoplasmic localizations when K12-14 were exchanged with alanines within $GNL1^{1–25}$ (Fig. 3f). The replacement of positively charged arginines and lysines at similar positions in the context of full-length GNL1 completely altered the localization of all mutant proteins (Fig. 3i). For example, exchange of R3,K4-5A resulted in a diffused nuclear and cytoplasmic localization pattern but was excluded from the nucleolus (Fig. 3i). Similar patterns of localization were observed for GNL1 variants carrying mutations at K12–14, R22K23, and R95,96,

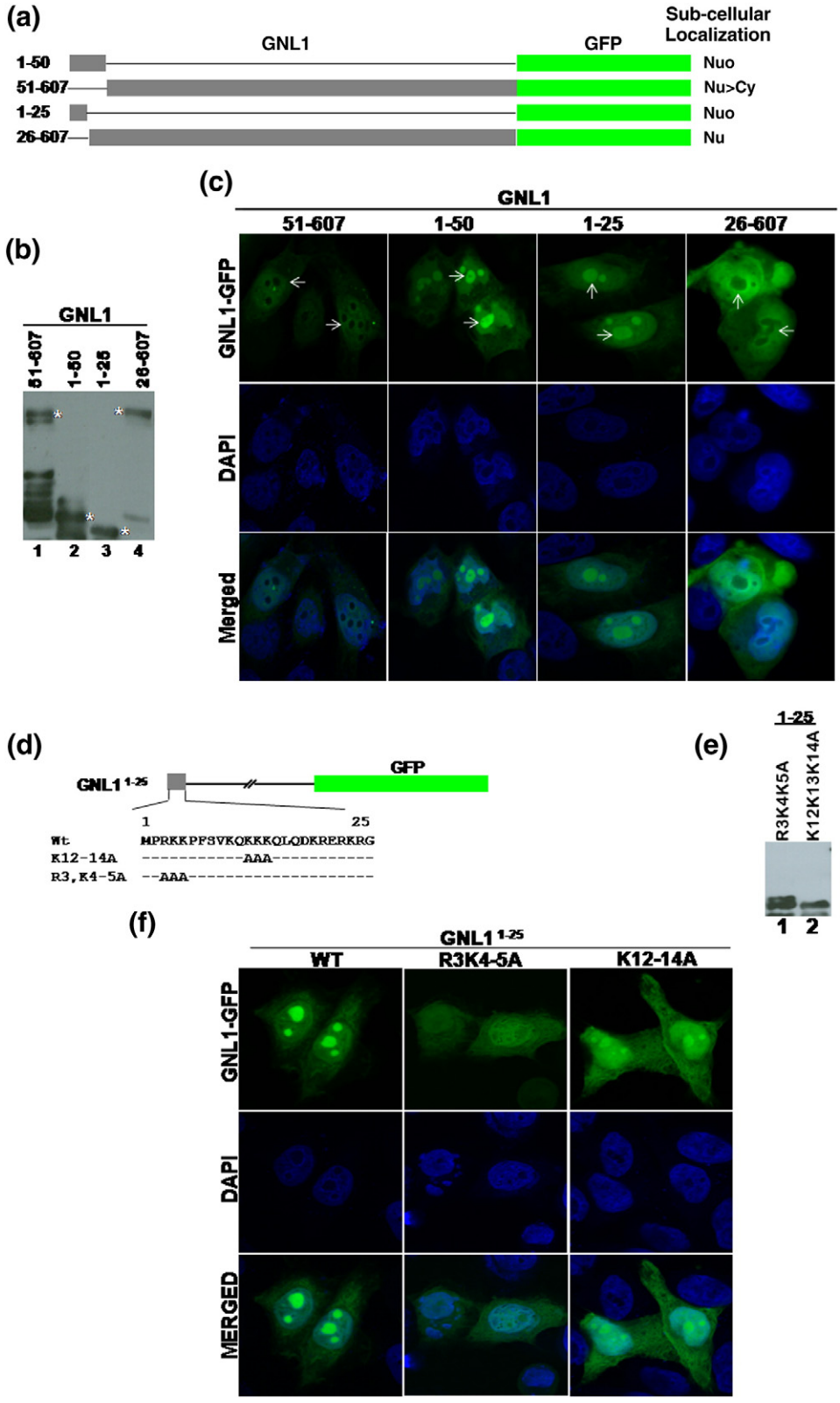


**Fig. 3** (*legend on page 354*)

K97. Quantitative analysis by counting 200 fluorescence-positive cells from three independent experiments showed 8.99%, 1.13% ($p < 0.0001$), 5.25% ($p < 0.0001$), and 1.1% ($p < 0.0001$) of cells with nucleolar localization patterns for wild type or mutants K12–14A, R22, K23A, and R95, 96, K97A (Figs. 2c and 3k–m), respectively. In contrast, no nucleolar staining was observed for the R3,K4-5A mutant of GNL1 (Fig. 3j). These data suggest that, despite the importance of R3,K4-5A for nucleolar retention, multiple repeats of positively charged arginine and lysine residues are critical for forming a functional NLS/NoLS for the efficient targeting and retention of GNL1 in the nucleolar compartment.

To further understand the mechanism of GNL1 nuclear/nucleolar transport, we sought transport

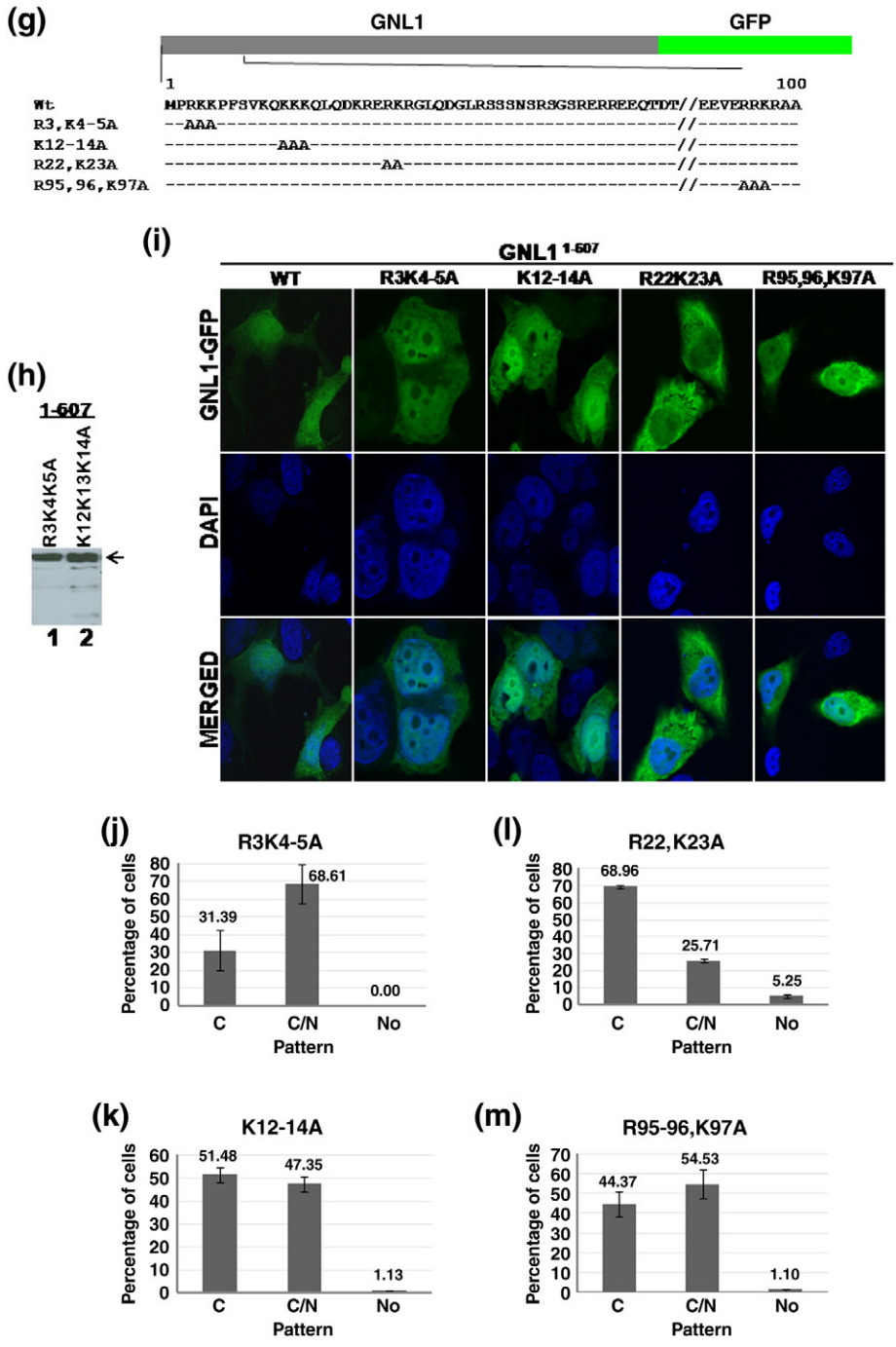


**Fig. 3** (*legend on next page*)

receptors that specifically interact with GNL1. A detailed analysis of the NoLS sequence of GNL1 with other known nucleolar transport signals suggests the presence of arginine/lysine-rich repeats, which are known to interact with receptors in classical protein transport pathways. Interestingly, proteins that are localized in the nucleolus preferentially interact with importin-β directly without the involvement of importin-α.[29,30] In order to identify the receptor involved in the transport of GNL1 to the nucleolus, we first tested whether GNL1 interacts with importin-α or importin-β by a series of glutathione *S*-transferase (GST) pull-down assays. 293T cell lysates containing equal amounts of GNL1/GFP and GFP were mixed with glutathione-Sepharose beads that had been prebound to either GST or GST fused to importin-α or importin-β. Four hours after incubation, the beads were washed, and the bound proteins and a fraction of the input proteins were examined by 10% SDS-PAGE, followed by Western blot analysis, using anti-GFP antibodies. Interactions between importin-β and HIV-1 Rev (known to interact with importin-β) and between importin-α and SV40 large T Ag (known to interact with importin-α) were readily detected (Fig. S2). Surprisingly, GNL1 did not interact with either importin-β or importin-α. These data suggest that GNL1 may interact with a novel nucleolar transport pathway for translocation into the nucleolar compartment.

### GNL1 is a nucleocytoplasmic shuttling protein

As described above, GNL1 may interact with a novel nuclear transport pathway and translocate to the nuclear/nucleolar compartments. Interestingly, GNL1 localized in different cellular compartments when expressed in unsynchronized HeLa cells, suggesting the possibility that it may be shuttling between nuclear and cytoplasmic compartments or localized in different cellular compartments in a cell-cycle-dependent manner. In order to understand the mechanism of GNL1 localization in different cellular compartments, we first tested whether it is a nucleocytoplasmic shuttling protein by treating the transfected cells with the fungal metabolite leptomycin B (LMB), a well-known inhibitor of CRM1-mediated nuclear export pathway. Surprisingly, LMB did not alter the subcellular distribution of GNL1 (data not shown). Recent reports suggest that a large number of cellular proteins are exported from the nucleus via a CRM1-independent pathway.[58–60] Nucleocytoplasmic shuttling of GNL1 was confirmed by heterokaryon assay in which GNL1/GFP-transfected HeLa cells were fused with untransfected NIH 3T3 cells, as described in Materials and Methods. The cells were treated with the translational inhibitor cycloheximide (CHX) throughout the assay to prevent *de novo* protein synthesis. Should nuclear export occur, GNL1 would exit the human nucleus (HeLa cell), traverse the cytoplasm, and enter the mouse nucleus (NIH3T3 cells). Interestingly, we observed the GNL1 signal in both HeLa and NIH 3T3 nuclei in the heterokaryon assay (Fig. 4a). Mouse nuclei can be readily distinguished from human nuclei by a punctate fluorescence pattern upon staining with Hoechst 33342 (Fig. 4a, middle). Taken together, these results provided evidence that GNL1 shuttles between the nucleus and the cytoplasm by an energy-dependent signal-mediated process. The unaltered cellular distribution of GNL1 in the presence of LMB leads to the hypothesis that it may be exported from the nucleus by a novel unknown export pathway independent of CRM1.

Based on the results of the subcellular localization analysis, addition of amino acids 201–300 with $GNL1^{1–200}$ resulted in cytoplasmic distribution otherwise localized in the nucleus (Fig. 2g). These data suggest the possibility that a signal residing within the region comprising amino acids 201–300 may be responsible for GNL1 cytoplasmic localization. In order to identify the signal that is critical for GNL1 export from the nucleus, we generated

**Fig. 3.** Identification of minimal nucleolar targeting signal within the amino-terminal region of GNL1. (a) Schematic representation of the amino-terminal deletion fragments of GNL1 as GFP fusion. (b) HeLa cells were transfected with all the indicated variants of GNL1 as described in Materials and Methods. Transfected cell lysates were subjected to Western blot analysis using anti-GFP antibody. (c) Subcellular localization of GNL1 amino-terminal variants. Mammalian expression vector carrying all the indicated mutants of GNL1 were transfected in HeLa cells in chamber slides as described in Materials and Methods. After 16–24 h, the cells were fixed with 3% paraformaldehyde in 1× PBS, the nucleus was stained with DAPI, and the images were captured by a Carl Zeiss laser scanning confocal microscope using Zen2009 software. Schematic representation of expression vectors containing mutations within $GNL1^{1–25}$ (d) and $GNL1^{1–607}$ (g). All the site-specific mutants were generated by QuickChange site-directed mutagenesis as described in Materials and Methods. The expression of various $GNL1^{1–25}$ (e) and $GNL1^{1–607}$ (h) mutant proteins from the total transfected cell lysates was examined by Western blot analysis using anti-GFP antibodies (1:1000 dilutions). Variants of $GNL1^{1–25}$ (f) and $GNL1^{1–607}$ (i) were transfected in HeLa cells, and subcellular distribution was determined by fluorescence microscopy as described in Materials and Methods. Percent fluorescent cells representing different subcellular localization patterns [cytoplasmic (C), nucleolar (No), and nucleocytoplasmic (C/N)] for all the indicated GNL1 variants (j, k, l, and m) were counted in three different independent experiments. Results from the subcellular localization analysis suggest that clusters of positively charged arginine and lysine residues at amino acid positions 3–5 within NoLS are critical for efficient translocation/retention of GNL1 in the nucleolar compartment.

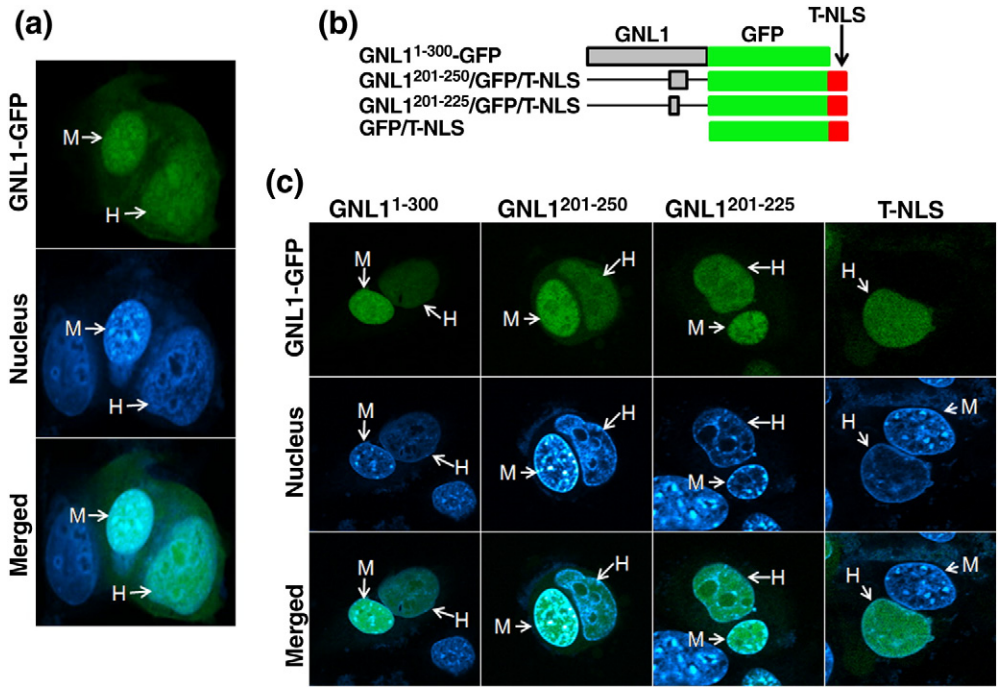


**Fig. 4.** GNL1 is a nucleocytoplasmic shuttling protein, and export from the nucleus is a signal-mediated process. (a) HeLa cells were transfected with GNL1/GFP expression vector, and heterokaryon assay was performed 24 h after transfection as described in Materials and Methods. Subcellular distribution of GNL1 was determined by fluorescence microscopy. The green signal represents the GNL1 protein. Hoechst 33342 was used to stain the nucleus. Murine nuclei (NIH3T3) are readily distinguished from human nuclei (HeLa) by their punctate appearance. GNL1 shuttling was observed in a heterokaryon containing a transfected HeLa cell nucleus and an untransfected NIH3T3 nucleus. H: human; M: mouse. (b) Schematic diagram of various GNL1/GFP/T-NLS fusion constructs. (c) Functional NES residing within amino acids 201–225 is critical for GNL1 export from the nucleus. HeLa cells were transfected with indicated GNL1/GFP/T-NLS fusion constructs, and their export was determined by heterokaryon assay as described above. Our data suggest that GNL1 is a nucleocytoplasmic shuttling protein, and the domain between amino acids 201 and 225 may be sufficient to export GNL1 from the nucleus.

variants of GNL1 as fusion with GFP/T-NLS (Fig. 4b). Since the functional NoLS resided within amino acids 1–300 of GNL1, it is directly fused with GFP (without T-NLS), and all the other indicated fragments of GNL1, such as amino acids 201–225 and 201–250 (without GNL1 NoLS), are fused with GFP/T-NLS. All the fusion constructs were transfected into HeLa cells, and expression was determined by Western blot analysis using anti-GFP antibodies. The results indicate that all variants expressed correct size polypeptides (data not shown). To define the minimal sequences critical for GNL1 export from the nucleus, we performed heterokaryon assay as described above using all the indicated GNL1 variants. Interestingly, results in Fig. 4c demonstrate that fusion proteins ($GNL1^{1–300}$, $GNL1^{201–250}$, and $GNL1^{201–225}$) containing amino acids 201–225 are exported from the transfected HeLa cell nucleus and imported into the untransfected NIH 3T3 cell nucleus. In contrast, no fluorescence signal was observed in the NIH 3T3 cell nucleus when it was fused with HeLa cells expressing only GFP/T-NLS (Fig. 4c). These data demonstrate that the signal located between amino acids 201 and 225 is sufficient to efficiently export GNL1 from the nucleus by a signal-mediated pathway. A comparative analysis of the sequences within this 25-amino-acid domain reveals the presence of a large number of hydrophobic residues, particularly leucines and valines. It is well known that hydrophobic amino-acid-rich motifs are involved in interacting with CRM1 and export the cargo from the nucleus; this export pathway is specifically blocked by the fungal metabolite LMB.[61] Despite the presence of hydrophobic residues within the identified export signal of GNL1, it is insensitive to LMB. Interestingly, Epstein–Barr virus EB2 protein,[58] steroid receptors,[59] and bovine herpesvirus type 1 UL47 protein[60] were exported to the cytoplasm from the nucleus independent of the CRM1 receptor, similar to GNL1. Collectively, these data suggest that amino acids other than hydrophobic residues within the export signal may dictate the specificity of its interaction with the export receptor in a novel unknown pathway for the export of GNL1 from the nucleolus.

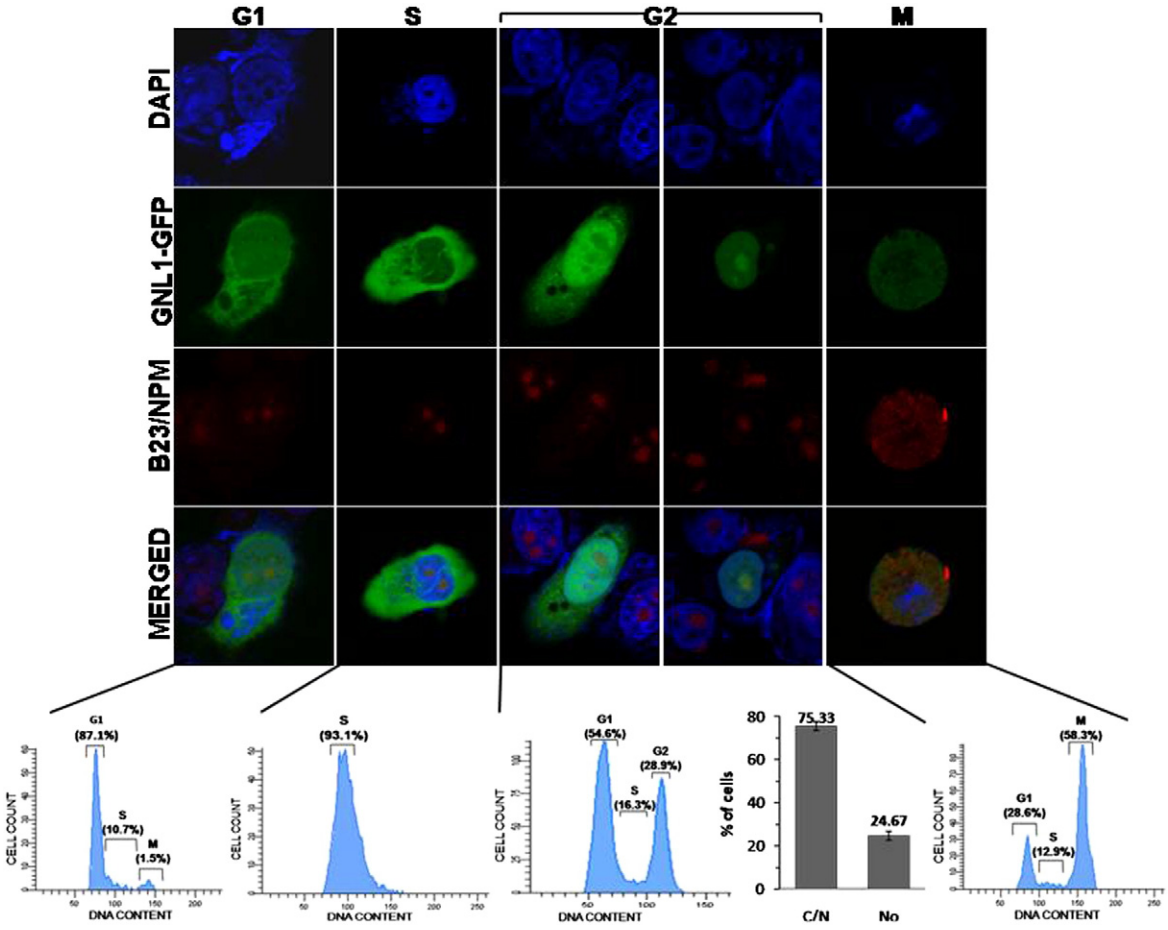


**Fig. 5.** Cell division cycle G2-dependent nucleolar translocation of GNL1. Subcellular localization of GNL1 was determined at different stages of the cell cycle. HeLa cells were transfected with GNL1/GFP and arrested at the G1, S, G2, and M phases of the cell cycle as described in Materials and Methods. Accumulation of cells at the G1, S, G2, and M phases of the cell division cycle was confirmed by flow cytometry analysis (bottom). The localization of GNL1 (green) in cells arrested at the G1, S, G2, and M phases was determined by confocal microscopy (top). A well-known nucleolar protein, B23/NPM, was also visualized by probing the cells with anti-B23 antibodies, followed by Alexa 594 staining (red). Data suggest that GNL1 translocated to the nucleolus only during the G2 stage of the cell cycle.

### Cell cycle G2-phase-dependent nucleolar localization of GNL1

One of the leading hypotheses is that the newly identified nucleolar GTP binding proteins play a critical role in cell cycle regulation but that the mechanism remains unknown. Results from our subcellular localization studies indicate that GNL1 is localized in different cellular compartments in cycling cells. To find out whether the differential localization of GNL1 is a cell cycle-dependent process, we examined the specific cellular localization of GNL1 at different stages of the cell cycle. HeLa cells were transfected with GNL1/GFP and synchronized at the G1, S, G2, and M phases of the cell cycle as described in Materials and Methods. Flow cytometry analyses confirmed that all the cells were synchronized at the indicated stages of the cell cycle (Fig. 5). As shown in Fig. 5, GNL1 underwent dramatic redistribution during the different stages of the cell cycle. We noticed complete cytoplasmic staining for GNL1 during the G1 and S phases of the cell cycle. In contrast, a different GNL1 staining pattern was observed in G2-arrested cells (nucleolus; a nucleus with faint cytoplasm but excluded from the nucleolus) (Fig. 5, top). Quantitative analysis of GNL1 cellular distribution in G2-arrested cells by visual counting of fluorescence-positive cells suggests that 75.33% of cells showed a nucleus with faint cytoplasmic pattern compared with the nucleolar localization in 24.67% of cells (Fig. 5, bottom). Surprisingly, a punctate pattern of GNL1 localization around the chromosome was observed in cells arrested at the M phase of the cell cycle (Fig. 5). Nucleolar localization of a well-known nucleolar protein, B23/NPM, was not altered despite the

altered cellular distribution of GNL1 at different stages (G1, S, and G2) of the cell cycle (Fig. 5). However, B23 colocalized with GNL1 in M-phase-arrested cells. Interestingly, immunofluorescence and cell cycle analyses indicate that the number of cells showed that the nucleolar localization (24.67%) of GNL1 corresponds to the amount of cells accumulated in G2 phase (28.9%) after cell cycle arrest. These data suggest that GNL1 localization in the nucleolar compartment is dependent on the G2 stage of the cell cycle.

### Three-dimensional structural model of GNL1

The Ylqf GTPase from *Bacillus subtilis* was used to model the three-dimensional structure of GNL1

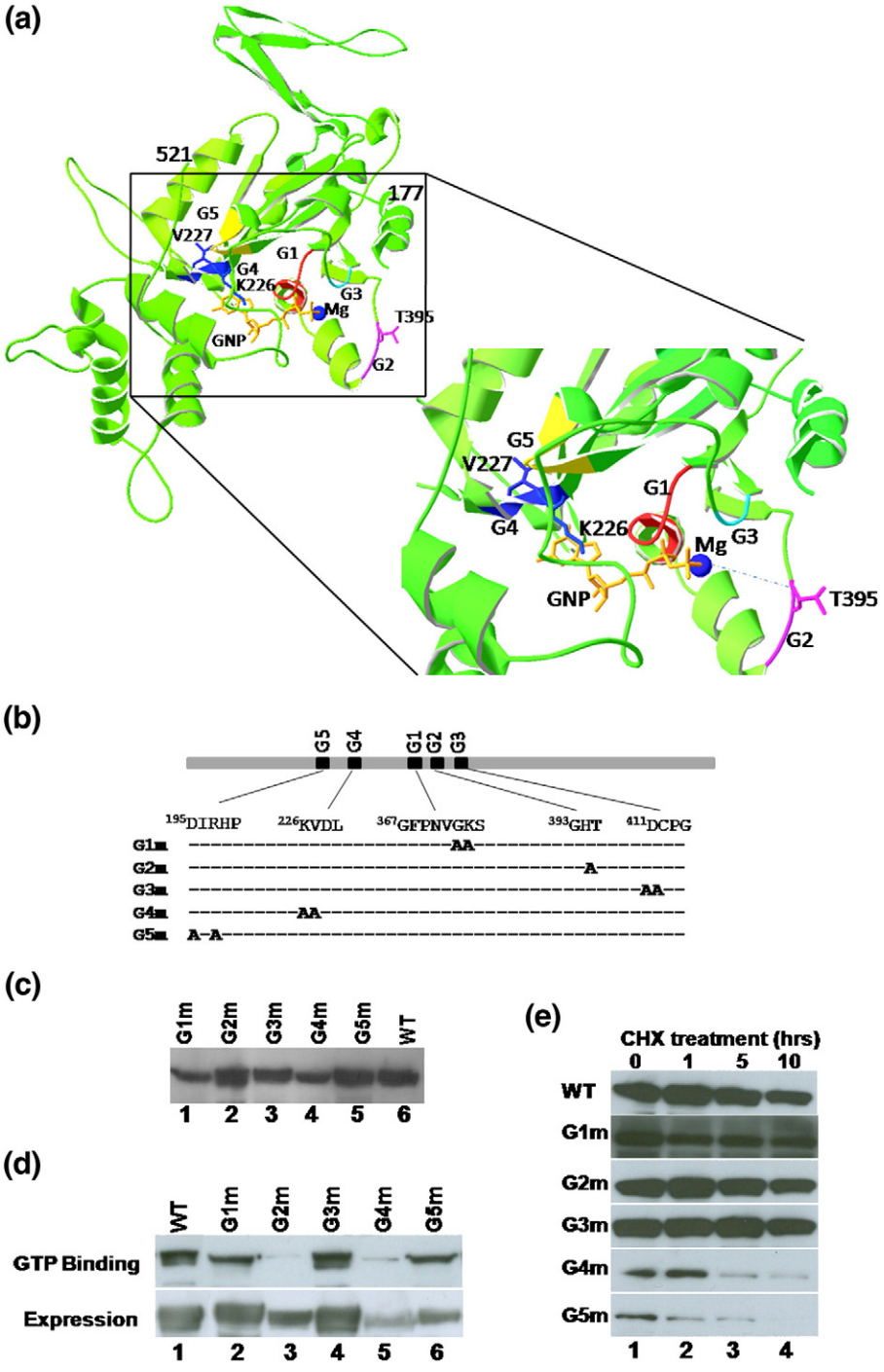


**Fig. 6** (*legend on next page*)

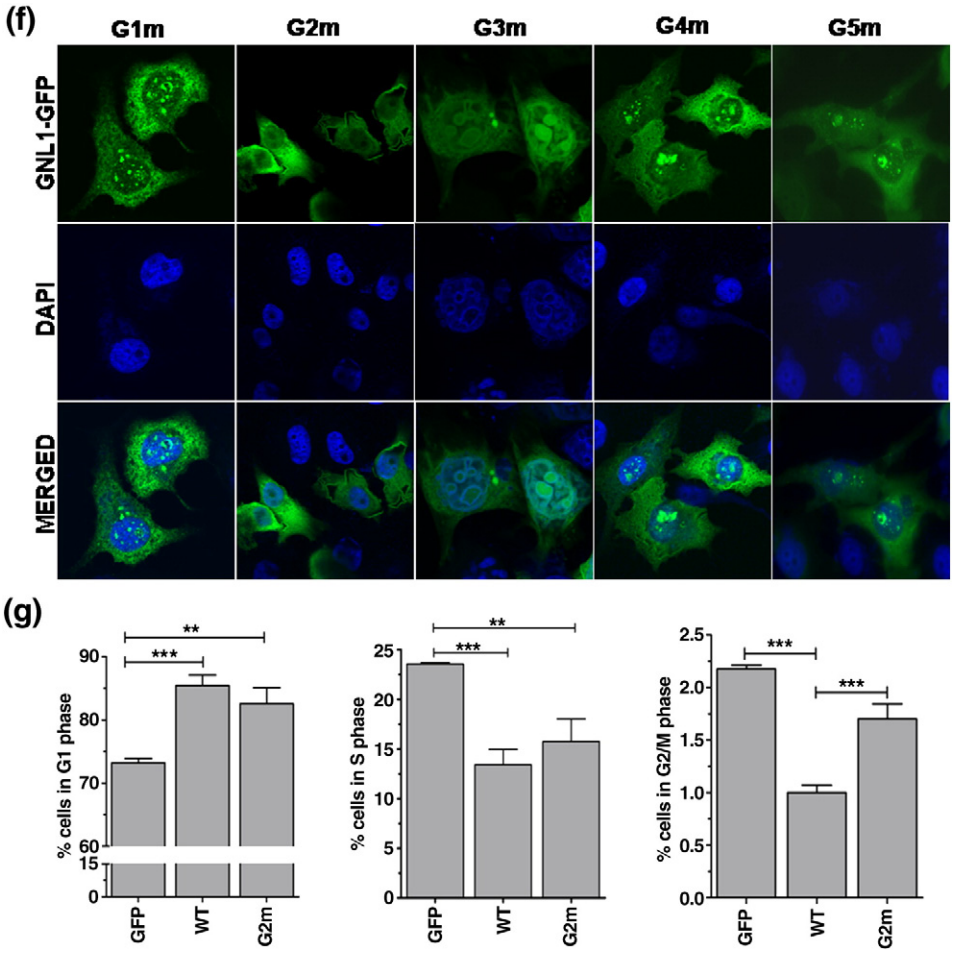


**Fig. 6.** G-domains are critical for GNL1 function. (a) A ribbon diagram of the modeled structure of GNL1 residues 177–521. G-domains are indicated in different colors: motifs G1 (red), G2 (pink), G3 (sky blue), G4 (gray), and G5 (blue), along with GNP and a magnesium ion (blue sphere). The figure is prepared using the program SETOR. (b) Different G-domain mutants of GNL1 were generated by QuickChange mutagenesis to understand their role in GNL1 function. All the indicated G-domain variants were sequenced to verify integrity. (c) All of the indicated G-domain mutants of GNL1 were transfected in HeLa cells, and expression was determined by Western blot analysis using anti-GFP antibodies as described in Materials and Methods. (d) GNL1 binds to GTP. Expression vectors containing the wild type and the various indicated G-domain mutants of GNL1 were transfected in HeLa cells, and the lysates containing equal amounts of total cellular proteins were incubated with GTP agarose beads. Bound proteins were resolved by SDS-10%PAGE and Western blot analysis as described above. The results suggest that the G2 motif is critical for the GTP binding activity of GNL1. (e) Expression vectors containing the wild type and the indicated G-domain mutants of GNL1 were transfected into HeLa cells. After 16 h of transfection, the cells were treated with the translational inhibitor CHX for various time periods. Cell lysates were resolved by SDS-10%PAGE, followed by Western blot analysis, using anti-GFP antibodies as described above. The results indicated that domains G4 and G5 are important for the stability of GNL1. (f) Expression vectors containing the wild type and the various G-domain mutants of GNL1 were transfected into HeLa cells. After 16 h, the transfected cells were fixed, and the subcellular distribution of GNL1 G-domain mutants was determined by fluorescence microscope. The respective G-domain mutants are indicated on top. Data suggest that the G2 motif is essential for GNL1 translocation/retention to the nucleolus. (g) GNL1 promotes M-phase transition. HeLa cells were transfected with 6 μg of wild type, G2m mutant of GNL1, or the pEGFP vector using Lipofectin as described above. After 24 h of transfection, single-cell suspension was prepared and fixed using 2% paraformaldehyde/PBS. DNA was stained with 5 μg/ml Hoechst 33342 at 37 °C for 1 h, and the cell cycle was analyzed by FACS Canto II (BD Biosciences) using laser lines with 488-nm and 407-nm wavelengths to measure GFP and DNA content, respectively. Cell cycle analysis was performed with GFP-positive cells using FACSDiva software (BD Biosciences). The significance of the data was confirmed with one-way analysis of variance among G1 cells ($P=0.0004$), S cells ($P=0.0006$), and G2/M cells ($P<0.0001$). $^{**}P<0.01$; $^{***}P<0.001$.

using the SWISS-MODEL server, as described elsewhere.[62] The final model (Fig. 6a) was derived using amino acid residues 177–521 of GNL1, with a 15.62% sequence identity to Ylqf GTPase. The validity of the model could be readily checked with the conservation of residues in the hydrophobic core

of the molecule and the exposure of hydrophilic residues to the solvent. The G-domain has the characteristic fold of P-loop-containing nucleoside triphosphate hydrolases that belong to the G-protein family.[49] The active site—as identified using the crystal structure of Ylqf GTPase in complex with GNP, a nonhydrolyzable GTP analog, and a magnesium ion (Protein Data Bank code: 1PUJ-A)—is located in the G-domain with several highly conserved residues surrounding GNP and a single magnesium ion. This further confirms the feature of the model, since active-site residues originate from different parts of the primary structure of the protein. For example, T395 interacts with the guanine moiety of GNP, and K226 is involved in binding with phosphate groups of GNP. The conserved residues within all G-domains were mutated to understand the role of these motifs in GNL1 functions.

### Conserved G-domains G4 and G5 are essential for the stability of GNL1

To understand the role of GTP binding motifs in the steady-state levels of GNL1, we constructed variants of GNL1 (G1m [G372A/K373A], G2m [T395A], G3m [D411A/C412A], G4m [K226A/V227A], and G5m [D195A/R197A]) in which the indicated residues within G-domains were replaced by alanines (Fig. 6b). Amino acids selected for GNL1 mutagenesis are conserved in different putative nuclear GTPases from diverse eukaryotic organisms (Fig. 1). Western blot analysis of the transfected cell lysates showed that exchange of conserved residues within G-domains did not alter the expression of GNL1 mutant polypeptides (Fig. 6c). To further understand the importance of the GTP binding status of GNL1, we first characterized the GTP binding ability of various G-domain GNL1 mutants. 293T cell lysates containing indicated G-domain mutants of GNL1 were mixed with GTP-conjugated agarose beads, and the bound proteins were analyzed by Western blot analysis using anti-GFP antibodies. Results demonstrate that a specific retention of GNL1 by the GTP-conjugated agarose (Fig. 6d). The GTP binding ability of GNL1 depends on the G2 motif, as amino acid substitutions within the G2 domain severely altered the GTP binding ability of GNL1 mutant proteins (Fig. 6d). These results, together with the molecular modeling (Fig. 6a), suggest that the G2 motif forms a platform for GTP binding within GNL1. The known nucleolar GTP binding protein nucleostemin was used as positive control and GFP was used as negative control (data not shown) to ensure the integrity of the assay. We next tested whether GTP binding or intact G-domains are critical for the steady-state levels of GNL1. To this end, 293T cells were transfected with the indicated G-domain variants of GNL1 and treated with the translational inhibitor CHX for different time intervals, as described in Materials and Methods. Equal amounts of CHX-treated cell lysates were subjected to Western blot analysis using anti-GFP antibodies. As shown in Fig. 6e, wild-type GNL1 was stable at 10 h after CHX treatment. Replacement of conserved residues within G-domains G1, G2, and G3 did not alter the steady-state levels of GNL1 mutant proteins (Fig. 6e). However, exchange of conserved residues within the G4 and G5 domains alters the stability of mutant proteins (Fig. 6e). Interestingly, more than 50% of G5 mutant protein degraded within 1 h of CHX treatment despite its interaction with GTP, similar to the wild-type protein (Fig. 6d). Together, these results suggest that exchange of conserved residues within the G4 and G5 domains may have altered the conformation of the mutant proteins, which may favor easy access to protease activity and resulted in faster protein degradation. In addition, mutation within the G2 domain abrogated the interaction of mutant GNL1 with GTP (Fig. 6d) without altering the stability of the mutant protein (Fig. 6e), suggesting that GTP binding is not critical for the steady-state levels of GNL1. These data suggest that the proper conformation of G-domains is critical for maintaining the steady-state levels of GNL1.

### G-domains are important for efficient nucleolar retention of GNL1

We next tested the role of GTP binding activity in the retention of GNL1 in the nucleolar compartment. All the G-domain mutants of GNL1 were transfected in HeLa cells and subjected to immunofluorescence analysis as described in Materials and Methods. As shown in Fig. 6f, GNL1 mutants G1m, G3m, G4m, and G5m continued to localize in the nucleolus, in addition to intense cytoplasmic staining. Exchange of conserved residues within the G2 domain (G2m) resulted in the mutant protein being localized both in the nucleus and in the cytoplasm but being excluded from the nucleoli (Fig. 6f). These results suggest that the GTP binding motif G2 plays a critical role in the efficient nucleolar retention of GNL1. However, the G2 mutant of GNL1 retained stability like the wild-type protein but failed to bind with GTP and to translocate to the nucleolus. It is worth noting that mutations within the G2 domain completely blocked the nucleolar localization of G2m even in the presence of functional NoLS, indicating the importance of G-domains in GNL1 nucleolar translocation. These results demonstrate that NoLS and the wild-type GTP binding domains play a critical role in the efficient nucleolar retention of GNL1. To further support these data, we next investigated whether altering intracellular GTP levels would affect the nucleolar residence of GNL1. To deplete the intracellular GTP levels, we treated the GNL1-expressing cells with mycophenolic acid (MPA), which blocks the rate-limiting

enzyme inositide 5′-monophosphate dehydrogenase of *de novo* guanine nucleotide biosynthesis.[63] Immunofluorescence analysis of MPA-treated cells indicates that GNL1 localized mainly in the cytoplasm with faint nuclear staining but was completely excluded from the nucleolus compared with untreated cells (Fig. S3). These results support our mutagenesis data and further provide evidence that GNL1 shuttling receives regulatory inputs from intracellular signals. Thus, GNL1 may utilize its GTP binding activity as a molecular switch to regulate the transition between the nucleolus-localized state and the nucleoplasm-localized state involving the interaction between the NoLS and GTP binding domains.

To understand the importance of GTP binding activity and GNL1 nucleolar localization in cell cycle regulation, we transfected the wild type or G2 mutant (G2m) of GNL1 in HeLa cells. Single-cell suspension was prepared and labeled with Hoechst 33342 (Sigma-Aldrich, USA) 24 h posttransfection, as described in Materials and Methods. The labeled cells were subjected to flow cytometry for cell cycle analysis. Interestingly, results in Fig. 6g suggest that a lesser number of cells accumulated in the G2/M phase of the cell cycle in wild-type GNL1, in contrast to G2m-transfected and GFP-transfected cells. However, we observed that a greater number of cells accumulated in G1 phase in wild type compared to G2m-transfected cells (Fig. 6g). As described above, G2m protein was not able to interact with GTP (Fig. 6d) and failed to localize to the nucleolus (Fig. 6f) despite wild-type levels of stability (Fig. 6e). Together, these data provide evidence that GTP binding is critical for the nucleolar translocation of GNL1, which in turn regulates G2/M phase transition.

## Discussion

Human GNL1 is a novel nucleolar G-domain-containing protein that is highly conserved in the genomes of bacteria to humans. In the present investigation, we have demonstrated that GNL1 is localized in different cellular compartments and further showed that it harbors a novel arginine/lysine-rich NoLS that is sufficient to independently translocate a heterologous protein to the nucleolus. Our data also provided evidence that GNL1 shuttles between the nucleus and the cytoplasm via a CRM1-independent pathway. In addition, our results indicate that GTP binding to GNL1 acts as a molecular switch to control its transition between the nucleolus and the nucleoplasm, suggesting the possibility of cross-talk between NoLS and GTP binding domains. Furthermore, our data suggest that G-domains play a critical role in maintaining the proper conformation of GNL1, which in turn regulates stability and its retention in the nucleolus. Finally, the present investigation provided evidence that nucleolar translocation of GNL1 is critical for G2/M transition during cell proliferation.

The NoLS of GNL1, which is rich in arginine and lysine residues, is able to translocate the heterologous protein to the nucleus/nucleolus via a pathway that is independent of importin-α and importin-β. To our knowledge, it has been shown that a transport signal rich in lysine residues translocates the cargo to the nucleus by interacting with importin-α of the importin-α/importin-β complex, as noted with NLSs of SV40 and p53.[27,28] However, the localization signal is rich in arginine residues, directly interacts with importin-β, and transports the cargo to the nucleolus without the involvement of importin-α.[29,30,64] Surprisingly, the newly identified NLS/NoLS, GNL1$^{1-25}$, is rich in both arginine and lysine residues and transports the heterologous protein to the nucleolus by a novel pathway without interacting with importin-α and importin-β (see Fig. S1). These data suggest the possibility that amino acids other than lysine and arginine residues within NoLS may be responsible for the specificity of interaction with novel transport receptors despite the basic residues being critical for the nucleolar translocation of GNL1. It is likely that, upon entry into the nucleolus, GNL1 may interact with other nucleolar proteins or RNA for it to be retained in the nucleolus. Such view is supported by the altered subcellular localization of GNL1 in Act-D-treated cells (see Fig. 2e). This observation leads us to conclude that ongoing transcription may be required for the efficient retention of GNL1 in the nucleolus. It is well established that arginine-rich domains are known to interact with RNA, in addition to its role in nucleolar transport.[65] The presence of arginine clusters within NoLS may promote GNL1 interaction with RNA and supports its retention in the nucleolus. Interestingly, subcellular distribution analysis in the synchronized cell population suggests a G2-stage-dependent nucleolar translocation of GNL1. It should be noted that GNL1 localized only in the cytoplasm in the G1 and S stages, in contrast to a punctate pattern surrounding the chromosomes in the M phase of the cell division. These data lead to the speculation that translocation of GNL1 into the nucleolus during G2 arrest may be critical to promoting M-phase transition. Further studies are warranted to identify the receptors involved in GNL1 nucleolar transport and to define whether GNL1 interacts with RNA or other nucleolar components to regulate cell proliferation.

Using a combination of heterokaryon and immunofluorescence analyses, the present studies provide evidence that GNL1 is a nucleocytoplasmic shuttling protein and that export from the nucleus is an energy-dependent signal-mediated process. Mutational analysis suggests that GNL1$^{201-225}$ was

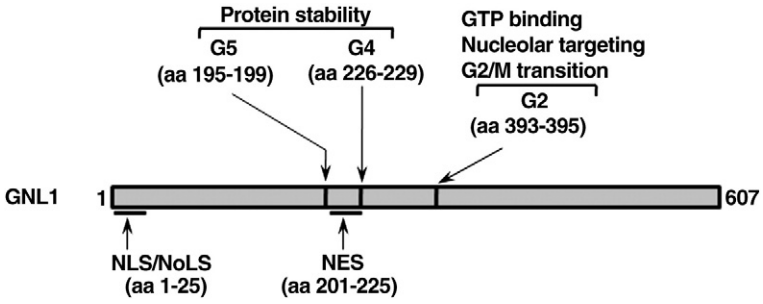


**Fig. 7.** GNL1 encodes multiple functional domains. Combination of mutational, cell biological, and protein–protein interaction analyses suggests that GNL1 encompasses multiple functional domains. An amino-terminal domain containing amino acids 1–25 encodes a nuclear/nucleolar transport signal, G-domains G4 and G5 play a critical role in GNL1 stability, and amino acids 201–225 are essential for the nucleocytoplasmic shuttling activity of GNL1. G2 domain plays an important role in GNL1 interaction with GTP.

sufficient to export the fusion protein containing GFP and SV40T-NLS from the nucleus, indicating the presence of a functional transferable nuclear export signal (NES). Comparative sequence analysis suggests the presence of leucine clusters within GNL1 NES, but it appears that there is no consensus with any one of the known NESs.[66,67] In addition, replacement of hydrophobic leucines within NES did not alter the export of GNL1 from the nucleus, suggesting the presence of a novel noncanonical export signal. This notion is supported by the fact that LMB (known to block the classical CRM1-dependent nuclear export pathway[61]) treatment did not alter the export of GNL1 from the nucleus. These data lead to the hypothesis that GNL1 may interact with a novel export receptor. It is interesting to note that many proteins of cellular and viral origin export from the nucleus via a CRM1-independent pathway and are insensitive to LMB.[58–60] Further experiments are needed to identify the receptor involved in GNL1 export from the nucleus and its relevance to cell proliferation.

The present study suggests that both the NoLS and the GTP binding motifs regulate the nucleolar translocation of GNL1. Interestingly, the GNL1 mutant that failed to bind with GTP localized in the cytoplasm and the nucleoplasm (excluded from the nucleolus). GNL1$^{1–25}$ was sufficient to target the GFP to the nucleolus despite the additional requirement of the GTP binding motifs for an efficient nucleolar localization of full-length GNL1. Recent reports suggest that a GTP-bound state regulates the nucleolar retention of nucleostemin,[48] GNL3L,[68,69] and GNL2.[69,70] Together, these findings indicate that an inhibitory mechanism gates the nucleolus targeting activity of GNL1. Although it is presently unknown whether the GTP-bound or unbound GNL1 represents the active form, our data demonstrate that nucleolar accumulation of GNL1 appears to be dependent on its GTP-bound conformation. Exchange of conserved residues within the G4 (decreased GTP binding) and G5 (wild-type GTP binding) domains altered the stability of GNL1. Surprisingly, we observed a wild-type level of stability for G2m despite its failure to interact with GTP. These data suggest that a proper conformation of G-domains, not GTP binding activity, is critical for maintaining the steady-state level of GNL1. Taken together, the present investigation demonstrates that multiple nonoverlapping domains regulate the different functions of GNL1 (Fig. 7). Interestingly, GTP binding and the nucleolar translocation properties of GNL1 are critical for regulating G2/M-phase transition during cell proliferation.

The nucleolus functions as a factory for assembling ribosomal subunits. The discovery of a surprising variety of macromolecules in the nucleolus with no apparent ribosomal function indicates that the nucleolus may play a role in nuclear export, sequestering regulatory molecules, modifying small RNAs, assembling ribonucleoproteins, and controlling aging. In these novel events, the nucleolus serves as a privileged site for both recruitment and exclusion of regulatory complexes. Does the nucleolus serve as a "sequestration center" for the newly identified nucleolar putative GTPase GNL1? One of the current hypotheses is that certain nucleolar proteins up-regulate cell proliferation during tumorigenesis by modulating ribosome biogenesis. Growing evidence suggests that alteration of ribosome biogenesis may lead to uncontrolled cell proliferation and tumorigenesis. Nucleolar proteins have also been shown to regulate cell proliferation and growth by modulating the ribosomal and nonribosomal functions of the nucleolus.[11,71–73] Further studies are required to understand whether GNL1 can participate in the regulation of the nucleolar sequestration of proteins with nonribosomal functions to control cell proliferation.

## Materials and Methods

### Plasmids

GNL1 open reading frame was amplified from HeLa cDNA using appropriate primers (Table 1). The amplified product was digested with HindIII and BglII and cloned into a modified pcDNA3 vector (Invitrogen, USA) as fusion with GFP. Various truncation and site-specific

**Table 1.** Primers used for the construction of GNL1 variants

| Name[a] | Restriction site[b] | Sequence (5′–3′)[c] |
|---|---|---|
| GNL1 WT + | HinDIII | TGCGAC***AAGCTT***GCCGCCACCATGCCGAGGAAGAAGCCATTC |
| GNL1 WT – | BglII | TACAGG***AGATCT***GCACTCATCCTCACCCAGCA |
| GNL1 25 – | EcorV | GCTACT***GATATC***CCCCTCTCTTCCGCTCCCGTTTGTCCTG |
| GNL1 50 – | EcorV | GCTACT***GATATC***GGTGTCGGTCTGTTCCTCGCCGCTCC |
| GNL1 100 – | EcorV | TTTGG***GATATC***GGCTGCTCTCTTTCTCCTCTCT |
| GNL1 200 – | KpnI | GCATGA***GGTACC***TGGATGTCGGATATCAGTGA |
| GNL1 300 – | KpnI | GCATGA***GGTACC***CCCCACAGTGATGGCTTCAG |
| GNL1 400 – | KpnI | GCATGA***GGTACC***GGTCTGAAAGTATCGGGTAT |
| GNL1 500 – | KpnI | GCATGA***GGTACC***TCTGTACACATCATTCCGAG |
| GNL1 26 + | HinDIII | TGCGAC***AAGCTT***ATGCTTCAAGATGGGCTGCGCTCCAGT |
| GNL1 51 + | HinDIII | ACTGAC***AAGCTT***ATGTCGGACGGGGAGTCTGTGACCCATCAT |
| GNL1 101 + | HinDIII | ATCCTA***AAGCTT***GCCGCCACCATGCGGGAGCAAGTTCTACAGCCG |
| GNL1 201 + | HinDIII | GATCTA***AAGCTT***ATGGTTGTGAATTTCCCGCCAGCA |
| GNL1 301 + | HinDIII | GATCTA***AAGCTT***ATGAAAGTGGACTTGAGCAGCTGG |
| GNL1 401 + | HinDIII | GATCTA***AAGCTT***ATGTACTTTCTTACCCCCTCTGTG |
| GNL1 501 + | HinDIII | GATCTA***AAGCTT***ATGGCAGCCAACAGTCTCTTGCGG |
| GNL1 R3K4K5A + | | GCCGCCACCATGCCGGCCGCCGCCCCATTCAGCGTGAAG |
| GNL1 R3K4K5A – | | CTTCACGCTGAATGGGGCGGCGGCCGGCATGGTGGCGGC |
| GNL1 K12K13K14A + | | TTCAGCGTGAAGCAGGCCGCCGCCCAGTTGCAGGAC |
| GNL1 K12K13K14A – | | GTCCTGCAACTGGGCGGCGGCCTGCTTCACGCTGAA |
| GNL1 R22K23A + | | CAGGACAAACGGGAGGCCGCCAGAGGGCTTCAAGAT |
| GNL1 R22K23A – | | ATCTTGAAGCCCTCTGGCGGCCTCCCGTTTGTCCTG |
| GNL1 R95R96K97A + | | CAGCAGGGAGGAGGTAGAGGCTGCAGCCAGAGCAGCCCGGGAGCAAG |
| GNL1 R95R96K97A – | | CTTGCTCCCGGGCTGCTCTGGCTGCAGCCTCTACCTCCTCCCTGCTG |
| GNL1 G1m + | | GGTTTCCCTAATGTGGCCGCCTCCTCGCTGATCAATGG |
| GNL1 G1m – | | CCATTGATCAGCGAGGAGGCGGCCACATTAGGGAAACC |
| GNL1 G2m + | | GAACCCCGGGCCATGCCCGATACTTTC |
| GNL1 G2m – | | GAAAGTATCGGGCATGGCCCGGGGTTC |
| GNL1 G3m + | | CTCTGTGAAGCTCTGTGCCGCCCCAGGCCTCATCTTCC |
| GNL1 G3m – | | GGAAGATGAGGCCTGGGGCGGCACAGAGCTTCACAGAG |
| GNL1 G4m + | | CTGGTGCTGGTTTTGGCCGCCGTGGATCTGGCCCCG |
| GNL1 G4m – | | CGGGGCCAGATCCACGGCGGCCAAAACCAGCACCAG |
| GNL1 G5m + | | CTGCTTATCACTGCCATCGCCCATCCAGTTGTGAATTTCC |
| GNL1 G5m – | | GGAAATTCACAACTGGATGGGCGATGGCAGTGATAAGCAG |

[a] (+) and (–) indicate forward and reverse primers, respectively. Numbers indicate the amino acid number in the full-length sequence.
[b] Restriction sites in the primer are used for subsequent cloning.
[c] Restriction sites are shown in bold-italics.

mutants of GNL1 were generated with full-length GNL1 as template, using appropriate primers (Table 1). All of the constructs were sequenced to verify the integrity of each clone.

### Cell culture, transfection, and Western blot analysis

293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic (Invitrogen, USA). GNL1 full-length or relevant mutant was transfected into HeLa and 293T cells by Lipofectin (Invitrogen, USA) and $CaCl_2$, respectively. For Western blot analysis, transfected cells were solubilized in loading buffer [62.5 mM Tris–HCl (pH 6.8), 0.2% SDS, 5% 2-mercaptoethanol, and 10% glycerol] and resolved on 10% SDS-PAGE. The proteins were then transferred onto Hybond-P (GE Healthcare, Sweden) and probed with mouse monoclonal anti-GFP antibodies (1:1000 dilution; Santa Cruz, USA). The antibody-bound proteins were probed with goat horseradish-peroxidase-conjugated anti-mouse secondary antibodies (1:3000 dilution; Southern Biotechnology, USA) and detected using Enhanced Chemiluminescence Plus Detection System (GE Healthcare, Sweden).

### Cell cycle analysis

HeLa cells were arrested at different stages of the cell cycle, as described elsewhere.[74,75] HeLa cells were transfected with GNL1/GFP and GFP using Lipofectin as described above. Double-thymidine block was performed to arrest the cells at S phase. Briefly, at 24 h posttransfection, complete DMEM (with 10% FBS and 1% antibiotic–antimycotic) with 2 mM thymidine (Sigma-Aldrich, USA) was added to the cells and incubated for 19 h. The medium was then removed and washed thrice with phosphate-buffered saline (PBS) before the fresh complete DMEM was added and incubated for an additional 9 h. Following this, DMEM containing 2 mM thymidine was added to the cells and incubated for 16 h. To arrest the cells at G2 phase, we blocked the transfected cells with thymidine for 24 h, as described above, and incubated the cells for 3 h with fresh DMEM. Following this, DMEM containing 10 μg/ml Hoechst 33342 (Sigma-Aldrich, USA) was added to the cells and incubated for 12 h. S-phase-arrested cells were washed thrice with PBS before fresh DMEM was added and incubated for an additional 3 h. Following this, the cells were washed, and DMEM containing 100 ng/ml nocodazole (Sigma-Aldrich, USA) was added to the cells and incubated

for 12 h for blocking at M phase.[74] G1 arrest was performed by incubating the transfected cells with DMEM (without FBS) for 48–72 h. After all the different treatments, cells were trypsinized, and single-cell suspension was prepared and fixed using 2% paraformaldehyde/PBS. DNA was stained with 5 μg/ml Hoechst 33342 at 37 °C for 1 h, and the cell cycle was analyzed by FACS Canto II (BD Biosciences, USA) using 488-nm and 407-nm laser lines to measure GFP and DNA content, respectively. The cell cycle profiles were analyzed only with GFP-positive cells using FACSDiva software (BD Biosciences, USA). Subcellular distribution of GNL1 at different stages of the cell division cycle was determined by laser scanning spectral confocal microscopy (Carl Zeiss, Germany).

### Fluorescence microscopy

HeLa cells in chamber culture slides (BD Biosciences, USA) were infected with vTF7-3, a vaccinia virus expressing T7-RNA polymerase, at a multiplicity of infection of 10 and transfected with GNL1 expression plasmids using Lipofectin as described above. Cells were fixed with 3% paraformaldehyde/PBS for 15–20 min at 12–16 h posttransfection to determine the subcellular localization of GNL1/GFP fusion proteins. Cells were mounted in a mounting medium (Vector Laboratories, USA) containing 4′,6-diamidino-2-phenylindole (DAPI) to stain the nuclei. Samples were viewed, and the images were captured by a laser scanning spectral confocal microscope (Carl Zeiss) and processed using Zen2009 software.

### Protein interaction assays

GST–importin-α and GST–importin-β fusion proteins were expressed and purified in accordance with Imamoto *et al.*[76] Briefly, importin-α and importin-β expression vectors were transformed into *Escherichia coli* BL21(DE3) and grown at 37 °C in an ampicillin-containing medium to an optical density of ~0.9 at 600 nm prior to induction with 1 mM IPTG for 16 h at 18 °C. Cells were harvested and suspended in 1× *E. coli* lysis buffer and disrupted by vigorous sonication on ice. Glutathione-Sepharose 4B beads (GE Healthcare, USA) were washed three times with ice-cold PBS, and the mixtures were rocked for 60 min at 4 °C, followed by four washes of protein-bound beads with ice-cold NP-40 binding buffer [25 mM Hepes (pH 7.9), 150 mM KCl, 0.1% NP-40, 5% glycerol, 0.5 mM DTT, 0.4 mM PMSF, 1 mM Na-fluoride, 1 mM Na-orthovanadate, and protease inhibitors]. Bound proteins were eluted in elution buffer [5 mM reduced glutathione in 50 mM Tris–Cl (pH 7.4)], and the integrity of fusion proteins was resolved on 10% SDS-PAGE, followed by staining with Coomassie blue.

### GTP binding assay

The wild type and the various G-domain mutants of GNL1 were transfected in 293T cells. Twenty-four hours posttransfection, cells were lysed with lysis buffer [20 mM Hepes (pH 7.9), 20 mM $MgCl_2$, 300 mM NaCl, 0.5% NP-40, 1 mM DTT, 0.4 mM PMSF, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin], and lysates were precleared with Ni-NTA agarose beads. These precleared lysates were then incubated with GTP-agarose resins (Innova Biosciences, UK) for 3 h at 4 °C with rocker, followed by four washes of protein-bound beads with ice-cold NP-40 binding buffer [25 mM Hepes (pH 7.9), 150 mM KCl, 0.1% NP-40, 5% glycerol, 0.5 mM DTT, 0.4 mM PMSF, 1 mM Na-fluoride, 1 mM Na-orthovanadate, and protease inhibitors]. Bound proteins were resolved by 10% SDS-PAGE, followed by Western blot analysis, using anti-GFP antibodies.

### Cycloheximide (CHX) treatment

The wild type and the various G-domain mutants of GNL1 were transfected in HeLa cells. Forty-eight hours posttransfection, DMEM supplemented with 10% FBS, 1% antibiotic–antimycotic, and 30 μg/ml CHX was added, and the cells were collected at different times after CHX treatment. The cells were lysed with lysis buffer [20 mM Hepes (pH 7.9), 20 mM $MgCl_2$, 300 mM NaCl, 0.5% NP-40, 1 mM DTT, 0.4 mM PMSF, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin], and an equal quantity of protein from each sample was separated on 10% SDS-PAGE, followed by Western blot analysis, using anti-GFP antibodies.

### Heterokaryon assay

HeLa cells in chamber culture slides (BD Biosciences, USA) were infected with vTF7-3, a vaccinia virus expressing T7-RNA polymerase, at a multiplicity of infection of 10 and transfected with GNL1 expression plasmids as described above. Sixteen hours posttransfection, cells were treated with DMEM containing 30 μg of CHX for 3 h. Untransfected NIH3T3 cells were then layered onto the CHX-treated transfected HeLa cells and incubated for 4 h in DMEM containing CHX. Cell–cell fusion was achieved by treating the cells with 50% wt/vol polyethylene glycol 3350 for 2 min, followed by washing with PBS three times. Cells were then incubated in DMEM containing CHX for 1 h and fixed with 3% paraformaldehyde, and the nucleus was stained using 5 μg/ml Hoechst 33342. Distribution of GNL1 was determined by a laser scanning confocal microscope.

### Structural modeling of GNL1

GNL1 structure was modeled based on the crystal structure of Ylqf GTPase from *B. subtilis* (Protein Data Bank ID: 1PUJ-A) using Web-based SWISS-MODEL software.[62] These two proteins are known to be evolutionarily related, with a sequence identity of 15.62%. The final model was obtained for a part of GNL1 containing amino acid residues 177–521. Important structural domains of GNL1, such as G-motifs and the characteristic fold of P-loop, were modeled.

Supplementary materials related to this article can be found online at doi:10.1016/j.jmb.2011.12.066

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