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NVL2 Is a Nucleolar AAA-ATPase that Interacts with Ribosomal Protein L5 through Its Nucleolar Localization Sequence

Masami Nagahama, Yoshimitsu Hara, Akihiro Seki, Takeshi Yamazoe, Yumiko Kawate, Takashi Shinohara, Kiyotaka Hatsuzawa,* Katsuko Tani, and Mitsuo Tagaya†

School of Life Science, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo 192-0392, Japan

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NVL (nuclear VCP-like protein), a member of the AAA-ATPase family, is known to exist in two forms with N-terminal extensions of different lengths in mammalian cells. Here, we show that they are localized differently in the nucleus; NVL2, the major species, is mainly present in the nucleolus, whereas NVL1 is nucleoplasmic. Mutational analysis demonstrated the presence of two nuclear localization signals in NVL2, one of which is shared with NVL1. In addition, a nucleolar localization signal was found to exist in the N-terminal extra region of NVL2. The nucleolar localization signal is critical for interaction with ribosomal protein L5, which was identified as a specific interaction partner of NVL2 on yeast two-hybrid screening. The interaction of NVL2 with L5 is ATP-dependent and likely contributes to the nucleolar translocation of NVL2. The physiological implication of this interaction was suggested by the finding that a dominant negative NVL2 mutant inhibits ribosome biosynthesis, which is known to take place in the nucleolus.

INTRODUCTION

The AAA (ATPase associated with various cellular activities) proteins constitute a large family of ATPases, which is characterized by the presence of one (Type I) or two (Type II) conserved ATP-binding modules (AAA modules), each consisting of ~200 amino acid residues (Neuwald *et al.*, 1999). To date, many members of the AAA family have been identified in a wide range of organisms, ranging from bacteria to mammals. They play important roles in various cellular processes, including proteolysis, membrane fusion, cytoskeletal regulation, protein folding, and DNA replication (Neuwald *et al.*, 1999; Ogura and Wilkinson, 2001). Although the biological functions of the various members of this family seem unrelated, it has been proposed that they commonly modulate the assembly and disassembly of macromolecular complexes as energy-dependent molecular machinery (Neuwald *et al.*, 1999; Vale, 2000; Ogura and Wilkinson, 2001).

NVL (nuclear VCP-like protein) was first identified as a gene product that exhibits a high level of amino acid similarity with an AAA protein, VCP/p97. Two alternatively spliced isoforms, NVL1 (a short isoform) and NVL2 (a long one), are produced through the utilization of different methionines as translation initiation sites (Figure 1; Germain-Lee *et al.*, 1997). NVL and VCP/p97, both of which are Type II AAA ATPases, comprise three domains, i.e., an N-terminal domain, followed by two tandem AAA modules (termed D1 and D2). VCP/p97 and its yeast counterpart, Cdc48p, are involved in various cellular processes, including cell cycle regulation, organelle membrane fusion events, and ubiquitin-dependent proteolysis (Patel and Latterich, 1998; Lord *et al.*, 2002). More recently, a nucleolar function of VCP/p97 involving interaction with Werner syndrome protein was also suggested (Partridge *et al.*, 2003; Indig *et al.*, 2004). Despite the high sequence similarity between VCP/p97 and NVL, the two proteins exhibit different subcellular localizations. While VCP/p97 is distributed in the cytoplasm, nucleus, and endoplasmic reticulum membrane, NVL has been shown to be exclusively localized in the nucleus (Germain-Lee *et al.*, 1997), suggesting its specialized role in this subcellular compartment.

Previously, *smallminded* (*smid*), a *Drosophila* gene encoding a protein homologous to NVL, was isolated and characterized. Disruption of the *smid* gene led to larval lethality with an abnormally small CNS and imaginal discs (Long *et al.*, 1998b). In situ DNA synthesis analysis indicated that this phenotype is probably due to defective mitosis of postembryonic neuroblasts and their subsequent death due to apoptosis (Long *et al.*, 1998a). Mac-1, an NVL homologue in *Caenorhabditis elegans*, was identified as a protein that interacts with CED-4, a homologue of mammalian Apaf-1, and shown to prevent cell death in specific circumstances (Wu *et al.*, 1999). MAC-1 has also been shown to be essential for larval development of the worm independently of its apoptosis-related function. Recently, a putative homologue of NVL in *Saccharomyces cerevisiae*, Rix7p, was shown to be required for the biogenesis and/or nuclear export of the 60S ribosomal subunit (Gadal *et al.*, 2001).

In this study, to gain insights into the cellular function of mammalian NVL, we first examined the subcellular localization of the two isoforms of NVL. We demonstrated that

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* Present address: Institute of Biomedical Sciences, Fukushima Medical University School of Medicine, Fukushima 960-1295, Japan.

† Corresponding author. E-mail address: tagaya@ls.toyaku.ac.jp.

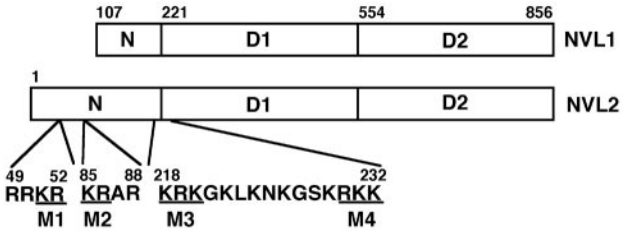

Figure 1. Schematic representation of human NVL1 and NVL2. The numbering of amino acids starts from the initiation methionine of NVL2. The translation of NVL1 starts from the residue corresponding to the second methionine (residue 107) of NVL2. The amino acid sequences of potential nuclear and nucleolar localization signals are indicated. The residues mutated in this study are underlined, and named M1, M2, M3, and M4, respectively.

NVL1 and NVL2 show different subnuclear localizations, and that NVL2, the major species, is mainly localized in the nucleolus. We also found that NVL2 specifically associates with ribosomal protein L5, a component of the 60S large ribosomal subunit, in a manner dependent on its nucleolar localization signal (NoLS). Furthermore, expression of a dominant negative form of NVL2 in cells significantly reduced the level of 60S ribosomes, implying its role in ribosome biogenesis.

MATERIALS AND METHODS

Plasmid Construction

Full-length cDNA for human NVL2 (Germain-Lee *et al.*, 1997) was amplified from a human leukocyte cDNA library (Clontech, Palo Alto, CA) by PCR and then subcloned into pFLAG-CMV-5 (Sigma-Aldrich, St. Louis, MO) and pGBT9 (Clontech) to construct pFLAG-NVL2 and pGBT-NVL2, respectively. pFLAG-NVL1, pGBT-NVL1, and pTrx-NVL1 were constructed by inserting PCR-generated fragments corresponding to the coding region for NVL1 into pFLAG-CMV-5, pGBT9, and pET-32 (Novagen, Madison, WI), respectively. Plasmids encoding the N- (residues 1–220), D1- (residues 221–553), D2- (residues 554–856), and ND1- (residues 1–553) domains of NVL2 were constructed by amplifying cDNA fragments corresponding to the individual coding regions and then inserting them into pFLAG-CMV-5 and pGBT9, respectively. The K628M, M1, M2, M3, and M4 mutants (Figure 1) and mutants with combinations of the mutations were generated by PCR-based mutagenesis. Full-length cDNA for L5 was amplified by PCR from a human kidney cDNA library (Clontech) and then subcloned into pEBG (Tanaka *et al.*, 1995) and pGEX-4T (Amersham Biosciences, Piscataway, NJ) to express L5 as a glutathione *S*-transferase (GST) fusion protein in mammalian cells and *Escherichia coli*, respectively. PCR-generated fragments corresponding to the entire coding region and amino acid residues 81–297 of L5 were inserted into pEGFP-N1 (Clontech) for its expression as green fluorescent protein (GFP) fusion proteins. The sequences of constructs generated by PCR were all confirmed by nucleotide sequencing.

Yeast Two-hybrid Screening and Assay

The yeast two-hybrid screening and β-galactosidase filter assay were performed essentially according to the manufacturer's protocols (Clontech). pGBT-NVL2, as a bait plasmid, was transformed into yeast strain Y190. Trp^+ transformants were isolated and subsequently transformed with a human kidney cDNA library in *LEU*2 marker plasmid pACT2 (Clontech). Transfor-

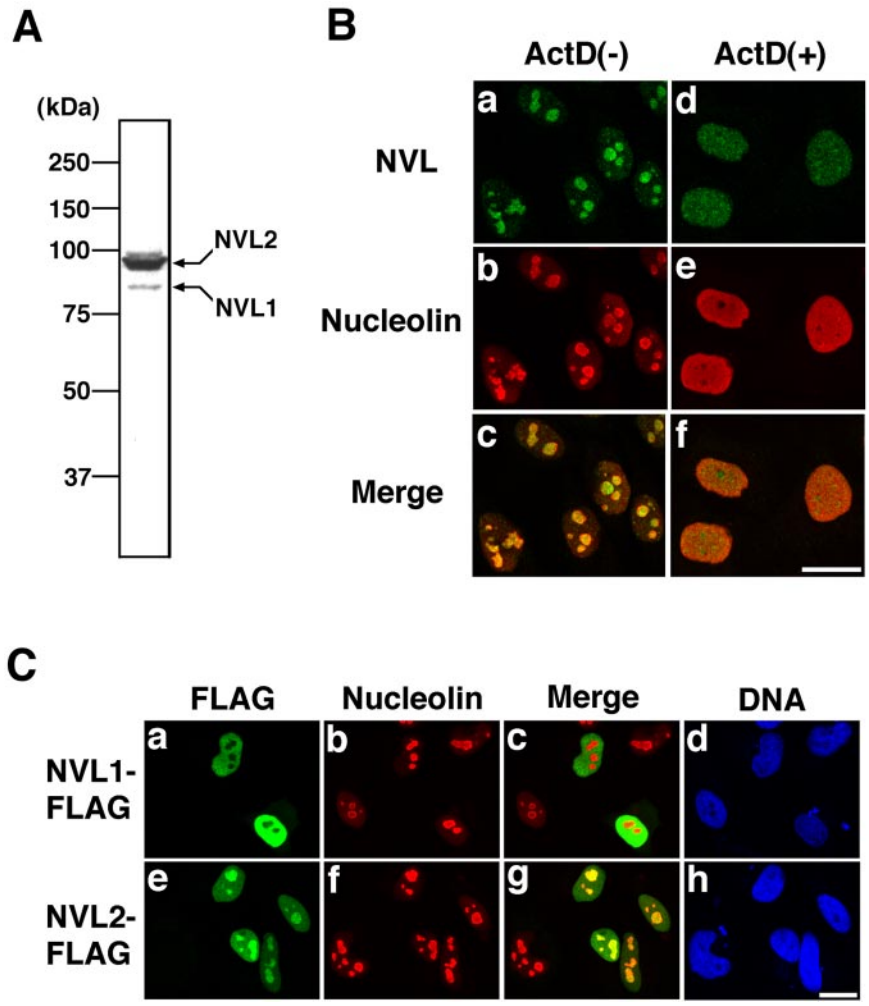

Figure 2. Differential subnuclear localization of NVL isoforms. (A) A 293T cell lysate was analyzed by Western blotting using an anti-NVL antibody. The molecular size markers are indicated on the left. (B) HeLa cells were untreated or treated with ActD (50 ng/ml) for 3 h. The localization of endogenous NVL proteins and nucleolin was determined by fluorescence microscopy after double staining with anti-NVL and anti-nucleolin antibodies. (C) Immunofluorescence analysis of the localization of NVL isoforms. HeLa cells were transiently transfected with FLAG-tagged NVL1 and NVL2. After 20 h, the cells were fixed and double-stained with anti-FLAG and anti-nucleolin antibodies. Nuclei were visualized with DAPI (DNA). Bars, 20 μm.

mants were plated on SD-Trp/-Leu/-His medium containing 25 mM 3-aminotriazole, and colonies that grew on this medium were assayed for β-galactosidase activity. Prey plasmids in the positive colonies were isolated and sequenced.

Expression of Recombinant Proteins and Antibody Production

To express NVL1 as an N-terminal thioredoxin-hexahistidine fusion protein (Trx-His_6-NVL1), expression plasmid pTrx-NVL1 was transformed into *E. coli* strain BL21-CodonPlus(DE3)-RP (Stratagene, La Jolla, CA). The expressed recombinant protein was purified from a soluble bacterial lysate using Ni-nitrilotriacetic acid-agarose (Qiagen, Chatsworth, CA). The isolated thioredoxin-His_6-NVL1 was used to produce polyclonal antiserum in rabbits. Antibodies reactive to thioredoxin-His_6 in the antiserum were removed by passage through a column of thioredoxin-His_6 immobilized on Sepharose beads, and then the anti-NVL antibody was affinity-purified using thioredoxin-His_6-NVL1-immobilized beads. L5 protein was expressed as an N-terminal GST fusion protein in *E. coli*. The recombinant protein was purified with glutathione-Sepharose 4B beads (Amersham Biosciences) and then used to produce polyclonal antiserum in rabbits. Antibodies reactive to GST in the antiserum were removed by passage through a column of GST-immobilized beads, and then the anti-L5 antibody was affinity-purified with beads bearing a GST fusion protein of L5.

Cell Culture, Transfection, and Immunofluorescence

HeLa cells were cultured in α-minimum essential medium supplemented with 10% fetal bovine serum. 293T cells were cultured in DMEM containing 10% fetal bovine serum. Transfection was performed with LipofectAMINE PLUS reagent (Invitrogen, Carlsbad, CA) according to the supplier's instructions. Immunofluorescence staining of HeLa cells was performed as described previously (Tagaya *et al.*, 1996). Fluorescence images were obtained with an Olympus Fluoview 300 laser-scanning confocal microscope (Tokyo, Japan).

Protein Interaction Assays

The interaction between NVL2 and L5 was studied by coimmunoprecipitation analysis of extracts prepared from 293T cells transiently transfected with pFLAG-NVL2. At 24 h posttransfection, cells were lysed on ice for 20 min in lysis buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM EDTA, 1% Nonidet-P40, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM dithiothreitol, 15 mM $MgCl_2$, 1 mM ATP, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 1% Trasylol [aprotinin solution; Bayer, Leverkusen, Germany] and 1 mM phenylmethyl sulfonylfluoride). After brief sonication, the lysate was centrifuged at 14,000 rpm for 20 min at 4°C, and the supernatant was incubated with an anti-FLAG antibody (Sigma-Aldrich) for 1 h at 4°C and then with protein G-Sepharose Fast Flow (Amersham Biosciences) for 2 h at 4°C with gentle rotation. After incubation, the beads were collected by centrifugation, washed four times with lysis buffer, and then boiled for 5 min in SDS-PAGE sample buffer. Proteins in the immunoprecipitate were analyzed by SDS-PAGE and Western blotting with enhanced chemiluminescence reagent (Amersham Biosciences). For GST pull-down assay, a lysate of cells transiently expressing GST-L5 was incubated with glutathione-Sepharose 4B beads for 2 h at 4°C and then processed as for coimmunoprecipitation analysis.

Ribosomal Profiles on Sucrose Gradient Fractionation

Fractionation of cytoplasmic extracts was performed as described previously with slight modifications (Strezoska *et al.*, 2000). To fractionate ribosomes of 293T cells, a cytoplasmic extract containing proteins and RNAs was loaded onto a linear sucrose gradient (10–40%) in 10 mM Tris-HCl (pH 7.2), 60 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mg/ml heparin and then centrifuged for 3 h at 36,000 rpm in a Beckman SW-40 rotor (Fullerton, CA). After centrifugation, the sucrose gradient was fractionated and the absorbance at 254 nm was determined.

RESULTS

Different Subnuclear Localization of NVL1 and NVL2

A previous article has reported that two isoforms of the NVL protein, NVL1 and NVL2, with different N-terminal extensions are present in the nucleus. To determine the localization of the NVL proteins more precisely, a polyclonal antibody was raised against the entire NVL1 and affinity-purified. On Western blotting of an extract of cultured cells, the affinity-purified antibody recognized two bands, a major 95-kDa band and a minor 83-kDa one (Figure 2A). Densitometoric analysis revealed that the ratio of the former species to the latter one was ~20:1. Judging from their molecular masses, the major and minor species most likely correspond to NVL2 and NVL1, respectively (Germain-Lee *et al.*, 1997).

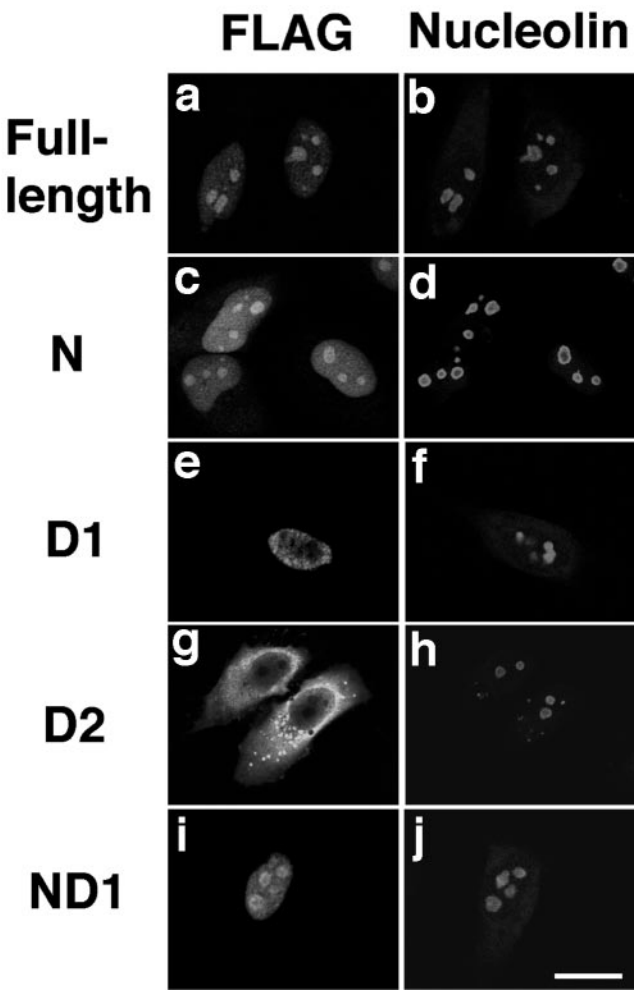

Figure 3. Subcellular localization of each domain of NVL2. HeLa cells were transiently transfected with full-length FLAG-tagged NVL2 or a truncation mutant representing the N- (residues 1–220), D1- (residues 221–553), D2- (residues 554–856), or ND1- (N- and D1-) domain. After 20 h, the cells were fixed and double-stained with anti-FLAG and anti-nucleolin antibodies. Bars, 20 μm.

Immunofluorescence analysis with the purified antibody showed that endogenous NVL is mainly localized in subnuclear foci, with some in the nucleoplasm, in HeLa cells (Figure 2Ba). The subnuclear foci were labeled with an anti-nucleolin antibody (Figure 2Bb), indicating that they are nucleoli. Like other nucleolar proteins such as nucleolin, NVL exhibited a uniformly dispersed distribution throughout the nucleus upon inhibition of RNA polymerase I with actinomycin D (ActD; Figure 2B, d–f). We assumed that this immunoreactivity distribution reflects the localization of NVL2 because of its abundance compared with NVL1.

To examine the localization of each isoform of NVL, the two proteins were individually tagged with a FLAG epitope at their carboxy terminus and then transiently expressed in HeLa cells. As expected, NVL2 was localized in the nucleolus and nucleoplasm (Figure 2Ce). In contrast, NVL1 was localized in the nucleoplasm and not in the nucleolus (Figure 2Ca). These results suggest that NVL1 and NVL2 are

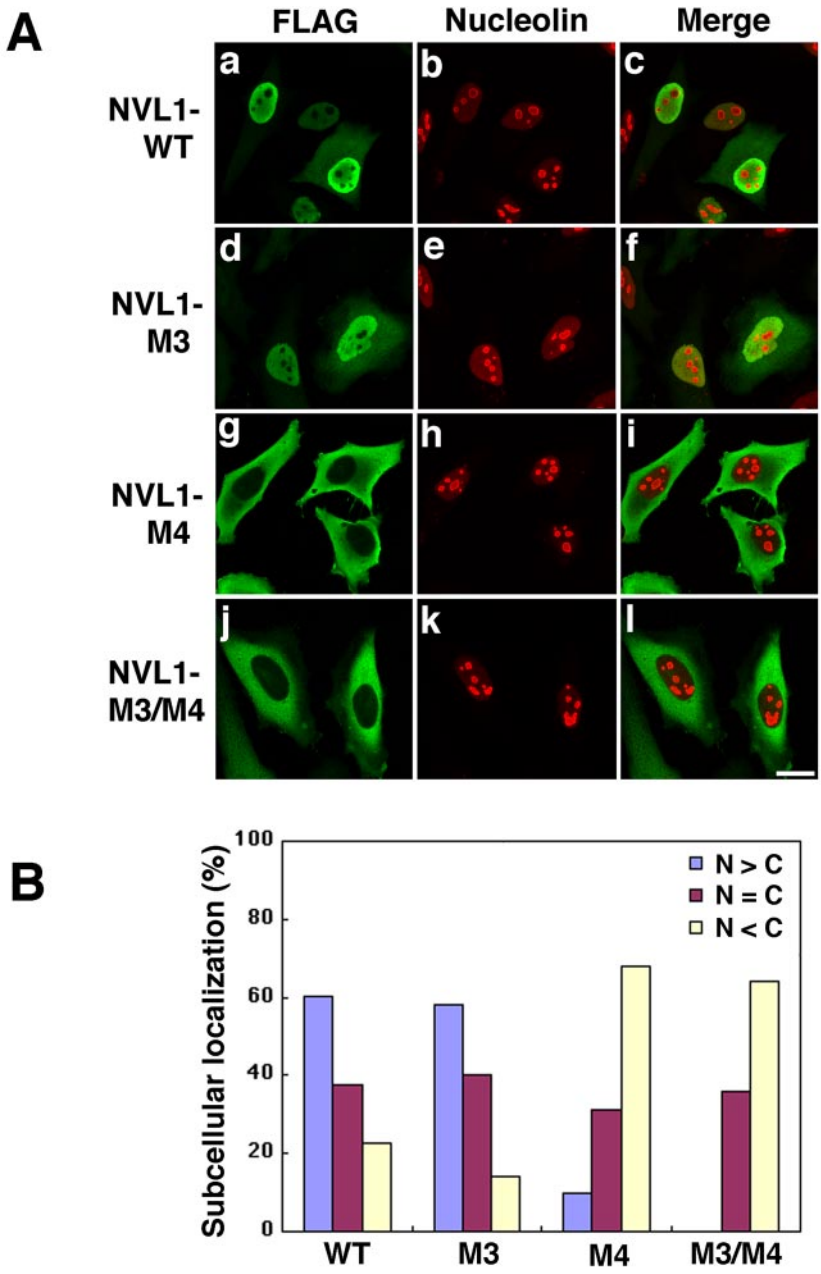

Figure 4. Identification of a nuclear localization signal in NVL1. (A) HeLa cells were transiently transfected with the FLAG-tagged wild-type (WT) NVL1, NVL1-M3, NVL1-M4, and NVL1-M3/M4. After 20 h, the cells were fixed and double-stained with anti-FLAG and anti-nucleolin antibodies. Bars, 20 μm. (B) Quantitative analysis of the results in A. The cellular localization of the indicated constructs was scored as follows: cells showing more intense nuclear than cytoplasmic staining (N > C), cells showing a nearly equal distribution between the cytoplasm and nucleus (N = C), and cells showing predominantly cytoplasmic staining (N < C). At least 100 cells were scored for each construct.

differently distributed in the nucleus and that predominantly expressed NVL2 is mainly localized in the nucleolus.

Identification of a Nuclear Localization Signal in NVL1

Like other Type II AAA proteins, NVL comprises two AAA domains (D1 and D2) with an N-terminal domain (N-domain), which differs in length between NVL1 and NVL2 (Figure 1). To gain insights into the mechanism underlying the nuclear/nucleolar localization of NVL proteins, we first examined the localization of each domain of NVL2 by using immunofluorescence microscopy. Full-length FLAG-tagged NVL2 or a truncation mutant representing the N- (residues 1–220), D1- (residues 221–553), D2- (residues 554–856), or ND1- (N- and D1-) domain was expressed in HeLa cells and then double-stained with anti-FLAG and anti-nucleolin antibodies (Figure 3). The N- and ND1-domains retained the ability to be localized in the nucleolus and nucleoplasm, whereas the D2-domain showed a diffuse distribution throughout the cells. The D1-domain was localized in the nucleus, but it was excluded from the nucleolus. These results suggest that the N-domain and D1-domain of NVL2 individually contain information for targeting of the protein to the nucleus and that information for nucleolar targeting resides only in the N-domain of NVL2.

The import of proteins into the nucleus is directed by relatively short basic sequences called nuclear localization signals (NLSs; Dingwall and Laskey, 1991; Hicks and Raikhel, 1995). On looking at the amino acid sequences of the N- and D1-domains of NVL proteins, we found clusters of basic amino acids, residues 218–220 (KRK) and residues 229–232 (KRKK), at the boundary between the two domains, which might function as NLSs (Figure 1). We also found clusters of basic amino acids, residues 49–52 (RRKR) and residues 85–88 (KRAR), in the N-terminal extended part of

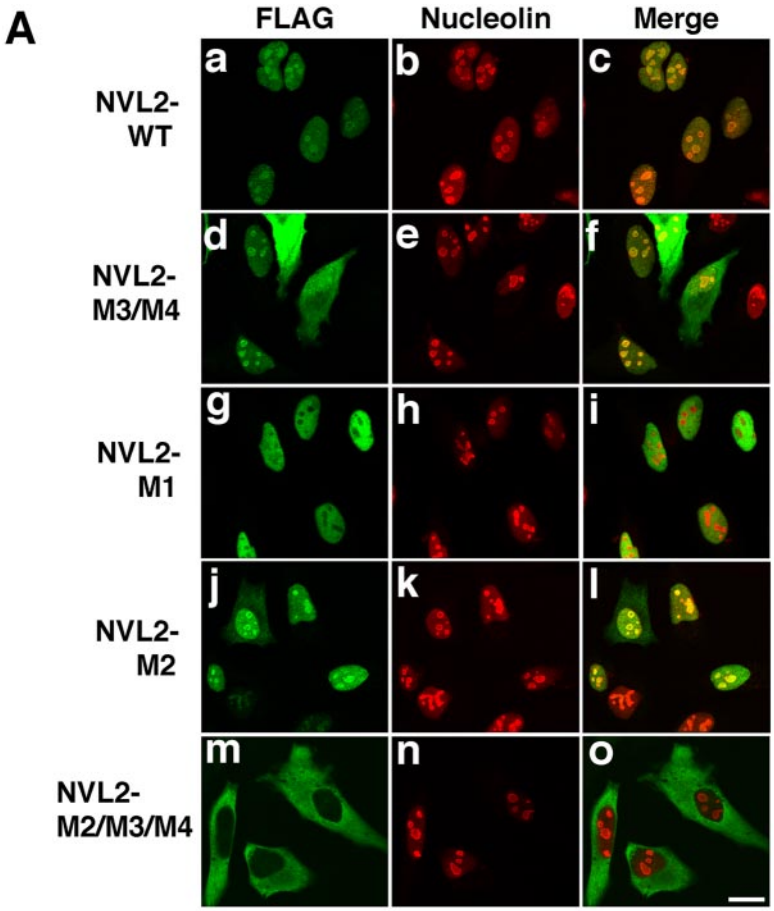

Figure 5. Identification of nuclear and nucleolar localization signals in NVL2. (A) HeLa cells were transiently transfected with the FLAG-tagged wild-type (WT) NVL2, NVL2-M3/M4, NVL2-M1, NVL2-M2, and NVL2-M2/M3/M4. After 20 h, the cells were fixed and double-stained with anti-FLAG and anti-nucleolin antibodies. Bars, 20 μm. (B) Quantitative analysis of the results in A. At least 100 cells were scored for each construct.

NVL2. To identify residues that are required for the nuclear localization of NVL1, we constructed mutant forms of NVL1 (M3, M4, and M3/M4), in which residues 218–220 (KRK) and 230–232 (RKK), and all of them were replaced by alanines, respectively. FLAG-tagged forms of these mutants were transiently expressed in HeLa cells and then their subcellular localization was examined by immunofluorescence with an anti-FLAG antibody (Figure 4). NVL1-M3 was present in the nucleoplasm, and there was no marked difference in subcellular distribution between NVL1-M3 and wild-type (WT) NVL1. For NVL1-M4, the percentage of cells showing more intense nuclear than cytoplasmic staining was significantly decreased. For NVL1-M3/M4, clear nuclear localization of NVL1 was not observed. These results suggest that residues 230–232 (RKK) in the D1-domain function as a basic type NLS, although residues 218–220 (KRK) may subtly contribute to the nuclear localization of NVL1.

Identification of Nuclear and Nucleolar Localization Signals in NVL2

Next, to determine the contributions of residues 218–220 and 230–232 to the nuclear localization of NVL2, we constructed a mutant form of NVL2, NVL2-M3/M4, in which residues 218–220 (KRK) and 230–232 (RKK) were all replaced by alanines. Immunofluorescence analysis showed that NVL2-M3/M4 could still be localized in the nucleus, although the percentage of cells showing more intense nuclear than cytoplasmic staining was decreased to some extent (Figure 5). These results suggest that these basic residues contribute to the nuclear localization of NVL2, but other basic resides in the N-terminally extended part of NVL2 might act as an additional NLS. To address this possibility, we further constructed mutant forms of NVL2, NVL2-M1 and NVL2-M2, in which residues 51–52 (KR) and

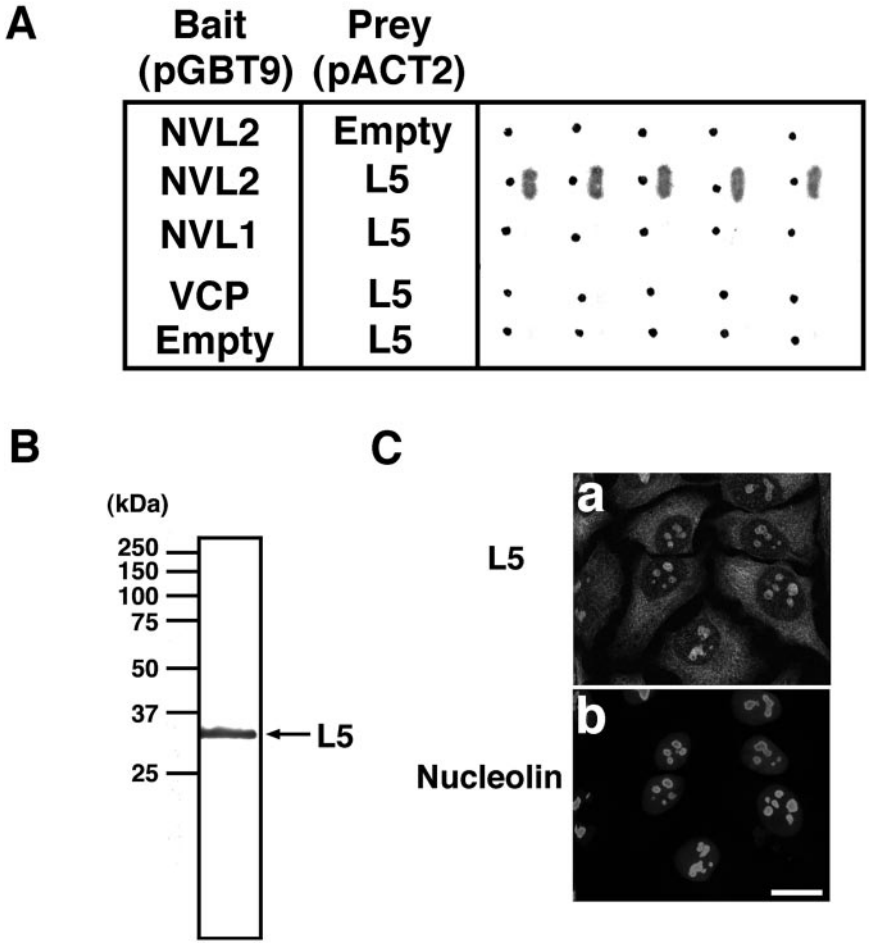

Figure 6. L5 is an interaction partner of NVL2. (A) For yeast two-hybrid analysis, *S. cerevisiae* Y190 cells were transformed with bait vector pGBT9 containing NVL2, NVL1, or VCP/p97, or pGBT9 alone as a control (Empty), and prey vector pACT2 containing L5 (residues 81–297) or pACT2 alone as a control (Empty). The interaction was examined by monitoring β-galactosidase activity on a filter. Five independent transformants were examined for each pair of constructs. (B) A 293T cell lysate was analyzed by Western blotting using an anti-L5 antibody. The molecular size markers are indicated on the left. (C) The localization of endogenous L5 and nucleolin was determined by fluorescence microscopy after double-staining with anti-L5 and anti-nucleolin antibodies. Bars, 20 μm.

85–86 (KR) were replaced by alanines, respectively, and then their subcellular localization was examined by immunofluorescence analysis (Figure 5). For NVL2-M1, the efficiency of nuclear localization was not affected, but it was excluded from the nucleoli. This suggests that basic residues 49–52 act as a nucleolar localization signal (NoLS) rather than as an NLS. For NVL2-M2, the percentage of cells showing more intense nuclear than cytoplasmic staining was decreased, as observed for NVL2-M3/M4. Combined mutation of residues 85–86 (KR), 218–220 (KRK), and 230–232 (RKK) of NVL2 resulted in complete prevention of the nuclear localization of the resultant mutant, NVL2-M2/M3/M4. These data suggest that basic residues 85–88 in the N-terminal portion of NVL2 function as an NLS together with another NLS at the boundary between the N-domain and D1-domain and that NVL2 transported to the nucleus through the action of these NLSs is further targeted to the nucleolus by using an NoLS distinct from the NLSs.

Ribosomal Protein L5 Is an Interaction Partner of NVL2

To facilitate understanding the role of NVL2 in the nucleolus, we sought nucleolar proteins that specifically interact with NVL2 by using the yeast two-hybrid system. We screened a human kidney cDNA library with full-length NVL2 as bait. Sixty-three positive clones were initially selected based on their survival on selective medium (-Trp, -Leu, -His) and β-galactosidase activity. Among them, 14 clones were found to encode ribosomal protein L5, a component of the 60S ribosomal large subunit. To determine the specificity of the interaction, NVL2, NVL1, and VCP/p97, the AAA protein that is most closely related to NVL, were examined as to their ability to cause reporter activation in cooperation with L5. Besides the interaction of L5 with NVL2, no interaction was observed with NVL1 or VCP/p97, demonstrating the specificity of the interaction (Figure 6A).

We next raised a polyclonal antibody against the entire L5 to detect endogenous L5 in cells. The affinity-purified antibody specifically recognized a single band on Western blotting of a cell lysate (Figure 6B). We then determined the distribution of endogenous L5 in HeLa cells by immunofluorescence microscopy. As previously shown for ectopically expressed epitope-tagged L5 (Michael and Dreyfuss, 1996; Claussen *et al.*, 1999; Rosorius *et al.*, 2000), endogenous L5 was mainly present in the cytoplasm and nucleoli (Figure 6C).

The interaction between NVL2 and L5 observed in the yeast two-hybrid assay was further substantiated by immunoprecipitation experiments using mammalian cells. For this, FLAG-tagged NVL2 or NVL1 was expressed in 293T cells and then cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody. Because the interaction of AAA proteins with their binding partners is generally sensitive to the nucleotide bound state, the interaction was examined in the presence and absence of ATP. The results showed that endogenous L5 was specifically coimmunoprecipitated with NVL2, and not with

NVL1 (Figure 7A). In the presence of ATP, the interaction was greatly enhanced. As a reverse experiment, L5 was expressed in cells as a GST fusion protein (GST-L5) and then pull-down assay was performed. Endogenous NVL2 was pulled down from the cell lysate with GST-L5, whereas a control pull-down assay with GST failed to precipitate endogenous NVL2 (Figure 7B).

NoLS of NVL2 Is Required for the Interaction with L5

To investigate the implication of the NVL2/L5 interaction, we first mapped the L5-binding domain of NVL2 by using the yeast two-hybrid assay. Yeast strain Y190 was transformed with pGBT9 containing the full-length NVL2, or its ND1-, N-, D1-, or D2-domain together with pACT2-L5. The interaction was examined by monitoring β-galactosidase activity on a filter. The results showed the interaction of L5 with the N- and ND1-domains as well as with the full-length NVL2, although the D2-domain or D1-domain alone did not show any interaction (Figure 8A). The interaction with the N-domain was somewhat weaker than that with the full-length NVL2 or the ND1-domain. These results indicate that L5 specifically interacts with the N-domain of NVL2, and that the D1-domain may somewhat contribute to the interaction.

It is thought that the nucleolar localization of proteins is mediated by a variety of molecular interactions involving NoLS (Scheer and Hock, 1999; Carmo-Fonseca *et al.*, 2000). The specific interaction of L5 with the N-terminal region of NVL2, where the NoLS resides, raised the possibility that L5 may be an NoLS-interacting protein. To test this possibility, we examined whether or not the NoLS-disrupted form of NVL2, NVL2-M1, can associate with L5. The wild-type or mutant form of NVL2 was expressed as a FLAG-tagged protein in 293T cells, and then immunoprecipitation with an anti-FLAG antibody was performed (Figure 8B). Although endogenous L5 was coprecipitated with the wild-type NVL2, it was not precipitated with NVL2-M1 (lanes 8 and 9). On the contrary, the NLS-disrupted form of NVL2, NVL2-M2/M3/M4, interacted with L5 as efficiently as the wild-type NVL2 (lane 10). These results indicate that residues 51 and 52 of NVL2 are important not only for the nucleolar localization but also for the interaction with L5.

L5 Determines the Subnuclear Localization of NVL2

The results so far obtained suggest that the nucleolar localization of NVL2 is established through interaction with L5. Therefore, we investigated if the two proteins affect each other's localization. For this purpose, we first examined the distribution of endogenous NVL2 in cells overexpressing a L5-GFP fusion protein. Similar to endogenous L5, L5-GFP was mainly localized in the nucleolus, with some in the nucleoplasm and cytoplasm. The expressed L5-GFP did not affect the localization of endogenous NVL (Figure 9A, a–c). We next expressed residues 81–297 of L5, which correspond to the sequence encoded by the longest clone obtained through two-hybrid screening, as a GFP-fusion protein. Interestingly, this construct, L5(81–297)-GFP, was localized to small punctate structures in the nucleoplasm. In these cells, endogenous NVL was redistributed from the nucleolus to the small punctate structures, where it was colocalized with L5(81–297)-GFP (Figure 9A, d–f), although the localization of nucleolin was not affected (Figure 9A, g–i). On the other hand, overexpression of the wild-type or NLS-disrupted form (M2/M3/M4) of NVL2 did not have an obvious effect on the localization of endogenous L5 (Figure 9B). These results suggest that L5 is able to coordinate the localization of NVL2, but not vice versa.

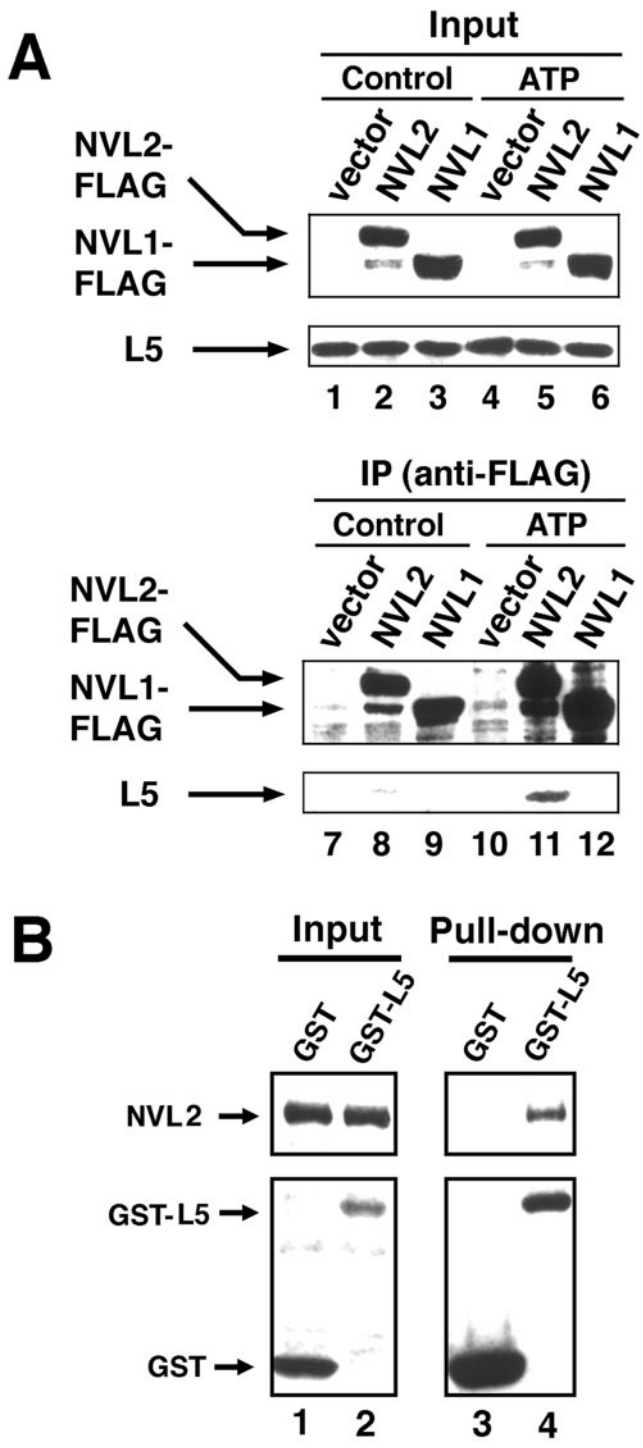

Figure 7. NVL2 interacts with L5 in an ATP-dependent manner. (A) 293T cells were transfected with FLAG-tagged forms of NVL1 or NVL2. After 24 h, cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody in the presence or absence (Control) of ATP. The immunoprecipitates (IP) and 2% of the starting material (Input) were resolved by SDS-PAGE and then analyzed by Western blotting. (B) A cell lysate of 293T cells expressing GST-L5 or GST as a control was subjected to GST pull-down assay with glutathione beads. ATP was included during the incubation. The proteins bound to glutathione beads (Pull-down) and 2% of the starting material (Input) were resolved by SDS-PAGE and then analyzed by Western blotting.

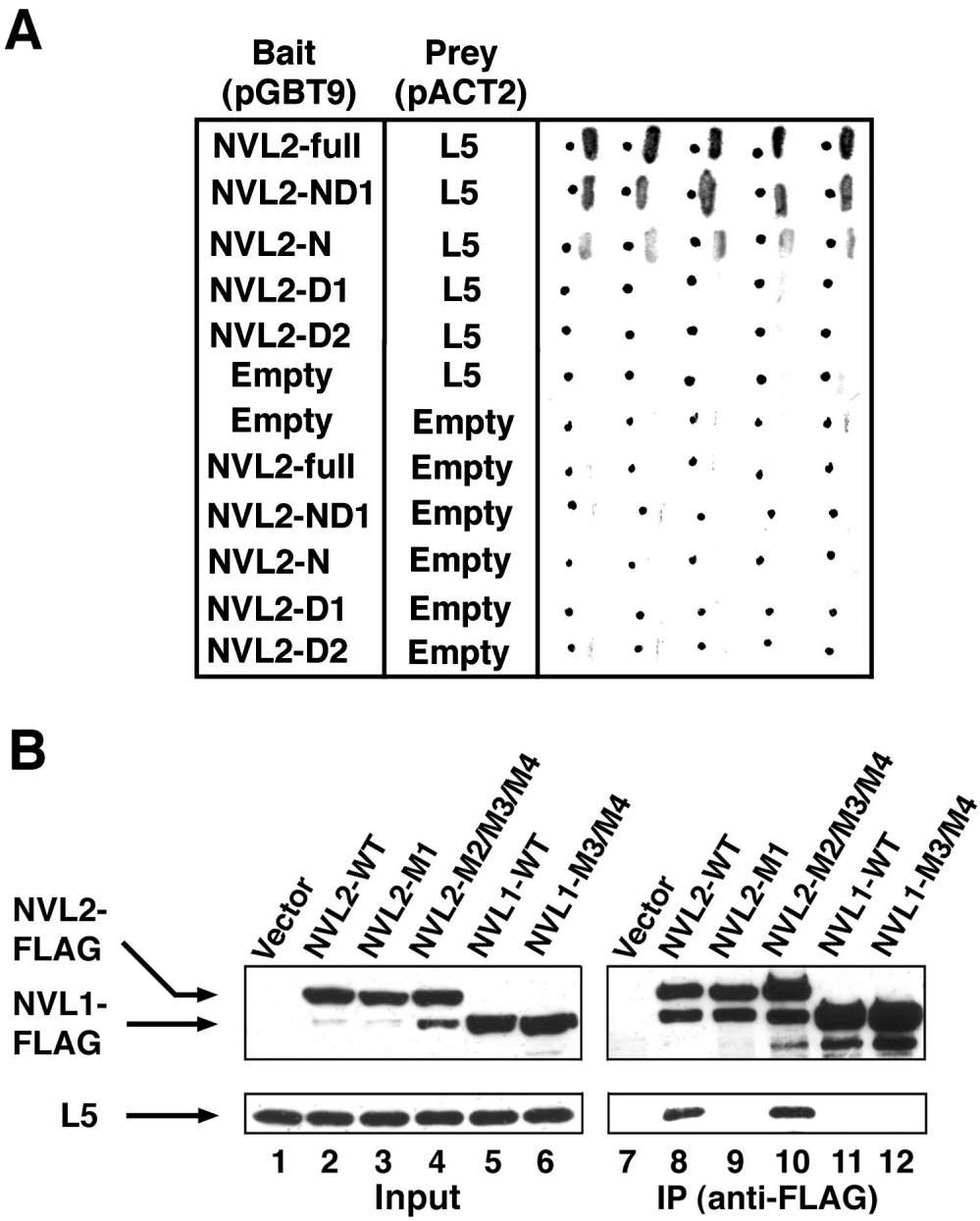

Figure 8. The NoLS of NVL2 is required for its interaction with L5. (A) For yeast two-hybrid analysis, *S. cerevisiae* Y190 cells were transformed with bait vector pGBT9 containing the indicated NVL2 domain, or pGBT9 alone as a control (Empty), and prey vector pACT2 containing L5 (residues 81–297) or pACT2 alone as a control (Empty). The interaction was examined by monitoring β-galactosidase activity on a filter. Five independent transformants were examined for each pair of constructs. full, full-length. (B) 293T cells were transfected with the indicated FLAG-tagged mutant of NVL1 or NVL2. After 24 h, cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody. ATP was included during the incubation. The immunoprecipitates (IP) and 2% of the starting material (Input) were resolved by SDS-PAGE and then analyzed by Western blotting.

NVL2 Is Involved in 60S Ribosomal Subunit Biogenesis

Given the interaction between NVL2 and L5, and their colocalization in the nucleolus, we reasoned that NVL2 would be involved in ribosome biogenesis. To examine this possibility, we used the dominant negative mutant strategy. A conserved lysine residue in the D2 domain (Lys-628), which is supposed to be important for ATP binding in analogy with the case of VCP/p97 (Ye *et al.*, 2003), was substituted with a methionine residue to generate the NVL2(K628M) mutant. This mutant was tagged with a C-terminal FLAG epitope and then expressed in 293T cells. The mutant was expressed with similar efficiency to that of the wild-type NVL2 and was shown to interact with endogenous L5 on coimmunoprecipitation analysis (Figure 10A).

To examine the effects of this mutant on ribosome biogenesis, the mutant construct as well as the wild-type NVL2 was expressed in 293T cells, and then the ribosome profiles were examined by sucrose gradient centrifugation (Figure 10B). The ribosome profile in an extract prepared from cells expressing the wild-type NVL2 was similar to that in one prepared from cells transfected with the con-

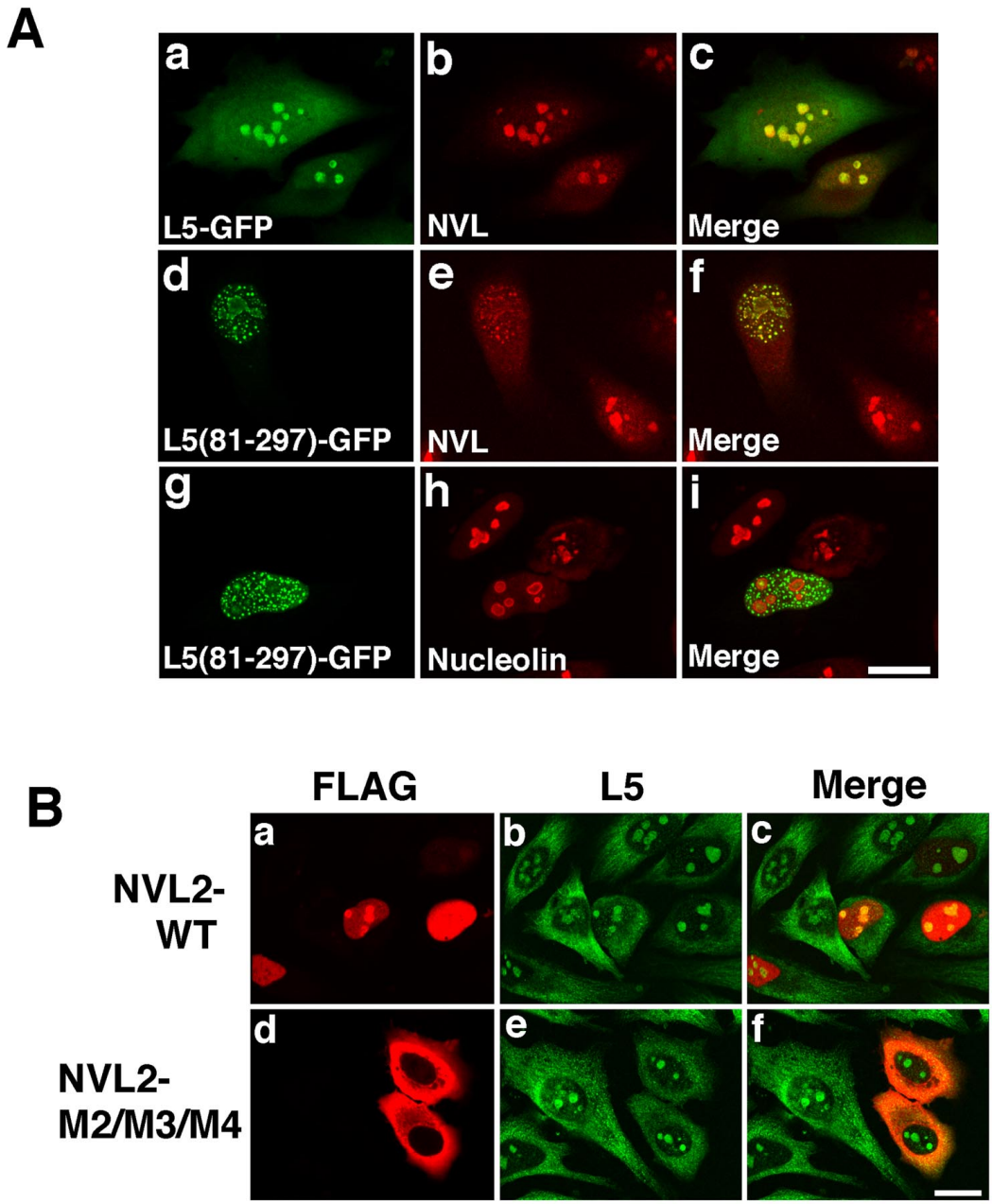

Figure 9. N-terminally truncated L5(81–297) relocalizes NVL2 from the nucleolus to nucleoplasmic punctate structures. (A) HeLa cells were transiently transfected with L5-GFP or L5(81–297)-GFP. After 20 h, the cells were fixed and stained with an anti-NVL antibody (panels b and e) or anti-nucleolin antibody (panel h). L5-GFP (panel a) or L5(81–297)-GFP (panels d and g) was visualized directly. Merged images are shown in the right panels. (B) HeLa cells were transfected with FLAG-tagged forms of the wild-type (WT) NVL2 and the NVL2-M2/M3/M4 mutant. After 20 h, the cells were fixed and double-stained with an anti-FLAG antibody and anti-L5 antibody. Merged images are shown in the right panels. Bars, 20 μm.

trol vector; both exhibited a pattern reflecting the presence of ribosomal subunits with sedimentation coefficients of 40S, 60S, and 80S. The 60S:40S ratio determined from the UV absorption was ~1.6 for both the control cells and wild-type NVL2-overexpressing cells. When the dominant negative NVL2(K628M) mutant was expressed, the 60S and 80S peaks were drastically reduced, whereas the 40S peak was not affected. The 60S:40S ratio was 0.81 for the mutant-expressing cells. The marked reduction in the amount of the 60S subunit suggests that NVL2 functions in 60S subunit synthesis.

DISCUSSION

In this study, we first showed that two NVL isoforms are differently localized in the nucleus: NVL2 is mainly confined to the nucleolus, whereas NVL1 is distributed throughout the nucleoplasm. Moreover, we identified an NoLS consisting of a stretch of basic amino acids in the N-terminal region of NVL2, which specifically associates with ribosomal protein L5. Although the N-domain of NVL2 is sufficient for the interaction with L5, the interaction was weaker than that observed with the full-length NVL2 or ND1-domain (Figure

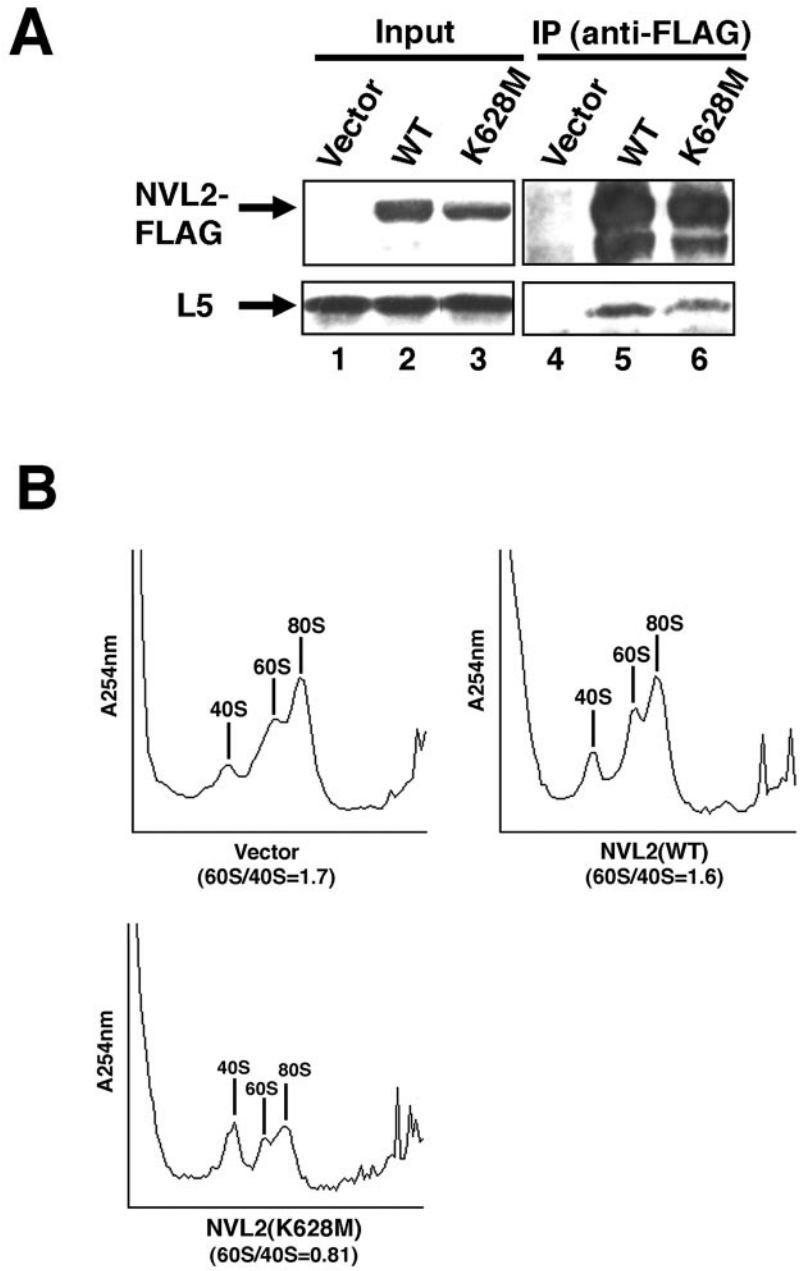

Figure 10. Overexpression of the K628M mutant of NVL2 impairs 60S subunit biogenesis. (A) 293T cells were transfected with FLAG-tagged forms of the wild-type NVL2 (WT) and the K628M mutant. After 24 h, cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody in the presence of ATP. The immunoprecipitates (IP) and 2% of the starting material (Input) were resolved by SDS-PAGE and then analyzed by Western blotting. (B) The ribosome profiles ($A_{254\ nm}$) on sucrose gradient centrifugation of extracts of cells transfected with the control vector and the expression plasmids for the FLAG-tagged wild-type NVL2 (WT) and K628M mutant are shown. The positions of the 40S, 60S, and 80S ribosomal subunits are indicated.

8A). In addition, the interaction of NVL2 with L5 is markedly enhanced in the presence of ATP (Figure 7A). These results suggest that the D1-domain, which contains an ATP-binding module, also contributes to the interaction with L5. On the contrary, ATP-binding at the D2-domain does not affect the interaction, as indicated with the K628M mutant of NVL2 (Figure 10A). This kind of regulation through ATP-binding has also been observed for other AAA proteins. For example, ATP-binding at the D1- but not the D2-domain of VCP/p97 is essential for the substrate binding in the pathway of protein retrotranslocation across the ER membrane (Ye *et al.*, 2003). These observations suggest that the interaction between NVL2 and L5 is mechanistically regulated through an ATP-binding/hydrolysis cycle, which may be associated with nucleolar functions.

Our analysis showed that the nuclear import and nucleolar accumulation of NVL2 are mediated by distinct signals, although they both consist of stretches of basic amino acids. This is consistent with the view that the nuclear import and nucleolar targeting of proteins are accomplished through distinct molecular interactions. Proteins destined for the nucleolus are first transported from the cytoplasm into the nucleus via nuclear pores in association with cytoplasmic import receptors through the function of NLSs (Mattaj and Englmeier, 1998; Gorlich and Kutay, 1999; Yoneda, 2000). The imported proteins are then accumulated in the nucleolus through interaction between their NoLSs and other nucleolar components (Scheer and Hock, 1999; Carmo-Fonseca *et al.*, 2000). Our finding that NVL2 interacts with L5 via the NoLS raises the possibility that L5 may be a nucleolar component that contributes to the recruitment of NVL2 to the nucleolus. This idea is supported by the fact that expression of N-terminally truncated L5-GFP, which is mislocalized to the nucleoplasm, leads to the redistribution of NVL2 from

the nucleolus to nucleoplasmic punctate structures positive for the expressed protein (Figure 9A).

Recently, ribosomal protein L11 was proposed to be a nucleolar component that contributes to the nucleolar targeting of other proteins such as proto-oncoprotein HDM2 and tumor-suppressor protein PML (Lohrum *et al.*, 2003; Bernardi *et al.*, 2004). There is accumulating evidence that ribosomal proteins are not only structural components of ribosomes, but also have extraribosomal functions (Wool, 1996; Mazumder *et al.*, 2003). Our findings may reveal a new function of L5 as a factor recruiting NVL2 to the nucleolus. However, we cannot completely exclude the possibility that NVL2 per se or factor(s) other than L5 mediate or support the nucleolar localization of NVL2. To verify the critical requirement of L5 for nucleolar targeting of NVL2, it is necessary to fully deplete L5 in cells. However, our efforts to deplete L5 by RNA interference were not successful. With several RNA duplexes tested, the L5 protein level was reduced by only 30~40% compared with that in control cells. This is perhaps due to the abundance and relatively high stability of L5. In cells partially depleted of L5, the nucleolar localization of NVL2 was not significantly affected.

Previous reports on analysis of NVL homologues in a fly, a nematode, and yeast suggested multiple cellular functions for this chaperone-like protein, such as cell cycle regulation, apoptosis, and ribosomal biogenesis (Long *et al.*, 1998a, 1998b; Wu *et al.*, 1999; Gadal *et al.*, 2001). We showed that expression of a dominant negative form of NVL2 markedly reduced the level of the 60S ribosomal subunit, but not that of the 40S subunit. This observation, together with other data obtained in this study, suggests that the major isoform of NVL, like yeast Rix7p (Gadal *et al.*, 2001), is involved in 60S ribosome biosynthesis. Thus, NVL2 is structurally and functionally homologous to yeast Rix7p.

Ribosome biogenesis is a process that takes place primarily in the nucleolus through a series of precise and complicated RNA-processing and protein-assembly event (Melese and Xue, 1995; Kressler *et al.*, 1999), in which molecular chaperones participate (Tschochner and Hurt, 2003). Because AAA ATPases are chaperone-like proteins that generally modulate the assembly and disassembly of macromolecular complexes (Neuwald *et al.*, 1999; Vale, 2000; Ogura and Wilkinson, 2001), it is likely that NVL2 may participate in ribosome formation by modulating various molecular interactions. Alternatively, NVL2 may specifically regulate the function of L5. It is known that L5 acts to deliver 5S rRNA to the maturing pre-60S ribosomal particle (Steitz *et al.*, 1988; Scripture and Huber, 1995). In yeast, the recruitment of 5S rRNA to the pre-60S ribosomal particle is important for the efficient processing of the 27SB rRNA precursor (Dechampesme *et al.*, 1999). Because the N-terminal region of L5, which is involved in the binding of 5S rRNA (Michael and Dreyfuss, 1996; Claussen *et al.*, 1999; Rosarius *et al.* 2000), is not required for NVL2-interaction (Figures 6A and 9A), NVL2 may bind to the L5–5S rRNA complex and coordinate the integration of 5S rRNA into the maturing pre-60S particle.

At present, the physiological significance of NVL1 remains unclear. Although it does not seem to be a dominant species, transcripts that specifically generate NVL1 are also present in the mouse EST database (e.g., GenBank accession numbers AA153910, BB660279, BY139725, and BY717981). NVL1 may be involved in cellular functions in particular tissues or cells or in specific circumstances such as limited developmental stages. Further studies are needed to elucidate the function of NVL1.

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