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Nup211, the Fission Yeast Homolog of Mlp1/Tpr, Is Involved in mRNA Export

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Synthetic lethal mutants have been previously isolated in fission yeast *Schizosaccharomyces pombe*, which genetically interact with *spmex67*, in order to identify the genes involved in mRNA export. The *nup211* gene was isolated by complementation of the growth defect in one of the synthetic lethal mutants, SLMex2, under synthetic lethal condition. We showed that Nup211, fission yeast homolog of Mlp1/Mlp2/Tpr, is essential for vegetative growth and Nup211-GFP proteins expressed at endogenous level are localized mainly in nuclear periphery. The accumulation of poly(A)$^+$ RNA in the nucleus is exhibited when expression of *nup211* is repressed or over-expressed. These results suggest that the Nup211 protein plays a pivotal role of mRNA export in fission yeast.

***Keywords*:** mRNA export, *Schizosaccharomyces pombe*, *nup211*

In eukaryote, mRNA that is transcribed and processed within nucleus should be exported to cytoplasm for translation. The nuclear export of mRNA is a complex multistep process involving a lot of mRNA export factors, which is evolutionally conserved and is linked to the other steps of mRNA metabolism (transcription, 5'-end capping, splicing, and 3'-end cleavage and polyadenylation) and surveillance for mRNA quality control (Reed and Hurt, 2002; Erkmann and Kutay, 2004; Saguez *et al*., 2005; Köhler and Hurt, 2007).

Heterodimeric Mex67p-Mtr2p in yeast *Saccharomyces cerevisiae* (TAP/NXF-p15/NXT in metazoan) is a conserved mRNA export receptor, which plays essential roles in bulk mRNA export through the nuclear pore complexes (NPCs) (Segref *et al*., 1997; Grüter *et al*., 1998). The NPC is a huge assembly embedded in the nuclear envelope and functions as the sole gates for the exchange of macromolecules between the nucleus and the cytoplasm (Tran and Wente, 2006). Translocation of the mRNA export receptor with its mRNA cargo through NPCs is mediated primarily by weak interactions between the receptor and phenylalanine-glycine (FG) repeats of nucleoporins (proteins composing NPC) lining central transport channel of NPC (Rodriguez *et al*., 2004; Cole and Scarcelli, 2006). Although the mRNA export receptor can bind directly to mRNA with low affinity, it is recruited to transcripts by adaptor proteins that are typically mRNA-binding proteins. So far, Yra1 (Aly/REF in metazoans), poly(A) RNA-binding protein Nab2, SR-like protein Npl3, and Sac3 are thought to be essential mRNA adaptors in *S. cerevisiae* (Strässer and Hurt, 2000; Fischer *et al*., 2002; Green *et al*., 2002; Gilbert and Guthrie, 2004).

In addition to targeting the receptor to mRNA, the adaptors may also contribute to efficient mRNA export through facilitating interactions with constituents of the NPC. Two *S. cerevisiae* myosin-like proteins, Mlp1p and Mlp2p, are thought to act as a docking platform for mRNA-protein (mRNP) complexes and other mRNA export adaptors (Green *et al*., 2003). Mlp1p and Mlp2p are able to directly interact with mRNA export adaptors such as Yra1p, Nab2p, and Npl3p (Green *et al*., 2003; Vinciguerra *et al*., 2005; Fasken *et al*., 2008). Both Mlp1p and Mlp2p are localized to the nucleoplasmic side of the NPC through the association with nucleoporin Nic96p (Strambio-de-Castillia *et al*., 1999; Kosova *et al*., 2000). In vertebrate, the protooncogenic protein Tpr (translocated promoter region) that is homologous to Mlp1p/Mlp2p is localized to both the nuclear basket of the NPC and within nucleus (Frosst *et al*., 2002; Krull *et al*., 2004). Consistent with their localization, Tpr/Mlp proteins are known to be involved in RNA biogenesis, mRNA export, spindle pole assembly, telomere organization, and especially nuclear retention of unspliced mRNAs (Galy *et al*., 2000, 2004; Niepel *et al*., 2005; Vinciguerra *et al*., 2005).

In *Schizosaccharomyces pombe* (*S. pombe*), *mex67* (*spmex67*) gene was isolated as a multicopy suppressor of *rae1-167 nup184-1* synthetic lethal mutant that showed the mRNA export defect under synthetic lethal condition (Yoon *et al*., 2000). Although spMex67p is not essential for cell viability and mRNA export, it is able to partially suppress a temperature sensitive mutation of *rae1* that is essential for growth and mRNA export, indicating that spMex67p is also involved in mRNA export (Brown *et al*., 1995). Moreover, *spmex67* null allele is synthetically lethal with mutant alleles of some nucleoporins that are involved in mRNA export, and with soluble mRNA export factors such as *npp106*, *nup97*, *nup184*, *dss1*, *elf1*, *mlo3*, *rae1*, and *rsm1* (Yoon *et al*., 1997, 2000; Kozak *et al*., 2002; Yoon, 2004; Thakurta *et al*., 2005).

In this study, we described the isolation of the essential *nup211* gene by complementation of the growth defect in the Δ*spmex67 slmex2* synthetic lethal mutant. Repression or

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over-expression of the essential *nup211* was shown to cause defects in both growth and mRNA export. In addition, Nup211p is localized mainly nuclear periphery. These results suggested that *nup211* is also involved in the mRNA export in *S. pombe*.

Materials and Methods

Strains and culture

The basic genetic and cell culture techniques used have been described (Moreno *et al*., 1991; Alfa *et al*., 1993). The *S. pombe* strains used were of wild type AY217: *h*$^-$ *leu1-32 ura4-d18* (Yoon *et al*., 1997); SP286: *h*$^+$/ *h*$^+$ *leu1-32/leu1-32 ura4-d18/ura4-d18 ade6-210/ ade6-216* (Matsumoto and Beach, 1991); SLMex2: *h*$^-$ *leu1-32 ura4-d18 slmex2* Δ*spmex67::kan*^{r} /pREP81X-*spmex67*$^+$ (Yoon, 2003); ΔNup211: *h*$^-$ *leu1-32 ura4-d18* Δ*nup211:: ura4*$^+$/ pREP81X-Nup211 (this study). Yeast extract plus supplement (YES) medium was used for general propagation of *S. pombe* cells and appropriately supplemented EMM medium was used to express genes from the pREP series plasmids containing the *nmt* promoter (Basi *et al*., 1993; Maundrell, 1993). The *nmt1* promoter was repressed by the addition of 15 μM thiamine in EMM medium (Forsburg, 1993). *Escherichia coli* TOP10 strain was used for plasmid propagation and selection.

Isolation of the *nup211* gene that complement the growth defect of SLMex2

SLMex2 cells were transformed with a partial *Sau*3A genomic library that was cloned into the *Sal*I site of pUR18 (Barbet, 1992). Transformants that could grow in the presence of thiamine at 28°C were isolated. Plasmids were rescued and retransformed into SLMex2 for confirmation. A plasmid that complemented synthetic lethality of SLMex2 was sequenced at both ends of insert and the DNA sequence was used to search the *S. pombe* genome database (http://www.sanger. ac.uk/Projects/S_pombe/, Sanger Center, UK). An 8 kb DNA fragment containing the *nup211* gene was subcloned into pDW232 and the resulting plasmid was transformed into SLMex2 to confirm the complementation of the growth defect of SLMex2 in the presence of thiamine.

Construction of plasmids

For construction of pNup211, a 7.3 kb genomic DNA fragment containing the *nup211* gene was inserted into the plasmid pDW232 at *Sph*I site. The cloning of entire ORF of *nup211* into pREP series vectors, pREP3X, pREP41X, and pREP81X (Maundrell, 1993), was conducted by first creating *Sal*I and *Sma*I sites immediately upstream of the initiation codon and downstream of the stop codon of *nup211*, respectively, by PCR using the genomic clone as a temperate. The *Sal*I-*Sma*I digested PCR product was then

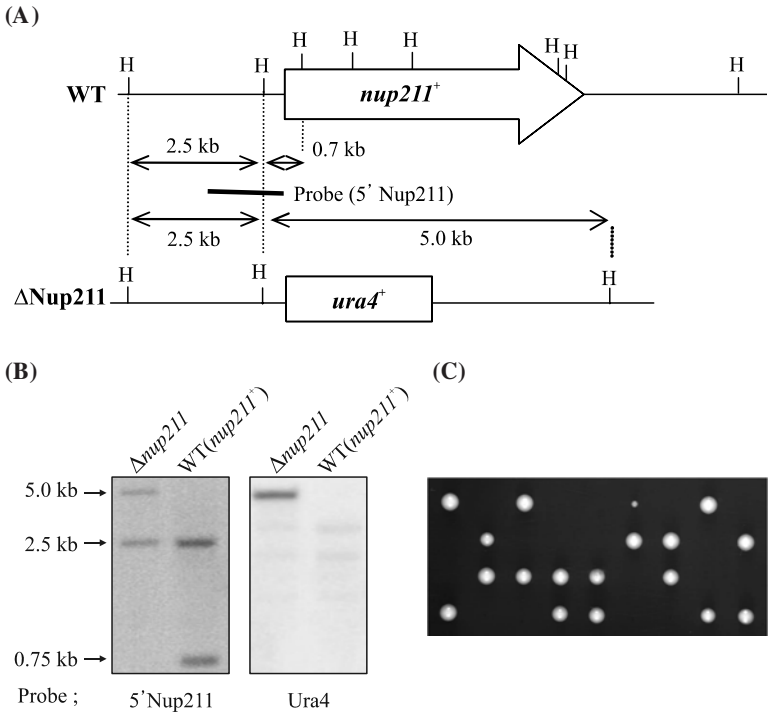

Fig. 1. The *nup211* gene is essential for vegetative growth. (A) A schematic diagram representing the construct of the *nup211* null allele in *S. pombe*. Most of the *nup211* ORF region was replaced by the marker gene, *ura4*$^+$. The expected sizes of Southern blotting using *Hind*III (H)-digested chromosomal DNA are also denoted. (B) Confirmation of the deletion of the *nup211* locus in haploid cells by Southern blotting. Genomic DNAs isolated from wild type (WT) and Δ*nup211* haploid with 81X-Nup211 plasmid (Δ*nup211*) strains were digested with *Hind*III. A 1 kb fragment (5' Nup211) used as probe is denoted in (A). (C) Tetrad analysis. The *h*$^+$/ *h*^{90} heterozygous diploids cells (*nup211*$^+$/Δ*nup211*) were sporulated on ME plates. Ten tetrads were dissected on YES plates and were incubated for 4 days at 28°C.

inserted into pREP series vectors cut with *Xho*I and *Sma*I. The Nup211p-GFP fusion in pZA69U and pREP41/EGFP C vectors (Craven *et al*., 1998) were constructed by insertion of *Sal*I-*Sma*I fragment bearing full-length *nup211* ORF into the vectors cut with *Sal*I and *Sma*I.

Construction of *nup211* null strain and genomic integrated nup211-gfp strain

The Δ*nup211::ura4*$^+$ null mutation was constructed by double-joint PCR (Yu *et al*., 2004). Total *nup211* ORF was replaced by maker gene, *ura4*$^+$. After third round PCR, the amplified Δ*nup211::ura4*$^+$ fragment was purified and transformed into the SP286 diploid strain. Ura$^+$ transformants were selected by PCR and Southern blotting for the disruption of one of the *nup211* alleles. The selected heterozygous diploid cells were sporulated, and ten tetrads were dissected (Fig. 1).

Because the *nup211* gene is essential for vegetative growth, we constructed a Δ*nup211::ura4*$^+$ haploid strain harboring 81X-Nup211 plasmid, in which *nup211* expression is under the control of the weak *nmt1* promoter. A haploid strain AY217 with 81X-Nup211 plasmid was transformed with the amplified Δ*nup211::ura4*$^+$ fragment. Ura$^+$ Leu$^+$ transformants were then selected and confirmed the deletion of genomic *nup211* gene by PCR and Southern blotting (Fig. 1).

For chromosomal integration of Nup211p-GFP, the *Pst*I-*Sac*I fragment containing C-terminal *nup211*-GFP was inserted into a derivative of pDW232 lacking the ARS sequence (pDW234). The resulting plasmid was linearized with *Bsp*MI and integrated into chromosomal *nup211* locus by transformation into AY217 strain. Ura$^+$ transformants were screened for proper insertion of this fusion by PCR and Southern blotting.

In situ hybridization

In situ hybridization was performed as previously described (Yoon, 2000). Oligo-$(dT)_{50}$ carrying an α-digoxygenin at the 3' end was used as the hybridization probe. FITC-anti-digoxygenin Fab antibody (Roche Applied Science, Germany) was used for detecting the hybridization probe by fluorescence microscopy. 4', 6-Diamidino-2'-phenyindole (DAPI) was used for observing DNA.

Results and Discussion

Complementation of growth defect of SLMex2 by Nup211

We have previously isolated three synthetic lethal mutants, SLMex1, SLMex2, and SLMex3, using *mex67* knockout allele in *S. pombe* (Yoon, 2003). These synthetic lethal mutants carry the *mex67* knockout allele and a synthetic lethal mutation in another gene, respectively, as well as a plasmid (pREP81X-Mex67) that expresses *mex67*$^+$ from the weak

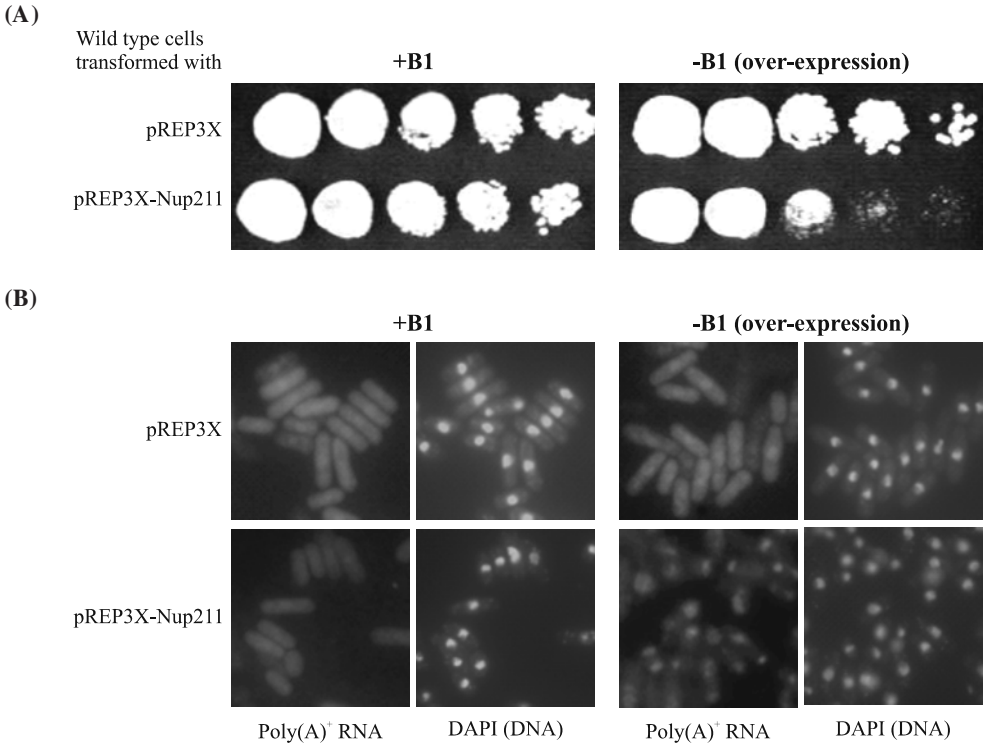

Fig. 2. (A) Over-expression of *nup211* results in growth retardation. Haploid wild-type (AY217) cells transformed with pREP3X plasmid or pREP3X-Nup211 were grown to saturation, serially diluted in 10-fold dilutions, and spotted onto EMM agar in the absence (-B1) and the presence (+B1) of thiamine. Cells were incubated for 4 days at 28°C. (B) Over-expression of *nup211* caused the defect in mRNA export. Cells were grown to the mid-log phase in appropriately supplemented EMM medium in the presence of thiamine (+B1) at 28°C. The cells were then washed and shifted to EMM medium without (-B1) or with thiamine (+B1), and were grown for 18 h. Coincident DAPI staining is shown in the right panels.

thiamine-repressible *nmt1* promoter (Basi *et al*., 1993). Under permissive conditions (in the absence of thiamine) when *mex67*$^+$ was expressed from the plasmid, these mutant cells grew normally. However, under synthetic lethal conditions when *mex67*$^+$ expression was repressed by the addition of thiamine, these mutant cells showed growth defects that were accompanied by bulk poly(A)$^+$ RNA accumulation in the nucleus (Yoon, 2003). The corresponding synthetic lethal genes for SLMex1 and SLMex3 were identified as *rsm1* and *nup97*, respectively (Yoon, 2004; Cho *et al*., 2007).

In this study, we isolated a genomic clone that complement the growth defect of SLMex2 cells under synthetic lethal condition, as mentioned in 'Materials and Methods'. Both ends of insert DNA of this clone were sequenced and the obtained DNA sequence was used to search the *S. pombe* genome database (Sanger Center, UK). The *S. pombe* genome database and restriction enzyme digestion patterns of the clone revealed that this genomic clone contained one full-length open reading frame (ORF) and two truncated ORFs found in the cosmid c162 (chromosome III). The full-length ORF (SPCC162.08c) was subcloned into pDW232 vector and the resulting plasmid could restore the growth defect of SLMex2.

The SPCC162.08c gene contains no intron and encodes an 1837 amino-acid protein, Nup211p, with predicted molecular weight of 211.4 kDa. The Nup211 protein had significant homology with NPC-associated protein Mlp1/Mlp2 in *S. cerevisiae* and Tpr in mammal. Alignment of amino-acid sequences in these proteins revealed that structural and sequence similarities are not restricted in certain region but extend throughout the entire sequences. All of these proteins contain a long coiled-coil N-terminal domain that anchors them to nuclear basket of NPC, and a globular C-terminal domain that harbors NLS and binds mRNA export factors (Green *et al*., 2003; Hase and Cordes, 2003; Vinciguerra *et al*., 2005; Fasken *et al*., 2008).

The *nup211* gene is essential for growth

In order to determine the phenotype of the *nup211* knockout, a deletion mutant in a stable h^+/h^+ diploid strain was generated by replacing the *nup211*-coding region with an *ura4*$^+$ gene using a one-step gene disruption method (Fig. 1A). The stable Ura$^+$ transformants were screened by PCR for the selection of diploid cells, in which one of two *nup211* loci was substituted with *ura4*$^+$. The heterozygous diploids heterozygous were allowed to sporulate en tetrads were dissected and gave 2:2 segregation patterns for viability (Fig. 1C). The two growing haploid progeny in tetrads were all uracil auxotroph, indicating that they carry wild-type *nup211*$^+$ allele (data not shown). Microscopic inspection of haploid cells bearing Δ*nup211::ura4*$^+$ allele revealed that the spores did not germinate, or germinated but stopped growth at small abnormal cells as mentioned before (Chen *et al*., 2004). This result confirmed that the *nup211* knockout

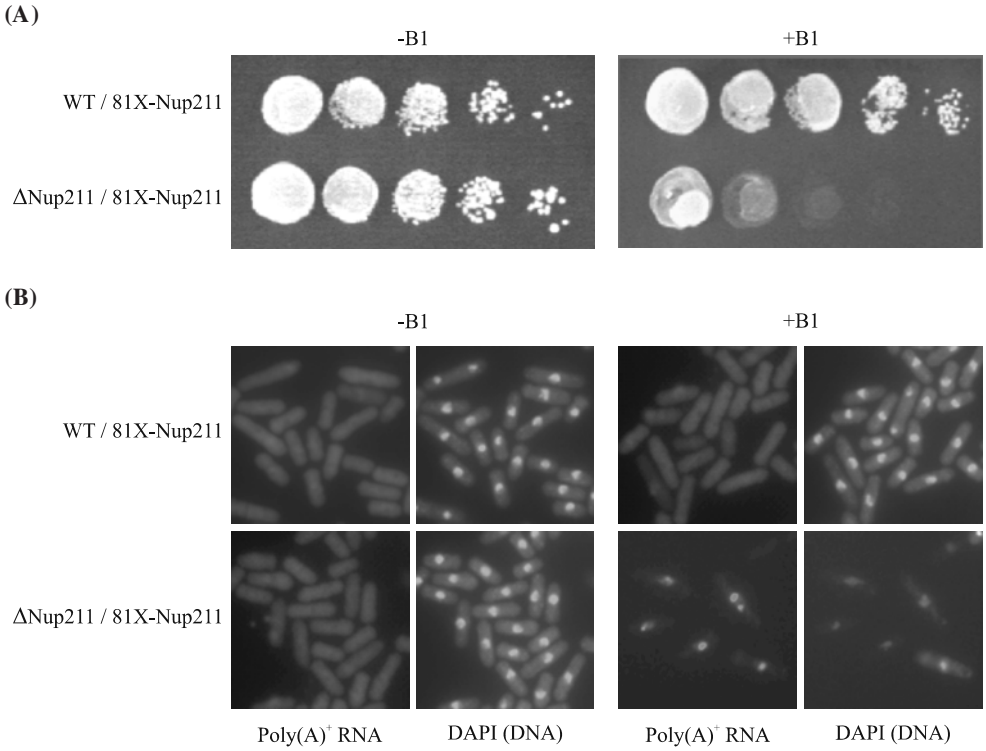

Fig. 3. Δ*nup211* mutants showed the defects of growth and mRNA export. (A) Growth of wild type (AY217) and ΔNup211 haploid strains carrying 81X-Nup211 plasmid were monitored by spot assay for 4 days at 28°C with (+B1) and without (-B1) expression of *nup211*. (B) Poly(A)$^+$ RNA localization in the Δ*nup211* mutants. Cells were grown to the mid-log phase in appropriately supplemented EMM medium in the absence of thiamine (-B1) at 28°C. The cells were then shifted to EMM medium containing thiamine (+B1) and were grown for 18 h. Coincident DAPI staining is shown in the right panels.

allele is lethal to cells in *S. pombe*. In contrast, two *S. cerevisiae* homologs, Mlp1p and Mlp2p, are not essential for cell growth, respectively. Moreover, *mlp1*Δ *mlp2*Δ double deletion mutant is also viable and shows no clear growth defects (Strambio-de-Castillia *et al*., 1999; Kosova *et al*., 2000). It is therefore possible that Nup211 protein plays unique and pivotal roles of cell viability in *S. pombe*.

Over-expression or repression of *nup211* inhibits growth and mRNA export

S. cerevisiae strains lacking Mlp1p, Mlp2p, or both did not exhibit any defects of mRNA export as well as growth (Strambio-de-Castillia *et al*., 1999). However, over-expression of Mlp1p in yeast and Tpr in mammalian cells caused some poly(A)$^+$ RNA accumulation in the nucleus (Bangs *et al*., 1998; Kosova *et al*., 2000). Thus, we wanted to check whether over-expression of Nup211p also affects mRNA export. For this, we constructed pREP3X-Nup211 vector in which *nup211* is expressed under the control of a strong *nmt1* promoter in the absence of thiamine (Forsberg, 1993). When *nup211* was over-expressed from pREP3X-Nup211 in addition to expression from genomic *nup211* locus, we tested if cell growth and nuclear export of poly(A)$^+$ RNA is inhibited in the wild-type cells. As shown in Fig. 2A, over-expression of Nup211p in the absence of thiamine (-B1) cause retardation of growth and this inhibition was accompanied by accumulation of poly(A)$^+$ RNA in nucleus (Fig. 2B). However, when Nup211p expression from plasmid was repressed in the presence of thiamine (+B1), cell growth and the distribution of poly(A)$^+$ RNA looked normal as like a negative control, wild-type cells transformed with empty vector, pREP3X. These results suggest that over-expressed Nup211p likely interact with and titrate out other proteins that are essential for growth and nuclear export of mRNA.

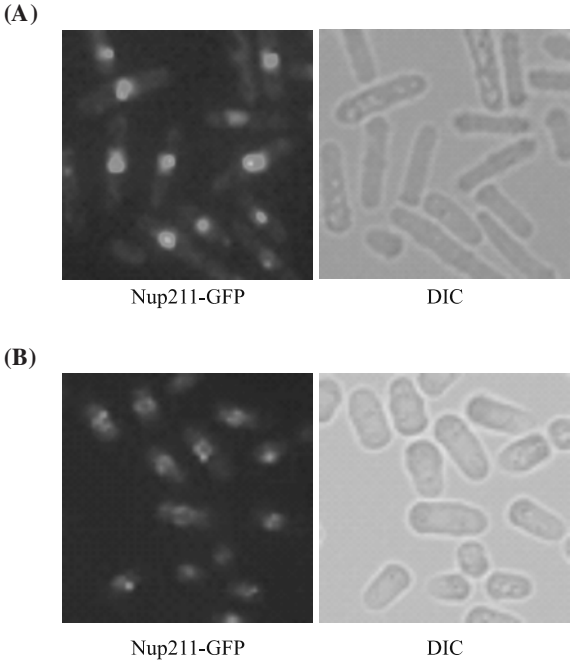

Fig. 4. Localization of Nup211p fused to GFP. (A) Localization of the ectopically expressed Nup211p-GFP fusions from *nmt1* promoter. (B) Localization of Nup211p-GFP fusions expressed at endogenous level from its own promoter. Cells were grown to the mid-log phase in appropriately supplemented EMM medium at 28°C. Coincident differntial interference contrast (DIC) images are also shown in the right panels.

On the other hand, we also wanted to examine whether Nup211p plays crucial roles of mRNA export in fission yeast, because the *nup211* gene is essential for viability in contrast to *Mlp* genes that are not essential. For cell growth, we constructed a haploid Δ*nup211* null strain harboring an 81X-Nup211 plasmid and confirmed the strain by Southern blotting (Fig. 1B). In this strain, *nup211* is expressed only from the plasmid under the control of the attenuated version of *nmt1* promoter that has reduced activity compared to the wild-type *nmt1* promoter (Basi *et al*., 1993). As shown in Fig. 3A, this strain grew normally in the absence of thiamine (-B1), suggesting that expression level of *nup211* from the weak *nmt1* promoter is enough to retain the normal growth rate. However, when *nup211* expression was turned off by the addition of thiamine (+B1), the cells showed the cessation of cell growth. These results confirmed again that *nup211* is essential for vegetative growth. To determine whether the growth defect is accompanied by mRNA export, poly(A)$^+$ RNA distribution was examined in this strain. As expected, repression of *nup211* in the presence of thiamine resulted in extensive poly(A)$^+$ RNA accumulation in the nucleus and a decrease of poly(A)$^+$ RNA in the cytoplasm (Fig. 3B). However, when *nup211* expression was turned on by the absence of thiamine, poly(A)$^+$ RNA is distributed throughout the whole cell as like wild-type cells. These results suggested that *nup211* plays the pivotal role of bulk mRNA export from the nucleus to the cytoplasm.

In *S. cerevisiae*, the clue to the function of Mlp proteins in mRNA export came from the observation that over-expression of Mlp1p causes poly(A)$^+$ RNA accumulation within the nucleus (Kosova *et al*., 2000), and its C-terminal domain that interacts directly with essential mRNA export factors is enough to inhibit mRNA export when over-expressed (Green *et al*., 2003). Mlp proteins, however, seem to play an only auxiliary function in mRNA export, because the null strains of *Mlp1*, *Mlp2*, or both did not show any defects of bulk mRNA export (Strambio-de-Castillia *et al*., 1999). Rather, they seem to play a pivotal role in mRNP surveillance at the nuclear side of the NPC by mediating nuclear retention of intron-containing mRNA and by blocking the export of improperly assembled mRNP complex (Galy *et al*., 2004; Vinciguerra *et al*., 2005). In contrast to Mlp, it is possible that Nup211 in *S. pombe* play a direct role in mRNA export by acting as a docking platform of The NPC for mRNP complex export, or by mediating intranuclear mRNA processing and transport step(s) that might be crucial for mRNA export.

Nup211-GFP fusion protein predominantly localized in the nucleus pheriphery

To determine the intracellular localization of Nup211p, we constructed the plasmids in which Nup211 was tagged with GFP (green fluorescent protein) at its amino-terminal or carboxy-terminal end. The ectopic expression of both Nup

211p-GFP fusions from *nmt1* promoter caused the appearance of an extremely bright fluorescence in the nucleus, respectively (Fig. 4A). These results were in contrast to a previous finding, in which Nup211p-GFP was located in nuclear rim (Chen *et al*., 2004). So, we integrated Nup211-GFP construct at the authentic gene locus to replace endogenous *nup211* by *nup211-GFP*, and confirmed the strain by PCR and Southern blotting (data not shown). When observed with fluorescence microscope, Nup211-GFP protein expressed at endogenous level from its own promoter was concentrated predominantly at the nuclear periphery in a punctuate pattern, but also detected in cytoplasm and nucleus. This localization pattern is similar to that of Mlp/Tpr proteins.

Mlp/Tpr proteins contain NLS (nuclear localization signal) in globular C-terminal domain and the C-terminal domain can target GFP into nucleus (Bangs *et al*., 1998; Kosova *et al*., 2000). Nup211p also contained putative bipartite NLS in C-terminal domain, and it was observed within the nucleus. Moreover, over-expressed Nup211p is accumulated strongly within the nucleus in addition to the nuclear periphery. It is therefore possible that Nup211p may play a role for mRNA export within the nucleus as well as at the NPC.

Acknowledgements

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