RESEARCH PAPER

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Characterization of nuclear pore complex components in fission yeast *Schizosaccharomyces pombe*

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Abbreviations: FG repeat, Phe-Gly repeat; HILO microscopy, highly inclined and laminated optical sheet microscopy; HU, hydroxyurea; NE, nuclear envelope; NPC, nuclear pore complex; TBZ, thiabendazole

The nuclear pore complex (NPC) is an enormous proteinaceous complex composed of multiple copies of about 30 different proteins called nucleoporins. In this study, we analyzed the composition of the NPC in the model organism *Schizosaccharomyces pombe* using strains in which individual nucleoporins were tagged with GFP. We identified 31 proteins as nucleoporins by their localization to the nuclear periphery. Gene disruption analysis in previous studies coupled with gene disruption analysis in the present study indicates that 15 of these nucleoporins are essential for vegetative cell growth and the other 16 nucleoporins are non-essential. Among the 16 non-essential nucleoporins, 11 are required for normal progression through meiosis and their disruption caused abnormal spore formation or poor spore viability. Based on fluorescence measurements of GFP-fused nucleoporins, we estimated the composition of the NPC in *S. pombe* and found that the organization of the *S. pombe* NPC is largely similar to that of other organisms; a single NPC was estimated as being 45.8–47.8 MDa in size. We also used fluorescence measurements of single NPCs and quantitative western blotting to analyze the composition of the Nup107-Nup160 subcomplex, which plays an indispensable role in NPC organization and function. Our analysis revealed low amounts of Nup107 and Nup131 and high amounts of Nup132 in the Nup107-Nup160 subcomplex, suggesting that the composition of this complex in *S. pombe* may differ from that in *S. cerevisiae* and humans. Comparative analysis of NPCs in various organisms will lead to a comprehensive understanding of the functional architecture of the NPC.

Introduction

The cell nucleus is enclosed by the nuclear envelope (NE), a double-membrane structure, which physically separates the nucleoplasm as a transcription space and the cytoplasm as a translation space, in eukaryotes. Molecules in the nucleus and the cytoplasm can be transported across the NE through the nuclear pores. The nuclear pore is an enormous protein complex, named the nuclear pore complex (NPC), that penetrates the NE. The molecular mass of the NPC is estimated to be ~125 MDa in vertebrate cells[1] and ~50 MDa in yeast cells.[2] The NPC is an 8-fold rotational symmetrical structure composed of multiple copies of approximately 30 different protein components called nucleoporins.[2,3] A few transmembrane- and several scaffold-nucleoporins form the NPC-core structure that penetrates the nuclear membrane, and another group of nucleoporins form the NPC-peripheral layer which associates with the NPC-core structure. The core and peripheral layer are connected by adaptor nucleoporins. The NPC structure and most nucleoporins are conserved among eukaryotes.[2-11]

One of the subcomplexes forming the NPC-core structure is the Nup107-Nup160 subcomplex, composed of Nup160, Nup133, Nup107, Nup85, Nup96, Sec13, and Seh1, and depending on the species, additional nucleoporins Nup37, Nup43, and ELYS.[12-15] Structural analysis of this complex has revealed that recombinant Nup107-Nup160 subcomplex proteins form a Y-shaped structure, and thus the complex is also referred as the Y-complex.[16,17] The existence of the Y-complex is well conserved among eukaryotes.[4,9,10,12,13,18-20] One of the important functions of the Nup107-Nup160 subcomplex is to assemble other nucleoporins or NPC subcomplexes into a complete and functional NPC after mitosis. In addition, the Nup107-Nup160

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complex plays a role in kinetochore function.[15,21-23] Thus, the Nup107-Nup160 complex is indispensable for NPC organization and chromosome segregation in eukaryotes.

The fission yeast *Schizosaccharomyces pombe* is an invaluable model experimental organism used for the elucidation of fundamental biological processes. The function and architecture of the NPC is also studied in *S. pombe*,[19,24-34] but compared with budding yeast and humans in which NPC organization and function are intensively studied, knowledge of the *S. pombe* NPC is limited. In the present study, we generated *S. pombe* strains expressing individual GFP-tagged nucleoporins. In these strains, the genomic sequence coding the target nucleoporin was replaced with a GFP tagged nucleoporin sequence resulting in the GFP tagged nucleoporin being expressed under the regulation of its natural promoter as the sole endogenous protein. Using these strains, we confirmed the localization of 26 nucleoporins listed in the *S. pombe* database (http://www.pombase.org/) to the nuclear periphery. We also confirmed the localization of the predicted nucleoporin, Nup37, to the nuclear periphery. In addition we characterized 3 other *S. pombe* proteins as nucleoporins: Tts1, Ely5, and Amo1. The organization of the *S. pombe* NPC is largely similar to that of other organisms. Intriguingly, however, our fluorescence and biochemical analysis in *S. pombe* revealed some variation in the composition of the Nup107-Nup160 subcomplex among eukaryotes that suggests possible variation in the composition of the *S. pombe* Nup107-Nup160 subcomplex. In addition, we systematically disrupted the genes of the 9 nucleoporins that had not been individually characterized previously. Our results coupled with the previous reports indicate that 15 of the 31 proteins we identified as nucleoporins are essential for vegetative cell growth. Finally, disruption of the 16 non-essential nucleoporins revealed that 11 of these nucleoporins are essential for meiosis and normal spore formation and/or spore viability.

Results

Nucleoporins in *S. pombe*

To comprehensively understand the nuclear pore complex (NPC) of *S. pombe*, we first selected 30 proteins as nucleoporins based on the *S. pombe* database (http://www.pombase.org/): 21 genes were selected based on experimental evidence and amino acid sequence similarities to proven nucleoporins,[19,24-31,35] and the other 9 genes were selected based on amino acid sequence similarities alone (see Materials and Methods for details). Our previous study identified and named spEly5 and spNup37 (in this report we add the prefix "sp" for *S. pombe*) as nucleoporins in *S. pombe*,[32] and during the course of the present study, Bilokapic and Schwartz independently characterized spEly5 and spNup37 as non-essential components of the *S. pombe* NPC.[33] Thus, spEly5 and spNup37 are included in the group of "experimentally-identified nucleoporins" in **Table S1**. To examine whether these genes encode nucleoporins, individual target genes were replaced with GFP tagged sequences, as described in Materials and Methods, resulting in the GFP tagged nucleoporin being expressed under the regulation of its natural promoter as the sole endogenous protein. In these strains, since the gene is transcribed under its natural promoter, the GFP-fusion gene is expressed at physiological levels. Our strains expressing GFP-tagged nucleoporins were all viable, but four of them (spNup45-GFP, spNup184-GFP, GFP-spRae1, and spNup189n-GFP) showed growth deficiencies, and in one strain (spNup189n-GFP) localization of the GFP fluorescence was unusual; it showed clustered localization to the nuclear periphery with some bright spots (see "spNup189n" in **Fig. 1A**) and often showed additional dots inside the nucleus as well.

Fluorescence microscopy of these strains revealed that 29 proteins, all of our target genes except for spSec13, were located at the nuclear periphery with non-uniform dots characteristics of NPC localization (**Fig. 1A**), suggesting they are nucleoporins. Sec13 is conserved among eukaryotes and a component of the Nup107-Nup160 complex in humans and *S. cerevisiae*;[14,16,36,37] spSec13 is also conserved in *S. pombe*.[38] spSec13, however, did not localize to the nuclear periphery when fused with GFP either at its N- or C-termini (**Fig. 1B**). To further test if spSec13 is a structural component of the NPC in *S. pombe*, we examined its localization in cells lacking spNup132. In these cells, the NPCs make a cluster on one side of the nucleus,[19] and this clustering of NPCs provides a unique opportunity to identify structural components of the NPC. In cells lacking spNup132, GFP-spSec13 did not co-localize with spCut11-mCherry (**Fig. 1B**): the nucleoporin spCut11 fused with mCherry at its C-terminus was used as a marker of the NPC. These results suggest that spSec13 may not be a structural component of the NPC in *S. pombe*.

In addition to the 29 proteins identified as nucleoporins, two other nuclear membrane proteins in the *S. pombe* database, spAmo1 and spTts1, were selected as candidate nucleoporins because they had amino acid sequence similarities to the nucleoporins hsNlp1 (also known as hCG1 and NUPL2)/scNup42 (also known as scRip1)[39] and hsTMEM33/scPom33/scPer33,[40] respectively. spAmo1-GFP localized to the nuclear periphery similarly to the other nucleoporins. spTts1-GFP localized to the nuclear envelope and endoplasmic reticulum, as reported previously.[41] These results suggest that these proteins are associated with the NPC in *S. pombe*. To further affirm that spAmo1 and spTts1 are structural components of the NPC in *S. pombe*, we examined their localization in cells lacking spNup132 as described above.[19] In these cells, spAmo1-GFP co-localized with the NPC marker spCut11-mCherry (**Fig. 1C**). spTts1-GFP also co-localized with spCut11-mCherry, although a fraction stayed on the NPC-free region of the NE (**Fig. 1D**). These results suggest that spAmo1 and spTts1 are nucleoporins.

We searched for novel nucleoporins in *S. pombe* using two different GFP fusion libraries, in which *S. pombe* genes are individually fused to the GFP gene,[42,43] but failed to find any proteins with a localization characteristic of a nucleoporin. In the *S. pombe* genome database, at least 6 uncharacterized genes (SPAC18G6.13, SPAC17A2.07c, SPBC409.11, SPCC70.04c, SPCC1919.04, and SPBC25B2.08) are registered as genes encoding nuclear envelope proteins according to the genome wide protein localization study of *S. pombe*.[44] However, they

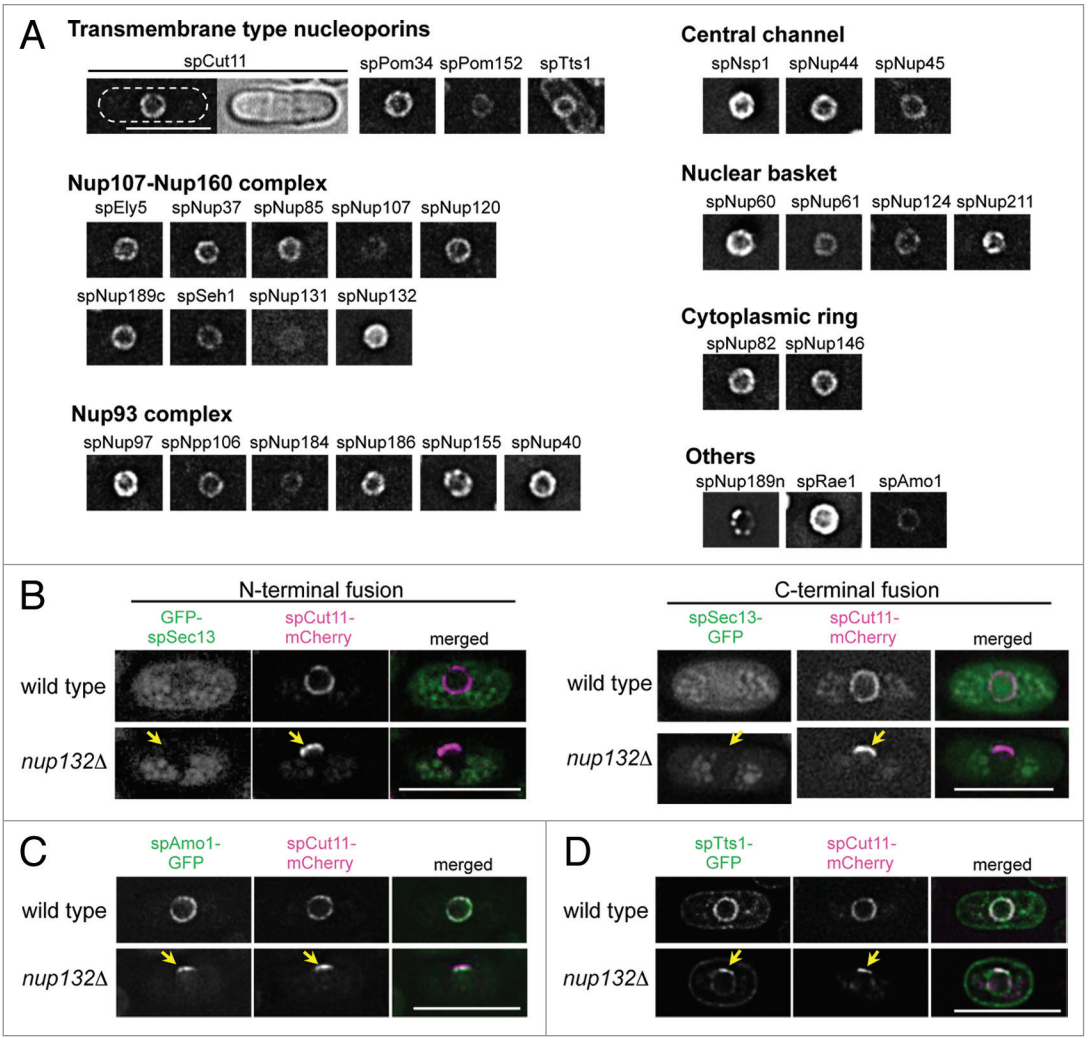

Figure 1. *S. pombe* nucleoporins fused to GFP. (**A**) Subcellular localization of GFP-tagged nucleoporins. The different images were obtained with the same acquisition times and processed in parallel. Deconvolved images are shown. The top left two panels show fluorescence (left) and bright field (right) images of an spCut11-GFP expressing cell. Nucleoporins were classified into seven groups according to localization in the NPC inferred from localization of the budding yeast orthologs. The scale bar represents 10 μm. (**B-D**) Localization of GFP-spSec13 (**B**), spAmo1-GFP (**C**), and spTts1-GFP (**D**) in wild type and cells lacking spNup132 (*nup132*Δ); in (**B**), GFP was fused with N-terminus (left) and C-terminus (right) of spSec13. spCut11-mCherry was simultaneously observed as a known nucleoporin marker. Yellow arrows indicate NPC-clustering regions in *nup132*Δ cells (lower panels). The scale bars represent 10 μm.

are *S. pombe*-specific genes and have no apparent orthologs in other eukaryotes. Thus, in this study we designate 31 proteins as nucleoporins in *S. pombe* based on their localization (**Table S1**). The domain organization of each nucleoporin in *S. pombe* is indicated in **Figure S1**.

Quantification of the nucleoporins based on fluorescence intensity

As the expression of GFP-tagged nucleoporins is under the control of the endogenous promoter, it is assumed that these proteins are generated in similar amounts as those of the native protein and that the composition of the NPC is the same in the strains generated in this study and wild-type cells. To determine the amount of the nucleoporins constituting the NPC, the fluorescence intensity of GFP in each strain was measured in living cells (see Materials and Methods for details): use of fluorescence intensity allows quantification of proteins present in the nuclear rim as opposed to quantifying the levels of proteins present throughout the cell. As expected, the fluorescence intensity of the different nucleoporins varied (**Fig. 2A**). Therefore, we estimated the relative values of the nucleoporins based on their fluorescence intensities. The fluorescence intensities and the relative values of the nucleoporins are listed in **Table 1**. Although spNup131, spNup107, and spSeh1 were also thought to form the Nup107-Nup160 subcomplex, they were separated from the other constituents because of their low fluorescence intensity (**Fig. 2**; **Table 2**). Notably, spNup131 had an extremely low fluorescence intensity. Because spNup131 has a paralog (spNup132), which shows relatively high fluorescence intensity, the small amount of spNup131 may be sufficient for its function in combination with spNup132 as an scNup133/hsNup133 homolog in *S. pombe*.

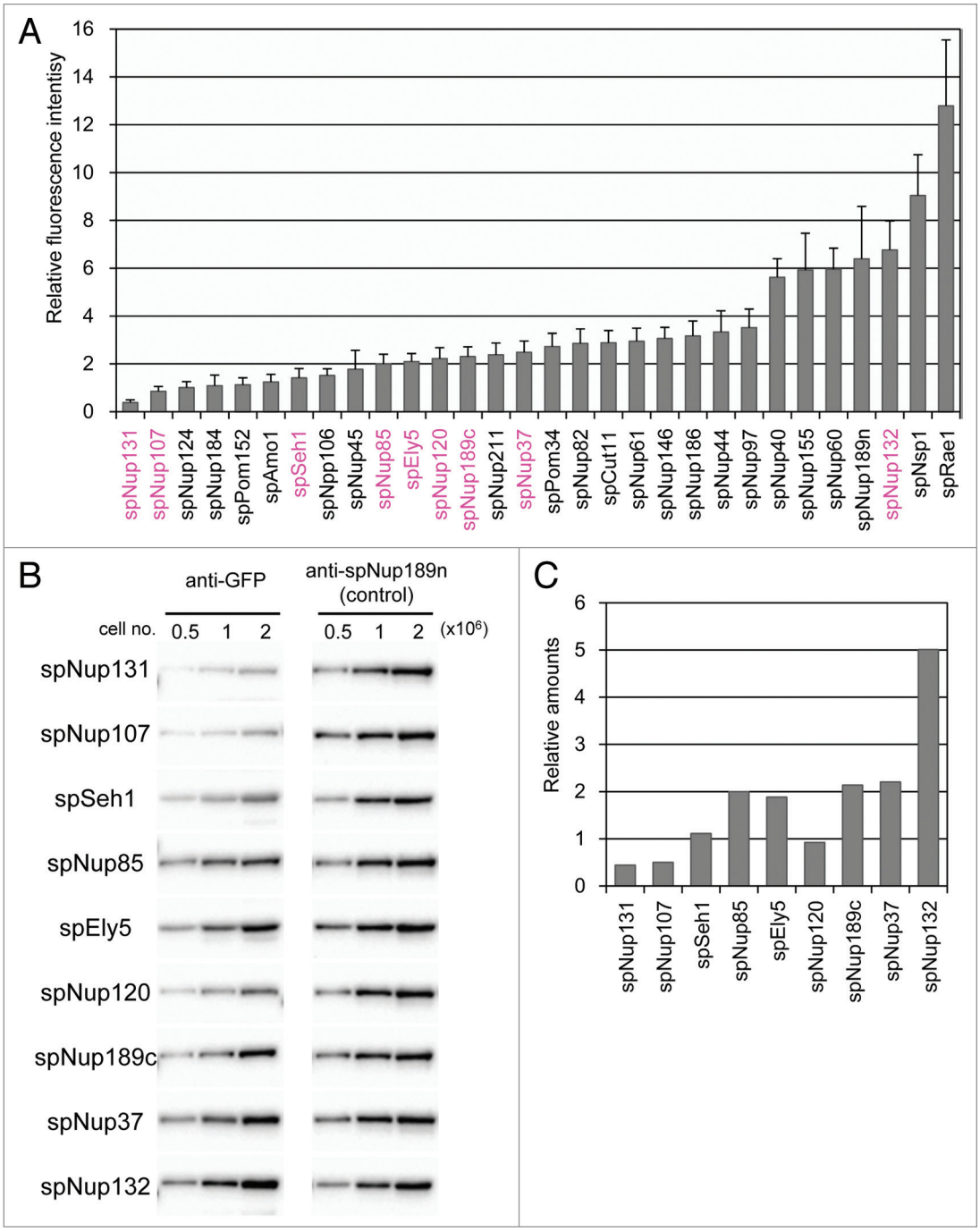

Figure 2. Quantification of GFP-tagged nucleoporins. (**A**) Fluorescence intensities of GFP-tagged nucleoporins. Fluorescence intensities of about 50 cells from each strain cultured at 26 °C were measured. Average values after background subtraction are shown in the bar graph. Error bars represent standard deviations. Components of the Nup107-Nup160 subcomplex are shown in magenta. (**B**) Quantitative western blot analysis of Nup107-Nup160 subcomplex nucleoporins. GFP-fused nucleoporins were fractioned by SDS-PAGE and detected by anti-GFP antibody (left). The membranes were stripped and reprobed to detect endogenous spNup189n as an internal control (right). The numbers above the images indicate cell numbers used to prepare the whole cell extract for each lane. (**C**) The relative amounts of Nup107-Nup160 subcomplex nucleoporins based on quantitative western blot analysis. Protein band intensities for 1×10^6 cells in (**B**) were measured for quantification. See Materials and Methods for detail.

Alternatively, it is possible that not all NPCs contain spNup131. The low fluorescence intensities of two other nucleoporins, spNup107 and spSeh1, may reflect a different Nup107-Nup160 subcomplex composition in *S. pombe* compared with that in *S. cerevisiae* and humans.

Quantification of Nup107-Nup160 subcomplex components based on western blotting

We also determined the approximate amounts of Nup107-Nup160 subcomplex proteins by quantitative western blot analysis (**Fig. 2B**); development of the membranes yielded single protein

Table 1. Nucleoporins in *S. pombe*

Name	ORF name	predicted MW	fluorescence intensity (unit)	Relative amount
spCut11	SPAC1786.03	69.3	100.32	2.89
spPom34 /Mug31	SPAC1002.02	25.8	94.83	2.73
spPom152	SPBC29A10.07	139.5	39.31	1.13
spTts1	SPBC1539.04	31.5	ND	ND
spEly5	SPBC29A10.06c	35.1	73.02	2.10
spNup37	SPAC4F10.18	42.7	86.60	2.49
spNup85	SPBC17G9.04c	78	69.54	2.00
spNup107	SPBC428.01c	93.2	29.75	0.86
spNup120	SPBC3B9.16c	129.7	77.40	2.23
spNup131	SPBP35G2.06c	131.4	13.32	0.38
spNup132	SPAC1805.04	132	235.30	6.77
spNup189c	SPAC1486.05	94.6	80.09	2.30
spSeh1	SPAC15F9.02	38.5	49.13	1.41
spNup97 /Mug87	SPCC1620.11	97.5	122.35	3.52
spNpp106	SPCC1739.14	105.7	52.71	1.52
spNup184	SPAP27G11.10c	176.9	37.90	1.09
spNup186	SPCC290.03c	186.4	110.10	3.17
spNup155	SPAC890.06	147.6	206.04	5.93
spNup40	SPAC19E9.01c	40.2	195.44	5.62
spNsp1	SPAC26A3.15c	60.7	314.31	9.04
spNup44	SPBC19G7.15	44.4	115.94	3.33
spNup45	SPAC22G7.09c	44.9	60.53	1.77
spNup82	SPBC13A2.02	90.6	99.41	2.86
spNup146	SPAC23D3.06c	145.7	106.41	3.06
spNup60	SPCC285.13c	80.2	207.13	5.96
spNup61	SPCC18B5.07c	59.1	102.42	2.95
spNup124	SPAC30D11.04c	123.9	35.09	1.01
spNup211	SPCC162.08c	211.4	82.91	2.38
spNup189n	SPAC1486.05	95	222.33	6.39
spRae1	SPBC16A3.05c	38.6	444.89	12.80
spAmo1	SPBC15D4.10c	51.7	43.34	1.25

bands corresponding to each GFP-nucleoporin, suggesting that none of GFP-nucleoporins tested were degraded (**Fig. S2**). For these membranes, endogenous spNup189n was used as an internal control for normalization of the relative amounts of the GFP-nucleoporins; the specific spNup189n antibody clone 13C2[45] was used in the determination of the band intensity of endogenous spNup189n (**Fig. 2B**). The results of our western blot analysis agreed with the results of our fluorescence-based analysis (compare **Fig. 2A and C**): the *S. pombe* NPC contains low amounts of Nup107 and Nup131 and high amounts of Nup132 (**Fig. 2A and C**). This result suggests that the *S. pombe* Nup107-Nup160 subcomplex may have a different composition from that in *S. cerevisiae* and humans.

Estimation of nucleoporin amounts in a single NPC

Gross estimation by fluorescence and biochemistry suggested that the composition of the *S. pombe* NPC is different. Especially, the smaller amount of spNup107 is unique because Nup107 is an essential component for cell growth and is thought to be a constituent of a conserved core structure of the NPC in other organisms. The estimation by fluorescence was based on fluorescence intensities from a whole single nucleus and estimation by biochemistry was based on whole cell extracts. Consequently, the low amount of spNup107 in the *S. pombe* NPC may reflect diverse compositions of individual NPCs: some NPCs may harbor a higher number of spNup107 proteins, similar to the levels in the NPCs of *S. cerevisiae* and humans,

Table 2. Essentiality of *S. pombe* nucleoporins

Name	ORF name	Individual analyses (references)		Comprehensive deletion analysis (Kim et al.)[46]	This study
spCut11	SPAC1786.03	e	(West et al.)[26]	e	
spPom34 /Mug31	SPAC1002.02			n	n
spPom152	SPBC29A10.07			n	n
spTts1	SPBC1539.04	n	(Zhang et al.)[41]	n	
spEly5	SPBC29A10.06c	n	(Bilokapic and Schwartz)[33]	n	n
spNup37	SPAC4F10.18	n	(Bilokapic and Schwartz)[33]	n	n
spNup85	SPBC17G9.04c	e	(Baï et al.; Chen et al.)[19,29]	e	
spNup107	SPBC428.01c	e	(Baï et al.; Chen et al.)[19,29]	e	
spNup120	SPBC3B9.16c	n	(Baï et al.)[19]	n	
spNup131	SPBP35G2.06c	n	(Baï et al.)[19]	n	
spNup132	SPAC1805.04	n	(Baï et al.; Chen et al.)[19,29]	n	
spNup189c	SPAC1486.05				e
spSeh1	SPAC15F9.02	n	(Baï et al.; Chen et al.)[19,29]	n	
spNup97 /Mug87	SPCC1620.11	e	(Cho et al.)[30]	e	
spNpp106	SPCC1739.14	n	(Yoon et al.)[25]	n	
spNup184	SPAP27G11.10c	n	(Whalen et al.)[28]	n	
spNup186	SPCC290.03c	e	(Chen et al.)[29]	e	
spNup155	SPAC890.06			n	e
spNup40	SPAC19E9.01c	n	(Chen et al.)[29]	n	
spNsp1	SPAC26A3.15c	e	(Chen et al.)[29]	e	
spNup44	SPBC19G7.15	e	(Chen et al.)[29]	e	
spNup45	SPAC22G7.09c	e	(Chen et al.)[29]	e	
spNup82	SPBC13A2.02			n	e
spNup146	SPAC23D3.06c	e	(Chen et al.)[29]	e	
spNup60	SPCC285.13c			n	n
spNup61	SPCC18B5.07c	n	(Chen et al.)[29]	n	
spNup124	SPAC30D11.04c	n	(Balasundaram et al.)[27]	n	
spNup211	SPCC162.08c	e	(Chen et al.)[29]	e	
spNup189n	SPAC1486.05				e
spRae1	SPBC16A3.05c	e	(Brown et al.)[24]	e	
spAmo1	SPBC15D4.10c	n	(Pardo and Nurse)[48]	n	

Essential nucleoporins are shown in bold according to individual analyses and this study. "e" and "n" represent "essential" and "non-essential," respectively.

while others may harbor lower numbers. To clarify the amounts of spNup107 present in individual NPCs, we measured fluorescence intensities of selected nucleoporins for single NPCs using highly inclined and laminated optical sheet (HILO) microscopy, which is a modification of total internal reflection fluorescence microscopy.[46] The fluorescence of individual NPCs on the surface of the NE can be imaged using HILO microscopy (**Fig. 3A**). In this experiment, we used spNup85 as a standard and set the relative value of its fluorescence intensity as two. The number of spNup107-GFP foci in a single nucleus was similar to that of spNup85-GFP foci, but the fluorescence intensity per single NPC was lower than that of spNup85 (**Fig. 3A and B**). These results suggest that most or all *S. pombe* NPCs contain small amounts of spNup107. We also measured the amounts of spNup131-GFP and spNup132-GFP in single NPCs. The relative values of spNup131-GFP and spNup132-GFP were 0.4 and 4.8, respectively. These results suggest that the constituents of the Nup107-Nup160 subcomplex may be present in a different ratio in *S. pombe* than *S. cerevisiae* or humans.

Our estimations, based on fluorescence intensities and biochemistry, suggest that some of the *S. pombe* nucleoporins, including spNup107, spSeh1, and spNup131, are present in the NPC in smaller amounts than in *S. cerevisiae*, although we cannot exclude the possibility that their intensities were underestimated for technical reasons. Based on the relative amounts obtained from the fluorescence intensities of the GFP-tagged nucleoporins,

we estimate a molecular mass of a single *S. pombe* NPC as 45.8–47.8 MDa, similar to the value of ~50 MDa for *S. cerevisiae*.[2,47]

Essentialities of *S. pombe* nucleoporins

Previous studies on individual nucleoporins have reported 11 essential nucleoporins and 11 non-essential nucleoporins in *S. pombe*,[19,24-30,41,48] and 6 additional non-essential nucleoporins were identified by comprehensive deletion analysis (see **Table 2**).[49] Here we examined the 9 nucleoporins that had not been individually characterized previously (spEly5, spNup37, spNup189c, spNup155, spNup82, spNup60, spNup189n, spPom152, and spPom34) by gene disruption analysis using PCR-based gene targeting methods.[50] We found that spNup155 and spNup82 were essential for vegetative cell growth and that spEly5, spNup37, spNup60, spPom152, and spPom34 were not essential. An alternative method was used for spNup189n and spNup189c because they are encoded by a single *nup189*$^{+}$ gene transcribed as one transcript (HA and TH, unpublished result) and consequently endogenous *nup189*$^{+}$ gene deletion results in deletion of both spNup189c and spNup189n. Therefore, nup189n was integrated into the *lys1*$^{+}$ locus when analyzing spNup189c and nup189c was integrated into the *aur1*$^{+}$ locus when analyzing spNup189n (see Materials and Methods for details). We found that cells were viable only when spNup189n and spNup189c were simultaneously expressed. Thus, we concluded that spNup189n and spNup189c were both essential for cell growth. Two of the nucleoporins, spNup155 and spNup82, reported to be nonessential by Kim et al.,[49] were essential in our analysis (**Table 2**).

Taken together, gene disruption analysis in the previous studies combined with the gene disruption analysis of our study identified 15 nucleoporins essential for vegetative growth in *S. pombe* (**Table 2**). *S. pombe* homologs of essential nucleoporins in *S. cerevisiae* are also essential in cell growth in S. *pombe* (**Table 3**), suggesting that the NPC structure is largely similar among eukaryotes.

Functions of non-essential nucleoporins

We analyzed the 16 nucleoporins non-essential for vegetative cell growth. First, the growth of strains deleted for these nucleoporins was examined under various temperatures or in the presence of a DNA replication inhibitory drug, hydroxyurea (HU), or a microtubule-depolymerizing drug, thiabendazole (TBZ). In this assay, the colony size and colony number on agar plates were tested using a dilution series to compare growth rate and viability; strains deleted for Cds1 and Mph1 were used for positive controls to see the defects in the presence of HU and TBZ, respectively. The results are summarized in **Table 4**. Almost all strains could grow between 20 and 36 °C, but the strain lacking spNup120 showed severe temperature sensitivity: it could not grow at 36 °C, and formed small colonies even at 20 °C. The strain lacking spNpp106 or spAmo1 showed weak temperature sensitivity. Most strains showed growth inhibition on plates containing TBZ or HU, as judged by either small or fewer colonies. These results suggest that non-essential nucleoporins can be required to maintain cell viability when cell cycle progression is disturbed at DNA synthesis or mitosis.

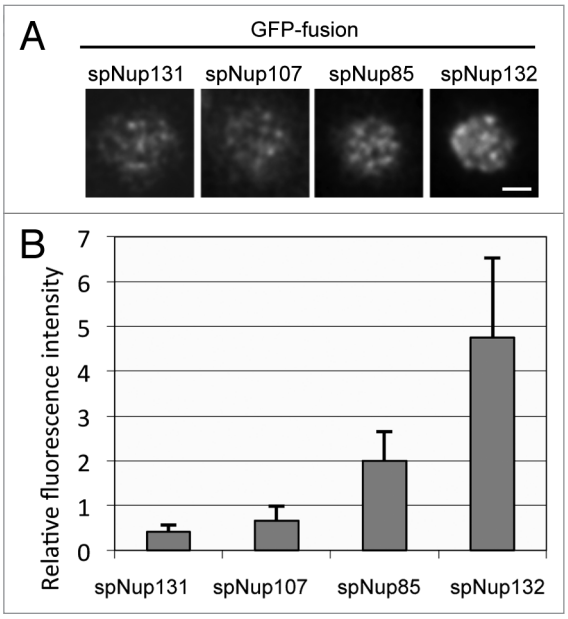

Figure 3. Fluorescence intensities of GFP-tagged nucleoporins measured by HILO microscopy. (**A**) HILO microscopy images of cells expressing spNup131-GFP, spNup107-GFP, spNup85-GFP, and spNup132-GFP. The nuclear region of a single cell is shown. The scale bar indicates 1 μm. (**B**) Fluorescence intensity of nucleoporins in single NPCs. Average and standard deviation are shown.

Next we examined meiotic functions of non-essential nucleoporins. In order to check meiotic phenotypes, deletion strains were induced to undergo meiosis, and spore formation and viability were examined. The wild type cell usually forms four spores following meiosis in *S. pombe*; however, in strains deleted for spNup132, spNup37, spEly5, spNup120, spPom152, spNpp106, spNup124, or spNup61 a fraction of the cells formed less than four spores (**Fig. 4A**). Furthermore, spore viability was low in strains lacking spNup131, spNup37, spSeh1, spEly5, spPom152, spPom34, spNpp106, spNup124, spNup60, or spNup61 (**Fig. 4B**); in cells lacking spNup120, spore viability was not determined because spore formation was severely defective. This result suggests an unidentified function of 11 non-essential nucleoporins in spore formation and/or spore viability in *S. pombe*.

Discussion

The *S. pombe* NPC consists of evolutionary conserved nucleoporins: it includes an ELYS homolog and almost the same set of essential components as metazoans. Indeed, spEly5 and spNup37, which are nucleoporins lacking in the *S. cerevisiae* NPC, were recently reported to participate in the assembly of the *S. pombe* Nup107-Nup160 subcomplex and the crystal structure of spNup37 in the complex has been recently solved.[33,34] This allows *S. pombe* to be used as a model organism for studies of the NPC.

Table 3. Correspondence table of nucleoporins

S. pombe	*S. cerevisiae*	*A. nidulans*	*H. sapiens*	*A. thaliana*
spCut11	**scNdc1**	*An-Ndc1*	hsNdc1	At1g73240
spPom34/Mug31	*scPom34*	*An-Pom34*	-	-
spPom152	*scPom152*	*An-Pom152*	-	-
spTts1	*scPom33*	AN6689.2	hsTMEM33	At3g02420
	scPer33			
-	-	-	hsGp/Nup210	atGp210
-	-	-	hsPom121	-
spEly5	-	*An-ELYS*	hsELYS	atElys/HOS1
spNup37	-	*An-Nup37*	hsNup37	-
spNup85	**scNup85**	*An-Nup85*	hsNup75/85	atNup75
spNup107	*scNup84*	*An-Nup84*	hsNup107	atNup107
spNup120	*scNup120*	*An-Nup120*	hsNup160	atNup160
spNup131	*scNup133*	*An-Nup133*	hsNup133	atNup133
spNup132				
spNup189c	**scNup145c**	**SonBc^{Nup96}**	hsNup96	atNup96/PRECOZ
spSeh1	*scSeh1*	*An-Seh1*	hsSeh1/Sec13L	atSeh1
—*	**scSec13**	**An-Sec13**	hsSec13R	atSec13
spNup97/Mug87	**scNic96**	**An-Nic96**	hsNup93	atNup93a
spNpp106				atNup93b
spNup184	*scNup188*	**An-Nup188**	hsNup188	atNup188
spNup186	**scNup192**	**An-Nup192**	hsNup205	atNup205
spNup155	*scNup157*	**An-Nup170**	hsNup155	atNup155
	scNup170			
spNup40	*scNup53*	-	hsNup35 (MP-44)	atNup35
	scNup59			
spNsp1	**scNsp1**	**An-Nsp1**	hsNup62	atNup62
spNup44	**scNup57**	*An-Nup57*	hsNup54	atNup54
spNup45	**scNup49**	*An-Nup49*	hsNup58	atNup58
-	-	-	hsNup45	-
-	-	-	hsNup358	-
spNup82	**scNup82**	**An-Nup82**	hsNup88	atNup88
spNup146	**scNup159**	**An-Nup159**	hsNup214	atNup214
spNup60	*scNup60*	-	-	-
spNup61	*scNup2*	**An-Nup2**	hsNup50	atNup50a
				atNup50b
spNup124	**scNup1**	**-**	hsNup153	atNup136/Nup1
spNup211	*scMlp1*	**An-Mlp1**	hsTpr	atTpr/NUA
	scMlp2			
spNup189n	*scNup100*	**SonBn^{Nup98}**	hsNup98	atNup98a
	scNup116			
	scNup145n			atNup98b

Essential and non-essential nucleoporins reported in *S. pombe*, *S. cerevisiae*, and *A. nidulans* are indicated in bold and italics, respectively. *Sec13 does not show nuclear periphery localization in *S. pombe*, thus it is not included in the "*S. pombe*" column and is represented as (-). Essentiality of AN6689.2 of *A. nidulans* is not clarified to date.

Table 3. Correspondence table of nucleoporins (continued)

S. pombe	*S. cerevisiae*	*A. nidulans*	*H. sapiens*	*A. thaliana*
spRae1	*scGle2*	**SonA^{Gle2}**	hsRAE1	atRAE1
spAmo1	*scNup42/Rip1*	*An-Nup42*	hsNlp1/hCG1/NUPL2	-
-	-	-	hsALADIN	atALADIN
-	-	-	hsNup43	atNup43

Essential and non-essential nucleoporins reported in *S. pombe*, *S. cerevisiae*, and *A. nidulans* are indicated in bold and italics, respectively. *Sec13 does not show nuclear periphery localization in *S. pombe*, thus it is not included in the "*S. pombe*" column and is represented as (-). Essentiality of AN6689.2 of *A. nidulans* is not clarified to date.

Organization and specific features of the *S. pombe* NPC

We constructed a series of strains in *S. pombe* in which nucleoporin genes and genes similar to known nucleoporins were tagged with GFP. Fluorescence microscopy of the strains identified 31 nucleoporins (listed in **Table 1**) defined by their localization to the nuclear periphery. In this study, in addition to verifying 27 nucleoporins listed in the *S. pombe* database (http://www.pombase.org/) and the predicted nucleoporin spNup37 as nucleoporins, we characterized Tts1, Ely5, and Amo1 as nucleoporins. spNup37 and spEly5 have also been characterized as nucleoporins in other studies.[32-34] Higher-order structures of nucleoporins are conserved, such as α-solenoid, β-propeller, and unstructured FG repeats, rather than amino acid sequences, consequently, there is poor similarity in amino acid sequence of *S. pombe* nucleoporins to human and budding yeast nucleoporins.

The organization of the NPC has been intensively studied in humans and budding yeast, and more recently in the nematode *Caenorhabditis elegans*,[4] the filamentous fungus *Aspergillus nidulans*,[6,20] the plant *Arabidopsis thaliana*,[10] the divergent eukaryote *Trypanosoma brucei*,[9] and the ciliate *Tetrahymena thermophile*.[8,51] These studies have revealed that while there are species-specific differences in the NPC, the organization of the NPC is generally similar in the eukaryotes studied so far. This is also true of the *S. pombe* NPC. One of the features of the *S. pombe* NPC is that it includes spEly5/hsELYS and spNup37/hsNup37.[20,32-34] These nucleoporins were originally identified as components of the Nup107-Nup160 subcomplex and thought to be included only in higher eukaryotic NPCs, as they are not found in the budding yeast. However, the ortholog of hsELYS and hsNup37 has been identified in the filamentous fungus *Aspergillus nidulans*,[20] and now, in this study and others,[32-34] in *S. pombe*. However, like *S. cerevisiae* and *A. nidulans* the *S. pombe* NPC lacks orthologs for hsNup45, hsNup358, hsALADIN and hsNup43. The unique characteristics of the *S. pombe* NPC likely reflect *S. pombe*-specific nuclear dynamics and/or life cycle.

Composition of the Nup107-Nup160 complex

We estimated the amount of endogenous nucleoporin by biochemistry and fluorescence microscopy of strains expressing physiological amounts of GFP-tagged nucleoporins. Our analysis suggests a unique organization of Nup107-Nup160 subcomplex in *S. pombe*. In *S. cerevisiae* and humans, each component of the Nup107-Nup160 subcomplex is thought to exist in equal amounts: one molecule of each nucleoporin is assembled into the Nup107-Nup160 subcomplex of the NPC, with two Nup107-Nup160 subcomplexes included in one NPC. In *S. pombe*, however, the relative ratios of spNup107 and spNup131 are lower compared with the other components, and the ratio of spNup132 is higher. Another feature in the *S. pombe* Nup107-Nup160 subcomplex is that spSec13 is lacking: GFP-spSec13 did not show localization to the nuclear periphery. Consistent with this result, biochemical isolation of the Nup107-Nup160 subcomplex did not identify spSec13.[19] These data suggest that spSec13 is not associated with the Nup107-Nup160 subcomplex in *S. pombe*. It is possible that some other, unidentified protein, may substitute for Sec13 in the *S. pombe* Nup107-Nup160 subcomplex, but our analysis indicates that the organization of the *S. pombe* Nup107-Nup160 subcomplex may be different. Although we cannot

Table 4. Function of non-essential nucleoporins for various growth conditions

genotypes	26 °C	30 °C	36 °C	20 μg/ml TBZ	10 mM HU
wild type	++++	++++	++++	+++	+++
Δ*pom34*	++++	++++	++++	+++	+++
Δ*pom152*	++++	++++	++++	+++S	+++
Δ*ely5*	++++	++++	++++	++	+
Δ*nup37*	++++	++++	++++	+++	+++
Δ*nup120*	+++S	++SS	-	-	-
Δ*seh1*	++++	++++	++++	+++	+++
Δ*nup131*	++++	++++	++++	+++	+++
Δ*nup132*	++++	++++	++++	+SS	+S
Δ*nup131* Δ*nup132*	++++	++++	++++	+SSS	+S
Δ*nup40*	++++	++++	++++	+++	+++
Δ*nup184*	++++	++++	++++	+++	+++
Δ*npp106*	++++	++++S	+++SS	+	+++
Δ*nup60*	++++	++++	++++	+++	++SS
Δ*nup61*	++++	++++	++++	+++	+++
Δ*nup124*	++++	++++	++++	++SS	++SS
Δ*amo1*	++++S	+++S	+++S	+++	+++
Δ*mph1*	++++	++++	++++	-	++S
Δ*cds1*	++++	++++	++++	+++	-

Each cell strain was cultured to midlog phase in liquid YES. 5-fold serial dilutions of each transformant were spotted onto YES plates with or without HU or TBZ. Viable colony number is indicated by -, +, ++, +++, and ++++: "-" indicates no colony formation. Colony size (growth rate) is indicated by S, for small; SS, for smaller; SSS, for smallest colonies. Δ*mph1* and Δ*cds1*strains were used as positive controls sensitive to TBZ and HU, respectively. For Δ*nup120* strains, TBZ sensitivity and HU sensitivity were assayed at 26 °C.

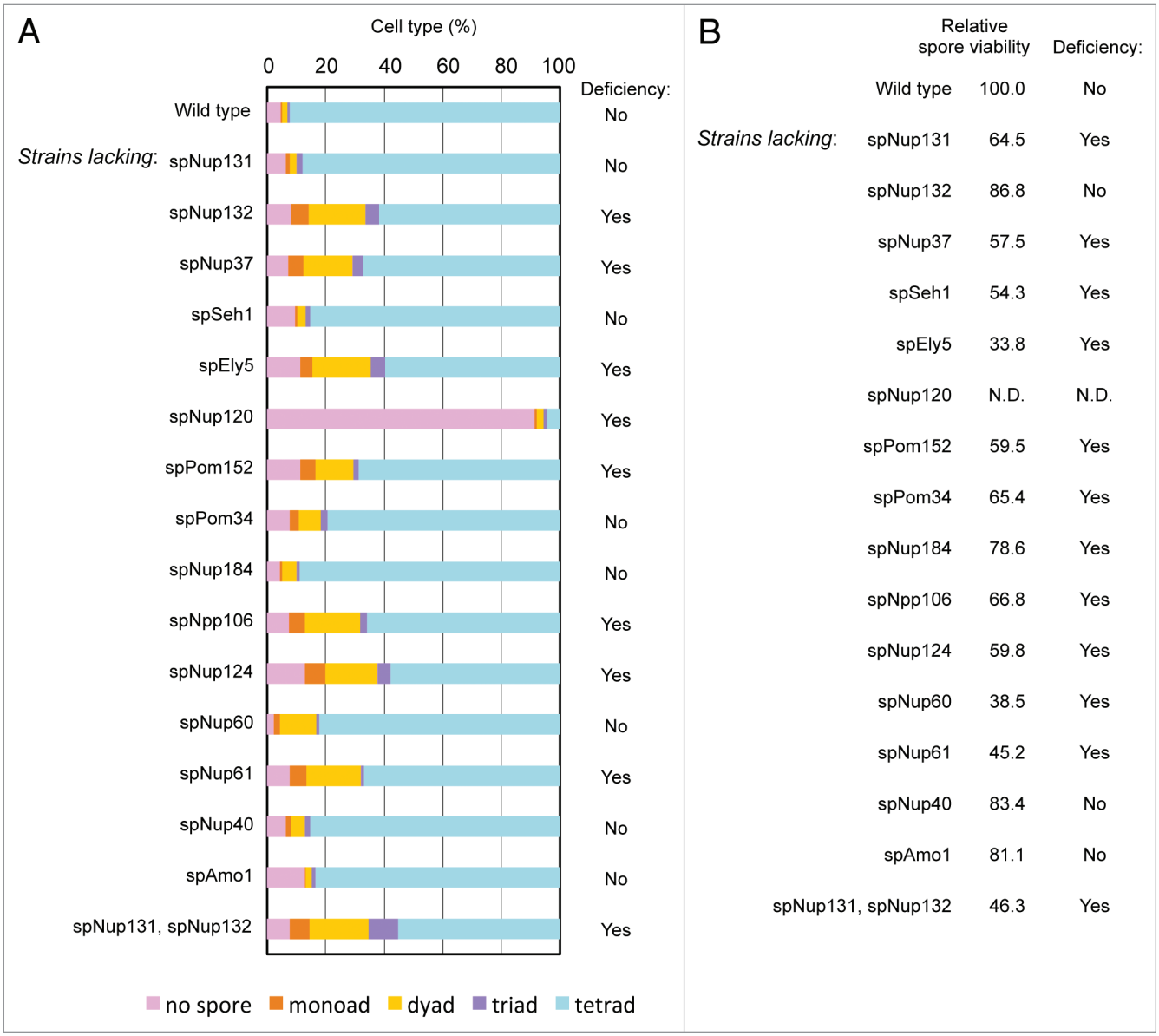

Figure 4. Effect of gene disruption of non-essential nucleoporins on spore formation. **(A)** Spore numbers in wild type and nucleoporin mutants. Cells were cultured on sporulation medium for two days, and zygotic cells containing spores were differentially scored. "Yes" and "No" indicate cell strains with a deficiency and no deficiency in spore formation, respectively. **(B)** The numbers indicate the spore viability of wild type and nucleoporin mutants. Cells forming spores were treated with β-glucuronidase to digest vegetative cells, and the resistant spores were counted and spread on growth media. After colony formation, colony numbers were counted and spore viability was calculated. The value was normalized with that of the wild type, which was given a value of 100. "Yes" and "No" indicate cell strains with a deficiency and no deficiency in spore viability, respectively.

exclude the possibility of artificial effects of GFP fusion and technical limitations of fluorescence intensity measurement, the unique organization of the *S. pombe* Nup107-Nup160 subcomplex suggests an evolutional diversity of the NPC and/or a species-specific function of the NPC in this organism. It will be important to follow the results of future proteomic and other parallel studies to see if they also support a potential difference in the stoichiometry of the *S. pombe* nuclear pore.

Functions of non-essential nucleoporins

While about half of the *S. pombe* nucleoporins are not essential for vegetative cell growth under normal laboratory conditions, they may be essential under certain circumstances. Strains deleted for non-essential nucleoporins enabled us to examine the effect of nucleoporin disruption on cell growth in the presence of a DNA replication inhibitory drug, HU, or a microtubule-depolymerizing drug, TBZ. In the presence of HU or TBZ, the non-essential nucleoporins spPom152, spEly5, spNup120, spNup132, spNpp106, spNup60, and spNup124 were required for cell growth, suggesting they have a role in chromosome function. An ELYS mutant caused decreased interaction of Mcm2, a protein required for DNA replication, with chromatin in zebrafish,[52] and ELYS is required for genome stability in intestinal epithelium

progenitor cells in the mouse,[53] also suggesting a chromatin-related function for this nucleoporin. A role of NPCs in chromosome function has also been reported for many eukaryotes (for a review see refs. 54, 55). Further analysis is required to clarify whether the defects observed in the strains deleted for spPom152, spEly5, spNup120, spNup132, spNpp106, spNup60, or spNup124 are dependent on the nucleocytoplasmic transport function of the NPCs, a transport-independent function of the NPC, or an NPC-independent function of specific nucleoporin molecules.

In addition, the effect of non-essential nucleoporin gene disruption on the meiotic process was analyzed. Interestingly, most non-essential nucleoporins (11 out of 16) were required for meiosis and spore formation or spore viability. In particular, spNup60, spNup61, and spNup124, whose budding yeast orthologs localize at nucleoplasmic side of the NPCs,[2] were all required for this processes in *S. pombe*. This result suggests important functions for nucleoplasmic components of the NPC in meiosis and spore formation. Consistent with this concept, the spNup61 ortholog is highly expressed in rat testis, suggesting a meiotic or developmental role.[56] In *A. thaliana*, the mutant of the spNup124 ortholog, atNup136, displays early flowering and low fertility, indicating a function in plant development.[10]

Curiously, the strain lacking spNup131, which is thought to be a paralog of spNup132, showed low spore viability, but it did not show a defect in cell growth in the presence of HU or TBZ. The strain lacking spNup132, in contrast to the strain lacking spNup131, had a defect in spore formation but normal spore viability, and exhibited growth defects in the presence of both HU and TBZ. *S. pombe* is the only eukaryote that has two orthologs (spNup131 and spNup132) for hsNup133, and our analysis suggests they have distinct roles.

Meiosis and spore formation in *S. pombe* are thought to be an example of gametogenesis or cell differentiation that involves regulation of nutrition sensing, signal transduction and expression of specific transcription factors.[57] The orthologs of nucleoporins required for meiosis and spore formation in *S. pombe* are also involved in cell differentiation in other eukaryotes. Nup133 (spNup131/spNup132 ortholog) is reported to have a role in neural cell differentiation in the mouse embryo;[58] ELYS/Flo is required for progenitor cell proliferation in certain tissues in the zebrafish;[54,59] Seh1 function is essential in fly oogenesis;[60,61] the membrane-integrated nucleoporins, spPom34 and spPom152, are required for meiosis and spore formation in *S. pombe*; gp210/Nup210, a membrane-integrated nucleoporin in mammals, is expressed during oogenesis and is essential for myogenic and neuronal differentiation in the mouse;[62-64] and gp210 is required for embryogenesis in *Arabidopsis thaliana*.[65] On one hand, the cell differentiation process may require nucleocytoplasmic transport of specific molecules, including transcription factors. On the other hand, it is reported that the composition of the NPC may be altered during cell differentiation.[52,64] A recent large-scale proteomics study of various types of human cells revealed varied composition of the NPC.[66] Thus, the abnormal meiosis and spore formation observed in mutant *S. pombe* strains may be caused by failure of nucleocytoplasmic transport or by transport-independent functions of the NPC.

Investigation of nucleoporin function in *S. pombe* meiosis and spore formation may shed light on developmental roles of nucleoporins and provide information to help elucidate NPC and nucleoporin function not only in nucleocytoplasmic transport but also in cell differentiation in other eukaryotes.

Materials and Methods

Cell culture

YES medium was used as routine culture medium, and ME was used as sporulation medium. For measurement of fluorescence intensity of GFP-fused nucleoporins, strains were precultured in YES at 26 °C and then transferred to EMM2 medium at 26 °C.[67] For subcellular localization analysis in cells lacking spNup132, cells were cultured on YES plate medium at 30 °C. To prepare drug-containing media, stock solutions of hydroxyurea (Sigma), 1 M in sterile water, and thiabendazole (Sigma), 20 mg/mL in dimethyl sulfoxide, were added to YES plates to the indicated final concentrations. Sporulation was induced at 26 °C.

Identification of nucleoporin genes in *S. pombe*

Candidates *S. pombe* nucleoporin genes were searched for in the *S. pombe* genome database (pombase, http://www.pombase.org/). We found 21 genes (genes encoding spCut11, spNup85, spNup107, spNup120, spNup131, spNup132, spNup189c, spSeh1, spNup97/Mug87, spNpp106, spNup186, spNup40, spNsp1, spNup44, spNup45, spNup146, spNup61, spNup124, spNup211, spNup189n, and spRae1) that were experimentally characterized as nucleoporins, and 9 genes (genes encoding spEly5, spNup37, spPom34/Mug31, spPom152, spNup184, spNup155, spNup82, spNup60, and spSec13) that were not experimentally characterized as nucleoporins but which share homologies with known nucleoporins in other eukaryotes. In addition, spAmo1 and spTts1 were identified as candidate nucleoporins because of homologies with known nucleoporins and their localization to the nuclear periphery.[41,48]

Generation of cell strains expressing fluorescent protein-tagged nucleoporins

We generated 31 cell strains, each with a different nucleoporin tagged with GFP, using AY160–14D (*h^{90} ade6–216 ura4-D18 leu1–32 lys1–131*) as a host strain. Sixteen nucleoporins (spCut11, spNup40, spNup44, spNup45, spNup61, spNup82, spNup85, spNup107, spNup120, spNup124, spNup131, spNup132, spNup146, spNup189c, spPom152, and spSeh1) were tagged with GFP-HA at their C-termini in a previous study.[43] In this study, two nucleoporins (spEly5 and spTts1) were tagged with GFP-HA at their C-termini by a two-step PCR method described previously:[43,68] these 18 GFP-HA tagged nucleoporins are denoted "-GFP" in the text. Ten nucleoporins (spNpp106, spNup155, spNup184, spNup186, spNup211, spAmo1, spNup37, spNup60, spNup189n, and spPom34) were tagged with GFP alone at their C-termini. spNup97 and spNsp1 were tagged with GFP at their N-termini: tagging these nucleoporins at the C-terminus was unsuccessful. In addition, spNup40, spNup124, spNup131, and spNup132 were also tagged with GFP at their N-termini: the fluorescence intensities of the C-terminus GFP-tagged proteins

were weak and tagging with GFP at their termini N-termini increased the fluorescence signal. For spNup189n, we inserted an additional copy of the promoter sequence of spNup189 into the 3′ end of the marker module to ensure the physiological expression of downstream spNup189c. The GFP fusion constructs for each nucleoporin are summarized in **Table S1**. The pFA6a-mCherry-hphMX6 plasmid was used for tagging spCut11 with mCherry using the same PCR-based method as for tagging with GFP.[69]

Gene disruption

Gene disruption was performed with an *ura4*$^+$ marker using PCR-based gene targeting methods.[50] A diploid strain (*h*$^+$/*h*$^-$ *ade6-210/ade6-216 ura4-D18/ura4-D18 leu1-32/leu1-32 lys1-131/lys1-131*) was used as the host strain. Diploid cells heterozygously deleted for a nucleoporin gene were induced to sporulate, and tetrad spores were dissected. Four viable spores in a tetrad set indicated that the nucleoporin gene was dispensable; two viable spores in a tetrad set indicated that the gene was essential.

To examine the importance of spNup189n and spNup189c in vegetative growth, a single entire ORF of the *nup189*$^+$ gene was deleted in the diploid host strain, and then either *nup189n* or *nup189c*, or both, was integrated at the *lys1*$^+$ or *aur1*$^+$ locus. The spores generated in the diploid cell were dissected by micromanipulation and examined for cell growth. All of the haploid progeny were examined for ectopically-integrated spNup189n or spNup189c by checking their marker genes.

Image acquisition of GFP-nucleoporins by fluorescence microscopy

Living cells were mounted between coverslips.[70] A DeltaVision microscope system (Applied Precision, Inc) based on an Olympus wide-field fluorescence microscope IX70 (Olympus Corp) was used for imaging. Images were obtained using an interline CoolSNAP HQ^2 CCD camera (Photometrics, Tucson, USA) as an image detector through an oil-immersion objective lens (PlanApo 60×, NA, 1.4, Olympus). For measurement of fluorescence intensities, an oil-immersion objective lens (UApo 40×, NA, 0.65–1.35, Olympus) was used at NA = 0.65 (see below). Z-stack images were obtained at 0.4 μm interval for 8 Z-steps, and subjected to deconvolution to improve FM images by removing out-of-focus images.[71]

Estimation of nucleoporin amount based on fluorescence intensity

For measurement of fluorescence intensities, images of the cells expressing each GFP-nucleoporin were taken using a DeltaVision system as described above, using an oil-immersion objective lens (UApo 40×, NA, 0.65–1.35, Olympus) at NA = 0.65; simultaneously, images of the cell expressing spNup82-GFP were also taken as a control. The best-focused image of the eight optical sections was selected for quantification. The total fluorescence intensity of each nucleus was calculated as the sum of the intensities located in the nuclear region on the raw image using the SoftWoRx software equipped with the DeltaVision microscope system. The intensity values were normalized with that of spNup82-GFP. Fluorescence intensities of about 50 cells were measured in each strain, and average values were calculated after background subtraction. spNup85-GFP fluorescence intensity was set to two in calculating relative fluorescence intensities.

Western blot analysis

For quantitative western blot analysis, yeast whole cell extracts were prepared as follows. Yeast cells were cultured to log phase and incubated with 1 mM PMSF in the culture medium for 10 min. Five × 10^7 cells were then harvested and resuspended in 800 μl ice-cold water. The cell suspension was mixed with 150 μl of 1.85 N NaOH and incubated for 10 min on ice. Then, 150 μl of 55% (w/v) trichloroacetic acid was added and the mixture was incubated for 10 min on ice. The cells were centrifuged at 10 000 rpm for 2 min at 4 °C, and the pellet was washed with cold acetone. The pellet was then resuspended in sample buffer (200 mM Tris [pH 6.8], 8 M urea, 5% SDS, 0.1 mM EDTA, 100 mM DTT) and used as whole cell extract. Volume equivalents for cell number were calculated, and three different amounts of extract were applied and run by 8% SDS-PAGE. Proteins were transferred to a PVDF membrane in 25 mM Tris, 192 mM glycine, 10% methanol by wet transfer. The membrane was blocked with 5% skim milk in PBS containing 0.1% Tween20 and incubated with a rabbit polyclonal anti-GFP antibody (1:4000; Rockland Immunochemicals Inc) for 2 h at room temperature. The membrane was washed with PBS three times, and then incubated with secondary antibodies (1:2000) for 2 h at room temperature: HRP-conjugated goat anti rabbit IgG (GE Healthcare) was used as the secondary antibody. To ensure equal loading, the same membranes were reprobed for endogenous spNup189n using a mouse monoclonal anti-Nup98 antibody (13C2; 1:10 000)[45] and HRP-conjugated goat anti-mouse IgG as described above. For quantitative western blot analysis, the band intensity of spNup189n was used for normalization. Protein bands were detected by chemiluminescence using ChemiDoc MP imaging system (Bio-Rad), and band intensities were measured using Image Lab software (Bio-Rad). Band intensity of spNup85 was set to two in calculating relative band intensities.

Measurement of nucleoporin amounts per a single NPC

Living cells were mounted in a glass-bottom culture dish coated with 2 mg/mL concanavalin A[70] and were imaged with HILO microscopy as previously described.[46] A solid-state laser (488 nm, 20 mW; Sapphire 488-20-OPS, Coherent) and an inverted microscope (IX-81, Olympus) equipped with an oil-immersion objective lens (PlanAPO 100×, NA 1.45, Olympus) were used. Images were captured with a back-thinned electron multiplying charge-coupled device camera (C9100-13; Hamamatsu Photonics) at a frame rate of 30 frames per second, and recorded on a digital hard-disk recorder (AQUACOSMOS, Hamamatsu Photonics). Fluorescence intensities were measured using Image J software.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/nucleus/article/28487

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