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Pml39, a Novel Protein of the Nuclear Periphery Required for Nuclear Retention of Improper Messenger Ribonucleoparticles[D]

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Using a genetic screen, we have identified a previously uncharacterized *Saccharomyces cerevisiae* open reading frame (renamed *PML39*) that displays a specific interaction with nucleoporins of the Nup84 complex. Localization of a Pml39-green fluorescent protein (GFP) fusion and two-hybrid studies revealed that Pml39 is mainly docked to a subset of nuclear pore complexes opposite to the nucleolus through interactions with Mlp1 and Mlp2. The absence of Pml39 leads to a specific leakage of unspliced mRNAs that is not enhanced upon *MLP1* deletion. In addition, overexpression of *PML39-GFP* induces a specific trapping of mRNAs transcribed from an intron-containing reporter and of the heterogenous nuclear ribonucleoprotein Nab2 within discrete nuclear domains. In a *nup60*Δ mutant, Pml39 is mislocalized together with Mlp1 and Mlp2 in intranuclear foci that also recruit Nab2. Moreover, *pml39*Δ partially rescues the thermosensitive phenotypes of messenger ribonucleoparticles (mRNPs) assembly mutants, indicating that *PML39* deletion also bypasses the requirement for normally assembled mRNPs. Together, these data indicate that Pml39 is an upstream effector of the Mlps, involved in the retention of improper mRNPs in the nucleus before their export.

INTRODUCTION

Compartmentalization of eukaryotic cells allows the physical separation of the genetic material from its sites of expression into proteins. Part of the nucleocytoplasmic flux of genetic information is mediated by messenger RNAs (mRNAs), which are exported from the nucleus through nuclear pore complexes (NPCs). mRNA export to the cytoplasm exhibits unique features that distinguishes it from other transport pathways. First, export of most mRNAs is not directly dependent on the nucleocytoplasmic gradient of the small GTPase Ran. Second, the mRNA is not exported as such but packaged within a battery of proteins—the so-called heterogenous nuclear ribonucleoprotein (hnRNPs)—giving rise to the messenger ribonucleoparticle (mRNP). Finally, mRNA export is tightly coupled with transcription and with the different posttranscriptional processing events, e.g., 5′-capping, splicing, 3′-cleavage, and polyadenylation (reviewed in Jensen et al., 2003; Rodriguez et al., 2004). For example, the yeast shuttling mRNA-binding protein Nab2, is required both for poly-A tail length control and proper mRNA export (Hector *et al*., 2002). In addition, cotranscriptional loading of a set of proteins onto the pre-mRNA facilitates the subsequent binding of mRNA export factors. In yeast, the THO complex (Hpr1–Tho2-Mft1–Thp2), associated with actively transcribed genes, recruits the mRNA export adaptors Yra1 and Sub2 within a large conserved multiprotein complex called TREX (for transcription and mRNA export; Strasser *et al*., 2002). As an alternative pathway, the Sus1 protein, a component of the SAGA histone acetylase complex, is able to bind the Sac3–Thp1 complex required for efficient mRNA export (Rodriguez-Navarro *et al*., 2004).

mRNP assembly systematically concludes in the recruitment of the conserved mRNA export receptor Mex67/Mtr2 (TAP/p15 in vertebrates; Segref *et al*., 1997). Binding of Mex67 to the mRNP is thought to depend on direct interactions with alternate adaptors, mainly Yra1 (Strasser and Hurt, 2000), but also Sac3 (Fischer *et al*., 2002) or the SR-like hnRNP Npl3 (Gilbert and Guthrie, 2004). In turn, Mex67-Mtr2 is able to dock export competent mRNPs to the NPC through interaction with a subset of nucleoporins characterized by their repeated phenylalanine-glycine (FG) motifs (reviewed in Rodriguez *et al*., 2004). In addition, mRNA export requires some nucleoporins devoid of FG motifs, possibly involved in the integrity of NPC architecture. One such structural building block is the conserved Nup84 NPC subcomplex, made of seven subunits in budding yeast (Nup120, Nup85, Nup145-C, Nup84, Nup133, Seh1, and a fraction of Sec13) (Lutzmann *et al*., 2005, and references therein); indeed, yeast strains deleted for most of the sub-

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Abbreviations used: FISH, in situ fluorescence hybridization; mRNP, messenger ribonucleoparticle; NPC, nuclear pore complex; SPB, spindle pole body.

units of the complex display mRNA export defects. The last step of mRNA export is the delivery of the mRNP particle to the cytoplasm, and it requires Dbp5, a conserved helicase associated with the cytoplasmic face of NPCs that could provoke the remodeling of the exported mRNP and consequently ensure the directionality of the process (reviewed in Rodriguez *et al*., 2004).

To prevent the nuclear export and translation of mRNAs carrying defects in their nucleotide sequence and/or in their packaging into mRNPs, eukaryotic cells have evolved quality control mechanisms that prevent the synthesis of dysfunctional proteins (reviewed in Jensen *et al*., 2003 and Vinciguerra and Stutz, 2004). For example, nonsense-mediated mRNA decay (NMD) allows the elimination of mRNA-harboring defects such as premature stop codons. In the nucleus, the completion of mRNA surveillance is partially performed by Rrp6, an exonuclease specific for nuclear exosome and involved in the degradation of unprocessed mRNAs as well as aberrant mRNPs (Hilleren *et al*., 2001). Another aspect of mRNA surveillance is the active retention of intron-containing mRNAs in the nucleus. In budding yeast, *cis*- and *trans*-acting mutations affecting the splicing process were first shown to enhance pre-mRNA export in the cytoplasm (Legrain and Rosbash, 1989). In addition, genes involved specifically in pre-mRNA retention but not in splicing, were identified. One of them, *MSL5*, encodes a protein recognizing intronic sequences at the level of the branchpoint (BP) (Rutz and Seraphin, 2000). More recently, a biochemical purification of splicing complexes led to the identification of the trimeric *re*tention and *s*plicing (RES) complex. Interestingly, deletion of one of its subunits, encoded by the *PML1* gene, gave rise to an mRNA leakage phenotype without affecting the splicing process (Dziembowski *et al*., 2004). Finally, at the level of the nuclear envelope barrier, the NPC-associated protein Mlp1 (myosin-like protein 1) is required for nuclear retention of intron-containing mRNAs (Galy *et al*., 2004). In addition, genetic and physical interactions between Mlp1 and its paralogue Mlp2, and the mRNP components Yra1 and Nab2, have revealed the more general involvement of Mlps in the nuclear retention of improperly assembled mRNPs (Green *et al*., 2003; Vinciguerra *et al*., 2005). These data strongly emphasize the importance of quality control steps before the export of mRNAs out of the nucleus.

Here, we report the functional characterization of a nonessential open reading frame (ORF) of *Saccharomyces cerevisiae*, *YML107c*, identified on the basis of a strong genetic interaction with constituents of the Nup84 complex. We demonstrate that this protein, renamed Pml39 (for *p*re-*m*RNA *l*eakage; 39 kDa), is anchored by the Mlp1-2 proteins to a subset of NPCs, facing the chromatin. The *pml39* deletion mutant exhibits unspliced mRNA leakage and genetic interactions with mutations affecting the mRNP assembly factors *YRA1* and *NAB2*. Conversely, Pml39 overexpression leads to a specific trapping of the Nab2 hnRNP as well as mRNAs transcribed from intron-containing genes within discrete nuclear foci. Together, our data demonstrate that the Pml39 protein is an upstream effector of the Mlp-controlled pathway required for retention of improper mRNPs.

MATERIALS AND METHODS

Media and Growth Conditions

Yeast growth in standard YPD or SC media, transformation, mating, and sporulation were performed as described previously (Loeillet *et al*., 2005). Plasmid shuffling was carried out on SC plates containing 50 mg/l uracil and 1 g/l 5-fluoroorotate (Toronto Research Chemicals, North York, Ontario, Canada). For gene induction, 2% glucose or galactose was added to cells cultured in glycerol-lactate (0.17% YNB, 0.5% ammonium sulfate, 0.05% glucose, 2% lactate, and 2% glycerol) supplemented with the required nutrients. Except when indicated, cells were grown at 30°C.

Yeast Strains and Plasmids

The genotypes and origins of the strains used are listed in Supplemental Table 1. All strains are isogenic to S288c, except the *YRA1* and *NAB2* shuffle strains, which are W303 derivatives. Most strains were obtained by successive crosses between single-gene deletants obtained from EUROSCARF (Frankfurt, Germany) (Winzeler *et al*., 1999), green fluorescent protein (GFP)-tagged BY derivatives from the GFP collection (Huh *et al*., 2003; purchased from Invitrogen, Carlsbad, CA), and red fluorescent protein (RFP) reference strains kindly provided by Won-Ki Huh (Seoul National University, Korea). Complete ORF deletion and GFP or monomeric red fluorescent protein (mRFP) tagging at the locus were achieved through homologous recombination using cassettes amplified from pFA6a derivatives (Longtine *et al*., 1998; Huh *et al*., 2003) or the pOM42 vector (Gauss *et al*., 2005). N-terminal GFP tagging was followed by Cre-Lox-mediated pop-out of the selection marker (Gueldener *et al*., 2002). Auxotrophy marker conversion was performed using KanMX::URA3 modifier (Loeillet *et al*., 2005) or other marker swap plasmids according to described procedures (Voth *et al*., 2003). Genotypes were checked by PCR and sets of isogenic strains were systematically considered for phenotypic analysis.

Plasmids used in this study and details of their construction are listed in Supplemental Table 2. Sequences of the primers used in this study are available upon request.

Synthetic Lethal and Two-Hybrid Screens

*nup133*Δ and *pml39*Δ synthetic genetic array screen were performed as reported previously (Loeillet *et al*., 2005) using MATα haploids from the EUROSCARF deletion collection. Candidates were further characterized by tetrad analysis.

The two-hybrid screen was carried out by a mating strategy using the FRYL genomic library as described previously (Fromont-Racine *et al*., 1997). Approximately 10^8 diploids were screened for histidine prototrophy and β-galactosidase activity. Genomic prey inserts from all positive diploids were characterized by direct sequencing. pACTII prey plasmids were rescued in *Escherichia coli* and transformed back in Y-187 yeast cells. Interactions were finally confirmed by mating followed by selection on histidine-free SC medium containing 5 mM 3-aminotriazole, and X-Gal lift assay.

Cell Imaging

Fluorescence in situ hybridization (FISH) was performed essentially as described previously (Long *et al*., 1995), using an equimolar mixture of o-LacZ1- and o-LacZ2 CY3-conjugated oligonucleotides. An ultimate 30-min wash in 0.5× SSC was included, and coverslips were finally mounted on Moviol (Calbiochem, San Diego, CA).

For live cell imaging, exponentially growing cells were washed twice, resuspended in minimal medium supplemented with the required nutrients, and mounted on a glass slide. Images were acquired as described previously (Bai *et al*., 2004; Loeillet *et al*., 2005) using the MetaMorph 6.2.6 software (Molecular Devices, Sunnyvale, CA) and were further processed using Adobe Photoshop CS (Adobe Systems, Mountain View, CA). Quantification of fluorescence intensity was performed using MetaMorph.

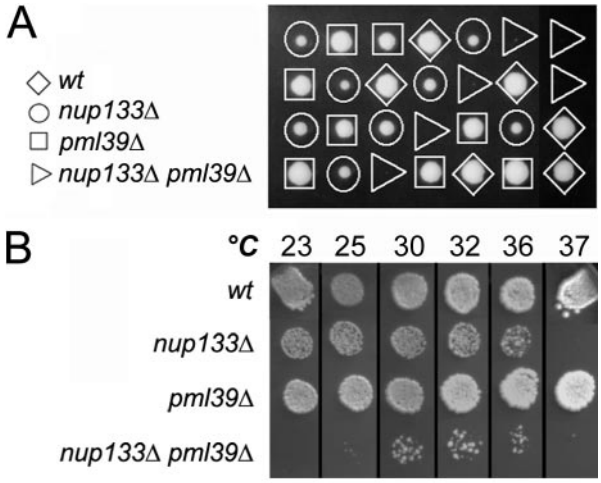
Splicing and Leakage Assays

All experiments were performed on pools of colonies. β-Galactosidase assays were performed as described previously (Legrain and Rosbash, 1989), except that yeast cells extracts were obtained by bead-beating in Z buffer followed by a 10-min clarification at 10,000 × *g*.

RESULTS

PML39 Encodes a Previously Uncharacterized Protein of the Nuclear Periphery and Displays a Strong Genetic Interaction with Members of the Nup84 NPC Subcomplex

In *S. cerevisiae*, Nup84 complex mutants display similar phenotypes, e.g., thermosensitivity, NPC aggregation within the nuclear envelope, mRNA export defects, and DNA double-strand break accumulation (Siniossoglou *et al*., 1996; Loeillet *et al*., 2005). To gain further insight into these processes, we undertook a systematic synthetic lethal screening of the collection of nonessential gene deletions using the *nup133*Δ mutation as a bait. Among the candidate genes of unknown function, one corresponded to the *YML107c* ORF, which encodes a 39-kDa protein localized to the nuclear periphery according to the general annotation of yeast protein local-



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nup120Δ	*pml39Δ*	strong synergistic
nup133Δ	*pml39Δ*	strong synergistic
nup84Δ	*pml39Δ*	n.d.
seh1Δ	*pml39Δ*	n.d.
nup133ΔN	*pml39Δ*	viable
pom34Δ	*pml39Δ*	viable
pom152Δ	*pml39Δ*	viable
nup42Δ	*pml39Δ*	viable
nup157Δ	*pml39Δ*	viable
nup170Δ	*pml39Δ*	viable
nup100Δ	*pml39Δ*	viable
nup2Δ	*pml39Δ*	viable
nup60Δ	*pml39Δ*	viable
mlp1Δ	*pml39Δ*	viable
mlp2Δ	*pml39Δ*	viable
mlp1Δ mlp2Δ	*pml39Δ*	viable
nup159-1	*pml39Δ*	viable

Figure 1. The *PML39* gene displays a strong genetic interaction with the Nup84 complex. (A) Tetrad analysis of the *nup133*Δ/+ *pml39*Δ/+ diploid. Spores were germinated at 30°C and genotypes were inferred from marker segregation analysis. (B) Growth properties of *wt*, *nup133*Δ, *pml39*Δ, and *nup133*Δ *pml39*Δ mutants. Equivalent amounts of cells were spotted and grown at the indicated temperatures. (C) Genetic interactions of *pml39*Δ mutation with other nucleoporins mutants. A *pml39*Δ strain (YV615) was used as a bait for a secondary synthetic lethal screening of nucleoporin deletants issued from the EUROSCARF *MATα* collection except *nup157*Δ (YV726) and *nup159-1* (LGY101). Interactions with *nup133*Δ*N* (YV529) and *mlp1*Δ *mlp2*Δ (YV726) mutants were scored by crosses with *pml39*Δ (Y16507). n.d., not determined; attempts to analyze these genetic interactions were not conclusive because of the poor germination and overall spore viability of the heterozygous diploids.

ization (Huh *et al*., 2003; http://yeastgfp.ucsf.edu/). We therefore initiated the characterization of this gene subsequently termed *PML39* (used hereafter).

The genetic interaction between *NUP133* and *PML39* deletion was first confirmed by sporulation of the *nup133*Δ/+ *pml39*Δ/+ heterozygote (Figure 1A). Marker segregation revealed that *nup133*Δ *pml39*Δ spores form microcolonies at 30°C and showed a strong growth defect at all temperatures assayed (Figure 1B).

To characterize the specificity of the interaction between *PML39* and *NUP133*, we conducted a secondary screen by combining the *pml39*Δ mutation with most of the nucleoporin disruption available in the EUROSCARF collection. Among these, only members of the Nup84 complex, *nup133*Δ and *nup120*Δ, displayed a strong synergistic interaction with *pml39*Δ (Figure 1C). The other members of the Nup84 complex could not be tested because their deletion is either lethal in this background (*NUP85*, *NUP145*, and *SEC13*) or leads to poor sporulation and germination of the heterozygous diploids (*pml39*Δ/+ *nup84*Δ/+, and *pml39*Δ/+ *seh1*Δ/+). In addition, no synergistic interaction could be scored between *pml39*Δ and *nup60*Δ, *sac3*Δ *or nup159-1* mutant strains (see Supplemental Figure 1, A and B) that strongly affect mRNA export (Gorsch *et al*., 1995; Fischer *et al*., 2002). This suggests that alteration of the mRNA export process is not sufficient to induce a synergistic interaction with *pml39*Δ. Finally, the *nup133*-Δ*N* separation-of-function mutant that only affects NPC distribution (Doye *et al*., 1994) did not show any genetic interaction with the *PML39* deletion. This indicates that Pml39, although functionally linked to the Nup84 complex, is unlikely to be involved in this process. Indeed, NPC distribution is not altered in *pml39*Δ cells (our unpublished data).

Pml39 Is Associated with a Subset of Nuclear Pore Complexes

In agreement with the previously annotated nuclear envelope localization of Pml39-GFP (Huh *et al*., 2003), fluorescence analysis of a similarly constructed *PML39-GFP* strain revealed a perinuclear staining (Figure 2A, left). This GFP fusion seemed to be functional because, unlike *nup133*Δ *pml39*Δ, the growth properties of *nup133*Δ *PML39-GFP* were comparable with those of *nup133*Δ (our unpublished data). To determine whether Pml39 is associated with nuclear pores, we used the classical assay of NPCs clustering in *nup133*Δ cells (Doye *et al*., 1994). As shown in Figure 2A (right), *nup133*Δ *PML39-GFP* cells displayed a dot-like staining characteristic of NPC clusters that occur in *nup133*Δ cells, indicating that Pml39 behaves as an NPC-associated protein. However, the Pml39-GFP staining was not continuous throughout the nuclear envelope in the wild-type context, and NPC aggregates labeled by the Pml39-GFP fusion in *nup133*Δ cells seemed to be less extended compared with the aggregates classically observed with other GFP-tagged nucleoporins.

To further characterize the NPC localization of Pml39, we constructed a *PML39-mRFP* strain and transformed it with a reporter plasmid encoding a fusion between GFP and the Nup49 nucleoporin. As shown in Figure 2B, Pml39-mRFP localization seemed to be restricted to a limited region of the nuclear envelope (NE), whereas the Nup49 staining was homogeneous throughout the whole nuclear periphery. Observation of a strain expressing, in addition to Pml39-GFP, the nucleolar protein Sik1 tagged with mRFP (Huh *et al*., 2003) revealed that Pml39 was only present in the portion of the nuclear envelope opposite to the nucleolus (Figure 2C). Such a U-shaped distribution within the nuclear envelope has already been reported for a few NPC-associated proteins, including Mlp1 and Mlp2 (Galy *et al*., 2004). Observation of Pml39-mRFP localization in *MLP1-GFP* or *MLP2-GFP* strains indicated that these proteins indeed colocalize within a subset of NPCs (Figure 6B, insets). Consistently, in a *nup133*Δ context, an Mlp2-GFP fusion localized within NPC aggregates with a similar pattern compared with Pml39-GFP (see Supplemental Figure 2).

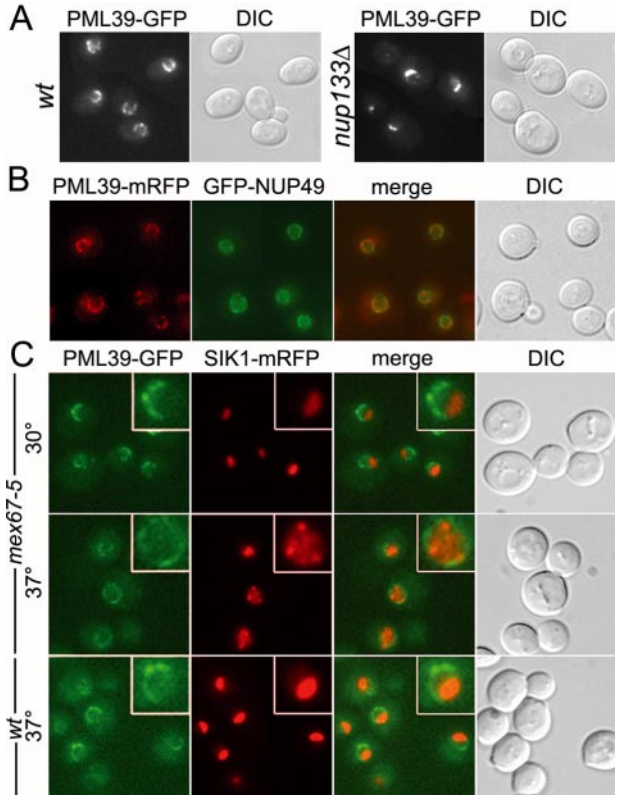

Figure 2. Pml39 is associated with a subset of nuclear pore complexes. (A) *PML39-GFP* (*wt*) and *nup133*Δ *PML39-GFP* strains were analyzed by fluorescence microscopy. Differential interference contrast (DIC) images are also shown. (B) The *PML39-mRFP* strain transformed with a GFP-Nup49-expressing plasmid was analyzed for localization of Pml39 relative to nuclear pores. (C) *PML39-GFP SIK1-mRFP*-expressing strains either *wt* or carrying the *mex67-5* mutation were grown at 30°C or shifted to 37°C for 30 min and examined for Pml39-GFP localization relative to the nucleolus (SIK1-mRFP). Insets show magnifications of typical nuclei. Overlay image of mRFP and GFP signals (merge) and DIC are also shown.

In an attempt to disrupt this restricted localization, we took advantage of the *mex67-5* mutation that induces nucleolar disintegration at restrictive temperature (Segref *et al*., 1997). In *mex67-5 SIK1-mRFP PML39-GFP* cells shifted for 30 min to 37°C, Pml39-GFP redistributed throughout the whole nuclear envelope, concomitantly with the nucleolar disruption, leading to the appearance of intranuclear Sik1-mRFP spots (Figure 2C). Hence, Pml39 nucleolar exclusion and retention in the chromatin neighboring area are dynamic processes.

Pml39 Is Anchored to NPCs through the Nup60/Mlp1-2 Pathway

To identify the nuclear pore components involved in the targeting of Pml39 to the NPC, we analyzed Pml39-GFP localization in various nucleoporin mutant strains. Mlp1 and Mlp2 were good candidates because they exhibit, like Pml39, a restricted NPC localization. As shown in Figure 3A, Pml39-GFP was lost from the nuclear envelope in *mlp1*Δ cells. A less pronounced phenotype was observed in a *mlp2*Δ background (arrowhead), whereas the *mlp1*Δ *mlp2*Δ double mutant also exhibited a total mislocalization of the protein. The differential effect of *MLP1* and *MLP2* deletions on Pml39-GFP localization prompted us to investigate the localization of Mlp1-GFP and Mlp2-GFP in *mlp2*Δ and *mlp1*Δ backgrounds, respectively. Whereas *MLP2* deletion did not affect Mlp1-GFP localization, the level of Mlp2-GFP present at the nuclear periphery was strongly reduced in *mlp1*Δ cells (Figure 3B). This may explain why *MLP1* deletion more efficiently mislocalizes Pml39 from the nuclear envelope, compared with the *mlp2*Δ context where Mlp1 is still able to recruit Pml39.

Mlp1 and Mlp2 are anchored to nuclear pores through interaction with Nup60 (Feuerbach *et al*., 2002; Galy *et al*., 2004). Consistently, in *nup60*Δ cells, Pml39-GFP staining was strongly reduced at the nuclear envelope and concentrated in one or two foci (Figure 3A; see below). In contrast, Pml39-GFP targeting to the nuclear periphery was not affected in the other nucleoporin mutants assayed, including *nup2*Δ, *pom34*Δ, *pom152*Δ, *nup100*Δ, *nup157*Δ, and *nup170*Δ (Figure 3A; our unpublished data). Finally, *PML39* deletion did not affect the nuclear envelope localization of Nup133-GFP, Nup49-GFP, Nup60-GFP, Mlp1-GFP, or Mlp2-GFP fusions (our unpublished data). Together, these data demonstrate that Pml39 is a peripheral NPC-associated protein physically anchored to their nuclear side by Mlp1 and Mlp2.

Interactions between Pml39 and Mlp1/Mlp2

To identify potential partners of Pml39, we used full-length Pml39 as a bait to screen a yeast genomic library by the two-hybrid technique (Fromont-Racine *et al*., 1997). Strikingly, out of 34 recovered genomic inserts able to mediate interaction with Pml39, most encoded N-terminal domains of either Mlp1 (14 clones, corresponding to 7 distinct fragments) or Mlp2 (13 clones, corresponding to 3 overlapping inserts). The only other candidate identified in the screen more than twice was Nup157 (4 clones, corresponding to 3 overlapping fragments). Because this nucleoporin has been recently identified in close physical relationship with the Nup84 complex (Lutzmann *et al*., 2005), it may represent an NPC component physically or functionally linked to Pml39. However, because *NUP157* deletion affects neither Pml39-GFP localization nor the viability of *pml39*Δ cells (our unpublished data), the relevance of this interaction was not further investigated.

Sequence analysis of the recovered Mlp1-2 inserts revealed two minimal domains of Mlp1 (N1, aa 7–143; N2, aa 287–584) and one domain of Mlp2 (aa 1–120) required for Pml39 interaction (Figure 3C). Conversely, to identify the regions of Pml39 required for Mlp proteins binding, N-terminal (aa 1–125) and C-terminal (aa 126–334) portions of Pml39, defined with help of the DOMCUT software (Suyama and Ohara, 2003), were cloned in the two-hybrid bait vector. Only a weak interaction was found between the C-terminal moiety of Pml39 and the Mlp1 287-584 (N2) domain (Figure 3C). In conclusion, this two-hybrid analysis demonstrates that Pml39 may bind Mlp1-2 by two different manners: the interaction with the extreme N-terminal region of Mlp1 (N1) or Mlp2 requires the whole Pml39 protein, whereas the more internal (N2) domain of Mlp1 weakly interacts with the C-terminal region of Pml39. However, the later interaction is not sufficient for Pml39 localization, because a fusion of the C-terminal moiety of Pml39 with GFP is not targeted to the nuclear envelope (our unpublished data).

PML39 Deletion Causes Unspliced mRNA Leakage from the Nucleus

Perinuclear Mlp1-2 have been reported to be involved in a variety of nuclear processes, including definition of transcriptionally inactive nuclear subdomains, maintenance of

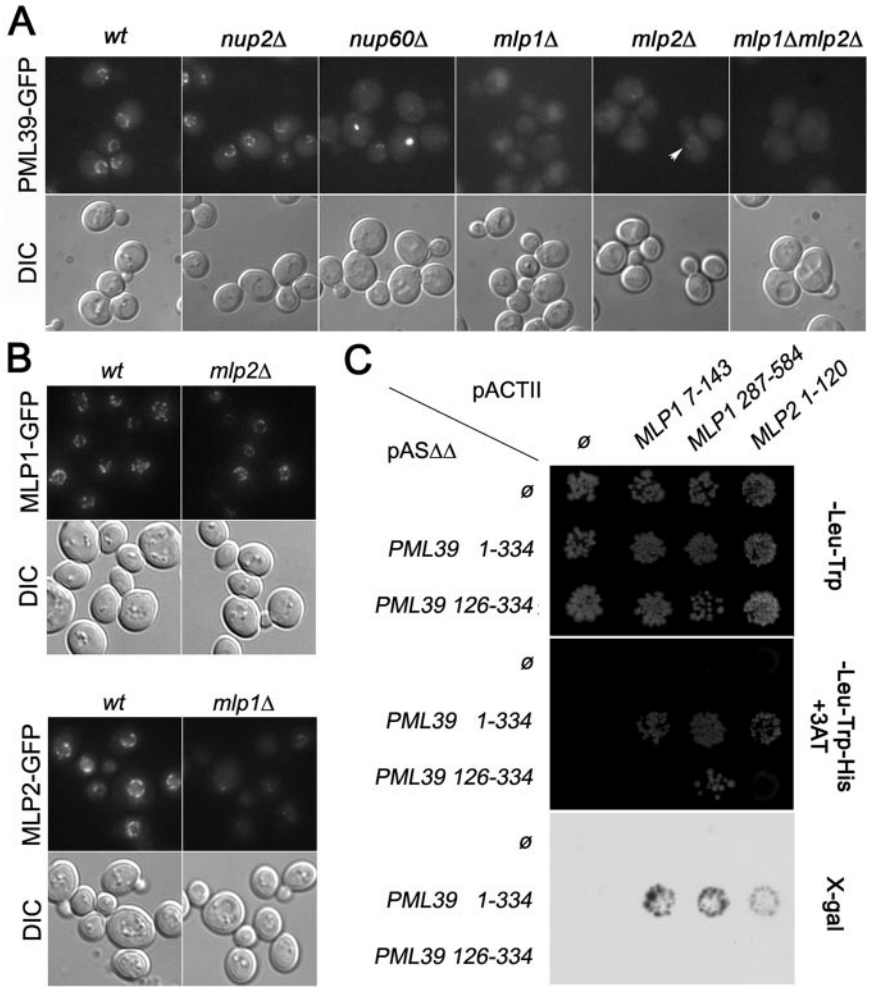

Figure 3. Pml39 is anchored to NPCs through interaction with Mlp1 and Mlp2. (A) *PML39-GFP* localization was examined in *wt*, *nup100*Δ, *nup2*Δ, *nup60*Δ, *mlp1*Δ, *mlp2*Δ, and *mlp1*Δ *mlp2*Δ backgrounds. (B) Mlp1-GFP and Mlp2-GFP localizations were examined in isogenic *wt* and *mlp2*Δ or *mlp1*Δ background, respectively. DIC images are also shown. (C) Two-hybrid interaction of Pml39 and Mlp1/Mlp2. CG1945 cells transformed with pASΔΔ bait vectors either empty (Ø) or encoding full-length Pml39 (aa 1–334) or its C-terminal moiety (aa 126–334) were mated with Y-187 cells transformed with pACTII prey vectors either empty (Ø) or encoding the indicated domains of Mlp1 or Mlp2. Diploids were grown on SC-Leu-Trp (top) or SC-Leu-Trp-His supplemented with 5 mM 3-aminotriazole (middle). Cells grown on SC-Leu-Trp plates were replicated on nitrocellulose membrane for β-galactosidase assay (bottom).

genomic stability, and control of telomere length (Feuerbach *et al*., 2002; Hediger *et al*., 2002). More recently, Mlp1-2 have been reported to be involved in a quality control step before the export of mRNPs (Green *et al*., 2003; Galy *et al*., 2004; Vinciguerra *et al*., 2005). In particular, *MLP1* deletion, although not affecting splicing or bulk mRNA export, gave rise to a specific leakage of intron-containing mRNAs out of the nucleus. In *pml39*Δ mutants, FISH using oligo(dT) probes did not reveal any global nuclear accumulation of poly(A)$^+$ RNA (our unpublished data). To determine whether Pml39 could be involved in unspliced mRNA retention, *pml39*Δ cells were transformed with reporter constructs previously used to monitor this process (Legrain and Rosbash, 1989; Rain and Legrain, 1997) (Figure 4A). In the splicing reporter (pJCR51), an intron disrupts the reading frame of the LacZ coding sequence, and only spliced mRNAs can be translated into a functional enzyme (Figure 4A, top). β-Galactosidase activity, normalized to the activity obtained with an intron-less control pLGSD5 plasmid, therefore, reflects the extent of splicing. In cells transformed with the unspliced mRNA leakage reporter plasmid (pJCR1), LacZ translation only occurs in the absence of splicing (Figure 4A, bottom). Normalized β-galactosidase activity thus reflects the export of unspliced mRNAs. This analysis revealed that neither the expression of the control intronless reporter (our unpublished data), nor the splicing process (Figure 4B, pJCR51) is significantly affected by *PML39* deletion. In contrast, the pJCR1 leakage reporter indicates that unspliced mRNA are exported and translated into β-galactosidase in *pml39*Δ cells to significant levels compared with wild-type (*wt*) cells (Figure 4B). The extent of pre-mRNA leakage was similar to the one occurring in an isogenic *mlp1*Δ strain (Figure 4B), indicating that Pml39 is also specifically involved in the nuclear retention of unspliced mRNAs.

Pioneer studies demonstrated that nuclear retention of pre-mRNA depends upon *cis*-elements within both the 5′ splicing site (5′SS) and the intron BP (Legrain and Rosbash, 1989). Accordingly, leakage of pre-mRNAs with weakened 5′SS or BP sequences is significantly enhanced in *wt* cells (Rain and Legrain, 1997; Figure 4B). It is noteworthy that the extent of leakage of these mutated splicing reporters was not increased further in *pml39*Δ cells compared with *wt* cells (Figure 4B). This suggests that *PML39*-mediated mRNA retention relies on the integrity of both 5′ splicing site and branchpoint. In our genetic background, only a modest, but statistically insignificant increase in mRNA leakage was observed with the mutBP reporter in *mlp1*Δ cells. This contrasts with previous studies, in which an additive effect of the *mlp1*Δ mutation on the mutBP reporter was observed, leading to the proposal that *MLP1*-mediated pre-mRNA retention is independent from an intact branchpoint (Galy *et al*., 2004). This discrepancy may reflect quantitative differences attributable to different genetic backgrounds. Indeed, the extent of the *mlp1*Δ leakage phenotype was slightly weaker in our study compared with the previously reported study. Consistently, although deletion of *PRP18*, which encodes a

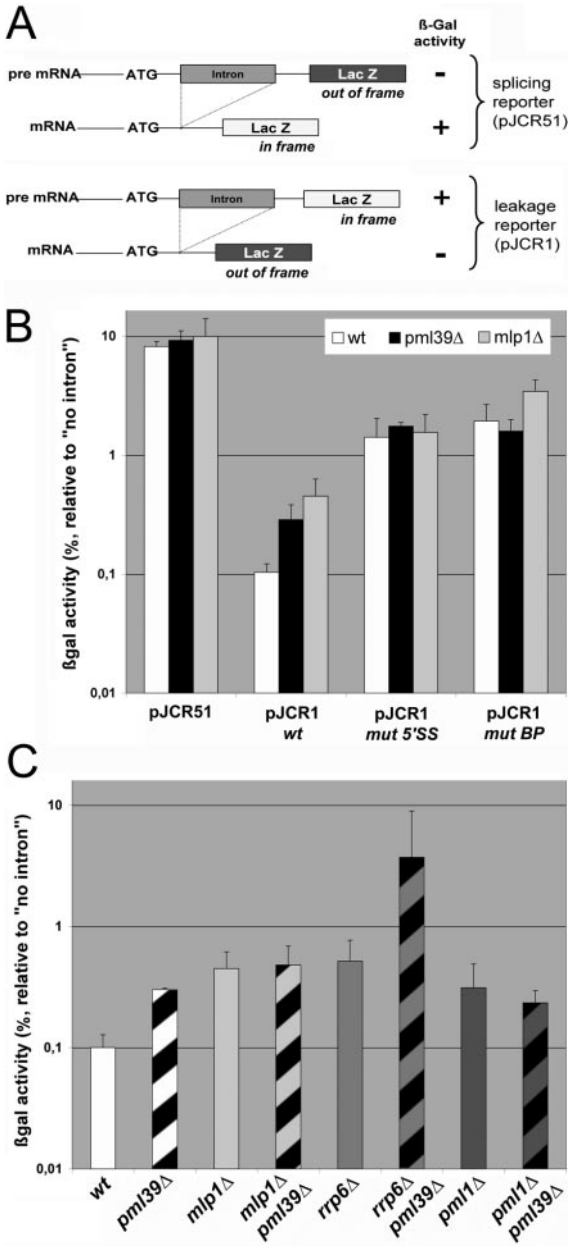

Figure 4. *PML39* deletion gives rise to a pre-mRNA leakage phenotype. (A) Schematic representation of the splicing (pJCR51) and leakage (pJCR1) reporters. Adapted from Rain and Legrain (1997). (B) Quantification of pre-mRNA splicing and leakage in *wt*, *pml39*Δ, and *mlp1*Δ mutants. Cells were transformed with pJCR51 or with pJCR1 either *wt* or mutated in its 5′ splicing site (*mut 5′SS*) or branchpoint (*mut BP*). For each construct, the percentage of β-galactosidase activity relative to the one obtained with the pLGSD5 intron-less control plasmid is represented. Values represent the means and standard deviations from five independent experiments. (C) pre-mRNA leakage in different mutant strains. *wt*, *pml39*Δ, *mlp1*Δ, *mlp1*Δ *pml39*Δ, *rrp6*Δ, *rrp6*Δ *pml39*Δ, *pml1*Δ, and *pml1*Δ *pml39*Δ isogenic strains carrying the pJCR1 *wt* reporter construct were assessed for pre-mRNA leakage as in B. Values arise from three independent experiments, and standard deviations are indicated.

factor required for splicing as well as pre-mRNA retention, was reported to be lethal in a *mlp1*Δ mutant background (Galy *et al*., 2004), both *prp18*Δ *mlp1*Δ and *prp18*Δ *pml39*Δ strains were fully viable in our genetic background.

Pml39, Mlp1, and Pml1 Are Involved in a Common Pre-mRNA Retention Pathway

Unspliced mRNA retention requires perinuclear Mlp1 (Galy *et al*., 2004) and a nucleoplasmic protein, Pml1 (Dziembowski *et al*., 2004). To determine whether Pml39 is involved in the same mRNA retention pathway as these factors, leakage was assayed in strains combining these mutations. *mlp1*Δ *pml39*Δ as well as *pml1*Δ *pml39*Δ double mutants did not exhibit an enhanced leakage compared with single mutants, demonstrating that Pml39, Mlp1 and Pml1 act in a common pathway of unspliced mRNA retention (Figure 4C).

The exosome subunit Rrp6 is an exonuclease involved in degradation of improper nuclear mRNAs (Hilleren *et al*., 2001). *RRP6* deletion increases nuclear pre-mRNA levels, independently of splicing (Bousquet-Antonelli *et al*., 2000), and therefore leads to an apparent leakage phenotype, probably because of an overall increase in unprocessed or inaccurately processed RNAs (Galy *et al*., 2004; Figure 4C). As reported previously for the *mlp1*Δ *rrp6*Δ double mutant, combination of *RRP6* and *PML39* deletions gave rise to a synergistic effect on the levels of translated and accordingly cytoplasmic unspliced mRNAs (Figure 4C). This indicates that Pml39 is also required for retention of pre-mRNA accumulated independently of any splicing defects. On the basis of these results, we have renamed the original *YML107c* ORF as *PML39* (for *p*re-*m*RNA *l*eakage, *39* kDa).

Overexpression of Pml39 Traps mRNA Transcribed from Intron-containing Genes in Discrete Nuclear Foci

Because *PML39* deletion gives rise to a leakage of unspliced mRNAs, we wondered whether its overexpression could lead to an enhanced mRNA retention within the nucleus. To test this hypothesis, the *PML39-GFP* fusion was overexpressed under the control of the GAL1 inducible promoter in a *pml39*Δ strain. As shown in Figure 5A, Pml39-GFP is properly addressed to the nuclear periphery after 30 min of induction and then accumulates in the whole nucleus, with many cells exhibiting one or two Pml39-GFP foci at the nuclear periphery (≈30% after 1 h of induction). At this time point, Pml39-GFP is 10- to 40-fold overexpressed compared with the same fusion driven by its endogenous promoter as determined by quantification of fluorescence intensities.

Then, plasmids carrying the LacZ gene containing or not an artificial intron upstream of its coding sequence (pLGSD5, without intron; pJCR51, with intron) were used to monitor the localization of specific transcripts by FISH. Cells were induced for production of both the LacZ transcripts and Pml39-GFP by a 2-h shift in galactose-containing medium. As reported previously (Long *et al*., 1995; Vinciguerra *et al*., 2005), LacZ mRNAs accumulate within a few nucleoplasmic foci probably corresponding to the sites of transcription (Figure 5B). Interestingly, the Pml39-GFP foci were found to colocalize with the LacZ mRNA-containing foci, an event that was significantly favored in the case of transcripts issued from intron-containing constructs (Figure 5, B and C). Additional LacZ-dependent FISH foci were frequently seen outside of the Pml39-GFP clusters, a fact that may reflect the presence of additional intranuclear retention sites or nondetectable Pml39-GFP clusters. In conclusion, overexpression of Pml39-GFP leads to its accumulation within discrete nuclear foci that are able to trap preferentially mRNAs issued from intron-containing genes.

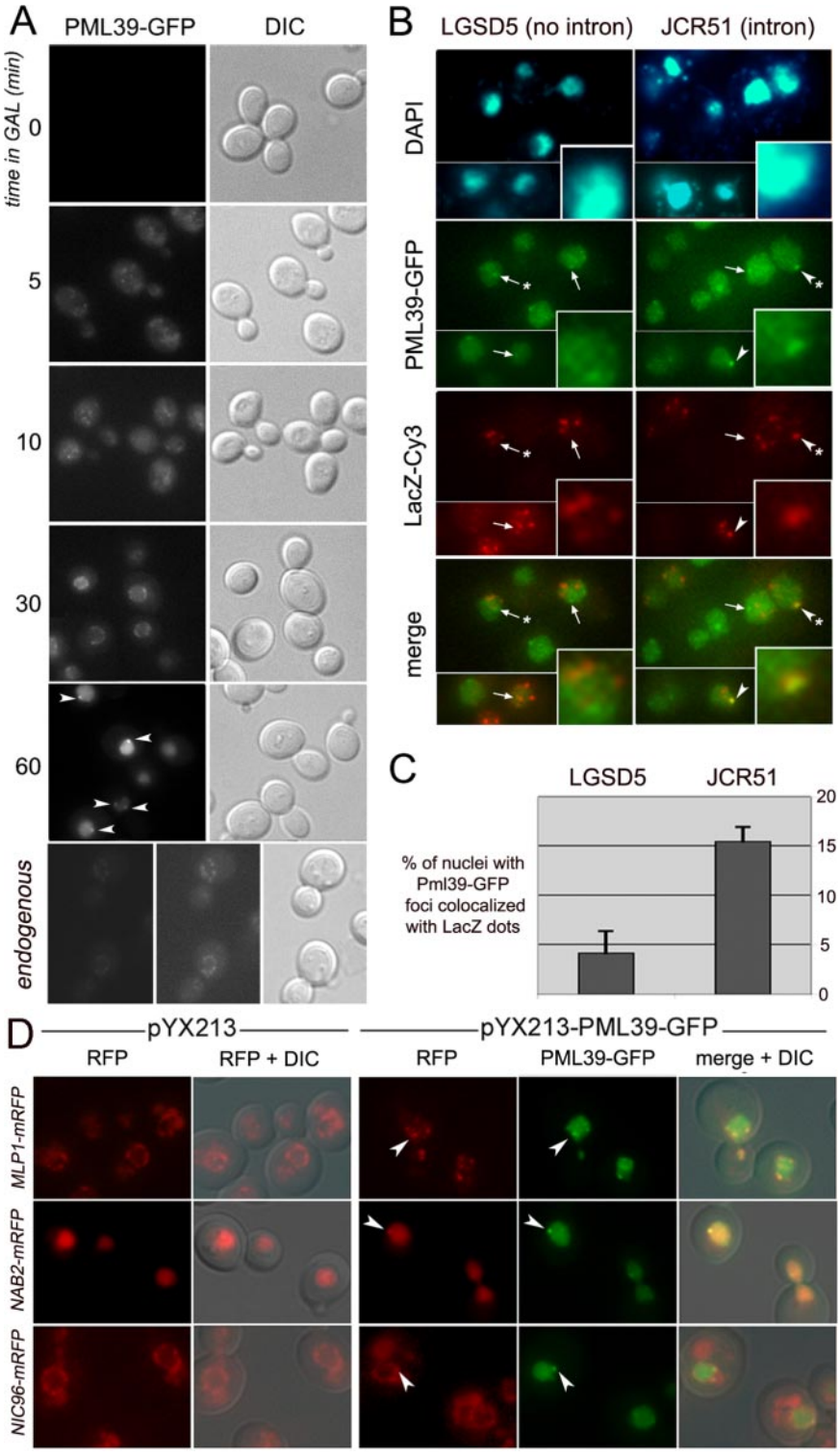

Figure 5. Analysis of PML39 overexpression. (A) *pml39*Δ cells transformed with the *pYX213-PML39-GFP* construct were induced in galactose for the time indicated (minutes) and analyzed for Pml39-GFP localization. Arrowheads point to Pml39-GFP foci. Cells expressing Pml39-GFP under the control of its *endogenous* promoter are shown as control (left, same acquisition time as for the other panels; middle, enhanced signal to show proper localization of the fusion). (B) FISH analysis of LacZ transcripts in Pml39-GFP-overexpressing cells. *pml39*Δ cells transformed with *pYX214-PML39-GFP* and either *pLGSD5* (intronless LacZ reporter) or *pJCR51* (intron-containing LacZ reporter) were induced for 2 h in galactose and analyzed for localization of LacZ transcripts by FISH using Cy3-conjugated probes specific to the LacZ sequences. GFP and Cy3 images are two-dimensional projections from z-stacks. DNA staining of the nuclei (4,6-diamidino-2-phenylindole) as well as overlay images of GFP and Cy3 signals (merge) are shown. Arrowheads and arrows respectively point to Pml39-GFP foci containing or not LacZ transcripts. Insets (bottom right corners) show a threefold magnification of nuclei exhibiting typical Pml39-GFP and LacZ transcripts localizations (indicated by a star on the original image). (C) Quantification of the colocalization events from B. The number of nuclei displaying a Pml39-GFP dot merged with one of the LacZ foci was counted among nuclei exhibiting detectable signals for both species. The values represents the means and standard deviations obtained from two independent experiments; total numbers of counted cells were 285 (pLGSD5) and 363 (pJCR51). (D) Analysis of Pml39-containing foci. Strains expressing mRFP-tagged versions of Mlp1, Nab2, or Nic96 were transformed with either *pYX213* or *pYX213-PML39-GFP* and Pml39 overexpression was induced in galactose for 1 h. Overlay images of GFP, mRFP signals, and differential interference contrast (DIC) are shown (merge + DIC). Arrowheads point to Pml39-GFP foci.

Pml39 Nuclear Foci Contain Mlp Nucleoporins and the mRNP Component Nab2

To determine whether Pml39-GFP was able to trap specific proteins in the foci occurring upon its overexpression, the protein expression was induced in strains carrying various mRFP fusions. As shown in Figure 5D, Pml39 overexpression disturbed the nuclear envelope localization of Mlp1 and provoked its accumulation in the nuclear Pml39-GFP foci. A similar observation was made in Nab2, an mRNA export factor shown to associate with Mlp1 (Green *et al*., 2003), which was also partially recruited to the Pml39-GFP foci upon Pml39 overexpression. This last phenomenon was not observed in all cells, possibly reflecting a dependence on the level of Pml39 overexpression (Figure 5D). The recruitment of Mlp1 and Nab2 to Pml39 foci was specific because an unrelated nucleoporin, Nic96, was unaffected by the Pml39

overexpression and was not recruited into the Pml39-GFP foci. This result further indicates that these foci do not correspond to NPC aggregates (Figure 5D, bottom).

As mentioned above, Pml39-GFP also accumulates within bright foci in *nup60*Δ cells (Figure 3A). Analysis of various strains deleted for *NUP60* and coexpressing a *PML39-mRFP* fusion together with different nuclear GFP fusions confirmed this localization and further indicated that the Pml39 foci are localized at the nuclear periphery (Figure 6B). These foci did not colocalize with the nucleolus labeled with Sik1-mRFP (Figure 6A). Although functional links have recently been suggested between spindle pole bodies (SPBs) and NPCs (Fischer *et al*., 2004; Niepel *et al*., 2005), the Pml39-GFP foci did not merge with the Spc42-mRFP labeled SPBs (Figure 6A).

These Pml39 foci were reminiscent to the Mlp1-2 localization in *nup60*Δ cells (Feuerbach *et al*., 2002; Galy *et al*., 2004) and indeed colocalized with the Mlp1-GFP and Mlp2-GFP foci occurring upon *NUP60* deletion (Figure 6B). Such a localization was recently demonstrated to be shared by another Mlp1-2-anchored protein, Mad1 (Scott *et al*., 2005). In contrast, although the NE localization of another Mlp-interacting protein, the ubiquitin-like protease Ulp1 (Zhao *et al*., 2004), was altered in *nup60*Δ cells, Ulp1-GFP was not enriched in the Pml39-mRFP foci (our unpublished data). This may reflect its previously reported degradation (Zhao *et al*., 2004), and/or the presence of additional Ulp1 binding sites at the NPC (Panse *et al*., 2003). Finally, a fraction of Nab2-GFP was also recruited within the Pml39-mRFP foci. In contrast, no detectable recruitment of other mRNA export factors components (Yra1 and Npl3) or known pre-mRNP retention factors (Msl5 and Pml1) could be observed (Figure 6B; our unpublished data).

In summary, upon its overexpression or its mislocalization due to *NUP60* deletion, Pml39 localizes within perinuclear foci containing Mlp1-2 and Nab2. This result emphasizes the link existing between Mlp1-2 and Pml39 and further strengthens the connection between mRNPs assembly factors—such as Nab2—and the Mlp–Pml39 complex.

PML39 Deletion Bypasses the Requirement for mRNPs Assembly Factors

The previous result suggested a link between Pml39 and mRNPs components. Interestingly, recent data established that *MLP1* or *MLP2* deletions can rescue the temperature-sensitive phenotypes of mRNPs mutants such as *GFP-yra1-8* or Δ*N-nab2* alleles (Vinciguerra *et al*., 2005). We therefore analyzed the effect of *PML39* deletion on the growth properties of both mutants. In the *pml39*Δ mutant, the thermosensitive *GFP-yra-1-8* mutation was suppressed, allowing growth of the cells at the restrictive temperatures of 34 and 37°C, to the same extent as in a *mlp2*Δ mutant (Figure 7A). Similarly, growth of the Δ*N-nab2* mutant was substantially improved at 30 and 37°C in the *pml39*Δ context (Figure 7B). The *PML39* deletion is therefore able to rescue sublethal phenotypes of mRNPs mutants, indicating that it can bypass the requirement for normal mRNPs assembly factors.

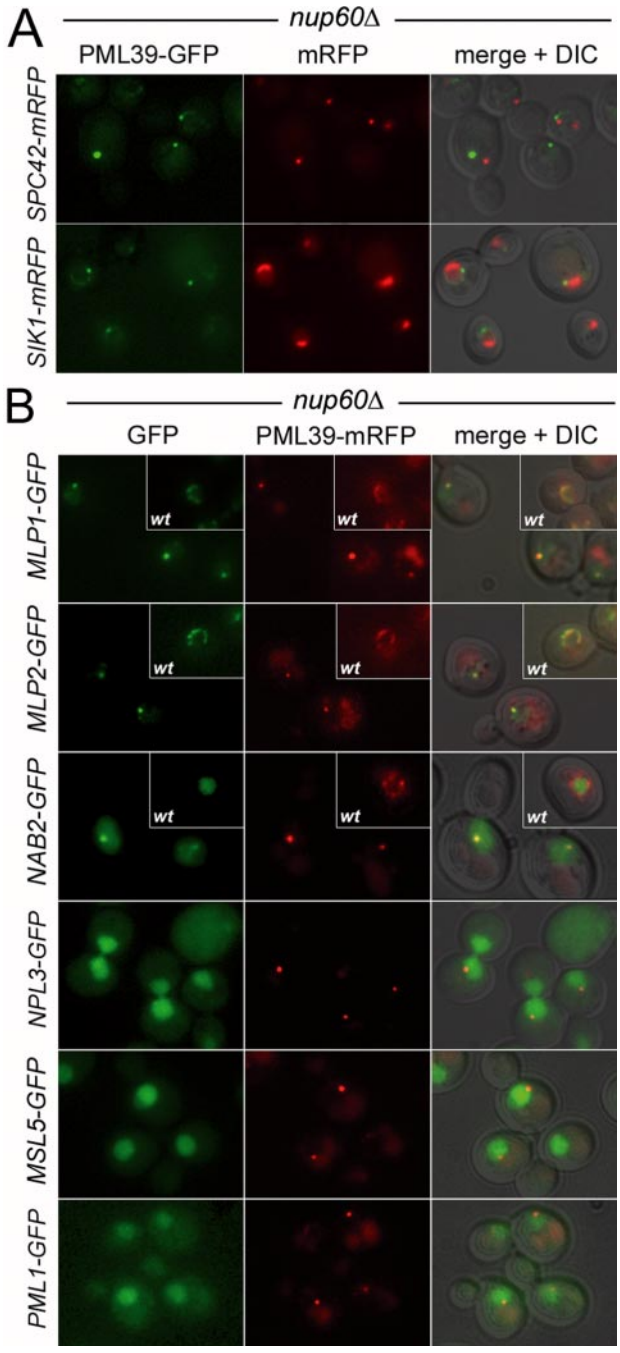

Figure 6. Pml39 form nuclear foci upon *NUP60* deletion. (A) Fluorescence microscopy analysis of *PML39-GFP nup60*Δ cells expressing either Spc42-mRFP (SPBs) or Sik1-mRFP (nucleolus). (B) The localization of GFP-tagged Mlp1, Mlp2, Nab2, Npl3, Msl5, or Pml1 was analyzed in *PML39-mRFP nup60*Δ cells. Insets show the localization of the GFP-tagged protein and of Pml39-mRFP in a *wt* context. Overlay images of GFP, mRFP signals and differential interference contrast (DIC) are shown.

DISCUSSION

Asymmetric NPC Anchoring of Pml39 Is Mediated by Mlp1-2

Using a systematic synthetic lethal screen, we have identified a novel NPC-associated protein that we named Pml39, because the deletion of the corresponding gene affects pre-mRNA retention. Our data demonstrate a physical connection between Pml39 and Mlp1-2: 1) these proteins share a restricted U-shaped NPC localization opposite to the nucleolus and colocalize into perinuclear foci upon *NUP60* deletion; 2) overexpression of Pml39 leads to its nuclear accumulation, a feature anticipated for a nucleoporin localized on the nuclear side of the NPCs; under these conditions,

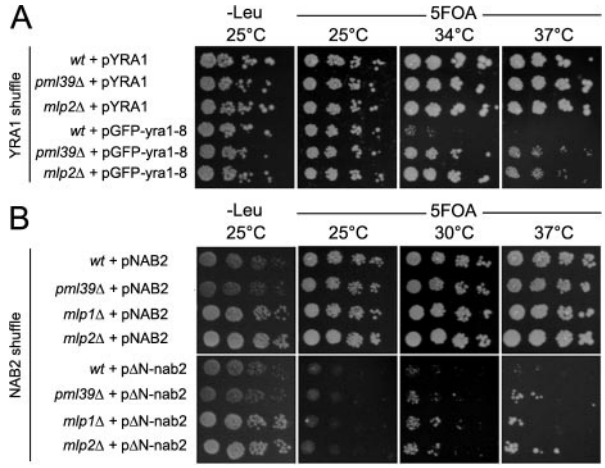

Figure 7. *PML39* deletion rescues the temperature-sensitive phenotypes of *GFP-yra1-8* and Δ*N-nab2* mutants. (A) The *YRA1* shuffle strains as such or deleted for *PML39* or *MLP2* were transformed with the *LEU2*-containing wt *YRA1* or *GFP-yra1-8* plasmids. Transformants were spotted as fivefold dilutions on 5-fluoroorotic acid to select against the wt pURA3-*YRA1* plasmid or on −Leu as control. Plates were incubated at 25, 34, or 37°C. (B). The *NAB2* shuffle strains as such or deleted for *PML39*, *MLP1*, or *MLP2* were transformed with the *LEU2*-containing wt *NAB2* or Δ*N-nab2* plasmids. Growth of the transformants was analyzed as in A.

Pml39 accumulates within intranuclear foci able to specifically recruit a fraction of Mlp1; 3) the N-terminal domains of Mlp1 and Mlp2 were frequently found in a two-hybrid screen using Pml39 as a bait (27 of 34 candidates); 4) deletion of Mlp1, and to a lesser extent of Mlp2, leads to the mislocalization of Pml39, whereas neither Mlp1 nor Mlp2 is mislocalized in *pml39*Δ cells, indicating that Mlp1-2 anchor Pml39 to NPCs. Unlike Mlp1 and Mlp2, Pml39 was not previously identified in proteomic studies of the yeast NPCs (Rout *et al*., 2000). Its Mlp-dependent association with NPCs might thus be either transient or unstable under biochemical purification conditions. Indeed, we were not so far able to confirm these interactions under classical immunoprecipitation conditions. Nevertheless, our data establish that Pml39 is anchored to a subset of NPCs, through interaction with the N-terminal domains of Mlp1 and Mlp2.

Upon nucleolar fragmentation, Pml39, and most likely its NPC-anchoring determinants Mlp1-2, are redistributed throughout the whole nuclear periphery. This could reflect either the dynamic exchange of these proteins between NPCs or the redistribution of a specific subset of NPCs by lateral diffusion. The fact that Mlp1 remains asymmetrically localized in a *pml39*Δ mutant (our unpublished data) indicates that Pml39 is not directly involved in this process. However, recent studies have established functional links between NPC constituents, including Mlps, and the transcriptional machinery (Vinciguerra and Stutz, 2004, and references therein). NPCs facing the transcribed chromatin may thus be specialized in the export of specific mRNPs and subsequently in the quality control steps required before their export.

Plm39 Is an Upstream Effector of Mlp1 and Mlp2 in the Retention of Improper mRNP Particles

In addition to its Mlp-dependent NPC anchoring, our data demonstrate that Pml39 is involved in the Mlp-dependent pre-mRNPs retention process (Figure 8): 1) both *PML39* and *MLP1* deletions lead to similar levels of unspliced mRNA translation that are enhanced in the absence of Rrp6, a nuclear component of the exosome. Conversely, combined deletion of *PML39* and *MLP1* does not increase leakage, suggesting that they act in a common pathway of pre-mRNA retention; 2) both Pml39 and Mlp1 overexpression lead to the specific retention of mRNA transcribed from intron-containing genes (Galy *et al*., 2004; this study); 3) loss of Mlp1, Mlp2, or Pml39 proteins notably improves the survival of mRNP assembly mutants such as *GFP-yra1-8* or Δ*N-nab2*. This suggests that the Mlp/Pml39-dependent inhibition of improper mRNP export becomes fatal when the proportion of such mRNPs is severely increased. It is noteworthy that deletion of *PML39* allows the survival of *GFP-*

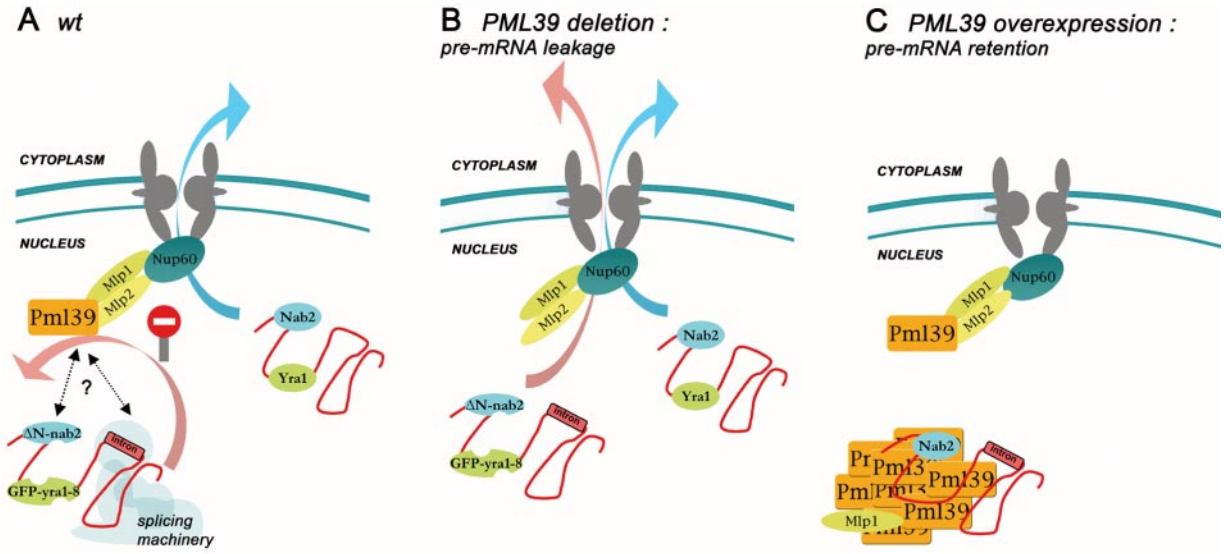

Figure 8. Model for Pml39 function in improper mRNP retention in the nucleus. The mRNP trafficking is represented in the *wt* situation (A), upon *PML39* deletion (B) or overexpression (C). Black dotted arrows point to potential interactions between Pml39 and the mRNP or the splicing machinery.

yra1-8 cells at 37°C, a feature shared by Mlp2 but not Mlp1 (Vinciguerra *et al*., 2005; this study). Because Mlp2 is partially mislocalized in *mlp1*Δ cells (Figure 3), Mlp2 could be the main actor in a Yra1-sensitive mRNP surveillance process. In contrast, Mlp2 does not seem to affect nuclear retention of unspliced mRNAs (Galy *et al*., 2004). Accordingly, our data indicate that Pml39 recapitulates both the Mlp1- and Mlp2-dependent phenotypes in terms of mRNA surveillance. In contrast, Pml39 does not seem to play a role in the Ulp1-dependent functions of Mlp1-2, such as clonal lethality caused by increased levels of 2μ circle DNA or defects in DNA repair (Zhao *et al*., 2004). In particular, unlike Mlp1-2, its disruption does not lead to the formation of nibbled colonies (our unpublished data). Considering the recently demonstrated SUMOylation of Ku70 (Zhao and Blobel, 2005), the other so far-reported Mlp functions in telomere length regulation, clustering, or silencing (Feuerbach *et al*., 2002; Hediger *et al*., 2002) may also involve the desumoylating enzyme Ulp1 rather than Pml39.

As anticipated for a gene solely involved in a checkpoint function, *PML39* deletion does not affect cell growth or viability in a wild-type context. In addition, despite the enhanced levels of unspliced cytoplasmic mRNA in *pml39*Δ *rrp6*Δ cells, genetic analyses did not reveal any synergistic interaction between *pml39*Δ and *rrp6*Δ. Similarly, *PML39* deletion did not impair the viability or the growth properties of NMD mutants cells (*xrn1*Δ, *upf1*Δ, *upf2*Δ, and *upf3*Δ; see Supplemental Figure 1C), indicating that cytoplasmic accumulation of unspliced mRNA occurring in *pml39*Δ cells does not affect cell viability when NMD is compromised. Yet, *PML39* function can be revealed in specific genetic backgrounds, such as (as unraveled in our screen) alteration of the Nup84 complex integrity. In this respect, our *nup133*Δ synthetic lethal screen identified, besides nucleoporins, double-strand break metabolism genes (Loeillet *et al*., 2005), and mRNA export factors (Sac3, Thp1, and THO complex genes; our unpublished data), several components of mRNA decay pathways including Rrp6. This suggests that affecting at the same time the bulk mRNA export process (*nup133*Δ) and a quality control step in mRNP export (*pml39*Δ) or decay (*rrp6*Δ) may be deleterious for the cell as the ratio of proper mRNAs reaching the cytoplasm becomes limiting. However, *NUP133* deletion is in contrast not synergistic with the deletion of another pre-mRNA retention factor, *PML1*. Because combination of nucleoporin mutations often gives rise to synthetic lethality, the association of Pml39 with NPCs might also contribute to its genetic interaction with *NUP133*. Yet, the molecular mechanisms underlying these genetic interactions are likely to be more complex, because *PML39* deletion does not affect the viability of *nup159-1* mutants, which also affects a nucleoporin involved in mRNA export.

Molecular Links between Pml39 and the mRNP Retention Pathway

Although we could demonstrate that Pml39 acts as an upstream effector of Mlp1-2 in their mRNA quality control function, the molecular mechanisms ensuring the recognition and trapping of improper mRNPs still remain to be unraveled. Because RNA binding motifs have not been found in Pml39 amino acid sequence, mRNA-associated proteins are likely to serve as landmarks to recruit Pml39 onto improper mRNPs. In this respect, the identification of Nab2 in intranuclear Pml39-containing foci is intriguing. Although our two-hybrid screen revealed that Pml39 interacts with the N-terminal domains of Mlp1 and Mlp2, Nab2 binds directly to the C-terminal domain of Mlp1 (Green *et al*., 2003). Mlp1 may therefore bind simultaneously Nab2 and Pml39 and thus mediate Nab2 recruitment in Plm39-containing foci.

Remarkably, the C-terminal region of Pml39 contains a short domain (aa 173–214) displaying 54% similarity with the unusual WW-domain of human HYPA/FBP11, a protein–protein interaction motif characterized by the presence of a central block of tyrosine residues ($Y_{189}Y_{190}$ in Pml39). In HYPA/FBP11, this domain mediates the interaction with proteins of the splicing machinery containing a proline-glycine-methionine-rich motif such as SF1, the human orthologue of yeast Msl5 (Bedford *et al*., 1998). Such a domain may thus be involved in targeting Pml39 to unspliced mRNAs through interactions with splicing factors. In contrast, the lack of synergism between *pml1*Δ and *pml39*Δ in term of pre-mRNA retention suggests that Pml1 could directly or indirectly interact with Pml39. Although we could not so far detect any stable interaction between Pml39 and Msl5 or Pml1, more systematic biochemical and microscopic studies, performed in various mutant backgrounds, might help in the future to define the molecular link between recognition of improper mRNPs and their Pml39-mediated retention.

Besides in closely related fungi, *PML39* seems to have diverged quickly. Yet, a putative orthologue, *rsm1*, was found in *Schizosaccharomyces pombe* genome. Interestingly, combination of the nonessential *rsm1* and *spmex67* deletions confers synthetic lethality accompanied by a severe mRNA export defect (Yoon, 2004). Although further functional studies of *rsm1* will be required to validate the relevance of this hypothesis, the Pml39 protein and its functions might have been conserved through evolution.

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