Intranuclear dynamics of the Nup107-160 complex

Stéphanie Morchoisne-Bolhy, Marie-Claude Geoffroy, Imène B. Bouhlel, Annabelle Alves, Nicolas Audugé, Xavier Baudin, Kevin Van Bortle, Maureen A. Powers, and Valérie Doye

INSTITUTIONS

1Institut Jacques Monod, CNRS UMR7592–Université Paris Diderot, Sorbonne Paris Cité, 75205 Paris, France; 2Ecole Doctorale Gènes Génomes Cellules, Université Paris Sud, 91405 Orsay, France; 3ImagoSeine Imaging Facility, Institut Jacques Monod, 75205 Paris, France; 4Department of Cell Biology and Biochemistry, Cell and Developmental Biology Graduate Program, Emory University School of Medicine, Atlanta, GA 30322

ABSTRACT Nup98 is a glycine-leucine-phenylalanine-glycine (GLFG) repeat–containing nucleoporin that, in addition to nuclear transport, contributes to multiple aspects of gene regulation. Previous studies revealed its dynamic localization within intranuclear structures known as GLFG bodies. Here we show that the mammalian Nup107-160 complex (Y-complex), a major scaffold module of the nuclear pore, together with its partner Elys, colocalizes with Nup98 in GLFG bodies. The frequency and size of GLFG bodies vary among HeLa sublines, and we find that an increased level of Nup98 is associated with the presence of bodies. Recruitment of the Y-complex and Elys into GLFG bodies requires the C-terminal domain of Nup98. During cell division, Y-Nup–containing GLFG bodies are disassembled in mitotic prophase, significantly ahead of nuclear pore disassembly. FRAP studies revealed that, unlike at nuclear pores, the Y-complex shuttles into and out of GLFG bodies. Finally, we show that within the nucleoplasm, a fraction of Nup107, a key component of the Y-complex, displays reduced mobility, suggesting interaction with other nuclear components. Together our data uncover a previously neglected intranuclear pool of the Y-complex that may underscore a yet-uncharacterized function of these nucleoporins inside the nucleus, even in cells that contain no detectable GLFG bodies.

INTRODUCTION

Nuclear pore complexes (NPCs) are elaborate structures embedded in the nuclear envelope (NE) that provide the main route for bidirectional transport of a variety of molecules between the cytoplasm and the nucleus. They have a dual function as sieves that limit passive diffusion to small molecules less than ∼40 kDa and as highly selective gates that facilitate the active import or export of large cargoes bearing specific targeting signals recognized by soluble nuclear transport receptors (reviewed in Wente and Rout, 2010; Floch et al., 2014). NPCs are composed of ∼30 different proteins called nucleoporins (Nups), each present in multiple copies due to the eightfold rotational symmetry of this structure, leading to an ∼125-MDa assembly in vertebrates (reviewed in Floch et al., 2014). Transmembrane Nups anchor the NPCs within the NE through their interaction with two core scaffold complexes: the central Nup93 complex, comprised of Nup53/Nup35, Nup93, Nup155, Nup188, and Nup205 in vertebrates (reviewed in Vollmer and Antonin, 2014), and the Nup107-160 complex, also termed the Y-complex because of its Y-shaped structure (reviewed in Gonzalez-Aguilera and Askjaer, 2012).

Y-complexes in vertebrates are composed of nine distinct subunits: Nup160/Nup120, Nup133, Nup107, Nup96, Nup85/Nup75, Nup43, Nup37, Seh1, and Sec13 (Loiodice et al., 2004). They are symmetrically localized to both the cytoplasmic and nuclear faces of the NPCs; those on the nuclear side interact with the chromatin-binding nucleoporin partner Elys/MEL-28 (Belgareh et al., 2001;
Rasala et al., 2006; Bui et al., 2013). Of importance, the interaction with Elys contributes to the recruitment of the Y-complex to chromatin at mitotic exit, a key step in the postmitotic reassembly of NPCs (Rasala et al., 2006; Gillespie et al., 2007). In addition, the entire Y-complex and Elys localize at kinetochores in mitosis and are required for proper cell division in mammalian cells (Loiodice et al., 2004; Rasala et al., 2006; reviewed in Wozniak et al., 2010). Fluorescence recovery after photobleaching (FRAP) and turnover studies further revealed that the central structure of the NPC, notably the entire Y-complex, is composed of long-lived proteins that are very stably anchored within the NPCs in interphase, consistent with a function as a structural scaffold (Belgareh et al., 2001; Rabut et al., 2004; D’Angelo et al., 2009; Savas et al., 2012). In contrast, peripheral pore components exhibit a more dynamic behavior (Rabut et al., 2004; D’Angelo et al., 2009; Savas et al., 2012).

Several of the peripheral Nups contain a domain formed of multiple phenylalanine-glycine (FG) motifs separated by spacer sequences. These unstructured and hydrophobic FG domains dynamically interact with nuclear transport receptors, giving to the FG-Nups a key function in active nucleocytoplasmic transport. In addition, certain FG domains associate via low-affinity, cohesive intramolecular and intermolecular interactions to form the permeability barrier of the pore (reviewed in Terry and Wente, 2009; Walde and Kehlenbach, 2010; Schmidt and Gorlich, 2015). Unique among vertebrate FG-Nups, Nup98 features a highly cohesive glycine-leucine-FG (GLFG) subtype of FG domain (Radu et al., 1995; Powers et al., 1995; Xu and Powers, 2013; Schmidt and Gorlich, 2015, and references therein). Significantly, chromosomal translocations involving Nup98 have been described in patients with leukemias, especially acute myeloid leukemia (reviewed in Gough et al., 2011). Such translocations result in expression of fusion proteins that juxtapose the N-terminal GLFG/FG domain of Nup98 to the C-terminus of various partner proteins. Although still debated, the aberrant localization and function of the chimeras, as well as their interaction with endogenous Nup98, appear to contribute to leukemogenesis (reviewed in Gough et al., 2011; Franks and Hetzer, 2013; see also Sarma and Yaseen, 2013; Salsi et al., 2014).

Endogenous Nup98 is predominantly generated through autoproteolytic cleavage of a Nup98-Nup96 precursor that also generates Nup96, a subunit of the Y-complex. However, Nup98 can also be expressed independently of Nup96 from a differently spliced mRNA (Nup98-6kDa; Fontoura et al., 1999; Rosenblum and Blobel, 1999; see later schemes in Figures 2C and 3A). After cleavage, the C-terminal autoproteolytic domain of Nup98 (amino acids [aa] 676–863) can interact with the N-terminal domain of Nup96 and Nup88 (Hodel et al., 2002; Griffis et al., 2003), thereby contributing to the anchorage of Nup98 to both sides of the NPC (Chatel et al., 2012). Nup98 also directly interacts with Pom121 (Mitchell et al., 2010), and binding of its N-terminal GLFG/FG repeat domain to Nup93 contributes to the dynamic association of Nup98 with NPCs (Xu and Powers, 2013).

The N-terminal GLFG/FG domain of Nup98 (aa 1–506) also serves as a docking site for several nuclear transport receptors, notably the nuclear protein export factor CRM1 (Oka et al., 2010) and the RNA export factors TAP/NXF1 (Blevins et al., 2003). Within this FG-containing region, a GEBS domain allows Nup98 interaction with another RNA export factor, the nucleoporin Rae1/Gle2 (Pritchard et al., 1999). Nup98 has been demonstrated to move on and off the nuclear pore in a transcription-dependent manner (Griffis et al., 2002) and to contribute to multiple aspects of gene regulation. Via its GLFG/FG domain, Nup98 interacts with the histone-modifying enzyme CBP/p300 and the histone deacetylase 1 (Kasper et al., 1999; Bai et al., 2006). Nup98 also regulates posttranscriptional expression of defined p53 target genes (Singer et al., 2012) and was recently shown to associate dynamically with the human genome, thereby regulating developmental gene expression programs (reviewed in Franks and Hetzer, 2013; see also Liang et al., 2013).

Within the nucleus, Nup98 was previously shown to localize in distinct intranuclear bodies termed GLFG bodies. These bodies have been detected consistently in two cell lines (Xenopus XL177 and a HeLa subline termed HeLa-C; Griffis et al., 2002; Xu and Powers, 2010), in Xenopus A6 cells, and in ∼5% of HeLa CCL-2 cells (unpublished results). However, GLFG bodies can be induced in other cell lines upon Nup98 overexpression (Griffis et al., 2002). Although the physiological significance of GLFG bodies remains elusive, previous studies addressing the dynamics of these structures have helped to elucidate several aspects of intranuclear Nup98 function, notably in mRNA and protein export (Griffis et al., 2002; Oka et al., 2010). However, besides the presence of the nuclear export factor CRM1 (Oka et al., 2010), the composition of the GLFG bodies had not been previously investigated.

In the present study, we show that the Y-complex and its partner Elys dynamically colocalize with Nup98 and CRM1 into endogenous GLFG bodies present in specific HeLa sublines. FRAP studies further reveal the existence of a nucleoplasmic pool of Y-Nups outside of the nuclear bodies, suggesting a potential, as-yet-uncharacterized intranuclear function of these scaffold nucleoporins.

RESULTS

The Y-complex and Elys colocalize with Nup98 in intranuclear bodies

Nup107, a component of the Y-complex, has been characterized as a key structural component of the NPC during interphase (Belgareh et al., 2001). However, anti-Nup107 antibodies also stained a few discrete intranuclear bodies, most frequently localized in the vicinity of the nucleoli, in ∼20–30% of the cells from the HeLa subline we used (subsequently referred as HeLa-E; see Materials and Methods; Figure 1A). Unlike cytoplasmic annulate lamellae (membrane stacks containing NPC-like structures) or nuclear envelope invaginations (Fricker et al., 1997), these Nup107-stained intranuclear bodies were not labeled by mAb414, which in mammalian cells recognizes several FG-Nups but not Nup98. Analysis of live or fixed HeLa-E cells stably expressing green fluorescent protein (GFP–Nup107 or GFP–Nup43) another constituent of the Y-complex) similarly revealed the presence of these two GFP-Nups within intranuclear bodies (Figure 1A; see later images in Figure 5A for live cells). Elys, a well-characterized Y-complex partner (Rasala et al., 2006), was also enriched in these bodies (Figure 1A), a localization that mirrors the intranuclear staining recently observed for GFP-Elys (Bilokapic and Schwartz, 2013). These nuclear bodies were reminiscent of the previously described Nup98- and CRM1-containing GLFG bodies (Griffis et al., 2002; Xu and Powers, 2010; Oka et al., 2010). In support of this identification, we found Nup107, GFP–Nup43, and Elys colocalized with Nup98 and CRM1 in HeLa-E cells (Figure 1A).

These results prompted us to assess the localization of various Y-complex constituents (subsequently referred to as Y-Nups) in another HeLa subline, HeLa-C, in which a very high frequency of GLFG bodies was previously reported (Xu and Powers, 2010). In this cell line, >90% of the cells displayed intranuclear foci stained by Y-Nups (Nup107, Nup133, Nup96), Elys, and Nup98 (Figures 1B and 2A). In addition, transfection of GFP fusions in HeLa-C cells revealed that all tested Y-Nups and Elys were targeted to GLFG bodies (Figures 1C and Supplemental Figure S1A; unpublished data).
FIGURE 1: The Y-complex colocalizes with Nup98 and Elys in intranuclear bodies in HeLa cells. (A) HeLa-E cells transiently transfected with GFP-Nup98 (left) or stably expressing GFP-Nup43 (middle) or GFP-Nup107 (right) were stained with the indicated antibodies and DAPI. The GFP-Nups signals are also shown. (B) HeLa-C cells were stained with the indicated antibodies and DAPI. (C) HeLa-C cells transiently transfected with the indicated GFP fusions were fixed 3 d after transfection and stained with anti-Nup98 antibody and DAPI. Top, merge signals between the GFP (green) and the Nup98 staining (red). Arrowheads point to foci localized within the nucleolus that are stained with Nup98 but not GFP-Nup37 or Elys. Bars, 10 μm.
siently overexpressing Nup98 (Griffis et al., 2002), no colocalization could be detected in HeLa-E or HeLa-C cells with markers of other well-characterized intranuclear structures, including coilin, SC35, PML, Rad51, or centromeric proteins (Table 1; unpublished data). Taken together, these results revealed that in addition to their localization at NPCs, the Y-complex and Elys are found inside the nucleus, where they can be specifically recruited, together with Nup98 and CRM1, to GLFG bodies.

Unlike Y-Nups and Elys, the other nucleoporins assayed using antibodies (Nup205, Nup153, Tpr, and RanGAP1, as well as the FG-Nups recognized by mAb414) or GFP fusions (Nup58, Nup93, and Aladin) were not detected in these intranuclear bodies (Figure 1, B and C, and Table 1). Although lack of epitope accessibility cannot be formally excluded for some Nups, these data clearly indicate that not all nucleoporins are recruited to these bodies. Finally, and in agreement with the initial characterization of GLFG bodies in cells transiently overexpressing Nup98 (Griffis et al., 2002), no colocalization could be detected in HeLa-E or HeLa-C cells with markers of other well-characterized intranuclear structures, including coilin, SC35, PML, Rad51, or centromeric proteins (Table 1; unpublished data).

Taken together, these results revealed that in addition to their localization at NPCs, the Y-complex and Elys are found inside the nucleus, where they can be specifically recruited, together with Nup98 and CRM1, to GLFG bodies.
transient overexpression of Nup98 was previously reported to induce its accumulation within GLFG bodies, we compared Nup98 levels in HeLa-K and HeLa-C cell lines. Western blot analyses revealed approximately twofold increase of Nup98 protein levels in HeLa-C as compared with HeLa-K cells (Figure 2). In contrast, neither Elys nor any of the Y-Nups assayed showed an increased level in HeLa-C cells. We next performed quantitative reverse transcription PCR (qRT-PCR) on total RNAs extracted from these two cell lines, using primers recognizing either all Nup98-containing transcripts or only the short (Nup98-6kDa) or long (Nup98-Nup96) mRNAs. This analysis revealed that the levels all Nup98-encoding mRNAs were increased by twofold to threefold in HeLa-C as compared with HeLa-K cells (Figure 2C). This study also revealed that in both HeLa sublines, the long Nup98-Nup96 transcripts were much more abundant than the short ones solely encoding Nup98 (as also originally described by Fontoura et al., 1999). Taken together, these results indicate that in HeLa-C cells, the increased protein level of Nup98 is linked to increased transcription or stability of the Nup98-Nup96 mRNAs and not to major changes in alternative splicing favoring the expression of the short Nup98-6kDa mRNAs.

The increased levels of Nup98 in HeLa-C cells prompted us to determine the consequences of Nup98 overexpression in cells largely devoid of GLFG bodies. As shown in Figure 3B, immunofluorescence analysis of HeLa-K cells stably expressing mCherry-Nup107 revealed the recruitment of Elys and mCherry-Nup107 into most of the GLFG bodies induced by GFP-Nup98 overexpression. Similar recruitment of Elys into these GLFG bodies was also observed in HeLa-K cells (Supplemental Figure S2). Together these data thus revealed that increased Nup98 levels are sufficient to recruit Elys and Y-Nups into the induced GLFG bodies.

The C-terminal domain of Nup98 contributes to the recruitment of Elys and the Y-complex to nuclear bodies

We next sought to determine how Nup98 recruits the Y-complex and Elys to the GLFG bodies. We therefore overexpressed in HeLa-K cells a GFP-Nup98 N-terminal construct (GFP-Nup981-469) that carries most of the cohesive GLFG/FG repeat domain of Nup98. This truncated form lacks the C-terminal domain of Nup98 and is not favored upon overexpression with CRM1 into intranuclear bodies (Griffis et al., 2003). As a control, cells were transfected with a C-terminally tagged form of Nup98 (GFP-Nup981-469) in HeLa-K cells did not lead to recruitment of Elys or mCherry-Nup107 into the newly formed nuclear bodies (Figure 3B and Supplemental Figure S2). These data thus indicate a critical role for the C-terminal domain of Nup98 in the recruitment of Elys and the Y-complex into GLFG bodies. Of note, HeLa-C cells overexpressing GFP-Nup981-469 most often displayed fewer Elys-positive GLFG bodies than did nontransfected cells (Supplemental Figure S2). Together these data thus revealed that increased Nup98 levels are sufficient to recruit Elys and Y-Nups into the induced GLFG bodies.

The C-terminal domain of Nup98, which is absent from GFP-Nup981-469, includes the region required for interaction with the N-terminus of Nup96 (Griffis et al., 2003). To determine whether this interaction contributes to the recruitment of Nup96 to the GLFG bodies, we transfected HeLa-C cells with an N-terminal epitope-tagged form of Nup96 (GFP-Nup96) that was reported to abolish its binding to Nup96 (Griffis et al., 2003). As a control, cells were transfected with a C-terminally tagged form of Nup96 (Nup96-GFP). As shown in Figure 3C, both GFP-Nup96 and Nup96-GFP were properly targeted to the NPCs, indicating that these fusions can be assembled within Y-complexes. However, unlike Nup96-GFP, the GFP-Nup96 fusion was not targeted to the existing GLFG bodies in

TABLE 1: Nucleoporins, transport factors, and nuclear proteins analyzed for localization in Nup98-containing nuclear bodies.

<table>
<thead>
<tr>
<th>Y-complex + Elys</th>
<th>Ab</th>
<th>GFP</th>
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<tbody>
<tr>
<td>Nup160</td>
<td>+</td>
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<td>Nup133</td>
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<td>Nup107</td>
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<td>(1A,B)</td>
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<td>Nup96</td>
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<td>Nup85</td>
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<td>Nup37</td>
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<tr>
<td>Seh1</td>
<td>+</td>
<td>(1C)</td>
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<tr>
<td>Elys</td>
<td>+</td>
<td>(1A,C)</td>
</tr>
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Other Nups and transport factors

| Nup205            | –  | (1B) |
| Nup93             | –  | (1B) |
| Nup58             | –  | (1B) |
| Aladin            | –  | (1B) |
| mAb414            | +  | (S1B) |
| “FG-Nups”         | +  | (S1B) |
| Nup153            | –  | (1B) |
| Tpr               | –  | (1B) |
| RanGAP1           | –  | (1B) |
| CRM1              | –  | (1B) |
| Gle2/Rae1         | –  | (1B) |

Markers from other nuclear compartments

| CGoBs (GFP-CPEB1) | +  | (S1B) |
| Coiled bodies (colin) | – |
| Species (SC35) | –  |
| PML bodies (PML) | –  |
| DNA repair foci (Rad51) | –  |
| Centromeres (CREST serum) | –  |

The last two columns indicate localizations that were analyzed using either specific antibodies (Ab) and/or transfected GFP fusions (GFP), +, colocalization with Nup98 in GLFG bodies; –, no detectable localization in intranuclear bodies. The parentheses in the last two columns indicate the figures or supplemental figures in which the corresponding localization is shown.

**GFP-CPEB1** is a translational regulator previously reported to colocalize with Nup98 in GLFG bodies; –, no detectable localization in intranuclear bodies. The parentheses in the last two columns indicate the figures or supplemental figures in which the corresponding localization is shown.

**TABLE 1: Nucleoporins, transport factors, and nuclear proteins analyzed for localization in Nup98-containing nuclear bodies.**

Increased level of Nup98 is key to the appearance of Elys- and Y-complex-containing GLFG bodies

Unlike in the HeLa-E and HeLa-C sublines, endogenous GLFG bodies were rarely detected in cells from the recently sequenced HeLa subline HeLa-K (Landry et al., 2013; Figure 2A). Because...
We next investigated the localization of the Y-complex and Elys upon treatment with drugs previously reported to affect GLFG bodies. Leptomycin B (LMB), a specific inhibitor of the nuclear export receptor Crm1, was previously reported to interfere with the GLFG bodies in HeLa-K cells (Figure 3C). (Note that the presence of endogenous Nup96 likely accounts for the persistence of Elys in GLFG bodies in GFP-Nup96–expressing HeLa-C cells.) Together these data indicate that the Nup98-C terminal domain contributes to the recruitment or maintenance of the Y-complex and Elys in intranuclear GLFG bodies, most likely through its interaction with Nup96.

**Behavior of Elys and Y-Nups compared with Nup98 in intranuclear bodies**

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or α-amanitin led to the nucleolar accumulation of Nup98 and its progressive disappearance from the intranuclear bodies. In contrast, Elys did not accumulate in nucleoli, and its localization within nuclear foci was less affected by these treatments (Figure 4A). In addition, the fraction of Nup98 present in intranuclear bodies was readily solubilized by 1% Triton extraction, whereas Elys, Nup96, GFP-Nup107, and GFP-Nup43 staining in nuclear bodies was resistant to sequential extraction with 1% Triton, DNase I, 2 M NaCl, and RNase A (Buckler-White et al., 1980; Figure 4B; unpublished data). This indicates that once recruited to intranuclear foci, Elys and Y-complex constituents become associated with a stable nuclear structure, whereas the bulk of Nup98 might be more dynamically associated.

**Dynamics of Y-Nups–, Elys–, and Nup98-containing nuclear bodies during the cell cycle**

To characterize further these Y-complex–containing intranuclear bodies, we monitored their dynamics throughout the cell cycle. Time-lapse sequences of live HeLa-E cells stably expressing GFP-Nup107 (Figure 5A) or GFP-Nup43 (unpublished data) revealed a progressive disassembly of the intranuclear bodies that was completed in most cells ∼30 min before NPC and nuclear envelope disassembly (Figure 5, A and B). Similarly, immunofluorescence studies in HeLa-C cells revealed that Elys and Nup98 signals within foci decreased both in number and intensity in late G2 cells, as characterized by a discontinuous phospho-H3 staining (Hendzel et al., 1997). GLFG bodies were no longer detectable in prophase cells (defined by a strong and continuous phospho-H3 staining but persistent staining of Elys at the nuclear rim; Figure 5C). After mitosis, reassembly of these bodies was progressive in HeLa-E cells (Figure 5, A and B) but likely occurs faster in HeLa-C cells, as revealed by the presence of multiple intranuclear bodies in most cells (Figure 5C).

GLFG bodies, including the Y-complex Nups and Elys, are therefore disassembled and reformed during cell cycle progression. Of importance, this dynamic behavior highlights the fact that these structures are not simply a result of aggregation, but instead that specific regulations can modulate these protein assemblies in a cell cycle–dependent manner.

**Y-Nups shuttle between nuclear bodies and the nucleoplasm**

We next characterized the intranuclear dynamics of Y-Nups in interphase, taking advantage of our HeLa-E cell lines stably expressing GFP-Nup107 or GFP-Nup43. Consistent with previous studies (Rabut et al., 2004), photobleaching of the nuclear envelope did not lead to any detectable recovery of the GFP-Y-Nups signals over

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**FIGURE 4:** Compared behavior of Elys, Nup98, and Y-Nups upon LMB, Act-D, or α-amanitin treatment or after sequential cellular extractions. (A) HeLa-C cells were treated for 2 h with 20 nM LMB or actinomycin-D at 50 ng/ml (low) or 5 μg/ml (high) or for 4 h with 50 μg/ml α-amanitin. Cells were then fixed and immunostained with anti-Elys, anti-Nup98, and anti-Nop52. DAPI staining was used to visualize nuclei. (B) HeLa-C cells (left) or HeLa-E cells stably expressing GFP-Nup107 (right) were submitted to in situ extraction with 1% Triton X-100 (+1% Tx100), followed by sequential DNase I, 2 M NaCl, and RNase A extractions before fixation. Cells were then immunostained with the indicated antibodies. The efficiency of chromatin digestion is attested by the disappearance of the DAPI staining. Bars, 5 μm.
FIGURE 5: Cell cycle dynamics of GFP-Nup107–, Elys–, and Nup98-containing nuclear bodies. (A) Representative time frames of a HeLa-E cell stably expressing GFP-Nup107 analyzed by spinning-disk confocal microscopy at various stages of the cell cycle. One single Z-plane is shown. Time 0 was set at mitotic entry. Scale bar, 10 μm. See also Figure 5_movie .mov. (B) Quantification of the kinetics of foci disassembly and reassembly in HeLa-E cells stably expressing GFP-Nup107. The number of cells imaged is indicated. Mitotic entry was defined as the first time frame at which cell rounding was observed and mitotic exit as the first frame where two distinct nuclei were detectable. The disappearance and reappearance of foci was manually recorded on individual dividing cells. Box plots were generated using KaleidaGraph (see Materials and Methods). (C) HeLa-C cells triple stained with anti-Elys, anti-Nup98, and anti–phospho-histone H3 and DAPI. Typical stages of the cell cycle are shown. The star denotes a late-prophase cell in which kinetochores staining by Elys becomes detectable. Scale bar, 10 μm.
et al., 2003; Rasala et al., 2006; Bilokapic and Schwartz, 2013; Schwartz et al., 2015). Here the use of specific HeLa cell lines displaying high frequency of GLFG bodies provides the first evidence of the colocalization of all Y-complex subunits analyzed and Elys, along with Nup98 and CRM1, within a subset of GLFG bodies (Figure 1). The presence of Y-Nups and Elys in GLFG bodies seems quite specific, as none of the other Nups analyzed using antibodies or GFP fusions were detectable within these bodies (Table 1).

We further demonstrate that the increased occurrence of GLFG bodies in HeLa-C compared with HeLa-K cells is correlated with increased Nup98 protein levels. However, despite approximately two-fold increase in mRNA level observed for the Nup98-Nup96 precursor (which represents the major Nup98-encoding transcript in all HeLa cell lines we analyzed), Nup96 protein levels were not higher in HeLa-C cells than in HeLa-K cells (Figure 2). Although other cellular mechanisms cannot be excluded, this discrepancy between Nup98 and Nup96 mRNAs versus protein levels may be linked to posttranslational regulations of Nup96 that lead to its proteasome-mediated degradation during mitosis (Chakraborty et al., 2008).

Consistent with the increased levels of Nup98 in HeLa-C cells, we further showed that transient overexpression of Nup98 in HeLa-K cells is sufficient to induce the recruitment of the Y-complex and Elys to most of the induced GLFG bodies (Figure 3B and Supplemental Figure S2). Conversely, conditions known to induce the disassembly of GLFG bodies, such as LMB (Oka et al., 2010), or overexpression of a GFP-Nup98-HoxA9 fusion led to the disappearance of Elys and the Y-complex subunits from intranuclear bodies in HeLa-C cells (Figure 4A, Supplemental Figure S2, and unpublished data). Our data further indicate that the C-terminal domain of Y-Nups able to exchange dynamically with the fraction present in the nuclear bodies.

To better characterize this nucleoplasmic pool, we next photo-bleached half of the nucleus and watched the subsequent recovery of fluorescence (Figure 7 and Supplemental Figure S5). Under those conditions, the signal within the nuclear bodies recovered with kinetics comparable to the ones observed when only the intranuclear bodies were bleached. Although the overall recovery within the rest of the nucleoplasm appeared faster, with ~35% of the signal recovering within the first 10 s, subsequent intranuclear recovery was far slower, reaching ~80% within only 5–10 min, and was not complete after 30 min. This indicates that within the nucleoplasm, Nup107 exists in multiple fractions: a very mobile one, and fractions with far more restricted diffusion.

DISCUSSION

Previous studies pointed to the existence of intranuclear fractions of several Y-complex subunits and Elys in human cells (Enninga et al., 2003; Rasala et al., 2006; Bilokapic and Schwartz, 2013; Schwartz et al., 2015). Here the use of specific HeLa cell lines displaying high frequency of GLFG bodies provides the first evidence of the colocalization of all Y-complex subunits analyzed and Elys, along with Nup98 and CRM1, within a subset of GLFG bodies (Figure 1). The presence of Y-Nups and Elys in GLFG bodies seems quite specific, as none of the other Nups analyzed using antibodies or GFP fusions were detectable within these bodies (Table 1).

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30 min. In contrast, GFP-Nup107 and GFP-Nup43 recovery was clearly detectable when nuclear bodies were bleached (Figure 6A and Supplemental Figures S3A and S4). After quantifications (Figure 6B and Supplemental Figure S3, B and C), the GFP-Nup107 recovery curves of the bleached bodies were fitted using a reaction-dominant model involving a unique binding state (Supplemental Figure S3D; see Materials and Methods). This analysis revealed that most of the GFP-Nup107 fluorescence in the nuclear bodies recovers with a half-time of ~3–4 min (Figure 6C). Thus, in contrast to their behavior at the NPCs, Y-Nups are dynamic within the GLFG bodies. These findings further highlight the existence of a nucleoplasmic pool of Y-Nups able to exchange dynamically with the fraction present in the nuclear bodies.

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DISCUSSION

Previous studies pointed to the existence of intranuclear fractions of several Y-complex subunits and Elys in human cells (Enninga et al., 2003; Rasala et al., 2006; Bilokapic and Schwartz, 2013; Schwartz et al., 2015). Here the use of specific HeLa cell lines displaying high frequency of GLFG bodies provides the first evidence of the colocalization of all Y-complex subunits analyzed and Elys, along with Nup98 and CRM1, within a subset of GLFG bodies (Figure 1). The presence of Y-Nups and Elys in GLFG bodies seems quite specific, as none of the other Nups analyzed using antibodies or GFP fusions were detectable within these bodies (Table 1).

We further demonstrate that the increased occurrence of GLFG bodies in HeLa-C compared with HeLa-K cells is correlated with increased Nup98 protein levels. However, despite approximately two-fold increase in mRNA level observed for the Nup98-Nup96 precursor (which represents the major Nup98-encoding transcript in all HeLa cell lines we analyzed), Nup96 proteins levels were not higher in HeLa-C cells than in HeLa-K cells (Figure 2). Although other cellular mechanisms cannot be excluded, this discrepancy between Nup98 and Nup96 mRNAs versus protein levels may be linked to posttranslational regulations of Nup96 that lead to its proteasome-mediated degradation during mitosis (Chakraborty et al., 2008).

Consistent with the increased levels of Nup98 in HeLa-C cells, we further showed that transient overexpression of Nup98 in HeLa-K cells is sufficient to induce the recruitment of the Y-complex and Elys to most of the induced GLFG bodies (Figure 3B and Supplemental Figure S2). Conversely, conditions known to induce the disassembly of GLFG bodies, such as LMB (Oka et al., 2010), or overexpression of a GFP-Nup98-HoxA9 fusion led to the disappearance of Elys and the Y-complex subunits from intranuclear bodies in HeLa-C cells (Figure 4A, Supplemental Figure S2, and unpublished data). Our data further indicate that the C-terminal domain of...
Nup98 clearly contributes to the recruitment or stability of the Y-complex and Elys to the GLFG bodies, a process that likely involves the interaction between Nup98 and Nup96 (Griffis et al., 2003; Figure 3, B and C, and Supplemental Figure S2). However, other determinants, possibly including Y-complex-independent interactions between Elys and Nup98 (Schwartz et al., 2015), also appear to contribute to this process. Of interest, Elys encompasses within its C-terminal disordered region a putative AT-hook DNA-binding motif and an additional domain required for chromatin binding. In addition, it physically associates on chromatin with the Mcm2-7 replication-licensing proteins (Gillespie et al., 2007; Rasala et al., 2008). These properties of Elys and/or additional partners of the Y-complex or Elys may explain their persistence within nuclear bodies upon extraction conditions defined as “nuclear matrix” preparation that lead to the removal of most if not all Nup98 (Figure 4B).

Of note, Elys was also largely displaced from these bodies upon overexpression in HeLa-C cells of a GFP-Nup981-469 construct derived from the GFP-Nup98-HoxA9 fusion but lacking the HoxA9 domain. This result was somewhat unexpected, as this fusion still forms intranuclear bodies that should recruit endogenous Nup98. However, earlier FRAP experiments indicated that GFP-Nup981-469 recovered less quickly and thus was more tightly associated in GLFG bodies than was the full-length protein (Xu and Powers, 2010). Thus it is possible that Nup981-469 displaces the full-length protein, with the consequence that Y-complex interaction sites are reduced. Alternatively, this observation may reflect the existence of distinct sub-sets of GLFG bodies. Indeed, unlike CRM1 and Rae1/Gle2-GFP, Elys and Y-Nups were not detectable in a subset of Nup98-containing nuclear foci, most frequently those localized within the nucleolus (arrowheads in Figure 1C and Supplemental Figures S1 and S2). This may be caused by a lack of accessibility of the Nup98-binding site and/or an impaired accessibility of the Y-complex and Elys to specific nuclear domains. In line with this hypothesis, it was recently demonstrated that Nup98, when targeted to tandem copies of a LacO-containing DNA array in U2OS cells, does not recruit Y-Nups to this LacO array (Schwartz et al., 2015).
Our study of Y-complex dynamics revealed the progressive disassembly of these nuclear bodies that initiates ~1 h before mitotic entry, coincidently with histone H3 phosphorylation, and is completed ~20–30 min before NE breakdown (Figure 5). GLFG body disassembly thus seems to precede the weakening of the NE barrier that was previously correlated with a specific phosphorylation of Nup98 by multiple kinases (Laurell et al., 2011). However, that study also revealed that expression of a nonphosphorylatable form of Nup98 delays nuclear body disassembly and that a Nup98 phosphomimetic mutant is targeted with lower efficiency to intranuclear bodies (Laurell et al., 2011; Laurell and Kutay, 2011). It is thus possible that cell cycle–dependent phosphorylations on Nup98 affect both nuclear bodies and NPCs, albeit with different kinetics. In the future, it will be interesting to determine the extent to which changes in GLFG cohesiveness, Nup98-Nup96 interactions, and/or chromatin binding properties of Elys contribute to the dynamics of these nuclear bodies throughout the cell cycle.

Analysis of the mobility of GFP-Nup107 and GFP-Nup43 during interphase indicated that most of the fluorescence in the nuclear bodies recovers with a similar half-time of ~3 min (Figure 6 and Supplemental Figure S4), suggesting that the Y-complex is likely recruited as complete entity, possibly along with Elys, to these bodies. In contrast, this exchange rate appears to be far slower that the previously reported dynamics of Nup98, whose half-time of fluorescence recovery was found to be ~12 s with an immobile GFP-Nup98 fraction of ~25% (Griffis et al., 2002). Because in that study GFP-Nup98 recovery was followed over only 2–3 min, the “immobile” fraction may correspond to a slowly exchanging pool of Nup98 to which the Y-complex and Elys would bind. The existence of distinct pools of Nup98 within the GLFG bodies could possibly explain the apparent earlier dissociation from the nuclear bodies of a pool of Nup98 as compared with Elys upon transcription inhibition or in late G2 (Figures 4A and SC).

Because the detection within nuclear bodies, our FRAP experiments clearly established the presence of an intranuclear pool of Nup107 or Nup43 able to exchange dynamically with these bodies (Figures 6 and 7 and Supplemental Figure S4). Experiments performed on half-bleached nuclei further indicate that although a large fraction of GFP-Nup107 is highly dynamic, leading to a significant recovery within the first 10–20 s, the rest of the intranuclear pool recovers with a slower rate, similar to the one observed within the nuclear bodies. This less mobile fraction likely reflects the dynamic association of Nup107 embedded within Y-complexes, possibly along with Elys and Nup98, with dispersed intranuclear binding sites.

Because endogenous GLFG bodies have so far been detected only in the Xenopus A6 and XL177 cell lines and in several HeLa sublines, their physiological relevance remains elusive. However, our study demonstrates the existence of an intranuclear pool of the Y-complex, even in HeLa-K cells largely devoid of GLFG bodies, which likely underscores a more general function of this complex. At this stage, we can only speculate about the function of the intranuclear pool of the Y-complex during interphase. Although this fraction may possibly underlie the requirement of nuclear Y-complex for interphase NPC assembly (D’Angelo et al., 2006), we do not favor this hypothesis, since Elys is dispensable for NPC assembly at this stage of the cell cycle (Doucet et al., 2010). On the other hand, our data revealed that the Y-complex and Elys can interact with Nup98, at least within GLFG bodies. These nucleoporins may thus contribute to some of the previously described intranuclear functions of Nup98 during interphase. The fact that unlike Nup98, Y-Nups and Elys do not relocalize inside the nucleolus upon transcription inhibition indicates that these Nups do not contribute to all Nup98 functions in RNA processing and trafficking (Oka et al., 2010). Similarly, whereas a role for a non–NPC-bound fraction of Nup98 in transcriptional memory was established, the authors reported that Nup107 depletion does not affect this process (Light et al., 2013). In the future, it will be interesting to determine whether the intranuclear fraction of the Y-complex contributes to other known functions of Nup98 in transcriptional or posttranscriptional gene regulation (Singer et al., 2012; Liang et al., 2013) or in other nuclear processes.

Beyond the detection within nuclear bodies, our FRAP experiments clearly established the presence of an intranuclear pool of Nup107 or Nup43 able to exchange dynamically with these bodies (Figures 6 and 7 and Supplemental Figure S4). Experiments performed on half-bleached nuclei further indicate that although a large fraction of GFP-Nup107 is highly dynamic, leading to a significant recovery within the first 10–20 s, the rest of the intranuclear pool recovers with a slower rate, similar to the one observed within the nuclear bodies. This less mobile fraction likely reflects the dynamic association of Nup107 embedded within Y-complexes, possibly along with Elys and Nup98, with dispersed intranuclear binding sites.

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MATERIALS AND METHODS

Cell culture and plasmid transfections

HeLa cells were grown at 37°C in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, 1% l-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. HeLa-E is a subclone of HeLa cells initially received from F. Perez (Institut Curie, Paris, France) that was used in our initial studies (Belgareh et al., 2001). The HeLa-K cells were a gift from J. Ellenberg (EMBL, Heidelberg, Germany) and correspond to the recently sequenced HeLa subline (Landry et al., 2013). The HeLa-C subline, which features multiple endogenous GLFG bodies (Xu and Powers, 2010), was a kind gift from V. Cordes (MPIBPC, Göttingen, Germany).

Plasmids used in this study were either previously published or generated using standard molecular cloning techniques and are listed in Supplemental Table S1. Plasmid transfections were performed using Lipofectamine 2000 (Invitrogen). To establish stable cell lines (HeLa-E stably expressing GFP-Nup107 or GFP-Nup43 and HeLa-K stably expressing mCherry-Nup107), individual clones were isolated by G418 (1 mg/ml) selection.

Immunofluorescence analyses

The following primary antibodies were used for immunofluorescence in this study: anti-Nup98 rat monoclonal (2H10; ab50610; 1:2000; Abcam, Cambridge, MA); affinity-purified rabbit antibodies directed against human Nup107 and Nup133 (Belgareh et al., 2001; each at 1:400), Nup96/p87 (Fontoura et al., 1999; Lioidecio et al., 2004; serum provided by B. Fontoura, UT Southwestern, Dallas, TX; 1:1500), and Nup205 and Nup53 (Mansfeld et al., 2006; provided by U. Kutay, ETH, Zürich, Switzerland; 1:10,000 each); rabbit polyclonal anti-Elys/Mel28 antibody (FID1 serum, directed against histidine-tagged human Mel28 protein, aa 1208–1800; generously provided by R. Walczak and I. Mattaj, EMBL, Heidelberg, Germany; 1:1000); rabbit polyclonal immunoglobulin G anti-CRM1 (sc-5595, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal antibody mAb414, recognizing several FG-Nups other than Nup98 (BAbCO, Richmond, CA; 1:5000), anti-Nup153 (SA1; Bodoor et al., 1999, provided by B. Burke, IMB, Singapore; 1:5); and anti-phospho-histone H3 Ser-10 (ab14955; 1:2000; Abcam); human serum C13, recognizing Nop52 (Savino et al., 1999; provided by D. Hernandez-Verdún, IJM, Paris, France; 1:500). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) or Molecular Probes (Eugene, OR).

Fluorescence signals within the intranuclear bodies were best detected upon preextraction of cells, followed by a fixation-extraction procedure. Briefly, cells were quickly preextracted for 15 s in PHEM buffer (20 mM K–1,4-piperazinediethanesulfonic acid, 10 mM K–ethylene glycol tetraacetic acid, 1 mM MgCl₂, pH 6.8) containing 0.2% Triton and then fixed for 10 min in the same buffer further...
containing 4% paraformaldehyde supplemented with 0.85% of 1 N NaOH to the buffer to improve the detection of some antigens (Loidice et al., 2004).

To analyze the behavior of proteins within foci, monolayers of HeLa cells were submitted to in situ sequential extraction with 1% Triton X-100, 50 U/ml DNase I, 2 M NaCl, and 50 μg/ml RNase A before fixation as described (Ernoult-Lange et al., 2009; a procedure referred to as “nuclear matrix” preparation). The efficiency of chromatin devastation was attested by the disappearance of the 4',6-diamidino-2-phenylindole (DAPI) staining.

Wide-field fluorescence images were acquired using a microscope (DM6000B; Leica, Wetzlar, Germany) with a 100×, numerical aperture 1.4 (HCX Plan-Apo) oil immersion objective and a charge-coupled device (CCD) camera (CoolSNAP HQ; Photometrics, Tucson, AZ). Rapid and precise Z-positioning was accomplished by a piezoelectric motor mounted underneath the objective lens. Image stacks were acquired without camera binning, with a plane spacing of 0.2 μm. A single unique plane is shown in all images.

**Western blot analysis**

For Western blot analysis, whole-cell lysates solubilized in SDS-PAGE sample buffer were separated on 10% or 4–12% SDS–PAGE gels and transferred to nitrocellulose filters. The resulting blots were saturated with Tris-buffered saline, 0.1% Tween, and 5% dried milk and probed with the following antibodies: anti-Nup98 rat monoclonal (2H10; ab50610; 1/2000; Abcam), polyclonal rabbit anti-Elys (A300-166A; 1/2000; Bethyl Laboratories, Montgomery, TX), rabbit polyclonal anti-hNup133 (serum #759; 1:2500; Belgareh et al., 2001), anti-hNup107-Ctern (serum #520; 1/2000; Belgareh et al., 2001), rabbit polyclonal anti-Nup96 (A301-784A, 1/2000; Bethyl Laboratories, Texas), and monoclonal mouse anti-γ-tubulin (ab11316, 1/10,000; Abcam). After incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), signals were detected by enhanced chemiluminescence (SuperSignal Pico or Femto; Thermo Scientific, Lafayette, CO).

**qRT-PCR**

Total RNAs were isolated using the NucleoSpin RNAII kit (Macherey-Nagel, Düren, Germany). mRNAs were reverse transcribed using random hexamers and the AMV reverse transcriptase (Finnzymes, Espoo, Finland). Real-time PCR was performed with a LightCycler 480 system (Roche, Basel, Switzerland) by using SYBR Green incorporation (SYBR Green PCR-Master Mix; Applied Biosystems, Foster City, CA), and the Nup98-containing cDNAs were amplified using the following primers (see also Figure 2): F1 AATACCGGCCTGAAAACTGGT; R1 (AGGCCAAGGAGCAGTCTTCA); R2 (TACAGGATCTCATTCAAAATTC); F3 (CATCCACCTGCTGAAACACT); and R3 (AAAGATGCTGCTGTCGGGAGGT). The relative amounts of cDNAs in the samples were quantified according to the manufacturer’s instructions and normalized by reference to the TATA-binding protein (TBP) cDNAs (using the prime pair AGTGAAGAAACAGTCAGCAGT and CCAGGAAATAACTCTGCGTCCAT).

**Videomicroscopy and FRAP**

For spinning-disk confocal imaging, cells plated on 35-mm glass base dishes (Iwaki) were maintained at 37°C in DMEM F-12 without phenol red supplemented with 10% fetal calf serum and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. Images were acquired on a spinning-disk microscope (CSU22; Roper Scientific, Sarasota, FL) using a 491-nm laser and three band pass filters (S10AF23, 590DF35, and 670DF40). Acquisitions were performed using oil immersion objectives mounted on a piezoelectric motor (Physik Instruments, Irvine, CA) and a QuantEM:512SC electron-multiplying CCD camera (Photometrics). The whole setup was driven with MetaMorph software (Molecular Devices, Sunnyvale, CA). After acquisition, images were scaled equivalently using MetaMorph software and converted to 8-bit images before being imported to Photoshop software (Adobe, Mountain View, CA).

For cell cycle studies, acquisitions were performed using a Plan-Apochromat 63×/1.4 oil objective lens. Z-stacks (10 planes 1 μm apart covering the entire nucleus) were acquired at 10-min intervals during ~20 h. For quantifications, the timing of disappearance and reappearance of foci was manually recorded on individual dividing cells. Box plots were then generated using KaleidaGraph (Synergy Software, Reading, PA): each box encloses 50% of the values obtained, centered on the median value. The bars extending from the top and bottom of each box mark the minimum and maximum values within the data set falling within an acceptable range. Values falling outside of this range are displayed as an individual point.

FRAP experiments on GFP-Nup107 and GFP-Nup43 cells were performed using a Plan-Apochromat 63×/1.4 (FRAP on nuclear bodies) or a Plan-Apochromat 40×/1.25 oil objective lens. For each cell, three prebleach images were taken, and either a single 10-pixel (2.4-μm) spot containing a nuclear body (along with three spots covering the NE in some cells) or a square region covering approximately half of the nucleus and part of the cytoplasm was bleached with the 473-nm line at 100% transmission (three iterations). Z-stacks (nine planes 1 μm apart covering the entire nucleus) were acquired at 10-s intervals for 12 frames and then at 1-min intervals for 30 frames.

Quantifications of fluorescence recovery were performed using the MetaMorph software on additive projections of one to three consecutive relevant Z-planes. When required, sequences were aligned using the “turboreg” registration plug-in on ImageJ (National Institutes of Health, Bethesda, MD). For each cell, the mean fluorescence intensities within circular regions of 8 pixels covering the bleached nuclear body (I) and (in the case of half-bleached nuclei) within intranuclear regions apparently devoid of nuclear bodies were measured at each time point. After background (BG) subtraction, the fluorescence signals were subjected to double normalization using a manually defined region (T) encompassing the whole nucleus (excluding the NE) to take into account the loss of total nuclear signal due to the bleach pulse and bleaching during postbleach imaging as previously described, \( I(t) = \frac{(T - BG)/(T_{prebleach} - BG_{prebleach})}{(T - BG)/(T_{prebleach} - BG_{prebleach})} \) (Phair et al., 2004). These measurements were then normalized to 0 for the image taken immediately after photobleaching and to 1 for the steady-state distribution of fluorescence (mean of three images acquired just before photobleaching). The resulting graphs were generated using Excel (Microsoft).

The recovery curves for each cell were fitted to a monoeponential equation (also called reaction-dominant model, as described by Sprague et al., 2004). In this reaction-dominant scenario, diffusion occurs so rapidly that it is not taken in account in the model. The corresponding molecules are thus considered to be part of a “freely” diffusing population.

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