Nup98-Homeodomain Fusions Interact with Endogenous Nup98 during Interphase and Localize to Kinetochores and Chromosome Arms during Mitosis

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Chromosomal translocations involving the Nup98 gene are implicated in leukemias, especially acute myelogenous leukemia. These translocations generate chimeric fusion proteins, all of which have in common the N-terminal half of Nup98, which contains the nucleoporin FG/GLFG repeat motifs. The homeodomain group of Nup98 fusion proteins retain the C-terminus of a homeodomain transcription factor, including the homeobox responsible for DNA binding. Current models for Nup98 leukemogenesis invoke aberrant transcription resulting from recruitment of coregulators by the Nup98 repeat domain. Here we have investigated the behavior of Nup98-homeodomain fusion proteins throughout the cell cycle. At all stages, the fusion proteins exhibit a novel localization distinct from the component proteins or fragments. During interphase, there are dynamic interactions between the Nup98 fusions and endogenous Nup98 that lead to mislocalization of the intranuclear fraction of Nup98, but do not alter the level of Nup98 at the nuclear pore complex. During mitosis, no interaction between the fusion proteins and endogenous Nup98 is observed. However, the fusions are entirely concentrated at kinetochores and on chromosome arms, sites where the APC/C, a target of Nup98 regulation, is also found. Our observations suggest new possibilities for misregulation by which Nup98 translocations may contribute to cellular transformation and leukemogenesis.

INTRODUCTION

Chromosomal translocations involving the Nup98 gene found on chromosome 11 (11p15) have been repeatedly implicated in acute myelogenous leukemia (AML) and, less commonly, in myelodysplastic syndrome (MDS), chronic myelogenous leukemia, or T-acute lymphocytic leukemia (reviewed in Slape and Aplan, 2004; Moore *et al.,* 2007). These translocations generate chimeric fusion proteins, all of which have in common the N-terminal half of the Nup98 protein. The C-terminal partner in these chimeras can be one of a wide variety of proteins that can be divided into two general classes: 1) homeodomain transcription factors and 2) other, typically nuclear and often nucleic acid-binding proteins, such as topoisomerases or the putative RNA helicase, DDX10.

The Nup98 protein is a component of the nuclear pore complex (NPC; reviewed in Tran and Wente, 2006). Like approximately one-third of nucleoporins, Nup98 possesses a series of FG (phenylalanine-glycine) repeats nontandemly clustered within one domain of the protein. Unique among metazoan nucleoporins, Nup98 also contains numerous copies of the GLFG (glycine-leucine-phenylalanine-glycine) subtype of repeats; however, functional distinctions between the different types of repeats are not fully defined. Within the NPC, nucleoporin repeat domains constitute in-

teraction sites for nuclear transport receptors and, through homotypic interactions, are thought to form a permeability barrier that excludes macromolecules lacking nuclear-targeting signals from the nucleus. Nup98 is a dynamic nucleoporin; it is found both on and off the NPC and can move between nuclear and cytoplasmic compartments. Within the nucleus, Nup98 is often found at sites termed GLFG bodies because the GLFG repeats are required for targeting to these structures (Griffis *et al.,* 2002). At the NPC, Nup98 is associated with both faces of the pore and specific nucleoporin-binding partners for both the C-terminal (Vasu *et al.,* 2001; Hodel *et al.,* 2002; Griffis *et al.,* 2003) and N-terminal regions (Pritchard *et al.,* 1999; Xu and Powers, unpublished data) domains have been identified. Additionally, Nup98 interacts with multiple transport factors from both the karyopherin and NXF families (Radu *et al.,* 1995a,b; Fontoura *et al.,* 2000; Blevins *et al.,* 2003).

Misregulation of transcription is a common theme in AML translocations (reviewed in Scandura *et al.,* 2002). In keeping with this, Nup98 fusions identified in patients frequently involve homeodomain transcription factors, most commonly HoxA9. Chimeric fusion proteins retain the C-terminal sequences of the transcription factor, including the 60-amino acid homeobox responsible for binding to DNA. Additionally, the GLFG nucleoporin repeats of Nup98, which are always present in fusion proteins, were reported to possess cryptic transcriptional regulatory activity through recruitment of the coactivator, p300/CBP (Kasper *et al.,* 1999; Bai *et al.,* 2006; Wang *et al.,* 2007) or the corepressor, HDAC1 (Bai *et al.,* 2006). The same repeat domain was also found to couple the mobility of Nup98 within the nucleus to ongoing transcription; in the presence of the transcription inhibitor

actinomycin D, Nup98 is much less mobile (Griffis *et al.*, 2002).

Models for Nup98-homeodomain fusion-induced leukemogenesis cite aberrant transcriptional activation of novel target genes as the basis for cellular transformation. Microarray experiments showed that expression of Nup98-HoxA9 in cells induces both the HoxA9 and Meis genes, as well as numerous others (Ghannam *et al.*, 2004; Chung *et al.*, 2006; Takeda *et al.*, 2006). More recently, Wang *et al.* (2007) found that the Nup98/NSD1 fusion, a nonhomeodomain fusion, also up-regulated expression of several HoxA genes as well as Meis1, a homeodomain cofactor that works in concert with HoxA9 during development. Simultaneous overexpression of HoxA9 and Meis1 was previously shown to cause myelogenous leukemias in mice, and Meis1 can potentiate leukemias induced by Nup98/HoxA9 (Kroon *et al.*, 2001).

Fusion proteins generated by chromosomal translocations can contribute to leukemogenesis not only through the acquisition of novel or unregulated activities, but also through modification of the normal function of one or both constituent proteins (Scandura *et al.*, 2002). This aspect of Nup98 leukemogenesis has not been well explored. Here we have focused on the behavior of Nup98 fusion proteins throughout the cell cycle, in particular the Nup98-homeodomain protein fusions. We find that, during both interphase and mitosis, the fusion proteins exhibit striking differences in localization from the parental proteins or from the fragments that make up the fusions. These differences are most dramatic during mitosis when fusion proteins are concentrated on chromosome arms, whereas the endogenous Nup98 is found diffusely throughout the cell. During interphase, we find that there are dynamic interactions between the Nup98 fusions and the endogenous Nup98 protein that result in relocalization of the intranuclear population of Nup98, although Nup98 at the nuclear pore remains unaffected. During mitosis, interactions between the chromatin-associated fusion proteins and endogenous Nup98 are lost and the fusion has no apparent influence on localization of the endogenous protein. Our findings indicate that the leukemogenic proteins have strikingly novel properties and can also influence behavior of the endogenous wild-type protein, each of which may contribute to leukemogenesis.

MATERIALS AND METHODS

DNA Constructs

Enhanced green fluorescent protein (GFP) fusion constructs pGFP-Nup98 (full length, amino acids 1-920), pGFP-Nup98 N-term (amino acids 1-225), and pGFP-Nup98 GLFG (amino acids 221-504) plasmid constructs were described previously (Griffis *et al.*, 2002). pGFP-Nup98 (1-469) was made by the introduction of two stop codons following Ser469 in pGFP-Nup98. Fluorescent derivatives of Nup98/HoxA9 and HoxA9 (kind gifts of Dr. Jan van Deursen, Mayo Clinic, Rochester, MN, and Dr. Corey Largman, University of California, San Diego) were made by transfer of these genes into pEGFP or pEYFP (Clontech, Palo Alto, CA). pGFP-HoxA9 C-term (amino acids 63-271) was made by excision of the Nup98 fragment from pGFP-Nup98/HoxA9. To encode either GFP-HOXA9 or GFP-Nup98/HoxA9 without the homeobox, a stop codon was introduced after residue A203 of HoxA9 by site-directed mutagenesis.

pGFP-Nup98/PMX1 and pGFP-PMX1 were produced by transfer of each gene (kind gifts of Dr. Takuro Nakamura, Akita University, Akita, Japan) to pEGFP-C1. The pGFP-PMX1 C-terminus construct (amino acids 81-217) was made by introducing an EcoRI site after S469 of Nup98-PMX1 through site-directed mutagenesis. The PMX1 C-terminus was then excised from the mutant and ligated into pEGFP-C1. The enhanced cyan fluorescent protein (CFP)-tagged Nup98/PMX1 plasmid was produced by transfer of a Kpn1-BamH1 fragment from pGFP-Nup98/PMX1 into pCFP-Nup98.

The coding sequences of Nup98/HoxD13 or the HoxD13 C-terminus (amino acids 253-335) were produced by PCR from MSCV-Nup98/HoxD13 (the gift of Dr. Keith Humphries, BC Cancer Agency, Vancouver, Canada) and ligated into pEGFP-C1 to produce pGFP-Nup98/HoxD13 or pGFP-HoxD13 Cterm. Full-length HoxD13 was cut out from pRD67-HoxD13 and inserted into pEGFP-C2 to produce pGFP-HoxD13. All subcloning was carried out by standard techniques (Sambrook *et al.*, 1989). Site-directed mutagenesis was carried out by Stratagene QuikChange mutagenesis (La Jolla, CA).

Cell Culture, Synchronization, and Immunofluorescence

HeLa cells (HeLa CCL-2) and HeLa-C cells (the gift of Volker Cordes, Max Planck Institute, Goettingen, Germany) were cultured in high-glucose DMEM (Cellgro, Manassas, VA) supplemented with 10% FBS as described previously (Griffis *et al.*, 2002). Transient transfection was performed using either Fugene 6 (Roche Diagnostics, Indianapolis, IN) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Unless otherwise indicated, transcriptions typically used 0.2–0.5 μg of DNA per well of a six-well plate. For transcriptional inhibition, cells were treated for 4 h with 5 μg/ml actinomycin D1 (Roche).

To enrich for mitotic cells, HeLa cells transfected with various GFP-fusions using Lipofectamine 2000 were synchronized by thymidine/nocodazole block (Zhu and Jiang, 2005). Twenty hours after transfection, cells were blocked in S phase with 2 mM thymidine for 16 h. After release into fresh medium for 6 h, cells were blocked in M phase with 20 ng/ml nocodazole for 12 h. Mitotic cells were collected by shake-off, pelleted and washed with PBS, suspended in fresh medium, and transferred to wells containing poly-L-lysine–coated glass coverslips. Coverslips were collected and fixed for immunofluorescence every 20 min up to 1 h after release.

For immunofluorescence assays, cells were plated on coverslips in six-well plates. Fourteen to eighteen hours after transfection, cells were washed with PBS, fixed 10 min at RT with 4% formaldehyde (methanol-free; Polysciences, Warrington, PA) in PBS, permeabilized with 0.2% Triton X-100, and processed as described previously (Griffis *et al.*, 2002). The following antibodies were used in these experiments: anti-Nup98 (1:2000; Griffis *et al.*, 2002), monoclonal 414 (1:1000; Calbiochem, La Jolla, CA), CREST serum (1:250; Antibodies Incorporated, Davis, CA), and chicken anti-mouse Gle2 (1:200). Antibodies were detected using Alexa Fluor–conjugated secondary antibodies with the exception of chicken anti-Gle2, which was detected using rhodamine-conjugated donkey anti-chicken (1:500). For staining with CenpA antibody (1:400, Cell Signaling Technology, Danvers, MA), cells were fixed for 10 min at −20°C with 100% methanol.

Epifluorescence images were captured using a BX-60 microscope (Olympus, Tokyo, Japan) equipped with an eight-bit camera (Dage-MTI, Michigan City, IN) and IP Lab software (Scanalytics, Fairfax, VA). When indicated, TIFF files were deconvolved using Simple PCI software (Compix, Cranberry Township, PA) and a nearest-neighbor algorithm. For simultaneous detection of CFP and YFP, or CFP and GFP fusion proteins, a Zeiss LSM510 Meta-equipped confocal microscope was used (Carl Zeiss, Thornwood, NY). To simultaneously image CFP and YFP, excitation of CFP was at 458 nm, with emission monitored at 463–495 nm. YFP was excited at 514 nm, and emission monitored at 516–570 nm. For simultaneous imaging of CFP and GFP, excitation was at 458 nm, emission was monitored at 463–612 nm, and Zeiss online fingerprinting software was used to separate CFP and GFP images. The images in Figure 6B were taken from a z-stack captured using a three-dimensional microscopy system (Intelligent Imaging Innovations, Denver, CO) based on a 200M microscope (Zeiss) equipped with a 12-bit Cool Snap HQ camera (Photometrics, Tucson, AZ) and Slidebook software (Intelligent Imaging Innovations). Deconvolution (nearest neighbors algorithm) and surface rendering were performed using Slidebook.

Immunoprecipitations

GFP-Nup98 and GFP-Nup98/HoxA9 were transfected into Hela cells using TransIT-Hela Monster (Mirus, Madison, WI) according to manufacturer's instructions. For mitotic samples, cells were synchronized by incubation with 10 ng/ml nocodazole for 12 h beginning 20 hours after transfection. Mitotic cells were collected by shake-off. For interphase samples, cells were collected by trypsinization. Harvested cells were washed and resuspended in PBS containing aprotinin/leupeptin, pepstatin, and COMPLETE protease inhibitor mixture, and sonicated. Clarified supernatant (340 μg protein) was incubated for 3 h at 4°C with 30 μl of protein A beads containing bound anti-GFP. Beads were then washed with PBS and eluted in 50 μl protein gel sample buffer. Eluted protein (5 μl) was resolved on 10% SDS-PAGE and immunoblotted using anti-GFP (1:10,000; Synaptic Systems, Goettingen, Germany) or chicken anti-Gle2 (1:2000).

Cell Sorting and Western Blots

GFP constructs were transfected into HeLa cells using Lipofectamine 2000. Twenty-four hours after transfection, cells were released from dishes using 25 mM EDTA, pelleted, and washed twice with PBS. Cells were sorted by flow cytometry and 250,000 GFP-positive cells were collected, pelleted, and lysed in 62.5 μl lysis buffer (150 mM NaCl, 20 mM HEPES, pH 7.4, 5 mM EGTA, 5 mM MgCl$_2$, 0.1% Triton X-100, and COMPLETE protease inhibitor mixture; Roche) for 30 min on ice. An equal volume of Laemmli gel sample buffer was added, and 3000 cell equivalents were subjected to SDS-PAGE, transferred to PVDF (Roche), and immunoblotted with anti-Nup98 (1:1000; Griffis *et al.*, 2002), anti-α-tubulin (1:1000; Sigma, St. Louis, MO), or anti-GFP (1:10,000;

A

B

C
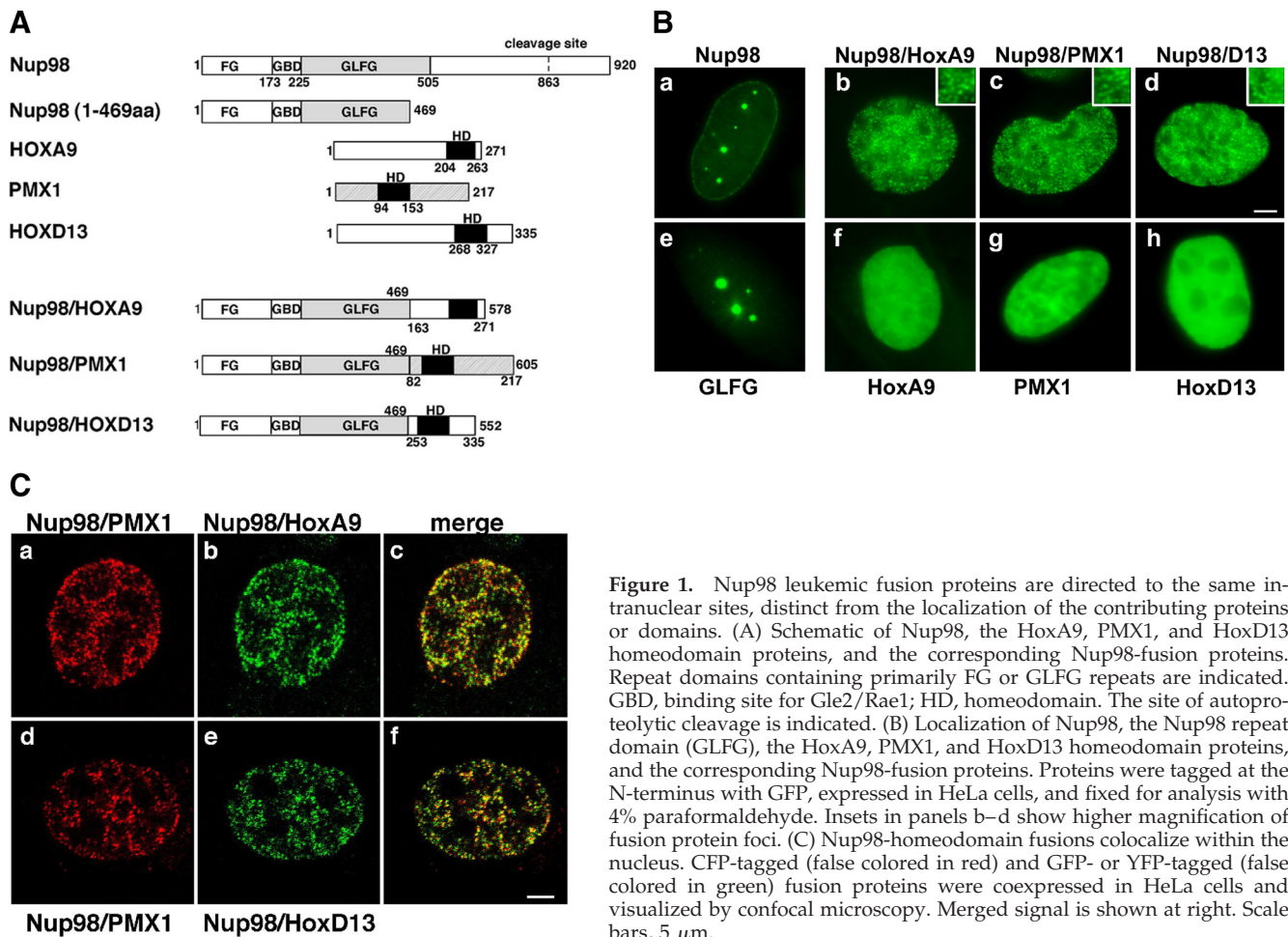
Figure 1. Nup98 leukemic fusion proteins are directed to the same intranuclear sites, distinct from the localization of the contributing proteins or domains. (A) Schematic of Nup98, the HoxA9, PMX1, and HoxD13 homeodomain proteins, and the corresponding Nup98-fusion proteins. Repeat domains containing primarily FG or GLFG repeats are indicated. GBD, binding site for Gle2/Rae1; HD, homeodomain. The site of autoproteolytic cleavage is indicated. (B) Localization of Nup98, the Nup98 repeat domain (GLFG), the HoxA9, PMX1, and HoxD13 homeodomain proteins, and the corresponding Nup98-fusion proteins. Proteins were tagged at the N-terminus with GFP, expressed in HeLa cells, and fixed for analysis with 4% paraformaldehyde. Insets in panels b–d show higher magnification of fusion protein foci. (C) Nup98-homeodomain fusions colocalize within the nucleus. CFP-tagged (false colored in red) and GFP- or YFP-tagged (false colored in green) fusion proteins were coexpressed in HeLa cells and visualized by confocal microscopy. Merged signal is shown at right. Scale bars, 5 μm.

Synaptic Systems). Blots were developed using ECL chemiluminescent substrate (Amersham, Piscataway, NJ).

Fluorescence Recovery after Photobleaching

Fluorescence recovery after photobleaching (FRAP) was performed as described previously (Griffis *et al.,* 2002). Briefly, HeLa cells were plated and transfected in LabTek II-chambered coverslips (Nalgene, Rochester, NY). Before imaging, the medium was replaced with imaging medium composed of indicator-free DMEM, supplemented with 20% FCS and 25 mM HEPES, pH 7.0. Photobleaching was carried out using a Zeiss LSM 510 confocal microscope and data analysis utilized Origin 6.1 software.

RESULTS

Localization of Nup98-Homeodomain Fusion Proteins

Previously, we found that the nucleoporin Nup98 is localized not only to the NPC but also to intranuclear bodies that we characterized by both deconvolution and electron microscopy (Griffis *et al.,* 2002). The prevalence of such bodies varies somewhat in different cell types. The central GLFG repeat domain of Nup98 is both necessary and sufficient for targeting to these structures. In contrast, efficient targeting of Nup98 to the NPC requires both the GLFG and the C-terminal domains (Griffis *et al.,* 2004).

In all Nup98 leukemogenic translocations, the N-terminal half of Nup98, containing the FG and GLFG repeats, is retained in the leukemic fusion protein (Figure 1A). We therefore asked whether Nup98 leukemic fusion proteins would be associated with the GLFG bodies along with the endogenous Nup98 or with a distinct intranuclear compart-

ment. We chose to focus our analysis on the homeodomain class of Nup98 fusion proteins as these are the most frequently detected in AML patients. The most common of this class is the Nup98/HoxA9 fusion, which consists of amino acids 1-469 of Nup98 joined to amino acids 163-271 of HoxA9 (Figure 1A). This protein encompasses the FG and GLFG repeats and the Rae1/Gle2-binding site of Nup98 joined to the homeodomain of HoxA9. To assess localization, GFP-tagged proteins were visualized in HeLa cells after transient transfection (Figure 1B). As previously reported (Griffis *et al.,* 2002), both the GFP-tagged Nup98 GLFG domain and the full-length protein were present in intranuclear bodies (Figure 1B, a and e). GFP-tagged HoxA9 protein was diffusely distributed throughout the nucleoplasm (Figure 1Bf). In contrast, GFP-Nup98/HoxA9 exhibited a unique localization, distinct from either of the component proteins. This fusion protein was found in finely punctate structures throughout the nuclear interior but was typically excluded from the nucleolus (Figure 1Bb and inset). The localization of the fragment of HoxA9 corresponding to the fusion protein was identical to full-length HoxA9, and the fragment of Nup98 (residues 1-469) was localized identically to the central GLFG domain (residues 226-505, data not shown). To investigate the contribution of the homeodomain, we truncated the Nup98/HoxA9 fusion just upstream of this domain. We found that the homeodomain made a substantial contribution to nuclear targeting or retention of the HoxA9 protein; in its absence HoxA9 accumu-

lated in the nucleus much less efficiently. Further, the homeodomain was required for the unique localization of the fusion protein (Supplemental Figure 1). Thus it appeared that the AML-associated juxtapositioning of sequences from Nup98 and HoxA9 resulted in a completely novel localization of the fusion protein, distinct from that of either wild-type protein or the component domains.

We then asked whether other Nup98-homeodomain fusion proteins would be similarly targeted to punctate intranuclear structures. To investigate this, we chose two other leukemic fusion proteins, Nup98/HoxD13 and Nup98/PMX1. HoxD13 is a class I homeogene residing in the Hox D gene cluster and is not normally expressed during hematopoietic cell differentiation. PMX1 is encoded by a class II homeogene found outside of the four genomic Hox gene clusters (Owens and Hawley, 2002; Argiropoulos and Humphries, 2007). Like the Nup98/HoxA9 fusion, each of these retains the N-terminal 469 amino acids of Nup98 as well as the homeodomain of the partner protein (Figure 1A). When expressed with a GFP tag, each of the native homeodomain proteins was diffusely distributed in the nucleus (Figure 1B, g and h). In contrast, each of the GFP-tagged Nup98 fusion proteins was again localized to finely punctate structures throughout the nucleoplasm (Figure 1B, c and d, and insets).

Although the leukemogenic fusions lack the NPC-targeting sequences in the C-terminus of Nup98, the finely punctate nature of the fusion protein localization patterns made it difficult to state definitively that none of the fusion protein was associated with the nuclear pore. We therefore tested each Nup98-homeodomain protein for colocalization with a marker of NPCs and found that these proteins were not associated with the nuclear pores (Supplemental Figure 2).

Because each Nup98 leukemogenic fusion protein exhibited a similarly punctate intranuclear distribution, we asked whether these proteins were in fact targeted to the same or similar intranuclear sites. To test this, we coexpressed Nup98/PMX1 tagged with CFP and Nup98/HoxA9 tagged with YFP. Localization patterns were determined using confocal fluorescence microscopy and meta-analysis to distinguish the signals (Figure 1C). We observed that the punctate localization patterns of the two proteins were substantially overlapping (Figure 1C, a–c). Similar results were obtained when we coexpressed Nup98/PMX1 and Nup98/HoxD13 (Figure 1C, d–f). Thus Nup98-homeodomain fusion proteins appear to all be distributed in similar, finely punctate patterns that exhibit significant, but not complete, overlap with each other. These novel localization patterns require contributions from each component, the homeodomain of the transcription factor and the repeat domains of Nup98.

Dynamics of Nup98 Fusions in Live Cells and Effect of Transcriptional Inhibition

Our previous photobleaching studies indicated that endogenous Nup98 is dynamically associated with the intranuclear GLFG bodies and moves rapidly in and out of these structures (Griffis et al., 2002). The rate of recovery of GFP-Nup98 is slower than the rate of diffusion, in keeping with transient interactions between Nup98 and other nuclear components throughout the nucleoplasm and especially within the GLFG bodies. We typically observe that 10–15% of Nup98 in GLFG bodies does not exchange or does so only very slowly (Figure 2A, left). To understand how the dynamics of Nup98 might be impacted in the leukemic fusions, we carried out FRAP (Lippincott-Schwartz et al., 2001) analyses of GFP fused to full-length HoxA9, HoxD13, or PMX1. We observed very rapid recovery of these proteins after

photobleaching of the nucleoplasm (Figure 2A, left). Although slower than the rate of diffusion, the Hox proteins move rapidly in the nucleoplasm, in keeping with very transient potential interactions with chromatin. The fragments of each of these proteins that are found in Nup98 fusions showed dynamics similar or identical to the corresponding full-length proteins (data not shown). Unexpectedly, we found that the fragment of Nup98 corresponding to the leukemogenic fusions (Figure 2A, right; Nup98 1-469) was less mobile than the full-length Nup98, as if some interactions are stabilized in the absence of additional residues.

Although their subnuclear localization patterns were virtually identical, we did observe differences in dynamics between the Nup98 fusions. Nup98/HoxA9 displayed recovery kinetics virtually identical to full-length Nup98 (Figure 2A, right), indicating that fusion to this homeodomain overcame the stabilization observed in the 1-469 fragment of Nup98. In contrast, fusion of the same Nup98 fragment to the homeodomain of either HoxD13 or PMX1 somewhat decreased its mobility in the nucleus.

We previously reported that inhibition of transcription by either actinomycin D or DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) dramatically reduced the mobility of Nup98 in GLFG bodies and, to a lesser extent, in the nucleoplasm (Griffis et al., 2002). This led us to ask whether the mobility of the leukemic fusion proteins might be similarly linked to transcription. We found that mobility of the Hox proteins themselves was moderately reduced when transcription was inhibited with actinomycin D, whereas both full-length Nup98 and the Nup98 fragment were strongly affected, as previously reported (Figure 2B, top panels).

The Nup98/HoxA9 fusion again differed somewhat from the other two homeodomain fusions in its response to transcriptional inhibition. Actinomycin D treatment did not alter the dynamics of the HoxA9 fragment, nor did fusion to HoxA9 increase the sensitivity of the Nup98 fragment to actinomycin D; the Nup98/HoxA9 fusion exhibited recovery kinetics that were slightly faster than the Nup98 fragment alone (Figure 2B, top right). In contrast, the fusion fragments of PMX-1 and HoxD13 appeared to contain the sequences responsible for the moderate response of the parental protein to actinomycin D treatment, as their recovery curves were similar to those seen for the full-length proteins (Figure 2B, bottom panels). When joined to the Nup98 fragment, the mobility of the resulting Nup98/PMX1 or Nup98/HoxD13 fusion proteins was somewhat more sensitive to actinomycin D treatment than the Nup98 fragment alone. Thus it appears both HoxD13 and PMX1 fragments stabilize interaction of the GLFG/FG repeats with a less mobile partner, particularly in the absence of ongoing transcription.

### Effects of Nup98-Homeodomain Fusions on Endogenous Nup98

The ability of leukemic fusion proteins to interact with and alter the normal targeting or function of their endogenous counterparts can be one contributing factor in leukemogenesis. In HeLa cells, ~5% of cells exhibit endogenous intranuclear GLFG bodies that stain with anti-Nup98. However, in a substrain of HeLa cells (HeLa-C; the kind gift of V. Cordes, Heidelberg) 95% of cells contain GLFG bodies detected by Nup98 antibody. The HeLa-C cells were transfected with each of the Nup98-homeodomain fusion proteins, and endogenous Nup98 was imaged using an antibody specific for the C-terminal domain of the protein; leukemic fusion proteins lack this domain and are not detected by the antibody. Strikingly, in cells expressing a Nup98-homeodomain fusion
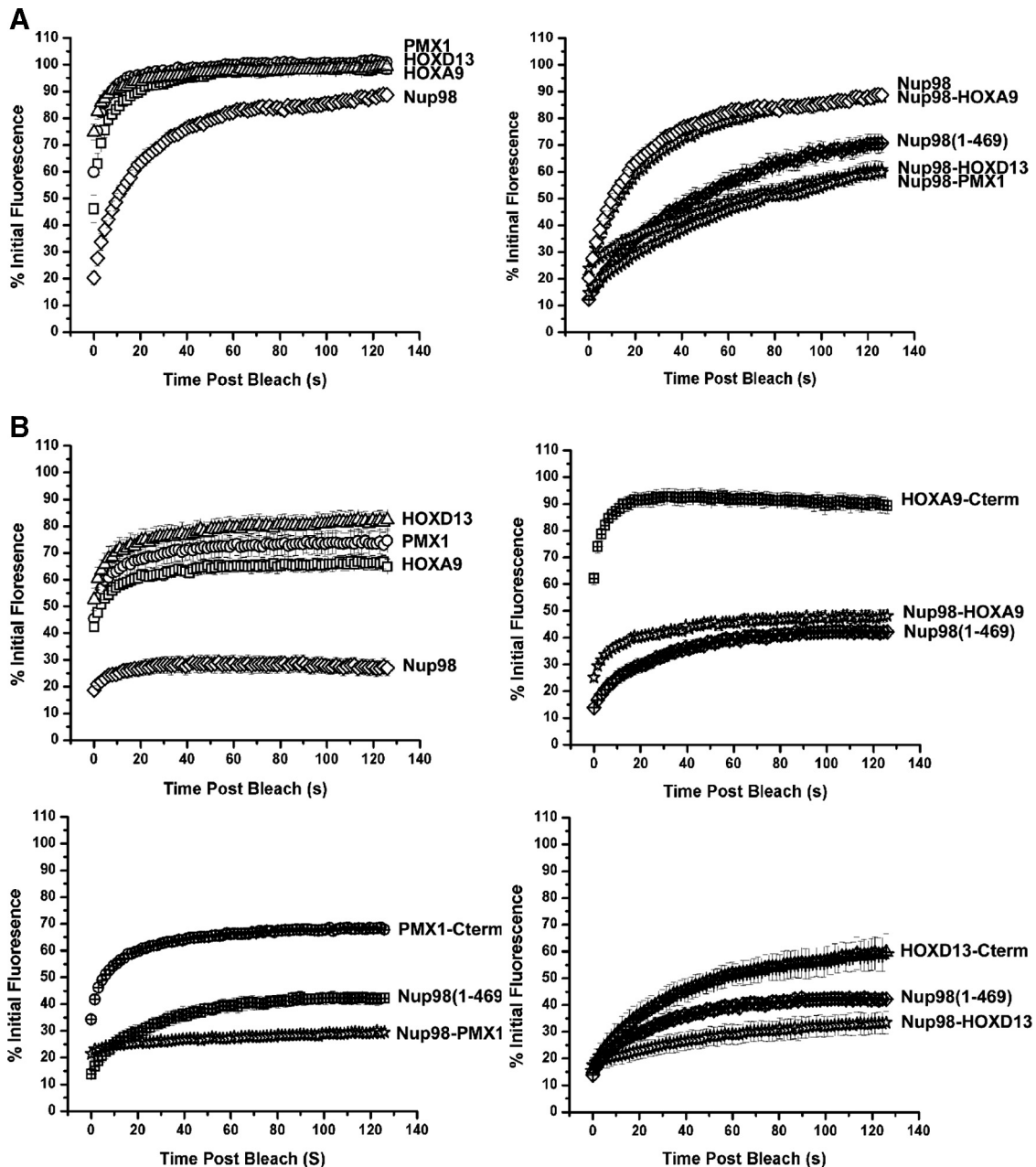
Figure 2. The dynamic behavior of Nup98-homeodomain fusions in live cells is distinct from that of the parental proteins. (A) Left, GFP-tagged Nup98 in GLFG bodies or GFP-tagged homeodomain proteins in the nucleoplasm were photobleached and fluorescence recovery was recorded over time. Right, GFP-Nup98, the GFP-tagged Nup98 fragment corresponding to the leukemic fusion proteins (amino acids 1-469), or GFP-tagged Nup98 fusions in intranuclear foci were photobleached and recovery was monitored over time. (B) The leukemic fusions and their component parts respond differently to inhibition of transcription with actinomycin D. Top left, the mobility of Nup98 is highly sensitive to inactivation of transcription, although the mobility of homeodomain proteins is only mildly effected. Top right, dynamic recovery of the Nup98/HoxA9 fusion and each of its component fragments in the absence of transcription. Note that the C-terminal HoxA9 fragment is fully mobile in the presence of actinomycin D, and the fusion protein shows the same mobility as the Nup98 fragment. Bottom left, dynamics of the Nup98/PMX1 fusion and each of its component fragments in the absence of transcription. Bottom right, dynamics of the Nup98/HoxD13 fusion protein and each of its component fragments in the absence of transcription. Note that the dynamics of both PMX1 and HoxD13 fusions are more sensitive to actinomycin D than either of their component fragments. Error bars, ±SEM; error bars are present in all curves although in some cases they are smaller than symbols and not visible. For each curve, n ≥ 10.

protein, the endogenous Nup98 GLFG bodies were largely absent, although Nup98 remained associated with the NPC (Figure 3A and Supplemental Figure 3). Thus expression of the Nup98-homeodomain fusions altered the normal intranuclear localization of the endogenous Nup98 protein.

To quantify the relative effect of the leukemic fusion proteins on endogenous Nup98, we transfected cells with different amounts of plasmid encoding GFP or GFP fused to either full-length Nup98 or the Nup98/HoxA9 fusion. For each condition, we scored 100 transfected cells for the pres-

A

B

C
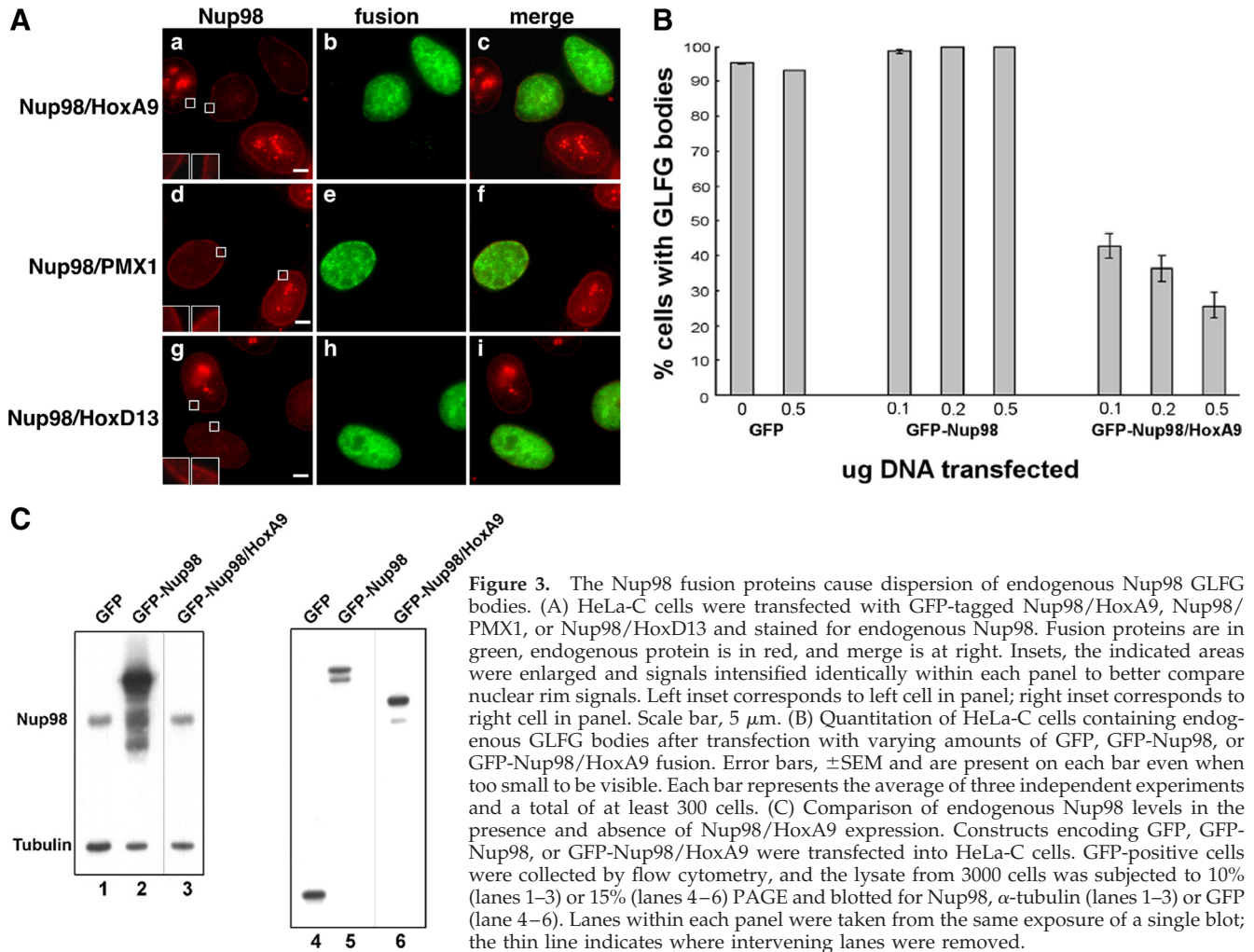
Figure 3. The Nup98 fusion proteins cause dispersion of endogenous Nup98 GLFG bodies. (A) HeLa-C cells were transfected with GFP-tagged Nup98/HoxA9, Nup98/PMX1, or Nup98/HoxD13 and stained for endogenous Nup98. Fusion proteins are in green, endogenous protein is in red, and merge is at right. Insets, the indicated areas were enlarged and signals intensified identically within each panel to better compare nuclear rim signals. Left inset corresponds to left cell in panel; right inset corresponds to right cell in panel. Scale bar, 5 μm. (B) Quantitation of HeLa-C cells containing endogenous GLFG bodies after transfection with varying amounts of GFP, GFP-Nup98, or GFP-Nup98/HoxA9 fusion. Error bars, $\pm$SEM and are present on each bar even when too small to be visible. Each bar represents the average of three independent experiments and a total of at least 300 cells. (C) Comparison of endogenous Nup98 levels in the presence and absence of Nup98/HoxA9 expression. Constructs encoding GFP, GFP-Nup98, or GFP-Nup98/HoxA9 were transfected into HeLa-C cells. GFP-positive cells were collected by flow cytometry, and the lysate from 3000 cells was subjected to 10% (lanes 1–3) or 15% (lanes 4–6) PAGE and blotted for Nup98, α-tubulin (lanes 1–3) or GFP (lane 4–6). Lanes within each panel were taken from the same exposure of a single blot; the thin line indicates where intervening lanes were removed.

ence of endogenous Nup98 GLFG bodies as detected by anti-Nup98 C-terminus. Strikingly, although expression of full-length Nup98 had no effect and indeed colocalized with endogenous protein (data not shown, but see Figure 4), expression of the Nup98/HoxA9 fusion protein led to loss of endogenous bodies in a dose-responsive manner (Figure 3B). Expression of the HoxA9, HoxD13, or PMX1 proteins had no effect on endogenous Nup98 (data not shown).

The disappearance of Nup98 intranuclear bodies could result from either degradation or mislocalization of the endogenous protein in the presence of the leukemic fusion. To assess whether the endogenous protein was being degraded, we transfected cells as above and selected GFP-positive cells by fluorescence-activated cell sorting. Protein from equivalent numbers of GFP-positive cells was analyzed by immunoblotting with anti-Nup98 C-terminus and anti-tubulin (Figure 3C, left) or anti-GFP (Figure 3C, right). To ensure selection of GFP-positive cells, many cells expressing low levels of GFP fusion protein are likely gated out, resulting in a population of cells that expresses relatively higher than average levels of the fusion protein. Nonetheless, we found that the level of endogenous Nup98 remained unaltered in the presence of even high levels of the Nup98/HoxA9 fusion protein.

If the endogenous Nup98 protein were being relocalized, it seemed likely to occur through recruitment of Nup98 to the site of the Nup98/HoxA9 protein. This would result in redistribution of the protein from relatively few intranuclear

sites to the very many foci observed with the Nup98/homeodomain fusions. We were unable to detect colocalization of endogenous Nup98 with the fusion protein, most likely because of dispersion of signal among many foci. We therefore coexpressed a small amount of GFP-Nup98 to act as a "tracer" for the endogenous protein. In the absence of the Nup98/PMX1 fusion, GFP-Nup98 colocalized with the endogenous protein both at the NPC and in GLFG bodies (Figure 4, a–c). However, when CFP-Nup98/PMX1 was simultaneously expressed, the intranuclear GFP-Nup98 pattern was altered, and both proteins were seen to localize to many small intranuclear foci (Figure 4, d–i). In many of these foci, colocalization of Nup98 and Nup98/PMX1, yielding a yellow signal, was observed. In others, separate foci of Nup98 and Nup98/PMX1 were closely apposed but were not coincident. Using the endogenous Nup98-specific antibody, we quantified the level of Nup98 at the NPC and found that it was unaltered by expression of the Nup98/PMX protein (Supplemental Figure 3). We therefore conclude that the leukemic fusion proteins can interact, either directly or indirectly, with the endogenous Nup98 protein. This interaction results in relocalization of the intranuclear population of Nup98, but does not affect Nup98 at the nuclear pore.

We were unable to demonstrate interaction between the fusions and endogenous Nup98 through coimmunoprecipitation from cells transfected with the fusion proteins. We were similarly unable to demonstrate direct binding be-

endog Nup98 **GFP-Nup98** **merge**

GFP-Nup98 **CFP-Nup98/PMX1** **merge**
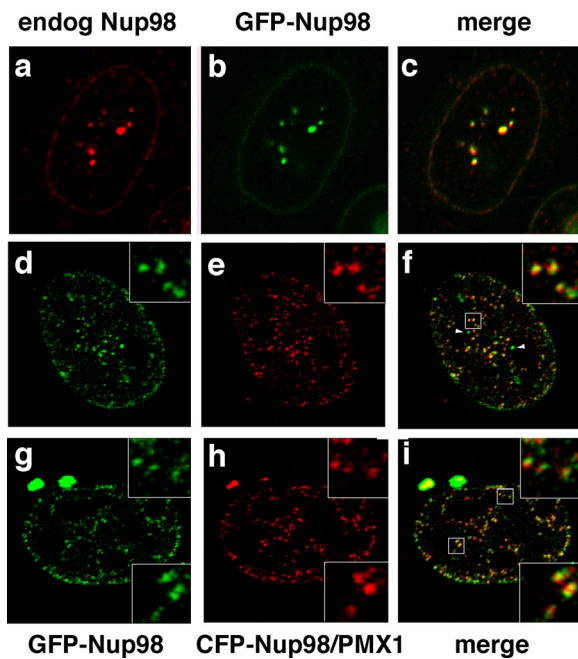
Figure 4. The Nup98 fusions disperse endogenous Nup98 by relocalization. (a–c) GFP-Nup98 acts as an indicator of endogenous Nup98 (top row). GFP-Nup98 (panel b) was expressed in HeLa-C cells where it colocalizes with endogenous Nup98 visualized with anti-Cterm antibody (a). (d–i) GFP-Nup98 in the nuclear interior was recruited to CFP-Nup98/PMX1 bodies (middle and bottom rows). GFP-Nup98 (d and g) and CFP-Nup98/PMX1 (e and h) were cotransfected in HeLa-C cells, and significant colocalization within the nucleus was visualized by confocal microscopy (f and i, merge). Arrowheads in panel f indicate two residual GLFG bodies located within nucleoli that seem unaffected by coexpression of the fusion protein. Regions boxed in the merge panels are enlarged as insets in each panel. Because of the low sensitivity of the Meta detection system required to separate GFP and CFP and the resulting higher background, images in panels d–i were enhanced by thresholding and Gaussian blur to better visualize signals. Scale bar, 5 μm.

tween the fusion proteins and Nup98 using in vitro–translated proteins (data not shown). This finding is not unexpected as we have been unable to detect dimerization of Nup98 by the same methods. Interaction between nucleoporin repeat domains is thought to occur via multiple, low-affinity binding interactions between the FG motifs. These interactions do not survive the conditions used for either immunoprecipitation or in vitro–binding experiments (Patel *et al.*, 2007).

*Association of Nup98 Fusions with Kinetochores and Chromatin during Mitosis*

During mitosis, the NPC is disassembled and most solubilized nucleoporins are found diffusely throughout the cell and are generally excluded from the chromatin. This localization pattern is observed for both endogenous Nup98 and exogenously expressed GFP-Nup98 (Figure 5A, a–d). The full-length homeodomain proteins, HoxA9, HoxD13, and PMX1, are similarly found throughout the mitotic cell, although both HoxA9 and PMX1 show some diffuse association with mitotic chromatin (Figure 5A, e–j). It was therefore most unexpected to find that each of the leukemic fusion proteins is highly concentrated on chromatin during mitosis. This association begins at prophase and continues through metaphase and anaphase (Figure 5B).

Because we had observed interaction between the Nup98 fusions and the endogenous Nup98 during interphase, we asked whether such interaction, as detected by the relocalization of endogenous Nup98, persisted during mitosis. Cells were transfected with the fusion proteins and stained with the Nup98 C-terminus antibody. Regardless of the level of Nup98 fusion protein expression, there was no recruitment of the endogenous Nup98 to chromosomes (Figure 5C). Thus we conclude that although there is interaction between the leukemic fusions and endogenous Nup98 during interphase, this interaction is lost during mitosis.

Within the repeat domain of Nup98 is the binding site for the Nup98 partner, Rae1/Gle2 (Brown *et al.*, 1995; Murphy *et al.*, 1996). Rae1 is a dynamic, WD40 repeat nucleoporin that moves between nucleus and cytoplasm (Pritchard *et al.*, 1999; Craige, Griffis and Powers, unpublished data) and has been implicated in both mRNA export and cell cycle regulation (Brown *et al.*, 1995; Murphy *et al.*, 1996; Babu *et al.*, 2003). Because the leukemic fusion proteins retain this site for Rae1 interaction, we asked whether we could observe binding between Nup98/HoxA9 and Rae1 in vivo and whether the pattern of Rae1 localization within the cell was influenced by the leukemic fusions. Lysates from cells expressing either GFP, GFP-Nup98, or GFP-Nup98/HoxA9 were used for immunoprecipitation with anti-GFP and assessed for the extent of Rae1 coprecipitation. We found no apparent difference between the level of Rae1 associated with Nup98 or with Nup98/Hoxa9 in either unsynchronized or synchronized mitotic cells (Figure 6A). Endogenous Rae1 was then localized in cells expressing either GFP-Nup98 or GFP-Nup98/HoxA9. As we previously reported (Blevins *et al.*, 2003), we detected Rae1 throughout the nucleus of interphase cells; Rae1 was not strikingly enriched at the NPC or in GLFG bodies. The same localization pattern was observed with Rae1-GFP (data not shown). During mitosis, Rae1 was found diffusely throughout the cytoplasm and was not associated with chromatin. These patterns did not vary regardless of whether Nup98 or Nup98/HoxA9 was coexpressed (Figure 6B). Thus we conclude that, although only a fraction of the total Rae1 appears to associate with Nup98 at any given time, the extent of interaction is equivalent for wild-type Nup98 and Nup98 fusion proteins.

Because the leukemic fusion proteins appeared as punctate dots associated with mitotic chromatin, we asked whether they might be associating, at least in part, with kinetochores. We transfected cells with each of the leukemic fusions tagged with GFP and synchronized the cells at the G2/M transition using nocodazole. Cells were fixed at short times after release of the nocodazole block and costained using CREST serum, a human autoimmune serum that recognizes kinetochores (Moroi *et al.*, 1980). Under these conditions, the fusions were not specifically enriched on kinetochores; rather each is found in finely punctate foci along the chromosome arms (Figure 7A, a–c, and Supplemental Figure 4). However, we noted that, in prophase or prometaphase cells expressing low levels of the Nup98-homeodomain fusions, the fusion protein was typically found in relatively few, paired dots (Figure 7A, d–f, and Supplemental Figure 4). In such low expressing cells, these paired foci of fusion proteins were very often adjacent to the CREST-stained kinetochores.

To further investigate this localization, we stained synchronized, GFP-Nup98/HoxA9–transfected cells with both CREST serum and antibody to CenpA, the kinetochore-specific histone H3 variant. CREST serum recognizes a subset of Cenp proteins, primarily CenpB, the centromeric DNA repeat-binding protein of the inner kinetochore. Under the
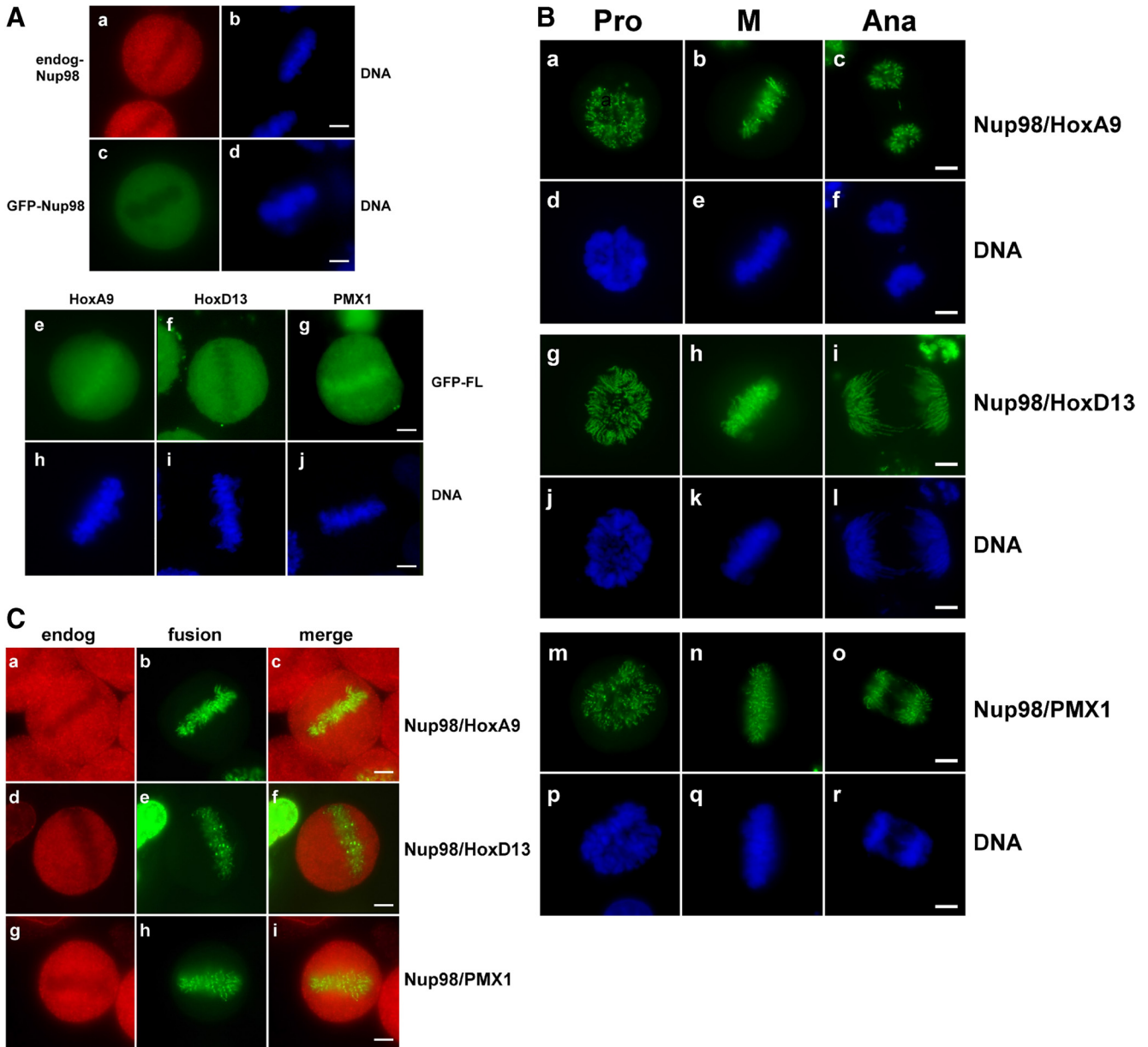
Figure 5. Unlike either component protein, Nup98 fusions are highly localized to chromosomes during mitosis but do not recruit endogenous mitotic Nup98. HeLa cells were transfected with GFP constructs as indicated. Cells were then synchronized in M phase by thymidine/nocodazole block. (A, a and b) Neither endogenous Nup98, detected by antibodies, nor GFP-Nup98 show any significant association with mitotic chromosomes. (c and d) GFP-HoxA9 and GFP-PMX1, but not GFP-HoxD13 exhibit some general association with mitotic chromosomes. (B) GFP-Nup98 fusions were highly concentrated on chromosomes during mitosis. All three Nup98 fusions exhibit a punctate staining pattern along mitotic chromosomes. (C) HeLa cells expressing GFP-Nup98 fusions were synchronized in M phase. In contrast to interphase, endogenous Nup98 (red) is not recruited to sites of GFP-Nup98 fusions during mitosis. Scale bar, 5 μm.

fixation conditions required for the CenpA antibody, these two inner kinetochore components can be seen to separate slightly (Figure 7B, e, g, and h). We found that Nup98/HoxA9 was always most closely associated with the CenpA staining (Figure 7B, red). Indeed, CenpB (as indicated by CREST, blue) and Nup98/HoxA9 were always found on opposing sides of CenpA. This arrangement can be most easily seen on the unattached kinetochores indicated by arrowheads in Figure 7B, panels a and e, and are rendered at higher magnification in panel h. When kinetochores are aligned at the metaphase plate, the blue CREST signal is always somewhat more central than is the CenpA signal

(Figure 6Be). From these patterns, we conclude that the Nup98-homeodomain fusions are most likely found in the outer kinetochore. Overall, the mitotic localization data suggest that the unique localization of the Nup98 fusions during mitosis may initiate at the outer kinetochore and then spread in a punctate manner along the chromosome arms.

DISCUSSION

It is a recurrent theme in AML that fusion proteins acquire novel intranuclear localizations as well as novel activities (McNeil *et al.*, 1999; Scandura *et al.*, 2002). Additionally,
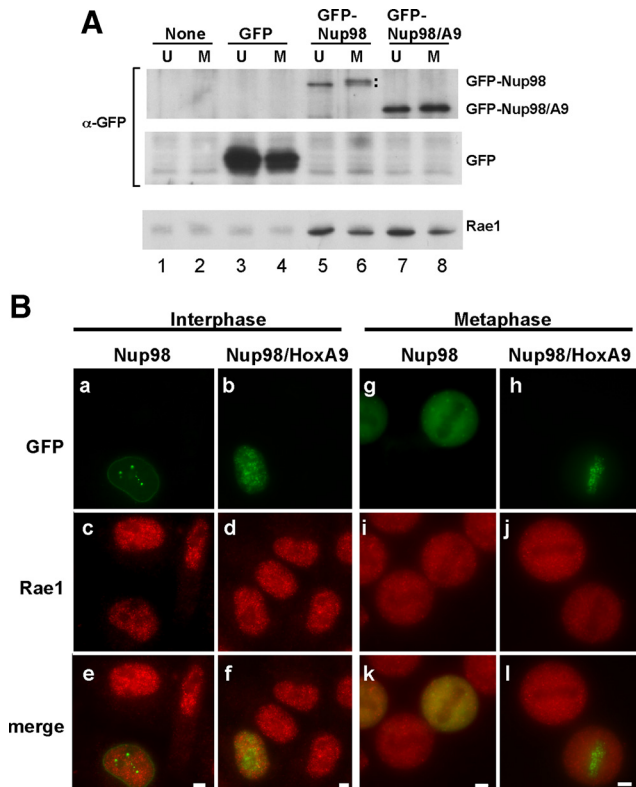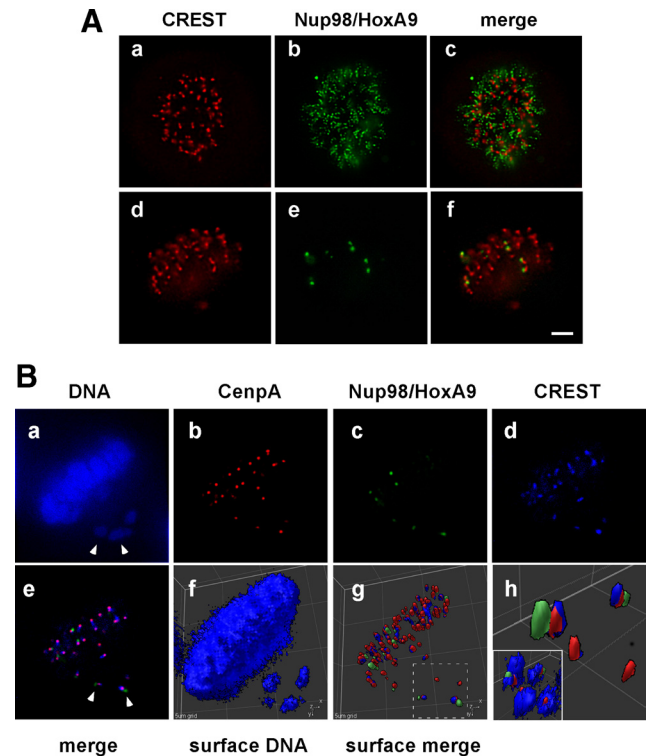
Figure 6. Interaction of Nup98/HoxA9 with Rae1 is similar to that of Nup98. HeLa cells were transfected and synchronized in M phase when indicated. U, unsynchronized cells. (A) Lysates were prepared from equivalent numbers of interphase or mitotic cells, immunoprecipitated using anti-GFP, and equivalent amounts of precipitate were immunoblotted using anti-GFP or anti-Rae1. The presence of shifted bands (dots, lane 6) due to phosphorylation of Nup98 confirms the mitotic nature of lysates from the synchronized cells. Similar amounts of Rae1 are immunoprecipitated with both GFP-Nup98 and GFP-Nup98/HoxA9. (B) Transfected cells expressing GFP-Nup98 (a, c, e, g, i, and k) or GFP-Nup98/HoxA9 (b, d, f, h, j, and l) were synchronized in M phase when indicated and fixed for immunofluorescence with anti-Rae1 (red). The Rae1 localization pattern is equivalent in the presence of either form of Nup98. Scale bars, 5 μm.

Figure 7. Nup98/HoxA9 is associated with the outer kinetochore during mitosis. HeLa cells were transfected, synchronized in M phase and fixed for immunofluorescence. (A) CREST serum was used to mark the inner kinetochores. Wide-field images were deconvolved using Simple PCI software. (a–c) GFP-Nup98 fusions (green) expressed at moderate levels were found in a punctate pattern along the chromosomes. CREST staining is shown in red. (d–f) When expressed at very low levels, GFP-Nup98/HoxA9 is found only at kinetochores during mitosis, overlapping or adjacent to CREST antigens. Scale bar, 5 μm. (B) HeLa cells expressing a low level of GFP-Nup98 (green) were fixed and stained for DNA (Hoechst, blue, a and f and inset in h), CenpA (red), and CREST (blue, d, e, g, and h). Arrowheads indicate unattached kinetochores positive for Nup98/HoxA9. (a–e) Deconvolved images from a single plane of a z-series. (f–h) Surface renderings of the entire z-stack. GFP-Nup98/HoxA9 (green) is found immediately adjacent to CenpA (red). The inset in panel h shows the DNA stain added to the image in the panel.

fusions such PML/RARα can bind to and alter the localization and/or function of their endogenous component proteins (Weis *et al.*, 1994). In the work presented here, we investigated the unique properties of several Nup98/Hox leukemogenic fusions. We found that, during interphase, these fusion proteins can recruit the intranuclear population of endogenous Nup98 although they do not affect the NPC-associated fraction. During mitosis, the fusions no longer interact with endogenous Nup98 and, unexpectedly, are concentrated at kinetochores and along chromosome arms.

We observed that three different Nup98-homeodomain fusion proteins exhibit similar finely punctate localization patterns within the interphase nucleus and indeed could be seen to significantly colocalize with each other, indicating that they are targeted, at least in part, to similar intranuclear sites. This localization is clearly distinct from that of the wild-type partner proteins or the component fragments and is consistent with intranuclear localizations reported previously for Nup98/HoxA9 and Nup98/PMX1 (Kasper *et al.*, 1999; Bai *et al.*, 2006). The leukemic Nup98 fusions are not found at the NPC; this is consistent with our previous find-

ing that the C-terminal domain of Nup98, which is absent from the fusions, provides a major NPC-targeting sequence (Griffis *et al.*, 2003). The localization of the fusion proteins required both the GLFG repeats of Nup98 and the homeodomain of the Hox protein; similarly, both fragments are required for leukemogenesis caused by Nup98-homeodomain fusions (Pineault *et al.*, 2003; Hirose *et al.*, 2008). We would propose therefore that combining the DNA-binding activity of the homeodomain with one or more binding interaction mediated by through the nucleoporin repeats of Nup98 acts synergistically to create unique protein targeting as well as a unique function that leads to leukemogenic transformation.

The pattern we observed for the leukemic fusion proteins is highly reminiscent of the localization pattern observed for sites of transcription (Misteli, 2007). However, when we performed BrUTP incorporation, we did not observe significant overlap between the transcription sites labeled by this technique and the Nup98-homeodomain fusions (Xu and Powers, unpublished data). The fusion proteins often ap-

peared to be near the nuclear rim, but their localization could be readily resolved from that of a marker for NPCs. This suggests that fusions might interact either with heterochromatin or with proteins associated with the inner nuclear envelope, although such an interaction remains to be demonstrated. Interestingly, although all the Nup98-homeodomain fusions we tested displayed virtually identical patterns, this localization is quite different from the intranuclear localization we observed for several nonhomeodomain Nup98 fusions (Xu and Powers, unpublished data).

In the presence of Nup98-homeodomain fusion proteins, endogenous Nup98 no longer associates in the characteristic GLFG bodies, but instead appears to disperse. Using a low level of GFP-Nup98 as a tracer, we showed that Nup98 was recruited from the GLFG bodies, to the many Nup98/HoxA9 foci. This effect is specific to the fusion proteins and is dose-dependent, increasing with increasing level of fusion protein expression. In contrast, we found no evidence that any of the fusion proteins reduced the level of Nup98 at the NPC. Association between repeat domains, as could occur between Nup98 and the Nup98 fusions, is thought to result from multiple, low-affinity, hydrophobic interactions between repeat motifs (Patel et al., 2007). Such binding could be sufficient to recruit Nup98 from intranuclear bodies where association also depends upon the repeat domain (Griffis et al., 2002). Studies carried out by others indicate that interactions between repeat motifs do not persist through the conditions of in vitro–binding assays (Patel et al., 2007), and similarly, we were unable to demonstrate direct binding between Nup98 and Nup98 fusions in this assay. In contrast, Nup98 association with the NPC is stabilized by additional, higher affinity interactions between the C-terminal domain and the nucleoporins Nup96 and Nup88 (Griffis et al., 2003; Ratner et al., 2007). Thus it is quite feasible that, despite its relocalization within the nucleus, endogenous Nup98 would remain unaltered at the NPC. We conclude that the fusion proteins do not impact Nup98 at the NPC, but rather they exert their effects in the nuclear interior. Nonetheless, because of the heterozygous nature of Nup98 translocations in leukemia patients and the lack of detectable fusion protein at the NPC, the NPC in these patients is presumably deficient in Nup98. Such a 50% reduction in Nup98 protein could impact nuclear transport in a manner independent of the fusion proteins themselves. However, the Nup98 +/− mouse is viable (Jeganathan et al., 2005), and in RNAi experiments, we have observed that the level of Nup98 can be reduced by more than 50% without a substantial impact on cell growth in culture, suggesting that nuclear transport is surprisingly tolerant of variation in the level of Nup98 (B. Hilbert, A. Pierce, and M. A. Powers, unpublished data).

The Nup98-homeodomain fusions display unique in vivo dynamics. Hox proteins, as well as the Hox fragments corresponding to the fusions, are highly mobile, significantly more so than Nup98. In contrast, the fragment of Nup98 found in the fusion proteins is substantially less mobile in the nucleus than is the full-length protein, as if its interaction with a more stationary binding partner is strengthened in the absence of downstream sequences. There are, however, notable differences between the dynamic behavior of individual fusion proteins: Nup98/HoxA9 is virtually indistinguishable from full-length Nup98, whereas the HoxD13 and PMX1 fusions are slightly less mobile than the Nup98 fragment, indicating that they do not inhibit binding by the GLFG/FG repeat domain, but rather they may act to strengthen binding to a less mobile partner, perhaps chromatin or a large transcriptional regulatory complex. This

finding suggests that there are differences either in the binding partners of the fusions or in the characteristics of their interaction with a common partner.

The most dramatic differences between Nup98 fusions and the component proteins were observed during mitosis. Endogenous Nup98 protein was diffuse throughout the cell during mitosis, a localization that was recapitulated by GFP-Nup98. Both HoxA9 and PMX1 show some diffuse interaction with mitotic chromosomes although HoxD13 appears to be largely excluded from chromatin. Remarkably, each of the Nup98-homeodomain fusions is concentrated in a punctate array along chromosome arms. It is intriguing that, at the lowest expression level, each of the fusions is found on a few, apparently paired kinetochores. Multiple nucleoporins including Nup358 and the Nup107 complex have been found to associate with mitotic kinetochores; however, only 10–15% of each nucleoporin is typically found at this site. Despite attempts with two independent antibodies as well as a GFP tag, we have never detected either endogenous or transfected Nup98 at the kinetochores or on chromatin (M. K. Cross and M. A. Powers, unpublished data). Thus it is particularly noteworthy that the Nup98 leukemic fusions were entirely associated with the kinetochores and chromosomes. Fusion proteins were detected on kinetochores regardless of whether the kinetochore showed bipolar attachment and alignment on the metaphase plate, suggesting that binding is not a function of the spindle checkpoint. The association of Nup98 fusions with the chromosome arms is detected in prophase and persists through metaphase and anaphase.

At the kinetochore, Nup98/HoxA9 was found closely juxtaposed to CenpA, most likely as a component of the outer kinetochore. This near association with CenpA, the centromeric histone 3 variant, is interesting because the nonhomeodomain Nup98 fusion, Nup98/JARID1A, was recently reported to specifically bind histone H3K4 me3 (Wang et al., 2009). However, in contrast to the PHD domain of JARID1A, a histone demethylase, there are no known histone H3-specific binding sites in either Nup98 or the Hox proteins. The apparent paired nature of kinetochores with associated Nup98 fusion proteins is intriguing. It might be that the Nup98 fusion protein was partially transferred to the daughter chromosome during replication; however, this possibility will require further investigation.

Nup98, together with its partner Rae1/Gle2, has been shown to bind Cdc27, a component of the APC/C (anaphase-promoting complex/cyclosome; Jeganathan et al., 2005, 2006). Cdc27 and other APC/C components have been detected at the kinetochore and in a punctate manner along chromosome arms during mitosis (Topper et al., 2002). The APC/C is the E3 ligase responsible for progression from metaphase to anaphase, in part through targeting the protein securin for degradation (reviewed in Peters, 2006). On degradation of securin, its binding partner separase becomes active and cleaves the cohesin complex, releasing sister chromatids to migrate to opposing poles in anaphase. Association of Nup98 and Rae1/Gle2 with the APC/C has an inhibitory effect on the degradation of securin and thus delays progression to anaphase (Jeganathan et al., 2005).

It is not established where in the mitotic cell the interaction between Nup98, Rae1/Gle2, and the APC/C occurs, although this interaction is likely to be transient, in keeping with our observation that, although some Nup98 and Rae1/Gle2 can be coprecipitated, their localization patterns are not identical. Our finding that, unlike endogenous Nup98, the Nup98/homeodomain fusions accumulate at sites where the APC/C is also present raises the possibility that aberrant,

possibly hyperstablized, interactions with the APC/C might influence sister chromatid separation or other anaphase events regulated by the APC/C. Nup98 and Rae1/Gle2 normally bind the APC/C only as a heterodimer; neither protein alone binds significantly. However, the binding site for Rae1/Gle2 is present in the Nup98 fusion proteins, raising the possibility that leukemic fusion proteins could act in concert with Rae1/Gle2 to influence APC/C activity. The combined Nup98+/−, Rae1+/− mouse showed a significant increase in aneuploidy although the Nup98 +/− mouse did not (Jeganathan *et al.,* 2005). However, the double heterozygous mouse did not show an increased frequency of spontaneous tumor formation (Jeganathan *et al.,* 2006). Thus, on its own, altered APC/C regulation by Nup98 and Rae1/Gle2 seems insufficient for spontaneous tumorigenesis. It is possible, however, that misregulation of the APC/C may play a contributory role when combined with aberrant transcriptional regulation.

The current model for Nup98 leukemogenesis has focused on misregulation of transcription due to coupling of DNA binding through the homeodomain with recruitment of chromatin modifying coregulators by the Nup98 repeat domain. This combination of activities leads to upregulation of homeodomain target genes, particularly HoxA9 and its cofactor, Meis. Here we have defined several new properties of the Nup98-homeodomain fusion proteins including their interactions with and mislocalization of endogenous Nup98 protein, and their strong association with mitotic chromatin and kinetochores. Our findings highlight several potential new prospects for misregulation by the Nup98-homeodomain fusions. Further exploration of these novel properties will continue to define their contributions to leukemogenesis.

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