

ORIGINAL ARTICLE

MLL is essential for NUP98-HOXA9-induced leukemia

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Rearrangements involving the *NUP98* gene resulting in fusions to several partner genes occur in acute myeloid leukemia and myelodysplastic syndromes. This study demonstrates that the second FG repeat domain of the NUP98 moiety of the NUP98-HOXA9 fusion protein is important for its cell immortalization and leukemogenesis activities. We demonstrate that NUP98-HOXA9 interacts with mixed lineage leukemia (MLL) via this FG repeat domain and that, in the absence of MLL, NUP98-HOXA9-induced cell immortalization and leukemogenesis are severely inhibited. Molecular analyses indicate that MLL is important for the recruitment of NUP98-HOXA9 to the *HOXA* locus and for NUP98-HOXA9-induced *HOXA* gene expression. Our data indicate that MLL is crucial for NUP98-HOXA9 leukemia initiation.

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INTRODUCTION

NUP98, a component of the nuclear pore complex (NPC), has an important role in molecular trafficking between the cytoplasm and nucleus. NUP98 has two Phe-Gly (FG) repeat domains, which are characteristic of NPC proteins.¹ In acute myeloid leukemia (AML) and myelodysplastic syndromes, the *NUP98* gene can be rearranged and fused to several partner genes, including *HOXA9* and *DDX10*.^{2,3} In all NUP98 fusion proteins reported to date, the NUP98 moiety is at the N-terminus, with the C-terminal region consisting of the fusion partner moiety.⁴ NUP98 rearrangements are associated with poor prognosis in leukemia.

NUP98-HOXA9 regulates the expression of several genes, including *Hoxa* genes and *Meis1*, to induce cell proliferation and block the differentiation of hematopoietic stem cells.⁵ Another NUP98 fusion protein, NUP98-NSD1, also regulates the expression of the *Hoxa* genes and *Meis1*.⁶ NSD1 is a histone methyltransferase that targets lysine-36 of histone H3, and H3K36 methylation of the *Hoxa* locus is important for NUP98-NSD1-mediated tumorigenesis. CBP/p300 interacts with the NUP98 moiety of NUP98-HOXA9, and in NIH3T3 cells, the transactivation function of NUP98 is impeded by an inhibitor of CBP/p300.⁷ The proteins CRM1 and AES also interact with the NUP98 moiety of NUP98 fusion proteins, and transcription factors exported to the cytoplasm by CRM1 in normal cells accumulate in the nucleus of NUP98 fusion protein-expressing cells.⁸ NUP98-HOXA9 and AES cooperatively regulate the undifferentiated state of human CD34 cells;⁹ however, the role of the NUP98 moiety in leukemogenesis and cell immortalization is poorly understood. By contrast, the role of HOXA9 in leukemia development is well established. HOXA9 is a homeodomain-containing transcription factor activated in AML. HOXA9 itself can transform hematopoietic cells;^{10,11} therefore, the HOXA9 moiety of NUP98-HOXA9 is thought to be particularly important for cell immortalization and leukemogenesis by NUP98-HOXA9.

MLL, the mixed lineage leukemia gene, is a homolog of *Drosophila* trithorax. MLL is one of the most important regulators of *Hox* genes^{12,13} and has a SET domain, which functions as a histone methyltransferase, targeting lysine-4 of histone H3, methylation of which is a mark of transcriptionally active chromatin states, and activates the expression of *Hox* genes.^{14,15}

MLL is also fused to a wide variety of partner genes in AML, resulting in MLL fusion proteins, which constitutively activate the expression of *Hox* genes. Together with several epigenetic factors, MLL fusion proteins promote leukemia development. CBX8, a Polycomb group protein, is required for MLL-AF9-induced leukemogenesis,¹⁶ and DOT1L, a histone methyltransferase, is also important for MLL-rearranged leukemia.^{17–20} MLL-AF9 acts in cooperation with wild-type MLL to induce leukemia.²¹

In this study, we show that the second NUP98 FG repeat domain of NUP98-HOXA9 is important for cell immortalization and leukemogenesis. NUP98-HOXA9 interacted with MLL via the FG repeat domain and both proteins colocalized to the *Hoxa* gene locus. During the analysis of *MLL* null mice, we also found that MLL is essential for the recruitment of NUP98-HOXA9 to the *Hoxa* gene locus and that NUP98-HOXA9 induces cell immortalization and leukemogenesis. Our data indicate that NUP98-HOXA9 and MLL cooperatively activate *HOXA* genes during leukemogenesis.

MATERIALS AND METHODS

Cell culture, infection and antibodies

293FT cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Plat-E cells were obtained from Dr T Kitamura (Tokyo University, Tokyo, Japan) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 10 µg/ml blasticidin and 1 µg/ml puromycin. Antibodies were purchased commercially: anti-HA (3F10, Roche, Mannheim, Germany), anti-FLAG (M2, Sigma, St Louis, MO, USA), anti-MLL (Bethyl, Montgomery, TX, USA), anti-GAL4 (Santa Cruz, Dallas, TX, USA), anti-DYKDDDDK (FLAG) (Biolegend, San Diego, CA, USA), anti-histone H3 (Abcam, Cambridge, UK), anti-histone H3 (tri methyl K4) (Abcam), normal rat IgG (Santa Cruz), and normal rabbit IgG (Santa Cruz).

Plasmids

NUP98-HOXA9, NUP98-DDX10 and NUP98 expression vectors were obtained from Dr Y Arai (National Cancer Center, Tokyo, Japan). HOXA9 cDNA was amplified by PCR from a human cDNA library generated from RNA from K562 cells. Deletion and point mutants were generated by PCR. MLL deletion mutants were described previously.²²

Mice

C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan). *MLL*-deficient mice were reported previously.²³ Fetal liver cells were obtained from E12.5 mouse embryos. Mouse experiments were carried out in a specific pathogen-free environment at the National Cancer Center animal facility according to institutional guidelines and with the approval of the National Cancer Center Animal Ethics Committee.

Serial replating assay

C-kit⁺ cells were selected from the femurs of C57BL/6 mice using CD117-specific MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) and transduced with retroviruses using RetroNectin (Takara, Shiga, Japan) as described previously.²⁴ The cells were plated in methylcellulose medium M3234 (StemCell Technologies, Vancouver, BC, Canada) supplemented with 10 ng/ml stem cell factor, 10 ng/ml interleukin-3, 10 ng/ml granulocyte-macrophage colony-stimulating factor and 1% penicillin-streptomycin. The cells were cultured and replated every 4–5 days in methylcellulose medium under G418 selection (the first and second rounds). Colony numbers were counted on the third to fifth rounds. Fetal liver cells were transduced with retrovirus and plated in methylcellulose medium under puromycin selection.

Transplantation

pMys-ires-GFP, pMys-NUP98-HOXA9-ires-GFP, pMys-NUP98-HOXA9-ΔFG1-ires-GFP, pMys-NUP98-HOXA9-ΔFG2-ires-GFP and pMys-FLT3-ITD-ires-hNGFR were transfected into Plat-E cells using GeneJuice (Millipore, Billerica, MA, USA), and supernatants containing the retrovirus were collected 48 h after transfection. C-kit⁺ cells were transduced with FLT3-ITD-ires-hNGFR and ires-GFP, NUP98-HOXA9-ires-GFP, NUP98-HOXA9-ΔFG1-ires-GFP or NUP98-HOXA9-ΔFG2-ires-GFP. Fetal liver cells were transduced with FLT3-ITD-ires-hNGFR and NUP98-HOXA9-ires-GFP. The infants were transplanted into sublethally irradiated (6 Gy) C57BL/6 mice.

Real-time PCR

Based on the result of mRNA microarray analysis (GSE93923), quantitative real-time PCR was performed using QuantStudio 12K Flex (Applied Biosystems, Waltham, MA, USA) with commercially purchased TaqMan Gene Expression Assays, except for *Hoxa9*. *Hoxa9* expression was detected using a Custom TaqMan Gene Expression Assay to avoid crossreacting with human HOXA9, including the following primer set and reporter: forward primer (5'-CTGTCCGTCTCCCTCTGTCT-3'), reverse primer (5'-CTGGGATTTAGAGCCCTTTGT-3'), and reporter (5'-CCCA GCCTCCACCCGC-3'). Values were normalized with respect to the expression of the *Tbp* gene.

Chromatin immunoprecipitation (ChIP) assay

Cells were fixed with 1% formaldehyde for 15 min at room temperature and incubated with 125 mmol/l glycine for a further 10 min to stop the crosslinking reaction. The cells were washed twice with phosphate-buffered saline containing 1 mmol/l phenylmethylsulfonyl fluoride and Complete protease inhibitor (Roche) and lysed in FA lysis buffer (50 mmol/l HEPES-KOH, pH 7.5; 140 mmol/l NaCl; 1 mmol/l EDTA; 1% Triton X-100; 0.1% deoxycholic acid; 0.1% sodium dodecyl sulfate (SDS) and 1 mmol/l phenylmethylsulfonyl fluoride) supplemented with Complete protease inhibitor. The lysates were sonicated using a Sonifier 450 (Branson, Danbury, CT, USA) or an S220 Focused-ultrasonicator (Covaris, Woburn, MA, USA), diluted 10-fold in FA wash buffer (50 mmol/l HEPES-KOH, pH 7.5; 150 mmol/l NaCl; 1 mmol/l EDTA and 1% Triton X-100) and incubated with antibodies and protein G Dynabeads (Invitrogen, Carlsbad, CA, USA) at 4 °C overnight. The immunoprecipitates were washed four times with FA wash buffer (anti-DYKDDDDK and anti-MLL antibodies) or twice with low-salt wash buffer (20 mmol/l Tris-HCl, pH 8.1; 150 mmol/l NaCl; 2 mmol/l EDTA; 0.1% SDS and 1% Triton X-100), once with high-salt wash buffer (20 mmol/l Tris-HCl, pH 8.1; 500 mmol/l NaCl; 2 mmol/l EDTA; 0.1% SDS and 1% Triton X-100) and finally twice with TE containing 0.1% Triton X-100 (anti-Histone H3 and anti-Histone H3K4me3 antibodies). The immunoprecipitated chromatin was eluted in elution buffer (1% SDS and 100 mmol/l NaHCO₃), and crosslinking was reversed by incubation overnight at 65 °C with the addition of 200 mmol/l NaCl to the elution buffer. The eluted chromatin was treated with proK buffer (40 mmol/l Tris-HCl, pH 6.5; 80 mg/l proteinase K and 10 mmol/l EDTA) at 45 °C for 1 h. Proteins were removed

using phenol/chloroform/isoamyl alcohol, and the DNA was ethanol-precipitated. Samples were analyzed by quantitative real-time PCR using FastStart Universal SYBR Green Master Mix (Roche) and QuantStudio 12K Flex. Primer sequences were as follows: *Hoxa9* promoter forward (5'-TGCC TTGGTTATCCTTGAAACAG-3'), *Hoxa9* promoter reverse (5'-TTCCTCCCGG GTTAATTTGTAG-3'), *Hoxa7* promoter forward (5'-TTGGGCGGACAGGTT ACAG-3'), *Hoxa7* promoter reverse (5'-TGTTCCGGCCCTCTCTGA-3'), *Hoxa7* intron forward (5'-AGCAGGACATTGTGGAACGT-3'), *Hoxa7* intron reverse (5'-GGGCAGAATTCACACTTTCAACT-3'), *Hoxa7* backward forward (5'-GTT CCTACCTCATTCCATTCAA-3'), *Hoxa7* backward reverse (5'-CCCCAAGGT GACCTAATTTCC-3'), between *Hoxa4* and *Hoxa5* forward (5'-TGACCAT GTCCCTGTCAA-3') and between *Hoxa4* and *Hoxa5* reverse (5'-TGTTA GTGGAGTCGCAGGTG-3'). ChIP-seq analysis was performed by Filgen, Inc (Aichi, Japan).

Immunoprecipitation and western blotting

293FT cells were transfected with the desired vectors and lysed as described previously.²⁴ Cell lysates and immunoprecipitates were fractionated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Amersham, Little Chalfont, UK). The membranes were incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The immune complexes were visualized by the ECL or ECL-Plus technique (Amersham), and the images were analyzed by ImageGauge (FUJIFILM, Tokyo, Japan) or Fusion SL (Vilber Lourmat, Marne La Vallée, France).

RESULTS

The HOXA9 moiety of NUP98-HOXA9 is essential for binding to the *Hoxa* gene locus

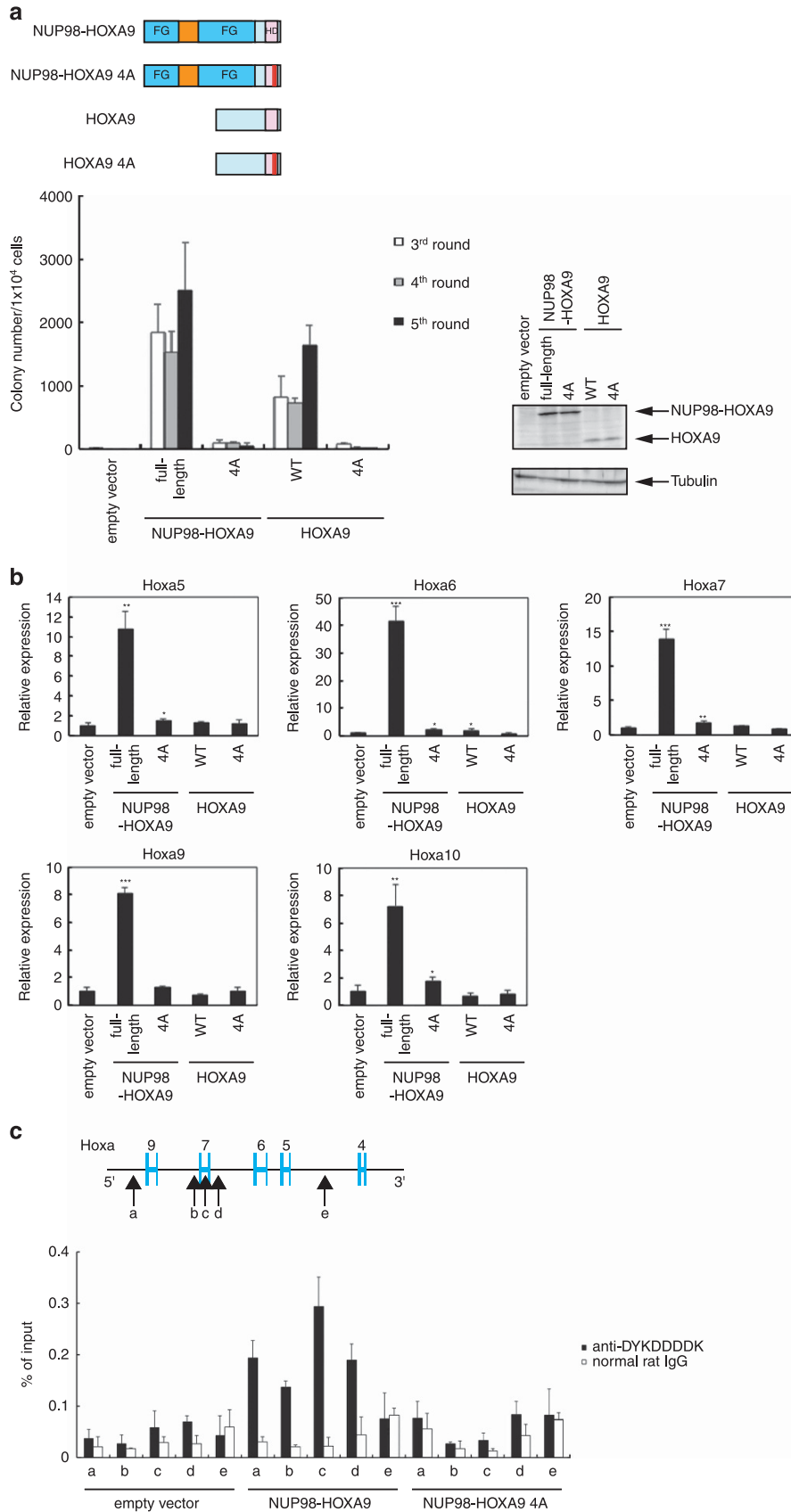
The NUP98-HOXA9 fusion protein retains the HOXA9 homeodomain, a characteristic of homeobox proteins and an important element in their DNA binding. To investigate the role of the HOXA9 moiety of NUP98-HOXA9, we generated mutant versions of NUP98-HOXA9 and HOXA9 in which four amino acids in the homeodomain essential for DNA binding were substituted with alanine residues²⁵ (Figure 1a). The ability of these mutant proteins to immortalize murine cells was assessed by transducing them into normal murine hematopoietic progenitor cells and performing a serial replating assay. Both wild-type NUP98-HOXA9 and HOXA9 immortalized the cells, as previously reported (Figure 1a)^{10,26}; however, NUP98-HOXA9-4A and HOXA9-4A could not immortalize the cells, despite the fact that the 4A mutants were expressed as well as the wild type. We examined the effect of NUP98-HOXA9, HOXA9 and the 4A mutants on *Hoxa* gene expression. Full-length NUP98-HOXA9 induced the expression of *Hoxa* genes, whereas wild-type HOXA9, NUP98-HOXA9-4A or HOXA9-4A could not (Figure 1b). It has been reported that wild-type HOXA9 does not strongly activate *Hoxa* gene expression.¹¹ To examine whether the homeodomain of NUP98-HOXA9 is needed for its localization to the *Hoxa* gene locus, we performed a ChIP assay. The results indicated that NUP98-HOXA9 localized to the *Hoxa* gene locus, whereas NUP98-HOXA9-4A did not (Figure 1c). These results suggest that the homeodomain of NUP98-HOXA9 is essential for localization at the *Hoxa* gene locus and activation of *Hoxa* gene expression.

The second FG repeat domain of NUP98-HOXA9 is important for its cell immortalization activity

The FG repeat domain is characteristic of NPC proteins. Most NUP98 fusion proteins, including NUP98-HOXA9, retain both NUP98 FG repeat domains. To investigate the role of NUP98 moiety in NUP98-HOXA9, we generated deletion mutants of NUP98-HOXA9 lacking the first FG repeat domain (ΔFG1), second FG repeat domain (ΔFG2) or entire NUP98 moiety (HOXA9-Δ1-163) (Figure 2a). To assess the ability of these mutant proteins to immortalize cells, they were transduced into normal murine hematopoietic progenitor cells and a serial replating assay was performed. Transduction with NUP98-HOXA9 led to cell

immortalization (Figure 2a); however, neither NUP98 alone nor HOXA9- Δ 1-163 immortalized the murine progenitor cells. These results indicate that the NUP98 moiety of NUP98-HOXA9 is

essential for immortalization activity of NUP98-HOXA9. Furthermore, NUP98-HOXA9- Δ FG1 immortalized the murine cells, whereas NUP98-HOXA9- Δ FG2 did not (Figure 2a), suggesting that



the second FG repeat domain of the NUP98 moiety is important for cell immortalization. We also investigated the ability of the NUP98-HOXA9 mutants to promote leukemogenesis *in vivo*. Leukemogenesis by NUP98-HOXA9 was shown to have a long latency period in a mouse model.²⁶ In fact, when NUP98-HOXA9-expressing cells were transplanted into sublethally irradiated mice, all of the animals survived for at least 250 days after transplantation (Supplementary Figure S1). However, co-expression of Fms-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD) enhances the activity of the NUP98 fusion gene in inducing leukemia²⁷; therefore, we adopted a combination of NUP98-HOXA9 and FLT3-ITD to induce leukemia in mice. As shown in Figure 2b, full-length NUP98-HOXA9 and NUP98-HOXA9-ΔFG1 in combination with FLT3-ITD rapidly induced death of mice in which they were expressed, whereas the effects of the expression of NUP98-HOXA9-ΔFG2 were comparatively delayed. These results suggest that the second FG repeat domain in the NUP98 moiety is important, not only for cell immortalization *in vitro* but also for leukemogenesis *in vivo*.

The second FG repeat domain of NUP98-HOXA9 is required to induce *Hoxa* gene expression

As shown above, the second FG repeat domain of NUP98-HOXA9 is important for the activity of the fusion protein in cell immortalization and leukemogenesis. Next, we performed mRNA microarray analysis using material from cells expressing full-length and mutant NUP98-HOXA9. Fifteen genes were upregulated more than twofold in full-length NUP98-HOXA9- and ΔFG1-expressing cells but not in NUP98-HOXA9-ΔFG2-expressing cells, compared with empty vector-transduced cells (Supplementary Table S1). Next, we performed quantitative PCR to confirm the results of the microarray analysis, demonstrating that 14 genes were upregulated in full-length NUP98-HOXA9- and ΔFG1-expressing cells compared with cells containing the an empty vector control (Figure 3a). In ΔFG2-expressing cells, *Hoxa6*, *Erv3*, *Cd27*, *B4galt4*, *Cd28*, *Rhoj*, *Mansc1* and *Rassf6* genes were slightly but significantly more activated than in cells containing the empty vector control (Figure 3a). However, the second FG repeat domain was needed for strong upregulation of these genes. In full-length NUP98-HOXA9-expressing cells, *Hoxa9*, *Hoxa10* and *Meis1* were upregulated (Supplementary Figure S2a). Surprisingly, in NUP98-HOXA9-ΔFG1-expressing cells, *Hoxa9*, *Hoxa10* and *Meis1* were not upregulated (Supplementary Figure S2a) and NUP98 alone did not induce the expression of *Hoxa* or *Meis1* (Supplementary Figure S2b). We next performed a ChIP assay to determine the direct targets of NUP98-HOXA9 among the 14 upregulated genes. ChIP-seq analysis showed that the *Hoxa7* and *B4galt4* gene loci were occupied by full-length and ΔFG1 NUP98-HOXA9 (Supplementary Figures S2c and d). HOXA9 alone did not activate the *Hoxa* gene expression (Figure 1b) nor did it localize to the *Hoxa* gene locus (Supplementary Figure S2c). We also examined whether NUP98-DDX10, another NUP98 fusion protein, regulates the 14 genes described above. NUP98-DDX10

upregulated *Hoxa5*, *Hoxa6*, *Hoxa7*, *Rhoj* and *Mansc1* (Supplementary Figure S2e). These data indicate direct and indirect regulation of genes by the NUP98 fusion protein and suggest that

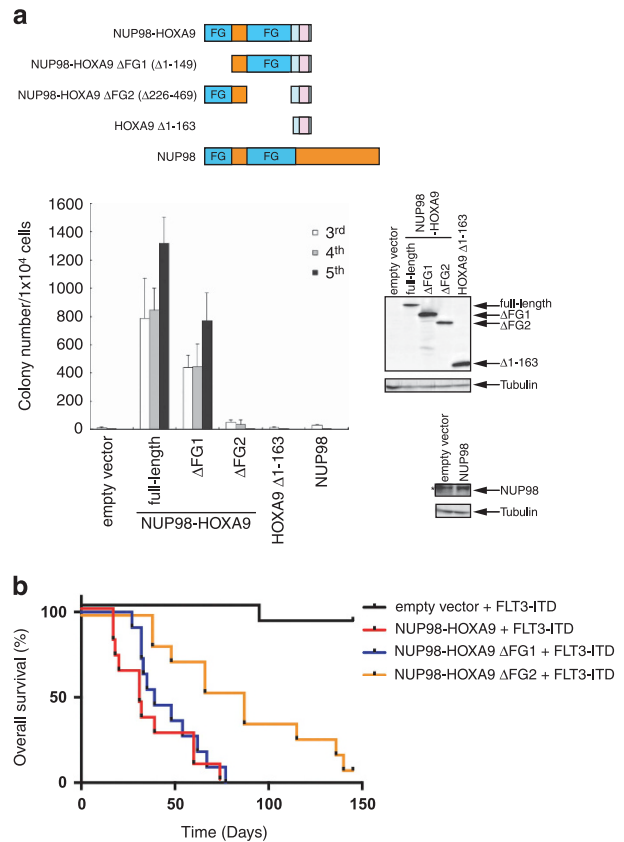


Figure 2. The second FG repeat domain of NUP98-HOXA9 is important for cell immortalization and leukemogenesis. **(a)** Diagram of full-length NUP98-HOXA9 and its mutants (upper). C-kit⁺ cells were infected with empty vector, full-length pMSCV-HA-NUP98-HOXA9, ΔFG1, ΔFG2, pMSCV-HA-HOXA9-Δ1-163 or pMSCV-FLAG-NUP98 and plated in methylcellulose medium (lower). The colony numbers from the third to fifth rounds are indicated (left panel). Values represent mean ± s.e.m. from four independent experiments. Full-length NUP98-HOXA9, ΔFG1, ΔFG2, HOXA9 Δ1-163 and NUP98 expression in the lysates of the first-round colonies was detected by immunoblotting with anti-HA or anti-FLAG antibody (right panel). The asterisk indicates non-specific band. **(b)** C-kit⁺ cells were infected with empty vector, full-length NUP98-HOXA9, ΔFG1 or ΔFG2, together with FLT3-ITD, and transplanted into sublethally irradiated mice (1 × 10⁶ cells/mouse). The overall survival times of the mice were analyzed (n = 11). P < 0.001 (full-length), P < 0.01 (ΔFG1), compared with ΔFG2.

Figure 1. The homeodomain of NUP98-HOXA9 is important for its cell immortalization activity. **(a)** Diagram of NUP98-HOXA9, HOXA9 and their mutant variants (upper). The FG repeat domain (FG) and homeodomain (HD) are indicated. C-kit⁺ cells were infected with empty vector, pMSCV-FLAG-NUP98-HOXA9, pMSCV-FLAG-NUP98-HOXA9-4A, pMSCV-FLAG-HOXA9 or pMSCV-FLAG-HOXA9-4A and plated in methylcellulose medium (lower). The colony numbers from the third to fifth rounds are indicated (left panel). Values represent mean ± s.e.m. from four independent experiments. NUP98-HOXA9, NUP98-HOXA9-4A, HOXA9 and HOXA9-4A expression in the lysates of the first-round colonies of cells was detected by immunoblotting with anti-FLAG antibody (right panel). **(b)** The first-round colonies of cells transduced with empty vector, full-length NUP98-HOXA9 or the 4A mutant or wild-type HOXA9 or the 4A mutant were collected and analyzed for the expression levels of *Hoxa5*, *Hoxa6*, *Hoxa7*, *Hoxa9* and *Hoxa10* by quantitative real-time PCR. Values represent mean ± s.e.m. from four independent experiments. ***P < 0.001, **P < 0.01, *P < 0.05, compared with empty vector (value normalized to 1). **(c)** *Hoxa9* promoter (a), *Hoxa7* promoter (b), *Hoxa7* intron (c), *Hoxa7* backward (d) and between *Hoxa4* and *Hoxa5* (e) primer sets are indicated (top). The first-round cells described in panel (b) were collected, fixed and lysed. The lysates were sonicated using Sonifier 450. The complexes of wild-type NUP98-HOXA9, HOXA9 or the 4A mutants and associated DNA were purified using an anti-DYKDDDDK (FLAG) antibody. Samples were analyzed by quantitative real-time PCR (bottom). ChIP experiments were performed three independent times, and the data from one representative experiment are shown.

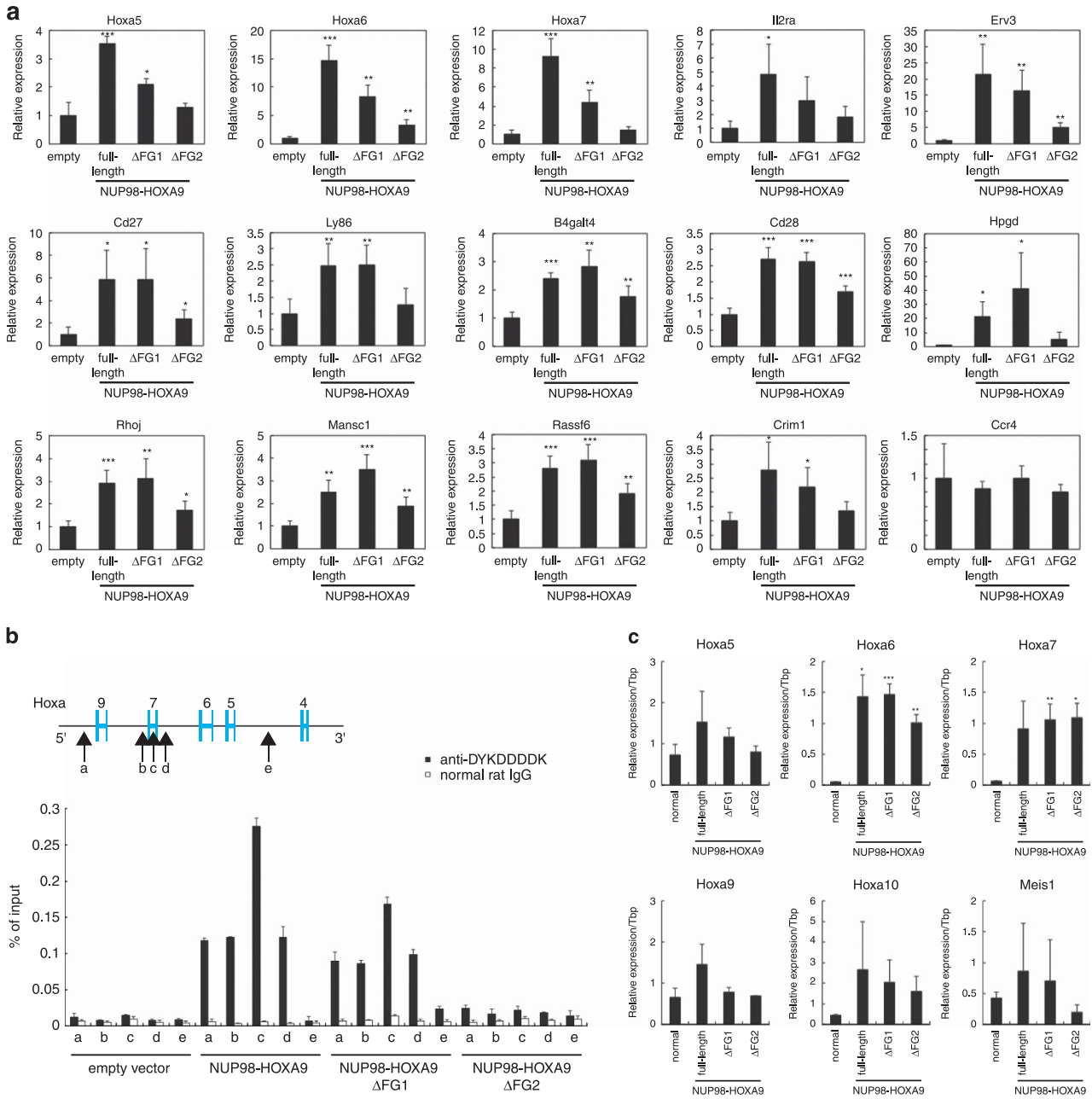


Figure 3. The second FG repeat domain of NUP98-HOXA9 is required for the regulation of *Hoxa* gene expression. **(a)** The first-round colonies of cells transduced with empty vector, full-length NUP98-HOXA9, Δ FG1 or Δ FG2 were collected and analyzed for the expression levels of *Hoxa5*, *Hoxa6*, *Hoxa7*, *Il2ra*, *Erv3*, *Cd27*, *Ly86*, *B4gal4*, *Cd28*, *Hpgd*, *Rhoj*, *Mansc1*, *Rassf6*, *Crim1* and *Ccr4* by quantitative real-time PCR. Values represent mean \pm s.e.m. from five independent experiments. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$, compared with empty vector (value normalized to 1). **(b)** The cells described in panel (a) were collected, fixed and lysed. The lysates were sonicated using Sonifier 450. The complexes of full-length NUP98-HOXA9 or its mutants and associated DNA were purified using anti-DYKDDDDK antibody. Samples were analyzed by quantitative real-time PCR (bottom). ChIP experiments were performed three independent times. The data from one representative experiment are shown. **(c)** The expression levels of *Hoxa5*, *Hoxa6*, *Hoxa7*, *Hoxa9*, *Hoxa10* and *Meis1* in the normal and leukemic bone marrow cells were examined by quantitative real-time PCR. Values represent mean \pm s.e.m. from three independent mouse bone marrow cell samples. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$, compared with empty vector.

direct regulation of the expression of *Hoxa* genes, in particular *Hoxa5*, *Hoxa6* and *Hoxa7*, by the second FG repeat domain of the NUP98 fusion protein is important for its immortalization activity. Therefore, we used primer sets around the *Hoxa7* gene (Figure 1c) to perform a ChIP assay to confirm the results of the ChIP-seq analysis. Full-length NUP98-HOXA9 and the Δ FG1 mutant occupied the *Hoxa7* gene region, whereas NUP98-HOXA9- Δ FG2 did not (Figure 3b). We also designed primer sets around the *Hoxa6* gene,

but occupation of the *Hoxa6* locus by NUP98-HOXA9 and NUP98-HOXA9- Δ FG1 was not detected (data not shown). NUP98-DDX10 also occupied the *Hoxa7* gene locus (Supplementary Figure S2f). As shown in Figure 3c, the expression of *Hoxa6* and *Hoxa7* was increased in leukemic bone marrow cells collected from mice expressing full-length NUP98-HOXA9 or deletion mutants alongside FLT3-ITD (Figure 2b). The data in Figure 3 indicate that the second FG repeat domain of NUP98-HOXA9 has an important role in the

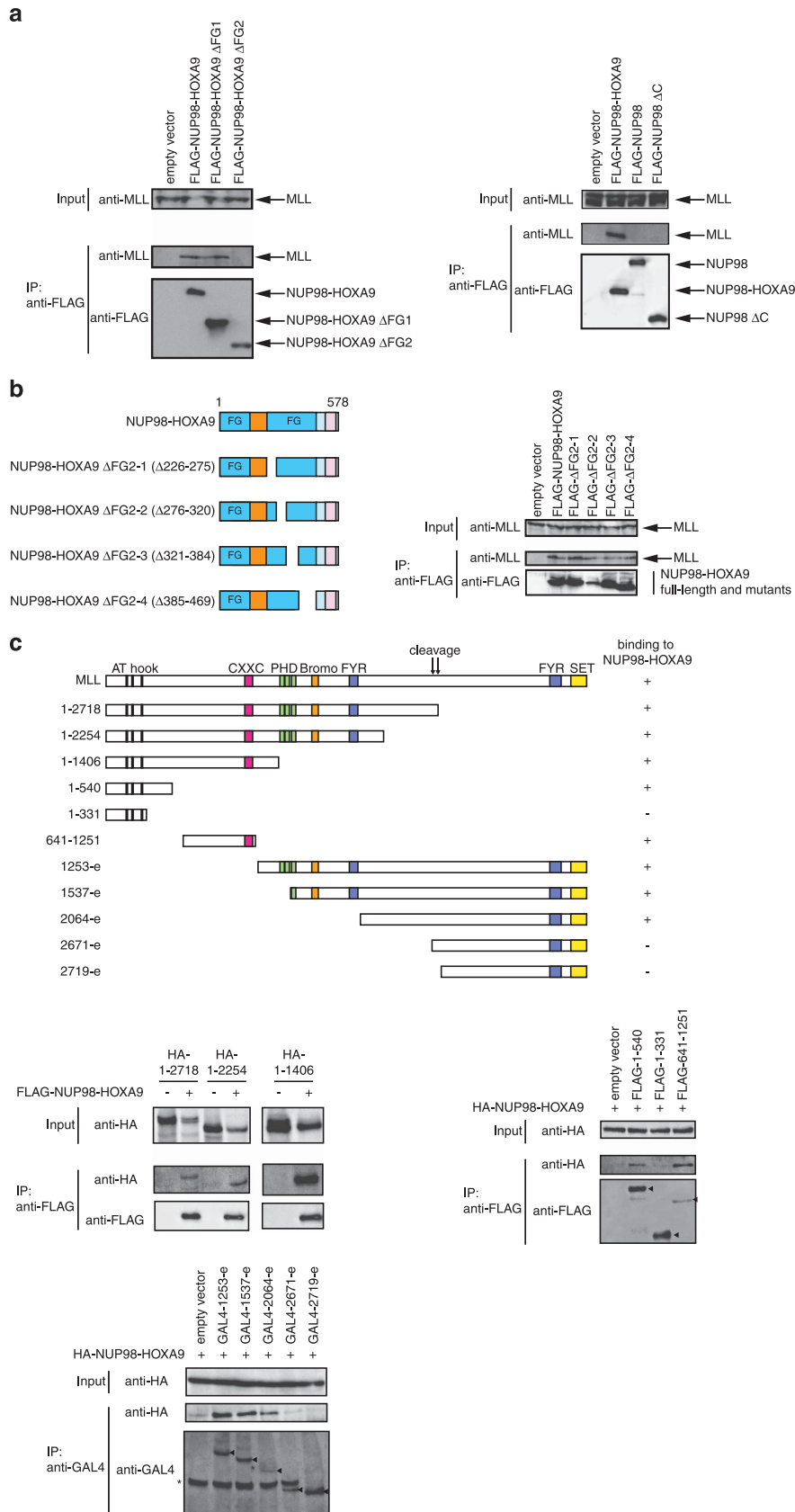


Figure 4. For caption see next page.

direct regulation of the expression of *Hoxa* genes, including *Hoxa6* and *Hoxa7*.

NUP98-HOXA9 interacts with MLL

Our results demonstrating that wild-type HOXA9 did not localize to the *Hoxa* locus nor induce the expression of *Hoxa* genes led us to hypothesize that the second FG repeat domain of NUP98-HOXA9 is required for localization to the *Hoxa* gene locus. The FG repeat domain is important for binding to other proteins¹; therefore, we hypothesized that other proteins that interact with NUP98-HOXA9 via its FG repeat domain are required for recruitment of NUP98-HOXA9 to the *Hoxa* gene locus. To identify proteins interacting with NUP98-HOXA9 via its second FG repeat domain, we performed mass spectrometric analysis. We identified MLL family proteins in complexes with NUP98-HOXA9 and NUP98-HOXA9-ΔFG1 but not in the NUP98-HOXA9-ΔFG2 complex (Supplementary Figure S3a and Supplementary Tables S2–S4). MLL is a histone H3 lysine 4 methyltransferase and directly regulates *Hoxa* gene expression.^{12,14,15} To confirm the results of the mass spectrometric analysis, we performed an immunoprecipitation assay. As shown in Figure 4a, full-length NUP98-HOXA9 and the ΔFG1 mutant interacted with MLL, whereas NUP98-HOXA9-ΔFG2 did not. Recently, Nup98 was reported to interact with Trx and regulate *Hox* gene expression in *Drosophila*.²⁸ In this study, we found that NUP98-HOXA9 interacted with MLL; therefore, we analyzed the interaction between NUP98 and MLL in mammalian cells. NUP98 alone did not interact with MLL and nor did the NUP98 moiety derived from the NUP98-HOXA9 fusion (mutant NUP98-ΔC) (Figure 4a). On the other hand, NUP98-DDX10, another NUP98 fusion protein, interacted with MLL (Supplementary Figure S3b). These results indicate that interaction with MLL in mammalian cells requires the fusion of NUP98 with a partner gene.

A previous study suggested the number of FG repeats is important for the transformation activity of NUP98-HOXA9.⁷ In this study, NUP98b-HOXA9, whose structure is similar to that of NUP98-HOXA9-ΔFG2, transformed NIH3T3 cells.⁷ On the other hand, in our study, NUP98-HOXA9-ΔFG2 failed to transform murine hematopoietic progenitor cells (Figure 2a). Thus we generated deletion mutants of NUP98-HOXA9 lacking a few FG repeats in the second FG repeat domains (Figure 4b). All of the deletion mutants (NUP98-HOXA9-ΔFG2-1, 2-2, 2-3 and 2-4) interacted with MLL (Figure 4b), immortalized murine cells (Supplementary Figure S3c) and occupied the *Hoxa* gene region (Supplementary Figure S3d). As previously reported, these results suggest that the number of FG repeats in the second FG repeat domain might be important for cell immortalization and all of the second FG repeat domain is needed for interaction with MLL.

Next, we used MLL deletion mutants to identify the region of the protein responsible for the interaction with NUP98-HOXA9 (Figure 4c). MLL is proteolytically cleaved to produce N- and

C-terminal fragments that interact with each other.^{29,30} NUP98-HOXA9 interacted with the N-terminal (residues 1–2718), but not the C-terminal (2719–e), fragment of MLL (Figure 4c). NUP98-HOXA9 also interacted with amino acids 1–540, but not with 1–331, and with 641–1251 and 2064–e, but not with 2671–e. These results indicate that NUP98-HOXA9 interacts with at least three regions of the N-terminal fragment of MLL between amino acids 332 and 540, between 641 and 1251 and between 2064 and 2670.

MLL is important for cell immortalization and induction of leukemogenesis by NUP98-HOXA9

The interaction of NUP98-HOXA9 with MLL (Figure 4) led us to hypothesize that NUP98-HOXA9 and MLL cooperate to induce leukemia. To identify the role of MLL in NUP98-HOXA9-induced leukemia, we used *Mill* null (*Mill*^{-/-}) fetal liver cells. *Mill*^{-/-} fetal liver cells were transduced with NUP98-HOXA9 and replated in methylcellulose medium. The replating activity of the *Mill*^{-/-} cells expressing NUP98-HOXA9 was much lower than that of *Mill*^{+/+} cells (Figure 5a, left panel). The replating activity of the *Mill*^{-/-} cells expressing MOZ-TIF2 was also severely inhibited (Figure 5a, right panel). On the other hand, the activity of the *Mill*^{-/-} cells expressing PML-RARα was maintained (Figure 5a, middle panel). The expression levels of *Hoxa5*, *Hoxa6*, *Hoxa7*, *Hoxa9* and *Hoxa10* were lower in *Mill*^{-/-} than in *Mill*^{+/+} first-round colony cells expressing NUP98-HOXA9 (Figure 5b). In an *in vivo* transplantation assay, *Mill*^{+/+} fetal liver cells transduced with NUP98-HOXA9 and FLT3-ITD-induced leukemia within 46 days, whereas *Mill*^{-/-} cells did not induce leukemia for at least 100 days after transplantation (Figure 5c). These results indicate that MLL is essential for NUP98-HOXA9-induced leukemia development.

MLL is required for recruitment of NUP98-HOXA9 to the *Hoxa* gene locus

Our results demonstrate that MLL interacts with NUP98-HOXA9 and is important for NUP98-HOXA9-regulated *Hoxa* gene expression (Figures 4 and 5). Therefore, we hypothesized that NUP98-HOXA9 would be recruited to the *Hoxa* gene locus by MLL. First, we examined whether NUP98-HOXA9 and MLL colocalized to the *Hoxa* gene locus to activate *Hoxa* gene expression. ChIP analysis showed that MLL colocalized at the *Hoxa* gene locus with NUP98-HOXA9 (Figure 3b and Supplementary Figure S2c) in NUP98-HOXA9-expressing murine cells (Figure 6a, upper panel). Furthermore, histone H3 lysine 4 was trimethylated at this locus in the NUP98-HOXA9-expressing cells (Figure 6a, lower panel). These results suggest that NUP98-HOXA9 and *Mill* cooperatively and directly activate *Hoxa* gene expression. Next, to examine whether *Mill* is required for NUP98-HOXA9 localization at the *Hoxa* gene locus, a ChIP assay was performed using the *Mill*^{-/-} fetal liver cells (Figure 5a). As there were very few *Mill*^{-/-} fetal liver cells in the

Figure 4. NUP98-HOXA9 interacts with MLL. **(a)** 293FT cells were transfected with pLNCX (empty vector), pLNCX-FLAG-NUP98-HOXA9, pLNCX-FLAG-NUP98-HOXA9-ΔFG1 or pLNCX-FLAG-NUP98-HOXA9-ΔFG2 (left panel) or with pLNCX (empty vector), pLNCX-FLAG-NUP98-HOXA9, pLNCX-FLAG-NUP98 or pLNCX-FLAG-NUP98-ΔC (right panel). The expression of MLL in the lysates of transfectants was detected by immunoblotting using anti-MLL antibody (Input). The lysates of the transfectants were incubated with anti-FLAG antibody. Immunoprecipitates were analyzed by immunoblotting with anti-MLL and anti-FLAG antibodies (IP). **(b)** Diagram of NUP98-HOXA9 deletions in the second FG repeat domain (left panel). 293FT cells were transfected with pMSCV (empty vector), pMSCV-FLAG-NUP98-HOXA9 or each pMSCV-FLAG-NUP98-HOXA9 mutant (right panel). The expression of MLL in the lysates of the transfectants was detected by immunoblotting with anti-MLL antibody (Input). The lysates of the transfectants were incubated with anti-FLAG antibody. Immunoprecipitates were analyzed by immunoblotting with anti-MLL and anti-FLAG antibodies (IP). **(c)** Diagram of wild-type MLL and mutants (upper). The results of the interaction with NUP98-HOXA9 are represented. 293FT cells were transfected with pLNCX (-) or pLNCX-FLAG-NUP98-HOXA9 and pLNCX-HA-MLL 1-2718, 1-2254 or 1-1406 (middle left), with pLNCX-HA-NUP98-HOXA9 and pCMV5 (empty vector), pCMV5-FLAG-MLL 1-540, 1-331 or 641-1251 (middle right) or with pLNCX-HA-NUP98-HOXA9 and pLNCX (empty vector), pLNCX-GAL4-MLL 1253-e, 1537-e, 2064-e, 2671-e or 2719-e (lower). Arrowheads and asterisk indicate specific and non-specific bands, respectively.

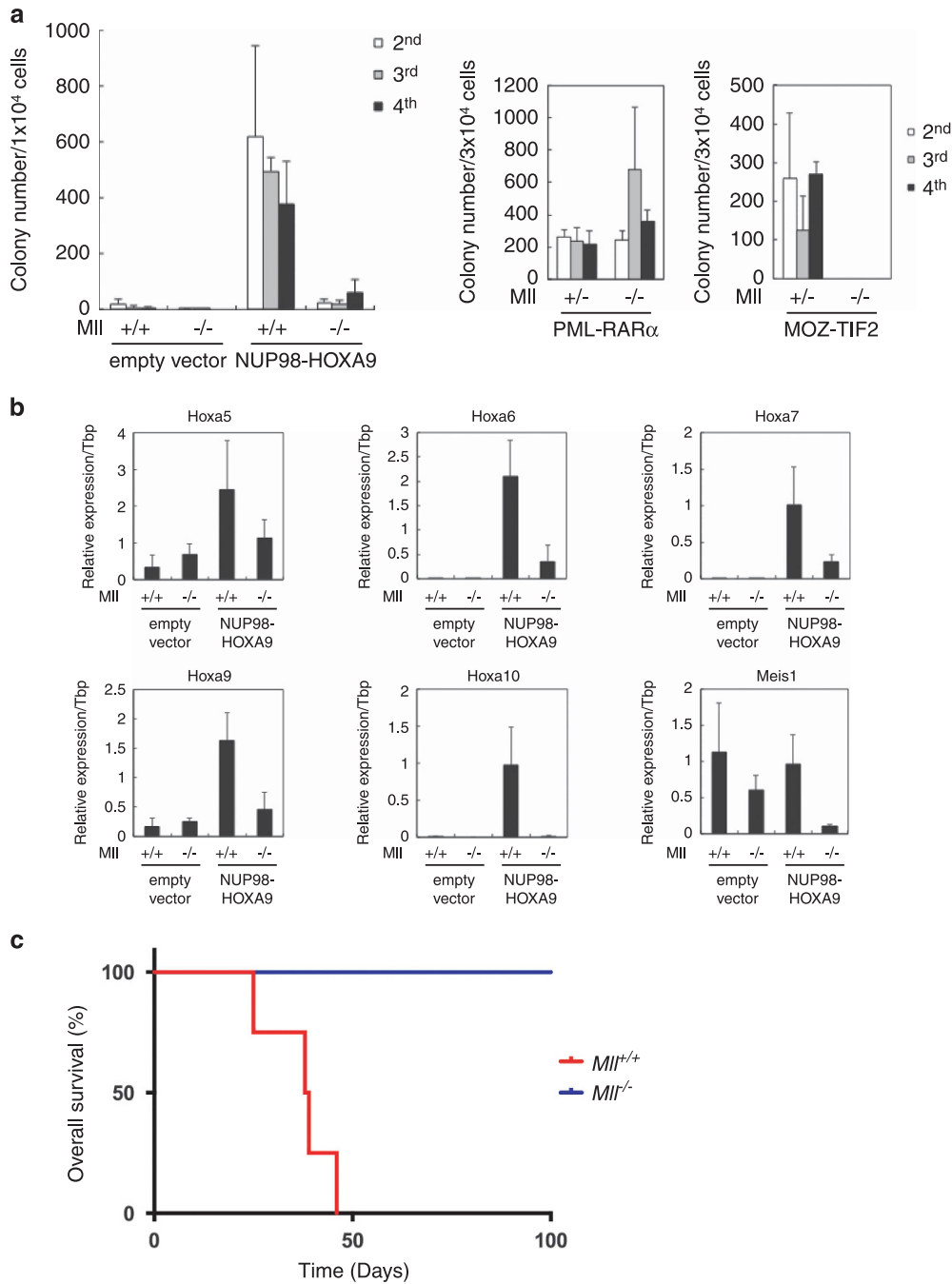


Figure 5. *Mll* is essential for NUP98-HOXA9-induced leukemogenesis. **(a)** E12.5 fetal liver cells from *Mll*^{+/+} or *Mll*^{-/-} mice were infected with empty vector or pMSCV-FLAG-NUP98-HOXA9 and plated in methylcellulose medium. PML-RAR α - or MOZ-TIF2-expressing *Mll*^{+/+} or *Mll*^{-/-} fetal liver cells were also cultured in methylcellulose medium. The colony numbers from the second to the fourth rounds are indicated. Values represent mean \pm s.e.m. from four (NUP98-HOXA9) or two (PML-RAR α or MOZ-TIF2) independent experiments. **(b)** The first-round colonies of cells transduced with empty vector or NUP98-HOXA9 were collected and analyzed for the expression levels of *Hoxa5*, *Hoxa6*, *Hoxa7*, *Hoxa9*, *Hoxa10* and *Meis1* by quantitative real-time PCR. Values represent mean \pm s.e.m. from four independent experiments. **(c)** E12.5 fetal liver cells from *Mll*^{+/+} or *Mll*^{-/-} mice were infected with NUP98-HOXA9 together with FLT3-ITD and transplanted into sublethally irradiated mice. Overall survival times of the mice were analyzed (*Mll*^{+/+}: *n* = 4, *Mll*^{-/-}: *n* = 7).

colony, we were only able to perform the analysis using the *Hoxa7* intron primer set (Figure 6a). As shown in Figure 6b, NUP98-HOXA9 was recruited to the *Hoxa7* gene locus in *Mll*^{+/+} cells, but its recruitment was severely inhibited in *Mll*^{-/-} cells. These results indicate that MLL is required for recruitment of NUP98-HOXA9 to the *HOXA* gene locus. Overall, our data suggest that the recruitment of NUP98-HOXA9 and MLL to the *HOXA* gene

locus is essential for NUP98-HOXA9-induced leukemogenesis (Figure 6c).

DISCUSSION

The *NUP98* gene is subject to rearrangement and fusion to a variety of partner genes in AML and myelodysplastic syndromes. However,

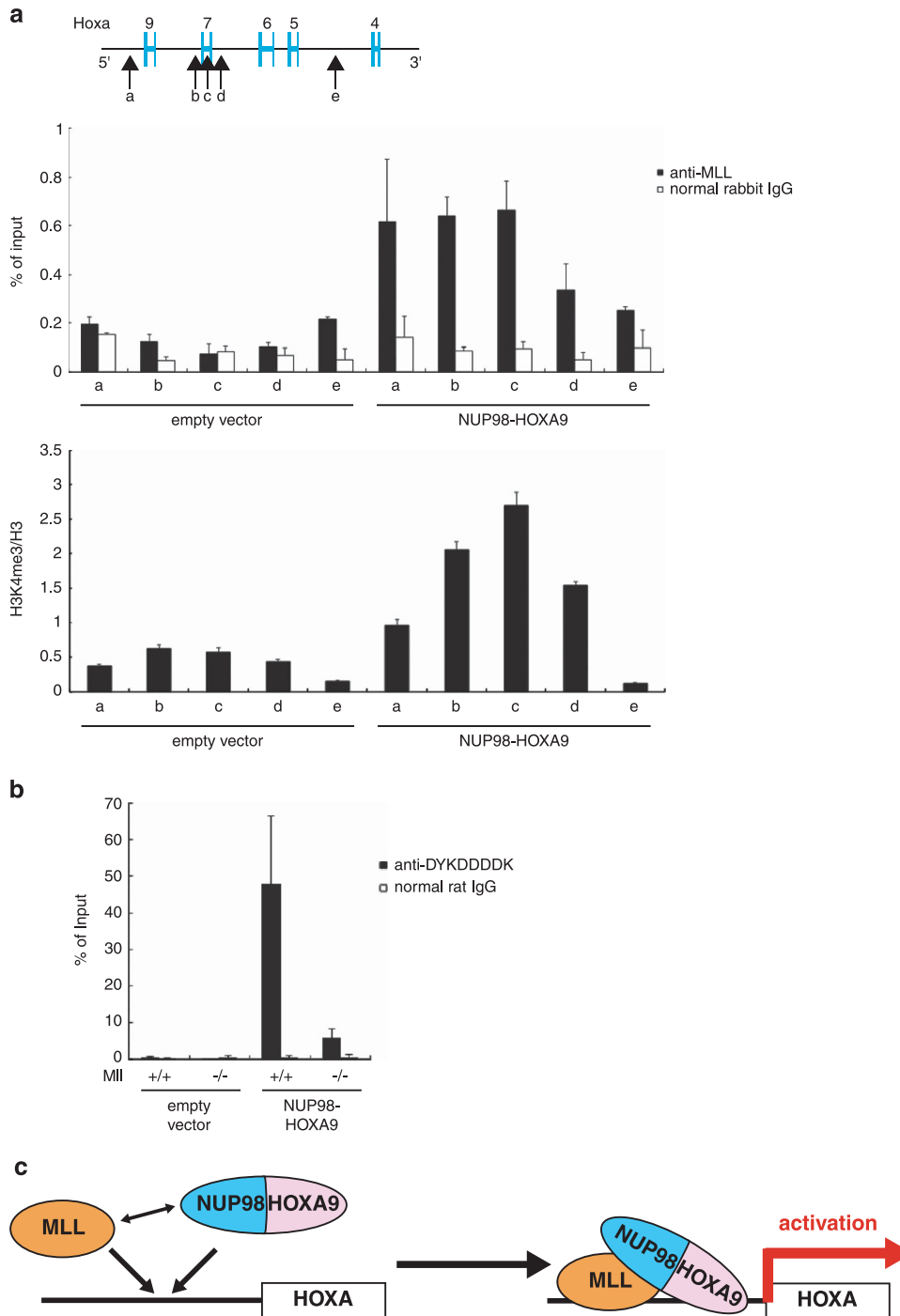


Figure 6. *Mll* is required for recruitment of NUP98-HOXA9 to the *Hoxa* locus. **(a)** The first-round colonies of cells transduced with empty vector or NUP98-HOXA9 were collected and fixed. The *Mll*-DNA complex was purified using an anti-MLL antibody (top). Chromatin including histone H3 or histone H3 trimethylated at K4 was purified using anti-histone H3 or anti-histone H3 (tri methyl K4) antibodies (bottom). Samples were analyzed by quantitative real-time PCR. ChIP experiments were performed three independent times. The data from one representative experiment are shown. **(b)** The cells of the first-round colonies derived from *Mll*^{+/+} or *Mll*^{-/-} fetal liver cells were collected, fixed and lysed. The lysates were sonicated using the S220 Focused-ultrasonicator. A ChIP assay was performed using anti-DYKDDDDK or normal rat IgG and the *Hoxa7* intron primer set. The data from one representative experiment are shown. **(c)** A model for NUP98-HOXA9-initiated leukemia. NUP98-HOXA9 and MLL are cooperatively recruited to the *HOXA* locus. When NUP98-HOXA9 and MLL colocalize at the locus, they strongly activate the expression of *HOXA* genes, particularly *HOXA6* and *HOXA7*, to initiate leukemia.

the role of the NUP98 moiety in leukemogenesis is not well understood. In this study, we found that NUP98-HOXA9 interacts with MLL via its NUP98 moiety, although it is unknown whether the interaction is direct or indirect. NUP98-DDX10 also interacted with MLL. In the absence of MLL as well as MLL-AF9,²¹ NUP98-HOXA9

and MOZ-TIF2 could not immortalize cells, but PML-RAR α immortalized cells (Figure 5a). These results suggest that HOXA gene regulation by MLL is important for leukemia initiation by NUP98-HOXA9, MOZ-TIF2 and MLL-AF9. In fact, in the absence of MLL, the recruitment of NUP98-HOXA9 to the *Hoxa* gene locus and

activation of *Hoxa* genes by NUP98-HOXA9 were severely inhibited (Figures 5b and 6b); therefore, the interaction with MLL would be required to recruit NUP98-HOXA9 to the *Hoxa* gene locus. For NUP98-fusions to activate *HOXA* genes, a chromosomal rearrangement associated with the *NUP98* gene (for example, t(7,11)) may be necessary in cells in which MLL is present at the *HOXA* gene locus. In AML, leukemia-initiating cells are generated from hematopoietic stem cells or myeloid progenitor cells,^{31–33} in which MLL maintains *HOXA* gene expression.^{12,13}

By contrast, wild-type NUP98 and NUP98-ΔC did not interact with MLL (Figure 4a), even though these proteins have the first and second FG repeat domains. In *Drosophila*, wild-type Nup98 interacts with Trx.²⁸ Together with previous reports, our data suggest that fusion with partner proteins is required for interaction of NUP98 with MLL in mammalian cells. In fact, wild-type NUP98, which could not interact with MLL, did not induce *Hoxa* or *Meis1* expression (Supplementary Figure S2b).

NUP98-HOXA9 is also reported to interact with CBP/p300 proteins, which are acetyltransferases.⁷ In this study, CBP/p300 proteins were not on the list of those that interacted with NUP98-HOXA9 identified by mass spectrometry. In *MLL*^{-/-} cells, the expression of *Hoxa* genes was severely inhibited; however, they were slightly activated in NUP98-HOXA9-expressing cells compared with those containing only the empty vector control (Figure 5b). These results indicate that MLL is an essential factor in NUP98 fusion protein-induced cell immortalization and leukemogenesis and suggest that NUP98 fusion proteins might use not only MLL but also other epigenetic factors, such as CBP/p300, to facilitate recruitment to the *HOXA* gene locus. From the data in Figures 1 and 6, once NUP98-HOXA9 is recruited to the *HOXA* gene locus, the HOXA9 moiety of NUP98-HOXA9 appears to be important for DNA binding to anchor the fusion protein to the *HOXA* gene locus, facilitating more effective and strong localization of epigenetic factors, such as MLL, to the *HOXA* region via their interaction with NUP98-HOXA9.

Although NUP98-HOXA9-ΔFG1 localized to the *Hoxa* gene locus (Figure 3b and Supplementary Figure S2c), *Hoxa9* was not upregulated (Supplementary Figure S2a). This result suggests that the first FG repeat domain of NUP98-HOXA9 is important for direct or indirect upregulation of *Hoxa9* gene expression and that factors that interact with NUP98-HOXA9 via this first FG repeat domain might be needed for this upregulation. NUP98-HOXA9-ΔFG1 immortalized cells without *Hoxa9* or *Hoxa10* activation (Supplementary Figure S2a). In CALM-AF10-mediated leukemia, *Hoxa5* is important for leukemogenesis.³⁴ *Hoxa7* and *Hoxa9* are upregulated in MLL-AF9-expressing leukemic stem cells.³⁵ Most individual *HOXA* genes transform hematopoietic cells.¹¹ Taken together with the data presented in this paper, these reports suggest that *Hoxa5*, *Hoxa6* and *Hoxa7* upregulation is sufficient for NUP98-HOXA9-ΔFG1-induced cell immortalization and leukemogenesis.

NUP98-HOXA9-ΔFG2 demonstrated a reduced ability to induce leukemia compared with full-length NUP98-HOXA9 and ΔFG1; however, leukemia was still induced (Figure 2). It is interesting to note that all mouse models of leukemia expressing full-length or deletion mutants of NUP98-HOXA9 and FLT3-ITD demonstrated strong upregulation of *Hoxa6* and *Hoxa7* expression in their leukemic bone marrow cells (Figure 3c). In the mouse model, *Hoxa9* expression in NUP98-HOXA9-ΔFG1-induced leukemia cells did not differ from that in normal bone marrow cells, and, in the colony-formation assay, NUP98-HOXA9-ΔFG1 immortalized murine progenitor cells in the absence of upregulation of *Hoxa9* expression. By contrast, *Hoxa6* and *Hoxa7* expression was upregulated in NUP98-HOXA9-ΔFG1-expressing cells. Cells transformed with NUP98-HOXA9 differ from those transformed with HOXA9.³⁶ These results suggest that the regulation of *HOXA6* and *HOXA7* expression is more important than that of *HOXA9* for leukemogenesis in t(7,11) AML. We do not

understand well why *Hoxa6* and *Hoxa7* expression was upregulated to induce leukemia in NUP98-HOXA9-ΔFG2-expressing cells (Figure 3c). However, *Hoxa6* was the only *Hoxa* gene whose expression was upregulated by NUP98-HOXA9-ΔFG2 (Figure 3a) and slightly upregulated by HOXA9 *in vitro* (Figure 1b). Full-length NUP98-HOXA9 strongly activated *Hoxa6* expression (Figure 3a). These results suggest that *Hoxa6* expression is regulated synergistically by the NUP98 and HOXA9 moieties of NUP98-HOXA9.

Full-length NUP98-HOXA9, but not ΔFG1 or ΔFG2, activated *Meis1* (Supplementary Figure S2a). These results indicate that both the first and second FG repeats are required for *Meis1* activation. *Meis1* was not strongly activated (Figure 3c and Supplementary Figure S2a). This might be because *Meis1* expression was already near its maximum level in the control cells or because FLT3-ITD was used to induce leukemia in the mouse model.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Leukemia website (<http://www.nature.com/leu>)