

Functions of leukemia-associated NUP214-fusion proteins

(白血病関連 NUP214 融合タンパク質の機能)

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北里大学

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Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
CC	Coiled coil
ChIP	Chromatin immunoprecipitation
CRM1	Chromosomal Maintenance 1
DAPI	4,6-diamidino-2-phenylindole
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
FG	Phenylalanine-Glycine
HA	Hemagglutinin
HD	Homeodomain
HOX	Homeobox
IF	Immunofluorescence
IP	Immunoprecipitation
IPO	Importin
MLL	Mixed lineage leukemia
NE	Nuclear envelope
NES	Nuclear export signal
NLS	Nuclear localization sequence
NPC	Nuclear pore complex
NTR	Nuclear transport receptor
NUP	Nucleoporin
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
SDS-PAGE	Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis
WB	Western blot
XPO	Exportin

Chapter 1 Introduction

1.1 Cancer

Cancer is a genetic disorder caused by DNA mutations that are obtained spontaneously or stimulated by environmental factors [1]. Besides, cancers frequently exhibit epigenetic variations, such as altered DNA methylation and histone modifications, which may result from acquired mutations in genes that regulate such modifications. These genetic and epigenetic changes alter the expression or function of key genes that regulate fundamental cellular processes, such as proliferation, differentiation, and apoptosis [2]. Cancer is one of the major causes of death in the world [3].

1.2 Leukemia

Leukemia is a cancer of blood cells, which starts in the bone marrow, and causes large numbers of immature blood cells to be produced and enter the bloodstream. Leukemia is subdivided into different subtypes according to cellular maturity (acute or chronic) and cell type (lymphoid or myeloid) [4]. The acute leukemias, which have the more aggressive presentation, are grouped into acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). Acute leukemia is an extremely heterogeneous disease, characterized by blocked differentiation and uncontrolled expansion of hematopoietic progenitor cells [5, 6].

1.3 Hematopoiesis and Leukemogenesis

Hematopoiesis refers to the commitment and differentiation processes that lead to the production of blood cells, leukemogenesis is the process by which blood cells become cancerous. Leukemia starts in the bone marrow and causes the production of large numbers of immature blood cells [7, 8]. While hematopoietic stem cells (HSCs) differentiate into functionally specified blood cells, leukemic stem cells (LSCs) are arrested in an undifferentiated state (Figure 1.1).

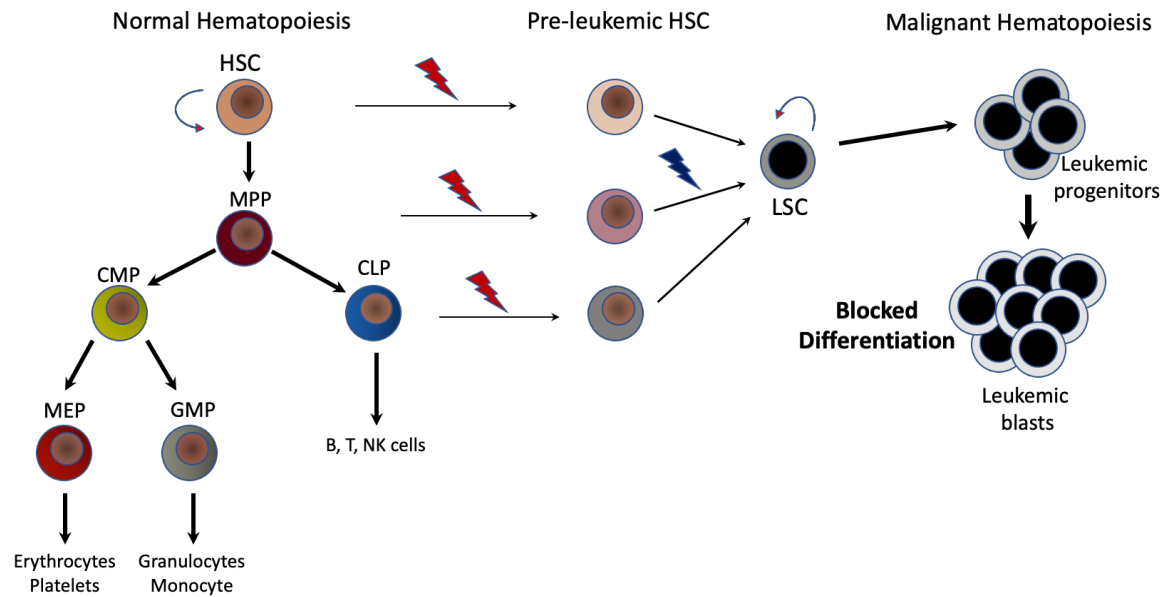
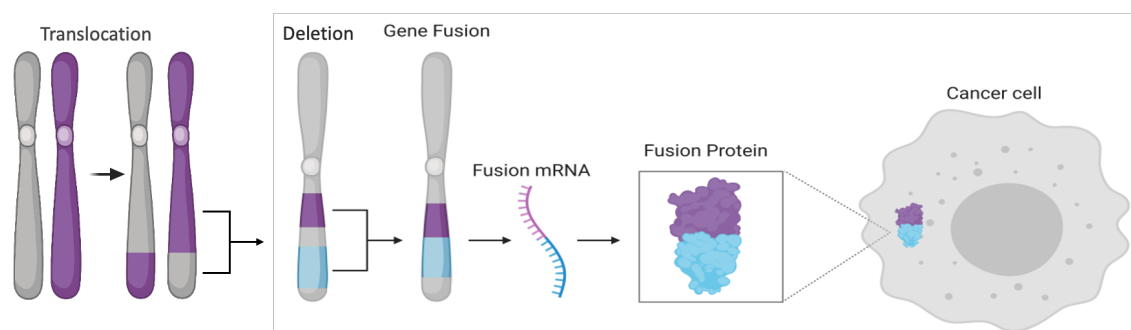


Figure 1.1. Normal and malignant hematopoiesis.

1.4 Gene Fusions in Leukemia

Chromosomal translocation and interstitial deletion result in the generation of gene fusion from which fusion mRNA is transcribed and then translated to the fusion protein, which can transform a normal cell into a cancer cell. Gene fusions, resulting from chromosomal rearrangements, generate either a deregulated partner gene through juxtaposition of the coding sequences with the regulatory sequences or a chimeric protein (Figure 1.2) with new properties; e.g. altered transcriptional regulation or constitutive kinase activity (Table 1.1).

A. chromosomal rearrangements



B. functional outcomes

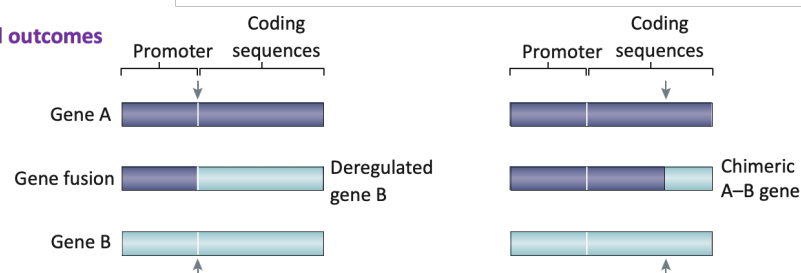


Figure 1.2. Chromosomal rearrangements and functional consequences of gene fusions [9]

Table 1.1. Recurrent fusion genes in acute leukemia [10, 11]

Disease	Fusion Gene	Original function	Chromosomal Aberration	Frequency (%)
Acute Lymphocytic Leukemia (ALL)	<i>ETV6-RUNX1</i>	TF	t(12;21)	20-25
	<i>BCR-ABL1</i>	TK	t(9;22)	20
	<i>TCF3-PBX1</i>	TF	t(1;19)	5
	<i>KMT2A-AFF1</i>	HMT	t(4;11)	2-5
	<i>SET-NUP214</i>		del(9) / t(9;9)	3-10 (T-ALL)
Acute Myeloid Leukemia (AML)	<i>RUNX1- RUNX1T1</i>	TF	t(8;21)	10
	<i>CBFB-MYH11</i>	TF	inv(16)	5-10
	<i>KMT2A-MLLT3</i>	HMT	t(9;11)	2-5
	<i>RPN1-MECOM</i>	TF	inv(3)	1-2
	<i>DEK-NUP214</i>		t(6;9)	1
Acute Promyelocytic Leukemia (APL)	<i>PML-RARA</i>	TF	t(15;17)	90
Chronic Myelocytic Leukemia (CML)	<i>BCR-ABL1</i>	TK	t(9;22)	95

TF: transcription factor; TK: tyrosine kinase; HMT: Histone methyltransferase

1.5 NUP214 fusion proteins

Recurrent genetic abnormalities associated with nucleoporins found in acute leukemia often have a poor prognosis. Four nucleoporins, NUP98, NUP214/CAN, NUP358/RanBP2, and Tpr, have been reported to form chimeric proteins by chromosomal rearrangements leading to hematological malignancies, in particular acute leukemia [12, 13]. NUP98 and NUP214 rearrangements share some common characteristics, such as HOXA activation, which may account for the compromised differentiation of the hematopoietic progenitor cells and their uncontrolled proliferation [14-18].

Chromosomal rearrangements involving the *NUP214* locus are recurrent in acute leukemia and frequently fuse the C-terminal region of NUP214 with SET and DEK, two chromatin remodeling proteins with roles in histone chaperoning and transcription regulation [14]. Histone chaperones facilitate the assembly and disassembly of nucleosomes in an ATP-independent manner [19]. SET and DEK are suggested to be histone chaperones for histone H1 and H3, respectively. The acidic amino acid region of SET and DEK is indispensable for their histone chaperone activity [20, 21]. SET-NUP214 and DEK-NUP214 fusion proteins consist of almost the entire SET and DEK, and two-thirds of NUP214, which is a component of nuclear pore complex and thereby localized in the nuclear envelope. The histone chaperones, SET and DEK, have histone binding ability through their acidic region and thus diffused in the nucleus. On the other hand, SET-NUP214 and DEK-NUP214 are localized in the nucleus as granular dots (Figure 1.3).

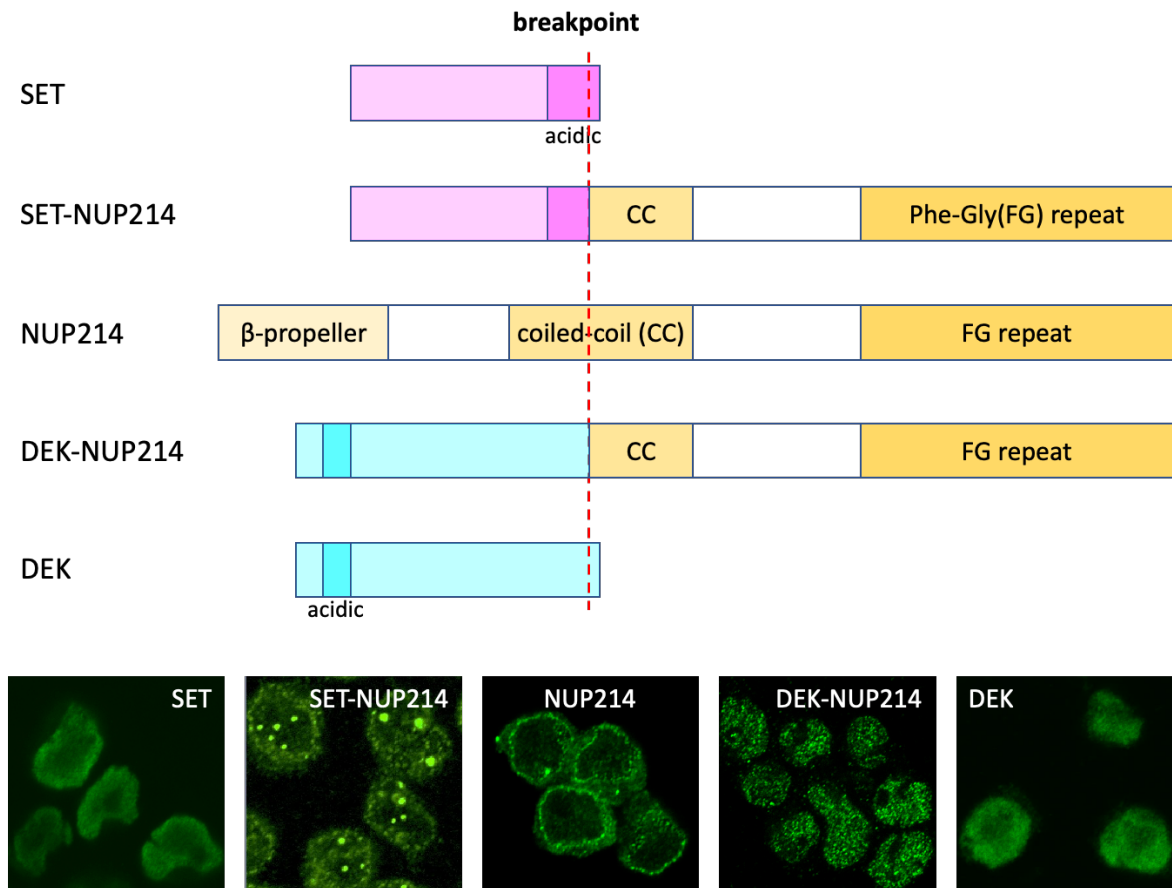


Figure 1.3. NUP214 fusion proteins and their normal counterparts.

SET-NUP214 is predominantly associated with ALL, whereas *DEK-NUP214* exclusively results in AML [14]. *SET-NUP214* fusion gene is caused by del(9)(q34.11q34.13) or occasionally by t(9;9)(q34; q34) and *DEK-NUP214* fusion gene results from t(6;9)(p23;q34). *SET/DEK-NUP214* associated leukemia often predicts a poor outcome since it is highly aggressive, and patients are frequently resistant to treatment. *SET-NUP214* has been found to bind to the *HOXA* locus and activate its expression [17]. *SET-NUP214* causes expansion of hematopoietic progenitors and blocks cell differentiation [22]. *DEK-NUP214* develops leukemia in mice [23].

1.6 Nuclear pore complex and NUP214

Nuclear pore complexes (NPCs) are large protein assemblies located at the nuclear pore. NPC consists of more than 30 different proteins called nucleoporins (NUPs). NPCs form selective passageways between nucleoplasm and cytoplasm [24]. Most proteins and RNAs translocate through NPCs by the help of shuttling nuclear transport receptors (NTRs) that interact with phenylalanine-glycine (FG)-repeat-containing NUPs. NUP214 is one of the FG-repeat-containing nucleoporins located on the cytoplasmic side and assists nuclear-cytoplasmic transport of proteins and RNAs through interaction with NTRs (Figure 1.4).

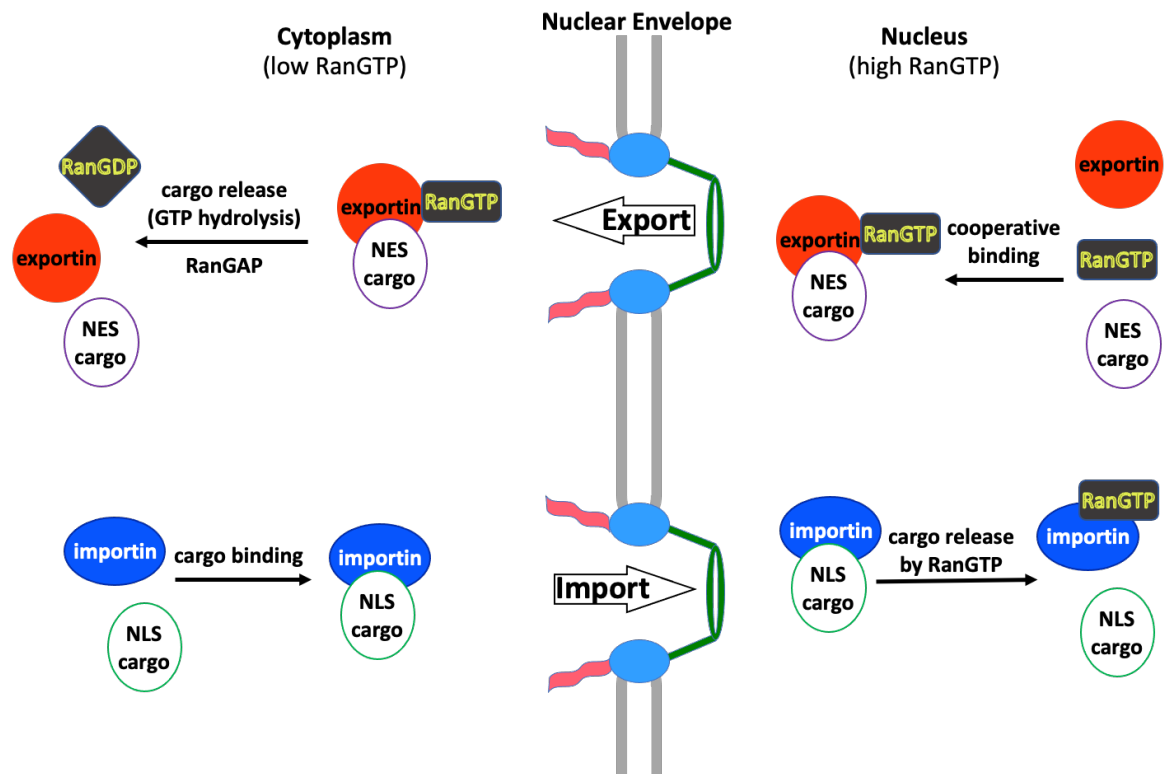
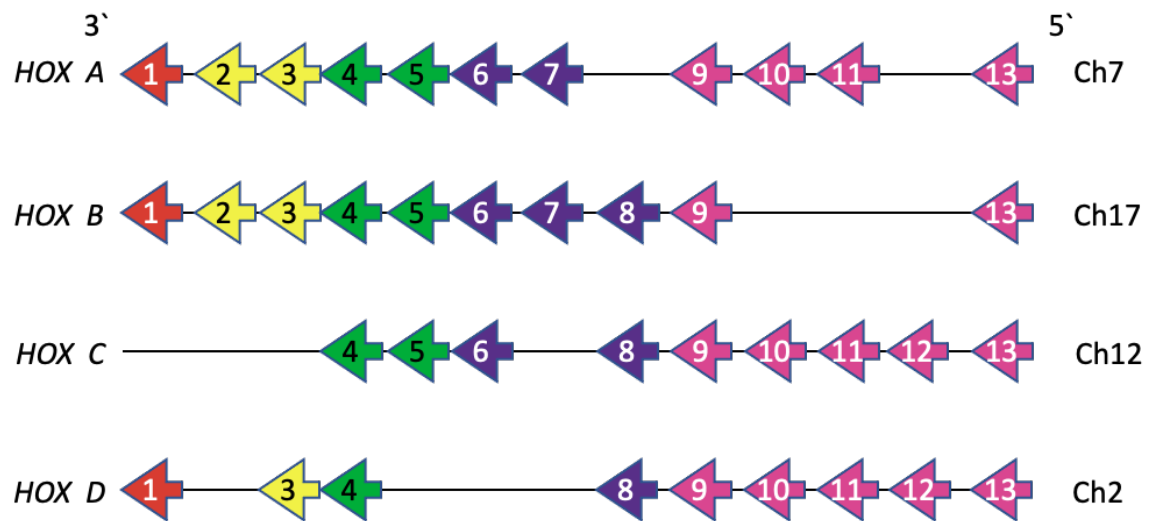


Figure 1.5. Nuclear-cytoplasmic transport, modified from [27].

1.8 Homeobox genes and their regulation

Homeobox (HOX) genes, which encode HOX proteins, are distributed as clusters (*HOXA* to *HOXD*) on four different chromosomes. HOX proteins are DNA-binding proteins with low sequence specificity. They regulate the downstream gene expression program during hematopoietic differentiation through complex formation with cofactors such as MEIS1 to confer sequence specificity and selectivity [28]. Despite a variety of genetic abnormalities, gene expression profiles showed that expression of *HOXA* cluster genes is often increased in AML and T-ALL. Therefore, *HOX* gene dysregulation is a dominant mechanism of leukemogenesis [29]. The expression of *HOX* genes in leukemia is regulated by histone modification enzymes, such as MLL/KMT2A and DOT1L, which mediate the methylation of histone H3K4 and K79, respectively.

Clustered *Hox* gene organization



Hox as transcription factor

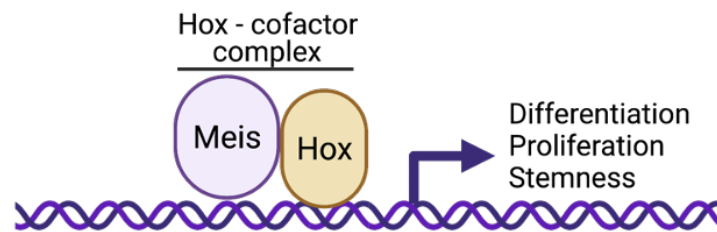


Figure 1.6. HOX genes and their regulation.

1.9 The aim of this study

SET-NUP214 and *DEK-NUP214* fusion genes, are driver mutations in acute leukemia. However, the detailed oncogenic mechanism of NUP214 fusion protein-driven leukemia is unclear. This study aims to reveal the functions of SET-NUP214 and DEK-NUP214 fusion proteins. For this aim, I investigated the proteins that interact with these fusion proteins and their roles in leukemogenesis.

Chapter 2 Leukemia-associated NUP214-fusion proteins disturb XPO1-mediated nuclear-cytoplasmic transport pathway and-thereby NF-κB signaling pathway

2.1 Abstract

Nuclear-cytoplasmic transport through nuclear pore complexes is mediated by nuclear transport receptors. Previous reports suggested that aberrant nuclear-cytoplasmic transport by mutations or overexpression of nuclear pore complexes and nuclear transport receptors is closely linked to diseases. NUP214, a component of nuclear pore complexes, has been found as chimeric fusion proteins in leukemia. Among various NUP214-fusion proteins, SET-NUP214 and DEK-NUP214 were shown to be engaged in tumorigenesis, but their oncogenic mechanism remains unclear. In this study, I examined the function of the NUP214-fusion proteins by focusing on their effects on nuclear-cytoplasmic transport. I found that SET-NUP214 and DEK-NUP214 interact with XPO1/CRM1 and NXF1/TAP, which mediate leucine-rich NES-dependent protein export and mRNA export, respectively. SET-NUP214 and DEK-NUP214 decreased XPO1-mediated nuclear export of NES proteins such as cyclin B and proteins involved in the NF-κB signaling pathway by tethering XPO1 onto nuclear dots where NUP214-fusion proteins are localized. I also demonstrated that SET-NUP214 and DEK-NUP214 expression inhibited NF-κB-mediated transcription by abnormal tethering of the complex containing p65 and its inhibitor, IκB, in the nucleus. These results implicate that SET-NUP214 and DEK-NUP214 perturb gene expression regulation through alteration of the nuclear-cytoplasmic transport system.

2.2 Introduction

Biological macromolecules are transported between the nucleus and the cytoplasm in response to extracellular signals. Transport of molecules with a molecular mass greater than 40 kDa does not occur by simple diffusion but is generally facilitated by nuclear transport receptors (NTRs) through nuclear pore complexes (NPCs) embedded in the nuclear envelope [24, 30, 31]. Controlled nuclear-cytoplasmic transport plays important roles in maintaining cellular integrity in eukaryotic cells. It is reported that aberrant subcellular localization of some proteins is associated with various cancer cases [32]. p53 has nuclear localization signal (NLS) and nuclear export signal (NES), and the accumulation of p53 in the cytoplasm has been reported to be a prognostic indicator in cancer [33]. Nuclear factor-kappa B (NF-κB) transcription factor is mainly observed in the cytoplasm in normal cells, whereas in many cancer cells it is largely localized in the nucleus [34].

In addition to aberrant subcellular localization of proteins in cancer, mutations of genes encoding NTRs and NPC components are found in various types of cancer [35]. Mutations of Exportin-5 (XPO5) and Exportin-1 (XPO1)/CRM1, members of export receptors, [36-38] are found in solid

cancer and leukemia, respectively. Four nucleoporins, NUP98, NUP214/CAN, NUP358/RanBP2, and Tpr, have been reported to form chimeric proteins by chromosomal translocations mainly in leukemia [15, 39-42]. NUP214 located at the cytoplasmic filament of NPC interacts with NTRs to control macromolecular transport. *SET-NUP214* and *DEK-NUP214* were identified in acute undifferentiated leukemia and acute myeloid leukemia (AML), respectively [43, 44], and recently found in several T cell acute lymphoid leukemia (T-ALL) and AML patients [45, 46]. *SET-NUP214* is found to bind to the *HOXA* locus and activate its expression [17]. Ectopic expression of *SET-NUP214* causes expansion of hematopoietic progenitors [22, 47], and blocks cell differentiation [48]. Expression of *DEK-NUP214* leads to acceleration of protein synthesis [49], cell proliferation [50], and development of leukemia in mice [51]. However, the detailed functions of these fusion proteins in leukemogenesis remain unclear.

NUP214 interacts with various NTRs such as Importin- β (IPOB)[52, 53], Exportin-T (XPOT)[54], XPO1[55], Nuclear RNA export factor 1 (NXF1)/TAP [56-59], NXF2, and NXF3 [60]. Export of both mRNAs and proteins is severely reduced when the level of NUP214 expression is decreased [61-63], and ectopic overexpression of truncated NUP214 causes accumulation of NES proteins in the nucleus in mammals [64]. On the other hand, in *Drosophila*, *NUP214* deletion enhances export of GFP fused with NES [65]. These results indicate that appropriate expression of NUP214 is critical for regulated export of macromolecules and that generation of fusion genes containing NUP214 by chromosomal translocation may affect the nuclear-cytoplasmic transport system [66].

Previously in our laboratory, it was reported that *SET-NUP214* interacts with XPO1 and causes accumulation of GFP with artificial NES into the nucleus (Figure 2.1). However, it is not known whether the subcellular localization and function of endogenous NES proteins are affected by the expression of NUP214 fusion proteins. Moreover, the effect of NUP214 fusion proteins on other NTRs remains to be elucidated to reveal the mechanism in detail.

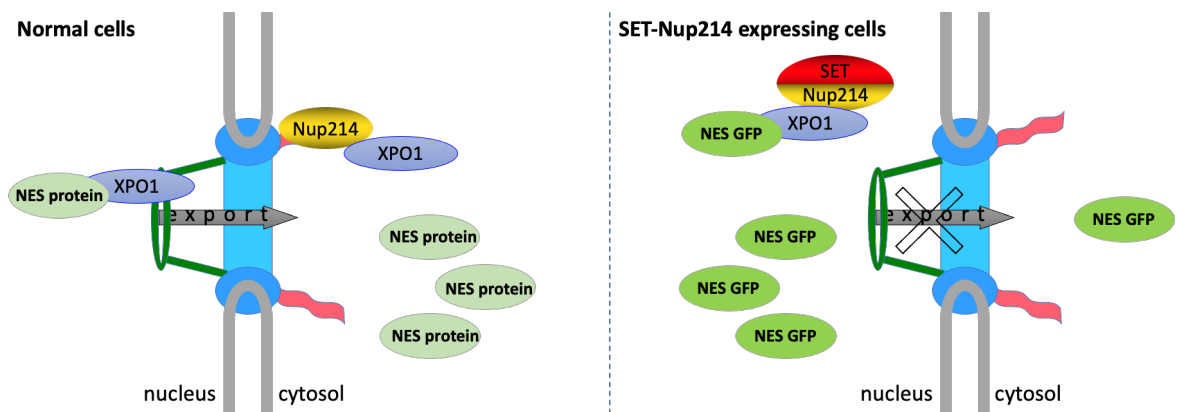


Figure 2.1. Effect of *SET-NUP214* on nuclear transport of NES proteins.

In previous studies, we and another group reported that both SET-NUP214 and DEK-NUP214 interact with XPO1. This interaction causes a change in XPO1 localization and consequently impairs correct localization of an artificial model protein containing NES [53, 67, 68]. Here, I comprehensively analyzed the effects of SET-NUP214 and DEK-NUP214 expression on the functions of NTRs and their cargoes. I found that SET-NUP214 and DEK-NUP214 interact with NXF1 and XPO1, and abrogates the XPO1 function to dampen nuclear export of endogenous NES proteins such as cyclin B and proteins involved in the NF- κ B pathway. In addition, I demonstrated that SET-NUP214 and DEK-NUP214 expression inhibited NF- κ B-mediated transcription due to abnormal retention of complexes containing p65 and its inhibitor, I kappa B (I κ B), in the nucleus. These results suggest the possibility that perturbation of proper nuclear-cytoplasmic shuttling of macromolecules by the expression of the NUP214-fusion proteins lead to various hematologic disorders.

2.3 Materials and Methods

2.3.1 Cell culture and transfection

HeLa cells and HEK293T cells were grown in DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin. For transfection assays, GeneJuice (Merck KGaA, Germany) (IF assay, luc assay) or *Polyethylenimine, Linear (MW 25,000)* (Polysciences, Inc.) (IP assay, ChIP assay) was used.

2.3.2 Plasmids

For construction of NTR expression vectors, cDNAs were prepared from total RNA derived from HeLa and HEK293T cells by revers-transcription with ReverTra Ace (TOYOBO Co., Ltd, Japan) and oligo dT₂₀. PCR amplification was performed using KOD FX (TOYOBO Co., Ltd). PCR fragments were inserted into pCHA [69]. pCAGGS-HA-XPO1 was made by inserting HA-tagged XPO1 fragment obtained by PCR using pXHC1 as a template into pCAGGS. pCAGGS-SET-NUP214-3Flag and pCAGGS-DEK-NUP214-3Flag were made by inserting amplified PCR fragment (C terminal fragment of NUP214 fused with three Flag-tags) using pCAGGS-SET-NUP214 as a template, into pCAGGS-SET-NUP214 or pCAGGS-DEK-NUP214. For pCAGGS-3Flag-NUP214 (1057-2090) and pCAGGS-3Flag-SET-NUP214 (1637), NUP214 (1057-2090) and SET-NUP214 (1637) was digested from pCAGGS-NUP214 (1057-2090) and pCAGGS-SET-NUP214 (1637), and inserted into pCAGGS-3Flag. pmKate2-C-SET-NUP214 and pmKate2-C-DEK-NUP214 were constructed by excising pmKate2-C (Evrogen) and ligated with SET-NUP214 and DEK-NUP214 excised from pCAGGS-SET-NUP214 and pCAGGS-DEK-NUP214. To construct pNF- κ B40-firefly luciferase, Interferon Stimulated Response Element (ISRE) of pISRE-TA-luc (Clontech Laboratories, Inc.) was removed, and a fragment of NF- κ B binding element from

pNF- κ B-SEAP (Clontech Laboratories, Inc.) was inserted. To construct pTA-Renilla luciferase, ISRE of pISRE-TA-luc vector was removed, and the firefly luciferase region was replaced with a fragment of the Renilla luciferase region, which was obtained from pRL-SV40 (Promega). Sequences of all fragments obtained by PCR were confirmed by sequencing analysis.

2.3.3 IP assay and WB analysis

IP assays and western blot analyses were performed as described previously [70]. To detect chemiluminescence in western blot analysis, Chemi-Lumi One L (Nacalai tesque, Inc., Japan) or ImmunoStar LD (Wako Pure Chemical Industries, Ltd., Japan) was used, and signals were observed using LAS-4000mini (GE Healthcare UK Ltd.), and processed by Adobe Photoshop Elements (Adobe Systems).

2.3.4 IF assay, oligo dT-FISH, and PLA

IF assays were performed as described previously [70]. For DNA staining, TO-PRO-3 Iodide (Life Technologies) (1:5000) was used. For IF assays of spleen sections, sample sections with 2 μ m in thickness were examined. Paraffin-embedded sections were deparaffinized and rehydrated in xylene and ethanol. Samples were autocleaved for 5 min in 10 mM citrate buffer solution. After washing with PBS(-), samples were subjected to IF assays. *In situ* hybridization using oligo dT was performed according to the protocol as described previously [71]. PLA was performed using Duolink In Situ PLA (Sigma-Aldrich Co. LLC) according to the manufacturer's instructions after incubation with primary antibodies. After PLA, samples were incubated with Alexa 488-conjugated anti-mouse IgG and Alexa 633-conjugated anti-rabbit IgG antibodies for 30 min to observe I κ B α and p65, respectively. Samples were observed by LSM5Exciter confocal microscope with Plan-Apochromat 63x objective lens (Carl Zeiss Microscopy GmbH, Germany). Pictures were processed by ZEN software (Carl Zeiss Microimaging GmbH, Germany). Statistical analyses were performed by Student's t-test.

2.3.5 RNA extraction and RT-qPCR

Total RNA was extracted using MagExtractor -RNA- (TOYOBO Co., Ltd). The experimental procedure was as described in the manufacturer's protocol. Total RNA (0.5 μ g) was reverse-transcribed by ReverTra Ace (TOYOBO Co., Ltd) and oligo dT₂₀ for 60 min at 42°C. For qPCR, Fast SYBR Green Master (Roche Diagnostics GmbH) was mixed with reverse-transcribed samples and primers, and PCR was carried out by Thermal Cycler Dice Real Time System (TAKARA BIO Inc. Japan). Primer sequences used in this study were as follows: for A20, 5'-AAG CTG TGA AGA TAC GGG AGA-3' and 5'-CGATGAGGGCTTTGTGGATGAT-3', for I κ B α /NFKBIA, 5'-CTCCGAGACTTTTCGAGGAAATAC-3' and 5'-GCCATTGTAGTTGGTAGCCTTCA-3', and

for GAPDH, 5'-AGCCAAAAGGGTCATCATCTC-3' and 5'-GGACTGTGGTCATGAGTCCTTC-3'. Statistical analyses were performed by Student's t-test.

2.3.6 ChIP assay

ChIP assays were carried out according to the manual supplemented from Merck except for sonication buffer used in figure 7D. Sonication buffer; 640 mM KCl, 30 mM NaCl, 1% Triton X-100, 10 mM EDTA, 20mM Tris-HCl (7.9), and 20% glycerol. qPCR analysis was performed as described for RT-qPCR. Primer sequences used in this study were as follows: for A20, 5'-CAGCCCGACCCAGAGAGTCAC-3' and 5'-CGGGCTCCAAGCTCGCTT-3', and for IκBα/NFKBIA, 5'-ATTCAAATCGATCGTGGGAAAC-3' and 5'-GGGAATTTCCAAGCCAGTCA-3'.

2.4 Results

2.4.1 SET-NUP214 and DEK-NUP214 interact with nuclear transport receptors

Nucleoporins can be categorized into three groups; transmembrane nucleoporins, scaffold nucleoporins, and nucleoporins containing phenylalanine-glycine repeats (FG-NUPs). NUP214 is one of FG-NUPs and interacts with several NTRs through its FG repeat region. Because both SET-NUP214 and DEK-NUP214 contain the intact FG repeat region of NUP214 (Figure 2.2A), it is possible that these fusion proteins interact with NTRs. Indeed, SET-NUP214 and DEK-NUP214 were shown to interact with XPO1 [67, 68]. To verify whether SET-NUP214 and DEK-NUP214 bind to several NTRs other than XPO1, first immunoprecipitation (IP) assays were performed. We assessed seven well-known NTRs as follows; Importin-β1 (IPOB) (import of NLS-containing proteins), Importin-7 (IPO7) (import of proteins such as histone and MAPK), XPO1 (export of NES-containing proteins, snRNAs, and snoRNAs), Exportin-2 (XPO2) /CSE1L (export of Importin α), Exportin-5 (XPO5) (export of small RNAs), Exportin-3 (XPO3) /Exportin-t (export of tRNAs), and NXF1 (export of mRNAs). Among these NTRs, XPO1 and NXF1 bind to SET-NUP214 and DEK-NUP214 efficiently (Figure 2.2B). Because SET-NUP214 and DEK-NUP214 have a shared NUP214 portion (813-2090 a.a.) which is composed of coiled-coil and FG repeat domains, we addressed whether the binding of SET-NUP214 and DEK-NUP214 with NTRs was mediated by the NUP214 portion. The C-terminally truncated NUP214 (1057-2090) efficiently bound to XPO1 and NXF1 (Figure 2.2B), demonstrating the interaction between SET-NUP214 or DEK-NUP214 and XPO1 or NXF1 depends on the NUP214 part. Reciprocal IP experiments confirmed the interaction between SET-NUP214, DEK-NUP214, or NUP214 (1057-2090) and endogenous XPO1 or NXF1 (Figure 2.2C).

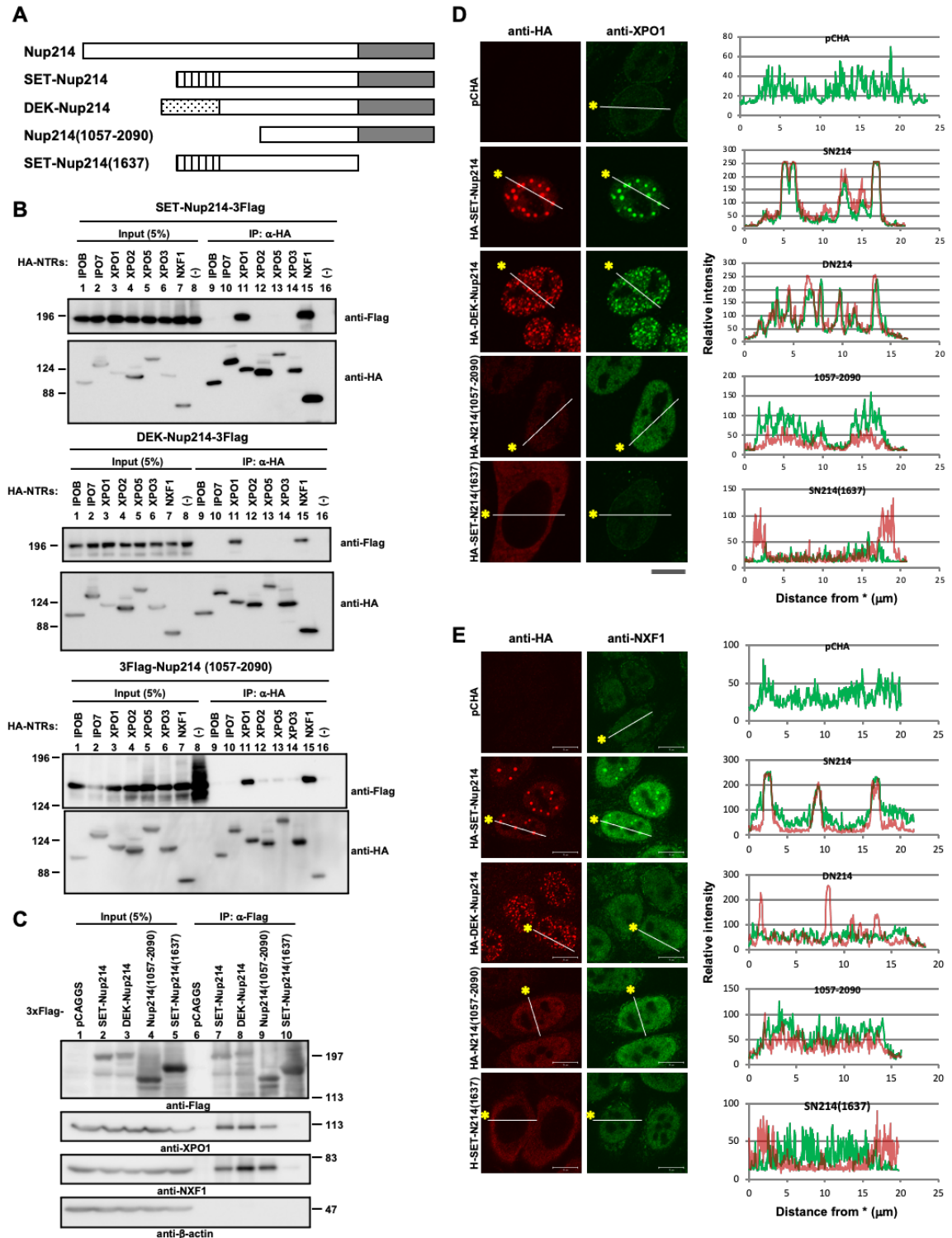


Figure 2.2. Interaction between NUP214 fusion proteins and NTRs.

(A) Schematic representation of SET-NUP214, DEK-NUP214, NUP214 (1057-2090), and SET-NUP214 (1637) used in our study and full length NUP214. Box with vertical bars, SET portion; box with black dots, DEK portion; gray box, FG repeat region. (B) HEK293T cells cultured in 6-well plates were transfected with 1 μ g of pCHA-nuclear transport receptors (NTRs) or pCAGGS and 1 μ g of pCAGGS-SET-NUP214-3Flag, DEK-NUP214-3Flag, or 3Flag-NUP214 (1057-2090). At two days after transfection, cells were collected and subjected to IP assays with 300 ng of anti-HA (3F10) High Affinity antibody (Roche Diagnostics GmbH), and immunocomplexes were recovered by nProtein A Sepharose Fast Flow (GE Healthcare UK Ltd.). After IP assays, proteins in input lysates and immunoprecipitated samples were separated by 6% SDS-PAGE, and western blot analyses were performed using anti-Flag M2 (2 μ g/ml) (Sigma-Aldrich Co. LLC), and anti-HA

(3F10) (1:1000) antibodies. Prestained molecular weight markers (kDa) (Nacalai tesque, Inc., Japan) are indicated in the left. (C) HEK293T cells cultured in 10-cm dishes were transfected with 5 µg of pCAGGS, SET-NUP214-3Flag, DEK-NUP214-3Flag, 3Flag-NUP214 (1057-2090), and 3Flag-SET-NUP214 (1637). At two days after transfection, cells were collected, and cell lysates were subjected to immunoprecipitation with anti-Flag M2 Agarose Affinity Gel (Sigma-Aldrich Co. LLC.). Proteins in input lysates and immunoprecipitated samples were separated by 6.5%SDS-PAGE, and western blot analyses were performed using anti-Flag, anti-XPO1 (H-300) (1:1000) (Santa Cruz Biotechnology, Inc.), anti-NXF1 (53H8, Santa Cruz Biotechnology, Inc.) (1:500), and anti-β-actin (AC-15, Sigma-Aldrich Co. LLC) (1:5000) antibodies. Prestained molecular weight markers (kDa) are indicated in the left. (D, E) HeLa cells cultured in 35-mm dishes were transfected with pCHA, HA-SET-NUP214, HA-DEK-NUP214, HA-NUP214 (1057-2090), and HA-SET-NUP214 (1637). At two days after transfection, cells were subjected to IF assays. For primary antibody, anti-HA (3F10) (1:100) (D), anti-XPO1 (1:20) (D), anti-HA rabbit (1:500) (E), and anti-NXF1 (1:20) (E) antibodies were used. Right graphs represent relative intensities of HA-tagged protein and XPO1 (D) or NXF1 (E) along a line. Bar: 10 µm.

2.4.2 SET-NUP214 and DEK-NUP214 affect subcellular localization of NTRs

As we and another group reported previously [67, 68], SET-NUP214 and DEK-NUP214 are mainly localized in the nucleus as granular dots and influence subcellular localization of XPO1. Because NXF1 was also co-immunoprecipitated with SET-NUP214 and DEK-NUP214, I next examined subcellular localization of both NTRs by indirect immunofluorescence (IF) assays. Endogenous XPO1 was located in the nucleus and the nuclear envelope, whereas it was localized markedly as granular dots upon expression of SET-NUP214 and DEK-NUP214 (Figure 2.2D). XPO1 localization at the nuclear envelope in cells expressing NUP214 (1057-2090) was reduced and mainly localized in the nucleus where NUP214 (1057-2090) existed. Endogenous NXF1 was observed in the nucleus in control cells. On the other hand, in cells expressing SET-NUP214, NXF1 was found in nuclear dots where SET-NUP214 accumulated (Figure 2.2E), albeit the accumulation was less than XPO1. In addition, in DEK-NUP214 expressing cells, I could not find clear accumulation of NXF1 to the sites where DEK-NUP214 was accumulated. It was previously reported that the FG repeat region of NUP214 plays crucial roles in binding with NTRs. Therefore, I next examined the importance of the FG repeat region of the fusion proteins for the localization changes of XPO1 and NXF1 using the deletion mutant of SET-NUP214 termed SET-NUP214 (1637), which lacks the FG repeat region of SET-NUP214. This mutant neither interacted with nor changed the localization of both endogenous XPO1 and NXF1 (Figure 2.2C, lane 10, Figures 2.2D and 2.2E). These results indicate that expression of SET-NUP214 and DEK-NUP214 affects the localization pattern of both XPO1 and NXF1 by their physical interaction through the FG repeat region of NUP214.

2.4.3 NUP214 fusions affect subcellular localization of endogenous proteins harboring NES

As XPO1 was accumulated in nuclear dots in cells expressing SET-NUP214 and DEK-NUP214 (Figure 2.2D), it was reasonable to hypothesize that intracellular availability of XPO1 may be decreased. We have previously found that EGFP fused to the NES of cAMP-dependent protein kinase inhibitor (PKI) was accumulated in the nucleus in cells expressing SET-NUP214 [68].

However, it is not known whether subcellular localization of endogenous NES proteins is actually affected by the expression of both SET-NUP214 and DEK-NUP214. Hence, I performed IF assays to observe endogenous XPO1 cargo proteins, I κ B α and cyclin B1. I κ B α (Figure 2.3A) and cyclin B1 (Figure 2.3C) were localized mainly in the cytoplasm in cells that did not express SET-NUP214 or DEK-NUP214. In contrast, they were uniformly accumulated in the nucleus upon expression of SET-NUP214 and DEK-NUP214. Because NF- κ B transcription factor p65/RelA binds to I κ B α in unstimulated cells, I examined the localization of p65 in cells expressing SET-NUP214 and DEK-NUP214. Interestingly, the cytoplasmic localization of p65 was also disturbed by SET-NUP214 and DEK-NUP214. Quantitative analyses revealed that the ratio of fluorescent intensity of I κ B α or p65 in the nucleus to that in the cytoplasm was increased significantly as intensity of SET-NUP214 and DEK-NUP214 increased (Figure 2.3A). When the C-terminal region of NUP214 (NUP214 (1057-2090)) was expressed, nuclear accumulation of I κ B α and p65 was also observed (Figure 2.3B). It indicated that the C-terminal region of NUP214 can function as a dominant negative mutant of endogenous NUP214 as previously reported [64]. From these observations, I conclude that SET-NUP214 and DEK-NUP214 change subcellular localization of endogenous proteins harboring NES by inhibiting the endogenous NUP214 function.

2.4.4 XPO1 overexpression rescue nuclear accumulated I κ B α induced by NUP214 fusions

To address whether inhibition of NF- κ B signaling pathway by SET-NUP214 and DEK-NUP214 is mediated via XPO1 trapping, the effects of XPO1 over-expression on the NF- κ B signaling pathway were examined by immunofluorescence assays. We found that overexpression of XPO1 reduced nuclear I κ B α that was accumulated by SET-NUP214 expression, DEK-NUP214 expression, and LMB treatment (Figure 2.4). This result indicates that over-expression of XPO1 rescues the I κ B α localization pattern that was changed by the NUP214 fusion proteins, and supports our hypothesis that nuclear accumulation of I κ B α was caused by decreased availability of active XPO1 in the cells expressing SET-NUP214 or DEK-NUP214. However, we found that the over-expression of XPO1 did not rescue the NF- κ B transcription activity in these cells, and that it diminished the NF- κ B activity in control cells where SET-NUP214 or DEK-NUP214 was not expressed. This could be because that p65 was excluded from the nucleus upon XPO1 over-expression (Figure 2.5). Although the rescue experiment by XPO1 over-expression did not work in terms of NF- κ B transcription activity, these data suggest that XPO1 trapping is one of the major causes of the inhibition of the NF- κ B activity in SET-NUP214 and DEK-NUP214 expressing cells.

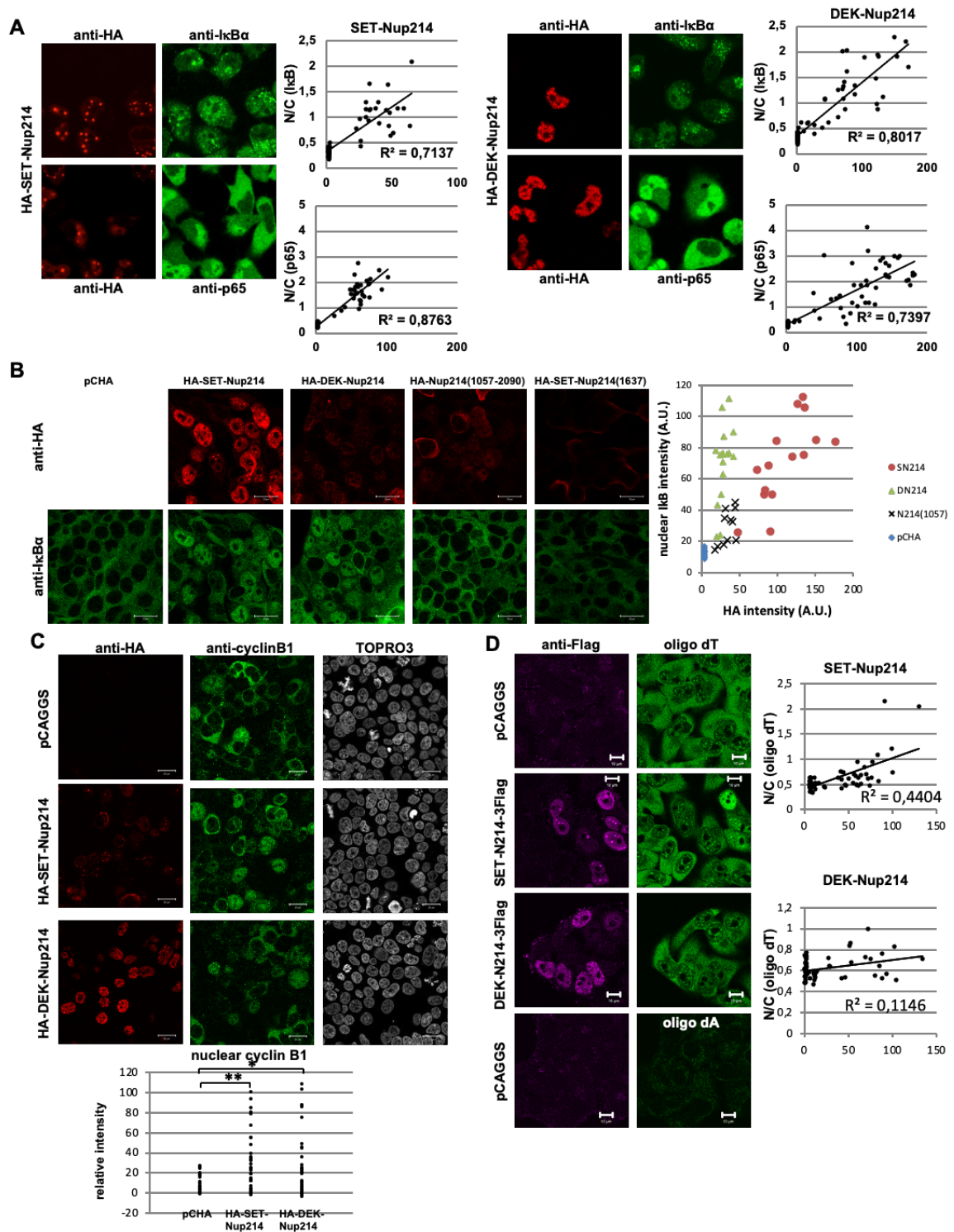


Figure 2.3. Localization of endogenous proteins harboring NES and mRNA.

(A) (Pictures) HeLa cells were transiently transfected with pCHA-SET-NUP214 or HA-DEK-NUP214, and subjected to IF assays using anti-HA (3F10), anti-IkBa (C-21, Santa Cruz Biotechnology, Inc.) (1:100) or anti-p65 (PC137, Calbiochem) (1:100) antibodies. (Graphs) Fluorescence intensity was quantitatively determined using ImageJ software. Nuclear and cytoplasmic areas were selected manually. X axis=mean (intensity of HA-tagged protein in the nucleus)-mean(background), and Y axis=N/C=(mean(intensity of IkBa or p65 in the nucleus)-mean (background))/(mean (intensity of IkBa or p65 in the cytoplasm)-mean(background)). (B) 293T cells were transfected with pCHA, HA-SET-NUP214, HA-DEK-NUP214, HA-NUP214 (1057-2090), HA-SET-NUP214 (1637). Two days later, cells were subjected to immunofluorescence assay using anti-HA (3F10) and anti-IkBa (L35A5, CST, Inc.) (1:20) antibodies.

Fluorescence intensities of nuclear IκBα and HA-tagged protein in each sample were quantitatively determined using ImageJ software and plotted. Bar: 20 μm. (C) HeLa cells were transiently transfected with pCHA-SET-NUP214 or DEK-NUP214, and subjected to IF assays using anti-HA (3F10), and anti-cyclinB1 (4138, CST Inc.) (1:20) antibodies. Fluorescence intensity of nuclear cyclin B1 in each cell was quantitatively determined using ImageJ software and plotted. Bar: 20 μm. **P* < 0.005, ***P* < 0.0005. (D) HeLa cells were transiently transfected with pCAGGS, SET-NUP214-3Flag, or DEK-NUP214-3Flag, and subjected to IF assays using anti-Flag M2, and *in situ* hybridization assay with 10 ng/μl biotinylated oligo dT₄₅ or oligo dA₄₅ as probes. In the dot plots, fluorescence intensity was quantified using ImageJ software as described in Figure 3A. Bar: 10 μm.

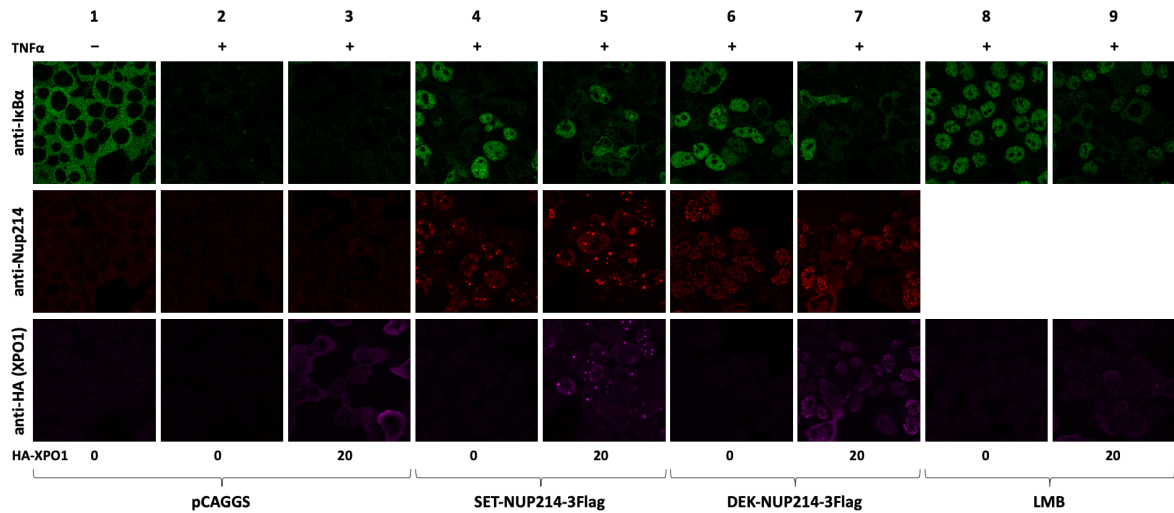


Figure 2.4. Subcellular localization of IκBα (rescue experiment by XPO1 over-expression)

HEK293T cells (1×10^5) cultured in 12-well plates were transfected with 200 ng of pCAGGS, SET-NUP214-3Flag (panels 4, 5) or DEK-NUP214-3Flag (panels 6, 7) with 20 ng of pCHA-XPO1 (panels 3, 5, 7, 9). At two days after transfection, cells were incubated with 5 ng/ml LMB (panels 8, 9) for 1 hour, and TNF-α was added at the final concentration as 20 ng/ml (panels 2-9). After TNF-α incubation for 30 min, cells were collected and immunofluorescence assays were performed using rabbit anti-IκBα (L35A5), anti-NUP214, and anti-HA (3F10) antibodies.

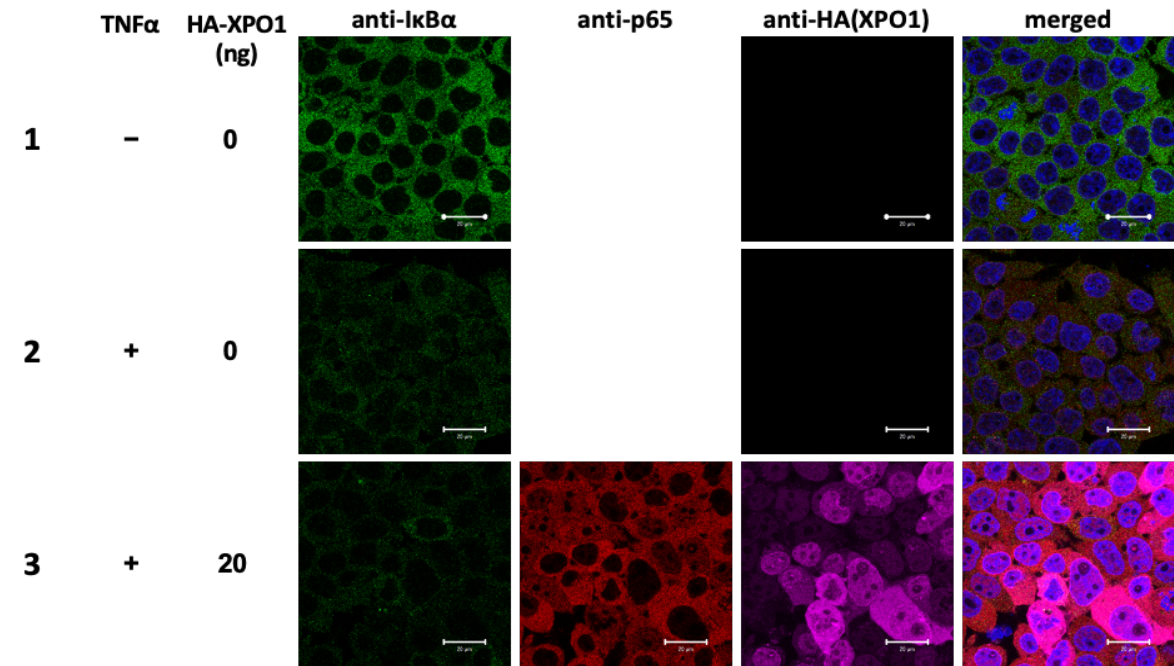


Figure 2.5. Subcellular localization of NF-κBp65 (rescue experiment by XPO1 over-expression)

HEK293T cells (6×10^5) cultured in 6-cm dishes were transfected with 20 ng or 200 ng of pCHA-XPO1 (panels 2, 3). At two days after transfection, cells were incubated with 20 ng/ml TNF- α for 30 m (panels 2-4) and IF assays were performed using anti-I κ B α (L35A5), anti-p65 (ab7970), and anti-HA (3F10) antibodies. Scale bar: 20 μ m.

2.4.5 SET-NUP214 has a small effect on poly A mRNA localization

In addition to XPO1, the expression of SET-NUP214 affected the localization of NXF1, an NTR for mRNA (Figure 2.2E). We assessed subcellular localization pattern of mRNA by fluorescence *in situ* hybridization assays using oligo dT as a probe (Figure 2.3D). In control cells, oligo dT signal was observed both in the nucleus and the cytoplasm, and the cytoplasmic intensity was higher than the nuclear intensity. In some of SET-NUP214 expressing cells, the signal intensity ratio of oligo dT in the nucleus to that in the cytoplasm is higher than that in control cells. Quantitative analyses showed that cells highly expressing SET-NUP214 were prone to mRNA accumulation in the nucleus. However, the accumulation of mRNAs was less clear than that of proteins harboring NES, suggesting that the effect of SET-NUP214 on the NXF1 function is lower than that on the XPO1 functions. In addition, we found that there was little difference of oligo dT staining pattern in control cells and cells expressing DEK-NUP214.

2.4.6 SET-NUP214 and DEK-NUP214 affect NF- κ B transcription activity

I presumed that a cause of oncogenesis by the expression of SET-NUP214 and DEK-NUP214 is due at least in part to the deregulation of gene expression caused by aberrant localization of proteins and/or RNAs. Subcellular localization of p65 and I κ B α was changed upon expression of SET-NUP214 and DEK-NUP214 (Figure 2.3A). It was reported NUP98-fusion proteins stimulate NFAT- and NF- κ B-mediated transcription activities by impairing the XPO1 function [72]. Thus, I examined the effect of these NUP214-fusion proteins on the NF- κ B signaling pathway. The transcriptional activity of NF- κ B was assessed first by reporter assays using firefly luciferase under the control of NF- κ B (κ B-FLuc). In the absence of TNF- α , the fusion proteins did not affect the luciferase activity (Figure 2.6A, left graph), although the fusion proteins induced nuclear accumulation of p65 (Figure 2.3A). TNF- α treatment dramatically increased the reporter activity of κ B-FLuc. This increase of the transcriptional activity was markedly inhibited by the expression of SET-NUP214 and DEK-NUP214 (Figure 2.6A, right graph). As reported [73] [74] [75], LMB treatment showed an inhibitory effect on the luciferase activity of NF- κ B-mediated transcription in the presence of TNF- α (Figure 2.6A, right graph), while no significant effect was observed in the absence of TNF- α (Figure 2.6A, left graph). The effects of SET-NUP214 and DEK-NUP214 expressions on the pTA-Renilla luciferase reporter under the control of the minimal promoter (TATA-RLuc) was less clear than those on the κ B-luciferase activity, suggesting that the effect of the expression of SET-NUP214 and DEK-NUP214 on the NF- κ B-mediated transcription was specific. I next evaluated the effect of SET-NUP214 and DEK-NUP214 on the transcription of

endogenous NF- κ B target genes A20 and I κ B α by RT-qPCR. Consistent with the reporter assays above, *SET-NUP214* and *DEK-NUP214* diminished the mRNA amounts of A20 and I κ B α in a dose-dependent manner (Figure 2.6B). Collectively, these results demonstrate that SET-NUP214 and DEK-NUP214 impair the NF- κ B transcription activity and this impairment occurs when the NF- κ B signaling pathway is activated.

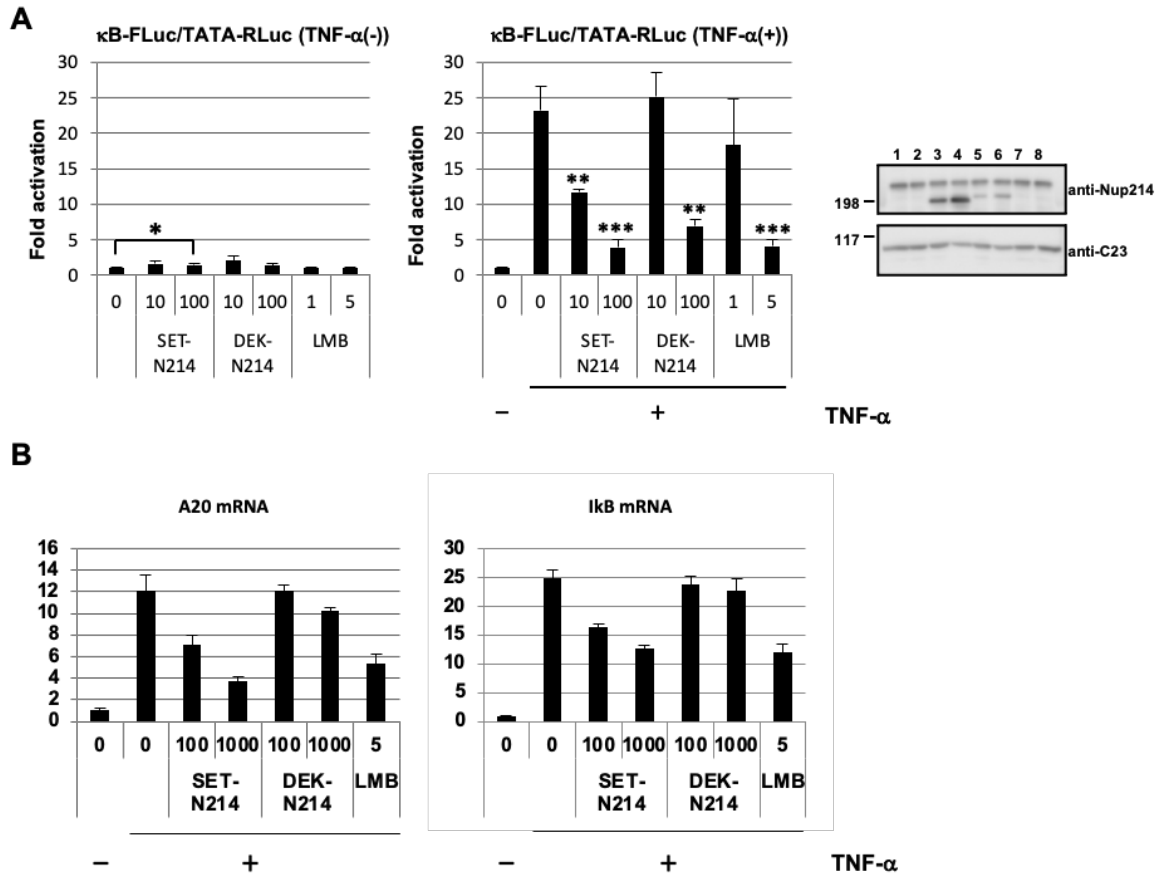


Figure 2.6. Effects of SET-NUP214 and DEK-NUP214 on NF- κ B transcription activity.

(A) HEK293T cells (3×10^4) cultured in 24-well plates were transfected with pNF- κ B40-firefly luciferase (10 ng), and pCAGGS-SET-NUP214 (SET-N214) or DEK-NUP214 (DEK-N214) (10, 100 ng). pTA-Renilla luciferase (100 ng) was co-transfected for normalization of transfection efficiency. At two days after transfection, cells were incubated with 1 ng/ml (lane 6 in left graph and lane 7 in right graph) or 5 ng/ml LMB (lane 7 in left graph and lane 8 in right graph) for 30 min. Then, recombinant human TNF- α (300-01A, PeproTech) was added, at the final concentration as 20 ng/ml (lanes 2-8 in right graph), incubated for 3-4 h, and cell lysates were subjected to luciferase assays using Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions. Luminescence was measured by CentroXS³ LB960 (Berthold Japan K.K.). Normalized luciferase activity (A) were expressed as fold activation relative to lane 1. Data are presented as the mean \pm SD of three independent experiments. *P* value was calculated with lane 1 (left graph) or lane 2 (right graph). **P* < 0.05, ***P* < 0.005, ****P* < 0.001. Western blot analyses were performed using lysate prepared for luciferase assays in the presence of TNF- α . Anti-NUP214 and anti-C23 antibodies were used as primary antibodies. Prestained molecular weight markers (kDa) are indicated in the left. (B) HEK293T cells (3×10^5) cultured in 6-well plates were transfected with pCAGGS-SET-NUP214 (SET-N214) or DEK-NUP214 (DEK-N214) (100, 1000 ng). At two days after transfection, cells were incubated with 5 ng/ml LMB (lane 7) for 30 min, and TNF- α was added at the final concentration as 20 ng/ml (lanes 2-7). After TNF- α incubation for 3-4 h, cells were collected, and isolated RNAs were subjected to RT-qPCR to measure A20 and I κ B α mRNAs. These mRNA expression levels were normalized to the level of β -actin

mRNA. Data are presented as the mean \pm SD of three independent experiments. *P* value was calculated with lane 2. **P* < 0.05, ***P* < 0.005, ****P* < 0.001.

2.4.7 NUP214 fusions induce nuclear accumulation of the p65-I κ B α complex

In unstimulated cells, the majority of NF- κ B transcription factors such as p65 and p50 interact with I κ B. As p65 and p50 have NLS, and I κ B α has NLS and NES, the NF- κ B-I κ B α complex shuttles between the nucleus and the cytoplasm in an XPO1-dependent manner and is mainly observed in the cytoplasm [73, 74, 76] [34, 75, 77, 78]. In cells expressing SET-NUP214 and DEK-NUP214, the NF- κ B transcription activity remained inactive (Figure 2.6A, left graph), despite p65 being located in the nucleus (Figure 2.3A). As I κ B α is also localized in the nucleus, I hypothesized that the interaction between p65 and I κ B α was maintained in the nucleus, and thus NF- κ B was kept inactive. To test this, we performed *in situ* proximity ligation assays (PLA) and IP assays (Figures 2.7A and B). In control cells, p65 and I κ B α were observed in the cytoplasm, and cytoplasmic PLA signals were detected, indicating the proximity of p65 and I κ B α in the cytoplasm. When cells were transfected with *SET-NUP214* or *DEK-NUP214*, both p65 and I κ B α were found in the nucleus (Figure 2.3A), and nuclear PLA signals were observed in these cells (Figure 2.7A). By IP assays, p65 was found to interact with I κ B α , and this interaction was not affected by the absence or presence of SET-NUP214 and DEK-NUP214 (Figure 2.7B). These results suggest that SET-NUP214 and DEK-NUP214 induce nuclear accumulation of the p65-I κ B α complex, but that the NF- κ B signaling pathway was kept inactive, as the fusion proteins did not affect the interaction between p65 and I κ B α . Binding of I κ B α to p65 causes a release of p65 from DNA [79]. Therefore, it was presumed that nuclear p65 bound by I κ B α in the presence of SET-NUP214 and DEK-NUP214 could not bind to the target gene promoter. To confirm this notion, I performed chromatin immunoprecipitation assays. The level of p65 that bound to A20 and I κ B α promoter regions was increased by TNF- α treatment. However, p65 binding to these promoters was not enhanced by SET-NUP214 and DEK-NUP214 (Figure 2.7C), although p65 was localized in the nucleus (Figure 2.3A). These results support our notion that p65 was kept inactive in cells expressing SET-NUP214 and DEK-NUP214.

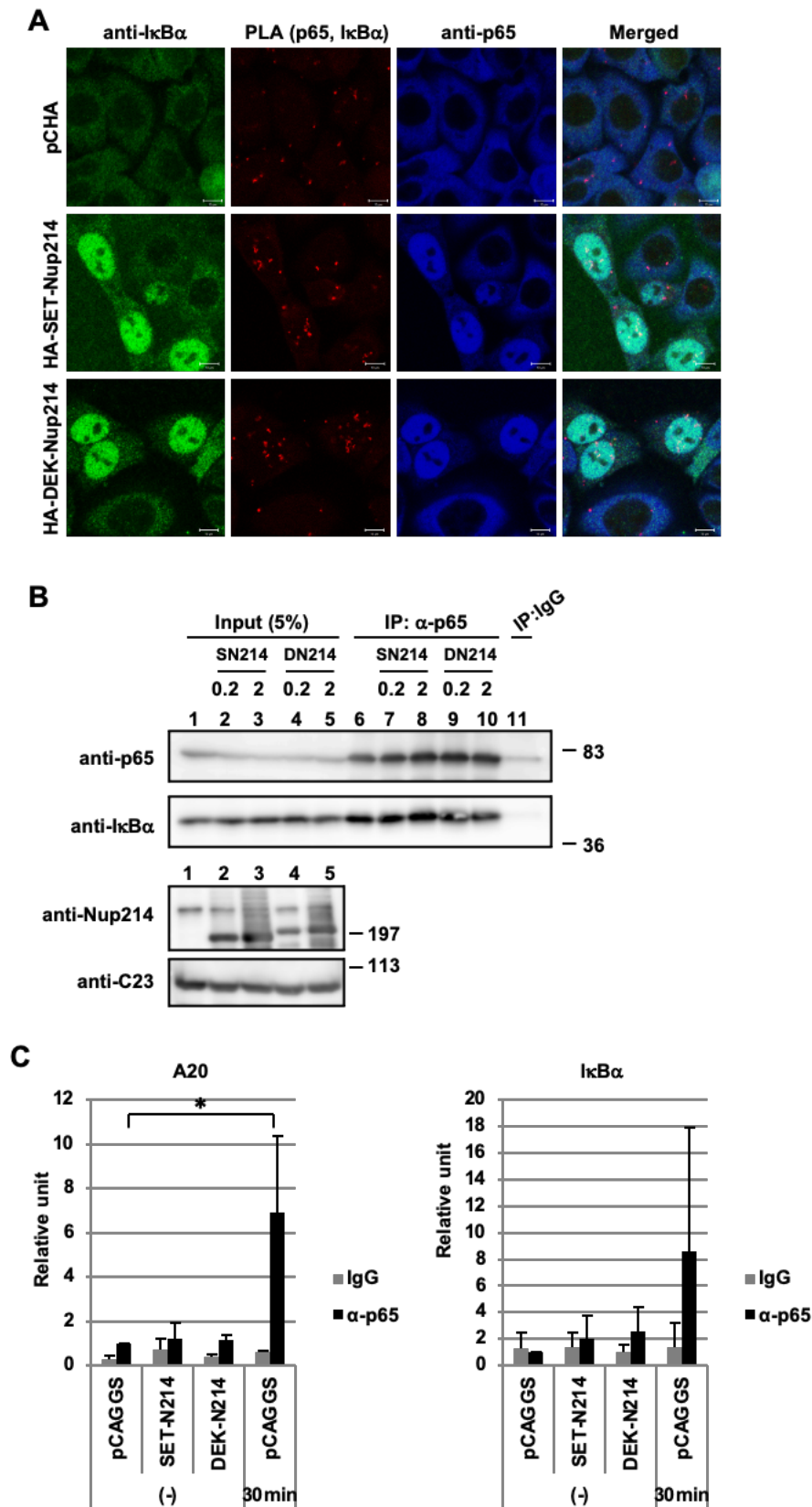


Figure 2.7. Interaction of p65 with I κ B α or chromatin in the nucleus.

(A) HeLa cells cultured in 6-cm dishes were transfected with 2 μ g pCHA, HA-SET-NUP214 (SET-N214), or HA-DEK-NUP214 (DEK-N214). At two days after transfection, cells were collected, and subjected to IF assays and PLA. Anti-p65 (ab7970, Abcam) (1:100) and anti-I κ B α (L35A5, CST, Inc.) (1:30) antibodies were used for primary antibodies. Merged is a composite picture stained with Alexa 488, Detection Reagents

Red (for PLA), and Alexa 633. Bar: 10 μ m. (B) HEK293T cells were transfected with pCAGGS, pCAGGS-SET-NUP214 (SN214), pCAGGS-DEK-NUP214 (DN214) (0.2, 2 μ g), and incubated for 2 days. Cells were collected, and IP assays were conducted using anti-p65 (ab7970) and rabbit IgG polyclonal antibodies (PP64B) (Merck KGaA, Germany). Proteins in input lysates and immunoprecipitated samples were separated by 10% or 5% SDS-PAGE, and western blot analyses were performed using anti-p65, anti-I κ B α , anti-NUP214, and anti-C23 antibodies. Molecular weights (kDa) of prestained markers are indicated in the right. (C) HEK293T cells were transfected with 5 μ g pCAGGS, pCAGGS-SET-NUP214 (SET-N214), or pCAGGS-DEK-NUP214 (DEK-N214). At two days after transfection, cells were treated with or without TNF- α (20 ng/ml) for 30 min, and then subjected to ChIP assays using 2 μ g anti-IgG or anti-p65 (ab7970) antibodies to measure p65 binding to A20 and I κ B α promoter regions. The immunoprecipitated DNA levels were normalized to the input DNA level and shown as fold activation relative to immunoprecipitated DNA from pCAGGS-transfected lysates by anti-p65 antibody in the absence of TNF α . Data are presented as the mean \pm SD of three independent experiments. * P < 0.05.

2.4.8 p65-I κ B α complex is inactive in the stimulated cells expressing NUP214 fusions

Reporter assays and RT-qPCR showed that SET-NUP214 and DEK-NUP214 down-regulate the NF- κ B transcription activity in the presence of TNF- α (Figure 2.6A, right graph). I κ B α is phosphorylated in the cytoplasm upon stimulation, followed by degradation by the ubiquitin proteasome system [80]. NF- κ B transcription factors are then freed from I κ B, localized in the nucleus, and execute target gene transcription. I predicted that NF- κ B-I κ B α complex is maintained by the expression of SET-NUP214 and DEK-NUP214 even after TNF- α addition. To test this, the localization pattern of I κ B α and p65 was monitored, and PLA and co-immunoprecipitation assays were performed. After addition of TNF- α , I κ B α in the cytoplasm of both control and SET-NUP214- and DEK-NUP214-expressing cells was markedly reduced (Figure 2.8A), indicating degradation of cytoplasmic I κ B α . On the contrary, I κ B α in the nucleus of SET-NUP214- and DEK-NUP214-expressing cells was visible after TNF- α treatment (Figure 2.8A). Restored expression level of I κ B α by SET-NUP214 and DEK-NUP214 was also confirmed by western blotting analysis (Figure 2.8C, lanes 1-6). In cells expressing nuclear p65 and I κ B α , PLA signals were detected both 0 and 30 minutes after TNF- α treatment, demonstrating that the interaction between p65 and I κ B α was maintained in the nucleus after stimulation (Figure 2.8B). The p65-I κ B α complex formation in stimulated cells expressing SET-NUP214 or DEK-NUP214 was confirmed by IP assay (Figure 2.8C, lanes 7-13). Finally, to examine whether the recruitment of p65 to its target genes was affected by SET-NUP214 or DEK-NUP214, ChIP assay was performed. It was demonstrated that p65 binding to the A20 and I κ B α promoter regions was impaired in the presence of SET-NUP214 or DEK-NUP214 (Figure 2.8D). These results indicate that nuclear-localized I κ B α induced by SET-NUP214 and DEK-NUP214 is escaped from its phosphorylation and degradation and keeps p65 inactive.

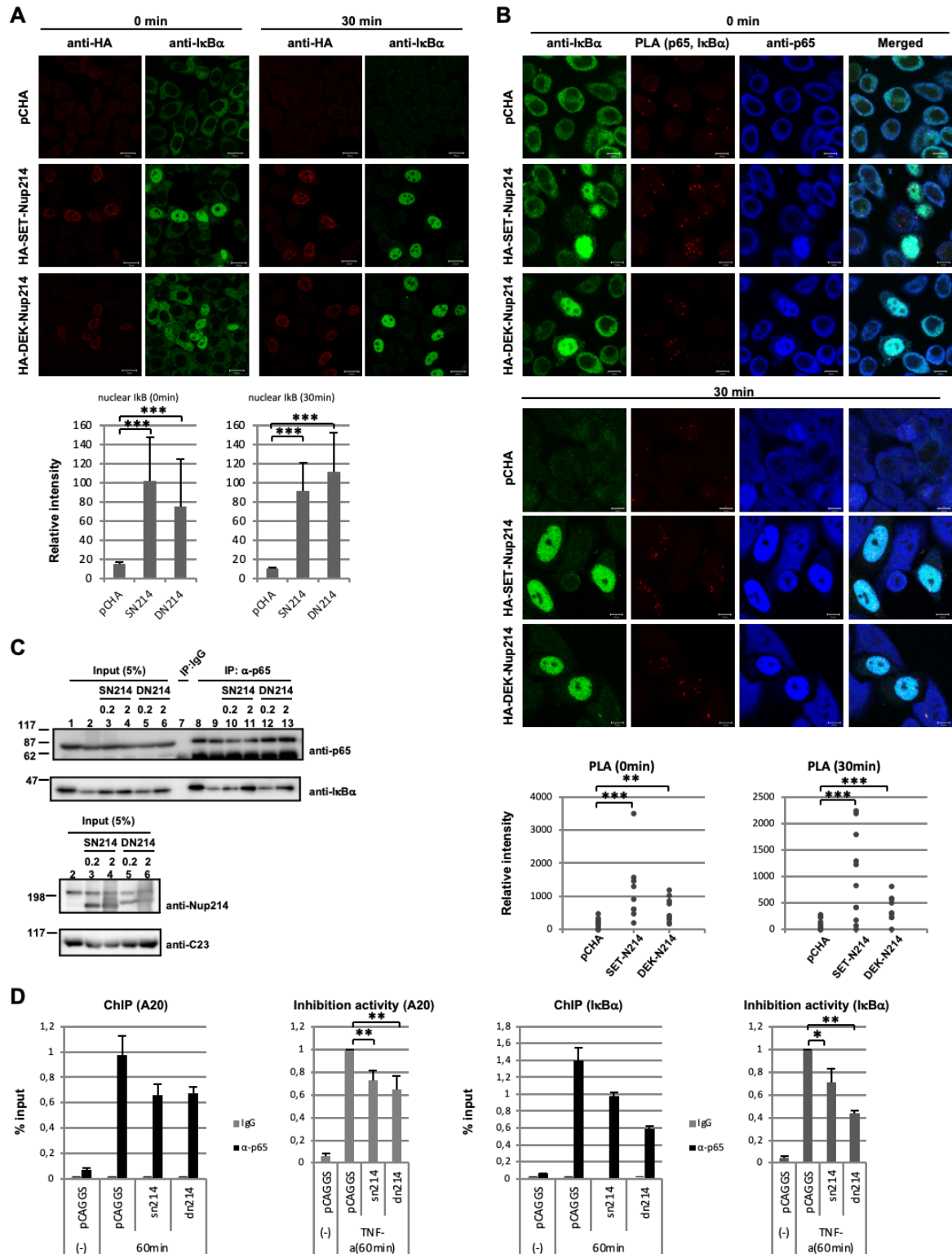


Figure 2.8. Interaction of p65 with IκBα or chromatin in the presence of stimuli.

(A) HeLa cells were transfected with 1 μg of pCHA, HA-SET-NUP214, or HA-DEK-NUP214. At two days after transfection, cells were treated with TNF-α (10 ng/ml) 30 min, and IF assays were performed using anti-HA rabbit and anti-IκBα (L35A5) antibodies. Fluorescence intensity of nuclear IκBα in control cells, SET-NUP214 expressing cells, and DEK-NUP214 expressing cells was quantitatively determined using ImageJ software. Bar: 20 μm. *** $P < 0.001$. (B) The protocol was the same as one for Figure 2.8A. After incubation with anti-p65 (ab7970) and anti-IκBα (L35A5), PLA were performed. Merged is a composite picture stained with Alexa 488, Detection Reagents Red (for PLA), and Alexa 633. Sum of fluorescence

intensity of PLA dots in each cell was quantitated using ImageJ software. Bar: 10 μ m. $**P < 0.005$, $***P < 0.001$. (C) HEK293T cells were transfected with 1 μ g pCAGGS, pCAGGS-SET-NUP214 (SN214), pCAGGS-DEK-NUP214 (DN214) (0.2, 2 μ g). At two days after transfection, cells were treated with TNF- α (20 ng/ml) 30 min, collected, and IP assays were conducted using anti-p65 (ab7970) and rabbit IgG polyclonal antibodies. Proteins in input lysates and immunoprecipitated samples were separated by 12.5% or 5% SDS-PAGE, and western blot analyses were performed using anti-p65, anti-I κ B α , anti-NUP214, and anti-C23 antibodies. Molecular weights (kDa) of prestained markers are indicated in the right. (D) HEK293T cells were transfected with 2 μ g pCAGGS, pCAGGS-SET-NUP214 (SET-N214), or pCAGGS-DEK-NUP214 (DEK-N214). At two days after transfection, cells were treated with or without TNF- α (20 ng/ml) for 60 min, and then subjected to ChIP assays same as Figure 2.7C. Left graph represents the typical example and the right graph represents fold inhibition relative to immunoprecipitated DNA from pCAGGS-transfected lysates by anti-p65 antibody in the presence of TNF- α . Right graphs are presented as the mean \pm SD of three independent experiments. $*P < 0.05$, $**P < 0.01$.

2.4.9 Subcellular localization of XPO1 and its cargos in *SET-NUP214* transgenic mice

Previously, we generated a transgenic mouse expressing *SET-NUP214* [22]. Although this mouse did not develop leukemia, it shows severe anemia and a halt in hematopoietic differentiation, both of which are frequently associated with leukemia. In order to understand the biological relevance of the results obtained from *in vitro* cell culture studies, I assessed the localization pattern of XPO1 and its cargos using spleen sections from *SET-NUP214* transgenic mice. I observed that SET-NUP214 and XPO1 are co-localized in the nucleus as granular dots (Figure 2.9A). In addition, it was found that I κ B α (Figure 2.9B) and p65 (Figure 2.9C) are also localized in the dots. These results demonstrate that the localization pattern of XPO1 is affected and its function could be impaired by SET-NUP214 *in vivo* as well.

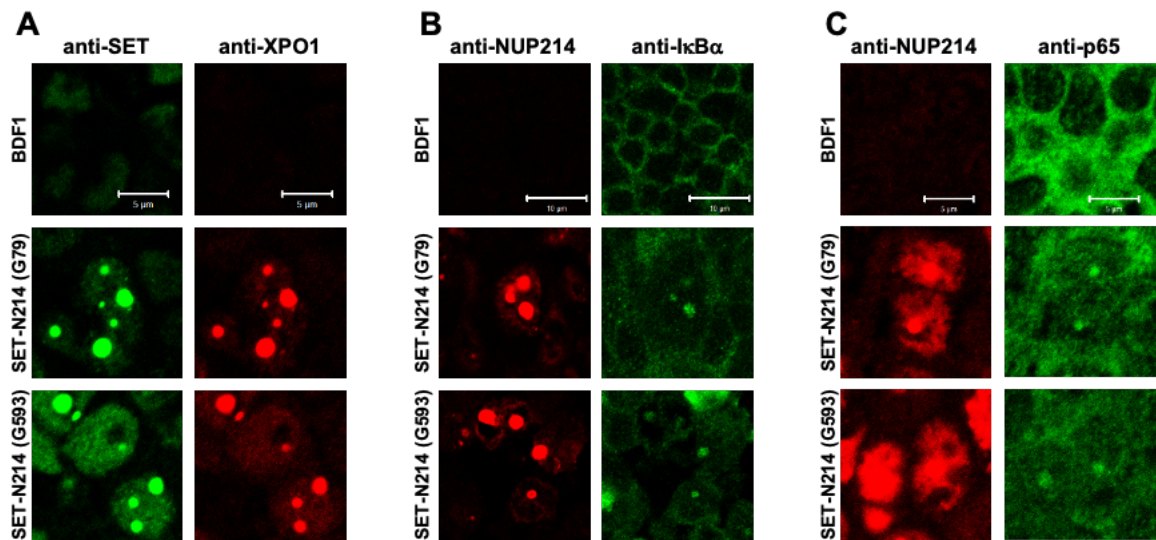


Figure 2.9. Subcellular localization of XPO1, I κ B α , and p65 in spleen of *SET-NUP214* mice.

Spleen sections of wild-type BDF1 and *SET-NUP214* transgenic mice (lines G79 and G593) were subjected to IF assays. Anti-SET/TAF-I β (KM1721) (1:20) (A), anti-XPO1 (1:100) (A), anti-NUP214 (1:100) (B, C), anti-I κ B α (L35A5) (1:20) (B), and anti-p65 (F6) (1:20) (C), antibodies were used as primary antibodies. Bar: 5 μ m (A, C), 10 μ m (B).

2.5 Discussion

2.5.1 Interaction of SET-NUP214 and DEK-NUP214 with XPO1 and NXF1

In this study, I examined the function of SET-NUP214 and DEK-NUP214 in terms of their effects on nuclear-cytoplasmic transport of proteins and RNAs. I found that among several NTRs, SET-NUP214 and DEK-NUP214 interact preferentially with not only XPO1 but also NXF1 (Figure 2.2B). These interactions were dependent on the FG repeat region of SET-NUP214 and DEK-NUP214 (Figure 2.2C). It is shown that each FG-NUP binds with different affinity to some of the NTRs, which contain multiple binding sites for FG-repeats [81, 82]. Hence, it is supposed that the affinities of the FG repeat region of SET-NUP214 and DEK-NUP214 to NTRs are also to be varied, and the affinity difference generates binding preference. NUP214 interacts with XPO1 rather than Xpo-t, NXF1, or XPO2/CAS [54, 83]. Among various NTRs, ectopic expression of truncated NUP214 containing FG repeat region has an inhibitory effect on subset of NTRs functions including XPO1 [64]. Our results are consistent with previous ones, and imply that the SET and DEK portions of the SET-NUP214 or DEK-NUP214 do not affect the structure and function of NUP214 portion for association with NTRs.

2.5.2 Effects of SET-NUP214 and DEK-NUP214 on the functions of XPO1 and NXF1

I have demonstrated that SET-NUP214 and DEK-NUP214 associate with both XPO1 and NXF1. However, the effect of NUP214-fusion proteins on XPO1 function was different from that on NXF1. We showed that SET-NUP214 and DEK-NUP214 cause accumulation of XPO1 cargos in the nucleus (Figure 2.3A and C). In contrast, mRNA, which is an NXF1 cargo, was not accumulated in the nucleus of cells expressing SET-NUP214 and DEK-NUP214 (Figure 2.3D). XPO1 was mainly incorporated in the dots where SET-NUP214 and DEK-NUP214 are located, whereas nuclear-diffused NXF1 was observed in cells expressing SET-NUP214 and DEK-NUP214 (Figures 2.2D and E). This differential localization of XPO1 and NXF1 in cells expressing SET-NUP214 and DEK-NUP214 could explain the different effects of SET-NUP214 and DEK-NUP214 on the XPO1 and NXF1 functions. It is assumed that SET-NUP214-XPO1 and DEK-NUP214-XPO1 complexes could be more stable than SET-NUP214-NXF1 and DEK-NUP214-NXF1 complex.

2.5.3 SET-NUP214 and DEK-NUP214 form stable complexes with XPO1

A question is raised as to how SET-NUP214 and DEK-NUP214 can form a stable complex with XPO1 to induce accumulation of NES proteins in the nucleus. The interaction between NUP214 and XPO1 is stabilized when both RanGTP and NES proteins are incorporated [63, 84, 85]. Consistent with this, we found that inhibition of the interaction between XPO1 and NES proteins by LMB leads to disappearance of dots formed in the presence of SET-NUP214 and DEK-NUP214

(data not shown). From these results, we speculate that the nuclear dots are formed by the quaternary stable complex containing SET-NUP214 or DEK-NUP214, NES proteins, XPO1, and RanGTP. Furthermore, it is possible that other proteins play roles in the dot formation. Complex formation of RanGTP-XPO1-NES proteins is enhanced by NUP98 [86] and RanBP3 [87] [88] [89]. NUP214 functions as a scaffold for the recruitment of several nucleoporins such as NUP88, NUP358, NUP62, and NUP98 [90] [63] [65, 91]. These proteins may facilitate stable complex formation induced by SET-NUP214 and DEK-NUP214. In spleen cells, these dots are much larger than those in cultured cells. It is likely that high expression of proteins constituting these dots in mouse spleen enlarges the SET-NUP214 nuclear dots.

2.5.4 Deregulation of transcription by SET-NUP214 and DEK-NUP214 and oncogenesis

We found that I κ B α is localized in the nucleus regardless of the presence or absence of TNF- α (Figure 2.8A) in cells expressing SET-NUP214 and DEK-NUP214. It is presumed that nuclear-accumulation of I κ B α is escaped from IKK β -mediated phosphorylation after TNF- α addition, and thus the NF- κ B transcription activity is repressed by the interaction with I κ B α in the nucleus. In general, NF- κ B induces transcription of various genes related to inflammation, cell proliferation, invasion, and so on. NF- κ B inactivation is known to counteract oncogenesis or tumorigenesis and NF- κ B is an efficient therapeutic target for cancer [92] [93]. In contrast, NF- κ B has also been reported to have anti-oncogenic activities, such as induction of cellular senescence. p53^{-/-} MEFs bypass senescence [94], and p53-downregulated mouse lymphoma become chemoresistance by escape of senescence [95]. SET-NUP214 and DEK-NUP214 impair the NF- κ B pathway (Figure 2.6). On this line, it is speculated that SET-NUP214 and DEK-NUP214 may promote to bypass senescence via suppression of the NF- κ B signaling pathway. In addition, several studies have documented the importance of the NF- κ B pathway for hematopoiesis. Conditional knockout of IKK β gene upregulates IL-1 production and stimulates proliferation of neutrophil progenitor, leading to neutrophilia and splenomegaly [96] [97]. In addition, it was reported that conditional knockout of p53 or IKK β induces cell cycling of hematopoietic stem cells and increases their number [98] [99]. These observations suggest that inhibition of the NF- κ B pathway is one potential cause of the SET-NUP214-induced differentiation block of hematopoietic progenitor cells observed in SET-NUP214 transgenic mice. Furthermore, it was previously demonstrated that the differentiation of U937 was inhibited by the expression of SET-NUP214 [48]. Similarly, the differentiation of U937 was inhibited by the presence of IKK β inhibitor [100] [101]. These results also suggest that the inhibition of the NF- κ B pathway by SET-NUP214 is, at least in part, a potential cause of U937 differentiation block induced by SET-NUP214.

In conclusion, SET-NUP214 and DEK-NUP214 interact with NTRs, and the interaction of either SET-NUP214 or DEK-NUP214 with XPO1 leads to a malfunction of transcription regulation by NF- κ B. Until now, many proteins have been identified as XPO1 cargos [102] [103]. In addition to I κ B α and cyclin B1, these various cargos might accumulate in the nucleus of the case in which either SET-NUP214 or DEK-NUP214 is present. In fact, β -catenin was reported to accumulate in the nucleus of *SET-NUP214* transgenic mouse [47]. Because appropriate nuclear-cytoplasmic transport is required to cellular integrity, these localization disturbances of various proteins may synergistically lead to onogenesis by SET-NUP214 and DEK-NUP214. To know which NES proteins are responsible for SET-NUP214- and DEK-NUP214-mediated onogenesis, comprehensive post-genome type analyses are required.

Chapter 4 Further Discussions, Conclusions and Perspectives

4.1 NUP214 fusion proteins disturb nuclear export and transcription

NUP214 interacts with several NTRs through its FG-repeat region. DEK-NUP214 and SET-NUP214 bear FG-repeats region located in the NUP214 moiety. To verify whether DEK-NUP214 and SET-NUP214 bind to NTRs, immunoprecipitation assays were first performed. Among several NTRs, XPO1 and NXF1 were found to bind to both DEK-NUP214 and SET-NUP214. XPO1 and NXF1 are NTRs of proteins containing leucine-rich nuclear export signals (NES) and mRNA, respectively. Because DEK-NUP214 and SET-NUP214 are present in granular dots in the nucleus, it was hypothesized that the NTRs associated with the fusion proteins were abnormally accumulated to the granular dots containing the fusion proteins. Consistent with this hypothesis, I found that XPO1 and NXF1 were accumulated to the granular dots where DEK-NUP214 or SET-NUP214 are accumulated. Consequently, cells expressing DEK-NUP214 or SET-NUP214 showed mislocalization of the endogenous NES proteins such as I κ B (Inhibitor of NF κ B). In control cells, the NES proteins were efficiently exported to the cytoplasm by XPO1, whereas they were accumulated in the nucleus upon the expression of DEK-NUP214 or SET-NUP214. I further examined whether DEK-NUP214 and SET-NUP214 affect the NF- κ B transcription activity because the activity of NF- κ B is regulated by nuclear-cytoplasmic transport. In control cells, the p65 subunit of NF- κ B and I κ B are imported to the nucleus via the nuclear localization signal (NLS) of both proteins but efficiently exported by the NES of I κ B, and therefore the NF- κ B-I κ B complex is mainly detected in the cytoplasm. Upon various stimulation including an inflammatory cytokine, TNF α , I κ B is phosphorylated and degraded by the ubiquitin-proteasome system, and the released NF- κ B is accumulated to the nucleus. Interestingly, I found that when DEK-NUP214 or SET-NUP214 was expressed, both p65 and I κ B were found in the nucleus, and the NF- κ B-I κ B complex was maintained in the presence or absence of TNF α . These results suggest that nuclear-localized I κ B induced by DEK-NUP214 and SET-NUP214 via XPO1 inhibition is escaped from its phosphorylation and degradation and keeps the NF- κ B signaling pathway inactive. A supposed model is shown below.

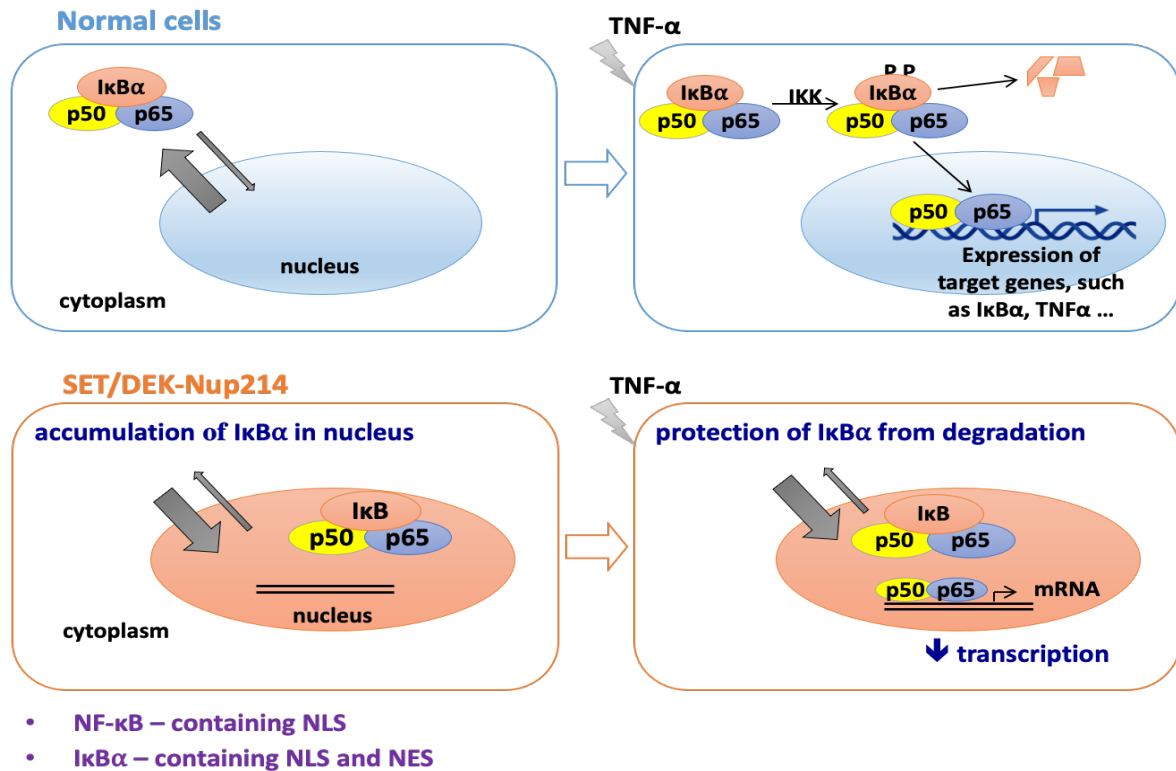


Figure 4.1. A model for downregulation of NF-κB pathway caused by NUP214 fusion proteins.

4.2 SET-NUP214 and MLL cooperatively enhance *HOXA10* gene promoter activity

HOX (homeobox) genes, which encode HOX proteins, are distributed as clusters (*HOXA* to *HOXD*) on four different chromosomes. HOX proteins are DNA-binding proteins with low sequence specificity. They regulate the downstream gene expression program during hematopoietic differentiation through complex formation with cofactors such as MEIS1 to confer sequence specificity and selectivity. Despite a variety of genetic abnormalities, gene expression profiles showed that expression of *HOXA* cluster genes is often increased in acute leukemia. Therefore, *HOX* gene dysregulation is a dominant mechanism of leukemogenesis. SET-NUP214 has been found to bind to the *HOXA* locus and activate its expression. It is currently not well understood, however, how SET-NUP214 is specifically recruited to the *HOXA* clusters and how the expression of the *HOXA* gene is regulated.

In this section, I focused on the function of MLL in SET-NUP214 mediated *HOX* gene expression, because MLL was previously reported to enhance the HOX genes, and it was also demonstrated to bind to SET. I have demonstrated by immunoprecipitation assays that SET-NUP214 interacts with MLL via the SET acidic region of SET-NUP214. To gain insight into the biological significance of the interactions between MLL and SET-NUP214, the effect of these proteins on *HOX* gene expression was examined. SET-NUP214 and MLL cooperatively enhanced the promoter activity of the *HOXA10* gene. Neither the SET region alone nor the NUP214 region alone sufficiently

enhanced the *HOXA10* gene promoter. These results will contribute to understanding the molecular mechanism by which *HOX* genes are abnormally expressed in leukemia.

4.3 Putative model of gene regulation by NUP214 fusion proteins

The distinct functions of SET and DEK (as parts of NUP214 fusions) in transcription regulation and chromatin remodeling may drive malignant hematopoietic transformation towards ALL or AML. Respective interacting proteins of SET and DEK might give some clues about different leukemogenic driver mechanisms and may contribute to the different leukemia outcomes. On the other hand, shared functions of NUP214 fusion proteins seem to be related to NUP214 portion of the fusion proteins.

To this end, I demonstrated that leukemia-associated NUP214 fusion proteins interact with some crucial gene regulators and cause deregulation of gene expression either through a direct epigenetic pathway distinct to only SET-NUP214 or an indirect nuclear-cytoplasmic transport defect pathway common to both NUP214 fusion proteins (Figure 4.2).

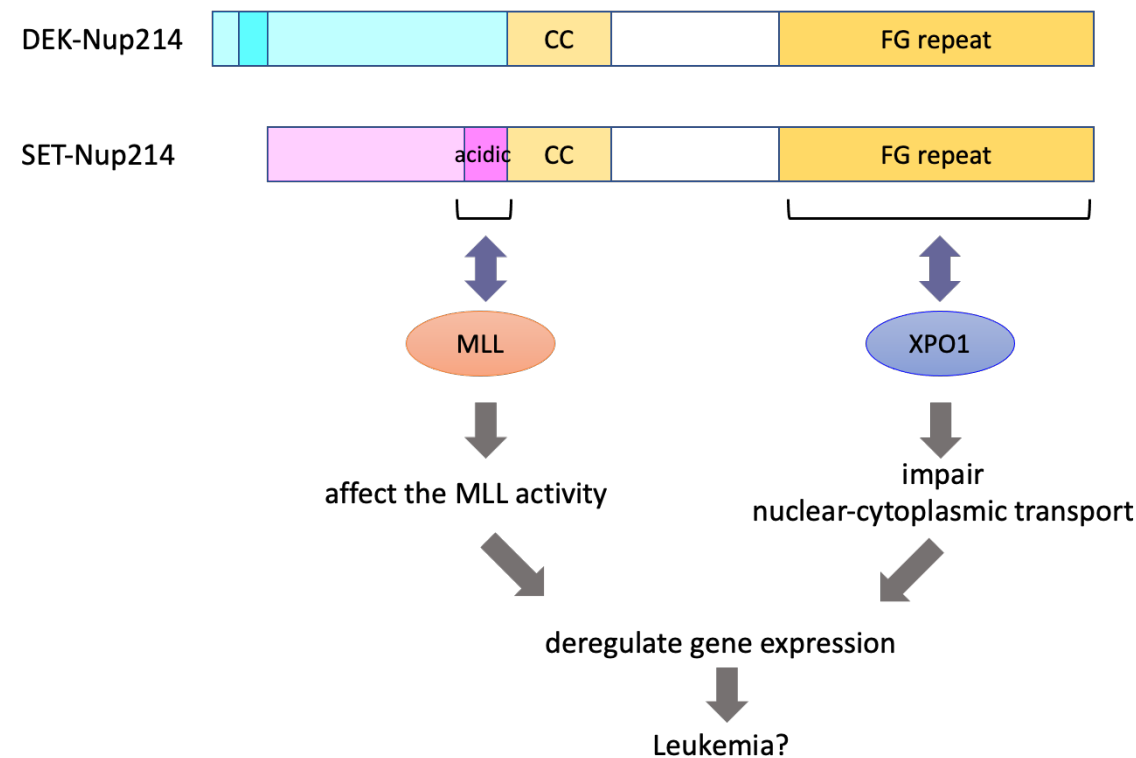


Figure 4.2. Leukemogenic mechanisms of NUP214 fusions through interacting proteins.

4.4 Limitations and perspectives

To reveal the oncogenic mechanism of NUP214 fusion proteins through gene regulation, it is critical to express NUP214 fusion proteins in hematopoietic cells with the actual cell stage representing the leukemia-initiating cells. However, it is difficult to establish this system *in vitro* cell culture. The transgenic mice generated with the emerging technologies and precisely mimicking leukemia-associated NUP214 fusion genes loci may reveal critical gene regulators related to leukemogenesis if the bone marrow cells are analyzed by new technologies such as single cell sequencing. Furthermore, comprehensive interactome analysis for NUP214 fusion proteins could reveal additional networking proteins, which may complete the missing pieces of the puzzle.

Mislocated activity of gene regulators appears to be a prominent driver of leukemogenesis. Inhibiting the interaction of NUP214 fusion proteins with gene regulators may provide a pharmacologic basis for therapeutic intervention in leukemia. Small molecule inhibitors of XPO1, MLL, DOTL1 or other interacting proteins might be bona fide candidates as clinical approaches.

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