

Functions of leukemia-associated NUP214-fusion proteins

(白血病関連 NUP214 融合タンパク質の機能)

2022

Sadık Çiğdem

北里大学

博士（薬科学）学位論文

Functions of leukemia-associated NUP214-fusion proteins

(白血病関連 NUP214 融合タンパク質の機能)

2022

北里大学薬学研究科

Sadık Çiğdem

Contents

Contents	i
Figures	iii
Tables	iv
Abbreviations	v
Chapter 1 Introduction	1
1.1 <i>Cancer</i>	1
1.2 <i>Leukemia</i>	1
1.3 <i>Hematopoiesis and Leukemogenesis</i>	1
1.4 <i>Gene Fusions in Leukemia</i>	2
1.5 <i>NUP214 fusion proteins</i>	3
1.6 <i>Nuclear pore complex and NUP214</i>	4
1.7 <i>Nuclear-cytoplasmic transport</i>	5
1.8 <i>Homeobox genes and their regulation</i>	6
1.9 <i>The aim of this study</i>	7
Chapter 2 Leukemia-associated NUP214-fusion proteins disturb XPO1-mediated nuclear-cytoplasmic transport pathway and-thereby NF-κB signaling pathway	8
2.1 <i>Abstract</i>	8
2.2 <i>Introduction</i>	8
2.3 <i>Materials and Methods</i>	10
2.3.1 Cell culture and transfection	10
2.3.2 Plasmids	10
2.3.3 IP assay and WB analysis	11
2.3.4 IF assay, oligo dT-FISH, and PLA	11
2.3.5 RNA extraction and RT-qPCR	11
2.3.6 ChIP assay	12
2.4 <i>Results</i>	12
2.4.1 SET-NUP214 and DEK-NUP214 interact with nuclear transport receptors	12
2.4.2 SET-NUP214 and DEK-NUP214 affect subcellular localization of NTRs	14
2.4.3 NUP214 fusions affect subcellular localization of endogenous proteins harboring NES.....	14
2.4.4 XPO1 overexpression rescue nuclear accumulated I κ B α induced by NUP214 fusions.....	15
2.4.5 SET-NUP214 has a small effect on poly A mRNA localization	18
2.4.6 SET-NUP214 and DEK-NUP214 affect NF- κ B transcription activity	18
2.4.7 NUP214 fusions induce nuclear accumulation of the p65-I κ B α complex.....	20
2.4.8 p65-I κ B α complex is inactive in the stimulated cells expressing NUP214 fusions	22
2.4.9 Subcellular localization of XPO1 and its cargos in <i>SET-NUP214</i> transgenic mice	24
2.5 <i>Discussion</i>	25
2.5.1 Interaction of SET-NUP214 and DEK-NUP214 with XPO1 and NXF1	25
2.5.2 Effects of SET-NUP214 and DEK-NUP214 on the functions of XPO1 and NXF1	25
2.5.3 SET-NUP214 and DEK-NUP214 form stable complexes with XPO1	25
2.5.4 Deregulation of transcription by SET-NUP214 and DEK-NUP214 and oncogenesis	26

Chapter 3	SET-NUP214 and MLL cooperatively regulate the promoter activity of the HOXA10 gene .	28
3.1	<i>Abstract</i>	28
3.2	<i>Introduction</i>	28
3.3	<i>Materials and Methods</i>	29
3.3.1	Cell culture	29
3.3.2	Plasmids	29
3.3.3	Immunoprecipitation assay and western blot analysis	30
3.3.4	Luciferase assay and immunofluorescence analyses	30
3.4	<i>Results</i>	31
3.4.1	MLL is attracted to the SET-NUP214-granular dots by interactions with SET-NUP214	31
3.4.2	SET-NUP214 and MLL enhance the promoter activity of <i>HOX</i> genes	32
3.5	<i>Discussion</i>	37
Chapter 4	Further Discussions, Conclusions and Perspectives	39
4.1	<i>NUP214 fusion proteins disturb nuclear export and transcription</i>	39
4.2	<i>SET-NUP214 and MLL cooperatively enhance HOXA10 gene promoter activity</i>	40
4.3	<i>Putative model of gene regulation by NUP214 fusion proteins</i>	41
4.4	<i>Limitations and perspectives</i>	42
References		43
Acknowledgements		50

Figures

Figure 1.1. Normal and malignant hematopoiesis.....	2
Figure 1.2. Chromosomal rearrangements and functional consequences of gene fusions [9]	2
Figure 1.3. NUP214 fusion proteins and their normal counterparts.	4
Figure 1.4. NPC and NUP214, modified from [25].	5
Figure 1.5. Nuclear-cytoplasmic transport, modified from [27].	6
Figure 1.6. HOX genes and their regulation.	7
Figure 2.1. Effect of SET-NUP214 on nuclear transport of NES proteins.	9
Figure 2.2. Interaction between NUP214 fusion proteins and NTRs.....	13
Figure 2.3. Localization of endogenous proteins harboring NES and mRNA.....	16
Figure 2.4. Subcellular localization of I κ B α (rescue experiment by XPO1 over-expression).....	17
Figure 2.5. Subcellular localization of NF- κ Bp65 (rescue experiment by XPO1 over-expression).17	
Figure 2.6. Effects of SET-NUP214 and DEK-NUP214 on NF- κ B transcription activity.....	19
Figure 2.7. Interaction of p65 with I κ B α or chromatin in the nucleus.	21
Figure 2.8. Interaction of p65 with I κ B α or chromatin in the presence of stimuli.	23
Figure 2.9. Subcellular localization of XPO1, I κ B α , and p65 in spleen of <i>SET-NUP214</i> mice.	24
Figure 3.1. Interaction and colocalization of SET-NUP214 and MLL.	32
Figure 3.2. SET-NUP214 and MLL enhance the promoter activity of <i>HOX</i> genes.....	34
Figure 3.3. SET-NUP214 interacts with the N-terminal region of MLL through acidic region.	36
Figure 4.1. A model for downregulation of NF- κ B pathway caused by NUP214 fusion proteins. ..	40
Figure 4.2. Leukemogenic mechanisms of NUP214 fusions through interacting proteins.....	41

Tables

Table 1.1. Recurrent fusion genes in acute leukemia [10, 11] 3

Table 3.1. Oligonucleotides used in this study.....30



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).

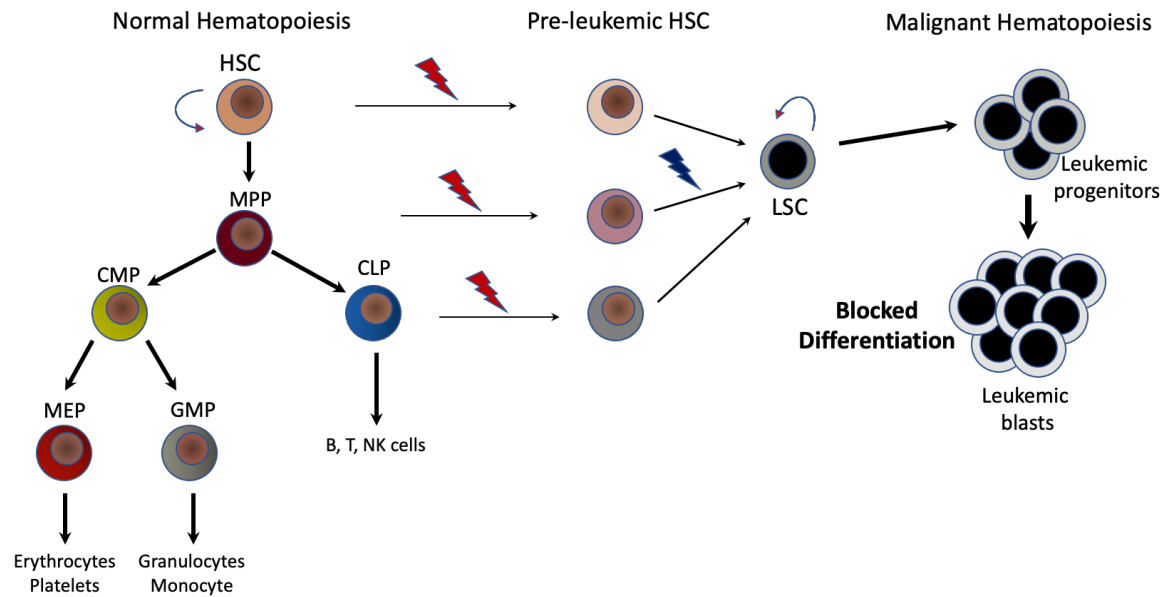
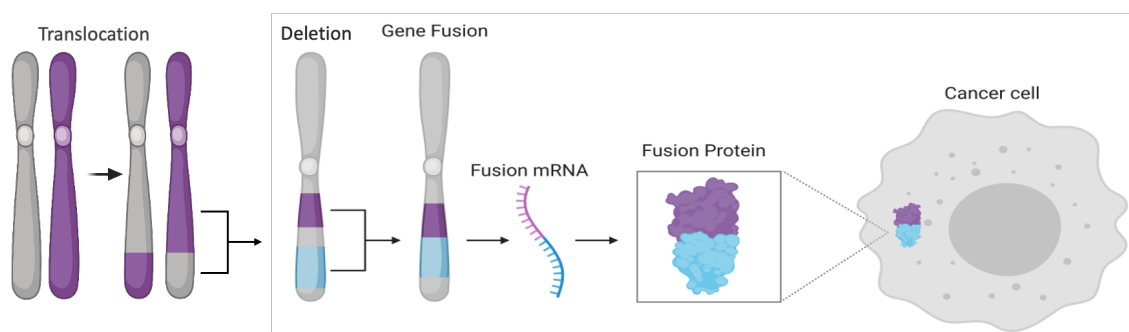


Figure 1.1. Normal and malignant hematopoiesis.

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

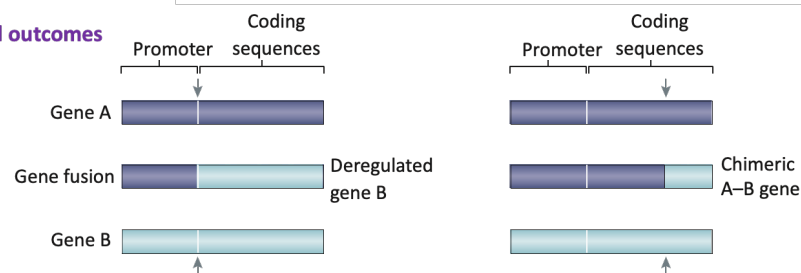


Figure 1.2. Chromosomal rearrangements and functional consequences of gene fusions [9]

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).

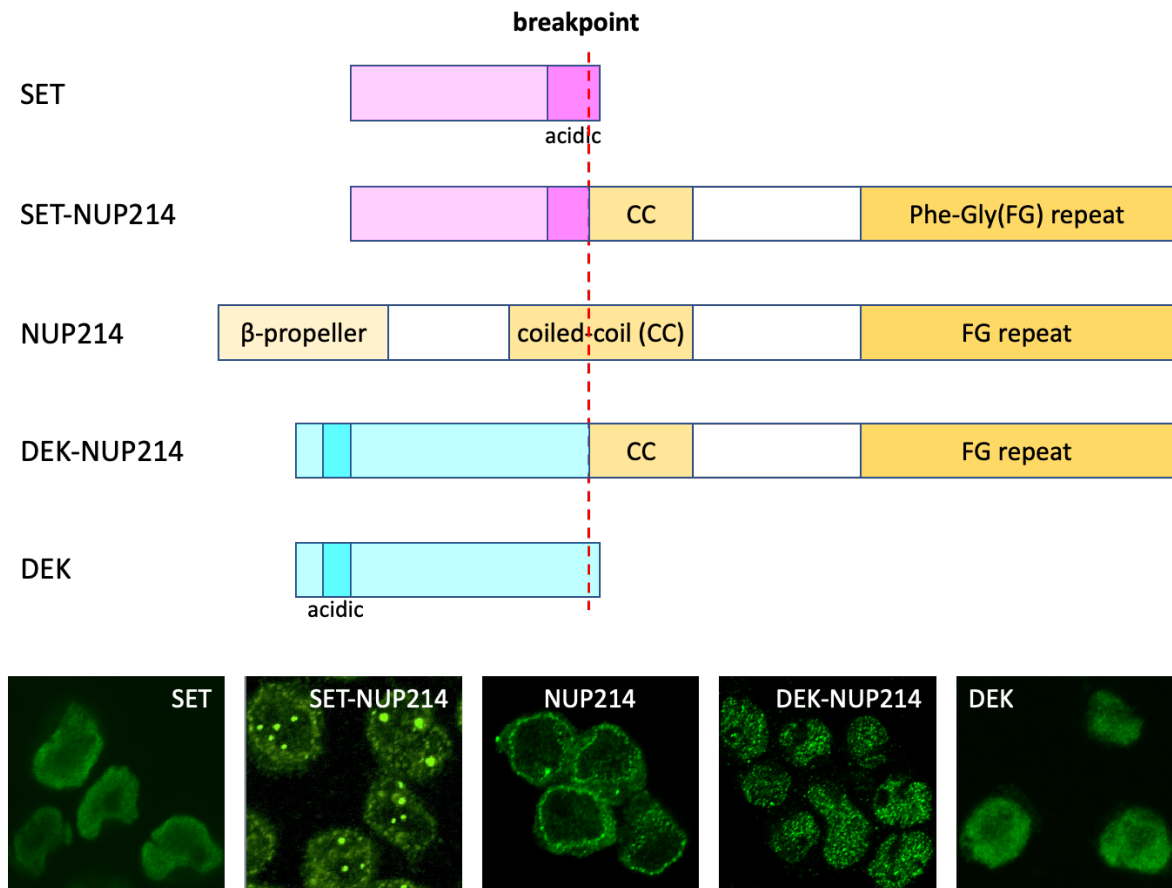


Figure 1.3. NUP214 fusion proteins and their normal counterparts.

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).

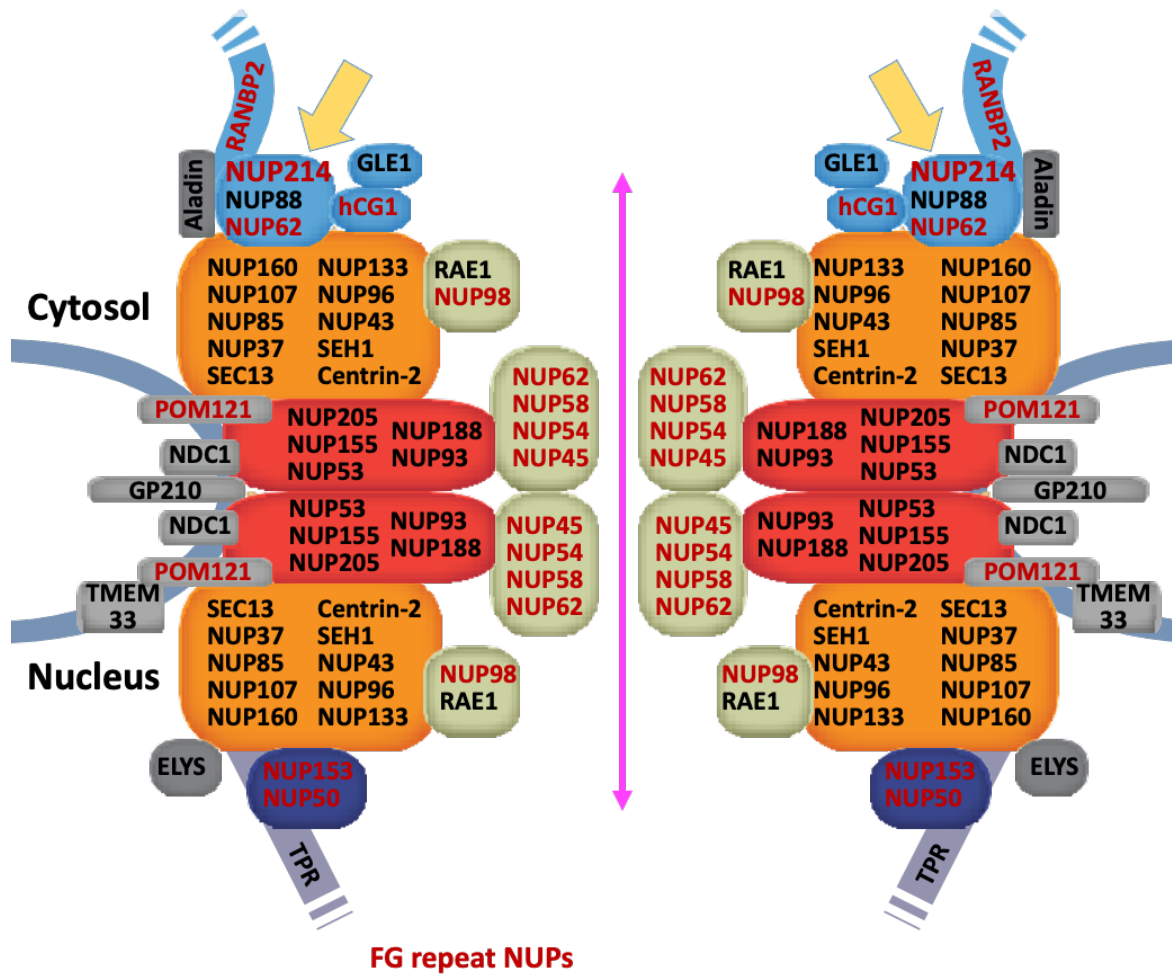


Figure 1.4. NPC and NUP214, modified from [25].

1.7 Nuclear-cytoplasmic transport

Nuclear transport occurs through nuclear pore complexes, the sole gates through which macromolecules are exchanged between cytosol and nucleus. Cargos with nuclear localization signal (NLS) are recognized and imported into the nucleus via importins, the nuclear import receptors. In the nucleus, they are dissociated by RanGTP. Cargos with nuclear export signal (NES) are recognized and exported from the nucleus via exportins, the nuclear export receptors. The hydrolysis of RanGTP to RanGDP results in dissociation of cargo and exportin in the cytoplasm [26]. Therefore, directionality and energetics of the nuclear transport is Ran dependent (Figure 1.5). The study of the NPC, nucleoporins, and the nucleocytoplasmic transport has revealed an unexpected layer to pathogenic disease onset and progression. Recent advances in the study of the regulation of NPC composition and function suggest that the precise control of this structure is necessary to prevent diseases from arising or progressing.

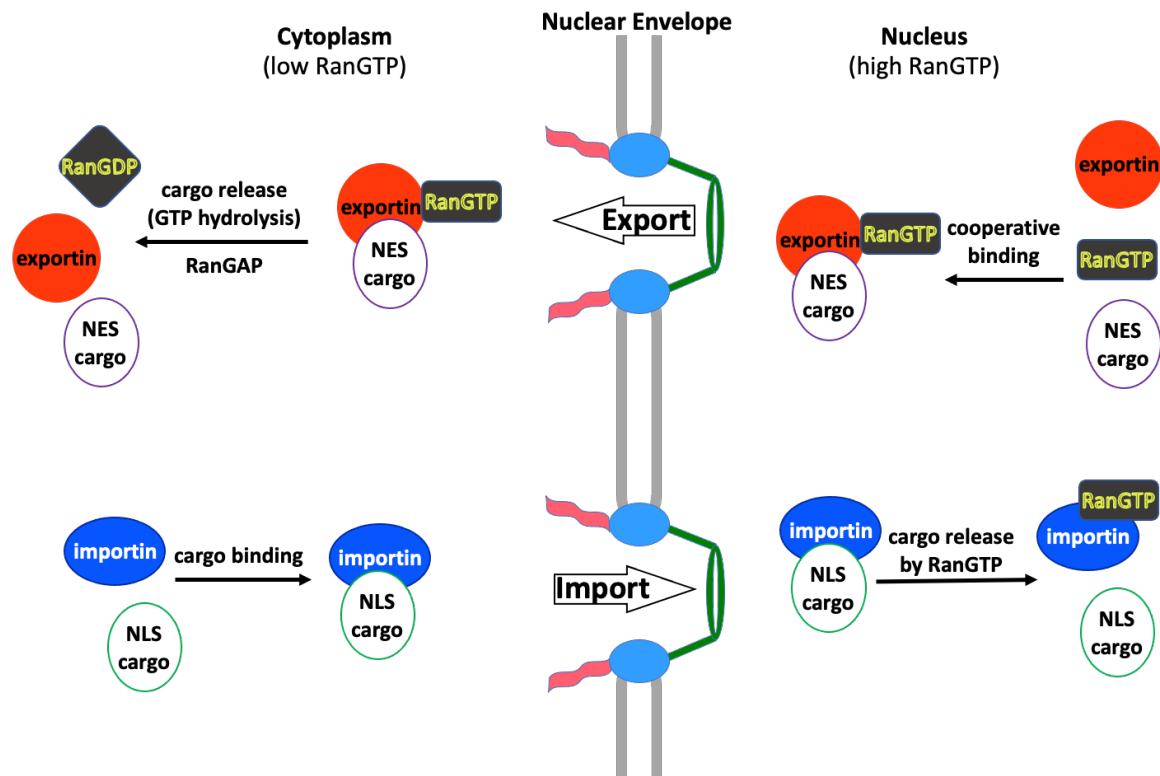
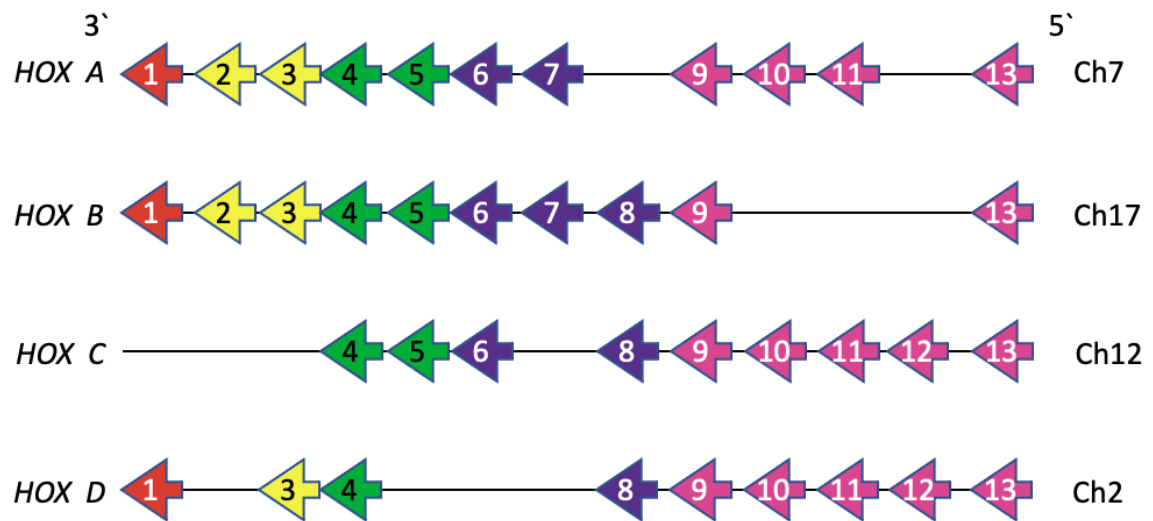


Figure 1.5. Nuclear-cytoplasmic transport, modified from [27].

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

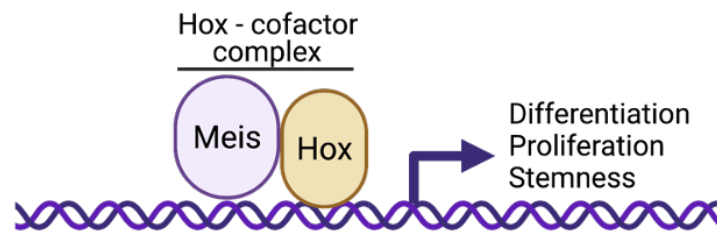


Figure 1.6. HOX genes and their regulation.

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- κ B 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- κ B 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- κ B-mediated transcription by abnormal tethering of the complex containing p65 and its inhibitor, I κ B, 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- κ B) 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.

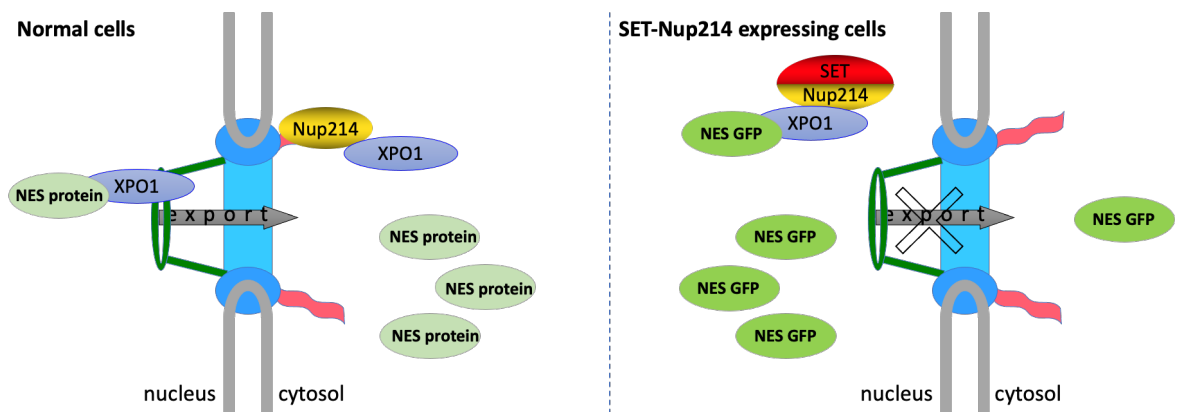


Figure 2.1. Effect of *SET-NUP214* on nuclear transport of NES proteins.

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).

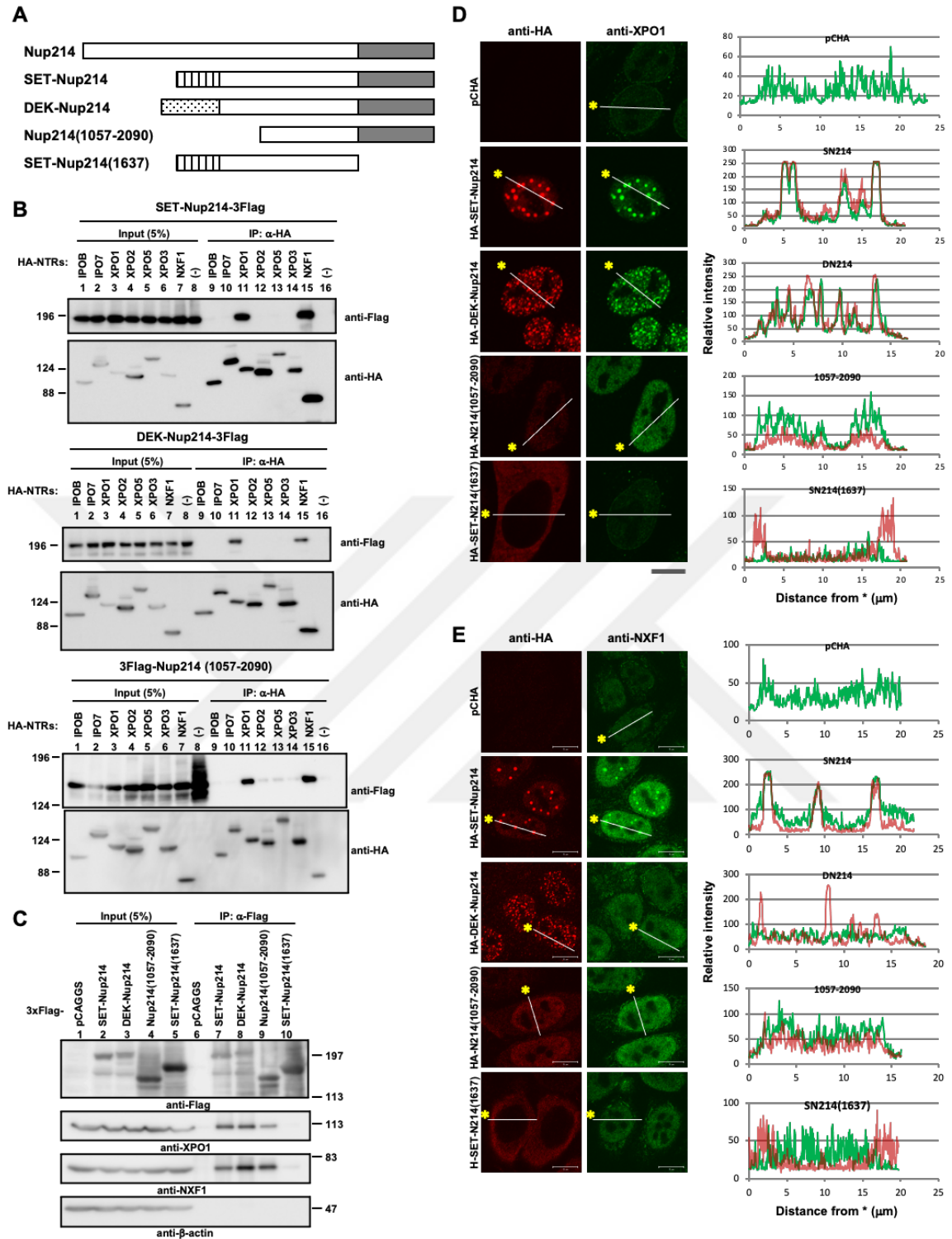


Figure 2.2. Interaction between NUP214 fusion proteins and NTRs.

(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 μ g 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 μ m.

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.

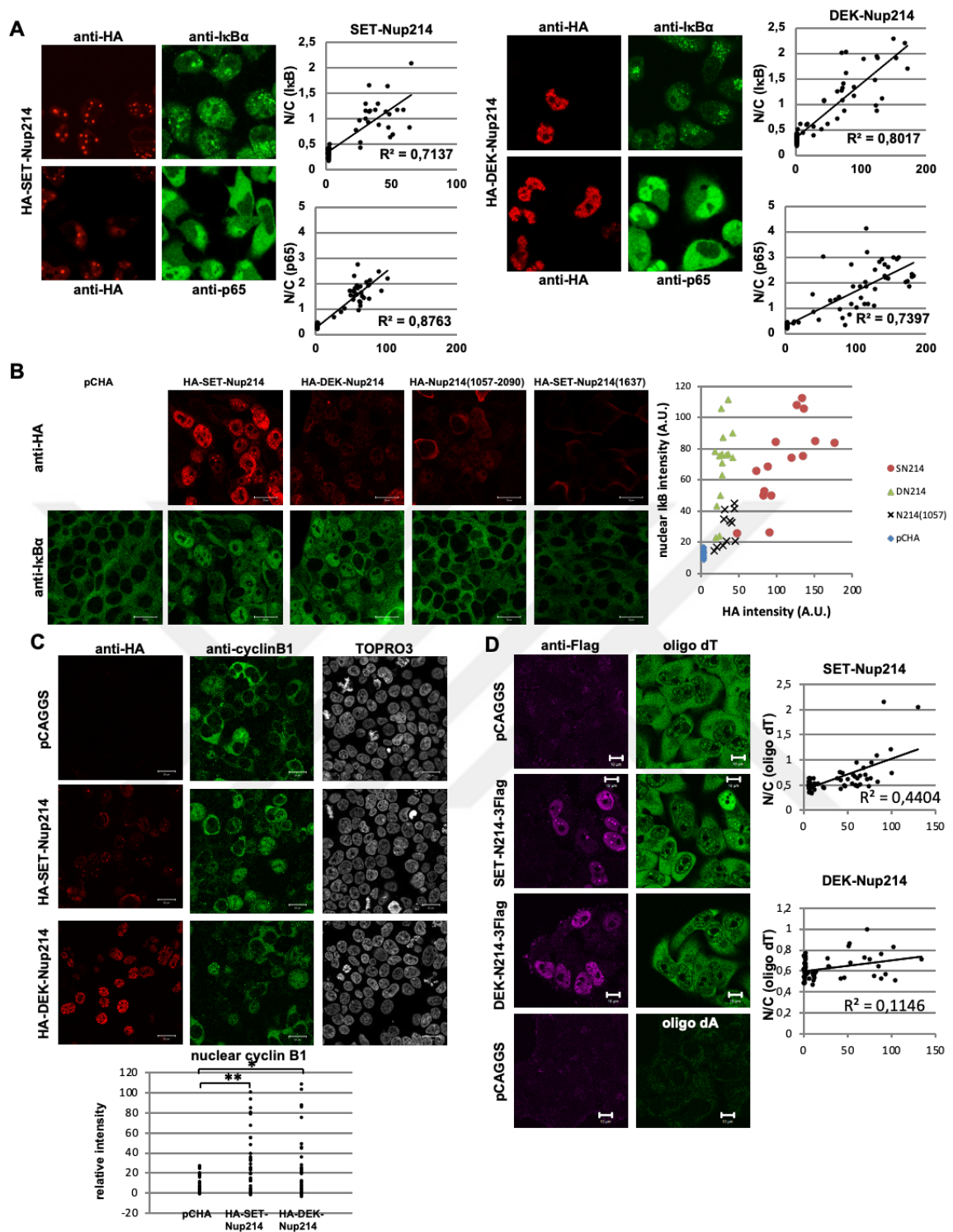


Figure 2.3. Localization of endogenous proteins harboring NES and mRNA.

(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.

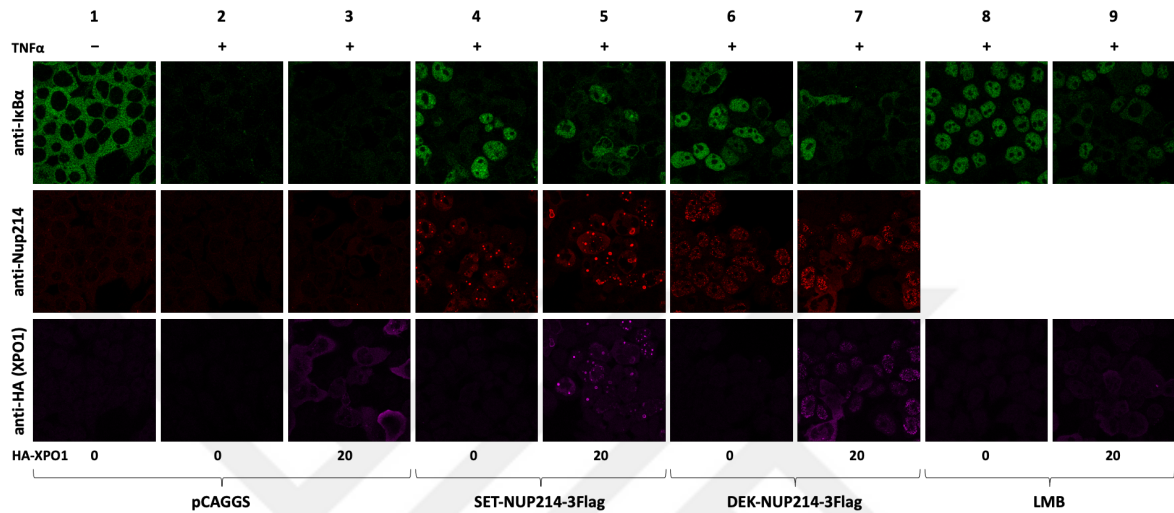


Figure 2.4. Subcellular localization of IκBα (rescue experiment by XPO1 over-expression)

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.

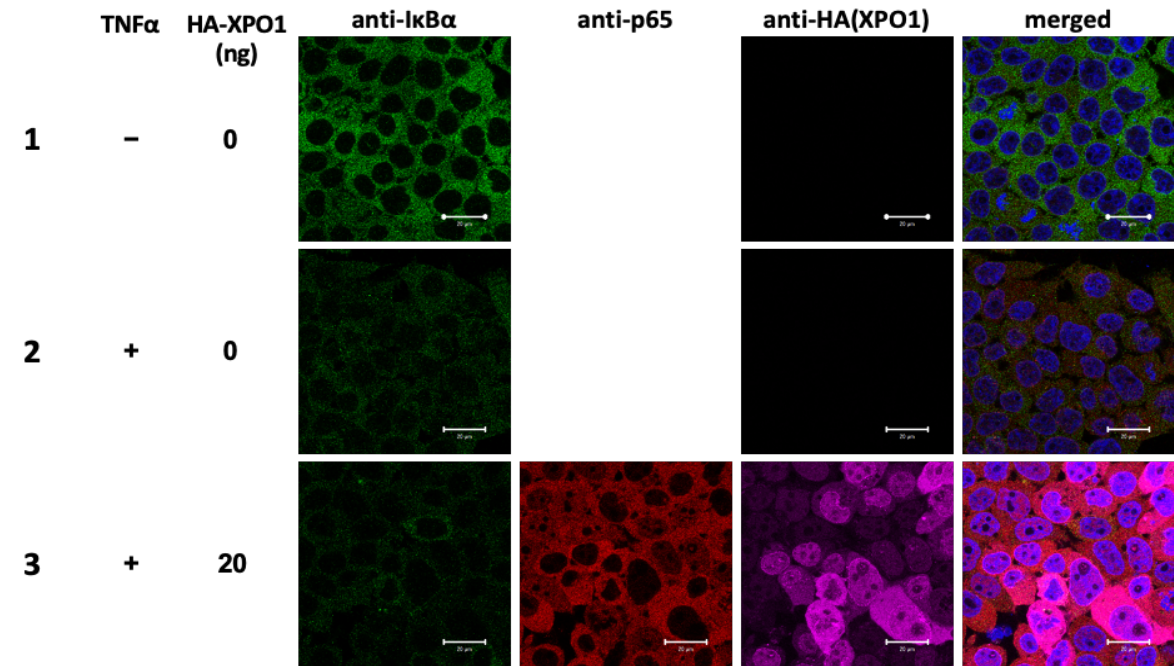


Figure 2.5. Subcellular localization of NF-κBp65 (rescue experiment by XPO1 over-expression)

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.

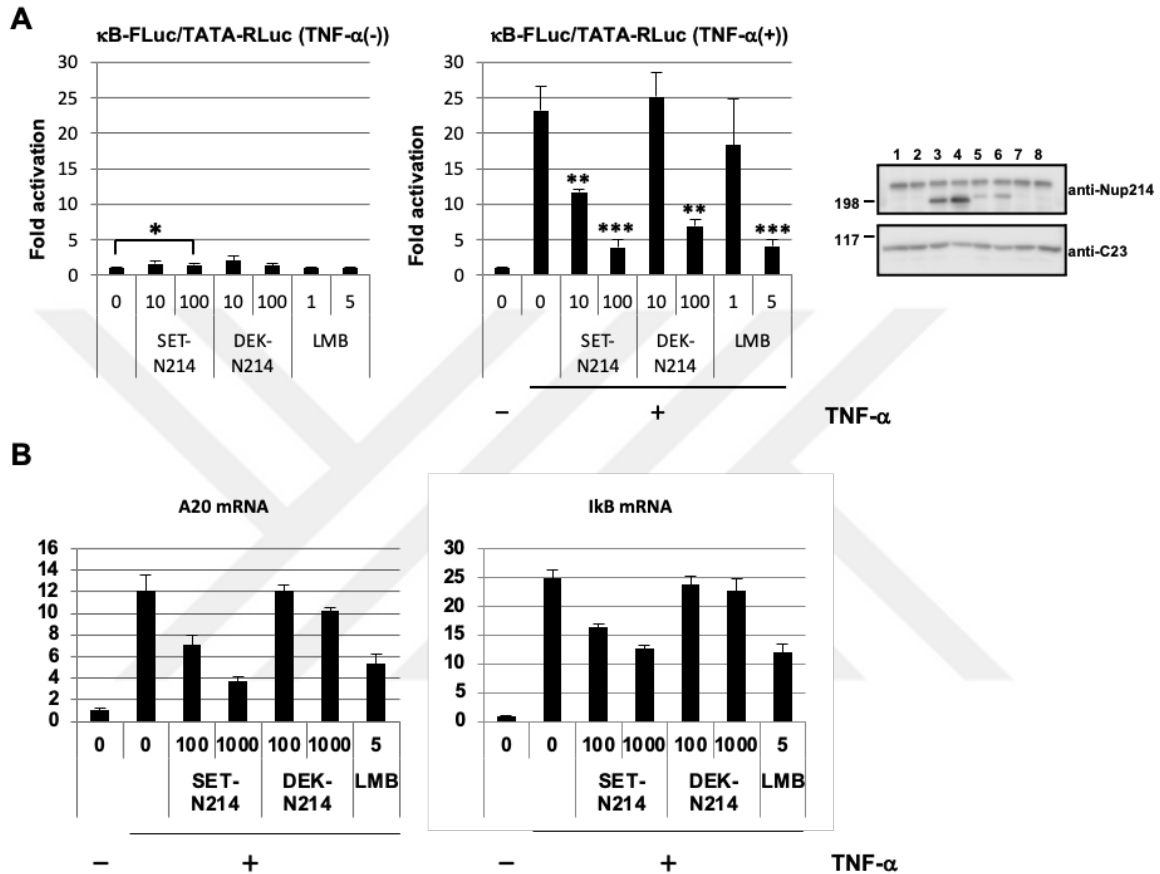


Figure 2.6. Effects of SET-NUP214 and DEK-NUP214 on NF- κ B transcription activity.

(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.

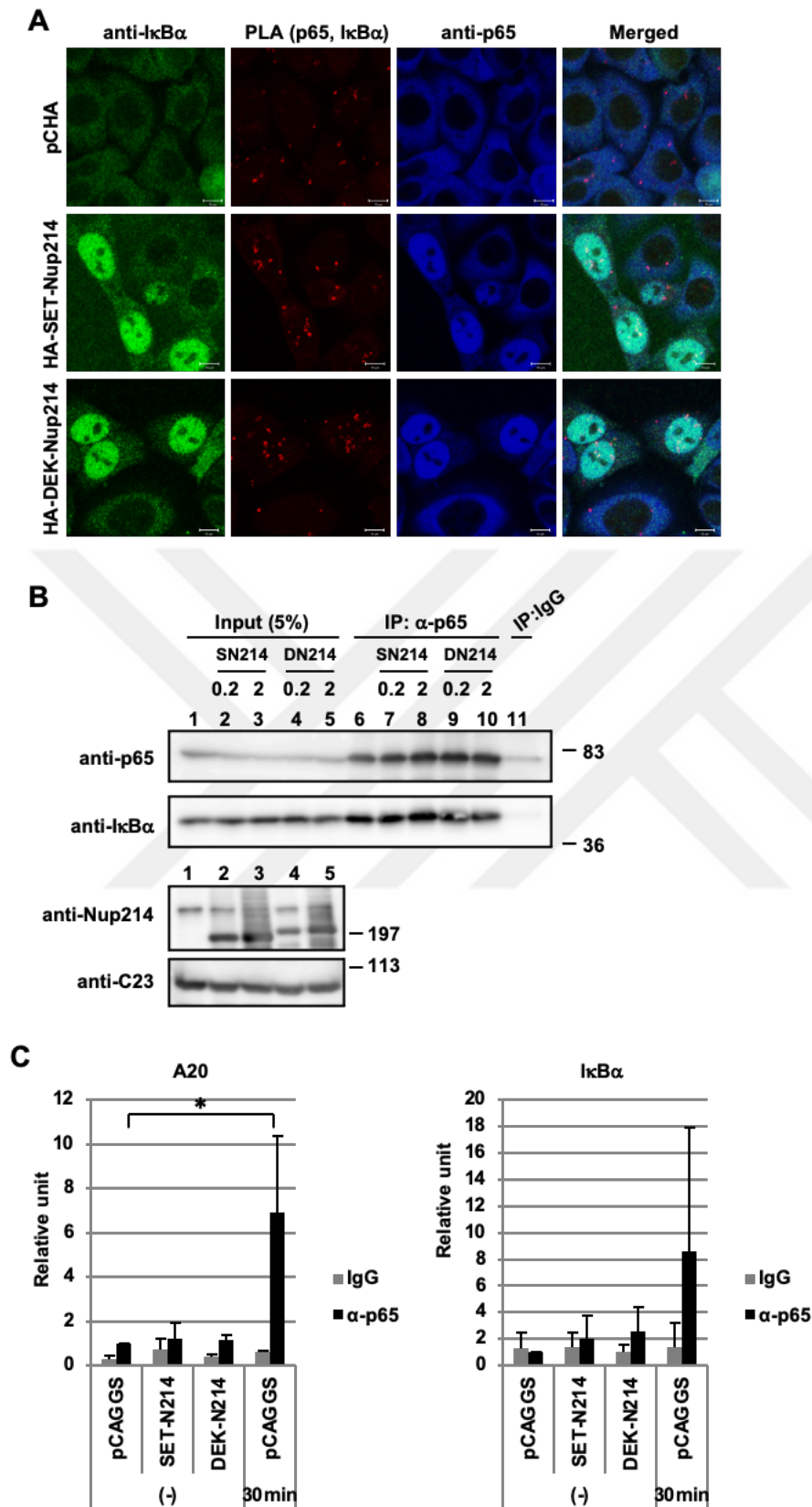


Figure 2.7. Interaction of p65 with I κ B α or chromatin in the nucleus.

(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.

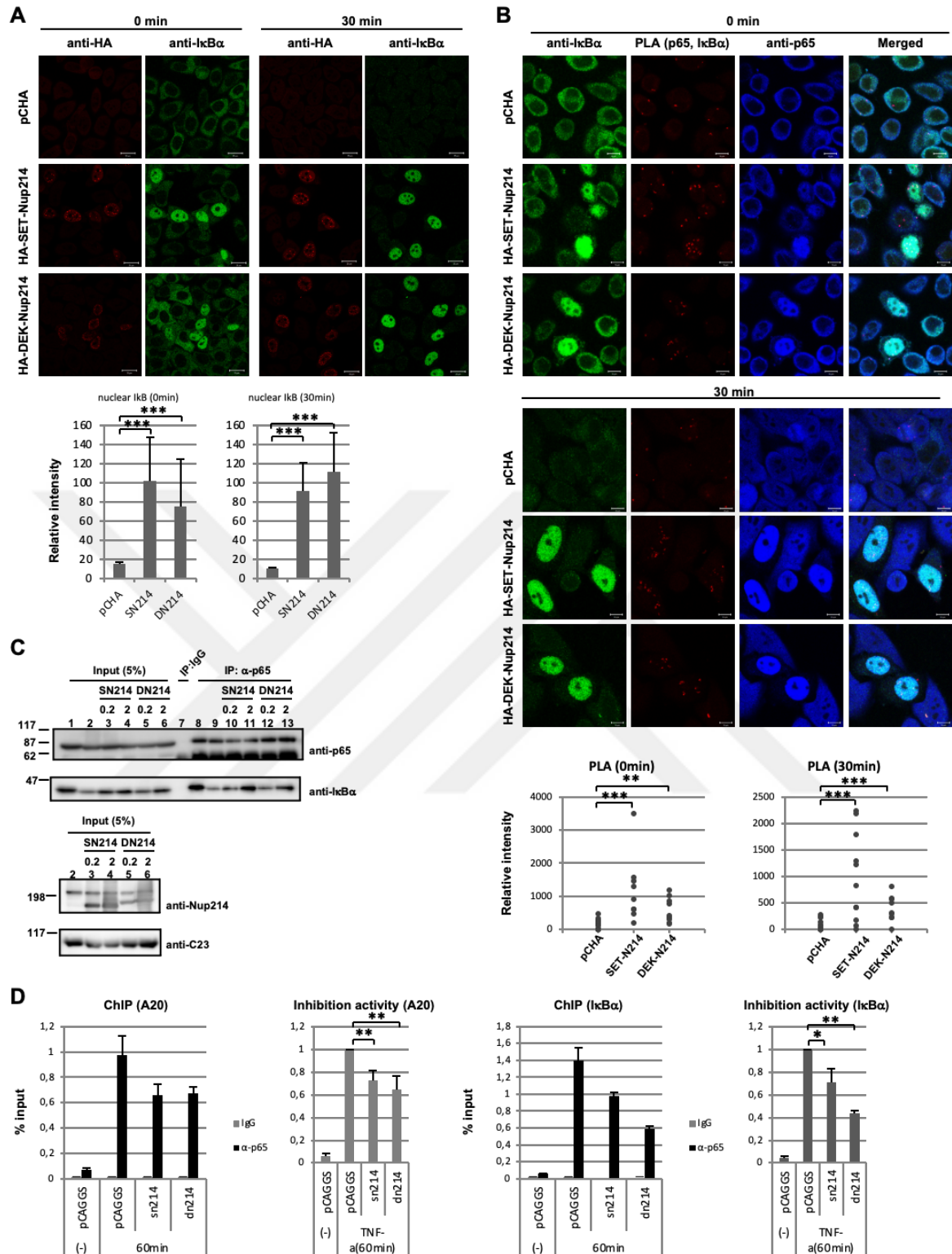


Figure 2.8. Interaction of p65 with IκBα or chromatin in the presence of stimuli.

(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.

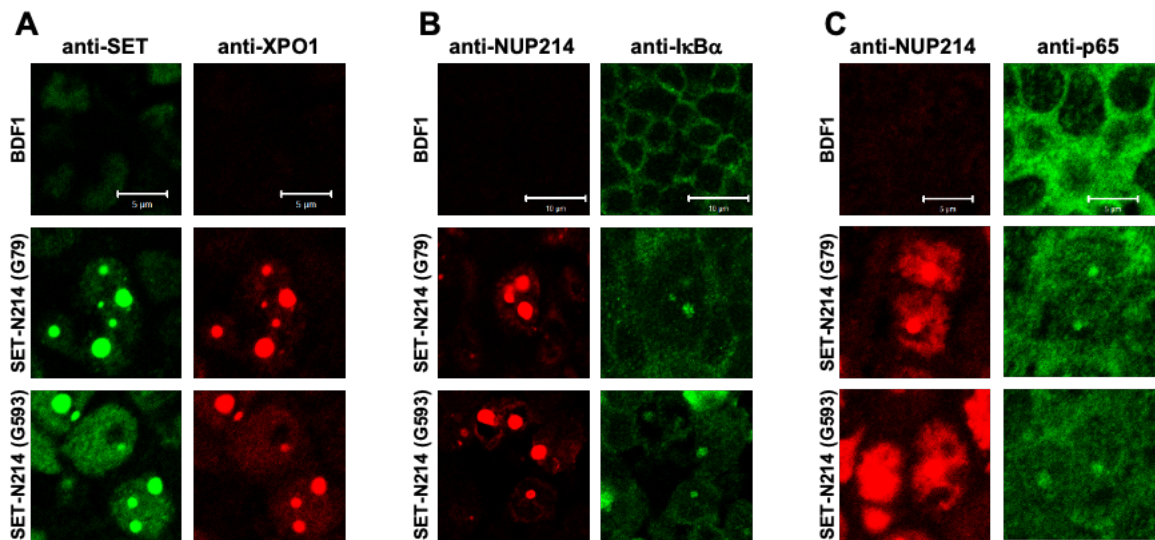


Figure 2.9. Subcellular localization of XPO1, I κ B α , and p65 in spleen of *SET-NUP214* mice.

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 3 SET-NUP214 and MLL cooperatively regulate the promoter activity of the HOXA10 gene

3.1 Abstract

SET-NUP214 is a recurrent fusion gene that is mainly observed in T-cell acute lymphoblastic leukemia (T-ALL). Dysregulation of *homeobox (HOX)* genes is frequently observed in patients with leukemia. Consistent with this, *HOXA* genes are upregulated in the *SET-NUP214*⁺ T-ALL cell line and patients. Although SET-NUP214 has been reported to be recruited to the promoter regions of *HOXA* genes, the detailed mechanisms of how SET-NUP214 specifically binds to *HOXA* gene promoters and regulates *HOXA* gene expression are not known. In this study, I demonstrated that SET-NUP214 interacts with MLL via the SET acidic region of SET-NUP214. SET-NUP214 and MLL cooperatively enhance the promoter activity of the *HOXA10* gene. Neither the SET region alone nor the NUP214 region alone sufficiently enhanced the *HOXA10* gene promoter. Our results indicated that the SET portion of the SET-NUP214 fusion protein is important for interactions with MLL and transcription enhancement of the *HOXA10* gene. Thus, our study will contribute to the understanding of how SET-NUP214 and MLL disturb the expression of *HOXA10* gene in leukemia.

3.2 Introduction

Leukemia is heterogeneous and many gene alterations have been found to be related to this disease. NUP214/CAN is a component of the nuclear pore complex and rearrangement of the *NUP214* gene is recurrently found in acute leukemia [14, 104]. *SET/TAF-I β -NUP214* is one of the *NUP214*-fusion genes, and it is found mainly in T-cell acute lymphoblastic leukemia (T-ALL). SET-NUP214 comprises almost the entire SET protein (1–270 among 277 amino acids) and two-thirds of the NUP214 C-terminal region (813–2090 among 2090 amino acids) [44]. A well-established function of SET/TAF-I β is its core and linker histone chaperone activity [105] [106]. The frequency of *SET-NUP214* positive leukemia among T-ALL is 3.3%–10.3% and *SET-NUP214* positive leukemia has been reported to be chemotherapy-resistant [107] [108]. It is therefore crucial to determine the molecular mechanism by which SET-NUP214 induces malignant transformation. To gain insight into SET-NUP214-induced leukemogenesis, *SET-NUP214* transgenic mice were generated and showed that hematopoietic differentiation is impaired by SET-NUP214 expression [109] [110]. This suggests that *SET-NUP214* fusion gene expression is a driver mutation in leukemia.

The HOX proteins, which are encoded by four different chromosomes (*HOXA* to *HOXD* clusters), are DNA-binding proteins that have low sequence specificity. They regulate the downstream gene expression program during hematopoietic differentiation through complex formation with cofactors

that confer sequence specificity, such as Meis1 [111]. Despite a variety of genetic abnormalities, gene expression profiles showed that expression of *HOXA* cluster genes is often increased in acute myeloid leukemia (AML) and T-ALL subgroups [112]. Therefore, the *HOXA* genes are likely to be critical regulators of leukemogenesis. *HOXA* gene expression in leukemia is regulated by histone modification enzymes, such as MLL/KMT2A and DOT1L, which mediate the methylation of histone H3K4 and K79, respectively [113] [114]. A chromatin-binding protein, CTCF [115], and a Parahox gene product, CDX2 [116] [117], have also been shown to regulate *HOXA* cluster gene expressions. In cells expressing SET-NUP214, the expression of the *HOXA* genes is reported to be enhanced by XPO1-mediated recruitment of SET-NUP214 to the *HOXA* gene promoters [118] [119]. However, it is currently not well understood how SET-NUP214 regulates the expression of the *HOXA* genes.

In this study, I demonstrated that SET-NUP214 and MLL interact with each other and cooperatively enhance promoter activity of the *HOXA10* gene. Our results suggest that the SET portion of the SET-NUP214 fusion protein is important for interactions with MLL and transcription enhancement of the *HOXA10* gene.

3.3 Materials and Methods

3.3.1 Cell culture

HeLa cells and HEK293T cells were grown in DMEM (Nissui) supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37°C in 5% CO₂.

3.3.2 Plasmids

To construct pGL3-HOXA vectors, the upstream regions of the *HOXA7* (-2928 to +130), *A9* (-1928 to +79), and *A10* (-1987 to +14) genes (where the transcription start sites are set to +1) were obtained by PCR using HeLa cell genomic DNAs as a template. The amplified fragments were inserted into pGL3-basic vector. pCHA-SET-NUP214, pCHA-DEK-NUP214, pCHA-SET, and pCAGGS-Flag-MLL-N constructs were described previously [68, 69, 120, 121]. To construct pCAGGS-SETDC-NUP214, *SETDC*-portion was prepared by PCR using pBS-SET-NUP214 as a template, followed by digestion with *XhoI* and *NarI*. *NUP214*-portion was obtained by pBS-SET-NUP214 digestion by *NarI* and *MluI*. Obtained fragments were inserted into *XhoI*- and *MluI*-digested pCHA vector. To construct MLL expression vectors, MLL fragments were obtained by PCR using pCA-FLA-Hrx as a template, followed by digestion with *ClaI*. Digested fragments were inserted into *ClaI*-digested pCAGGS-3Flag vector. Primers are listed in Table 3.1.

Table 3.1. Oligonucleotides used in this study

name	sequence (5'-3')	vector
MluI-HOXA7pro F-2928	GCGGACGCGTAGGCCTCAGGTAGGTGCAG	pGL3basic-HOXA7(-2928)
XhoI-HOXA7pro R+130	GCGGCTCGAGAATTTTGACCTGTGATTGTTGTCC	pGL3basic-HOXA7(-2928)
BglII-hHOXA9up F4821	GCAGATCTGACACGGTGGACTGTGGTGG	pGL3basic-HOXA9(-1928)
HindIII-hHOXA9up R6827	GCAAGCTTCACCGTGCCAGCGCCTGG	pGL3basic-HOXA9(-1928)
BglII-hHOXA10up F5448	GCAGATCTGAGAGTTAATGATCTCTTGGCACAG	pGL3basic-HOXA10(-1987)
HindIII-hHOXA10up R7448	GCAAGCTTTTCTGTGCAAAACATGCTGAATAC	pGL3basic-HOXA10(-1987)
BglII hHA10pro F1750	GCATGAGATCTCAGCCACGTTGGGGCAGCCC	pGL3basic-HOXA10(-238)
BglII-HOXA10pro F-150	GCGGAGATCTGGGCTCTGAGGGCGC	pGL3basic-HOXA10(-136)
BglII-HOXA10pro F-50	GCGGAGATCTCCGCGGTGCGGGGGG	pGL3basic-HOXA10(-36)
kozak SET	GGAATTCTCGAGCGGCCGCCACCATGTCGGCGCCGGCGGC	pCAGGS-SETDC-NUP214
SET C3bp	TGATGAAGGCGCCGAATTTCCATATCGGGAACCAAGTAGTA	pCAGGS-SETDC-NUP214
ClaI hMLL 6262F	GCCCATCGATCCGATATCAACAGCACTGTTGA	pCAGGS-3Flag-MLL(2081-2750)
ClaI hMLL 8271R stop	GCCCATCGATTCATGTTCCATTTCTTTACCATTCTTT	pCAGGS-3Flag-MLL(2081-2750)
ClaI hMLL 8272F	GCCCATCGATGAGAACTTAAAGATTGATAGACCTGAAGATGC	pCAGGS-3Flag-MLL(2751-3969)
ClaI hMLL 11931R	GCCCATCGATTTAGTTTAGGAACTTCCGGCATTCTT	pCAGGS-3Flag-MLL(2751-3969)

3.3.3 Immunoprecipitation assay and western blot analysis

Immunoprecipitation assays and western blot analyses were performed as described previously [121]. Antibodies used for western blotting were anti-Flag (M2, Sigma, 1:1000), anti-HA (3F10, Roche, 1:1000), anti-NUP214 (ab70497, Abcam, 1:1000), anti-DDDDK-tag (PM020, MBL, 1:1000) anti-b-actin (C4, Santa Cruz, 1:1000), and anti-MLL1 (A300-374A-M, Bethyl laboratories, 1:1000).

3.3.4 Luciferase assay and immunofluorescence analyses

HEK293T cells ($0.5\text{--}1.0 \times 10^5$) in a 24-well plate were transfected with vectors with GeneJuice (Merck) according to the manufacturer's manual. Two days after transfection, cells were collected and the lysates were subjected to luciferase assays using the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activity was normalized to the *Renilla* luciferase activity. Immunofluorescence analyses were performed as described previously [121]. For primary antibodies, anti-Flag, anti-HA, anti-NUP214 described above and anti-SET (KM1720, 1:20) [69] were used.

3.4 Results

3.4.1 MLL is attracted to the SET-NUP214-granular dots by interactions with SET-NUP214

SET-NUP214 is involved in the regulation of *HOXA* cluster gene expression. It was previously reported that SET interacts with the N-terminal region of MLL through its acidic region [122, 123], which is included in the SET region of the SET-NUP214 fusion protein. This observation led to the investigation of the possible interactions between SET-NUP214 and MLL and the effects of potential interactions on the *HOXA* gene expression program. To address this possibility, I examined the interaction between SET-NUP214 and MLL by immunoprecipitation assays with the cell extracts expressing HA-tagged SET-NUP214 and Flag-tagged MLL (Fig. 3.1A). Flag-tagged MLL was immunoprecipitated with anti-Flag antibodies and coimmunoprecipitation of HA-tagged proteins was detected with western blotting. I determined that transiently expressed HA-SET-NUP214 was coimmunoprecipitated with Flag-MLL (Fig. 3.1A). DEK-NUP214 is a leukemic fusion protein, which is found in AML, and is composed of a nuclear protein DEK and the NUP214 portion of SET-NUP214. I also examined the interaction between MLL and HA-tagged DEK-NUP214 and found that DEK-NUP214 failed to co-immunoprecipitated MLL, suggesting that the SET portion of SET-NUP214 is important for the interaction with MLL.

We previously showed that SET-NUP214 accumulates and forms granular dots in the nucleus, whereas DEK-NUP214 remains distributed throughout the nucleus by forming very small foci [124]. To assess whether subcellular localization of MLL was affected by SET-NUP214 or DEK-NUP214, I performed immunofluorescence analyses with U2OS cells expressing Flag-MLL with or without NUP214-fusion proteins (Fig. 3.1B). U2OS cells were used in this experiment because they are thin and flat cells with a large cytoplasmic area, which allowed us to clearly visualize the localization of cellular proteins. MLL was distributed throughout the nucleus by forming relatively large dots. Of interest is that we observed MLL in the granular dots where SET-NUP214 had accumulated. On the other hand, MLL localization was not clearly affected by the expression of DEK-NUP214. These results support the notion that SET-NUP214, but not DEK-NUP214, interacts with MLL. To test whether MLL and SET-NUP214 are colocalized in leukemic cells, we performed immunofluorescence analyses with Loucy cell that expresses the SET-NUP214 fusion protein (Fig. 3.1C). The results demonstrated that MLL was partially accumulated to the large dots containing SET-NUP214.

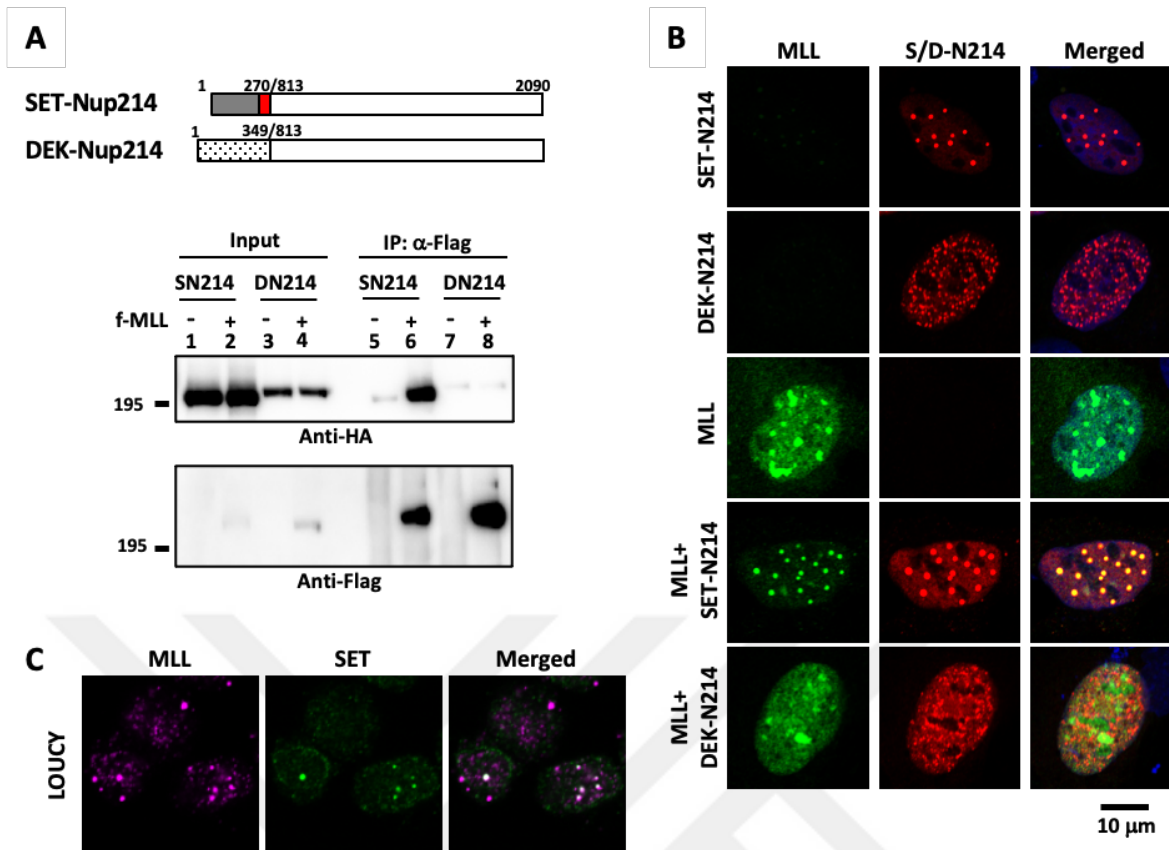


Figure 3.1. Interaction and colocalization of SET-NUP214 and MLL.

A. Interaction between SET-NUP214 and MLL. HEK293T cells were transfected with the expression vector for HA-SET-NUP214 (SN214) or HA-DEK-NUP214 (DN214) without or with the vector for Flag-MLL (f-MLL). Two days after transfection, cells were collected and immunoprecipitation assay was performed by using Flag-M2 gel. The samples were subjected to a 5% SDS-PAGE followed by western blotting with anti-HA and anti-Flag antibodies. SET-NUP214 and DEK-NUP214 structures are schematically represented at the top of the figure. **B.** Localization of MLL in the presence of the NUP214 fusion proteins. HA-SET-NUP214, HA-DEK-NUP214, and Flag-MLL alone (top 3 panels) or in combination (4th and 5th panels) were expressed in U2OS cells and immunofluorescence analysis was performed by using anti-HA and anti-Flag antibodies. Bar; 10 μ m. **C.** Localization of endogenous MLL in a leukemia cell line expressing SET-NUP214. Loucy cells (expressing SET-NUP214) were centrifuged and collected onto glass coverslips. Localizations of SET-NUP214 and MLL were monitored using antibodies against SET and MLL.

3.4.2 SET-NUP214 and MLL enhance the promoter activity of *HOX* genes

To gain insight into the biological significance of interactions between MLL and SET-NUP214, I next examined the effect of these proteins on *HOX* gene expression. I created luciferase reporter plasmids containing the promoter regions of *HOXA7*, *HOXA9*, and *HOXA10* genes [2928, 1928, and 1987 base pairs (bp) upstream of putative transcription start sites (+1), respectively] to evaluate the effects of MLL and SET-NUP214 on the promoter activities of *HOX* genes (Fig. 3.2A). The reporter luciferase activity under the control of either a *HOXA7*, -*A9*, or -*A10* promoter was significantly increased with the expression of MLL, and the effect of MLL on *HOXA10* promoter activity was the most prominent. SET-NUP214 also enhanced *HOXA10*-luciferase activity, whereas this enhancement was inefficient for the *HOXA7*- and *HOXA9*-luciferase activities. To determine the optimal amount of SET-NUP214, the *HOXA10*-reporter activity was examined in the

presence of an increasing amount of SET-NUP214 expression vectors (Fig. 3.2B). The *HOXA10*-luciferase activity was enhanced up to 5–6 fold when a low amount of SET-NUP214 expression vector was introduced, whereas that was diminished when an excess amount of SET-NUP214 expression vector was introduced (Fig. 3.2B, lanes 2–4). I also determined the optimal amounts of MLL expression vector in the absence or presence of an optimal amount of SET-NUP214 expression vector (Fig. 3.2C). MLL alone increased *HOXA10*-luciferase activity in an MLL expression vector dose-dependent manner. SET-NUP214 further enhanced the activity, although the effect of SET-NUP214 was decreased by increasing amounts of MLL expression vector.

It should be noted that the effects of MLL and SET-NUP214 on *HOXA10*-promoter activity were additive even with a low amount of MLL expression vector. Therefore, I next sought to determine the *cis*-regulatory elements along the *HOXA10* gene promoter region that was required for maximal effects of MLL and SET-NUP214. For this purpose, I prepared deletion constructs of the *HOXA10* promoter region containing sequences that were 36, 136, and 238 bp upstream of the transcription start site in addition to the original reporter vector (A10-1987) that contained 1,987 bp upstream of the transcription start site (Fig. 3.2D). The reporter activity of the *HOXA10* promoter was left out by deletion of the sequence between –239 to –1987 (A10-238) (compare Fig. 3.2D lanes 1 and 4), but it was recovered to similar activity as in A10-1987 when the sequence between –137 and –238 was deleted (A10-136) (lane 7). This suggests that the sequence between –137 and –238 contains a putative *cis*-regulatory sequence that is recognized by some transcription inhibitory factors. The promoter activity was again dropped by deletion of the sequence between –136 and –37 (lane 10), indicating that the sequence between –136 and –37 is required for promoter activity of the *HOXA10* gene. When SET-NUP214 or MLL was added to the reporter assay, enhanced promoter activities of A10-1987, A10-238, and A10-136 (lanes 1–9) were found. However, they failed to enhance the activity of A10-36 (lanes 10–12). These results suggested that the *cis*-regulatory elements affected by SET-NUP214 and MLL are similar, and these two proteins cooperatively regulate the *HOXA10* promoter by forming a complex in cells.

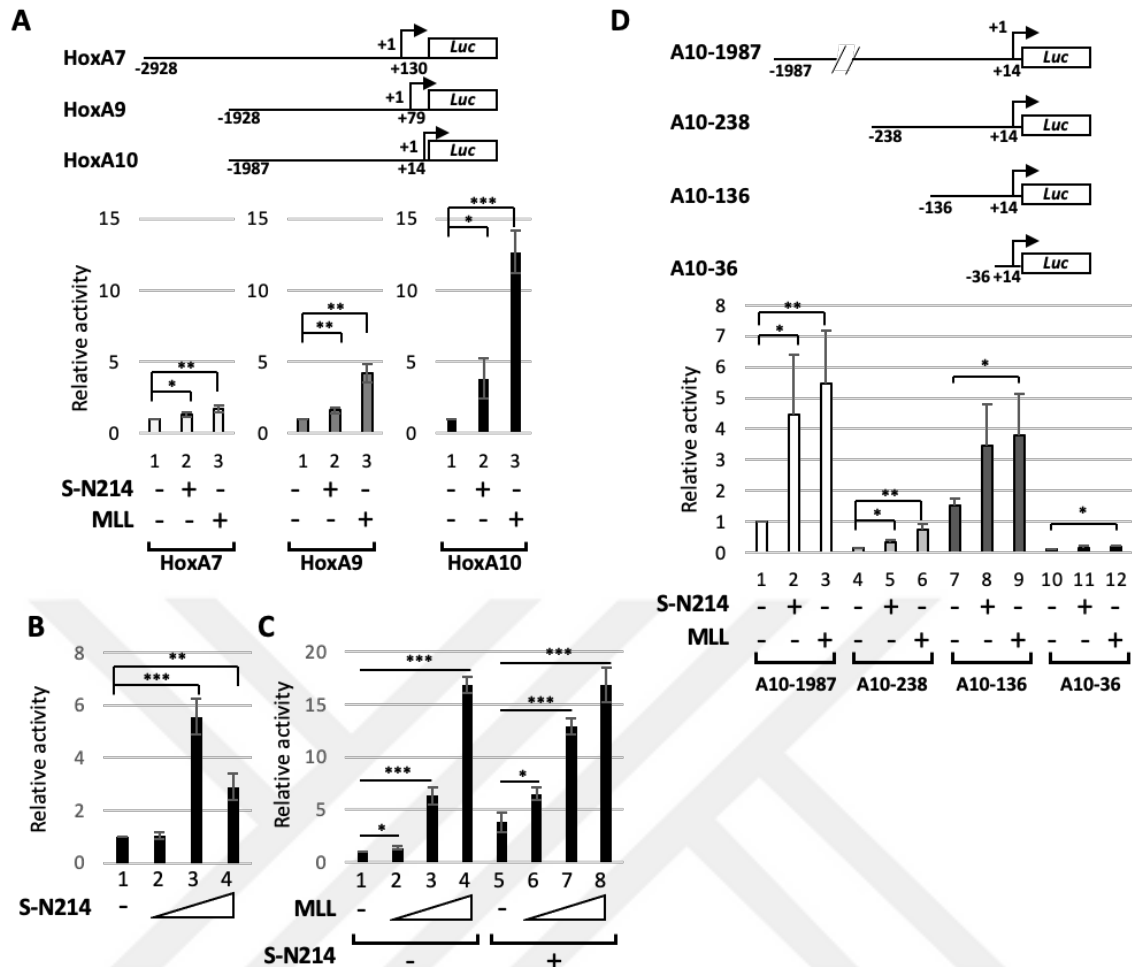


Figure 3.2. SET-NUP214 and MLL enhance the promoter activity of *HOX* genes.

A. The effects of SET-NUP214 and M7L on the promoter activities of *HOX A7*, *A9*, and *A10* genes. Ten ng pGL3 vectors containing the proximal *cis*-acting elements of *HOXA7*, *HOXA9*, and *HOXA10* were transfected with 5 ng of SET-NUP214 (S-N214) or 125 ng of Flag-MLL into HEK293T cells. Two days after transfection, cells were collected and the luciferase assay was performed. Relative activity is shown as fold activation relative to lane 1. **B, C.** The effect of SET-NUP214 and MLL on the *HOXA10* promoter activity. 293T cells were transfected with 10 ng pGL3-*HOXA10* (-1987 to +14), and SET-NUP214 (1, 5, and 25 ng for B and 5 ng for C) and MLL (5, 25, and 125 ng for lanes 2–4 and 6–8) expression vectors. Luciferase assay was performed as in A. **D.** Identification of the *cis*-regulatory element on the *HOXA10* gene promoter regulated by SET-NUP214 and MLL. Luciferase assay with a series of several *HOXA10* upstream regions shown at the top with MLL (25 ng) or SET-NUP214 (5 ng) was performed. Relative activity was expressed as fold activation relative to lane 1. For all luciferase assays shown, the data are average of three independent experiments, and error bars indicate $\pm$ SD. The data were statistically analyzed by student *t*-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The promoter regions tested are schematically represented at the top of A and D.

To further clarify the effect of the interaction between MLL and SET-NUP214 on promoter activity of the *HOXA10* gene, I measured the activity of SET Δ C-NUP214 in which the SET acidic region of SET-NUP214 was deleted by the MLL-enhanced *HOXA10* promoter activity (Fig. 3.3A). Using immunoprecipitation assays, I confirmed that SET-NUP214, but not SET Δ C-NUP214, was efficiently precipitated with Flag-MLL (Fig. 3.3B). The *HOXA10*-promoter luciferase activity enhanced by MLL expression (Fig. 3.3C, lanes 1–3) was further enhanced by co-expression of SET-NUP214 (lanes 4–6). Interestingly, SET Δ C-NUP214 did not stimulate the MLL-enhanced

HOXA10 promoter activity (lanes 7–9). Furthermore, I determined that neither DEK-NUP214 nor SET had a positive effect on the MLL-enhanced *HOXA10* promoter activity (lanes 10-15). These results suggested that the acidic region-mediated interaction with MLL is required for the stimulatory effect of SET-NUP214 on *HOXA10* promoter activity and that neither the SET region alone nor the NUP214 region alone is sufficient for stimulating this activity.

If the interaction with MLL is required for maximal transcriptional stimulation of the *HOXA10* promoter by SET-NUP214, it is hypothesized that this stimulation could be impaired by the addition of the SET interaction domain of MLL as a decoy (Fig. 3.3D). Given that SET interacts with the N-terminal region of MLL [123], the N-terminal region of MLL (MLL-N) was expressed and its effect on *HOXA10* promoter activity was analyzed. I showed that the N-terminal region of MLL did not affect *HOXA10* promoter activity when SET-NUP214 was not expressed (Fig. 3.3D, lanes 1, 3, 5, and 7). Conversely, it decreased SET-NUP214-stimulated transcription activity in a dose-dependent manner (lanes 2, 4, 6, and 8). To confirm the specific inhibitory effect of MLL-N on the SET-NUP214-stimulated transcription activity of *HOXA10* promoter, we also examined the effect of MLL fragment between amino acids 2081 and 2750 (Figs. 3.3A and 3.3E). I demonstrated that MLL-N decreased the SET-NUP214-stimulated *HOXA 10* promoter activity (lanes 2–4), whereas MLL (2081–2750) did not. The expression level of endogenous MLL and MLL-N was examined by western blotting (Figs. 3.3F and G). MLL is translated as a long precursor protein with 3969 amino acids and is processed at amino acids 2666 or 2718 by caspase 1, which generates two MLL fragments termed MLL^{N320} and MLL^{C180} for N- and C-terminal regions of MLL, respectively[125]. The two fragments form a heterodimer and function as a transcription regulator. A specific antibody recognizing the MLL^{C180} efficiently detected both endogenous MLL^{C180} and exogenous Flag-tagged MLL(2751–3969). By comparing the expression levels of Flag-MLL-N and Flag-MLL(2751–3969), the expression level of Flag-MLL-N relative to that of endogenous MLL (MLL^{C180}) could be indirectly estimated. The expression level of MLL-N with 125 ng plasmid vector was estimated to be 10 to 20 times higher than that of endogenous MLL (Figs. 3.3F and 3.3G), which supported the idea that MLL-N functions as a decoy to inhibit the MLL- and SET-NUP214-mediated enhancement of the *HOXA10* gene promoter (Fig. 3.3H). Taking these results together, we concluded that SET-NUP214 stimulates the *HOXA10* promoter activity through its interaction with MLL.

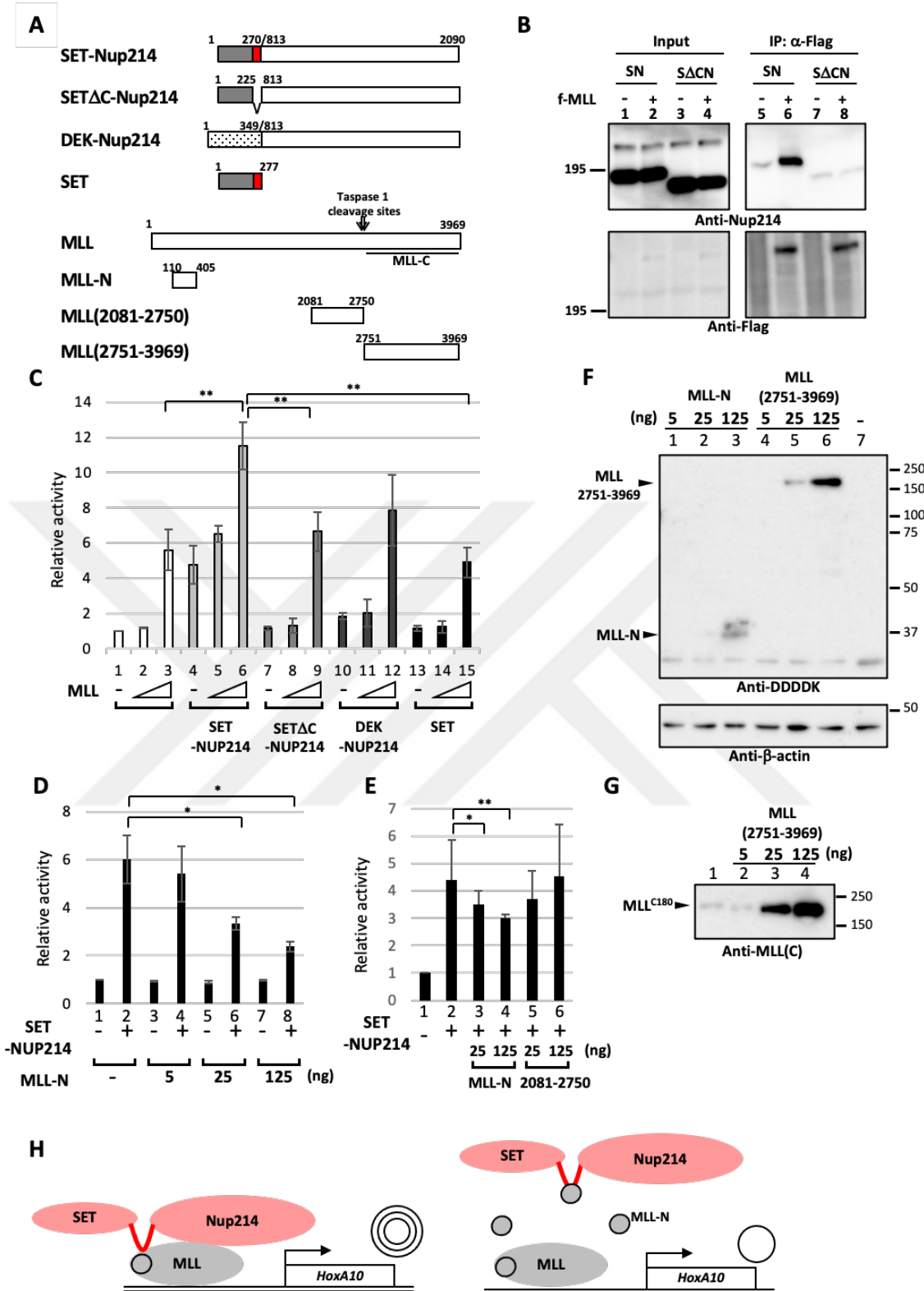


Figure 3.3. SET-NUP214 interacts with the N-terminal region of MLL through acidic region.

A. Schematic structure of NUP214-fusion proteins, SET, MLL, and their mutants. SET portion (grey), the acidic region of SET portion (red), and DEK portion (dots) are highlighted. **B.** The acidic region of SET portion is important for the interaction of SET-NUP214 with MLL. HEK293T cells were transfected with plasmids for HA-SET-NUP214 (SN) or SETDC-NUP214 (SDCN) without or with a plasmid for Flag-MLL (f-MLL). Two days after transfection, cells were collected and immunoprecipitation assay was performed by using Flag M2 gel. The samples were subjected to 5% SDS-PAGE followed by western blotting with anti-NUP214 and anti-Flag antibodies. **C.** Effects of SET-NUP214 and its mutants on *HOXA10* transcription

activity. Luciferase assay was performed in the presence of 10 ng pGL3-*HOXA10* (-1987 to +14), and 5 ng of empty vector, SET-NUP214, SETDC-NUP214, DEK-NUP214, or SET expression vectors with the increasing amount of MLL expression vector (5, 25 ng) as indicated. Relative activity is expressed as fold activation relative to lane 1. **D.** MLL-N diminishes the SET-NUP214-enhanced *HOXA10* promoter activity. Cells were transfected with 10 ng pGL3-*HOXA10* (-1987 to +14), SET-NUP214 (5 ng), and MLL-N (5, 25, and 125 ng for +, ++, and +++) expression vectors and luciferase assays were performed. Relative activity was expressed as fold activation relative to lane 1. **E.** Effects of MLL fragment on *HOXA10* promoter activity. HEK293T cells were transfected with 10 ng pGL3-*HOXA10* (-1987 to +14), 5 ng SET-NUP214, and MLL-N or MLL(2081–2750) (25 and 125 ng) expression vectors and luciferase assays were performed. Relative activity was expressed as fold activation relative to lane 1. **F, G.** Expressions of MLL fragments and endogenous MLL. HEK293T cells were transfected with MLL-N or MLL (2751–3969) expression vectors. Two days after transfection, western blot analyses were performed by using anti Flag (F), anti-b-actin (F), and anti-MLL (G) antibodies. For all luciferase assays shown, the data are average of three independent experiments, and error bars indicate $\pm$ SD. The data were statistically analyzed by student *t*-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. 293T cells were transiently transfected with MLL-N or MLL (2751–3969) expression vectors (25, 125, and 500 ng). The data shown in this figure is schematically represented at panel F. The N-terminal region of MLL represented by the gray ball directly interacts with the acidic region of the SET portion of the SET-NUP214.

3.5 Discussion

In this study, we demonstrated SET-NUP214 cooperatively regulates the *HOXA10* promoter activity with a histone methyltransferase KMT2A/MLL. As shown in Figure 3.3D, the enhancement of the *HOXA10* promoter activity by SET-NUP214 was impaired in the presence of the N-terminal domain of MLL (MLL-N). This suggested that transcription activation by SET-NUP214 is, at least in part, mediated by MLL. MLL may stimulate the *HOXA10* gene using two independent mechanisms, *i.e.*, chromatin structure-dependent and DNA sequence-dependent mechanisms. As shown in Figure 3.2D, the sequences between -136 and -37 of the *HOXA10* gene promoter are required for MLL-mediated enhancement of the reporter activity. We found that the sequence between -1 to -150 bp is rich in CpG sequences. The CXXC domain of MLL is known to associate with non-methylated CpG sequences for transactivation [126]. These observations led us to speculate that MLL directly binds to the *HOXA10* promoter region and recruits SET-NUP214 to enhance transcription.

The *HOXA10* promoter analyses shown in Fig. 3.2D suggest that there is a negative regulatory element between -238 to -136 of the *HOXA10* promoter region. To predict a possible transcription factor that decreases the promoter activity of *HOXA10* gene, we took advantage of the transcription factor binding profile database JASPAR [127] and found a sequence element recognized by a transcription factor YY1 in the region between -238 and -136 of *HOXA10* gene. YY1 functions as both a transcription activator and repressor. Further investigation is needed to clarify the biological significance of this inhibitory sequence.

The mechanism by which SET-NUP214 enhances the *HOXA10* gene promoter activity is undetermined. Two possibilities are under consideration; first, that SET-NUP214 may stabilize MLL binding to the *HOXA10* promoter and, second, SET-NUP214 may enhance MLL function to

recruit transcription activators including the PAF1 complex and CBP/p300 [128, 129]. I also found that SET failed to enhance the HOXA10 promoter activity (Fig. 3.3C), although it associates with MLL [123]. This implies that the NUP214 domain of SET-NUP214 is required for transcription activation of the *HOXA10*-luciferase activity. XPO1, a nuclear export receptor, may play a role in the regulation of the *HOXA10* gene expression cooperatively with MLL and SET-NUP214, because it was shown to associate with SET-NUP214 and is involved in the regulation of *HOX* genes [119]. Further studies are required to clarify the function of SET-NUP214 and MLL in the *HOXA10* gene expression program.



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.

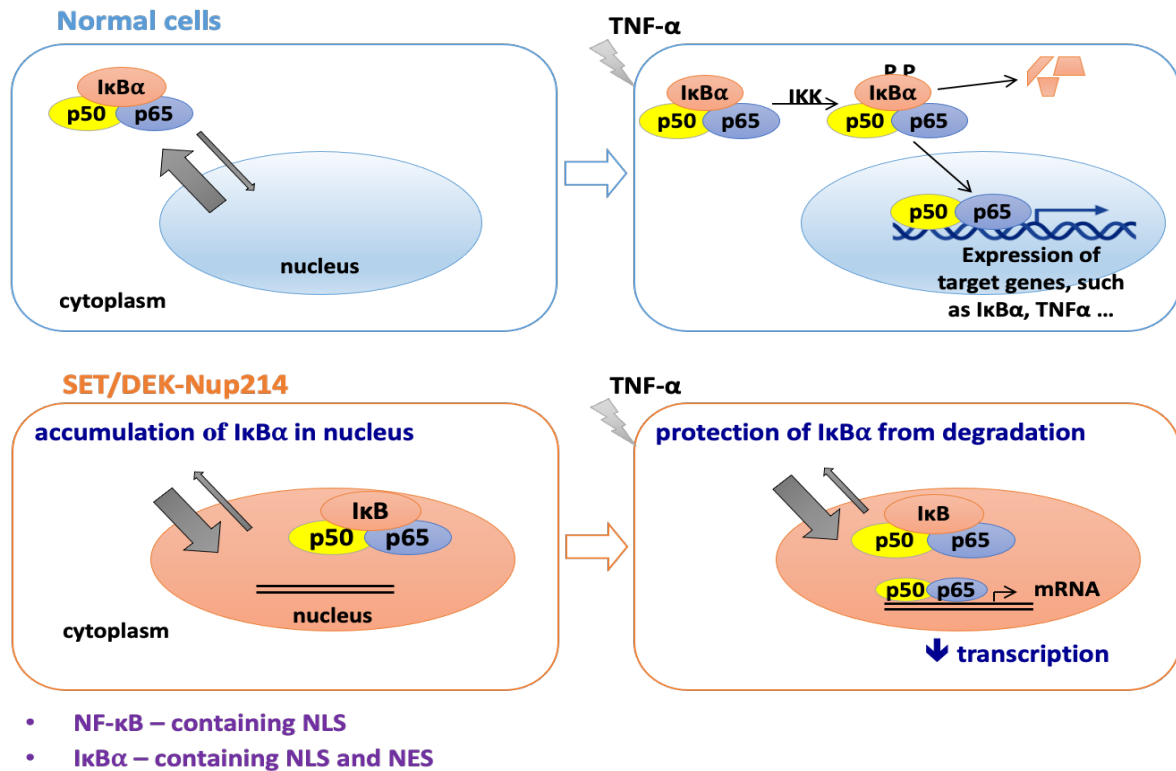


Figure 4.1. A model for downregulation of NF-κB pathway caused by NUP214 fusion proteins.

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).

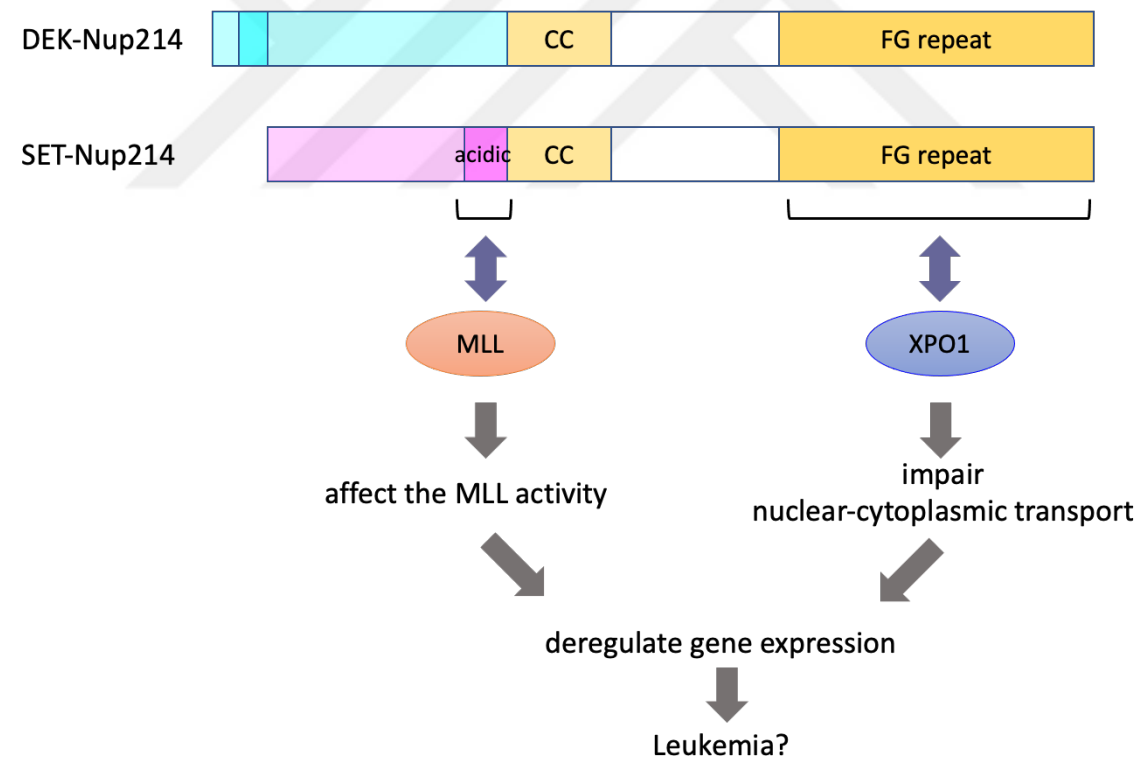


Figure 4.2. Leukemogenic mechanisms of NUP214 fusions through interacting proteins.

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.

References

1. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation*. Cell, 2011. **144**(5): p. 646-74.
2. Chen, J., O. Odenike, and J.D. Rowley, *Leukaemogenesis: more than mutant genes*. Nat Rev Cancer, 2010. **10**(1): p. 23-36.
3. Sung, H., et al., *Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries*. CA Cancer J Clin, 2021. **71**(3): p. 209-249.
4. *Nature Portfolio*. [21 March 2022]; Available from: <https://www.nature.com/subjects/leukaemia>.
5. Lowenberg, B., J.R. Downing, and A. Burnett, *Acute myeloid leukemia*. N Engl J Med, 1999. **341**(14): p. 1051-62.
6. Pui, C.H., M.V. Relling, and J.R. Downing, *Acute lymphoblastic leukemia*. N Engl J Med, 2004. **350**(15): p. 1535-48.
7. Long, N.A., et al., *Acute Myeloid Leukemia Stem Cells: Origin, Characteristics, and Clinical Implications*. Stem Cell Rev Rep, 2022.
8. Chopra, M. and S.K. Bohlander, *The cell of origin and the leukemia stem cell in acute myeloid leukemia*. Genes Chromosomes Cancer, 2019. **58**(12): p. 850-858.
9. Mertens, F., et al., *The emerging complexity of gene fusions in cancer*. Nat Rev Cancer, 2015. **15**(6): p. 371-81.
10. Wang, Y., et al., *Recurrent Fusion Genes in Leukemia: An Attractive Target for Diagnosis and Treatment*. Curr Genomics, 2017. **18**(5): p. 378-384.
11. Mitelman, F., B. Johansson, and F. Mertens, *Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer*. 2021.
12. Nofrini, V., D. Di Giacomo, and C. Mecucci, *Nucleoporin genes in human diseases*. Eur J Hum Genet, 2016. **24**(10): p. 1388-95.
13. Fahrenkrog, B., *Nucleoporin Gene Fusions and Hematopoietic Malignancies*. New Journal of Science, 2014. **2014**: p. 468306.
14. Mendes, A. and B. Fahrenkrog, *NUP214 in Leukemia: It's More than Transport*. Cells, 2019. **8**(1).
15. Xu, S. and M.A. Powers, *Nuclear pore proteins and cancer*. Semin Cell Dev Biol, 2009. **20**(5): p. 620-30.
16. Oka, M., et al., *Chromatin-prebound Crm1 recruits Nup98-HoxA9 fusion to induce aberrant expression of Hox cluster genes*. Elife, 2016. **5**.
17. Van Vlierberghe, P., et al., *The recurrent SET-NUP214 fusion as a new HOXA activation mechanism in pediatric T-cell acute lymphoblastic leukemia*. Blood, 2008. **111**(9): p. 4668-80.
18. Mendes, A., et al., *Disclosing the Interactome of Leukemogenic NUP98-HOXA9 and SET-NUP214 Fusion Proteins Using a Proteomic Approach*. Cells, 2020. **9**(7).
19. Keck, K.M. and L.F. Pemberton, *Histone chaperones link histone nuclear import and chromatin assembly*. Biochim Biophys Acta, 2012. **1819**(3-4): p. 277-89.
20. Kajitani, K., K. Kato, and K. Nagata, *Histone H1 chaperone activity of TAF-I is regulated by its subtype-dependent intramolecular interaction*. Genes Cells, 2017. **22**(4): p. 334-347.

21. Sawatsubashi, S., et al., *A histone chaperone, DEK, transcriptionally coactivates a nuclear receptor*. Genes Dev, 2010. **24**(2): p. 159-70.
22. Saito, S., et al., *Impairment of erythroid and megakaryocytic differentiation by a leukemia-associated and t(9;9)-derived fusion gene product, SET/TAF-Ibeta-CAN/Nup214*. J Cell Physiol, 2008. **214**(2): p. 322-33.
23. Qin, H., et al., *Transformation of human CD34+ hematopoietic progenitor cells with DEK-NUP214 induces AML in an immunocompromised mouse model*. Oncogene, 2016. **35**(43): p. 5686-5691.
24. Wentz, S.R. and M.P. Rout, *The nuclear pore complex and nuclear transport*. Cold Spring Harb Perspect Biol, 2010. **2**(10): p. a000562.
25. Rothballer, A. and U. Kutay, *SnapShot: The nuclear envelope I*. Cell, 2012. **150**(4): p. 868-868 e1.
26. Yang, W., *Nuclear-cytoplasmic transport*. Nucleic acids and molecular biology, volume 33. 2018, Cham, Switzerland: Springer.
27. Gorlich, D., *Transport into and out of the cell nucleus*. EMBO J, 1998. **17**(10): p. 2721-7.
28. Argiropoulos, B. and R.K. Humphries, *Hox genes in hematopoiesis and leukemogenesis*. Oncogene, 2007. **26**(47): p. 6766-76.
29. He, H., X. Hua, and J. Yan, *Epigenetic regulations in hematopoietic Hox code*. Oncogene, 2011. **30**(4): p. 379-88.
30. Walde, S. and R.H. Kehlenbach, *The Part and the Whole: functions of nucleoporins in nucleocytoplasmic transport*. Trends Cell Biol, 2010. **20**(8): p. 461-9.
31. Strambio-De-Castillia, C., M. Niepel, and M.P. Rout, *The nuclear pore complex: bridging nuclear transport and gene regulation*. Nat Rev Mol Cell Biol, 2010. **11**(7): p. 490-501.
32. Kau, T.R., J.C. Way, and P.A. Silver, *Nuclear transport and cancer: from mechanism to intervention*. Nat Rev Cancer, 2004. **4**(2): p. 106-17.
33. O'Brate, A. and P. Giannakakou, *The importance of p53 location: nuclear or cytoplasmic zip code?* Drug Resist Updat, 2003. **6**(6): p. 313-22.
34. Oeckinghaus, A. and S. Ghosh, *The NF-kappaB family of transcription factors and its regulation*. Cold Spring Harb Perspect Biol, 2009. **1**(4): p. a000034.
35. Capelson, M. and M.W. Hetzer, *The role of nuclear pores in gene regulation, development and disease*. EMBO Rep, 2009. **10**(7): p. 697-705.
36. Melo, S.A., et al., *A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells*. Cancer Cell, 2010. **18**(4): p. 303-15.
37. Puente, X.S., et al., *Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia*. Nature, 2011. **475**(7354): p. 101-5.
38. Jeromin, S., et al., *SF3B1 mutations correlated to cytogenetics and mutations in NOTCH1, FBXW7, MYD88, XPO1 and TP53 in 1160 untreated CLL patients*. Leukemia, 2014. **28**(1): p. 108-17.
39. Kohler, A. and E. Hurt, *Gene regulation by nucleoporins and links to cancer*. Mol Cell, 2010. **38**(1): p. 6-15.
40. Funasaka, T. and R.W. Wong, *The role of nuclear pore complex in tumor microenvironment and metastasis*. Cancer Metastasis Rev, 2011. **30**(2): p. 239-51.

41. Simon, D.N. and M.P. Rout, *Cancer and the nuclear pore complex*. Adv Exp Med Biol, 2014. **773**: p. 285-307.
42. Zhou, M.H. and Q.M. Yang, *fusion genes in acute leukemia (Review)*. Oncol Lett, 2014. **8**(3): p. 959-962.
43. von Lindern, M., et al., *The translocation (6;9), associated with a specific subtype of acute myeloid leukemia, results in the fusion of two genes, dek and can, and the expression of a chimeric, leukemia-specific dek-can mRNA*. Mol Cell Biol, 1992. **12**(4): p. 1687-97.
44. von Lindern, M., et al., *Can, a putative oncogene associated with myeloid leukemogenesis, may be activated by fusion of its 3' half to different genes: characterization of the set gene*. Mol Cell Biol, 1992. **12**(8): p. 3346-55.
45. Ben Abdelali, R., et al., *SET-NUP214 is a recurrent gammadelta lineage-specific fusion transcript associated with corticosteroid/chemotherapy resistance in adult T-ALL*. Blood, 2014. **123**(12): p. 1860-3.
46. Sandahl, J.D., et al., *t(6;9)(p22;q34)/DEK-NUP214-rearranged pediatric myeloid leukemia: an international study of 62 patients*. Haematologica, 2014. **99**(5): p. 865-72.
47. Ozbek, U., et al., *SET-CAN, the product of the t(9;9) in acute undifferentiated leukemia, causes expansion of early hematopoietic progenitors and hyperproliferation of stomach mucosa in transgenic mice*. Am J Pathol, 2007. **171**(2): p. 654-66.
48. Kandilci, A., E. Mientjes, and G. Grosveld, *Effects of SET and SET-CAN on the differentiation of the human promonocytic cell line U937*. Leukemia, 2004. **18**(2): p. 337-40.
49. Ageberg, M., et al., *Identification of a novel and myeloid specific role of the leukemia-associated fusion protein DEK-NUP214 leading to increased protein synthesis*. Genes Chromosomes Cancer, 2008. **47**(4): p. 276-87.
50. Sanden, C., et al., *Forced expression of the DEK-NUP214 fusion protein promotes proliferation dependent on upregulation of mTOR*. BMC Cancer, 2013. **13**: p. 440.
51. Oancea, C., et al., *The t(6;9) associated DEK/CAN fusion protein targets a population of long-term repopulating hematopoietic stem cells for leukemogenic transformation*. Leukemia, 2010. **24**(11): p. 1910-9.
52. Moroianu, J., et al., *Mammalian karyopherin alpha 1 beta and alpha 2 beta heterodimers: alpha 1 or alpha 2 subunit binds nuclear localization signal and beta subunit interacts with peptide repeat-containing nucleoporins*. Proc Natl Acad Sci U S A, 1995. **92**(14): p. 6532-6.
53. Boer, J., J. Bonten-Surtel, and G. Grosveld, *Overexpression of the nucleoporin CAN/NUP214 induces growth arrest, nucleocytoplasmic transport defects, and apoptosis*. Mol Cell Biol, 1998. **18**(3): p. 1236-47.
54. Kuersten, S., et al., *Steady-state nuclear localization of exportin-t involves RanGTP binding and two distinct nuclear pore complex interaction domains*. Mol Cell Biol, 2002. **22**(16): p. 5708-20.
55. Fornerod, M., et al., *The human homologue of yeast CRM1 is in a dynamic subcomplex with CAN/Nup214 and a novel nuclear pore component Nup88*. EMBO J, 1997. **16**(4): p. 807-16.
56. Katahira, J., et al., *The Mex67p-mediated nuclear mRNA export pathway is conserved from yeast to human*. EMBO J, 1999. **18**(9): p. 2593-609.
57. Bachi, A., et al., *The C-terminal domain of TAP interacts with the nuclear pore complex and promotes export of specific CTE-bearing RNA substrates*. RNA, 2000. **6**(1): p. 136-58.

58. Levesque, L., et al., *RNA export mediated by tap involves NXT1-dependent interactions with the nuclear pore complex*. J Biol Chem, 2001. **276**(48): p. 44953-62.
59. Wiegand, H.L., et al., *Formation of Tap/NXT1 heterodimers activates Tap-dependent nuclear mRNA export by enhancing recruitment to nuclear pore complexes*. Mol Cell Biol, 2002. **22**(1): p. 245-56.
60. Herold, A., et al., *TAP (NXF1) belongs to a multigene family of putative RNA export factors with a conserved modular architecture*. Mol Cell Biol, 2000. **20**(23): p. 8996-9008.
61. van Deursen, J., et al., *G2 arrest and impaired nucleocytoplasmic transport in mouse embryos lacking the proto-oncogene CAN/Nup214*. EMBO J, 1996. **15**(20): p. 5574-83.
62. Bernad, R., et al., *Nup214-Nup88 nucleoporin subcomplex is required for CRM1-mediated 60 S preribosomal nuclear export*. J Biol Chem, 2006. **281**(28): p. 19378-86.
63. Hutten, S. and R.H. Kehlenbach, *Nup214 is required for CRM1-dependent nuclear protein export in vivo*. Mol Cell Biol, 2006. **26**(18): p. 6772-85.
64. Roloff, S., C. Spillner, and R.H. Kehlenbach, *Several phenylalanine-glycine motives in the nucleoporin Nup214 are essential for binding of the nuclear export receptor CRM1*. J Biol Chem, 2013. **288**(6): p. 3952-63.
65. Xylourgidis, N., et al., *The nucleoporin Nup214 sequesters CRM1 at the nuclear rim and modulates NFkappaB activation in Drosophila*. J Cell Sci, 2006. **119**(Pt 21): p. 4409-19.
66. Takeda, A. and N.R. Yaseen, *Nucleoporins and nucleocytoplasmic transport in hematologic malignancies*. Semin Cancer Biol, 2014. **27**: p. 3-10.
67. Fornerod, M., et al., *Interaction of cellular proteins with the leukemia specific fusion proteins DEK-CAN and SET-CAN and their normal counterpart, the nucleoporin CAN*. Oncogene, 1996. **13**(8): p. 1801-8.
68. Saito, S., M. Miyaji-Yamaguchi, and K. Nagata, *Aberrant intracellular localization of SET-CAN fusion protein, associated with a leukemia, disorganizes nuclear export*. Int J Cancer, 2004. **111**(4): p. 501-7.
69. Nagata, K., et al., *Cellular localization and expression of template-activating factor I in different cell types*. Exp Cell Res, 1998. **240**(2): p. 274-81.
70. Numajiri Haruki, A., et al., *Interferon-inducible antiviral protein MxA enhances cell death triggered by endoplasmic reticulum stress*. J Interferon Cytokine Res, 2011. **31**(11): p. 847-56.
71. Herold, A., T. Klymenko, and E. Izaurralde, *NXF1/p15 heterodimers are essential for mRNA nuclear export in Drosophila*. RNA, 2001. **7**(12): p. 1768-80.
72. Takeda, A., et al., *Inhibition of CRM1-mediated nuclear export of transcription factors by leukemogenic NUP98 fusion proteins*. J Biol Chem, 2010. **285**(21): p. 16248-57.
73. Johnson, C., D. Van Antwerp, and T.J. Hope, *An N-terminal nuclear export signal is required for the nucleocytoplasmic shuttling of IkappaBalpha*. EMBO J, 1999. **18**(23): p. 6682-93.
74. Rodriguez, M.S., et al., *Nuclear retention of IkappaBalpha protects it from signal-induced degradation and inhibits nuclear factor kappaB transcriptional activation*. J Biol Chem, 1999. **274**(13): p. 9108-15.
75. Huang, T.T., et al., *A nuclear export signal in the N-terminal regulatory domain of IkappaBalpha controls cytoplasmic localization of inactive NF-kappaB/IkappaBalpha complexes*. Proc Natl Acad Sci U S A, 2000. **97**(3): p. 1014-9.
76. Tam, W.F., et al., *Cytoplasmic sequestration of rel proteins by IkappaBalpha requires CRM1-dependent nuclear export*. Mol Cell Biol, 2000. **20**(6): p. 2269-84.

77. Malek, S., et al., *IkappaBbeta, but not IkappaBalpha, functions as a classical cytoplasmic inhibitor of NF-kappaB dimers by masking both NF-kappaB nuclear localization sequences in resting cells.* J Biol Chem, 2001. **276**(48): p. 45225-35.
78. Hayden, M.S. and S. Ghosh, *Signaling to NF-kappaB.* Genes Dev, 2004. **18**(18): p. 2195-224.
79. Baeuerle, P.A. and D. Baltimore, *I kappa B: a specific inhibitor of the NF-kappa B transcription factor.* Science, 1988. **242**(4878): p. 540-6.
80. Kanarek, N. and Y. Ben-Neriah, *Regulation of NF-kappaB by ubiquitination and degradation of the IkappaBs.* Immunol Rev, 2012. **246**(1): p. 77-94.
81. Allen, N.P., et al., *Proteomic analysis of nucleoporin interacting proteins.* J Biol Chem, 2001. **276**(31): p. 29268-74.
82. Terry, L.J. and S.R. Wente, *Flexible gates: dynamic topologies and functions for FG nucleoporins in nucleocytoplasmic transport.* Eukaryot Cell, 2009. **8**(12): p. 1814-27.
83. Kang, Y., H.P. Bogerd, and B.R. Cullen, *Analysis of cellular factors that mediate nuclear export of RNAs bearing the Mason-Pfizer monkey virus constitutive transport element.* J Virol, 2000. **74**(13): p. 5863-71.
84. Askjaer, P., et al., *RanGTP-regulated interactions of CRM1 with nucleoporins and a shuttling DEAD-box helicase.* Mol Cell Biol, 1999. **19**(9): p. 6276-85.
85. Kehlenbach, R.H., et al., *A role for RanBP1 in the release of CRM1 from the nuclear pore complex in a terminal step of nuclear export.* J Cell Biol, 1999. **145**(4): p. 645-57.
86. Oka, M., et al., *The mobile FG nucleoporin Nup98 is a cofactor for Crm1-dependent protein export.* Mol Biol Cell, 2010. **21**(11): p. 1885-96.
87. Englmeier, L., et al., *RanBP3 influences interactions between CRM1 and its nuclear protein export substrates.* EMBO Rep, 2001. **2**(10): p. 926-32.
88. Lindsay, M.E., et al., *Ran-binding protein 3 is a cofactor for Crm1-mediated nuclear protein export.* J Cell Biol, 2001. **153**(7): p. 1391-402.
89. Koyama, M., N. Shirai, and Y. Matsuura, *Structural insights into how Yrb2p accelerates the assembly of the Xpo1p nuclear export complex.* Cell Rep, 2014. **9**(3): p. 983-95.
90. Bernad, R., et al., *Nup358/RanBP2 attaches to the nuclear pore complex via association with Nup88 and Nup214/CAN and plays a supporting role in CRM1-mediated nuclear protein export.* Mol Cell Biol, 2004. **24**(6): p. 2373-84.
91. Schwartz, M., et al., *Analysis of the initiation of nuclear pore assembly by ectopically targeting nucleoporins to chromatin.* Nucleus, 2015. **6**(1): p. 40-54.
92. Prasad, S., J. Ravindran, and B.B. Aggarwal, *NF-kappaB and cancer: how intimate is this relationship.* Mol Cell Biochem, 2010. **336**(1-2): p. 25-37.
93. Perkins, N.D., *Integrating cell-signalling pathways with NF-kappaB and IKK function.* Nat Rev Mol Cell Biol, 2007. **8**(1): p. 49-62.
94. Wang, J., et al., *RelA/p65 functions to maintain cellular senescence by regulating genomic stability and DNA repair.* EMBO Rep, 2009. **10**(11): p. 1272-8.
95. Chien, Y., et al., *Control of the senescence-associated secretory phenotype by NF-kappaB promotes senescence and enhances chemosensitivity.* Genes Dev, 2011. **25**(20): p. 2125-36.

96. Hsu, L.C., et al., *IL-1beta-driven neutrophilia preserves antibacterial defense in the absence of the kinase IKKbeta*. Nat Immunol, 2011. **12**(2): p. 144-50.
97. Mankan, A.K., et al., *TNF-alpha-dependent loss of IKKbeta-deficient myeloid progenitors triggers a cytokine loop culminating in granulocytosis*. Proc Natl Acad Sci U S A, 2011. **108**(16): p. 6567-72.
98. Stein, S.J. and A.S. Baldwin, *Deletion of the NF-kappaB subunit p65/RelA in the hematopoietic compartment leads to defects in hematopoietic stem cell function*. Blood, 2013. **121**(25): p. 5015-24.
99. Zhang, J., et al., *Loss of IKKbeta but Not NF-kappaB p65 Skews Differentiation towards Myeloid over Erythroid Commitment and Increases Myeloid Progenitor Self-Renewal and Functional Long-Term Hematopoietic Stem Cells*. PLoS One, 2015. **10**(6): p. e0130441.
100. Dai, Y., M. Rahmani, and S. Grant, *An intact NF-kappaB pathway is required for histone deacetylase inhibitor-induced G1 arrest and maturation in U937 human myeloid leukemia cells*. Cell Cycle, 2003. **2**(5): p. 467-72.
101. Song, M.G., et al., *NRF2 Signaling Negatively Regulates Phorbol-12-Myristate-13-Acetate (PMA)-Induced Differentiation of Human Monocytic U937 Cells into Pro-Inflammatory Macrophages*. PLoS One, 2015. **10**(7): p. e0134235.
102. Fu, S.C., et al., *ValidNESs: a database of validated leucine-rich nuclear export signals*. Nucleic Acids Res, 2013. **41**(Database issue): p. D338-43.
103. Thakar, K., et al., *Identification of CRM1-dependent Nuclear Export Cargos Using Quantitative Mass Spectrometry*. Mol Cell Proteomics, 2013. **12**(3): p. 664-78.
104. Zhou, M.H. and Q.M. Yang, *NUP214 fusion genes in acute leukemia*. Oncology Letters, 2014. **8**(3): p. 959-962.
105. Kawase, H., et al., *NAP-I is a functional homologue of TAF-I that is required for replication and transcription of the adenovirus genome in a chromatin-like structure*. Genes Cells, 1996. **1**(12): p. 1045-56.
106. Kato, K., M. Okuwaki, and K. Nagata, *Role of Template Activating Factor-I as a chaperone in linker histone dynamics*. Journal of Cell Science, 2011. **124**(19): p. 3254-3265.
107. Ben Abdelali, R., et al., *SET-NUP214 is a recurrent gamma delta lineage-specific fusion transcript associated with corticosteroid/chemotherapy resistance in adult T-ALL*. Blood, 2014. **123**(12): p. 1860-1863.
108. Zhu, H.H., et al., *B-cell acute lymphoblastic leukemia associated with SET-NUP214 rearrangement: A case report and review of the literature*. Oncology Letters, 2016. **11**(4): p. 2644-2650.
109. Ozbek, U., et al., *SET-CAN, the product of the t(9;9) in acute undifferentiated leukemia, causes expansion of early hematopoietic progenitors and hyperproliferation of stomach mucosa in transgenic mice*. American Journal of Pathology, 2007. **171**(2): p. 654-666.
110. Saito, S., et al., *Impairment of erythroid and megakaryocytic differentiation by a leukemia-associated and t(9;9)-derived fusion gene product, SET/TAF-I beta-CAN/NUP214*. Journal of Cellular Physiology, 2008. **214**(2): p. 322-333.
111. Argiropoulos, B. and R.K. Humphries, *Hox genes in hematopoiesis and leukemogenesis*. Oncogene, 2007. **26**(47): p. 6766-6776.
112. Fröhling, S., et al., *HOX gene regulation in acute myeloid leukemia: CDX marks the spot?* Cell Cycle, 2007. **6**(18): p. 2241-5.
113. Thiel, A.T., et al., *MLL-AF9-induced leukemogenesis requires coexpression of the wild-type Mll allele*. Cancer Cell, 2010. **17**(2): p. 148-59.

114. Chen, C.W. and S.A. Armstrong, *Targeting DOT1L and HOX gene expression in MLL-rearranged leukemia and beyond*. Experimental Hematology, 2015. **43**(8): p. 673-684.
115. Luo, H.C., et al., *CTCF boundary remodels chromatin domain and drives aberrant HOX gene transcription in acute myeloid leukemia*. Blood, 2018. **132**(8): p. 837-848.
116. Rawat, V.P., R.K. Humphries, and C. Buske, *Beyond Hox: the role of ParaHox genes in normal and malignant hematopoiesis*. Blood, 2012. **120**(3): p. 519-27.
117. Collins, C.T. and J.L. Hess, *Deregulation of the HOXA9/MEIS1 axis in acute leukemia*. Current Opinion in Hematology, 2016. **23**(4): p. 354-361.
118. Van Vlierberghe, P., et al., *The recurrent SET-NUP214 fusion as a new HOXA activation mechanism in pediatric T-cell acute lymphoblastic leukemia*. Blood, 2008. **111**(9): p. 4668-4680.
119. Oka, M., et al., *Chromatin-bound CRM1 recruits SET-Nup214 and NPM1c onto HOX clusters causing aberrant HOX expression in leukemia cells*. Elife, 2019. **8**.
120. Shimoyama, T., et al., *Synergistic action of MLL, a TRX protein with template activating factor-I, a histone chaperone*. FEBS Lett, 2005. **579**(3): p. 757-62.
121. Saito, S., et al., *Leukemia-Associated Nup214 Fusion Proteins Disturb the XPO1-Mediated Nuclear-Cytoplasmic Transport Pathway and Thereby the NF-kappaB Signaling Pathway*. Mol Cell Biol, 2016. **36**(13): p. 1820-35.
122. Adler, H.T., et al., *HRX leukemic fusion proteins form a heterocomplex with the leukemia-associated protein SET and protein phosphatase 2A*. Journal of Biological Chemistry, 1997. **272**(45): p. 28407-28414.
123. Shimoyama, T., et al., *Synergistic action of MLL, a TRX protein with template activating factor-I, a histone chaperone*. Febs Letters, 2005. **579**(3): p. 757-762.
124. Saito, S., et al., *Leukemia-Associated Nup214 Fusion Proteins Disturb the XPO1-Mediated Nuclear-Cytoplasmic Transport Pathway and Thereby the NF-kappa B Signaling Pathway*. Molecular and Cellular Biology, 2016. **36**(13): p. 1820-1835.
125. Yokoyama, A., et al., *Leukemia proto-oncoprotein MLL is proteolytically processed into 2 fragments with opposite transcriptional properties*. Blood, 2002. **100**(10): p. 3710-8.
126. Ayton, P.M., E.H. Chen, and M.L. Cleary, *Binding to nonmethylated CpG DNA is essential for target recognition, transactivation, and myeloid transformation by an MLL oncoprotein*. Molecular and Cellular Biology, 2004. **24**(23): p. 10470-10478.
127. Fornes, O., et al., *JASPAR 2020: update of the open-access database of transcription factor binding profiles*. Nucleic Acids Res, 2020. **48**(D1): p. D87-D92.
128. Ernst, P., et al., *MLL and CREB bind cooperatively to the nuclear coactivator CREB-binding protein*. Molecular and Cellular Biology, 2001. **21**(7): p. 2249-2258.
129. Muntean, A.G., et al., *The PAF Complex Synergizes with MLL Fusion Proteins at HOX Loci to Promote Leukemogenesis*. Cancer Cell, 2010. **17**(6): p. 609-621.

Acknowledgements

I would like to thank my supervisor Dr. Shoko SAITO for the great support and motivation she gave from the beginning to the end of my doctoral studies, who patiently contributed to this thesis without withholding his knowledge, experience, time, and assistance.

Also, I would like to express my endless thanks to my academic advisor Dr. Mitsuru OKUWAKI, I am very grateful to you for your kindness and beautiful mind. Thanks to you, I was able to easily overcome many problems.

Stating that my doctorate in Japan as an important turning point for me, I would like to thank my academic advisor at the University of Tsukuba, Dr. Kyosuke NAGATA, for his initiatives and mind improving scientific challenges.

I would like to thank members at the Laboratory of Biochemistry at Kitasato University; namely Mogi, Yoshi, Nishi, Hashi ... and the members at the Laboratory of Molecular Virology at University of Tsukuba, nick namely; Kaz, Non, Cha, KenS, Noby, Randy, Koh, Moe, Poly, Kuma, Dogan ...様 for all their experience, scientific discussion and support throughout my thesis work.

Finally, I would like to thank my dear parents, siblings, wife, and joyful children who have always supported me.

I would like to thank Dr. Minoru YOSHIDA (RIKEN) for providing pHCF1 vector and Dr. Masao SETO (Aichi Cancer Center Research Institute) for MLL vector. This work was supported by by grants from JSPS KAKENHI (24790309 to SS, 25291001 to KN and 17K08620 to SS) and Takeda Science Foundation (SS).

In memory of my relatives, whom I lost due to cancer and various reasons, who encouraged my motivation in the realization of this study.