Role of the oncoprotein FOXM1 in NPM-ALK+ anaplastic large cell lymphoma

by

Moinul Haque

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Department of Laboratory Medicine and Pathology

University of Alberta

ABSTRACT

Forkhead Box M1 (FOXM1) is a transcription factor which belongs to the evolutionary conserved Forkhead protein family. FOXM1 has been implicated in the pathogenesis of several solid and hematologic cancers. In this study, the significance of FOXM1 in NPM-ALK-positive anaplastic large cell lymphoma (NPM-ALK+ ALCL)—a childhood cancer—was assessed, with a focus on how FOXM1 interacts with NPM-ALK, a key oncogenic driver in these tumors. FOXM1 was highly expressed in NPM-ALK+ ALCL cell lines, patient samples and tumors arising in NPM-ALK transgenic mice. FOXM1 was found localized in the nuclei and confirmed to be transcriptionally active in NPM-ALK+ ALCL cells. Inhibition of FOXM1 in NPM-ALK+ ALCL cells using shRNA or a pharmacologic agent (thiostrepton) resulted in significant reductions in cell growth and soft-agar colony formation, which were associated with apoptosis and cell-cycle arrest. FOXM1 is functionally linked to NPM-ALK, as FOXM1 enhanced the phosphorylation of the NPM-ALK/STAT3 axis. NPM-ALK was found to influence the transcriptional activity of FOXM1. Specifically, FOXM1 failed to effectively bind to DNA when NPM-ALK was siRNA-silenced or when kinase-dead NPM-ALK variant was tested. Moreover, FOXM1 was found to coimmunoprecipitate with NPM-ALK, which was previously shown to exist as NPM-ALK:NPM1 heterodimers in the nuclei. Further studies showed that this binding of FOXM1 to NPM-ALK hinges on the meditator nucleophosmin (NPM1) and the phosphorylation status of NPM-ALK. In conclusion, this study identified FOXM1 as an important oncogenic protein in NPM-ALK+ ALCL. This study exemplified that NPM-ALK can exert oncogenic effects in the nuclei by regulating FOXM1 and illustrated an additional role of NPM1 in the context of NPM-ALK pathobiology.

PREFACE

This thesis is an original work by Moinul Haque. The research project of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name: STUDY OF BIOLOGY OF ALK IN HUMAN ALK+ CANCERS, No. Pro00062737, Feb 17th of 2016). Some of the research conducted for this thesis forms part of an international research collaboration with Professor Suzanne Turner at the University of Cambridge and Professor Adam Karpf at the University of Nebraska Medical Center, with Professor Raymond Lai being the lead collaborator at the University of Alberta.

The research in this thesis has been accepted for publication as: “Haque M, Li J, Huang Y, Almowaled M, Barger C, Karpf A, Wang P, Chen W, Turner S and Lai R. NPM-ALK is a key regulator of the oncoprotein FOXM1 in ALK-positive anaplastic large cell lymphoma.” *Cancers* 2019. I wrote the manuscript, performed most of the experimental work and prepared data and figures. J.L., W.H. and M.A. provided assistance with preparation of the data and figures, particularly with Figures 1B and 1C; Figure 3B; Figure 4C and 4H and Figure 5A. C.J.B. and A.R.K. provided material including the FOXM1 expression and reporter vectors and provided significant support for the project. A.R.K., P.W., and W.C. provided intellectual input into the preparation of the manuscript. S.D.T contributed to the writing of the manuscript and provided necessary reagents, namely the murine tumor sections expressing NPM-ALK. R.L. supervised the study and contributed significantly to the writing of the manuscript.

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LIST OF ABBREVIATIONS

AKT - Protein Kinase B

ALCL - Anaplastic Large Cell Lymphoma

ALK - Anaplastic Lymphoma Kinase

ALK+ ALCL – Anaplastic Lymphoma Kinase Positive Anaplastic Large Cell Lymphoma

ALK- ALCL – Anaplastic Lymphoma Kinase Negative Anaplastic Large Cell Lymphoma

AML - Acute Myeloid Leukemia

AURKA - Aurora A Kinase

B-ALL - B-cell Acute Lymphoblastic Leukemia

CCNB1 - Cyclin B1

CDC25 - Cyclin Division Cycle 25

CDK - Cyclin Dependent Kinase

CENPA - Histone H3-like centromeric protein A

CENPB - Histone H3-like centromeric protein C

CENPF - Histone H3-like centromeric protein F

CFSE - Carboxyfluorescein succinimidyl ester

CHOP - Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone

CREB - cAMP response element-binding protein

CSK1 - Serine/threonine-protein Kinase csk1

DLBCL - Diffuse Large B-Cell Lymphoma

DMNT1 - DNA (cytosine-5)-methyltransferase 1

DN - Double Negative

DP - Double Positive

EML4-ALK - Echinoderm microtubule-associated protein-like 4 - Anaplastic Lymphoma Kinase

FDA - Federal Drug Administration

FDBD - Forkhead DNA Binding Domain

FHRE - Forkhead Response Element

FOX - Forkhead Box

FoxM1 - Forkhead Box M1 (mouse)

FOXM1 - Forkhead Box M1 (human)

GFP - Green Fluorescent Protein

HDAC1 - Histone Deacetylase 1

ISP - Immature Single Positive

JAK/STAT - Janus Kinases/Signal Transducer and Activator of Transcription proteins

JNK1 - c-Jun N-terminal Kinases

LTK - Leukocyte Tyrosine Kinase

MAPK (RAS-RAF-MEK-ERK) - Mitogen-Activated Protein Kinase Cascade

MK - Midkine

MMP-2 - Matrix Metalloproteinase-2

MMP-9 - Matrix Metalloproteinase-9

MYC - Myelocytomatosis protein

NPM1 - Nucleophosmin 1

NPM-ALK - Nucleophosmin 1-Anaplastic Lymphoma Kinase

NRD - N-terminal Negative Regulatory Domain

NSCLC - Non-Small Cell Lung Cancer

OD - Oligomerization Domain

OS - Overall Survival

PARP - Poly-ADP Ribose Polymerase

PBMC - Peripheral Blood Mononuclear Cells

PI3K - Phosphoinositide 3-kinase

PLK1 - Polo Like Kinase 1

PTN - Pleiotrophin

RB - Retinoblastoma Protein

SCF - Skp1/Cullin/F-box protein

SHP1 - Src homology region 2 domain-containing phosphatase-1

shRNA - Short Hairpin RNA

siRNA - Short Interfering RNA

SKP2 - S-phase Kinase Associated Protein 2

SOX2 -SRY (sex determining region Y)-box 2

STAT3 - Signal Transducer and Activator of Transcription 3

TAD - C-terminal Acidic Transactivating Domain

TCR - T Cell Receptor

TKD - Tyrosine Kinase Domain

TPM3 - Tropomyosin 3

VGEF - Vascular Endothelial Growth Factor

Chapter 1: Introduction

1.1 FOXM1 Overview

Forkhead Box M1 (FoxM1) is a member of the *FOX* transcription factor family and was the originally named as Trident during its first discovery in 1997 (1). FoxM1 was originally identified as an important factor driving the proliferation of lymphocytes (2). Since then, FOXM1 has been identified to have a variety of functions in mammalian biology and possessing important roles in human disease (reviewed in (3, 4)). In the first section of this Chapter, the following items will be discussed: pertinent information about FOXM1's protein family; FOXM1 structure and isoforms; FOXM1's regulation of the cell cycle; FOXM1 in the context of development; FOXM1's role of solid and hematopoietic cancers; diagnostic, prognostic and therapeutic inhibition surrounding FOXM1 will be discussed.

1.1.1 FOX Protein Family

Forkhead box (FOX) family of proteins are a group of evolutionary conserved transcription factors (5). The name forkhead originates from the *Drosophila melanogaster* gene fork head (*fkh*) (6). Hundreds of *Fox* genes have been identified in mammals which have been further classified into over 40 subfamilies (7). This ranges from *FoxA* to *FoxP*, with each gene subfamily possessing a unique influence in mammalian cell biology (8). The shared feature between *Fox* genes is the highly conserved ~100-residue forkhead DNA-binding domain (FDBD), usually located in the center of the protein (5). The FDBD binds the Forkhead binding elements (FHRE) in mammalian genomes (5). The FDBD consists of three α-helices (H1, H2 and H3), three β-sheets (S1, S2 and S3) and two wing regions (W1 and W2) which borders the third β-sheet. These domains are arranged as such: H1–

S1–H2–turn–H3–S2–W1–S3–W2. The two wing regions flank the third helix H3, such of that as a butterfly, which is why Fox proteins are termed to contain the winged-helix motif (5). Besides the FDBD, *Fox* genes do not share significant sequence similarity elsewhere in their genes (5). Due to this reason, *Fox* genes display a set diverse set of evolutionary functions. For example, *Fox* genes control multiple aspects of embryonic development, including cell cycle progression, differentiation of T lymphocytes and formation of body structures such as the inner ear (7, 8). Alterations of *FOX* genes in humans can result in a range of phenotypes in disease, including cancer formation, glaucoma and language impediments (7, 8).

1.1.2 Forkhead Box M1 Discovery

Structurally, the FOXM1 protein contains the N-terminal negative regulatory domain (NRD), winged helix DNA binding domains, and C-terminal acidic transactivating domains (TAD) (3, 5, 9). Various cellular proteins, in some cases FOXM1 itself, bind and modify the NRD/TAD domains through phosphorylation or other modifications, which fine tune FOXM1 transcriptional activity. The consensus sequence of FOXM1 through which it binds FHRE is believed to be AT/CAAAAT/CA; T/C are interchangeable (3, 10).

Three FOXM1 isoforms have been identified: a, b, c (11, 12). The difference between the isoforms are due to alternative splicing of two exon cassettes (A1 and A2) . *FOXM1A* contain the additional exons A1 and A2, whereas *FOXM1C* contains only the A1 exon. *FOXM1B* does not contain either exons. The presence of the A2 domain in the FOXM1A protein interferes with its ability to bind DNA. Thus, FOXM1A is inactive as it fails to

activate transcription. *FOXM1C*, contains one of the isoforms is partially active. *FOXM1B* which doesn't possess either of these exons is believed to be the isoform that is most transcriptionally active (11, 12).

1.1.3 Role of FOXM1 in Cell Cycle Regulation

FOXM1 was originally identified as an essential factor for cell-cycle regulation (2). The expression of FOXM1 is normally restricted in proliferating cells, and its expression is completely absent in quiescent and differentiated cells (13). In normal cells, the expression of FOXM1 is tightly regulated, often requiring some form of an external stimulus from an upstream signal cascade for it to become activated (13). During cell cycle progression, the presence of FOXM1 expression is noticeable starting from late G1 and sustained throughout the S, G2 and M phases (14). The expression of FOXM1 is highest during the G2 and M phases. During this period, FOXM1 controls the expression and activity of cell- genes governing the cell cycle such as of *CDC25A/B, CCNB1*, *PLK1* and *AURKA* (15). FOXM1 also controls the transcription of *SKP2* and *CKS1*, to form the Skp1/cullin/F-box protein (SCF) complex (16-18). By doing so, FOXM1 can dictate for the ubiquitinylation and degradation of cell cycle inhibitors. FOXM1 also controls the expression of genes responsible for the integrity of chromosomes and segregation during mitosis such as *CENPA*, *CENPB* and *CENPF* (16). Therefore, it is no surprise that the most common phenotype observed with the ablation of FOXM1 is mitotic cell death—chromosomal misalignment and duplication defects, lack of spindle formation, cytokinesis failure—as cells are unable to properly conduct cell division (19).

The transcriptional activity of FOXM1 is repressed by ubiquitination and at residues located at the N-terminal region during in G1 and early S phases (9, 20). In contrast, phosphorylation of FOXM1 at its C-terminus, such as the Thr596 residue, by cyclin-CDK complexes initiate its transcriptional activity (21-23). Phosphorylation of FOXM1 at this residue recruits the transcriptional co-activator proteins p300/CREB, which enhances FOXM1 transcriptional activity (22, 23). Furthermore, phosphorylation of FOXM1 via Raf/MEK/MAPK pathway at serine residues (S331 and S701) stimulates FOXM1 nuclear translocation and thereby its transcriptional activity between the G2 and M phases (14). Lastly, the expression of FOXM1 forms a positive feedback loop as FOXM1 is dependent on one of its downstream targets, PLK1, for its phosphorylation and activation (24, 25). The structure and regulatory regions are demonstrated in Figure 1.1

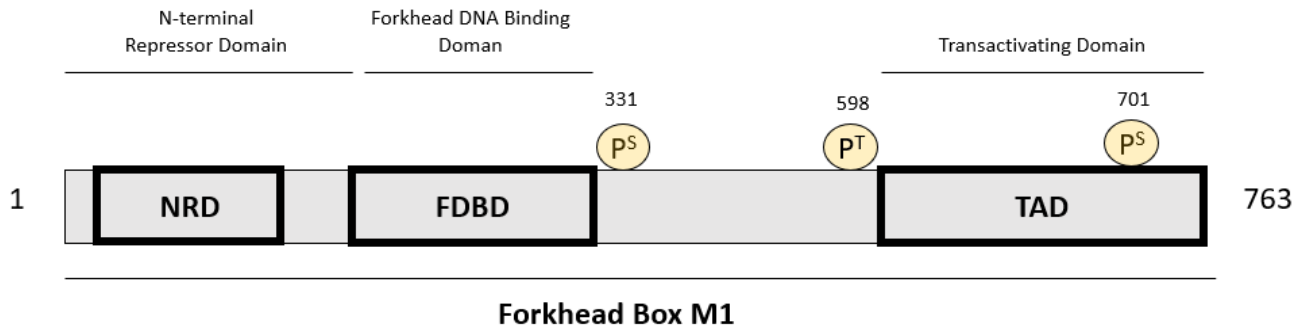

Figure 1.1 Structure of Forkhead Box M1 (FOXM1). The regulatory elements NRD (N-terminal Repressor Domain), Forehead DNA Binding Domain (FBDB) and Transactivating Domain (TAD) are highlighted with black boxes. Important regulatory residues at serine and threonine sites have been indicated.

1.1.4 Expression of FoxM1 in Early Development

During embryonic development of animals, such as in mice, the expression of *FoxM1* is believed to be ubiquitous; *FoxM1* null mice are embryonically lethal (26). The human FOXM1 and mouse FoxM1 ortholog share 79% sequence similarity (1). As an organism is conceived the expression levels of FoxM1 in most tissues decrease and its expression becomes spatially restricted (14, 27). In adult mice, the expression of *FoxM1* is only present in tissues with high proliferative index such as the colon, small intestine, testis and thymus (14). In addition, FOXM1 expression can be found in the progenitor cells of almost all which show high self-renewal capacity (28). In contrast, FOXM1 is usually not present in quiescent or terminally differentiated cells (29). It is held that in normal tissue, only the FOXM1c isoform can be identified (14).

Hou *et al* (2015) identified that the deletion of FoxM1 from hematopoietic stem cells significantly affected the amount of white blood cells, including lymphocytes (29). Ablation of FoxM1 resulted in a delayed entry of cells into S and M phases of the cell cycle (29). However, deletion of FoxM1 was not associated with a reduction of stem cell factors such as Sox2 or downstream target genes of β-catenin (29).

1.1.5 Expression and Biological Role of FoxM1 in T-Cell Development

A dynamic change of FoxM1 expression and activity occurs during the development of T lymphocytes. While FoxM1 is not present in early double negative (DN) T lymphocytes, characterized by the CD44+/CD25- cell markers, the expression of FoxM1 begins in the

DN T cells when both CD44+/ CD25+ cell surface markers are acquired (30). During this stage of lymphocyte development, T-cells are actively proliferating in the medulla of the thymus (31). FoxM1 expression continues to increase throughout DN3 (CD44-/CD25+) and DN4 (CD44-/CD25-) stages of T-cell development (2). The expression level of FoxM1 reaches a peak when immature single positive (ISP) cells are produced (2). At this stage of T-cell development, cells are undergoing rapid cell division (31). The expression level of FoxM1 decreases as T lymphocytes reach the double positive (DP) CD4+/CD8+ stage, when cells are undergoing TCRα rearrangements (31). T cells do not possess high proliferation rates during the DP stage (31). As T lymphocytes undergo migrate and selection in the medulla of the thymus, the expression levels of FoxM1 disappears (2). FoxM1 is not present in mature CD4+/CD3+ or CD8+/CD3+ T cells (30). This process is summarized in Figure 1.2.

In resting peripheral blood lymphocytes (which are G0 cells), FoxM1 expression is not present (2). However, upon stimulation of lymphocytes with agents such as phytohaemagglutinin (PHA), FoxM1 expression can be significantly increased within 24 hours (2). This suggests that FoxM1 is important for dividing lymphocytes.

The activity of FoxM1 is tightly regulated during T cell development. The ablation of FoxM1 from different stages of T cell development in transgenic mice produces unique and somewhat unrelated effects (30). When FoxM1 deleted from DN cells (early stage, before the TCR mediated proliferation takes place between DN3 and DN4 stages, a 2-fold reduction takes place in the number of total and DP thymocytes (30). Interestingly,

the number of DN cells remains the same. Differences can only start to be seen when immature single positive cells are produced (30).

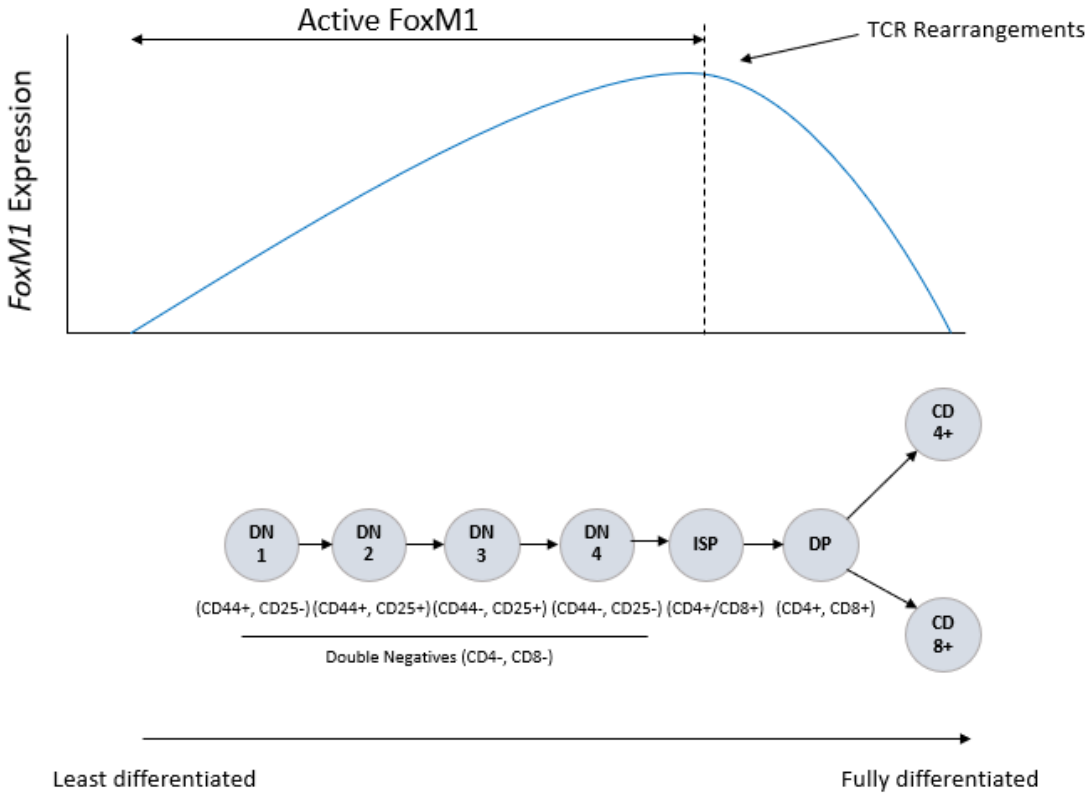

Figure 1.2 The expression and activity of FoxM1 through T-cell development. *FoxM1* expression and activity begins in the double negative (DN) stages. Expression of *FoxM1* is at maximum during the immature Single Positive (ISP) cells where cells are either CD4+ and CD8+. The expression and activity of *FoxM1* are significantly lower following TCR Rearrangements during which period immature thymocytes undergo selection to become mature, differentiated CD4+ Helper T and CD8+ cytotoxic T cells.

1.2 Expression FOXM1 in Cancers

Research in the past last two decades have largely focused on the significance of FOXM1 in driving tumorigenesis. Recently, FOXM1 has been a popular topic in the molecular biology of cancer, as in the past five years alone, at least 70% of the publications involving FOXM1 has been published. Unlike most FOX proteins which contain role anti-apoptotic roles in cancer (8), the sustained expression of FOXM1 in cancer can regulate several oncogenic signalling molecules, giving cancer cells a proliferative and survival advantage (32). Indeed, in most cancer models, the blockade of FOXM1 through genetic and pharmacological means significantly reduces cancer growth potential (32). In this section, the expression of FOXM1 in solid tumors, hematological malignancies, prognostic, diagnostic and pharmacological inhibitors of FOXM1 will be discussed.

1.2.1 FOXM1 in Solid Tumors

In cancer, FOXM1 was initially studied in the infamous HeLa (human cervical carcinoma) cell line (33). Since then, the presence of FOXM1 (presumably FOXM1b) has been noted in most human solid cancers. From breast cancer (with highest expression in triple negative breast cancer) (34-69), ovarian cancer (70-82), lung cancer (83-100), colon cancer (101-104), liver cancer (105-116), pancreatic cancer (104, 117-127), prostate cancer (128-133), glioblastoma (134-142) and neuroblastoma (143-147), FOXM1 expression has been confirmed in both primary cancer cells and in cell line models. The inhibition of FOXM1 through genetic modulators like siRNA/shRNA or through the chemical inhibitors of FOXM1, is usually associated with decreased in vitro cell growth,

proliferation, migration, invasion, self-renewal capacity, angiogenesis and in vivo tumor propagating ability in cell lines, mouse xenograft models and transgenic mouse models (4). Therefore, roles of FOXM1 in cancer fit the hallmarks of cancer development. Interestingly, while FOXM1 is found in many cancer types, genome wide studies have rarely associated genetic aberrations such as mutations or amplifications of FOXM1 in cancer (148).

FOXM1 contributes to cell proliferation and growth in most cancers. Some specific examples include the following: in lung, liver and colon cancer mouse models, deletion of the FOXM1 gene contributes to significantly decreased tumor size (4). FOXM1 knockdown mice formed significantly smaller tumor size and showed prolonged survival glioblastoma (134). The reduction of FOXM1 by siRNA/shRNA in various cancer cell lines (lung, prostate, liver, breast, colon and cervix) reduces cell proliferation and colony forming ability (4). In a hepatocellular carcinoma, constitutive enforced expression of FoxM1B did not induce cancer, however, the presence of FoxM1B resulted in significant proliferation of neoplastic cells following tumorigenesis (26). On the other hand, it has been known that xenografts containing *FoxM1* overexpression showed increased tumor number and size in liver, prostate and colon cancer models (4). These effects are likely since FOXM1 is a master regulation of cell cycle proteins, and its knockdown is associated with decreased expression of Cyclin A2, Cyclin B1, and Cdc25 phosphatases, and increased expression of the cell cycle inhibitors p21 Waf1/Cip1 and p27Kip1 (4). These findings indicate that the sustained expression of FOXM1 can be a mechanism by which oncogenes provide a proliferative advantage to cancer cells.

The reason why FOXM1 is found and activated in most cancer types could be due to its relationship with the p53 and Rb tumor suppressor pathways (149). It is a known fact that most human cancers have defects or mutations in the p53 and RB tumor suppressor pathways (150). FOXM1 has been found to be a gene that is directly repressed by the p53 pathway. Inhibiting the p53 pathways, activates FOXM1. Moreover, in turn activated FOXM1 can repress p53 (150). Likewise, FOXM1 is also repressed by the RB protein (151). Consequently, defects RB in turn activate E2F which has been shown to upregulate FOXM1. Furthermore, FOXM1 has been also shown to interact with and influence the localization of many other important signal transducers in cancer including that of STAT3 and β-catenin (139, 142, 152-154).

Apart from roles in proliferation, the expression of FOXM1 in cancer has been also associated with an inhibition of cell senescence. The downregulation of FOXM1 contributed to increased p53, p16 and p27Kip1 expression in a gastric cancer model (155). Furthermore, it has been shown that FOXM1 can override cellular senescence induced by hydrogen peroxide by upregulating the Myc/Bmi-1 pathway (156). Lastly, mouse fibroblasts with deleted FoxM1 experience spontaneous cellular senescence (155). FOXM1 has also noted to promote the Warburg effect and alter the biochemical pathways of metabolism (74, 157).

FOXM1 may also hold important functions in maintaining populations of cancer stem cells. The overexpression of FOXM1 suppressed oral keratinocyte differentiation and induced the expansion of stem cell-like cell (158). In another lung cancer model FoxM1

overexpression expanded the stem like Clara cells (159). In a neuroblastoma cell line, it was found that FoxM1 is involved in maintaining the tumorigenicity of neuroblastoma cells by upregulating the transcription factor SOX2 (143). Moreover, the expression of SOX2 by FOXM1 has been linked to chemoresistance in a glioblastoma model (141). Lastly, a report in a human teratocarcinoma model showed that FOXM1 was directly responsible for stem cell renewal and prevention of differentiation through the expression of the pluripotency genes Oct4, Nanog and Sox2 (160).

FOXM1 has roles in tissue injury repair through which it may contribute to cancer. Additionally, FOXM1 has been shown to modulate the levels of VEGF secretion which is responsible for angiogenesis in a brain, breast, gastric, hepatic, colon and pancreatic cancer model (119, 161-164). One study identified that FOXM1 can directly regulate expression of *VGEF* by binding to FHREs in its promoter region (165). that Lastly, FOXM1 contributes to cancer cell migration and metastasis by the induction of metalloproteinase proteins (MMP-2 and MMP-9) which aid in epithelial to mesenchymal transition (EMT) and the spread of cancer cells. The regulation of MMP-2 and MMP-9 by FOXM1 has been revealed in numerous studies (84, 166-171). According to Chen *et al* (2013) FOXM1 also directly controls the expression of MMP-2 by binding to its promoter (167). In contrast, the expression of MMP-9 indirectly through its downstream target gene, JNK1 (172). Furthermore, FOXM1 was demonstrated to drive cancer metastasis in hepatocellular carcinoma by enhancing EMT. It was shown that in absence of Arf, FOXM1 enhances EMT through the PI3K/AKT pathway and influencing several cytoskeletal proteins (106, 173). Lastly, there has been several additional studies that the expression FOXM1 can

lower sensitivity of anti-cancer drugs. The overexpression of FOXM1 lead to the development of acquired drug resistance. It is believed that the interaction of FOXM1 with FOXO3a the PI3K/AKT pathway is often the point that is altered when drug resistance takes place (37, 174-176). The role of FOXM1 in cancer is summarized in Figure 1.3

1.2.2 FOXM1 in Hematological Malignancies

The expression and biological significance of FOXM1 in hematologic malignancies has not been extensively investigated. Few published studies have specifically addressed the functional importance of FOXM1 in hematologic cancers. These include precursor B-cell acute lymphoblastic leukemia (B-ALL) (177, 178), plasma cell myeloma (179), diffuse large B cell lymphoma (DBLCL) (180) or acute myeloid leukemia (AML) (181, 182). In these studies, experimental results from *FOXM1* knockout mice and *in vitro* studies in which FOXM1 was inhibited by a using pharmacologic agent such as thiostrepton, or gene knockdown support the concept that FOXM1 contributes to the oncogenicity of these cancers. Nonetheless, the mechanism by which FOXM1 mediates its oncogenic effects is not well-understood.

In the B-ALL study, FOXM1 was found to have high expression in the pre-B-cell receptor checkpoint but were found to be dispensable for normal B-cell development (177). Compared with normal B-cell populations, FOXM1 levels were found to be 2- to 60-fold higher in ALL cells. The high expression of FOXM1 was predictive of poor outcome in ALL patients (177). Furthermore, the study found that FOXO3A negatively regulates FOXM1 is negatively regulated by FOXO3A. The study found FOXM1 supports cell

survival, drug resistance, colony formation and proliferation in vitro, and promotes leukemogenesis in vivo (177).

In the plasma cell myeloma model, overexpression of FOXM1 prognosticates inferior outcome in a subset of patients (179). In experimental cell line models, the enforced expression of FOXM1 increased growth, survival, clonogenicity, and xenograft formation of myeloma cells, whereas knockdown of FOXM1 had the opposite effect (179). The study further found that FOXM1 coregulated and co-immunoprecipitated with of cyclin-dependent kinase 6 (CDK6) and NIMA-related kinase 2 (NEK2), which are important factors in cancer model (179).

In diffuse large B-cell lymphoma (DBLCL) tumors, FOXM1 expression was detected in 84.6% and found to be positively associated with proliferative tumor marker Ki67, MMP-9 and SKP2 and negatively associated with p27 (183, 184). In cell line models, siRNA or thiostrepton treatment reduced expression of the tissue invasion markers MMP-2 and MMP-9 (183). Additionally, the knockdown of FOXM1 was found to decrease invasive and migratory capability, and induced caspase dependent apoptosis via activation of the mitochondrial apoptotic pathway (183).

In acute myeloid leukemia (AML), nuclear expression of FOXM1 was determined to be an independent clinical predictor of chemotherapeutic resistance using multivariate analysis (185). In cell line models, knockdown of FOXM1 significantly reduce the

clonogenicity of AML cells; furthermore, in a transgenic mouse model mimicking AML, constitutive overexpression of FOXM1 in this model induces chemoresistance (185). The use of the proteasome inhibitor ixazomib, which reduces FOXM1 activity, synergized with the chemotherapy drugs cytarabine and 5-azacitidine in reducing tumor growth in these animal models (185).

1.2.3 FOXM1 as a Prognostic Factor

A recent meta-analysis of 23 studies has shown that FOXM1 overexpression is associated with poor prognosis of breast cancer, colorectal cancer, gastric cancer, hepatic cancer, lung cancer, melanoma, and ovarian cancer (186). The expression of FOXM1 was significantly associated with worse 3-year overall survival (OS) (OR = 3.30, 95% CI = 2.56 to 4.25, $P < 0.00001$) 5-year OS (OR =3.35, 95% CI = 2.64 to 4.26, $P < 0.00001$) and 10-year OS (OR = 5.24, 95% CI = 2.61 to 10.52, $P < 0.00001$) in all solid tumors (187). High expression level of FOXM1 also predicted advanced tumor stage. Therefore, FOXM1 could be a useful predictor of cancer progression and may be an effective therapeutic target for cancer treatment.

1.2.4 FOXM1 Inhibitors

Due to the above-mentioned roles of FOXM1 in human cancer, many inhibitors (small molecules and aptamers) are currently being investigated (4). As FOXM1 forms an autoregulatory loop, the inhibition of FOXM1 is associated with a decrease of protein levels over time (188). This is hypothesized due to the upregulation of a negative regulator of FOXM1 (189). Siomycin A was one of the first thiazole compounds to be identified as an inhibitor for FOXM1 (190). Siomycin A decreases the transcriptional activity of FOXM1 and reduces FOXM1 target gene expression (190, 191). Moreover, this compound can reduce clonogenicity of cancer cells and increase apoptosis (192). Thiostrepton, like Siomycin A is a thiazole antibiotic that has been commonly used in the literature to study FOXM1 (189, 191). Some studies have suggested that thiostrepton physically binds to FOXM1 (193). Other proteasome inhibitors such as MG-132 and Bortezomib inhibit the expression of FOXM1 (190). More recently, DNA aptamers of FOXM1 are being developed to inhibit the function of FOXM1 in human cells (194). In general, the inhibition of FOXM1 through these inhibitors showed reduced cell growth, induce apoptosis and reduce tumor volume when xenografts were made in nude mice. The role of FOXM1 in cancer is summarized in Figure 1.3

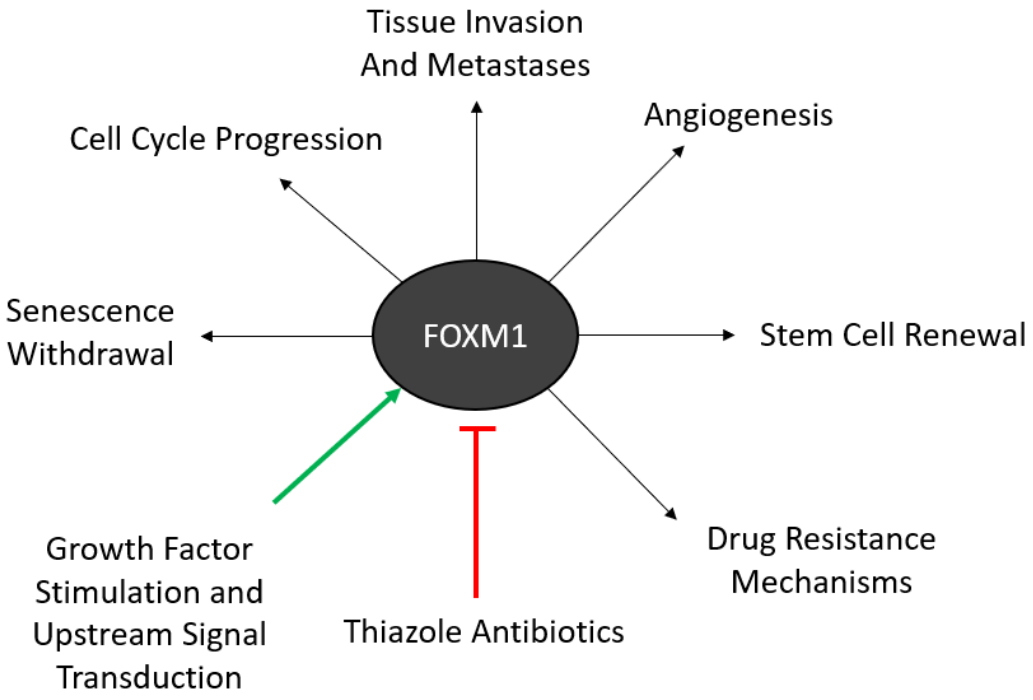

Figure 1.3 Regulators and biological impact of FOXM1 in cancer.

1.3 Anaplastic Lymphoma Kinase Overview

Nearly three decades have passed since the discovery and identification of Anaplastic Lymphoma Kinase (ALK) as a potential cancer-causing gene (195). Years of research into the biology of ALK have resulted in significant clinical advancements for those afflicted with an ALK-implicated cancer. However, the current understanding of ALK continues to evolve as new research sheds light into the role of ALK in an increasing number of human cancers. It is now clear that there is a multi-faceted and complex mechanism through which ALK mediates oncogenesis (195). The targeting of ALK through specific small molecule inhibitors have been approved for ALK positive lung cancers due to their high efficacy and low toxicity, and ALK inhibitor therapy continues to be explored in preclinical and clinical models for several cancer types (195). In this section, the following items will be discussed: background of ALK; significance in human cancers with a focus on anaplastic large cell lymphoma (ALCL); signalling pathways deregulated in cancer; discussion of ALK inhibitors.

1.3.1 Background of ALK

The name of ALK itself reveals details regarding its initial discovery and function. Initially, the high expression of ALK was discovered in ALCL, a rare type of non-Hodgkin's lymphoma (196). This was a significant finding as ALK is not expressed in normal lymphoid cells and served as the ideal diagnostic marker to identify ALCL (197). It was soon found that ALK encoded for a receptor tyrosine kinase which possessed transformation ability when mutated (198).

Structurally, ALK belongs to the insulin receptor tyrosine kinase family (199), sharing the most sequence similarity to the leukocyte tyrosine receptor kinase (LTK) (200). The ALK Tyrosine Kinase Domain (TKD) is evolutionary conserved and contains the YxxxYY motif, which is also found in the Insulin receptor (200, 201). The full length ALK protein contains a ligand binding domain located extracellularly—composed of a low-density lipoprotein receptor domain class A region lying in between two meprin, A-5 protein, multiple receptor protein-tyrosine phosphatase mu regions—followed by a transmembrane domain, and an intracellular tyrosine kinase domain (201). Although ALK is still considered to be an orphan receptor, several ligands of ALK have been suggested such as pleiotrophin (PTN) and midkine (MK) (202, 203). PTN and MK were suggested to activate the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways (202, 204). However, later studies failed to confirm that PTN and MK directly bind to ALK and have suggested an indirect mechanism through by which these ligands activate ALK (205). More recently, chains of heparin, FAM150A and FAM150B have also been suggested as novel ligands that activate the full length ALK receptor (206).

While ALK has been found to be evolutionarily conserved in mammals, suggesting a critical role in mammalian cells, its normal biological function is still not fully clear. In adults, the expression of ALK is only weakly present in certain regions of the brain, small intestine and testis (200, 207). Some studies in model organisms have provided insight that during embryonic development, ALK may have functional roles in the proliferation, survival and maturity of early neuronal progenitors (200, 208).

1.3.2 Genetic Abnormalities of ALK

ALK has been recognized to possess a variety of genetic changes in cancer. These include chromosomal rearrangements, mutations, amplifications and increase of copy number (195). These changes often result in aberrant phosphorylation and constitutive activation or of ALK. Chromosomal rearrangements of ALK are the prevalent genetic abnormality found in ALK+ cancers, whereby the 3' located tyrosine kinase domain of ALK-at the proximity of exon 20 or within intron 19-is fused to the 5' portion of another corresponding gene (195). Common features of the 5' partner include constitutive expression and possessing protein domains that assist in ALK dimerization (209). Structural studies show that most chromosomal rearrangements of ALK result in ligand independent activation of the Tyrosine Kinase Domain (TKD) (209). It should be noted that not that all ALK fusion proteins possess the same degree of oncogenic potential as differences in the 5' partner impacts ALK's dimerization and signaling ability (210). Similarly, certain mutations of full length ALK have been associated with increased phosphorylation potential of ALK by making it ligand independent or by disrupting its auto-inhibitory function (210). Amplifications of ALK significantly increase the overall expression and activity thereby increasing its oncogenic potential (210). Increased copy number of ALK have been also suggested to increase the phosphorylation of ALK, however this does not always correlate with ALK protein expression (210).

1.3.3 ALK in Anaplastic Large Cell Lymphoma

Anaplastic Large Cell Lymphoma was first discovered in 1985 and described as a neoplastic proliferation of lymphoid cells containing large and anaplastic cytology, sinusoidal growth pattern, and strong expression of Ki-1 antigen (CD30) (211). ALK+ ALCL is a type of non-Hodgkin's lymphoma, which primarily affects children and young adults. ALK+ ALCL has higher prevalence in males and comprises about 10-30% of all pediatric lymphomas (212). During disease onset, swollen & enlarged lymph nodes (lymphadenopathy) are observed in the periphery, abdomen and mediastinum (chest). In some cases, extra nodal involvement with the bone, bone marrow and skin are also observed (212). However, the prognosis of ALK+ALCL is good as the five-year survival is around 85%, and for the most part is a curable disease (212). ALK+ ALCL is commonly treated with the CHOP regimen (cyclophosphamide, doxorubicin, vincristine, and prednisone) of chemotherapy (212).

ALK+ ALCL is believed to have originated from activated cytotoxic CD8+T cells, although some cases of ALK+ ALCL do not possess the T-cell antigens and are described as the "null" phenotype (213). In addition, ALK+ ALCL often show reduction or silencing in the expression of the CD3 and the T-cell receptor (TCR) complex. Around 80%-90% of ALK+ALCL show improper TCR gene rearrangement (212).

1.3.4 NPM-ALK and Other ALK Fusion Partners in ALCL

Morris et al (1994) was the first to discover a chromosomal rearrangement of ALK in an ALCL cell line through a positional cloning strategy (196). This was the (2;5)(p23;q35) chromosomal rearrangement, whereby Anaplastic Lymphoma Kinase ALK on 2p23 is fused with NPM1 on 2p23 (196). Nearly 80% of ALCL contain the NPM-ALK translocation, dubbed as NPM-ALK+ ALCL (214). It was shown that enforced expression of NPM-ALK caused transformation of fibroblasts in vitro and can caused lymphoma in *NPM-ALK/CD4* transgenic mice (215, 216). Additionally, the downregulation of NPM-ALK from ALK+ ALCL cell lines dramatically reduce their tumorigenic potential (217). These findings highlighted the of ALK in maintaining the growth and survival of the majority ALCL, due to which NPM-ALK has been dubbed as a major oncogenic driver.

Nucleophosmin 1 (NPM1) is a multifunctional protein that is ubiquitously expressed in mammalian cells (218). Its localization can be detected in both the cytoplasm, nucleus and nucleolus. The canonical understanding of NPM1 is in its role of ribosomal shuttling between the cytoplasm and the nucleus (218). However, it has been noted NPM plays other important roles in gene transcription, DNA repair and maintaining genomic stability (218). The N-terminus domain of NPM1 contains a coiled-coil oligomerization domain which is why NPM1 exists as a homodimer (218). Furthermore, this domain is also represented in NPM-ALK, allowing for the dimerization of NPM-ALK (218). Dimerization

of NPM-ALK results in its auto-phosphorylation, and therefore constitutive activation of the ALK TKD (209). The tyrosine 338, 342 and 343 are crucial phosphotyrosine residues of NPM-ALK (219). The mutation of even one these tyrosine residues to phenylalanine (Y→F) results in partial loss of NPM-ALK phosphorylation, or total loss of activity such as in the triple FFF variant (219). Activation of NPM-ALK regulates numerous downstream signaling pathways that control key cellular processes such as cell cycle progression, survival, cell migration and invasiveness (220). Thus, the oncogenic potential of NPM-ALK is derived from both the dimerization ability of NPM1 and the kinase activity of ALK. The structure of NPM-ALK is shown in Figure 1.4.

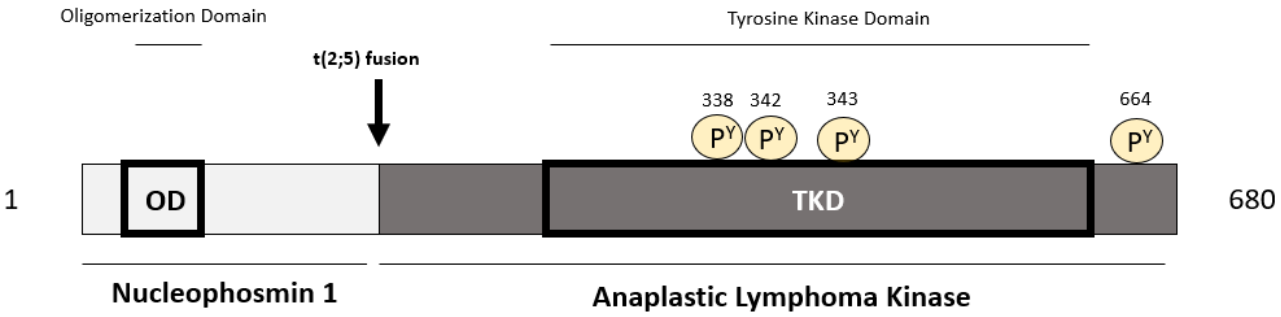

Figure 1.4 Structure of NPM-ALK. The indicated light grey region is the Nucleophosmin region (NPM1) and the dark grey region is the Anaplastic Lymphoma Kinase (ALK) portion of NPM-ALK. The highlighted with black boxes are on the oligomerization domain of NPM1 and the tyrosine kinase domain of ALK. Important regulatory residues at tyrosine sites have been indicated.

Interestingly, unlike most fusion partners which display cytoplasmic localization, NPM-ALK displays both localization in both cytoplasmic and nuclear portions of the cell. This is attributed to NPM1, which contains a nuclear translocalization signal, allowing it to shuttle between the nucleus and cytoplasm (218). While this nuclear translocalization signal is missing from NPM-ALK, due to the oligomerization of NPM-ALK to NPM1, NPM-ALK is able to enter the nucleus (218, 221, 222). Thus, in the cytoplasm, NPM-ALK normally exists as NPM-ALK—NPM-ALK homodimers, while in the nucleus, NPM-ALK typically exists as NPM-ALK—NPM1 heterodimers (218).

Another significant fusion in ALK+ ALCL is TPM3-ALK, contributing to 12%-18% of ALK+ ALCL (223). Several other ALK fusion partners in ALCL have been identified at lesser frequencies (<2%) including TFG, TRK, CLTC, ATIC and TPM4 (214).

1.3.5 NPM-ALK Signalling Pathways

NPM-ALK regulates a host of signalling pathways through which it mediates oncogenesis. The most commonly extensively studied are the JAK/STAT, PI3K/AKT and MAPK (RAS-RAF-MEK-ERK) pathways:

The activation of Signal Transduction and Activator of Transcription 3 (STAT3) by NPM-ALK is one of the most thoroughly studied pathways in ALK+ ALCL. NPM-ALK directly phosphorylates STAT3 at the Y705 residue (224). The phosphorylation of STAT3 leads

to dimerization and nuclear localization upon which STAT3 can regulate numerous genes involved in cell proliferation (Cyclin D, cMYC, B-catenin) and cell survival (Bcl-2, Survivin) (224-228). Furthermore, the activation of STAT3 epigenetically silences the Src homology-2 domain containing protein tyrosine phosphatase 1 (SHP1), which is a negative regulator of the JAK/STAT pathway (229). Activation of STAT3 leads to methylation of the SHP1 (230). STAT3 achieves these functions by upregulating the gene transcription of DNA methyltransferase 1 (DNMT1) and the histone deacetylase 1 (HDAC 1) (231). Indeed, the inhibition of NPM-ALK dramatically impacts the phosphorylation and thereby activation of STAT3 in in ALK+ ALCL.

In the PI3K/AKT pathway, NPM-ALK binds p85 through its phosphorylated Y664 residue, resulting in the phosphorylation of PI3K, and ultimately the activation of the serine/threonine kinase AKT, also known as Protein Kinase B (232). The PI3K/AKT pathway enhances survival of ALK+ALCL cells by communicating anti apoptotic signals (232). Furthermore, activated NPM-ALK induces activation of the PI3K/AKT which results in the hyperphosphorylation and inactivation of the transcription factor FOXO3A (233). The inactivation of FOXO3A inhibits the transcription of the cell cycle progression negative regulator p27Kip1, further contributing to cell cycle progression (233).

Finally, NPM-ALK activates the MAPK pathways through the adapter proteins IRS-1, SHC and Grb-2 (208, 234, 235). RAS activation by NPM-ALK results into phosphorylation of AP-1 transcription factors complex. Ultimately, the effector proteins ERK1/2 proteins are phosphorylated and provide increased cell proliferation and cell viability in these tumors

through enhancing the expression of the anti-apoptotic protein Bcl-xL and the cyclin dependent kinase CDK4 (236).

1.3.6 ALK in Non-Small Cell Lung Cancer

In 2007, the EML4-ALK chromosomal rearrangement was discovered in non-small cell lung cancer (NSCLC) at a frequency of about 6.8% (237). The rearrangement occurs due to a small inversion of chromosome 2 which fuses the echinoderm microtubule-associated protein-like 4 (EML4) gene to ALK, also located on chromosome 2 (237). The EML4-ALK fusion is important to NSCLC diagnostically as it is usually mutually exclusive to EGFR, KRAS and ERBB2 mutations, which may suggest that these genes signal through similar pathways (238).

Interestingly, numerous EML4-ALK variants have been reported in NSCLC. These EML4-ALK variants differ in the location at where they are fused to ALK (239). Very much like NPM-ALK, studies in preclinical models that EML4-ALK possess strong oncogenic properties and ALK+ NSCLC are sensitive to its inhibition (240). Interestingly, several researchers have suggested that the EML4-ALK variants possess varying sensitivity to ALK inhibitors (238). Therefore, understanding the fusion partner of ALK is significant when studying an ALK rearranged cancer. Apart from EML4, other fusion partners of ALK in NSCLC occurring in lesser frequency include SEC31A, HIP1, KLC, KIF5B and TFG (241).

1.3.7 ALK in Other Cancers

The expression of the full length ALK receptor is known to be present in many other cancer types including neuroblastoma, medulloblastoma, inflammatory myofibroblastic tumors, spitzoid tumors, ovarian and thyroid cancers (210). In these malignancies, mutations of ALK at specific residues have been found (210). These mutations result in constitutive phosphorylation of ALK without requiring a ligand molecule (210). For example, in neuroblastoma, mutations of the full length ALK are detected in about 7% of cases (242-245). In the case of familial neuroblastoma, over 50% of cases can be attributed to an ALK mutation (245). R1275Q is the most common germline mutation in familial neuroblastoma (245). The F1174L mutation is the most common mutation from sporadic neuroblastoma cases (244). Furthermore, the expression wild-type ALK has been widely demonstrated in several cancer types (210). In neuroblastoma, an estimated 50-90% of cases possess the expression of wild-type ALK, and the high expression of ALK is linked to poor prognosis (210). Although, whether ALK is an oncogenic driver in neuroblastoma and other cancer is highly disputed and is a subject of further study.

1.3.8 ALK Inhibitors

More than a dozen ALK inhibitors have been created and investigated over the last decade (210). ALK inhibitors are generally small molecule compounds that interfere with the ATP binding cassette, in other words ATP competitive, of ALK's TKD. Thus, ALK is prevented from being phosphorylated. Crizotinib was the first ALK inhibitor to be approved

by the FDA as it showed superior effectivity and safety over conventional treatments (246). In one clinical trial, Crizotinib showed an overall response rate of 65% as compared to 20% in conventional based chemotherapies (247). In another clinical study, Crizotinib treated led to 10.9 months of progression free survival as compared 7 months in conventional therapy (248).

Unfortunately, resistance to Crizotinib was found to be a common feature in ALK positive NSCLC (210). While the exact reasons of resistance are fully understood, some mechanisms of resistance include secondary mutations within of the ALK kinase which prevent the drug's efficacy, amplifications of the fusion kinase, or activation of alternative or bypass signalling pathway(s) (210). Interestingly, the majority of Crizotinib resistant tumors remain sensitive to other ALK inhibitors. Due to this fact, structurally distinct second generation ALK inhibitors have been developed, some of which have been approved by the FDA. These drugs include Certinib and Alectinib, which can overcome drug resistance to Crizotinib (249, 250). In-vitro and in-vivo models have shown that secondary ALK inhibitors much greater specificity and potency towards ALK, albeit being slightly more toxic than Crizotinib (251). Second generation inhibitors also can cross the blood brain carrier and treat metastatic ALK-rearranged NSCLC (210). However, even second generation ALK inhibitors ultimately face resistance (252). As a result, third generation ALK inhibitors have been also developed and are currently under clinical trial. These include Lorlatinib, Brigatinib, ASP3026, Entrectinib and X-396 (210).

1.4 Project Rationale and Objectives

While FOXM1 has been studied extensively in solid malignancies, its functions and roles has not been determined in many hematologic cancers, including NPM-ALK+ ALCL. FOXM1 is known to have an important biological role during embryonic development as well as T-cell differentiation—the cells from NPM-ALK+ ALCL are believed to be derived. Thus, the role of this protein in this cancer could be of importance. It is possible for FOXM1 to be a downstream effector gene that is crucial in the pathogenesis of NPM-ALK+ ALCL. Furthermore, recent studies indicated FOXM1 and NPM-ALK could form a functional relationship based on numerous mutual interacting partners and convergent signalling pathways.

Based on these observations, I hypothesized that FOXM1 was an important oncogenic transcription factor required for the growth and survival of NPM-ALK+ ALCL cells. I further hypothesized that FOXM1 and NPM-ALK demonstrate protein-protein interaction, which influence on their respective function and activity (i.e phosphorylation and/or DNA binding).

The objective of this thesis was to: 1) evaluate the expression and nature of FOXM1 expression in NPM-ALK+ derived cancers 2) demonstrate the biological effect of NPM-ALK+ cancers upon knockdown and inhibition of FOXM1 3) ascertain any functional relationships (i.e protein-protein interactions) between the two oncoproteins NPM-ALK and FOXM1.

Chapter 2: Methods[1]

[1] Jing Li, Yung-Hsing Huang and Meaad Almowaled assisted with cell culture, western blotting, immunoprecipitations and lentiviral transductions

2.1 Cell Lines and Tissues

All NPM-ALK+ ALCL and Jurkat cell lines were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA). HEK 293 and 293T cells were maintained in high glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA). Media were supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA). All cell lines were maintained in a 5% CO_2 atmosphere at 37 °C. Standard tissue culture and aseptic techniques were always used when handling all cell lines.

Suspension cells were constantly maintained at a sub-confluent density, ~200,000-400,000 cells/ml. Roughly every 6 weeks, late passage NPM-ALK+ ALCL cells were discarded, and early passage suspension cells were thawed and grown for up to 2 weeks of recovery period before subject to experimentation. Adherent Cells were passaged at 80% confluence every 3-4 days and grown for maximum of 4 passages.

All doxycycline-inducible stable cell lines were maintained in 10% tetracycline approved fetal bovine serum (Clonetech, Mountain View, CA, USA), 1% penicillin and streptomycin and 0.5-1 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA).

The 21 ALK+ALCL primary tumors used in this study for immunohistochemistry were diagnosed at the Cross-Cancer Institute as per the World Health Organization Classification Scheme. The four mouse ALK+ALCL tumors were a gift of Dr. Suzanne D. Turner from the University of Cambridge and has been previously characterized (253).

The use of these tissues was approved by Dr Raymond Lai laboratory Institutional Ethics Committee.

2.2 Peripheral Blood Mononuclear Cells Isolation

Human blood was obtained from a healthy individual and stored in EDTA treated tubes. Blood sample with diluted to 1 in 2 with PBS. Ficoll-Paque (GE Healthcare) was then carefully applied on top of blood. Centrifugation was then performed 400xg for 40 minutes at room temperature. The uppermost layer containing blood plasma was removed. The second layer containing PBMCs (lymphocytes, monocytes and thrombocytes) was then transferred into a new tube and mixed with PBS containing 0.2% FBS. The cell pellet was then removed. To remove platelets, the cell suspension was resuspended in additional buffer, followed by two consecutive low speed centrifugations at 200xg for 15 minutes. The final pellet contained PBMCs with lymphocytes and monocytes.

2.3 Pharmacological Inhibitors and Treatment Conditions

Crizotinib and doxycycline were all purchased from Sigma-Aldrich. Thiostrepton and doxorubicin were a gift of from the Velazquez and Lavasanifer laboratories, respectively. All drugs were sterilized using a 0.22 μm filter (EndMillipore, Cork, Ireland) before use in tissue culture. Crizotinib and thiostrepton were dissolved in DMSO at a working concentration of 1 mM and stored -20 C after being aliquoted in microcentrifuge tubes. Doxorubicin was dissolved in ultrapure water at a working concentration of 1 mg/ml and

stored in the dark at -20 C. Doxycycline was dissolved in DMSO and used at a working concentration of 1 µg/ml and stored in the dark at -20 C.

2.4 Immunohistochemistry

For immunohistochemistry, formalin-fixed, paraffin-embedded tissue sections were first cut at 4-µM thickness on Apex^{TM} Superior Adhesive Slides (Leica, Mount Waverly, Germany). Slides were baked at 60 degrees for 1 hours followed by deparaffinization with xylene and gradual re-hydration with decreasing concentrations of ethanol. Slides were then rinsed with water. Heat induced epitope retrieval was performed using a microwave with Tris EDTA pH 9 buffer for 20 minutes. Following epitope retrieval, slides were again washed with water and then blocked for endogenous peroxidase using hydrogen peroxide (Fischer Scientific, Geel, Belgium). After a rinse with water, slides were blocked with antibody dilution buffer (EnVision; Dako) with incubated with primary antibody overnight at 4 C. For the 21 NPM-ALK+ ALCL patient tumor sections, an anti-FOXM1 rabbit antibody (1:500, Abcam, #207298) was used. For NPM-ALK+ ALCL transgenic mouse sections, an anti-FOXM1 mouse antibody (1:500, SC, #376471) was used. Reactive human tonsils sections were used as a negative control. On the second day, the primary antibody was carefully removed, and slides were incubated with Dako Envision+ Dual Link System-HRP (Dako, Caprinteria, CA) for 1 hour. Following washes with TBST and TBS, slides were stained with Dako Liquid DAB+ Substrate Chromagen System (Dako, Caprinteria, CA) for up to 3 minutes. Slides were washed with water, followed by sequential dipping in copper sulfate, water and lithium carbonate. Finally, slides were

dehydrated with increasing concentrations in ethanol and an incubation in xylene. Slides were mounted with coverslips followed by imaging with a Zeiss Axioscope 2 microscope (Carl Zeiss).

2.5 Cell Viability

The CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used to measure cell viability. Drug treatments were administrated in 48-well plates at a cell density of 100,000 cells/ml in 1 ml of fully supplemented medium. Cells were assayed with 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (i.e MTS reagent) 48 hours after drug treatment. Measurements were obtained at a wavelength of 450 nM using a FLUOstar Omega Microplate Reader (BMG Labtech). The trypan blue exclusion assay (Amresco, Solon, OH, USA) was used to measure cell viability for time-dependent experiments.

2.6 RNA Extraction and cDNA Synthesis

To isolate total RNA from cell lines, the RNeasy Mini Kit (Qiagen, Valencia, CA) was used. On column DNA digestion with DNAse I (Invitrogen, Carlsbad, CA) was performed to remove trace DNA. RNA concentration was measured using a Du 730 UV spectrophotometer (Beckerman Coulter). The High-Capacity cDNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA) was used to prepare of cDNA from 1 µg of total RNA according to manufacturers instructions.

2.7 Reverse Transcriptase Polymerase Chain Reaction

For Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), cDNA was mixed with ultra pure water, primers and 2x PCR master mix (Fischer Scientific, Carlsbad, CA). Sequence of primers were:

FOXM1B/C F- 5-CACCCCAGTGCCAACCGCTART-3 and

FOXM1B/C R- 5-AAAGAGGAGCTATCCCCTCCTCAG-3

GAPDH F -5-GGTCTCCTCTGACTTCAACAGCG-3 and

GAPDH R- 5-ACCACCCTGTTGCTGTAGCCAA-3

PCR parameters included an initial denaturing step at 95°C for 10 minutes followed by 30 cycles of 95°C for 45 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 30 seconds, followed by a final extension step at 72°C for 10 minutes. PCR products were then analyzed by 2% agarose gel electrophoresis.

2.8 Quantitative Real Time PCR

Quantitative Real Time PCR (qPCR) reactions were prepared in 96-well plates (Invitrogen, Carlsbad, CA) with the PowerUp™ SYBR™ Green Master Mix (Invitrogen, Carlsbad, CA) according to manufacturers protocol. The Mastercycler® ep Realplex system (Eppendorf, Hamburg, Germany) was used for monitoring of the qPCR. The following primers, FOXM1: F- 5-CGTCGGCCACTGATTCTCAAA-3 & R- 5-GGCAGGGGATCTCTTAGGTTC-3, GAPDH: F- 5-GGTCTCCTCTGACTTCAACAGCG-

3 & R- 5-ACCACCCTGTTGCTGTAGCCAA-3 were used. qPCR cycling was setup as denaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, annealing & extension at 60°C for 1 minute. The relative gene expression was determined using the ΔΔ-CT method. Normalization of target gene expression was made to GAPDH and then to the experimental control group.

2.9 Plasmids and siRNA

Short hairpin RNA (shRNA) plasmids for FOXM1 (pLKO.1) purchased from Dharmacon. The NPM1 shRNA (pLKO.1) was purchased from Sigma-Aldrich. The GFP shRNA plasmid was purchased from Addgene (#30323). Vectors for doxycycline inducible overexpression of FOXM1B/C vectors were from made with the pCW57.1 vector and purchased from Addgene (#68811 and 68810) (254). The empty vector luciferase reporter and FOXM1 luciferase reporter containing FOXM1 consensus sequences were based on the pGL4.10 backbone, provided by Dr. Cater J Barger and Dr. Adam Karpf from the University of Nebraska Medical Center (148). The NPM-ALK (pcDNA3.1) plasmid was a gift from Dr. Stephan Morris. NPM-ALK^{WT} and NPM-ALK^{FFF} (HB tagged) expression vectors were previously created by members of the Lai laboratory and has been described in (219). Short interfering RNA (siRNA) for scrambled siRNA and ALK smartpool siRNA were purchased from Dharmacon.

2.10 General Transfections

The electro square electroporator BTX ECM 800 (225 V, 8.5 ms, 6 pulses) was used for transient transfections of ALK+ ALCL cell lines with siRNA and plasmid vectors. Per 10 million ALK+ ALCL cells, 1 nanomole of siRNAs were used. Western blots were used to determine the efficiency of the knockdown.

Transient transfections of 293T cells were performed in 6-well plates. 1.5 µg of plasmid or 200 picomole of siRNA were combined with 5 µl Lipofectamine and added to each well. Cells were harvested 24 hours following transfection.

2.11 Lentivirus Preparation and Transduction of Lymphomas

A lentivirus-based transduction strategy optimized for T-lymphocytes was used to stably transfect ALK+ ALCL cell lines (255). Lentivirus production was achieved in 100 mm dishes by transfecting 293T cells with 9 µg transfer vector, 9 µg psPax2 and 3 µg pMD2.G plasmids (Addgene) using 30 µl Lipofectamine (Life Technologies, Carlsbad, CA, USA). After 48 hours, viral supernatant was collected and filtered. 2 million ALK+ ALCL cells were then mixed with 1 ml of viral supernatant containing 0.8 µg/ml polybrene (Sigma-Aldrich) in a 6-well plate. The plate was then spun at 1000g and 32°C at 2 hours to facilitate viral infection. Following the spin infection, 1 ml of fresh medium was added to each well. To increase the transduction efficiency, the spin infection step was repeated

the next day with fresh virus supernatant. 24 hours after the final infection, cells were washed twice with cold PBS and resuspended in fresh medium. Cells were then collected for subsequent assays and western blot analysis. For the development of monoclonal stable cell lines, cells were expand followed by selection with increasing concentrations of puromycin, increasing from 0.5 µg/ml to 3 µg/ml. To isolate monoclonal populations of lymphoma stably expressing, single cell selection was performed with serial dilution in 96-well plates. The clones were expanded, and responsiveness to doxycycline was validated using western blotting.

2.12 Flow Cytometric Analyses

The CellTrace™ CFSE Cell Proliferation Kit was purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). Briefly, 5 nmol of CFSE (carboxyfluorescein succinimidyl ester) dye (in DMSO) was incubated with 1 million cells for 30 minutes. Flow cytometry was conducted, and viable cells were gated using forward and side scatter parameters. Over the next three days, 200,000 cells were analyzed each day for loss of CFSE staining.

To determine apoptosis and conduct cell cycle analysis of NPM-ALK+ALCL cell lines, cells were stained with the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, Franklin Lakes, NJ, USA) according to manufacturer's recommendations.

For all flow cytometry experimentations, the BD LSR FORTESSA X-20 instrument at the Flow Cytometry core, University of Alberta was used. The BD FACSDiva software was

used to record and analyze flow cytometry data. Flow cytometry experimentation results are representative of at least two or more biological replicates.

2.13 Soft Agar Colony Formation Assay

For soft agar colony formation assays, 6-well plates were first layered with the bottom 0.7% low melting point (LMP) agar in complete RPMI 40 medium. Following experimental treatments, 10,000 cells were seeded into each well of a 6-well in the upper layer, at a concentration of 0.3% LMP agar in complete RPMI 40 medium. Soft agar experiments were performed in triplicates. Colonies were grown for about two weeks followed by staining of the plates with 0.05% crystal violet (Sigma-Aldrich). Images of colonies were taken using a fluorescent microscope (Leica Biosystems).

2.14 Western Blotting

Cell lysates were prepared by first pelleting cells at 300xg for 5 minutes and then washing cells with PBS. An appropriate volume of 1x RIPA buffer supplemented with protease inhibitor, phosphatase inhibitor and PMSF was added to the cell pellet. Cells were then ruptured by rigorous pipetting on ice. Following a 30-minute incubation period on ice, cells were centrifuged on maximum speed at 4 C for 10 minutes on a table-top centrifuge. The supernatant was collected and transferred to a freshly chilled tube. Protein concentration of lysates were assessed using the BCA assay according to manufacturers recommendations. The assay plate was read on FluoStar machine. Cell lysates were then

combined with 4x loading dye (90 mM Tris-HCl (pH 7.9), 2% SDS, 10% glycerol, 5 mM EDTA, 125 mg/mL urea, 0.1 M dithiothreitol, 0.02% bromophenol blue) and boiled at 100 C for 10 minutes. Cell lysates were then electrophoresed on 8% or gradient SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (GE Healthcare) Following transfer the membranes were blocked with 5% milk in TBST buffer (20 mM Tris-HCL, pH 7.6, 150 mM NaCl, 0.02% Tween 20) for 30 minutes. Primary antibodies were then added to the membranes and incubated overnight at 4 C. The next day, membranes were washed with TBST, following by addition of secondary antibody conjugated with the horseradish peroxidase (Cell Signalling Technologies). Further TBST washes were performed. Proteins were detected using the enhanced chemiluminescence detection kit (Thermo Scientific) with the use of x-ray films (Diamed) for band development.

2.15 Antibodies

The primary antibodies used for western blot in this study included: anti-FOXM1 (1:500, Santa Cruz (SC), #271746; 1:500, Abcam, #207298), anti-pALK Y1604 (1:500, Cell Signalling technologies (CST), #3341S), anti-ALK (1:1000, CST, #3633), anti-pSTAT3 (Y705) (1:2000, CST, #9145), anti-STAT3 (1:1000, CST, #124H6), anti-Cyclin B1 (1:1000, SC, #752), anti-Actin (1:8000, SC, #47778), anti-PARP (1:1000, CST, #9542), anti-Caspase 3(1:1000, CST, #9662) anti-Survivin (1:1000, CST, #), anti-NPM1 (1:2000, Milipore Sigma, clone 3C9) anti-Vinculin (1:500, SC, #25336), anti HDAC-1 (1:500, SC, #81598), and anti-α-actinin (1:500, SC, 17829). Secondary antibodies used were HRP-conjugated anti-mouse (1:2000, CST, #7076) and anti-rabbit (1:2000, CST, #7074).

2.16 Co-Immunoprecipitation

For co-immunoprecipitation assays, at least 5-10 adherent or 20-40 million lymphoma cells were lysed with 1ml of Cell-Lytic M buffer supplemented with protease and phosphatase inhibitors. Cells were thoroughly lysed via vigorous pipetting. The cells were then pelleted, and cell supernatant was collected, followed by BCA protein estimation. Next, 1 mg of protein lysate was diluted with Cell-Lytic M and 2-4 µg of an immunoprecipitation grade antibody was added. For ALK, SC-6344 and for FOXM1 SC-271746 was used. The mixture was then rotated overnight at 4 C. The next day, 50 ul of protein G plus protein A slurry was added, and the mixture was rotated at 4 C for further 6 hours. Next, the beads were washed by first pelleting at 0.2xg for 1 minute, removal of the supernatant, and rotation with cold PBS for 10 minutes. This step was repeated for a total of 4 times for of 40 minutes washing. In the final wash step, Cell-Lytic M was used for additional stringency. Following removal of Cell-Lytic buffer, 20 µl of 4x loading dye was added and the samples were boiled at 100 C for 10 minutes. Precipitated lysates were then centrifuged at maximum speed (14000xg) and the supernatant was subjected to western blotting.

2.17 Luciferase Assay

Luciferase reporter assay was performed using Luciferase Assay System kit (Promega, Corporation, Madison, USA) according to the manufacturer's protocol. In brief, cells were transiently transfected with either the empty or FOXM1 luciferase reporter, as mentioned

above. Cells were lysed with Passive Lysis Buffer followed by estimation of protein concentration. Equal amounts of protein for each sample were then assessed for luciferase activity using the FLUOstar Omega multi-mode microplate reader (BMG Labtech, Ortenburg, Germany) to read and analyze data.

2.18 Subcellular Fractionation and DNA Pulldown Assay

Cells were washed with cold PBS and then separated into cytoplasmic and nuclear fractions using the Pierce NE-PER kit (Fisher Scientific Canada, Ottawa, ON, Canada) according to the manufacturer's instructions.

For DNA pulldown (streptavidin/biotinylated DNA immunoprecipitation), a previously published study was followed (256). 400 µg nuclear protein was incubated with or without 300 pmol of a 5' biotin-labeled probe (Integrated DNA Technologies, Edmonton, AB) containing FOXM1 consensus sequences. The sequence of the FOXM1 probe with the consensus site underlined is as follows: AAACAAACAAACAATCAAACAA. Mutant DNA and unbiotinylated DNA probes were used as negative controls to optimize the protocol. Following addition of the biotinylated probe, the mixture was then incubated with rotation for 30 minutes at room temperature. High capacity streptavidin agarose beads (50 µl, Fisher Scientific) were then added to each sample, which were then incubated with rotation overnight at 4°C. The following day, the samples were collected by centrifugation at low speed and the supernatant was discarded. The beads were then washed three

times with ice cold PBS. Protein elution was achieved by boiling the beads at 100°C in 4x sample loading buffer, before then assessing for protein binding using western blots.

2.19 Chromatin Immunoprecipitation

ChIP was performed according to the manufacturer's instructions (Upstate-Millipore Chromatin Immunoprecipitation Assay Kit (Temecula, CA, USA)). Briefly, HEK 293 cells were plated in 15 cm plates. Transient transfections in HEK 293, as mentioned above, were performed with GFP, FOXM1, NPM-ALK^{WT} and NPM-ALK^{FFF} cDNA-containing expression vectors for 48 hours. At approximately 90% confluency, cells were fixed for with 1% formaldehyde (in culture media) for 20 minutes at room temperature with rocking. Formaldehyde was then quenched by the addition of glycine and incubated for an additional 10 minutes. Cells were collected by centrifugation, washed and lysed with SDS lysis buffer (1% SDS, 10 mM EDTA). Chromatin was sheared by sonication on ice using a Fisher Scientific Sonic Dismembrator Ultrasonic Processor Model 705 (Fisher Scientific) at 25% power with 10 pulses of 20 s sonication and 30 s rest to give optimized DNA fragments of between 200 bp and 1 kb. Chromatin was subsequently suspended in ChIP Dilution Buffer and incubated with 5 µg of either normal rabbit IgG (Cell Signaling) or anti-FOXM1 antibody (Santa Cruz) overnight at 4 °C. Next, Protein A agarose beads were added to the chromatin/FOXM1 or rabbit IgG antibody for an additional 6 hours at 4 °C. The immunoprecipitated material bound to agarose beads was then washed once with a low salt wash buffer, a high salt wash buffer, LiCl wash buffer, and then twice in TE buffer. To elute the immunoprecipitated material from the beads, the bead-bound chromatin was

incubated with ChIP Elution Buffer. Input chromatin and DNA/protein formaldehyde crosslinks were then reversed with 5 M NaCl at 65 °C overnight. Proteins were digested with Proteinase K (Ambion, Invitrogen, Burlington, ON, Canada) at 55 °C for 2 hours and DNA was purified using a PCR Purification kit (Qiagen). The resulting ChIP DNA and input DNA were then amplified by PCR with previously validated Cyclin B1 (*CCNB1*) ChIP primers:

Cyclin B1 Prom 1 F 5-CGCGATCGCCCTGGAAACGCA-3

Cyclin B1 Prom 1 R 5-CCCAGCAGAAACCAACAGCCGT-3

Cyclin B1 Prom 2 F 5-CCTCCAACCCAGAGAGTTGTTGC-3

Cyclin B1 Prom 2 R 5-AGCCAAGGACCTACACCCAGCA-3

2.20 Statistical Analyses

Numerical data have been expressed as the mean ± standard error of the mean obtained from the number of replicates mentioned in the figure legends. The two-tailed Student's t test and ANOVA was used to determine the significance of samples with $\alpha=0.05$. Statistical analysis was performed with GraphPad software (La Jolla, CA, USA).

Chapter 3: Results

3.1 Assessment of FOXM1 Protein Levels in NPM-ALK+ ALCL Cells

NPM-ALK+ ALCL cell lines were assessed for expression of FOXM1 by western blot. As shown in Figure 3.1, FOXM1, detected at approximately 110 kd, which is concordant with its previously reported molecular weight (3), was highly expressed in all 5 cell lines examined. In contrast, cell lysates harvested from peripheral blood mononuclear cells (PBMC) from a healthy individual showed no detectable FOXM1. Jurkat (a T-cell acute lymphoblastic leukemia cell line), which has previously been reported to express a high level of FOXM1 (257), was found to express FOXM1 at a level comparable to that of NPM-ALK+ ALCL cell lines. Vinculin, which shares a similar molecular weight as FOXM1, was used as a loading control.

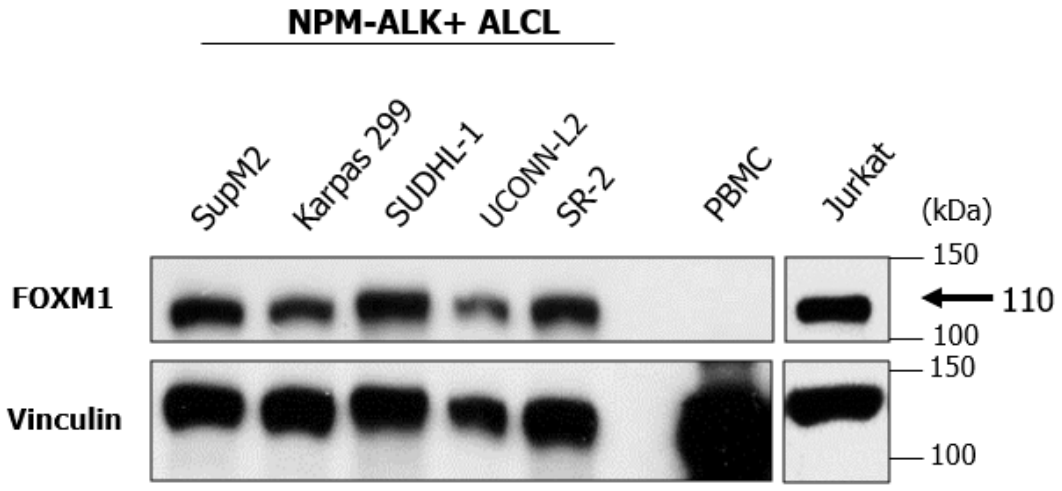

Figure 3.1 Western blot analysis of FOXM1 protein (mw: 110 kDa) expression in NPM-ALK+ ALCL cell lines. Peripheral blood mononuclear cell (PBMC) protein lysate from a healthy individual was used as a negative control for FOXM1 expression. Jurkat (acute lymphoblastic leukemia) was used as a positive control. Vinculin (mw: 110 kDa) was used as a loading control

3.2 Expression of FOXM1B and FOXM1C Isoforms in NPM-ALK+ ALCL Cells

In order to determine whether both oncogenic FOXM1 isoforms (i.e. FOXM1B and FOXM1C) are expressed in NPM-NPM-ALK+ ALCL, RT-PCR was performed using a specific primer set that is designed to detect both FOXM1B and FOXM1C (14) (since these isoforms are known to share a similar molecular weight and cannot be readily distinguished from each other by western blot). Expression of both isoforms was detectable in all 4 NPM-ALK+ ALCL cell lines tested but not PBMC (Figure 3.2). Jurkat cells and the PCR products from commercially available FOXM1B and FOXM1C expression vectors served as positive controls.

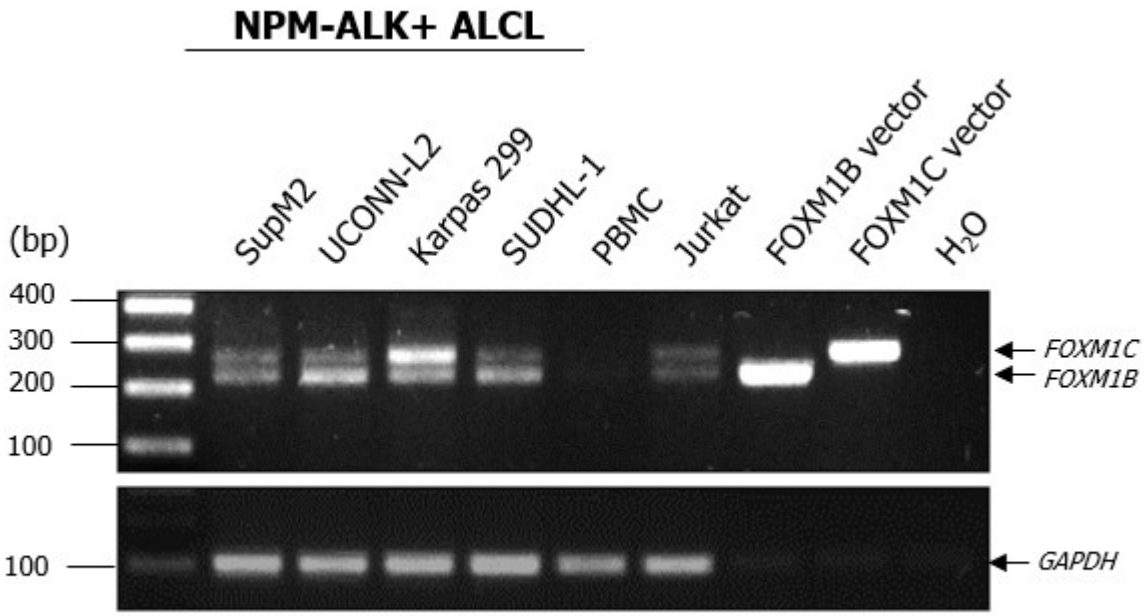

Figure 3.2 RT-PCR for transcript levels of *FOXM1B* and *FOXM1C* mRNA. PBMC was used as a negative control for *FOXM1* gene expression. Commercial FOXM1 expression vectors for FOXM1B and FOXM1C were used as controls for this experiment. GAPDH was used as a loading control. The final lane included was a reaction with H_2O instead of cDNA for a PCR reaction negative control.

3.3 Transcriptional Activity of FOXM1 in NPM-ALK+ ALCL

Since FOXM1 is a transcription factor, the transcriptionally activity of this protein in NPM-ALK+ ALCL was evaluated. In support of the concept that FOXM1 is transcriptionally active, FOXM1 was found to be localized to the nuclei of NPM-ALK+ ALCL cells, as shown in by nuclear/cytoplasmic fractionation experiments (Figure 3.3A). Histone deacetylase (HDAC1) and α-actinin served as the controls for the efficiency of the nuclear/cytoplasmic fractionation protocol.

Furthermore, following transfection of a FOXM1 luciferase reporter, containing FOXM1 consensus sequences, into NPM-ALK+ ALCL cells, a significantly higher level ($p<0.005$) of luciferase activity was observed compared to the negative control (Figure 3.3B).

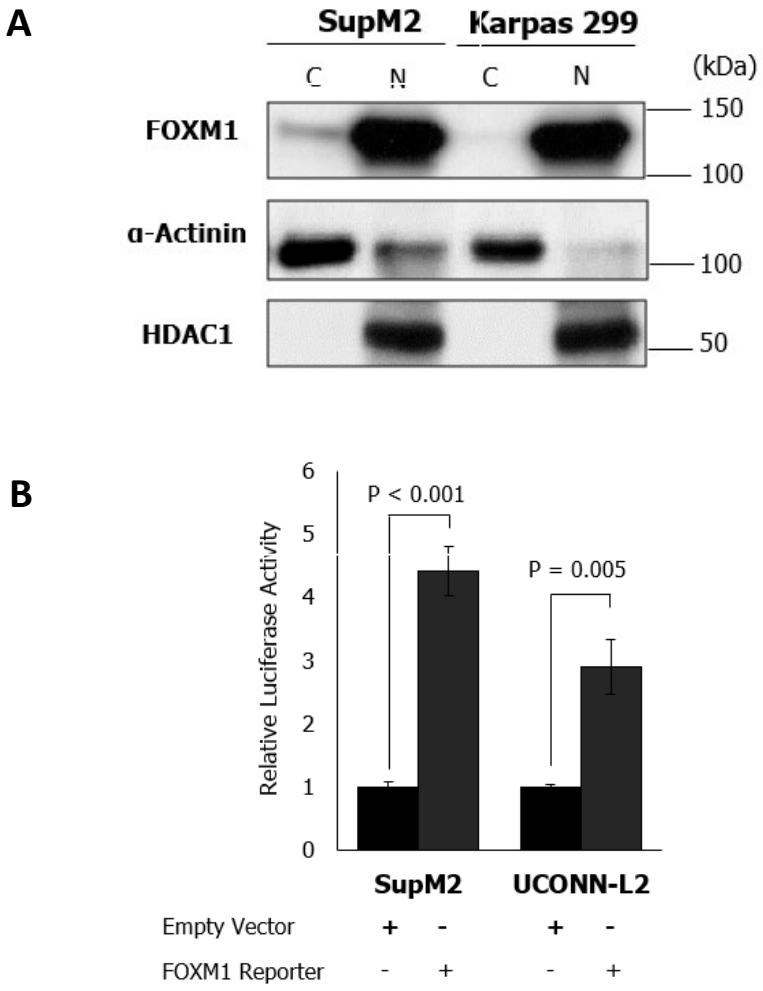

Figure 3.3. FOXM1 subcellular fractionation and protein activity. A) Subcellular fractionation of FOXM1 was performed in the two NPM-ALK+ cell lines, SupM2 and Karpas 299. (C) = cytoplasmic, (N) = nuclear protein. α-Actinin was used as a control for cytoplasmic protein while HDAC1 was used a control for nuclear proteins. B) SupM2 and UCONN-L2 cell lines were either transfected with an empty luciferase reporter (Empty Vector) or 6x FOXM1 luciferase reporter (FOXM1 reporter). Experiments were performed independently in triplicates. Bars indicate mean±SEM.

3.4 FOXM1 Immunocytochemistry and Immunohistochemistry in NPM-ALK+ ALCL Cases

FOXM1 immunocytochemistry in cell line blocks from 3 NPM+ALK ALCL cell lines (SupM2, Karpas 299 and SUDHL-1) showed further illustrated the expression of FOXM1 (Figure 3.4). The intensity of FOXM1 expression varied among individual cells, suggesting not all NPM-ALK+ cells express an equal level of FOXM1. Moreover, FOXM1 expression was restricted largely to the nucleus, although some cytoplasmic expression of FOXM1 could be also identified.

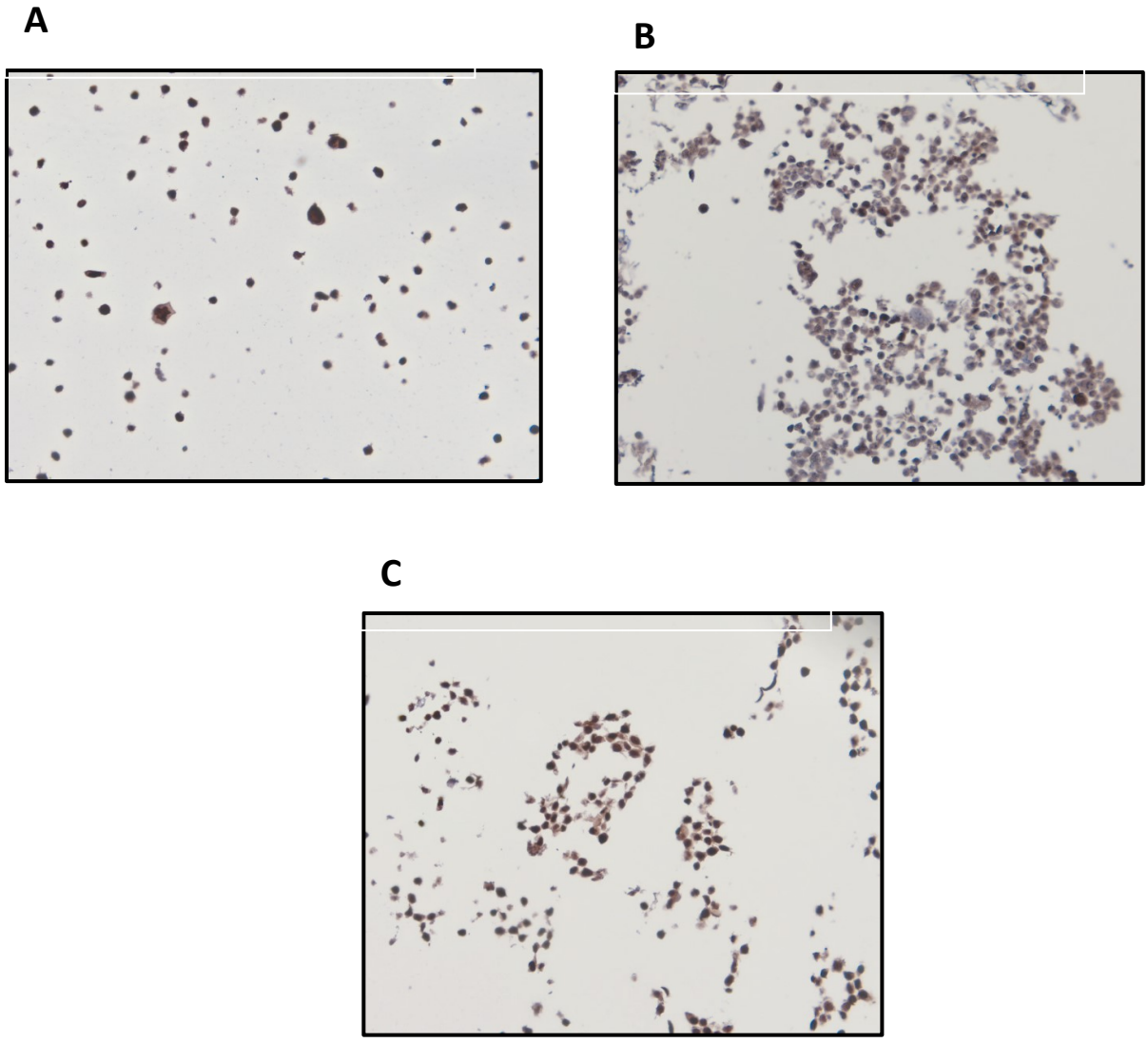

Figure 3.4. Immunocytochemistry of FOXM1 protein in NPM-ALK+ ALCL cell lines. Positive staining is denoted by brown colour development. Blue areas indicate weak to no staining. A) SupM2 B) SUDHL-1 C) Karpas 299

To evaluate FOXM1 expression in primary NPM-ALK+ ALCL tumors, immunohistochemistry (IHC) was conducted of 21 cases of formalin-fixed, paraffin-embedded tissue. FOXM1 expression was identified all case 21 cases. In all cases, high expression of nuclear FOXM1 staining was identified in virtually all neoplastic lymphoid cells whereas the surrounding non-malignant small lymphocytes showed no definitive staining (Figures 3.5A and 3.5B).

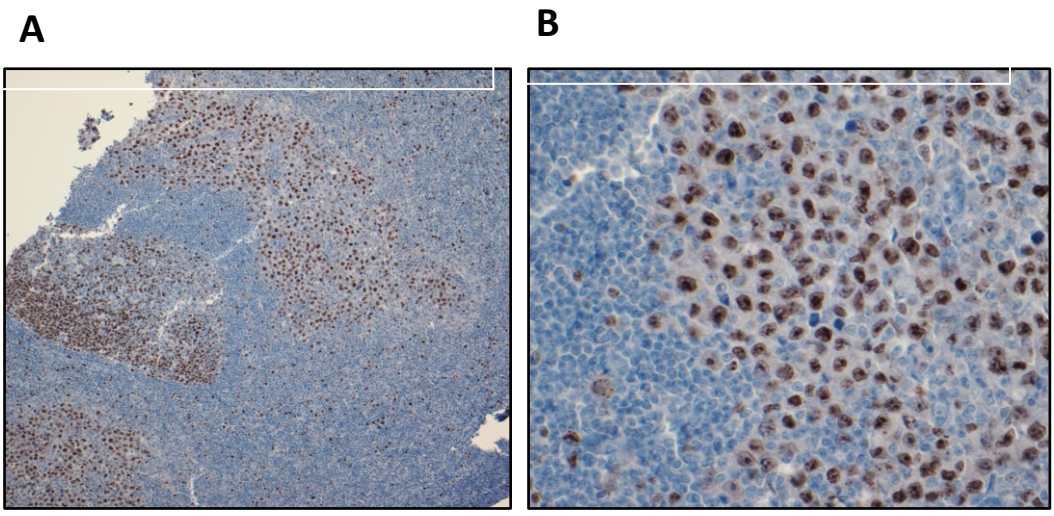

Figure 3.5. Immunohistochemistry of FOXM1 expression in a case of NPM-ALK+ ALCL tumor. Note that the expression of FOXM1 was localized in the nucleus of the infiltrating lymphoma cells (purple stain) whereas surrounding small lymphocytes were negative for FOXM1 (blue stain) A) (immunoperoxidase, 100x) B) (immunoperoxidase, 400x)

In a reactive tonsil, FOXM1 immunostaining was located mostly in centroblasts in the germinal centers and small lymphocytes in the mantle zones, but the inter-follicular spaces were largely negative (Figure 3.6A and 3.6B).

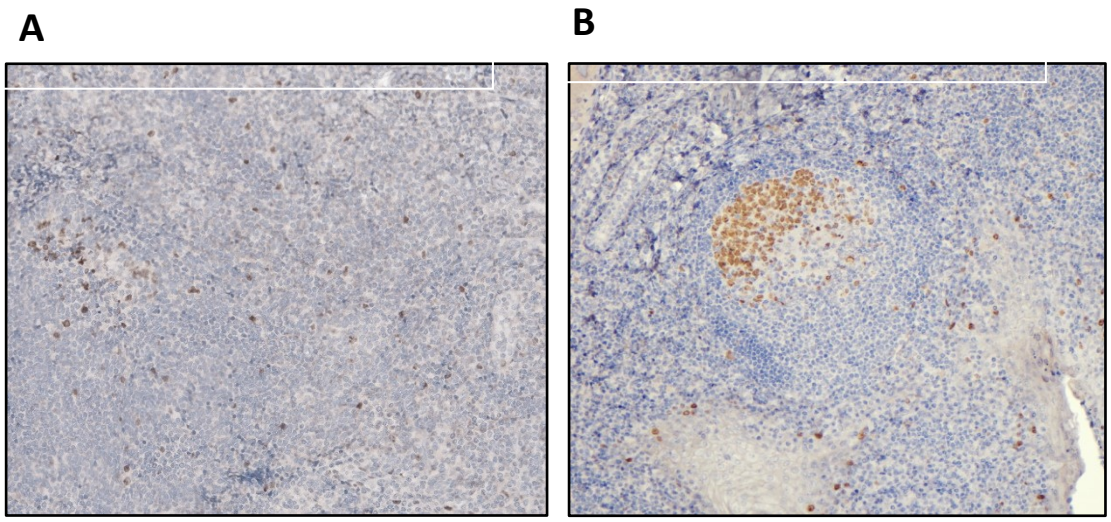

Figure 3.6. Immunohistochemistry of FOXM1 expression in a case of tonsil. Weak FOXM1 staining was found scattered across the tissue in mantle zones. However, intense FOXM1 staining could be identified in centroblasts of the germinal centers A) (immunoperoxidase, 100x) B) (immunoperoxidase, 400x)

Similar results were obtained on IHC of four tumors arising in NPM-ALK+ transgenic mice, a study model previously published (253); FOXM1 was found to be highly expressed in the nuclei of these ALK-expressing tumor cells with the surrounding benign cells being negative (Figures 3.7A and 3.7B). Note that the morphology of the infiltrating lymphocytes appears distinct, that is spindle shaped, compared to the lymphocyte morphology found in human ALK+ ALCL.

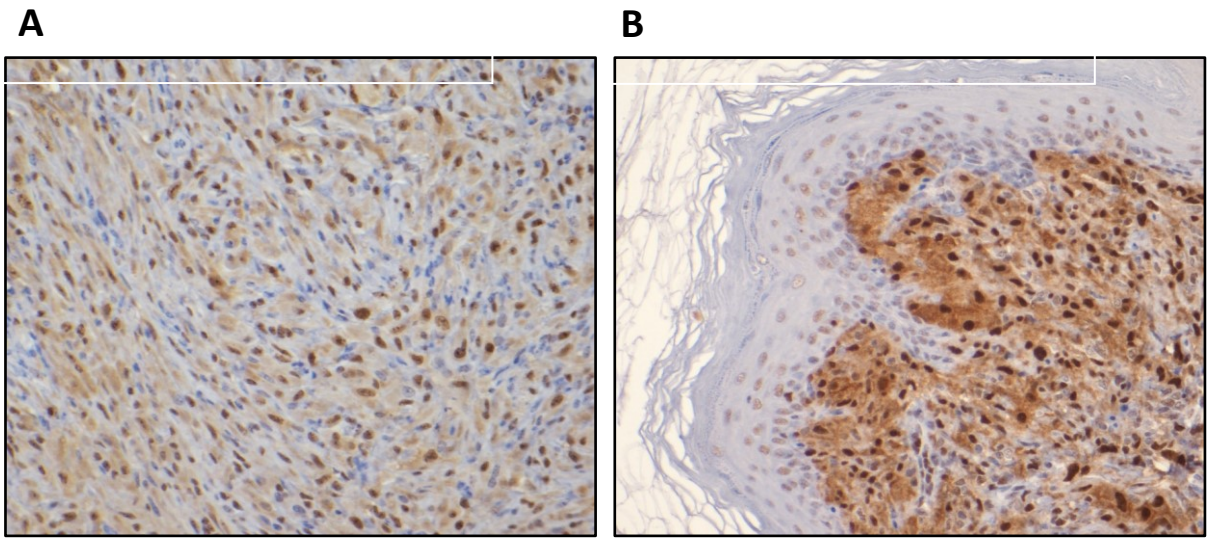

Figure 3.7. Immunohistochemistry of FOXM1 expression in NPM-ALK+ mouse tumors. Two cases of transgenic mouse expressing NPM-ALK+ tumors are shown. Weak cytoplasmic and strong nuclear staining of FOXM1 was found in both cases. A) (immunoperoxidase, 100x) B) (immunoperoxidase, 100x)

3.5 Inhibition of Expression of FOXM1 on Cell growth and the Clonogenicity of NPM-ALK+ ALCL Cell Lines

To understand the biological significance of FOXM1 in NPM-ALK+ ALCL, FOXM1 expression was suppressed in SupM2 and UCONN-L2 cells using two different short hairpin RNAs (shRNA). As shown in Figures 3.8A and 3.8B, efficient FOXM1 knockdown was achieved by using both shRNA species, although the efficiency of knockdown was higher in SupM2 cells as compared to UCONN-L2 cells.

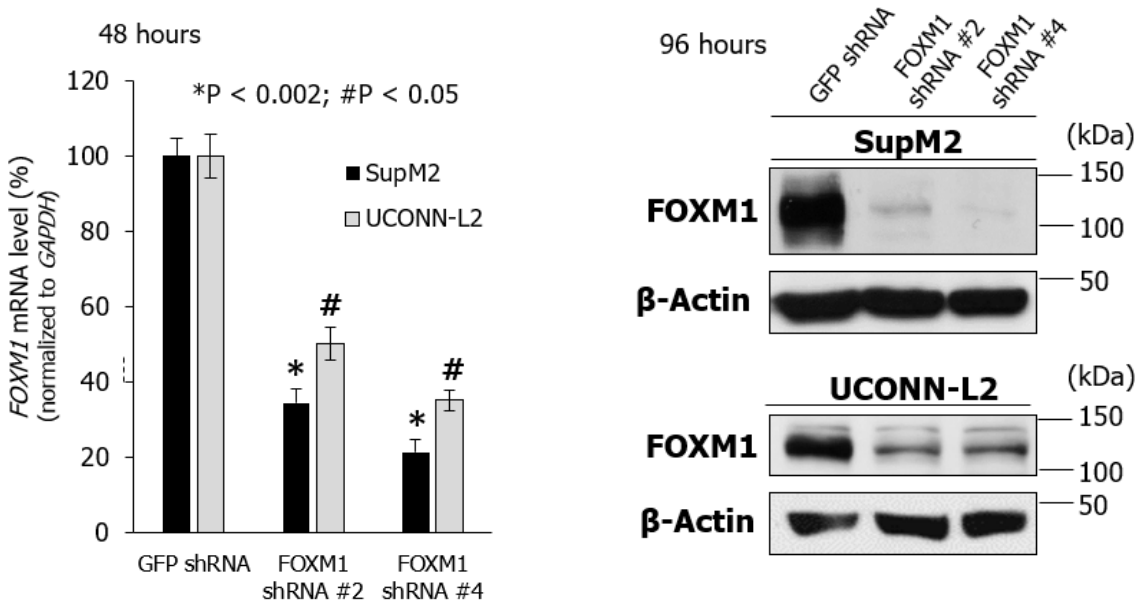

Figure 3.8 FOXM1 mRNA and protein levels following shRNA mediated knockdown. A) *FOXM1* mRNA levels were assessed by qPCR following knockdown of FOXM1 with two different FOXM1 shRNA species in SupM2 and UCONN-L2 cells. Expression was normalized to GAPDH. B) FOXM1 protein expression in SupM2 and UCONN-L2 cell lines following knockdown of FOXM1 using the same two FOXM1 shRNA.

shRNA-induced downregulation of FOXM1 resulted in inhibition of the growth of SupM2 and UCONN-L2 cells on days 4 and 6, as determined following trypan blue exclusion (Figure 3.9A and 3.9B). Cells infected with shRNA against the green fluorescence protein (GFP) served as the negative control. On day 6, the decrease in the number of viable SupM2 cells was in the range of 80-90%. shRNA mediated knockdown of FOXM1 in UCONN-L2 cells also resulted in a significant decrease in cell growth (~50% on day 6), although the extent was not as profound as that seen in SupM2 cells, probably since inhibition of FOXM1 in this cell line was not as efficient.

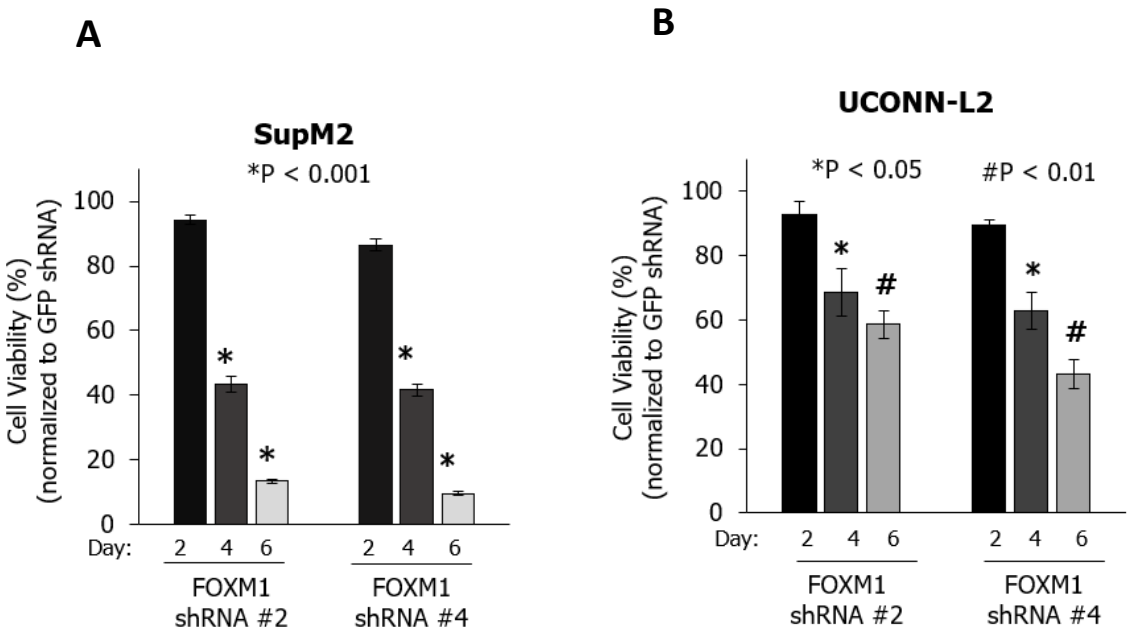

Figure 3.9 Cell viability of SupM2 and UCONN-L2 following knockdown of FOXM1. Trypan blue exclusion assay was used to determine the viability of SupM2 (A) and UCONNL-2 (B) cells at 2, 4 and 6 days following transduction to force expression of either GFP or two FOXM1 shRNAs. Results are normalized to the values of the GFP shRNA for each day.

In addition to inducing a reduction in cell viability, inhibition of FOXM1 expression resulted in a significant increase in apoptosis of both SupM2 and UCONN-L2 cells, as defined by positive staining for both Annexin V and propidium iodide (PI) ($p<0.05$, Figure 3.10A). These functional effects correlate with downregulation of expression of Survivin, as well as increased cleavage of caspase 3 and PARP, consistent with apoptosis (Figure 3.10B)

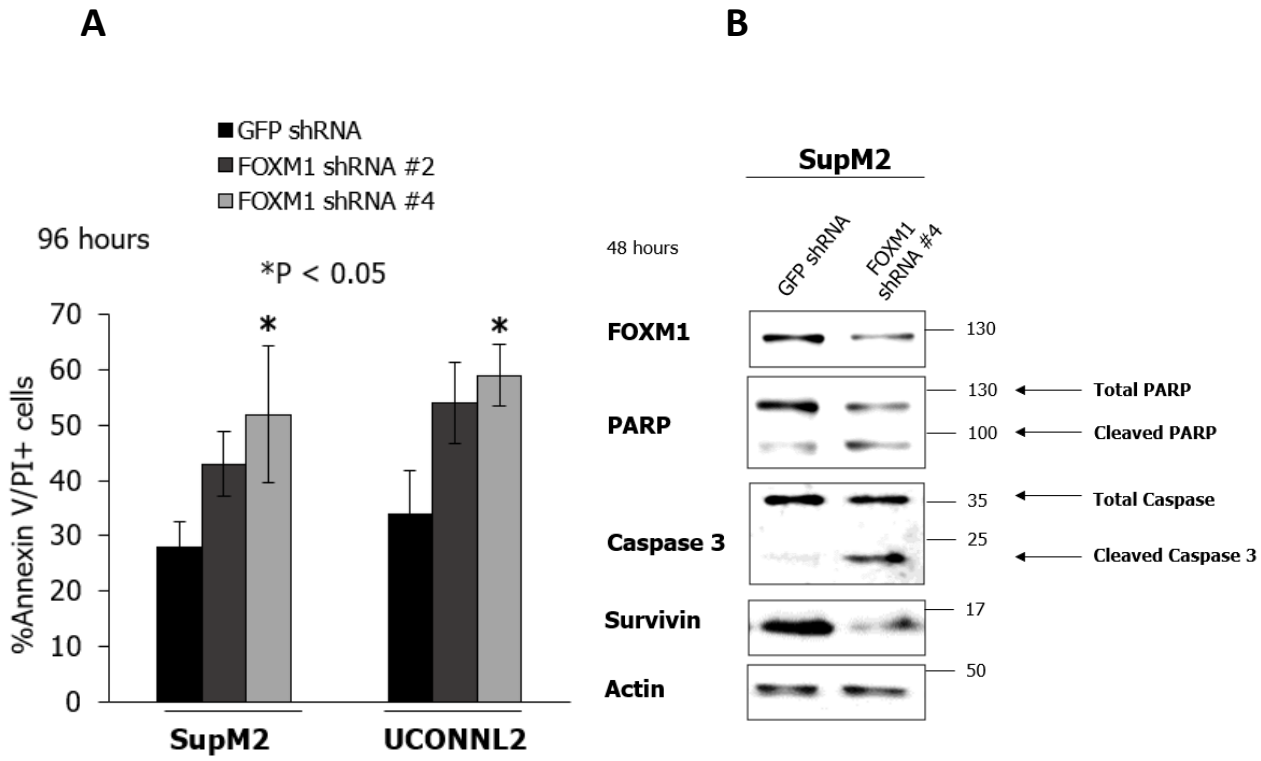

Figure 3.10 Assessment of apoptosis in NPM-ALK+ cells following FOXM1 knockdown. A) Annexin V/PI staining of FOXM1 following lentiviral mediated knockdown of FOXM1 in SupM2 and UCONN-L2 cells. The percentage of cells positive for Annexin V+ and PI+ was gated from 3 independent experiments. Results are presented as mean±SEM. B) Western blot analysis of SupM2 cells infected with FOXM1 shRNA for 48 hours showed cleavage of PARP and caspase 3, markers of apoptosis. Moreover, the expression of an anti-apoptotic protein, Survivin, was dramatically reduced. β-actin served as the loading control. Data are representative of two biological replicates.

Since FOXM1 is known to be a facilitator of cell cycle progression, the impact of FOXM1 knockdown on cell proliferation was assessed using the CFSE assay. As shown in Figure 3.11A and 3.11B, the frequency of cell division, which is inversely proportional to the CFSE content, was decreased following suppression of FOXM1 expression in both cell lines.

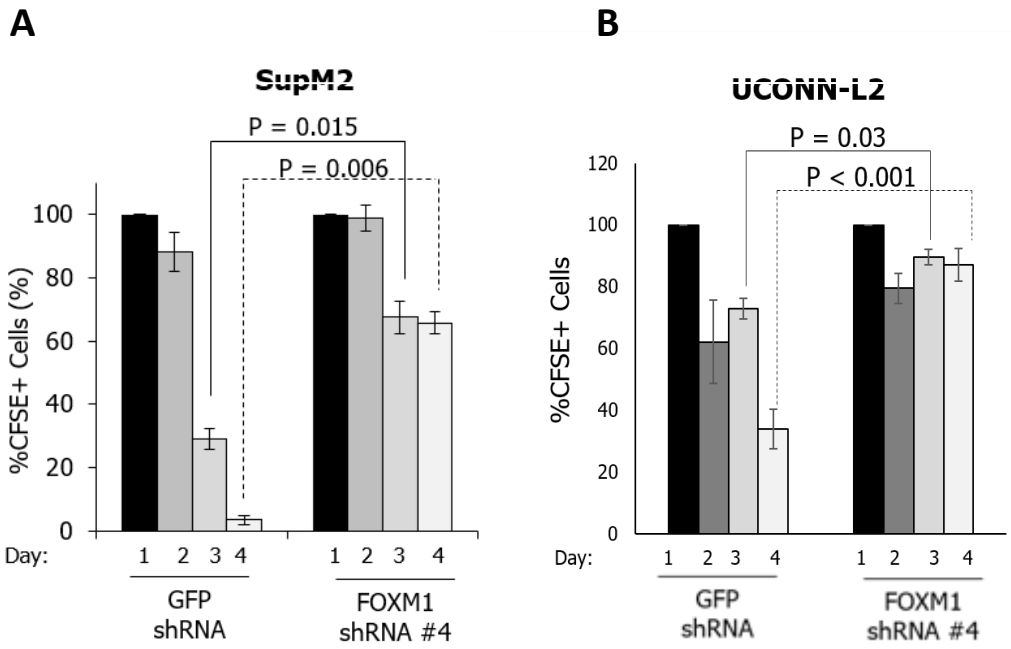

Figure 3.11 Assessment of cell division following knockdown of FOXM1. A) SupM2 and B) UCONN-L2 cells were transduced with either GFP or FOXM1 shRNA #4 followed by staining of cells with CFSE. The loss of CFSE (inversely proportional to cell division frequency) was measured using flow cytometry for four consecutive days. Results are presented as mean±SEM.

Furthermore, expression of the FOXM1 shRNA in either cell line resulted in a significant reduction in col ony formation compared to cells expressing GFP shRNA (Figure 3.12).

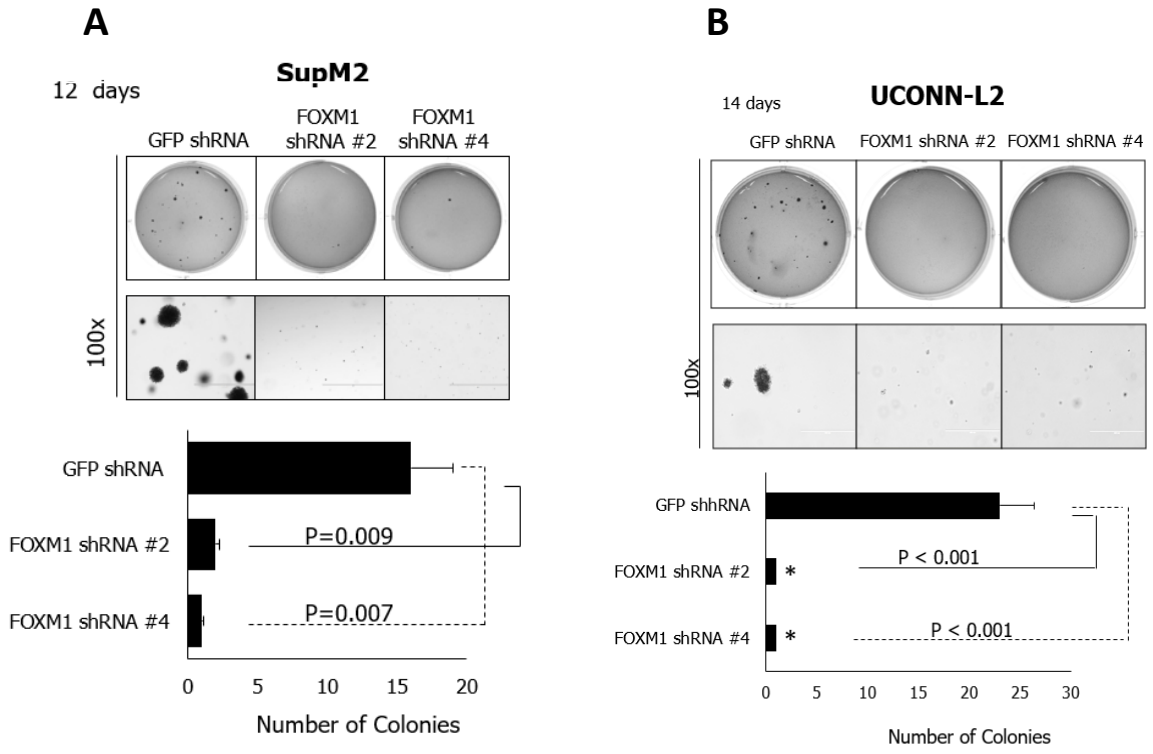

Figure 3.12. Soft agar colony formation assay following knockdown of FOXM1. SupM2 (A) and UCONN-L2 cells (B) were first transduced with FOXM1 shRNA and then grown in soft agar plates for about 2 weeks. The upper panel shows an image of a representative well of the soft agar. The middle panel shows a zoomed 100x image of the colonies. The bottom panel shows quantification of the colonies by counting. Results are presented as mean±SEM.

Moreover, FOXM1 knockdown sensitized cells to doxorubicin-induced cell growth inhibition on exposure to either 50 ng/mL or 100 ng/mL of doxorubicin for SupM2 cells, and 100ng/ml for UCONN-L2 cells (Figure 3.13A and 3.13B). For SupM2 cells the, IC_{50} of doxorubicin was 87 ng/ml with GFP whereas compared to 51 ng/ml with FOXM1 shRNA #4. For UCONN-L2 cells, it was 64 ng/ml (GFP shRNA) compared to 55 ng/ml (FOXM1 shRNA #4).

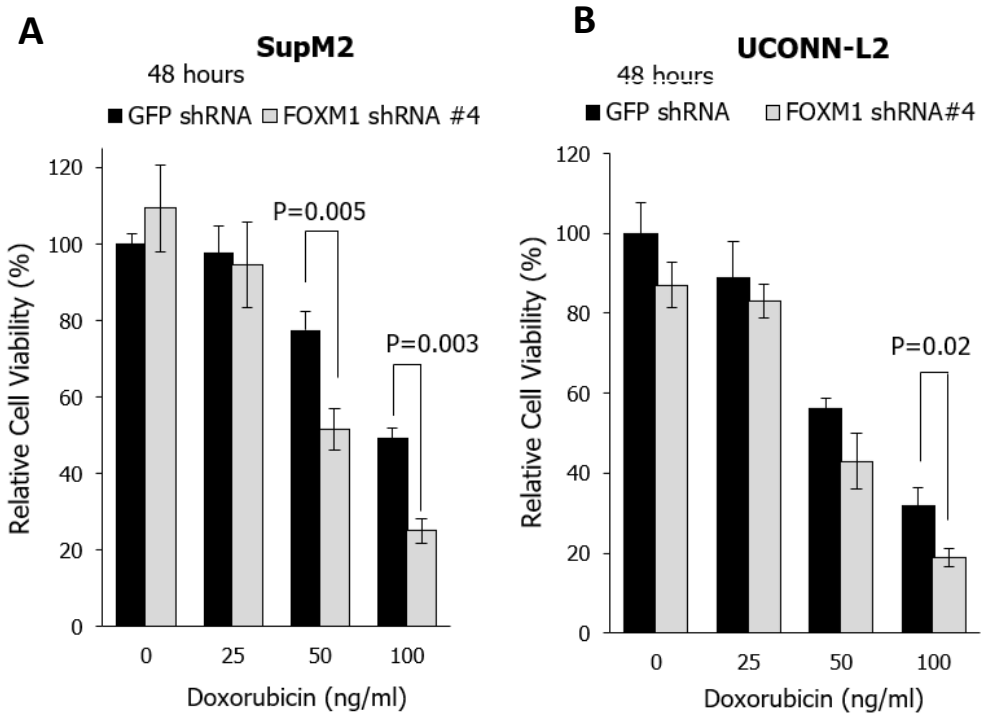

Figure 3.13. Sensitivity to doxorubicin following knockdown of FOXM1. SupM2 (A) and UCONN-L2 (B) cells were transduced with FOXM1 shRNA #4 followed by treatment of cells with increasing dose of doxorubicin for 48 hours. Cell viability was measured by MTS assay. Results are presented as mean±SEM.

3.6 Pharmacological Inhibition of FOXM1 on Cell Growth and Clonogenicity of NPM-ALK+ ALCL Cell Lines

Thiostrepton, a commonly used pharmacological inhibitor of FOXM1, potently inhibited the growth of SupM2 and UCONN-L2 cells with IC_{50} of 0.89 μM and 1.22 μM respectively, following 48 hours of exposure to the compound (Figure 3.14A). Thiostrepton led to a potent and dose-dependent reduction in expression of the FOXM1 protein in SupM2 and UCONN-L2 cell lines (Figure 3.14B) with complete inhibition of FOXM1 expression seen by 48 hours (Figure 3.14C).

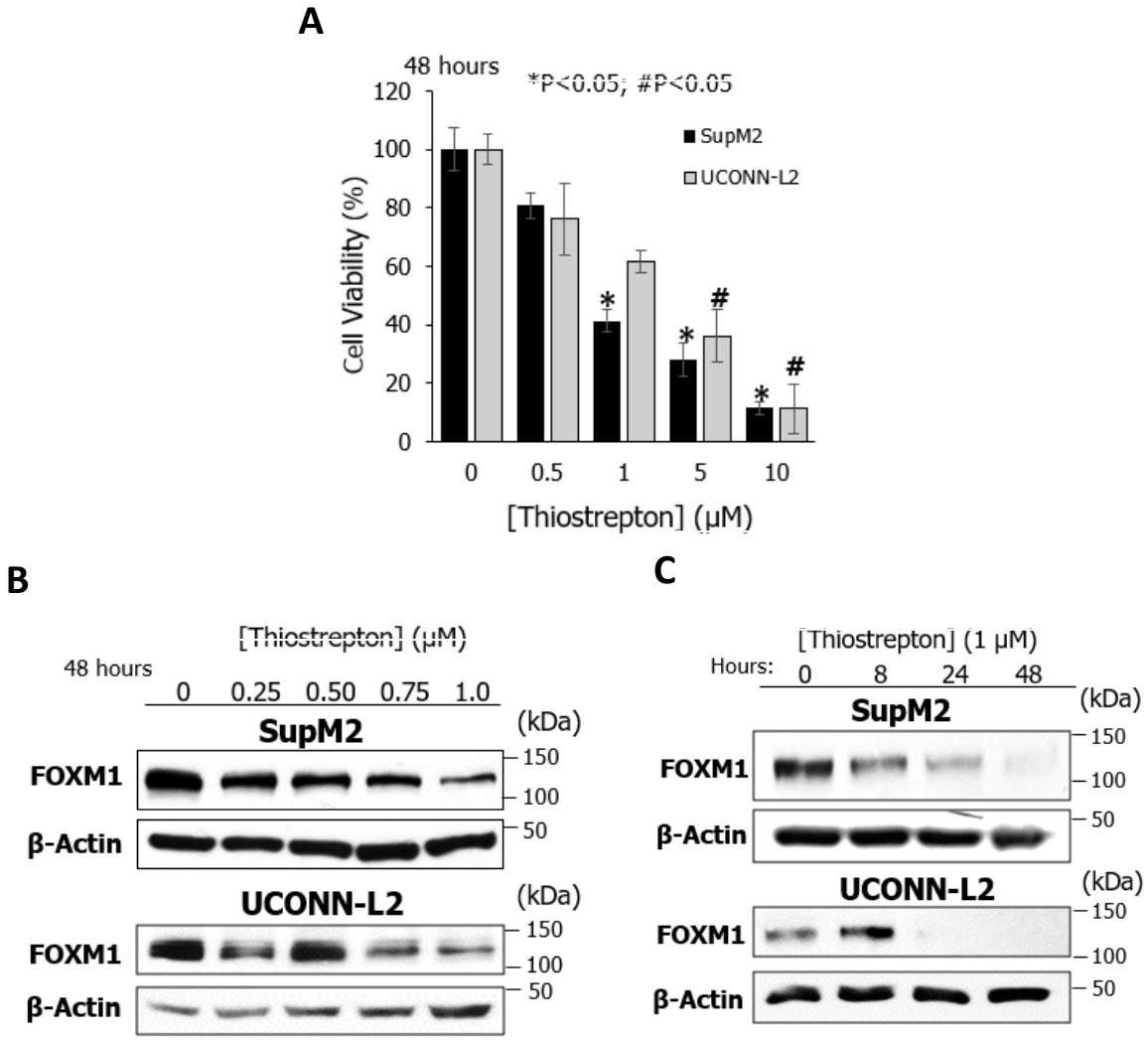

Figure 3.14 Thiostrepton reduces cell viability and FOXM1 levels in NPM-ALK+ ALCL. A) Dose response curve of SupM2 and UCONN-L2 to increasing amounts of thiostrepton. Cell viability was measured using MTS assay at 48 hours. Results are presented as mean±SEM from at least three biological replicates. B) Western blot analysis of FOXM1 protein levels in SupM2 and UCONN-L2 cells following reduction of FOXM1 with increasing doses of thiostrepton. C) Western blot analysis of FOXM1 protein levels in SupM2 and UCONN-L2 cells following thiostrepton treatment over time.s

Moreover, on transfection of a luciferase reporter construct containing FOXM1 consensus sequences, a significant and dose-dependent reduction in luciferase activity was observed upon treatment with thiostrepton (Figure 3.15).

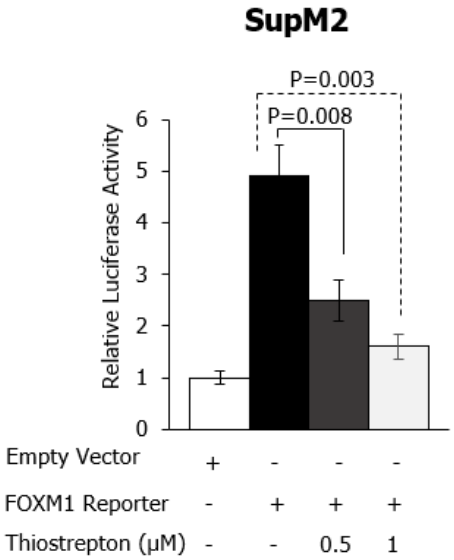

Figure 3.15 Luciferase reporter activity of FOXM1 following its inhibition by thiostrepton. SupM2 cells were first electroporated with either empty vector or FOXM1 reporters. The next day, cells were treated with thiostrepton. Luciferase reporter activity was measured after 48 hours. Results are presented as mean±SEM.

Concomitantly, thiostrepton induced apoptosis (Figure 3.16) as well as inhibition of cell division (i.e. CSFE staining) and soft agar colony formation of NPM-ALK+ ALCL cell line (Figures 3.17A-D).

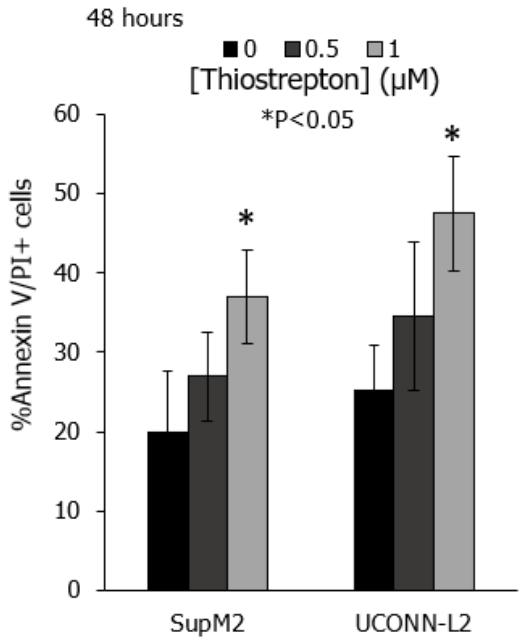

Figure 3.16. Assessment of apoptosis by Annexin V and PI staining following thiostrepton treatment. SupM2 and UCONN-L2 were treated with two doses of thiostrepton for 48 hours. Cells positive for both Annexin V and PI were gated. Results are presented as mean±SEM.

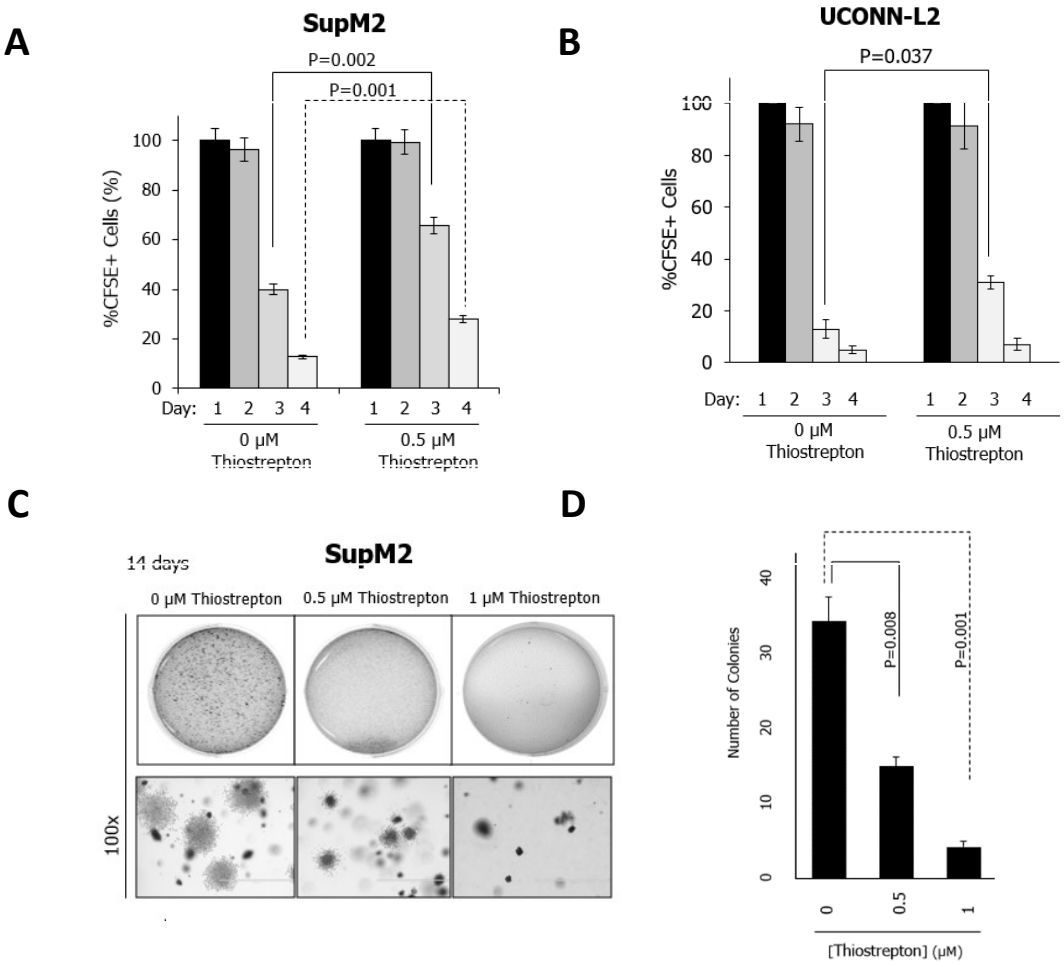

Figure 3.17 Thiostrepton negatively influences NPM-ALK+ cell lines A) The frequency of cell division following thiostrepton treatment was assessed using the CFSE assay. SupM2 and UCONNL-2 were first stained for CFSE, followed by treatment of cells with thiostrepton. Cells were then analyzed for loss for CFSE stain for four consecutive days. B) Soft agar colony formation was assessed in SupM2 cells with two different concentrations of thiostrepton following treatment of cells for 48 hours. The right panel indicates the relative number of colonies counted between the control and treated samples. Bars indicate mean±SEM

3.7 FOXM1 on the Phosphorylation of NPM-ALK and STAT3 Pathway

In order to investigate the mechanisms by which FOXM1 mediates its oncogenic effects in NPM-ALK+ ALCL, the status of the NPM-ALK/STAT3 signaling axis, a key oncogenic driving force in NPM-ALK+ ALCL was investigated (220). As shown in Figure 5A, lentiviral-transduced shRNA-mediated suppression of FOXM1 expression in SupM2 and UCONN-L2 cells resulted in down-regulation of Cyclin B1, a known downstream target of FOXM1 (21). At the same time, phosphorylation of NPM-ALK at Y664 was inhibited as was phosphorylation of STAT3 at Y705 in both cell lines. Interestingly, expression of STAT3 was not substantially affected in SupM2 whereas both pSTAT3 and total STAT3 levels decreased concurrently in UCONN-L2 cells expressing FOXM1 shRNA #4, suggesting that FOXM1 might regulate expression of STAT3 as well as its phosphorylation. Indeed, both pSTAT3 and total STAT3 expression was elevated in cells over-expressing FOXM1C as was expression of Cyclin B1 and phosphorylated NPM-ALK confirming the ability of FOXM1 to regulate the expression and/or phosphorylation of these proteins in NPM-ALK+ ALCL (Figure 3.18).

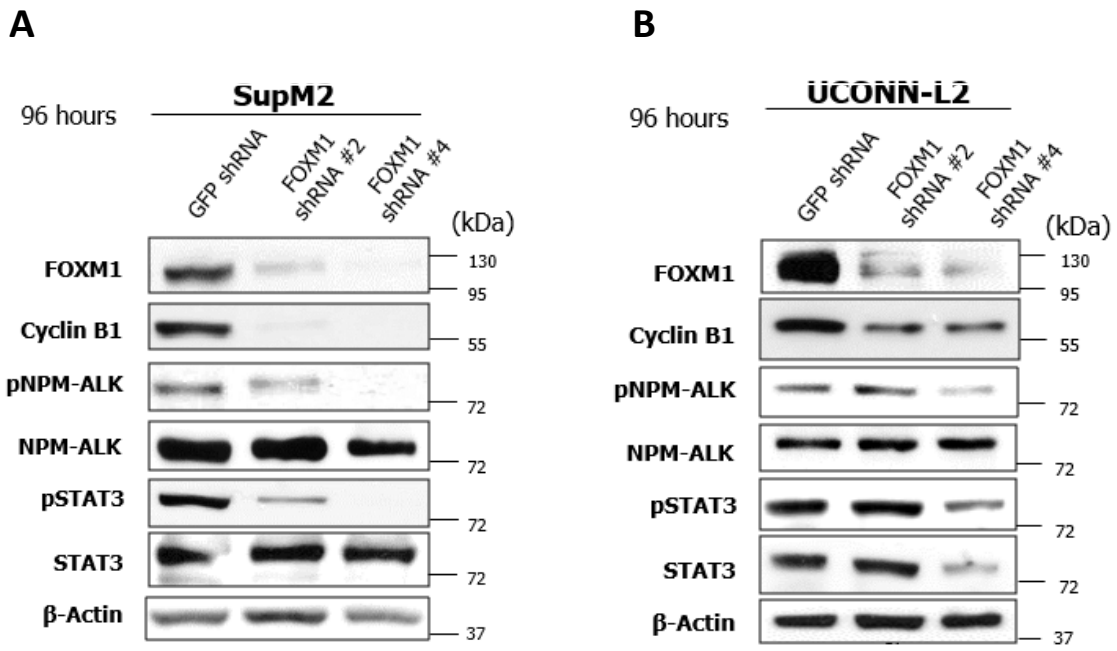

Figure 3.18 Effect of FOXM1 knockdown on signalling pathways in NPM-ALK+ ALCL. SupM2 and UCONN-L2 cells were transduced with 2 different species of FOXM1 shRNA. FOXM1 knockdown was assessed with anti-FOXM1 antibody and by probing the FOXM1-mediated target gene product Cyclin B1. pNPM-ALK detected the Y664 residue of NPM-ALK and pSTAT3 detected Y705 residue. β-actin was used as a loading control.

To determine whether this FOXM1-mediated activity is specific to ALCL, 293T cells were stably transfected with a FOXM1C doxycycline-inducible vector in the presence or absence of NPM-ALK (Figure 3.19). With increasing doses of doxycycline, a dose-dependent increase of FOXM1 expression was observed, in both the absence or presence of NPM-ALK in the latter case correlating with a dose-dependent increase in phosphorylation of NPM-ALK. Consistent with previous observations, STAT3 was phosphorylated in the presence of NPM-ALK although the presence of FOXM1 did not increase this further, yet an appreciable elevation in the expression of total STAT3 was detected. Interestingly, in the absence of NPM-ALK (lanes a-c), upregulation of FOXM1 also resulted in appreciable increases in both pSTAT3 and STAT3. The results indicate FOXM1 can activate the STAT3 signaling pathway in an NPM-ALK—independent manner in 293T cells.

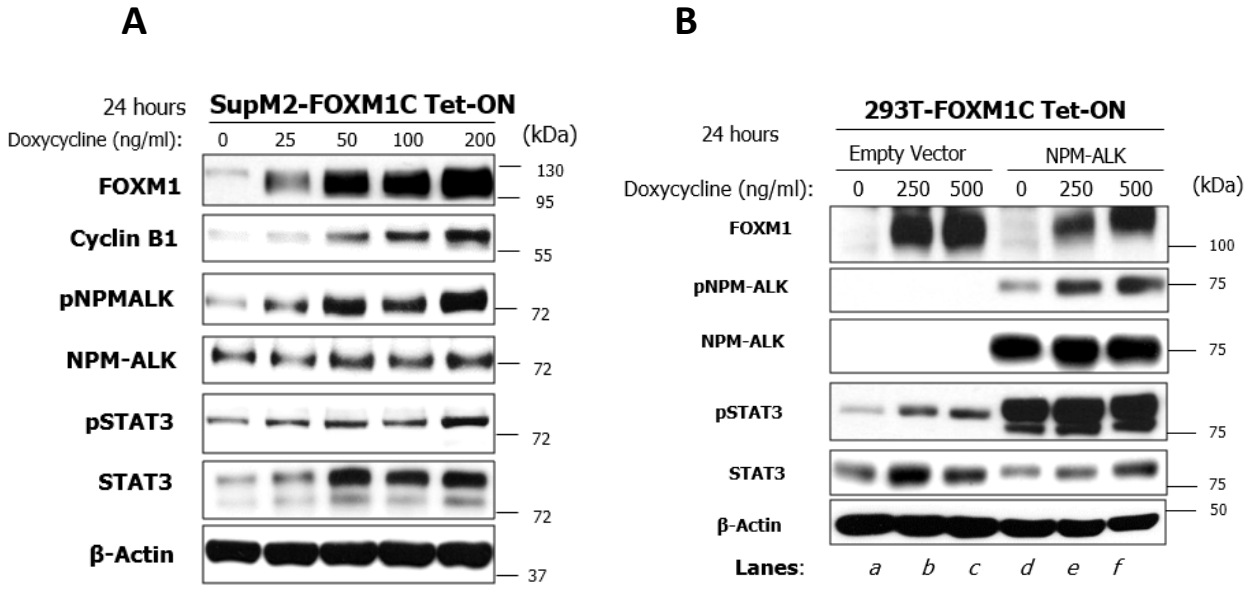

Figure 3.19. FOXM1 overexpression alters the signalling of NPM-ALK. A) SupM2-FOXM1C Tet-ON were induced to overexpress FOXM1 followed by downstream assessment of signalling molecules. B) 293T-FOXM1C Tet-ON cells were transfected with either empty vector or NPM-ALK overexpression constructs. Western blotting of downstream signalling proteins was then performed. β-actin was used as a loading control. Results are representative of at least two biological replicates

3.8 Influence of NPM-ALK on Transcriptional Activity of FOXM1

To determine whether NPM-ALK plays a role in regulating the transcriptional activity of FOXM1, a FOXM1 consensus sequence-luciferase reporter construct was transiently transfected into SupM2 cells. Following siRNA-mediated downregulation of NPM-ALK luciferase activity was abrogated, suggesting that NPM-ALK is required for FOXM1 to function as a transcription factor (Figure 3.20A). In keeping with this concept, siRNA-induced downregulation of NPM-ALK expression in SupM2 cells resulted in a dramatic decrease in FOXM1 protein bound to a biotinylated DNA probe containing FOXM1 consensus sequences, while the total FOXM1 protein level in the cell lysate (i.e. the input) was largely unaltered (Figure 3.20B). In the same experiment, western blot studies showed that siRNA -mediated inhibition of NPM-ALK expression led to a substantial decrease in Cyclin B1, a well-known target of FOXM1 transcriptional activity (258).

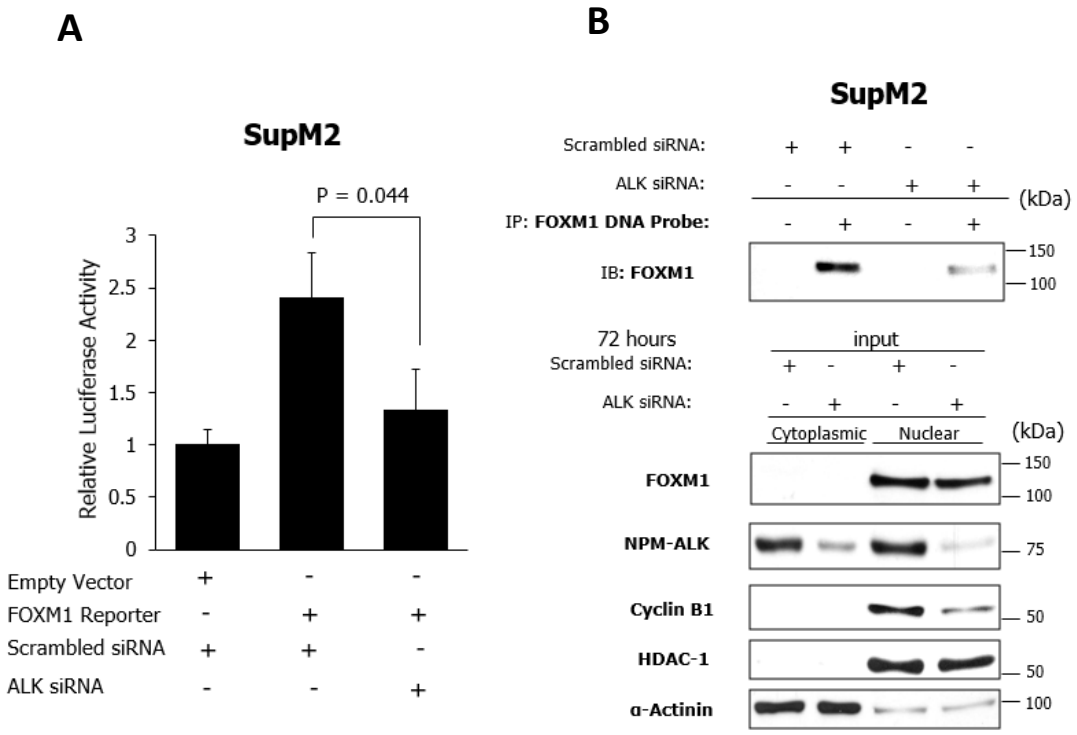

Figure 3.20. Impact of FOXM1 activity upon downregulation of NPM-ALK. A) SupM2 cells were electroporated with either the Empty Vector or FOXM1 reporter, in conjunction with either scrambled siRNA or ALK siRNA. Luciferase reporter activity was then measured in triplicates. B) SupM2 cells were electroporated with either scrambled siRNA or ALK siRNA followed by cytoplasmic/nuclear fraction (lower panel). DNA pulldown of FOXM1 following ALK knockdown is shown on the upper panel

In order to determine whether the effect of NPM-ALK on the transcriptional activity of FOXM1 is dependent on the kinase activity of NPM-ALK, HEK 293 cells were transfected with a FOXM1 reporter luciferase construct together with either wild-type NPM-ALK or a kinase-dead mutant form of NPM-ALK. As shown in Figures 3.21A and 3.21B, transfection of wild-type NPM-ALK (NPM-ALK^{WT}), but not the kinase-dead form (NPM-ALK^{FFF}), led to a significant increase in luciferase activity in the presence of exogenous FOXM1 ($p=0.02$).

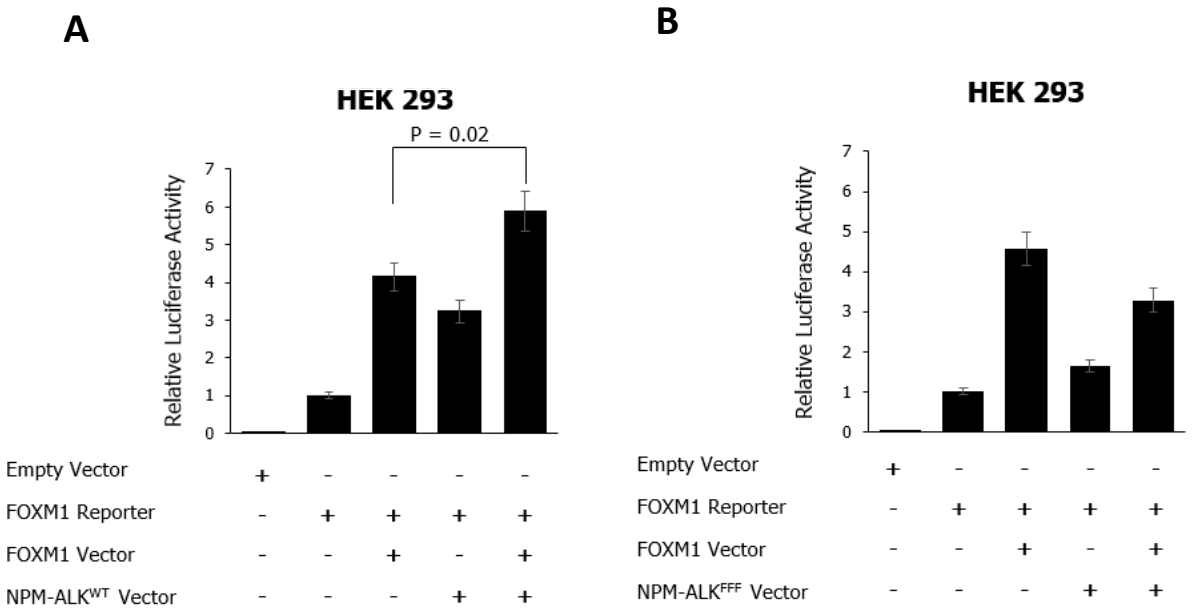

Figure 3.21. Effect of NPM-ALK activation on the transcriptional activity of FOXM1 A) HEK 293 cells were transfected with either Empty Vector or FOXM1 Reporter in conjunction with either FOXM1 or NPM-ALK^{WT} vectors. B) HEK 293 cells were transfected with either Empty Vector or FOXM1 Reporter in conjunction with either FOXM1 or NPM-ALK^{FFF} vector.

In addition, FOXM1 Chromatin Immunoprecipitation (ChIP) in the presence of NPM-ALK^{WT} but not the kinase-dead variant, NPM-ALK^{FFF}, showed binding of exogenous FOXM1 to Cyclin B1 (*CCNB1*) promoter regions (Figure 3.22).

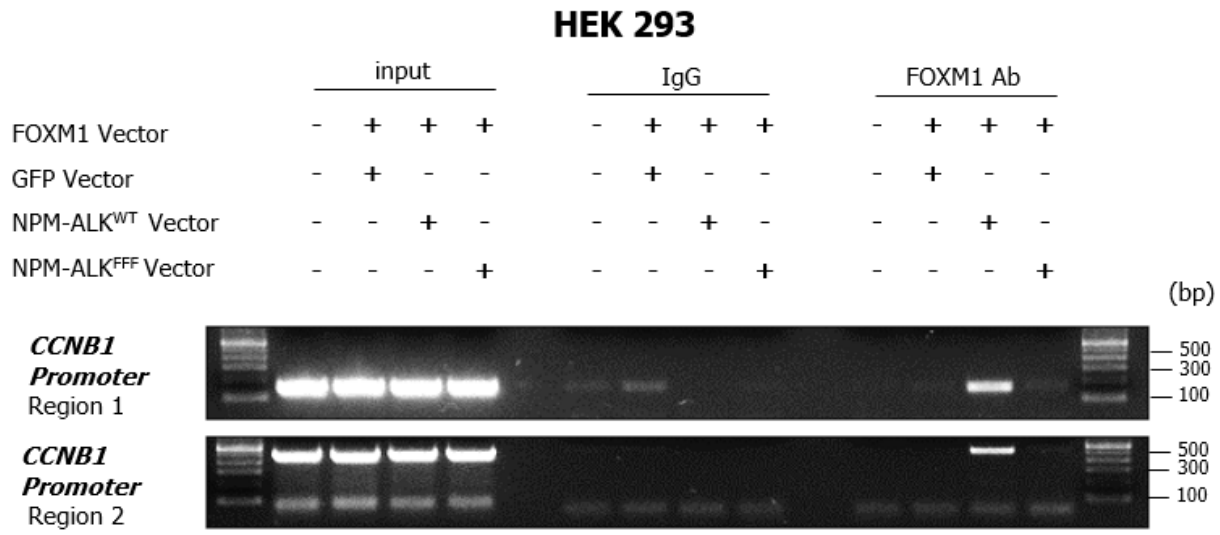

Figure 3.22 Influence of NPM-ALK on FOXM1 transactivation as assessed by chromatin immunoprecipitation. PCR amplification of two promoter sites of *CCNB1* (Cyclin B1 promoter) was performed following ChIP with a control IgG or FOXM1 antibody in HEK 293 cells. Cells were co-transfected with FOXM1 and either wild-type NPM-ALK (*NPM-ALK^{WT}*) or kinase-dead NPM-ALK (*NPM-ALK^{FFF}*). Data are representative of two replicates.

3.9 Role of NPM1 in Mediating NPM-ALK—FOXM1 binding

In order to delineate the mechanism by which NPM-ALK facilitates the transcriptional activity of FOXM1, co-immunoprecipitation was conducted revealing that FOXM1 complexes with NPM-ALK in SupM2 and Karpas 299 cells (Figure 3.23A). Furthermore, NPM-ALK was detected bound to a biotinylated DNA probe containing FOXM1 consensus sequences when expressed in ALCL cell lines, as was FOXM1 (Figure 3.23B). Taken together, these results are consistent with the hypothesis that formation of a complex involving NPM-ALK and FOXM1 is critical for FOXM1 to carry-out its transcriptional regulatory function.

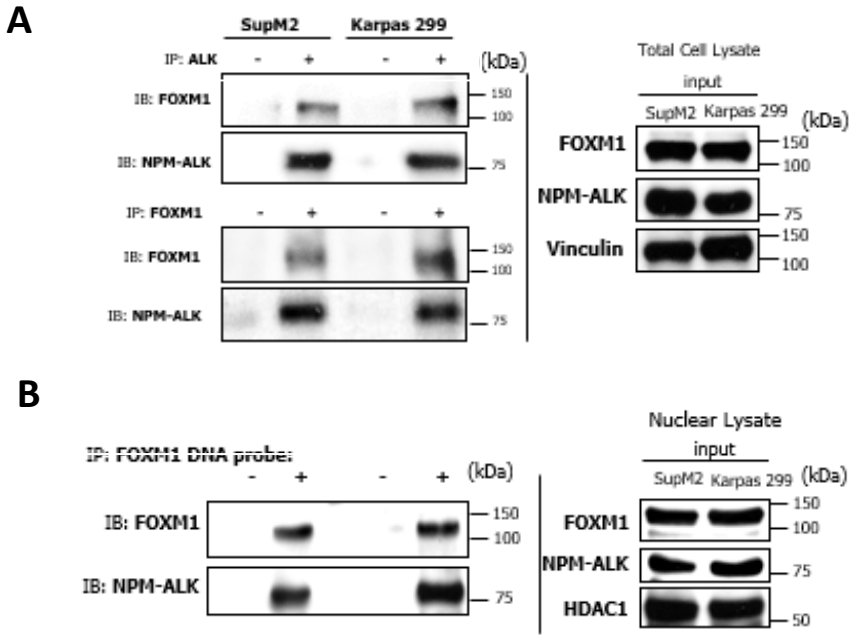

Figure 3.23 Evaluation of FOXM1 and NPM-ALK as evaluated by reciprocal co-immunoprecipitation. In the top panel, an ALK antibody was used to pulldown ALK in SupM2 and Karpas 299. In the middle panel, a FOXM1 antibody was used to pulldown FOXM1 (B) A biotinylated DNA probe consisting of FOXM1 consensus sequences was immunoprecipitated from nuclear cell lysates of SupM2 and Karpas-299 cell lines, followed by western blotting to asses the indicated proteins.

In the nuclei of NPM-ALK+ ALCL cells, it has been previously published that NPM-ALK is present predominantly as NPM-ALK—NPM1 heterodimers (218). Given that in acute myeloid leukemia, NPM1 physically binds to FOXM1 (259) via the portion of NPM1 that is not retained in the NPM-ALK fusion (218), shRNA was employed to inhibit expression of wild-type NPM1. A decrease in co-immunoprecipitation of NPM-ALK and FOXM1 was observed (Figure 3.24).

Since it is known that shNPM1 could readily decrease the protein expression of FOXM1 in several cell types as well as FOXM1 as illustrated (lane 2 of Figure 3.24), the SupM2 Tet ON FOXM1 model was employed for this experiment. Doxycycline was used to induce the expression of FOXM1 in the SupM2 Tet ON model which dramatically increased the protein level of FOXM1. Therefore, a fairer comparison of ALK immunoprecipitated with FOXM1 could be evaluated using this system.

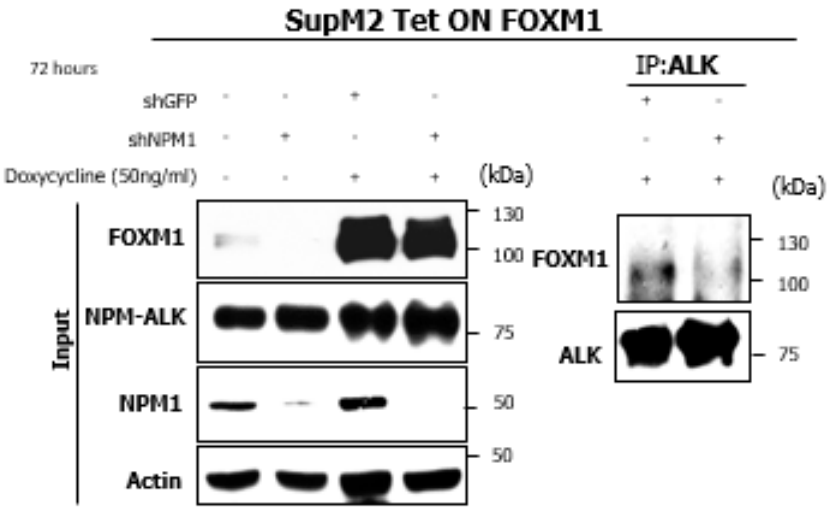

Figure 3.24 Influence of NPM1 on the interaction between FOXM1 and NPM-ALK. Immunoprecipitation of NPM-ALK was performed in SupM2 FOXM1 Tet-ON cells after treatment with doxycycline following shRNA mediated knockdown of NPM1 for 72 hours.

In order to confirm that binding is mediated via wild type NPM1 and the NPM portion of NPM-ALK, HEK 293 cells were transfected to express variant ALK fusion proteins including NPM-ALK, EML4-ALK and full length wild-type ALK. Compared to all three ALK forms tested, only NPM-ALK efficiently co-immunoprecipitated with FOXM1 (Figure 3.25).

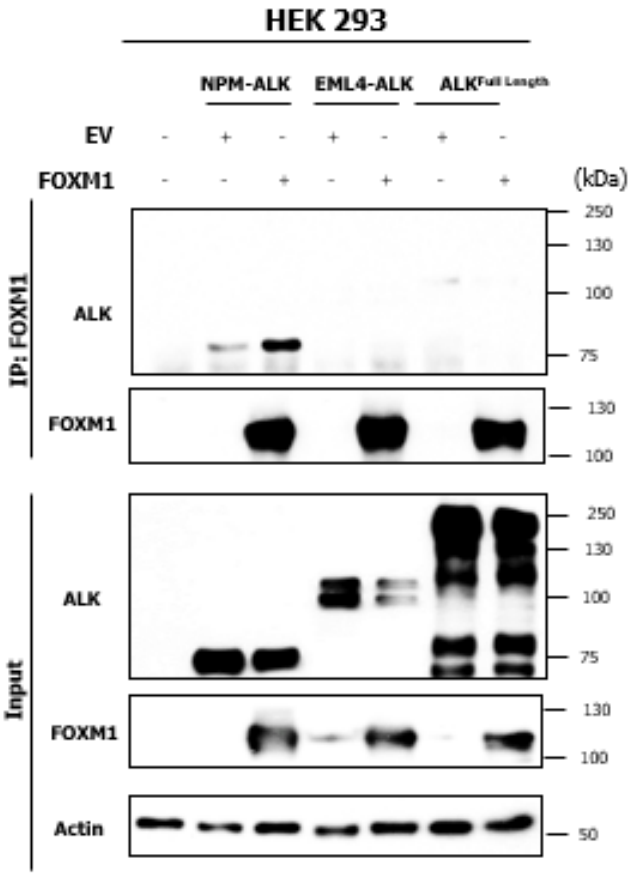

Figure 3.25. Evaluation of FOXM1 binding to known ALK forms in various human cancers. Co-immunoprecipitation was performed with FOXM1 antibody in HEK 293 cells following transfection of cells with FOXM1 and with either *NPM-ALK*, *EML4-ALK* or *ALK* (full length) expression vectors.

As shown in Figure 3.22, phosphorylation and hence activation of NPM-ALK is required to facilitate the DNA binding and transcriptional activity of FOXM1. In order to determine whether this activity is also required to enable the formation of a complex involving NPM1, NPM-ALK and FOXM1, HEK293 cells were transfected to express wild-type NPM-ALK (NPM-ALK^{WT}) or kinase-dead NPM-ALK (NPM-ALK^{FFF}). Figure 3.26 shows that NPM-ALK^{WT} was found to co-immunoreacted significantly more with FOXM1 in comparison to NPM-ALK^{FFF}.

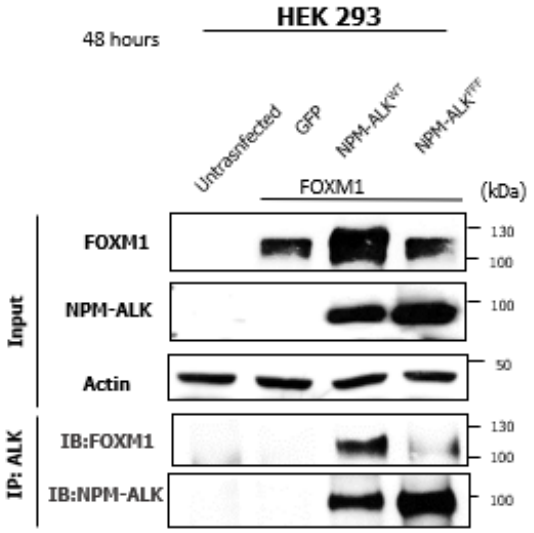

Figure 3.26. Influence of NPM-ALK activation on the binding with FOXM1. HEK 293 cells were transfected with FOXM1 and either *GFP*, *NPM-ALK^{WT}* or *NPM-ALK^{FFF}* expression vectors followed by co-immunoprecipitation with a FOXM1 antibody and western blotting of the indicated proteins.

Chapter 4: Discussion

4.1 General Discussion

In solid tumors, FOXM1 has been extensively studied, and it is known to contribute to the initiation, proliferation, tumorigenicity, angiogenesis, chemo-resistance, metastatic capabilities and stemness of malignancies (4). It is believed that FOXM1 mediates its oncogenic effects via a number of molecular mechanisms, including promotion of the nuclear translocation of β-catenin (134), upregulation of a host of cell-cycle facilitators (260), increasing the expression of stem cell-related proteins (e.g. SOX2 and MYC)(28), inhibition of tumor suppressors including p53 (149, 261) and increasing the level of VEGF in promoting angiogenesis (262). In comparison, the biological significance of FOXM1 in hematologic malignancies is less well understood: In an early study, it was found that FOXM1 promotes the growth of thymic lymphoma in a p53-null mouse model (263); In another study, conditional knockdown of FOXM1 in precursor B-cell lymphoblastic leukemia cell lines was found to significantly prolong the survival of mice xenografted with leukemic cells (264); In a study of plasma cell myeloma, overexpression of FOXM1 in cell lines resulted in a significantly higher tumor volume of xenografts formed in mice (179); a high level of FOXM1 expression detectable by immunohistochemistry was found in ~85% cases of diffuse large B-cell lymphoma, and pharmacologic inhibition of FOXM1 in lymphoma cell lines substantially decreased invasiveness in vitro, which correlated with a reduction in expression of Ki-67 and two epithelial-to-mesenchymal transition markers (MMP-2 and MMP-9) (184); Lastly, in two studies of acute myeloid leukemia, the silencing and reduction of FOXM1 led to decreased tumorigenic properties in both in vitro and in vivo models (182, 185).

4.2 Expression of FOXM1 in Aberrant and Important for NPM-ALK+ ALCL

The expression of FOXM1 in NPM-ALK+ ALCL was found to be consistent across all human tissues tested in this study. Elevated FOXM1 expression was found in 5/5 NPM-ALK+ ALCL cell lines in comparison to lymphocytes obtained from a healthy adult. Moreover, in 6/6 tumor blocks NPM-ALK+ ALCL blocks, the expression of FOXM1 was found to be localized in neoplastic cells whereas surrounding benign cells displayed no expression or little expression of FOXM1. These findings indicate that the expression of FOXM1 is highly relevant in NPM-ALK+ ALCL pathobiology. Furthermore, FOXM1 was found in this study to be localized mostly in the nuclei of NPM-ALK+ ALCL cells. Some expression of FOXM1 could be detected in the cytoplasm. This was especially the in the tumor blocks from the *NPM-ALK* transgenic mice.

The expression of FOXM1 in NPM-ALK+ ALCL, whose normal counterpart is believed to be mature T-cells (265), is an aberrant event. In this regard, it has been shown that the expression of FOXM1 mRNA is tightly regulated during T-cell development, with the expression of FOXM1 first detectable in double-negative (i.e. negative for CD4 and CD8) thymocytes, reaching its peak at the stage of immature double positive (i.e. positive for both CD4 and CD8) thymocytes, dramatically decreasing on becoming single-positive (e.g. positive for CD4 or CD8) thymocytes, and becoming undetectable in fully mature lymphocytes in the periphery (2). In keeping with these findings, PBMC cells, which include mature T-cells, had no detectable FOXM1 protein. Based on these observations, it is possible that the normal mechanism that is responsible for ‘silencing’ FOXM1 protein

expression at the mature T-cell stage has become defective during the oncogenesis of NPM-ALK+ ALCL.

The use of the luciferase reporter containing FOXM1 sequences (i.e FOXM1 reporter) helped verify the transcriptional activity of FOXM1 in NPM-ALK+ ALCL. Furthermore, the use of thiostrepton reduced the luciferase activity in cells transfected with the FOXM1 reporter. Additionally, nuclear/cytoplasmic fractionation of the two NPM-ALK+ ALCL cell lines (SupM2 and UCONNL-2) and in the immunocytochemistry of the three NPM-ALK+ ALCL cell lines, the expression of FOXM1 was found to be strictly localized in the nuclei. Indeed, the nuclear localization of FOXM1 is believed to be a strong indicator of positive FOXM1 transcriptional activity in human cancer. Thus, FOXM1 is likely to be highly involved in regulation of gene expression in NPM-ALK+ ALCL.

The downregulation of FOXM1 through both shRNA and a pharmacological agent greatly reduced the tumorigenic potential of NPM-ALK+ ALCL cells. Cells with ablated FOXM1 demonstrated slower cell divisions and an increase of apoptotic cells. Consistent results were obtained with two different species of FOXM1 shRNA and with the FOXM1 inhibitor, thiostrepton. These results suggest that FOXM1 is an important growth and survival factor in NPM-ALK+ ALCL. Furthermore, the finding that FOXM1 knockdown can affect the soft agar colony formation ability of NPM-ALK+ ALCL may suggest that FOXM1 can regulate self-renewal capabilities of this cancer. It is possible that the FOXM1 contributes to other tumorigenic properties such as escaping cellular senescence and allowing for cancer cell metastasis; however, these properties could not be tested in this study.

4.3 FOXM1 Regulates the NPM-ALK/STAT3 Axis

The exact mechanism in how FOXM1 is essential for NPM-ALK+ ALCL seems to be multifaceted. Indeed, experimental manipulation of the expression level of FOXM1 resulted in the expected changes in Cyclin B1, a well-known FOXM1 downstream target. Moreover, FOXM1 contributes to the activation/phosphorylation of NPM-ALK. Similarly, the activation/phosphorylation level of STAT3 mirrored that of pALK. The exact mechanism by which FOXM1 contributes to increased pALK and pSTAT3 levels remain a mystery. In this regard, it is known that FOXM1 can regulate several cellular signalling pathways through multiple mechanisms. For example, it has been shown that FOXM1 upregulates the expression and activity of STAT3 in a β-catenin-dependent manner in glioblastoma cells (266). In osteosarcoma cells, FOXM1 was also known to transcriptionally regulate the expression and phosphorylation c-Jun N-terminal kinase (JNK1) to drive cell proliferation (15).

Modulation of the NPM-ALK/STAT3 pathway by FOXM1 could be an important reason for the biological effects observed with FOXM1 knockdown in NPM-ALK+ ALCL. It has been well demonstrated previously that both NPM-ALK and STAT3 are required for the growth and survival of this cancer. As outlined in the Chapter 1, both NPM-ALK and STAT3 regulate key biological processes, including cell cycle progression, evasion of apoptosis and self-renewal. Furthermore, it is possible FOXM1 regulates several other cellular proteins and pathways which could not be fully examined in this work. In

summary, FOXM1 exerts some sort of biological influence of the NPM-ALK/STAT3 pathway by regulating their phosphorylation and perhaps expression.

4.4 NPM-ALK Regulates FOXM1 transcriptional activity

One of the key findings of this study is that NPM-ALK promotes the DNA binding and transcriptional activity of FOXM1. This conclusion is based on the observations that knockdown of NPM-ALK significantly reduce FOXM1 reporter activity and its binding to the FOXM1 DNA probe. Furthermore, results from the ChIP assay provided further support and validation to this conclusion. The functional role of NPM-ALK localized to the nucleus is largely unknown. The data presented here, considering that FOXM1 is largely localized to the nucleus, suggests that NPM-ALK can exert oncogenic effects in the nuclei of NPM-ALK+ ALCL cells, by promoting the biological activity of oncogenic transcription factors such as FOXM1.

The experimental results also have shed light into how NPM-ALK regulates the transcriptional activity of FOXM1. Specifically, the data points to a model in which the NPM-ALK facilitated DNA binding (and hence the transcriptional activity) of FOXM1 requires physical interaction between NPM-ALK and FOXM1. How exactly FOXM1 binds to NPM-ALK was thoroughly examined in this study. In this regard, two important features of NPM-ALK localized in the nucleus of NPM-ALK+ ALCL cells are highly relevant. First, NPM-ALK has been reported to exist predominantly as NPM-ALK—NPM1 heterodimer in the nuclei of NPM-ALK+ ALCL cells (39). Second, a previously published study showed

that FOXM1 can bind to the heterodimer domain of NPM1 (23), a segment close to the C-terminus of NPM1 which is known to be not represented in the fusion NPM-ALK protein (12, 40). This experimental data is consistent with the hypothetical model depicted in Figure 8. It was found that the NPM1 portion of NPM-ALK is crucial to binding to FOXM1, as EML-ALK and full length ALK were unable to bind to FOXM1. In this regard, this data suggests that the ALK portion alone cannot efficiently interact with FOXM1. Thus, this study found further evidence of the pathogenetic role of NPM1, a protein highly implicated in the pathogenesis of acute myeloid leukemias (AML) (218), in NPM-ALK+ ALCL. Interestingly, this is in parallel to how NPM1 is believed to promote tumorigenesis. Specifically, NPM1 has been shown to facilitate DNA binding and transcriptional activity of oncoproteins such as c-MYC and NFκB-p65 (267, 268).

It appears that the phosphorylation status of NPM-ALK is important in regulating the biological activity of FOXM1. This conclusion is based on the observation that transfection of NPM-ALK^{WT}, but not the kinase-dead NPM-ALK^{FFF}, significantly promoted the transcription activity of FOXM1. This is rather intriguing, as it has been reported that most NPM-ALK proteins present in the nuclei of NPM-ALK+ ALCL cells exist in the form of NPM-ALK—NPM1, which is not as highly phosphorylated as NPM-ALK dimer present in the cytoplasm of these cells (222). Nonetheless, a weak but definitive level of phosphorylated ALK can be detected in the nuclear fraction of NPM-ALK+ ALCL (222). It is possible that this relatively low level of NPM-ALK phosphorylation may have resulted in an optimal three-dimensional conformation in the NPM-ALK—NPM1 dimer, facilitating the physical interaction of NPM1 and FOXM1. This low level of NPM-ALK phosphorylation

also may have attracted the binding of other proteins to the NPM-ALK—NPM1—FOXM1 complex, facilitating the DNA binding of FOXM1.

In keeping with data presented in this study, FOXM1 was found to form a complex with NPM-ALK only in the presence of wild-type NPM-ALK. Taken together, these data suggest that NPM-ALK facilitates the DNA binding and transcriptional activity of FOXM1, and this process requires the phosphorylation of NPM-ALK as well as the presence of NPM1 as summarized in Figure 4.1.

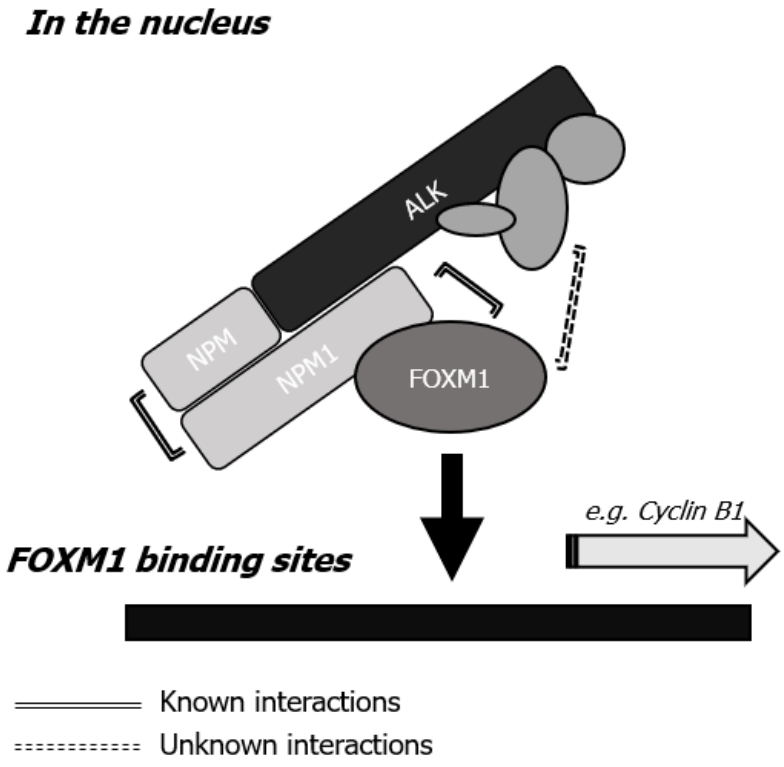

Figure 4.1 Hypothesized depiction of interaction between NPM-ALK and FOXM1. NPM-ALK binds to FOXM1 through mutual interactions with NPM1. The phosphorylation of NPM-ALK further potentiates its ability to bind to FOXM1 and influence FOXM1 transcriptional activity.

4.2 Limitations

One of the major limitations of NPM+ ALCL and several other lymphoma cell line models is the challenge of gene transfer efficiency. In this study, SupM2 and UCONN-L2 cell lines were found to have the highest transduction efficiency with lentivirus. Transduction of lentivirus expressing *green fluorescent protein* (GFP) achieves about 70% in SupM2 and 30% in UCONN-L2, respectively. Other NPM-ALK cell lines including Karpas 299, SUDHL-1, SR2 were found to have extremely poor gene transfer in the range of 1-5%. Thus, the study of FOXM1 and NPM-ALK could only restricted to SupM2 and UCONN-L2 cell lines. Whether or not the other NPM-ALK+ cell lines carry the same mechanisms as identified in SupM2 remains a mystery.

As mentioned in the *Introduction*, ALK+ ALCL presents itself with numerous *ALK* fusion partners besides NPM-ALK. Whether or not these fusion partners have an influence on FOXM1 function and transcriptional activity could not be evaluated in this study. NPM-ALK is the only fusion protein known in ALK+ALCL to be localized in both the nucleolus and nucleus. Other ALK fusion proteins do not share this same characteristic. These other ALK fusion proteins could serve as an excellent negative control when evaluating the functional properties between NPM-ALK and FOXM1 in test models.

To directly prove the protein-protein interactions of FOXM1, NPM1 and NPM-ALK, a system where proteins are absent would be the best suitable to conduct an appropriate study. However, this is difficult to achieve as the expression levels of FOXM1 and NPM1

are elevated in most immortal and cancer cell lines. One possible solution to this problem is to use the yeast two-hybrid system which could be used to evaluate the direct functional interaction between these three proteins.

4.3 Future Directions

In this study, one of the major discoveries was that FOXM1 could influence the phosphorylation and therefore the signalling of NPM-ALK+ ALCL cell lines. However, the exact mechanism of how FOXM1 influences the NPM-ALK/STAT3 signalling axis remains unknown. To understand exactly how FOXM1 controls the phosphorylation of NPM-ALK, STAT3 and several other possible proteins, ELISA or other phospho-proteomic analysis could be performed. These assays could potentially answer questions about exactly how FOXM1 regulates NPM-ALK and open new areas for evaluation.

Another possible tool that could be used is immunoprecipitation of FOXM1 followed by mass spectrometric analysis of proteins bound to FOXM1. These techniques could discern and identify the exact protein partners of FOXM1 in NPM+ ALCL. Lastly, RNA sequencing analysis or chromatin immunoprecipitation sequencing analysis could also help identify the exact nature of FOXM1 on the genome regulation of NPM-ALK+ ALCL. These assays could answer questions about whether, how and when FOXM1 is crucial to the development of NPM-ALK+ ALCL. For example, comparing the regulation of FOXM1 gene expression in mouse early thymocytes versus that of mouse possessing *NPM-ALK* thymocytes could reveal important details regarding how this protein mediates the oncogenesis of NPM-ALK+ lymphoma.

Additionally, the expression of FOXM1 in other ALK- ALCL could not be determined in this study. Future studies could compare the level of expression of FOXM1 in ALK+ vs in ALK- ALCL, and determine whether FOXM1 is relevant

4.4 Conclusions

In conclusion, this study reveals for the first time the importance of FOXM1 in NPM-ALK+ ALCL. The oncogenic effects of FOXM1 appears to be closely linked to NPM-ALK, the association of which contributes to the oncogenesis in these tumors. Furthermore, FOXM1 may be a potential therapeutic target in ALK+ALCL, and disruption of the binding between FOXM1 and NPM1 in the NPM-ALK—NPM1 heterodimers may serve as a highly specific anti-cancer therapeutic approach for NPM-ALK+ ALCL.

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