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The Role of Oncogenic Tyrosine Kinase NPM-ALK in Anaplastic Large Cell Lymphoma Pathobiology

by

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In the name of Allah, the Most-Merciful, the All-Compassionate

To my parents
Abdelghany Hegazy and Houriya Gad

To my husband Yasser
and my two lovely kids Essraa and Abdulrahman

ABSTRACT

Anaplastic lymphoma kinase expressing anaplastic large cell lymphoma (ALK^+ALCL) is an aggressive type of T/null cell lymphoma that mainly affects children and young adults and represents up to 40% of non-Hodgkin's lymphoma in children. These lymphomas are characterized by the abnormal chromosomal translocations involving the *ALK* gene resulting in the aberrant expression of an oncogenic fusion protein with constitutive activation of the ALK tyrosine kinase domain. In up to 80% of ALK^+ALCL cases, this fusion protein is nucleophosmin-anaplastic lymphoma kinase (NPM-ALK). NPM-ALK conducts its transforming ability through activation of many molecular mechanisms. Despite the well-known role of NPM-ALK in the pathogenesis of ALK^+ALCL as demonstrated by several *in vitro* and *in vivo* studies, there is evidence argue that this protein does not act alone in these tumors. Further studies are required to identify additional molecular defects in ALK^+ALCL, which could contribute to or potentiate NPM-ALK oncogenicity.

The first objective of this thesis examined the detailed mechanism of interaction between NPM-ALK and the tumor suppressor protein tyrosine phosphatase SHP1, a protein normally absent in most of ALK^+ALCL. Furthermore, the biological importance of this binding was demonstrated.

The second objective examined the aberrant expression of the embryonic stem cells (ESCs) transcription factor Sox2 in ALK^+ALCL cell lines and tumors. Despite its ubiquitous expression, Sox2 transcription activity was detected only in a small subset of tumor cells. Sox2 was demonstrated to contribute to ALK^+ALCL tumorigenesis, and its oncogenic potential correlated with its transcriptional activity.

The third objective examined the over-expression of the Wnt pathway members in ALK^+ALCL. The over-expression of disheveled proteins 2 and 3 (Dvl-2 and Dvl-3), was detected in ALK^+ALCL cell lines and tumors. I demonstrated that Dvl-2 and Dvl-3 play a significant biological role in ALK^+ALCL, and signal through the Wnt non-canonical pathway. Furthermore, cross talk between NPM-ALK and Wnt pathway through Dvl proteins was identified.

Overall, the identification of these novel signaling defects and their mechanisms in ALK^+ALCL oncogenesis furthered our current understanding of the pathobiology of these tumors and provided a framework for the development of multi-target therapies for these malignancies.

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LIST OF ABBREVIATIONS

17-AAG - 17-allylamino demethoxygeldanamycin

ABL - Abelson

ALCL- Anaplastic large cell lymphoma

ALK - Anaplastic lymphoma kinase

AML - Acute myeloid leukemia

APC - Adenomatous polyposis coli

Arg - Arginine

Asp - Aspartame

ATP - Adenosine tri phosphate

Bcl2 - B-cell lymphoma-2

BCR-Abl – Breakpoint cluster region-Abl

CamKII- Calcium-calmodulin-dependent kinase II

CARS–ALK - Cysteinyl- tRNA synthase - anaplastic lymphoma kinase

CDK4 - Cyclin-dependent kinase 4

CE - Convergent Extension

CK2- Casein kinase 2

CLL - Chronic lymphatic leukemia

CLTC1–ALK - Clathrin heavy chain like 1 - anaplastic lymphoma kinase

CML - Chronic myelogenous leukemia

CSCs- Cancer Stem Cells

CSF1 – Colony stimulating factor 1

C-SH2 – C terminal Src homology 2

Cys - Cysteine

DAG - Diacylglycerol

DEP - Disheveled, Egl-10, Pleckstrin

DIX - Disheveled, Axin

DMEM – Dulbecco’s Modified Eagle’s medium

DNMT1 - DNA methyltransferase 1

Dsh – *Drosophila* Disheveled

Dvl – Human Disheveled protein

Dvl-1 - Disheveled protein - 1

Dvl-2 - Disheveled protein - 2

Dvl-3 - Disheveled protein - 3

EGF - Epidermal growth factor

EMA - Epithelial membrane antigen

EML4 - Echinoderm microtubule-associated protein-like4

EML4–ALK - Echinoderm microtubule-associated protein-like4 - anaplastic lymphoma kinase

Epo-R - Erythropoietin receptor

ERK - Extracellular-signal-regulated kinase

ESCs - Embryonic stem cells

FBS - Fetal bovine serum

Fz - Frizzled

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GFP - Green fluorescent protein

GSK-3 - Glycogen Synthase Kinase-3

HDAC 1- Histone deacetylase 1

hLTK – Human leukocyte tyrosine kinase

HMG - High Mobility Group DNA binding domain

HSP-90 - Heat shock protein - 90

IGF-R1- Insulin growth factor receptor - 1

IL – Interleukin

IP_3 - Inositol trisphosphate

ips - Inducible pluripotent stem cells'

ITIM - Immunoreceptor tyrosine-based inhibition motifs

JAK/ STAT - Janus Kinase / Signal transducer and activator of transcription

JAK3/STAT3 - Janus Kinase 3 / Signal transducer and activator of transcription 3

JNK - c-Jun NH-terminal kinases

KAL – Kinase activation loop

LRP - Low density lipoprotein-related proteins

LTK - Leucocyte tyrosine kinase

Lys - Lysine

MCL1 - Mantle cell lymphoma 1

mCMV - Minimal cytomegalovirus

MMTV - Mouse mammary tumor virus

MSN–ALK – Moesin - anaplastic lymphoma kinase

mTOR - Mammalian target of rapamycin

mTORC1 - Mammalian target of rapamycin complex-1

mTORC2 - Mammalian target of rapamycin complex-2

MYH9–ALK - Non-muscle myosin heavy chain - anaplastic lymphoma kinase

NaCl – Sodium chloride

NFAT - Nuclear factor associated with T cells

NF-ƙB - Nuclear factor kappa B

NPM - Nucleophosmin

NPM-ALK -Nucleophosmin-anaplastic lymphoma kinase

N-SH2 – N-terminal Src Homolgy 2

PAR-1 – Protease activated receptor -1

PARP - Poly (ADP-ribose) polymerase

PBMCs - Peripheral blood mononuclear cells

PBS - Phosphate buffered saline

PCR - Polymerase chain reaction

PDGFR - Platelet-derived growth factor receptor

PDZ - Postsynaptic density 95, Discs Large, Zonula occludens-1

Phe - Phenylalanine

PI3K - Phosphatidylinositol 3-kinases

PKC - protein kinase C

PLC- γ - Phospholipase C- γ

PTCL-NOS - Peripheral T cell lymphoma – not otherwise specified

PTK - Protein tyrosine kinase

PTP – Protein tyrosine phosphatase

RANBP2–ALK - RAN binding protein 2 - anaplastic lymphoma kinase

RT-PCR - Reverse transcriptase -polymerase chain reaction

RYK - Related to tyrosine kinase

SCID – severe combined immune deficient

SDS - Sodium dodecyl sulphate

SEC31L1–ALK - SEC31 homologue A (*S. cerevisiae*) - *anaplastic lymphoma kinase*

Ser - Serine

SH2 - Src homology 2

SH3 - Src homology 3

SHH - Sonic hedgehog

SHP - SH2-containing phosphatase-1

siRNA - Short interfering RNA

Sox - Sex determining region Y-Box

STAT3 - Signal transducer and activator of transcription-3

STAT5A - Signal transducer and activator of transcription-5A

STAT5B- Signal transducer and activator of transcription-5B

TCF/LEF - T-cell factor/Lymphocyte enhancer factor

TCR - T-cell receptor

TFG–ALK - TRK-fused gene - anaplastic lymphoma kinase

TGF-β - Tumor growth factor - β

TPM3–ALK - Tropomyosin 3 - anaplastic lymphoma kinase

TRK-T3 – Tropomyosin receptor kinase –T3

Tyr- Tyrosine

WCP - Wnt canonical pathway

Wg - Wingless

WHO - World health organization

- # Chapter 1

General Introduction

1.1. Introduction

Historically, anaplastic large cell lymphoma (ALCL) has passed through several evolutionary stages. ALCL was first described in 1985 (1), as a neoplastic proliferation of lymphoid cells that are large and display anaplastic cytology, sinus growth pattern, and strong expression of Ki-1 antigen, which is now known as CD30 (1,2). Due to these morphological criteria ALCL was frequently misdiagnosed as metastatic carcinoma or malignant histocytosis. The fact that CD30 is broadly expressed by other types of malignancies, as well as reactive disease made the diagnosis of ALCL as a distinct entity more difficult. In early 1990s, the discovery of the recurrent chromosomal translocation t(2;5) that involves the receptor tyrosine kinase anaplastic lymphoma kinase (*ALK*) on 2p23 and nucleophosmin (*NPM*) on 5q35, made ALCL a clinically and biologically specific entity(3). Based on ALK expression, which is not normally expressed in lymphoid tissues, ALCL are categorized into ALK expressing or ALK positive (ALK^{+}ALCL) and ALK negative (ALK^{-}ALCL) (4,5).

Currently, according to the World Health Organization (WHO) 2008 classification (6), there are three distinct types of T cell lymphoma: ALK^{+}ALCL, ALK^{-} ALCL and primary cutaneous T-cell lymphoma. Thus according to this classification ALK^{+}ALCL is a distinct clinicopathological and molecular entity.

1.2. ALK- expressing Anaplastic Large Cell Lymphoma

1.2.1. An overview

ALK^{+} ALCL is an aggressive type of T/null-cell non-Hodgkin's lymphomas that primarily affects children and young adults, with more male predominance and it constitutes 10-30% of all pediatric lymphomas (7). However, ALK^{-} ALCL patients are mainly adults with a lower male predominance (8).

ALK^{+}ALCL is thought to derive from activated mature cytotoxic T cells (6) and the neoplastic transformation of these cells is due to the constitutive activation of the oncogenic ALK fusion proteins that are deriving from chromosomal rearrangements involving the *ALK* gene(9).

Approximately 60% - 70% of patients present with advanced stage disease (stage III/ IV), due to peripheral and abdominal lymphadenopathy. Mediastinal lymphadenopathy is detected in up to 40% of cases. Extranodal disease is detected in 40% to 50% of cases, with the skin, bone and soft tissues being the most common to be involved. Advanced stage disease and bone marrow involvement are much more frequent in ALK^{+}ALCL in comparison to ALK negative neoplasms (8,10). Bone marrow involvement is detected in up to 61% of cases of ALK^{+}ALCL at diagnosis, especially in the small cell variant and is associated with high risk of relapse (11-13) .

In pediatric patients, there is uncommon extranodal involvement of the perirectal soft tissues and muscles (14-16), hard palate and nasal cartilages (17) , ovary (18), bronchi (19), and the eye lid (20). Involvement of the central nervous system and the gastrointestinal tract is rare in ALK^{+}ALCL (21-24).

Except for the small cell variant, the prognosis of ALK^{+}ALCL is good with a five year survival of approximately 85%. ALK^{+}ALCL prognosis is favourable than that of ALK^{-}ALCL (8,25). This favourability in prognosis is attributed to the younger age of patients in ALK^{+}ALCL, since there is no

significant prognostic difference between ALK^{+}ALCL and ALK^{-} ALCL in patients older than 40 years (8).

1.2.2. Morphology and Immunophenotype

A) Morphology

There are three main morphological variants for ALCL (Figure 1.1). These are: common, small cell and lympho-histocytic type (6). Other rare histologic patterns including, giant cell rich, Hodgkin-like, sarcomatoid (26), monomorphic (27), signet ring- like (28,29) and hypocellular(30) are existing. However these patterns are not considered as distinctive variants (6,7). Despite this broad morphological variability, the presence of the "hallmark cells" (Figure 1.1A) is a characteristic feature of all the variants (31,32). These cells are present with a variable percentage among the different types. The "hallmark cells" are large lymphocytes with an eccentric lobulated "horse shoe" or "kidney – shaped" nuclei and abundant cytoplasm, with intense eosinophilic staining that corresponds to Golgi apparatus. In the small cell variant (Figure 1.1B) these cells are relatively smaller, with a distinct cytoplasmic membrane and they are mixed with medium-size and large lymphoid cells (33). In the lymphohistocytic variant (Figure 1.1C), the malignant cells are admixed with a predominant population of reactive histiocytes, that could hinder the detection of the malignant cells. More than one morphologic variant could also be seen in the same lymph node biopsy, and it is referred to as a composite pattern (Figure 1.1D) (6). The giant cell rich pattern is characterized by large, multinucleated cells that resemble Reed Sternberg cells (6,32).

In general, the diagnosis of anaplastic large cell lymphoma based on the morphology could be challenging and the differential diagnosis of other lesions should be considered. This differential diagnosis should include viral and bacterial inflammatory lesions, hemophagocytic syndrome, non lymphoid malignancies, peripheral T cell lymphoma – not otherwise specified PTCL-NOS, and primary cutaneous ALCL (6). The morphologic

criteria alone do not distinguish between ALK^{+} and ALK^{-} ALCL, although the small variant is mainly ALK^{+} (34).

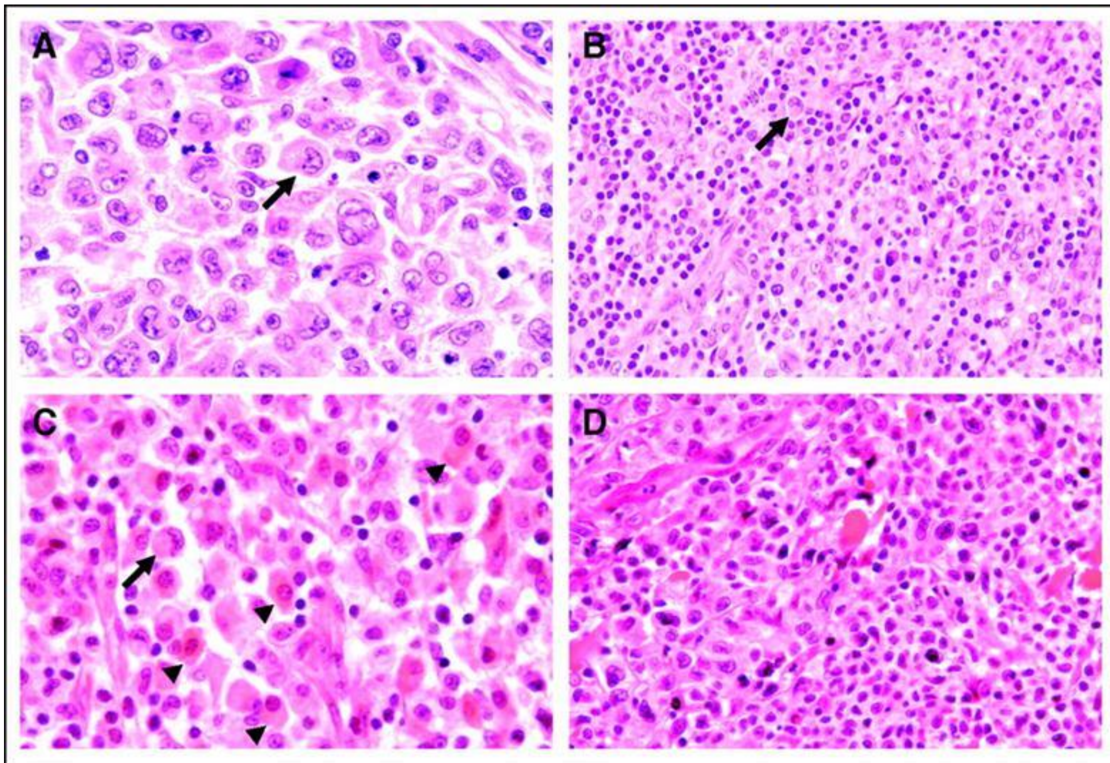

Figure 1.1 Histological features of ALK^{+}ALCL

(A) Anaplastic large-cell lymphoma, common variant. Predominant population of large cohesive cells. Characteristic to all the variants is the hallmark cells, with eccentric, horseshoe, or kidney-shaped nuclei, with a juxtanuclear eosinophilic region (arrow; hematoxylin and eosin [HE]; magnification, x400). (B) ALCL, small-cell variant. Predominant population of small cells with irregular nuclei and scattered hallmark cells (arrow; HE; magnification, x200). (C) ALCL, lymphohistiocytic variant. Malignant cells (arrow) are admixed with a predominant population of reactive histiocytes (arrow head) and are sometimes difficult to detect (HE; magnification, x400). (D) Composite ALCL. Association in a single biopsy of areas of common pattern (left side) and small-cell pattern (right side; HE; magnification, x400).

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B) Immunophenotype

ALK^+ALCL exhibits strong immunostaining for CD30 (1,35). CD30 is a cytokine receptor of tumor necrosis factor receptor superfamily. Immunostaining for CD30 in ALK^+ALCL is typically membranous, with more intense staining within the Golgi area (Figure 1.2) (35) . The pathognomonic feature of ALCL is the presence of lymphatic sinuses and perivascular infiltration by malignant cells which is detectable in approximately 75% of cases (31). This infiltrative pattern could be highlighted by immunostaining against CD30 (Figure 1.3) (31). However, the expression of CD30 is not exclusive for ALK^+ALCL; as it is also expressed by normal activated lymphocytes, Reed-Sternberg, Hodgkin cells, and a subset of B and T cell lymphomas (35). A diffuse pattern of staining for CD30 could also be seen in carcinomas, including pancreatic, nasopharyngeal, mesothelioma, malignant melanoma and undifferentiated carcinoma (35).

Epithelial membrane antigen (EMA), is expressed by neoplastic cells of both ALK^+ALCL and ALK^-ALCL as well as many types of carcinoma, thus it is not really useful on its own to differentiate ALK^+ALCL from carcinoma (31).

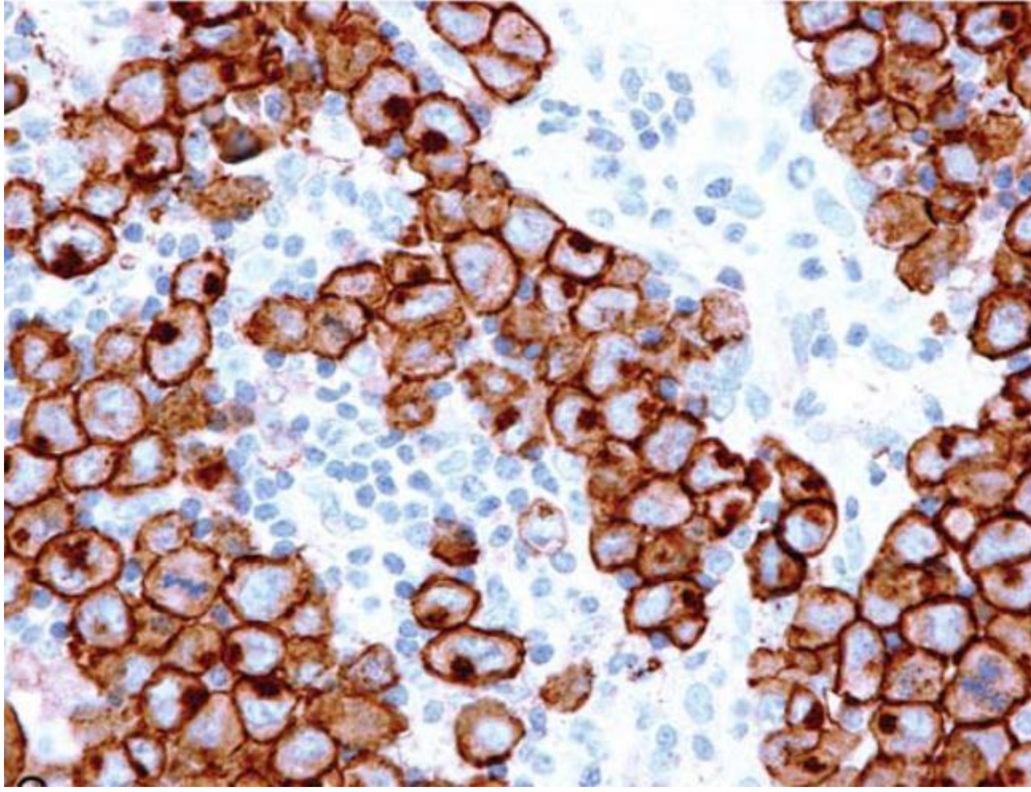

Figure 1.2. Expression of CD30 in ALK^{+}ALCL.

Tumor cells of the common histologic variant of ALK^{+}ALCL express a strong staining of CD30 in a membranous and Golgi-associated pattern.

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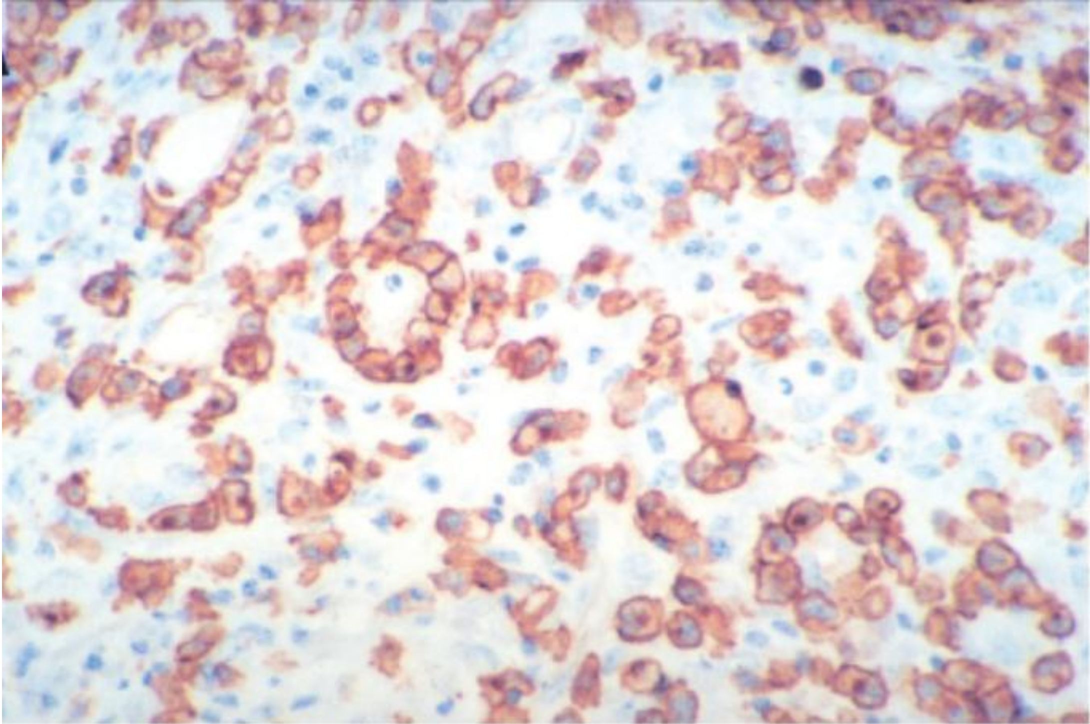

Figure 1.3. Perivascular infiltrative pattern of ALK^{+}ALCL

The perivascular infiltrative pattern by the malignant cells of ALK^{+}ALCL as highlighted by CD30 immunostaining is a pathognomonic feature.

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Dysregulation of signaling pathways in ALK$^+$ALCL as a type of T-cell lymphoma, results into loss of pan-T-cell antigens, particularly, CD3 and the αβ T-cell receptor complex are either decreased or absent(36,37). Around 80%-90% of ALK$^+$ALCL have T-cell receptor (TCR) gene rearrangement, even in the cases of “null” cell origin (i.e. cells that do not express T –cell and B -cell antigens). (38,39). T-cell lineage markers are expressed by ALK$^+$ALCL as detected by immunostaining. Among these markers CD2 is the most frequent to be detected, while CD3, CD5, CD7, and CD45RO are less detectable (40). The expression of CD4 and CD8 is variable. Despite the neoplastic cells in most of the cases are of CD4+/CD8- phenotype, CD8+ cases have been described. Cytotoxic molecules such as granzyme B, perforin and T-cell- restricted intracellular antigen-1 are almost always expressed (38,41) . Approximately 80% of ALK$^+$ALCL express CD99, which is also expressed by Ewing sarcoma and acute lymphoblastic leukemia (42,43).

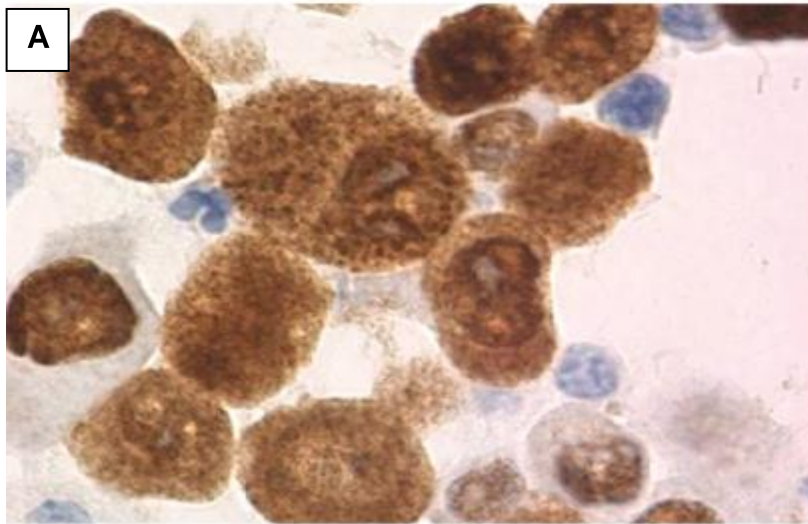
Expression of myeloid antigens (CD13 and CD33) could be detected in up to 100% of ALK$^+$ALCL (44). Due to the high positivity of myeloid antigens, in addition to the decrease or lack of the expression of pan –T-cell antigens and the weak expression of CD45RB, ALK$^+$ALCL could be mistaken with acute myeloid leukemia (10).

The discovery of ALK fusion genes and proteins in ALCL made a breakthrough in the diagnosis of these tumors (3). The use of specific monoclonal antibodies generated against ALK proteins, facilitated the immunohistochemical based detection of ALK proteins in fixed paraffin embedded tumor samples (Figure 1.4) (5,45). Approximately, 85% of ALK$^+$ ALCL express the chimeric protein NPM-ALK (46). These cases show ALK positivity in the cytoplasm and nucleus of lymphoma cells. However in case of other ALK variant fusion proteins, ALK staining is restricted to the cytoplasm of the malignant cells (46-48). In cases of ALCL with clathrin-

ALK expression, ALK staining is granular cytoplasmic, while in cases that express meosin-ALK, ALK staining is membranous (47,48).

Of all the morphologic variants, the small and lymphohistocytic ones are considered to be the most important, because they could be easily misdiagnosed with other lesions. The small cell variant may be misdiagnosed as PTCL-NOS due to the predominance of the small cell component (6). The abundance of histocytes in the lympho-histocytic variant, frequently masks the minor component of the neoplastic cell population, thus this variant may be misdiagnosed as atypical inflammatory lesions or hemophagocytic syndrome (6). Immunostaining for ALK protein expression in these tumors ensures an accurate diagnosis (Figure 1.5). In the small cell variant the large cells that are ALK^{+}, $CD30^{+}$, show a perivascular distribution rather than being randomly scattered (7).

The use of ALK staining with CD30 is proved to be of high diagnostic importance, especially in pediatric and young adult cases with subtle or focal nodal involvement (10). Bone marrow involvement is more common in the small cell variant of ALK^{+} ALCL, thus ALK staining of the bone marrow aspirate smears is necessary to detect such involvement (Figure 1.6) (49,50) .



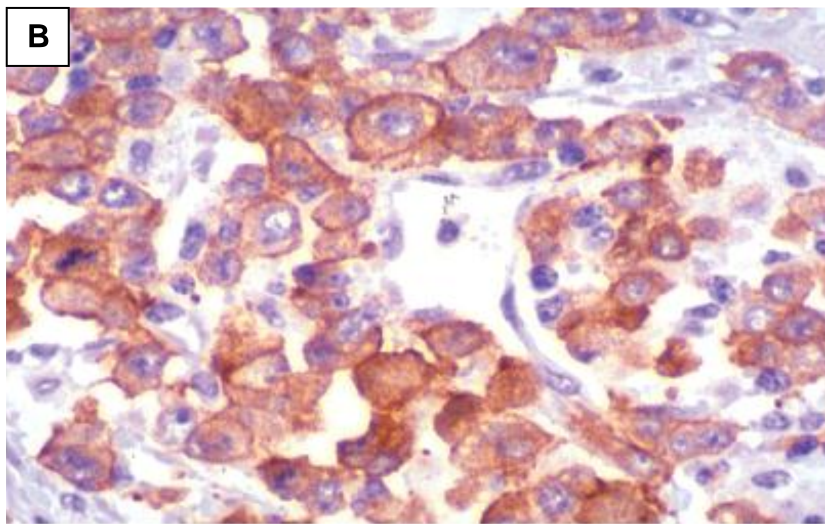


Figure 1.4. ALK immunostaining in ALK$^+$ ALCL

(A) ALK staining with ALK1 antibody. Nuclear, nucleolar, and cytoplasmic staining associated with the t(2;5) (p23;q35) translocation (expression of the NPM-ALK protein; magnification, x1,000). (B) ALK staining with ALK1 antibody. Restricted cytoplasmic staining with membrane reinforcement associated with the t(1;2)(q25;p23) translocation (expression of the TPM3-ALK protein; magnification, x400).

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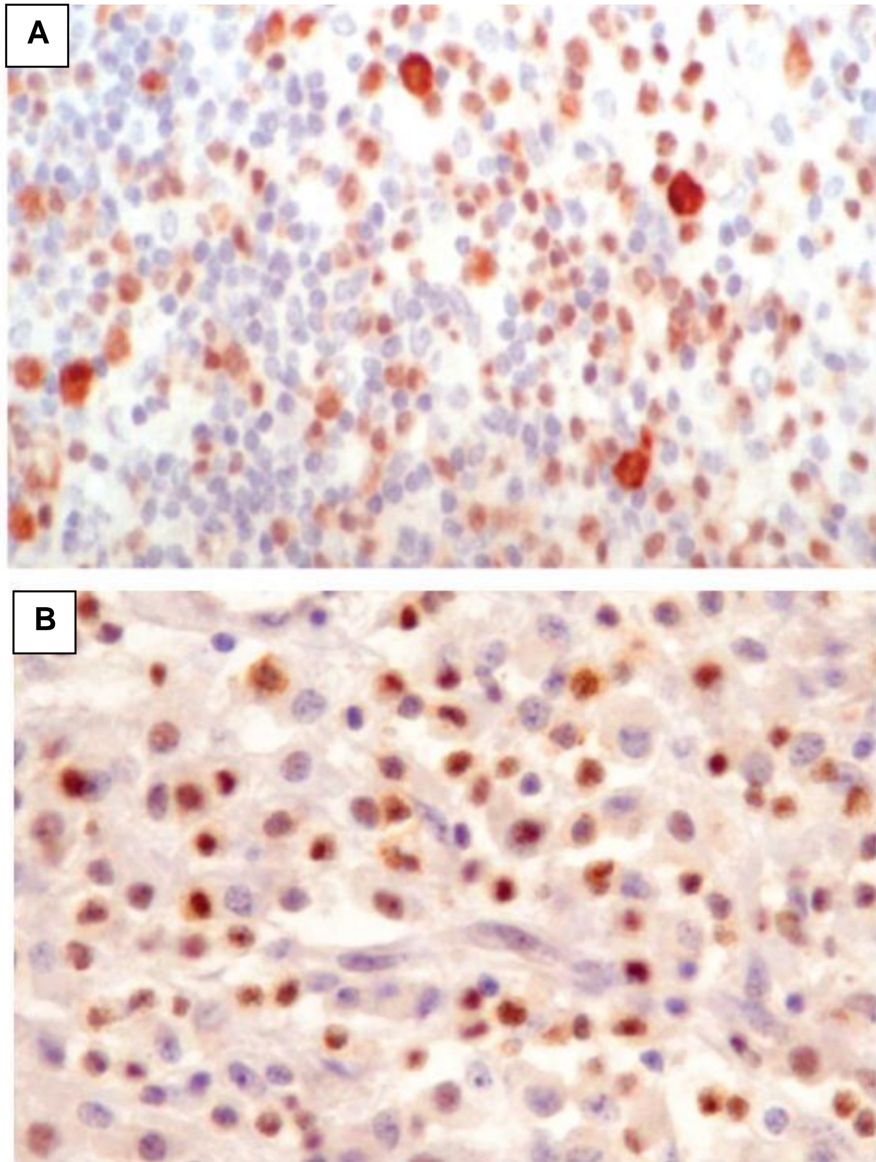

Figure 1.5. Diagnostic significance of ALK immunostaining in the differential diagnosis of small and lymphohistocytic variants of ALK$^+$ALCL

(A) In the small-cell pattern of ALK$^+$ALCL, ALK staining is frequently restricted to the nucleus (magnification, x200). (B) In the lymphohistiocytic pattern of ALCL, tumor cells are often small to medium sized. ALK staining is frequently restricted to the nucleus of small tumor cells (magnification, x400).

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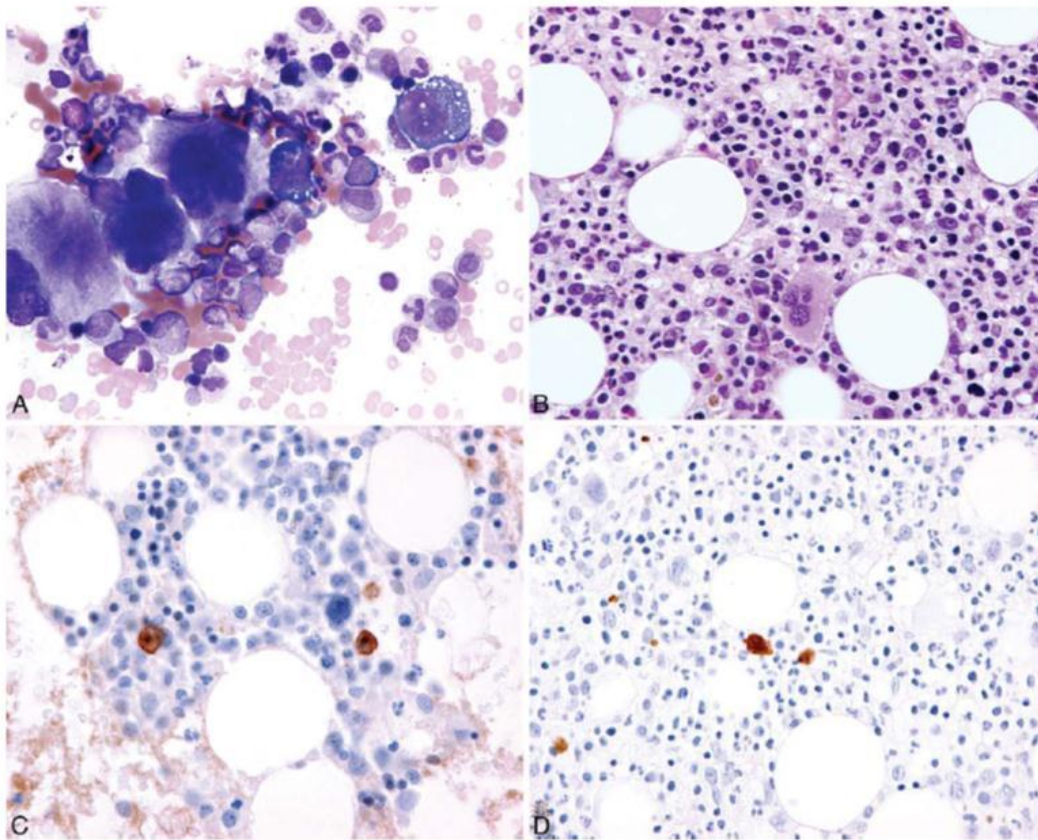

Figure 1.6. ALK immunostaining in the diagnosis of bone marrow involvement by ALK^{+}ALCL .

Bone marrow aspirate smear and tissue sections with subtle involvement by ALK^{+}ALCL A) With Wright stain, tumor cells have very characteristic dense blue cytoplasm with fine vacuoles, facilitating detection of even subtle involvement when the bone marrow aspirate is carefully examined. B) No mass lesions are often seen in bone marrow tissue sections, only scattered large tumor cells are present. C) Immunostaining for CD30. D) ALK-1 highlights the rare lymphoma cells (Wright, original magnification ×600 [A]; hematoxylin-eosin, original magnification ×600 [B]; CD30, original magnification ×600 [C]; ALK-1, original magnification x600 [D]).

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1.2.3. Genotype (*ALK* fusion genes)

ALK^+ ALCL are characterized by displaying chromosomal translocations involving the *ALK* gene. *ALK* encodes a receptor tyrosine kinase that belongs to the insulin growth factor receptor superfamily (51). The full length ALK displays the characteristic structural features of a receptor tyrosine kinase, with an extracellular (ligand binding) domain, a transmembrane domain and an intracellular tyrosine kinase domain (Figure 1.7.)(52). The domain structure of ALK is highly homologous to that of the leukocyte tyrosine kinase (LTK), thus both kinases form a specific subgroup within the insulin receptor superfamily (53,54) . The structure of ALK is highly conserved throughout evolution, especially for the kinase domain. The ALK kinase domain contains the YxxxYY motif that is also displayed by the Insulin receptor (55,56).

ALK expression is lacking in tissues outside the nervous system. It is expressed at relatively high levels in the nervous system during embryogenesis and early postnatal life in mice. It is also maintained to be expressed at low levels in certain areas of the mice adult brain (53). In humans a similar pattern of expression as that in mice, was observed using immunohistochemistry studies (45). This pattern of expression suggests a physiological role of ALK in normal neurological development and function (53,54). The ability of ALK to induce neurite outgrowth and synapse formation in both *in vitro* and *in vivo* models, suggests its role in neuronal differentiation (57,58). All the ALK chimeras are derived from genomic breakpoints within the intron between exon 19 and 20 of *ALK*, resulting into the fusion of the distal portion of the *ALK* gene (encoding for the intracytoplasmic domain of ALK) with the promoter region and the proximal domain of the gene encoding for the fusion partner(9,59). In ALK fusion proteins the N-terminal binding partners act as dimerization domains (9,59). Most of ALK^+ALCL express the NPM-ALK fusion protein that it is derived from t(2;5) (p23;q25) translocation (32,60). Recently, it

has been suggested that the dysregulation of several genes (*FRA2*, *ID2* and *CSF1*) near the breakpoints involving *ALK*, by unknown mechanisms, may predispose for the occurrence of these translocations. Specifically, it has been shown that dysregulation of *FRA2* (located on 2p23) and *CSF1* (located on 5q33.1), spatially juxtapose their chromosomes in close proximity and results into the occurrence of t (2;5) translocations involving *NPM1* and *ALK* (61).

NPM is a multifunctional and ubiquitously expressed protein. Besides its involvement in the pre-ribosomal shuttling between the cytoplasm and the nucleus; NPM plays an important role in transcription, DNA repair and genomic stability (62). The N-terminus domain of the NPM partner is a coiled-coil oligomerization domain, thus results into dimerization of the NPM-ALK with subsequent autophosphorylation and constitutive activation of the ALK kinase. This constitutive activation is responsible for activation of multiple downstream signaling pathways (Figure 1.8), which control key cellular processes such as cell cycle progression, survival, cell migration and invasiveness. (9,63-65). Both the dimerization ability of NPM and the kinase activity of NPM-ALK are critical for the transforming ability of this chimera (64).

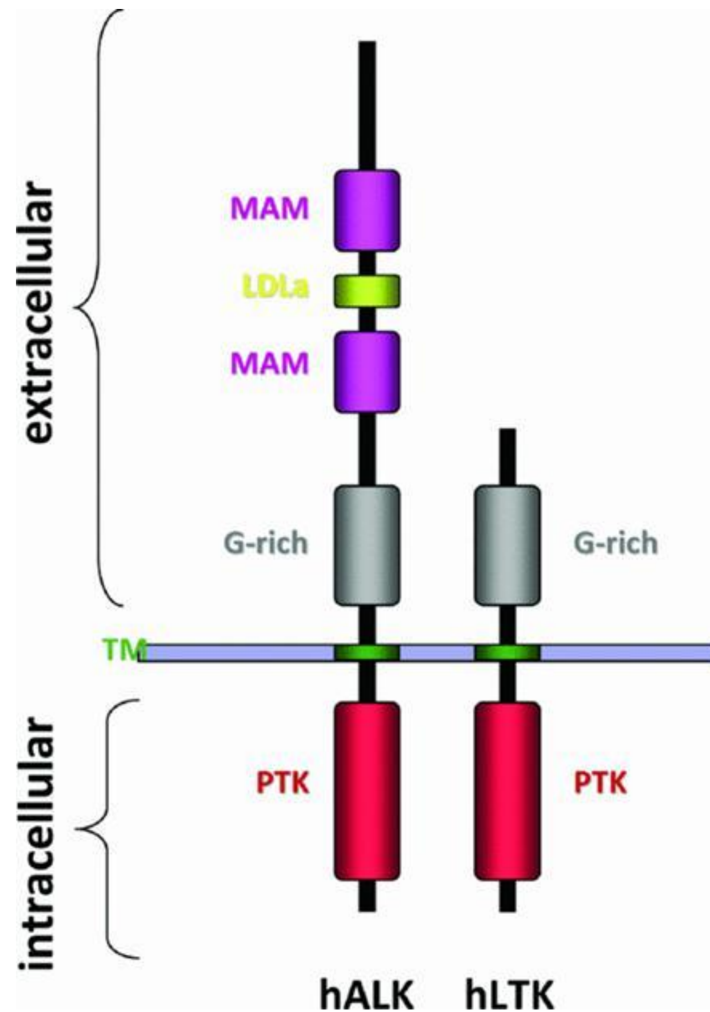

Figure 1.7. Domain structure of human ALK and human Leukocyte Tyrosine Kinase (hLTK).

The N-terminal region of human ALK (hALK) comprises two MAM domains, one LDLa (low-density lipoprotein class A) domain and a glycine rich (G-rich) region. A transmembrane (TM)-spanning segment, connects the extracellular region with the protein tyrosine kinase (PTK), domain (amino acids1116–1383)-containing intracellular region. The closest family member is the LTK.

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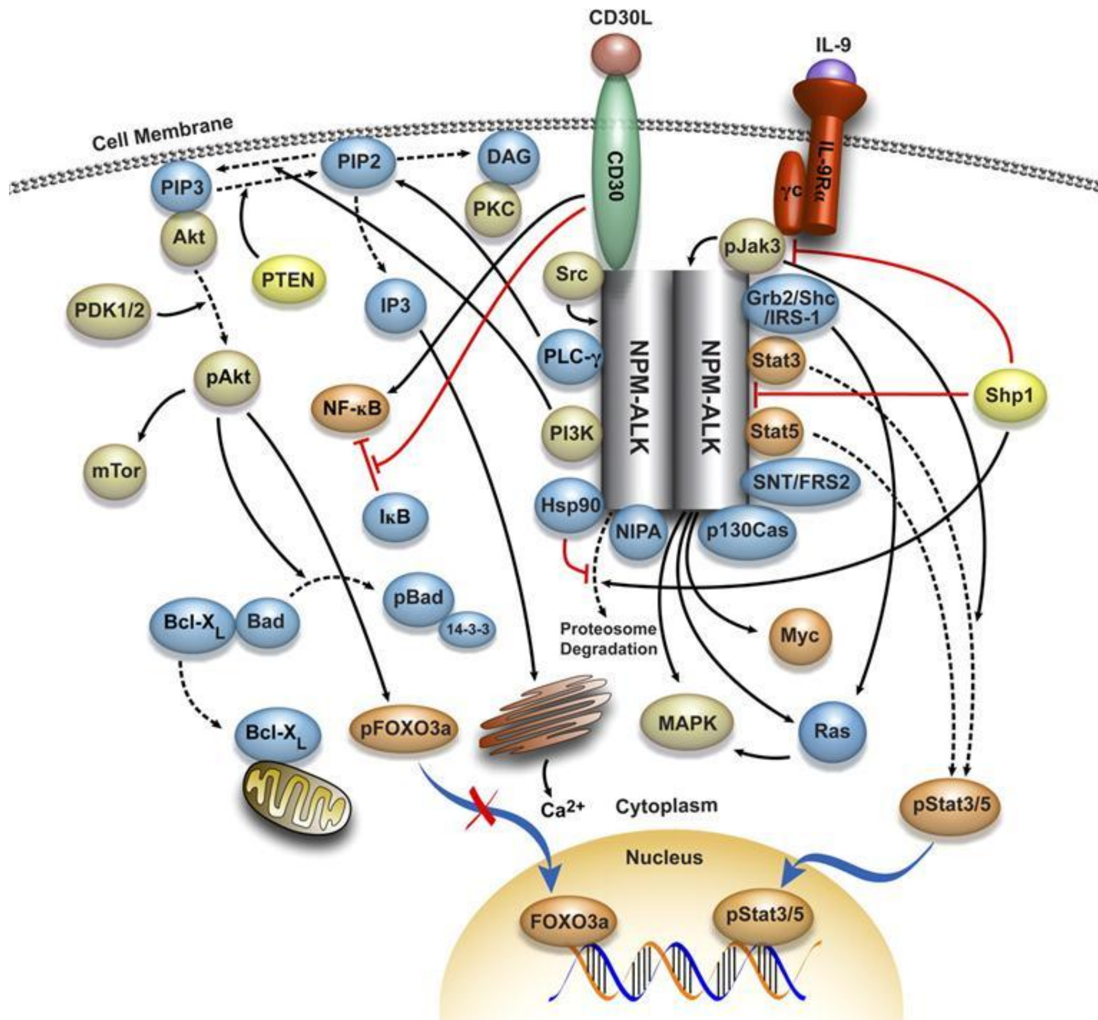

Figure 1.8. Molecular network interacting with NPM-ALK.

A complex network of protein kinases, protein phosphatases, transcription factors, apoptosis and cell-cycle regulators, adaptor proteins, and other molecules has been proposed to interact with NPM-ALK.

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Numerous other fusion ALK proteins exist in ALK^{+}ALCL (Table 1.1). All the fusion partners to ALK, share common characteristics: (i) The promoter of the gene of the ALK partner protein drives the transcription of the fusion gene; (ii) The subcellular localization of the fusion protein is determined by the ALK partner protein (Figure 1.9); (iii) The ALK partner is responsible for the oligomerization and the consecutive autophosphorylation of the chimera (66-68).

As mentioned above, the subcellular localization of the ALK protein is governed by the identity of the fusion partner protein. In case of NPM-ALK, it is detected in the cytoplasm, nucleus as well as the nucleolus. This subcellular localization is unique for NPM-ALK in comparison to the other types of ALK fusion proteins which mainly show only a cytoplasmic localization (Table 1.1 and Figure 1.9.). This difference in the pattern of subcellular localization is attributed to the presence of a nuclear localization domain in the wild type NPM which is able to form heterodimers with NPM-ALK, thus enabling the NPM/NPM-ALK heterodimer to enter to the nucleus (67). The truncated NPM in NPM-ALK chimera is lacking this nuclear localization signal, thus the NPM-ALK homodimers are mainly cytoplasmic (64). The nuclear localization signal is absent in the other fusion partners of the ALK chimeric proteins (67). For example, in case of TPM3-ALK, the TPM3/TPM3-ALK heterodimers and TPM3-ALK/TPM3-ALK homodimers are cytoplasmic. Also, both CLTC/CLTC-ALK heterodimers and CLTC-ALK/CLTC-ALK homodimers are located within the clathrin-coated vesicules of the neoplastic cell to give the characteristic granular distribution of this fusion protein in the cell (66,67).

Besides their expression in ALCL, ALK fusion proteins are also expressed in other types of malignancies (Table 1.1). The full length of ALK itself has been reported to be oncogenic in several tumors, either through overexpression or gain of function mutations (69-78).

The fact that ALK cDNAs are expressed in the peripheral blood as well as in the lymphoid tissues of healthy individuals (79,80) , is questioning the ability of these proteins to be oncogenic on their own and suggests that other events are required to be involved in order for these ALK chimeric proteins to reach their full oncogenic transformation.

Table 1.1. Chromosomal translocations involving ALK in cancers
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Chromosomal translocation	**Partner protein**	**Frequency (%)**	**Fusion protein (KDa)**	**Cellular localization**	**Type of tumor**
t(2;5)(p23;q35)	Nucleophosmin (NPM)	75–80	NPM–ALK (80)	Nucleus, nucleolus and cytoplasm	ALK^+ ALCL and ALK^+ DLBCL
t(1;2)(q25;p23)	Tropomyosin 3 (TPM3)	12-18	TPM3–ALK (104)	Cytoplasm	ALK^+ ALCL and IMT
t(2;3)(p23;q21)	TRK-fused gene (TFG)	2	TFG–ALK (113, 97, 85)	Cytoplasm	ALK^+ ALCL
inv(2)(p23;q35)	ATIC	2	ATIC–ALK (96)	Cytoplasm	ALK^+ ALCL and IMT
t(2;17)(p23;q23)	Clathrin heavy chainlike 1 (CLTC1)	2	CLTC1–ALK (250)	Granular cytoplasmic	ALK^+ ALCL, IMT and ALK^+ DLBCL
t(2;X)(p23;q11–12)	Moesin (MSN)	<1	MSN–ALK (125)	Cell-membrane associated	ALK^+ ALCL
t(2;19)(p23;p13)	Tropomyosin 4 (TPM4)	<1	TPM4–ALK (95–105)	Cytoplasm	ALK^+ ALCL and IMT
t(2;17)(p23;q25)	ALO17	<1	ALO17–ALK (ND)	Cytoplasm	ALK^+ ALCL
t(2;2)(p23;q13) or inv(2)(p23;q11-13)	RAN binding protein 2 (RANBP2)	<1	RANBP2–ALK (160)	Periphery of the nucleus	IMT
t(2;22)(p23;q11.2)	Non-muscle myosin heavy chain (MYH9)	<1	MYH9–ALK (220)	Cytoplasm	ALK^+ ALCL
t(2;11;2)(p23;p15;q31)	Cysteinyl-tRNA synthetase (CARS)	<1	CARS–ALK (130)	Unknown	IMT
ins(3′ALK)(4q22-24)	Unknown	<1	Unknown	Granular cytoplasmic	ALK^+ DLBCL
t(2;4)(p23;q21)	SEC31 homologue A (*S. cerevisiae*) (SEC31L1)	<1	SEC31L1–ALK (ND)	Cytoplasm	IMT
inv(2)(p21;p23)	Echinoderm microtubule-associated protein-like4 (EML4)	6	EML4–ALK (ND)	Unknown	NSCLC

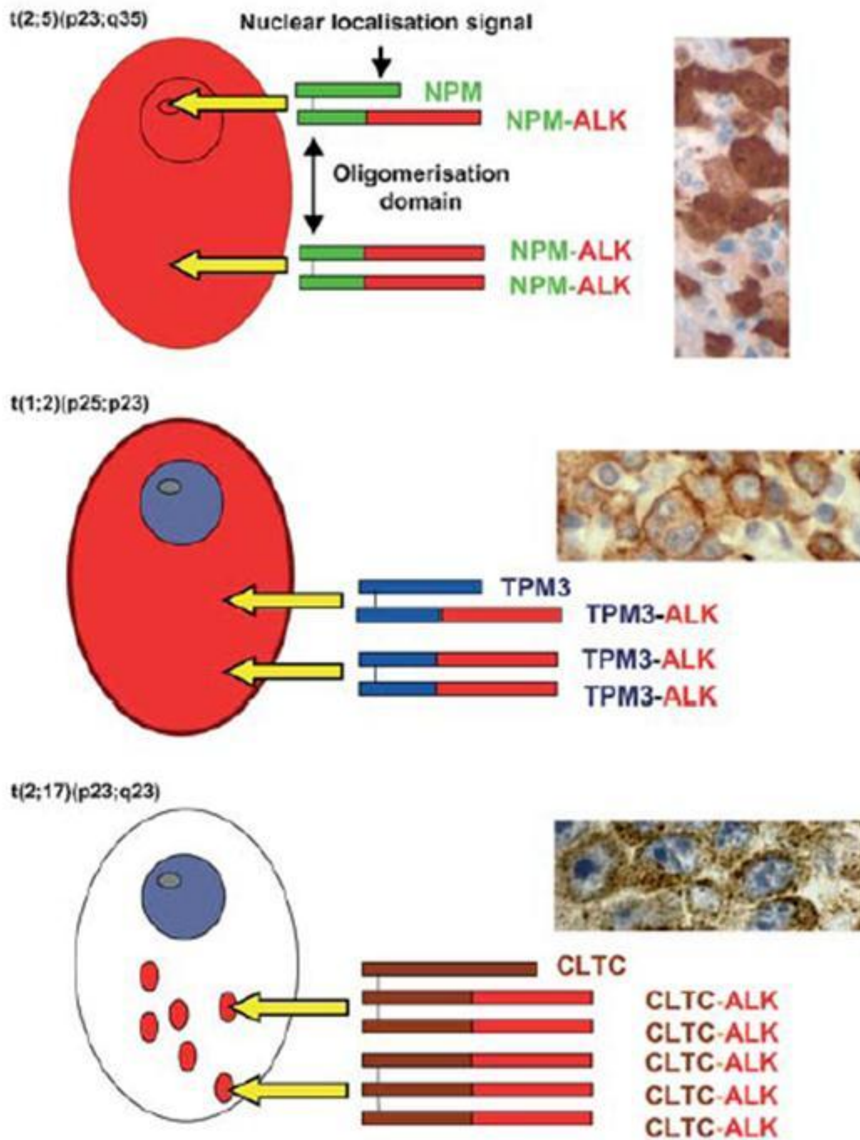

Figure 1.9. Subcellular localization of ALK is governed by ALK fusion partner in ALK^{+}ALCL.

“Springer and Birkhäuser Verlag/ Cellular and Molecular Life Sciences, 61(23), 2004, 2941, The emerging normal and disease-related roles of anaplastic lymphoma kinase, Pulford K, Lamant L, Espinos E, Jiang Q,Xue L, Turturro F, Delsol G, and Morris SW , figure 2, © Birkhäuser Verlag, Basel, 2004, with kind permission from Springer Science and Business Media”

1.2.4. Pathogenesis

There are several pieces of evidence suggest that ALK$^+$ALCL are mainly dependent on the continuous growth promoting cellular signals provided by ALK chimeric proteins. Such dependence suggests ALK$^+$ALCL to be "ALK addicts" (10,40).

It is postulated that ALK$^+$ALCL (being ALK dependent), may require a limited number of additional genetic derangements to occur in order to reach full transformation (40). On contrary, ALK$^-$ ALCL may require accumulation of more genetic defects in order for their full transformation to happen. In fact this idea has been supported by cytogenetic and genomic profiling studies. These studies revealed a difference in the secondary genomic aberrations between ALK positive and ALK negative ALCL, with higher and more heterogeneous karyotyping in the ALK negative tumors. This difference in genomic profiling between ALK$^+$ALCL and ALK$^-$ALCL, suggests that they correspond to different genetic entities (81,82) .

The oncogenic potential of ALK fusion proteins was first demonstrated *in vivo* in mice undergoing bone marrow transplantation with NPM-ALK transduced cells (83). Other following studies using mice models have further confirmed the oncogenic properties of ALK fusion proteins (84,85).

The oncogenic properties and the cellular signaling defects induced by ALK fusion proteins in ALK$^+$ALCL, have been extensively delineated using genomic, proteomic and pharmacologic approaches (9,10). Since the great majority of ALK$^+$ALCL are expressing the fusion protein NPM-ALK (46), thus it is the most extensively studied protein in terms of the dysregulation of its downstream and cross-talking signaling pathways. Very few studies have focused on other ALK fusion proteins in ALK$^+$ALCL; these are including ATIC-ALK and TFG-ALK fusion proteins (86,87) .

It is now well established that NPM-ALK and other ALK fusion proteins can interact with and activate many adapter molecules that are involved in the activation of critical signaling pathways such as Janus activated kinase/signal transducer and activator of transcription (JAK/STAT), phosphatidylinositol 3 kinase (PI3K/AKT), RAS/MEK/extracellular signal-related kinase (ERK), phospholipase C γ, and c-Src (10,40,65).

1.2.4.1. Janus activated kinase/ Signal transducer and activator of transcription (JAK/STAT) pathway

Janus activated kinase 3/ Signal transducer and activator of transcription 3 (JAK3/STAT3) pathway is one of the most studied and the best characterized dysregulated pathway in ALK^+ ALCL. The importance of this pathway in ALK^+ALCL pathobiology is underlined by the fact that the transcription factor STAT3 has been proven to be oncogenic by itself (88,89).

Several studies have reported the strong correlation between NPM-ALK activation and the STAT3 phosphorylation and activation (90-95). Supportive of such correlation is that inhibition of ALK activity dramatically reduced STAT3 phosphorylation (96-98).

Upon activation, STAT3 forms dimers and translocate to the nucleus where it regulates the transcription of a number of genes known to be involved in apoptosis (e.g. Bcl-2, Bcl-xL, survivin, and MCL1) and cell proliferation (e.g. cyclin D3 and c-Myc (88,89,92).

The critical role of STAT3 activation in mediating NPM-ALK tumorigenicity in ALK^+ALCL has been demonstrated in cell lines and animal models (90-92,95,99). In addition to its tumorigenic potential in ALK^+ALCL, STAT3 activation induced by NPM-ALK, contributes to the immune evasion of these malignancies. Particularly, STAT3 induces the expression of the immunosuppressive proteins interleukin 10 (IL-10) and the transforming growth factor-beta (TGF-β) (100). It also induces the expression of the tolerogenic cell membrane-bound protein CD274 (101).

The activation of STAT3 in ALK^{+}ALCL has been proven to be multifactorial. JAK3, which is a physiologic upstream activator of STAT3 has been shown to be physically interacting with NPM-ALK (94,102), and JAK3 activity is strongly associated with ALK expression and STAT3 phosphorylation in ALK^{+}ALCL tumors (103). Furthermore inhibition of JAK3 results into downregulation of STAT3 activation mediated by NPM-ALK (94,102). Contributing to STAT3 activation in ALK^{+}ALCL, is the overexpression of the protein phosphatase 2A, which is a STAT3 interacting protein that is necessary for STAT3 activation (91).

STAT family of transcription factors were first characterized for their role in cytokine signaling (89). The role of cytokines in the activation of STAT family of proteins, especially STAT3 is well established (89). Two recent studies from our laboratory have reported the aberrant expression of interleukin 21(IL 21) and interleukin 22 (IL22) in ALK^{+}ALCL (104,105). These two studies have clearly demonstrated the tumorigenic roles of these cytokines in ALK^{+}ALCL through activation of the JAK3/STAT3 pathway. The aberrant expression of IL 22 receptor has been shown to be attributed to NPM-ALK expression (104), a finding that revealed a novel and precise mechanism of NPM-ALK induced STAT3 activation.

The mechanisms by which the activated STAT3 pathway contributes to transformation in ALK^{+}ALCL are not only restricted to its ability to activate cell proliferation and cell survival pathways, but also extends to its ability to downregulate or silence the expression of its own physiological negative regulators. The Src homology-2 domain containing protein tyrosine phosphatase 1 (SHP1), is a well-known negative regulator of JAK/STAT activation (106). The loss of SHP1 activation in most of the ALK^{+}ALCL tumors is one of the mechanisms that contribute to STAT3 activation in these tumors (107,108). The expression of SHP1 has been shown to be epigenetically silenced in ALK^{+}ALCL through DNA methylation induced by

STAT3 activation (109). STAT3 stabilizes the binding of two members of the epigenetic silencing machinery to the promoter of *SHP1* gene. These two proteins are the DNA methyltransferase 1 (DNMT1) and the histone deacetylase 1 (HDAC 1). In addition STAT3 also, induces the expression of *DNMT1* gene, keeping a continuous supply of the machinery responsible for maintaining the epigenetic silencing of *SHP1* (110).

It is interesting that STAT3 activation in ALK^{+}ALCL suppresses the expression of other STAT family members of known tumor suppressive functions. For example the expression of *STAT5A*, which is another member of the STAT family of proteins, has been reported to be epigenetically silenced via methylation induced by STAT3 (111). STAT5A displays tumor suppressive functions in ALK^{+}ALCL, as restoration of STAT5A expression results into decreased expression of NPM-ALK in ALK^{+}ALCL cell lines through binding to NPM-ALK promoter (111). On contrary to STAT5A, STAT5B is activated by STAT3 and contributes to the tumorigenicity of ALK^{+}ALCL both *in vitro and in vivo* animal models (112).

1.2.4.2. Phospholipase C-γ (PLC- γ)

One of the pathways that are activated by NPM-ALK and hence mediating its enhancement of cellular proliferation, is the phospholipase C- γ (PLC-γ). NPM-ALK tyrosine residue 664 (Y664) is the site of docking of PLC- γ to NPM-ALK. This docking results into activation of PLC- γ by NPM-ALK (113). Activated PLC- γ induces the hydrolysis of phosphatidylinositol (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). These molecules modulate the release of Ca^{2+} from the intracellular stores and activate the serine/threonine protein kinase C (PKC)(114). The importance of PLC- γ in mediating the NPM-ALK oncogenic effect has been demonstrated by studies using NPM-ALK ^{Y664F} mutant. This mutant is not able to bind to and activate PLC- γ- in transforming Ba/F3 (pro-B cell line that depends on IL3 for growth and survival). Ba/F3 cells transfected with

this mutant are not able to grow in the absence of IL3, while those cells transfected with NPM-ALK are able to grow independent of IL3 (113).

1.2.4.3. PI3K/AKT pathway

The PI3K/AKT pathway has been found to be activated by NPM-ALK in ALK^{+}ALCL (115,116). NPM-ALK binds to the p85 which is the regulatory subunit of PI3K resulting into phosphorylation of PI3K and subsequent activation of the serine/threonine kinase AKT through the PI3K catalytic subunit (p110) (115).

Activation of PI3K/AKT pathway enhances survival of ALK^{+}ALCL cells via exerting anti apoptotic mechanisms involving phosphorylation and inhibition of BAD and caspase 9 and expression of FAS ligand(9). NPM-ALK induced activation of PI3K/AKT, results into hyperphosphorylation and activation of the transcription factor FOXO3A. FOXO3A activation inhibits the transcription of the cell cycle progression negative regulator p27 (Kip1), thus contributes to cell proliferation of ALK^{+}ALCL (117,118). Furthermore, FOXO3A contributes to cell proliferation capacity and survival of ALK^{+}ALCL through up regulation of the cyclin D2 and BIM-1 expression respectively (118).

The sonic hedgehog (SHH) expression is reported to be elevated in ALK^{+}ALCL through mechanisms dependent on PI3K activation induced by NPM-ALK (119). Inhibition of SHH pathway activity results in reduction of cell viability and tumorigenicity in ALK^{+}ALCL cell lines (119).

1.2.4.4. RAS/ MEK/ERK pathway

This pathway is activated by all ALK fusion proteins through engaging the adapter proteins IRS-1, SHC and Grb-2(120). RAS activation by NPM-ALK results into phosphorylation of AP-1 transcription factors complex, that contributes to maintaining the neoplastic phenotype and enhances the expression of CD30 (121,122).

ERK1/2 are found to be phosphorylated in ALK^+ALCL as well as tumor samples (63,123). This phosphorylation is induced by NPM-ALK, and the activated ERK1/2 proteins contribute to cell proliferation and cell viability in these tumors through enhancing the expression of the anti-apoptotic protein Bcl-xL and the cell cycle promoting protein CDK4(123).

1.2.4.5. Src

Src is a tyrosine kinase that plays an important role in cell proliferation, growth and migration. Under normal conditions, Src is found to be in a catalytically inactive conformation through intramolecular interactions between its SH2 and SH3 domains (124). Src kinase (pp60) is activated by NPM-ALK after its docking to NPM-ALK tyrosine residue 418(125). The interaction between Src and NPM-ALK is important for mediating NPM-ALK oncogenesis as loss of this interaction or inhibition of Src results in inhibition of NPM-ALK mediated cell proliferation (125).

Another piece of evidence for the biological importance of Src activation in ALK^+ALCL came from the study by Ambrogio *et al.* (126). In this study Src- family kinases have been shown to contribute to the activation of VAV1 leading to sustained activation of Cdc42, which regulates ALCL cell shape and migration *in vitro* and maintains cell growth of lymphoma cells *in vivo* (126).

1.2.4.6. The mammalian target of rapamycin pathway (mTOR)

The serine/threonine kinase mTOR activation is induced by NPM-ALK in ALK^+ALCL (127). The ability of NPM-ALK to activate mTOR is mediated by the MEK/ERK and the PI3K/AKT signaling pathways. The biological importance of mTOR activation in ALK^+ALCL is demonstrated by the effect of mTORC1 inhibitor rapamycin treatment of ALK^+ALCL cell lines, as it dramatically decreases cell proliferation and increases apoptosis (127). mTOR associates with other proteins; raptor and mLST8 to form mTORC1 and mTORC2 complexes, respectively. These complexes activate protein

synthesis including proteins involved in cell cycle progression, gene expression and metabolism (128).

1.2.5. Therapeutic targeting in ALK- expressing Anaplastic Large Cell Lymphoma

Generally ALK^{+}ALCL have a better prognosis in comparison to ALK^{-} ALCL. Cyclophosphamide, hydroxydaunorubicin (doxorubicin), oncovin (vincristine), and prednisone (CHOP) -based therapeutic regimens are the most commonly applied for ALK^{+}ALCL patients, with 60% - 80% cure rate (47,129).

However, relapses and resistance occur in up to 40% of cases (130,131). Despite that allogenic transplantation has been reported to be an effective therapeutic tool for relapsed cases of ALK^{+}ALCL (132) , these cases need more specific therapeutic targeting.

Several studies have explored the efficacy of specific therapeutic targeting of certain molecules implicated in the pathogenesis of ALK^{+}ALCL. These studies paved the way for novel therapeutic strategies for this type of malignancy. These strategies could be either based on individually targeting the signaling molecules downstream to NPM-ALK or targeting them in combination with ALK inhibitors.

(A) STAT3

Considering the multifactorial activation and the critical oncogenic role of STAT3 in ALK^{+}ALCL, direct therapeutic targeting of STAT3 in combination with ALK could be of optimal therapeutic effectiveness in this type of malignancy. Small molecule inhibitors that prevent the dimerization of STAT3 have been synthesized (133), thus providing a potential modality that could be employed in treating ALK^{+}ALCL.

(B) HSP90

17-allylamino demethoxygeldanamycin (17-AAG) is an analog of geldanamycin and has been undergoing phase I/II clinical trials since 1999(134). 17-AAG is an ansamycin derivative that bind to heat shock protein -90 (HSP-90) and interferes with its chaperone activity (134). The stability of NPM-ALK has been shown to be greatly dependent on the chaperone activity of HSP-90. Treatment of ALK^+ALCL cell lines with 17-AAG facilitated the proteasomal degradation of NPM-ALK, proposing 17-AGG as a specific therapeutic target for ALK^+ ALCL (135).

(C) SHP1

As previously mentioned, there is epigenetic silencing of the tumor suppressor genes *SHP-1* and *STAT5A* in ALK^+ALCL (107,111). Thus the use of the DNMT inhibitors 5'-azacytidine and 5'-aza-2'-deoxycytidine (Decitabine), in therapeutic management of ALK^+ALCL proposes a novel therapeutic approach. In fact, treatment of ALK^+ALCL cell lines with 5'-aza-2'-deoxycytidine results in restoration of SHP-1 expression and induces cell cycle arrest via mechanisms involving downregulation of the JAK3/STAT3 activation(136). It is worthy to mention that decitabine has been already successfully used in treating patients with myelodysplastic syndromes (137,138). However, this drug is not specific for one gene, thus it could influence the expression of genes that have undesirable effect.

(D) CD30

The use of anti-CD30 monoclonal antibody could be one of the avenues of specific therapeutic targeting in ALK^+ALCL (139). An anti-CD30-ribonuclease fusion protein has been shown to significantly inhibit the growth of ALK^+ALCL cell line Karpas 299 (140).

(E) Cyclins and cyclin- dependent kinases

The cyclin-dependent kinase inhibitor flavopiridol has been demonstrated to be effective against ALK^{+}ALCL (141).

(F) P53

P53 protein is not mutated in ALK^{+}ALCL, however it is inactivated by JNK-MDM2-dependent mechanisms (142), thus the MDM2 inhibitor Nultin-3a could be another therapeutic approach as it induced apoptosis in ALK^{+}ALCL cell lines by reactivation of p53 and p73(142,143).

(G) IGF-R1

The insulin growth factor receptor (IGF-R1) has been implicated in the pathogenesis of ALK^{+}ALCL through inducing survival of the tumor cells (144). A small molecule inhibitor (GSK 1838705A) has been recently developed against IGF-R1 and has been shown to promote complete tumor regression in NPM-ALK mice xenografts (145) .

(H) ALK

Based on the aberrant expression of ALK and its crucial role in the pathogenesis of ALK^{+}ALCL, more specific therapeutic strategies that directly target ALK have been exploited in both *in vitro* and *in vivo* models. These strategies included immune based therapy against ALK and the depletion of ALK protein levels through silencing its mRNA levels. Immune based therapeutic approaches are designed based on the fact that ALK fusion proteins are antigenic and able to induce both ALK specific humoral and cellular immune responses (146-149).

Vaccination of animal models with human ALK before injection of ALK^{+} lymphoma cells made these animals immune against tumor development (150). The immune responses against ALK^{+} lymphoma cells could be also

enhanced by pharmacological inhibitors of STAT3 and NPM-ALK through alleviating STAT3 and NPM-ALK mediated immune evasion (128).

Ribosome-mediated silencing of NPM-ALK using an adenovirus-expressing shRNA in ALK^+ALCL cell lines and mouse models resulted in G_1 cell cycle arrest and apoptosis as well as tumor growth inhibition and regression(151).

Since the constitutive tyrosine kinase activity of ALK fusion proteins in ALK^+ALCL is the main cause underlying the pathogenesis of this type of malignancy, small molecule inhibitors of ALK kinase activity provide a very promising therapeutic tool for these tumors. These inhibitors are designed to target the ATP binding pocket of the ALK kinase domain resulting in blocking of ATP binding (152). Several studies have demonstrated the efficacy of different small molecule inhibitors against ALK either *in vitro* and/or *in vivo*. One of these inhibitors; TAE684 has been shown to prevent the development of tumors in mice models xenografted with NPM-ALK positive ALCL cell line Karpas 299, the same inhibitor also resulted into regression of the pre-induced tumors(98). TAE684 induced growth inhibition and apoptosis in some neuroblastoma cell lines (153).

PF-02341066 (Crizotinib), which is a significant ALK inhibitor (154), was the first ALK inhibitor to enter into clinical trials and has been approved by the United States Food and Drug Administration for the treatment of non-small cell lung carcinoma that abnormally express the *ALK* gene (155). PF-02341066 treatment of NPM-ALK xenografts resulted in complete regression of all the NPM-ALK induced tumors (154). It has been reported recently that the use of Crizotinib in relapsed patients of ALK^+ALCL resulted in their complete cure (156). Despite the therapeutic success of the ALK inhibitors, the probability of occurrence of relapse and resistance against these inhibitors still could not be excluded. It has been shown

recently that non-small cell lung cancer cells expressing the gate keeper mutant EML4-ALK L1196M confer resistance to kinase inhibitors (157). The newly developed small molecule inhibitor CH5424802 is a highly selective ALK inhibitor that has been shown to block the EML4-ALKL1196M- driven cell growth (157). Similar mutations could occur in ALK^{+}ALCL, mandating the development of more specific ALK inhibitors such as CH5424802 and other molecules that specifically target multiple pathway defects in these tumors.

1.3. Cytoplasmic Tyrosine Phosphatase SHP1 (An overview)

It is well established that the equilibrium between protein phosphorylation and dephosphorylation is one of the key mechanisms for regulating eukaryotic cellular signaling pathways. The phosphorylation of tyrosine residues in proteins is precisely controlled by two types of enzymes, protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). PTKs catalyze the phosphorylation of tyrosine residues, while, PTPs dephosphorylate the phosphotyrosine residues. (106). Deregulation of this tight balance results into constitutive tyrosine kinase activity of PTK with subsequent accumulation of tyrosine phosphorylated proteins leading to abnormalities of cellular processes including cell proliferation and differentiation. These abnormal cellular processes result into development of diseases including cancers (158).

SHP1 is a SH2 containing cytosolic PTP and is considered one of the key proteins in regulating the intracellular phosphotyrosine levels in lymphocytes. In lymphocytes, SHP1 binds the immunoreceptor tyrosine based inhibition motif (ITIM) of the inhibitory receptors, such as CD22, CD72, and FcγRIIB via its SH2 domains to dephosphorylate the downstream proteins resulting into either termination of the activated signal or activation of other pathways such as apoptosis (106). Thus, impairment of SHP1 expression or function in lymphocytes induces lymphoma, leukemia and other related diseases.

1.3.1. SHP1 domain structure and regulation of its function

SHP1 contains two tandem SH2 domains (N-terminal and C-terminal SH2 domains), a central catalytic phosphatase domain (PTP) and a C-terminal tail that is rich in tyrosine and serine residues (Figure 1.10) (159,160).
By solving the crystal structure of SHP1, the role of its N-terminal domain in regulating its catalytic function has been revealed (160,161).

The current proposed model for the regulation of SHP1 activity is demonstrated in (Figure 1.11) (160). In this model, the N-SH2 domain of SHP1 associates with the PTP domain in the resting state. Ligand binding to the SH2 domains results into conformational change of the N-SH2 domain, leading to the dissociation of the N-SH2 domain from the PTP domain. This release of the N-SH2 domain triggers the rotation of the C-SH2 domain, thus facilitating the movement of the N-SH2 domain to the other side of the C-SH2 domain and opening-up of the active site for ligand binding. The intermolecular interaction between both of the SH2 domains, results in stabilization of an open conformation of SHP1.

The role of the C-terminus in the regulation of SHP1 activity is well established. Truncation and proteolysis of the C-terminal tail of SHP1 is associated with enhancement of the phosphatase activity of SHP1 *in vitro* (162,163). Phosphorylation of Tyr 536 of SHP1 has been shown to be associated with rapid autophosphorylation of SHP1 (164,165). However phosphorylation of Ser 591 of the C-terminal tail results into downregulation of SHP1 activity in platelets and T-cells (166,167).

The active site of the catalytic domain of SHP-1 contains three important amino acid residues. The Cys455 acts as a nucleophile to attack the substrate; Arg459, stabilizes the negative charge of the phosphotyrosine substrate; and Asp421, function as the proton donor and proton acceptor (168). Biochemical studies have shown that the catalytic domain of SHP1 prefers the substrate with the consensus sequence (D/E)X(L/I/V)X1–2pYXX(L/I/V)(168).

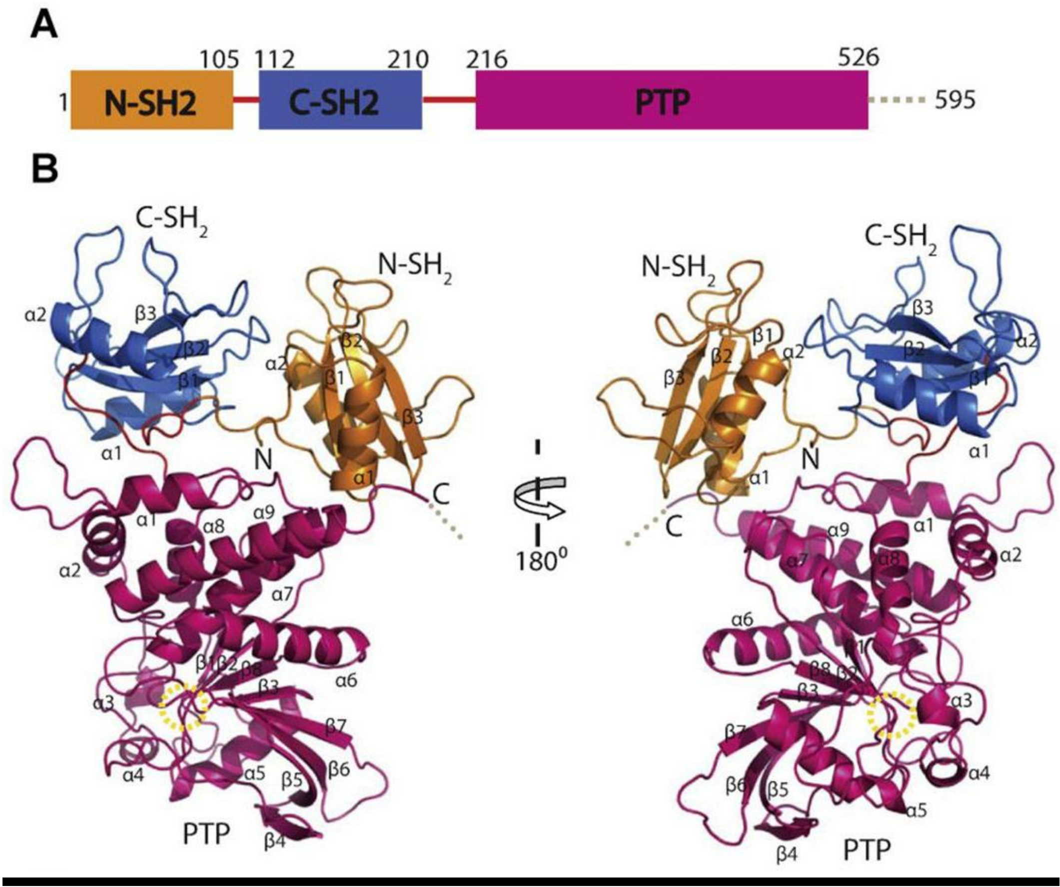

Figure 1.10. Domain structure of SHP1

A. The domain organization of SHP-1. The dashed line represents the C-terminal tail which is disordered in the structure. B. The overall structure of SHP-1. Two SH2 domains are like horns of the PTP domain. The yellow dashed circle shows the position of the active site.

(Crystal structure of human protein tyrosine phosphatase SHP-1 in the open conformation. J Cell Biochem, 112(8), 2011, 2062-2071. © 2011 Wiley-Liss, Inc. “This material is reproduced with permission of John Wiley & Sons, Inc.”)

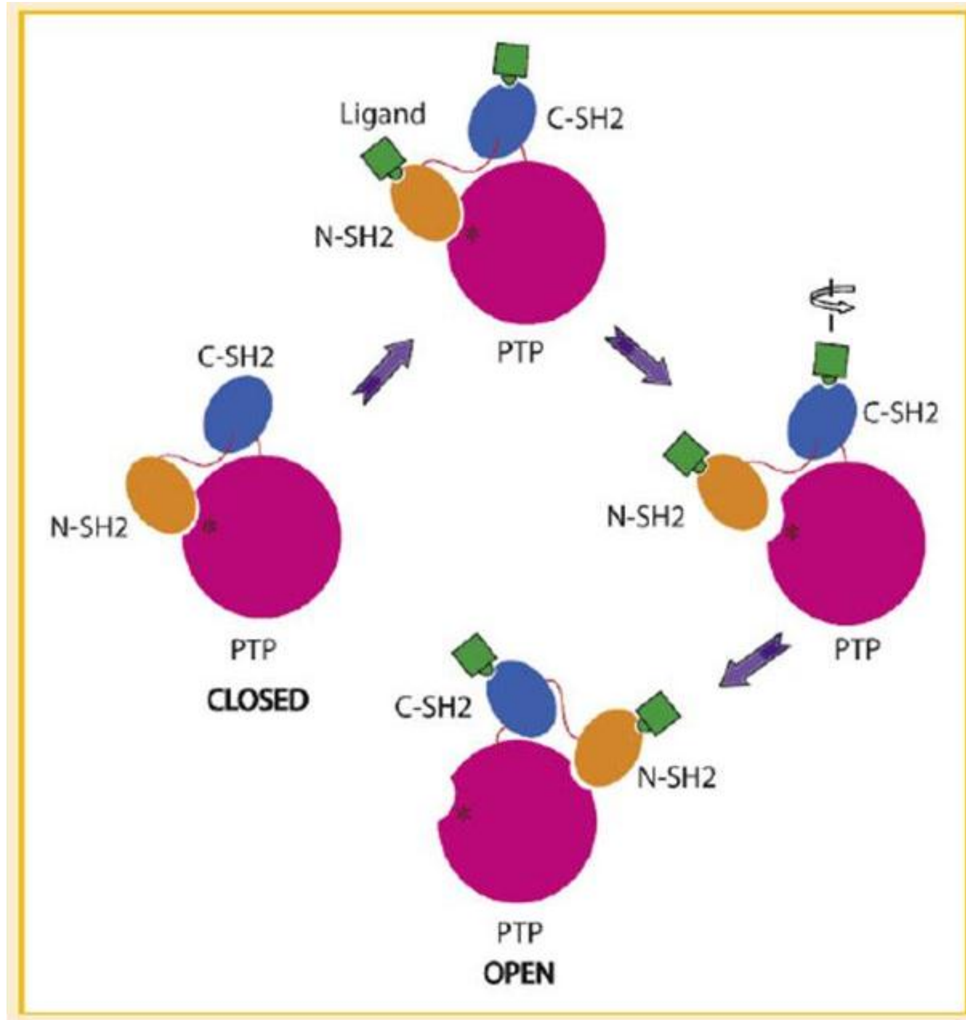

Figure 1.11. Proposed model for SHP1 activation

(Crystal structure of human protein tyrosine phosphatase SHP-1 in the open conformation. J Cell Biochem , 112(8), 2011, 2062-2071. © 2011 Wiley-Liss, Inc. "This material is reproduced with permission of John Wiley & Sons, Inc.")

1.3.2. SHP1 and lymphoma

SHP1 is predominantly expressed in hematopoietic cells, where it down-regulates intracellular signaling of the transmembrane receptors and growth factor receptors with intrinsic tyrosine kinase activity (e.g. c-kit, CSF-1, TrkA and EGF)(169-171) , cytokine receptors (e.g. Epo-R, and IL-3R) (172,173), and receptors involved in the immune response such as the TCR complex(174,175). Thus absence of the SHP1 expression or down regulation of its function is expected to be correlated with hematopoietic tumors including lymphomas.

In lymphoma and leukemia, absence or down regulation of SHP1 protein correlates with malignant cell transformation and invasiveness. Using tissue microarray studies, Oka et al. demonstrated the absence of SHP1 cDNA in up to 100% of cases of malignant lymphomas including (Hodgkin's, follicular lymphoma, and T-cell leukemia/lymphoma) (176). In contrast to the malignant cells, normal lymphocytes and cells of the associated reactive hyperplasia expressed high levels of SHP1(176).

Numerous studies have proved that the silencing of SHP1 expression in different types of leukemia and lymphoma (including ALK^{+}ALCL) is mainly due to epigenetic modifications of *SHP1* promoter by methylation (107,109,176-179).

1.4. Embryonic stem cell factors (An overview)

The embryonic stem cell factors are a group of proteins that are important for maintaining pluripotency of embryonic stem cells (ESCs). These factors are including Oct4, Sall4, Sox2, Klf4 and Nanog (180). The biological importance of these ESC proteins is highlighted by their ability of re-programming terminally differentiated cells and converting them back to so-called 'inducible pluripotent stem cells' or iPS (181-183). The expression of these proteins is mainly restricted to ESCs and a small subset of somatic stem cells (e.g. CD34+ hematopoietic stem cells) (184). However, these proteins have been found to be aberrantly expressed in specific types of cancer, and their expression may correlate with increased tumor aggressiveness and cell growth. For instance, the expression of Klf4 in pancreatic cancer has been shown to promote cell cycle progression via downregulation of p27 and p21 (185). Despite that several studies have addressed the expression of these factors in different types of cancer; the biological significance of these ESC proteins in cancer biology is incompletely understood. The regulation of the expression and functions of these proteins in cancer cells also has not been extensively studied (186-195).

1.4.1. Embryonic stem cell factors and cancer stem cells

The term cancer stem cell(s) (CSCs) is defined as a cancer cell that has the ability to self-renew to give rise to another malignant stem cell as well as a cell that will give rise to the other phenotypic cancer cells (196,197). These phenotypic cells are believed to form the tumor bulk that is responsive to conventional therapy, while the cancer stem-like cells are more resistant to therapy and can repopulate the tumor (197,198). Several properties are shared between CSCs and ESCs, namely the potential of self-renewal, the high proliferative capacity, the long population doubling time, increased telomerase activity and high nuclear to cytoplasmic ratios (199). In addition, both cell types share the same signaling mechanisms

and common pathways of “stemness”. It has been recently demonstrated that ESCs and multiple types of human cancer cells share the same patterns of gene expression that were originally repressed in normal differentiated somatic cells (200,201). Of these gene expression patterns that have been shown to be reactivated in several human cancers and to be strongly correlated with metastasis and reduced survival rate is the ESC-like transcriptional genes (200,201). Activation of ESC-like transcriptional program in adult differentiated cells might induce the pathological self-renewal characteristic of CSCs (201). The poorly differentiated tumors have been shown to preferentially over-express genes that are normally excessively expressed in ESCs; these are including Sox2, Nanog, Oct4, c-Myc and their target genes (200). Several studies also, have demonstrated that certain signaling pathways (e.g. notch, sonic hedgehog, Wnt and fibroblast growth factor-2) that regulate normal ESCs’ functions play a role in oncogenesis (202).

1.4.2. The embryonic stem cell factor Sox2

The Sox (stands for Sex determining region Y-Box) family of proteins that are known to be transcriptional factors involved in the regulation of embryonic development and determination of cell fate (203,204).

The Sox proteins bind to DNA in a highly sequence-specific manner through their High Mobility Group DNA binding domain (also known as HMG box domain) (205). This domain is a highly conserved DNA binding domain of ~ 80 amino acids. The mouse and human genomes contain 20 orthologous pairs of *Sox* genes localized on different chromosomes (206). Sox2 is the earliest protein expressed in the inner mass cells (207). Members of the Sox family are involved in various critical developmental processes of several body organs including brain, heart and lymphocytes.(205,208). In ESC, Sox2 works in concert with other proteins, particularly members of the Oct family to maintain self-renewal and pluripotency of these cells. The Sox-Oct cis-regulatory element, to which

Sox2 and Oct4 bind synergistically, is often contained in genes that are transcriptionally regulated by Sox2 (209,210). Thus, this Sox-Oct element contains specific DNA sequences recognized by the HMG domain of Sox2 and the POU domain of the Oct proteins.

1.4.3. The role of Sox2 in cancer biology

Despite the fact that Sox2 expression is normally restricted to ESCs and somatic stem cells, Its aberrant expression has been reported in various types of solid tumors, including cancers of the stomach (211), breast(212),and colorectum (213). However, most of these studies only described the expression of Sox2 in these tumors without characterizing the role of Sox2 in their pathogenesis. Only few recent studies have provided an evidence for the importance of Sox2 in cancer biology. Specifically, the aberrant expression of Sox2 has been recently shown to increase the migration and proliferation of lung cancer cells (214,215). One very recent report described that the CSC population of a lung cancer cell line expresses a relatively high level of Sox2; siRNA knockdown of Sox2 in these CSCs significantly decreased their tumorigenicity (188).

1.5. Wnt signaling pathway

The identification of Wnt genes and proteins was the result of studies performed to identify the site of integration of the mouse mammary tumor virus (MMTV) in virally induced breast tumors. This integration site is found to be in the promoter of a gene which was called *Int-1* (for integration) (216,217). Further studies proved the oncogenic role of *Int-1*, as forced expression of the Int-1 protein in transgenic mice models induced the development of mammary tumors (218). By sequencing analysis studies, *Int-1* gene was found to be orthologous to *Drosophila* gene *Wingless* (*Wg*), hence the name Wnt came from the combination of *Wg* and *Int-1*(219,220).

In humans the Wnt proteins are a family of 19 members of highly conserved secreted glycoproteins that function as ligands for receptor mediated signaling pathways that play a role in a variety of cellular processes implicated in development, normal physiological functions as well as diseases including cancers (221). These cellular processes include embryonic cell patterning, proliferation, differentiation, orientation, adhesion, survival, and apoptosis (222).

Frizzled (Fz) receptors family of proteins (currently ten members) are considered the main receptors for Wnt proteins (223). Frizzled proteins are seven transmembrane domain cell surface receptors that belong to the large family of G protein-coupled receptors (223). Frizzled receptors activation by Wnt ligand proteins mediates both canonical and non-canonical Wnt-Frizzled signaling pathways leading to specific biological functions (222). In addition to Frizzled proteins, single-pass transmembrane proteins, such as low-density-lipoprotein receptor-related protein (LRP) 5 and LRP6 are also functioning as co-receptors for Wnt

signaling (222,223). Wnt ligands have been shown to activate the receptor tyrosine kinases ROR2 and RYK (224,225).

Deregulation of the Wnt pathway signaling during embryonic development, results in abnormal morphogenesis in animal models (226-228) and congenital defects in humans (229,230). However, in mature tissues, the Wnt pathway is involved in the self-renewal of stem cells and the maintenance of many normal tissues (231-235).

1.5.1. Wnt canonical pathway

The first to be discovered and the most extensively studied of the Wnt signaling pathways is the Wnt-β-catenin [also known as the Wnt canonical pathway (WCP)] (Figure1.12) which is highly conserved among species (236). In the absence of Wnt signaling, the transcription factor β-catenin remains in a cytoplasmic protein complex [comprised of adenomatous polyposis coli (APC) and Axin], where it is phosphorylated initially by casein kinase I, and then by the serine/threonine kinase glycogen synthase kinase-3 (GSK-3). Phosphorylation of β-catenin, targets it for ubiquitination and proteasomal degradation (237).

Binding of canonical Wnt ligands, (such as Wnt1, Wnt2, Wnt3, Wnt3a, Wnt8, and Wnt8a) to their cell-surface receptor complex consisting of Fz and LRP5/6, activates the cytoplasmic phosphoprotein disheveled and inhibits GSK-3β-mediated phosphorylation of β-catenin. This inhibition of phosphorylation induces the stabilization and accumulation of cytoplasmic β-catenin and its entry into the nucleus, where it activates the transcription factor T-cell factor (TCF)/lymphoid enhancer factor (LEF) and stimulates the transcription of target genes such as *c-Myc* and *cyclin D-1* (222,223,236).

Activation of this pathway during normal development directs cell proliferation and survival and governs cell fate (223). Deregulation of this pathway predispose for development of diseases including cancers (223,236).

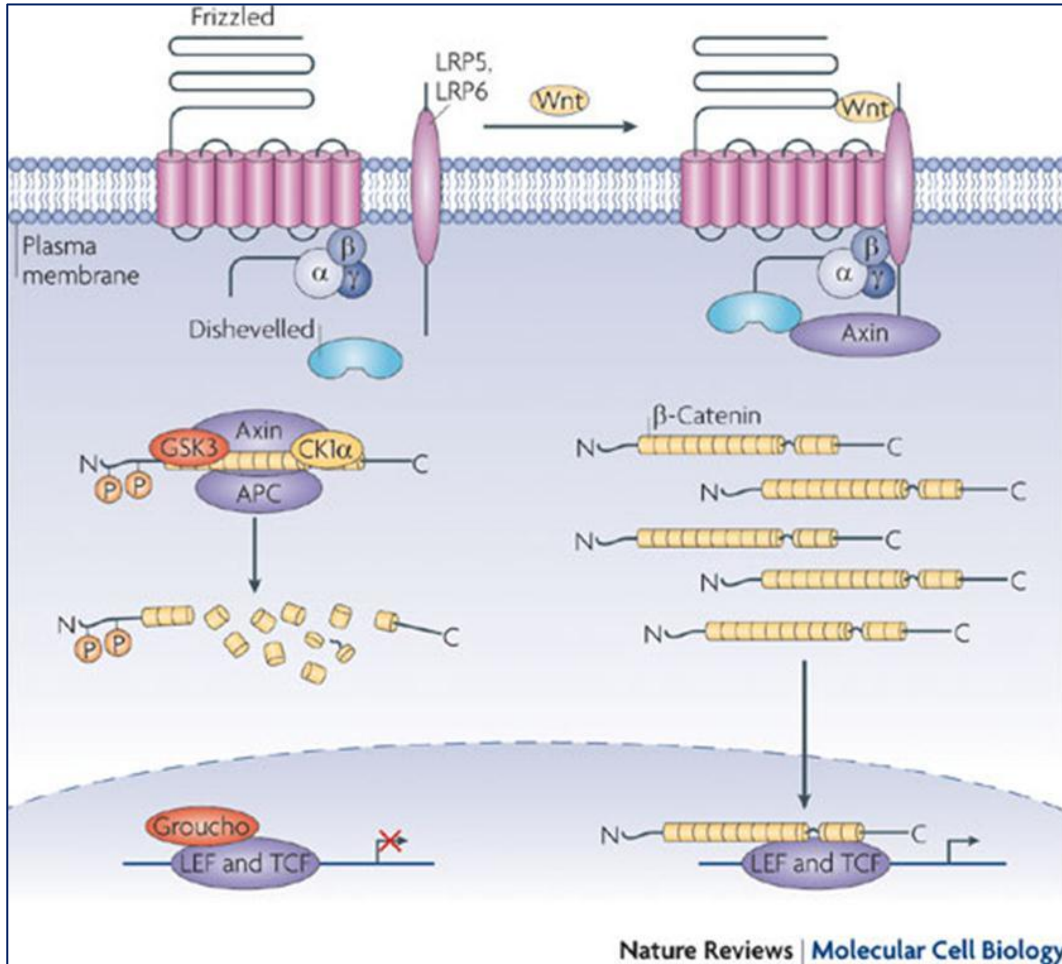

Figure 1.12. Overview of Wnt canonical pathway signaling

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1.5.2. Wnt non-canonical pathway

Some members of the Wnt family (Wnt 4, Wnt 5a,Wnt 5b,Wnt 6, Wnt7a, and Wnt 11) of proteins activate other signaling pathways that are not dependent on β-catenin stabilization (also known as Wnt- non-canonical pathways) (222,236).

The main two Wnt-non-canonical pathways that have been originally identified and extensively studied are the Wnt/planar cell polarity (Wnt/PCP) and the Wnt/calcium (Wnt/Ca^{2+}) signaling pathways (Figure1.13) (223,238,239). In vertebrates, activation of the Wnt-non-canonical pathway inhibits the WCP signaling in physiological conditions, including tissue regeneration, limb development and survival of progenitor thymocytes (240-242).

(A) Wnt/PCP pathway

This pathway has been studied extensively in *Drosophila.* The polarized arrangement of cells in an epithelial tissue sheet is known as planer cell polarity (PCP) (243). This polarity of cells is governed by local signals that are transmitted between cells and mediated by Wnt signal transduction through Fz and disheveled (Dvl), independently of β-catenin (243).

PCP signaling is mandatory for establishing and maintaining cell polarity in the orientation of wing hairs, the organization of multifaceted eye structures, and the orientation of bristles in *Drosophila* (244).

A similar pathway in vertebrates is the Wnt/ jun-N-terminal kinase has been identified and it is involved in the regulation of polarized cell movements during gastrulation, neural crest migration and cardiac outflow tract formation (243). These polarized cell movements are also known as convergent extension (CE) (245). Disruption of this pathway during the gastrulation of vertebrates results into CE defects which are characterized by embryos that are shortened along the body axis and widened in the lateral axis because of defective cell migration (246).

Activation of the PCP pathway results into activation of Rho-family GTPases and JNK (247-250). Wnt/PCP is also involved in the regulation of cell adhesion and cell motility (244). In mammals, activation of this pathway by Wnt5a is required for the uniform and symmetrical orientation of the stereocilia on the sensory hairs of the mammalian cochlea, cochlear extension and the closure of the neural tube (251).

(B) Wnt/Ca^{2+} pathway

The other main Wnt-non-canonical pathway is the Wnt/Ca^{2+} pathway (223,238). This pathway has been originally described in *Zebrafish* and *Xenopus* developmental models. Intracellular calcium waves have been detected at the margin of *Zebrafish* embryos during gastrulation (252,253), and dorsal explants of gastrulating *Xenopus* embryos (253-255). In *Xenopus* embryos, overexpression of Wnt5a or Wnt11 activates the calcium-sensitive protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CamKII) (246). In addition, inhibition of this calcium wave pharmacologically results in CE defects without affecting cell fate which is governed by WCP (255).

Similar to the Wnt/PCP pathway, the Wnt/Ca^{2+} pathway has also been demonstrated to play a crucial role in cell adhesion and/or cell movements during gastrulation (256). Certain Wnt ligands, such as Wnt5a and Wnt11, stimulate the release of intracellular calcium by utilizing G-proteins and Dvl in certain cell types (257-259) .

Increased levels of intracellular calcium modulate calcium-sensitive proteins such as CamKII and PKC (259,260). The increase in intracellular calcium stimulates the nuclear factor of Activated T-cells (NFAT) and other transcription factors (246,261,262). The activated NFAT translocates to the nucleus to regulate multiple processes such as T-cell proliferation and differentiation (263). The calcineurin– NFAT pathway is one of the major non-canonical Wnt signaling pathways in vertebrates, and its

dysregulation leads to several diseases including immune system abnormalities, multiple organs defects and cancer (264,265)

Dysregulation of the Wnt/Ca^{2+} pathway is implicated in carcinogenesis. For instance, overexpression of Wnt5a in melanoma cells induces actin reorganization and increased cell invasion, through the activation of PKC induced by calcium release (266). Similar mechanisms have been described in human mammary epithelial cells (267) and mouse F9 teratocarcinoma cells (268).

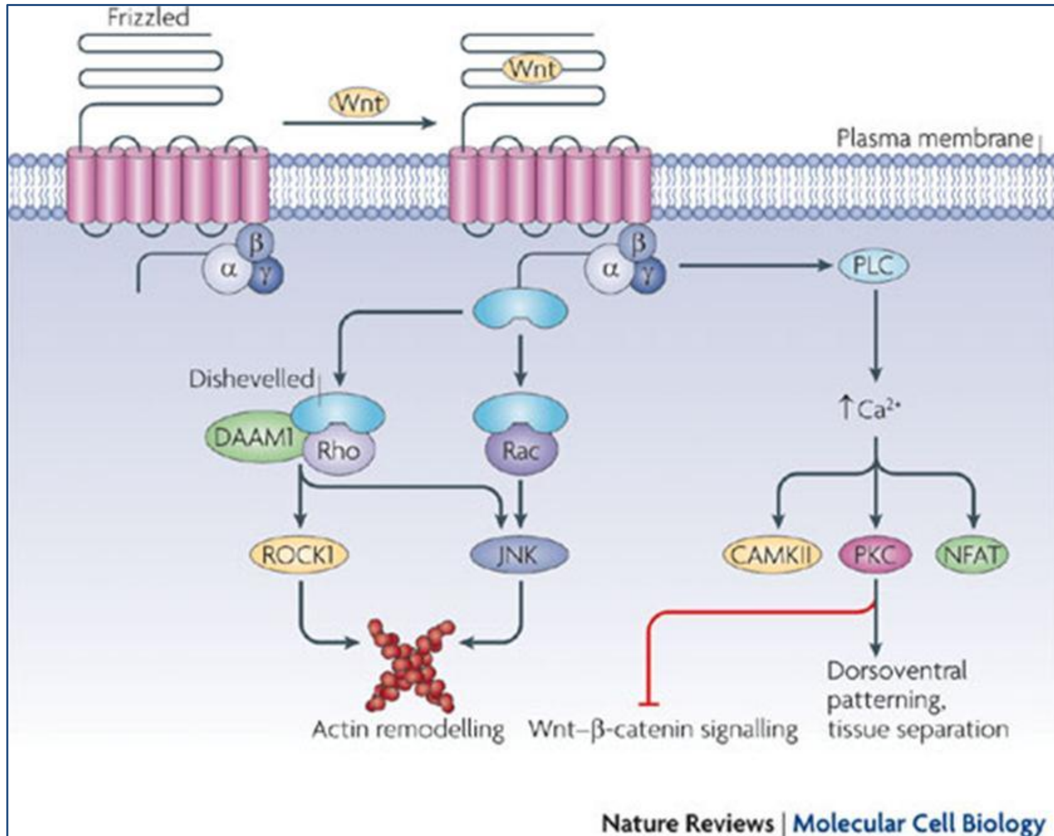

Figure 1.13. Overview of Wnt non-canonical pathway signaling

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1.5.3. Disheveled proteins (The hub of Wnt signaling)

The *Drosophila melanogaster* disheveled (Dsh) is the first member of disheveled proteins to be discovered. It was identified based on the presence of a recessive viable allele (*dsh*[1]) in the fruit fly (269,270). These viable alleles displayed defects in the orientation of the fly's photoreceptor cell clusters, sensory bristles and wing cell protrusions. These features resembled the disorganized state, hence the name disheveled (271). Disheveled proteins have been identified in other model organisms with active Wnt signaling; these are including *Hydra*, *Caenorhabditis elegans*, *Xenopus*, *mouse* and *human* (272).

Three Dsh orthologues have been identified in mice and humans, namely Dvl-1, Dvl-2 and Dvl-3(273-279). Disheveled proteins are approximately 600-700 amino acids, with conserved three domains (DIX, PDZ and DEP) structure and high sequence homology among them (278,279). The most N-terminal domain is ~80 amino acids and is known as DIX (Disheveled, Axin) domain. It mediates association between disheveled and Axin, resulting into sequestration or displacement of Axin from β-catenin destruction complex and subsequent cytosolic accumulation of β-catenin (280). The central domain is ~90 amino acids and is termed PDZ (Postsynaptic density 95, Discs Large, Zonula occludens-1) domain. This domain directly interacts with the Fz receptors and is important for signal transduction by Dvl from these receptors to the downstream effector molecules of the Wnt pathway (281,282). The carboxy-terminal DEP (Disheveled, Egl-10, Pleckstrin) domain is ~80 amino acids. DEP domain has been shown to be critical for planar cell polarity (283,284) .

Another two conserved regions of disheveled proteins are implicated in phosphorylation and protein-protein interactions have been identified. These are the basic region with positively charged amino acids (N-terminal to the PDZ domain) and the proline rich region (C-terminal to the PDZ domain). The former region contains several serine and threonine residues that are targets of phosphorylation by CK2 and PAR-1 (285,286).

The latter region represents a putative Src homology 3 (SH3) binding domain (287).

With their characteristic domains structures, disheveled proteins have the ability to distinguish and interact with a wide range of signaling proteins ensuring both spatial and temporal specificity of signaling pathways they are involved in (Figure 1.14). Thus, they are considered the central coordinators between WCP and Wnt-non-canonical pathway signaling (288).

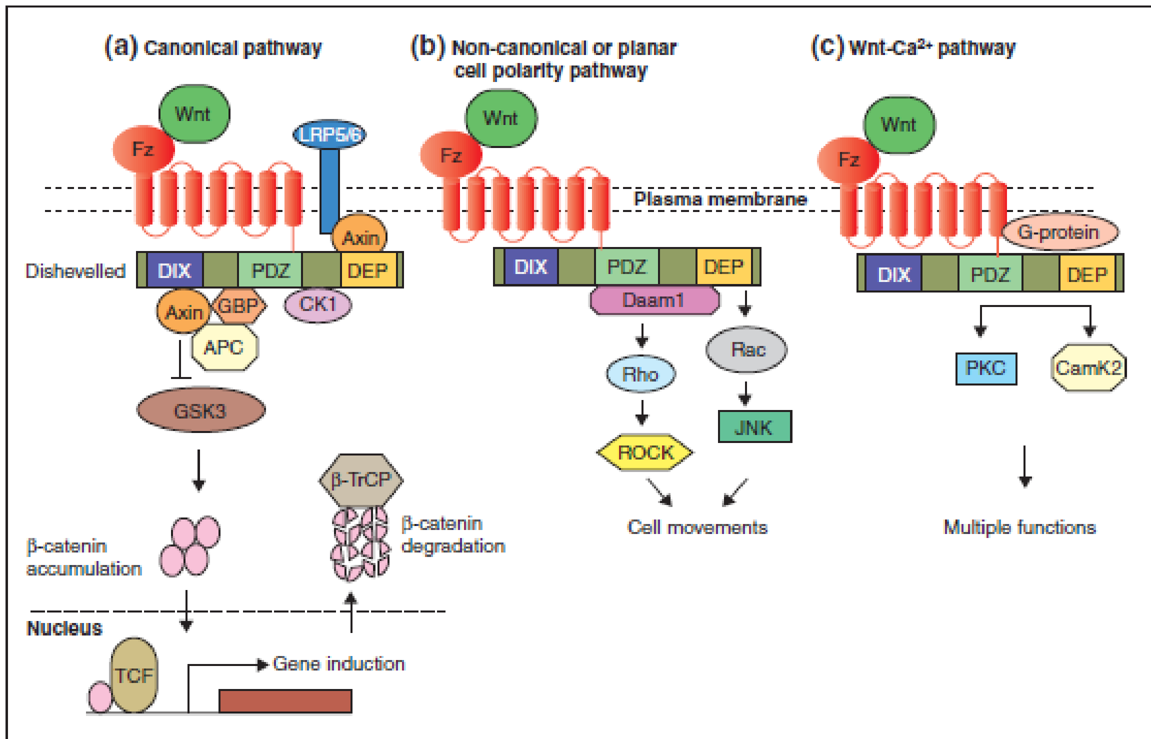

Figure 1.14. Disheveled role in different Wnt signaling pathways

Habas R, Dawid IB. Dishevelled and Wnt signaling: is the nucleus the final frontier? J Biol 2005;4(1):2. (Courtesy of BioMed Central) (289)

(A) Disheveled proteins in Wnt canonical pathway

The binding of Wnt ligands to their cell surface receptors (Fz), or (LRP5/6), results into recruitment of Dvl to the receptor complex (mediated by its PDZ domain), with subsequent dissociation of the β-catenin proteolytic destruction complex (281). Thus β-catenin is no longer directed to proteasomal degradation and it accumulates in the cytosol, for its subsequent nuclear localization and activation of TCF/LEF target genes.

Recent studies revealed a role of Dvl in clustering and polymerization of Wnt receptors Fz and LRP5/6(290,291). Dvl mutants that blocked this polymerization had a dominant –negative effect on Wnt signaling (291). Axin is one of the crucial proteins, involved in β-catenin destruction complex. In the absence of Wnt signaling, Axin through its association with glycogen synthase kinase 3β (GSK-3 β), facilitates phosphorylation of β-catenin and its proteasomal degradation (292). The exact role and mechanism of Dvl in antagonizing the effect of Axin on β-catenin has been revealed by a series of studies (280,293,294). It has been demonstrated that the DIX domain of Dvl is important for Dvl oligomerization at the plasma membrane and the transduction of Wnt signaling. (280) .

Moreover, the recruitment of Axin into Dvl-2 polymers changes the dynamic properties of Axin, resulting into inhibition of the stimulatory effect of Axin on GSK-3 β. These changes in Axin dynamics include induction of a direct conformational change of Axin, or relocation of Axin to the plasma membrane and/or its phosphorylation by a Dvl-2-associated kinase (280).

(B) Disheveled proteins in Wnt-non-canonical pathway

(i) Wnt- Planar cell polarity pathway

Mutations in the DEP domain of Dsh which is required for PCP but dispensable for the β-catenin-dependent pathway have also been found to affect the process of convergent extension in gastrulating vertebrate embryos (256).

As mentioned previously, PCP requires remodeling of the cytoskeleton which is governed by the activity of ROCK kinase and dependent on Rho-GTP complex (295,296). Dvl associates with the small GTPase Rho via the adapter protein Dvl associated activator of morphogenesis 1 (Daam1), since Daam1 harbours an N-terminal GTPase binding domain. The binding of Dvl and Daam is mediated by Dvl's PDZ and DEP domains, resulting into activation of Daam1 through disruption of the inhibitory intramolecular interactions (297). The complex of Dvl and activated Daam 1 interacts with the Rho guanine nucleotide exchange factor leading to the formation of Rho-GTP complex, which activates ROCK kinase that mediates cytoskeletal remodeling (295). Disheveled activates Rac, resulting in JNK activation that is important for PCP signaling in vertebrates (247,296,298).

(ii) Wnt/Ca^{2+} pathway

The role of Dvl in Wnt/Ca^{2+} signaling has been demonstrated by the DvlΔDIX (the DIX domain deletion mutant of Dvl) that was not able to activate the β-catenin-dependent pathway, but it stimulated Ca^{2+} activity in a heterotrimeric G protein-dependent manner in *Xenopus* embryos(259).

1.5.4. Role of Wnt pathway in lymphopoiesis and lymphoid malignancies

Wnt signaling plays an important role in T-cell development. Through loss of functions studies in mice models, it has been demonstrated that Wnt signalling (mediated by *Tcf1* and *Lef1*) is mandatory for maintenance of thymocytes (299,300). However, in mature T cells, Wnt signaling enhances the survival of these cells (301). Knockdown of Wnt1 and Wnt4 in mice models is correlated with hypocellularity of immature thymocytes (302).

Evidence of the importance of the Wnt pathway in B cell lymphogenesis came from the studies that demonstrated stimulation of pro-B cells by Wnt3a and the differential expression of Wnt receptors and coreceptors during different stages of progenitor B-cell maturation (232). Recently, it has been shown that the non-canonical Wnt5a increased B-cell lymphopoiesis in mice models (303).

Since Wnt signaling is important for lymphogenesis, it is role in lymphoid malignancies upon dysregulation is expected. Up regulation of Wnt target genes was associated with β-catenin nuclear localization in acute lymphoblastic leukemia and treatment of these cells with Wnt inhibitors induced apoptosis (304). However, *Wnt5a* has been shown to be epigenetically hypermethylated in up to 43% of acute lymphoblastic leukemia cases (305). In chronic lymphocytic leukemia (CLL), inhibition of GSK-3β enhanced the survival of CLL cells (306). In the same type of malignancy, it has been shown that Wnt5a enhances tumor cell viability by induction of ROR1 and NF-ƙB(307).

In acute myeloid leukemia (AML), aberrant expression of various components of the Wnt signalling, in addition to methylation of the Wnt antagonists have been reported (308-310). In the chronic myeloid

leukemia (CML) cell line K362, knockdown of Fz8 results in downregulation of the NFAT transcription activity as detected by a reporter construct, suggesting that Fz8 mediates the Wnt-non-canonical signaling in CML through the calcium pathway to regulate NFAT activity (311). In the same study, NFAT has been shown to protect CML cells from imatinib induced cell death via induction of IL-4 expression (311).

In lymphomas, β- catenin dysregulation was detected in 21% of cases of primary cutaneous B-cell lymphomas and in 42% of primary cutaneous T-cell lymphoma (312). A recent study reported the constitutive activation of WCP in mantle cell lymphoma (MCL), through the expression of various Wnt ligands and nuclear accumulation of β-catenin in MCL cell lines and patient samples (313). Most recently, it has been reported that β-catenin is transcriptionally active in ALK^{+}ALCL cell lines. In the same study, β-catenin has been shown to have a significant role in ALK^{+}ALCL biology, as down-regulation of β-catenin using siRNA significantly reduced the growth of ALK^{+}ALCL cells (314).

1.6. Thesis overview and objectives

ALK$^+$ALCL is an aggressive type of non-Hodgkin's lymphoma that is considered to be one of the most common forms of pediatric cancers (65). Despite that complete remission occurs in up to 95% of cases in response to conventional chemotherapy, relapses occur in up to 40% of cases (47,129). These lymphomas are characterized by the aberrant expression of ALK fusion proteins, with NPM-ALK expression in 80% of cases (32,60). Numerous studies have proven the oncogenic role and the transforming abilities of NPM-ALK in both *in vitro* and *in vivo* models (64,83,84,113,115,116,125,144,315-319). Despite this well established role of NPM-ALK in ALK$^+$ALCL pathobiology, more than one source of evidence suggests that NPM-ALK is not the only factor that governs the characteristic features and the oncogenic properties of ALK$^+$ALCL. This evidence is based on the fact that ALK fusion genes are aberrantly expressed by the peripheral blood cells as well as the lymphoid tissues of healthy individuals (79,80). It is also based on the results of *in vivo* studies in which animals transduced with NPM-ALK; develop lymphomas of the non T cell/null origins (84,315-317,320).

Thus, based on these findings I hypothesized that dysregulations of other signaling pathways contribute to NPM-ALK oncogenic abilities and are required to be involved in order for NPM-ALK to reach its full oncogenic transformation potential in ALK$^+$ALCL.

The scope of this thesis is to further our understanding of the pathobiology of ALK$^+$ALCL. Using NPM-ALK expressing ALCL as a study model, I aimed at further highlighting the biological importance of the already well known signaling pathways' defects through identifying their detailed mechanistic interaction with NPM-ALK. Furthermore, I aimed at identifying novel signaling pathways that contribute to the pathogenesis of this malignancy and that mediate their oncogenic effect either independently or through working in concert with NPM-ALK.

In chapter 2, I examined the detailed mechanistic interactions of NPM-ALK with SHP1, a well-known negative regulator of tyrosine kinase signaling that is epigenetically silenced in most of ALK^{+}ALCL cases (107,108,321). The biological importance of these mechanistic interactions was examined.

In chapter 3, I identified the aberrant expression of Sox2, one of the critical transcription factors for maintaining pluripotency and self-renewal of embryonic stem cells, in ALK^{+}ALCL cell lines and patients' tumors. Sox2 was demonstrated to be transcriptionally active in a small subset of the tumor cell population, and this activity is correlating significantly with its tumorigenicity in both *in vitro* and *in vivo* models. In addition Sox2 transcription activity in ALK^{+}ALCL has been shown to correlate with ALK^{+}ALCL cell lines sensitivity to chemotherapeutic agents.

In chapter 4, the over-expression and activation of disheveled proteins 2 and 3 as mediators of the Wnt non canonical pathway signaling was reported in ALK^{+}ALCL for the first time. The biological importance of this activation was demonstrated. Furthermore the cross talk between NPM-ALK and the Wnt non-canonical pathway represented by disheveled proteins was examined.

In conclusion, by identifying novel signaling defects in ALK^{+}ALCL, using NPM-ALK expressing ALCL as a study model, I have furthered our understanding of the pathobiology of this type of malignancy. My work demonstrated that the pathogenesis of ALK^{+}ALCL is more complex than what has been previously thought and it is not only related to the aberrant expression of ALK fusion proteins, but also to other signaling pathway defects. Furthermore, these findings provide a framework for the tailoring of novel therapeutic strategies for ALK^{+}ALCL.

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➢ Chapter 2

The tyrosine^{343} residue of nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) is important for its interaction with SHP1, a cytoplasmic tyrosine phosphatase with tumor suppressor functions

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The tyrosine^{343} residue of nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) is important for its interaction with SHP1, a cytoplasmic tyrosine phosphatase with tumor suppressor functions. *J Biol Chem.* 2010 Jun 25;285(26):19813-20.

2.1. Introduction

ALK-positive anaplastic large cell lymphoma (ALK^+ALCL) is a distinct type of aggressive lymphoma of T/null cell immunophenotypes (1). Approximately 80% of these neoplasms have the chromosomal translocation *t*(*2*;*5*)(*p23*;*q35*), which brings the *nucleophosmin* (*NPM*) gene at *5q35* in juxtaposition with the *anaplastic lymphoma kinase* (*ALK*) gene at *2p23*, leading to the formation of NPM-ALK (2–5). ALK is normally a receptor-tyrosine kinase expressed exclusively on the cell surface of embryonic neuronal cells (6). It has been shown that the oligomerization domain of the NPM portion in this fusion gene protein induces dimerization of ALK, which results in autophosphorylation and constitutive activation of the ALK tyrosine kinase (7, 8). By virtue of its constitutively active tyrosine kinase, it is believed that NPM-ALK promotes tumorigenesis by aberrantly phosphorylating various tyrosine residues of a host of cellular signaling proteins, thereby deregulating a large number of cellular signaling pathways (9). In addition to the expression of NPM-ALK, recent studies also revealed the existence of multiple coexisting biochemical defects in NPM-ALK-expressing lymphomas (10 –15).

The loss of SHP1, a cytoplasmic tyrosine phosphatase, serves as an example (14). SHP1 is normally expressed at the highest level in hematopoietic cells and it is known to act as a negative regulator of various cell signaling pathways, such as the JAK/ STAT pathway and that of the T-/B-cell receptor (16). The biological importance of SHP1 is highlighted by the phenotype of the so-called “moth-eaten” mice; specifically, homozygous mutations of the *SHP1* gene, which results in a complete loss of SHP1 expression, are associated with severe dysregulation of the leukocyte development and systemic autoimmunity (17,18). SHP1 also has been shown to have tumor suppressor functions. Specifically, loss of SHP1 has been demonstrated in a proportion of T-cell lymphomas, including the majority of ALK^+ALCL cases (10, 12, 19). Importantly, restoration of SHP1 expression has been shown to decrease

the growth of ALK^{+}ALCL cells *in vitro*. In another study, decreased expression of SHP1 has been shown to be associated with the progression of chronic myeloid leukemia (20). Previous studies have shown that SHP1 is physically associated with NPM-ALK, and SHP1 dephosphorylates NPM-ALK (14, 21). However, the molecular details underlying the physical and functional interaction between SHP-1 and NPM-ALK are incompletely understood. Because NPM-ALK is known to interact with its binding partners via the phospho-Tyr/SH2 motif (7, 22–24), one of the main objectives of this study was to identify the exact tyrosine residue of NPM-ALK involved in mediating its interaction with SHP1. We focused our search on the 11 tyrosine residues of NPM-ALK that have been previously found to be phosphorylated (25). Similarly, because SHP1 is known to interact with its substrates via one or both of its two SH2 domains, commonly termed N-SH2 (*i.e.* close to the N terminus) and C-SH2 (*i.e.* close to the C terminus) (26), we focused our studies on the roles of these two domains of SHP1 in the NPM-ALK/SHP1 binding. After we identified the exact tyrosine residue of NPM-ALK that is important for the physical interaction between NPM-ALK and SHP1, we examined the functional importance of this physical interaction in SHP1-mediated suppression on NPM-ALK.

2.2. Materials and Methods

2.2.1. Cell Lines and Tissue Culture

An ALK^{+}ALCL cell line, Karpas299, was used. These cells were from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 (Invitrogen) containing 2 mM L-glutamine supplemented with 10% fetal bovine serum (FBS) (Invitrogen). GP293, a human embryonic kidney cell line, was cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 4 g/liter of glucose supplemented with 10% FBS.

2.2.2. Vectors and Plasmids

The wild-type *SHP1* plasmid has been previously described (14). *SHP1* mutants with Arg→Lys mutation of one or both of the two functionally important arginine residues (Arg^{32} and Arg^{138}) located in the N-terminal and C-terminal SH2 domains, respectively, were kind gifts from Dr. F. Böhmer (Friedrich-Schiller-University, Jena, Germany) (27). Phosphatase inactive *SHP1* (*SHP1*^{C445S}) that has a point mutation (Cys →Ser) at the critical cysteine residue in the phosphatase domain was a kind gift from Dr. Zhenbao Yu (National Research Council of Canada, Montreal, Canada) (28). The *NPM-ALK* expression vector, a kind gift from Dr. S. Morris (St. Jude's Children Research Hospital, Memphis, TN), was cloned into the pcDNA3.1 (+) vector (Invitrogen). The use of the "kinase-dead" *NPM-ALK* (*NPMALK*K^{210R}) has been previously described (29). The *NPMALK*^{FFF} mutant, which was generated by mutating all three tyrosine residues in the kinase activation loop of ALK (Tyr^{338}, Tyr^{342}, and Tyr^{343}) in a pcDNA3.1 (+)/His-tagged *NPM-ALK* backbone, has also been described (25). Similarly, the single Tyr→Phe mutants of NPM-ALK (*i.e.* site-directed mutagenesis of Tyr^{138}, Tyr^{152}, Tyr^{156}, Tyr^{191}, Tyr^{419}, Tyr^{567}, Tyr^{644}, and Tyr^{664}) were constructed on a pcDNA3.1 (+)/*NPM-ALK* backbone. The coding sequences of *NPM-ALK* and all mutants were confirmed to ensure that no artificial mutations were acquired.

2.2.3. Gene Transfection

GP293 cells were plated in a 100-mm cell culture dish 24 h before gene transfection to obtain cell confluence of 70%. Gene transfection of *NPM-ALK* alone or in combination with *SHP1* was done using TurboFect™ *in vitro* Transfection Reagent (Fermentas, Burlington, Ontario, Canada) according to the manufacturer's protocol. Transient transfection of Karpas 299 cells (4 $x10^6$ cells) with 10 µg of DNA of wild-type *SHP1* or different *SHP1* mutants were performed using the Electro square electroporator,

BTX ECM 800(San Diego, CA) at 225 V (8 ms, 1 sec pulse length, 03 pulses).

2.2.4. Co-immunoprecipitation, Antibodies, and Western Blot Analysis

Cell lysates were prepared 24 h after gene transfection. For immunoprecipitation, a standard protocol was used as previously described (30). Briefly, cells were washed with cold phosphate-buffered saline and lysed using Cell Lytic Buffer M (Sigma) supplemented with 0.1 mM phenylmethylsulfonyl fluoride (Sigma), a protease inhibitor mixture (Nacalai Inc., SanDiego, CA), and phosphatase inhibitor mixture (Calbiochem, EMD Biosciences, Darmstadt, Germany). After incubating the lysate on ice for 30 min, it was centrifuged at 15,000 x*g* for 15 min. Two micrograms of the primary antibody was added to 500 µg of protein lysate and rotated overnight at 4 °C. Negative control samples with the primary antibody omitted were included. 50 µl of protein (A/G Plus-agarose) beads (Santa Cruz Biotechnology, Santa Cruz, CA) was added to both the test and control lysates and rocked for 2 h at 4 °C. The beads were then washed 3 times with cold phosphate-buffered saline. For co-immunoprecipitation experiments, the final wash was done using cold cell lysis buffer. For immunoprecipitation experiments, the final wash was done using RIPA buffer. Proteins were then eluted from the beads in 20µl of SDS protein loading buffer by boiling for 5 min at 100 °C. The complex was then subjected to SDS-polyacrylamide gel electrophoresis and western blotting, and the proteins were visualized using chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo Scientific, Rockford, IL). The following antibodies were used for immunoprecipitation and immunoblotting: goat polyclonal anti-ALK, rabbit polyclonal anti-SHP1 (both were from Santa Cruz Biotechnology), monoclonal anti-ALK (Dako, Glostrup, Denmark), mouse anti-SHP1 monoclonal antibody (BD Transduction Laboratories, Mississauga,

Ontario, Canada), rabbit polyclonal anti-phospho-ALK (Tyr1604), antiphosphotyrosine (both were from Cell Signaling, Danvers, MA), and anti-β-actin (Sigma). Western blot analysis is described as follows: cells were washed with cold phosphate-buffered saline, and cellular proteins were precipitated using RIPA buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris (pH 8), which was supplemented with 40 μg/ml of leupeptin, 1 mM pepstatin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 0.1 mM phenylmethylsulfonyl fluoride. The protein concentration of the samples was determined using the BCA Protein assay kit (Pierce, Thermo Fisher Scientific Inc.). Cell lysates were then electrophoresed on 8 or 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). Subsequently, the membranes were blocked with 5% milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.6, 150 mM NaCl), 0.1% Tween buffer for 1 h and then incubated with the primary antibodies overnight at 4 °C. After 3 washes with Tris-buffered saline, 0.1% Tween, the membranes were incubated with the isotype-specific secondary antibody conjugated with the horseradish peroxidase (Cedarlane Laboratories, Burlington, Ontario, Canada) for 1 h at room temperature. This was followed by 3 washes with Tris-buffered saline, 0.1% Tween, and the protein was detected using the chemiluminescence detection kit (Pierce).

2.2.5. Colony Formation in Soft Agar

The soft agar consisted of two layers, both of which were prepared from a stock 1.2% Bacto-agar (Difco, Detroit, MI) dissolved in distilled water and autoclaved. For the bottom layer, Dulbecco's modified Eagle's medium supplemented with 10% FBS was added to the stock agar to achieve a 0.6% agar concentration. For the top layer, cell suspension (20,000 cells in 2 ml of Dulbecco's modified Eagle's medium supplemented with 10% FBS) was mixed with the stock agar to achieve a final agar concentration of 0.3%. Cells in the agar were fed with 200 μl of Dulbecco's modified

Eagle's medium, 10% FBS every 2 days. Colonies were stained and visualized with 0.05% crystal violet after 4 weeks of culture.

2.2.6. Statistical Analysis

The association between SHP1 and the number of colonies formed in soft agar was evaluated using Student's *t* test. A *p* value ≤ 0.05 was considered statistically significant.

2.3. Results

2.3.1. SHP1 Interacts with NPM-ALK but Not the NPM-ALK^{K210R} Kinase-dead Mutant

Based on a number of previously published studies (23, 29, 31, 32), the binding between NPM-ALK and partners is typically dependent on the autophosphorylation and activation status of NPM-ALK. Thus, we asked if the activation status of NPM-ALK is important for the physical interaction between SHP1 and NPM-ALK. To address this question, we co-transfected GP293 cells with *SHP1* and *NPM-ALK* or the enzymatically inactive *NPM-ALK*^{K210R} mutant. As shown in Figure 2.1, SHP1 co-immunoprecipitated with NPM-ALK (*lane 2*); did not bind the NPM-ALK^{K210R} mutant (*lane 3*). We performed similar experiments using another NPM-ALK mutant, in which all three functionally important tyrosine residues in the kinase activation loop (KAL) of ALK have been mutated (labeled as FFF) (25, 33). As shown in *lane 4*, SHP1 also did not associate with the FFF mutant.

2.3.2. The Importance of the KAL of ALK for the NPM-ALK/SHP1 Binding

We have previously shown that mutations of one or more of the three tyrosine residues in the KAL of ALK result in a dramatic decrease in the number of phosphorylated tyrosine residues on NPM-ALK, its ability to

phosphorylate substrates and its tumorigenicity (25). Our previously published data showed that Tyr^{338} is the first tyrosine residue in the KAL to be phosphorylated and it appears to be functionally more important than Tyr^{342} and Tyr^{343} (25). With this background, we assessed how mutation of each one of these three tyrosine residues in the KAL affects the NPM-ALK/SHP1 binding. As shown in Figure. 2.2, mutation of Tyr^{338}, which was previously shown to dramatically decrease the overall level of tyrosine phosphorylation of NPM-ALK and the ability of NPM-ALK to phosphorylate various downstream targets, did not result in any substantial change in the binding between NPM-ALK and SHP1 (*lane 2*). Similarly, a relatively strong SHP1 binding was evident with the Tyr^{342} mutant (*lane 3*). In contrast, mutation of Tyr^{343} resulted in a complete loss of SHP1 binding detectable by our co-immunoprecipitation experiments (*lane 4*). Double mutation of Tyr^{342} and Tyr^{343} also abrogated the binding (*lane 5*), highlighting the importance of the Tyr^{343} residue in mediating the NPM-ALK/SHP1 interaction.

2.3.3. Tyr343 Falls into a Consensus Sequence That Is Recognized by SHP1

To explain why mutation of Tyr^{343} results in the dramatic loss of SHP1 binding, we performed peptide sequence analysis, and assessed if Tyr^{343} falls into any of the known consensus sequences that can be recognized by SHP1. In this regard, we found a consensus sequence, (*XX*pY(Y/F)K/R) (34), present in a segment spanning Tyr^{343} (namely ASY342Y343R). In contrast, Tyr^{338} of NPM-ALK was not found to fall into any specific consensus sequences recognizable by SHP1. This finding further supports that Tyr^{343} represents the crucial binding site between NPM-ALK and SHP1, and explains why mutation of Tyr^{343}, but not Tyr^{338} or Tyr^{342}, results in a dramatic change in the binding between NPM-ALK and SHP1.

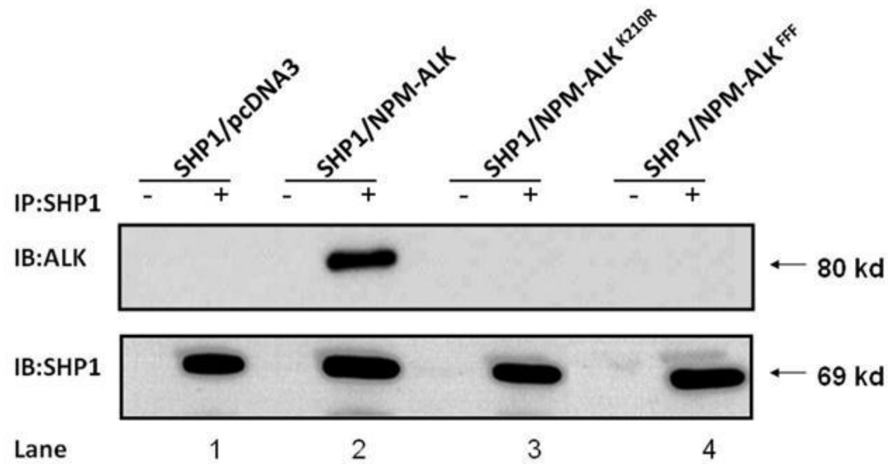

Figure 2.1. The activation status of NPM-ALK is important for its binding to SHP1.

Co-immunoprecipitation experiments using GP293 cells co-transfected with *NPM-ALK* (or its mutants) and *SHP1* revealed binding of SHP1 to NPM-ALK (lane 2), but not the enzymatically inactive NPM-ALK^{K210R} mutant (lane 3) or the NPM-ALK^{FFF} mutant (lane 4). Immunoblotting with anti-SHP1 revealed a relatively equal amount of immunoprecipitated SHP1 proteins. Negative control reactions (-) were performed by omitting the use of anti-SHP1 antibody. Cells co-transfected with *SHP1* and empty vector *(i.e. pcDNA3*) were used as a negative control. Results shown are representative of three independent experiments.

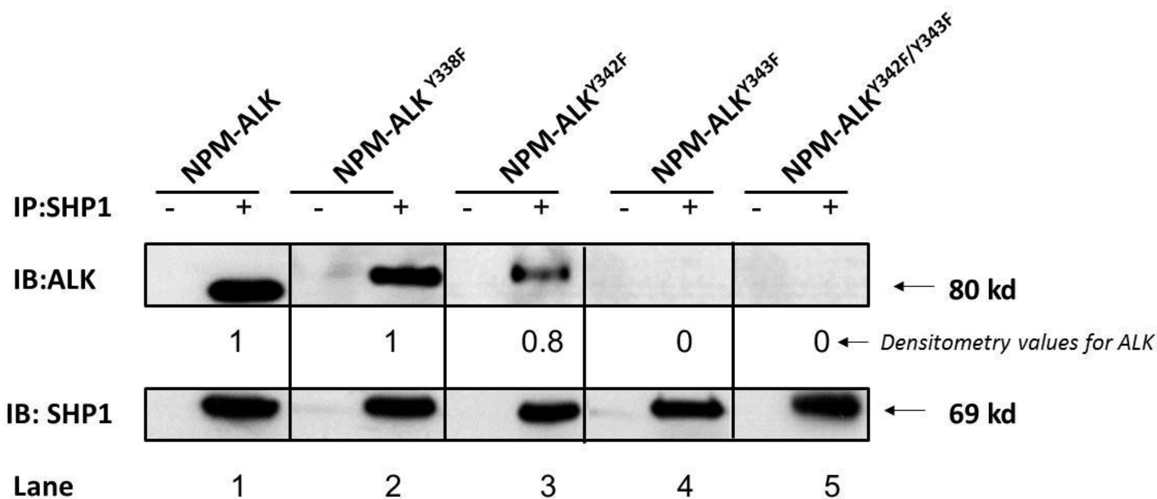

Figure 2.2. The importance of the three tyrosine residues in the KAL of ALK for the binding between NPM-ALK and SHP1.

Single mutation of the Y338 of the KAL showed no detectable change in its binding to SHP1 (lane 2), as compared to NPM-ALK (lane 1). A readily detectable SHP1 binding was also evident for the Y342 mutant (lane 3). In contrast, the Y343 mutant did not show detectable SHP1 binding (lane 4). In addition, double mutant NPM-ALK$^{Y342F/Y343F}$ also showed a complete loss of SHP1 binding. The degree of SHP1 binding seen in each lane was assessed by densitometry and the results were normalized to the SHP1 protein level and determined relative to the SHP1 binding to unaltered NPM-ALK (lane 1). Results shown are representative of three independent experiments. Of note, the *NPM-ALK* vectors used for lane 2-5 contained a His-tag, which explains the slightly slower migration pattern seen in these lanes.

2.3.4. The Loss of SHP1 Binding Is Specific for the NPM-ALK$^{Tyr\text{-}343}$ Mutant

We then asked if the loss of SHP1 binding can be seen in mutations outside the KAL of ALK. Because our recent mass spectrometry studies revealed only 11 tyrosine residues (including the three in the KAL of ALK) that are phosphorylated (25), we performed site-directed mutagenesis of each of the remaining 8 tyrosine residues outside the KAL, including Tyr^{138}, Tyr^{152}, Tyr^{191}, Tyr^{156}, Tyr^{419}, Tyr^{567}, Tyr^{644}, and Tyr^{664}. Using co-immunoprecipitation experiments, we found that mutations in each of these 8 tyrosine residues individually resulted in readily detectable binding between NPM-ALK and SHP1 (Figure 2.3A), and these findings are in sharp contrast with that for the Tyr^{343} mutant (Figure 2.2). We also examined a tyrosine residue of NPM-ALK that is not phosphorylated, namely Tyr^{67}; no loss of SHP1 binding was detected (Figure 2.3B).

2.3.5. Tyrosine Dephosphorylation of NPM-ALK by SHP1 Is Dependent on Their Physical Interaction

Because we identified Tyr^{343} of NPM-ALK as the crucial binding site for SHP1, we then asked if the SHP1- mediated tyrosine dephosphorylation of NPM-ALK is dependent on their physical interaction. A dependence on the physical interaction suggests that SHP1 directly dephosphorylates NPM-ALK. On the other hand, an independence could suggest that SHP1 dephosphorylates NPM-ALK indirectly (*i.e.* involves another mediator). As shown in Figure 2.4A, the expression of SHP1 and NPM-ALK resulted in a 50% reduction of the tyrosine phosphorylation level of NPM-ALK. This level of tyrosine dephosphorylation induced by SHP1 is similar to that seen previously (14, 35). In contrast, SHP1 did not result in any detectable change in the tyrosine phosphorylation level of the Tyr^{343} mutant (Figure 2.4B). We repeated similar experiments using other NPM-ALK mutants, including (i) the Tyr^{644} mutant, which is known to be phosphorylated and resides outside the KAL; and (ii) the Tyr^{342} mutant, which is known to be

phosphorylated and resides in the KAL. As shown in Figure 2.4C and D, SHP1 dephosphorylated these mutants as effectively as it did for NPM-ALK.

2.3.6. The SHP1/NPM-ALK Binding Is Dependent on Both SH2 Domains of SHP1, but Not Its Tyrosine Phosphatase Activity

As shown in Figure 2.5, in GP293 cells co-transfected with *SHP1* and *NPM-ALK*, SHP1 co-immunoprecipitated with NPMALK (*lane 2*). In contrast, as shown in *lanes 3-5*, all three SHP1 mutants (*i.e.* with mutations in the N-SH2 domain, the C-SH2 domains, or both the N-SH2 and C-SH2 domains) had reduced binding to NPM-ALK. Thus, it is evident that both the N-SH2 and C-SH2 domains of SHP1 are important in mediating its binding to NPM-ALK. Using the same experimental approach, we assessed whether the tyrosine phosphatase activity of SHP1 is important for the SHP1/NPM-ALK binding. As shown in Figure 2.6, NPM-ALK co-immunoprecipitated with the $SHP1^{C445S}$ mutant (*lane 3*), previously reported to be "phosphatase- dead" (28).

2.3.7. Mutation of the SH2 Domains of SHP1 Results in a Partial Loss of Its Inhibitory Effects on NPM-ALK

We then asked if mutations of the SH2 domain of SHP1 also affect its ability to dephosphorylate NPM-ALK. To address this question, we transfected *SHP1* or its double SH2 mutant into Karpas 299 cells, a SHP1-negative ALK^{+}ALCL cell line (14). As shown in Figure 2.7, mutations of both N-SH2 and C-SH2 domains of SHP1 resulted in a partial decrease in the level of tyrosine phosphorylation of NPM-ALK, as compared with the parental SHP1 construct. This partial decrease was expected, as mutation of the SH2 domain of SHP1 did not completely abrogate the SHP1/NPM-ALK binding (Figure 2.5).

2.3.8. Soft Agar Clonogenic Assay

Using a soft agar clonogenic assay, we assessed how the physical interaction between SHP1 and NPM-ALK affects NPM-ALK-driven tumorigenicity. As shown in Figure 2.8, the tumorigenicity of NPM-ALK in cells coexpressing SHP1 was significantly less than that seen in cells co-expressing an empty vector (p value = 0.0032, Student's t test). In contrast, there was no significant difference between tumorigenicity of the NPM-ALK Y343F mutant in cells coexpressing either empty vector or SHP1 ($p > 0.05$, Student's t test).

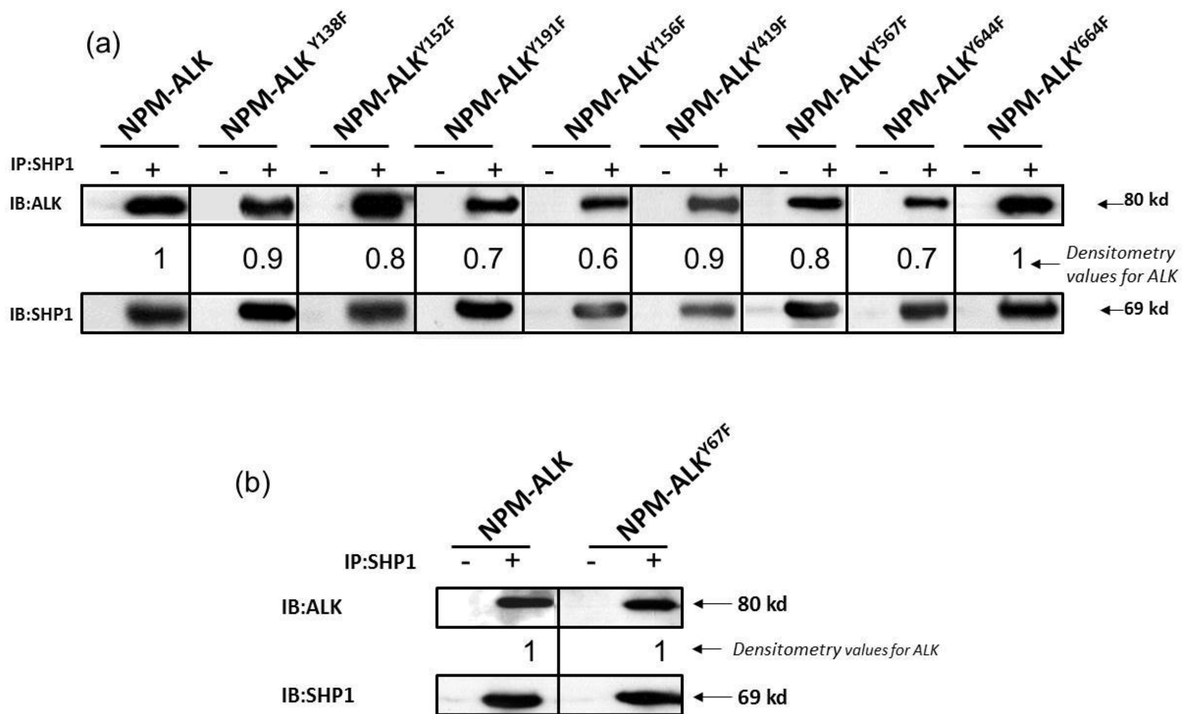

Figure 2.3. The loss of SHP1 binding is specific for the NPM-ALK^{Y343} mutant.

(a) Co-immunoprecipitation experiments using GP293 cells co-expressing SHP1 and 8 different NPM-ALK mutants (each of which contained a single mutation of the 8 remaining tyrosine residues known to be phosphorylated) showed no substantial or relatively minimal loss of SHP1 binding. (b) A randomly selected mutant Y67, which has been shown previously not to be phosphorylated, showed no loss of binding to SHP1. Results shown are representative of three independent experiments. The degree of SHP1 binding seen in each lane was assessed by densitometry, and the results were normalized to the SHP1 protein level and determined relative to the SHP1 binding to unaltered NPM-ALK.

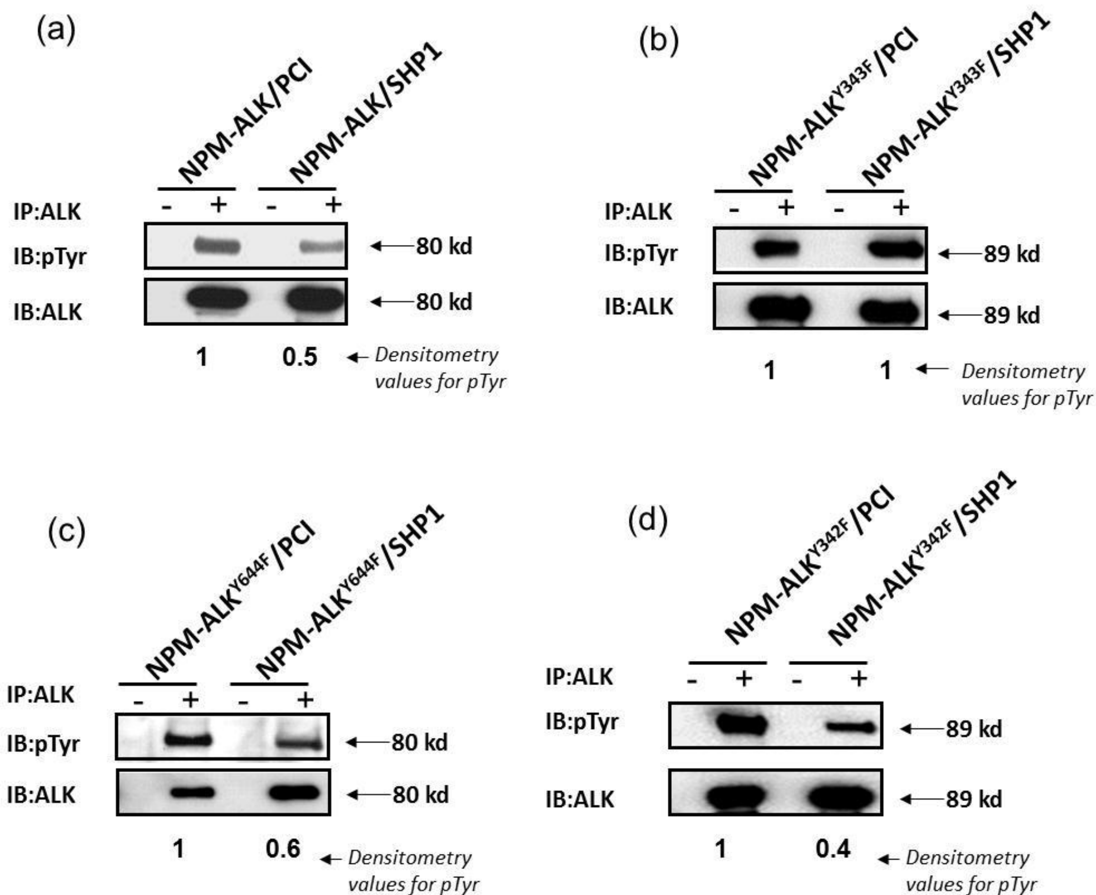

Figure 2.4. Tyrosine dephosphorylation of NPM-ALK by SHP1 is dependent on their physical interaction.

GP293 cells co-transfected with *NPM-ALK* (or its mutants) and *SHP1* (or the empty vector, *PCI*) were used for immunoprecipitation of NPM-ALK, followed by detection of the phosphorylation level of NPM-ALK using phosphotyrosine antibody. There is almost a 50% reduction of NPM-ALK tyrosine phosphorylation when SHP1 was coexpressed (a). In contrast, no detectable difference in the tyrosine phosphorylation of NPM-ALK^{Y343F} when it was coexpressed with SHP1 or an empty vector (b). The NPM-ALK^{Y644F} and NPM-ALK^{Y342F} showed similar results as NPM-ALK (c and d). Results shown are representative of three independent experiments.

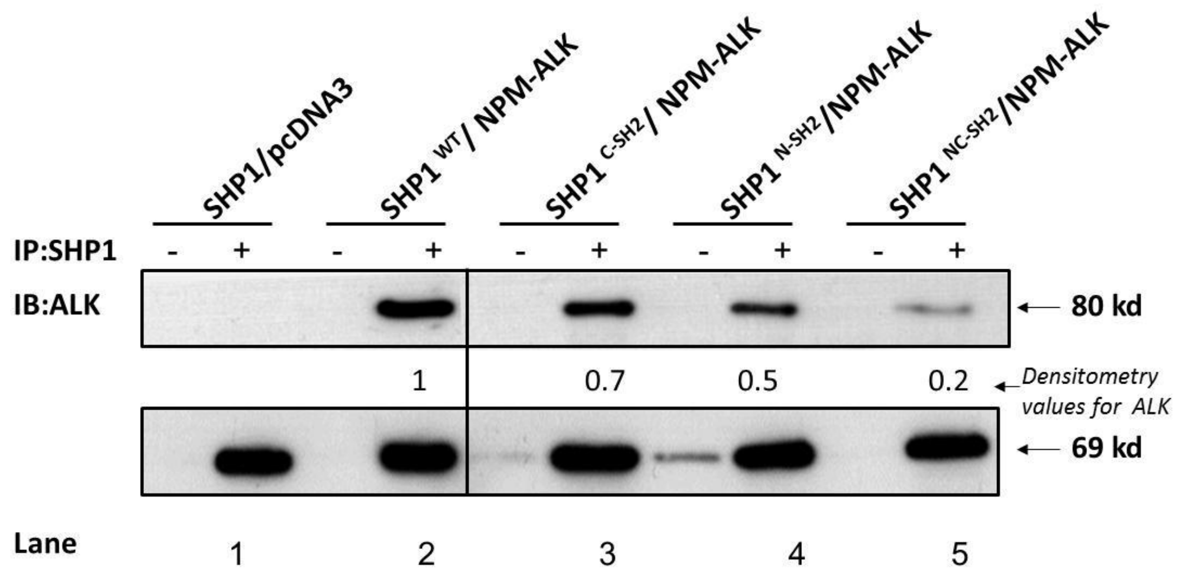

Figure 2.5. The SHP1/NPM-ALK binding is dependent on both of the SH2 domains of SHP1.

Co-immunoprecipitation experiments using GP293 cells co-expressing NPM-ALK and SHP1 or its SH2 mutants showed reduced NPM-ALK/SHP1 interaction in all three mutants (lanes 3-5). Results shown are representative of three independent experiments.

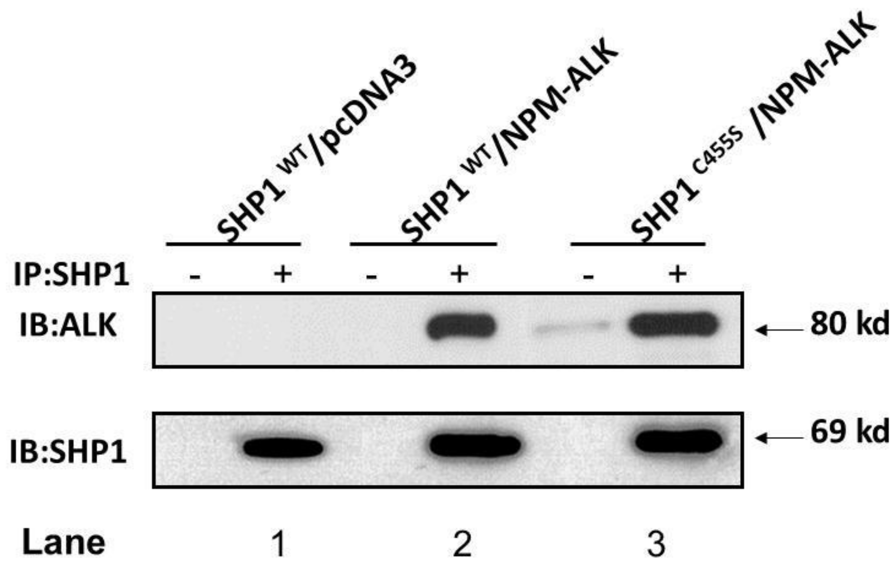

Figure 2.6. The SHP1/NPM-ALK binding is not dependent on the phosphatase activity of SHP1.

Co-immunoprecipitation experiments using GP293 cells showed that the NPM-ALK/SHP1 binding was the same between NPM-ALK/SHP1 (lane 2) and NPM-ALK/SHP1^{C455S} (lane 3). Results shown are representative of three independent experiments.

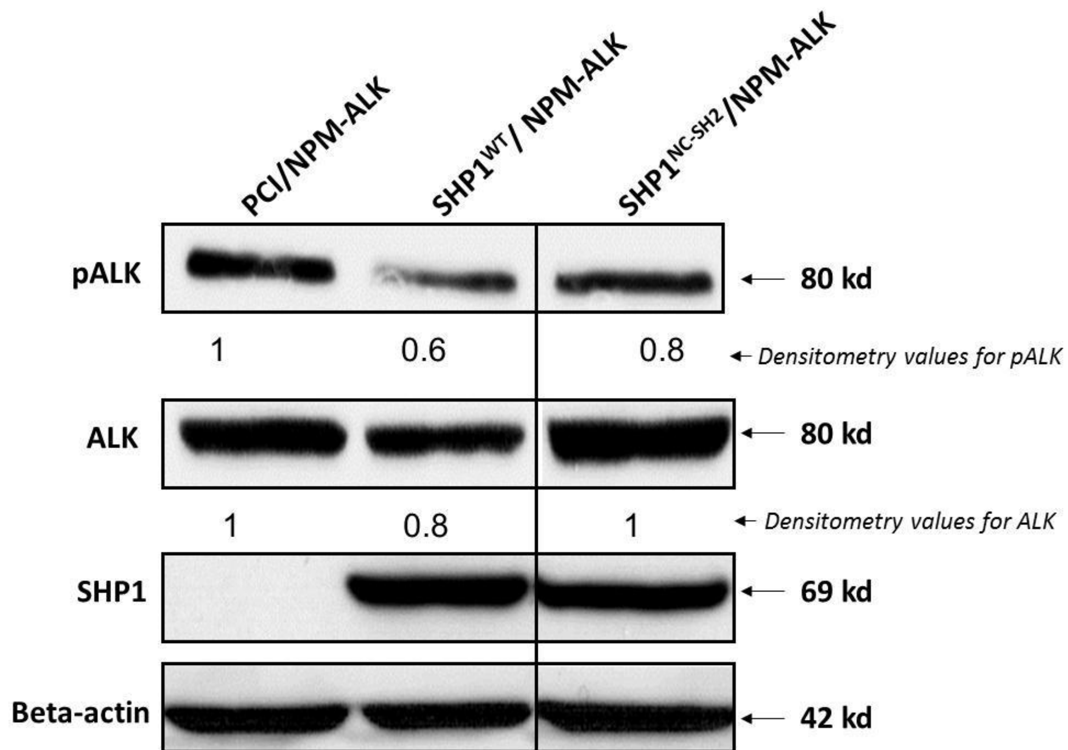

Figure 2.7. Mutation of the SH2 domains of SHP1 results in a partial loss of its inhibitory effects on NPM-ALK.

Transfection of *SHP1* in ALK^{+}ALCL cell line Karpas 299 resulted in a dramatic down- regulation of phosphorylated NPM-ALK as well as the total ALK protein level. In contrast, the SHP1 mutant (mutation in both the N-SH2 and C-SH2 domains, $SHP1^{NC\text{-}SH2}$) resulted in a partial decrease in the level of tyrosine phosphorylation of NPM-ALK, as compared to SHP1. The densitometry values were determined after normalization to the β-actin band. Results shown are representative of three independent experiments.

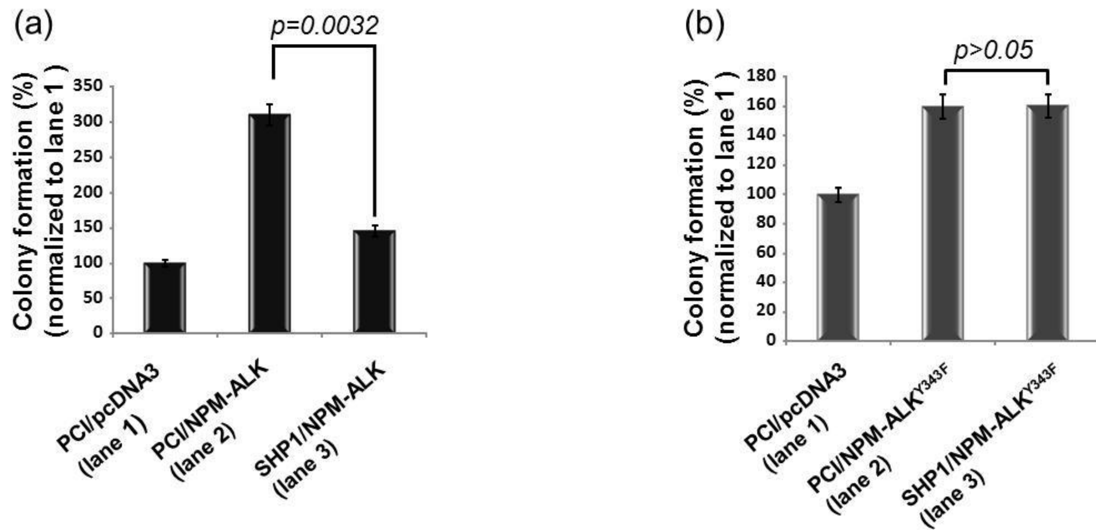

Figure 2.8. The tumorigenicity of NPM-ALK, but not that of NPM-ALK^{Y343F}, was suppressed by SHP1, as assessed by soft agar clonogenic assay.

The colony formation induced by NPM-ALK in GP293 cells was significantly reduced by SHP1 ($p= 0.0032$). In contrast, the colony formation induced by NPM-ALK^{Y343F} was not significantly affected by SHP1 ($p>0.05$). These experiments were performed in triplicates.

2.4. Discussion

In normal cells, the tyrosine phosphorylation status of various proteins is tightly regulated by interaction of a host of kinases and phosphatases (36, 37). SHP1, a cytoplasmic tyrosine phosphatase largely expressed in hematopoietic cells, is known to dephosphorylate and inhibit various proteins including cytokine receptors (38–42) as well as cell-surface receptors involved in immune response (43–47). By virtue of the two SH2 domains, SHP1 physically interacts with specific phosphotyrosine residues in its substrates. Upon binding to these substrates, SHP1 undergoes intramolecular changes that set its tyrosine phosphatase domain free from the hindering effects of its N-SH2 domain (48). The "exposed" tyrosine phosphatase domain is able to dephosphorylate the substrates and thereby down-regulate their activities. Reduction or loss of SHP1 expression has been found in a number of hematopoietic neoplasms (12, 20, 49–51). This loss of expression can be attributed to gene methylation, and restoration of SHP1 expression by 5-azathioprine has been shown in a number of hematopoietic cell lines (35, 50). In ALK^{+}ALCL, the loss of SHP1 expression can be identified in up to 80% of these tumors, and this finding correlates with gene methylation of *SHP1* (12). Its tumor suppressor function and inhibitory effects on NPM-ALK in these tumors have also been shown previously. Specifically, gene transfection of *SHP1* down-regulated the activation/phosphorylation level of NPM-ALK and inhibited its cell growth-promoting effects (14, 21, 35). Although the molecular events underlying the interactions between SHP1 and various substrates in normal cells have been extensively studied, how SHP1 binds and regulates oncogenic tyrosine kinases is incompletely understood. To our knowledge, there are only three published studies in which the physical and/or functional interactions between SHP1 and different oncogenic tyrosine kinases were examined (52–54). In the first study, Lim *et al.* (52) examined the effect of SHP1 on BCR-ABL and concluded that the physical binding between SHP1 and BCR-ABL is important for down-

regulating the phosphorylation level and tumorigenicity of BCR-ABL. In contrast with our study, this study employed an artificially created fusion protein containing the catalytic domain of SHP1 and the ABL binding domain of RIN1 (a known binding partner of c-ABL). Thus, whether the SH2 domains of SHP1 are required for this physical interaction could not be determined. In addition, the tyrosine residue(s) of BCR-ABL involved in this interaction was not determined. In the second study, Hennige *et al.* (53) examined the inhibitory effect of SHP1 on Ret, an oncogenic tyrosine kinase expressed in medullary thyroid carcinoma. Although it is nicely demonstrated that SHP1 reduces the phosphorylation status of Ret and its oncogenic activity, details of the physical interaction of these two proteins and the importance of this interaction were not examined. Finally, Roccato *et al.* (54) identified the site of interaction between SHP1 and TRK-T3 (an oncogenic tyrosine kinase characteristic for papillary thyroid tumors) although the functional significance of the physical interaction between these two proteins was not addressed. The requirement for both SH2 domains of SHP1 for its optimal interaction with NPM-ALK is in contrast with the previous finding that only the C-SH2 domain is important for mediating the physical interaction between SHP1 and FcγRIIb1 (43). Nevertheless, the requirement for both SH2 domains of SHP1 has been described for the SHP1-epidermal growth factor receptor interaction (27). Thus, the relative roles of N-SH2 and C-SH2 of SHP1 appear to vary among different substrates. Because mutations of both SH2 domains of SHP1 did not completely abrogate its binding to NPM-ALK, it is likely that other portions of SHP1 may contribute to the binding. In this regard, it has been previously reported that SHP1 binds the insulin receptor via a unique sequence located in the C-terminal tail of SHP1 (55). Because ALK is a member of the insulin receptor subfamily, this portion of SHP1 may also contribute to the NPM-ALK/SHP1 binding.

A recent study from our laboratory revealed that only 11 tyrosine residues in NPM-ALK are phosphorylated (25). Three of these 11 tyrosine residues

are found in the KAL (Tyr^{338}, Tyr^{342}, and Tyr^{343}). The other eight tyrosine residues were found to be confined to the ALK portion of the fusion protein, and they include Tyr^{138}, Tyr^{152}, Tyr^{191}, Tyr^{156}, Tyr^{419}, Tyr^{567}, Tyr^{644}, and Tyr^{664}. In the same study, we found that mutation of any of the three tyrosine residues in the KAL did not affect the phosphorylation status of the remaining two tyrosine residues. Furthermore, the tyrosine phosphorylation pattern outside the KAL is identical between the Tyr^{338} mutant and the Tyr^{343} mutant. These findings are relevant to this study, because we can conclude that, as compared with the Tyr^{338} mutant, the dramatic loss of SHP1 binding seen in the Tyr^{343} mutant is not because of a loss of phosphorylation in any tyrosine residue other than Tyr^{343} itself. Because Tyr^{343} is located in the KAL of ALK, one may have to consider the possibility that this loss of SHP1/NPM-ALK binding is related to the loss of the overall activity of NPM-ALK. We consider this possibility to be unlikely, because the Tyr^{338} or Tyr^{342} mutants, both of which have been shown to have significantly lower tumorigenicity than the Tyr^{343} mutant (25), did not show a substantial loss of SHP1 binding. To further show that Tyr^{343} is the specific binding site of SHP1, we found no substantial loss of SHP1 binding when we introduced mutations of each of the remaining 8 different tyrosine residues known to be involved in the phosphorylation of NPM-ALK. Of note, because the enzymatically inactive NPM-ALK^{K210R} mutant resulted in abrogation of the SHP1/NPM-ALK binding, some degree of (but not full) phosphorylation and activation of NPM-ALK is required for its physical interaction with SHP1. The requirement for at least some degree of tyrosine phosphorylation is in keeping with the observation that SHP1 generally binds only the phosphorylated substrates (16). Based on the presented data, we have developed a hypothetical model to describe the SHP1/NPM-ALK interaction. We speculate that one of the two SH2 domains of SHP1 directly binds Tyr^{343} of NPM-ALK, whereas the second SH2 domain of SHP1 is responsible for providing the optimal three-dimensional conformation for this interaction. This second

SH2 domain may also interact with another specific tyrosine residue of NPM-ALK, although this interaction by itself is so weak that mutation of this specific tyrosine residue alone did not result in a dramatic effect in our co-immunoprecipitation experiments. This model explains why mutation of either SH2 domain of SHP1 resulted in a reduction in the NPM-ALK/SHP1 binding.

In conclusion, we identified Tyr^{343} of NPM-ALK as the crucial binding site for SHP1. Furthermore, the inhibitory effects of SHP1 on NPM-ALK require some degree of (but not full) activation of NPM-ALK. Their physical interaction is also partly dependent on the two SH2 domains of SHP1. Our results support the model that SHP1 exerts its inhibitory effects directly on NPM-ALK. To our knowledge, this is one of the most comprehensive studies of how SHP1 physically and functionally interacts with an oncogenic tyrosine kinase.

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➢ Chapter 3

Aberrant expression and biological significance of Sox2, an embryonic stem cell transcriptional factor, in ALK-positive anaplastic large cell lymphoma

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Aberrant expression and biological significance of Sox2, an embryonic stem cell transcriptional factor, in ALK-positive anaplastic large cell lymphoma

* The first two authors have equal contribution to this work.

3.1. Introduction

The Sox (stands for Sex determining region Y-Box) family of proteins that includes a host of transcriptional factors that are known to play crucial roles in embryogenesis and development (1-2). Members of the Sox family have been reported to regulate a diversity of developmental processes, including the maintenance of pluripotency of embryonic stem cells (ESCs)(Sox2), testis determination (Sry), chondrogenesis (Sox5, Sox6, Sox9), as well as the development of the cardiac and lymphoid systems (Sox4), lens (Sox1 and Sox2), neural tissues and the brain (Sox1, Sox3, Sox11, Sox14, Sox21) (3-4).

The biological importance of Sox2 is highlighted by the observation that *Sox2* homozygous-null mouse embryos die soon after implantation (5). In humans, mutations of the *Sox2* gene have been linked to optic nerve hypoplasia and syndromic microphthalmia (6). Sox2 is believed to work in concert with other ESC proteins, particularly Oct4, to maintain self-renewal and pluripotency of ESCs (5). Similar to the other Sox family members, Sox2 binds to DNA in a highly sequence-specific manner (3). Genes that are transcriptionally regulated by Sox2 often contain a contiguous composite *Sox-Oct* cis-regulatory element to which Sox2 and Oct4 bind synergistically (7-8). Based on results of the ChIP-on-chip studies, it appears that the Sox2-Oct4 regulatory complex up-regulates a large number of genes important for the maintenance of pluripotency of ESCs and down-regulates another set of genes responsible for the initiation of differentiation (9-10).

Recent studies have implicated Sox2 in cancer biology. Sox2 has been reported to be highly expressed in a number of solid tumors, including cancers of the prostate (11), stomach (12-13), breast (14), colorectum (15), brain (16-17) and testicles (18). The vast majority of these reports focus on the correlation between Sox2 expression and various clinicopathologic parameters. Mechanistic studies of Sox2 in cancer cells are relatively scarce, and the roles of Sox2 in cancer have not been clearly

delineated. Nevertheless, results from a few recent publications have provided evidence to support that Sox2 indeed contributes to tumorigenesis and invasiveness. Specifically, Sox2 was found to enhance the migratory properties and cell proliferation in lung cancer cells (19-20). In another study, inhibition of Sox2 expression using siRNA was shown to decrease cell proliferation and tumorigenicity in glioblastoma cell lines (16). To our knowledge, Sox2 expression has not been previously reported in hematologic malignancies. In a study in which previously published gene array data was reviewed and summarized, Schenhals *et al.* did not identify Sox2 expression in a variety of hematologic malignancies (21).

ALK-positive anaplastic large cell lymphoma (ALK^{+}ALCL), a distinct type of non-Hodgkin's lymphoma of T/null-cell immunophenotype, primarily affects children and young adults and constitutes 10-30% of all pediatric lymphomas (22). The normal cellular counterpart of ALK^{+}ALCL is believed to be mature cytotoxic T-cells (22). Most of these tumors carry the *t(2;5)(p23;q35)* cytogenetic abnormality, which places the *ALK* (*anaplastic lymphoma kinase*) gene under the regulation of the *NPM* (*nucleophosmin*) gene promoter. The resulting fusion protein (NPM-ALK) has constitutively active tyrosine kinase activity, which has been shown to be critically important for its transformation property (23). NPM-ALK is known to bind to and activate a host of cell signaling pathways, including those of JAK/STAT3 (24-25), Ras/ERK (26) and PI3K/AKT (27), all of which are known to regulate important cellular functions such as cell cycle progression and cell survival. Of these pathways, the STAT3 pathway is the best characterized and the NPM-ALK/STAT3 signaling axis is believed to be central to the pathogenesis of ALK^{+}ALCL (28-29). Since STAT3 has been shown to contribute to Sox2 expression in neural precursor cells (30), we hypothesized that Sox2 expression may be induced by the relatively high level of STAT3 activation in ALK^{+}ALCL cells. To be detailed

in this manuscript, this turns out to be the case. In this study, we went on to characterize the biological effects of Sox2 in ALK$^+$ALCL.

3.2. Materials and Methods

3.2.1. ALK$^+$ALCL cell lines and patient samples

Three ALK$^+$ALCL cell lines were used in this study (Karpas 299, SUP-M2 and UCONN-L2), and their characteristics have been described previously (31). Ntera-2, a human teratocarcinoma cell line, and Hela, a human cervical cancer cell line, were purchased from American type Culture Collection (Manassas, VA, USA) and cultured in DMEM (Gibco, Carlsbad, CA, USA) containing 2 mM of L-glutamine supplemented with 10% FBS (Gibco). All ALK$^+$ALCL tumors and non-ALK T-cell neoplasms used in this study were diagnosed at the Cross Cancer Institute and the diagnostic criteria were based on those described in the World Health Organization Classification Scheme (22). The use of these human tissue samples has been approved by our Institutional Ethics Committee. Peripheral blood mononuclear cells were obtained from healthy individuals and isolated using Ficoll-paque (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA). T lymphocytes were then enriched using the EasySep® human T-cell enrichment kit (Catalog #19051) from Stem Cell Technology (Vancouver, British Columbia, Canada) following the manufacturer's recommendation. The quality of purification, as evaluated by CD3 immunostaining and flow cytometry, was >99.5%.

3.2.2. Antibodies and drugs

Anti-ALK was purchased from Dako (1:500, Glostrup, Denmark) and anti-Sox2 was purchased from R&D Systems Inc (1:1000, Minneapolis, MN, USA). Anti-c-myc (1:500), anti-PDGFR-alpha (1:500), anti-BCL2 (1:500) and anti-β-actin (1:1000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All of the following antibodies were purchased from Cell Signaling (Danvers, MA, USA): anti-ERK (1:1000), anti-p-ERK

(1:1000), anti-STAT3 (1:1000), anti-pSTAT3 (1:1000), anti-cyclin D3 (1:1000), anti-AKT (1:1000), anti-pAKT (1:1000), anti-p38α (1:1000), anti-p38β (1:1000) and anti-notch1(1:1000). Doxorubicin was purchased from LC Laboratories (Woburn, MA, USA).

3.2.3. Subcellular protein fractionation, Western blots and Immunohistochemistry

For subcellular protein fractionation, we employed a kit purchased from Active Motif (Carlsbad, CA, USA) and followed the manufacturer's instructions. Western blots and immunohistochemistry were performed using standard techniques as described in our previous publications (25, 32-33).

3.2.4. Immunofluorescence and confocal microscopy

Cells were grown on cover slip coated with poly-L-lysine (Sigma Aldrich, St Louis, MO, USA) placed in a 6-well plate. Prior to the staining, cells were fixed with 3% paraformaldehyde in PBS (pH 7.4). Subsequently, cells were rinsed three times with PBS, permeabilized with triton, washed again with PBS, and incubated with 200 μL of anti-Sox2 antibody (1:50, R&D) overnight at room temperature in a humidified chamber. After washings in PBS, cells were incubated with a secondary antibody conjugated with Alexa Fluor 488 (Invitrogen, Burlington, Ontario, Canada, 1:250) for 1 hour at room temperature. After washing in PBS, cover slips were mounted on slides using the mounting media (Dako). Cells were visualized with a Zeiss LSM 510 confocal microscope at the Core Cell Imaging Facility, Cross Cancer Institute.

3.2.5. Short Interfering RNA (siRNA) and gene transfection

To down-regulate the expression of Sox2, Karpas 299 and SUP-M2 cells ($5x10^6$ cells in 0.5 ml of culture medium) were transfected with (200 pmol/ $1x10^6$ cells) of SMARTpool-designed siRNA against *Sox2*. Cells

transfected with scrambled siRNA (Dharmacon, Lafayette, CO, USA) were used as negative controls. To down-regulate the expression of NPM-ALK, we transiently transfected ALK^+ALCL cells with SMARTpool-designed siRNA specific for *NPM-ALK*. Cells transfected with scrambled siRNA (Dharmacon) were used as negative controls. To down-regulate STAT3 expression, specific siRNA against STAT3 and scrambled siRNA were purchased from Qiagen (Mississauga, Ontario, Canada). All siRNA transfection experiments were performed using the Electro square electroporator, BTX ECM 800 (Holliston, MA, USA) at 225 V (8.5 ms, 03 pulses).

3.2.6. Generation of ALK^+ALCL cells stably transduced with the Sox2 reporter construct

Lentiviral particles were generated by transfecting the 293T packaging cell line with the *pGreenFire1-mCMV-EF1-Puro* lentiviral vector (SBI System Biosciences, Mountain View, CA, USA) or the *pGreenFire1-Sox2SRR2-mCMV-EF1-Puro* lentiviral vector (SBI System Biosciences). Characterization of the transcriptional response element in the Sox2 reporter (labeled as Sox2SRR2 in the vector) has been previously characterized and published (34-35). Briefly, as illustrated in figure 3.1., the Sox2 reporter vector contains three tandem transcriptional response elements, each of which contains a *Sox2* consensus binding sequence *CATTGTG*. Cells with Sox2 transcriptional activity express the green fluorescence protein (GFP), detectable by flow cytometry. The reporter vector containing mCMV without the *Sox2SRR2* segment served as the negative control; cells transfected with this negative control vector did not show any GFP expression detectable by flow cytometry (Figure 3.2.).

Sox2 TRE reporter construct

A.

Sox2 consensus site: ***CATTGTG***

B.

The transcriptional response element:

TAATTAATGCAGAGACTCTAAAAGAATTTCCCGGGCTCGGGCAGC**CATTGTG**ATGCATATAGGATTATTCACGTGGTAATG

C.

The Sox2 reporter construct sequence:

5'TAATTAATGCAGAGACTCTAAAAGAATTTCCCGGGCTCGGGCAGC***CATTGTG***ATGCATATAGGATTATTCACGTGG
TAATGTAATTAATGCAGAGACTCTAAAAGAATTTCCCGGGCTCGGGCAGC***CATTGTG***ATGCATATAGGATTATTCACGTGGT
AATG CCAGTCCAAGCTAGGCAGGTTCCCCTCTAATTAATGCAGAGACTCTAAAAGAATTTCCCGGGCTCGGGCAGC***CATTGT***
GATGCATATAGGATTATTCACGTGGTAATGAGCACAGTCGACAGTTCTTGC-3'

Figure 3.1. Details of the Sox2 reporter construct used in this study.

Panel A provides the *Sox2* binding consensus sequence. Panel B provides information regarding the *Sox2* transcriptional response element (TRE). Panel C indicates the presence of three tandem *Sox2* TREs included in the reporter construct. The *Sox2* consensus sequences are highlighted in bold and underlined.

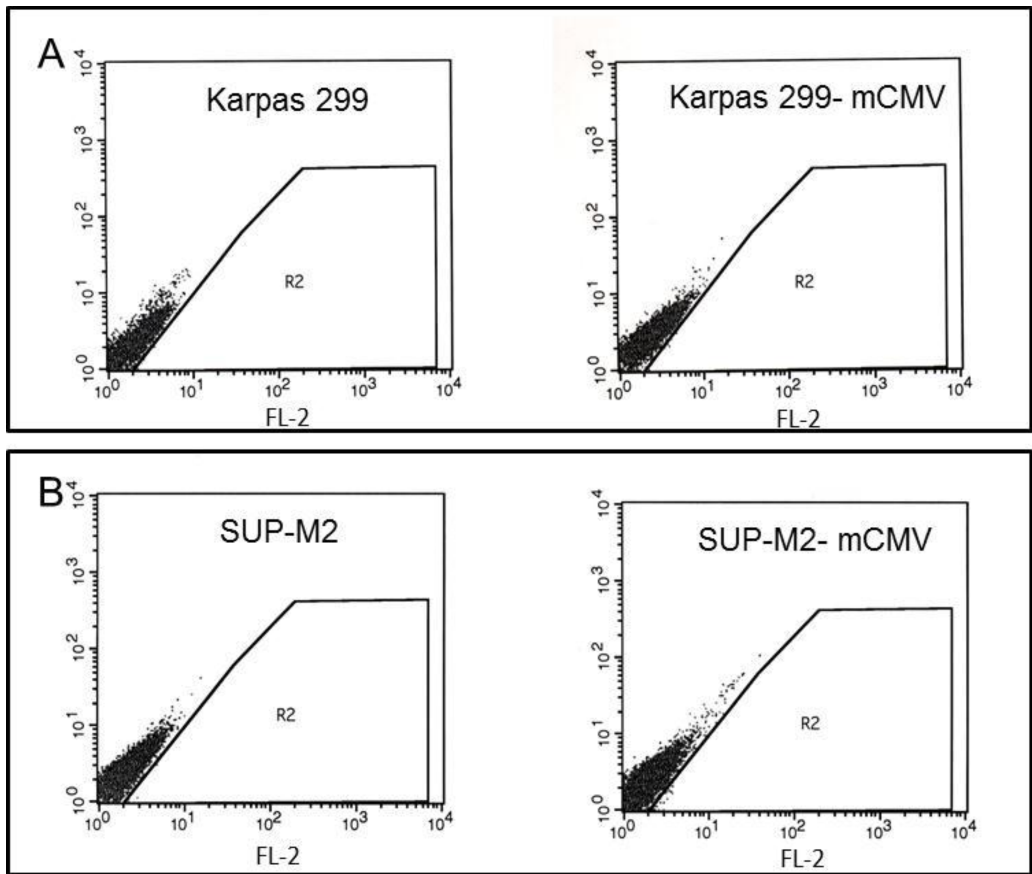


Figure 3.2. The reporter vector containing mCMV without the *Sox2SRR2* segment served as the negative control.

ALK^{+}ALCL cell lines, Karpas 299 and SUP-M2, showed no evidence of GFP signal detectable by flow cytometry. Similarly, cells stably transduced with the negative control lentiviral vector (i.e. containing *mCMV* but not the *Sox2* transcriptional response element) also did not show evidence of GFP expression.

To generate the viral particles required for the experiments, 293T cells were cultured at 37°C, in the presence of 5% CO_2, in 100 mm tissue culture dishes (Corning Life Sciences, Lowell, MA, USA) containing DMEM (Gibco), 10% FBS (Sigma), 2 mM glutamine (Gibco), and 100 units/ml penicillin with 100 g/ml streptomycin (Gibco).

Gene transfection was performed using 10 μg per dish of lentiviral vectors diluted in Opti-MEM (Gibco) and the lipofectamine 2000 reagent (Invitrogen). After 16 hours, 293T cells were placed in the regular culture medium. The viral supernatant was harvested at 48 hours post-transfection, centrifuged at 2000*g* for 5 minutes, and filtered through a 0.45 μm acetate filter (Millipore, Billerica, MA, USA). Two ALK^{+}ALCL cell lines, Karpas 299 and SUP-M2, were infected with the generated viral supernatant in the presence of polybrene (8 μg/ml)(Sigma-Aldrich). At 24 hours post-infection, cells were washed and cultured in the presence of puromycin selection at all times (2 μg/ml). The puromycin dose was determined by following the manufacturer's recommendation. Immediately before each experiment, ALK^{+}ALCL cells were placed in puromycin free culture media.

3.2.7. Flow cytometry and cell sorting

To obtain purified $Sox2^{active}$ and $Sox2^{inactive}$ cell subsets derived from Karpas 299 or SUP-M2 cells, cells stably transfected with the Sox2 reporter were subjected to flow cytometric cell sorting (Aria Cell Sorter, Becton Dickinson Biosciences, Franklin Lakes, NJ, USA). The purity of the resulted $Sox2^{active}$ and $Sox2^{inactive}$ cell subsets derived from Karpas 299 or SUP-M2 cells was >98%.

3.2.8. Reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative RT-PCR

Total cellular RNA was extracted from Karpas 299, SUP-M2, and UCONN-L2, Ntera-2 and Hela cell lines using TRIzol extraction according to

manufacturer's protocol (Gibco). RT was performed using 500 ng total RNA in the first strand cDNA synthesis reaction with superscript reverse transcriptase as recommended by the manufacturer (Invitrogen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as an internal control. PCR was performed by adding 2 μl of RT product into 20 μL volume reaction containing 1X PCR buffer, 200 μM of each dNTPs, 0.5 μM of oligonucleotide primer mix, and 0.4 μl of Phire™ Hot Start II DNA Polymerase (Fisher Scientific, Ottawa, Ontario, Canada). cDNA amplification was performed according to the recommended manufacturer protocol for Phire™ Hot Start II DNA Polymerase using a thermal cycler (Applied Biosystems, Foster City, CA, USA). Amplified products were analyzed by DNA gel electrophoresis in 1.5% agarose and visualized by the Alpha Imager 3400 (Alpha Innotech, San Leandro, CA, USA). For detection of *luciferase* and *GFP* in the stably created Karpas 299 and SUP-M2 clones, genomic DNA was extracted from Sox2^{active} and Sox2^{inactive} cells using QIAmp DNA mini kit (Qiagen) according to the manufacturer protocol. Genomic DNA amplification was performed using *Taq* DNA polymerase (Qiagen) according to the recommended manufacturer protocol, in a thermal cycler (Applied Biosystems). Amplified products were analyzed by DNA gel electrophoresis in 1.5% agarose and visualized by the Alpha Imager 3400 (Alpha Innotech). For real-time PCR experiments, total RNA was extracted as previously described from Karpas 299 cells, (transfected with either scrambled or siRNA against NPM-ALK or STAT3) at 48 hours after transfections, then cDNA was synthesized as previously described. Real-time PCR was performed using SYBR green (Qiagen), and the primer sets used for (Sox2, STAT3, NPM-ALK and GAPDH) (Table 3.1). The PCR conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Samples were run on a *Realplex* apparatus (Fisher). Results were examined using Qiagen software, and the relative expression levels of *Sox2, STAT3* and *NPM-ALK* were calculated by normalizing the cycle

threshold (CT) with those of *GAPDH*. Fold differences between cells treated with STAT3 siRNA (or NPM-ALK siRNA) versus those treated with scrambled siRNA were calculated. All the primer pairs used in this study were designed by Primer3 Input 0.4.0. The primer sequences are illustrated in Table 3.1.

3.2.9. Assessment of Luciferase activity

The luciferase enzymatic activity was assessed using a commercially available kit from Promega (Madison, WI, USA).

3.2.10. Assessment of cell growth and cell cycle analysis

ALK^{+}ALCL cells were transfected with Sox2 specific siRNA or scrambled siRNA, as described above. Cells were then plated at a density of 10,000 or 20,000/mL and cultured for 5 days. Cell count was done after 48 hours by using trypan blue staining (Sigma-Aldrich) and results are expressed as the percentage of the results obtained from the negative controls. Triplicate experiments were performed. For cell-cycle analysis, cells at a concentration of 10^6 cells/ml were prepared. Cells were washed twice with PBS and fixed with 70% cold ethanol for 2 h. These cells were then subjected to RNase treatment and propidium iodide (PI) staining. DNA content was determined using a FACSCalibur flow cytometer (BD Biosciences). Data acquisition was gated to exclude cell doublets and the cell-cycle stage distribution was determined using the CellQuest program (20,000 events were counted).

3.2.11. Cell invasiveness assay

Assessment of cell invasiveness was performed using the CytoSelect™ 96-well cell invasion assay, basement membrane (Cell Biolabs Inc., San Diego, CA, USA), and the procedures were carried out following the manufacturer's suggested protocol. Briefly, $1X10^5$ of purified $Sox2^{active}$ or $Sox2^{inactive}$ cells were plated onto the 96 invasive well plates. After 24

hours, quantification of fluorescence signals was performed using the Fluostar Optima fluorometer (BMG Labtech, Cary, NC, USA) at excitation 480 nm/emission 520 nm. Results are expressed in RFU (relative factor unit), which is a measure of the cell number. Triplicate experiments were performed.

3.2.12. Methylcellulose colony formation assay

Methylcellulose based media was purchased from R&D. Briefly, purified $Sox2^{active}$ or $Sox2^{inactive}$ cells were plated into a 6 well tissue culture plate at 100 or 500 cells per 1 mL of 1.2% methylcellulose, 30% FBS, 1% bovine serum albumin, 10^{-4} M 2-mercaptoethanol and 2 mM L-glutamine. Cells were incubated for 7 days at 37°C in the presence of 5% CO_2. The number of colonies containing >30 cells were counted using an inverted phase contrast microscope. Triplicate experiments were performed.

3.2.13. SCID mouse xenograft studies

CB-17 strain SCID mice were purchased from Taconic (Hudson, NY, USA). These animals were kept under sterile conditions. Briefly, $2x10^7$ purified $Sox2^{active}$ or $Sox2^{inactive}$ cells growing exponentially were injected into the right flank of 4 week-old male mice. These animals were euthanized when a tumor of >10 mm in the greatest dimension became palpable.

3.2.14. Statistical Analysis

Data is expressed as mean +/- standard deviation. Statistical significance is determined using Student's *t*-test. A *p*-value of less than 0.05 was considered to be statistically significant.

Table 3.1. List of primers used in Sox2 study

Primer	Forward (5' TO 3')	Reverse (5' TO 3')
GFP	aggacagcgtgatcttcacc	cttgaagtgcatgtggctgt
Luciferase	gctgggcgttaatcagagag	tcgcggttgttacttgactg
STAT3	cagtcagtgaccaggcagaa	gctgcaactcctccagtttc
NPM-ALK	gttcagggccagtgcatatt	atgatcagggcttccatgag
Sox2	cacaactcggagatcagcaa	gttcatgtgcgcgtaactgt
Genomic DNA control	acccagaagactgtggatgg	ttctagacggcaggtcaggt

3.3. Results

3.3.1. Aberrant expression of Sox2 in ALK$^+$ALCL cell lines

As shown in figure 3.3 all 3 ALK$^+$ALCL cell lines examined expressed the *Sox2* transcript of 294 bp detectable by RT-PCR. Using Western blots and subcellular fractionation, we were able to detect the protein expression of Sox2 at 37 kDa in all 3 cell lines (Figure 3.4A). Ntera-2 (derived from teratocarcinoma) and Hela (derived from cervical carcinoma) cells were used as the positive and negative control, respectively, as previously described (36-37). Normal peripheral blood T-cells had no Sox2 detectable by Western blots (Figure 3.4A). Using subcellular fractionation, we found that Sox2 protein was largely restricted to the nuclear fraction of the cell lysates of ALK$^+$ALCL cells (Figure 3.4B), and this finding correlated with the confocal microscopy results that Sox2 was localized to the nuclei of ALK$^+$ALCL cells (Figure 3.5A and 3.5B). The finding of the nuclear localization of Sox2 is in keeping with the fact that Sox2 is a transcriptional factor. Of note, confocal microscopy showed that the Sox2 protein expression was detectable in virtually all cells in SUP-M2 and Karpas 299 (Figure 3.5A and 3.5B).

3.3.2. Nuclear expression of Sox2 is found in ALK$^+$ALCL tumor patient cells

To show that the aberrant expression of Sox2 is not restricted to the ALK$^+$ALCL cell lines; we assessed Sox2 expression using immunohistochemistry and archival paraffin- embedded patient samples. As illustrated in Figure 3.6, 8 of 10 cases of tumor cells examined had readily detectable nuclear staining. In contrast, benign lymphocytes in reactive tonsils showed no definitive nuclear or cytoplasmic staining. In the remaining two ALK$^+$ALCL tumors in which no definitive nuclear staining was detectable, the levels of the Sox2 cytoplasmic staining were relatively high, which may have obscured any nuclear Sox2 staining, if present. Interestingly, in our survey of 10 cases of T-cell lymphomas that were

ALK-negative, we found strong Sox2 nuclear staining in 2 of 2 transformed mycosis fungoides, whereas all 5 cases classified as peripheral T-cell lymphoma, not further classified, showed no definitive nuclear staining in the cells (Figure 3.7).

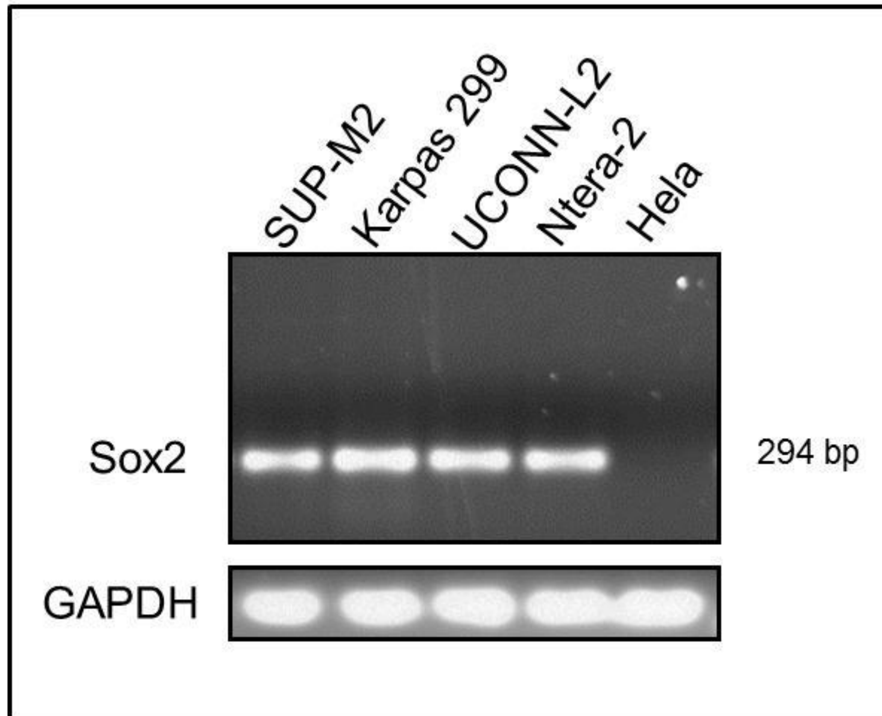


Figure 3.3. Aberrant expression of Sox2 mRNA in ALK^{+}ALCL cell lines.

RT-PCR studies demonstrated the expression of *Sox2* mRNA (at 294 base pairs) in three ALK^{+}ALCL cell lines. Ntera-2 (a teratocarcinoma cell line) and Hela (a cervical cancer cell line) were used as the positive and negative control, respectively.

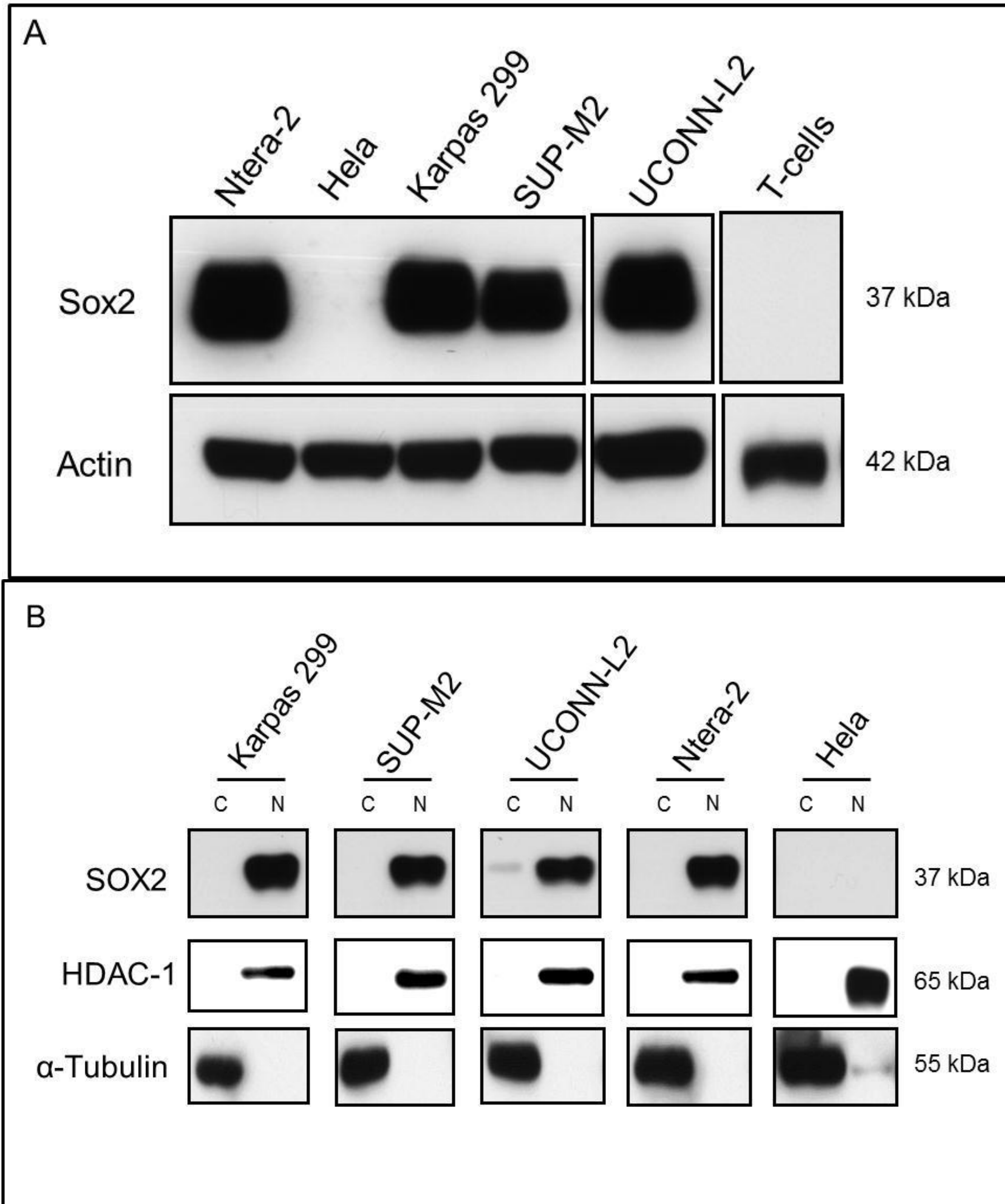


Figure 3.4. Aberrant expression of Sox2 protein in ALK$^+$ALCL cell lines.

A) The expression of Sox2 protein in ALK$^+$ALCL cell lines was detectable by Western blots. Ntera-2 and Hela cells were used as the positive and negative control, respectively. T-cells isolated from the peripheral blood of healthy donors were negative for Sox2 protein. B) Nuclear/cytoplasmic fractionation experiments showed the nuclear localization of Sox2 in ALK$^+$ALCL cells**.** Ntera-2 and Hela cells were used as the positive and negative control, respectively.

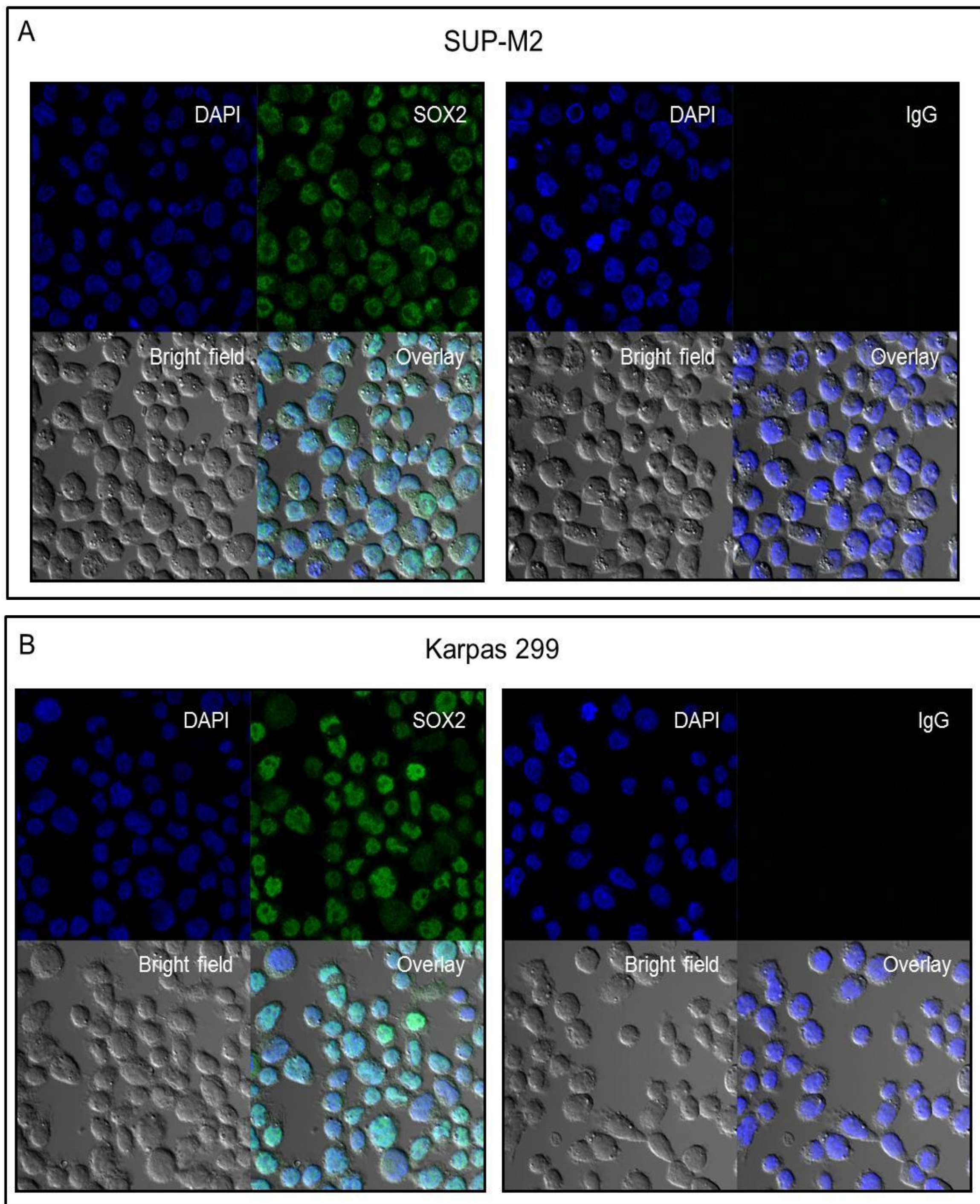


Figure 3.5. Immunofluorescence staining of Sox2 in ALK$^+$ALCL cell lines.

A and B. Confocal immunofluorescence microscopy studies showed that virtually all SUP-M2 and Karpas 299 cells had Sox2 expression, which was localized to the nuclei.

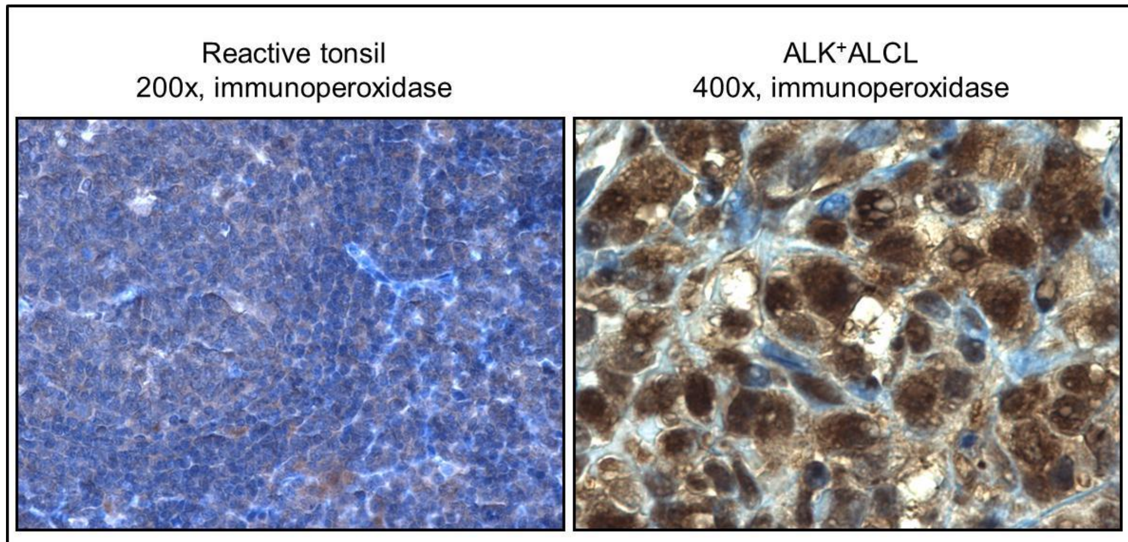


Figure 3.6. Aberrant expression of Sox2 protein in ALK^{+}ALCL tumors.

Immunohistochemical staining of paraffin-embedded tissue sections revealed that, ALK^{+}ALCL tumor cells expressed Sox2, which had a predominantly nuclear pattern. This was in contrast with the lack of definitive Sox2 staining in benign tonsillar lymphocytes.

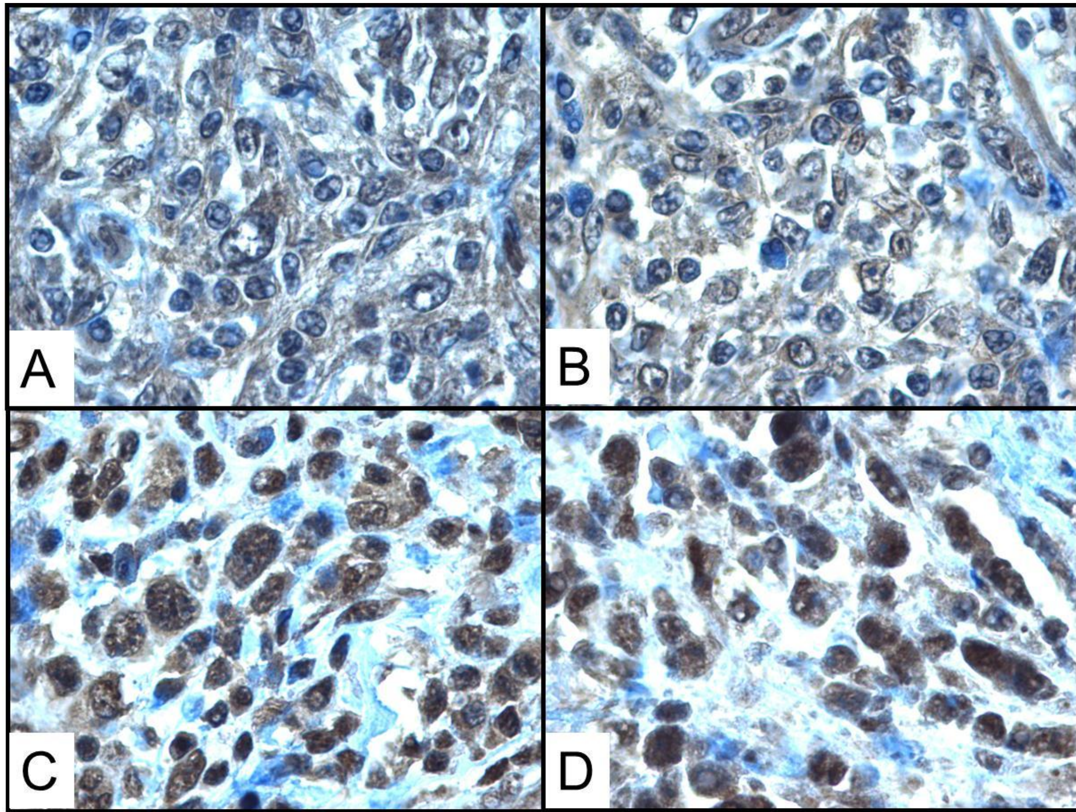


Figure 3.7. Expression of Sox2 in other T-cell lymphomas.

Immunohistochemistry was performed using 10 cases of T-cell malignant lymphomas. All 5 classified as peripheral T-cell lymphoma, not further classified, showed no definitive Sox2 nuclear staining (illustrated in A and B). In contrast, the neoplastic cells from 2 of 2 cases of transformed mycosis fungoides showed strong nuclear reactivities for Sox2. Cells from one case of T-cell lymphoma arising in the post-transplantation setting also showed strong Sox2 nuclear staining (illustrated in C and D).

3.3.3. Aberrant Sox2 expression in ALK$^+$ALCL cells can be attributed to NPM-ALK and STAT3 signaling

As mentioned, the NPM-ALK/STAT3 signaling axis is the central pathogenic factor for ALK$^+$ALCL. Thus, we asked if NPM-ALK and STAT3 are responsible for the aberrant expression of Sox2 in these cells. As illustrated in Figure 3.8A and 3.8B, siRNA-induced down-regulation of NPM-ALK in Karpas 299 and SUP-M2 cells resulted in a dramatic down-regulation of pSTAT3, a known surrogate marker for the NPM-ALK oncogenic activity (38-39). In the same experiment, the protein level of Sox2 was also dramatically decreased. As shown in Figure 3.8C and 3.8D, we found evidence that the expression of Sox2 was also dependent on STAT3, as knock-down of STAT3 using siRNA resulted in a dramatic down-regulation of Sox2. To investigate if the regulation of Sox2 by the NPM-ALK/STAT3 axis occurs at the transcriptional level, we compared the mRNA levels of *Sox2* before and after the knock-down of NPM-ALK or STAT3. We found that downregulation of STAT3 or NPM-ALK expression was followed by a significant decrease in the *Sox2* mRNA level, and the results are shown for Karpas 299 cell line (Figure 3.8E and 3.8F). We also asked the question as to whether the presence of NPM-ALK is sufficient to induce Sox2 expression. Thus, we transfected *NPM-ALK* into two ALK-negative lymphoma cell lines that do not express Sox2, namely Jurkat (a T-cell lymphoblastic lymphoma) and DG75 (a Burkitt's lymphoma cell line). Despite the expression of NPM-ALK and the activation of STAT3, no Sox2 expression was detectable. These results suggest that Sox2 expression in ALK$^+$ALCL cells is cell-type specific (Figure 3.8G and 3.8H).

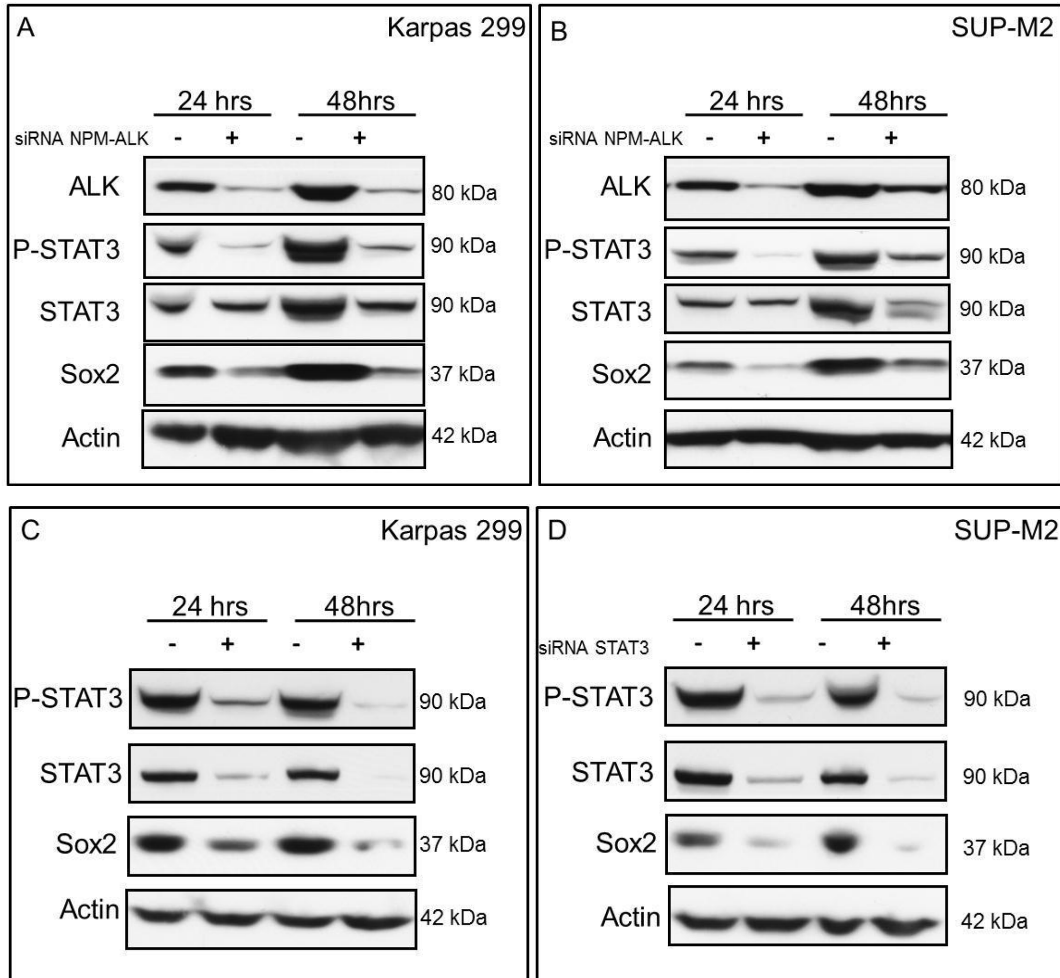


Figure 3.8. Sox2 expression in ALK$^+$ALCL can be attributed to NPM-ALK and STAT3

Down-regulation of NPM-ALK using specific siRNA decreased the Sox2 protein levels in Karpas 299 (A) and SUP-M2 (B) cells. Similar effects were observed with siRNA down-regulation of STAT3 in Karpas 299 (C) and SUP-M2 (D) cells. Results shown are representative of three independent experiments. (continued in the next page)

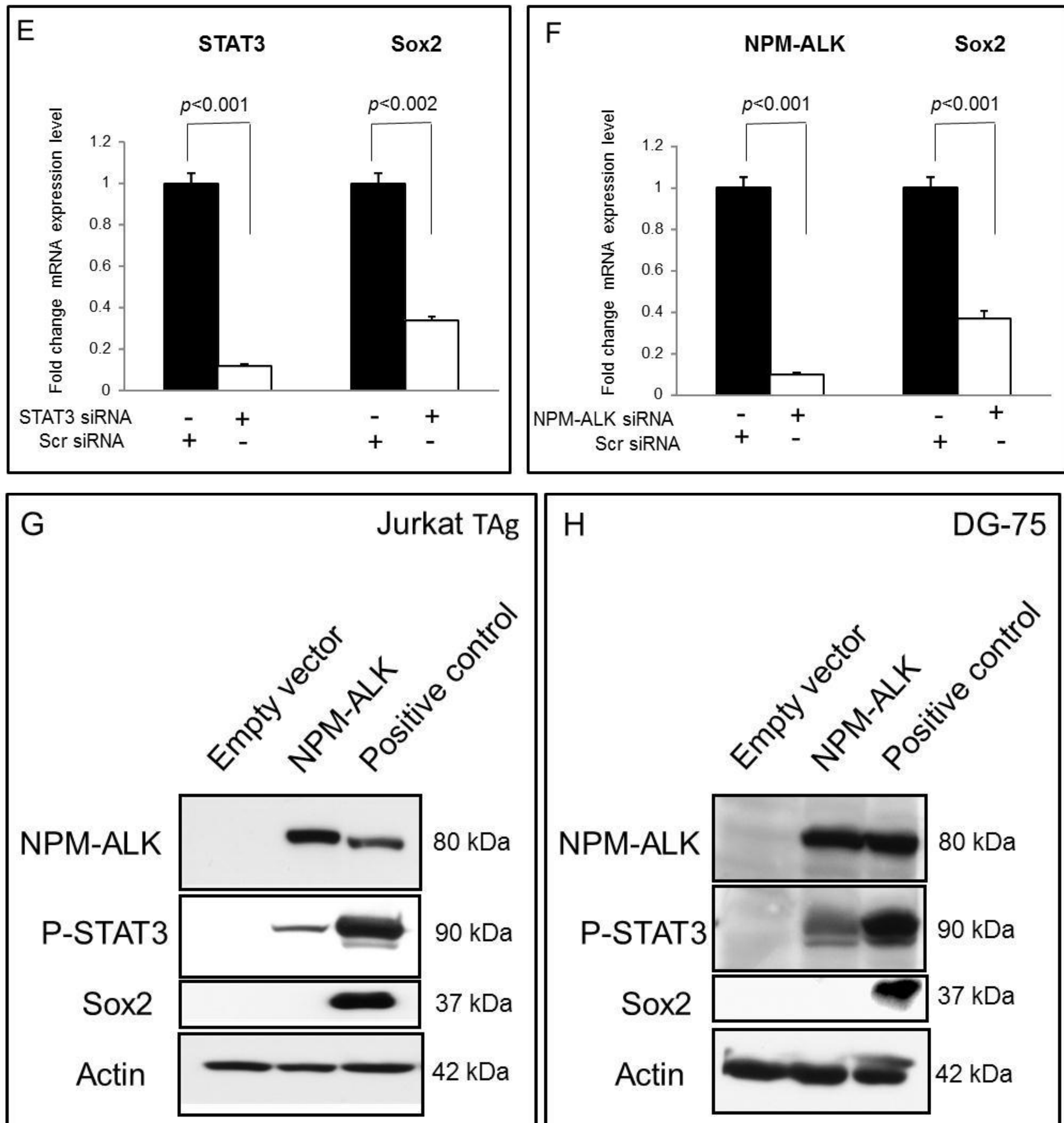


Figure 3.8. Sox2 expression in ALK^{+}ALCL can be attributed to NPM-ALK and STAT3. (continued)

E. and F. The levels of Sox2 mRNA in Karpas 299 cells treated with siRNA to knock-down STAT3 or NPM-ALK were significantly decreased, as compared to cells treated with scrambled siRNA (Scr siRNA). Quantitative RT-PCR using an Eppendorf Realplex apparatus and the Qiagen QuantiTect SYBR Green PCR kit was employed. G. and H. Two lymphoma cell lines, Jurkat TAg and DG75, were transfected with the *NPM-ALK*. While NPM-ALK expression and STAT3 activation occurred, no Sox2 expression was detectable in these cells. Cells transfected with the empty vectors served as the negative controls. Cell lysates from Karpas 299 served as the positive control.

3.3.4. Sox2 is transcriptionally active in subsets of ALK$^+$ALCL cell lines

We then assessed if Sox2 is transcriptionally active in ALK$^+$ALCL cells. Two ALK$^+$ALCL cell lines (Karpas 299 and SUP-M2) were infected with lentiviral vectors containing either the negative control reporter construct (i.e. mCMV) or the Sox2 reporter construct (i.e. contains three tandem Sox2 transcriptional response elements). To facilitate our studies, we generated stable cell clones for Karpas 299 and SUP-M2. Assessment of GFP expression using flow cytometry revealed that approximately 10% of the cells in Karpas 299 stably transduced with the reporter construct were GFP-positive, whereas 30% of the SUP-M2 cells stably transduced with the reporter construct were GFP-positive (Figure 3.9A and 3.9B). The transcriptional activity of Sox2 was further confirmed using the luciferase assay, as the Sox2 reporter construct contains the *luciferase* gene (Figure 3.9C and 3.9D). In support that the expression of GFP is specific for Sox2, we found that GFP expression in purified Sox2^{active} cells decreased significantly (i.e. 7 fold) when Sox2 was down-regulated using siRNA (Figure 3.9E and 3.9F). In long-term culture, the percentage of GFP-positive cells in purified Sox2^{active} cells gradually decreased, whereas no appreciable gain of GFP-positive cells was found in the long-term culture of Sox2^{inactive} cells (Figure 3.10).

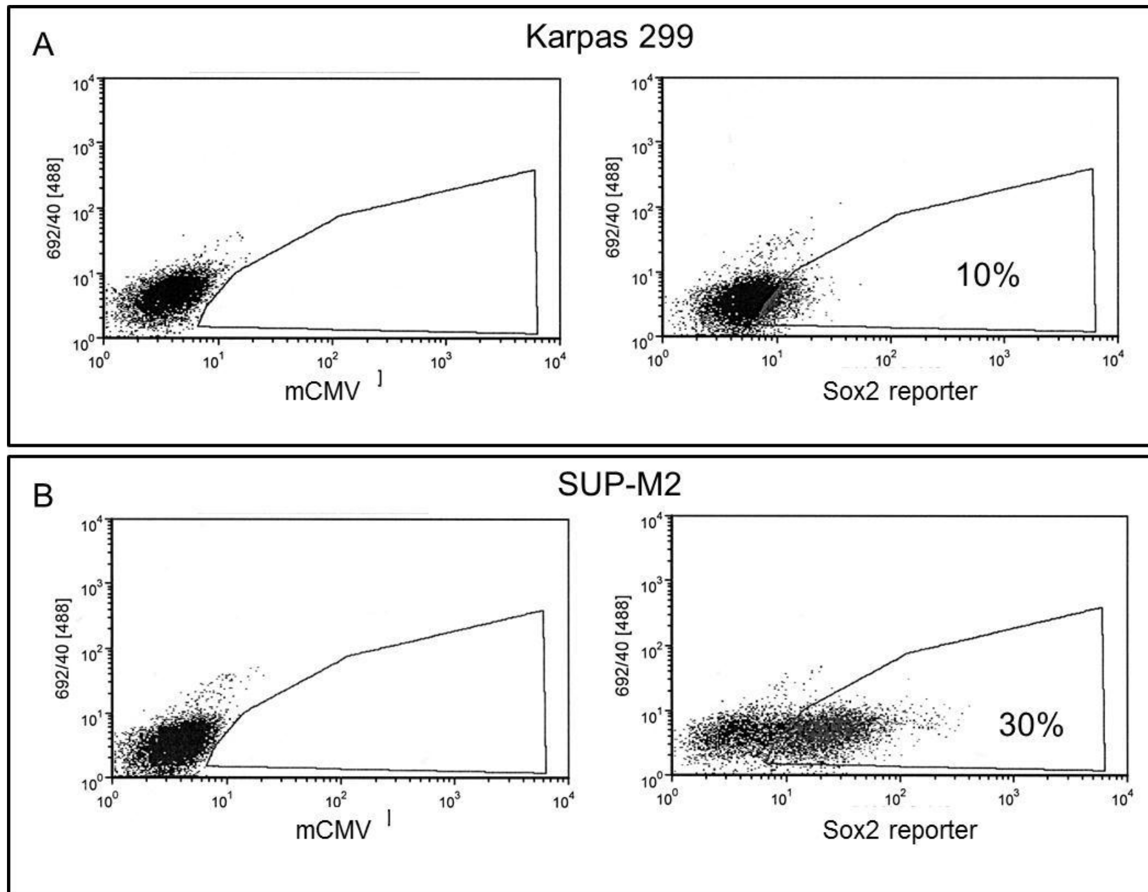


Figure 3.9. Sox2 is transcriptionally active in relatively small subsets of ALK⁺ALCL cell lines.

The percentage of GFP-positive cells in Karpas 299 cells stably transduced with the Sox2 reporter was approximately 10% (A), whereas the percentage of GFP-positive cells in SUP-M2 cells stably transduced with the Sox2 reporter was approximately 30% (B). The results are representative of two different clones for each cell line. Results shown are representative of three independent experiments. (continued in the next page).

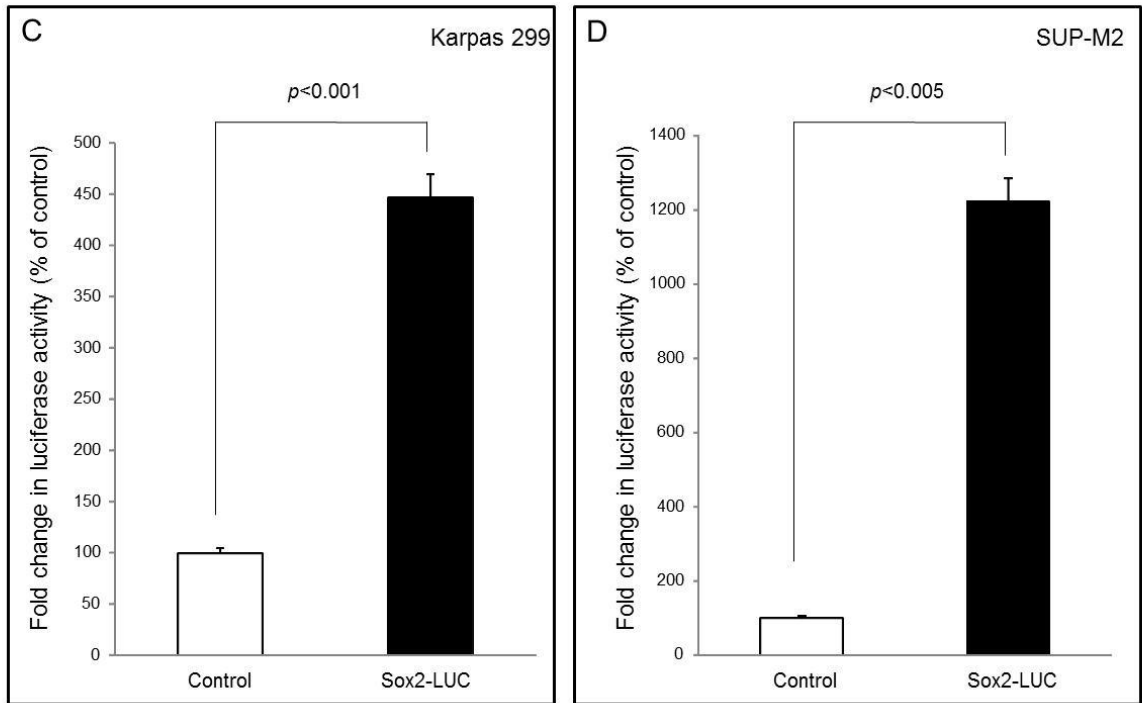


Figure 3.9. (continued) Sox2 is transcriptionally active in relatively small subsets of ALK$^+$ALCL cell lines.

In addition to the expression of GFP, purified Sox2^{active} cell subsets in Karpas 299 (C) and SUP-M2 (D) expressed significantly higher levels of luciferase than cells transduced with the negative control lentiviral vector (i.e. mCMV without the *Sox2* transcriptional response element). (continued in the next page).

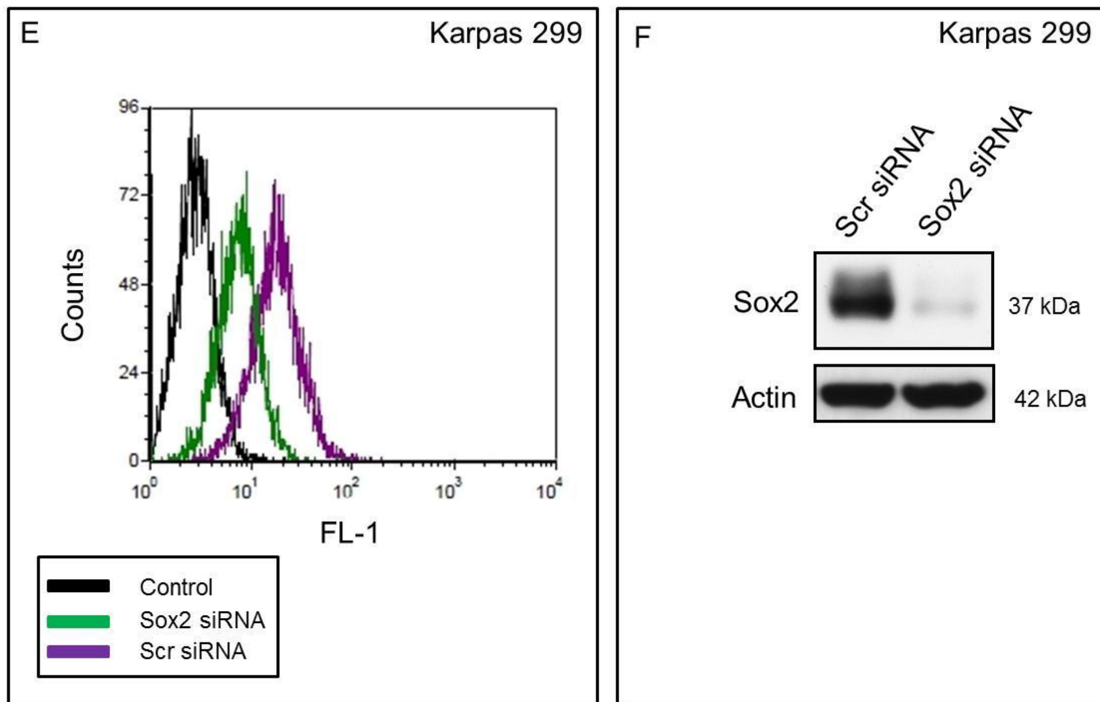


Figure 3.9. (continued) Sox2 is transcriptionally active in relatively small subsets of ALK^{+}ALCL cell lines.

(E) To confirm the validity of Sox2 reporter assay, $Sox2^{active}$ Karpas 299 cells (i.e. GFP-positive) were transfected with siRNA targeting Sox2, and we found a substantial decrease in the level of green fluorescence by 7 fold as assessed by flow cytometry. (F) Sox2 expression was confirmed to be dramatically decreased after treatment with siRNA Sox2 by Western blot. Results shown are representative of three independent experiments.

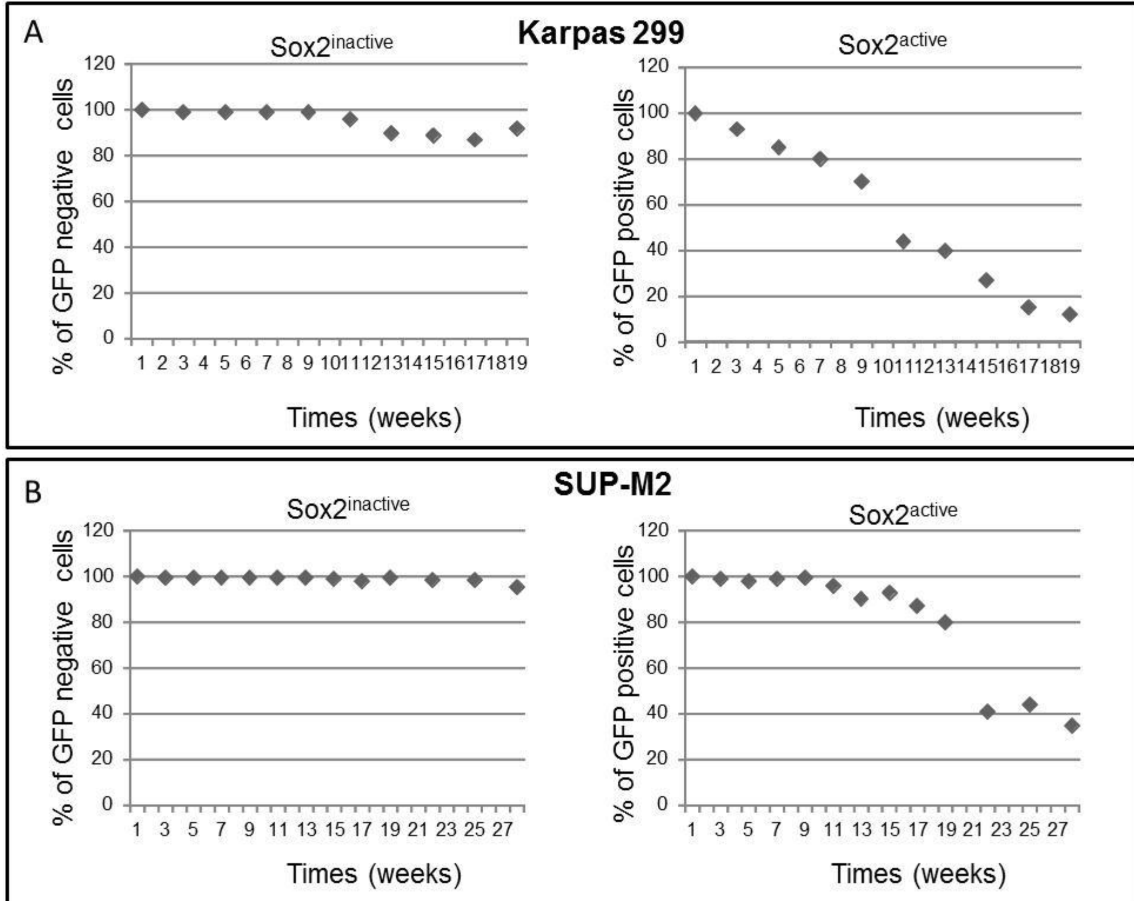


Figure 3.10. Sox2^{active} cells give rise to Sox2^{inactive} cells in long term cell culture.

By cytometry, we found that the proportions of GFP-positive cells in the Sox2^{active} subsets gradually decreased in both Karpas 299 cells (A) and SUP-M2 cells (B) (right panel). In contrast, purified Sox2^{inactive} cells did not show any detectable increase in the proportions of GFP-positive cells (left panel).

3.3.5. Sox2^{active} and Sox2^{inactive} cell subsets are biologically different

To determine the biological significance of the transcriptional activity of Sox2 in ALK^{+}ALCL cells, we performed a number of assays comparing the biological properties of purified Sox2^{active} cells with those of Sox2^{inactive} cells. Prior to these experiments, the purities of the two cell subsets were confirmed to be >98%, as assessed by flow cytometry.

a) Biochemical analysis of the Sox2^{active} and Sox2^{inactive} cells

Our first task was to ensure that the lack of GFP expression in Sox2^{inactive} cells was not due to the absence of the Sox2 reporter in these cells. We considered this scenario to be highly unlikely as these cells grew in the presence of the selection antibiotics. Two additional pieces of evidence support this. First, we re-infected the Sox2^{inactive} cells with the lentiviral vector carrying the *Sox2* reporter construct, and no GFP-expressing cells were detected (Figure 3.11A). Second, by PCR, we were able to detect the presence of the *GFP* and *luciferase* genes, both of which are included in the Sox2 reporter construct (Figure 3.11B and C). We then asked if the lack of the Sox2 activity in Sox2^{inactive} cells was due to a lower protein expression level of Sox2, NPM-ALK, and/or the downstream targets of NPM-ALK. By Western blots, the only consistent difference we identified was p-ERK (Figure 3.12A and 3.12B). However, the increased ERK signaling is not linked to Sox2 in these cells, since down-regulation of Sox2 with siRNA in the Sox2^{active} subset did not result in any appreciable difference in the protein level of p-ERK (Figure 3.12C). Subcellular fractionation experiments were also performed, and we found no difference in the nuclear localization of Sox2 in these two cell subsets (Figure 3.13A-3.13D).

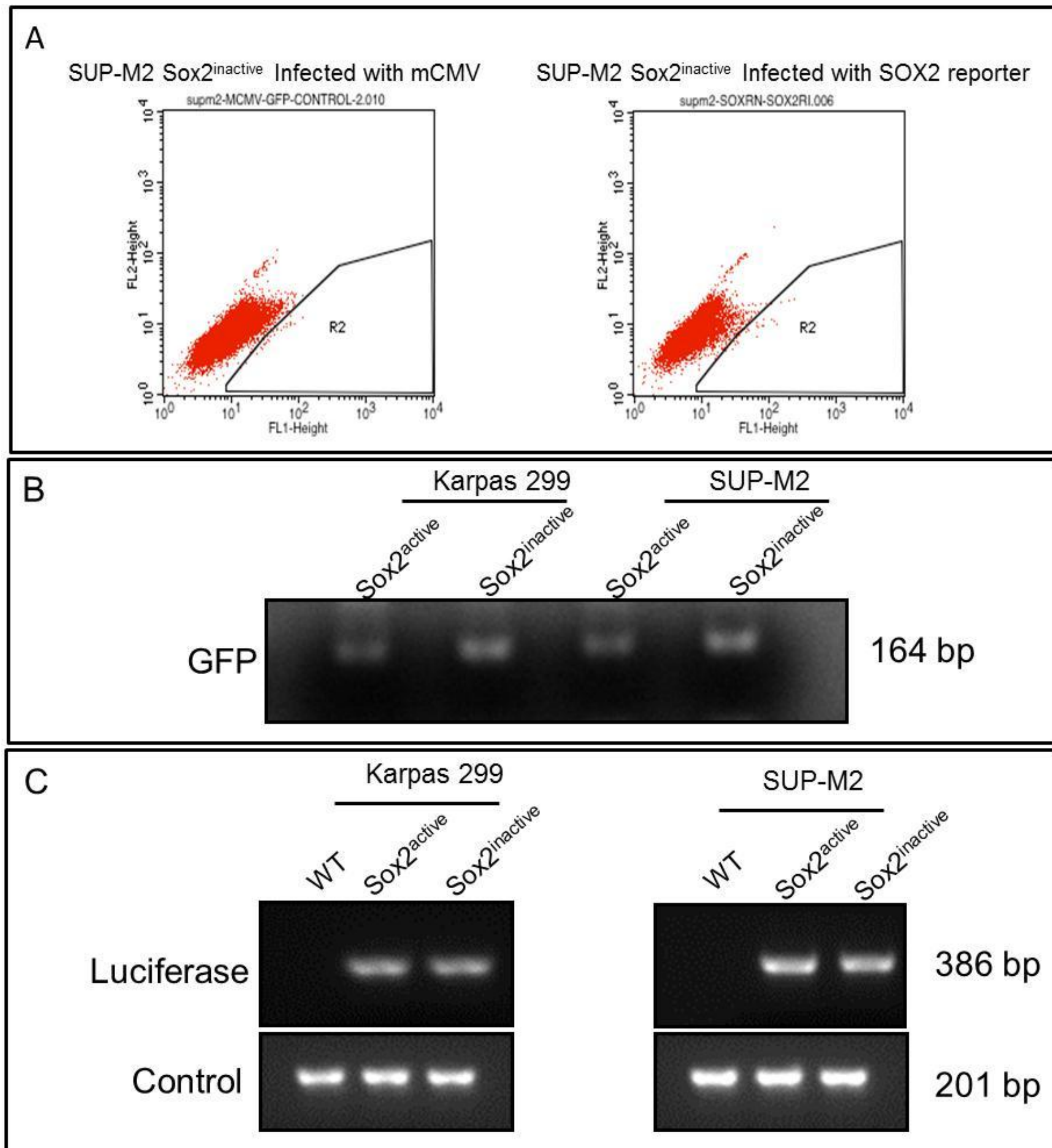


Figure 3.11. The lack of GFP expression in Sox2^{inactive} cells is not due to the absence of the reporter construct.

A) Sox2^{inactive} cells re-infected with Sox2 reporter or the mCMV reporter did not show any change in GFP expression as detected by flow cytometry. B) and C) PCR was performed on genomic DNA extracts from Sox2^{active} and Sox2^{inactive} cells. As demonstrated, both the *GFP* and *luciferase* genes were detectable in both Sox2^{active} and Sox2^{inactive} cell subsets.

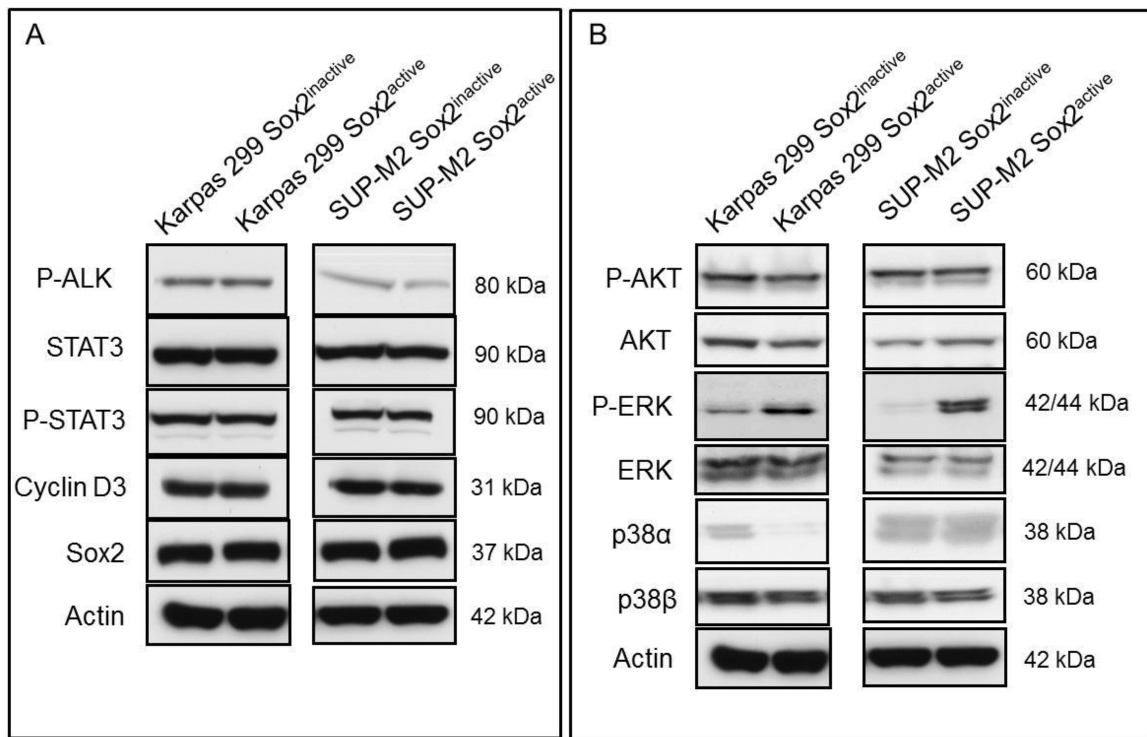


Figure 3.12. Biochemical comparison of Sox2^{active} and Sox2^{inactive} cells.

By Western blots, there was no substantial difference in the total protein levels and the activation/phosphorylation levels of NPM-ALK, STAT3, Akt and p38 (A and B). However, a slightly higher level of phospho-ERK was found in the Sox2^{active} cell subset (continued in the next page).

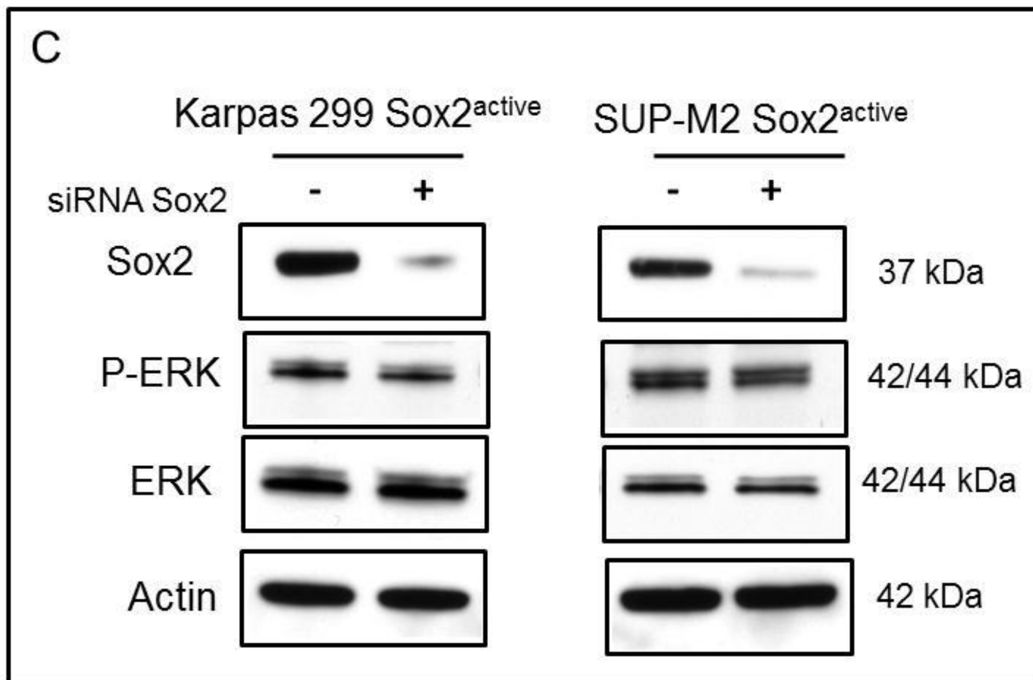


Figure 3.12. Biochemical comparison of Sox2^{active} and Sox2^{inactive} cells (continued).

C) Western blots revealed that Knockdown of Sox2 in Sox2^{active} Karpas 299 and SUP-M2 cells did not result in any appreciable difference in the protein level of phospho-ERK.

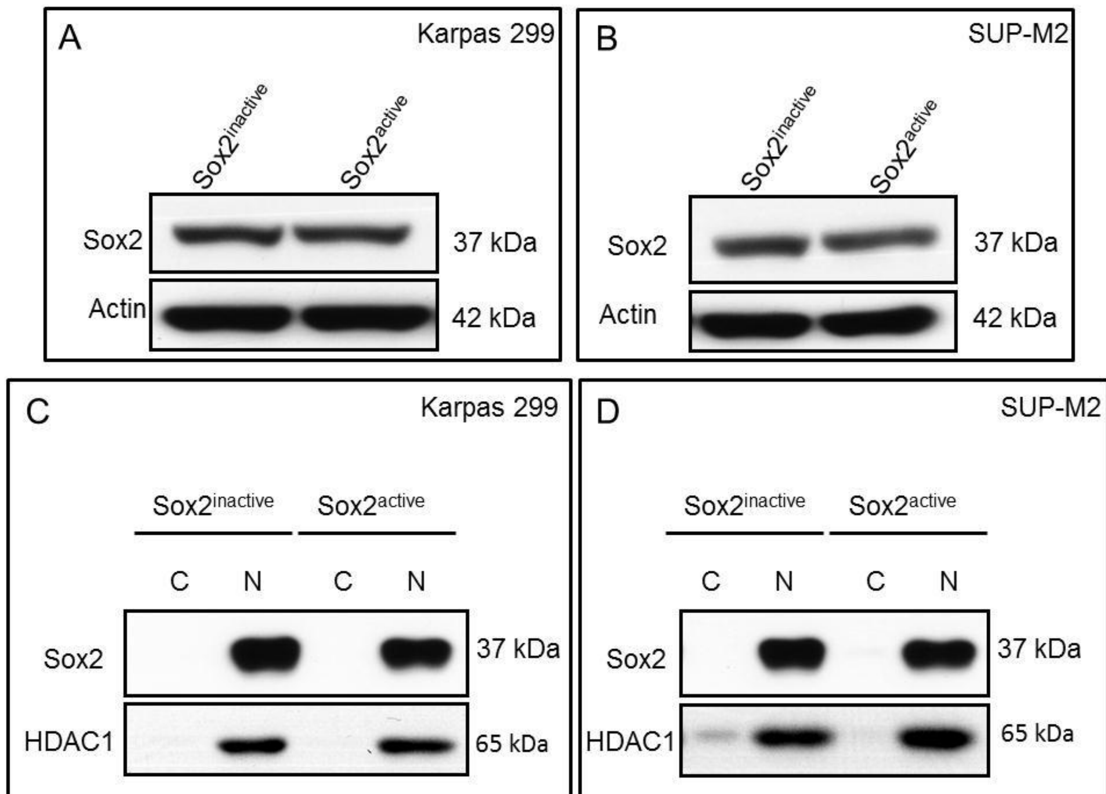


Figure 3.13. Sox2 transcriptional activity is not due to a difference in Sox2 protein level, or nuclear localization.

As shown in A and B, by Western blots, the expression levels of Sox2 protein were similar between the two cells subsets in both cell lines. As shown in C and D, there was no appreciable difference in the subcellular localization of Sox2 in both cell subsets.

To assess if the Sox2 transcriptional activity in ALK^{+}ALCL cells is associated with any substantial differences in the expression of potential Sox2 targets, we performed quantitative PCR and Western blots and found that Notch1 and PDGFR-alpha were expressed at higher levels in $Sox2^{active}$ cells, whereas BCL2 was expressed at a higher level in $Sox2^{inactive}$ cells; these findings were confirmed by Western blots (Figure 3.14). Lastly, we assessed if Sox2 regulates the NPM-ALK/STAT3 signaling axis. Down-regulation of Sox2 in $Sox2^{active}$ cells using siRNA did not result in any appreciable change to the levels of ALK, p-ALK, STAT3 and pSTAT3 (Figure 3.15).

b) Cell growth

To investigate if the Sox2 transcription activity promotes cell growth in ALK^{+}ALCL, we blocked Sox2 expression in Karpas 299 and SUP-M2 using siRNA in the $Sox2^{active}$ and $Sox2^{inactive}$ cell subsets. Down-regulation of Sox2 expression in the $Sox2^{active}$ subset resulted in a significant decrease in cell growth in both Karpas 299 and SUP-M2, as assessed by the trypan blue cell count assay (Figure 3.16A and 3.16C). However, no significant difference was observed for the $Sox2^{inactive}$ subset (Figure 3.16B and 3.16D).

c) $Sox2^{active}$ cells are more resistant to doxorubicin-induced apoptosis

To further assess the biological significance of Sox2; we tested if Sox2 expression contributes to the resistance to chemotherapeutic agents. We subjected the $Sox2^{active}$ and $Sox2^{inactive}$ subsets to varying concentrations of doxorubicin. We found that $Sox2^{active}$ cells were significantly more resistant to doxorubicin than $Sox2^{inactive}$ cells in two ALK^{+}ALCL cell lines (Figure 3.17A and B).

d) Sox2active cells are more sensitive to the ALK inhibitor Crizotinib

To further investigate if Sox2 transcription activity confers any specific response to the ALK inhibitor Crizotinib, we subjected Sox2^{active} and Sox2^{inactive} cells (derived from Karpas 299 and SUP-M2) to varying concentrations of this specific ALK inhibitor. In contrast to the response to doxorubicin, Sox2^{inactive} cells were significantly more resistant than Sox2^{active} cells (Figure 3.18A and B).

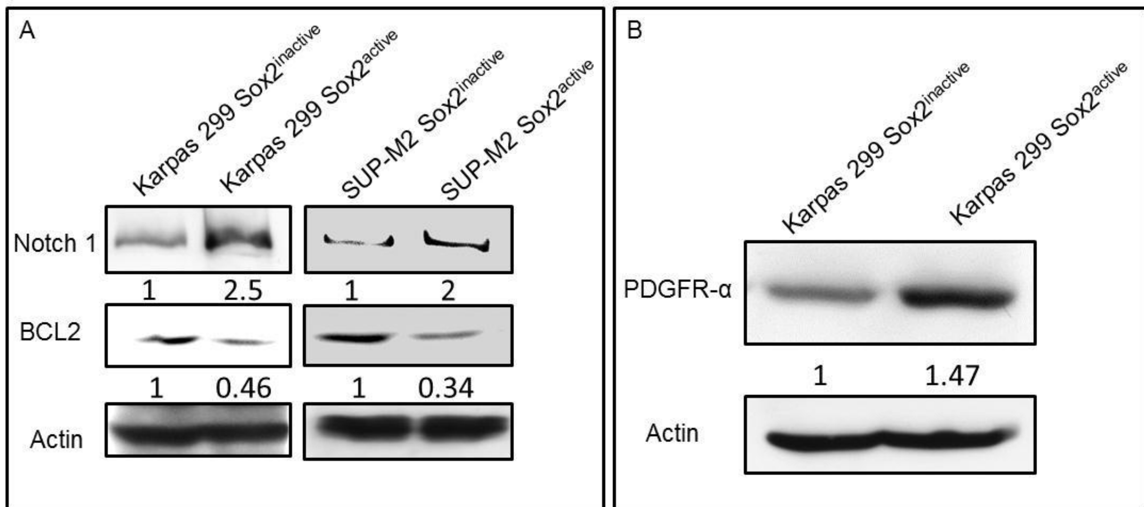


Figure 3.14. Sox2 transcriptional activity in ALK$^+$ALCL cells is associated with substantial differences in the expression of potential Sox2 targets

Western blots in both Karpas 299 and SUP-M2 revealed that the expression of notch 1 and PDGFR-alpha were higher in Sox2^{active} cells, whereas BCL2 was higher in Sox2^{inactive} cells. Of note, PDGFR-alpha was not expressed in SUP-M2 and a comparison between the two cell subsets in this cell line could not be made.

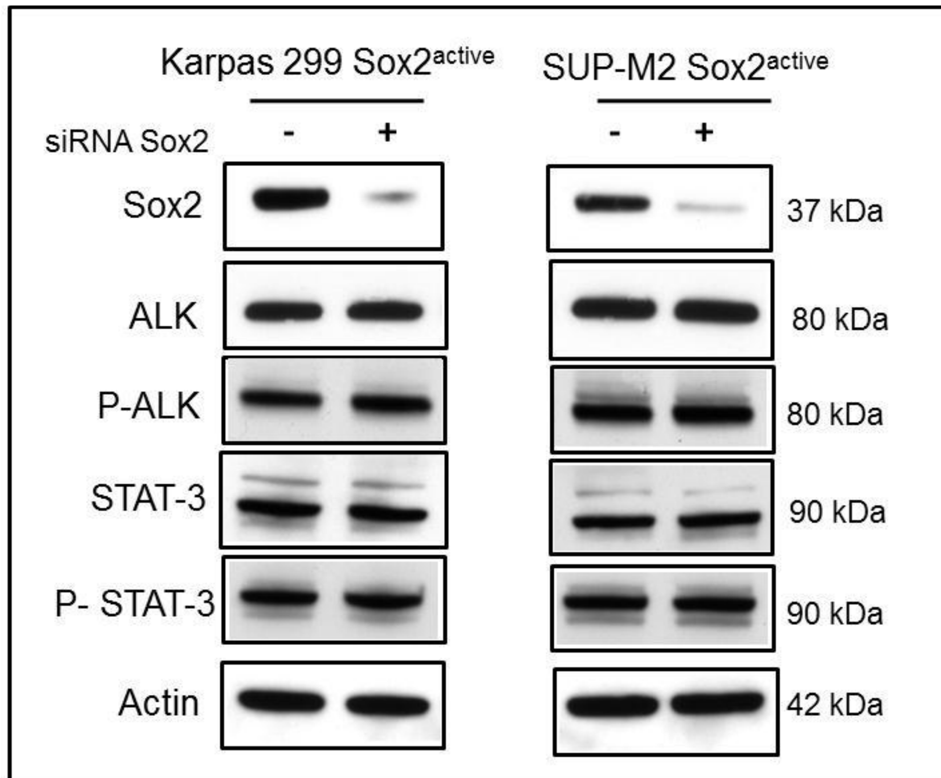


Figure 3.15. Sox2 transcriptional activity does not regulate NPM-ALK/STAT3 signaling axis in ALK^{+}ALCL cells

Western blots revealed that Knockdown of Sox2 in Sox2^{active} Karpas 299 and SUP-M2 cells did not result in any appreciable difference in the protein levels of ALK, p-ALK, STAT3 and pSTAT3.

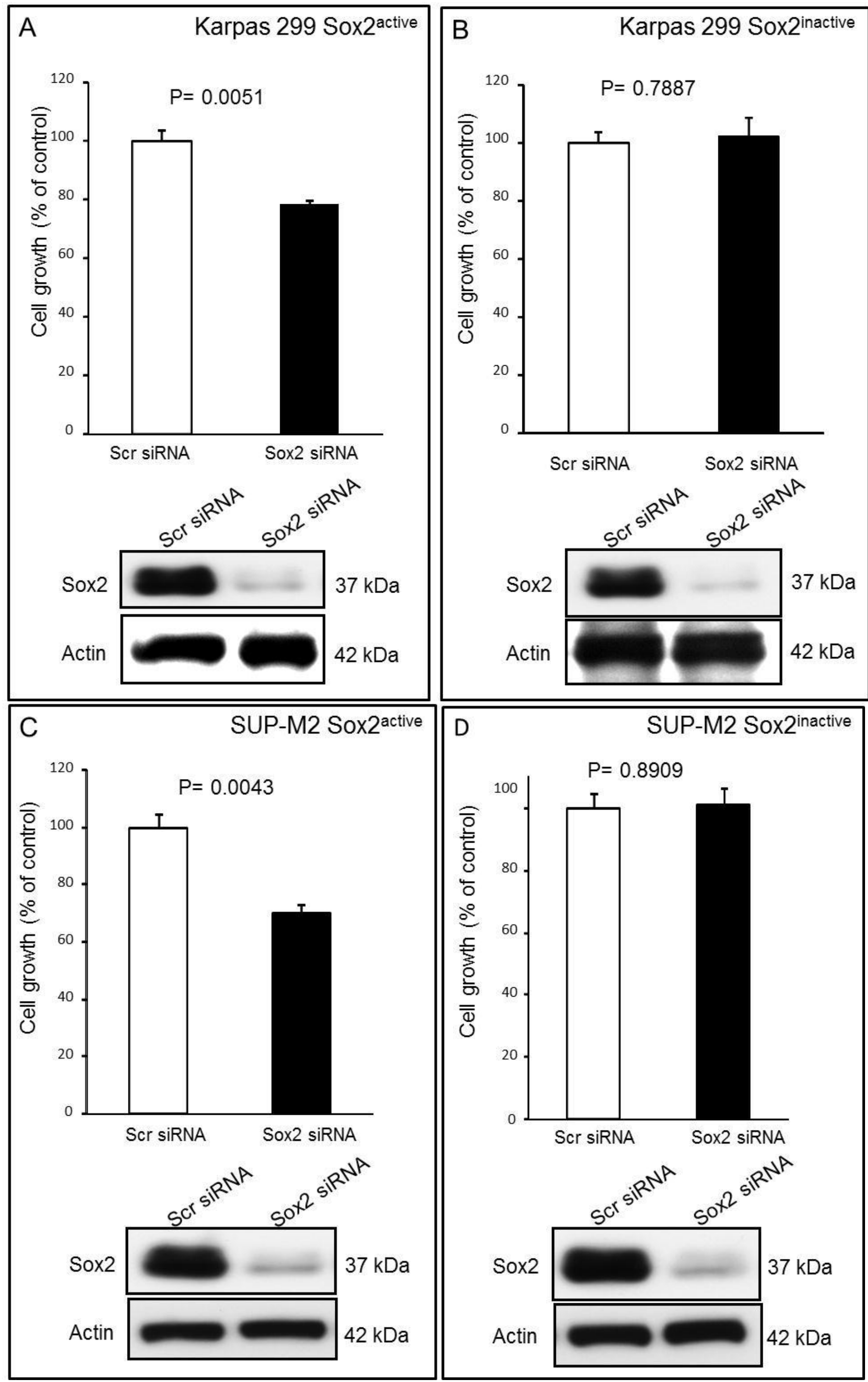


Figure 3.16. Sox2 transcription activity promotes cell growth in ALK$^+$ALCL

Down-regulation of Sox2 expression using siRNA in the Sox2^{active} cell subset resulted in a significant decrease in cell growth in both Karpas 299 (A) and SUP-M2 (C). Down-regulation of Sox2 expression in the Sox2^{inactive} cell subset resulted in no significant change in cell growth in both Karpas 299 (B) and SUP-M2 (D). Results shown are representative of three independent experiments.

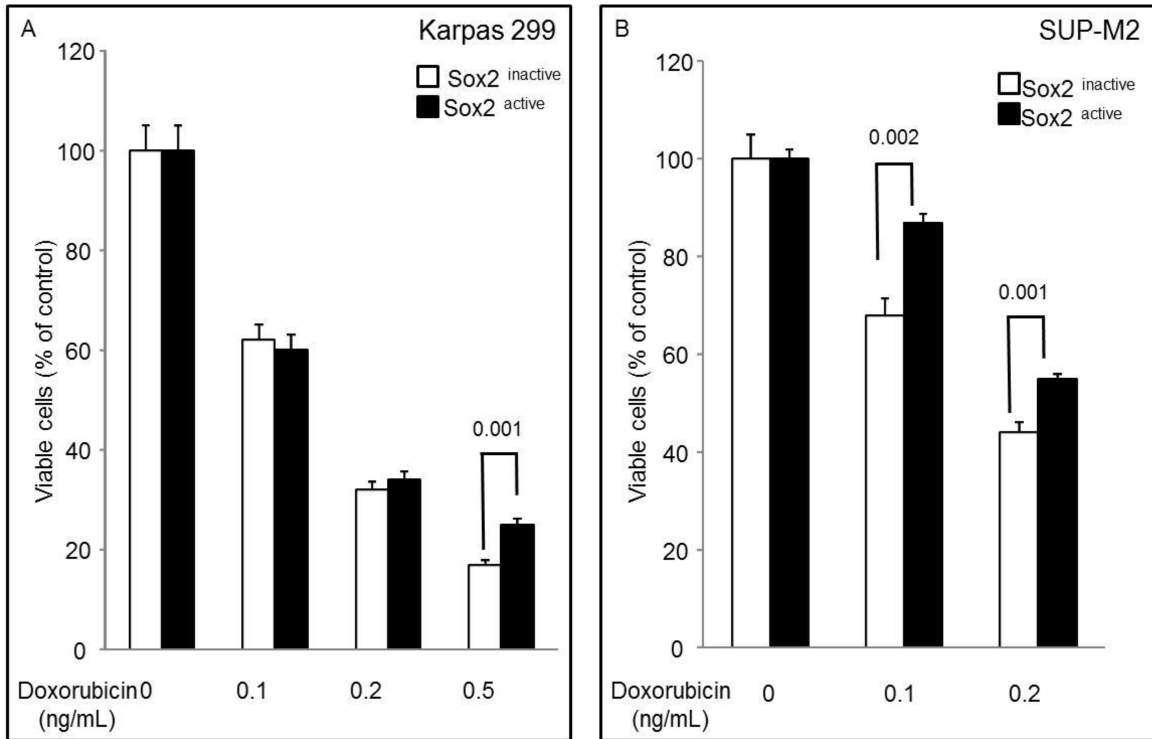


Figure 3.17. Sox2^{active} cells are more resistant to doxorubicin-induced apoptosis.

Sox2^{active} subset of cells in both Karpas 299 (A) and SUP-M2 (B) were more resistant to doxorubicin as compared to the Sox2^{inactive} subset. Results shown are representative of three independent experiments.

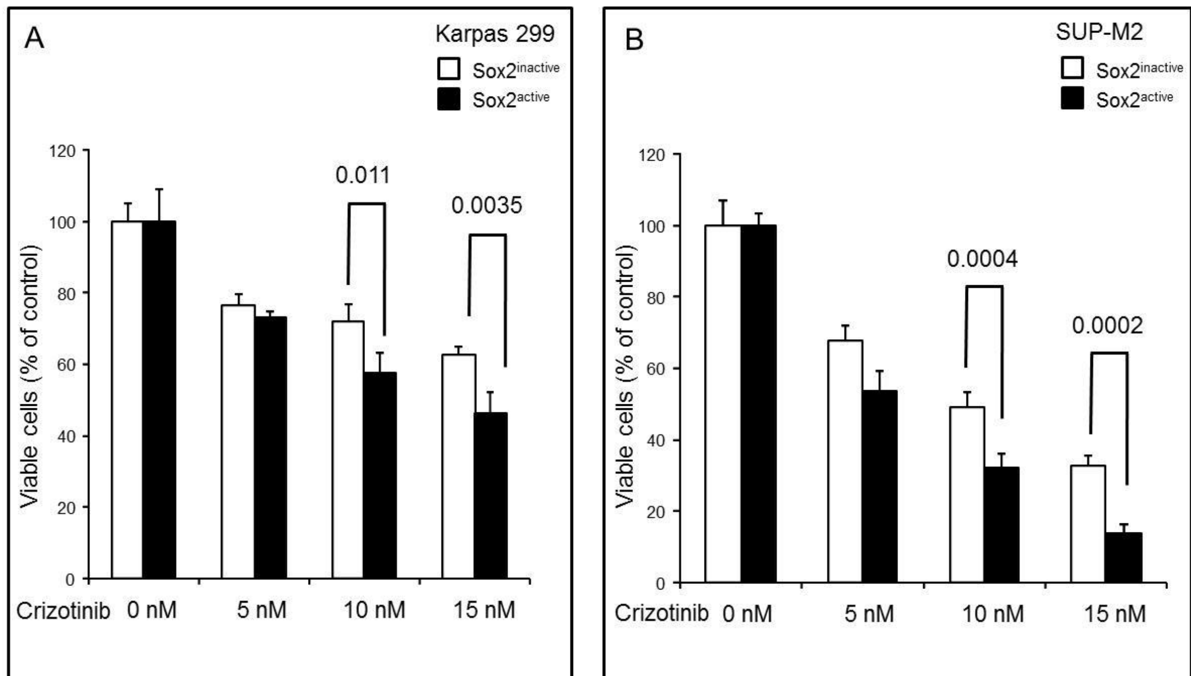


Figure 3.18. Sox2^{active} cells are more sensitive to the ALK inhibitor Crizotinib

Sox2^{active} subset of cells in both Karpas 299 (A) and SUP-M2 (B) were more sensitive to the specific ALK inhibitor Crizotinib as compared to the Sox2^{inactive} subset. Results shown are representative of three independent experiments.

e) The Sox2 transcriptional activity is associated with increased invasiveness of ALK^{+}ALCL cells

We then assessed if the transcriptional activity of Sox2 in ALK^{+}ALCL cells contributes to their invasiveness. As illustrated in Figure 3.19A and 3.19C, down-regulation of Sox2 expression using siRNA in the $Sox2^{active}$ cell subset led to a significant decrease in cell invasiveness in both Karpas 299 and SUP-M2 cells. However, no significant difference was observed when the same experiment was repeated using $Sox2^{inactive}$ cells (Figure 3.19B and 3.19D).

f) The Sox2 transcriptional activity increases tumorigenecity in ALK^{+}ALCL cells

Using methylcellulose colony formation assay, we tested if $Sox2^{active}$ cells differ from $Sox2^{inactive}$ cells in promoting tumorigenecity. We found a significant difference in the number of colonies between $Sox2^{active}$ and $Sox2^{inactive}$ cells for both Karpas 299 and SUP-M2 (Figure 3.20A and 3.20B). Down-regulation of Sox2 using siRNA in the $Sox2^{active}$ subset led to a significant decrease in the number of colonies in both Karpas 299 and SUP-M2 cells (Figure 3.20C and 3.20E). However, no significant difference was observed when the same experiment was repeated using $Sox2^{inactive}$ cells (figure 3.20D and 3.20F).

g) $Sox2^{active}$ cells are more tumorigenic than $Sox2^{inactive}$ cells in the SCID mouse xenograft model

$Sox2^{active}$ or $Sox2^{inactive}$ cells derived from SUP-M2 were injected intra-peritoneal in SCID mice of 4 weeks of age. Mice were euthanized when a sizable (estimated to be at least 1 cm in greatest dimension) tumor became palpable. Of the 3 SCID mice xenografted with $Sox2^{active}$ cells, all 3 developed palpable tumors on day 28. The necropsy findings were similar among the three animals; there was a large subcutaneous tumor in the abdominal region, with an average tumor size of 3.0 cm in the greatest

dimension. The gross appearance of the tumor is illustrated in Figure 3.21A. The spleen was enlarged in all 3 animals xenografted with $Sox2^{active}$ cells and confirmed to be involved by ALK^{+}ALCL microscopically. Of the 3 SCID mice xenografted with $Sox2^{inactive}$ cells, 2 developed palpable tumors on day 35. On necropsy, both of these two animals were found to have a subcutaneous tumor in the abdominal region measuring 1.0 cm in the greatest dimension. The remaining animal xenografted with $Sox2^{inactive}$ cells was found on necropsy (day 35) to have a minute tumor in the abdominal subcutaneous region measuring up to 0.2 cm in the greatest dimension. Overall, the difference in the tumor size between the two groups is statistically significant ($p=0.014$, Student *t*-test) (Figure 3.21B). Histologically, tumors cells from all xenografts appeared to be similar (illustrated in Figure 3.21C and 3.21D). Fresh tumor cells from each tumor were harvested and subjected to flow cytometry to determine the proportions of $Sox2^{active}$ and $Sox2^{inactive}$ cells. As shown in Figure 3.21E, 30% of $Sox2^{inactive}$ (i.e. GFP-negative) cells were found in the xenografts derived from isolated $Sox2^{active}$ cells. No $Sox2^{active}$ (i.e. GFP-positive) cells were found in the xenografts derived from isolated $Sox2^{inactive}$ cells (Figure 3.21F).

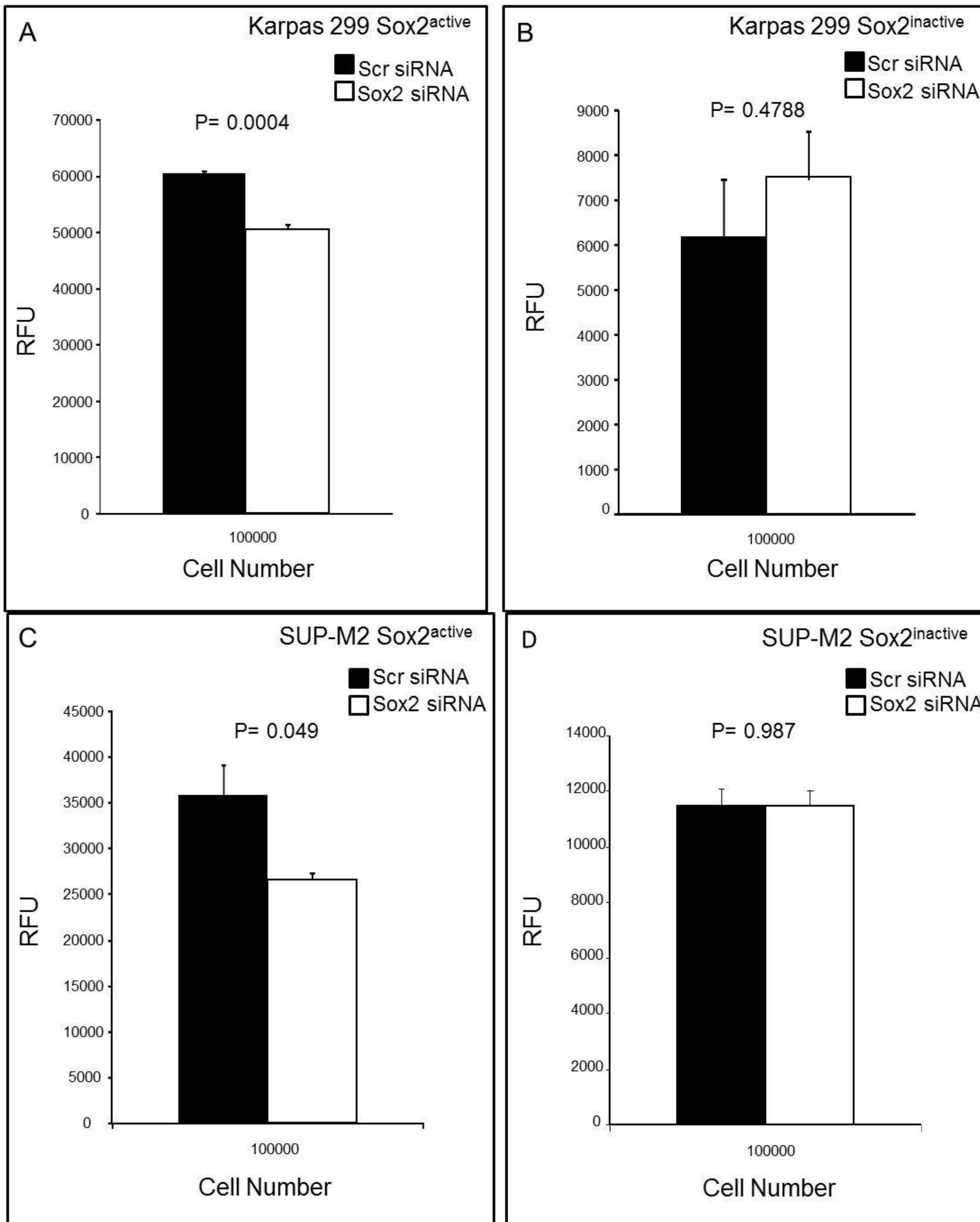


Figure 3.19. Sox2 transcriptional activity correlates with increased invasiveness of ALK^{+}ALCL cell lines.

Down-regulation of Sox2 expression in the Sox2^{active} subset of Karpas 299 and SUP-M2 cells led to a significant decrease in cell invasiveness (A and C). However, down-regulation of Sox2 expression in the Sox2^{inactive} subset resulted in no significant change (B and D). Results are expressed in RFU (relative factor unit), which is a measure of cells that have invaded the membrane.

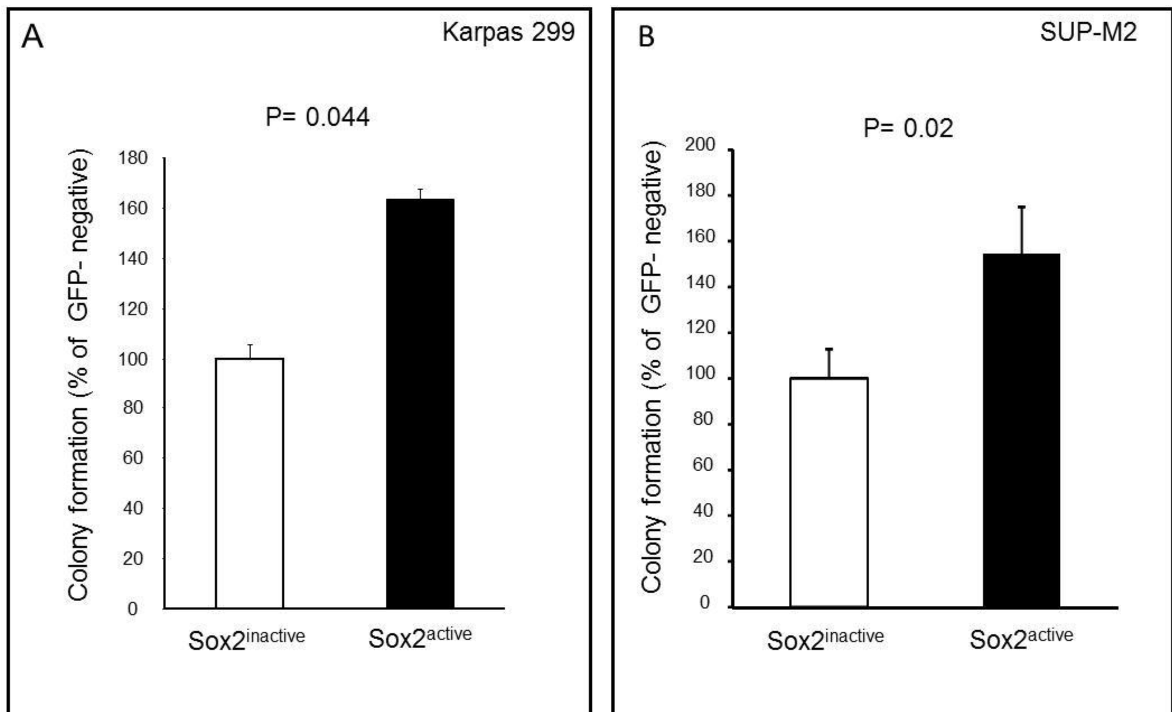


Figure 3.20. Sox2 transcriptional activity correlates with increased tumorigenicity of ALK^{+}ALCL cell lines.

The Sox2^{active} subsets of both Karpas 299 (A) and SUP-M2 (B) showed a significantly higher number of colonies in methylcellulose colony formation assay, as compared to the Sox2^{inactive} subsets. Data is presented as percentages of the control (i.e. Sox2^{inactive} cells). Results shown are representative of three independent experiments. (continued in the next page)

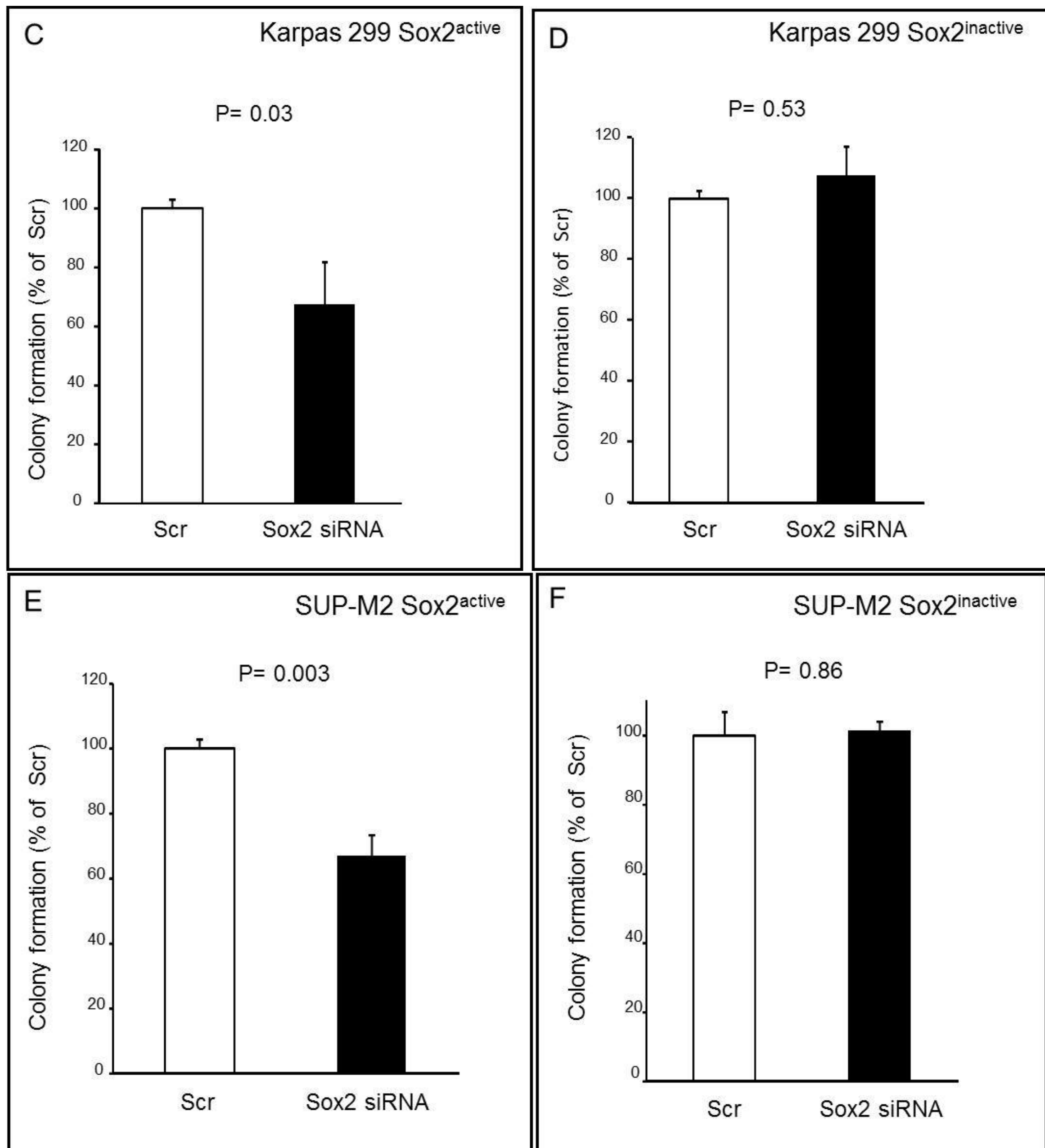
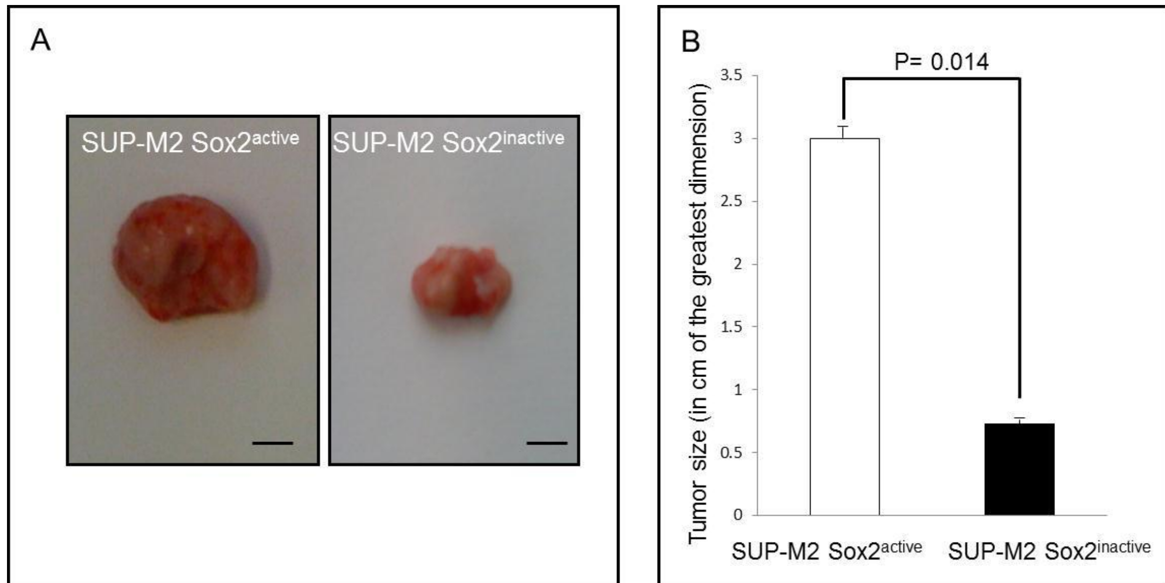


Figure 3.20. Sox2 transcriptional activity correlates with increased tumorigenicity of ALK^{+}ALCL cell lines (continued)

Down-regulation of Sox2 expression in the $Sox2^{active}$ subset of both Karpas 299 (C) and SUP-M2 (E) led to a significant decrease in colony formation. However, down-regulation of Sox2 expression in the $Sox2^{inactive}$ subset resulted in no significant change in both Karpas 299 (D) and SUP-M2 (F). Results shown are representative of three independent experiments.



C. Subcutaneous tumor, 100X, H&E

D. Splenic tumor, 100X, H&E

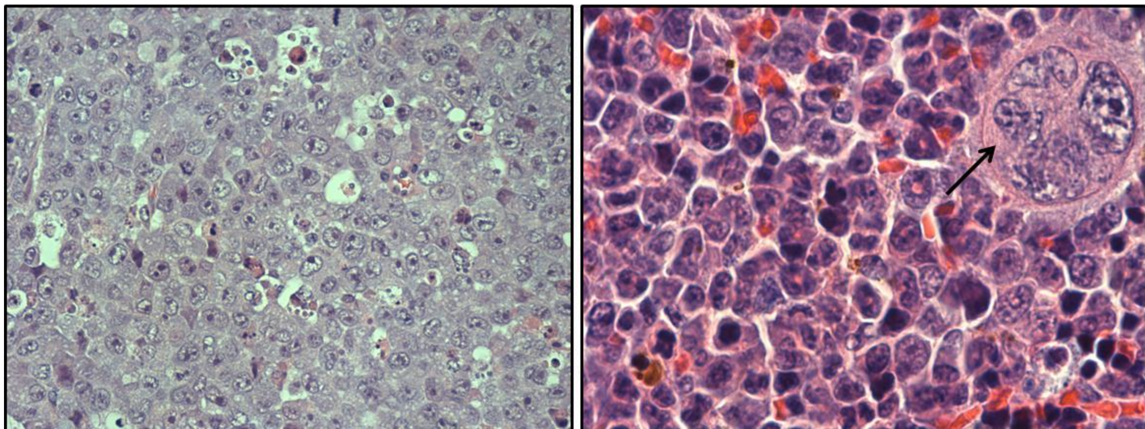


Figure 3.21. Sox2 transcriptional activity correlates with tumorogenicity in the SCID-xenograft mouse model.

Xenografts derived from the Sox2^{active} subset of SUP-M2 cells were significantly larger than those derived from the Sox2^{inactive} subset (A and B). A H&E section of the subcutaneous tumor harvested from an animal xenografted with Sox2^{active} cells reveals sheets of anaplastic lymphoma cells (C). A high magnification of the splenic tumor from the same animal is illustrated; a megakaryocyte is highlighted by a black arrow (D). (continued in the next page)

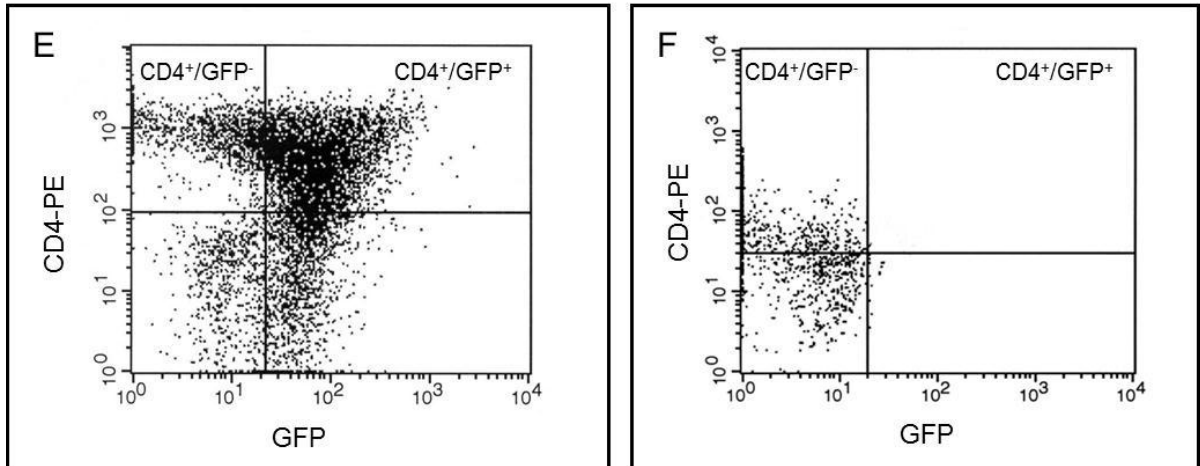


Figure 3.21. Sox2 transcriptional activity correlates with tumorogenicity in the SCID-xenograft mouse model. (continued)

By flow cytometry, xenografts derived from $Sox2^{active}$ cells contained a substantial proportion of $Sox2^{inactive}$ cells (E). In contrast, $Sox2^{active}$ cells were not detectable in the xenografts derived from $Sox2^{inactive}$ cells (F). Lymphoma cells were gated by virtue of their CD4 expression. The experiments have been performed on three mice for each group of the $Sox2^{active}$ and $Sox2^{inactive}$ xenografts.

3.4. Discussion

In this study, we have shown that Sox2 protein expression is a consistent feature in ALK^{+}ALCL, being detectable in all 3 cell lines and 10 of 10 tumors. To our knowledge, ALK^{+}ALCL represents the first example of a hematologic malignancy that manifests this feature. In contrast to ALK^{+}ALCL cells, we did not find Sox2 expression in benign lymphocytes, including those present in peripheral blood and benign tonsils. Correlating with our findings, a recent study performed by Cimpean *et al*. also found no evidence of Sox2 expression in normal lymphocytes (40). Nevertheless, it has been reported that Sox2 is expressed in CD34+ hematopoietic stem cells (41-42). Taken together, the expression of Sox2 in ALK^{+}ALCL cells represents an aberrant event. Of note, our immunohistochemical studies suggest that Sox2 expression is not limited to ALK^{+}ALCL in the spectrum of hematologic malignancies, as cases of transformed mycosis fungoides and one case of T-cell lymphoma arising in the setting of post-transplantation manifested this phenotype. Further studies are required to confirm whether Sox2 in these T-cell neoplasms without ALK expression is indeed transcriptionally active.

The link between Sox2 and STAT3 has been previously suggested in a study of neural precursor cells (30). Sox2 expression was shown to be up-regulated by STAT3, and the promoter region of the Sox2 gene was found to carry multiple STAT3 binding consensus sequences (30). Since one of the key characteristics of ALK^{+}ALCL cells is the high level of constitutive STAT3 activation (23), we hypothesized that the aberrant expression of Sox2 in this cell type can be attributed to STAT3. Our data supports this concept. As the oncogenic tyrosine kinase NPM-ALK is known to be the major activator of STAT3 in ALK^{+}ALCL, it is not surprising to observe that siRNA down-regulation of NPM-ALK also substantially decreased Sox2 expression. In other words, the NPM-ALK/STAT3 signaling axis, which is

considered to be the key oncogenic driving force in ALK^{+}ALCL, is primarily responsible for the aberrant expression of Sox2 in this cancer cell type.

We were rather surprised with the finding that the transcriptional activity of Sox2 is heterogeneous in ALK^{+}ALCL cell lines, since Sox2 protein is found in virtually all cells in the two ALK^{+}ALCL cell lines, as evidenced by our confocal microscopy experiments. To our knowledge, the observation that the transcriptional activity of Sox2 is heterogeneous in cancer cells has never been previously described. The validity of this novel finding is supported by the following. First, ALK^{+}ALCL cell lines stably transduced with the Sox2 reporter construct were cultured in the presence of puromycin selection at all times; thus, it is highly unlikely that the lack of the Sox2 activity in the Sox2^{inactive} subset is due to a loss of the reporter construct. Second, to confirm that the Sox2^{inactive} cells were truly Sox2-inactive, we re-infected these cells with the Sox2 reporter construct. The observation that no increase in GFP expression in these cells was detected further support that the Sox2^{inactive} cells are genuinely Sox2^{inactive}. Third, we were able to detect the GFP and luciferase genes in the Sox2^{inactive} cells. Forth, the transcriptional activity of Sox2 is associated with different biological characteristics, including cell growth, invasiveness and tumorigenesis. Based on our finding that Sox2 transcriptional activity, rather than its sheer protein expression, dictates oncogenic potential, we suggest that future studies of Sox2 in cancer should include the transcriptional activity of Sox2 as a major parameter.

An obvious question that emerges from our observations is related to how the Sox2 transcriptional activity is regulated. As mentioned above, the differential activity of Sox2 is not due to a difference in Sox2 protein expression, as our confocal microscopy results showed that virtually all cells in both ALK^{+}ALCL cell lines express this protein. Recently, it was demonstrated that the nuclear localization of Sox2 is regulated by

phosphorylation through the AKT signaling (43). Thus, we speculated that the activity of Sox2 is dependent on whether Sox2 is localized to the nuclei where it functions as a transcriptional factor. However, we did not observe any difference in the subcellular localization of Sox2 between the $Sox2^{active}$ and $Sox2^{inactive}$ subsets. In another recent study, Van Hoof *et al.* identified that serine phosphorylation of Sox2 can modulate its activity (44). Again, we did not observe any difference in the serine phosphorylation of Sox2 between the two subsets (Figure 3.22). Considering the fact that the transcriptional activity of Sox2 is tightly regulated by a number of co-factors (4, 45), it is likely that one or more of these cofactors are important in regulating the transcriptional activity of Sox2 in ALK^{+}ALCL cells. In this regard, we had investigated the expression of Oct4a, the most studied Sox2 cofactor (5). However, using Western blot and an anti-Oct4a antibody, we did not detect any Oct4a expression in ALK^{+}ALCL cell lines (Figure 3.23). Overall, further studies are needed to delineate this important regulatory process by which the transcriptional activity of Sox2 is regulated in cancer cells.

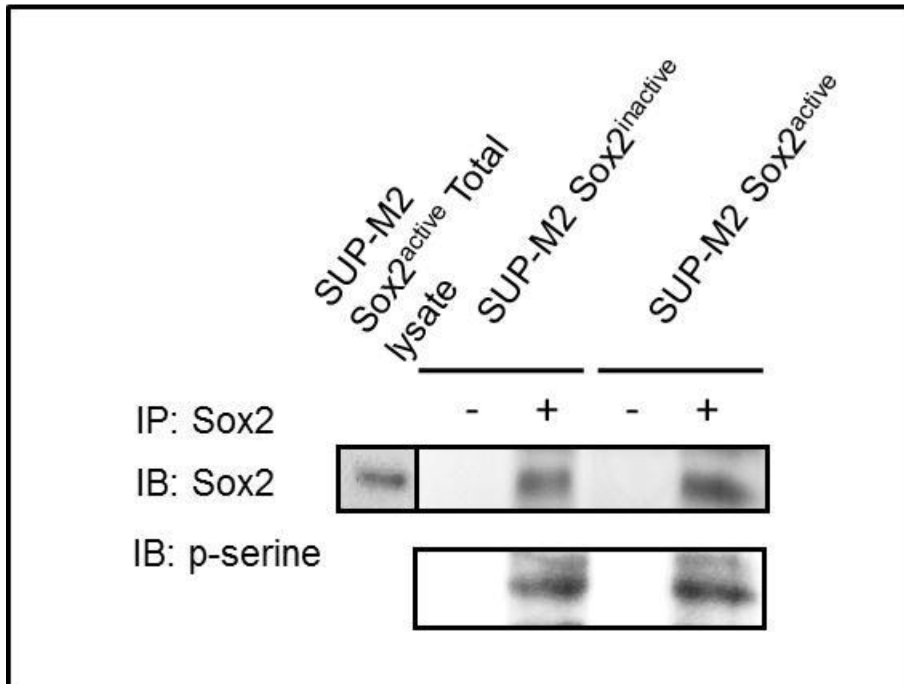


Figure 3.22. Sox2 transcriptional activity is not due to a difference in Sox2 phosphorylation.

Serine phosphorylation of Sox2 in Sox2^{active} and ^{inactive} cell subsets was examined. In order to confirm that the transcriptional activity of Sox2 is not related to a difference in serine phosphorylation of Sox2, we performed immunoprecipitation. No difference in the level of serine phosphorylation of Sox2 was seen between the two subsets. A similar experiment was done to investigate the status of Sox2 tyrosine phosphorylation; no detectable difference in Sox2 tyrosine phosphorylation was detectable between the two cell subsets (not shown).

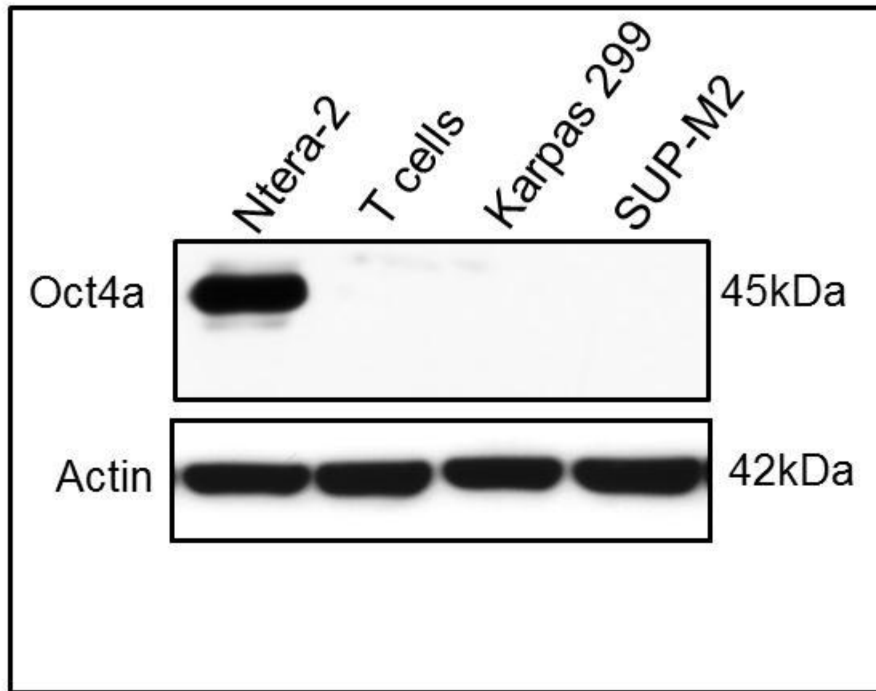


Figure 3.23. Oct4a is not expressed in ALK^{+}ALCL cell lines

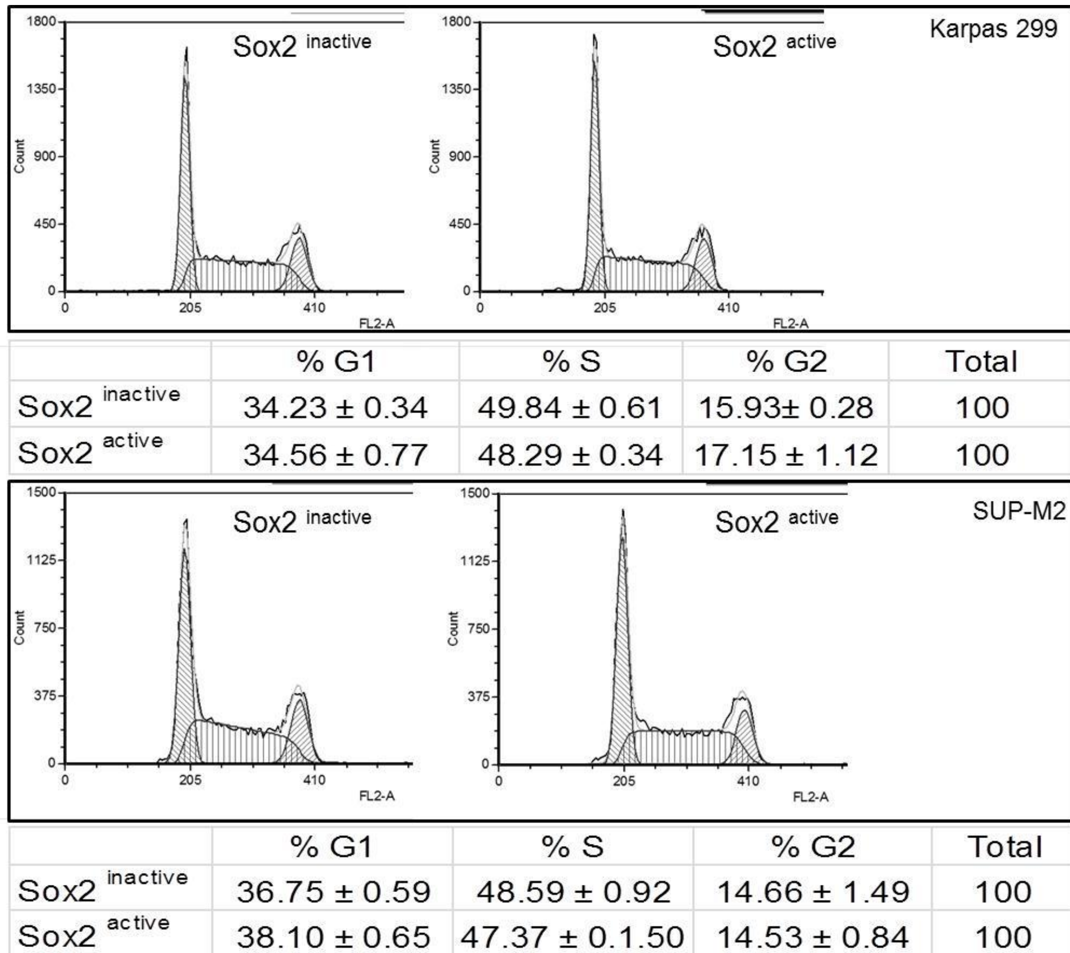
The expression of Oct4a protein in Karpas 299 and SUP-M2 cell lines was not detectable by Western blots. Ntera-2 cells were used as a positive control. T-cells isolated from the peripheral blood of healthy donors were negative for Oct4a protein.

In this study, we found that the transcriptional activity of Sox2 correlated with the sensitivity to doxorubicin, a conventional chemotherapeutic agent targeting DNA. Interestingly, Mallanna *et al.* (46) have previously reported that Sox2 interacts with DNA repair proteins such as members of the replication protein A family. Thus, it is possible that the mechanisms that regulate the transcriptional activity of Sox2 may also regulate how Sox2 interacts with DNA repair proteins, thereby modulating the efficiency of DNA repair. Alternatively, some of the downstream targets of Sox2 are responsible for DNA repair; thus, up-regulation of these genes in the $\text{Sox2}^{\text{active}}$ subset contributes to enhanced DNA repair and a resistance to doxorubicin.
In contrast with the previous findings, we found that $\text{Sox2}^{\text{active}}$ cells are more sensitive to Crizotinib, an ALK inhibitor. While it is unclear how the transcriptional activity of Sox2 correlates with a higher sensitivity to this agent, the observation that $\text{Sox2}^{\text{active}}$ and $\text{Sox2}^{\text{inactive}}$ subsets have differential sensitivities to agents working via different pathways has provided rationale of using a combination therapeutic approach to treat cancer with Sox2 expression.

We have examined the possible mechanisms by which Sox2 exerts its oncogenic effects. We first asked if Sox2 regulates the expression and activation of the NPM-ALK/STAT3 axis. As described, siRNA knockdown of Sox2 in $\text{Sox2}^{\text{active}}$ subset did not result in any detectable change to the levels of NPM-ALK, pALK, STAT3 and pSTAT3. The other approach used was to compare the expression and activation status of NPM-ALK and its downstream targets between the $\text{Sox2}^{\text{active}}$ and $\text{Sox2}^{\text{inactive}}$ cells. A comparison of these two cell subsets showed no substantial difference in the expression/activation status of NPM-ALK, STAT3 and a host of other cellular signaling proteins known to be activated by NPM-ALK. The only exception is p-ERK, which was expressed at a slightly higher level in

Sox2^{active} subset. As described in the results, this increase in p-ERK is not apparently linked to Sox2, since siRNA down-regulation of Sox2 in the Sox2^{active} subset did not result in any appreciable change in p-ERK. To address this question using a different angle, we asked if the Sox2 downstream targets in ESCs are differentially expressed between the two cell subsets. As shown in Figure 3.14, we confirmed the differential expression of 3 known Sox2 downstream targets including Notch1, BCL2 and PDGFR-alpha (47-48). These findings further support the validity of our findings of two cell subsets in ALK^{+}ALCL cells based on their differential Sox2 transcriptional activity. Of interest, both Notch1 and PDGFR-alpha have been previously implicated in the pathobiology of ALK^{+}ALCL (49-50).

One interesting observation is that xenografts derived from isolated Sox2^{active} cells contained a significant proportion of Sox2^{inactive} cells. This was a consistent finding in all three mice xenografted with Sox2^{active} cells. Since these animals were all euthanized 28 days after the initiation of the experiment, this ‘conversion’ appears to be relatively rapid, and we believe that it is highly unlikely that it is a result of a few contaminating Sox2^{inactive} cells, which should be $<2\%$. Furthermore, when we performed cell cycle studies using fractionated SUP-M2 cells, we did not observe any significant difference between the Sox2^{active} and Sox2^{inactive} cell fractions ($p>0.05$) (Figure 3.24). Taken together, we believe that the most likely scenario is that Sox2^{active} cells gave rise to Sox2^{active} as well as Sox2^{inactive} cells as they proliferate. This scenario is reminiscent of how stem cells maintain a pool of cells for self-generation and multi-potency and a pool of cells for differentiation. This model is supported by our observation that no Sox2^{active} cells were detectable in the xenografts derived from isolated Sox2^{inactive} cells. These findings raise the possibility that the Sox2^{active} cell fraction contains the tumor progenitor or cancer stem cells in this cancer type.



	% G1	% S	% G2	Total
Sox2 inactive	34.23 ± 0.34	49.84 ± 0.61	15.93± 0.28	100
Sox2 active	34.56 ± 0.77	48.29 ± 0.34	17.15 ± 1.12	100

	% G1	% S	% G2	Total
Sox2 inactive	36.75 ± 0.59	48.59 ± 0.92	14.66 ± 1.49	100
Sox2 active	38.10 ± 0.65	47.37 ± 0.1.50	14.53 ± 0.84	100

Figure 3.24. No significant difference in cell-cycle analysis between the Sox2^{active} and Sox2^{inactive} subsets of ALK^{+}ALCL cell lines.

Cell cycle analysis by flow cytometry using propidium iodide showed no appreciable significant difference between the Sox2^{active} and Sox2^{inactive} cell fractions of Karpas 299 and SUP-M2 cell lines ($p>0.05$). All experiments were performed in triplicate and representative results are shown.

To conclude, we have demonstrated that ALK$^+$ALCL aberrantly expresses Sox2, one of the master transcriptional factors in ESCs. We have also shown that Sox2 protein expression is dependent on STAT3 activation. Interestingly, we have observed that the transcriptional activity of Sox2 is restricted to a relatively small subset of cells, despite the fact that Sox2 protein is expressed in virtually all ALK$^+$ALCL cells. In view of the fact that the transcriptional activity, rather than the sheer protein expression, of Sox2 is important in mediating tumorigenesis, we believe that further studies of this regulatory mechanism will be highly worthwhile.

3.5. References

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➢ Chapter 4

Disheveled proteins promote cell growth and tumorigenicity in ALK-positive anaplastic large cell lymphoma

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Disheveled proteins promote cell growth and tumorigenicity in ALK-positive anaplastic large cell lymphoma**.**

4.1. Introduction

ALK-positive anaplastic large cell lymphoma (ALK^{+}ALCL) is a distinct non-Hodgkin lymphoma of T/null-cell immunophenotype recognized by the World Health Organization Classification for hematopoietic tumors (1). These tumors express oncogenic fusion proteins generated by chromosomal translocations involving the gene encoding for the ALK tyrosine kinase. The most common type of these translocations is NPM-ALK, which is a result of the reciprocal chromosomal translocation t(2;5)(p23;q35), leading to the juxtaposition of the nucleophosmin (NPM) gene at 5q35 with the ALK gene at 2p23 (2,3). It is well established that NPM-ALK is oncogenic, and it directly contributes to the pathogenesis of ALK^{+}ALCL through its constitutive tyrosine kinase activity (4,5). NPM-ALK activates a host of cellular signaling pathways, including the Janus kinase/STAT3 (JAK/STAT3) (6,7), Ras/ERK (8) and PI3K/AKT (9,10) pathways, which regulate important cellular functions such as cell-cycle progression and sensitivity to apoptosis.

Recently, our group published that β-catenin, a transcriptional factor and a downstream mediator of the Wnt canonical pathway (WCP) (11,12), is constitutively active in ALK^{+}ALCL and contributes to the growth of ALK^{+}ALCL cell lines in-vitro (13). In our further studies of the WCP in ALK^{+}ALCL, we employed pathway-specific oligonucleotide arrays and detected relatively high mRNA expression levels of disheveled proteins (Dvls). Dvls are upstream components of Wnt signaling (14), and the human Dvl family of proteins consists of three members, Dvl-1, Dvl-2, and Dvl-3 (14). All three Dvl genes are expressed during early embryonic development in mice (15,16), and the biological importance of the Dvl proteins is illustrated by the fact that Dvl2-/- and Dvl3-/- mice die perinatally (17,18). Although the Dvls possess no enzymatic activity, they serve as a critical link between the Wnt receptors and cytoplasmic components of the Wnt signaling pathways (14). In the WCP, activation of the Dvls upon the engagement of Wnt receptors by Wnts, results in the

disruption of the β-catenin destruction complex. This leads to decreased proteasomal degradation of β-catenin, resulting in corresponding increases in its total protein levels, nuclear translocation, and gene transcriptional activity (11). In addition, the Dvls also play important roles in the Wnt non-canonical pathways (WNCPs), which are defined as Wnt-mediated signaling that is independent of β-catenin (14,19). The best characterized WNCPs are the Wnt/planer cell polarity pathway and the Wnt/Ca^{2+} pathway. In the former pathway, the Dvls regulate cytoskeletal rearrangements by signaling through small GTPases (such as Rac and Rho), which subsequently activate the JNK and p38 kinases (20). In the latter pathway, Wnt-induced changes in intracellular Ca^{2+} levels activate protein kinase C, Ca^{2+}–calmodulin-dependent protein kinase II, and nuclear factor of activated T cells (NFAT) (20).

The Dvls have been implicated in the pathogenesis of various types of solid tumors. For example, expression of the Dvls has been reported in non-small cell lung carcinoma, and elevated expression of these proteins is associated with increased tumor cell invasiveness and a worse clinical outcome (21-23). As well, down-regulation of Dvl-3 suppressed the growth of mesothelioma cells and sensitized these cells to cisplatin (24). More recently, Dvl-2 signaling has been reported to promote self-renewal and tumorigenicity of human gliomas through mechanisms involving both the WCP and WNCP (25). Nevertheless, only a small number of studies have been performed to characterize how the Dvls mediate their oncogenic effects, and most of these studies showed that these proteins exert their effects via β-catenin and the WCP. To our knowledge, there are only two studies reporting that Dvls may promote oncogenesis via WNCPs. Specifically, expression of Dvl-3 in the lung adenocarcinoma cell line, A549, was found to activate the JNK and p38 kinases, and increase the invasiveness of these cells; importantly, both β-catenin protein expression and transcriptional activity were not affected by Dvl-3 expression in these cells (22). In a more recent study, depletion of Dvl-2 in the human

glioblastoma cell line, U87, was found to suppress cell proliferation and induce differentiation; again, this effect was β-catenin–independent since transfection of a constitutively active β-catenin could not rescue the effects of Dvl-2 depletion (25). Thus far, all published studies demonstrating oncogenic roles for the Dvls are in solid tumors, and the function of Dvls in hematologic malignancies is largely unknown. One report demonstrated that inhibition of the Wnt16b induced apoptosis in human lymphoblastoid leukemia cells, and this inhibition correlated with down-regulation of Dvl-2 and β-catenin protein levels (26). In view of our finding that the Dvls are highly expressed at the mRNA level in ALK^{+}ALCL, and the fact that little is known whether Dvls play a role in hematological malignancies, we examined whether the Dvls were important in this lymphoma. We found that the Dvls exert oncogenic effects in ALK^{+}ALCL. Interestingly, our data pointed to the involvement of the WNCPs rather than the WCP.

4.2. Materials and Methods

4.2.1. Cell lines and tissue culture

The characteristics of the ALK^{+}ALCL cell lines, Karpas 299, SU-DHL-1 and SUPM2, have been previously described (27). Cells were grown at 370C and 5% CO2 and maintained in RPMI medium (Sigma-Aldrich, St. Louis, MO). The culture media contain 2 mM L-glutamine and was enriched with 10% fetal bovine serum (Gibco, Carlsbad, CA). Ficoll-Paque (GE Health care, Quebec, Canada) was used to isolate peripheral blood mononuclear cells (PBMC) from healthy donors. Following PBMC isolation, T-cells were purified using a commercial available kit (Stem Cell Technologies, BC, Canada).

4.2.2. Subcellular protein fractionation, Immunoprecipitation and Western blotting

For subcellular protein fractionation, we employed a kit purchased from Active Motif (Carlsbad, CA, USA) and followed the manufacturer's instructions.

For co-immunoprecipitation experiments, cells were washed in phosphate buffered saline (PBS) and lysed using Cell Lytic Buffer M (Sigma-Aldrich) supplemented with 0.1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), a protease inhibitor mixture (Nacalai Inc., San Diego, CA), and a phosphatase inhibitor mixture (Calbiochem, EMD Biosciences, Darmstadt, Germany). After incubating the lysate on ice for 30 min., it was centrifuged at 15,000 x g for 15 min. Two micrograms of the primary antibody was added to 500 µg of protein lysate and rotated overnight at 4°C. Controls where the primary antibody was omitted were also performed. The next day, 30 µl of Protein G Plus/Protein A-Agarose suspension beads (Calbiochem) (anti-ALK immunoprecipitations) or Protein A-Agarose suspension beads (anti-Dvl-2 and Dvl-3 immunoprecipitations) was then added, and allowed to incubate for an additional 2 h at 4°C. The beads were then washed 4 times with cold PBS. For co-immunoprecipitation experiments, the final wash was done using cold cell lysis buffer. For immunoprecipitation experiments, the washes were done using RIPA buffer. Proteins were then eluted from the beads in 20 µl of SDS protein loading buffer by boiling for 5 min. at 100 °C. Eluates were then subjected to SDS-polyacrylamide gel electrophoresis and western blotting. Preparation of cell lysates for Western blots was done as previously described (28). Antibodies employed in this study included those reactive with Dvl-2, Dvl-3, phospho-Src (Tyr 416), phospho-GSK-3β, GSK-3β, PARP, cleaved caspase 3, cyclin D3, p21 and phospho-tyrosine (Cell Signaling Technology, Danvers, MA); anti- ALK mouse monoclonal antibody (Dako, Glostrup, Denmark); anti- β-catenin (BD Biosciences Pharmingen, San Diego, CA, USA); mouse monoclonal anti- β-actin,

mouse monoclonal anti- phospho-STAT3, and rabbit polyclonal anti-STAT3, anti-Src and anti-p27 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibodies used for immunoprecipitation were rabbit polyclonal antibodies against Dvl-2 or Dvl-3, and a goat polyclonal anti-ALK antibody (all from Santa Cruz Biotechnology).

4.2.3. Short interfering RNA (siRNA)

siRNAs for Dvl2 and Dvl3 were purchased from Invitrogen (Burlington, ON, Canada), while siRNAs for ALK were purchased from Sigma-Aldrich. Scrambled siRNA was purchased from Dharmacon (Lafayette, CO, USA). Transfection of siRNA was carried out using a square wave, BTX ECM 800 electroporator (Holliston, MA, USA) with the following settings: 225V, 8 ms pulse length, 3 pulses, 1 s between pulses). The concentration of siRNA used was 200 pmole1x106 cells in 500 µl of RPMI media, and cells were harvested 48 hours after transfection. Protein levels for Dvl-2, Dvl-3 and ALK were assessed by western blot analysis to evaluate the efficiency of knock-down.

4.2.4. Methylcellulose colony formation assay

Methylcellulose-based media was purchased from R&D Systems (Minneapolis, MN, USA) and assays were performed essentially as described in the manufacturer’s protocol. Briefly, cells transfected with either scrambled siRNA or siRNA against Dvl-2, Dvl-3 or both, were plated 24 hours post-transfection into a 6 well tissue culture plate at 100 or 500 cells/mL in 1.2% methylcellulose, 30% fetal bovine serum, 1% bovine serum albumin, 10-4 M 2-mercaptoethanol and 2mM L-glutamine. The cells were incubated for 7 days at 37°C and 5% CO_2. The number of colonies containing >30 cells were counted using an inverted phase contrast microscope. Triplicate experiments were performed.

4.2.5. Measurement of cell viability and cell-cycle analysis

Cells transfected with scrambled siRNA or siRNA against Dvl-2, Dvl-3, or both, were plated after transfection at a concentration of 50,000 cells/ ml of culture medium. Plating was performed in triplicates. Trypan blue (Sigma-Aldrich) exclusion assay was performed every 24 hours for up to 3 days following transfections. At least three independent experiments were performed. All experiments were performed in triplicate. For cell-cycle analysis, cells at a concentration of 10^6 cells/ml were prepared 48 h after siRNA transfection. Cells were washed twice with PBS and fixed with 70% cold ethanol for 2 h. These cells were then subjected to RNase treatment and propidium iodide (PI) staining. DNA content was determined using a FACSCalibur flow cytometer (BD Biosciences). Data acquisition was gated to exclude cell doublets and the cell-cycle stage distribution was determined using the CellQuest program (20,000 events were counted).

4.2.6. NFAT transcriptional activity

To assess the transcriptional activity of NFAT in ALK^+ALCL cell lines after knock-down of the Dvl proteins, we co-transfected the NFAT-responsive firefly luciferase reporter plasmid and the Renilla reporter plasmid (Promega, Madison, WI, USA), together with either scrambled siRNA, Dvl-2 siRNA, or Dvl-3 siRNA. Forty-eight hours post-transfection, cells were harvested and cell extracts were prepared using a lysis buffer purchased from Promega. The firefly luciferase activity and Renilla luciferase activity were assessed using a dual-luciferase reagent (Promega).

4.2.7. Statistical analysis

Data are expressed as mean +/- standard derivation. Statistical significance was determined using two-tailed Student's t-tests and statistical significance was considered achieved when the p value was <0.05.

4.3. Results

4.3.1. Dvl-2 and Dvl-3 are phosphorylated/activated in ALK$^+$ALCL cells

We first validated our oligonucleotide study results by western blotting. We found that Dvl-2 and Dvl-3 were highly expressed in ALK$^+$ALCL cell lines relative to their expression in normal peripheral blood T cells (Figure 4.1A). Importantly, we observed higher molecular weight species that likely correspond to the phosphorylated/active forms of Dvl-2 and Dvl-3 in ALK$^+$ALCL cell lines (arrows), but not in normal peripheral blood T cells (Figure 4.1A). Mouse embryonic stem cells serve as the positive control for Dvl protein expression and the presence of the higher molecular weight species of these proteins. We also found that Dvl-2 and Dvl-3 were expressed, and present as higher molecular weight forms, in ALK$^+$ALCL patient samples (Figure 4.1B).

4.3.2. siRNA knock-down of Dvl-2 and Dvl-3 inhibits cell growth and induces cell-cycle arrest in ALK$^+$ALCL cell lines

To investigate whether the Dvls are biologically important in ALK$^+$ALCL, we assessed whether siRNA knockdown of Dvl-2 or Dvl-3 will exert an impact on the growth or tumorigenicity of ALK$^+$ALCL cell lines. As shown in Figure 4.2A, transfection of siRNAs against Dvl-2 or Dvl-3 into two ALK$^+$ALCL cell lines (Karpas 299 and SUP-M2) resulted in a considerable down-regulation of these two proteins. Correlating with reduced Dvl-2 and 3 protein expression, we observed a significant decrease in the growth in both cell lines (Figure 4.2B). Of note, siRNA knock-down of Dvl-2 and Dvl-3 simultaneously resulted in a more dramatic decrease in the cell growth than knock-down of Dvl-2 or Dvl-3 alone. The decrease in the viable cell count was not associated with any detectable increase in trypan blue-positive dead cells, and we observed no cleavage of PARP or caspase 3 (Figure 4.3) suggesting that the cells were not undergoing apoptosis. Cells treated with staurosporine as an apoptosis-inducing agent served as

positive controls for apoptotic markers. To further characterize the mechanism by which the Dvls promote cell growth in ALK^{+}ALCL, we performed cell-cycle analysis. siRNA knock-down of both Dvl-2 and Dvl-3 induced a significant G0/1 cell-cycle arrest in Karpas 299 and SUP-M2 cells, compared to cells transfected with scrambled siRNA (Figure 4.4). The percentage of cells in G0/1 phase was (31.09 ± 2.2 vs 37.00 ± 1.22) for Karpas 299 and (34.06 ± 0.59 vs 38.48 ± 0.47) for SUP-M2. These results were statistically significant (*p* values, 0.04 and 0.004 for Karpas 299 and SUP-M2, respectively). To further support that treatment with siRNA against Dvls does not induce apoptosis in ALK^{+}ALCL, we did not detect any appreciable increase in the proportion of cells in the sub G0/1 phase in both cell lines. All experiments were performed in triplicate and representative results are shown in Figure 4.4. We then assessed whether the Dvl proteins have a role in regulating the expression of negative cell-cycle regulators such as, p21 and p27. As shown in Figure 4.5, we found an up-regulation of p27 in both Karpas 299 and SUP-M2 after knock-down of both Dvl-2 and Dvl-3, whereas we were able to detect an up-regulation of p21 in SUP-M2 but not in Karpas 299 cells.

4.3.3. siRNA knock-down of Dvl-2 and Dvl-3 significantly decreased colony formation in soft agar

Using a methylcellulose colony formation assay, we tested whether the Dvls contribute to the tumorigenicity of ALK^{+}ALCL cell lines. As shown in Figure 4.6A and 4.6B, siRNA knock-down of either Dvl-2, Dvl-3, or both simultaneously resulted in a significant reduction in the number of colonies that Karpas 299 and SUP-M2 cells formed in soft agar, compared to cells treated with scrambled siRNA.

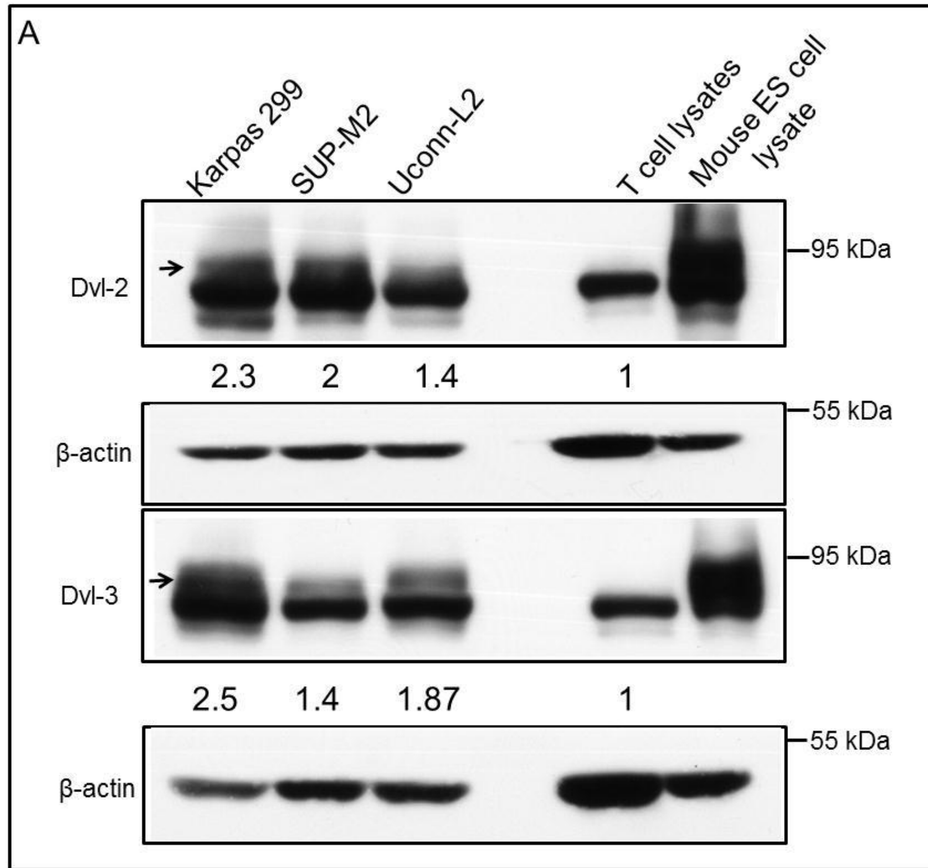


Figure 4.1. Dvl-2 and Dvl-3 are highly expressed and phosphorylated/activated in ALK⁺ALCL cell lines and tumors.

A) Western blots revealed a relatively strong expression of Dvl-2 and Dvl-3 proteins in all 3 ALK⁺ALCL cell lines Karpas 299, SUPM2 and Uconn-L2 compared to T-cells from healthy donors. Lysates from mouse embryonic stem (ES) cells were used as a positive control. The high molecular weight forms (arrows), which likely represent the phosphorylated/active Dvls, were detectable only in ALK⁺ALCL cell lines and mouse ES cells but not in normal T-cells. The electrophoretic mobility of molecular mass stands is indicated to the right of western blots. (Continued in the next page).

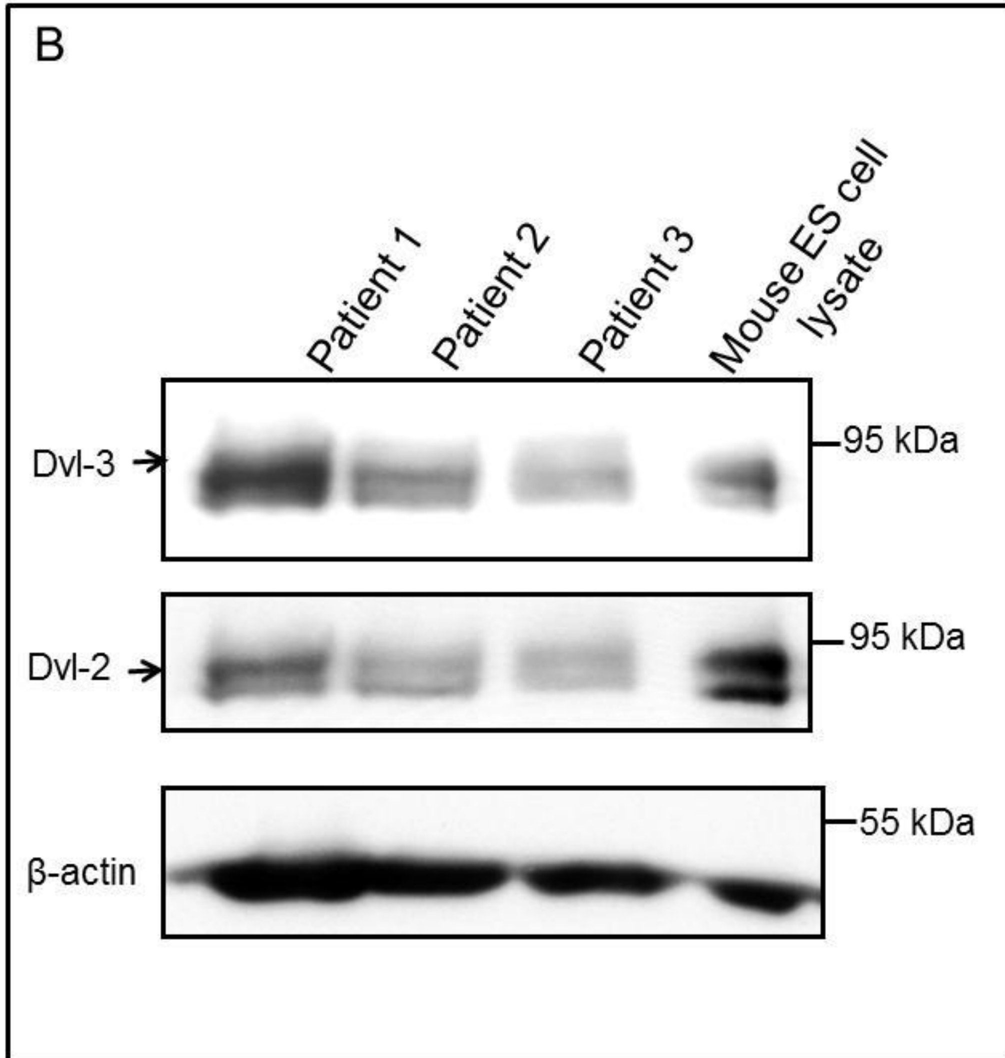


Figure 4.1. Dvl-2 and Dvl-3 are highly expressed and phosphorylated/activated in ALK⁺ALCL cell lines and tumors (continued).

B) Western blot showed the expression of Dvl-2 and Dvl-3 proteins in ALK⁺ALCL tumor samples. Again, the high molecular weight forms were detectable (arrows).

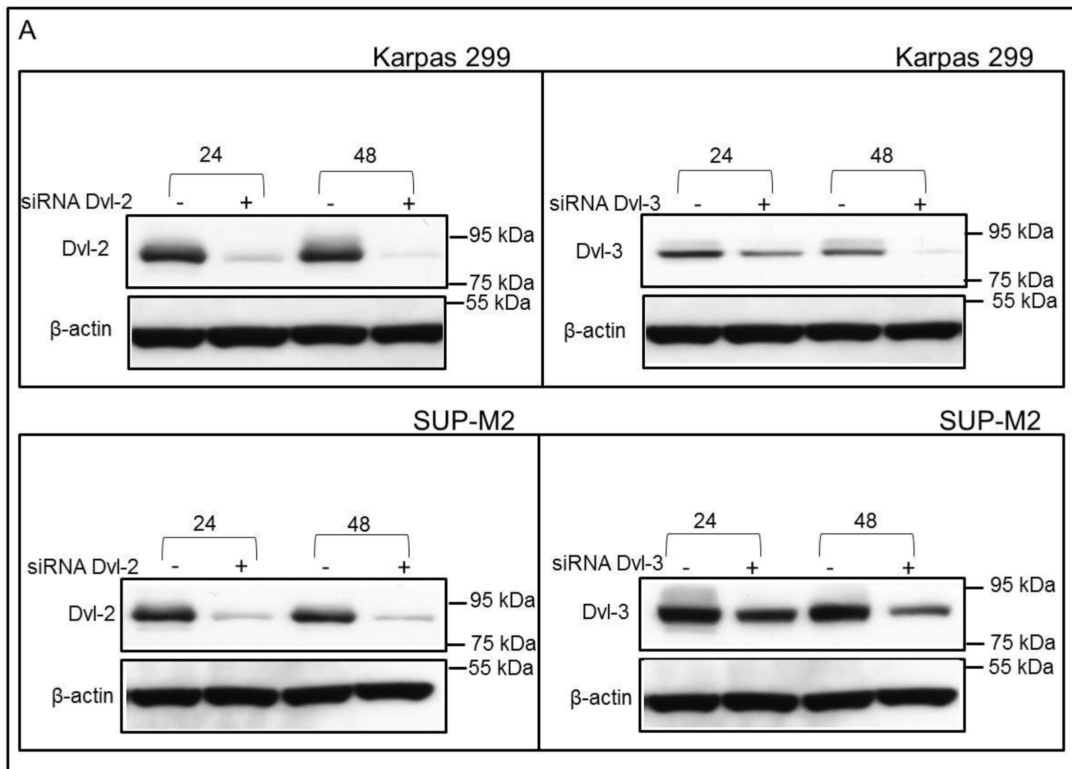


Figure 4.2. siRNA knock-down of Dvl-2 and Dvl-3 in ALK^{+}ALCL cells inhibits cell growth.

A) Western blots showed a significant downregulation of the Dvl-2 and Dvl-3 protein levels in Karpas 299 and SUP-M2 cells after transfection with specific siRNA for Dvl-2 or Dvl-3. (Continued in the next page)

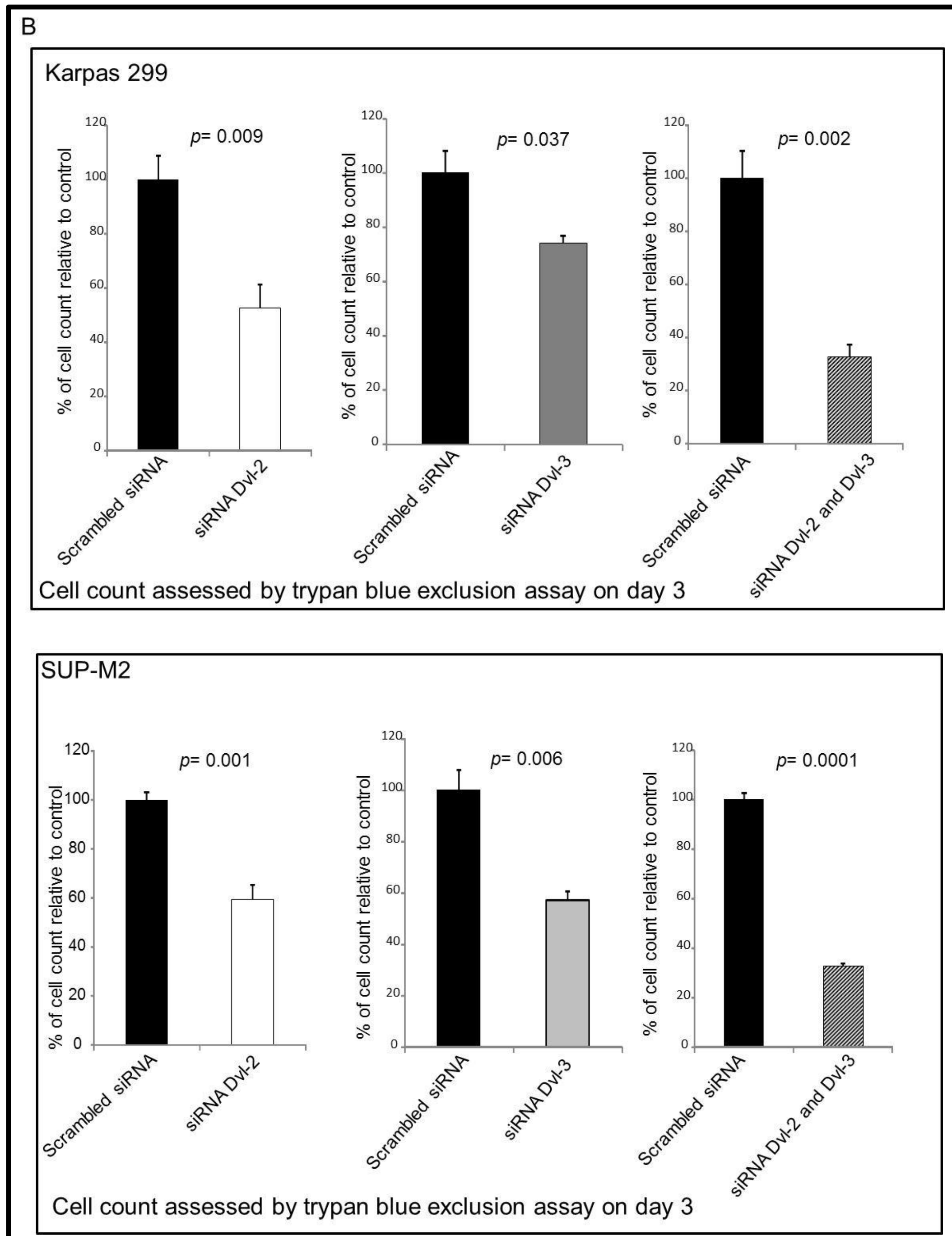


Figure 4.2. siRNA knock-down of Dvl-2 and Dvl-3 in ALK$^+$ALCL cells inhibits cell growth (continued).

B) Knockdown of Dvl-2, Dvl-3 or both induced a significant decrease in the growth of Karpas 299 and SUP-M2 cells at day 3 post-transfection, as assessed by trypan blue exclusion assay ($p<0.05$). Three independent experiments were performed.

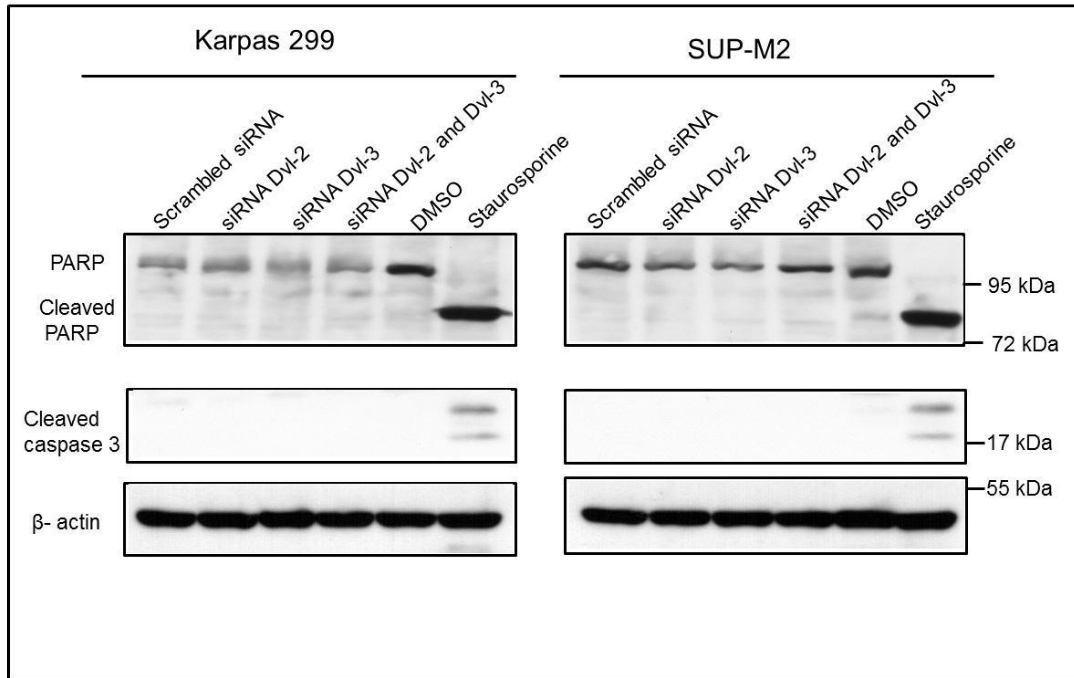
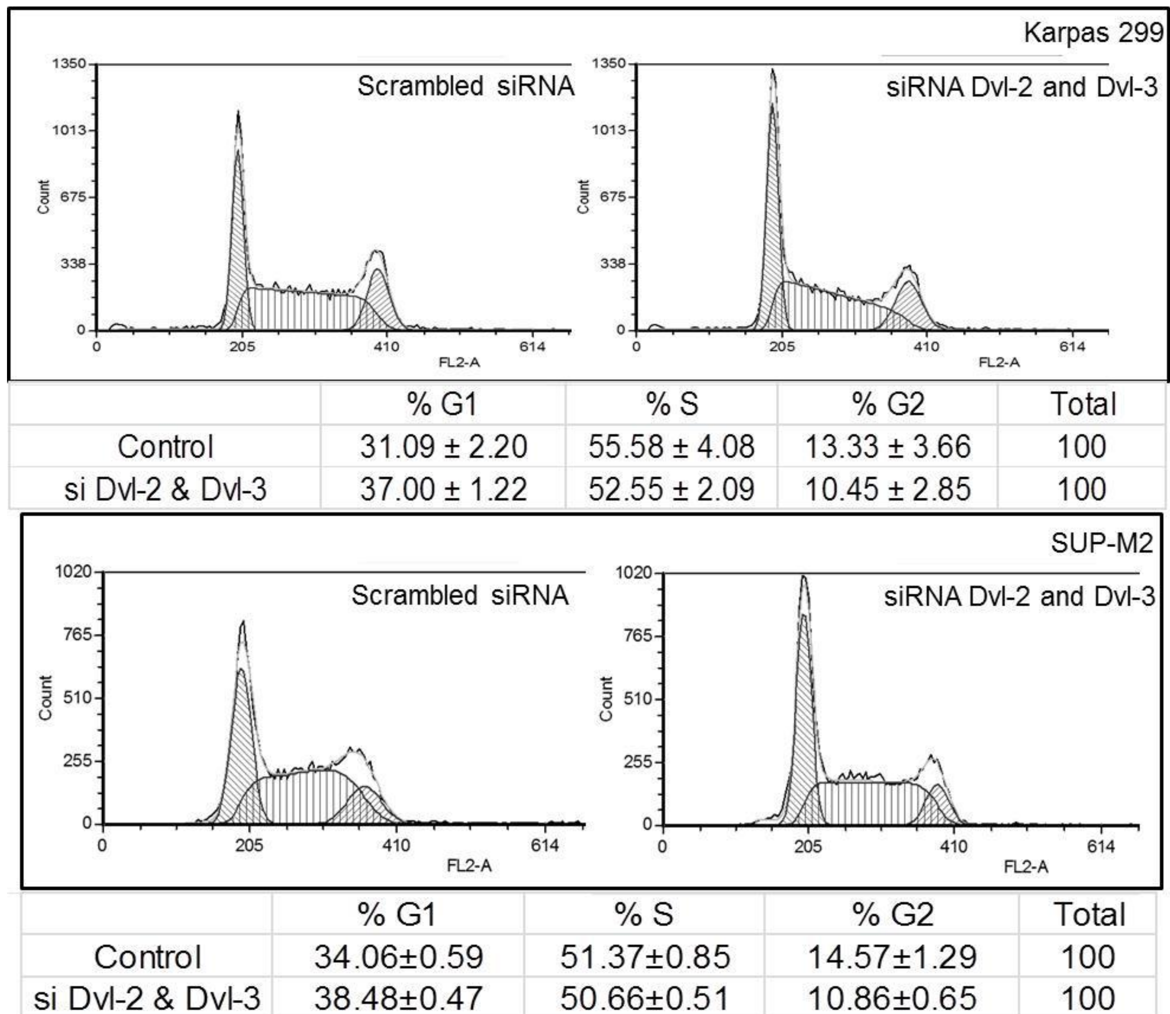


Figure 4.3. siRNA knock-down of Dvl-2 and Dvl-3 does not induce apoptosis in ALK^{+}ALCL cells.

Western blot showed absence of cleaved caspase 3 and cleaved PARP in Karpas 299 and SUP-M2 cells after transfections with siRNAs against Dvl-2, Dvl-3 or both. Evidence of apoptosis is present only in cells treated with staurosporine.



	% G1	% S	% G2	Total
Control	31.09 ± 2.20	55.58 ± 4.08	13.33 ± 3.66	100
si Dvl-2 & Dvl-3	37.00 ± 1.22	52.55 ± 2.09	10.45 ± 2.85	100

	% G1	% S	% G2	Total
Control	34.06±0.59	51.37±0.85	14.57±1.29	100
si Dvl-2 & Dvl-3	38.48±0.47	50.66±0.51	10.86±0.65	100

Figure 4.4. siRNA knock-down of Dvl-2 and Dvl-3 induces cell-cycle arrest in ALK^{+}ALCL cells.

Cell cycle analysis by flow cytometry using propidium iodide showed significant G0/1cell cycle arrest in Karpas 299 and SUP-M2, following siRNA transfection against Dvl-2 and Dvl-3. Of note, no appreciable increase in the fraction of cells in the sub G0/1 was detected. All experiments were performed in triplicate and representative results are shown.

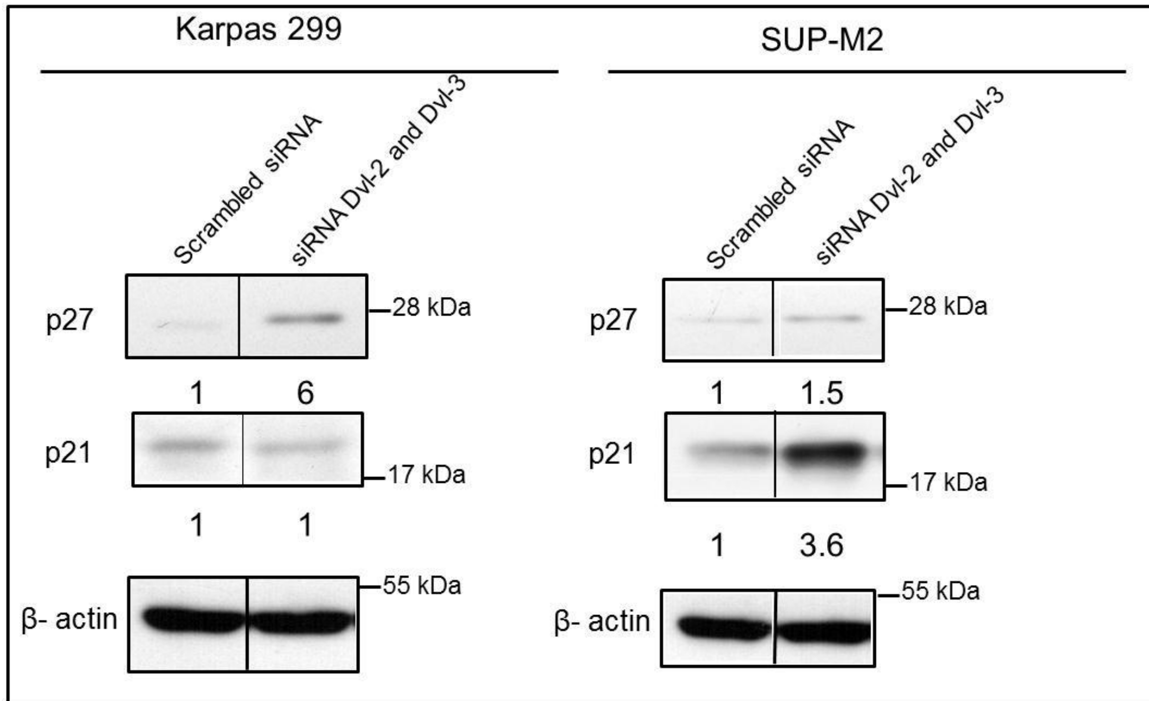


Figure 4.5. siRNA knock-down of Dvl-2 and Dvl-3 is correlated with upregulation of p27 and p21 in ALK$^+$ALCL cell lines.

Western blots showed an upregulation of p27 in Karpas 299 and SUP-M2 induced by transfection of siRNA against the Dvls. There was an upregulation of p21 in SUP-M2 cells after the same treatment; however, no appreciable difference in the protein levels of p21 was detected in Karpas 299 cells.

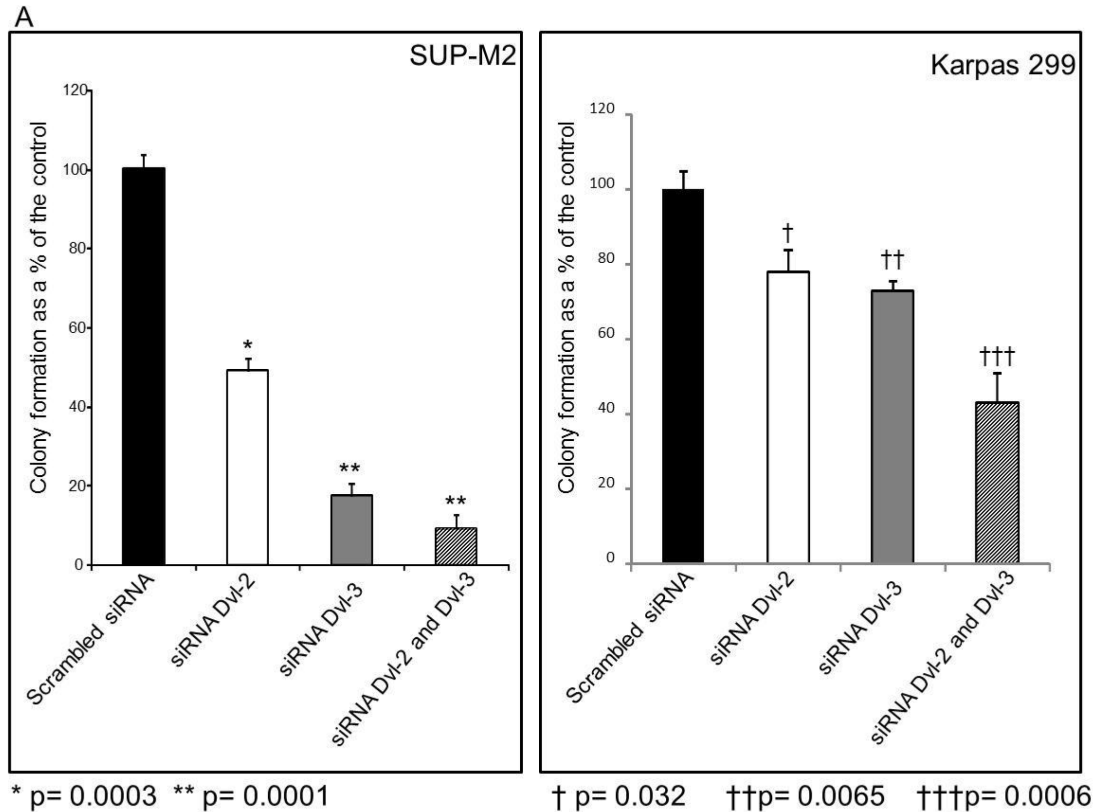


Figure 4.6. siRNA knock-down of Dvl-2 and Dvl-3 significantly decreases colony formation on methylcellulose colony formation assay.

A) Both Karpas 299 and SUP-M2 cells transfected with siRNA against Dvl-2, Dvl-3 or both showed a significantly lower number of colonies in methylcellulose colony formation assay, as compared to cells transfected with scrambled siRNA. Data is presented as percentages of the negative control (i.e. cells transfected with scrambled siRNA). Results shown are representative of three independent experiments. (continued in the next page).

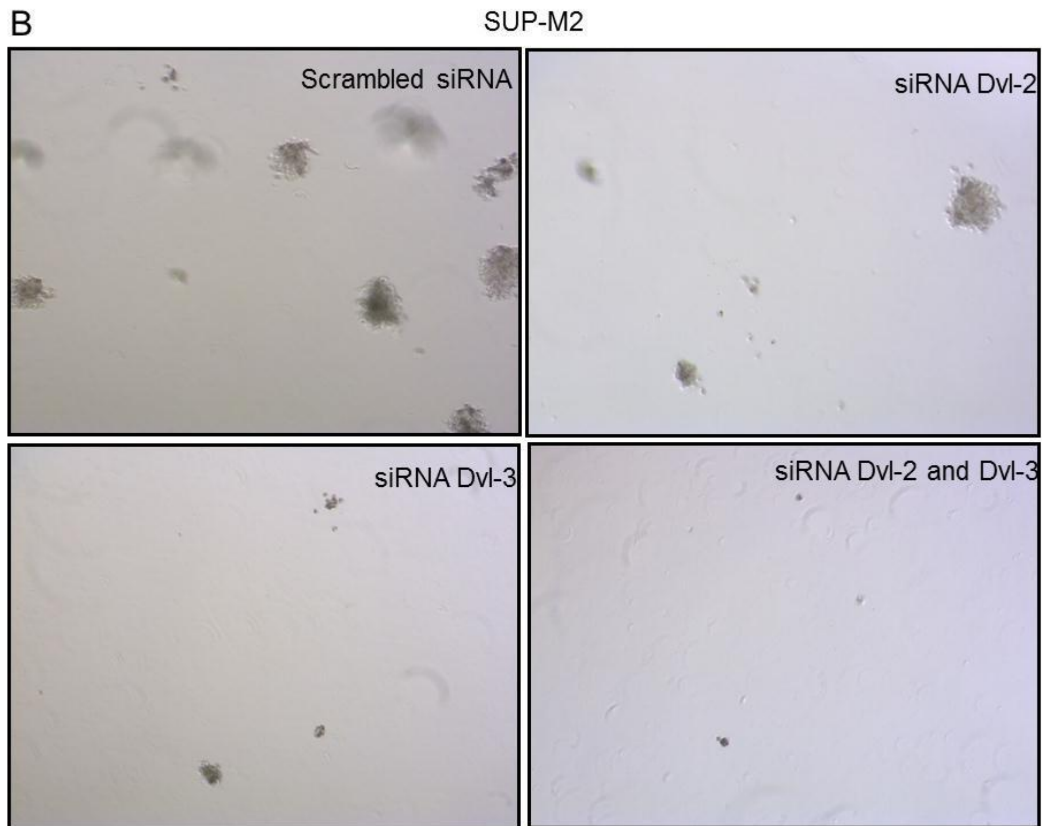


Figure 4.6. siRNA knock-down of Dvl-2 and Dvl-3 significantly decreases colony formation on methylcellulose colony formation assay (continued).

B) Images of SUP-M2 cells transfected with scrambled versus siRNA against Dvl-2, Dvl-3, or both. A significant decrease in cell colonies was observed in samples transfected with siRNA in comparison to control. Results shown are representative of three independent experiments.

4.3.4. The biological effects of Dvl-2 and Dvl-3 in ALK^{+}ALCL cells are independent of the Wnt canonical pathway

We next sought to determine the signaling pathways through which the Dvls were exerting their biological effects in ALK^{+}ALCL. As shown in Figure 4.7A, siRNA knock-down of Dvl-2, Dvl-3 or both simultaneously in ALK^{+}ALCL cell lines, did not result in a detectable change in the protein levels of β-catenin or its nuclear localization. We also examined whether siRNA knock-down of Dvls modulated phospho-GSK-3β levels, an upstream regulator of β-catenin and a surrogate marker commonly used to assess the activation status of the WCP (29,30) . As shown in Figure 4.7B, siRNA knock-down of Dvl-2, Dvl-3, or a combination of Dvl-2 and -3 did not result in detectable change in the phospho-GSK-3β levels. As it has been previously shown that β-catenin regulates the expression of STAT3 in ALK^{+}ALCL cells at the transcriptional level (13), we also investigated whether knockdown of the Dvls affected STAT3 protein levels or phosphorylation. We did not observe a significant effect on STAT3 protein levels or phosphorylation in either Karpas 299 or SUP-M2 cells after Dvls knock-down (Figure 4.7A). Together, these findings strongly argue that the Dvls do not exert their oncogenic effects via the WCP.

4.3.5. Dvl-2 and Dvl-3 regulate the transcriptional activity of NFAT in ALK^{+}ALCL

Since our results suggested that the Dvls are functioning through β-catenin–independent mechanisms, we explored the possibility that the WNCPs are involved. As mentioned, the Wnt/Ca^{2+} signaling pathway is one of the better characterized WNCPs (20). The transcriptional activity of the NFAT is regulated by changes in the intracellular calcium levels, and NFAT is a key down-stream effector of the Wnt/Ca^{2+} pathway in several cell types (20,31). Therefore, we investigated whether siRNA knockdown of Dvls had any impact on NFAT activity. As shown in Figure 4.8, siRNA knockdown of Dvls resulted in a significant down-regulation of the

transcriptional activity of NFAT in both Karpas 299 and SUP-M2 cells, as assessed by a NFAT-responsive luciferase reporter construct. These findings suggest that Dvls may mediate their oncogenic effects via activating the Wnt/Ca^{2+} pathway.

4.3.6. Down-regulation of Dvl-2 and Dvl-3 suppresses Src activation

In view of a recent report that Wnt3-induced stimulation of Dvl-2 activates Src in a murine teratocarcinoma cell line (32), and the fact that Src contributes to the pathogenesis of ALK^{+}ALCL (33), we hypothesized that the Dvls may also exert their oncogenic effects by enhancing Src activation. As shown in Figure 4.9, transfection of siRNA against Dvls in Karpas 299 and SUP-M2 cells resulted in a significant down-regulation of phospho-SrcY416, a surrogate marker commonly used to assess Src activation (33,34).

4.3.7. NPM-ALK interacts with and promotes the tyrosine phosphorylation of Dvl-2 and Dvl-3

The NPM-ALK fusion protein is an oncogenic tyrosine kinase that is central to the pathogenesis of ALK^{+}ALCL. Thus, we asked whether NPM-ALK contributes to the activation of the Dvls in this lymphoma. Since it has been reported that phosphorylation of Dvls is associated with their activation status (35,36), we evaluated whether NPM-ALK modulates the tyrosine phosphorylation of the Dvls. As shown in Figure 4.10A, siRNA knockdown of NPM-ALK resulted in a dramatic decrease in the tyrosine phosphorylation of Dvl2 (as shown for SUP-M2) and Dvl-3 (as shown for Karpas 299). Since NPM-ALK is known to exert its oncogenic effects in part by binding and activating a number of signaling proteins, we examined whether NPM-ALK associates with the Dvls. As shown in Figure 4.10B, NPM-ALK was found to co-immunoprecipitate with Dvl-2 and Dvl-3, demonstrating a physical interaction between NPM-ALK and the Dvls.

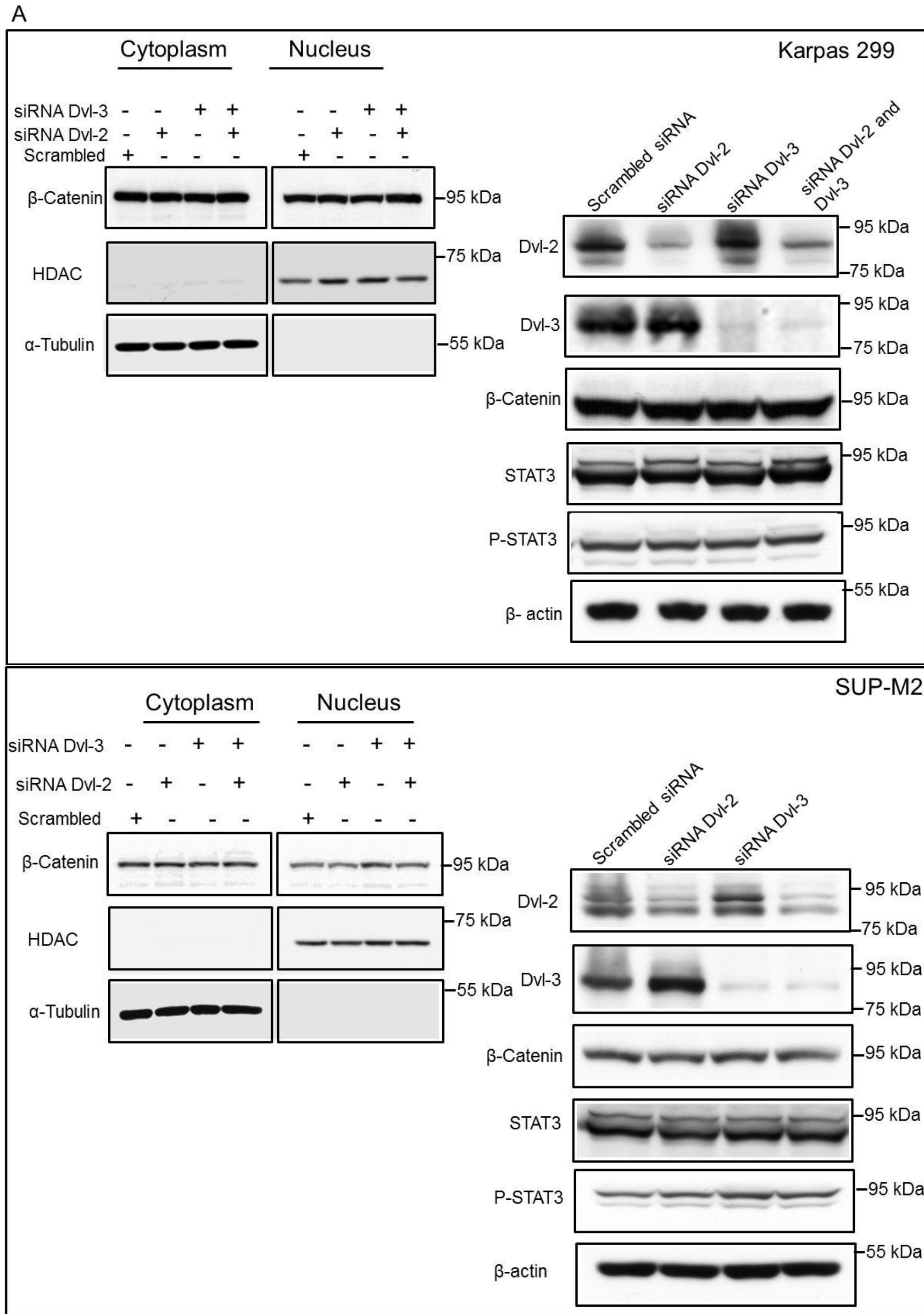


Figure 4.7 The biological effects of Dvl-2 and Dvl-3 are independent of the Wnt canonical pathway (continued in the next page).

A) Transfection of siRNAs against Dvl-2, Dvl-3 or both did not result in any appreciable change in the total protein levels as well as the nuclear protein levels of β-catenin. In addition, there was no appreciable change in the protein levels of STAT3 and phospho-STAT3 in Karpas 299 and SUP-M2.

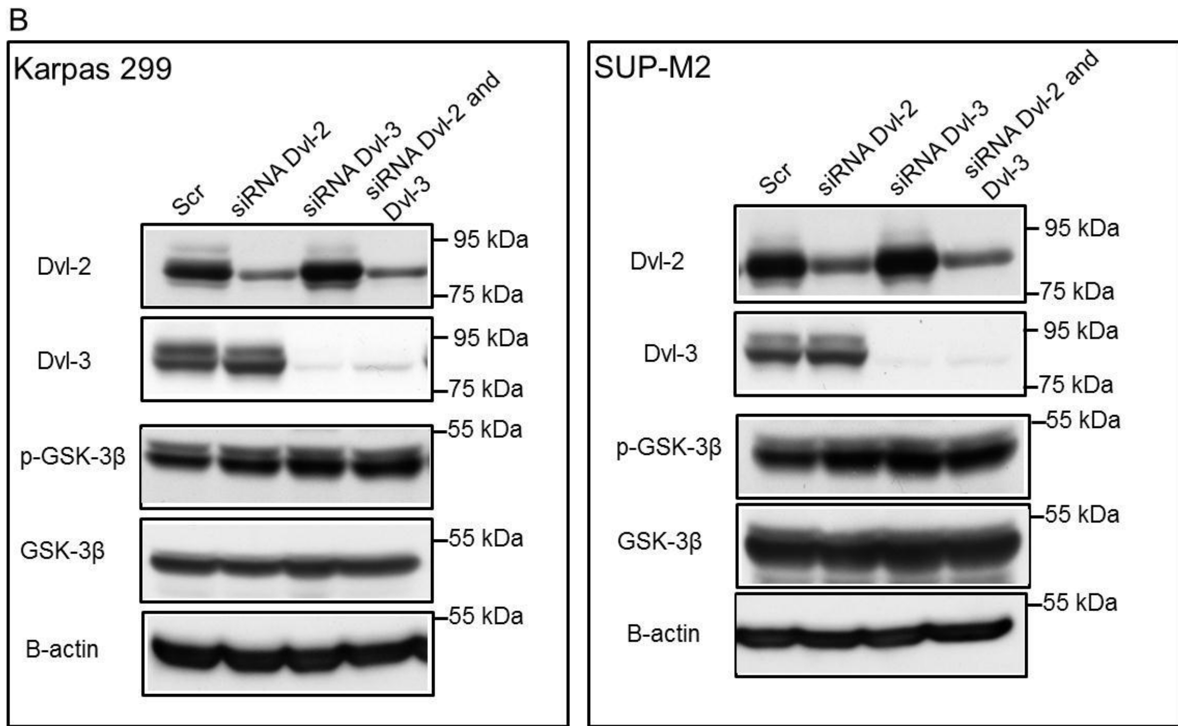
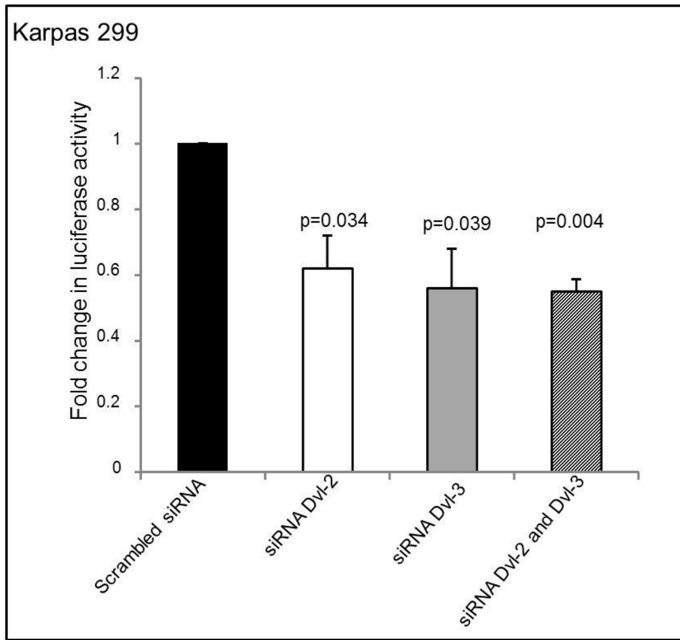


Figure 4.7 The biological effects of Dvl-2 and Dvl-3 are independent of the Wnt canonical pathway (continued).

B) Transfection of siRNAs against Dvl-2 Dvl-3 or both did not result in any appreciable change in the protein level of phospho- GSK-3β in both Karpas 299 and SUP-M2.



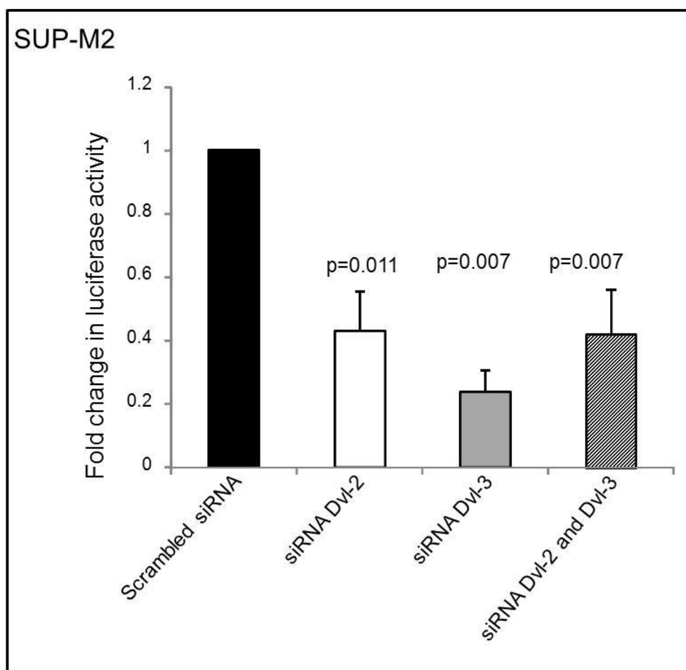


Figure 4.8. Dvl-2 and Dvl-3 regulate the NFAT transcriptional activity in ALK^{+}ALCL.

Using a NFAT-responsive firefly luciferase reporter plasmid, we measured the NFAT transcriptional activity in ALK^{+}ALCL treated with either scrambled siRNA or siRNA against the Dvls. There was a significant downregulation of the NFAT transcriptional activity ($p<0.05$) in both Karpas 299 and SUP-M2 cells after siRNAs knock-down of the Dvls.

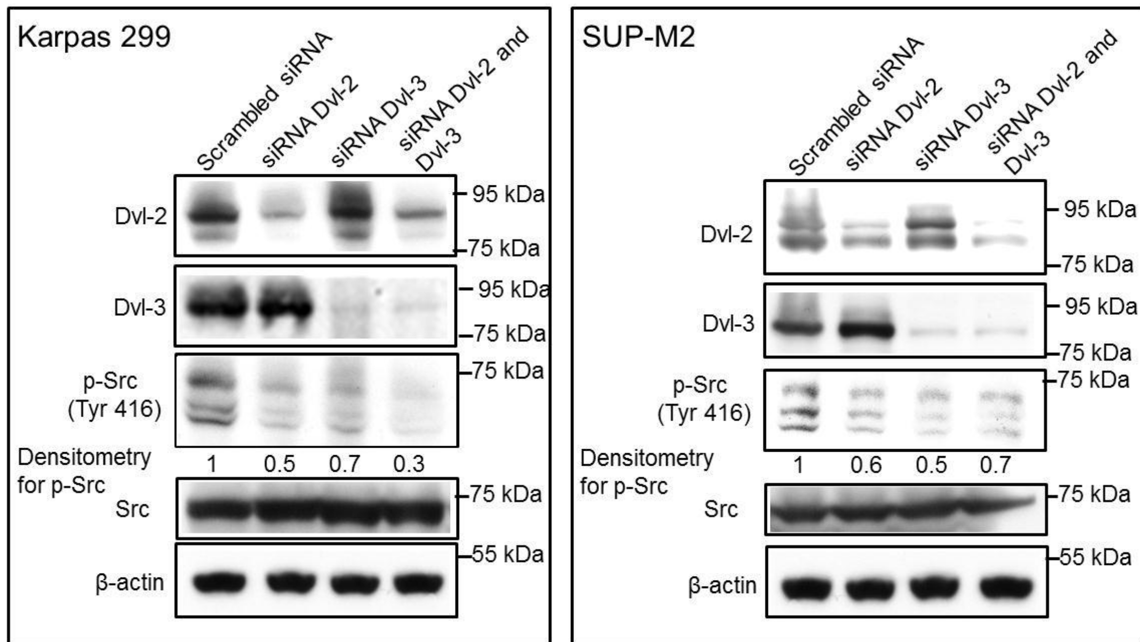


Figure 4.9. siRNA knockdown of Dvl-2 and Dvl-3 suppresses Src activation in ALK^{+}ALCL.

Western blots revealed a substantial downregulation of the protein levels of p-SrcTyr416 in both Karpas 299 and SUP-M2 cells after siRNA knockdown of the Dvls.

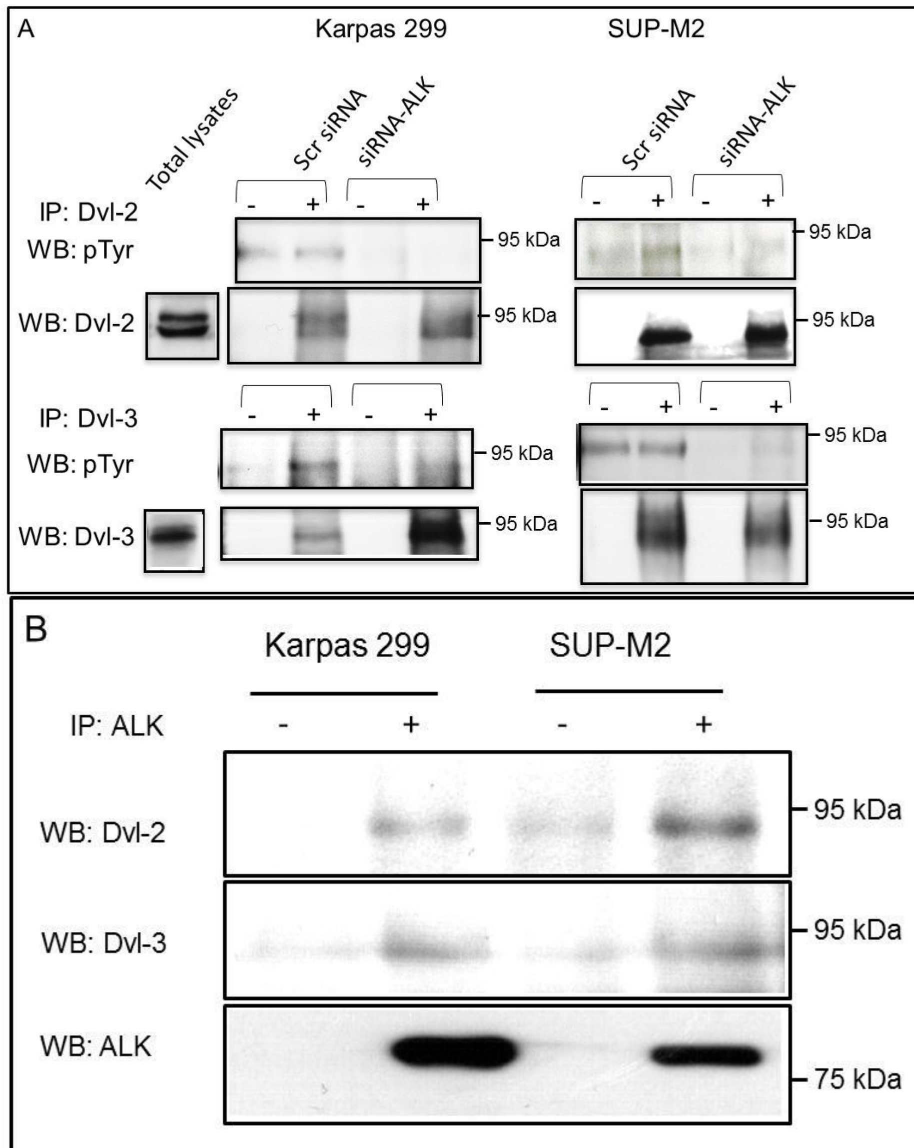


Figure 4.10. NPM-ALK interacts with Dvl-2 and Dvl-3 and contributes to their tyrosine phosphorylation.

A) Immunoprecipitation studies using anti-Dvl-2 (upper panel) or anti-Dvl-3 (lower panel) antibody was performed. Western blots revealed that the levels of phospho-tyrosine of the Dvls were reduced in response to siRNA knockdown of ALK. '+' denotes experiments in which anti-Dvl antibody was used for immunoprecipitation and '-' denotes experiments in which the anti-Dvl antibody was omitted. B) Co-Immunoprecipitation experiments were performed using anti-ALK antibody. Western blots showed the pull-down of Dvl-2 as well as Dvl-3, in Karpas 299 and SUP-M2 cells. '+' denotes experiments in which anti-ALK antibody was used for co-immunoprecipitation and '-' denotes experiments in which the anti-ALK antibody was omitted.

4.4. Discussion

The role of the Dvl proteins in oncogenesis has been an active area of study in recent years. Most of our current knowledge is based on a few studies in solid tumors, such as cancers of the colon (37-39), breast (37,40) and lungs (21,22,41).

We believe that this current study is the first to directly examine whether the Dvl proteins play an oncogenic role in a hematologic malignancy. We demonstrate that Dvl-2 and Dvl-3 are highly expressed in ALK^{+}ALCL. More importantly, we have demonstrated that the Dvls are tyrosine phosphorylated in ALK^{+}ALCL cell lines. Furthermore, we have provided evidence that the Dvls mediate their oncogenic effects in this lymphoma via the WNCPs, as no evidence for the involvement of β-catenin was detected. Lastly, we have showed that the tyrosine phosphorylation of the Dvls in ALK^{+}ALCL can be attributed to NPM-ALK signaling, demonstrating a novel link between an oncogenic tyrosine kinase and the Dvls.

The Wnt/Ca^{2+} pathway is one of the best characterized of the WNCPs (20). Upon Wnt activation, there is a release of intracellular calcium that leads to the activation of calcineurin, a calcium-sensitive phosphatase. Calcineurin, in turn, dephosphorylates NFAT which then migrates to the nucleus where it activates the transcription of several genes including cytokines known to promote T cell proliferation (42,43). NFAT has a well-established role in T cell biology as it regulates a wide range of processes including thymocyte development, as well as T cell activation, differentiation, proliferation and self-tolerance (44). In a previous study, evidence of NFAT activation in ALK^{+}ALCL was presented, and this was believed to contribute to the activated T cell phenotype of this lymphoma (8). Our results directly link the Dvls to the transcriptional activity of NFAT in ALK^{+}ALCL. In view of the well-established connection between calcium and the NFAT transcriptional activity, we hypothesize that the Dvls modulate intracellular calcium levels, and thus, NFAT activity. In support of

this idea, a previously published study demonstrated that the Dvls regulate calcium signaling in Xenopus embryos (45).

In our search for additional down-stream effectors of the Dvls in ALK^{+}ALCL, we examined Src. There are two major reasons for the inclusion of Src in this study. First, Src is activated and biologically important in ALK^{+}ALCL (33). Second, the relationship between Src and the WNCPs has been established in normal physiological processes in animal models (46-48). For example, Src is the down-stream mediator of the anti-apoptotic effect provided by Wnt5a in normal osteoblasts (46). In addition, the Src family kinases have been shown to regulate convergent extension cell movement, an event known to be mediated by the WNCP (48-51). The role of Src in the pathogenesis of ALK^{+}ALCL is well established. It has been shown that Src is a substrate of NPM-ALK (33). Furthermore, targeting Src in ALK^{+}ALCL cell lines, either with a chemical inhibitor or siRNA, resulted in a significant decrease in cell proliferation (33). With this background, we tested the hypothesis that the Dvls mediate their biological effects through a second WNCP via Src. Our findings that siRNA-mediated knockdown to the Dvls down-regulated p-SrcY416 support this notion. As all previous studies demonstrating the Dvls regulate Src activity were done in the context of normal development, we believe that these findings represent the first evidence that the Dvls and Src collaborate in human cancer. Lastly, as the Dvls do not possess intrinsic tyrosine kinase activity, their effects on Src phosphorylation are most likely indirect.

An important novel finding of this study is that NPM-ALK and the Dvl proteins interact, and that NPM-ALK enhances the tyrosine phosphorylation of the Dvls. In a recent publication, it was demonstrated that the tyrosine phosphorylation of Dvls is important for their biological activity. For instance, mutants of dsh (a Drosophila orthologue of the Dvls) that cannot be phosphorylated at tyrosine residue 473 failed to rescue the

Wnt non-canonical planar cell polarity signaling defects observed in dsh null mutants of Drosophila (52). In another study, phosphorylation of the same tyrosine residue in dsh induced by the tyrosine kinase, Abelson (Abl), is important in mediating the biological effects of Abl in Drosophila (53). We believe that the tyrosine phosphorylation of the Dvls by NPM-ALK is biologically important, and likely enhances its signaling through the WNCPs in ALK^+ALCL.

In conclusion, our study has demonstrated an important biological role for the Dvl proteins in the pathogenesis of ALK^+ALCL, and provided the first example of a hematologic malignancy in which the Dvls contribute to cell growth and tumorigenicity. In addition, our results argue that the Dvls transduce signals via the WNCPs, namely via the NFAT and Src, in ALK^+ ALCL. We have also demonstrated that the Dvl proteins are novel NPM-ALK interacting proteins and possibly NPM-ALK substrates.

4.5. References

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➢ Chapter 5

General Discussion

5.1. Thesis overview

In this thesis I have furthered our understanding of the pathobiology of ALK$^+$ALCL through identifying new signaling defects in this type of malignancy and by demonstrating that these deregulated signaling pathways are contributing to the pathogenesis of these tumors either by functioning independently of or in concert with NPM-ALK, the master oncogenic protein in these tumors.

Several previous studies have focused on proving the oncogenic role of NPM-ALK and demonstrating its transforming abilities using both in vitro and in vivo models (1-20). A significant number of these studies have demonstrated that NPM-ALK is mediating its oncogenic abilities through its interaction with and activation of several oncogenic molecules. However, only a few of these studies have addressed the exact mechanistic requirements for the occurrence of such interaction between NPM-ALK and its interacting partner and consequently the activation of this partner (2,11,16).

Studies using transgenic mouse models have shown that the enforced expression of NPM-ALK leads to the development of lymphomas. A significant number of these lymphomas are of plasmablastic or B-cell immunoblastic morphology and phenotype, even when NPM-ALK expression was driven by T-cell–associated antigen promoters (3-8).

Using a very sensitive RT-PCR method, NPM-ALK cDNA has been detected in the peripheral blood cells as well as in the lymphoid tissues of healthy individuals (21,22). Interestingly, this aberrant expression of NPM-ALK cDNA was detected in B lymphocytes and monocytes subpopulations, but not in T cells (21).

Based on these observations from studies of in vivo models and healthy individuals, I hypothesize that although the well-known role of NPM-ALK in promoting lymphoma genesis, it is not the only factor that governs the morphologic, immunophenotypic, and clinical features of NPM-ALK –expressing ALCL and that deregulation of other signaling pathways are required to be involved in order for NPM-ALK to reach its full oncogenic transformation potential.

The main objectives of this thesis are to increase our understanding of the pathobiology of ALK^{+}ALCL, by identifying novel molecular defects that may contribute to the pathogenesis of this type of malignancy, and that may mediate their oncogenic effects either independent of or in cooperation with NPM-ALK. In addition I aimed at further highlighting the biological importance of the already well known signaling pathways' defects (exemplified by SHP1) through examining the detailed mechanistic requirements for their interaction with NPM-ALK.

Through the identification of novel signaling defects and addressing their biological importance in ALK^{+}ALCL, my work in this thesis furthered our current understanding of the complexity of the pathogenesis of this type of malignancy and proposed novel potential therapeutic targets for multi-targeting therapeutic strategies against this tumor.

5.2. The importance of studying NPM-ALK/SHP1 interaction

One of the key mechanisms for maintaining signaling homeostasis is the tight regulation of proteins' phosphorylation mediated by kinases and their dephosphorylation mediated by phosphatases (23,24). Considerable evidence indicates that increased signaling by protein tyrosine kinases result in enhancement of several signaling pathways that contribute to oncogenesis(24). Protein tyrosine phosphatases (PTP) represent one of the most important regulators for the tyrosine kinase activity. Depending

on the cellular context, PTPs either exert oncogenic or tumor suppressor functions (25). Thus, it is mandatory to have a better understanding of the basic biology of PTPs in different types of malignancies in order to help choose the appropriate approach for targeting PTPs in cancer therapeutics.

A) In the context of ALK^{+}ALCL

According to our hypothetical model, other molecular defects are contributing to the tumorigenicity of NPM-ALK in ALK^{+}ALCL. The absence of expression of the protein tyrosine phosphatase SHP1 in the vast majority of ALK^{+}ALCL due to epigenetic modifications is one of the best examples for such signaling defects (26). It is considered one of the major signaling defects that contribute to the tumorigenicity of NPM-ALK by leaving its constitutive tyrosine kinase activity unchecked.

Previous studies by our research group and others revealed the biological importance of SHP1 restoration in ALK^{+}ALCL (27-29). In some of these studies, SHP1 has been shown to bind to NPM-ALK. However the detailed mechanisms of such interaction and its direct effect on the biological function of SHP1 have not been elucidated. Thus, one of the primary aims of this thesis was to delineate the detailed molecular requirements in both NPM-ALK and SHP1 for such interaction to occur between these two proteins.

As demonstrated in chapter 2, by overexpression of different combinations of NPM-ALK mutants and SHP1 mutants in GP293 cells, followed by co-immunoprecipitation studies, I identified that at least part of the tyrosine phosphorylation of NPM-ALK is required for its binding to SHP1; in addition I identified the specific tyrosine residue (Tyr 343) of NPM-ALK that mediates this interaction. Furthermore as regard of SHP1, I demonstrated that SHP1 mainly requires both of its SH2 domains to

mediate its interaction with NPM-ALK. The results of the clonogenic assay studies in the same chapter clearly emphasize the requirement of a direct physical interaction between SHP1 and NPM-ALK in order for the former to exert its tumor suppressive functions on the latter. Specifically, I demonstrated that the tumorigenicity of NPM-ALK in cells co-expressing SHP1 was significantly less than that seen in cells co-expressing NPM-ALK and SHP1 empty vector. In contrast, there was no significant difference between tumorigenicity of the Y343F mutant of NPM-ALK in cells co-expressing either empty vector or SHP1.
The study performed in chapter 2 is considered the first to extensively delineate the detailed mechanisms required for a mutual interaction between an oncogenic tyrosine kinase such as NPM-ALK and a phosphatase of tumor suppressive functions such as SHP1. Furthermore, this study is the first to directly address the biological importance of this physical interaction and shed the light on the importance of SHP1 therapeutic targeting in ALK^{+}ALCL.

Contrary to SHP1 which exerts tumor suppressor functions in ALK^{+}ALCL, the related tyrosine phosphatase SHP2 contributes to the tumorigenesis of this malignancy (20). Although it has been demonstrated that SHP2 binds to NPM-ALK and contributes to its tumorigenicity, the detailed mechanisms of such interaction has not been elucidated (20). It would be plausible to study the exact mechanisms of interaction between NPM-ALK and SHP2 to get a better understanding of how this phosphatase mediates NPM-ALK oncogenic potential.

B) In the context of other malignancies

Most of the studies reported the down-regulation or absence of SHP1 expression in hematopoietic tumors, highlighting the tumor suppressive functions of SHP1 in this category of malignancies (26,30-32). In contrary to hematopoietic tumors, SHP1 has been reported to be overexpressed in

solid tumors including breast, ovarian and pancreatic cancers (33-36). However the exact role of SHP1 contribution to the oncogenesis of these malignancies has not been extensively studied. One possibility that requires further studies is that SHP1 might potentiate signaling via tyrosine kinases in these tumors. Thus I believe that the study in chapter 2 also sets a model to be followed in future studies to examine how phosphatases in general and SHP1 in particular exert their tumor suppressive or potentiating effects on oncogenic kinases in different types of malignancies.

5.3. Aberrant expression of the embryonic stem cell factor Sox2 and its biological importance in ALK^+ALCL

In keeping with the main objective of this thesis, to identify novel signaling defects in ALK^+ALCL, in chapter 3, I identified the aberrant expression of the stem cell transcription factor Sox2 in ALK^+ALCL and tumor samples.

A) Implications in ALK^+ALCL anaplastic morphology

The expression of Sox2 in ALK^+ALCL cells is considered 'aberrant' because Sox2 expression in hematopoietic cells is normally restricted to the CD34+ stem cells (37), and normal peripheral T lymphocytes do not express this protein. Thus, in normal T-cell development, Sox2 expression is turned off by the time of initiation of T-cell differentiation.

The results from the study in chapter 3 showed that the aberrant expression of Sox2 in ALK^+ALCL is mediated by NPM-ALK. Based on this finding, a question might arise regarding the relation between Sox2 aberrant expression and the characteristic anaplastic morphology of these tumors. More specifically, "Does Sox2 aberrant expression contribute to the characteristic anaplastic morphology of ALK^+ALCL?" One of the speculated answers for this question might be that the aberrant expression of Sox2 in the differentiated T cells coverts them back to their

dedifferentiated state. This speculation is highly supported by the ability of Sox2 to induce iPS in terminally differentiated cells (37,38). Future studies are mandatory to examine the effect of Sox2 aberrant expression on the highly anaplastic morphology of ALK^{+}ALCL and to delineate the exact mechanisms underlying this effect.

B) Importance of addressing the transcriptional activity of Sox2

The expression of Sox2 in these tumors is nuclear; a finding that is keeping with the fact that Sox2 is a transcription factor. By creating stable clones of ALK^{+}ALCL cell lines expressing a Sox2 reporter construct, I showed that despite the ubiquitous nuclear expression of Sox2 in all the tumor cell populations, its transcription activity is only limited to a small fraction of cells in these populations. This finding is of great interest for many reasons; first it makes the study performed in chapter 3, the first to investigate the role of Sox2 in cancer biology based on its transcriptional activity rather than its sheer protein expression. Second, it implies that this small cell fraction might serve as the CSCs or the tumor initiating cells which normally represent a small bulk of tumors (39,40). Third, it mandates the performance of further studies in order to delineate the exact mechanisms underlying this difference in the transcription activity of Sox2 despite its ubiquitous expression by all the tumor cells.

The functional studies I performed in chapter 3, demonstrated the significant correlation between the transcription activity of Sox2 and several biological parameters. Specifically, the transcription activity of Sox2 has been shown to promote ALK^{+}ALCL invasiveness and tumorigenicity (both *in vitro* and *in vivo*). In addition the cell population in which Sox2 is transcriptionally active is more resistant to conventional chemotherapeutic agents as doxorubicin. These findings clearly demonstrate that the $Sox2^{active}$ cells in ALK^{+}ALCL are exhibiting a biological profile that is reminiscent of that of CSCs or tumor initiating cells. In further support of this notion is the finding that the biological

difference between $Sox2^{active}$ and $Sox2^{inactive}$ cells is not attributed to a difference in the expression/activation of the signaling pathways which are well known to be implicated in ALK^{+}ALCL pathobiology, particularly NPM-ALK and STAT3. Thus the difference in the previously mentioned biological attributes that puts the ALK^{+}ALCL $Sox2^{active}$ cells in the category of CSCs is genuine for the Sox2 transcription activity in these cells.

In both the *in vitro* and *in vivo* studies performed in chapter 3, I found that $Sox2^{active}$ cells give rise to $Sox2^{inactive}$ cells. This finding further supports the speculation that $Sox2^{active}$ cells might represent the tumor initiating cells in ALK^{+}ALCL.

The mechanisms underlying the biological differences between $Sox2^{active}$ and $Sox2^{inactive}$ cells could be attributed to preferential expression of certain proteins in the $Sox2^{active}$ population but not in the $Sox2^{inactive}$ one and these preferentially expressed proteins are encoded by genes that are expected to be regulated by the transcription activity of Sox2. In chapter 3, I have identified the differential expression of three proteins that their encoding genes are known to be regulated by Sox2. These proteins are BCL2, Notch1 and PDGFR-alpha (44,45); of these three the last two were expressed at higher levels in the $Sox2^{active}$ population and have been previously shown to be implicated in ALK^{+}ALCL pathogenesis(46,47). However, through further studies, the characterization of the full proteomic profile downstream to the Sox2 transcription activity will give a better understanding for the biological difference between $Sox2^{active}$ and $Sox2^{inactive}$ cells. Taking into consideration the fact that Sox2 in ESCs functions in concert with other pluripotent transcription factors(41,42) and has been recently shown to bind to approximately 60 binding partners in ESCs(43), one might hypothesize that the profile of the binding partners and co-factors of Sox2 in the $Sox2^{active}$ cells should be different from the corresponding profile in the $Sox2^{inactive}$ cells of ALK^{+}ALCL. Further studies are required to address this point.

C) Therapeutic implications of Sox2 in ALK$^+$ALCL

The findings that Sox2^{active} cells are more resistant to conventional chemotherapeutic agents and more sensitive to the specific ALK inhibitor Crizotinib are in agreement with the notion that these cells could be the tumor initiating cells or the CSCs in ALK$^+$ALCL. The same finding encourages future studies for designing specific therapies that target Sox2 for the treatment of ALK$^+$ALCL, and further supports the importance of multi-target therapeutic approaches for the treatment of this type of malignancy.

D) Relation to the hypothetical model of this thesis

Overall, the study in chapter 3 provides several evidences to support the general hypothetical model of this thesis. First, the aberrant expression of Sox2 is due to STAT3 activation at the transcription level, and the activation of STAT3 is well known to be multifactorial in ALK$^+$ALCL. Despite the over-expression of NPM-ALK in two different lymphoma cell lines and the subsequent induction of phosphorylated STAT3 in these cells, Sox2 expression is not induced. This finding suggests that other factors in the regulatory network controlling Sox2 expression are specifically functioning in ALK$^+$ALCL. This finding further supports the general hypothesis of this thesis that NPM-ALK requires other molecular deregulations specific to ALK$^+$ALCL in order to reach its full oncogenic abilities.

Second, the differential transcriptional activity of Sox2 between Sox2^{active} and Sox2^{inactive} cells could not be simply attributed to NPM-ALK/STAT3 expression, as there is no difference in NPM-ALK/STAT3 expression/activation between the two cell populations. Instead other factors namely, Sox2 binding partners or Sox2 transcription co-factors must be differentially expressed/activated among these two populations to contribute to such preferential transcription activity of Sox2 in Sox2^{active} cells. Third, the difference in the biological characteristics of these two cell

populations might be related to the preferential expression of Sox2 downstream target genes in the Sox2^{active} cells. At this point, I identified that the Sox2^{active} population express higher levels of Notch1 and PDGFR-alpha. Interestingly, the fact that Notch1 expression in ALK^{+}ALCL is independent of NPM-ALK expression further supports our findings and our general hypothetical model (46).

5.4. Activation of disheveled proteins as mediators of the Wnt non-canonical pathway and their biological importance in ALK^{+}ALCL

One of the fundamental objectives of this thesis was to identify novel cellular signaling defects that might contribute to the pathogenesis of ALK^{+}ALCL. The Wnt signaling pathway is of great biological importance in both normal development and cancer (48). As mentioned in chapter 1, the Wnt signaling has a well-established role in the regulation of hematopoiesis and lymphocyte development (49,50). This specific implication of the Wnt pathway in regulating lymphocyte development, made the exploration of this pathway in ALK^{+}ALCL of special interest as regard to the scope of this thesis.

Several studies demonstrated the role of Wnt pathway deregulation in the pathogenesis of cancers including hematopoietic tumors (48,49,51,52). In ALK^{+}ALCL specifically, a recent study has identified that β-catenin, which is known as the downstream effector of the WCP to be constitutively active in ALK^{+}ALCL (53). In addition, β-catenin contributes to cell growth in these tumors. The findings of this study gave me an insight to explore the status of other Wnt pathway members in ALK^{+}ALCL.

In this thesis, a preliminary study using a Wnt oligoarray was done to identify other members of the Wnt pathway that are expressed in ALK^{+}ALCL. A number of proteins that belong to the Wnt pathway were identified. Of these proteins, disheveled proteins 2 and 3 were of interest to me to pursue my studies on, as disheveled proteins represents the hub of Wnt signaling (54). In other words disheveled proteins have the ability to

function in different Wnt pathways, either canonical or non-canonical depending on the cell type and the presence or absence of other components of the Wnt pathway. In addition, the biological significance of Dvls' activation has not been previously described in hematologic malignancies including ALK^+ALCL. In chapter 4, I reported the expression and activation of Dvl-2 and Dvl-3 in ALK^+ALCL cell lines and tumor samples and demonstrated that these proteins contribute to cell growth and tumorigenicity of ALK^+ALCL. Furthermore, I demonstrated that Dvl-2 and Dvl-3 mediate their biological effects through the Wnt non-canonical pathway (β-catenin- independent), particularly the Wnt/Ca^{2+} pathway.

One of the novel findings in this study is that Dvls are functioning through β-catenin- independent mechanisms. Specifically, downregulation of Dvls did not result in any changes in the protein levels of β-catenin or its inhibitor phospho-GSK-3β. This finding raises the question of how β-catenin is constitutively active in ALK^+ALCL. Several possibilities could be considered regarding this point. One of these possibilities might be the presence of an activating mutation of β-catenin that prevents its phosphorylation and proteasomal degradation, as the case in some of the gastrointestinal tumors (55). Another possibility to be considered is the presence of inactivating mutations of the proteins normally involved in the β-catenin destruction complex such as APC or Axin, which is the scenario in a subset of colorectal tumors (55). Thus, future studies are required to explore the exact mechanisms underlying the constitutive activation of β-catenin in ALK^+ALCL.

Another novel finding of the study performed in chapter 4, is that Dvl proteins mediate the phosphorylation of Src in ALK^+ALCL. Dvl proteins have no kinase activity and they function as scaffolding proteins (54). Of note NPM-ALK has been previously shown to associate with and activate Src kinase pp60src and this association and activation is important for

mediating NPM-ALK oncogenesis(11). Thus, Dvl proteins might contribute to Src phosphorylation through their interaction with NPM-ALK and being part of the protein complex that involves Src, Dvl and NPM-ALK. In other words, Dvl proteins through their interactions with NPM-ALK and Src might bring these two proteins in close proximity to each other, where Src could be phosphorylated by NPM-ALK. Another possible mechanism that could explain Dvl mediated Src phosphorylation is that Dvl proteins might interact with Src and result in the release of the intramolecular auotinhibitory mechanisms that govern Src activation. The release of Src autoinhibitory mechanisms may facilitate Src activation by other kinases, including NPM-ALK. This speculation is supported by the findings of a recent study in which Dvl-2 has been demonstrated to bind to the SH3 domain of Src, thus disrupting the Src SH2/SH3 interaction and releasing the Src autoinhibition(56). Overall, the finding that Dvl proteins contribute to Src activation in ALK^{+}ALCL represents a point of cross talk between the Wnt pathway and one of the critical proteins that function downstream to NPM-ALK. This finding gives insights on the complexity of cellular signaling involved in NPM-ALK oncogenic network and places Src for the first time as one of the downstream effectors of Wnt pathway in human cancer.

In regard to the finding that NPM-ALK contributes to Dvls tyrosine phosphorylation, it is worthy to mention that the activation/phosphorylation of Dvl proteins is not only through tyrosine phosphorylation but also phosphorylation of serine and threonine residues contribute to the activation/phosphorylation of these proteins (57). Disheveled proteins become hyperphosphorylated up on Wnt pathway activation, specifically Wnt binding to its Fz receptors, results in disheveled activation by Fz through an unknown mechanism (48,58). In the preliminary oligonucleotide array study, both canonical (Wnt 3) and non-canonical (Wnt 7b and Wnt 11) ligands have been identified, in addition to different

members of the Fz receptors. Thus it is likely that the activation/phosphorylation status of disheveled proteins in ALK^{+}ALCL is mediated by the activation of the Wnt pathway, and part of this activation/phosphorylation is mediated by NPM-ALK through its cross talk to the Wnt pathway.One recent study have identified the exact phosphorylated residues in Drosophila Dsh using a mass-spectroscopic approach, and in the same study, the functional significance of these phosphorylations has been described using in vivo models(57). Similar future studies are required to be performed in order to delineate the phosphorylation profile of human Dvl proteins in ALK^{+}ALCL and its functional significance specific to the phosphorylation of certain residues.

One of the novel findings of the Dvl study is the identification of NFAT as another point of cross talk between the Wnt non canonical pathway and NPM-ALK. Based on the fact that NPM-ALK contribute to NFAT activation (59), and our finding that downregulation of Dvls correlates with downregulation of NFAT transcription activity, we hypothesize that NPM-ALK and Wnt pathway contribute to NFAT activation in ALK^{+}ALCL. Since the transcriptional activity of NFAT is regulated by changes in intracellular calcium levels, we used an NFAT reporter construct to assess the changes in NFAT transcription activity as a read out for the activation of Wnt/Ca^{2+} pathway in ALK^{+}ALCL. Recently, a similar approach has been used to assess the activation of Wnt/Ca^{2+} pathway in CML(60). Considering the well-established role of NFAT in T cell biology (61), it is speculative that NFAT activation in ALK^{+}ALCL contributes to the tumorigenesis of this malignancy through regulation of several genes involved in T cell proliferation and activation. Further future studies are mandatory to specifically delineate the exact NFAT target genes in ALK^{+}ALCL that might contribute to the pathogenesis of these tumors.

The biological significance of Dvls as described in chapter 4, proposes these proteins as novel therapeutic targets in ALK^{+}ALCL. Up to date, there is no known pharmacological agent to target Dvls in clinical trials. Few groups have developed small molecule inhibitors and targeting peptides to block the PDZ domain of Dvl proteins resulting in inhibition of the interaction between Dvl and the Fz receptors (62-64). However, the fact that there are approximately 500 PDZ domains in the human proteome with many of these domains having similar binding sites (65), makes it challenging for the development of small inhibitors that target specific PDZ domains. Besides, the PDZ domain of Dvls is not the only domain that mediates their scaffolding function (66). The involvement of other domains of Dvl proteins in mediating their functions has been demonstrated. For example the most N-terminal domain DIX has been proven to be important for Dvl binding to Axin and hence mediating the WCP. For the Wnt non canonical pathways; Wnt/PCP and Wnt/Ca^{2+} pathways, the Dvl DEP domain is involved (66). Thus further studies are required to help in the design of small molecule inhibitors that specifically target different domains of the Dvl protein.

Overall, the findings of the study in chapter 4 are in agreement with our hypothetical model that other signaling defects represented by the activation of Wnt non-canonical pathway contribute to the tumorigenicity of ALK^{+}ALCL and this pathway mediates its biological effects in concert with NPM-ALK.

5.5. Closing remarks

Overall, this thesis provided insight into the complexity of signaling in ALK$^+$ALCL, and set a model for studying the complexity of cancer pathogenesis. In this thesis, I identified dysregulation of novel signaling pathways in ALK$^+$ALCL (Figure 5.1) and demonstrated the biological importance relevant to this dysregulation in the pathogenesis of this type of malignancy.

In the first study , I delineated in details the molecular requirement of a phosphatase with tumor suppressive functions (SHP1) to bind to an oncogenic kinase (NPM-ALK) and the reflection of such binding on the tumor biology of ALK$^+$ALCL. In my second study, I reported for the first time the aberrant expression of the stem cell marker Sox2 in ALK$^+$ALCL. Most importantly I demonstrated that the biological role of Sox2 in these tumors is correlated with its transcription activity that was detected only in a small subset of these tumors. In the third study I identified for the first time the activation of the Wnt non-canonical pathway in ALK$^+$ALCL and demonstrated that this pathway mediates its biological effects through the activation of disheveled proteins. In addition this pathway functions in concert with NPM-ALK to contribute to the pathogenesis of ALK$^+$ALCL.

I believe that the work that has been done in this thesis furthers our knowledge of the pathobiology of ALK$^+$ALCL and sheds the light on the complexity of cancer pathogenesis in general and the pathogenesis of ALK$^+$ALCL in particular. By demonstrating the collaborative role of different components of different signaling pathways of the molecular network in ALK$^+$ALCL, I've proved my hypothesis that the pathobiology of these tumors is multifactorial and that deregulation of other signaling pathways contribute to the oncogenesis mediated by NPM-ALK.

The study model that has been introduced in this thesis provides the rationale for the design of novel multi-target therapeutic approaches for ALK^{+}ALCL in order to disrupt the additive and/or synergistic effects of these collaborative signaling defects.

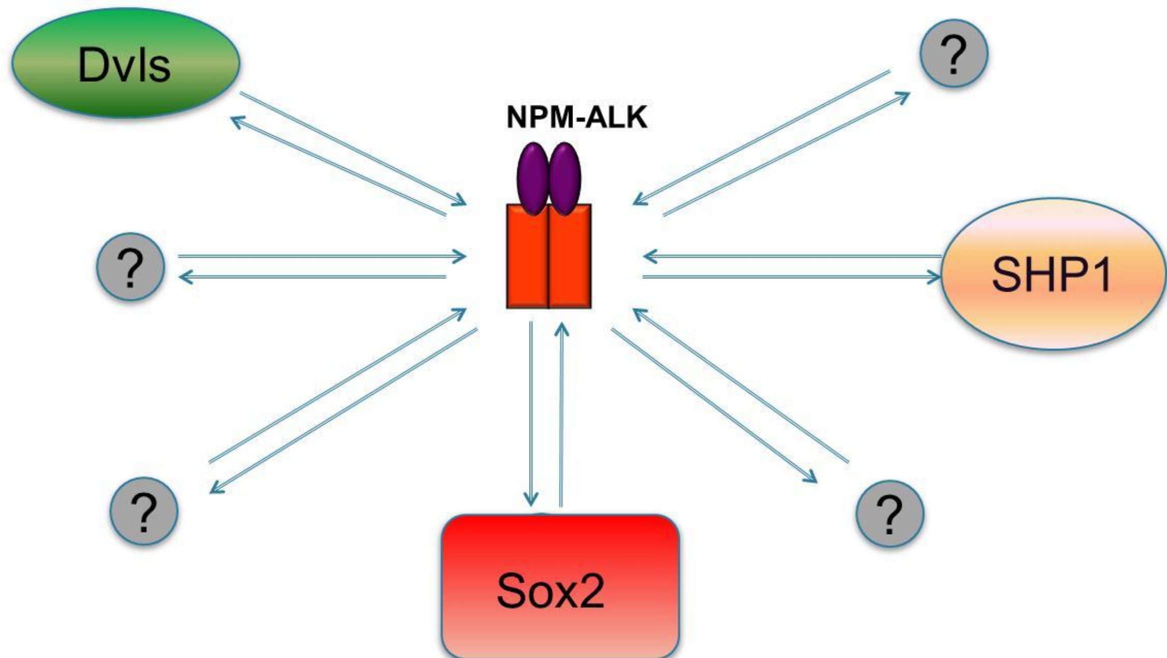

Figure 5.1. Summary of the different dysregulated signaling pathways studied in the current thesis.

According to the hypothetical model of this thesis, dysregulation of multiple signaling pathways is required for NPM-ALK to reach its full oncogenic potential. SHP1 has to interact with NPM-ALK in order to regulate its signaling. Sox2 is aberrantly expressed under NPM-ALK/STAT3 signaling and the heterogenic transcription activity of Sox2 correlates with its tumorigenic potential. Dvls as modulators of the Wnt pathway interact with NPM-ALK and contribute to the pathogenesis of ALK^{+}ALCL through the WNCP.

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