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CHARACTERIZATION OF THE INTERACTION BETWEEN NUMB AND THE ANAPLASTIC LYMPHOMA KINASE (ALK)

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CHARACTERIZATION OF THE INTERACTION BETWEEN NUMB AND THE ANAPLASTIC LYMPHOMA KINASE (ALK)

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By

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Abstract

Originally identified as a cell fate determinant, Numb has been found to also act as an epithelial polarity protein, an adaptor, an endocytotic regulator and a tumor suppressor. Recently, anaplastic lymphoma kinase (ALK) was identified as a potential Numb-interacting protein in our lab. ALK is a receptor tyrosine kinase and is expressed mainly within the neonatal brain. Thus ALK likely plays an important role in development of the nervous system. In addition, mutated ALK is associated with a number of cancer cell lines and tumors. In this thesis, the interaction of Numb with ALK was confirmed and was found to involve the Numb-PTB domain. Two Numb putative binding motifs NMAF (1477-1480) and NYGY (1583-1586) were identified on ALK. The Numb-ALK interaction was enhanced upon ALK activation and attenuated upon ALK inhibition. Further investigations suggested that Numb regulated ALK protein expression levels, its cleavage, nuclear localization and downstream Erk signaling.

Keywords: Numb, ALK, RTK, interaction, activation, tyrosine phosphorylation

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List of Abbreviations

Akt	v-akt murine thymoma vial oncogene homolog 1
ALCL	Anaplastic large cell lymphoma
ALK	Anaplastic lymphoma kinase
aPKC	atypical protein kinase C
APP	Amyloid precursor protein
ATIC	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase
BSA	Bovine serum albumin
CDK5	Cyclin-dependent kinase 5
CNS	Central nervous system
DCM	Dichloromethane
DLBCLs	Diffuse large B-cell lymphomas
DLG	Discs large
DMEM	Dulbecco's modified eagle's medium
DMF	Dimethylformamide
DPF	Asp-Pro-Phe
ECL	Enhanced chemiluminescence
EHD	ESP15-homology-domain containing
EPS	Epidermal growth factor receptor pathway substrate
ERB2	v-erb-b2 erythroblastic leukemia viral oncogene homology 2

ERK	Extracellular signal-regulated kinase
EVH1	Ena-VASP homology
FBS	Fetal bovine serum
FPLC	Fast protein liquid chromatography
FRS2	Fibroblast growth factor receptor substrate 2
Gli 1	Glioma-associated oncogene family zinc finger 1
GMC	Ganglion mother cell
GST	Glutathione S- transferase
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IMTs	Inflammatory myofibroblastic tumors
IP	Immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	Insulin receptor
IRS	Insulin receptor substrate
JAK	Janus kinase
JAM	Junctional adhesion molecule
LC/MS	Liquid chromatography/mass spectrometry
LDLa	Low density lipoprotein receptor class A
LGL	Lethal giant larvae
Lnx	Ligand of numb-protein X

PC12	Pheochromocytoma
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PI3K	Phosphoinositide 3-kinase
PLC	Phospholipase-C
PVDF	Polyvinylidene fluoride
PRR	Proline rich region
PRRi	PRR region with sequence insertion
PRRo	PRR region without sequence insertion
PSSM	Position-specific scoring matrix
PTB	Phosphotyrosine binding
PTBi	PTB domain with sequence insertion
PTBo	PTB domain without sequence insertion
PTN	Pleiotrophin
RIPK5	Receptor-interacting serine/threonine-protein kinase 5
Rme	Required for receptor-mediated endocytosis
ROS1	v-ros UR2 sarcoma virus oncogene homolog 1
RPTPζ/β	Receptor protein tyrosine phosphatase-ζ/β
RTK	Tyrosine receptor kinase
SCC	Squamous cell carcinoma
SCRIB	Scribble

SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
SHC	Src homology 2 domain-containing-transforming protein C
SMALI	Scoring matrix-assisted ligand identification
SOP	Sensory organ precursor
STAT	Signal transducer and activator of transcription
Sypt I	Synaptogamin-1
TAP	Tandem affinity purification
TBST	Tris buffered saline with Tween
TFA	Trifluoroacetic acid
TFG	TRK-fused gene
TPM3	Tropomyosin 3
TRK	Neurotrophic tyrosine kinase
WB	Western blot
WT	Wild type
WW	Trp-Trp
ZO	Zonula occludens

Chapter 1: Introduction

1.1 Numb

1.1.1 The discovery of Numb as a neuronal fate determinant protein

Numb was originally found to be involved in the determination of sensory organ precursor (SOP) cell asymmetric division in *Drosophila melanogaster* (1). SOPs are neural precursors in the *Drosophila* peripheral nervous system, and each sensory organ develops from a sensory organ precursor through several rounds of asymmetric cell division. A SOP of the external sensory bristle organ divides asymmetrically along the anterior-posterior axis and generates a IIa and a IIb cell of different developmental potential. During mitosis, Numb localizes to posterior cell membrane and is inherited by the IIb cell that gives rise to a neuron and a sheath cell upon an additional round of division (2, 3) (Fig. 1.1). Loss of Numb function causes symmetric division of the SOP cell and the generation of two IIa daughter cells (4). Numb also plays an important part in the development of central nervous system (CNS). Numb was found to localize asymmetrically in dividing *Drosophila* neuroblasts. Numb is preferentially segregated into the ganglion mother cell (GMC), as a neuroblast divides asymmetrically to generate another neuroblast and a GMC (2, 5). This function of Numb in asymmetric cell division appears to be conserved in mammals since, in asymmetrical division of neuroepithelial cells in the mouse cortex and rat retina, the Numb-inherited daughter cell has a distinct fate from the cell that lacks Numb (3).

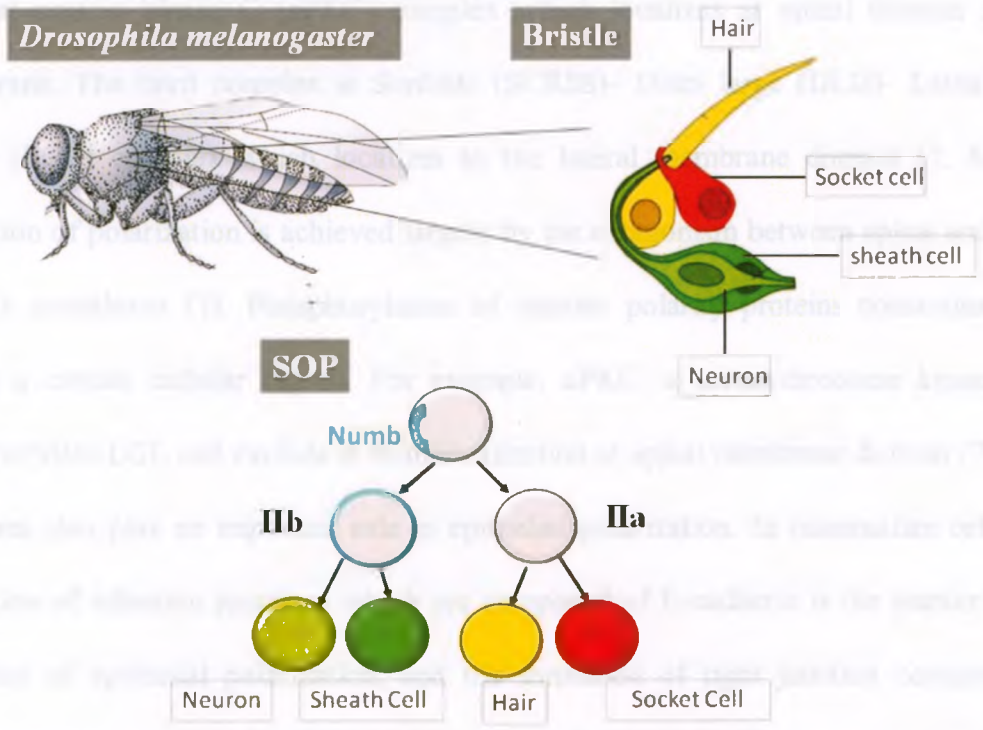

Figure 1.1 Sensor organ precursor (SOP) in *Drosophila melanogaster*. During the development of the *Drosophila* peripheral nervous system, Numb is asymmetrically distributed; the SOP cell asymmetrically divides twice, generating the four cells of a sensory bristle: a hair, a socket, a neuron and a sheath cell.

1.1.2 An epithelial polarity protein

Cell polarization is a very important physiology trait, which significantly contributes to oriented cell migration, asymmetric divisions of neural precursor cells and stem cells during development (6), and epithelial cell polarization. In epithelial cells, three major polarity complexes participate in the epithelial polarization. The first complex is Crumbs- Protein associated with Lin-7 1(PALS1) (Stardust, in *Drosophila*)- Protein associated to tight junction (PATJ) complex. The second complex is Partitioning defective 3 (PAR3) (Bazooka, *in Drosophila*)- Partitioning defective protein 6 (PAR6)-

atypical protein kinase C (aPKC) complex which localizes at apical domain of cell membrane. The third complex is Scribble (SCRIB)- Discs large (DLG)- Lethal giant larvae (LGL) complex which localizes to the lateral membrane domain (7, 8). The formation of polarization is achieved largely by the antagonism between apical and basal polarity complexes (7). Phosphorylation of various polarity proteins constrains them within a certain cellular region. For example, aPKC, a serine/threonine kinase, can phosphorylate LGL and exclude it from localization at apical membrane domain (7). Cell junctions also play an important role in epithelial polarization. In mammalian cells, the formation of adhesion junctions which are composed of E-cadherin is the marker of the initiation of epithelial polarization, and the formation of tight junction composed of transmembrane proteins (occludin, claudins, and Junctional adhesion molecule (JAM)) and peripheral membrane proteins (e.g. Zonula occludens (ZO)) signals the establishment of cell polarity. In addition, interactions also exist between polarity complexes and cell junctions. For example, the PAR3-PAR6-aPKC complex localizes at tight junctions through the interaction between PAR3 and JAM (8, 9).

Abundant evidence has shown that Numb is critical for cell polarity, especially in asymmetric cell division. In mammalian epithelial cells, endogenous Numb is localized primarily to the basolateral cortex, indicating that Numb maintains a polarized distribution in non-mitotic cells (10). Additionally, more and more evidence indicates that Numb also has an important role in epithelial cell polarization. Actually, previous work in our lab and Nishimura's work show that Numb can interact with Par3 and aPKC in epithelial Madin Darby canine kidney (MDCK)II cells, Hela cells and rat brain (11, 12). Therefore, it is evident that Numb could form a complex with PAR3-PAR6-aPKC in

the epithelial cell. In addition, phosphorylated by aPKC, Numb is constrained mainly to the basolateral cortex in mammalian epithelial cells (3). Furthermore, it can interact with E-cadherin and localize to the adherens junction in radial glial cells (4) and MDCKII epithelial cells (11).

1.1.3 The structure and isoforms of Numb

Structurally, Numb acts as an adaptor protein. Numb proteins from all the species share an N-terminal phosphotyrosine binding (PTB) domain, a proline rich region (PRR) and C-terminal Asp-Pro-Phe (DPF) and Asn-Pro-Phe (NPF) motifs (Epidermal growth factor receptor pathway substrate (EPS)15 homology domain binding motif) (Fig. 1.2) (13). Only one Numb protein isoform is expressed in *Drosophila*, while four are expressed in mammalian cells. The mammalian isoforms represent combinations of two different PTB domains (with or without sequence insertions) and two different PRRs (with or without sequence insertions) (13) (Fig. 1.3).

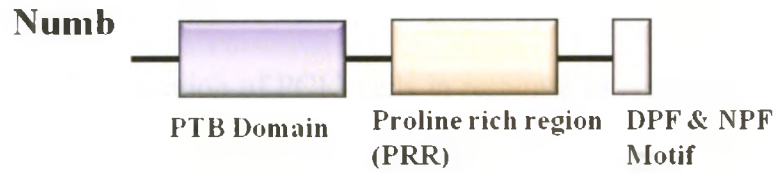

Figure 1.2 Numb protein domains. The Numb protein contains three distinct regions: phosphotyrosine binding (PTB) domain, proline-rich region (PRR), and C-terminal DPF and NPF motifs.

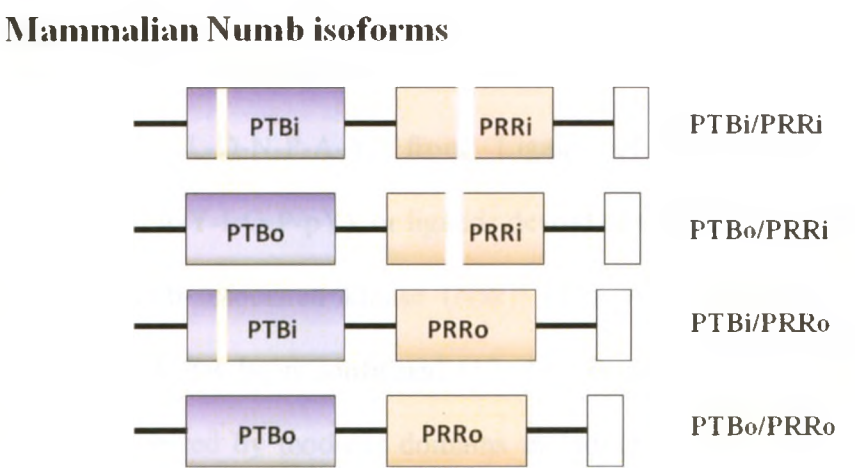

Figure 1.3 Four mammalian Numb isoforms. Mammalian Numb has four isoforms: PTBi/PRRi, PTBo/PRRi, PTBi/PRRo, PTBo/PRRo, where i and o represent domains with or without an insert, respectively.

Different mammalian Numb isoforms have distinct cellular localization and function. Numb isoforms with an insertion in the PTB domain (PTBi-Numb) associate with plasma membrane, while the isoform without the insert (PTBo-Numb) is cytoplasmic in polarized MDCK cells (13). It has also been shown that PTBo-Numb enhances neural differentiation of PC12 cells in response to Nerve growth factor (NGF) and increases apoptosis upon NGF withdrawal (14). On the other hand, Numb isoforms containing the short PRR domain (PRRo-Numb) promote differentiation, while the isoforms with long PRR domain (PRRi-Numb) promote proliferation (15).

1.1.4 Numb mediates the formation of multi-protein complexes

The Numb PTB domain can interact with a variety of peptide ligands containing either tyrosine (e.g., L-D-N-P-A-Y, from Ligand of numb-protein X (Lnx)), phosphotyrosine (e.g., A-Y-I-G-P-pY), or ligands devoid of tyrosine (e.g., G-F-S-N-M-S-F-E-D-F-P, from Numb-associated kinase (Nak)) (16). And the interaction between Numb and Lnx or Nak has been confirmed (17, 18). In addition, the PRR regions of Numb could be recognized by modular domains including the Src homology 3 (SH3) domain, the Trp-Trp (WW) and the Ena-VASP homology (EVH1) domains (19). The interaction between Numb and SH3 domains of SRC-family tyrosine kinases has been identified (20).

1.1.5 The endocytic function of Numb

The function of Numb in endocytosis was discovered when both Eps15, a component of the endocytic machinery and α adaptin, a major component of clathrin-coated pits were shown to interact with Numb via the C-terminal DPF and NPF motifs (21, 22). Subsequently, the interaction between Numb and ESP15-homology-domain containing (EHD)/ Required for receptor-mediated endocytosis (Rme)-1/Pincher family of endocytic proteins was demonstrated (23). Numb has been shown to control the endocytosis of some transmembrane proteins, including the Notch receptor (24), the cell adhesion receptor integrin (12), the leukocyte adhesion receptor P-selectin (25), Sanpodo (26), and amyloid precursor protein (APP) (27). The endocytosis-dependent function of Numb is isoform-dependent. For example, only Numb3 (PTBo/PRRi-Numb) can bind to and promote the internalization of the P-selectin receptor (25). In addition, the expression

of the PTBo-Numb isoforms significantly increased the accumulation of APP in early endosomal and recycling compartments, while the expression of PTBi-Numb resulted in marked reduction and lysosome mediated degradation of cellular APP (27).

1.1.6 The tumor suppressive function of Numb

Numb has been shown to be a tumor suppressor in some tumors. Loss of Numb is observed in breast cancer (28) and salivary gland carcinomas (29). In *Drosophila*, mutation of Numb causes differentiation-deficiency and tumor-like proliferation of neuroblasts (30). Three mechanisms have been suggested to account for the tumor suppression function of Numb. First, Numb can stabilize the p53 tumor suppressor and protect it from ubiquitination and subsequent degradation (31). Second, Numb can antagonize Notch signaling by promoting Notch receptor endocytosis. Notch is a widely recognized oncogene in breast cancer and plays a significant role in preserving the stem cell phenotype and preventing differentiation (32). Third, Numb could suppress the Hedgehog signaling pathway by targeting the transcription factor Glioma-associated oncogene family zinc finger 1 (Gli1) for ubiquitination. In the external germinal layer, Hedgehog signaling keeps the progenitor-cell population in an undifferentiated state while promoting cell expansion. Aberrantly regulated Hedgehog signaling is responsible for the development of medulloblastoma (33).

1.2 Numb-interacting proteins

1.2.1 Protein modular domains and the PTB domain

Regulatory proteins play important roles in cellular signal transduction. These proteins frequently contain modular domains that either have a catalytic function (such as kinase activity), or interact with other proteins, phospholipids, nucleic acids or small molecule second messengers (34). Isolated interaction domains can fold independently, and can be incorporated into a larger polypeptide leaving their ligand-binding surface available (35). The modular domains are able to recognize and bind to conserved amino acids, providing a mechanism for the formation of specific signaling complexes (34, 35).

One of these modular domains is the PTB domain, which was first discovered as a phosphotyrosine binding domain in 1994 (36). However, further studies revealed that the PTB domain could also bind ligands in a phosphorylation-independent manner or even in the absence of a tyrosine residue (16). PTB domains exist in a variety of signaling proteins, and they provide interaction surfaces between the signaling proteins and the phospho-tyrosine residues in the cytoplasmic region of tyrosine kinase receptors (37). The Numb PTB domain has the unique property of being capable of binding to either pTyr-containing or Nxx[Y/F] motifs (38, 39). This characteristic makes the Numb PTB domain a versatile interaction module involved in a variety of cellular processes. Besides binding Notch, the Numb PTB domain has also been shown to interact with Nak, Mdm2, Lnx (17, 18, 40) and many other proteins (11, 12, 21, 23, 25, 26, 40, 41, 42, 43, 44, 45, 46, 47) (Fig. 1.4). It is highly likely that the function of Numb is more diverse than mediated by the above interactions. One of the main goals of my thesis research is to

identify novel interactions mediated by the Numb PTB domain and characterize their functions using biochemical and cell biology approaches.

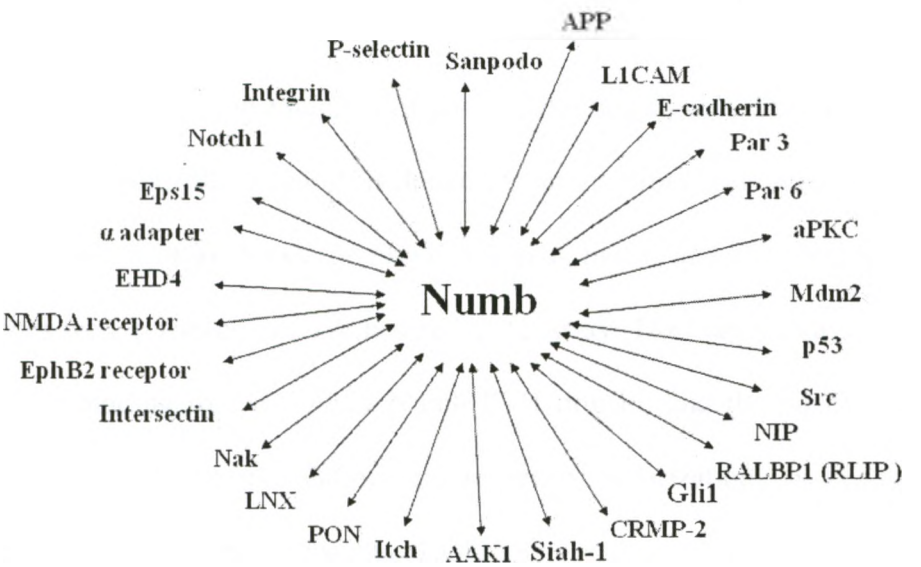

Figure 1.4 Referenced Numb interacting proteins

1.2.2 Peptide array and protoarray proteomic screening for Numb binding proteins

As the PTB domain is the only modular domain in Numb and is indispensable for its function, the Numb-PTBo domain was used to screen for potential Numb interacting partners. The work flow is shown in Fig. 1.5. An Oriented Peptide Array Library (OPAL) approach (48) was used to identify the consensus binding motif recognized by the Numb PTB domain. The identified motifs are NxxF and NxxY. The SMALI (scoring matrix-assisted-ligand identification) program was then employed to search the SwissProt database for potential binding partners using a position-specific scoring matrix (PSSM) derived from the OPAL binding data. The top peptides were synthesized as a peptide

array and probed with the purified Numb PTB domains. This work (carried out by Dr. Chenggang Wu) produced a number of potential binding partners for Numb.

In parallel experiments, an Invitrogen Protoarray (a protein array, containing 8000 proteins) was probed with either the *Drosophila* Numb-PTB or the mouse Numb-PTBo domain, which resulted in a number of positive binding signals. The proteins identified as potential binders for the Numb PTB domain from the ProtoArray screening were scanned for the presence of an NxxY or NxxF minimal motif, and the corresponding peptide sequences were retrieved and synthesized as an array. This target array was subsequently probed for binding to *Drosophila* Numb PTB domain and the positive peptides were further evaluated by in-solution fluorescence polarization assay to obtain the corresponding dissociation constants (Table 1.1).

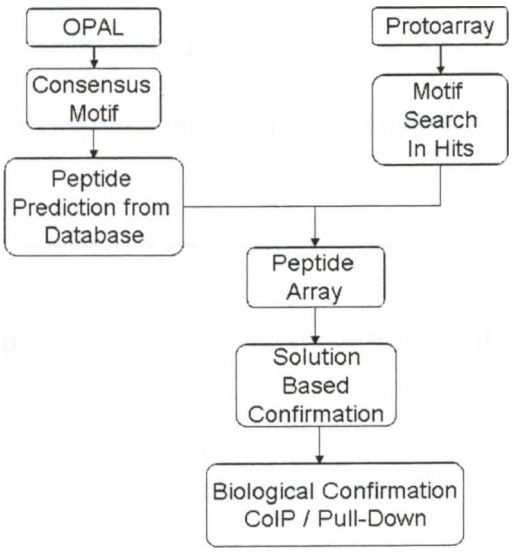

Figure 1.5 A flow chart of the proteomic screening for Numb PTB domain binding partners

Table 1.1 Hits identified from ProtoArray screen and the corresponding peptide binding affinities in solution measured by fluorescence polarization

Name	Binding sequences	Kd value (μM)
NAKAA (Positive Control)	TGFSNAAFEDF	0.1+/-0.01
v-ros UR2 sarcoma virus oncogene homolog 1 (ROS1)	SGVINESFEGE	0.7+/-0.02
Chromosome 10 open reading frame 91	QGFRNAAFEA	2.5+/-0.08
v-erb-b2 erythroblastic leukemia viral oncogene homology 2 (ERB2)	PAFDNLYYWDQ	9.4+/-0.3
Anaplastic lymphoma kinase (ALK)	GGHVNMAFSQS	0.7+/-0.04
Anaplastic lymphoma kinase (ALK)	FPCGNVNYGYQ	0.5+/-0.02
Cyclin-dependent kinase 5 (CDK5)	KVQPNSSYQNN	7.1+/-1.9
v-akt murine thymoma vial oncogene homolog1 (Akt), transcript variant 3	HSEKNVVYRDL	10.1+/-4.4
c-jun-amino-terminal kinase-interacting protein 4	KGIENKAFDRN	0.9+/-0.05

1.2.4 Validation of potential binding partners for Numb

The OPAL and subsequent peptide array screening identified a number of potential binding partners for Numb which included Ezrin, P21-Activating Kinase 2 (PAK2), Receptor-interacting serine/threonine-protein kinase 5 (RIPK5) and Synaptogamin-1 (Sypt I). To validate the Ezrin-, PAK2-, RIPK5- and Sypt I-Numb interactions, co-IP and/or GST pulldown experiments were carried out in cells. However,

none of these potential interactions can be convincingly recapitulated by co-IP or GST pull down, suggesting that Numb did not bind to these proteins in cells or interacted only weakly.

In contrast to the peptide array, the ProtoArray was based on full-length proteins. The identification of high-affinity PTB binding sites in the potential interacting proteins (Table 1.1), supports the possibility that the interactions are likely physiologically relevant. From the in solution binding assay, two putative Numb binding sites on ALK were found to have high affinity binding (<10 μM) sites, both Numb putative binding sites on ALK showed high binding affinity and therefore ALK was selected for further study.

1.3 ALK

1.3.1 The discovery of ALK

In 1994, ALK was first discovered as a fusion protein, Nucleophosmin (NPM)-ALK, which contains 5' portion of NPM and 3' portion of ALK (49). NPM is a highly conserved nucleolar phosphoprotein and it shuttles between the nucleus and cytoplasm (50). NPM is involved in ribosome biogenesis and acts as a histone chaperone (51). The NPM portion of NPM-ALK mediates oligomerization and constitutive activation of ALK (52). In 1997, full-length ALK was found in both mouse and human (53, 54).

1.3.2 The structure of ALK

ALK is a tyrosine receptor kinase (RTK) (53). Based on sequence similarity, ALK is most closely related to leukocyte tyrosine kinase (LTK), and together they form a

subgroup within the insulin receptor (IR) subfamily (53, 54)). ALK displays the classical RTK structure, with an extracelluar ligand-binding region, a transmembrane-spanning domain and a cytoplasmic catalytic region (53) (Fig. 1.6). The N-terminal extracellular region of ALK contains two Meprin/A5-protein/PTPmu (MAM) domains, one low density lipoprotein receptor class A (LDLa) domain and a glycine rich (G-rich) region. MAM domains of ALK are supposed to participate in cell-cell interactions and the LDL domain has a potential role in ligand binding to ALK (55) (Fig. 1.6). The human full-length ALK protein contains 1620 amino acids and has an approximate molecular weight of 220 kDa (53). Human ALK also exists as a 140 kDa species which results from a cleavage within the extracellular region of the full-length ALK (56).

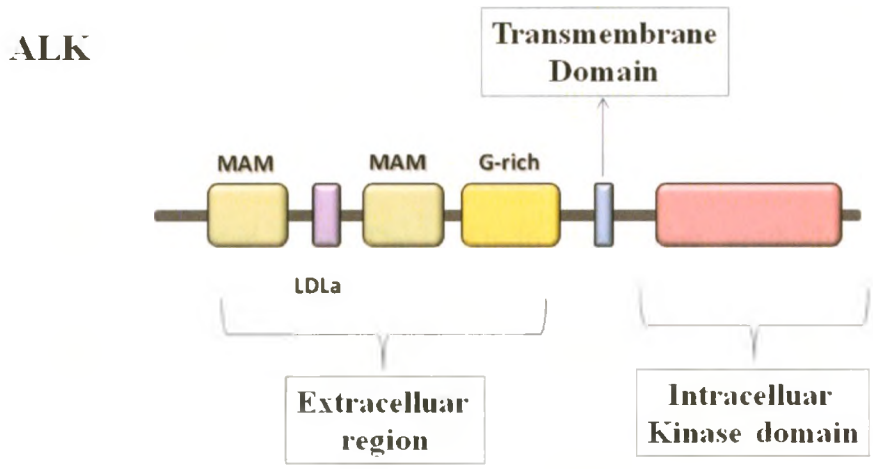

Figure 1.6 ALK protein structure. ALK contains an extracellular region, a transmembrane domain and an intracellular kinase domain. The extracellular region of the ALK protein contains two MAM domains, a LDLa domain and a G-rich region. This figure is adapted from Palmer (2009) (55)

1.3.3 The activation of ALK

Physiologically, all the tyrosine RTKs are activated by the ligands which can induce dimerization and subsequent autophosphorylation on the cytoplasmic side of the RTKs. Activated RTKs induce binding between specific phosphotyrosine sites and different adapter proteins such as Src homology 2 (SH2) domain-containing or PTB domain-containing proteins. The activation of RTKs further regulates a variety of downstream cellular signaling pathways involved in mitogenesis, differentiation, proliferation and apoptosis (37, 57). Although growth factors Midkine (MK) and pleiotrophin (PTN) are the potential ligands of the ALK, physiological ligands of ALK receptor are still under investigation (58, 59, 60). In addition, both PTN and MK can also bind receptor protein tyrosine phosphatase-ζ/β (RPTPζ/β) and syndecans (61).

Because of the uncertainty and multiplicity of ligand-receptor interaction, ALK receptor specific agonists are useful for studying the mechanism of ALK receptor activation. In this study, mouse monoclonal antibodies were used to either activate or inhibit ALK receptor (56). The mAb-46 (activating antibody) and the mAb-49 (inihibiting antibody) were kindly provided by Dr Marc Vigny, Institut du Fer à Moulin, Paris, France.

1.3.4 ALK tissue expression patterns and function in nervous system development

Characterization of the mRNA and protein expression of ALK during mouse and chick development suggests that ALK is predominantly expressed in the brain and spinal cord (53, 62). Additionally, ALK is expressed in special regions of nervous system such as the thalamus, mid-brain, sympathetic and dorsal root ganglia and olfactory bulb (53,

62). The intensity of ALK transcript and protein is highest in the neonatal mouse brain, and diminishes afterwards, reaching the lowest level at 3 weeks of age, and is maintained at low levels in the adult brain (53). The expression of ALK is also found in the eye, nasal epithelium, tongue, skin, tissue surrounding the esophagus, stomach and midgut. In addition, ALK is also detected in testis and ovary (63). The spatial-temporal expression pattern of ALK mRNA and protein in discrete regions of the developing central and peripheral nervous system of mammals indicates that this protein is likely to play an important role in the development of the nervous system.

The role of ALK in neuronal development is also supported by *in vitro* studies. Substitution of the extracellular domain of ALK by mouse IgG Fc fragment, which generates a constitutively active transmembrane ALK-IgG Fc, could induce neuronal differentiation of Pheochromocytoma (PC12) cells (64). Moreover, it has been shown that ALK is required for downstream Extracellular signal-regulated kinase (ERK)1/2 activation and neurite extension either in PC12 or neuroblastoma cell lines, when employing antibodies to activate ALK (61, 65).

1.3.5 ALK in oncogenesis

As mentioned above, ALK was initially discovered as part of the NPM-ALK oncogenic fusion protein in patients suffering from anaplastic large cell lymphoma (ALCL) in 1994 (49). Since then, many additional translocations in which ALK is fused to other partners have been discovered, such as Tropomyosin 3 (TPM3)-ALK (66), Neurotrophic tyrosine kinase (TRK)-fused gene (TFG)-ALK (67), Moesin (MSN)-ALK (68), 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP

cyclohydrolase (ATIC)-ALK (69), and etc. All ALK fusion proteins are oncogenic and capable of spontaneous dimerization induced by the different fusion partners, resulting in constitutive transphosphorylation and activation of ALK tyrosine kinase. In addition to ALCL, ALK fusion proteins have also been observed in inflammatory myofibroblastic tumours (IMTs) (70), non-small cell lung cancer (NSCLC) (71), diffuse large B-cell lymphomas (DLBCLs) (72), and squamous cell carcinoma (SCC) of the oesophagus (73).

Besides forced oligomerization of ALK kinase as forms of fusion proteins in malignancies, intact form of ALK can also be constitutively activated by point mutation in neuroblastoma (74). Knockdown of mutated ALK in neuroblastoma cell lines suppresses their proliferation (75), and two ALK mutants (F1174L and K1062M), are able to induce tumors formation in nude mice (76).

The oncogenic effects of ALK can also be manifested through its overexpression. Excessive expression of full-length ALK has also been reported in cell lines and tumours, such as thyroid carcinoma, NSCLC, breast cancer, melanoma, neuroblastoma, glioblastoma, astrocytoma, retinoblastoma, ewing sarcoma and rhabdomyosarcoma (55).

1.3.6 ALK-activated signaling pathway

The most extensively studied signaling pathway of ALK is NPM-ALK signaling. NPM-ALK signals through the Ras/MAPK, Phospholipase-C (PLCγ), Phosphoinositide 3-kinase (PI3K), and Janus kinase (JAK)/(Signal transducer and activator of transcription) STAT pathways (55). The Ras/MAPK and PLCγ pathways are involved in cell division and proliferation, whereas PI3K and JAK/STAT pathways primarily promote cell survival and oppose apoptosis (77, 78).

NPM–ALK interacts with Insulin receptor substrate (IRS)-1, Src homology 2 domain-containing-transforming protein C (SHC) and Grb2, suggesting that the RAS/MAPK pathway may be the downstream signal of the fusion kinase (79). However, it is noteworthy that the direct effects on Ras-MAPK signaling by NPM-ALK have been scarcely reported (78). PLCγ interacts with NPM-ALK through its SH2 domain, and this interaction is important for the transforming properties of NPM-ALK (80).

NPM-ALK interacts and activates PI3K in ALCL cells, resulting in the activation of its downstream kinase Akt. PI3K inhibitors induce apoptosis in NPM-ALK expressing cells (81). NPM-ALK can either directly phosphorylate STAT3 or indirectly activate STAT3 through its interacting partner JAK3 (82, 83, 84). In NPM-ALK expressing cell lines, JAK3 inhibitor AG490 reduces STAT3 activity and increases cellular apoptosis (83).

Compared with the information of NPM-ALK signaling, much less is known of the signaling events mediated by the full-length ALK. Activation of full-length ALK by agonist monoclonal antibodies leads to sustained activation of MAP kinase, ERK1/2, and specific activation of STAT3, but has no effect on the PI3K/Akt pathway (56, 61). Further study shows that different signaling pathways can be activated depending on distinct subcellular localization of the ALK kinase domain. In PC 12 cells, membrane attachment of ALK kinase domain induces MAP kinase activation and cell differentiation, while the cytosolic form of ALK kinase domain fails to activate MAP kinase but promotes PI3K/Akt dependent cell growth (85).

1.4 Purpose of thesis

Based on protoarray- screening ALK was identified as a potential Numb-binding partner. The expression patterns of Numb and ALK (Numb in subventricular zone (SVZ) neuroprogenitors (86) and ALK in the neonatal brain(53)) further support the possibility of a Numb-ALK interaction. Based on two pieces of previously documented evidence, we hypothesized that the binding of Numb to ALK may antagonize ALK signaling. First, abnormally (constitutively) activated ALK is oncogenic, while Numb is a known tumor suppressor. Second, activated tyrosine kinase receptors are rapidly internalized and eventually delivered to the lysosomes for degradation. The endocytosis of a tyrosine kinase receptor is an important mechanism by which to attenuate RTK signaling (37, 87). Numb is an endocytic protein, which can antagonize Notch signaling by endocytosis of the Notch receptor (45). As a tyrosine receptor kinase, ALK signaling may be negatively regulated by the association of ALK with Numb, and consequently the endocytosis of ALK.

To address our hypothesis, reciprocal co-immunoprecipitation of ALK and Numb was first performed to confirm a Numb and ALK interaction. To predict which region of Numb interacts with ALK, GST pull-down assays using Numb-GST fusion protein constructs were exploited. To identify amino-acid motifs of ALK involved in the Numb-ALK interaction, an alanine scanning peptide array was generated based on Numb potential binding motifs within ALK. Monoclonal antibodies (mAbs) directed against the extracellular domain of ALK were utilized to control the activation of ALK in the absence of clearly established ligands. Under different stimulatory conditions, Numb and ALK interaction intensities were investigated by co-immunoprecipitation. ALK

expression levels and ALK downstream Erk signaling were determined by western blot. In addition, ALK subcellular localization was examined by cell fractionation under different conditions of mAbs stimulation combined with or without Numb transfection.

Chapter 2: Materials and Methods

2.1 Plasmids

Plasmids were amplified in *E. coli* DH5α and purified using a standard Maxiprep kit (Invitrogen, San Diego, CA). Information of the plasmids used in the study is listed below (Table 2.1).

2.2 Site directed mutagenesis

2.2.1 Primers

The mutagenic oligonucleotide primers were designed according the following criteria and procedures:

A. Both of the mutagenic primers contained the desired mutation and annealed to the same sequence on opposite strands of the plasmid.

B. Primers were between 25 and 45 bases in length, with a melting temperature (Tm) of ≥78°C. The following formula was used for estimating the *T*m of primers:

T_m=81.5 + 0.41(%GC) - 675/ N - % mismatch (N: the primer length in bases)

C. The primers optimally had a minimum GC content of 40% and terminated in one or more C or G bases.

Oligonucleotides used in the study were purchased from Sigma (Mississauga, ON, Canada). Sequences of primers are listed in Table 2.2.

Table 2.1 List of plasmids

Plasmid ID	Coding proteins	Host	Source
pGEX4T2-dNumb-PTB	*Drosophila* Numb PTB domain	*E. coli*	Li Lab
pGEX4T2-dNumb-76	*Drosophila* Numb N-terminal region (residues 1-76)	*E. coli*	Li Lab
pGEX4T3-dNumb-C	*Drosophila* Numb C-terminal region (residues 206-556)	*E. coli*	Li Lab
pGEX4T2-mNumb-PTBi	Mouse Numb PTBi domain	*E. coli*	Li Lab
pGEX4T2-mNumb-PTBo	Mouse Numb PTBo domain	*E. coli*	Li Lab
pcDNA-mNumb PTBi/PRRi	Mouse Numb	Mammalian cells	Li Lab
pcDNA-mNumb PTBi/PRRi F162V	Mouse Numb with F162V mutation	Mammalian cells	Constructed by the author
pcDNA 3.1	Empty vector	Mammalian cells	Li Lab
pRc/CMV-vSrc	v-Src protein	Mammalian cells	Li Lab

Table 2.2 Lists of Primers

Primers	Sequences (5'to 3')	Plasmid involved
Numb PTBi/PRRi F162V mutation primer 5'	CCGTGGGCTGTGCTGTTG CAGCCTGTTTAG	pcDNA-mNumb PTBi/PRRi
Numb PTBi/PRRi F162V mutation primer 3'	CTAAACAGGCTGCAACAG CACAGCCCACGG	pcDNA-mNumb PTBi/PRRi

2.2.2 Polymerase chain reaction (PCR) preparation

PCR reactions were prepared according to the follow recipe (Table 2.3). All the reagents were purchased from Fermentas (Burlington, ON, Canada).

Table 2.3 PCR mixture

Ingredients	Stock concentration	Amount
***pfu* PCR buffer with $MgCl_2$ and $(NH_4)_2SO_4$**	10×	5 µl
dNTP	2 mM	10 µl
5' primer	125 ng/µl	2 µl
3' primer	125 ng/µl	2 µl
DNA template	100 ng/µl	2 µl
***Pfu* DNA polymerase**	5 U/µl	1 µl
H_2O		28 µl

2.2.3 PCR cycling parameters

PCR reactions were performed in a thermocycler (iCycler - BioRad, Hercules, CA) using the parameters outlined below:

Cycle 1: Denaturation: 95℃ 2 minutes

Cycle 2 (× 18): Denaturation: 95℃ 50 seconds

Anealing: 55℃ 50 seconds

Extension: 68℃ 30 minutes

Cycle 3: Extension: 68℃ 30 minutes

Cycle 4: 4℃ hold

2.2.4 Dpn I Digestion of PCR products

1 µl of *Dpn* I (Stratagene, La Jolla, CA) (10 U/µl) was added directly to each amplification reaction. Then the reaction mixture was gently and thoroughly mixed by pipetting the solution up and down several times. Finally, the reaction mixture was centrifuged for one minute and immediately incubated at 37°C overnight.

2.2.5 Transformation and plasmid amplification

10 µl *Dpn* I-treated DNA from each sample reaction was transferred to 100 µl competent cells following the transformation protocol described in 2.3.1. For plasmid amplification, 100 ml *E.coli* culture was used to purify plasmids with Maxiprep kit (Invitrogen, San Diego, CA). Agarose gel electrophoresis and UV-visible spectroscopy were used to assess the quality of DNA samples.

2.3 Transformation of *E.coli*, protein expression and purification

2.3.1 Transformation

E.coli competent cells were thawed on ice and 80 µl to 100 µl competent cells were added to a pre-chilled 10 ml falcon tube which contained 1 µg plasmid DNA. The competent cells and plasmid DNA mixture were mixed gently and incubated on ice for 30 minutes. Then the mixture was heat shocked at 42℃ for 90 seconds, and then placed on ice for two minutes. 1 ml 2×TY buffer was added into the falcon tube containing the cells and DNA mixture, and the tube was incubated at 37 ℃ for one hour. After spreading 100 µl cells onto an LB plate containing 50 µg/ml Ampicillin or 30 µg/ml of kanamycin, the plate was incubated at 37 ℃ overnight.

2.3.2 GST or GST fusion protein expression

E.coli BL-21 cells were transformed following the protocol described above. A single colony was selected from the LB-agar plate and was inoculated into 2 ml of culture medium. After an overnight growth at 37 ℃, 1 ml turbid culture was diluted into the 1 L ampicillin or kanamycin selective LB medium and the mixture was shaken at 37 ℃. When the culture reached 0.4-0.6 of OD_{600}, 1 M IPTG stock was added into the culture at a dilution ratio of 1:1000. The bacterial culture was then incubated at 18 ℃ overnight and harvested by centrifugation at 4,000 g for 20 minutes the next day.

2.3.3 GST protein purification

E.coli BL21 bacterial pellets expressing GST or GST fusion protein were put into -80 ℃ for 30 minutes and then 50 ml bacterial pellet was resuspended in 2 ml cold

bacterial lysis buffer. After lysing bacteria for 20 minutes on ice, the bacterial debris was centrifuged out at 16,100 g for 20 minutes at 4 ℃. The supernanant was taken and an appropriate amount of a 50:50 slurry of glutathione-sepharose beads in PBS lysis buffer was added into it. 1 ml of glutathione-Sepharose beads bind approximately 5 mg GST protein, and normally 200 µl 50:50 slurry of glutathione-Sepharose beads were added into 2 ml supernatant. After rotating the tube end over end for one hour at 4 ℃, the sample was centrifuged at 1000 g for three minutes at 4 ℃. The supernatant was removed and the glutathione-Sepharose beads were washed four times with 10 bed volumes of 1×PBS. The purified GST protein was either added directly to mammalian cell lysate for the pull down assay or eluted by the elution buffer.

2.3.4 Fast protein liquid chromatography (FPLC)

The eluted GST protein was purified by FPLC, which was performed by a Superdex 75 HR30/10 prepacked column connected to an ÄKTA FPLC system (GE healthcare, Little Chalfont Buckinghamshire, UK). First, pump A was washed at a 0.5 ml/minute flow rate. Then the column was washed with 30 ml PBS at a 0.5 ml/minute flow rate. After washing the sample loop, 500 µl sample was injected into the loop. 1.5 column volume of length of elution and 0.5 to 0.7 ml/min of flow rate were set up. Finally, the fractions were collected using a Frac-900 collector.

2.3.5 Measurement of Protein Concentration

Two methods were used to measure protein concentration. One involved measuring the protein's intrinsic UV absorbance, and the other is the Bradford dye assay.

The UV absorbance was used to measure FPLC purified protein concentration for Far Western. And the Bradford dye assay was used to measure the concentration of mammalian cell extract protein concentration and purified GST protein while attached to glutathione-sepharose beads.

The UV absorvance method was performed by diluting purified GST protein in water and estimating the protein concentration by the following equations:

A_{280} = 1 A (mL/cm mg) x [Conc.] (mg/mL) x 1 (cm)

(A = absorbance coefficient (or extinction coefficient) was calculated according to the protein sequence by ProtParam tool located at: http://ca.expasy.org/)

The Bradford assay was run according to Bio-Rad manual. Since this assay is linear over a short range, typically from 2 µg/ml to 120 µg/ml, the protein sample was diluted at an appropriate ratio in the dye reagent which was prepared by diluting 1 part concentrated dye reagent with 4 parts distilled deionized water. The OD value was measured in a GENESYSTM spectrophotometer (Spectronic Unicam, Rochester, NY, USA) at a wavelength of 595 nm. A BSA standard curve was used to determine protein concentration.

2.4 Cell culture, transfection and fractionation

2.4.1 Cell Culture

H370 (a kind gift from Dr Marc Vigny, Institut du Fer à Moulin, Paris, France) is an HEK-293 stable cell line expressing full-length human ALK. H370 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine

serum (FBS), 2 mg/ml glutamine, 100 units of penicillin-streptomycin and 2 μg/ml puromycin (Sigma) at 37 ℃ with 5% CO_2.

Jurkat cells were cultured in RPMI 1640 medium with 10% FBS, 2 mg/ml glutamine, and 100 units of penicillin-streptomycin at 37 ℃ with 5% CO_2.

Hela and HEK-293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2 mg/ml glutamine, 100 units of penicillin-streptomycin at 37 ℃ with 5% CO_2.

2.4.2 Transfection

H370 cells were transiently transfected using PEI (Polyethylenimine). H370 cells were plated in a 10 cm tissue culture dish at a density of 5×10^6, and grown to 60-70% confluence. 8 μg plasmid DNA was diluted in 440 μl of Opti-MEM medium and 60 μl PEI (1 mg/ml) was added. The solution was mixed immediately for 10 seconds and then incubated for 10 minutes at room temperature. The transfection mixture was completed by mixing 500 μl of DNA/PEI solution into 4.5 ml complete medium without antibiotics. Old medium was removed from the 10cm tissue culture plate and 5 ml transfection mixture was gently transferred into the plate. After incubating for six hours at 37 ℃, another 5 ml of antibiotic free complete medium was added to each plate followed by culturing for an additional 18 hours.

2.4.3 Pervanadate treatment

0.1 M Sodium metavanadate and 30% H_2O_2 were diluted in PBS to the final concentration of 3mM H_2O_2 and 0.1 mM Vanadate. The mixture was incubated at 37 ℃

for 10 minutes to generate pervanadate. Then the culture medium was removed and 10 ml pervanadate mixture was added to the 10 cm tissue culture dish. The cells were treated with pervanadate, and placed in 37 ℃ CO_2 incubator for 20 minutes before being lysed.

2.4.4 Serum starvation and mAbs treatment

H370 cells plated at a density of 2×10^6 in a 10 cm dish were cultured for two days and then the culture medium was removed. After being washed with 10 ml DMEM, the cells were maintained in serum free medium (DMEM supplemented with 100 units of penicillin-streptomycin and 2 μg/ml puromycin) for 16 hours. The cells were then treated with the indicated concentrations of mAbs in serum free medium for another two hours.

2.4.5 Cell fractionation

H370 cells of different physiological conditions were separately fractionated into cytosolic, membrane and nuclear portions according to the protocol provided in the FractionPREP cell fractionation kit (BioVision Research).

2.5 GST pull down, immunoprecipitation and Western blot

2.5.1 Preparation of cell extracts

The following procedures were performed on ice and all buffers were pre-chilled. Mammalian cell lysis buffer was prepared by supplementing RIPA buffer with 1 mM sodium orthovanadate, 2 μg/ml aprotinin, 10 μM leupeptin, 1 μM E64, 1 μM bestatin, 1 μM pepstatin A and 1 mM PMSF. Cells in a 10 cm tissue culture plate were washed once with 5ml PBS buffer, and were lysed in 700 μl lysis buffer. A rubber policeman was used

to scrape the cells. The cell lysate was transferred into a 1.5 ml eppendorf tube and rotated on ice at 4 ℃ for 30 minutes. Then the cell lysate was centrifuged at 16,000 g for 20 minutes at 4 ℃ and the supernatant was taken for subsequent experiments.

2.5.2 GST pull down

Mammalian cell lysates, which express the potential GST fusion protein binding partners, were incubated with glutathione-sepharose beads for two hours and then the glutathione-sepharose beads were washed with 10 bed volumes of mammalian cell lysis buffer. 2x SDS Laemmli sample buffer was used to elute the proteins bound to the glutathione-sepharose beads.

2.5.3 Immunoprecipitation (IP)

0.5 mg-1 mg total protein of cell lysate was incubated with 1 μg antibodies for two hours at 4°C. Then 40 μl protein G sepharose beads were added into and incubated with the cell lysate for another hour at 4 °C. Antibody-antigen-Protein G complexes were washed by mammalian cell lysis buffer three times and collected by 1000 g centrifugation for three minutes at 4 ℃. 2x SDS Laemmli sample buffer was used to elute antigen.

2.5.4 Western Blot (WB)

Protein samples and pre-stained protein makers were loaded onto a 4-12% Bis-Tris gradient gel (BioRad, Hercules, CA, #345-0125). The gel was run in MES buffer at 110V for one hour, and then transferred to PVDF membrane with a semi-dry blot

transferring system (BioRad, Hercules, CA) set to 20 V for 20 minutes. The PVDF membrane was blocked in 5% skim milk in Tris buffered saline with Tween (TBST) overnight in the cold room. Then the membrane was incubated with a primary antibody in blocking buffer for one hour at room temperature. The primary antibody was diluted according to the optimized conditions listed in Table 2.4. The membrane was rinsed three times and washed 3×5 minutes with TBST. After incubating with a Horse radish peroxidase (HRP) conjugated secondary antibody (diluted at the ratio of 1:5000 in 5% skim milk) for 40 minutes at room temperature, the membrane was again rinsed three times and washed 1×15 minutes following 3×5 minutes with TBST. The membrane was incubated with an enhanced chemiluminescence (ECL) detection solution (Perkin Elmer, Waltham, MA). Signals were captured with X-ray film (Kodak, Rochester, NY) over a range of five seconds to 10 minutes and developed using a Kodak M35A X-OMAT processor.

2.5.5 PVDF stripping and reprobing

The PVDF membrane was washed for 3×5 minutes in TBST. Then the membrane was incubated for 30 minutes at 50 ℃ in the stripping buffer. After washing the membrane for 5×5 minutes in TBST, the membrane was ready for reuse. The re-probing procedure began with a blocking step and processed according to established Western blotting procedures.

Table 2.4 Antibodies used in this study

Antibody	Spieces	Source	Optimized conditions
Numb (H-70)	Rabbit	Santa Cruz Biotech (Catalogue number: sc-25668)	1:1000 dilution in 5% skimmed milk for WB; 1 μg for IP
HA-probe (F-7)	Mouse	Santa Cruz Biotech (Catalogue number: sc-7392)	1:1000 dilution in 5% skimmed milk for WB
ALK	Mouse	From Dr Marc Vigny	1 μg for IP
Anti-Phosphotyrosine, clone 4G10	Mouse	Millipore (Catalogue number: 05-321)	1:5000 dilution in 5% skimmed milk for WB
P44/42 MAP (Erk1/2) kinase	Mouse	Cell Signaling (Catalogue number: 4696)	1:1000 dilution in 5% skimmed milk for WB
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Mouse	Cell Signaling (Catalogue number: 9106)	1:1000 dilution in 5% skimmed milk containing 25mM NaF for WB
Akt	Rabbit	Cell Signaling (Catalogue number: 9272)	1:1000 dilution in 5% skimmed milk for WB
Phospho-Akt (Ser473)	Mouse	Cell Signaling (Catalogue number: 4051)	1:1000 dilution in 5% skimmed milk containing 25mM NaF for WB
β Tubulin (H-235)	Rabbit	Santa Cruz Biotech (Catalogue number: sc-	1:1000 dilution in 5% skimmed milk for WB

		9104)	
Pak2	Rabbit	Cell Signaling (Catalogue number: 2608)	1:1000 dilution in 5% skimmed milk for WB
Synaptotagmin I/II (D-18)	Goat	Santa Cruz Biotech (Catalogue number: sc-12466)	1:1000 dilution in 5% skimmed milk for WB; 1 µg for IP
Ezrin (4A5)	Mouse	Santa Cruz Biotech (Catalogue number: sc-32759)	1:1000 dilution in 5% skimmed milk for WB; 1 µg for IP
RIPK5	Rabbit	ABGENT (Catalogue number: AP7194a)	1:500 dilution in 5% skimmed milk for WB; 1 µg for IP
v-Src (Ab-1)	Mouse	Calbiochem (Catalogue number: OP07)	1:1000 dilution in 5% skimmed milk for WB; 1 µg for IP
GST (HRP)	Rabbit	Abcam (Catalogue number: ab3416)	1:5000 dilution in 3% BSA for WB
Na^+/K^+-ATPase α (M7-PB-E9)	Mouse	Santa Cruz Biotech (Catalogue number: sc-58628)	1:100 dilution in 5% skimmed milk for WB
LaminA/C (N-18)	Goat	Santa Cruz Biotech (Catalogue number: sc-6215	1:1000 dilution in 5% skimmed milk for WB

2.5.6 Quantification of data

Western blot films were scanned and quantified by Scion Image software (Scion Corporation, Frederick, MD). Results were given as means ± standard deviation (SD). Each data point was representative of two independent experiments. Statistical analysis was performed with two-tailed Student's *t*-test.

2.6 Peptide synthesis and Far Western

2.6.1 Peptide array synthesis

The peptide array was synthesized on a TFA-soluble cellulose membrane (Intavis AG) using membrane supports (SPOT) technology (78) by an Auto-Spot ASP 222 Robot (Intavis AG). Before being cut with TFA, the membrane was sequentially washed with a deprotection solution 3×5 minutes, Dimethylformamide (DMF) 3×2 minutes, and ethanol 3×2 minutes. Then the membrane was incubated first with 30 ml 50% TFA cocktail (15 ml TFA cocktail in 15ml H_2O) rocked for one hour, followed by another bout of rocking in 30 ml of TFA cocktail. Afterwards, the membrane was sequentially washed with dichloromethane (DCM) 3×2 minutes, DMF 3×2 minutes, and ethanol 3×2 minutes. The membrane was air-dried.

2.6.2 Far Western

The peptide membrane was immersed in 100% ethanol. Then distilled water was added into ethanol slowly until a final concentration of 50% water was reached. The membrane was incubated in 50% ethanol for five minutes at room temperature, followed by 1×5 minutes in water, and 3×5 minutes in TBST. After incubation in 3% BSA

(containing 1 mM DTT) overnight in the cold room, the membrane was probed with 0.2 μM GST protein in 3% BSA at room temperature for one hour. HRP conjugated GST antibody was added into 3% BSA blocking buffer. The membrane was rocked at room temperature for another hour. Finally, after TBST washes for 3×15 minutes and 3×5 minutes, the membrane was incubated with ECL solution and exposed to x-ray film.

2.7 Solutions and buffers

Table 2.5 Solutions and buffers

Solutions	Components
1×TAE buffer (pH8.5)	Tris base 40 mM, EDTA 1 mM, Glacial acetic acid adjusting pH to 8.5
1× TBST	Tris base 20 mM, NaCl 137 mM, Tween20 0.05% (v/v)
1× PBS buffer	KH_2PO_4 1 mM, Na_2HPO_4 10 mM, KCl 2.7 mM, NaCl 137 mM
5× SDS-PAGE (Laemmli) Sample buffer	Tris base (pH 6.8) 312 mM, SDS 10%, α-mercaptoethanol 25%, Bromophenol Blue 0.05%, Glycerol 50%
Bacteria lysis buffer	Triton X-100 1%, Lysozyme 1 mg/ml, Benzonase nuclease (25units/μl) 12.5 U/ml, PMSF 1 mM, Bestatin 1 μM, E64 1 μM, Pepstatin A 1 μM in 1× PBS buffer
Coomassie blue staining solution	Coomassie brilliant blue R250 0.25% (w/v), Methanol 45% (w/v), Glacial acetic acid 10% (w/v)

Deprotection solution	Piperidine 20% (v/v) in DMF
GST protein elution buffer (pH 8.0)	Reduced L-Glutathione 25 mM, Tris-HCl (pH 8.0) 75 mM, NaCl 100 mM
MES buffer	MES 50 mM, Tris Base 50 mM, SDS 0.1%, EDTA 1 mM
RIPA buffer (pH7.8)	Triton X-100 1%, NaPi (pH7.8) 10 mM, NaCl 60 mM, β - glycerol phosphate 25 mM, Glycerol 10%, NaF 50 mM
Stripping buffer (pH 6.8)	β-mercaptoethanol 100 mM, SDS (w/v) 2%, Tris-HCl (pH 6.8) 62.5 mM
TFA cocktail	Trifluoroacetic acid (TFA) 95%, Tri-isopropylsilane (TIPS) 3%, H_2O 2%
Western blot transfer buffer	Methanol 20%, Tris base 24 mM, Glycine 194 mM

Chapter 3: Results

3.1 Interaction between Numb and ALK *in vitro*

To confirm the Numb and ALK interaction, we took advantage of a cell line (H370) that stably expresses HA-tagged full-length human ALK in HEK-293 cells. Two species of ALK protein were detected as reported in previous studies (56, 65): the full-length ALK (220 kDa) and a shorter form of ALK (140 kDa) (Fig. 3.1.1a). H370 cells were transiently transfected with a pcDNA-Numb PTBi/PRRi plasmid containing insertions in both the PTB domain and PRR region. Under the conditions of robust expression of Numb and ALK in H370 cells, Numb reciprocally coimmunoprecipitated with ALK (Fig. 3.1.1b), while neither Numb nor ALK was immunoprecipitated by an IgG negative control.

3.2 Prediction of the region of Numb required for its interaction with ALK

The PTB domain is the only identified modular domain of Numb and is responsible for a number of interactions between Numb and its binding partners. It was therefore logical to first investigate whether PTB domain is involved in the Numb-ALK interaction. GST pull-down assays were carried out using the GST-fused contructs of the mouse Numb PTB domains with or without the insert (Numb-PTBi and Numb-PTBo, respectively). The Numb PTB domains were purified using glutathione sepharose beads, then incubated with the lysate of H370 cells (HEK-293 cells stably expressing exogenous HA tagged full-length human ALK). As shown in Fig. 3.2.1, HA-tagged ALK expressed in H370 interacted with immobilized mouse Numb PTBo and Numb PTBi domains,

while it did not interact with the control GST protein. Coomassie blue staining indicated that approximately equal amounts of the proteins were used in this assay.

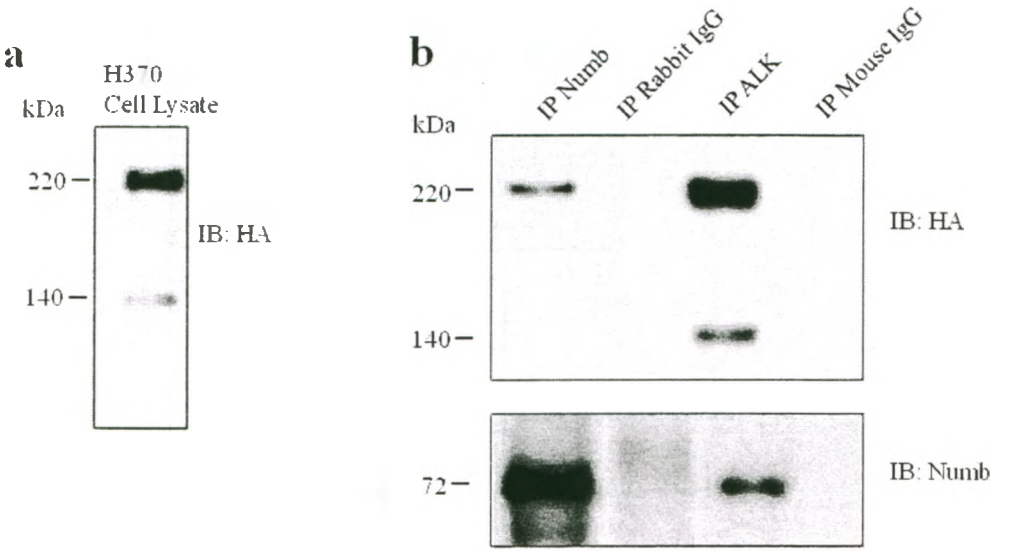

Figure 3.1.1 Interaction between Numb and ALK. a. Stable expression of HA-tagged ALK in H370 cells. H370 cells were lysed in mammalian cell lysis buffer. The whole cell lysate (20 µg) was resolved on a 4-12% Bis-Tris gradient gel, transferred to PVDF membrane and analyzed by Western blot using a mouse anti-HA antibody. Corresponding molecular weights (in kDa) were labeled on the left of the figure. **b.** Reciprocal immunoprecipitation of ALK and Numb. H370 cells transfected with the mouse Numb PTBi/PRRi isoform were lysed in mammalian cell lysis buffer and 1 mg of total protein was immunoprecipitated using the following antibodies: 1 µg of rabbit anti-Numb, 3 µg of mouse anti-ALK, 1 µg of rabbit IgG (negative control) and 1 µg of mouse IgG (negative control). Western blot analysis was performed using a mouse anti-HA antibody (top panel) or rabbit anti-Numb antibody (bottom panel).

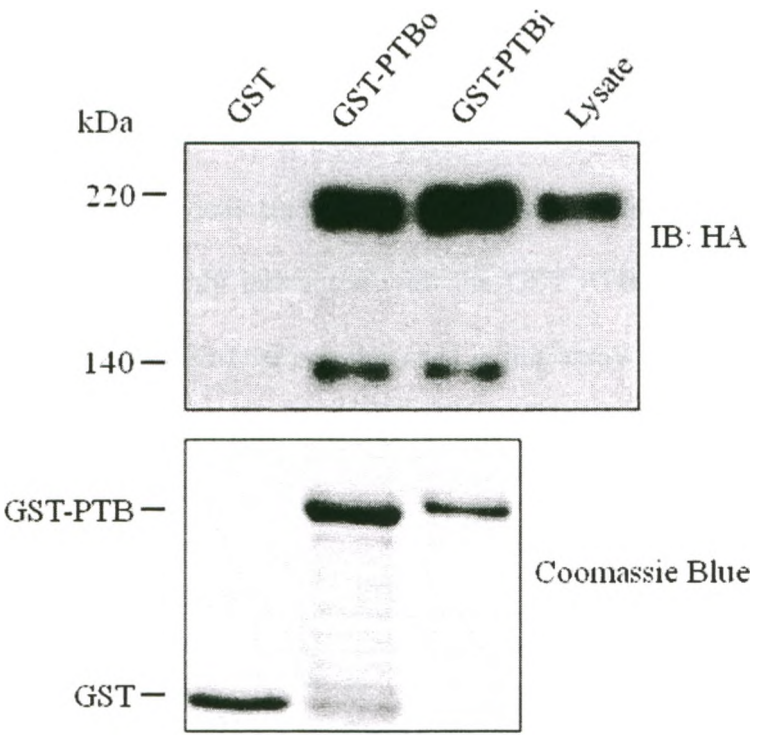

Figure 3.2.1 The PTB domain of Numb binds to ALK. H370 cell lysates were incubated with 200 μg of the GST-fusion proteins (GST as a negative control, GST-Numb-PTBo, and GST-Numb-PTBi). The pulldown complexes as well as 10 μg of the H370 cell lysate were resolved on a 4-12% Bis-Tris gradient gel. Western blot analysis was performed using a mouse anti-HA antibody (upper panel). For protein quality control, 5 μg of each protein was loaded on a 12% polyacrylamide gel, and visualized by staining the gel with Coomassie blue (lower panel).

To determine if any other regions of Numb are involved in the Numb-ALK interaction, different regions of *Drosophila* Numb fused to GST were used in a pull down assay. These regions corresponded to the region N-terminal to the PTB domain (GST-N, residues 1-76), the PTB domain itself (GST-PTB, residues 77-205) and the region C-terminal to the PTB domain (GST-C, residues 206-556) (Fig. 3.2.2). These constructs were made for previous studies and were readily available for this study. As is shown in Fig. 3.2.3, ALK only interacted with the GST-PTB domain. Coomassie blue staining showed the amounts of the proteins used in this assay.

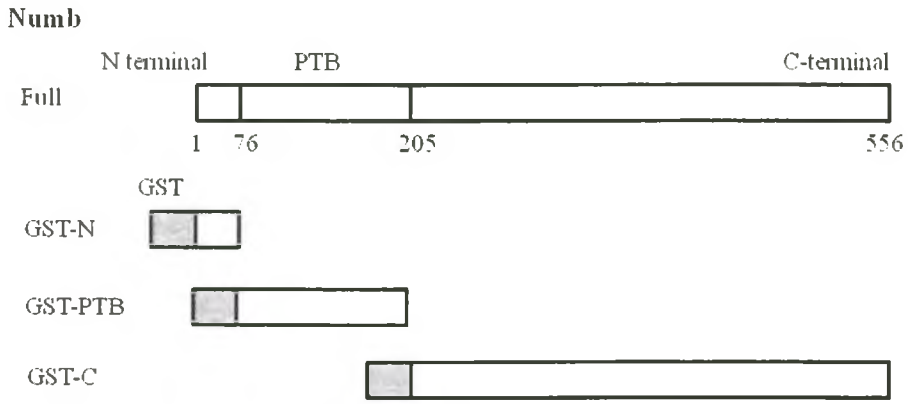

Figure 3.2.2 Schematic representation of full-length *Drosophila* Numb and its mutants. Three *Drosophila* Numb mutants were expressed as GST-fusion proteins: the GST-fused Numb N-terminal region (GST-N, residues: 1-76), the GST-fused Numb-PTB domain (GST-PTB, residues: 77-205) and the GST-fused Numb C-terminal (GST-C, residues: 206-556).

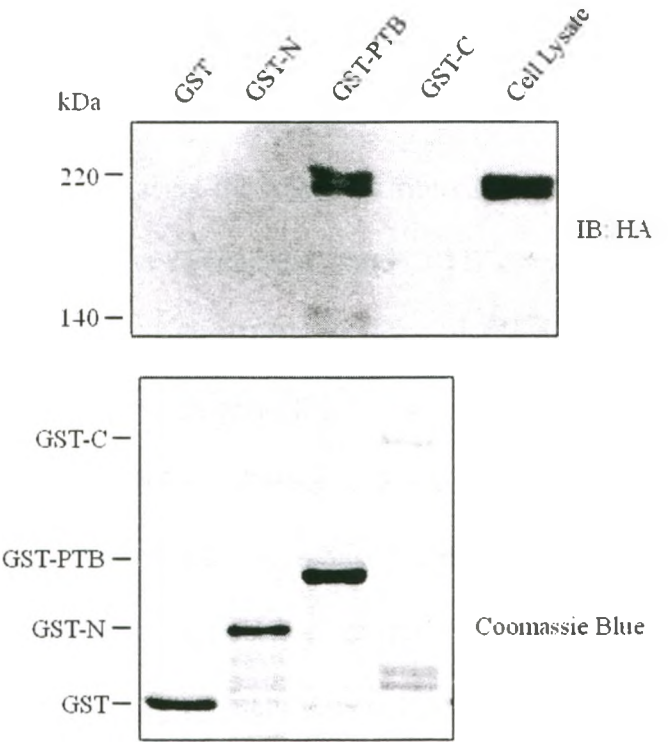

Figure 3.2.3 Interactions of ALK with the truncated *Drosophila* Numb proteins. H370 cell lysates were incubated respectively with: 200 µg of GST, GST-N, GST-PTB or GST-C. The complexes pulled down as well as 10 µg of H370 cell lysate were resolved on a 4-12% Bis-Tris gradient gel. The Western blot analysis was performed using a mouse anti-HA antibody (upper panel). 5 µg of each sample was loaded on a 12% polyacrylamide gel and visualized by Coomassie blue staining (lower panel).

It has been shown that several critical amino acids in the Numb PTB domain are important for binding to its ligand peptide including the Nak-c or the GPpY peptide (88). In particular, mutation of phenylalanine (amino acid residue 195) to valine in the *Drosophila* Numb PTB (dNumb-PTB) domain resulted in drastic loss of binding affinity for ligand peptides (decreasing the relative affinity from 100% to less than 1%) (88). A sequence alignment of the *Drosophila* Numb PTB domain (dNumb-PTB) and mouse Numb PTBi domain (mNumb-PTB) indicates the sequences are highly conserved, sharing approximately 80% identity (Fig. 3.2.4). As shown by *asterisks* in Fig. 3.2.4, a critical F_{195} in the dNumb-PTB domain is conserved in the mNumb-PTB domain and corresponds to F_{162} in the full-length mouse Numb PTBi/PRRi. To further confirm that the Numb-PTB domain is responsible for the Numb-ALK interaction, site -directed mutagenesis was performed to obtain a mouse Numb PTBi/PRRi mutant containing the point mutation F162V. ALK was then subjected to immunoprecipitation by either wild-type (WT-Numb) or mutant (F162V-Numb) protein from H370 cells. Fig. 3.2.5 showed that the interaction between Numb and ALK was greatly attenuated when Numb was mutated to valine at the F_{162} position in the PTB domain.

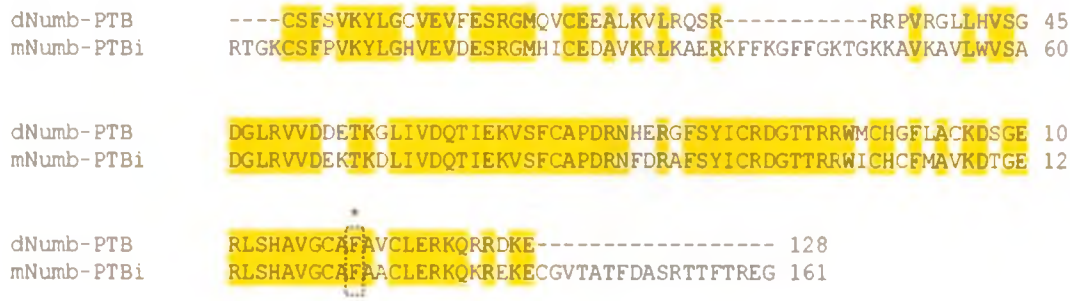

Figure 3.2.4 Sequence alignment of the Numb PTB domain in *Drosophila melanogaster* and the PTBi domain in mouse. The sequences of the *Drosophila* Numb PTB (residues 81-208 in the *Drosophila* Numb) and mouse Numb PTBi (residues 33-193 in the mouse Numb +/+ isoform) domains were aligned using online software ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Conserved regions between the two species are highlighted in yellow. An *asterisk* indicates the conserved F_{195} in the *Drosophila* Numb and F_{162} in the mouse Numb +/+.

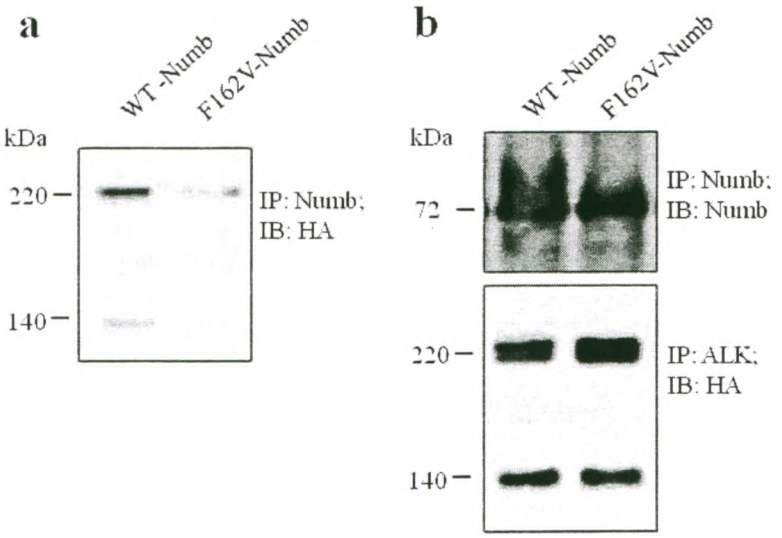

Figure 3.2.5 F_{162} is important for the interaction of Numb with ALK. H370 cells transfected with either the mouse Numb PTBi/PRRi isoform or the F162V Numb mutant were lysed in mammalian cell lysis buffer. 1 mg of protein was then immunoprecipitated using 1 μg of rabbit anti-Numb or 3 μg of mouse anti-ALK antibody. The western blot was performed using a mouse anti-HA antibody or a rabbit anti-Numb antibody. **a.** The western blot showed immunoprecipitation of HA-ALK with Numb. **b.** The western blot showed immunoprecipitation of Numb with anti-Numb (upper panel) or immunoprecipitation of HA-ALK with anti-HA (lower panel).

3.3 Prediction of the ALK sequences important for the Numb-ALK interaction

To determine the amino-acid sequence(s) of ALK involved in the Numb-ALK interaction, an alanine scanning peptide array was generated for the potential Numb binding motifs within the cytoplasmic region of the ALK receptor. Previous OPAL (Oriented Peptide Array Library) screening in our laboratory has identified the consensus NxxY or NxxF as the minimal sequence required for binding of the Numb PTB domain. The cytoplasmic sequence of ALK was examined for possible binding sites for the Numb PTB domain. Four motifs bearing either the NxxY or NxxF motif were identified: G_{1473}GHVNMAFSQS$_{1483}$, F_{1577}PCGNVNYGYQ$_{1587}$, P_{1499}TSLWNPTYGS$_{1509}$, M_{1099}TDYNPNYCFA$_{1109}$ (Fig. 3.3.1). Peptides with the wild type sequences or with alanine, phenylalanine or phosphotyrosine substitutions were synthesized on a cellulose membrane which was subsequently probed with the Numb PTBo domain. GFSNMSFEDFP, which is a known Numb PTB domain binding peptide (88), served as a positive control.

The Numb PTB domain bound two peptides (G_{1473}GHVNMAFSQS$_{1483}$ and F_{1577}PCGNVNYGYQ$_{1587}$) (Fig. 3.3.2), suggesting these two motifs may be involved in the interaction between ALK and Numb. Within the G_{1473}GHVNMAFSQS$_{1483}$ peptide, either the N1477A or F1480A mutation abolished binding to the Numb PTB domain. As for the F_{1577}PCGNVNYGYQ$_{1587}$ peptide, both the N1583A and Y1586A mutation greatly attenuated binding of the Numb PTB domain to the peptide. Phosphorylation of Y_{1586} moderately attenuated the peptide binding capacity with the Numb PTB domain as shown by the pY1586Y substitution. In contrast, the Y1586F mutant showed a stronger binding signal than the wild type peptide, indicating that the benzene ring may be critical for the

interaction between the peptide and the PTB domain. The pY1584Y substitution displayed a dramatically decreased binding signal while the Y1584F mutation showed a similar binding signal compared with the wild type peptide. The above results suggest that phosphorylation of Y_{1584} and Y_{1586} play a role in regulation of the Numb-ALK interaction. Taken together, the result shows that N_{1477}, F_{1480}, N_{1583} and Y_{1586} are likely the amino-acids critical for binding; phosphorylation of Y_{1584} and Y_{1586} may disrupt the Numb-ALK interaction.

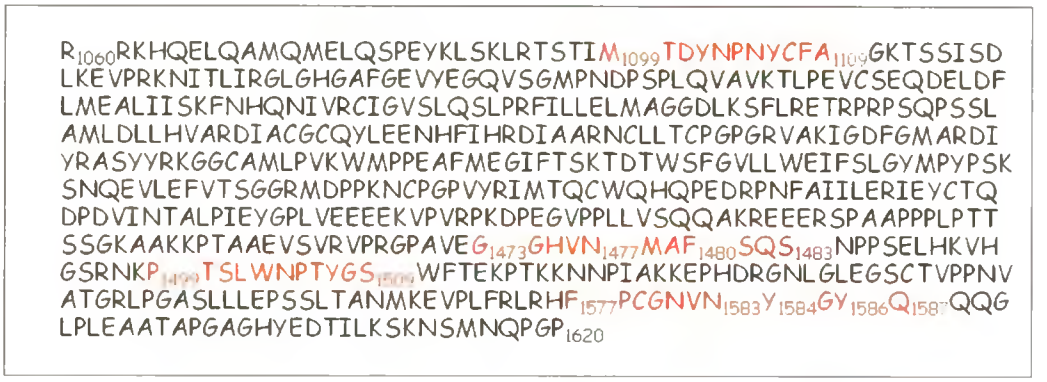

Figure 3.3.1 Amino acid sequence of the cytoplasmic region of Human ALK. Full-length of ALK contains 1620 amino acids with the cytoplasmic region starting from position 1060. Within the ALK cytoplasmic sequence, four potential Numb PTB domain binding sites were identified (red).

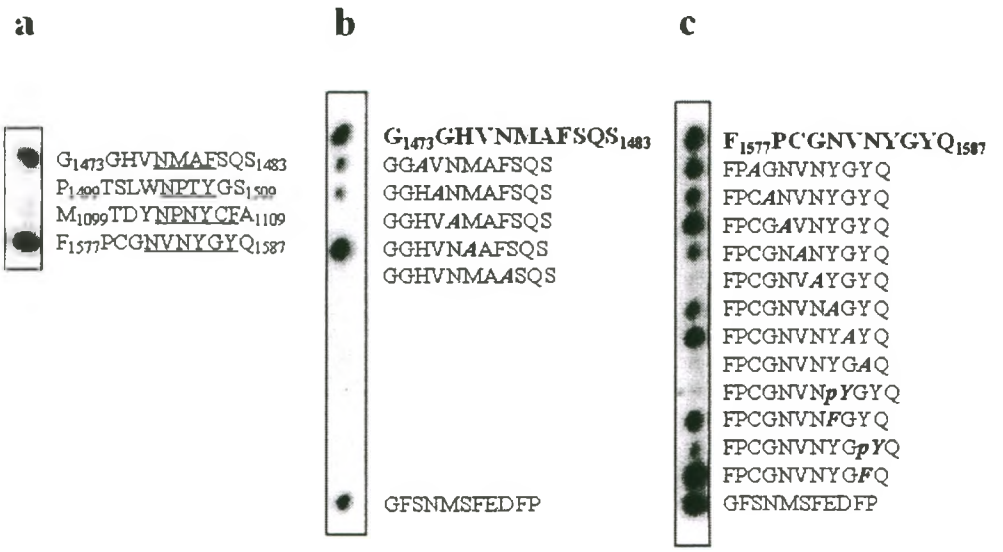

Figure 3.3.2 Peptide array to identify potential Numb binding sites on ALK. **a.** Identification of potential Numb binding motifs. NxxF/Y motifs in wild type ALK (underlined) were probed with 0.2 μM GST-mouse Numb-PTBo. **b.** The positive binder G_{1473}GHVNMAFSQS$_{1483}$ (in bold) was further analyzed by an alanine scanning array where each spot represented an alanine-subsituted (in italic) analogue of the wild-type peptide. The GFSNMSFEDFP peptide was used as a positive control. The peptide array was probed with 0.2 μM GST-mouse Numb-PTBo. **c.** The positive binder F_{1577}PCGNVNYGYQ$_{1587}$ (in bold) was further analyzed by a scanning array where each spot represented an alanine-, phenylalanine- or phosphotyrosine- substituted (in italic) analogue of the wild-type peptide. The peptide array was probed with 0.2 μM GST-mouse Numb-PTBo.

3.4 ALK-Numb interaction is tyrosine phosphorylation dependent.

According to the alanine scanning peptide array results, interactions between ALK and Numb appeared to be decreased by phosphorylation of ALK at Y_{1584} and Y_{1586} and possibly other potential binding motifs on ALK. Previous studies have shown that Numb interacts with E-cadherin (a transmembrane protein) in a tyrosine phosphorylation-dependent manner (11); tyrosine phosphorylation of ALK is required for the recruitment of Shc and FRS2 and subsequent activation of the downstream signaling ERK MAP kinase pathway (65). It was therefore important to investigate whether tyrosine phosphorylation of ALK would regulate its interaction with Numb *in vivo* and therefore affect Erk signaling. To test this hypothesis, the regulation of the ALK-Numb interactions was first investigated *in vivo*.

It has been reported that ALK can either be activated or inhibited by mouse monoclonal antibodies directed against its extracellular domain (56). Specifically mAb-46 antibody is able to activate the ALK receptor and therefore increase the level of tyrosine phosphorylation. In contrast, mAb-49 antibody inhibits ALK tyrosine phosphorylation (56). To explore the relationship between ALK activity and the Numb-ALK interaction, mAb-46 and mAb-49 antibodies were obtained from Dr. Vigny's lab and used to control the activation of ALK.

To confirm the activation of ALK by mAb-46, the H370 cell line was treated with the mAb-46 antibody titrated at different concentrations (0 μg/ml, 0.5 μg/ml, 1 μg/ml and 2 μg/ml). The tyrosine phosphorylation level was determined by Western blot with a 4G10 anti-phosphotyrosine antibody against immunoprecipitated ALK. The basal

tyrosine phosphorylation of both species of ALK-HA(220 kDa and 140 kDa) was increased upon mAb-46 treatment (Fig. 3.4.1).

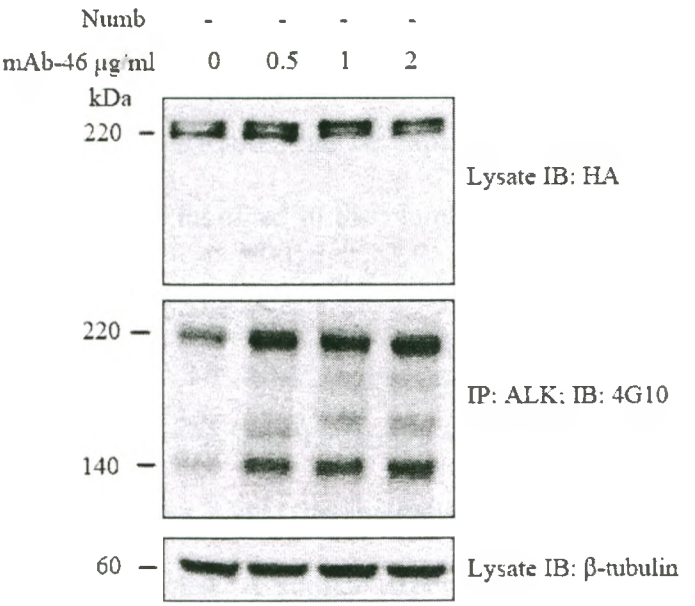

Figure 3.4.1 Effects of the mAb-46 antibody on activation of ALK phosphorylation. Western blot was performed on H370 cells treated with various concentrations of the mAb-46 activating antibody. H370 cells were serum-starved for 16 hours and then treated with mAb-46 at different concentrations (0 μg/ml, 0.5 μg/ml, 1 μg/ml and 2 μg/ml) for 2 hours at 37℃. H370 cells were lysed in mammalian cell lysis buffer and 500 μg of total protein was then immunoprecipitated using 1 μg of a mouse anti-ALK antibody. 20 μg of lysate and the IP samples were loaded on a 4-12% Bis-Tris gradient gel. Western blot was performed using mouse anti-HA, mouse anti- phosphotyrosine (4G10) and rabbit anti-β tubulin antibodies.

After confirmation of ALK activation by the mAb-46 antibody, the mouse Numb PTBi/PRRi isoform was transiently expressed in H370 cells followed by treatment with the mAb-46 antibody at the same concentrations mentioned above. Immunoprecipitation of Numb followed by immunoblotting for the HA tag showed that the interaction between Numb and either full-length (220kDa) ALK or short length (140kDa) ALK was increased upon ALK activation (Fig. 3.4.2). This result seems to contradict with the peptide array results, but the paradox can be explained by the possibility that multiple tyrosine residues on ALK are involved in the Numb-ALK interaction and the interaction is dynamically regulated by phosphorylation, which will be discussed in the following chapter.

In order to further confirm that the Numb-ALK interaction was dependent on the ALK tyrosine phosphorylation level, the interaction under inhibition of ALK by the mAb-49 antibody was examined. A similar experimental procedure was performed as with ALK activation above. H370 cells were treated with the mAb-49 antibody at the concentration of (2 μg/ml, 4 μg/ml and 8 μg/ml). The antibody was able to inhibit ALK at any concentration used (Fig. 3.4.3). Next, H370 cells were transfected with the mouse Numb +/+ isoform and treated with 2 μg/ml of mAb-49. Compared to the control, inhibition of ALK phosphorylation decreased the amount of full-length ALK immunoprecipitated with Numb. Furthermore, the interaction of Numb and short species of ALK was no longer detected when ALK was inhibited (Fig. 3.4.4).

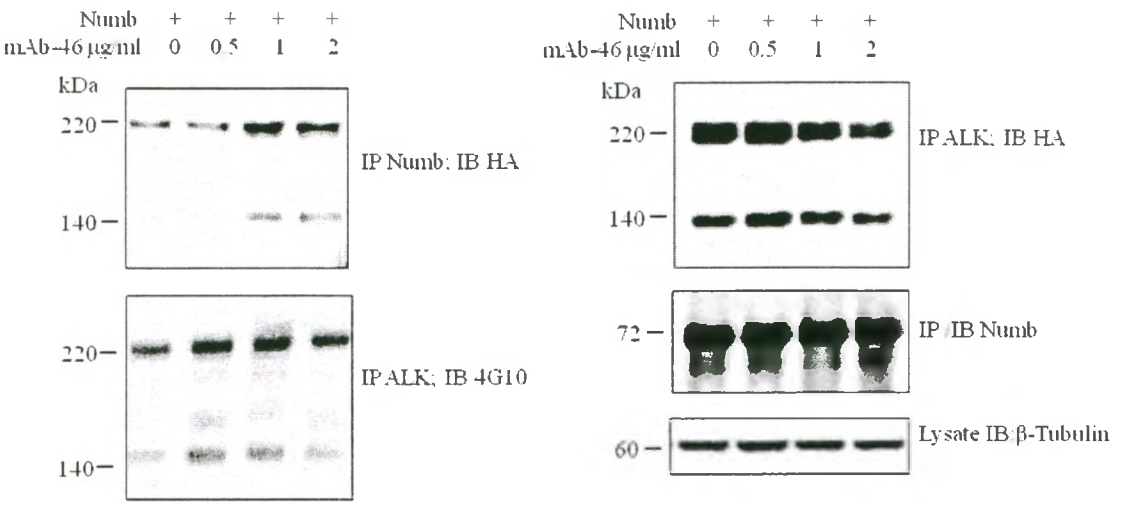

Figure 3.4.2 Activation of ALK increased the Numb-ALK interaction. H370 cells were transiently transfected with the mouse Numb PTBi/PRRi isoform. One day after transfection, the cells were serum-starved for 16 hours and then treated with 0.5 μg/ml, 1 μg/ml and 2 μg/ml mAb-46 (or no antibody) for 2 hours at 37 ℃. The cells were then lysed and 500 μg of total protein was immunoprecipitated with 1 μg rabbit anti-Numb antibody or 1 μg mouse anti-ALK antibody. 20 μg of lysate and the IP samples were loaded on a 4-12% Bis-Tris gradient gel. Western blot was performed using mouse anti-HA, mouse anti-phosphotyrosine (4G10), rabbit anti-Numb and rabbit anti-β tubulin antibodies.

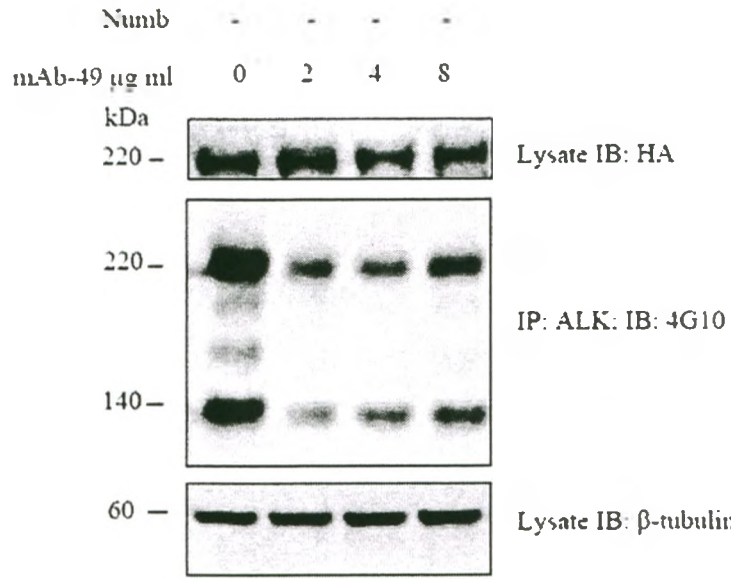

Figure 3.4.3 Effects of the mAb-49 antibody on inhibition of ALK phosphorylation. Western blot was performed on H370 cells treated with the ALK-inhibiting antibody mAb-49. H370 cells were serum-starved for 16 hours and then treated with mAb-49 at various concentrations (0 µg/ml, 2 µg/ml, 4 µg/ml and 8 µg/ml) for 2 hours at 37℃. Cells were then lysed and 500 µg of total protein was immunoprecipitated using 1 µg of a mouse anti-ALK antibody. 20 µg of cell lysate and the IP samples were loaded on a 4-12% Bis-Tris gradient gel. Western blot was performed using mouse anti-HA, mouse anti-phosphotyrosine (4G10) and rabbit anti-β tubulin antibodies.

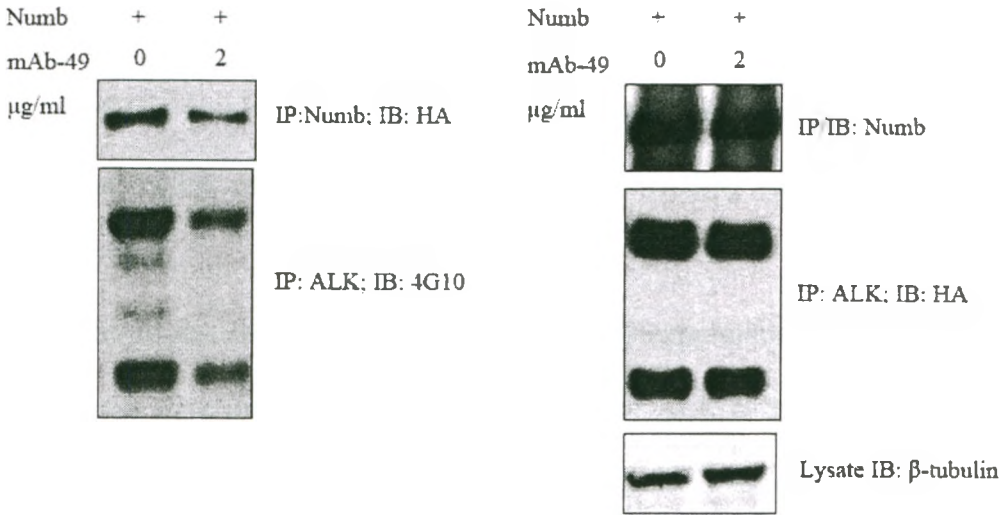

Figure 3.4.4 Inhibition of ALK decreased the Numb-ALK interaction. H370 cells were transfected with the mouse Numb PTBi/PRRi isoform. One day after transfection, the cells were serum-starved for 16 hours and then treated with 2 μg/ml of mAb-49 (or untreated) for 2 hours. The cells were then lysed using mammalian cell lysis buffer and 500 μg of total protein was immunoprecipitated with 1 μg rabbit anti-Numb antibody or 1 μg mouse anti-ALK antibody. 20 μg of lysate and the IP samples were loaded on a 4-12% Bis-Tris gradient gel. Western blot was performed using mouse anti-HA, mouse anti-phosphotyrosine (4G10), rabbit anti-Numb and rabbit anti-β tubulin antibodies.

3.5 Numb affects expression and/or cleavage of ALK

It has been shown that, upon growth factor stimulation, some receptor tyrosine kinases such as the EGFR can be rapidly degraded by endocytosis (37). As Numb is an endocytotic protein involved in the degradation of a number of transmembrane proteins, it is likely that Numb may regulate ALK-activated signaling pathways by controlling ALK levels *in vivo*.

In order to test this hypothesis, the total ALK expression level was determined when H370 cells were treated with various amounts of ALK activation antibody (mAb-46), combined with or without Numb transfection. Fig. 3.5.1 showed that in the absence of Numb in H370 cells, ALK expression levels and patterns remained unchanged with increasing amounts of mAb-46. However, when Numb was expressed in H370 cells activated with 1 μg/ml or 2 μg/ml of mAb-46 antibody, the level of full-length ALK expression was decreased, while the expression levels of the short form of ALK (140 KDa species) increased. Blotting immunoprecipitated ALK with 4G10 confirmed that ALK was indeed activated. β-tubulin immunoblotting confirmed equal loading of samples. In parallel sets of experiments, H370 cells were treated with various amounts of the inhibition antibody mAb-49 together with Numb transfection. In Fig. 3.5.2, the Western blot with the 4G10 antibody indicated that tyrosine phosphorylation levels of ALK were inhibited. Western blot against cell lysates demonstrated that the full-length ALK expression level remained constant regardless of inhibition. The short form of ALK was not detected when ALK was inhibited, suggesting ALK cleavage or processing is required for its activation.

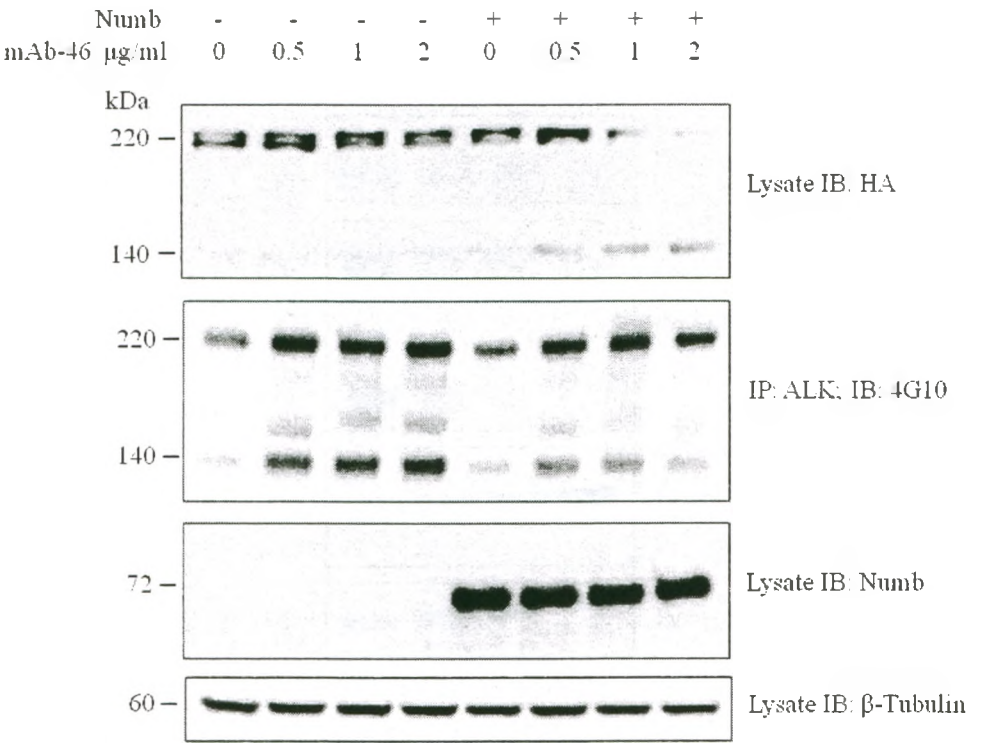

Figure 3.5.1 Numb affected the expression and/or stability of ALK upon ALK activation. H370 cells were transfected with either the mouse Numb PTBi/PRRi isoform or empty vector (pcDNA3.1). One day after transfection, the cells were serum starved for 16 hours. The cells from either group were treated with increasing concentrations of the activating antibody mAb-46 (0µg/ml, 0.5 µg/ µg ml, 1 µg/ml and 2 µg/ml) for 2 hours. The cells were then lysed and 500 µg of total protein was immunopreciptated using 1 µg of a mouse anti-ALK antibody. Cell lysates (20 µg) and IP samples were loaded on a 4-12% Bis-Tris gradient gel. Western blot analysis was performed using mouse anti-HA, mouse anti-phosphotyrosine (4G10), rabbit anti-Numb and rabbit anti-β tubulin antibodies.

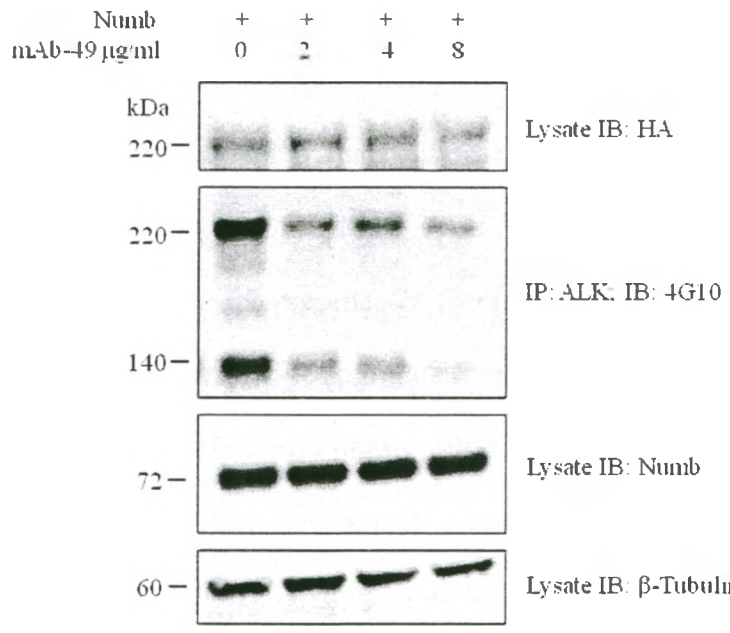

Figure 3.5.2 Numb did not affect the expression and/or stability of ALK upon ALK inhibition. H370 cells were transfected with the mouse Numb +/+ isoform. One day after transfection, the cells were serum starved for 16 hours. The cells were then treated with the increasing concentrations of inhibiting antibody mAb-49 (0 µg/ml, 2 µg/ µg ml, 4 µg/ml and 8 µg/ml) for 2 hours. The cells were lysed and 500 µg of total protein was immunopreciptated using 1 µg of a mouse anti-ALK antibody. Cell lysates (20 µg) and IP samples were loaded on a 4-12% Bis-Tris gradient gel. Western blot was performed using mouse anti-HA, mouse anti-phosphotyrosine (4G10), rabbit anti-Numb and rabbit anti-β tubulin antibodies.

3.6 Numb inhibits Erk activation downstream of ALK

Upon ALK activation, Erk, which is involved in the mitogen-activated protein kinase pathway, is dramatically activated (56). To further investigate whether Numb is involved in this signaling pathway downstream of ALK, Erk phosphorylation (pErk) levels were tested when H370 cells were treated with the ALK-activation antibody, mAb-46, combined with or without Numb transfection (Fig. 3.6.1: HA, 4G10 and Numb immunoblots same as in Fig. 3.5.1). When ALK was activated, the level of pErk appeared to decrease slightly in the presence of Numb. Further quantification confirmed that when ALK was activated by 2μg/ml of mAb-46, Numb reduced the Erk phosphorylation level and therefore may play a role in inhibition of the mitogen-activated protein kinase pathway (Fig. 3.6.2). Interestingly, when ALK was activated with 1 μg/ml or 2 μg/ml of mAb-46, the level of Erk expression decreased in the presence of Numb.

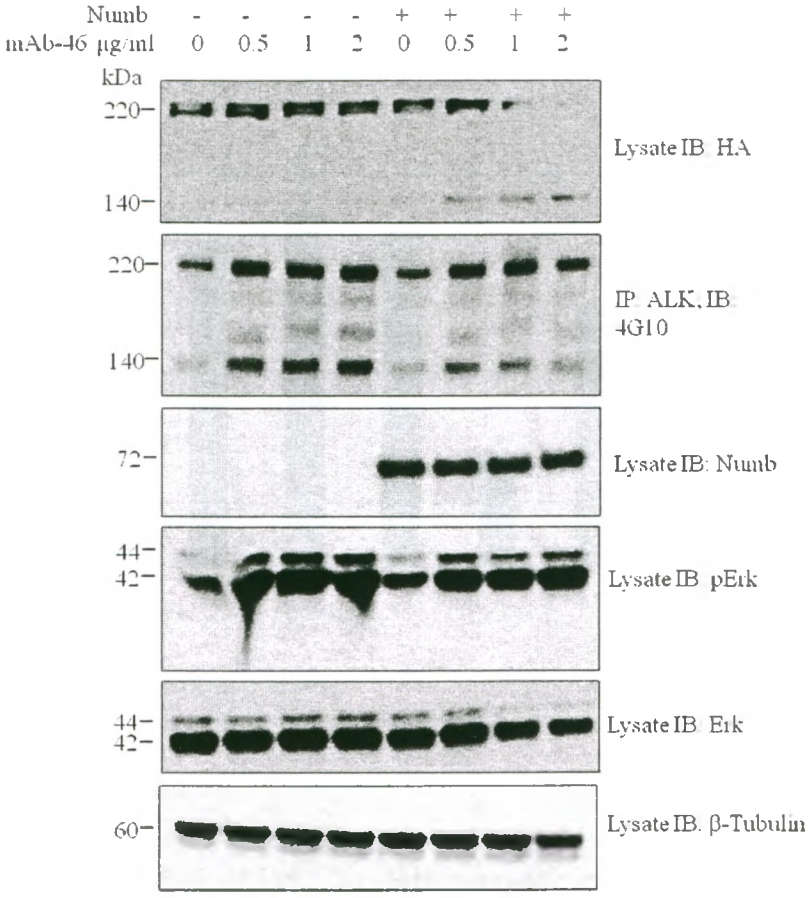

Figure 3.6.1 Numb inhibits Erk activation downstream of ALK. H370 cells were transfected either with Numb or empty vector (pcDNA3.1). One day after transfection, cells were serum starved for 16 hours. The cells from either transfection group were treated with increasing concentrations of the ALK-activating antibody mAb-46 (0 µg/ml, 0.5 µg/ ml, 1 µg/ml and 2 µg/ml) for 2 hours. 500 µg of total protein from each type of cell lysate was immunopreciptated using 1 µg of a mouse anti-ALK antibody. Cell lysates (20 µg) and IP samples were loaded on a 4-12% Bis-Tris gradient gel. Western blot analysis was performed using mouse anti-HA, rabbit anti-Numb and rabbit anti-β tubulin antibodies. The Western blot was also performed using a mouse anti- phospho-Erk1/2 (pErk) antibody and then was reprobed with a mouse anti-Erk1/2 antibody.

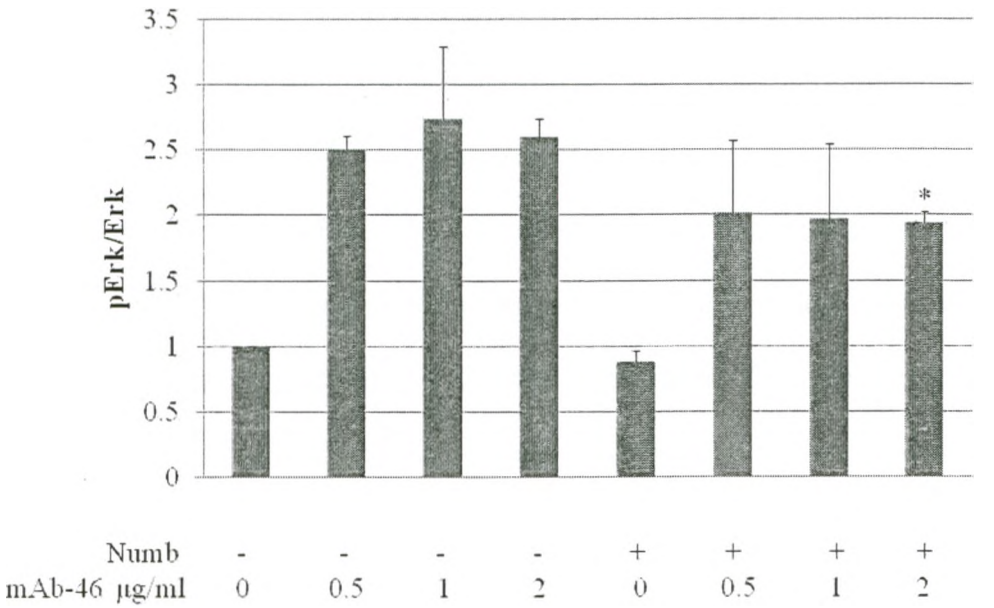

Figure 3.6.2 Quantification of Erk1/2 relative phosphorylation levels. The intensity of each band was measured by Scion Image software (Scion Corporation, Frederick, MD). pErk/Erk ratio was calculated and expressed as the mean ±SD of 2 separate experiments. *$p<0.04$, compared with control sample: H370 treated with 2 µg/ml of mAb-46 without Numb transfection.

3.7 Numb promotes Akt phosphorylation independent of ALK activation

Although activation of full-length ALK by agonist monoclonal antibodies has no effects on Akt signaling (56, 61), constitutively activated NPM-ALK or the full-length ALK mutants F1174L and K1062M activate Akt phosphorylation (pAkt) (81, 89). Therefore, pAkt levels were also examined when H370 cells were treated with the mAb-46 antibody, with or without Numb expression (Fig. 3.7.1: HA, 4G10 and Numb immunoblots same as in Fig. 3.5.1). The level of Akt phosphorylation did not change upon ALK activation, however the expression of Numb resulted in an increase in the level of Akt phosphorylation in two separate experiments. The quantification of pAkt/Akt was shown in Fig. 3.7.2. Therefore, although Akt was not involved in ALK activation signaling; the increase of pAkt indicates an additional function of Numb other than regulating the ALK signaling pathway.

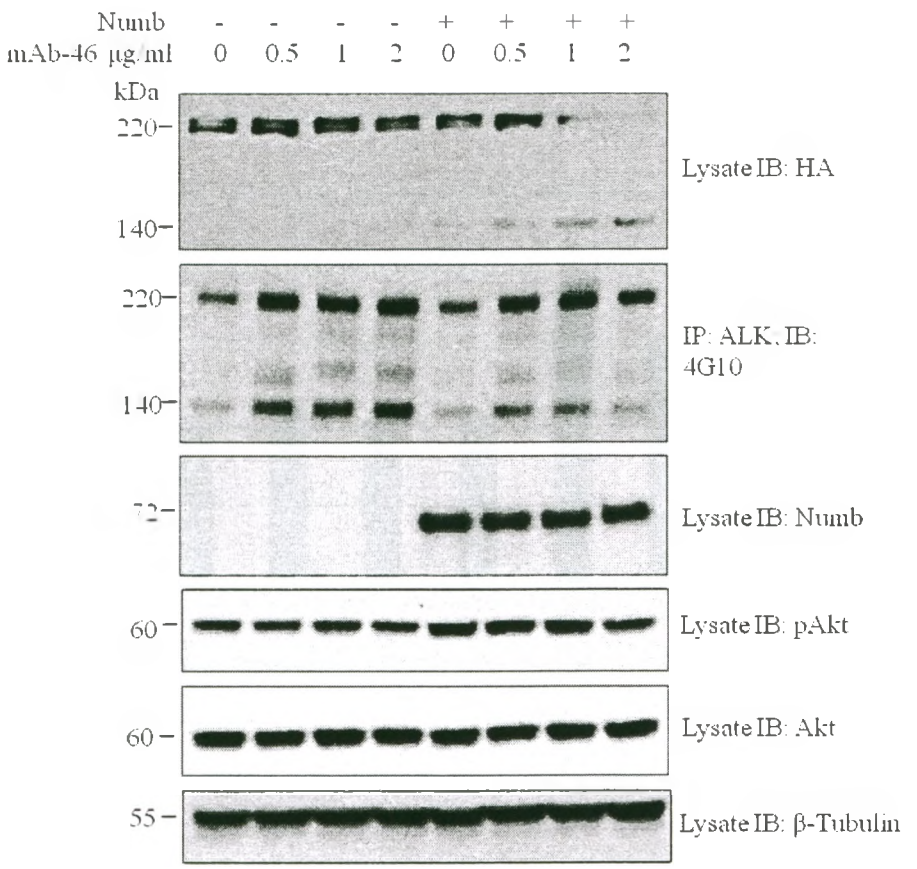

Figure 3.7.1 Enhancement of Akt phosphorylation by Numb overexpression. H370 cells were transfected either with Numb or empty vector (pcDNA3.1). One day after transfection, cells were serum starved for 16 hours. The cells from either transfection group were treated with increasing concentrations of the ALK-activating antibody mAb-46 (0 μg/ml, 0.5 μg/ ml, 1 μg/ml and 2 μg/ml) for 2 hours. 500 μg of total protein from each type of cell lysate was immunoprecipitated using 1 μg of a mouse anti-ALK antibody. Cell lysates (20 μg) and IP samples were loaded on a 4-12% Bis-Tris gradient gel. Western blot analysis was performed using mouse anti-HA, rabbit anti-Numb and rabbit anti-β tubulin antibodies. Western blot was also performed using a mouse anti-phosphor-Akt (pAkt) antibody and then was reprobed with a rabbit anti- Akt antibody.

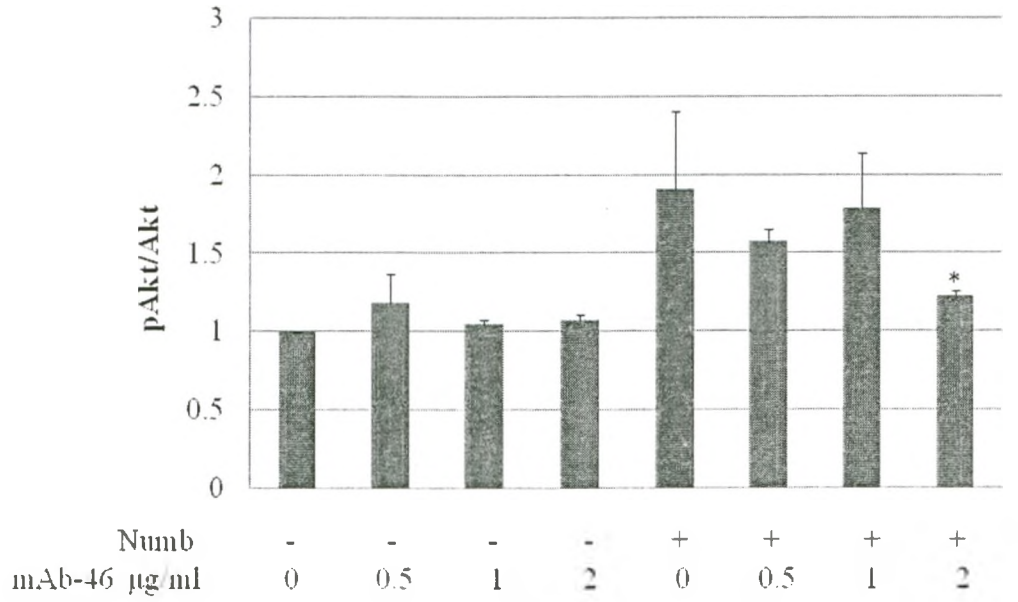

Figure 3.7.2 Quantification of Akt relative phosphorylation level. The intensity of each band was measured by Scion Image software (Scion Corporation, Frederick, MD). pAkt/Akt ratio was calculated and expressed as the mean ±SD of 2 separate experiments. *$p<0.05$, compared with control sample: H370 treated with 2 μg/ml of mAb-46 without Numb transfection.

3.8 Subcellular localization of ALK

Since Numb affects ALK cleavage upon ALK activation, it is probable that cleaved ALK has some biological functions. Some receptors such as the Notch receptor, can be cleaved by proteases and then translocated into the nucleus as transcription factors (90). Predicted by PSORT (http://psort.ims.u-tokyo.ac.jp/), the $R_{1060}RKH_{1063}$ motif within

human ALK was a potential nuclear localization signal (NLS). Therefore, it is probable that Numb might regulate ALK biological function(s) in the nucleus. In order to investigate this possibility, the subcellular ALK expression was examined at various conditions either activated by antibody mAb-46 or not, combined with co-expresssion of wild type (WT) Numb, the F162V Numb mutant (which greatly decreased the Numb-ALK interaction) or empty vector (pcDNA3.1). H370 cells were fractionated and the resulting subcellular components (cytosol, membrane and nucleus) were analyzed. β tubulin, Na+/K+-ATPase α and Lamin A/C were used as the markers for cytosolic, membrane and nuclear fractions repectively.

When ALK was not activated, the expression of wild type Numb enhanced the level of 140 kDa-ALK protein species in the nucleus (Fig. 3.8.1). Upon ALK activation, the amount of 140 kDa-ALK protein also increased in the nucleus regardless of the vector (empty, Numb or Numb mutant vector) transfected (Fig. 3.8.1).

Interestingly, full-length ALK existed in the nuclear fraction regardless of the ALK activation state or the presence/absence of Numb. However, the amount of full-length ALK in the nucleus increased either in the presence of WT Numb or upon ALK activation.

In summary, it is probable that both the activation of ALK and the presence of Numb have an effect in promoting the location of either the short form or full length of ALK in the nucleus.

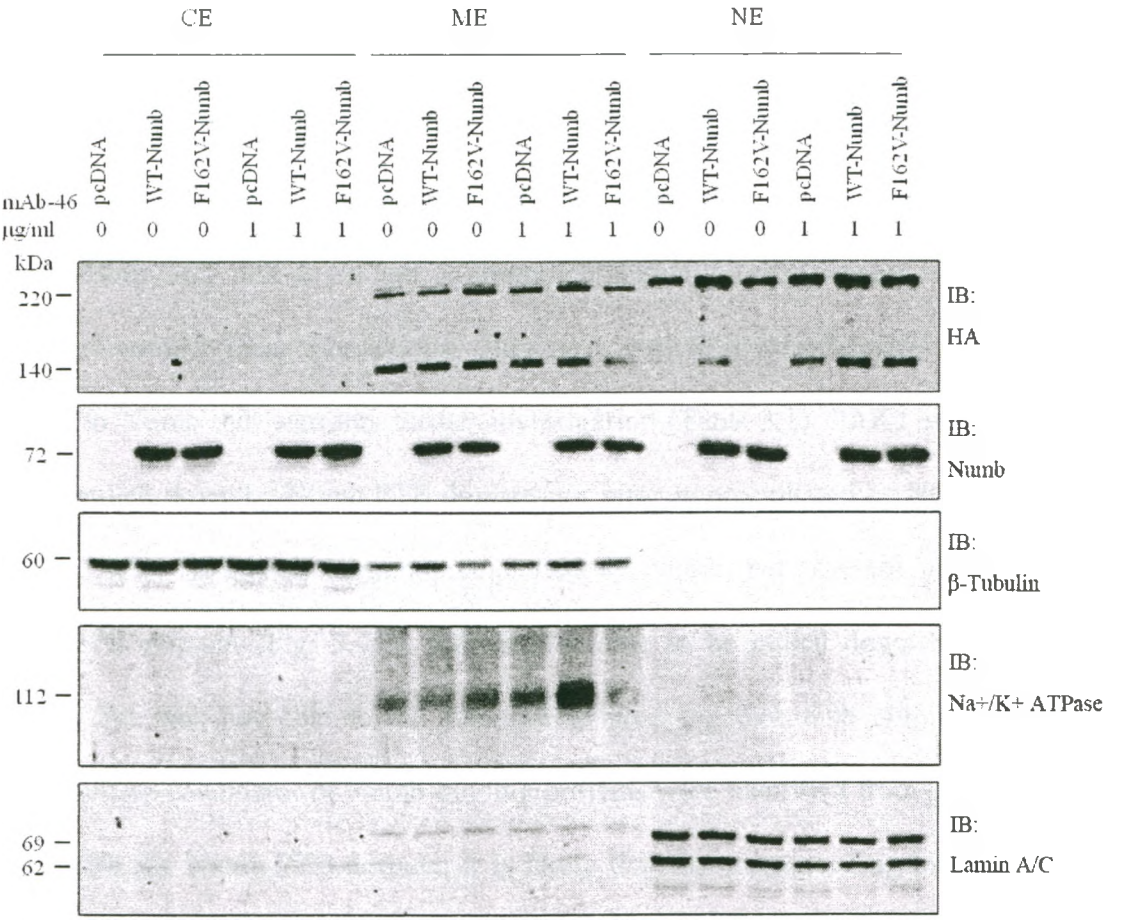

Figure 3.8.1 ALK subcellular localization. H370 cells were transfected with empty vector (pcDNA 3.1), WT Numb or the F162V Numb mutant. One day after transfection, the cells were serum starved for 16 hours. The cells from each group were either treated with the activating antibody mAb-46 at 1 μg/ml for two hours or left untreated. Cells were then fractionated using the FractionPREP cell fractionation kit. Each fraction of the cells was loaded on a 4-12% Bis-Tris gradient gel. Western blot was performed using mouse anti-HA, rabbit anti-Numb antibody, rabbit anti-β tubulin, mouse anti- Na+/K+-ATPase α, goat anti-Lamin A/C antibodies. β tubulin: cytosol marker; Na+/K+-ATPase α: membrane marker; Lamin A/C: nuclear marker. CE: fraction from cytosol extraction; ME: fraction from membrane extraction; NE: fraction from nuclear extraction.

3.9 Examination of Numb binding to other proteins than ALK

At the same time when the Numb-ALK interaction was investigated, several other potential Numb-interacting candidates were also examined, including PAK2, Sypt1, Ezrin and RIPK5. These proteins were identified from proteomic screens by colleagues in the lab. Both GST pull-down and co-immunoprecipitation experiments were used in an attempt to confirm these interactions. However, none of these candidates showed strong binding to Numb that warrants further investigation (Table 3.1). PAK2 and Sypt I were neither pulled down by Numb PTB domain nor immunoprecipitated by Numb (Fig. 3.9.1 & Fig. 3.9.2). Ezrin was immunoprecipitated by Numb, but was not pulled down by Numb PTB domain (Fig. 3.9.3). RIPK5 was able to be pulled down by Numb PTB domain, but was not able to be immuprecipitated together with Numb (Fig. 3.9.4). Because these candidates of Numb-binding proteins were identified from probing peptide arrays with the Numb PTB domain, it is likely that the corresponding binding sites are not accessible in the context of the full-length proteins.

Table 3.1 Examination of Numb binding to other proteins than ALK

Protein	Pulled down by Numb PTB domain	Immunoprecipitated by Numb
PAK2	NO	NO
Sypt I	NO	NO
Ezrin	NO	YES
RIPK5	YES	NO

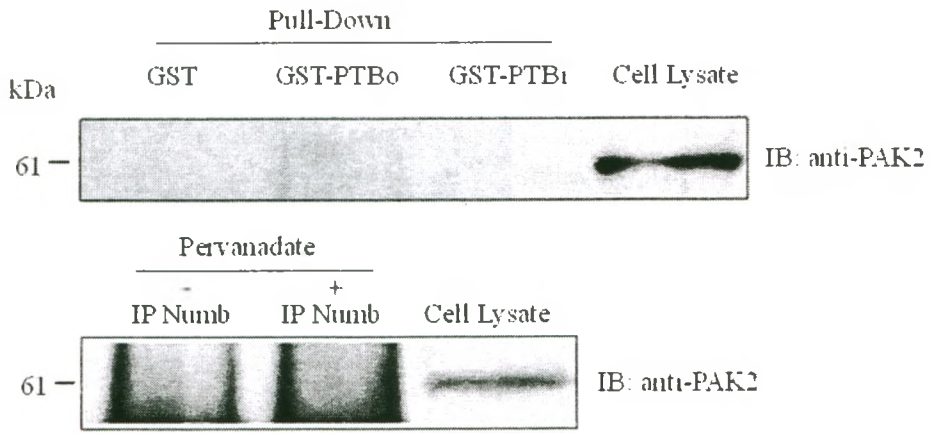

Figure 3.9.1 Examination of the Numb-PAK2 interaction. Hela cell lysates were incubated with 200 μg of GST protein, (GST as a negative control, GST-Numb-PTBo, or GST-Numb-PTBi) for the pull-down assay. Western blot was performed using a rabbit anti-PAK2 antibody (upper panel). Hela cells were treated with or without pervanadate, then lysed in mammalian cell lysis buffer.1 mg of total protein from each cell lysate was then immunoprecipitated using 1 μg of a rabbit anti-Numb antibody. Western blot was performed using a rabbit anti-PAK2 antibody (lower panel).

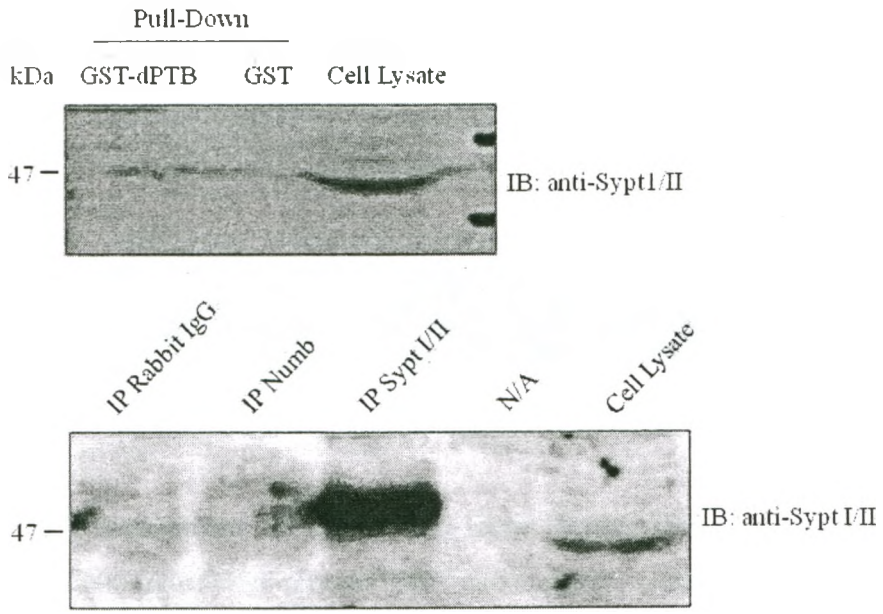

Figure 3.9.2 Examination of the Numb-Sypt I interaction. Jurkat cell lysates were incubated with 200 μg GST or GST-dNumb-PTB (*Drosophila* Numb PTB domain) for the pull-down assay. Western blot analysis was performed using a goat anti-Synaptotagmin (Sypt) I/II antibody (upper panel). Jurkat cells were lysed and 1 mg of total protein was then immunoprecipitated using 1 μg of a rabbit anti-Numb, 1 μg of a goat anti Sypt I/II and 1 μg of a rabbit IgG (negative control). Western blot was performed using a goat anti-Sypt I/II antibody (lower panel).

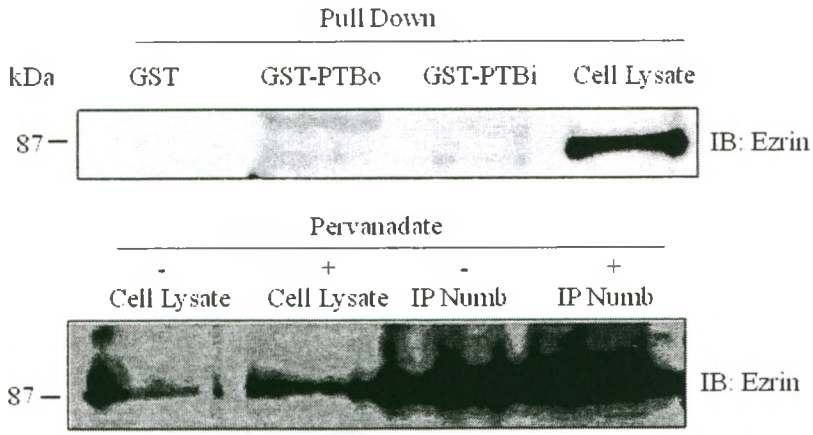

Figure 3.9.3 Examination of the Numb-Ezrin interaction. Hela cell lysates were incubated with 200 μg of GST protein (GST as a negative control, GST-Numb-PTBo and GST-Numb-PTBi) for the pull-down assay. Western blot was performed using a mouse anti-Ezrin antibody (upper panel). Hela cells were treated with or without pervanadate and then lysed in mammalian cell lysis buffer.1 mg of total protein from each cell lysate was then immunoprecipitated using 1 μg of a rabbit anti-Numb antibody. Western blot was performed using a mouse anti-Ezrin antibody (lower panel).

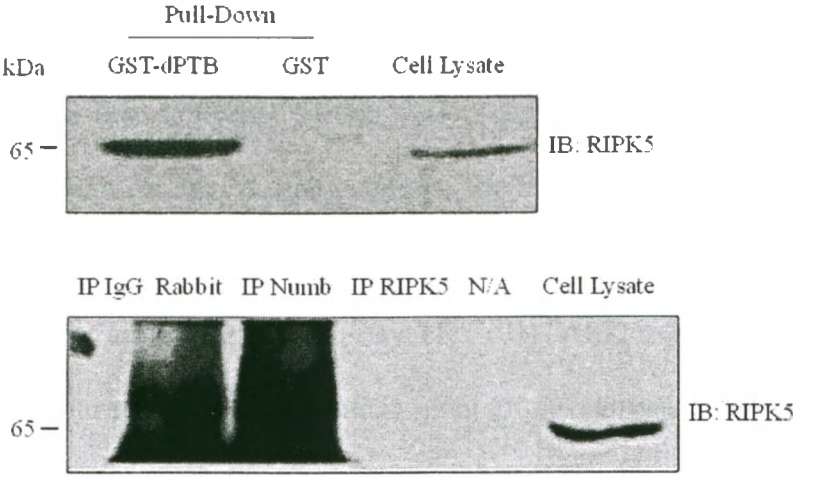

Figure 3.9.4 Examination of the Numb-RIPK5 interaction. Jurkat cell lysates were incubated with 200 μg of GST protein or GST-dNumb-PTB (*Drosophila* Numb PTB domain) for the pull-down assay. Western blot was performed using a rabbit anti-RIPK5 antibody (upper panel). Jurkat cells were lysed and 1 mg of total protein was then immunoprecipitated using 1 μg of a rabbit anti-Numb antibody, 1 μg of a rabbit anti-RIPK5 antibody and 1 μg of a rabbit IgG (negative control). Western blot was performed using a rabbit anti-RIPK5 antibody (lower panel).

Chapter 4: Discussion

A number of studies have been focusing on Numb and ALK separately, however, none has investigated a relationship between Numb and ALK. The interaction between Numb and ALK is first confirmed in this study, when both proteins are overexpressed. Numb is an important protein in neurogenesis; the homozygous loss-of-function Numb mouse shows severe defects in cranial neural tube closure and premature neuron production and dies around embryonic day 11.5 (91). ALK is expressed mainly in the nervous system during early mammalian embryo development, suggesting ALK is involved in the development of the nervous system (53, 62). Therefore it is possible that a role for the Numb-ALK interaction is in the regulation of neural development.

4.1 Numb and ALK interaction

Both PTB domain isoforms (PTBi and PTBo) of mouse Numb can pull down ALK, which indicates that the interaction between Numb and ALK is not Numb PTB domain isoform-dependent. However, whether the PTB domain is the only region of Numb involved in Numb-ALK interaction still requires further investigation. Although ALK was only pulled down by GST–PTB when PTB domain was present, it is hard to conclude that the Numb C-terminal construct (GST-C), which contained the last 351 residues of the protein (starting from the end of the PTB domain to the end of the protein), was not involved in the interaction. The expression of GST-C was very low compared to the other GST fusion proteins.

Previous studies have investigated Numb PTB domain binding to its ligands *in vitro* and *in vivo* (88,93), and from these studies several possible reasons arise for why

the F162V Numb mutant was unable to completely abrogate the Numb-ALK interaction. First, the F162V mutation in the Numb-PTB domain resulted in a drastic decrease instead of complete loss of binding-affinity to ligand peptides such as the Nak-c or the GPpY peptide (88). In addition, a homologous F to V mutation on the SHC PTB domain resulted in a decrease, but not complete loss of binding to the EGF receptor (92). Second, a peptide ligand itself may not totally simulate the original ligand and therefore may result in variable binding patterns compared with the native ligand. Third, several other amino acids in the PTB have been shown to be important for its binding to the ligands and its function (88, 92, 93); those amino acids likely cooperate with F_{162} for Numb-PTB domain to bind with its partner. Since the F162V Numb mutant only resulted in a decrease in the Numb-ALK interaction rather than a complete loss of binding in this study, it indicates that the Numb-PTB domain is very important for binding to ALK, but may not be solely responsible for the interaction.

From the peptide array results, two potential Numb binding sites on ALK were predicted (N_{1477}MAF and N_{1583}YGY) with key residues playing a critical role in binding. Alanine substitution at residues N_{1477} or F_{1480} resulted in a complete loss of binding to the NMAF-containing peptide, while alanine substitution at residues N_{1583} or Y_{1586} greatly attenuated Numb binding to the peptides containing the N_{1583}YGY motif. However, further *in vivo* experiments to conclude whether the N_{1477}MAF and/or N_{1583}YGY motifs are the Numb-binding sites still requires further work. Currently, ALK site-directed mutagenesis plasmids containing the N1447A or N1583A mutation have been completed. Next, the N1477A/N1583A double mutant will be generated. The interaction between

Numb and the mutated ALK proteins will be tested with co-immunoprecipitation in the future.

4.2 Phosphorylation regulated interaction between Numb and ALK

Results from the peptide array screening showed that phosphorylation of tyrosine at position 1584 greatly attenuated binding of Numb to the $N_{1583}Y_{1584}GY$ peptide and phosphorylation of tyrosine at position 1586 moderately attenuated Numb binding to the $N_{1583}YGY_{1586}$ peptide. However when ALK was activated using an activating antibody (mAb-46 (56)), instead of decreasing the interaction between Numb and ALK, the Numb-ALK interaction was increased.

There are thirteen tyrosine residues within the intracellular region of human ALK which could potentially be phosphorylated upon ALK activation (55). Y_{1584} and Y_{1586} are only two of them, and which tyrosine(s) are actually phosphorylated upon mAb-46 stimulation remains unknown. It is possible that tyrosine residues other than Y_{1584} and Y_{1586} become phosphorylated upon ALK activation by mAb-46. It has been reported that Y_{1507} was phosphorylated upon ALK activation, and this phosphotyrosine residue recruits SHC through its PTB domain onto the ALK receptor (65). Furthermore, besides interacting with NxxF/Y motifs, the Numb PTB domain also binds to phosphotyrosine containing peptides such as GPpY (88). Therefore, besides potentially interacting with $N_{1583}YGY$ and $N_{1477}MAF$ motifs, the Numb PTB domain likely interacts with other phosphotyrosine residues within the intracellular regions of the ALK receptor. To test this, additional arrays containing peptides of all the intracellular tyrosine sites of ALK either with unphosphorylated or phosphorylated tyrosine residues will be synthesized.

Probing this array with the Numb PTB domain will provide insight into the possibility of additional Numb binding sites on ALK. Regardless of the outcome of this experiment, it is likely that the Numb-ALK interaction is dynamically regulated by phosphorylation, ie., the phosphorylation of certain tyrosine residues may enhance binding while the phosphorylation of other tyrosine residues may attenuate binding. Identifying the kinases or conditions under which these phosphorylations may be regulated is instrumental to the understanding of ALK function, in particular with regard to Numb.

4.3 The possibility of Numb regulating ALK protein levels by endocytosis

When stimulated by growth factors, a number of RTKs, such as the EGFRs (94), are rapidly endocytosed and degraded to attenuate their signaling. The endocytosis of a chimeric ALK receptor (the extracellular and transmembrane domain of ALK was substituted for the corresponding regions of epidermal growth factor receptor) has been reported, when it was stimulated by the growth factor EGF (95). In addition, the cytoplasmic region of ALK also contains several putative internalization codes, (NPNY at residues 1093-1096, NPTY at residues 1504-1507, and YGPL at residues 1401-1404) and several di-leucine motifs (95). Therefore it is possible that ALK can undergo endocytosis. On the other hand, Numb is an endocytotic protein, involved in the endocytosis of a variety of transmembrane proteins, such as the Notch 1 receptor and amyloid precursor protein (96, 97). Numb antagonizes Notch1 by inducing Notch1 ubiquitination and endocytic degradation of Notch intracellular domain (NICD) (45). Numb isoforms with insertion in the PTB domain also reduce amounts of APP by endocytosis followed by lysosomal degradation (27).

In the present study, upon ALK activation, Numb expression decreased the full-length ALK protein level, which correlated well with an increase in the Numb-ALK interaction (Fig 3.5.1 & Fig. 3.4.2). It is possible that Numb regulates ALK protein levels by endocytosis. As discussed in the previous section 4.2, it is possible that Numb dynamically binds to phosphotyrosine residue(s) depending on the activity of ALK. Upon ALK activation, tyrosine residue(s) Y_{1096}, Y_{1507} or Y_{1401} within the internalization code of ALK is likely phosphoylated, therefore recruiting Numb to the phosphotyrosine residue(s) and potentially regulating ALK endocytosis and degradation (Fig. 4.1).

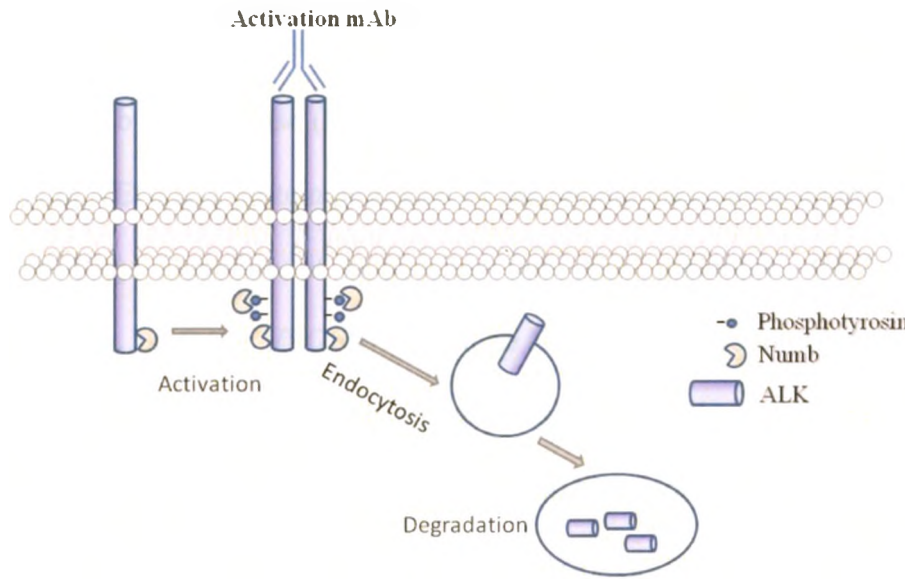

Figure 4.1 Numb may regulate ALK protein level by endocytosis. Upon ALK activation by mouse monoclonal antibody, tyrosine residues with endocytic motifs become phosphorylated, resulting in the recruitment of additional Numb protein to these sites on ALK, regulating protein level by endocytosis and degradation.

4.4 ALK cleavage

When Numb is expressed in H370 cells that are treated with ALK-activation antibody mAb46, there is a decrease in full-length ALK (220 kDa) and an increase in a shorter ALK fragment (140 kDa) (Fig. 3.5.1). A band of approximately 85 kDa was also observed when the exposure time of the film in Fig. 3.5.1 (uppermost panel) was increased (Fig. 4.2). The amount of the 85 kDa fragment did not significantly change either with ALK activation or Numb expression. A similar fragment of ALK has been also identified in Tadashi Yamamoto's lab (61). There are numerous reports of cleavage of receptors such as Notch, ErbB4 and VEGFR-1 upon the activation by ligands. These cleavages share similar characteristics: an initial cleavage in the extracellular region of the receptors by Metalloproteinase TACE/ADAM, followed by a second cleavage at the plasma membrane by γ-secretase (98, 99, 100) (Fig. 4.3). The molecular weight of the Notch, ErbB4 or VEGFR-1 protein produced by the second cleavage is approximately 80 kDa (98, 99, 100). Therefore, it is possible that ALK undergoes similar protease cleavages as Notch, ErbB4 or VEGFR-1. Because Numb overexpression led to increased level of the 140 kDa Alk band, Numb probably took part in the regulation of the first cleavage step of the ALK receptor (producing the 140 kDa protein), but not in the second cleavage step (producing the 85 kDa protein). However, it is not apparent how Numb accomplishes this task as it is present in the cytoplasm or the nucleus while the 1st cleavage site in ALK presumably lies at the extracellular domain. It is possible that Numb first promotes ALK endocytosis and this is followed by cleavage of ALK in a specific vesicular compartment. Endocytosis-dependent proteolysis has been observed for

the nerve growth factor receptors (101). A similar mechanism was shown to be responsible for the cleavage of activated Notch receptor by γ-secretase (102).

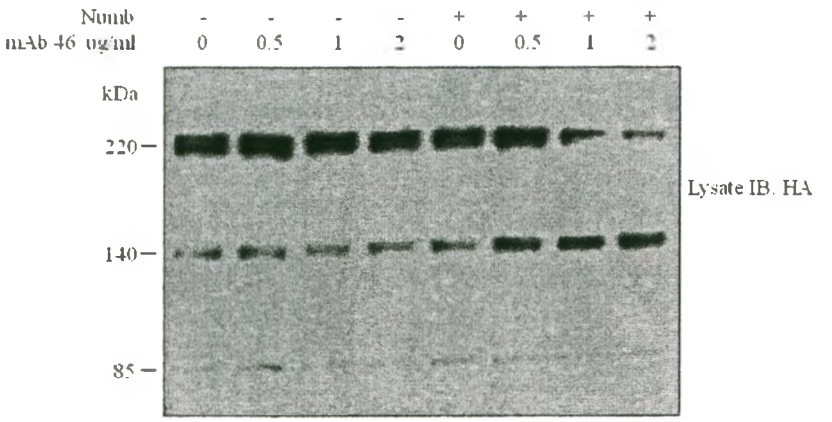

Figure 4.2 Longer exposure of the x-ray film in Fig. 3.5.1 (uppermost panel). H370 cells were treated with titrated concentrations (0 μg/ml, 0.5 μg/ μg ml, 1 μg/ml and 2 μg/ml) of the activating antibody mAb-46 with or without Numb expression. Western blot analysis was performed using a mouse anti-HA antibody.

4.5 ALK nuclear translocation

The cell fractionation results showed that the 220 kDa and 140 kDa species of ALK existed both in the membrane and in the nucleus (Fig. 3.8.1). Additionally, ALK activation or Numb expression dramatically increased the full-length ALK protein level in the nucleus. It is known that full-length EGFR, ErbB-2 and ErbB-3 can be detected in nucleus (103). Upon activation by a ligand, these ErbB proteins can be transported into the nucleus by endosome-mediated nuclear translocation (103, 104). These nuclear ErbB proteins can either regulate transcription or act as protein kinases (103). As an endocytic

protein, it will be interesting to investigate whether Numb regulates ALK nuclear translocation by endocytosis, and therefore regulates potential ALK nuclear functions such as transcription or as a nuclear tyrosine kinase.

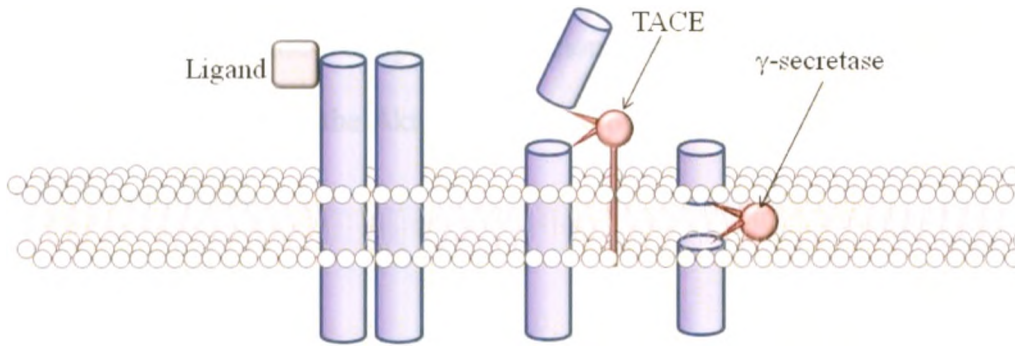

Figure 4.3 The cleavage of Notch, ErbB4 and VEGFR-1 receptors. Upon ligand binding, TACE protease initially cleaves the extracellular region of the receptor followed by cleavage by γ-secretase within the transmembrane region of the receptor. This figure is adapted from Boulton (2008) (98).

4.6 Erk and Akt

Erk is a downstream signal of ALK in the MAPK signaling pathway, with Erk phosphorylation levels increasing upon ALK activation. However, when Numb is present, the Erk phosphorylation level decreased. This result suggests that Numb interferes with the activation of the downstream MAPK signaling pathway, possibly by reducing the expression levels of ALK. Consistent with this possibility, the activation of ALK and the subsequent phosphorylation of Erk has been shown to promote neural differentiation (64, 65), while the Numb isoforms with the insertion in the PTB domain (which was used in this study) actually decreased Erk phosphorylation levels and did not have any effect on

neural differentiation (105). Future studies that express various isoforms of Numb together with ALK activation or inhibition would be useful in delineating the isoform-specific effect of Numb in Erk activation.

In contrast to Erk, the phosphorylation levels of Akt did not show any significant change in HEK-293/ALK stable cell line (H370) upon ALK activation. Yet Numb seemed to slightly increase Akt phosphorylation, independently of ALK activation. Therefore, Numb may affect the Akt signaling pathway via a different mechanism from its interaction with ALK.

4.7 Numb isoform effects

Originally, Numb was discovered as a neuronal fate determinant which promotes neural differentiation. Previous studies report that ALK promotes PC12 cells to differentiate into neurons, suggesting ALK is also involved in neural differentiation. However, results from the present study suggest that Numb antagonizes ALK biochemically and probably physiologically. The apparently conflicting functions of Numb could be explained by the presence of different Numb isoforms. Mammalian Numb has four isoforms: PTBi/PRRi, PTBi/PRRo, PTBo/PRRi, and PTBo/PRRo (PTBi/PTBo: PTB with/without insertion; PRRi/ PRRo: PRR with/without insertion, respectively). It has been reported that the PTBo-Numb isoform increases Erk phosphorylation in PC12 cells treated with NGF and therefore promotes neural differentiation of PC12 cells, while the PTBi-Numb isoform decreases Erk phosphorylation in NGF treated PC12 cells and did not have any effect on neural differentiation of PC12 cells (105). It was also found that the PRRi-Numb isoforms were

expressed in undifferentiated cortical progenitors cells and promoted the proliferation of neural crest stem cells, while the PRRo-Numb isoforms were expressed prominently during differentiation and contributed to an enhanced neuronal differentiation (106). The Numb isoform prominently used in this thesis was PTBi/PRRi-Numb. According to the reports on the functions of different Numb isoforms mentioned above, PTBi/PRRi-Numb plays a role in undifferentiated neural progenitor proliferation and inhibition of Erk activation, which is consistent with results obtained from this study.

4.8 Conclusions and Future directions

In summary, Numb is able to interact with ALK likely through its PTB domain. Two putative Numb binding motifs were identified on ALK, namely NMAF (1477-1480) and NYGY (1583-1586). However, there may exist other binding motifs for Numb on ALK and the roles of these various motifs in the Numb-ALK interaction await further investigation. The Numb-ALK interaction is enhanced upon ALK activation, and is attenuated upon ALK inhibition. The interaction between Numb and ALK led to a decrease in the level of the full-length ALK but an increase in the 140 KDa short species of ALK, suggesting Numb may be involved in endocytosis and/or cleavage of ALK upon its activation. Numb facilitates translocation of ALK (either the 140 kDa short form or the full-length species) from the plasma membrane into the nucleus. Furthermore, decreased level of the full length ALK in the presence of Numb was accompanied with decreased phosphorylation of Erk, the downstream target of activated ALK. Thus the interaction of Numb with ALK may serve as a negative regulator to prevent sustained activation of the ALK signaling pathway.

References

1. Uemura, T., Shepherd, S., Ackerman, L., Jan, L. Y., and Jan, Y. N. (1989) Numb, a Gene Required in Determination of Cell Fate during Sensory Organ Formation in Drosophila Embryos. *Cell. 58*, 349-360.

2. Jan, Y. N., and Jan, L. Y. (2001) Asymmetric Cell Division in the Drosophila Nervous System. *Nat. Rev. Neurosci. 2*, 772-779.

3. Cayouette, M., and Raff, M. (2002) Asymmetric Segregation of Numb: A Mechanism for Neural Specification from Drosophila to Mammals. *Nat. Neurosci. 5*, 1265-1269.

4. Rhyu, M. S., Jan, L. Y., and Jan, Y. N. (1994) Asymmetric Distribution of Numb Protein during Division of the Sensory Organ Precursor Cell Confers Distinct Fates to Daughter Cells. *Cell. 76*, 477-491.

5. Knoblich, J. A., Jan, L. Y., and Jan, Y. N. (1995) Asymmetric Segregation of Numb and Prospero during Cell Division. *Nature. 377*, 624-627.

6. Macara, I. G. (2004) Parsing the Polarity Code. *Nat. Rev. Mol. Cell Biol. 5*, 220-231.

7. Margolis, B., and Borg, J. P. (2005) Apicobasal Polarity Complexes. *J. Cell. Sci. 118*, 5157-5159.

8. Shin, K., Fogg, V. C., and Margolis, B. (2006) Tight Junctions and Cell Polarity. *Annu. Rev. Cell Dev. Biol. 22*, 207-235.

9. Wang, Q., and Margolis, B. (2007) Apical Junctional Complexes and Cell Polarity. *Kidney Int. 72*, 1448-1458.

10. Smith, C. A., Lau, K. M., Rahmani, Z., Dho, S. E., Brothers, G., She, Y. M., Berry, D. M., Bonneil, E., Thibault, P., Schweisguth, F., Le Borgne, R., and McGlade, C. J. (2007) APKC-Mediated Phosphorylation Regulates Asymmetric Membrane Localization of the Cell Fate Determinant Numb. *EMBO J. 26*, 468-480.

11. Wang, Z., Sandiford, S., Wu, C., and Li, S. S. (2009) Numb Regulates Cell-Cell Adhesion and Polarity in Response to Tyrosine Kinase Signalling. *EMBO J. 28*, 2360-2373.

12. Nishimura, T., and Kaibuchi, K. (2007) Numb Controls Integrin Endocytosis for Directional Cell Migration with aPKC and PAR-3. *Dev. Cell. 13*, 15-28.

13. Dho, S. E., French, M. B., Woods, S. A., and McGlade, C. J. (1999) Characterization of Four Mammalian Numb Protein Isoforms. Identification of Cytoplasmic and Membrane-Associated Variants of the Phosphotyrosine Binding Domain. *J. Biol. Chem. 274*, 33097-33104.

14. Pedersen, W. A., Chan, S. L., Zhu, H., Abdur-Rahman, L. A., Verdi, J. M., and Mattson, M. P. (2002) Numb Isoforms Containing a Short PTB Domain Promote Neurotrophic Factor-Induced Differentiation and Neurotrophic Factor Withdrawal-Induced Death of PC12 Cells. *J. Neurochem. 82*, 976-986.

15. Toriya, M., Tokunaga, A., Sawamoto, K., Nakao, K., and Okano, H. (2006) Distinct Functions of Human Numb Isoforms Revealed by Misexpression in the Neural Stem Cell Lineage in the Drosophila Larval Brain. *Dev. Neurosci. 28*, 142-155.

16. Siegal, G. (1999) The Surprisingly Flexible PTB Domain. *Nat. Struct. Biol. 6*, 7-10.

17. Nie, J., McGill, M. A., Dermer, M., Dho, S. E., Wolting, C. D., and McGlade, C. J. (2002) LNX Functions as a RING Type E3 Ubiquitin Ligase that Targets the Cell Fate Determinant Numb for Ubiquitin-Dependent Degradation. *EMBO J. 21*, 93-102.

18. Chien, C. T., Wang, S., Rothenberg, M., Jan, L. Y., and Jan, Y. N. (1998) Numb-Associated Kinase Interacts with the Phosphotyrosine Binding Domain of Numb and Antagonizes the Function of Numb in Vivo. *Mol. Cell. Biol. 18*, 598-607.

19. Li, S. S. (2005) Specificity and Versatility of SH3 and Other Proline-Recognition Domains: Structural Basis and Implications for Cellular Signal Transduction. *Biochem. J. 390*, 641-653.

20. Verdi, J. M., Schmandt, R., Bashirullah, A., Jacob, S., Salvino, R., Craig, C. G., Program, A. E., Lipshitz, H. D., and McGlade, C. J. (1996) Mammalian NUMB is an Evolutionarily Conserved Signaling Adapter Protein that Specifies Cell Fate. *Curr. Biol. 6*, 1134-1145.

21. Salcini, A. E., Confalonieri, S., Doria, M., Santolini, E., Tassi, E., Minenkova, O., Cesareni, G., Pelicci, P. G., and Di Fiore, P. P. (1997) Binding Specificity and in Vivo Targets of the EH Domain, a Novel Protein-Protein Interaction Module. *Genes Dev. 11*, 2239-2249.

22. Santolini, E., Puri, C., Salcini, A. E., Gagliani, M. C., Pelicci, P. G., Tacchetti, C., and Di Fiore, P. P. (2000) Numb is an Endocytic Protein. *J. Cell Biol. 151*, 1345-1352.

23. Smith, C. A., Dho, S. E., Donaldson, J., Tepass, U., and McGlade, C. J. (2004) The Cell Fate Determinant Numb Interacts with EHD/Rme-1 Family Proteins and has a Role in Endocytic Recycling. *Mol. Biol. Cell. 15*, 3698-3708.

24. Jafar-Nejad, H., Norga, K., and Bellen, H. (2002) Numb: "Adapting" Notch for Endocytosis. *Dev. Cell. 3*, 155-156.

25. Schluter, T., Knauth, P., Wald, S., Boland, S., and Bohnensack, R. (2009) Numb3 is an Endocytosis Adaptor for the Inflammatory Marker P-Selectin. *Biochem. Biophys. Res. Commun. 379*, 909-913.

26. Hutterer, A., and Knoblich, J. A. (2005) Numb and Alpha-Adaptin Regulate Sanpodo Endocytosis to Specify Cell Fate in Drosophila External Sensory Organs. *EMBO Rep. 6*, 836-842.

27. Kyriazis, G. A., Wei, Z., Vandermey, M., Jo, D. G., Xin, O., Mattson, M. P., and Chan, S. L. (2008) Numb Endocytic Adapter Proteins Regulate the Transport and Processing of the Amyloid Precursor Protein in an Isoform-Dependent Manner: Implications for Alzheimer Disease Pathogenesis. *J. Biol. Chem. 283*, 25492-25502.

28. Pece, S., Serresi, M., Santolini, E., Capra, M., Hulleman, E., Galimberti, V., Zurrida, S., Maisonneuve, P., Viale, G., and Di Fiore, P. P. (2004) Loss of Negative Regulation by Numb Over Notch is Relevant to Human Breast Carcinogenesis. *J. Cell Biol. 167*, 215-221.

29. Maiorano, E., Favia, G., Pece, S., Resta, L., Maisonneuve, P., Di Fiore, P. P., Capodiferro, S., Urbani, U., and Viale, G. (2007) Prognostic Implications of NUMB Immunoreactivity in Salivary Gland Carcinomas. *Int. J. Immunopathol. Pharmacol. 20*, 779-789.

30. Bowman, S. K., Rolland, V., Betschinger, J., Kinsey, K. A., Emery, G., and Knoblich, J. A. (2008) The Tumor Suppressors Brat and Numb Regulate Transit-Amplifying Neuroblast Lineages in Drosophila. *Dev. Cell. 14*, 535-546.

31. Carter, S., and Vousden, K. H. (2008) A Role for Numb in p53 Stabilization. *Genome Biol. 9*, 221.

32. Shi, W., and Harris, A. L. (2006) Notch Signaling in Breast Cancer and Tumor Angiogenesis: Cross-Talk and Therapeutic Potentials. *J. Mammary Gland Biol. Neoplasia. 11*, 41-52.

33. Di Marcotullio, L., Ferretti, E., Greco, A., De Smaele, E., Po, A., Sico, M. A., Alimandi, M., Giannini, G., Maroder, M., Screpanti, I., and Gulino, A. (2006) Numb is a Suppressor of Hedgehog Signalling and Targets Gli1 for Itch-Dependent Ubiquitination. *Nat. Cell Biol. 8*, 1415-1423.

34. Pawson, T., Raina, M., and Nash, P. (2002) Interaction Domains: From Simple Binding Events to Complex Cellular Behavior. *FEBS Lett. 513*, 2-10.

35. Pawson, T., and Nash, P. (2003) Assembly of Cell Regulatory Systems through Protein Interaction Domains. *Science. 300*, 445-452.

36. Kavanaugh, W. M., and Williams, L. T. (1994) An Alternative to SH2 Domains for Binding Tyrosine-Phosphorylated Proteins. *Science. 266*, 1862-1865.

37. Schlessinger, J. (2000) Cell Signaling by Receptor Tyrosine Kinases. *Cell. 103*, 211-225.

38. Li, S. C., Songyang, Z., Vincent, S. J., Zwahlen, C., Wiley, S., Cantley, L., Kay, L. E., Forman-Kay, J., and Pawson, T. (1997) High-Affinity Binding of the Drosophila Numb Phosphotyrosine-Binding Domain to Peptides Containing a Gly-Pro-(p)Tyr Motif. *Proc. Natl. Acad. Sci. U. S. A. 94*, 7204-7209.

39. Li, S. C., Zwahlen, C., Vincent, S. J., McGlade, C. J., Kay, L. E., Pawson, T., and Forman-Kay, J. D. (1998) Structure of a Numb PTB Domain-Peptide Complex Suggests a Basis for Diverse Binding Specificity. *Nat. Struct. Biol. 5*, 1075-1083.

40. Colaluca, I. N., Tosoni, D., Nuciforo, P., Senic-Matuglia, F., Galimberti, V., Viale, G., Pece, S., and Di Fiore, P. P. (2008) NUMB Controls p53 Tumour Suppressor Activity. *Nature. 451*, 76-80.

41. Zhong, W., Feder, J. N., Jiang, M. M., Jan, L. Y., and Jan, Y. N. (1996) Asymmetric Localization of a Mammalian Numb Homolog during Mouse Cortical Neurogenesis. *Neuron. 17*, 43-53.

42. Nishimura, T., Yamaguchi, T., Tokunaga, A., Hara, A., Hamaguchi, T., Kato, K., Iwamatsu, A., Okano, H., and Kaibuchi, K. (2006) Role of Numb in Dendritic Spine Development with a Cdc42 GEF Intersectin and EphB2. *Mol. Biol. Cell. 17*, 1273-1285.

43. Sorensen, E. B., and Conner, S. D. (2008) AAK1 Regulates Numb Function at an Early Step in Clathrin-Mediated Endocytosis. *Traffic. 9*, 1791-1800.

44. Susini, L., Passer, B. J., Amzallag-Elbaz, N., Juven-Gershon, T., Prieur, S., Privat, N., Tuynder, M., Gendron, M. C., Israel, A., Amson, R., Oren, M., and Telerman, A. (2001) Siah-1 Binds and Regulates the Function of Numb. *Proc. Natl. Acad. Sci. U. S. A. 98*, 15067-15072.

45. McGill, M. A., and McGlade, C. J. (2003) Mammalian Numb Proteins Promote Notch1 Receptor Ubiquitination and Degradation of the Notch1 Intracellular Domain. *J. Biol. Chem. 278*, 23196-23203.

46. Nishimura, T., Fukata, Y., Kato, K., Yamaguchi, T., Matsuura, Y., Kamiguchi, H., and Kaibuchi, K. (2003) CRMP-2 Regulates Polarized Numb-Mediated Endocytosis for Axon Growth. *Nat. Cell Biol. 5*, 819-826.

47. Rosse, C., L'Hoste, S., Offner, N., Picard, A., and Camonis, J. (2003) RLIP, an Effector of the Ral GTPases, is a Platform for Cdk1 to Phosphorylate Epsin during the Switch Off of Endocytosis in Mitosis. *J. Biol. Chem. 278*, 30597-30604.

48. Li, L., Wu, C., Huang, H., Zhang, K., Gan, J., and Li, S. S. (2008) Prediction of Phosphotyrosine Signaling Networks using a Scoring Matrix-Assisted Ligand Identification Approach. *Nucleic Acids Res. 36*, 3263-3273.

49. Morris, S. W., Kirstein, M. N., Valentine, M. B., Dittmer, K. G., Shapiro, D. N., Saltman, D. L., and Look, A. T. (1994) Fusion of a Kinase Gene, ALK, to a Nucleolar Protein Gene, NPM, in Non-Hodgkin's Lymphoma. *Science. 263*, 1281-1284.

50. Lim, M. J., and Wang, X. W. (2006) Nucleophosmin and Human Cancer. *Cancer Detect. Prev. 30*, 481-490.

51. Frehlick, L. J., Eirin-Lopez, J. M., and Ausio, J. (2007) New Insights into the nucleophosmin/nucleoplasmin Family of Nuclear Chaperones. *Bioessays. 29*, 49-59.

52. Bischof, D., Pulford, K., Mason, D. Y., and Morris, S. W. (1997) Role of the Nucleophosmin (NPM) Portion of the Non-Hodgkin's Lymphoma-Associated NPM-Anaplastic Lymphoma Kinase Fusion Protein in Oncogenesis. *Mol. Cell. Biol. 17*, 2312-2325.

53. Iwahara, T., Fujimoto, J., Wen, D., Cupples, R., Bucay, N., Arakawa, T., Mori, S., Ratzkin, B., and Yamamoto, T. (1997) Molecular Characterization of ALK, a Receptor Tyrosine Kinase Expressed Specifically in the Nervous System. *Oncogene. 14*, 439-449.

54. Morris, S. W., Naeve, C., Mathew, P., James, P. L., Kirstein, M. N., Cui, X., and Witte, D. P. (1997) ALK, the Chromosome 2 Gene Locus Altered by the t(2;5) in Non-Hodgkin's Lymphoma, Encodes a Novel Neural Receptor Tyrosine Kinase that is Highly Related to Leukocyte Tyrosine Kinase (LTK). *Oncogene. 14*, 2175-2188.

55. Palmer, R. H., Vernersson, E., Grabbe, C., and Hallberg, B. (2009) Anaplastic Lymphoma Kinase: Signalling in Development and Disease. *Biochem. J. 420*, 345-361.

56. Moog-Lutz, C., Degoutin, J., Gouzi, J. Y., Frobert, Y., Brunet-de Carvalho, N., Bureau, J., Creminon, C., and Vigny, M. (2005) Activation and Inhibition of Anaplastic Lymphoma Kinase Receptor Tyrosine Kinase by Monoclonal Antibodies and Absence of Agonist Activity of Pleiotrophin. *J. Biol. Chem. 280*, 26039-26048.

57. Fantl, W. J., Johnson, D. E., and Williams, L. T. (1993) Signalling by Receptor Tyrosine Kinases. *Annu. Rev. Biochem. 62*, 453-481.

58. Stoica, G. E., Kuo, A., Powers, C., Bowden, E. T., Sale, E. B., Riegel, A. T., and Wellstein, A. (2002) Midkine Binds to Anaplastic Lymphoma Kinase (ALK) and Acts as a Growth Factor for Different Cell Types. *J. Biol. Chem. 277*, 35990-35998.

59. Powers, C., Aigner, A., Stoica, G. E., McDonnell, K., and Wellstein, A. (2002) Pleiotrophin Signaling through Anaplastic Lymphoma Kinase is Rate-Limiting for Glioblastoma Growth. *J. Biol. Chem. 277*, 14153-14158.

60. Perez-Pinera, P., Zhang, W., Chang, Y., Vega, J. A., and Deuel, T. F. (2007) Anaplastic Lymphoma Kinase is Activated through the pleiotrophin/receptor Protein-Tyrosine Phosphatase beta/zeta Signaling Pathway: An Alternative Mechanism of Receptor Tyrosine Kinase Activation. *J. Biol. Chem. 282*, 28683-28690.

61. Motegi, A., Fujimoto, J., Kotani, M., Sakuraba, H., and Yamamoto, T. (2004) ALK Receptor Tyrosine Kinase Promotes Cell Growth and Neurite Outgrowth. *J. Cell. Sci. 117*, 3319-3329.

62. Hurley, S. P., Clary, D. O., Copie, V., and Lefcort, F. (2006) Anaplastic Lymphoma Kinase is Dynamically Expressed on Subsets of Motor Neurons and in the Peripheral Nervous System. *J. Comp. Neurol. 495*, 202-212.

63. Vernersson, E., Khoo, N. K., Henriksson, M. L., Roos, G., Palmer, R. H., and Hallberg, B. (2006) Characterization of the Expression of the ALK Receptor Tyrosine Kinase in Mice. *Gene Expr. Patterns. 6*, 448-461.

64. Souttou, B., Carvalho, N. B., Raulais, D., and Vigny, M. (2001) Activation of Anaplastic Lymphoma Kinase Receptor Tyrosine Kinase Induces Neuronal Differentiation through the Mitogen-Activated Protein Kinase Pathway. *J. Biol. Chem. 276*, 9526-9531.

65. Degoutin, J., Vigny, M., and Gouzi, J. Y. (2007) ALK Activation Induces Shc and FRS2 Recruitment: Signaling and Phenotypic Outcomes in PC12 Cells Differentiation. *FEBS Lett. 581*, 727-734.

66. Lamant, L., Dastugue, N., Pulford, K., Delsol, G., and Mariame, B. (1999) A New Fusion Gene TPM3-ALK in Anaplastic Large Cell Lymphoma Created by a (1;2)(q25;p23) Translocation. *Blood. 93*, 3088-3095.

67. Hernandez, L., Pinyol, M., Hernandez, S., Bea, S., Pulford, K., Rosenwald, A., Lamant, L., Falini, B., Ott, G., Mason, D. Y., Delsol, G., and Campo, E. (1999) TRK-Fused Gene (TFG) is a New Partner of ALK in Anaplastic Large Cell Lymphoma Producing Two Structurally Different TFG-ALK Translocations. *Blood. 94*, 3265-3268.

68. Tort, F., Campo, E., Pohlman, B., and Hsi, E. (2004) Heterogeneity of Genomic Breakpoints in MSN-ALK Translocations in Anaplastic Large Cell Lymphoma. *Hum. Pathol. 35*, 1038-1041.

69. Trinei, M., Lanfrancone, L., Campo, E., Pulford, K., Mason, D. Y., Pelicci, P. G., and Falini, B. (2000) A New Variant Anaplastic Lymphoma Kinase (ALK)-Fusion Protein (ATIC-ALK) in a Case of ALK-Positive Anaplastic Large Cell Lymphoma. *Cancer Res. 60*, 793-798.

70. Griffin, C. A., Hawkins, A. L., Dvorak, C., Henkle, C., Ellingham, T., and Perlman, E. J. (1999) Recurrent Involvement of 2p23 in Inflammatory Myofibroblastic Tumors. *Cancer Res. 59*, 2776-2780.

71. Soda, M., Choi, Y. L., Enomoto, M., Takada, S., Yamashita, Y., Ishikawa, S., Fujiwara, S., Watanabe, H., Kurashina, K., Hatanaka, H., Bando, M., Ohno, S., Ishikawa, Y., Aburatani, H., Niki, T., Sohara, Y., Sugiyama, Y., and Mano, H. (2007) Identification of the Transforming EML4-ALK Fusion Gene in Non-Small-Cell Lung Cancer. *Nature. 448*, 561-566.

72. Arber, D. A., Sun, L. H., and Weiss, L. M. (1996) Detection of the t(2;5)(p23;q35) Chromosomal Translocation in Large B-Cell Lymphomas Other than Anaplastic Large Cell Lymphoma. *Hum. Pathol. 27*, 590-594.

73. Du, X. L., Hu, H., Lin, D. C., Xia, S. H., Shen, X. M., Zhang, Y., Luo, M. L., Feng, Y. B., Cai, Y., Xu, X., Han, Y. L., Zhan, Q. M., and Wang, M. R. (2007) Proteomic Profiling of Proteins Dysregulted in Chinese Esophageal Squamous Cell Carcinoma. *J. Mol. Med. 85*, 863-875.

74. Janoueix-Lerosey, I., Lequin, D., Brugieres, L., Ribeiro, A., de Pontual, L., Combaret, V., Raynal, V., Puisieux, A., Schleiermacher, G., Pierron, G., Valteau-Couanet, D., Frebourg, T., Michon, J., Lyonnet, S., Amiel, J., and Delattre, O. (2008) Somatic and

Germline Activating Mutations of the ALK Kinase Receptor in Neuroblastoma. *Nature. 455*, 967-970.

75. Mosse, Y. P., Laudenslager, M., Longo, L., Cole, K. A., Wood, A., Attiyeh, E. F., Laquaglia, M. J., Sennett, R., Lynch, J. E., Perri, P., Laureys, G., Speleman, F., Kim, C., Hou, C., Hakonarson, H., Torkamani, A., Schork, N. J., Brodeur, G. M., Tonini, G. P., Rappaport, E., Devoto, M., and Maris, J. M. (2008) Identification of ALK as a Major Familial Neuroblastoma Predisposition Gene. *Nature. 455*, 930-935.

76. Chen, Y., Takita, J., Choi, Y. L., Kato, M., Ohira, M., Sanada, M., Wang, L., Soda, M., Kikuchi, A., Igarashi, T., Nakagawara, A., Hayashi, Y., Mano, H., and Ogawa, S. (2008) Oncogenic Mutations of ALK Kinase in Neuroblastoma. *Nature. 455*, 971-974.

77. Chiarle, R., Voena, C., Ambrogio, C., Piva, R., and Inghirami, G. (2008) The Anaplastic Lymphoma Kinase in the Pathogenesis of Cancer. *Nat. Rev. Cancer. 8*, 11-23.

78. Pulford, K., Morris, S. W., and Turturro, F. (2004) Anaplastic Lymphoma Kinase Proteins in Growth Control and Cancer. *J. Cell. Physiol. 199*, 330-358.

79. Fujimoto, J., Shiota, M., Iwahara, T., Seki, N., Satoh, H., Mori, S., and Yamamoto, T. (1996) Characterization of the Transforming Activity of p80, a Hyperphosphorylated Protein in a Ki-1 Lymphoma Cell Line with Chromosomal Translocation t(2;5). *Proc. Natl. Acad. Sci. U. S. A. 93*, 4181-4186.

80. Bai, R. Y., Dieter, P., Peschel, C., Morris, S. W., and Duyster, J. (1998) Nucleophosmin-Anaplastic Lymphoma Kinase of Large-Cell Anaplastic Lymphoma is a Constitutively Active Tyrosine Kinase that Utilizes Phospholipase C-Gamma to Mediate its Mitogenicity. *Mol. Cell. Biol. 18*, 6951-6961.

81. Bai, R. Y., Ouyang, T., Miething, C., Morris, S. W., Peschel, C., and Duyster, J. (2000) Nucleophosmin-Anaplastic Lymphoma Kinase Associated with Anaplastic Large-Cell Lymphoma Activates the Phosphatidylinositol 3-kinase/Akt Antiapoptotic Signaling Pathway. *Blood. 96*, 4319-4327.

82. Zamo, A., Chiarle, R., Piva, R., Howes, J., Fan, Y., Chilosi, M., Levy, D. E., and Inghirami, G. (2002) Anaplastic Lymphoma Kinase (ALK) Activates Stat3 and Protects Hematopoietic Cells from Cell Death. *Oncogene. 21*, 1038-1047.

83. Amin, H. M., Medeiros, L. J., Ma, Y., Feretzaki, M., Das, P., Leventaki, V., Rassidakis, G. Z., O'Connor, S. L., McDonnell, T. J., and Lai, R. (2003) Inhibition of JAK3 Induces Apoptosis and Decreases Anaplastic Lymphoma Kinase Activity in Anaplastic Large Cell Lymphoma. *Oncogene. 22*, 5399-5407.

84. Marzec, M., Kasprzycka, M., Ptasznik, A., Wlodarski, P., Zhang, Q., Odum, N., and Wasik, M. A. (2005) Inhibition of ALK Enzymatic Activity in T-Cell Lymphoma Cells Induces Apoptosis and Suppresses Proliferation and STAT3 Phosphorylation Independently of Jak3. *Lab. Invest. 85*, 1544-1554.

85. Gouzi, J. Y., Moog-Lutz, C., Vigny, M., and Brunet-de Carvalho, N. (2005) Role of the Subcellular Localization of ALK Tyrosine Kinase Domain in Neuronal Differentiation of PC12 Cells. *J. Cell. Sci. 118*, 5811-5823.

86. Kuo, C. T., Mirzadeh, Z., Soriano-Navarro, M., Rasin, M., Wang, D., Shen, J., Sestan, N., Garcia-Verdugo, J., Alvarez-Buylla, A., Jan, L. Y., and Jan, Y. N. (2006) Postnatal Deletion of Numb/Numblike Reveals Repair and Remodeling Capacity in the Subventricular Neurogenic Niche. *Cell. 127*, 1253-1264.

87. Wiley, H. S., and Burke, P. M. (2001) Regulation of Receptor Tyrosine Kinase Signaling by Endocytic Trafficking. *Traffic. 2*, 12-18.

88. Zwahlen, C., Li, S. C., Kay, L. E., Pawson, T., and Forman-Kay, J. D. (2000) Multiple Modes of Peptide Recognition by the PTB Domain of the Cell Fate Determinant Numb. *EMBO J. 19*, 1505-1515.

89. Takita, J., and Ogawa, S. (2009) Oncogenic Mutations of ALK are Associated with Tumorigenesis of Neuroblastoma. *Tanpakushitsu Kakusan Koso. 54*, 742-747.

90. Kopan, R. (2002) Notch: A Membrane-Bound Transcription Factor. *J. Cell. Sci. 115*, 1095-1097.

91. Zhong, W., Jiang, M. M., Schonemann, M. D., Meneses, J. J., Pedersen, R. A., Jan, L. Y., and Jan, Y. N. (2000) Mouse Numb is an Essential Gene Involved in Cortical Neurogenesis. *Proc. Natl. Acad. Sci. U. S. A. 97*, 6844-6849.

92. Yajnik, V., Blaikie, P., Bork, P., and Margolis, B. (1996) Identification of Residues within the SHC Phosphotyrosine binding/phosphotyrosine Interaction Domain Crucial for Phosphopeptide Interaction. *J. Biol. Chem. 271*, 1813-1816.

93. Yaich, L., Ooi, J., Park, M., Borg, J. P., Landry, C., Bodmer, R., and Margolis, B. (1998) Functional Analysis of the Numb Phosphotyrosine-Binding Domain using Site-Directed Mutagenesis. *J. Biol. Chem. 273*, 10381-10388.

94. Sorkin, A., and Goh, L. K. (2008) Endocytosis and Intracellular Trafficking of ErbBs. *Exp. Cell Res. 314*, 3093-3106.

95. Serresi, M., Piccinini, G., Pierpaoli, E., and Fazioli, F. (2004) A Ligand-Inducible Anaplastic Lymphoma Kinase Chimera is Endocytosis Impaired. *Oncogene. 23*, 1098-1108.

96. McGill, M. A., Dho, S. E., Weinmaster, G., and McGlade, C. J. (2009) Numb Regulates Post-Endocytic Trafficking and Degradation of Notch1. *J. Biol. Chem. 284*, 26427-26438.

97. Kyriazis, G. A., Wei, Z., Vandermey, M., Jo, D. G., Xin, O., Mattson, M. P., and Chan, S. L. (2008) Numb Endocytic Adapter Proteins Regulate the Transport and Processing of the Amyloid Precursor Protein in an Isoform-Dependent Manner: Implications for Alzheimer Disease Pathogenesis. *J. Biol. Chem. 283*, 25492-25502.

98. Boulton, M. E., Cai, J., and Grant, M. B. (2008) Gamma-Secretase: A Multifaceted Regulator of Angiogenesis. *J. Cell. Mol. Med. 12*, 781-795.

99. Muraoka-Cook, R. S., Feng, S. M., Strunk, K. E., and Earp, H. S.,3rd. (2008) ErbB4/HER4: Role in Mammary Gland Development, Differentiation and Growth Inhibition. *J. Mammary Gland Biol. Neoplasia. 13*, 235-246.

100. Le Borgne, R., Bardin, A., and Schweisguth, F. (2005) The Roles of Receptor and Ligand Endocytosis in Regulating Notch Signaling. *Development. 132*, 1751-1762.

101. Zupan, A. A., and Johnson, E. M.,Jr. (1991) Evidence for Endocytosis-Dependent Proteolysis in the Generation of Soluble Truncated Nerve Growth Factor Receptors by A875 Human Melanoma Cells. *J. Biol. Chem. 266*, 15384-15390.

102. Gupta-Rossi, N., Six, E., LeBail, O., Logeat, F., Chastagner, P., Olry, A., Israel, A., and Brou, C. (2004) Monoubiquitination and Endocytosis Direct Gamma-Secretase Cleavage of Activated Notch Receptor. *J. Cell Biol. 166*, 73-83.

103. Wang, S. C., and Hung, M. C. (2009) Nuclear Translocation of the Epidermal Growth Factor Receptor Family Membrane Tyrosine Kinase Receptors. *Clin. Cancer Res. 15*, 6484-6489.

104. Wells, A., and Marti, U. (2002) Signalling Shortcuts: Cell-Surface Receptors in the Nucleus? *Nat. Rev. Mol. Cell Biol. 3*, 697-702.

105. Pedersen, W. A., Chan, S. L., Zhu, H., Abdur-Rahman, L. A., Verdi, J. M., and Mattson, M. P. (2002) Numb Isoforms Containing a Short PTB Domain Promote Neurotrophic Factor-Induced Differentiation and Neurotrophic Factor Withdrawal-Induced Death of PC12 Cells. *J. Neurochem. 82*, 976-986.

106. Bani-Yaghoub, M., Kubu, C. J., Cowling, R., Rochira, J., Nikopoulos, G. N., Bellum, S., and Verdi, J. M. (2007) A Switch in Numb Isoforms is a Critical Step in Cortical Development. *Dev. Dyn. 236*, 696-705.