



HAL
open science

Anaplastic lymphoma kinase is a dependence receptor whose proapoptotic functions are activated by caspase cleavage.

Jaouhar Mourali, Alan Bénard, Filipe Calheiros Lourenço, Céline Monnet, Catherine Greenland, Christel Moog-Lutz, Claire Racaud-Sultan, Daniel Gonzalez-Dunia, Marc Vigny, Patrick Mehlen, et al.

► To cite this version:

Jaouhar Mourali, Alan Bénard, Filipe Calheiros Lourenço, Céline Monnet, Catherine Greenland, et al.. Anaplastic lymphoma kinase is a dependence receptor whose proapoptotic functions are activated by caspase cleavage.: Dual effects of ALK on apoptosis: a new dependence receptor. *Molecular and Cellular Biology*, 2006, 26 (16), pp.6209-22. 10.1128/MCB.01515-05 . inserm-00111959

HAL Id: inserm-00111959

<https://inserm.hal.science/inserm-00111959>

Submitted on 7 Nov 2006

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



**ALK IS A DEPENDENCE RECEPTOR WHOSE PROAPOPTOTIC FUNCTIONS
ARE ACTIVATED BY CASPASE CLEAVAGE**

**Jaouhar Mourali^{1,4}, Alan Bénard^{1,4}, Filipe Calheiros Lourenço², Céline Monnet¹,
Catherine Greenland¹, Christel Moog-Lutz³, Claire Racaud-Sultan¹, Daniel Gonzalez-
Dunia¹, Marc Vigny³, Patrick Mehlen², Georges Delsol¹ and Michèle Allouche^{1*}**

¹ INSERM U563, CPTP, CHU Purpan, BP 3028, 31024 Toulouse Cedex 3, France

² Apoptosis, Cancer and Development Laboratory, Equipe labellisée 'La Ligue', CNRS FRE2870, Centre Leon Bérard, Lyon, France

³ INSERM U706, UPMC, IFM, 17 rue du Fer à Moulin, Paris, France

⁴ These authors contributed equally to this work

Running title : Dual effects of ALK on apoptosis : a new dependence receptor

Keywords : ALK, receptor tyrosine kinase, dependence receptor, apoptosis, caspase

***Correspondence:** Dr Michèle ALLOUCHE, INSERM U563, CPTP, CHU Purpan, BP3028, 31024 Toulouse Cedex 3, FRANCE.

Phone : 33-5 62 74 45 29; Fax : 33-5 62 74 45 58; e-mail : allouche@toulouse.inserm.fr

Word count Mat and methods: 1861

Word count introduction, results and discussion: 4352

ABSTRACT

ALK (anaplastic lymphoma kinase) is a receptor tyrosine kinase, initially discovered as part of the NPM-ALK fusion protein, resulting from the t(2 ;5) translocation that is frequently associated with anaplastic large cell lymphomas. The native ALK protein is normally expressed in the developing and, at a weaker level, adult nervous system. We recently demonstrated that the oncogenic, constitutively kinase activated NPM-ALK protein was antiapoptotic when expressed in Jurkat lymphoblastic cells treated with cytotoxic drugs. In contrast, we now show that Jurkat cells overexpressing the wild type ALK receptor are more sensitive to doxorubicin-induced apoptosis than parental cells. Moreover, the ALK protein is cleaved during apoptosis in a caspase-dependent manner. Mutation of aspartic residues to asparagine allowed us to map the caspase cleavage site in the juxta-membrane region of ALK. In order to assess the role of ALK in a neural derived tissue, we transiently expressed ALK in the 13.S.1.24 rat neuroblast immortalized cell line. ALK expression led to apoptotic cell death of the neuroblasts. ALK ligation by specific activating antibodies decreased ALK-facilitated apoptosis in both lymphoid and neuronal cell lines. Moreover, ALK transfection reduced the survival of primary cultures of cortical neurons. Thus, ALK has a proapoptotic activity in the absence of ligand, whereas it is antiapoptotic in the presence of its ligand and when the kinase is intrinsically activated. These properties place ALK in the growing family of dependence receptors.

INTRODUCTION

Anaplastic lymphoma kinase (ALK) is a 200 kDa receptor tyrosine kinase (RTK) encoded by the *ALK* gene on chromosome 2p23. ALK was first identified as part of the NPM-ALK oncogenic fusion protein, resulting from the (2;5)(p23;q35) translocation that is frequently associated with anaplastic large cell lymphoma (ALCL) (30). This translocation produces a fusion gene that encodes a soluble chimeric transforming protein comprising the N-terminal portion of the phosphoprotein nucleophosmin (NPM) linked to the cytoplasmic portion of ALK. It has been demonstrated that the NPM portion is responsible for the dimerization of the fusion protein leading to constitutive activation of the kinase and to oncogenicity (5). PLC-gamma, PI3K, STATs and Src appear to be important downstream targets of NPM-ALK that contribute to its mitogenic and anti-apoptotic activities (2, 3, 10, 33, 46). ALK is also involved in different variant chromosomal translocations (see (35) for review) all leading to the expression of fusion proteins with a constitutively active kinase.

Full-length ALK has the typical structure of an RTK, with a large extracellular domain, a lipophilic transmembrane segment, and a cytoplasmic tyrosine kinase domain (21, 31). ALK is highly homologous to leukocyte tyrosine kinase (LTK) and belongs to the insulin receptor superfamily. Expression of the normal *ALK* gene in hematopoietic tissues has never been detected. It is, however, dominantly expressed in the neural system. *In situ* hybridization analysis performed in rodents showed that the *ALK* mRNA is essentially and transiently expressed in specific regions of the central and peripheral nervous systems such as the thalamus, mid-brain, olfactory bulb and peripheral ganglia and that it is mainly localized in neuronal cells (21, 31). Since ALK expression is maintained, albeit at a lower level, in the adult brain, it might play an important role both in the normal development and function of

the nervous system. Expression of the ALK protein has also been detected in tumors derived from the nervous system, such as neuroblastomas (23). Yet the function of ALK in adult normal tissue or in carcinogenesis is largely unknown. Several studies have recently indicated that pleiotrophin (PTN) and midkine, two heparin-binding growth factors with pleiotrophic activities involved in normal development and tumor growth (27, 45), may serve as possible ligands for ALK in mammals (38, 39). Although they appeared to induce the functional activation of ALK, it is still unclear whether these molecules are indeed the physiological ligands of ALK (11, 12, 28, 32).

Recent developments in cancer therapy are aimed at inactivating a key molecule in the mechanism of tumorigenesis, as demonstrated for Gleevec. This tyrosine kinase inhibitor is used in the treatment of chronic myeloid leukemia carrying the t(9;22), responsible for the constitutive activation of another oncogenic chimeric tyrosine kinase, BCR-ABL (41). We have previously shown that ALK, expressed under its chimeric form NPM-ALK, has antiapoptotic effects in Jurkat human T lymphoblastic leukemia cells treated with the chemotherapeutic drugs doxorubicin and etoposide. Moreover the ALK kinase activity is essential for this antiapoptotic effect, as kinase-dead NPM-ALK expressing cells were not protected against doxorubicin-induced apoptosis (19). One approach to treat ALK-positive tumors similarly aims at inactivating the kinase. It therefore seemed important to investigate the effect of ALK expression on apoptosis, in the presence or absence of ALK kinase activation.

In this paper we used two cell lines of either lymphoid or neuronal origin as models to express the wild type ALK receptor. Apoptosis was induced by doxorubicin in Jurkat T lymphoblastic cells stably expressing ALK, or triggered by serum deprivation in transiently ALK-transfected

13.S.1.24 murine immortalized olfactory neuronal cells. Our results show for the first time that ALK expression enhances apoptosis in both lymphoid and neuronal cells. In addition we found that the ALK protein was cleaved intracellularly by caspases during apoptosis, exposing a potentially proapoptotic region within the juxta-membrane intra-cytoplasmic segment of ALK. Point mutation of the aspartic acid residue in position 1160, which appears to be the caspase cleavage site, reversed the proapoptotic effect of ALK. Activating ALK-specific antibodies, used as ALK ligands, also counteracted the proapoptotic effect of ALK. Finally, ALK expression also reduced the survival of primary cortical neurons.

MATERIALS AND METHODS

Reagents and antibodies

Doxorubicin (doxorubicine Dakota^R pharm) was obtained from Sanofi Winthrop (Gentilly, France), geneticin (G418 sulphate) from GIBCO BRL (Life Technologies, Grand Island, NY), and ³²P-γATP from Amersham (Les Ulis, France), zVAD-fmk from R & D (Minneapolis, MN), and purified activated caspase-3 was a kind gift of G.S. Salvesen (The Burnham Institute, La Jolla, CA). All other chemicals were from Sigma Chemical Co. (St Louis, MO).

Several anti-ALK antibodies were used: the ALK1 (34) and ALKc (15) mouse monoclonal antibodies (mAbs) recognize the intracellular, kinase-containing region of ALK. The REAB polyclonal rabbit antiserum, mouse mAbs 46 and 48 (28), and rat mAb 16-39 (32) are directed against the extra-cellular domain of ALK. The anti-phosphotyrosine (clone 4G10) mAb was purchased from Upstate Biotechnology (Lake Placid, NY), and anti-actin mAb (clone AC-10) from Sigma. Peroxidase-conjugated goat anti-mouse and goat anti-rabbit Ig antisera were from Biorad (Hercules, CA). Alexa Fluor 594 goat anti-mouse IgG was obtained from Molecular Probes (Eugene, USA).

Cell lines

The Jurkat (clone E6.1, TIB 152) human T lymphoblastic leukemia cell line was purchased from the ATCC (Rockville, MD). Cells were maintained in RPMI1640 (GIBCO BRL) containing 10% fetal calf serum (FCS, GIBCO BRL), 2 mM glutamine, 100 Units/ml penicillin, 100 μg/ml streptomycin and 1 mM sodium pyruvate (GIBCO BRL). Transfected

Jurkat cells were cultured in the continuous presence of 2 mg/ml G418. The t(2;5)-positive ALCL cell line SU-DHL1 (29) was cultured in Iscove's medium supplemented with 15% FCS, 2 mM glutamine, 100 Units/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium pyruvate. The 13.S.1.24 immortalized olfactory neuronal rat cell line (9) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, and 0.3 mg/ml gentamycin.

Culture and transfection of primary cortical neurons

Primary cortical neurons were prepared from 18-day-old Sprague-Dawley rat embryos (Janvier). Briefly, cerebral cortices were dissected and dissociated by mechanical trituration and digestion in PBS with 0.3% *Aspergillus* protease (Sigma) for 10 min at room temperature. After addition of 35% FCS to stop the digestion, the suspension was filtered through a 70 µm cell strainer and centrifuged at 1000 rpm for 10 min. After resuspension in neurone medium (Neurobasal medium supplemented with 0.5 mM glutamine and 2% B-27 supplement (Gibco)), the cell suspension was centrifuged at 1000 rpm through a 4% BSA cushion for 10 min. Neurons were used immediately for transfection. Transfections were performed with the rat Neuron Nucleofector Kit (Amaxa), according to the manufacturer's protocol. We used 3µg of ALK plasmid and 0.5µg of a GFP-expression vector per 5×10^6 cells in DMEM containing 10% FCS. Thereafter, cells were seeded on poly-D L-ornithine (Sigma), laminin (Roche)-coated glass coverslips or plastic petri dishes. The medium was replaced by neurone medium 4 hours after transfection and plating. Culture medium was renewed (one half of the volume) every other day and transfected neurons were analyzed after 3 to 8 days. Neuronal cultures contained > 80 % neurons, as assessed by staining with neuron-specific markers (data not shown).

Site directed mutagenesis and plasmid construction

The pcDNA3 expression vector (InVitrogen, Groningen, The Netherlands), containing the cDNA for the wild type *ALK* (*ALK-wt*) and *NPM-ALK*, as well as the kinase dead *NPM-ALK* (named K210R, corresponding to K1150R in the ALK complete protein sequence) mutant, have been previously described (5, 30, 31). Point mutations resulting from the substitution of asparagine to aspartic residues were done using the QuickChange site-directed mutagenesis system (Stratagene, Amsterdam, The Netherlands). The following mutants were constructed and inserted into the pcDNA3 vector using the following primers: *D1141N* (5'-CCGGAATGCCCAACAACCCAAGCCCCCTGC-3' and 5'-GCAGGGGGCTTGGGTTGTTGGGCATTCCGG-3'), *D1160N* (5'-GTGCTCTGAACAGAACGAACTGGATTCCTC-3' and 5'-GAGGAAATCCAGTTCGTTCTGTTCAGAGCAC-3'), *D1163N* (5'-GAACAGGACGAACTGAATTCCTCATGGAAG-3' and 5'-CTCCATGAGGAAATTCAGTTCGTCCTGTTC-3'), *D1225N* (5'-CTCCCTGGCCATGCTGAACCTTCTGCACGTG-3' and 5'-CACGTGCAGAAGGTTTCAGCATGGCCAGGGAG-3'), and the *D1160-1163N* double mutant (5'-GAACAGAACGAACTGAATTCCTCATGGAAG-3' and 5'-CTCCATGAGGAAATTCAGTTCGTTCTGTTC-3').

In addition, a stop codon was inserted to replace the codon 1160 in order to generate a C-terminally truncated ALK protein (*ALK-stop1160*) using the following primers: 5'-TGCTCTGAACAGTAAGAACTGGATTC-3' and 5'-GAAATCCAGTTCTTACTGTTCAGAGCA-3'. Deletion of the 33 juxta-membrane amino acids 1057-1089 was further introduced into the *ALK-stop1160* construct by digestion with

BspHI to create the *ALK-delta33-stop1160* construct. Another truncated form of ALK was generated by the insertion of a stop codon to replace the codon 1126 (*ALK-stop1126*) using the following primers: 5'-CTGGGCCATGGCTGATTTGGGGAGGTG-3' and 5'-CACCTCCCCAAATCAGCCATGGCCCAG-3'. The cDNA coding for the ALK C-terminal fragment comprised between amino acids 1161-1620 (*ALK-Cter*) was inserted into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen) using the following PCR primers: 5'-CACCATGGAACTGGATTTCCTCATGGAA-3' and 5'-TCAGGGCCCAGGCTGGTTCAT-3'.

Inducible expression vectors were constructed by subcloning the full length ALK-wt coding sequence or the *ALK-Cter* (using HindIII and XbaI sites) from the pcDNA3 vectors into the pMTCB6+-derived expression vector (pMT) (8). Gene expression was under the control of the zinc-inducible sheep metallothionein promoter, resulting in the pMT-ALK and pMT-ALK-Cter vectors, respectively. All the mutant constructions were verified by sequencing.

DNA transfection of cell lines

Stable transfection of *ALK* cDNA into Jurkat cells was performed by electroporation with a Biorad (Ivry-sur-Seine, France) Gene Pulser apparatus at 270 volts, 950 μ F. After 48 hours of culture, transfected cells were selected and further cultured with 2 mg/ml G418. Cells expressing the transgene were cloned by limiting dilution. Cells transfected with control vector (Jurkat/neo) or with *NPM-ALK* cDNA have been previously described (19). Transient transfection of *ALK* constructs into 13.S.1.24 cells was performed using the Fugene 6 reagent (Roche Diagnostics, Meylan, France) according to the manufacturer's instructions.

Immunodetection by immunocytochemistry and immunofluorescence

Transfected Jurkat cells were cytospun onto glass slides coated with silane, fixed in acetone for 10 minutes, and stained using ALK1 mAb in a three-stage immunoperoxidase technique (4). For the detection of ALK at the cell surface, exponentially growing cells were centrifuged at 400 g for 5 minutes, washed in PBS and incubated for 30 minutes at 4°C with the REAB antibody. After two washes in PBS containing 1% bovine serum albumin and 0.1% sodium azide, fluorescein isothiocyanate (FITC)-coupled goat anti-rabbit Ig (Jackson ImmunoResearch, West Grove, Pa) antiserum was added for 30 minutes at 4°C as the secondary reagent. Cells were then washed twice and fixed in 0.5% formaldehyde. For intracellular ALK detection, cells were fixed and permeabilized using the Intra-Prep reagent (Immunotech, Marseille, France) according to the manufacturer's protocol, then labeled with ALK1 mAb, followed by FITC-coupled goat anti-mouse Ig (Jackson). Detection of active caspase-3-expressing cells after apoptosis induction was performed with an FITC-coupled caspase-3-specific mAb from BD Pharmingen (BD Biosciences, San Jose, CA). Fluorescence was analyzed on an XL4C Coulter (Beckman Coulter, Hialeah, FL) flow cytometer (excitation wavelength at 488 nm, and emission at 525 nm for FITC).

Transfected neurons grown on glass coverslips were fixed for 20 min at room temperature with 4% paraformaldehyde, permeabilized with 0.1% Triton X100 in PBS for 5 min at room temperature, rinsed with PBS, and blocked overnight at 4 °C in PBS with 2.5% normal goat serum. Incubation with ALKc mAb diluted in PBS with 1% BSA for 1 h at room temperature was followed, after several washes in PBS, by a 1 h incubation at room temperature with Alexa Fluor 594 goat anti-mouse IgG. After extensive washing, coverslips were mounted using DAPI-containing Prolong Gold (Invitrogen). Cells were examined using an LSM 510 confocal microscope (Zeiss axiovert 100) equipped with a 63X immersion objective.

Western blot analysis

Cells were washed once with phosphate-buffered saline (PBS), pelleted and extracted in Passive Lysis Buffer (Promega, Madison, WI) containing 10 µg/mL leupeptin, 2 µg/mL aprotinin, 1 mM 4-2 aminoethyl-benzenesulfonyl fluoride (AEBSF), 1 mM sodium orthovanadate and 4 mM sodium fluoride for 30 minutes on ice. Cell extracts were centrifuged at 10,000 *g* for 20 minutes at 4°C. The supernatant was recovered and its protein content was quantified using the Biorad protein assay. For Western blotting, 50-150 µg of total cell proteins were subjected to SDS-PAGE in a 7.5% or 10% gel under reducing (most cases) or non-reducing (for detection with the anti-ALK mAb 48) conditions, and transferred for 1 h at 100 V onto a nitrocellulose membrane.

ALK protein was detected using alternatively ALKc (1/50), ALK1 (1/200), "48" (1/1000) mouse mAbs, followed by horseradish peroxidase (HRP)-coupled goat anti-mouse Ig antiserum (1/6000), or REAB rabbit antibody (1/4000), followed by HRP-coupled goat anti-rabbit Ig antiserum (1/6000). Tyrosine phosphorylated proteins were detected using the 4G10 (1/1000) mouse mAb and HRP-coupled anti-mouse Ig antiserum. Signal detection was performed with an enhanced chemoluminescence kit (ECL, Amersham).

Immunoprecipitation

Cell pellets were suspended in lysis buffer (50mM Tris HCl, 150mM NaCl, 1% Triton X100) containing 1mM sodium orthovanadate, 4mM sodium fluoride, 10µg/ml leupeptin, 2µg/ml aprotinin, 2µg/ml pepstatin A, and 1mM AEBSF at 4°C for 30 minutes. Samples were precleared at 4°C for 1 hour using prewashed protein G-Sepharose beads. The supernatant

was then incubated with ALK1-precoated protein G-Sepharose beads at 4°C for 1 hour. After 5 washes in lysis buffer, the beads were heated at 95°C for 4 minutes. Protein separation was performed by SDS-PAGE, followed by Western blot analysis with the anti-phosphotyrosine or ALKc mAb.

***In vitro* kinase assay**

Immunoprecipitation with ALK1 mAb was carried out as described above. Sepharose-bound immune complexes in lysis buffer were washed and resuspended in kinase buffer (20 mM Hepes, pH7.4, 10 mM MnCl₂, 10 mM sodium fluoride, 1 mM sodium orthovanadate), before incubation with 5 μCi $\gamma^{32}\text{P}$ -ATP (Redivue, Amersham) at 25°C for 15 minutes. Samples were boiled at 95°C for 4 minutes and separated on a 10% gel by SDS-PAGE prior to autoradiography.

Induction of apoptosis

Exponentially growing cells were seeded at 4×10^5 cells/ml, and incubated for various periods of time (up to 24 hours) with doxorubicin (0.8-2.4 μM) as an inducer of apoptosis or with culture medium for controls.

Measure of cell viability

Cell viability was determined by three methods: trypan blue dye exclusion, the “CellTiter 96 Aqueous one solution cell proliferation” colorimetric assay (Promega), based on enzyme

conversion of the MTS dye by mitochondria of living cells, or the “CellTiter Glo” luminescence assay (Promega), to measure the ATP content in living cells.

Quantification of apoptosis by annexin V-FITC labeling and caspase activity

Annexin V-FITC binding to phosphatidylserine, a membrane phospholipid exposed at the surface of apoptotic cells (22), was measured at the end of apoptosis induction. Cells were washed twice in PBS and resuspended in 100 μ l annexin buffer (10 mM Hepes/NaOH, pH7.4, 140 mM NaCl, 5 mM CaCl_2) containing annexinV-FITC (1/100) (Roche Diagnostics, Meylan, France) and 1 μ g/ml propidium iodide, a DNA-intercalating agent. Propidium iodide is used to discriminate between apoptotic and necrotic cells, which bind annexin due to loss of membrane integrity. After incubation for 15 minutes at room temperature, annexin buffer (400 μ l) was added to samples and cell fluorescence was immediately analyzed on an XL4C Coulter (Beckman Coulter, Hialeah, FL) flow cytometer (excitation wavelength at 488 nm, and emission at 525 and 640 nm for FITC and propidium iodide, respectively). Cells labeled with annexin V-FITC and negative for propidium iodide were scored as apoptotic cells.

Caspase-3 activity was determined using the “Caspase glo 3/7” luminescence assay (Promega) according to the manufacturer’s instructions. Caspase activation is shown as the ratio between the caspase activity of the treated sample and the activity of the corresponding untreated cells (relative caspase activity index).

Statistical analysis

A paired Student’s *t* test was used to compare apoptosis values in different cell populations. A *p* value < 0.05 was considered significant.

RESULTS

Characterization of ALK expression in transfected Jurkat cells

Jurkat T-lymphoid cells, transfected with *ALK* cDNA or with control vector (Jurkat/neo cells), were selected with geneticin for stable gene expression and cloned by limiting dilution. Immunohistochemical and immunofluorescence analysis with ALK-specific antibodies directed against the intra-cytoplasmic (ALK1) and extra-cellular (REAB) portion of ALK revealed both cytoplasmic and membrane staining, respectively (Figure 1A and 1B), confirming that ALK was expressed as a transmembrane receptor. To further characterize the protein expressed in Jurkat/ALK cells, Western blot analysis using the ALKc mAb, directed against the C-terminal domain of ALK, revealed a major protein band (sometimes seen as a doublet, see below) at approximately 200 kDa (Figure 1C). Neither Jurkat (not shown) nor Jurkat/neo (Figure 1C) cells expressed the ALK protein. In addition, immunoprecipitation of ALK followed by an *in vitro* kinase assay showed that ALK (200 kDa) expressed in Jurkat cells was capable of autophosphorylation (Figure 1D), similarly to the NPM-ALK fusion protein (80 kDa), shown here as a positive control. Thus, Jurkat/ALK cells stably expressed a potentially functional full size ALK protein. However ALK immunoprecipitation followed by Western blot with the 4G10 mAb revealed that, in contrast to NPM-ALK (19), the ALK protein expressed in Jurkat was not phosphorylated on tyrosine (Figure 1E).

Doxorubicin-induced apoptosis is enhanced in ALK-expressing Jurkat cells

In a previous paper, we demonstrated that NPM-ALK expression in Jurkat cells led to a significant inhibition of doxorubicin-induced apoptosis, compared to Jurkat/neo cells (19). In

order to compare the respective effects of full size ALK and NPM-ALK on apoptosis, we treated Jurkat/neo (vector), Jurkat/NPM-ALK and Jurkat/ALK cells with 2 μ M doxorubicin for 16 hours. Apoptosis was measured using the annexin V-FITC labeling technique. Surprisingly, we found that the percentage of annexin-positive Jurkat/ALK cells was enhanced almost two-fold compared to control Jurkat/neo cells, and four- to five-fold compared to Jurkat/NPM-ALK cells (Figure 2A). For each cell line, caspase-3 activity in doxorubicin-treated vs untreated cells closely paralleled the corresponding variations observed in annexin labeling (Figure 2B). Thus in following experiments, we used the annexin technique to measure apoptosis unless otherwise indicated. Figure 2C and 2D show that the enhancement of doxorubicin-induced apoptosis in ALK-expressing cells was clearly dose- and time-dependent. Note that spontaneous apoptosis in untreated cells (incubated with culture medium alone) did not exceed 3% annexin-positive cells in all experiments (Figure 2A).

We and others have previously shown that doxorubicin-induced apoptosis in Jurkat cells is a caspase-dependent process that involves the mitochondrial apoptosis signaling pathway (19). We show here that the pan-caspase inhibitor zVAD-fmk is able to inhibit approximately 85% of doxorubicin-induced apoptosis in Jurkat/ALK cells, indicating that it is a caspase-dependent process, as in Jurkat/neo and Jurkat/NPM-ALK cells (Figure 2 A).

Caspase-dependent ALK degradation during drug-induced apoptosis

As many critical proteins are degraded in the course of caspase-dependent apoptosis, we followed ALK expression by Western blot with different ALK-specific antibodies directed against the extra-cellular (REAB) or intra-cellular (ALK1 and ALKc) portion of ALK. The intensity of the 200-220 kDa doublet corresponding to full size ALK decreased significantly

after doxorubicin treatment of Jurkat/ALK cells when examined with all three antibodies (Figure 3A), indicating that ALK was degraded. Moreover, ALK degradation was caspase-dependent, as it was inhibited by the addition of the broad caspase inhibitor zVAD-fmk in association with doxorubicin (Figure 3A). The ALKc mAb was used to study the kinetics of ALK degradation in drug-treated cells and to identify ALK-specific bands of lower molecular mass than the wild type protein that could correspond to fragments resulting from caspase cleavage. As shown in Figure 3B, a band of approximately 60 kDa appeared in a time-dependent manner and was maximal at 24 hours of treatment with doxorubicin. A longer blot exposure was necessary to visualize this band, we were therefore unable to see on the same image the decrease in the full size 200kDa ALK protein. The 60 kDa product disappeared in the presence of 10 μ M zVAD-fmk (Figure 3C, lane “dox + zVAD”). Another ALK-specific band of 140 kDa, was also frequently detected in untreated cells (Figure 3B and 3C). This band most probably results from an extra-cellular cleavage or processing of ALK, as indicated by other groups (28, 32). A distinct band of 50 kDa (Figure 3B) was inconsistently detected, did not vary in intensity and was probably not specific, as it could sometimes be seen in overexposed blots of control Jurkat/neo cells (not shown).

We hypothesized that the 60 kDa fragment could result from a caspase cleavage within the intra-cellular domain of ALK. In order to see whether ALK cleavage could be due to the major effector caspase, caspase-3, we incubated protein extracts from Jurkat/ALK cells with purified activated caspase-3 for 30 minutes at 37°C. Western blot with the ALKc mAb revealed that ALK was indeed cleaved *in vitro* by active caspase-3, giving rise to 2 major bands of 150 kDa and 60 kDa, respectively (Figure 3D). As ALKc recognizes an epitope in the C-terminal region of ALK, it appears that the larger band results from a cleavage within the extra-cellular domain of ALK (irrelevant *in vivo*, as caspases are intra-cellular enzymes),

whereas the smaller fragment of 60 kDa appears to be identical to the one detected after *in vivo* cell treatment with doxorubicin (seen in Figure 3B and 3C, arrowhead).

The caspase cleavage site of ALK is D1160

We then examined the intra-cellular protein sequence of ALK in search of a potential caspase cleavage site. The 1160-1163 amino acids of ALK are “DELD”, a motif similar to the “DEVD” caspase-3 consensus cleavage site (Figure 4A). Moreover, potential cleavage of ALK at this site would yield a protein fragment of 60 kDa, which matches our previous findings. In order to map the caspase cleavage site, we replaced aspartic acid residues by asparagine (D_N mutations) in positions 1141, 1160, 1163, and 1225, and also made a double mutant (1160-1163). Jurkat cells were stably transfected with these constructs and cloned by limiting dilution as described above. The pattern of ALK degradation after doxorubicin treatment in cells expressing the 1141, 1163 and 1225 D_N ALK mutant was the same as in the wild type ALK expressing cells, giving rise to the 60 kDa fragment (Figure 4B). In contrast, this fragment was undetectable in cells expressing the D1160N ALK mutant (Figure 4B), indicating that the 1160 aspartic acid residue is the site of ALK cleavage by caspase-3. In agreement with these findings, *in vitro* treatment of protein extracts from ALK-D1160N expressing cells with active caspase-3 failed to release the 60 kDa fragment, whereas the D1163N ALK mutant was still cleaved (Figure 4C). The 1160-1163 D_N double mutant responded like the D1160N mutant *in vivo* and *in vitro* (data not shown).

Kinase activation impairs NPM-ALK cleavage by caspase-3

Since the DELD motif belongs to the intra-cellular region of ALK, which is conserved in the NPM-ALK hybrid protein, we expected NPM-ALK to undergo caspase cleavage after doxorubicin treatment of Jurkat/NPM-ALK cells. Indeed, western blot analysis revealed a weak 60 kDa band after 16 hours of cell incubation with the drug (Figure 5A, left panel), instead of 3 hours for Jurkat/ALK cells (Figure 3B). Therefore the generation of the 60 kDa fragment was both impaired and delayed in Jurkat/NPM-ALK vs Jurkat/ALK cells. In contrast, the kinase-dead (K210R) NPM-ALK, mutated on the ATP fixation site (lysine (K) 1150 of the full length ALK sequence, Figure 4A) was cleaved as early as 6 hours after the onset of treatment (Figure 5A, right panel). Moreover, our published (19) and present results (Figure 5A) show that the kinase-dead mutant was expressed at a lower level than the wild type NPM-ALK. Therefore the relative ratio between the 60 kDa cleavage fragment and the full size 80 kDa protein appears more important in the kinase-dead mutant than in the wild type NPM-ALK. Active caspase-3 *in vitro* treatment of protein extracts from Jurkat/NPM-ALK cells and SU-DHL1 ALCL cell line, confirmed that NPM-ALK was cleavable, generating a 60 kDa fragment, as expected (Figure 5B). Taken together, our results suggest that activation/phosphorylation of the ALK kinase protects the protein from caspase-dependent degradation. It should be noted that ALK cleavage on residue 1160 disrupts the kinase domain of ALK, that spans between residues 1122 and 1376, but preserves lysine 1150, the ATP-binding site (but not necessarily the secondary structure). Thus caspase cleavage of ALK probably inactivates the kinase.

The D1160N mutation abrogates ALK-mediated enhancement of apoptosis

We next examined the effect of the various D_N mutations of ALK on doxorubicin-induced apoptosis, to see whether a relationship existed between caspase-dependent ALK cleavage and the extent of cell death. The 1141, 1163 and 1225 ALK mutants transfected into Jurkat cells responded like wild type ALK, *i.e.*, enhanced doxorubicin-induced apoptosis by 1.5 to two-fold compared to Jurkat/neo cells (Figure 6). In contrast, the D1160N mutant conferred resistance to apoptosis, similarly to NPM-ALK, reducing the percentage of annexin-positive cells to half that observed in Jurkat/neo (Figure 6). In addition, the 1160-1163 double mutant also protected cells from drug-induced apoptosis (not shown). As observed in ALK-wt-expressing cells, we could not detect tyrosine phosphorylation in the non cleavable D1160N ALK mutant, either before or after cell treatment with doxorubicin (data not shown), suggesting that the abrogation of the proapoptotic activity of this mutant was not due to kinase activation. Notably, doxorubicin-induced apoptosis was enhanced in NPM-ALK kinase-dead expressing cells (Figure 6), to the same extent as in Jurkat/ALK cells, and we have shown that this mutant was relatively sensitive to caspase cleavage (Figure 5A, right panel). Altogether these data strongly suggest that ALK cleavage by caspase at D1160 confers an active signal for enhancement of drug-induced apoptosis.

Mapping of a proapoptotic segment of ALK to the juxta-membrane region

We have found that ALK-mediated facilitation of drug-induced apoptosis depended on its intra-cellular cleavage by caspase-3 at D1160. These data suggest that either the 60 kDa fragment released is proapoptotic, or the intra-cellular segment upstream of D1160 contains a structure or sequence with proapoptotic properties. In a first approach, we constructed a

pcDNA3 vector containing the coding sequence of the 1161-1620 ALK C-terminal fragment. However, we repeatedly failed to obtain clones in permanent transfection of Jurkat cells or to detect the peptide in transient transfections (data not shown). At this point in the study, this result could be due either to a potent proapoptotic effect of the fragment or to unresolved technical problems. To investigate the presence of a potential proapoptotic sequence within the N-terminal juxta-membrane part of the ALK intra-cellular region, we constructed a mutant with a stop codon in position 1160 (ALK-stop1160). Doxorubicin-induced apoptosis in these cells was enhanced to the same extent as in Jurkat/ALK cells (Figure 7, middle panel). These data indicate that ALK might contain a proapoptotic domain in its intra-cytoplasmic juxta-membrane region between amino acids 1058 and 1160. In order to map this putative domain, we generated two additional ALK mutants, deleting the N- or C-terminal third of this 103 amino acid peptide segment: ALK delta33_stop1160 (deleting amino acids 1057 to 1089) and ALK-stop1126, respectively. All the C-terminally truncated ALK proteins expressed in Jurkat cells were detected through Western blotting using the mAb 48 directed against the extra-cellular domain of ALK (Figure 7, lower panel). Doxorubicin-induced apoptosis was also increased in cells expressing these mutant ALK proteins (Figure 7), strongly suggesting that a proapoptotic region lies between amino acids 1090 and 1125 of the ALK intra-cellular domain.

ALK ligation by activating antibodies inhibits ALK-mediated enhancement of apoptosis

The activation of RTKs typically requires ligand-induced receptor oligomerization, which results in tyrosine autophosphorylation of the receptors (37). Since our *in vivo* and *in vitro* experiments suggest that activation/phosphorylation of the ALK kinase protects the protein from caspase-dependent degradation and the cells from doxorubicin-induced apoptosis, we

tried to induce ALK receptor activation using a specific ligand. In our hands, commercially available recombinant PTN (Sigma) failed to activate the receptor. In addition, although cotransfection of Jurkat cells with *ALK* and *PTN* decreased drug-induced apoptosis, this effect was not ALK-specific as it was also observed in PTN-only-expressing cells (unpublished results). The use of ALK-specific activating mAbs, recently developed by two independent research groups (28, 32), was therefore a potent tool to activate the ALK receptor. Coincubation of Jurkat/ALK cells with doxorubicin and either the 16-39 or 46 mAb reversed the proapoptotic effect of ALK in a dose-dependent manner, with a maximal effect at 1-2 $\mu\text{g/ml}$ of purified antibody (Figure 8A). Note that mAb 16-39 decreased the level of apoptosis (measured by annexin labeling) of Jurkat/ALK cells to that of control Jurkat/neo cells (100% apoptosis inhibition, Figure 8A). In addition, the activating effect of mAb 46 on ALK kinase was confirmed, as cell incubation with this antibody induced tyrosine phosphorylation of ALK (Figure 8B).

ALK induces cell death in a neuroblast cell line

The ALK receptor is expressed during the development of the nervous system (21, 31). In order to determine whether ALK could have a proapoptotic effect in a more physiological context, we transiently expressed ALK in an immortalized murine neuroblast cell line, 13.S.1.24, which does not express endogenous ALK (not shown). Twenty-four hours after transfection, cells were deprived of serum (a stimulus which *per se* does not induce cell death in this cell line) and further incubated 24 hours in the absence or presence of 20 $\mu\text{g/ml}$ anti-ALK mAb 16-39. The transfection efficiency was approximately 50% in all experiments (evaluated by transfection with a green fluorescent protein expression vector and flow cytometry analysis of cell fluorescence). As shown in Figure 9A, ALK transfection

(determined by Western blot, not shown) induced significant cell death (lower left panel), compared to control vector-transfected cells (upper left panel). In contrast, incubation of ALK-transfected cells with mAb 16-39 rescued the cells from apoptosis triggered by serum deprivation (Figure 9A, lower right panel). A normal rat IgG, used as negative control for the mAb 16-39, had no effect. Cell viability was measured using an MTS assay (Figure 9B), and apoptosis by assessing the percentage of active caspase-3- positive cells in the different culture conditions (Figure 9C), confirming that the activating anti-ALK mAb 16-39 decreased apoptosis triggered by ALK in serum-deprived neuroblasts.

Since the ALK-stop1160 mutant was proapoptotic in Jurkat cells, it was important to assess that it had the same effect in 13.S.1.24 neuroblasts. Indeed, cell viability was decreased to the same extent after ALK-stop1160 and ALK-wt transfection in neuroblasts (Figure 9D). Protein expression was checked by Western blot (not shown).

As in the Jurkat cell model, transfection of 13.S.1.24 neuroblasts with a pcDNA3-ALK-Cter vector did not lead to detectable expression of the C-terminal ALK 60 kDa fragment (data not shown). We then tried another strategy and inserted the coding sequence for ALK-Cter (1161-1620), or for ALK-wt into a pMTCB6+-derived expression vector (8) under the control of the zinc-inducible sheep metallothionein promoter. Cells were transfected with pMT vector, pMT-ALK-wt or pMT-ALK-Cter in the absence of serum, induced or not with 100 μ M ZnSO₄ (an optimal dose to minimize toxicity for cells and obtain gene expression) 5 h later. Both proteins were detected in transfected neuroblasts by Western blot in the presence (Figure 9E, top), but not in the absence (not shown) of zinc induction. Cell viability was measured by trypan blue exclusion 48h post-induction. Zinc-induced ALK-wt expression significantly decreased the number of viable cells in the culture ($p<0.05$), compared to the control pMT

vector. The slight decrease of cell viability observed with ALK-Cter did not appear to be statistically significant (Figure 9E, lower part).

ALK induces cell death in primary cortical neurons

We finally addressed the issue of the potential proapoptotic effect of ALK on primary cortical neurons, which do not express endogenous ALK ((31) and unpublished results). Primary cortical neurons were prepared from 18-day-old rat embryos (note that these cells are post-mitotic and do not proliferate in culture) and were transfected with *ALK-wt* inserted either in the pcDNA3 or the zinc-inducible pMT vector. In both cases, *ALK* transfection decreased the number of viable cells observed after 6 to 8 days of culture, compared to the corresponding empty vectors (Figure 10A and 10B). The expression of ALK was determined by indirect immunofluorescence on permeabilized neurons using the ALKc mAb (Figure 10C).

DISCUSSION

We present here data supporting that in the absence of ligand, the ALK receptor kinase is not tyrosine phosphorylated, and actively enhances or triggers apoptosis, whereas kinase activation, induced by a ligand or constitutive as in NPM-ALK, decreases apoptosis. Unligated/ non-activated ALK receptor facilitates apoptosis via its own cleavage by caspases at D1160, a phenomenon allowing the exposure of a proapoptotic juxta-membrane intra-cellular domain (located between residues 1058 and 1160), most likely through the association with proapoptotic effectors. Caspases have been shown to associate to death receptors such as Fas/CD95, TNFR1 or TRAIL via intracellular adaptor proteins through different motifs within the death-like domain superfamily: death domain (DD), death-effector domain (DED) or caspase-recruitment domain (CARD) (18, 36). We searched databases but were unable to find any such domain within the juxta-membrane intra-cytoplasmic sequence (1058-1160) of ALK. Finer mapping allowed us to restrict the proapoptotic domain to a sequence lying between aminoacids 1090 and 1125 of ALK. The direct or indirect interaction of ALK with proapoptotic effector molecules will be the object of further studies. Yet preliminary data show that expression of several Bcl-2 family members is modulated in ALK-expressing Jurkat cells (data not shown).

This dual effect on apoptosis has been described for several receptors involved in development and tumorigenesis, composing the so-called functional family of “dependence receptors”. Members include p75NTR, the low affinity nerve growth factor receptor (7), the DCC (deleted in colorectal cancer) and UNC5H netrin receptors (16, 17, 24, 25), the integrin $\alpha\beta3$ (40), as well as the RET (rearranged during transfection) RTK, a receptor for glial cell-derived neurotrophic factor (6). Such receptors create states of dependence to their respective

ligands by inducing apoptosis when the ligand is absent, and inhibiting apoptosis in the presence of ligand. Moreover these receptors appear to be cleaved by caspases during apoptosis (7), as is the case for ALK. Recently, another RTK was identified as a receptor displaying several common traits with dependence receptors: MET is cleaved by caspases, this cleavage is inhibited by the presence of its ligand, hepatocyte growth factor/scatter factor and the cleaved fragment was shown to be proapoptotic (43). However, the authors failed to detect a pro-apoptotic activity of the full-length receptor while both RET or ALK are clearly proapoptotic in the absence of their respective ligand. Whether this is related to the experimental system or to the fact that MET is not a dependence receptor remains to be determined. However, it is intriguing to see that various kinase receptors could potentially be considered as dependence receptors. The physiological relevance of this phenomenon has yet to be discovered.

Multiple oncogenes have the ability to trigger apoptosis when expressed in an inappropriate manner, and this is thought to restrict tumor formation by eliminating potentially malignant cells that may have acquired a mutation that stimulates proliferation. For example, deregulated expression of c-myc promotes proliferation, but can also either induce or sensitize cells to apoptosis (14). A parallel could be made between c-myc and ALK. Inappropriate expression of c-myc under conditions which inhibit growth and down-regulate endogenous c-myc expression, including serum deprivation and exposure to cytotoxic agents, usually results in programmed cell death in many different cell types (reviewed in (13)). We show here that inappropriate expression of ALK, in the absence of ligand and/or in the wrong cellular context, also triggers or facilitates apoptosis. On the other hand, mechanisms that inhibit apoptosis should enhance or promote tumorigenesis. Indeed when ALK is in its oncogenic form NPM-ALK, the propapoptotic effect of ALK due to its cleavage by caspases is

counteracted by the proliferative and pro-survival effect of the constitutively activated tyrosine kinase. We propose that apoptosis associated with inappropriate ALK expression could limit the tumorigenic effect of the ALK proto-oncogene.

It is possible that ALK does not trigger apoptosis directly but that it acts as a sensitizer to another existing trigger. Two signals would thus be necessary to enhance apoptosis. In the lymphoid Jurkat cells, we have observed that a certain degree of caspase activation is present in both parental and transfected cells (data not shown). Moreover other authors have shown that lymphocytes can survive limited caspase activation (1), and that active caspase-3 was detected in ALK-positive ALCL tumors (42). If the level of caspase activation reaches a certain threshold, we propose a model, that may vary according to the cell type and the cell environment, in which ALK is cleaved by caspase-3 (or possibly a related subfamily caspase member), exposing a proapoptotic region and creating a positive feedback loop for apoptosis enhancement. This model could also apply to the neuronal cell, where serum deprivation, a stimulus that *per se* does not induce cell death in the 13.S.1.24 immortalized neuroblasts, “pushes” ALK to induce cell death.

Like several dependence receptors (26), ALK is involved in neural development (21, 31) and possibly morphogenesis, in spite of the lack of a lethal or abnormal phenotype in ALK-knock-out mice (Morris, personal communication and (12)). Interestingly, two recent papers analyzed the temporal spatial expression of the ALK receptor during the development of mice (44) and chicken (20), showing that ALK is dynamically expressed on subsets of neurons of both the central and peripheral nervous system. It is noteworthy that the expression of the ALK transcript in spinal motoneurons overlaps temporally with the period of programmed cell death (which affects 50% of these neurons) in the chick embryo, suggesting that ALK

could play a role in this phenomenon (20). Our results showing a pro-death effect of ALK in both murine immortalized neuroblasts and in *ex vivo* primary neurons support the potential proapoptotic role of ALK under certain physiological conditions that remain to be determined.

In conclusion, ALK is a receptor involved in both neural development and oncogenesis, with dual effects on apoptosis depending on the availability of the ligand. Therefore this is the first demonstration that ALK could be a new member of the dependence receptor family.

ACKNOWLEDGEMENTS

We are grateful to Fatima L'Faqihi and Sophie Allart from the Services Communs de Cytométrie et d'Imagerie de l'IFR Purpan, respectively, Catherine Guix for excellent technical help, Marie-Claire Bordeaux for precious help for initial constructions, Karen Pulford, Bruno Falini and Tadashi Yamamoto for the gift of anti-ALK mAbs. This work was partly funded by grants from the Conseil Régional Midi-Pyrénées, the Cancéropole Grand Sud-Ouest and the Association pour la Recherche sur le Cancer (ARC). Jaouhar Murali and Filipe Calheiros Lourenço are the recipients of PhD fellowships from the French Embassy in Tunisia (Institut Français de Coopération) and from the Portuguese Ministry of Science (FCT), respectively. Céline Monnet has a postdoctoral fellowship from the INSERM. Catherine Greenland was supported in part by an ARC fellowship.

REFERENCES

1. **Alam, A., L. Y. Cohen, S. Aouad, and R. P. Sekaly.** 1999. Early activation of caspases during T lymphocyte stimulation results in selective substrate cleavage in nonapoptotic cells. *J Exp Med* **190**:1879-90.
2. **Bai, R. Y., P. Dieter, C. Peschel, S. W. Morris, and J. Duyster.** 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**:6951-61.
3. **Bai, R. Y., T. Ouyang, C. Miething, S. W. Morris, C. Peschel, and J. Duyster.** 2000. Nucleophosmin-anaplastic lymphoma kinase associated with anaplastic large-cell lymphoma activates the phosphatidylinositol 3-kinase/Akt antiapoptotic signaling pathway. *Blood* **96**:4319-27.
4. **Benharroch, D., Z. Meguerian-Bedoyan, L. Lamant, C. Amin, L. Brugieres, M. J. Terrier-Lacombe, E. Haralambieva, K. Pulford, S. Pileri, S. W. Morris, D. Y. Mason, and G. Delsol.** 1998. ALK-positive lymphoma: a single disease with a broad spectrum of morphology. *Blood* **91**:2076-84.
5. **Bischof, D., K. Pulford, D. Y. Mason, and S. W. Morris.** 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**:2312-25.
6. **Bordeaux, M. C., C. Forcet, L. Granger, V. Corset, C. Bidaud, M. Billaud, D. E. Bredesen, P. Edery, and P. Mehlen.** 2000. The RET proto-oncogene induces apoptosis: a novel mechanism for Hirschsprung disease. *Embo J* **19**:4056-63.
7. **Bredesen, D. E., P. Mehlen, and S. Rabizadeh.** 2004. Apoptosis and dependence receptors: a molecular basis for cellular addiction. *Physiol Rev* **84**:411-30.

8. **Cook, D. M., M. T. Hinkes, M. Bernfield, and F. J. Rauscher, 3rd.** 1996. Transcriptional activation of the syndecan-1 promoter by the Wilms' tumor protein WT1. *Oncogene* **13**:1789-99.
9. **Coronas, V., F. Feron, R. Hen, G. Sicard, F. Jourdan, and E. Moyses.** 1997. In vitro induction of apoptosis or differentiation by dopamine in an immortalized olfactory neuronal cell line. *J Neurochem* **69**:1870-81.
10. **Cussac, D., C. Greenland, S. Roche, R. Y. Bai, J. Duyster, S. W. Morris, G. Delsol, M. Allouche, and B. Payrastre.** 2004. Nucleophosmin-anaplastic lymphoma kinase of anaplastic large-cell lymphoma recruits, activates, and uses pp60c-src to mediate its mitogenicity. *Blood* **103**:1464-71.
11. **Dirks, W. G., S. Fahnrich, Y. Lis, E. Becker, R. A. MacLeod, and H. G. Drexler.** 2002. Expression and functional analysis of the anaplastic lymphoma kinase (ALK) gene in tumor cell lines. *Int J Cancer* **100**:49-56.
12. **Duyster, J., R. Y. Bai, and S. W. Morris.** 2001. Translocations involving anaplastic lymphoma kinase (ALK). *Oncogene* **20**:5623-37.
13. **Evan, G., and T. Littlewood.** 1998. A matter of life and cell death. *Science* **281**:1317-22.
14. **Evan, G. I., A. H. Wyllie, C. S. Gilbert, T. D. Littlewood, H. Land, M. Brooks, C. M. Waters, L. Z. Penn, and D. C. Hancock.** 1992. Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69**:119-28.
15. **Falini, B., B. Bigerna, M. Fizzotti, K. Pulford, S. A. Pileri, G. Delsol, A. Carbone, M. Paulli, U. Magrini, F. Menestrina, R. Giardini, S. Pilotti, A. Mezzelani, B. Ugolini, M. Billi, A. Pucciarini, R. Pacini, P. G. Pelicci, and L. Flenghi.** 1998. ALK expression defines a distinct group of T/null lymphomas ("ALK lymphomas") with a wide morphological spectrum. *Am J Pathol* **153**:875-86.

16. **Forcet, C., E. Stein, L. Pays, V. Corset, F. Llambi, M. Tessier-Lavigne, and P. Mehlen.** 2002. Netrin-1-mediated axon outgrowth requires deleted in colorectal cancer-dependent MAPK activation. *Nature* **417**:443-7.
17. **Forcet, C., X. Ye, L. Granger, V. Corset, H. Shin, D. E. Bredesen, and P. Mehlen.** 2001. The dependence receptor DCC (deleted in colorectal cancer) defines an alternative mechanism for caspase activation. *Proc Natl Acad Sci U S A* **98**:3416-21.
18. **Green, D. R., and G. I. Evan.** 2002. A matter of life and death. *Cancer Cell* **1**:19-30.
19. **Greenland, C., C. Touriol, G. Chevillard, S. W. Morris, R. Bai, J. Duyster, G. Delsol, and M. Allouche.** 2001. Expression of the oncogenic NPM-ALK chimeric protein in human lymphoid T-cells inhibits drug-induced, but not Fas-induced apoptosis. *Oncogene* **20**:7386-97.
20. **Hurley, S. P., D. O. Clary, V. Copie, and F. Lefcort.** 2006. Anaplastic lymphoma kinase is dynamically expressed on subsets of motor neurons and in the peripheral nervous system. *J Comp Neurol* **495**:202-12.
21. **Iwahara, T., J. Fujimoto, D. Wen, R. Cupples, N. Bucay, T. Arakawa, S. Mori, B. Ratzkin, and T. Yamamoto.** 1997. Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. *Oncogene* **14**:439-49.
22. **Koopman, G., C. P. Reutelingsperger, G. A. Kuijten, R. M. Keehnen, S. T. Pals, and M. H. van Oers.** 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* **84**:1415-20.
23. **Lamant, L., K. Pulford, D. Bischof, S. W. Morris, D. Y. Mason, G. Delsol, and B. Mariame.** 2000. Expression of the ALK tyrosine kinase gene in neuroblastoma. *Am J Pathol* **156**:1711-21.
24. **Llambi, F., F. Causeret, E. Bloch-Gallego, and P. Mehlen.** 2001. Netrin-1 acts as a survival factor via its receptors UNC5H and DCC. *Embo J* **20**:2715-22.

25. **Mehlen, P., and L. Mazelin.** 2003. The dependence receptors DCC and UNC5H as a link between neuronal guidance and survival. *Biol Cell* **95**:425-36.
26. **Mehlen, P., F. Mille, and C. Thibert.** 2005. Morphogens and cell survival during development. *J Neurobiol* **64**:357-66.
27. **Mitsiadis, T. A., M. Salmivirta, T. Muramatsu, H. Muramatsu, H. Rauvala, E. Lehtonen, M. Jalkanen, and I. Thesleff.** 1995. Expression of the heparin-binding cytokines, midkine (MK) and HB-GAM (pleiotrophin) is associated with epithelial-mesenchymal interactions during fetal development and organogenesis. *Development* **121**:37-51.
28. **Moog-Lutz, C. H., J. L. Degoutin, J. Y. Gouzi, Y. B. Frobert, N. C. Brunet-De Carvalho, J. F. Bureau, C. E. Creminon, and M. R. Vigny.** 2005. Activation and inhibition of ALK receptor tyrosine kinase by monoclonal antibodies and absence of agonist activity of pleiotrophin. *J Biol Chem* **280**:26039-48.
29. **Morgan, R., S. D. Smith, B. K. Hecht, V. Christy, J. D. Mellentin, R. Warnke, and M. L. Cleary.** 1989. Lack of involvement of the c-fms and N-myc genes by chromosomal translocation t(2;5)(p23;q35) common to malignancies with features of so-called malignant histiocytosis. *Blood* **73**:2155-64.
30. **Morris, S. W., M. N. Kirstein, M. B. Valentine, K. G. Dittmer, D. N. Shapiro, D. L. Saltman, and A. T. Look.** 1994. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non- Hodgkin's lymphoma. *Science* **263**:1281-4.
31. **Morris, S. W., C. Naeve, P. Mathew, P. L. James, M. N. Kirstein, X. Cui, and D. P. Witte.** 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) [published erratum appears in *Oncogene* 1997 Dec 4;15(23):2883]. *Oncogene* **14**:2175-88.

32. **Motegi, A., J. Fujimoto, M. Kotani, H. Sakuraba, and T. Yamamoto.** 2004. ALK receptor tyrosine kinase promotes cell growth and neurite outgrowth. *J Cell Sci* **117**:3319-29.
33. **Nieborowska-Skorska, M., A. Slupianek, L. Xue, Q. Zhang, P. N. Raghunath, G. Hoser, M. A. Wasik, S. W. Morris, and T. Skorski.** 2001. Role of signal transducer and activator of transcription 5 in nucleophosmin/ anaplastic lymphoma kinase-mediated malignant transformation of lymphoid cells. *Cancer Res* **61**:6517-23.
34. **Pulford, K., L. Lamant, S. W. Morris, L. H. Butler, K. M. Wood, D. Stroud, G. Delsol, and D. Y. Mason.** 1997. Detection of anaplastic lymphoma kinase (ALK) and nucleolar protein nucleophosmin (NPM)-ALK proteins in normal and neoplastic cells with the monoclonal antibody ALK1. *Blood* **89**:1394-404.
35. **Pulford, K., S. W. Morris, and F. Turturro.** 2004. Anaplastic lymphoma kinase proteins in growth control and cancer. *J Cell Physiol* **199**:330-58.
36. **Reed, J. C., K. S. Doctor, and A. Godzik.** 2004. The domains of apoptosis: a genomics perspective. *Sci STKE* **2004**:re9.
37. **Schlessinger, J.** 2000. Cell signaling by receptor tyrosine kinases. *Cell* **103**:211-25.
38. **Stoica, G. E., A. Kuo, A. Aigner, I. Sunitha, B. Souttou, C. Malerczyk, D. J. Caughey, D. Wen, A. Karavanov, A. T. Riegel, and A. Wellstein.** 2001. Identification of anaplastic lymphoma kinase as a receptor for the growth factor pleiotrophin. *J Biol Chem* **276**:16772-9.
39. **Stoica, G. E., A. Kuo, C. Powers, E. T. Bowden, E. B. Sale, A. T. Riegel, and A. Wellstein.** 2002. Midkine binds to anaplastic lymphoma kinase (ALK) and acts as a growth factor for different cell types. *J Biol Chem* **277**:35990-8.

40. **Stupack, D. G., X. S. Puente, S. Boutsaboualoy, C. M. Storgard, and D. A. Cheresh.** 2001. Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. *J Cell Biol* **155**:459-70.
41. **Talpaz, M., R. T. Silver, B. J. Druker, J. M. Goldman, C. Gambacorti-Passerini, F. Guilhot, C. A. Schiffer, T. Fischer, M. W. Deininger, A. L. Lennard, A. Hochhaus, O. G. Ottmann, A. Gratwohl, M. Baccarani, R. Stone, S. Tura, F. X. Mahon, S. Fernandes-Reese, I. Gathmann, R. Capdeville, H. M. Kantarjian, and C. L. Sawyers.** 2002. Imatinib induces durable hematologic and cytogenetic responses in patients with accelerated phase chronic myeloid leukemia: results of a phase 2 study. *Blood* **99**:1928-37.
42. **ten Berge, R. L., C. J. Meijer, D. F. Dukers, J. A. Kummer, B. A. Bladergroen, W. Vos, C. E. Hack, G. J. Ossenkoppele, and J. J. Oudejans.** 2002. Expression levels of apoptosis-related proteins predict clinical outcome in anaplastic large cell lymphoma. *Blood* **99**:4540-6.
43. **Tulasne, D., J. Deheuninck, F. C. Lourenco, F. Lamballe, Z. Ji, C. Leroy, E. Puchois, A. Moumen, F. Maina, P. Mehlen, and V. Fafeur.** 2004. Proapoptotic function of the MET tyrosine kinase receptor through caspase cleavage. *Mol Cell Biol* **24**:10328-39.
44. **Vernersson, E., N. K. Khoo, M. L. Henriksson, G. Roos, R. H. Palmer, and B. Hallberg.** 2006. Characterization of the expression of the ALK receptor tyrosine kinase in mice. *Gene Expr Patterns*. Jan 31; [Epub ahead of print] PMID: 16458083
45. **Zhang, N., and T. F. Deuel.** 1999. Pleiotrophin and midkine, a family of mitogenic and angiogenic heparin-binding growth and differentiation factors. *Curr Opin Hematol* **6**:44-50.

46. **Zhang, Q., P. N. Raghunath, L. Xue, M. Majewski, D. F. Carpentieri, N. Odum, S. Morris, T. Skorski, and M. A. Wasik.** 2002. Multilevel dysregulation of STAT3 activation in anaplastic lymphoma kinase-positive T/null-cell lymphoma. *J Immunol* **168**:466-74.

FIGURE LEGENDS

Figure 1. Characterization of ALK expressed in stably transfected Jurkat cells.

(A) Jurkat cells, transfected with *ALK* cDNA or with control vector (Jurkat/neo cells), were selected with geneticin for stable gene expression and cloned by limiting dilution. Jurkat/neo cells (top) or an ALK-transfected Jurkat clone (bottom) were cytopun onto glass slides. Immunohistochemical analysis of the ALK protein was done using the ALK1 mAb in a three-stage immunoperoxidase technique. Magnification, x 800. (B) Intact (left panel) or permeabilized (right panel) Jurkat/ALK cells were stained with the REAB or ALK1 antibodies, followed respectively by FITC-coupled goat anti-rabbit or anti-mouse Ig. Immunofluorescence analysis of surface (left) and intracellular (right) ALK staining was analysed by flow cytometry (colored histograms). Background cell fluorescence with an isotopic control antibody is shown as a black line histogram. (C) 50µg of protein from total cell lysates of Jurkat/neo (vector) or Jurkat/ALK (ALK-wt) cells were separated by 7.5% SDS-PAGE and analyzed by Western blotting using the ALKc mAb. ALK is detected as a 200 kDa band. Molecular mass markers are indicated on the left. The filter was stripped and reprobed using an anti-actin mAb to assess comparable loading. (D) *In vitro* kinase assay of anti-ALK immunoprecipitates from total cell extracts of two ALK- (labeled 1 and 2, ALK-wt) and one NPM-ALK-expressing clones, showing autophosphorylation of the proteins. (E) Cell lysates from vector- or ALK-wt-transfected Jurkat cells and from the NPM-ALK-expressing ALCL cell line SU-DHL1 were immunoprecipitated using the ALK1 mAb, then analysed through Western blotting using the antiphosphotyrosine mAb 4G10. Tyrosine phosphorylation was detected in NPM-ALK-, but not in ALK-expressing cells. Molecular mass markers are indicated.

Figure 2. Doxorubicin-induced apoptosis is enhanced in ALK-expressing Jurkat cells.

(A) Jurkat cells stably transfected with vector or cDNA for NPM-ALK or ALK were seeded at 4×10^5 cells/ml, and incubated for 16 hours with culture medium (white bars) or 2 μ M doxorubicin (black bars) as apoptosis inducer. The caspase inhibitor zVAD-fmk (10 μ M) was added in the absence (grey bars) or presence (hatched bars) of doxorubicin. Apoptosis was measured using the annexin V-FITC labeling technique. The percentage of annexin-positive cells was significantly enhanced in Jurkat/ALK and decreased in Jurkat/NPM-ALK cells, respectively, compared to control vector-transfected cells ($p < 0.001$). Doxorubicin-induced apoptosis was also significantly inhibited by zVAD-fmk in all cases ($p < 0.001$). Histograms represent the mean \pm SEM of 3-6 independent experiments. (B) Correlation of apoptosis detection by 2 methods within a representative experiment. Cells were treated for 16 hours with doxorubicin (2 μ M) as in (A) and the percentage of annexin-positive cells was determined (black histograms). Moreover at the end of apoptosis induction, an aliquot of the culture was assayed for caspase-3/7 activity using the “Caspase glo 3/7” luminescence assay (Promega) according to the manufacturer’s instructions. Caspase activation is shown as the ratio between the caspase activity of the treated sample and of the corresponding untreated cells (relative caspase activity index, grey histograms, right side scale). (C) Dose-response curve and (D) time course analysis of doxorubicin-induced apoptosis in Jurkat/neo (black square symbols) and Jurkat/ALK (black triangles) cells. (C) displays one representative experiment of two and (D) the mean \pm SEM of 3 independent experiments.

Figure 3. Caspase-dependent ALK degradation during drug-induced apoptosis.

(A) Jurkat/ALK cells were treated with 2 μ M doxorubicin for 24 hours, in the presence or absence of 10 μ M zVAD-fmk, or incubated with culture medium alone. Cells were lysed and 50 μ g of protein from total cell extracts were separated by 7.5% SDS-PAGE and analyzed on

replicate gels through Western blotting using antibodies directed against the extra-cellular (REAB), or intra-cellular (ALK1 and ALKc) domain of ALK. The filters were stripped and reprobed using an anti-actin mAb to assess comparable loading. The intensity of the 200 kDa ALK band (seen as a doublet) decreases in doxorubicin-treated cells, a decrease partially inhibited by the caspase inhibitor zVAD-fmk. **(B)** Western blot analysis of time-dependent degradation of ALK using the ALKc mAb, showing the appearance of a 60 kDa band (arrowhead). The decrease of the 200 kDa band is not visible because of a longer exposure of the blot, necessary to visualize the 60 kDa band. Molecular mass markers are shown. **(C)** Jurkat/ALK cells were treated as in (A) and ALK degradation was analyzed by Western blotting using the ALKc mAb. Appearance of the 60 kDa ALK fragment (arrowhead) is inhibited by zVAD-fmk. **(D)** Total protein cell lysates from untreated Jurkat/ALK cells were incubated *in vitro* with purified activated caspase-3 for 30 minutes at 37°C and analyzed by Western blot using the ALKc mAb. The arrowhead indicates the 60 kDa ALK fragment. Lysates from equivalent numbers of cells were loaded onto a 10% SDS-PAGE gel.

Figure 4. The caspase cleavage site of ALK is D1160. **(A)** Portion of the ALK intra-cellular sequence showing the caspase-3 consensus-like cleavage site (DELD) and the position of aspartic acids chosen for experimental D_N mutation, as well as the ATP-binding site (K1150). **(B)** Jurkat cells were permanently transfected with cDNAs coding for ALK mutants displaying D_N mutations at position 1141, 1160, 1163 and 1225 and cloned by limiting dilution. One representative cell clone is shown for each mutant. Cells were treated with 2 µM doxorubicin for 24 hours, in the presence or absence of 10 µM zVAD-fmk, or left untreated. The arrowhead indicates the 60 kDa ALK fragment, absent from the ALK D1160N mutant-expressing cells. The filters were stripped and reprobed using an anti-actin mAb to assess comparable loading. **(C)** Total protein cell lysates from untreated Jurkat cells expressing the

1160 and 1163 D_N ALK mutants were incubated *in vitro* with purified activated caspase-3 for 30 minutes at 37°C and analyzed by Western blot using the ALKc mAb. The arrowhead indicates the 60 kDa ALK fragment. Lysates from equivalent numbers of cells were loaded onto a 7.5% SDS-PAGE gel.

Figure 5. Kinase activation impairs NPM-ALK cleavage by caspase-3. (A) Jurkat cells expressing the wild type or kinase-dead NPM-ALK mutant were treated for the indicated times (hours) with 2 μ M doxorubicin. Cells were lysed and 50 μ g of protein from total cell extracts were separated by 10% SDS-PAGE and analyzed by Western blot using ALKc. The filters were stripped and reprobed using an anti-actin mAb to assess protein loading. The arrow indicates the 60 kDa ALK fragment. (B) Total protein cell lysates from untreated Jurkat/NPM-ALK cells and from the ALCL line SU-DHL1 were incubated *in vitro* with purified activated caspase-3 for 30 minutes at 37°C and analyzed by Western blot using the ALKc mAb. The arrow indicates the 60 kDa ALK fragment. Lysates from equivalent numbers of cells were loaded onto a 10% SDS-PAGE gel.

Figure 6. The D1160N mutation abrogates ALK-mediated enhancement of apoptosis. Jurkat cells expressing wild type or mutant ALK displaying D_N mutations at position 1141, 1160, 1163 and 1225, and wild type or kinase-dead NPM-ALK, were treated with 2 μ M doxorubicin for 16 hours. Apoptosis was measured using the annexin V-FITC labeling technique. One representative cell clone is shown for each cell line. The percentage of annexin-positive cells in all groups was statistically different ($p < 0.05$) from that in control vector transfected cells (white bar). Apoptosis was increased in all groups (with no significant difference between them) except for the ALK D1160N mutant and the NPM-ALK wt

expressing cells, in which apoptosis was inhibited in comparison to control vector-transfected cells. Bars represent the mean \pm SEM of 3 independent experiments.

Figure 7. Mapping of a proapoptotic segment of ALK to the juxta-membrane region.

Upper panel: Schematic representation of wild type and C-terminally truncated ALK proteins: the extra-cellular (EC), transmembrane (TM), tyrosine kinase (TK) and intra-cellular (IC) domains are indicated. Middle panel: Jurkat cells, stably transfected with pcDNA3 vector (white bar) or cDNA coding for wild type (A) or C-terminally truncated ALK proteins (black bars) ALK-stop1160 (B), ALK-delta33-stop1160 (C) or ALK-stop1126 (D), were treated with 2 μ M doxorubicin for 16 hours. Apoptosis was measured using the annexin V-FITC labeling technique. Results are represented as an index of the percentage of annexin-positive control vector-transfected cells (mean \pm SEM of 3 independent experiments). Lower panel: Western blot detection of wt and truncated ALK proteins (indicated by arrowheads) using the mAb 48 directed against the extra-cellular domain of ALK. 7.5% SDS-PAGE was run under non reducing conditions.

Figure 8. ALK ligation by activating antibodies inhibits ALK-mediated enhancement of apoptosis.

(A) Jurkat/ALK cells were treated with 2 μ M doxorubicin for 16 hours in the presence of increasing doses of the ALK-specific activating mAbs 46 (black square symbols) or 16-39 (black triangles). Apoptosis was measured using the annexin V-FITC labeling technique. Results are expressed as the percentage of apoptosis inhibition in Jurkat/ALK cells relative to the control Jurkat/neo (vector) cells treated in the same conditions. One representative experiment of 3 is shown. (B) Jurkat/ALK cells were serum-starved for 24 hours and incubated for various times with 10 μ g/ml mAb 46 or control isotypic mouse IgG. Lysates from equivalent numbers of cells were loaded onto a 7.5% SDS-PAGE gel. ALK

phosphorylation was analyzed by Western blot with the 4G10 anti-phosphotyrosine mAb (Ptyr). The blot was stripped and sequentially reprobed with ALKc and REAB antibodies, to visualize total ALK expression, and with anti-actin to assess comparable protein loading.

Figure 9. ALK induces cell death in neuroblasts. (A) ALK cDNA or empty pcDNA3 vector was transiently transfected into the 13.S.1.24 immortalized murine neuroblast cell line,. Twenty-four hours after transfection, cells were serum-starved and incubated in the presence or absence of 20 µg/ml ALK-specific activating mAb 16-39 for a further 24 hours. Cell morphology was observed by phase contrast microscopy under an inverted microscope. ALK-transfected cells exhibited significant cell death (lower left panel), but could be rescued by the activating anti-ALK mAb (lower right panel). (B) Cells from cultures shown in (A) were assessed for viability, by the MTS assay (CellTiter 96 Aqueous cell proliferation assay from Promega), (C) and apoptosis, measuring the percentage of active caspase-3-expressing cells by flow cytometry. The control (set arbitrarily at 100%) in both graphs is the vector-transfected neuroblast cell line. Results represent the mean ± SEM of triplicate measures within one representative experiment of 3. (D) In addition to vector and ALK-wt, the truncated ALK-stop1160 mutant cDNA was transfected into 13.S.1.24 cells. Cell viability was measured as in (B). Bars represent the mean ± SEM of 3 independent experiments. The difference between ALK-wt or ALK-stop1160 and the vector-transfected cells was statistically significant (* : $p < 0.05$, Student's *t* test). (E) The zinc-inducible metallothionein promoter-containing pMTCB6+-derived vector (pMT vector) was used to express ALK-wt or the ALK C-terminal peptide 1161-1620 (ALK-Cter) in 13.S.1.24 neuroblasts, respectively. Cells were transfected in the absence of serum, and treated with 100µM ZnSO₄ 5 h later. Both proteins were detected in transfected neuroblasts by Western blot (upper panel), and cell viability was measured by trypan blue exclusion 48 h post-induction (lower panel). Bars

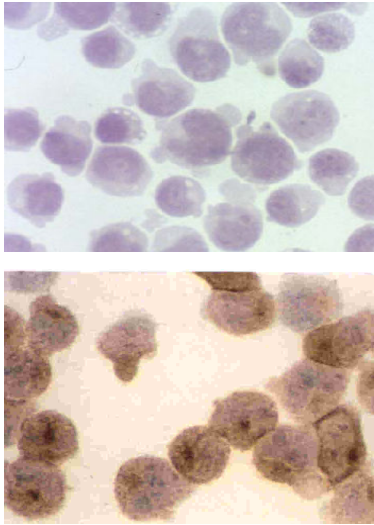
represent the mean \pm SEM of 4 independent experiments. Zinc-induced ALK-wt expression significantly decreased the number of viable cells in the culture (* : $p < 0.05$), compared to the control pMT vector, whereas the Cter peptide did not have a significant effect.

Figure 10. ALK induces cell death in primary cortical neurons

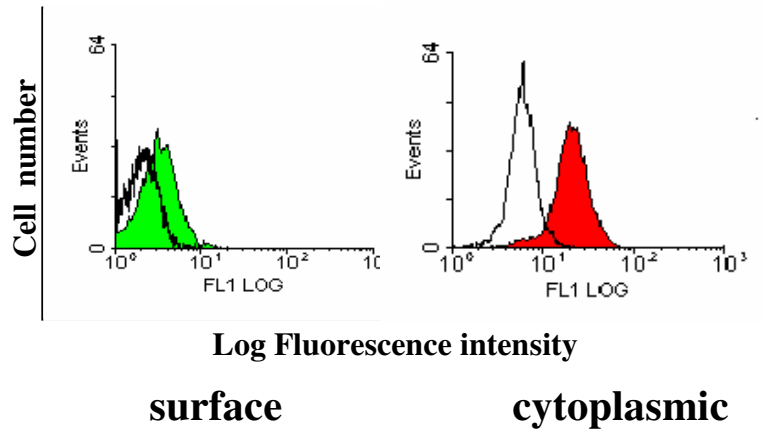
Primary cortical neurons prepared from 18-day-old embryonic Sprague Dawley rats were transfected with ALK cDNA inserted into the pcDNA3 or the zinc-inducible pMT vector, together with a GFP-expression vector as control, as described in material and methods. Zinc (100 μ M Zn SO₄) was added at day 2, 3 and 6 post-transfection for pMT vectors. Transfected neurons were analyzed after 6 to 8 days. **(A)** Phase contrast microscopy of cultures at day 6, showing a lower density of neurons in ALK-transfected cultures. **(B)** Cell viability was measured at day 7 post-transfection using the CellTiterGlo luminescence assay. Results represent the mean \pm SD of quadruplicate measures within one representative experiment of 2. **(C)** Immunofluorescence analysis of transfected neurons (day 8) grown on glass coverslips, permeabilized, stained with ALKc mAb followed by an Alexa Fluor 594 goat anti-mouse IgG (right panels). Left panels show the analysis of GFP expression in transfected neurons.

Figure 1

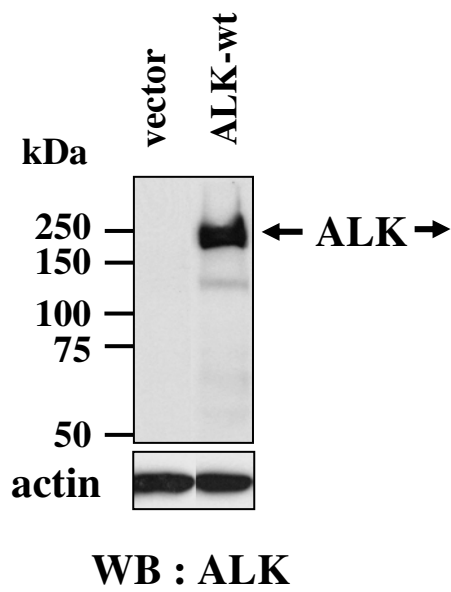
A



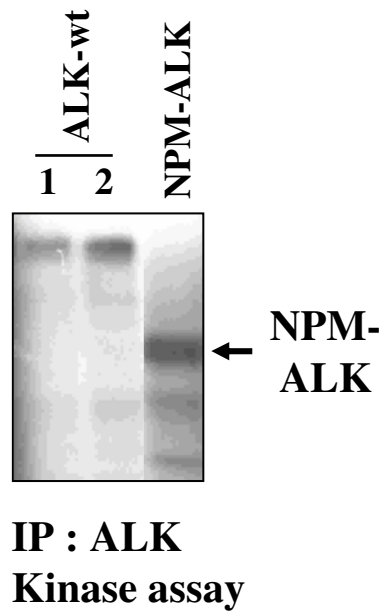
B



C



D



E

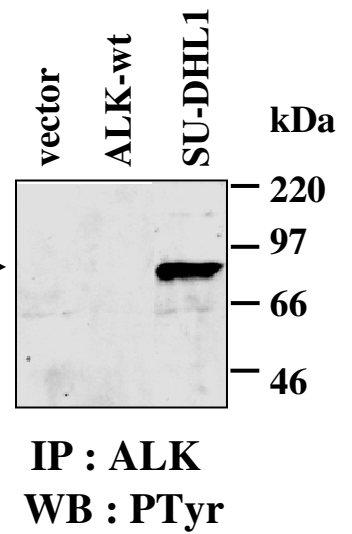


Figure 2

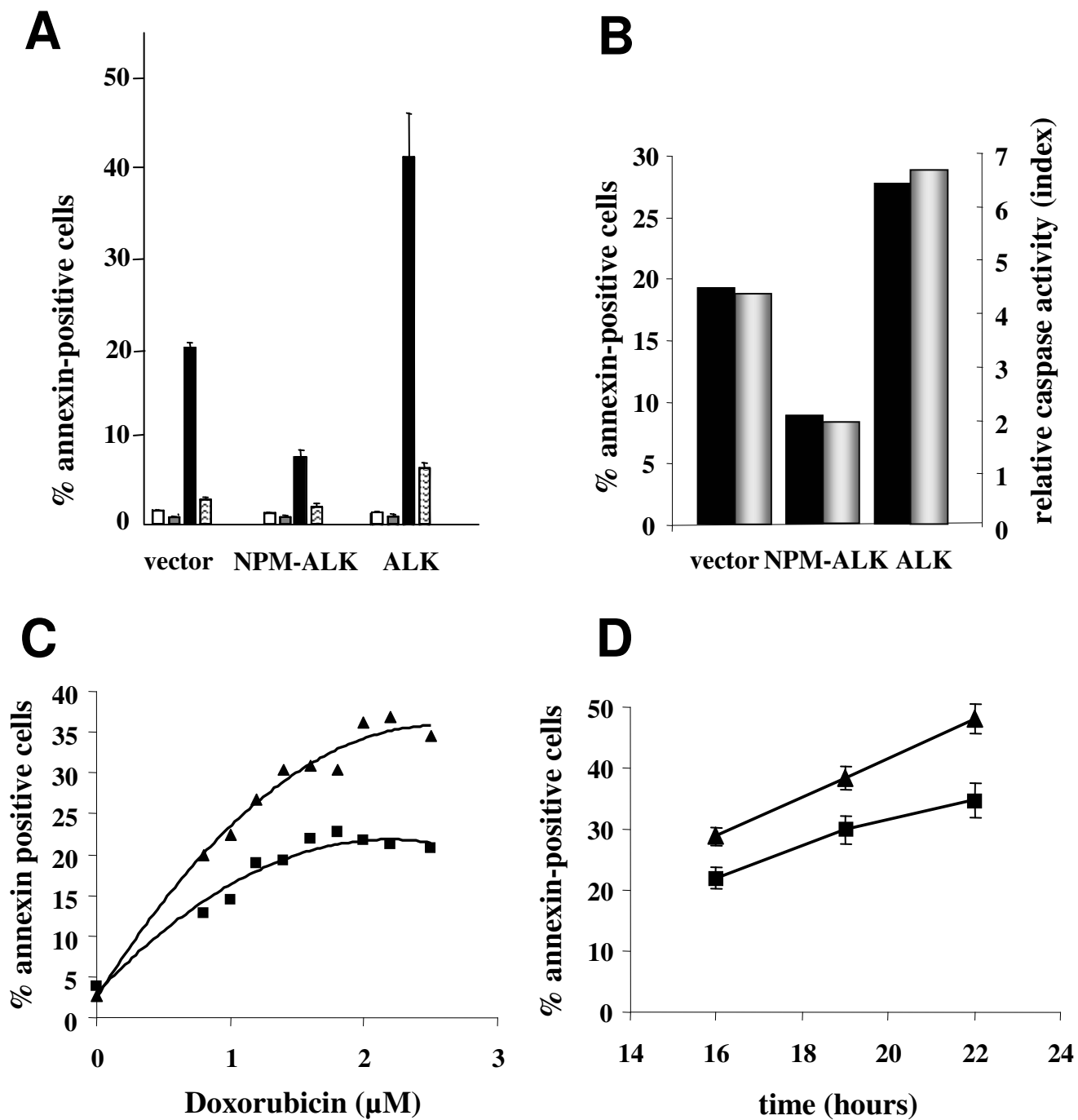


Figure 3

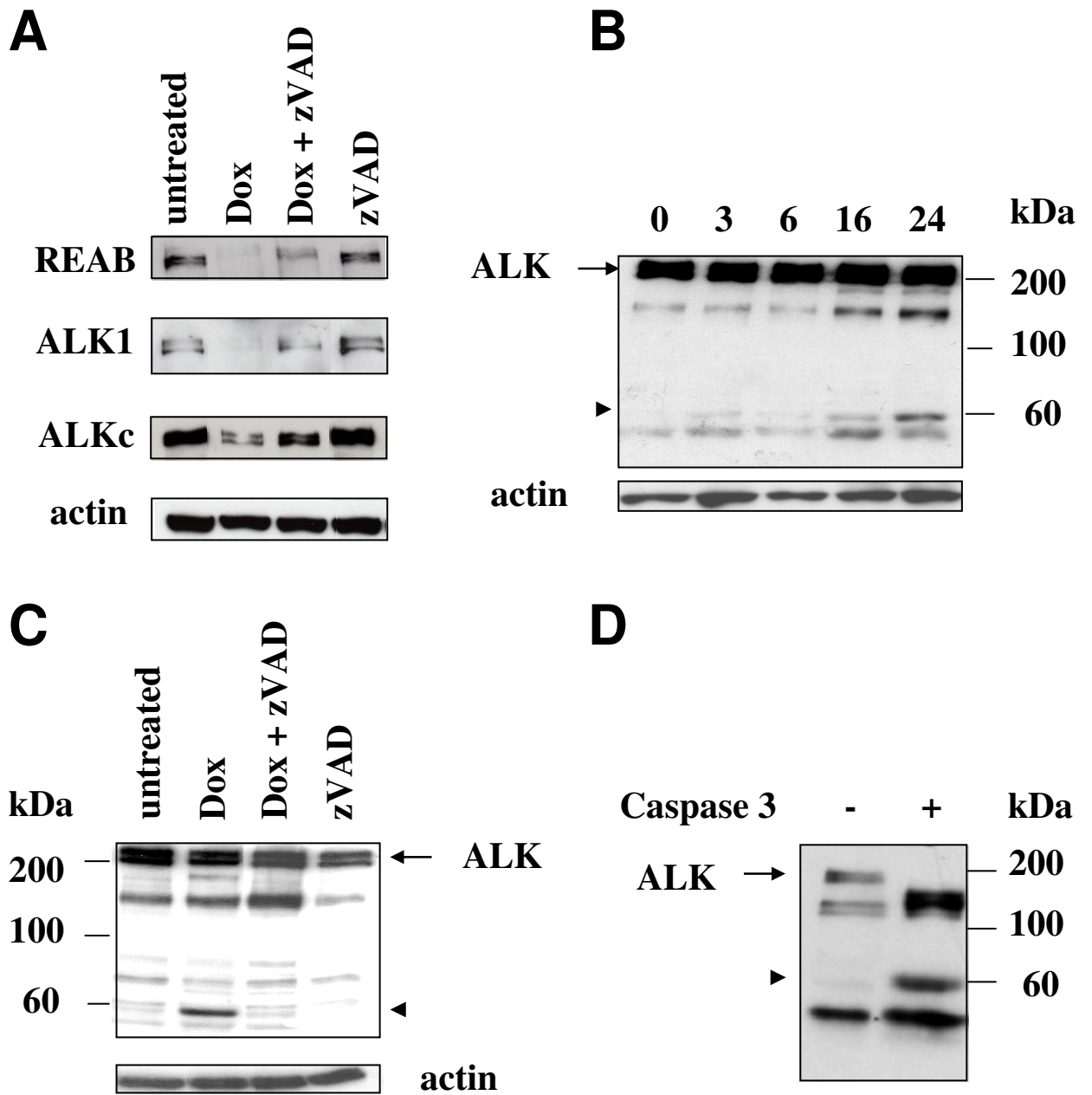


Figure 4

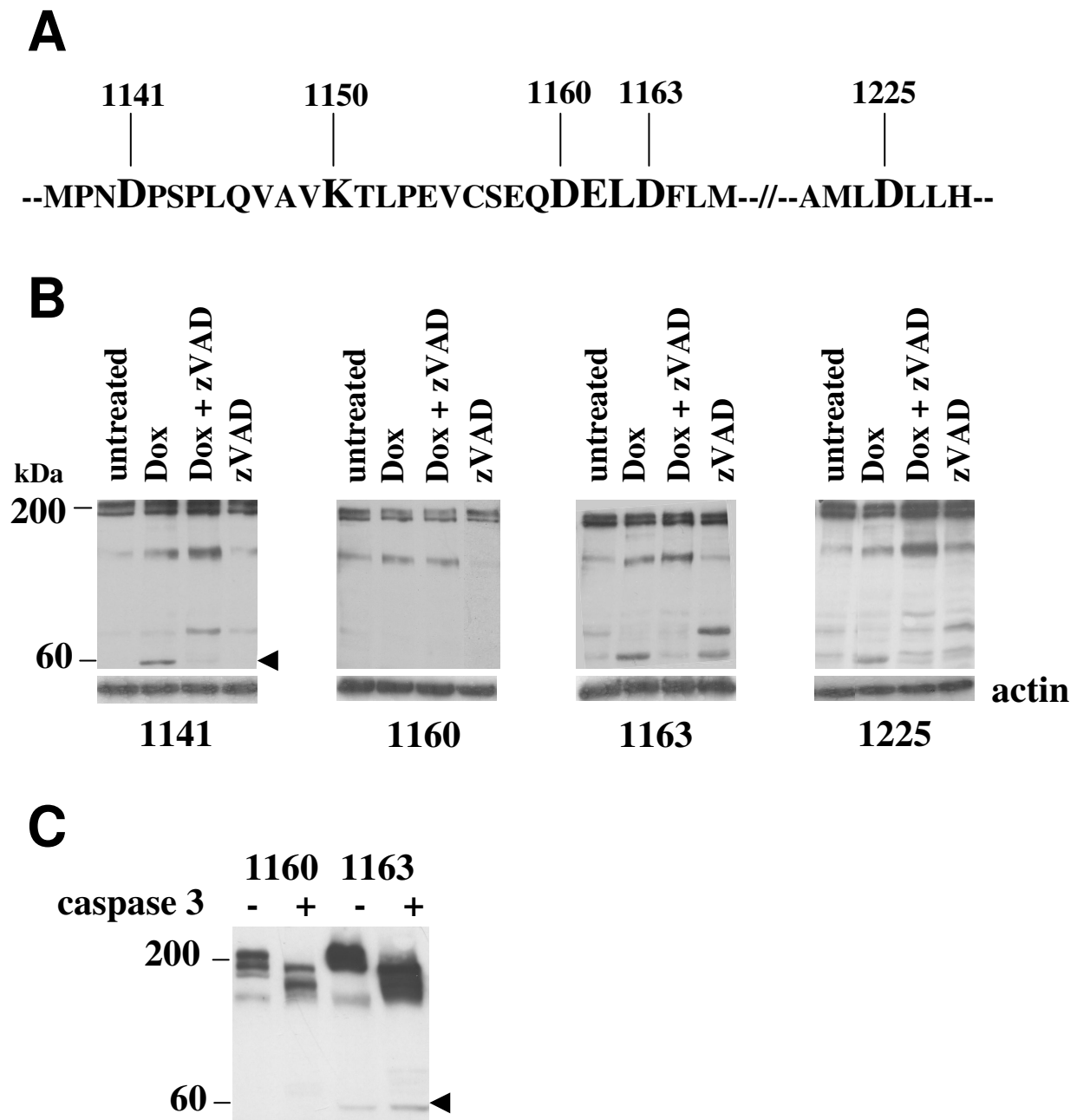


Figure 5

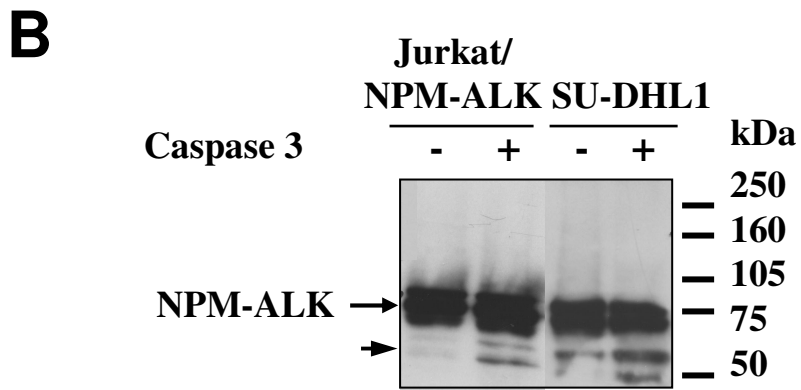
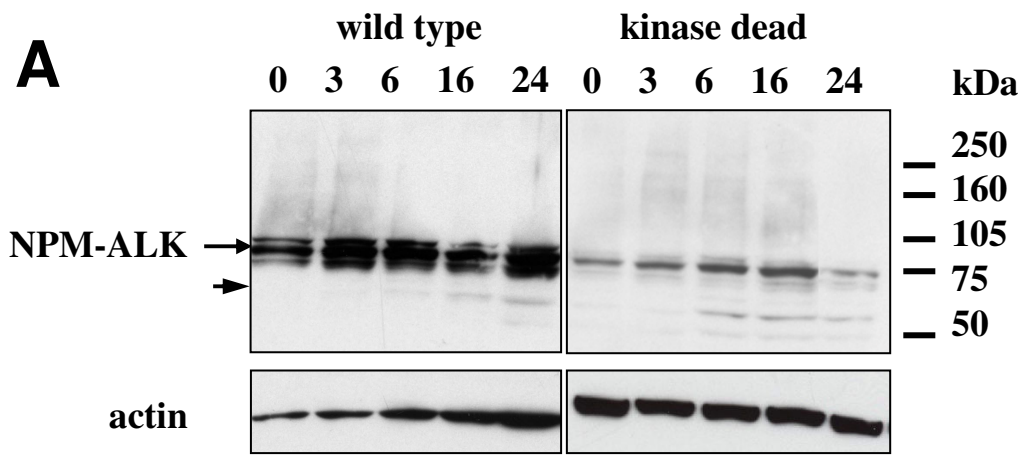


Figure 6

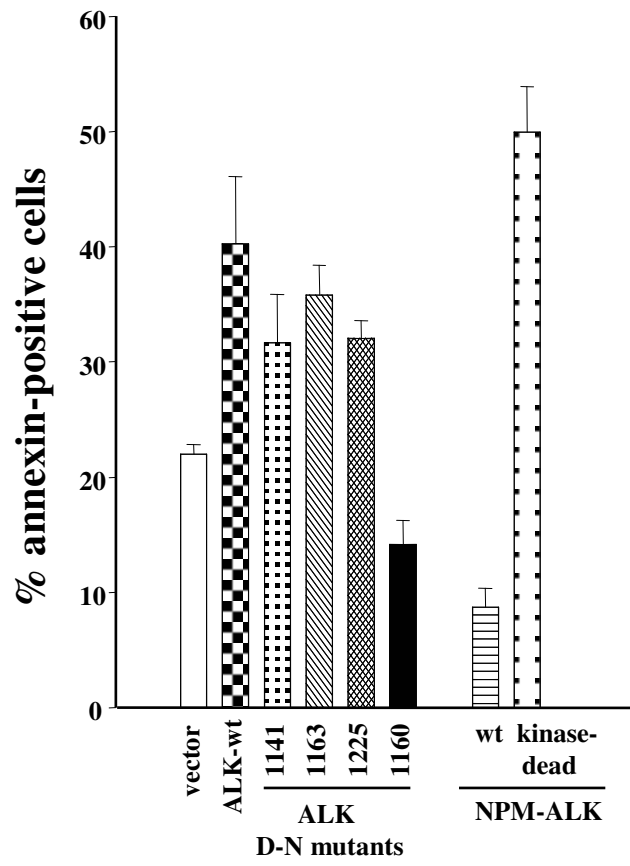


Figure 7

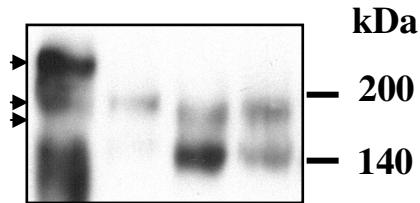
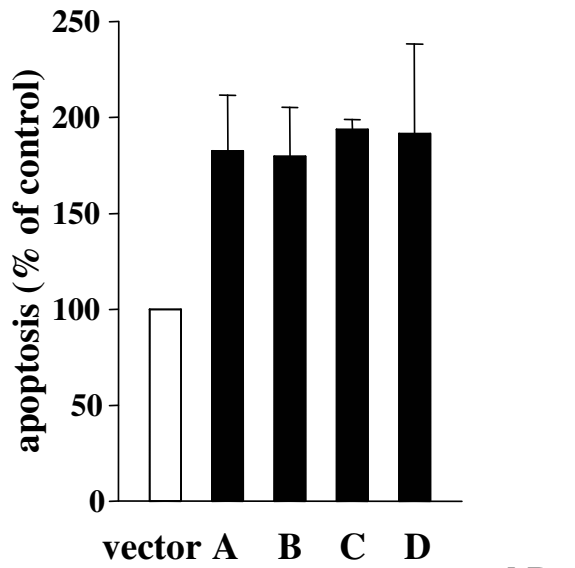
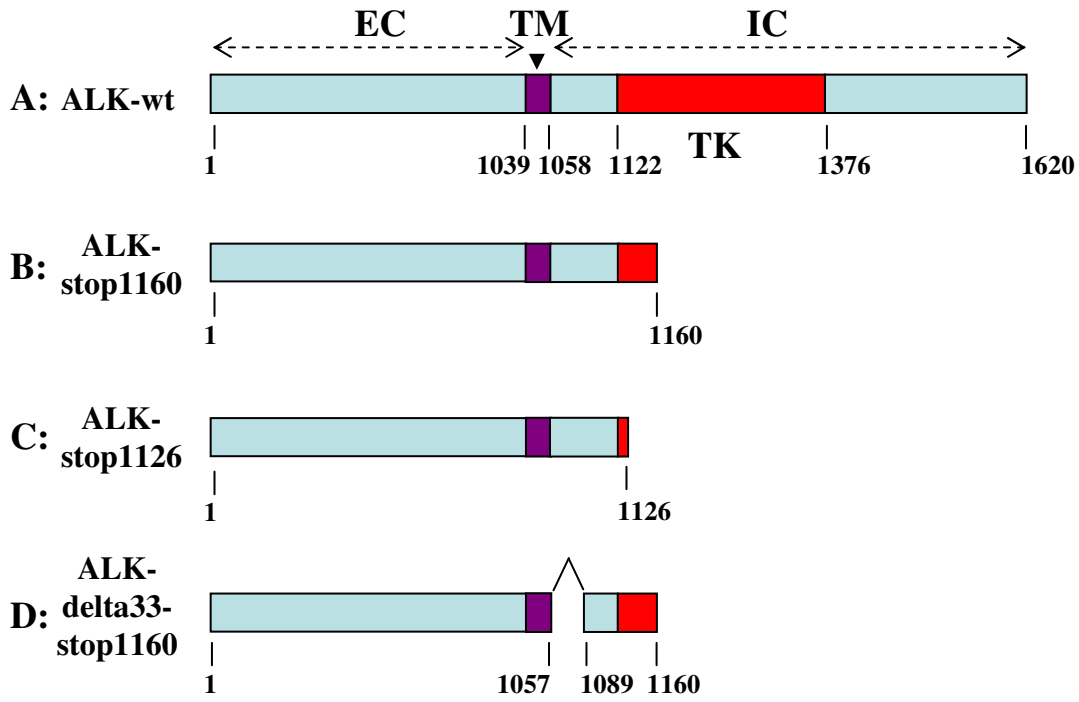


Figure 8

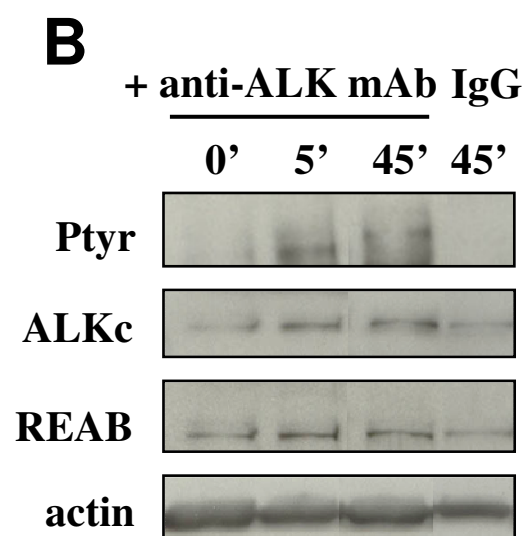
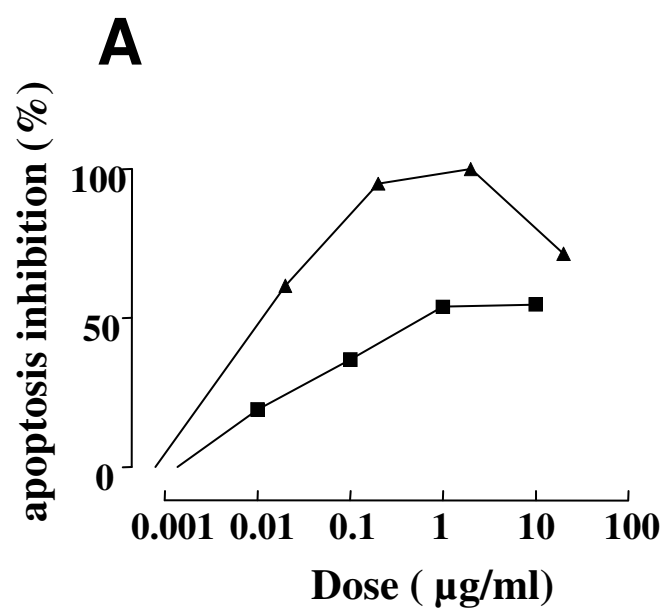
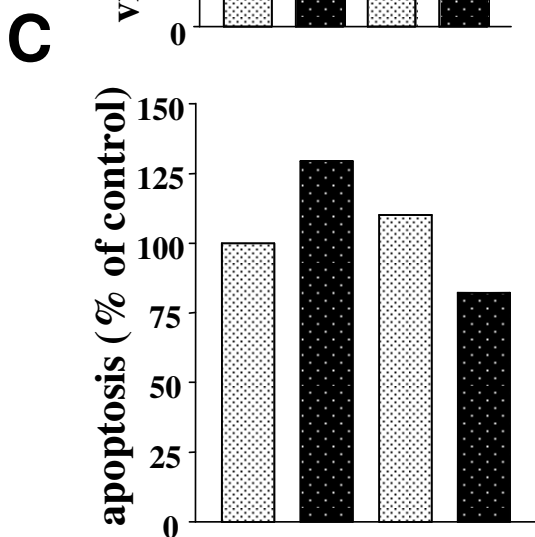
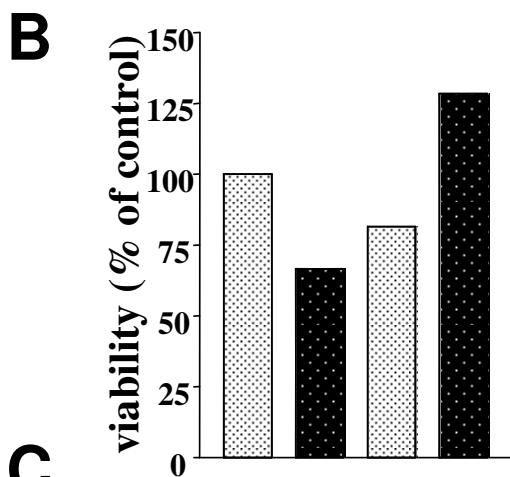
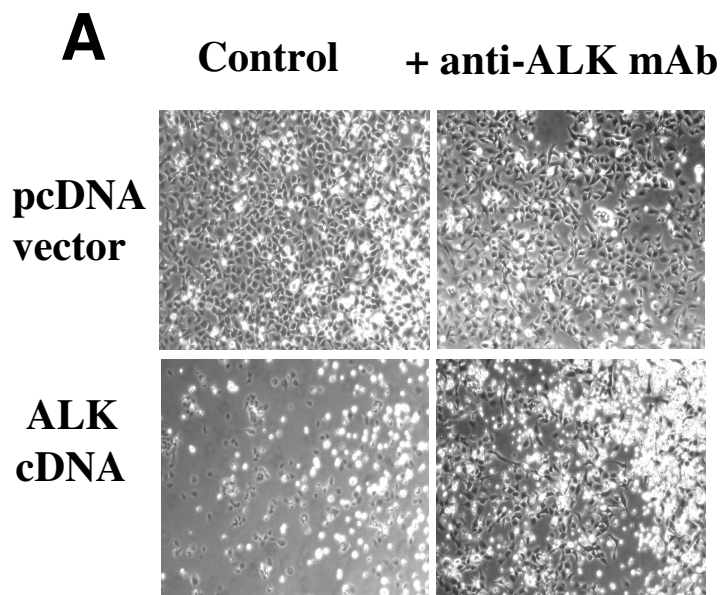


Figure 9



ALK cDNA	-	+	-	+
anti-ALK mAb	-	-	+	+

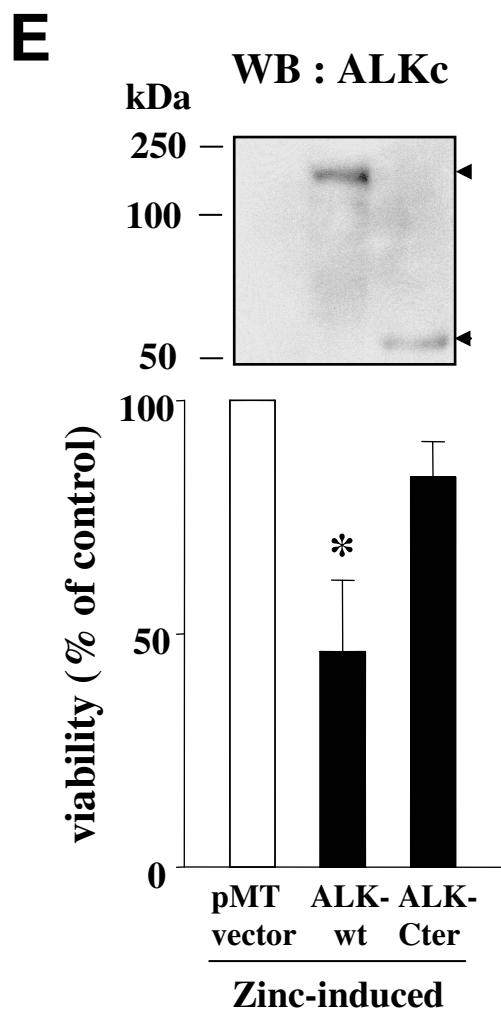
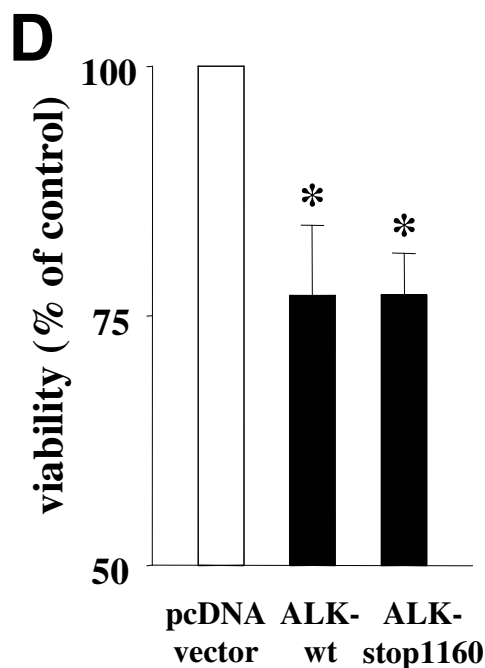


Figure 10

