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Bcl-X_L downregulation suppresses the tumorigenic potential of NPM/ALK *in vitro* and *in vivo*

Running Title: Bcl-X_L inhibition in ALK^+ ALCL lymphoid cells

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ABSTRACT

Deregulated apoptosis is a common finding in tumorigenesis. The oncogenic tyrosine kinase NPM/ALK delivers a strong survival signal in Anaplastic Large Cell Lymphomas (ALCLs). Although NPM/ALK activates multiple antiapoptotic pathways, the biological relevance and therapeutic potential of more downstream apoptotic effectors are mostly unknown. In this report, the NPM/ALK-mediated induction of Bcl-X_L (but not of Bcl-2) was identified in human ALCL-derived cells. NPM/ALK kinase activity was required to promote Bcl-X_L expression and its protective effect on mitochondrial homeostasis. Downregulation of Bcl-X_L significantly reduced the antiapoptotic potential of NPM/ALK in both transformed murine Ba/F3 pro-B cells and human ALCL-derived KARPAS-299 cells. To elucidate the role of Bcl-X_L *in vivo*, Ba/F3-NPM/ALK$^+$ cells expressing a doxycycline (Dox)-inducible *Bcl-X_L* antisense transgene (pTet-ON) were injected into nude mice. Doxycycline administration prevented a fatal systemic disease in 15/15 intravenously injected mice and the appearance of subcutaneous tumor xenografts in 9/12 mice: *in vivo* downregulation of Bcl-X_L was also documented. Our results show a pivotal role for Bcl-X_L in ALK-mediated oncogenicity: a single protein placed downstream of a known oncogene can be crucial for the survival of neoplastic cells both *in vitro* and *in vivo*. Bcl-X_L deserves further investigation as a possible therapeutic target in ALK$^+$ ALCLs.

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INTRODUCTION

The broad category of non Hodgkin's lymphomas (NHLs) includes distinct neoplasms arising from clonal expansion of B- or T-lymphocytes at different stages of maturation and differentiation [1]. The CD30/Ki-1^{+} Anaplastic Large Cell Lymphomas (ALCLs) account for about 20% of high-grade NHLs presenting unique immunophenotypical, morphological and genetic features [2]. Approximately 70% of ALCLs express an 80 kDa hybrid oncoprotein, named NPM/ALK, arising from the chromosomal translocation t(2;5)(p23;q35) [3]. The NPM/ALK chimera contains the N-terminal portion of the ubiquitously expressed nucleolar protein NPM (Nucleophosmin) fused to the kinase domain of ALK (Anaplastic Lymphoma Kinase) [4]. ALK is a member of the insulin receptor tyrosine kinase superfamily, whose expression is restricted to the central nervous system mainly during the neonatal period [5].

As a consequence of the t(2;5) rearrangement, the strong *NPM* gene promoter drives the high-level ectopic expression of ALK in lymphoid ALCL cells [6,7]. In addition, the oligomerization domain contained in the NPM portion promotes the constitutive *trans*-phosphorylation and activation of the ALK kinase domain in NPM/ALK [8]. Thus, NPM/ALK can exert its oncogenic potential via abnormal stimulation of multiple cellular signaling cascades, enhancing growth factor-independent proliferation and prolonging viability. Several groups described a direct recruitment and activation by NPM/ALK of numerous intracellular signal transduction effectors such as phospholipase C-γ (PLC-γ) [9], pp60 c-Src kinase [10], phosphatidylinositol-3-kinase (PI-3kinase) [11,12], Janus kinases (Jaks) [13-15] and Signal

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Transducers and Activators of Transcription (Stats) [13,16,17], but the precise biological implication of their activation in ALK^+ ALCL cells remains unclear.

The Bcl-2 family members are important modulators of mitochondrially initiated apoptosis [18]. This still expanding family contains both pro-survival (Bcl-2, Bcl-X_L, Mcl-1) and pro-apoptotic (Bad, Bak, Bax) factors which share the presence of one to four Bcl-2 homology (BH) domains [19]. Of these domains, BH3 represents a key element in regulating their homo-/heterodimerization and subcellular localization. Bcl-2 and Bcl-X_L exhibit both structural and functional similarity to prokaryotic pore-forming proteins, acting as ionic channels in the outer mitochondrial membrane for the control of mitochondrial swelling and loss of transmembrane potential [20,21]. The "BH3-only" pro-apoptotic Bad, which lacks the mitochondrial-membrane insertion C-terminal signal, is reversibly regulated by phosphorylation at multiple serine (Ser132, Ser136 and Ser155) residues [22-24]. In particular, phosphorylation at Ser-136 is known to create a binding site for the phospho-specific binding of 14-3-3 proteins, which retain Bad in the cytoplasm preventing its translocation to the mitochondrial membrane [23,25]. In fact, upon a death signal, such as withdrawal of IL-3 [26] or insulin-like growth factor-1 [27], dephosphorylated Bad uses its BH3-death-domain to bind the hydrophobic groove in the non-membrane inserted portion of Bcl-X_L, which is then inactivated [28,29]. The collapse of mitochondrial transmembrane potential caused by the Bcl-X_L/Bad association occurs early in the apoptotic process, before other changes develop, such as nuclear DNA fragmentation and the exposure of phosphatidylserine (PS) on the outer surface of the cell membrane [18,19]. This early event in the commitment to apoptosis

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leads to the subsequent release of cytocrome *c* from mitochondria and activation of the intracellular apoptogenic effectors caspases [28,30].

Recently, it has been demonstrated that NPM/ALK blocks the activation of caspase-3 by preventing the cytosolic accumulation of cytocrome *c* [31,32]. In addition, transcriptional induction of Bcl-X_L is elicited by NPM/ALK through the constitutive activation of the Jak3/Stat3 pathway, protecting cells from drug-induced apoptosis [13,15].

In this report, the expression and biological relevance of Bcl-X_L as potential therapeutic target was evaluated *in vitro* and *in vivo* in NPM/ALK-transformed lymphoma cells.

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MATERIALS AND METHODS

Cell cultures and reagents. The murine pro-B Ba/F3 cells stably transformed by NPM/ALK and the SUDHL-1 and SUP-M2 cell lines established from human T-ALCL carrying the t(2;5) were a kind gift from Dr. Stephan W. Morris (St. Jude Research Hospital, Memphis, TN, USA). The t(2;5)-positive KARPAS-299 ALCL cells were purchased from DSMZ, Berlin, Germany. Cells were cultured in RPMI-1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, GibcoBRL, Paisley, UK). Parental Ba/F3 cells were maintained in RPMI-1640 supplemented with 10% FBS and 0.2% WEHI-3B-conditioned medium as a source of interleukin-3 (IL-3) as described previously [14]. All culture media contained 100 Units/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine (GibcoBRL, Paisley, UK) and the cells were incubated at 37°C with 5% CO_2 atmosphere.

LY294002 (PI-3kinase pathway inhibitor) and Herbimycin A (HA) (non-specific tyrosine kinase inhibitor) were purchased from Calbiochem, La Jolla, CA. Etoposide was from Sigma Chemical Co., St. Louis, MO. All reagents were resuspended in dimethyl-sulfoxide (DMSO, Sigma Chemical Co.) used as a solvent control in some experiments.

Plasmids and DNA transfections. The pcDNA3.1-neo expression vector (Invitrogen, Groningen, The Netherlands) bearing the human cDNA for a kinase-defective mutant of NPM/ALK, named NPM/ALK.K210R (kindly provided by Dr. Stephan W. Morris, St. Jude Research Hospital, Memphis, TN, USA) [9], was transiently transfected into Ba/F3-NPM/ALK$^+$ cells by electroporation with a Biorad Gene Pulser apparatus (Biorad Laboratories, Hercules, CA) at 270 V, 1060 μF. The human Bcl-X_L expression vector pRSV-*Bcl-X_L* was a kind gift from Dr. Fabrice Gouilleux (ICGM-INSERM,

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France). The full-length *Bcl-X_L* cDNA was then subcloned into a pEGFP vector (Clontech, Palo Alto, CA) in a reverse 3' to 5' orientation. The resulting *Bcl-X_L* antisense expression construct was checked by sequencing and restriction enzyme digestion and transiently transfected into KARPAS-299 cells using Lipofectamine2000 Transfection kit (Invitrogen, Groningen, The Netherlands) according to manufacturer's instructions. In order to enhance pEGFP-CMV promoter activity and antisense transgene expression, Karpas 299 cells were incubated with PHA-L and PMA at final concentration of 1μg/mL and 50 ng/mL, respectively, for 12 hrs following transfection. A sequence of 496 base pairs derived from *Bcl-X_L* cDNA was ligated in an antisense orientation into pTRE vector (Clontech), containing a doxycycline (Dox)-responsive promoter (pTRE.AS*Bcl-X_L*). To obtain an inducible expression of the antisense *Bcl-X_L* transgene, Ba/F3-NPM/ALK$^+$ cells stably expressing a reverse Tet-repressor (rTetR) encoded by the pTet-ON vector (Clontech) were electroporated with 30μg pTRE empty vector (pEV) or pTRE.AS*Bcl-X_L* (pAS), respectively, and 5μg pTK-Hygro vector (Clontech). Double stable Tet-cell lines (Ba/F3-N/A-pEV or Ba/F3-N/A-pAS, respectively) were selected for 3 weeks with hygromycin-B (Boehringer Mannheim, Indianapolis, IN) and individual clones were obtained by limiting dilution. Selective antisense *Bcl-X_L* transcription was confirmed by RT-PCR after addition of 1μg/mL of Dox in culture.

Antibodies and Western blot analysis. The monoclonal anti-ALK1 antibody was kindly provided by Dr. Karen Pulford (John Radcliffe Hospital, Oxford, UK). The mouse monoclonal anti-phosphotyrosine 4G10 antibody and the rabbit polyclonal total anti-Bad or anti–phosphoserine-136-Bad antibodies were purchased from Upstate

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Biotechnology Inc., Lake Placid, NY. Polyclonal antibody against Bcl-X_L (H-62) and goat polyclonal antibody anti-β-actin were from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Anti-Bcl-2 (Ab-2) rabbit polyclonal was from Oncogene Research Products, Boston, MA. Protein lysates, prepared as previously reported [14], were separated by 8-16% SDS-PAGE (Biorad, Hercules, CA) and blotted onto Immobilon-P nitrocellulose membrane (Millipore Corp., Bedford, MA). Membranes were blocked for 1 hour at room temperature in TBS-T buffer containing 5% dried milk (for anti-ALK, -Bcl-X_L, -Bad, -phophoserine-136-Bad blots) or with 2% bovine serum albumin (for 4G10 and anti-Bcl-2 blots). Primary antibody incubation times were 1 to 16 hours. Secondary incubations were for 1 hour and antibodies used were HRP-conjugated anti-mouse or anti-rabbit (Amersham, Arlington, Heights, IL). Proteins were visualized by chemiluminescence as recommended by the manufacturer (Super Signal, Pierce, Rockford, IL). Relative protein levels of Bcl-X_L were quantified by immunoblotting and densitometric analysis of films was carried out on an Eagle Eye II Photodensitometer (Stratagene, La Jolla, CA).

Preparation and analysis of mitochondria extracts. The isolation of mitochondria was adapted from a previously described method [33]**.** Briefly, cells washed twice with ice-cold PBS were resuspended in a cold hypotonic buffer (10mM KCl; 1 mM $MgCl_2$; 10% glycerol; 0.5 mM dithiothreitol (DTT); 1mM phenylmethylsulfonyl-fluoride (PMSF); 15 mg/ml aprotinin; 3 mg/ml leupeptin/pepstatin; 2mM Na_3V0_4; 0.2% Nonidet P-40 (NP-40) with 20mM HEPES, pH 7.9) and homogenized by using a Dounce homogenizer. The homogenate was mixed with an iso-osmotic MSE buffer (210mM mannitol; 70 mM sucrose; 5mM Tris-HCl and 1mM ethylenediaminetetraacetic acid [EDTA] pH 7.5) and centrifuged at 750*g* for 20

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minutes at 4°C to pellet nuclei and unbroken cells. The pellet containing the mitochondria was resuspended in the MSE buffer and stored at –80°C. Protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL) and 100μg of protein was immunoprecipitated for 16 hours with 4μg of anti-Bcl-X_L polyclonal antibody (H-62). Bcl-X_L-immunocomplexes were captured with 60μg of protein A-Sepharose beads (Pharmacia Biotech, St. Alban, UK) for 4 hours at 4°C, separated on 16% SDS-PAGE and analysed by western blotting as described above.

Mitochondrial permeability transition detection. Changes in the mitochondrial transmembrane potential (MTP) were detected using a fluorescent cationic dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzamidazolocarbocyanin iodide, commonly known as JC-1-fluorochrome (BIOMOL Research Laboratories Inc., PA). Inside healthy cells the lipophilic positively charged JC-1 dye aggregates in mitochondria and under fluorescescent light emits a red signal (J-aggregates emit at 590 nm). Upon reduction in MTP, the JC-1 fluorochrome converts to a monomeric form which fluoresces green (monomeric dye emits at 490 nm). Cells were incubated with 2μg/ml JC-1 in media for 30 min at 37°C. All stained samples were analyzed by using a FACScalibur and Cell-quest software (Becton Dickinson, San Josè, CA).

Treatment with Bcl-X_L antisense oligonucleotides. The optimal conditions for Bcl-X_L protein depletion was established previously [34]. NPM/ALK positive Ba/F3 cells were incubated for 3 days in the presence of either sense (S), antisense (AS) or nonsense (NS) oligodeoxynucleotides (ODNs) targeting the coding region 5' to the translation initiation codon site for the *Bcl-X_L* gene (100% sequence identity between mouse and human) and extending 3' downstream for a total of 18 bases. The DNA oligonucleotides with a phosphorothioated backbone were synthesized and purified by

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HPLC (MWG-Biotech, AG). The antisense AS-ODN sequence used was 5'-CCGGTTGCTCTGAGACAT-3'. Two additional control ODNs were used: (A) a complementary sequence to the antisense in the reverse 5' to 3' order, (S: 5'-ATGTCTCAGAGCAACCGG-3'), (B) a missense sequence containing a 4-base mismatched as compared with the antisense sequence (NS: 5'-CCGGTTGCT**G**T**C**A**C**A**G**AT-3'). The lyophilised ODNs were reconstituted in sterile distilled water to 100 mM, filter-sterilized and stored in aliquots at –20°C as stock solutions. Fresh oligonucleotides were added at 0, 16, 24, 32, 48 and 60 hours.

Cell viability and apoptosis assays. Apoptosis was assessed by several criteria.

The percentage of apoptotic cells was determined by enumerating cells undergoing a reduction of cell volume with chromatin condensation or micronuclear fragmentation in a total of at least 200 cells using the intercalating fluorescent DNA binding dye, acridine orange (AO, Sigma Chemical Co.). Cells were stained with 5μM AO dissolved in PBS and examined using a Zeiss fluorescence microscope.

DNA degradation was further assessed by measuring the DNA content of individual cells by flow cytometry. Cells were washed with ice-cold PBS and fixed in 70% cold ethanol. Samples were then treated with Dnase-free Rnase (Boehringer Mannheim, Indianapolis, IN) and stained with 10μg/ml propidium iodide (Sigma Chemicals Co., St. Louis, MO). Apoptosis was also detected by Annexin V-FITC binding using the Apoptosis Detection Kit purchased from Bender MedSystems Diagnostic GmbH, Austria. All samples were prepared following manufacturer's instructions and analyzed using FACScalibur and Cell-quest software (Becton Dickinson, San Josè, CA).

Animals and treatments. Female CD-1 nu/nu mice (7-9 weeks old) were supplied by Charles River (Calco, Como, Italy) and kept under standard laboratory conditions

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according to the guidelines of our Institute. Animal studies were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori, Milan, Italy. Mice were implanted with Ba/F3 cells transformed by NPM/ALK and transfected with the inducible antisense *Bcl-X_L* transgene (Ba/F3-N/A-pAS) or bearing an empty vector as control (Ba/F3-N/A-pEV) either intravenously ($10x10^6$ cells/mouse) or subcutaneously ($20x10^6$ cells/mouse). Therapeutic efficiency of the pAS vector was assessed by the addition of doxycycline hydrochloride in the drinking water (5% sucrose, 0.2 mg Dox/mL) which was changed twice a week. Mice were monitored every other day for the appearance of tumors and for signs of disease (weight loss, adenopathies). To analyze *in vivo* Bcl-X_L protein levels, subcutaneous tumor-bearing mice were killed and non-necrotic tumor tissue was extracted and homogenized in a fivefold volume of sodium dodecyl sulphate (SDS)-loading buffer (50 mM Tris-HCl [pH6.8], 2% SDS, and 5% β-mercaptoethanol). After sonication for 3 minutes, samples were centrifugated at 15,000*g* for 15 minutes, heated at 95°C for 10 minutes and analyzed in western blot as described above.

Statistical analysis. Statistical analysis was performed with Student's test by use the GraphPad software analysis program (Prism, San Diego, CA). P values of less 0.05 were considered to be statistically significant and were derived from two-sided statistical tests. All data are presented as the mean (95% confidence interval [CI]). CIs are displayed when they exceed 10% of the respective mean. Correlation Coefficient (CC) was calculated using the Pearson method.

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RESULTS

The Bcl-2 family members in ALK$^+$ ALCL lymphoid cells.

Bcl-2 family members are well known cell death regulators [18]. Bcl-2 and Bcl-X_L protect cells from apoptosis whereas Bad increases susceptibility to cell death [20,23]. The expression levels of Bcl-X_L and of Bcl-2 were evaluated in human NPM/ALK$^+$ ALCL-derived cells carrying the t(2;5) translocation and in pro-B murine Ba/F3 transformed by NPM/ALK (Ba/F3-N/A) [9,12]. Analysis on total lysates from Ba/F3 cells showed that NPM/ALK promotes an IL-3-independent Bcl-X_L protein induction of approximately 2,2 folds (as measured by band densitometry) (Figure 1, lane C), compared to parental cells starved of IL-3 for 18 hours (Figure 1, lane A). Bcl-X_L expression is induced in parental Ba/F3 cells after stimulation with the growth-factor IL-3, as previously reported by Dumon *et al.* [35], (Figure 1, lane B compared to lane A). In three ALK$^+$ ALCL-derived cell lines Bcl-X_L protein is detected at levels higher than in Ba/F3-N/A$^+$ cells. A direct relationship between Bcl-X_L expression levels and the intensity of NPM/ALK bands appears to be present. (Figure 1, lanes C-F). The basal level of Bcl-2 in parental Ba/F3 cells remains low and unchanged after ectopic overexpression of NPM/ALK or IL-3 stimulation. These Bcl-2 levels are comparable to those detected in ALCL ALK$^+$ cells, suggesting that NPM/ALK does not markedly affect Bcl-2 levels in these cells. The hybridization of the same filter with an anti-total Bad rabbit polyclonal antibody reveals that Bad migrates as a doublet of approximately 26 kDa, as previously described [24-25]. It has been proposed that these two distinct forms of Bad could reflect a hyperphosphorylated (pp-Bad) and hypophosphorylated (p-Bad)

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status of the protein which can be modified at multiple serine residues [25]. In Ba/F3 parental cells, two hours following withdrawal of IL-3, the hyperphosphorylated Bad was eliminated (Figure 1B, lane A), whereas 30 min readdition of IL-3, restored the slower-migrating form of Bad (Figure 1B, lane B). The serine 136 has been identified as the major site of Bad phosphorylation by AKT [23,26]. Treatment with an irreversible inhibitor of PI-3kinase, LY294002, in presence of IL-3, impaired AKT activation at serine 473 and phosphorylation of Bad at serine-136, as indicated by using two phospho-site specific antibodies (Figure 1B, lane C). Likewise, treatment of Ba/F3-N/A$^+$ cells and ALK$^+$ ALCL Karpas 299 (K-299) with LY294002 inhibited AKT and Bad phosphorylation and decreased but not completely abolished the hyperphosphorylated form of Bad.

It can be concluded that NPM/ALK, when expressed in ALCL cells as a result of the t(2;5) chromosomal translocation or after transfection of *NPM/ALK* cDNA into Ba/F3 cells is able to upregulate Bcl-X_L (but not Bcl-2), and to promote an hyperphosphorylation of Bad which can be partially modulated by PI-3kinase/AKT signaling.

Inhibition of NPM/ALK tyrosine kinase activity induces apoptosis via modulation of the Bcl-X_L/Bad death effectors.

The tyrosine kinase activity of NPM/ALK is essential to elicit growth-factor independent proliferation and resistance to apoptosis in Ba/F3 cells. In fact, it is known that a kinase-defective mutant of NPM/ALK, named N/A.K210R, characterized by a point mutation in the critical ATP-binding site (K210R) fails to induce a transformed phenotype in Ba/F3 cells after IL-3 withdrawal [9]. We used this construct as a dominant

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negative inhibitor of ALK kinase activity and determined its effect on Bcl-X_L expression and survival in NPM/ALK-transformed cells. The N/A.K210R mutant was transiently overexpressed in Ba/F3-N/A cells. 48 hours after transfection anti-ALK (ALK) and anti-phosphotyrosine (PY) immunoblots were performed to evaluate the expression and activation status of NPM/ALK (Figure 2A, *upper panel*). Decreased tyrosine phosphorylation of NPM/ALK, as well as of several other proteins was observed in N/A.K210R transfectants confirming the inhibitory action of N/A.K210R on wilde-type NPM/ALK. The transient transfection of N/A.K210R also caused a substantial downregulation of Bcl-X_L expression but did not change the Bcl-2 protein levels (Figure 2A, *lower panel*). In addition, N/A.K210R failed to promote Bad phosphorylation on serine 136 without affecting its total expression levels (Figure 2A, *lower panel*). Upon Bad dephosphorylation, Bcl-X_L/Bad dimerization plays a significant role in promoting apoptosis by inactivating mitochondrial Bcl-X_L [28,29]. Correlating with the inactivation of Bad and downregulation of Bcl-X_L in Ba/F3-N/A.K210R-expressing cells, an increase in the sub-G_1 peak, indicative of apoptotic cells with subdiploid DNA (42,4%) was detected in these cells compared to control vector Ba/F3-N/A.EV transfectants (5,2%) (Figure 2B). In addition, morphological changes related to the exposure of phosphatidylserine (PS) on the outer surface of Ba/F3-N/A.K210R-transfected cells undergoing apoptosis (7.8% + 29,2%) were detected by using an annexinV-propidium iodide staining (Figure 2C).

Treatment of ALK^+ ALCLs with LY294002 [11,12] as well as the non-specific tyrosine kinase inhibitor Herbimycin A (HA) [32,36] has been shown to promote induction of apoptosis. Therefore, we asked whether Bad dephosphorylation on serine 136, upon

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PI-3kinase/AKT pathway inactivation, was important to promote Bcl-X_L/Bad heterodimerization altering the mitochondria homeostasis (Figure 3). Mitochondrial extracts from Karpas 299 cells untreated (NT) or incubated for 8 hours with vehicle alone (DMSO), 10μM HA or 25μM LY294002 were immunoprecipitated using the anti-Bcl-X_L antibody and immunoblotted with anti-Bad and anti-Bcl-X_L antibodies. Both proteins were detected in samples treated with HA, and to a higher extent, with LY294002, whereas in untreated and vehicle alone samples only Bcl-X_L was observed (Figure 3A). Western analysis on total lysates confirmed that Bad, mainly in its hypophosphorylated form, was coimmunoprecipitated with an anti-Bcl-X_L antibody upon LY294002 treatment, while in DMSO-treated cells Bad remained detectable as a doublet in the supernatant depleted of Bcl-X_L complexes (Figure 3A). This finding confirmed that a specific inhibition of PI-3kinase activity appears to dictate subcellular localization of Bad and its capacity to associate with Bcl-X_L. As a consequence of Bcl-X_L/Bad heterodimerization, an early event in the commitment to cell death is mitochondrial membrane depolarization, which occurs as a result of the opening of permeability transition pores [21,28,37]. We therefore analyzed the collapse of the mitochondrial transmembrane potential ($\Delta\Phi m$) in KARPAS-299 cells after exposure to LY294002 for 8 hours using the JC-1 dye (Figure 3B). Blocking the PI-3kinase/AKT pathway caused cells to fall into the R5 region, indicating a loss of mitochondrial homeostasis as JC-1 converts from the red-aggregated form to the green-monomeric form.

These data indicates that NPM/ALK blocks the onset of apoptosis by preventing Bcl-X_L/Bad heterodimerization and associated pre-apoptotic mitochondrial

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perturbations. Therefore, NPM/ALK can deliver its antiapoptotic signal through modulation of the expression levels of Bcl-X_L and of its active/inactive conformational states via PI-3kinase/AKT/Bad signaling pathway.

Inhibition of Bcl-X_L induces apoptosis in NPM/ALK-transformed lymphoid cells *in vitro*.

The above reported results provide evidence for the hypothesis that NPM/ALK expression protects cells from cell death through the Bcl-X_L pathway.

Since antisense ODNs represent a potent approach to downmodulate the expression of specific genes [38], we employed antisense strategies targeted to Bcl-X_L in order to biologically validate this death regulator as a potential therapeutic target in NPM/ALK$^+$ malignancies. Ba/F3-NPM/ALK$^+$ cells were treated for 72 hours with increasing concentrations of antisense *Bcl-X_L* ODN corresponding to the AUG containing region of *Bcl-X_L* mRNA. A dose-dependent inhibition of Bcl-X_L protein levels was observed with an almost complete inhibition at 100µM AS-ODN (Figure 4A). There was no effect of control ODNs representing sense (S) or nonsense scrambled (NS, four base mismatched) ODNs on Bcl-X_L expression. The two control oligonucleotides had some non-specific effects on cell survival at 50µM and 100µM (data not shown). To avoid such non-specific toxic effects all subsequent experiments have been performed with 30µM ODN. A time course experiment showed downregulation of Bcl-X_L protein expression after 60 hours of incubation with *Bcl-X_L* antisense ODN (Figure 4B). This slightly preceded the massive cell death induction in Ba/F3-NPM/ALK$^+$ cells at 96

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hours (Figure 4C). A considerable fraction of NPM/ALK-expressing cells incubated with *Bcl-X_L* antisense ODN were found in either early (17,6%) or late (54,7%) stages of apoptosis, whereas control ODNs failed to modify the baseline proportion of apoptotic cells compared to the untreated cultures (NT). Figure 4D shows that treatment with Bcl-X_L antisense makes cells more sensitive to killing by etoposide, an antineoplastic drug currently used in the treatment of ALCL. When Ba/F3-N/A^+ cells incubated with a non-sense oligonucleotide or no oligo were treated with etoposide, a dose-dependent induction of apoptosis occurred with 25% dead cells at 10μM drug concentration. In contrast the combination of the Bcl-X_L antisense oligonucleotide and 10μM etoposide resulted in over 40% cell death.

To determine the effect of Bcl-X_L downregulation also in ALK^+ ALCL cells, KARPAS-299 were transiently transfected with a plasmid encoding full-length *Bcl-X_L* antisense cDNA (AS*Bcl-X_L)*. Figure 5A shows a time-dependent inhibition of Bcl-X_L expression with a maximal effect at 96 hours after transfection. The percentage of apoptotic cells was analyzed by an annexin V and propidium iodide staining. Figure 5B shows that downregulation of Bcl-X_L caused approximately 40% of the cell population (13,2% + 26,6%) to undergo cell death (Figure 5B, *left panels*).

Furthermore, Bcl-X_L downregulation was also associated with an increased susceptibility of cells to the genotoxic compound etoposide (Figure 5B, *right panels*) while vehicle alone had little effect (Figure 5B, *middle panels*), suggesting a synergistic effect of DNA damage and Bcl-X_L downregulation on cell death induction.

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Conditional suppression of Bcl-X_L expression impairs the tumorigenic activity of NPM/ALK *in vivo.*

The doxycycline (Dox)-inducible reverse tetracycline transactivator (rtTA) is frequently used for manipulations of transcription levels in a temporally regulated fashion *in vivo* [39]. Using a "pTet-ON" antisense system, murine Ba/F3-NPM/ALK$^+$ cells were stably transfected with a plasmid encoding a *Bcl-X_L* antisense sequence under the control of a doxycycline (Dox)-responsive promoter (Ba/F3-N/A.pAS). After 96 hours of Dox treatment (1μg/mL) *in vitro*, Ba/F3-N/A.pAS (N/A-pAS) cells were found to have <10% of the initial steady-state level of Bcl-X_L (Figure 6A) while the NPM/ALK expression levels were unaffected by the presence of Dox. At the same time cell viability was significantly decreased (approximately 90% apoptotic cells) as indicated by acridine orange staining, while Ba/F3-NPM/ALK$^+$ cells transfected with an empty vector control (N/A-pEV) did not show any cell death induction in presence of Dox (Figure 6B).

Based on these *in vitro* results, we examined the ability of Dox-driven *Bcl-X_L* antisense transcription to inhibit the tumorigenic potential of NPM/ALK-transformed Ba/F3 cells *in vivo.* $10x10^6$ Ba/F3-NPM/ALK cells bearing a Dox-inducible empty vector (N/A-pEV) or a *Bcl-X_L* antisense (N/A-pAS) transcript were inoculated intravenously into nude mice (Figure 7A). Half of the mice from each group was maintained under continuous administration of Dox (0.2 mg/mL) in the drinking water. The N/A-pEV and N/A-pAS cell lines induced a fatal disease in 7/8 (white squares) and in 15/15 (black squares) control animals not receiving Dox, respectively, within a similar time frame (4-8 weeks). The animals showed weight loss, poor fur status and inguinal/axillary adenopathies. Macroscopic examination of dead animals showed

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massive paraaortic adenopathies and splenomegaly (*data not shown*). Doxycycline administration prevented the occurrence of this wasting syndrome in 15/15 mice injected with N/A-pAS cells (black circles), while it did not affect the appearance and growth of NPM/ALK$^+$ tumors in the control group (N/A-pEV) (white circles). The inhibitory effect of Dox lasted throughout the 2 months of its administration and was also stable after withdrawal of Dox for a further 2 months. Cells (N/A-pEV and N/A pAS) were also injected subcutaneously, producing a local growth within 8 days (*data not shown*). In Table 1, Dox administration for 21 days (starting 24 hours after tumor cell injections) prevented the appearance of tumors in 9/12 mice inoculated with N/A pAS cells, with no effect on tumor growth in mice injected with N/A-pEV cells. To further document the *in vivo* downregulation of Bcl-X_L by Dox, animals bearing measurable subcutaneous N/A-pEV and N/A-pAS tumors were also treated with Dox. Figure 7B shows that Dox administration for 3 days reduced the expression of Bcl-X_L in two N/A-pAS regressing tumors without effects in control (N/A-pEV) treated animals. NPM/ALK protein levels are used as loading controls and did not show substantial variations.

These findings reveal a crucial role for Bcl-X_L in mediating the *in vivo* transforming potential of NPM/ALK.

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DISCUSSION

Protection from cell death after cytokine withdrawal or exposure to conventional anticancer drugs has been reported in human NPM/ALK$^+$ ALCLs [9,31]. In particular, NPM/ALK exerts its antiapoptotic effect delaying cytosolic accumulation of cytocrome *c* and activation of caspase-3 [31,32]. These findings support a mitochondrial control of the apoptogenic proteolytic cascade, although the involvement of Bcl-2 family members in ALK$^+$ ALCL remains unclear.

In the present report we provide direct evidence of a functional link between Bcl-X_L and the survival potential of NPM/ALK. Several antisense strategies have been employed to directly validate Bcl-X_L as a crucial cell fate regulator associated to the transforming ability of NPM/ALK *in vitro* and *in vivo*. Tyrosine kinase activity of NPM/ALK is required to upregulate Bcl-X_L protein levels in pro-B murine Ba/F3 cells as well as in human ALK$^+$ ALCL-derived cells, apparently without effects on Bcl-2 expression. The immunohistochemically undetectable Bcl-2 expression in human ALK-positive ALCLs has been recently correlated with the favourable clinical outcome of these lymphomas compared to ALK-negative ALCLs [40,41]. However, the expression of Bcl-X_L [13,15] and myeloid cell-leukemia-1 (Mcl-1) antiapoptotic protein [42], another Bcl-2-like member, could represent important prognostic factors for those ALK$^+$ ALCL patients refractory to the current highly toxic conventional therapy [43]. Our findings in human ALK$^+$ ALCL-derived cell lines indicate that Bcl-X_L may provide alternative mechanisms resulting in prolonged tumor cell survival. The involvement of Bcl-X_L in protecting human Karpas 299 cells from apoptosis-inducing agents was investigated *in*

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vitro showing that Bcl-X_L downregulation significantly increased their susceptibility to etoposide. In addition, upregulation of Bcl-X_L in murine Ba/F3 cells transformed by several hybrid oncogenes, including NPM/ALK, has been recently proposed to work in concert with the overexpression of RAD51 to delay the G_2/M phase transition of the cell-cycle, thereby promoting drug resistance [44]. Therefore, these findings support the possibility that inappropriate Bcl-X_L expression or its functional modulation may represent an important indicator of the outcome of disease treatment and also has important therapeutic implications in NPM/ALK-mediated oncogenesis. In fact, a few resistant NPM/ALK-expressing cells may be selected during remission by cytotoxic drug, since a third of "ALKoma" patients experience multiple relapses [45,46].

Bcl-X_L has been identified to be involved in the neoplastic transformation caused by several oncogenes such as BCR/ABL [47,48]. Increased expression of Bcl-X_L has been reported in AML and multiple myeloma patients with poor clinical response [49,50]. The Jak/Stat and PI-3kinase/AKT activation pathways have been involved in Bcl-X_L regulation in multiple hematopoietic cell lineages [51,52]. Two recent reports using cell lines derived from ALK^+ ALCLs, have shown that Bcl-X_L, but not Mcl-1 or survivin, can be downmodulated by treatment with the Jak/Stat inhibitor AG490 [13,15]. In particular, Bcl-X_L expression rate seems to be strictly related to the NPM/ALK-induced high-level of STAT3 protein as well as its constitutive activation in ALK^+ ALCLs [13].

We also investigated the possibility that post-translational mechanisms, other than transcriptional induction of Bcl-X_L, might influence the balance of competing death/life of ALK^+ ALCLs toward survival.

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In our experiments, we were unable to detect any coimmunoprecipitation with NPM/ALK and phosphorylated form of Bcl-X_L (data not shown), as instead previously described for Bcl-2 [53]. In contrast, the Bcl-X_L-antagonist Bad has been found to be primarily regulated by phosphorylation. A PI-3kinase/AKT-mediated phosphorylation of Bad on serine 136 is required for coupling NPM/ALK-delivered survival signals to the mitochondrial death-machinery. In fact, LY294002-induced Bad dephosphorylation was able to promote association of Bcl-X_L/Bad heterodimers triggering the onset of commitment to apoptosis as indicated by the sudden mitochondrial membrane depolarization. Other than AKT serine/threonine kinase [23,26], the reversible phosphorylation of Bad can be modulated by survival-promoting kinases including Ras-induced MAP kinases [22], p21-activated kinase 1 [54], PKA [55] as well as phosphatases, such as PP1 [56] and a PP2a-like enzyme [57]. This could explain the slower mobility form of Bad which does not appear to be influenced by inhibition of PI-3/AKT kinases in ALK^+ ALCL cells. However, further studies are warranted to clarify the biological implications of these pathways in terms of NPM/ALK-enhanced survival and regulation of Bcl-2 family proteins.

The significant *in vitro* and *in vivo* apoptotic effects of Bcl-X_L downregulation in different cell types including human ALK^+ ALCL-derived cell lines highlight another important point: the selection of therapeutic targets downstream of known oncogenes. It is known that NPM/ALK activates several signal transduction pathways, therefore it could be hypothesized that the downregulation of a single protein (or its inhibition) could be easily bypassed by activation of parallel pathways. The data presented here indicate that in the context of NPM/ALK-mediated oncogenicity the

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presence of Bcl-X_L remains crucial and accounts for most of the survival potential of neoplastic cells. This point is of general importance because primary oncogenes are not always easy targets for therapies, especially when they do not code for an enzyme.

It is important to note in this regard, that there is presently considerable interest in discovery of small-molecule inhibitors targeted at the BH3-binding hydrophobic groove of Bcl-X_L, which can antagonize its pore-forming activity [58,59] and protein-protein interactions [60]. These attempts are based on the hypothesis that Bcl-X_L downregulation in normal cells will not result in significant toxicity, an important aspect that was not investigated here and will require further analysis.

In conclusion, our results provide evidence that Bcl-X_L is positively regulated by NPM/ALK in human lymphoid cells and could represent a relevant target for a new generation of therapeutics for the treatment of ALK^+ ALCLs. By altering the ratio of pro-survival and pro-apoptotic proteins through Bcl-X_L downregulation or inhibition, it may still be possible to antagonize the pleiotropic antiapoptotic cascades activated by NPM/ALK in lymphoma cells.

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LEGENDS TO FIGURES.

Figure 1. Expression and activation status of Bcl-2 family members in NPM/ALK-expressing cells. A. Expression and tyrosine-phosphorylation of NPM-ALK were evaluated with an anti-ALK monoclonal and anti-phosphotyrosine (PY)antibodies in total lysates from pro-B Ba/F3 parental cells cultured in absence or presence of IL-3 for 18 hours (lane A and B), in NPM/ALK-expressing cell lines including Ba/F3 cells transfected with NPM/ALK (Ba/F3-N/A, lane C) and the human ALCL-derived cell lines SUP-M2 (lane D), KARPAS-299 (lane E) and SUDHL-1 (lane F). Expression levels of Bcl-X_L, Bcl-2, and Bad were also assessed. B. Ba/F3 parental cells were starved without IL-3 for 2 hours (lane A) and then stimulated with IL-3 in absence (lane B) or presence of 25μM 294002 for 30 min (lane C). Ba/F3-N/A^+ and Karpas-299 ALCL cells were incubated with the same drug concentration (lane E and G) or vehicle alone (lane D and F) for 30 min. Expression and activation status of AKT and Bad were evaluated from total lysates with anti-total AKT and Bad polyclonal antibodies as well as by using anti-phosphoserine specific antibodies (phospho-Ser473 for AKT and phospho-Ser136 for Bad).

Figure 2. Tyrosine kinase activity of NPM/ALK upregulates Bcl-X_L and promotes Bad phosphorylation. Ba/F3-NPM/ALK^+ cells were transiently transfected with NPM/ALK kinase defective mutant (N/A.K210R), an empty control vector (N/A.EV) or electroporated in the absence of DNA (Ba/F3.N/A). **A.** At 48 hours from transfection total lysates were separated by 7.5% SDS-PAGE and analyzed by immunoblotting with anti-ALK (ALK) and anti-phosphotyrosine (PY) monoclonal antibodies (*upper panel).* The same cellular lysates were also separated by 12% SDS-PAGE and analyzed by

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immunoblotting with antibodies specifically recognizing total Bcl-X_L, Bcl-2, Bad and a phosphoserine-136 (p136-Bad) residue of Bad (*lower panel).* Levels of β-actin were reported as a protein loading control (*lower panel).* **B.** Cell-cycle distribution of the transfected cells was assessed 48 hours after transfection by propidium iodide-staining and flow cytometric analysis. The sub-G_1 peak is indicative of the presence of apoptotic cells with subdiploid DNA content. **C.** Occurrence of apoptosis was also performed by a propidium iodide/annexin V-staining at 48 hours after transfection.

Figure 3. Inhibition of NPM/ALK signaling promotes Bcl-X_L/Bad heterodimerization and disruption of mitochondrial homeostasis. A. KARPAS-299 cells were incubated with vehicle alone (DMSO), Herbimycin A (10μM), LY294002 (25μM) or left untreated (NT) for 8 hours. Mitochondrial extracts (*left panels*) and total lysates (*right panels*) were immunoprecipitated by using an anti-Bcl-X_L polyclonal antibody. Supernatant (IP:sup) depleted of Bcl-X_L complexes and immunoprecipitates (IP: Bcl-X_L) were analyzed by western blot with anti-Bcl-X_L and Bad antibodies **B.** Disruption of mitochondrial transmembrane potential (ΔΦm) was assessed after an 8 hour incubation with LY294002 (25 μM) or the equivalent amount of DMSO by FACS analysis of JC-1 dye-stained cells. Apoptotic cells fall into the R5 region *(right panel*) showing a reduced red FL2-H-fluorescence; R5 = 2.1%, in the control sample (*upper right panel)* and 47.6% in the LY294002 treated sample (*lower right panel).* The left panels show cells plotted as forward scatter (FSC) against side scatter (SSC) showing no substantial alteration in cell morphology at this early stage of apoptosis induction.

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Figure 4. Specific antisense oligonucleotide-mediated downregulation of Bcl-X_L increased apoptosis of NPM/ALK$^+$ cells *in vitro*. A. Ba/F3-NPM/ALK$^+$ cells were incubated for 72 hours with increasing concentrations of either sense (*S*), nonsense scrambled (*NS*) or antisense (*AS*) phosphorothioated oligonucleotides (ODNs) targeting the *Bcl-X_L* AUG region. Fresh oligonucleotides were added at 0, 16, 24, 32, 48 and 60 hours. Western blot analysis was performed 72 hours after treatment. Blots were reprobed with an anti-β-actin specific antibody as control for protein loading. **B.** Total cell lysates from Ba/F3-NPM/ALK$^+$ cells treated with 30μM *Bcl-X_L* AS-ODN for 48, 52 and 60 hours were compared with those of cultures incubated with sense (S) and nonsense scrambled (NS) ODNs for 60 hours for Bcl-X_L expression by immunoblotting. **C.** The fraction of apoptotic cells was evaluated by annexin V/ propidium iodide staining and flow cytometric analysis after 96 hours of treatment with ODNs or in the untreated control (NT). The values indicate the percentages of cells in early (*lower right quadrant*) and late apoptosis (*upper right quadrant*). **D.** Ba/F3-N/A$^+$ cells treated for 48 h with Bcl-X_L antisense, control nonsense scrambled or no oligo were incubated with increasing concentration of etoposide for additional 18 hours. Apoptosis was evaluated by AO staining at the end of drug incubation, the data are represented as the mean +/- standard deviation (S.D.) of three independent experiments.

Figure 5. Effect of Bcl-X_L downregulation on cell survival in KARPAS-299 cells. KARPAS-299 cells were transiently transfected with a pEGFP control vector (*left panels*) or AS*Bcl-X_L*-GFP plasmid (*right panels*) encoding full-length *Bcl-X_L* antisense cDNA. Cultures containing at least 60% of transfected cells were used for further

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analysis by western blot and apoptosis determination. **A.** Western blot analysis of total lysates for the expression of Bcl-X_L was performed at the indicated time points after transfection. NPM/ALK levels are presented as a protein loading control. **B.** At 80 hours after transfection, cultures were treated for 16 hours with DMSO (*middle panels*), 10μM etoposide (*right panels*) or left untreated (*left panels*). Apoptosis induction was assessed using an Annexin V binding assay and flow cytometric analysis.

Figure 6. Dox-inducible downregulation of Bcl-X_L in stable pTet-ON transfectants *in vitro*. A. Western blot analysis of Bcl-X_L expression in a representative Ba/F3-NPM/ALK$^+$ clone (N/A-pAS) cultured in absence/presence of a single dose of doxycycline (Dox 1μg/mL) for the indicated times. **B.** A Ba/F3-N/A.AS*Bcl-X_L* antisense inducible clone (N/A-pAS) and a Ba/F3-NPM/ALK$^+$ clone bearing an empty vector (N/A-pEV) were grown in triplicate in the absence (-Dox) or presence of doxycycline (+Dox) for 4 days. A single dose of doxycycline (1μg/mL) was added in the culture medium at the beginning of the experiment and replaced after 2 days. Apoptosis was evaluated by AO staining as reported in figure 4D.

Figure 7. Impaired growth of NPM/ALK-expressing cells *in vivo* after Bcl-X_L downregulation. A. $10x10^6$ Ba/F3-NPM/ALK$^+$ cells bearing an empty vector control (N/A-pEV, white squares) and a Dox-inducible AS*Bcl-X_L* antisense transgene (N/A-pAS, white circles), were injected intravenously into nude mice. Half of the mice from each group (N/A-pEV, black squares; N/A-pAS, black circles) was maintained under continuous administration of doxycycline (0.2 mg/mL) in the drinking water. Survival

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of the animals was monitored every other day. **B.** Animals injected with N/A-pEV and N/A-pAS cells and bearing measurable nodules (mean tumor weight was 235mg; 95% confidence interval [CI]= 200-320mg) were treated with Dox (0.2 mg/mL) in the drinking water. After 3 days of Dox treatment, mice were killed and tumors extracted and analyzed in western blot with anti- Bcl-X_L and anti-ALK antibodies.

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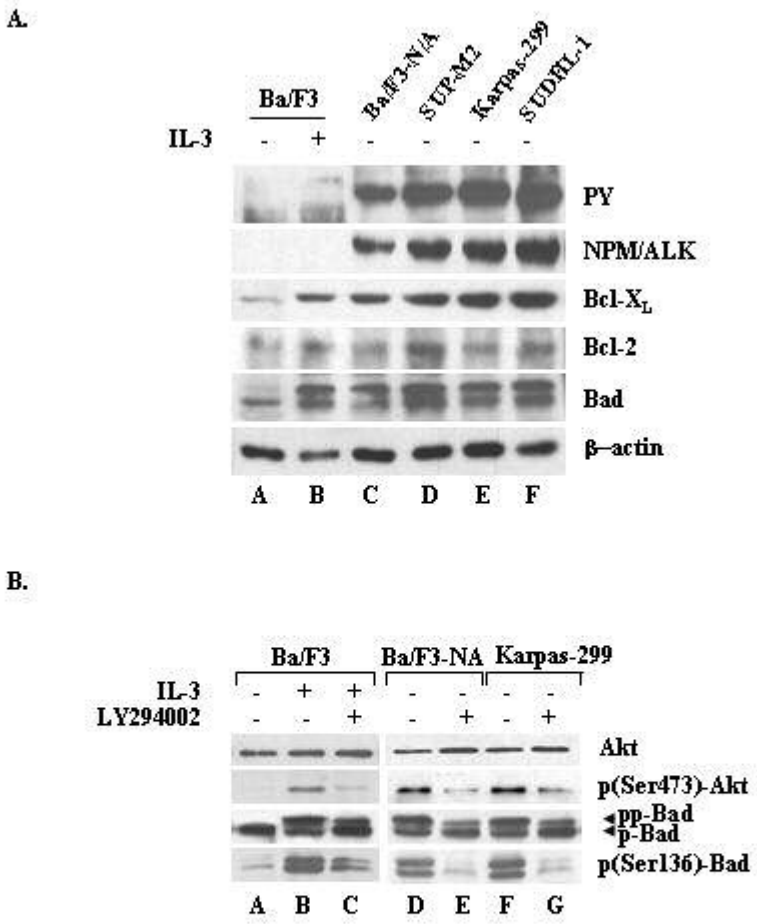

Coluccia et al.;
Figure 1

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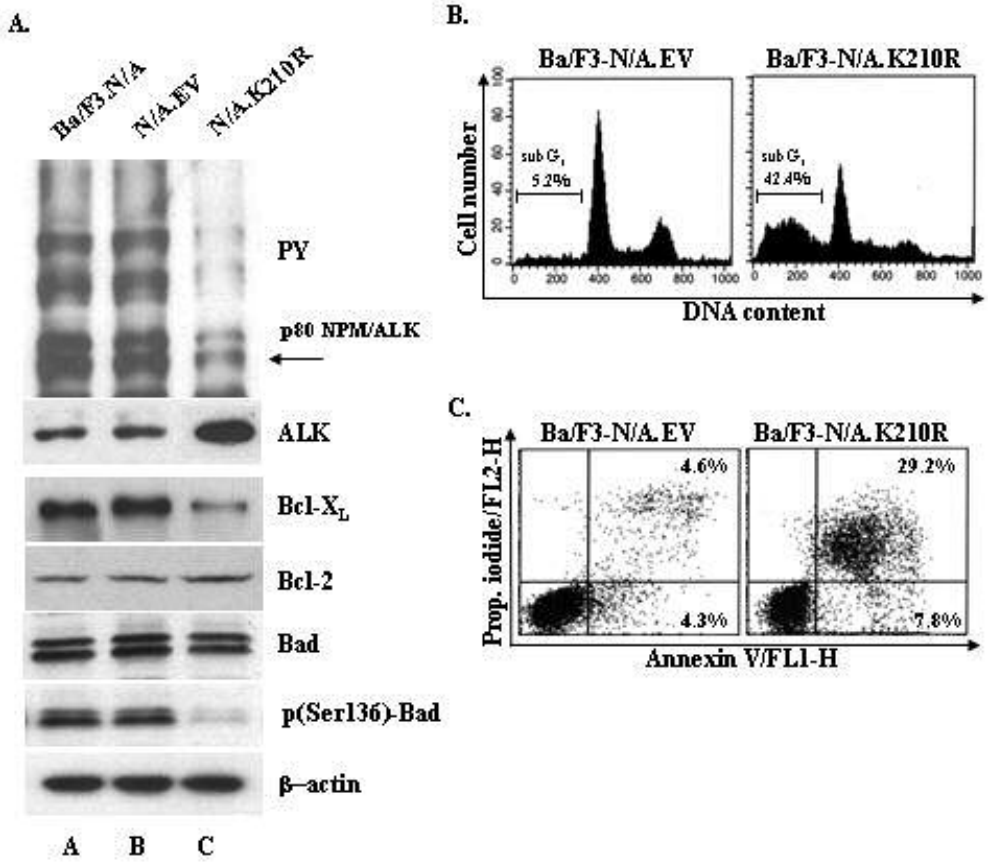

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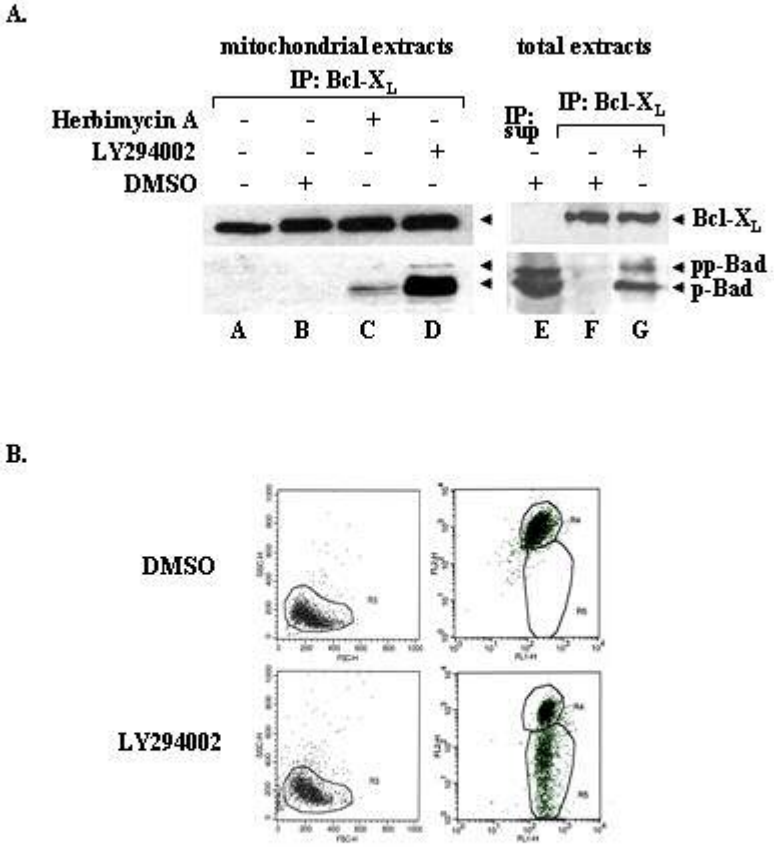

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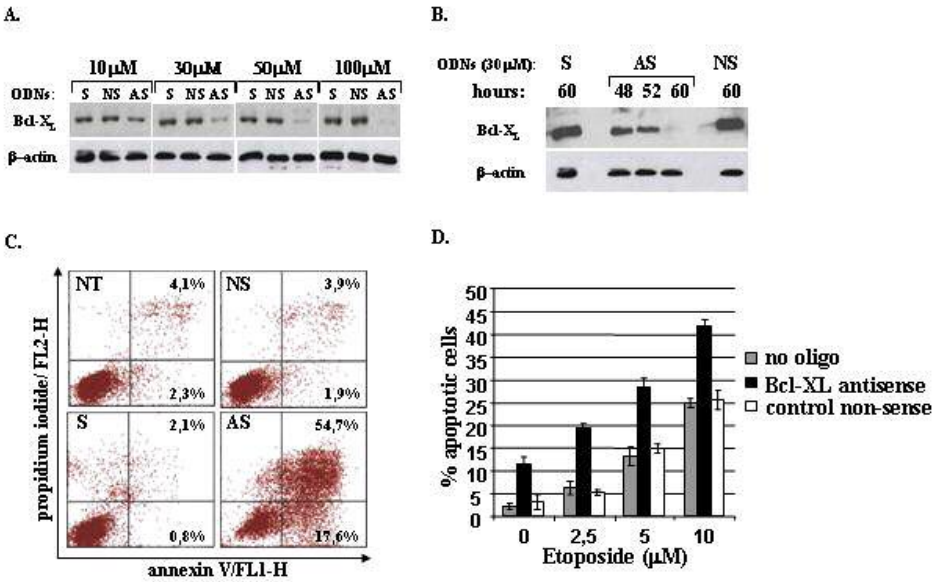

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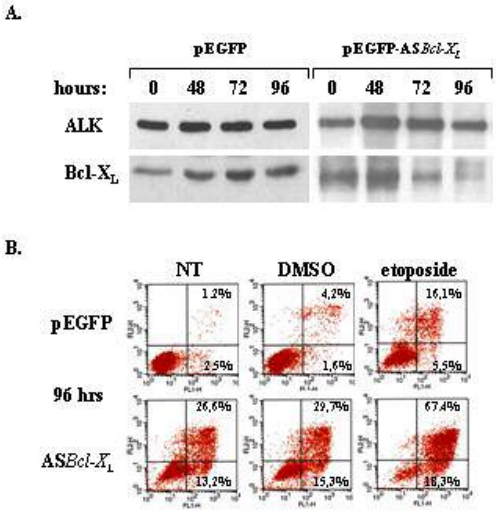

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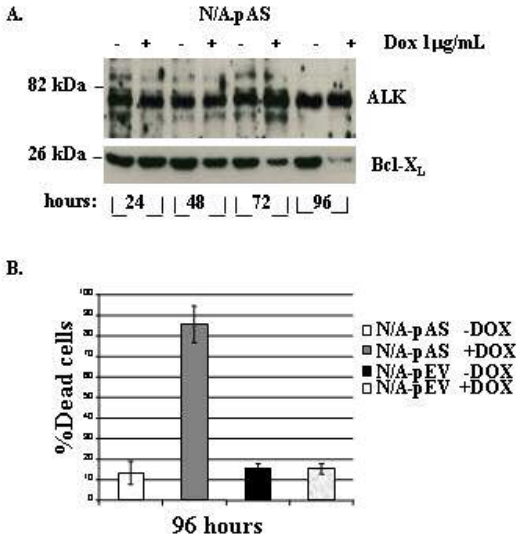

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Figure 6

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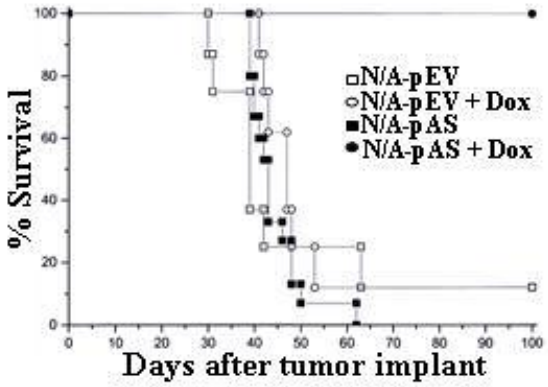
A.



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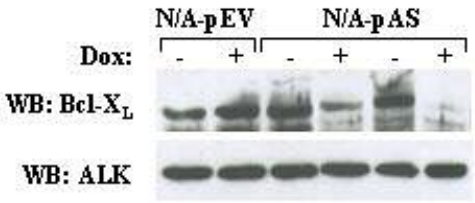

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Table 1. Downregulation of Bcl-X_L prevents growth of subcutaneous ALK^+ tumors xenografts.

Cells	Growth properties *in vitro*		Tumorigenicity in nude mice Tumors/n. of injections		Dox treatment (days)
	- DOX	+ DOX	- DOX	+ DOX	
N/A.pEV	+	+	12/12	12/12	21
N/A.pAS	+	-	12/12	3/12	21

In vitro growth and *in vivo* tumorigenic potential of N/A.pEV and N/A.pAS cells. $5x10^5$ cells were cultured for 4 days in absence/presence of Dox 1μg/mL. Cell viability was evaluated by acridine orange staining as indicated in figure 6B. $20x10^6$ N/A.pEV and N/A.pAS cells were injected subcutaneously in nude mice. 24 hours after injection, half of each group was maintained in the presence of Dox (0.2 mg/mL) in the drinking water and treatment lasted for 21 days. Mice were monitored daily for the appearance of tumors.

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Bcl-X_L downregulation suppresses the tumorigenic potential of NPM/ALK *in vitro* and *in vivo*

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