

# Characterization of Some Molecular Mechanisms Governing Autoactivation of the Catalytic Domain of the Anaplastic Lymphoma Kinase\*

Received for publication, July 24, 2007, and in revised form, November 27, 2007. Published, JBC Papers in Press, December 10, 2007, DOI 10.1074/jbc.M706067200

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NPM/ALK is an oncogenic fusion protein expressed in ~50% of anaplastic large cell lymphoma cases. It derives from the t(2;5)(p23;q35) chromosomal translocation that fuses the catalytic domain of the tyrosine kinase, anaplastic lymphoma kinase (ALK), with the dimerization domain of the ubiquitously expressed nucleophosmin (NPM) protein. Dimerization of the ALK kinase domain leads to its autophosphorylation and constitutive activation. Activated NPM/ALK stimulates downstream survival and proliferation signaling pathways leading to malignant transformation. Herein, we investigated the molecular mechanisms of autoactivation of the catalytic domain of ALK. Because kinases are typically regulated by autophosphorylation of their activation loops, we systematically mutated (Tyr → Phe) three potential autophosphorylation sites contained in the “YXXYY” motif of the ALK activation loop, and determined the effect of these mutations on the catalytic activity and biological function of NPM/ALK. We observed that mutation of both the second and third tyrosine residues (YFF mutant) did not affect the kinase activity or transforming ability of NPM/ALK. In contrast, mutation of the first and second (FFY), first and third (FYF), or all three (FFF) tyrosine residues impaired both kinase activity and transforming ability of NPM/ALK. Furthermore, a DFF mutant, in which the aspartic residue introduces a negative charge similar to a phosphorylated tyrosine, possessed catalytic activity similar to the YFF mutant. Together, our findings indicate that phosphorylation of the first tyrosine of the YXXYY motif is necessary for the autoactivation of the ALK kinase domain and the transforming activity of NPM/ALK.

The anaplastic lymphoma kinase (ALK)<sup>2</sup> is a receptor tyrosine kinase that belongs to the insulin receptor (IR) kinase subfamily of receptor tyrosine kinases (1). ALK is normally expressed in both the central and peripheral nervous systems during embryogenesis (2, 3). Expression of ALK is down-regulated before birth and in the adult ALK expression is restricted to specific cells in the central and peripheral nervous system (4). However, ALK is also aberrantly expressed in cancer as a result of chromosomal rearrangements involving the *alk* gene located on the 2p23 chromosome. ALK fusion proteins have been detected in anaplastic large cell lymphoma, diffuse large B-cell lymphoma, and in inflammatory myofibroblastic tumors (5–7).

The most commonly expressed ALK fusion protein in anaplastic large cell lymphoma is NPM/ALK, which derives from the t(2;5)(p23;q35) chromosomal translocation that fuses the kinase domain of ALK to the dimerization domain of nucleophosmin (NPM) (5). Dimerization of NPM/ALK stimulates autophosphorylation and activation of the ALK kinase domain (8, 9) and, subsequently, phosphorylation of other tyrosine residues within NPM/ALK that are important for the activation of oncogenic signaling pathways. Phosphorylated tyrosine residues act as docking sites for SH2 and PTB domains contained within signaling molecules or adaptor proteins (5, 6, 10–12). Several docking sites in NPM/ALK have already been described: pY156 for IRS-1, pY567 for SHC, and pY664 for phospholipase C- $\gamma$  (13). The docking sites for other important downstream targets of NPM/ALK, the Janus tyrosine kinases and STATs, remain to be identified (14–16).

ALK, as a member of the IR kinase subfamily, is characterized by the “YXXYY” motif contained in the activation loop (A-loop). The IR kinase has been extensively studied and provides a model for the autoregulation of the kinase domain by autoinhibition (17). In the inactive conformation, the A-loop traverses the cleft between the N- and C-terminal lobes of the kinase domain occupying both the substrate and ATP binding sites. The second tyrosine in the YXXYY motif (Tyr-1162) interacts with the catalytic loop (Asp-1132) by H-bonds and

\* This work was supported by the Associazione Italiana per la Ricerca sul Cancro, Min. San. Ricerca Finalizzata (2003); the CNRS and Ministry of Education, University, and Research-COFIN and PRIN programs (2003, 2004, and 2005); Fondazione Cariplo; the European Union (Prokinase Network 503467); the Canadian Fund for Innovation; and the National Cancer Institute of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>2</sup> The abbreviations used are: ALK, anaplastic lymphoma kinase; IR, insulin receptor; NPM, nucleophosmin; STAT, signal transducers and activators of transcription; IL-3, interleukin-3; FITC, fluorescein isothiocyanate; WT, wild type; MAPK, mitogen-activated protein kinase.

## Autoactivation Mechanisms of the ALK Catalytic Domain

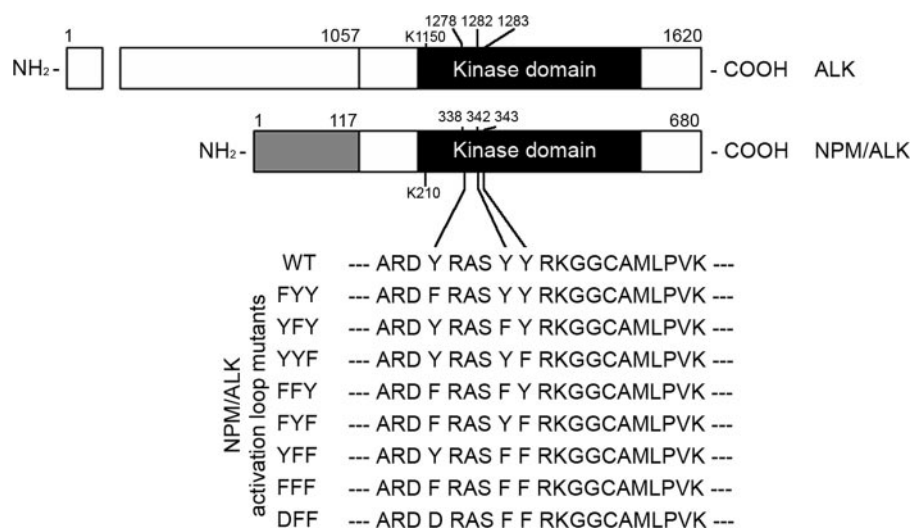


FIGURE 1. **NPM/ALK activation loop mutants.** NPM/ALK schematic representation compared with ALK full-length protein is shown. The position of the Lys residue, the mutation of which causes catalytically inactive kinase (K210R), is also indicated. Details of the YXXYY motif of the ALK A-loop are shown as well as the A-loop sequence of mutants generated by site-directed mutagenesis of pcDNA3-NPM/ALK-WT.

competes with peptide substrate for binding. Because Tyr-1162 is crucial for stabilizing the inactive conformation, it is not surprising that this second tyrosine is the preferred substrate of the IR kinase and is autophosphorylated first when the kinase undergoes autoactivation. Autophosphorylation of the A-loop tyrosines occurs in the order, second (Tyr-1162), first (Tyr-1158), and then third (Tyr-1163) (18). Sequential phosphorylation of the A-loop is accompanied by partial and then full activation of the kinase domain, when the A-loop is in the trisphosphorylated state. Conformational changes induced by phosphorylation leads to the opening of the A-loop, allowing access of ATP and substrates to the active site.

As ALK contains the YXXYY motif in its A-loop (Tyr-1278, Tyr-1282, and Tyr-1283, respectively) it can be expected that ALK may be autoregulated in a similar fashion to the IR kinase. However, we have previously demonstrated that the amino acid sequence of the ALK A-loop differs significantly from the consensus sequence for the IR kinase family (19). In addition, we observed that purified ALK kinase domain preferentially phosphorylates the first tyrosine residue of the YXXYY motif in synthetic peptides reproducing the ALK A-loop (19). These *in vitro* results suggest that the mechanism of autoregulation of ALK might differ from that of the IR kinase and that the first tyrosine in the A-loop may play a central role in its autoactivation. To test this hypothesis we assessed the role of the three tyrosine residues in the activation of full-length NPM/ALK (Tyr-338, Tyr-342, and Tyr-343). We generated mutants of NPM/ALK (Fig. 1) in which the A-loop tyrosine residues were replaced by either the neutral and non-phosphorylatable amino acid, phenylalanine, or by a negatively charged, acidic amino acid, aspartate. NPM/ALK mutants were stably expressed in BaF3 cells and the effect of these mutations on the NPM/ALK kinase activity and *in vitro/in vivo* transforming abilities was determined.

## EXPERIMENTAL PROCEDURES

**Mutagenesis and Generation of Stable BaF3 Cell Transfectants**—Full-length human NPM/ALK cDNA, subcloned in the expression vector pcDNA3, was kindly provided by Dr. S. W. Morris (Dept. of Pathology and Hematology-Oncology, St. Jude Children's Research Hospital, Memphis, TN). Mutations in the NPM/ALK A-loop were generated using the QuikChangeXL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The kinase-dead mutant was generated by mutating the residue Lys-210 to Arg (K210R) (9). The IL-3-dependent murine pro-B BaF3 cell line was transfected by electroporation of  $5 \times 10^6$  cells with 10  $\mu$ g of linearized plasmid DNA at 260 V and 960

microfarads using the Gene Pulser Electroporator (Bio-Rad, Hercules, CA). Transfected cells were selected by culturing in the presence of G418 (Sigma) at 800  $\mu$ g/ml for 2 weeks. The expression of NPM/ALK protein was verified by immunoblotting. Single cell clones were obtained for each mutant by limiting dilution in 96-well plates, and clones with comparable expression levels of NPM/ALK were selected. Mutations were verified by reverse transcription-PCR on total RNA extracted from 10,000 cells and DNA sequencing as previously described (20). BaF3 cells transfected with empty vector or mutated NPM/ALK were maintained in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine, and 0.2% supernatant from Chinese hamster ovary cells expressing IL-3 growth factor. BaF3 cells expressing wild-type NPM/ALK were grown in the absence of IL-3. All cells were cultured in a humidified atmosphere at 37 °C and 5% CO<sub>2</sub>.

**Immunoblotting Analysis**—Cells ( $2 \times 10^6$ ) were collected by centrifugation, washed once with ice-cold phosphate-buffered saline, and lysed for 30 min in lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 1 mM NaVO<sub>4</sub>, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml of Pepstatin-A, leupeptin, and aprotinin). Lysates were clarified by centrifugation at  $13,000 \times g$  for 15 min at 4 °C. Protein concentration was determined in the supernatant using the Bio-Rad Protein Assay (Bio-Rad). Proteins (150  $\mu$ g) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. NPM/ALK expression was detected using the anti-ALK#1 monoclonal antibody, kindly supplied by Dr. K. Pulford (Nuffield Department of Clinical Laboratory Sciences, John Radcliff Hospital, Oxford, UK). Tyrosine-phosphorylated proteins were detected using the mouse monoclonal anti-phosphotyrosine 4G10 antibody (anti-PY) from Upstate Biotechnology (Lake Placid, NY). Signaling molecules were detected using anti-STAT3, anti-phospho-STAT3, anti-p70S6K, anti-phospho-

p70S6K, anti-MAPK, anti-phospho-MAPK, anti-Akt, anti-phospho-Akt, and anti-Bcl-x<sub>L</sub> antibodies from Cell Signaling Technology (Danvers, MA), and the anti-p27 antibody from Santa Cruz Biotechnology (Santa Cruz, CA). Protein loading was controlled using the anti-actin antibody (Sigma). The horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies were obtained from GE Healthcare (Princeton, NJ). Proteins were visualized by enhanced chemiluminescence (ECL) as recommended by the manufacturer (GE Healthcare).

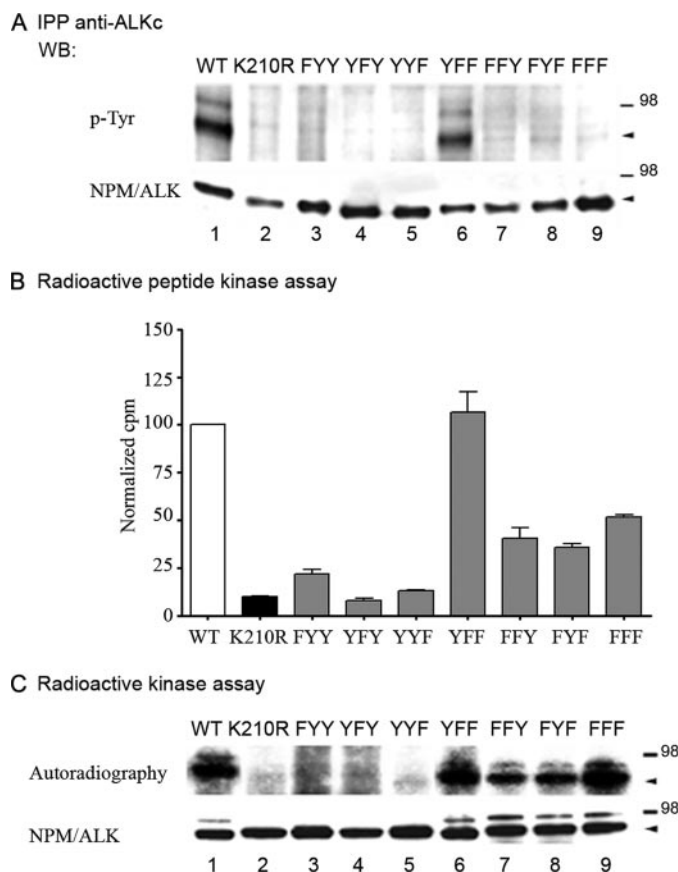
**Radioactive Kinase Assay**—NPM/ALK was immunoprecipitated from 2 mg of protein from total cell lysate using the anti-ALKc antibody, kindly supplied by Dr. B. Falini (University of Perugia, Italy), and Gammabind<sup>TM</sup> G-Sepharose (GE Healthcare). Immunocomplexes were washed five times in lysis buffer and once in kinase buffer (25 mM Hepes, pH 7.0, 5 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>).

Phosphorylation of exogenous peptide substrate was measured by incubating immunocomplexes with kinase buffer, 1 mM dithiothreitol, 30 μM ATP, 1 μCi of [γ-<sup>32</sup>P]ATP, and 200 μM peptide substrate (ARDIYRASFFRKGCCAMLPVK) (19) at 30 °C for 15 min. The reaction was stopped by spotting the reaction mix onto P81 phosphocellulose paper (2 × 2 cm). Filters were washed three times for 10 min with 0.75% phosphoric acid and once with acetone for 5 min. After drying cpm values were measured by scintillation using the Wallac Betacounter System (PerkinElmer Life Sciences). Autophosphorylation of NPM/ALK was measured by incubating immunocomplexes with kinase buffer, 1 mM dithiothreitol, 30 μM ATP, and 10 μCi of [γ-<sup>32</sup>P]ATP at 30 °C for 15 min. The reaction was stopped by the addition of SDS-gel loading buffer and heating at 95 °C for 10 min. Samples were resolved by SDS-PAGE and visualized by autoradiography.

**Proliferation Assay**—Cells were washed twice in IL-3-free medium and plated (10,000 cells/well) in 96-well plates. At 24, 48, 72, and 96 h after IL-3 withdrawal, cells were labeled with 1 μCi/well [<sup>3</sup>H]thymidine (GE Healthcare) for 8 h. Cells were harvested onto glass fiber filters (Printed Filtermat, PerkinElmer Life Sciences) and [<sup>3</sup>H]thymidine incorporation was measured using a filter scintillation counter (1430 Micro-Beta, PerkinElmer Life Sciences).

**Annexin V Apoptosis Assay**—Apoptosis in BaF3 cell transfectants after 24, 48, and 72 h IL-3 withdrawal was detected using the Annexin V-FITC Assay kit (Bender Med System, Vienna, Austria) according to the manufacturer's instructions. Briefly, BaF3 cells (1 × 10<sup>4</sup>) were incubated with Annexin V-FITC and propidium iodide for 10 min at room temperature. Samples were analyzed using Q-Cell software and FACSCalibur (BD Biosciences, Mountain View, CA).

**Animal Studies**—Female CD-1 *nu/nu* mice (7–9 weeks old) were supplied by Charles River (Calco, Lecco, Italy) and kept under standard laboratory conditions according to the local ethical guidelines. Mice were subcutaneously injected with BaF3-transfected cells (10 × 10<sup>6</sup> cells/mouse) expressing different NPM/ALK mutants. Mice that developed tumors were sacrificed and tumor tissue was extracted and homogenized in 15 ml of RPMI medium. Cells were collected and lysed, and the expression of NPM/ALK protein was assessed by immunoblotting. Mutations were verified by reverse transcription-PCR and DNA sequencing.

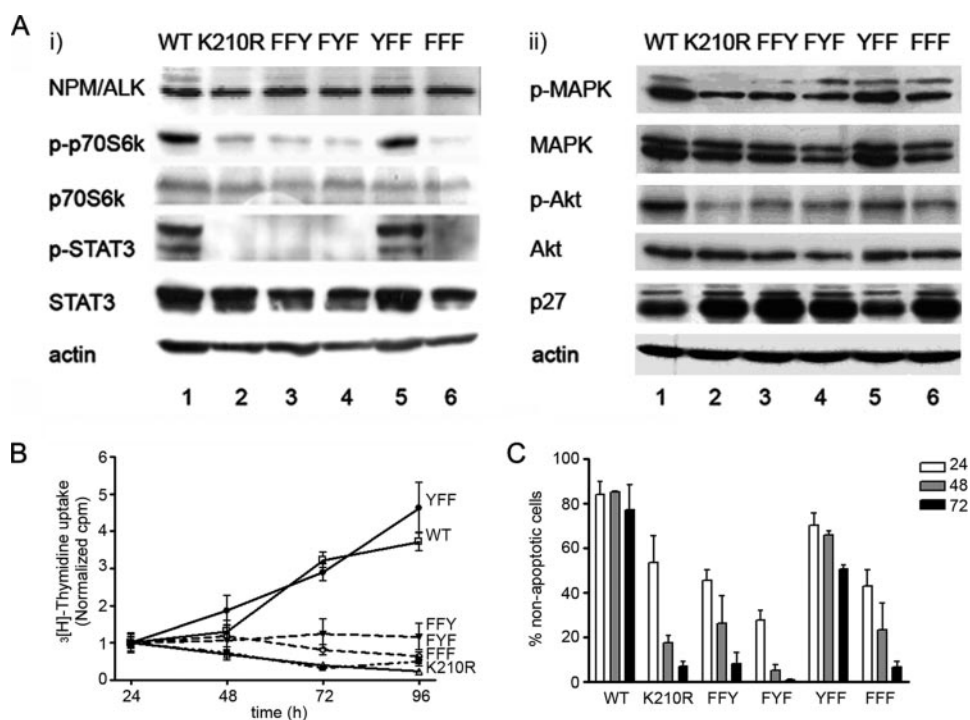


**FIGURE 2. Catalytic activity of NPM/ALK activation loop mutants.** WT and mutated NPM/ALK, stably expressed in BaF3 cells, were immunoprecipitated using the anti-ALKc antibody. **A**, the level of tyrosine phosphorylation inside cells was assessed by SDS-PAGE and anti-phosphotyrosine immunoblotting (upper panel). NPM/ALK loading was controlled by immunoblotting with the anti-ALK#1 antibody (lower panel). The gel was loaded as follows: WT NPM/ALK (lane 1); kinase-dead NPM/ALK (K210R, lane 2); FYY (lane 3); YFY (lane 4); YYF (lane 5); YFF (lane 6); FFY (lane 7); FYF (lane 8); and FFF (lane 9). The arrowhead indicates NPM/ALK protein. **B**, the activity of immunoprecipitated NPM/ALK mutants against exogenous substrate was assessed in the *in vitro* radioactive peptide kinase assay. The <sup>32</sup>P-labeled peptide was quantified by scintillation. The graph shows the normalized versus WT cpm values (mean ± S.D.; n = 3) of a single experiment, representative of three independent repeats. **C**, autophosphorylation of immunoprecipitated NPM/ALK was stimulated in presence of [γ-<sup>32</sup>P]ATP. Proteins were resolved by SDS-PAGE and visualized by autoradiography (upper panel). NPM/ALK loading was controlled by immunoblotting with the anti-ALK#1 antibody (lower panel). Gel was loaded as in **A**. Arrowheads indicate NPM/ALK protein.

## RESULTS

**In Vitro Kinase Activity of NPM/ALK Activation Loop Mutants**—We systematically mutated tyrosine residues to phenylalanine in the YXXXYY motif of the NPM/ALK A-loop (summarized in Fig. 1) and determined the effect of these mutations on ALK activity. The level of NPM/ALK tyrosine phosphorylation in BaF3-stable transfectants was assessed by anti-PY immunoblotting (Fig. 2A). The YFF mutant (Fig. 2A, lane 6) displayed a tyrosine phosphorylation level similar to WT NPM/ALK (Fig. 2A, lane 1). The FFY, FYF, and FFF mutants (Fig. 2A, lanes 7, 8, and 9, respectively) displayed a very low level of tyrosine phosphorylation that was only visible in an overexposed film (data not shown). The single mutants (Fig. 2A, lanes 3–5) were not tyrosine-phosphorylated, similar to the kinase-dead mutant, K210R (Fig. 2A, lane 2).

## Autoactivation Mechanisms of the ALK Catalytic Domain



**FIGURE 3. Stimulation of downstream signaling pathways and cellular transformation by NPM/ALK mutants.** A, BaF3 cells stably expressing WT NPM/ALK, K210R NPM/ALK, FFY, FYF, YFF, or FFF A-loop mutants were lysed after overnight IL-3 withdrawal, and proteins were resolved by SDS-PAGE. Monoclonal anti-ALK#1 (NPM/ALK), anti-phospho-p70S6 kinase (p-p70S6K), anti-phospho-STAT3 (p-STAT3) (A, panel i), anti-phospho-MAPK (p-MAPK), anti-phospho-Akt (p-Akt), and anti-p27 immunoblots with respective controls (A, panel ii), and anti-actin as a loading control were performed. B, the *in vitro* transforming ability of different NPM/ALK A-loop mutants was assessed by the [<sup>3</sup>H]thymidine uptake proliferation assay after 24, 48, 72, and 96 h of IL-3 withdrawal. Results are presented as the mean  $\pm$  S.D. ( $n = 6$ ) of a single experiment, representative of three independent repeats. C, annexin V apoptosis assay was performed on different A-loop mutants after 24, 48, and 72 h of IL-3 withdrawal. The graph shows the percentage of non-apoptotic (annexin-negative) cells (mean  $\pm$  S.E.;  $n = 3$ ) and is representative of three independent repeats.  $p$  value (24 h) YFF versus WT = 0.125;  $p$  value (48–72 h) YFF versus WT < 0.05.

We assessed the ability of the NPM/ALK mutants to phosphorylate an exogenous peptide substrate (Fig. 2B). The YFF mutant displayed a phosphorylation activity comparable to WT NPM/ALK, whereas the FFY, FYF, and FFF mutants showed an impaired activity (35–50% of WT). The single mutants (FYY, YFY, and YFF) as well as the kinase-dead mutant were inactive. Similar results were also obtained in an *in vitro* radioactive autophosphorylation assay (Fig. 2C). In this semi-quantitative assay we observed that the activity of YFF mutant (Fig. 2C, lane 6) was comparable to WT NPM/ALK (Fig. 2C, lane 1), whereas the single mutants (FYY, YFY, and YFF) were inactive (Fig. 2C, lanes 3–5). The FFY and FYF mutants displayed reduced autophosphorylation activity (Fig. 2C, lanes 7 and 8, respectively). Interestingly, the autophosphorylation of FFF mutant appeared equivalent to WT NPM/ALK in this assay. This reflects autophosphorylation occurring outside the A-loop and is symptomatic of an active conformation of this mutant.

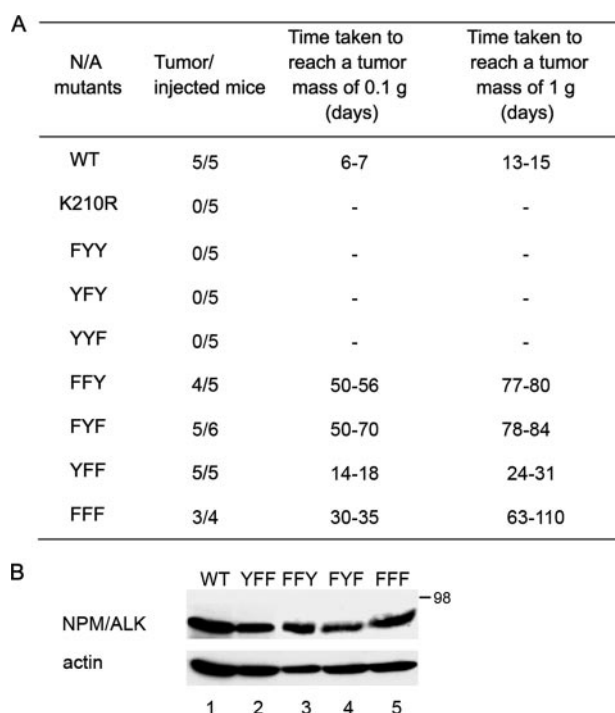
Together these data suggest that the presence of the first tyrosine alone in the YXXXXY motif of the A-loop is necessary for the ALK kinase domain to adopt a fully active conformation. Furthermore, the activity data of YFY and YFF mutants on one side (inactive despite the presence of the first tyrosine) as opposed to those of FFY, FYF, and FFF mutants on the other (active despite their lack of the first tyrosine) (Fig. 2, B and C) suggest that the second and third tyrosines may play a role in

stabilizing an inactive conformation. The absence of all three tyrosine residues in the A-loop appears to cause the destabilization of the inactive conformation leading to a partially active conformation, whose activity does not require the phosphorylation of the first tyrosine.

**Stimulation of Downstream Signaling Pathways and Cellular Transformation by NPM/ALK Activation Loop Mutants**—To determine if the YFF mutant, in addition to possessing a fully activable kinase domain, was capable of activating downstream effectors of NPM/ALK in cells, we analyzed the phosphorylation of STAT3, p70S6K, MAPK, Akt, and p27 in total cell lysates after overnight IL-3 withdrawal. STAT3 phosphorylation has been associated with NPM/ALK activation in numerous studies and is crucial for NPM/ALK-mediated transformation (21–27). The p70S6K is a downstream target of the phosphatidylinositol 3-kinase/Akt pathway, which is also a key mediator of NPM/ALK transformation (28, 29). In addition the MAPK pathway has been implicated in NPM/ALK signaling (13, 30). Recent studies have

shown that, at least in part, NPM/ALK mediates oncogenesis through phosphorylation/activation of Akt (12, 31). We observed that the YFF mutant was able to promote the phosphorylation of both STAT3 and p70S6K to an extent comparable to WT (Fig. 3A, panel i, lanes 5 and 1). Furthermore, the YFF mutant was able to induce phosphorylation of Akt and to reduce the expression of p27, similarly to NPM/ALK WT (Fig. 3A, panel ii, lanes 5 and 1). In contrast, other NPM/ALK mutants (including FFF) were defective in the activation of these pathways. Consistent with these data, only the YFF mutant promoted growth and survival of BaF3 cells in the absence of IL-3, an indication of cellular transformation (Fig. 3, B and C).

To determine if the A-loop mutants possessed *in vivo* transforming activity, nude mice were subcutaneously injected with BaF3 cells stably expressing similar levels of different NPM/ALK A-loop mutants. All mice injected with YFF-BaF3 cells developed tumors, although with a 7-day delay with respect to WT (Fig. 4A). Mice injected with FFY-BaF3 cells, FYF-BaF3 cells or FFF-BaF3 cells also developed tumors, albeit with a long delay (Fig. 4A) compared with WT. The FFF-positive tumors appeared after ~30–35 days, whereas the FFY- and FYF-positive tumors appeared after 50–70 days. All growing tumors were examined by Western blotting for the presence of NPM/ALK (Fig. 4B) and found to be positive. The kinetics of tumor



**FIGURE 4. *In vivo* transforming ability of NPM/ALK activation loop mutants.** The *in vivo* transforming ability of A-loop mutants was assessed by subcutaneous injection of  $10 \times 10^6$  stably transfected BaF3 cells in nude mice. **A**, the table summarizes tumor development. **B**, tumors were analyzed for NPM/ALK protein expression by anti-ALK#1 immunoblotting on total cell lysates, and anti-actin was used as the loading control.

development for these mutants reflects well their catalytic activity measured in the radioactive kinase assays. In contrast, all mice injected with BaF3 cells expressing single mutants or kinase dead NPM/ALK did not develop tumors (Fig. 4A).

These data suggest that the YFF mutant is fully capable of oncogenic transformation, although with slower kinetics compared with the WT. This may be attributable to a lack of signaling directed by the second and third tyrosines, which, although not essential for tumor development, may contribute to oncogenic signaling.

**Function of the First Tyrosine of the NPM/ALK Activation Loop**—We hypothesized that the first tyrosine can represent an important autophosphorylation site for the regulation of ALK activity. To test this hypothesis, we generated an A-loop mutant in which the first tyrosine was replaced by aspartic acid. Aspartate has a negatively charged side chain and therefore has been used to mimic phosphotyrosine (18). We generated a DFF mutant that mimics the NPM/ALK A-loop phosphorylated on the first tyrosine residue. As judged from *in vitro* autophosphorylation, the DFF mutant proved active (Fig. 5A, panel i, lane 3). In contrast, the FYY mutant, which has the potential to be phosphorylated on both the second and third tyrosines, was not (Fig. 2C, lane 3). These findings suggest that the conformation of the A-loop induced by the introduced negative charge in DFF, can allow the *in vitro* autophosphorylation of ALK. Similarly, when we analyzed the level of tyrosine phosphorylation in cells, DFF was phosphorylated to similar extent to YFF (Fig. 5A, panel ii, lanes 3 and 4, respectively). In addition, the DFF mutant was able to phosphorylate substrate (63% of WT) in a radioactive peptide kinase assay as the YFF mutant (Fig. 5A,

panel iii). These results suggest that in cells the DFF mutant can mimic the YFF mutant resulting in phosphorylated NPM/ALK. Therefore, the negative charge of the aspartic residue can correctly mimic a phosphorylated tyrosine, causing the A-loop to adopt an open/active conformation as seen in the YFF mutant and WT NPM/ALK.

The ability of the DFF mutant to activate anti-apoptotic pathways (Fig. 5, B and C) was also assessed. The annexin V assay was performed on different mutants after IL-3 withdrawal. Interestingly, the DFF mutant displayed a significant ( $p$  value = 0.027) level of protection from apoptosis at 48 h compared with the kinase-dead mutant (Fig. 5B). A key mediator of NPM/ALK induced survival is the anti-apoptotic protein, Bcl-x<sub>L</sub> (32). After 48 h of IL-3 withdrawal we performed an immunoblotting analysis with the anti-Bcl-x<sub>L</sub> antibody. The DFF mutant (Fig. 5C, lane 2) displayed Bcl-x<sub>L</sub> expression levels comparable to YFF and WT NPM/ALK (Fig. 5C, lanes 3 and 1, respectively), in agreement with the annexin V results (Fig. 5B).

The FFF mutant was also used as a control of the DFF one. Kinase assay showed that FFF displayed *in vitro* kinase activity (Fig. 5A, panel i, lane 5 and panel iii). This is likely the result of a structural destabilization of the A-loop resulting in an open/active conformation. However, the FFF mutant was not able to be phosphorylated in cells (Fig. 5A, panel ii), did not induce Bcl-x<sub>L</sub>, and could not protect cells from apoptosis (Fig. 5, B and C).

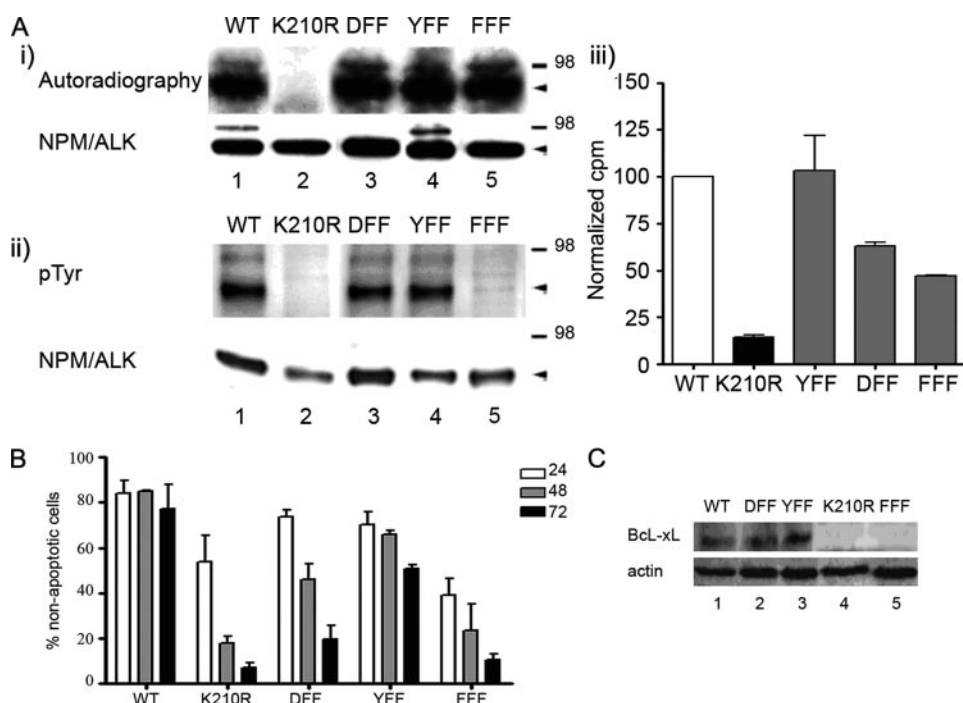
We also assessed the ability of the DFF mutant to promote growth of BaF3 cells in the absence of IL-3 (Fig. 6A). In contrast to the YFF mutant, the DFF mutant did not support IL-3-independent growth of BaF3 cells as the FFF mutant. We observed that the DFF mutant (Fig. 6B, lane 4) was unable to phosphorylate the downstream effectors STAT3 and p70S6K similarly to the FFF and K210R mutants (Fig. 6B lanes 5 and 2, respectively). In addition, p27 was down-regulated in cells expressing NPM/ALK WT and YFF (Fig. 6B, lanes 1 and 3) but not in cells expressing DFF or FFF mutants (Fig. 6B, lanes 4 and 5, respectively). Together, these results suggest that introducing a negative charge to activate the kinase is sufficient for inducing some downstream signaling such as induction of Bcl-x<sub>L</sub>, and protection from apoptosis, but not others such as STAT3 phosphorylation, which may require direct phosphorylation of this residue.

To assess if the first tyrosine is a docking site for signaling molecules involved in proliferative pathways a co-immunoprecipitation with the anti-ALKc antibody was performed (Fig. 6C). Interestingly, the DFF mutant did not co-immunoprecipitate with STAT3 (Fig. 6C, lane 3) in contrast to WT NPM/ALK and the YFF mutant, suggesting that the first tyrosine might play an important role in the interaction of NPM/ALK and STAT3.

## DISCUSSION

The human genome contains 90 tyrosine kinase genes encoding 58 receptor tyrosine kinases grouped into 20 subfamilies and 32 non-receptor tyrosine kinases divided into 10 subfamilies (1, 33). Tyrosine phosphorylation is essential for the autoactivation of the majority of these kinases and the stimulation of their downstream signaling pathways. The activation of

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**FIGURE 5. The catalytic activity and anti-apoptotic activity of the DFF NPM/ALK activation loop mutant.** A, NPM/ALK immunoprecipitated from stably transfected BaF3 cells expressing WT (lane 1), kinase-dead (K210R, lane 2), or the DFF, YFF, and FFF A-loop mutants (lanes 3–5, respectively) was assessed for kinase activity. *i*, autophosphorylation was stimulated in presence of [ $\gamma$ - $^{32}$ P]ATP. Proteins were resolved by SDS-PAGE and visualized by autoradiography (first panel). NPM/ALK loading was controlled by immunoblotting with the anti-ALK#1 antibody (second panel). *ii*, the level of tyrosine phosphorylation inside cells was also assessed by anti-phosphotyrosine immunoblotting (first panel). NPM/ALK loading was controlled by immunoblotting with the anti-ALK#1 antibody (second panel). Arrowheads indicate NPM/ALK protein. *iii*, the activity of immunoprecipitated NPM/ALK mutants against exogenous substrate was assessed in the *in vitro* radioactive peptide kinase assay. The  $^{32}$ P-labeled peptide was quantified by scintillation. The graph shows the normalized versus WT cpm values (mean  $\pm$  S.D.;  $n = 3$ ) of a single experiment, representative of three independent repeats. B, annexin V apoptosis assay was performed on different A-loop mutants after 24, 48, and 72 h of IL-3 withdrawal. The graph shows the percentage of non-apoptotic (annexin negative) cells (mean  $\pm$  S.E.;  $n = 3$ ) and is representative of three independent repeats. C, activation of anti-apoptotic signaling pathways was assessed in total cell lysates. Anti-Bcl-x<sub>L</sub> immunoblotting was performed after 48 h of IL-3 withdrawal. Protein loading was controlled by anti-actin immunoblotting.

downstream pathways is important for cellular processes in both normal and pathological conditions (34–37).

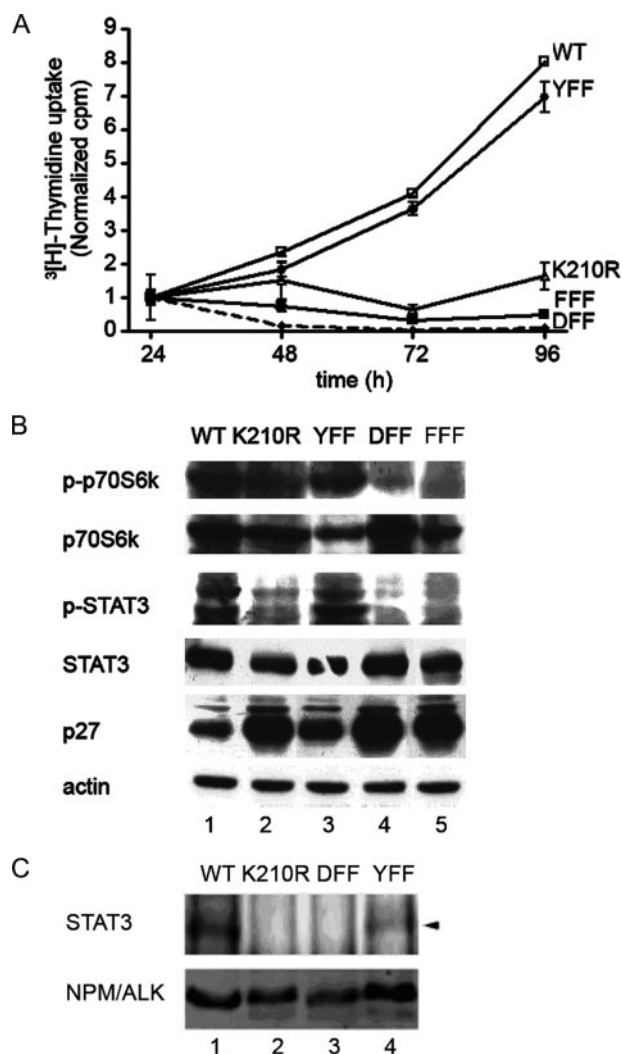
Here, we assessed the role of potential autophosphorylation sites in the NPM/ALK A-loop in the autoactivation of the kinase domain. We demonstrated that the first tyrosine is necessary for triggering the kinase activity of NPM/ALK and for stimulating its downstream signaling pathways, as demonstrated by the YFF mutant. This function of the first tyrosine is likely to be mediated by its phosphorylation, considering its unique susceptibility to ALK-catalyzed phosphorylation *in vitro* (19) and the cellular phosphorylation of the YFF mutant (Fig. 2A), at variance with all other mutants. In addition, the introduction of a negative charge at the position of the first tyrosine in the DFF mutant led to autophosphorylation *in vitro* and tyrosine phosphorylation in cells similar to that of the YFF mutant (Fig. 5A). This finding suggests that the aspartic acid residue is able to mimic a phosphorylated tyrosine residue and induce conformational changes in the A-loop that stabilize an open/active conformation. In accordance with our results, phosphopeptide mapping of NPM/ALK derived from SUDHL1 and Karpas cell lines has identified four ALK phosphopeptides containing the YXXXXY motif of the A-loop: all four peptides displayed phosphorylation of the first tyrosine in the YXXXXY

motif, while only one peptide was phosphorylated on the second tyrosine, and none were phosphorylated of the third tyrosine residue (38). These results suggest that for the ALK kinase the order of tyrosine phosphorylation inside the A-loop could be first, second, and third.

The presence of the first tyrosine is not a sufficient condition for activity, however, as disclosed by the failure of the mutants YFY and YYF to undergo autophosphorylation as well as phosphorylate the specific peptide substrate *in vitro* (Fig. 2, B and C). These findings indicate that the fully open/active conformation of the A-loop cannot be stabilized in these mutants. It is possible that the conformation of the A-loop in these mutants does not render the first tyrosine susceptible to autophosphorylation, suggesting a negative effect of the second and third residues on ALK activation. Alternatively, the inactivity of these mutants might reflect the inability of the first tyrosine, once phosphorylated in YFY and YYF contexts, to sustain an active conformation.

The FFY, FYF, and FFF mutants, whose cellular phosphorylation levels and biological function are severely impaired (Fig. 2A), are nevertheless catalytically active *in vitro* (Fig. 2, B and C). These findings are consistent with a partially active conformation of the A-loop in these mutants, which under *in vitro* experimental conditions can be pushed toward a more active conformation, whereas in the cellular context a more inactive conformation is adopted. The observation that these mutants are capable of developing tumors *in vivo*, albeit with delayed kinetics (Fig. 4), despite such low catalytic activity in cells, may reflect the potency of NPM/ALK as an oncogene or may possibly be attributed to the accumulation of other oncogenic mutations.

Our results suggest that ALK is characterized by a different mechanism of autoactivation from that of the IR kinase. In fact, assuming that the starting point of trans-phosphorylation is the existence for a short period of time of a non-phosphorylated protein in the active conformation, then, based on the models of ALK kinase domain and three-dimensional structure of IR kinase, it is plausible that the first tyrosine is phosphorylated and the most exposed one in the inactive conformation. The second tyrosine is more prone to cis-phosphorylation. In the active conformation, it is also conceivable, looking at the surroundings of the first tyrosine, that it can stabilize the active conformation by forming H-bonds with neighbor residues, which differ from those present in the IR kinase.



**FIGURE 6. *In vitro* transforming ability and activation of signaling pathways by the DFF A-loop mutant.** *A*, *in vitro* transforming ability of BaF3 cells was assessed for WT, K210R, DFF, YFF, and FFF NPM/ALK by the [ $^3$ H]thymidine uptake proliferation assay after 24, 48, 72, and 96 h of IL-3 withdrawal. Results are presented as the mean  $\pm$  S.D. ( $n = 6$ ) of a single experiment, representative of three repeats. *B*, activation of NPM/ALK downstream signaling pathways was analyzed in total cell lysates. Anti-phospho-p70S6 kinase, anti-phospho-STAT3, and anti-p27 immunoblots with respective controls and anti-actin as loading control were performed. *C*, NPM/ALK was immunoprecipitated from BaF3 cells expressing WT, K210R, DFF, and YFF NPM/ALK using the anti-ALKc antibody after 24 h of IL-3 withdrawal. STAT3 co-immunoprecipitating with NPM/ALK was detected by Western blotting.

In addition to its crucial role in the autoactivation of NPM/ALK, the first tyrosine residue in the YXXXYY motif might also be directly involved in the activation of downstream signaling pathways. Our data demonstrate that cells expressing the DFF mutant were partially protected from apoptosis (Fig. 5, *B* and *C*), although unable to proliferate in the absence of IL-3 (Fig. 6*A*). Our results also show that the first tyrosine, which remains exposed in the modeled ALK-active conformation, may be involved in the docking and activation of STAT3 (Fig. 6*C*).

Investigating the role of tyrosine residues of the NPM/ALK A-loop has improved our knowledge of the mechanisms regulating the autoactivation of ALK kinase domain. We have demonstrated that phosphorylation of the first tyrosine plays a cru-

cial role during autoactivation of NPM/ALK and may also mediate the activation of downstream pathways involved in cellular proliferation.

*Acknowledgments*—We are grateful to Dr. Karen Pulford (Nuffield Department of Clinical Laboratory Sciences, John Radcliff Hospital, Oxford, UK) for monoclonal anti-ALK#1 antibody and Prof. Brunangelo Falini (University of Perugia, Italy) for polyclonal anti-ALKc antibody. We also thank Dr. Oriano Marin (Dept. of Biochemistry, University of Padua, and Venetian Institute for Molecular Medicine, Padua, Italy) for the synthesis of the peptide and Dr. Loredana Cleris for *in vivo* experiments (Dept. of Experimental Oncology, Foundation National Cancer Institute, Milan).

## REFERENCES

- Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) *Science* **298**, 1912–1934
- Hurley, S. P., Clary, D. O., Copie, V., and Lefcort, F. (2006) *J. Comp. Neurol.* **495**, 202–212
- Vernersson, E., Khoo, N. K., Henriksson, M. L., Roos, G., Palmer, R. H., and Hallberg, B. (2006) *Gene Expr. Patterns* **6**, 448–461
- Pulford, K., Morris, S. W., and Turturro, F. (2004) *J. Cell Physiol.* **199**, 330–358
- Morris, S. W., Kirstein, M. N., Valentine, M. B., Dittmer, K. G., Shapiro, D. N., Saltman, D. L., and Look, A. T. (1994) *Science* **263**, 1281–1284
- Cook, J. R., Dehner, L. P., Collins, M. H., Ma, Z., Morris, S. W., Coffin, C. M., and Hill, D. A. (2001) *Am. J. Surg. Pathol.* **25**, 1364–1371
- Rudzki, Z., Rucinska, M., Jurczak, W., Skotnicki, A. B., Maramorosz-Kurianowicz, M., Mruk, A., Pirog, K., Utych, G., Bodzioch, P., Srebro-Stariczek, M., Wlodarska, L., and Stachura, J. (2005) *Pol. J. Pathol.* **56**, 37–45
- Cordell, J. L., Pulford, K. A. F., Bigerna, B., Roncador, G., Banham, A., Colombo, E., Pelicci, P. G., Mason, D. Y., and Falini, B. (1999) *Blood* **93**, 632–642
- Bischof, D., Pulford, K., Mason, D. Y., and Morris, S. W. (1997) *Mol. Cell Biol.* **17**, 2312–2325
- Coluccia, A. M., Gunby, R. H., Tartari, C. J., Scapozza, L., Gambacorti-Passerini, C., and Passoni, L. (2005) *Expert Opin. Ther. Targets* **9**, 515–532
- Pulford, K., Lamant, L., Espinos, E., Jiang, Q., Xue, L., Turturro, F., Delsol, G., and Morris, S. W. (2004) *Cell Mol. Life Sci.* **61**, 2939–2953
- Bai, R. Y., Ouyang, T., Miething, C., Morris, S. W., Peschel, C., and Duyster, J. (2000) *Blood* **96**, 4319–4327
- Bai, R. Y., Dieter, P., Peschel, C., Morris, S. W., and Duyster, J. (1998) *Mol. Cell Biol.* **18**, 6951–6961
- Nieborowska-Skorska, M., Slupianek, A., Xue, L., Zhang, Q., Raghunath, P. N., Hoser, G., Wasik, M. A., Morris, S. W., and Skorski, T. (2001) *Cancer Res.* **61**, 6517–6523
- Lai, R., Rassidakis, G. Z., Lin, Q., Atwell, C., Medeiros, L. J., and Amin, H. M. (2005) *Hum. Pathol.* **36**, 939–944
- Amin, H. M., Lin, Q., and Lai, R. (2006) *Lab. Invest.* **86**, 417–419
- Hubbard, S. R. (2002) *Front. Biosci.* **7**, d330–d340
- Zhang, B., Tavare, J. M., Ellis, L., and Roth, R. A. (1991) *J. Biol. Chem.* **266**, 990–996
- Donella-Deana, A., Marin, O., Cesaro, L., Gunby, R. H., Ferrarese, A., Coluccia, A. M., Tartari, C. J., Mologni, L., Scapozza, L., Gambacorti-Passerini, C., and Pinna, L. A. (2005) *Biochemistry* **44**, 8533–8542
- Gunby, R. H., Cazzaniga, G., Tassi, E., Le Coutre, P., Pogliani, E., Specchia, G., Biondi, A., and Gambacorti-Passerini, C. (2003) *Haematologica* **88**, 408–415
- Chiarle, R., Simmons, W. J., Cai, H., Dhall, G., Zamo, A., Raz, R., Karras, J. G., Levy, D. E., and Inghirami, G. (2005) *Nat. Med.* **11**, 623–629
- Kasprzycka, M., Marzec, M., Liu, X., Zhang, Q., and Wasik, M. A. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 9964–9969
- Khoury, J. D., Medeiros, L. J., Rassidakis, G. Z., Yared, M. A., Tsioli, P., Leventaki, V., Schmitt-Graeff, A., Herling, M., Amin, H. M., and Lai, R. (2003) *Clin. Cancer Res.* **9**, 3692–3699

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24. Lai, R., Rassidakis, G. Z., Medeiros, L. J., Ramdas, L., Goy, A. H., Cutler, C., Fujio, Y., Kunisada, K., Amin, H. M., and Gilles, F. (2004) *Am. J. Pathol.* **164**, 2251–2258
25. Marzec, M., Kasprzycka, M., Ptasznik, A., Wlodarski, P., Zhang, Q., Odum, N., and Wasik, M. A. (2005) *Lab. Invest.* **85**, 1544–1554
26. Zamo, A., Chiarle, R., Piva, R., Howes, J., Fan, Y., Chilosi, M., Levy, D. E., and Inghirami, G. (2002) *Oncogene* **21**, 1038–1047
27. Zhang, Q., Raghunath, P. N., Xue, L., Majewski, M., Carpentieri, D. F., Odum, N., Morris, S., Skorski, T., and Wasik, M. A. (2002) *J. Immunol.* **168**, 466–474
28. Chiang, G. G., and Abraham, R. T. (2005) *J. Biol. Chem.* **280**, 25485–25490
29. Vega, F., Medeiros, L. J., Leventaki, V., Atwell, C., Cho-Vega, J. H., Tian, L., Claret, F. X., and Rassidakis, G. Z. (2006) *Cancer Res.* **66**, 6589–6597
30. Fujimoto, J., Shiota, M., Iwahara, T., Seki, N., Satoh, H., Mori, S., and Yamamoto, T. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 4181–4186
31. Slupianek, A., Nieborowska-Skorska, M., Hoser, G., Morrione, A., Majewski, M., Xue, L., Morris, S. W., Wasik, M. A., and Skorski, T. (2001) *Cancer Res.* **61**, 2194–2199
32. Coluccia, A. M., Perego, S., Cleris, L., Gunby, R. H., Passoni, L., Marchesi, E., Formelli, F., and Gambacorti-Passerini, C. (2004) *Blood* **103**, 2787–2794
33. Robinson, D. R., Wu, Y. M., and Lin, S. F. (2000) *Oncogene* **19**, 5548–5557
34. Cohen, P. (2001) *Eur. J. Biochem.* **268**, 5001–5010
35. Gunby, R. H., Sala, E., Tartari, C. J., Puttini, M., Gambacorti-Passerini, C., and Mologni, L. (2006) *Anticancer Agents Med. Chem.* **7**, 594–611
36. Hubbard, S. R. (1999) *Prog. Biophys. Mol. Biol.* **71**, 343–358
37. Hubbard, S. R., and Till, J. H. (2000) *Annu. Rev. Biochem.* **69**, 373–398
38. Rush, J., Moritz, A., Lee, K. A., Guo, A., Goss, V. L., Spek, E. J., Zhang, H., Zha, X. M., Polakiewicz, R. D., and Comb, M. J. (2005) *Nat. Biotechnol.* **23**, 94–101