Delocalization and Destabilization of the Arf Tumor Suppressor by the Leukemia-Associated NPM Mutant

Emanuela Colombo, Paola Martinelli, Raffaella Zamponi, Danielle C. Shing, Paola Bonetti, Lucilla Luzi, Sara Volorio, Loris Bernard, Giancarlo Pruneri, Myriam Alcalay, and Pier Giuseppe Pelicci

1Department of Experimental Oncology and 2Division of Pathology, European Institute of Oncology; 3IFOM Institute; and 4Department of Medicine, Surgery, and Dentistry, University of Milan, Milan, Italy

Abstract

One third of acute myeloid leukemias (AMLs) are characterized by the aberrant cytoplasmic localization of nucleophosmin (NPM) due to mutations within its putative nucleolar localization signal. NPM mutations are mutually exclusive with major AML-associated chromosome rearrangements and are frequently associated with a normal karyotype, suggesting that they are critical during leukemogenesis. The underlying molecular mechanisms are, however, unknown. NPM is a nucleocytoplasmic shuttling protein that has been implicated in several cellular processes, including ribosome biogenesis, centrosome duplication, cell cycle progression, and stress response. It has been recently shown that NPM is required for the stabilization and proper nucleolar localization of the tumor suppressor p19Arf. We report here that the AML-associated NPM mutant localizes mainly in the cytoplasm due to an alteration of its nucleus-cytoplasmic shuttling equilibrium, forms a direct complex with p19Arf, but is unable to protect it from degradation. Consequently, cells or leukemic blasts expressing the NPM mutant have low levels of cytoplasmic Arf. Furthermore, we show that expression of the NPM mutant reduces the ability of Arf to initiate a p53 response and to induce cell cycle arrest. Inactivation of p19Arf, a key regulator of the p53-dependent cellular response to oncogene expression, might therefore contribute to leukemogenesis in AMLs with mutated NPM.

Introduction

The gene encoding for nucleophosmin (NPM; also known as B23, NO38, or numatrin) is involved in several tumor-associated chromosome translocations, which result in the formation of fusion proteins retaining the NH₂ terminus of NPM [NPM-ALK (1), NPM-RAR (2), and NPM-MLF1 (3)]. It is generally thought that the NPM component does not contribute biological signaling to the transforming potential of these fusion proteins but instead provides a dimerization interface for the oligomerization and the oncogenic conversion of the various NPM partners (ALK, RAR, and MLF1; ref. 4). More recently, mutations of NPM have been described in about 35% of acute myeloid leukemias (AMLs; ref. 5). NPM mutations are usually associated with a normal karyotype (85%) and are mutually exclusive with the other AML-associated major genetic abnormalities, suggesting that they represent an initiating event in myeloid leukemogenesis, and that alterations of NPM might contribute directly to the transformation process. The underlying mechanism(s) remain, however, unknown.

NPM is an abundant and ubiquitously expressed nucleolar phosphoprotein, which functions as a molecular chaperone (6) and shuttles between nucleus and cytoplasm (7). It regulates cell proliferation (8, 9), although its specific effect remains controversial, and stimulates cell survival after DNA damage (10, 11). NPM physically interacts with many cellular proteins, including the tumor suppressors p53 (8) and Arf (ref. 12; p19Arf in mouse and p14ARF in human). We (13) and others (14) have recently shown that the nucleolar Arf protein is delocalized and markedly unstable in the absence of NPM expression, thus suggesting that one physiologic function of NPM is to ensure the proper localization of Arf and to protect it from degradation. We report here our investigations aimed at defining whether the leukemia-associated mutant of NPM (NPMmut) alters localization and/or stability of Arf.

Materials and Methods

Cell culture, transfection, and infection. Mouse embryo fibroblasts (MEF), Phoenix, and NIH cells were cultured at 37°C and 5% CO₂ in DMEM supplemented with 10% fetal bovine serum. Transient transfections were done using the standard calcium phosphate precipitate method for Phoenix, FuGENE 6 (Roche, Indianapolis, IN) for MEFs and LipofectAMINE (Invitrogen, Carlsbad, CA) for NIH-ST3 cells. For infection experiments, we used a Pinco-based vector expressing green fluorescent protein (GFP; control vector), GFP-MLM1, or GFP-mutant NPM fusion proteins. Empty or recombinant retroviral vectors were transfected into Phoenix packaging cell lines, and after 48 hours, the supernatants were used to infect target cells. Lineage minus (lin−) hematopoietic cells were purified from the bone marrow of 8- to 12-week strain 129SvEv mice by depletion of cells expressing myeloid, erythroid, and lymphoid differentiation markers, using a StemSep Murine Progenitor Enrichment kit (Stem Cell Technologies, Vancouver, British Columbia, Canada). Lin− cells were grown for 2 to 3 days in RPMI containing interleukin-3 (20 ng/mL), interleukin-6 (20 ng/mL), and stem cell factor (100 ng/mL). The cells were then plated onto retinectin-coated (Takara-Shuzo, Shiga, Japan), non–tissue culture–treated plates and exposed to the supernatant of packaging, ecotropic Phoenix cells transiently transfected with the indicated retroviral vectors (Pinco or Pinco-NPMmut). Infected, GFP-positive cells were sorted using a Becton Dickinson (Franklin Lakes, NJ) FACS Vantage instrument. Cytospins were done of the infected lin− cells immediately after sorting. The cells were fixed with PBS containing 4% paraformaldehyde and stained for immunofluorescence analysis.

Plasmids. We amplified the mutated form of NPM (NPMmut) directly from the mRNA of one patient using the following primers: forward, 5-GTTTTGTCTCTGGAAGCAAGTTTCT; reverse, 5-ATGGCAGATAT-CAACTGTTACAG. The amplified product was cloned into pCR2.1TOPO (Invitrogen) and then subcloned into the Pinco retroviral vector or reamplified using two primers containing a XbaI HI site at the 5′-term (forward, 5-ATGGCGATCCCTCGATGGAAGATCAG) and a XbaI

Note: E. Colombo and P. Martinelli contributed equally to this work.

Requests for reprints: Pier Giuseppe Pelicci, Department of Experimental Oncology, European Institute of Oncology, Via Ripamonti 435, Milan 20141, Italy. Phone: 39-02-5748-9853; E-mail: piegiuseppe.pelicci@fom-ies-campus.it. ©2006 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-05-2378
Results

Expression of NPM1, NPM2, and NPMmut. Approximately one third of AMLs are characterized by the aberrant cytoplasmic localization of NPM due to mutations in the most 3' of its coding exons (5). Although molecularly heterogeneous, all mutations lead to a frameshift in the region encoding for the COOH terminus of the NPM protein. We cloned a full-length mutant NPM cDNA from the leukemic blasts of one AML patient carrying cytoplasmic NPM (data not shown) and a duplication of the TCTG tetranucleotide at positions 956 to 959 of the NPM nucleotide sequence (Fig. 1A). This insertion, which is representative of the most frequent type of NPM

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Figure 1. Expression of NPM1, NPM2, and NPMmut. A, DNA and protein sequence of WT and NPMmut. Tetranucleotide insertion and the novel mutant amino acid residues (red). Stop codons (*). B, schematic representation of NPM isoforms and mutant. NoLS, nucleolar localization signal. HoD, homodimerization domain. Right, amino acid positions (aa) of relevant protein regions. C, MEFs from NPM-deficient mice (p53-/- NPM-/-; zkO; see text) were transfected with flag-NPM1, flag-NPM2, and HA-flag-NPMmut; lysed; and analyzed by Western blotting using antibodies against flag or NPM, as indicated. Control, lysates from p53-/- fibroblasts. The faster migrating anti-NPM polyepitope seen in the NPM1, NPM2, and NPMmut lanes might represent an NH2-terminal degradation product, which is not recognized by the anti-Flag antibody. D, p53-/- MEFs were transfected with GFP-NPM1, GFP-NPM2, GFP-NPMmut, or infected with untagged NPMmut (pinco vector). Infected cells expressed the GFP (green fluorescence merged with 4',6-diamidino-2-phenylindole [dapi]) as marker, and the NPMmut protein, as revealed by using the NPMa antibody (red fluorescence).

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mutations found in AMLs (5), causes a frameshift that substitutes the last seven amino acids (WQQWRKSL) of the NPM protein with 11 novel residues (CLAVEEVSLRK; Fig. 1A-B). The NPMmut cDNA was then tagged with hemagglutinin (HA), HA/flag, or GFP and cloned within different mammalian expression vectors (the Pinco retrovirus or cytomegalovirus promoter-based plasmids). As controls, we used cDNAs representative of the two alternative transcripts of the NPM locus, which encode proteins differing at their extreme COOH terminus (NPM1 or NPM2; Fig. 1B; ref. 16).

Expression of tagged NPM1, NPM2, and NPMmut cDNAs in NPM knockout MEFs yielded polypeptides of the expected size, which were specifically recognized by anti-NPM or anti-tag antibodies (see Fig. 1C for representative results). GFP-tagged constructs were then transfected into MEFs and analyzed by direct epifluorescence. GFP-NPM1 showed a nucleolar localization pattern, GFP-NPM2 localized to both nucleoplasm and nucleoli, whereas the GFP-NPMmut was detected in the nucleoli, nucleoplasm, and cytosol (Fig. 1D). This localization pattern of the NPMmut protein was not influenced by fusion of NPMmut with GFP, because it was also seen with nontagged (Fig. 1D) and HA/flag- (data not shown) NPMmut. The same intracellular localization pattern was observed upon retrovirus-mediated expression of NPMmut into hematopoietic precursor cells (data not shown). It seems, therefore, that as reported (5), the NPMmut has the aberrant property of localizing within the cytoplasm.

**NPM mutant induces delocalization and destabilization of Arf protein.** NPM is required for the proper nuclear localization of Arf as well as for its stability (13, 14). We thus investigated the effects of NPMmut expression on Arf localization and stability. Expression of the NPMmut but not NPM1 or NPM2 in MEFs provoked delocalization of p19<sup>Arf</sup> to the cytoplasm (Fig. 2A, left) in virtually all the transfected cells (Fig. 2A, right). This effect was evident also upon retrovirus-mediated expression of untagged NPMmut into hematopoietic precursor cells (Fig. 2B). Finally, immunohistochemical analysis of bone marrow biopsies from AML patients carrying mutations and cytoplasmic delocalization of NPM revealed the presence of weak anti-p14<sup>ARF</sup> cytoplasmic staining in three of three positive cases (Fig. 2C), suggesting that cytoplasmatic delocalization of p14<sup>ARF</sup> is a characteristic of AMLs with NPM mutations.

The overall anti-Arf staining intensity seemed lower in MEFs expressing the NPMmut (Fig. 2A) or in leukemia blasts from AML patients carrying mutations of NPM (Fig. 2C). To investigate whether Arf protein levels are decreased following NPMmut expression, NPMmut and NPM1 were cotransfected with p19<sup>Arf</sup> into p19<sup>Arf</sup>-/- MEFS (together with a β-galactosidase expressing vector to normalize transfection efficiency) and their expression levels analyzed by Western blotting. Consistently, lower levels of Arf were found in cells cotransfected with the NPMmut (Fig. 3A, left) but not with wild-type (WT) NPM1 (Fig. 3A, middle). Similar results were obtained using one clone of NIH 3T3 carrying a conditional (Zn inducible) p19<sup>Arf</sup> allele (MT-Arf ref. 17). Indeed, levels of Zn-induced p19<sup>Arf</sup> expression (and of the p53 target p21 gene) were lower in MT-Arf cells expressing GFP-NPMmut (Fig. 3A, right).

To preliminary investigate the mechanisms underlying the effects of NPMmut on levels of p19<sup>Arf</sup> expression, we investigated the effects of NPMmut on p19<sup>Arf</sup> protein stability. MT-Arf cells were first infected with a control retrovirus or with a retrovirus expressing NPMmut and then treated with Zn for 24 hours, to induce Arf expression. At the end of the Zn treatment, cells were treated with cycloheximide, to block de novo protein synthesis, and p19<sup>Arf</sup> levels analyzed by Western blotting at different time points (tubulin was used to normalize for protein levels, due to its relatively high stability; ref. 13). As shown in Fig. 3B, levels of p19<sup>Arf</sup> remained constant up to 8 hours after cycloheximide treatment in the control cells, whereas at the same time point they were significantly reduced in the presence of GFP-NPMmut expression, thus suggesting that NPMmut induces a reduction of the half-life of p19<sup>Arf</sup>.

**Figure 2. NPMmut delocalizes p19<sup>Arf</sup>**. A, left, immunofluorescence analysis of p53<sup>−/−</sup> MEFs transfected with expression vectors for GFP-NPM1, GFP-NPM2, and GFP-NPMmut, as indicated. Cells were grown in the presence or absence of Zn for 24 hours, to induce Arf expression. At the end of the Zn treatment, cells were treated with cycloheximide, to block de novo protein synthesis, and p19<sup>Arf</sup> levels analyzed by Western blotting at different time points (tubulin was used to normalize for protein levels, due to its relatively high stability; ref. 13). As shown in Fig. 3B, levels of p19<sup>Arf</sup> remained constant up to 8 hours after cycloheximide treatment in the control cells, whereas at the same point time they were significantly reduced in the presence of GFP-NPMmut expression, thus suggesting that NPMmut induces a reduction of the half-life of p19<sup>Arf</sup>.
Expression of p19\textsuperscript{Arf} induces cell cycle arrest of MT-Arf fibroblasts (17). Therefore, to investigate the biological effects of NPMmut on p19\textsuperscript{Arf} activity, we evaluated, by the BrdUrd incorporation test, the number of cycling cells after Zn treatment in MT-Arf cells infected with the control retrovirus, or with retroviruses expressing GFP-NPMmut or GFP-NPM1. As shown in Fig. 3C, expression of the NPMmut reduced significantly ($P = 0.003$) the ability of p19\textsuperscript{Arf} to block S-phase entry, whereas the WT NPM protein did not show any effect. The effects of GFP-NPM1 or GFP-NPMmut on the levels of p19\textsuperscript{Arf} and p53 expression in this experiment are shown in Fig. 3D. Taken together, these data suggest that expression of NPMmut causes delocalization and destabilization of Arf, thus antagonizing its ability to induce cell cycle arrest.

NPMmut forms a complex with p19\textsuperscript{Arf}. NPM forms, in vivo and in vitro, homo-oligomeric complexes containing five or six molecules (18). Because the NPMmut retains the homodimerization domain of NPM (Fig. 1B), we investigated whether the effect of NPMmut on Arf was due to its interaction with endogenous NPM. To this end, we first evaluated the effect of NPMmut on the localization of endogenous NPM [using a monoclonal antibody (NPMc) directed against the portion of NPM that is lost in the NPMmut; see Materials and Methods]. Surprisingly, in cells expressing NPMmut, endogenous NPM localizes predominantly in the nucleolus and is only marginally delocalized in the cytoplasm (Fig. 4A, b). Because Arf is instead predominantly cytoplasmic in the same cells (Fig. 4A, a), NPMmut might delocalize Arf through a direct interaction. Therefore, we investigated the effects on Arf protein localization upon expression of the NPMmut (and NPM1 or NPM2, as controls) in MEFs derived from NPM null mice. To this end, we used cells derived from double NPM$^{-/-}$/p53$^{-/-}$ mice (dKO). In fact, we have shown that lack of NPM leads to accumulation of DNA damage and p53-dependent apoptosis, thus preventing the possibility of culturing NPM$^{-/-}$ cells, whereas the transfer of the NPM$^{-/-}$ mutation into a p53$^{-/-}$ background (dKO) allows propagation of NPM-deficient cells in vitro (13). As expected, exogenously expressed GFP-NPM1 or GFP-NPM2 localized in the nucleolus or in the nucleoplasm, respectively (Fig. 4A, l and o). Strikingly, instead, the NPMmut was exclusively cytoplasmic in the dKO cells (Fig. 4A, g) and caused the cytoplasmic delocalization of Arf (Fig. 4A, h), suggesting that the NPMmut interacts directly with Arf. This was then confirmed by coimmunoprecipitation experiments showing that NPMmut, NPM1, or NPM2 all form stable complexes with Arf in the dKO cells (Fig. 4B). Moreover, the restoration of NPM1 (Fig. 4A, m) or NPM2 (Fig. 4A, p) expression in

**Figure 3.** Decreased stability and reduced biological activity of cytoplasmic p19\textsuperscript{Arf}. A, left and middle, Western blotting analysis of p19\textsuperscript{Arf} and NPM expression in NIH3T3 cells cotransfected with p19\textsuperscript{Arf} and a control vector (Arf) or p19\textsuperscript{Arf} and flag-tagged NPMmut (left) or flag-tagged NPM1 (middle). Transfection efficiency was evaluated and normalized using a cotransfected β-galactosidase expressing plasmid. Representative of three that gave comparable results. Right, Western blotting analysis of p19\textsuperscript{Arf}, NPM, p21, and vinculin expression in MT-Arf cells infected with a control retrovirus (NPMmut: $-$) or with a retrovirus expressing GFP-NPMmut (NPMmut: $+$) and treated (Zn: $+$) or not (Zn: $-$) for 24 hours with 80 μmol/L ZnSO\textsubscript{4}. B, Western blotting analysis of p19\textsuperscript{Arf} and tubulin expression in M-Arf cells infected with the control retrovirus or with a retrovirus expressing NPMmut, as indicated. Cells were treated 24 hours with 80 μmol/L ZnSO\textsubscript{4}, and then with 100 μmol/L cyclohexamide (CHX). Cells were harvested at the indicated time points, lysed, and analyzed by Western blotting, as indicated. C, left, evaluation of BrdUrd-positive cells in MT-Arf cells infected with a control retrovirus (Ctrl.), with retroviruses expressing GFP-NPMmut (NPMmut) or GFP-NPM1 (NPM1), and treated (Zn: $+$) or not (Zn: $-$) for 24 hours with 80 μmol/L ZnSO\textsubscript{4}. The percentage of BrdUrd positive cells in the ZnSO\textsubscript{4}-treated samples has been normalized with that of the corresponding untreated cells (considered as 100%).*, $P = 0.0031$, statistical significance of the difference in BrdUrd-positive cells in the control and NPMmut cells after Zn induction. The experiment has been done twice, each time in triplicate; ~200 cells have been counted for each sample. Right, the same cell samples as described in the left panel have been analyzed by Western blotting for the indicated proteins.
NPM-deficient cells caused a marked increase in the levels of nucleolar or nucleoplasmic Arf, respectively, whereas NPMmut had no effects on p19Arf protein levels (Fig. 4A, h). Taken together, these findings indicate that the effects of the NPMmut on Arf localization is not exerted through its interaction with endogenous NPM and support a model, whereby NPMmut competes with WT NPM for Arf binding and targets Arf to the cytoplasm, where it becomes more susceptible to degradation.

**Altered nuclear-cytoplasmic shuttling of NPM.** In the absence of endogenous NPM (dKO cells), the NPMmut localized exclusively in the cytoplasm, without any accumulation in the nucleoli or in the nucleoplasm (Fig. 5A). The lack of nucleolar staining of the NPMmut was expected, because the region of NPM1 responsible for its nucleolar localization is partially lost in the NPMmut (Fig. 1B). Consistently, NPM2, which lacks this same region, is also nuclear diffuse in dKO cells (Fig. 5A). However, the inability of NPMmut to enter the nucleus is surprising, because it retains the two NPM nuclear localization signals (Fig. 1B). NPM is a protein that continuously shuttles between nucleus and cytoplasm, and that, at the steady state, is predominantly localized in the nucleus. Therefore, we investigated whether NPMmut retains the ability of WT NPM to shuttle between nucleus and cytoplasm. Controls and dKO cells were transfected with GFP-NPMmut and then treated with leptomycin, a drug that inhibits nuclear export (19). Strikingly, treatment with leptomycin leads to a massive accumulation of the NPMmut in the nucleus, both in the presence and in the absence of endogenous NPM (Fig. 5B). Likewise, cytoplasmic Arf was entirely relocated to the nucleus (Fig. 5C). These findings show that NPMmut is still able to shuttle between nucleus and cytoplasm, and that the leukemia-associated mutation imposes a predominant cytoplasmic localization at the steady state. Because NPMmut forms a stable complex with Arf, it is then expected that also Arf becomes cytoplasmic in cells expressing NPMmut (as shown in Fig. 5C).

**Discussion**

Arf is a potent tumor suppressor that protects cells from oncogenic conversion, through either p53-dependent and p53-independent pathways (20). Biologically, activation of Arf induces cell cycle arrest or apoptosis, two cellular responses that depend on intact p53 and require inactivation of the Mdm2 oncogene, a critical factor in the termination of the p53 response. Although the underlying mechanisms are not yet fully elucidated, they involve binding of Arf to Mdm2 in the nucleus, thus allowing dissociation of the p53/mdm2 complex and stabilization of p53 (21).

It has been hypothesized that disruption of the ARF/Mdm2/p53 pathway is a critical step during transformation. Indeed, genetic alterations leading to inactivation of p53 or Arf, or to increased expression of Mdm2, are found in most human tumors (22). A notable exception is represented by hematopoietic tumors, including AMLs carrying mutations of NPM (23), where...
genetic alterations of members of the ARF/Mdm2/p53 pathway are relatively rare. In these tumors, however, other genetic alterations might be selected, which lead to the functional inactivation of the ARF/Mdm2/p53 pathway. For example, the leukemia-associated bcr-abl or PML-RAR oncogenes have been shown to activate pathways leading to functional inactivation of p53 (24, 25). We reported here that the ability of Arf to stabilize p53 is diminished when Arf is expressed in the presence of the mutated form of NPM, and that under these circumstances, the resulting levels of the p53 target gene p21 are reduced, as well as the ability of p19Arf to induce cell cycle arrest. These findings show that cytoplasmic NPM interferes with the biological activity of p19Arf, thus suggesting that one possible mechanism through, which mutated NPM contributes to leukemogenesis in AMLs is by antagonizing the tumor suppressor function of p19Arf.

We showed that mutated NPM exerts two distinct effects on p19Arf: cytoplasmic delocalization and decreased protein stability, both of which might well affect the function of p19Arf. The AML-associated mutations of NPM create a de novo nuclear export signal at its NH2 terminus, which leads to increased Crm1-dependent export of the altered protein to the cytoplasm (26). The NPM mutation, however, does not affect the ability of NPM to form a complex with p19Arf in vivo, as shown by the fact that the mutated NPM forms a complex with p19Arf in cells lacking expression of WT NPM. Therefore, it seems that the mutated NPM sequesters p19Arf in the cytoplasm by competing with the WT protein, which remains instead mainly localized in the nucleus.

Figure 5. NPMmut shows altered nucleus-cytoplasmic shuttling properties. A, fluorescence analysis of dKO cells transfected with GFP-NPMmut (a), GFP-NPM1 (e), and GFP-NPM2 (g), or infected with untagged NPMmut (pinco vector; c). The infected cells expressed GFP (green staining merged with 4,6-diamidino-2-phenylindole (dapi)) as marker and the NPMmut protein (as revealed by staining with the NPMa antibody; red staining). B, immunofluorescence analysis of p53-/- and dKO MEFs transfected with GFP-NPMmut treated or not with leptomycin for 8 hours, as indicated. C, immunofluorescence analysis of p53-/- cells transfected with GFP-NPMmut, treated or not for 8 hours with leptomycin and stained with an anti-p19Arf specific antibody (red), as indicated.
nucleus. Because p19Arf prevents Mdm2-mediated ubiquitination (and degradation) of p53 by interacting with Mdm2 in the nucleus (27), it is expected that the delocalization of p19Arf to the cytoplasm by mutated NPM antagonizes Arf-mediated activation of p53.

We have recently shown that the half-life of p19Arf is markedly reduced in cells deficient for NPM expression, suggesting that NPM contributes to the stabilization of p19Arf (13). Because NPM possesses chaperone-like activities and forms high-stoichiometry complexes with p19Arf (28), it is possible that NPM functions as a chaperone for p19Arf, allowing its correct folding and/or protecting it from degradation. This function of NPM, however, is lost in the mutant NPM, despite its ability to form a stable complex with p19Arf. Treatment of cells expressing mutant NPM with leptomycin induces the nuclear relocalization of both NPMmut and p19Arf, and, notably, increases the levels of p19Arf, thus suggesting that the degradation of p19Arf in the presence of the mutated NPM is due to its cytoplasmic localization. Although further studies are required to elucidate the molecular mechanisms involved in the degradation of p19Arf in AMLs carrying mutated NPM, they might provide novel targets for the reactivation of the p19Arf/p53 pathway in AMLs with mutated NPM.

Acknowledgments

Received 7/11/2005; revised 10/31/2005; accepted 1/11/2006.

Grant support: Associazione Italiana per la Ricerca sul Cancro, Ministero dell'Istruzione, dell'Università e della Ricerca, and EC (P.G. Pelicci).

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