Nucleophosmin and its AML-associated mutant regulate c-Myc turnover through Fbw7γ

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Mutations leading to aberrant cytoplasmic localization of nucleophosmin (NPM) are the most frequent genetic alteration in acute myelogenous leukemia (AML). NPM binds the Arf tumor suppressor and protects it from degradation. The AML-associated NPM mutant (NPMmut) also binds p19Arf but is unable to protect it from degradation, which suggests that inactivation of p19Arf contributes to leukemogenesis in AMLs. We report here that NPM regulates turnover of the c-Myc oncoprotein by acting on the F-box protein Fbw7γ, a component of the E3 ligase complex involved in the ubiquitination and proteasome degradation of c-Myc. NPM was required for nucleolar localization and stabilization of Fbw7γ. As a consequence, c-Myc was stabilized in cells lacking NPM. Expression of NPMmut also led to c-Myc stabilization because of its ability to interact with Fbw7γ and delocalize it to the cytoplasm, where it is degraded. Because Fbw7γ induces degradation of other growth-promoting proteins, the NPM–Fbw7γ interaction emerges as a central tumor suppressor mechanism in human cancer.

Introduction

Mutations of the nucleophosmin (NPM) gene occur in ~35% of acute myelogenous leukemias (AMLs) and are mutually exclusive with the major AML-associated genetic abnormalities, which suggests that they represent an initiating event in myeloid leukemogenesis (Falini et al., 2005). The underlying mechanisms, however, remain unknown.

NPM is a ubiquitously expressed nucleolar protein that functions as a molecular chaperone (Okuwaki et al., 2001) and shuttles between the nucleus and cytoplasm (Borer et al., 1989). It is part of a high–molecular weight complex and physically interacts with many cellular proteins including p53 (Colombo et al., 2002), Mdm2 (Kurki et al., 2004), and Arf (Itahana et al., 2003). NPM binds Arf and protects it from degradation (Kuo et al., 2004). In NPM−/− cells, the Arf protein looses its nucleolar localization and becomes markedly unstable, which suggests that NPM is required for correct localization and stability of Arf (Colombo et al., 2005). This function of NPM is lost in mutant NPM (NPMmut), which contains a de novo nuclear export signal and mainly localizes in the cytoplasm (Mariano et al., 2006). NPMmut competes with wild-type (WT) NPM for Arf binding and targets Arf to the cytoplasm, where it becomes more susceptible to degradation (Colombo et al., 2006).

Preliminary evidence suggests, however, that NPM controls other intracellular pathways that negatively regulate cell proliferation. In NPM−/− embryos, cells proliferate more actively, accumulate DNA damage, and undergo p53-dependent apoptosis (Colombo et al., 2005), a picture that is reminiscent of the DNA damage response induced by oncogene expression in primary cells (Bartkova et al., 2005). NPM−/− cells also show aberrant mitotic figures with multiple centrosomes (Grisendi et al., 2005) and are more susceptible to transformation by activated oncogenes such as Myc and Ras (Colombo et al., 2005). Consistently, NPM± mice show accelerated Myc-induced lymphomagenesis (Grisendi et al., 2005). We report here that NPM and its AML-associated mutant regulate the stability of the c-Myc protein.

Results and discussion

We initially analyzed levels of c-Myc in lysates from NPM−/− and WT whole embryos. Western blotting (WB) revealed a

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de novo protein synthesis widely used to measure c-Myc protein half-life; Popov et al., 2007) and lysed at different time points to follow c-Myc protein degradation. As an internal control, we monitored expression of \(\beta\)-tubulin (Colombo et al., 2005).

WB in the \(p53^{-/-}\) MEFS confirmed that c-Myc is an unstable protein with a half-life of \(20\) min (Gregory and Hann, 2000). In the dKO cells, instead, the c-Myc protein appeared to be more stable, with a calculated half-life of \(40\) min (Fig. 1f). It appears, therefore, that NPM regulates stability of the c-Myc protein. To confirm the NPM dependency, we reconstituted NPM expression in dKO cells and measured levels of c-Myc protein, c-Myc protein stability, and c-Myc target gene expression.

Notably, NPM reexpression in \(NPM^{-/-}\) MEFS reduced the half-life of the c-Myc protein (Fig. 1g, left; and Fig. 1h), its steady-state and serum-stimulated levels (Fig. 1g, middle), and expression of c-Myc target genes after serum stimulation (Fig. 1g, right). Finally, to investigate the effects of p53 on c-Myc expression, we evaluated levels of c-myc mRNA/protein and c-Myc protein in WT and \(p53^{-/-}\) MEFS. As shown in Fig. S1a (available at http://www.jcb.org/cgi/content/full/jcb.200711040/DC1), a lack of p53 led to increased levels of c-Myc mRNA and protein, as previously described (Ho et al., 2005). Notably, marked increase of the c-Myc protein in the \(NPM^{-/-}\) samples (Fig. 1a, left). Levels of c-myc mRNA, instead, were equivalent in the two samples (Fig. 1a, right), which suggests that the increased levels of c-Myc protein in the \(NPM^{-/-}\) embryos were caused by enhanced protein stability. Accordingly, we observed a moderate increase in mRNA expression for several c-Myc target genes in \(NPM^{-/-}\) embryos (Fig. 1b).

We then examined c-Myc levels in cultured fibroblasts (mouse embryonic fibroblasts [MEFs]) from double knockout \(NPM^{-/-}; p53^{-/-}\) embryos (dKO) and, as controls, MEFs from \(p53^{-/-}\) embryos. \(NPM^{-/-}\) MEFS, in fact, do not grow in culture due to the accumulation of DNA damage and rapid acquisition of a p53-dependent senescence-like phenotype (Colombo et al., 2005). c-Myc protein was elevated in dKO MEFs as compared with the \(p53^{-/-}\) cells (Fig. 1c, left) in the absence of significant variations of the amount of c-myc transcripts (Fig. 1c, right). dKO MEFs also showed increased binding of c-Myc to target promoters, as assayed by chromatin immunoprecipitation (ChIP; Fig. 1d), and an increased induction of c-Myc target genes after serum stimulation of starved cells (Fig. 1e).

To measure the half-life of the c-Myc protein, dKO and control cells were treated with cycloheximide (CHX; an inhibitor of
its stabilization (Fig. 2 a), and binding to target promoters (Fig. 2 b). Overexpression of Fbw7γ reduced Myc-ER protein stabilization and promoter occupancy in p53−/− cells (Fig. 2, a and b) but had no effects in dKO cells (Fig. 2, a and b). The same experiments were repeated in the dKO and p53−/− parenteral cells. Overexpression of Fbw7γ slightly yet consistently reduced levels of endogenous c-Myc protein and c-Myc occupancy at target promoters in the p53−/− cells but not in dKO cells (Fig. 2, c and d). It appears, therefore, that NPM expression is critical for the ability of Fbw7γ to regulate c-Myc protein levels.

We then analyzed whether NPM interacts physically with Fbw7γ and affects the localization and expression levels of Fbw7γ. Coimmunoprecipitation experiments revealed that NPM forms a complex in vivo with ectopically expressed tagged Fbw7γ (Fig. 2 e, left). The interaction is likely to be direct, as recombinant GST-NPM bound in vitro translated 35S-labeled Fbw7γ or Fbw7α and equal amounts of GST or GST-NPM proteins. Data represent the mean of three determinations ± SEM.
Fbw7γ/H9253, instead, had no effect on OHT-treated dKO;Myc-ER cells (Fig. 3 e). In summary, NPM positively regulates the nucleolar localization and stability of the Fbw7γ/H9253 protein and is essential for the ability of Fbw7γ to promote c-Myc degradation and limit cell transformation. The effect of NPM on c-Myc protein stability appears to be specifically exerted through Fbw7γ/H9253, as we found no effects of NPM on expression and localization of Skp2 (Fig. S1 f).

c-Myc is frequently overexpressed in human cancers (Adhikary and Eilers, 2005). c-Myc protein and/or RNA are also frequently overexpressed in AMLs, though the underlying molecular mechanisms are unknown (Hoffman et al., 2002). We thus investigated whether expression of the AML-associated mutant of NPM leads to increased c-Myc expression. Ectopic expression of NPMmut in WT MEFs increased the steady-state because Fbw7α and Fbw7β showed unchanged localization patterns in dKO and control cells (Fig. 3 a, bottom left and bottom right). Fbw7γ levels in the dKO MEFs were also consistently lower than in the control p53−/− cells (Fig. 3 b, left), whereas mRNAs were comparable (Fig. 3 b, right). CHX treatment revealed that the half-life of Fbw7γ in p53−/− and dKO MEFs was ~80 min but was reduced in the dKO to ~30 min (Fig. 3 c). The half-life of Fbw7γ protein was not influenced by expression of p53 (Fig. S1 e). Accordingly, Fbw7γ-dependent ubiquitination of c-Myc was reduced in NPM-null cells (Fig. 3 d). Finally, we analyzed the ability of Fbw7γ to inhibit c-Myc-dependent transformation. Overexpression of Fbw7γ induced a significant attenuation of the transformed phenotype in OHT-treated p53−/−-Myc-ER cells, as shown by their reduced ability to form colonies in semisolid medium (Fig. 3 e). Overexpressed Fbw7γ, instead, had no effect on OHT-treated dKO;Myc-ER cells (Fig. 3 e). In summary, NPM positively regulates the nucleolar localization and stability of the Fbw7γ protein and is essential for the ability of Fbw7 to promote c-Myc degradation and limit cell transformation. The effect of NPM on c-Myc protein stability appears to be specifically exerted through Fbw7γ, as we found no effects of NPM on expression and localization of Skp2 (Fig. S1 f).

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Moreover, in the presence of NPMmut, levels of Fbw7/H9253 protein were almost undetectable without changes of its mRNA (Fig. 5a). Treatment of the same cells with the MG132 proteasome inhibitor for 2 h was sufficient to reconstitute Fbw7/H9253 expression at levels similar to those observed in control cells without NPMmut (Fig. 5b). It appears, therefore, that NPMmut induces destabilization of Fbw7/H9253 and, as a consequence, stabilization of c-Myc.

Finally, we investigated the mechanism through which NPMmut affects Fbw7/H9253 protein stability. Coimmunoprecipitation experiments in NPM-deficient MEFs revealed that NPMmut is able to form a complex with Fbw7/H9253 both in the absence (Fig. 5c) or presence (not depicted) of WT NPM. IF analysis of the intracellular localization of Fbw7/H9253 in p53/H11002/dKO showed that expression of NPMmut causes, in both cell types, a partial displacement of Fbw7/H9253 from the nucleus to the cytoplasm (Fig. 5d and Fig. S2a, available at http://www.jcb.org/cgi/content/full/jcb.200711040/DC1). These results were confirmed by WB analysis of the levels of Fbw7γ in nuclear and cytoplasmic fractions of p53/H11002 and dKO cells in the presence or absence of NPMmut expression (Fig. 5e and Fig. S2b).

The ability of NPMmut to delocalize Fbw7γ and to form a complex in cells also in the presence of the WT NPM suggest that NPMmut competes with the WT protein to bind Fbw7γ. Consistently, we observed that up-regulation of c-Myc protein levels of the c-Myc protein in the absence of significant variations of the amount of c-Myc transcripts (Fig. 4a) and the expression of several Myc-target genes (Fig. 4b). These effects were maintained in Arf/H11002 MEFS (Fig. 4e) and were thus independent on the presence of Arf in the nucleolus or its interaction with Myc (Qi et al., 2004).

To gain evidence that this effect of NPMmut on c-Myc is biologically relevant, we analyzed growth of fibroblasts ectopically expressing NPMmut. Overexpression of c-Myc induces DNA damage (Dominguez-Sola et al., 2007) and, in primary fibroblasts, a p53-dependent cellular checkpoint mainly characterized by a reduction of cell proliferation; when cells are grown in low serum, it also induces apoptosis (Zindy et al., 1998). Ectopic expression of the NPMmut in MEFs induced no modifications of Arf protein levels (Fig. S1g), it increased the levels of γH2AX (a marker of DNA damage) and phosphorylated p53 (Fig. S1g), decreased the proliferation rate, and, in low serum conditions, induced massive apoptosis (Fig. 4d). Together, these results suggest that apoptosis induced by NPMmut expression follows c-Myc stabilization, induction of DNA damage, and activation of p53.

We then investigated whether the effect of NPMmut on c-Myc stability is mediated by Fbw7γ. The ability of overexpressed Fbw7γ to down-regulate Myc-ER levels was completely lost in the presence of NPMmut expression (Fig. 5a). Moreover, in the presence of NPMmut, levels of Fbw7γ protein were almost undetectable without changes of its mRNA (Fig. 5a). Treatment of the same cells with the MG132 proteasome inhibitor for 2 h was sufficient to reconstitute Fbw7γ expression at levels similar to those observed in control cells without NPMmut (Fig. 5b). It appears, therefore, that NPMmut induces destabilization of Fbw7γ and, as a consequence, stabilization of c-Myc.

Finally, we investigated the mechanism through which NPMmut affects Fbw7γ protein stability. Coimmunoprecipitation experiments in NPM-deficient MEFs revealed that NPMmut is able to form a complex with Fbw7γ both in the absence (Fig. 5c) or presence (not depicted) of WT NPM. IF analysis of the intracellular localization of Fbw7γ in p53/H11002 and dKO showed that expression of NPMmut causes, in both cell types, a partial displacement of Fbw7γ from the nucleus to the cytoplasm (Fig. 5d and Fig. S2a, available at http://www.jcb.org/cgi/content/full/jcb.200711040/DC1). These results were confirmed by WB analysis of the levels of Fbw7γ in nuclear and cytoplasmic fractions of p53/H11002 and dKO cells in the presence or absence of NPMmut expression (Fig. 5e and Fig. S2b).

The ability of NPMmut to delocalize Fbw7γ and to form a complex in cells also in the presence of the WT NPM suggest that NPMmut competes with the WT protein to bind Fbw7γ. Consistently, we observed that up-regulation of c-Myc protein

Figure 4. Mutant NPM stabilizes the c-Myc protein. (a, left) WB analysis in WT and NPM± MEFS infected with retroviruses expressing NPMmut (Mut) or control (EV) retroviruses. (right) WB analysis in the same cell. (b) QPCR analysis of the indicated c-Myc target genes. (c) Logarithmic growth curves of NPM± MEFS infected with retroviruses expressing NPMmut or control (EV) retroviruses. 10^6 cells were plated in presence of 10% serum (high serum) or 0.5% serum (low serum) as indicated. (d) Percentage of apoptosis in the same cell lines maintained in low serum culture conditions for 24 h. (e) QPCR analysis of the indicated c-Myc target genes in NPM± MEFS infected with EV or NPMmut retroviruses. (e, left) WB analysis in Arf/H11002, p53/H11002, and dKO MEFS infected with retroviruses expressing NPMmut (mut) or control (EV) retroviruses. (right) c-Myc mRNA levels in the same samples. (f) QPCR analysis of the indicated c-Myc target genes in the same cells as in panel e. Data represent the mean of three determinations ± SEM.
levels (Fig. 4 a) and Myc-target gene expression (Fig. 4 d) by NPMmut were facilitated in NPM+/− MEFs, which carry reduced levels of NPM (Grisendi et al., 2005).

To investigate if the cytoplasmic displacement of Fbw7γ correlates with its degradation, we analyzed levels of the Fbw7γ protein upon treatment of p53−/− and dKO MEFs with the nuclear-export inhibitor leptomycin B (LMB) as compared with treatment with the proteasome inhibitor MG132. As shown in Fig. 5 f and Fig. S2 c, LMB and MG132 increased the levels of Fbw7γ to the same extent in both cell types. However, despite the fact that NPMmut induced degradation of Fbw7γ in dKO cells (Fig. 4 e), it did not increase c-Myc protein levels in the same cells, which is consistent with the observation that, in the absence of WT NPM, the function of Fbw7γ is lost, regardless of its nuclear or cytoplasmic localization (Fig. 2, a–c). These data are consistent with the reported chaperone activity of WT NPM in maintaining the proper folding and activity of its interactors (Szébeni and Olson, 1999). In summary, we observed that expression of NPMmut induces delocalization and accelerated degradation of Fbw7γ also in the absence of the WT NPM protein, thus demonstrating that the effect of the mutant NPM on Fbw7γ is direct. Furthermore, because LMB treatment induced nuclear relocalization of NPMmut and Fbw7γ and restoration of physiological levels of Fbw7γ protein, aberrant cytoplasmic localization of the NPMmut appears critical for the degradation of Fbw7γ.

In conclusion, our findings demonstrate that NPM regulates c-Myc protein stability through its effect on the γ-isofrom of the F-box E3 ubiquitin ligase Fbw7. They are consistent with a model whereby NPM binds directly to Fbw7γ and serves as a molecular chaperone to ensure its proper folding, nucleolar localization, and to prevent its degradation. This effect of NPM on Fbw7γ is relevant for the regulation of one of its substrates, c-Myc, which, in fact, accumulates in the absence of NPM.

The effect of NPM on Fbw7 regulation, however, might be more complex. Other intracellular proteins are also Fbw7-substrates, including Notch, cyclin E, and c-Jun (Nakayama and Nakayama, 2005). Because NPM does not regulate the Fbw7α and Fbw7β isoforms, it might allow regulation of Fbw7γ-specific substrates or the nucleolar site of degradation of Fbw7γ common substrates. Close inspection of the regulation of Notch, cyclin E, and c-Jun protein stability and function in NPM−/− cells might help to elucidate this question.

The leukemia-associated mutant of NPM interacts with Fbw7γ and delocalizes it to the cytoplasm, thus favoring its degradation. As a consequence, c-Myc protein levels are increased in cells expressing the NPMmut. Notably, c-Myc protein is frequently overexpressed in AMLs and stabilized in several leukemia cell lines (Hoffman et al., 2002), and c-Myc overexpression favors myeloid leukemogenesis in mouse models (Luo et al., 2005). Thus, the pathway of c-Myc degradation is relevant for human cancer, which suggests that elevated
c-Myc protein expression might contribute to leukemogenesis in the NPMmut AMLs. Oncogene-induced hyperproliferation activates an Arf- and p53-dependent intracellular checkpoint that leads to cell cycle arrest or apoptosis (Di Micco et al., 2006). Remarkably, the NPMmut also favors Arf degradation, which suggests that in AMLs, a single genetic mutation might have the double effect of activating proliferation and attenuating the resulting checkpoint response. This would probably result in accelerated leukemogenesis and reduced pressure for accumulation of further genetic abnormalities. Interestingly, AMLs with NPMmut usually have normal karyotypes (Falini et al., 2005).

Materials and methods

qChIP and quantitative PCR (QPCR) analysis
MEFs were grown and processed for qChIP analysis as described previously (Frank et al., 2001) using anti-Myc (N-262, Santa Cruz Biotechnology, Inc.) antibodies. Immunoprecipitated DNA from ~1 × 10^6 cell equivalents was resuspended in 300 μl of 10 mM Tris at pH 8.0. Real-time PCR was performed with 6 μl of DNA per reaction and 200 nM of primers, diluted in a final volume of 20 μl of SYBR Green reaction mix (PerkinElmer). Data represent the mean of three determinations ± SEM. A list of the used primers is shown in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200711040/DC1).

Total RNA was extracted from MEFs with an RNAeasy MiniKit (QIA-GEN), including a DNase treatment before elution from the column, and processed as described previously (Frank et al., 2001). Each PCR reaction contained 10 ng of cDNA template and primers at a concentration of 200 nM for SYBR Green reaction mix (PerkinElmer). Data represent the mean ± SEM of three determinations for each gene. A list of the used primers is shown in Table S1.

Cell culture, transfection, and infection
MEFs, Phoenix, and 293T packaging cell lines were cultured at 37 °C and 5% CO_2. Cell culture, transfection, and infection used primers is shown in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200711040/DC1). Viral vector. The mutated form of Fbw7/H9251, Fbw7/H9253, and Fbw7/308 were kindly provided by B. Clurmann (Fred Hutchinson Cancer Research Center, Seattle, WA).

Immunoblotting, immunoprecipitation (IP), and IF
WB, IP, and IF experiments were performed as described previously (Colombo et al., 2002). All protein total lysates for WB analysis were performed in Laemmli buffer. The primary antibodies used for WB were: monoclonal anti-NPM (NPMa that recognizes both WT and mutant NPM, and NPMc that recognizes only the WT NPM1 isoform; Cordell et al., 1999), monoclonal anti-NPMmut specific (homemade according to the protocol described in Quentmeier et al., 2005), anti-Myc (provided by S. Hann, Vanderbilt University School of Medicine, Nashville, TN), anti-flag (Sigma-Aldrich), monoclonal anti-HA (Covance), anti-actin (Sigma-Aldrich), anti-tubulin (Santa Cruz Biotechnology, Inc.). The primary antibodies used for IPs were: monoclonal anti-NPM (Invitrogen), monoclonal anti-NPMmut, monoclonal anti-flag M2 (Sigma-Aldrich), anti-hBcl2 (Santa Cruz Biotechnology, Inc.), and monoclonal anti-HA (Covance). Secondary antibodies conjugated to FITC or Cy3 fluorochromes were used for immunofluorescence. Images were acquired at room temperature using a camera (C4742-95; Hamamatsu) on a microscope (BX61; Olympus) with 60 × 1.40 NA oil objective lenses (Olympus). The acquisition software was Cell F (Olympus).

Plasmids
Fbw7α, Fbw7β, and Fbw7γ flag-tagged plasmids were provided by B. Clurmann (Fred Hutchinson Cancer Research Center, Seattle, WA). Fbw7α and Fbw7γ cDNAs were subcloned in the pCDNA3.1 mammalian expression vector (Invitrogen). Fbw7γ was also subcloned in the pBabe-flag-HA retroviral vector. The mutated form of NPM (NPMmut) was cloned in the pRLLsin.hPGK.IRES.EGFPtr10-hr vector (Follenz et al., 2000) using BamHI restriction sites. The plasmid expressing HA-ubiquitin was kindly provided by S. Pol (Istituto IRCC di Oncologia Molecolare [IFOM], Milan, Italy).

Apoptosis assay
Cells were harvested and double fixed in 1% formaldehyde and then cold ethanol. After permeabilization in 0.1% Triton X-100 for 10 min, cells were stained with anti-cleaved caspase3 antibody (Cell Signaling Technology), washed, and incubated with cy3-conjugated secondary antibody. The percentage of positive cells was evaluated by FACS analysis.

Nuclear and cytoplasmic fractionation
MEFs were washed twice in ice-cold hypotonic buffer (20 mM Hepes, pH 7, 5 mM potassium-acetate, 0.5 mM MgCl2, and 0.5 mM DTT) supplemented with protease inhibitors, and then swollen on ice for 10 min. After buffer removal, cells were collected by scraping and disrupted by vigorous pipetting. The integrity of nuclei was checked with the microscope after trypan blue staining. Nuclei were pelleted at 4,000 rpm for 5 min, and supernatants were stored as cytoplasmic fraction. The nuclear fraction was washed twice with hypotonic buffer and then lysed in Laemmli buffer. Equal volumes of nuclear and cytosolic fractions were loaded on an SDS-polyacrylamide gel for WB analysis.

Statistical analysis
Statistical significance was evaluated by nonparametric Mann-Whitney U test (Wilcoxon/Kruskal Walli) to analyze variables that were not normally distributed. Significance was defined at P < 0.05 (two-tailed test).

Online supplemental material
Fig. S1 shows control experiments regarding the role of p53 in regulating c-Myc and Fbw7γ half-lives, the role of NPM in controlling Skp2 protein localization and stability, and the expression levels of different proteins upon NPMmut expression in MEFs. Fig. S2 shows the effect of NPMmut expression in dKO MEFs on Fbw7γ localization and stability. Table S1 shows all the primers used in the QPCR experiments. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200711040/DC1.

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