

# Nucleophosmin and its AML-associated mutant regulate c-Myc turnover through Fbw7 $\gamma$

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**M**utations 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 Fbw7 $\gamma$ , 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 Fbw7 $\gamma$ . 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 Fbw7 $\gamma$  and delocalize it to the cytoplasm, where it is degraded. Because Fbw7 induces degradation of other growth-promoting proteins, the NPM–Fbw7 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*<sup>−/−</sup> cells, the Arf protein loses 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*<sup>−/−</sup> 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*<sup>−/−</sup> 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* $\pm$  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.

P.G. Pelicci and E. Colombo contributed equally to this paper.

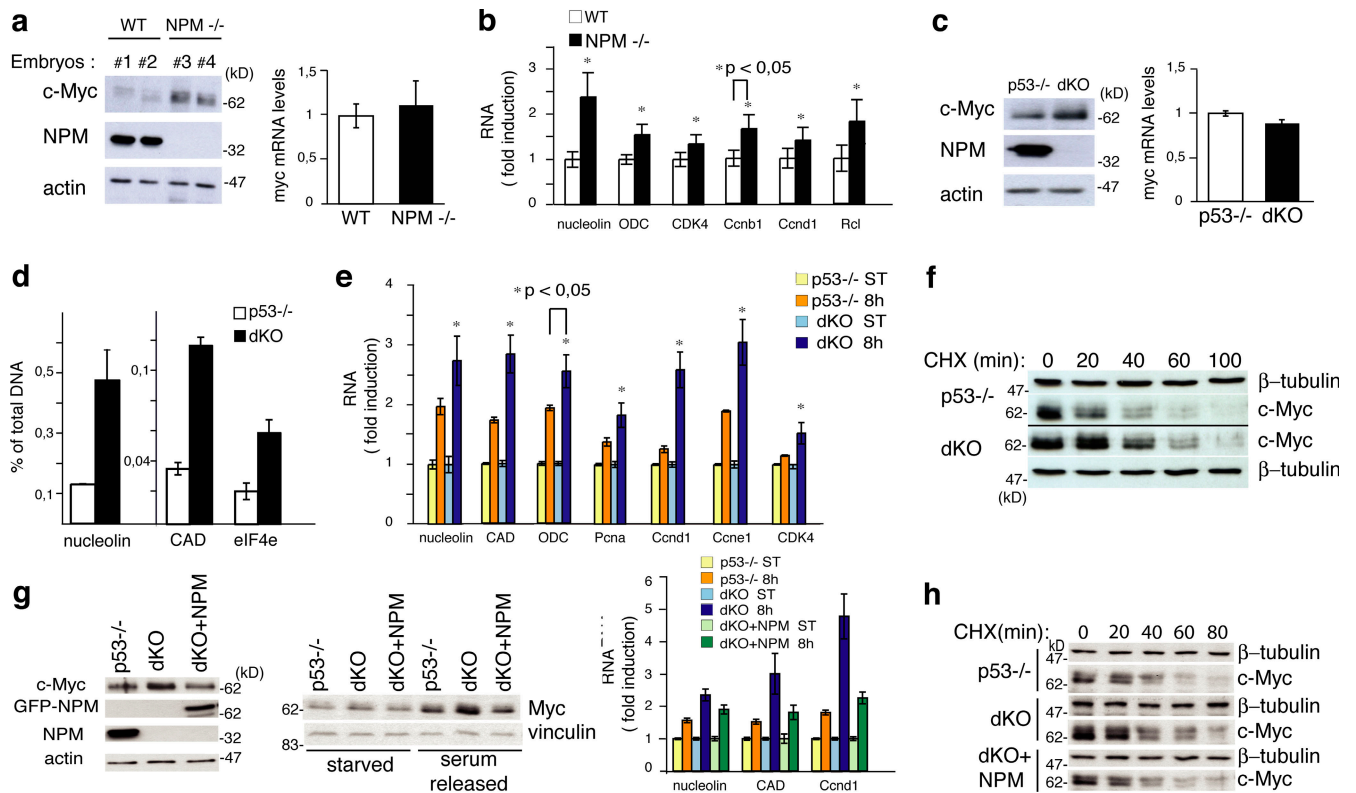
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Abbreviations used in this paper: AML, acute myelogenous leukemia; ChIP, chromatin immunoprecipitation; CHX, cycloheximide; LMB, leptomycin B; MEF, mouse embryonic fibroblast; Myc-ER, Myc–estrogen receptor fusion protein; NPM, nucleophosmin; NPMmut, NPM mutant; OHT, hydroxytamoxifen; QPCR, quantitative PCR; WB, Western blot; WT, wild type.

The online version of this paper contains supplemental material.

## Results and discussion

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

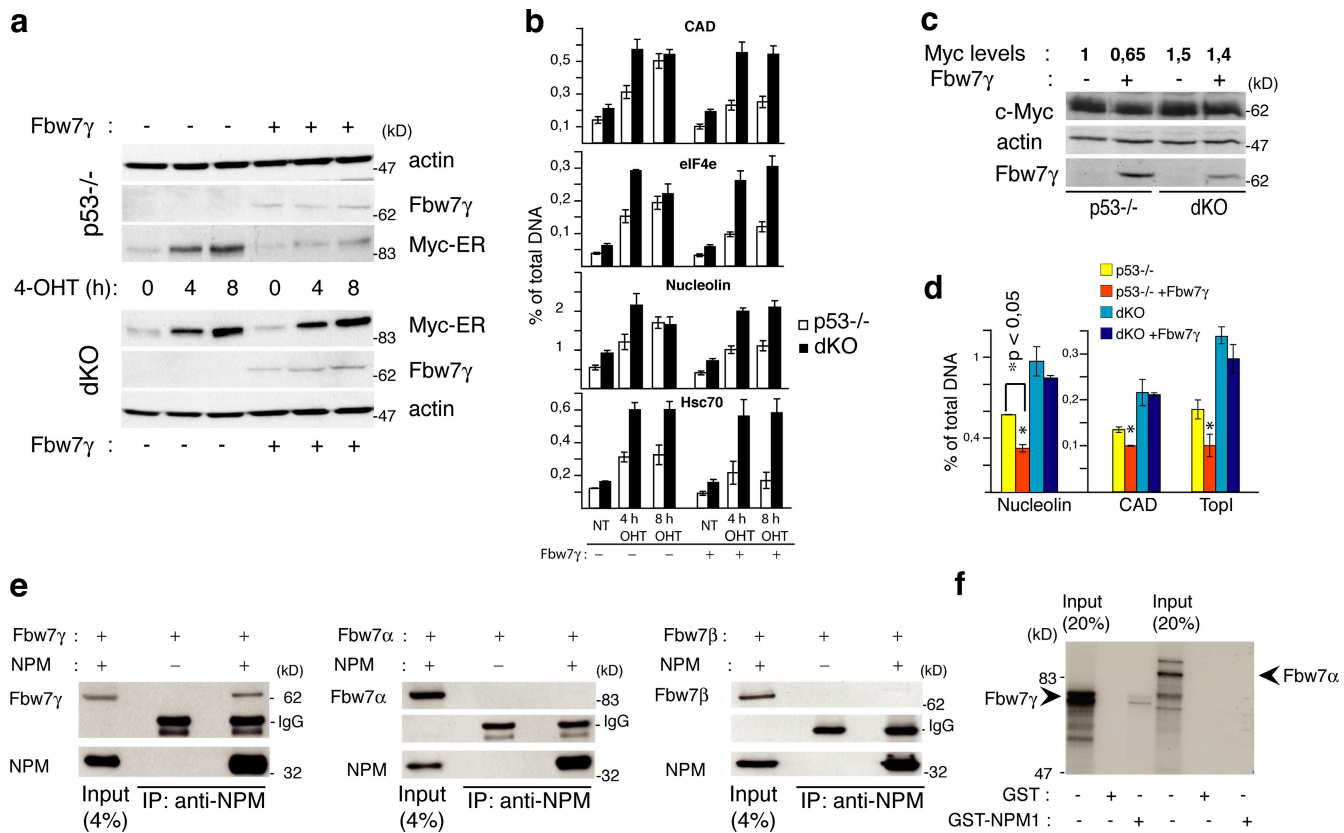


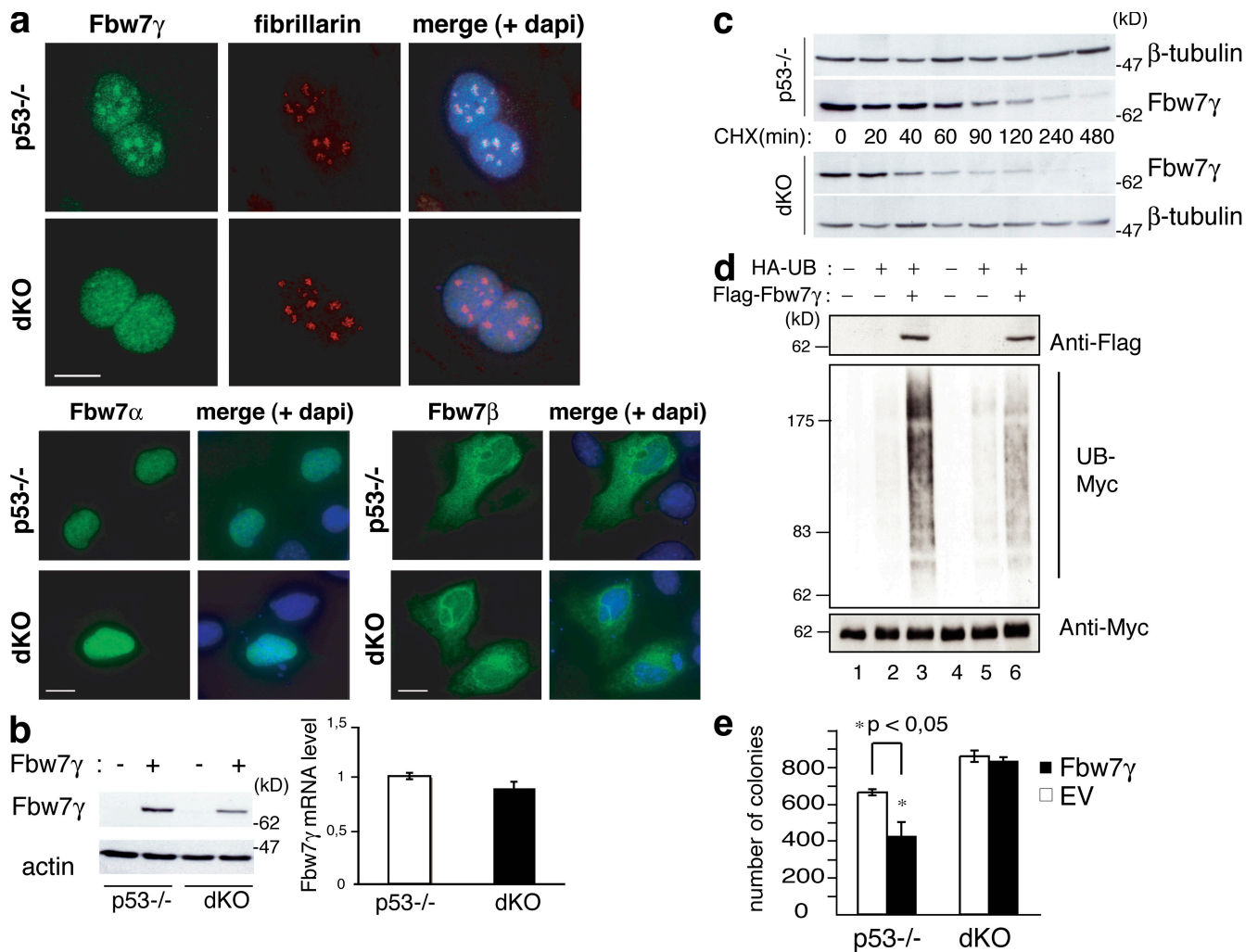
**Figure 1. NPM regulates c-Myc protein stability.** (a, left) WB analysis of two NPM WT (Nos. 1 and 2) and knockout (KO; Nos. 2 and 3) embryos at 10.5 d post coitum (Colombo et al., 2005). (right) *c-Myc* mRNA levels in the same embryos evaluated by QPCR (results are normalized against WT samples). (b) Expression of *c-Myc* target genes analyzed by QPCR using mRNA from WT and NPM KO embryos. Expression was standardized with ubiquitin and normalized against the control WT RNAs. (c) *c-Myc* protein levels (left) and *c-Myc* mRNA levels (right) in *p53*<sup>-/-</sup> and dKO MEFs. (d) QPCR of anti *c-Myc* ChIP. The percentage of DNA bound to *c-Myc* was calculated as described previously (Frank et al., 2001). (e) QPCR analysis of *c-Myc* target genes at 8 h after serum treatment. Results analyzed as in panel b. (f) *c-Myc* protein level in *p53*<sup>-/-</sup> and dKO MEFs. Cells were treated with CHX and harvested at the indicated time points. (g, left) *c-Myc* protein levels in *p53*<sup>-/-</sup> and dKO MEFs infected with retroviruses expressing the GFP-NPM1 protein (+NPM) or control retroviruses. (middle) WB analysis of the same cells during serum starvation and upon serum release. (right) QPCR analysis of *c-Myc* target gene expression after serum treatment (8 h) as in panel d. (h) *c-Myc* protein level in *p53*<sup>-/-</sup>, dKO, and dKO + GFP-NPM MEFs as described in panel f. Data represent the mean of three determinations  $\pm$  SEM.

marked increase of the *c-Myc* protein in the *NPM*<sup>-/-</sup> samples (Fig. 1 a, left). Levels of *c-myc* mRNA, instead, were equivalent in the two samples (Fig. 1 a, right), which suggests that the increased levels of *c-Myc* protein in the *NPM*<sup>-/-</sup> embryos were caused by enhanced protein stability. Accordingly, we observed a moderate increase in mRNA expression for several *c-Myc* target genes in *NPM*<sup>-/-</sup> embryos (Fig. 1 b).

We then examined *c-Myc* levels in cultured fibroblasts (mouse embryonic fibroblasts [MEFs]) from double knockout *NPM*<sup>-/-</sup>;*p53*<sup>-/-</sup> embryos (dKO) and, as controls, MEFs from *p53*<sup>-/-</sup> embryos. *NPM*<sup>-/-</sup> 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*<sup>-/-</sup> cells (Fig. 1 c, left) in the absence of significant variations of the amount of *c-myc* transcripts (Fig. 1 c, right). dKO MEFs also showed increased binding of *c-Myc* to target promoters, as assayed by chromatin immunoprecipitation (ChIP; Fig. 1 d), and an increased induction of *c-Myc* target genes after serum stimulation of starved cells (Fig. 1 e). To measure the half-life of the *c-Myc* protein, dKO and control cells were treated with cycloheximide (CHX; an inhibitor of

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  $\gamma$ -tubulin (Colombo et al., 2005). WB in the *p53*<sup>-/-</sup> MEFs confirmed that *c-Myc* is an unstable protein with a half-life of  $\sim$ 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  $\sim$ 40 min (Fig. 1 f). 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*<sup>-/-</sup> MEFs reduced the half-life of the *c-Myc* protein (Fig. 1 g, left; and Fig. 1 h), its steady-state and serum-stimulated levels (Fig. 1 g, middle), and expression of *c-Myc* target genes after serum stimulation (Fig. 1 g, right). Finally, to investigate the effects of *p53* on *c-Myc* expression, we evaluated levels of *c-myc* mRNA/protein and *c-Myc* protein stability in WT and *p53*-null MEFs. As shown in Fig. S1 a (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,





**Figure 3. NPM is required for Fbw7 $\gamma$  nucleolar localization and stability.** (a) IF analysis of Fbw7 $\gamma$  (top), Fbw7 $\alpha$  (bottom left), and Fbw7 $\beta$  (bottom right) localization in p53 $^{-/-}$  and dKO MEFs transiently transfected with the corresponding flag-Fbw7 constructs (green staining). Anti-fibrillarin staining (red) is shown as a marker for nucleoli. Bars, 10  $\mu$ m. (b, left) WB analysis in p53 $^{-/-}$  and dKO MEFs infected with retroviruses expressing HA-Fbw7 $\gamma$  (+) or control retroviruses (-) as indicated. (right) Fbw7 $\gamma$  mRNA levels in p53 $^{-/-}$  and dKO MEFs. (c) HA-Fbw7 $\gamma$  protein stability in p53 $^{-/-}$  and dKO MEFs. Cells were treated with CHX and harvested at the indicated time points. (d) Effects of Fbw7 $\gamma$  expression on Myc ubiquitination in p53 $^{-/-}$  and dKO cells. p53 $^{-/-}$  (lanes 1-3) and dKO (lanes 4-6) MEFs were transfected with expression vectors for HA-tagged ubiquitin and flag-tagged Fbw7 $\gamma$ , as indicated. Cell lysates were IPed with an anti-Myc antibody and blotted with anti-HA (to identify ubiquitinated myc; UB-myc) and anti-Myc (as control) antibodies. Levels of flag-Fbw7 $\gamma$  in the input were analyzed with an anti-flag antibody. (e) Methylcellulose colony assay of p53 $^{-/-}$  and dKO MEFs expressing Myc-ER protein and infected with control (EV) or HA-Fbw7 $\gamma$ -expressing retroviruses. 4-OHT was added every 3 d; colonies were counted after 15 d. Data represent the mean of three determinations  $\pm$  SEM. \*, P < 0.045.

because Fbw7 $\alpha$  and Fbw7 $\beta$  showed unchanged localization patterns in dKO and control cells (Fig. 3 a, bottom left and bottom right). Fbw7 $\gamma$  levels in the dKO MEFs were also consistently lower than in the control p53 $^{-/-}$  MEFs (Fig. 3 b, left), whereas mRNAs were comparable (Fig. 3 b, right). CHX treatment revealed that the half-life of Fbw7 $\gamma$  in p53 $^{-/-}$  MEFs was  $\sim$ 80 min but was reduced in the dKO to  $\sim$ 30 min (Fig. 3 c). The half-life of Fbw7 $\gamma$  protein was not influenced by expression of p53 (Fig. S1 e). Accordingly, Fbw7 $\gamma$ -dependent ubiquitination of c-Myc was reduced in NPM-null cells (Fig. 3 d). Finally, we analyzed the ability of Fbw7 $\gamma$  to inhibit c-Myc-dependent transformation. Overexpression of Fbw7 $\gamma$  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

Fbw7 $\gamma$ , 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 Fbw7 $\gamma$  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 Fbw7 $\gamma$ , 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

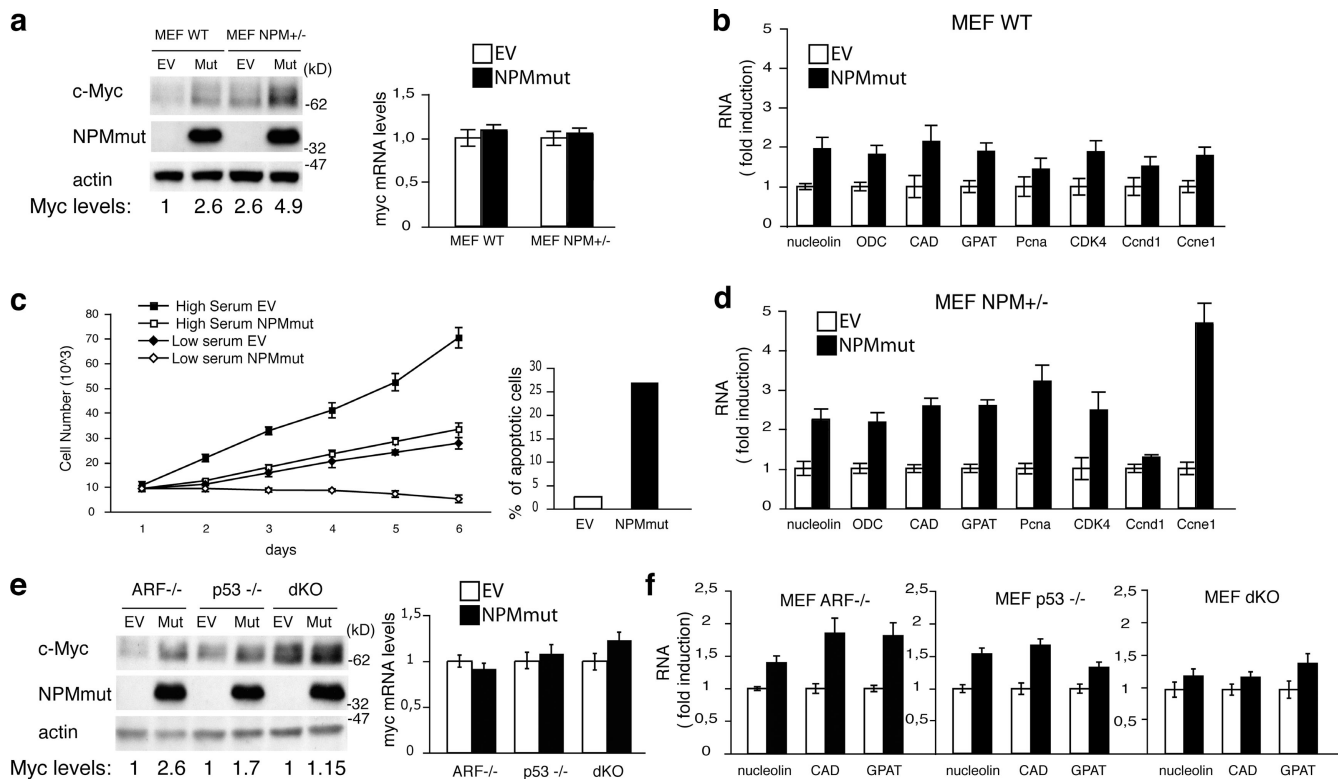


Figure 4. **Mutant NPM stabilizes the c-Myc protein.** (a, left) WB analysis in WT and *NPM* $\pm$  MEFs infected with retroviruses expressing NPMmut (Mut) or control (EV) retroviruses. (right) c-Myc mRNA levels in the same cell. (b) QPCR analysis of the indicated c-Myc target genes. (c, left) Growth curves of *NPM* $\pm$  MEFs infected with retroviruses expressing NPMmut or control (EV) retroviruses.  $10^4$  cells were plated in presence of 10% serum (high serum) or 0.5% serum (low serum) as indicated. (right) The percentage of apoptosis in the same cells maintained in low serum culture conditions for 24 h. (d) QPCR analysis of the indicated c-Myc target genes in *NPM* $\pm$  MEFs infected with EV or NPMmut retroviruses. (e, left) WB analysis in *ARF* $^{-/-}$ , *p53* $^{-/-}$ , 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  $\pm$  SEM.

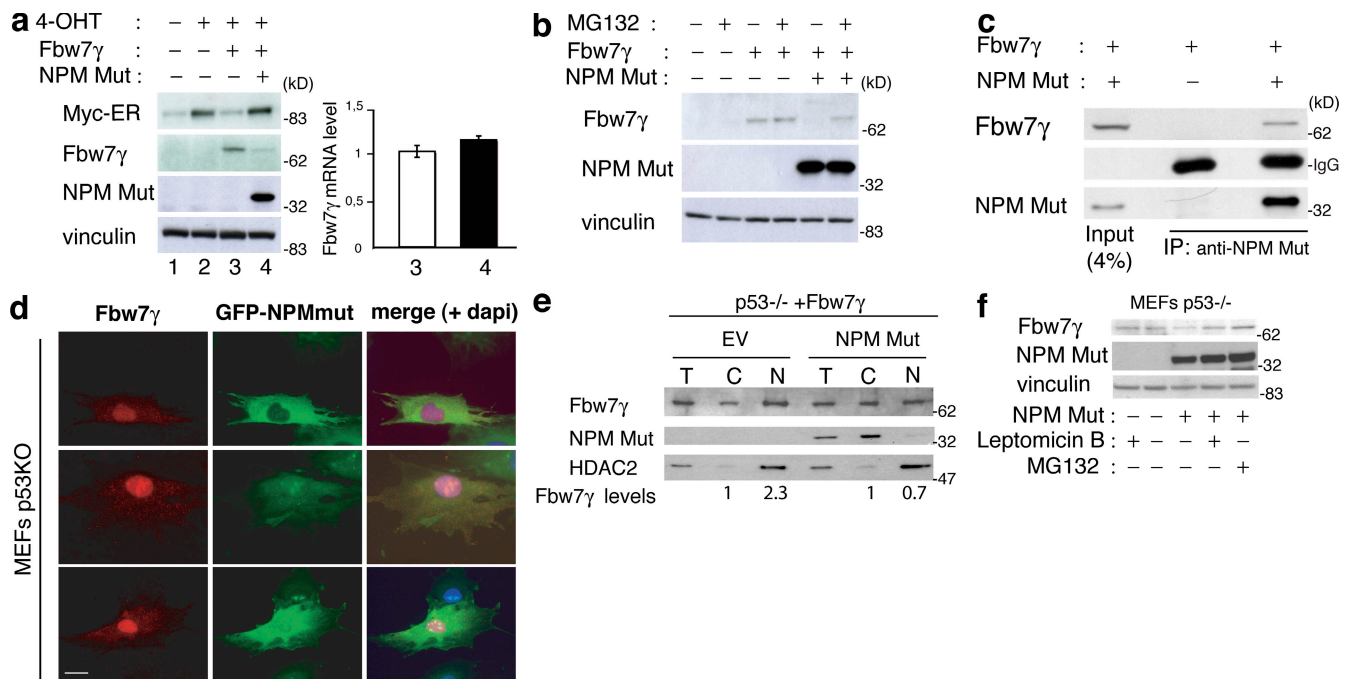
levels of the c-Myc protein in the absence of significant variations of the amount of c-Myc transcripts (Fig. 4 a) and the expression of several Myc-target genes (Fig. 4 b). These effects were maintained in *Arf* $^{-/-}$  MEFs (Fig. 4 e) and were thus independent on the presence of Arf in the nucleolus 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. S1 g), it increased the levels of  $\gamma$ H2AX (a marker of DNA damage) and phosphorylated p53 (Fig. S1 g), decreased the proliferation rate, and, in low serum conditions, induced massive apoptosis (Fig. 4 c). 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 Fbw7 $\gamma$ . The ability of overexpressed Fbw7 $\gamma$  to down-regulate Myc-ER levels was completely lost in the presence of NPMmut expression (Fig. 5 a).

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

Finally, we investigated the mechanism through which NPMmut affects Fbw7 $\gamma$  protein stability. Coimmunoprecipitation experiments in *NPM*-deficient MEFs revealed that NPMmut is able to form a complex with Fbw7 $\gamma$  both in the absence (Fig. 5 c) or presence (not depicted) of WT NPM. IF analysis of the intracellular localization of Fbw7 $\gamma$  in *p53* $^{-/-}$  and dKO showed that expression of NPMmut causes, in both cell types, a partial displacement of Fbw7 $\gamma$  from the nucleus to the cytoplasm (Fig. 5 d and Fig. S2 a, available at <http://www.jcb.org/cgi/content/full/jcb.200711040/DC1>). These results were confirmed by WB analysis of the levels of Fbw7 $\gamma$  in nuclear and cytoplasmic fractions of *p53* $^{-/-}$  and dKO cells in the presence or absence of NPMmut expression (Fig. 5 e and Fig. S2 b). The ability of NPMmut to delocalize Fbw7 $\gamma$  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 Fbw7 $\gamma$ . Consistently, we observed that up-regulation of c-Myc protein



**Figure 5. Mutant NPM delocalizes and destabilizes Fbw7 $\gamma$ .** (a, left) WB analysis in *p53*<sup>-/-</sup>;Myc-ER cells infected with empty (No. 2) or HA-Fbw7 $\gamma$ -expressing (No. 4) retroviruses. HA-Fbw7 $\gamma$ -infected cells were reinfected with empty or NPMmut-expressing lentiviruses (also expressing GFP as marker). Cells were treated for 2 h with 4-OHT (Nos. 2–4). (right) QPCR analysis of *Fbw7 $\gamma$*  mRNA levels in samples Nos. 3 and 4. Data represent the mean of three determinations  $\pm$  SEM. (b) WB analysis in the same cells as in panel a. Cells were or were not treated with MG132 for 2 h, as indicated. (c) dKO MEFs were cotransfected with plasmids expressing flag-Fbw7 $\gamma$  and NPMmut or the empty vector, as indicated. Total lysates and IPs were blotted with antibodies against NPMmut or the flag epitope. (d) IF analysis of *p53*<sup>-/-</sup> MEFs infected with GFP-NPMmut- and flag-Fbw7 $\gamma$ -expressing constructs. A merge of the NPMmut, Fbw7 $\gamma$ , and DAPI staining is also shown. Bar, 10  $\mu$ m. (e) Nucleus–cytoplasm fractionation in *p53*<sup>-/-</sup> MEFs expressing flag-Fbw7 $\gamma$  infected with GFP-NPMmut or control (EV) retroviruses. Total cell lysates (T) or cellular fractions (C, cytoplasm; N, nucleus) were analyzed by WB as indicated. (f) The same cells as in panel e were or were not treated with 1  $\mu$ M LMB or MG132 for 3 h, as indicated. Expression of Fbw7 $\gamma$  was analyzed by WB on total cells lysates using anti-flag antibodies.

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 Fbw7 $\gamma$  correlates with its degradation, we analyzed levels of the Fbw7 $\gamma$  protein upon treatment of *p53*<sup>-/-</sup> and dKO cells 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 Fbw7 $\gamma$  to the same extent in both cell types. However, despite the fact that NPMmut induced degradation of Fbw7 $\gamma$  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 Fbw7 $\gamma$  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 (Szebeni and Olson, 1999). In summary, we observed that expression of NPMmut induces delocalization and accelerated degradation of Fbw7 $\gamma$  also in the absence of the WT NPM protein, thus demonstrating that the effect of the mutant NPM on Fbw7 $\gamma$  is direct. Furthermore, because LMB treatment induced nuclear relocalization of NPMmut and Fbw7 $\gamma$  and restoration of physiological levels of Fbw7 $\gamma$  protein, aberrant cytoplasmic localization of the NPMmut appears critical for the degradation of Fbw7 $\gamma$ .

In conclusion, our findings demonstrate that NPM regulates c-Myc protein stability through its effect on the  $\gamma$ -isoform of the F-box E3 ubiquitin ligase Fbw7. They are consistent with a model whereby NPM binds directly to Fbw7 $\gamma$  and serves as a molecular chaperone to ensure its proper folding, nucleolar localization, and to prevent its degradation. This effect of NPM on Fbw7 $\gamma$  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 Fbw7 $\alpha$  and Fbw7 $\beta$  isoforms, it might allow regulation of Fbw7 $\gamma$ -specific substrates or the nucleolar site of degradation of Fbw7-common substrates. Close inspection of the regulation of Notch, cyclin E, and c-Jun protein stability and function in *NPM*<sup>-/-</sup> cells might help to elucidate this question.

The leukemia-associated mutant of NPM interacts with Fbw7 $\gamma$  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  $\sim 1 \times 10^7$  cell equivalents was resuspended in 300  $\mu$ l of 10 mM Tris at pH 8.0. Real-time PCR was performed with 6  $\mu$ l of DNA per reaction and 200 nM of primers, diluted in a final volume of 20  $\mu$ l of SYBR Green reaction mix (PerkinElmer). Data represent the mean of three determinations  $\pm$  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 RNeasy MiniKit (QIAGEN), 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 800 nM in a final volume of 20  $\mu$ l of SYBR Green reaction mix. Data represent the mean  $\pm$  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<sub>2</sub> in DME supplemented with 10% fetal bovine serum. Empty or recombinant retroviral or lentivirus vectors were transfected into phoenix or 293T packaging cell lines, respectively, and after 48 h, the supernatants were used to infect target cells. Transient transfections were performed using the standard calcium phosphate precipitate method for Phoenix, 293T, and Fugene 6 (Roche) for MEFs.

### 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-fibrillarlin (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 $\times$  1.40 NA oil objective lenses (Olympus). The acquisition software was Cell<sup>+</sup>F (Olympus).

### Plasmids

*Fbw7 $\alpha$* , *Fbw7 $\beta$* , and *Fbw7 $\gamma$*  flag-tagged plasmids were provided by B. Clurmann (Fred Hutchinson Cancer Research Center, Seattle, WA). *Fbw7 $\alpha$*  and *Fbw7 $\gamma$*  cDNAs were subcloned in the pCDNA3.1 mammalian expression vector (Invitrogen). *Fbw7 $\gamma$*  was also subcloned in the pBabe-flag-HA retroviral vector. The mutated form of NPM (NPMmut) was cloned in the pRR1-sin.hPGK.IRES.EGFP.Wpre vector (Follenzi et al., 2000) using BamHI restriction sites. The plasmid expressing HA-ubiquitin was kindly provided by S. Polo (Istituto FIRC 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 cy5-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 MgCl<sub>2</sub>, 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 Wallis) 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 Fbw7 $\gamma$  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 Fbw7 $\gamma$  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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