Cytoplasmic FANCA-FANCC Complex Interacts and Stabilizes the Cytoplasm-dislocalized Leukemic Nucleophosmin Protein (NPMc)*

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Wei Du‡,1, Jie Li‡, Jared Sipple§, Jianjun Chen‡, and Qishen Pang‡,2

From the §Division of Experimental Hematology and Cancer Biology, the Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio 45229, the ¶Section of Hematology/Oncology, the University of Chicago, Chicago, Illinois 60637, and the ¶‡Department of Pediatrics, the University of Cincinnati College of Medicine, Cincinnati, Ohio 45229

Eight of the Fanconi anemia (FA) proteins form a core complex that activates the FA pathway. Some core complex components also form subcomplexes for yet-to-be-elucidated functions. Here, we have analyzed the interaction between a cytoplasmic FA subcomplex and the leukemic nucleophosmin (NPMc). Exogenous NPMc was degraded rapidly in FA acute myeloid leukemia bone marrow cells. Knockdown of FANCA or FANCC in leukemic OCI-AML3 cells induced rapid degradation of endogenous NPMc. NPMc degradation was mediated by the ubiquitin-proteasome pathway involving the IBR-type RING-finger E3 ubiquitin ligase IBRDC2, and genetic correction of FA-A or FA-C lymphoblasts prevented NPMc ubiquitination. Moreover, cytoplasmic FANCA and FANCC formed a cytoplasmic complex and interacted with NPMc. Using a patient-derived FANCC mutant and a nuclearized FANCC, we demonstrated that the cytoplasmic FA NPMc complex was essential for NPMc stability. Finally, depletion of FANCA and FANCC in NPMc-positive leukemic cells significantly increased inflammation and chemoresistance through NF-κB activation. Our findings not only reveal the molecular mechanism involving cytoplasmic retention of NPMc but also suggest cytoplasmic function of FANCA and FANCC in NPMc-related leukemogenesis.

Fanconi anemia (FA)3 is a genetic disorder associated with bone marrow (BM) failure and progression to acute myeloid leukemia (AML) and other cancers (1, 2). FA is caused by a deficiency in any of at least 14 genes, which encode the FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ/BRIP1, FANCL, FANCN, FANCM, FANC/PALB2, and FANCO/RAD51C proteins (3–7). Some of the core complex components also form distinct subcomplexes among themselves or with factors outside the core complex (3, 8, 9). The function of these FA subcomplexes has yet to be elucidated. In the present study, we show that FANCA and FANCC form a cytoplasmic subcomplex, which interacts with and stabilizes the leukemic nucleophosmin (NPM).

Mutation of the NPM gene in exon 12 creates a nuclear export signal and generates an aberrantly cytoplasm-dislocalized NPMc protein, which has been identified in ~35% of adult patients and 6.5% of children with AML (10, 11). The cytoplasmic mutated NPMc is generally associated with a subset of AML characteristic of a normal karyotype and clinically a better response to chemotherapy and a favorable prognosis (11, 12). We performed sequence analysis of BM samples from 46 FA patients and found that NPMc mutations were excluded from FA patients. Significantly, our study indicates that the cytoplasmic FA NPMc subcomplex is required for the stability of the leukemic NPMc.

EXPERIMENTAL PROCEDURES

Patient Samples

A total of 46 FA BM samples were obtained from the Cincinnati Children’s Hospital Medical Center (CCHMC) Fanconi Anemia Comprehensive Care Center in accordance to guidelines from the Institutional Review Board of CCHMC (IRB protocol 2008–1644). A total of 37 AML samples including 28 AML primary patient samples and 9 AML cell lines were included for the study. The AML patient samples were obtained at the time of diagnosis from the University of Chicago Medical Center in accordance to guidelines from the Institutional Review Board of University of Chicago (IRB protocol 6609).

Genomic DNA Sequencing

Genomic DNA from each cell line and bone marrow sample was extracted by using a DNA extraction kit (Qiagen, Valencia, CA) in accordance with the manufacturer’s instructions. NPMc mutation analysis was performed as described previously (13). All PCR products were purified using the Qiagen PCR purifica-
tion kit and directly sequenced. All sequences were compared with the GenBank NPM cDNA sequence.

**Cell Culture**

The HSC 536 lymphoblasts and OCI/AML3 cells were maintained in RPMI 1640 medium with 10% fetal bovine serum, 2 mM glutamine, and 10 units/ml penicillin, 10 μg/ml streptomycin at 37 °C and 5% CO₂. Cells were treated with cycloheximide (CX; 10 μg/ml), doxycycline (Dox; 15–40 ng/ml), MG132 (25 μM), or etoposide (40 μM), and harvested at indicated time points.

**Mutagenesis and Construction of Retroviral Expression Vectors**

The FANCA or FANCC cDNA was amplified by PCR, using Pfu DNA polymerase (Stratagen, La Jolla, CA). The resulting PCR fragments were subcloned into StuI and NotI sites of MIEG3 vector to create MIEG3-FANCA or -FANCC. The NLS sequence (CCGAAGAAGAAGCGAAGGTA) was introduced to the FANCC cDNA by site-directed mutagenesis. The FLAG-tagged NPMc mutagenesis: forward, 5'-TTTGACTACTTTGGCAGAAGAAGTTTGAAAGAAGGA-3', reverse, 5'-GTCCTAGATGCTGCTGAGATGCTGCTGAGATGCTGCTGAC-3'. Sequencing: NPM1-112R (5'-CCTGGGACACCATTTATCAACACACTGC-3').

**NPM and FANCC Mutagenesis**—FLAG-NPM subcloning: forward, 5'-ATTAGGCTGTGAGTCTGACGATGACTGATGACTGATGACTGACAAGA-3'; reverse, 5'-ATAAGGCTGAGTCTGACGATGACTGATGACTGATGACTGAC-3'. FLAG-FANCC-NPM mutation (14) was introduced to the plasmid by PCR mutagenesis. The FLAG-tagged IBRD2 cDNA was subcloned into the EcoRI and Xhol sites of MIEG3 vector to create MIEG3-HF-NPM. The NPMc mutation (14) was introduced to the plasmid by PCR mutagenesis. The FLAG-tagged IBRD2 cDNA was subcloned into the EcoRI and Xhol sites of MIEG3 vector to create MIEG3-HF-IBRD2.

**Retrovirus-mediated Gene Transfer**

Retroviruses were prepared by the Vector Core of Cincinnati Children's Research Foundation (Cincinnati Children’s Hospital Medical Center, Cincinnati, OH). Retroviral supernatant was collected at 36, 48, and 72 h, respectively, after transfection. For retroviral transduction, cells were seeded in fibronectin (8 μg/ml) and an equal volume of medium was then added to the culture. Transduction was repeated two times, and clones stably expressing WT or mutant NPM were selected in medium containing 1 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO).

**Knockdown of FANCA, FANCC, and E3 Ligases by shRNA**

pTRIPZ lentiviral vector targeting human FANCA, FANCC, KPC1, KPC2 were purchased from OpenBiosystems (Huntsville, AL). Hairpin sequence targeting luciferase (CTTCAGCT-GAGTACTTTCA), SFPWBT (ACCTTCCTGGAGAGAGA-AATGC), Nedd4-1 (TTTGAGATAGTTCTTCTAGTGA), or IBRD2 (CGGGTTTATATCGAAGCAGAAT) was also cloned into pTRIPZ empty vector. Cells were transduced with the pTRIPZ shRNA vectors and induced with Dox (Clontech) after puromycin selection and then incubated for 72 h prior to experimentation. For p65 knockdown, cells were transduced with the pGFP-V-RS vector expressing shRNA targeting p65 (purchased from OriGene, Rockville, MD). GFP-positive cells were then sorted and then subjected to experimentation.

**Primer List**

**Genomic Sequencing**—First NPM amplification: NPM1-ex12F (5'-GCCAAATCTTGGCACACTCTA-3'), NPM1-ex12R (5'-TTTACAAGACTATTTGGCACCATTCC-3'). Second NPM amplification: NPM1-ex12F (5'-TTAACTCTCTGGGTAGAATG-3'), NPM1-ex12R (5'-CAAGACTCTTTGGCACCATTCTAAC-3'). Sequencing: NPM1-112R (5'-CCTGGGACACCATTTATCAACACACTGC-3').

**NPM and FANCC Mutagenesis**—FLAG-NPM subcloning: forward, 5'-ATTAGGCTGTGAGTCTGACGATGACTGATGACTGATGACTGACAAGA-3'; reverse, 5'-ATAAGGCTGAGTCTGACGATGACTGATGACTGATGACTGAC-3'. FLAG-FANCC-NPM mutation (14) was introduced to the plasmid by PCR mutagenesis. The FLAG-tagged IBRD2 cDNA was subcloned into the EcoRI and Xhol sites of MIEG3 vector to create MIEG3-HF-IBRD2.

**Immunoblotting and Immunoprecipitation**

Nuclear and cytosolic fractions of the cell extracts were prepared using a nuclear extraction kit (BD Biosciences) according to the manufacturer’s instructions. Approximately 50 μg of cell extracts were resolved on SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblots were probed with antibodies for FLAG (M2; Sigma-Aldrich), NPM (Sigma-Aldrich), NPMc (Novus Bioloficals, Littleton, CO), ubiquitin (Enzo Life Sciences International, Plymouth Meeting, PA), IBRD2 (Abcam, Cambridge, MA), p65 (Abcam), or β-actin (Sigma-Aldrich, St. Louis, MO). For immunoprecipitation, cells were lysed in Nonidet P-40 lysis buffer (20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.15 units/ml aprotinin, 1 mM PMSF, 20 μM leupeptin, 5 μM sodium vanadate). Nuclear and cytoplasmic extracts were prepared and incubated with the antibodies specific for the FLAG tag (M2) for 2 h at 4 °C. The immunocomplexes were examined directly by SDS-PAGE and immunoblotted with antibodies specific for indicated antibodies.
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**Immunocytochemistry**

Cells were cytopspun onto slides and fixed in ice-cold methanol for 5 min in −20 °C. After air drying, cells were blocked for 1 h with 5% normal serum. Then cells were incubated with anti-FLAG antibody (M2) in PBS with 2% normal serum at room temperature for 1 h. After extensive washes, cells were incubated with secondary antibodies (Jackson Immunoresearch, Bar Harbor, ME). DNA was then labeled with DAPI (4,6-diamidino-2-phenylindole; Sigma-Aldrich). Slides were finally mounted in mounting medium (Vector, Burlingame, CA).

Immunofluorescence-labeled cells were viewed and photographed using a Leica DM IRB microscope, and images were captured and processed using a digital camera and the OpenLab 3.1 software (Improvision, Waltham, MA).

**Protein Degradation Assay**

Cell lysates from indicated cells were extracted in the presence of 25 μM MG132 for 4 h followed by immunoprecipitation using anti-FLAG (M2) antibody. The immunocomplexes were then resolved by SDS-PAGE and immunoblotted with antibody specific for ubiquitin (Enzo Life Sciences International, Plymouth Meeting, PA).

**NF-κB Activation**

Nuclear protein extracts were prepared from OCI/AML3 cells using a Transfactor Extraction kit (BD Biosciences). Nuclear extracts were incubated with DNA specific for the NF-κB consensus sequence, and the DNA binding activity of NF-κB was measured using a Transfactor kit.

**Analysis of Inflammatory Gene Expression**

Total RNA was prepared with RNeasy kit (Qiagen) following the manufacturer’s procedure. Following treatment with RNase-free DNase, RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen). Real-time PCR was performed on a ABI PRISM 7700 sequence detection system (Applied Biosystems) with SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer’s instructions, using the primers listed below. Samples were normalized to the level of GAPDH mRNA, and the relative expression levels were determined by the standard curve method.

**Apoptosis Assay**

Cells were cultured in the presence of Dox (40 ng/ml) for 2 days and then treated with vehicle or etoposide (40 μM) for 12 h and harvested for immunostaining using antibody specific for allophycocyanin-conjugated Annexin V and 7-amino-actinomycin (7-AAD) (BD Biosciences) followed by flow cytometry analysis. Annexin V-positive/7-AAD-negative populations were determined as apoptotic cells using FACS Canto (BD Biosciences).

**RESULTS**

**NPMc Mutation Is Excluded from FA Patients**—Because FA can be considered as a preleukemia and patients with FA develop exclusively AML (15), we performed genomic sequencing analysis to screen the leukemic NPMc mutations in FA patients. Totally, 46 primary FA BM samples, 10 cell lines derived from FA patients, and 37 adult non-FA AML samples were examined. The NPMc-positive OCI/AML3 leukemic cell line was used as a positive control (16). Although some non-FA AML samples showed a TCTG duplication (type A NPMc mutation) as described previously (Fig. 1A) (10–12), no NPMc mutations were found in FA patients (Fig. 1B).

**RAPID DEGRADATION OF NPMc IN FA AML PATIENT BM CELLS**—To study NPMc function in FA AML cells, we attempted to express FLAG-tagged NPMc in BM cells from FA-A and FA-C AML patients. We found that NPMc was extremely unstable in FA AML cells. The NPMc-positive OCI/AML3 leukemic cell line was used as a positive control (16). Although some non-FA AML samples showed a TCTG duplication (type A NPMc mutation) as described previously (Fig. 1A) (10–12), no NPMc mutations were found in FA patients (Fig. 1B).

**Rapid Degradation of NPMc in FA AML Patient BM Cells**—To study NPMc function in FA AML cells, we attempted to express FLAG-tagged NPMc in BM cells from FA-A and FA-C AML patients. We found that NPMc was extremely unstable in FA AML cells. In contrast, the wild-type (WT) NPM protein was relatively stable in both FA-A- and FA-C-deficient cells (Fig. 2B). We also confirmed the cytoplasmic localization of ectopically expressed NPMc by immunofluorescence using anti-FLAG antibody (Fig. 2C). Thus, the cytoplasmic NPMc may undergo rapid proteasome-mediated degradation in FA AML cells.

To demonstrate further that FA deficiency was responsible for rapid degradation of NPMc, we used an inducible shRNA lentivirus to knock down FANCA or FANCC in NPMc-positive OCI/AML3 leukemic cells (16). As shown in Fig. 2D, Dox at 20 or 40 ng/ml induced efficient knockdown of FANCA and FANCC in OCI/AML3 leukemic cells. Consistent with this, the level of cytoplasmic NPMc was greatly decreased in FANCA- or FANCC-knockdown cells compared with cells expressing the luciferase control shRNA (Fig. 2D). These results suggest that the FA proteins may be required for NPMc stability. Moreover, we found that the proteasome inhibitor MG132 effectively stabilized NPMc in the FANCA or FANCC knock-down OCI/AML3 cells (Fig. 2E), which further indicates that NPMc degradation is mediated by the ubiquitin-proteasome pathway.

**FIGURE 1. NPMc mutations are excluded from FA patients. A, NPMc mutations in exon 12 of NPM1 gene exclusive from FA patients. Chromatograms of NPM exon 12 sequences from genomic WT cells, OCI/AML3 cells as well as bone marrow samples or cell lines derived from FA patients are shown. Primers specific for NPM1 gene exon 12 were used for first and second amplifications followed by direct DNA sequencing. All sequences were compared with the germ line NPM cDNA sequence (GenBank accession no. NM_002520, version 4). B, summary of genomic DNA sequencing for NPMc mutation. A total of 46 primary FA patient BM samples, 10 FA lymphoblast cell lines, and 37 AML samples including 28 AML primary patient samples (10 with normal karyotype whereas the remaining 18 bearing common chromosomal translocations) and 9 AML cell lines (all bearing common chromosomal translocations) were screened. No NPMc mutations were found in FA cells. Primers specific for NPM1 gene exon 12 (listed under “Experimental Procedures”) were used for first and second amplifications followed by direct DNA sequencing. All sequences were compared with the germ line NPM cDNA sequence (GenBank accession no. NM_002520, version 4).
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Forced Nuclear Localization of FANCC Induces NPMc Degradation—The rapid degradation of NPMc in FA AML cells prompted us to investigate the mechanism of NPMc maintenance. The majority of FANCC is localized in cytoplasm (1, 17). We reasoned that depletion of cytoplasmic FANCC by forcing its dislocalization to the nucleus would render NPMc susceptible to degradation. To test this, we created a FANCC mutant with a nuclear localization signal (FANCC-NLS) (Fig. 3A) and confirmed the expression (Fig. 3B) and the nuclear localization of FANCC-NLS in FANCC-deficient (FA-C) HSC536 cells by both immunostaining (Fig. 3C) and Western blot analysis (Fig. 3D). FANCC-NLS retained its nuclear function, as it could rescue FANCD2 monoubiquitination in HSC 536 cells treated with hydrogen peroxide or hydroxyurea (Fig. 3E) (1, 2, 4). However, FANCC-NLS failed to stabilize NPMc (Fig. 3F), as the level of NPMc was significantly reduced in cells expressing the FANCC-NLS mutant protein after 30 min of CX treatment. Thus, these results indicate that NPMc stability requires cytoplasmic FANCC.

FANCA and FANCC Prevent NPMc Ubiquitination—The observation that ectopically expressed NPMc was stable in the presence of the proteasome inhibitor MG132 in FA AML cells indicates that NPMc underwent ubiquitin-mediated degradation (18). Indeed, we observed significantly increased ubiquitinated NPMc in mutant FA-A and FA-C cells compared with normal cells in the presence of proteasome inhibitor MG132 (Fig. 4A). Complementation of the mutant cells with a functional FANCA or FANCC by retroviral gene transfer prevented NPMc ubiquitination, as the amount of ubiquitinated NPMc in genetically corrected FA-A or FA-C cells was reduced to the basal level shown in normal cells (Fig. 4B). These results suggest that the FA proteins stabilize NPMc through a mechanism involving ubiquitination.

Ubiquitination is a prerequisite to proteosomal targeting (18). To determine whether FA deficiency affects the ubiquitination process, we used shRNAs to knock down several E3 ligases including SCF<sup>F<sub>W87</sub></sup>, IBRDC2, KPC1, KPC2, and Nedd4-1 (19–24) and examined NPMc ubiquitination and stability in FA-A cells. As shown in Fig. 4C, knockdown of IBRDC2, an IBR-type RING-finger E3 ubiquitin ligase (21), significantly reduced NPMc ubiquitination and restored NPMc stability in FA-A cells (Fig. 4C). Conversely, overexpression of IBRDC2 in NPMc-positive OCI/AML3 cells induced NPMc degradation (Fig. 4D). To demonstrate further the involvement of IBRDC2, we determined NPMc ubiquitination in IBRDC2-overexpressing OCI/AML3 cells in the presence of the proteasome inhibitor MG132. As shown in Fig. 4E, overexpression of IBRDC2 resulted in a significant increase in NPMc ubiquitination compared with the vector control. These data indicate that IBRDC2 is the E3 ligase ubiquitinating the cytoplasmic NPMc and provide a potential mechanistic explanation of the
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increased accumulation of ubiquitinated NPMc in FA-deficient cells.

Cytoplasmic FANCA-FANCC Complex Interacts with NPMc—To investigate further how the FA proteins maintain NPMc stability, we determined whether NPMc interacted with cytoplasmic FANCA or FANCC. We expressed FLAG-tagged NPMc in normal lymphoblasts and performed immunoprecipitation with the anti-FLAG antibody. We observed that NPMc interacted with FANCA and FANCC in the cytoplasmic extracts but not in the nuclear fractions (Fig. 5A). To demon-
the nucleus (Fig. 3C). Cells expressing WT-FANCC formed FANCA-FANCC complex in both cytoplasmic and nuclear fractions; whereas the complex was only detectable in nuclear fraction in cells expressing FANCC-NLS (Fig. 5B). As expected, FANCC-L554P failed to interact with FANCA in either cytoplasm or the nucleus (Fig. 5B). We next determined the kinetics of NPMc degradation in HSC 536 cells expressing WT-FANCC, FANCC-L554P, or FANCC-NLS. Significantly, the formation of cytoplasmic FANCA-FANCC complex was required for NPMc stability, as FANCC-L554P and FANCC-NLS failed to prevent NPMc from rapid degradation (Fig. 5C). Together, these results suggest that FANCA and FANCC stabilize NPMc through a mechanism involving the formation of a cytoplasmic complex and interaction with NPMc.

To determine whether other FA mutations affect NPMc stability, we examined the effect of FANCD2 or FANCE deficiency on the formation of cytoplasmic FANCA-FANCC-NPMc complex and consequently stability of the cytoplasmic NPMc. It has been shown that FANCD2 is absolutely required for activation of the FA pathway (26) and that FANCE interacts directly with FANCA in either cytoplasm or the nucleus (Fig. 3C). Cells expressing WT-FANCC formed FANCA-FANCC complex in both cytoplasmic and nuclear fractions; whereas the complex was only detectable in nuclear fraction in cells expressing FANCC-NLS (Fig. 5B). As expected, FANCC-L554P failed to interact with FANCA in either cytoplasm or the nucleus (Fig. 5B). We next determined the kinetics of NPMc degradation in HSC 536 cells expressing WT-FANCC, FANCC-L554P, or FANCC-NLS. Significantly, the formation of cytoplasmic FANCA-FANCC complex was required for NPMc stability, as FANCC-L554P and FANCC-NLS failed to prevent NPMc from rapid degradation (Fig. 5C). Together, these results suggest that FANCA and FANCC stabilize NPMc through a mechanism involving the formation of a cytoplasmic complex and interaction with NPMc.

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FA Deficiency Activates NF-κB and Inflammatory Signaling in NPMc-positive Leukemia—We recently showed that NPM activated NF-κB and inflammatory signaling in Fanc−/− leukemic cells (28). Simultaneously, Cilloni et al. reported that NPMc interacted with NF-κB in the cytoplasm and prevented the nuclear translocation and thus the activation of the transcription factor, which might contribute to increased chemosensitivity observed in NPMc-positive AML patients (29). To investigate the functional relevance of NPMc stabilization by cytoplasmic FANCA and FANCC, we depleted FANCA
or FANCC in NPMc-positive OCI/AML3 leukemic cells by inducible shRNA lentivirus and determined NF-κB activation, inflammatory cytokine expression, and chemotherapeutic agent-induced apoptosis. Correlated with increased NPMc degradation (Fig. 2C), we observed significantly increased the activity of NF-κB (Fig. 6A), the expression of inflammatory cytokines TNF-α and IL-1β (Fig. 6B), and resistance to etoposide-induced apoptosis (Fig. 6C) in OCI/AML3 leukemic cells transduced with either FANCA or FANCC shRNA lentivirus. Furthermore, knockdown of the NF-κB subunit p65 (Fig. 6D) reduced expression of the inflammatory cytokines (Fig. 6E) and increased etoposide-induced apoptosis (Fig. 6F) in the FANCA- and FANCC-knockdown cells. These data provide further evidence that the increased inflammation and apoptotic resistance in the FANCA- or FANCC-knockdown NPMc-positive leukemic cells are due to increased NF-κB activity. Taken together, we conclude that the FANCA and FANCC deficiencies contribute to inflammation and chemotherapeutic resistance in NPMc-positive leukemic cells.

**DISCUSSION**

The present study identifies a potential role of FANCA and FANCC in maintaining the stability of the leukemic NPMc, likely through the formation of a cytoplasmic FANCA–FANCC complex and interaction with NPMc protein. Whether the interaction between cytoplasmic FANCA–FANCC and NPMc blocks proteasome-associated ubiquitination and degradation of NPMc remains to be seen; however, it is clear that NPMc stability requires the formation of a cytoplasmic FANCA–FANCC subcomplex. Certain components of the FA core complex also form distinct subcomplexes among themselves or with factors outside the core complex (3, 8, 9). For example, it has been reported that the members of the eight-component FA core complex form two discrete subcomplexes, one composed of FANCA, FANCB, FANCG, FANCL, and FANCM, and the other consisting of FANCC, FANCE, and FANCF (9). Outside the FA core complex, FANCA, FANCC, and FANCG are found to associate with a variety of cellular factors that primarily function in redox-related processes (30). In the context of DNA damage repair, the FANCA protein has been shown to interact with BRCA1 (31), and the FANCG protein has been shown to interact with XRCC3 (32). Although the exact function of these subcomplexes is not clear, their formation is consistent with the...
presumption that the core complex proteins may also perform other functions in addition to regulation of FANCD2 mono-ubiquitination. In this context, the cytoplasmic FANCA-FANCC complex represents a novel FA protein subcomplex that may function to influence leukemogenesis.

The NPMc mutations are created by two characteristic changes at the C terminus of the NPM gene (33): one is the generation of an additional leucine-rich NES motif, and the other is the loss of Trp285 and Trp290 or Trp290, which are required for NPM nuclear localization. These alterations result in aberrant cytoplasmic NPM localization, which can easily be detected by immunohistochemistry (Fig. 2C). The NPMc mutations have been identified in ~35% of adult patients and 6.5% of children with AML (10, 11). Because the NPMc mutations are AML-specific and because leukemia developed in FA patients is uniformly AML, we screened FA patients for the NPMc mutations. We found no NPMc mutations in FA patients. Interestingly, we found that deficiency in FANCA or FANCC gene led to rapid degradation of NPMc in FA AML patients and NPMc-positive AML cells (Fig. 2). Biochemically, we use a patient-derived FANCC mutant and a nuclearized FANCC to demonstrate that the interaction between FANCA-FANCC and NPMc in the cytoplasm is essential for NPMc stability. However, the functional consequence of FANCA or FANCC deficiency in NPMc-associated leukemogenesis remains to be defined.

The extremely unstable NPMc in FA-deficient cells can be mitigated by the presence of the proteasome inhibitor MG132, suggesting that NPMc degradation is mediated by the ubiquitin-proteasome pathway. We showed that the cytoplasmic NPMc was ubiquitinated in mutant FA-A or FA-C lymphoblasts (Fig. 4A). Furthermore, we observed that genetic correction of the mutant FA-A or FA-C cells with a functional FANCA or FANCC gene, respectively, prevented NPMc ubiquitination (Fig. 4B). These results are consistent with the notion that the FA proteins somehow regulate the ubiquitination process. However, it remains to be established as to whether the FA proteins function in modification of NPMc (through their interaction with NPMc) (Fig. 5A) or regulation of the activity of the responsible ubiquitin ligase (Fig. 4, C–E).

The ubiquitin-proteasome system controls the abundance of many cellular proteins (28). The E3 ligase responsible for NPMc ubiquitination was not known. In the present study, we made an effort to identify the E3 ligase responsible for ubiquitinating NPMc. We found that knockdown of IBRDC2, an IBR-type RING-finger E3 ubiquitin ligase (21), significantly reduced NPMc ubiquitination and restored NPMc stability in FA-deficient cells (Fig. 4C), although overexpression of IBRDC2 in NPMc-positive OCI/AML3 cells induced NPMc ubiquitination and degradation (Fig. 4, D and E). These data not only indicate that IBRDC2 is the E3 ligase ubiquitinating the cytoplasmic NPMc, but also provide a possible mechanistic explanation of the increased accumulation of ubiquitinated NPMc in FA-deficient cells.

To demonstrate that the stabilization of the cytoplasmic NPMc specifically requires FANCA and FANCC, we have examined the formation of cytoplasmic FANCA-FANCC complex and stability of the cytoplasmic NPMc in cells derived from patients carrying FANCD2 or FANCE mutation. FANCD2 is absolutely required for activation of the FA pathway (28), whereas FANCE interacts directly with FANCC in the nucleus (29). Unlike FANCA or FANCE mutation, we observed an undisturbed cytoplasmic FANCA-FANCC complex (Fig. 5D) as well as stable NPMc (Fig. 5E) in the FANCD2 or FANCE mutant cells. This finding further substantiates the notion that stabilization of the cytoplasmic NPMc specifically requires FANCA and FANCC.

We also show that depletion of FANCA or FANCC in NPMc-positive leukemic cells significantly increased inflammation and chemoresistance through a mechanism involving the transcription factor NF-κB (Fig. 6). NF-κB regulates the expression of anti-apoptotic genes and activates many pro-inflammatory cytokines and chemokines, which constitutes a key molecular link between inflammation and tumorigenesis as well as chemoresistance (34). In the context of leukemogenesis, NF-κB activation is known to confer resistance to chemotherapy in AML patients (35). We have recently demonstrated that NPM activated NF-κB and inflammatory signaling in Fanc−/− leukemic cells (28). Simultaneously, another group reported that NPMc interacted with NF-κB in the cytoplasm and thus reduced active nuclear NF-κB, which might contribute to increased chemosensitivity of NPMc-positive leukemic cells (29). Our data demonstrate that depletion of FANCA or FANCC in NPMc-positive OCI/AML3 leukemic cells resulted in NF-κB activation, leading to increased inflammatory cytokine expression and resistance to chemotherapeutic agent-induced apoptosis (Fig. 6, B and C). Conversely, knockdown of p65 reduced expression of the inflammatory cytokines and increased etoposide-induced apoptosis in the FAANCA and FANCC knockdown cells (Fig. 6, E and F). Therefore, the suppression of NF-κB-mediated cell signaling may offer an exciting opportunity for therapeutic intervention in FA AML patients. In the meantime, this also raises the issue of whether these results are physiologically relevant in AML. We argue that from a clinical point of view, NPMc-positive AML has a better response to chemotherapy and a functional FANCA-FANCC may contribute to this favorable prognosis through regulation of NPMc/NF-κB/inflammation. In the context of AML biology, stabilization of NPMc by cytoplasmic FANCA-FANCC would lead to the retardation of NF-κB in the cytoplasm and thus ensure the sensitivity of the AML cells to chemotherapy. During the course of leukemic evolution, a second-hit event may inactivate FANCA or FANCC in NPMc-positive AML cells. This would result in NF-κB activation leading to increased inflammation and resistance to chemotherapeutic agent-induced apoptosis, consequences presented in the present study. In conclusion, our findings not only reveal the potential molecular mechanism involving cytoplasmic retention of the leukemic NPMc but also suggest a cytoplasmic function of FANCA and FANCC in inflammation and NPMc-related leukemogenesis.

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