

FAST TRACK

## Fanconi Anemia Protein Complex Is a Novel Target of the IKK Signosome

Tetsuya Otsuki,<sup>1</sup> David B. Young,<sup>2</sup> Dennis T. Sasaki,<sup>2</sup> Matthew P. Pando,<sup>3</sup> Jianwu Li,<sup>2</sup> Anthony Manning,<sup>2</sup> Merl Hoekstra,<sup>2</sup> Maureen E. Hoatlin,<sup>4</sup> Frank Mercurio,<sup>2</sup> and Johnson M. Liu<sup>1\*</sup>

<sup>1</sup>Hematology Branch, Bethesda, Maryland 20892

<sup>2</sup>Signal Research Division, Celgene Corporation San Diego, California 92121

<sup>3</sup>The Salk Institute, La Jolla, California 92037

<sup>4</sup>Division of Molecular Medicine, Oregon Health Sciences University, Portland, Oregon 97201

**Abstract** Fanconi anemia (FA), a genetic disorder predisposing to aplastic anemia and cancer, is characterized by hypersensitivity to DNA-damaging agents and oxidative stress. Five of the cloned FA proteins (FANCA, FANCC, FANCE, FANCF, FANCG) appear to be involved in a common functional pathway that is required for the monoubiquitination of a sixth gene product, FANCD2. Here, we report that FANCA associates with the I $\kappa$ B kinase (IKK) signosome via interaction with IKK2. Components of the FANCA complex undergo rapid, stimulus-dependent changes in phosphorylation, which are blocked by kinase-inactive IKK2 (IKK2 K > M). When exposed to mitomycin C (MMC), cells expressing IKK2 K > M develop a cell cycle abnormality characteristic of FA. Thus, FANCA may function to recruit IKK2, thus providing the cell a means of rapidly responding to stress. *J. Cell. Biochem.* 86: 613–623, 2002. © 2002 Wiley-Liss, Inc.

**Key words:** Fanconi anemia; FANCA; IKK signosome; NF-kappa B

Fanconi anemia (FA) is an autosomal recessive disorder clinically manifested by aplastic anemia, the variable presence of birth defects, and cancer susceptibility [Joenje and Patel, 2001]. The hallmark of the mutant FA cell is hypersensitivity to DNA cross-linking agents such as mitomycin C (MMC) and to oxygen. Although rare, FA has generated disproportionately great interest because it serves as a genetic model for hematopoietic failure and neoplasia, and because the function of FA proteins involves the cellular response to certain types of stress. FA can be caused by mutations in any of at least eight genetic loci, FA-A, FA-B, FA-C, FA-E, FA-F, FA-G, FA-D1, and FA-D2. The genes mutated in FA-A [FANCA, FA/BC Consortium, 1996; Lo Ten Foe et al., 1996], FA-C [FANCC, Strathdee et al., 1992], FA-E [FANCE, de Winter et al., 2000a], FA-F [FANCF, de Winter et al., 2000b], and FA-G [FANCG, de

Winter et al., 1998] patients have been identified, but the biochemical function of each encoded protein is unknown, as no significant homologies exist between each FA protein and proteins of known function. Attempts to understand the molecular function of the FA gene products have been further frustrated because FA gene knock-out mice do not spontaneously develop the marrow failure characteristic of the human disease [Joenje and Patel, 2001].

Since the first five cloned FA genes are not homologous with each other and yet mutation in each gene leads to FA, FA proteins have been postulated either to function in a common pathway (e.g., as members of an enzymatic cascade), to physically associate, or to interact in some indirect manner [Joenje and Patel, 2001]. Recently, direct binding between FANCA and FANCG proteins was demonstrated [Garcia-Higuera et al., 1999; Waisfisz et al., 1999], with varying amounts of the complex in FA cells of different complementation groups [Waisfisz et al., 1999]. Although the details are as yet unclear, FANCA, FANCC, FANCE, FANCF, and FANCG appear to be involved in a common functional pathway that is required for the monoubiquitination of a sixth gene product, FANCD2 [Garcia-Higuera et al., 2001; Timmers

\*Correspondence to: Johnson M. Liu, Hematology Branch, NHLBI Bldg. 10, Rm. 7C103, Bethesda, Maryland 20892. E-mail: liuj@nhlbi.nih.gov

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et al., 2001]. When activated by this biochemical modification, FANCD2 colocalizes with the breast cancer susceptibility protein, BRCA1, in ionizing radiation-induced foci and in synaptonemal complexes of meiotic chromosomes.

At the cellular level, mutation in FA genes induces intrinsic slowing or arrest in G2 phase of the cell cycle, which is accentuated by high oxygen tension or by treatment with MMC [reviewed in Joenje and Patel, 2001]. FA cells also generally exhibit increased sensitivity to oxygen, at least as assessed by a number of in vitro parameters, leading to the suggestion that FA is a pro-oxidant state [Ruppitsch et al., 1997]. Consistent with this hypothesis is the recent finding that wild-type (WT) FANCC binds to and attenuates the activity of NADPH cytochrome-P450 reductase [Kruyt et al., 1998], a microsomal enzyme involved in electron transfer. Disruption of this normal interaction by mutation in FANCC would be predicted to enable unopposed generation of reactive oxygen intermediates (ROI). Overexpression of the pro-inflammatory cytokine tumor necrosis factor- $\alpha$  [TNF- $\alpha$ , Rosselli et al., 1994] in FA cells may also reflect oxidative stress. Recently, FANCC has been found to retard apoptosis in hematopoietic cells through a novel mechanism of redox regulation of glutathione *S*-transferase P1-1 [Cumming et al., 2001].

We report here that FANCA associates with I $\kappa$ B kinase-2 (IKK2), a critical mediator of cellular response to stress, including oxidative stress, DNA damage, exposure to chemotherapeutic agents and pro-inflammatory cytokines [Mercurio and Manning, 1999]. The cellular functions that have been well documented for the IKK/NF- $\kappa$ B signaling pathway are remarkably similar to those proposed for FA proteins. IKK2 (IKK $\beta$ ) and IKK1 (IKK $\alpha$ ) are kinase components of a multi-protein complex termed the IKK signalsome [DiDonato et al., 1997; Mercurio et al., 1997; Woronicz et al., 1997; Zandi et al., 1997]. The IKK signalsome is responsive to myriad exogenous and endogenous stressors, and exerts its protective effects by modulating the activity of the transcription factor NF- $\kappa$ B [Mercurio and Manning, 1999], which also responds to ROI signaling and to DNA damage. NF- $\kappa$ B-regulated genes, in turn, play a key role in redox regulation and DNA repair, and include superoxide dismutase, gamma-glutamylcysteine synthetase and several anti-apoptotic genes. We propose that, in

addition to activation of the NF- $\kappa$ B pathway, the IKK signalsome functions to protect the cell from specific types of stress through regulation of proteins comprising the FA pathway.

## MATERIALS AND METHODS

### Yeast Two-Hybrid Screening

Full-length *FANCA* cDNA or (1–1953 nt), (1801–3441 nt), and (3271–4368 nt) *FANCA* fragments were fused in-frame downstream of the DNA-binding domain of GAL4 in the vector pGBT9 (Clontech, Palo Alto, CA). The yeast strain HF7c was transfected with pGBT9-*FANCA* plasmids (MATCHMAKER Two Hybrid System; Clontech). The full-length *FANCA* construct or the (1–1953 nt) fragment alone showed transcriptional activity. The (1801–3441 nt) and (3271–4368 nt) plasmids were, therefore, used for transfection with 200  $\mu$ g of pACT cDNA library plasmid (Clontech) and selected on SD plates. Colonies that grew in the absence of leucine, tryptophan, and histidine were tested for their ability to activate the *lacZ* reporter gene. Plasmids recovered from positive colonies were used to transform *Escherichia coli*. The inserts obtained were sequenced by the 310 Genetic Analyzer (ABI PRISM) and analyzed by the BLAST program of the NCBI database.

### Expression Constructs

Full-length *FANCA* cDNA was fused in-frame upstream of the *myc*-His epitope tag in the vector pcDNA3.1/*myc*-His (Invitrogen, Carlsbad, CA). Flag-tagged full-length human IKK2, pCMV-Flag IKK2, was previously described [Mercurio et al., 1997]. Flag-tagged IKK2  $\Delta$ CT was generated by site-directed mutagenesis of pCMV-Flag IKK2, changing leucine 708 to a stop codon.

### Recombinant Proteins

SF9 cells were infected at a multiplicity of infection (MOI) of 10 with recombinant baculovirus encoding His-tagged IKK2 (PharMingen, La Jolla, CA). Whole cell lysate was prepared and the His-tagged IKK proteins purified using Ni-NTA resin (Qiagen).

### Purification of IKK Signalsome

Whole cell extracts (WCEs,  $\sim$ 1 g of total protein) were prepared from TNF- $\alpha$ -stimulated (20 ng/ml, 7 min induction) HeLa cells. Purifica-

tion of the endogenous IKK complex was as described [Mercurio et al., 1999]. Specifically, IKK2-containing complexes were immunoprecipitated with IKK2-specific antibodies, eluted via incubation with excess IKK2 peptide to which the antibody was generated, and subsequently fractionated by gel filtration chromatography. Isolated fractions were analyzed by immunoblot analysis using the indicated antibody.

#### Generation of Anti-FANCA Antiserum

Rabbit anti-FANCA antibodies were directed against a peptide that corresponds to the N-terminal region, NH<sub>2</sub>-MSDSWVPNSASGQD-PGRRRRAC-COOH (Alpha Diagnostics, Inc.). Antibodies were affinity-purified using specific peptide columns.

#### Purification of FANCA Complex

Whole cell lysate was prepared from unstimulated HeLa cells [Mercurio et al., 1999]. Affinity purified anti-FANCA antibody was added to the lysate (1:150 dilution) and incubated at 4°C for 2 h. Subsequently, 5 ml of Protein A agarose (Calbiochem) was added and the mixture incubated for an additional 2 h. After washing, the immunoprecipitate was made into a thick slurry by the addition of 3 ml of PD buffer, 8 mg of the specific FANCA peptide to which the antibody was generated (Alpha Diagnostics, Inc.), and incubated overnight at 4°C. The eluted fraction was analyzed by Western blot analysis for the presence of FANCA and IKK1 (PharMingen IKK $\alpha$  antibody, San Diego, CA).

#### Co-Transfection of FANCA and IKK2

HeLa cells were co-transfected with Myc-tagged FANCA and Flag-tagged IKK2, and subsequently stimulated with TNF- $\alpha$  (20 ng/ml) for the indicated times (minutes). Immunoprecipitation with anti-Myc antibodies was performed with whole cell lysates of the transfected HeLa cells. The IP samples were then analyzed for I $\kappa$ B $\alpha$  kinase activity with GST I $\kappa$ B $\alpha$  1-54 added exogenously (2  $\mu$ g per reaction) as the substrate. Whole-cell lysates were subjected to Western blot analysis to determine the level of expression of Flag-IKK2 and Myc-FANCA.

#### Immunoprecipitations

For small-scale immunoprecipitations, whole cell lysate (0.3–1.0 mg) from HeLa cells ( $\pm$  TNF-

$\alpha$  stimulation) was prepared and diluted to 0.5 ml with PD buffer, and the indicated antibody was added. Each reaction was incubated on ice for 1–2 h with gentle rotation, followed by the addition of 10  $\mu$ l of protein A or G beads and left to incubate for an additional 1 h at 4°C. After washing, the immune precipitate was either resuspended in sample buffer and subjected to Western analysis or washed 1  $\times$  with kinase buffer without ATP and subjected to an in vitro kinase assay.

#### Kinase Assay

FANCA or control immunoprecipitates were subjected to an IKK2 in vitro kinase assay. Kinase assays were performed in kinase buffer (20 mM HEPES, pH 7.7, 2 mM MgCl<sub>2</sub>, 10 mM ATP, 10 mM -glycerophosphate, 10 mM NaF, 10 mM PNPP, 300  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 1 mM benzamide, 1 mM DTT, 1  $\times$  complete protease inhibitor cocktail [Boehringer Mannheim, GmbH, Germany]), at 30°C for 30 min in the presence of 2 Ci [ $\gamma$ -<sup>32</sup>P] ATP and 100 ng of purified baculovirus-expressed IKK2 protein. The kinase reaction was terminated, subjected to SDS-PAGE, and visualized by autoradiography.

#### Adenoviral Vector-Mediated Expression of IKK Dominant-Negative Mutants

IKK adenovirus vectors were constructed by blunt ligation into the replication-deficient vector pAxCA. Virus stocks were amplified to high titer (Quantum Biotechnologies, Montreal, Canada). The concentration of viral particles (VP) was determined by OD<sub>260</sub> measurement. Plaque assay and TCID<sub>50</sub> assays to determine infectious virus units (IU) gave a VP:IU ratio of less than 100:1. Adenovirus preparations were re-titered using human umbilical vein endothelial cells to determine the optimum MOI.

#### Metabolic Labeling Studies

HeLa cells were infected with the indicated adenovirus for 4 h and washed. After overnight incubation, the cells were washed extensively with phosphate-free DMEM medium and allowed to incubate for 30 min with phosphate-free DMEM. This medium was replaced with fresh phosphate-free medium containing 1 mCi/ml <sup>32</sup>P orthophosphate and the cells left to incubate an additional 4 h. Cells  $\pm$  TNF- $\alpha$  (20 ng/ml) were rapidly harvested on ice, WCEs were prepared, and the endogenous FANCA complex immunoprecipitated with affinity purified anti-FANCA

antibodies (as above). The FANCA immune complex was subjected to SDS-PAGE and visualized by autoradiography.

### Retroviral FANCA Gene-Corrected Lymphoblastoid Cell Lines

HSC 72 cells are EBV-transformed lymphoblastoid cells derived from a FA-A patient. Mock-infected HSC 72 cells, HSC 72 cells transduced and complemented by retroviral FANCA gene transfer, and lymphoblastoid cells from a normal individual were kind gifts from Dr. C. Walsh, University of North Carolina, Chapel Hill.

### I $\kappa$ B Degradation Assays

HSC 72 cells, either mock-infected or complemented with WT FANCA, were stimulated with PMA (50 ng/ml)/ionomycin (2  $\mu$ M) for the indicated times, and whole cell lysate was prepared from each condition. Equal aliquots of the respective lysates (50  $\mu$ g/lane) were resolved on SDS-PAGE gels and subsequently subjected to Western blot analysis with either anti-I $\kappa$ B $\beta$  or anti-I $\kappa$ B $\alpha$  antibodies (Santa Cruz Biotechnology, Inc, Santa Cruz, CA).

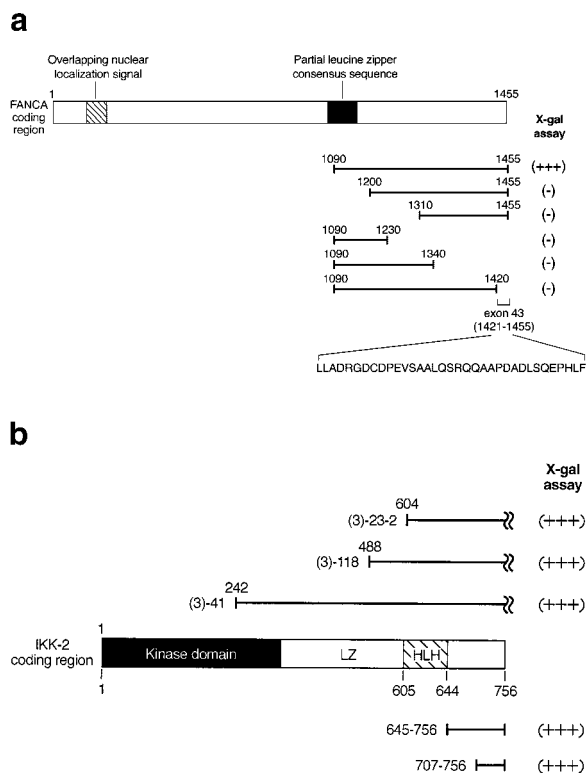
### Cell Cycle Analyses

Cells were mock-infected or infected with the indicated adenovirus for 4 h. After washing and incubation with fresh media, cells were harvested 24 h post-infection. One milliliter of Accutase (Innovative Cell Technology, Inc., La Jolla, CA) was added. Cells were centrifuged and resuspended in 1 ml of propidium iodide solution. After analysis by flow cytometry, data were analyzed using the Dean, Jett, Fox, or Watson Pragmatic Algorithm. Each study was repeated at least three times with nearly identical results.

## RESULTS

### C-Terminus of FANCA Interacts With IKK2

The FANCA protein has a molecular mass of 163 kDa and has a partial consensus leucine zipper binding site. FANCA was previously demonstrated to associate with FANCC and FANCG [Garcia-Higuera et al., 1999]. To look for additional protein-protein interactions, we constructed overlapping fragments of FANCA as "bait" in the two-hybrid screen (Fig. 1a). From the primary screens, we isolated three independent clones that strongly interacted



**Fig. 1.** (a) IKK2 interaction with FANCA. Interestingly, homozygous deletion of exon 43 has been described as a pathogenic FANCA mutation. (b) Yeast two-hybrid cloning of IKK2 and its binding site for FANCA. Three independent pACT-IKK2 fusions were identified as binding to the FANCA bait (1090–1455): (3)-23-2, (3)-118, and (3)-41. The coding region of the three plasmids coincided with the HLH domain of IKK2. The HLH domain, however, was not essential for binding between FANCA and IKK2.

with the C-terminus of FANCA. The sequence of each clone matched that of the I $\kappa$ B kinase-2 (IKK2), which with IKK1, is a component of a multi-protein complex termed the IKK signalosome. Multiple regions of FANCA appeared to be required for interaction with IKK2, as deletion of residues 1090–1200, 1090–1310 or any of a series of C-terminal deletions disrupted interaction (Fig. 1a). Conversely, the region of IKK2 required for interaction with FANCA consisted of the C-terminal 50 amino acid residues (Fig. 1b).

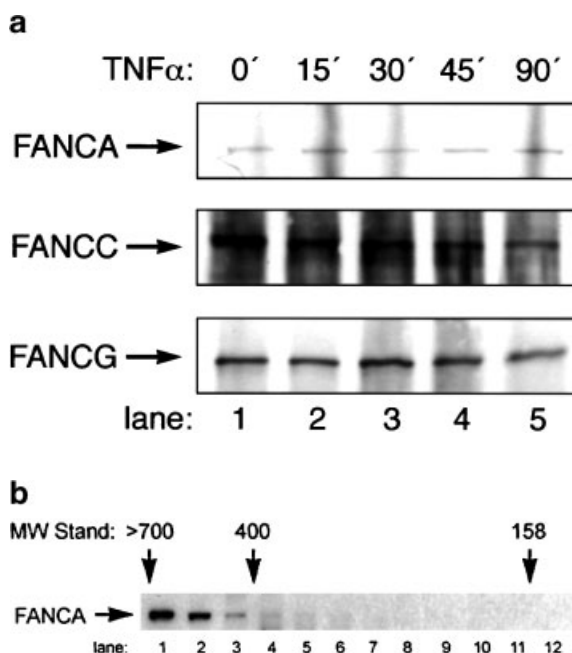
The interaction between FANCA and IKK2 was confirmed by co-immunoprecipitation studies. HeLa cells were cotransfected with mammalian expression vectors encoding Myc-tagged FANCA (Myc-FANCA) and either full-length Flag-tagged IKK2 (Flag-IKK2) or Flag-tagged IKK2 lacking the FANCA binding domain (Flag-IKK2  $\Delta$ CT). Whole cell lysates were



subsequently analyzed to establish the presence of FANCA and IKK1 (Fig. 2c). Here, we observed the presence of IKK1 in the endogenous FANCA complex. (Interaction between FANCA and IKK1 seemed to be independent of TNF- $\alpha$ , since these results were obtained with FANCA complex derived from cells not stimulated with TNF- $\alpha$ .) Hence, we demonstrated by several stringent assays that FANCA physically associates *in vivo*, with IKK2 and the IKK signalsome.

Next, we wanted to identify possible functional connections between the FA and IKK pathways. Previous reports had shown that FANCC and FANCG associate with FANCA *in vivo*. We wanted to determine whether these associations were subject to modification by IKK stimuli. To this end, we examined the association of FANCC and FANCG with FANCA in response to TNF- $\alpha$  stimulation. Whole cell lysates were prepared from HeLa cells with or without TNF- $\alpha$  stimulation; the FANCA complex was immunoprecipitated and directly examined for the presence of all three proteins (Fig. 3a). Our studies suggested that in contrast to FANCG, the amount of FANCC associated with the FANCA complex was reduced following treatment with TNF- $\alpha$ . We also sought to confirm previous reports that the majority of FANCA exists in a high molecular weight complex. Endogenous FANCA was isolated from HeLa whole cell lysate by immunoprecipitation with anti-FANCA antibodies. We eluted the FANCA complex with the FANCA-specific peptide to which the antibodies were derived, and fractionated it further by gel filtration chromatography. Fractions were subjected to immunoblot analysis using anti-FANCA antibodies. Most of the endogenous FANCA was found to exist in a high molecular weight complex of greater than 700 kDa (Fig. 3b).

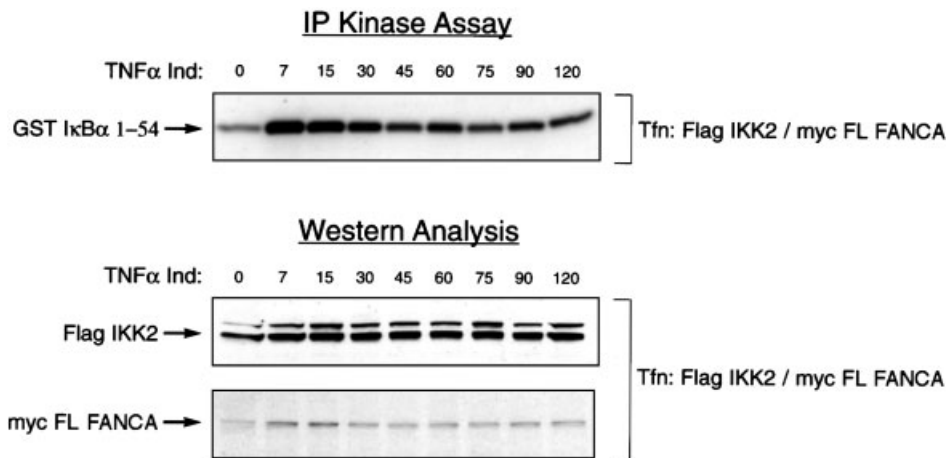
One initial concern we wanted to address is whether FANCA-associated IKK2 is fully functional. We, therefore, assessed IKK2 kinase activity by co-expressing IKK2 and FANCA. The transfected cells were then induced with TNF- $\alpha$ , and the FANCA complex was subjected to an immune kinase assay using I $\kappa$ B $\alpha$  1–54 aa, which contains the serine residues phosphorylated by IKK2, as the substrate (Fig. 4). We observed TNF- $\alpha$ -induced IKK2 kinase activity with typical activation kinetics, suggesting that FANCA association with IKK2 did not alter its ability to undergo TNF- $\alpha$ -induced activation.



**Fig. 3.** (a) The amount of FANCC associated with FANCA is reduced in response to treatment with TNF- $\alpha$ . HeLa cells stimulated with TNF- $\alpha$  for the indicated times were assayed for the association of FANCG and FANCC with FANCA. The immune complex was divided into three equal aliquots and probed with anti-FANCA, anti-FANCC, or anti-FANCG antibodies. (b) Most of the endogenous FANCA was found to exist in a high molecular weight complex of greater than 700 kDa. MW Stand, molecular weight standards.

#### Components of the Endogenous FANCA Complex Undergo IKK-Dependent Changes in Phosphorylation

FANCA and FANCG have been demonstrated to exist as phosphoproteins [Yamashita et al., 1998; Futaki et al., 2001]. However, whether other components of the FANCA complex undergo phosphorylation is unknown. In order to determine whether IKK1 or IKK2 modulates phosphorylation of components of the endogenous FANCA complex, we performed  $^{32}$ P metabolic labeling studies. HeLa cells were infected with adenovirus vectors expressing a dominant negative IKK1 (IKK1 K > M) or IKK2 mutant (IKK2 K > M), or green fluorescent protein (GFP) as a negative control. Twenty-four hours after infection, cells were metabolically labeled with  $^{32}$ P-orthophosphate, with or without subsequent stimulation with TNF- $\alpha$ . Whole cell lysates were subjected to immunoprecipitation with affinity-purified antiserum directed



**Fig. 4.** FANCA association with IKK2 did not alter its ability to undergo TNF- $\alpha$ -induced activation. HeLa cells were co-transfected with Myc-tagged full-length (FL) FANCA and Flag-tagged IKK2, and subsequently stimulated with TNF- $\alpha$  for the indicated times (minutes). Immunoprecipitation with anti-Myc antibodies was performed with whole cell lysates of the transfected HeLa

cells. The IP samples were then analyzed for I $\kappa$ B $\alpha$  kinase activity with GST I $\kappa$ B $\alpha$  1-54 added exogenously as the substrate (**top panel**). Whole-cell lysates were subjected to Western blot analysis to determine the level of expression of Flag-IKK2 (**bottom panel**) and Myc-FANCA (**bottom panel**).

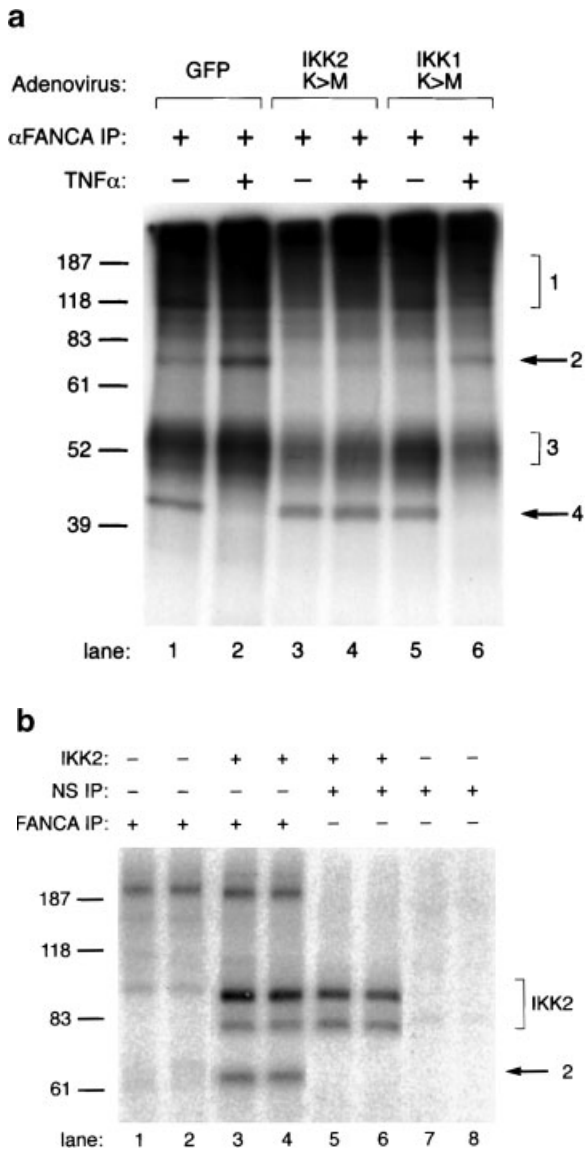
against endogenous FANCA. The phosphorylation state of several FANCA-associated proteins was altered, as a function of the presence of either kinase-inactive IKK1 K > M or IKK2 K > M (Fig. 5a). This was apparent for both unstimulated and TNF- $\alpha$ -induced cells. The most striking changes were IKK2-K > M-mediated differences in phosphorylation of a 68 kDa and a ~42 kDa component of the FANCA complex. Our studies could not definitely distinguish whether the 42-kDa protein undergoes TNF- $\alpha$ -dependent dephosphorylation, is inducibly degraded, or undergoes regulated dissociation.

We next examined whether any component(s) of the FANCA complex was a direct substrate for IKK2. HeLa whole cell lysates were prepared and subjected to immunoprecipitation, with either affinity-purified anti-FANCA antibodies or non-specific antibodies, and the immune complexes were washed with 1 M urea to remove loosely associated contaminating proteins. The immune complexes were then used in an *in vitro* kinase reaction, either alone or with purified recombinant IKK2 protein. First, we noted that the FANCA complex contained detectable kinase activity (Fig. 5b, lanes 1 and 2). More interestingly, we also observed that IKK2 directly phosphorylated a 68 kDa component of the FANCA complex. Although similar in size [de Winter et al., 1998], the 68 kDa

component could not be confirmed as FANCG and remains unidentified currently.

#### Kinetics of Stimulus-Dependent Degradation of I $\kappa$ B $\beta$ , But Not I $\kappa$ B $\alpha$ , Are Altered by Complementations of Mutant FA-A Cells

Several studies were performed to discern whether FANCA plays a direct role in IKK activation. Although we were unable to discern any direct effect on IKK activation by FANCA in response to a variety of inducers (Fig. 4 and data not shown), it remained possible that mutations in FANCA might lead to aberrant NF- $\kappa$ B signaling. We, therefore, examined whether mutant FA-A lymphoblastoid cells displayed properties of I $\kappa$ B degradation distinct from cells complemented with WT FANCA. Mutant FA-A, complemented FA-A, and WT lymphoblastoid cells, were stimulated with PMA/ionomycin for the indicated times, and whole cell lysates were prepared. Immunoblot analysis was performed using I $\kappa$ B $\alpha$ - and I $\kappa$ B $\beta$ -specific antibodies (Fig. 6). In these experiments, we can only discern meaningful changes in the pattern of degradation of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ , whereas differences in the absolute intensities of each I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  band (such as those seen between WT and FANCA cells) may reflect only differences in protein transfer or may be due to variation between cell lines. Consequently, we conclude



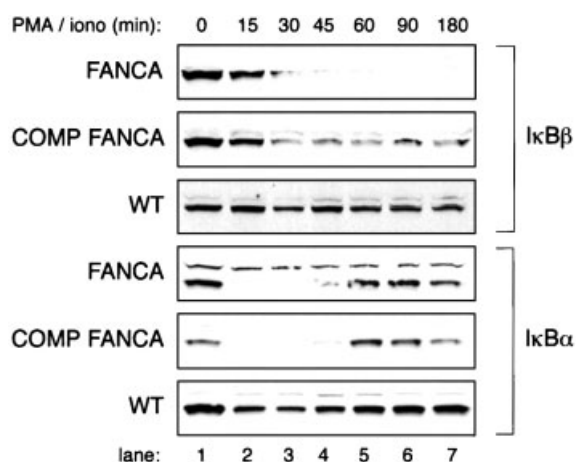
**Fig. 5.** Components of the endogenous FANCA complex undergo TNF- $\alpha$ -dependent changes in phosphorylation that are mediated, in part, by IKK signalsome. **(a)** Kinase-inactive IKK1 and IKK2 alter TNF- $\alpha$ -induced phosphorylation of FANCA complex components. HeLa cells were infected with adenovirus vectors encoding for dominant-negative IKK1 (IKK1 K>M) or IKK2 (IKK2 K>M) or GFP (as a negative control). Cells were metabolically labeled with  $^{32}$ P orthophosphate with or without subsequent stimulation with TNF- $\alpha$ . Molecular weight markers are indicated to the left, and FANCA complex components which undergo an IKK-dependent change in phosphorylation are indicated to the right (numbered 1–4). **(b)** IKK2 directly phosphorylates a 68 kDa component of the FANCA complex. Lanes 1, 3, 5, and 7 are proteins derived from unstimulated HeLa cell lysates, whereas lanes 2, 4, 6, and 8 are derived from TNF- $\alpha$ -stimulated HeLa cell lysates. The phosphoproteins resulting from IKK2 autophosphorylation are labeled “IKK2.” The IKK2-dependent phosphorylation of a 68 kDa protein is indicated by the label “2,” which corresponds in size to the 68 kDa phosphoprotein associated with the endogenous FANCA complex in part (a) above.

from these experiments that I $\kappa$ B $\alpha$  displayed identical kinetics of degradation for both cell types. In contrast, we observed a reduction in the prolonged, sustained degradation of I $\kappa$ B $\beta$  in the complemented cell line as compared with the mutant FA-A cell line. Although we observed only a modest change in the levels of I $\kappa$ B $\beta$ , for chronic conditions such as FA, this may be sufficient to generate aberrant NF- $\kappa$ B signaling. We believe the effect of FANCA mutation on PMA/ionomycin-induced I $\kappa$ B $\beta$  degradation is not a direct result of FANCA modulating IKK activity. We have performed several studies to examine a role for FANCA in the regulation of IKK activity and, in all cases, FANCA does not alter IKK activation (data not shown). Rather, we believe that mutation in FANCA leads to chronically elevated levels of ROI which, in turn, lead to reduced levels of I $\kappa$ B $\beta$  protein, ultimately resulting in elevated constitutive NF- $\kappa$ B activity (for which there is precedent in the literature [Ruppitsch et al., 1997]).

#### Inactivation of IKK2 Induces FA-Like G2 Cell Cycle Arrest With MMC

FA cells display a characteristic propensity to accumulate in the G2 phase of the cell cycle, particularly following exposure to MMC [reviewed in Joenje and Patel, 2001]. To further substantiate a role for IKK2 in FA function, we explored whether kinase-inactive versions of IKK1 or IKK2 induce features of the FA cellular phenotype, such as G2 phase accumulation. For these experiments, we again infected HeLa cells with our IKK1 K>M, IKK2 K>M, and GFP adenovirus vectors. We used a combination of immunofluorescence and GFP studies to test the efficiency of adenovirus expression and found that > 80% of cells expressed the protein of interest (data not shown). We also included, as a second control, an adenovirus that encodes for the so-called “I $\kappa$ B $\alpha$  super-repressor” of NF- $\kappa$ B, in which serines 32 and 36 are mutated to alanines, rendering I $\kappa$ B refractory to stimulus-dependent degradation. In this manner, we could distinguish between direct IKK-mediated effects on the FANCA complex versus indirect effects from inactivation of NF- $\kappa$ B-mediated gene expression. Here, with 1 mM MMC treatment, we observed a significant increase in the number of cells in G2 phase for IKK2 K>M, but not for IKK1 K>M or, more importantly, the I $\kappa$ B $\alpha$  super-repressor (Fig. 7). Hence, consistent with the in vivo phosphorylation results,



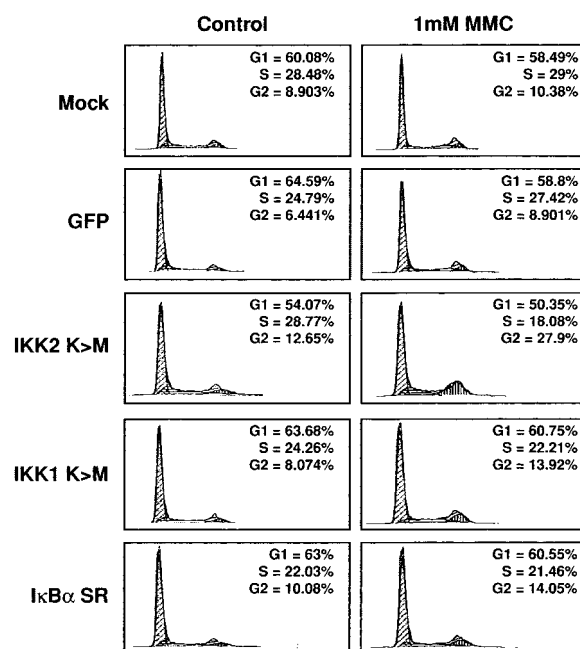


**Fig. 6.** The kinetics of stimulus-dependent degradation of  $I\kappa B\beta$ , but not  $I\kappa B\alpha$ , are altered by complementation of FA lymphoblastoid cells with WT FANCA. Whole cell lysates were prepared from either WT, FANCA (FANCA), or FANCA-complemented (COMP FANCA) EBV-transformed lymphoblastoid cells stimulated with PMA (50 ng/ml)/ionomycin (2  $\mu$ M) for the indicated times. Equal aliquots of the respective lysates (50  $\mu$ g/lane) were resolved on SDS-PAGE and subsequently subjected to Western blot analysis with either anti- $I\kappa B\beta$  or anti- $I\kappa B\alpha$  as indicated to the right of the figure. The cell type is indicated to the left of the figure.

IKK2 appeared to play a functional role in FA biology.

## DISCUSSION

In this article, we describe a novel interaction between the IKK signosome and the FANCA complex. FANCA physically contacts IKK2 and the IKK signosome. Components of the FANCA complex undergo rapid, TNF- $\alpha$ -induced changes in phosphorylation, which are blocked by the kinase-inactive IKK2 mutant, IKK2 K>M. Specifically, TNF- $\alpha$ -induced phosphorylation of a 68 kDa component is blocked by IKK2 K>M. In vitro kinase studies suggest that this FANCA component is a direct substrate of IKK2. Inactivation of IKK2 also is associated with TNF- $\alpha$ -induced dephosphorylation, degradation or regulated dissociation of a 42 kDa FANCA complex component. These studies suggest a functional role for the IKK signosome in the biological pathway mediated by FA proteins. Consistent with these actions on the FANCA complex, loss of IKK2 activity induces a FA-like cell cycle abnormality with MMC. As this phenotype is specific to inactivation of IKK2, there may be divergence between the functional loss of IKK2 and the loss of NF- $\kappa$ B.



**Fig. 7.** Inactivation of IKK2 induces an FA-like cell cycle abnormality. HeLa cells were infected with adenoviruses encoding for either IKK1 K>M, IKK2 K>M, GFP, or the  $I\kappa B\alpha$  super-repressor ( $I\kappa B\alpha$  SR). With 1 mM MMC treatment, a significant increase in the number of cells in G2-phase was observed for IKK2 K>M, but not for IKK1 K>M or the  $I\kappa B\alpha$  super-repressor.

Current models propose that the biologic function of FA proteins involves response to ROI and/or DNA damage. In regards to the latter, a monoubiquitinated isoform of FANCD2 has been found to colocalize with BRCA1 in ionizing radiation-induced foci and in synaptonemal complexes of meiotic chromosomes [Garcia-Higuera et al., 2001]. Although our studies do not precisely elucidate the mechanism by which FA proteins function, they do provide compelling data linking FA-mediated signaling events with those of the IKK signosome, which has been shown to play a prominent role in redox regulation and response to DNA damage, such as the formation of DNA double strand breaks (DSBs) following ionizing radiation [Huang et al., 2000; Li et al., 2001]. Thus, regardless of whether FA proteins play a role in DNA repair or ROI processing, perturbation of either cellular process results in IKK activation and consequently directly activates the FA pathway. We propose that the IKK signosome possesses the capacity to modulate at least two distinct stress-response pathways, namely the NF- $\kappa$ B and FA pathways. Activation of the IKK/NF- $\kappa$ B pathway promotes its

protective effects via induction of anti-apoptotic and redox-regulating gene expression [Mercurio and Manning, 1999]. In contrast, the IKK/FA pathway likely provides the cell with a mechanism by which to directly activate an enzymatic cascade in response to cellular insult. This would provide the cell with an exquisite mechanism to activate non-redundant, temporally distinct, signaling pathways.

Work by other investigators had previously established that FANCA is itself also phosphorylated [Yamashita et al., 1998]. IKK2 does not appear to directly phosphorylate FANCA, however, nor is FANCA's own phosphorylation state directly regulated by TNF- $\alpha$ . According to our studies, the FANCA complex is also associated with a constitutive kinase activity (Fig. 5b, lanes 1 and 2); we are actively investigating the role of this additional kinase.

We have thus far focused on the actions of the IKK signalsome on the FANCA complex. Conversely, however, we have little evidence to suggest that FANCA (or other FA gene products) directly participates in the signaling pathway that regulates IKK activity. Moreover, we do not believe that FANCA is a core component of the IKK signalsome, in that it is found at sub-stoichiometric quantities within the complex. (Most likely, only a small fraction of the IKK complex will associate with the FANCA complex at any given time.) However, it is possible that FA cells display aberrant IKK/NF- $\kappa$ B regulation through the indirect effects of chronically elevated levels of ROI [Ruppitsch et al., 1997] or accumulated DNA damage.

In this article, we have proposed that the IKK signalsome acts on FA gene products. In support of this notion, disruption of IKK2 activity by expression of a dominant negative mutant induces a FA-like MMC/G2 cell cycle arrest. We and others had previously documented that hematopoietic progenitor cells from *Fancc* knockout mice are also abnormally sensitive to cell death induced by TNF- $\alpha$  and Fas receptor ligation [Otsuki et al., 1999]. Disruption of *Ikk-2* by targeted mutation results in the death of the homozygous embryo at mid-gestation due to massive hepatocyte apoptosis [Li et al., 1999b,c; Tanaka et al., 1999]. Fetal liver hematopoietic colony-forming cells from the *Ikk-2* knockout mice were also more sensitive to TNF- $\alpha$  [Tanaka et al., 1999], a result that parallels our findings in *Fancc* knockout mice [Otsuki et al., 1999]. *Fanca* knockout mice have only recently been

reported [Cheng et al., 2000], but they would be predicted to exhibit sensitivity to the TNF death ligands. An as yet untested corollary of our hypothesis is that targeted mutation of *Ikk1* [Hu et al., 1999; Li et al., 1999a; Takeda et al., 1999] or *Ikk2* [Li et al., 1999b,c; Tanaka et al., 1999] genes might lead to changes in the phosphorylation state of FANCA-associated proteins and to a (partial) FA phenotype.

Establishing the interaction between the endogenous IKK signalsome and the FA complex represents a significant advance that should aid in our understanding of the cellular function of FA proteins. One outstanding question, however, is the relationship (if any) between the IKK pathway and the recently described FANCD2/BRCA1 link. The FA protein complex has been proposed to function as a multi-protein ubiquitin ligase [Garcia-Higuera et al., 2001]. It is possible that the IKK signalsome may phosphorylate FA proteins that have some enabling role in the monoubiquitination of FANCD2. In this and many other respects, the FA proteins can be compared to ATM, the well-known multifunctional protein kinase whose activity is stimulated by DSBs. Patients suffering from the genetic disorder ataxia telangiectasia (AT), caused by mutations in the *ATM* gene, are highly sensitive to inducers of DSBs, such as ionizing radiation. In turn, ATM plays a complex role in the phosphorylation of BRCA1 and other members of the DSB repair complex. On the other hand, ATM is also essential for NF- $\kappa$ B activation in response to DSBs, and this activity is mediated via the IKK complex [Huang et al., 2000; Li et al., 2001].

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