

The Interferon Consensus Sequence Binding Protein (ICSBP/IRF8) Activates Transcription of the *FANCF* Gene during Myeloid Differentiation*

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The interferon consensus sequence binding protein (ICSBP) is an interferon regulatory transcription factor with leukemia-suppressor activity. ICSBP regulates genes that are involved in phagocyte function, proliferation, and apoptosis. In murine models ICSBP deficiency results in a myeloproliferative disorder (MPD) with increased mature neutrophils. Over time this MPD progresses to acute myeloid leukemia (AML), suggesting that ICSBP deficiency is adequate for MPD, but additional genetic lesions are required for AML. The hypothesis of these studies is that dysregulation of key target genes predisposes to disease progression under conditions of decreased ICSBP expression. To investigate this hypothesis, we used chromatin co-immunoprecipitation to identify genes involved in the ICSBP-leukemia suppressor effect. In the current studies, we identify the gene encoding Fanconi F (*FANCF*) as an ICSBP target gene. *FanCF* participates in a repair of cross-linked DNA. We identify a *FANCF* promoter cis element, which is activated by ICSBP in differentiating myeloid cells. We also determine that DNA cross-link repair is impaired in ICSBP-deficient myeloid cells in a *FanCF*-dependent manner. This effect is observed in differentiating cells, suggesting that ICSBP protects against the genotoxic stress of myelopoiesis. Decreased ICSBP expression is found in human AML and chronic myeloid leukemia during blast crisis (CML-BC). Our studies suggest that ICSBP deficiency may be functionally important for accumulation of chromosomal abnormalities during disease progression in these myeloid malignancies.

The interferon consensus sequence binding protein (ICSBP)² is an interferon regulatory factor (also known as IRF8) that is

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² The abbreviations used are: ICSBP, interferon consensus sequence binding protein; MPD, myeloproliferative disorder; BC, blast crisis; MMC, mitomycin C; FA, Fanconi anemia; IRF, interferon regulatory factor; M-CSF, macrophage colony-stimulating factor; GM-CSF, granulocyte-macrophage CSF; CML, chronic myeloid leukemia; BC, blast crisis; AML, acute myeloid leukemia; shRNA, short hairpin RNA; IFN, interferon; WT, wild type; IL, interleukin; IECE, IRF/ets composite element; ISRE, interferon-stimulated response element; PRDI, positive regulatory domain I; EICE, ets/IRF composite element; SCF, stem cell factor.

expressed in CD34+ bone marrow progenitor cells and in differentiating and mature myeloid and B cells (1, 2). ICSBP functions as an activator or repressor of gene transcription, depending upon the sequence of the cis element and cellular context. For example, during myeloid differentiation, ICSBP activates transcription of the genes encoding the phagocyte oxidase proteins gp91^{PHOX} and p67^{PHOX}, the Toll-like receptor 4, and interleukin-12 (3–6). Therefore, ICSBP contributes to functional differentiation of phagocytic cells.

ICSBP also activates transcription of the genes encoding Neurofibromin (Nf1) and Ink4b in differentiating myeloid cells (7, 8). ICSBP-induced expression of Nf1 and Ink4b decreases the proliferative effects of cytokines such as GM-CSF and SCF. In contrast, ICSBP represses transcription of the gene encoding Fas-associated phosphatase (Fap1; the *PTPN13* gene) in myeloid progenitors (9). Because Fap1 antagonizes Fas-induced apoptosis, a decrease in ICSBP-induced repression of *PTPN13* transcription during myelopoiesis increases the sensitivity of differentiating cells to Fas (10–12). Therefore, ICSBP-deficient myeloid cells would be anticipated to exhibit apoptosis resistance, cytokine hypersensitivity, and phagocyte functional defects.

Consistent with this hypothesis, studies in murine models identified a leukemia suppressor function for ICSBP. In one model the gene encoding ICSBP (the *IRF8* gene) was disrupted by homologous recombination (13, 14). Mice with homozygous ICSBP knock-out develop an MPD characterized by increased granulocytes in the peripheral blood and tissues. 80% of ICSBP^{-/-} mice develop myeloid differentiation block and progress to fatal blast crisis over 6–8 months (13, 15).

These results suggest that ICSBP deficiency is sufficient for myeloproliferation but that development of AML (*i.e.* blast crisis) requires accumulation of additional mutations. In another model, mice were transplanted with bone marrow expressing *Bcr/abl*; the CML oncoprotein. These mice develop a CML-like MPD that progresses to blast crisis (BC) with time (16). Decreased ICSBP expression is observed in the bone marrow of these mice (16). Overexpression of ICSBP in *Bcr/abl*⁺ bone marrow decreases MPD and delays progression to blast crisis in this model (16), suggesting ICSBP deficiency in CML contributes to myeloproliferation and predisposes to acquisition of genetic defects leading to BC.

Studies of human leukemia also identified an association between ICSBP deficiency and myeloid malignancy. Various investigators found decreased ICSBP expression in up to 70% of

human AML (17). Decreased ICSBP expression is observed in myeloid blasts, total bone marrow cells, and in CD34⁺ cells from the bone marrow of subjects with AML and myelodysplastic syndrome (2, 17). Also, decreased ICSBP expression is found in bone marrow samples from subjects with uncontrolled, chronic phase CML (18, 19). ICSBP expression increases during remission but decreases during progression to CML-BC (18, 19).

These results indicate the importance of normal ICSBP expression for myelopoiesis. However, cytokine-induced post-translational modification also regulates ICSBP function. For example, activation of the genes encoding gp91^{PHOX}, p67^{PHOX}, and Nf1 by ICSBP requires phosphorylation of conserved tyrosine residues in the interferon regulatory factor (IRF) domain of this protein (20, 21). Also, repression of *PTPN13* transcription by ICSBP is decreased by tyrosine phosphorylation (9). This is significant because ICSBP tyrosine phosphorylation increases during myeloid differentiation (20, 21). Therefore, both abundance and post-translational modification of ICSBP influence phagocyte functional competence, cytokine-induced proliferation, and apoptosis in differentiating myeloid cells.

The tendency for ICSBP^{-/-} mice to progress from MPD to blast crisis suggests that ICSBP deficiency predisposes to the accumulation of genetic lesions. Possible candidate genes for such an effect would include genes involved in DNA repair. To identify target genes involved in the ICSBP-leukemia suppressor effect, we used chromatin co-immunoprecipitation coupled with sequence analysis or CpG island microarray screening. In previously reported studies, we used identified genes encoding Fap1, Nf1, and various inflammatory mediators as ICSBP target genes using this approach (7, 9). In the current studies we report that the *FANCF* gene, which encodes the Fanconi complementation group F protein, is an ICSBP target gene of potential relevance to disease progression in myeloid malignancy.

Fanconi F is one of eight identified Fanconi proteins (A, B, C, D2, E, F, G, L, and M) that participate in repair of double-stranded DNA breaks (22, 23). Deficiency in any Fanconi protein increases susceptibility to DNA damage by cross-linking agents such as mitomycin C (MMC) or diepoxybutane (24). Congenital absence of any of these proteins results in Fanconi anemia (FA), a bone marrow failure disorder characterized by excess apoptosis of bone marrow progenitor cells due to DNA damage (25). Many subjects with FA develop AML due to acquisition of mutations which impair the normal apoptotic response to DNA damage (25). Acquired Fanc deficiency has been found in human AML and in CML-BC (19, 26). Fanc proteins A, C, E, F, and G form the Fanconi "nuclear complex." FancF is an adaptor protein that associates the A-G and C-E heterodimers (22). This complex permits ubiquitination of FancD2, which interacts with BRCA2 and ATM at the site of DNA damage. Therefore, FancF would be a reasonable candidate for the rate-limiting protein for this pathway.

In these studies we investigate the possibility that *FANCF* is a functionally significant ICSBP target gene in myeloid cells. Identification of *FANCF* as a target gene suggests the possibility that ICSBP deficiency in human myeloid malignancy leads to an increase in susceptibility to DNA cross-link damage. This would be consistent with the tendency of chromosomal trans-

locations to accumulate with disease progression in primary AML or with progression of CML to blast crisis.

MATERIALS AND METHODS

Plasmids

Protein Expression Vectors—The ICSBP cDNA was obtained from Dr. Ben Zion-Levi (Technion, Haifa, Israel), and the full-length cDNA was generated by PCR and subcloned into the mammalian expression vector pcDNA (27). The human FancF cDNA was obtained by reverse transcriptase and PCR using RNA from U937 myeloid cells. The cDNA was compared with published sequences and subcloned into the pcDNA expression vector and pMSCVpuro retroviral vector (Stratagene, La Jolla, CA).

shRNA Expression Vectors—ICSBP- and FancF-specific shRNA and scrambled control sequences were designed with the assistance of the Promega website. Double-stranded oligonucleotides representing the complementary sequences separated by a hairpin loop were subcloned into the pLKO.1puro vector (a gift from Dr. Kathy Rundell, Northwestern University, Chicago, IL). Several sequences were tested, and the most efficient for ICSBP or FancF were used in the experiments.

***FANCF* Reporter Vectors**—Various fragments of the *FANCF* 5'-flank were obtained from U937 chromatin by genomic PCR. The fragments were sequenced to ensure identity with the published sequence and subcloned into the pCATE reporter vector (Promega, Madison, WI).

Oligonucleotides

Oligonucleotides were custom-synthesized by MWG Biotech (Piedmont, NC). Double-stranded oligonucleotides used in DNA affinity purification experiments represented the proximal 30- or 20-bp sequence of the *FANCF* promoter. Double-stranded oligonucleotides used in electrophoretic mobility shift assays represent the proximal 60 bp of the *FANCF* promoter. Other oligonucleotides used in these assays include sequences from the ICSBP binding sites in the *NFI* (5'-ggatcccactccgg-ggc-3') (9) or *CYBB* (5'-ctgctgttttcatttctcattggaagaagaagcatag-3') (3) promoters. An oligonucleotide with the α globin gene CCAAT box was used as a control (5'-ccgggctccgcgccagcaat-gagcgcggc-3') (3).

Myeloid Cell Line Culture

The human myelomonocytic leukemia cell line U937 (28) was obtained from Andrew Kraft (Hollings Cancer Center, Medical University of South Carolina, Charleston, SC). Cells were maintained as described (3, 4). U937 cells were treated for 48 h with 500 units/ml human recombinant IFN γ for differentiation (Roche Applied Science).

Primary Murine Bone Marrow Studies

Animal studies were performed according to a protocol approved by the Animal Care and Use Committees of Northwestern University and Jesse Brown Veterans Affairs Medical Center.

Bone Marrow Harvest and Culture—Bone marrow mononuclear cells were obtained from the femurs of WT or ICSBP^{-/-}

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C57/BL6 mice (13). Sca1⁺ cells were separated using the Miltenyi magnetic beads (Miltenyi Biotechnology, Auburn, CA). Bipotential myeloid progenitor cells were cultured (at a concentration of 2×10^5 cells per ml) for 48 h in Dulbecco's modified Eagle's media supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, 10 ng/ml murine GM-CSF (R&D Systems Inc., Minneapolis, MN), and 10 ng/ml murine recombinant IL-3 (R&D Systems). Cells were either maintained in GM-CSF plus IL3 for 48 h or were differentiated over 72 h in 10 ng/ml G-CSF or 10 ng/ml murine M-CSF.

Bone Marrow Retroviral Transduction—Retrovirus was generated with the *FancF*/MSCV plasmid or control MSCV using the Phoenix cell packaging line according to manufacturer's instructions (Stratagene). The average concentration of producer cell supernatants was 10^7 pfu/ml. Bone marrow mononuclear cells were obtained as above and cultured for 24 h in 10 ng/ml IL3, 10 ng/ml GM-CSF, and 100 ng/ml SCF. Cells were transduced by incubation with retroviral supernatant in the presence of Polybrene (6 μ g/ml) as described (7). Transduced cells were selected for 48 h in puromycin, differentiated with M-CSF, and used for mitomycin C cross-link studies, described below. Transgene expression was confirmed by real time PCR.

Quantitative Real Time PCR

RNA was isolated using the Triazol reagent (Invitrogen) and tested for integrity by denaturing gel electrophoresis. Primers were designed with Applied Biosystems software, and real time PCR was performed using SYBR green according to the "standard curve" method. Results were normalized to 18 S (for mRNA determination) or input chromatin (for chromatin immunoprecipitation studies).

Murine DNA Cross-link Assays

Bone marrow was harvested from WT or ICSBP^{-/-} mice between 20 and 26 weeks of age. At this point, the majority of ICSBP^{-/-} mice have MPD but have not progressed to AML. Peripheral blood counts were performed, and mice were selected with less than 10% blasts in the peripheral blood. Murine bone marrow cells were separated and cultured in GM-CSF, IL3, and SCF for 48 h, as above. Other cells were cultured for 24 h in GM-CSF, IL3, and SCF followed by 48 h in M-CSF. Some cells were treated with mitomycin C (20 ng/ml) for the final 24 h of incubation. Chromosome spreads were made according to standard techniques. Chromosomes were analyzed microscopically for breaks and radials.

Chromatin Immunoprecipitation and Gene Discovery

U937 cells were cultured with or without IFN γ for 48 h (3, 4). Cells were incubated briefly in media supplemented with formaldehyde to generate DNA-protein cross-links. For sequence analysis studies, cell lysates were sonicated to generate chromatin fragments with an average size of 2.0 kb (29). Lysates underwent two rounds of immunoprecipitation with ICSBP anti-serum (29). Antibody to ICSBP was a kind gift of Dr. Stephanie Vogel (University of Maryland, Baltimore, MD). Precipitated chromatin was recovered as described (29). The chromatin was modified to have blunt ends using Klenow enzyme, and BamHI linkers were added. The linker ligated chromatin was digested

with BamHI and subcloned into Bluescript plasmid, and individual clones were sequenced using non-high-throughput approaches. Sequences were analyzed by individual GenBankTM search to identify potential target genes.

Identified genes were confirmed by independent chromatin immunoprecipitation experiments. For these studies chromatin was co-immunoprecipitated from U937 lysates with antibody to ICSBP or preimmune serum. Lysates were sonicated to generate chromatin fragments of 500 bp to 1.5 kb. This precipitated chromatin was PCR-amplified using primer sets to various 500-bp sequences in the 5'-flank or first exon. PCR products were separated by agarose gel electrophoresis. In other studies cell lysates were sonicated to generate chromatin fragments of 200–500 bp. This chromatin was used in quantitative real time PCR.

Myeloid Cell Line Transfections and Assays

Stable Transfectants—U937 cells were transfected by electroporation with equal amounts of an ICSBP expression vector or empty vector control (ICSBP/pcDNAamp or pcDNAamp) plus a vector with a neomycin phosphotransferase cassette (pSR α) (30 μ g each). Stable pools of cells were selected in G418 (0.5 mg/ml), and aliquots were tested for ICSBP expression by Western blot. Other cells were transfected by electroporation with a construct to express an ICSBP-specific shRNA or scrambled control shRNA using the pLKO.1puro vector. Stable pools of transfected cells were selected in puromycin (1.2 μ g/ml) and tested for ICSBP expression by Western blot. In some experiments U937 cells that were stably transfected with either ICSBP expression vector or empty vector control were co-transfected with a vector to express either a *FancF*-specific shRNA or scrambled control shRNA or ICSBP-specific shRNA or scrambled control shRNA. Cells were selected in both G418 and puromycin.

DNA Cross-link Repair Assays—DNA cross-links were generated in a plasmid with the chloramphenicol acetyltransferase reporter linked to a cytomegalovirus promoter by incubation with mitomycin C (40 μ M) (24). U937 cells (32×10^6 /ml) were co-transfected with MMC-treated plasmid or sham-treated control and vectors to overexpress ICSBP or control (ICSBP/pcDNAamp or pcDNAamp) and *FancF*-specific shRNA or scrambled control (sh*FancF*/pLKO or shcontrol/pLKO) (50 μ g). Reporter assays were performed with or without 24 h of IFN γ treatment (400 units/ml) as described (3, 4). Efficiency of DNA repair was indicated by chloramphenicol acetyltransferase reporter gene expression. Cells were also transfected with a β -galactosidase reporter vector to control for transfection efficiency (Rous sarcoma virus/ β -galactosidase).

***FANCF* Reporter Assays**—U937 cells were co-transfected with a construct with various *FANCF* 5'-flank sequences linked to a chloramphenicol acetyltransferase reporter (1.6 kb and 500, 200, 100, 50, 30, or 20 bp *FANCF*/pCAT or empty pCAT control) (70 μ g) and a vector to overexpress ICSBP or vector control (50 μ g). Reporter assays were performed with or without 24 h of treatment with IFN γ (400 units/ml). Cells were also transfected with a β -galactosidase reporter vector to control for transfection efficiency.

Western Blots

Cells were lysed by boiling in 2× SDS sample buffer. Lysate proteins (50 μg) were separated by SDS-PAGE (8% acrylamide) and transferred to nitrocellulose. Western blots were serially probed with antibodies to ICSBP, FancF, and glyceraldehyde-3-phosphate dehydrogenase or tubulin (to control for loading). Each experiment was repeated three times with different batches of lysate proteins, and representative blots are shown.

In Vitro DNA Binding Assays

Isolation of Nuclear Proteins—Nuclear proteins were extracted from U937 cells by the method of Dignam (31) as described (3, 4).

DNA Affinity Purification Assays—Nuclear proteins (300 μg) were incubated with biotin-labeled double-stranded oligonucleotide probe representing the –20- or –30-bp *FANCF* promoter overnight in DNA affinity purification assay buffer (25 mM HEPES, pH 7.6, 60 mM KCl, 5 mM MgCl₂, 7.5% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.25% Triton X-100). DNA-protein complexes were precipitated with 50 μl of a 50% slurry of neutravidin-coated agarose beads (Pierce). Proteins bound to the beads were eluted, separated by SDS-PAGE, and transferred to nitrocellulose. Western blots were probed with antibody to ICSBP (Santa Cruz Biotechnology, Santa Cruz, CA).

Electrophoretic Mobility Shift Assays—Nuclear proteins (2 μg) were incubated with nuclear extract binding buffer, poly(dI·C) and a radiolabeled probe representing the –20- to –30-bp sequence from the *FANCF* promoter as described (3, 4). Unlabeled, competitor oligonucleotide was added to some binding reactions (200-fold molar excess). Antibody to ICSBP (Santa Cruz Biotechnology) or control antibody (anti-glutathione *S*-transferase; Santa Cruz Biotechnology) was added to other binding reactions. Proteins were separated by native acrylamide gel electrophoresis, and DNA probes were identified by autoradiography.

Genomic Sequence Analysis

Conserved genomic sequences and consensus sequences for IRF protein DNA binding were identified using the VISTA software (Genomics Division of the Lawrence Berkley National Laboratory (Berkeley, CA) (32–34).

Statistical Analysis

Statistical significance was determined by Student’s *t* test and analysis of variance methods using SigmaPlot and SigmaStat software.

RESULTS

Identification of FANCF as an ICSBP Target Gene—To identify target genes involved in the leukemia suppressor function of ICSBP, we used chromatin co-immunoprecipitation followed by sequence analysis. A GenBank™ search was employed to identify putative ICSBP target genes represented by co-precipitating sequences. Because ICSBP can function as either a repressor or activator, we were interested in target genes in both immature and differentiating myeloid cells.

TABLE 1

Genes identified by sequence analysis of ICSBP-co-precipitating chromatin are listed

<i>APC</i>
<i>C4ST</i>
<i>CLM</i>
<i>CST5</i>
<i>CTBS</i>
<i>FANCF</i>
<i>GLUCY1A3</i>
<i>IRF2</i>
<i>NF1^a</i>
<i>PCDC10</i>

^a Previously reported.

In these studies we used U937 cells, a myeloid leukemia line that undergoes differentiation in response to various cytokines, including IFNγ (28). Over a 48-h period, differentiating U937 cells acquire functional capabilities characteristics of mature phagocytes such as respiratory burst activity and phagocytosis (3, 4, 28). U937 differentiation is also characterized by cell cycle arrest by 24 h and programmed cell death over 48–96 h. These cells express IRFs, including ICSBP (3, 4). In previous studies we found differentiation stage-specific regulation of ICSBP target genes in these cells (3, 4, 9, 27). Therefore, U937 cells represent a reasonable model to study events influenced by ICSBP during myelopoiesis.

Chromatin co-immunoprecipitation was performed with an antibody to ICSBP (or control, pre-immune serum) and U937 cells (with or without IFNγ differentiation). Co-precipitating chromatin fragments were individually subcloned into plasmid vectors and subjected to sequence analysis. Using this approach we identified a 5′-flank sequence from a number of potential ICSBP target genes, including *FANCF* (Table 1).

The *FANCF* gene encodes a protein in the Fanconi family of DNA repair proteins designated F (FancF). The identified *FANCF* sequence included ~300 bp in the proximal 5′-flank of the gene (Fig. 1A). Independent chromatin co-immunoprecipitation experiments were performed to confirm interaction of ICSBP with the *FANCF* 5′-flank and locate a binding site. Chromatin that co-precipitated with ICSBP (or preimmune serum) was amplified with primers representing various *FANCF* sequences (Fig. 1B). In experiments with IFNγ-differentiated U937 cells, 0 to –1.5 kb, 0 to –500 bp, and 0 to –150 bp of the *FANCF* 5′-flank specifically co-precipitated with ICSBP. These sequences did not co-precipitate with ICSBP from undifferentiated cells. These results suggest that ICSBP might regulate *FANCF* transcription in response to the genotoxic stress of myelopoiesis. Other tested *FANCF* sequences did not co-precipitate with ICSBP.

We next investigated the *FANCF* 5′-flank for ICSBP binding consensus sequences. IRF proteins bind to DNA through interferon-stimulated response elements (ISREs), ets/IRF composite elements (EICEs), closely related IRF/ets composite elements (IECEs), or positive regulatory domain I elements (PRDIs). We searched the proximal 2.0-kb *FANCF* 5′-flank for these sequences and identified a number of potential binding sites which we characterized by sequence type (Table 2). Three potential sites were identified within the proximal 150 bp of *FANCF* 5′-flank (Fig. 1A).

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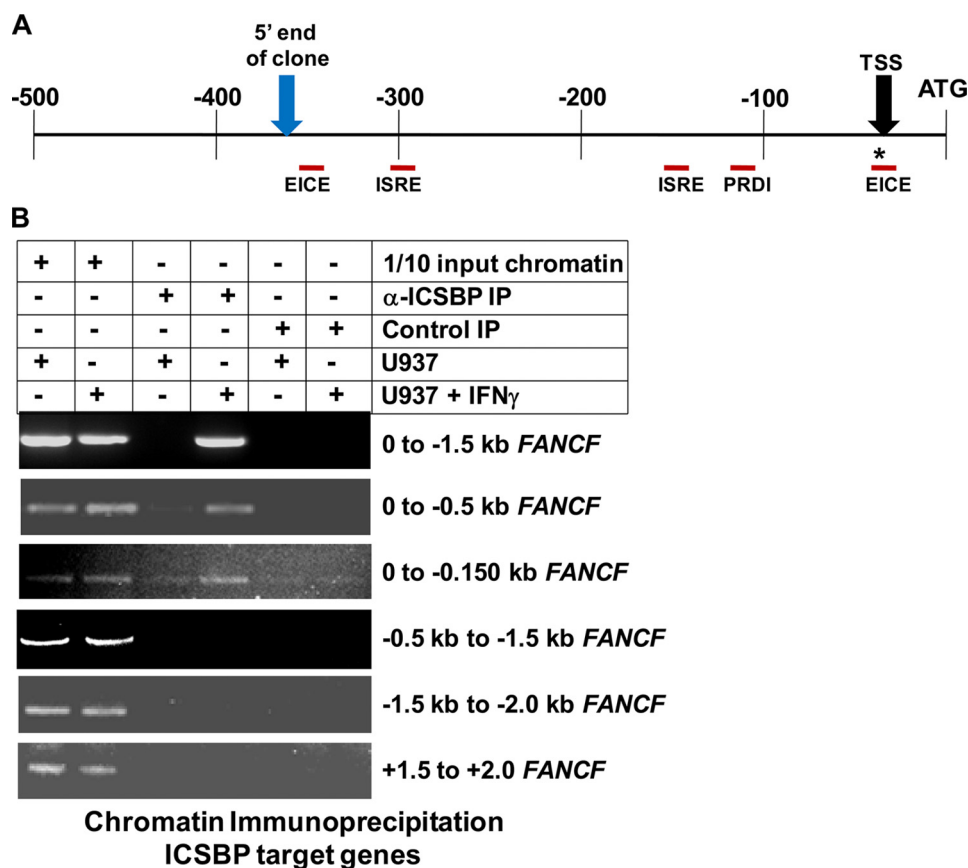


FIGURE 1. *FANCF* is an ICSBP-target-gene in differentiating myeloid cells. *A*, a schematic representation of consensus sequences for IRF-protein binding in the proximal *FANCF* 5'-flank is shown. The sequence of the proximal *FANCF* 5'-flank was analyzed for IRF DNA binding consensus sequences (underlined in red). The ICSBP binding cis element is indicated by an asterisk. The red arrow indicates the 5' end of the sequence of the clone obtained by ICSBP co-immunoprecipitation. The black arrow indicates the transcription start site. TSS, transcription start site. *B*, ICSBP interacts with the proximal 5'-flank of the *FANCF* gene *in vitro* in IFN γ -differentiated, but not untreated, U937 myeloid cells. Chromatin co-immunoprecipitation (IP) was performed with U937 myeloid leukemia cells (with or without IFN γ -induced differentiation) and an antibody to ICSBP (or irrelevant control antibody). Co-precipitating chromatin was amplified with primers encompassing 0 to -1.5 kb, 0 to -0.5 bp, 0 to -150 bp, -0.5 bp to -1.5 kb, or -1.5 to -2.0 kb in the proximal *FANCF* 5'-flank or +1.5 to +2.0 kb (in exon I).

TABLE 2

Location	Sequence	Homology
-21 to -30	GAAAGCGGAA	IECE
-29 to -35	TCACCTT	PRDI
-136 to -146	GAACACGGATA	ISRE
-180 to -188	GAAAGTAAA	ISRE
-297 to -304	GAAAGGAA	IECE
-321 to -329	GTAGAAA	EICE
-423 to -431	AATGAAA	ISRE
-542 to -537	TCAGTT	PRDI
-594 to -602	GCAAAGGAA	EICE
-642 to -651	GAAAACGAAA	EICE
-697 to -706	GGAATGGGATT	IECE
-882 to -889	AATGAA	ISRE
-898 to -906	GGAAGGAAA	EICE

*ICSBP Activates a *FANCF* Cis Element in Differentiating Myeloid Cells*—To determine the functional significance of ICSBP binding to the *FANCF* 5'-flank, we performed promoter/reporter assays. For these studies reporter constructs were generated with 1.6 kb to 20 bp of the *FANCF* 5'-flank. These constructs (or empty reporter vector) were co-transfected into U937 cells with a vector to overexpress ICSBP (or vector control). Reporter expression was determined with or without IFN γ differentiation.

We found that IFN γ treatment increases activity of the all constructs except for the 20-bp construct (Fig. 2A). We found that ICSBP overexpression significantly increases reporter activity from constructs with 1.6 kb to 30 bp of *FANCF* 5'-flank in differentiated transfectants but not undifferentiated transfectants. This is consistent with our initial chromatin immunoprecipitation data. Expression from the empty reporter vector control was not influenced by ICSBP overexpression or IFN γ -induced differentiation and was subtracted as background.

Reporter activity of constructs containing between 1.6 kb and 100 bp of 5'-flank is equivalent in IFN γ -differentiated, ICSBP-overexpressing transfectants (Fig. 2A). However, reporter activity in IFN γ -treated, ICSBP-overexpressing transfectants with constructs containing 50 or 30 bp *FANCF* promoter is significantly less. Despite the difference in total reporter activity between these two groups, the increment in promoter activity due to ICSBP overexpression is equivalent for all constructs with 30 bp or more of *FANCF* 5'-flank (~2.4-fold, $p = 0.9$, $n = 6$). In undifferentiated transfectants, reporter activity is not significantly different for any of the constructs with or without ICSBP overexpression.

These studies indicate that differentiation of U937 cells increases ICSBP-induced *FANCF* promoter activity and *in vivo* ICSBP binding. Although ICSBP is equivalently expressed in untreated and differentiating U937 cells, we previously found that ICSBP tyrosine phosphorylation increases binding to and activation of other target genes (*CYBB*, *NCF2*, *NF1*) (3, 4, 7, 15). ICSBP is minimally tyrosine-phosphorylated in undifferentiated U937 cells, and tyrosine phosphorylation increases by 12 h of differentiation with IFN γ and is maximal by 48 h (15). Therefore, we investigated the role of ICSBP tyrosine phosphorylation in *FANCF* transcription.

U937 cells were co-transfected with a *FANCF* promoter construct and vectors to overexpress either WT ICSBP or a form of ICSBP with all tyrosine residues mutated to phenylalanine (Y-mut ICSBP). We previously demonstrated that these two proteins are equivalently stable in U937 transfectants (15). IFN γ -treated transfectants were analyzed for reporter activity (Fig. 2B). Unlike transfectants overexpressing WT ICSBP, reporter activity is not significantly different in Y-mut ICSBP-overexpressing transfectants in comparison to control ($p = 0.8$, $n = 3$).

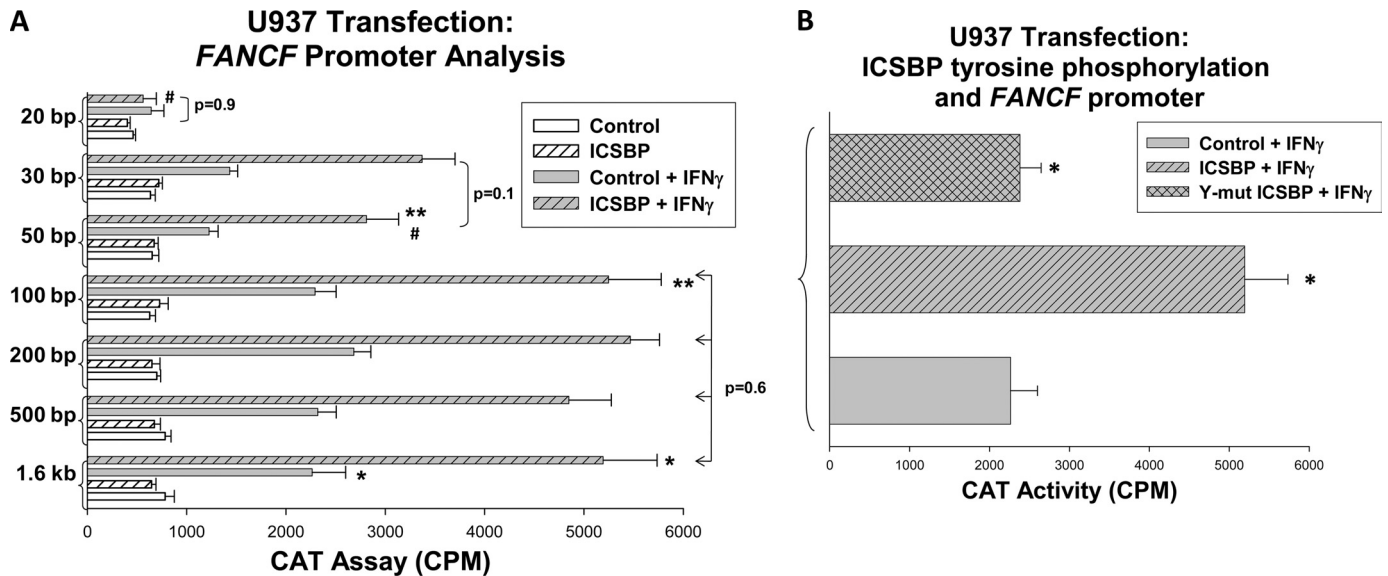


FIGURE 2. ICSBP activates *FANCF* transcription in differentiating myeloid cells. A, ICSBP activates a cis element found between -20 and -30 bp in the proximal *FANCF* 5'-flank in differentiating U937 cells. U937 cells were transfected with a series of plasmid constructs containing ICS sequences from the *FANCF* 5'-flank (1.6 kb, and 500, 200, 100, 50, 30, and 20 bp) linked to a reporter. Cells were co-transfected with a vector to overexpress ICSBP or empty vector control. Reporter gene assays were performed with or without IFN γ -induced differentiation of the transfectants. A statistically significant difference in reporter gene expression with versus without ICSBP overexpression in IFN γ -differentiated transfectants is indicated by an asterisk. Statistically significant differences in reporter gene expression in ICSBP overexpressing, differentiated transfectants with 100 bp of *FANCF* versus 50 bp is indicated by double asterisks (**), and for 50 bp and 20 bp is indicated by the number symbol (#). CAT, chloramphenicol acetyltransferase. B, ICSBP tyrosine phosphorylation is necessary for *FANCF* promoter activation. U937 cells were co-transfected with a reporter vector with the proximal 1.6 kb of *FANCF* 5'-flank (or empty control reporter vector) and a vector to overexpress ICSBP, a mutant form of ICSBP with all tyrosine residues mutated to phenylalanine (Y-mut ICSBP) or empty vector control. Reporter gene assays were performed after IFN γ -induced differentiation. Statistically significant difference in reporter activity in transfectants with WT versus Y-mut ICSBP overexpression is indicated by an asterisk.

We performed additional studies to verify ICSBP binding to the proximal *FANCF* promoter. Because overexpressed ICSBP increases activity from a 30-bp *FANCF* promoter construct but not a 20-bp construct, we hypothesized that there is an ICSBP-interacting cis element between -20 and -30 bp. This region of the *FANCF* 5'-flank includes an IECE. Therefore, we investigated *in vitro* ICSBP binding to this sequence using a DNA affinity purification assay.

For this study, biotin-labeled, double-stranded oligonucleotide probes with the proximal 20- or 30-bp sequence from the *FANCF* 5'-flank were incubated with nuclear proteins from untreated or IFN γ -differentiated U937 cells. DNA-bound proteins were co-purified by affinity to avidin-linked beads, and Western blots were probed with anti-ICSBP antibody. An aliquot of input protein was analyzed by Western blot with antibody to ICSBP and laminin A (a loading control) (Fig. 3A). These studies confirmed ICSBP binding to the proximal 30 bp of *FANCF* 5'-flank but not the 20-bp sequence.

We also investigated ICSBP binding to the proximal *FANCF* promoter using electrophoretic mobility shift assays. For these studies, radiolabeled, double-stranded oligonucleotide probe with 60 bp of proximal *FANCF* 5'-flank was incubated with U937 nuclear proteins. We identified a shifted complex that is more abundant in assays with nuclear proteins from differentiated U937 cells (Fig. 3B). To determine the specificity of this complex, some binding reactions were preincubated with excess unlabeled oligo nucleotide competitors representing -20 to -30 bp or -30 to -60 bp of the *FANCF* promoter. We found that only the former competes for binding of the shifted complex. Other binding reactions were preincubated with

competitor oligonucleotides representing ICSBP binding sites from the *NF1* or *CYBB* genes. Both of these oligonucleotides competed for binding of the shifted complex (Fig. 3B). To verify that ICSBP is a component of the complex, some binding reactions were preincubated with antibody to ICSBP. We found that this antibody specifically disrupts the complex (Fig. 3B). These experiments were repeated with at least two batches of proteins.

We also investigated ICSBP binding to this putative *FANCF* cis element *in vivo* by chromatin immunoprecipitation. In these studies lysates were sonicated to generate chromatin fragments of 200–500 bp size. Chromatin was amplified by quantitative real time PCR using primers designed to flank the proximal 60-bp *FANCF* 5'-flank. We found significantly more *in vivo* binding of endogenous ICSBP to this sequence in differentiated U937 cells in comparison to untreated cells (Fig. 3C).

These studies suggest that ICSBP interacts with a positive cis element in the proximal *FANCF* promoter and activates transcription in differentiating myeloid cells. However, these studies did not determine the impact of altered ICSBP expression on endogenous *FancF* expression. We addressed that issue directly.

FancF Expression Is ICSBP-dependent in Differentiating Myeloid Cells—We initially investigated the impact of ICSBP deficiency on *FancF* expression in ICSBP $^{-/-}$ mice. For these studies murine bone marrow was Sca1-selected and cultured in GM-CSF, IL3, and SCF. We previously found that such cultures consist of predominantly bi-potential myeloid progenitor cells (for either WT or ICSBP $^{-/-}$ bone marrow) (15, 21). Some cells were *ex vivo* differentiated to monocytes with M-CSF or gran-

ICSBP Activates FANCF Transcription

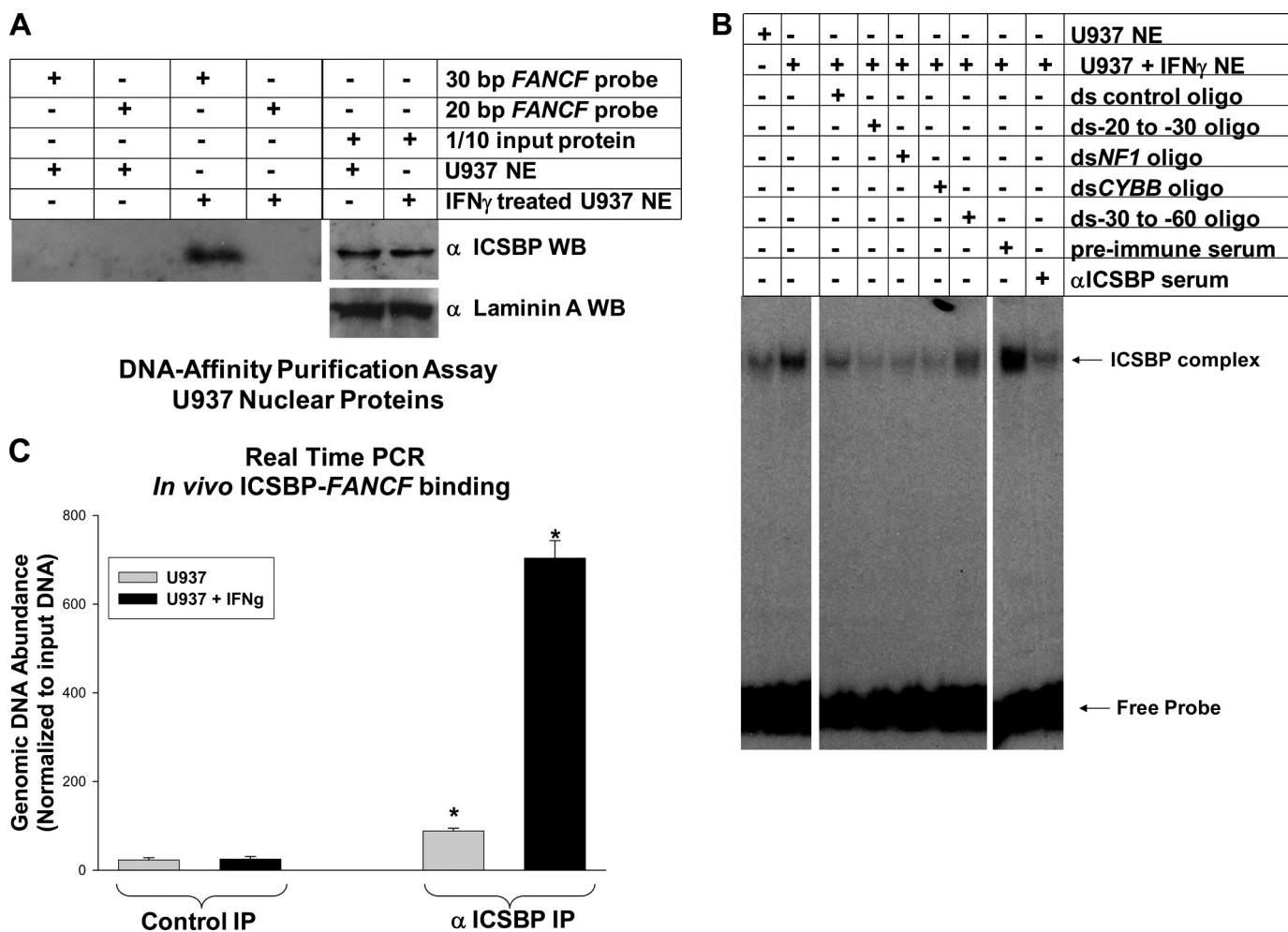


FIGURE 3. ICSBP binds to a sequence in the proximal FANCF promoter *in vitro* and *in vivo*. *A*, ICSBP interacts with the -20 to -30 bp sequence from the FANCF promoter *in vitro* in DNA affinity purification assays with nuclear proteins from differentiated U937 cells. Nuclear proteins were isolated from U937 cells with or without IFN γ -induced differentiation. DNA affinity purification assays were performed using biotin-labeled probes representing the proximal 20 or 30 bp from the FANCF 5'-flank. Western blots (WB) of co-precipitating proteins were probed with antibody to ICSBP. Control Western blots were performed with 1/10 amounts of nuclear proteins used in the affinity purification experiments. These blots were sequentially probed with antibody to ICSBP and with laminin A (as a loading control for nuclear proteins). *B*, ICSBP interacts with the -20 to -30 bp sequence from the FANCF promoter *in vitro* in electrophoretic mobility shift assays with nuclear proteins from differentiated U937 cells. The nuclear proteins described above were also analyzed by electrophoretic mobility shift assays using a radiolabeled DNA probe representing the proximal 60 bp of FANCF 5'-flank. Some binding reactions were preincubated with double-stranded (ds) oligonucleotide competitors (200-fold molar excess) representing the -20 to -30 bp or -30 to -60 bp sequences from the FANCF promoter, the ICSBP binding cis element from the CYBB or NF1 genes, or an irrelevant oligonucleotide as indicated. Other binding reactions were preincubated with antibody to ICSBP or preimmune serum, as indicated. The shifted complex, which is cross-immunoreactive with ICSBP, is indicated by the upper arrow, and the free probe is indicated by the lower arrow. Shifted bands in the middle panel are less intense in comparison to the flanking panels because less nuclear protein was used ($1 \mu\text{g}$ versus $2 \mu\text{g}$), and the exposure was shorter. *C*, ICSBP interacts with the -20 to -30 bp sequence from the FANCF promoter *in vivo* in chromatin co-immunoprecipitation (IP) assays with differentiated U937 cells. Chromatin co-immunoprecipitation was performed with U937 cells (with or without IFN γ -induced differentiation) and an antibody to ICSBP (or irrelevant control antibody). Co-precipitating chromatin was amplified by real time PCR with primers flanking the -20 to -30 -bp sequence in the FANCF promoter. Results were normalized to total input chromatin. Statistically significant differences in the amount of co-precipitating chromatin in undifferentiated versus differentiated U937 cells is indicated by an asterisk. NE, nuclear extract.

ulocytes with G-CSF (as in Refs. 15 and 21). FancF expression was determined by real time PCR.

We found that FancF expression is not significantly different in WT versus ICSBP $^{-/-}$ myeloid progenitors cultured in GM-CSF, IL3, and SCF (Fig. 4A). For WT cells, differentiation with either M-CSF or G-CSF significantly increases FancF-expression. In contrast, there is no increase in FancF expression in ICSBP $^{-/-}$ progenitors treated with G-CSF or M-CSF. These results suggest that ICSBP is involved in regulation of FancF expression in differentiating myeloid cells. However, ICSBP $^{-/-}$ cells have a constitutive absence of ICSBP and may have developed compensatory mechanisms related to this deficiency.

Therefore, we tested the impact of directly manipulating ICSBP expression on FancF.

For these studies we used U937 myeloid cells. We found an increase in FancF mRNA (Fig. 4B) and protein (Fig. 4C) during U937 differentiation, consistent with the FANCF promoter activity studies. We next investigated the impact of altered ICSBP abundance on FancF expression. For these studies U937 cells were stably transfected with vectors to overexpress ICSBP (or control vector) or an ICSBP-specific shRNA (or scrambled control shRNA). Stable transfectants were analyzed for FancF mRNA (Fig. 4D) and protein (Fig. 4E). We found that ICSBP overexpression increases FancF in differentiating, but not undifferentiated, U937

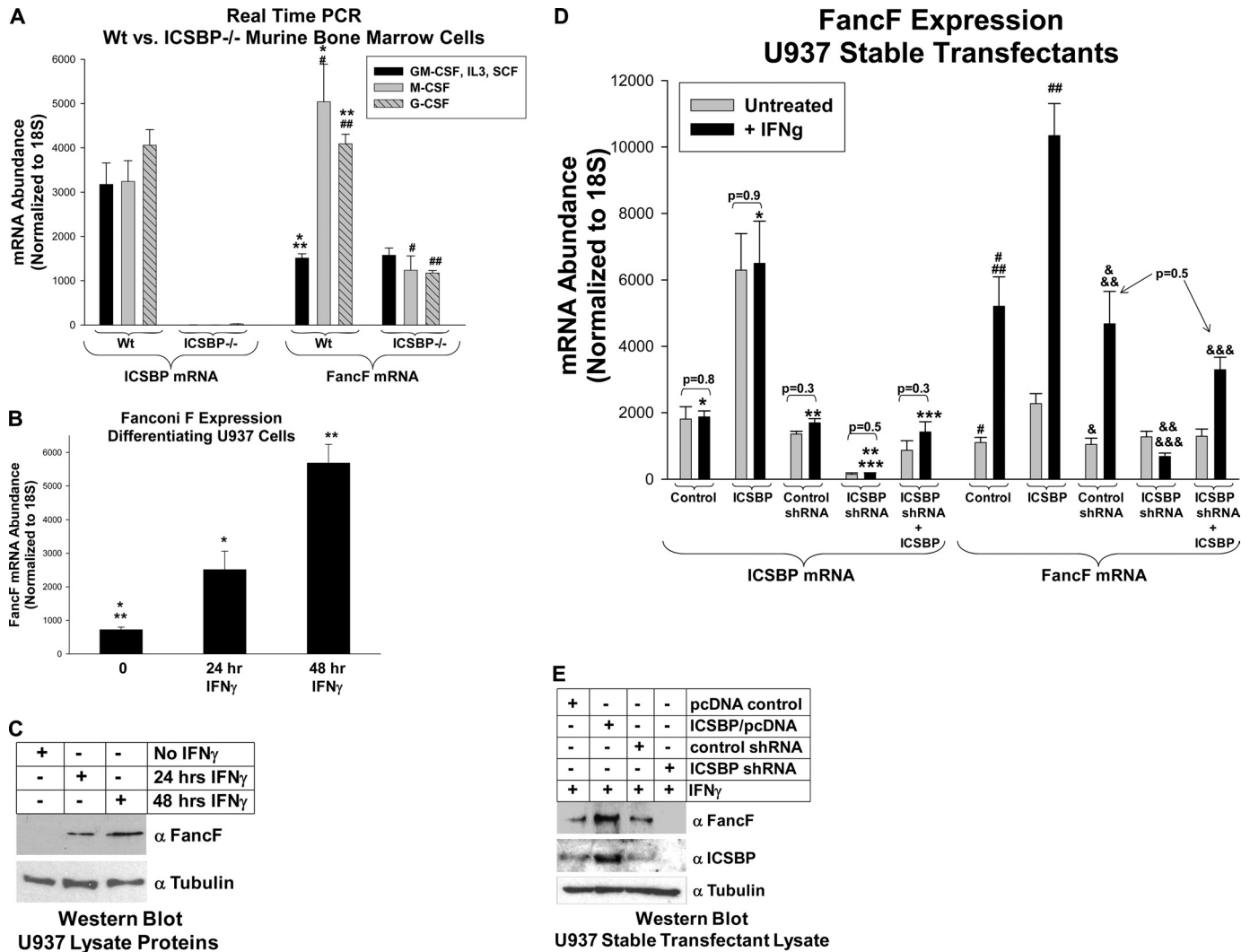


FIGURE 4. FancF expression is differentiation stage-specific and ICSBP-dependent. *A*, FancF mRNA expression is differentiation stage-specific and ICSBP-dependent in primary murine myeloid progenitor cells. Bone marrow was harvested from WT or ICSBP^{-/-} mice and Sca1-separated and cultured in GM-CSF, IL3, and SCF (bi-potential myeloid progenitors). Some of these cells were differentiated to granulocytes with G-CSF or monocytes with M-CSF. RNA was isolated from the cells and analyzed by quantitative real time PCR for FancF expression. Results were normalized to 18 S. Statistically significant differences in FancF mRNA expression in WT myeloid progenitors in comparison to M-CSF- or G-CSF-differentiated cells are indicated by *single* and *double asterisks*, respectively. Statistically significant differences in FancF expression between WT *versus* ICSBP^{-/-} cells differentiated with M-CSF or G-CSF are indicated by *single* and *double number symbols* (# and ##), respectively. *B*, FancF mRNA expression increases during IFN γ -induced differentiation of U937 myeloid leukemia cells. U937 cells were treated with IFN γ for 0, 24, or 48 h. RNA was isolated and analyzed for FancF mRNA expression by quantitative real time PCR. Results were normalized to 18 S. Statistically significant differences in FancF expression at 24 or 48 h in comparison to untreated cells are indicated by *single* and *double asterisks* and, respectively. *C*, FancF protein expression increases during IFN γ -induced differentiation of U937 myeloid leukemia cells. Proteins isolated from the U937 cells, described above, were analyzed by Western blot for FancF expression. The blot was re-probed with antibody to tubulin as a loading control. *D*, FancF mRNA expression is increased by ICSBP overexpression and decreased by ICSBP knockdown in differentiating U937 myeloid leukemia cells. Stable transfectants of U937 cells were generated with various combinations of vectors to overexpress ICSBP (or empty pcDNA vector control) or an ICSBP-specific shRNA (or with scrambled shRNA control). RNA isolated from the stable transfectants (with or without IFN γ -induced differentiation) was analyzed by quantitative real time PCR for FancF mRNA expression. ICSBP mRNA expression was also determined as a control for overexpression or knockdown. Statistically significant ICSBP overexpression or knockdown is indicated by *one* or *two asterisks*, respectively. Statistically significant differences in ICSBP expression in cells with ICSBP knockdown with *versus* without ICSBP re-expression is indicated by *three asterisks*. A statistically significant increase in FancF expression in control vector-transduced cells during differentiation is indicated by the *number symbol* (#) or *ampersand*. Statistically significant differences in FancF expression in differentiated cells with ICSBP overexpression *versus* vector control is indicated by the *double number symbol* (#). Statistically significant differences in FancF expression in differentiated cells with ICSBP knockdown *versus* control shRNA is indicated by a *double ampersand*. Statistically significant differences in FancF expression in cells with ICSBP knockdown with *versus* without ICSBP re-expression is indicated by *three ampersands*. *E*, FancF protein expression is increased by ICSBP overexpression and decreased by ICSBP knockdown in differentiating U937 myeloid leukemia cells. Proteins isolated from the U937 cells, described above, were analyzed by Western blot. The blots were sequentially probed with antibodies to ICSBP, FancF, and tubulin (as a loading control).

cells. Conversely, ICSBP knockdown blocks the increase in FancF expression in differentiating U937 cells. ICSBP expression was not altered by U937 differentiation, consistent with previous results (3, 4, 7, 15). In control experiments, re-expression of ICSBP rescued FancF expression in U937 cells with ICSBP-shRNA (Fig. 4D).

These studies suggest that ICSBP is functionally relevant to increased FancF expression during myelopoiesis. However, this did not provide a functional association between ICSBP deficiency, decreased FancF expression, and impaired DNA repair. We addressed this directly.

ICSBP Activates FANCF Transcription

ICSBP-deficient Murine Bone Marrow Cells Have Defective DNA Cross-link Repair—Previous studies in ICSBP^{-/-} mice indicate that ICSBP deficiency induces MPD and predisposes to additional mutations that lead to blast crisis. However, abnormalities in DNA repair have not been specifically investigated in these mice. Based on identification of *FANCF* as an ICSBP target gene, we hypothesized that ICSBP deficiency increases sensitivity to DNA cross-link agents.

To investigate this, bone marrow was harvested from ICSBP^{-/-} and WT mice and cultured in GM-CSF, IL3, and SCF, as above. Because we were interested in abnormalities leading to AML and not in mutations arising during disease progression, ICSBP^{-/-} mice in the MPD phase of the disease were selected. Some cells were cultured for 48 h under these cytokine conditions with or without MMC for the last 24 h. Because our studies indicate that ICSBP regulates *FANCF* transcription in differentiating myeloid cells, some cells were incubated for 24 h in GM-CSF, IL3, and SCF followed by 24 h of differentiation with M-CSF with or without MMC for the last 24 h. Chromosome spreads were analyzed for DNA breaks and radial formation.

We found only rare DNA breaks or radials in WT or ICSBP^{-/-} cells in the absence of MMC. In experiments with MMC-treated WT or ICSBP^{-/-} myeloid progenitors (in GM-CSF, IL3, SCF), we found few DNA breaks (Fig. 5A). Similarly, few DNA radials were generated by MMC treatment of WT or ICSBP^{-/-} myeloid progenitors cultured in GM-CSF, IL3, and SCF (Fig. 5B).

The number of DNA breaks was not significantly different in MMC-treated WT cells in GM-CSF, IL3, and SCF *versus* M-CSF ($p = 0.6$, $n = 4$). In contrast, there were significantly more DNA breaks in MMC-treated ICSBP^{-/-} cells undergoing M-CSF differentiation in comparison to ICSBP^{-/-} progenitors or WT cells under either cytokine condition ($p = 0.002$, $n = 4$). The number of DNA radials in MMC-treated WT or ICSBP^{-/-} cells undergoing differentiation was significantly greater than in WT or ICSBP^{-/-} myeloid progenitors. And significantly more radials were identified in MMC-treated, ICSBP^{-/-} cells undergoing M-CSF-induced differentiation in comparison to WT cells under the same conditions ($p = 0.003$, $n = 4$). These results suggest that ICSBP deficiency is associated with impaired repair of DNA cross-link damage (Fig. 5C). However, these studies do not determine the role of decreased FancF in this effect.

To investigate this we determined if FancF re-expression rescues DNA repair. For these studies, bone marrow cells were isolated from ICSBP^{-/-} mice and cultured in GM-CSF, IL3, and SCF, as above. Cells were transduced with a retroviral vector to overexpress FancF or with vector control. FancF transgene expression was confirmed by real time PCR. Cells were differentiated with M-CSF, and chromosome spreads of MMC-treated cells were analyzed. We found that ICSBP^{-/-} cells transduced with the FancF expression vector exhibited significantly fewer breaks (Fig. 5A) and radials (Fig. 5B) in comparison to cells with control vector. This supports our hypothesis regarding the role of FancF in DNA repair in ICSBP-deficient cells.

However, this murine model represents constitutive loss of ICSBP. Although we analyzed bone marrow from ICSBP^{-/-} mice that had not developed AML, it is possible that the observed DNA repair defects were secondary abnormalities. Therefore, we performed additional experiments to directly analyze the impact of manipulating ICSBP expression on cross-link repair.

ICSBP Influences DNA Cross-link Repair in a FancF-dependent Manner in U937 Cells—We investigated repair of cross-link DNA damage using U937 stable transfectants with ICSBP overexpression *versus* knockdown, discussed above. For these studies we determined the ability of the stable transfectants to repair a cross-linked reporter plasmid. In this assay, the reporter plasmid was treated *in vitro* with MMC to generate DNA cross-links. Cells were transfected with the cross-linked plasmid or sham-treated control plasmid. Reporter activity from the MMC-treated plasmid (in comparison to sham treated plasmid) reflects the efficiency of DNA repair (24).

We found efficient repair of the MMC-treated reporter plasmid in undifferentiated U937 transfectants with or without ICSBP overexpression. IFN γ treatment significantly decreases activity of the MMC-treated reporter construct in control transfectants ($p = 0.02$, $n = 4$) but does not decrease activity of sham-treated reporter plasmid ($p = 0.8$, $n = 5$) (Fig. 6A). However, reporter activity of the MMC-treated plasmid in ICSBP-overexpressing transfectants is not significantly different with or without IFN γ differentiation ($p = 0.9$, $n = 4$) (Fig. 6A). Conversely, transfectants expressing an ICSBP-specific shRNA exhibited decreased repair of the cross-linked reporter plasmid in differentiating transfectants ($p = 0.01$, $n = 3$). This defect could be rescued by re-expression of ICSBP (Fig. 6A). Cross-linking of the MMC-treated plasmid was demonstrated by electrophoresis (Fig. 6B). These studies provided another line of evidence suggesting that ICSBP influences DNA cross-link repair in differentiating myeloid cells.

We next investigated the impact of FancF expression on DNA cross-link repair. For these studies we generated stable transfectants with overexpression or knockdown of FancF (Fig. 6C). These cells were co-transfected with MMC-treated reporter plasmid or sham-treated plasmid, and reporter assays were performed with IFN γ -induced differentiation. In FancF-overexpressing, IFN γ -treated U937 cells, we found no significant difference in reporter expression from MMC-treated *versus* sham-treated plasmids (Fig. 6A). Reporter activity from the MMC-treated or sham-treated plasmids was decreased in cells stably expressing a FancF-specific shRNA in comparison to control (Fig. 6A). This decrease was reversed by re-expression of FancF.

To determine whether DNA-cross-link repair is FancF-dependent in ICSBP-overexpressing cells, stable U937 transfectants were generated with a vector to overexpress ICSBP and knockdown FancF. These cells were co-transfected with MMC-treated or control reporter constructs. We found that expression of a FancF-specific shRNA prevents ICSBP overexpression from rescuing DNA cross-link repair in IFN γ -differentiated transfectants (Fig. 6A). These studies suggest that increased FancF expression is at least one mechanism by which ICSBP

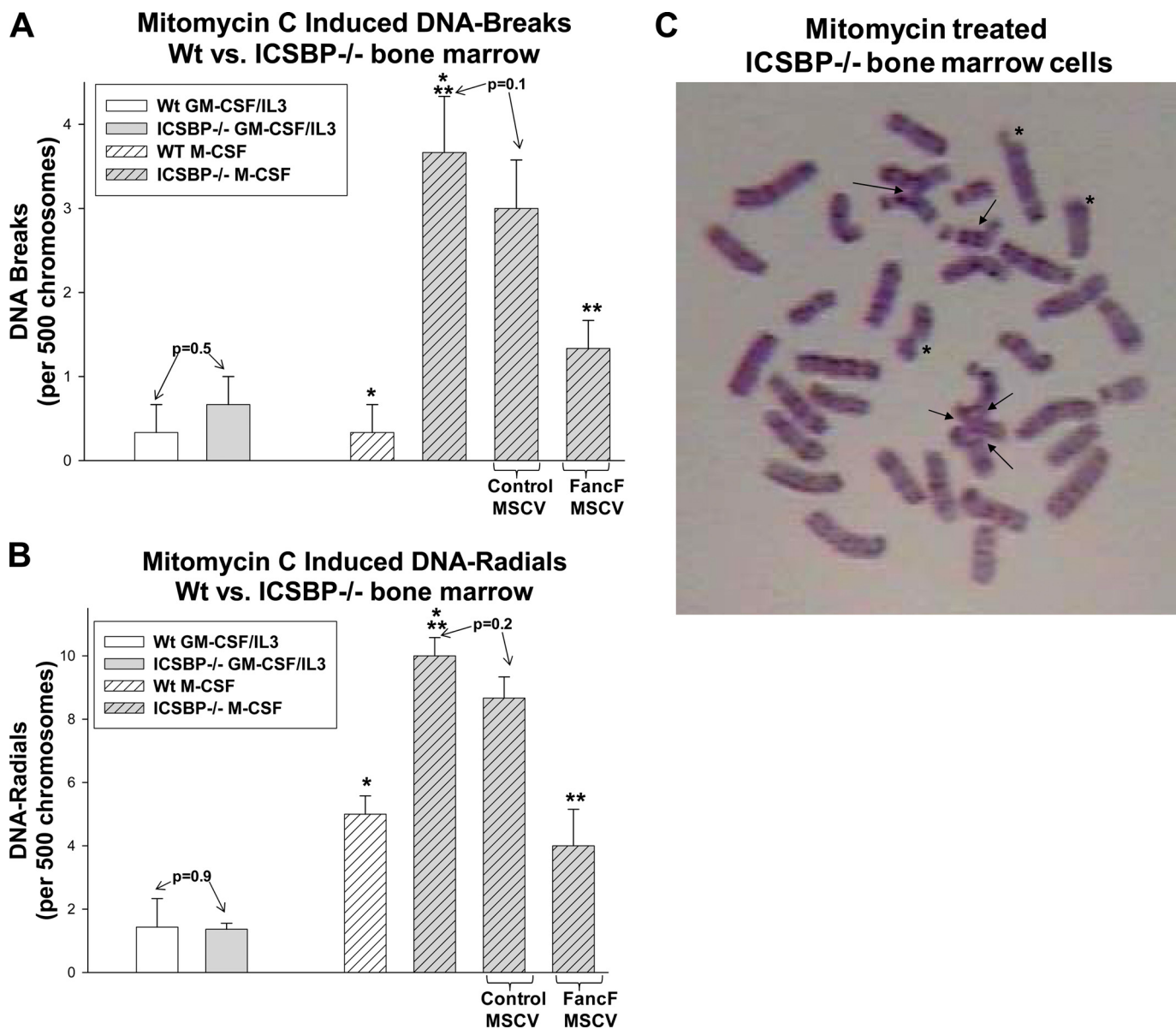


FIGURE 5. ICSBP deficiency increases sensitivity to mitomycin C-induced DNA damage in primary murine bone marrow cells in a FancF-dependent manner. A, ICSBP deficiency increases sensitivity to mitomycin C-induced DNA breaks in myeloid progenitor cells during M-CSF-induced differentiation in a FancF-dependent manner. Bone marrow cells were harvested from WT or ICSBP^{-/-} mice and cultured in GM-CSF, IL3, and SCF for 48 h or in GM-CSF, IL3, and SCF for 24 h followed by 24 h in M-CSF. MMC was added during the last 24 h of culture. Chromosome spreads were analyzed microscopically for DNA breaks (indicated as the number of breaks per 500 chromosomes). Statistically significant difference in DNA breaks for WT versus ICSBP^{-/-} cells is indicated by an asterisk. Other ICSBP^{-/-} bone marrow cells were cultured in GM-CSF, IL3, and SCF and transduced with a retroviral vector to express FancF (*FancF/MSCV*) or empty vector control (*MSCV*). After 48 h of antibiotic selection, cells were differentiated with M-CSF and treated with MMC, and chromosome spreads were analyzed for DNA breaks. Statistically significant differences in DNA breaks with versus without FancF re-expression are indicated by double asterisks. B, ICSBP deficiency increases sensitivity to mitomycin C-induced DNA radial formation in myeloid progenitor cells during M-CSF-induced differentiation. The chromosomes described above were also analyzed microscopically for the presence of DNA-radial forms (indicated as the number of radials per 300 chromosomes). A statistically significant difference in DNA radials for WT versus ICSBP^{-/-} cells is indicated by an asterisk and for ICSBP^{-/-} cells with versus without FancF re-expression is indicated by double asterisks. C, chromatin spread from ICSBP^{-/-} bone marrow cells with DNA breaks and radials. A representative chromatin spread from MMC-treated, M-CSF-differentiated ICSBP^{-/-} bone marrow cells. DNA breaks are indicated by an asterisk, and DNA radials are indicated by an arrow.

influences DNA cross-link repair in differentiating myeloid cells.

DISCUSSION

AML and CML-BC are characterized by ongoing accumulation of genetic lesions, leading to disease progression and worsening prognosis. This progression involves chromosomal breaks and translocations, as would be found with defective

double-stranded DNA-break repair (*i.e.* increased sensitivity to cross-link damage). Consistent with this, an acquired decrease in expression of Fanconi proteins is found in AML and CML-BC (19, 26). Expression of ICSBP is also decreased in human AML and CML-BC (2, 17, 18). In a murine model, ICSBP deficiency is sufficient for development of MPD (13, 15). Identification of ICSBP target genes involved in regulation of cytokine-induced proliferation (*i.e.* genes encoding the Ras-

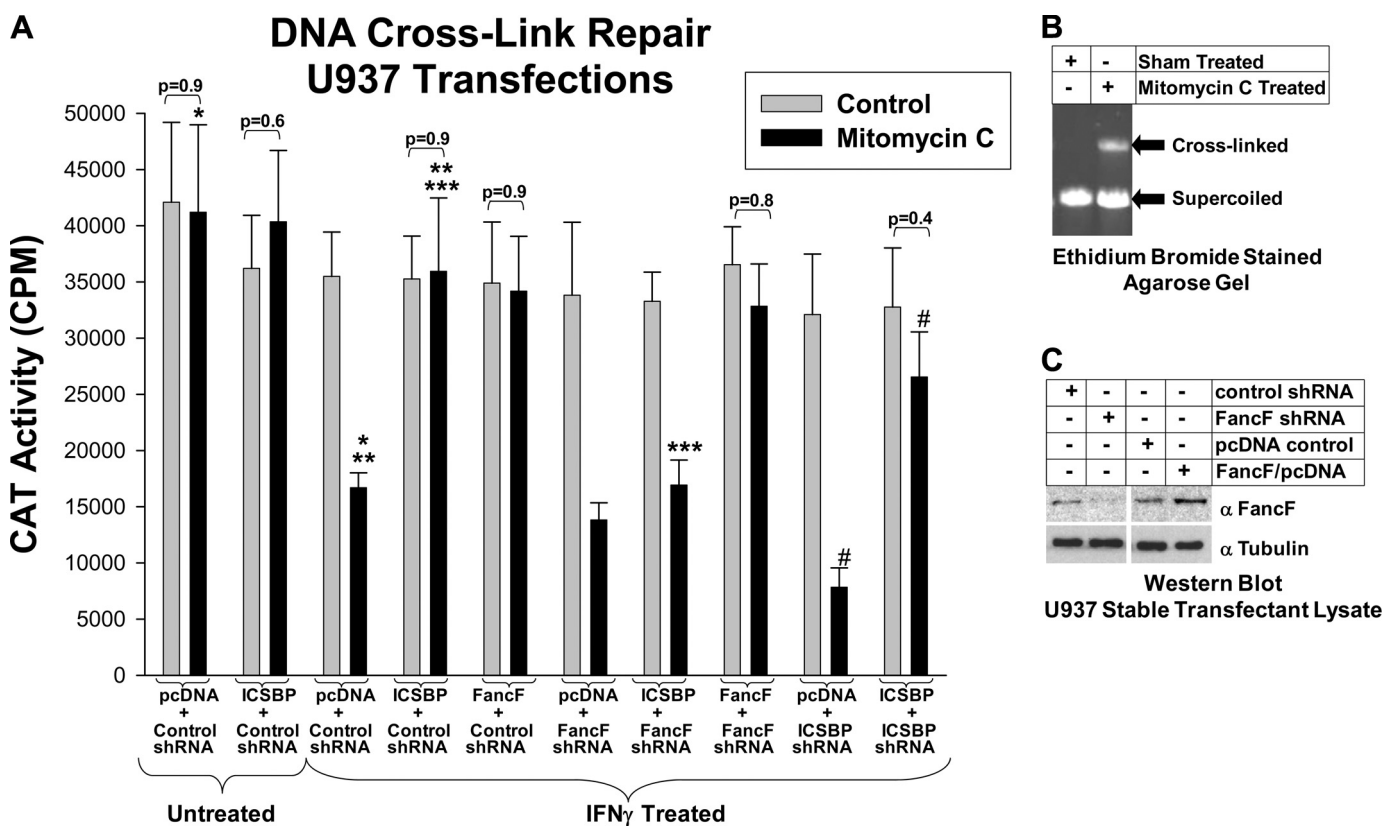


FIGURE 6. ICSBP overexpression increases DNA-cross-linked repair in differentiating U937 cells in a FancF-dependent manner. A, ICSBP overexpression improves the repair of mitomycin C-treated plasmid in differentiating U937 myeloid leukemia cells in a FancF-dependent manner. U937 stable transfectants were generated with various combinations of vectors to overexpress ICSBP or FancF (or with empty vector control) or with vectors to express an ICSBP or FancF-specific shRNA (or scrambled shRNA control). These transfectants were co-transfected with MMC-treated or sham-treated reporter plasmid, and reporter assays were performed with or without IFN γ -induced differentiation. A statistically significant difference in reporter expression from MMC-treated plasmid in control cells with versus without differentiation is indicated by an asterisk. CAT, chloramphenicol acetyltransferase. Statistically significant difference in MMC-treated reporter gene activity in differentiated transfectants with vector control versus ICSBP overexpression is indicated by double asterisks. Statistically significant difference in reporter gene activity from MMC-treated plasmid in ICSBP-overexpressing, IFN γ -treated transfectants with versus without FancF-specific shRNA are indicated by three asterisks. Statistically significant difference in reporter gene activity from MMC-treated plasmid in IFN γ -treated transfectants with ICSBP-knock down with versus without re-expression of ICSBP is indicated by the number symbol (#). B, mitomycin C treatment results in DNA-cross-link generation *in vitro*. Reporter plasmid DNA (chloramphenicol acetyltransferase reporter with a cytomegalovirus promoter) was treated *in vitro* with MMC (versus sham-treated control). Plasmid DNA was analyzed by native agarose gel electrophoresis and ethidium bromide staining to verify the presence of cross-linked DNA. C, FancF expression in stable transfectants of U937 myeloid leukemia cell with FancF overexpression or knock down. U937 stable transfectants were generated with a vector to overexpress FancF (or empty vector control) or with a vector to express a FancF-specific shRNA (or scrambled control shRNA). Western blots of lysates from differentiated stable transfectants were serially probed with antibodies to FancF and tubulin (as a loading control).

Gap Nf1 and Ink4b) and apoptosis (*i.e.* the gene encoding the Fas antagonist Fap1) suggest mechanisms for this effect. The lag time between development of MPD and AML in ICSBP^{-/-} mice suggests that additional mutations are required for differentiation block and disease progression. Because progression inevitably occurs in these mice, this model also suggests that ICSBP deficiency predisposes to ongoing DNA mutation.

In these studies, we identify the gene encoding Fanconi F as an ICSBP target gene. We define a FANCF cis element that is activated by ICSBP in differentiating myeloid cells. We determine that ICSBP-induced FancF expression protects myeloid cells from DNA cross-link damage during the genotoxic stress of differentiation. Therefore, these studies identify a unique role for ICSBP during myelopoiesis.

In these investigations we used chromatin co-immunoprecipitation to identify ICSBP target genes. ICSBP co-precipitating chromatin was subjected to sequence analysis or used to screen a CpG island microarray to identify potential target genes, as previously reported (7, 9). Using the first approach, we

identified the gene that encodes Nf1 as an ICSBP activation target in myeloid cells (9). In that study we determined that Nf1 deficiency is a mechanism for cytokine hypersensitivity in ICSBP-deficient cells. By the second approach we identified the gene encoding Fap1 as an ICSBP repression target (7). We demonstrated that increased Fap1 expression is a mechanism for Fas resistance in ICSBP-deficient myeloid cells. In the latter study we also reported a set of potential ICSBP target genes that encode proteins involved in the inflammatory response (7). In the current studies we report on a target gene that implicates ICSBP in a novel function; that is, regulation of DNA repair.

We identified ~300 bp of sequence from the FANCF 5'-flank/first intron in chromatin that co-precipitated with ICSBP from differentiated U937 cells. Although the assay conditions involve generation of 2.0-kb genomic DNA fragments by sonication, we found that the average size of subcloned inserts sequenced in our studies was not that large. However, based on the initial size of the chromatin fragments used in co-precipitation studies, we investigated the proximal 2.0 kb of FANCF

5'-flank for potential ICSBP binding cis elements. We used additional independent chromatin co-immunoprecipitation studies to further define the ICSBP/*FANCF* interaction.

Although the average size of sonicated chromatin fragments in these studies was 500 bp to 1.5 kb, PCR conditions were modulated to enrich for a specific 500 bp sequence across this locus. We analyzed the 5'-flank for IRF-protein DNA binding site consensus sequences and performed promoter assays to specify a functional, ICSBP binding cis element. These functional studies were confirmed by ICSBP co-precipitation studies using chromatin sheared to less than 500 bp to permit more specific evaluation of a potential binding site.

The proximal *FANCF* promoter includes a number of IRF DNA binding consensus sequences. These are of various types, including ISRE (5'-gaaanngaaact-3'), EICE (5'-ggaanngaaa-3'), IECE (5'-gaaann(n)ggaa-3'), and PRDI (5'-tcactt-3') sequences. In previous studies we identified an EICE in the genes encoding gp91^{PHOX} and p67^{PHOX} (the *CYBB* and *NCF2* genes, respectively) that is activated by a protein complex comprised of PU.1, IRF1, ICSBP, and the CREB (cAMP-response element-binding protein)-binding protein (3, 4). We also identified an IECE in the gene encoding Nf1 that is activated by PU.1, IRF2, and ICSBP (9). Assembly of these multiprotein complexes requires tyrosine phosphorylation of the IRF proteins during myelopoiesis.

In the current studies we identified an IECE at -21 to -30 bp in the proximal *FANCF* promoter that is activated by ICSBP in differentiating cells. Consistent with these data, the ICSBP binding cis elements from the *CYBB* and *NF1* genes compete for binding of the ICSBP-containing protein complex to this *FANCF* sequence. Also similar to the cis elements from these other two target genes, ICSBP tyrosine phosphorylation is required for *FANCF* transcriptional activation. These results suggest the possibility that ICSBP interacts with PU.1 or another ets protein to activate *FANCF* transcription. The partner proteins for ICSBP binding to *FANCF* are a subject of current investigation in the laboratory.

The functional IECE identified in the *FANCF* promoter at -21 to -30 bp corresponds to the reported location of the transcription start site. ICSBP binding cis elements in other target genes are generally within 30–100 bp of the transcription start site. The current studies represent the first identification of an ICSBP binding cis element immediately adjacent to the transcription start site. Other investigators determined that PU.1 interacts with TFIID and can substitute for a TATA box and initiate transcription (30). It is of interest because the *FANCF* promoter does not include a strong TATA-box sequence. If PU.1 interacts with ICSBP at this IECE, recruitment of basal transcription factors to this complex will be of interest.

The requirement for ICSBP tyrosine phosphorylation for *FANCF* transcription during myelopoiesis is consistent with regulation of other target genes. These studies suggest that both protein abundance and post-translational modification regulate the activity of ICSBP during myelopoiesis. In the current studies we found that FancF expression during IFN γ -induced differentiation of U937 cells follows a similar time course to ICSBP tyrosine phosphorylation in these cells (4). Similarly,

both FancF expression and ICSBP tyrosine phosphorylation increase during M-CSF-induced differentiation of primary murine myeloid progenitor cells (21). The requirement for specific ICSBP tyrosine residues for *FANCF* transcription will be determined in future studies in the context of partner proteins.

Increased expression of FancF in differentiating myeloid cells may identify a mechanism to protect cells from the genotoxic stress of differentiation. *In vivo*, stimulation of bi-potential granulocyte-monocyte progenitors with differentiating cytokines such as G-CSF or M-CSF occurs in response to infections challenge. These cytokines induce initial proliferation of progenitor cells followed by phenotypic and functional differentiation during "stress" or "emergency" myelopoiesis. Our results suggest that the Fanconi pathway may protect differentiating cells from DNA damage during this process.

In the Fanconi protein complex, FancF associates heterodimers of FancA·C with hetero dimers of FancG·E (22). This complex binds DNA and associates with FancD2. As the bridge protein, FancF would be a logical candidate for the rate-limiting factor for this interaction. Alternatively, ICSBP may regulate multiple Fanconi genes. These issues are currently under investigation in the laboratory. Fanconi proteins are not restricted in expression to hematopoietic cells. Involvement of a lineage specific transcription factor such as ICSBP in *FANCF* transcription suggests that regulation of this gene may involve different transcription factors in cells of various lineages.

Therefore, these studies identify *FANCF* gene regulation as a potentially important leukemia suppressor function of ICSBP. However, there are several important questions to be answered, including whether decreased ICSBP expression in AML or CML-BC correlates with decreased FancF expression. Our current studies suggest that decreased FancF expression in ICSBP-deficient myeloid leukemia cells may be a marker of adverse prognosis and portend disease progression. These studies also suggest that the pathway that regulates this function of ICSBP may be a rationale target for therapeutic approaches to prevent disease progression in myeloid malignancy.

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