

SIGNALING DEFECTS IN FANCONI ANEMIA GROUP C CELLS

By

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LIST OF COMMON ABBREVIATIONS

FA	Fanconi Anemia
MMC	mitomycin C
DEB	Diepoxybutane
IFN γ	Interferon gamma
JAK	Janus Kinase
STAT	Signal Transducer and Activator of Transcription
HPC	hematopoietic progenitor cells
LDBMC	low density bone marrow cells
<i>FAC, FACC, FANCC</i>	The Fanconi Anemia group C gene
Th1	T helper type I cells
Th2	T helper type II cells
BFU-E	Burst forming unit-erythroid
CFU-GM	Colony forming unit-granulocyte macrophage
IL	interleukin
LIF	leukemia inhibitory factor
SIE	Steel factor, IL-3 and Erythropoietin

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Abstract

In 1927, Swiss Pediatrician Guido Fanconi described a family with three children affected by a combination of congenital abnormalities and progressive aplastic anemia [1]. Since that time, numerous cases of what is now known as Fanconi anemia (FA) have been reported. Fanconi anemia was later classified as a chromosomal breakage disorder following findings that metaphase chromosomes show increased spontaneous breaks [2]. Despite marked advances in technology, the cloning of six of the disease causing genes and numerous research articles, the understanding of the pathogenesis of Fanconi anemia still remains elusive. Life expectancy for Fanconi anemia patients is markedly reduced with the average length being twenty years. The two main causes of death are bone marrow failure and cancer, particularly acute myeloid leukemia [3]. The relationship between chromosomal instability and bone marrow failure is unclear. All cell types exhibit the characteristic chromosomal instability, however the most deadly aspects of the disease derive from dysfunction of hematopoietic tissue [4,5]. Moreover, other chromosomal breakage syndromes do not result in progressive bone marrow failure [6]. Therefore, this thesis work has focused precisely on those cells that are most consistently affected in Fanconi anemia, namely hematopoietic cells. This work will demonstrate that (1) the Fanconi anemia group C protein, FANCC, affects an important hematopoietic cellular signaling pathway, (2) Fanconi anemia group C lymphoid cells are defective in their ability to differentiate into specific subpopulations, likely a result of aberrant cellular signaling and (3) the abnormal balance of lymphocyte subpopulations negatively affects growth of hematopoietic progenitor cells.

Chapter 1:

Introduction

1-1: Clinical Features of Fanconi Anemia

Fanconi anemia (FA) is an autosomal recessive disease with an estimated occurrence of 1 per 360,000 births [7]. FA is a pleiotropic disorder characterized by cellular hypersensitivity to DNA cross-linking agents, multiple congenital abnormalities, progressive bone marrow (BM) failure and a high incidence of malignancy. The most common morphologic features of FA include skin anomalies, short stature, abnormal limb, kidney and gastrointestinal development and male hypogonadism [8].

1-2: Diagnosis

Because of the wide phenotypic variations in FA patients and the overlap of many of these symptoms with other disorders, diagnosis strictly on clinical features is difficult. Approximately 33% of FA patients present with no congenital abnormalities [9]. A large number of patients present with either aplastic anemia or malignancy [10]. FA was originally classified as a chromosomal instability syndrome following the discovery that replicating FA cells have an increased number of spontaneous breaks [2]. It was later reported that FA cells are hypersensitive to bifunctional DNA alkylating agents [11], which form adducts between two adjacent guanidine residues on DNA, forming both intra- and inter-strand crosslinks. This is the basis for the current diagnostic test. Replicating cells are cultured and exposed to low doses (doses that generally have little or no effect on normal cells) of the bifunctional alkylating agents diepoxybutane (DEB) or mitomycin C (MMC). At these doses, FA cells exhibit a marked increase in chromosome breakage and radial formation, whereas chromosomes from normal cells are not affected [12,13]. A positive DEB or MMC test, especially in conjunction with hematological or congenital abnormalities, is generally considered to confirm the diagnosis of FA.

1-3: Hematological Abnormalities

Functionally, the bone marrow is the only organ that universally fails in FA patients. Complications due to bone marrow failure are the leading cause of death [4]. The majority of FA patients develop pancytopenia early in life (usually within the first decade). Patients often present with thrombocytopenia or anemia, which then progresses to pancytopenia [3]. In addition, FA patients may express high levels of fetal hemoglobin indicating stress erythropoiesis [14].

While all somatic FA cells exhibit sensitivity to DNA crosslinking agents, human hematopoietic FA cells seem to exhibit a unique apoptotic phenotype [15]. Clonogenic progenitor cell growth is decreased in virtually all cases, and, in humans, these cells are extraordinarily sensitive to apoptotic cues [16]. Hematopoietic FA cells have a high apoptotic index and are hypersensitive to several cytokines known to induce apoptosis including combination of IFN γ , TNF α and double stranded RNA [17-20]. In addition, overexpression of FANCC in factor-dependent progenitor cell lines can suppress apoptosis induced by growth factor withdrawal [21]. Understanding the pathogenesis of hematopoietic-specific apoptosis in FA may reveal why bone marrow alone fails.

1-4: Treatment

Currently the only treatment for the hematological abnormalities in FA is bone marrow transplantation. However, the FA patient presents some unique challenges for transplant. First, because of the increased sensitivity of FA cells to clastogenic agents, the transplant conditioning regimen which generally includes alkylating agents and radiation, has necessarily been altered for FA patients. Protocols using cytoablation combined with low doses of

radiation and chemotherapy have been a successful treatment for the hematological manifestations, when an HLA-matched sibling donor is available [22]. However, many patients do not have a HLA matched sibling. Transplant with an unrelated marrow or cord blood donor with a similar regimen has resulted in a high rate of graft rejection (reported to be 15-37%) and a very low survival rate (29-43%). In both cases, FA patients experience high rates of graft versus host disease (GVHD) (45-51%) [22,23]. Further complications include abnormally high levels of infection. One report of 29 transplants found an extraordinarily high rate of infection in the FA patient following transplant. This study found a 32% occurrence of invasive aspergillus infection by day 100 despite patients achieving early hematopoietic recovery and having little or no GVHD [24].

Current transplant protocols have improved outcomes for unrelated donors by utilizing fludarabine for immunosuppression, which has significantly lowered the rate of graft rejection, and T-cell depletion to combat GVHD [25,26]. Use of these two methods appear to have improved survival rates of unrelated donor and HLA mismatched bone marrow transplants in FA [27-29], however, large scale long term studies have not been reported.

1-5: Cancer in the FA patient

Those patients that escape death due to complications of bone marrow failure generally succumb to malignancy. Approximately twenty percent of FA patients develop malignant neoplasms. The majority of these are myeloid leukemias. A portion of these AML cases (10-25%) are preceded by myelodysplasia, with monosomy 7 being a typical one of many nonrandom cytogenetic abnormalities [3,30,31]. The risk of leukemia in FA patients reaches a plateau of 20% by age 25 [32]. FA patients also develop solid tumors at an exceptionally high

rate. FA patients develop head and neck cancer, esophageal cancer, liver tumors and gynecological cancers at exceedingly high rates compared to the general population. Risk of non-hepatic solid tumor was estimated at 75% by age 45 without a plateau [32]. Interestingly, many of these solid tumors are known to be virally induced [33-35], however, no studies have been conducted on susceptibility of FA patients to virally induced carcinoma.

1-6: Immune Cell Defects in FA

FA is generally thought to be a disease affecting myeloid cell growth. Very little literature exists on lymphoid defects in FA. Although some reports indicate an increased rate of infections in FA, this is generally attributed to pancytopenia [36]. Preliminary studies indicate possible subtle defects of lymphoid subsets, especially those involved in cell-mediated immunity [37,38]. First, one small study reported that FA patients have reduced natural killer (NK) cell activity [39]. Second, another study reported that a portion of FA patients had an inadequate response to certain vaccinations [40]. Third, some sporadic case reports indicate that a fraction of FA patients are immunodeficient [41,42]. Fourth, FA patients have significantly high levels of TNF α [43], which is produced mainly by lymphoid cells and macrophages [44,45], in plasma. Fifth, a more recent study involving 10 FA patients found significantly reduced levels of total lymphocyte counts, CD4⁺ (T helper) cells and B-lymphocytes and a reduced CD4⁺/CD8⁺ ratio [46]. Sixth, analysis of V(D)J recombination, an important step in proper immune system function, found FA cells have a several fold increase in frequency of aberrant rearrangements in association with V(D)J coding joint formation [47]. Finally, FA patients undergoing transplantation suffer from complications specifically caused

by defects in the lymphoid system. Patients suffer a higher rate of infection, especially aspergillus infection, severe graft versus host disease (GVHD) and high levels of graft rejection [24,48]. Despite this circumstantial evidence implicating subtle immune defects in the pathogenesis of FA, no large-scale studies have been performed. Certainly, immune cell defects may affect predisposition of FA cells to certain tumors since a large number of cancers known to be virally-induced occur at significantly higher rates in FA patients [32,49]. In addition, it is clear, that many acquired aplastic anemias are caused by defective immune cell function [50-52]. Therefore, it is very likely immune cell defects may affect progression of bone marrow failure in FA patients. Taken together, these findings emphasize the need for more specific detailed analysis of immune cell functions among FA complementation groups.

1-8: The FA genes

FA is a heterogeneous disorder consisting of at least 8 complementation groups designated FA-A, FA-B, FA-C, FA-D1, FA-D2, FA-E, FA-F, and FA-G. There is some evidence that indicates the severity of the disease, with regard to hematological and developmental abnormalities, is dependent on the FA subtype [53]. To date six genes have been cloned and sequenced (Table 1-1). However, the gene sequences reveal no strong homology to one another or to any other known gene products and have not yielded significant insight into the function of these proteins.

FANCA (FAA) is the gene mutated in the FA-A complementation group and is responsible for approximately 65% of all cases of FA [54]. *FANCA* is found on chromosome 16q24.3 [55]. The gene contains 43 exons and encodes a 163 kilodalton (Kd) protein. *FANCA* is mainly nuclear and contains two overlapping bipartite nuclear localization signals

Table 1-1: The Fanconi Anemia Genes				
Gene	Chromosome Location	Exon	Amino Acid	Evolutionary Conservation
FANCA	16q24.3	43	1455	Fish
FANCC	9q22.3	14	558	Fish
FANCD2	3p25.3	44	1451	<i>A. Thaliana</i> <i>C. elegans</i> <i>D. Melanogaster</i>
FANCE	6p21.3	10	536	Fish
FANCF	11p15	1	374	Fish
FANCG	9p13	14	622	Fish

[56,57]. More than 100 mutations have been found in FANCA, the majority being deletions, and no mutational hotspots have been identified [58,59]. Murine studies have found that *Fanca* is expressed at low levels in most tissues and at high levels in lymphoid tissues (spleen, thymus and lymph nodes), testis and ovaries [60].

The first gene cloned corresponds to the FA-C complementation group and is designated *FANCC* (*FAC*, *FACC*). *FANCC* contains 24 exons, is localized to chromosome 9q22.3 and encodes a 558 amino acid protein with a molecular weight of 63 Kd [61]. *FANCC* is mainly cytoplasmic, however, a small amount of *FANCC* (approximately ten percent of the total cellular protein) is found in the nucleus [62]. Overexpression studies of *FANCC* indicate fluctuation through the cell cycle peaking at G2/M transition followed by proteosome dependent degradation in G1 [63-65].

The *FANCD2* gene is localized to 3p25.3 [66], contains 44 exons and encodes a 1451 amino acid protein with a molecular weight of 166 Kd. *FANCD2* is evolutionarily conserved, and is the only FA protein with known non-vertebrate homologues. Homologues have been found in *Arabidopsis thaliana*, *Caenorhabditis elegans* and *Drosophila melanogaster* [67]. Two isoforms of *FANCD2* exists in normal cells; the higher molecular weight form is

monoubiquitinated. The monoubiquitinated form of FANCD2 accumulates in nuclear foci in response to DNA damaging agents, indicating a possible role for FANCD2 in DNA repair [68].

FANCE localizes to 6p21.3 and contains 10 exons that encode a 536 amino acid protein containing two potential nuclear localization signals [69]. Recently, FANCE was shown to interact directly with FANCC, however, little else is known regarding the function of FANCE [70].

FANCF gene localizes to 11p15 and contains one exon that encodes a 374 amino acid protein [71]. A region of FANCF has loose homology to the RNA-binding protein ROM; however, this appears to be insignificant, because mutations in that region of FANCF do not affect its function [69].

FANCG (XRCC9) was isolated by its ability to partially complement the chinese hamster ovary (CHO) cell line, UV40, which is hypersensitive to UV, γ -irradiation, simple alkylating agents and DNA cross-linking agents [72,73]. *FANCG* is localized to 9p13, contains 14 exons and encodes a 622 amino acid protein. 18 mutations have been identified in FANCG which account for approximately 97% of known FA-G cases [74].

1-9: Murine Models

Mice with targeted disruption in *Fanca* (*Fanca*^{-/-}), *Fancc* (*Fancc*^{-/-}) and *Fancg* (*Fancg*^{-/-}) have been generated. Consistent with the clinical phenotype, these mice have hypogonadism and reduced fertility and their cells are hypersensitive to MMC and DEB. However, unlike FA patients, no macroscopic developmental abnormalities have been detected in FA knockout mice. In addition, these mice do not exhibit progressive bone marrow failure and they do not get cancer at an accelerated rate [18,75-77]. Mild hematopoietic defects have been documented in the *Fancc*^{-/-} mice including reduced colony forming capacity of committed progenitors and reduced hematopoietic stem cell repopulating ability [18,78,79]. However, it is unclear what redundant or

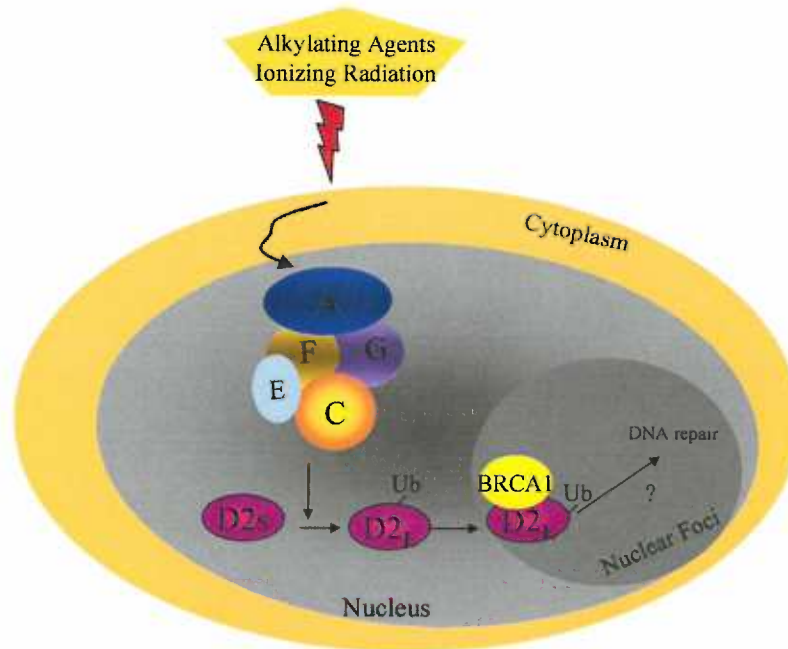
differential pathways exist in the mouse to allow it to bypass the most severe clinical manifestations of the disease.

1-10: The role of FA proteins in preventing DNA Damage: The “Linear” Model

Immunoprecipitation and yeast two hybrid studies have demonstrated that the majority of the FA proteins form a multiprotein nuclear complex. Strong interactions have been found with FANCA and FANCG, FANCF and FANCG, FANCC and FANCE and weaker interactions occur between FANCE and FANCA and FANCE and FANCG [70,80]. Some controversy exists whether a direct interaction occurs between FANCA and FANCC, however it is clear that both are part of the large complex [80-82]. FANCG binds the amino terminal NLS of FANCA and this interaction is required for stability and nuclear accumulation of both proteins [83]. The nuclear FA complex is disrupted in FA-A, FA-B, FA-C, FA-E, FA-F and FA-G complementation groups, however formation of the complex occurs normally in both FA-D1 and FA-D2 cells indicating FANCD1 and FANCD2 are not required for assembly and stabilization of the complex [82]. The FA complex appears to be constitutively present in the nucleus and is not affected by the cell cycle or DNA damage. However, it does appear that the complex is compartmentalized within the nucleus with a fraction of the complex translocating into the chromatin compartment following cellular exposure to interstrand cross linking (ICL) damage [84].

FANCD2 is not part of this complex and is thought to act downstream of the complex in response to DNA cross-linking agents and ionizing radiation (IR). FANCD2 can be monoubiquitinated on lysine 561 yielding two forms, FANCD2-L and FANCD2-S. FANCD2-L, the monoubiquitinated form, is easily recognized on immunoblot analysis as a higher molecular mass moiety. It is thought that monoubiquitination of FANCD2 signals the protein

Figure 1-1: Linear Repair Model



Upon stimulation with ionizing radiation or alkylating agents, FANCD2 is monoubiquitinated and moves to nuclear foci complexing with BRCA1. A functional complex is required for monoubiquitination of FANCD2

to become involved in some aspect of DNA repair. Indeed, FANCD2-L, accumulates and moves to nuclear foci following genotoxic stress, most notably IR and DNA cross linkers, and, upon exposure to ionizing radiation, FANCD2 co-localizes and co-immunoprecipitates with BRCA1 [68]. BRCA1 is involved in aspects of DNA repair, linking, for the first time, the FA gene products to functional DNA repair. However, the nature of the BRCA1/FANCD2 interaction is unclear, and the precise function of FANCD2 is unknown.

These important studies have begun to clarify the nature of chromosomal instability in FA cells. One point of view that exists is that the linear model [FA-complex → FANCD2-Ub] accounts for all phenotypic features of FA, in which case the nuclear complex would necessarily serve some specific hematopoietic function. However, this model fails to explain several aspects of FA, including the hematological manifestations of the disease. In addition, other chromosomal breakage syndromes such as Ataxia Telangiectasia (AT) fail to process ionizing radiation signals. However, AT patients do not develop bone marrow failure [6,85].

Why do FA patients get bone marrow failure? Why do FA patients develop acute myeloid leukemia (AML) at such a high rate compared to the general population? Furthermore, it is unclear how this model accounts for the pleiotropic nature of FA and why the different FA proteins, which are part of the complex, affect the severity of the hematological and developmental manifestations [53]. Moreover, given that the majority of the FANCC protein (90%) exists in the cytoplasm [62] and that the FA complex is strictly nuclear [86], it is likely FANCC is serving some function in the cytoplasm, outside of the complex. Therefore, this thesis work has focused on the involvement of FANCC in hematopoietic apoptotic and cellular signaling.

1-11: Non-FA FANCC Binding Partners

Several proteins, the majority of which are cytoplasmic, have been reported to bind to FANCC. However, there does not seem to be a consistent trend among interacting partners and their role remains unclear. One of the earliest described interacting partners discovered was the molecular chaperone, GRP94. The binding of FANCC to GRP94 requires amino acid residues 103-308. Inactivation of GRP94 resulted in reduced levels of FANCC suggesting that GRP94 might stabilize FANCC [87]. GRP94 is a member of the HSP90 family of stress-induced proteins. It resides in the ER and binds proteins involved in the secretory pathway by recognizing advanced folding intermediates or incompletely assembled proteins. GRP94 has also been shown to bind peptides and can act as a tumor vaccine, delivering the peptides for presentation to T lymphocytes [88,89]. However, the role played by the FANCC:GRP94 interaction is unclear.

NADPH cytochrome p450 reductase is a microsomal membrane protein found to bind FANCC. Cytochrome p450 reductase is involved in oxidative metabolism of various drugs, xenobiotics and endogenous substrates such as fatty acids, steroids and prostaglandins. FANCC was shown to bind NADPH cytochrome 450 reductase in transfected COS-1 cells and normal

murine liver cells, and FANCC suppressed activity of the enzyme. Some attribute FA defects to defective oxygen metabolism, and there is some evidence to support an inability of FANCC to properly metabolize oxygen by an as yet unknown mechanism [90,91]. Finding interaction between FANCC and NADPH p450 cytochrome reductase further implicates FA in oxygen metabolism and may indicate that the characteristic cytogenetic instability found in FA-C cells resulted from a “pre-repair” defect [92,93]. However, formal proof of this hypothesis will require additional studies.

More recently, FANCC was found to interact with glutathione S-transferase P1-1 (GSTP1) [21]. GSTP1 is a key protein that catalyzes the detoxification of xenobiotics and the by-products of oxidative stress. In addition, GSTP-1 acts as an anti-apoptotic factor, at least in the context of factor withdrawal [21,94,95]. FANCC was shown to increase GSTP1 catalytic activity and prevent GSTP1 oxidation during apoptosis [21]. These studies further implicate FANCC as an anti-apoptotic signaling factor possibly playing a role in oxygen metabolism.

FANCC was also reported to co-immunoprecipitate with the cyclin dependent kinase cdc2 [96]. Since FA-C cells have a prolonged G2/M phase, it was thought that cdc2 may affect this checkpoint [63]. However, cdc2 may affect FANCC function in other ways. Like FA-C cells, cells expressing dominant negative cdc2 are hypersensitive to *fas*-mediated apoptosis [97]. Also, cdc2 interacts with the zinc finger transcriptional repressor promyleocytic zinc finger protein, PLZF [98], and FANCC associates with a related protein, FAZF [99]. However, the precise nature and effects of the cdc2:FANCC interaction is unclear.

FAZF is a novel protein containing a conserved N-terminal bric-a-brac/tramtrack/broad complex (BTB)/poxvirus, zinc finger (POZ) domain and three C-terminal PLZF-like C2H2 zinc fingers. FAZF was cloned by three independent groups. Using FANCC as bait in a yeast two hybrid screen, Hoatlin *et al* found an association between FANCC and FAZF [99]. An

identical protein, TZFP, was identified as a testis specific factor [100]. Repressor of GATA, ROG, was isolated as a murine GATA-3 interacting protein [101], and was recently found to be identical to murine Tzfp [102]. TZFP/FAZF/ROG is expressed predominately in testis [100], but is rapidly induced in T helper cells upon stimulation with anti-CD3 [101]. TZFP binds to the promoter of *Aie1*, a serine/threonine protein kinase which may have a role in spermatogenesis, and a consensus sequence core motif, termed *tbs*, was defined [102]. A *tbs* core motif was also found on the *lex A* operator and the IL-3 receptor promoter, and, not surprisingly, FAZF/TZFP binds to those elements as well, although how binding affects transcription is unclear [99,102]. Functional data were obtained by the group who cloned ROG. This group found that ROG/TZFP/FAZF is induced rapidly in T helper cells by anti-CD3 stimulation. ROG represses the function of GATA-3 in part by preventing binding of GATA-3 to its cognate DNA sequence. This is important because PLZF represses transcription by recruitment of the nuclear co-repressor SMRT and the histone deacetylase complex, however, FAZF/TZFP/ROG did not appear to function in that manner, at least in the case of repressing GATA-3-induced transactivation [101]. Overexpression of ROG interfered with production of T helper type I and T helper type II specific cytokines [101], indicating FAZF/ROG/TZFP may regulate effector functions of T helper cells. Given that so little literature exists on FAZF/TZFP/ROG, more studies are warranted to determine the precise effects the FAZF:FANCC interaction has on hematopoietic and T helper cell growth, spermatogenesis and chromatin structure.

1-12: FA-C cells are hypersensitive to pro-apoptotic factors

Because of the uniquely apoptotic phenotype of FA hematopoietic cells, one key question was whether FA hematopoietic progenitor cells were hypersensitive to apoptotic cues or insensitive to anti-apoptotic cues. Early studies in two independent laboratories revealed that

progenitor cells derived from the *Fancc* knockout mouse are indeed more sensitive to Interferon-gamma (IFN γ)-induced inhibition [17,18]. Furthermore, later studies revealed that these cells are also hypersensitive to another pro-apoptotic factor, tumor necrosis factor-alpha (TNF- α), indicating FANCC is playing a protective role against apoptotic signaling [103-106]. Further studies have revealed that IFN γ is capable of priming *FANCC* mutant (FA-C) hematopoietic cells to respond with excessive vigor to *fas* ligation. Specifically, exposure to low doses (doses that have no effect on normal hematopoietic progenitors) of IFN γ *in vitro*, primes a substantial fraction of FA-C progenitor cells to undergo apoptosis after subsequent exposure to *fas*-ligand [19]. Moreover, this involves excessive caspase 8-dependent activation of caspase 3 [107].

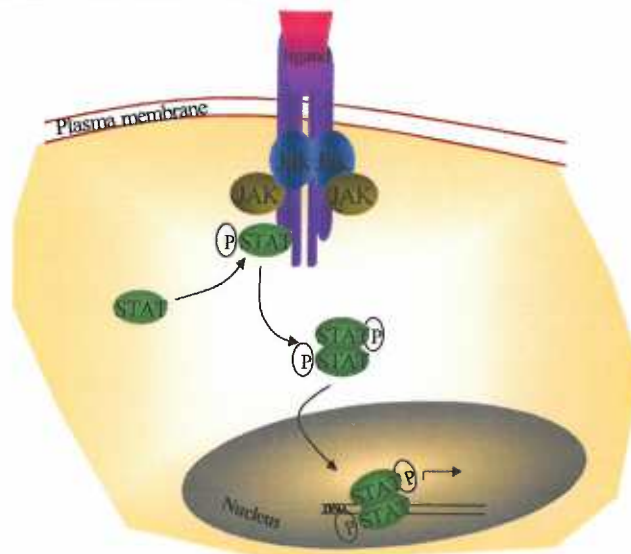
IFN γ is involved in a broad range of mammalian biological functions including immunity, inflammation, hematopoiesis, cell proliferation and differentiation [108-110]. In hematopoietic progenitor cells, IFN γ induces apoptosis [15,106,111-115], and there is compelling indirect evidence that it plays a pathophysiologically significant role in acquired aplastic anemias [109,116]. For the most part, IFN γ signals are carried out through the Janus Kinase/Signal Transducer and Activator of Transcription pathway (JAK/STAT) [117]. Details of the JAK/STAT pathway are discussed below.

Finding hypersensitivity to IFN γ in FA-C cells led us to question whether FA-C cells express high levels of downstream effectors. The first objective of this thesis work was to determine if IFN γ -inducible genes are aberrantly regulated in FA-C cells. These studies revealed that FA-C hematopoietic and embryonic cells, but not mature fibroblasts, express high levels of specific IFN γ -inducible genes. These results are discussed in detail in Chapter 3.

1-13: The JAK/STAT pathway

The JAK/STAT pathway was originally described as the signal-transducing pathway for interferons [117]. It is now clear this pathway is utilized by many cytokines especially those involved in hematopoietic and immune cell signaling. There are four known JAK proteins: JAK1, JAK2, JAK3 and TYK2, and seven known STAT proteins: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 [118]. For the most part, signaling by JAK and STAT molecules follows the same general trend (Fig 1-1). JAK proteins are constitutively bound to the intracellular portion of receptors. Upon ligand binding to its cognate receptor and dimerization of the receptor, JAK proteins are activated by auto- and cross-phosphorylation of tyrosine residues. JAKs then phosphorylate tyrosine residues on the receptor creating a docking site for STAT molecules. STATs then dock at the receptor and are phosphorylated by JAKs. Following phosphorylation, STATs dissociate from the receptor, form hetero- or homodimers and translocate to the nucleus where they bind elements of DNA and, in most cases, activate transcription of downstream genes [119].

Figure 1-2: JAK/STAT Signaling



JAKs constitutively associate with the intracellular portion of the receptor. JAKs phosphorylate STAT proteins upon cytokine stimulation. Upon Tyrosine phosphorylation, STATs dissociate from the receptor, dimerize and translocate to the nucleus and activate transcription of downstream genes.

It is unclear how STAT proteins move to and from the receptor and how they translocate to the nucleus [150]. The mechanism for activation of transcription is beginning to be uncovered. STATs bind to known transcriptional enhancers such as SP1 and c-jun [151]. In addition, STATs bind to the cAMP-response-element-binding protein (CREB)-binding protein (CBP)/p300 family of histone acetyl transferases [152]. All STATs, except STAT2, bind N-myc interacting protein 1 (Nmi-1) which enhances CBP recruitment [153]. Recently, it was found that STAT1 associates with the nuclear phosphoprotein, BRCA1, which is involved in DNA repair [154]. Moreover, STAT1 and STAT2 can bind directly to the initiator transcription factor, TFII-I [151,155]. Therefore, STAT proteins may recruit complexes that alter chromatin dynamics resulting in greater access to cellular transcriptional machinery and subsequent activation of transcription.

1-14: *Jak* knockout mice

The biological importance of JAK and STAT proteins, especially pertaining to hematopoietic and immune cell development, is demonstrated in murine knockout models. An overview of these knockouts is listed in Table 1-2. *Jak1*-deficient mice die perinatally and exhibit profound defects in lymphoid development. *Jak1* deficient mice are defective in response to IL-2, IL-4, IL-6, IL-7, IL-9, IL-10, IL-13, IL-15, leukemia inhibitory factor (LIF) and type I and type II IFNs [120]. Loss of *Jak2* is embryonic lethal due to the absence of definitive erythropoiesis correlating with *in vitro* data that demonstrate a significant role for *Jak2* in erythropoietin (EPO), thrombopoietin (TPO), IL-3 and IL-5 signaling [121,122]. *Jak3* knockout mice have severe combined immune deficiency (SCID) due to an indispensable role for JAK3 in signaling of the common cytokine receptor γ chain (γ_c) [123-126]. However, these

for JAK3 in signaling of the common cytokine receptor γ chain (γ_c) [123-126]. However, these mice survive and are fertile. *Tyk2* knockout mice survive, show no overt developmental abnormalities and are fertile. However, cells from these mice demonstrate impaired (but not absent) responses to low levels of IFN- α , - β and - γ and IL-12. Moreover, T-helper cells (Th) from these mice fail to differentiate in the T-helper type I subset (Th1) leading to an impaired ability of these mice to handle specific viral pathogens [127,128].

1-15: Stat knockout mice

For the most part, *Stat* knockout models have demonstrated more specificity to certain cytokine signaling pathways than *Jak* knockouts. *Stat1* deficient mice have no apparent

Table 1-2: The JAK/STAT proteins

Gene	Activating cytokines	Knockout Phenotype
JAK1	Type I & II IFNs, G-CSF, LIF, IL-2 like cytokines	Perinatal death, no lymphopoiesis
JAK2	IFN γ , EPO, TPO, IL-3, GM-CSF	Embryonic lethal, no definitive erythropoiesis
JAK3	IL-2, -4, -7, -15	combined immunodeficiency
TYK2	Type I IFNs, IL-12	reduced STAT activation in response to IFN- α , - β , - γ & IL-12, viral susceptibility defective Th differentiation
STAT1	Type I & II IFNs, growth hormone, SCF	Defective responses to IFN- α , - β , - γ , defective anti-viral responses
STAT2	Type I IFNs	Defective IFN- α , - β responses, susceptibility to viral and bacterial infection
STAT3	IL-6, gp130 cytokines, G-CSF, Leptin	Early embryonic lethal
STAT4	IL-12, Type I IFNs	Defective IL-12 signaling and T helper type I cell development
STAT5a	growth hormone, prolactin, EPO, TPO	Impaired mammary gland development and lactation
STAT5b	growth hormone, prolactin, EPO, TPO	loss of sexually dimorphic growth
STAT5a/b	growth hormone, prolactin, EPO, TPO	no NK cells, defective IL-2 induced T cell Proliferation, female infertility
STAT6	IL-4, IL-13	Impaired IL-4/IL-14 responses and T helper type 2 development
SOCS1	Type I & II IFNs, G-CSF	perinatal death, increased IFN γ production liver degeneration, lymphoid deficiencies

immune responses against viruses and microbial pathogens [129,130]. *Stat2* knockout mice are deficient in type I IFN signaling leading to increased susceptibility to viral infection [131]. Similarly, *in vitro* studies demonstrate that the type I IFN (IFN α and β) signaling pathway utilizes STAT1/STAT2 heterodimers and the type II IFN (IFN γ) signaling utilizes STAT1 homodimers [117,132-134]. *Stat3* deficient mice have a severe developmental defect resulting in early fetal death [135]. However, conditional knockout models have revealed a vital role for Stat3 in IL-2, IL-6 and IL-10 signaling [136]. *Stat4* deficient mice have impaired IL-12 signaling leading to severe defects in Th1 cell development [137,138]. STAT5a and STAT5b share more than 90% homology at the amino acid level [139]. Murine models have found both distinct and overlapping roles for the two proteins. *Stat5a* mice have defective mammary gland development, implicating Stat5a in prolactin signaling [140]. Cells from *Stat5a*^{-/-} mice also have defects in IL-2-induced proliferation [141-143]. *Stat5b*^{-/-} mice have impaired growth hormone signaling resulting in loss of sexually dimorphic growth [144]. These mice also exhibit impaired defects in IL-2 signaling. *Stat5a/Stat5b* double knockout mice have been generated and demonstrate severe immune cell signaling defects. These mice have no NK-cell development and their T-cells fail to proliferate following anti-CD3 stimulation [145,146]. *Stat6* deficient mice have diminished Th2 and enhanced Th1 responses emphasizing the role of STAT6 in IL-4 and IL-13 signaling [147-149].

1-16: The SOCS family: inhibitors of JAK/STAT signaling

Recently, a family of proteins that inhibit the JAK/STAT pathway was identified. Three groups simultaneously cloned identical proteins capable of inhibiting the JAK/STAT pathway and called it SOCS-1 (suppressor of cytokine signaling-1) [156], JAB (Jak binding protein)

[157] and SSI-1 (STAT-induced STAT-inhibitor) [158]. Members of the SOCS/JAB/SSI-1 (SOCS1-SOCS7 and CIS) family contain N-terminal regions of variable length, a central SH2 domain and a C-terminal 40 residue motif called a SOCS box. In addition, database searches revealed that more than twenty proteins contain the C-terminal SOCS box [159]. SOCS-1, SOCS-2, SOCS-3 and CIS are induced rapidly by a wide variety of cytokines. This induction is mediated by STAT induced transcription of SOCS proteins. SOCS proteins then inhibit cytokine signaling creating a classical negative feedback loop [160]. SOCS-1 inhibits cytokine signaling by associating with JAK1, JAK2, JAK3 and TYK2 and inhibiting their catalytic activity [157,158,161,162]. In addition, some SOCS family members have been shown to bind to both STAT proteins and cytokine receptors [163]. *Socs1*^{-/-} mice are runted and die at three weeks of age. Their pathology demonstrates severe lymphopenia, fatty degeneration of the liver and macrophage infiltration of major organs [164-167]. The lymphopenia has been attributed to upregulation of the pro-apoptotic factor, bax [165]. The complex phenotype of *Socs1*^{-/-} mice appears to be a consequence of augmented signaling by IFN γ because (1) a similar phenotype can be induced in wild type neonatal mice by administration of IFN γ [161], and (2) the disease phenotype can be prevented in *Socs1*^{-/-} mice using a neutralizing anti-IFN γ antibody or by crossing the mice on an IFN γ -negative background [167]. Moreover, thymic T cell development of *Socs1*^{-/-} mice is perturbed and peripheral T cells exhibit an activated phenotype in these animals indicating SOCS1 may regulate T cell development. Consistent with this, *Socs1*^{-/-} that lack RAG2, which is required for lymphocyte development, exhibit normal levels of IFN γ and survive [167]. Therefore, it appears SOCS1 regulates both IFN γ signaling and T cell differentiation preventing emergence of a population of T cells that secrete high levels of IFN γ . SOCS proteins may also be involved in ubiquitin targeting of proteins.

SOCS proteins associate with a complex containing elongins B and C through their SOCS box [168]. The elongin BC complex binds a putative E3 ubiquitin ligase termed cullin-2. Some have postulated that SOCS proteins act as adapters bringing ubiquitin ligases into the vicinity of activated signaling proteins causing their ubiquitination and degradation [169]. However, at this point it is unclear whether degradation is the ultimate outcome and whether SOCS proteins themselves are ubiquitinated and degraded, although recently it was found that CIS can be monoubiquitinated and accumulates in the presence of proteasome inhibitors [170]. Thus, it appears that in addition to their important role as specific inhibitors of cytokine signaling, SOCS proteins play additional roles in development, apoptosis and protein stability.

1-17: The JAK/STAT pathway in FA-C cells

Given the essential role the JAK/STAT pathway plays in IFN γ signaling and that FA-C cells are hypersensitive to IFN γ and overexpress several IFN γ -inducible genes, the second goal of this thesis work was to determine how FANCC influences IFN γ -induced JAK/STAT signaling molecules. These results are discussed in detail in Chapter 4. Surprisingly, activation of STAT1 is reduced in response to IFN γ in FA-C hematopoietic and embryonic cells, but no differences in activation of JAK1, JAK2 or the IFN γ receptor alpha chain (IFN γ R α) are observed. Moreover, FANCC interacts with STAT1 following stimulation with IFN γ and is required for STAT1 to dock at the IFN γ R α . Finding defective STAT signaling raised two very important questions. First, if FANCC plays a role in STAT1 signaling in response to IFN γ , are other STATs or modulators of STAT signaling dysfunctional in FA-C cells? This is an extremely important question given the fact that most hematopoietic cytokines and growth factors utilize some combination of the JAK/STAT pathway for cellular signaling, and defects

in these pathways could quite conceivably affect hematopoietic cell growth. Secondly, what is the specific pathway that is hyperactive in FA-C cells allowing for the hypersensitivity to IFN γ and overexpression of IFN γ -inducible genes even in the face of defective STAT signaling. This thesis work has touched on some aspects of the second question with regard to specific STAT inducible inhibitors of IFN γ signaling (Chapter 5). In addition, Qishen Pang in our laboratory has found that the double stranded RNA (dsRNA) dependent protein kinase apoptotic pathway is constitutively active in FA-C cells accounting, at least in part, for the IFN γ hypersensitivity of FA-C cells (discussed in Chapter 5). However, the majority of this thesis work has focused on the first question regarding JAK/STAT signaling in response to other cytokines and the impact that defects in these pathways may have on FA-C hematopoietic cell growth and differentiation (Chapter 6 and 7).

1-18: Type I versus Type II IFNs

IFNs are a heterogeneous family of cytokines originally identified on the basis of their ability to induce cellular resistance to viral infections. In addition, both types of IFNs have anti-proliferative effects as well [108]. Type I IFNs include members of the IFN α family (IFN- α_1 , - α_2 , - ω , - τ) and IFN β . Type II IFN includes only IFN γ and is structurally unrelated to type I IFNs and mechanisms that regulate its production are also different [171]. Type I IFNs are produced in many cell types upon viral infection. IFN γ , on the other hand, is produced mainly in cells that are derived from hematopoietic stem cells, namely T cells and natural killer cells, following antigen or cytokine stimulation. In IFN γ signaling, JAK1 and JAK2 activate STAT1. STAT1 then forms a homodimer, translocates to the nucleus and binds the gamma activation sequence or GAS element. Type I IFNs utilize JAK1 and TYK2 which in turn

activate STAT1 and STAT2. STAT1 and STAT2 then complex with ISGF3 γ (p48, IRF-9) to form the ISFG3 complex. ISGF3 then translocates to the cell nucleus and binds the Interferon Stimulated Response Element (ISRE) [117]. In addition, Type I IFNs have also been shown to activate STAT3, STAT5 and in some cell types STAT4. However, the downstream affects of these proteins in response to type I IFNs is less well characterized [172,173].

Despite differences in structure and regulation, it was recently found that type I and type II receptors are localized in the caveolar membrane and that there is cross-talk between signaling components of theses two pathways. Specifically, cells derived from IFN α receptor 1 (IFN α R1) null mice (*IFN α R1*^{-/-}) have impaired IFN γ -induced antiviral responses and IFN γ -induced DNA binding of STAT1 is reduced 6 to 7 fold in *IFN α R1*^{-/-} cells. This work also found that IFN γ enhances phosphorylation of IFN α R1 and STAT1/STAT2 recruitment to the receptor, and that the IFN α R1 interacts with IFN γ R2 [174].

Given the cross-talk that exists between type I and type II IFNs and that FA-C cells are defective in IFN γ signaling through STAT1, the next logical step of this work was to analyze components of the IFN α / β signaling pathway. We found that phosphorylation of STAT1, STAT3 and STAT5 are reduced in response to type I IFNs in FA-C cells. In addition, these studies revealed, for the first time, a defect upstream of STATs where phosphorylation of the janus kinase, TYK2 (important for optimal STAT signaling and T helper differentiation), is diminished in FA-C cells. These results are discussed in detail in Chapter 6.

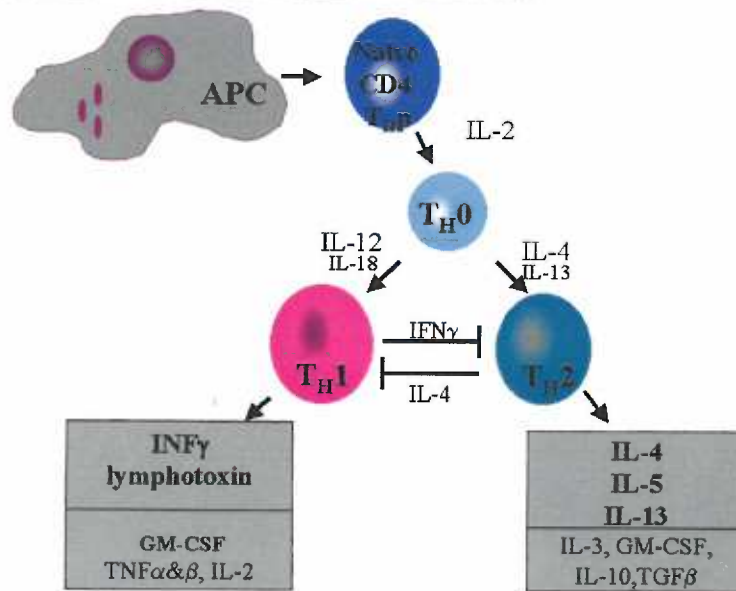
1-19: T helper Cells

The discovery of T helper (Th) subsets provided a link between adaptive and innate immunity. Naïve CD4⁺ T cells can differentiate upon activation into either Th1 or Th2 cells.

The two subsets differ in the cytokines they produce upon activation, and, thus, vary in the way they affect surrounding cells and pathogens [175,176]. A proper balance between Th1 and Th2 is necessary to maintain normal functions of not only the immune system, but also for proper maintenance of hematopoietic cells and tumor surveillance [177-184]. Th1 cells are responsible for cell-mediated immunity and the main Th1-secreted cytokine is IFN γ . However, these cells can also produce IL-2, GM-CSF, TNF- α , TNF- β , and lymphotoxin. Th2 cells are mainly involved in humoral responses and IL-4 influences Th2 differentiation and is produced by Th2 cells. Th2 cells can also produce IL-5, IL-9, IL-10, IL-13 and GM-CSF [185-188]. The factors that determine whether a proliferating CD4 $^{+}$ T cell will differentiate into Th1 or Th2 is not fully understood. However, it is clear that the cytokine elicited by the infectious agent, the co-stimulators used to drive the response and the nature of the peptide:MHC ligand all have an effect [185,189].

Following differentiation and emigration from the thymus to the peripheral immune organs, CD4 $^{+}$ T helper cells are termed naïve T helper precursors (Thp). Thp cells are functionally immature and capable of only secreting IL-2. Thp activation and differentiation to Th subsets requires at least two separate signals. First, signal is delivered by the TCR/CD3 after interaction with antigen presented on the surface of major histocompatibility (MHC) complex of a professional antigen presenting cell (APC), followed by other costimulatory molecules. Specific cytokine stimulation plays the most critical role in T helper cell polarization. Th1 and Th2 differentiation depends mostly on IL-12 and IL-4, respectively [190]. IL-12, which is secreted by APC, activates STAT4 and augments transcription of genes important for Th1 lineage development such as *T-bet* [137,138,191,192]. IL-18 and INF γ also influence Th1 development [193-196]. IL-4 is produced by Thp cells and many other cell

Figure 1-3: CD4+ T helper differentiation



Upon contact with a professional antigen presenting cell, Naïve CD4+ precursor cells are induced to secrete IL-2 and other cytokines depending upon the stimulus. The cells then differentiate into Th1 or Th2 subsets and secrete specific cytokines. The “hallmark” Th1 cytokine is IFN γ and the “hallmark” Th2 cytokine is IL4.

types. IL-4 induces STAT6 activation, which then initiates transcription of genes important for Th2 differentiation such as *GATA3* [147,197-199]. In addition, the biological function of IL-13 partially overlaps with IL-4, and, in some cases, drives Th2 development in a IL-4 independent manner [200,201]. Regulation of cytokines, important for both Th differentiation and Th function, appears to be regulated on the level of chromatin structure, as well. Cytokine genes undergo changes in chromatin structure that allow access to gene-specific transcription factors. There is evidence of chromatin remodeling for both the IL-4 and IFN γ locus suggesting that another level of control exists in T helper differentiation [202-205].

More recently, it has been discovered that, while the classical differentiation (IL-12/Th1, IL-4/Th2) pathway of T helper subsets is conserved between mice and humans, a second

Th1 differentiation pathway induced by type I IFNs is specific to humans [206,207]. Human type I IFNs induce phosphorylation of STAT4 by recruitment of STAT4 to the IFN α R1 via STAT2 [208]. However, murine Stat2 lacks a C-terminal sequence necessary for the recruitment of Stat4. Therefore, murine type I IFNs are unable to activate Stat4 and induce Th1 differentiation [131,209]. Given the differences between murine and human T helper differentiation, murine models may be sufficient to analyze the classical Th1 differentiation process, but may not be sufficient when analyzing all the overall effects of defective Th1 development on other elements of the human system.

Given that: (1) loss of certain STATs and TYK2 results in defective T helper differentiation [127,128,210], (2) FA-C cells have diminished STAT and TYK2 signaling (Chapter 6) and (3) FANCC interacts with factors involved in T helper differentiation [99,211], the next logical step of this thesis work was to determine if FA-C T helper cells differentiate into Th1 and Th2 subsets properly. Indeed, we found that the ability to differentiate into the Th1 subset is impaired in Th cells from *Fancc*^{-/-} mice. We also demonstrate that abnormal FA-C lymphocyte development affects the activity of committed hematopoietic progenitors. These results are discussed in Chapter 7.

1-20: Thesis Overview

The major catalyst for this thesis work was the observation that FA-C hematopoietic progenitor cells are hypersensitive to the inhibitory effects of IFN γ [18,19]. Further analysis of the effects of IFN γ on FA-C cells revealed that FANCC suppresses expression of certain key IFN γ -inducible genes in hematopoietic and embryonic cells [212] (Chapter 3). Furthermore, analysis of the IFN γ signaling pathway revealed, surprisingly, that activation of STAT1 is

defective in FA-C hematopoietic and embryonic cells [212,213] (Chapter 4). This finding has driven the remainder of this thesis work.

First, how is it possible that IFN γ -inducible genes are expressed at high levels even in the absence of optimal STAT1 activation? The answer to this question appears to be complex and due to; (1) loss of IFN γ -antagonist signaling pathways such as IL-4 induced STAT6 activation (Chapter 5) and (2) hyperactivation of known apoptotic pathways such as PKR [20]. Second, is signaling of other cytokines through STAT molecules defective in FA-C cells? Indeed, activation of type I IFN-induced STAT activation is diminished in FA-C cells. However, defective STAT phosphorylation is not a global defect in FA-C cells and is specific to certain cytokines. In addition, activation of the janus kinase, TYK2, is defective. Correlating with this, expression of SOCS-1, a known TYK2 inhibitor, is upregulated in FA-C cells (Chapter 6). In order to evaluate the functional consequence of JAK/STAT defects in FA-C cells, we analyzed differentiation of T helper cells in *Fancc*^{-/-} mice. Loss of *Fancc* results in impaired Th1 differentiation, and, for the first time, reveals specific functional lymphoid defects in FA. Since, the clinically significant manifestations of FA occur in bone marrow and preliminary studies indicate an involvement of Th1 cells in proper maintenance and differentiation of myeloid progenitor cells [177], the final objective of this thesis was to determine whether aberrant FA-C lymphocyte subpopulations abnormally affected proper myeloid cell development. Indeed, these studies reveal that defective lymphopoiesis in FA plays a role in hematopoietic stem cell development (Chapter 7).

Chapter 2

Materials and Methods

The following methods were employed in the studies described in the remainder of this thesis:

2-1: Cell culture and cytokine Stimulation

Epstein-Barr virus (EBV)-transformed human lymphoblasts were maintained in RPMI media 1640 (Life Technologies, Grand Island, NY) supplemented with 15% heat-inactivated fetal calf serum (FCS) and grown in a humidified 5% CO₂-containing atmosphere at 37°C. The FA-A B-lymphoblast lines, HSC72 and HSC72/FANCC were a generous gift from Dr. Walsh (University of North Carolina, Chapel Hill, NC). The lymphoblast lines, JY (normal), HSC536N (*FANCC* mutant), HSC536N/FAC and HSC536N/neo were described previously [19]. Briefly, HSC536N/FANCC were derived by transducing the HSC536N with a retrovirus encoding both *FANCC* and neomycin phosphotransferase (*neo*). The HSC536N/neo line was derived by transducing the HSC536N with a vector encoding for neomycin phosphotransferase alone.

Murine embryonic fibroblasts (MEFs) were established from *Fancc* knockout and wild type mice as previously described [18] and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum. MEFs were transformed and immortalized by SV40 large T-antigen [214]. The Fanconi fibroblast lines (PD134 (FA-C), PD426/FANCA (FA-C expressing FANCA), PD426/FANCC (FA-C corrected), PD331.T (FA-C), PD720/FANCC (FA-A expressing FANCC), PD720/FANCA (FA-A corrected), ML7334 (FA-G), ML7334/FANCG (FA-G corrected)) were received from the Oregon Health Sciences University Fanconi Anemia Cell Repository. Human primary fibroblasts cell lines were established using standard procedures. The PD331.T line was immortalized as previously described [215]. Cells were grown in MEM- α (Life Technologies, Grand Island, NY) supplemented with 20% heat-inactivated fetal bovine serum.

Correction of PD134 and PD331 was done using a retroviral vector carrying the human FANCC cDNA as previously described [19] Cells were incubated with virus for 3 hours, twice daily over a 5 day period, and then selected with G418.

ICSBP expressing HSC536N cells were made using a PLXSN based retroviral vector expressing the human ICSBP cDNA. pLXSN was a generous gift of Dr. A.D. Miller (Fred Hutchinson Cancer Research Center, Seattle, WA). pTarget/ICSBP was a generous gift supplied by Dr. Ben-Zion Levi (Technion-Israel Institute of Technology, Haifa, Israel) and contains the entire human ICSBP cDNA. pLXSN/ICSBP was made as follows: pTarget/ICSBP was digested with EcoRI, a 1.3 Kb insert was isolated and cloned into pLXSN. Proper orientation was determined using a KpnI/NotI digest. Viruses were made as previously described [19]. HSC536N were incubated with pLXSN/ICSBP or pLXSN virus plus 8 µg/ml polybrene for 3 hours, twice daily for five days. Cells were then selected with G418.

2-2: Cytokine treatment

MEFs were serum starved for 24 hours and treated with the indicated amount of recombinant murine IFN γ (rmIFN γ) (R& D systems, Minneapolis, MN) and rmLIF (R & D systems) for the indicated times at 37°C, 5% CO $_2$. B-lymphoblasts and human primary fibroblasts were plated 1 day prior to treatment and incubated with the indicated amounts of recombinant human IFN γ (R& D systems), rhIL-6 (R & D systems) at 37°C, 5% CO $_2$. Recombinant human IFN α 2 β (Schering Corporation, Kentworth, NJ) was used at 1000 U/ml. Recombinant human IFN β (Biogen, Cambridge, MA) was used at 1000 U/ml. Recombinant human IL-4 (R & D systems) was used at 1ng/ml.

2-3: Preparation of total cell lysates and nuclear extracts

Total cell lysates were made by washing cells twice with phosphate-buffered saline, and cell pellets were solubilized in RIPA [10 mmol/L Tris-HCl [pH 7.6], 150 mmol/L NaCl, 1% (w/v) SDS, 1% (v/v) aprotinin, 2mM Na₃VO₄, leupeptin (1µg/ml), pepstatin (1µg/ml) and 1mM phenylmethsulfonylfluoride (PMSF)]. Lysates were centrifuged at 16000g for 15 minutes at 4° C, and protein concentrations were determined using a protein microassay of the Bradford method (BioRad, Hercules, CA). Nuclear extracts were prepared as previously described [216]. Briefly, cells were washed in ice-cold PBS and resuspended in cold hypotonic buffer (10mM HEPES, pH 7.9, 1.5mM MgCl₂, 10mM KCl, 0.2mM PMSF, 0.5mM DTT, 2 µg/ml aprotinin, and 2 µg/ml leupeptin). Cells were allowed to swell on ice 10 minutes, then lysed with a Dounce B homogenizer. Nuclei were pelleted at 3300g for 20 minutes at 4° C, then resuspended in low-salt buffer (20mM HEPES, pH 7.9, 25% glycerol, 1.5mM MgCl₂, 20mM KCl, 0.2mM EDTA, 0.2mM PMSF, 0.5mM DTT, 2 µg/ml aprotinin, and 2 µg/ml leupeptin). High-salt buffer (0.6M KCl) was added dropwise, and nuclear proteins were extracted by incubation at 4° C for 30 minutes and centrifuged at 13,000g for 30 minutes at 4° C. Supernatants were dialyzed at 4° C for 3-5 hours against a 250-fold volume excess of 20mM HEPES, pH7.9, 20% glycerol, 100mM KCl, 0.2mM EDTA, 0.2mM PMSF, 0.5mM DTT, 2µg/ml aprotinin, and 2 µg/ml leupeptin.

2-4: Immunoblot analysis

Total cell lysates and nuclear extracts were mixed with Laemmli sample buffer [217], heated at 94°C for 5 minutes. Equivalent amounts of protein were subjected to SDS-

polyacrylamide gel electrophoresis (PAGE), and electroblotted onto Bio-Blot (Costar, Cambridge, MA) as previously described [218]. Blots were then subjected to *Ponceau S* staining to demonstrate equivalent loading. Nonspecific binding was blocked for 1 hour at room temperature in Tris-buffered saline plus 0.05% Tween 20 (TBS-T) containing 5% milk. Blots were incubated with primary antibody, washed with TBS-T, then incubated with secondary antibody, and washed again with TBS-T. Antibody-reactive proteins were detected using Enhanced Chemiluminescence (ECL) (Amersham, Arlington Heights, IL). The anti-SOCS1 antibody was a generous gift from Dr. Jianguo-Zhang and Dr. Douglas Hilton (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). IRF-1 antibody (1:1000), p21^{WAF1} antibody (1:500), ISGF3 γ (BD pharmingen) (1:250), p27^{KIP1} (BD pharmingen) (1:500), p53 (1:1000), IRF-2 (1:1000) total STAT1 (1:2000), STAT2 (1:1000) total STAT3 (1:1000), total STAT5 (1:500), total STAT6 (1:1000), Tyk2 (Transduction laboratories, Lexington, KY) (1:1000), anti-JAK1 (Transduction Laboratories) (1:500), anti-JAK2 (Upstate Biotechnology, New York, NY) (1:1000) were used at the indicated dilutions and incubated with the blots for 1 hour at room temperature. Donkey anti-rabbit IgG horseradish peroxidase (HRP) secondary antibody (1:5000) (Amersham, Arlington Heights, IL) was used against these primary antibodies. ICSBP antibody was diluted 1:500 for 1 hour at room temperature. Rabbit anti-goat HRP secondary antibody (1:2000) (Pierce, Rockford, IL) was used against ICSBP primary antibody. Phospho-specific STAT1 (New England BioLabs, Beverly, MA) (1:500), Phospho-specific STAT3 (New England Biolabs, Beverly, MA) (1:1000), phospho-specific STAT5 (New England Biolabs, Beverly MA) (1:1000), phospho-specific STAT6 (New England Biolabs, Beverly, MA) and phospho-specific Tyk2 (New England Biolabs, Beverly, MA) were incubated at 4°C overnight. Antibodies were used at the indicated dilutions and purchased from Santa Cruz Biotechnology, Santa Cruz, CA

unless otherwise indicated.

2-5: Immunoprecipitation

Following treatment with IFN γ cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with Nonidet P-40 (NP-40) lysis buffer (1% NP-40, 20 mM Tris-HCl [pH 8.0], 137 mM NaCl, 10% glycerol) or digitonin lysis buffer (1% digitonin, 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 50 mM NaF, 5 mM sodium pyrophosphate). Both lysis buffers were supplemented with the following freshly made protease inhibitors: 1% aprotinin, 1 μ g of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM sodium orthovanadate. After rocking at 4°C for 20 min (60 min when cells were lysed with digitonin buffer), cell lysates were cleared by centrifugation at 13,200 rpm for 20 min at 4°C, and protein concentrations were determined by the Bradford method using a protein microassay reagent (Bio-Rad, Hercules, Calif.). Whole cell extracts (WCE) (about 1 mg of total proteins) were precleared with 50 μ l of 50% protein A-Sepharose suspension (Pharmacia Biotech, Inc.) for 1 h at 4°C. After separation of the protein A-Sepharose from the lysate by centrifugation at 1,000 rpm for 1 min at 4°C, the lysates were incubated with either anti-P-Tyr antibody 4G10 (a generous gift from Brian Druker, Oregon Health Sciences University) (12 μ g/ml), anti-IFN γ R1 (Antigenix America Antibodies, New York, NY) (2 μ g/ml), anti-STAT1 (Santa Cruz Biotechnology) (1 μ g/ml) or anti-IFN α R1 (Research diagnostics Inc, Flanders, NF) for 3 to 5 h at 4°C with constant agitation. Immunocomplexes were then bound to protein A-Sepharose beads (50 μ l of 50% slurry) during a 1- to 2-hour incubation at 4°C. The immunoprecipitates were recovered by centrifuging at 1,000 rpm for 1 min at 4°C. If cells were lysed in NP-40 lysis buffer, the immunocomplexes were washed three times with 0.05% NP-40 wash buffer (0.05% NP-40 in 1 $\times$ PBS). If cells were lysed in digitonin lysis buffer, the immunocomplexes were then washed with 0.1% digitonin wash buffer (same as digitonin lysis buffer except that the concentration of digitonin was 0.1%). Immunoprecipitates were then run on

SDS PAGE, electroblotted and probed as standard immunoblots.

2-6: Electromobility Shift Assay (EMSA)

Sequences for oligonucleotides used in binding reactions were as follows: human IRF-1 GAS, 5'-ACAACAGCCTGATTTCCTCCGAA-3'; murine IRF-1 GAS, 5'-CCTGATTTCCTCCGAAATGATG-3'; murine ICSBP GAS, 5'-AGTGATTCTCGGAAAGAGAG-3'. Oligonucleotides were synthesized at the Molecular Biology Core Lab (Portland VAMC), then labeled with [γ^{32} P-ATP] to 25,000 cpm/ng using T4 polynucleotide kinase (Boehringer Mannheim). Binding reactions (20 μ l) contained 5.0-7.5 μ g nuclear extracts, 0.2 ng labeled oligo, 2 μ g poly (dI-dC), and 10 μ g BSA in 10 mM Tris-Cl, pH 7.4, 50 mM NaCl, 1mM dithiothreitol, 1mM EDTA, and 10% glycerol. Reactions were incubated at room temperature for 30 minutes, then resolved on a 4% polyacrylamide gel in 25 mM Tris, 190 mM glycine and 1 mM EDTA. Gels were dried and autoradiographed with intensifying screens at -80°C.

2-7: RNA isolation, cDNA synthesis and RTQ-PCR

Total RNA was isolated from cells with TriReagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. cDNA synthesis was performed with 1 μ g total RNA, 200ng random hexamers (Life Technologies, Grand Island, New York), and Superscript II reverse transcriptase (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The cDNAs were then diluted to an approximate concentration of 20 ng/ml before performing the amplification reactions. Amplifications were performed in an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA) using 100ng cDNA, 7.5 pmoles of each target primer, 2.5 pmoles of the target probe, 2.5 pmoles of

each housekeeping primer, and 2.5 pmoles of the housekeeping probe (h18S). As recommended by the manufacturer, the TaqMan PCR kit (PE Applied Biosystems) was used as directed. The primer and probe sequences, designed using ABI Primer Express software (PE Applied Biosystems) and synthesized by Integrated DNA Technologies (Coralville, Iowa) are: hMxA (forward primer) 5'-TGGTGGTGGTCCCCAGTAAT-3', hMxA (reverse primer) 5'-CGTCAAGATTCCGATGGTCC-3', hMxA (dual labeled probe) 5'-FAM-CCACCACAGAGGCTCTCAGCATGG-TAMRA-3', h18S (forward primer) 5'-CGGCTACCACATCCAAGGAA-3', h18S (reverse primer) 5'-GGGCTCTGA AAGAGTCCTGT-3', and 18S Probe 5'-VIC-CAGCAGGCGCGCAAATTACCCA-TAMRA-3'.

2-8: Th1/Th2 assay

Mice were euthanized by cervical dislocation. Spleens were removed, minced and passed through 50 mesh screens. Single cell suspensions were made by passaging cells through 18 G needle. Cells were incubated on plate bound anti-CD3 plates in RPMI containing 15% Fetal Calf Serum (FCS) alone or under conditions conducive for either Th1 or Th2. Th1 conditions included 10 ug/ml anti-IL-4 (R & D systems) and 40 ng/ml rmIL-12 (R & D systems) were added immediately and 100 U/ml rmIL-2 added the following day. Th2 conditions included anti-IFN γ (R & D systems) (10 ug/ml) added immediately, and 10 ng/ml rmIL-4 and 100 U/ml rmIL-2 were added the following day. After 3 days, medium and cytokines were replaced and cells were cultured for 3 more days. Cells were then washed and equal numbers of cells were replated on anti-CD3 bound plates in RPMI with 15% FCS with no additional cytokines for 24 hours. Supernatants were harvested for ELISA and cells were harvested for IFN γ capture assay.

2-9: IFN γ secretion assay

Mouse IFN γ secretion assay kits (Miltenyi Biotec, Auburn, CA) were run as per manufacturer's instructions. Cells were kept on ice and washed with cold wash buffer (PBS containing 2.5% BSA and 2mM EDTA). Cells were incubated with cytokine catch reagent (CD45 antibody conjugated to anti-IFN γ) for 45 minutes at 37 °C and then washed twice with cold wash buffer. Cells were incubated for 10 minutes with an IFN γ -specific detection antibody conjugated to PE. Cells were then stained for 10 minutes with an antibody specific for CD4 conjugated to FITC. Cells were washed twice with cold buffer. Immediately prior to flow cytometric analysis, cells were stained with propidium iodide.

2-10: ELISA

Quantitative sandwich enzyme immunoassay kits for murine IFN γ and murine IL-4 were obtained from R and D systems. Procedures were run as per manufacturer's recommendations. Briefly, standards, controls and samples were pipetted into IFN γ or IL-4 pre-coated microplate wells. Any unbound substrate was washed away and an enzyme-linked polyclonal antibody specific for mouse IFN γ or IL-4, respectively, was added to the wells. Wells were washed to remove any unbound antibody-enzyme reagent. A solution containing enzyme substrate was added to the wells yielding a colored product. The reaction was stopped by addition of a stop solution and color intensity was measured on a microplate reader. Quantitation of IFN γ or IL-4 levels was then determined by the generated standard curve.

2-11: Colony Forming Assay

Femoral marrow samples were obtained from wild type mice following cervical dislocation, washed twice and resuspended in RPMI 1640 (Life Technologies, Grand Island) for viable cell counts (trypan blue exclusion). Spleens were removed from *Fancc*^{-/-} or *Fancc*^{+/-} mice and passed through sterile screens. Following sequential passage through a series of 21-, 23- and 25 guage needles to yield a single cell suspension, cells were washed twice and resuspended in RPMI 1640 for viable cell counts.

Equivalent amounts of unfractionated marrow cells and splenocytes were mixed and cultured for 7 days at 37 °C in 5% CO₂. 1x10⁵ cells were then cultured in 1 mL of MethoCult H4230 (Stem Cell Technologies, Vancouver, BC, Canada), penicillin-streptomycin (Life Technologies), in the presence and absence of: human erythropoietin (2 U/ml; Amgen, Thousand Oaks, CA), murine Steel Factor (10 ng/ml; R & D Systems) and murine IL-3 (10 ng/ml; R & D Systems). CFU-GM and BFU-E were counted after 7 and 14 days. Colony growth results were expressed as mean (of triplicate plates) ± standard deviation, colonies and bursts per plate.

Chapter 3

Functional Correction of FA-C Cells with *FANCC* Suppresses the Expression of IFN γ -Inducible Genes in a Cell Type Specific Manner

These results were published in part in:
Fagerlie *et al.* (2001) Blood 97:3017
and
Fagerlie *et al.* (2001) Exp Hematol 29:1371

3-1: Overview

Because hematopoietic cells derived from Fanconi Anemia (FA) patients of the C-complementation group (FA-C) are hypersensitive to the inhibitory effects of interferon- γ (IFN γ), we quantified the products of certain IFN γ -inducible genes known to influence hematopoietic cell survival. We found high constitutive expression of the IFN γ -inducible genes, interferon-stimulated gene factor 3 gamma subunit (ISGF3 γ), interferon regulatory factor-1 (IRF-1), and the cyclin dependent kinase (cdk) inhibitor p21^{WAF1} in *FANCC* mutant B-lymphoblasts, low density bone marrow cells and murine embryonic fibroblasts, but not in mature fibroblasts. Thus, the *FANCC* protein functions in a cell type specific manner to modulate expression of a family of genes that in normal cells are only inducible by specific environmental cues for apoptosis or mitogenic inhibition.

3-2: Introduction

Hematopoietic precursor cells from children with FA of the C complementation group (FA-C) are excessively apoptotic and hypersensitive to a variety of cytokines known to induce apoptosis, including interferon- γ (IFN γ) [17-19]. IFN γ is a cytokine involved in host defense against viral infections and regulation of cell growth [108]. IFN γ clearly plays a role in hematopoietic suppression and has been implicated in the pathogenesis of acquired aplastic anemia [108-111,219-226]. Cells with inactivating *FANCC* mutations are hypersensitive to the apoptotic effects of IFN γ , an effect that depends in part on an intact *fas/fas*-ligand pathway [18,19,104-106,113,227]. Specifically, exposure to low doses of IFN γ *in vitro*, primes a

substantial fraction of FA-C progenitor cells to undergo apoptosis after subsequent exposure to *fas*-ligand. Such doses of IFN γ have no effect on normal hematopoietic progenitor cells [18,19]. Because of the influence of IFN γ on the *fas* pathway, we sought to determine whether other IFN γ -inducible genes are hyperactive in FA-C cells. We carried out studies designed to analyze expression of IFN γ -inducible genes known to promote apoptosis and/or mitogenic inhibition in hematopoietic progenitor cells. We describe here studies revealing that the transactivators, interferon regulatory factor-1 (IRF-1) and interferon stimulated gene factor 3 gamma subunit (ISGF3 γ) and the cyclin dependent kinase (cdk) inhibitor, p21^{WAF1}, are expressed at constitutively higher levels in FA-C B-lymphoblasts, low density bone marrow cells (LDBMC) and murine embryonic fibroblasts (MEFs) compared to cells expressing a normal *FANCC*. However, these proteins are expressed at normal levels in mature FA-C fibroblasts, suggesting some degree of tissue or developmental stage specificity of FANCC function in control of these genes. Thus, FANCC is required to suppress expression of IFN γ -inducible genes in a cell type specific manner.

3-3: Results

Constitutive overexpression of IFN γ -inducible genes in mutant FA-C cell lines

Because FA-C cells are hypersensitive to the inhibitory effects of IFN γ [18,19,104], we quantified protein levels of transcriptional activators known to be downstream of the IFN γ signaling pathway by immunoblot analysis of total cell lysates or nuclear extracts. Analysis of the IFN γ -inducible transactivating factors IRF-1 and ISGF3 γ , consistently revealed high constitutive expression levels in nuclear extracts and whole cell lysates derived from the *Fancc*

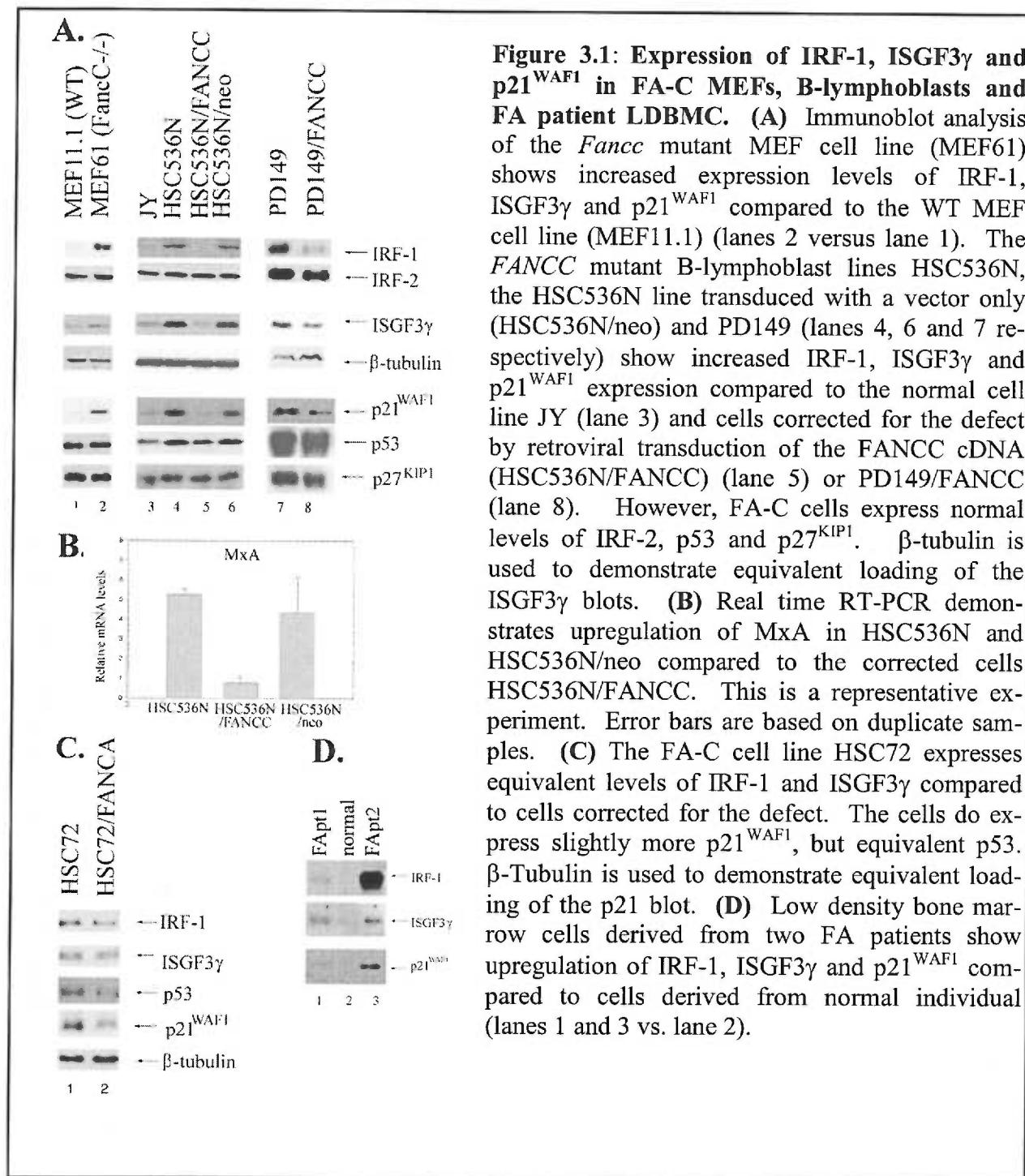


Figure 3.1: Expression of IRF-1, ISGF3γ and p21^{WAF1} in FA-C MEFs, B-lymphoblasts and FA patient LDBMC. (A) Immunoblot analysis of the *Fancc* mutant MEF cell line (MEF61) shows increased expression levels of IRF-1, ISGF3γ and p21^{WAF1} compared to the WT MEF cell line (MEF11.1) (lanes 2 versus lane 1). The *FANCC* mutant B-lymphoblast lines HSC536N, the HSC536N line transduced with a vector only (HSC536N/neo) and PD149 (lanes 4, 6 and 7 respectively) show increased IRF-1, ISGF3γ and p21^{WAF1} expression compared to the normal cell line JY (lane 3) and cells corrected for the defect by retroviral transduction of the *FANCC* cDNA (HSC536N/FANCC) (lane 5) or PD149/FANCC (lane 8). However, FA-C cells express normal levels of IRF-2, p53 and p27^{KIP1}. β-tubulin is used to demonstrate equivalent loading of the ISGF3γ blots. **(B)** Real time RT-PCR demonstrates upregulation of MxA in HSC536N and HSC536N/neo compared to the corrected cells HSC536N/FANCC. This is a representative experiment. Error bars are based on duplicate samples. **(C)** The FA-C cell line HSC72 expresses equivalent levels of IRF-1 and ISGF3γ compared to cells corrected for the defect. The cells do express slightly more p21^{WAF1}, but equivalent p53. β-Tubulin is used to demonstrate equivalent loading of the p21 blot. **(D)** Low density bone marrow cells derived from two FA patients show upregulation of IRF-1, ISGF3γ and p21^{WAF1} compared to cells derived from normal individual (lanes 1 and 3 vs. lane 2).

mutant MEF cell line (MEF61) compared to the wild type (WT) MEF cell line (MEF11.1). We observed the same phenomenon using (a) whole cell extracts and nuclear extracts from the EBV-transformed *FANCC* mutant human B-lymphoblasts lines, HSC536N, (b) nuclear extracts from the EBV-transformed B-lymphoblast line PD149, relative to levels in those cells

corrected for the defect by pLXSN based retroviral transduction of the normal *FANCC* cDNA (Fig. 3-1A), and (c) whole cell lysates from LDBMC derived from FA patients compared to LDBMC derived from a normal person (Fig 3-1D). In addition, we have previously found constitutively high expression of IRF-1 mRNA also occurs in diploid bone marrow cells from children with FA-C but not in normal bone marrow cells [19]. However, no differences in IRF-2 expression levels were found between FA-C cells and normal cells (Fig. 3-1A). In addition, mRNA levels of the IFN γ -inducible GTPase, MxA are reduced in the HSC536N B-lymphoblast line compared to corrected cells (Fig. 3-1B). We found no differences in IRF-1 and ISGF3 γ expression in the FA-A lymphoblasts line, HSC72 (Fig. 3-1C).

We next quantified levels of the cell cycle modulator, p21^{WAF1}, which is known to be induced by IRF-1 and IFN γ [228-230]. FA-C B-lymphoblasts, MEFs and LDBMC express high levels of p21^{WAF1} compared to normal or corrected cells (Fig. 3-1A and 3-1D). The FA-A cell line, HSC72, also expresses higher levels of p21^{WAF1} compared to corrected cells, but does not overexpress IRF-1 or ISGF3 γ (Fig. 3-1C). This suggests that overexpression of the transactivators, IRF-1 and ISGF3 γ and the cell cycle inhibitor, p21^{WAF1}, may be due to abnormalities in different pathways, one of which is influenced by both FANCA and FANCC and the other by FANCC alone. We found no differences in p53 binding to

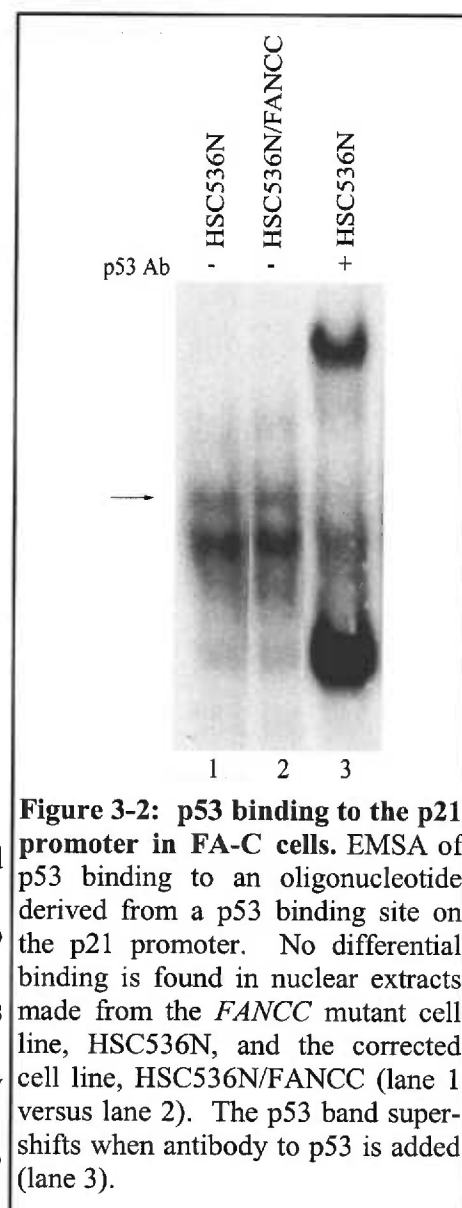


Figure 3-2: p53 binding to the p21 promoter in FA-C cells. EMSA of p53 binding to an oligonucleotide derived from a p53 binding site on the p21 promoter. No differential binding is found in nuclear extracts made from the *FANCC* mutant cell line, HSC536N, and the corrected cell line, HSC536N/FANCC (lane 1 versus lane 2). The p53 band super-shifts when antibody to p53 is added (lane 3).

its p21 binding site (Fig 3-2) and minimal differences in p53 expression levels, indicating that p21^{WAF1} overexpression is p53 independent [231] (Fig. 3-1A). No differences in expression levels of the CDK inhibitor p27^{KIP1} were found (Fig 3-1A).

Mature FA-C fibroblasts express normal levels of IFN γ -inducible genes

No differences in protein levels of IRF-1, ISGF3 γ and p21^{WAF1} in mature primary human FA fibroblasts were found (Fig. 3-3). Whole cell extracts were made and run on immunoblots probed with antibodies to IRF-1, ISGF3 γ and p21 revealing equivalent levels of gene expression between mutant cells and cells corrected for the defect by retroviral transduction of the *FANCC* cDNA. Finding molecular differences between mature fibroblasts and hematopoietic cells is not surprising given that hematopoietic abnormalities represent the

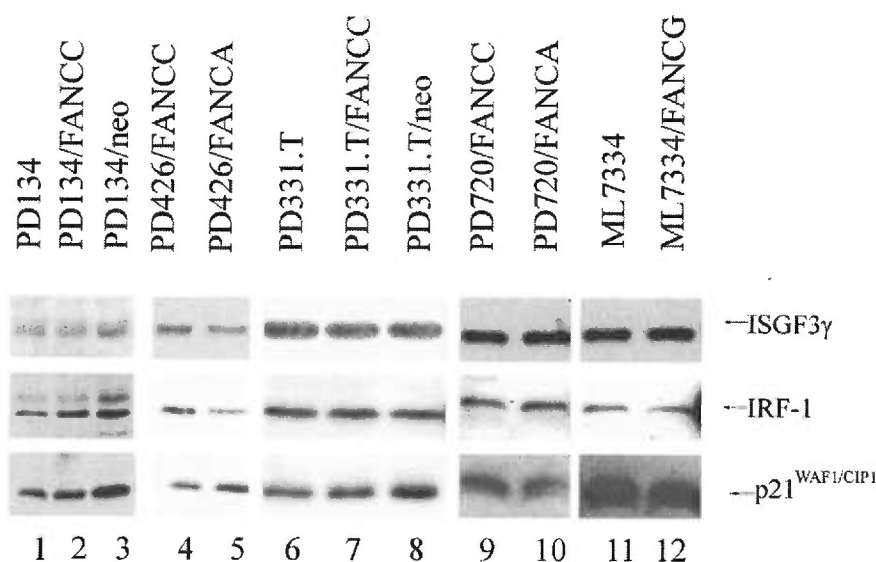


Figure 3-3: Human FA Fibroblasts express equivalent levels of IRF-1, ISGF3 γ and p21^{WAF1}. Immunoblot analysis of human FA-C fibroblasts PD134 (lane 1), PD134/neo (lane 3), PD426/FANCA (lane 5), PD331.T (lane 6), and PD331.T/neo (lane 8); the FA-A fibroblasts PD720/FANCC (lane 9); and the FA-G fibroblast line ML7334 (lane 11) express equivalent levels of ISGF3 γ , IRF-1 and p21^{WAF1} compared to those cells corrected for the defect by retroviral transduction of the appropriate FA cDNA (PD134/FANCC (lane 2), PD426/FANCC (lane 4), PD331.T/FANCC (lane 7), PD720/FANCA (lane 10), ML7334/FANCG (lane 12)).

dominant phenotype in FA patients. In addition, finding that a normal FANCC is required for proper regulation of IRF-1, p21^{WAF1} and ISGF3 γ in hematopoietic cells and embryonic fibroblasts but not in mature fibroblasts suggests that MEFs have retained more plasticity and may exhibit responses more reflective of hematopoietic and germ cells than adult fibroblasts.

3-4: Discussion

Interferons are involved in a broad range of mammalian biological functions including immunity, inflammation, hematopoiesis, cell proliferation and differentiation [108,110]. In hematopoietic progenitor cells, IFN γ induces apoptosis [15,106,111-115], and there is compelling indirect evidence that it plays a pathophysiologically significant role in acquired aplastic anemias [108,109,111,219-221]. Because FA-C cells are hypersensitive to IFN γ [18,19,104], we believe that IFN γ hypersensitivity and bone marrow failure may be linked in FA. Accordingly, we have focused our attention on the IFN γ -induced gene expression in FA-C cells as a way of deciphering the function of the FANCC protein in modulating the apoptotic response to extracellular cues.

The effects of IFN γ are mediated, at least in part, through transcriptional regulation of downstream genes, which include members of the IRF family of transcription factors. We find that certain IRF family members are constitutively overexpressed in specific FA-C cell types compared to normal cells. ISGF3 γ is constitutively expressed at higher levels in *FANCC* mutant B-lymphoblasts, MEFs and patient LDBMC and is of particular interest because it is the only protein of the IRF family known to complex with STAT molecules [232]. ISGF3 γ exerts its transcriptional activation only in conjunction with STAT1 and STAT2 [233,234] and plays a crucial role in the regulation of IFN β gene expression [235-238], a finding of relevance

because some have suggested that IFN γ -induced IFN β gene expression is required for the hematopoietic suppressive effects of IFN γ [239].

IRF-1 is required for carrying out the apoptotic effects of IFN γ signaling. The ratio of IRF1 to IRF2 expression is important for proper cell cycle control [240-242]. IRF-1 expression is significantly increased in FA-C hematopoietic and embryonic cells, but no differences in IRF-2 expression levels were found (Fig. 3-1); upsetting the proper balance of IRF-1 and IRF-2 levels in FA-C cells. IRF-1 also cooperates with p53 to induce expression of the cdk inhibitor p21^{WAF1} [228,243,244]. Although equivalent p53 expression levels were found between *Fancc*^{-/-} and *Fancc*^{+/+} MEFs and only minimal differences in p53 expression were found in human FA-C B-lymphoblasts compared to normal cells, FA-C cells expressed significantly greater amounts of p21^{WAF1} (Fig. 3-1). We find no difference in p53 binding to its p21^{WAF1} binding site (Fig 3-2), and expression of the IRF-1 independent cdk inhibitor p27^{KIP1} was equivalent (Fig. 3-1). Thus, it is possible that IRF-1 upregulation contributes to the overexpression of p21^{WAF1}. However, given that we find high constitutive p21^{WAF1} in FA-A cells and these cells do not overexpress IRF-1, it is more likely that upregulation of p21^{WAF1} and the IRF transactivators may be due to abnormalities in different pathways; one of which is influenced by both FANCA and FANCC or the entire FA complex and the other by FANCC alone.

In addition to IRF-1, ISGF3 γ and p21^{WAF1}, Li and Yousouffian have found that IFN γ -inducible GTPase, MxA, which confers intracellular antiviral activity in IFN-induced cells [245], is also constitutively expressed in FA cells, a finding we confirm here (Fig. 3-1B) Thus, several IFN γ -inducible factors that are important in regulation of apoptosis and mitogenic inhibition are constitutively upregulated in FA-C cells in a cell type specific manner.

FA cells both *in vitro* and *in vivo* are not hardy. They grow slowly and have a high apoptotic fraction [15,19,113]. Studies in our laboratory have focused, in large part, on clarifying molecular determinants of the death pathways in FA cells and to begin to establish an ordered relationship of these death pathways to discrete FA proteins. We have identified roles for the fas/fasL pathway [19], caspases 8 and 3 [107], and the double stranded RNA dependent protein kinase (PKR) [20]. In this work, while we cannot yet clarify the cause, it is clear that even without being exposed to environmental cues for apoptosis or mitotic arrest, at a gene-expression level, FA-C cells act as-if they have been stimulated by such factors. This is similar to another chromosomal instability syndrome, Ataxia Telangiectasia (AT). AT cells are hypersensitive to γ -irradiation and constitutively overexpress several γ -irradiation-inducible factors [6]. In view of the apoptotic and mitogenic inhibitory activities of these proteins we suggest that they contribute to the characteristic apoptotic phenotype in FA bone marrow cells and may contribute to the IFN-“hypersensitive” phenotype by specifically setting ground-state levels of these proteins higher than in normal cells. Clarification of the signaling pathways by which aberrantly expressed genes are controlled in FA-C cells should lead to an understanding of why such cells are hypersensitive to both biological and chemical inducers of apoptosis and may reveal clearer functions for the *FANCC* gene product.

Chapter 4

FANCC Binds to and Facilitates Interferon γ -induced Activation of STAT1 in a Cell Type Specific Manner

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and
Pang, Fagerlie *et al.* (2000) Mol. Cell. Biol. 20:4724

4-1: Overview

Hematopoietic progenitor cells from Fanconi Anemia (FA) group C (FA-C) patients display hypersensitivity to the apoptotic effects of interferon gamma (IFN γ) [18,19] and constitutively express high levels of a variety of IFN γ -dependent genes in a cell type specific manner (Chapter 3). Therefore, we tested whether the main IFN γ signaling pathway, namely the JAK/STAT pathway, functions normally in FA-C cells. Paradoxically, however, we find that IFN γ -induced activation of STAT1 is suppressed in precisely those cells that have high constitutive expression levels of IFN γ -inducible genes. We therefore sought to define the specific role of FANCC plays in IFN γ signaling through the JAK/STAT pathway. Expression and phosphorylation levels of the IFN γ receptor α chain (IFN γ R α , IFN γ R1), and the tyrosine kinases, JAK1 and JAK2, were equivalent in both normal and FA-C cells. However, STAT1 does not dock at the IFN γ R in FA-C cells, an abnormality corrected by transduction of the *FANCC* gene. Moreover STAT1 bound to glutathione S-transferase fusion proteins encoding a normal FANCC but not FANCC containing a naturally occurring inactivating point mutation (L554P). Therefore, FANCC is required for proper activation of STAT1 and may function as a chaperone facilitating the docking of STAT1 at IFN γ R α .

4-2: Introduction

Interferon- γ (IFN γ) utilizes the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway to deliver its extracellular signals to the nucleus [246]. The IFN γ receptor (IFN γ R) is composed of two chains: Interferon- γ receptor 1 (IFN γ R1) and Interferon- γ receptor 2 (IFN γ R2) (these chains are also known as IFN γ R α and IFN γ R β ,

respectively). The IFN γ R lacks intrinsic kinase activity, but constitutively binds the Janus kinases, JAK1 and JAK2. JAK1 and JAK2 bind the IFN γ R1 and IFN γ R2 chains, respectively [247]. Upon ligand binding and dimerization of the receptor, JAK1 and JAK2 are activated by auto and cross phosphorylation. JAK1 then phosphorylates IFN γ R1 creating a docking site for the src homology 2 (SH2) domain of STAT1 [117,248,249]. The factors that govern the traffic of cytoplasmic STAT1 molecules to the docking site on IFN γ R1 are unknown. Following docking on IFN γ R1, STAT1 is phosphorylated at Tyrosine 701 by JAK1, dissociates from the receptor and forms a homodimer which translocates to the nucleus and binds gamma activation sequence (GAS) and upregulates transcription of a distinct set of genes, including IRF-1, ISGF3 γ , p21^{WAF1} and MxA [236,246,250-252].

Hematopoietic progenitor cells (HPC) from Fanconi Anemia (FA) group C (FA-C) patients and *Fancc* null mice (*Fancc*^{-/-}) are hypersensitive to the apoptotic effects of IFN γ [19]. [17,18] Furthermore, *FANCC* mutant hematopoietic and embryonic cells express high levels of key IFN γ -inducible genes (discussed in Chapter 3). Given the hypersensitivity of FA-C HPC to IFN γ and the finding of high-level constitutive expression of IFN γ -inducible genes, a natural extension of this work was to examine the signaling pathway involved. We therefore hypothesized that the JAK/STAT pathway would be hyperactive in FA-C cells accounting for increased sensitivity to IFN γ and overexpression of IFN γ -inducible genes.

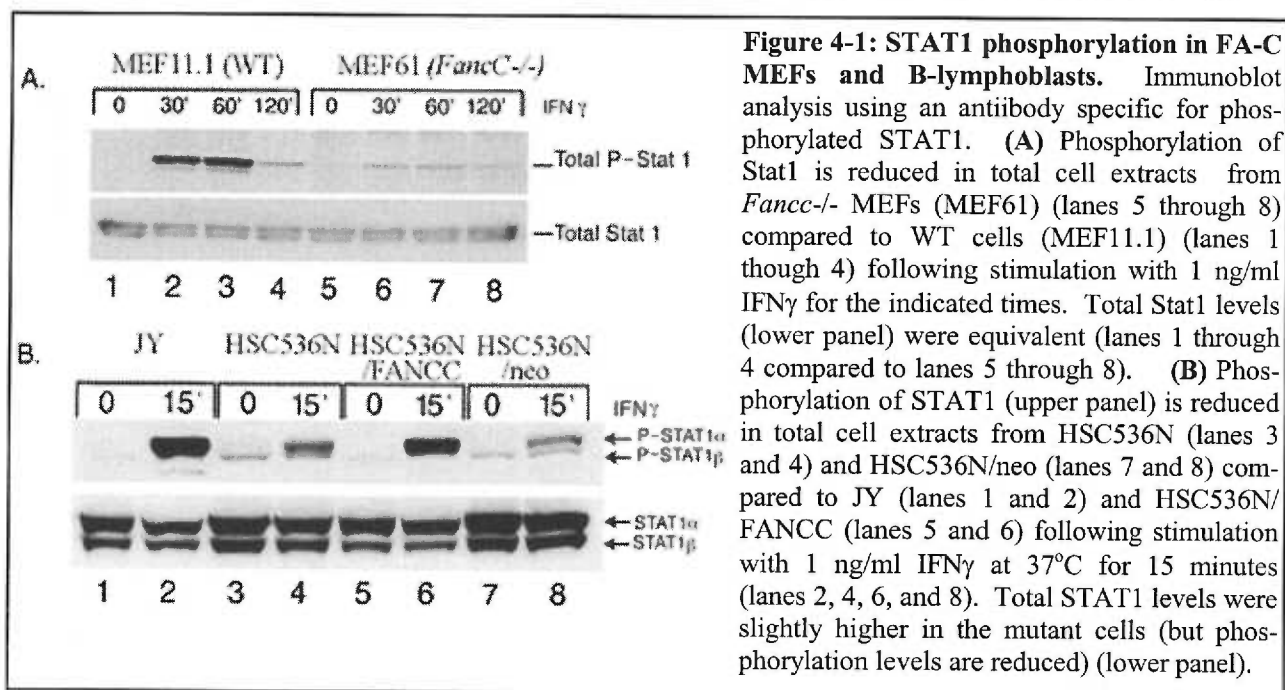
To test this hypothesis we analyzed phosphorylation levels of components of the JAK/STAT pathway. Surprisingly, we found that phosphorylation of STAT1 is significantly reduced in precisely those FA-C cells expressing constitutive high levels of IFN γ -inducible genes. Moreover, levels of phosphorylated nuclear STAT1 are reduced, as is binding to an IRF-1 derived GAS element. Expression and phosphorylation of JAK1, JAK2 and IFN γ R1 occurs

normally in these cells, but STAT1 does not dock at IFN γ R1. Expression of normal *FANCC* cDNA in mutant cells results in normal STAT1 docking and phosphorylation, as well as normal binding to IRF-1 GAS and normal expression of IRF-1. Further analysis revealed that in normal cells, upon stimulation with IFN γ , STAT1 associate associates with gluathione S-transferase (GST) fusion proteins encoding wild type FANCC but not a naturally occurring inactivating mutant FANCC (L554P). These studies indicate FANCC is a control factor for STAT1 traffic to IFN γ R1 and, ultimately, optimal activation and signaling of STAT1.

4-3: Results

Phosphorylation of STAT1 is reduced in FA-C hematopoietic and embryonic cells

FA-C HPC are hypersensitive to the apoptotic affects of IFN γ [17-19]. Furthermore, specific FA-C cells have constitutive high-level expression of IFN γ -inducible genes (Chapter 3). Therefore we sought to determine the activity of the JAK/STAT pathway in FA-C cells. We analyzed levels of IFN γ -induced STAT1 phosphorylation by immunoblot analysis using an



antibody specific for STAT1 phosphorylated at Tyrosine 701 in isogenic EBV-transformed B-lymphoblasts and murine embryonic fibroblast cell lines derived from WT and *Fancc*^{-/-} mice. MEFs were serum starved for 24 hours and then incubated at 37°C with 1 ng/ml of murine IFN γ for the indicated time periods. Surprisingly, the level of phosphorylated STAT1 was significantly decreased in *Fancc* mutant cells compared to the WT cells. When these blots were stripped and re-probed for total STAT1, levels of total STAT1 were equivalent (Fig. 4-1A). In addition, analysis of total cell lysates from the *FANCC* mutant EBV-transformed B-cell lines, HSC536N and HSC536N/neo, demonstrate that levels of phosphorylated STAT1 is diminished compared to the normal EBV-transformed B-lymphoblast line, JY, and the *FANCC* transduced (thus corrected for the FA defect) cell line, HSC536N/*FANCC*. When the same blots were stripped and re-probed for STAT1, total STAT1 were slightly increased in the mutant cells (but phosphorylated levels were decreased) (Fig. 4-1).

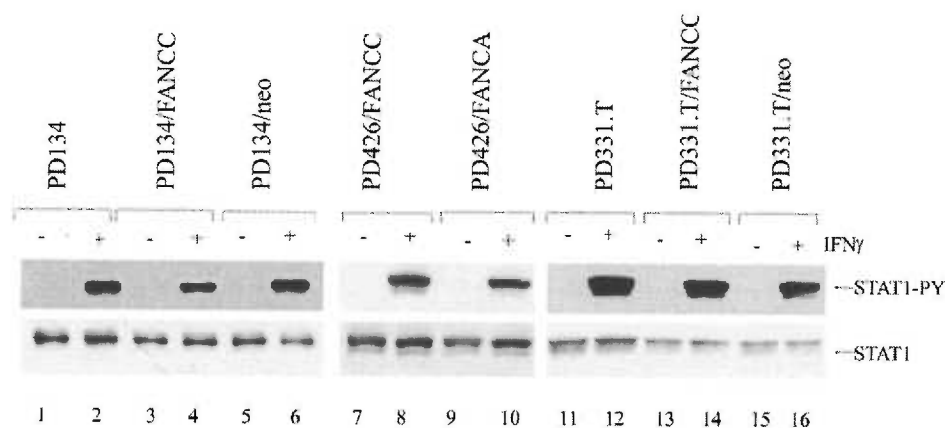


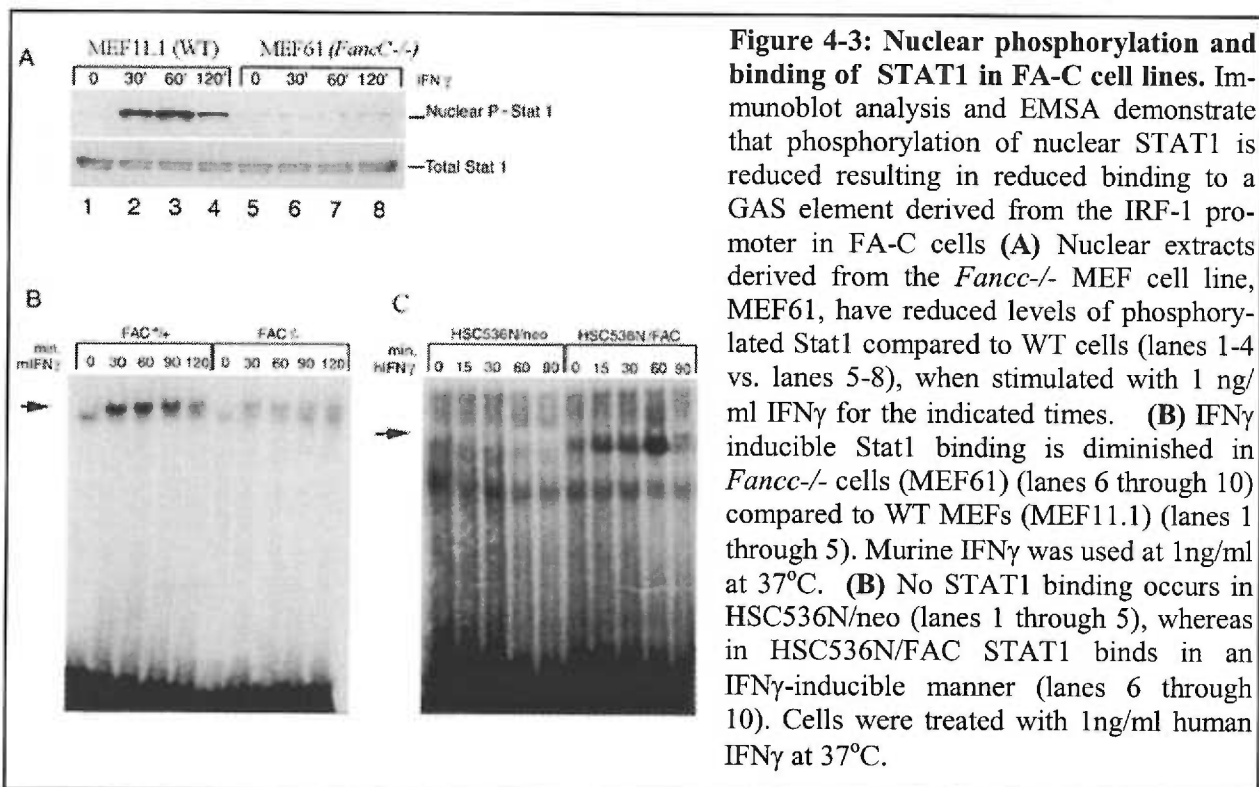
Figure 4-2: Phosphorylation of STAT1 in mature fibroblasts. Immunoblot analysis of human FA-C fibroblasts using an antibody specific for phosphorylated STAT1 demonstrates that STAT1 is phosphorylated equivalently between *FANCC* mutant and corrected cells when stimulated with 1 ng/ml IFN γ for 15 min. (top panel). Total STAT1 is expressed at approximately equal levels (bottom panel).

Mature fibroblasts express normal levels of phosphorylated STAT1

Given that only specific cell types overexpress IFN γ -inducible genes, we tested whether decreased STAT1 phosphorylation was cell type specific. We analyzed phosphorylation of STAT1 by immunoblot analysis probing with a phospho-specific STAT1 antibody. STAT1 phosphorylation was found to be normal in all mature human FA fibroblast lines tested (Fig. 4-2). Thus, in FA cells STAT1 phosphorylation defects are specific to certain cell types, and, paradoxically, those cell types defective in STAT1 activation have high constitutive expression of several IFN γ -inducible genes (Chapter 3).

Reduced nuclear phospho-STAT1 and binding to the GAS element in FA-C cells

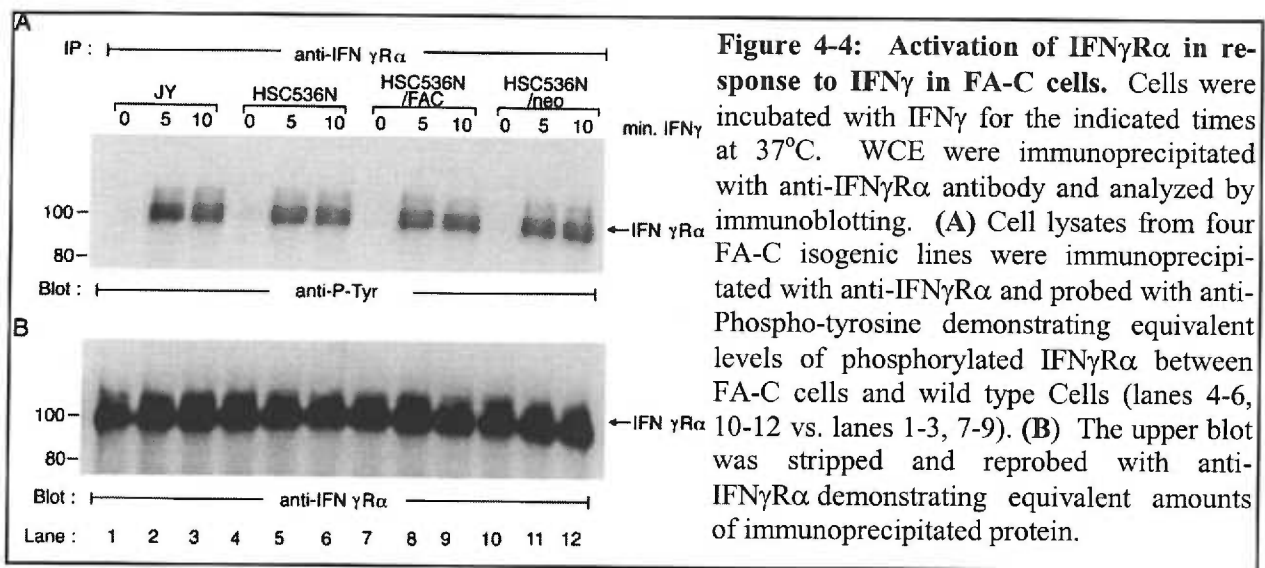
It was theoretically possible that a more complete and rapid nuclear translocation of STAT1 in FA-C cells might have given an appearance of reduced STAT1 activation. Consequently, we analyzed nuclear STAT1 phosphorylation levels and asked whether



apparently reduced levels of STAT1 phosphorylation were associated with increased binding to the STAT1 response element, the Gamma Activation Sequences (GAS). We first analyzed IFN γ -induced STAT1 phosphorylation levels in the nucleus by preparing nuclear extracts and running them on SDS PAGE, electroblotting and probing with phosph-specific STAT1. We found, like total STAT1, levels of nuclear phosphorylated STAT1 are reduced in *Fancc*^{-/-} MEFs (MEF61) compared to the WT MEFs (MEF11.1) (Fig 4-3A). Using an oligonucleotide derived from the GAS element of murine IRF-1 for EMSA analysis of murine MEF lines, we found that IFN γ -inducible binding to the GAS element was significantly reduced in the *Fancc*^{-/-} MEF line compared to the WT MEF line [231] (Fig. 4-3B). Similarly, IFN γ -inducible binding to an oligonucleotide representing a human IRF-1 GAS element was not detectable in mutant *FANCC* human B-lymphoblasts, whereas normal inducible binding was observed in the *FANCC* complemented cell line (Fig. 4-3C). These results indicate that the diminished ability to phosphorylate STAT1 in FA-C cells results in decreased activated STAT1 nuclear translocation and binding to GAS.

Tyrosine phosphorylation of IFN γ R α occurs normally in FA-C B-lymphoblasts

Cellular responsiveness to IFN γ requires the activation of IFN γ R α (IFN γ R1) through phosphorylation on tyrosine residues. To analyze more specifically where in the JAK/STAT pathway the defect occurs, we asked whether ligand-induced IFN γ R α tyrosine phosphorylation was different in FA-C cells. Cells were treated with IFN γ for the indicated time, whole cell extracts (WCE) were co-immunoprecipitated using an anti-IFN γ R α antibody. Immunoblots were performed and probed with antibody specific for phosphorylated tyrosines. In the absence of IFN γ , no tyrosine phosphorylated IFN γ R α was detected. Following IFN γ treatment,



equivalent levels of IFN γ R α was observed in all four isogenic cell lines [253] (Fig. 4-4A). The amounts of the receptor proteins loaded in each lane were comparable as determined by re-probing the membrane with anti-IFN γ R α antibody (Fig 4-4B) [253].

Tyrosine phosphorylation of JAK1 and JAK2 is normal in FA-C cells

JAK1 and JAK2 are activated by IFN γ through tyrosine phosphorylation, and these activated kinases play an essential role in the transduction of IFN γ signal [120,122,254]. To determine whether IFN γ -induced tyrosine phosphorylation of the JAK kinases occurs normally in FA-C cells, whole cell extracts from normal and FA-C cells were immunoprecipitated with an antibody specific for phosphorylated tyrosines (4G10) and then immunoblotted and probed with antibodies specific for JAK1 or JAK2 [253] (Fig 4-5). Tyrosine phosphorylation of JAK1 and JAK2 was not detected in unstimulated cells, and no difference in tyrosine phosphorylation was detected following IFN γ stimulation (Fig 4.5A and 4.5C). To analyze total cellular JAK levels, immunoblot analysis was performed on whole cell extracts (WCE). No differences in expression levels of JAK1 or JAK2 were found between FA-C and normal cells (Fig 4-5B and 4-5D) [253].

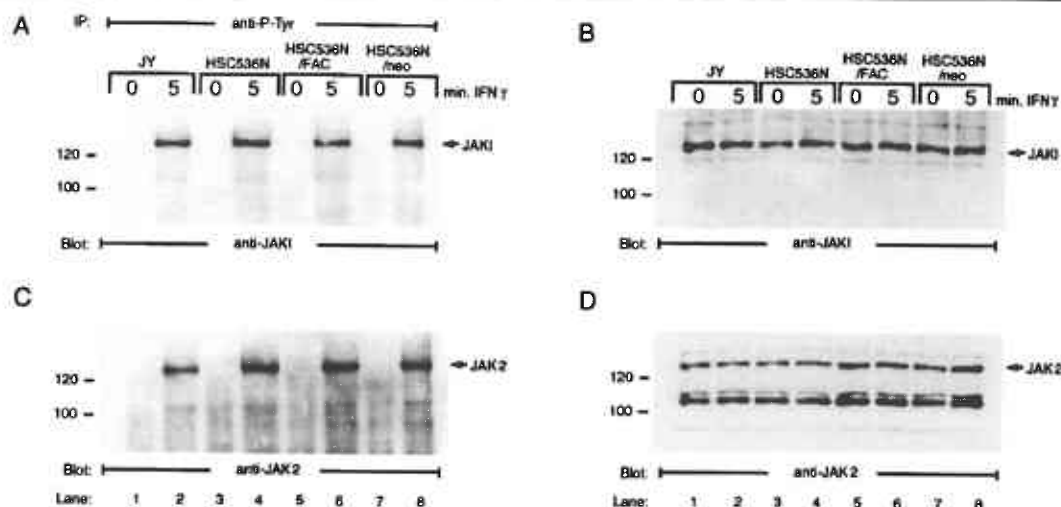


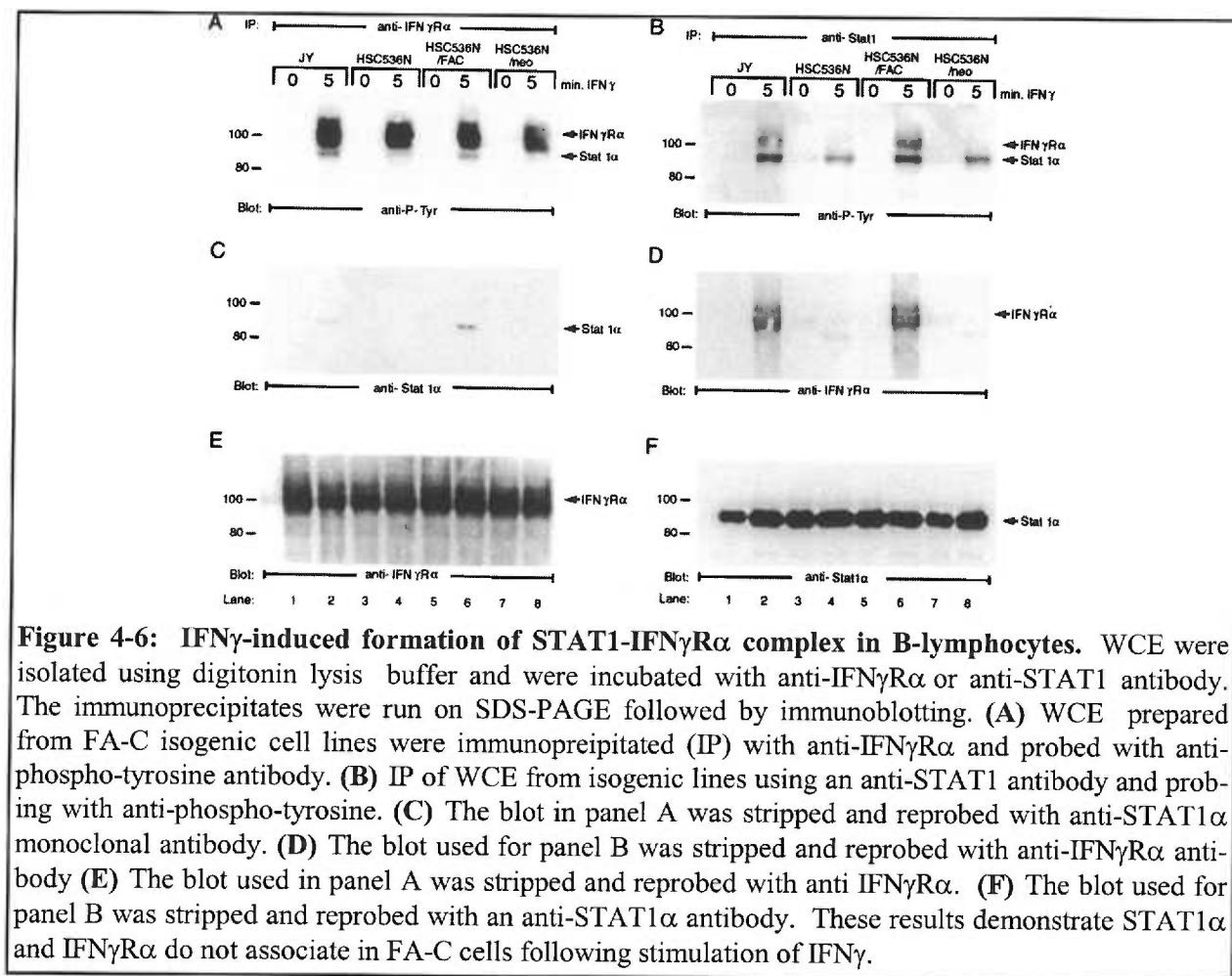
Figure 4-5: IFN γ -induced tyrosine phosphorylation of JAK1 and JAK2 in FA-C cells. WCE were either directly analyzed by immunoblotting with anti-JAK1 or -JAK2 antibodies or immunoprecipitated with anti-phospho-tyrosine antibody and immunoprecipitates were analyzed by immunoblotting. (A) Cell lysates were immunoprecipitated with anti-phospho-tyrosine antibody, and the immunoprecipitates were probed with anti-JAK1 demonstrating no difference in phosphorylation levels of JAK1 between FA-C cells and normal or corrected cells (lanes 3,4 & 7,8 vs. 1,2 & 5,6) in response to IFN γ (B) WCE were directly immunoblotted with anti-JAK1 antibody demonstrating equivalent expression levels. (C) WCE from FA-C isogenic lines were immunoprecipitated with anti-phospho-tyrosine and probed with anti-JAK2 demonstrated equivalent phosphorylation levels of JAK2 in response to IFN γ in FA-C cells (lanes 3,4 & 7,8 vs. lanes 1,2 & 5,6). (D) WCE were directly immunoblotted with anti-JAK2 and demonstrate equivalent expression of JAK2 in FA-C cells.

A functional FANCC is required for STAT1 docking at IFN γ R1

IFN γ -induced tyrosine phosphorylation of IFN γ R1 is thought to create a docking site for STAT1, which, after docking, is phosphorylated by the JAK kinases [255,256]. In support of this model, STAT1 has been found to associate with tyrosine phosphorylated IFN γ R1 both *in vitro* and *in vivo* [257,258]. Having established that IFN γ -induced phosphorylation of IFN γ R1, JAK1 and JAK2 occurs normally, but phosphorylation of STAT1 is diminished in FA-C cells, we next asked whether STAT1 is recruited to the activated IFN γ R in these cells. IFN γ -stimulated isogenic cells were solubilized with 1% digitonin buffer, and protein complexes were immunoprecipitated using antibody to IFN γ R1. These immunoprecipitates were then run on

western blots and probed with anti-phospho-tyrosine antibody or STAT1. When immunoprecipitates from FA-C isogenic cell lines were analyzed with the anti-phospho-tyrosine antibody, a diffusely migrating phosphoprotein of ~100 kDa was detected in all four cell lines only when cells were treated with IFN γ . This 100-kDa phosphoprotein was identified as IFN γ R1 by reprobing the blot with anti-IFN γ R α . In addition to the tyrosine-phosphorylated IFN γ R1, a distinct phospho-protein of ~90 kDa illuminated on the immunoblot. This phospho-protein precipitated by anti-IFN γ R α in lysates prepared from IFN γ treated normal (JY) and complemented FA-C (HSC536N/FANCC) cell lines but not in those from stimulated FA-C (HSC536N and HSC536N/neo) cell lines (Fig 4-6A, lanes 2 and 6 versus 4 and 8) [253]. Reprobing the blot with a monoclonal antibody specific to STAT1 revealed that the 90-kDa band was STAT1 α . No signal was detected in the anti-STAT1 α immunoblot in anti-IFN γ R1 immunoprecipitates prepared from IFN γ -treated mutant cell lines HSC536N and HSC536N/neo (Fig 4-6A, lanes 4 and 8) or from cells that had not been incubated with IFN γ .

To confirm the above observations, digitonin lysed cell extracts were co-immunoprecipitated with an anti-STAT1 α antibody, blotted and probed with the anti-phospho-tyrosine antibody. Two tyrosine-phosphorylated proteins are evident in both IFN γ -treated normal (JY) and complemented (HSC536N/FANCC) cell lines, but not in stimulated mutant FA-C (HSC536N and HSC536N/neo) cell lines (Fig. 4-6B, lanes 2 and 6 versus 4 and 8). The upper band proved to be IFN γ R α as indicated in (Fig. 4-6D lanes 2 and 6) by re-probing the membrane with anti-IFN γ R1. Although a small amount of phosphorylated STAT1 precipitated with anti-STAT1 α antibody in the FA-C cell lines HSC536N and HSC536N/neo, no co-precipitating IFN γ R1 protein was detected [253] (Fig 4-6B and D, lanes 4 and 8), indicating reduced STAT1 does not associate with the receptor in FANCC mutant cells.



As an important technical note, we emphasize that we were unable to detect the STAT1: IFN γ R1 association in cell lysates prepared with RIPA buffer or NP-40 lysis buffer (see Chapter 2). Washing of the immunoprecipitate in 1% Triton X-100, 1% NP-40, or 1% digitonin lysis buffer resulted in the disruption of the STAT1-IFN γ R1 complex, suggesting that interaction between STAT1 and the receptor protein may be weak or unstable. Finally, Western blot analysis of the anti-IFN γ R1 and STAT1 immunoprecipitates demonstrated the presence of equivalent IFN γ R1 and STAT1 proteins in all four cell lines at 5 min of IFN γ incubation compared to unstimulated cells (Fig. 4-6D and 4-6F). This indicates that both the receptor and STAT1 proteins are constitutively expressed at equivalent levels in all isogenic cell lines. Taken

together, these results demonstrate an unambiguous requirement for the FANCC protein in the formation of IFN γ -induced STAT1 α -IFN γ R1 complexes.

FANCC associates with STAT1 following IFN γ stimulation

Since FANCC is required for recruitment of STAT1 to the activated IFN γ R, this suggested that the activation of STAT1 might result from a direct interaction of the FANCC protein with STAT1. Therefore, we sought to identify a binding interaction between FANCC and STAT1. JY cells were stimulated with IFN γ or left untreated. WCE were incubated with glutathione-Sepharose bead-bound GST fusion proteins containing either WT FANCC (GST-FANCC) or a naturally occurring mutant FANCC (GST-L554P). FANCC-interacting cellular proteins were then isolated from WCL by glutathione-Sepharose affinity precipitation. STAT1 Western blot analysis of the affinity-precipitated proteins (Fig 4-7, top panel) demonstrated that STAT1 binds strongly to the WT FANCC fused to GST (lane 4) but not to GST alone (lanes 1 and 2). STAT1 bound weakly to the GST fusion protein containing the L554P mutation (lane 6). Reprobing the blot with antibodies to FANCC revealed comparable amounts of GST-fused FANCC and L554P

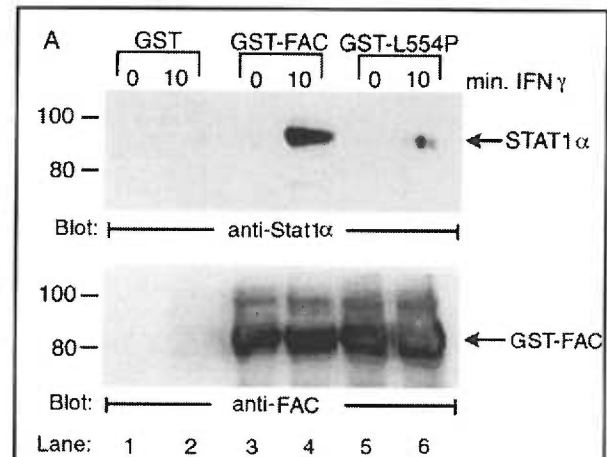


Figure 4-7: FANCC and STAT1 associate following stimulation with IFN γ . WCE derived from the normal lymphoblast cell line, JY $\pm$ IFN γ , were incubated with yeast-expressed GST fusion proteins (10 ug) bound to glutathione-sepharose beads. The glutathione Sepharose affinity precipitates were analyzed by SDS-PAGE following followed by immunoblotting with STAT1 (upper panel). The blot was then reprobed with an antibody to FANCC to verify equivalent input of GST (lower panel). These results demonstrate that IFN γ induces an association between WT FANCC and STAT1 (lane 4), but the naturally occurring mutant FANCC, L554P, binds STAT1 at a much lower affinity (lane 6).

proteins were input in the binding reactions (Fig 4-7, bottom panel). It is important to note that FANCC-STAT1 interaction was detected only in lysates prepared from IFN γ -stimulated cells (Fig 4-7, lanes 3 and 5 versus 4 and 6), suggesting that the association is initiated by early activation elements of the IFN γ signaling pathway [253].

4-4: Discussion

Hematopoietic progenitor cells from mice nullizygous at the *FANCC* locus and children of the C complementation group are hypersensitive to the apoptotic effects of IFN γ [17-19]. Because most of the known effects of IFN γ are ignited via the JAK/STAT pathway, in particular through STAT1 [130,259,260], we initially expected that this pathway would be hyperactive in FA-C cells. Surprisingly, however, we find that IFN γ -induced activation of STAT1 is markedly reduced in precisely those cells that contain high-level expression of key IFN γ -inducible genes (Fig 4-1, 4-2 and Chapter 3). We find no difference in expression and phosphorylation of IFN γ R1, JAK1 or JAK2 in these cells (Fig 4-4, 4-5), indicating that reduced phosphorylation of STAT1 results neither from reduced expression nor activation of JAK1, JAK2 or IFN γ R1. This suggests that the defect of FA-C cells occurred somewhere between phosphorylation of the receptor (which occurs normally in FA-C cells) and phosphorylation of STAT1 (which is diminished in FA-C cells).

To further investigate the nature of the STAT1 phosphorylation defect in FA-C cells, we tested whether STAT1 associates properly with IFN γ R α in FA-C cells. However, IFN γ R α and STAT1 co-immunoprecipitate only in WCE derived from normal and corrected cells; this association did not occur in lysates derived from FANCC mutant cells (Fig 4-6). Our observation that STAT1 forms a complex with IFN γ R α in normal cells stimulated with IFN γ is

consistent with the previous reports that STAT1 physically associates with IFN γ R α both *in vitro* and *in vivo* [257,261,262]. In these reports, STAT1 was shown to specifically recognize a phosphorylated tyrosine residue in the cytoplasmic domain of the receptor protein and to co-immunoprecipitate with IFN γ R1 in stimulated cell lysates. Once recruited into the dimeric IFN γ R1/2 complex, STAT1 becomes activated by phosphorylation at tyrosine 701 [248,263]. We found that co-immunoprecipitation of STAT1 with IFN γ R1 was ligand dependent and additionally depended on the presence of an intact FANCC protein (Fig 4-7). Moreover, the difference in STAT1-IFN γ R1 co-immunoprecipitation between normal and FA-C cells was not due to the varying expression levels of STAT1, since STAT1 expression levels were slightly increased in mutant cells (Fig 4-1). Therefore, it appears that the presence of a functional FANCC protein somehow facilitates the recruitment of STAT1 to the activated IFN γ R complex.

The mechanism by which cytoplasmic STAT1 is recruited to IFN γ R1 is not understood. Given that our data indicates FANCC is required for recruitment of STAT1 to IFN γ R1, we postulated that FANCC may govern trafficking of STAT1 to the receptor, and that this would likely require FANCC to associate with STAT1. To test this notion, GST fusion proteins were made encoding wild type FANCC and a naturally occurring mutant FANCC (L554P). STAT1 clearly bound to wild type FANCC following stimulation with IFN γ and binding to the mutant FANCC was significantly reduced. These studies suggest that FANCC may function to deliver STAT1 to the receptor following IFN γ stimulation, in part, through formation of a FANCC-STAT1 complex.

Dr. Qishen Pang and others in our lab have extended these studies. First, Dr. Pang has performed kinetic binding assays which indicate the FANCC mutation directly causes the impaired STAT1-FANCC association and is not due to modulations in the ability of STAT1 to

bind wild type FANCC. Second, he has determined that the FANCC-STAT1 association precedes tyrosine phosphorylation of STAT1. Third, he has demonstrated that STAT1 and wild type FANCC, but not mutant FANCC, associate in the factor dependent myeloid precursor cell in response to not only IFN γ , but also in response to Interferon alpha (IFN α), granulocyte macrophage colony stimulating factor (GM-CSF) and stem cell factor (SCF) [213]. These studies further implicate FANCC in the trafficking of STAT1 to the receptor and indicate responses to cytokines, particularly those cytokines important for hematopoietic cell development, may be blunted in FA-C cells.

The mechanism by which STAT1 fails to appear at its docking site on IFN γ R and subsequently reduced tyrosine phosphorylation in FA-C cells is unclear. One possibility that exists is that FANCC may function as a chaperone for delivery of STAT1 molecules to receptors following extracellular stimulation. This is supported by findings that FANCC and STAT1 associate very rapidly and prior to STAT1 phosphorylation [213]. Given that STAT1 has been found to interact with factors involved in chromatin remodeling including the CREB-binding protein CBP/p300, and FANCC interacts with STAT1, as well as FAZF, another protein postulated to be involved in chromatin remodeling, another possibility is that FANCC facilitates proteins involved in chromatin remodeling and ultimately transcription of factors that govern STAT1 docking and activation. A third possibility is that FANCC may modulate function or gene expression of factors known to inhibit STAT1 signaling. One such likely candidate is SOCS1. SOCS1 is a STAT1-responsive gene whose product interdicts IFN γ -induced activation of STAT1, and is an appealing candidate, particularly since SOCS1 nullizygous mice like FANCC nullizygous mice exhibit IFN γ hypersensitivity [156,164]. However, this molecule functions by reducing JAK activation, and the studies described herein demonstrate that FANCC mutations result in STAT1 inactivation without affecting JAK1 or

JAK2 phosphorylation. Consequently, repression of SOCS1 expression or function per se, is an unlikely explanation for our results. Nonetheless, while their function is unclear to date, a number of proteins with C-terminal SOCS domains have been identified, and it remains quite possible that such proteins or others like them function to inhibit STAT1 activation and are also encoded by genes responsive to transcriptional repressors with which FANCC interacts.

Finding reduced activation of STAT1 in FA-C cells creates somewhat of a paradox, given that FA-C cells are hypersensitive to IFN γ and overexpress several IFN γ -inducible genes. Thus creating a disconnect between classical IFN γ signaling and downstream activation of IFN γ -inducible genes in FA-C cells, and indicating that the FANCC protein is multifunctional in its capacity to suppress hematopoiesis. Therefore, given the apoptotic phenotype of FA-C hematopoietic cells and the involvement of STAT1 in signaling of important hematopoietic growth factors, these studies raise two very important questions. First, what is the alternative pathway that is hyperactive in FA-C cells resulting in the highly apoptotic phenotype? Second, are other STAT signaling pathways defect in FA-C cells and does loss of these pathways result in decreased ability of FA-C cells to respond to growth signals?

From the hematopoietic standpoint, FA-C cells are hypersensitive to a variety of mitogenic inhibitory molecules and apoptotic cues, including cytokines that utilize pathways not known to involve STAT1 [257]. Consequently, we do not argue that the function of FANCC vis-à-vis STAT1 is sufficient to account for every element of the FA phenotype. If this were the case, we would need to propose that STAT1 null mice would share the same phenotype, and they do not [130]. It is clear, however, that FA-C cells, whose receptor complexes are disconnected from STAT1 signaling pathways, are likely deprived of survival signals that utilize this pathway. With STAT1 deficiency alone, this state of disconnection from survival cues may

not be sufficient to cause BM failure (a universal finding in FA), but when combined with the constitutive expression of mitogenic inhibitory and apoptotic factors, and hypersensitivity to IFN γ and tumor necrosis factor- α [17,19], the impact of STAT1 disconnection in hematopoietic cells may be substantial.

Chapter 5

Loss of Inhibitors of Interferon- γ Signaling in FA-C cells

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5-1: Overview

Fanconi Anemia group C (FA-C) hematopoietic and embryonic cells overexpress several key IFN γ -inducible genes [212] (Chapter 3). Paradoxically, these cells do not activate signal transducer and activator of transcription (STAT) 1 properly [212,213] (Chapter 4). Seeking to clarify mechanisms by which FA-C cells overexpress IFN γ -inducible genes in the face of defective STAT1 phosphorylation, we reasoned that decreased levels of activated STAT1 might result in reduced expression of a hematopoietic IFN γ -responsive protein that normally modulates expression of other IFN γ -responsive genes. We quantified levels of the IFN γ -inducible factor, interferon consensus sequence binding protein (ICSBP), a negative trans-acting regulator of some IFN γ -inducible genes [264]. We find that ICSBP levels are reduced in FA-C B-lymphoblasts and MEFs. However, enforced expression of ICSBP fails to downregulate IRF-1, ISGF3 γ and p21^{WAF1}, indicating that reduction in ICSBP expression does not govern the overexpression of other IFN γ -inducible genes in FA-C cells. We next sought to identify defects in other pathways that might govern the aberrant regulation of these genes in FA-C cells. In normal cells, Interleukin 4 (IL-4) inhibits IFN γ -induced IRF-1 expression via STAT6 [265]. Therefore, we quantified levels of IL-4 induced STAT6 activation in FA-C cells. We found that activation of STAT6 is diminished in FA-C cells, suggesting that defective STAT6 signaling in FA-C cells contributes to the upregulation of IFN γ -inducible genes.

5-2: Introduction

Hematopoietic progenitor cells (HPC) derived from Fanconi Anemia (FA) group C patients and mice nullizygous at the *Fancc* locus are hypersensitive to Interferon gamma (IFN γ)

[17-19] and have high constitutive expression of key IFN γ -inducible genes including Interferon Regulatory Factor 1 (IRF-1), Interferon Stimulated gene factor 3 gamma (ISGF3 γ) and p21^{WAF1} (Chapter 3). However, paradoxically, FA-C cells are defective in their ability to properly activate STAT1 (Chapter 4), an abnormality that occurs in precisely those cell types that overexpress these IFN γ -inducible genes. Therefore, it is highly likely that loss of a cell type specific STAT1-inducible transcriptional repressor may contribute to the upregulation of IFN γ -inducible genes in these cells. An ideal candidate is the transcriptional repressor Interferon Consensus Sequence Binding Protein (ICSBP).

ICSBP is a hematopoietic specific IFN γ -inducible transcriptional repressor known to inhibit expression of specific IFN γ -inducible genes [264]. ICSBP modulates functions of other IFN γ -inducible genes including the IRF family members IRF-1 and ISGF3 γ [266]. Furthermore, stable expression of the DNA binding domain of ICSBP blocks IRF-1 transactivation and also inhibits binding of STAT1 to its core binding sequence. We found that binding to the gamma activation sequence (GAS) derived from the ICSBP promoter is reduced in FA-C cells, therefore, we analyzed expression of ICSBP and found significant reduction in constitutive and inducible ICSBP levels. In order to more clearly assess the role of ICSBP in the constitutive high expression levels of IFN γ -inducible genes, we enforced ICSBP expression in FA-C cells. However, increased levels of ICSBP failed to influence IRF-1, ISGF3 γ or p21 levels, indicating ICSBP does not govern the aberrant expression levels of IFN γ -inducible genes in FA-C cells.

Given that ICSBP expression did not influence high-level expression of IFN γ -inducible genes, we focused our attention of other signaling pathways known to inhibit IFN γ -induced

gene expression. One such pathway is the IL-4 signaling pathway. IL-4 and IFN γ are two mutually antagonistic cytokines that have opposing biological influences [267,268]. This occurs in part through opposing effects on gene transcription [265]. While IFN γ upregulates IRF-1 through activation of STAT1, IL-4 inhibits IFN γ -induced IRF-1 gene expression through activation of STAT6 [265]. Given the antipodal relationship between IL-4 and IFN γ , and, that IL-4 signaling, like IFN γ signaling, utilizes STAT molecules (STAT1 in the case of IFN γ and STAT6 for IL-4), we hypothesized that IFN γ -inducible genes are upregulated in FA-C cells because inhibitory signals that ordinarily depend on IL-4 activation are defective in FA-C cells. Therefore we analyzed levels of IL-4 activated STAT6 in FA-C cells. We find that phosphorylation of STAT6 is significantly decreased in FA-C cell lines. In addition, we find STAT6 abnormalities in low density bone marrow cells (LDBMC) derived from FA-C patients, an abnormality that is lost in cells from a patients who have progressed to myelodysplastic syndrome (MDS). Thus, defective IL-4-induced STAT6 signaling likely contributes to the upregulation of IFN γ -inducible genes in FA-C cells. Moreover, our findings demonstrate that FANCC is required for proper activation of at least two different STAT proteins in response to at least two different cytokines suggesting FANCC may have widespread involvement in the regulation of STAT signaling and STAT responses.

5-3: Results

Expression of the IFN γ response modulator, ICSBP, is reduced in the FA-C cell lines

Seeking to identify potential molecular points of control involved in the aberrant expression of IRF-1, p21^{WAF1} and ISGF3 γ in FA-C cells, we examined ICSBP, an interferon responsive transcription factor known to modulate IFN-responses in hematopoietic cells

[269,270]. Electromobility shift assay (EMSA) demonstrates that binding to an ICSBP derived GAS element is significantly reduced in FA-C cells compared to WT cells [231] (Fig. 5-1), suggesting that ICSBP expression may be affected by reduced STAT1 phosphorylation in FA-C cells. Immunoblot analysis demonstrates that ICSBP expression levels are constitutively low in the FA-C B-lymphoblast cell lines, HSC536N and HSC536N/neo, compared to the normal cell line, JY, and the FANCC complemented line HSC536N/FANCC (Fig. 5-2). In addition, although

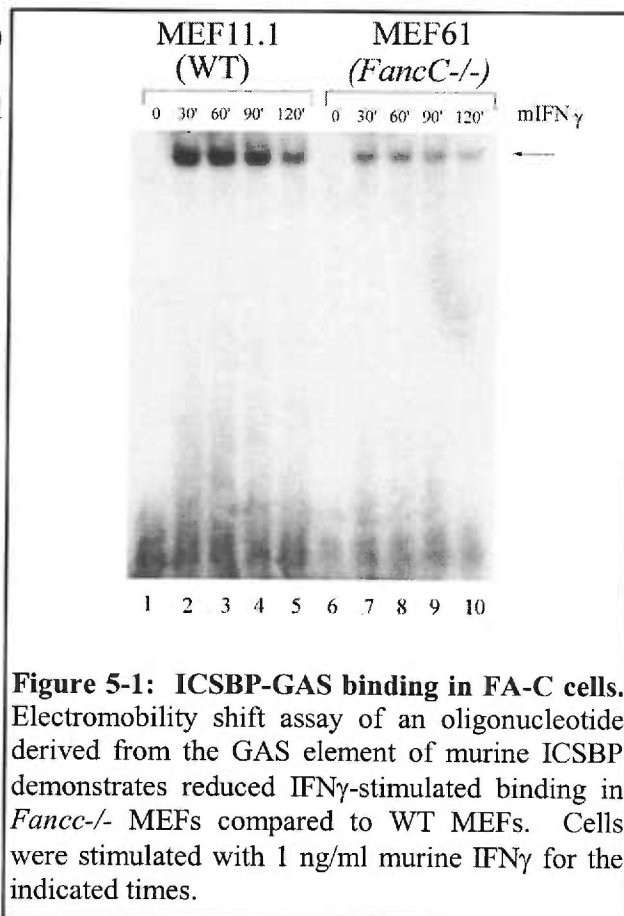


Figure 5-1: ICSBP-GAS binding in FA-C cells. Electromobility shift assay of an oligonucleotide derived from the GAS element of murine ICSBP demonstrates reduced IFN γ -stimulated binding in *FancC*^{-/-} MEFs compared to WT MEFs. Cells were stimulated with 1 ng/ml murine IFN γ for the indicated times.

ICSBP is reported to be expressed mainly in hematopoietic cells, we found that ICSBP expression is consistently inducible in a transformed murine embryonic fibroblast (MEF) cell line derived from a wild type (WT) mouse, however, expression of IFN γ -inducible ICSBP is significantly reduced in a transformed MEF cell line derived from a *Fancc* knockout (*Fancc*^{-/-})

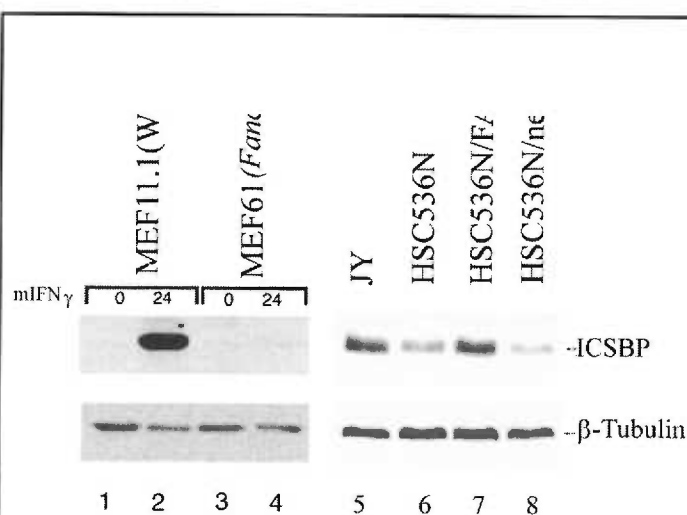
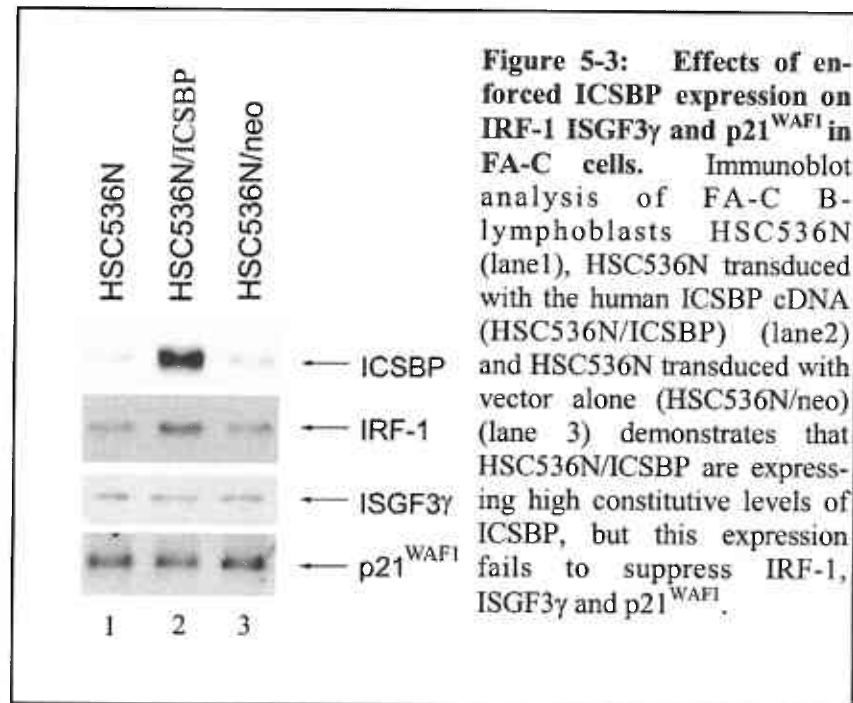


Figure 5-2: Expression of ICSBP FA-C cells. Immunoblot analysis demonstrates that ICSBP is expressed inducibly (50 ng/ml IFN γ for 24 hours) in wild type MEFs (MEF11.1) (lane 2) at high levels. ICSBP expression in the *Fancc*^{-/-} MEF line (MEF61) is significantly reduced compared to the wild type cells (lane 4 vs. lane 2). Constitutive expression of ICSBP is reduced in FA-C B-lymphoblasts compared to normal and corrected cells (lanes 6 and 8 vs. lanes 5 and 7). The same blot was stripped and re-probed with β -tubulin to demonstrate equivalent loading (lower panel).

mouse embryo. Therefore, a functional FANCC is necessary for optimal constitutive and inducible expression of ICSBP.

Enforced expression of ICSBP does not suppress IRF-1, p21^{WAF1/CIP1} or ISGF3 γ

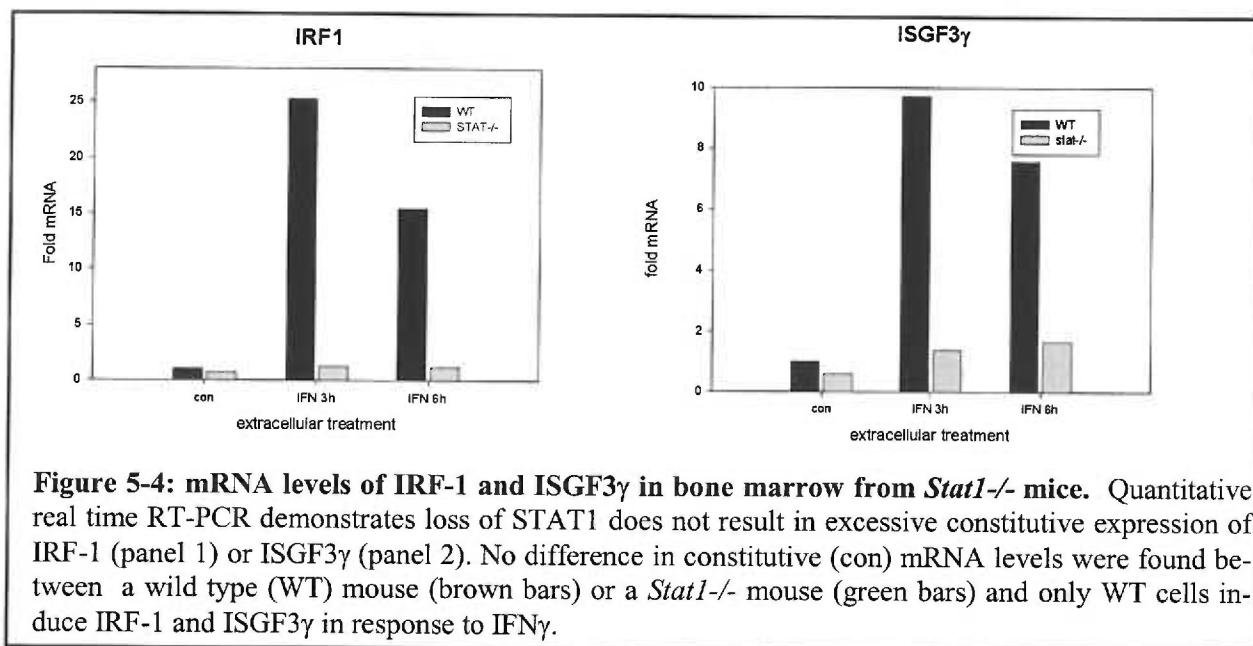
To examine the linkage of reduced ICSBP with overexpression of IRF-1, ISGF3 γ or p21^{WAF1}, we transduced the HSC536N B-lymphoblast cell line with a retroviral vector carrying the human ICSBP cDNA. Although immunoblot analysis



demonstrates that these cells express high levels of ICSBP expression, ICSBP expression failed to suppress the expression of IRF-1, p21^{WAF1} and ISGF3 γ (Fig. 5-3). In fact; expression of IRF-1 was slightly increased in cells expressing ICSBP. Taken together these data indicate that upregulation of IRF-1, ISGF3 γ and p21^{WAF1} in FA-C cells is not directly linked to reduced levels of ICSBP expression.

Cells derived from STAT1^{-/-} mice do not overexpress IRF-1 or ISGF3 γ

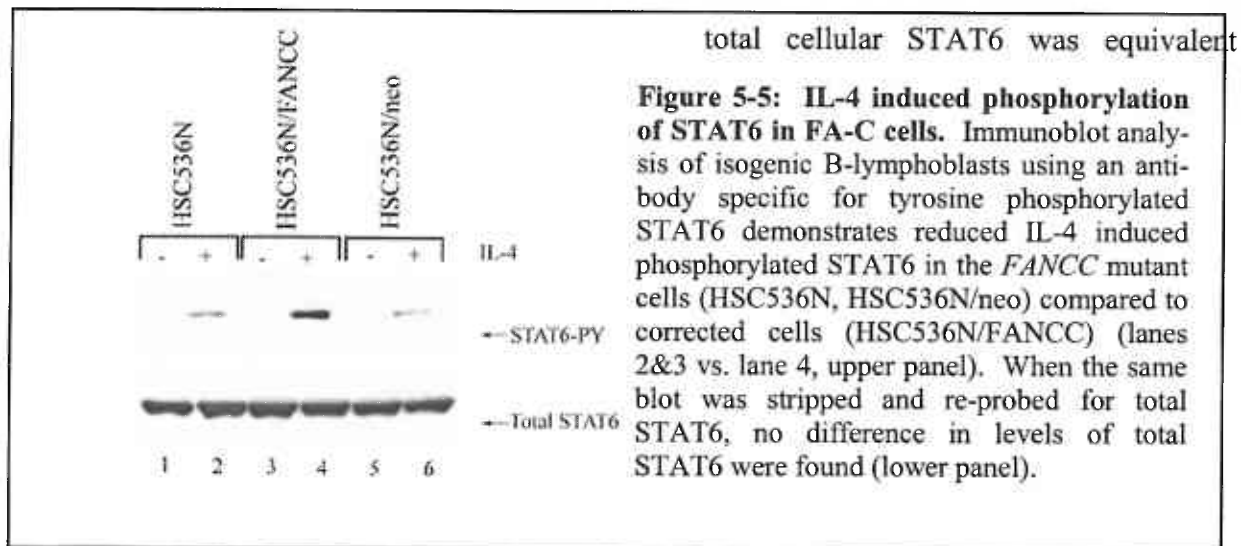
Given that ICSBP is not directly linked to upregulation of IRF-1, ISGF3 γ and p21^{WAF1}, we next analyzed whether reduced STAT1 contributes at all to high level constitutive expression of IFN γ -inducible genes. To test this we removed whole bone marrow cells from



mice nullizygous for *Stat1* (*Stat1*^{-/-}) or WT mice and then performed real time RT-PCR using probes specific for IRF-1 and ISGF3 γ [271]. Loss of STAT1 does not affect constitutive mRNA expression of IRF-1 and ISGF3 γ , implying that aberrant expression of IRF-1 and ISGF3 γ expression in FA-C cells is not due to a reduction in STAT1 and is likely the result of a defective STAT1-independent pathway (Fig 5-4).

IL-4-induced STAT6 phosphorylation is reduced in FA-C cells

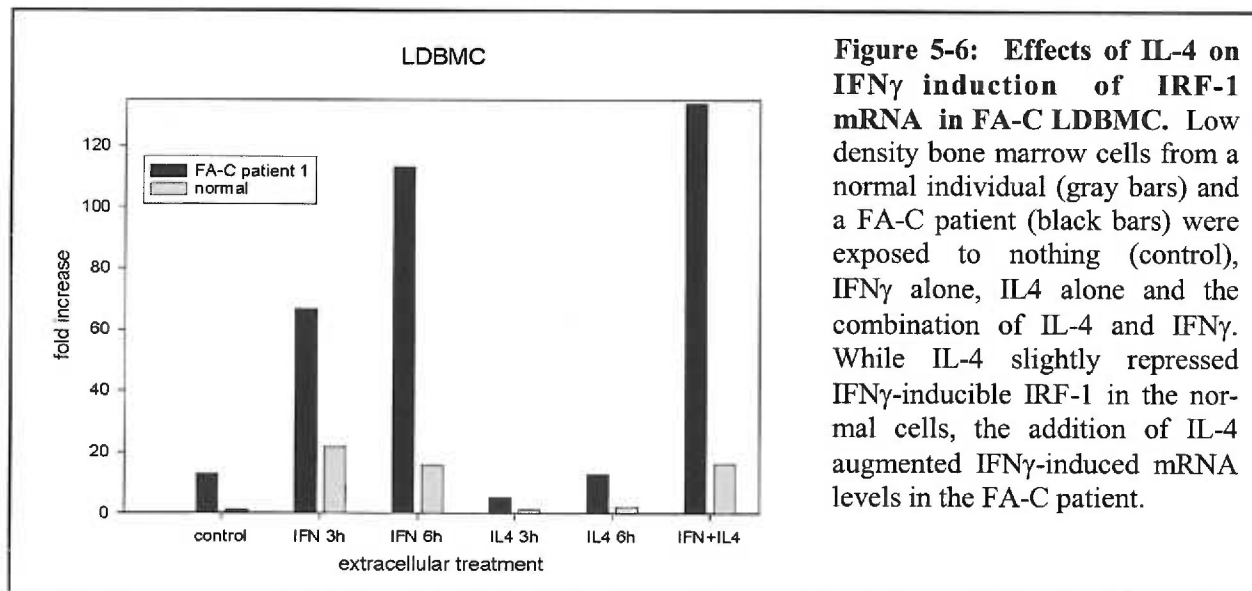
Given that loss of STAT1 does not appear to be the root cause for high constitutive expression of IFN γ -inducible genes, we focused on inhibitory pathways whose signaling occurs independent of STAT1. IL-4 and IFN γ are two mutually antagonistic cytokines. Furthermore IL-4 is known to inhibit IFN γ -induced IRF-1 expression via STAT6 [265]. Therefore, we analyzed levels of STAT6 phosphorylation in FA-C cells. Using immunoblot analysis and an antibody specific for STAT6 phosphorylated at tyrosine 641, we determined levels of IL-4 induced phosphorylation of STAT6 in isogenic cell lines. The FANCC mutant cell lines HSC536N and HSC536N/neo, have reduced levels of IL-4-induced STAT6 phosphorylation compared to the FANCC corrected line HSC536N/FANCC (Fig 5-5, top panel). Expression of



between mutant FANCC and normal cells (Fig 5-5, lower panel), indicating the defect is phosphorylation specific.

IL-4 fails to inhibit IFN γ -induced IRF-1 expression in FA-C patient derived LDBMC

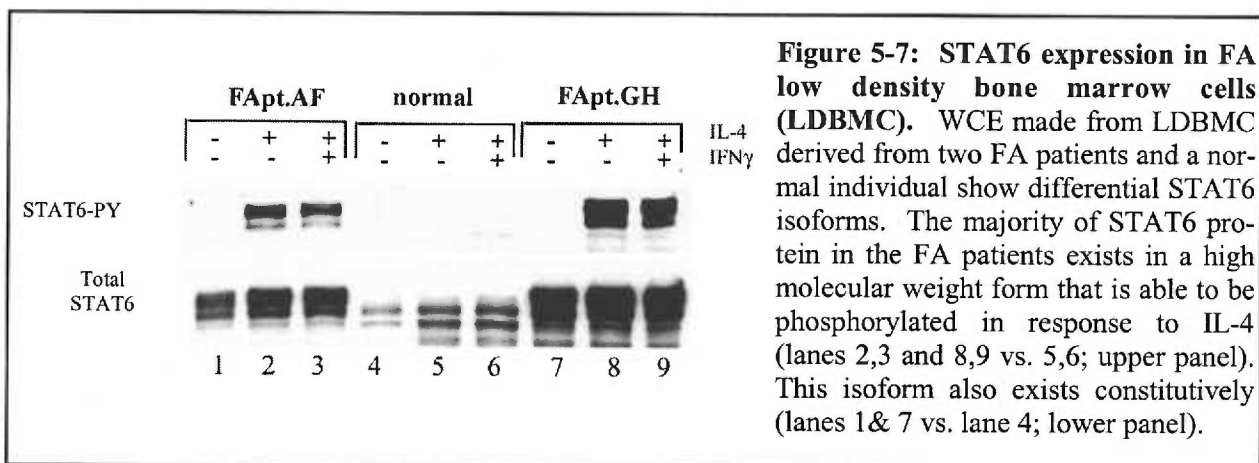
In order to determine if IL-4 inhibits IRF-1 expression in FA-C patient low density bone marrow cells, we performed real time quantitative RT-PCR on normal and FA-C patient derived LDBMC exposed to nothing, IFN γ alone, IL-4 alone and the combination of IL-4 and IFN γ . Clearly, IRF-1 mRNA is found at high levels in the FA-C LDBMC compared to normal



LDBMC at all time points. In normal LDBMC the combination of IL-4 and IFN γ found that addition of IL-4 slightly inhibited IFN γ -induced IRF-1 mRNA expression. However, the combination of IL-4 and IFN γ treatment on the FA-C LDBMC, not only did not inhibit IFN γ -induced IRF-1 mRNA expression, but, rather augmented IFN γ -induced IRF-1 mRNA expression in what appears to be a synergistic affect (Fig 5-6), indicating that FANCC mutant cells not only misinterpret inhibitory signals, but may appear to read them as stimulatory, contributing to high level expression of downstream genes.

FA-C patient derived LDBMC express a high molecular weight form of STAT6

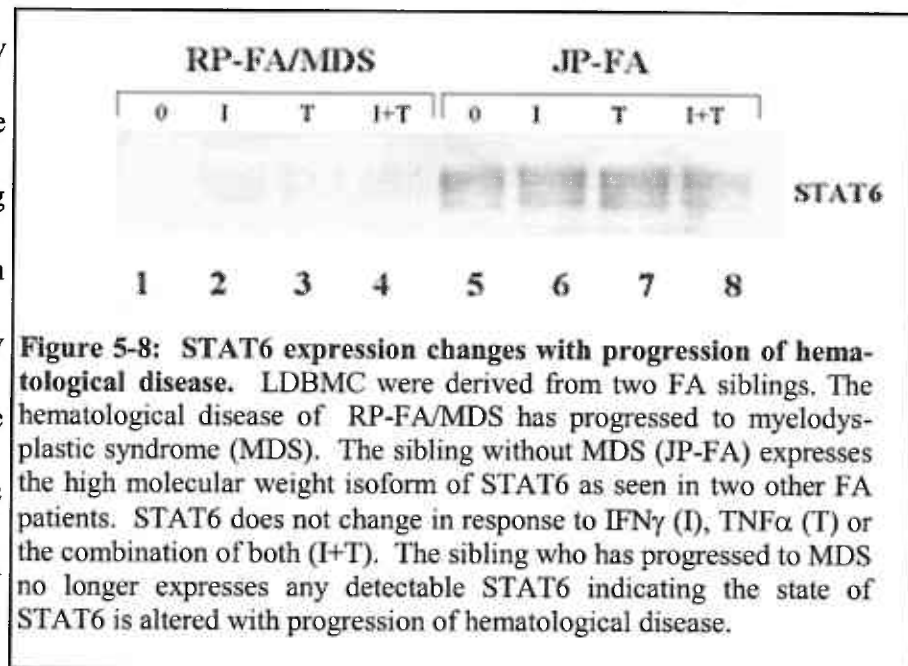
Given the abnormal response FA-C LDBMC have in response to IL-4, we next sought to determine if STAT6 activation levels were aberrant in LDBMC. Bone marrow aspirates were taken from two FA patients and a normal control. Aspirates were then run on a ficoll gradient and low density bone marrow cells were derived. LDBMC were treated for 15 minutes with IL-4, IFN γ or the combination. Whole cell extracts (WCE) were made and run on immunoblots. These blots were probed for both phosphorylated and total STAT6. Surprisingly, we find multiple STAT6 isoforms in both WT and FA-C LDBMC. However, the majority of STAT6 in FA-C LDBMC exists in a high molecular weight form that is absent in



WT LDMBC. Moreover, this aberrant STAT6 isoform can be phosphorylated in response to IL-4 (Fig 5-7).

Progression to MDS results in loss of the high molecular weight form of STAT6

To further analyze the expression of this high molecular weight STAT6 isoform in FA patients, we compared expression of STAT6 in two FA siblings, one of which has progressed to myelodysplastic syndrome (MDS), by immunoblot analysis. Although the FA sibling without MDS also expressed the high molecular weight STAT6, the sibling who had progressed to MDS did not express any detectable STAT6 (figure 5-8), suggesting modulations of STAT6 in bone marrow cells may change as the hematopoietic manifestations of FA progress [272].



5-4: Discussion

IFN γ induces FANCC to associate with STAT1 and is required for proper docking of STAT1 on the IFN γ receptor, therefore, FANCC mutant cells are defective in IFN γ -induced STAT1 signaling (Chapter 4). Paradoxically, however, FANCC mutant hematopoietic cells are hypersensitive to the mitogenic inhibitory effects of IFN γ , and express constitutively high levels of several IFN γ -inducible genes (effects abrogated by transduction of normal *FANCC* cDNA)

response, even in the face of defective STAT1 signaling? Because mature human fibroblasts do not have defective STAT1 phosphorylation and do not overexpress IFN γ -inducible genes, we reasoned that the overexpression of IRF-1, ISGF3 γ and p21^{WAF1} occurs only in FA-C cells defective in their ability to activate STAT1. The overexpression of these genes in FA-C hematopoietic cells appeared to require that (a) FANCC be inactivated and (b) the particular *FANCC* mutation suppresses STAT1 activation. One possible explanation for this was that loss of a hematopoietic STAT1-inducible modulator of IFN γ responses contributed to the upregulation of IRF-1, p21, ISGF3 γ and MxA.

One factor of interest to us was the transcriptional repressor, ICSBP, a member of the IRF family [273]. ICSBP interacts with both IRF-1 and IRF-2 *in vitro* and *in vivo* and inhibits the DNA binding activity of ISGF3 γ [274]. ICSBP binds to IRF-1 and suppresses some IFN γ -induced gene transcription [273]. Stable expression of the DNA binding domain of ICSBP blocks IRF-1 transactivation and also inhibits binding of STAT1 to GAS [275]. In FANCC mutant B-lymphoblasts we found less ICSBP expression than normal cells (Fig. 5-1). In fact, ICSBP is the only IFN γ -inducible protein of those we have tested that is reduced in FA-C cells. Surprisingly, ICSBP is also expressed in an inducible manner in MEFs and this expression is significantly reduced in *FancC* mutant MEFs. Low expression levels of ICSBP in FA-C cells may be a direct result of a deficiency in activated STAT1, given that binding to the GAS element from ICSBP is significantly reduced in FA-C cells. Because ICSBP is decreased in those cells with high level IFN γ -inducible gene expression, we next reasoned that loss of ICSBP contributes to the upregulation of these genes. However, enforced expression of ICSBP into these cells failed to downregulate IRF-1, ISGF3 γ and p21^{WAF1}. We conclude that in FA-C hematopoietic and embryonic cells expression of IFN γ -inducible genes is increased even in the

face of defective STAT1 phosphorylation but is not linked directly with reduced levels of ICSBP. Although reduced ICSBP levels is not directly linked to expression of IFN γ -inducible genes, it may still play a role in some aspect of the FA phenotype, especially given the hematopoietic cell specific expression of ICSBP.

In order to determine if loss of STAT1 contributed at all to high level constitutive expression of IFN γ -inducible genes, we analyzed mRNA levels in bone marrow cells derived from *Stat1*^{-/-} mice. Constitutive levels of IRF-1 and ISGF3 γ did not appear to be defective in a *Stat1* null background, indicating IFN γ hypersensitivity in FA-C cells occurs in a STAT1 independent manner. These data suggest that FANCC functions to indirectly suppress expression of IFN γ -inducible genes in a cell type specific STAT1-independent manner, possibly by modulating responses of a STAT1 independent IFN γ inhibitory pathway.

IFN γ and IL-4 have opposing biological influences that occurs, in part, through opposing influences on gene transcription [268]. IL-4 has been found to inhibit IFN γ -induced gene activation through activation of another STAT family member, STAT6 [265]. Given the antagonistic relationship of IL-4 and IFN γ , and that IL-4, like IFN γ , signals through STAT molecules (STAT1 for IFN γ and STAT6 for IL-4), we hypothesized that IFN γ -inducible genes may be upregulated in FA-C cells because inhibitory signals that ordinarily depend on IL-4 activation are defective. We first analyzed levels of activated STAT6 in FA-C cell lines and found that phosphorylation of STAT6 is defective in these cells. We next determined the effect of IL-4 on IFN γ -induced IRF-1 mRNA expression in low density bone marrow cells, and found that the combination of IL-4 and IFN γ not only failed to inhibit IFN γ -induced IRF-1 mRNA, but, rather, synergistically upregulated IRF-1 mRNA levels in FA-C patient LDBMC.

Moreover, immunoblot analysis of STAT6 in FA patient LDBMC demonstrated that a novel high molecular weight isoform exists specifically in FA patients, and this high molecular moiety is lost as bone marrow abnormalities progress to MDS.

STAT6 carries out signals induced by IL-4 and the similar cytokine IL-13 and has mainly been implicated in the differentiation of T helper type 2 (Th2) cells [276]. STAT6 can exist in different isoforms depending on the tissue or cell type. Two naturally occurring mutants of human STAT6 have been described and appear to arise by alternative splicing. The STAT6b isoform has a deletion at the amino terminus of full length STAT6 and STAT6c lacks a portion of the SH2 domain. Both of these isoforms can attenuate various IL-4 responses, and STAT6c behaves effectively as a dominant negative by reducing tyrosine phosphorylation of wild type STAT6 [277]. Recently another truncated form of STAT6 was found to be coexpressed in Mast cells with wild type STAT6. The origin of this form is unclear, but it does bind DNA and may also function as a dominant negative [278]. We find multiple STAT6 isoforms in LDBMC. However, the majority of STAT6 that exists in FA LDBMC appears to be a higher molecular weight protein. To our knowledge, no such high molecular weight STAT6 has been described before. Currently, it is unclear what role this high molecular weight isoform is playing in FA cells. This form is clearly able to be phosphorylated (Fig 5-7) and may bind DNA possibly acting as a dominant negative to normal STAT6. More studies are warranted to understand the precise nature this high molecular weight STAT6 plays in the pathogenesis of FA.

IL-4 induced STAT6 responses are defective in FA-C cells and this likely contributes to the high level expression of IFN γ -inducible genes. However, it would be naïve to presume that IL-4 induced STAT6 is the sole cause of IFN γ hypersensitivity in FA-C cells. It is more likely

that the issue of IFN γ hypersensitivity in FA-C hematopoietic cells is a complex one, possibly involving an independent hyperactive pathway combined with loss of an inhibitory pathway. Moreover, evidence is mounting that the effects IFN γ hypersensitivity in FA-C cells are exacerbated when IFN γ acts in collaboration with other apoptotic factors [17,19,20,211].

Dr. Qishen Pang has found that the double stranded RNA (dsRNA) dependent protein kinase, PKR, is aberrantly activated in FA-C cells. PKR is a well recognized mediator of apoptosis optimally activated in normal cells by a variety of stimuli including the combinations of dsRNA and IFN γ or IFN γ and TNF α [20,279]. PKR functions to induce apoptotic responses, in part, by inducing phosphorylation of translation initiation factor eIF2 α , a phenomenon that results in translational inhibition and subsequent apoptosis. Dr. Pang recently demonstrated that PKR and eIF-2 α are excessively activated in FA-C cells. Moreover, inhibition of PKR function in FA-C cells by introduction of a dominant negative PKR significantly reduced IFN γ hypersensitivity, suggesting PKR is an essential mediator of IFN γ -induced hypersensitivity in FA-C cells [20]. Therefore, FA-C cells abnormally activate a signaling pathway known to induce apoptosis. Moreover, studies presented here demonstrate that these cells do not have the ability to induce certain inhibitory pathways appropriately. It is likely that hypersensitivity to IFN γ and overexpression of IFN γ -inducible genes in FA-C cells is complex and involves both loss of inhibitory signals, such as IL-4-induced inhibition, combined with hyperactive apoptotic signaling, such as PKR, and, together, defects in these pathways synergistically contribute to the constitutive apoptotic phenotype of FA-C cells.

Chapter 6

FANCC Is Required for Optimal Activation of TYK2 and Multiple STAT Proteins in Response to Type I Interferons and Suppresses Expression of SOCS1

6-1: Overview

The FA protein, FANCC, interacts with Signal Transducer and Activator of Transcription 1 (STAT1) following stimulation with Interferon- γ (IFN γ) and is required for proper docking of STAT1 at the IFN γ receptor alpha chain (IFN γ R α , IFN γ R1). Thus, loss of a functional FANCC results in decreased activation of STAT1 following IFN γ stimulation [213] (Chapter 4). In addition, IL-4-induced activation of STAT6 is also defective in *FANCC* mutant (FA-C) cells (Chapter 5). Because of the intimate involvement of STAT proteins in growth and differentiation of hematopoietic cells and the role of STAT proteins in these responses, we investigated whether activation of other STAT proteins is compromised in FA-C cells. Recently it was recognized that cross talk exists between type I and type II Interferon (IFN) receptors [174], therefore, we analyzed responses of FA-C cells to type I IFNs. Using phospho-specific antibodies or immunoprecipitation we find that activation of STAT 1, 3 and 5 in response to type I IFNs is diminished in FA-C cells. Furthermore, unlike JAKs 1 and 2 in response to IFN γ , phosphorylation of the Janus Kinase, TYK2, is defective in response to IFN α and IFN β . In addition, the known TYK2 inhibitor, SOCS1, is expressed at abnormally high levels, indicating a possible mechanism for decreased TYK2 activation.

6-2: Introduction

Members of the STAT family of latent transcription factors are used by multiple cytokines and growth factors to transmit extracellular signals from the receptor (via Janus Kinases) to the nucleus. The majority of hematopoietic cytokines and growth factors utilize this signaling pathway [280]. Studies in our lab have demonstrated that a functional FANCC is required for proper signaling of STAT proteins in response to IFN γ (Chapter 4). Preliminary

studies indicated that the defect might be isolated to STAT1 because FANCC bound to STAT1 in response to multiple factors but did not bind to STAT5 or STAT3 [213]. However, more recent studies found that phosphorylation of STAT6 is compromised in response to IL-4 in FA-C cells (chapter 5), suggesting that defects in STAT signaling in FA-C cells is not isolated to STAT1 and interferon gamma ($\text{IFN}\gamma$) and may represent a more global defect in STAT activation.

Signaling components of the structurally unrelated type I and type II IFN pathways communicate with one another. Cells derived from $\text{IFN}\alpha$ receptor 1 ($\text{IFN}\alpha\text{R1}$) null mice ($\text{IFN}\alpha\text{R1}^{-/-}$) have impaired $\text{IFN}\gamma$ -induced responses [174]. Type I IFNs include members of the $\text{IFN}\alpha$ family ($\text{IFN-}\alpha_1, -\alpha_2, -\omega, -\tau$) and $\text{IFN}\beta$. Type II IFN includes only $\text{IFN}\gamma$. $\text{IFN}\gamma$ binding to its receptor results in activation of JAK1 and JAK2 which subsequently activate STAT1 by phosphorylation on tyrosine residues. STAT1 then forms a homodimer, translocates to the nucleus and binds the gamma activation sequence or GAS element [117]. Type I IFNs utilize JAK1 and TYK2, which in turn activate STAT1 and STAT2. STAT1 and STAT2 then complex with ISGF3 γ (p48, IRF-9) to form the ISFG3 complex. ISGF3 translocates to the cell nucleus and binds the Interferon Stimulated Response Element (ISRE) [259,281,282]. In addition Type I IFNs have also been shown to activate STAT3, STAT5 and in some cell types STAT4, however, the downstream affects of these proteins in response to type I IFNs is less well characterized [246,283,284].

Given the cross-talk that exists between type I and type II IFNs and that FA-C cells are defective in $\text{IFN}\gamma$ signaling through STAT1, we analyzed components of the $\text{IFN}\alpha/\beta$ signaling pathway. We find phosphorylation of STAT1, STAT3 and STAT5 is reduced in response to type I IFNs in FA-C cells. However, these STAT phosphorylation defects are specific to

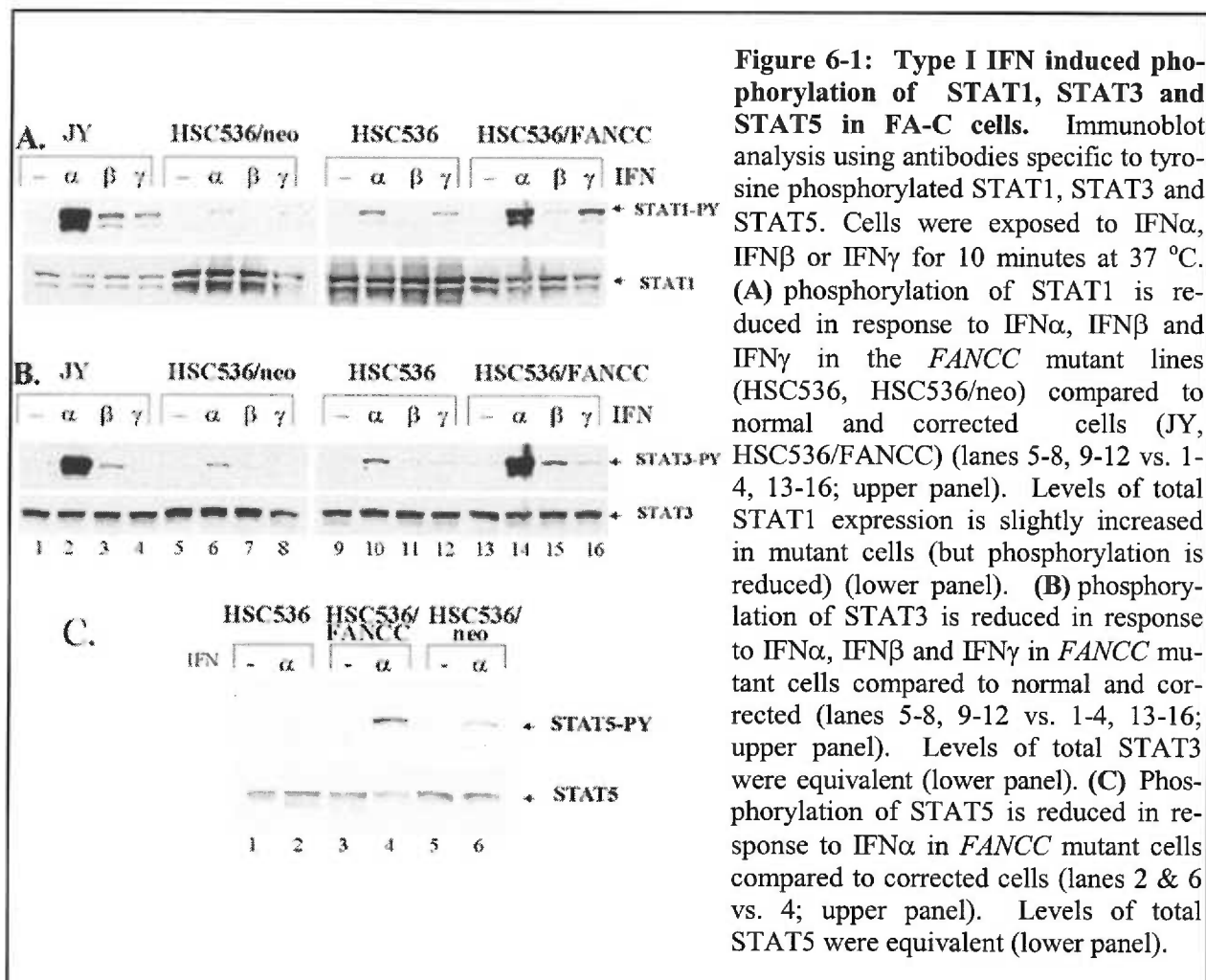
certain cytokine signaling pathways because phosphorylation of STAT3 occurred normally in FA-C cells in response to leukemia inhibitory factor (LIF) or Interleukin-6 (IL-6). Further analysis of the IFN α/β signaling pathway revealed, for the first time, a signaling defect upstream of STAT signaling. Phosphorylation of the janus kinase, TYK2, is significantly reduced in FA-C cells in response to type I IFNs. Interestingly, similar to FA-C cells, cells derived from *Tyk2* null (*Tyk2*^{-/-}) mice have reduced, but not absent, phosphorylation of specific STAT proteins in response to both type I and type II Interferons [128]. Taken together these data determine that subnormal STAT signaling in FA-C cells is more widespread than previously thought, however, this defect is not a global defect and appears to be specific to certain cytokine signaling pathways. Moreover, diminished STAT activation correlates with decreased levels of the upstream Janus Kinase, Tyk2, and may be a direct result of a TYK2 deficiency in FA-C cells.

One potential mechanism for diminished TYK2 activation in FA-C cells is loss of an inhibitor of Tyk2 activation such as SOCS1. Suppressor of cytokine signaling-1 (SOCS1) (JAB/SSI-1) is a member of a family of proteins containing a central SH2 domain and a C-terminal 40 residue domain termed a SOCS box [159]. SOCS proteins were cloned by their ability to inhibit cytokine signaling through the JAK/STAT pathway [156,285]. SOCS1 has been found to inhibit activation of multiple JAKs including TYK2 [162,285,286]. To test this relationship between FANCC and SOCS1, we quantified levels of SOCS1 in FA-C cells and found high constitutive SOCS1 expression. Therefore, high SOCS1 expression may be an underlying mechanism of decreased TYK2 activation in FA-C cells.

6-2: Results

Phosphorylation of STAT1, STAT3 and STAT5 is diminished in response to type I IFN

FANCC is required for proper IFN γ -induced STAT1 and IL-4 induced STAT6 phosphorylation (Chapter 4 and Chapter 5). Given that crosstalk occurs between type I (IFN α/β) and type II (IFN γ) IFNs, specifically, type I IFN signaling influences IFN γ induction of STAT1 [174], we analyzed levels of phosphorylated STAT proteins in response to type I IFNs. Cells were treated with 1000 U/ml IFN α , 1000U/ml IFN β or 1 ng/ml IFN γ for 15 minutes. Whole cell extracts (WCE) were made and immunoblot analysis was performed. Blots were probed with antibodies specific for the tyrosine phosphorylated forms of STAT1, STAT3 and STAT5. Phosphorylation of STAT1 is decreased in response to IFN α , IFN β and



IFN γ in FANCC mutant B-lymphoblasts, HSC536N and HSC536N/neo compared to isogenic cells corrected for the defect by retroviral transduction of the *FANCC* cDNA, HSC536N/FANCC and normal B-lymphoblasts, JY. Levels of total STAT1 are slightly increased in HSC536N and HSC536N/neo compared to corrected and normal cells (but phosphorylation levels are reduced) (Fig 6-1A). Phosphorylation of STAT3 was also decreased in response to IFN α , IFN β and IFN γ in the FA-C cells, HSC536N and HSC536N/neo, compared to wild type (WT) and corrected cells, JY and HSC536N/FANCC. Levels of total cellular STAT3 were equivalent between mutant and corrected cells (Fig 6-1B). Phosphorylation of STAT5 in response to IFN α was also reduced in mutant cells, HSC536N and HSC536N/neo, compared to the corrected cell line, HSC536N/FANCC. No differences in total cellular STAT5 were detected (Fig 6-1C). Thus, phosphorylation of multiple STATs is defective in FA-C cells.

FA-C cells have low-level constitutive STAT2 phosphorylation

EBV-transformed B-lymphoblasts were treated with 1000 U/ml IFN α for 15 minutes. Subsequently whole cell extracts were made and cells were immunoprecipitated with an antibody specific for phosphorylated tyrosines. These extracts were run on SDS-PAGE, electoblotted and probed with an antibody specific for STAT2. These studies consistently

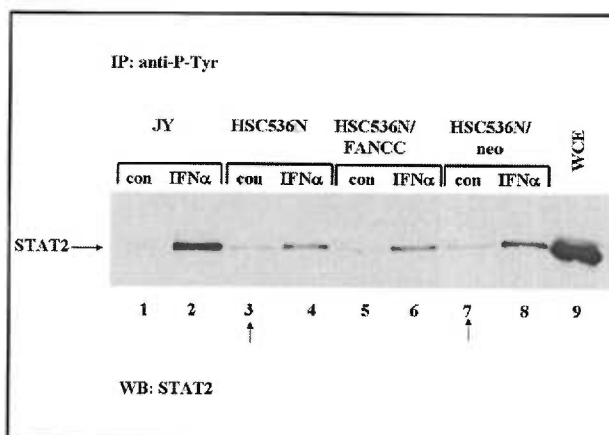


Figure 6-2: Phosphorylation of STAT2 in FA-C cells. WCE were immunoprecipitated (IP) with an anti-phospho-tyrosine antibody and immunoblotted using a STAT2 specific antibody. These studies consistently revealed low level constitutive phosphorylated STAT2 in response to IFN α in FA-C cells (lanes 3 & 7 vs. 1 & 5). Phosphorylation of STAT2 was clearly inducible in both mutant and normal cells and no consistent differential inducible phosphorylated STAT2 could be detected between mutant and normal cells (lanes 4 & 6 vs. lane 2 & 9).

revealed no differential IFN α -induced phosphorylation between the FANCC mutant cells HSC536N and the corrected cell HSC536N/FANCC (Fig 6.2, lanes 2 and 6). However, we consistently found low level constitutive activation of STAT2 in mutant cells (fig 6-2 lanes 1 and 5) compared to normal cells (fig 6-2, lane 3). Therefore, STAT2 is constitutively active in FA-C cells at low levels. However, this does not result in abnormal amounts of inducible activated STAT2 in FA-C cells.

STAT3 phosphorylation occurs normally in response to LIF and IL-6

Signaling of multiple STAT molecules is decreased in FA-C cells in response to multiple extracellular signals including type I IFNs, IFN γ and Interleukin 4. We therefore questioned whether decreased STAT activation is a global effect in FA-C cells. We analyzed phosphorylation of STAT3 in response to other cytokines including Interleukin-6 (IL-6) and Leukemia Inhibitory Factor (LIF) by immunoblot analysis using an antibody specific for the phosphorylated form of STAT4. However we find no differential phosphorylation of STAT3 between the FA-C cells, HSC536N and HSC536N/neo, and the corrected isogenic cell line,

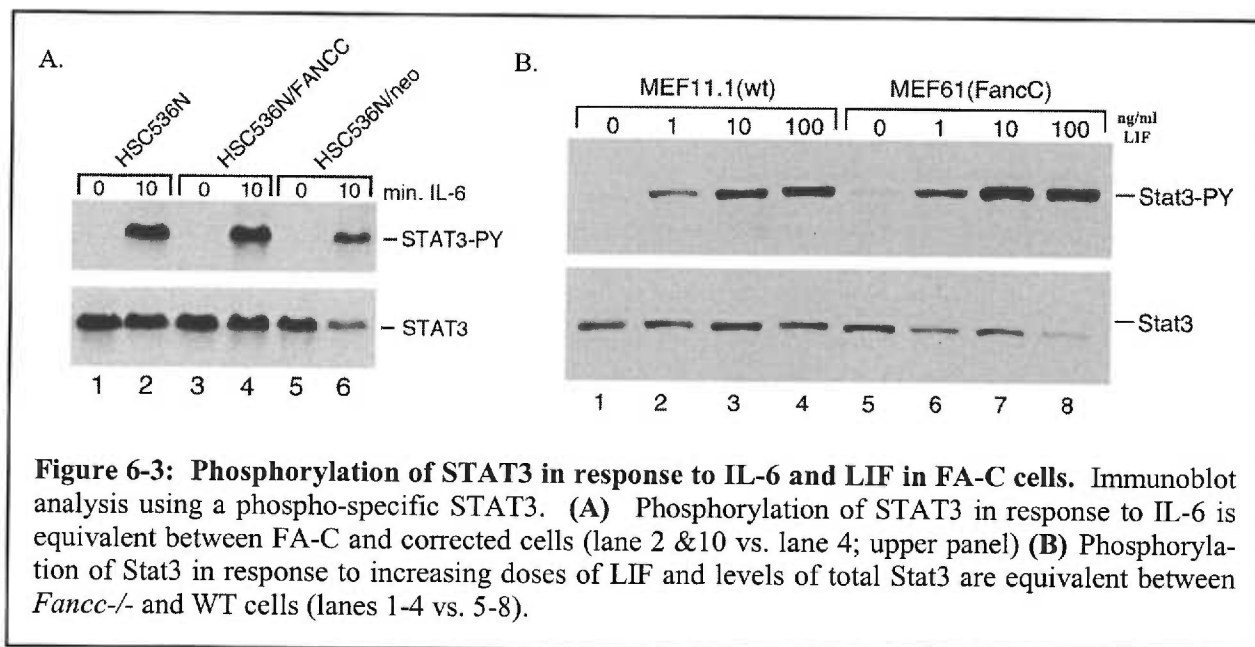
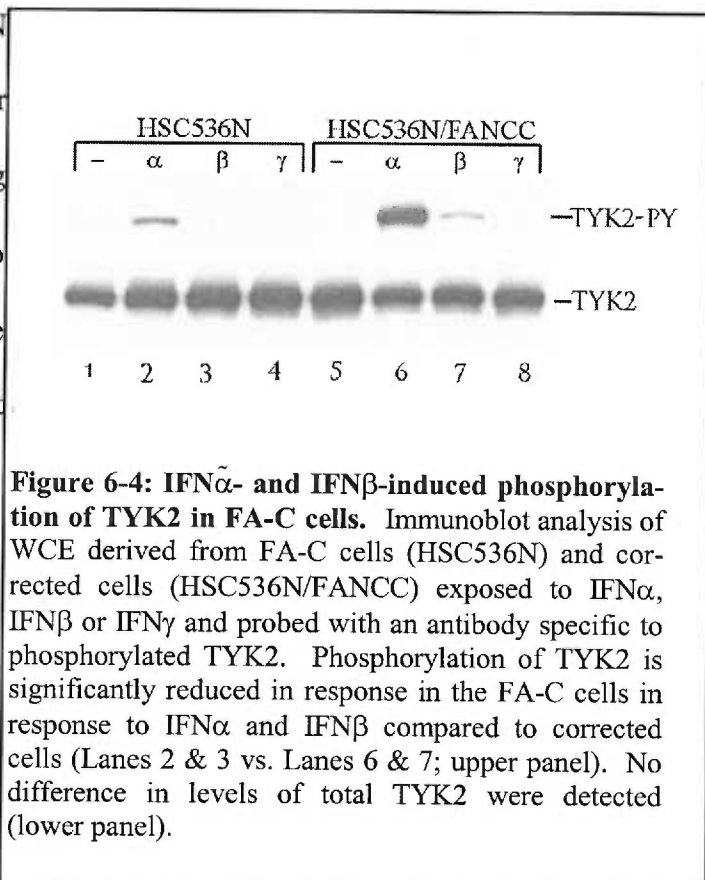


Figure 6-3: Phosphorylation of STAT3 in response to IL-6 and LIF in FA-C cells. Immunoblot analysis using a phospho-specific STAT3. (A) Phosphorylation of STAT3 in response to IL-6 is equivalent between FA-C and corrected cells (lane 2 & 10 vs. lane 4; upper panel) (B) Phosphorylation of Stat3 in response to increasing doses of LIF and levels of total Stat3 are equivalent between *Fancc*^{-/-} and WT cells (lanes 1-4 vs. 5-8).

HSC536N/FANCC, in response to IL-6 (Fig 6-3A). Moreover, phosphorylation of LIF-induced STAT3 occurred normally in an SV40 transformed murine embryonic cell line (MEF61) derived from a *Fancc* null (*Fancc*^{-/-}) mouse compared to a transformed cell line (MEF11.1) derived from a wild type (WT) mouse (Fig 6.3B). These studies indicate that abnormal STAT phosphorylation in FA-C cells is not a global defect and is specific to certain extracellular signals.

Phosphorylation of Tyk2 is decreased in FA-C cells

Since diminished STAT phosphorylation in FA-C cells is specific to certain extracellular signals including type I IFN signaling, we next examined other components of the the IFN α / β signaling pathway. IFN α / β carries its signals to STAT molecules, in part, through the Janus Kinase, TYK2. Using immunoblot analysis and an antibody specific for tyrosine phosphorylated TYK2, we determined the phosphorylation state of TYK2 in FA-C cells. Surprisingly, unlike JAK1 and JAK2 in response to IFN γ , phosphorylation of TYK2 in



response to IFN α and IFN β is consistently reduced in FA-C mutant B lymphoblasts (HSC536N) compared to normal cells (HSC536N/FANCC) (Fig 6-4, upper panel). Levels of

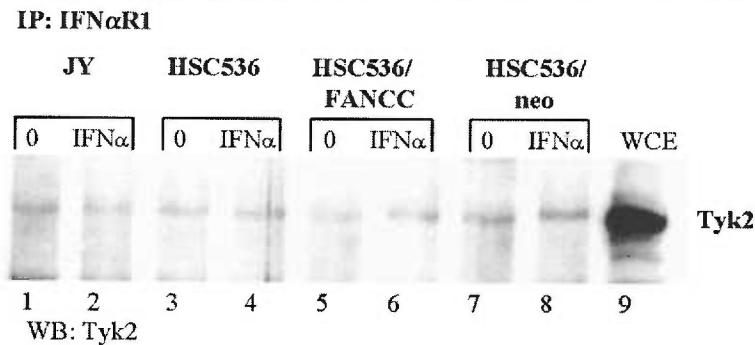


Figure 6-5: Association of Tyk2 with IFN α R1 in FA-C cells. WCE were immunoprecipitated with an antibody specific for IFN α R1. Immunoprecipitates were run on SDS-PAGE and immunoblotted with an antibody specific for Tyk2. Tyk2 and IFN α R1 associate constitutively and no difference was detected between normal and FA-C cells.

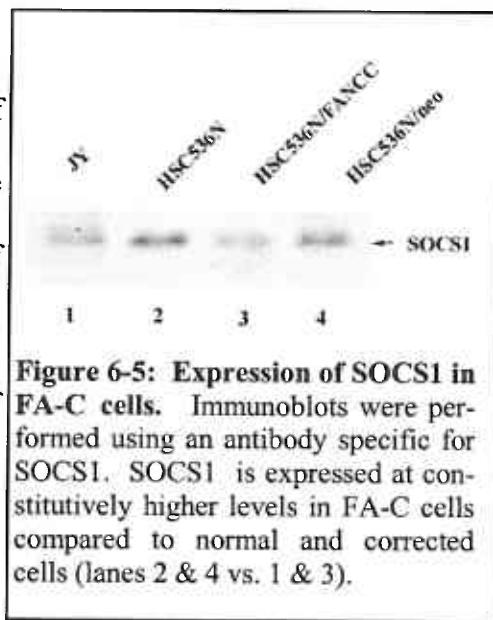
total cellular TYK2 were equivalent between mutant and corrected cells (Fig 6-4, lower panel). These studies for the first time reveal a defect in a JAK/STAT pathway upstream of STAT proteins.

Tyk2 binds normally to the IFN alpha receptor

To further investigate potential reasons for reduced TYK2 phosphorylation in response to type I IFNs we analyzed whether Tyk2 associated properly with the Interferon alpha receptor 1 chain (IFN α R1). Cells were treated with nothing or 1000U/ml IFN α for 10 minutes. Whole cell extracts were made and used for immunoprecipitation with an antibody specific for IFN α R1. Immunoprecipitates were then run on SDS PAGE transferred to nitrocellulose and probed using an antibody specific for TYK2. TYK2 associates with IFN α R1 constitutively, an association that is not affected by IFN α treatment in all cells tested including the FANCC mutant B-lymphoblasts, HSC536N and HSC536N/neo (Fig 6-5). These studies suggest the defect in TYK2 activation is not due to altered binding of TYK2 to the intracellular receptor.

FA-C cells overexpress SOCS-1

Seeking to identify potential molecular points of control for decreased TYK2 phosphorylation, we examined levels of SOCS-1, a known inhibitor of TYK2 [162,285]. Using immunoblot analysis and an antibody specific for SOCS-1, we found that levels of SOCS-1 are significantly elevated in the FA-C cell line, HSC536N, compared to cells corrected for the defect, HSC536N/FANCC (Fig 6-6). Given that SOCS1 is a



known inhibitor of TYK2 [285], SOCS-1 is a potential upstream modulator of defective TYK2 and STAT phosphorylation in FA-C cells.

6-5: Discussion

In the 1950's Issacs and Lindenmann discovered that incubation of chick embryo membranes with inactive influenza virus and subsequent incubation of that same membrane with live virus resulted in interference of growth with the live virus [287]. It was surmised that the membrane produced this inhibitory activity and the interfering agent was called Interferon. Subsequently several IFNs have been identified and are divided into type I and type II categories based on their structure and receptor specificity. Type I IFNs include members of the IFN α family (IFN- α_1 , - α_2 , - ω , - τ) and IFN β and type II consists of only IFN γ . IFNs are involved in a broad range of mammalian functions including antiviral responses, inflammation, hematopoiesis, cell proliferation and differentiation [284,288]. Until recently it was unclear why, despite being structurally unrelated and signaling through different receptors, type I and

type II IFNs share many overlapping biological functions [289]. Reasons for this have become more evident recently in light of findings that type I and type II IFNs affect signaling of one another [173,174].

FANCC associates with STAT1 following IFN γ stimulation and facilitates docking of STAT1 on IFN γ R1 [213]. Therefore, loss of a functional FANCC results in absence of STAT1 docking and subsequent decreased phosphorylation of STAT1 (Chapter 5). Given that proper IFN γ signaling requires an intact type I IFN receptor [174], we dissected signaling of type I IFNs in FA-C cells. Phosphorylation of STAT1, STAT3 and STAT5 is significantly reduced in response to IFN α and IFN β (Figure 6-1). Therefore, in FA-C cells activation of multiple STAT proteins is compromised in response to multiple cytokines including STAT1 and STAT3 in response to IFN α , IFN β and IFN γ , STAT5 in response to IFN α (Figure 6-1) and STAT6 in response to Interleukin 4 (Chapter 6). However, defective STAT phosphorylation does not appear to be a global defect because, although STAT3 phosphorylation is decreased in FA-C cells in response to IFN α , IFN β and IFN γ , phosphorylation of STAT3 occurs normally in response to LIF and IL-6. Thus, diminished phosphorylation of STAT proteins is specific to certain extracellular signals. Further analysis is warranted to determine specifically what other STAT signals are defective in FA-C cells and what factors are not. Especially important are those factors known to affect growth and differentiation of early progenitor cells, in particular Thrombopoietin (Tpo), Erythropoietin (Epo) Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Stem Cell Factor (SCF) and Interleukin 3 (IL-3). However, analysis of these pathways will be difficult to assess in FA-C cells, given the limited availability of hematopoietic progenitor cells. There is, however, preliminary evidence indicating that at least some of these early hematopoietic signaling pathways will be defective in FA-C cells. Using

the factor dependent leukemia cell line, MO7e, Dr. Pang has demonstrated that GST fusion proteins encoding a WT FANCC bound STAT1 in response to IFN α , GM-CSF and SCF while a FANCC naturally occurring mutant either bound STAT1 at extremely low levels or not at all. Moreover, *Fancc*^{-/-} bone marrow is hyporesponsive to SCF-induced granulocyte-macrophage colony formation (CFU-GM) compared to *Fancc*^{+/-} bone marrow, further implicating SCF signaling defects in FA-C hematopoietic progenitor cells. WT FANCC did not bind STAT5 in response to GM-CSF or STAT3 in response to Epo [213]. However, binding of FANCC to STAT molecules may not be the optimal readout for defective phosphorylation since FANCC has only been determined to bind to STAT1, and it is entirely possible defects in other STAT proteins are not the result of direct binding. In order to decipher the mechanism by which FANCC functions to affect phosphorylation of multiple (but not all) STATs, the defective pieces of each pathway will need to be more thoroughly studied.

In order to more completely understand what defects of type I IFN signaling lead to diminished activation of multiple STAT proteins in FA-C cells, we asked whether other components of this pathway are defective. Unlike JAK1 and JAK2 in response to IFN γ , phosphorylation of the Janus Kinase, TYK2, is reduced in FA-C cells in response to IFN α and β (Figure 6-4). TYK2 is predominately involved in signaling of IL-12 and IFN α/β . Similar to FA-C cells, murine embryonic fibroblasts derived from *Tyk2* knockout (*Tyk2*^{-/-}) mice show reduced, but not absent, phosphorylation of STAT proteins to not only IL-12 and IFN α/β , but also to IFN γ . Moreover, despite *in vitro* evidence implicating TYK2 in IL-6 and LIF signaling, like FA-C cells, IL-6 and LIF induced phosphorylation of STAT3 occurred normally in *Tyk2*^{-/-} cells [128]. Therefore, specific FA-C STAT activation defects correlate precisely with defective TYK2 phosphorylation in FA-C cells.

In order to more precisely understand the nature of TYK2 activation defects in FA-C cells, we analyzed binding of TYK2 to IFN α R1 and found Binding of TYK2 to IFN α R1 occurred normally in FA-C cells (Figure 6-5). Given that expression levels of TYK2 are equivalent in FA-C cells and TYK2 associates normally with IFN α R1, defective TYK2 activation is likely due to either (1) a defective TYK2 activating Tyrosine kinase or (2) an overactive TYK2 inhibitor or phosphatase. SOCS1 (JAB/SSI-1) is a member of a family of proteins induced by cytokine stimulation, subsequently these proteins negatively regulate cytokine signaling forming a classical negative feedback loop [156-158]. SOCS1 is an important regulator of IFN γ as determined by generation of SOCS1 null mice (*SOCS1*^{-/-}) [164-167]. SOCS1 regulates T cell differentiation and prevents the emergence of a population of T cells that secrete high levels of IFN γ [167]. In addition to IFN γ signaling, SOCS1 has been shown to negatively regulate IL-4 and IFN α/β signaling [290]. Moreover, SOCS1 interacts with and inhibits phosphorylation of TYK2 [158,285]. Therefore, we analyzed SOCS-1 expression in FA-C and found FA-C cells express significantly higher levels of SOCS-1 compared to normal cells (Fig 6-6), indicating that reduced phosphorylation of TYK2 may be due, at least in part, to high levels of the TYK2 inhibitor, SOCS1.

How SOCS1 is upregulated in FA-C cells is unclear at this time. One possible mechanism may be via STAT2. As indicated in Figure 6-2, FA-C cells express low-level activated STAT2 in the absence of any extracellular treatment. STAT2 is unique among STAT family members in its size, sequence divergence between mouse and human and the fact that it is the only STAT member who does not form homodimers, but instead associates with STAT1 and ISGF3 γ to form the ISGF3 complex [151]. STAT1 β can form ISGF3 and this complex can induce transcription in response to IFN α , despite the fact that STAT1 β homodimers have not

been found to activate transcription [291]. FA-C cells express low level constitutively active STAT1 β and high constitutive levels of ISGF3 γ , therefore it is possible that a constitutively active ISGF3 complex containing STAT1 β exists in FA-C cells and activates transcription of SOCS1. Another possible effect of constitutively active STAT2 may be in activation of PKR. However, no literature exists on PKR and STAT2, despite a physical association of PKR and STAT1 [292]. More studies are needed to determine the precise affects of constitutively active STAT2.

The biological effects of low TYK2 activation and high SOCS1 expression in FA-C cells are unclear at this time. Murine models have found important roles for TYK2 and SOCS1 in T cell development [127,167,293,294]. Although FA patients reveal no gross immunoligical defects, there is some evidence indicating subtle immune system defects [36,39,46,47,295], and more detailed analysis of the FA lymphoid system is warranted.

Chapter 7

Secondary Defects of Myelopoiesis in *Fancc*^{-/-} Mice Result from Defective Differentiation of Specific Regulatory Lymphocyte Subsets

7-1: Overview

FA-C cells do not properly activate TYK2 in response to type I Interferon (IFN) and overexpress an inhibitor of TYK2 phosphorylation, Suppressor of Cytokine Signaling 1 (SOCS-1). In addition, multiple STAT molecules are defective in FA-C cells correlating with loss of TYK2. Evidence from murine knockout models demonstrates important roles for STAT proteins, TYK2 and SOCS1 in T-Cell development, in particular in differentiation of T helper (Th) cells into Th1 or Th2 subsets [127,128,167,293,296]. Moreover, these T helper subsets secrete cytokines that are important for survival and differentiation of myeloid progenitor cells. Therefore, we analyzed T helper differentiation in *Fancc* deficient cells and find that FA-C cells are impaired in their ability to differentiate into Th1 subpopulations. We hypothesized that differences in FA-C lymphocyte subpopulations may differentially affect normal myeloid progenitor activity compared to normal lymphocyte populations. Indeed, *Fancc* null lymphocytes aberrantly affected normal myeloid progenitor cell activity. These data indicate that specific defects of cellular signaling in FA cells may differentially affect auxiliary populations, and, through the actions of these cells, profoundly affect myeloid progenitor cell growth, survival and differentiation.

7-2: Introduction

Mice nullizygous for *Tyk2* (*Tyk2*^{-/-}) show no overt developmental abnormalities. However, cells derived from these mice have reduced (but not absent) responses to Interferon (IFN) α , IFN β , IFN γ and IL-12, as demonstrated by reduced phosphorylation of Stat1, Stat3 and Stat4 in response to these cytokines. Responses to other cytokines thought to utilize Tyk2 signaling by *in vitro* studies, including Interleukin 6 (IL-6), Leukemia Inhibitory Factor (LIF)

and IL-10 were unaffected in *Tyk2*^{-/-} mouse cells. *Tyk2*^{-/-} mice have multiple subtle immune defects including; the failure of *Tyk2*^{-/-} macrophages to produce nitric oxide following LPS induction (whereas LPS-induced TNF α production is unimpaired), the inability of splenic T-helper (Th) cells from *Tyk2* deficient mice to differentiate into IFN γ -producing-Th1 subsets and an increased susceptibility to specific viral infections [127,128].

T-helper lymphocytes are CD4⁺ effector cells, and can differentiate from naïve T helper cells into two distinct subsets which are defined by both their function and the cytokines they secrete. T helper 1 (Th1) cells are involved in cell-mediated immunity. The “hallmark” cytokine secreted by Th1 cells is IFN γ . The main Th2-secreted cytokine is IL-4, but Th2 cells also secrete IL-5, IL-9, IL-10 and IL-13 [297].

Cells lacking functional FANCC do not properly activate TYK2. Moreover, these cells do not properly activate STAT1, STAT3, STAT5 and STAT6 in response to specific cytokines and overexpress Suppressor of Cytokine Signaling 1 (SOCS1, JAB, SSI-1), a known inhibitor of TYK2 phosphorylation. TYK2 along with certain STAT proteins and SOCS1 are required for proper differentiation of naïve CD4⁺ cells into Th subsets. Therefore, we hypothesized that specific signaling defects in FA-C cells may lead to a relative Th deficiency. To test this we removed splenocytes from mice nullizygous (*Fancc*^{-/-}) or heterozygous (*Fancc*^{+/-}) at the *Fancc* locus, and placed them under conditions conducive for either Th1 or Th2 differentiation. We find that *Fancc*^{-/-} splenocytes are impaired in their ability to properly differentiate in Th1 cells.

Recent studies suggest Th1 cells affect myeloid progenitor cell activity and that loss of Th1 results in reduced colony forming capacity of committed progenitors [177]. Given that the majority of hematological manifestations of Fanconi Anemia (FA) occur in myeloid cell types,

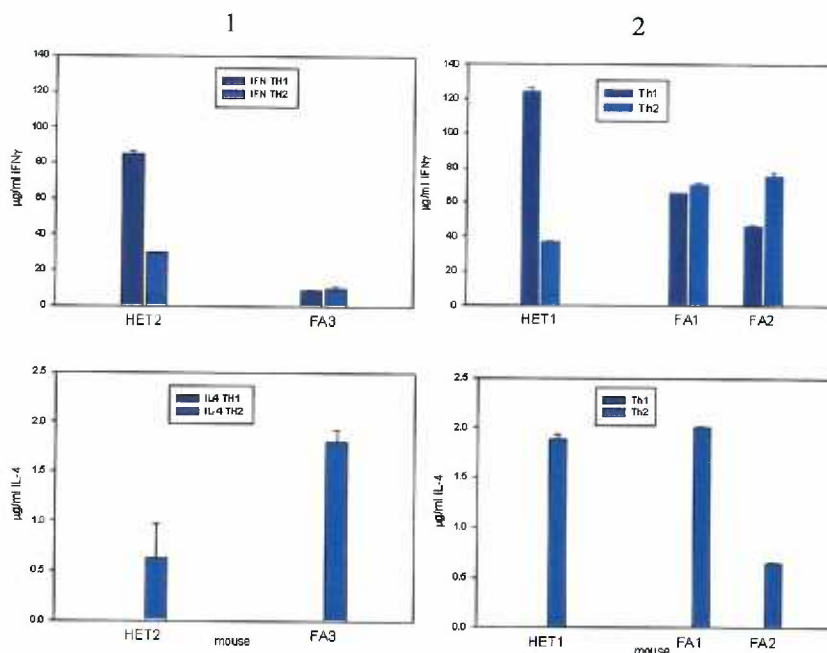
we sought to determine if *Fancc*^{-/-} lymphocytes abnormally affect growth of normal myeloid progenitor cells. We find that *Fancc*^{-/-} lymphocytes profoundly suppress granulopoiesis and erythropoiesis compared to *Fancc*^{+/-}. These studies suggest that specific cellular signaling defects in FA-C cells leads to abnormal growth and differentiation of specific lymphocyte populations which in turn has negative affects on myeloid progenitor cells, thus, contributing to bone marrow failure in FA.

7-3: Results

Fancc^{-/-} T helper cells fail to differentiate properly into the Th1 subpopulation

TYK2, STAT1, STAT4, STAT6 and SOCS-1 all have important roles in differentiation and maintenance of T helper subsets [127,128,210,293]. Therefore, we analyzed whether T lymphocytes from *Fancc*^{-/-} mice differentiate properly into T helper subsets. WT, *Fancc*^{+/-} and *Fancc*^{-/-} mice were euthanized by cervical dislocation and spleens were removed and a single cell suspension was made. Any animal displaying any signs of infection or an abnormal spleen size was removed from the study. Splenocytes were activated with murine plate-bound anti-CD3 and placed under conditions conducive for Th1 or Th2 differentiation (See Chapter 3). Cells were then washed and incubated with plate bound anti-CD3 in the absence of any additional cytokines for 20 hours prior to harvest. Supernatants were removed from cells and used for ELISA. IFN γ secreting cells were analyzed using the IFN γ secretion assay and flow cytometry. These studies consistently revealed reduced numbers CD4⁺ IFN γ producing cells in splenocytes derived from the *Fancc*^{-/-} cells and placed under Th1 conducive conditions. While 96% of the CD4⁺ *Fancc*^{+/-} splenocytes become IFN γ producing when placed under Th1 conditions only 22% of the *Fancc*^{-/-} CD4⁺ splenocytes become IFN γ -producing cells (Figure 7-1A). Analysis of IFN γ production in supernatants by ELISA consistently shows that *Fancc*^{-/-}

A.



B.

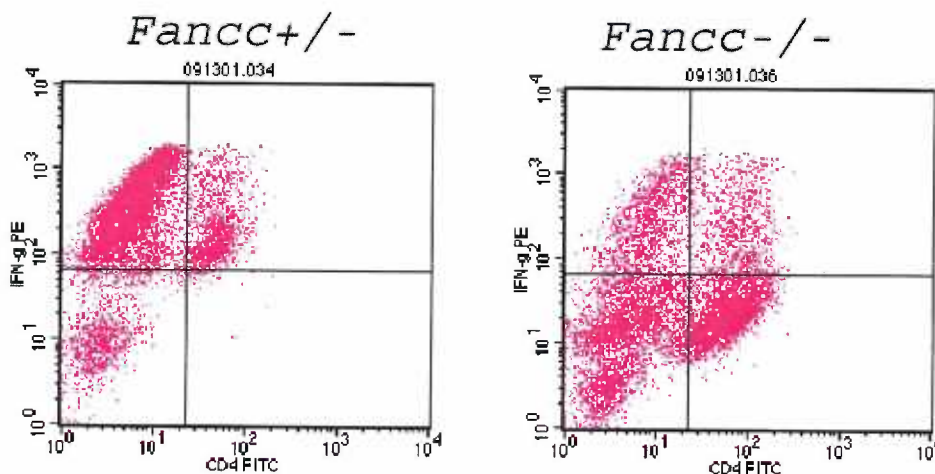
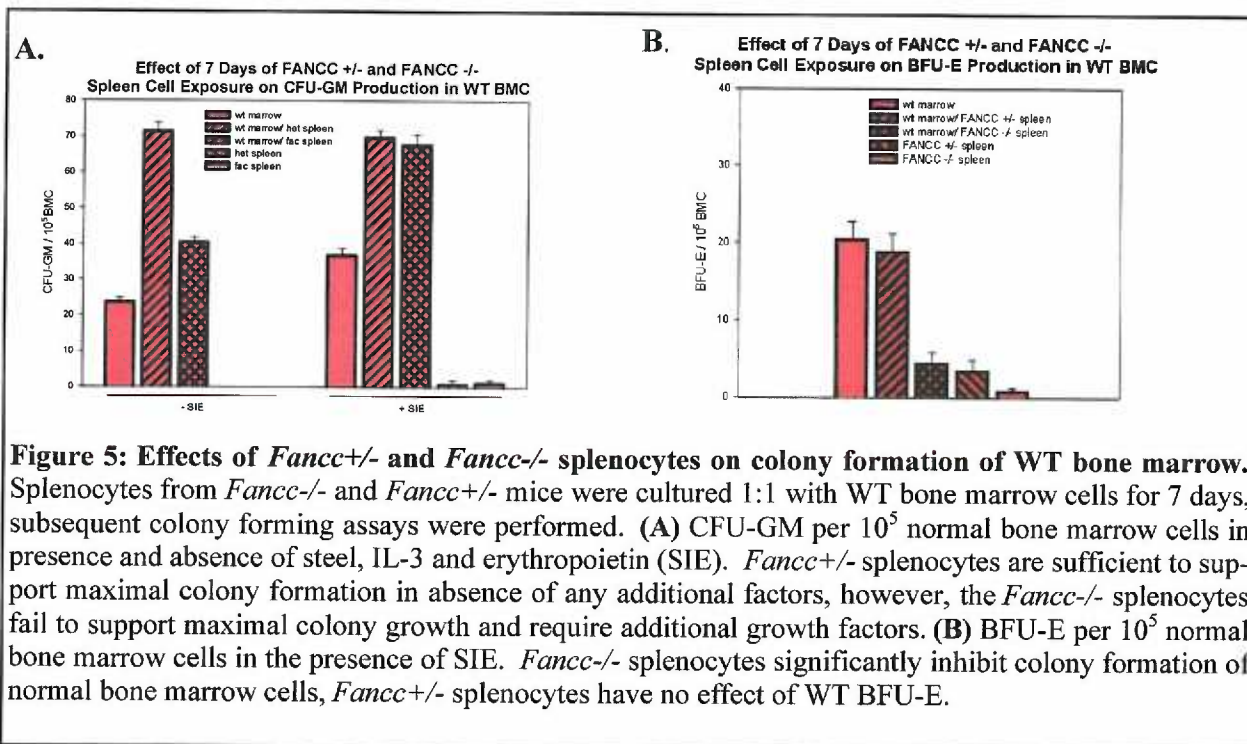


Figure 7-1: T helper differentiation in *Fancc*^{-/-} splenocytes. Splenocytes derived from *Fancc*^{-/-} and *Fancc*^{+/-} mice were subjected to conditions conducive for Th1 or Th2 differentiation and then stimulated with plate bound anti-CD3. (A) ELISA assays of two independent experiments on supernatants of splenocytes from *Fancc*^{+/-} mice (HET1, HET2) and *Fancc*^{-/-} mice (FA1, FA2, FA3) placed under conditions conducive for Th1 or Th2. The *Fancc*^{-/-} mice under Th1 conditions (dark blue bar) produce significantly lower levels of IFN-γ compared to *Fancc*^{+/-} Th1 cells. Moreover, *Fancc*^{-/-} cells under Th2 conditions (light blue bar) produce equivalent levels as those *Fancc*^{-/-} cells placed under Th1 conditions (upper panel). Analysis of IL-4 production did not yield any significant difference between *Fancc*^{-/-} and *Fancc*^{+/-} Th2 cells (lower panel). (B) Flow cytometric analysis of IFN-γ secreting cells and CD4 demonstrates significantly reduced numbers of *Fancc*^{-/-} CD4⁺ IFN-γ secreting (Th1) cells compared *Fancc*^{+/-} CD4⁺ IFN-γ secreting cells (upper right quadrant, panel 1 vs. upper right quadrant, panel 2).

splenocytes placed under Th1 conditions produce less IFN γ than *Fancc*^{+/-} splenocytes, and the level of IFN γ production of *Fancc*^{-/-} splenocytes under Th1 conditions is no different than the levels of cells from the same mice placed under Th2 conditions. Whereas, *Fancc*^{+/-} splenocytes produce significantly higher levels of IFN γ when placed under Th1 conditions compared to cells placed under Th2 conditions [298] (Figure 7-1B, left panel). These data indicate that CD4⁺ cells from *Fancc*^{-/-} mice fail to properly differentiate into Th1 subsets. No consistent differences in IL-4 production could be detected in cells placed under Th2 conditions.

Fancc^{-/-} lymphocytes negatively affect myeloid differentiation of normal bone marrow

Recent studies indicate CD4⁺ Th1 cells are required for optimal myelopoiesis [177]. To determine if abnormal lymphocyte subsets in *Fancc*^{-/-} splenocytes abnormally affects normal myelopoiesis, we mixed splenocytes from *Fancc*^{-/-} mice or *Fancc*^{+/-} mice with bone marrow cells derived from Wild Type mice and performed colony assays to determine levels of colony



forming units granulocyte-macrophage (CFU-GM) and burst forming units erythroid (BFU-E). An equal number of splenocytes and bone marrow cells were cultured for 7 days. *Fancc*^{+/-} splenocytes are sufficient to support maximal colony formation in absence of any additional factors, however, the *Fancc*^{-/-} splenocytes fail to support maximal colony growth and require additional the additional factors of Steel Factor, Interleukin-3 and erythropoietin (SIE) [299] (Fig 8-2A). *Fancc*^{-/-} splenocytes significantly inhibit BFU-E of normal bone marrow cells, whereas the *Fancc*^{+/-} splenocytes had no effect (Figure 8-2B). These studies indicate that abnormal *Fancc*^{-/-} lymphocyte subpopulations create a sub-optimal environment for normal myelopoiesis.

7-4: Discussion

It has only been thirteen years since the discovery that naïve CD4⁺ cells have the potential to differentiate into two distinct subsets in mice [175]. Since that time numerous research articles have been published and the role of T helper cell subsets in the immune system are beginning to be elucidated [297]. However, less evident, is the role T helper cells play in supporting myeloid growth and differentiation. It is clear the T helper subsets not only secrete cytokines important for immune cell function, the also secrete cytokines important for growth and differentiation of myeloid progenitor cells. Moreover, recent evidence suggests that Th1 cells are required for normal myeloid progenitor cell activity [177].

FA-C cells have defective signaling of TYK2 and several STAT proteins, as well as SOCS1 (Chapter 6). All of these proteins are involvement in some aspect of proper T-helper differentiation [127,128,293,296]. In addition two FANCC interacting proteins, HSP70 and ROG (the murine form of FAZF) are also important in Th1 differentiation and control of T

helper cytokine expression respectively [99,101,211,300]. Therefore, we analyzed the ability of CD4⁺ T cells from *Fancc*^{-/-} mice to differentiate into T helper subsets. We find that *Fancc*^{-/-} T helper cells are impaired in their ability to become T helper type I cells. We were unable to detect any consistent impairment in *Fancc*^{-/-} Th2 differentiation under our assay conditions. Seeking to identify potential biological effects of defective Th1 differentiation in *Fancc*^{-/-} mice, we analyzed the affects *Fancc*^{-/-} lymphocytes have on normal bone marrow. *Fancc*^{-/-} lymphocytes alone were not sufficient to induce optimal granulopoiesis and required additional growth factors. In addition, *Fancc*^{-/-} lymphocytes inhibited erythropoiesis of normal bone marrow while *Fancc*^{+/-} lymphocytes had no effect. These data indicate that defects in specific lymphocyte populations of *Fancc*^{-/-} mice profoundly affect normal hematopoietic progenitor cell growth, survival and differentiation

We emphasize that these studies were conducted in mouse cells, and significant differences exist between mice and human in differentiation of Th1 cells [301]. The classical pathway for Th1 differentiation occurs by IL-12 induction of STAT4 [137,210]. The most dramatic difference between mouse and human is that in human cells, type I IFNs can also induce Th1 differentiation in addition to the classical IL-12 induction pathway. Human type I IFNs induce phosphorylation of STAT4 by recruitment of STAT4 to the IFN α R1 via STAT2. However, murine Stat2 lacks a C-terminal sequence necessary for the recruitment of Stat4, therefore, murine type I IFNs are unable to activate Stat4 and induce Th1 differentiation [131,208]. *Fancc*^{-/-} mice do not mimic the severity of the FA phenotype. *Fancc*^{-/-} mice show no overt physical anomalies, do not get bone marrow failure nor do they exhibit an increased risk of cancer. It is entirely possible that the Th1 defect in mice is less pronounced than that in humans, given that we know IFN α signaling does not occur properly in human lymphocytes.

We show here that *Fancc*^{-/-} lymphocytes abnormally affect normal myeloid progenitor cell growth. If Th1 defects are more pronounced in humans, this may ultimately have even more devastating effects on human myeloid progenitor activity. One study does point to decreased numbers of CD4⁺ cells in FA patients [46], however, no analysis was done on differential subsets. Additional detailed analysis on CD4 populations, as well as, other specific lymphocyte subsets, in multiple FA patients is required. Moreover, analysis of the effects human T helper cells have on myeloid progenitor activity will need to be assessed to determine if this mechanism is conserved from mice to humans. Reasons for the phenotypic differences between *Fancc*^{-/-} mice and FA patients remains a mystery. However, for the first time, these studies place FANCC in a functional pathway that affects mice and humans differentially.

Recent studies have found that the Th1:Th2 ratio is important in the progression and outcome of neoplastic disease [302-305]. Moreover, cytokines secreted by Th1 cells are known to have potent antitumor and antimetastatic activities [306-310]. Defective Th1 differentiation, leading to improper Th1:Th2 ratio in FA cells, may not only have an effect on immune function and myeloid progenitor cell activity, but may also be reflected in the increased risk of cancer in the FA population, particularly in solid tumors. FA patients are at a significantly increased risk of squamous carcinoma of the head and neck, esophageal, liver and gynecological cancers compared to the general population [32,49]. Interestingly, most of these cancers are associated with virus induction, in particular Human Papilloma Virus (HPV) [35,311-313]. Recent evidence points to an intimate involvement in T helper cells in protecting cells against HPV and eradicating HPV induced tumor cells although the precise mechanism is unclear [314,315]. To date, no study exists on viral induced cancers in FA patients. Given the Th1 defects in *Fancc*^{-/-} mice large scale detailed studies on viral involvement in FA cancers is warranted.

These studies find a specific hematopoietic abnormality in the *Fancc*^{-/-} mouse. However, the defect occurs in the lymphoid lineage rather than the myeloid lineage, forcing us to rethink FA as a myeloid specific defect. Moreover, these studies demonstrate, that the environment created by specific lymphoid subsets affect myeloid differentiation, and the environment created by FA lymphocytes appears to be less favorable for proper myelopoiesis. These studies emphasize the importance of analyzing both lymphoid and myeloid lineages to clarify mechanisms underlying bone marrow failure and cancer development in FA patients.

Chapter 8

Summary

These studies originated from the observation that hematopoietic progenitor cells derived from FA-C patients and *Fancc* null mice are hypersensitive to inhibitory effects of IFN γ [18,19]. Beginning by quantification of downstream genes important for carrying out the effects of IFN γ , we revealed that the IRF family members IRF-1 and ISGF3 γ (p48, IRF-9), the cyclin dependent kinase inhibitor, p21^{WAF1}, and the GTPase, MxA, are expressed at extraordinarily high constitutive levels. Interestingly, these abnormalities are specific to certain hematopoietic and embryonic cells (Chapter 3). This is extremely important given that the most profound manifestations of FA occur in bone marrow cells, and a major goal of FA research is to understand why the disease affects hematopoietic cells so severely.

The next step in this thesis work was to determine if IFN γ -induced signaling through the JAK/STAT pathway occurred normally in FA-C cells. Naturally, given that FA-C cells are hypersensitive to IFN γ and overexpress key downstream genes, we expected this pathway to be hyperactive in FA-C cells. However, much to our surprise, we found that activation of STAT1 is defective in FA-C cells. Moreover, this occurred precisely in those cell types that overexpress IFN γ -inducible genes (Chapter 5). Finding defective STAT1 activation in FA-C cells has driven the remainder of this thesis work.

Following our finding of defective IFN γ -induced STAT1 activation, we first asked how, in the face of defective STAT1 activation, FA-C cells upregulate IFN γ -inducible genes. Initially we planned to answer this by doing promoter/reporter analysis on IRF-1 and p21^{WAF1}. However, FA-C B-lymphoblasts were extremely resistant to transient transfection (although cells did not die at a higher rate), and we were unable to answer the question in this manner. Therefore, we focused our attention on other signaling pathways that may, in some instances, influence expression of IRF-1 and p21^{WAF1}. Preliminary studies on both PTen and the Map

kinase pathways did not reveal any significant differences, so we turned our attention to STAT-inducible pathways known to modulate IFN γ responses.

We first evaluated levels of ICSBP, a STAT1-inducible modulator of IFN γ responses that is expressed specifically in hematopoietic cells [264], and found ICSBP levels to be significantly reduced. However, exogenous expression of ICSBP by retroviral transduction did not suppress expression of IRF-1, ISGF3 γ and p21^{WAF1} indicating defective expression of these genes in FA-C cells is not due to reduced ICSBP levels.

We next turned to a pathway known to inhibit IFN γ -induction of IRF-1. IL-4, an antagonist of IFN γ , inhibits IFN γ -induced IRF-1 expression through activation of another STAT protein, STAT6 [265]. Analysis of IL-4 induced STAT6 revealed decreased activation of STAT6 in response to IL-4. Moreover, data on low density bone marrow cells from FA-C patients revealed that IL-4 augmented rather than inhibited IFN γ -induced IRF-1 expression. In addition, we find FA patients express an abnormal high molecular weight form of STAT6, indicating STAT6 may be involved in progression of bone marrow abnormalities in FA (Chapter 5). While these studies were being conducted, Qishen Pang found that the double stranded RNA dependent protein kinase, PKR, is constitutively activated in FA-C cells. PKR is a well recognized mediator of apoptosis, optimally activated by a variety of stimuli including combinations of dsRNA and IFN γ and IFN γ and TNF α [20,279], and more recent studies indicate that the most profound differences between FA and normal cells are found when IFN γ is combined with another cytokine [17,19,107]. Overriding PKR activation with a dominant negative PKR significantly reduced IFN γ hypersensitivity. Therefore, defective IFN γ sensitivity in FA-C cells appears to be complex, involving both hyperactive apoptotic pathways, such as PKR through eIF2 α , and defective inhibitory pathways, such as IL-4 via STAT6.

The second part of this work driven by finding defective IFN γ -induced STAT1 phosphorylation was to determine if other STAT pathways are defective in FA-C cells. Early studies analyzing binding of STATs to FANCC indicated only those pathways utilizing STAT1 would be defective [213]. However, finding reduced activation of STAT6 in response to IL-4 negated that hypothesis. Further analysis revealed that STAT1 and STAT3 are defective in response to IFN α , IFN β and IFN γ . Moreover, STAT5 is defective in response to IFN α . However, diminished STAT signaling is not a global defect because STAT3 activation is defective in response to type I and type II IFNs but not in response to IL-6 or LIF (Table 8-1).

Further studies of the type I IFN signaling in FA-C cells revealed that, in addition to defective STAT signaling, phosphorylation of the Janus Kinase, TYK2, is also defective. Type I and type II IFNs communicate, and a functional IFN α R is required for proper IFN γ signaling. Moreover, it is reported that IFN α R1 and IFN γ R2 interact [174]. We were unable to detect any interaction in normal or FA-C cells between the two chains even in low stringency conditions in

Table 8-1: Summary of JAK/STAT Phosphorylation Defects in FA-C Cells

Molecule	P-	Cytokines	Cytokines that work
STAT1	Reduced	IFN α , β , γ	
STAT2	constitutive	IFN α	
STAT3	Reduced	IFN α , β , γ	LIF, IL-6
STAT5	Reduced	IFN α	
STAT6	Reduced	IL-4	
JAK1	Normal	IFN γ	
JAK2	Normal	IFN γ	
TYK2	Reduced	IFN α , β	

the B-lymphoblast lines. Analysis of TYK2 binding to IFN α R found normal binding in FA-C cells. In an effort to more fully understand defective TYK2 activation in FA-C cells, we quantified expression levels of SOCS1. SOCS1 is intimately involved in IFN γ signaling, but its role in type I IFN signaling is less well studied [160]. However, SOCS1 does inhibit TYK2 activation *in vitro*, and, given the recent findings of cross talk between type I and type II IFN signaling, may be an important regulator of type I IFN signaling as well [285]. We find high levels of SOCS1 expression in FA-C cells correlates with reduced TYK2 activation (Chapter 6).

Seeking to identify potential biological effects of improper cytokine signaling in FA-C cells, we studied the potential for *Fancc*^{-/-} CD4⁺ T helper cells to differentiate into type I or type II subsets. We demonstrate that the differentiation of CD4⁺ Th1 cells is impaired in the absence of *Fancc*, likely leading to a Th1:Th2 imbalance. In order to further understand how loss of Th1 contributes to the FA phenotype, we analyzed the affects of *Fancc*^{-/-} splenocytes on normal bone marrow and found that *Fancc*^{-/-} splenocytes created a less favorable environment for myeloid progenitor cell activity. Thus, one potential effect of loss of Th1 is decreased myeloid progenitor cell activity. Less clear is how defective Th1 differentiation affects the immune system in FA patients. More detailed analysis of FA patient lymphoid subsets and analysis of the effects of viral infections on *Fancc*^{-/-} mice will be important in assessing possible subtle FA immune system defects.

This thesis work has begun to decipher the role FANCC plays in cytokine signaling through the JAK/STAT pathway and specific defects likely resulting from these defects. In addition, structure/function studies from our laboratory indicate that FANCC is multifunctional [316]. Specific FANCC mutations, while able to complement MMC hypersensitivity and FANCD2 monoubiquitination, fail to restore HSP70-FANCC interactions, permit docking of

STAT1 at IFN γ R1 and protect FA-C cells from apoptosis induced by combinations of dsRNA and IFN γ or IFN γ and TNF α .

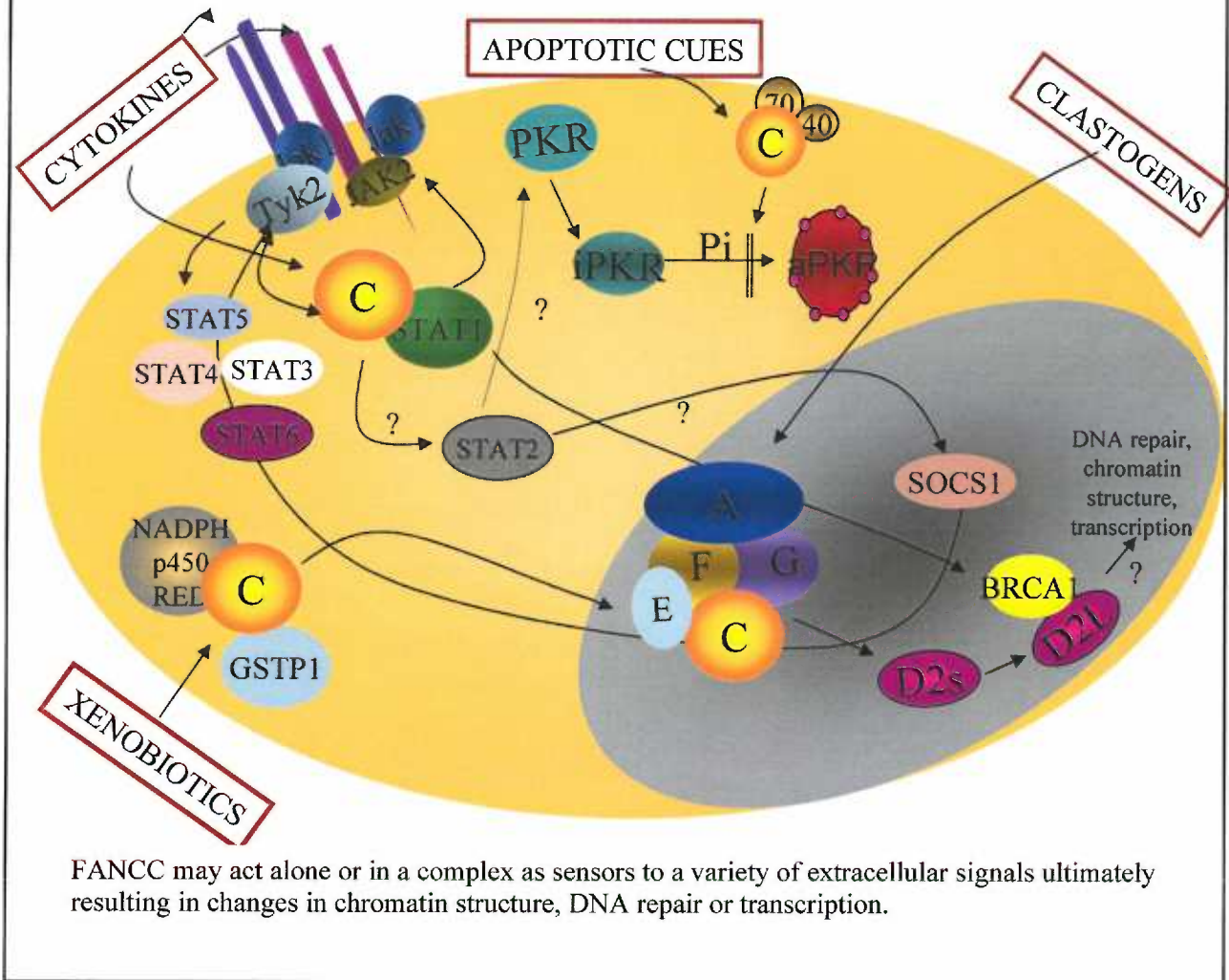
Overexpression of IRF-1 and ISGF3 γ do not occur in FA-A cells and appears to be specific to FA-C cells, whereas the overexpression of p21^{WAF1} occurs across complementation groups. This indicates that upregulation of IRF-1 and ISGF3 γ and p21^{WAF1} may occur through different pathways, one involving FANCC alone and the other involving the entire FA complex. This is not surprising given the multifunctionality of FANCC and that the severity of FA hematological abnormalities differ among complementation groups [53]. p21^{WAF1} is implicated in aspects of DNA repair, especially with respect to its interaction with PCNA [228,317]. Moreover, regulation of p21^{WAF1} expression may involve chromatin structure, in particular structures maintained by histone acetylation [318,319]. Indeed, we have performed preliminary studies using the inhibitor of histone deacetylation, Trichostatin A (TSA) and found that activation of p21^{WAF1} transcription by histone acetylation is more pronounced in normal cells than in FA-C cells (Appendix A) indicating the FA-C cell may be constitutively hyperacetylated. However, additional studies could not confirm a chromatin structure defect in FA cells.

Further elements support a role for FANCC in maintenance of chromatin structure including the finding that FAZF (ROG, TZFP) interacts with FANCC. FAZF is related to PLZF, a known modulator of histone acetylation and subsequent chromatin structure. FAZF is cell type specific, expressed in testis, lymphocytes and inducible in T helper cells. If the FA complex is involved in regulation of chromatin structure via FAZF, it would clarify, to some degree, cell type specific abnormalities in FA. Unfortunately, the data to date do not implicate FAZF in histone acetylation, but functioning through competition for its DNA binding site

[101]. More studies need to be conducted on FAZF and alterations in the chromatin structure of FA cells to fully determine if defects in chromatin fidelity underlie some of the phenotypic consequences of mutations in the FA disease causing genes.

Clearly, FANCC plays an important role in the FA complex and monoubiquitination of FANCD2 [68]. It is likely the DNA repair and cytokine signaling are not mutually exclusive. Several items link cytokine signaling with DNA repair molecules and/or chromatin. First, STATs associate with the histone acetylase, CBP/p300 [151], and FANCC associates with a putative recruiter of histone deacetylase [99]. Second, BRCA1 and IFN γ -activated STAT1 interact and synergize to upregulate p21^{WAF1} [154]. Therefore, in BRCA1 null cells, IFN γ is unable to induce p21^{WAF1}, implicating a function for BRCA1 in IFN γ -dependent antiproliferative responses [154]. It would be interesting to find what other IFN γ responses are defective in BRCA1 null cells, given that, like FA cells, BRCA1 cells are hypersensitive to MMC [86]. Third, SOCS proteins may function in protein ubiquitination. All SOCS proteins tested associate with a complex containing elongins B and C through their SOCS box. In turn, the elongin BC complex binds to a putative E3 ubiquitin ligase called cullin-2 [168]. The precise role SOCS plays in the ubiquitination pathway is unclear at this time and any affect this may have on cytokine signaling has not been reported. However, this is interesting given that others have postulated that the FA complex may be acting as a ubiquitin ligase facilitating monoubiquitination of FANCD2 [86]. Fourth, T helper cell differentiation requires chromatin remodeling for proper differentiation. This may not occur properly in FA cells contributing to the impaired Th1 response and further linking defective cytokine signaling, DNA repair/ chromatin rearrangement and hematopoietic cell development [204]. Analysis of the effects of cytokine signaling on the FA complex in addition to the effects on chromatin rearrangement in

Figure 8-1: Model of FANCC as an Extracellular Sensor



FA cells may finally link the hematopoietic specific abnormalities in FA with chromosomal instability.

Data presented in this thesis indicate that FANCC senses specific types of cytokine stimulation and facilitates signaling via the JAK/STAT pathway, suggesting that FANCC is required for proper alignment and positioning of specific JAK, STAT and SOCS proteins upon extracellular stimulation of specific cytokines. In addition, a large body of literature indicates that the FA proteins are affected by a variety of extracellular stimuli including cytokine stimulation, ionizing radiation, DNA cross-linking and oxidative stress [21,68,90,320]. One

model that allows for these apparently diverse observations is that FANCC functions both alone and as a complex acting as a sensor for extracellular signaling including growth and apoptotic signaling by cytokines, as well as responses to oxidative stress and DNA damaging agents, ultimately resulting in changes in chromatin structure and/or fidelity (Figure 8-1).

These thesis studies have clarified defective pathways involved in FA bone marrow failure. Beginning with the observation that FA-C hematopoietic cells are hypersensitive to the cytokine IFN γ , further analysis revealed specific signaling defects in an extremely important hematopoietic signaling pathway. Evaluation of the functional consequences of these signaling defects revealed a previously undescribed defect in T helper type I differentiation in *Fancc*^{-/-} mice. The significance of defective Th1 development in FA-C cells is threefold. First, finding a defective lymphocyte subpopulation in FA-C cells forces the FA researcher to re-evaluate FA as a myeloid specific defect, and emphasizes the need for more detailed studies on lymphopoiesis. Second, recent studies indicate Th1 cells support normal myelopoiesis [177], therefore, loss of Th1 likely contributes, in part, to pancytopenia in FA patients. Indeed, we find that auxiliary cell support of normal hematopoiesis is profoundly reduced in the *Fancc*^{-/-} mice. Third, given the importance of Th1 cells in tumor surveillance and viral clearance, loss of Th1 may contribute to the specific subset of solid tumors found in FA patients. Taken together, these studies have uncovered a potentially novel mechanism contributing to both bone marrow failure and cancer development in FA patients.

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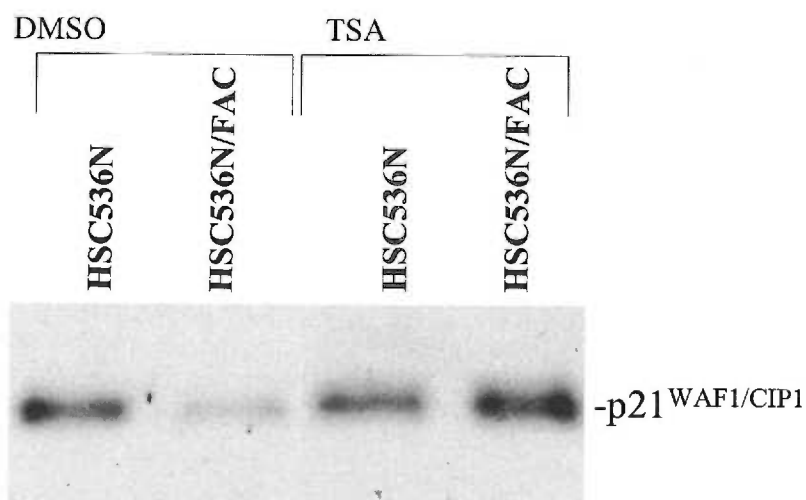
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Appendix A



Appendix A: Effects of the histone deacetylase inhibitor, Trichostatin A (TSA) on p21 expression in FA-C cells. *FANCC* mutant and corrected cells were exposed to DMSO or TSA for 24 hours. Cell lysates were then harvested and run on immunoblots probing with p21 demonstrating that TSA significantly upregulates p21 expression in corrected cells, but has no affect on the already high levels of p21 expression in mutant cells.

Appendix B

Published papers resulting from this work

Functional correction of FA-C cells with *FANCC* suppresses the expression of interferon γ -inducible genes

Sara R. Fagerlie, Jane Diaz, Tracy A. Christianson, Kelli McCartan, Winifred Keeble, Gregory R. Faulkner, and Grover C. Bagby

Because hematopoietic cells derived from Fanconi anemia (FA) patients of the C-complementation group (FA-C) are hypersensitive to the inhibitory effects of interferon γ (IFN γ), the products of certain IFN γ -inducible genes known to influence hematopoietic cell survival were quantified. High constitutive expression of the IFN γ -inducible genes, IFN-stimulated gene factor 3 gamma subunit (ISGF3 γ), IFN regulatory factor-1 (IRF-1), and the cyclin-dependent kinase inhibitor p21^{WAF1} was found in *FANCC* mutant B lymphoblasts, low-density bone marrow cells, and murine embryonic fibroblasts. Para-

doxically, these cells do not activate signal transducer and activator of transcription (STAT) 1 properly. In an attempt to clarify mechanisms by which FA-C cells overexpress IFN γ -inducible genes in the face of defective STAT1 phosphorylation, it was reasoned that decreased levels of activated STAT1 might result in reduced expression of a hematopoietic IFN γ -responsive protein that normally modulates expression of other IFN γ -responsive genes. Levels of the IFN γ -inducible factor IFN consensus sequence binding protein (ICSBP), a negative trans-acting regulator of some IFN γ -inducible genes,

were quantified. ICSBP levels were reduced in FA-C B lymphoblasts and MEFs. However, enforced expression of ICSBP failed to down-regulate IRF-1, ISGF3 γ , and p21^{WAF1}. Thus, the *FANCC* protein functions to modulate expression of a family of genes that in normal cells are inducible only by specific environmental cues for apoptosis or mitogenic inhibition, but it does so independently of the classic IFN-STAT1 pathway and is not the direct result of reduced ICSBP expression. (Blood. 2001;97:3017-3024)

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Introduction

Fanconi anemia (FA) is an autosomal recessive disease characterized by progressive bone marrow failure, multiple congenital anomalies, and a high incidence of acute myelogenous leukemia.¹⁻⁴ Cells from FA patients are hypersensitive to the effects of DNA cross-linking agents, such as mitomycin C and diepoxybutane.^{5,6} The disorder is genetically heterogeneous, with at least 7 different complementation groups.^{7,8} The genes corresponding to group A (*FANCA*), C (*FANCC*), E (*FANCE*), F (*FANCF*), and G (*FANCG*) have been cloned.⁹⁻¹³ Fanconi gene sequences show no significant homology to any known genes, and the functions of their gene products are unknown.^{14,15}

Hematopoietic precursor cells from children with FA of the C complementation group (FA-C) are excessively apoptotic and hypersensitive to a variety of cytokines known to induce apoptosis, including interferon γ (IFN γ). IFN γ is a cytokine involved in host defense against viral infections and regulation of cell growth.¹⁶ IFN γ clearly plays a role in hematopoietic suppression and has been implicated in the pathogenesis of acquired aplastic anemia.¹⁶⁻²² Cells with inactivating *FANCC* mutations are hypersensitive to the apoptotic effects of IFN γ , an effect that depends in part on an intact *fas*/*fas*-ligand pathway.²³⁻²⁶ Specifically, exposure to low doses of IFN γ in vitro primes a substantial fraction of FA-C progenitor cells to undergo apoptosis after subsequent exposure to *fas* ligand. Such doses of IFN γ have no effect on normal hematopoietic progenitor cells.^{23,24} Because of the influence of IFN γ on the *fas* pathway, we sought to determine whether other

IFN γ -inducible genes are hyperactive in FA-C cells. We carried out studies designed to analyze expression of IFN γ -inducible genes known to promote apoptosis and/or mitogenic inhibition in hematopoietic progenitor cells. We describe here studies revealing that the transactivators interferon regulatory factor-1 (IRF-1) and IFN-stimulated gene factor 3 gamma subunit (ISGF3 γ) and the cyclin-dependent kinase (cdk) inhibitor p21^{WAF1} are expressed at constitutively higher levels in FA-C B lymphoblasts, low-density bone marrow cells (LDBMCs), and murine embryonic fibroblasts (MEFs), compared with cells expressing a normal *FANCC*. However, these proteins are expressed at normal levels in mature mutant fibroblasts, suggesting some degree of tissue or developmental stage specificity of *FANCC* function in control of these genes.

IFN γ exerts its effects, at least in part, through the Jak/signal transducer and activator of transcription (STAT) signaling pathway.^{27,28} However, FA-C cells are defective in their ability to properly activate STAT1.²⁹ We show here that up-regulation of IFN γ -inducible genes occurs precisely in those cell types that fail to properly activate STAT1. For this reason, we hypothesized that loss of a STAT1-inducible transcriptional repressor may result in up-regulation of IFN γ -inducible genes in FA-C cells. Indeed, we found expression of the transcriptional repressor IFN consensus sequence binding protein (ICSBP) is decreased in both FA-C B lymphoblasts and murine embryonic fibroblasts. However, enforced expression of ICSBP failed to suppress IRF-1, ISGF3 γ , or p21^{WAF1}.

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We conclude that in FA-C hematopoietic and embryonic cells, expression of IFN γ -inducible genes is increased even in the face of defective STAT1 phosphorylation but is not linked directly with reduced levels of ICSBP. We suggest that FANCC functions to indirectly suppress expression of IFN γ -inducible genes in a cell-type-specific, STAT1-independent manner.

Materials and methods

Cell culture and IFN γ stimulation

Epstein-Barr virus (EBV)-transformed human lymphoblasts were maintained in RPMI media 1640 (Life Technologies, Rockville, MD) supplemented with 15% heat-inactivated fetal calf serum and grown in a humidified 5% CO $_2$ -containing atmosphere at 37°C. The FA-A B-lymphoblast lines HSC72 and HSC72/FANCC were a generous gift from Dr Walsh (University of North Carolina, Chapel Hill). The lymphoblast lines JY (normal), HSC536N (FANCC mutant), HSC536N/FANCC, and HSC536N/neo were described previously.²⁴ Briefly, HSC536N/FANCC was derived by transducing the HSC536N with a retrovirus encoding both FANCC and neomycin phosphotransferase (neo). The HSC536N/neo line was derived by transducing the HSC536N with a vector encoding for neomycin phosphotransferase alone.

MEFs were established from *FancC* knockout and wild-type mice as previously described²³ and grown in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum. MEFs were transformed and immortalized by SV40 large T antigen.³⁰

The following Fanconi fibroblast lines were received from the Oregon Health Sciences University Fanconi Anemia Cell Repository: PD134 (FA-C); PD426/FANCA (FA-C-expressing FANCA); PD426/FANCC (FA-C corrected); PD331.T (FA-C); PD720/FANCC (FA-A-expressing FANCC); PD720/FANCA (FA-A corrected); ML7334 (FA-G); and ML7334/FANCG (FA-G corrected). Human primary fibroblasts cell lines were established by means of standard procedures.³¹ The PD331.T line was immortalized as previously described.³¹ Cells were grown in MEM- α (Life Technologies) supplemented with 20% heat-inactivated fetal bovine serum. Correction of PD134 and PD331 was done by means of a retroviral vector carrying the human FANCC complementary DNA (cDNA) as previously described.²⁴ Cells were incubated with virus for 3 hours twice daily over a 5-day period and then selected with G418.

ICSBP-expressing HSC536N cells were made by means of a PLXSN-based retroviral vector expressing the human ICSBP cDNA. The pLXSN was a generous gift of Dr A. D. Miller (Fred Hutchinson Cancer Research Center, Seattle, WA). The pTarget/ICSBP was a generous gift supplied by Dr Ben-Zion Levi (Technion-Israel Institute of Technology, Haifa, Israel) and contains the entire human ICSBP cDNA. The pLXSN/ICSBP was made as follows: pTarget/ICSBP was digested with EcoRI; a 1.3-kilobase insert was isolated and cloned into pLXSN. Proper orientation was determined by means of a KpnI/NotI digest. Viruses were made as previously described.²⁴ HSC536N were incubated with pLXSN/ICSBP or pLXSN virus plus 8 μ g/mL polybrene for 3 hours twice daily for 5 days. Cells were then selected with G418.

IFN γ treatment

MEFs were serum starved for 24 hours and treated with the indicated amount of recombinant murine IFN γ (R&D Systems, Minneapolis, MN) for the indicated times in 5% CO $_2$ at 37°C. B lymphoblasts and human primary fibroblasts were treated with the indicated amounts of recombinant human IFN γ (R&D Systems) in 5% CO $_2$ at 37°C.

Preparation of total cell lysates and nuclear extracts

Total cell lysates were made by washing cells twice with phosphate-buffered saline (PBS), and cell pellets were solubilized in RIPA: 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% (wt/vol) sodium dodecyl sulfate

(SDS), 1% (vol/vol) aprotinin, 2 mM Na $_3$ VO $_4$, 1 μ g/mL leupeptin, 1 μ g/mL pepstatin, and 1 mM phenylmethylsulfonylfluoride (PMSF). Lysates were centrifuged at 16 000g for 15 minutes at 4°C, and protein concentrations were determined by means of protein micro-assay of the Bradford method (BioRad, Hercules, CA). Nuclear extracts were prepared as previously described.³² Briefly, cells were washed in ice-cold PBS and resuspended in cold hypotonic buffer: 10 mM Hepes, pH 7.9, 1.5 mM MgCl $_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM dithiothreitol (DTT), 2 μ g/mL aprotinin, and 2 μ g/mL leupeptin. Cells were allowed to swell on ice 10 minutes and then lysed with a Dounce B homogenizer (Bellco Glass, Vineland, NJ). Nuclei were pelleted at 3300g for 20 minutes at 4°C and then resuspended in low-salt buffer: 20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM MgCl $_2$, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, 2 μ g/mL aprotinin, and 2 μ g/mL leupeptin. High-salt buffer (0.6 M KCl) was added dropwise, and nuclear proteins were extracted by incubation at 4°C for 30 minutes and centrifuged at 13 000g for 30 minutes at 4°C. Supernatants were dialyzed at 4°C for 3 to 5 hours against a 250-fold volume excess of 20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, 2 μ g/mL aprotinin, and 2 μ g/mL leupeptin.

Immunoblot analysis

Total cell lysates and nuclear extracts were mixed with Laemmli sample buffer,³³ heated at 94°C for 5 minutes. Equivalent amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis and electroblotted onto Bio-Blot (Costar, Cambridge, MA) as previously described.³⁴ Blots were then subjected to Ponceau S. staining to demonstrate equivalent loading. Nonspecific binding was blocked for 1 hour at room temperature in Tris-buffered saline plus 0.05% Tween 20 (TBS-T) containing 5% milk. Blots were incubated with primary antibody, washed with TBS-T, then incubated with secondary antibody, and washed again with TBS-T. Antibody-reactive proteins were detected by means of Enhanced Chemiluminescence (Amersham, Arlington Heights, IL). IRF-1 antibody (1:1000), p21^{WAF1} antibody (1:500), ISGF3 γ (BD Pharmingen) (1:250), p27^{KIP1} (BD Pharmingen) (1:500), p53 (1:1000), IRF-2 (1:1000), and total STAT1 (1:2000) were used at the indicated dilutions and incubated with the blots for 1 hour at room temperature. Donkey antirabbit immunoglobulin G horseradish peroxidase (HRP) secondary antibody (1:5000) (Amersham) was used against these primary antibodies. ICSBP antibody was diluted 1:500 for 1 hour at room temperature. Rabbit anti-goat HRP secondary antibody (1:2000) (Pierce, Rockford, IL) was used against ICSBP primary antibody. Phospho-specific STAT1 (New England Biolabs, Beverly, MA) (1:500) was incubated at 4°C overnight. Donkey antirabbit HRP (New England Biolabs) (1:2000) secondary antibody was used against phosphorylated STAT1 primary antibody. Antibodies were used at the indicated dilutions and purchased from Santa Cruz Biotechnology (CA) unless otherwise indicated.

Electromobility shift assay

Sequences for oligonucleotides used in binding reactions were as follows: human IRF-1 gamma activation sequence (GAS), 5'-ACAACAGCCTGATTCCCGAA-3'; murine IRF-1 GAS, 5'-CCTGATTCCCGAAATGATG-3'; murine ICSBP GAS, 5'-AGTGATTTCTCGGAAAGAGAG-3'. Oligonucleotides were synthesized at the Molecular Biology Core Laboratory (Portland, OR, Veterans Affairs Medical Center), then labeled with [γ -³²P-ATP] to 25 000 cpm/ng by means of T4 polynucleotide kinase (Roche Molecular Biochemical, Indianapolis, IN). Binding reactions (20 μ L) contained 5.0 to 7.5 μ g nuclear extracts, 0.2 ng labeled oligo, 2 μ g poly (dI-dC), and 10 μ g bovine serum albumin in 10 mM Tris-Cl, pH 7.4, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 10% glycerol. Reactions were incubated at room temperature for 30 minutes and then resolved on a 4% polyacrylamide gel in 25 mM Tris, 190 mM glycine, and 1 mM EDTA. Gels were dried and autoradiographed with intensifying screens at -80°C.

RNA isolation, cDNA synthesis, and real-time reverse-transcriptase polymerase chain reaction

Total RNA was isolated from cells with TriReagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. We

performed cDNA synthesis with 1 μ g total RNA, 200 ng random hexamers (Life Technologies), and Superscript II reverse transcriptase (Life Technologies) according to the manufacturer's instructions. The cDNAs were then diluted to an approximate concentration of 20 ng/mL before performing the amplification reactions. Amplifications were performed in an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA) by means of 100 ng cDNA, 7.5 pmol each target primer, 2.5 pmol target probe, 2.5 pmol each housekeeping primer, and 2.5 pmol housekeeping probe (h18S). As recommended by the manufacturer, the TaqMan polymerase chain reaction (PCR) kit (PE Applied Biosystems) was used as directed. The primer and probe sequences, designed by means of ABI Primer Express software (PE Applied Biosystems) and synthesized by Integrated DNA Technologies (Coralville, IA), are as follows: hMxA (forward primer) 5'-TGGTGGTGGTCCCCAGTAAT-3'; hMxA (reverse

primer) 5'-CGTCAAGATTCCGATGGTCC-3'; hMxA (dual labeled probe) 5'-FAM-CCACCACAGAGGCTCTCAGCATGG-TAMRA-3'; h18S (forward primer) 5'-CGGCTACCACATCCAAGAA-3'; h18S (reverse primer) 5'-GGGCCTCGA AAGAGTCTGT-3'; and 18S Probe 5'-VIC-CA GCAGGCGCGCAAATTACCCA-TAMRA-3'.

Results

Constitutive overexpression of IFN γ -inducible genes in mutant FA-C cell lines

Because FA-C cells are hypersensitive to the inhibitory effects of IFN γ ,^{23,24} we quantified protein levels of transcriptional activators known to be downstream of the IFN γ /Jak/STAT pathway by immunoblot analysis of total cell lysates or nuclear extracts. Analysis of the IFN γ -inducible transactivating factors IRF-1 and ISGF3 γ consistently revealed high constitutive expression levels in nuclear extracts and whole cell lysates derived from the *FancC* mutant MEF cell line (MEF61) compared with the wild-type (WT) MEF cell line (MEF11.1). We observed the same phenomenon using (1) whole cell lysates and nuclear extracts from the EBV-transformed *FANCC* mutant human B-lymphoblast line HSC536N; (2) nuclear extracts from the EBV-transformed B-lymphoblast line PD149, relative to levels in those cells corrected for the defect by retroviral transduction of the normal *FANCC* cDNA (Figure 1A); and (3) whole cell lysates from LDBMCs derived from FA patients compared with LDBMCs derived from a normal person (Figure 1D). In addition, we have previously found constitutively high expression of IRF-1 messenger RNA (mRNA) also occurs in diploid bone marrow cells from children with FA-C but not in normal bone marrow cells.²⁴ However, no differences in IRF-2 expression levels were found between FA-C cells and normal cells (Figure 1A). In addition, mRNA levels of the IFN γ -inducible guanosine triphosphatase (GTPase) MxA are reduced in the HSC536N B-lymphoblast line compared with corrected cells (Figure 1B). We found no differences in IRF-1 and ISGF3 γ expression in the FA-A lymphoblast line HSC72 (Figure 1C).

We next quantified levels of the cell cycle modulator p21^{WAF1}, which is known to be induced by IRF-1 and IFN γ .^{35,36} FA-C B lymphoblasts, MEFs, and LDBMCs express high levels of p21^{WAF1} compared with normal or corrected cells (Figure 1A,D). The FA-A cell line HSC72 also expresses higher levels of p21^{WAF1} compared with corrected cells, but does not overexpress IRF-1 or ISGF3 γ (Figure 1C). This suggests that overexpression of the transactivators IRF-1 and ISGF3 γ and of the cell cycle inhibitor p21^{WAF1} may

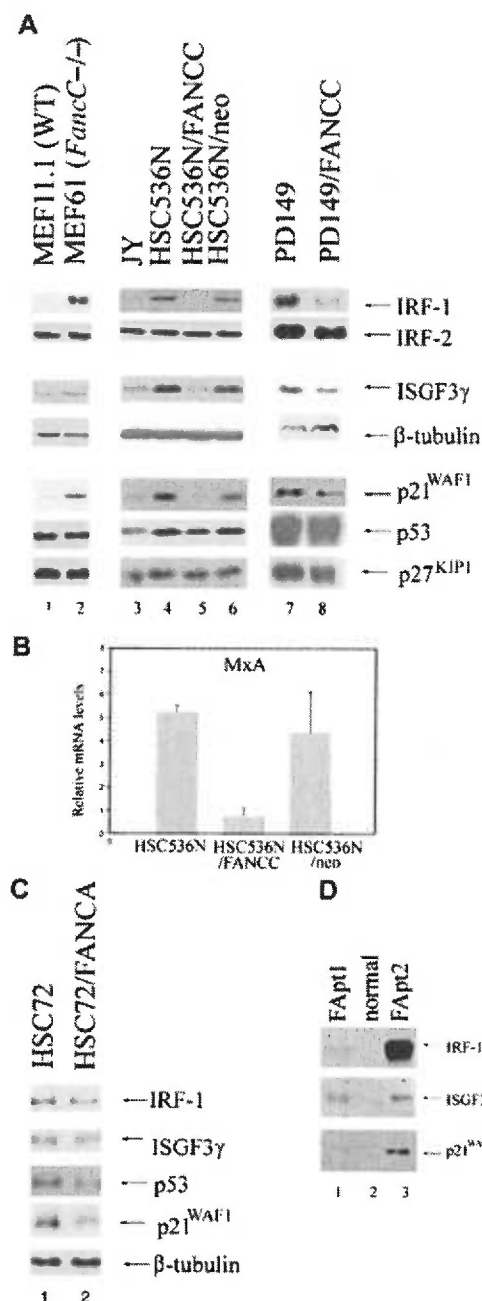


Figure 1. Expression of IRF-1, ISGF3 γ , and p21^{WAF1} is reduced in FA-C MEFs, B lymphoblasts, and FA patient LDBMCs. (A) Immunoblot analysis of the *FancC* mutant MEF cell line (MEF61) shows increased expression levels of IRF-1, ISGF3 γ , and p21^{WAF1} compared with the WT MEF cell line (MEF11.1) (lane 2 vs lane 1). The *FANCC* mutant B-lymphoblast line HSC536N, the HSC536N line transduced with a vector only (HSC536N/*neo*), and PD149 (lanes 4, 6, and 7, respectively) show increased IRF-1, ISGF3 γ , and p21^{WAF1} expression compared with the normal cell line JY (lane 3) and with cells corrected for the defect by retroviral transduction of the *FANCC* cDNA (HSC536N/*FANCC*) (lane 5) or PD149/*FANCC* (lane 8). However, FA-C cells express normal levels of IRF-2, p53, and p27^{KIP1}. To demonstrate equivalent loading of the ISGF3 γ blots, β -tubulin is used. (B) Real-time reverse transcriptase PCR demonstrates up-regulation of MxA in HSC536N and HSC536N/*neo* compared with the corrected cells HSC536N/*FANCC*. This is a representative experiment. Error bars are based on duplicate samples. (C) The FA-C cell line HSC72 expresses equivalent levels of IRF-1 and ISGF3 γ compared with cells corrected for the defect. The cells do express slightly more p21^{WAF1}, but equivalent p53. To demonstrate equivalent loading of the p21 blot, β -tubulin is used. (D) LDBMCs derived from 2 FA patients show up-regulation of IRF-1, ISGF3 γ , and p21^{WAF1} compared with cells derived from normal individual (lanes 1 and 3 vs lane 2).

be due to abnormalities in different pathways, one of which is influenced by both FANCA and FANCC and the other by FANCC alone. We found no differences in p53 binding to its p21 binding site (data not shown) and minimal differences in p53 expression levels, indicating that p21^{WAF1} overexpression is p53-independent (Figure 1A). No differences in expression levels of the cdk inhibitor p27^{KIP1} were found (Figure 1A).

We found no differences in protein levels of IRF-1, ISGF3 γ , and p21^{WAF1} in mature primary human FA fibroblasts (Figure 2). Finding molecular differences between mature fibroblasts and hematopoietic cells is not surprising given that hematopoietic abnormalities represent the dominant phenotype in FA patients. In addition, finding that a normal FANCC is required for proper regulation of IRF-1, p21^{WAF1}, and ISGF3 γ in hematopoietic cells and embryonic fibroblasts but not in mature fibroblasts suggests that MEFs have retained more plasticity and may exhibit responses more reflective of hematopoietic and germ cells than adult fibroblasts.

FA-C cells overexpressing IFN γ -inducible genes are deficient in STAT1 phosphorylation

FA-C cells have been shown to be defective in STAT1 phosphorylation, despite having normal activation of the IFN γ receptor, JAK1, and JAK2.²⁹ We sought to determine if this occurred in cells that overexpress IFN γ -inducible genes and those that do not. We analyzed the levels of phosphorylated STAT1 in IFN γ -induced cells. MEFs were serum starved for 24 hours and then incubated at 37°C with 1 ng/mL of murine IFN γ for the indicated time periods. Using immunoblot analysis, we determined that in both total cell lysates and nuclear extracts from MEFs, the level of phosphorylated STAT1 was significantly decreased in *FancC* mutant cells compared with the WT cells. When these blots were stripped and reprobed for total STAT1, levels of total STAT1 were equivalent (Figure 3A). In addition, analysis of total cell lysates from the mutant *FANCC* B-cell lines HSC536N and HSC536N/neo demonstrated that the amount of phosphorylated STAT1 was diminished when compared with the normal cell line JY and the *FANCC*-transduced cell line HSC536N/FANCC. When the same blots were stripped and reprobed for STAT1, total STAT1 was slightly increased in the mutant cells (but phosphorylated levels were

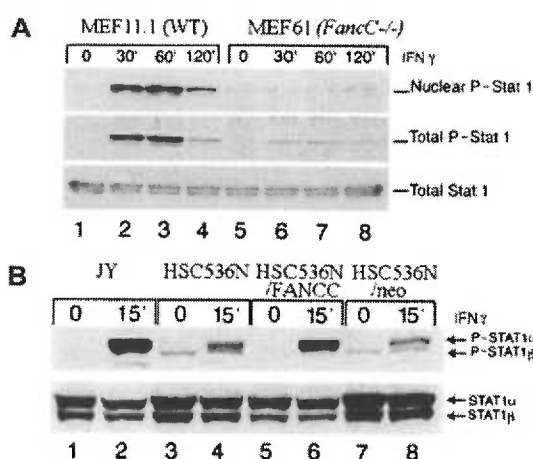


Figure 3. STAT1 phosphorylation at tyrosine 701 is reduced in MEFs and B lymphoblasts. (A) Immunoblot analysis demonstrates that phosphorylation of STAT1 is reduced in both nuclear extracts and total cell extracts (upper and middle panel respectively) from *FancC*^{-/-} MEFs (MEF61) (lanes 5 through 8) compared with WT cells (MEF11.1) (lanes 1 through 4) following stimulation with 1 ng/mL IFN γ for the indicated times. Total STAT1 levels (lower panel) were equivalent (lanes 1 through 4 compared with lanes 5 through 8). (B) Phosphorylation of STAT1 (upper panel) is reduced in total cell extracts from HSC536N (lanes 3 and 4) and HSC536N/neo (lanes 7 and 8) compared with JY (lanes 1 and 2) and HSC536N/FANCC (lanes 5 and 6) following stimulation with 1 ng/mL IFN γ at 37°C for 15 minutes (lanes 2, 4, 6, and 8). Total STAT1 levels were slightly higher in the mutant cells (but phosphorylation levels are reduced) (lower panel).

decreased) (Figure 3B). However, STAT1 phosphorylation was found to be normal in all mature human FA fibroblast lines tested (Figure 4). Thus, in FA cells, STAT1 phosphorylation defects are specific to certain cell types, and paradoxically, those cell types defective in STAT1 activation have high constitutive expression of several IFN γ -inducible genes.

Reduced binding to the GAS element in FA-C cells

It was theoretically possible that a more complete and rapid nuclear translocation of STAT1 in FA-C cells might have given an appearance of reduced STAT1 activation. Consequently, we asked whether apparently reduced levels of STAT1 phosphorylation were associated with increased binding to the STAT1 response element, GAS. Using an oligonucleotide derived from the GAS element of murine IRF-1 for electrophoretic mobility shift assay (EMSA) analysis of murine MEF lines, we found that IFN γ -inducible binding to the GAS element was significantly reduced in the *FancC*^{-/-} MEF line compared with the WT MEF line (Figure 5A). Using oligonucleotides representing the human IRF-1 GAS element, we found that

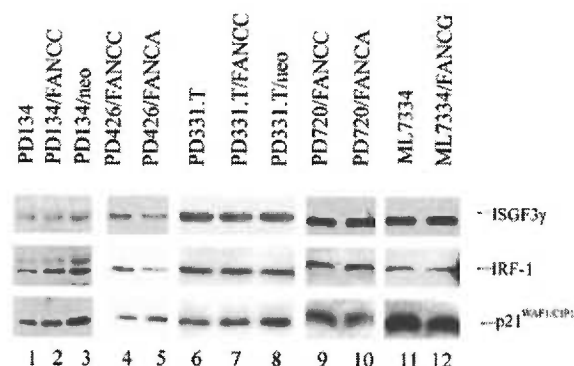


Figure 2. Human FA fibroblasts express equivalent levels of IRF-1, ISGF3 γ , and p21^{WAF1}. Immunoblot analysis of human FA-C fibroblasts PD134 (lane 1), PD134/neo (lane 3), PD426/FANCA (lane 5), PD331.T (lane 6), and PD331.T/neo (lane 8); the FA-A fibroblasts PD720/FANCC (lane 9); and the FA-G fibroblast line ML7334 (lane 11) express equivalent levels of ISGF3 γ , IRF-1, and p21^{WAF1} compared with those cells corrected for the defect by retroviral transduction of the appropriate FA cDNA: PD134/FANCC (lane 2), PD426/FANCC (lane 4), PD331.T/FANCC (lane 7), PD720/FANCA (lane 10), ML7334/FANCC (lane 12).

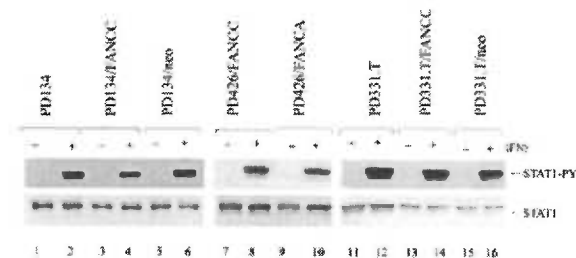


Figure 4. Normal phosphorylation of STAT1 in mature FA-C fibroblasts. Immunoblot analysis of human FA-C fibroblasts by means of an antibody specific for phosphorylated STAT1 demonstrates that STAT1 is phosphorylated equivalently between mutant and corrected IFN γ -stimulated cells (1 ng/mL IFN γ) (top panel). In addition, total STAT1 is expressed at approximately equal levels (bottom panel).

IFN γ -inducible binding was not detectable in mutant *FANCC* human B cells, whereas normal inducible binding was observed in the *FANCC*-complemented cell line (Figure 5B). These results indicate that the diminished ability to phosphorylate STAT1 in FA-C cells results in decreased activated STAT1 nuclear translocation and binding to GAS.

Expression of the IFN γ response modulator ICSBP is reduced in the FA-C cell lines

Seeking to identify potential molecular points of control involved in the aberrant expression of IRF-1, p21^{WAF1}, and ISGF3 γ in FA-C cells, we quantified ICSBP, an interferon-responsive transcription factor known to modulate IFN responses in hematopoietic cells.³⁷⁻³⁹ ICSBP levels were constitutively low in HSC536N and HSC536N/neo cell lines compared with the normal cell line JY and the complemented line HSC536N/FANCC (Figure 6A). In addition, although ICSBP is reported to be expressed mainly in hematopoietic cells, we found ICSBP expression to be consistently inducible in WT MEFs as well. Expression of ICSBP is significantly decreased in *FANCC* mutant MEFs. Therefore a functional *FANCC* is necessary for optimal constitutive expression of ICSBP. In addition, we find reduced binding to the GAS element derived from murine ICSBP (Figure 6B), suggesting that decreased ICSBP expression may be a direct result of reduced STAT1 phosphorylation.

Enforced expression of ICSBP does not suppress IRF-1, p21^{WAF1/CIP1}, or ISGF3 γ

To examine the linkage of reduced ICSBP with overexpression of IRF-1, ISGF3 γ , or p21^{WAF1}, we transduced the HSC536N B-lymphoblast cell line with a retroviral vector carrying the human *ICSBP* cDNA. Although these cells express high levels of ICSBP, ICSBP expression failed to suppress the expression of IRF-1, p21^{WAF1}, or ISGF3 γ (Figure 7). In fact, expression of IRF-1 was slightly increased in cells expressing ICSBP. Therefore, up-regulation of IRF-1, ISGF3 γ , and p21^{WAF1} is not directly linked to reduced ICSBP expression.

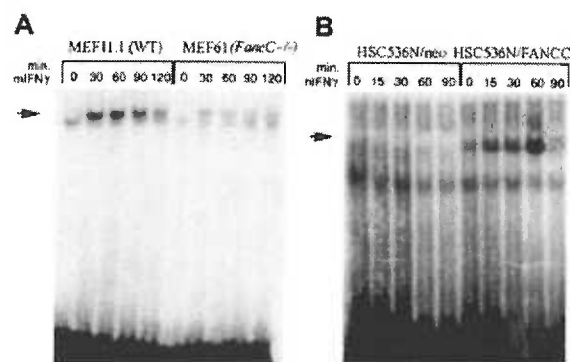


Figure 5. STAT1 binding to the IRF-1 GAS element is reduced in FA-C cell lines. EMSA of STAT1 binding to oligonucleotides corresponding to the GAS element in the IRF-1 promoter. (A) IFN γ -inducible STAT1 binding is diminished in *FancC*^{-/-} cells (MEF61) (lanes 6 through 10) compared with WT MEFs (MEF11.1) (lanes 1 through 5). Murine IFN γ was used at 1 ng/mL at 37°C. (B) No STAT1 binding occurs in HSC536N/neo (lanes 1 through 5), whereas in HSC536N/FANCC, STAT1 binds in an IFN γ -inducible manner (lanes 6 through 10). Cells were treated with 1 ng/mL human IFN γ at 37°C.

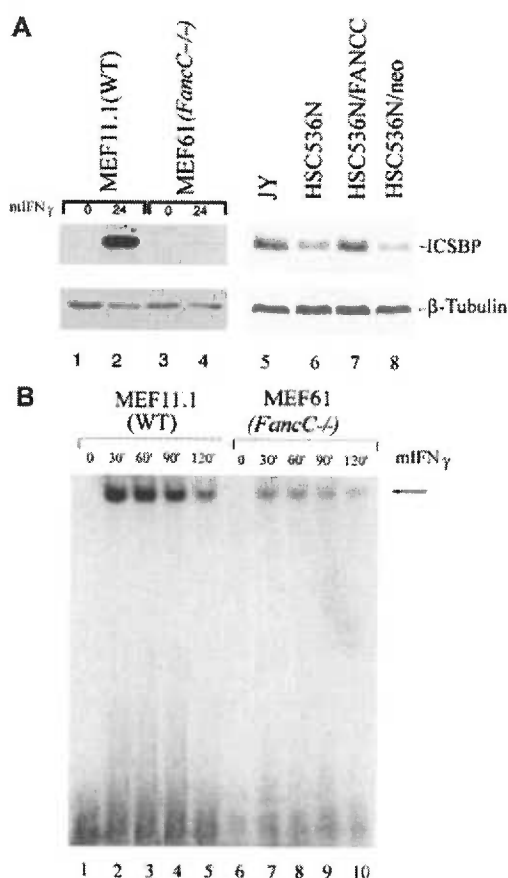


Figure 6. ICSBP is expressed at reduced levels in FA-C cells. (A) Immunoblot analysis demonstrates that ICSBP is expressed inducibly (50 ng/mL IFN γ for 24 hours) in wild-type MEFs (MEF11.1) (lane 2) at high levels. ICSBP expression in the *FancC*^{-/-} MEF line (MEF61) is significantly reduced compared with the wild-type cells (lane 4 vs lane 2). Constitutive expression of ICSBP is reduced in FA-C B lymphoblasts compared with normal and corrected cells (lanes 6 and 8 vs lanes 5 and 7). The same blot was stripped and reprobed with β -tubulin to demonstrate equivalent loading (lower panel). (B) EMSA of the GAS element of murine ICSBP demonstrates reduced IFN γ -stimulated binding in *FancC*^{-/-} MEFs compared with WT MEFs. Cells were stimulated with 1 ng/mL murine IFN γ for the indicated times.

Discussion

Interferons are involved in a broad range of mammalian biological functions, including immunity, inflammation, hematopoiesis, cell proliferation, and differentiation.^{16,17} In hematopoietic progenitor cells, IFN γ induces apoptosis,^{21,26,40,41} and there is compelling indirect evidence that it plays a pathophysiologically significant role in acquired aplastic anemias.^{17-19,21,41} Because FA-C cells are hypersensitive to IFN γ ,^{23,24} we believe that IFN γ hypersensitivity and bone marrow failure may be linked in FA. Accordingly, we have focused our attention on the IFN γ signaling pathway in FA-C cells as a way of deciphering the function of the *FANCC* protein in modulating the apoptotic response to extracellular cues.

The effects of IFN γ are mediated, at least in part, through transcriptional regulation of downstream genes, which include members of the IRF family of transcription factors. We find that certain IRF family members are constitutively overexpressed in specific FA-C cell types compared with normal cells. ISGF3 γ , which is constitutively expressed at higher levels in *FANCC* mutant

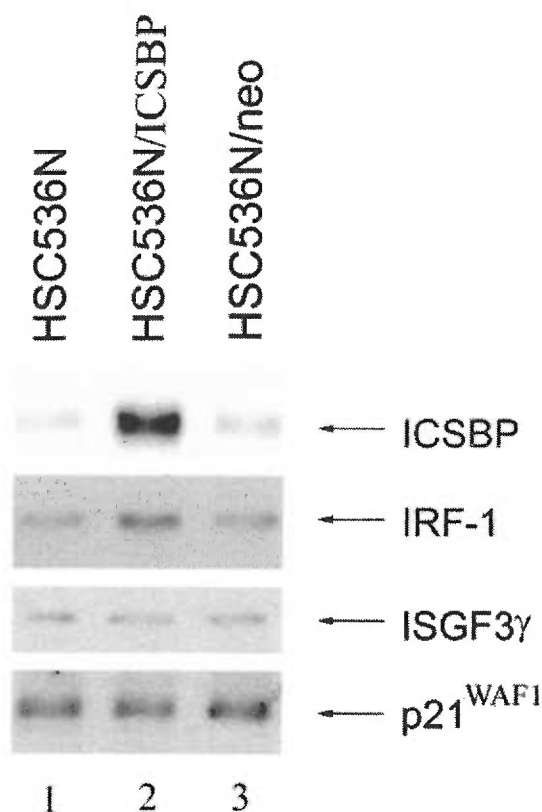


Figure 7. Enforced expression of ICSBP fails to down-regulate IRF-1 ISGF3 γ or p21^{WAF1}. Immunoblot analysis of FA-C B lymphoblasts HSC536N (lane1), HSC536N transduced with the human ICSBP cDNA (HSC536N/ICSBP) (lane2), and HSC536N transduced with vector alone (HSC536N/neo) (lane 3) demonstrates that HSC536N/ICSBP are expressing high constitutive levels of ICSBP, but this expression fails to suppress IRF-1, ISGF3 γ , or p21^{WAF1}.

B lymphoblasts, MEFs, and patient LDBMCs, is of particular interest because it is the only protein of the IRF family known to complex with STAT molecules.⁴² ISGF3 γ exerts its transcriptional activation only in conjunction with STAT1 and STAT2⁴³ and plays a crucial role in the regulation of IFN- β gene expression,⁴⁴⁻⁴⁶ a finding of relevance because some researchers have suggested that the induced expression of IFN- β is required for the hematopoietic suppressive effects of IFN γ .⁴⁷ IRF-1 is required for carrying out the apoptotic effects of IFN γ signaling. The ratio of IRF1 to IRF2 expression is important for proper cell cycle control.^{48,49} IRF-1 expression is significantly increased in FA-C hematopoietic and embryonic cells, but no differences in IRF-2 expression levels were found (Figure 1), upsetting the proper balance of IRF-1 and IRF-2 levels in FA-C cells. IRF-1 also cooperates with p53 to induce expression of the cdk inhibitor p21^{WAF1}.^{35,50} Although equivalent p53 expression levels were found between *FancC*^{-/-} MEFs and *FancC*^{+/+} MEFs and only minimal differences in p53 expression were found in human FA-C B lymphoblasts compared with normal cells, FA-C cells expressed significantly greater amounts of p21^{WAF1} (Figure 1). There was no difference in p53 binding to its p21^{WAF1}-binding site (data not shown), and expression of the IRF-1 independent cdk inhibitor p27^{KIP1} was equivalent (Figure 1). Thus, it is possible that IRF-1 up-regulation contributes to the overexpression of p21^{WAF1}. However, given that we find high constitutive p21^{WAF1} in FA-A cells and that these cells do not overexpress IRF-1, it is more likely that up-regulation of p21^{WAF1} and the IRF

transactivators may be due to abnormalities in different pathways, one of which is influenced by both FANCA and FANCC or the entire FA complex and the other by FANCC alone.

In addition to IRF-1, ISGF3 γ , and p21^{WAF1}, others have found that IFN γ -inducible GTPase, MxA, which confers intracellular antiviral activity in IFN-induced cells,^{51,52} is also constitutively expressed in FA cells,⁵³ a finding we confirm here (Figure 1B). Thus, several IFN γ -inducible factors that are important in regulation of apoptosis and mitogenic inhibition are constitutively upregulated in FA-C cells in a cell-type-specific manner.

Surprisingly, though FA-C hematopoietic and embryonic cells (1) are hypersensitive to the mitotic inhibitory effects of IFN γ and (2) express constitutively high levels of several IFN γ -inducible genes (effects abrogated by transduction of normal *FANCC* cDNA), activation of STAT1 is impaired.²⁹ We sought to determine if loss of STAT1 activation occurs only in those cell types exhibiting high constitutive expression of IFN γ -inducible genes. We found STAT1 phosphorylation to be defective in FA-C B lymphoblasts and MEFs, but not in mature human fibroblasts (Figures 3, 4). Because mature human fibroblasts do not overexpress these genes, we reason that the overexpression of IRF-1, ISGF3 γ , and p21^{WAF1} occurs only in FA-C cells defective in their ability to activate STAT1. Up-regulation of these genes in these cells appears to require that (1) FANCC be inactivated and (2) the particular *FANCC* mutation suppress STAT1 activation. Therefore, constitutive expression of IFN γ -responsive genes in *FANCC* mutant cells is due to either ground-state activation of a pathway that does not require STAT1 phosphorylation or loss of a hematopoietic IFN γ -responsive protein that normally modulates expression of other IFN-responsive genes. One IFN γ -responsive modulator of interest to us was the transcriptional repressor ICSBP, a member of the IRF family expressed mainly in hematopoietic cells.³⁸

ICSBP interacts with both IRF-1 and IRF-2 in vitro and in vivo and inhibits the DNA-binding activity of ISGF3 γ .³⁷ ICSBP binds to IRF-1 and suppresses some IFN γ -induced gene responses.³⁸ Stable expression of the DNA binding domain of ICSBP blocks IRF-1 transactivation and also inhibits binding of STAT1 to GAS.⁵⁴ In *FANCC* mutant B lymphoblasts we found less ICSBP expression than normal cells (Figure 6). In fact, ICSBP is the only IFN γ -inducible protein of those we have tested that is reduced in FA-C cells. Surprisingly, ICSBP is also expressed in an inducible manner in MEFs, and this expression is significantly reduced in *FancC* mutant MEFs. We first hypothesized that low expression levels of ICSBP in FA-C cells may be a direct result of a deficiency in STAT1 phosphorylation in FA-C cells. Indeed, STAT1 binding to the GAS element from ICSBP is significantly reduced in FA-C cells (Figure 6). Because ICSBP is decreased in those cells with up-regulated IFN γ -inducible genes, we next reasoned that loss of ICSBP contributes to the up-regulation of these genes. However, enforced expression of ICSBP into these cells failed to down-regulate IRF-1, ISGF3 γ , or p21^{WAF1} (Figure 7). Therefore, up-regulation of these IFN γ -inducible factors in FA cells is not directly linked with reduced ICSBP expression levels.

FA cells, both in vitro and in vivo, are not hardy. They grow slowly and have a high apoptotic fraction.^{21,24,26} Studies in our laboratory have focused, in large part, on clarifying molecular determinants of the death pathways in FA cells and on beginning to establish an ordered relationship of these death pathways to discrete FA proteins. We have identified roles for the *fas*/*fas*-ligand pathway,²⁴ caspases 8 and 3,⁵⁵ and the double-stranded RNA-dependent protein kinase.⁵⁶ In this work, although we cannot yet clarify the cause, it is clear that even without being exposed to

environmental cues for apoptosis or mitotic arrest, at a gene-expression level, FA-C cells act as if they have been stimulated by such factors. This is similar to another chromosomal instability syndrome, ataxia telangiectasia (AT). AT cells are hypersensitive to γ irradiation and constitutively overexpress several γ -irradiation-inducible factors.^{57,58} In view of the apoptotic and mitogenic inhibitory activities of these proteins, we suggest that they contribute to the characteristic apoptotic phenotype in FA bone marrow cells and may contribute to the IFN-hypersensitive phenotype by specifically setting ground-state levels of these proteins that are higher than those in normal cells. Clarification of the signaling pathways by which aberrantly expressed genes are controlled in FA-C cells should lead to an understanding of why such cells are

hypersensitive to both biological and chemical inducers of apoptosis and may reveal clearer functions for the *FANCC* gene product.

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The Fanconi Anemia Protein FANCC Binds to and Facilitates the Activation of STAT1 by Gamma Interferon and Hematopoietic Growth Factors

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Hematopoietic progenitor cells from Fanconi anemia (FA) group C (FA-C) patients display hypersensitivity to the apoptotic effects of gamma interferon (IFN- γ) and constitutively express a variety of IFN-dependent genes. Paradoxically, however, STAT1 activation is suppressed in IFN-stimulated FA cells, an abnormality corrected by transduction of normal FANCC cDNA. We therefore sought to define the specific role of FANCC protein in signal transduction through receptors that activate STAT1. Expression and phosphorylation of IFN- γ receptor α chain (IFN- γ R α) and JAK1 and JAK2 tyrosine kinases were equivalent in both normal and FA-C cells. However, in coimmunoprecipitation experiments STAT1 did not dock at the IFN- γ R of FA-C cells, an abnormality corrected by transduction of the FANCC gene. In addition, glutathione *S*-transferase fusion genes encoding normal FANCC but not a mutant FANCC bearing an inactivating point mutation (L554P) bound to STAT1 in lysates of IFN- γ -stimulated B cells and IFN-, granulocyte-macrophage colony-stimulating factor- and stem cell factor-stimulated MO7e cells. Kinetic studies revealed that the initial binding of FANCC was to nonphosphorylated STAT1 but that subsequently the complex moved to the receptor docking site, at which point STAT1 became phosphorylated. The STAT1 phosphorylation defect in FA-C cells was functionally significant in that IFN induction of IFN response factor 1 was suppressed and STAT1-DNA complexes were not detected in nuclear extracts of FA-C cells. We also determined that the IFN- γ hypersensitivity of FA-C hematopoietic progenitor cells does not derive from STAT1 activation defects because granulocyte-macrophage CFU and erythroid burst-forming units from STAT1^{-/-} mice were resistant to IFN- γ . However, BFU-E responses to SCF and erythropoietin were suppressed in STAT1^{-/-} mice. Consequently, because the FANCC protein is involved in the activation of STAT1 through receptors for at least three hematopoietic growth and survival factor molecules, we reason that FA-C hematopoietic cells are excessively apoptotic because of an imbalance between survival cues (owing to a failure of STAT1 activation in FA-C cells) and apoptotic and mitogenic inhibitory cues (constitutively activated in FA-C cells in a STAT1-independent fashion).

Gamma interferon (IFN- γ) induces transcription of a distinct set of genes by activating STAT1, one member of a family of latent cytoplasmic transcription factors that are activated via phosphorylation on tyrosine residues (6). The IFN- γ receptor (IFN- γ R) lacks intrinsic tyrosine kinase activity, but on ligand binding, receptor multimerization results in reciprocal activation of JAK1 and JAK2, two Janus tyrosine kinases noncovalently attached to the IFN- γ R α and β chains (2, 20, 22). Phosphorylation of the tyrosine residue on IFN- γ R α by JAK molecules creates a docking site for the Src homology 2 domain of STAT1, which is then phosphorylated on tyrosine and ultimately forms a homodimeric transcription factor that translocates to the nucleus (6, 21). The factors that govern the traffic of cytoplasmic STAT molecules to the docking site on the IFN- γ R are unknown. We have recently found that the activation of STAT1 in response to IFN- γ is suppressed in hematopoietic cells from children with Fanconi anemia of type C (FA-C) and in mice nullizygous at the FA-C locus. However, in the ground state (uninduced by IFN), IFN response factor 1 (IRF-1) is expressed at high levels in mutant FA-C cells (35), suggesting that a non-STAT1 pathway is involved in constitu-

tive activation of IRF-1 in FA cells. In addition, complementation of the defect by retrovirus mediated transfer of normal FANCC cDNA reconstitutes the normal STAT1 response (10, 38).

Linkage of FANCC function with that of STAT1 provided us with an opportunity to test whether the relationship of these two molecules was direct or indirect. We report herein results of experiments in which the assembly of the fully activated IFN- γ R complex, including STAT1, JAK1, and JAK2, was examined in isogenic murine and human FA-C cells. We report that in IFN- γ -stimulated FA-C cells, phosphorylation of JAK1, JAK2, and IFN- γ R α occurs normally, but STAT1 does not dock at the receptor α chain. In FA-C cells nuclear STAT1 is reduced, and IFN fails to induce STAT1-specific DNA-binding complexes and expression of IRF-1. Expression of the normal FANCC cDNA in mutant cells results in normal STAT1 docking and phosphorylation as well as normal induction of nuclear STAT1-DNA complex and normal induction of IRF-1. We also find that a variety of cytokines and hematopoietic growth factors stimulate the association of STAT1 with glutathione *S*-transferase (GST) fusion proteins encoding the normal FANCC but not a naturally occurring inactivating mutant FANCC (L554P) and that the association occurs rapidly and prior to STAT1 phosphorylation on Y⁷⁰¹. Coimmunoprecipitation experiments confirmed the IFN-inducible association of

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endogenous FANCC and STAT1 in normal but not mutant cells. We propose that FANCC is a control factor for STAT1 traffic to receptor docking sites, and that mutations that inactivate FANCC function interdict this function. We propose that the hematopoietic defects in FA derive, at least in part, from a loss of balance between mitogenic cues (owing to a reduction of signals transduced through receptors for growth factors that activate STAT1) and mitogenic inhibitory cues (secondary to FANCC-dependent, STAT1-independent constitutive activation of mitotic inhibitory factors), in particular IRF-1, a factor known to effect apoptotic responses in hematopoietic progenitor cells (43).

MATERIALS AND METHODS

Cell cultures and IFN- γ stimulation. Epstein-Barr virus-transformed lymphoblasts were maintained in RPMI 1640 medium (RPMI; Life Technologies, Grand Island, N.Y.) supplemented with 15% heat-inactivated fetal calf serum and grown in a humidified 5% CO₂-containing atmosphere at 37°C. The lymphoblast lines JY and HSC536N (FA-C) have been described elsewhere (38). Unless otherwise indicated, cells were stimulated with recombinant human IFN- γ (1 ng/ml; R & D Systems, Minneapolis, Minn.) for the indicated time periods. For detection of STAT1 phosphorylation, cells were serum starved for 2 h prior to IFN- γ treatment. The leukemia cell line MO7e and its high erythropoietin receptor (EPOR)-expressing subline MO7ER (51) were maintained in RPMI containing 20% fetal calf serum and granulocyte-macrophage colony-stimulating factor (GM-CSF; 100 U/ml; Genetics Institute, Cambridge, Mass.) or EPO (2 U/ml; Amgen Inc., Thousand Oaks, Calif.). Before stimulation, cells were washed and incubated for 18 h at 37°C in serum-free RPMI containing 0.5% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). After starvation, cells were washed and then exposed to IFN- γ (10 ng/ml), IFN- α (20,000 U/ml; Schering Corp., Kenilworth, N.J.), GM-CSF (100 U/ml), SCF (50 ng/ml; R & D Systems), and EPO (40 U/ml) for 10 min.

Retroviral infection and transduction of FA cell lines. FANCC cDNA (47) was subcloned into the retroviral vector pLXSN as described previously (38). Plasmid pLXSN-FANCC (10 μ g) was transfected by the calcium phosphate precipitation method into PA12 producer cells expressing the vesicular stomatitis virus G envelope protein (26). Retroviral supernatants were collected and used for transduction of FA-C cell lymphoblasts. HSC536N cells were exposed to the filtered supernatants containing virus in the presence of Polybrene (8 μ g/ml; Sigma). Sets of isogenic lines were selected in G418 and then exposed to multiple doses of mitomycin C in cytotoxicity (trypan blue viability) and apoptosis (TUNEL [terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling] and annexin V binding) assays to ensure that the mutant cells transduced with the normal cDNA were fully complemented. HSC536N/FANCC cells are HSC536N cells transduced by the FANCC vector. HSC536N/neo cells are HSC536N cells transduced by the vector alone.

Preparation of whole-cell lysates (WCL). Following treatment with IFN- γ , cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with either radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.6], 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS]) or Nonidet P-40 (NP-40) lysis buffer (1% NP-40, 20 mM Tris-HCl [pH 8.0], 137 mM NaCl, 10% glycerol). Both lysis buffers were supplemented with the following freshly made protease inhibitors: 1% aprotinin, 1 μ g of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 mM sodium orthovanadate. For cell lysates used specifically for assessment of protein-protein interactions, cells were lysed in digitonin lysis buffer (1% digitonin, 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 50 mM NaF, 5 mM sodium pyrophosphate) containing the protease inhibitors aprotinin, leupeptin, and PMSF. After rocking at 4°C for 20 min (60 min when cells were lysed with digitonin buffer), cell lysates were cleared by centrifugation at 13,200 rpm for 20 min at 4°C, and protein concentrations were determined by the Bradford method (4) using a protein microassay reagent (Bio-Rad, Hercules, Calif.).

Electromobility shift assay (EMSA). Nuclear extracts were prepared by the method of Dignam et al. (8). A human IRF-1 IFN- γ activation sequence (GAS) oligonucleotide (5'-ACAACAGCTGATTTCGCCGAA-3') was synthesized in the Molecular Biology Core Lab (Portland VA Medical Center) and then labeled with [γ -³²P]ATP to 2.5 $\times$ 10⁴ cpm/ng, using T4 polynucleotide kinase (Boehringer Mannheim). The binding reaction mixture (20 μ l) contained 5.0 to 7.5 μ g of nuclear extracts, 0.2 ng of labeled oligonucleotide 2 μ g of poly(dI-dC), and 10 μ g of bovine serum albumin in 10 mM Tris-Cl (pH 7.4)-50 mM NaCl-1 mM dithiothreitol (DTT)-1 mM EDTA-10% glycerol. Reaction mixtures were incubated at room temperature for 30 min and then resolved on a 4% polyacrylamide gel in 25 mM Tris, 190 mM glycine, and 1 mM EDTA. Gels were dried and autoradiographed with intensifying screens at -80°C.

Reverse transcription-PCR. Total RNA was isolated from cells using Tri Reagent (Molecular Research Center, Inc., Cincinnati, Ohio) in accordance with the manufacturer's instructions. First-strand cDNA was reverse transcribed from the indicated RNA using random hexanucleotide primers (Gibco BRL) and

mouse mammary tumor virus RNase H⁻ reverse transcriptase (Gibco BRL) as previously described (46). The cDNA was then amplified by PCR for 35 cycles (denatured at 94°C for 30 s, primer annealed at 53°C for 30 s, and primer extended at 72°C for 30 s). Control reactions in which no cDNA (i.e., water only) was added to the PCR mixture were analyzed with each experiment. For IRF-1 amplification, the primers used were 5'-GAGCTGGGCCATTCACACAG-3' and 5'-CATGGCGACAGTGTGGAGT-3', which produced an amplicon with a predicted length of 390 nucleotides. The amplification products were separated by electrophoresis on 1.5% agarose, stained with ethidium bromide, and visualized by UV transillumination.

Antibodies. Antibodies used included antiphosphotyrosine (anti-P-Tyr) antibody 4G10, provided by Brian Druker (Oregon Health Sciences University, Portland), anti-JAK1 monoclonal antiserum (Transduction Laboratories, Lexington, Ky.), anti-JAK2 polyclonal antiserum (Upstate Biotechnology, New York, N.Y.), anti-IFN- γ R α polyclonal antibody (Antigenix America Antibodies, New York, N.Y.), anti-STAT1 α monoclonal antiserum from Santa Cruz Biotechnology (Santa Cruz, Calif.), rabbit polyclonal anti-Stat3, anti-Stat5, and antibodies specific for the tyrosine-phosphorylated forms of STAT1 (P-STAT1), Stat3, and Stat5 (New England Biolabs, Beverly, Mass.), anti-FANCC polyclonal antiserum as described previously (52), and sheep anti-mouse and donkey anti-rabbit horseradish peroxidase-conjugated antibody (Amersham, Arlington Heights, Ill.). All antibodies were used in accordance with the manufacturer's instructions.

Immunoprecipitation. WCLs (about 1 mg of total proteins) were precleared with 50 μ l of 50% protein A-Sepharose suspension (Pharmacia Biotech, Inc.) for 1 h at 4°C. After separation of the protein A-Sepharose from the lysate by centrifugation at 1,000 rpm for 1 min at 4°C, the lysate was incubated with either anti-P-Tyr antibody 4G10 (12 μ g/ml), anti-IFN- γ R α (2 μ g/ml), or anti-STAT1 (1 μ g/ml) for 3 to 5 h at 4°C with constant agitation. Immunocomplexes were then bound to protein A-Sepharose beads (50 μ l of 50% slurry) during a 1- to 2-h incubation at 4°C. The immunoprecipitates were recovered by centrifuging at 1,000 rpm for 1 min at 4°C. If cells were lysed in NP-40 lysis buffer, the immunocomplexes were washed three times with 0.05% NP-40 wash buffer (0.05% NP-40 in 1 $\times$ PBS). If cells were lysed in digitonin lysis buffer, the immunocomplexes were then washed with 0.1% digitonin wash buffer (same as digitonin lysis buffer except that the concentration of digitonin was 0.1%).

Immunoblotting. WCL, nuclear extracts, or immunocomplexes were prepared as above and subjected to immunoblot analyses. Samples were heated at 94°C for 5 min in 2 $\times$ Laemmli SDS sample buffer, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto Bio-Blot nitrocellulose membranes (Costar, Cambridge, Mass.). Nonspecific binding sites on the membranes were blocked with Tris-buffered saline plus 0.05% Tween 20 containing 5% nonfat dry milk for 1 h at room temperature or overnight at 4°C. Immunoblots were subsequently incubated with the indicated primary antibodies at a dilution according to the manufacturer's instruction. After stringent washing, the blots were incubated with appropriate secondary antibodies (linked to horseradish peroxidase) for 30 min at room temperature and developed by using an enhanced chemiluminescence kit (Amersham).

GST-FANCC fusion protein purification and in vitro binding assays. The GST-FANCC and GST-L554P fusion constructs were created by insertion of the entire open reading frame of FA-C and its mutant version, L554P, into the *Bam*HI and *Sma*I sites of yeast overexpression vector pGST (31). The plasmids were transformed into yeast strain Sc334 (19) using a Frozen-EZ yeast transformation kit (ZYMO Research, Orange, Calif.). Expression and purification of the yeast GST fusion proteins were performed as described elsewhere (31, 32), with minor modifications. The GST fusion protein was prepared by suspending the yeast cells containing pGST, pGST-FANCC, or pGST-L554P in an equal volume of buffer A (37.5 mM K₂PO₄ [pH 9.5], 7.5 mM DTT, 1.5 mM EDTA, 1 M NaCl, 2.5% glycerol, 7.5% ethylene glycol, 0.03% silicon antifoam), and the cells were broken by vortexing with an equal volume of glass beads (eight 15-s bursts). The lysate was removed from the beads, and Triton X-100 was added to 1%. After being clarified twice by centrifugation, the supernatant was incubated for 30 min at 4°C with 0.1 ml of glutathione-Sepharose 4B (Pharmacia Biotech, Piscataway, N.J.) 50% slurry equilibrated with buffer B (50 mM KPO₄ [pH 7.2], 1 mM DTT, 0.5 mM EDTA, 1 M NaCl, 10% glycerol, 1% Triton X-100). The Sepharose beads were washed three times with buffer B containing 0.5 M NaCl. For binding, about 10 μ g of GST, GST-FANCC, or GST-L554P fusion protein bound to glutathione-Sepharose beads was incubated with WCL from untreated or IFN- γ -treated JY cells (1 mg of total proteins) prepared in 1% digitonin buffer supplemented with 1% aprotinin, 1 μ g of leupeptin per ml, 1 mM PMSF, and 2 mM sodium orthovanadate. The final concentration of digitonin in the binding mixture was adjusted to 0.5%. After shaking at 4°C for 40 min, the beads were recovered and washed three times with 0.1% digitonin buffer before boiling in Laemmli SDS sample buffer and immunoblotting as described above. For WCL input controls, 60 to 100 μ g of total WCL proteins was loaded in each lane.

In vivo phosphate labeling. Cells were starved for 90 min in phosphate-free RPMI containing 15% dialyzed fetal bovine serum, and [³²P]orthophosphate (150 μ Ci/ml; DuPont NEN) was added to the medium. After labeling for 3 h, cells were incubated with IFN- γ (10 ng/ml) for the indicated time points. WCL prepared as described above were subjected to affinity precipitation with GST-FANCC and analyzed by SDS-PAGE followed by autoradiography. West-

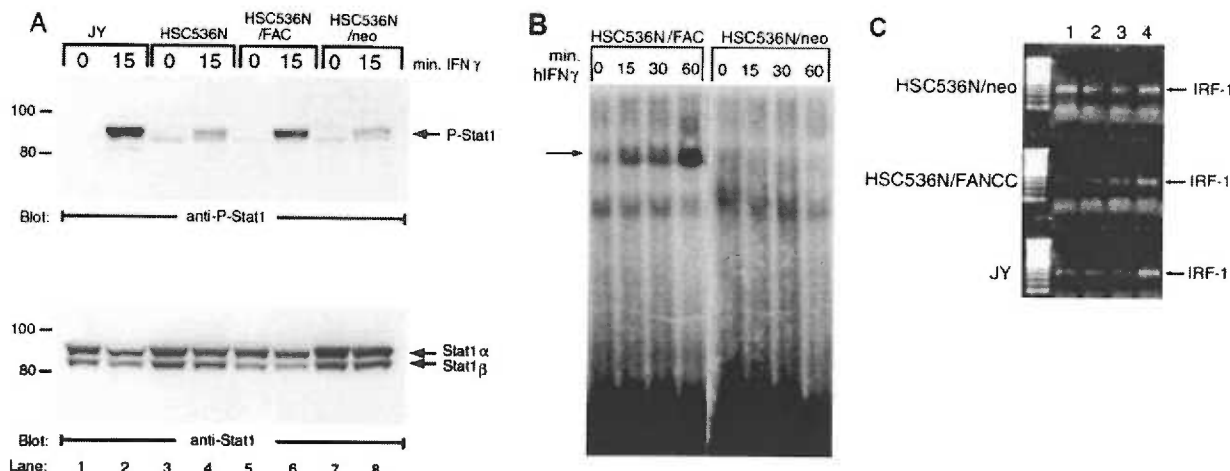


FIG. 1. Defective STAT1 tyrosine phosphorylation in IFN- γ -stimulated FA-C cells. (A) Cells from the indicated FA-C isogenic lines were serum starved for 2 h, incubated with IFN- γ for the indicated time periods at 37°C, and then solubilized in RIPA lysis buffer as described in Materials and Methods. The WCL were separated by SDS-PAGE and blotted with antibodies to P-STAT1. (B) The blot described above, stripped and reprobed with anti-STAT1. Here and in subsequent figures, the label "FAC" represents FANCC, and the positions of migration of protein standards (in kilodaltons) are indicated on the left. (C) EMSA of STAT1 binding to oligonucleotides corresponding to the STAT1-specific GAS element in the IRF-1 promoter. Nuclear extracts were prepared from HSC536N/FANCC (lanes 1 to 4) and HSC536N (lanes 5 to 8) cells untreated (lanes 1 and 5) or treated with 1 ng of IFN- γ per ml (lanes 2 to 4 and 6 to 8). Arrow denotes STAT1-GAS complexes. (D) IFN- γ -induced expression of IRF-1 transcript. Horizontal rows represent amplification from individual cell lines as indicated. Vertical columns show samples from untreated cells at time zero (lane 1), untreated cells at 3 h (lane 2), cells treated with IFN- γ (0.5 ng/ml) for 3 h (lane 3), and cells treated with IFN- γ (50 ng/ml) for 3 h (lane 4).

crn blot analysis was performed on these precipitates to determine the quantity of both tyrosine-phosphorylated and total STAT1 bound to GST-FANCC.

Murine clonal BM cell cultures. Bone marrow (BM) samples were obtained from an equal number of FANCC^{-/-} and FANCC^{+/+} mice (55) and STAT^{-/-} and STAT^{+/+} mice (9, 28) for each experiment. Femoral marrow samples obtained from mice after cervical dislocation were washed and resuspended in RPMI medium for viable counts (trypan blue dye exclusion). Unfractionated BM cells (10^5) were cultured in 1 ml of methoCult H4230 (Stem Cell Technologies, Vancouver, British Columbia, Canada), penicillin-streptomycin (Life Technologies), and either murine SCF (from 0.10 to 10 ng/ml; R & D Systems), murine EPO (2 ng/ml), or both SCF and EPO. A combination of three growth factors was used as a positive control: murine Steel factor (10 ng/ml; R & D Systems), murine interleukin-3 (IL-3; 10 ng/ml; R & D Systems), and human EPO (2 U/ml; Amgen). Granulocyte-macrophage CFU (CFU-GM) were cultured in 35-mm-diameter tissue culture dishes at 37°C in 5% CO₂ in air and counted after 7 and 14 days, using a dissecting microscope. Colony growth results were expressed as mean (of triplicate plates) colonies per plate $\pm$ standard deviation (SD). Between-group comparisons were made using one-way analysis of variance.

RESULTS

Deficient STAT1 α activation in FA-C cells. We have recently shown that IFN- γ -induced STAT1 tyrosine phosphorylation is reduced in FA-C cells (38). Equal numbers of cells from each isogenic line were serum starved for 2 h followed by stimulation with IFN- γ at 37°C for the indicated time periods. WCL were prepared in RIPA buffer (see Materials and Methods) and analyzed by immunoblotting with antisera specific to P-STAT1. As shown in Fig. 1, the FA-C mutant cell lines HSC536N and HSC536N/neo contained less P-STAT1 than the normal B-cell line JY following IFN- γ stimulation (Fig. 1A, lane 2 versus lanes 4 and 8). Introduction of the normal FANCC gene into FA-C cells (HSC536N/FANCC) corrected the STAT1 activation defect (Fig. 1A, lane 6), suggesting that STAT1 activation via tyrosine phosphorylation is dependent on expression of a normal FANCC gene product. Reprobing the blot with anti-STAT1 antibody showed that STAT1 protein was expressed at comparable levels in all four cell lines, as indicated in the lower panel.

To ensure that FA-C cells had reduced STAT1 function, we sought to identify nuclear STAT1 and found none at any time

point from 5 to 120 min after IFN- γ stimulation (data not shown). Using an EMSA with a STAT1-specific oligonucleotide as a probe, we observed STAT1-DNA complexes in the nuclear extracts from IFN-stimulated HSC536N/FANCC cells (Fig. 1B, lanes 1 to 4). In contrast, no constitutive or inducible STAT1-specific DNA-binding activity was detected in the nuclear extracts from mutant FA-C cells (Fig. 1B, lanes 5 to 8). In addition, IFN- γ induction of IRF-1 was also abolished in mutant cells (Fig. 1C) as we have described elsewhere (38).

Tyrosine phosphorylation of IFN- γ R α in IFN- γ -stimulated B lymphocytes. Cellular responsiveness to IFN- γ requires the activation of IFN- γ R α through phosphorylation on tyrosine residues (12, 13, 22). We therefore asked whether ligand-induced IFN- γ R α tyrosine phosphorylation was different quantitatively and temporally in FA-C cells. Cells were treated with IFN- γ for the indicated time, and lysates were incubated with anti-IFN- γ R α antibodies. In the absence of IFN- γ , no tyrosine-phosphorylated IFN- γ R α was detected in any of the isogenic cells (Fig. 2A, lanes 1, 4, 7, and 10). Following addition of ligand, equivalent IFN- γ R α tyrosine phosphorylation was observed in all four isogenic cell lines (Fig. 2A, lanes 2, 3, 5, 6, 8, 9, 11, and 12). The kinetics of induced IFN- γ R α tyrosine phosphorylation, which was maximal at 5 min and remained at maximal levels within 10 min of exposure to IFN- γ , was similar in all four cell lines. The amounts of the receptor proteins loaded in each lane were comparable, as determined by reprobing the membrane with anti-IFN- γ R α antibody (Fig. 2B).

Tyrosine phosphorylation of JAK1 and JAK2 kinases in FA-C cells. JAK1 and JAK2 are activated by IFN- γ through tyrosine phosphorylation, and these activated kinases play an essential role in transduction of the IFN- γ signal, at least in part, by phosphorylating STAT1 (25, 30, 33, 35, 39, 54). To determine whether IFN- γ -induced tyrosine phosphorylation of the JAK kinases was normal in FA-C cells, WCL prepared from normal and FA-C cells were first immunoprecipitated with anti-P-Tyr antibody 4G10 and then immunoblotted with

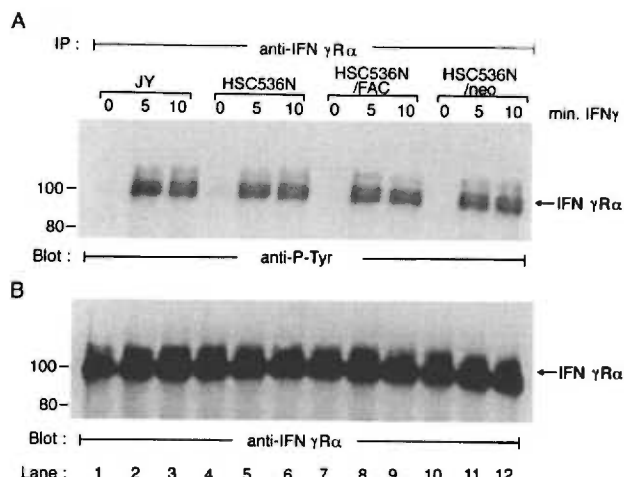


FIG. 2. Activation of IFN- γ R α in response to IFN- γ in FA-C cells. Cells were incubated with IFN- γ for the indicated time periods at 37°C and then solubilized in NP-40 lysis buffer as described in Materials and Methods. The WCL were immunoprecipitated (IP) with anti-IFN- γ R α antibody. The immunoprecipitates were then analyzed by immunoblotting. (A) Cell lysates prepared from four FA-C isogenic lines were immunoprecipitated with anti-IFN- γ R α and probed with anti-P-Tyr; (B) the blot described above was stripped and reprobed with anti-IFN- γ R α .

antibodies against JAK1 and JAK2. As shown in Fig. 3, tyrosine phosphorylation of JAK1 (Fig. 3A) and JAK2 (Fig. 3C) was evident in all four isogenic cell lines after 5 min of IFN- γ treatment. Tyrosine phosphorylation of JAK1 or JAK2 was not detected in unstimulated cells (Fig. 3A and C, lanes 1, 3, 5, and 7). The kinetics of induced JAK phosphorylation were comparable in normal and FA-C cell lines. To ensure that expression of JAK1 and JAK2 proteins was equivalent in both normal and FA-C cells, we performed immunoblotting with anti-JAK1 and -JAK2 antibodies, using WCL from the same cell lines. As expected, both the untreated and IFN- γ -treated samples prepared from each cell line were found to contain similar levels of JAK1 and JAK2 (Fig. 3B and D). These results indicate that

after ligation of IFN- γ to its receptor, activation, by tyrosine phosphorylation, of JAK1 and JAK2 was normal in FA-C cells.

IFN- γ -induced coimmunoprecipitation of IFN- γ R α with STAT1 requires the FANCC protein. IFN- γ -induced tyrosine phosphorylation of IFN- γ R α creates a docking site for latent STAT1, which, after docking, is then activated by the JAK kinases (6, 18, 22, 58). In support of this model, it has been shown that STAT1 associates with tyrosine-phosphorylated IFN- γ R α both in vitro and in vivo (12, 13, 42). Having established the normal pattern of IFN- γ -induced tyrosine phosphorylation of IFN- γ R α and the two JAK kinases in FA-C cells, we next asked whether STAT1 was recruited to the activated IFN- γ R complex. IFN- γ -treated isogenic cells were solubilized with 1% digitonin buffer, and protein complexes were immunoprecipitated using antibody to IFN- γ R α . The presence of STAT1 in anti-IFN- γ R α immunoprecipitates was assessed by immunoblotting with either anti-P-Tyr or anti-STAT1 α monoclonal antibody. When material precipitated using lysates from FA-C isogenic cell lines was analyzed with the anti-P-Tyr antibody, a diffusely migrating phosphoprotein of ~100 kDa was detected in all four cell lines only when cells had been treated with IFN- γ (Fig. 4A, lanes 2, 4, 6, and 8). This 100-kDa phosphoprotein was identified by reprobing the blot with anti-IFN- γ R α (Fig. 4E). In addition to the tyrosine-phosphorylated IFN- γ R α , a distinct phosphoprotein of ~90 kDa recognized by anti-P-Tyr immunoblotting was also precipitated by anti-IFN- γ R α in lysates prepared from IFN- γ -treated normal (JY) and complemented FA-C (HSC536N/FANCC) cell lines but not in those from stimulated FA-C (HSC536N and HSC536N/neo) cell lines (Fig. 4A, lanes 2 and 6 versus 4 and 8). Reprobing the blot with a monoclonal antibody specific to STAT1 α revealed that the 90-kDa band was STAT1 α (Fig. 4C, lanes 2 and 6). No signal was detected in the anti-STAT1 α immunoblot when anti-IFN- γ R α immunoprecipitates had been prepared from IFN- γ -treated mutant cell lines HSC536N and HSC536N/neo (Fig. 4C, lanes 4 and 8) or from cells that had not been incubated with IFN- γ (Fig. 4C, lanes 1, 3, 5, and 7).

To confirm the above observations, we conducted coimmunoprecipitation experiments using anti-STAT1 antibody. As shown in Fig. 4B, on the blot probed with anti-P-Tyr, two

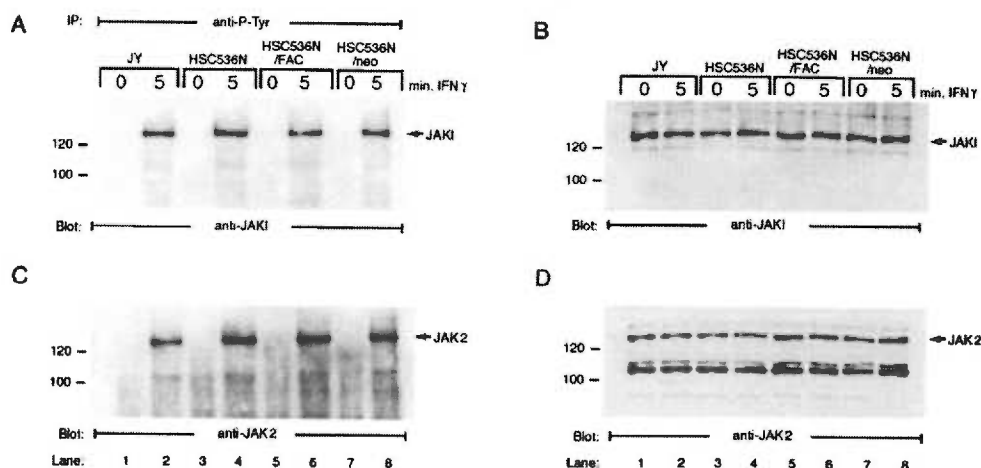


FIG. 3. IFN- γ -induced tyrosine phosphorylation of JAK1 and JAK2 in FA-C cells. Cells were stimulated with IFN- γ for various time periods and lysed in NP-40 lysis buffer. The WCL were either directly analyzed by immunoblotting with anti-JAK1 and -JAK2 antibodies or immunoprecipitated (IP) with anti-P-Tyr antibody, and the immunoprecipitates were then analyzed by immunoblotting. (A) Cell lysates prepared from four FA-C isogenic lines were immunoprecipitated with anti-P-Tyr and probed with anti-JAK1 antibody; (B) cell lysates were directly immunoblotted with anti-JAK1 antibody; (C) cell lysates prepared from FA-C isogenic lines were immunoprecipitated with anti-P-Tyr and probed with anti-JAK2 antibody; (D) cell lysates were directly immunoblotted with anti-JAK2 antibody.

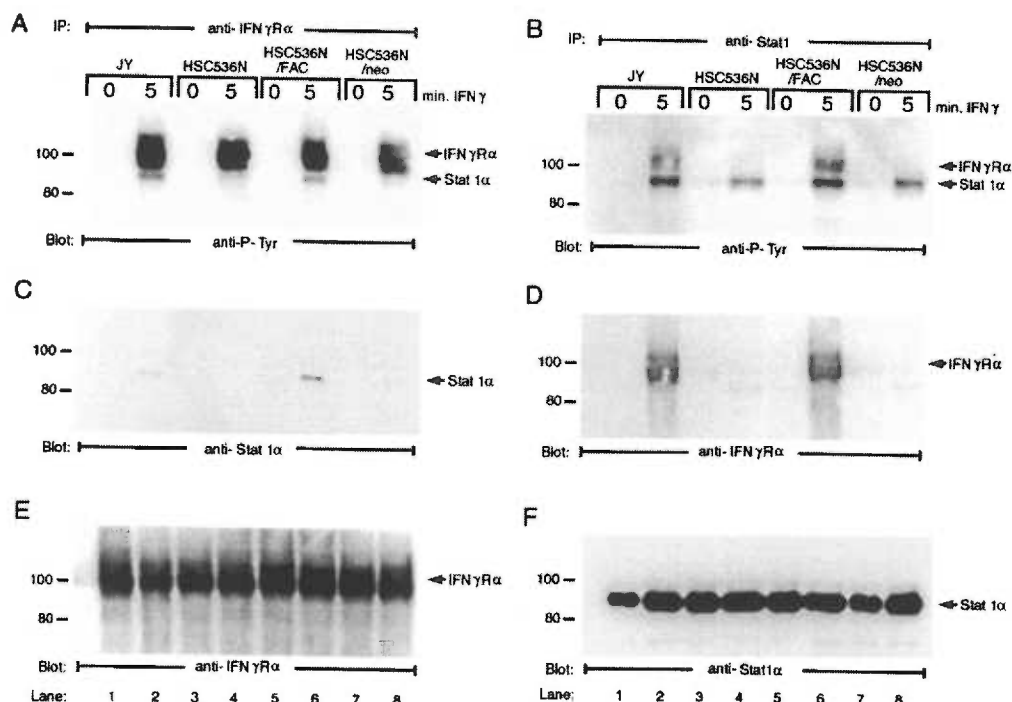


FIG. 4. IFN- γ -induced formation of STAT1 α -IFN- γ R α complex in B lymphocytes. Cells were stimulated with IFN- γ for up to 10 min and solubilized in digitonin lysis buffer as described in Materials and Methods. The WCL were then incubated with anti-IFN- γ R α or anti-STAT1 antibody. The immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting. (A) Cell lysates prepared from FA-C isogenic lines were immunoprecipitated (IP) with anti-IFN- γ R α and probed with anti-P-Tyr antibody; (B) cell lysates prepared from FA-C isogenic lines were immunoprecipitated with anti-STAT1 and probed with anti-P-Tyr antibody; (C) the blot used for panel A was stripped and reprobed with anti-STAT1 α monoclonal antibody; (D) the blot used for panel B was stripped and reprobed with anti-IFN- γ R α monoclonal antibody; (E) the blot used for panel A was stripped and reprobed with anti-IFN- γ R α ; (F) the blot used for panel B was stripped and reprobed with anti-STAT1 α .

tyrosine-phosphorylated proteins are evident in both IFN- γ -treated normal (JY) and complemented (HSC536N/FANCC) cell lines but not in stimulated mutant FA-C (HSC536N and HSC536N/neo) cell lines (lanes 2 and 6 versus 4 and 8). The upper band proved to be IFN- γ R α , as indicated in Fig. 4D (lanes 2 and 6), when we reprobbed the membrane with antibody to the receptor protein. Although a small amount of phosphorylated STAT1 was precipitated by anti-STAT1 antibody in FA-C cell lines HSC536N and HSC536N/neo, no co-precipitating IFN- γ R α receptor protein was detected (Fig. 4B and D, lanes 4 and 8), indicating reduced association of STAT1 with the receptor in mutant cells.

As an important technical note, we emphasize that we were unable to detect STAT1 in anti-IFN- γ R α or IFN- γ R α in anti-STAT1 immunoprecipitates from cell lysates prepared with RIPA buffer or NP-40 lysis buffer (see Materials and Methods). Washing of the immunoprecipitate in 1% Triton X-100, 1% NP-40, or 1% digitonin lysis buffer resulted in the disruption of the STAT1-IFN- γ R α complex, suggesting that interaction between STAT1 and the receptor protein may be weak or unstable. Finally, Western blot analysis of the anti-IFN- γ R α and -STAT1 immunoprecipitates demonstrated the presence of equivalent IFN- γ R α and STAT1 proteins in all four cell lines at 5 min of IFN- γ incubation compared to unstimulated cells (Fig. 4E and F), indicating that both the receptor and STAT1 proteins were constitutively expressed at equivalent levels in all isogenic cell lines. Taken together, these results demonstrate an unambiguous requirement for the FANCC protein in the formation of IFN- γ -induced STAT1 α -IFN- γ R α complexes.

IFN- γ -induced association of STAT1 with FANCC. The coimmunoprecipitation result (Fig. 4) demonstrated that FANCC is required for recruitment of STAT1 to the activated IFN- γ R complex. Because this suggested that the activation of STAT1 might result from a direct interaction of the FANCC protein with STAT1, we sought to identify a binding interaction between FANCC and STAT1. JY cells were stimulated with IFN- γ or left untreated, and WCL were incubated with glutathione-Sepharose bead-bound GST fusion proteins containing the normal FANCC (GST-FANCC) or a patient-derived mutant FANCC (GST-L554P). FANCC-interacting cellular proteins were then isolated from WCL by glutathione-Sepharose affinity precipitation. STAT1 Western blot analysis of the affinity-precipitated proteins (Fig. 5A, top) showed that STAT1 bound strongly to the normal FANCC fused to GST (lane 4) but not to GST alone (lanes 1 and 2). STAT1 bound weakly to the GST fusion protein containing the L554P mutation (lane 6). Reprobing the blot with antibodies to FANCC revealed comparable amounts of GST-fused FANCC and L554P proteins were input in the binding reactions (Fig. 5A, bottom). It is important to note that FANCC-STAT1 interaction was detected only in lysates prepared from IFN- γ -stimulated cells (lanes 3 and 5 versus 4 and 6), suggesting that the association is initiated by early activation elements of the IFN- γ signaling pathway.

Because STAT1 interacted in vitro with GST-FANCC, we sought to determine whether similar association also occur within cells. To detect complexes formed between endogenous FANCC and STAT1 in IFN- γ -stimulated lymphocytes, we performed immunoprecipitation with an affinity-purified

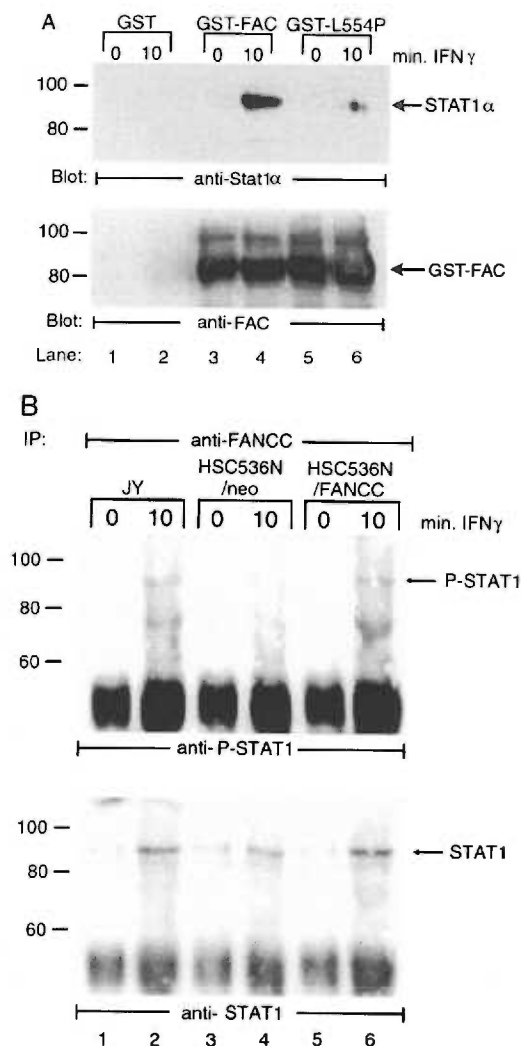


FIG. 5. IFN- γ -induced association of FANCC and STAT1 in B lymphocytes. (A) WCL (1 mg of protein) of JY cells stimulated with IFN- γ for up to 10 min were incubated with yeast-expressed GST fusion proteins (10 μ g) bound to glutathione-Sepharose beads. The glutathione-Sepharose affinity precipitates were analyzed by SDS-PAGE followed by immunoblotting with antibodies to STAT1 α (top) and reprobed with anti-FANCC to verify the input of the GST fusion proteins. (B) Coimmunoprecipitation (IP) of endogenous FANCC and STAT1. WCL prepared from JY and HSC536N cells (500 μ l of each, containing 1 mg of total proteins) were incubated with FANCC-specific antibody in the presence of protein A-agarose. The immunocomplexes were then analyzed by Western blotting with antibody specific for P-STAT1 (top) and for total STAT1 (bottom).

FANCC-specific antiserum. The precipitates were analyzed by Western blotting with antibodies against either P-STAT1 or total STAT1. As shown in Fig. 5B, a detectable P-STAT1 signal was obtained in precipitates from IFN- γ -stimulated JY and HSC536N/FANCC cells, which was absent in samples from the stimulated mutant HSC536N cells (lanes 2 and 6 versus 4). There was no detectable P-STAT1 in precipitates from unstimulated cells (lanes 1, 3, and 5). This result was further substantiated by reprobing the blot with an antibody against total STAT1. The lower panel of Fig. 5B clearly demonstrated that FANCC coimmunoprecipitated with STAT1 in the extracts from the normal JY and corrected HSC536N/FANCC cells treated with IFN- γ (Fig. 5B, bottom, lanes 2 and

6). We also found that there was significantly reduced total STAT1 bound to FANCC in the mutant HSC536N/neo extracts (lane 4), consistent with our observation that STAT1 bound weakly to the GST fusion protein containing the mutant FANCC L554P (Fig. 5A, lane 6). Faint bands were detected in the precipitates from unstimulated cell extracts when the blot was reprobed with antibody against total STAT1 (Fig. 5B, bottom, lanes 1, 3, and 5). This was probably due to a slight reactivity of the antiserum with a protein that was migrating at the same position as the STAT1 protein, as we observed the same faint band in a preimmune precipitation (data not shown). To assess whether the FANCC mutation directly caused a failure of STAT1-FANCC binding, we performed a binding assay using WCL from HSC536N cells. As shown in Fig. 6B, STAT1 from IFN-stimulated HSC536N cells was able to bind to a normal GST-FANCC with association kinetics comparable to that observed in the normal JY cells (Fig. 6A), indicating that the limiting factor for STAT1 complex formation in IFN-stimulated FA-C mutant cells is FANCC itself.

IFN- γ initially induces FANCC association with STAT1 form not phosphorylated on Y⁷⁰¹. We further examined kinetically whether the interaction of FANCC-STAT1 in induced cells depended on prior tyrosine phosphorylation of STAT1. We stimulated JY cells with IFN- γ for various periods of time and performed GST fusion protein affinity binding assays. FANCC-bound STAT1 could be detected as early as 30 s after IFN- γ treatment, reached its maximal level at 5 min, and decreased at 30 min after stimulation (Fig. 6A, top). Interestingly, the amounts of total STAT1 proteins associated with FANCC were comparable between 30 s and 1 min of IFN- γ treatment, but the amount of phosphorylated STAT1 bound to FANCC at the 30-s time point was negligible compared to that at the 1-min time point (Fig. 6A, top and middle, compare lanes 4 and 5). While we cannot rule out the possibility that the antibody against total STAT1 may have higher affinity than the one specific for P-STAT1, comparison of P-STAT1 and total STAT1 at the 30-s, 1-min, and 5-min time points clearly demonstrated an increase in the phosphorylated form that occurs only following full binding of the nonphosphorylated form of STAT1. Because the kinetic appearance of STAT1 at the docking site occurs at the time of phosphorylation, our results are most compatible with the notion that IFN- γ initiates the association of FANCC with STAT1 before STAT1 becomes phosphorylated and that the association of FANCC with P-STAT1 results from the delivery by FANCC to the IFN- γ R docking site. Because STAT1 is also phosphorylated on serines (e.g., Ser⁷²⁷), additional studies using [³²P]orthophosphate-labeled cells were conducted to confirm that the initial association of STAT1 with FANCC is with an unphosphorylated form. Figure 6C (top) shows that significantly more P-STAT1 was bound to GST-FANCC in IFN-stimulated JY cells at 20 min than at the 1-min time point. However, the amounts of FANCC-bound P-STAT1 and total STAT1 were comparable between these two time points (Fig. 6C, middle, compare lanes 2 and 3). This suggests that the more intense signal obtained at 20 min of IFN- γ stimulation is most likely due to serine phosphorylation. In HSC536N cells, there was no P-STAT1 bound to GST-FANCC at 1 min of IFN stimulation, but P-STAT1 was evident at the 20-min time point. Interestingly, however, total bound STAT1 could be detected in equal amount at both 1- and 20-min time points even though none of these bound STAT1 proteins were tyrosine phosphorylated. Taken together, these results suggest that the association of STAT1 with FANCC precedes STAT1 phosphorylation.

FANCC-STAT1 association in MO7e cells and its functional relevance. We next sought to determine whether FANCC-

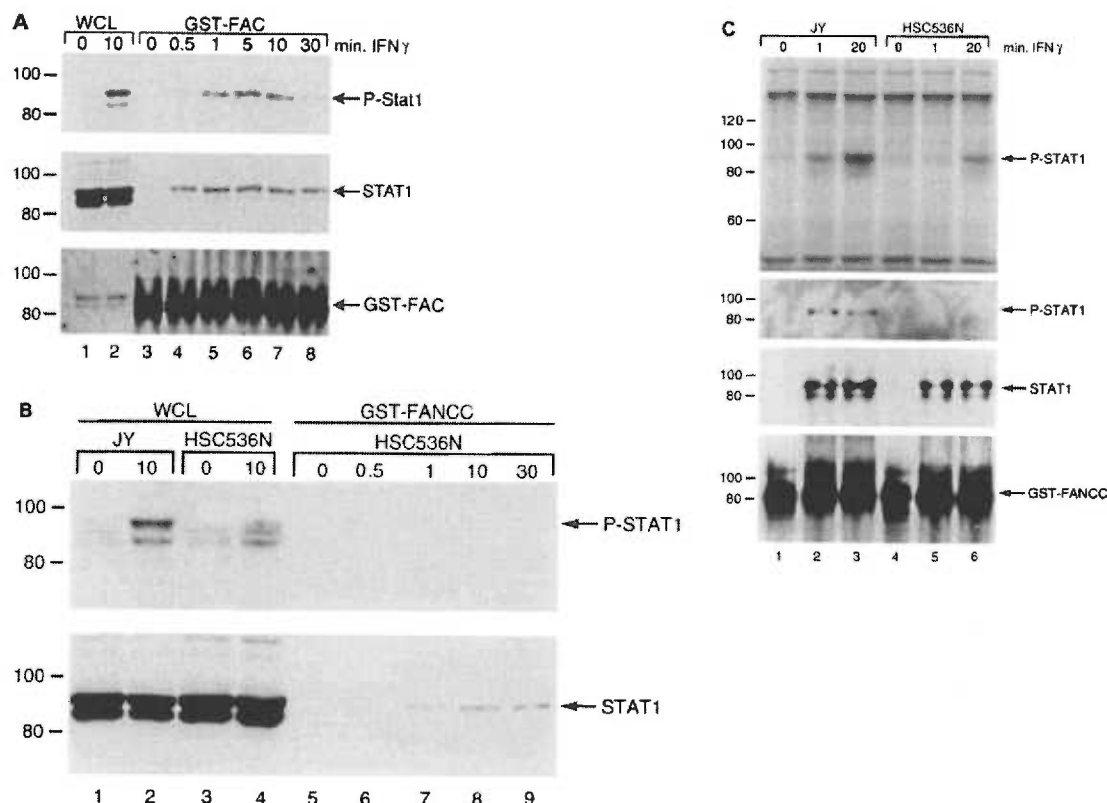


FIG. 6. Kinetics of FANCC-STAT1 association. WCL (1 mg of total proteins) of JY (A) and HSC536N (B) cells stimulated with IFN- γ (10 ng/ml) for various periods of time were incubated with GST-FANCC (10 μ g) bound to glutathione-Sepharose beads, and the glutathione-Sepharose affinity precipitates were analyzed by immunoblotting with antibodies to P-STAT1 (A and B, top), STAT1 α/β (A, middle; B, bottom), and FANCC (A, bottom). Lanes 1 and 2 in panel A and lanes 1 to 4 in panel B are the input controls, each of which was loaded with 100 μ g of total WCL proteins. The double bands in lanes 1 and 2 of the bottom gel in panel A are nonspecific. (C) In vivo phosphorylation of STAT1 in cells stimulated with IFN- γ . JY and HSC536N cells were metabolically labeled with [^{32} P]orthophosphoric acid for 4 h followed by IFN- γ treatment for the indicated time points. WCL were prepared and affinity precipitated with GST-FANCC and analyzed by SDS-PAGE and autoradiography (top). Western blot analysis, performed on these affinity precipitates to determine the amounts of tyrosine-phosphorylated and total STAT1 proteins, is shown in the lower three panels, which were probed with antibodies specific for P-STAT1, STAT1, and FANCC, respectively. The order of samples is the same in all panels.

STAT1 association occurs in normal myeloid precursor cells. MO7e cells were incubated in the presence of IFN- γ , and analysis of the WCL by Western blotting indicated that IFN- γ induced tyrosine phosphorylation of STAT1 in MO7e cells (Fig. 7A, top, lane 2). A GST fusion protein affinity binding assay showed that IFN- γ induced association of STAT1 with the normal FANCC protein but not with the mutant L554P protein fused to GST (Fig. 7A, top and middle, lanes 4 versus 6). Figure 7A demonstrates that comparable amounts of total STAT1 protein (middle, lanes 1 and 2) and GST fusion proteins (bottom, lanes 3 to 6) were loaded in the binding reactions.

Although the STAT1-FANCC association is clear-cut, and while we can state with reasonable certainty that the hypersensitivity does not require STAT1 activation, the mechanism of IFN hypersensitivity in FA-C cells is less clear and seems even counterintuitive. The STAT1 activation defect in FA cells may play a more direct role in signaling for survival or self-replication. Certain growth and survival factors for hematopoietic progenitor cells and stem cells are known to activate STAT1. These include SCF (5, 7), GM-CSF (37), G-CSF (1, 47), and EPO (24, 36). Our hypothesis that the STAT1 defect in FA cells might result in a functional disconnection from survival cues required, first, that FANCC and STAT1 associate in growth factor-responsive hematopoietic cells after stimulation

with growth and survival factors other than IFN- γ . To this end, we stimulated MO7e cells with IFN- α , GM-CSF, and SCF, all of which have been shown to induce tyrosine phosphorylation of STAT1 (6, 7, 24) and stimulated MO7ER cells with EPO. As shown in Fig. 7B, IFN- α , GM-CSF, and SCF induced activation of STAT1 by tyrosine phosphorylation (lane 2) and association of STAT1 and FANCC proteins (lane 4). The intensity of the signal owing to STAT1 phosphorylation was greatest for IFN- α and least for GM-CSF (Fig. 7B, lane 2, compare between panels). As has been described by others (24), we also noted low-level STAT1 phosphorylation in MO7ER cells in response to EPO (data not shown). To extend the above observations, we sought to determine whether the FANCC protein also interacts with other STAT molecules. MO7e and MO7ER cells were treated with GM-CSF and EPO, respectively. Both GM-CSF and EPO have been reported to activate STAT3 (7), and the former also induces activation of STAT5 (34) in these cells. Figure 7C demonstrates that GM-CSF and EPO activate Stat5 and Stat3, respectively (lane 2), but that no FANCC-Stat5 or FANCC-Stat3 association was detected in these cells.

Because Steel factor deficiencies account for both bone marrow and germ cell deficiencies in mice, both of which occur in FANCC knockout mice, we sought to test the hypothesis that

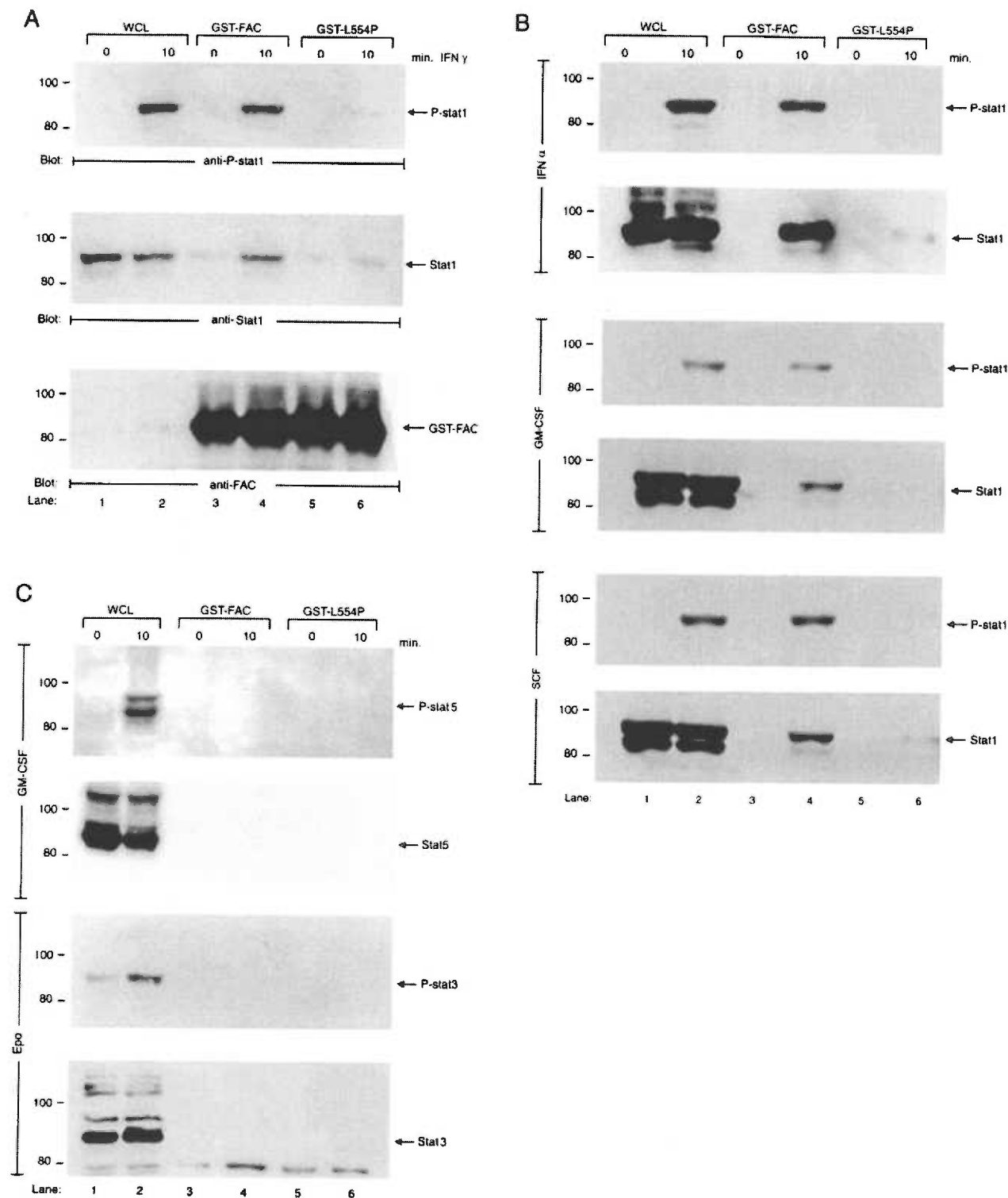


FIG. 7. FANCC-STAT1 association in stimulated MO7e cells. (A) WCL of MO7e cells stimulated with IFN- γ (10 ng/ml) for 10 min were used for either direct Western blotting (60 μ g of total WCL proteins; lanes 1 and 2) or affinity precipitation (1 mg of total WCL proteins) with yeast-expressed GST fusion proteins (10 μ g) and then analyzed by immunoblotting (lanes 3 to 6). The blot was probed with anti-P-STAT1 (top), anti-STAT1 (middle), or anti-FAC (bottom). (B) MO7e cells were stimulated with IFN- α (20,000 U/ml), GM-CSF (100 U/ml), or SCF (50 ng/ml) for 10 min, and WCL was subjected to either direct Western analysis (100 μ g of total WCL proteins; lanes 1 and 2) or GST affinity binding (1 mg of total WCL proteins; 10 μ g of GST fusion proteins; lanes 3 to 6) as described above. All IFN- α , GM-CSF, and SCF blots were first probed with anti-P-STAT1 and then stripped and reprobed with anti-STAT1. (C) MO7e and MO7ER cells were treated with GM-CSF (100 U/ml) and EPO (40 U/ml), respectively, for 10 min. WCL was then subjected to either direct Western blotting (100 μ g of total WCL proteins; lanes 1 and 2) or GST affinity binding (1 mg of total WCL proteins; 10 μ g of GST fusion proteins; lanes 3 to 6) as described for panel A. The GM-CSF blot was probed first with anti-P-Stat5 and then anti-Stat5; the EPO blot was probed first with anti-P-Stat3 and then anti-Stat3.

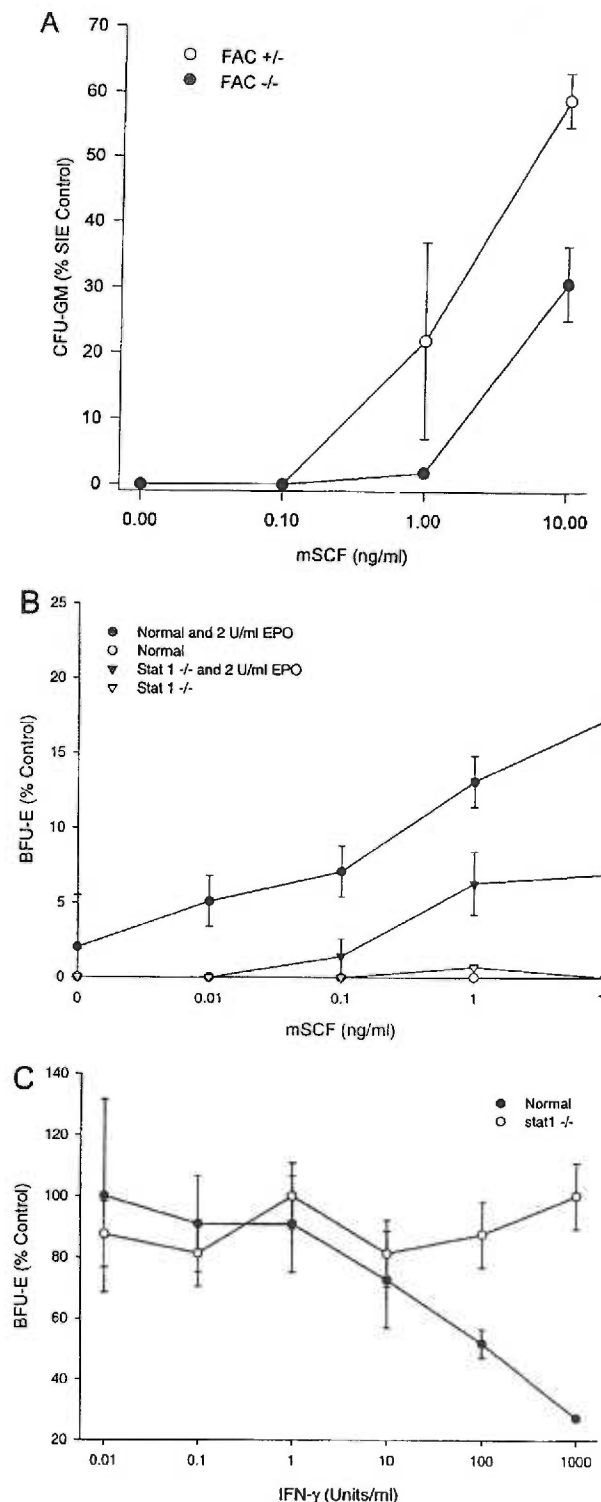


FIG. 8. SCF induces CFU-GM growth of murine BM cells. (A) BM cells from $FANCC^{+/+}$ and $FANCC^{-/-}$ mice were exposed to either recombinant murine SCF (0 to 10 ng/ml) or a combination of three growth factors (SIE): murine Steel factor (10 ng/ml), murine IL-3 (10 ng/ml), and human EPO (2 U/ml). CFU-GM and BFU-E were counted after 14 days. Data points represent mean colony growth ($\pm$ SD) of triplicate cultures. (B) Suboptimal responses of erythroid burst-forming units (BFU-E) from $STAT1^{-/-}$ to EPO and SCF. Results of a single representative experiment (or two independent experiments) are shown. Data points represent mean ($\pm$ SD) clonal growth derived from colony

$FANCC$ mutations might result in unresponsiveness to Steel factor, a survival factor for hematopoietic cells that induces $STAT1$ activation (5, 7). We treated BM cells from heterozygotes and $FANCC$ knockout mice with graded doses of Steel factor in the presence of EPO or IL-3 in clonal assays. As shown in Fig. 8A, both normal (heterozygote $FANCC^{+/+}$) and mutant ($FANCC^{-/-}$) BM cells proliferated in response to the hematopoietic growth factor SCF, but clonal growth of hematopoietic progenitor cells from $FANCC^{-/-}$ animals was consistently less responsive to SCF stimulation, confirming earlier preliminary reports (3). We found the same to be true in clonal assays from marrows of $STAT1^{-/-}$ mice (Fig. 8B). However, clonal growth of progenitor cells from $STAT1^{-/-}$ mice differed from that from $FANCC^{-/-}$ mice in that they were completely resistant to IFN-mediated suppression (Fig. 8C).

DISCUSSION

We have recently demonstrated that hematopoietic progenitor cells from mice nullizygous at the $FANCC$ locus and children with FA-C are hypersensitive to the antiproliferative effect of IFN- γ (38, 55). Because most of the known effects IFN- γ are ignited via $STAT1$ activation and nuclear translocation of $STAT1$ homodimers, we initially expected that at IFN- γ doses too low to activate $STAT1$ in normal cells, FA-C cells would contain high levels of phosphorylated $STAT1$. We were surprised to discover that in FA-C cells, $STAT1$ phosphorylation in response to IFN- γ was reduced markedly (10, 38). We ruled out the possibility that reduced $STAT1$ activation per se was the cause of progenitor cell hypersensitivity to IFN- γ by quantifying growth of clonogenic progenitor cells from the marrow of $STAT1^{-/-}$ mice. Progenitors from these mice were completely resistant to the growth-suppressive activity of IFN- γ (Fig. 8C), indicating that the hypersensitivity derived not from the failure of $STAT1$ activation but from another biological effect of $FANCC$ mutation. Like FA-C progenitors, however, progenitor cells from $STAT1^{-/-}$ mice were suboptimally responsive to combinations of Steel factor (SCF) and EPO (Fig. 8B), indicating that inactivating $FANCC$ mutations may blunt responses to survival factors for hematopoietic progenitor cells.

We therefore sought to more fully understand the function of the $FANCC$ protein vis-à-vis the JAK/STAT signaling pathway, using the IFN- γ signaling pathway as a paradigm. We also investigated factor-dependent hematopoietic cell lines, seeking to reveal $FANCC$ -JAK-STAT relationships by using other receptor molecules including SCF, EPO, and GM-CSF. We reasoned that the normal $FANCC$ protein, localized primarily in the cytoplasm (16, 56, 57), might functionally interact with key components of the JAK/STAT cytokine signaling cascades induced by survival factors for hematopoietic cells, and that mutations of $FANCC$ might interdict such responses. We have confirmed that notion in this study. We used isogenic cells derived from patients and from nullizygous mice carrying inacti-

counts in three replicate plates. Control BFU-E growth was defined in cultures containing IL-3 (10 ng/ml), Steel factor (10 ng/ml), and EPO (2 U/ml). Control BFU-E growth was equivalent in heterozygotes (31 colonies/ 10^5 cells) and $STAT1^{-/-}$ mice (48 colonies/ 10^5 cells). (C) Suppression of BFU-E growth by IFN- γ is $STAT1$ dependent. Optimally stimulated (IL-3, SCF, and EPO) clonal cultures of erythroid marrow cells from $STAT1^{-/-}$ mice and control animals were exposed to the indicated doses of IFN- γ throughout the culture period. Results shown are from a single representative experiment. Each point represents mean ($\pm$ SD) BFU-E growth expressed as percent control. Control colony growth was equivalent (see above), but IFN- γ failed to suppress clonal growth in $STAT1^{-/-}$ mice, even at supraphysiological levels.

vating mutations in the FANCC gene to dissect the ligand-induced assembly of the multimeric IFN- γ R-JAK-STAT complex.

We confirmed that STAT1 tyrosine phosphorylation was deficient in FA-C cells (Fig. 1) but determined that the activation kinetics of IFN- γ R α (Fig. 2) and JAK1 and JAK2 (Fig. 3) were normal in FA-C cells. In addition, immunoblot analysis of total cell lysates prepared from unstimulated and IFN- γ -treated cells indicated that both normal and FA-C cells constitutively expressed equivalent levels of IFN- γ R α , JAK1, and JAK2. These results suggest that the deficiency in STAT1 α tyrosine phosphorylation results neither from reduced expression nor activation of the JAK molecules or IFN- γ R α . However, STAT1 α was detected only in the IFN- γ R complexes of cell lysates from IFN-stimulated normal and corrected FA-C cell lines (Fig. 4). Moreover, GST-fused FANCC was shown to bind STAT1 contained in IFN- γ -induced cell lysates, but STAT1 did not bind to a FANCC mutant protein (a point mutant known to inactivate the function of FANCC) fused to GST (Fig. 5). Using activation state-specific antibodies and orthophosphate labeling studies, we have concluded that FANCC-STAT1 association did not require STAT1 phosphorylation, on either tyrosine or serine residues (Fig. 6). We further demonstrated that GST-FANCC-STAT1 association was also induced by IFN- γ , IFN- α , GM-CSF, and SCF in MO7e cells and that mutant GST-FANCC-L554P did not associate with STAT1 in cells stimulated with these factor (Fig. 7). That survival of committed progenitor cells exposed to graded doses of SCF was suppressed in FA-C progenitor cells from FANCC knockout mice confirmed that FANCC augments responses to SCF and EPO in hematopoietic cells (Fig. 8).

Our observation that STAT1 α forms a complex with IFN- γ R α in normal cells stimulated with IFN- γ is consistent with the previous reports that STAT1 α physically associates with IFN- γ R α both in vitro and in vivo (12, 13, 42). In these reports, STAT1 was shown to specifically recognize a phosphorylated tyrosine residue in the cytoplasmic domain of the receptor protein and to coimmunoprecipitate with IFN- γ R α in stimulated cell lysates. Once recruited into the dimeric IFN- γ R α / β complex, STAT1 becomes activated by tyrosine phosphorylation (4, 50, 52). We found that coimmunoprecipitation of STAT1 with IFN- γ R α was ligand dependent and additionally depended on the presence of an intact FANCC protein (Fig. 4). The difference in STAT1-IFN- γ R α coimmunoprecipitation between normal and FA-C cells (Fig. 4A and B) was not due to the varying levels of the STAT1 proteins, since STAT1 was expressed at comparable levels (Fig. 1B and 4F). Therefore, it appeared that the presence of a functional FANCC protein somehow facilitated the recruitment of STAT1 to the activated IFN- γ R complex.

The reason for the complete failure of STAT1 to appear at the docking site of the IFN- γ R and subsequently to be activated by tyrosine phosphorylation in FA-C cells is not completely understood. Three possible mechanisms may account for our observation. First, because FANCC and STAT1 associate very rapidly and prior to STAT1 phosphorylation (Fig. 6), FANCC itself might function as a chaperone for STAT1 molecules, moving them from cytoplasm to the receptor docking sites. This shuttling function of FANCC might occur directly or with the help of intermediary molecules. Second, that FANCC interacts with transcription factors including STAT1 in the studies described here and PLZF homologs (17) is compatible with the notion that in FA cells genes whose products govern STAT1 docking and activation might be derepressed. Third, FANCC may suppress the function of proteins that function to inhibit STAT1 activation. There is growing evidence that FANCC interacts with other FA proteins to form a functional

nuclear complex (11, 53). It is possible that such nuclear complex functions to modulate gene expression in response to external signals such as IFNs or other cytokines and growth factors. SOCS1, a STAT1-responsive gene whose product interdicts IFN-induced activation of STAT1, is an appealing candidate, particularly since SOCS1 nullizygous mice like FANCC nullizygous mice exhibit IFN- γ hypersensitivity (15, 29). However, this molecule functions by reducing JAK activation (41), and the studies described herein demonstrate that FANCC mutations result in STAT1 inactivation without reduction in JAK1 and JAK2 phosphorylation. Consequently, derepression of SOCS1 expression per se is an unlikely explanation for our results. Nonetheless, while their function is unclear to date, a number of proteins with C-terminal SOCS domains have been identified (15), and it remains quite possible that such proteins or others like them function to inhibit STAT1 activation and are also encoded by genes responsive to transcriptional repressors with which FANCC interacts.

Because the initial association of FANCC and STAT1 did not require tyrosine phosphorylation, we sought to determine whether the initially bound STAT molecule was phosphorylated on other residues. We found that FANCC-STAT1 association required neither tyrosine nor serine phosphorylation (Fig. 6A and C). We also found that STAT1 from induced FA-C cells was capable of binding to normal GST-FANCC (Fig. 6B), demonstrating that early IFN-induced signals influence the receptivity of STAT1 to FANCC, even in mutant cells. Thus, although the nature of the early IFN-induced signaling pathway is unclear at this time, the limiting factor for STAT1 phosphorylation in FA-C cells is clearly FANCC itself, not a FANCC-dependent cofactor. The association of FANCC with STAT1 prior to receptor docking is one of the earliest reported responses to IFN- γ .

While IFN- γ was used as our paradigm, we were also able to demonstrate the association of STAT1 with FANCC in cells exposed to hematopoietic growth and survival factors (Fig. 7A and B). Our results also demonstrate reasonable specificity of FANCC-STAT1 interaction and potential functional consequence of this interaction. While the FANCC-STAT1 binding phenomenon occurred in both myeloid and lymphoid cells (Fig. 7A and B) and occurred in cells stimulated with IFN- γ , IFN- α , GM-CSF, and SCF (Fig. 7A and B), FANCC did not associate with activated STAT3 and STAT5 (Fig. 7C). Therefore, these results suggest that although other STAT molecules are present and activated in appropriately stimulated cells, STAT1 is a specific FANCC partner.

Others have reported that SCF-stimulated BM colony growth is defective in FA patients (3). Since we have shown that SCF induces FANCC-STAT1 interaction in MO7e cells, our results are consistent with a role of the interaction in cell proliferation induced by SCF and certain other hematopoietic growth factors. This notion is further strengthened by our findings that BM progenitor cells from FANCC $^{-/-}$ and STAT1 $^{-/-}$ mice exhibited blunted responses to SCF. This suggests a potential role of the FANCC-STAT1 interaction in stem cell and progenitor cell survival mediated by SCF and possibly other growth factors.

That constitutive activation of IFN-responsive genes like IRF-1 and MxA (9, 27) is disconnected with STAT1 activation in FA-C cells not only indicates that the FANCC protein is multifunctional in its capacity to suppress hematopoiesis but also explains why FA patients are not susceptible to opportunistic infections as might be predicted by extrapolation from observations of STAT1 $^{-/-}$ mice (9, 28). That is, some of the constitutively expressed proteins in FA mediate the antiviral state (27, 37, 46) and would likely be protective. While the

mechanisms by which FANCC mutations result in constitutive activation of ordinarily repressed genes are unclear, that FANCC binds to a PLZF homolog (17) suggests that the failure of FAZF-FANCC interaction in mutant cells may result in selective gene derepression.

From the hematopoietic standpoint, FA-C cells are hypersensitive to a variety of mitogenic inhibitory molecules and apoptotic cues, including cytokines that utilize pathways not known to utilize STAT1 (13). Consequently, we do not argue that the function of FANCC vis-à-vis STAT1 is sufficient to account for every element of the FA phenotype. If this were the case, we would need to propose that STAT1^{-/-} mice should share this phenotype, and they do not. It is clear, however, that FA-C cells, whose receptor complexes are disconnected from STAT1 signaling pathways, are likely deprived of survival signals that utilize this pathway. With STAT1 deficiency alone, this state of disconnection from survival cues may not be sufficient to cause BM failure (a universal finding in FA), but when combined with the constitutive expression of mitogenic inhibitory and apoptotic factors, and hypersensitivity to IFN and tumor necrosis factor (38), the impact of STAT1 disconnection in hematopoietic cells may be substantial. Additional studies are warranted on the structural elements of FANCC required for STAT1 activation and both IRF-1 and MxA repression. We also argue that a comprehensive investigation of molecular hematopoietic control is warranted in STAT1^{-/-} mice, particularly using factors known to influence expansion, survival, and responsiveness of myeloid and erythroid progenitor cells.

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Role of double-stranded RNA-dependent protein kinase in mediating hypersensitivity of Fanconi anemia complementation group C cells to interferon γ , tumor necrosis factor- α , and double-stranded RNA

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Hematopoietic cells bearing inactivating mutations of Fanconi anemia group C (FANCC) are excessively apoptotic and demonstrate hypersensitivity not only to cross-linking agents but also to interferon γ (IFN- γ) and tumor necrosis factor- α . Seeking essential signaling pathways for this phenotype, this study quantified constitutive and induced RNA-dependent protein kinase (PKR) activation in Fanconi anemia cells of the C complementation group (FA-C). PKR was constitutively phosphorylated and exhibited an increased binding affinity for double-stranded RNA (dsRNA) in FANCC^{-/-} cells. FANCC^{-/-} cells were hyper-

sensitive to both dsRNA and the combination of dsRNA and IFN- γ in that these agents induced a higher fraction of apoptosis in FANCC^{-/-} cells than in normal cells. Overexpression of wild-type PKR sensitized FANCC^{-/-} cells to apoptosis induced by IFN- γ and dsRNA. Conversely, inhibition of PKR function by enforced expression of a dominant-negative inhibitory mutant of PKR (PKR Δ 6) substantially reduced the IFN and dsRNA hypersensitivity of FANCC^{-/-} cells. Two PKR target molecules, I κ B- α and IRF-1, were not differentially activated in FANCC^{-/-} cells, but enforced expression of a nonphosphorylatable form of eukaryotic transla-

tion initiation factor-2 α reversed the PKR-mediated block of messenger RNA translation and partially abrogated the PKR-mediated apoptosis in FANCC^{-/-} cells. Because no evidence was found of a PKR/FANCC complex in normal cells, it was concluded that an essential function of FANCC is to suppress, indirectly, the activity of PKR and that FANCC inactivation results in IFN hypersensitivity, at least in part, because this function of FANCC is abrogated. (Blood. 2001;97:1644-1652)

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Introduction

The hallmark of the rare autosomal recessive disorder Fanconi anemia (FA) is progressive bone marrow failure, deriving, at least in part, from excessive apoptosis in committed progenitor cells.¹⁻⁴ Hematopoietic progenitor cells from mice nullizygous at the FA group C (FANCC) locus and children with FA of the C complementation group (FA-C) are hypersensitive to the apoptosis-inducing effects of interferon γ (IFN- γ).^{1,2,5} Although most of the known effects of IFN- γ are thought to be transduced through signal transducer and activator of transcription-1 (STAT1) activation,^{6,7} and although it seemed reasonable, initially, to expect that STAT1 might be inappropriately activated in FA cells, STAT1 signaling is paradoxically suppressed in FANCC^{-/-} cells because optimal STAT1 activation depends on the presence of a normal FANCC gene product.⁸ Consequently, the excessive apoptotic response to IFN- γ in FANCC^{-/-} cells is STAT1 independent.

If the STAT1 pathway is not directly involved in these aberrant responses of FA-C cells, what signaling pathway is? Any viable candidates should meet the standards of being activatable or inducible by both IFN- γ and tumor necrosis factor (TNF) and should also influence fas activity and activate caspase 3 and 8.^{2,7} The IFN-inducible, double-stranded RNA

(dsRNA)-dependent protein kinase (PKR) meets these criteria.^{9,10} We carried out studies, reported herein, designed to test the hypothesis that PKR is excessively activated in FANCC^{-/-} cells. We found that FANCC^{-/-} cells are hypersensitive to dsRNA alone and in combination with IFN- γ and that PKR, a known mediator of apoptosis, the expression of which is induced by IFN- γ and the activation of which occurs on exposure to dsRNA and TNF,¹¹ is constitutively activated in FANCC^{-/-} cells. This aberrant activation state is suppressed by expression of the normal FANCC gene. Inhibition of PKR function significantly reduced IFN- γ hypersensitivity of FANCC^{-/-} cells, leading to the conclusion that PKR is an essential mediator of IFN hypersensitivity in FANCC^{-/-} cells.

The capacity of PKR to induce apoptosis can depend on its capacity to activate interferon-responsive factor-1 (IRF-1)¹² and to inactivate either the eukaryotic translation initiation factor 2 α (eIF-2 α)^{13,14} or the transcription factor NF- κ B inhibitor (I κ B- α),^{14,15} or both. We also report here that IRF-1 is not excessively activated by PKR in FANCC^{-/-} cells, that dominant negative mutant forms of eIF-2 α partially correct the IFN- γ hypersensitivity effect, and that dominant negative mutants of I κ B- α do not.

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Materials and methods

Cell culture and treatments

Murine embryonic fibroblasts (MEFs) were established from FANCC knockout (FANCC^{-/-}) and wild-type (FANCC^{+/+}) mice¹ and maintained in Dulbecco modified Eagle medium (DMEM) (Gibco Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS). These FANCC^{-/-} and FANCC^{+/+} MEFs were transformed and immortalized by SV40 large T-antigen.¹⁶ Human fibroblasts derived from FA patients with complementation groups A (PD720f), C (PD134f), and G (PD758f) were maintained in DMEM supplemented with 20% FBS. Unless otherwise indicated, cells were stimulated with 10 ng/mL of recombinant murine or human IFN- γ (R & D Systems, Minneapolis, MN) for the indicated time periods. For dsRNA treatment, 5×10^5 MEFs were plated in 60-mm tissue culture dishes and incubated at 37°C in 5% CO₂ in air overnight before being transfected with 100 μ g/mL of poly(I).poly(C) (Boehringer Mannheim, Indianapolis, IN), using LipofectAMINE (Gibco Life Technologies). The lipo-poly(I).poly(C) complexes were allowed to form for 30 to 45 minutes in serum-free medium before being added to the culture dishes. After incubation at 37°C for 7 hours, the medium was replaced with medium containing 10% serum. Cells were further incubated for 16 to 18 hours, followed by cell viability and flow cytometry analysis.

Construction of retroviral expression vectors and transduction of MEFs

The full-length human PKR (kindly provided by Dr G. N. Barber [Emory University, Atlanta, GA]; GenBank sequence accession number NM002759)¹⁷ was amplified by polymerase chain reaction (PCR), using Pfu DNA polymerase (Stratagene, La Jolla, CA) and a primer pair of 5'-CCGGCTCGAGATGGCTGGTATCTTTCAGCA-3' and 5'-CGCGATCCCTAACATGTGTGTCGTTCA-3'. The resulting PCR fragment was subcloned into the *Xho*I and *Bam*HI sites of vector pCITE-2a (Novagen, Madison, WI). The 1260-base pair (bp) *Stu*I-*Bsu*36I fragment of amplified PKR sequence in this plasmid was then replaced with the corresponding fragments from the original complementary DNAs (cDNAs) of both wild-type PKR and its dominant negative mutant PKR Δ 6.⁹ Finally, the replaced cDNAs were inserted into the retroviral vector pLXSN¹⁸ to create pLXSN-PKR and pLXSN-PKR Δ 6, respectively. The cDNAs of wild-type eIF-2 α , I κ B- α , and their nonphosphorylatable mutants eIF-NP and I κ B-M (kindly provided by Dr John W. B. Hershey [University of California, Davis] and Dr R. T. Hay [University of St. Andrews, United Kingdom], respectively) were tagged with the influenza hemagglutinin (HA) epitope (YPYDVPDYA) at the amino-terminus of each protein by PCR. The primers for eIF-2 α and eIF-NP were 5'-CCGCTCGAGTACCCATACGATGTTCTGACTATGCGCCGGGTCTAAGTTGTA-3' and 5'-CGCGGATCCCTAATCTTCAGCTTTGGCTTCCA-3'. For HA-tagged I κ B- α and I κ B-M, the primers were 5'-CCGCTCGAGTACCCATACGATGTTCTGACTATGCGTTCCAGGCGGCGAG-3' and 5'-CGCGGATCCTCATAACGTCAGACGCTGGCC-3'. The amplified cDNA products were subcloned into the *Xho*I and *Bam*HI sites of the retroviral vector pLXSH,¹⁸ which is different from pLXSN in that the former provides hygromycin resistance, whereas the latter is neomycin resistant. FANCC cDNA¹⁹ was subcloned into pLXSN as described previously.² The pLXSN and pLXSH plasmids (10 μ g each) were transfected, by the method of calcium phosphate precipitation, into Ψ 2 and the resulting retrovirus was used to infect PA12 cells.²⁰ MEFs were exposed to the retroviral supernatants in the presence of 8 μ g/mL polybrene (Sigma Chemical Co, St Louis, MO) and selected in G418 (300 μ g/mL) or hygromycin (300 μ g/mL). FANCC-transduced cells were exposed to multiple doses (0, 10, 25, 50, 100, and 250 nM) of mitomycin C in cytotoxicity (trypan blue viability) to ensure that the mutant cells transduced with the normal cDNA were fully complemented.

Analysis of cell viability and apoptosis

Cell viability was measured by trypan blue exclusion analysis. To quantify apoptotic cells, we used a polyclonal antibody to the active form of caspase

3 in a flow cytometric assay to detect MEFs in the early stages of apoptosis. Caspase 3 is activated during apoptosis to varying degrees in different cell types, and recent studies from our laboratory³ indicate that the apoptotic response of FANCC^{-/-} cells is caspase 8 and 3 dependent. The active, cleaved form of caspase 3, therefore, provides an instructive and biologically relevant marker of FANCC^{-/-} cells undergoing programmed cell death. We observed that some cells lost adherence to the tissue culture dish after incubation with LipofectAMINE and dsRNA, and many of the apoptotic cells were in the nonadherent fraction. Therefore, assays were performed on pools of adherent and nonadherent cells. Specifically, the cell culture medium containing nonadherent MEF cells was removed and reserved in a separate tube. The adherent cells were then detached by trypsinization. The adherent and suspension cells were then pooled, washed with phosphate-buffered saline and resuspended at a concentration of 1×10^6 /mL in staining buffer containing phosphate-buffered saline/2% FCS/0.1% Na Azide. This cell suspension (400 μ L) was then added to 400 μ L of Cytofix/Cytoperm (Pharmingen, San Diego, CA) and incubated for 20 minutes on ice to fix and permeabilize the cells. The cells were then washed with 2 mL Perm/Wash buffer (Pharmingen) and resuspended in 100 μ L Perm/Wash buffer. Purified rabbit immunoglobulin G (IgG; 10 μ L; Pharmingen) was then added to each sample as a blocking antibody to prevent the nonspecific uptake of fluorochrome-conjugated antibody. After 15 minutes, 20 μ L of phycoerythrin-conjugated antiactive Caspase 3 antibody (Pharmingen) was added, and samples were incubated for 30 minutes in the dark at room temperature. Cells were washed with 2 mL Perm/Wash buffer, resuspended in 500 μ L of staining buffer, and analyzed by flow cytometry, using a FACSCalibur (Becton Dickinson, San Jose, CA). Camptothecin-treated cells were used as a positive control. Cells were exposed to camptothecin at a concentration of 60 μ M for 9 hours at which time caspase 3 activation was optimal.

Immunoprecipitation and immunoblotting

Cells were lysed with Nonidet P-40 (NP-40) lysis buffer (1% NP-40, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, and 10% glycerol). The lysis buffer was supplemented with 1% aprotinin, 1 μ g/mL leupeptin, 1 mM phenylmethylsulfonylfluoride (PMSF), and 2 mM sodium orthovanadate. Cell lysates were cleared by centrifugation at 13 200 rpm for 20 minutes at 4°C, and protein concentrations were determined by the Bradford method.²¹ For immunoprecipitations with anti-mPKR and anti-eIF-2 α antibodies (generous gifts from Dr J. C. Bell of Ottawa Regional Cancer Center Research Laboratories, Ottawa, and Dr B. Datta of University of Nebraska, Lincoln, respectively), whole cell lysates (about 1 mg of total proteins) were precleared with 50 μ L of 50% protein A-Sepharose suspension (Pharmacia Biotech, Piscataway, NJ) for 1 hour at 4°C and then incubated with either anti-mPKR or anti-eIF-2 α at 4°C for 3 to 5 hours. Immunocomplexes were recovered by incubation with 50 μ L of protein A-Sepharose beads for 1 to 2 hours at 4°C. For immunoblotting, whole cell lysates or immunocomplexes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed with the indicated antibodies. To determine whether PKR interacts directly with FA proteins, whole cell lysates (1 mg of total proteins) were incubated with the indicated antibody against the specified FA protein, and the resulting immunocomplexes were analyzed using an anti-PKR antibody.

Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from cells by using Tri Reagent (Molecular Research Center, Cincinnati, OH). First, strand cDNA was reverse transcribed from the indicated RNA by using random hexanucleotide primers (Gibco BRL) and MMLV RNase H⁻ reverse transcriptase (Gibco BRL) as previously described.²² The cDNA was then amplified by polymerase chain reaction (PCR) for 35 cycles (denatured at 94°C for 30 seconds, primer annealed at 53°C for 30 seconds, primer extended at 72°C for 30 seconds). For fas amplification, the primers used were 5'-ACAGACAAAGCCATTTC-3' (upstream primer) and 5'-TTGC-CACTGTTTCAGGATT-3' (downstream primer), which produced an amplicon with a predicted length of 328 nucleotides.

Electrophoretic mobility shift assay

Nuclear extracts were prepared by the method of Dignam et al.²³ A degenerate IRF family consensus oligonucleotide (5'-GAAAA^{G/C/T}/CGAAA^{G/C/T}/CGAAA^{G/C/T}/CG-3') or a kB sequence (5'-CGGGCCGGG-GAATCCCGCTAA-3') was labeled with γ -³²P-ATP to 2.5×10^4 cpm/ng by using T4 polynucleotide kinase (Boehringer Mannheim). Binding reactions (20 μ L) contained 5 μ g nuclear extract, 0.2 ng labeled oligo, 1 μ g poly (dI-dC), and 10 μ g BSA in 10 mM Tris-Cl, pH 7.4, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 10% glycerol. Reactions were incubated at room temperature for 30 minutes, then resolved on a 4% nondenaturing polyacrylamide. For supershift assays, reactions were incubated for a further 45 minutes in the presence of antibodies against IRF-1 (5 μ g; Santa Cruz Biotechnology, Santa Cruz, CA), p50 (3 μ L; Upstate Biotech, New York, NY), or p65 (3 μ g; Santa Cruz Biotechnology).

dsRNA binding assay

Whole cell lysates (200 μ L) that contained 500 μ g of total proteins were mixed with 50 μ L of poly(I).poly(C)-agarose (Pharmacia) beads and rocked at 4°C for 60 minutes. The beads were then washed 3 times with 500 μ L of NP-40 lysis buffer. Proteins bound to beads were eluted from the beads by heating the samples at 94°C for 5 minutes in 2 $\times$ Laemmli SDS sample buffer, separated by SDS-PAGE, and subjected to immunoblot analysis by using anti-mPKR.

In vivo ³²P and ³⁵S labeling of proteins

Cells were starved for 60 minutes in phosphate-free DMEM that contained 10% dialyzed FBS and were treated with 10 ng/mL of murine recombinant IFN- γ and 100 μ g/mL of poly(I).poly(C). Labeling was performed in the same medium by addition of [³²P] orthophosphate (150 μ Ci/mL; DuPont NEN, Boston, MA). After labeling for 3 hours, whole cell lysates were prepared and subjected to immunoprecipitation with antibodies specific for mPKR or eIF-2 α . [³²P]-phosphate-incorporated immunocomplexes were analyzed by SDS-PAGE, followed by autoradiography. Immunoblot analysis was performed on these samples to determine the quantity of PKR or eIF-2 α precipitated by the respective antibody. For ³⁵S labeling, 0.5×10^6 MEFs were seeded in a 60-mm dish and cultured overnight. Cells were rinsed with methionine-cysteine-free DMEM and treated with dsRNA and IFN- γ where indicated. Labeling was again performed in the same medium containing 50 μ Ci/mL of [³⁵S]methionine-cysteine labeling mix (DuPont NEN) and incubated at 37°C for 60 minutes. Protein synthesis was measured by the incorporation of [³⁵S]methionine and [³⁵S]cysteine into trichloroacetic acid-precipitable proteins.

Results

FANCC mutants are hypersensitive to apoptosis induced by IFN- γ and dsRNA

Having previously described hypersensitivity of FANCC^{-/-} cells to IFN- γ -induced apoptosis,^{1,5} we sought to determine whether the IFN- γ -inducible, dsRNA-dependent PKR plays a role in the FA apoptotic pathway. We initially chose bone marrow cells because such hematopoietic progenitor cells from FANCC knockout mice and from children with FA-C are hypersensitive to IFN- γ and TNF- α -induced apoptosis.¹⁻⁵ However, we were unable to transfect these bone marrow cells with dsRNA, which is most commonly used as a PKR activator. Recently, MEFs from PKR knockout mice have been successfully used to study dsRNA-dependent PKR-induced apoptosis.^{10,24} Therefore, we employed MEFs derived from normal (FANCC^{+/+}) and FANCC knockout mice (FANCC^{-/-})

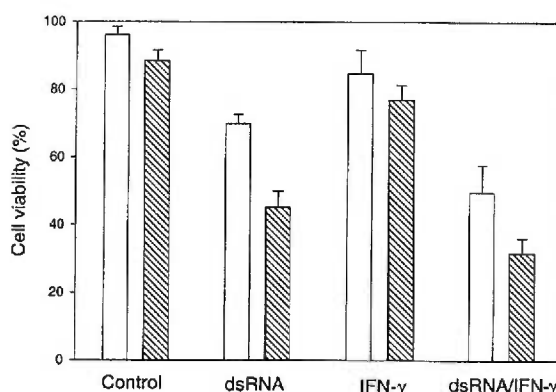


Figure 1. FANCC^{-/-} MEFs are hypersensitive to apoptosis induced by IFN- γ and dsRNA. Equivalent numbers of cells were incubated with murine recombinant IFN- γ (10 ng/mL) for 15 hours, followed by transfection with or without 100 μ g/mL of poly(I).poly(C) in the presence of LipofectAmine. Mock exposed cells were incubated with LipofectAmine only. After 24 hours, cells were subjected to viability determination by trypan blue exclusion assay. ■, FANCC^{-/-}; □, FANCC^{+/+}. Data are the mean $\pm$ standard deviations of triplicate determinations.

to study the involvement of PKR in the FA apoptotic pathway. As shown in Figure 1A, a greater fraction of FANCC^{-/-} cells were killed after 24 hours of treatment with dsRNA or IFN- γ in combination with dsRNA when compared to the normal MEFs. Specifically, cell viability in FANCC^{-/-} cells treated with dsRNA alone was reduced to 43% compared to 72% in FANCC^{+/+} cells (Figure 1A, column dsRNA). Treatment with a combination of dsRNA and IFN- γ caused an increase in cell death of nearly 2-fold in FANCC^{-/-} cells (column dsRNA/IFN- γ).

Caspase 3 activation in single cells, analyzed by flow cytometry, mirrored the cell viability findings. As shown in Table 1, IFN- γ (0.78%) or dsRNA (2.1%) alone did not cause a significant increase in caspase 3 activation in normal MEF cells compared to untreated cells (0.46%). The combination of dsRNA and IFN- γ did, however, cause significant induction of caspase 3 (14.5%) in normal MEFs. In FANCC^{-/-} cells, significant caspase 3 activation was induced with IFN- γ alone (9.35%), dsRNA alone (35.98%), and the combination of both (50.95%).

PKR is constitutively activated in FANCC^{-/-} cells

The above results suggested a possible involvement of PKR in the FA apoptotic pathway. We then asked whether FANCC^{-/-} cells expressed higher levels of PKR. Figure 2A shows that IFN- γ but not dsRNA induced PKR expression in both normal and FANCC^{-/-}

Table 1. Flow cytometric analysis of fractional apoptotic responses in murine embryonic fibroblasts treated with interferon γ and double-stranded RNA

Cells	% Apoptotic cells			
	Untreated	IFN- γ	dsRNA	IFN- γ /dsRNA
FANCC ^{+/+}	0.46 (3937)	0.78 (4000)	2.10 (4000)	14.53 (4000)
FANCC ^{-/-}	0.25 (4000)	9.35 (4000)	35.98 (4000)	50.95 (4000)

Cells were incubated with murine recombinant IFN- γ (10 ng/mL) for 15 hours before transfection with or without 100 μ g/mL of poly(I).poly(C) in the presence of LipofectAmine and harvested 24 hours after transfection for flow cytometric analysis as described in "Materials and methods." The untreated cells were incubated with LipofectAmine only. Treatment of FANCC^{+/+} murine embryonic fibroblasts with camptothecin was used as a positive control (60 μ M for 9 hours) and induced 11.8% positivity (2 916 events). Numbers in parentheses are total events analyzed.

IFN- γ indicates interferon γ ; dsRNA indicates double-stranded RNA; FANCC, Fanconi anemia group C.

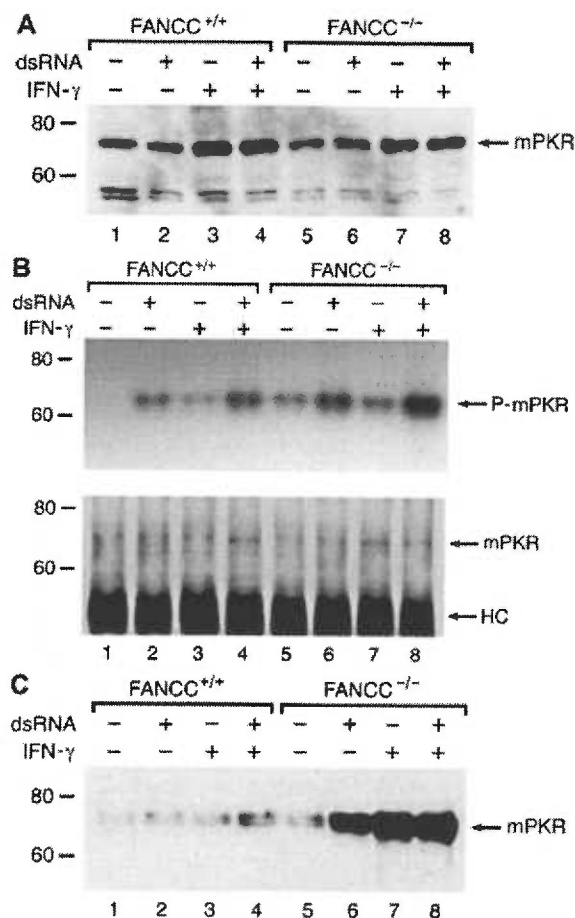


Figure 2. PKR is constitutively activated in FANCC^{-/-} cells. (A) Expression of PKR in MEFs in response to IFN-γ and dsRNA. Whole cell lysates were prepared 48 hours after incubation with IFN-γ (10 ng/mL) and 24 hours after transfection with 100 μg/mL of poly(I).poly(C), separated on a 7.5% SDS-PAGE, and immunoblotted with anti-mPKR antibody. (B) In vivo phosphorylation of PKR in MEFs treated with IFN-γ and dsRNA. FANCC^{+/+} and FANCC^{-/-} MEFs were labeled, 24 hours after incubation with IFN-γ (10 ng/mL) and 6 hours after transfection with 100 μg/mL of poly(I).poly(C), with [³²P]orthophosphate (150 μCi/mL) for an additional 3 hours. Cells were lysed, and equal amounts of whole cell lysates (500 μg of total proteins each sample) were immunoprecipitated with antibody specific for mPKR. [³²P]phosphate-incorporated PKR immunocomplexes were analyzed by SDS-PAGE, followed by autoradiography (top panel). To ensure additionally that equivalent amounts of the immunocomplexes were measured, immunoblot analysis of equal fractions of the PKR immunoprecipitates was performed by using the mPKR monoclonal antibody (bottom panel). P-mPKR, phosphorylated mouse PKR; HC, IgG heavy chain. (C) dsRNA-agarose affinity chromatography showing significantly more PKR from FANCC^{-/-} cell lysates binding to dsRNA. Equal amounts of whole cell lysates (500 μg of total proteins each sample) were applied to a poly(I).poly(C)-agarose column. The bound proteins were eluted and analyzed by SDS-PAGE and immunoblot, using anti-mPKR antibody.

MEFs and that the levels of PKR proteins were not significantly different between normal and mutant cells. With the reason that there might be differences in the ground-state activation of PKR in these cells, we measured PKR phosphorylation in isogenic FANCC cells. Cells were labeled with [³²P]-orthophosphate, and levels of ³²P-labeled PKR were quantified by using immunoprecipitation. As shown in Figure 2B, PKR activation was undetectable in untreated normal MEFs (top panel, lane 1). Although IFN-γ significantly induced expression of the PKR protein as described in Figure 2A, activated PKR was only slightly increased in these IFN-γ-treated cells (Figure 2B, top panel, lanes 3,7). However, both normal and FANCC^{-/-} MEFs showed greatly elevated levels of activated PKR

after treatment with dsRNA (lanes 2,6). This is consistent with previous observations that phosphorylative activation of PKR is dsRNA dependent.¹¹ Significantly, there was approximately 2-fold more phosphorylated PKR precipitated from FANCC^{-/-} cells than normal MEFs treated with dsRNA or dsRNA plus IFN-γ (compare lanes 2 and 4 with 6 and 8). Of equal importance, a significant amount of PKR was constitutively activated in FANCC^{-/-} MEFs (lane 5). Furthermore, the degree to which increased PKR was phosphorylated correlated positively with the fraction of apoptotic cells induced by dsRNA and IFN-γ (compare Table 1 with Figure 2B). Immunoblots confirmed that all immunoprecipitates contained similar amounts of PKR proteins (Figure 2B, bottom panel).

Considering that the higher activation state of PKR might be caused by a higher affinity of PKR for dsRNA in mutant cells, we tested the binding capacity of PKR in isogenic cell lines. RNA affinity binding was performed by using equal amounts of total proteins from treated and untreated normal and FANCC^{-/-} MEFs. Bound PKR was analyzed in eluates by immunoblotting. For reasons that remain to be clarified, the dsRNA binding capacity of PKR was higher in FANCC^{-/-} MEFs compared to the normal cells (Figure 2C).

Overexpression of PKR sensitizes FANCC^{-/-} MEFs to dsRNA- and IFN-γ-induced apoptosis

The results described above support the hypothesis that the state of PKR activation may contribute to the IFN-γ hypersensitivity in FA cells. To test this idea, we overexpressed human wild-type PKR (hPKR) and a dominant negative mutant, PKRΔ6 (catalytically inactive^{9,25}), in both normal and FANCC^{-/-} cells and subsequently quantified dsRNA- and IFN-γ-induced apoptosis. After 24 hours of dsRNA transfection, cells were examined for hPKR protein expression by Western blot analysis, for cell viability by trypan blue staining, and for induction of apoptosis by quantification of the fraction of exposed cells containing activated caspase 3. As shown in Figure 3A, endogenous murine PKR (approximately 65 kD) was induced by treatment of IFN-γ but not dsRNA. The expression of hPKR, which migrated at approximately 70 kD on SDS-PAGE, was equivalent between transduced cell lines and was not responsive to IFN-γ, likely reflected the lack of regulatory regions in our constructs. Cell viability assays (Figure 3B) showed that approximately 90% of wild-type PKR-expressing FANCC^{-/-} cells underwent apoptosis after exposure to dsRNA and IFN-γ compared to 50% of normal cells (column PKR wt). Overexpression of PKRΔ6 increased survival of FANCC^{-/-} cells treated with dsRNA and IFN-γ from 30% to more than 50% (compare column VEC with PKRΔ6). Although expression of the human PKR was not induced by IFN-γ and dsRNA treatment (Figure 3A), cell death was substantial in FANCC^{-/-} cells (Figure 3B, column PKRwt). It is possible that overexpression of hPKR increases the amount of total PKR proteins available for activation by dsRNA. Another explanation may pertain to the activation state and binding capacity of the overexpressing hPKR protein in these MEFs. On the latter point, we did observe higher dsRNA-binding capacity by the overexpressed human form of PKR than by the endogenous murine PKR in untreated FANCC^{-/-} cells (data not shown). This may partially explain why overexpression of hPKR alone can induce apoptosis in FANCC^{-/-} MEFs (Table 1, Figure 3B).

To confirm the role of PKR in apoptotic responses of FANCC^{-/-} MEFs, we carried out flow cytometry for caspase 3 activation. Table 2 shows that after exposure either to dsRNA

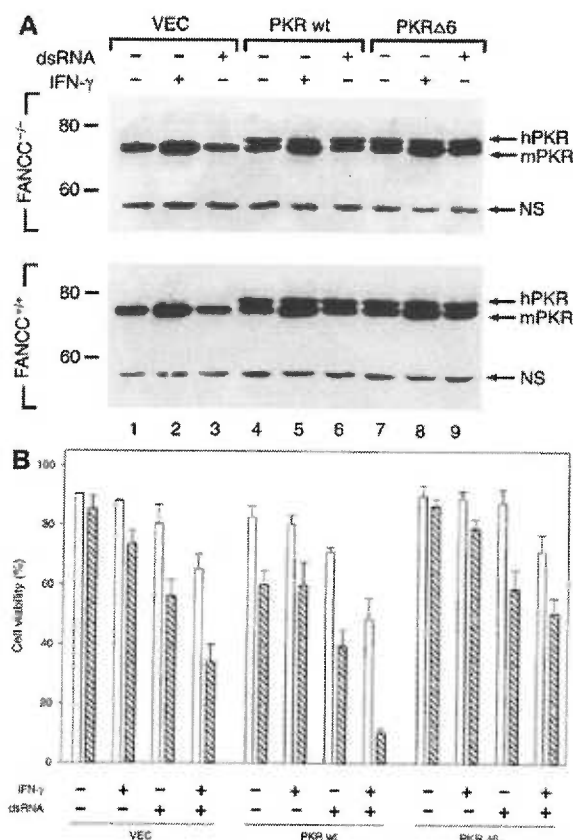


Figure 3. Enforced expression of PKR-sensitized FANCC^{-/-} cells to IFN-γ and dsRNA-induced apoptosis. (A) Retrovirally expressed human PKR proteins in MEFs: control cells carrying vector alone (VEC) or cells expressing wild-type human PKR (PKRwt) or PKRΔ6 were treated with or without IFN-γ (10 ng/mL) or dsRNA (100 μg/mL), lysed, and analyzed by Western blotting by using a polyclonal antibody that reacted with both human PKR (hPKR) and mouse PKR (mPKR) proteins. NS, nonspecific. (B) Cell viability of MEFs carrying vector alone (VEC) or expressing wild-type hPKR (wtPKR) or PKRΔ6, in response to IFN-γ and/or dsRNA treatments; data represent the means (standard deviations of triplicate determinations). ■, FANCC^{-/-}; □, FANCC^{+/+}.

alone or the combination of dsRNA and that IFN-γ caspase 3-positive FANCC^{-/-} cells expressing wild-type PKR were increased 5- to 7-fold over normal MEFs. When compared to cells containing the empty vector, the percentage of apoptosis induced by dsRNA and dsRNA plus IFN-γ in PKR-expressing FANCC^{-/-} cells increased by 12.3- and 8.7-fold, respectively, over the PKR-expressing normal cells. In addition, expression of PKRΔ6 gave more protection to FANCC^{-/-} cells than to normal cells. When compared to cells containing the empty vector, the percentage of apoptosis induced by dsRNA or dsRNA and IFN-γ in PKRΔ6-expressing FANCC^{-/-} cells was reduced by 23.9% and 82.4%, respectively. Less remarkable reductions were seen in PKRΔ6-expressing normal cells (12.1% and 34.5%, respectively). Collectively, these data clearly demonstrate the involvement of PKR in IFN-γ- and dsRNA-induced apoptosis in FANCC^{-/-} MEFs.

PKR-induced apoptosis in cells treated with a variety of cellular stressors, including dsRNA and IFN-γ occurs, at least in part, through up-regulation of Fas expression.^{10,26} Indeed, IFN-γ-induced apoptosis involves priming of the Fas pathway in FANCC^{-/-} hematopoietic progenitor cells.¹ In addition, Otsuki et al⁴ reported that apoptosis induced by TNF-α in FANCC^{-/-}

hematopoietic progenitor cells involved Fas ligation.⁴ Therefore, although we have not found that Fas expression increases in cells treated with IFN-γ alone,²⁷ we sought to confirm that Fas mRNA levels were unaltered in response to both IFN-γ and dsRNA by reverse transcriptase-PCR, using Fas mRNA-specific primers. We did not observe significant changes in the levels of Fas mRNA in both normal and FANCC^{-/-} MEFs expressing wild-type PKR or PKRΔ6, treated with or without IFN-γ and dsRNA (data not shown).

Li and Yousoufian²⁸ demonstrated that an IFN-responsive gene, MxA, was up-regulated in several FA complementation group mutant cells, including groups A, B, C, and D. By having established the involvement of PKR in IFN-γ and dsRNA-induced apoptosis in FANCC^{-/-} MEFs, we wondered whether other FA group mutant cells were also susceptible to PKR-mediated apoptosis. We treated fibroblasts derived from FA patients of complementation groups A (FANCA^{-/-}), C (FANCC^{-/-}), and G (FANCG^{-/-}) with IFN-γ and dsRNA and determined the sensitivity of these mutant cells in response to the reagents. We found that in contrast to FANCC^{-/-} fibroblasts, which were hypersensitive to IFN-γ and dsRNA treatment, FANCA^{-/-} and FANCG^{-/-} fibroblasts exhibited no more sensitivity than the normal fibroblasts (data not shown).

An eIF-NP mutant inhibits PKR-induced apoptosis in FANCC^{-/-} cells

PKR signaling results in phosphorylation of at least 3 separate substrates that affect apoptotic responses: phosphorylation of IRF-1¹² and phosphorylation inactivation of either translation initiating factor eIF-2α or the transcription factor inhibitor IκB-α.^{14,29} To test the respective roles of the latter 2 substrates in the apoptotic response of FANCC^{-/-} cells, we expressed wild-type eIF-2α, IκB-α, and their nonphosphorylatable mutants, eIF-NP and IκB-M, as HA-tagged recombinant proteins in both normal and FANCC^{-/-} MEFs. Immunoblot analysis revealed that HA-tagged eIF-2α, eIF-NP, IκB-α, and IκB-M were equally overexpressed in resting normal and FANCC^{-/-} MEFs (Figure 4A,B). These cells were then cotransduced with a retroviral wild-type PKR vector to study the interaction of PKR and its 2 substrates in apoptosis in FANCC^{-/-} cells. When normal MEFs were cotransduced with PKR and empty vector alone, treatment with dsRNA or a combination of

Table 2. Flow cytometric analysis of fractional apoptotic responses in RNA-dependent protein kinase-overexpressing murine embryonic fibroblasts treated with interferon γ and double-stranded RNA

Cells	% Apoptotic cells		
	Untreated	dsRNA	IFN-γ/dsRNA
Vector			
FANCC ^{+/+}	0.48 (9940)	0.15 (9950)	1.15 (9820)
FANCC ^{-/-}	0.28 (9906)	1.84 (9967)	10.01 (9645)
PKR			
FANCC ^{+/+}	0.14 (9956)	0.61 (9909)	1.91 (9821)
FANCC ^{-/-}	1.35 (9818)	4.22 (9675)	10.86 (9445)
PKRΔ6			
FANCC ^{+/+}	0.93 (9925)	0.13 (9930)	0.75 (9878)
FANCC ^{-/-}	3.69 (9621)	1.40 (9496)	1.76 (9660)

Murine embryonic fibroblasts carrying vector alone (pLXSH) or expressing (wtPKR) or PKRΔ6 were treated with murine recombinant IFN-γ (10 ng/mL) and/or 100 μg/mL of poly(I).poly(C) as described in Table 1. Numbers in parentheses are total events analyzed.

dsRNA indicates double-stranded RNA; IFN-γ, interferon γ; FANCC, Fanconi anemia group C; wtPKR, wild-type human RNA-dependent protein kinase.

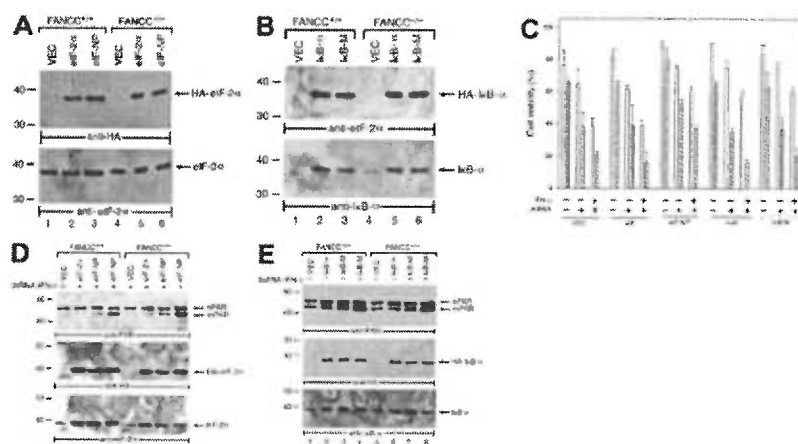


Figure 4. Enforced expression of eIF-NP inhibits PKR-induced apoptosis in FANCC^{-/-} MEFs. (A) Expression of HA-eIF proteins in FANCC^{+/+} and FANCC^{-/-} cells. Whole cell lysates (80 μ g of total proteins) were separated on a 7.5% SDS-PAGE and immunoblotted with the anti-HA antibody (top panel) or the anti-eIF-2 α antibody (bottom panel). (B) Expression of HA-I κ B proteins in FANCC^{+/+} and FANCC^{-/-} cells. Total proteins (80 μ g) were analyzed as described in (A), using the anti-HA antibody (top panel) or the anti-I κ B- α antibody (bottom panel). (C) Cell viability of PKR-expressing MEFs carrying vector alone (VEC), wild-type eIF-2 α (eIF), a nonphosphorylatable mutant eIF-NP, wild-type I κ B- α (I κ B), or a nonphosphorylatable mutant I κ B-M; data points were determined by trypan blue exclusion assay as described in Figure 1 and presented as the mean (standard deviations of triplicate determinations). $\blacksquare$, FANCC^{-/-}; $\square$, FANCC^{+/+}. (D) Expression of hPKR and eIF proteins in FANCC^{+/+} and FANCC^{-/-} cells. Total proteins (80 μ g) were analyzed by Western blotting with anti-PKR (top panel), anti-HA (middle panel), or anti-eIF-2 α antibody (bottom panel). (E) Expression of hPKR and I κ B proteins in FANCC^{+/+} and FANCC^{-/-} cells. Total proteins (80 μ g) were analyzed by immunoblotting with anti-PKR (top panel), anti-HA (middle panel), or anti-I κ B- α antibody (bottom panel).

dsRNA and IFN- γ induced apoptosis (Figure 4C, column VEC). Cotransduction of wild-type eIF-2 α neither sensitized nor relieved PKR-induced cytotoxicity (compare column eIF with VEC), but overexpression of the nonphosphorylatable mutants eIF-NP and I κ B-M partially abrogated the effects of dsRNA and dsRNA plus IFN- γ (Figure 4C, columns eIF-NP and I κ B-M). As has been reported by others,^{14,30} we also observed partial relief of cytotoxicity by overexpression of wild-type I κ B- α (Figure 4C, column I κ B). Results differed in studies on FANCC^{-/-} cells. In FANCC^{-/-} cells transduced with empty vector and wild-type eIF- α , treatment with dsRNA or dsRNA plus IFN- γ caused more cell death than in normal MEFs (columns VEC and eIF). Overexpression of eIF-NP in FANCC^{-/-} cells reduced, but not completely, the percentage of apoptotic cells compared to vector alone or wild-type eIF-2 α (Figure 4C, compare column eIF-NP with VEC and eIF). However, expression of either wild-type I κ B- α or nonphosphorylatable I κ B-M in FANCC^{-/-} cells did not relieve cytotoxicity caused by treatment of dsRNA or dsRNA plus IFN- γ (column I κ B and I κ B-M). We also quantified fractional apoptosis in these cells by flow cytometry. Consistent with the cell viability studies described above, overexpression of eIF-NP, but not that of I κ B-M, significantly blocked PKR-mediated apoptosis in FANCC^{-/-} MEFs (data not shown). Immunoblot analysis demonstrated that the levels of retrovirally expressed PKR proteins were similar in all samples (Figure 4D,E, top panels). The bottom panels of Figure 4D and E show the levels of eIF-2 α and eIF-NP, and I κ B- α and I κ B-M, respectively.

To assure ourselves that PKR-induced apoptosis in FANCC^{-/-} cells does not specifically involve differential NF- κ B activation, we performed an electrophoretic mobility shift assay (EMSA) for NF- κ B. As shown in Figure 5, overexpression of PKR alone induced nuclear NF- κ B activity in normal cells (lane 1), and coexpression of I κ B-M substantially suppressed this activity (lane 2). Although we observed higher NF- κ B DNA binding in PKR-expressing FANCC^{-/-} cells than in normal cells (lane 3 versus lane 1), overexpression of I κ B-M did not inhibit NF- κ B activity in these mutant cells (compare lane 1 with 2 and lane 3 with 4). Antibody

supershift analysis indicated that the NF- κ B DNA complexes contained the p50 and p65 subunits (lanes 7,8).

PKR-mediated apoptosis in FANCC^{-/-} MEFs is associated with translational inhibition

To determine whether excessive apoptosis in FANCC^{-/-} cells treated with dsRNA and IFN- γ is induced by excessive PKR-mediated phosphorylation of eIF-2 α , we performed *in vivo* analysis of eIF-2 α phosphorylation in MEFs. The eIF proteins were

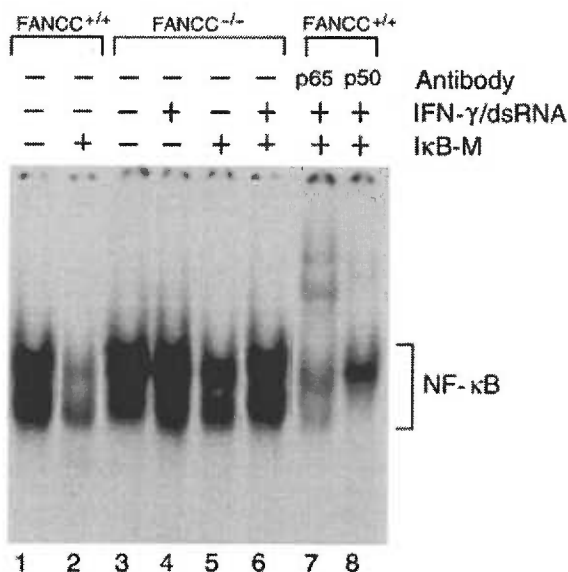


Figure 5. EMSA analysis of NF- κ B activation in I κ B-M-expressing MEFs. I κ B-M-expressing normal and FANCC^{-/-} MEFs were treated with IFN- γ (10 ng/mL) and dsRNA (100 μ g/mL), and EMSA was performed by using 5 μ g of nuclear extracts and a κ B oligonucleotide sequence as described in "Materials and methods." The final 2 lanes indicate that binding reactions were incubated with 3 μ g of anti-p65 (lane 7) or 3 μ L of anti-p50 (lane 8) antibody.

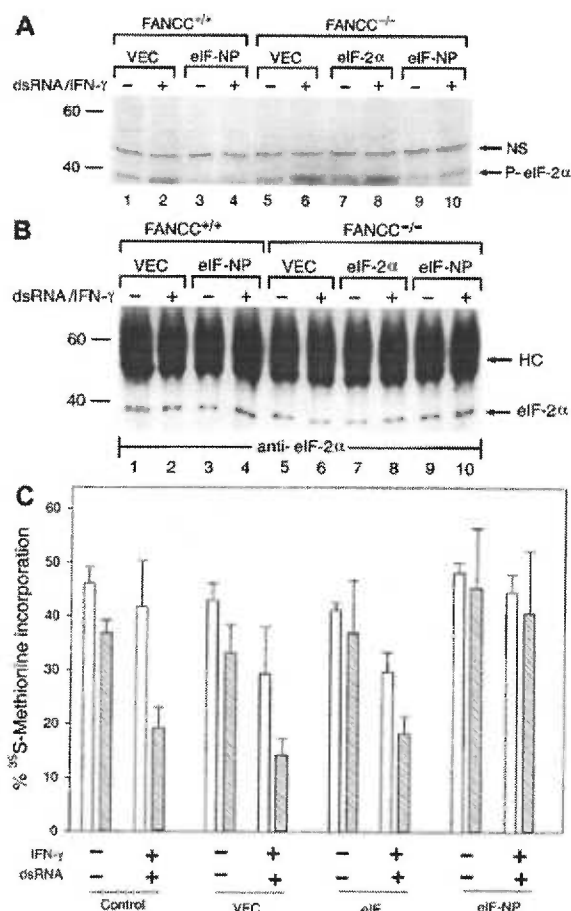


Figure 6. Expression of the nonphosphorylatable mutant eIF-NP rescued PKR-mediated inhibition of protein synthesis in FANCC^{-/-} cells. (A) Analysis of phosphorylated eIF-2α levels in PKR-expressing MEFs carrying vector alone (VEC), wild-type eIF-2α (eIF), and a nonphosphorylatable mutant eIF-NP; cells were pretreated with 10 ng/mL of murine recombinant IFN-γ, transfected with 100 μg/mL of poly(I).poly(C), and labeled in a methionine-cysteine-free medium containing 50 μCi/mL of [³⁵S]methionine-cysteine labeling mix for 60 minutes. Whole cell lysates were prepared, and 800 μg of total proteins from each sample were subjected to immunoprecipitation with anti-eIF-2α antibody. The immunocomplexes were analyzed by SDS-PAGE followed by autoradiography. NS, nonspecific. (B) Immunoblot analysis of a fraction of the same immunoprecipitates described in (A) using anti-eIF-2α antibody. HC, IgG heavy chain. (C) Rate of protein synthesis in PKR-expressing MEFs carrying vector alone (VEC), wild-type eIF-2α (eIF), and a nonphosphorylatable mutant eIF-NP. Cells were pretreated with 10 ng/mL of murine recombinant IFN-γ, transfected with 100 μg/mL of poly(I).poly(C), and labeled in a methionine-cysteine-free medium containing 50 μCi/mL of [³⁵S]methionine-cysteine labeling mix for 60 minutes. Protein synthesis was measured by the incorporation of [³⁵S]methionine and [³⁵S]cysteine into trichloroacetic acid-precipitable proteins. The control cells did not carry any expression vectors. ■, FANCC^{-/-}; □, FANCC^{+/+}. Data represent means ± standard deviations of 2 independent experiments.

immunoprecipitated from [³²P]-orthophosphate-labeled normal and FANCC^{-/-} MEFs that coexpressed PKR and wild-type eIF-2α or eIF-NP. Treatment of PKR-expressing FANCC^{-/-} cells with dsRNA and IFN-γ resulted in significantly more phosphorylation of eIF-2α than in normal cells (Figure 6A, compare lanes 1 versus 2 and 5 versus 6). Expression of eIF-NP dramatically reduced eIF-2α phosphorylation in treated FANCC^{-/-} cells compared to FANCC^{-/-} cells bearing the wild-type eIF-2α or empty vector (Figure 6A, compare lanes 10 with 6 and 8). Phosphorylation of eIF-2α in normal MEFs treated with dsRNA plus IFN-γ (Figure 6A, lane 4) was also inhibited by eIF-NP, albeit to a lesser extent. Immunoblot

analysis showed that the amount of eIF-2α immunoprecipitated was not significantly different between samples (Figure 6B).

We wished to confirm that the excessive phosphorylation of eIF-2α had a functional consequence in FANCC^{-/-} cells. Because phosphorylation of eIF-2α results in translational inhibition,³¹ we reasoned that global translational activity should be reduced to a greater extent in FANCC^{-/-} cells than in normal cells exposed to IFN-γ and dsRNA. As can be seen in Figure 6C, treatment with dsRNA plus IFN-γ reduced protein synthesis by approximately 60% in FANCC^{-/-} cells compared to approximately 25% in normal MEFs (columns Control and VEC). The percentages of [³⁵S] methionine and [³⁵S] cysteine incorporation in eIF-2α-expressing cells were similar to those in MEFs carrying vector only (column eIF), consistent with the eIF-2α phosphorylation results described above (Figure 6A). Expression of eIF-NP restored protein synthesis to 44% and 50% in PKR/eIF-NP coexpressed FANCC^{-/-} cells treated with and without dsRNA plus IFN-γ, respectively (Figure 6C, column eIF-NP). Normal MEFs coexpressing PKR and eIF-NP also partially rescued the inhibition of protein synthesis.

IRF-1 activation is similar in normal and FANCC^{-/-} cells

PKR has been implicated in regulating the activity of transcription factor IRF-1.^{9,24} We sought to determine whether constitutive activation of PKR in FANCC^{-/-} cells resulted in up-regulation of IRF-1 activity. EMSA using an IRF consensus oligonucleotide probe showed that treatment of MEFs with IFN-γ and dsRNA greatly increased DNA-binding activity of IRF-1; however, no difference was observed in IRF-1 activity between FANCC^{-/-} and normal cells or between wild-type PKR- and PKRΔ6-expressing FANCC^{-/-} cells (data not shown).

Discussion

Hematopoietic progenitor cells from patients with FA and FANCC knockout mice are hypersensitive to IFN-γ and TNF-α,¹⁻⁵ agents known to induce apoptotic responses in hematopoietic cells^{32,33} and may, therefore, play an important role in the pathogenesis of bone marrow failure in patients with FA.¹ MEFs from FANCC^{-/-} mice also demonstrate this hypersensitivity, although fibroblasts from fully developed mice do not. The mechanism underlying this hypersensitivity is not clear. We originally hypothesized that constitutive STAT1 activation might account for this phenotype but discovered that this was not the case. Surprisingly, STAT1 activation was suppressed by certain mutations of the FANCC gene, the product of which is normally required for optimal STAT1 activation.⁸ With the reasoning that the hypersensitive phenotype might reflect constitutive activation of other IFN-responsive factors not directly linked to STAT1 activation, we tested the notion that PKR plays such a role. PKR, an IFN-induced protein, activated by dsRNA or by single-stranded RNAs that possess substantial secondary structure, plays an important role in the antiviral effects of the interferons and in control of cell survival.¹¹ Other features of PKR function, especially its inducibility by TNF-α and certain chemicals,^{10,24,34} factors that are also excessively toxic to FA cells, made PKR a particularly attractive molecule to evaluate functionally in FA cells.

We report here that cultured embryonic fibroblasts derived from FANCC^{-/-} mice⁵ are also hypersensitive to dsRNA and that PKR is a key mediator of the high level of apoptosis induced by IFN-γ

and dsRNA in FANCC^{-/-} cells. We argue that these results reflect, in large part, the distinctly abnormal high-level constitutive activation of PKR in FANCC^{-/-} cells (Figure 2B). Furthermore, treatments with IFN- γ and dsRNA induced a significantly higher level of activated PKR in FANCC^{-/-} cells than in normal MEFs. With the use of a dominant negative mutant of human PKR (PKR⁶), we showed that excessive apoptosis in FANCC^{-/-} MEFs treated with IFN- γ and dsRNA requires PKR activation. Our results also confirm recent studies demonstrating that PKR is a general transducer of the apoptotic response.^{10,13,26,35,36} In addition, our studies showed that priming with IFN- γ greatly sensitized FANCC^{-/-} MEFs to apoptosis following treatment with dsRNA (Figure 1).

It is known that IFN primes or enhances dsRNA-mediated cytotoxicity in NIH3T3 cells,¹³ and that IFN- γ treatment of MEFs leads to a posttranslational modification of PKR (by phosphorylation).^{24,37} One possibility for the hypersensitive response in FA cells is that normal FANCC modulates induced expression of PKR, and, therefore, IFN- γ more effectively induces expression of PKR in FA cells. In this circumstance, PKR would be more substantially activated by treatment with dsRNA because there is simply more substrate. However, our results indicate that this is not the case. PKR expression was equally induced in both normal and FANCC^{-/-} MEFs in response to IFN- γ stimulation (Figure 2A), indicating that FANCC does not govern expression of PKR and that the constitutive activation (Figure 2B) and high affinity (Figure 2C) of PKR, rather than its overproduction, is unique to the FANCC^{-/-} cells. We argue that this represents a pathogenically important phenotype linked to the FANCC mutation.

The capacity of PKR to influence cell survival involves inactivation of at least 2 factors critical to the survival of normal cells, eIF-2 α and I κ B- α .^{13-15,38} To identify which of these PKR substrates is necessary to effect hypersensitivity of FA-C cells to IFN- γ and dsRNA, we coexpressed PKR and nonphosphorylatable forms of eIF-2 α (eIF-NP) or I κ B- α (I κ B-M). We found that excess apoptosis in FANCC^{-/-} cells correlated only with increased phosphorylation of eIF-2 α (Figures 4,6). Collectively, our results indicate that hypersensitivity of FANCC^{-/-} cells to IFN- γ involves translational inhibition through aberrant activation of the dsRNA-dependent protein kinase PKR. It is important to note that, whereas overexpression of the dominant negative mutant I κ B-M failed to block PKR-mediated apoptosis in FANCC^{-/-} MEFs, probably as a result of its inability to inhibit NF- κ B activation (Figure 5), we cannot exclude an additional role of high-level activated NF- κ B on PKR-induced apoptosis in these mutant cells. Further experiments are required to determine the functionality of the overexpressing I κ B-M protein in FANCC^{-/-} cells, namely, its capacity to compete with endogenous I κ B- α as the substrate for PKR.

That the dominant negative eIF-NP construct was less capable of completely abrogating induced apoptosis in FANCC^{-/-} cells than was the dominant negative mutant of PKR, suggests that additional PKR substrates may be involved. One such candidate is the transcription factor IRF-1. IRF-1, a

known effector of programmed cell death in hematopoietic cells,³⁹ is constitutively expressed in FA cells, including MEFs, from FANCC knockout mice⁴⁰ and in bone marrow cells from children with inactivating mutations of FANCC.¹ FANCC^{-/-} cells are known to be hypersensitive to IFN- γ and TNF- α ,¹⁻⁵ and expression of both PKR and IRF-1 is induced by both IFN- γ and TNF- α .^{24,36} In fact, there is evidence that IRF-1 cannot function unless a phosphorylation signal is provided, potentially by PKR.¹² Studies by Der et al¹⁰ have also shown that PKR is required for DNA-binding activity of IRF-1 in MEFs treated with stress-related agents, including TNF- α and dsRNA. Therefore, constitutive activation of PKR combined with known constitutive expression of IRF-1 in FANCC^{-/-} cells would be expected to have serious apoptotic consequences. We found no differences in constitutive or induced nuclear IRF-1 DNA-binding activity between FANCC^{-/-} and normal cells in EMSAs.

The mechanism by which FANCC protein suppresses PKR activation in normal cells is not clear. We did not find that the FANCC protein associates with PKR in normal cells. However, FANCC exists in a complex with at least 2 other FA proteins,⁴¹ and the same is true of PKR that is known to complex with a variety of other proteins, including itself.^{42,43} Consequently, further studies are warranted because one of the other FANCC-binding proteins might associate with one of the PKR-associated proteins, thus suppressing PKR activation. It is clear that phosphorylation of eIF-2 α is involved in the FANCC phenotype, but it is also apparent that other priming mechanisms may be involved. Recent studies implicating certain MAP kinase pathways in PKR responses⁴⁴ suggest that p38 MAPK and JNK might also be involved in some cell types. PKR-induced apoptosis occurs at least in part through up-regulation of fas expression,^{10,26} a pathway we and others have shown to be involved in IFN- γ and TNF- α responses of FANCC^{-/-} hematopoietic progenitor cells.^{1,4} However, we did not observe significant changes in the levels of fas mRNA in both PKR-expressing normal and FANCC^{-/-} MEFs treated with or without IFN- γ and dsRNA.

Although the signaling pathways responsible for constitutive activation of PKR and PKR-dependent apoptosis in FANCC^{-/-} cells are not yet clear, our study does provide unambiguous evidence of a pivotal role of PKR in the characteristic hypersensitivity of FANCC^{-/-} cells to IFN. We speculate that appropriately targeted inhibitors of PKR activity might prevent bone marrow failure in children with this disease.

Acknowledgments

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The Fanconi anemia group C gene product: Signaling functions in hematopoietic cells

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Seventy years ago, the Swiss pediatrician Guido Fanconi reported a family in which three children had not only aplastic anemia but a number of congenital defects as well [1]. Since that time, the syndrome has been reported many times and its clinical heterogeneity is now widely recognized. In fact, Fanconi anemia (FA) is sometimes diagnosed in adulthood and should be considered in the differential diagnosis of hypoplastic or aplastic anemias in young adults as well as in children. The disease is rare, with a prevalence of 1–5 per million (reviewed in [2]), but is complicated by life-threatening bone marrow failure, an extraordinary risk of myelodysplasia [3], and acute nonlymphocytic leukemia, and an increased risk of epithelial malignancies [4,5].

FA has been classified as a chromosomal instability syndrome since the discovery by Schroeder et al. [6] that metaphases from FA cells showed an increased number of spontaneous breaks. A decade later, Sasaki and Tonomura [7] reported the phenomenon of hypersensitivity to cross-linking agents that was to become the foundation of the current diagnostic test for FA. Cytogenetic analysis of metaphase cells, usually lymphocytes cultured with phytohemagglutinin, after exposure to low doses of diepoxybutane (DEB) [8] or mitomycin C (MMC) [9] reveal characteristically abnormal chromosomes. Auerbach reported that metaphases from affected cells treated with 0.1 micrograms/mL DEB exhibited a 175-fold increase in chromosome breakage compared with metaphases of normal lymphocytes [8]. A remarkable abnormality, termed “radial” chromosome forms (Fig. 1), are most noticeable in MMC-treated cells and less so with DEB. Some cytogenetics laboratories, ours included, use both agents in every case for the sake of completeness.

Genetic heterogeneity

Somatic cell fusion studies have shown that FA is genetically heterogeneous, with at least eight complementation groups (A through G and including D1 and D2) having been identified thus far [10]. The genes corresponding to groups A (*FANCA*), C (*FANCC*), G (*FANCG*), D2 (*FANCD2*), E (*FANCE*), and F (*FANCF*) have been cloned [11–16]. However, the FA gene products reveal strong homologies neither to one another nor to any known protein. This has led to a multi-institutional push to identify the function of these novel proteins in both somatic and germ cells.

FA protein complex formation

Initially, the first two FA gene products studied (*FANCA* and *FANCC*) were thought to be cytoplasmic proteins [17–19]. Later, cell fractionation and confocal microscopic studies revealed that both these proteins are present in the nucleus as well as the cytoplasm, with the majority (90%) of *FANCC* being cytoplasmic [20,21]. *FANCG* and *FANCF* are reported to be largely nuclear [22] but in our hands can be found in cytoplasmic fractions as well (unpublished). *FANCE* binds to *FANCC* [22] and given the high percentage of *FANCC* found in the cytoplasm, it is possible that *FANCE* exists in the cytoplasm as well. This has not yet been tested formally but should be, because *FANCE* has two potential nuclear localization signals [15] and could conceivably be largely nuclear in location.

With the exception of *FANCD2*, most of the FA proteins are thought to form a large multi-protein complex [21,23–29]. Strong evidence of FA complex formation has been derived from immunoprecipitation studies as well as yeast-two-hybrid analyses [22]. It seems likely that cytoplasmic and nuclear complexes consisting of *FANCA*, C, G, E, and F proteins likely exist in vivo. *FANCD2* does not participate in the formation of this complex. Of more relevance to the functional importance of the FA complex, patient-derived inactivating mutations of *FANCA*, *FANCC*, and *FANCG*

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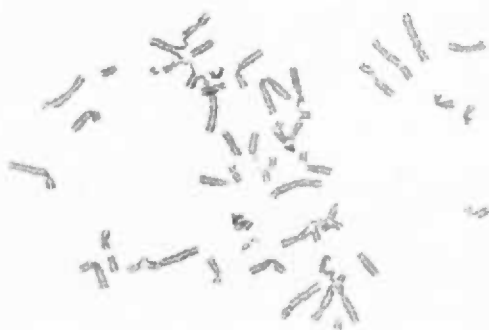


Figure 1. A metaphase from a FA-C cell treated with mitomycin C. Shown are the characteristic broken chromosomes and interchromosomal attachments that form "radial" structures.

proteins reduce assembly, stability, and/or nuclear translocation of the multi-subunit FA protein complex [21,26,30] and mutations of any one of these reduce downstream ubiquitinylation of FANCD2 [23].

Function of the Fanconi protein complex

The role the FA complex plays in protecting cells from cross-linking agent-induced damage may soon become clearer. The cloning of the FANCD2 gene [16] allowed D'Andrea's group to develop antibodies to the FANCD2 protein. With these antibodies D'Andrea's group demonstrated that FANCD2 is not part of the FANCC, -A, -F, -G complex and exists as a nuclear protein that can be monoubiquitinated. Furthermore, monoubiquitinylation of FANCD2 occurs only if the FA protein complex is intact and located in the nucleus, further implicating FANCD2 as a downstream effector. The ubiquitinated form of FANCD2, easily recognized on immunoblots as a higher molecular mass moiety, is the form that moves to discrete nuclear foci after genotoxic stress and associates with BRCA1 (Fig. 2) [23]. While the function of FANCD2 in this induced nuclear-focus context is as yet unknown, in view of its association with BRCA1 it is reasonable to suggest that the FANCD2 protein participates in repair of DNA, maintenance of chromosomal stability, or activation of the ubiquitin ligase or hydrolase activity of BRCA1 protein complexes [31,32]. In addition, the ubiquitinated form of FANCD2 may serve as a reasonable way to make the diagnosis of Fanconi anemia in the future because mutations of FANCC, FANCA, FANCF, and FANCG all interdict FA complex formation and thereby prevent this post-translational change.

The notion of a common functional pathway

The recent work defining this linear pathway has opened a variety of exciting new doors for investigation of the nature of chromosomal instability in FA cells and per-

haps in studies on the bone marrow failure as well. One point of view is that this [FA-complex→FANCD2-Ub] linear pathway serves to account for all phenotypic features of Fanconi anemia, in which case the nuclear complex would necessarily serve some specific hematopoietic function. Taking into account the apoptotic phenotype of FA bone marrow cells [33–42], the complex might well serve to govern the expression of genes that encode intra- or extracellular survival factors. An alternative view is that the Fanconi proteins have different functions, some of which involve the formation of a nuclear complex, others of which may involve activities of single FA proteins that either function in a cytoplasmic FA complex, or perhaps even work entirely on their own. If this multifunctional model for FA proteins proves to be the case, the FANCC gene product might represent a scientific bellwether for the other non-D FA proteins. We subscribe to the view that FANCC is a multifunctional protein and will review the evidence in support of this model. To do so, we will focus on the major point of our own investigations: a molecular assessment of the pathogenesis of bone marrow failure in children and adults with Fanconi anemia.

Pathogenesis of marrow failure in Fanconi anemia

While all somatic FA cells exhibit mitomycin C sensitivity, hematopoietic cells in humans seem to exhibit a uniquely apoptotic phenotype [43]. Functionally the bone marrow is the only organ that seems to overtly fail with consistency in FA patients. Clonogenic progenitor cell growth is decreased in virtually all cases [44] and in humans these cells are extraordinarily sensitive to extracellular apoptotic cues including combinations of interferon (IFN)- γ and double-stranded RNA (dsRNA) or combinations of IFN- γ and tumor necrosis factor (TNF)- α [39,40]. Therefore, if the linear pathway of nuclear FA protein function accounts for all elements of the phenotype, it follows that the linear pathway must be intersected by pathways specific to hematopoietic lineages.

Alternatively, a single nuclear complex pathway may be particularly relevant to cross-linking agent resistance, but may not play a major role in governing the responses of hematopoietic cells to biological cues. Although the final answer is unclear, some evidence now suggests that the nuclear pathway that leads to FANCD2 monoubiquitinylation does not account for all phenotypic features of Fanconi anemia, particularly those that have to do with the survival of hematopoietic progenitor cells. Two types of complementary approaches have been taken to define the functions of the Fanconi proteins. The first has been to identify interacting partners and the second, to define consistent perturbations in cell populations that can be complemented by the normal Fanconi gene, and then to investigate the signaling pathways of relevance to that particular abnormality.

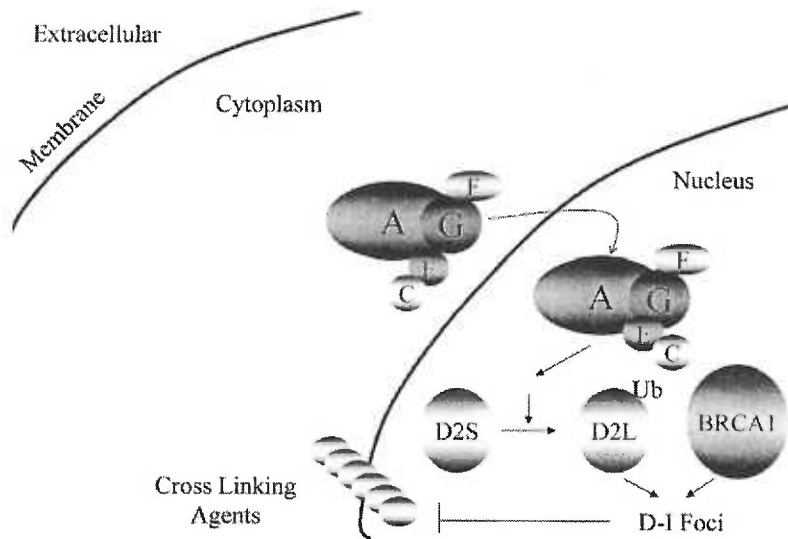


Figure 2. The Fanconi anemia nuclear protein complex influences posttranslational modification of FANCD2. The monoubiquitylation of FANCD2 reportedly permits the association of this nuclear protein with BRCA1 in cells exposed to cross-linking agents such as mitomycin C. While the function of the FANCD2/BRCA1 complex is not known at this time, that the two proteins associate in large multimeric complexes in response to “damage,” suggests that the complex serves to prevent or repair structural genotoxic lesions. Reports by Youssoufian’s group [50] arguing that the FA defect is a “pre-repair” abnormality, raise the possibility that the nuclear damage-induced (D-I) foci functions somehow to prevent DNA damage. The pathway outlined in this figure is clearly tentative but takes into account the compelling models proposed by Garcia-Higuera et al. [23,26,27] and the recent FA protein interaction studies of Medhurst et al. [22]. The role played by this linear pathway in maintaining survival of hematopoietic cells is unclear. Indeed, recent data (reviewed below) suggest that the FANCC protein is multifunctional and has important possibly independent nonnuclear functions in hematopoietic cells.

Non-FA binding partners of FANCC

The FANCC protein has been found to bind to and regulate the function of a variety of proteins other than Fanconi proteins. Hoshino et al. [45] reported that in yeast two-hybrid analysis, FANCC bound to the chaperone GRP94 in a manner that required residues 103–308. Inactivation of GRP94 resulted in reduced levels of FANCC, suggesting that GRP94 might stabilize FANCC. The role played by FANCC vis-à-vis GRP94 is unclear, as are preliminary reports that FANCC associates with the cyclin-dependent kinase *cdc2* [46]. However, certain other pathophysiological abnormalities in FA cells suggest that the capacity of FANCC protein to bind to *cdc2* may influence its function. First, FA-C cells are hypersensitive to Fas-mediated apoptosis as are cells in which p34*cdc2* activity is suppressed with dominant negative mutants of *cdc2* [47]. Secondly, *cdc2* is known to associate with the zinc finger transcriptional repressor protein PLZF [48], and FANCC has been reported to associate with a related protein FAZF [49]. The role of FAZF is unclear since little is known regarding function of this protein. However, FAZF is expressed at high levels in both testis and specific lymphoid lineages and may provide a link to *cdc2* and the cell type specificity of FA.

NADPH cytochrome p450 reductase is a FANCC-binding microsomal membrane protein. Youssoufian’s group proposed NADPH cytochrome p450 may be modulated by FANCC binding, and provided compelling evidence that the characteristic cytogenetic instability found in FA cells re-

sulted from a “pre-repair” defect [50]. They demonstrated that FANCC did bind to NADPH cytochrome P450 reductase in transfected COS-1 cells and in normal murine liver cells. Moreover, FANCC also suppressed the cytochrome-c reductase activity of the enzyme. Formal proof of the “pre-repair defect” will require a quantitative and dynamic assessment of cross-link induction and repair rates, experiments that demand, first, a clear explication of the specific types of cross-link repair pathways (homologous recombination or end-joining, or both) defective in FA cells.

Of particular interest to our laboratory has been the capacity of FANCC to interact with signaling proteins that govern cell survival and the activation of apoptotic pathways in FA-C hematopoietic and embryonic cells. Most notable among these binding partners are STAT and heat shock proteins [39,51,52].

Apoptosis in hematopoietic cells

The first hint that the Fanconi anemia marrow cells were constitutively apoptotic was from work published by Maciejewski et al. reporting that CD34⁺ cells from three children with Fanconi anemia expressed high levels of the death receptor fas [43]. Subsequently a number of investigators have reported that Fanconi anemia cells are hypersensitive to a variety of extracellular biological apoptotic cues [33,35,39–41,52–54]. Furthermore, overexpression of FANCC in the hematopoietic factor-dependent progenitor cell lines can

suppress apoptosis induced by growth factor withdrawal [33]. Once again the function of the Fanconi anemia group C gene product has been the most well studied in this regard, in large part because FANCC was the first Fanconi gene cloned and the *fanc*c knockout mouse was the first to have been created.

The role of IFN- γ

Early studies on the *fanc*c knockout mouse provided an opportunity to test the notion that progenitor cells were hypersensitive to apoptotic cues. The most consistent finding in our hands was that clonal growth of committed progenitor cells was more sensitive to interferon- γ -induced inhibition in these mutant mice. Work by Haneline et al. [36] confirmed this finding and also confirmed the findings of Liu's group that FANCC mutant cells were also hypersensitive to TNF- α [53]. It may be important to recognize that the interferon-hypersensitive phenotype was discovered in a *fanc*c knockout mice on a mixed-strain background (C57BL/129). However, because JAK/STAT signaling is impaired in FA cells, it has become clear that cytokine hypersensitivity and excessive apoptosis in FA cells is not a direct result of hyperactivity of the classical STAT1-dependent interferon signaling pathway. Indeed, using transcriptomal analyses of STAT1^{-/-} cells exposed to IFN, it has become clear that generally IFN- γ signaling can proceed quite nicely in the absence of STAT1 (although the induced set of transcripts are clearly different compared to those of wild-type cells) [55,56]. Nonetheless, based on studies from our laboratory, in which the most profound differences between FA and normal cells are noted when IFN is combined with another cytokine, it is likely that the effect of IFN- γ on FA-C cells involves a collaboration with other factors including *fas* and other elements of TNF receptor signaling pathways [39,40,41,52,53].

There is also suggestive indirect evidence that the IFN- γ signaling pathways is involved somehow in the FA phenotype. A series of studies in our laboratory confirmed the notion that the IFN- γ pathway is aberrantly regulated, and FA-C murine embryonic fibroblasts and hematopoietic cells express high constitutive levels of IFN-inducible genes [35]. In large part because of these findings we focused our attention initially on signaling pathways with which IFN- γ is directly involved. Recognizing that interferon might influence the expression of TNF-receptor family members, we tested that notion directly and discovered that it had no such effect [37]. However, interferon- γ is capable of priming mutant cells to respond with excessive vigor to *fas* ligation [41] and involves the caspase 8-dependent activation of caspase 3 [54].

The idea that interferon- γ requires additional factors to reveal the full apoptotic phenotype of Fanconi cells is further supported by the observations that the classical interferon- γ signaling pathway [57] is paradoxically suppressed in Fanconi cells. We initially expected to find excessive STAT1 activation. In fact, we found the reverse.

Specifically, STAT1 activation in response to interferon- γ and other cytokines is paradoxically suppressed in mutant hematopoietic and embryonic cells [35,52]. Moreover, FANCC and STAT1 physically interact in cells stimulated with cytokines and this interaction is reduced in mutant cells. FANCC mutations result in the failure of STAT1 to associate with the IFN receptor α chain, and, consequently, STAT1 phosphorylation is reduced [52]. Therefore, either interferon alone is not a sufficient stimulus to discriminate FA and normal hematopoietic cells, or there exists a non-JAK-STAT IFN- γ signaling pathway constitutively active in FA cells. Notwithstanding the reduction in STAT1 activity, FA-C cells in the ground state act as if they have been exposed to interferon. It seems clear that FANCC modulates pathways with which interferon interacts but that wild-type FANCC does not act as a direct suppressor of classical IFN- γ signaling.

These findings raised two very important questions. First, if FANCC plays a role in STAT signaling in response to IFN- γ , are other Stats or modulators of STAT signaling dysfunctional in Fanconi cells? This is an extremely important question given the fact that most hematopoietic cytokines and growth factors utilize some combinations of the Jak/Stat pathway for cellular signaling, and defects of many of these pathways could quite conceivably affect hematopoietic cell survival. Work in progress in our laboratory has revealed multiple defects of STAT signaling in response to some cytokines and growth factors (but not all) as well as an abnormality of a JAK molecule Tyk2 (unpublished). The role of these signaling molecules in bone marrow failure will be difficult to assess in murine models because no evidence of spontaneous bone marrow failure has been reported in these mice. Furthermore, some Jak/Stat signaling pathways are specific to human cells and do not occur in mice (i.e., IFN- α activation of STAT4) [58]. Understanding the differential hematopoietic FA-linked signaling pathways in mice and humans will be important in elucidating the specific human defects that lead to bone marrow failure in FA patients but not in murine models.

It is also possible that defects in other cell types may modulate progenitor cells in FA patients. Apoptosis of progenitor cells can be lymphocyte-dependent [59–61], so there exists the possibility that FA lymphocytes may, in part, mediate cytokine and *fas* hypersensitivity and modulate hematopoietic cell survival. Indeed, it has been known for quite some time that serum levels of TNF- α are elevated in the serum of children with Fanconi anemia [62] and perhaps lymphocyte subset abnormalities reflecting STAT molecule defects actually account for this phenomenon. Clearly, interactions of auxiliary cells and progenitors should be carefully studied.

Given that STAT activation is suppressed in FA cells, the second question that evolved from the work related to the identity of the specific apoptotic pathway that is so hyperactive in FA cells. It was clear that this pathway should

1) be influenced by interferon- γ but not dependent on STAT1 activation, 2) influence fas activation responses, 3) be influenced by ligation of TNF receptors, and 4) involve activation of caspase 8 and caspase 3. This led our group to test the role of double-stranded RNA-dependent protein kinase (PKR), a molecule that met all of the above litmus tests [63–65].

High-level PKR activation in FA-C cells

PKR is a well recognized mediator of apoptosis optimally activated in normal cells by a variety of stimuli including combinations of dsRNA and IFN- γ or IFN- γ and TNF [66]. It functions to induce apoptotic responses in part by inducing phosphorylation of translation initiation factor eIF-2 α , a phenomenon that results in translational inhibition and subsequent apoptosis [67]. Recently we determined that PKR and eIF-2 α are excessively activated in FANCC cells. FANCC^{-/-} cells are hypersensitive to dsRNA alone and in combination with IFN- γ , and PKR is constitutively activated in FANCC^{-/-} cells, an activation state suppressed by expression of the normal FANCC gene [40]. The functional test of the role of PKR activation in FA cells was to utilize a dominant negative mutant of PKR [68] seeking to complement the apoptotic phenotype in FA cells. Indeed, inhibition of PKR function significantly reduced IFN-hypersensitivity of FANCC^{-/-} cells [40], leading to the conclusion that PKR is an essential mediator of IFN- γ hypersensitivity in FANCC^{-/-} cells. Furthermore, we found no PKR abnormalities in fibroblasts derived from FA-C patients. The only PKR defects were confined to murine embryonic fibroblasts and hematopoietic cells, and it is precisely these cells that act as if they have been stimulated by cytokines [35]. That this function of FANCC was not a global one was precisely the kind of finding we would expect if FANCC is an independent regulator of hematopoietic cell survival. The finding energized our search for a role of FANCC in modulating constitutive and induced PKR activity. We were equally excited by reports that PKR is induced by chemicals including benzene, a known inducer of marrow toxicity and leukemia [69], which suggested the possible involvement of PKR in the apoptotic response of selected cells to chemical toxins as well.

Modulation of PKR by HSP-70

It is not likely that the capacity of FANCC to interact with STAT molecules accounts for FANCC's capacity to suppress PKR. Therefore, we sought to identify such factors using affinity chromatography. Normal lymphoblasts were metabolically labeled, stimulated with interferon and TNF- α , and lysed. The lysates were placed in contact with GST-FANCC and GST mutant FANCC (L554P). Proteins pulled down by the mutant and wild-type proteins were analyzed by gel electrophoresis. Peptide bands unique to the wild-

type protein were isolated and sequenced. One inducible band was found to associate with the wild-type protein but not the mutant (L554P) protein. This proved to be hsp70. Hsp40 also bound to these columns but not differentially. We confirmed by co-immunoprecipitation that FANCC and hsp70 were induced to physically associate in cells exposed to the combination of IFN- γ and TNF- α . FANCC and hsp70 also associated in a cell-free system but required additional factors, specifically, ADP and hsp40. The hsp70-binding domain of FANCC was shown to reside between residues 105 and 424. Evidence that the heat shock binding function of FANCC plays a defining role in suppressing apoptotic responses to IFN- γ and TNF- α was developed using antisense constructs to hsp70. Specifically, exposure of normal human lymphoblasts IFN- γ and TNF- α resulted in substantial increases in apoptosis but suppression of hsp70 by these antisense constructs had no impact on apoptotic responses of FA cells, compatible with the notion that FA-C cells had already functionally inactivated hsp70 because of their failure to associate with FANCC [39].

The importance of the FANCC-hsp70 interaction may reach beyond cytokine hypersensitivity. More than 5 years ago, Liu et al. [70] reported that induction of hsp70 expression by protein alkylation requires glutathione depletion, induction of oxidative stress, and increased lipid peroxidation. Others have provided indirect evidence that the effect of MMC in FA cells is mediated by oxidative stress [71], tending to confirm the long held view of some investigators that the most fundamental defect of FA cells, generally, is intolerance to oxidative stress [72]. Further support for this view was the recent report of Buchwald's group that FANCC enhances the function of GSTP1 in cells exposed to inducers of apoptosis. GSTP1 is an enzyme that detoxifies by-products of redox stress and xenobiotics [34]. FANCC does not directly interact with GSTP1 but we speculate that a FANCC complex containing hsp70 with or without FANCE may bind to GSTP1 and suppress protein oxidation in normal cells.

FANCC is multifunctional

The discovery of FANCD2 and subsequent characterization of the protein was a very significant step forward in understanding the responses of Fanconi cells to genotoxic stress, specifically with cross-linking agents. However, this defect is found in all somatic cells and may not account for the specific responses of FA hematopoietic cells to biological extracellular cues. Clearly a structure-function study was required in which distinct structural motifs in FANCC were sought; motifs that might have a role in a critical pathway for one response but not the other. To accomplish this, we designed FANCC mutants and tested their capacity to affect STAT activation, interact with hsp70, suppress PKR activation, induce resistance to mitomycin C, facilitate ubiquitination of FANCD2, and induce resistance to double-stranded

RNA. We aligned the human, rat [73], bovine [73], and murine [74] FANCC cDNA sequences and discovered three perfectly conserved domains (Fig. 3). Targeting two residues in each of these three domains for alanine mutations, we discovered that while all of the Ala mutant constructs were capable of complementing FA-C cell hypersensitivity to mitomycin C, the two Ala mutants in domain II (S249A and E251A) did not associate with hsp70, did not permit binding of STAT-1 to the IFN- γ receptor α chain docking site in interferon-stimulated cells, did not protect against apoptosis induced by combinations of double-stranded RNA and interferon or interferon and TNF- α , and did not reduce constitutive activation of PKR [51]. In addition, one FANCC mutation that leads to a much more mild hematopoietic phenotype, 322delG, is associated with a reinitiation of translation and a putatively smaller, albeit partially active, form of FANCC [75]. This mutant FANCC protein would be expected to retain domain II and is known to be associated with a milder hematopoietic phenotype. In fact, we have shown that this mutant, which fails to correct MMC responses in FA-C cells, does, in fact, correct the aberrant STAT responses in stimulated cells. Therefore, a wild-type domain II sequence is necessary and, in the context of an N-terminal truncation, sufficient to support the anti-apoptotic function of FANCC in cytokine stimulated cells [51].

Summary

Structure-function analysis of FANCC demonstrates that this protein is multifunctional. The FA proteins bind together in a complex and influence the function of FANCD2 in as yet unspecified ways. FANCC functions not only as a requisite member of a protein complex that facilitates this posttranslational change of FANCD2, but also protects cells from the apoptotic cues from their biological environment.

FANCC probably protects hematopoietic cells in at least two ways (Fig. 4). First, FANCC suppresses apoptosis at least in part by suppressing PKR through its hsp40-dependent direct interaction with hsp70. Secondly, wild-type FANCC promotes cell survival by facilitating signaling through the JAK/STAT pathway in response to multiple cytokines and growth factors. Using domain analysis of FANCC we have demonstrated that the role played by FANCC in protecting cells against MMC-induced injury is distinct from that which it plays in protecting hematopoietic cells from apoptotic cues. Therefore, FANCC is a multifunctional FA protein. A critical functional domain for control of the signaling function of FANCC in the center of the molecule is a highly conserved region encompassing residues 248 to 260. Because the L554P mutation (near the c-terminus of the protein) preserves domain II, yet reduces association of hsp70 and FANCC, the c-terminal portion of FANCC likely plays a role in the maintenance of a proper FANCC conformation, a conformation that may change in response to stimulation with extracellular apoptotic cues.

Implications and future studies

Clinical diagnostics: FA without MMC hypersensitivity?

We have not yet discovered naturally occurring mutations of FANCC that retain the MMC resistance function of that protein. Theoretically, however, such mutations might occur but would not have been ascertained (Fanconi anemia would have been ruled out based on DEB or MMC testing). If such cases can be identified by sequencing marrow or blood samples from children with bone marrow failure and negative MMC tests, we would have an opportunity to dissect the respective roles of genomic instability and cytokine hypersensitivity in the pathogenesis of both leukemia and cancer in this population. Naturally, other explanations

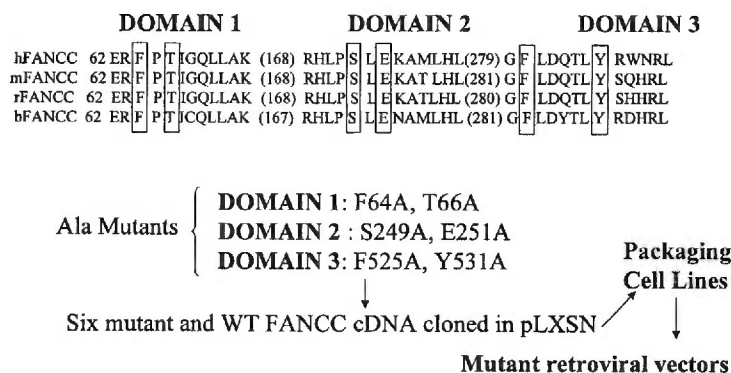


Figure 3. There exist three highly conserved domains in FANCC. Shown here are the amino acid sequences of human, murine, rat, and bovine FANCC. We introduced Ala mutations at two positions in each domain and used these cDNAs to attempt complementation FA-C cells using retroviral-mediated gene transfer. In these experiments we asked whether the responses to MMC and responses to apoptotic cytokines might be structurally distinguished. The results of these studies, reviewed below, showed that Ala mutants in domain 2 failed to correct defective induced STAT and hsp70 binding but did correct MMC hypersensitivity [51].

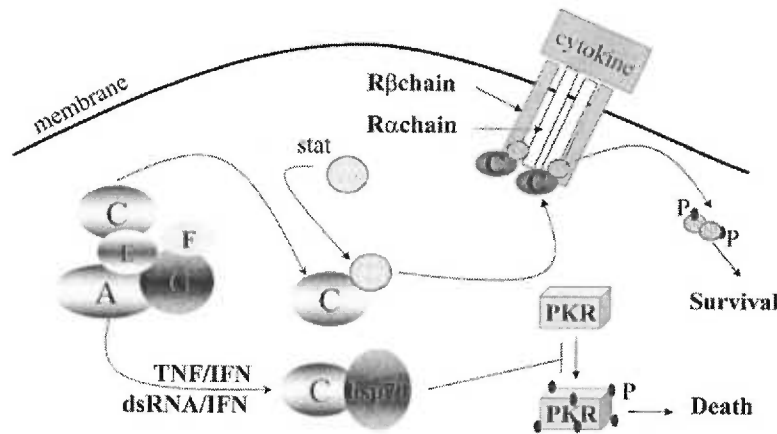


Figure 4. Signaling functions of FANCC. Of the many proteins with which FANCC binds, two clearly play a role in governing apoptotic responses in hematopoietic cells. In both cases, binding of FANCC to the signaling protein is induced. In the first pathway, in cytokine stimulated cells, FANCC leaves the FA complex within 10–15 seconds to associate with STAT molecules. These then appear at the cytokine receptor to become phosphorylated, dimerize, then translocate to the nucleus to govern transcriptional responses that effect cell survival. Mutation of FANCC prevents its association with STAT molecules and fewer of them appear at the receptor docking site. Fewer STAT molecules are phosphorylated and fewer of them appear in the nucleus of FA-C cells [52]. The second molecule with which FANCC associates is hsp70. Most of the association of this molecule with FANCC is induced (by either combinations of TNF and IFN or dsRNA and IFN). The association with hsp70 indirectly suppresses the activation or effects the rapid inactivation of double-stranded RNA-dependent protein kinase (PKR). Mutant FANCC molecules fail to associate with hsp70 and FA-C cells have excessive PKR activation (serine phosphorylation) and excessive apoptosis. The central role of PKR in transducing death signals in FA-C cells is best demonstrated by the capacity of dominant negative mutants of PKR to abrogate the excessive apoptotic responses of FA-C cells to TNF and IFN or dsRNA and IFN [40].

should be sought for MMC resistance in suspected cases of FA. Such explanations might include clonal evolution to myelodysplasia or acute myeloid leukemia [76,77] or somatic mosaicism [78,79]. On this point, it is worth noting that blood counts are commonly low in patients with mosaicism and while this might reflect the incapacity of the revertant stem cell to fully repopulate the FA marrow, it might also be reflective of incomplete complementation in which MMC hypersensitivity is relieved but cytokine hypersensitivity is not. High-throughput methods for mutation screening of both hematopoietic and nonhematopoietic cells from children with aplastic anemia would rule out these two possibilities.

Some patients with such mutations may already have been inadvertently described. Giampietro et al. described four children with certain clinical findings compatible with Fanconi anemia (FA), but whose lymphocytes were not hypersensitive to DNA cross-linking agents [80]. Additional unreported cases are often the subject of discussion at the scientific meetings of the Fanconi Anemia Research Fund. While Giampietro's group suggested that their cases represented examples of a previously undescribed autosomal recessive syndrome, based upon our findings with the ala mutants of FANCC, they may represent children with mutations of Fanconi anemia genes that knock out signaling functions of the protein without influencing the capacity of the protein to protect cells from cross-linking agents. Although these cases were ascertained prior to the description of the posttranslational changes of FANCD2 effected by the

non-D FA protein complex [23] it seems likely, given our finding that the MMC phenotype and the FANCD2 ubiquitinylation sort together, that in these cases immunoblots for FANCD2 might show both FANCD2_S and FANCD2_L.

Treatment of bone marrow failure

Currently, the strongest evidence for an apoptotic signaling pathway in FA is that of PKR activation. While small molecule inhibitors of PKR exist, the potential risks of their clinical use are substantial, taking into account the role of PKR in controlling viral infections [66,81,82] and perhaps even malignant transformation [83] in mammalian cells. In our view, understanding the precise molecular mechanisms by which PKR is activated in FA cells will lead to a clearer understanding of therapeutic target opportunities. If, for example, aberrant FA complexes bind to PKR and prevent it from binding to inhibitory factors, one might propose that the apoptotic phenotype could be reversed by small molecules that disrupt the aberrant complex, permitting a reassociation of the inhibitor with PKR. This would also assure that proper regulation of PKR would be preserved. Whether or not inhibiting PKR in FA cells is sufficient to overcome all of the defects in cellular signaling and processing of survival cues remains to be tested directly. However, since FA cells do exhibit suppression of STAT activation, it is possible that blocking the constitutive apoptotic pathway will be sufficient to protect FA hematopoietic cells in a clinically relevant manner.

Gonadal dysfunction

While the *fanca* and *fancc* knockout mice lack some features of the human syndrome (although a state of chronic bone marrow failure can be induced by exposing the mice to MMC [84,85], they do not develop bone marrow failure spontaneously), they clearly exhibit a severe germ cell failure phenotype [86–89]. The functional expression of certain survival factor pathways in gonadal tissues and hematopoietic tissues are similar [90,91] but it is known that redundancies can include some tissues but exclude others. For example, although the *bcl-2* family member *bcl-w* is expressed widely in murine tissues, loss of *bcl-w* function in mice seems only to impair spermatogenesis [92]. We argue that evaluating shared and distinct survival pathways in FA knockout mice may reveal why hematopoietic stem and progenitor cell function seem less perturbed than gonadal germ cells in this animal model.

Clonal evolution in

patients with FA and in patients without FA

Myelodysplasia and acute myelogenous leukemia are major predictors of clinical outcome in children with Fanconi anemia [3]. As described above, we believe that in the context of a mutator phenotype (a marker of which is MMC hypersensitivity) the cytokine hypersensitivity of many FA cells (linked to the PKR pathway) may create a selective pressure for the emergence of resistant clones [77]. What pathways are silenced in these clones that allow them to escape the consequences of the FANCC mutation? Which pathways are activated? Is MMC hypersensitivity always retained in the clones? If so, how?

Myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML) also occur in patients who have been treated with alkylating agents (known as secondary AML/MDS). In fact, the cytogenetic defects in such patients are precisely the same as those found in children with FA who have evolved to MDS and AML [93–97]. Could the complex cytogenetic picture found in patients with secondary leukemia reflect loss of function of the FA pathway? A survey study describing immunoblots for FA proteins suggested that FA proteins are dysfunctional in certain leukemic cells lines and in a small number of patients with AML, but there was no evidence that the cells had aberrant MMC or survival signaling responses [98] so the significance of the findings is unclear at this time. Preliminary studies in our laboratory indicate that full-blown FA pathway dysfunction may occur in marrow cells from patients with secondary AML/MDS, and we share the view that a comprehensive multidisciplinary analysis of cells from patients with primary and secondary AML/MDS is warranted.

Gene therapy

We share the view of a number of investigators that Fanconi anemia is a leading candidate for gene therapy. The apoptotic phenotype would suggest that it is possible that stem

cells transduced with the appropriate normal FA gene will simply outcompete the mutant untransduced stem cells and that extraordinary measures (for example, administration of alkylating agents to “force” selection of transduced cells) might not be required at all. To date, this has not been possible to test. Clearly, murine FA marrow cells are hypersensitive to MMC [84], and in murine transplantation models heterozygous stem cells have an advantage over homozygous ones [99], but even in these models the most clear expression of this advantage required the administration of MMC. Because the murine models of FA have not demonstrated bone marrow failure, this notion cannot be fully tested without using murine/human pre-clinical models and human experimentation. Further development of the murine model is needed to discover why marrow failure does not occur and to develop biological strategies to enforce it. In that context, models of selection that utilize biological agents that suppress only hematopoietic activity could be developed to reduce the need for exposure of stem cell recipients to MMC (a chemical factor to which all somatic cells are hypersensitive, not merely hematopoietic cells).

The past decade of Fanconi anemia research has been particularly robust. Most of the genes have been cloned and the description of the D’Andrea model of FA nuclear complex formation has been extraordinarily helpful in describing a coherent model of how mutations of these disparate proteins can lead to the same aberrant response to MMC. There is less certainty on the pathogenesis of the hematopoietic defects, but it is clear that the FA proteins are part of a cluster of survival signaling functions, some of which require participation of the other FA proteins, others of which may well not. Taking into account the emerging evidence that somatic mutations of FA proteins may play a role in disease pathogenesis in patients who do not have classical FA and clear evidence that the FANCD2 protein associates with an important tumor suppressor gene, one thing is very clear indeed: although Fanconi anemia is not a common disease, studies on the function of these genes is extraordinarily relevant to normal mammalian development and a broad spectrum of human disorders.

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