SIGNALING FUNCTIONS OF FANCONI ANEMIA PROTEINS

By

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CERTIFICATE OF APPROVAL

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List of Common Abbreviations

FA	Fanconi anemia
TLR	toll-like receptor
TNF	tumor necrosis factor
STAT	signal transducer and activator of transcription
STAT-P	tyrosine phosphorylated STAT
NF-κB	nuclear factor-κB
IκB	inhibitor of NF-κB
IKK	IκB kinase
NLS	nuclear localization signal
IRAK	interleukin-1 receptor associated kinase
TRAF	TNF-receptor-associated factor
TAK1	transforming growth factor-activated kinase-1
LPS	lipopolysacharride
JAK	Janus kinase
Hsp	heat shock protein

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Abstract

Fanconi anemia (FA) is a recessive inherited disease characterized by bone marrow failure, congenital abnormalities, and a predisposition to cancer. Since 1992 when the first FA gene (FANCC) was cloned, a total of 13 genes have been identified. Because hypersensitivity to cross-linking agents is a consistent feature of this disorder, most research in the field has been and continues to be focused on the role of the FA gene products in maintaining chromosomal stability. In contrast, the molecular pathogenesis of marrow failure in this disorder, the leading cause of death, has not been clarified. The focus of my thesis work has been to define the potential roles of these gene products in supporting the replication and survival of hematopoietic cells. Several pathophysiological mechanisms have been defined by our laboratory. First, FA hematopoietic cells are hypersensitive to tumor necrosis factor (TNF)-α induced apoptosis. Second, for reasons as yet unexplained, TNF-α production is increased in hematopoietic cells from FA patients and Fancc-/- knockout mice. The first project of this thesis addressed this problem using systems biology approaches followed by biochemical studies. This project describes the discovery that FANCC modulates the activation potential and activation state of toll-like receptor (TLR) 8 and that the overproduction of TNF-α by FA-C cells devolves from the unmodulated state of this particular TLR molecule. The second project has established a key survival signaling function for FANCC, FANCG and FANCD2. Specifically, we identified STAT5 activation defects in cells with inactivating mutations of FANCC, FANCG and FANCD2. Based upon the biochemical studies we have completed, we report that activation of the STAT5 signaling pathway requires inducible intermolecular interactions involving STAT5, FANCC and FANCD2 and that these

interactions require FANCG. Moreover, we provide evidence that these proteins influence specifically the nuclear translocation of tyrosine phosphorylated STAT5. Because STAT5 is a key signaling molecule for survival and replication of hematopoietic stem and progenitor cells, we argue that failure of the STAT5 signaling pathway is partly responsible for the hematopoietic phenotype of FA. Because both of the functions I define for these gene products are not dependent upon the nuclear "core complex" (which participates in the responses of normal cells to cross-linking agents), our work confirms that at least three FA genes are multifunctional. We anticipate that the other ten FA genes will also be shown to encode proteins that function in survival signaling pathways of relevance to hematopoietic cells.

Chapter 1

Introduction

1-1: Clinical Features of Fanconi Anemia (FA)

FA is a rare genetic disease with an estimated prevalence of 1 per 360,000. FA is characterized by bone marrow failure (BMF), congenital abnormalities, and a predisposition to cancer. Congenital anomalies frequently include radial ray abnormalities such as thumb hypoplasia and absent radius, macular hyperpigmentation of skin (café au lait spots), low birth weight, shortness of stature, renal abnormalities and genital defects. However, as many as one-third of FA patients do not display obvious congenital defects (1) and, in such cases, a diagnosis is only made following the diagnosis of an affected sibling or after hematological defects or cancers develop. The most prevalent FA malignancies include acute myeloid leukemia (AML) and upper aerodigestive tract cancers. BMF is the major cause of mortality, typically developing in the first decade of life.

1-2: Hematological Abnormalities

Defects of the hematological system are the most consistent clinical feature of FA. Bone marrow failure in FA patients is characterized by an inability to adequately produce blood cells (mature erythrocytes, neutrophils and platelets) so pancytopenia is common. Elevated infection rates in FA patients have been considered secondary to neutropenia. However, FA patients undergoing stem cell transplantation have higher rates of aspergillus infection than non-FA patients (2). Obligate intracellular parasites of this type are controlled significantly by the innate and adaptive immune system. Therefore, the reason for increased susceptibility to infection is still not resolved. This thesis

addresses a pathogen-induced signaling pathway that is aberrantly activated in FA patients. Although it is beyond the scope of this thesis which is focused on regulation of hematopoiesis, we believe that the observations we describe may ultimately lead to clarification of immune defects in the disease.

1-3: Diagnostic Test

The hallmark of FA cells is their hypersensitivity to DNA crosslinking agents, including mitomycin C (MMC), cisplatin and diepoxybutane (DEB). This hypersensitivity forms the basis of the current diagnostic test, whereby FA and normal cells are treated *in vitro* with MMC or DEB, followed by cytogenetic analysis of chromosomal breakage and quadri-radial formation. It is significant that this test for FA only reveals deviances in DNA damage and repair processes. A more comprehensive analysis of other signaling abnormalities in FA cells, the cornerstone of this thesis, may ultimately identify cases of FA that previously defied detection.

1-4: FA genes

FA is genetically heterogeneous, with at least 13 complementation groups (*A, B, C, D1, D2, E, F, G, I, J, L, M* and *N*). All of these except *FANCJ* have been cloned. *FANCA* is the most common complementation group (66%), followed by *FANCC* (10%) and *FANCG* (9%) (3). *FANCD1*, *FANCN* and *FANCJ* are identical to the previously

known breast cancer susceptibility (BRCA) genes *BRCA2*, *PALB2* and *BRIP1*, respectively.

1-5: Canonical FA Pathway

Although little is understood about the underlying causes of FA, monoubiquitinylation of the protein encoded by the FANCD2 gene appears to be a prerequisite for its interaction with BRCA2 and other repair proteins in foci of nuclear damage. Recruitment of these proteins to nuclear foci seems to be required for either resolution of or prevention of double-strand breaks. Ubiquitinylation of FANCD2 is dependent upon the formation of an FA "core complex" consisting of FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM. In cells bearing FA mutations, the functional core complex (and FANCD2 ubiquitinylation) is disrupted. Recently, FANCI was also shown to be monoubiquitinylated at a lysine residue that is conserved in FANCD2. Like FANCD2, FANCI localizes to chromatin in response to DNA damage and is believed to form a complex with FANCD2 to direct downstream repair events.

1-6: Multifunctionalitiy of FA Proteins

Published studies on the function of FA proteins have emphasized the canonical role of the "core complex" in protection against genotoxic stress, at least in part by facilitating the monoubiquitinylation of FANCD2. However, there is increasing evidence that FA proteins function in other signaling pathways. FA proteins have been reported to

bind to many non-FA proteins, many of which are cytoplasmic and not associated with DNA damage and repair processes. As shown in Table 1, FANCC is an example of an FA protein whose binding partners are functionally diverse. As one specific example, we have shown that FA proteins (FANCC and FANCD2) associate with certain signal transducer and activator of transcription (STAT) proteins and facilitate their activation. STAT proteins belong to a family of transcription factors that function to link ligand-bound cytokine receptors and oxidative stressors to specific transcriptional responses in mammalian cells.

Table 1. FANCC-interacting proteins.

Protein Name	**Functional Relevance**	**Reference**
STAT1	Facilitates STAT activation, promotes cell survival	(4)
STAT5		This thesis
Hsp70	FANCC/Hsp70 suppresses PKR activation, promotes cell survival	(5-7)
PKR		
Hsp90	Protein maturation/ activation	This thesis
Grp94	Protein maturation/ activation	(8)
GSTP1	Maintains the reduced /active state of GSTP1, promotes cell survival	(9)
FAZF	Transcription factor involved in hematopoiesis	(10-12)
Alpha spectrin	DNA repair	(13;14)
Cdc2	Cell cycle, G2/M progression	(15)
NADPH Cyt P450 reductase	Xenobiotic biotransformation, redox homeostasis	(16)

1-7: Hematopoiesis

The bone marrow is the only organ that universally fails in FA patients. It is not clear what the underlying molecular mechanisms are that make the bone marrow the most susceptible organ in FA. Understanding the molecular pathways involved in defective hematopoiesis in FA is the major focus of the laboratory in which my work, described herein, was conducted. Although all somatic FA cells are sensitive to DNA crosslinking agents, FA hematopoietic cells are uniquely sensitive to apoptotic cues, particularly double-stranded RNA, interferon-γ and tumor necrosis factor (TNF)-α.

1-8: TNF-alpha

TNF-α is a pro-inflammatory cytokine that has many cellular effects, including proliferation, differentiation, necrosis, apoptosis and induction of other cytokines. When overproduced in marrow cells, TNF-α plays a major role in suppressing hematopoiesis and is known to play an important role in the pathogenesis of acquired aplastic anemia (17;18). Previous studies conducted in our laboratory and in those of other groups have demonstrated that FA cells have increased sensitivity to TNF-α mediated apoptosis, consistent with a possible role for TNF-α in the pathogenesis of bone marrow failure in FA (19-21).

Signaling pathways that lead to the production of TNF-α may be relevant to FA, based on data from FA patients that showed elevated TNF-α levels in bone marrow cells

and serum (22-24). The source of increased TNF-α in FA bone marrow is not yet known. While many cells can produce TNF-α, the dominant producers of TNF-α are myeloid cells and T cells. Myeloid-lineage cells produce TNF-α upon stimulation of Toll-like receptors, activation via cytokines, and induction by leukotrienes. Hematopoietic cells have been shown to be large contributors of systemic TNF-α when exposed to endotoxin (25;26).

The biochemical alterations responsible for increased TNF-α production in FA have yet to be explained. There are many regulated steps involved in the production of TNF-α *in vivo* (Figure 1). Nuclear factor–κB (NF– κB) is a key transcription factor

Figure 1. Simplified model of NF-kB activation and expression of TNF-α. Engagement of TNF receptors (TNFRs), TLR/IL-1Rs or B-cell receptors/T-cell receptors (BCR/TCR) leads to IκB kinase (IKK) activation. Upon phosphorylation of IkB, active NF-kB dimers translocate to the nucleus and upregulate the expression of TNF-α. The production of mature TNF-α is also regulated post-transcriptionally by 1) decay of unstable mRNA and 2) cleavage of the membrane-bound TNF-α precursor in the secretory pathway to form active TNF-α.

regulating TNF-α production. Constitutive NF- κB activity, determined by measuring levels of nuclear NF κB protein, has been reported to be elevated in SV40-transformed FA fibroblasts (27). Another study analyzed genes expressed in EBV-transformed FA-C lymphoblasts, compared to normal transformed lymphoblasts, and found that many of the

genes upregulated in FA-C cells are genes that are expressed in normal cells during inflammation (28). Because NF-κB is believed to be the major inflammatory transcription factor, it was suggested that some of the upregulated genes might be due to constitutive activation of NF-κB. It remains to be shown whether NF-κB is activated in primary FA hematopoietic cells or myeloid cells, which are likely sources of TNF-α in FA patients.

Activation of NF-κB requires its translocation from the cytoplasm to the nucleus. NF-κB complexes are retained in the cytoplasm by a family of inhibitory proteins known as inhibitors of NF-κB (IκBs). NF-κB is released from these inhibitory factors upon phosphorylation of IκBs by the IκB kinase (IKK) complex. Phosphorylated IκBs are subsequently targeted for polyubiquitinylation and proteasomal degradation, and the release of NF-κB is rapidly followed by nuclear translocation.

Most of the diverse signaling pathways that activate NF-κB converge on IKK. IKK has received much attention in recent years as an attractive therapeutic target for modulation of inflammation. The IKK complex contains the catalytic kinases IKKα and IKKβ, and the regulatory scaffold protein NEMO (NF-κB essential modifier; also called IKKγ). One of the catalytic subunits, IKKβ, has been shown to associate with FANCA in HeLa cells by co-immunoprecipitation, although the functional significance of this interaction is not yet known (29).

TNF-α production can also be regulated at the mRNA level. The mRNAs of many pro-inflammatory proteins, such as TNF-α, contain AU-rich elements (AUREs) in

the 3'-untranslated regions. Messenger RNAs tagged with AUREs, are the target of exonucleases and are highly unstable. This stability can be regulated by non-nuclease proteins that bind to AURE sequences.

An additional point of TNF regulation is post-translational. For example, the 17 kDa secreted form of TNF-α must undergo processing from a 26 kDa membrane-bound precursor. Cleavage of the immature form is regulated by the activity of TNF-α converting enzyme (TACE) or matrix metalloproteinase 7 (MMP-7). MMP-7 is overexpressed in FA cells and TNF-α production in FA cells is in part dependent on MMP-7 activity (30).

Although TNF-α overproduction in FA cells might depend partly on elevated levels of MMP-7, the results of my studies, reviewed in Chapter 2, demonstrate clear-cut hyperactivation of TNF transcription owing to the influence of FANCC on the activation state of TLR8.

1-9: JAK/STAT Pathway

The JAK/STAT pathway represents a signaling pathway that regulates the expression of genes that are critical to the survival, self-renewal and differentiation of hematopoietic cells. A unique feature of STAT transcription factors is their ability to directly transmit a signal from the cell membrane, via recruitment of their SH2 domains to phosphorylated cytokine receptors, to the nucleus, where they modulate gene transcription. The pathway is stimulated when cytokines and growth factors bind to their

cell surface receptor. The receptor-bound tyrosine kinase JAK is thereby activated and phosphorylates tyrosine residues on intracellular portions of the receptor. Phosphotyrosines provide docking sites for proteins with SH2 domains, primarily STATs. Bound STAT molecules are then phosphorylated, by JAK molecules, on conserved tyrosine residues near the C-terminus leading to dimerization and translocation of STAT dimers to the nucleus, where they bind to promoters and regulate the transcription of specific target genes. Activated STAT proteins have also been reported to form tetramers, possibly influencing the affinity and specificity of STATs for particular target genes (31).

There are seven proteins that belong to the STAT family: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. STAT5A and STATB have 95% sequence identity and are likely the consequence of a gene duplication (32). STATs contain 750-850 amino acids and 6 conserved domains: N-terminal domain, coiled-coil domain, DNA-binding domain, linker domain, SH2 domain, and transactivation domain. The SH2 domain is the most highly conserved STAT domain, an indication of the essential function of phosphotyrosine recognition, which is required for the association of STATs with phosphorylated cytokine receptors. The SH2 domain is also required for STAT dimerization, which depends on the interaction of the SH2 domain of one STAT monomer with the C-terminal phosphotyrosine motif of another STAT monomer, resulting in both homo- and heterodimerization.

The mechanism of nuclear transport of STAT proteins is not fully understood. Tyrosine phosphorylation and STAT dimerization likely induces a conformation that is recognizable by transport machinery. Proteins that are targeted to the nucleus usually

contain a nuclear localization signal (NLS) that binds to importin-α. Importin-α, along with its cargo, binds to importin-β, which targets the complex to the nuclear pore, where it is translocated into the nucleus. Although classical NLSs have not been identified in STATs, NLSs with unconventional sequences have been identified in STAT1, STAT2 and STAT3. The NLSs belonging to these STATs are required for nuclear import and are only active as dimers in the case of STAT1/2, with one NLS element present in each monomer (33). In the case of STAT3, a monomeric NLS is functional (34).

1-10: STAT5

It is evident that STATs play diverse biological roles. For example, whereas STAT1 is involved in innate immunity, based upon genetic studies STAT5 plays critical roles in hematopoiesis and mammary gland differentiation (35;36). Our research has particularly emphasized STAT5 signaling because of its key role in hematopoiesis, and therefore its potential involvement in bone marrow failure in FA patients.

STAT5 activation in murine embryonic stem cells facilitates their differentiation into multipotent hematopoietic stem cells (37). Activated STAT5 is critically important for erythropoiesis and myelopoiesis (38). STAT5 activation plays a key role in the clonal expansion of stem cells (39). The differentiation of human stem/progenitor cells is affected by a wide variety of hematopoietic growth factors and cytokines that activate STAT5, including FLT3-L, SCF, G-CSF, GM-CSF, IL-3, EPO, and TPO (40-46). STAT5 dimers bind to γ-interferon activated sequence (GAS) motifs, containing the sequence TTC(T/C)N(G/A)GAA (31). Examples of STAT5 target genes important for

hematopoiesis include Bcl-xL, Pim, c-myc, OncostatinM, SOCS, D-type cyclins, *p21*, *c-fos, Id-1*, and *CIS.*

STAT5 proteins are encoded by two closely related genes, STAT5a and STAT5b. The amino acid sequences of STAT5a and STAT5b are 91% identical. STAT5a and STAT5b can form both homo- and heterodimers (47). Activation of Stat5a and Stat5b requires phosphorylation on Tyr-694 and Tyr-699, respectively. Interestingly, there is evidence that activated STAT5a can bind DNA as a tetramer as well as a dimer (31).

1-11: Involvement of FA proteins in STAT signaling

FANCC interacts with STAT1 and is required for activation of STAT1 in hematopoietic cells following IFN-γ stimulation (4). It was later observed that phosphorylation of STAT1, STAT3 and STAT5 are all reduced in response to type I IFNs in FA-C cells (48). This was significant considering the critical importance of STAT5 in hematopoiesis. The third chapter of this thesis will describe work I have conducted to address key unanswered questions regarding the intersection of FA proteins with STATs. Specifically, these questions include: 1) are other FA proteins besides FANCC involved in STAT activation, 2) are FA cells hyporesponsive to other modulators of STAT signaling and 3) how do FA proteins influence STAT function (i.e. trafficking of STATs into the nucleus)?

1-12: Thesis Overview

The results presented in this thesis further emphasize the multi-functionality of FA proteins. Chapter 2 represents the first studies ever conducted to examine whether FA proteins are involved in the ubiquitinylation of proteins other than FANCD2. Interestingly, the results of these studies, strongly supported by both transcriptomal and proteomics data, have clarified a transcriptional mechanism that accounts for TNF-α overproduction in FA-C cells. Specifically, the experiments led to the discovery that the toll-like receptor 8-dependent signaling pathway is hyperactivated in FA-C lymphoblasts, possibly due to ubiquitinylation of TLR8. Promiscuous activation of this pathway results in NF-κB activation and elevated TNF-α production, which is consistent with the elevated TNF-α levels observed in patients with FA.

The molecular and biochemical studies included in Chapter 3 also reveal non-canonical roles of FA proteins in regulating STAT5 signaling. We have defined components of large multiprotein complexes involved in STAT5 signaling, which consist of FANCD2, FANCC, STAT5 and Hsp90. We are the first to report that FANCD2, previously considered to be strictly a nuclear protein, participates in cytosolic signaling pathways as well. This surprising role for FANCD2 in the cytoplasm may have far-reaching implications in the field of FA research, which has largely ignored the possibility that FANCD2 is involved in processes other than DNA damage and repair.

The STAT5 paper (Chapter 3) has been submitted for publication and the TLR8 studies summarized here will (Chapter 2) be submitted promptly. They reveal discrete mechanisms by which hematopoietic cells are unfit. That is, FA hematopoietic cells are pro-apoptotic in part because they cannot transduce STAT5 signals, in part because they are hypersensitive to apoptotic cytokines (including TNFα), and in part because cell

populations in hematopoietic tissues overproduce TNFα, precisely the cytokine responsible for marrow damage. Because the studies have not yet been validated specifically in primary hematopoietic stem cells using knockout mice, more work must be done to determine which of these mechanisms plays the key role in stem cells per se. However, this task has been made much easier now that the specific molecular defects have been clarified. Finally, studies recently published from our laboratory have confirmed the adaptive nature of clonal evolution in Fancc-/- mice (49). These studies showed that ongoing exposure of hematopoietic stem cells to TNF-α *ex vivo* resulted in the evolution of pre-leukemic clones which were, not surprisingly, highly resistant to TNF-α (49).

Chapter 2

Aberrant Modulation of TLR8

Responses in FA-C cells

2-1: Introduction

Rationale

The FA "core complex," containing 8 FA proteins, is required for the monoubiquitinylation of FANCD2. Because it seems unlikely that all 8 proteins evolved to perform that single function, we hypothesized that the FA proteins participate in the ubiquitinylation of other substrates. A supportive clue came from the results of an expression microarray study of RNA derived from low density marrow cells from 20 FA patients and 11 normal volunteers, which revealed an over-representation of genes in the ontological category, "ubiquitin cycle" (Z=4.73). To directly test this hypothesis, we used proteomics-based methods to identify ubiquitinylated proteins in mutant FA-C and isogenic complemented FA-C/C cells. The ubiquitinylation and purification strategy employed is based on a previously described method (50), which involves *in vitro* ubiquitinylation of cell lysates with 6xHis-tagged ubiquitin, followed by purification using nickel chromatography.

The FA-C B-lymphoblast cell line used in the ubiquitinylation studies, as I will show later in this chapter, produces abundant TNF-α in the ground-state. In fact the level of TNF-α secreted from the FA-C cells is approximately 6-fold greater than the TNF-α secreted from complemented FA-C/C cells. The proteomics screens we performed were designed in the expectation that we would discover ubiquitinylated proteins in complemented cells that were not present in mutant FA cells, and therefore represent proteins whose ubiquitinylation requires an intact FA "core complex." Additionally,

because the approach was unbiased, we also found a number of proteins in mutant FA-C cells that were aberrantly ubiquitinylated (ones that were not ubiquitinylated in complemented cells). Significantly, one of these was TLR8, a known point of control for TNF production. Based on the potential link of TLR8 activation state with high level TNF production in FA cells, we pursued this observation biochemically.

Summary of Findings

TNF-α is elevated in bone marrow and serum from FA patients. However, the mechanism of increased TNF-α production is unclear. It will be demonstrated in this chapter that regulation of TNF-α synthesis in FA-C cells occurs transcriptionally and results from hyper-activation of a canonical TLR signaling pathway in FA-C cells. We are the first to observe that TLR8, an inducer of NF-κB dependent TNF-α expression, is hyper-activated in FA-C cells. TLR8 is either directly or indirectly ubiquitinylated as well. It will be described later in this chapter how we developed this hypothesis based on unbiased transcriptomal studies on marrow cells from FA patients and on proteomic studies that examined the role of FANCC in protein ubiquitinylation.

Toll-Like Receptor Structure and Function

TLRs are a family of transmembrane proteins that sense pathogen-derived molecules and initiate inflammatory responses, including the production of cytokines such as type I interferons and TNF-α (Figure 2). TLRs are abundantly expressed in

various hematopoietic-derived immune cells, such as macrophages, dendritic cells, and B lymphocytes. TLRs are also expressed in freshly isolated human bone marrow CD34+ progenitor cells, particularly TLR4, TLR7 and TLR8 (51).

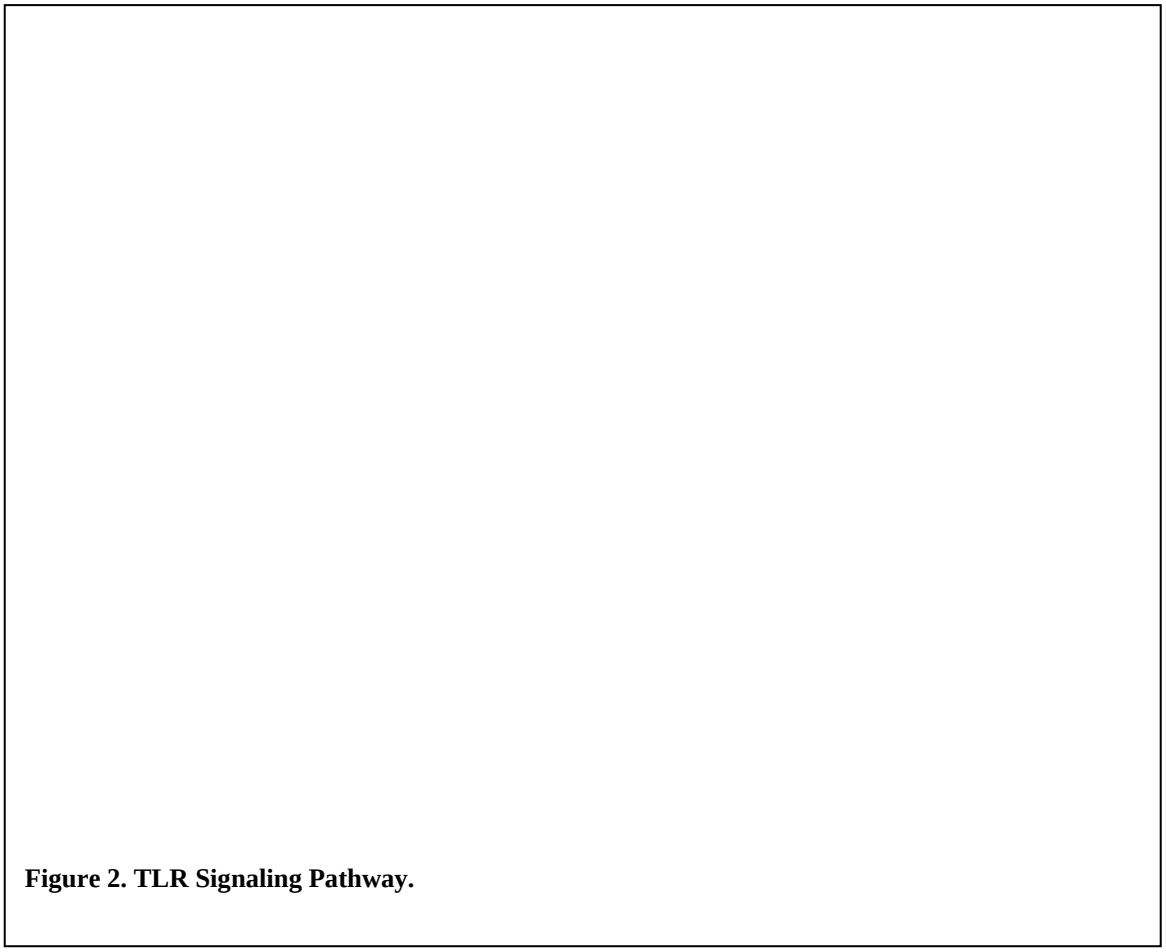


Figure 2. TLR Signaling Pathway.

TLRs are type I membrane proteins, characterized by an ectodomain containing multiple leucine-rich repeats (LRRs) that recognize microbial protein, carbohydrate and nucleic acids, and a cytoplasmic motif known as the Toll/IL-1 receptor (TIR) domain because of its homology with the type-1 IL-1 receptor. Ligands include bacterial lipoproteins (TLR2 heterodimerized with TLR1 or TLR6), double-stranded RNA (TLR3), lipopolysaccharide (TLR4), bacterial flagellin (TLR5), single-stranded RNA (TLR7 and TLR8), and pathogen-associated unmethylated CpG motifs in DNA (TLR9).

Nucleic acid-sensing TLRs (TLR3, TLR7, TLR8, and TLR9) are located within endosomal compartments, whereas the other TLRs are expressed at the cell surface.

Ligand binding induces TLR dimerization and conformational changes that allow the binding of adapter molecules to the cytoplasmic Toll/Interleukin-1 receptor (TIR) domain, via TIR-TIR interactions. These adaptors recruit downstream signaling molecules, activating signaling pathways, which are generally characterized as MyD88-dependent and MyD88-independent (TRIF-dependent) pathways (Figure 2). This leads ultimately to the activation of transcription factors, in particular NF-κB and members of the IRF family, resulting in the production of pro-inflammatory cytokines and type-1 interferons.

All of the TLRs except TLR3 use MyD88 as an adaptor. TLR3 uses the adaptor TRIF, and TLR4 is unique in its ability to recruit both MyD88 and TRIF. Upon binding of MyD88 to the TIR domain of the TLR, MyD88 interacts with IRAK4 and IRAK1 via death domain (DD)-DD interactions. IRAK4 activates IRAK1, leading to IRAK1 phosphorylation and dissociation from MyD88. Dissociated IRAK1 then binds to TNF-receptor-associated factor 6 (TRAF6). TRAF6 is an ubiquitin E3 ligase that catalyzes the synthesis of lysine 63-linked polyubiquitin chains on itself. Regulation of TLR signaling by ubiquitinylation and de-ubiquitinylation is a common theme (Figure 2). Polyubiquitinylated TRAF6 recruits and activates transforming growth factor-activated kinase-1 (TAK1) complex, which consists of TAK1, TAB2 and TAB3. TAB2 and TAB3 are regulatory subunits that contain nuclear zinc finger (NZF) motifs, which interact with K63-linked polyubiquitin chains. TAK1 then phosphorylates and activates the IκB kinase (IKK) complex, which includes the catalytic subunits IKKα and IKKβ, and the

regulatory subunit IKKγ/NEMO. TAK1 also activates the MAPK family. Induction of MAPK signaling activates the transcription factor AP-1. It has recently been shown that IRAK1 undergoes Lys63-linked polyubiquitinylation, like TRAF6, in response to IL-1 (52). The same authors proposed that IKK is activated following its association in a large multimeric complex containing IKK (IKKa, IKKβ and IKKγ), TAK1 (TAK1, TAB1 and TAB2/3), polyubiquitinylated TRAF6 and polyubiquitinylated IRAK1.

The activated IKK complex then phosphorylates the substrate IκB. The phosphorylation and subsequent degradation of IκB results in the activation of NF-κB. NF-κB subunits include NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (p65), RelB and c-Rel. They function as hetero- and homo-dimers, p50:p65 being the most studied dimer. NF-κB dimers are usually inactive in resting cells, sequestered in the cytoplasm by inhibitory proteins, IκBα, IκBβ, IκBγ, IκBε, or NF-κB precursors p100 or p105. The NF-κB activation pathway is dependent on the activity of IKKβ or IKKα: IKKβ functions to phosphorylate the IκB proteins, IκBα, IκBβ, IκBγ, IκBε and p105, and IKKα is responsible for p100 phosphorylation. Phosphorylation of IκBs leads to polyubiquitinylation and subsequent degradation by the 26S proteasome. The release of the inhibitor IκB from NF-κB dimers, such as the canonical p50:p65, permits NF-κB nuclear translocation.

P100 and p105 contain ankyrin repeats in their C-terminal halves. These ankyrin repeats, which have high homology to the ankyrin repeats in the IκB proteins, are required for the binding and sequestering of NF-κB dimers. IκBγ expression is thought to result from alternative splicing of *nfκb1*, the gene that encodes p105. Interestingly, IκBγ

has been reported to bind FANCA. Because the protein sequence of IκBγ is identical to the C-terminal 607 amino acids of p105, it's conceivable that p105 is an interacting partner of FANCA as well. This would place FANCA in a protein complex that contains both the kinase IKKβ and one of its substrates, IκBγ (or p105).

Negative Regulation of TLRs

It is critically important that the pro-inflammatory response to invading pathogens is turned off once the threat is gone because unbridled inflammatory signaling can be toxic to the host. Tissue debris produced from dying cells can also generate endogenous ligands, namely nucleic acids, that trigger TLRs. Excessive TLR signaling has been implicated in a variety of autoimmune and chronic inflammatory diseases, such as rheumatoid arthritis, lupus and cardiovascular disease (53-56).

Many proteins have been discovered that repress TLR signaling pathways. Suppressor of tumorigenicity 2 (ST2) is a membrane protein that contains a TIR domain, allowing it to inhibit TLR signaling by binding via TIR-TIR interactions to the adaptors MyD88 and Mal. SIGIRR is another membrane protein that interferes with TLR signaling by binding to IRAK and TRAF6. The expression of another membrane protein, TRAILR, decreases TLR signaling as well by a less understood mechanism.

There are numerous intracellular regulators of TLR signaling. MyD88 short (MyD88s) is a splice variant of MyD88 that forms MyD88s-MyD88 heterodimers (57). It was demonstrated that MyD88s prevents recruitment of IRAK4 to the IL-1R signaling complex (58). Sterile-alpha and Armadillo motif containing protein (SARM) negatively

regulates the MyD88-independent TLR pathway, possibly due to its interaction with TRIF, preventing the recruitment of downstream signaling molecules (59). Two IRAK family members, IRAK-M and IRAK-2, lack kinase activity and modulate TLR signaling: IRAK-M inhibits dissociation of IRAK-4/IRAK-1 from the MyD88 receptor complex (60) and IRAK-2 (specifically isoforms IRAK-2c and IRAK-2d) regulate TLR signaling due to the absence of N-terminal DDs (61).

Additional roles of ubiquitinylation in TLR pathway signaling

Ubiquitinylation and de-ubiquitinylation is a mechanism for rapid regulation of TLR signaling. A20 is a de-ubiquitinylating enzyme induced by TLR ligands and TNF-α. It acts in a negative feedback loop that restricts TLR and TNF receptor-induced signals. A20 removes ubiquitin moieties from TRAF6, resulting in TLR signal termination and recycling of TRAF6 (62). A20 also functions to remove K63-linked ubiquitin moieties from NEMO and RIP. Not surprisingly, NF-κB activation is exaggerated in A20-deficient macrophages (63). It is interesting that A20 knockout mice, similar to FA mice, are highly susceptible to endotoxin, and wild-type hematopoietic cells restore normal endotoxin response.

TLR signaling is also regulated by the E3 ubiquitin ligase Triad3A, which appears to target TIR domain-containing proteins for degradation (64;65). Known targeted proteins include TLR4, TLR9, RIP-1, TRIF and Mal (64;65). Mal is also polyubiquitinylated and targeted for proteasomal degradation by the E3 ligase SOCS-1 (66).

2-3: Results

Transcriptomal analysis.

To identify genes that were either under-expressed or over-expressed in FA cells, we conducted a genome wide transcriptomal analysis of bone marrow RNA from 20 FA patients in the aplastic phase (no evidence of clonal evolution cytogenetically and no evidence of acute leukemia) and 11 normal volunteers. We found a total of 1003 genes that were significantly overexpressed by 1.4-fold or greater in FA marrow cells and 2739 genes suppressed in FA samples by 1.4 fold or greater. We found the ubiquitin cycle to be over-represented both in the suppressed gene set and the over-expressed list (Appendix Figure 1). Specifically, sixty five features on the chip represented the biological process category of "Regulation of ubiquitin protein ligase activity" and 29 of these genes were suppressed in the FA samples, a significant over-representation of this ontological category (Z=5.57). Of the 1003 genes overexpressed in the FA samples, gene products that participate in "ubiquitin dependent catabolic processes" were also over-represented (Z=3.47).

The observation that genes involved in ubiquitin-related processes were over-represented, not only in the list of suppressed genes but in that of over-expressed genes as well, is compatible with the view that a more general defect in the control of ubiquitinylation may exist in FA cells. To test this notion, we conducted a proteomic analysis of the ubiquitome in FA cells.

In vitro ubiquitin mapping

The proteomics-based method I am using to isolate ubiquitinylated proteins is modified from a published procedure (50). Specifically, hexahistidine-tagged ubiquitin, an ATP-recycling system, and inhibitors of the proteasome and de-ubiquitinylating enzymes, are added to lysed FA cells and normal cells (Figure 1). This method uses endogenous enzymes systems (E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes and E3 ubiquitin-protein ligases) present in the cell lysates. Following the ubiquitinylation reaction, ubiquitinylated proteins are affinity purified by nickel chromatography, digested and analyzed by 2D capillary LC-MS/MS. We identified 99 proteins that were uniquely ubiquitinylated in the FA complemented (FA-C/C) cell lysate but not the FA-C cell lysate, and therefore represent proteins that require an intact FA core complex for ubiquitinylation (Appendix Table 2). The identified proteins could be classified ontologically into many functional categories. The prevalence of proteins involved in ubiquitinylation provided confirmation that our assay could reliably identify proteins that were either directly ubiquitinylated or associated with

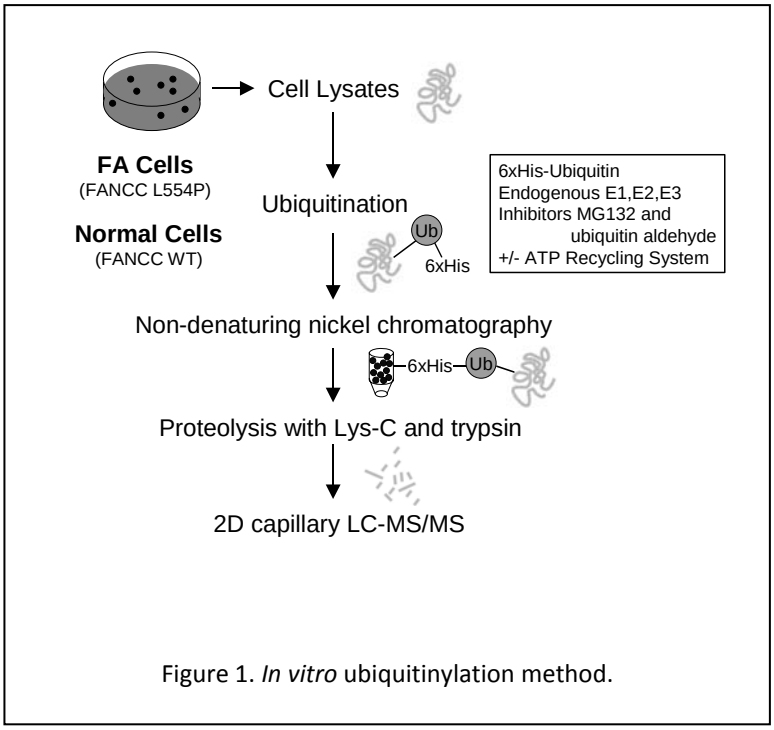


Figure 1. *In vitro* ubiquitinylation method.

ubiquitinylated proteins. The observed diversity of cellular substrates for ubiquitinylation is in agreement with other studies conducted in our laboratory that suggest FA proteins participate in a variety of cellular processes.

In the course of analyzing the results from the ubiquitinylation experiments, we also identified 90 proteins that were ubiquitinylated in the Fanconi anemia cell lysate but not in the normal cell lysate (Appendix Table 1). We have followed up experimentally on one of these proteins, TLR8. The TLR8 peptide sequences identified are shown in Figure 2. We hypothesize that ubiquitinylation of some of these proteins is due to alterations in the macromolecular structure of the FA core complex, which allow the ubiquitinylation machinery to ubiquitinylate proteins promiscuously. There is also the possibility that mutations in Fanconi proteins indirectly alter the activity of E3 ligases that are not part of (or associated with) the "core complex" of proteins. For example, the overproduction of TNF-α in FA-C cells may result in activation of the TNF receptor pathway, which has been linked to downstream polyubiquitinylation of TRAF2, RIP, TAK1 and IKK.

It is possible to confirm direct ubiquitinylation of a protein because tryptic digestion of ubiquitin leaves the two C-terminal glycine residues (114 Da) attached to the target protein, which is revealed in the identification of a protein with an

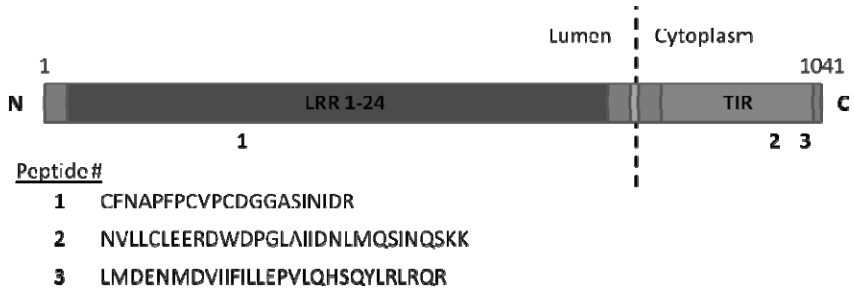


Figure2. Schematic of Toll-like receptor 8 functional domains and peptide sequences identified by LC-MS/MS

added mass of 114 Da. Using this method of analysis, we identified 18 ubiquitinylated proteins in FA-C cells and 11 ubiquitinylated proteins in FA-C/C cells (Appendix Table 3).

Confirmation of TLR8 ubiquitinylation in FA-C cells

We hypothesized that ground state hyperactivation of the TLR8 signaling pathway in FA-C cells was due to ubiquitinylation of TLR8 itself or a protein associated with TLR8. By co-immunoprecipitation (using antibodies against ubiquitin and TLR8), it appears that TLR8 or an associated protein is indeed more ubiquitinylated in mutant FA-C than complemented cells, in agreement with the proteomic data (Figure 3).

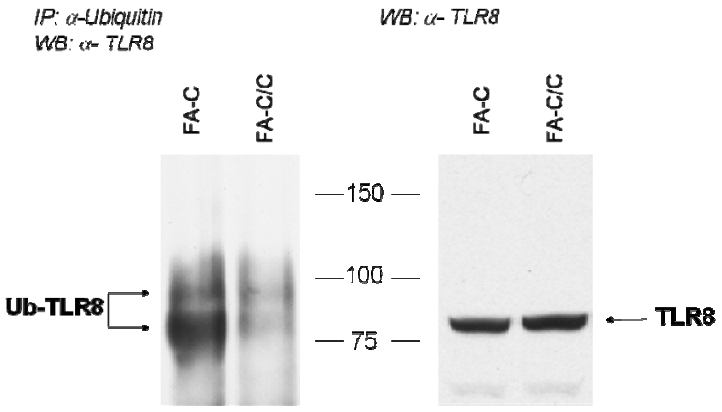


Figure 3. TLR8 associates either with ubiquitin or a ubiquitinylated protein. Co-immunoprecipitation revealed more ubiquitinylation of TLR8 (or an associated protein) in FA-C (mutant) cells than in FA-C/C (complemented) cells.

Ground state TNF-α gene expression is elevated in FA-C cells

Because TLR8 signaling is important for the production of TNF-α, we compared TNF-α released from mutant FA-C and normal FA-C/C cells, measured in culture media by ELISA. We observed a 6-fold increase in TNF-α produced by the FA-C cells,

suggesting that ubiquitinylation of TLR8 in FA-C cells might be an activation event, leading to cytokine expression (Figure 4). We have confirmed this result in primary cells by showing that low-density bone marrow cells from an FA patient also overproduced TNF-α protein (data not shown). At the level of mRNA, we demonstrated by real-time PCR that TNF-α mRNA from FA cells is also overproduced (Figure 5), suggesting that TNF-α overproduction in FA cells is due to some molecular alteration prior to translation of mRNA. I evaluated mRNA stability by measuring the rate of TNF-α mRNA degradation in the presence of the transcription inhibitor, actinomycin D (Figure 6). The rate of decay of TNF-α mRNA was equivalent for FA-C and normal cells, indicating that FA cells have increased TNF-α gene transcription, a defect complemented by the transduction of normal FANCC cDNA.

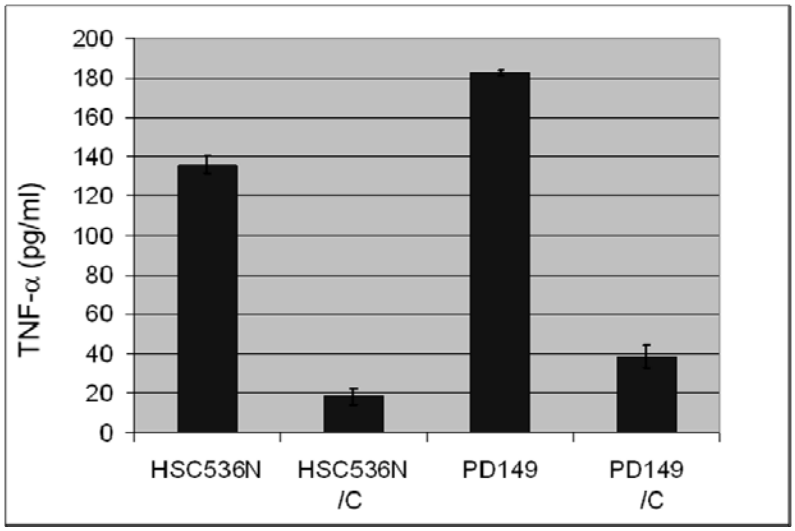


Figure 4. TNF-α gene expression is elevated in FA-C lymphoblasts. Mutant FA-C lymphoblasts (HSC536N and PD149) produce more TNF-α than isogenic complemented FA-C/C cells. Secreted TNF-α was measured in culture media by ELISA assay.

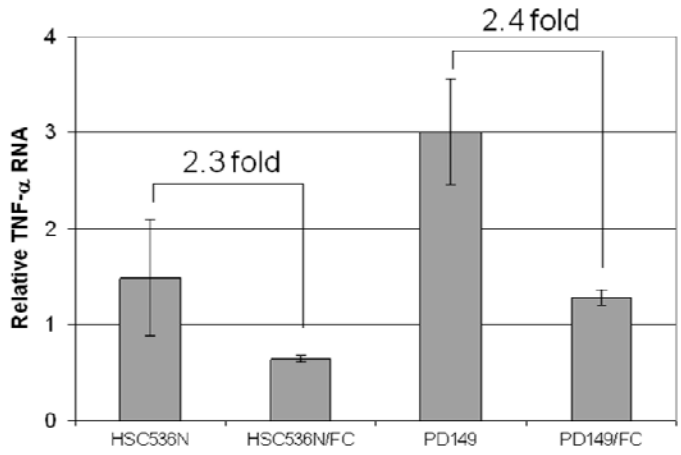


Figure 5. TNF-α RNA is elevated in FA-C lymphoblasts Increased TNF-α mRNA in mutant FA-C cells is also observed at the mRNA level. TNF-α mRNA was analyzed by real time PCR.

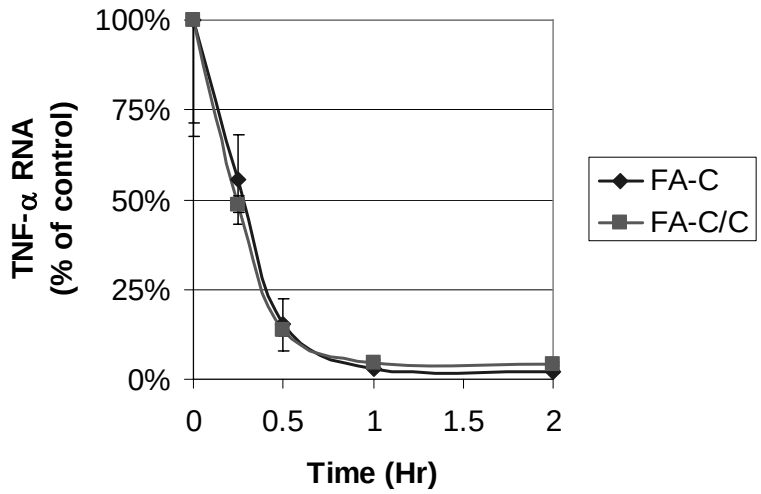


Figure 6. TNF-α mRNA decay rates are equal. To rule out differential TNF-α mRNA decay as an explanation for the increase in mutant cells, mRNA stability was measured by real-time PCR following treatment of cells with the transcription inhibitor actinomycin-D. The mRNA decay rates were identical, demonstrating that the differences in mRNA levels between FA-C and FA-C/C cells is due to differences in mRNA production rather than differences in mRNA stability.

TNF-α production in FA-C cells is TLR8-dependent

Based on the above results, we hypothesized that elevated levels of transcription of TNF-α in FA-C cells is mediated by hyperactivation of TLR pathways. To demonstrate hyperactivation of TLR8 in FA-C cells, we transfected siRNA against TLR8. In agreement with our model, inhibition of TLR8 expression in mutant FA-C cells led to decreased production of TNF-α mRNA (Figure 7).

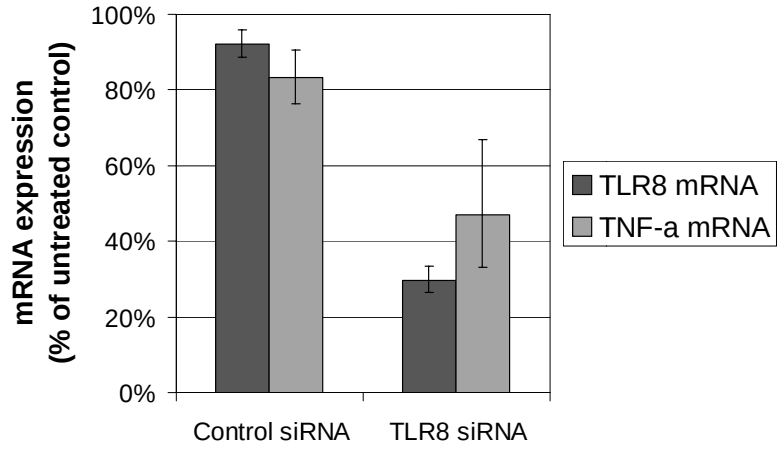


Figure 7. TLR8 siRNA decreases TNF-α mRNA synthesis in FA-C cells. TNF-α mRNA was lowered in FA-C cells treated with siRNA against TLR8, demonstrating that TNF-α production in these cells is TLR8-dependent.

IKK and IRAK-1 are constitutively activated in FA-C Cells

To confirm activation of the toll-like receptor pathway in FA-C cells, we monitored phosphorylation of two kinases, IKK and IRAK-1 by western blot. As shown in Figure 8, IKK phosphorylation is apparent in FA-C cells. Because the antibody used to detect P-IKK recognizes the phosphorylated forms of both IKK-α and IKK-β, the specific IKK subunit that is hyperphosphorylated in FA-C cells is not yet known.

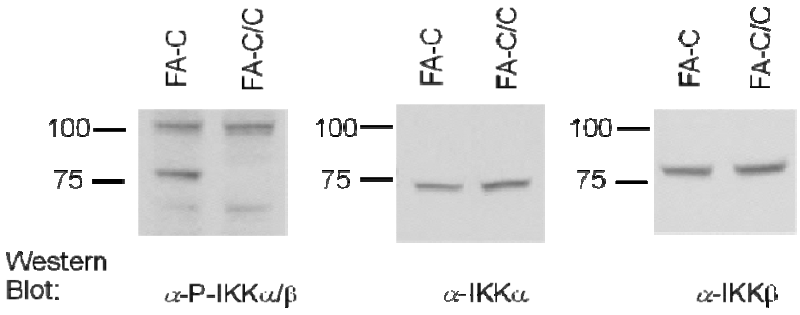


Figure 8. IKK-α/β is phosphorylated in FA-C cells. IKK-α or IKK-β is phosphorylated (approx. 75 kDa band) in FA-C cells as demonstrated by western blot analysis.

Because many signaling pathways converge on IKK activation, we decided to determine the activation state of IRAK-1, which would implicate upstream activation by TLRs or IL-1Rs. IRAK-1 phosphorylation was measured by western blot using an antibody that recognizes phosphorylated Ser-376 of IRAK-1. The apparent molecular weight of P-IRAK-1 in Figure 9 is between 150 and 250 kDa. This is well above the apparent MW of unmodified IRAK-1, which runs at approximately 80 kDa. The high apparent molecular weight and "smeared" nature of P-IRAK-1 suggests that the protein might be ubiquitinylated. Phosphorylation of IRAK-1 has been demonstrated to precede its polyubiquitinylation (67). Recently, Lys63-linked polyubiquitinylation of IRAK-1 was shown to be an activating modification (52). As shown in Figure 9, removing vanadate, a tyrosine phosphatase inhibitor, from the lysis buffer results in a reduction of P-IRAK-1, demonstrating that serine phosphorylation of IRAK-1 is tyrosine kinase dependent. To date, it is unknown if IRAK-1 can be activated by tyrosine phosphorylation. However, IRAK-1 was recently shown to bind to SHP-1, a protein tyrosine phosphatase (68). The authors showed that in bone marrow-derived dendritic cells from SHP-1 deficient mice TNF-α is substantially increased upon LPS or poly(I:C), compared to wild type mice. It can therefore be theorized that in FA-C cells, SHP-1 may not properly bind to and dephosphorylate IRAK-1, allowing hyperactivation of IRAK-1.

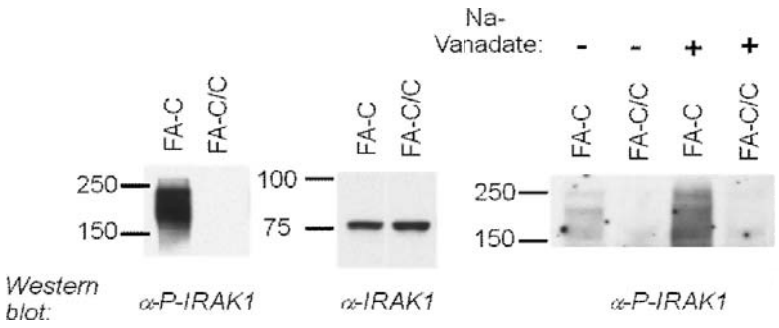


Figure 9.

IRAK1 is phosphorylated in FA-C cells. Phospho-IRAK1 is detected in FA-C cells in the ground state by western blot using an antibody specific for the phosphorylated form of IRAK1. Phospho-IRAK1 levels are diminished when vanadate is left out of the lysis buffer.

We attempted to confirm phosphorylation of IRAK-1 by treating cell lysates with lambda phosphatase and analyzing P-IRAK-1 by western blot. IRAK-1 phosphorylation was not reduced when FA-C cell extracts were treated with lambda phosphatase (data not shown). This suggests that 1) the anti-P-IRAK1 antibody is detecting a non-specific band or 2) additional modifications of IRAK-1 (such as ubiquitinylation) prevent access to lambda phosphatase due to steric hindrance.

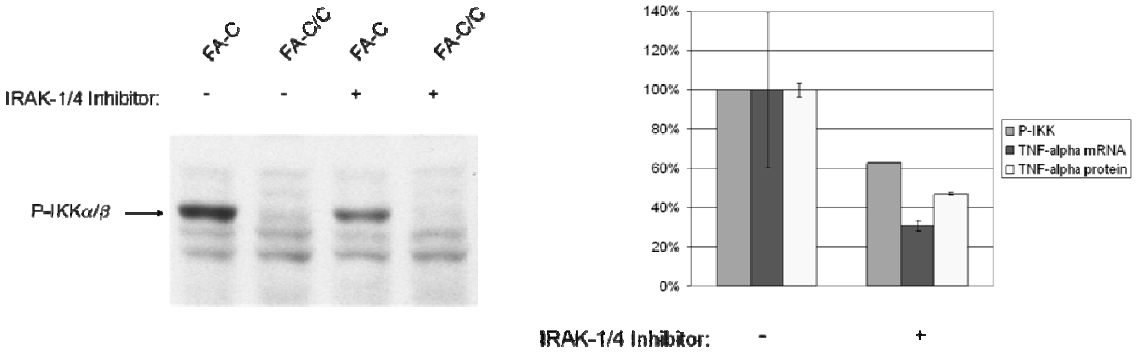


Figure 10. Phosphorylation of IKKα/β and TNF-alpha expression are reduced in FA-C Cells Treated with an IRAK-1/4 Inhibitor. Cells treated with an IRAK1/4 inhibitor (N-(2-Morpholinylethyl)-2-(3-nitrobenzoylamido)-benzimidazole) showed reduced phosphorylation of IKK-α/β as measured by western blot (A). The inhibitor also lowered TNF-α mRNA and protein (B). P-IKK was measured by western blot / densitometry, TNF-α mRNA was determined by real-time PCR, and TNF-α protein was measured by ELISA.

Because western blot data suggested that IRAK-1 might be hyperactivated in the ground state in FA-C cells, we tested the effects of an IRAK-1/4 inhibitor on FA-C cells. As shown in Figure 10A, the IRAK-1/4 inhibitor reduced ground state phosphorylation of IKK α/β in FA-C cells, illustrating that ground-state phosphorylation of IKK in FA-C cells is at least partly due to IRAK activation. We also determined whether the IRAK-1/4 inhibitor affected TNF-α production, at both the mRNA and protein level (Figure 10B). The inhibitor not only reduced phosphorylation of IKK, calculated by densitometry of the western blots shown in Figure 10A, but in addition lowered both TNF-α mRNA and protein. Therefore, IKK phosphorylation and TNF-α synthesis in FA-C cells is at least partly dependent on IRAK-1 activity.

High Ground state activation of NF-κB in FA-C cells

We have described thus far evidence for a constitutively activated TLR8-dependent signaling pathway in FA-C cells involving the activation of two kinases, IRAK-1 and IKK, which leads to the biosynthesis of TNF-α. The transcription of TNF-α is regulated by numerous transcription factors, including NF-κB, IRF family members (IRF), AP-1, Ets, NF-AT, SP-1, cAMP response element-binding protein, C/EBPb, ATF-2, Jun and LITAF. We decided to evaluate the state of NF-κB activation in the ground state in FA-C and FA-C/C cells. Electromobility shift assay (EMSA) experiments demonstrated greater constitutive NF-κB activation in two different FA-C cell lines, compared to the corrected FA-C/C cells (Figure 11). The DNA-bound NF-κB complexes contained both p50 and p65, evident by supershifting with anti-p50 and anti-p65

antibodies. Based on the density of the supershifted bands, p50 is a major component of the NF-κB complex activated under basal conditions.

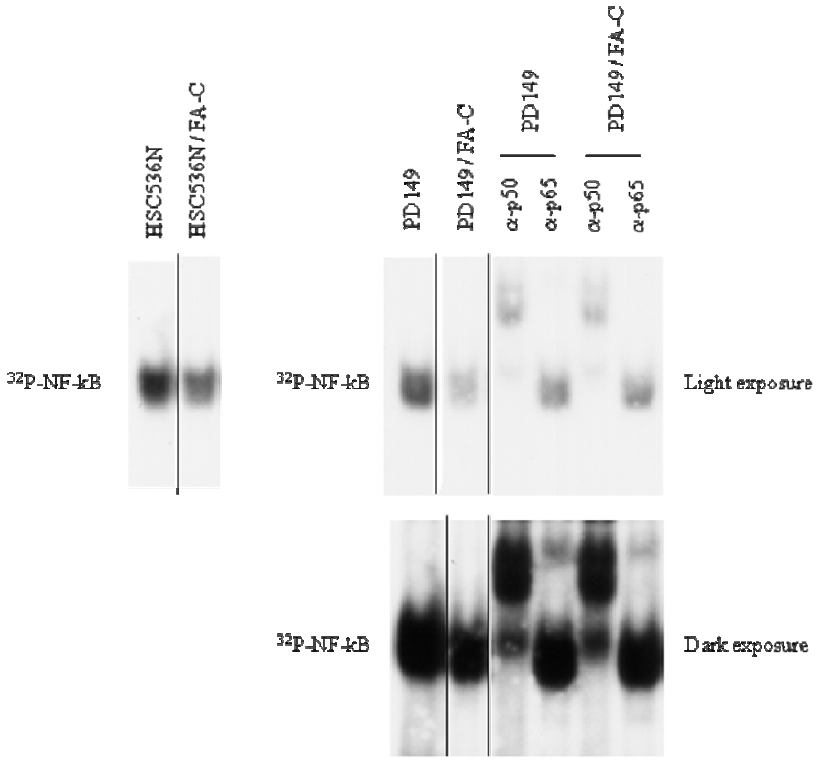


Figure 11. Activation of NFκB in FA-C Cells is Demonstrated by EMSA. Ground-state activation of NF-κB was seen in two different FA-C lymphoblast cell lines. Supershifting was observed with anti-p50 antibody, and with anti-p65 antibody upon longer exposure.

NF-κB activation requires the phosphorylation, ubiquitinylation and degradation of inhibitory IκB proteins. To evaluate the phosphorylation state of IκBα, IκBβ and IκBε in FA-C cells, we performed western blots using antibodies against the phosphorylated forms of those proteins (Figure 12). We analyzed total protein levels by western blot as well. As shown in Figure 12, IκBα appears to be constitutively phosphorylated in the FA-C and FA-C/C cell lines used for these studies, and there isn't a significant difference in phosphorylation level between the two cell lines. The phosphorylation of IκBβ and IκBε on the other hand was barely detectable. To determine if the low levels of phospho- and total IκBβ and IκBε were due to proteasome-dependent degradation, the same proteins were analyzed in cells that were treated with the proteasome inhibitor MG132. However,

there was no significant difference in any of the protein levels with or without MG132 (data not shown). Based on this data, we conclude that the pathway to NF-κB hyperactivation in FA-C cells does not involve the inhibitor proteins IκBα, IκBβ or IκBε.

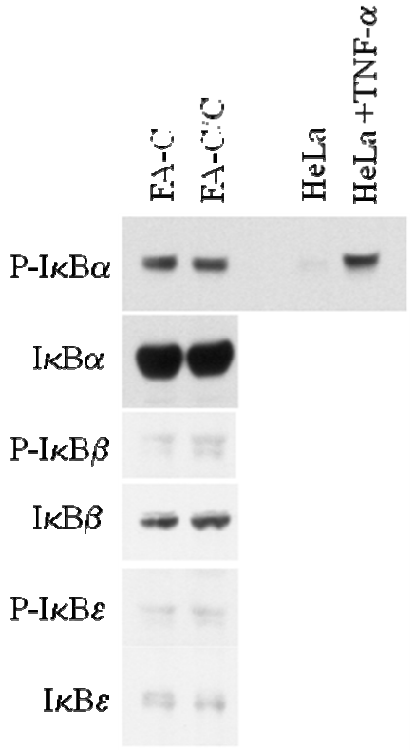


Figure 12. Western blot analysis of IκBα, IκBβ and IκBε in FA-C and FA-C/C cells. The levels of phosphorylated IκBα, IκBβ and IκBε, as well as the total levels of these proteins don't vary significantly between FA-C and FA-C/C cells, suggesting they are not involved in the hyperactivation of NF-κB observed in FA-C cells.

Confirming the role of FANCC in modulating TLR8 activation state: Loss of Function studies.

FA-C mice are hypersensitive to lipopolysacharride (LPS)-induced shock and LPS induces TNF-α dependent marrow failure (26). This is important because activation of the immune response in normal cells is tightly controlled to prevent exaggerated stimulation and toxicity. Based on the combination of our results and the reported LPS responsiveness in FA cells, we hypothesized that FA cells might be *generally* hyper-

responsive to various TLR agonists. To test this possibility, FA-C and FA-C/C lymphoblast cell lines were treated with TLR agonists LPS, R-848, CL-075 and ssRNA. Unfortunately, none of these agents induced expression of TNF-α (data not shown) in these cell lines. Given our experience with these cell lines, this was not unexpected because they are not generally responsive to various small molecules and nucleic acids because of poor uptake.

The human monocytic cell line THP-1 is routinely used for studying toll-like receptor signaling and has the advantage over lymphoblast cell lines of being responsive to many TLR agonists. In addition, a THP-1 derivative, THP-1 Blue, expresses an NF-κB-inducible reporter. Using this system, we have inhibited FANCC expression in THP-1 cells by stable expression of FANCC shRNA, as shown in Figure 13A, and monitored responses to various TLR agonists. FANCC expression was reduced by 90% in cells expressing FANCC shRNA (#371), compared to untreated cells or cells expressing non-target shRNA (NT). The effectiveness of FANCC knockdown was confirmed by analyzing FANCD2 monoubiquitinylation (Figure 13B). Whereas MMC treatment of control cells (treated with non target shRNA or no shRNA) resulted in FANCD2 monoubiquitinylation (upper band), cells expressing FANCC shRNA showed very little response to MMC as expected.

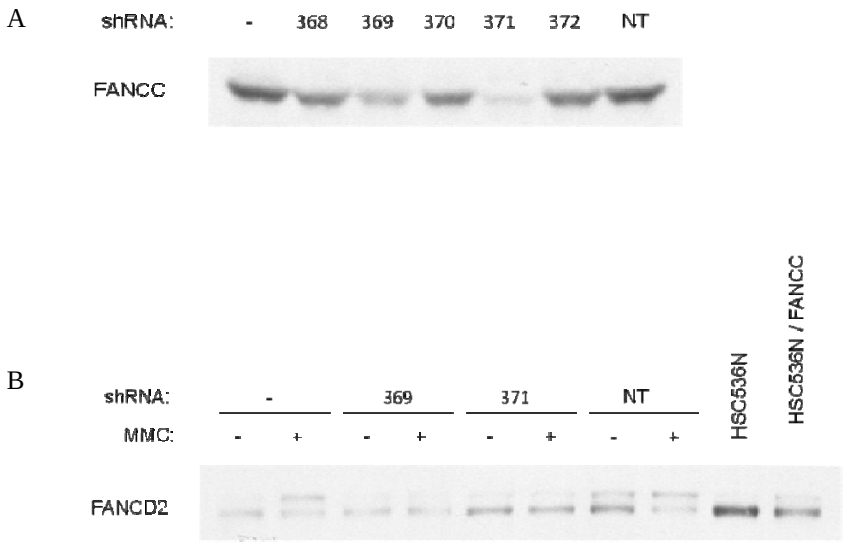
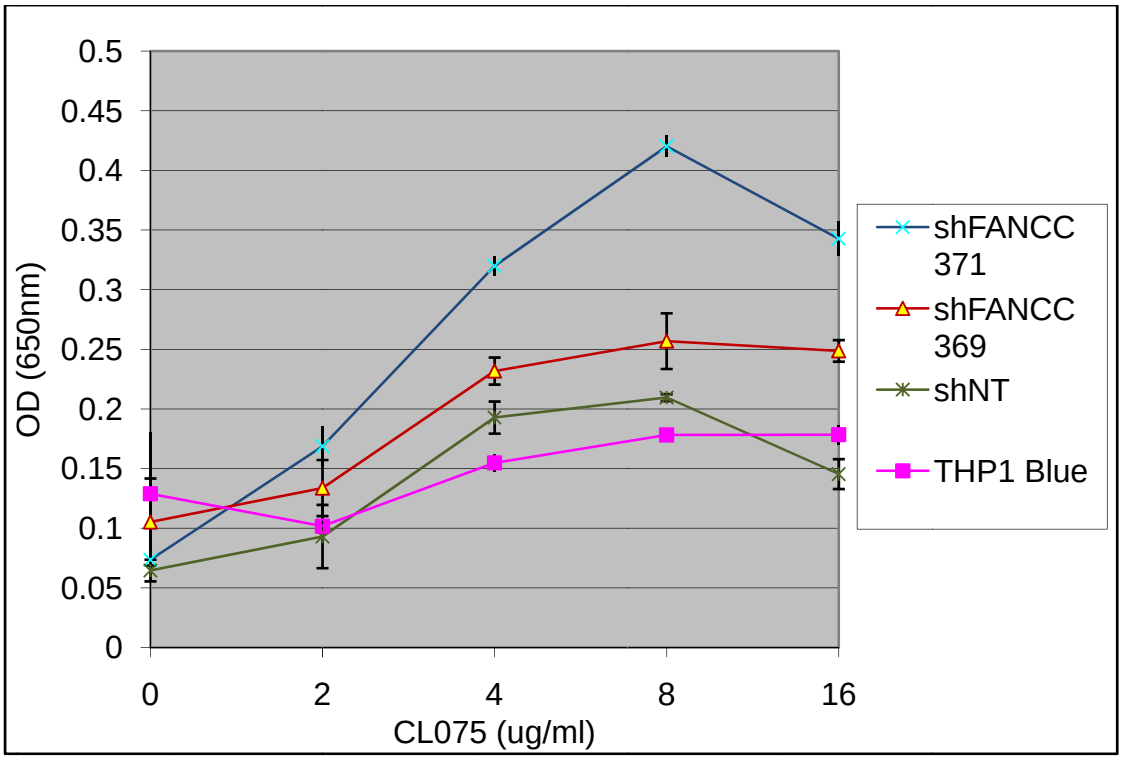


Figure 13. ShRNA Effectively Reduces FANCC protein in THP1 Monocytes. (A) Different shRNAs were screened by western blot for their ability to reduce FANCC protein. ShRNA #371 lowered FANCC 90% (by densitometry). (B) Suppression of FANCC by shRNA limited the ability of mitomycin-C to induce FANCD2 monoubiquitinylation (upper band). NT=non-target shRNA.

We initially sought to confirm that TLR8-dependent activation of NF-κB in the THP-1 Blue cells was modulated by FANCC. To do this, we treated cells with the two ligands, R-848 (Resiquimod) and CL075 (3M002). R-848 and CL075 are small-molecular-weight synthetic compounds belonging to the imidazoquinoline family that activate TLR7 and TLR8. However, CL075 induces TLR8 to a greater extent than TLR7 (approximately 10-fold more CL075 is required to activate NF-κB in TLR7-transfected HEK293 cells than in TLR8-transfected HEK293 cells). As shown in Figure 14, reduced FANCC expression resulted in elevated sensitivity to both CL075 and R-848. We believe the observed differences in sensitivity are due to the ability of FANCC to suppress TLR-dependent signaling.

Based on the reported sensitivity of FA mice to the TLR4 agonist LPS, we also treated THP-1 Blue cells, expressing shRNA against FANCC, to a range of other TLR ligands (Figure 15). Cells were incubated with agonists to specifically target TLR1-9. The only ligands capable of inducing FANCC-dependent NF-κB activation were

molecules that target TLR7 and/or TLR8. We also tested the TLR7-specific agonist imiquimod, but none of the cells responded to this molecule. This imiquimod-insensitivity suggests that THP-1 Blue cells might preferentially express TLR8 over TLR7. We conclude that FANCC modulates the activation state or potential of TLR8 but does not influence the state of other molecules in the Toll like receptor family. Thus, we have shown that the TLR8 pathway distinguishes itself by being modulated by FANCC. This observed TLR8-specificity fits our original model, i.e., that TLR8 itself might be regulated by ubiquitinylation. This seems quite likely considering that the canonical signaling cascades activated by various TLRs are quite similar. It is also apparent that further studies of the biochemical effect of FANCC on the downstream IRAK/IKK/NF-κB signaling pathway should include non-canonical proteins.



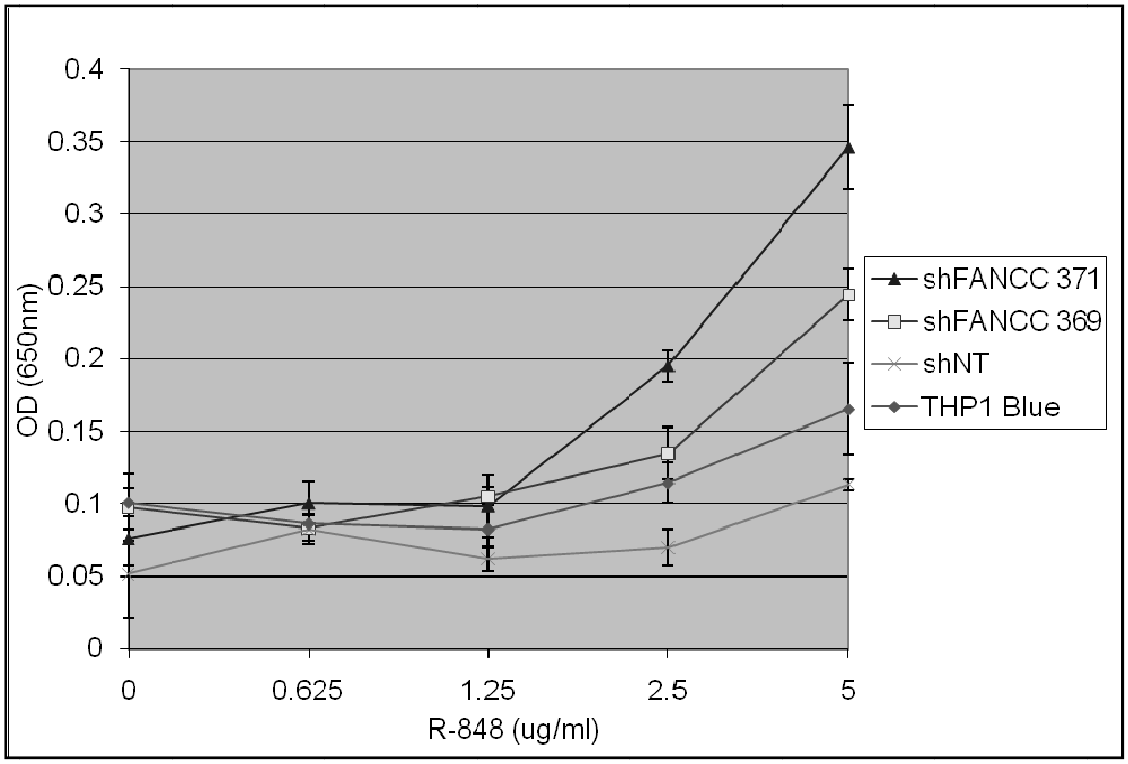


Figure 14. NF-κB activation by TLR7/8 ligands in THP-1 Blue cells is FANCC-dependent. Cells were incubated 24 hr with various concentrations of CL075 and R-848. Expression of the NF-κB reporter was monitored spectrophotometrically at 650 nm.

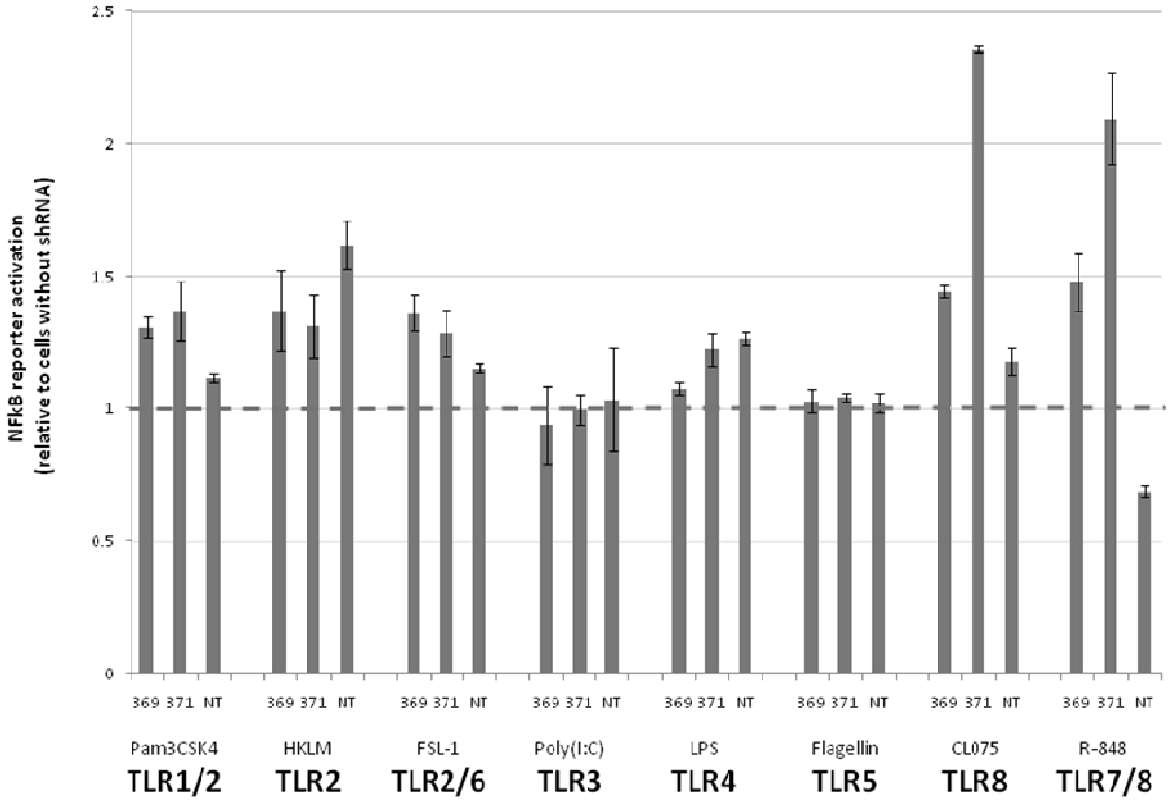


Figure 15. Profile of NFκB Activation with Various TLR Ligands

THP-1 Blue cells expressing FANCC shRNAs (369 and 371) and non-target shRNA (NT) were treated for 24 hr with the following TLR agonists:

Pam3CSK4	(Pam3CysSerLys4)	1 ug/ml
HKLM	(heat-killed preparation of *Listeria monocytogenes*)	10^8 cells/ml
FSL-1	(fibroblast-stimulating lipopeptide-1)	1 ug/ml
Poly(I:C)	(polyinosinic:polycytidylic acid)	1 ug/ml
LPS	(lipopolysacharride from *E. coli* K12)	1 ug/ml
Flagellin	(flagellin from *S. typhimurium*)	1 ug/ml
CL075		8 ug/ml
R-848		5 ug/ml

Dose-response curves were generated for each compound. The above graph shows the levels of NF-κB stimulation (at the ligand concentrations that gave the peak activation of NF-κB), relative to NF-κB activation in cells without shRNA (y-axis =1). Cells were insensitive to imiquimod (TLR7) and ODN2006 (TLR9).

FANCC suppresses phosphorylation of the NFκB inhibitor p105 in THP-1 Blue cells treated with R-848

We began to dissect the TLR-dependent signaling pathway activated in THP-1 blue cells expressing shRNA against FANCC. Although we previously investigated the basal phosphorylation state of IκBα, IκBβ and IκBε in FA-C cells, we realized that the non-canonical NF-κB inhibitor p105 should also be considered. Phosphorylation of p105 was measured in THP-1 Blue cells expressing non-target shRNA (shNT) or shRNA against FANCC (shFANCC) (Figure 16). R-848 induced phosphorylation of p105 in cells expressing either shNT or shFANCC, but to a greater extent in shFANCC-expressing cells. This result is in line with our hypothesis that p105, an inhibitor of NF-κB, is phosphorylated in cells with low FANCC expression. Whether phosphorylation of p105 in these cells also leads to an increase in p50, the proteolytic cleavage product of p105, remains to be tested.

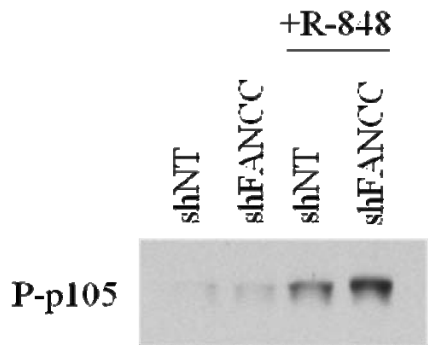


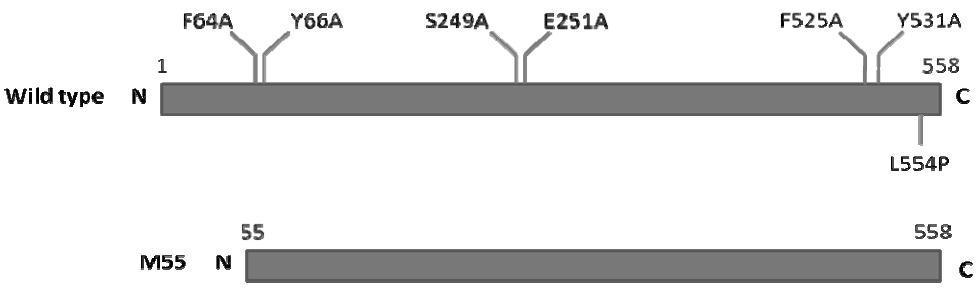
Figure 16. Phosphorylation of p105 in response to R-848 is influenced by FANCC expression level. THP-1 Blue cells expressing non-target (NT) shRNA or FANCC shRNA were treated 24 h with 5 μg/ml R-848. More P-p105 was detected by western blot in induced cells expressing FANCC shRNA than NT shRNA.

Structure-function analysis indicates that several conserved regions of FANCC are required for normal TNF-α production.

Although the precise mechanism for FANCC's effect on TNF-α production is unknown, we investigated whether several conserved regions of FANCC were required

for maintaining low cellular production of TNF-α. We reasoned that conserved residues across the length of the protein might differ in their participation in TNF-α synthesis. This would be analogous to our previous findings that demonstrated FANCC's multifunctionality: To test the contribution of various residues on TNF-α production, six point mutants and M55 (Figure 17), a naturally occurring FANCC truncation mutant whose translation initiates at methionine 55, were expressed in FA-C lymphoblasts that already harbor the FANCC L554P mutation. Unlike wild type FANCC, which reverses TNF-α overproduction when expressed in L554 cells, none of the mutants tested were capable of normalizing TNF-α production levels. This suggests that residues F64, Y66, S249, E251, F525, Y531, the N-terminal 54 residues, and of course L554 are all required for normal TNF-α production. Interestingly, the S249A mutant but not the E251A mutant partially lowered TNF-α production, suggesting that S249 may be slightly less important for regulating TNF-α synthesis, compared to the neighboring residue, E251.

A



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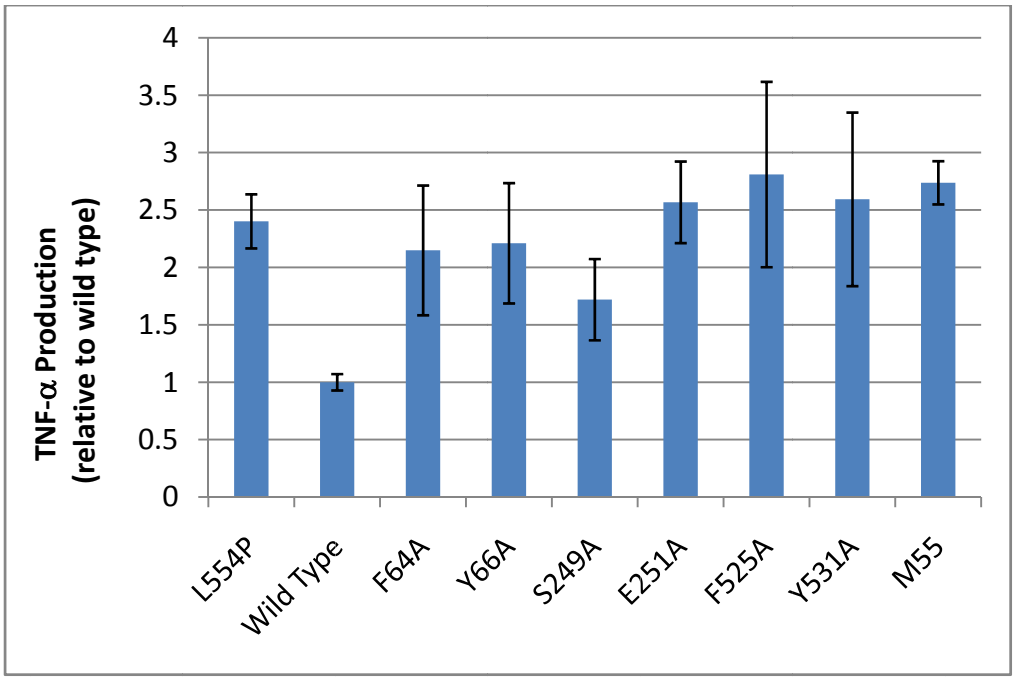

Figure 17. Conserved regions of FANCC are required for normal TNF-α production. (A) Locations of FANCC alanine substitutions. M55 is a naturally occurring truncation mutant. (B) TNF-a levels remain high in all of the cells expressing the alanine mutants and M55.

FANCA associates with TRAF6 and TAK1 by Co-immunoprecipitation.

Seeking to identify the specific biochemical effect of FANCC on the TLR8 pathway, we conducted coimmunoprecipitation experiments designed to test the hypothesis that proteins in the TLR signaling pathway bind directly to FANCC, or indirectly to FANCC through association with another

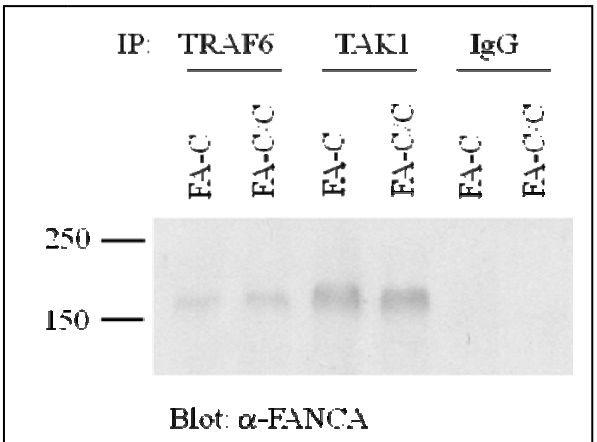

Figure 18. FANCA binds to TRAF6 and TAK1 by Co-IP. Protein from FA-C and FA-C/C cells was immunoprecipitated with anti-TRAF6 antibody, anti-TAK1 antibody or normal IgG, and blotted with anti-FANCA antibody

FA protein. Because FANCA has been shown to associate with IKKβ and IκBγ, we asked whether other TLR-activated proteins might associate with FANCA. By co-immunoprecipitation, we found that TRAF6 and TAK1 both associate with FANCA (Figure 18). Interestingly, there was no difference between mutant FA-C cells and corrected FA-C/C cells in the amount of FANCA bound to TRAF6 and TAK1. This data suggests that FANCC might interact with TRAF6 or TAK1 via FANCA, and that FANCA-TRAF6 and FANCA-TAK1 associations are not dependent on fully-functional FANCC proteins.

2-3: Discussion

The increased TNF-α production observed in FA patients is significant given the high level toxicity of TNF-α in FA cells. We describe herein the first evidence that FANCC modulates the activation potential of TLR8 and mutations of FANCC result in TLR8 hyperactivation leading to elevated TNF-α production in FA cells. We confirm using both gain- and loss-of-function analyses that TLR8 induces the synthesis of TNF-α in FA-C cells by activation of a concatenated signaling pathway that includes IRAK1, IKK, and NF-κB.

Protein ubiquitinylation is a common theme in TLR pathway regulation. It is therefore not surprising, given our biochemical results, that we identified differences in ubiquitinylation between FA-C and FA-C/C cells. The differential ubiquitinylation of TLR8 provides a clue as to the mechanism by which TLR signaling is altered in FA-C

cells. One of the most intriguing questions that remain unanswered is whether the TLR8 ubiquitinylation that we observed was direct ubiquitinylation of TLR8 or if the ubiquitinylation was of a TLR8-associated protein. TLR8 was recently shown to be ubiquitinylated in normal cells stimulated with the TLR7/8 agonist 3M-003 (69). The authors of this paper demonstrated ubiquitinylation of overexpressed TLR8 in HEK-293 cells by mass spectrometry, but did not identify the site(s) of ubiquitinylation or any consequence of this modification on TLR8 function. This report does however confirm that TLR8 can be ubiquitinylated, as our results suggested. Although the site(s) of ubiquitinylation in TLR8 have yet to be identified, it should be possible to do so in the future by expressing tagged-TLR8 in cells, followed by its purification and proteolytic digestion. The digested TLR8 sample can then be analyzed by mass spectrometry and evidence of direct ubiquitinylation can be verified, and the site of ubiquitinylation can be determined (based on the 114 Da additional mass of a diglycine-modified peptide). This result can also be verified by follow up experiments to express TLR8, containing a mutation at the determined site of ubiquitinylation, in FA-C and FA-C/C cells to study the effect of ubiquitinylation on the cellular function of TLR8.

The possibility still remains that our studies have revealed differential ubiquitinylation of a protein associated with TLR8. Other potential binding partners of TLR8 (or a TLR8-containing complex) include MyD88, Mal, IRAK4, IRAK1, and other TLRs (TLR8 has been shown to interact with TLR7 and TLR9) (70). TLR8, or associated proteins, may also bind to negative regulators, including SIGIRR, ST2L, MyD88s, SOCS1, Tollip, IRAKM, IRAK2c/d, and TRIAD3A. If a TLR8-associated ubiquitinylated protein is present in FA-C cells rather than ubiquitinylated TLR8 itself,

all of these proteins should be considered as possible targets for this key post-translational modification.

Indeed, in this regard I sought to identify non TLR binding partners for FANCC and modulators of TLR activity, to investigate in detail the precise molecular event(s) that give rise to aberrant TLR signaling. I have performed co-immunoprecipitations using antibodies against known members of TLR signaling complexes, namely TLR8, Myd88, IRAK-1, phosphorylated IRAK-1, IRAK-4 and TRAF6 (data not shown). I have also examined the interaction of two FA proteins, FANCA and FANCC, with TLR signaling complexes. Based on these experiments, we have evidence for associations between 1) FANCA and TRAF6, and 2) FANCA and TAK1. Other laboratories have found associations between 1) FANCA and IKK-β and 2) FANCA and IκBγ (or p105). Based on these results, we hypothesize that FANCA may act as a scaffold that facilitates a series of protein-protein interactions and activation steps that link TRAF6 activation to NF-κB activation.

As a protein that binds to FANCA, we hypothesize that FANCC may “sense” activation of the TLR pathway and participate in a negative feedback loop that modulates upstream TLR pathway signaling (Figure 19). If this negative modulation is reflected in the ubiquitinylation level of TLR8 or an associated protein, FANCC would most likely function to suppress ubiquitinylation (or promote deubiquitinylation), since in mutant FA-C cells, increased levels of ubiquitinylation of TLR8 or an associated protein is observed. We believe it will be important to thoroughly investigate whether known negative regulators are activated by FANCC. A20 is of particular interest because it inhibits both TLR and TNF receptor signaling pathways. This might explain why FA

cells demonstrate both hyperactivation of the TLR pathway and hypersensitivity to TNF-α. As a deubiquitinylating enzyme, A20 is also significant because its activation affects ubiquitinylation levels of proteins in the TLR and TNF receptor (TNFR) pathways. Studies on the influence of FANCC on A20 function are in progress.

Figure 19. Proposed functions of FANCA and FANCC.

Co-immunoprecipitation data suggests that FANCA may act as a scaffold protein, facilitating a series of activation events between TRAF6 and NF-κB. We propose a model by which FANCC, through its interaction with FANCA, may function in a negative feedback loop that results in deubiquitinylation of TLR8 or another component of the receptor complex (such as IRAK-1).

TRAF proteins have also been shown to interact with members of the TNF receptor superfamily. TRAF6 appears to be unique among TRAF family proteins because it participates in signaling induced by both TLRs and TNFRs (71). If FANCA does in fact associate with TRAF6, which may be a central point of interaction between the TLR and TNFR pathways, this might explain the observed sensitivity of FA cells to both TLR agonists and

TNF-α.

Chaperones and TLR Function

The associations between Fanconi anemia proteins and heat shock proteins are known to promote survival signaling pathways. In particular, Hsp70 and FANCC associate, and cooperate to suppress the activity of pro-apoptotic interferon-inducible double-stranded RNA-dependent protein kinase (PKR). The association between Hsp90 and two FA proteins, FANCC and FANCD2, will also be described in detail in Chapter 3 of this thesis.

The endoplasmic reticulum chaperone grp94 (gp96, HSP90b1) is a Hsp90 homolog that has been shown to regulate the level of functional TLRs, including TLR1, TLR2, TLR3, TLR4, TLR5, TLR7, TLR8 and TLR9 (72;73). Interestingly, grp94 has been reported to associate with FANCC and FANCA (8;74). Future experiments will determine whether grp94 is required for the basal TLR8 activation seen in FA-C cells, and if so, whether FANCC or FANCA modulates the interaction between TLR8 and grp94.

2-4: Materials & Methods

Antibodies and Reagents

The following rabbit polyclonal antibodies were purchased from Cell Signaling Technology (Boston, MA): anti-phospho-IKK-α/β (Ser176/180), anti-IKK-α, anti-IKK-β, anti-phospho-IRAK1 (Ser376), anti-IRAK1, anti-phospho-IκBα, anti-IκBα, anti-phospho-IκBβ (Thr19/Ser23), anti-IκBβ, anti-phospho-IκBε (Ser18/22) and anti-IκBε. The anti-phospho-p105 (Ser933) rabbit monoclonal antibody was also obtained from Cell Signaling Technology. Other rabbit polyclonal antibodies used include anti-ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-TLR8 (Abcam, Cambridge, MA). The following monoclonal antibodies were also purchased from Santa Cruz Biotechnology: anti-FANCD2, anti-TRAF6 and anti-TAK1. The anti-TLR8 monoclonal antibody was obtained from MBL International (Woburn, MA). Actinomycin D (Sigma-Aldrich, St. Louis, MO) was used at 5 μg/ml. The IRAK1/4 inhibitor N-(2-Morpholinylethyl)-2-(3-nitrobenzoylamido)-benzimidazole (Sigma-Aldrich, St. Louis, MO) was used at 50 μM. All TLR ligands except R-848 were obtained from InvivoGen (San Diego, CA). R-848 was purchased from Alexis Biochemicals (San Diego, CA).

Cell lines

Epstein–Barr virus (EBV)-transformed lymphoblast cell lines HSC536N (FA-C) and PD149 (FA-C), and the corrected counterparts HSC536N/FANCC (FA-C/C) and PD149/FANCC (FA-C/C) were previously described (75;76). HSC536N (FA-C) cells expressing FANCC with site-directed mutations (F64A, T66A, S249A, E251A, F525A and Y531A) were described previously (20). THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and THP-1 Blue cells were

obtained from InvivoGen (San Diego, CA). All cell lines were grown in RPMI 1640 supplemented with 15% FBS, 1% glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin at 37°C, and 5% CO2 in a humidified atmosphere.

In vitro $(His)_6$-Ubiquitin Conjugation and Affinity Chromatography

Subconfluent HSC536N and HSC536N/FA-C cells were washed twice with ice-cold Dulbecco's Phosphate-Buffered Saline (PBS) (GIBCO, Grand Island NY). Cells were then lysed in buffer containing 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 1% Triton X-100, 1 mM DTT, 2 mM sodium orthovanadate, 1mM phenylmethysulfonyl fluoride (PMSF), 1% leupeptin, 1% pepstatin and 1% aprotinin. Cell extracts were centrifuged 15 min at 4°C. Supernatants (11 mg) were incubated for 2 hr at 20°C with 1 mg hexahistidine-tagged ubiquitin (BostonBiochem, Cambridge, MA), 5 μM ubiquitin aldehyde (BostonBiochem, Cambridge, MA), and 10 μM MG132 (BostonBiochem, Cambridge, MA) in a final volume of 2.5 ml. Reactions were performed with and without energy regeneration solution (BostonBiochem, Cambridge, MA), which contains MgCl2, ATP and ATP regenerating enzymes. The samples were then desalted with PD10 desalting columns (Amersham Biosciences, Piscataway, NJ) and eluted in buffer containing 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 1% Triton X-100, 300 mM NaCl and 10 mM imidazole. The eluents were loaded onto HisTrap HP columns (Amersham Biosciences, Piscataway, NJ) and washed with buffer containing 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 1% Triton X-100, 300 mM NaCl and 40 mM imidazole. Resin containing the bound His-tagged proteins was then removed from the columns by

centrifugation and proteolytically digested with Lys-C endoproteinase and modified trypsin as described previously (50). The microcapillary 2D LC-MS/MS methodology was also described previously. Briefly, digested samples were injected into a biphasic microcapillary HPLC column and separated by cation-exchange in the first dimension and by reversed phase in the second dimension.

Immunoprecipitations

Total proteins were extracted on ice in buffer containing 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 1% Triton X-100, 2 mM sodium orthovanadate, 1mM phenylmethysulfonyl fluoride (PMSF), 1% leupeptin, 1% pepstatin and 1% aprotinin. Cell extracts were centrifuged 15 min at 4°C to remove cellular debris. For immunoprecipitations, supernatants (1 mg of total protein) were precleared by adding 30 µl (50% v/v) of Sepharose and incubated for 45 min at 4°C. The extracts were then incubated with indicated antibodies overnight at 4°C. The immune complexes were then bound to protein A (or G)-sepharose (1 h, 4°C) and washed two times with lysis buffer, followed by one wash with PBS. Samples were eluted in 15 µl of 2x Laemmli buffer [1M Tris-HCl pH 6.8, 4% sodium dodecyl sulfate (SDS), 40% glycerol, and 4% 2-mercaptoethanol].

Immunoblotting

Proteins were separated in SDS-PAGE gels under reducing conditions. The proteins were subsequently transferred to nitrocellulose and blocked with 5% nonfat milk

(Nestle USA, INC., Solon, OH) in TBST (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.4, 0.05% Tween-20). Blots were incubated with indicated antibodies in 1% milk overnight at 4°C. 5% BSA was used rather than 1% milk in incubations with phospho-specific antibodies. Following incubations with appropriate horseradish peroxidase-coupled secondary antibodies (Bio-Rad, Hercules, CA), proteins were detected by Enhanced Chemiluminescence Kit (Amersham Biosciences, Piscataway, NJ).

ELISA

TNF-α levels in culture supernatants were measured with ELISA kits from R&D Systems (Minneapolis, MN) according to the manufacturer's protocol.

RNA measurements by real time-PCR

Total RNA was prepared from 1-5x10^6 cells using the RNeasy Mini kit (Qiagen, Valencia, CA). Complementary DNA synthesis and real-time PCR were performed as described previously (77). Predesigned primer and probe sets for *TNF-α* (Hs00174128_m1) were purchased as Taqman Gene Expression Assays from Applied Biosystems.

Nucleofection of siRNA

Cells were transfected using the Amaxa nucleofection technology (Amaxa Inc, Gaithersburg, MD, USA). SiRNA against FANCC (SMARTpool) and control siRNA were purchased from Dharmacon (Chicago, IL). Following Amaxa protocols, $4x10^6$ cells were suspended in 0.1 ml solution V, mixed with 200 pmol of siRNA, and transfected using program A-23 of the Nucleofector device. After transfection, cells were immediately transferred to 0.5 ml of RPMI medium without serum in six-well plates at 37°C. After 15 min incubation, 1.5 ml of complete medium was added and the cells were cultured for 72 hours.

***Electromobility Shift A*ssay (EMSA)**

The sequence of the oligonucleotide used in the binding reactions was the following: human IRF-1 NF-kB recognition site, 5'-CGG GCC GGG GAA TCC CGC TAA G-3'. The oligonucleotide was synthesized at the Molecular Biology Core Lab (Portland VAMC) and labeled with [γ^{32}P-ATP] using T4 polynucleotide kinase (Boehringer Mannheim). Binding reactions were performed as described previously (4) and then resolved on 4% polyacrylamide gels. Gels were dried and autoradiographed with intensifying screens at -80°C.

Stable Expression of shRNA with Lentivirus

Lentiviral particles containing FANCC and non-target shRNA was purchased from Sigma-Aldrich (St. Louis, MO). ShRNA transductions into THP-1 and THP-1 Blue cells were performed as described by the manufacturer. The media was replaced one day

after transduction. After two more days, shRNA-expressing cells were selected with 0.6 μg/ml puromycin for 2-3 weeks, at which time the cells were maintained in media containing 0.3 μg/ml puromycin.

THP-1 Blue NF-κB Reporter Assay

THP-1 Blue cells express the secreted embryonic alkaline phosphatase (SEAP) gene under the control of a promoter inducible by NF-κB. SEAP expression is maintained by growing the cells in media containing the selectable marker Zeocin (InvivoGen, San Diego, CA). Upon NF-κB activation, the expression and secretion of SEAP is monitored by the colorimetric enzyme assay QUANTI-Blue (InvivoGen, San Diego, CA), which produces a purple-blue color that is measurable on a spectrophotometer at 650 nm.

Chapter 3

Fanconi Anemia Proteins Facilitate Nuclear Translocation of Stat5a

FANCONI ANEMIA PROTEINS FACILITATE NUCLEAR TRANSLOCATION OF STAT5a

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Running title: Fanconi anemia proteins facilitate STAT5 activation

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3-1: ABSTRACT

We tested the notion that FA proteins facilitate activation of STAT5, an essential signaling molecule for survival and replication of hematopoietic cells. Because the numbers of hematopoietic progenitor cells are greatly diminished in FA patients and there exist no informative FA hematopoietic cell lines that respond to defined growth factors we screened widely available FA fibroblast cell lines. We identified STAT5 activation defects by quantifying tyrosine phosphorylated STAT5 (STAT5-P) in H_2O_2-exposed cells using cell fractionation and deconvolution microscopic methods. In normal fibroblasts and in isogenic complemented cell lines from three complementation groups (FA-C, -G, and -D2) H_2O_2 induced JAK2-dependent STAT5 phosphorylation and nuclear translocation at least in part by activating EGFR and IGFR. In fibroblasts deficient in FANCC, FANCG and FANCD2, cytoplasmic STAT5 was phosphorylated, but not translocated to the nucleus and apoptotic responses to H_2O_2 were exaggerated. In normal cells but not in FA cells, H_2O_2 induced formation of multimeric complexes containing STAT5, FANCD2, Hsp90, and FANCC. Indeed, inactivation of any one of the three FA proteins interdicted the inducible association of the other two with STAT5 and prevented nuclear translocation of STAT5-P. Because we find that nuclear STAT5-P is reduced in FA fibroblasts exposed to EGF and in an erythropoietin responsive cell line exposed to EPO after FANCD2 knockdown, we conclude that activation of a JAK2-dependent STAT5a signaling pathway requires inducible intermolecular interactions involving STAT5, FANCC, and FANCD2 and reason that failure of this signaling pathway may contribute to the Fanconi anemia phenotype.

3-2: INTRODUCTION

Fanconi anemia is a rare inherited genetic instability disease characterized by bone marrow failure, developmental anomalies, a high incidence of myelodysplasia (MDS), and acute non-lymphocytic leukemia (AML) (78). Eight of the proteins encoded by the 13 FA genes (78-81) function to protect cells from genotoxic stress induced by chemical cross-linking agents by forming nuclear complexes with each other (82-84). Inactivation of any one of these eight (*FANCA, FANCB, FANCC, FANCE, FANCF, FANCG/XRCC9, FANCL,* and *FANCM*) "nuclear core complex" proteins disrupts the genoprotective function of the entire complex (82;85). The eight "core complex" FA proteins accomplish this function, at least in part, by facilitating the monoubiquitylation of FANCD2 (84;86) thereby permitting FANCD2 to co-localize with BRCA1 in nuclear damage foci following genotoxic stress (84;87;88).

It is not at all clear that the nuclear damage functions of the FA proteins contribute to the maintenance of hematopoiesis. Indeed, it is more likely that additional functions of FA proteins (89) some of which involve the formation of alternative complexes in both the nucleus and cytoplasm (74;90), play a key role. FANCC, for example, modulates apoptotic responses to extracellular cues by suppressing the ground activation state of the pro-apoptotic double-stranded RNA-dependent protein kinase (PKR) (91-93), thereby modulating responses to double-stranded RNA, TNF-α, and IFN-γ. Likewise, FANCA, FANCG, and FANCC also modulate responses to IFN-γ (94). A recent report has identified FANCL as a TGFβ-receptor type I binding protein (95). Finally, FANCC forms complexes with and facilitates the activation of STAT1 in IFN-γ-stimulated cells (4). However, it is not clear that FA proteins directly facilitate activation of STAT5, a

signaling molecule of critical importance to the replication (96-98), repopulation potential (99) and differentiation (100-102;102;103;103;104) of hematopoietic stem and progenitor cells (101;105) and a molecule required for the transduction of signals induced by a variety of hematopoietic growth factors (38;96;106-108).

The biochemical functions of FA proteins vis-à-vis STAT5 function in hematopoietic cells has been difficult to establish using primary cells because the numbers of hematopoietic stem and progenitor cells are markedly diminished in FA patients (78). In addition, while FA fibroblast cell lines are readily available there are no available informative FA hematopoietic cell lines that respond to defined growth factors. Reasoning that the intersection of the FA and STAT5 pathway might be defined using widely available fibroblast cell lines and taking into account that hydrogen peroxide activates STAT molecules (109), we reasoned that H_2O_2 exposed fibroblasts might represent a reasonable screening tool for defining mechanisms that could then be applied to less available primary hematopoietic cells. We quantified differential STAT responses to hydrogen peroxide in fibroblast cell lines representing four complementation groups and isogenic cell lines complemented with wild type cDNA. A secondary objective of our studies was to test the hypothesis that defects in STAT activation may contribute to their hypersensitivity to H_2O_2. Hypersensitivity to oxidative stress is a commonly reported abnormality in FA cells (110-112) and recent biochemical evidence attributes this hypersensitivity, at least in FA-C and FA-G cells, to losses of extranuclear signaling functions of those FA proteins (20;113;114).

We report herein results that confirm that FANCC, FANCD2 and FANCG participate directly in a canonical signal transduction response. Specifically, H_2O_2

induces JAK2-dependent phosphorylation of STAT5a in both normal and FA cells but translocation of STAT5-P to the nucleus requires these three FA proteins. We also provide evidence that these proteins form mutually interdependent H_2O_2-inducible multimeric signaling complexes that include both STAT5 and Hsp90 (known to be required for STAT5 activation(115;116)). Because we also find that nuclear phospho-STAT5 is reduced in EGF-stimulated FANCD2 deficient fibroblasts and in erythropoietin- stimulated FANCC- and FANCD2-deficient MO7E-epoR cells, we suggest that hydrogen peroxide exposure of non-hematopoietic cells provides clues to biochemical functions of FA proteins in hematopoietic progenitor cells, cells that are impossible to obtain in sufficient numbers from patients with bone marrow failure to conduct biochemical studies of this kind.

3-3: RESULTS

Fanconi anemia cells are hypersensitive to hydrogen peroxide.

FA-D2, FA-G, FA-C, and retrovirally complemented isogenic fibroblasts (FA-D2/D2, FA-G/G, and FA-C/C, respectively) were exposed to H_2O_2 and the fraction of apoptotic cells was quantified using annexin V and 7-AAD staining. As shown in Figure 1A-C, all FA cells were hypersensitive to H_2O_2 compared to complemented cells. The differences in viability between mutant cells and complemented cells was observed at doses between 100 μM and 1mM H_2O_2, and was evident between 2 h and 12 h post-exposure Apoptotic responses to increasing doses of H_2O_2 were exaggerated in all three mutant cell types ($P<0.001$ by nonlinear regression analysis; Supplementary data 1) To rule out artifacts that might have been introduced by the immortalization process (SV40 large T-antigen) we confirmed that primary embryonic fibroblasts (MEF) from *Fancc*$^{-/-}$ mice were also hypersensitive to H_2O_2 (Figure 1D).

H_2O_2 exposure induces rapid nuclear accumulation of phosphorylated STAT5 in complemented cells.

FA and isogenic complemented fibroblasts were treated with 1mM H_2O_2 and nuclear extracts were prepared at time points ranging from 0 – 120 min (Figure 2A). Immunoblot analyses were initially carried out with antibodies to total STAT1, 3, or 5 and to their tyrosine phosphorylated forms. Nuclear accumulation of phosphorylated STAT5 (STAT5-P) was suppressed in FA mutant cells but accumulation of phosphorylated STAT1 and STAT3 was not (data not shown). Nuclear STAT5-P

accumulation was detected within 5 min of exposure and was also suppressed in H_2O_2-exposed primary *Fancc*$^{-/-}$ MEFs (Figure 2B). In contrast to the results using nuclear extracts, H_2O_2 induced equivalent cytoplasmic phosphorylation of both STAT5 and JAK2 in both normal and FA cells (Figure 2C). The JAK2 inhibitor AG490 reduced both JAK2 and STAT5 phosphorylation in response to H_2O_2 (Figure 2D).

Because H_2O_2 is known to activate JAK2 by activating receptor tyrosine kinases (117;118) we sought evidence for activation of the insulin receptor (IR), insulin like growth factor receptor (IGF-1R), epidermal growth factor receptor (EGFR), and the platelet derived growth factor receptor 1 (PDGF-R1) in mutant and complemented FA-D2 cells. Only EGFR and IGF-1R were phosphorylated in response to H_2O_2 (Table 1) and there were no differences in the state of activation of these receptors between mutant and complemented cells. In addition, EGF-exposed FA fibroblasts contained less nuclear Stat5-P than did complemented cells (Figure 3C) as did EPO exposed MO7e/EPOR cells treated with FANCD2 siRNA (Figure 3D). Preincubation with either the EGFR inhibitor AG1478 or the IGF-1R inhibitor AG1024 partially suppressed H_2O_2-induced STAT5 phosphorylation (Table 1). Because JAK2 and STAT5 cytoplasmic phosphorylation did not differ between FA and complemented cells, we focused on explaining the differential nuclear accumulation of STAT5-P

Reduced nuclear accumulation of STAT5-P in induced FA cells was confirmed by electrophoretic mobility shift analysis (EMSA) (Figure 3A), deconvolution microscopy (Figure 3B), and by measuring accumulation of the product of the STAT5 responsive Bcl-XL gene (101) using immunoblotting (Figure 2D). Two supershifting antibodies were utilized in the EMSA experiments, one with specificity for both STAT5a

and STAT5b and the other with specificity for only STAT5b. The dual specificity antibody influenced the migration of the shifted STAT5 oligonucleotide signal (Figure 3A) but the STAT5b antibody did not (data not shown). To confirm the specific activation of STAT5a, levels of total STAT5a and STAT5b were quantified in nuclear extracts of FA-D2 and complemented cells exposed for 0, 5, 15, 30, 60, and 120 minutes to H_2O_2. Greater amounts of STAT5a were found in nuclear extracts obtained from induced complemented cells but STAT5b levels were equivalent at all time points (Supplementary Figure 2B). In addition, using a labeled oligonucleotide probe specific for STAT5b (sequence 5'-CAGAATTTCTTGGGAAAGAAAAT-3', Panomics, Inc Redwood City, CA) we confirmed using EMSA assays that STAT5b activation did not occur on exposure to H_2O_2 (data not shown).

Theoretically, excessive nuclear degradation or nuclear export could account for the low levels of nuclear STAT5-P but in H_2O_2-stimulated FA-D2 cells neither the proteasomal inhibitor MG132 (Boston Biochem, Cambridge, MA) nor Leptomycin B (Sigma-Aldrich), an inhibitor of nuclear export, enhanced STAT5-P nuclear accumulation (data not shown). We detected no constitutive or induced STAT5-importin interactions in normal or FA cells by co-immunoprecipitation, using anti-STAT5 and anti-importin α (Sigma-Aldrich) antibodies (data not shown). Taken together with the rapidity of the H_2O_2-induced response in normal cells, the results indicate that the primary defect in the mutant cells reflected a failure of nuclear import of JAK2 activated STAT5-P.

STAT5a phosphorylation enhances survival of H_2O_2 –exposed cells.

We used both gain- and loss-of-function analyses to test the hypothesis that STAT5 activation serves a pro-survival function in H_2O_2-exposed cells. Ectopic expression of the constitutively active STAT5a mutant, STAT5aN642H, or the dominant negative STAT5a mutant, STAT5aΔ750, in the FA-D2 cell line resulted in a decrease and increase, respectively, in the apoptotic fraction after exposure to H_2O_2 (Figure 4A). We confirmed that the STAT5aN642H mutant construct was constitutively active in FA-D2 cells (Figure 4B) and that it did result in translocation to the nucleus even in FA-D2 and FA-C mutant cells (Figure 4C). Finally, to confirm that STAT5 activation was required for survival in the face of oxidative stress, transformed *Stat5a*$^{-/-}$ *b*$^{-/-}$ MEFs were tested. In the absence of STAT5, H_2O_2 significantly increased cell death (Figure 4D). Bcl-xL induction only occurred in H_2O_2-exposed wild type cells and was in fact decreased in exposed mutant cells (Figure 4E).

FANCD2, FANCC, STAT5 and Hsp90 participate in formation of H_2O_2-inducible molecular complexes.

Based on our prior observations that FANCC interacts with STATs and recent reports that FANCG has an extranuclear antioxidant function (114) we expected to detect STAT abnormalities in FA-C and FA-G cells. At the time we designed our studies the field accepted the dogma that FANCD2 was strictly located in the nucleus so we included FANCD2 mutant cells expecting that they would serve as reasonable negative controls. Interestingly, we discovered during the course of these studies, using both cytosolic and

nuclear fractionation methods and immunohistochemistry, that the non-ubiquitinylated form of FANCD2 is present in both the nucleus and cytoplasm (Figure 5A and supplementary Figure 5) (Savoia and her colleagues have recently reported having discovered FANCD2 in the cytosol (119) as has Meetei's group [personal communication]) and that FANCD2 is important in facilitating the formation of functional FANCC-STAT5 complexes. We detected no monoubiquitinylated FANCD2 in the cytoplasmic fractions.

The rapid formation of inducible complexes was clearly seen in cytoplasmic fractions in which complemented cells (but not FA-D2 cells) form STAT5/FANCD2 complexes and STAT5/FANCC complexes within 1 min, peaking at 5 min (Figure 5B). Co-immunoprecipitation experiments using whole cell lysates from all three FA cell lines prepared before and after exposure to 500 μM H_2O_2 for 15 min revealed like results (Figure 5C and E). In complemented (FA-D2/D2) cells, H_2O_2 induced FANCD2/STAT5, FANCC/STAT5, and FANCD2/Hsp90 complex formation (Figure 5C, rows 1, 2 and 5). Although Hsp90/STAT5 and FANCC/Hsp90 complexes were more abundant in complemented cells, the formation of neither complex absolutely required FANCD2 (Figure 5C, rows 3 and 4). Therefore, FANCD2 not only participates in formation of complexes with STAT5 and Hsp90, it was also required for the formation of the STAT5-FANCC complex. This result demonstrates that the STAT5/FANCC association was not simply a non-specific protein:protein cross-linking phenomenon induced by H_2O_2. That inhibition of JAK2 by AG490 blocked formation of STAT5/FANCC and STAT5/FANCD2 complexes suggests that the inducibility of both complexes is

dependent upon either JAK2 activation, STAT5 phosphorylation or both (data not shown).

Results were nearly identical when FA-G cells were used (Figure 5D). That is, FANCD2/STAT5 and FANCC/STAT5 complex formation was FANCG-dependent (Figure 5D, rows 1 and 2). FA-G cells differed slightly from FANCD2 mutant cells in that FANCC/Hsp90 complex formation was FA-G dependent (Figure 5D, row 4).

Because the inducible complex that was consistently dependent upon both FANCD2 and FANCG was that of FANCC/STAT5 we wondered whether an inactive (disease-associated) mutation of FANCC might fail to bind to STAT5 in induced FANCC cells. As shown in Figure 5E there was no detectable inducible interaction between STAT5 and FANCD2 (row 1, lane three) or FANCD2 and Hsp90 in H_2O_2 -induced FA-C cells. However, the minimally truncated functionally inactive mutant FANCC molecule (bearing the R548X mutation) did bind to STAT5 (Figure 5E, row 2) but not to Hsp90 (Figure 5E, row 4). Nor did FANCD2 bind to STAT5 in these FANCC mutant cells. Therefore, just as the inducible FANCC/STAT5 complex requires FANCD2, the FANCD2/STAT5 association depends upon a fully functional FANCC molecule (once more ruling out a non-specific cross-linking effect). In addition, nuclear accumulation of STAT5-P also required fully functional FANCC (Figure 3B).

STAT5aN642H bypasses the requirement for functional FA proteins.

In complemented FA-D2 cells both STAT5-P and FANCC accumulated in the nucleus in H_2O_2 -stimulated cells and STAT5 and FANCC co-immunoprecipitate in

nuclear lysates (Supplemental Figure 3), compatible with the view that the STAT5/FANCC complex is essential for nuclear transport of STAT5-P. Moreover, while FANCC did not form complexes with STAT5 in FA-D2 cells (Figure 5C) under any condition, the constitutively-active STAT5aN642H did form complexes with FANCC in these cells even in the ground state (Supplemental Figure 4). One interpretation of this observation might be that the ground-state interaction of the mutant STAT5 and FANCC was sufficient to allow nuclear translocation. This is unlikely however because STAT5aN642H is also perfectly capable of translocating to the nucleus in a cell that expresses only a functionally inactive mutant of FANCC (Figure 4C). Although others have speculated that STAT5aN642H stabilizes STAT5a dimers by enhancing binding of the SH2 domain and phosphorylated tyrosines or protects the molecule from tyrosine-phosphatases (120), the mechanisms that directly result in basal activation are unknown. How this point mutation of STAT5a enhances translocation to the nucleus in FA mutant cells is also unknown but our results indicate generally that the STAT5 activating mutation not only results in constitutive phosphorylation but also bypasses the requirements that constrain nuclear translocation of wild type STAT5-P.

Co-localization of STAT5, Hsp90 and FA proteins in high molecular weight complexes.

FANCC, -G, and D2 were interdependent in forming inducible FANCD2/STAT5, FANCC/STAT5, FANCD2/Hsp90, and FANCC/Hsp90 complexes (Figures 5C, D, and E), suggesting that these inducible complexes might represent large multimers. This

would be in keeping with that of the nuclear core FA protein complex model, the function of which requires that all eight of its FA components be functionally intact (85;121). Therefore, to rule out the null hypothesis (that immunoprecipitating complexes we have identified are simply dimers consisting of only the two co-immunoprecipitating proteins) size exclusion chromatography was performed using whole cell lysates of FA-D2 mutant and complemented (FA-D2/D2) cells exposed or not to H_2O_2. STAT5 and FANCC co-eluted in a fraction of approximately 400 kDa (fraction 8), suggesting that STAT5 and FANCC exist as members of a larger multimeric protein complex (Figure 6A). STAT5 and FANCC also partially co-elute with Hsp90, FANCD2, and FANCG in 600 – 400 kDa fractions (fractions 6-8). Therefore, 600-400 kDa fractions contain STAT5, Hsp90, FANCD2, FANCC, and FANCG. While the results do not prove that there exists a single multimeric complex containing all five proteins they are at least consistent with that notion and clearly rule out simple dimerization. As expected (because FANCC and Hsp90 are Hsp70 binding proteins (92;122)) Hsp70 eluted in some of the same fractions as FANCC and Hsp90 (Figure 6A). The elution profiles for these proteins from whole cell lysates were identical to those from cytoplasmic extracts.

Importantly, in the cytoplasm of H_2O_2-stimulated cells STAT5-P was found in much larger complexes than unphosphorylated STAT5 (Figure 6B) and overlapped with the fractions in which FANCD2 was found. Interestingly, however, the inducible appearance of STAT5-P in high molecular weight fractions was also observed in mutant FA-D2 cells, and therefore its entry into these fractions did not depend upon the presence of FANCD2 (data not shown). We confirmed H_2O_2 induced STAT5-P and FANCD2

association in cytoplasmic preparations of complemented FA-D2 cells by immunoprecipitation (Figure 6C).

3-4: DISCUSSION

STAT5 is a critical signaling molecule for most hematopoietic lineages including pluripotent hematopoietic stem cells (96;98;123;124). STAT5a/b nullizygous mice have impaired hematopoiesis and lymphopoiesis (125). Erythroid and megakaryocytic responses to erythropoietin (38) and thrombopoietin (126;127) respectively, require STAT5. STAT5 signaling is also required for development of Foxp3+ regulatory T cells in response to IL-2 (128), and for lymphoid responses to IL-7 and IL-9 (103;129;130).

Some degree of hematopoietic failure is universal in patients with Fanconi anemia and tends to progress over time (78). T-cell defects have also been reported in a murine model of FA (131;132). In light of the capacity of FANCC to facilitate the activation of STAT1 (4) and in view of the critical roles played by STAT5 in hematopoietic cells, including pluripotent stem cells, we reasoned that FA proteins may facilitate STAT5 activation and therefore sought to define a biochemical role for FANCA, FANCG, FANCC and FANCD2 in this pathway.

The ideal cell type for these studies would naturally be primary non-neoplastic non-transformed hematopoietic progenitor cells or stem cells. However, the number of cells required for biochemical studies exceeds the numbers of cells available by orders of magnitude. The requirement for the development of isogenic controls would reduce the available cell numbers even further. In light of the broad availability of fibroblast lines from FA patients along with isogenic cells complemented with wild type FA cDNA, we chose to examine the STAT5 activation response in those cells after exposure to H_2O_2. We determined that H_2O_2 does activate STAT5 in both mutant and complemented cells

of the C, G, and D2 complementation groups in a JAK2 dependent fashion (Figure 2D) but that nuclear translocation of tyrosine phosphorylated STAT5 occurs only in complemented cells (Figure 3). That the defect in FA cells was specifically related to the nuclear accumulation of STAT5-P rather than more rapid nuclear export was confirmed by demonstrating: (a) normal STAT5 phosphorylation responses in the cytosol of mutant cells (Figure 2C), (b) reduced nuclear STAT5-P (Figure 2A, 3B) and (c) that nuclear STAT5-P in H_2O_2-induced mutant cells was not restored by inhibiting nuclear export with Leptomycin B. In addition, that the full transcriptional activation potential of STAT5 was influenced by the FA proteins was confirmed by the lack of induction of Bcl-xL in cells bearing inactivating mutations of either STAT5 or FANCD2 (Figure, 2D, 4E) and the failure to detect STAT5 bound to its cognate DNA binding site in FA-D2 cells (Figure 3A).

We confirmed the STAT5 activation defect in primary *Fancc*$^{-/-}$ MEFs (Figure 2B), ruling out the possibility that the phenotype was influenced by SV40 transformation of the human FA cell lines we utilized. Chemical inhibitors of JAK2, IGFR and EGFR activation suppressed STAT5 activation in mutant and complemented cells suggesting that the H_2O_2-induced STAT5 response is JAK2-dependent and may involve H_2O_2-induced receptor dimerization.

We also found that STAT5 activation provided some measure of protection from H_2O_2 (Figure 1). That STAT5 activation protects cells from cytotoxicity of extracellular H_2O_2 was confirmed by our finding that H_2O_2-exposed *Stat5a*$^{-/-}$*b*$^{-/-}$ MEFs were extraordinarily sensitive to cell death induced by H_2O_2 exposure (Figure 4D). The role of STAT5 was also confirmed using both gain- and loss-of-function analyses. Specifically,

the FA-D2 cell line ectopically expressing the constitutively active STAT5 mutant STAT5aN642H increased resistance to H_2O_2 and FA-D2 cells expressing the dominant negative mutant STAT5aΔ750 were more sensitive (Figure 4A). Cells expressing the constitutively active mutant form of STAT5a exhibited, as expected (120), ground state STAT5 phosphorylation and enhanced STAT5 phosphorylation in response to H_2O_2 (Figure 4B). It also accumulated in the nucleus even in mutant cells (Figure 4C) indicating that this particular STAT5 mutation permitted not only phosphorylation in the ground state but also permitted translocation to the nucleus without requiring FANCD2. Interestingly, the activating STAT5 mutant did bind to FANCC in FA-D2 cells even though the STAT5 complex with wild type FANCC required intact FANCD2. The mechanisms by which this mutant form is functionally activated are currently unknown (120), but our results suggest that this STAT5 mutant is capable of overcoming constraints imposed upon it by scaffolds of FA proteins. In fact, the capacity to form novel ground state complexes with other proteins "without help" may play a direct role in its capacity to enter the nucleus.

In light of our prior observations that FANCC forms an interferon-inducible complex with STAT1 (4), we tested the hypothesis that FANCC associates with STAT5 in response to H_2O_2 using co-immunoprecipitation. We included experiments that examined the potential involvement of Hsp90 in STAT5 complexes in light of prior reports that STAT3 and STAT5 form inducible complexes with Hsp90 in a variety of cell types (133), (115). At the time we conducted our initial experiments, the prevailing view was that FANCD2 was an exclusively nuclear protein involved solely in the DNA damage response (121). For that reason we carried out FANCD2/STAT5 co-

immunoprecipitations because we expected that these molecules would not associate. As we discovered herein, and as has now been reported by others (119), there is readily detectable FANCD2 in the cytoplasm as well as the nucleus of the cells we studied and in certain highly proliferative tissues (a demonstrative immunohistochemical view of FANCD2 localization is presented in supplementary Figure 5). In fact, not only did cytoplasmic FANCD2 and STAT5 associate, the FANCD2/STAT5 and FANCD2/Hsp90 associations were clearly prerequisites for optimal survival of normal and complemented FA-D2 cells (Figure 5C), complemented FA-G cells (Figure 5D), and complemented FA-C cells (Figure 5E) exposed to H_2O_2. FANCD2/STAT5 and FANCC/STAT5 complexes were found in the cytoplasm of induced complemented cells (Figure 5B) but were not observed in any FA mutant cell type we studied. This is the first report of a cytoplasmic signaling function of FANCD2 and demonstrates that it, like FANCC, is a multi-functional protein that facilitates both genoprotective responses to cross-linking agents and survival signal transduction pathways.

That formation of FANCC/STAT5 and FANCD2/STAT5 complexes depends upon FANCC, FANCD2 and FANCG, is significant technically for two reasons. First, this mutual dependency provides assurance that the differential molecular associations we measured in complemented cells (e.g. the formation of a FANCC/STAT5 complex in complemented FA-D2 cells) were not artifacts of non-specific H_2O_2-induced protein crosslinking. That is, while there is plenty of FANCC in FA-D2 cells and plenty of FANCD2 in FA-C cells both proteins associate with STAT-5 *only* in induced complemented cells and none associate in mutant cells. Secondly, consistent with the well-developed nuclear core complex model in which many FA proteins can co-

precipitate with each other (121), these inducible complexes may represent large multimers containing and depending upon the function of each of the three (or more) FA proteins. Thus, our observations are consistent with a model in which, in normal cells, but not FA cells, H_2O_2 induces STAT5-P to bind to FANCD2, FANCC, and Hsp90. We also suggest that the FANCD2, FANCC, and Hsp90 molecules represent a pre-formed multimeric scaffold.

That FA proteins participate in a variety of ground-state multimeric protein complexes has been described recently (74;90), but the function of the complexes described is unknown and dynamic complex formation in response to environmental cues has not been reported. The co-immunoprecipitation experiments shown in Figure 5 were compatible with the idea that preformed complexes of FANCC/Hsp90 and STAT5/Hsp90 exist in normal cells and that H_2O_2 induced the formation of multimeric complexes containing at least FANCD2, STAT5, Hsp90, and FANCC. In support of this notion are results of size-exclusion chromatography experiments in which fractions of approximately 500 kDa contained STAT5, FANCD2, Hsp90, FANCC, and FANCG, compatible with our view that the key elements of the H_2O_2–induced STAT5 response form large multimeric signaling complexes. The majority of cytoplasmic FANCD2 was found in fractions representing higher molecular weights than the other FA proteins (Figure 6A and 6B). Interestingly, once phosphorylated, STAT5 shifted from low molecular weight fractions to higher fractions (4 and 5), perfectly overlapping with the location of FANCD2. That this shift also occurred in mutant FA-D2 cells indicated that an interaction with FANCD2 was not a prerequisite and that other interactors must be involved.

In response to H_2O_2 there was no substantial shift in the fractional distribution of cytoplasmic FANCD2, FANCC and FANCG (Figure 6A) suggesting one or more of the following; (a) that the H_2O_2-induced protein interactions observed by co-immunoprecipitation involve a minority of the total protein molecules of each partner, (b) that there exist multiple complexes in the high molecular weight fractions and that individual signaling molecules of similar size may shuttle between them, thereby not significantly altering the apparent molecular weight of each complex, or (c) that there exists a single preformed multimeric complex in which the binding affinities between members of the complex differ before and after stimulation; affinities that were apparent using co-immunoprecipitation but not size-exclusion chromatographic methods.

Having confirmed that the nuclear STAT5 we detected (Figure 2) corresponded directly with its transcriptional activation function (using EMSA and an immunoblot for Bcl-xL), our observations indicate that FANCD2, FANCC, and FANCG each play a role in coordinating a key STAT5-mediated survival response to H_2O_2 and that FA-D2, FA-C, and FA-G cells are hypersensitive to oxidative stress in part because STAT5 survival signals are suppressed. In parallel studies we found that FANCA is not required for STAT5 activation in response to oxidative stress in fibroblasts (data not shown). Therefore, while others have reported that oxidative stress induces multimerization of FANCG and FANCA proteins (134), it is unlikely that this particular interaction is of direct relevance to the STAT5 response studied here. The observations (Figure 1C) are also consistent with reports that FANCC mutant cells are more sensitive to oxidative stress than FANCA cells (75).

The inducible formation in normal cells of multimeric signaling complexes containing STAT5, Hsp90, and FANCD2 and the failure of complex formation in FA-D2, -C, and -G cells, position FANCD2 and FANCC squarely as central coordinators of the STAT5 responses. In light of the minimal shift to the right in H_2O_2 sensitivity we found in complemented cells (Supplemental Figure 1), notwithstanding that the curves are statistically different for each complementation group, we do not argue that STAT5 defects underlie the reported hypersensitivity of FA cells to oxidative stress. In fact, biochemical evidence supports a direct role for FA proteins in control of alternative responses to oxidative stress through: (1) the redox-dependent ASK1 pathway (113) and (2) failure of a FANCG/peroxiredoxin-3 complex formation (114).

We do not yet know whether any of the other nine FA proteins participate in this cytoplasmic response either to hematopoietic growth factors or H_2O_2, we have not identified the precise makeup of the STAT5 complex that traverses the nuclear membrane (although STAT5-P and FANCC do associate in the nucleus by immunoprecipitation), and do not know whether there exists only one or several multimeric high molecular weight complexes containing STAT5, Hsp90, FANCD2, and FANCC. We have, however, demonstrated that the phosphorylation of STAT5 in response to H_2O_2 is JAK2-dependent, that STAT5 participates in the formation of inducible high molecular weight complexes with FANCD2 and FANCC, and that nuclear translocation of STAT5-P requires that STAT5 form a complex with functional FANCC and FANCD2 proteins in a FANCG dependent manner. Our observations also confirm a recent report that FANCD2 can exist in both the nuclear and cytoplasmic compartments (119) and

represents the first evidence that cytoplasmic FANCD2 may participate in canonical cytoplasmic signaling functions.

Although hundreds of FA cell lines are available, they are almost exclusively lymphoblastoid or fibroblasts and express no lineage specific receptors for myeloid or erythroid growth factors. The aberrant STAT5 responses we have observed in FA fibroblasts in response to extracellular H_2O_2 must be confirmed in primary hematopoietic cells but having identified discrete inducible protein-protein interaction patterns in fibroblasts the task will be easier to accomplish. In light of the promising results in EGF stimulated FA fibroblasts and in a FANCD2 knocked-down erythropoietin-responsive hematopoietic cell line, such studies are clearly warranted.

3-5 MATERIALS AND METHODS

Cells and cell lines

PD20 (FA-D2) and PD331T (FA-C) are SV40 large T-antigen transformed human skin fibroblasts. PD829 (FA-G) cells are primary human skin fibroblasts. These lines were complemented by retroviral transfer of the appropriate FA cDNA in the Fanconi Anemia Cell Repository (OHSU, Portland OR). The cells were maintained in Minimum Essential Medium (MEM) α medium (GIBCO, Grand Island, NY) supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, Utah) and penicillin (100 units/ml) /streptomycin (100 μg/ml) (Sigma, St. Louis, MO). Primary MEFs were isolated from embryonic day 13.5 wild-type and *Fancc*$^{-/-}$ C57BL6/J embryos and were cultured in MEM α Medium supplemented with 10% FBS. Primary *Stat5a*$^{-/-}$ *5b*$^{-/-}$ MEFs, kindly provided by Dr. James Ihle (St. Jude Children's Research Hospital, Memphis, TN), were transformed with SV40 large T antigen from U19-5 line (135).

Retroviral vectors and FA mutant constructs

The construction and activity of the constitutively active (pcDNA3STAT5aN642H) and dominant negative (pMXSTAT5aΔ750) cDNA molecules have been described previously (120;136). The constitutively active STAT5a mutant plasmid was transfected using the calcium phosphate precipitation method. Ground-state phosphorylation and nuclear translocation of this mutant was confirmed using immunoblotting and confocal microscopy. The dominant negative mutant STAT5aΔ750 plasmid was subcloned into the EcoRI and Hpal sites of retroviral vector pLXSN and was transfected, by the method of calcium phosphate precipitation, into ψ-2 and the resulting virions were used to infect PA12 cells (137). Supernatants containing the STAT5aΔ750 retroviral vectors were utilized to transduce FA-D2 cells by methods described previously (76).

Antibodies and Reagents

Anti-phosphotyrosine STAT5 (Tyr694), anti-STAT5, anti-phosphotyrosine JAK2 (Tyr1007/1008), anti-phosphotyrosine EGFR (Tyr845 or 992), anti- EGFR, anti-phosphotyrosine IGF-1R (Tyr1131), anti-IGF-1R, anti-phosphotyrosine IR-β (Tyr1345 or1361), anti-IR-β, anti-phosphotyrosine PDGFR-β (Tyr740, or 751, or 771, or 1021), anti-PDGFR-β, anti-Bcl-xL and anti-histone deacetylase I (HDAC1) polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-STAT5a and anti-STAT5b were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-FANCD2, anti-Hsp70, and anti-Hsp90 monoclonal antibodies were purchased

from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-TATA binding protein TBP monoclonal antibody was purchased from Abcam (Cambridge, MA). The affinity purified anti-FANCC peptide antibody was generated by our group (138). The FANCG antiserum was kindly provided by Maureen Hoatlin (Oregon Health and Science University, Portland, OR). AG490, AG1478, and AG1024 were obtained from CalBiochem (Darmstadt, Germany). Hydrogen peroxide (H_2O_2) was purchased from Sigma (St. Louis, MO). All the other reagents, unless otherwise specified, were obtained from Sigma-Aldrich (St. Louis. MO).

Apoptosis assays

Cells were treated with H_2O_2 (100μM -1mM) for 5 to 12 h. Flow cytometric assays for apoptotic and necrotic cells were performed with the PCA-96 Nexin Kit (Guava Technologies Inc, Hayward, CA) according to the manufacturer's protocol with minor modifications. Briefly, $5x10^4$ cells harvested from the culture plates were incubated with 10 μl annexinV-PE, 5 μl Nexin 7-AAD, and 135 μl "Nexin buffer" in a microplate plate for 20 min at room temperature in the dark. Apoptotic fractions of cultured cells were quantified using the PCA-96 instrument (Guava Technologies Inc, Hayward, CA). Results were expressed as mean percent non-apoptotic viable cells (both Annexin V- and 7-AAD-negative) $\pm$ S.D.

Nuclear and cytosolic extracts

Subconfluent cells were incubated with 1 mM H_2O_2 for 0, 5, 15, 30, 60, and 120 min. The cells were washed with ice-cold Dulbecco's Phosphate-Buffered Saline (PBS) (GIBCO, Grand Island NY) twice. Nuclear and cytosolic extracts were prepared using the Active Motif Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Potential contamination of cytosolic extracts with nuclear material was monitored using immunoblots for histone deacetylase I (HDAC1) and TATA binding protein (TBP). These molecules were detected in nuclear extracts but not in cytoplasmic extracts (supplementary Figure 5). In some experiments the conditions of nuclear extraction and lysis were altered to omit nonidet P-40 (NP-40).

Immunoprecipitation

Subconfluent cells were treated with 500 μM H_2O_2 for from 1 to 15 min. The cells were washed with ice-cold PBS twice and lysed with modified-RIPA buffer (m-RIPA) [50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 1% NP-40, 0.1% deoxycholic acid, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 4 mM ethylenediaminetetracetic acid (EDTA), 2 mM sodium orthovanadate, 1 mM phenylmethysulfonyl fluoride (PMSF), 1% leupeptin, 1% pepstatin and 1% aprotinin]. For immunoprecipitations, cell extracts (1 mg of total protein) were precleared by adding 1 μg of rabbit or mouse IgG (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) and 10 μl (50% v/v) of Sepharose conjugated Protein A or Protein G and incubated for 45 min at 4^0C. The supernatants were then incubated with indicated antibodies overnight at 4^0C. The immune complexes were then bound to protein A (or G)-Sepharose (2 h, 4^0C) and washed four times with m-

RIPA lysis buffer. Samples were eluted in 50 μl of 2x Laemmli buffer [1M Tris-HCl pH 6.8, 4% sodium dodecyl sulfate (SDS), 40% glycerol, and 4% 2-mercaptoethanol].

Immunoblotting

Nuclear or cytoplasmic extracts and immune complexes were denatured by incubation with an equal volume of 2X Laemmli buffer for 5 min at 94^0C prior to electrophoresis on SDS-page gels under reducing conditions. The proteins were subsequently transferred to nitrocellulose and blocked with 5% nonfat milk (Nestle USA, INC., Solon, OH) in TBST (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.4, 0.05% Tween-20). Blots were incubated with indicated antibodies overnight at 4^0C. Proteins were detected by Enhanced Chemiluminescence Kit (Amersham Biosciences, Piscataway, NJ) after incubation with an appropriate horseradish peroxidase-coupled secondary antibody (Bio-Rad, Hercules, CA). In experiments with AG490, cells were treated with the inhibitor (50 μM) for 16 h before H_2O_2–exposure. For receptor tyrosine kinase activity assays, cells were exposed for 2 h first to the chemical inhibitor (20 μM AG1024 or 25 μM AG1478) then for 5 min to H_2O_2. Total and tyrosine phosphorylated receptor forms were detected using specific antibodies. When phosphorylation of a specific receptor in response to H_2O_2 was detected, the effect of a pharmacologic inhibitor for that receptor was tested using STAT5 and JAK2 phosphorylation as measurements of the degree to which the particular receptor was linked to the H_2O_2 response.

Deconvolution Microscopy

Mutant FA-D2 (or FA-C, or FA-D2 STAT5aN642H), and complemented FA-D2/D2 (or FA-C/C) cells were plated on chamber slides (Nalge Nunc International, Naperville, IL) and treated with or without 300 μM H_2O_2 for 10 min, fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 15 min and permeabilized with ice-cold methanol for 10 min. After 1 h incubation in blocking buffer containing 10% horse serum (ICN Biomedicals, Inc, Costa Mesa, CA), 1% bovine serum albumin (Sigma, St. Louis, MO) and 0.02% NaN3 (Amresco, Solon, Ohio), the cells were incubated with 5 μg/ml phospho-STAT5 antibody in 1% BSA in PBS overnight at 4^0C. The next day, Alexa Fluor 488-conjugated secondary antibody (1:200 dilution; Molecular Probes, Eugene, OR) was added for 30 min. Nuclei were counterstained with DAPI (4, 6 diamidino-2-phenylindole; Molecular Probes, Eugene, OR) before treating with Prolong Gold Antifade mounting medium (Molecular Probes, Eugene, OR). Images were acquired with The Applied Precision "Image Restoration System" that included a motorized XYZ stage, a Nikon TE200 inverted fluorescent microscope with standard filter sets, halogen illumination with API light homogenizer, a CH350L Camera (500 KHz, 12-bit, 2 Mp, KAF 1400 GL, 1317x1035, liquid cooled) and DeltaVision software (Issaquah, WA). The iterative constrained algorithm of Sedat and Agard (139) was used for deconvolution and additional image processing was performed on a Dell XPS workstation with Bitplane Imaris software system (Bitplane Inc, Saint Paul, MN).

Immunohistochemical staining for FANCD2

Ovarian cancer tissue sections were analyzed for FANCD2 expression. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 5 minutes. Sections were incubated with FANCD2 antibody (Novus Biological, Littleton, Colorado), diluted to 1:100, at room temperature. The biotin-free HRP enzyme labeled polymer of the Envision plus detection system (Dakocytomation, CA-USA) was used as a secondary reagent. The diaminobenzidine (DAB) complex was used as chromogen. For negative controls, normal goat serum was used instead of the primary antibody.

Size Exclusion Chromatography

Size exclusion chromatography was used to fractionate multimeric complexes in both whole cell lysates and cytoplasmic extracts. FA-D2 and complemented FA-D2/D2 cells (10^7 cells, 70-80% confluent) were washed twice in cold PBS and lysed at 4^0C in m-RIPA buffer. For whole cell lysates, after centrifugation at 16,000 x g for 15 min, protein concentrations were determined and supernatants (1 mg) were loaded onto a Tricorn Superose 6 10/300 GL column (Amersham Biosciences, UK) connected to a Hewlett-Packard 1100 HPLC system (Hewlett-Packard, Palo Alto, CA). Proteins were eluted in 0.5 ml fractions at 0.5 ml/min in buffer containing NP-40 (1% NP-40, 150 mM NaCl and 50 mM Tris-HCl pH 7.4). Fractions were analyzed by immunoblot and apparent molecular weights were determined after column calibration with a set of molecular mass standards between 29 kDa and 2000 kDa (MW-GF-1000, Sigma, St. Louis, MO).

Electrophoretic Mobility Shift Assay

Nuclear proteins were prepared as described above. A double-stranded biotin-labeled STAT5 oligonucleotide 5'-AGATTTCTAGGAATTCAATCC-3' was purchased from Panomics, Inc (Redwood City, CA) and the EMSA was performed according to the manufacturer's suggested protocol. Briefly, protein-DNA binding reactions were carried out for 20 min at room temperature in 10 μl reaction volumes containing 1 μg of poly (dI:dC), 10 μg of nuclear protein extract, and 10 ng biotin-labeled STAT5 probe. For EMSA supershift assays, nuclear extracts were preincubated with anti-STAT5 polyclonal antibody that recognized both STAT5a and b and an antibody that identified only STAT5b for 1 h at 4^0C. Binding complexes were resolved in native 6% polyacrylamide gels (Tris/Borate/EDTA buffer). Samples were transferred to a nitrocellulose membrane. The membrane was crosslinked in a UV oven (Stratagene, La Jolla, CA) for 3 min at 1200 μjoules and then subsequently incubated with streptavidin-HRP conjugate diluted according to the manufacturer's instructions. After washing, the samples were visualized with chemiluminescence (Panomics Inc. Redwood City, CA).

Statistical analysis

Numerical data are presented as means (from experiments performed using no fewer than three replicates from each sample) $\pm$ S.D. In addition, each experiment, including size exclusion chromatography, was repeated thrice with similar results. Statistical calculations were made using the Excel statistical software package and SigmaSTAT (Systat, San Jose, CA). The student's *t*-test was utilized to determine the

significance of measured differences. Dose response ID_{50}s were compared using analysis of variance.

RNA interference

To determine whether FANCD2 influences erythropoietin-induced nuclear accumulation of STAT5-P we used SMARTpool FANCD2 siRNA (Dharmacon, Inc., Lafayette, CO). Introduction of human FANCD2 siRNA (siGenome on-target plus SMARTpool, Catalog number NM_001018115) and SMARTpool non-target siRNA negative control (catalog number D-001206013020) into MO7e/EPOR cells were carried out by nucleofection (Amaxa Inc. Gaithersburg, MD) according to the manufacturer's instructions. Briefly, a total 5x 10^6 cells in 100 µl R-solution with 100 nM siRNA was nucleofected with the S-002 program. After nucleofection, cells were incubated with 1 ml pre-warmed serum-free and antibiotic-free medium for 15 min, and then the cells were grown in regular medium for 24 h. The cells were treated similarly on two more consecutive days after which nuclear extracts and whole cell lysates were prepared.

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3-6: FIGURES

Figure 1

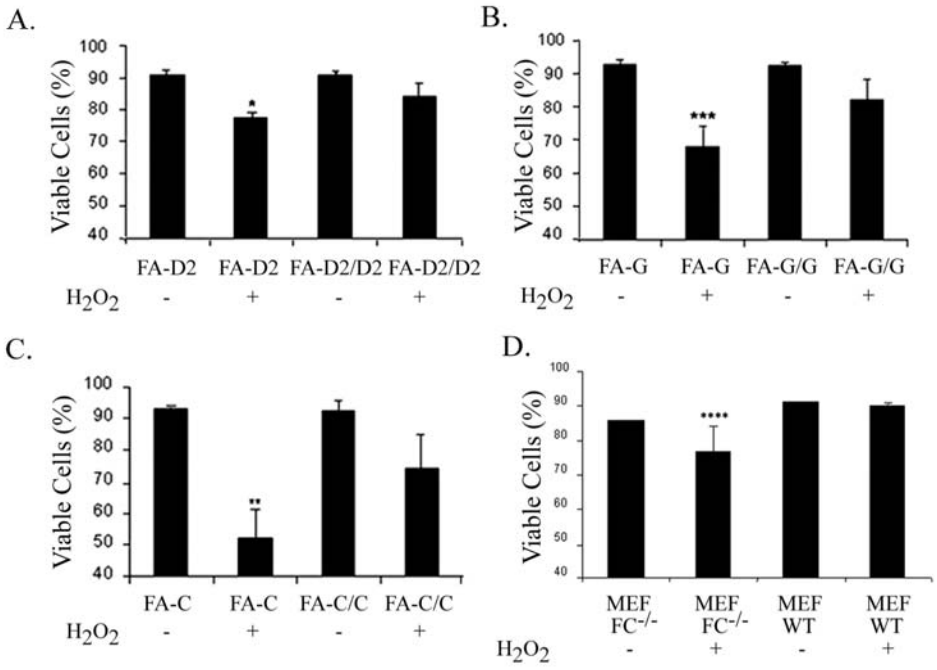


Figure 1 Legend. Fanconi anemia (FA) cells are hypersensitive to hydrogen peroxide.

The FA cell lines and retrovirally complemented isogenic cell lines were exposed to 500 μM H_2O_2 for 5 h. Viable cells (defined as non-apoptotic cells that are Annexin V- and 7-AAD-negative) were quantified by flow cytometry. While the non-viable cell populations were equivalent in mutant and complemented cells in the ground state, there

was a significant ($P < 0.05$) decrease in the viable fraction of mutant cells after exposure to H_2O_2 when compared to the exposed complemented cells. (A) D2 mutant cell line FA-D2 and complemented cell line FA-D2/D2. (B) G mutant cell line FA-G and complemented cell line FA-G/G. (C) C mutant cell line FA-C and complemented cell line FA-C/C. (D) Primary MEFs from *Fancc*$^{-/-}$ and wild-type C57BL6/J embryos. *Fancc*$^{-/-}$ MEFs were intolerant of H_2O_2. Asterisks represent significant ($P<0.05$) inhibition of survival in exposed mutant cells when compared to exposed complemented cells (the fourth bar in each graph). The three mutant cell lines were also hypersensitive in dose (H_2O_2) response (survival) curves (Supplementary Figure 1).

Figure 2

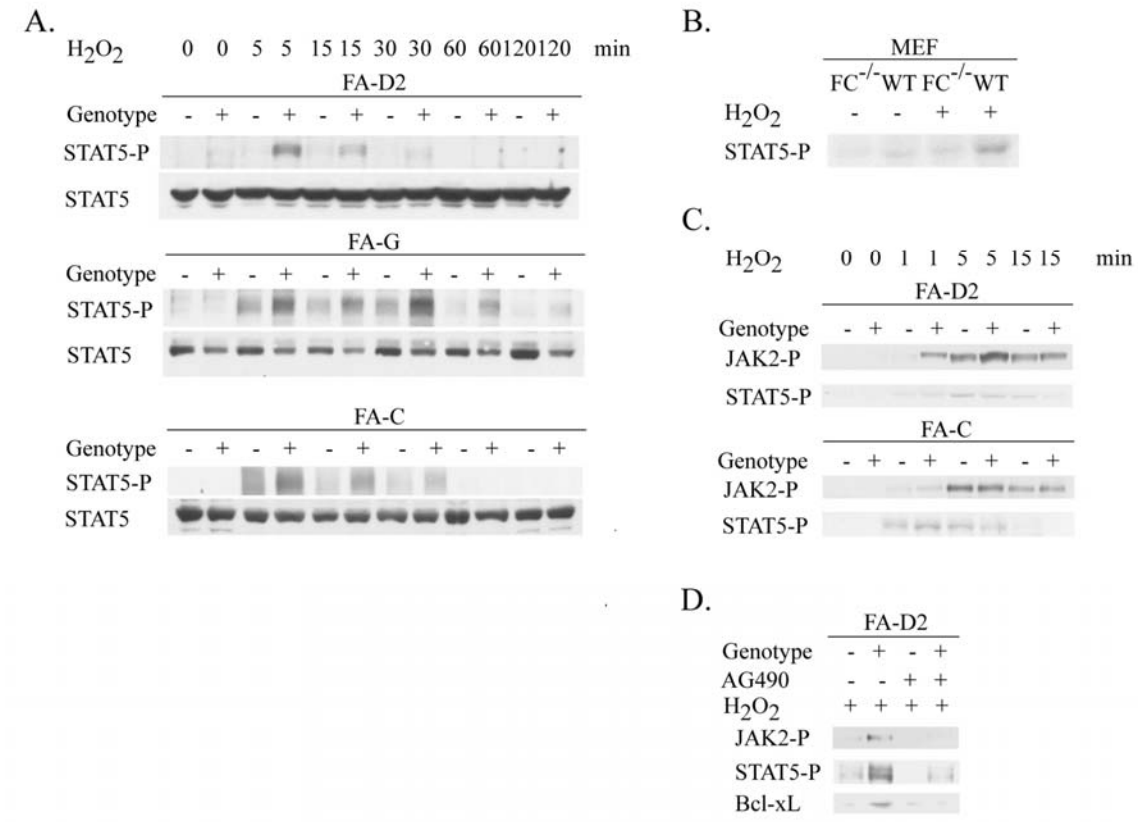


Figure 2 Legend. H_2O_2 exposure rapidly activates nuclear STAT5 phosphorylation (Tyr694). (A) Immunoblot analyses were carried out on nuclear extracts obtained from mutant and complemented cells at multiple time points following exposure to H_2O_2.

Antibodies specific for STAT5 and the tyrosine-phosphorylated form of STAT5 (STAT5-P) were used. Nuclear STAT5 phosphorylation levels were consistently induced in complemented cells. STAT5 phosphorylation responses were optimal at 5 min post exposure in FA-D2/D2 cells and FA-C/C cells. The duration of optimal STAT5 phosphorylation was prolonged in FA-G/G cells. (B) Wild-type primary MEFs exposed to H_2O_2 exhibited nuclear STAT5 phosphorylation. There is no H_2O_2 induced STAT5 phosphorylation in *Fancc*$^{-/-}$ cells. (C) H_2O_2 induced cytoplasmic STAT5 and JAK2 activation in FA cells. Immunoblot analysis of cytoplasmic extracts demonstrated H_2O_2-induced phosphorylation of JAK2 and STAT5 that peaked at 5 min and declined thereafter. STAT5 phosphorylation had declined to baseline by 15 minutes at which time nuclear STAT5 was maximal. (D) H_2O_2 induced STAT5 phosphorylation is JAK2 dependent. JAK2, STAT5 phosphorylation, and Bcl-xL level were examined after 15 min exposure to H_2O_2, and the effect of 20 μM AG490 (given 16 h prior to H_2O_2) was tested. H_2O_2 increased JAK2-P, STAT5-P, and Bcl-xL level in FA-D2/D2 cells. This effect was almost completely inhibited by AG490.

Figure 3.

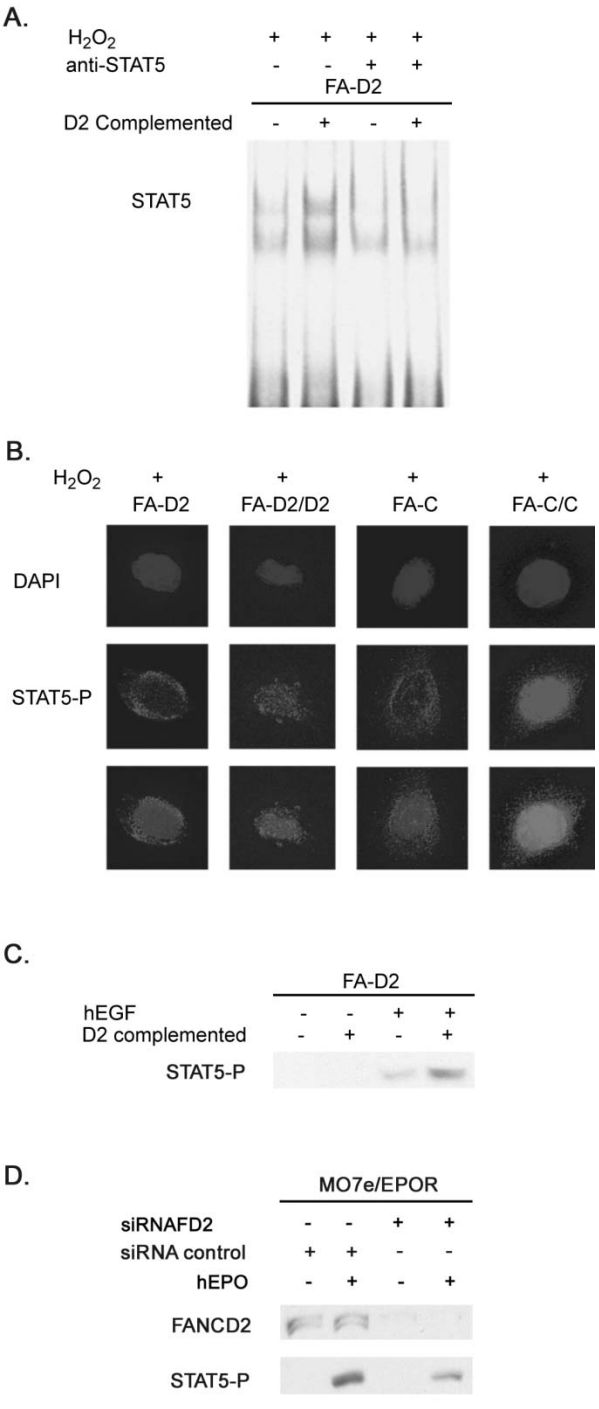

A.
H_2O_2 + + + +
anti-STAT5 - - + +
FA-D2
D2 Complemented - + - +
STAT5
B.
H_2O_2 +
FA-D2
+
FA-D2/D2
+
FA-C
+
FA-C/C
DAPI
STAT5-P
C.
FA-D2
hEGF - - + +
D2 complemented - + - +
STAT5-P
D.
MO7e/EPOR
siRNAFD2 - - + +
siRNA control + + - -
hEPO - + - +
FANCD2
STAT5-P

Figure 3 Legend. Reduced nuclear accumulation of STAT5-P in H_2O_2-stimulated mutant cells was also confirmed using electrophoretic mobility shift analysis (EMSA), and deconvolution microscopy. (A) EMSA was performed using nuclear extracts from H_2O_2 –exposed (15 min) FA-D2 cells and complemented cells (FA-D2/D2) and demonstrated an enhanced interaction of STAT5 with a consensus STAT5 binding site in nuclear extracts of FA-D2/D2 cells. Nuclear protein binding to STAT5 probe was induced only in FA-D2/D2 cells and the STAT5 band disappeared in lysates pretreated with an anti-STAT5 antibody. (B) Mutant FA-D2 (or FA-C), and complemented FA-D2/D2 (or FA-C/C) cells were studied by deconvolution microscopy before and after treatment with 300 μM H_2O_2 for 10 min. The deconvoluted images show STAT5-P (green) only in the cytoplasm of mutant FA-D2 and FA-C cells, but STAT5-P was present in both nucleus and cytoplasm of complemented FA-D2/D2 or FA-C/C cells. DAPI (blue) is a nuclear stain. C. Nuclear STAT5 phosphorylation. Nuclear extracts were obtained from FA and complemented FA cells after 10min hEGF treatment. Immunoblot analyses were carried out by using the tyrosine-phosphorylated form of STAT5. After hEGF exposure, nuclear STAT5-P levels increased to a larger extent in complemented cells than in FA cells. D. FANCD2 siRNA suppresses FANCD2 and erythropoietin (EPO)-induced nuclear STAT5 phosphorylation in MO7E-epoR cells. After 3 transfections of siRNA targeted to FANCD2 or of control siRNA, MO7E-epoR cells were exposed to EPO for 30 minutes after which nuclear extracts were prepared for STAT5 immunoblotting.

Figure 4

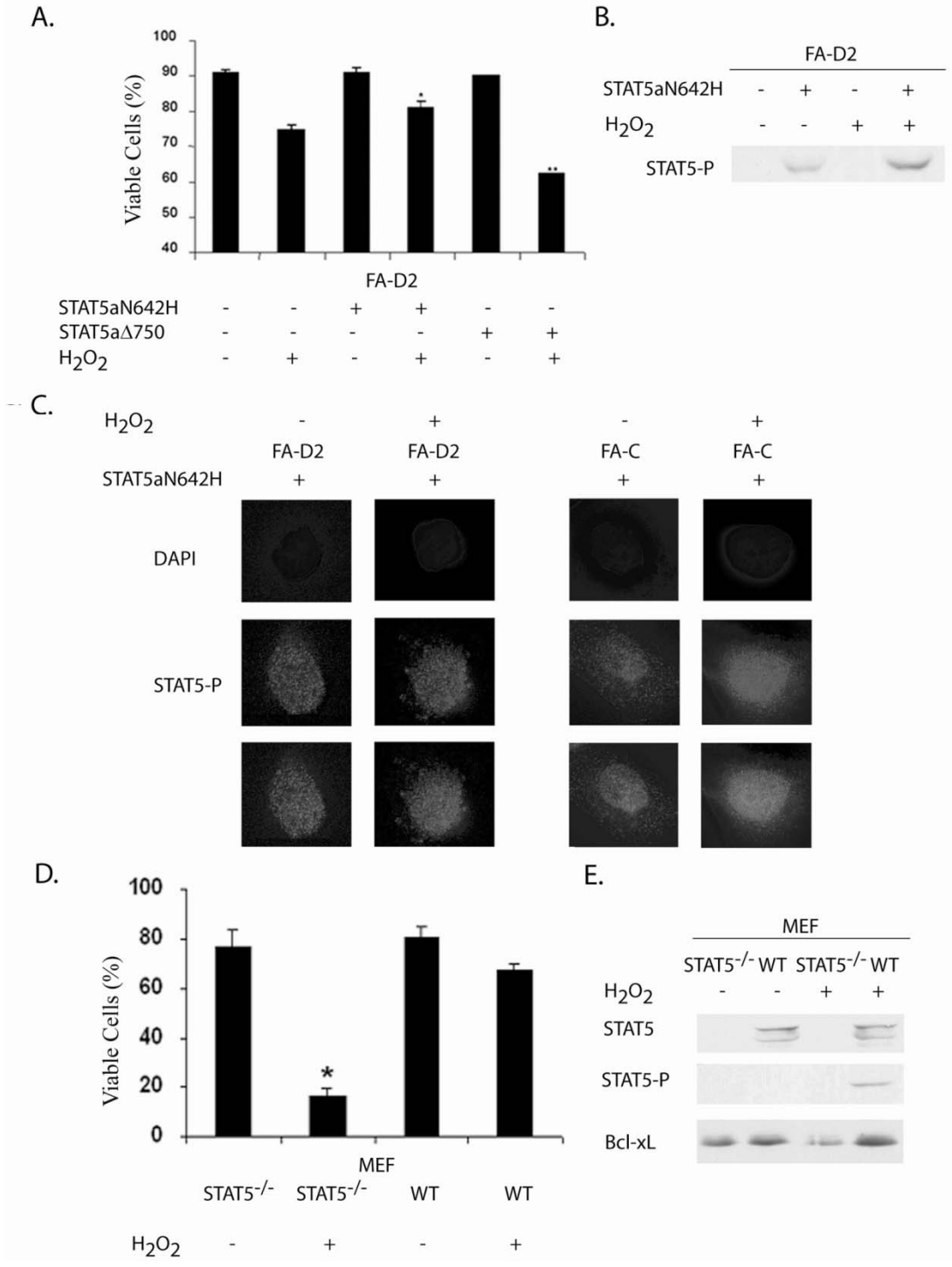

A.
Viable Cells (%)
100
90
80
70
60
50
40
FA-D2
STAT5aN642H - - + + - -
STAT5aΔ750 - - - - + +
H2O2 - + - + - +
B.
FA-D2
STAT5aN642H - + - +
H2O2 - - + +
STAT5-P
C.
H2O2 - + - +
FA-D2 FA-D2 FA-C FA-C
STAT5aN642H + + + +
DAPI
STAT5-P
D.
Viable Cells (%)
100
80
60
40
20
0
MEF
STAT5-/- STAT5-/- WT WT
H2O2 - + - +
E.
MEF
STAT5-/- WT STAT5-/- WT
H2O2 - - + +
STAT5
STAT5-P
Bcl-xL

Figure 4 Legend. H_2O_2–induced STAT5 activation enhances survival. (A) FA-D2 cells were transfected or transduced with vectors expressing either the constitutively active STAT5 mutant STAT5aN642H or the dominant negative mutant STAT5aΔ750. The cells were exposed to 500 μM H_2O_2 for 5 h after which apoptotic and non-viable cells were quantified by flow cytometry. The constitutively active mutant STAT5aN642H increased the fraction of viable cells (* $p<0.01$ compared to treated mutant cells [bar 2]) and the dominant negative mutant STAT5aΔ750 decreased viability of mutant cells (** $p<0.001$ compared to treated mutant cells [bar 2]) after exposure to H_2O_2. (B) Immunoblot analyses confirmed baseline and induced phosphorylation of STAT5 in the FA-D2 cells transduced with the constitutively active mutant STAT5aN642H. (C) Deconvolution microscopic images show STAT5-P (green) present in both nucleus and cytoplasm with and without H_2O_2 stimulation in STAT5aN642H transfected FA-D2 or FA-C cells. (D) Transformed MEFs and $STAT5a^{-/-}$ $5b^{-/-}$ MEFs (MEF $STAT5^{-/-}$) were incubated with 500 μM H_2O_2 for 5 h. MEF $STAT5^{-/-}$ cells are more sensitive to H_2O_2 than treated WT cells ($p<0.001$). (E) Immunoblot analysis confirmed that H_2O_2 induced STAT5 phosphorylation and Bcl-xL only in wild-type MEFs.

Figure 5

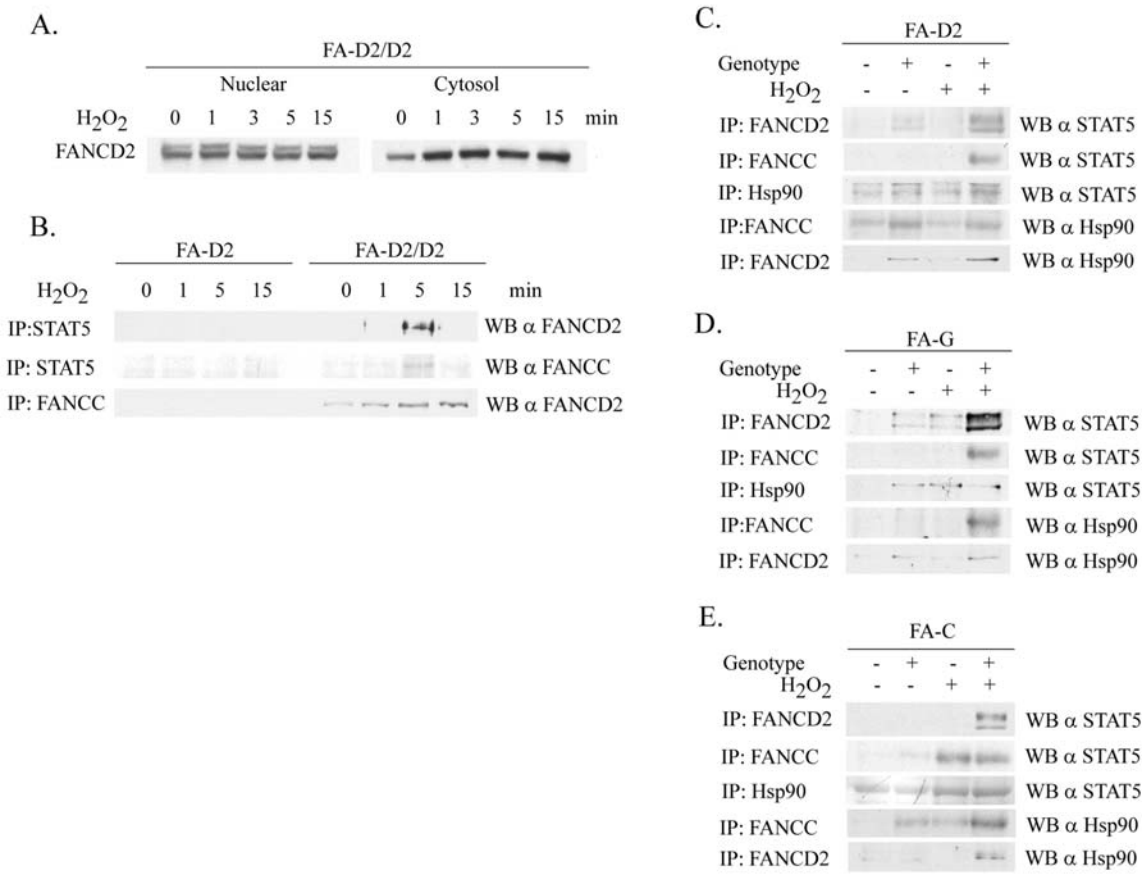


Figure 5 Legend. Inducible protein complex formation in FA and complemented cells. (A) FA-D2 and FA-D2/D2 cells were treated with 500 μM H_2O_2 for the indicated times. Nuclear extracts were prepared as described above (methods). The non-ubiquitinated form of FANCD2 is found in both nuclear and cytoplasmic fractions but ubiquitinated form only in nuclear fractions. (B) Co-immunoprecipitation experiments were performed with cytoplasmic fractions. The rapid formation of inducible complexes is only seen in which complemented cells (but not FA-D2 cells) form STAT5/FANCD2 complexes and

STAT5/FANCC complexes within 1 min, peaking at 5 min. (C. D. E) Co-immunoprecipitation experiments were carried out using lysates of FA mutant cells and complemented cells (with or without exposure to 500 μM H_2O_2 for 15 min). (C) Protein complex formation in mutant FA-D2 and complemented FA-D2/D2 cells. Rows 1 and 5 demonstrate that FANCD2 forms complexes with STAT5 and Hsp90 in complemented FA-D2/D2 cells, and the complexes were inducible by H_2O_2. FANCC also formed a dynamic complex with STAT5 after exposure to H_2O_2 in FA-D2/D2 cells. STAT5 does form complexes with Hsp90 even in mutant FA-D2 cells (row 3), but in the ground-state, more of this complex is found in complemented (FA-D2/D2) cells. Ground state association of FANCC and Hsp90 is greater in FA-D2/D2 cells than in mutant FA-D2 cells (row 4). (D) Protein complex formation in mutant FA-G and complemented FA-G/G cells. FANCD2/STAT5 and FANCD2/Hsp90 complexes were detected in both the ground state and induced state of FA-G/G cells, and the interaction was optimally induced by H_2O_2 (row 1, row 5). As was the case with the other complementation groups studied (Figure 5C. E), STAT5/Hsp90 complexes did form in both mutant and complemented cells (row 3), but FANCC/STAT5 (row 2) and FANCC/Hsp90 (row 4) complexes were seen only in induced FA-G/G cells. (E) Protein complex formation in mutant FA-C and complemented FA-C/C cells. Inducible formation of FANCD2/STAT5 and FANCD2/Hsp90 complexes were observed only in H_2O_2 –stimulated FA-C/C cells (rows 1 and 5), but FANCC/STAT5 and FANCC/Hsp90 complexes develop equally in both induced FA-C and induced FA-C/C cells (row 2, row 4) (this FA-C cell line bears an inactivating point mutation of FANCC and expresses an intact protein).

Figure 6

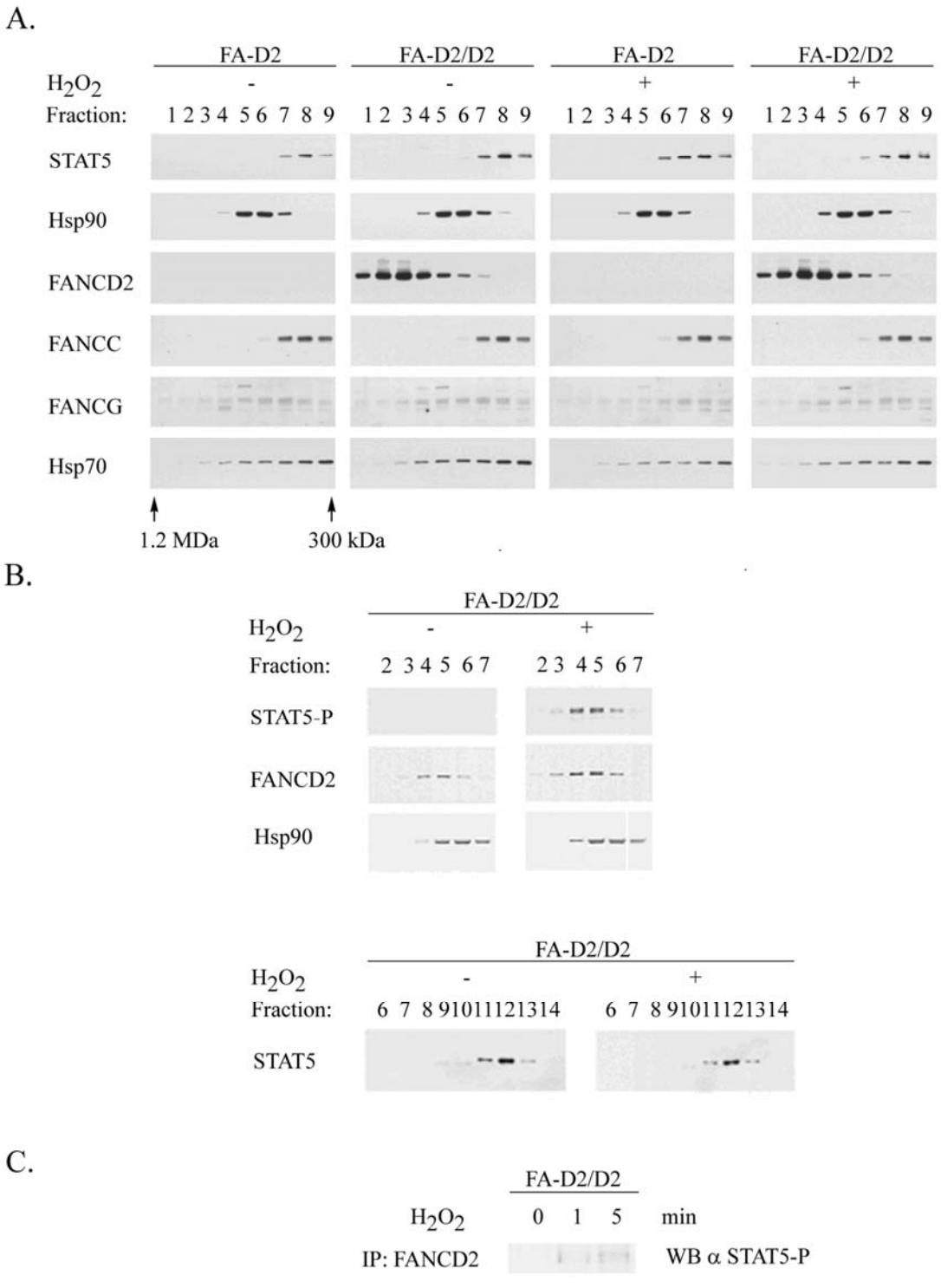

A.
FA-D2
FA-D2/D2
FA-D2
FA-D2/D2
H2O2
-
-
+
+
Fraction:
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
STAT5
Hsp90
FANCD2
FANCC
FANCG
Hsp70
1.2 MDa
300 kDa
B.
FA-D2/D2
H2O2
-
+
Fraction:
2 3 4 5 6 7
2 3 4 5 6 7
STAT5-P
FANCD2
Hsp90
FA-D2/D2
H2O2
-
+
Fraction:
6 7 8 9 10 11 12 13 14
6 7 8 9 10 11 12 13 14
STAT5
C.
FA-D2/D2
H2O2
0 1 5 min
IP: FANCD2
WB α STAT5-P

Figure 6 Legend. FANCD2, STAT5, Hsp90, FANCC and FANCG co-localize in high molecular weight fractions. Size exclusion chromatography was performed using whole cell lysates and cytoplasmic fractions of FA-D2 mutant and complemented cells, incubated in the absence (-) or presence (+) of H_2O_2, and the content of each protein in each fraction was analyzed by immunoblotting. (A) Whole cell lysate fractions ranged from 1.2 MDa to 300 kDa (lanes 1-9). Consistent with the known STAT-binding capacity of FANCC, STAT5 and FANCC co-eluted in a fraction of approximately 400 kDa (fraction 8). STAT5 and FANCC also partially co-eluted with Hsp90, FANCD2, and FANCG in 600 – 400 kDa fractions (fraction 6-8). FANCD2 signals increased in fractions 4, 5, and 6 after H_2O_2 stimulation of complemented cells. (B) In the cytoplasm, STAT5-P is found in fractions ranging from 1 MDa to 600 kDa (lane 3-7). STAT5 phosphorylation was detected in mutant FA-D2 (not shown) and complemented cells with H_2O_2 stimulation. In FANCD2 complemented cells, induced tyrosine phosphorylated STAT5 increased substantially in fractions 4 and 5, precisely the fractions that contained the most FANCD2. (C) Co-immunoprecipitation experiments showing FANCD2 and STAT5-P binding in cytosol of complemented FA-D2 cells is H_2O_2 – inducible.

Figure 7.

Figure 7 Legend. Involvement of FANCC and FANCD2 in STAT5 activation. We propose that in the ground state, STAT5 (“5”) and Hsp90 exist in preformed multimeric complexes and that FANCC (“C”) and FANCD2 (“D2”) also form ground state complexes with hsp90. However, FANCC and FANCD2 form complexes with STAT5 only after exposure to H_2O_2 and only in the presence of FANCG and on the condition that JAK2 is activated. STAT5 is tyrosine phosphorylated in response to H_2O_2 in a JAK2-dependent manner and then enters into a large macromolecular complex in which it is tightly bound to both FANCD2 and FANCC. This complex facilitates either directly or

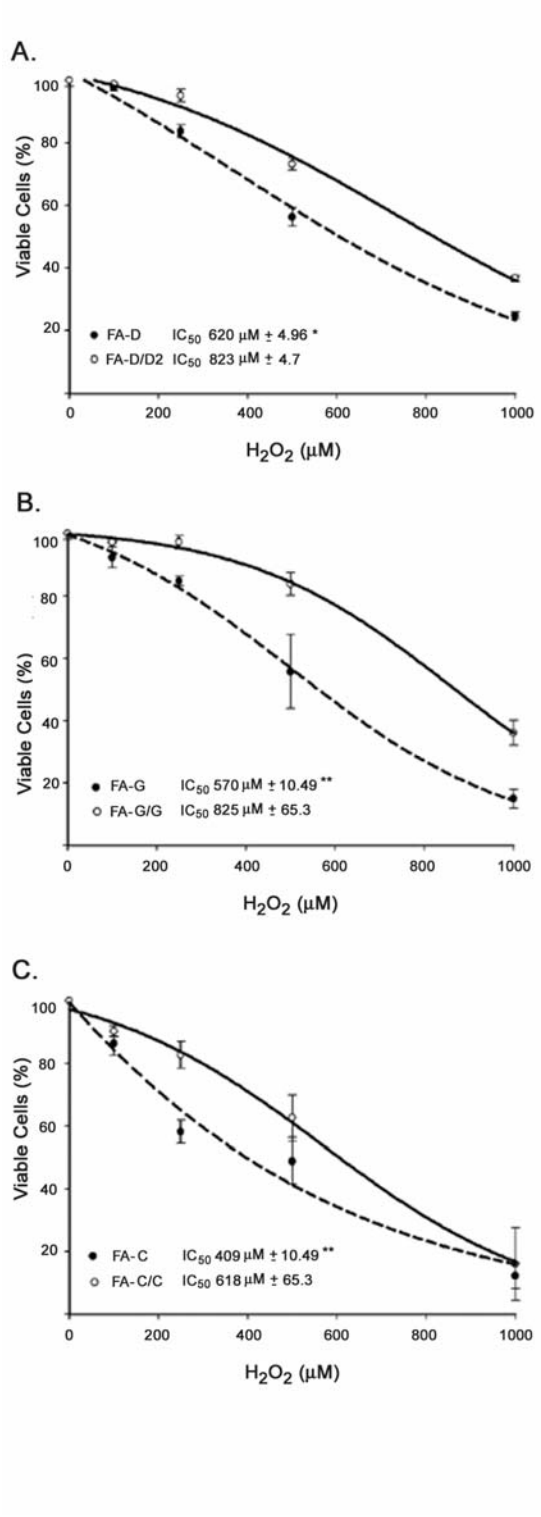
indirectly the nuclear translocation of phosphorylated STAT5. Following exposure to H_2O_2 in cells bearing inactivating mutations of *FANCD2*, *FANCC*, or *FANCG*, JAK2 dependent cytoplasmic phosphorylation of STAT5 does occur and the phosphorylated molecule does enter a macromolecular complex. However it does not bind to FANCC or FANCD2 and is not translocated to the nucleus.

TABLE 1. Receptor tyrosine kinases involved in the H_2O_2 response. Mutant and complemented FA-D2 cells were exposed to H_2O_2 (500 μM) for 5 min. Total and tyrosine phosphorylated forms of four receptors were detected using immunoblots. Only EGFR and IGF-1R were phosphorylated in response to H_2O_2. Prior to H_2O_2 exposure, cells were incubated for 2 hours with the EGFR inhibitor AG1478 or the IGF-1R inhibitor AG1024. Each inhibitor partially suppressed H_2O_2-induced STAT5 phosphorylation. "+" denotes detected. "-"denotes undetectable. ND, not done.

Receptor	Receptor detected by immunoblot	Receptor phosphorylation induced by H_2O_2	Effect of receptor-specific inhibitor on H2O2-induced STAT5 activation
EGFR	+	+	Partial Inhibition
IGF-1R	+	+	Partial Inhibition
PDGFR	+	-	N.D.
IR	-	-	N.D.

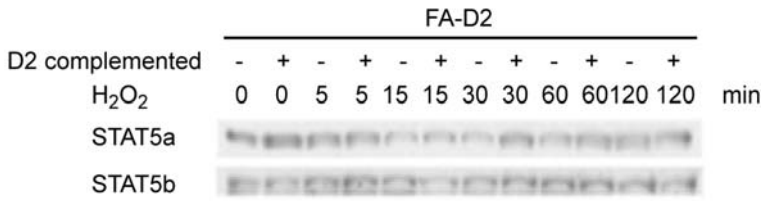
3-7: SUPPLEMENTAL FIGURES

Supplementary Figure 1.



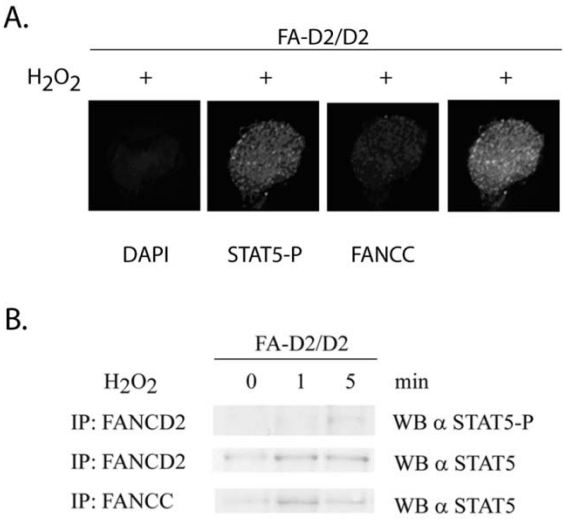
Supplemental Figure 1 Legend. The FA cell lines and retrovirally complemented isogenic cell lines were exposed to 100μM to 1mM H_2O_2 for 12 h. Viable cells (non-apoptotic cells that are Annexin V- and 7-AAD-negative) were quantified by flow cytometry. While the non-viable cell populations were equivalent in mutant and complemented cells in the ground state, there was a significant ($P < 0.05$, analysis of variance) decrease in the viable fraction of mutant cells after exposure to H_2O_2 when compared to the exposed complemented cells. Apoptotic responses to increasing doses of H_2O_2 were exaggerated in all three mutant cell types.

Supplementary Figure 2.



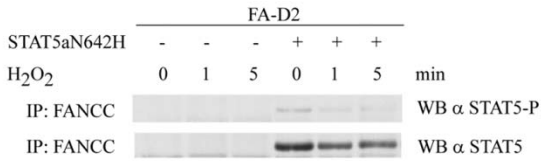
Supplemental Figure 2 Legend. Nuclear STAT5a and STAT5b in mutant FA-D2 and complemented FA-D2/D2 cells before and after exposure to H_2O_2. Nuclear extracts were obtained from FA and complemented FA cells at multiple time points following exposure to H_2O_2. Immunoblot analyses were carried out by using antibodies specific for STAT5a and STAT5b. After H_2O_2 exposure, nuclear STAT5a levels increased in complemented cells but nuclear STAT5b levels did not.

Supplementary Figure 3



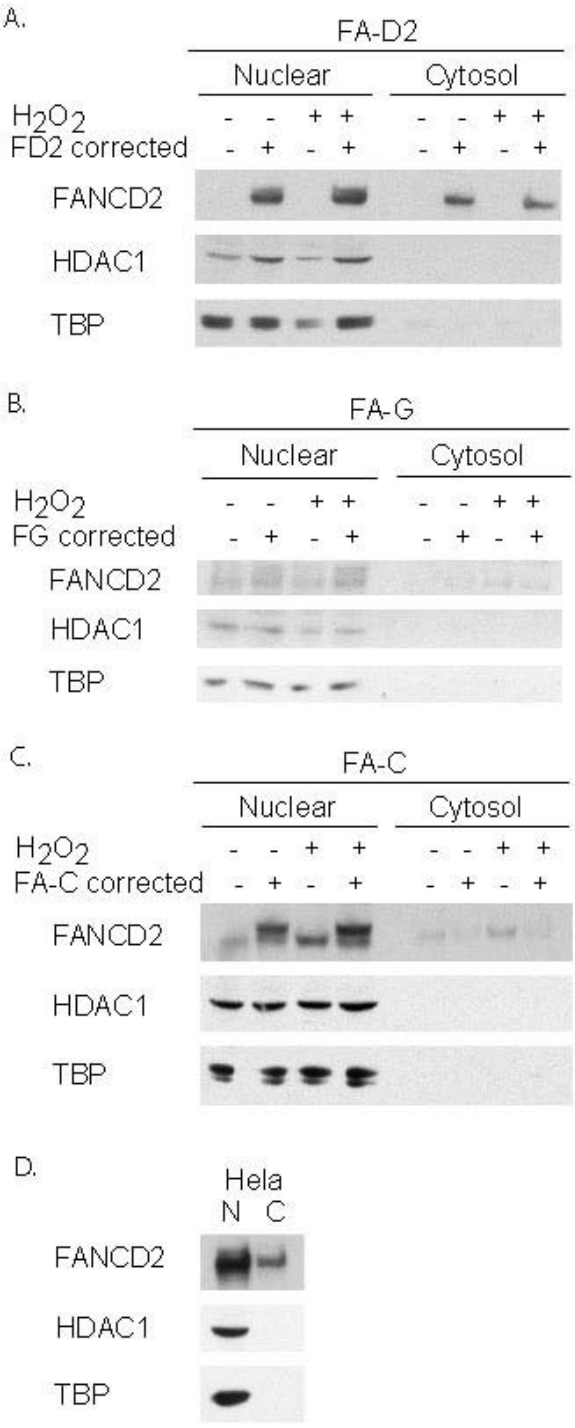
Supplemental Figure 3 Legend. STAT5-P associates with FANCC and FANCD2 in nuclear extracts. (A) FA-D2/D2 cells were examined by deconvolution microscopy after treatment with 300 μM H_2O_2 for 10 min. The deconvoluted images show STAT5-P (green) and FANCC in both nuclear and cytoplasm of complemented FA-D2/D2 cells. DAPI (blue) is a nuclear stain. (B) Co-immunoprecipitation experiments were performed using nuclear lysates. The formation of H_2O_2 -inducible complexes (FANCD2/STAT5-P, FANCD2/STAT5, and FANCC/STAT5) is seen in complemented cells.

Supplementary Figure 4

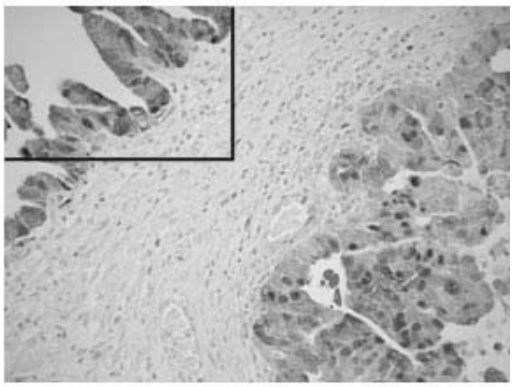


Supplemental Figure 4 Legend. The constitutively active STAT5a mutant forms a complex with FANCC. FA-D2 cells ectopically expressing STAT5aN642H were treated with or without 500 μM H_2O_2 for 1 or 5 min. Co-immunoprecipitation experiments were performed with whole cell lysates. STAT5-P and STAT5 did form complexes with FANCC in STAT5aN642H transfected FA-D2 cells even in the ground state. The amount of total STAT5 was equivalent in the STAT5aN642H expressing and control cells (not shown).

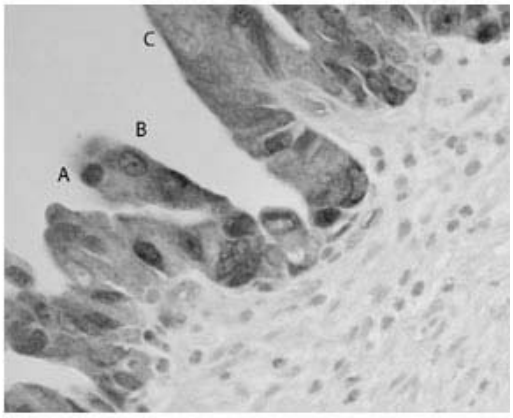
Supplementary Figure 5



E.



F.



Supplemental Figure 5 Legend. Non-ubiquitinylated form of FANCD2 is present in both the nucleus and cytoplasm. Immunoblots were carried out using nuclear extracts obtained from mutant and complemented cells (A, B, and C) or HeLa cells (D) following exposure to H_2O_2 for 10 min. Antibodies specific for FANCD2, and two nucleus-specific proteins, histone deacetylase I (HDAC1) and TATA binding protein TBP were used. The non-ubiquitinylated form of FANCD2 is detected in both the nucleus and cytoplasm, but HDAC1 and TBP are only detected in nuclear extracts. Similar results were observed using two other antibodies specific for FANCD2 (not shown): polyclonal anti-FANCD2 from Santa Cruz Biotechnology (Santa Cruz, CA) and polyclonal anti-FANCD2 provided by Maureen Hoatlin (Oregon Health and Science University, Portland, OR). (E, F) Immunohistochemical staining of an ovarian epithelial carcinoma sample shows both nuclear and cytoplasmic signals in epithelial cells but only rare nuclear positivity in stromal cells. Panel F is a higher magnification of the boxed region of panel E. The cell marked A shows a strong nuclear FANCD2 signal, and the cell marked B and the column of cells marked C reveal largely cytoplasmic signals.

Chapter 4

Summary

In the recent past, the role of FA proteins has been a significant point of controversy between those who believe FA proteins function primarily in a DNA damage response pathway (facilitated by the monoubiquitinylation of FANCD2), and others who believe that the FA proteins are multifunctional and that, in addition to their nuclear DNA-damage-response function, the cytoplasmic signaling functions of FA proteins are of critical importance for hematopoietic cell survival. This second point of view has gained increasing acceptance based on cumulative scientific evidence. For example, cells from FA patients 1) overproduce apoptotic cytokines (such as TNF-α), 2) are hypersensitive to apoptotic factors (such as TNF-α) and 3) are hypo-responsive to survival factors (such as erythropoietin). Chapter 1 of this thesis summarizes the current state of the field. Chapters 2 and 3 describe experimental evidence for novel cytoplasmic signaling pathways, which involve FA proteins, but have little or nothing to do with the DNA damage response (and FANCD2 monoubiquitinylation). These pathways are aberrant in cells from FA patients, and might be key to understanding why FA patients suffer from defective hematopoiesis.

Chapter 2: Mechanisms involved in the over-production of TNF-α in FA-C cells

Transcriptomal analysis of FA bone marrow revealed the over-representation of transcripts that encode proteins involved in ubiquitinylation and the ubiquitin cycle, supporting the theory that a general ubiquitinylation defect exists in FA cells. Using an unbiased proteomics method, we demonstrate in chapter 2 that TLR8 or a protein associated with TLR8 is ubiquitinylated in mutant FA-C lymphoblasts. We hypothesized

that ubiquitinylation in FA cells activated the toll-like receptor pathway, leading to the overproduction of TNF-α. Our findings supported this hypothesis: In FA-C lymphoblasts in the ground state, IRAK-1, IKK and NF-κB are hyperactivated, and TNF-α gene expression is high. We also demonstrate that loss of FANCC by shRNA resulted in exaggerated NF-κB activity in response to TLR8 agonists but did not enhance NF-kB activity in response to agonists with specificity for other TLRs. We conclude that FANCC modulates the activation state of TLR8 and that failure to modulate TLR8 results in TNF-α overproduction. This represents the first report identifying the pathway responsible for this key phenotype, one that was reported over thirty years ago.

Our findings and methods have also created a technical opportunity for follow-up studies designed to identify the mechanism by which FANCC negatively regulates the TLR8 signaling pathway. For example, our group intends to apply proteomic methods to identify FANCC associated proteins in cells before and after exposure to the TLR8 ligand R-848. In addition, having developed the THP-1/shRNA method, it will be possible to identify proteins that are differentially ubiquitinylated *in vivo* in THP-1 cells. Although the *in vitro* ubiquitinylation method led to our discovery of TLR8 as a key candidate for TNF control, a method based on the *in vivo* ubiquitinylation of proteins has several advantages. First, the results are more likely to be physiologically relevant. Second, the use of live cells allows the dynamic changes in ubiquitinylation to be measured that arise in cells exposed to TLR agonists, various forms of extracellular or intracellular stress, or other environmental cues. Technically, the experiments will utilize 2xFLAG tagged ubiquitin (we found that the 6xHis tag was associated with a high-non-specific binding rate). Proteins conjugated to 2xFLAG-ubiquitin can then be purified, digested and

analyzed by 2D capillary LC-MS/MS. Finally, given the role of TNF-α in molecular pathogenesis, TLR8 may be a good therapeutic target. Other studies beyond the scope of this thesis could involve the identification of TLR8 inhibitors. The effects of TLR8 inhibition in hematopoietic cells could be tested directly by transplantation of murine hematopoietic stem cells with retroviral vectors expressing TLR8 shRNA.

Chapter 3: Abnormalities of JAK2 dependent STAT5 activation.

In chapter 3, we show that in normal cells FANCD2, FANCC and FANCG facilitate STAT5 nuclear translocation in response to H_2O_2, EGF and erythropoietin. In normal cells but not in FA cells, H_2O_2 induced formation of multimeric complexes containing STAT5, FANCD2, Hsp90 and FANCC in the cytosol. Because FANCD2 was previously considered to function exclusively in the nucleus, the participation of FANCD2 in a cytosolic signaling pathway was both unexpected and exciting. We expect this discovery to open up new areas of study concerning the role of FA proteins in the cytoplasm, in both normal and mutant cells.

As explained in chapter 3, primary hematopoietic cells from FA patients are difficult to obtain, and hematopoietic cell lines don't respond to defined growth factors. Based on prior work demonstrating robust activation of STAT molecules by H_2O_2 exposure, we reasoned that more widely available fibroblast lines exposed to H_2O_2 might represent convenient surrogates for hematopoietic cells exposed to hematopoietic growth factors. Indeed, having defined aberrant STAT5 activation responses in fibroblasts, we confirmed that the same abnormalities occurred in erythropoietin responsive cells treated

with FANCD2 and FANCC siRNA. We conclude that STAT5 activation requires interactions of STAT5, FANCC, FANCD2 and Hsp90 in both hematopoietic and non-hematopoietic cells and propose that defects of this pathway underly, at least in part, the pathogenesis of bone marrow failure in Fanconi anemia.

As was the case in chapter 2, future studies will focus on (a) further refining the mechanisms by which FANCC and FANCD2 facilitate STAT5 nuclear translocation and (b) evaluating the relevance of the STAT5 pathway in whole animals.

a. We must determine the exact composition of the STAT5 complex that crosses the nuclear membrane. To accomplish this, as was the case in chapter 2, proteomics methods will be used in which FANCC or FANCD2 is immunoprecipitated from stimulated cells and associated proteins are identified by mass spectrometry. The identification of importin-α, importin-β or any nuclear pore complex proteins, for example, would be suggestive of their involvement in the nuclear targeting of STAT5/FANCC/FANCD2/Hsp90 complexes. Alternatively, STAT5 could be immunoprecipitated from induced normal cells (and cells bearing FA mutations for comparison), and associated proteins could then be identified by mass spectrometry.

b. If the marrow failure phenotype is related to STAT5 defects in FA-C cells, transduction of a constitutively active form of STAT5 that does not require interaction with FA molecules (see chapter 3) into murine hematopoietic stem cells could be informative. Specifically these transduced cells would be transplanted into irradiated recipient mice seeking to correct at least some of the marrow defects *in vivo*. Secondly, if STAT5 defects are more important in the

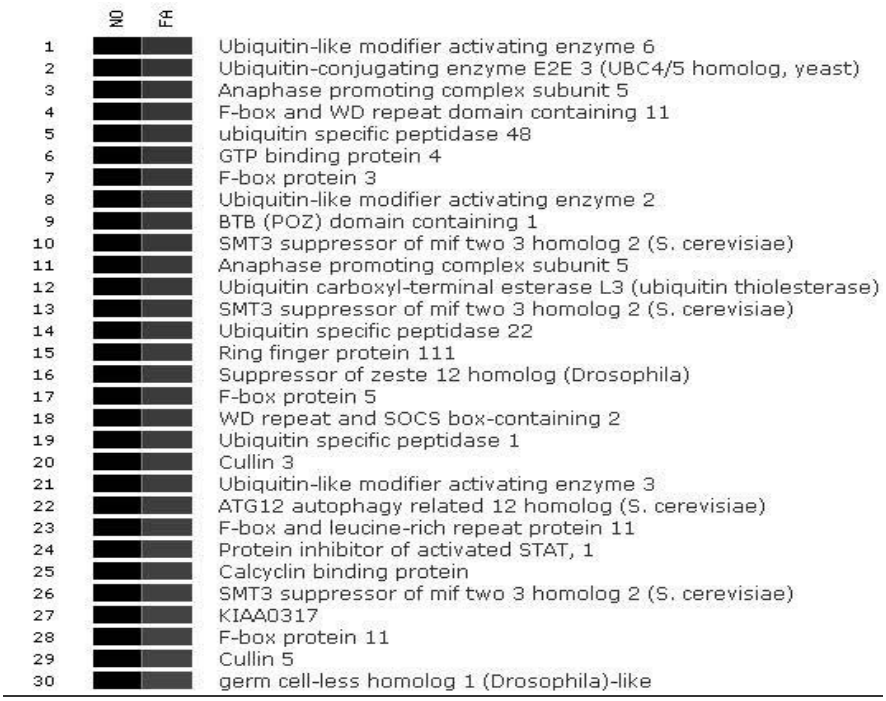
hematopoietic phenotype than the DNA-damage phenotype, transplantation of a FANCC mutant form that corrects the DNA-damage phenotype but not the STAT5 defect should not change the abnormal hematopoietic phenotype. These studies, obviously beyond the scope of this thesis are planned as a component of the program project competitive renewal application to be submitted to the NHLBI in October 2008.

In conclusion, this thesis describes aberrancies of two novel signaling pathways in FA cells, both of which may play an important role in bone marrow failure. We also believe that both pathways are particularly good targets for future therapeutic intervention designed on the one hand to reducing TNF-α release and on the other to facilitate nuclear translocation of STAT5-P in mutant cells.

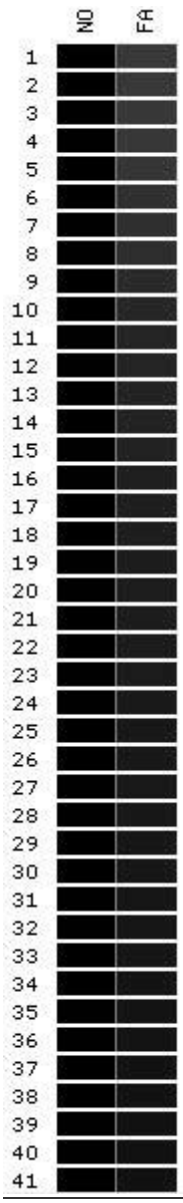
Appendix

Figure 1. Heat Maps. Ontology: Ubiquitin Cycle

Underexpressed in FA Cells



Overexpressed in FA Cells



	NO	FA	Gene
1			G1 to S phase transition 1
2			G1 to S phase transition 1
3			Membrane-associated ring finger (C3HC4) 8
4			Seven in absentia homolog 2 (Drosophila)
5			Ubiquitin-conjugating enzyme E2H (UBC8 homolog, yeast)
6			Ubiquitin-conjugating enzyme E2O
7			Ubiquitin-conjugating enzyme E2H (UBC8 homolog, yeast)
8			Ring finger protein 14
9			Cell division cycle 34 homolog (S. cerevisiae)
10			Ring finger protein 11
11			Ring finger protein 14
12			F-box protein 7
13			Membrane-associated ring finger (C3HC4) 2
14			Tumor necrosis factor, alpha-induced protein 3
15			Zer-1 homolog (C. elegans)
16			Ring finger protein 123
17			Ubiquitin-conjugating enzyme E2B (RAD6 homolog)
18			Ubiquitin-conjugating enzyme E2B (RAD6 homolog)
19			Tumor necrosis factor, alpha-induced protein 3
20			Ubiquitin specific peptidase 12
21			Zer-1 homolog (C. elegans)
22			Ubiquitin specific peptidase 15
23			Glutamate-cysteine ligase, catalytic subunit
24			Ubiquitin protein ligase E3 component n-recognin 2
25			Tripartite motif-containing 23
26			Ubiquitin-conjugating enzyme E2B (RAD6 homolog)
27			Zer-1 homolog (C. elegans)
28			Glutamate-cysteine ligase, catalytic subunit
29			Ubiquitin B
30			Neural precursor cell expressed, developmentally down-regulated 4-like
31			Ubiquitin specific peptidase 15
32			Ubiquitin-conjugating enzyme E2 variant 1
33			Chromosome 12 open reading frame 51
34			Ubiquitin-conjugating enzyme E2 variant 1
35			Tripartite motif-containing 23
36			F-box protein 9
37			Hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC1
38			Fizzy/cell division cycle 20 related 1 (Drosophila)
39			Cullin 4A
40			Ubiquitin specific peptidase 32
41			Fizzy/cell division cycle 20 related 1 (Drosophila)

METHODS (Transcriptome)

Patients: Samples from FA patients were obtained in one of four transplant centers: Oregon Health and Science University, Portland, OR, Hospital de Clinicas, Federal University of Parana, Curitiba, Brazil; Children's Hospital Medical Center, Cincinnati, OH, and University of Minnesota, Minneapolis, MN. Fanconi anemia patients eligible for this study met the following on-study criteria: 1) a positive chromosomal breakage tests on exposure of either lymphoblasts or fibroblasts to either diepoxybutane or mitomycin C or both (140), and 2) a bone marrow cytogenetics study using conventional Giemsa banding methods on metaphase preparations within 18 months of accrual to the study. All patients who had received a stem cell transplant were ineligible. Patients with mosaicism (reversion of the FA mutation in hematopoietic cells (141;142)) were excluded.

Subjects and Marrow Cells. Bone marrow samples were aspirated in heparinized syringes from 11 normal volunteers and 33 FA patients. Low density marrow mononuclear cells were prepared from heparinized bone marrow aspirates using Ficoll-Paque. RNA was prepared immediately and ethanol precipitates stored at -80^{o}C and were shipped on dry ice to Portland.

RNA isolation and processing: Total RNA was extracted by using the RNeasy Micro kit (QIAGEN) and treated with DNase I. Prior to hybridization to a GeneChip genome array, a small portion of the cRNA target was analyzed by microfluidic separation using the RNA 6000 LabChip (Caliper Technologies, Mountain View, CA) and the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). Effective assessment of target quality prior to hybridization on the genome array helps minimize costs associated with failed samples in the expression assay and identified questionable targets early in the analysis process. Two of the 33 FA samples but none of the 11 normal samples were excluded from further analysis on these grounds.

Gene Expression Profiling. Gene expression profiling was performed by the OHSU Affymetrix Microarray Core using GeneChip Human Genome HG-U133A (22,283 probe sets) arrays. The HG-U133A array represents a total of 22,000 transcripts from known genes and EST clusters The array also includes probe sets representing exogenous bacterial and bacteriophage genes (BioB, BioC, BioD, cre) that are used as assay controls, as well as probe sets representing endogenous housekeeping genes (e.g., GAPDH and actin). These housekeeping genes are represented by 3 probe sets designed to assess the representation of full-length transcripts in the final target material (5', M and 3' probe sets). Each RNA sample was labeled and amplified following the recommended protocol in the GeneChip Expression Analysis Technical Manual p.2.1.14-2.1.23. In the first step, mRNA was converted to double-stranded cDNA using Reverse Transcriptase (Invitrogen, Carlsbad, CA) and an oligo-dT primer linked to a T7 RNA polymerase binding site sequence (IDT, Coralville, IA). In the second step, the cDNA was converted

to labeled cRNA (the target) using T7 RNA polymerase in the presence of biotinylated UTP and CTP (Enzo, Farmingdale, NY). This step resulted in a linear amplification of the labeled material. The target was fragmented to produce a uniform distribution of short cRNAs. Fragmented targets were combined with hybridization control oligomer (Affymetrix, Santa Clara, CA) and control cRNAs (1.5 pM BioB, 5.0 pM Bioc, 25 pM BioD and 100 pM cre; Affymetrix, Santa Clara, CA), in hybridization buffer and applied to the array which was packaged in a self-contained cartridge. The arrays were hybridized in a GeneChip Hybridization Oven 640 (Affymetrix, Santa Clara, CA) for 16 hours at 45°C, followed by washing, staining with streptavidin-phycoerythrin (SAPE; Molecular Probes, Eugene, OR), signal amplification with biotinylated anti-streptavidin antibody (Vector Labs, Burlingame, CA), and a final staining with SAPE. The staining and washing was performed using two GeneChip Fluidic Stations (Affymetrix, Santa Clara, CA). The distribution of fluorescent material on the processed array was determined using the GeneArray laser scanner (Affymetrix, Santa Clara, CA).

The Microarray Analysis Suite Version 5 (MAS 5.0) was used for image analysis, signal quantification and normalization. The data were normalized using the MAS 5.0 global scaling method with the target intensity of 325. The sample performance was evaluated using QC/QA parameters including: background, noise (Q), average signal intensity, and ratio of signal intensities for probe sets representing the 5’ and 3’ ends of actin and GAPDH transcripts. Targets that did not meet pre-determined thresholds were remade whenever possible or discarded from further analysis. In addition, each array was

visually inspected for scratches and non-uniform intensity patterns that may affect chip performance.

Statistical Analysis. Raw expression data was normalized with Robust Multi-array Averaging (RMA). Initially the data were examined visually using the box-plot of each sample, hierarchical clustering of samples, multidimensional scaling (MDS) of samples based on all 22,283 gene transcripts. After the initial visual assessment of the data, we performed a two-sample t-test for each gene separately using GeneSifter and P values were adjusted for multiple comparisons using the False Discovery Rate (FDR) of Benjamini and Hochberg (143). Ontological analyses were performed using the Gene Ontology functions of GeneSifter. All statistical analyses were performed using Statistical Analysis System (SAS) (144). Data visualization was performed using STATISTICA, GeneSifter, and Partek.

Table 1. Summary of Putative Ubiquitinylated Proteins in Mutant FA-C Cells.

Protein Name	Accession Number	# of Identified Peptides	Probability of best peptide	Molecular Function
splicing factor proline/glutamine rich	NP_005057	2	0.99	RNA splicing
ubiquitin-specific protease otubain 1	NP_060140	2	0.99	Ubiquitin cycle
splicing factor 3b, subunit 4	NP_005841	1	0.99	RNA splicing
H2B histone family, member S	NP_059141	1	0.99	Nucleosome assembly
ubiquitin-conjugating enzyme E2L 3 isoform 1	NP_003338	1	0.99	Ubiquitin cycle
tubulin, alpha 1a	NP_006000	1	0.98	Microtubules
DEAD box polypeptide 42 protein	NP_031398	3	0.98	RNA helicase
histone cluster 1, H2ai	NP_003500	1	0.97	Nucleosome component
ribosomal protein S4, X-linked X isoform	NP_000998	2	0.97	Protein translation
ribosomal protein S18	NP_072045	1	0.97	Protein translation
tubulin, beta	NP_821133	1	0.95	Microtubules
ribosomal protein S15	NP_001009	1	0.92	Protein translation
paraspeckle protein 1	NP_060752	1	0.91	Transcription
ubiquitin-activating enzyme E1	NP_003325	2	0.89	Ubiquitin cycle
splicing factor 3a, subunit 1	NP_005868	2	0.87	RNA splicing
ribosomal protein S4, Y-linked 2	NP_620413	1	0.87	Protein translation
gamma-aminobutyric acid (GABA) A receptor, beta 2 isoform 2	NP_000804	1	0.87	Ion transport
inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	NP_001547	4	0.86	Regulation of NF-kB

coiled-coil-helix-coiled-coil-helix domain containing 2	NP_057223	1	0.86	
solute carrier family 26, member 1 isoform a	NP_998778	1	0.85	Sulfate transport
chromosome 16 open reading frame 7	NP_004904	1	0.85	Transporter activity
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 15	NP_001349	1	0.84	RNA helicase, RNA splicing
heterogeneous nuclear ribonucleoprotein L	NP_001524	3	0.83	RNA processing
splicing factor 3b, subunit 1 isoform 1	NP_036565	3	0.82	RNA splicing
rotatin	NP_775901	2	0.80	Early developmental processes
tubulin, alpha 4a	NP_005991	1	0.80	Microtubules
hypothetical protein FLJ35709	NP_775860	2	0.79	
H2A histone family, member X	NP_002096	1	0.78	Nucleosome assembly
cyclin K isoform 2	NP_003849	1	0.77	Transcription
zinc finger protein 650	NP_742067	3	0.76	Ubiquitin cycle
transforming, acidic coiled-coil containing protein 2 isoform a	NP_996744	4	0.75	Centrosomal Microtubules
chromosome 1 open reading frame 9 protein isoform 1	NP_055098	2	0.75	
similar to p33 ringo	XP_370631	1	0.75	
heat shock 70kDa protein 5; GRP78	NP_005338	1	0.75	Protein folding/ complex assembly
A-kinase anchor protein 9 isoform 1	NP_671700	12	0.74	Scaffolding protein
cystathionase isoform 1	NP_001893	2	0.73	Amino acid metabolic process
MDN1, midasin homolog	NP_055426	13	0.72	Protein folding/ complex assembly
multiple inositol polyphosphate histidine phosphatase, 1	NP_004888	2	0.72	Protein dephosphorylation
bone morphogenetic protein 7 precursor	NP_001710	1	0.70	BMP signaling pathway
olfactomedin-like 2A	NP_872293	1	0.70	

desmoglein 1 preproprotein	NP_001933	1	0.69	Cell-cell adhesion
NIMA (never in mitosis gene a)-related kinase 10 isoform 2	NP_689747	4	0.69	Protein phosphorylation
protein tyrosine phosphatase, non-receptor type 13 isoform 2	NP_006255	6	0.67	Protein dephosphorylation
poly A binding protein, cytoplasmic 4	NP_003810	2	0.67	RNA processing
lamin A/C isoform 2	NP_005563	1	0.67	Nuclear lamina component
notch1 preproprotein	NP_060087	2	0.66	Notch signaling pathway
neuron navigator 3	NP_055718	11	0.65	
ribosomal protein L19	NP_000972	2	0.65	Translation
transmembrane protein 49	NP_112200	1	0.65	Vacuole formation, cell-cell adhesion
eukaryotic translation initiation factor 3, subunit 8	NP_003743	2	0.64	Translation
CREB regulated transcription coactivator 2	NP_859066	1	0.64	Transcription regulation
syntaxin 8	NP_004844	1	0.64	Vesicle trafficking
lamin A/C isoform 3	NP_733822	1	0.63	Nuclear lamina component
calcium homeostasis endoplasmic reticulum protein	NP_006378	2	0.62	Calcium homeostasis
SPARC-like 1	NP_004675	1	0.62	
RNA binding motif protein 25	NP_067062	3	0.61	mRNA splicing
similar to MGC52970 protein	XP_377586	1	0.61	
hypermethylated in cancer 1	NP_006488	1	0.61	Regulation of transcription
connective tissue growth factor	NP_001892	5	0.60	
acetylserotonin O-methyltransferase-like	NP_004183	1	0.59	melatonin biosynthetic process
PQQ repeat and WD repeat domain containing	NP_775750	3	0.59	
tubulin tyrosine ligase-like family, member 5	NP_055887	5	0.59	

sulfite oxidase	NP_000447	1	0.57	Sulfite oxidase activity
solute carrier family 35, member A5	NP_060415	2	0.57	
radical S-adenosyl methionine and flavodoxin domains 1	NP_060734	4	0.55	wybutosine biosynthesis pathway
solute carrier family 44, member 3 isoform 2	NP_689582	4	0.55	
toll-like receptor 8	NP_619542	3	0.55	Toll-like receptor pathway
aryl hydrocarbon receptor nuclear translocator-like 2	NP_064568	2	0.55	Transcription factor activity
BMS1-like, ribosome assembly protein	NP_055568	2	0.55	Ribosome assembly
interferon induced transmembrane protein 5	NP_001020 466	1	0.55	
postreplication repair protein hRAD18p	NP_064550	2	0.54	Ubiquitin cycle, DNA repair
protein phosphatase 1, regulatory (inhibitor) subunit 13B	NP_056131	1	0.54	
p53-associated parkin-like cytoplasmic protein	NP_055904	2	0.53	
suppression of tumorigenicity 5 isoform 1	NP_005409	1	0.53	
parvin, alpha	NP_060692	1	0.53	
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex	NP_004991	1	0.53	
myeloid/lymphoid or mixed-lineage leukemia 2	NP_003473	15	0.52	
mannosidase, alpha, class 1A, member 2	NP_006690	6	0.52	
hypothetical protein BC001339	NP_612493	2	0.52	
semenogelin I	NP_002998	1	0.52	
similar to RIKEN cDNA 2900024C23	XP_371269	1	0.52	
protein tyrosine phosphatase, receptor type, C isoform 1 precursor	NP_002829	4	0.52	
gamma-aminobutyric acid (GABA) B receptor 1 isoform a precursor	NP_001461	4	0.51	
odorant-binding protein 2B	NP_055396	1	0.51	

MyoD family inhibitor	NP_005577	1	0.51	
ryanodine receptor 2	NP_001026	12	0.50	
centrosomal protein 1	NP_008949	2	0.50	
proteasome 26S ATPase subunit 1	NP_002793	1	0.50	

Table 2: Summary of Putative Ubiquitinylated Proteins in Corrected FA-C/C Cells

Protein Name	Accession Number	# of Identified Peptides	Probability of best peptide	Molecular Function
ribosomal protein S15	NP_001009	3	0.99	Translation
proteasome 26S non-ATPase subunit 7	NP_002802	1	0.99	Proteasome complex
splicing factor proline/glutamine rich (polypyrimi	NP_005057	1	0.99	RNA splicing
DNA topoisomerase I	NP_003277	1	0.99	DNA topoisomerase activity
ets variant gene 6	NP_001978	1	0.99	Transcription factor activity
ubiquitin and ribosomal protein S27a precursor	NP_002945	9	0.98	Protein ubiquitinylation
ring finger protein 20	NP_062538	1	0.96	Protein ubiquitinylation
sequestosome 1	NP_003891	1	0.94	Ubiquitin-binding, IKK/NF-kB cascade
paraspeckle protein 1	NP_060752	2	0.93	Transcription regulation
similar to bA92K2.2 (similar to ubiquitin)	XP_210488	2	0.93	Ubiquitin homolog
M-phase phosphoprotein 9	NP_073619	1	0.93	
ribosomal protein L19	NP_000972	1	0.93	Translation
ribosomal protein S4, X-linked X isoform	NP_000998	1	0.92	Translation
growth factor receptor-bound protein 2	NP_002077	1	0.92	EGFR signaling pathway
ribosomal protein L10	NP_006004	1	0.91	Translation
non-POU domain containing, octamer-binding	NP_031389	3	0.9	RNA splicing

ribosomal protein S4, Y-linked 2	NP_620413	2	0.89	
ubiquitin specific protease 28	NP_065937	1	0.89	
hypothetical protein LOC285989	NP_998768	1	0.89	
similar to tripartite motif-containing 51	XP_374917	2	0.88	
nuclear receptor subfamily 2, group C, member1	NP_003288	1	0.88	
ribosomal protein S11	NP_001006	1	0.87	
interleukin 17B precursor	NP_055258	1	0.87	
integrin alpha 5 precursor	NP_002196	1	0.87	
interleukin 6 signal transducer isoform 1 precurs	NP_002175	1	0.87	
UV excision repair protein RAD23 homolog A	NP_005044	1	0.87	
cyclin K	NP_003849	1	0.87	
EGF-like-domain, multiple 3	XP_031401	3	0.86	
small conductance calcium-activated potassiumcha	NP_002239	2	0.86	
hypothetical protein XP_211028	XP_211028	4	0.85	
cullin 7	NP_055595	3	0.85	
zinc finger protein 200	NP_003445	1	0.85	
lymphocyte-specific protein tyrosine kinase	NP_005347	1	0.85	
similar to Centromeric protein E	XP_062871	10	0.84	
hypothetical protein XP_378667	XP_378667	1	0.84	
ribosomal protein L26	NP_000978	2	0.83	
zinc finger, SWIM domain containing 2	NP_872327	2	0.83	

hypothetical protein FLJ20276	NP_060208	1	0.83	
WD repeat domain 20 isoform 2	NP_653175	1	0.83	
solute carrier family 17 (sodium phosphate), membe	NP_005826	3	0.81	
thyroid stimulating hormone receptor	NP_000360	1	0.81	
angiotensin I converting enzyme isoform 1 precurso	NP_000780	1	0.81	
modulator of apoptosis 1	NP_071434	1	0.81	
protein kinase, lysine deficient 1	NP_061852	6	0.79	
hypothetical protein XP_296173	XP_296173	2	0.79	
low density lipoprotein receptor-related protein	XP_035037	1	0.79	
F-box protein 16	NP_758954	2	0.78	
breast cancer 2, early onset	NP_000050	14	0.77	
hypothetical protein FLJ33868	NP_689787	2	0.77	
mitogen-activated protein kinase kinase 2	NP_109587	1	0.77	
utrophin	NP_009055	15	0.75	
AMPK-related protein kinase 5	XP_374996	4	0.75	
similar to seven transmembrane helix receptor	XP_293581	3	0.75	
similar to KIAA1201 protein	XP_370660	2	0.75	
similar to LanC lantibiotic synthetase component	XP_374439	1	0.75	
ribosomal protein S26	NP_001020	1	0.75	
centromere/kinetochore protein zw10 homolog	NP_004715	5	0.74	
zinc finger protein 179	NP_009079	4	0.72	

ataxia telangiectasia mutated protein isoform1	NP_000042	2	0.72	
secretin preproprotein	NP_068739	2	0.72	
doublesex and mab-3 related transcription factor	NP_870987	4	0.71	
membrane frizzled-related protein	NP_113621	2	0.71	
BTB and CNC homology 1, basic leucine zippertran	NP_068585	3	0.7	
hypothetical protein FLJ30525	NP_653185	1	0.7	
cadherin 3, type 1 preproprotein	NP_001784	1	0.69	
kinase insert domain receptor (a type III recepto	NP_002244	7	0.68	
DKFZP434F2021 protein	NP_056227	2	0.68	
CUB and Sushi multiple domains 3 isoform 1	NP_937756	7	0.67	
KIAA0853	NP_055885	6	0.67	
euchromatic histone methyltransferase 1	NP_079033	5	0.67	
RAD51-like 1 isoform 1	NP_002868	3	0.67	
apolipoprotein C-III precursor	NP_000031	1	0.67	
requiem	NP_006259	1	0.67	
hemicentin	NP_114141	4	0.66	
ceruloplasmin (ferroxidase)	NP_000087	2	0.66	
GRB2-related adaptor protein 2	NP_004801	1	0.66	
centromere protein F (350/400kD)	NP_057427	19	0.63	
similar to RING finger protein 18 (Testis-specifi	XP_208043	1	0.63	
hyperpolarization activated cyclic	NP_066550	3	0.62	

nucleotide-gat				
FLJ22794 protein	NP_071357	2	0.62	
hypothetical protein XP_297622	XP_297622	3	0.61	
lectin, mannose-binding, 1 precursor	NP_005561	1	0.6	
similar to Tripartite motif protein 16 (Estrogen-	XP_085722	5	0.59	
hypothetical protein DKFZp761B107	NP_775734	1	0.59	
matrix metalloproteinase 8 preproprotein	NP_002415	2	0.58	
plasma carboxypeptidase B2 isoform a preproprotein	NP_001863	1	0.58	
KIAA1554 protein	XP_290768	12	0.57	
breast cancer 2, early onset	NP_000050	9	0.57	
ectonucleotide pyrophosphatase/phosphodiesterase	NP_848638	2	0.57	
nucleolar protein family A, member 2	NP_060308	1	0.57	
septin 1	NP_443070	2	0.56	
MAWD binding protein	NP_071412	1	0.56	
F-box and leucine-rich repeat protein 11	NP_036440	7	0.55	
DEAD box polypeptide 42 protein	NP_031398	1	0.55	
dipeptidylpeptidase 9	NP_631898	1	0.54	
hypothetical protein FLJ20272	NP_060205	1	0.53	
ribosomal protein L24	NP_000977	1	0.52	
zinc finger protein 500	XP_085507	4	0.5	
kelch-like 6	NP_569713	1	0.5	

Table 3. Summary of Proteins with Identified Ubiquitinylation Sites

Mutant		Corrected	
Protein Name	**Molecular Function**	**Protein Name**	**Molecular Function**
Protein tyrosine phosphatase, non-receptor type 13	Protein de-phosphorylation	Matrix metalloproteinase 1 preprotein	Peptidolysis
Ankyrin 3 isoform 1	Cytoskeletal anchoring	Glutamate receptor KA1 precursor	Glutamate signaling
Hypothetical protein FLJ34969		Hypothetical protein DKFZp761G058	
Nuclear factor (erythroid-derived 2)-like 1	Transcription factor activity	FLJ11171	
Zinc finger protein 509		Solute carrier family 17	
PiggyBac transposable element		Ubiquitin-protein isopeptide ligase (E3)	
Ubiquitin specific protease 53		Cas-Br-M (murine) ecotropic retroviral transforming sequence b	
KIAA1726		similar to bA442O18.2 (novel protein)	
LOC126248		Phospholipase C, beta 2	
FLJ21069		SH3-domain binding protein 5 (BTK-associated) isoform a	
KIAA0478		Similar to ret finger protein-like	
Tubulin, beta polypeptide 4, member Q			
Apolipoprotein B			
WD repeat domain 6 protein isoform 1			
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit E	Proton transport		
DnaJ (Hsp40) homolog, subfamily C, member 6			
restin isoform a			

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