Functional Characterization of the Fanconi Anemia Group C (FANCC) Protein During Apoptosis in Factor-Dependent Hematopoietic Progenitor Cells.

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Molecular and Medical Genetics
University of Toronto

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Functional Characterization of the Fanconi Anemia Group C (FANCC) Protein During Apoptosis in Factor-Dependent Hematopoietic Progenitor Cells.

Doctor of Philosophy (2001)

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Abstract

The rare autosomal recessive disease Fanconi Anemia (FA) is characterized by progressive bone marrow failure, congenital malformations and elevated cancer susceptibility. Cells derived from FA patients exhibit chromosomal instability, hypersensitivity to DNA cross-linking agents and oxygen and an increased predisposition to undergo apoptosis. FA is genetically heterogeneous, with at least seven complementation groups (FA-A through FA-G). The gene defective in FA-C patients (FANCC) lacks significant homology with other known genes and its function is poorly defined. Here I demonstrate that the FANCC gene plays an essential role in ensuring the survival of hematopoietic cells following growth factor deprivation. Using retroviral-mediated gene transfer, I present evidence that overexpression of the FANCC gene in the interleukin-3 (IL-3) dependent hematopoietic progenitor cells lines, 32D and MO7e, suppresses apoptosis induced by IL-3 withdrawal. Overexpression of FANCC in both
cell lines promotes increased viability rather than proliferation after growth factor deprivation.

In order to elucidate the role of the FANCC protein in IL-3 deprivation-induced apoptosis, a 25 kDa FANCC-interacting protein was purified from 32D cell lysates and identified as Glutathione S-Transferase P1-1 (GSTP1). Overexpression of GSTP1 by retroviral mediated gene transfer significantly delays the onset of apoptosis in 32D cells following IL-3 deprivation. Coexpression of both FANCC and GSTP1 in 32D cells confers dramatically higher resistance to apoptosis compared to cells expressing FANCC or GSTP1 alone. GST activity is significantly elevated in 32D cells overexpressing FANCC, particularly after IL-3 withdrawal. Furthermore, overexpression of either FANCC or GSTP1 prevents the loss of the endogenous antioxidant glutathione (GSH) during apoptosis. However, depletion of GSH abolishes the ability of GSTP1 but not FANCC to inhibit apoptosis. FANCC functions by inhibiting the oxidation of GSTP1 during apoptosis, even in the absence of GSH. These findings identify GSTP1, an enzyme involved in the detoxification of xenobiotics and by-products produced during oxidative stress, as a redox sensitive anti-apoptotic protein regulated by FANCC. Moreover, the prevention of disulfide bond formation by FANCC reveals a new pathway for redox regulation of proteins.
“Nothing in life comes easy. 
But if it does, it’s probably not worthwhile.”

Edward H. Edge
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>apoptotic protease activating factor-1</td>
</tr>
<tr>
<td>BER</td>
<td>base excision repair</td>
</tr>
<tr>
<td>BSO</td>
<td>buthionine-[S,R]-sulfoximine</td>
</tr>
<tr>
<td>cdc2</td>
<td>cyclin dependent kinase 2</td>
</tr>
<tr>
<td>cdc25</td>
<td>cell division cycle 25</td>
</tr>
<tr>
<td>CDNB</td>
<td>1-chloro-2,4,-dinitrobenzene</td>
</tr>
<tr>
<td>DEB</td>
<td>diepoxybutane</td>
</tr>
<tr>
<td>DSB</td>
<td>double-strand break</td>
</tr>
<tr>
<td>EA</td>
<td>ethacrynic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular receptor kinase</td>
</tr>
<tr>
<td>FA</td>
<td>Fanconi anemia</td>
</tr>
<tr>
<td>FA-C</td>
<td>Fanconi anemia complementation group C</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>FANCC</td>
<td>Fanconi anemia complementation group C gene</td>
</tr>
<tr>
<td>FANCC</td>
<td>Fanconi anemia complementation group C protein</td>
</tr>
<tr>
<td>FAZF</td>
<td>Fanconi anemia zinc finger</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GPX</td>
<td>glutathione peroxidase</td>
</tr>
<tr>
<td>GRX</td>
<td>glutatredoxin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
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</tr>
<tr>
<td>GRP94</td>
<td>glucose-regulated protein 94</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>glutathione disulfide</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>GSTP1</td>
<td>glutathione S-transferase P1-1 isozyme</td>
</tr>
<tr>
<td>HPC</td>
<td>hematopoietic progenitor cells</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>IκB</td>
<td>inhibitor of κB</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitor of κ-kinase</td>
</tr>
<tr>
<td>IL-3</td>
<td>interleukin-3</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun N-terminal kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MKK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MMC</td>
<td>mitomycin C</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>NER</td>
<td>nucleotide excision repair</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>NHEJ</td>
<td>nonhomologous end-joining</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>superoxide anion</td>
</tr>
<tr>
<td>ODN</td>
<td>oligodeoxynucleotide</td>
</tr>
<tr>
<td>PARP</td>
<td>poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PDI</td>
<td>protein disulfide isomerase</td>
</tr>
</tbody>
</table>
PICOT protein kinase C-interacting cousin of thioredoxin
P450-RED NADPH cytochrome P450 reductase
ROS reactive oxygen species
SOD superoxide dismutase
STAT signal transducers and activators of transcription
TNF tumor necrosis factor
TRX thioredoxin
TUNEL terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
8OhdG 8-hydroxy-2’-deoxyguanine
$\Delta \Psi_m$ loss of mitochondrial transmembrane potential
Chapter 1

Introduction
1.1. Recognition of an inherited blood disorder with associated birth defects as Fanconi Anemia

In 1927, Guido Fanconi described a family in which three brothers, all between 5 and 7 years of age, displayed pancytopenia and various birth defects (1). Subsequent reports revealed a consistent association of progressive aplastic anemia with congenital abnormalities involving the skeleton and skin, which formed a recognizable syndrome. In 1931, Naegeli proposed the name Fanconi Anemia (FA) to describe patients with pancytopenia, congenital physical anomalies and familial occurrence. Subsequently it was recognized that the FA phenotype is extremely variable, making diagnosis difficult on the basis of clinical manifestations alone. An objective test was necessary that could be used in conjunction with clinical data to confirm the diagnosis of FA. In 1964, Schroeder and colleagues discovered that cells derived from FA patients displayed higher levels of spontaneous chromosomal aberrations when compared to cells derived from unaffected individuals (2). This cellular phenotype of FA cells is even more pronounced following exposure to DNA crosslinking agents such as diepoxybutane (DEB) or mitomycin C (MMC). The hypersensitivity of FA cells to DNA cross-linking agents is now recognized as a unique cellular marker for the disorder, and is also an essential diagnostic tool for the disease (3-7).

1.1.1. Clinical Features

More than 800 cases of FA have now been reported in varying detail in the literature (8) since Fanconi first recognized this disease as an inherited disorder. Most of the documented cases, particularly the earlier ones, were made when aplastic anemia developed in individuals with characteristic physical abnormalities; thus these reports are undoubtedly biased toward the most severe clinical cases. More recently it has been recognized that the FA phenotype is so variable that diagnosis on the basis of clinical features alone is difficult and often unreliable (9, 10). In order to study the full spectrum of clinical manifestations of the disease, The International Fanconi Anemia Registry (IFAR) was established at The Rockefeller University in 1982 to collect clinical, genetic and hematologic information from a large number of FA patients. The information derived from this database, has been reported in the literature in order to facilitate earlier diagnosis of FA and to increase
clinicians’ awareness of the complete phenotypic spectrum of the syndrome (11-14). Due to the extreme variability in the clinical phenotype, the diagnosis of FA now depends on the finding of increased chromosomal breakage after incubation of the patient’s cells with clastogenic crosslinking agents such as DEB (11). DEB testing is also used for prenatal diagnosis of FA (15).

1.1.2. Congenital Malformations

FA patients can exhibit a wide range of developmental abnormalities. Congenital malformations observed in FA can involve one or more systems including the skeletal, skin, genital, renal, gastrointestinal, ocular, auditory and central nervous systems (9). However, approximately one-third of FA patients do not manifest any congenital abnormalities (13). FA is associated with abnormal growth parameters both prenatally and postnatally and FA patients are typically short in stature (16). Other morphometric abnormalities involve mainly the head and face in the form of microcephaly, micrognathia, and microstomia, which produces a characteristic elfin appearance of children with FA (8). The most frequently encountered skeletal anomalies affect the radial ray and thumb. The thumb may be absent, hypoplastic, misshapen, supernumerary, bifid, or misplaced (17, 18). Polydactyly may be seen and less commonly, syndactyly. In a minority of FA patients, the radius is either absent or hypoplastic and is always associated with the absence or hypoplasia of the corresponding thumb (17, 19). Interestingly, the radial ray abnormalities can be bilateral or unilateral, however, even patients with bilateral abnormalities usually exhibit asymmetry, having limbs with different specific anomalies (11). Approximately 20% of FA patients suffer from various other skeletal defects including congenital hip, vertebral and rib abnormalities as well as spina bifida (18).

Skin pigmentation anomalies due to increased or decreased melanin deposition are the most common physical feature in FA patients (13). Café-au-lait patches of hyperpigmentation are often seen on the trunk, neck and groin area. Hypopigmentation is also observed and consists of smaller vitiligo-like patches, which may occur alone or in conjunction with café-au-lait spots (20). The skin appears to be otherwise normal and does not display increased sensitivity to ultraviolet light. However, the skin is more sensitive to irradiation and alkylating agents (21).
Genitourinary tract defects are also common in FA patients, mainly in the form of ectopic, pelvic or horseshoe kidneys and double ureters (8, 18). Hypogenitalia is frequently found in up to 51% of patients of both sexes (9). In males, hypoplastic and undescended testes are common, while in females, sparse menses, infantile uterus and hypoplastic ovaries are frequently observed. Gastrointestinal anomalies consist of oesophageal, duodenal or jejunal atresia and imperforate anus (8). Various other abnormalities involving the eye (microphthalmia and strabismus) and ear (deafness and structural deformities) are also associated with FA (8). Central nervous system malformations have been found in 7% of FA patients including hydrocephalus and other structural anomalies, which may be associated with an increased incidence of learning disabilities and/or mental retardation (13, 22). The wide range of congenital malformations associated with FA suggests that the gene(s) defective in FA patients are involved in embryonic development.

1.1.3. Hematological Aspects

Undoubtedly the most severe defects associated with FA involve the hematopoietic system. Hematological abnormalities are usually not detected at birth. Pancytopenia develops slowly, occurring mostly between the ages of 5 and 10 with a mean age of onset of approximately 7 years (14). Statistical analysis has shown that 98% of all FA patients diagnosed with FA will develop hematological problems during their lifetime, although the age of onset can be highly variable (14). The progression of hematological complications is usually fatal with a predicted median survival age of 16 years (10). The presenting cytopenia is usually thrombocytopenia, with granulocytopenia and red cell anemia developing more slowly (10, 23). Eventually, severe pancytopenia develops in most cases. Erythrocytes usually appear large or macrocytic, even in FA patients with normal blood counts (pre-anemic or treatment-responsive) (8). In addition, FA erythrocytes exhibit an irregular shape and surface blebbing typical of acanthocytes (24, 25). FA patients have "stress erythropoiesis" with the production of erythrocytes with fetal characteristics including increased fetal hemoglobin and i antigen production (26, 27). The reduction in white blood cells involves mainly granulocytes whereas lymphoid cells appear to be unaffected until the latter stages of the disease (28). Quite frequently FA patients die of infection and some studies have shown deficient T-cell function indicating that FA patients
may be immunodeficient (29, 30). This hypothesis is supported by the observation that FA patients have frequent relapses of hematological problems after viral infections (31).

As pancytopenia develops, the bone marrow becomes increasingly hypocellular and fatty with few hematopoietic elements (8). *In vitro* studies of hematopoietic cells from FA patients revealed that multipotent or erythroid precursor cells (colony-forming unit-granulocyte, erythroid, monocyte, macrophage [CFU-GEMM], CFU-granulocyte-macrophage [CFU-GM] and burst-forming unit erythroid [BFU-E]) were decreased or absent in FA patients with aplastic anemia, even in a small number of patients without signs of anemia (32-36). At present, bone marrow transplantation has been the only long-term treatment for bone marrow failure in FA patients (37). The *in vitro* data, as well as cures with bone marrow transplantation suggest that the principal defect in FA resides in the pluripotent hematopoietic stem cell compartment. Cultured FA bone marrow cells failed to form more colonies in the presence of Stem cell factor (SCF) in one study, prompting the suggestion that FA cells have an intrinsic inability to respond to cytokines (35). However, other studies showed that FA marrow and blood progenitor cells did indeed respond to SCF and granulocyte-macrophage colony-stimulating factor (GM-CSF) *in vitro* and *in vivo* (38-41). Although FA marrow cells do respond to stimulatory cytokines, the effect is highly variable and is not an effective clinical treatment. Several studies have demonstrated that FA marrow cells produce low levels of interleukin-6 (IL-6) and GM-CSF *in vitro* compared to normal marrow cells under the same culture conditions (42-44). However, it is uncertain if these low cytokine levels relate to the primary defect of FA or are merely a secondary effect.

1.1.4. Cancer predisposition

FA patients are highly predisposed to developing cancer. Acute myeloid leukemia (AML) is the most common neoplasm that develops, although the risk of multiple forms of cancer is generally increased in FA (45). The risk of AML in FA children is substantial and is estimated to be 15,000 times greater than that of the general population (12). Leukemia occurs in approximately 10% of FA patients (46). Preleukemic, myelodysplastic and/or cytogenic clonal abnormalities are frequently observed in FA marrow biopsies (12). The vast majority of leukemias are myeloid in origin, suggesting that the molecular mechanisms
defective in FA cells may be of particular importance in normal myeloid development. Although FA patients have the highest risk of developing AML they are also at risk of developing solid tumors involving the liver, oropharynx, gastrointestinal tract and gynecological systems (45, 47, 48). Patients that develop solid tumors tend to be older than the average FA patient but far younger than the average age at which these types of tumors are seen in individuals without FA (45). Thus, patients that fail to succumb to earlier complications like anemia, are at a higher risk of dying of cancer.

1.2. Cellular Features of FA

1.2.1. DNA Repair and Genomic Instability

The human genome is susceptible to attack by a wide array of DNA-damaging agents of both exogenous and endogenous origin. DNA repair enzymes continuously monitor chromosomes to excise and replace damaged nucleotide residues, thereby counteracting potentially mutagenic and cytotoxic genomic events (49). DNA damage can include lesions such as pyrimidine dimers, single- and double-stranded breaks, deletions, base changes, adducts, and cross-links (50). At the cellular level, DNA lesions interfere with essential processes including transcription and DNA replication resulting in cell-cycle arrest, genomic instability and cell death. DNA lesions are repaired by specific sets of enzymes that function through multiple pathways including direct reversal of the damage, excision of the lesion and by recombination and rejoining pathways that specifically repair DNA double-strand breaks (DSB) (50) (49, 51). In addition, tolerance mechanisms have evolved that allow DNA polymerase to bypass certain lesions, reinitiate replication of DNA downstream, and allow post-replicative repair of the gap to occur after DNA synthesis (50). A number of human autosomal recessive disorders exist that are directly attributed to defects in the cellular response to DNA damage. For example, xeroderma pigmentosa (XP), Cockayne’s syndrome (CS) and trichothiodystrophy (TTD) are caused by mutations in nucleotide excision repair (NER) genes (51). In addition, the genes mutated in ataxia telangiectasia (AT) and Nijmegen breakage syndrome and their respective protein products are implicated in the recognition of and response to DNA DSB and the coordination of
nonhomologous end-joining (NHEJ) repair (52). All of these disorders are associated with chromosomal instability and elevated cancer predisposition.

Although FA exhibits features of typical DNA repair disorders, the exact cellular defect is unknown. Initial work by Sasaki and Tonamura revealed that FA cells are sensitive to MMC but are not sensitive to the closely related compound decarbamoyl mitomycin C (DCMMC) (3). MMC is a bifunctional alkylating agent whereas DCMMC is a monofunctional alkylating agent. MMC can generate interstrand and intrastrand DNA cross-links whereas DCMMC only generates mono-adducts. Clinical use of cyclophosphamide (also a cross-linking agent) as a conditioning agent for bone marrow transplantation, revealed that FA patients were also hypersensitive to this compound (53). Cytogenetic studies of cells derived from FA patients revealed an increase in the number of spontaneous chromosomal breaks, gaps, and sister chromatid exchanges (SCE) (2, 3, 54). Furthermore, chromosomal aberrations in FA cells increase in response to treatment with DNA cross-linking agents, such as MMC, DEB, nitrogen mustard and cisplatin (3, 55-58). The unique sensitivity of FA cells to chemical cross-linking agents suggests that the recognition, removal or tolerance of the lesions caused by these agents may be defective in FA.

DNA cross-links are typically repaired by the NER pathway (49). The steps in the NER pathway include recognition of the DNA damage, incision of the DNA strand containing the lesion, excision of the defective strand, DNA synthesis, and ligation of the newly synthesized strand (49, 51). XP is a well-studied inherited disorder that is directly linked to defects in a NER pathway involved in the removal pyrimidine dimers cause by ultraviolet (UV) light exposure (51). Although FA cells are not sensitive to UV light, they are highly sensitive to bifunctional cross-linking agents, indicating that a NER pathway specific for chemically induced cross-links may be defective in FA. A number of studies have examined excision repair in FA by comparing DNA repair profiles in FA cells to those of normal cells following cross-linking agent exposure. The rate of induction of cross-links occurs at similar levels in both FA and normal cells (59, 60). However, initial studies showed that FA fibroblasts have an impaired capacity for removing DNA interstrand crosslinks induced by MMC, suggesting that a defect in the incision of cross-links is responsible for chromosomal damage in FA (61-63). Similar studies found a deficiency in
the incision of 8-methoxypsoralen in conjunction with long-wave ultraviolet light (8-MOP+UVA) induced cross-links in a FA cell line (59). Further support for an incision repair defect came from studies in which the activity of an endonuclease complex with specificity for cross-links was lower in nuclear extracts from a subset of FA cell lines compared to normal cell lines (64-68).

In contrast, other studies using different FA cell lines, found no deficiency in cross-link incision compared to normal cells (69-72). In addition, the repair defect in FA may not be a stable property as some FA cell lines exhibit competency for DNA cross-link repair during early passages, but become deficient in cross-link repair during later passages in vitro (73). FA fibroblasts transfected with a chloramphenicol acetyltransferase (CAT) expression vector cross-linked in vitro using either nitrogen mustard, psoralen plus UVA or cisplatin revealed no difference in the repair dependent reactivation between normal or FA cells (74, 75). Some of the conflicting results between laboratories may be attributed to the extensive genetic heterogeneity associated with FA (discussed later).

Several studies have indicated that a recombination repair mechanism may be defective in FA cells. Papadopoulo et al demonstrated that the frequency of 8-MOP+UVA induced mutations is lower at two different loci in two FA cell lines than in normal cells (76, 77). Analysis of cross-linker induced mutations at the HPRT locus revealed that the majority of lesions in FA cells are large deletions and rearrangements, whereas point mutations predominate in normal cells (77). One interpretation of these data is that FA cells are deficient in a postreplication repair pathway that operates during the bypass of unexcised lesions. One possible pathway activated by cross-links in normal cells involves the stalling of DNA polymerase upon encountering a DNA lesion, resumption of replication downstream of the lesion, and postreplicative filling of the gap by either mismatch repair (MMR) or through a recombination mechanism that is poorly understood (78). In FA cells, deletions may occur due to a deficiency in the filling of postreplication gaps by a recombination process. In support of this theory, a DNA end-joining assay revealed that in FA cells the error-free processing of blunt ended DSB is markedly decreased, resulting in a higher deletion frequency (79). The higher frequency of deletions in FA cells may be attributed to a defect in error-free NHEJ of blunt ended DSB, leading to a channeling towards an error-prone mechanism, or may be due to the lack of a factor that is involved in
maintaining the fidelity of rejoining. The increase in chromosomal breaks and genomic instability in FA cells may be related to a recombination repair defect in which DSB are not properly processed.

Alternatively, the chromosomal instability and elevated MMC sensitivity in FA cells may be attributed to an ineffective response to DNA damaging reactive oxygen intermediates that are generated during normal metabolic processes or during the activation of cross-linking drugs (80, 81). The hydroxyl radical readily reacts with DNA bases, and attempts to remove these bases by base excision repair (BER) can result in a DNA DSB (49, 82). The formation of 8-hydroxy-2'-deoxyguanine (8OHdG) is a sensitive biomarker of oxidative DNA damage (82, 83). Takeuchi and Morimoto provided evidence for excess production of 8OHdG in FA cells challenged with H₂O₂ that was related, at least in part, to a decreased ability to decompose H₂O₂ (84). Ex vivo studies of freshly drawn lymphocytes from FA patients, revealed an excess production of ROS as detected by luminol-dependent chemiluminescence (LDCL), in addition to increased 8OHdG levels that were significantly correlated with LDCL as well as with chromosomal instability (85, 86). Mutagenesis studies using a SupF shuttle plasmid revealed a significantly higher oxygen-dependent mutation rate in FA cells when compared to normal controls that was augmented with MMC exposure (87). FA lymphoblasts transfected with a CAT expression vector, that was oxidatively damaged extracellularly, revealed a significant decrease in repair dependent reactivation of the vector in FA cells compared to normal cells (88). These observations have raised the notion that the FA defect might be attributed to a mutation in an enzyme specialized for oxidized DNA repair or that mutations exist in other FA proteins involved in the regulation of such an enzyme. The ultimate conclusion from the variable and frequently conflicting data on aberrant repair processes in FA cells is that at least one mechanism of DNA repair is most likely deficient in FA. However, it is now more accurate to refer to this disease as a chromosomal instability disorder rather than a DNA repair disorder.

1.2.2. Role of oxygen in FA

The basis of the enhanced chromosomal instability in FA cells is poorly understood. Because elevated oxygen radicals are known to cause chromosome breaks (89, 90), a defect in oxygen metabolism has been proposed as one potential underlying mechanism leading to
the pathophysiology of FA. Reactive oxygen species (ROS) are free radicals associated with the oxygen atom or their equivalents. The family of ROS resulting from the incomplete reduction of oxygen includes the superoxide radical \( \text{O}_2^- \), hydrogen peroxide \( \text{H}_2\text{O}_2 \) and the hydroxyl radical \( \text{HO}^- \) (91). Other biologically important free radicals include lipid hydroperoxides \( \text{ROOH} \), lipid alkoxyl radicals \( \text{RO}^- \), nitric oxide \( \text{NO} \) and singlet oxygen \( \text{^1O}_2 \). Small amounts of ROS are constantly generated in mammalian cells in response to both external and internal stimuli. Cellular energy metabolism is based on the production of ATP through the electron-transport pathway in which \( \text{O}_2 \) ultimately accepts electrons and \( \text{H}^+ \) resulting in the production of water. However, at least two sites have been identified in the electron-transport chain (Complex I and ubisemiquinone) where electrons may leak out to react directly with oxygen molecules, resulting in the formation of superoxide (91). Another site of \( \text{O}_2^- \) production is at the endoplasmic reticulum where leakage of electrons from NADPH cytochrome P450 reductase occurs (92). ROS can also be produced by high oxygen tension, gamma irradiation, near-UV radiation and drugs that are bioactively reduced. (89). \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) are only moderately reactive with other biological molecules. However, these molecules are readily converted into \( \text{HO}^- \), which is highly reactive. At elevated levels, free radicals are highly cytotoxic and can cause oxidative damage to macromolecules leading to lipid peroxidation, oxidation of amino acid side chains (especially cysteine), formation of protein-protein cross-links, DNA damage and DSB (93, 94).

As protection against increased levels of ROS, referred to as oxidative stress, cells possess several anti-oxidants or reductants that maintain the cytoplasmic redox environment in a highly reduced state. Several families of antioxidant enzymes have evolved for the elimination of ROS. Superoxide dismutases (SOD) convert \( \text{O}_2^- \) into \( \text{H}_2\text{O}_2 + \text{O}_2 \). Catalase can then detoxify \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} + \text{O}_2 \). Glutathione (GSH), the principal intracellular non-protein thiol, can be present in cells at concentrations as high as 10 mM and provides a primary defence against oxidative stress by its ability to scavenge free radicals or participate in the reduction of \( \text{H}_2\text{O}_2 \) (95, 96). Glutathione peroxidases are selenium-dependent enzymes that catalyze the reduction of \( \text{H}_2\text{O}_2 \) and organic hydroperoxides to water and
alcohols respectively, with the concurrent generation of oxidized glutathione (GSSG) (96). Glutathione S-transferases (GST) are a family of selenium-independent enzymes that can reduce organic hydroperoxides in addition to detoxify xenobiotics via a GSH conjugation mechanism (97). Table 1-1 shows several of the relevant equations with regard to oxygen metabolism.

The possibility that FA cells have a defect in oxygen metabolism was first suggested by Nordenson, who noted a decrease in the level of spontaneous chromosomal aberrations in FA cells treated with either catalase or SOD (98). SOD, catalase and L-cysteine (a GSH precursor) were subsequently shown to exert a protective effect on both spontaneous and DNA cross-linker induced chromosomal breakage in FA fibroblasts (99, 100). Pilot clinical trials using recombinant SOD have suggested a beneficial effect on chromosomal breakage in lymphocytes and growth of bone marrow progenitor cells from FA patients (101, 102). A partial correction of either DEB or peroxide induced chromosomal instability was observed in FA lymphoblast lines treated with various antioxidants including L-cysteine, 2-mercaptoethanol and GSH (103). Complementing these studies, a positive correlation between oxygen tension and spontaneous chromosomal aberrations has been documented for FA cells. The rate of spontaneous chromosomal breaks in FA cells increases with increasing oxygen tension (104-106). Furthermore, the effect of increased oxygen tension also augments MMC induced chromosomal aberrations in FA cells (105). During hyperoxic conditions, the rate of electron leakage from the mitochondria, and associated ROS formation, is in direct proportion to the oxygen tension (91). The oxygen tension in most tissues in situ is in the range of 1% to 5% (107). However, cells cultured in ambient oxygen are exposed to 20% oxygen tension. FA cells tend to grow poorly under standard culture conditions, and exhibit slow doubling times associated with a G2 phase cell cycle delay (106, 108, 109). However, the poor growth and G2 phase delay observed in FA cells is partially corrected in 5% oxygen culture conditions (106) (110). The increased sensitivity to oxygen has led to the hypothesis that either the cellular overproduction or the decreased removal of ROS may be the primary defect in FA cells (75, 80, 104).

Several laboratories have reported 10% to 40% decreased SOD activity in erythrocytes from FA patients, suggesting that this is the direct cause of spontaneous chromosomal breakage (111-115). However, other studies have shown no significant
Table 1-1. Summary of reactions catalyzed by the major antioxidant enzymes. (1) Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide anion (O$_2^-$) to oxygen (O$_2$) and to the less reactive species hydrogen peroxide (H$_2$O$_2$). (2) Catalase (CAT) reacts with H$_2$O$_2$ to form water (H$_2$O) and molecular oxygen (O$_2$). Glutathione peroxidase (GPX) is a selenium-containing enzyme that catalyzes the reduction of both H$_2$O$_2$ and organic hydroperoxides (ROOH) to H$_2$O (3) and alcohols (ROH) (4) respectively. In the process, reduced glutathione (GSH) is oxidized (GSSG). Glutathione S-transferase (GST) also catalyzes the reduction of ROOH to ROH in a selenium-independent manner (4).
difference in SOD levels in erythrocytes even though high levels of superoxide exist (116). FA erythrocytes also exhibit a faster rate of GSH decay and a higher ratio of oxidized to reduced glutathione (GSSG/GSH) than normal erythrocytes (24, 117). In order to relate cellular SOD deficiency to chromosomal breakage, several labs have looked for a deficiency in nucleated FA cells. SOD levels in FA lymphocytes are only marginally lower than normal (115, 118). However, FA fibroblasts appear to contain more MnSOD, catalase and GSH peroxidase than normal cells, especially when cultured for relatively long periods (119). The elevation of these antioxidant enzymes may indicate that FA cells are in a "prooxidant state" which may lead to an increased level of certain protective enzyme activities. Purified SOD from FA cells is indistinguishable from the wild-type enzyme by gel electrophoresis and isoelectric focusing, and has a normal activity per unit of enzyme, so it is unlikely that a mutation in the SOD genes exists in FA cells (107).

Pathways for the bioactivation of MMC suggest a potential interrelationship with oxygen metabolism. Cellular enzymes reductively activate the semiquinone form of MMC, which can then be reoxidized by molecular oxygen under aerobic conditions (120-122). The reoxidized form can undergo further futile cycles of reduction and oxidation, with the cyclic generation of intracellular ROS (81). Thus, the generation of ROS during MMC bioactivation may be the direct cause of MMC induced cytotoxicity in FA cells. In support of this theory, MMC hypersensitivity in FA cells is attenuated when cells are cultured under decreased oxygen levels that inhibit redox cycling of MMC and ROS production (80). In addition, overexpression of thioredoxin, an antioxidant protein, decreases the cytotoxic and DNA damaging effect of MMC and DEB in FA fibroblasts (123). These findings imply a direct role for oxygen free radicals, rather than DNA cross-linking, in the sensitivity of FA cells to MMC.

1.3. Genetics of FA

1.3.1. Identification of FA complementation groups

FA is a rare disorder, with a prevalence of 1 in 350,000 in North America (47, 124). FA segregates in a simple autosomal recessive manner with no significant deviations from the predicted Mendelian inheritance (125, 126). The extensive clinical and cellular
heterogeneity observed in FA is paralleled by genetic heterogeneity, implying a role for several genes in the pathophysiology of the disease. Genetic heterogeneity studies have exploited the hypersensitivity of FA cells to the cytotoxic action of DNA cross-linking agents. FA fibroblast lines were initially classified into two complementation groups based on the correction of their chromosomal instability after somatic cell fusion (127). Complementation of the FA phenotype was based on correction of spontaneous and MMC induced chromosomal breakage and growth inhibition by MMC treatment in the cell hybrids. Initial studies using a panel of somatic cell hybrids, created by fusing together lymphoblast cell lines derived from six unrelated FA patients, revealed at least two complementation groups that were designated group A and non-A (formally group B) (128). Subsequent analysis of the non-A lines has led to the identification of six other complementation groups (129-133). Among the FA complementation groups, group A (FA-A) is the most prevalent, accounting for 66% of all FA patients (134). The identification of seven complementation groups (FA-A through FA-G) provides evidence for the existence of at least seven FA disease genes. A defect in any of the seven possible FA genes leads to a similar clinical phenotype.

13.2. Cloning of FA genes

The first FA gene, belonging to complementation group C (FANCC), was cloned in 1992 by functional complementation of the MMC hypersensitivity phenotype in FA-C cells using a cDNA expression library (135). Cloning of the second FA gene belonging to complementation group A (FANCA) was achieved independently by both positional cloning and by functional complementation (136, 137). The group E (FANCE), group F (FANCF) and group G (FANCG) genes were also successfully cloned by functional complementation (138-140). The chromosomal region containing the group D gene (FANCD) was localized to chromosome 3p25.3 using microcell-mediated chromosome transfer and functional complementation (141, 142). The major characteristics of the FA genes are summarized in Table 1-2.

With the exception of FANCF, sequence analysis of all the FA genes at both the nucleotide and amino acid level has revealed no significant homologies with any other genes. The predicted FANCF protein possesses a small region of homology, 60 amino acids
<table>
<thead>
<tr>
<th>FA gene</th>
<th>Chromosome location</th>
<th>Genomic Structure</th>
<th>Size of open reading frame</th>
<th>Predicted size of reading frame</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>FANCA</td>
<td>16q24.3</td>
<td>43 exons spanning 80 kb</td>
<td>4.4 kb</td>
<td>1455 aa (162 kD)</td>
<td>136, 137, 148</td>
</tr>
<tr>
<td>FANCB</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>FANCC</td>
<td>9q22.3</td>
<td>17 exons (14 coding + 3 noncoding) spanning 150 kb</td>
<td>1.7 kb</td>
<td>558 aa (63 kD)</td>
<td>135, 150</td>
</tr>
<tr>
<td>FANCD</td>
<td>3p25.3</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>FANCE</td>
<td>6p21-22</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>FANCF</td>
<td>11p15</td>
<td>lacks introns</td>
<td>1.3 kb</td>
<td>374 aa (42 kD)</td>
<td>139</td>
</tr>
<tr>
<td>FANCG</td>
<td>9p13</td>
<td>Nd</td>
<td>1.9 kb</td>
<td>622 aa (69kD)</td>
<td>140, 144</td>
</tr>
</tbody>
</table>

**Table 1-2.** Summary of the major characteristics of the FA genes. Nd, not determined; aa, amino acids.
long, with the prokaryotic RNA-binding protein ROM (143). The significance of this homology or relevance to FA is unknown. FANCG is identical to XRCC9, a gene originally isolated by partial correction of the chromosomal instability and MMC-sensitive phenotype of the Chinese hamster ovary (CHO) mutant cell line UV40, although its precise function is unknown (144). Interestingly, the FA proteins have no homology amongst each other.

Cloning of the murine homologue of FANCA (Fanca) has revealed an identity of 74% at the nucleotide level and 65% identity at the protein level with its human counterpart (145). Comparison of the murine and human FANCC proteins revealed a 68% amino acid sequence identity (146). A cross species comparison of the human, mouse, rat and bovine FANCC amino acid sequences has revealed no significant areas of highly conserved residues common across all species (147). However, eight cysteine residues were conserved across species, especially in the N-terminal portion of the FANCC protein, suggesting that structurally important disulfide bridges may exist (147). Despite the moderate conservation in coding sequences between the human and mouse homologues of the FA genes, both murine Fanca and Fancc cDNA can complement the MMC-sensitive phenotype of human FA-A and FA-C lymphoblasts respectively, suggesting a fundamental conservation of function (145, 146). The lack of significant stretches of highly conserved sequences in both FANCA and FANCC has hindered identification of important functional motifs or domains. Furthermore, database searches of the Saccharomyces cerevisiae, Drosophila melanogaster and Caenorhabditis elegans genomes have not revealed any homologies to any of the cloned FA genes. Thus, the family of known FA genes appear to participate in a novel cellular pathway of defence or repair that arose at a later point in evolution.

1.3.3. Mutations in FA genes

In FA-A patients, the FANCA gene contains a highly heterogeneous spectrum of mutations consisting of substitutions, large intragenic deletions and microinsertions/microdeletions of 1-5 nucleotides (148, 149). Furthermore, these mutations are scattered throughout the gene, with most resulting in the absence of the FANCA protein (149). In the case of FA-C patients, phenotype-genotype correlations have enabled FANCC mutations to be classified into three subgroups: (1) patients, predominately of Ashkenazi Jewish ancestry, with a splice site mutation in exon 4 (IVS4+4); (2) patients with at least
one exon 14 mutation (R548X or L554P); and (3) patients with at least one exon 1 mutation (322delG or Q13X) and no known exon 14 mutation (150). Patients with mutated alleles in exon 4 or 14 have a more severe phenotype as defined by the early onset of hematologic abnormalities, presence of major congenital malformations and a reduced life expectancy. Both exon 4 and 14 mutations result in the production of either a slightly truncated or aberrant full length FANCC polypeptide respectively (151). These findings suggest that specific FANCC mutant proteins may interact in a negative manner with other cellular proteins, and implies a functional role for the C-terminal portion of the protein. In support of this theory, overexpression of a C-terminal mutant protein (L554P) in wild-type cells induces MMC-hypersensitivity reminiscent of FA cells, and is believed to act through competition with endogenous FANCC for interactions with FANCC-binding proteins (152). Patients with exon 1 mutations generally have a milder phenotype with later onset of hematological problems, and absence of congenital malformations. The milder phenotype of the exon 1 subgroup may be attributed to the presence of a truncated FANCC protein isoform caused by an alternate downstream translation initiation site, where a second methionine is located (151). A single study on FANCG mutations revealed a majority of splice-site mutations and short deletions (140).

1.4. The FA Protein Complex

1.4.1. FANCA

Examination of the primary sequence of the FA proteins has revealed few clues about structure or function. The FANCA protein is approximately 160 kD and possesses two overlapping bipartite nuclear localization signals (NLS) (136, 137). More recently, computational analysis of FANCA revealed an area with homology to a primitive peroxidase domain, although no experimental data supports this observation (153). Several studies have shown that FANCA is primarily localized to the nucleus, with about 10% present in the cytoplasm, and that the NLS motifs are functional and necessary for FANCA activity (154–157). The FANCA protein is phosphorylated and accumulates in the nucleus in normal cells but not in cells from any of the FA complementation groups (with the exception of FA-D), indicating that FANCA function requires the activity of the other FA
proteins (158). In addition, nuclear localization appears to be essential for FANCA function because its forced removal, using a nuclear export signal tag, abolishes its complementing ability in FA-A cells (154).

1.4.2. FANCC

The predicted open reading frame (ORF) of FANCC encodes a 63 kD protein with a preponderance of hydrophobic amino acids, but no identifiable transmembrane domains (135). *In vitro* transcription and translation of the cDNA produces a product with an apparent molecular weight of 60 kD (159). A protein of similar size is immunoprecipitated from lymphoblasts and COS-7 cells transfected with FANCC cDNA using anti-FANCC specific antibodies (154, 160, 161). Initial studies on the cellular localization of FANCC revealed that it was primarily cytoplasmic with a small fraction associated with membranes (160, 161). However, more recent studies have shown that a fraction of the FANCC protein (approximately 10%) is also detected in the nucleus of human 293 and lymphoblast cell lines (162). However, cytoplasmic localization appears to be important for FANCC function because forced nuclear localization, using a nuclear localization signal tag, abolishes its complementing ability in FA-C cells (163).

1.4.3. Interaction of the FA proteins

Because FA patients from different complementation groups have similar clinical and cellular phenotypes, it is possible that FA proteins act either sequentially within a common molecular pathway or jointly as a multi-subunit complex. Accumulating evidence now supports the later hypothesis. Coimmunoprecipitation studies revealed that FANCA and FANCC interact in both the cytoplasm and nucleus (155, 164). Furthermore, coimmunoprecipitation analysis of either FANCA amino-terminal truncated mutant proteins expressed in FA-A cells or endogenous FANCA in various FA cell lines revealed that phosphorylation and nuclear accumulation of FANCA requires the presence of wild-type FANCC (155, 158). However, it is unclear if FANCA is phosphorylated before or after it accumulates in the nucleus (158). In addition, *in vitro* translated FANCA and FANCC proteins failed to bind directly and yeast two-hybrid analysis failed to detect an interaction between the two proteins, suggesting that the interaction may require additional adapter
proteins or posttranslational modifications (154, 155, 158, 165). The FANCA/FANCC complex is not detected in cells from other complementation groups (except FA-D) suggesting that binding of FANCA with FANCC may be regulated by proteins encoded by other FA genes (155, 158). One of these adapter proteins is likely FANCG, which complexes with FANCA in vitro and within the cell (166, 167). Unlike the FANCA/FANCC complex, FANCA and FANCG interact directly through the N-terminal region of FANCA (166, 167). FANCG is found in both the cytoplasm and nucleus, and like FANCA, it is primarily localized to the nucleus (166, 167). The FANCA/FANCG complex is not detected in FA-A and FA-G cell lines, and is substantially reduced in cells from complementation groups B, C, F, and H (167). Furthermore, introduction of FANCG cDNA into FA-G cells restores the formation of the FANCA/FANCG complex (166), as well as FANCA/FANCC/FANCG binding (166). The FANCD protein is thought to function independently of the other FA proteins, because the FA protein complex assembles normally in FA-D cells (158, 167). These recent findings support the view that FANCA, FANCC and FANCG form part of a larger protein complex containing other FA and possibly non-FA proteins, whereas the FANCD protein appears to function either separately or downstream of the FA complex.

1.4.4. Non-FA protein interactions with FANCC

Aside from FANCA and FANCG, several non-FA proteins have been shown to interact with FANCC. Initial studies using radiolabeled lysates from wild-type mammalian cells indicated that three unknown proteins with molecular masses of 65, 50 and 35 kDa coprecipitated with a recombinant chimeric FANCC protein (168). Furthermore, endogenous FANCC and the three proteins, co-fractionated within cytosolic and membrane extracts from Dami cells (168). Coimmunoprecipitation studies using HeLa cell extracts revealed that a 34 kDa cyclin-dependent kinase (cdc2) binds to endogenous FANCC (169). This interaction is mediated by the C-terminus of FANCC as the L554P FANCC mutant protein in FA-C lymphoblasts fails to coimmunoprecipitate cdc2 (169). Because cdc2 dependent phosphorylation is required for progression of the cell cycle through G2, a disruption of the FANCC/cdc2 interaction may be related to the G2 delay observed in FA-C
However, FANCC is not phosphorylated by cdc2 and the significance of the FANCC/cdc2 binding interaction remains unclear (169).

The molecular chaperone GRP94 was identified as a FANCC interacting protein by a yeast two-hybrid screen (170). GRP94 is a stress-inducible glycoprotein that regulates the intracellular level of FANCC (but not vice versa). Ribozyme-mediated inactivation of GRP94 leads to reduced levels of FANCC and a concomitant hypersensitivity to MMC in rat NRK cells (170). GRP94 is localized primarily in the lumen of the endoplasmic reticulum (ER) in addition to a small transmembrane fraction that spans the ER membrane and extends into the cytoplasm (171). GRP94 functions as a chaperone that aids in the proper folding of proteins by preventing the formation of mispaired disulfide bonds (172). Interestingly, GRP94 binds to an area of FANCC that contains several highly conserved cysteine residues (147) suggesting that it may mediate proper folding of FANCC.

FANCC was also shown to interact with NADPH cytochrome P450 reductase (P450-RED) by co-transfection of both genes into COS-1 cells followed by coimmunoprecipitation studies and by yeast two-hybrid analysis (173). P450-RED is a microsomal membrane protein that activates cytochrome P450 enzymes that in turn bioreductively activate xenobiotics including MMC (174). P450-RED contains binding sites for flavin mononucleotide (FMN), flavin dinucleotide (FAD), and NADPH. Electrons donated by NADPH are initially transferred internally from FAD to FMN, then externally to one of the cytochrome P450 enzymes or cytochrome c. FANCC competes with FMN for binding to P450-RED and interferes with electron transfer to cytochrome c (173). Because P450-RED bioreductively activates MMC under aerobic conditions, an inhibition of P450-RED by FANCC is consistent with the MMC and oxygen hypersensitivity observed in FA-C cells.

A novel zinc finger transcription factor named FAZF (Fanconi Anemia Zinc Finger) was recently found to interact with FANCC by yeast two-hybrid screening (175). The interaction was confirmed by transient transfection of epitope-tagged FANCC and FAZF expression vectors into 293 EBNA cells followed by coimmunoprecipitation analysis. FAZF is homologous to the promyelocytic leukemia zinc finger (PLZF) protein, a transcriptional repressor implicated in oncogenesis, limb morphogenesis, hematopoiesis and proliferation (176). Following transfection, FAZF and FANCC colocalize in the nucleus of
293 EBNA cells suggesting that a function of FANCC may be linked to a transcriptional repression pathway. Recently, the structural protein α spectrin II was shown to form a chromatin-associated complex with both FANCA and FANCC in normal but not FA-A lymphoblast cell lines (68). Levels of endogenous α spectrin were shown to be significantly reduced in FA-A, FA-B, FA-C and FA-D cell lines (68). The cleavage of spectrin II (also known as fodrin) during programmed cell death in a variety of tissues is associated with the characteristic morphological changes that occur during this form of cell death (177, 178). It is uncertain if the FA proteins (including FANCC) prevent cleavage or proteolysis of fodrin.

1.5. Determining the function of the FANCC protein

1.5.1. Expression of Fancc

The analysis of gene expression can yield essential information about the role of a gene, such as the sites and levels where the gene functions. Initial analysis of FANCC mRNA expression revealed that both the human and mouse genes are expressed ubiquitously at low levels in adult tissues (135, 146). However, during mouse embryogenesis high Fancc expression is observed in undifferentiated mesenchymal cells and its derivatives with osteogenic potential 8-10 days p.c. (179). Starting at 13 days, expression becomes restricted to regions containing rapidly replicating chondrocyte and osteocyte progenitors, that persists until later stages (15—19.5 days), except in regions where differentiation has taken place. As bone development proceeds, expression is seen in osteogenic and hematopoietic cells in the zone of calcification. Fancc expression is also detected in the rapidly dividing ependymal cell layer of the developing brain that gives rise to neuronal and glial cell precursors (179). Analysis of Fancc expression, using PCR-derived cDNA libraries from single hematopoietic cells, revealed that high levels of expression are observed in less differentiated (multilineage progenitors) and that Fancc expression drops dramatically in differentiated cells (single lineage progenitors) (180). Thus, in general, high Fancc expression is observed in relatively undifferentiated cells (i.e. mesenchymal, ependymal and hematopoietic progenitor cells) and diminishes as cells mature and differentiate.
The expression profile in undifferentiated bone marrow cells suggests that FANCC expression is important for hematopoietic progenitor cell growth. In support of this notion, Segal et al demonstrated that inhibition of FANCC expression in normal cells using an antisense oligodeoxynucleotide (ODN) complementary to FANCC mRNA inhibited the growth of erythroid and granulocyte-macrophage progenitor cells but did not inhibit the growth of fibroblasts, lymphoblasts or endothelial cells (181). Normal lymphoblasts treated with antisense ODN had higher levels of chromosomal breaks after MMC exposure compared to sense ODN treated controls. In addition, antisense ODN treatment did not inhibit the production of growth factors like IL-6, GM-CSF or G-CSF. Thus, FANCC antisense molecules directly inhibited clonal growth or differentiation of progenitor cells. In a complementary study, Walsh et al demonstrated that infection of hematopoietic progenitor cells from FA-C patients with FANCC viral vectors resulted in a marked increase in colony formation both in the absence or presence of MMC (182, 183). These findings indicate that FANCC expression is necessary for viability of hematopoietic progenitor cells under normal growth conditions but plays a lesser role in maintaining viability of differentiated cells, except under the stress conditions associated with DNA damage.

1.5.2. Mice with targeted disruption of the Fanc gene

In order to understand the in vivo role of the murine Fanc gene, two groups have generated mice homozygous for a targeted deletion of the Fanc locus in either exon 8 or exon 9 (Fanc−/−) (184, 185). The mutant mice have normal neonatal viability, gross morphology and fail to shown any spontaneous hematologic abnormalities. However, both homozygous male and female mice have markedly reduced fertility associated with impaired gametogenesis. Histological analysis of Fanc−/− mice revealed testicular atrophy in males associated with degeneration of seminiferous tubules and ovarian hypoplasia in females associated with reduced or absent germ cells. Spleen and fibroblast cells derived from Fanc−/− mice exhibit spontaneous chromosomal breakage and hypersensitivity to DNA cross-linking agents (186). Furthermore, a significant accumulation of cells in the G2 phase of the cell cycle occurs after MMC exposure in mutant mice, similar to the cell cycle abnormalities in FA patients (185).
Fancc−/− mice exposed to levels of MMC that are non-toxic in wildtype mice develop bone marrow failure and eventually die within a few days of treatment (187). However, progressive pancytopenia can be induced in Fancc−/− mice by treatment with sequential nonlethal doses of MMC, which results in a reduced bone marrow cellularity and decreased numbers of early and committed hematopoietic progenitor cells. Flow cytometric analysis of progenitor cell surface markers revealed a significant decrease in the CD34+ population of bone marrow cells obtained from Fancc−/− mice (188). The CD34 cell surface antigen is a marker that is closely associated with hematopoietic progenitor cells (189). Fancc−/− mice have 40 to 70% fewer CD34+ cells under non stressed conditions and 92% fewer CD34+ cells after non-lethal MMC treatment compared to wild-type mice (188). Furthermore, bone marrow from Fancc−/− mice has reduced ability for primary and secondary long-term repopulation of myeloablated recipient mice compared to marrow from wild-type or heterozygous mice (188). Hematopoietic stem cell function can also be assessed in vivo using a competitive repopulation assay (190, 191). Using this assay, the degree of chimerism in irradiated recipient mice was dramatically lower in mice transplanted with Fancc−/− marrow compared to wild-type marrow during both short-term and long-term repopulation periods (192). These results suggest that the Fancc gene product may be necessary for both the maintenance of normal numbers and development of hematopoietic progenitor cells, especially after DNA damage.

1.6. The role of FANCC in apoptosis

1.6.1. Features of apoptosis

Apoptosis or programmed cell death is morphologically distinct from necrotic cell death and is designed to remove unwanted and potentially harmful cells. Apoptosis is an active gene-directed mechanism that plays an important role in removing surplus cells and sculpting the developing embryo in addition to maintaining a homeostatic balance of cells in the hematopoietic system (193, 194). A growing body of evidence has revealed that inappropriate regulation of apoptosis is associated with a variety of diseases including neurodegenerative disorders, AIDS, anemia and cancer (195-197). Cells undergoing apoptosis exhibit a series of morphological features, including a decrease in cell volume,
chromatin condensation, blebbing of the plasma membrane, and cleavage of DNA at internucleosomal sites (198, 199). During apoptosis the nucleus often breaks up into membrane bound apoptotic bodies containing nuclear fragments (199). Although cleavage of DNA into oligonucleosomal-sized fragments is a hallmark of apoptosis, single-strand nicks and cleavage into 50-200 Kb-sized DNA may occur before or in the absence of oligonucleosome fragmentation (200-203). Apoptosis is generally characterized by one or more of these morphological changes, but may vary with cell type and mode of induction.

The role of mitochondria in initiation of apoptosis was first demonstrated in a cell-free system in which spontaneous nuclear condensation and DNA fragmentation were found to be dependent on the presence of mitochondria (204). Subsequent studies revealed that the mitochondrial transmembrane potential ($\Delta \Psi_m$) collapses and the opening of a large conductance channel known as the mitochondrial permeability transition (PT) pore occurs during apoptosis (205-207). The opening of the PT pore and loss of $\Delta \Psi_m$ results in dissipation of the electrochemical gradient within the mitochondrial intermembrane space and osmotic swelling. These events either together or independently result in the redistribution of intermembrane proteins to the cytosol, which in turn promote apoptosis. Two mitochondrial intermembrane proteins, cytochrome c and apoptosis-inducing factor (AIF), have recently been identified as critical molecules for the induction of apoptosis (208, 209). The release of both these proteins from the mitochondria is prevented by the anti-apoptotic protein Bcl-2 or its close family member Bcl-XL (210, 211). Cytochrome c release from the mitochondria is associated the generation of ROS, decreased production of ATP and activation of the caspase cascade (207).

Caspases comprise a family of cysteine proteases that are activated in response to a variety of apoptotic stimuli (212). Once activated, caspases can transduce or amplify signals by mutual activation and self cleavage (213). Caspase activation involves proteolytic processing of an inactive precursor molecule (zymogen) to two smaller subunits, which then associate into a heterotetrameric active enzyme complex (214). Caspases are highly specific proteases that cleave substrate proteins after aspartic acids residues. The specificity of caspases is determined by the recognition of at least four amino acids N-terminal to the cleavage site, resulting in a select set of proteins cleaved during apoptosis (215). Caspases act as key effector molecules for the disassembly of the cell during
apoptosis by cleaving proteins involved in the maintenance of the cytoskeleton, DNA repair, mRNA splicing and DNA replication (216-218). For example, caspases can cleave nuclear lamins (219), poly(ADP-ribose) polymerase (PARP) (220), and DNA-dependent protein kinase (DNA-PK) (221).

Cytosolic cytochrome c is an essential component of the apoptosome, which is composed of cytochrome c, Apaf-1 (apoptotic protease activating factor-1) and procaspase-9. Apaf-1 interacts with procaspase-9 but requires the presence of cytochrome c and ATP for activation of caspase-9 (222). Active caspase-9 can then process and activate other downstream caspases, like caspase-3, leading to the apoptotic destruction of the cell (222). Like cytochrome c, AIF is released from the mitochondria into the cytosol during apoptosis. Cytosolic AIF induces nuclear condensation and large scale (~50kb) DNA fragmentation in a caspase-independent manner (209). The mechanism by which AIF induces apoptosis is unknown, however, AIF shares significant homology with plant ascorbate reductases and bacterial NADH-oxidoreductases indicating that it may have a redox function (209).

The initiation of apoptosis can occur by multiple signals, which can originate either from within the cell or from the extracellular environment. Three classic pathways of apoptotic signaling in mammalian cells have emerged. The first pathway is initiated by DNA damage and is in part regulated by p53. The second pathway involves signaling by cell surface death receptors such as tumor necrosis factor (TNFR) and Fas receptor, which, through adapter molecules, can recruit and activate caspases. The final well-established apoptosis pathway is initiated by the withdrawal of growth factors and is regulated by the Bcl-2 family of proteins. The relationship of these pathways to FA and in particular the FANCC protein is discussed below.

1.6.2. The role of p53 in DNA damage induced apoptosis

p53 is a transcription factor that mediates the expression of a variety of genes that induce growth arrest or apoptosis in response to DNA damage. Thus, p53 maintains genomic integrity by ensuring that DNA damage will be repaired during growth arrest, or the damaged cells will be eliminated by apoptosis. p53 can act as a “fail-safe” mechanism by inducing cell death and reducing the propensity for growth-deregulating mutations and
the expansion of potentially malignant cells following DNA damage. Overexpression of wild-type p53 can either induce cell cycle arrest in the G1 phase (223, 224) or can induce apoptosis (225, 226). Following DNA damage by X-rays, UV radiation or DNA cross-linking agents, the p53 protein rapidly accumulates and becomes transcriptionally active (227-229). Accumulation of p53 results in the transactivation various genes implicated in cell cycle regulation such as p21^{WAF1} and 14-3-3 sigma (230, 231).

Depending on the cell type and the extent or nature of DNA damage, accumulation of p53 may also initiate apoptosis. Furthermore, p53-dependent apoptosis does not necessarily require induction of G1 cell cycle check point genes such as Waf1 (232). The separation of p53-dependent pathways for growth arrest and apoptosis may be based on selective induction of genes specific for each pathway. For example, p53 can transactivate pro-apoptotic genes such as Bax, the product of which can antagonize the anti-apoptotic activity of Bcl-2 (233), and Fas/APO1, a member of the death receptor family (234).

1.6.3. p53 and FANCC

Several groups have investigated the relationship between p53 activation and apoptosis induction in FA cells following DNA damage. In the first study, Rosselli et al. found lack of p53 activation and reduced apoptosis induction in two FA cell lines following exposure to X-rays, UV-B irradiation or MMC compared to normal cells, even though FA cells exhibited normal G1 arrest (235). However, a separate study, using different MMC concentrations and exposure times from Rosselli et al., found that p53 induction in FA cells was more pronounced and was induced by lower levels of MMC treatment than in normal cells (236). Furthermore, FA-C cells were more susceptible to MMC induced apoptosis which was suppressed by ectopic expression of FANCC cDNA. However, overexpression of a dominant-negative p53 mutant in FA lymphoblasts failed to protect FA cells from MMC induced apoptosis, indicating that alterations in the p53 response in FA cells did not contribute to the induction of apoptosis following DNA damage (236). Analysis of p53 induction in two isogenic FA-C cell lines, one mock transfected and one complemented by transfection with wild-type FANCC cDNA, revealed that p53 was induced in mock transfected cells at lower MMC concentrations than in complemented cells (237). However, ionizing radiation treatment induced p53 levels equally in both cell lines. In addition,
ectopic expression of FANCC cDNA in FA-C cells rescued MMC induced G2 arrest (237). In a similar study, Marathi et al demonstrated that overexpression of FANCC cDNA in FA-C lymphoblasts prevented nitrogen mustard induced growth inhibition, G2 arrest and apoptotic DNA fragmentation (238). However, cytoprotection by FANCC was not conferred against the effect of non-cross-linking mustards, which induce G1 arrest and apoptosis equally for both normal and FA cells. These studies showed that although the p53 response is altered in FA cells, the apoptotic pathway initiated by DNA damaging agents is specific for bifunctional cross-linking agents, is not dependent on p53 accumulation and results in apoptosis in the G2 phase of the cell cycle.

Several studies have shown that the response initiated by cross-linker exposure in FA cells results in G2 arrest and apoptosis (236-239). A key step in regulating the progression of cells from G2 into mitosis is the activation of the protein kinase cdc2 (240). Cdc2 is regulated by posttranslational events including phosphorylation and dephosphorylation. Phosphorylation of cdc2 by wee1 results in decreased cdc2 kinase activity and G2 arrest (241, 242), whereas dephosphorylation by the phosphatase cdc25 results in increased cdc kinase activity and progression of the cell cycle from G2 to M phase (243, 244). Expression of the FANCC protein is regulated during the cell cycle and peaks at the G2/M transition period in HeLa cells (169). The G2 arrest and associated apoptosis observed in FA-C cells after MMC treatment is mediated by sustained hyperphosphorylation of cdc2 which is not apparent in FA-C cells complemented with wild-type FANCC cDNA (239). FANCC co-immunoprecipitates with the cdc2 complex suggesting that it may either directly or indirectly regulate the phosphorylation state of cdc2 (169). A direct interaction between FANCC and cdc2 has not been detected and it is unknown if FANCC binds to other members of the cdc2 complex (169). Nevertheless, the available evidence indicates that FANCC affects a p53-independent G2-M checkpoint possibly by modifying either protein kinases or phosphatases, which in turn regulate the activity of the cdc2 kinase complex.

1.6.4. TNF/Fas/CD95 mediated apoptosis

Tumor necrosis factor (TNF) is a cytokine that promotes diverse cellular responses such as immunity, inflammation, growth and cytotoxicity via two distinct cell surface
receptors of approximately 55 kD (TNFR1) and 75 kD (TNFR2) (245, 246). Both TNFRs are members of the larger TNFR superfamily, which consists of more than 15 different molecules including Fas (also called CD95 or Apo1), nerve growth factor (NGF) receptor, death receptor 3 (DR3; also called Apo3), death receptor 4 (DR4) and death receptor 5 (DR5) (247, 248). The ligands that activate these receptors, with the exception of NGF, are all structurally related molecules that belong to the TNF superfamily (249). Fas ligand binds to Fas, TNFα and lymphotoxinα (LTα) bind to TNFR1, Apo3 ligand binds to Apo3 (DR3), and Apo2 ligand binds to DR4 and DR5 (247).

Death receptors are cell surface receptors that transmit apoptotic signals initiated by specific death ligands. The death receptor members of the TNF receptor family include TNFR1, Fas, NGFR, DR3, DR4 and DR5. All of these receptors possess a cytoplasmic motif termed a “death domain” (DD), which is a protein-protein interaction motif that is necessary for the receptors to transmit apoptotic signals (248). Binding of an extracellular ligand such as TNFα or FasL to its respective receptor, induces trimerization of the receptor. Because death domains have a propensity to associate with one another, ligand binding leads to clustering of the receptors’ cytoplasmic death domains and transduction of the signal (250, 251). Through the death domains, receptors associate with a number of intracellular signal transduction proteins that themselves contain the death domain. Once TNFR1 is activated, an adapter protein termed TRADD (TNFR-associated death domain) binds through its own death domain to the clustered receptor death domains (252). TRADD then functions as a platform to recruit several signaling proteins to the activated receptor. These proteins include TNRF-associated factor-2 (TRAF2) (253, 254) and receptor-interacting protein (RIP) (255). Both proteins can stimulate pathways ultimately leading to activation of nuclear factor kB (NF-κB) or the stress-activated c-Jun N-terminal kinase (JNK) (247, 251). TRAF2 and RIP activate JNK through activation of the mitogen-activated protein kinase (MAPK) family (256, 257). Activation of NF-κB or JNK following TNF exposure has primarily been associated with cell survival or proliferation and is dependent on the presence of TRAF-2 and RIP (256, 258, 259). Apoptosis initiated by TNF occurs through a TNFR1-TRADD interaction with an adapter protein called FADD (Fas-associated death domain; also called Mort1) (260). Both TNFR1 and Fas trigger cell death via the FADD molecule (261) (See Figure 1-1).
Figure 1-1. Signaling by TNFR1 and Fas. TNF binds to TNFR1 resulting in trimerization of the receptor and recruitment of TRADD via interactions between the death domains (DD). The DD of TRADD recruits RIP and TRAF2, which together activate c-Jun and NF-κB leading to cell proliferation or survival. TRADD also binds FADD leading to the recruitment and activation of caspase 8 via the death effector domains (DED). Binding of FasL to Fas induces trimerization of the Fas receptors which recruits caspase-8 via an adaptor, FADD. Activated caspase-8 can then activate downstream effector caspases leading to apoptosis.
Binding of FasL to the Fas receptor leads to a clustering of the receptors’ death domains and binding of FADD through its own death domain (262, 263). The N-terminal region of FADD contains a death effector domain (DED) and is responsible for initiating apoptotic signalling (261). The DED of FADD facilitates binding to the DED of procaspase-8 (also called FLICE or MACH) which leads to activation of the caspase by self-processing (264). Activated caspase-8 then activates downstream caspases, which can cleave many cellular substrates including structural proteins, signaling proteins and regulators of DNA replication or repair, ultimately leading to apoptosis (265). FADD is an essential shared signaling molecule in both the TNFR1 and Fas apoptotic pathways because it also interacts with TRADD and is the principal molecule that mediates TNF induced apoptosis (260). Thus the signal from Fas is primarily restricted to apoptosis via FADD activation, whereas the signal from TNFR1 can lead to either cell survival or proliferation via NF-κB or apoptosis via FADD. The activation of apoptosis by TNF appears to be cell type specific and depends on cross-talk between the components of the survival and death signaling pathways within the cell (266).

1.6.5. FANCC inhibits Fas and TNF mediated apoptosis in hematopoietic progenitor cells

Several groups have demonstrated that hematopoietic progenitor cells (HPCs) derived from the bone marrow of FA-C patients or Fancc/- mice are hypersensitive to the mitotic inhibitory effects of interferon-γ (IFN-γ) (185, 267, 268). IFN-γ suppresses growth of bone marrow progenitor cells and induces apoptosis at least in part through its capacity to induce Fas expression in these cells (269-271). The increased apoptosis in HPCs from FA-C patients and Fancc/+ mice after IFN-γ treatment is mediated by the Fas pathway because neutralizing anti-Fas antibodies inhibit IFN-γ hypersensitivity in these cells (267). Clonogenic growth of Fancc/+ derived HPCs is significantly inhibited by both TNF-α and Fas ligation compared to normal HPCs and is associated with increased apoptosis (268, 272). Fancc/+ mice bred against transgenic mice overexpressing TNF-α yield decreased numbers of erythroid precursor cells compared to purebred TNF-α transgenic mice (272). In contrast, HPCs from transgenic mice overexpressing the human FANCC gene are up to
10-fold more resistant to Fas induced apoptosis compared to normal mice (273). The ability of FANCC to modulate apoptotic responses to TNF-α and Fas ligand is not due to alteration of the expression of the TNFR superfamily of genes because neither constitutive nor IFN-γ induced expression of these receptors is influenced by the absence of a functional FANCC gene product in either human lymphoblast lines or Fancc−/− derived HPCs (274). These findings indicate that although FANCC suppresses apoptotic responses initiated by either TNF-α or Fas ligand, it does not do so by suppressing expression of the TNFR superfamily receptors. Therefore, the FANCC protein appears to modulate intracellular apoptotic signaling pathways initiated by either Fas ligand or TNFα.

1.6.6. Induction of apoptosis by growth factor withdrawal

Withdrawal of extracellular signals can induce apoptosis in many fetal and adult tissues (275). Growth factors and cytokines are considered to be key regulators of hematopoiesis, mainly by stimulating cell cycle progression, proliferation, and differentiation, as well as inhibiting apoptosis (276, 277). Interleukin-3 (IL-3) is a key cytokine that promotes the survival and proliferation of bone marrow-derived HPCs (278, 279). IL-3 is produced and secreted by activated T-cells and is capable of inducing the growth and differentiation of multipotential hematopoietic stem cells, neutrophils, eosinophils, megakaryocytes, macrophages, lymphoid and erythroid cells (280, 281). IL-3 belongs to a family of cytokines that include interleukin-5 (IL-5) and granulocyte/macrophage colony stimulation factor (GM-CSF). Although the IL-3/IL-5/GM-CSF cytokines can have distinct effects on different target cells, they can also stimulate cells responsive to all three cytokines by virtue of the fact that they share a common signaling receptor subunit, the common β chain (βc) (282). All three cytokine receptors consist of a cytokine-specific α chain (IL-3Rα, IL-5Rα and GM-CSFRα) linked to the βc subunit (282, 283). The α chains binds its cognate ligand with low affinity, whereas the βc subunit does not directly bind ligand, but when linked to the α chain, forms a high affinity signaling receptor (278, 284). The murine IL-3 receptor has an additional β chain that uniquely binds IL-3 (βIL-3) and exhibits extensive sequence homology with the βc subunit (285). In contrast, the human IL-3R only possesses the common β chain (286).
The binding of IL-3 to the IL-3 receptor results in dimerization of the IL-3α and βc subunits and the subsequent oligomerization of the entire IL-3 receptor with adjacent receptors (287). Although the IL-3 receptor does not possess intrinsic kinase activity, tyrosine phosphorylation of many cellular substrates is observed after receptor activation (279). The Janus tyrosine kinase, Jak2, is preassociated with the membrane proximal region of the βc chain and is activated following ligand-induced heterodimerization of IL-3α and βc (288). Activated Jak2 can then phosphorylate a number of substrates including the cytoplasmic region of the IL-3 receptor βc chain (288). The phosphorylated tyrosine residues of the receptor then serve as docking sites for Src homology 2 (SH-2) containing adaptor molecules, the most important of which are the STAT (signal transducers and activators of transcription) family of transcription factors (289). When brought in close proximity with activated Jak2, STAT5 becomes phosphorylated, dimerizes and then translocates to the nucleus and activates gene expression (290-292). IL-3 dependent growth is dependent on STAT5 activation because dominant negative versions of this protein interfere with proliferation and activation of immediate early response genes implicated in cell growth such as cyclin D1, c-myc, c-fos and c-jun (291, 293-295). Thus IL-3 induced proliferation is mainly mediated by the Jak2-STAT5 pathway.

In addition to activation of STATs, IL-3 also activates multiple signal transduction pathways involved in cell survival and suppression apoptosis, which include the Ras, Raf-1, mitogen activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) pathways (see Figure 1-2). Mutational studies have shown that the membrane distal domain of βc is important for mediating survival signals from the IL-3 receptor (296, 297). After IL-3 binding, the adapter protein Shc binds to the phosphorylated tyrosine 577 residue of βc (298). Phosphorylated Shc interacts with the Grb2 (growth-factor-receptor-bound protein-2) adaptor protein, which in turn interacts with mSOS (mammalian son of sevenless homologue), the GTP exchange factor that activates Ras (299-302). Activated Ras can then promote the sequential activation of Raf-1, MEK and MAP (ERK1 and ERK2) kinases (303). Activation of this cascade results in the increased expression of the transcription factors c-jun and c-fos and is important for mediating cell survival signals (297, 304). Other
Figure 1-2. Schematic representation of signal transduction pathways activated by the IL-3 receptor.
MAPKs are also activated by IL-3 signaling including p38 and JNK, although the mechanism through which these pathways are activated is not completely clear (305-307).

IL-3 also induces a rapid activation of phosphatidylinositol-3 kinase (PI3K) via the same region of the βc chain that is responsible for Ras activation (302, 308). This interaction appears to be mediated by a novel adaptor protein (p80), which interacts with βc and PI3K in IL-3 stimulated cells (309). PI3K may also be activated via a Ras-dependent pathway (310, 311). Activated PI3K phosphorylates inositol phospholipids, which then bind to and activate Akt kinases (312, 313). Akt can then phosphorylate Bad, a pro-apoptotic member of the Bcl-2 family of proteins (314, 315). Akt mediated phosphorylation of Bad at serine 136 is associated with the IL-3 response and is necessary for inhibition of the pro-apoptotic activity of Bad (314, 315).

Bad is a pro-apoptotic protein that is a member of the Bcl-2 family of proteins. The Bcl-2 protooncogene was initially identified as a cellular gene located at the site of a frequent chromosomal translocation in follicular B cell lymphomas (316, 317). The t(14;18) chromosomal translocation results in the juxtaposition of the Bcl-2 gene near the enhancer elements of the immunoglobulin heavy chain locus, which results in elevated expression of the Bcl-2 protein (317, 318). Deregulated Bcl-2 expression contributes to malignant transformation by blocking apoptosis in cells that are normally destined to die (319-322). Although the primary amino acid sequence of Bcl-2 is not homologous to any known enzymes, a number of related proteins have been isolated and together they constitute the Bcl-2 protein family (323). Functional studies have shown that the different members of the Bcl-2 protein family act to either suppress (Bcl-2, Bcl-XL, Mcl-1, A1) or promote (Bak, Bad, Bid, Bik, Bok, Bcl-Xα) apoptosis (323). The Bcl-2 proteins function in a concentration-dependent manner by forming homo- and heterodimers with other family members. For example, if excess Bcl-2 protein exists in the cell then the formation of Bcl-2/Bax heterodimers is favored and apoptosis is inhibited whereas Bax homodimers are formed and apoptosis occurs when the Bax protein is in excess (324).

Many of the Bcl-2 related proteins, including Bcl-2, Bcl-XL and Bax are localized to the outer mitochondrial membrane (325-327). The three dimensional structure of the Bcl-XL protein revealed strong similarities with the pore-forming domains of bacterial toxins such as diphtheria toxin and the colicins (328). Subsequent studies have shown that Bcl-XL, Bcl-
2 and Bax all have ion channel-forming ability when studied in artificial lipid membranes (329-331). The localization of Bcl-2 family members to the outer mitochondrial membrane and their ability to form pores has implicated these proteins in the regulation of the permeability of the mitochondria to ions and other large molecules. The pores formed by Bax exhibit properties different from those by Bcl-2 (331, 332). Furthermore, the channel-forming activity of Bax is inhibited by heterodimerization with Bcl-2 (331). Following IL-3 withdrawal, Bax homodimerizes, translocates to the mitochondria, where it causes loss of $\Delta \Psi_m$ and release of cytochrome c (333). Once cytochrome c is released from the mitochondria, it forms a complex with procaspase-9 and Apaf-1, which results in the processing of inactive procaspase-9 to active caspase-9 (222, 334). Active caspase-9 then promotes the processing and activation of other caspases, ultimately leading to apoptosis (207, 222).

The inhibition of apoptosis by IL-3 signaling is in part mediated by the anti-apoptotic members of the Bcl-2 family. Both Bcl-2 and Bcl-X_L block Bax-mediated $\Delta \Psi_m$ loss, cytochrome c release and activation of caspases (331, 335). The pro-apoptotic protein Bad forms heterodimers with either Bcl-2 or Bcl-X_L thereby freeing Bax to translocate to the mitochondria (336). Overexpression of Bad can inhibit the anti-apoptotic effects of Bcl-2 in IL-3 deprived cells (336). However, IL-3 signaling triggers Akt-mediated phosphorylation of Bad on serine 136, which promotes binding of Bad to 14-3-3 proteins (315, 337). Unphosphorylated Bad forms heterodimers with Bcl-2 and Bcl-X_L whereas the phosphorylated form is associated with 14-3-3 proteins (337). The sequestration of Bad by 14-3-3 frees up Bcl-2 and Bcl-X_L, allowing them to bind Bax and inhibit apoptosis. The importance of Bcl-2 and Bcl-X_L in promoting cell survival was shown by studies in which overexpression of either protein suppressed apoptosis following IL-3 withdrawal in various hematopoietic cell lines (319, 338-340).

There have been no direct studies examining the role of the FANCC protein in IL-3 mediated proliferation or suppression of apoptosis in hematopoietic cells. Inhibition of FANCC expression in wild-type HPCs by antisense ODN treatment represses in vitro colony growth of progenitor cells, even in the presence of exogenous growth factors including IL-3 (181). In addition, HPCs derived from FANCC transgenic mice are more resistant to Fas-mediated apoptosis (273). However, the effect of overexpression of
FANCC on cell survival following growth factor withdrawal has not been determined. Several studies examining the effect of ectopic expression of FANCC on the suppression of MMC-induced apoptosis have been performed using Epstein-Bar virus (EBV) immortalized lymphoblasts derived from FA-C patients (80, 236, 237). However, these findings are difficult to interpret due to the pleiotropic effects of MMC treatment and the possibility that the increased apoptosis observed in MMC treated FA cells is a secondary rather than primary defect. The generation of a hematopoietic cell culture model to examine the role of FANCC during IL-3 deprivation-induced apoptosis (a representative form of cell death found in vivo), could provide more informative clues about the function of this protein than apoptosis initiated by drug induced DNA damage.

1.7. Objectives of this study

IL-3 is an important cytokine that can maintain the growth and viability of HPCs (341). The role of FANCC in IL-3 mediated proliferation or suppression of apoptosis is unknown. Since the cloning of the FANCC gene, several studies have indicated that this gene is necessary for survival of HPCs, especially during cellular stress. The objective of this study was to (1) determine if overexpression of the FANCC protein affects survival in IL-3 deprived HPCs (2) Identify FANCC interacting proteins in HPCs and (3) determine the role of FANCC and its interacting protein partners during apoptosis induced by IL-3 withdrawal.

1.8. References


with Fanconi’s Anemia and Their Parents. *Biochem Biophys Res Commun.* 273: 899-901


84. Takeuchi, T., K. Morimoto. 1993. Increased formation of 8-hydroxydeoxyguanosine, an oxidative DNA damage, in lymphoblasts from Fanconi's anemia patients due to possible catalase deficiency. *Carcinogenesis.* 14: 1115-20


Classification of Fanconi anemia patients by complementation analysis: evidence for a fifth genetic subtype. **Blood.** 86: 2156-60


134. Buchwald, M. 1995. Complementation groups: one or more per gene?. **Nat Genet.** 11: 228-30


186. Tomkins, D. J., M. Care, M. Carreau, M. Buchwald. 1998. Development and characterization of immortalized fibroblastoid cell lines from an FA(C) mouse model. Mutat Res. 408: 27-35


caspase-3 in vitro and is cleaved by a caspase-3-like protease during Fas-mediated apoptosis. *Embo J.* 16: 6346-54.


Chapter 2

Suppression of apoptosis in hematopoietic factor-dependent progenitor cell lines by expression of the FANCC gene

The work described in this chapter forms the basis of the paper:
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I performed all the work presented in this chapter with the exception of the generation of the G1FASVNa retroviral packaging cell line which was supplied by J.M. Liu and the anti-FANCC antibody which was supplied by H. Youssoufian.
2.1. Abstract

The hypersensitivity to DNA cross-linking agents and chromosomal instability observed in FA cells has led to the traditional view that FA is a DNA repair disorder. However, the exact cellular defect in FA cells has not been identified. The FANCC protein lacks homology with other proteins and is primarily localized to the cytoplasm, indicating that FANCC may either play an indirect role in DNA repair or is involved in a cellular pathway independent of DNA damage. Several studies have demonstrated that cells lacking functional FANCC more readily undergo apoptosis following exposure to FasL, TNF, γ-IFN or MMC. However, the role of FANCC in apoptosis induced by growth factor deprivation is unknown. The results presented in this study showed that expression of FANCC in the hematopoietic factor-dependent progenitor cell lines 32D and MO7e by retroviral mediated gene transfer resulted in the suppression of apoptosis induced by growth factor withdrawal. Flow cytometry and morphological analysis of propidium iodide stained cells revealed significantly lower levels of apoptosis in FANCC-transduced MO7e and 32D cells following growth factor deprivation. Expression of FANCC in both cell lines promoted increased viability rather than proliferation, which is consistent with other apoptosis inhibiting genes such as Bcl-2. Three clonally derived 32D cell lines overexpressing FANCC displayed an identical anti-apoptotic phenotype. These findings imply that FANCC may act as a mediator of apoptosis initiated by growth factor withdrawal. Furthermore, the congenital malformations and hematological abnormalities characterizing FA may be related to an increased predisposition of FA progenitor cells to undergo apoptosis, particularly in the absence of extracellular signals.
2.2. Introduction

The production of mature blood cells is dependent on a small pool of self-renewing pluripotent stem cells located mainly in the bone marrow. Hematopoietic stem cells develop gradually from pluripotent to unipotent, committed progenitor cells and during this process they lose their self-renewal capacity (1). Hematopoiesis is a constant continuum in development since most mature blood cells are short lived and must be replaced throughout adult life. However, to maintain hematopoietic homeostasis, the expanded population of mature blood cells must be quickly eliminated after the presence of these cells is no longer required (2). The hematopoietic system is one of the most active cell systems in adult animals in which the rate of proliferation of mature blood cells is offset by an equivalent rate of apoptosis which ensures that hematopoietic homeostasis is maintained (3).

Viability and proliferation of hematopoietic cells is dependent on stromal cells or the presence of a family of soluble glycoproteins referred to as cytokines. The cytokine family includes the interleukins, interferons, colony-stimulating factors and erythropoietin (1, 4). In the absence of stromal cells or cytokines, hematopoietic cells cease to proliferate and die by apoptosis (5, 6). IL-3 is an important cytokine that has the capacity to ensure the survival and self-renewal of primitive erythroid and myeloid hematopoietic stem cells and at the same time stimulate the production of unipotent progenitors (7-9). Apoptosis occurs in IL-3 dependent hematopoietic cell lines or in primary cultures of IL-3 responsive bone marrow cells following IL-3 removal (5, 10). Therefore, IL-3 plays a critical role in stimulating proliferation and at the same time promoting survival of hematopoietic stem cells.

Apoptosis is known to be involved in many biological processes, including embryonic development and hematopoiesis (3, 11, 12). Withdrawal of extracellular signals can induce apoptosis in many fetal and adult tissues (11, 13). Although the diverse phenotypes of FA appear unrelated, the defects in FA cells may be explained by an increased predisposition to undergo apoptosis, particularly in the absence of extracellular signals. Indeed, several reports have shown that cultured FA lymphoblasts produce a reduced level of interleukin 6 (IL-6)
compared to normal lymphoblasts and that addition of IL-6 to the culture medium reduces
the sensitivity of FA cells to MMC and DEB and decreases the number of spontaneous
chromosomal breaks (14, 15). IL-6 has been shown to suppress apoptosis induced by
cytokine deprivation or cytotoxic drug exposure in plasma cells and p53-induced apoptosis in
myeloid leukemic cells (16-18).

Several studies have indicated that the FANCC protein may play a role in the survival
of hematopoietic progenitor cells. Antisense oligodeoxynucleotides (ODN) complementary
to FANCC mRNA inhibited the in vitro clonal growth of normal human erythroid and
granulocyte-macrophage progenitor cells, even in the presence of exogenous growth factors
(19). Peripheral blood CD34+ cells isolated from FA-C patients were either deficient in
colony growth or formed very few colonies in vitro, even in the presence of IL-3 and stem
cell factor (SCF) (20, 21). However, infection of CD34+ cells derived from FA-C patients
with either a recombinant adeno-associated virus or retroviral vector containing FANCC
cDNA, restored the growth of progenitor colonies formed in vitro (20, 21). These results
suggest that the FANCC gene may play a direct role in the survival or viability of
hematopoietic progenitor cells.

The ability of anti-apoptotic genes to suppress apoptosis, as in the case of Bcl-2 and
Bcl-X
t, have been accomplished by overexpressing such genes in factor dependent
hematopoietic cell lines (22-24). These experiments demonstrated the potential of a gene to
prevent or delay apoptosis after withdrawal of the growth factor. Similar studies have shown
that over expression of a pro-apoptotic gene like wild-type p53 in a myeloid progenitor cell
line can accelerate apoptotic cell death following IL-3 withdrawal (25). 32D is a myeloid
cell line established from a murine long term bone marrow culture and is dependent on IL-3
for survival (26, 27). MO7e is a human cell line derived from a megakaryocytic leukemia
and is dependent on either IL-3 or GM-CSF for survival (28, 29). Both cell lines die by
apoptosis if deprived of IL-3. The results presented in this study revealed that high
expression of the FANCC gene in 32D and MO7e cells using retroviral gene transfer delayed
the onset of apoptosis in both cell lines following IL-3 withdrawal, without the abolition of long term factor dependence.

2.3 Materials and Methods

Cell lines and culture conditions.

The immature murine myeloid cell 32D.3 is a subclone generated from the 32D C13 cell line (30), and was a gift from L. Penn (Ontario Cancer Institute, Toronto, Ontario, Canada). This cell line was grown in Iscove’s modified Dulbeco’s medium (IMDM) supplemented with 10% fetal calf serum and 1% conditioned media from X63Ag8-653 cells (which have been transfected with a murine IL-3 cDNA vector, and, thus constitutively secrete IL-3) (31). The human cell line, MO7e (29), is a subline of MO7 cells, originally isolated from a patient with acute megakaryocytic leukemia (28) and was a gift from J. Dick (The Hospital for Sick Children, Toronto, Ontario, Canada). This cell line was maintained in α-minimum essential medium (α-MEM) supplemented with 10% fetal calf serum and 10 units/ml recombinant IL-3 (Amgen, Toronto, Ontario, Canada). The retroviral packaging cell line PA317 (32) was grown in α-MEM supplemented with 10% FCS. All cell lines were tested and found to be free of mycoplasma.

Infection and subcloning of 32D cells.

The FANCC retroviral vector used to infect 32D cells (G1FASVNa) contains a 1.7 kb human FANCC cDNA insert including the entire open reading frame (ORF) but lacks any 5’ or 3’ untranslated sequences. The FANCC cDNA is under the control of the Moloney Murine Leukemia Virus (MoMuLV) 5’ LTR, and is linked in tandem with a SV40 driven Neomycin gene (21). The FANCC retroviral vector used to infect MO7e cells (pBabe-FAC) was based on the pBabe-Neo backbone (33) and consisted of a 2.4 kb FANCC cDNA insert containing the entire ORF and both 5’ and 3’ UTR sequences, under the control of the
MoMuLV 5' LTR, in addition to the Neo gene driven from an internal SV40 promoter. The FANCC cDNA insert was obtained by digesting the pFAC3 plasmid (34) with SmaI then subcloning this fragment into the SnaB1 restriction site of pBabe-Neo. Both vectors as well as pBabe-Neo were transfected into the amphotropic packaging cell line PA317 for production of retroviral particles. For infection of 32D cells, packaging cell lines were grown to near confluence and the culture supernatants were used for infection in the presence of 5 mg/ml polybrene (Aldrich, Milwaukee, WI) and IL-3. After infection, cells were grown for 48 hours, then plated at 5 X 10^5 cells/ml in Iscove's media, 20% FCS, 1% methyl cellulose (Fluka, Buchs, Switzerland), 3% X-63 conditioned media and 1 mg/ml G418 (GIBCO, Gaithersburg, MD). After 7 to 9 days discrete colonies were picked and expanded in media in the presence of IL-3. Only clones that showed intact proviral integration, as detected by southern blot hybridization, were kept for further studies. MO7e cells were infected using the culture supernatant from packaging cell lines in the presence of 7mg/ml polybrene and IL-3. Bulk populations of infected cells were then selected in G418 (1 mg/ml) and expanded in the presence of IL-3.

Western blotting analysis.

Cells were washed once with cold phosphate buffered saline (PBS) and lysed by addition of lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 0.2 mmol/L phenylmethylsulfonyl fluoride). Insoluble material was removed by centrifugation at 10,000g for 2 minutes at 4°C. Protein concentrations were determined by using the Bradford reagent as recommended by the manufacturer (BIO-RAD, Richmond, CA). After normalization for protein content, 20 μg of each sample was separated in reducing conditions in a 10% SDS-PAGE gel and electroblotted onto Immobilon-P membranes (Millipore, Bedford, MA). After blocking for 2 hours at room temperature in a solution of tris-buffered saline (TBS) containing 5% milk and 0.5% Tween-20, membranes were incubated with the primary antibody in blocking buffer for
an additional 2 hours. The affinity purified FANCC polyclonal antibody, which was made against a recombinant FANCC fusion protein (35), was used at a concentration of 0.6 μg/ml. Commercial polyclonal antibodies were used for the detection of p53 and Bcl-2 (Santa Cruz Research, Santa Cruz, CA) at a dilution of 1:2000. After washing, blots were probed with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham, Arlington Heights, IL) at a dilution of 1:10,000 followed by further washing and detection using the Enhanced Chemiluminescence (ECL) detection system (Amersham)

**Cell proliferation assay.**

Cell proliferation was measured by [3H]thymidine incorporation. Cells (1 X 10^5) were suspended in 24-well plates in triplicate 1 ml cultures. Plates were incubated at 37°C in the absence of growth factor. After the indicated time, cultures were pulsed with 1 μCi of [3H]thymidine (Amersham) and harvested 14 to 16 hours later on glass fiber filters. Following several washes with PBS, the filters were air-dried and the radioactivity in each sample was determined by liquid scintillation. Results were expressed as mean cpm ± SD of triplicate cultures.

**Cell viability assay.**

For factor deprivation studies, cells were first grown logarithmically in the presence of IL-3 for 48 hours. The cell lines were then washed thoroughly three times using PBS and resuspended in media (5 X 10^5 cells/ml) supplemented with 10% FCS but without IL-3, in replicates of three. At 24 hour time points, aliquots were removed, mixed 1:1 in trypan blue and counted using a hemocytometer. Viable cells were assessed by their ability to exclude trypan blue after visualization by light microscopy.

**Flow cytometry analysis.**

Cells were collected by centrifugation and fixed using 75% ethanol at 4°C for 30 minutes. Cells were then pelleted and resuspended in 0.5 ml PBS containing 0.1mg/ml
propidium iodide (Sigma, St. Louis, MI) and 0.6% NP-40 for one hour. DNA fluorescence was analyzed microscopically or by quantitative flow cytometry (FACScan, Becton Dickinson). Data shown is representative of three separate experiments.

Electrophoretic analysis of DNA fragmentation.

Cells (4 X 10^5) were pelleted and lysed using 30 ml of lysis buffer [20mM EDTA, 100mM Tris (pH 8.0), 0.8% (w/v) sodium lauryl sarcosinate, 5 mg proteinase K/ml]. After a two hour incubation at 50°C, 0.2 mg/ml RNase A was added and the lysate was further incubated at 37°C for 30 minutes. The resulting DNA solution was resolved by electrophoresis in a 1.2% agarose gel for 16 hours at 20 volts, followed by staining with ethidium bromide.

2.4. Results

2.4.1. Retroviral-mediated transfer of the FANCC gene into 32D and MO7e cells.

Transfer of the FANCC gene into 32D cells was accomplished using the supernatant from a cell line producing a previously described FANCC retroviral vector (21, 36). The Neomycin retroviral vector used as a control contained only the Neomycin resistance gene under the control of the SV-40 promoter (33). Both vectors were previously transfected into the packaging cell line PA317 and generated titers ranging from 1 to 3 X 10^6 infectious particles/ml. To minimize heterogeneity, transduced 32D cells were subcloned by selection in methylcellulose containing G418 (1 mg/ml). MO7e cells were infected with a retroviral vector similar to the vector used to infect 32D cells except that it contained a 2.4 Kb FANCC cDNA insert composed of the FANCC reading frame and portions of the FANCC 5' and 3'UTR (Fig. 2-1). Following viral infection, MO7e cells were grown in suspension and selected by neomycin resistance for 10 days. Due to the poor ability of MO7e cells to form
Figure 2-1. FANCC retroviral vectors. (A) The retroviral vector used to transduce 32D cells (G1FASVNa) contains the coding region of the FANCC cDNA under the control of the 5' MoMuLV LTR linked in tandem with a SV-40 driven Neomycin (Neo) resistance gene. (B) A similar vector was used to transduce MO7e cells (pBabe-FAC) is based on the pBabe-Neo vector and contains the FANCC cDNA with portions of the 5' and 3' untranslated region (UTR). Polyadenylation signals for both vectors reside within the 3' LTR.
clones, bulk populations of infected cells, MO7e-Neo and MO7e-FANCC, were used in all subsequent experiments.

To ensure correct viral integration, genomic DNA from transduced cell lines was extracted and digested with restriction enzymes that cleaved exclusively in both the 5' and 3' LTR. Southern analysis using a FANCC-specific probe revealed a 4.6 kb and a 7.0 kb band in 32D and MO7e cells respectively, only in FANCC transduced cells, indicating an unrearranged integrated proviral genome (data not shown). Western blotting was performed to determine if FANCC transduced cells produced increased amounts of FANCC protein in the presence of IL-3 (Fig. 2-2). In addition, protein extracts of the parental and transduced cell lines were examined at various times following IL-3 deprivation. A rabbit polyclonal antibody produced against a FANCC bacterial expression product, which recognizes both human and mouse FANCC protein (35), was utilized to detect the retrovirally derived and endogenous FANCC protein. The parental 32D cell line produced little detectable endogenous Fancc protein in the presence or absence of IL-3, whereas FANCC-transduced 32D clones (32D-FANCC) produced abundant amounts of both a 60 and 55 kD protein product (Fig. 2-2A). The 60 kD protein conforms to the previously recognized molecular weight of FANCC (35, 37), whereas the 55 kD product may represent a smaller product derived from a potential downstream inframe translational start site (38). FANCC-transduced MO7e cells (MO7e-FANCC) had similar levels of the 60 kD FANCC protein as the parental cell line (Fig. 2-2B). However, in this case the protein was constitutively expressed following IL-3 withdrawal while the endogenous levels of FANCC in the parental cell line decreased until no detectable levels of FANCC were seen past the 72 hour time point.

2.4.2. FANCC-retroviral transduced cells have increased viability after IL-3 withdrawal.

To examine the effect of FANCC expression on cell viability following IL-3 withdrawal, control cells and FANCC-transduced cells were grown logarithmically for
Figure 2-2. Western blot analysis of FANCC expression in parental and FANCC-transduced cell lines. (A) FANCC expression in 32D cells and two FANCC subclones D2 and D5, in the presence of IL-3 (+) and following factor deprivation for the indicated times. Strong expression of a 60 kDa and 55 kDa FANCC protein products were seen only in the FANCC subclones with no detectable expression in the parental cell line. (B) Expression of a 60 kDa FANCC protein in MO7e and MO7e-FANCC cells before (+) and after IL-3 withdrawal. Only MO7e-FANCC cells constitutively expressed FANCC following factor deprivation. All immunoblots were probed with an anti-FANCC polyclonal antibody (0.6 μg/ml).
several days, then were thoroughly washed and resuspended in media lacking IL-3, in replicates of three. At 24 hour periods afterwards, cell viability was determined by trypan blue dye exclusion. 32D clones overexpressing FANCC showed increased viability compared to Neo controls (Fig. 2-3A). After two days, IL-3 deprived FANCC clones had between 40% and 60% cell survival whereas Neo clones had less than 5% viability. Unlike 32D-Neo cells, the majority of MO7e-Neo cells did not die within the first 48 hours, but rather after 48 hours. Following factor deprivation MO7e-FANCC cells had dramatically higher viability than MO7e-Neo cells (Fig. 2-3B). MO7e cells constitutively expressing FANCC exhibited 75% viability up to 4 days following IL-3 withdrawal whereas MO7e-Neo cells exhibited less than 20% viability. Three independent pools of Neo and FANCC-transduced MO7e cells were analyzed for increased survival following IL-3 withdrawal, with minimal variability seen between pools (data not shown).

To determine if the increased survival of FANCC-transduced cells was due to an enhancement of cellular proliferation, [³H]thymidine incorporation was monitored after IL-3 starvation. Both 32D cells overexpressing FANCC and mock infected cells displayed similar decreasing levels of [³H]thymidine incorporation (Fig. 2-3C). MO7e-Neo and MO7e-FANCC cells also showed similar decreasing levels of [³H]thymidine incorporation following IL-3 withdrawal, with MO7e-FANCC cells having only slightly higher levels of incorporation than control cells (Fig. 2-3D). Thus, the enhancement of cellular viability in FANCC-transduced cells is not due to an effect on proliferation, since both Neo and FANCC-transduced cells displayed features of growth arrest following factor withdrawal.

2.4.3 The increased viability of FANCC-transduced cells following IL-3 withdrawal is due to a delay in the onset of apoptosis.

To determine if the increased viability of FANCC-transduced cells, following IL-3 deprivation, was due to a suppression of apoptosis, flow cytometric analysis of propidium iodide-stained cells was performed. Apoptotic cells exhibited less propidium iodide
Figure 2-3. Retroviral mediated FANCC expression enhances viability without affecting cellular proliferation following IL-3 withdrawal. Survival of 32D (A) and MO7e (B) cells, transduced with either Neo or FANCC retroviral vectors, following IL-3 deprivation. Cells were thoroughly washed in PBS and resuspended in media lacking IL-3. Viability was determined at the indicated time points by trypan blue exclusion. The percent cell survival was determined by dividing the number of viable cells per milliliter by the initial cell concentration. Data expressed as mean ± SD of triplicate cultures.[^3H]thymidine uptake after IL-3 withdrawal in 32D (C) and MO7e (D) cells infected with either Neo or FANCC retroviral vectors. A total of $1 \times 10^5$ cells were seeded/well at time 0. Data expressed as mean ± SD of triplicate cultures.
Figure 2-4. DNA fluorescence histograms of propidium iodide-stained FANCC-transduced cells following cytokine deprivation. 32D (A) and MO7e (B) cells (1 X 10^4 cells in total), transduced with either Neo or FANCC retroviral vectors, were analyzed in each histogram. The percentage of apoptotic cells (sub-diploid peak) is indicated in brackets.
fluorescence than viable cells, owing to chromatin condensation and DNA cleavage, resulting in a subdiploid population. Following IL-3 withdrawal, all cells showed G1 arrest as indicated by the strong peak at a fluorescence intensity of 350 (Fig. 2-4). 32D cells expressing high levels of FANCC showed significantly fewer apoptotic cells than the Neo control, as shown by a comparison of the sub-diploid peaks (Fig. 2-4A). At 24 hours after IL-3 withdrawal, 32D-FANCC cells had fewer than 10% apoptotic cells, whereas 32D-Neo cells had levels of apoptosis ranging from 25% to 40%. Similarly, at 48 hours FANCC clones had approximately 50% fewer apoptotic cells than Neo clones. MO7e-Neo cells did not show significant levels of apoptosis until 48 hours after IL-3 withdrawal (Fig. 2-4B). At this time, approximately 50% of MO7e-Neo cells were apoptotic, whereas 25% of MO7e-FANCC cells displayed apoptotic fragmented DNA. At 72 hours of IL-3 deprivation, a similar 50% reduction in apoptotic cells was seen in the FANCC-transduced cells compared to the Neo control.

Propidium iodide stained 32D cells were also analyzed by fluorescence microscopy. Cells displaying characteristic features of apoptosis, such as condensed chromatin and subnuclear bodies, constituted approximately 65%±5% of the 32D-Neo clones deprived of IL-3 for 24 hours while significantly fewer (33%±11%, p<0.02) apoptotic cells were observed in parallel cultures of 32D-FANCC clones (Fig. 2-5).

To further demonstrate that 32D-FANCC clones had reduced levels of apoptosis, gel electrophoresis of genomic DNA was performed. Apoptosis typically results in degradation of nuclear DNA into fragments of integral multiples of the nucleosomal distances (~180bp) (39). Lysates of cells deprived of IL-3 for 18 hours were resolved by agarose gel electrophoresis and were then stained with ethidium bromide. 32D-Neo clones had marked DNA fragmentation resulting in the characteristic "DNA laddering" pattern indicative of apoptosis (Fig. 2-6). In contrast, 32D-FANCC clones showed little apoptotic DNA fragmentation.
Figure 2-5. 32D-FAC cells exhibit fewer morphological features of apoptosis following IL-3 withdrawal than 32D-Neo cells. Cells deprived of IL-3 for 24 hours were fixed in ethanol, stained with propidium iodide and visualized by fluorescence microscopy. 32D-Neo cells had significantly more apoptotic cells than 32D-FANCC cells. Apoptotic cells were identified by characteristic features such as chromatin condensation and nuclear fragmentation (arrows).
Figure 2-6. 32D-FAC cells have reduced levels of internucleosomal DNA cleavage after IL-3 withdrawal. 32D-Neo and 32-FANCC cells were deprived of IL-3 for 18 hours, then cell lysates were analyzed by agarose gel electrophoresis. Internucleosomal cleavage results in the appearance of 200 base pair incremental DNA fragments (DNA laddering), a hallmark of apoptosis. Lane 1: 1 Kb DNA ladder, Lanes 2 and 3: clones Neo-A3 and FANCC-D2 growing logarithmically in the presence of IL-3, Lanes 4-7: clones Neo-A3, FANCC-D2, FANCC-D5 and FANCC-E1, respectively, in the absence of IL-3.
2.4.4 Overexpression of the FANCC gene does not alter expression of either Bcl-2 or p53

Overexpression of the anti-apoptotic gene Bcl-2 has been shown to inhibit or delay apoptosis in IL-3 deprived hematopoietic cell lines (22, 23, 40). FANCC mediated suppression of apoptosis may act by up-regulating levels of the Bcl-2 protein. To examine this hypothesis, Bcl-2 protein levels were monitored by immunoblot analysis after IL-3 deprivation (Fig. 2-7A). Although Bcl-2 levels declined in 32D cells, a similar decrease was also observed in 32D-FANCC cells following IL-3 deprivation. Bcl-2 protein levels in MO7e cells slightly decreased over a long period of time after removal of IL-3. However, MO7e-FANCC cells also displayed a similar pattern of Bcl-2 expression. Thus, overexpression of FANCC does not affect the expression of Bcl-2 following IL-3 deprivation.

Down-regulation of wild-type p53 by expression of a dominant-negative p53 mutant protein has been shown to delay apoptosis in IL-3 dependent cells following factor deprivation (41). The effect of FANCC expression on endogenous p53 levels was monitored by immunoblot analysis. Levels of p53 were not significantly affected in 32D or 32D-FANCC cells in the presence or absence of IL-3 (Fig. 2-7B). p53 expression was slightly reduced in MO7e cells expressing FANCC in the presence of IL-3 compared to the parental cell line (Fig. 2-7B). However, p53 expression decreased both in MO7e and MO7e-FANCC cells, especially after 72 hours of IL-3 deprivation. Thus, p53 expression is not significantly affected by FANCC after initiation of apoptosis by factor withdrawal.

2.5. Discussion

Elucidation of the basic cellular defect in FA has been difficult. Analysis of both the FANCC nucleotide and amino acid sequences has provided no clues about the function of this gene. Furthermore, the FANCC protein has been localized primarily within the cytoplasm, indicating that FANCC is unlikely to play a direct role in DNA repair or chromosomal stability (35, 37). The data presented here suggests that the FANCC gene is
A

Bcl-2

+ 0 5 12 24 48 hrs

32D

32D FANCC-D2

+ 0 24 48 72 96 120 hrs

MO7e

MO7e-FANCC

B

p53

+ 24 48 hrs

32D

32D FANCC-D2

+ 24 48 72 96 120 hrs

MO7e

MO7e-FANCC
Figure 2-7. Overexpression of FANCC does not affect either Bcl-2 or p53 expression after IL-3 deprivation. Expression of Bcl-2 (A) and p53 (B) was examined in both 32D and MO7e cell lines in the presence (+) and absence of IL-3 at the indicated time points. Whole cell extracts were normalized for protein content using the Bradford Assay and 25 µg of each cell lysate was resolved by 10% SDS-PAGE. Bcl-2 and p53 proteins were detected by immunoblotting using specific polyclonal antibodies for each protein.
important in regulating the apoptotic pathway initiated in hematopoietic cells in response to growth factor deprivation.

Initial examination of FANCC expression using PCR and reverse transcribed RNA, revealed ubiquitous low level expression in all adult human tissues examined (34). However, RNA in situ hybridization analysis of developing mouse embryos detected high levels of Fancc transcripts in osteogenic progenitor cells in the perichondrial layer of developing bones, mesenchyme of the spinal cord and in the ependymal cell layer of the developing brain (42, 43). High levels of Fancc transcripts have also been detected in early pluripotent hematopoietic progenitor cells in a pattern similar to that of Bcl-2 (44). Thus, up-regulation of Fancc expression in a specific subset of cells appears to play a role in embryogenesis and hematopoietic development.

Overexpression of FANCC cDNA appears necessary to delay the onset of apoptosis in 32D cells. Interestingly, the parental 32D cell line expressed little endogenous Fancc protein. Retrovirally mediated expression of human FANCC resulted in the production of two protein products. The 60 kD product corresponds to the full length 558 amino acid protein, whereas the 55 kD product is either a cleavage product or a smaller protein resulting from an internal translation initiation at either methionine 48 or methionine 55. The latter hypothesis is favoured since in vitro transcription-translation experiments revealed both 60 kD and 55 kD bands (45). In addition, FA patients with a mild form of the disease have a missense mutation in exon 1 (delG322), and have reinitiation of translation at methionine 55 (38). The amino terminal truncated protein produced in patients with the delG322 mutation, can partially correct the MMC sensitivity of FA-C lymphoblasts (38).

The amount of FANCC protein in 32D-FANCC cells, appeared to correlate with the degree of inhibition of apoptosis. The parental cell line 32D expressed low levels of endogenous Fancc and displayed marked levels of apoptosis by 24 hours (Fig. 2-4A). The amount of FANCC protein in both the D2 and D5 clones was abundant in the presence of IL-3 (Fig. 2-2A). However, following factor deprivation, FANCC levels dropped off until only
minimal levels of FANCC were apparent at 48 hours. A significant reduction in cell viability was observed at this time point in FANCC-transduced 32D cells (Fig. 2-2A) with an increase in apoptotic death (Fig. 2-3A).

Retroviral-mediated expression of FANCC in MO7e cells did not result in a significant increase of FANCC protein above endogenous levels (Fig. 2-2B). *In vitro* protein expression studies in different human cell lines has revealed that expression of wild-type FANCC via both retroviral and episomal vectors results in similar or only slightly higher levels of FANCC protein when compared with its endogenous counterpart (46, 47). Post-translational mechanisms may prevent expression of high FANCC levels in certain cell types. Nevertheless, when comparing FANCC expression in MO7e-FANCC cells and the parental cell line, MO7e-FANCC cells constitutively express FANCC following IL-3 withdrawal, whereas MO7e cells display a marked reduction of protein until little or no FANCC is detectable at 120 hrs. Once again the level of FANCC is inversely related to the level of apoptosis. In the presence of IL-3 both MO7e and MO7e-FANCC cells have approximately equal levels of FANCC. However, following IL-3 withdrawal, MO7e cells have a significant reduction in FANCC levels by 48 hours whereas minimal reduction is seen in MO7e-FANCC cells. Comparing cell survival at 48 hours in Fig. 2-3, MO7e-Neo cells had 50% viability whereas MO7e-FANCC cells had 90% viability. The increased survival of FANCC-transduced cells at 48 hours was related to a suppression of apoptosis since levels of apoptotic DNA were 50% in mock infected cells, whereas only 25% in MO7e-FANCC cells (Fig. 4B). The trend of constitutive FANCC expression and suppression of apoptosis continues up to 120 hours after factor deprivation in MO7e-FANCC cells. 32D cells may die more readily than MO7e cells following IL-3 starvation, due to the fact that they express little or no endogenous Fancc, whereas MO7e cells express abundant amounts under normal growth conditions. Although FANCC expression suppressed the early onset of apoptosis in both 32D and MO7e cell lines, this suppression was not indefinite and long term factor-dependence was not abolished. Both 32D-FANCC and MO7e-FANCC cell lines stopped
proliferating following IL-3 withdrawal (Fig. 2-3C and 2-3D). Thus, FANCC expression promoted increased viability rather than proliferation.

The ability of FANCC to delay the onset of apoptosis in factor deprived cells closely resembles that of Bcl-2. Overexpression of Bcl-2 in IL-3 dependent cell lines has been shown to delay the onset of apoptosis after IL-3 deprivation, without leading to factor-independence (22, 40). Comparison of the survival curves of 32D cells overexpressing FANCC (Fig. 2-3A) with previously published survival curves of 32D cells expressing high levels of Bcl-2, reveals similar profiles following IL-3 deprivation (22, 48). Thus, FANCC can inhibit apoptosis in factor deprived 32D cells at a level comparable to that of Bcl-2. However, the ability of FANCC to suppress apoptosis is independent of Bcl-2 because no change in the BCL-2 protein expression pattern was seen in either control or FANCC-transduced cells, both in the presence or absence of IL-3 (Fig. 2-7A). Thus, FANCC expression does not upregulate BCL-2 protein levels. Although p53 has been implicated in growth-factor withdrawal induced apoptosis (25, 41), FANCC had no significant effect on p53 levels following IL-3 deprivation (Fig. 2-7B). Thus FANCC-mediated suppression of apoptosis in IL-3 deprived cells is independent of both Bcl-2 and p53.

Artificially overexpressing FANCC in hematopoietic precursor cell lines and preventing apoptosis may mimic the physiological role this gene plays in maintaining the viability of bone marrow precursors. Overexpression of FANCC, using a recombinant adeno-associated virus, in hematopoietic progenitor cells isolated from a FA-C patient, resulted in a dramatic increase in the growth potential of these cells (20). Conversely, inhibition of FANCC expression, using antisense oligodeoxynucleotides complementary to FANCC mRNA, inhibited the in vitro clonal growth of normal bone marrow cells (19). In one study, FA lymphoblasts were shown to die by apoptosis in response to MMC treatment, whereas expression of FANCC in FA-C lymphoblasts alleviated the MMC hypersensitivity of these cells and their predisposition to undergo apoptosis (49). However, these experiments did not determine if the primary defect of FA cells is an increased general predisposition
towards apoptosis or whether the increased apoptosis reflects the fact that FA cells are simply more sensitive to MMC. If the latter were the case, exposure of FA-C and wildtype cells to equitoxic doses should lead to similar levels of apoptosis, and it could be argued that the principal defect in FA cells is an inability to repair DNA damage or detoxify DNA damaging agents while apoptosis occurs as a consequence of these primary defects. In contrast to experiments using MMC, the evidence presented here demonstrates that expression of FANCC in both 32D and MO7e cells delays the onset of apoptosis following IL-3 deprivation, independent of an FA phenotype. Therefore, FANCC appears to act directly in an apoptotic pathway initiated in response to a normal physiological mechanism, that is, factor deprivation, as opposed to apoptosis initiated by drug induced DNA damage.

Apoptosis plays an important role in hematopoietic homeostasis by ensuring that the rate of new cell production in self-renewing progenitor cells is offset by a commensurate rate of cell death (3). The FANCC protein may act as a regulator by suppressing apoptosis in hematopoietic precursor cells during the short term factor deprivation that may accompany normal fluctuations of cytokines in the marrow microenvironment (50).

2.6. References


regulate the cell cycle status of very primitive hematopoietic cells in long-term human marrow cultures. II. Analysis of positive and negative regulators produced by stromal cells within the adherent layer. *Blood.* 78: 110-7
Chapter 3

Redox regulation of GSTP1 by FANCC leads to suppression of apoptosis in hematopoietic cells

The work described in this chapter forms the basis of the paper: Robert C. Cumming, Jeff Lightfoot, Kristin Beard, Hagop Youssoufian, Peter J. O’Brien and Manuel Buchwald. Redox regulation of GSTP1 by FANCC prevents apoptosis in hematopoietic cells. (Manuscript submitted)

I performed all of the work described in this chapter with the exception of the analysis of glutathione levels which was performed by K. Beard, the construction of the GST-mFancc fusion vector which was performed by J. Lightfoot and the generation of the anti-FANCC antibody which was supplied by H. Youssoufian
3.1 Abstract

Overexpression of the FANCC protein in hematopoietic cells delays the onset of apoptosis following growth factor withdrawal by an unknown mechanism. In order to elucidate the role of the FANCC protein in apoptosis, affinity purification of FANCC interacting proteins was performed using large scale lysates from 32D cells. A 25 kDa protein was isolated and identified as murine Glutathione S-Transferase P1-1 (GSTP1) by microsequencing. The FANCC specific binding of GSTP1 was confirmed by co-immunoprecipitation studies. Overexpression of GSTP1 by retroviral mediated gene transfer significantly delayed the onset of apoptosis in 32D cells following IL-3 deprivation. Coexpression of both FANCC and GSTP1 in 32D cells conferred dramatically higher resistance to apoptosis compared to cells expressing FANCC or GSTP1 alone. GST activity was significantly elevated in 32D cells overexpressing FANCC, particularly after IL-3 deprivation. Furthermore, overexpression of either FANCC or GSTP1 prevented the loss of the endogenous antioxidant glutathione (GSH). However, depletion of GSH, using an agent that inhibits its synthesis, abolished the ability of GSTP1 but not FANCC to inhibit apoptosis. Cytoplasmic proteins predominantly exist in a reduced state due to the presence of abundant levels of GSH. However, the efflux or oxidation of GSH that occurs during apoptosis can create an environment that may promote the formation of non-native disulfide bonds (protein oxidation). FANCC functions by inhibiting the oxidation of GSTP1 during apoptosis, even in the absence of GSH. This is the first example that a cytoplasmic protein such as GSTP1 can be inactivated by the generation of non-native disulfide bonds during apoptosis. Moreover, the prevention of disulfide bond formation within GSTP1 by FANCC, a protein lacking homology to thiol-disulfide reductases, reveals a new pathway for redox regulation of proteins.
3.2 Introduction

The FANCC gene product does not have any known functional motifs and the precise role of this protein is ill defined. Although the FANCC protein is part of a complex that includes other FA proteins, the mechanism by which this complex maintains chromosomal stability and protects cells from cross-linking agents and oxygen induced cytotoxicity is unknown (1-3). Furthermore, the other FA genes (FANCA, FANCE, FANCF and FANCG) lack homology to other known genes or proteins and have yielded few clues about the pathophysiology of FA (4-7). However, the identification of non-FA proteins that bind to FANCC has provided some insight into the function of this protein. Recent studies have shown that FANCC binds to several cytosolic proteins, including the molecular chaperone GRP94, nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P450 reductase (P450RED) and the cyclin dependent kinase cdc2 (8-10). The interaction between FANCC and P450RED is of particular interest because MMC, an agent that FA cells are highly sensitive towards, requires bioreductive activation by cellular enzymes including P450RED (11, 12). FANCC binds to the amino-terminal domain of P450RED and inhibits its catalytic activity, suggesting that FANCC may affect drug metabolism (9).

P450RED belongs to the Phase I category of drug metabolizing enzymes that catalyze the bioreduction of a wide range of lipophilic xenobiotics to toxic or carcinogenic electrophilic intermediates (13, 14). Phase II enzymes then detoxify these bioactivated drugs by frequently catalyzing conjugation reactions, creating more water soluble compounds, which are generally less toxic and more readily excretable (15). Glutathione S-transferases (GSTs) are classic Phase II enzymes which catalyze the conjugation of the endogenous nucleophile glutathione (GSH) with electrophilic xenobiotics (16). In addition, GSTs also possess selenium-independent GSH peroxidase activity and play an important role in the detoxification of lipid and nucleic acid hydroperoxides produced during oxidative stress (17, 18). Therefore, GSTs are important for detoxifying both endogenous and exogenous electrophilic compounds that would otherwise cause widespread cellular damage.

Mammalian cytosolic GSTs are classified into five main distantly related classes (designated alpha, mu, pi, sigma and theta) on the basis of their immunochemical properties and primary structure relatedness (16). The amino acid sequence identity between any two members within a class is typically greater than 70%, whereas an identity of less than 30% is generally observed between classes (16). These enzymes exhibit broad and overlapping
substrate specificities, however, some enzyme classes are more selective for certain substrates. For example, ethacrynic acid is a preferred substrate for pi-class GSTs, although at high concentrations it is an irreversible inhibitor (19, 20). The GST pi isozyme P1-1 (GSTP1) is of particular interest because of its expression is elevated in many tumor tissues relative to matched normal tissue (21). Increased GSTP1 expression has also been associated with preneoplasia and chemoresistance (22, 23). Recently, elevated expression of several GST isozymes have been implicated in resistance to apoptosis. These GSTs have been shown to suppress cell death induced by chemotherapeutic agents or ionizing radiation in mammalian cells and Bax expression in yeast cells (24-26). Elevated GST expression has also been implicated in p53 induced apoptosis (27). The association between elevated GST expression and inhibition of cell death may be related to the ability of these enzymes to detoxify products produced during the oxidative stress associated with apoptosis.

Oxidative stress has been implicated as an important mediator of apoptosis and degenerative diseases (28-30). Exposure of cultured cells to agents that produce reactive oxygen species (ROS), such as ionizing and ultraviolet radiation, H₂O₂ or chemotherapeutic agents, leads to apoptosis (31-33). Other apoptotic stimuli, such as treatment with TNFα, anti-Fas antibodies or growth factor withdrawal can stimulate ROS production (34-36). Inhibiting ROS formation by the addition of antioxidants or overexpression of thioredoxin (a thiol-disulfide reductase), can block or delay apoptosis, indicating that ROS are essential for signaling apoptosis (34, 35, 37, 38). GSH is the most abundant low molecular weight thiol within the cell and has a high capacity to neutralize free radicals, reduce disulfide bonds within proteins and detoxify xenobiotics (39, 40). The induction of apoptosis by activation of the Fas pathway or by growth factor withdrawal causes a significant decrease or efflux of GSH (41-43). Inhibition of GSH depletion by the addition of exogenous GSH or the GSH precursor N-acetylcysteine (NAC) blocks or delays apoptosis (41, 44, 45). In apoptotic resistant cells several genes involved in GSH metabolism are upregulated, leading to elevated levels of GSH within these cells (25). Therefore the redox state of the cell can strongly influence whether cells will undergo apoptosis or survive.

Overexpression of FANCC has been shown to suppress apoptosis induced by Fas-ligation or IL-3 withdrawal in hematopoietic progenitor cells (46, 47). However, the mechanism by which the FANCC protein inhibits apoptosis in hematopoietic cells is unknown. In order to elucidate the function of FANCC during apoptosis, FANCC
interacting proteins were purified using lysates from the factor dependent hematopoietic cell line 32D. In this study, the FANCC protein was shown to interact with and stabilize GSTP1 during IL-3 deprivation-induced apoptosis. Elevated expression of GSTP1 by either FANCC-mediated stabilization or by retroviral gene transfer of GSTP1 into 32D cells leads to suppression of apoptosis following factor withdrawal. FANCC appears to prevent the formation of disulfide bonds within GSTP1 during apoptosis, even under highly oxidative conditions. These findings reveal unique roles for FANCC as a redox regulator of proteins and GSTP1 as a newly indentified anti-apoptotic protein.

3.3. Materials and Methods

Cell lines and Culture Conditions

The 32D.3 cell lines overexpressing either the FANCC gene (originally designated 32D-FAC D2 and 32D-FAC E1) or the neomycin resistance gene (32D-Neo) were described previously (47). These cell lines were grown in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% fetal calf serum (FCS) and 1% (vol/vol) conditioned medium from the IL-3 producing cell line X63Ag8-653 (48) and continuously selected in 500 μg/ml G418 (Gibco). Epstein-Barr virus (EBV) transformed normal (HSC93) and FA-C (HSC622) lymphoblasts were maintained in RPMI media supplemented with 15% FCS. The retroviral packaging cell line PA317 (49) was maintained in α-minimum essential medium (α-MEM) supplemented with 10% FCS.

Generation and Purification of a Murine Fancc Fusion protein

A hybrid polypeptide was created consisting of full-length schistosoma japonicum GST (sjGST) at the amino terminus and all but the first amino acid of murine Fancc (GST-mFancc). The plasmid pmfac2 (50) was used as a source of the Fancc cDNA. A 5' ORF sequence of was amplified with forward and reverse oligonucleotide primers, respectively, as follows: 5'EcoR1-fac (5'-TCCCCCGGAAATTCTGGCTCAGGAGTCTGCAGACCTTGCT-3') and 3'BamH1-fac (5'-AACCCCGGATCCACGAGTTAAGGTATTAATCAACC-3'). The 340 bp product was subsequently digested with EcoR1 and BamH1. A 3' ORF sequence was amplified with forward and reverse oligonucleotide primers, respectively, as follows: 5'Ase1-fac (5'-'AGCTGAAGGTATTAATCAACC-3') and 3'EcoR1-fac (5'-ACGGAATTCCCTAGACCTGGGC-
TTGACAGCTCCTTAGGA-3'). The 320 bp product was then digested with AseI and EcoR1. The rest of the ORF was gel purified as a 1 kb BamH1/AseI fragment from pmfac2. All three fragments were ligated, and the resulting 1.7 kb mfac ORF was gel purified. The complete fragment was then ligated into the EcoR1 site of the prokaryotic expression vector pGEX2TK (Pharmacia) (51).

GST-Fancc was used to transform Escherichia coli strain BL21. Three liters of transformed cells were grown to an OD_{600} of 0.6 at 37°C and induced with 1mM isopropyl B-D-thiogalactopyranoside for 4 hrs. Bacteria pelleted by centrifugation were resuspended in 100 ml PBS, sonicated and clarified by centrifugation at 10,000 X g for 20 min. Approximately 2 ml of a 50% slurry (wt/vol) of glutathione sepharose was incubated with the sonicate for 3 hrs at 4°C. After extensive washing with PBS, bound fusion protein was eluted with 20 mM reduced glutathione (Sigma) overnight at 4°C. Approximately 1.2 mg of fusion protein per ml of swollen glutathione-sepharose beads was obtained. The eluted fusion protein was dialyzed against 0.1M HEPES (pH 7.0) and 0.1M NaCl, and was coupled to 0.1 ml (bed vol) Affigel 15/10 (2:1 ratio; Bio-Rad), according to the instructions of the manufacturer.

### Purification and Amino Acid Sequencing of a Fancc Associated Protein

The procedure for isolating Fancc associated proteins was based on an affinity purification strategy used to isolate tumor necrosis factor receptor (TNF-R2) associated proteins (52). Approximately 4 X 10^8 32D.3 cells were harvested and washed twice with cold PBS. The cells were lysed on ice in 8 ml of lysis buffer containing 50 mM Hepes (pH 7.0), 100 mM NaCl, 0.1% NP-40, 10% glycerol, 2 mM EDTA, 0.1mM PMSF, 1ug/ml benzamidine and 1 ug/ml aprotinin. After 30 min, the lysate was sheared by passing it through a 25.5 gauge needle 10 times. Cell debris was then clarified by centrifugation at 18,000 X g for 10 min at 4°C. The supernatant was then dialyzed against lysis buffer containing 500 mM NaCl overnight at 4°C. All subsequent purification steps were performed in lysis buffer containing 500 mM NaCl. The dialyzed cell lysate was precleared for 2 hr at 4°C using sjGST linked to an Affigel matrix (BioRad). The flow through was divided; half was applied to a 0.1 ml Affigel GST-Fancc fusion protein affinity column and as a control, the other half was passed through as similar column containing the sjGST fusion element only. After running the precleared lysate through the columns, the absorbance (280
nm) of the wash fractions was monitored until no protein was detectable. Bound proteins were eluted with 1.5 ml of ImmunoPure Gentle Ag/Ab Elution Buffer (Pierce, Rockford, IL). The elution fractions were precipitated with methanol-chloroform and resuspended in 50 µl Laemmli SDS sample buffer. A portion of each precipitated fraction (5µl) was separated by 4–20% SDS-PAGE under reducing conditions and visualized by silver staining.

The remaining portion of the elution fractions were separated by 10% SDS-PAGE, electroblotted onto PVDF sequencing membrane (Millipore) and the proteins were visualized by Coomassie brilliant blue R-250 (Sigma). A 25 kDa band identified on the PVDF membrane was excised and subjected to N-terminal analysis on a Porton Gas-phase Microsequencer Model 2090 equipped with a phenylthiohydantoin microanalyzer. The sequence obtained (Pro-Pro-Tyr-Thr-Ile-Val-Tyr-Phe-Pro-Val) had 100% homology to murine Glutathione S-Transferase P1-1 (Gstp1) (53).

**Construction of GSTP1 retroviral vectors**

Total RNA (3 µg) from the lymphoblast cell line HSC 93 was reverse-transcribed in a total volume of 20 µl using 1X first strand buffer (Gibco) 0.5 mM of each dNTP, 100 nmol of oligo(dT), 10 nM DTT and 200 units of SuperScript reverse transcriptase (Gibco). After 60 min at 37°C, the reaction mixture was heated at 95°C for 5 min then cooled on ice. Then 5 µl of the reaction mixture was added to 50 µl of 1X PCR buffer (Perkin Elmer) containing 50 pmol of each primer and 2 units of Vent polymerase (Perkin Elmer). The PCR was run for 30 cycles (with one full cycle consisting of three time and temperature settings as follows: 30 s denaturing at 95°C, annealing for 45 s at 58°C, and primer extension for 1 min at 72°C) after an initial step of 5 min at 95°C. The oligonucleotide primer pairs used for the PCR reaction (5’-CGGGAATTCCAGTCTTCCACCATGC-3’ and 5’ GACGAATTCGGTCTTGGAGAAAGGAAGGC-3’) were designed using the published sequence of hGSTP1 (54) and included EcoR1 restriction sites. The specifically amplified 710 bp PCR product was digested with EcoR1 and then cloned into the EcoR1 site of the retroviral vector pBabe-PURO (55). The cloned GSTP1 cDNA product was sequenced to ensure fidelity of the PCR reaction and to verify that the insert was in the correct orientation. For the generation of the C47A/C101A GSTP1 retroviral vector, a 288 bp cDNA fragment of the GSTP1 ORF was amplified by PCR using the pBabe-GSTP1 vector as a template. The forward primer (5’-
GGAGGTGGTGACCGTGGAGACGTGGCAGGAGGGCI'CACKAAAGCCTCCGCCCT ATACGGGC-3') and the reverse primer (5'-CAGTTGCCGGGAGCTGCCTTCCTAGTCATGCTTGCCCCTCATAGTGGGTAGATGGAGGATGTATGGGGCGGGA GGTCC-3') were designed so that the amplified fragment would contain altered codons at positions 142 (TGC to GCC) and 304 (UGC to GCC) and would encode alanine instead of cysteine residues. The amplified fragment was digested with BstEI and AvaI then gel purified and subcloned into the pBabe-GSTP1 vector previously digested with BstEI and AvaI. The resulting vector pBabe-GSTP1-C47A/C101A was sequenced to ensure that the altered sequences were incorporated.

Infection of 32D cell lines with GSTP1 retroviral vectors

The GSTP1 retroviral vectors pBabe-GSTP1, pBabe-GSTP1-C47A/C101A and the parent vector pBabe-Puro were transfected into the amphotropic packaging cell line PA317 for production of retroviral particles. After 1 week of selection in 2μg/ml puromycin, the culture supernatant from resistant cells were used for infection of various 32D cell lines in the presence of 5μg/ml Polybrene (Aldrich) and IL-3. After infection, 32D cells were grown for 48 hrs and then selected in puromycin (1μg/ml) and expanded in the presence of IL-3.

Immunoprecipitation and Immunoblotting

Cells (3 X 10^7) were washed with cold PBS and resuspended in 1ml of lysis buffer (50 mM Hepes [pH 7.0], 100mM NaCl, 1mM EDTA and 0.1% NP-40) followed by shearing with a 23.5 gauge needle 10 times. Cell debris was pelleted by centrifugation at 18,000 x g for 15 min at 4°C, and protein concentrations were determined using the Bio-Rad protein assay according to the instructions of the manufacturer (Bio-Rad). After normalization for protein content, cell lysates (2 mg) were mixed overnight at 4°C with 1 μg of either affinity purified anti-FANCC antisera (56) normal rabbit immunoglobulin, an anti-GSTP1 monoclonal antibody (Dako) or an anti-sjGST monoclonal antibody (Santa Cruz Biotech). For immunoprecipitation studies using the anti-GSTP1 or anti-sjGST antibodies, cells were lysed in a slightly modified lysis buffer (50 mM Hepes [pH 7.0], 100mM NaCl, 1mM EDTA, 0.025% NP-40). Immunocomplexes were precipitated by the addition of 15μl (bed volume) of Protein-A-sepharose beads for 2 hrs at 4°C. Beads were washed three times with lysis
buffer and bound proteins were eluted with 20 µl of ImmunoPure Gentle Ag/Ab Elution Buffer (Pierce). An equal volume of 2X Laemmli buffer containing 200 mM dithiothreitol (DTT) was added to the elution fractions, and the proteins were resolved by 10% SDS PAGE and transferred to Immobilon-P membranes (Millipore) by electroblotting. After blocking for 1 hr at room temperature in a solution of TBST (50 mM Tris [pH 7.5], 137 mM NaCl and 0.1% Tween-20) containing 5% nonfat milk, membranes were incubated with an anti-FANCC antibody (1 µg/ml) or an anti-GSTP1 antibody (Panvera) at a dilution of 1:2000 for an additional 2 hrs. After washing, blots were probed with either an anti-rabbit secondary antibody (Amersham) or Protein-A (Sigma) conjugated with horseradish peroxidase at a dilution of 1:10,000, followed by further washing and detection using the enhanced chemiluminescence detection system (Dupont).

For analysis of protein expression, 5 X 10^6 cells were washed once with cold PBS and lysed by the addition of 250 ul of lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 0.2 mM PMSF). After pelleting cell debris by centrifuging at 18,000 X g for 10 minutes, protein concentrations were determined using the Bio-Rad protein assay. An equal volume of 2X Laemmli buffer containing 200 mM DTT was added and 25 µg of each lysate was resolved by 10% SDS-PAGE, electroblotted and hybridized with either an anti-FANCC or anti-GSTP1 antibody as described above. For the detection of PARP, a commercial mouse anti-PARP antibody (clone C-2-10, PharMingen) was used for immunoblotting at a dilution of 1:2000 in 10 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween-20 and 5% nonfat milk overnight at 4°C. After washing, blots were probed with a horseradish peroxidase conjugated anti-mouse secondary antibody (Amersham) at a dilution of 1:5000, followed by further washing and detection using the enhanced chemiluminescence detection system (Dupont).
Cell Viability and Apoptosis Assays

Cell viability and detection of DNA laddering was performed as described previously (47). Apoptotic cells were labeled using the terminal deoxynucleotidyl transferase (tDNA)-mediated dUTP nick end labeling (TUNEL) assay (57) as specified by the manufacturer (Boehringer Mannheim, Indianapolis, IN). Briefly, cells were fixed in a paraformaldehyde solution (4% in PBS) for 30 minutes at room temperature. Cells were incubated with tDNA and dUTP-FITC at 37°C for 1 hour, counterstained with DAPI (2 µg/ml) for 5 minutes and washed twice with PBS. Three independent fields, each consisting of 200 nuclei were scored for each cell line, and the mean percentage of apoptotic cells was expressed as the number of TUNEL positive nuclei/total nuclei X 100%.

GST Activity Measurement

Cells were harvested and washed twice in PBS and resuspended in HEDG buffer (25 mM Hepes, 1.5 mM EDTA and 10% glycerol, pH 7.2). Cells were disrupted by 25 strokes in a tight fitting dounce homogenizer and centrifuged at 100,000 X g for 1 hr, with the resulting supernatant designated the cytosolic fraction. Cytosolic fractions were standardized to bovine serum albumin using the Bradford reagent and were stored at -80°C until use. GST catalytic activity was measured using standard spectrophotometric assays (58). All reactions were performed in 0.1M potassium phosphate (pH 6.5) at 25°C in a total volume of 1 ml using 200 µg of cytosol. Total GST activity was determined by monitoring GSH (2mM) conjugation with 1-chloro-2,4,-dinitrobenzene (CDNB) (2mM) at 340 nM over a period of 5 min, ε=9.6mM⁻¹cm⁻¹. GST-π selective activity was determined by monitoring GSH (0.2mM) conjugation to EA (0.2mM) at 270 nm, ε=5.0mM⁻¹cm⁻¹.

Determination of reduced GSH levels after IL-3 deprivation

Triplicate samples of trypan blue excluding cells (5 X 10⁶) were harvested at each time point after IL-3 deprivation, washed 1X with PBS, pelleted and stored at -80°C until analysis. Frozen pellets were resuspended in 400 µl water and 150 µl of 25% metaphosphoric acid was added to precipitate all proteins. After a 20 minute incubation at room temperature, the samples were centrifuged at 1350 rpm for 5 min. A volume of 450 µl of supernatant from each sample plus 50 µl of iodoacetic acid (15 mg/ml) were added
simultaneously to a tube containing an excess of solid sodium bicarbonate. The tubes were vortexed and incubated in the dark for at least 1 hour, or up to overnight. Following the incubation in the dark, 500 μl of 1.5% 2,4-dinitro-1-fluorobenzene (DNFB) (prepared in 95% ethanol) was added to each of the incubated samples and vortexed. The samples were incubated in the dark for 4 hours, centrifuged for 10 minutes and 200 μl aliquots of each sample were placed into reaction vials. Samples were run on a Waters high-performance liquid chromatography (HPLC) machine according to the method of Reed et al (59). The percent GSH was determined by comparing the amount of GSH/5 X 10⁶ cells at each time point with the amount of cellular GSH/5 X 10⁶ cells present immediately after IL-3 deprivation (Time 0).

**Detection of Oxidized GSTP1**

Cells (1 X 10⁷) were washed in PBS containing 40mM N-ethylmaleimide (NEM) to prevent disulfide exchange. Total cellular protein was precipitated in 10% (w/v) trichloroacetic acid then centrifuged at 18,000 X g for 15 min at 4°C. The precipitated proteins were then solubilized in 80 mM Tris-HCl (pH 6.8), 2% SDS, 1 mM PMSF and 40 mM NEM. Protein concentrations were determined by using the Lowry reagent (60) as recommended by the manufacturer (Bio-Rad). After normalization for protein content, 30 μg of each lysate was resolved by nonreducing SDS-PAGE followed by western blot analysis using an anti-GSTP1 polyclonal antibody (PanVera).

**Statistical Analysis**

Values are expressed as means ± s.d. Statistical analyses were performed using ANOVA. A *post hoc* analysis was made by the Student-Neuman-Keuls test.
3.4. Results

3.4.1. Identification of GSTP1 as an FANCC Interacting Protein

We sought to determine the mechanism by which FANCC inhibits apoptosis by identifying specific interacting proteins in 32D cells. Large scale purification of FANCC-associated proteins from logarithmically growing 32D cells was performed by affinity chromatography using a GST-mFancc fusion protein. As a negative control, a replicate experiment was performed using the sjGST fusion element alone. After excluding the bait proteins that were partially released during the elution process, several proteins with molecular weights of approximately 25, 35 and 120 kDa were identified as binding partners eluted specifically from the GST-mFancc fusion protein affinity column (Fig. 3-la) whereas no binding proteins were eluted from the sjGST negative control.

The 25 kDa mFancc binding protein was subjected to microsequence analysis revealing a 10 amino acid peptide (Pro-Pro-Tyr-Thr-Ile-Val-Tyr-Phe-Pro-Val) that exhibited 100% homology to the murine form of GSTP1-1 (Gstp1) (53). Mammalian GSTs are present in multiple isoforms and can form homodimers or heterodimers but only between subunits of the same class (16). In order to confirm this protein’s identity and exclude the possibility that Gstp1 formed a heterodimer with the sjGST fusion element, a portion of the elution fractions from both sjGST and GST-Fancc columns were subjected to immunoblot analysis using a polyclonal antibody that specifically recognizes Gstp1. Only the GST-mFancc elution fraction showed immunoreactivity with the Gstp1 specific antibody (Fig. 3-1b). Thus, the interaction between GST-mFancc and Gstp1 was directly mediated through mFancc and not through the sjGST affinity ligand. In addition, distinct electrophoretic mobilities exist between recombinant sjGST and Gstp1 after separation by SDS-PAGE, providing further evidence that the 25 kDa protein eluted from the GST-Fancc column was Gstp1 and not sjGST derived from the degraded fusion protein (Fig. 3-1c). Microsequence analysis of the 35 and 120 kDa mFancc-interacting proteins, was not performed due to the limited amount of affinity purified protein obtained.
Figure 3-1. Identification of Gstp1 as a Fancc-interacting protein following affinity chromatography. a. Fancc associated proteins, purified from 32D cell lysates using a GSTmFancc fusion protein, were resolved by 4-20% SDS-PAGE and visualized by silver staining. As a negative control, a replicate experiment was performed using the sjGST fusion element alone. Following microsequence analysis, the Fancc specific 25 kD protein was identified as murine GSTP1 (mGstp1). The unidentified Fancc binding proteins (*) were not microsequenced due to the limited amount of protein purified. b. A portion of the elution fractions were resolved by 10% SDS-PAGE, electroblotted and hybridized with an anti-GSTP1 antibody. Only GST-mFancc elution fractions showed immunoreactivity with anti-GSTP1. c. GSTP1 and sjGST exhibit distinct electrophoretic mobility patterns. Following expression in bacteria, 1µg of purified sjGST and GSTP1 was resolved by 10% SDS-PAGE and visualized by Coomassie Blue staining. sjGST migrates as a doublet with a molecular weight of approximately 27 and 29 kDa, whereas GSTP1 migrates as a 25 kDa monomer.
3.4.2. Confirmation of the interaction between GSTP1 with FANCC by co-immunoprecipitation assays

In order to examine the in vivo interaction of GSTP1 with FANCC, coimmunoprecipitation studies were performed using retroviral-transduced 32D cells and patient derived lymphoblast cell lines. A GSTP1-retroviral vector was used to generate stable 32D cell lines expressing high levels of the 25 kDa human GSTP1 protein. Incubation of cell extracts with a polyclonal FANCC-specific antibody which recognizes both human and mouse FANCC, resulted in the coimmunoprecipitation of both endogenous Gstp1 and ectopic GSTP1 in 32D cell lines overexpressing FANCC (Fig. 3-2a). In contrast, GSTP1 was not detected in Fancc immunocomplexes from 32D or 32D-GSTP1 cells, which express little endogenous Fancc nor was GSTP1 detected in extracts incubated with pre-immune serum (Fig. 3-2a). Immunoprecipitation using an anti-GSTP1 monoclonal antibody, followed by immunoblotting with FANCC antiserum further confirmed that FANCC interacts with both endogenous Gstp1 and ectopic GSTP1 in 32D cells (Fig. 3-2a).

Although the FANCC/GSTP1 complex was detected in 32D cells overexpressing both proteins, it could be argued this interaction is not reflective of physiological conditions. Therefore, we examined the interaction of endogenous FANCC and GSTP1 in both a normal and a FA-C lymphoblast cell line (Fig. 3-2b). The FA-C lymphoblast line, HSC622, does not express a full-length FANCC polypeptide. Immunoprecipitation with anti-FANCC serum resulted in co-immunoprecipitation of endogenous GSTP1 in normal but not in FA-C lymphoblast lysates. However, the FANCC/GSTP1 interaction was restored in HSC622 cells following transduction with a wild-type FANCC retroviral vector. Anti-FANCC immunoblotting revealed that FANCC co-immunoprecipitated with GSTP1 in both normal and corrected FA-C cells. A direct interaction between FANCC and GSTP1 has not been observed using the yeast two-hybrid system, in vitro translated proteins or recombinant proteins purified from E.coli (data not shown), indicating that the GSTP1/FANCC complex may require adapter proteins. The 35 kDa and 120 kDa Fancc binding proteins (Fig. 3-1a) may be such adapters.
Figure 3-2. Confirmation of the FANCC/GSTP1 interaction in 32D and lymphoblast cell lines by co-immunoprecipitation analysis. a. Lysates (2mg) from the indicated 32D cell lines, were immunoprecipitated (IP) with a either anti-FANCC or pre-immune serum. In the reciprocal experiment, lysates were incubated with either an anti-GSTP1 or anti-sjGST antibody. FANCC was detected by immunoblotting (IB) with an anti-FANCC antibody (upper panel), and GSTP1 was detected by immunoblotting with rabbit anti-GSTP1 (lower panel). The FANCC/GSTP1 complex was detected only in FANCC overexpressing cells. b. Co-immunoprecipitation of endogenous FANCC and GSTP1 in normal and complemented FA-C lymphoblasts. The FANCC/GSTP1 interaction was detected in normal (HSC93) but not FA-C (HSC622) lymphoblasts. The interaction was restored in FA-C cells transduced with a wild-type FANCC retroviral vector (HSC622+FANCC).
3.4.3. Overexpression of GSTP1 in 32D cells delays the onset of apoptosis

To examine the effects of GSTP1 overexpression on cell viability after IL-3 withdrawal, 32D and 32D-FANCC cells were transduced with the supernatant from a GSTP1 retroviral packaging cell line. GSTP1-transduced cells were grown logarithmically for several days and then were thoroughly washed and resuspended in media lacking IL-3. At 24 hour intervals, cell viability was determined by trypan blue dye exclusion. 32D cells overexpressing GSTP1 (32D Neo/GSTP1) displayed increased viability compared with the parental cell line (Fig. 3-3a). Interestingly, 32D Neo/GSTP1 cells displayed enhanced viability comparable to that of cell lines overexpressing FANCC. Furthermore, an additive effect between GSTP1 and FANCC was observed because 32D cells overexpressing both GSTP1 and FANCC (32D FANCC/GSTP1) had almost double an enhancement of cell viability compared to only single transformants. The increased viability in 32D FANCC/GSTP1 clones was not due to a proliferative effect, because no increase in viable cells, above the initial number after IL-3 removal, was observed over the course of the experiment. Four days after IL-3 withdrawal, only 10% of 32D Neo/Puro cells were alive whereas 80% of the 32D-FANCC/GSTP1 cells and approximately 30% of either 32D-FANCC and 32D-GSTP1 cells were still viable. Thus, FANCC and GSTP1 appear to work together in suppressing cell death in IL-3 deprived 32D cells.

To determine if the increased viability in GSTP1 transduced cells was due to a suppression of apoptosis, apoptotic cells were labeled using the terminal deoxynucleotidyl transferase (tDT)-mediated dUTP nick end labeling (TUNEL) assay (57). This assay takes advantage of the fact that endonucleases activated during apoptosis cleave genomic DNA yielding double-stranded, low molecular weight DNA fragments as well as single strand breaks in high molecular weight DNA (62). These DNA strand breaks can be identified by tDT labeling of free 3'-OH termini with fluorescein-conjugated dUTP. Twenty-four hours after IL-3 withdrawal, over 40% of 32D Neo/Puro cells were stained positive by TUNEL whereas less than 20% of cells expressing either FANCC or GSTP1 showed staining (Fig. 3b). Few cells expressing both FANCC and GSTP1 displayed TUNEL staining even 48 hours after IL-3 deprivation. DNA fragmentation analysis revealed similar results (Fig. 3-3c). 32D Neo/Puro cells exhibited pronounced DNA fragmentation within 24 hours of IL-3 withdrawal whereas cells expressing either GSTP1 or FANCC did not show significant DNA
Figure 3-3. Co-expression of GSTP1 and FANCC promotes increased survival and suppression of apoptosis. a. Viability of 32D cells expressing GSTP1, clonally derived cell lines expressing FANCC (FANCC-D2 and FANCC-E1) in addition to cell lines expressing both proteins together was examined following IL-3 removal by trypan blue staining. Expression of either FANCC or GSTP1 significantly delayed cell death (*, P < 0.05; **, P < 0.01) compared to 32D mock infected cells following IL-3 deprivation. Co-expression of both FANCC and GSTP1 dramatically (***, P < 0.001) delayed cell death compared to mock infected control cells at each time point following IL-3 deprivation. Data represent the averages of three independent experiments, each in triplicate wells. Error bars represent standard deviations (s.d.). b. Quantitative analysis of apoptosis was performed using TUNEL and DAPI staining. Three independent fields of nuclei (n=200) were scored using fluorescence microscopy, and the percentage of apoptotic cells was determined by comparing the number of TUNEL positive nuclei with the total number of all nuclei scored. Expression of either FANCC or GSTP1 significantly (**, P < 0.01; ***, P < 0.001) decreased the number of apoptotic cells when compared to 32D control cells following IL-3 withdrawal. Data is presented as mean ± s.d. (n=3) and is representative of three independent experiments. c. DNA laddering analysis of IL-3 deprived cell lines at the indicated time points. 32D cells expressing either FANCC or GSTP1 exhibited only moderate fragmentation after 24 hours, whereas cells expressing both proteins displayed no fragmentation up to 48 hours after factor deprivation.
fragmentation until after 24 hours. In contrast, 32D FANCC/GSTP1 cells did not exhibit any apoptotic DNA fragmentation, even after 48 hours of growth factor deprivation (Fig. 3-3c).

Characteristic features of apoptosis, besides DNA cleavage, include chromatin condensation and fragmentation of the nucleus into apoptotic bodies (63, 64). Staining of nuclei with DAPI and TUNEL revealed extensive chromatin condensation and nuclear fragmentation in 32D Neo/Puro cells after IL-3 withdrawal (Fig. 3-4a). Few nuclei from 32D cells expressing either FANCC or GSTP1 were TUNEL positive or showed fragmentation, (Fig. 3-4a). These findings indicate that FANCC and GSTP1 can suppress the overall number of cells undergoing apoptosis and nuclear fragmentation.

Several studies have shown that caspases are activated following IL-3 withdrawal-induced apoptosis in 32D cells (65-67). The 115 kDa nuclear protein poly(ADP-ribose) polymerase (PARP) is specifically cleaved by caspases to lower molecular weight fragments following initiation of apoptosis by various stimuli (68, 69). PARP is typically cleaved by caspase 3 at Asp-213 to form two fragments of molecular mass 89 and 25 kDa (69). PARP cleavage was examined in the 32D cell lines after IL-3 deprivation by immunoblotting using an anti-PARP monoclonal antibody. The full-length 115 kDa PARP protein showed signs of cleavage, as determined by the increased appearance of the 89 kDa fragment, in the 32D control cell line, especially after 24 hours (Fig. 3-4b). Cells overexpressing FANCC showed little cleavage at 24 hours, but following this time point levels of the full length 115 kDa PARP protein rapidly declined. Surprisingly, cleavage of PARP in 32D-FANCC cells resulted in the generation of a 42 kDa fragment instead of the 89 kDa fragment. 32D cells overexpressing GSTP1, and particularly cells overexpressing both FANCC and GSTP1 displayed strong inhibition of PARP cleavage, even up to 72 hours after IL-3 deprivation (Fig. 3-4b). These findings suggest that FANCC may only partially suppress PARP cleavage, whereas GSTP1 is a more effective inhibitor of either PARP cleavage or upstream caspases.

3.4.4. The FANCC and GSTP1 proteins stabilize one another following IL-3 withdrawal

The persistence of the FANCC protein has been shown to correlate with suppression of apoptosis after IL-3 withdrawal (47). Therefore FANCC and GSTP1 protein levels were monitored by immunoblot analysis after induction of apoptosis (Fig. 3-5).
Figure 3-4. 32D cells overexpressing either FANCC or GSTP1 display fewer morphological features of apoptosis and exhibit less PARP cleavage. a. Nuclear morphology of the 32D cell lines were stained by DAPI (top) or TUNEL (bottom) after 24 hours IL-3 deprivation. Mock infected 32D cells displayed a high degree of TUNEL staining, nuclear condensation and fragmentation (indicated by arrows) whereas FANCC and GSTP1 expressing cells exhibited low TUNEL staining and nuclear fragmentation. b. Whole cell extracts from 32D cell lines deprived of IL-3 at the indicated time points, were prepared and proteins (25 µg) were resolved by 10% SDS-PAGE, transferred to PVDF membrane, and hybridized with an anti-PARP monoclonal antibody (C-2-10). The locations of intact PARP (115 kDa) and the 89 and 42 kDa proteolytic fragments are indicated. Although GSTP1 was more effective at maintaining full length PARP, FANCC partially protected PARP from cleavage resulting in the appearance of a 42 kDa PARP fragment.
Figure 3-5. The FANCC and GSTP1 proteins stabilize each other during apoptosis when overexpressed in IL-3 deprived 32D cells. Expression of FANCC and GSTP1 was monitored after IL-3 deprivation by resolving whole cell extracts (25 µg) by 10% SDS-PAGE under reducing conditions, followed by western blot analysis using anti-FANCC and anti-GSTP1 antibodies. Endogenous Fancc is indicated by the small arrow and ectopic FANCC is indicated by the two large arrows. 32D-FANCC-D2/GSTP1 cells showed almost no loss of either protein suggesting that both proteins stabilize one another.
removal of IL-3, endogenous levels of both Fancc and Gstp1 dropped dramatically in the control cell line 32D-Neo/Puro, especially after 48 hours. Endogenous Gstp1 expression was maintained only in cells overexpressing FANCC. A significant decrease in the levels of the ectopically expressed 60 kDa and 55 kDa FANCC isoforms was observed in 32D-FANCC/Puro cells after IL-3 withdrawal, especially 48 hours after IL-3 withdrawal. In addition, multiple lower molecular weight FANCC protein fragments were present in 32D FANCC/Puro (Fig. 3-5). However, the lower molecular weight FANCC protein fragments were absent in 32D-FANCC/GSTP1 cells, and the levels of both the 60 kDa and 55 kDa isoforms did not decrease following IL-3 removal. These findings suggest that GSTP1 may suppress cleavage or proteolytic degradation of FANCC. Thus, co-expression of FANCC and GSTP1 results in the stabilization of both proteins following IL-3 withdrawal which correlates with increased suppression of apoptosis.

3.4.5. FANCC increases GSTP1 catalytic activity following induction of apoptosis

The additive effect of FANCC and GSTP1 on the suppression of hematopoietic cell death could be explained by the modulation of either a common or distinct apoptotic pathway. Because GSTs catalyze the conjugation of a variety of electrophilic substrates with the endogenous nucleophile GSH (16, 18), it was of interest to determine if FANCC could alter the catalytic activity of GSTP1, particularly after initiation of apoptosis. GSTs exhibit large variation in their substrate specificity, however, most of the enzymes can conjugate GSH with the substrate 1-chloro-2,4-dinitrobenzene (CDNB) (16). Cytosolic lysates from 32D cells overexpressing FANCC, GSTP1 or both proteins had approximately 1.9, 3 and 5 fold more total GST activity, respectively, than the parental cell line when grown in the presence of IL-3 (Table 3-1). Surprisingly, when cells were deprived of IL-3, total GST activity increased in FANCC expressing cells, whereas no induction was observed in mock infected cells and only weak induction was observed in 32D Neo/GSTP1 cells. 32D cells overexpressing FANCC displayed a two fold increase in GST activity in the presence of IL-3 and displayed 3 to 5 fold higher GST activity compared to the parental cell line at the same time points following IL-3 deprivation. Cells expressing both FANCC and GSTP1 exhibited approximately 2 to 3 fold higher levels of GST activity compared to cells expressing GSTP1 or FANCC alone after factor withdrawal (Table 3-1). Similar studies
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>+ IL-3</th>
<th>- IL-3 (24 Hrs)</th>
<th>- IL-3 (48 Hrs)</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>32D Neo/Puro</td>
<td>31 ± 5</td>
<td>32 ± 8</td>
<td>22 ± 9</td>
<td>CDNB</td>
</tr>
<tr>
<td>32D FANCC-D2/Puro</td>
<td>57 ± 11***</td>
<td>109 ± 23**</td>
<td>115 ± 25*</td>
<td>CDNB</td>
</tr>
<tr>
<td>32D Neo/GSTP1</td>
<td>92 ± 10***</td>
<td>114 ± 15**</td>
<td>117 ± 25*</td>
<td>CNDB</td>
</tr>
<tr>
<td>32D FANCC-D2/GSTP1</td>
<td>156 ± 13***</td>
<td>245 ± 40***</td>
<td>301 ± 87***</td>
<td>CDNB</td>
</tr>
<tr>
<td>32D Neo/Puro</td>
<td>2.4 ± 1</td>
<td>2.4 ± 0.6</td>
<td>2.5 ± 0.7</td>
<td>EA</td>
</tr>
<tr>
<td>32D FANCC-D2/Puro</td>
<td>3.8 ± 0.4</td>
<td>5.3 ± 0.8*</td>
<td>6.5 ± 0.7*</td>
<td>EA</td>
</tr>
<tr>
<td>32D Neo/GSTP1</td>
<td>4.3 ± 1</td>
<td>4.9 ± 0.7*</td>
<td>4.7 ± 0.6</td>
<td>EA</td>
</tr>
<tr>
<td>32D FANCC-D2/GSTP1</td>
<td>5.1 ± 0.6</td>
<td>9.5 ± 1.7**</td>
<td>11.6 ± 0.9***</td>
<td>EA</td>
</tr>
</tbody>
</table>

**Table 1:** FANCC increases GSTP1 catalytic activity following induction of apoptosis by factor deprivation. Total GST activity was measured using the substrate 1-chloro-2,4-dinitrobenzene (CDNB) and GST pi activity was measured using the substrate ethacrynic acid (EA) in cytoplasmic extracts from cells cultured in either the presence or absence of IL-3. Cells expressing FANCC or GSTP1 alone displayed elevated activity compared to mock infected cells, especially after the removal of IL-3. Cells expressing both FANCC and GSTP1 displayed higher activity than cells expressing each protein alone, especially after IL-3 deprivation (*, P < 0.05; **, P < 0.01; ***, P < 0.001). Enzyme activity (nmol/min/mg) was determined by monitoring the spectrophotometric changes that occur over time as a result of substrate conjugation with GSH. Results represent the average of three independent experiments performed in triplicate.
using the pi-class substrate ethacrynic acid (EA), revealed that overexpression of FANCC also increased GSH conjugation with the pi class substrate after removal of IL-3 (Table 3-1). Thus, FANCC increases GSTP1 catalytic activity, particularly after initiation of apoptosis.

3.4.6. GSTP1 relies on the presence of GSH to suppress apoptosis whereas FANCC can suppress apoptosis even in the absence of GSH

GSH, a cysteine containing tripeptide, is the most abundant low-molecular weight thiol within the cell (up to 10 mM) (70). GSH provides a source of reducing equivalents for the reduction of disulfide bonds within proteins and the neutralization of free radicals. GSH depletion is known to occur after the initiation of apoptosis by a variety of treatments, including IL-3 withdrawal, and is an indicator of oxidative stress (41-43, 71, 72). GSTs have recently been implicated in maintaining GSH homeostasis, especially in response to cellular stress (25, 26, 73). Therefore, it was of interest to examine the effect of GSTP1 and FANCC expression on GSH metabolism after induction of apoptosis. Following removal of IL-3 in the control cell 32D Neo/Puro, GSH levels dropped by more than 60% within the first three hours (Fig. 3-5a). This drop in GSH was followed by a sharp rise that peaked at 12 hours, then quickly fell to negligible levels by 48 hours. In contrast, ectopic expression of either GSTP1 or FANCC significantly attenuated the drop after 24 hours of IL-3 deprivation (Fig. 3-6a). Interestingly, GSH levels in 32D FANCC/GSTP1 cells did not drop at 3 hours nor decline following 24 hours (Fig. 3-6a), indicating that the FANCC/GSTP1 complex stabilizes GSH levels following initiation of apoptosis.

Artificially depleting GSH in various cell lines using the gamma-glutamylcysteine synthetase inhibitor, buthionine-[S,R]-sulfoximine (BSO), has been shown to induce or hasten apoptosis (43, 71, 72). 32D cells pretreated with 5 mM BSO for 8 hours had no detectable levels of GSH (Fig. 3-6a). After IL-3 withdrawal, BSO pretreated control cells were significantly less viable by 48 hours (Fig. 3-6b, T-test, P<0.01) than non-treated control cells (Fig. 3-3a). Moreover, the protective effect of GSTP1 alone was completely abolished in BSO treated 32D-Neo/GSTP1 cells. However, the anti-apoptotic effect conferred by FANCC was still present, albeit to a lesser extent, in BSO treated 32D-FANCC/Puro cells (Fig. 3-6c and 3-6d). While cells co-expressing both FANCC and GSTP1 were still the most viable, this level of protection more closely resembled that of cells expressing FANCC alone.
Figure 3-6. FANCC suppresses apoptosis even in the absence of GSH. a. Intracellular GSH levels were monitored over time following the removal of IL-3 and expressed as the percentage of GSH/5 X 10^6 cells at each time point relative to the level prior to IL-3 deprivation. Overexpression of either FANCC or GSTP1 attenuated the loss of GSH at 36 and 48 hours (*, P < 0.05) after IL-3 withdrawal compared to mock infected cells. Overexpression of both FANCC and GSTP1 maintained GSH levels at or above initial levels, especially at 36 and 48 hours (**, P < 0.01), following IL-3 withdrawal compared with the mock infected control cells. 32D Neo/Puro cells treated with the glutathione synthetase inhibitor BSO (5mM) exhibited a steady decrease in GSH levels until no GSH was detected by 12 hours following BSO exposure. Data represent averages ± s.d. of three independent experiments each performed in triplicate. b. Cell viability was monitored after BSO induced depletion of GSH and IL-3 withdrawal by trypan blue staining. Although no significant difference in viability existed between FANCC or FANCC/GSTP1 expressing cells at each time point, these cell lines exhibited a significant decrease in cell death at 48, 72 and 96 hours following IL-3 deprivation (*, P < 0.05; **, P < 0.001) compared to mock infected cells or cells expressing GSTP1 only. Data represent the averages ± s.d. of three independent experiments, each in triplicate wells. c. DNA laddering analysis of BSO treated cells before and after IL-3 deprivation. Control and GSTP1 expressing cells exhibited a similar degree of DNA fragmentation following GSH depletion and factor deprivation, whereas FANCC and FANCC/GSTP1 expressing cells displayed low levels of fragmentation up to 24 hours. d. BSO induced depletion of GSH before IL-3 withdrawal abolished the protective effect of GSTP1 but not FANCC on inhibition of apoptosis. Quantification of apoptotic cells was performed by TUNEL/DAPI staining. No significant difference in the number of apoptotic cells was detected between FANCC and FANCC/GSTP1 expressing cells following BSO treatment and IL-3 removal. However, FANCC and FANCC/GSTP1 expression resulted in significantly fewer (*, P < 0.01; **, P < 0.001) TUNEL positive cells at 24 and 48 hours post-IL-3 deprivation compared to either 32D Neo/Puro or 32D Neo/GSTP1 cells.
Thus, the ability of GSTP1 to delay apoptosis in the absence of high levels of FANCC is dependent on the presence of GSH, whereas high levels of GSH are not essential for FANCC to exert a protective effect. Furthermore, these findings suggest that FANCC may act through a pathway that is independent of GSH.

3.4.7. FANCC suppresses oxidation of GSTP1 during apoptosis

GSTP1 is unique amongst all known GST proteins in that it is rapidly inactivated by oxidizing agents such as $H_2O_2$ and disulfides (74-79). Human and rat GSTP1 have four cysteine residues (Cys-14, Cys-47, Cys-101 and Cys-169), whereas murine Gstp1 has three (Cys-14, Cys-47 and Cys-167), but none of these residues participate in the formation of disulfide bonds when the protein exists in its native structure (76, 80, 81). Site directed mutagenesis studies of purified rat GSTP1 treated with $H_2O_2$ revealed that an intramolecular disulfide bond forms preferentially between Cys-47 and Cys-101, and that the formation of this bond facilitates the formation of a second disulfide between Cys-14 and Cys-169 (76). Covalently linked dimers of GSTP1 also form by intersubunit disulfide bonding between Cys-47 residues in different subunits (76). In addition, covalent aggregates of GSTP1 form via disulfide bonding following $H_2O_2$ treatment; a process that is dependent on the Cys-101 residue (76). Thus, oxidation of GSTP1 results in the formation of intrasubunit and intersubunit disulfide bonds via cysteine residues, leading to conformational changes that obscure the catalytic sites and decrease protein stability (76, 78, 79). However, oxidative inactivation of GSTP1 is readily reversed by the addition of antioxidants, GSH or reducing agents such as dithiothreitol (DTT) (76-78, 82).

The finding that FANCC suppresses apoptosis in the absence of GSH suggests that it may act by preventing oxidation of GSTP1, thereby stabilizing this enzyme and maintaining its activity during apoptosis. To test this hypothesis, in vivo oxidation of GSTP1 was examined in 32D cell lysates using SDS-PAGE under non-reducing conditions followed by immunoblotting with a GSTP1 specific antibody to detect the altered mobilities of oxidized GSTP1 (Fig. 3-7a). The formation of intramolecular disulfide bonds within GSTP1 results in faster electrophoretic migration, whereas intermolecular disulfide bonds formed between GSTP1 subunits result in slower migration. Under reducing conditions only one 25 kDa band, corresponding to the native monomeric form of the protein, was evident in all the cell lines. However, under non-reducing conditions, 32D cells deprived of IL-3 displayed a
decrease in the level of the endogenous 25 kD reduced monomer and the appearance of a lower molecular weight product of approximately 18 kDa, particularly after BSO treatment and IL-3 withdrawal. The appearance of the 18 kDa lower molecular weight polypeptide is indicative of an oxidized form of the Gstp1 monomer containing an intramolecular disulfide bond (75, 76). In contrast, 32D-FANCC/Puro cells maintained a constant level of the reduced monomer after IL-3 withdrawal, even in the presence of BSO.

32D-Neo/GSTP1 cells showed an increase in several higher molecular weight bands following IL-3 removal (Fig. 3-7a). The appearance of the 37 kDa band corresponds to a non-native oxidized dimer formed by an intersubunit bond between Cys-47 residues in different GSTP1 subunits (76). The increased appearance of the 60 kDa band and several higher molecular weight bands observed above 98 kDa represent multimers of GSTP1 likely involving the Cys-101 residue, which is absent in murine Gstp1 (76). All multimers of GSTP1 were significantly increased in BSO treated 32D-Neo/GSTP1 cells after IL-3 withdrawal, indicating that depletion of GSH from the cytosol further promoted a shift towards oxidation of human GSTP1. As a positive control, recombinant GSTP1 treated with H2O2 resulted in the formation of higher and lower molecular weight products similar to those observed in 32D Neo/GSTP1 cells deprived of IL-3 (Fig. 3-7a). A 45 kDa band that was present in all the cell lines (Fig. 3-7a), including 32D cells expressing a cysteine mutant GSTP1 protein (see below) indicated that this band was most likely the result of a non-specific cross-reaction of the anti-GSTP1 antibody with an unrelated endogenous protein.

Oxidation of human GSTP1 during apoptosis was strongly inhibited by FANCC (Fig. 3-7a). No significant increases in the oxidized monomer or multimers were observed in 32D FANCC/GSTP1 cells after IL-3 deprivation. Co-expressing cells only displayed an increase in the 60 kDa product and a slight increase in the 37 kDa dimer after BSO treatment. Thus, FANCC can suppress oxidation of GSTP1 even under conditions that would favour the formation of disulfide bonds that disrupt the active conformation of GSTP1. These results are consistent with the finding that FANCC increases GSTP1 activity (Table 3-1) and can confer protection against apoptosis following IL-3 deprivation, even when GSH is depleted (Fig. 3-6b, 3-6c). Therefore, the ability of GSTP1 to delay apoptosis is dependent on the reducing activity of either GSH or FANCC. In the absence of GSH, the anti-apoptotic effect
Figure 3-7. The FANCC protein prevents oxidation of GSTP1 during apoptosis, even in the absence of GSH. a. Whole cell extracts (30 μg) from cells treated as indicated were resolved by nonreducing SDS-PAGE and immunoblotted using a GSTP1 specific antibody. Cells pretreated with BSO (5 mM) for 8 hours were deprived of IL-3 and maintained in the presence of BSO for 48 hours. As a control, lysates were compared with recombinant GSTP1 protein treated with 1 mM H₂O₂ for 30 minutes and oxidized GSTP1 isoforms are indicated with arrows. The reduced 25 kDa GSTP1 monomer was observed only in lysates treated with 100 mM DTT, 5% β-mercaptoethanol and boiled for 10 minutes. Overexpression of FANCC alone prevented the formation of oxidized endogenous Gstp1 monomers following IL-3 withdrawal, even after BSO treatment. Co-expression of FANCC with GSTP1 prevented the formation of oxidized multimers of GSTP1 following IL-3 deprivation. b. Overexpression of a mutant GSTP1 protein possessing alanine instead of cysteine residues at positions 47 and 101 (GSTP1-C47A/C101A) in 32D cells did not undergo oxidation following IL-3 withdrawal, even after depletion of GSH using BSO. The GSTP1 antibody exhibited a non-specific cross-reaction with an unrelated 45 kDa endogenous protein under non-reducing conditions (*). Data are representative of two separate analyses.
of GSTP1 appears to be dependent on the total amount of FANCC available to maintain GSTP1 in a reduced state.

In order to verify that the higher and lower molecular weight bands observed under non-reducing conditions were due to the formation of disulfide bonds, a mutant GSTP1 retroviral vector was made with the critical cysteine residues at positions 47 and 101 replaced with alanine residues (C47A/C101A). Cells overexpressing the C47A/C101A mutant did not form any oxidized GSTP1 multimers or monomers following IL-3 deprivation, even in the presence of BSO (Fig. 3-7b). Thus, the cysteine residues that were replaced are essential for disulfide bond formation.

3.5. Discussion

The results presented here reveal that FANCC interacts with and regulates GSTP1, a redox sensitive protein traditionally implicated in xenobiotic detoxification. This is the first direct example of a cytosolic protein (GSTP1) undergoing oxidation during apoptosis induced by a physiological stimulus (IL-3 withdrawal). Previous in vitro studies have shown oxidation of purified or recombinant GSTP1 only after treatment with H2O2 or disulfides in the absence of a cellular background (75, 76, 78, 79, 82, 83). In addition, the work presented here is the first to directly demonstrate that overexpression of a mammalian GST can influence cellular GSH levels during apoptosis. Furthermore, the ability of FANCC to maintain GSTP1 in a reduced state, even in the absence of GSH, reveals a previously unrecognized function of FANCC.

A direct interaction was not detected between FANCC and GSTP1, indicating that adapter or intermediate proteins are required. We are currently trying to identify the other proteins that were eluted from the Fancc affinity column (Fig. 3-1a), as they may represent critical adapter proteins necessary for the disulfide reductase activity associated with FANCC. Several studies have shown that FANCC indirectly interacts with other FA proteins in a multisubunit complex, suggesting that adapter proteins are also necessary for this interaction (1-3). In addition, FANCC indirectly interacts with the non-FA protein cdc2, further indicating that the formation of a multisubunit complex may be necessary for FANCC function (10). The possibility that GSTP1 is part of the FA complex and may interact with FANCC via other FA proteins is currently being investigated.
The interaction between FANCC and GSTP1 appears to stabilize both proteins following initiation of apoptosis by IL-3 withdrawal (Fig. 3-4). Recent studies have shown that the FANCC protein is regulated at the posttranscriptional level by proteasome-dependent proteolysis (84). Interestingly, several lower molecular weight FANCC proteins present in cells overexpressing FANCC were not apparent in cells overexpressing both FANCC and GSTP1 (Fig. 3-5). Thus, GSTP1 may prevent proteolytic degradation of FANCC during apoptosis. A correlation between elevated FANCC protein levels and suppression of apoptosis has been previously demonstrated (47). A similar correlation between GSTP1 expression and suppression of apoptosis was also observed in this study (Fig. 3-5). Elevated expression of GSTP1, either by stabilization of the protein via a FANCC interaction or by retroviral-mediated gene transfer of the GSTP1 gene, conferred transient protection from apoptosis induced by IL-3 deprivation. Furthermore, an additive effect on the suppression of cell death was observed in cells overexpressing both proteins (Fig. 3-3).

Although the combined expression of GSTP1 and FANCC conferred higher suppression of apoptosis than cells expressing each gene individually, it could be argued that each gene product affects a distinct rather than common apoptotic pathway. The finding that GSTP1 suppresses PARP cleavage more effectively than FANCC (Fig. 3-4b) suggests that GSTP1 may affect apoptotic pathways independently of FANCC. However, enzymatic assays indicated that FANCC augmented GST catalytic activity, especially after induction of apoptosis (Table 3-1). Although these findings do not exclude the possibility that FANCC or GSTP1 can suppress apoptosis through separate pathways, it strongly indicates that FANCC acts, at least in part, by stabilizing GSTP1 and enhancing its enzymatic and/or anti-apoptotic activity. A number of recent studies have implicated GSTs and glutathione metabolizing enzymes in apoptotic pathways. Using differential display, a protein with homology to a theta class GST was isolated from a mouse lymphoma cell line that had gained resistance to apoptotic cell death induced by either ionizing radiation or chemotherapeutic drug exposure (24). In a separate study, analysis of an apoptosis resistant lymphoma cell line using DNA microarrays revealed a 24 fold increase in the expression of the same GST theta homologue in addition to highly elevated expression of genes involved in the generation or maintenance of GSH (25). A theta class GST homologue was also isolated in a genetic screen to identify plant cDNAs that could rescue Bax induced cell death in yeast (26). This GST could also enhance resistance to H2O2 induced cell death and prevent the Bax-induced depletion of
cellular GSH (26). A detailed analysis of genes induced by p53 expression, before the onset of apoptosis, revealed a strong induction of a microsomal GST homologue (PIG12) in addition to an enzyme involved in GSH metabolism (PIG4) (27). These studies provided evidence that GSTs play an important role in GSH homeostasis and the prevention of apoptosis.

Because GSTs bind GSH, these enzymes may be important for the sequestration of cytosolic GSH with a concomitant impact upon the cellular redox status (73). Apoptosis can be inhibited by preventing GSH depletion, through the addition of exogenous GSH or the GSH precursor N-acetylcysteine (NAC) (33, 71, 85). Overexpression of the anti-apoptotic genes Bcl-2 and Bcl-XL has been shown to suppress GSH loss during apoptosis (42, 86-88). Expression of either FANCC or GSTP1 prevented the loss of GSH after factor withdrawal, suggesting that the FANCC/GSTP1 complex functions by regulating GSH levels (Fig. 3-6a). However, depletion of GSH using BSO abolished the protective effect of GSTP1 but only partially decreased the ability of FANCC to inhibit apoptosis (Fig. 3-6b). These findings suggest that GSTP1 depends on the presence of GSH to exert its anti-apoptotic effects, whereas FANCC is not as dependent on GSH levels. These findings must be put into context when one considers the interaction of GSH with GSTP1.

X-ray crystallography has revealed that cytosolic GST subunits, including GSTP1, are composed of two separate domains of different structure. For GSTP1, Domain I is located at the N-terminus (residues 1 to 74) and contains the GSH binding site (G-site) (80). Domain II or the C-terminal domain (residues 81 to 207), contains the second substrate-binding site (H-site) to which hydrophobic electrophilic compounds bind (80). The catalytic activity of GSTs is based on the ability of the enzyme to lower the pKₐ of the sulphydryl group of reduced GSH when bound in the active site (89). GSTs bind the neutral thiol form of GSH, then ionization of the GSH thiol group to the thiolate anion (GS-) occurs at the G-site (90, 91). Therefore, GSH exists predominantly as a thiolate (GS-) anion at neutral pH when bound by the G-site (91, 92). Several residues in the N-terminal domain of GSTP1 are involved in stabilizing GS- through hydrogen bonding (80). Once GS- is formed in the G-site of GST, it becomes capable of reacting spontaneously, by nucleophilic attack, with electrophilic xenobiotics or compounds that are situated in close proximity within the H-site (16).
GSTP1, unlike other GSTs, is very susceptible to oxidation, mainly through its highly reactive Cys-47 residue (74, 76, 93). Although Cys-47 is not part of the active site, it is located close to the G-site and is highly susceptible to chemical alkylation (80, 83). Under oxidative conditions, intrasubunit disulfide bonds form between Cys-47 and Cys-101 residues resulting in steric hindrance for GSH binding and loss of enzymatic activity (74, 94). The Cys-47 and Cys-101 residues have also been implicated in the formation of intersubunit disulfide bonds between different GSTP1 subunits with an associated loss of activity (76). However, addition of high amounts of GSH can reduce disulfide bonds within GSTP1 by a thiol/disulfide exchange mechanism, thereby returning the reactive cysteines to a sulfhydryl state and restoring enzyme activity (78, 82). In addition, binding of GSH to the G-site results in a conformational change which buries Cys-47 in a hydrophobic pocket and makes it inaccessible to oxidation (81, 95). Thus, in the absence of GSH, GSTP1 is very susceptible to oxidation and inactivation.

Interestingly, FANCC inhibits the oxidation of endogenous Gst1 after IL-3 withdrawal, even in the absence of GSH (Fig. 3-7a). FANCC also inhibited the oxidation of ectopically expressed human GSTP1 following IL-3 deprivation (Fig. 3-7a). However, FANCC only partially prevented oxidation of ectopically expressed GSTP1 following depletion of GSH and initiation of apoptosis. One interpretation of these findings is that the ability of FANCC to prevent oxidation of GSTP1 is dependent on the stoichiometric ratio that exist between the two proteins. In 32D cells overexpressing both proteins, there is presumably more endogenous and ectopic GSTP1 than FANCC protein. In the absence of GSH, the ability of FANCC to protect GSTP1 is dependent on the amount of FANCC available to interact with GSTP1. This hypothesis is supported by the observation that GSH depleted 32D FANCC/GSTP1 cells were no longer strongly resistant to apoptosis induced by factor deprivation, but instead showed a suppression of apoptosis comparable to that observed in cells expressing FANCC alone (Fig. 3-6c,d). Because overexpression of GSTP1 can prevent GSH depletion (Fig. 3-6a), GSTP1 may autoregulate its own redox state by maintaining the environment of the cytosol under reducing conditions. However, after artificially depleting GSH by BSO treatment, the number of reduced GSTP1 monomers present is dependent on the amount of FANCC available.

The effect of covalent modification by disulfide bond formation on protein activity during oxidative stress is poorly understood. It is generally assumed that cytoplasmic
proteins predominantly exist in a reduced state due to the presence of GSH (40, 96) or members of the thioredoxin thiol-disulphide oxidoreductase family (97). However, the efflux or oxidation of GSH that occurs during apoptosis (42, 43, 71, 72) can create an environment that may promote the formation of mispaired protein disulfide bonds. The results presented here are the first to directly demonstrate that the formation of non-native disulfide bonds can occur within a cytoplasmic protein during apoptosis and can lead to loss of enzymatic activity or function.

Interestingly, FANCC bears no resemblance to any known proteins including thiol-disulfide oxidoreductases but can still maintain GSTP1 in a reduced state during oxidative stress. FA has traditionally been regarded as a DNA repair disorder based on the observation that FA cells are hypersensitive to alkylating agents such as MMC and exhibit chromosomal instability (98, 99). However, a link between oxidative stress and the FA cellular phenotype has long been established, starting with a study by Nordenson, who reported an improvement of chromosomal instability following addition of catalase or superoxide dismutase (SOD) to FA cell cultures (100). Subsequent studies revealed that SOD, catalase and L-cysteine (a GSH precursor) prevented both spontaneous and alkylating agent induced chromosomal breakage in FA fibroblasts (101, 102). In addition, a direct association between elevated oxygen tension and both spontaneous and alkylating agent induced chromosomal aberrations in FA cells has been shown (103, 104). Hyperinduction of apoptosis by MMC exposure in FA-C cells is attenuated when cells are cultured under decreased oxygen levels that inhibit redox cycling of MMC and free radical production (105). A recent study demonstrated that overexpression of thioredoxin eliminated the hypersensitivity of FA cells to MMC and DEB (106). Thus, either the prevention or removal of reactive oxygen species (ROS), or the elevation of thiol reducing agents can compensate for the basic defect in FA. The role of FANCC as a novel regulator of GSTP1, a redox sensitive protein that detoxifies xenobiotics and ROS, is consistent with the abnormal oxygen metabolism associated with FA cells. A hypothetical model in which FANCC promotes the detoxification of xenobiotics and organic hydroperoxides by maintaining GSTP1 in an active reduced state is presented (Fig. 3-8).

GSTP1 is expressed at high levels in both spontaneous and chemically induced preneoplastic hepatic lesions in rats (22). Some preneoplastic lesions regress, whereas others persist and develop into hepatocellular carcinomas that are resistant to the cytotoxic effects of a variety of drugs (22). GSTP1 is also present at elevated levels in preneoplastic and
neoplastic lesions in a wide variety of organs in man (21, 107). These findings have led to the suggestion that GSTP1 be classified as a neoplastic marker. The correlation between elevated GSTP1 expression and preneoplasia may be attributed to GSTP1-mediated suppression of apoptosis and, subsequently, an increased potential for cellular transformation. Indeed, preneoplastic GSTP1-positive hepatocytes exhibit a poor apoptotic response after exposure to chemotherapeutic agents or anti-Fas antibody (108-110). The inhibition of IL-3 deprivation-induced apoptosis by overexpression of GSTP1, provides further evidence that this enzyme may contribute to neoplastic development.

Although GSTP1 may suppress apoptosis after chemotherapeutic drug exposure by enzymatic detoxification of the drug, the mechanism by which GSTP1 suppresses apoptosis induced by physiological stimuli (i.e. anti-Fas treatment, IL-3 withdrawal) is unknown. Furthermore, the ability of FANCC to maintain GSTP1 in a reduced state and suppress apoptosis even in the absence of GSH, a GSTP1 substrate, suggests that the FANCC/GSTP1 complex may function through a non-enzymatic mechanism. Recent studies have shown that GSTP1 can affect stress kinase pathways during oxidative stress (111, 112). The reduced monomeric form of GSTP1 directly binds to Jun N-terminal kinase (JNK) and acts as an inhibitor through a non-enzymatic process (111). However, in mouse fibroblasts treated with H₂O₂ or UV, GSTP1 multimerizes and dissociates from JNK (111). In a separate study, increased expression of GSTP1 in mouse NIH 3T3 cells, via a tetracycline regulated promoter, protected against H₂O₂-induced apoptosis and led to the activation of mitogen-activated protein (MAP) kinase kinase 4, p38, extracellular receptor kinase (ERK), and inhibitor of κ-kinase (IKK), and the inhibition of JNK (112). Thus, the coordinated regulation of stress kinases by GSTP1 during oxidative stress contributes to the protection against H₂O₂-induced cell death (112)
- Oxidative Stress
- MMC Activation
- Apoptosis (Factor Withdrawal)
- GSH Depletion

GSTP1 Oxidation

Reduced Monomer

Enzymatic Pathway

GSH conjugation with:
- Xenobiotics
- Lipid hydroperoxides
- DNA hydroperoxides

Survival

Non-Enzymatic Pathway

Modulation of Stress Kinases
- JNK
- p38
- IkK
- ERK

DNA Damage
- Lipid Peroxidation
- Stress kinase activation

Apoptosis
Figure 3-8. Potential mechanisms by which the FANCC/GSTP1 complex suppresses apoptosis. Oxidative stress leads to the formation of non-native disulfide bonds within (intrasubunit) or between (intersubunit) GSTP1 monomers. Oxidized GSTP1 is enzymatically inactive due to the loss of GSH binding and unable to detoxify xenobiotics or organic hydroperoxides, ultimately leading to widespread damage to cellular macromolecules. Oxidative inactivation of GSTP1 may also lead to inappropriate activation of stress kinases and triggering of apoptosis. High intracellular levels of GSH maintain GSTP1 in an active reduced state by thiol-disulfide exchange. The ability of FANCC to prevent GSTP1 inactivation during oxidative stress may contribute to the inhibition of apoptosis by both enzymatic and non-enzymatic pathways.
Elevated levels of GSTP1 protein have been implicated in multidrug resistance (MDR) in cancer cell lines and in cancer tissues resistant to chemotherapy (113-116). The heightened sensitivity of FA-C cell lines to alkylating agents may be related to an increased oxidation of GSTP1 in FA cells and an associated loss of drug detoxification. MMC is bioactively reduced and reoxidized by molecular oxygen resulting in cycles of reduction and oxidation with the concomitant generation of intracellular ROS (117). In the absence of a functional FANCC protein, GSTP1 may be susceptible to oxidation following MMC exposure, thereby losing its ability to detoxify xenobiotics, leading to increased DNA damage. However, alkylating agents, including MMC, can activate JNK and initiate apoptosis even in the absence of DNA damage (118-120). The redox state of the cell is critical for JNK activation by alkylating agents; while activation of JNK is blocked by treatment of the cells with reducing agents, such as GSH and NAC, depletion of intracellular GSH results in the superinduction of JNK following exposure to alkylating agents (120). The ability of FANCC to maintain GSTP1 in a reduced state, may facilitate the continued inhibition of JNK following MMC exposure, and the prevention of drug-induced apoptosis. Therefore, FANCC may inhibit apoptosis via GSTP1 by three mechanisms: (1) catalytically by a GSH conjugation mechanism with electrophilic compounds which leads to the detoxification of xenobiotics and DNA/lipid hydroperoxides, the prevention redox cycling and the accumulation of ROS, (2) non-catalytically via interactions with proteins like JNK or (3) through an unknown mechanism that leads to the differential activation or inhibition of stress kinases (Fig. 3-8). Future work will determine if FANCC regulation of GSTP1 directly alters the induction of stress kinases or the catalytic detoxification of ROS during apoptosis.
3.6. References


Chapter 4

Conclusions and Future Perspectives
As detailed in the previous chapters, the aim of this thesis was to determine the role of the FANCC gene during apoptosis in factor-deprived hematopoietic cells. In this chapter, the conclusions reached from each project will be briefly summarized in the context of the current understanding of the apoptotic pathways attributed to IL-3 deprivation, in addition to other pathways in which FA proteins have been implicated. The questions that have been raised by these studies will be discussed and the potential approaches to addressing them are proposed.

4.1. The function of the FANCC protein in hematopoietic cell death induced by IL-3 deprivation.

4.1.1. Overexpression of the FANCC protein does not stimulate cellular proliferation in the presence or absence of IL-3.

As discussed in Chapter 1, binding of IL-3 to the IL-3 receptor results in the activation of multiple signal transduction pathways that can generally be grouped into three categories: (1) activation of cellular proliferation genes, (2) suppression of apoptosis by inhibition of pro-apoptotic proteins or (3) activation of survival genes. In the case of the first category, it is unlikely that FANCC participates in the stimulation of cell growth because 32D or M07e cells overexpressing FANCC display doubling times similar to those of the parental cell lines in the presence of IL-3. Moreover, 32D-FANCC or M07e-FANCC cells both arrest in the G1 phase of the cell cycle after IL-3 withdrawal and exhibit a sharp decrease in [3H]thymidine incorporation, indicating a halt in DNA synthesis (Chapter 2). Thus, FANCC appears to inhibit apoptosis by promoting viability rather than proliferation.

4.1.2. The possible role of FANCC in the modulation of pro- and anti-apoptotic proteins

FANCC-mediated inhibition of apoptosis may occur through the modulation of either pro-apoptotic or anti-apoptotic proteins. Elevated levels of wildtype p53 hasten apoptosis after IL-3 withdrawal (1). However, overexpression of FANCC had no significant effect on p53 protein levels after IL-3 deprivation. Furthermore, FANCC
expression did not increase the levels of the anti-apoptotic protein Bcl-2 after factor withdrawal (Chapter 2). Thus, FANCC does not appear to affect the level of two well characterized proteins implicated in IL-3 withdrawal-induced apoptosis. However, these findings do not exclude the possibility that FANCC may affect other proteins that either promote or suppress apoptosis.

As discussed in Chapter 1, the Bcl-2 family of proteins are primarily involved in regulating the permeability of the mitochondrial membrane and play a significant role in regulating apoptosis following the removal of growth factors (2). The loss of the mitochondrial transmembrane potential ($\Delta \Psi_m$) results in the opening of the mitochondrial permeability transition (PT) pore and the release of pro-apoptotic proteins such as cytochrome c and apoptosis-inducing factor (AIF) (3). The release of cytochrome c is considered the main trigger for the activation of caspases following IL-3 deprivation (4-6). Although $\Delta \Psi_m$ loss and cytochrome c release was not directly examined in this study, IL-3 deprived 32D cells exhibited distinct signs of caspase activation as shown by PARP cleavage. Overexpression of FANCC resulted in only a modest inhibition of PARP cleavage (Chapter 3), indicating that $\Delta \Psi_m$ loss and caspase activation still occurred even in the presence of high levels of FANCC. A recent study showed that the loss of $\Delta \Psi_m$ following MMC induced apoptosis occurred equally in normal and FA lymphoblasts (7). Furthermore, overexpression of the FANCC protein in FA-C lymphoblasts inhibited apoptosis but not the decrease of $\Delta \Psi_m$ following MMC exposure. Thus, FANCC may inhibit apoptosis even when the loss of $\Delta \Psi_m$ occurs and caspases are activated.

Interestingly, overexpression of GSTP1 inhibited PARP cleavage more effectively than FANCC after the initiation of apoptosis in factor deprived 32D cells (Chapter 3). GSTP1 may prevent the loss of $\Delta \Psi_m$ and the subsequent activation of caspases in a manner independent of FANCC. In support of this hypothesis, a novel plant protein homologous to a class 0 GST (BI-GST) was shown to suppress cell death in yeast following overexpression of murine Bax (8). Bax-induced apoptosis in yeast is characterized by loss of $\Delta \Psi_m$ and release of cytochrome c (9). However, Bax-induced $\Delta \Psi_m$ loss is reversed by overexpression of either BI-GST or Bcl-2 (8). Therefore, GSTP1 may confer protection against apoptosis by suppressing $\Delta \Psi_m$ loss, cytochrome c release and the activation of apoptosis. Future
studies will focus on the effect of FANCC and GSTP1 expression on ΔΨm loss, cytochrome c release and caspase activation in IL-3 deprived cells to determine if these proteins alter mitochondrial events during apoptosis either together or independently of one another.

4.1.3. FANCC-mediated stabilization of GSTP1 may affect JNK activation following alkylation agent exposure

Recently, the monomeric form of GSTP1 was shown to interact directly with Jun N-terminal kinase (JNK) and inhibit its activation under non-stressed conditions (10). However, UV irradiation or H2O2 treatment causes GSTP1 oligomerization and dissociation of the GSTP1-JNK complex (10). JNK, also known as stress activated protein kinase (SAPK), belongs to the mitogen activated protein kinase (MAPK) family, and is activated by various environmental stresses (UV irradiation, heat shock, X-ray irradiation, H2O2, DNA damaging agents) and by proinflammatory cytokines (TNF-α and IL-1) (11). JNK is also transiently activated in response to stimulation of the IL-3 receptor (12-15). Like other kinases in the MAPK family, JNK is activated by phosphorylation on threonine and tyrosine residues by dual-specific MAPK kinases (MKK) known as MKK4 and MKK7 (16, 17). The activation of JNK then leads to the phosphorylation of the transcription factor c-Jun, resulting in an increase in its transcriptional activity (16). JNK activation has been implicated in both apoptosis and survival signaling (11). For example, JNK activation is necessary for neuronal cell death in response to neurotrophic factor withdrawal (18). In contrast, JNK activation is involved in the proliferative response of IL-3 dependent hematopoietic cells (15). Furthermore, inhibition of JNK by overexpression of a dual-specific threonine/tyrosine phosphatase does not confer protection against apoptosis following IL-3 deprivation in factor dependent cells (15). Therefore, it is unlikely that FANCC-mediated stabilization of GSTP1 affects JNK activity following IL-3 deprivation.

The specific role of JNK activation in mediating apoptosis appears to be inducer- and cell type-specific. Although JNK activation is not involved in apoptosis induced by IL-3 withdrawal in hematopoietic cells, it is strongly activated by bifunctional alkylating agents, including cis-platinum (CDDP) and mitomycin C (MMC) in fibroblasts (19, 20). In addition, the monofunctional alkylating agent methyl methanesulfonate (MMS) is also a potent activator of JNK and the p38 MAPK, and can induce apoptosis in a variety of cells
JNK activation is independent of a nuclear signal because cells lacking a nucleus (cytoplasts) still exhibit JNK activation after MMS treatment (21). Furthermore, the induction of JNK by MMS is redox sensitive; raising the intracellular level of glutathione (GSH) inhibits, whereas depletion of GSH causes hyperinduction of JNK activity (21). MMS-induced cytotoxicity is also associated with GSH loss and oxidative stress (24). MMC exposure causes hyperinduction of apoptosis in FA-C cells, which is linked to oxygen radical generation rather than DNA crosslinking (25). Furthermore, MMC sensitivity in FA cells is attenuated by the addition of interleukin-6 (IL-6) to the culture medium (26). IL-6 inhibits JNK activation in multiple myeloma cell lines and prevents apoptosis induced by serum starvation, dexamethasone treatment or triggering of the Fas death pathway (27, 28). Thus, the hypersensitivity of FA-C cells to MMC may be attributed to increased activation of JNK following drug exposure. In the absence of a functional FANCC protein, GSTP1 may be more susceptible to oxidative inactivation with a concomitant loss of JNK inhibition following MMC exposure. In support of this hypothesis, preliminary studies have shown that overexpression of GSTP1 in FA-C lymphoblasts can alleviate the MMC hypersensitivity of these cells (data not shown). Furthermore, inhibition of GSTP1 by treatment with ethacrynic acid (EA) leads to increased MMC sensitivity in wild-type but not FA-C lymphoblasts (data not shown). These observations indicate that GSTP1 activity is deficient in FA-C cells and that restoration of activity may be accomplished by overexpression of GSTP1.

To examine the hypothesis that FANCC, via GSTP1, inhibits JNK activation following MMC exposure, several experiments would be performed. Firstly, co-immunoprecipitation experiments using anti-JNK, Jun and FANCC specific antibodies would be used to determine if FANCC is part of the JNK-Jun complex. Secondly, in vitro kinase assays and immunoblot analysis with anti-phospho-JNK antibodies would be used to examine MMC-induced JNK activation in FA-C cells and compared with the JNK activity of MMC treated FA-C cells complemented with wild-type FANCC cDNA. If JNK activity was less in complemented FA-C cells, then the role of GSTP1 in JNK activation in FA-C cells would be examined through the use of GSTP1 specific inhibitors. Complemented and non-complemented FA-C cells would be treated with EA or glutathione analogs that specifically inhibit pi class GSTs (29) and MMC-induced JNK activity would be monitored.
in both cell types. If FANCC was found to mediate JNK inhibition, then the pharmacological inhibitor of JNK, CEP-1347 (30), would be used to determine if chemical inhibition of JNK in FA-C cells can mimic the protective effect of FANCC expression. In a similar set of experiments, overexpression of a dominant-negative form of JNK could be used to rescue the MMC hypersensitivity phenotype of FA-C cells.

4.1.4. The role of FANCC and GSTP1 in the regulation of MAP kinases during apoptosis induced by IL-3 withdrawal

MAP kinases (MAPKs) are stress-responsive kinases involved in the regulation of a wide range of cellular responses, including cell proliferation, differentiation and survival (31). Several distinct MAPKs have been identified that participate in independent signaling pathways. This family includes JNK, the p42/p44 extracellular signal-regulated kinases (ERK1/ERK2) and p38 MAPK (32). As discussed above, depending on the cell type and stimuli, activation of JNK and p38 MAP kinases is generally associated with the promotion of apoptosis. In contrast, ERK activation is associated with the inhibition of apoptosis induced in response to a wide range of stimuli including growth factor withdrawal and H2O2 or chemotherapeutic agent treatment (33-35). ERK is phosphorylated by MAPK kinases (MEK1/2), MEK1/2 is activated by Raf-1, and Raf-1 is activated by Ras (36). The balance between activation or inhibition of different MAPKs is important in determining whether a cell survives or undergoes apoptosis. For example, initiation of apoptosis by withdrawal of nerve growth factor (NGF) in PC12 cells requires activation of JNK and p38, with a concurrent inhibition of ERK (18). ERK1 and ERK2 have been shown to be activated by IL-3 in several hematopoietic cell lines (13, 15, 37, 38). Several studies have shown that signaling via Ras and ERK MAP kinases is not required for proliferation induced by IL-3, suggesting that the ERK pathway mainly mediates cell survival in hematopoietic cells (39, 40). Activation of the Ras pathway by the expression of a constitutively active form of Ras (RasG12V) prevents apoptosis in IL-3 dependent cells, and requires the activation of ERK (39-41). In addition, enforced expression of a constitutively active version of raf (v-raf) suppresses apoptosis in 32D cells but does not lead to factor independence following the withdrawal of IL-3 (42). Interestingly, 32D cells overexpressing v-raf have many features similar to 32D cells overexpressing either FANCC or GSTP1. For example, although v-raf
transduced cells are more viable following IL-3 deprivation, nuclear condensation still occurs (42). In addition, steady state levels of Bcl-2 and p53 remain unchanged in both the parental cell line and in the v-raf transduced 32D cell lines following factor-withdrawal induced apoptosis (42). These findings suggest that FANCC and GSTP1 may participate in the same pathways initiated by either ras or raf activation.

Recently, GSTP1 was shown to protect against H2O2-induced cell death by differentially regulating MAPKs (43). Increased expression of GSTP1 in NIH3T3 cells under non-stressed conditions resulted in the activation of M KK4, p38, ERK, and inhibitor of κ-kinase (IkK), and reduced activation of M KK7 and JNK. H2O2 treatment of control cells resulted in the induction of JNK, p38 and IkK, whereas cells expressing high levels of GSTP1 displayed an additional increase in ERK, p38, and IkK activities and a decrease in JNK activity (43). Furthermore, GSTP1-mediated protection from H2O2-induced death was attenuated by inhibition of p38, nuclear factor kB (NF-κB), or ERK dominant negative or pharmacological inhibitors (43). This study demonstrated that GSTP1 coordinates ERK/p38/IkK activation and JNK inhibition as part of a mechanism to suppress H2O2-induced cell death. Considering the role of ERKs in the survival of factor deprived hematopoietic cells, it is tempting to speculate that FANCC-mediated stabilization of GSTP1 increases or maintains ERK activity after IL-3 deprivation. To evaluate this hypothesis, ERK activity would be monitored in 32D cells expressing either FANCC or GSTP1 individually or together before and after IL-3 deprivation. If either FANCC or GSTP1 augmented ERK activity, then pharmacological inhibitors of upstream activators of ERK, such as PD098059, would be used to assess the importance of FANCC/GSTP1 modulation of ERK activity in the survival of IL-3 deprived 32D cells. If ERK activity was increased by either FANCC or GSTP1 then dominant-negative mutants of ERK1 and ERK2 could be expressed in all the 32D cell lines to determine if direct inactivation of ERK would abolish the protective effects of either FANCC or GSTP1.

4.2. Possible role of FANCC and GSTP1 in the development of leukemia

FA patients are at high risk of developing cancer, particularly acute myeloid leukemia (AML) (44). The risk of FA children developing AML is estimated to be 15,000 times greater than that of the general population (45). The high risk of AML in FA is
paradoxical because the lack of a functional FANCC protein is characterized by the progressive loss of hematopoietic cells in patients. Furthermore, overexpression of FANCC suppresses apoptosis and may allow the emergence of clones with an increased survival advantage, whereas the lack of wild-type FANCC renders hematopoietic cells more susceptible to apoptosis. However, this paradox may be reconciled if one considers the potential role of the FANCC/GSTP1 complex in the regulation of MAPKs. The role of MAPKs in leukemic transformation is best exemplified by the clonal myeloproliferative disorder caused by the t(9,22) Philadelphia chromosome translocation, which fuses Bcr to the c-Abl tyrosine kinase (46). The Bcr-Abl fusion protein is constitutively active and when expressed in hematopoietic cells causes cytokine independent proliferation, induces tumorigenic growth and prevents apoptosis in response to cytokine deprivation or DNA damage (46). Expression of Bcr-Abl leads to activation of Ras, whereas interruption of Ras function interferes with the transforming and anti-apoptotic ability of Bcr-Abl (47, 48). Bcr-Abl, via ras, activates JNK leading to increased transactivation of c-Jun (49). Dominant-negative mutants of c-Jun impair Bcr-Abl transforming activity (50). Furthermore, a cytoplasmic protein that binds to JNK, JIP-1, causes cytoplasmic retention of JNK, inhibition of JNK-regulated gene expression, and suppresses the effects of the JNK signaling pathway on cellular proliferation, including transformation by Bcr-Abl (51). These studies have shown that Bcr-Abl is dependent on JNK activation for its transforming ability.

The inhibitory effects of GSTP1 on JNK activation may contribute to the prevention of cellular transformation or deregulated proliferation. Analysis of mice with a targeted disruption of the Gstp gene cluster (Gstp1/p2<sup>−/−</sup>) revealed that in the absence of a functional Gstp protein, mice are highly susceptible to increased skin tumor formation following exposure to tumor promoting agents (52). In addition, fibroblasts derived from Gstp1/p2<sup>−/−</sup> mice exhibit higher basal and UV-induced JNK activity compared to wild-type fibroblasts (10). Interestingly, the glutathione analog TER199, a GSTP1 specific inhibitor, has myeloproliferative effects in mice (53). In wild-type mice, a single administration of TER199 results in a dose dependent increase in the number of granulocyte/macrophage colonies formed from extruded bone marrow (53). JNK activation is involved in the proliferation of hematopoietic progenitor cells in response to growth-factor stimulation (15).
Therefore, removal of JNK inhibition by chemical inactivation of GSTP1 by TER199, may allow increased proliferation of certain bone marrow progenitor cells in response to growth factors. In the absence of a functional FANCC protein in FA-C patients, GSTP1 may be more readily inactivated, thereby allowing uncontrolled JNK activation in a subset of hematopoietic cells. Deregulated JNK activation, in combination with elevated chromosomal instability, may lead to the increased possibility of cellular transformation in FA-C patients. Analysis of JNK activity in leukemic cells from FA-C patients may provide evidence to support this hypothesis. Neither Fancc−/− nor Gstp1/p2−/− mice spontaneously develop tumors, though a double mutant mouse strain may be more susceptible to cellular transformation. Crossbreeding both mouse strains to produce double mutant mice could then be examined for the appearance of tumors or hematological malignancies over time. The double mutant mice could also be challenged with sub-lethal doses of MMC to induce cellular transformation and compared with similarly treated heterozygote and wild-type littermates.

4.3. Lack of GSTP1 function may contribute to anemia in FA

Hereditary defects in the synthesis of glutathione are extremely rare and are associated with hemolytic anemia and, in some instances, neurological disorders and metabolic acidosis (54, 55). Interestingly, several cases of hemolytic anemia attributed to a hereditary glutathione synthetase deficiency are also associated with a GST deficiency (56). However, the GST deficiency is considered a secondary effect because the addition of high levels of GSH before enzyme analysis can restore GST activity in erythrocytes from these patients. The authors of this study concluded that the erythrocyte GST deficiency was due to an instability of the enzyme in the absence of adequate erythrocyte GSH levels (56). GSTP1 is the predominant GST isozyme found in red blood cells (57). Therefore, it is not surprising that low GSH levels in erythrocytes would lead to oxidative inactivation of GSTP1. However, in a separate study, a patient was found with mild hemolytic anemia that was attributed to a direct GST deficiency, although the defective GST isozyme was not identified (58). Because GSTP1 binds heme, it has been suggested that its function may be in the intracellular transport of heme within the developing erythroid cell (59, 60). The
absence of functional erythrocyte GST may lead to impaired hemoglobin synthesis, red cell alterations and increased hemolysis.

Because overexpression of GSTP1 in hematopoietic progenitor cells inhibits the loss of GSH during apoptosis, it may be possible that insufficient GSTP1 activity in FA erythrocytes may contribute to red cell anemia observed in these patients. Although decreased red cell survival plays only a minor role in the development of FA, erythrocytes from FA patients often appear macrocytic, exhibit an irregular shape or have surface blebbing (61-63). FA patients also exhibit stress erythropoiesis with the production of erythrocytes with fetal characteristics including increased fetal hemoglobin (61). Two recent studies indicated that the abnormalities in FA erythrocytes may be due to an altered redox balance because the ratio of oxidized to reduced glutathione (GSSG/GSH) in FA erythrocytes was significantly higher than that of normal controls (62, 63). Furthermore, significant modifications of the spectrin cytoskeleton network were found in FA erythrocytes but not in normal erythrocytes, indicating that oxidative modification, denaturation or crosslinking of cytoskeletal components occurred in FA red blood cells (62, 63). GSTs are only capable of binding the reduced form of glutathione (GSH) and not the oxidized form (GSSG). Therefore, as the predominant GST isozyme in erythrocytes, GSTP1 may participate in the regulation of the GSSG/GSH ratio by sequestering GSH and preventing either its oxidation or efflux. However, in the absence of FANCC, GSTP1 may be more susceptible to oxidative inactivation and unable to bind GSH. Analysis of the enzymatic activity and redox forms of GSTP1 in FA erythrocytes, particularly in group C patients, would provide evidence to support this hypothesis. GSTP1 activity in FA erythrocytes could be determined using EA as a specific substrate and the redox state of GSTP1 could be analyzed by non-reducing SDS-PAGE and immunoblotting with a GSTP1 specific antibody as described in Chapter 3.
4.4. The role of FANCC in the redox regulation of apoptosis

4.4.1. FANCC-mediated stabilization of GSTP1 may enhance detoxification of lipid hydroperoxides during apoptosis

Oxidative stress can be defined as a disturbance in the prooxidant-antioxidant balance within the cell resulting in the excess production of reactive oxygen species (ROS). ROS are capable of direct oxidative damage to macromolecules including DNA, protein, and lipid membranes (64). Several lines of evidence have clearly implicated ROS as mediators of apoptosis. Firstly, elevating ROS levels by the addition of agents that produce free radicals or by depleting endogenous antioxidants can induce apoptosis (65-67). Secondly, apoptosis can be inhibited in some instances by increasing the levels of endogenous or exogenous antioxidants (68, 69). Finally, apoptosis is frequently associated with increases in intracellular ROS levels and/or the depletion antioxidants such as GSH (69-71).

As discussed in Chapter 1, cells possess several anti-oxidant enzymes (catalase, SOD, GPx) or reductants (GSH, thioredoxin) that maintain the intracellular redox environment in a reduced state. GSTs, including GSTP1, are known to act as selenium-independent GSH peroxidases that catalyze the reduction of organic hydroperoxides (72). Suppression of apoptosis by overexpression of GSTP1 in 32D cells may partially be attributed to the increased detoxification of hydroperoxides. Although 32D cells transduced with a GSTP1 retroviral vector did not exhibit higher activity towards the classical organic peroxidase substrate cumene hydroperoxide (data not shown), it may be possible that GSTP1 reacts with lipid oxidative breakdown products produced in vivo during apoptosis. For example, GSTP1 exhibits high reactivity with α,β-unsaturated aldehydes derived from polyunsaturated fatty acids during lipid peroxidation (72). In addition, GSTP1 is particularly active in catalyzing GSH conjugation with adenine and thymine propenals, reactive purine and pyrimidine bases formed during oxidative damage to DNA (73). Furthermore, GSTP1 has an extremely high affinity for acrolein, an α,β-unsaturated aldehyde found in tobacco smoke, gasoline, and diesel exhaust, and produced intracellularly as a result of metabolic activation of the cytostatic drug cyclophosphamide (73).
Interestingly, FA cells are hypersensitive to cyclophosphamide and low doses of this drug must be used to pre-condition FA patients for bone marrow transplantation (74, 75).

Recent studies have shown that acrolein (CH\(_2\)=CH-CHO) is generated endogenously during lipid peroxidation associated with oxidative stress in cells and tissues, and is rapidly incorporated into proteins (76-78). In vitro, acrolein modifies axonal cytoskeletal proteins and reacts rapidly with and depletes GSH (79). Acrolein ranks among the most active substrates known for GSTP1 (80). GSTP1 has also been shown to confer protection against the cytotoxic effects of acrolein and cyclophosphamide in vivo (73, 81). It is possible that the FANCC-mediated increase in GSTP1 activity may contribute to the detoxification of reactive aldehydes formed during IL-3 withdrawal-induced apoptosis. IL-3 dependent cells treated with the aldehyde generating agent, methional, undergo apoptosis even in the presence of IL-3 (82). In addition, the endogenous levels of malondialdehyde, an aldehyde structurally related to acrolein, increase following IL-3 deprivation (82). Livers from mouse strains with targeted disruptions in both the Fancc (Fancc\(^{+}\)) and Cu,Zn-SOD (Sod\(^{+}\)) gene loci display significantly elevated levels of lipid peroxidation compared to livers from either wild-type, Fancc\(^{+}\) or Sod\(^{+}\) mice (F. Jirik, personal communication). These findings suggest that the FANCC protein may indirectly participate in the detoxification of ROS, in particular lipid hydroperoxides, via a GSTP1-mediated mechanism. The possible endogenous substrates produced during oxidative stress that may be detoxified by GSTP1 are shown in Figure 4-1.

In order to determine if the suppression of apoptosis afforded by either FANCC-mediated stabilization of GSTP1 or by direct overexpression of GSTP1 in 32D cells occurs by an enzymatic mechanism, several experiments would be performed. Firstly, acrolein-induced cytotoxicity would be examined in all the 32D cell lines and the effect of FANCC and GSTP1 overexpression on acrolein sensitivity would be determined. In vitro enzyme assays would then be performed using cell lysates to determine if either FANCC or GSTP1 overexpression in 32D cells increases the rate of conjugation of acrolein with GSH. Similar studies would be performed using patient-derived FA-C lymphoblasts with and without a complementing FANCC cDNA to evaluate the sensitivity of FA-C cells to acrolein, and the ability of wildtype FANCC to alleviate this sensitivity. Secondly, the effects of GSTP1 and FANCC expression on acrolein-induced GSH depletion would be assessed in 32D cells.
Figure 4-1. FANCC may enhance the ability of GSTP1 to detoxify endogenous substrates produced during oxidative stress. (a) Acrolein is a reactive aldehyde generated during lipid peroxidation and is detoxified by glutathione (GSH) conjugation. (b) Adenine propenal is a product of oxidative DNA damage and is also detoxified by GSH conjugation.
Lastly, site-directed mutagenesis of residues within the catalytic sites of GSTP1, such as Tyr 7, would be used to inactivate enzyme activity while maintaining the ability of GSTP1 to inhibit JNK (10). The catalytic inactive mutants would then be overexpressed in 32D cells and their ability to suppress either IL-3 withdrawal or acrolein-induced apoptosis would be determined. Catalytically inactive GSTP1 mutants that can still confer protection against apoptosis would support the hypothesis that GSTP1 can also exert protective effects through the modulation of stress kinases.

4.4.2. FANCC may act as a redox regulator of proteins involved in signal transduction and apoptosis

A growing number of studies have demonstrated that ROS can act as agents which initiate specific signaling pathways by modifying redox sensitive cysteine residues within proteins, rather than acting as agents which nonspecifically and irreversibly damage intracellular macromolecules (83). Disulfide bonds play an important structural role in many proteins, especially proteins in the extracellular environment or on the cell surface, reflecting the oxidizing conditions found there (84). In contrast, disulfide bonds are rarely found in cytoplasmic proteins due to the strongly reducing environment. In fact, when many exported proteins that ordinarily form disulfide bonds are expressed in the cytoplasm, they do not form these bonds (85). Cysteine residues are among the most easily oxidized residues in proteins, and the oxidation of cysteine thiol groups occurs readily during oxidative stress (86). The oxidation of thiol groups can result in the formation of protein disulfide bonds between cysteine residues within the same protein (intramolecular), between cysteine residues in separate proteins (intermolecular or mixed disulfides) or between cysteine residues and low-molecular weight thiols like GSH (protein S-thiolation) (87). Two major factors are responsible for maintaining a low redox potential and high thiol levels in the cytoplasm. The first is GSH, which is present in millimolar concentrations and is kept reduced by NADPH and glutathione reductase (88). The second factor is thioredoxin (TRX), a ubiquitous thiol-disulfide oxidoreductase which is reduced by electrons from NADPH via thioredoxin reductase (89).

Glutathione maintains cytoplasmic proteins in a reduced state by a thiol-disulfide exchange reaction that is catalyzed by glutaredoxin (GRX), also known as thiol-transferase...
In the process of reducing protein disulfide bonds, GSH itself becomes oxidized and is subsequently reduced by glutathione reductase in a NADPH dependent reaction. The reducing ability of TRX depends on two cysteine residues, which are located in close proximity to one another in a Cys-X₁-X₂-Cys active site. This active site is found in other members of the thioredoxin superfamily including GRX and protein disulfide isomerase (PDI) (91). The reduced form of TRX binds to substrate proteins containing a disulfide bond, and a dithiol-disulfide exchange reaction occurs in which the active site cysteine residues of TRX are oxidized while the cysteine residues in the substrate protein are reduced. The oxidized TRX active site is then reduced back to a dithiol state by TRX reductase. GSH and TRX are not only essential for maintaining the structural integrity and function of cytoplasmic proteins but also conferring protection against proteolytic attack by proteinases that more readily recognize oxidized proteins (86).

Oxidative stress can activate apoptotic pathways via the JNK and p38 kinases by inhibiting redox sensitive regulators of these kinases. For example, TRX binds to the apoptosis signal-regulating kinase (ASK1), an upstream activator of both JNK and p38, and under normal conditions inhibits its activity (92). However, serum withdrawal, TNFα or H₂O₂ treatment results in the dimerization of TRX, its dissociation from ASK1 and the activation of JNK and p38 kinases (92). The inhibitory effects of TRX on ASK1 activation can be mimicked by N-acetyl-L-cysteine, a potent antioxidant and GSH precursor (92, 93). Depending on the cell type, TRX can also activate or inhibit the transcription factor NF-κB (94). TRX appears to play dual and opposing roles in the regulation of NF-κB depending on its cellular location. In the cytoplasm, TRX interferes with the signals to IkB kinases and blocks the degradation of IkB, whereas in the nucleus, TRX enhances NF-κB transcriptional activities by enhancing its ability to bind DNA (95). Nuclear TRX maintains the Cys-62 residue within the p50 subunit of NF-κB in a reduced state, which is essential for its DNA binding activity (96). Overexpression of TRX prevents apoptosis in lymphoid cells induced by GSH depletion or by treatment by a variety of agents including dexamethasone, staurosporine and etoposide (97, 98). Thus, the TRX and GSH systems ensure that apoptotic pathways are not activated under normal conditions or during mild oxidative stress. However, initiation of apoptosis by a variety of stimuli can result in GSH depletion.
and TRX dimerization, leaving the cytosol in an unprotected state and allowing oxidation of proteins.

Recently, three-dimensional analysis of a number of proteins involved in thiol-disulphide interchange revealed a common structure among these proteins termed a thioredoxin fold (99). The thioredoxin fold consists of four central β strands surrounded by three α helices (99). The superfamily of proteins containing a thioredoxin fold can be grouped into six classes: thioredoxins, glutaredoxins, DsBA, protein disulfide isomerases, glutathione S-transferases, and glutathione peroxidases (99, 100). Although the sequence homology between these six classes is limited and no function or activity is common to all, there is a functional similarity shared with four of these members. The C-X$_1$-X$_2$-C active site motif is found in thioredoxins, DsBA, protein disulfide isomerases and glutaredoxins. The glutathione S-transferases and glutathione peroxidases lack the C-X$_1$-X$_2$-C motif but share with the glutaredoxins a specific interaction with GSH.

Among the recent additions to the thioredoxin superfamily is a protein that interacts with protein kinase C, termed PICOT (protein kinase C-interacting cousin of thioredoxin) (101). PICOT is similar to GSTP1 in the sense that it also has a thioredoxin fold but lacks a C-X$_1$-X$_2$-C active site motif (101, 102). Like GSTP1, transient overexpression of PICOT in cells reduces the basal activity of JNK but increases ERK activity (101). Interestingly, PICOT was recently identified as a FANCC-interacting protein in a yeast two-hybrid screen (M. Hoatlin, personal communication). The fact that GSTP1 and PICOT both share a thioredoxin fold suggests that FANCC may specifically interact with proteins containing this domain.

FANCC has been shown to form a complex with the cyclin dependent kinase Cdc2, although a direct interaction between these proteins was not detected (103). Expression of wild type FANCC protein in FA-C cells prevents the sustained hyperphosphorylation of Cdc2 and G$_2$/M arrest observed after MMC treatment (104). Phosphorylation of Cdc2 at Thr-161 promotes the association of Cdc2 with cyclin B, increases its kinase activity and facilitates the entry of cells into mitosis (105). However, Cdc2 is kept inactive by phosphorylation at Thr-14 and Tyr-15 throughout the cell cycle, except during the G$_2$/M phase (106). Activation of Cdc2 depends on the dual-specificity phosphatase Cdc25 which dephosphorylates both Thr-14 and Tyr-15 residues of Cdc2 during the G2/M phase of the
cell cycle (107). Cdc25 is a redox sensitive phosphatase with a small α/β catalytic domain that bears a strong resemblance to the thioredoxin fold domain (108). Like GSTP1, Cdc25 contains a redox sensitive cysteine residue within its catalytic site that can form an intramolecular disulfide bond with an N-terminal cysteine residue (108, 109). Furthermore, oxidation of the cysteine residue in the catalytic site during disulfide bond formation or by direct alkylation with N-ethylmaleimide inhibits the phosphatase activity of Cdc25 (108, 110). Interestingly, the study in which the 34 kDa Cdc2 protein was identified as a FANCC interacting partner by co-immunoprecipitation analysis using an anti-FANCC antibody, also revealed an abundant 80 kD interacting protein (103). Although the authors of this study did not identify this protein, it is possible that it may be Cdc25, which also has an electrophoretic mobility of 80 kDa (111). FANCC may regulate thioredoxin fold containing proteins that control stress kinase activation and cell cycle progression (Figure 4-2).

A FANCC/Cdc25 interaction may be necessary to maintain the phosphatase activity of Cdc25 during oxidative stress. In the absence of FANCC, Cdc25 may be susceptible to oxidative inactivation, especially during MMC-induced stress, and unable to dephosphorylate Cdc2, leading to a G2/M arrest or delay. To confirm that these proteins interact, co-immunoprecipitation studies using anti-FANCC and anti-Cdc25 antibodies would be performed. If an interaction was detected, then further studies using yeast-two hybrid assays and recombinant fusion proteins, would be used to identify the domains within each protein required for the interaction. Detection of oxidized Cdc25 in complemented and non-complemented FA-C cell lines would be performed using non-reducing SDS-PAGE followed by immunoblotting with an anti-Cdc25 antibody. The oxidized form of Cdc25 would be identified by its faster electrophoretic mobility when compared with the reduced form. If FANCC inhibits the oxidation of Cdc25, then redox insensitive mutants of Cdc25 would be made by replacing the cysteine residues involved in intramolecular disulfide bond formation with alanine residues. The Cdc25 cysteine mutants would then be expressed in FA-C cells in an attempt to rescue the hyperphosphorylation of Cdc2 and associated G2/M phase delay found in these cells.
Figure 4-2. FANCC may regulate stress kinase activation and cell cycle progression via proteins that contain a thioredoxin fold domain. FANCC may interact with proteins containing a thioredoxin fold domain (indicated by hatched area) and maintain cysteine residues that are essential for their function in a reduced state. Kinase regulating proteins affected by FANCC may include GSTP1 (glutathione S-transferase P1), PICOT (protein kinase C-interacting cousin of thioredoxin) and Cdc25 (cell division cycle 25).
4.4.3. **FANCC may directly participate in the oxidation/reduction of redox sensitive proteins**

The mechanism by which FANCC maintains GSTP1 in a reduced state is unknown. One possibility is that FANCC directly catalyzes thiol/disulfide exchange reactions with the proteins it interacts with. Although FANCC lacks any known functional motifs, it does possess a preponderance of highly conserved cysteine residues (112). Furthermore, the N-terminus of FANCC contains the sequence Ile-Trp-Cys-Leu-Cys-Cys-Leu (IWCLCCL) which is 100% conserved amongst four species, including human, mouse, rat and bovine (112). As discussed above, members of the thioredoxin family contain the active site C-X₁-X₂-C (where X₁ and X₂ represent any of the 19 commonly occurring noncysteine amino acids) (113). The identities of the two central residues (X₁ and X₂) vary considerably between the thioredoxin family of proteins, and have been shown to have substantial effects on the stability of the disulfide bond formed between the N-terminal and C-terminal cysteine residues within the active site (114). To date, no thioredoxin active sites have been identified containing the sequence C-X-C-C. The active site is usually found in an exposed loop that exists between an α-helix and β-strand within a thioredoxin fold structure (99). Solving the three-dimensional structure of FANCC by X-ray crystallography would greatly help in determining if the C-X-C-C motif is located in an exposed location and could function as a thioredoxin active site. Recently, Phosphoglycerate kinase (PGK), an enzyme traditionally involved in glycolysis, was found to reduce disulfide bonds in the serine proteinase plasmin (115). PGK reduces plasmin through a mechanism involving one or more of its cysteine residues, even though it lacks a C-X-X-C active site. Therefore, an alternative mechanism for reducing disulfide bonds must exist in proteins that lack conventional reductase active sites.

The theory that the FANCC protein may participate in a thiol-disulfide exchange mechanism is indirectly supported by several lines of evidence. Firstly, overexpression of TRX in FA fibroblasts can alleviate the hypersensitivity of these cells towards MMC and DEB (116). Secondly, extracellular addition of antioxidants (SOD, catalase) or reducing agents (DTT, L-cysteine) can inhibit both spontaneous and MMC-induced chromosomal breakage in FA fibroblasts (117-119). Finally, the molecular chaperone GRP94 was shown to interact with and stabilize FANCC (120). GRP94 belongs to the heat shock family of
proteins and, like protein disulfide isomerase, facilitates the folding of a variety of secretory and membrane bound proteins (121). GRP94 binds to the N-terminus of FANCC, within an area that contains several highly conserved cysteine residues and is immediately adjacent to the C-X-C-C motif (112, 120). Furthermore, inactivation of GRP94 in rat NRK cells leads to FANCC degradation and a concomitant hypersensitivity to MMC (120). Thus, GRP94 may stabilize FANCC by preventing the formation of mispaired disulfide bonds within FANCC and the premature degradation of the protein, particularly during oxidative stress.

The mechanism of thiol-disulfide exchange involves the formation of a transient mixed disulfide intermediates between thioredoxin and the substrate protein (122). Due to the rapid nature of this reaction, it is difficult to trap proteins with the ratios of thiol-disulfide-bonded species as they exist in vivo (91). However, using agents such as NEM, which alkylates free cysteines, and acid trapping techniques, it is possible to isolate mixed-disulfide intermediates (123). Using the techniques described in Chapter 3 for the detection of oxidized GSTP1 isoforms, it may be possible to detect proteins that interact with FANCC via a mixed disulfide. By taking measure to prevent thiol-disulfide exchange, FANCC mixed-disulfide complexes could be immunoprecipitated and resolved by non-reducing SDS-PAGE. Unique bands that are only present using non-reducing isolation techniques could be identified by microsequence analysis. This procedure could also be applied to analyzing FANCC mixed disulfides formed during apoptosis induced by IL-3 withdrawal or MMC treatment.

The regulation of protein activity in response to a changing environment includes well established mechanisms such as protein-protein interactions, allosteric changes generated by ligand binding and phosphorylation/dephosphorylation reactions. The regulation of intracellular protein activity by disulfide bond formation has largely been overlooked based on the assumption that cytoplasmic proteins predominantly exist in a reduced state due to the abundant presence of reducing equivalents within the cytoplasm. However, the efflux or oxidation of GSH and the dramatic rise in ROS that occurs during apoptosis initiated by a variety of stimuli can create an environment that can promote the formation of mispaired disulfide bonds within or between redox sensitive proteins. The observation that GSTP1 becomes oxidized following IL-3-withdrawal is the first example that a cytosolic protein can be inactivated by the generation of non-native disulfide bonds
during apoptosis initiated by a physiological relevant stimuli (i.e. factor withdrawal). Furthermore, the prevention of disulfide bond formation by FANCC, a protein that lacks homology with classical thiol-disulfide oxidoreductases, indicates that an alternative pathway for redox regulation of proteins exists. The identification of proteins that form mixed disulfides with FANCC, which may include other FA proteins, will provide valuable insight into the pathobiology of this devastating disease.

4.5. References


