Stat3 and CCAAT enhancer–binding protein β (C/ebpβ) activate Fanconi C gene transcription during emergency granulopoiesis.

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Abstract
Interferon consensus sequence–binding protein (Icsbp) is required for terminating emergency granulopoiesis, an episodic event responsible for granulocyte production in response to infections and a key component of the innate immune response. Icsbp inhibits expression of Stat3 and C/ebpβ, transcription factors essential for initiating and sustaining granulopoiesis, and activates transcription of Fanconi C (FANCC); a DNA repair protein. In prior studies, we noted accelerated bone marrow failure in Fancc-/- mice undergoing multiple episodes of emergency granulopoiesis, associated with apoptosis of bone marrow cells with unrepaired DNA damage. Additionally, we found increased expression of Fanconi C and F proteins during emergency granulopoiesis. These findings suggest that Icsbp protects the bone marrow from DNA damage by increasing activity of the Fanconi DNA repair pathway, but the mechanisms for FANCC activation during initiation of emergency granulopoiesis are unclear. In the current study, we observed that Stat3 and C/ebpβ activate FANCC transcription and contribute to DNA repair. Our finding indicate that FancC expression is increased during Stat3- and C/ebpβ-induced initiation of emergency granulopoiesis by these transcription factors, and is maintained through termination by Icsbp. Our work reveals that Stat3- and C/ebpβmediate FancC expression as a critical component for initiating and sustaining the key innate immune responses.

Introduction
The Fanconi DNA-repair pathway rescues collapsed or stalled replication forks during S phase of the cell cycle, protects chromatin common fragile sites (CFSs) during DNA replication, and effects repair DNA interstrand crosslinks (ICLs) (1-6). Fanconi proteins are categorized in three groups; core components, substrates and effectors. Assembly of the core components (Fanconi A, B, C, E, F, F, L, M) into a complex with ubiquitin ligase activity is the first step in activating the Fanconi pathway. This complex activates the substrate components (Fanconi D2 and I) through mono-ubiquitination. These substrate proteins recruit effectors to sites of DNA damage (Fanconi D1, J, O, P and Rad51) (1,3). Fanconi Anemia (FA) is an inherited disorder due to mutation of a gene in the Fanconi repair pathway. FA is clinically variable, but classically involves skeletal abnormalities, bone marrow failure (BMF) during childhood, and progression to acute myeloid leukemia (AML) in subjects surviving BMF (1,7,8). In prior studies, we found that BMF and/or clonal progression were accelerated in a murine model of FA (Fancc-/- mice) by repeated stimulation of an emergency (stress) granulopoiesis response (9).

Emergency granulopoiesis is an episodic process for production of granulocytes in response to infectious challenge and a key component of the innate immune response (10,11). In contrast, steady state granulopoiesis is a continuous process for replacement of granulocytes lost to normal programmed cell death. Studies murine models with gene disruptions demonstrated that emergency granulopoiesis requires IL1β, and an IL1β-induced expression of G-CSF at levels that are 10 fold
greater than steady state (11,12). In additional murine gene disruption studies, Stat3 and C/ebpβ were found to be necessary for initiation and maintenance of the emergency granulopoiesis response (13,14). Also, such studies determined that termination of emergency granulopoiesis requires the leukemia suppressor Icsbp (also known as interferon regulatory factor 8; Irf8) and the proto-oncogene HoxA10 (15,16). In contrast, steady state granulopoiesis requires Stat5 and C/ebpα and is facilitated by G-CSF and GM-CSF (12).

In prior studies, we found that Icsbp activated FANCC and FANCF gene transcription during emergency granulopoiesis (respectively encoding Fanconi C and F; FanC and FancF) (9,17). This was associated with Icsbp-dependent protection of bone marrow cells from DNA damage during in vivo stimulation of emergency granulopoiesis in mice, or ex vivo stimulation of bone marrow cells with IL1β or G-CSF (9,17). Also, we determined that Icsbp is required for decreased expression of Stat3 and C/ebpβ during termination of the emergency granulopoiesis response (15).

Emergency granulopoiesis is studied in mice by intraperitoneal (IP) injection with pathogens (Candida Albicans or encapsulated bacteria), or an antigen/adjuvant mixture referred to as “Alum” (ovalbumin antigen and aluminium hydroxide adjuvant) (9,11,15,18). In Wt mice, this results in immediate release of mature granulocytes from the bone marrow, followed by enhanced commitment of hematopoietic stem cells (HSC) to granulocytes, with maximal expansion of granulocyte/monocyte progenitors and differentiating granulocytes in the bone marrow by two weeks (9,15). By four weeks, the process has terminated and steady state resumed.

We found injecting Alum every four weeks repeated this process in Wt mice without resulting in morbidity or mortality (9,15). We performed repeated induction of emergency granulopoiesis to mimic repeated infectious challenges, due to environmental exposure to bacterial and fungal pathogens, which occur on an ongoing basis in humans.

Although Alum injection induced immediate granulocyte release from the bone marrow of Fancεm mice, there was no subsequent expansion of myeloid progenitors or production of mature granulocytes (9). Instead, repeated emergency granulopoiesis attempts resulted in progressive pancytopenia and death in most Fancεm mice; associated with apoptosis of bone marrow HSC and progenitors (9). Surviving mice developed clonal progression with a rapid rise of myeloid blasts in the bone marrow (9).

These studies suggest Icsbp protects the bone marrow from DNA-damage as emergency granulopoiesis is terminating by increasing activity of the Fanconi DNA-repair pathway (9,15,17). However, mechanisms to increase FANCC transcription during initiation of emergency granulopoiesis or at peak granulocyte production are not clear.

In this work, we investigate the roles of Stat3 and C/ebpβ in activation of the FANCC gene promoter. We determine that Stat3 and C/ebpβ increase FANCC transcription during initiation of emergency granulopoiesis, Icsbp cooperates with these two transcription factors for maximal FANCC transcription during peak granulocyte production, and Icsbp maintains FANCC expression as Stat3 and C/ebpβ levels fall during termination of this process. We also implicate a sustained increase in Stat3 and C/ebpβ proteins as the major mechanism for this activity during initiation and peak emergency granulopoiesis.

Results

Stat3 and C/ebpβ activate separate FANCC promoter cis elements. In prior studies, we identified an Icsbp binding cis element in the proximal FANCC promoter (-48 to -56 bp) (9). We hypothesize that Icsbp protects bone marrow stem and progenitor cells from DNA damage during termination of emergency granulopoiesis. Since Stat3 and C/ebpβ are required to initiate and sustain emergency granulopoiesis, we considered the possibility that these transcription factors activate the FANCC promoter early in this process.

To investigate this hypothesis, we assayed promoter activity of a set of reporter constructs with -1.0 kb to -400 bp of the FANCC 5’ flank linked to a firefly luciferase reporter (or empty control reporter vector) (Figure 1A). We determined activity of these constructs in U937 myeloid cells co-transfected with vectors to express Stat3 or C/ebpβ (vs empty control expression vector). For these studies, we considered two isoforms of C/ebpβ; Lap and Lip. The former is a transcriptional activator and the shorter, Lip isoform an antagonist of Lap (19). Since Stat3 and
Stat5 share similar binding site consensus sequences, we tested both proteins. Icsbp was a positive control for activation of the FANCC promoter. All cells were co-transfected with an internal control reporter vector (with a CMV promoter and renilla luciferase reporter) to normalize for transfection efficiency.

We found Stat3, C/ebpβ-Lap and Icsbp each significantly increased reporter activity from constructs with 1.0 kb or 500 bp of FANCC promoter (p<0.001, n=6) (Figure 1B). Both Icsbp and C/ebpβ-Lap activated a construct with 450 bp of FANCC promoter (p<0.001, n=6), but Stat3 did not. This suggested the presence of a Stat3-influenced cis element between 450 and 500 bp in the FANCC promoter.

Further truncation of the promoter to 400 bp abolished activation by C/ebpβ-Lap, identifying a potential cis element between -450 and -400 bp (Figure 1B). None of the FANCC promoter constructs were activated or repressed by Stat5 or C/ebpβ-Lip. None of these proteins influenced activity of an empty reporter control vector and this was subtracted as background.

Stat3 and C/ebpβ are required for emergency granulopoiesis and IL1β is the essential cytokine for this process. Therefore, we tested the effect of Stat3 and C/ebpβ on FANCC promoter activity during differentiation with IL1β (11). We assayed activity of the 1.0 kb FANCC promoter/luciferase reporter construct in U937 cells co-transfected with vectors to overexpress or knockdown Stat3 or C/ebpβ-Lap. We found that IL1β significantly increased FANCC promoter activity, with or without overexpression of Stat3 or C/ebpβ-Lap (p<0.0001, n=6) (Figure 1C). We also found knockdown of either Stat3 or C/ebpβ significantly decreased FANCC promoter activity (p<0.001, n=6), but only in IL1β-treated transfectants. This was consistent with a role for endogenous Stat3 and C/ebpβ in FANCC promoter activation during IL1β-induced differentiation.

We investigated possible additive or cooperative effects of these transcription factors on the FANCC promoter, despite interacting with discrete binding sites. For these studies, we co-transfected U937 cells with the 1.0 FANCC promoter/firefly luciferase reporter vector and various combinations of vectors to overexpress Stat3, C/ebpβ-Lap or Icsbp. Cells were co-transfected with an internal control reporter vector, described above. We found the combination of any two of these transcription factors were additive for FANCC promoter activity (p<0.001, n=6), and the effect of all three was greater than any two (p<0.001, n=6) (Figure 1C). The total amount of expression vector was maintained at a constant level in these studies (e.g. there is half the amount of Stat3 plasmid in Stat3 + C/ebpβ experiments compared to experiments with Stat3 alone). Therefore, these studies indicated cooperation, rather than redundancy, between the three transcription factors.

Stat3 and C/ebpβ bind to and activate discrete FANCC promoter cis element. To confirm that Stat3 and C/ebpβ activated cis elements within the promoter regions defined above, we performed sequence analysis (using Vista Tools for Comparative Software) (20,21).

We identified a tandem Stat consensus sequence from -442 to -464 bp in the FANCC promoter (separated by 2 bp). These sites were conserved in mouse and human, but slightly divergent from the derived consensus for Stat3 binding (consensus; 5'-T(T/G)N4-5GAA-3', proximal FANCC site; 5'-T GATTTGAA-3' and distal site; 5'-AGCGTTGAA-3') (23,24). We generated a reporter construct with one copy of this tandem site linked to a minimal promoter (-438 to -474 bp from the FANCC promoter in the pGL3-promoter vector). Additional constructs were generated with mutation of the proximal, distal or both putative Stat3 binding sites. Reporter activity was assayed in U937 cells co-transfected with vectors to express Stat3, Stat5 or empty control vector (and reporter vector to function as an internal control, as described above).

We found Stat3 significantly increased activity of the -438 to -474 bp FANCC/minimal-promoter construct (p<0.0001, n=6) (Figure 2A). Mutation of either consensus significantly decreased this activity (p<0.001, n=6), but activation by Stat3 was completely abolished by mutation of both (p=0.6, n=6 relative to minimal-promoter/reporter control) (Figure 2A). Stat5 had no effect on the -438 to -474 bp construct, and none of these proteins influenced the empty minimal-promoter/luciferase reporter vector (Figure 2A).

We identified a potential C/ebp binding site between -392 and -403 in the FANCC promoter (consensus; 5'-[TG]NNGNNAA[TG]-3', FANCC sequence; 5'-TAGGGGAAAATC-3') (26).
We generated a minimal-promoter/reporter construct with three copies of the -385 to -408 bp FANCC promoter sequence. An additional construct was generated with mutation of this consensus. Reporter activity was assayed in U937 cells co-transfected with vectors to express C/ebpβ-Lap, C/ebpβ-Lip or control vector (and the internal control reporter vector, described above). C/ebpβ-Lap increased activity of the -385 to -408 bp FANCC sequence significantly (p<0.0001, n=6) (Figure 2B). C/ebpβ-Lip did not activate this FANCC sequence, and neither protein activated the minimal-promoter/reporter control vector.

We investigated binding of Stat3 and C/ebpβ to these FANCC promoter regions by chromatin co-immunoprecipitation. For these studies, lysates of Lin-CD34+ murine bone marrow cells were immunoprecipitated with antibodies to Stat3, C/ebpβ or irrelevant control antibody (27). Some cells were differentiated with IL1β for 24 hours prior to cross linking and lysis. Co-precipitating chromatin was amplified by semi-quantitative PCR (Figure 2C) or quantitative real time PCR (using SYBR green and the standard curve method) (Figure 2D).

We found specific co-precipitation of the -380 to -410 bp sequence with C/ebpβ, and of the -430 to -470 bp sequence by Stat3 (Figure 2C). Co-precipitation of both proteins was significantly increased by IL1β. Neither protein co-precipitated irrelevant 5' flank sequence (not shown).

IL1β increased expression of FancC, Stat3 and C/ebpβ in murine bone marrow cells. Reporter assays in cell lines provide information regarding promoter activity, but should be interpreted carefully due to the transformed nature of these cells. To investigate FancC expression in a non-transformed setting, and possible contributions by Stat3 or C/ebpβ, we performed studies in primary myeloid progenitor cells from murine bone marrow. For these studies, Lin-CD34+ cells were isolated and some cells were differentiated with IL1β or G-CSF. The amount of G-CSF employed was consistent with serum levels in emergency granulopoiesis (9). Total cellular RNA was analyzed for gene expression by quantitative real time PCR. The standard curve method was used, so data is presented as mRNA abundance as per this technique.

We found significantly increased FancC mRNA in response to treatment with either cytokine (p<0.001, n=4) (Figure 3A). We also found a significant cytokine-induced increase in Stat3 and C/ebpβ mRNA in these cells (p<0.001, n=4) (Figure 3A). The relative increase in expression of Stat3 and C/ebpβ in response to either IL1β or G-CSF was not significantly different (p=0.2, n=4).

We next investigated the impact on Stat3 protein. For these studies, cells were analyzed with or without IL1β or G-CSF treatment for total Stat3, pY705-Stat3 or pS727-Stat3 by Western blot. We found that either cytokine increased total and phospho Stat3 protein (Figure 3B).

To quantify these results, we performed ELISA for total Stat3, pY705-Stat3 or pS727-Stat3. We found IL1β also significantly increased total Stat3 protein in this assay (p<0.001, n=3) (Figure 3C). Although phospho-tyrosine or phospho-serine Stat3 increased, the relative increase was significantly less than in total Stat3 protein (4 fold increase vs ~50% increase) (Figure 3C).

We similarly investigated the impact of emergency granulopoiesis on C/ebpβ protein (by Western blot). We found IL1β increased C/ebpβ expression, consistent with our mRNA studies (Figure 3D). In this experiment, only the C/ebpβ-Lap form was detected (35 kDa), not the smaller Lip form (~20 kDa).

Abundance of Stat3 protein influenced FANCC promoter activity. We also investigate the roles of tyrosine or serine phosphorylation of Stat3 on activation of the FANCC cis element. For these experiments, we co-transfected U937 cells with a vector to express Y705F-Stat3 or S727A-Stat3 and the -482 to -517 bp FANCC minimal-promoter/reporter vector (or control minimal-promoter/reporter vector). Cells were also co-transfected with the internal control reporter vector, as described above. Phosphorylation of Y705 enhances transcriptional activation of some target genes by Stat3 (23). Phosphorylation of S727 was found to either enhance or inhibit this effect, in a context dependent manner (25).

In initial studies, we performed Western blots to verify overexpression of these forms of Stat3 in U937 cells. We found that Wt, Y705F or S727A Stat3 were equivalently expressed under the assay conditions (Figure 4A).

We also found this FANCC cis element was equivalently activated by tyrosine mutant, serine mutant, or wild type (Wt) Stat3 (p≥0.2, n=6).
For these studies, we induced emergency granulopoiesis in mice by intraperitoneal (IP) injection of an ovalbumin/alum mixture (Alum) or saline (as a control for steady state granulopoiesis, n=6 mice per group) (9,15,18). Alum injection results in maximal expansion of myeloid progenitor cells and differentiating granulocytes in the bone marrow by two weeks and resumption of steady state by four weeks post injection (9,15,18). To investigate mRNA expression of FancC, Stat3, C/ebpβ and Icsbp at various points during this process, cohorts of mice were sacrificed at 0, 1, 2, 3 and 4 weeks after Alum injection and LinCD34+ bone marrow cells were analyzed.

We found significantly increased expression of Stat3 and C/ebpβ that was maximal two weeks post Alum injection (p<0.0001, n=3 relative to steady state), and began to decrease at three weeks (p<0.001, n=3 for comparison of one vs two or two vs three weeks) (Figure 5B). In contrast, Icsbp mRNA was maximally expressed three weeks post Alum injection (p<0.001, n=3 for comparison of two and three weeks) and was returning to steady state levels at four weeks (Figure 5B). FancC mRNA expression was significantly increased one week after Alum injection, maximal at 2 weeks (p<0.0001, n=3), and returning to baseline at 4 weeks (Figure 5B).

Stat3 or C/ebpβ rescues DNA-repair in a FancC dependent manner. We were interested in determining the impact of Stat3 or C/ebpβ on DNA repair in cells exposed to the stress of emergency granulopoiesis. To investigate this, we used a plasmid based DNA-repair assay that we previously employed to study the influence of Icsbp on this process. In these studies, a reporter plasmid is treated with mitomycin C to generate DNA-crosslinks (with untreated plasmid as a control) (9,17). Damaged or undamaged reporter plasmids are transfected into U937 cells and reporter activity represents the efficiency of DNA repair. Cells are co-transfected with a second reporter plasmid as an internal control for transduction efficiency (not MMC treated).

In prior studies, we determined that U937 cells efficiently repaired MMC-treated plasmid, but treatment of U937 cells with differentiating agents (retinoic acid/dimethyl formamide or Ifnγ) significantly impaired this activity (9,17). For the current studies, U937 cells were co-transfected with MMC-treated or untreated control plasmid (CMV-
firefly luciferase vector) and vectors to overexpress Icsbp, Stat3, C/ebpβ-Lap, combinations of these proteins, or empty control expression vector (with TK-renilla luciferase vector as a control for transfection efficiency). Transfectants were assayed after 24 hours of treatment with IL1β.

We found significantly less firefly luciferase reporter activity from the MMC-treated reporter vector compared to untreated, control reporter vector in IL1β-treated transfectants (p<0.001, n=6) (Figure 5B). Overexpression of Icsbp, Stat3 or C/ebpβ significantly increased reporter activity of the MMC-treated plasmid in IL1β transfectants (p<0.001, n=6), but had no effect on activity of reporter vector that had not been MMC treated (Figure 6A). Assays with combinations of the three proteins demonstrated effects were non-redundant, since the total amount of expression plasmid was held constant in these experiments.

In the absence of IL1β treatment of the transfectants, we found luciferase reporter activity from MMC-treated plasmid was not significantly different than activity of untreated plasmid (not shown), consistent with our prior studies (9,17).

We were interested in determining if the effects of Icsbp, Stat3 or C/ebpβ on activity of the MMC-damaged reporter plasmid was not significantly different than activity of untreated plasmid (not shown), consistent with our prior studies (9,17).

We were interested in determining if the effects of Icsbp, Stat3 or C/ebpβ on activity of the MMC-damaged reporter plasmid required FancC expression. To examine this, we co-transfected U937 cells with MMC-treated or un-treated reporter plasmid, vectors to overexpress Icsbp, Stat3 or C/ebpβ, vectors to express shRNAs specific to FancC (or scrambled shRNA control), and a plasmid to control for transfection activity (as above). Transfectants were assayed after 24 hours treatment with IL1β.

We found knockdown of FancC prevented Icsbp, Stat3 or C/ebpβ overexpression from rescuing reporter activity of the MMC-treated plasmid (Figure 4A). These results suggested these transcription factors were acting through a FancC-dependent mechanism to drive DNA crosslink repair.

Discussion

Emergency granulopoiesis is a high risk/high gain response to infectious challenge. During this process, rapid granulocyte production requires increased proliferation and faster differentiation of bone marrow stem and progenitor cells; resulting in genotoxic stress. This risk is further enhanced by apoptosis resistance and cell cycle shortening that occur during this process (9,15). In the current studies, we find Stat3 and C/ebpβ, essential transcription factors for initiating and sustaining emergency granulopoiesis, are involved in protecting the genome by increasing expression of Fanconi C.

In the current studies, we find Stat3, C/ebpβ and Icsbp are non-redundant for activation of the FANCC promoter. We also find IL1β increases FANCC promoter activity and enhances the effects of these transcription factors. Also, our studies demonstrate that IL1β increases binding of Stat3 and C/ebpβ to their respective FANCC cis elements; perhaps due to the ability of IL1β to increase expression of Stat3 and C/ebpβ mRNA and protein. We find IL1β treatment decreases the ability of U937 myeloid cells to repair DNA crosslinks. And, that DNA crosslink repair is rescued by Stat3 or C/ebpβ in a FancC dependent manner; similar to our prior results with Icsbp and FancC (9).

Our studies suggest that different expression levels of Stat3, C/ebpβ and Icsbp at various times during emergency granulopoiesis ensure sustained FancC expression throughout this process. At initiation of emergency granulopoiesis, Stat3 expression increases, followed by expression of C/ebpβ; correlating with increased FancC expression. All three transcription factors are expressed at peak granulocyte production during Alum-stimulated emergency granulopoiesis, correlating with maximal FancC expression and consistent with maximal protection from DNA damage at this point in the process.

We found expression of FancC was still increased, relative to steady state, 3 weeks after initiation of emergency granulopoiesis, despite decreasing Stat3 and C/ebpβ at this time. However, Icsbp expression is persistently elevated at this point. In prior studies, we found that emergency granulopoiesis failed to terminate in Icsbp−/− mice, associated with increased and sustained Stat3 and C/ebpβ expression (15). This implicated Icsbp in resetting Stat3 and C/ebpβ to steady state levels. These results determined that Icsbp antagonizes FancC expression by decreasing Stat3 and C/ebpβ, but compensates for this effect by activating the FANCC promoter, until resumption of steady state (Figure 5B) (15, 22).

We found Stat3, C/ebpβ and Icsbp each activate different FANCC promoter cis elements. We
previously found activation of the FANCC promoter by Icsbp was enhanced by tyrosine phosphorylation of this protein during emergency granulopoiesis; identifying roles for enhanced expression and post translational modification in this process (9). In the current studies, we find that neither tyrosine nor serine phosphorylation of Stat3 enhances FANCC promoter activation, suggesting protein expression is driving function. This is in contrast to some other Stat3 target genes, where tyrosine phosphorylation enhances transcriptional activation (23). The role of pS727 in Stat3 function is controversial and may be context dependent (25).

In addition to regulating FANCC transcription, Icsbp also enhances calpain activity through repression of the Growth Specific Arrest 2 (GAS2) gene; a calpain inhibitor (28). Since Stat3 is a calpain substrate, Icsbp may influence both Stat3 mRNA expression and Stat3 protein stability during termination of emergency granulopoiesis (29). Understanding cooperation vs antagonism between Icsbp and Stat3 is of interest to understanding the innate immune response, and a focus of ongoing investigations in the laboratory.

The Stat3-binding FANCC cis element identified in this work has a tandem binding consensus sequence and we found both copies were required for maximal cis element activity. This is consistent with interaction of Stat3 as a homodimer with such tandem binding sites in a number of pro-inflammatory genes (30).

Although this cis element was activated by Stat3, we found no effect of Stat5 on the FANCC promoter. Conversely, we previously found Stat5 repressed the distal IRF8 promoter in myeloid progenitor cells (31), but Stat3 had no effect. We also previously determined Icsbp regulated Stat5 protein stability (through Gas2/calpain), but did not influence Stat5 mRNA (31). Therefore, these two Stat proteins play discrete roles during myelopoiesis and are differentially regulated by Icsbp.

We found the C/ebpβ Lap isoform activated a cis element in the FANCC promoter, but the Lip isoform did not. These isoforms were originally described in regenerating liver cells and Lip (liver inhibitory protein) antagonized the effects of Lap (liver activating protein) in these cells (19). In our studies, we found Lap was the dominant C/ebpβ isoform in myeloid cell lines and differentiating murine bone marrow progenitors. Overexpression of Lip did not repress FANCC promoter activity in myeloid cell transfecants, but we also did not find an increase in Lip during termination of emergency granulopoiesis. This suggests other mechanisms, such as general repression of CEBPB transcription by Icsbp-dependent events, may regulate C/ebpβ activity during emergency granulopoiesis.

Increased expression of Stat3 and C/ebpβ is found in chronic myeloid leukemia (CML) (32,33). The function of these transcription factors in leukemogenesis may be consistent with their normal roles in expanding myeloid progenitor populations during emergency granulopoiesis. In contrast, Icsbp is a leukemia suppressor for CML with decreased expression in this disease (34,35). Therefore, regulation of emergency granulopoiesis may represent a paradigm for leukemia promotion versus suppression.

It is additionally possible that episodes of emergency granulopoiesis facilitate leukemogenesis under conditions with decreased Icsbp or enhanced expression of Stat3 or C/ebpβ. All three proteins enhance FancC expression, which would be anticipated to protect cells from DNA damage. However, we found significantly more FANCC promoter activity and FancC expression in the presence of all three transcription factors compared to Stat3 and C/ebpβ without Icsbp. It would be of interest to determine if repeated episodes of emergency granulopoiesis enhance drug resistance or progression to blast crisis in CML. Studies are currently being performed in the laboratory to address this issue.

**Experimental procedures**

**Protein expression vectors:** The Icsbp/Irf8 cDNA was obtained from Dr. Ben Zion-Levi (Technion, Haifa, Israel) and subeloned into the mammalian expression vector pcDNA (Stratagene, La Jolla, CA), as described (36). Wild type and Y705F mutant murine Stat3 cDNAs and C/ebpβ Lap and Lip cDNAs were obtained from Addgene and subeloned in to the pcDNA (for expression in myeloid cell lines) and MSCV (for generation of retrovirus) vectors. FancC specific shRNAs (and scrambled control shRNAs) were generated using the Promega website and subeloned into the pLKO retroviral vector.

**Reporter constructs:** The human FANCC 5’ flank (1.0 kb from the ATG codon) was generated
by PCR from the U937 myeloid cell line. The genomic clone was sequenced to ensure identity with the sequence in the ENSEMBL database (37). This sequence and additional truncations (-500, -450 or -400 bp) were subcloned into the pGL3-basic luciferase reporter vector (Promega; expressing firefly luciferase reporter gene). Other constructs were generated with one copy of the -387 to -403 bp FANCC promoter or with three copies of the -470 to -530 bp FANCC promoter subcloned into a minimal promoter-reporter vector (pGL3-promoter vector) (Promega, with the TK minimal promoter, expressing firefly luciferase reporter gene). Some of the -387 to -403 bp FANCC promoter/minimal-promoter/reporter constructs had mutation of the proximal, distal or both Stat consensus binding sequences.

Myeloid cell line culture: The human myelomonocytic leukemia cell line U937 (38) was obtained from Andrew Kraft (University of Arizona, Tucson). Cells were maintained as described (38).

Transfections and reporter gene assays: U937 cells were transfected with FANCC promoter/luciferase reporter constructs (or empty control reporter vector) and various combinations of vectors to overexpress Stat3, C/ebpβ or Icsbp (or empty expression vector), or specific shRNAs to knockdown Stat3 or C/ebpβ (or scrambled shRNA control vectors). Cells were also co-transfected with an internal control plasmid to normalize for transfection efficiency (Promega Dual Luciferase System, a CMV-luciferase reporter vector expressing renilla luciferase). Transfectants were assayed for luciferase activity per manufacturer’s instructions. Some transfectants were treated with IL1β (50 ng/ml for 24 hrs) prior to harvesting.

Luciferase activity from control empty reporter vectors was not influenced by IL1β treatment or overexpression or knockdown of any of these proteins and was subtracted as background. All reporter assays were repeated 6 times in independent experiments (and samples were assayed in duplicate) for each condition.

Efficacy of IL1β differentiation of U937 cells was verified for various batches of cytokine used in these studies by determining enhanced FANCC promoter activity in transfection assays or expression of endogenous mRNA for FancC and gp91phox in cells treated with the cytokine.

Some transfectants were studied after treatment for 24 hours with H7 (a serine kinase inhibitor for Stat3; 40) or Stattic (a specific inhibitor of Stat3 tyrosine phosphorylation; 41).

Mitomycin C treatment of plasmids and DNA repair assays: To generate DNA crosslinks, purified plasmid DNA (CMV-firefly luciferase from Promega) was incubated with mitomycin C (40 μM) for 12 hrs at room temperature. Plasmid DNA was recovered by phenol:chlooroform extraction followed by ethanol precipitation. DNA crosslinking was verified by non-denaturing agarose gel electrophoresis (17).

U937 cells were co-transfected with crosslinked or untreated control reporter plasmid, vectors to overexpress Icsbp, Stat3 or C/ebpβ (or control expression plasmid), and vectors expressing specific shRNAs to knockdown FancC (or scrambled control). Cells were also transfected with TK-renilla luciferase vector as an internal control for transfection efficiency. Lysates were analyzed simultaneously assayed for dual luciferase activity, as described above. Luciferase reporter activities were determined after 24 hrs treatment with IL1β. Reporter assays were repeated 6 times in duplicate, as described above.

Western blot and ELISA of lysates proteins: For Western blots, cells were lysed by boiling in 2X SDS sample buffer. Lysate proteins (50 μg) were separated by SDS-PAGE (10% acrylamide), transferred to nitrocellulose, and filters were serially probed with antibodies as described (39). Each experiment was repeated at least three times with different sets of lysates and a representative blot is shown.

In other experiments, total, serine phosphorylated or tyrosine phosphorylated Stat3 protein in cell lysates were quantified by commercially available ELISA (Abcam, Cambridge, MA). ELISAs were performed in duplicate on three independent sets of lysates and results were graphed as OD450 (per manufacturer’s instructions).

Chromatin immunoprecipitation: Cells were incubated briefly in media supplemented with formaldehyde, lysates were sonicated to generate chromatin fragments with an average size of 500 bp, and immune-precipitated with Stat3, C/ebpβ, or irrelevant control antibody (Abcam, Cambridge, MA) (26). Chromatin was amplified by quantitative real time PCR using SYBR green and the “standard curve” method (Thermo-Fisher Scientific,
according to manufacturer’s instructions). Primers were designed flanking the Stat3 or C/ebp consensus sequences in the *FANCC* promoter. The standard curve was generated using total chromatin from murine bone marrow cells. Input chromatin (not precipitated) from each sample was analyzed to normalize data between the samples. At least three independent immuno-precipitation experiments were performed and the samples were analyzed in triplicate.

**Quantitative real time PCR**: RNA was isolated using Trizol reagent (Gibco-BRL, Gaithersburg, MD) and tested for integrity by denaturing gel electrophoresis. Primers were designed with Applied Biosystems software and real time PCR performed using SYBR green by the “standard curve” method. The standard curve for these experiments was generated with cDNA from Wt cells cultured in GM-CSF, IL3 and stem cell factor. Result were normalized to 18S and actin and presented as mRNA abundance with 1 ng of cDNA set as 1000 in the standard curve. At least three independent samples were evaluated in triplicate.

**Animal use**: Mice for this study were C57 Black 6 and were maintained in an approved and accredited animal facility at Northwestern University. Mice were housed in a specified pathogen free, tightly regulated environment that includes; control of the flow of animals, equipment and personnel, use of micro-isolator cages, and use of husbandry procedures to minimize pathogen exposure and disease outbreak.

All work was reviewed and approved by the Animal Care and Use Committees of Jesse Brown VA and Northwestern University.

**In vitro murine studies**: For *in vitro* studies, bone marrow mononuclear cells were harvested by flushing femurs repeatedly with Hank’s Balanced Salt Solution until no additional cells were obtained. Washed cells were treated with ACK (ammonium-chloride-potassium) buffer to lyse red blood cells, then washed extensively. Lin-CD34+ cells were separated using a magnetic bead-based affinity chromatography based technique (according to manufacturer’s instructions, Miltenyi Biotech, San Diego, CA). Cells were cultured (2 X 10^5/ml) for 48 hrs in DME media supplemented with 10% fetal calf serum, 1% pen-strep, 10 ng/ml murine GM-CSF (R & D Systems Inc., Minneapolis, MN), 10 ng/ml murine recombinant IL-3 (R & D Systems Inc.), and 100 ng/ml of Stem Cell Factor (Scl; R & D Systems Inc.). Cells were maintained in GM-CSF, IL3, Scl for 24 hrs, or were stimulated with 50 ng/ml G-CSF (R & D Systems Inc.) or 20 ng/ml IL1β (R & D Systems Inc.) during this time period. Apoptotic cells were removed before analysis (Miltenyi “Dead cell clean up”, per manufacturer’s instructions). Some cells were transduced with retroviral vectors prior to analysis, according to techniques described in our prior work (9).

**In vivo murine emergency granulopoiesis assay**: Wt mice (18-20 weeks of age) were injected intraperitoneally (IP) an ovalbumin/alum mixture (referred to as Alum) or saline control (mice per group). Mice were randomly assigned to cohorts for injection of Alum or saline control. Alum was prepared as described in the following references and a volume of 0.5 ml injected (9,15,18).

Cohorts of mice (6 per group) were sacrificed weekly and bone marrow collected from both femurs. Successful induction of emergency granulopoiesis in Alum injected mice (compared to saline control) was verified by weekly peripheral blood granulocytes counts (using a Hemavet automated cell counter, Drew Scientific, Miami Lakes, FL). Blood count data was analyzed by an investigator who was blinded for the status of the mice as Alum vs saline injected.

**Statistical Analysis**: Statistical significance was determined by unpaired, two tailed Student’s t-test (comparing two conditions), or ANOVA (for more than two conditions) using SigmaPlot software. P value of <0.02 was considered statistically significant. In all graphs, error bars represent standard error.

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**Author contributions**: CAS performed chromatin immuno-precipitation, emergency granulopoiesis studies, reporter gene assays, DNA-repair studies, protein expression and gene expression studies; LB performed transfection experiments, protein expression and gene expression studies; LH generated reporter constructs and assisted with emergency granulopoiesis and DNA repair studies; DD assisted with gene expression and protein...
expression studies; LB and WQH assisted with animal studies; EAE planned the experiments, analyzed data and wrote the manuscript.

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Figure 1: C/ebpβ and Stat3 activate the FANCC promoter in myeloid cells. A. Alignment of the human andmurine FANCC promoters identified conserved consensus sequences for Stat and C/ebp binding. The human sequence is in black and the murine in blue. Conserved regions are indicated in gray. The tandem Stat consensus is in purple and the C/ebp consensus in red. Truncations for reporter assays are indicated. B. Stat3 and C/ebpβ activate discrete regions of the FANCC promoter, but C/ebpβ-Lip and Stat5 do not influence FANCC promoter activity. U937 cells transfected with a 1 kb FANCC promoter construct or truncated derivatives thereof were co-transfected with DNA plasmids driving expression of each of three different transcription factors indicated. Reporter constructs with truncations of the FANCC promoter were assayed for the effect of overexpressed Stat3, Stat5, C/ebpβ (Lap or Lip isoform), Icsbp (positive control) or empty vector. Statistically significant differences are indicated by *, **, *** or # (p<0.01, n=6 for all comparisons). C. Stat3, C/ebpβ-Lap and Icsbp are not redundant for FANCC promoter activation. The 1.0 kb of FANCC promoter/luciferase reporter construct was assayed in U937 transfectants for the effect of combinations of overexpressed Stat3, C/ebpβ (Lap) or Icsbp, or shRNA knockdown of Stat3 or C/ebpβ. Some transfectants were differentiated with IL1β prior to analysis. Statistically significant differences are indicated by *, **, *** or # (p<0.01, n=6 for all comparisons).
Figure 2: Stat3 and C/ebpβ-Lap interact with discrete FANCC promoter cis elements. A. Stat3 activates tandem cis elements in the FANCC promoter. U937 cells were transfected with a minimal promoter/luciferase reporter construct (designated pGL3p) with -438 to -474 bp of FANCC 5’ flank, or constructs with mutation in one or both of the Stat binding consensus sequences (or minimal-promoter/luciferase reporter control vector). Cells were co-transfected with vectors to overexpress Stat3 or Stat5 (vs control vector). Statistically significant differences are indicated by *, **, ***#, or ## (p<0.01, n=6 for all comparisons). B. C/ebpβ-Lap activates a cis element in the FANCC promoter, but C/ebpβ-Lip does not. U937 cells were transfected with a minimal promoter/luciferase reporter construct with three copies of the -385 to -408 bp of FANCC 5’ flank, or a construct with mutation of the C/ebp consensus sequence (or minimal-promoter/luciferase reporter control vector). Cells were co-transfected with vectors to overexpress Lap or Lip forms of C/ebpβ (vs control vector). Statistically significant differences are indicated by * or ** (p<0.001, n=6 for all comparisons). C. Stat3 and C/ebpβ bind to the FANCC promoter. Murine bone marrow myeloid progenitor cells were analyzed by chromatin immuno-precipitation with antibodies to Stat3, C/ebpβ or irrelevant control antibody. Some cells were differentiated for 24 hours with IL1β prior to analysis. Co-precipitating chromatin was amplified by PCR with primers flanking the cis elements activated by Stat3 or C/ebpβ and separated by acrylamide gel electrophoresis. D. IL1β increases Stat3 or C/ebpβ binding to FANCC promoter cis elements. Some co-immuno-precipitated chromatin was analyzed by quantitative real time PCR (data is presented using the standard curve method). Statistically significant differences are indicated by * or ** (p<0.01, n=4 for all comparisons).
Figure 3: Expression of Stat3, C/ebpβ and FancC increased IL1β or G-CSF differentiated cells. A. Differentiation of murine bone marrow myeloid progenitor cells with IL1β or G-CSF increased Stat3, C/ebpβ and FancC mRNA. Lin-CD34+ murine bone marrow cells were isolated and cells some were differentiated with IL1β or G-CSF for 48 hours prior to analysis. RNA expression was analyzed by real time PCR. Statistically significant difference are indicated by *, **, or *** (p<0.001, n=6 for all comparisons). B. Expression of total and phospho Stat3 protein was increased by IL1β-differentiation. Lin-CD34+ cells were isolated from bone marrow mononuclear cells from the femurs of mice. Some cells were differentiated with IL1β for 48 hrs prior to analysis. Cell lysates were analyzed by Western blot or C. ELISA for total Stat3, pY705-Stat3, or pS727-Stat3. Statistically significant differences are indicated by *, ** or *** (p<0.001, n=6 for all comparisons). D. C/ebpβ-Lap protein was increased by IL1β differentiation of these cells. Cells lysates were also analyzed by Western blots serially probed with antibodies to C/ebpβ or Tubulin (as a loading control). Representative blots are shown.
Figure 4: Stat3 protein abundance contributes to FANCC promoter activation. A. Stat3, Y705F-Stat3 and S727A-Stat3 are equivalently overexpressed in U937 cells. U937 cells were transfected with vectors to overexpress various Stat proteins. Protein expression was determined by Western blots serially probed with antibodies for Stat3, pY705-Stat3, pS727-Stat3 or Tubulin (as a loading control). A representative blot is shown. B. Increased abundance of Stat3 protein increases activity of the FANCC promoter Stat3-binding cis element. U937 cells were transfected with a minimal promoter/luciferase reporter construct with -438 to -474 bp of FANCC 5' flank (or minimal-promoter/luciferase reporter control vector). Cells were co-transfected with vectors to overexpress Stat3, S727A-Stat3 or Y705F-Stat3 (vs control vector). Some transfectants were treated with a serine kinase inhibitor (H7), a tyrosine kinase inhibitor (Stattic) or both, and some were differentiated with IL1β prior to analysis. Statistically significant differences are indicated by *, ** or *** (p<0.01, n=6 for all comparisons). C. H7 or Stattic alter Stat3 phosphorylation state, but not protein abundance. U937 cells were transfected and assayed under the conditions described for reporter gene assays. Total, pY705-Stat3, or pS727-Stat3 were analyzed by ELISA. Statistically significant differences are indicated by *, **, ***#, ##, ###, &, && or &&& (p<0.01, n=6 for all comparisons).
Figure 5: Stat3 and C/ebpβ influence FancC expression during emergency granulopoiesis. A. Knockdown of Stat3 or C/ebpβ decreases IL1β-induced expression of Fanconi C. Murine bone marrow myeloid progenitor cells were transduced with vectors to express shRNAs specific for Stat3 or C/ebpβ (or scrambled control shRNA) and analyzed for FancC expression. Bars for FancC mRNA are indicated by F, Stat3 by S, and C/ebpβ by C. Statistically significant differences are indicated by *, **, ***, #, ##, ### or & (p<0.01, n=6 for all comparisons). B. Expression of Stat3 and C/ebpβ increase early, Icsbp increases later, and FancC is increased throughout emergency granulopoiesis. Mice were injected with Alum (IP) to induce emergency granulopoiesis and bone marrow Lin−CD34+ cells were collected t=0, 1, 2, 3 or 4 weeks after injection. Gene expression was determined by quantitative real time PCR. Statistically significant differences are indicated by *, **, ***, #, ###, &, or && (p<0.01, n=6 for all comparisons).
Figure 6: Stat3 and C/ebpβ enhance DNA repair in a FancC-dependent manner in myeloid cells undergoing IL1β-induced differentiation. A. Icsbp, Stat3 and C/ebpβ rescue DNA repair during IL1β-induced differentiation of U937 cells, but this is reversed by FancC knockdown. U937 cells were co-transfected with a mitomycin C (MMC) crosslinked luciferase reporter plasmid (or untreated control luciferase reporter plasmid), vectors to express Icsbp, Stat3, C/ebpβ, alone or in combinations (or control vector). Other cells were transfected with vectors to express Icsbp, Stat3, or C/ebpβ and express FancC specific shRNAs (or scrambled control shRNA). Some transfectants were treated for 24 hours with IL1β before reporter activity was determined. Statistically significant differences are indicated by *, **, ***#, or ## (p<0.001, n=6 for all comparisons). B. Schematic of regulation of FancC expression by Icsbp, Stat3 and C/ebpβ during emergency granulopoiesis. Cross regulation of these transcription factors is also indicated.
Stat3 and CCAAT enhancer–binding protein β (C/ebpβ) activate Fanconi C gene transcription during emergency granulopoiesis.

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