MYC Promotes Bone Marrow Stem Cell Dysfunction in Fanconi Anemia

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SUMMARY

Bone marrow failure in Fanconi anemia (FA) patients is attributable to the dysfunctional hematopoietic stem and progenitor cells (HSPCs). Although the underlying mechanisms of dysfunctional HSPCs are unclear, the hyperactive p53 and TGFβ pathways have been implicated in their exhaustion. In order to identify additional determinants of HSPC impairment leading to the bone marrow failure, we profiled primary HSPCs from FA patients for single cell transcriptome (scRNA-seq). Trajectory analysis revealed that early hematopoietic differentiation potential is preserved in FA HSPCs. As expected, p53 and TGFβ pathway genes were overexpressed in HSPCs from FA patients. The oncogene *MYC* was also identified as one of the top over-expressed genes in FA HSPCs. Interestingly, we observed co-existence of "High-*TP53"* expressing HSPCs and High-*MYC* expressing HSPCs in FA bone marrow. Inhibition of *MYC* expression by the BET bromodomain inhibitor (+)-JQ1 reduced the clonogenic potential of primary HSPCs from FA patients but rescued the physiological/genotoxic stress in HSPCs from FA mice. The "High-*MYC*" expressing HSPCs exhibited a significant downregulation of cell adhesion genes, such as *CXCR4*. Consistently, HSPCs in FA patients showed a defect in adhesion to their bone marrow niche resulting in egression from the bone marrow into peripheral blood. We speculate that *MYC* overexpression impairs HSPC function and contributes to exhaustion of HSPCs in FA bone marrow.

KEYWORDS

Fanconi anemia, hematopoietic stem cells, bone marrow failure, single cell RNA sequencing, MYC, DNA damage, genotoxic stress, physiological stress, CXCR4

INTRODUCTION

Fanconi anemia (FA), a DNA repair disorder, is the most frequently inherited bone marrow failure (BMF) syndrome. Patients with FA suffer from early childhood onset of bone marrow (BM) failure, developmental abnormalities, and heightened susceptibility to solid tumors. Patients with FA also have a strong predisposition to myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML), with an overall risk of 5000 fold and 700-800 fold respectively, compared to the general population (Alter, 2014, Risitano et al., 2016). Allogeneic hematopoietic stem and progenitor cell (HSPC) transplantation is the long-term curative treatment for the hematologic manifestations in FA (Ebens et al., 2017, Shimamura and Alter, 2010, Guardiola et al., 2000). Alternative treatment approaches, such as gene therapy, are also underway (Rio et al., 2019).

FA is caused by biallelic mutations in one of twenty-three *FANC* genes, the protein products of which cooperate in the FA/BRCA DNA repair pathway and regulate cellular resistance to DNA cross-linking agents (Rodríguez and D'Andrea, 2017). Due to their underlying DNA repair defect, FA cells exhibit chromosomal instability and hypersensitivity to genotoxic DNA crosslinking agents, such as Mitomycin C (MMC) (Oostra et al., 2012). FA BM HSPCs are also hypersensitive to oxidative stress (Li et al., 2017) and inflammatory cytokines (Briot et al., 2008).

Patients with FA develop BMF due to HSPCs exhaustion. Progressive age-related attrition is observed in CD34+ cell content in FA patients (Ceccaldi et al., 2012). Additionally, both in FA patients and in FA mice bone marrow HSPCs exhibit hematopoietic stem and progenitor cell functional defects (Ceccaldi et al., 2012, Parmar et al., 2010, Parmar et al., 2009, Haneline et al., 1999). BMF in FA results from accumulation of DNA damage in HSPCs caused by the endogenous crosslinking agents such as endogenous aldehydes (Garaycoechea et al., 2018, Pontel et al., 2015) or from physiological stress (Walter et al., 2015). In response to genotoxic stress, FA HSPCs hyperactivate growth suppressive pathways, such as the p53 pathway (Ceccaldi et al., 2012) and the TGF-β pathway, further contributing to BMF (Zhang et al., 2016).

Despite the current progress in understanding FA, the molecular pathways in HSPCs leading to BMF and MDS/AML remain unknown. A greater understanding of the molecular mechanisms of pathogenesis of FA would help in designing novel therapies. Although primary HSPCs from BM of patients with FA provide a useful model system, studying these cells is challenging, due to their heterogeneity and low number. Sub-populations of HSPCs with heterogeneous transcriptional profiles may co-exist in the BM (BM) of a patient with FA – including *1)* a sub-population of stressed HSPCs sustaining hematopoiesis, *2)* a population of HSPCs committed to apoptosis due to accumulation of unrepaired DNA damage, and *3)* a sub-population of premalignant/malignant cells that eventually might outgrow until becoming a clinically detectable MDS or AML.

Due to the relative scarcity of FA HSPCs, most studies have been unable to explore the molecular mechanisms of BMF. In addition, traditional approaches have failed to uncover information from rare populations of HSPCs, due to technological restrictions and the lack of resolution of bulk protein and RNA level measurements. Single cell RNA sequencing (scRNA-seq) allows high-resolution whole-transcriptome profiling of individual cells and direct analysis of cell subpopulations from heterogeneous samples (Zheng et al., 2017) . The ability of scRNAseq to deconvolute cell sub-populations results in a wide range of applications, including detection and quantification of cell populations of interest, discovery of new cell populations, and comparisons of the relative abundance of cell populations from different groups of patients including the patients with BMF (Zheng et al., 2018, Watcham et al., 2019, Tikhonova et al., 2019, Zhao et al., 2017, Joyce et al., 2019).

In order to investigate novel mechanisms contributing to the pathogenesis of BMF in FA, we used scRNAseq to transcriptionally profile HSPCs from 7 patients with FA and 5 healthy donors. The HSPC compartment of patients with FA preserved the early multilineage commitment profile seen in healthy donors. Importantly, FA HSPCs overexpressed the MYC pathway seemingly as a counteracting force against the growth suppressive activities of the p53 and TGFβ pathways. In addition, we identified a gradient of opposing states co-existing in the FA HSPC pool, comprised of "High-*MYC"* expressing HSPCs, which have increased survival and proliferation, and "High-*TP53"* expressing HSPCs, previously shown to have a prominent cell cycle arrest and increased apoptosis (Ceccaldi et al., 2012).

The "High-*MYC"* expressing HSPCs from patients with FA also have a reduction in the expression of cell-adhesion genes, resulting in a propensity for egress from the BM. Consistently, increased trafficking of "High-*MYC"* HSPCs to the peripheral blood was observed in both patients with FA and in an FA mouse model of pI:pC induced BMF.

RESULTS

Early hematopoietic differentiation is preserved in FA HSPCs

Using state of the art scRNAseq technologies we isolated HSPCs from 7 patients with FA and 5 healthy donors and resolved the heterogeneity of their gene expression profile (A description of patients with FA and healthy donors studied by scRNAseq appears in **Supplementary Table 1**). After sequencing, we combined the sequenced FA and healthy HSPCs in a single bioinformatic pipeline (**Figure 1A**). Using Seurat (Satija et al., 2015), we deconvoluted the different clustering subpopulations which express CD34. Using gene expression profiles of human HSPCs subpopulations from healthy donors (Velten et al., 2017, Zheng et al., 2018, Setty et al., 2019), we identified different HSPC clusters. Thirteen different HSPCs clusters were identified, including a HSC-containing cluster (identified by green in **Figure 1B**) and two divergent progenitor populations (composed by several clusters). Clusters committed to the lympho-myeloid fate (identified by shaded blue colors in **Figure 1B**) and the megakaryocyte-erythroid fate (Identified by shaded red colors in **Figure 1B**) were also identified.

Expression levels of genes required for hematopoietic development were used for cluster identification. These genes included markers of HSC (*AVP*), differentiating progenitors (*CDK6*), early Megakaryocyte/Erythroid progenitors (*GATA2*), early Lymphoid progenitors (*SELL*), and downstream lineage progenitors. Specifically, we used *BLVRB* and *GATA1* levels for identifying Erythroid progenitors, *LMO4* for EBM progenitors, *PLEK* for megakaryocyte progenitors, *MPO* for neutrophil progenitors, *IRF8* for monocyte progenitors, and *LTB* for B-cell progenitors (Zheng et al., 2018, Velten et al., 2017) (**Supplementary Figure 1A**). Interestingly, FA and healthy cells clustered together in every subpopulation, demonstrating that lineage commitment during early hematopoietic differentiation is preserved in FA (**Figure 1C**). Although each cluster was evident in both FA and normal BM, an unusual cell cluster, enriched in FA-derived CD34 expressing cells, emerged: Unannotated-2 cluster (Identified by yellow in **Figure 1B**). This cluster has a unique gene expression profile of unknown significance. Because patients with FA have a strong predisposition to MDS and AML, we speculate this cluster may consist of pre-leukemic cells or leukemia stem cells. Besides this unannotated-2 cluster, there were two additional FA-specific sub-clusters; one in the B-progenitors and one in the MK/E cluster (identified by circles in **Figure 1C**). Both of these clusters were part of a larger cell-type cluster. Analysis of cells in these FA-specific sub-clusters revealed specific functional differences from the corresponding big clusters, such as the elevation of expression of genes involved in TNF-α pathway, Toll-like receptor pathway, and apoptosis pathway, consistent with ongoing cell death and leading to the bone marrow failure in FA patients.

Using StemNet (Velten et al., 2017), and the gene expression profile of each cell, we performed a lineage trajectory analysis and tracked the distinct differentiation pathways in FA and normal BM (**Figure 1D**). Cells positive for *AVP* and negative for *CDK6* expression in the HSC-containing cluster have the most primitive gene expression profile and were used as the root of the lineage trajectory analysis (Zheng et al., 2018). In concordance with the results of Velten et al., 2017, committed progenitors emerged early from primitive HSCs and not from intermediary progenitors. In addition, two very distinctive differentiation outcomes were identified. One pathway is composed of cells committed to the lympho-myeloid fate (identified by shades of blue in **Figure 1D**), and the other pathway is composed of cells committed to the megakaryocyte-erythroid fate (identified by shades of red in **Figure 1D**). Taken together, these results support the hypothesis that these cell populations are the major branches of differentiation emerging from the most primitive human HSCs. Expression of markers previously described (Velten et al., 2017, Zheng et al., 2018) were used for lineage trajectory analysis (**Supplementary Figure 1B**).

FA cells (red circles) follow the same differentiation trajectories as normal cells (blue circles), indicating that modifications in gene expression profiles acquired during differentiation are preserved in FA HSPC. FA HSPCs therefore appear to exhibit an appropriate hematopoietic differentiation program, despite the FA pathway deficiency (**Figure 1E**). Expression of CD38, a classic progenitor-commitment marker, is acquired by hematopoietic progenitors as they develop specialized gene expression profiles and differentiate into specific cell types (**Supplementary Figure 1C**). Individual analysis of

the proportion of different subpopulations detected by scRNAseq demonstrated that patients with FA have a decreased number of cells in the HSC-containing cluster and an increased frequency of cells in cluster Unannotated-2, in comparison to healthy donors (**Supplementary Figure 1D**). Quantitation of CD34-expressing cells and HSCs confirmed the decreased number of these populations in FA patients compared to healthy donors. Additionally, older FA patients had a reduced number of CD34-expressing cells compared to the younger patients (**Supplementary Figure 1E and 1F**). The reduced CD34-expressing cell content in FA patients did not correlate with their aplastic anemia status (**Supplementary Figure 1G**). Of note, patients with severe aplastic anemia were not used for the single cell RNAseq due to the limitation of the cell numbers. The inter-individual variability was observed among healthy individuals and that HSCs were low in some individuals.

The MYC pathway is overexpressed in most HSPC clusters from FA Bone Marrow

Inter-cluster gene expression analysis identified *MYC* as one of the top over-expressed genes in the CD34-expressing cells from patients with FA (**Figure 2A**). As a consequence, the subset of genes transcriptionally-regulated by *MYC* are also significantly over-expressed in FA across all of the HSPC clusters, as shown by single cell enrichment score of CD34-expressing cells (**Figure 2B**).

In addition to *MYC*, other genes over-expressed in the CD34 expressing cells from patients with FA include the members of the immunoglobulin super-family, previously linked to stimulatory division signals in premalignant states. Expression of *DYNLL1,* a

recently described gene regulating anti-resection and NHEJ, was also upregulated in FA cells (He et al., 2018), consistent with the well-known role of the hyperactive NHEJ in FA pathogenesis (Adamo et al., 2010, Pace et al., 2010, Eccles et al., 2018). *HSPA8*, was also upregulated, consistent with the observation that chaperone proteins are upregulated in FA (Karras et al., 2017); *RPL9*, a ribosome gene, exhibited increased expression, consistent with a nucleolar function of an FA protein in ribosome biogenesis (Sondalle et al., 2019). The top 100 differentially expressed genes (DEG) in FA HSPC versus HSPC from healthy donors, are shown in **Supplementary Figure 2A**.

MYC overexpression was validated through real-time PCR in progenitor-enriched fractions from patients with FA (**Figure 2C**). In addition to *MYC* overexpression, which varies greatly among patients with FA, we detected upregulation of the MYC paralog, *MYCN*, and *MAX*, the dimerization partner of MYC (Mathsyaraja and Eisenman, 2016) (**Supplementary Figure 2B**). This result suggests that upregulation of the MYC pathway can be achieved through alternative mechanisms. Inter-patient scRNAseq analysis demonstrated a clear overexpression of *MYC* in most of the samples derived from patients with FA. Interestingly, one healthy control exhibited increased levels of *MYC* expression demonstrating interindividual variability (**Supplementary Figure 2C**). No significant variation was observed in terms of *MYC* levels with respect to the age of the FA patients (**Supplementary Figure 2C**). However, the MYC phenotype was associated with the severity of the aplastic anemia status. FA patients with mild/moderate disease, but not the patients with severe disease, had high *MYC* levels in their bone marrow HSPCs (**Supplementary Figure 2D**).

MYC is a transcription factor regulating the expression of several genes (Nasi et al., 2001). Accordingly, RT-PCR was used to validate changes in the expression of previously recognized MYC targets in bulk HSPCs from eleven patients with FA. *EIF4A1* showed the highest upregulation level (**Figure 2D**), consistent with the known transcriptional regulatory pattern of MYC. Overexpression of *EIF4A1*, *ODC1,* and *TOP1*, in primary HSPCs may indicate that the patients are responding to inflammatory stimuli (**Figure 2D**).

Single cell enrichment score was performed in our scRNAseq dataset using AUCell to verify enrichment of other pathways previously known to be over-expressed in FA, including the p53 pathway (Ceccaldi et al., 2012) (**Supplementary Figure 2E**) and the TGFβ pathway (Zhang et al., 2016) (**Supplementary Figure 2F**).

HSPCs in two opposing functional states co-exist in the BM of patients with FA.

Overexpression of *TP53,* previously demonstrated in FA and linked to BM failure (Ceccaldi et al., 2012), was validated in the scRNAseq data set (**Supplementary Figure 2E**) and by real time-PCR in primary HSPC samples from patients with FA (**Supplementary Figure 3A**). Since MYC and TP53 are transcription factors with opposing activities (Aguda et al., 2011), cells with their differential outcomes may co-exist in the BM of patients with FA. By dividing the scRNAseq LogRatio of *TP53* expression by the LogRatio of *MYC* expression, we identified a gradient of High *TP53* expressing FA HSPC and High *MYC* expressing FA HSPC (**Figure 3A**). The TP53-MYC gradient was not exclusive of FA BM cells; however, both the "high TP53" and "high MYC" cell states

were exacerbated in FA. Bone marrow from healthy donors exhibited more "**balanced**" state whereas bone marrow samples from FA patients exhibited "**unbalanced**" expression of both the genes, with a clear indication of high MYC expression in FA. This gradient behavior was observed in every HSPC cluster from patients with FA (**Supplementary Figure 3B**) and along the entire trajectory of HSPC differentiation (**Supplementary Figure 3C**). The gradient suggests that HSPCs with an apoptotic state, dominated by *TP53* expression, or HSPCs with a proliferative state, dominated by *MYC* expression, may co-exist during early hematopoiesis and lineage commitment in FA bone marrow.

scRNAseq analysis of individual cells showed that, in every patient with FA, this dichotomy of cellular states is present (**Supplementary Figure 3D**). Of note, bone marrow from one of the healthy individuals also showed a moderate dichotomy of the cellular states. The dichotomic cellular state, orchestrated by *TP53* versus *MYC*, suggests that patients with FA have HSPC populations controlled by different cellular states throughout their lifespan. The prevalence of these states might be controlled by various genetic, epigenetic, and environmental factors.

To confirm the presence of the two populations of HSPCs, we stained the BM cells from FA patients versus healthy donors and evaluated, by flow cytometry, the protein expression of p53 and MYC in CD34 expressing cells (**Figure 3B *left panel***). Again, two leading populations of cells were identified – those with "High-p53" expression and those with "High-MYC" expression (**Figure 3B *right panel***). These results further support a

model of mutual exclusivity and suggest that some FA HSPCs are prone to apoptosis or quiescence (High-p53) and some are prone to enhanced proliferation and survival (High-MYC).

CD34+ cells were co-stained for DNA content in order to determine the stage of the cell cycle in which MYC protein is most highly expressed. As the amount of DNA content increased, the levels of MYC also increased (**Figure 3C *left panel***). Accordingly, CD34+ cells in S phase and G2 phase showed the highest levels of MYC, suggesting that MYC is required for progression into the cell cycle of FA CD34+ cells (**Figure 3C *right panel*)**.

MYC is required for proliferation of FA hematopoietic cells at the expense of DNA damage

We next examined the possible mechanisms of MYC upregulation in FA bone marrow. Previous studies have failed to detect MYC gene amplifications or translocations in the BM or leukemias derived from patients with FA (Quentin et al., 2011). Accordingly, we hypothesized that the increase in MYC expression in FA BM cells may result from transcriptional upregulation, perhaps via a super enhancer mechanism, and activation of the BET proteins (Bahr et al., 2018). We tested this hypothesis in several experimental models, using a BRD4 inhibitor with super enhancer inhibitory capacity, (+)-JQ1, known to suppress MYC transcription (Qi, 2014).

scRNAseq analysis of linage negative (Lin-) cells from bone marrow of FA mice (Fancd2-/- mice) showed a gradient of High *Trp53* expressing and High *Myc* expressing cells (**Supplementary Figure 4A**). In comparison to the bone marrow from FA patients,

however, the co-existence of *Trp53*-high and *Myc*-high HSPCs was less prominent in bone marrow of FA mice. This is consistent with the previous observations that most FA mice do not exhibit spontaneous bone marrow failure unless they are exposed to physiological stress-inducing agents (Parmar et al., 2009, Walter et al., 2015).

We next injected (+)- JQ1 or its inactive enantiomer (-)-JQ1 daily over a 1 month period to WT or *Fancd2-/-* mice (**Figure 4A**) and analyzed the effect on the long-term-HSC (LT-HSC) compartment, through the identification of the LSK CD150+CD48- cells (**Figure 4B**). Higher basal levels of Myc protein were observed in the LT-HSC from *Fancd2-/-* mice in comparison to WT mice, and (+)-JQ1 was able to reduce *in vivo* the levels of Myc during this period of treatment (**Figure 4C**). This effect was accompanied by a reduction in the percentage of LT-HSC (**Figure 4D**) and a reduction in the percentage of cycling Ki67 positive cells in the same compartment (**Supplementary Figure 4B**), suggesting that Myc pathway activity is required for proliferation of the hematopoietic stem cell pool in FA mice. As MYC is required for the proliferation of HSPCs and normal hematopoiesis (Wilson et al., 2004), the reduction in the percentage in LT_HSC and in cycling Ki67 was also observed in WT mice (**Figure 4D and Supplementary Figure 4B**). Accordingly, quantification of mature peripheral blood hematopoietic cells showed that the main impact in the reduction of LT-HSC numbers induced by (+)-JQ1 was a reduction in the production of cells from the lympho-myeloid blood compartment (**Supplementary Figure 4C**). (+)-JQ1 exposure in FA mice induced a CD48+CD150+ LSK cell population- namely, the HPC-2 population with megakaryocyte potential (**Figure 4B**) (Oguro et al., 2013).

Cell proliferation required for blood cell production needs several rounds of DNA replication. This process appears to be promoted by the Myc pathway and contributes to the production of physiologically-induced DNA damage in FA pathway deficient cells. Accordingly, the *in vivo* inhibition of the Myc pathway with (+)-JQ1 for one month reduced the amount of DNA damage in *Fancd2-/-* LT-HSC, as measured by a comet assay of sorted LT-HSC (**Figure 4E**). In line with the former, *in vitro* proliferation of LSK cells from *Fancd2-/-* mice was reduced by exposure to (+)-JQ1, as determined by a CFSE dilution assay, in comparison to WT LSK cells (**Supplementary Figure 4D**).

To determine whether human FA pathway-deficient HSPCs depend on MYC for cell division, we initially generated FA-like HSPCs, using a lentivirus expressing a short-hairpin RNA against *FANCD2,* with (+)-JQ1 (Zhang et al., 2016). FA-like HSPCs exhibited reduced clonogenic and cell division potential when exposed to (+)-JQ1, confirming that MYC enhances cell division (**Figure 4F**). We also used a CRISPR/Cas9 genome editing of primary human umbilical cord blood CD34+ human hematopoietic stem and progenitor cells (HSPCs) and generated *FANCG*-knockout (*FANCG*-KO) CD34+ cells. As expected, these *FANCG*-KO cord blood CD34+ cells also exhibited higher MYC and p53 expression compared to the control CD34+ cells (**Supplementary Figure 4E and 4F**). Interestingly, *FANCG*-KO CD34+ cells exhibited high MYC and high p53 expression profile similar to what was observed in primary HSPCs from FA patients (**Supplementary 4F and Figure 3B**). Knocking out MYC in the *FANCG*-KO cord blood CD34+ reduced their clonogenic potential and confirmed that MYC is required for survival of primary FA HSPCs (**Supplementary Figure 4G**). Next, Lin- BM cells (enriched in HSPCs) from patients with

FA and healthy donors, were cultured in methylcellulose and exposed to (+)-JQ1 over 14 days. The clonogenic potential of the primary FA HSPCs was reduced after (+)-JQ1 exposure (**Figure 4G and Supplementary Figure 4H**). Taken together, our results suggest that MYC upregulation has some benefits for FA HSPCs, resulting in enhancement of cell proliferation and survival.

Overexpression of MYC was validated by western blot in the FA lymphoblast cell line EUFA316+EV (*FANCG*-deficient), derived from a patient with FA and reversed by functional complementation of the cells with the FANCG cDNA (EUFA316+G) (**Figure 5A**). As expected, several genes of the MYC pathway were found overexpressed in the EUFA316+EV cell line (**Supplementary Figure 5A**) and overexpression of *MYC* was efficiently blocked by the BET bromodomain inhibitor (+)-JQ1, as assessed at the protein level (**Figure 5B**) and gene expression level (**Supplementary Figure 5B**).

Reduced proliferation capacity of FA pathway deficient cells exposed to (+)-JQ1 appeared to have protective effects against DNA damaging agents *in vivo* and *in vitro*. We therefore analyzed cell cycle status of the *FANCG*-deficient cell line, EUFA316+EV, when exposed to (+)-JQ1 and genotoxic agent Mitomycin C (MMC). As expected, FANCG+EV cells showed an increase in the number of cells in G2 after exposure with MMC (**Figure 5C**). Exposure of EUFA316+EV cells to (+)-JQ1 and MMC resulted in an increase in the number of cells in G1, with a concomitant reduction in the number of cells traversing S-phase (**Figure 5C**). DNA fiber assays also showed that exposure to (+)-JQ1 has a direct effect in cell proliferation by reducing the opening of new origins of replication

(**Figure 5D**) and reducing replication fork speed of EUFA316+EV cell line (**Figure 5E**), confirming that FA cells require MYC for progression through the cell cycle. Since EUFA316+EV cells expressed high levels of MYC, the major effect of (+)-JQ1 appears to be the reduction in MYC expression, leading to decreased fork speed and to an increase in the G1 fraction of the cell cycle. Interestingly, as previously shown, the wild-type cells (*FANCG*-corrected cells) exhibited an increase in fork speed following short-term (+)-JQ1 exposure, consistent with a previous study (Bowry et al., 2018).

We next determined whether MYC inhibition contributed to reduce replication stress. Replication stress from MYC upregulation is known to activate the ATR-CHK1 pathway, leading to the generation of single strand DNA and to the activation of replication stress biomarkers, including pRPA, pKAP1, and pCHK1 (Maya-Mendoza et al., 2015, Herold et al., 2009, Puccetti et al., 2019). Interestingly, (+)-JQ1 reduced the replication stress and DNA damage induced by HU or MMC as indicated by decreased phosphorylation of p-RPA and γH2AX in *FANCG*-deficient lymphoblast cells (**Figure 5F**, **Supplementary Figure 5C**). In addition, (+)-JQ1 improved the survival to MMC of FA cells in 5 days survival assays (**Figure 5G).** Of note, induction of DNA damage by both HU and MMC is dependent on active S-phase. As (+)-JQ1 treatment increases the number of cells in G1 and decreases cells in S phase (**Figure 5C**), the improved survival of FA cells under genotoxic stress may be attributed to the inhibition of toxic effects due to reduced replication fork speed and reduced replication origin firing. Collectively, inhibition of MYC improves survival and avoids DNA damage in FA cells by inhibiting the accumulation of toxic effects, which are S-phase dependent.

Physiological stress by the immunostimulant molecule pI:pC activates the MYC pathway in a mouse model of FA

Polyinosinic:polycytidylic acid (pI:pC) is a double-stranded RNA mimetic and immunostimulant that promotes the activation of inflammatory responses. pI:pC is a potent inductor of BM failure in FA mice since it prompts HSCs to enter into the cell cycle and accumulate DNA damage (Walter et al., 2015). We tested the potential of pI:pC to induce MYC activation by injecting WT and *Fancd2-/-* mice with pI:pC or water twice per week for 1 month (**Figure 6A**).

One month after pI:pC injection, BM cells from *Fancd2-/-* mice exhibited a pathological expansion of the LSK compartment accompanied by a reduction in the number of LT-HSCs, consistent with a previous report (Walter et al., 2015) (**Supplementary Figure 6A**). Also, a striking increase in Myc expression was observed in the hematopoietic compartments of *Fancd2-/-* mice, including the LT-HSC compartment (**Figure 6B**). Taken together, these data suggest that the Myc pathway can be enlisted among the intracellular mechanisms driving the expansion of LSK cells with the subsequent exhaustion of the LT-HSC pool in FA bone marrow.

Since pI:pC is a potent Myc pathway inducer, we tested whether (+)-JQ1 can reduce the HSC exhaustion *in vivo* by reducing the activation of Myc. We co-injected pI:pC and (+)-JQ1 into WT and *Fancd2-/-* mice (**Figure 6C**), and observed that 48 h after treatment initiation, (+)-JQ1 effectively was able to reduce the activation of a subset of pro-

inflammatory cytokines in *Fancd2-/-* mice, including MCP1 and TNFα (**Figure 6D**). (+)-JQ1 reduced the Myc expression in the HSPCs and LT-HSCs, as determined by flow cytometry (**Figure 6E and Supplementary Figure 6B**). In addition, (+)-JQ1 reduced *in vivo* the pI:pC induced pathologic expansion of HSPCs (LSK cells) in *Fancd2-/-* mice (**Supplementary Figure 6C**) and ameliorated the pI:pC-induced reduction of LT-HSC (**Supplementary Figure 6D**). Finally, (+)-JQ1 exposure improved the peripheral blood WBC counts in pI:pC-injected *Fancd2-/-* mice (**Supplementary Figure 6E**). In agreement with reduced expansion of the HSPC compartment after exposure to (+)-JQ1 upon pI:pC treatment, we observed a reduction in DNA damage as measured by γH2AX fluorescence per cell when *Fancd2-/-* mice were co-injected with (+)-JQ1 and pI:pC (**Supplementary Figure 6F**).

As the acute inflammatory response induced by pI:pC drove Myc pathway activation in FA mouse model, we returned to samples from patients with FA and explored inflammatory cues that might be responsible for MYC pathway activation in the human setting. Inflammatory cytokines are increased in the BM of patients with FA (Dufour et al., 2003, Briot et al., 2008, Garbati et al., 2016). By exploring the BM plasma from patients with FA (n=19) we confirmed the increased concentration of pro-inflammatory cytokines in the BM plasma of patients with FA with respect to healthy subjects (n=9), including IFNγ, MCP1 and TNFα (**Figure 6F *upper panel***). Interestingly, anti-inflammatory cytokines IL-5, IL-10, and IL-13, which have the capacity to mitigate the activation of immune cells (Banchereau et al., 2012, Couper et al., 2008), had significantly decreased concentrations in the BM plasma from patients with FA, a previously non-reported

observation that might contribute to a sustained proinflammatory BM environment in FA (**Figure 6F *lower panel***). In addition, historical records from patients with FA showed that C-reactive protein (CRP), a protein known to be elevated during inflammatory conditions (Sproston and Ashworth, 2018), was on average over reference values in children with FA (**Supplementary Figure 6G**). To confirm a role for inflammatory cytokines in the transcriptional activation of MYC, we exposed CD34+ cells *in vitro* to these pro-inflammatory cytokines. IFNγ increased the percentage of CD34+ cells expressing MYC. As expected, the addition of (+)-JQ1 counteracted this effect (**Figure 6G**). Since TNFα is implicated in the pathogenesis of FA (Rosselli et al., 1994, Dufour et al., 2003, Li et al., 2007), we next evaluated its effect in cord blood CD34+ cells with CRISPR knockout of *FANCA*. Interestingly, *FANCA*-KO HSPCs exhibited higher expression of MYC compared to the mock controls and this was even further increased after *in vitro* exposure to TNFα (**Figure 6H**). As expected, the addition of (+)-JQ1 counteracted this effect (**Figure 6H**). *In vitro* treatment with MCP1 did not induce any changes (data not shown).

MYC overexpression causes detachment of FA HSPCs from their bone marrow niche

Gene expression profiles of “High-*TP53”* expressing HSPCs and “High-*MYC”* expressing HSPCs from bone marrow of patients with FA revealed that these cellular states have opposite gene expression profiles (**Figure 7A**). High-*TP53* expressing HSPCs exhibited high expression of *CXCR4,* a pro-quiescence gene (Nie et al., 2008), whereas High-*MYC* expressing HSPCs had a strong downregulation of *CXCR4* and other cell adhesion

genes, such as *VIM*. *CXCR4* is a critical regulator of adhesion of stem cells to their BM niche (Karpova and Bonig, 2015) (**Figure 7A**). *MYC* overexpression is known to negatively impact the adhesion of HSPCs to their BM niche (Wilson et al., 2004). Taken together, these results suggest that, in addition to enhanced proliferation, "High-*MYC*" expressing HSPC have weak adhesion to their BM niche and a predisposition to detachment.

To explore the predisposition to detachment hypothesis, we explored whether there was an increase in the CD34+ cells trafficking in the peripheral blood of patients with FA with respect to healthy controls. Indeed, an increased percentage of circulating CD34+ cells, in comparison to healthy individuals, was observed (**Figure 7B**). Interestingly, although a few patients exhibited cytogenetic evidence of clonal hematopoiesis, none of them displayed pathologic evidence of AML transformation (**Supplementary Table 3).** Clonogenic capacity of these FA circulating CD34+ cells was assessed *in vitro* using several assays. CFU assays resulted in erythroid colonies, granulocyte colonies, and monocyte colonies. Colony numbers were reduced when the samples were obtained from patients with severe BM failure (**Supplementary Figure 7A**). In vitro differentiation assays indicated that circulating CD34+ cells from peripheral blood of FA patients exhibit lymphoid and myeloid differentiation potential (**Figure 7C**). Additional LTC-IC assays demonstrated that primitive HSC are more frequently found in the circulating CD34+ population from patients with FA than from healthy donors (**Supplementary Figure 7B**). Immunophenotype profiling of the FA circulating CD34+ cells revealed the presence of

pro-B cells, early lymphoid progenitors, and myeloid progenitors (**Supplementary Figure 7C**).

Validation of the CD34+ cells residing in the BM in a sub-set of eleven patients with FA confirmed that, as expected, patients with FA have a decreased percentage of CD34+ cells residing in the BM in comparison to healthy individuals, which worsens with the progression of the disease (**Supplementary Figure 7D**, ***left panel***). The opposite occurred in peripheral blood, where patients with FA have a higher percentage of circulating CD34+ cells in comparison to healthy controls. Importantly, the number of circulating CD34+ cells in patients with FA tends to decrease as the severity of BM failure worsened, correlating with the cellularity of the BM (**Supplementary Figure 7D, *right panel***).

When we compared the MYC expression in CD34+ cells from patients with FA, we found that circulating CD34+ cells have a significantly higher MYC fluorescence per cell in comparison to CD34+ cells residing in the BM from the same patients, and also in comparison to healthy BM CD34+ cells (**Figure 7D**). In addition, CD34+ cells from patients with FA, both from BM and peripheral blood, have reduced levels of CXCR4 per cell when compared to BM CD34+ cells from healthy controls (**Figure 7E**). Reduced levels on CXCR4 levels might be responsible for the reduced adhesion capacity that FA CD34+ cells to be retained in the BM niche and eased egression into the peripheral blood.

We have shown above that pI:pC induces Myc expression *in vivo*, and others have previously shown that pI:pC provokes the exit from dormancy of LT-HSC (Walter et al., 2015, Sato et al., 2009), which consequently should reduce adhesion of progenitor cells to their BM niche. We therefore tested if pI:pC exposure was able to increase the trafficking of mouse progenitor cells into the peripheral blood of WT and *Fancd2-/-* mice. Indeed, we found that upon pI:pC injection two main hematopoietic progenitor populations circulate in peripheral blood of WT and *Fancd2-/-* mice: LSK and LSK- cells, the LSK population is enriched in progenitor and LT-HSC cells, whereas the LSK- population has been reported to be an LSK derived population (Peng et al., 2012) (**Figure 7F left panel, Supplementary Figure 7E**). Moreover, the MNCs from the peripheral of blood of the pI:pC-treated mice had CFU capacity (**Figure 7F right panel**). Our results suggest that inflammatory conditions or physiological stress, such as pI:pC exposure, change the FA HSPC compartment from a quiescent state to a hyperproliferative state orchestrated by *Myc*. This transition results in increased replication stress and propensity to detachment of progenitor cells (**Fig. 7G**).

DISCUSSION

The consequences of MYC upregulation in FA hematopoietic cells

In the current study, we used single cell RNA sequencing to study the transcriptional patterns in BM hematopoietic cells derived from patients with FA. As expected, and consistent with our earlier studies, we found an increase in the transcription of genes from the p53 pathway and the TGFβ signaling pathway (Ceccaldi et al., 2012, Zhang et al.,

2016). We also found an increase in *MYC* mRNA expression across multiple HSPC lineages in FA.

The increased transcriptional activity of MYC in the FA BM cells has important consequences. On the one hand, MYC upregulation plays a beneficial role in HSPCs, providing a critical survival function and stimulates cell cycle progression (Bretones et al., 2015, Laurenti et al., 2008). Accordingly, inhibition of MYC transcription with the BET inhibitor, (+)-JQ1, reduces cell proliferation and colony number in clonogenic survival studies of FA bone marrow HSPCs.

On the other hand, MYC upregulation, resulting in chronic replication stress and increased DNA damage (Puccetti et al., 2019), might have a detrimental role in the BM of patients with FA. Consistent with this notion, inhibition of *MYC* expression with (+)-JQ1 reduced the percentage of cells in S phase, reduced replication stress biomarkers, reduced DNA damage, and improved the survival of FA cells upon genotoxic stress. For instance, we observed that in the human EUFA316 *FANCG*-deficient lymphoblasts, the MYC inhibitor (+)-JQ1 rescues the DNA damage resulting from MMC exposure. Also, in a *Fancd2-/-* mouse model, *in vivo* inhibition of *Myc* transcription reduces cell cycle entry and hyperproliferation of LT-HSCs that can result in replication stress, therefore reducing the amount of DNA damage. Multiple FA proteins function in mitophagy (Sumpter et al., 2016) and the absence of mitophagy may contribute to the survival of FA cells during genotoxic stress. BRD4 is a negative regulator of autophagy gene expression, and the BET inhibitor (+)-JQ1 induces an upregulation of autophagy gene expression, with an

increase in autophagy and lysosomal function (Sakamaki et al., 2017). Therefore, MYC inhibition by (+)-JQ1 may also result in survival of FA HSPCs by upregulation of autophagy.

In short, *MYC* upregulation in the HSPCs from FA patients contributes to chronic replication stress and increased induction of DNA damage. Previous studies have also demonstrated that FA cells have increased replication stress and replication fork instability (Balcerek et al., 2018).

MYC-induced release of BM HSPCs to the peripheral blood

Another potentially detrimental consequence of MYC upregulation in the FA HSPCs is the transcriptional downregulation in the "High-*MYC"* expressing cells of the cell surface adhesion protein, CXCR4 (Karpova and Bonig, 2015). Our scRNAseq and flow cytometry of HSPC confirmed that CD34+ cells from FA patients have reduced CXCR4 levels, moreover, we observed an increase in the number of HSPCs with clonogenic capacity in the peripheral blood of patients with FA and *Fancd2-/-* mice, in the later specifically under pI:pC physiologically induced proliferative stress, which exacerbated MYC induction. Therefore, chronic release of HSPCs from the FA BM, exacerbated by acute MYC induction, may contribute to the chronic state of BM failure in these patients.

CXCR4 has been clearly defined as a molecule needed for maintenance of HSPC in their BM niche and is also critical for maintenance of HSPC in quiescence (Nie et al., 2008), when CXCR4 expression is lost, cells enter into the cell cycle. On the other hand, MYC

promotes entrance into the cell cycle of HSPC, therefore both mechanisms, *MYC* upregulation and *CXCR4* downregulation, must be acting in a concerted manner; however, it remains to be defined if MYC acts upstream of CXCR4, which seems feasible given the transcription factor activity of *MYC*.

Mechanism of MYC upregulation in Fanconi anemia

In human cells, MYC can be upregulated by multiple mechanisms including gene amplification (Schaub et al., 2018), translocation (Boxer and Dang, 2001), or transcriptional enhancement (Lancho and Herranz, 2018). Previous studies have failed to detect MYC gene amplifications or translocations in the BM or leukemias derived from patients with FA (Quentin et al., 2011). Accordingly, we hypothesized that the increase in MYC expression in FA BM cells may result from transcriptional upregulation, perhaps via a super enhancer mechanism and BET activation (Wroblewski et al., 2018, Roe et al., 2015, Qi, 2014, Delmore et al., 2011, Bahr et al., 2018). Consistent with this hypothesis, the BET inhibitor (+)-JQ1 strongly downregulated *MYC* expression and acutely reduced many of the functional consequences of elevated MYC activity. The increased *MYC* mRNA expression in FA BM cells may also result from the local increased levels of inflammatory cytokines, such as IFNγ and TNFα (Lin et al., 2014, Ramana et al., 2000, Briot et al., 2008). Accordingly, the inhibition of these cytokine signaling pathways can reduce *MYC* expression and offer potential therapeutic opportunities.

Translational implications

Our results have translational implications for patients with FA. For instance, in acute episodes of stress, resulting from a viral infection and inflammation (Matatall et al., 2016, Walter et al., 2015, Sato et al., 2009), patients with FA may experience a strong MYC induction, leading to rapid cycling and HSPC loss. Indeed, patients with FA often exhibit a rapid decline in BM HSPCs numbers after an acute viral infection, such as a varicella infection. In this setting, HSPCs are likely to be released into the peripheral blood, due to the reduction in expression of the cell surface adhesion protein CXCR4 that accompanies MYC overexpression. Release of HSPCs to the peripheral blood may create a chronic state of BM failure. A BET inhibitor may reduce HSPC cycling during the acute viral episode, limit DNA damage, and preserve the number of BM HSPCs.

In contrast, the chronic use of BET inhibitors may have negative consequences for patients with FA. Chronic MYC loss or inhibition would be expected to reduce the overall survival of FA BM cells. Paradoxically, FA cells seem to require MYC to overcome the cell cycle blockade resulting from high p53 expression (Ceccaldi et al., 2012) and increased TGFβ signaling (Zhang et al., 2016).

Finally, since we have only evaluated a single BM sample from each patient with FA, we do not know if the percentage of High-*MYC* versus High-*TP53* expressing HSPCs changes over time. For instance, the percentage may vary, depending on the degree of BM failure. Also, chronic administration of (+)-JQ1 or another BET inhibitor might not be desirable, but it may be useful episodically to preserve the HSPC pool of patients with FA traversing an infection crisis.

Limitations of the study

Our study has limitations. During the development of our initial scRNAseq screening, we were limited to the specific FA patients coming into the clinics, as the scRNAseq experiments needed to be run on fresh samples. Working with a bank of frozen clinical bone marrow samples was not possible due to RNA degradation. We were therefore unable to perform the scRNAseq analysis of the bone marrow from FA patients with severe aplastic anemia, myelodysplastic syndrome, or acute myeloid leukemia. Also, we have mainly studied patients with pathogenic variants in *FANCA* and *FANCC* genes, as patients with mutations in genes downstream in the FA pathway are rare. Despite these limitations, we believe this study provides clear evidence of the coexistence of cells with High-TP53 levels and cells with High-MYC levels in the bone marrow of FA patients. This study uncovers a unique mechanism of the pathophysiology of Fanconi Anemia, using rare bone marrow samples derived from patients.

AUTHOR CONTRIBUTIONS

A.R., K.P. and A.D.D. designed experiments. K.Z., A.F. and S.H. developed bioinformatic tools and performed computational analysis and interpretation. A.R performed experiments with help from J.F., C.Y., M.V., G.G.M., K.MQ, L.A.S., B.M., L.T., M.G. and E.V. E.F., B.G.T. and A.S. provided samples and analyzed clinical data. R.P., M.G., S.F. and J.G. provided reagents and analyzed data. A.R., K.P. and A.D.D. analyzed and interpreted the data. A.R. K.P. and A.D.D. wrote the manuscript.

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DECLARATION OF INTERESTS

A.D. D'Andrea is a consultant/advisory board member for Lilly Oncology, Merck-EMD Serono, Intellia Therapeutics, Sierra Oncology, Cyteir Therapeutics, Third Rock Ventures, AstraZeneca, Ideaya Inc., Cedilla Therapeutics Inc., a stockholder in Ideaya Inc., Cedilla Therapeutics Inc., and Cyteir, and reports receiving commercial research grants from Lilly Oncology and Merck-EMD Serono.

FIGURE LEGENDS

Figure 1. Single cell RNA sequencing reveals that early hematopoiesis is not perturbed in FA.

A) Magnetically enriched Lin- cells from 7 patients with FA and 5 healthy donors (**Supplementary Table 1**) were individually captured, lysed and barcoded using the 10x Chromium^{TM} controller platform. Barcoded bulk cDNA was sequenced in an Illumina

platform. Unique Molecular Identifiers (UMIs) were used for sequencing deconvolution and clustering analysis, DEG analysis and lineage analysis.

B) t-SNE plot showing the clustering analysis for CD34 expressing HSPCs. Clustering analysis was performed using Seurat (Satija et al., 2015). FA and healthy CD34 expressing HSPCs were combined for clustering based on their gene expression profiles. A total of 13 clusters were identified spanning the different HSPC subpopulations described so far. Identities of the different clusters were assigned based on gene expression profiles, as previously described (Velten et al., 2017, Zheng et al., 2018). Identified clusters include a HSC-containing cluster (HSC-containing); clusters with megakaryocytic-erythroid identity include: EMP (Erythroid-megakaryocyte progenitor), MK (Megakaryocyte progenitor), MK/E (common megakaryocyte/erythroid cluster), E (erythroid progenitor) and BEM (basophil, eosinophil and mast cell progenitor); clusters with lympho-myeloid identity include LMPP (lymphoid-primed multi-potential progenitor), B-prog (B-cells progenitor), N (neutrophil progenitor), DC (dendritic cell progenitor) and M (monocyte progenitor). Two clusters with distinctive gene expression profiles, one of them enriched in FA cells, were assigned as "unannotated" since they required further research for identity definition.

C) FA-derived CD34 expressing HSPCs (red dots) cluster together with healthy donors derived CD34 expressing HSPCs (blue dots), indicating that hematopoietic cell types are preserved during FA hematopoiesis. Two FA-specific sub-clusters are highlighted with circles.

D) Lineage trajectory analysis was performed using StemNet (Velten et al., 2017) showing that HSPC differentiation follows a star-like path, rather than the conventional

tree-like path, similar to what has recently been proposed (Velten et al., 2017). HSC (green) located in the center region of this star follow at least two main differentiation pathways, one characterized by lympho-myeloid commitment (shades of blue), and the other characterized by megakaryocytic-erythroid commitment (shades of red). The lympho-myeloid commitment includes B, M, DC and N progenitors; the megakaryocytic-erythroid commitment spans MK, E and BEM progenitors.

E) Projection of FA HSPCs in the lineage trajectory analysis showing that FA HSPCs follow the same differentiation star-like profile as healthy cells do. FA HSPCs (red dots), healthy HSPCs (blue dots). See also Supplementary Figure 1.

Supplementary Table 1 describes the characteristics of heathy donors and FA patients whose bone marrow samples were used for the experiments described in panels A-E.

Figure 2. The MYC pathway is overexpressed in FA HSPCs.

A) Single cell gene expression analysis of the different HSPCs clusters shows that the proto-oncogene *MYC* is significantly overexpressed in FA cells in almost every cell compartment in comparison to healthy cells.

B) Single cell enrichment score analysis showing that the MYC pathway genes are significantly overexpressed in FA HSPCs in comparison to healthy HSPCs across the different identified cell clusters.

C) Quantitative real-time RT-PCR validation in progenitor cells showing that *MYC* is overexpressed in primary HSPCs from patients with FA, in comparison to HSPCs from healthy controls. Red bars indicate BM samples used in our initial scRNAseq screening,

black bars indicate additional FA bone marrow samples used for validation of *MYC* expression. The **Supplemental Table 1** and **Supplemental Table 2** describe the characteristics of healthy donors and FA patients whose bone marrow samples were used in this study. Complementation group of each patient with FA is indicated between parenthesis. UID: Unidentified pathogenic variant.

D) Real-time RT-PCR in bulk progenitor cell population showing changes in the expression of the MYC pathway genes in FA samples in comparison to healthy control samples (see **Supplementary Table 2** for the details about the samples).

p-values of <0.001 were considered extremely significant (***, ****). See also Supplementary Figure 2.

Figure 3. HSPCs expressing two opposing functional cell states exist in the BM of patients with FA.

A) "High-*TP53"* expressing HSPCs and "High-*MYC"* expressing HSPCs co-exist in the BM of patients with FA. The LogRatio of *TP53* expression was divided by the LogRatio of *MYC* expression per cell, resulting in a gradient spanning from "High-*TP53"* expressing HSPCs to "High-*MYC"* expressing HSPCs. The expression of *Myc* and *Trp53* per cell is shown.

B) Flow cytometry was used to confirm the co-existence of MYC and p53 expressing CD34+ cells from patients with FA. FACS plot for gating CD34+ cells is shown in left panel. FACS plot for MYC and p53 expression in CD34+ cells is shown in right panel. Two seemingly exclusive populations of FA CD34+ cells were detected, one with high MYC levels (shaded green box) and the other with high p53 levels (shaded pink box) in

comparison to healthy cells. A third population (shaded in orange box) in samples from patients with FA co-expresses medium-high levels of MYC and p53 in comparison to healthy CD34+ cells. Data are from pooled BM samples of two healthy controls and eight patients with FA.

C) FACS plot (left panel) and quantitation (right panel) of DNA content showing high MYC expression in FA CD34+ cells during S and G2/M phases of the cell cycle (The data are from pooled healthy BM controls (n=2) and patients with FA (n=8). See also Supplementary Figure 3.

Figure 4. (-)-JQ1 inhibits the growth of FA-deficient hematopoietic stem cells.

A) WT and *Fancd2-/-* mice were injected daily with (+)-JQ1 (50 mg/kg) for 1 month or the inactive enantiomer (-)-JQ1 (50 mg/kg) as a negative control (n=3 mice per group) and BM was analyzed

B) Sorting strategy for identification of LT-HSC in wild-type (WT) and *Fancd2-/-* mice exposed to (-)-JQ1 or (+)-JQ1. LSK (Lin-Sca-1+c-Kit+) population was analyzed for CD150 and CD48 expression and LT-HSCs (LSK CD150+CD48-) were identified. Flow cytometry plots are shown.

C) Quantitation of Myc expression by FACS analysis in LT-HSCs from wild-type (WT) and *Fancd2-/-* mice exposed to (-)-JQ1 or (+)-JQ1. The inactive enantiomer (-)-JQ1 was used as negative control (n=3 mice per group).

D) LT-HSC (Long-term HSC), MPP (multipotent progenitors), HPC (restricted hematopoietic progenitors) content in wild-type (WT) and *Fancd2-/-* mice exposed to (-)-JQ1 or (+)-JQ1 (n=3 mice per group).

E) DNA damage in LT-HSCs from wild-type (WT) and *Fancd2-/-* mice exposed to (-)-JQ1 or (+)-JQ1. DNA damage was measured by tail length in a comet assay (n=3 mice per group). Representative images of the alkaline comets (left panel) and quantitation of comet tail length (right panel) are shown.

F) CFU assay of FA-like CD34+ HSPCs, generated through infection with a shRNA lentivirus against *FANCD2*. FA-like HSPCs were cultured in triplicates in complete methylcellulose medium with or without (+)-JQ1 (50 nM) for 14 days and clonogenic growth was assessed. (+)-JQ1 reduces the clonogenic capacity of FA-like HSPCs.

G) CFU assay of HSPCs from bone marrow of healthy donors or FA patients. Healthy and FA BM derived Lin- cells (3000 cells for healthy BM and 5000 cells for FA BM) were plated in complete methycellulose with (+)-JQ1 (50 nM) or DMSO and CFU numbers were quantified after 14 days of culture. MYC inhibition with (+)-JQ1 reduces the clonogenic capacity of healthy and FA HSPCs. Note that the cells were plated at different time depending upon the availability of the samples.

p-values of 0.01 to 0.05 were considered significant (*), p-values of 0.001 to 0.01 were considered very significant (**) and p-values of <0.001 were considered extremely significant (***, ****). See also Supplementary Figure 4.

Figure 5. MYC inhibition prevents entry into S phase and reduces replication associated stress in FA lymphoblastoid cells.

A) Western blots of the lysates of FANCG-deficient human lymphoblastoid cells (EUFA316+EV) and FANCG-complemented lymphoblastoid cells (EUFA316+G) showing overexpression of the MYC protein in the FANCG-deficient cells.

B) Western blots of the lysates of FANCG-deficient lymphoblastoid cells (EUFA316+EV) exposed to the BET bromo-domain inhibitor (+)-JQ1 for 48 h showing that (+)-JQ1 inhibits the expression of the MYC protein.

C) EUFA316+G and EUFA316+EV cells were treated for 24h with Mitomycin C (MMC) (20 ng/ml) and (+)-JQ1 (50 nM) and cell cycle distribution was analyzed.

D) (+)-JQ1 reduces the amount of new replication origins in the FANCG-deficient lymphoblastoid cells. EUFA316+G and EUFA316+EV cells were exposed to (+)-JQ1 for 8 h and then incubated with the nucleotide analogs IdU for 30 min followed by CldU for 30 min in presence of (+)-JQ1. DNA fiber assay was carried out and the percentage of fibers with replication origins were quantified per condition.

E) (+)-JQ1 slows-down replication fork speed in the FANCG-deficient lymphoblastoid cells. EUFA316+G and EUFA316+EV cells were exposed to (+)-JQ1 for 8 h and then incubated with the nucleotide analogs IdU for 30 min followed by CldU for 30 min in presence of (+)-JQ1. DNA fiber assays were performed and the fiber length composed by IdU tracts plus CldU tracts was measured and divided by the incubation time in the presence of the analogs. At least 100 fibers were analyzed per sample.

F) (+)-JQ1 reduces the phosphorylation of replication stress markers in the FANCG-deficient lymphoblastoid cells. EUFA316+G and EUFA316+EV cells were exposed to (+)-JQ1 and the DNA damaging agent hydroxyurea (HU) for 24 h. HU increased the levels of replication stress and DNA damage associated markers (phospho-KAP1, phosphor-RPA32 and γH2AX) however (+)-JQ1 reduced the levels in EUFA316+EV cells.

G) Survival of the FANCG- deficient EUFA316+EV cells to MMC is improved by (+)-JQ1. EUFA316+G and EUFA316+EV cells cultured in presence of increased concentrations of

MMC and (+)-JQ1 (50 nM). Five days after, cells were harvested, and survival assessed. The experiment was performed three times and the data from a representative experiment are shown.

p-values of 0.01 to 0.05 were considered significant (*), p-values of 0.001 to 0.01 were considered very significant (**) and p-values of <0.001 were considered extremely significant (***, ****). See also Supplementary Figure 5.

Figure 6. Physiological/inflammatory stress activates the MYC pathway in *Fancd2-/-* mice and causes BM failure.

A) Wild-type (WT) and *Fancd2-/-* mice were injected with pI:pC (5 mg/kg) twice per week for 1 month and bone marrow was analyzed.

B) pI:pC treatment increases the Myc fluorescence in LT-HSC from *Fancd2-/-* mice in comparison to WT control. Flow cytometric plots (left panel) and the quantitation (right panel) for Myc expression in LT-HSCs are shown (n=3 mice per group).

C) Wild-type (WT) and *Fancd2-/-* mice were treated with pI:pC (5 mg/kg) and (+)-JQ1 and 48 h after the treatment BM was analyzed. (-)-JQ1 was used as a negative control. (n=3 mice per group)

D) (+)-JQ1 prevents the activation of a subset of pI:pC-induced pro-inflammatory cytokines, including MCP-1 and TNFα in *Fancd2-/-* mice. Mice were treated with pI:pC and (+)-JQ1 as shown in panel C and peripheral blood was analyzed.

E) (+)-JQ1 reduces *in vivo* the levels of Myc in LSK cells from *Fancd2-/-* mice both in basal conditions and after pI:pC injection. Mice were treated with pI:pC and (+)-JQ1 as

shown in panel C and Myc expression in LSK cells from BM was analyzed using flow cytometry.

F) Higher levels of pro-inflammatory cytokines in the BM plasma of patients with FA, including IFNγ, MCP-1 and TNFα (upper panel), and reduced levels of anti-inflammatory cytokines in comparison to healthy BM plasma (lower panel). FA *n=19*, Healthy *n=9*.

G) The *in vitro* exposure of BM CD34+ cells to pro-inflammatory cytokine IFNγ increases MYC expression which can be mitigated by (+)-JQ1. CD34+ cells from healthy donors were cultured *in vitro* for 24 h with IFNγ (20 ng/ml) and/or JQ1 (50 nM), and MYC expression was analyzed by flow cytometry. Flow cytometry plots for MYC staining in CD34+ cells (upper panels) and quantitation of MYC expression (lower panel) are shown. (n= 4)

H) The *in vitro* exposure of primary *FANCA*-KO cord blood CD34+ cells to pro-inflammatory cytokine TNF-α increases MYC expression which can be mitigated by (+)-JQ1. Cord blood CD34+ cells with CRISPR-mediated knockout of *FANCA* (*FANCA*-KO) were cultured *in vitro* for 24 h with TNF-α and/or (+)-JQ1, and MYC expression was analyzed by flow cytometry. Flow cytometry plots for MYC staining (left panels) and quantitation of MYC expression (right panel) in CD34+ cells are shown. Note that *FANCA*-KO CD34+ cells exhibited higher expression of MYC compared to the mock controls and this was even further increased after *in vitro* exposure to TNF-α.

p-values of 0.01 to 0.05 were considered significant (*), p-values of 0.001 to 0.01 were considered very significant (**) and p-values of <0.001 were considered extremely significant (***, ****). See also Supplementary Figure 6.

Figure 7. Physiological stress-induced MYC overexpression predisposes FA HSPCs to egress from the BM.

A) High *MYC* expression significantly correlates with reduced expression of the HSPC adhesion molecule CXCR4. Gene expression profiles of “High-*TP53”* and “High-*MYC”* expressing HSPCs were compared and DEG were obtained. Heat map for the gene expression in High-*TP53* and High-*MYC* expressing HSPCs is shown in the lower panel. Note that both cellular states have totally opposite gene expression profiles. “High-*MYC”* expressing cells downregulate several cell adhesion genes, including *CXCR4,* a critical HSPC adhesion gene, and *VIM,* encoding Vimentin.

B) Patients with FA have an increased percentage of CD34+ cells circulating in peripheral blood in comparison to healthy controls. *Left panel.* Representative FACS plot showing the percentage of CD34+ cells in peripheral blood of a healthy donor and a patient with FA. *Right panel.* The quantitation of the percentage of CD34+ cells in peripheral blood of healthy donors and patients with FA. **C)** FA circulating CD34+ cells have multi-lineage differentiation properties *in vitro*. Representative flow cytometry plots (left panel) and quantitation (right panel) of Lin+ cells from the CD34+ cells of peripheral blood MNC, (i) Lin+ cell production in liquid myeloid promoting culture conditions, (ii) NK-cell (CD56+) production in MS-5 supporting stroma. (iii) B-cell (CD19+) production in MS-5 supporting stroma, (iv) T-cell (CD3+) production in OP9-DL4 supporting stroma. Note that the circulating CD34+ cells from patients with FA shows multilineage potential while the healthy counterpart shows preferential T-cell potential.

D) FA circulating CD34+ cells have higher MYC levels in comparison to FA CD34+ cells residing in the BM and, in comparison to healthy CD34+ cells. MYC expression in CD34+ cells was analyzed by flow cytometry. *n*= 8 FA samples and 2 healthy samples.

E) Circulating CD34+ cells and BM CD34+ cells from FA patients express reduced levels of CXCR4, which may at least partially explain their presence in peripheral blood. *n*= 6 FA samples and 2 healthy samples.

F) pI:pC treatment increases the frequency of LSK and LSK- progenitor cells trafficking in the peripheral blood of *Fancd2-/-* mice. WT or Fancd2-/- mice were treated with pI:pC for two weeks and peripheral blood was analyzed. Quantitation of LSK- cells by flow cytometry is shown in left panel. Quantitation of LSK cells is shown in supplementary Figure 7E. CFU assay of peripheral blood showing the representative images of the hematopoietic colonies is shown in the right panel.

G) Working model: MYC has a pro-survival role for the HPSC pool of patients with FA, driving the cells through S phase at the expense of DNA damage. Inflammatory episodes might hyperactivate the MYC pathway and precipitate BM failure by increasing replicative stress and reducing adhesion of the HSPCs to their niche. In the FA context, MYC must be a counteracting force against the previously described growth suppressive activities of p53 and TGFβ pathways.

The **Supplemental Table 3** describes the characteristics of the FA patients whose bone marrow or peripheral blood samples were used in the experiments described in panels B-E.

p-values of 0.01 to 0.05 were considered significant (*), p-values of 0.001 to 0.01 were considered very significant (**) and p-values of <0.001 were considered extremely significant (***, ****). See also Supplementary Figure 7.

METHODS

Enrichment of human HSPCs

Whole BM and peripheral blood samples were obtained from patients with FA after informed consent of sample use for research. Fresh healthy BM samples were purchased from Lonza (1M-105, Lonza). Supplementary Tables 1, 2 and 3 describe characteristics of all the FA patients and normal donors whose bone marrow or blood samples were used for the analysis. Based on the DEB test or MMC sensitivity test, all the patient samples used in our study met clinical criteria for FA.

Red blood cell lysis was performed by incubating the samples with Ammonium Chloride (07800, StemCell Technologies) for 10 min on ice followed by washing with PBS. After red blood cell lysis, Lin- cells were enriched for mononuclear cells (MNCs) by negative selection using the EasySep kit (19056, StemCell Technologies), according to manufacturer's instructions. For isolation of CD34+ cells, MNCs were incubated 30 min on ice with anti-CD34+ antibody coupled to magnetic beads and FcR blocking reagent (Miltenyi). MNCs were washed with PBS and sieved with a MACS MultiStand isolation system (Miltenyi).

Capture of HSPCs and scRNAseq library preparation.

Chromium™ Single Cell 3’ Reagent Kits v2 were used following manufacturer’s instructions. GEM generation and barcoding were performed using Chromium™ Single Cell A Chip Kit (PN-120236). Enriched HSPCs were resuspended in 1X PBS with 0.04% bovine serum albumin (BSA) and 3000-5000 cells per individual were loaded into the single Cell 3’ Chip, individually captured and barcoded using the 10x Chromium™ Controller (10x Technologies). Post-GEM-RT clean-up and cDNA amplification reaction were performed using the Chromium™ Single Cell 3' Library & Gel Bead Kit v2 (PN-120237). Library construction was performed using the Chromium™ i7 Multiplex Kit (PN-120262). Sequencing was performed in a NextSeq High Output 75 Cycle Flow Cell in an Illumina platform.

Single cell RNA-seq data pre-processing and quality control

The raw base call (BCL) files were processed (demultiplexation, alignment, barcode assignment and UMI quantification) with Cell Ranger (version 2.1.1) pipelines. The reference index was built upon the GRCh38.d1.vd1 reference genome with GENCODE v25 annotation. To filter lower quality cells, we removed any cells expressing fewer than 2000 genes or with fewer than 80,000 UMIs or with greater than 4% expression originating from mitochondrial genes.

Clustering

UMI matrix was log-normalized with a scale factor of 10^4. Cells with normalized expression of CD34 higher than 0.125 were identified as $CD34^+$ cells. The latent variables, defined as the number of UMI per cell and the clinics batch of the samples,

were regressed out using a negative binomial model. For further downstream analysis, the highly variable genes were selected using FindVariableGenes (Seurat R package). The graph-based method from Seurat was used to cluster cells. PCA was selected as dimensional reduction technique to use in construction of Shared Nearest-Neighbor (SNN) graph. Differentially expressed genes were identified using FindMarkers function.

Single cell gene set enrichment score

Single cell enrichment score was run using AUCell (version 1.6.0) in R (Aibar et al., 2017) on normalized expression values and Molecular Signatures Database C2 (version 5) signatures and custom signatures.

StemNet

STEMNET (Velten et al., 2017) was used to reconstruct the differentiation continuum, in particular, a gradient commitment towards the B, MD, ME/EBM and Neutrophil lineages.

Clonogenic assays with human BM samples

Clonogenic potential of human HSPCs was assessed in colony-forming unit (CFU) assays, 3000 (for healthy samples) or 5000 (for FA samples) HSPCs were plated per triplicate in human methylcellulose MethoCult H4434 Classic (04434, StemCell Technologies). The BET bromodomain inhibitor (+)-JQ1 was added to a final concentration of 50 nM and hematopoietic colonies were scored after 14 days of culture at 37°C and 5% CO_2. Pictures were taken with the STEMvision System (StemCell Technologies).

Flow cytometric analysis of human bone marrow and blood samples

BM and peripheral blood MNCs were blocked with fetal bovine serum (FBS) for 15 min and incubated with anti-CD34-FITC antibody (Biolegend) and anti-CXCR4-PE-Cy7 antibody (Biolegend) for 30 min at room temperature. After antibody incubation cells were thoroughly resuspended and fixed with CytoFix/CytoPerm (BD) buffer during 30 min on ice, washed with PermWash buffer (BD) and permeabilized for 30 on ice with Transcription Factor Fix/Perm buffer (BD). After permeabilization cells were incubated at room temperature for 40 min with primary antibodies goat-anti-human-p53 (R&D) and rabbit-anti-human-MYC (Cell Signaling), washed with Transcription Factor Perm/Wash buffer (BD) and incubated for additional 40 min with secondary antibodies Donkey anti-goat-PE (Thermo-Fisher) and Donkey anti-rabbit-Brilliant Violet 421 (Biolegend). DNA was stained with DRAQ5 (Thermo-Fisher). Samples were acquired in a LSR Fortessa Analyzer (BD) and data analysis was performed in FlowJo software version 10.5.3.

Mice

Fancd2$^{-/-}$ mice in C57BL/6J background are previously described (Parmar et al., 2010). pI:pC injections were performed intraperitoneally at a concentration of 5 mg/kg twice per week for one month. (+)-JQ1 or the inactive enantiomer (-)-JQ1 were injected intraperitoneally at a 50 mg/kg concentration daily for one month. All experimental procedures were approved by the Animal Care and Use Committee of the Dana Farber Cancer Institute.

Isolation of mouse HSPCs

BM cells were harvested from tibia and femurs of mice by gentle flushing with HBSS++ buffer [Hanks balanced salt solution (10-547F, Lonza) + HEPES (BP299-100, Fisher Scientific) + Fetal bovine serum (F2442, Sigma) + penicillin-streptomycin (15140-122, GIBCO)]. Samples were filtered through a 70 μM filter and Lin- enrichment was performed using the lineage cell depletion kit (130-090-858, Miltenyi). LT-HSCs were recognized by being LSK (Lin-Sca-1+c-Kit+) and CD150+CD48- using PE-Cy7-Sca (Clone D7, 558162, BD Biosciences), APC-c-kit (Clone ACK2, 135108, BD Biosciences), Pacific Blue-CD150 (Clone TC15-12F12.2, 115924, Biolegend) and APC-Cy7-CD48 (Clone HM48-1, 47-0481-82, e-Bioscience).

Clonogenic assays with mouse BM cells

For clonogenic assays, Lin- cells were plated in Methocult GF M3434 methylcellulose (03444, Stem Cell Technologies) and cultured. The BET bromodomain inhibitor (+)-JQ1 was added to a final concentration of 50 nM and hematopoietic colonies were scored after 7 days of culture at 37 ^{o}C and 5% CO_2. Pictures were taken with the STEMvision System (StemCell Technologies).

Cytokines quantification

Plasma was collected from human whole BM blood or from mouse peripheral blood and subjected to a bead-based Multiplex Immunoassay for detection of human and mouse pro-inflammatory cytokines using the discovery assay of Eve Technologies (Eve

Technologies Corporation, Canada). The bead analyzer Bio-Plex 200 (BIORAD) was used for detection and results were quantified according to a standard curve.

Lentiviral transduction of CD34+ cells

Isolated human CD34+ cells were cultured in StemSpan SFEMII (09655, Stem Cell Technologies,) with 100ng/ml of recombinant human cytokines SCF (300-07, Peprotech), TPO (300-18, Peprotech), Flt3 (300-19, Peprotech) and IL-6 (200-06, Peprotech) at a density of 1-2 million cells/ml in non-tissue culture (non-TC) treated plates for 36 hours. After initial culture, cells were plated in non-TC treated 96 well plates with a density of 1-2x10^5 cells in 100-150 μl of new media with 8 μg/ml polybrene (TR-1003-G, Sigma) and shRNA producing lentivirus targeting human *FANCD2*. A MOI of 50 was used for the scrambled shRNA and a MOI of 100 was used for the *FANCD2* shRNA. Plates were spun down at 2300 rpm for 30 minutes at RT after addition of the viral prep. New media was added after 12-16 hours of incubation. Selection media with 1ug/ml puromycin (MIR 5940, MirusBio) was added to cultures 12-24 hours after viral infection. Puromycin selection was applied for 72 hours. Puromycin resistant cells were used for assays with FA-like CD34+ cells.

Gene targeting in $CD34^+$ umbilical cord blood (UCB) cells

Single donor $CD34^+$ UCB cells were purchased from commercial suppliers (Stemcell Technologies and Cincinnati Children’s Hospital Translation Core Laboratories). Unique $CD34^+$ UCB samples were used for each experimental replicate. Unless otherwise

indicated, cells were cultured in 5% CO_2 and 5% O_2 at 37°C at a cell density of ≤ $3x10^5$ cells/ml in StemSpan SFEM II (Stemcell Technologies) supplemented with 200mM L-glutamine, $1x10^3$ U/ml penicillin, 10mg/ml streptomycin, 25µg/ml amphotericin B, 10 µg/ml human LDL (Stemcell technologies), 35nM UM171 (Selleckchem) and 100ng/ml each human recombinant FLT3L, SCF, IL-6, TPO (Peprotech and Stemcell Technologies). For inflammatory cytokine cultures, targeted cells were plated in StemSpan SFEMII supplemented with 20ng/ml human recombinant TNFα, IFNγ or MCP1 (Peprotech) and 50nM (+)-JQ1 (ApexBio). Cells were plated at a density of ~$1x10^6$ cells/ml into round bottom 96 well plates (200µl/well), cultured for 24 hours at 37°C in 5% CO_2, washed twice in PBS, then fixed and permeabilized prior to staining. For colony forming assays, cells were plated in duplicate or triplicate into methocult H4434 classic (Stemcell Technologies).

The introduction of indels into $CD34^+$ UCB cells using Crispr/Cas9 was performed as described by (Bak et al., 2018). Specifically, chemically modified short guide RNAs (sg RNA) were purchased from Synthego (**Supplementary Table 4**) and complexed with Alt-R *S. pyogenes* Cas9 nuclease V3 (IDT, catalog 1081059) at a molar ratio of 1:2.5. Ribonucleoprotein complexes were introduced into cells using an Amaxa 4D nucleofector (program DZ100) and P3 primary cell solution (Lonza). Mock controls were nucleofected without RNPs. To evaluate targeting efficiency, genomic DNA was harvested from mock and targeted cells and the targeted region was amplified with Platinum SuperFi or Platinum SuperFi II DNA polymerase (Invitrogen) using the primers listed in **Supplementary Table 5**. Reactions were column purified (PureLink PCR purification kit, Thermofisher) or treated with Exo-SAP-IT express (Applied Biosystems) and then

sequenced using the primers in Table 5. Indel analysis was performed on sequenced DNA using the Synthego ICE Analysis Tool (https://ice.synthego.com/#/). Targeting efficiency data are presented in **Supplementary Table 6**.

Quantitative real-time PCR

RNA was extracted from human Lin- cells using the micro-RNA extraction kit (74034, QIAGEN) and from lymphoblast cell lines using the Mini-RNA extraction kit (74134, QIAGEN) following the manufacturer's instructions. cDNA was synthesized using the RT^2 PreAMP cDNA synthesis kit (33045, QIAGEN), target sequences were amplified using the RT^2 PreAMP Pathway primer Mix (PBM-029Z, 30241, QIAGEN) and detected using the qPCR Master Mix RT^2 SYBR® Green (330533, QIAGEN). Multiplex real-time PCR was performed with the MYC targets PCR array (QIAGEN) following manufacturer's instructions. PCR was performed in a QuantStudio 7 Flex real Time machine (Life Technologies).

Survival assays

For survival assays, EUFA316+EV and EUFA316+G cells were plated at a density of $1x10^3$ cells per well in 96-well plates and exposed to increasing doses of mitomycin C (MMC) and (+)-JQ1 (50 nM). Cell viability was assessed after 5 days of culture using the Cell Titer-Glo® Luminescent Cell Viability Assay (G7573, Promega).

Western blotting

Whole cell lysates were prepared using RIPA cell lysis buffer (9803, Cell Signaling) and 1 mM PMSF (8553s, Cell Signaling) and western blots were performed using the following antibodies MYC (Cell Signaling), pKAP1 (S824), KAP1, pCHK1 (S345), CHK1, pRPA32 (S33), RPA32, γH2AX and Vinculin (sc-25336, Santa Cruz).

Alkaline comet assay

Sorted LT-HSCs were mixed with low-melting-temperature agarose, plated on slides and incubated at 4°C overnight in lysis solution using the CometAssay kit (4250-050-K, Trevigen). Next day cells were subject to a current voltage of 12 V washed and stained with SYBR-Green dye (S7567, Invitrogen). Pictures were taken with a Zeiss Imager Z1 fluorescence microscope and analysis was performed using the OpenComet plugin in the software Image J.

Cell cultures

MYC induction in vitro. 60,000 BM CD34+ cells were seeded in alfa-MEM medium (Gibco) without FBS and exposed for 24 h to 50 nM (+)-JQ1 in combination with 20 ng/mL of human-TNFα, IFNγ or MCP-1 (Peprotech). After the incubation time, cells were stained with anti-CD34-FITC, fixed and permeabilized, and MYC was detected using a rabbit anti-MYC primary antibody and a Donkey anti-rabbit-Brilliant Violet or Donkey anti-rabbit-PE secondary antibody.

Differentiation assays. 60,000 cells derived from the CD34+ enriched fraction were seeded in 200 μl of alfa-MEM medium with 10% FBS and specific cytokines. For myeloid differentiation cells were seeded in 96-well round bottom plates supplemented with SCF,

GM-CSF, IL-6, IL-3 and FLT3-l (Peprotech). For B/NK lymphoid differentiation cells were seeded in 96-well flat bottom plates in co-culture with the mouse derived MS-5 stromal cell line and the medium was supplemented with FLT-3, SCF, IL-7 and IL-15 (Peprotech). For T lymphoid differentiation cells were seeded in 96-well flat bottom plates in co-culture with the mouse derived OP9-DL4 stromal cell line expressing the Delta-like 4 Notch ligand and supplemented with cytokines. Cells were maintained at 37ºC and 5% CO_2 until harvest. Myeloid cultures were harvested after 7 and 14 days of culture and cell differentiation status was analyzed with the following antibody mixture: CD34-APC, CD19-PE, CD3-PE, CD11b-PE, CD14-PE, CD56-PE and GlyA-PE. NK/B lymphoid cultures were harvested after 14 and 28 days of culture and cell differentiation status was analyzed with the following antibody mixture: CD34-APC, CD19-FITC and CD56-PE. T lymphoid cultures were harvested after 28 days of culture and cell differentiation status was analyzed with the following antibody mixture: CD34-APC and CD3-FITC.

Clinical information of the patients

Information from patients with FA was obtained from the clinical records of the Boston Children's Hospital and Instituto Nacional de Pediatría, Mexico. Absolute neutrophil count (ANC), Platelet count (PC), and Hemoglobin (Hb) levels were used for classifying the patients as having mild aplastic anemia if ANC <1,500/mm^3, PC 150,000-50,000/mm^3 and Hb≥8 g/dL; moderate aplastic anemia if ANC <1,000/mm^3, PC <50,000/mm^3 and Hb<8 g/dL; and severe aplastic anemia if ANC <500/mm^3, PC <30,000/mm3 and Hb<8 g/dL.

DNA fiber assay

DNA fiber assay was performed using the FiberComb machine (Genomic Vision). Cells were cultured in presence of IdU (Sigma I7125), CldU (Sigma C6891) and (+)-JQ1 according to the times indicated in the figures (Lim et al., 2018, Kais et al., 2016).

After treatment, cells were embedded in low melting point agarose plugs and incubated with proteinase K (Fisher E0491) overnight. Next day plugs were washed and digested with Agarase (BioLab M0392L). Agarase-treated samples were combed onto silanized coverslips in a FiberComb well. Slides were incubated with rat anti-BrdU antibody (clone BU1/75 (ICR1) specific to CldU, Abcam ab6326) and mouse anti-BrdU Antibody (specific to IdU, BD Biosciences 347580). Pictures were taken in a fluorescence microscope with at least 100 fibers per condition. DNA fibers were measured with ImageJ.

Study approval

Experimental procedures were approved by the Animal Care and Use Committee of the Dana Farber Cancer Institute. Use of patients' samples was approved by the ethics and research committee from Dana Farber Cancer Institute, Boston Children's Hospital and Instituto Nacional de Pediatría, Mexico. Written informed consent was received from participants prior to inclusion in the study.

Statistics

Normality was assessed using the D'Agostino-Pearson, Shapiro-Wilk and Kolmogorov Smirnoff normality tests. 2-way ANOVA and Tukey's multiple comparisons test were used for detection of differences between experimental groups. Two-tailed P values for

statistical analysis were obtained using Student's *t*-test. Graphpad Prism 8 was used for all statistical analysis.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Single cell level gene expression profile of FA HSPCs reflects the sub-population distribution and their differentiation commitment.

A) t-SNE plots showing the gene expression patterns of markers used for identification of hematopoietic subpopulations in our study. These markers have previously been described and validated (Velten et al., 2017, Zheng et al., 2018; Setty et al., 2019)

B) t-SNE plots showing the gene expression of the early hematopoietic markers for cell differentiation used for the reconstruction of lineage trajectory (according to Velten et al., 2017).

C) Projection of CD38-expressing HSPCs in the lineage trajectory analysis showing how the expression of CD38, a marker of progenitor commitment, is gradually acquired as the cells move apart from the HSC state (located in the center).

D) Pie charts showing the distribution of the hematopoietic cell sub-populations (clusters) per individual. Cells sub-populations are identified by color. One FA sample is not shown as an individual pie chart due to low capture of CD34 expressing cells. UID: Unidentified pathological variant.

E) Quantitation of CD34 expressing cells in the FA patients and healthy donors as determined by gene expression analysis in single cell RNA sequencing (left panel). The

CD34 expressing cell content was plotted according to the age of the individuals (right panel).

F) Quantitation of HSCs in the FA patients and healthy donors as determined by gene expression analysis in single cell RNA sequencing (left panel). The HSC content was plotted according to the age of the individuals (right panel).

G) Quantitation of CD34 expressing cells in the FA patients in relation to their aplastic anemia status as determined by gene expression analysis in single cell RNA sequencing.

Supplementary Figure 2. scRNAseq uncovers at single cell resolution an altered gene expression profile in FA HSPCs

A) Single cell resolution heatmap showing the top 100 DEG in FA HSPCs with respect to healthy HSPCs. FA cells are indicated in red and healthy cells are indicated in blue. Color-coded legend indicates the samples of origin.

B) Real time PCR showing expression of *MYCN*, the *MYC* paralog, and *MAX,* the dimerization partner of MYC and MYCN, with respect to healthy controls. *MAX* and *MYCN* are overexpressed in some FA samples that do not overexpress *MYC*. Note that a bulk population of cells was used for this analysis. **Supplementary Table 2** describes the characteristics of the FA patients whose bone marrow samples were used in this study.

C) *MYC* expression per donor at single cell resolution. Patients with FA are arranged according to increasing age. Note that the data are from single cell RNAseq analysis of *MYC* expression.

D) *MYC* expression per donor according to the degree of aplastic anemia in FA patients. Note that the *MYC* expression was determined by quantitative real time PCR in a bulk

population of cells. The red dots indicate that those samples were also used for single cell RNA sequencing. **Supplemental Table 2** describes the characteristics of the FA patients whose bone marrow samples were used in this study.

E) Single cell enrichment score showing that the p53 pathway is upregulated in FA HSPCs across the different hematopoietic clusters, as previously reported (Ceccaldi et al., 2012).

F) Single cell enrichment score showing that the TGFβ pathway is upregulated in FA HSPCs across the different hematopoietic clusters, as previously reported (Zhang et al., 2016).

Supplementary Figure 3. The TP53 pathway is also overexpressed in FA BM HSPCs

A) Real-time PCR in bulk primary HSPCs from patients with FA showing overexpression of *TP53* in comparison to the HSPCs from healthy controls. n= 9 FA samples and n=3 healthy controls

B) High *TP53* expressing HSPCs and high *MYC* expressing HSPCs exist in every HSPC cluster of patients with FA.

C) Projection of the FA High-*TP53* and FA High-*MYC* expressing HSPCs in the lineage trajectory analysis.

D) Interindividual comparison of the "High-*TP53"*-"High-*MYC"* expression gradient. Note that most patients with FA in this scRNAseq study have an HSPC compartment with "High-*TP53"* expressing cells co-existing with "High-*MYC"* expressing cells. These results suggest that two opposing cellular states in the HSPCs compartment might also render two main states for the HSPC compartment.

Supplementary Figure 4. The BET bromodomain inhibitor (+)-JQ1 reduces *MYC* expression and reduces the clonogenic capacity of FA HSPCs.

A) “High-*Trp53”* expressing HSPCs and “High-*Myc”* expressing HSPCs co-exist in the BM of FA mice. Single cell RNA sequencing was performed using Lin- cells from Wild-type mice or *Fancd2-/-* mice. The LogRatio of *Trp53* expression was divided by the LogRatio of *Myc* expression per cell, resulting in a gradient spanning from “High-*Trp53”* expressing HSPCs to “High-*Myc”* expressing HSPCs. The expression of *Myc* and *Trp53* per cell is shown**.**

B) Flow cytometry plots (left panel) and quantitation (right panel) of the cell cycle status of LT-HSCs in WT and *Fancd2-/-* mice one month after treatment with (+)-JQ1 (50 mg/kg). *In vivo* exposure to (+)-JQ1 increases the amount of BM LT-HSC in G0 cell cycle phase (quiescence) in both WT and in *Fancd2-/-* mice as measured by absence of Ki67 marker and haploid DNA content measured by DAPI.

C) Peripheral blood analysis of WT and in *Fancd2-/-* mice one month after *in vivo* injection of (+)-JQ1 (50 mg/kg).

D) (+)-JQ1 *in vitro* treatment (50 nM) reduces the proliferation capacity of LSK cells from *Fancd2-/-* mice as assessed in a CFSE dilution assay. LSK cells were stained with CFSE and set in culture, after 7 days of culture the fluorescence intensity of CFSE was measured as an *in vitro* surrogate marker for cell division. Flow cytometry plots (left panel) and quantitation (right panel) of the CFSE staining are shown. Higher CFSE fluorescence intensity indicates less cell division capacity. Division of WT cells was not affected by exposure to (+)-JQ1 since they have a reduction in CFSE fluorescence intensity similar

to untreated WT cells, in contrast, *Fancd2-/-* cells have reduced proliferative capacity in comparison to WT cells, which is exacerbated by exposure to (+)-JQ1, which increased the cells with high CFSE content and therefore high CFSE fluorescence (*Right panel*).

E) Increased MYC expression (left panel) and p53 expression (right panel) in primary FANCG-KO compared to the Mock control cord blood CD34+ cells. Human cord blood CD34+ cells with CRISPR-mediated knockout of FANCG (FANCG-KO) were analyzed for MYC or p53 expression by flow cytometry.

F) Flow cytometry was used to confirm the co-existence of MYC and p53 expressing *FANCG*-KO cord blood CD34+ cells. FACS plot for MYC and p53 expression is shown. Two seemingly exclusive populations of *FANCG*-KO CD34+ cells were detected, one with high MYC levels (shaded green box) and the other with high p53 levels (shaded pink box) in comparison to Mock control cells. A third population (shaded in orange box) in *FANCG*-KO cells co-expresses medium-high levels of MYC and p53 in comparison to control cells.

G) CFU-C content of the cord blood CD34+ cells with CRISPR knockout of *FANCG*, *MYC* or *FANCG* and *MYC*.

H) Representative photomicrograph of the colony assay showing that FA cells are hypersensitive to (+)-JQ1 (50 nM), in comparison to a healthy control sample. BM samples from patients with FA and healthy donors were enriched in primary progenitor cells through Lin- selection and cultured in triplicates in complete methylcellulose with or without (+)-JQ1 (50 nM) for 14 days and clonogenic growth was assessed.

Supplementary Figure 5. MYC inhibition prevents entry into S phase and reduces replication associated stress in FA cells.

A) Quantitative real time PCR showing the mean fold difference in expression of genes composing the MYC pathway in the *FANCG* deficient EUFA316+EV cell line in comparison to the corrected EUFA316+G cell line. The FA cells show upregulation of the MYC pathway core components, including *MYC, MAX, MAT2A* and *MYCN*.

B) Quantitative real time PCR showing that *in vitro* treatment of the EUFA316+EV (*FANCG* deficient) cells with the BET bromodomain inhibitor (+)-JQ1 (50 nM) for 48h inhibits the expression of *MYC* transcript.

C) Western blots of the lysates from FANCG-deficient EUFA316+EV cells and corrected EUFA316+FANCG cells after *in vitro* exposure to MMC and (+)-JQ1. (+)-JQ1 was able to reduce MMC-induced phosphorylation of certain replication stress associated markers, including pCHK1(S345) and γH2AX in EUFA316+EV cells.

Supplementary Figure 6. Physiological/inflammatory stress activates the MYC pathway in *Fancd2-/-* mice.

A) *In vivo* treatment with pI:pC induces a physiological stress response that expands the LSK population at the expense of reducing the amount of LT-HSCs, leading eventually to BM failure in *Fancd2-/-* mice (Walter et al, 2015). Flow cytometry plots of bone marrow cells from WT and *Fancd2-/-* mice stained for LT-HSC and LSK markers 48 hrs after treatment with pI:pC are shown.

B) MYC expression in LT-HSCs of WT and *Fancd2-/-* mice 48 hrs after treatment with pI:pC and (+)-JQ1. (+)-JQ1reduces *in vivo* the levels of Myc in LT-HSC from *Fancd2-/-* mice in basal conditions but not after pI:pC injection in a 48h time frame. Longer times

were not possible to assess due to toxicity of the combination treatment. Fluorescence per cell is presented.

C) LSK content of WT and *Fancd2-/-* mice at 48 hrs after treatment with pI:pC and (+)-JQ1.

D) Flow cytometry plots (left panel) and quantitation (right panel) of LT-HSCs, MPPs and HPCs of WT and *Fancd2-/-* mice 48 hrs after treatment with pI:pC and (+)-JQ1. (+)-JQ1 ameliorates pI:pC-induced reduction of LT-HSCs in *Fancd2-/-* mice.

E) White blood cell counts (WBCs) in peripheral blood of WT and *Fancd2-/-* mice 48 hrs after treatment with pI:pC and (+)-JQ1. (+)-JQ1 injection prevents the pI:pC-induced reduction of WBCs.

F) (+)-JQ1 reduces *in vivo* the DNA damage in the LT-HSC compartment of *Fancd2-/-* mice. DNA damage was measured by γH2AX staining in LT-HSCs of WT or *Fancd2-/-* mice at 48 hrs after treatment with pI:pC and (+)-JQ1.

G) C-reactive protein (CRP), a protein marker for inflammation, was found to be over the values of reference in almost every sample from patients with FA, confirming that patients with FA have a pro-inflammatory condition. n= 12 FA peripheral blood samples.

Supplementary Figure 7. The circulating CD34+ derived from patients with FA exhibit *in vitro* clonogenic potential.

A) Colony assays of total peripheral blood (PB) MNCs from normal healthy donors and FA patients. MNCs were cultured in complete methylcellulose and after 14 days of culture the total progenitor content was quantified. FA MNCs isolated from PB show CFU-C

capacity which seems to be reducing with BMF progression. Inset shows examples of (*i)* a monocyte colony, (*ii)* a granulocyte colony and (*iii)* an erythroid colony.

B) LTC-IC assays of total MNCs from peripheral blood of healthy donors and FA patients. Cells were cultured in M2-10B4 stroma for 5 weeks, plated in complete methylcellulose medium, and the CFU-C clonogenic capacity was quantified after 14 days of culture.

C) The circulating CD34+ population from FA patients contains committed progenitors. FACS plots (left panel) and quantitation (right panels) of circulating FA CD34+ cells expressing CD10 or CD19 markers. CD10 and CD19 staining, in addition to CD34, was used to classify progenitor types found in circulating CD34+ cell population. Pro-B progenitors were defined as CD34+CD10+CD19+, early lymphoid progenitors were defined as CD34+CD10+ and myeloid progenitors were defined as CD34+CD10+CD19-

D) Paired BM and PB samples were analyzed in 11 out 25 patients with FA and the percentage of CD34+ cells was contrasted with healthy controls. A reduction in circulating CD34+ cells accompanies BMF progression, suggesting that egression of HSPCs is an active process and promotes HSC pool exhaustion. *Left* panel shows the percentage of CD34+ cells in BM (relative to MNCs) and *right* panel shows the percentage of CD34+ cells in PB (relative to MNCs).

E) pI:pC induces in both WT and *Fancd2-/-* mice the egress into peripheral blood of LSK progenitor cells. WT and *Fancd2-/-* mice were treated with pI:pC for two weeks and LSK cells were analyzed in the peripheral blood.

The **Supplemental Table 3** describes the characteristics of the FA patients whose bone marrow or peripheral blood samples were used in the experiments described in panels A-E.

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