Published in final edited form as:

Exp Hematol. 2012 April ; 40(4): 295–306.e5. doi:10.1016/j.exphem.2011.12.004.

Mice heterozygous for *CREB binding protein* are hypersensitive to γ -radiation and invariably develop myelodysplastic/myeloproliferative neoplasm

Stephanie N. Zimmer^{*,1,2}, Madeleine E. Lemieux^{*,3}, Bijal P. Karia^{1,2}, Claudia Day², Ting Zhou^{1,2}, Qing Zhou², Andrew L. Kung³, Uthra Suresh², Yidong Chen^{4,2}, Marsha C. Kinney⁵, Alexander J. R. Bishop^{1,2}, and Vivienne I. Rebel^{1,2,6}

¹Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio (UTHSCSA), TX

²Greehey Children's Cancer Research Institute (GCCRI), University of Texas Health Science Center at San Antonio (UTHSCSA), TX

³Department of Pediatric Oncology, Dana-Farber Cancer Institute & Harvard Medical School, Boston, MA

⁴Department of Epidemiology and Biostatistics, University of Texas Health Science Center at San Antonio, TX

⁵Department of Pathology, University of Texas Health Science Center at San Antonio, TX

Abstract

Myelodysplastic syndrome (MDS) is a complex family of pre-leukemic diseases in which hematopoietic stem cell defects lead to abnormal differentiation in one or more blood lineages. Disease progression is associated with increasing genomic instability and a large proportion of patients go on to develop acute myeloid leukemia. Primarily a disease of the elderly, it can also develop following chemotherapy. We have previously reported that *CREB binding protein* (*Crebbp*) heterozygous mice have an increased incidence of hematological malignancies, and others have shown that *CREBBP* is one of the genes altered by chromosomal translocations found in patients suffering from therapy-related MDS. This led us to investigate whether hematopoietic tumor development in *Crebbp*^{+/-} mice is preceded by a myelodysplastic phase and whether we could uncover molecular mechanisms that might contribute to its development. We report here that *Crebbp*^{+/-} mice invariably develop myelodysplastic/myeloproliferative neoplasm within 9-12 months of age. They are also hypersensitive to ionizing radiation and show a marked decrease in PARP1 activity after irradiation. In addition, protein levels of XRCC1 and APEX1, key components of base excision repair machinery, are reduced in unirradiated *Crebbp*^{+/-} cells or upon targeted knock down of *CREBBP* levels. Our results thus provide validation of a novel myelodysplastic/myeloproliferative neoplasm mouse model and, more importantly, point to

© 2011 International Society for Experimental Hematology. Published by Elsevier Inc. All rights reserved.

⁶Corresponding author: Dr. V.I. Rebel, MD, PhD; GCCRI, UTHSCSA, 8403 Floyd Curl Drive, San Antonio, TX 78229 USA., Phone: 210-562-9096; FAX: 210-562-9014; rebel@uthscsa.edu.

*These authors contributed equally to this work.

Financial Disclosure Declaration The authors declare no conflicts of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

defective repair of DNA damage as a contributing factor to the pathogenesis of this currently incurable disease.

Keywords

CREBBP; MDS/MPN; DNA repair; radiation hypersensitivity; PARP1

Introduction

Myelodysplastic syndromes (MDS) is a complex family of pre-leukemic diseases in which hematopoietic stem cell (HSC) defects lead to abnormal differentiation in one or more blood lineages. Disease progression is associated with increasing genomic instability and a large proportion of patients go on to develop acute myeloid leukemia (AML) (reviewed in [1]). Primarily a disease of the elderly, MDS/AML can also develop following treatment with alkylating agents, radiation and topoisomerase II inhibitors [2,3]. The poor outcome and increasing incidence of MDS, due to an aging population and increasing numbers of cancer survivors, motivated our efforts to better understand the pathogenesis of this disease.

Studies in marrow or blood cells from patients suffering from AML or myeloproliferative neoplasms (MPNs) suggest that inadequate DNA repair may play an important role in the etiology of these diseases. It has been shown that some of the frequently-observed genomic aberrations in these diseases [4-7] cause excessive DNA damage by increasing the production of reactive oxygen species and/or usage of alternative, error-prone DNA repair pathways. This mechanism of genomic instability, or mutator phenotype, as proposed by Loeb [8], explains why progression of many of these diseases is associated with increasing genetic abnormalities. MDS patient samples have been less extensively investigated in this context; however, increased oxidative DNA damage has been observed in blood cells from MDS patients [9,10] and DNA repair deficiencies have been demonstrated in MDS patients with a high risk of progressing towards leukemia [9,11]. Moreover, children suffering from diseases due to mutated genes essential for DNA repair, such as Fanconi's anemia [12], Bloom's disease [13,14], and Rothmund-Thomson syndrome [15,16] have an increased risk of developing MDS.

CREB binding protein (CREBBP) interacts with DNA damage response/repair proteins such as TP53 [17,18] and BRCA1 [19], among others, to enhance their function. CREBBP also helps remodel chromatin through its histone acetyltransferase activity, thereby facilitating DNA repair [20,21]. Finally, CREBBP modulates the activity of poly(ADP-ribose) polymerase-1 (PARP1), an accessory factor in transcriptional regulation and base-excision repair (BER) (reviewed in [22]). Since the amount of CREBBP is dose-limiting within the cell [23,24], a decrease in its availability is likely to impair its ability to enhance DNA repair.

We previously reported that ~40% of *Crebbp* heterozygous mice develop hematological malignancies [25] and others have shown that *CREBBP* is one of the genes involved in chromosomal translocations found in patients suffering from therapy-related MDS [26]. We now report that *Crebbp*^{+/-} mice invariably develop myelodysplastic/myeloproliferative neoplasm (MDS/MPN) within 9-12 months of age and are hypersensitive to γ -radiation. Mechanistically, we find a marked decrease in PARP1 activity upon exposure to ionizing radiation and a reduction of key BER proteins in progenitor and stem cell-enriched bone marrow (BM), suggesting deficient DNA repair as a contributing factor to their disease.

Material and Methods

Mice

Crebbp^{+/-} mice [25] were fully backcrossed onto a C57BL/6 background. Wild-type (WT) littermates served as controls. Mice were bred and maintained under micro-isolator conditions at the animal facility of UTHSCSA. All animal procedures were in accordance with University policies regarding animal care and use.

Total body irradiation (TBI) and survival analysis

WT and *Crebbp*^{+/-} mice (3-6 month-old) received a total dose of 10 or 11 Gy (90-100 cGy/minute from a Co⁶⁰ source (Theratron T-780 unit, Atomic Energy of Canada Limited), delivered as two equal doses of 5 or 5.5 Gy, respectively, 5 hours apart. Kaplan-Meier curves and log rank survival statistics were generated using the R-project *survival* package [27,28].

Blood analysis, histology, flow cytometry and *in vitro* methylcellulose assays

Standard techniques were used. See Supplementary Materials and Methods for details.

shRNA knock-down of CREBBP in EML1 cells

A lentivirus-encoded shRNA targeting the sequence 5'-CAAGCACTGGGAATTCTCT-3' from mouse *Crebbp* was created by cloning oligonucleotides into the FSIPPW vector as previously described [29]. A lentivirus targeting EGFP (5'-AAGAACGGCATCAAGGTGAAGT-3') was used as a control. Both were packaged as previously described [30]. Co-transfection of 293TD cells was performed using lipofectamine 2000 as per the manufacturer's instructions (Invitrogen).

EML1 cells (CRL-11691; ATCC) [31], were cultured in IMDM medium supplemented with 20% FBS (StemCell Technologies) and rmSCF (100ng/ml) (R&D Systems) and were never carried for more than three months. Undifferentiated EML1 cells were split one day prior to infection. Virus-containing supernatant supplemented with 8μg/ml protamine was added to the cells and left until a complete medium change the next morning. At the end of day 2, another round of infection was performed using a flow-through infection protocol as previously described [32]. On day 3, infected cells were selected in puromycin (3μg/ml). EML1 cells were cloned in methylcellulose-based medium (M3234, StemCell Technologies) and expanded in liquid medium.

Gene expression and network analysis

Total RNA was isolated in two independent experiments from HSCs sorted from WT and *Crebbp*^{+/-} day 14.5 fetal livers as described in the Supplementary Material and Methods. For each, 12ng were amplified, in duplicate, using the Ovation RNA amplification kit (NuGen Technologies, Inc.) and hybridized to Affymetrix Gene Chip Mouse Genome 430 2.0. Data files are MIAME-compliant and available from the Gene Expression Omnibus (accession GSE18061). Arrays were normalized, corrected for background and analyzed using R and the Bioconductor *gcrma* package [27,33]. After averaging technical replicates, litter-paired T-tests with p-values <0.05 and a fold-change >1.5 were used as the cut-off for calling significant change. Quantitative RT-PCR (qRT) for two genes on 3 independent samples were consistent with the microarray results (*Klf6* average±SD: on microarray = 1.8±0.0, by qRT = 2.8±1.4; *Tcf4* average±SD on microarray = 1.1±0.03, by qRT = 1.1±0.09). To generate protein interaction networks (PINs), murine genes were mapped via the NCBI HomoloGene database (May 2009) to their human homologs (Table S1). The human proteins were used to retrieve direct binding partners from the human interactome

[34] where both binding partners were called “present” by Affymetrix MAS5 (Bioconductor *affy* package implementation [35]) in at least one sample, resulting in a reference “HSC PIN” of 4237 proteins and 14704 interactions. Similarly, the 93 distinct genes we found significantly altered in *Crebbp*^{+/-} HSCs relative to WT corresponded to 39 human homologs that were represented in the HSC PIN. Together, these 39 proteins and their direct interactors comprise the *Crebbp*-target PIN of 258 proteins and 257 interactions. Cytoscape [36] was used to visualize the resulting networks and its BinGO plugin [37] to determine Gene Ontology (GO) annotation enrichment. We used our HSC PIN as the background for enrichment with a p-value <0.01 cut-off.

Protein extracts

PB cells were obtained from >3 mice and pooled. Leukocytes were irradiated with 6 Gy (1 Gy/minute) using a Gammacell 40 Cesium Unit (Atomic Energy of Canada Limited). Post-irradiation cells were either put on ice directly or incubated for various times at 37°C to allow DNA repair to occur. Cells were lysed in RIPA buffer for CREBBP westerns or in NaCl lysis buffer (0.1M NaCl, 50mM Tris-HCl, pH7.2, 1mM DTT) containing phosphatase and protease inhibitors in other cases. After lysis, CREBBP and PARP1 samples were centrifuged at 14,000 rpm for 10 minutes at 4°C and supernatant protein concentrations determined by BCA protein assays (Pierce). For BER protein westerns, lysates were passed five times through a QIAshredder homogenizer (Qiagen) then centrifuged at 16,000 rcf for 10 minutes at 4°C and the supernatants concentrated for 40 minutes at 14,800 rcf in Amicon Ultra-0.5 filter devices (Millipore). After concentration, the protein lysates were diluted 1:1 with a 10mM Tris-HCl, pH7.5, 1mM EDTA, 1mM DTT, 20% glycerol solution and protein concentrations determined by Bradford assays (Sigma).

Western blots

Equal amounts of protein extract were separated on 12% Bis-Tris gels and transferred to nitrocellulose membranes (Invitrogen). Primary antibodies used: CREBBP (AC26 [38]), XRCC1 (SantaCruz) and from Abcam: ACTB, LIG1, POLB, APEX1, TRP53, phospho-TRP53 (Ser15), PARP1. They were visualized with horseradish peroxidase-coupled secondary antibodies (Cell Signaling) and ECL plus solution (Amersham) and quantified with densitometry using Image J software (NIH).

PARP1 activity assay

PARP1 activity in protein extracts from PB and BM (200ng/25μl), BM or EML1 cells (400ng/25μl) was measured using the HT Colorimetric PARP1/Apoptosis Assay Kit (Trevigen) following manufacturer’s instructions. The absorbance of the colorimetric substrate was read at 450nm on a Spectramax M5 spectrophotometer (Molecular Devices).

Statistical analysis

Unless otherwise indicated, Excel was used to perform T-tests. The R *stats* package (27) was used for the paired time-series T-tests of Fig. 6A and Kolmogorov-Smirnov distribution tests of Fig. 6E. In all cases, p-values < 0.05 were considered statistically significant.

Results

Myelodysplastic features of *Crebbp*^{+/-} mice

To determine whether *Crebbp*^{+/-} mice might harbor previously undetected MDS, we compared the hematopoietic system of 3-4 and 9-12 month-old *Crebbp*^{+/-} mice with that of age-matched WT controls. The number of cells harvested from two femurs was similar for both age groups and genotypes (Fig. 1A) but *Crebbp*^{+/-} mice were significantly smaller by

weight than their WT counterpart (Fig. 1B). When BM cellularity was corrected for weight, we found that the marrow of *Crebbp*^{+/-} mice was significantly more cellular than WT (Fig. 1C). At both ages, a mild but significant splenomegaly was also observed in *Crebbp*^{+/-} mice (Fig. 1D).

This marrow hypercellularity was not accompanied by an increase in the number of colony-forming cells (CFCs). On the contrary, 9-12 month-old *Crebbp*^{+/-} mice had significantly fewer CFCs in their marrow than WT mice, most notably granulocytic and monocytic CFCs (Fig. 1E). No significant differences were detectable between young *Crebbp*^{+/-} and WT mice (data not shown). A decrease in the numbers of myeloid CFCs in the context of an overall increase in BM cellularity and splenomegaly is indicative of abnormal myeloid differentiation.

Histological examination of blood smears and BM preparations revealed distinct dysplastic features [39,40] of *Crebbp*^{+/-} blood cell differentiation (Fig. 2), including hypersegmented granulocytes (Fig. 2B; 55% of 9-12 month-old mice) and leukocytes with a pseudo Pelger-Huët anomaly (Fig. 2C; 22%). *Crebbp*^{+/-} BM preparations confirmed the hypercellularity and showed an increased myeloid to erythroid ratio, mostly due to an excess of mature granulocytes (Fig. 2E). More than half of the 9-12 month-old *Crebbp*^{+/-} mice exhibited either increased numbers of megakaryocytes or abnormal forms such as hyperlobulated cells (Fig. 2F) or naked nuclei (Fig. 2G).

Unlike older mice, 3-4 month-old *Crebbp*^{+/-} mice displayed none of these characteristics; interestingly, however, 2 of 14 young animals examined had small clusters of immature cells in the center of the marrow space (Fig. 2H and inset). This atypical localization of immature precursors is indicative of very early stages of myelodysplastic hematopoiesis and possibly the onset of leukemia. Another common finding in MDS, particularly in its early stages, is an increase in apoptosis in marrow progenitors (reviewed in [1]). Consistent with this, Fig. 2I shows a significant increase in Annexin V⁺ cells in the lineage-depleted (Lin⁻) fraction of marrow enriched for stem- and progenitor cells but not in whole BM (WBM).

Altered numbers of HSCs and myeloid progenitors in *Crebbp*^{+/-} mice

FACS analysis at 3-4 months of age showed that the only significant, albeit small difference in hematopoietic cell populations between *Crebbp*^{+/-} and WT mice was an increased proportion of Gr-1^{lo}Mac-1⁺⁺ myeloid cells in the BM ($15.3 \pm 2.4\%$ versus $12.7 \pm 1.3\%$; $p=0.015$). By 9-12 months of age, however, the frequency of LT-HSCs was significantly lower in *Crebbp*^{+/-} BM compared to control (Fig. 3A), resulting in ~2-fold fewer LT-HSCs per femur (2300 ± 1100 vs 4400 ± 1900 , respectively; $p=0.007$). These mice furthermore showed a decrease in common myeloid progenitors (CMPs) and an expansion of granulocyte/macrophage progenitors (GMPs) (Fig. 3B). No differences between *Crebbp*^{+/-} and WT mice were found with respect to megakaryocyte/erythroid progenitors (MEPs) (Fig. 3B) or common lymphoid progenitors (CLPs) (Fig. 3C).

Because primitive BM progenitors represent only a very small proportion of the total BM content, the expansion of GMPs alone cannot explain the greater marrow cellularity observed in the older *Crebbp*^{+/-} mice relative to controls. Moreover, histological analysis suggested that the difference was due to an increase in mature myeloid cells in the marrow (Fig. 2E). Indeed, relative to controls, *Crebbp*^{+/-} marrow contained significantly more Gr-1^{lo}Mac-1⁺⁺ and Gr-1⁺⁺Mac-1⁺⁺ myeloid cells (Fig. 4A). Concurrent PB cell analysis of 9-12 month-old mice by FACS (Fig. 4B) and CBC (Fig. 4C) showed a significant increase in granulocytes while the lymphoid cell compartment contracted. In contrast, total leukocyte, erythrocyte and platelet numbers measured by CBC were similar to age-matched

controls (data not shown). By one year of age, the relative number of myeloid cells had therefore significantly increased in *Crebbp*^{+/-} mice at the expense of lymphoid cells.

Taken together, the hematopoietic characteristics of 9-12 month-old *Crebbp*^{+/-} mice are clearly different from those found in *Crebbp*^{+/-} mice suffering from myeloid leukemia as described previously [25] and shown in Supplementary Fig. S1. These features are reminiscent of human MDS, most consistent with MDS/MPN according to the current WHO classification [41].

Changes in apoptosis and DNA damage repair pathways predicted by expression profiling of *Crebbp*^{+/-} HSCs

As MDS is considered a stem cell disease, we compared gene expression profiles of *Crebbp*^{+/-} and WT HSCs in the hope of uncovering molecular mechanisms that might be at its root. We chose to isolate HSCs from fetal livers because at this stage there were no overt differences between *Crebbp*^{+/-} and WT hematopoiesis (data not shown). Any differences in gene expression found in *Crebbp*^{+/-} fetal liver HSCs would thus more likely reflect the initially altered genetic program of HSC regulation as opposed to adaptation to a compromised or failing hematopoietic system.

GO annotation analysis of the protein-coding genes differentially expressed in *Crebbp*^{+/-} HSCs (Table S1) showed little enrichment in any particular pathway or process. However, it has been shown that PINs can be used to predict loss-of-function phenotypes through so-called “guilt by association” [42]. Since there is better coverage of the human than of the murine interactome, we generated a reference PIN consisting of human proteins corresponding to all genes expressed in fetal liver HSCs (HSC PIN) and assumed that key hematopoietic pathways and processes are preserved in both species. We next generated a CREBBP PIN nucleated by the human homologs of genes differentially expressed in *Crebbp*^{+/-} fetal liver HSCs relative to WT (“seed proteins”, Fig. 5A larger nodes) and their co-expressed, direct binding partners (Fig. 5A smaller nodes). By comparing the HSC and CREBBP PINs, we sought to identify pathways that might be altered by “guilty” interactions between the primary CREBBP target genes and other HSC-expressed proteins.

GO term enrichment analysis of the CREBBP PIN identified two interesting pathways significantly enriched above the HSC PIN background. First, 28 proteins were annotated to the GO term “programmed cell death” (GO:0012501; black and green nodes in Fig. 5A and Table S2, $p\text{-value} = 5 \times 10^{-3}$). Of those, 20 were annotated to the more specific term “positive regulation of apoptosis” (GO:0043065). Second, 25 proteins were annotated to the GO term “response to DNA damage stimulus” (GO:0006974; blue and green nodes in Fig. 5A, Table S2, $p\text{-value} = 9 \times 10^{-3}$), 20 of which corresponded to the more specific term “DNA repair” (GO:0006281).

As a control, we randomly selected 10,000 sets of 39 proteins normally expressed in HSCs, retrieved their interaction partners as before and determined the proportion of cases in which the overlap was greater than for the CREBBP PIN as an empirical test of significance. For “programmed cell death” and “response to DNA damage stimulus”, the $p\text{-values}$ were 0.033 and 0.045, respectively, supporting our contention that both these pathways might be indirectly affected in *Crebbp*^{+/-} HSCs through direct interaction with primary CREBBP targets. The involvement of apoptotic pathways is consistent with our finding, described above, of increased apoptosis in *Crebbp*^{+/-} Lin⁻ cells (Fig. 2I). Analysis of purified adult BM HSCs revealed a similar, significant increase in apoptosis in *Crebbp*^{+/-} HSCs (Fig. 5B, paired T-test; $p=0.021$).

Increased hypersensitivity of *Crebbp*^{+/-} mice to ionizing radiation

The possibility that responses to DNA damage, and particularly DNA repair functions, might be altered in *Crebbp*^{+/-} HSCs was of considerable interest since this has only been linked fairly recently to the development of chromosomal abnormalities in MDS and its progression to AML [9,11]. If the CREBBP PIN prediction of impaired DNA repair were correct, one might expect *Crebbp*^{+/-} animals to have an altered sensitivity to DNA damaging agents.

We tested this hypothesis by subjecting groups of mice to a split-dose of 11 Gy TBI. Immediately following TBI, mice received various doses of BM cells (Fig. 5C) or no cells at all (Fig. 5D). As expected, WT controls not transplanted with a supportive hematopoietic graft all died 11-13 days after irradiation. All WT animals receiving a transplant of 5.0×10^6 marrow cells survived, as did 9/11 receiving a ~30-fold smaller graft of 1.5×10^5 cells. In contrast, 5/22 of *Crebbp*^{+/-} mice receiving the high dose graft died and only 1/11 recipients of the low dose BM cells survived the first 2 weeks following TBI (Fig. 5C). To rule out the possibility that differences in survival were due to the *Crebbp*^{+/-} microenvironment having a negative impact on homing of the transplanted cells, we also compared the intrinsic sensitivity of mice to a 10 Gy split-dose TBI in the absence of supporting BM cells (Fig. 5D). In this case, all *Crebbp*^{+/-} mice died before the first WT mouse succumbed to hematopoietic failure. Moreover, ~1/3 of the WT mice survived a 10 Gy split-dose TBI.

Decreased PARP1 activity and BER protein levels in *Crebbp*^{+/-} cells

Two notable players in the detection and repair of DNA lesions present in the CREBBP PIN by association with a direct target are TP53 and PARP1 (Fig. 5A). We decided to examine both more closely. Western blot analysis of PB leukocytes obtained before and after γ -irradiation showed no clear differences in total or Ser15-phosphorylated TRP53 levels between the two genotypes (Fig. S2A), nor did we find any clear differences in full-length or cleaved PARP1 protein levels (Fig. S2B). PARP1 activity was slightly, but not significantly, lower in *Crebbp*^{+/-} cells before irradiation (Fig. 6A). However, immediately after irradiation, *Crebbp*^{+/-} leukocytes showed significantly lower levels in enzymatic activity at several time points (Fig. 6A, asterisks). Comparing responses between *Crebbp*^{+/-} and WT cells over the entire 12-hour period revealed that PARP1 activity is significantly dampened by the reduction of CREBBP levels (T-test paired by condition and time-point, p-value = 8.8×10^{-6}). We next looked in unirradiated WBM and Lin⁻ BM to see whether PARP1 activity was reduced in more primitive hematopoietic cell populations. Similar to unirradiated PB, *Crebbp*^{+/-} BM samples showed decreased PARP1 activity compared to WT but not to a statistically significant level (Fig. 6B).

PARP1 plays an important, though not enzymatic, role in BER by protecting single-strand breaks until repair can proceed. Consistent with this, cells that lack PARP1 rapidly accumulate DNA breaks and *Parp1*^{-/-} animals are extremely sensitive to γ -irradiation (reviewed in [22]). Because we had observed the same radiation hypersensitivity in *Crebbp*^{+/-} mice, and because MDS patients are thought to have deficiencies in BER [9], we wondered whether other BER proteins were affected by a reduction in CREBBP. When we measured the abundance of a several key BER proteins in *Crebbp*^{+/-} and WT Lin⁻ BM cells, we found a modest reduction in XRCC1 and DNA polymerase beta (POLB) and a significant decrease in AP endonuclease (APEX1) abundance in *Crebbp*^{+/-} Lin⁻ cells (Fig. 6C). To determine whether the changes in PARP1 activity and BER protein levels were directly related to reduced CREBBP levels, we knocked down *Crebbp* expression in the multipotential hematopoietic EML1 cell line [31]. Consistent with the *Crebbp*^{+/-} Lin⁻ BM cells, shRNA-mediated reduction of CREBBP levels in unirradiated EML1 cells resulted in a marked decrease in XRCC1 levels and more modest changes in other BER protein levels

(Fig. 6D-E), whereas an irrelevant shRNA against eGFP had no effect. Decreased availability of CREBBP thus has a negative impact on the abundance of BER proteins, which may explain the increased radiosensitivity of *Crebbp*^{+/-} mice and their predisposition to developing MDS.

Discussion

Several MDS mouse models have been described to date (reviewed in [39]), each capturing some features of this notoriously variable disease. The histopathology, hematological characteristics and increased progenitor/stem cell apoptosis of our *Crebbp*^{+/-} mice constitute a good model for human MDS/MPN as defined in the current WHO classification [41].

Our protein network analysis predicted an impaired ability to respond to and repair DNA damage in *Crebbp*^{+/-} cells. Consistent with this, our study revealed a significant hypersensitivity of *Crebbp*^{+/-} mice to high dose ionizing TBI. TP53 and PARP1 are both present in the CREBBP PIN and their functions are known to be modulated through acetylation by CREBBP/EP300 [17,18,22]. However, a recent report showed that neither CREBBP nor its paralog EP300 are required for TRP53-dependent up-regulation of *Cdkn1a* and *Mdm2* following DNA damage [43]. Consistent with this observation, we found that neither total nor Ser15-phosphorylated TRP53 protein levels were altered in PB by a reduction in CREBBP levels. Nor could we detect any significant differences in PARP1 abundance between WT and *Crebbp*^{+/-} cells, before or after irradiation. In contrast, PARP1 enzymatic activity tended to be lower in cells with reduced levels of CREBBP and was dampened in PB cells in response to radiation. An interesting question arises from this observation: does acetylation by CREBBP/EP300 of PARP1 enhance not only PARP1's transcriptional coactivation functions [22] but also its enzymatic or repair-associated activities? Further studies will be required to formally address this.

The majority of endogenous DNA damage, such as caused by reactive oxygen species, is repaired by BER. PARP1 binds and protects single strand DNA breaks and further facilitates BER by recruiting other BER proteins such as APEX1, LIG3, POLB and XRCC1 to the site of damage (reviewed in [44]). In this study we demonstrate that a reduction in CREBBP causes these BER protein levels to decline, some more than others. There is evidence to suggest that even small changes in the relative abundance of these essential BER proteins can cause aberrant BER, which can lead to increased mutagenesis and genomic instability [44]. This raises the possibility that loss of a single copy of *Crebbp* constitutes a mutator phenotype [8] predisposing *Crebbp*^{+/-} animals to MDS/AML.

In summary, we have demonstrated a previously undetected but highly penetrant MDS/MPN in *Crebbp*^{+/-} mice. These animals are furthermore radiosensitive, a common feature of human MDS [11], although not shown yet for MDS/MPN in particular. Moreover, we find significantly decreased PARP1 enzymatic activity in *Crebbp*^{+/-} blood cells and an imbalance of BER proteins. Further studies will be required to fully elucidate the molecular mechanisms at play and determine the direct impact of these CREBBP-associated changes on BER activity and genomic instability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge Charles Thomas and Karla Moncada for their help with flow cytometry and Don McEwen for help acquiring the histology images.

Support This work was supported with funding from the GCCRI/UTHSCSA (VIR), NIH/NCI (P30 CA054174-17 to YC and the institutional flow cytometry core) and NIH/NCRR (1UL1RR025767 to YC).

References

1. Corey SJ, Minden MD, Barber DL, Kantarjian H, Wang JC, Schimmer AD. Myelodysplastic syndromes: the complexity of stem-cell diseases. *Nat Rev Cancer*. 2007; 7:118–129. [PubMed: 17251918]
2. Godley LA, Larson RA. Therapy-related myeloid leukemia. *Semin Oncol*. 2008; 35:418–429. [PubMed: 18692692]
3. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood*. 2002; 100:2292–2302. [PubMed: 12239137]
4. Sallmyr A, Fan J, Datta K, et al. Internal tandem duplication of FLT3 (FLT3/ITD) induces increased ROS production, DNA damage, and misrepair: implications for poor prognosis in AML. *Blood*. 2008; 111:3173–3182. [PubMed: 18192505]
5. Koptyra M, Falinski R, Nowicki MO, et al. BCR/ABL kinase induces self-mutagenesis via reactive oxygen species to encode imatinib resistance. *Blood*. 2006; 108:319–327. [PubMed: 16527898]
6. Nowicki MO, Falinski R, Koptyra M, et al. BCR/ABL oncogenic kinase promotes unfaithful repair of the reactive oxygen species-dependent DNA double-strand breaks. *Blood*. 2004; 104:3746–3753. [PubMed: 15304390]
7. Plo I, Nakatake M, Malivert L, et al. JAK2 stimulates homologous recombination and genetic instability: potential implication in the heterogeneity of myeloproliferative disorders. *Blood*. 2008; 112:1402–1412. [PubMed: 18515659]
8. Loeb LA. A mutator phenotype in cancer. *Cancer Res*. 2001; 61:3230–3239. [PubMed: 11309271]
9. Jankowska AM, Gondek LP, Szpurka H, Nearman ZP, Tiu RV, Maciejewski JP. Base excision repair dysfunction in a subgroup of patients with myelodysplastic syndrome. *Leukemia*. 2008; 22:551–558. [PubMed: 18059482]
10. Novotna B, Bagryantseva Y, Siskova M, Neuwirtova R. Oxidative DNA damage in bone marrow cells of patients with low-risk myelodysplastic syndrome. *Leuk Res*. 2009; 33:340–343. [PubMed: 18687469]
11. Kuramoto K, Ban S, Oda K, Tanaka H, Kimura A, Suzuki G. Chromosomal instability and radiosensitivity in myelodysplastic syndrome cells. *Leukemia*. 2002; 16:2253–2258. [PubMed: 12399970]
12. Alter BP, Giri N, Savage SA, et al. Malignancies and survival patterns in the National Cancer Institute inherited bone marrow failure syndromes cohort study. *Br J Haematol*. 2010; 150:179–188. [PubMed: 20507306]
13. Iwahara Y, Ishii K, Watanabe S, Taguchi H, Hara H, Miyoshi I. Bloom's syndrome complicated by myelodysplastic syndrome and multiple neoplasia. *Intern Med*. 1993; 32:399–402. [PubMed: 8400502]
14. Poppe B, Van Limbergen H, Van Roy N, et al. Chromosomal aberrations in Bloom syndrome patients with myeloid malignancies. *Cancer Genet Cytogenet*. 2001; 128:39–42. [PubMed: 11454428]
15. Narayan S, Fleming C, Trainer AH, Craig JA. Rothmund-Thomson syndrome with myelodysplasia. *Pediatr Dermatol*. 2001; 18:210–212. [PubMed: 11438000]
16. Pianigiani E, De Aloe G, Andreassi A, Rubegni P, Fimiani M. Rothmund-Thomson syndrome (Thomson-type) and myelodysplasia. *Pediatr Dermatol*. 2001; 18:422–425. [PubMed: 11737690]
17. Gu W, Roeder RG. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell*. 1997; 90:595–606. [PubMed: 9288740]
18. Gu W, Shi XL, Roeder RG. Synergistic activation of transcription by CBP and p53. *Nature*. 1997; 387:819–823. [PubMed: 9194564]
19. Pao GM, Janknecht R, Ruffner H, Hunter T, Verma IM. CBP/p300 interact with and function as transcriptional coactivators of BRCA1. *Proc Natl Acad Sci U S A*. 2000; 97:1020–1025. [PubMed: 10655477]

20. Karagiannis TC, Harikrishnan KN, El-Osta A. Disparity of histone deacetylase inhibition on repair of radiation-induced DNA damage on euchromatin and constitutive heterochromatin compartments. *Oncogene*. 2007; 26:3963–3971. [PubMed: 17213813]
21. Masumoto H, Hawke D, Kobayashi R, Verreault A. A role for cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response. *Nature*. 2005; 436:294–298. [PubMed: 16015338]
22. Krishnakumar R, Kraus WL. The PARP side of the nucleus: molecular actions, physiological outcomes, and clinical targets. *Mol Cell*. 2010; 39:8–24. [PubMed: 20603072]
23. Horvai AE, Xu L, Korzus E, et al. Nuclear integration of JAK/STAT and Ras/AP-1 signaling by CBP and p300. *Proc Natl Acad Sci U S A*. 1997; 94:1074–1079. [PubMed: 9037008]
24. Kamei Y, Xu L, Heinzel T, et al. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell*. 1996; 85:403–414. [PubMed: 8616895]
25. Kung AL, Rebel VI, Bronson RT, et al. Gene dose-dependent control of hematopoiesis and hematologic tumor suppression by CBP. *Genes Dev*. 2000; 14:272–277. [PubMed: 10673499]
26. Bedford DC, Kasper LH, Fukuyama T, Brindle PK. Target gene context influences the transcriptional requirement for the KAT3 family of CBP and p300 histone acetyltransferases. *Epigenetics*. 2010; 5:9–15. [PubMed: 20110770]
27. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2009.
28. Harrington DP, Fleming TR. A class of rank test procedures for censored survival data. *Biometrika*. 1982; 69:553–566.
29. Kanellopoulou C, Muljo SA, Kung AL, et al. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes Dev*. 2005; 19:489–501. [PubMed: 15713842]
30. Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science*. 2002; 295:868–872. [PubMed: 11786607]
31. Tsai S, Bartelmez S, Sitnicka E, Collins S. Lymphohematopoietic progenitors immortalized by a retroviral vector harboring a dominant-negative retinoic acid receptor can recapitulate lymphoid, myeloid, and erythroid development. *Genes Dev*. 1994; 8:2831–2841. [PubMed: 7995521]
32. Chuck AS, Palsson BO. Consistent and high rates of gene transfer can be obtained using flow-through transduction over a wide range of retroviral titers. *Hum Gene Ther*. 1996; 7:743–750. [PubMed: 8919596]
33. Gentleman RC, Carey VJ, Bates DM, et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol*. 2004; 5:R80. [PubMed: 15461798]
34. Rual JF, Venkatesan K, Hao T, et al. Towards a proteome-scale map of the human protein-protein interaction network. *Nature*. 2005; 437:1173–1178. [PubMed: 16189514]
35. Gautier L, Cope L, Bolstad BM, Irizarry RA. affy--analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics*. 2004; 20:307–315. [PubMed: 14960456]
36. Cline MS, Smoot M, Cerami E, et al. Integration of biological networks and gene expression data using Cytoscape. *Nat Protoc*. 2007; 2:2366–2382. [PubMed: 17947979]
37. Maere S, Heymans K, Kuiper M. BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. *Bioinformatics*. 2005; 21:3448–3449. [PubMed: 15972284]
38. Yao TP, Oh SP, Fuchs M, et al. Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. *Cell*. 1998; 93:361–372. [PubMed: 9590171]
39. Beachy SH, Aplan PD. Mouse models of myelodysplastic syndromes. *Hematol Oncol Clin North Am*. 2010; 24:361–375. [PubMed: 20359631]
40. Brunning, RD.; Orazi, A.; Germing, U.; Le Beau, MM. Myelodysplastic syndromes/neoplasms, overview. In: Swerdlow, SH.; Campo, E.; Harris, NL., et al., editors. WHO classification of tumours of haematopoietic and lymphoid tissues. Lyon: International agency for research on cancer (IARC); 2008. p. 88-93.

41. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*. 2009; 114:937–951. [PubMed: 19357394]
42. McGary KL, Lee I, Marcotte EM. Broad network-based predictability of *Saccharomyces cerevisiae* gene loss-of-function phenotypes. *Genome Biol*. 2007; 8:R258. [PubMed: 18053250]
43. Kasper LH, Thomas MC, Zambetti GP, Brindle PK. Double null cells reveal that CBP and p300 are dispensable for p53 targets p21 and Mdm2 but variably required for target genes of other signaling pathways. *Cell Cycle*. 2011; 10:212–221. [PubMed: 21220944]
44. Nemec AA, Wallace SS, Sweasy JB. Variant base excision repair proteins: contributors to genomic instability. *Semin Cancer Biol*. 2010; 20:320–328. [PubMed: 20955798]

Figure 1. Hypercellularity, mild splenomegaly and low CFC counts in *Crebbp*^{+/-} mice
 (A) Number of BM cells in 2 femurs ($\times 10^6$) and (B) body weights of 3-4 or 9-12 month-old mice, as indicated. (C) Relative BM cellularity and (D) spleen size (in mg) corrected for body weight (g). (E) Number and type of CFUs from an input of 1×10^4 BM cells from 9-12 month-old mice. Bars represent average ($\pm$ SD). Seven to 10 animals were used per group. Significant differences are indicated by p-values.

Figure 2. Histopathology and Annexin V staining of WT and *Crebbp*^{+/-} animals
 (A-C) Wright-stained PB cells from 9-12 month-old mice. (A) WT control. (B, C) *Crebbp*^{+/-} blood smears showing hypersegmented granulocytes (i.e., granulocytes with >6 segments of irregular size) (B, inset) and (C) a pseudo Pelger-Huët anomaly. (D-H) Bone marrow sections from 9-12 month-old mice. (D) WT control. (E-H) *Crebbp*^{+/-} sections. (E) Hypercellular marrow and an increased myeloid to erythroid ratio due to increased mature granulocytes, particularly near the bony trabeculae. (F, G) Abnormal megakaryocytic differentiation with hyperlobulated megakaryocytes (F, arrow and inset) and naked megakaryocytic nuclei (G, arrow and inset). (H, and inset) Clusters of immature precursor cells present in the middle of the marrow cavity. Magnification: 40X (A,B,D-H), 60X (C). (I) Quantification of apoptosis by Annexin V staining of whole (WBM) and lineage-depleted (Lin-) bone marrow isolated from WT and *Crebbp*^{+/-} mice (n=7). P-value indicated where significant.

Figure 3. Abnormal numbers of HSCs, CMPs and GMPs in *Crebbp*^{+/-} mice

(A-C) Left and middle panels depict the sorting strategy used for each BM population while bar graphs on the right show average percentages (+SD) for the corresponding cell population(s). (A) Long-term HSCs (LT-HSCs) are selected from mature lineage marker negative (Lin⁻) c-Kit⁺⁺Sca-1⁺ cells (sorting gate 1 of the dot plot) that are also CD34⁻ (histogram). (B) Immature myeloid progenitors are identified by separating Lin-Sca-1-c-Kit⁺⁺ BM cells on the basis of CD16/32 and CD34 expression as shown. (C) CLPs are purified from WBM based first on expression of IL-7R but not of other mature lineage markers. Cells in sorting gate 3 of the dot plot are then further analyzed for c-Kit and Sca-1 expression (contour plot). CLPs are c-Kit⁺ Sca-1(intermediate) cells. Significant differences are indicated by p-values with 9-12 mice used in each analysis.

Figure 4. Increased mature myeloid cells in the BM and PB of *Crebbp*^{+/-} mice
(A,B) Representative FACS profiles of WT (left) and *Crebbp*^{+/-} cells (middle) immunostained for Mac-1 and Gr-1 expression. Right panels show average percentages (+SD) from profiles of 5-8 mice for the indicated cell populations. **(A)** Identification of granulocytes (Mac-1⁺⁺Gr-1⁺⁺, right gate) and more immature myeloid cells (Mac-1⁺+Gr-1^{lo} cells, left dot plot gate) in BM. **(B)** Identification of granulocytes (as indicated by the gate) in PB. **(C)** Average proportion (+SD) of lymphocytes (LYMPH), granulocytes (GRAN) and monocytes (MONO) in PB measured by CBC (n=16-17). In all bar graphs, significant differences are indicated by p-values.

Figure 5. Decreased survival of *Crebbp*^{+/-} mice in response to ionizing radiation consistent with CREBBP PIN predictions

(A) Predicted interaction network of proteins encoded by genes differentially expressed in *Crebbp*^{+/-} HSCs relative to WT (larger circles) and their direct interaction partners (smaller circles). Red edges indicate interactions with proteins encoded by genes up-regulated in *Crebbp*^{+/-} HSCs while blue edges mark interactions with proteins encoded by down-regulated genes. Black nodes mark proteins implicated in programmed cell death and blue nodes those involved in responses to DNA damage. Green nodes are annotated to both processes and gray nodes to neither. See Tables S1 and S2 for full lists of genes. (B) Quantification of apoptosis by Annexin V staining in the stem cell compartment (LSK; Lin⁻;Sca-1⁺;cKit⁺⁺) of WT and *Crebbp*^{+/-} mice. Depicted is the data of two independent experiments. (C) Kaplan-Meier curves for WT (black and blue lines) and *Crebbp*^{+/-} mice (green lines) after exposure to 11 Gy of γ -radiation. Mice received either no cells (control, blue line (n=9)), a single dose of 1×10^5 BM cells (dashed lines; n=11) or 5×10^6 BM cells (solid lines, n=22). (D) Kaplan-Meier curves for WT (black line) and *Crebbp*^{+/-} (green line) mice after exposure to 10 Gy of γ -radiation with no injected cells. (C, D) Logrank p-values

for the difference in survival between WT (excluding control animals) and *Crebbp*^{+/-} as indicated.

Figure 6. PARP1 enzymatic activity and BER protein levels in cells with reduced CREBBP levels (A) PARP1 enzymatic activity measured in PB cells of WT and *Crebbp*^{+/-} mice before (no IR) and at various time points after 6 Gy of radiation exposure (post-IR). Presented are average values (+SEM) of 3 independent experiments. Asterisks indicate statistical significance at individual time points between WT and *Crebbp*^{+/-} cells (paired T-test; **p*<0.05). (B) PARP1 activity in non-irradiated WBM and Lin- cells from WT and *Crebbp*^{+/-} animals (*n*=3-4). In the boxplot, the horizontal line represents the median and the boxes extend from the 25th to 75th percentile. The whiskers extend to the maximum and minimum values. (C) Representative western blots (left) of the indicated BER proteins in unirradiated Lin- BM and corresponding average levels (+SD) relative to ACTB in 4 experiments (right). Probabilities that protein levels are significantly reduced in *Crebbp*^{+/-} cells (one-sided paired T-test) as shown. (D) Western blots of CREBBP and BER proteins in parental EML1 cells, control eGFP knock down cells and in 5 independent *Crebbp* knock down (KD) clones. (E) Quantification of CREBBP and BER proteins in the *Crebbp* KD clones color-coded as in (D) relative to eGFP control levels (dashed line). PARP1 activity in unirradiated cells in the corresponding clones is shown on the right. Horizontal bars indicate average values. Likelihood that values are normally distributed around 1 (*P*(*X* ~ Norm(1,1)), Kolmogorov-Smirnov Test) are indicated below the figure.