MYBL2 supports DNA double strand break repair in haematopoietic stem cells

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Running title: Low MYBL2 expression in MDS alters DNA DSB repair in HSCs

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Abstract

Myelodysplastic syndromes (MDS) are a heterogeneous group of diseases characterized by blood cytopenias that occur as a result of somatic mutations in hematopoietic stem cells (HSC). MDS leads to ineffective haematopoiesis, and as many as 30% of patients progress to acute myeloid leukaemia (AML). The mechanisms by which mutations accumulate in HSC during aging remain poorly understood. Here we identify a novel role for MYBL2 in DNA double-strand break (DSB) repair in HSC. In MDS patients, low MYBL2 levels associated with and preceded transcriptional deregulation of DNA repair genes. Stem/progenitor cells from these patients display dysfunctional DSB repair kinetics after exposure to ionizing radiation (IR). Haploinsufficiency of Mybl2 in mice also led to a defect in the repair of DSB induced by IR in HSC and was characterized by unsustained phosphorylation of the ATM substrate Kap1 and telomere fragility. Our study identifies MYBL2 as a crucial regulator of DSB repair and identifies MYBL2 expression levels as a potential biomarker to predict cellular response to genotoxic treatments in MDS and identify patients with defects in DNA repair. Such patients with worse prognosis may require a different therapeutic regimen to prevent progression to AML.

Significance

Findings suggest MYBL2 levels may be used as a biological biomarker to determine the DNA repair capacity of hematopoietic stem cells from MDS patients and as a clinical biomarker to inform decisions regarding patient selection for treatments that target DNA repair.
Introduction

Myelodysplastic syndrome (MDS) is an age-associated hematopoietic malignancy, characterized by abnormal blood cell maturation and a high propensity for leukemic transformation. It is a clonal disease thought to originate in the haematopoietic stem cell (HSC) [1]. Effective management and treatment of MDS is important, as early identification of patients who are likely to progress to malignant disease allows for an optimal therapeutic regime to be implemented. Genetic alterations are often present in MDS and a frequent chromosome abnormality is del20q, whose common deleted region only contains 5 genes expressed in HSCs, one of which is MYBL2 [2, 3]. The MYBL2 gene encodes a ubiquitously expressed protein belonging to the MYB family of transcription factors and has been shown to form part of different protein complexes such as the Myb-MuvB/DREAM complex [4] [5] [6] [7], Myb-Clafi complex [8] and the MRN complex [9], through which it exerts its vital role in cell cycle regulation and maintenance of genome stability [10-14]. Analysis of publicly available global gene expression data from CD34+ MDS patient cells [15] have confirmed that down regulation of MYBL2 expression correlates with poor prognosis; even in patients with a normal karyotype [2, 3]. This suggests that changes in MYBL2 expression could have significant consequences with regards to disease pathogenesis. Furthermore, it has been demonstrated that mice with low levels of MYBL2 develop haematological disorders during ageing that closely resemble the human disease, implying that MYBL2 functions as a haploinsufficient tumour suppressor gene [2, 3]. However, how low MYBL2 expression contributes to MDS during ageing remains unknown.

Given that HSCs must last for the entire lifetime of an individual to guarantee continuous blood cell production, this increases the dependence of these cells on DNA repair to maintain genomic integrity. Because HSCs are predominantly quiescent, they are thought
to primarily utilize non-homologous end joining (NHEJ) rather than homologous recombination (HR) to repair DNA double-strand breaks (DSBs) [16, 17]. Although mainly error-free, NHEJ-dependent DSB repair can also result in the generation of small genomic deletions at the repaired break site, leading to the hypothesis that HSCs accumulate somatic mutations over time. This is thought to precede the appearance of blood disorders such as MDS and acute myeloid leukaemia (AML) [18-20], although no direct link between DNA repair and the pathogenesis of MDS has been reported.

Since recent studies have shown that MDS originates from HSCs and that MYBL2 is known to play a role in maintaining genome stability [1, 21, 22], we hypothesized that low levels of MYBL2 may compromise the DNA repair capacity of the cell, resulting in the accumulation of genetic alterations to a sufficient level to induce HSC transformation. To test this, we used ionising radiation (IR) in vitro to induce DNA damage in MDS patient’s stem cells. Following treatment, we determined the ability of these cells to repair their DNA and correlated this with expression levels of MYBL2. To further study the role of MYBL2 in DNA repair, we utilized a Mybl2 haploinsufficient mouse model, which is susceptible to MDS development. Our findings uncover a novel role for MYBL2 in regulating DSB repair in the hematopoietic stem cell population.

Methods

Differential expression and reactome pathway enrichment analyses

To assess differential gene expression and pathway enrichment between MDS samples displaying higher and lower levels of MYBL2 expression, we used previously published microarray data [15] deposited in the NCBI Geo DataSets repository (GSE19429), and the BROAD Institute Gene Set Enrichment Analysis software (GSEA) [23, 24]. Differential
expression was assessed over 1000 permutations and ranked according to signal to noise ratio. A weighted enrichment statistic was applied. Gene sets comprising less than 15 genes were excluded from the analysis (the list of gene set used in the analysis is presented as supplementary material Extended Data Table 1 and Table 2). The adjustment of the FAB composition was done using a method of random sample removal. In order to balance the composition of the MYBL2hi and MYBL2lo sets, samples of specific diagnosis (for example RA or RAEB) were randomly removed when they were found to be over-represented in a set. Four different permutations were performed to verify that the results were not affected by the methodology.

Isolation and expansion of human peripheral blood cells

Peripheral blood samples from MDS patients were obtained in heparin-coated vacutainers. Peripheral blood mononuclear cells were isolated using Ficoll-Paque (GE Healthcare) and stored at -80°C. Cells were thawed and cultured for 8 days in expansion medium as previously described [25], with the exception that the base medium was StemSpan H3000 (Stem Cell Technologies). Medium was refreshed on day 3 and 6. On day 8, cells were harvested and CD34+ cells purified using microbeads (130-046-702, Miltenyi Biotec, Germany).

Quantitative Reverse Transcriptase-PCR (qRT-PCR)

For human gene expression assays, qRT-PCR for MYBL2 (Hs00942543_m1 MYBL2, Applied Biosystems, USA) was carried out using Taqman PCR master mix (Applied Biosystems, USA) and qRT-PCR for β-glucuronidase (HsGusB QT00046046, Quantitect primer assay, Qiagen, Germany) was carried out using SYBRGreen master mix (Thermo Fisher Scientific). For murine gene expression assays, qRT-PCR for p21
(Mm01303209_m1), Puma (Mm00519268_m1), Noxa (Mm00451763_m1), Bax (Mm00432051_m1), and β-2-microglobulin (Mm00437762_m1) were carried out using Taqman PCR master mix (Applied Biosystems, USA). Reactions were carried out in a Stratagene Mx3000P machine and samples were run in duplicate. Relative gene expression was calculated as $2^{-ΔΔCt}$ values relative to control genes (β-glucuronidase for human samples and β-2-microglobulin for murine samples).

Mice

Mice were maintained on a C57/BL6 background and genotyped by Transnetyx. For mouse studies, no specific randomization or blinding protocol was used during experimental protocols. Mice of both genders were used. Age and gender matched mice were used per experiment. 70 weeks old healthy mice were chosen to perform ageing studies. Disease free status of these animals was assessed based on behavior and physical appearance of the mice, normal values of white blood cell, red blood cell and platelets obtained from peripheral blood counts, and by internal organ examination after dissection, in particular no signs of splenomegaly.

Inhibitors

Inhibitors were dissolved in DMSO; KU60019 (10µM) was used for inhibition of ATM and NU7441 (1µM) was used for inhibition of DNA-dependent protein kinase (Tocris Bioscience, UK).

Flow cytometry and cell sorting

Single cell suspensions of bone marrow were prepared using standard techniques and red blood cells were depleted by ACK lysis (0.15M NH₄Cl, 1mM KHCO₃, 0.1 mM EDTA,
pH 7.4). Non-specific antibody binding to Fc receptors was blocked using anti-CD16/CD32 (93, eBioscience) and cells were stained with a combination of fluorochrome-conjugated antibodies including anti-mouse lineage; CD5, CD8a, CD11b, Gr-1, Ter119, B220 (APC or FITC), cKit (PeCy5 or e780 (2B8, eBioscience,), Sca-1 PeCy7 (D7, eBioscience), Flk2 PE (A2F10, eBioscience), CD48 APC (HM48-1, eBioscience), CD150 PEcy7 (TC15-12F12.2, BioLegend ), to allow identification of Flk2- haematopoietic stem cells (HSCs) (lineage- cKit+ Sca-1+ Flk2-) and long-term haematopoietic stem cells (SLAM staining) (lineage- cKit+ Sca-1+ CD48- CD150+). Some cells were analysed directly by flow cytometry using a CyAn ADP Analyzer (Beckman Coulter, USA) and some were sorted using a Cytomation XDP MoFlo machine (Beckman Coulter, USA). In both cases data were analysed using either Summit software (Dako, Denmark) or FlowJo software (FLOWJO, LLC, USA). When cells were required for sorting, a cKit+ enrichment using streptavidin microbeads (130-048-101, Miltenyi Biotec, Germany) and columns (130-042-201, Miltenyi Biotec, Germany) was performed prior staining.

**Proliferation and apoptosis assays**

For proliferation assays *in vivo* using BrdU, mice were given an intraperitoneal injection of 2 mg BrdU in PBS and 24 h later animals were sacrificed. cKit-enriched bone marrow cells were isolated using anti-mouse cKit biotin (eBioscience, USA) and streptavidin microbeads (Miltenyi Biotec, Germany) as per the manufacturer’s instructions. For proliferation assays *in vitro* using BrdU, expanded CD34+ cells were labelled with 10µM BrdU for 3.5 h. Cells were stained using the BrdU flow kit (8811-6600, BD Biosciences, USA) according to the manufacturer’s instructions. For proliferation assays using Ki67, cKit-enriched bone marrow cells were stained using the Ki67 flow kit (556027, BD Biosciences, USA) according to the manufacturer’s instructions. For colony assays, purified HSC (lineage- cKit+ Sca-1+ Flk2-)
were obtained by sorting. 500 HSCs were plated in Methocult (M3434, Stem Cell Technologies, Canada) supplemented with penicillin/streptomycin (Invitrogen, USA) in 35mm petri dishes and incubated for 6 days at 37°C in an atmosphere containing 5% CO₂. Colonies were counted using a standard light microscope with x10 objective. For G2/M checkpoint studies, cKit-enriched bone marrow cells were isolated as described previously. 1-2x10⁶ cKit-enriched cells were cultured for 18 h in Iscove’s modified Dulbecco’s medium (IMDM) containing 10% heat-inactivated foetal bovine serum (HIFBS), 3% penicillin/streptomycin, 1mM sodium pyruvate, 2mM L-glutamine, 0.1mM non-essential amino acids, 50µM 2-mercaptoethanol, 25ng/ml SCF, 10ng/ml IL-3, 25ng/ml IL-11, 25ng/ml TPO, 4U/ml EPO, 10ng/ml GMCSF and 25ng/ml FLT3L (complete cytokine medium). Medium was replaced and cells were cultured for 1 h with DMSO or 10µM KU60019 prior to irradiation (IR) in vitro (2 Gy). Cells were cultured for a further 5 h and stained with (1) cell surface markers antibodies to identify sub populations, (2) mouse anti-phospho histone H3 Ser10 (2 h staining) (clone 6G3, Cell Signaling Technologies, USA) and goat anti-mouse Alexa 488 (30 min staining) (A-11001, Molecular Probes, USA) and (3) Vybrant DyeCycle (Molecular Probes, USA). Fixation and permeabilization were carried out using buffers from the BrdU flow kit (BD Biosciences, USA) according to the manufacturer’s instructions. For apoptosis assays, whole bone marrow cells were stained with cell surface markers antibodies to identify sub populations as well as mouse anti-cleaved PARP (Asp214) (clone F21-852, BD Biosciences, USA).

**Immunofluorescence**

Purified murine cells (Flk2⁻ HSCs, or SLAM HSC) or human CD34+ cells were cytospun onto microscope slides for 5 min at 800RPM. For 53BP1, gamma-H2AX and MRE11 staining, cells were treated with CSK buffers (buffer 1 for 4 min at RT, buffer 2 for 1 min at
RT), washed in PBS and fixed in 4% PFA/PBS for 15 min at RT. Fixation was quenched with 50mM ammonium chloride for 5 min at RT and cells were permeabilized with 0.3% Triton-X 100/PBS for 5 min at RT. For pKap1, cells were fixed in 4% PFA/PBS for 10 min at RT, washed in PBS and permeabilized with ice-cold methanol for 10 min at RT. Cells were blocked with 3% BSA/10% HIFBS/1% goat serum/0.3% Triton-X 100/PBS (blocking buffer) for 1 h at RT. Cells were incubated with primary antibodies to 53BP1 (NB-100-904, Novus Biologicals), pKap1 (S824) (A300-767A, Bethyl Laboratories Inc) or rabbit IgG control (sc-2027, Santa Cruz Biotechnology) diluted in blocking buffer overnight at 4°C. Cells were washed 3 times in 0.1% Tween 20/PBS and incubated with goat anti-rabbit Alexa 488 secondary antibody diluted in blocking buffer for 1 h at RT. Cells were washed 3 times in 0.1% Tween 20/PBS, dipped in Milli-Q water and mounted with ProLong Gold AntiFade Reagent containing DAPI (Invitrogen). Microscopy imaging was performed using a Zeiss LSM 510 Meta confocal microscope (100x objective NA 1.4 lens) and images were analysed using ImageJ software. For all immunofluorescence staining, 30-50 cells were scored for each independent experiment. Experiments were performed at least three times and results represent a minimum of three animals. For 53BP1 staining on CD34+ cells from MDS patients, a minimum of 25 cells were scored per patient per condition.

**Comet Assays**

HSCs (Lin-/cKit+/Sca1+/Flk2-) were purified 0, 1, 5 and 24 h after IR *in vivo* (2Gy). Alkaline comet assays were performed as previously described [26]. Cells were stained with SYBR Safe (Invitrogen) for 1 h and imaged using a Leica DM6000 microscope (20x objective). Analysis was performed using ImageJ software using the Open Comet plugin and the head finding was selected to the brightest region. Olive moment values were
automatically generated by the software. Statistical significance was calculated using GraphPad Prism software utilizing the Mann-Whitney test.

**PNA-FISH**

Purified HSCs were obtained by cell sorting, exposed to ionising radiation *in vitro* (2 Gy) and cultured for a further 7 days in methylcellulose semi-solid medium containing cytokines (M3434). Colonies were dissociated and cultured with 100ng/ml colcemid for 3 h at 37°C to arrest cells in metaphase. PNA staining was performed as described previously [27]. Briefly, cells were exposed to hypotonic solution (0.56% KCl) for 16 min at 37°C and then fixed in methanol:acetic acid (3:1). After three changes of fixative solution, the cells were dropped on to slides that that been pre-treated with 1N HCl followed by 100% ethanol and finally fixative solution. FISH with FITC labelled (CCCTAA)<sub>3</sub> peptide nucleic acid (F1009-5, Panagene) was performed followed by mounting with ProLong Gold AntiFade Reagent containing DAPI (Invitrogen). Microscopy imaging was performed using a Leica DM6000 microscope (100x objective) and images were analysed blindly using ImageJ software. An average of 20-30 metaphases were scored per condition.

**Statistics**

All data shown are presented as mean±SEM. When comparing data sets between *Myb12<sup>+/+</sup>* and *Myb12<sup>+/Δ</sup>* animals, two-tailed unpaired Student’s t-test was applied using GraphPad Prism software, unless indicated. No statistical method was used to estimate the sample size. No specific randomization or blinding protocol was used. N indicates the numbers of independent experiments performed and was chosen to ensure adequate statistical power. P≤0.05 was considered statistically significant. Significance tests were performed on all
samples and therefore graphs lacking p values indicate results were not statistically significant.

Study approval

All animal experiments were performed under an animal project license in accordance with UK legislation. Human MDS patients were recruited from the clinic held at the Centre for Clinical Haematology, University Hospital Birmingham NHS Foundation Trust. All the subjects have read the patient information sheet and signed the consent form. The study was conducted as according to Good Clinical Practice (GCP) guidelines, consistent with the principles that have their origin in the Declaration of Helsinki. The study was approved by the West Midlands – Solihull Research Ethics Committee (10/H1206//58).

Results

Low MYBL2 expression associates with decreased expression of DNA repair genes in human MDS

Given the role of MYBL2 in transcription and in maintaining genome stability [10-14] and recent studies showing increased DNA damage in MDS [28, 29], we decided to determine whether MYBL2 levels were associated with altered expression of DNA repair genes in MDS patients. To do this, we first subdivided the patients into MYBL2lo and MYBL2hi populations based on a differential gene expression analysis on HSCs from MDS patients [15], using the 25th and 75th percentiles of MYBL2 expression to define each group. Comparing these two groups, genes were then ranked based on the signal to noise ratio for differential gene expression (DGE) (Supplementary Table 1A). This DGE scoring was then analysed against a collection of reactome gene sets (Supplementary Table 1B), to assess
their individual enrichment, which encompassed pathways with which MYBL2 has previously been associated (cell cycle, DNA replication) and pathways relevant to this study (DNA damage, DNA repair, chromosome maintenance and apoptosis). Consistent with previously published observations, the analysis (Supplementary Table 1C) confirmed the previously published association between MYBL2 levels and cell cycle progression in the context of MDS [2]. Remarkably, it also revealed a significant enrichment (NOM p-val=0.00065, FWER p-val=0.006) for the reactome gene set “DNA damaged pathway” (R-HAS-73894), indicating an overall downregulation of DNA repair pathways components in the MYBL2lo MDS cases (Figure 1A). Upon further inspection of the two populations, and confirming our previous results [2], we found that the MYBL2lo population was significantly enriched in MDS with excess blasts type 2 (MDS-EB2) cases with worse diagnosis (Figure 1B, upper table). To exclude the possibility that the observed link could primarily reflect an association between DNA damage pathway and advanced disease states rather than MYBL2 levels, we re-curated the compared subsets to display balanced diagnosis composition (Figure 1B, lower table), and repeated the gene expression analysis (Figure 1C). This unbiased analysis confirmed that expression of DNA repair genes correlates with MYBL2 levels in MDS (Figure 1C and Supplementary Table 2), independently of the disease state.

Low MYBL2 expression associates with poor DNA repair in human MDS

To determine whether the differential expression of DNA repair genes (Figure 1) had a functional consequence, we next assessed the DNA repair kinetics of CD34+ cells, a multipotent stem and progenitor cell population, from five MDS patients with differing prognoses (Table 1), without prior knowledge of MYBL2 expression levels. CD34+ cells were irradiated in vitro (2 Gy) and the prevalence of p53-binding protein 1 (53BP1) foci, a robust marker for DNA double-strand breaks (DSBs), was measured at 1, 3 and 5 h post-IR (Figure 2A and 2B). Cells from these patients showed a striking difference in their ability to...
repair IR-induced DSBs, as evidence by their differing ability to resolve 53BP1 foci. After
MYBL2 expression levels were measured (Figure 2C) in the same patients, it was evident that
DSB repair kinetics strongly correlated with MYBL2 mRNA expression (Figure 2D, R²=0.83,
p=0.0114). For example, a patient (patient 5) expressing similar MYBL2 levels to cells
isolated from a healthy individual could efficiently repair DSBs within 5 h, whereas in
contrast, patients expressing around 50% normal levels of MYBL2 (patients 3 and 4), or even
lower (patients 1 and 2) exhibited defective clearance of 53BP1 foci, suggesting the
persistence of unresolved DSBs. We also investigated whether proliferation rates correlated
with MYBL2 expression in MDS patient CD34+ cells by performing BrdU incorporation
assays. This revealed that MYBL2 expression in these patients did not correlate with
proliferation, (Supplementary Figure 1A+1B), nor did this correlate with the cell’s DNA
repair capacity (Figure 2E). These data suggest that reduced expression of DNA damage
genes in patients with compromised MYBL2 expression has a functional impact on the ability
of these cells to repair genetic damage. Furthermore, we propose that MYBL2 mRNA
expression levels may also be used as a potential biomarker predicting the cellular response
to DNA damage which could be of use for patient stratification.

Slow DSB DNA repair kinetics in Mybl2+/∆ HSCs after in vivo IR

Following the observation that MYBL2 expression levels correlated with DSB repair
kinetics in human MDS stem cells, we wanted to further investigate the involvement of
MYBL2 in regulating the DNA damage response. To do this, we used a Mybl2
haploinsufficient mouse model (Mybl2+/∆), known to be susceptible to MDS with ageing [30].
We decided to focus our studies on the HSC population, the population in which MDS
originates [1]. Importantly, these animals did not show any major differences in the numbers
of HSC/progenitor cells when compared to wild type mice prior to treatment (Supplementary
To investigate the DNA damage response, we assessed the clearance of 53BP1 foci, as a measure of repair kinetics, in both wild type and *Mybl2*+/Δ HSCs (defined as (Lin-/cKit+/Sca1+/Flk2-) following irradiation of the mice with 2 Gy of IR (Figure 3A and Supplementary Figure 3A). Whilst we did not observe any difference in the initial recruitment of 53BP1 1 h post-irradiation between wild type and *Mybl2*+/Δ HSCs, there were notable differences in the kinetics of 53BP1 foci resolution over time between the two genotypes (Figure 3B and 3C). Wild type HSCs showed a significant reduction in cells positive for 53BP1 foci at 5 h post-IR, whereas in contrast, more than 50% of *Mybl2*+/Δ HSCs still retained a significant number of 53BP1 foci-positive cells at this time point. Moreover, the absolute number of 53BP1 foci per cell was also increased in the *Mybl2*+/Δ HSCs (Figure 3B and 3C and Supplementary Figure 3B+C). To extend these observations to a more refined HSC population, we repeated this experiment with HSCs purified from young animals (7 weeks) using SLAM staining (KSL CD48-CD150+). This revealed that the retention of 53BP1 foci was also apparent in *Mybl2*+/Δ CD150 positive HSCs, but not in their wild type counterparts (Figure 3D). Moreover, to determine whether this effect was dose dependent, we measured the percentage of 53BP1 foci 5 h after 1 Gy of IR. This revealed that at a lower dose of IR *Mybl2*+/Δ HSCs exhibited a “wild-type” 53BP1 response (Supplementary Figure 3D), suggesting that there is an insufficient quantity of DNA repair proteins present in the *Mybl2*+/Δ HSCs required to cope with repairing high level DNA damage. Since aged haploinsufficient *Mybl2* mice develop an MDS-like disease, we investigated how aging affected DSB repair capacity of wild type and *Mybl2*+/Δ HSCs from young (7 weeks) and old mice (70 weeks). Unsurprisingly, 70 week-old HSCs exhibited a higher percentage of 53BP1 positive cells that 7 week-old HSCs. However, in keeping with a role for MYBL2 in regulating DNA repair, *Mybl2* haploinsufficiency exacerbated the age-associated decrease in genome stability (Figure 3B+C and Supplementary Figure 3C). Interestingly, levels of 53BP1...
positive cells in young Mybl2+/Δ HSCs were comparable to aged wild type HSCs, suggesting that these cells may demonstrate a premature aging phenotype. Together, these data indicate that Mybl2 haploinsufficiency is associated with defective repair of IR-induced DSBs in HSCs.

Since our data are only indicative of unrepaired DNA DSBs, we performed comet assays to directly measure the total amount of DNA damage remaining in these cells at different times post-irradiation. In keeping with a failure to properly repair IR-induced damage, Mybl2+/Δ HSCs displayed an increase in the olive tail moment 5 h post-irradiation when compared to wild type HSCs (Figure 3E). These differences in DNA repair kinetics were not the consequence of changes in cell cycle profile between wild type and Mybl2+/Δ HSCs, as HSCs from both genotypes showed a similar percentage of cells in G0/G1 prior to irradiation (Supplementary Figure 4), and the same percentage of cells in S-phase measured by in vivo BrdU incorporation (Supplementary Figure 5A+B). Importantly, we did not observe any changes in the absolute numbers of HSCs after in vivo IR (Supplementary Figure 5C), nor apoptosis, measured either by PARP1 cleavage (Supplementary Figure 5D) or the induction of p53-dependent apoptotic genes (Supplementary Figure 5E), that could account for our observations using the comet assay. Overall, these data demonstrate that Mybl2 haploinsufficient mice display a defect in the kinetics of DSB repair in response to IR, which is heightened during ageing, but that has no impact on HSC survival.

Mybl2+/Δ HSCs are highly dependent on DNA-PK for DNA DSB repair

To gain a mechanistic understanding of the DNA repair defect in Mybl2+/Δ HSCs, we used small molecule inhibitors to investigate the relationship of MYBL2 haploinsufficiency with two key proteins involved in the DSB response, namely DNA-dependent protein kinase
(DNA-PK) and ATM. Treatment of wild type HSCs with the DNA-PK inhibitor NU7441 (Figure 4A) [31] induced an increase in the percentage of cells positive for 53BP1 foci, in line with an expected defect in DSB repair due to inhibition of the NHEJ pathway (Figure 4B-D). In contrast, Mybl2+/∆ HSCs treated with the same inhibitor demonstrated a dramatic loss of 53BP1 foci formation at 5 h after IR (Figure 4B-D). These findings were recapitulated by analysis of the formation/retention of gamma-H2AX foci, a pan-DNA damage marker (Figure 4E-G). Nonetheless, 53BP1 foci were detected at 1 h post-irradiation in Mybl2+/∆ HSCs (Figure 4B-D), suggesting that our observations did not reflect a global inability to form 53BP1 foci. Furthermore, Mybl2+/∆ HSCs treated with DNA-PK inhibitor were proficient at sensing DNA damage, since MRE11 foci formed 1 h and 5 h after IR (Figure 4H+I). Moreover, the absence of 53BP1 and H2AXgamma foci 5 h post-irradiation was not because DNA repair had been completed, as Mybl2+/∆ HSCs exhibited increased olive tail moments by comet assay (Figure 4J). However, these breaks were eventually repaired, as by 24 h levels of DNA damage in Mybl2+/∆ HSCs was equal to that of wild-type cells (Figure 4J). Interestingly, it has been previously shown that DNA-PK and ATM are both required for efficient H2AX phosphorylation and 53BP1 recruitment to DSBs and that ATM+/ B cells are completely dependent on DNA-PK to sustain the phosphorylation of H2AX after gamma-irradiation [32]. Based on this, these data suggest that reliance that Mybl2+/∆ HSCs have on DNA-PK to mediate DSB signaling is indicative of an underlying defect in the ATM-dependent DNA damage response.

The slow repair kinetics in Mybl2+/∆ HSCs are epistatic with inhibition of ATM

Whilst previously published data has been suggestive of a link between MYBL2 and ATM signaling [9], we wanted to specifically determine if Mybl2+/∆ HSCs exhibit defective ATM-dependent signaling in response to DSBs. To address this, we treated WT and Mybl2+/∆...
HSCs with the ATM inhibitor KU60019 [33], and analyzed 53BP1 foci clearance (Figure 5A). These analyses revealed that although treatment with KU60019 delayed the clearance of 53BP1 foci in wild type cells, with ATM inhibition causing a >2-fold increase in the number of cells still displaying 53BP1 foci 5 h after IR (Figure 5B-5D), in line with the known requirement for ATM in DSB repair. In contrast, ATM inhibition in Mybl2+/∆ HSCs had little effect on 53BP1 clearance after IR (Figure 5C and Supplementary Figure 6 A). These data further support the prediction that the altered DSB repair kinetics observed in Mybl2+/∆ HSCs is potentially due to a defect in ATM-dependent signaling.

Given these findings, it is tempting to speculate that, since ATM inhibition in wild type HSCs mimics the 53BP1 foci clearance defect observed in Mybl2+/∆ HSCs, that a similar treatment would also lead defective cell survival as seen in Mybl2+/∆ cells. However, wild type HSCs transiently treated with ATM inhibitor did not display the same characteristics as untreated Mybl2+/∆ HSCs when assessed by colony assay (Supplementary Figure 6B) indicating that either short-term inhibition of ATM pathway does not have the same overall effect as Mybl2 haploinsufficiency, or that Mybl2+/∆ HSCs display additional defects that are not mimicked by ATM inhibitor.

A subset of ATM function is impaired in Mybl2+/∆ HSCs

To further investigate the defective ATM signaling in Mybl2+/∆ HSCs, we next assessed the phosphorylation of Kap1. It has been previously reported that approximately 10-15% of DSBs require ATM signaling to be repaired in G0/G1 phases of the cell cycle [34] and that this repair requires the phosphorylation of Kap1, which is known to be completely dependent on ATM [35]. Therefore, we examined Kap1 phosphorylation after exposure to IR in HSCs (Figure 6A). In line with previous reports, wild type HSCs displayed robust pan-
nuclear p-Kap1 staining within minutes following IR, which could be distinguished as discrete foci by 3 h post-IR (Figure 6B and 6C) [35]. Interestingly, although Mybl2+/Δ HSCs also exhibited rapid Kap1 phosphorylation immediately post-IR, they were unable to maintain this phosphorylation at later time points (Figure 6B and 6C). This further reinforced our findings that partial Mybl2 loss leads to defective ATM signaling, and also suggests that MYBL2 may be required to maintain rather than initiate ATM-dependent signaling during late stage repair. To confirm this prediction, we next stimulated HSCs to enter the cell cycle, and analyzed activation and maintenance of the ATM-dependent G2/M cell cycle checkpoint after IR. Interestingly, Mybl2+/Δ HSCs retained the ability to activate this checkpoint following exposure to IR (Supplementary Figure 7A-C), suggesting that these cells are not completely defective in ATM function. This suggests that MYBL2 is required to maintain, but not initiate, activation of a specific subset of ATM-dependent signaling pathways.

One of the characteristics of loss of ATM function is telomere instability [36]. We therefore postulated that lower MYBL2 levels in HSCs might lead to telomere fragility as a result of defective ATM signaling. To examine this possibility, HSCs from young and old mice were irradiated and cultured for seven days in a colony assay. Metaphase spreads were prepared from these cells and stained with telomere probes. These investigations revealed that telomere instability (defined as sister chromatid fusion or loss of telomere signal) was twice as frequent in the progeny derived from young Mybl2+/Δ HSCs compared to controls (Figure 6D). In fact, this percentage was similar between young Mybl2+/Δ HSCs and old wild type HSCs, in line with our earlier suggestion that Mybl2+/Δ HSCs display an ageing phenotype that could lead to neoplastic lesions.

In conclusion, we demonstrate that Mybl2+/Δ HSCs are defective in the maintenance of ATM-dependent DNA damage signaling at the sites of DSBs, leading to slower DSB
repair kinetics and a higher dependency on DNA-PK in the surviving cells. Overall, these data suggest that correct MYBL2 protein levels are required for a proper DNA damage response and appropriate DSB repair in the hematopoietic stem cell compartment. Deregulation of these levels leads to defective DSB repair, telomere instability, and likely contributes to the accumulation of genetic alterations in MDS.

**Discussion**

HSCs are the life-long pillars of continuous blood cell production. Maintenance of their genetic integrity is paramount to avert the accumulation of mutations that can contribute to the development of blood disorders such as MDS during the aging process. Our work demonstrates a previously undescribed role for MYBL2 in promoting efficient DSB repair in HSCs, possibly via regulation of the ATM kinase. Furthermore, our findings suggest that **MYBL2** levels may be used as a biological biomarker to determine the DSB repair capacity of CD34+ cells from MDS patients. Furthermore, **MYBL2** levels could also be used as a clinical biomarker to inform decisions regarding patient selection for transplantation or treatments which target DNA repair, highlighting the translational importance of this work.

In line with a role for MYBL2 in regulating ATM signaling, we have shown that **Mybl2** haploinsufficient HSCs display a delay in 53BP1 clearance after DNA damage induced by IR, which is exacerbated during ageing. In these cells, defective ATM signaling leads to loss of sustained Kap1 phosphorylation and telomere instability. This renders these cells reliant on other DNA repair pathways prevalent in non-cycling cells. As a result, **Mybl2**+/∆ HSCs are highly dependent on the NHEJ regulator DNA-PK for DSB signaling, since inhibition of this kinase leads to a failure to maintain γ-H2AX and 53BP1 at sites of damage. Moreover, since ATM-dependent phosphorylation of Kap-1 has been suggested to
be required for chromatin relaxation and the repair of DSBs within heterochromatin regions [35, 37], our data suggests a requirement for MYBL2 in repairing a subset of DSBs associated with heterochromatic chromosomal regions.

In agreement with our conclusions, studies using human cell lines have recently demonstrated that MYBL2 interacts with Nbs1, which is required for the activation of ATM in response to DSBs and also ATM-dependent heterochromatic DSB repair in G0/G1 [9]. Whilst on face value this may explain our observations Mybl2^{+/\Delta} HSCs, in stark contrast to the work of Henrich et al., we were unable to detect a G2/M checkpoint defect in our Mybl2-haploinsufficient HSCs, suggesting that at least some ATM-dependent signaling is intact in these cells. Moreover, Henrich and colleagues failed to observe any defects in DNA repair, leading them to conclude that MYBL2 does not have an essential role in the DNA repair response. In contrast, we have shown that MYBL2 does have a role for DSB repair in HSCs, and without sufficient MYBL2 expression cells show defective repair kinetics of IR-induced DSBs. These differences could be due to cell type differences, such as primary cells versus cell lines, or the use of different DNA damaging agents to induce DSBs (IR versus UV). Alternatively, the highly quiescent nature of HSCs in vivo may also account for these discrepancies, as this may make any defects in fast DSB repair by NHEJ more pronounced as they cannot utilize repair by HR.

The importance of an appropriate DNA damage response for the maintenance and protection of the HSC pool against functional decline during ageing has been well reported [38-41]. Quiescent HSCs cannot use the HR pathway and thus rely on NHEJ-dependent mechanisms to repair their DNA [16, 17]. A failure to repair DSBs by the canonical DNA repair pathways can be detrimental to the cell, as alternative pathways may allow the potential for genome instability [42, 43]. It has also been reported that reduction in or
mutation of splicing factors in MDS leads to altered splicing of DNA repair and telomere maintenance genes [44]. This then perturbs myeloid differentiation and contributes to disease development via a mechanism not necessarily involving chromosomal rearrangements. Equally, abnormal DNA damage signaling in Mybl2+/∆ HSCs could increase the mutational burden by facilitating the use of alternative, more error-prone signaling pathways. Indeed, after inducing irradiated Mybl2+/∆ HSCs to proliferate, we found evidence of telomere instability in their progeny. In vivo, this prospect is likely to have a severe impact, and may ultimately lead to HSC malfunction and the accumulation of cells that are primed for the development of blood disorders such as MDS. Recent work in the field of MDS suggests that telomere dysfunction is a potent driver of the disease phenotype [44] and telomere elongation using danazol treatment has been shown to improve haematological responses including reducing transfusion-dependency [45].

Importantly, we show that the association between low MYBL2 levels and impaired DNA repair also holds true in MDS patients. Thus, by directly measuring the DNA repair kinetics in CD34+ MDS cells, our data shows a correlation between MYBL2 levels and functional DSB repair. Moreover, in CD34+ cells from these patients, low MYBL2 levels largely associate with low expression of DNA-repair genes. Together, these data provide a molecular rationale for the accumulation of genetic anomalies in patients deficient for MYBL2, which could play a role in the progression of their disease. Importantly, this data represents the first direct study of repair kinetics in MDS patient CD34+ cells. Current treatment options for MDS patients are mostly based on cytotoxic agents prior to autologous transplant, and are challenged by the occurrence of clonogenic relapse, increased resistance to therapy and, in some cases, leukaemic transformation. Relapse of disease in these patients is typically driven by additional genetic lesions. Of note, delq20 clones are not uncommon in
patients following cytotoxic chemotherapy, with more than 20% of these patients having a
therapy-related myeloid neoplasm, conferring a high mortality [46]. Patients with therapy-
related myeloid neoplasms more frequently have clonal hematopoiesis with the originating
mutation being present prior to chemotherapy treatment [47]. We therefore hypothesize that
these clones are susceptible to DNA damaging agents and that DNA repair defects may be
involved in the aetiology of their subsequent myeloid neoplasm. Moreover, our work supports
the notion that \textit{Mybl2} haploinsufficiency results in changes in DNA repair kinetics and
defective ATM signaling. Both ATM signaling and NHEJ are known to be activated to repair
double-strand breaks induced by doxorubicin [48, 49], indicating that our findings with IR
are also likely to be applicable to the use of anthracyclines in chemotherapy.

In light of our findings, we propose that compromising the MYBL2-dependent DNA
damage response in HSCs can facilitate MDS development by allowing inefficient repair of
physiological DSBs, and promoting telomere instability, two processes known to contribute
to the generation of oncogenic transformation in the surviving HSC population. Furthermore,
we suggest that sustained low levels of the MYBL2 protein in the premalignant cells may
confer a susceptibility to disease progression through the accumulation of incorrectly repaired
DNA lesions.

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References


Table 1. Clinical data for the myelodysplastic syndrome patients used in this study.

Description of the clinical data for the five patients used in this study. IPSS-R = Revised International Prognostic Scoring System, WHO = World Health Organisation, NPM1 =
nucleophosmin, MDS-EB = MDS with excess blasts, MDS-SLD = MDS with single lineage
dysplasia, MDS-MD = MDS with multilineage dysplasia and AML = acute myeloid
leukaemia.

Figure Legends

Figure 1. Differential MYBL2 expression associates with a DNA-repair gene signature in
MDS. (a) Heat map of the reactome DNA repair gene set core signature represented as a
Blue-Pink O’ Gram in the space of the analyzed gene set. (b) Characteristics of the MYBL2hi
and MYBL2lo subsets. A two tailed Fisher's exact test (right column) was applied to assess
the dependence between the variables MYBL2 class and MDS World Health Organisation
(WHO) classification. (c) Enrichment plot for the DNA repair reactome gene set after
adjustment of the FAB composition of the compared sample sets (left panel); Heat map of the
reactome DNA repair gene set core signature represented as a Blue-Pink O’ Gram in the
space of the adjusted (unbiased) set (right panel). (See also Table S1, and Table S2).

Figure 2. MYBL2 expression correlates with kinetics of 53BP1 clearance in
myelodysplastic syndrome (MDS) patient CD34+ cells after irradiation. (a-e) Peripheral
blood cells from a healthy control and MDS patients were cultured for 8 days in cytokine
containing medium to expand CD34+ cells. Cells were harvested on day 8 and CD34+ cells
were purified using microbeads for performing PCR, cell cycle analysis and
immunofluorescence. Purified CD34+ cells were subject to 2 Gy irradiation (IR) in vitro and
cultured in cytokine containing medium. Cells were removed from culture at different time
points (1, 3 and 5 h), cytospun onto microscope slides and immunofluorescence for 53BP1
was performed. (a) Representative images of 53BP1 foci 5 h after IR in MDS patient CD34+
cells and CD34+ cells taken from a healthy individual. Scale bar = 5µm. (b) Average number
of 53BP1 foci at different time points in MDS patients and CD34+ cells taken from a healthy individual. Values are calculated as a percentage of the average number of 53BP1 foci present 1 h after IR. P= MDS patient number, HC = healthy control. (c) MYBL2 gene expression from purified CD34+ cells normalised using expression of GusB. (d) Negative correlation between number of 53BP1 foci 5 h after IR and MYBL2 expression levels in MDS patient CD34+ cells. (e) Purified CD34+ cells were labelled with 10μM BrdU for 3.5 h and stained with a fluorescent antibody directly conjugated to BrdU. No correlation was observed between the number of 53BP1 foci 5 h after IR and percentage of BrdU+ cells in MDS patient CD34+ cells. Error bars: mean±SEM.

Figure 3. MYBL2 deficient haematopoietic stem cells have altered kinetics of DNA double strand break repair. Mybl2+/+ and Mybl2+/∆ animals aged 7 and 70 weeks were exposed to 2Gy irradiation (IR) in vivo. Bone marrow (BM) cells were obtained at different time points after IR (1, 3, 5 and 24 h) and haematopoietic stem cells (HSCs) were purified using cell sorting. Immunofluorescence was performed (53BP1 and DAPI) and alkaline comet assays. (a) Experimental scheme for 53BP1 staining and comet assays using purified HSC subpopulations including Flk2- HSC (KSL Flk2-) and SLAM HSC (KSL CD48-CD150+). (b) Representative images of 53BP1 staining of Flk2- HSCs from young and old animals 5 h after IR (c) Percentage of Flk2- HSCs positive for 53BP1 foci at different time points in young animals (left panel) (n=4). Comparison of the percentage of Flk2- HSCs positive for 53BP1 foci 5 h after IR in young and old animals (right panel) (n=4). (d) Percentage of SLAM HSCs positive for 53BP1 staining in young animals 5 h after IR (n=3) (e) Representative images of alkaline comets from Flk2- HSCs isolated from young animals 5 h after 2Gy IR in vivo (left panel). Mean olive tail moment of alkaline comets of Flk2- HSCs isolated from young animals at different time points after 2Gy IR in vivo (right panel).
(Mybl2+/+: n=4 for 5 h and n=3 for 0,1 and 24 h, and Mybl2+/∆ n=5 for 5 h and n=3 for 0,1 and 24 h). Error bars: mean±SEM; p values included in the figure when using a two-tailed unpaired Student’s t-test.

Figure 4. DNA-dependent protein kinase is required to maintain normal kinetics of fast DNA double strand break repair in MYBL2 deficient haematopoietic stem cells. BM cells were obtained from Mybl2+/+ (n=3) and Mybl2+/∆ (n=3) animals aged 7 weeks and enriched for cKit using microbeads. cKit+ enriched cells were cultured for 1 h in medium containing cytokines and an inhibitor of DNA-dependent protein kinase (DNA-PK) (NU7441, 1 µM). Cells were exposed to 2Gy IR in vitro and cultured for a further 1, 5 or 24 h. Flk2- HSCs were purified using cell sorting and prepared for immunofluorescence and comet assays. (a) Experimental scheme for isolation and culture of cKit+ cells with DNA-PK inhibitor for immunofluorescence and comet assays. (b) Representative images of Flk2-HSCs stained with 53BP1. (c) Percentage of Flk2- HSCs positive for 53BP1. (d) Number of 53BP1 foci per cell. (e) Representative images of Flk2- HSCs stained with γH2AX. (f) Percentage of Flk2- HSCs positive for γH2AX. (g) Fluorescence intensity of γH2AX foci. (h) Representative images of Flk2- HSCs stained with MRE11 (scale bar 10 um). (i) Percentage of Flk2- HSCs positive for MRE11. (j) Mean olive tail moment of alkaline comets of Flk2-HSCs at different time points after 2Gy IR in vivo. -/+ indicates on axes indicates if cells were treated with DNA-PK inhibitor. Error bars: mean±SEM p values on the graphs obtained when using a two-tailed unpaired Student’s t-test.

Figure 5. ATM-signalling is affected in MYBL2 deficient haematopoietic stem cells. BM cells were obtained from Mybl2+/+ (n=3) and Mybl2+/∆ (n=3) animals aged 7 weeks and enriched for cKit using microbeads. cKit+ enriched cells were cultured for 1 h in medium.
containing cytokines and an inhibitor of ATM (KU60019, 10µM). Cells were exposed to 2Gy IR in vitro and cultured for a further 5 h. Flk2- HSCs were purified using cell sorting and immunofluorescence for 53BP1 and DAPI was performed. (a) Experimental scheme for isolation and culture of cKit+ cells with ATM inhibitor for immunofluorescence. (b) Representative images of 53BP1 staining of Flk2- HSCs treated with the ATM inhibitor KU60019. (c) Percentage of Flk2- HSCs positive for 53BP1 foci when treated with KU60019. (d) Number of 53BP1 foci per cell when Flk2- HSCs were treated with KU60019. -/+ indicates on axes indicates if cells were treated with ATM inhibitor. Error bars: mean±SEM . Samples were not statistically significant when using a two-tailed unpaired Student’s t-test.

**Figure 6.** MYBL2 deficient haematopoietic stem cells show a deficiency in the maintenance of ATM-dependent P-Kap1 and increased telomere fragility after irradiation. (a-c) BM cells were obtained from Mybl2+/+ (n=2) and Mybl2−/Δ (n=2) animals aged 7 weeks and enriched for cKit using microbeads. Cells were exposed to 2 Gy IR in vitro and cultured for a further 30 min, 3 h or 5 h. Flk2- HSCs were purified using cell sorting and immunofluorescence for P-Kap1 (S824) and DAPI was performed. (a) Experimental scheme for isolation and culture of cKit+ cells for immunofluorescence. (b) Representative images of P-Kap1 in Flk2- HSCs at different time points after IR. Scale bar = 5µm. (c) P-Kap1 corrected total cell fluorescence for each HSC at different time points after IR. (d) Purified Flk2- HSCs were obtained from Mybl2+/+ (n=2) and Mybl2−/Δ (n=2) animals aged 7 and 70 weeks by cell sorting. Cells were exposed to 2Gy IR in vitro and cultured for a further 7 days in methylcellulose semi-solid medium containing cytokines. Colonies were dissociated and cultured with colcemid to arrest cells in metaphase. Metaphase preparations were performed and chromosomes stained with telomere PNA and DAPI. Shown are examples of fragile
telomeres found in 7 and 70 weeks old cells. Table shows the number of chromatid ends scored and the percentage of chromatid ends with fragile telomeres. p values in the table were obtained using a Mann-Whitney test comparing numbers of fragile telomeres in Mybl2+/+ and Mybl2+/Δ of the same age. Error bars on graphs: mean±SEM and p values obtained when using a two-tailed unpaired Student’s t-test are indicated.
Table 1

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Figure 2.
A 2 Gy ionising radiation

1, 3, 5 and 24 h

7 or 70 week old Myb2+/+ and Myb2+/Δ mice

Extraction of bone marrow cells

cKit+ enrichment

Cell sorting

Fk2- HSC (KSL Fk2-)

SLAM HSC (KSL CD48-CD150+)

Immunofluorescence and comet assay

B

7 week
Myb2+/+
2 Gy 5 h

7 week
Myb2+/Δ
2 Gy 5 h

70 week
Myb2+/+
2 Gy 5 h

70 week
Myb2+/Δ
2 Gy 5 h

DAPI

53BP1

C

% of 53BP1 foci positive cells (>6 foci)

0 Gy

2 Gy 1 h

2 Gy 2 h

2 Gy 3 h

2 Gy 4 h

2 Gy 5 h

7 week Sh

70 week Sh

D

% of 53BP1 foci positive cells (>6 foci)

Myb2+/+

Myb2+/Δ

0 Gy

2 Gy 5 h

E

Myb2+/+

0 Gy

Myb2+/+

2 Gy 5 h

Myb2+/Δ

2 Gy 5 h

Mean olive tail moment

0 Gy

2 Gy 1 h

2 Gy 2 h

2 Gy 3 h

2 Gy 5 h

Figure 3.
Figure 4.
Figure 6.
MYBL2 supports DNA double strand break repair in haematopoietic stem cells

Rachel Bayley, Daniel Blakemore, Laila Cancian, et al.

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