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Role of lncRNA *Morrbid* in *PTPN11*(*Shp2*)^{E76K}-driven juvenile myelomonocytic leukemia

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Key Points

- Myeloid-specific *Morrbid* is hyperactivated in a mouse model of JMML (*Shp2*^{E76K}) and regulates the number of leukemic cells.
- Low expression of *Morrbid/MORRBID* is associated with improved survival in mice and humans.

Mutations in *PTPN11,* which encodes the protein tyrosine phosphatase SHP2, contribute to ~35% of cases of juvenile myelomonocytic leukemia (JMML). A common clinical picture in children with JMML is that it presents as a constitutive hyperinflammatory syndrome, partially reminiscent of chronic myelomonocytic leukemia in adults. Thus, a component of JMML is associated with a hyperinflammatory state and abundant innate immune cells such as neutrophils and monocytes. Recently, we showed that the evolutionarily conserved mouse lncRNA *Morrbid* is specifically expressed in myeloid cells and uniquely represses the expression of the proapoptotic gene *Bim* to regulate the lifespan of myeloid cells. However, its role in JMML has not been investigated. In this study, we characterized the role of *Morrbid* and its target *Bim*, which are significantly dysregulated in *Shp2*$^{E76K/+}$-bearing myeloid cells, in driving JMML. Loss of *Morrbid* in a mouse model of JMML driven by the *Shp2*$^{E76K/+}$ mutation resulted in a significant correction of myeloid and erythroid cell abnormalities associated with JMML, including overall survival. Consistently, patients with JMML who had *PTPN11*, *KRAS*, and *NRAS* mutations and high expression of *MORRBID* manifested poor overall survival. Our results suggest that *Morrbid* contributes to JMML pathogenesis.

Introduction

Juvenile myelomonocytic leukemia (JMML) is the most common myeloproliferative neoplasm (MPN) in childhood. JMML is characterized by mutations in *NF1*, *CBL*, *KRAS*, *NRAS*, or *PTPN11*, and cells from JMML patients show hypersensitivity to GM-CSF in vitro.[1-3] Traditional cytotoxic chemotherapeutic agents are ineffective in JMML, and the only curative modality is allogeneic hematopoietic stem cell transplantation.[4] As the most prevalent mutation in children with MPN, the activated gain-of-function mutation in *PTPN11*, which encodes the protein tyrosine phosphatase SHP2, contributes to ~35% of cases of JMML, and leads to hyperactive Ras-MAPK signaling.[5]

A common clinical picture in JMML is that it presents as a hyperinflammatory syndrome and is often indistinguishable from viral infections.[6] Unlike acute myeloid leukemia (AML) but similar to CMMLchronic myelomonocytic leukemia, JMML rarely progresses to a blast crisis; rather, death is caused by extramedullary myeloid cell expansion and enhanced survival, leading to organ failure, respiratory failure, bleeding, and infection.[1] *SHP2* mutations such as *SHP2*$^{E76K/+}$ and *SHP2*$^{D61Y/+}$ downregulate the expression of the proapoptotic protein Bim, which is associated with enhanced survival relative to controls.[7] Loss of Bim in hematopoietic progenitor cells enhances their survival and shows resistance to apoptosis.[8,9]

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Original data are available by e-mail request to either of the corresponding authors.

The full-text version of this article contains a data supplement.

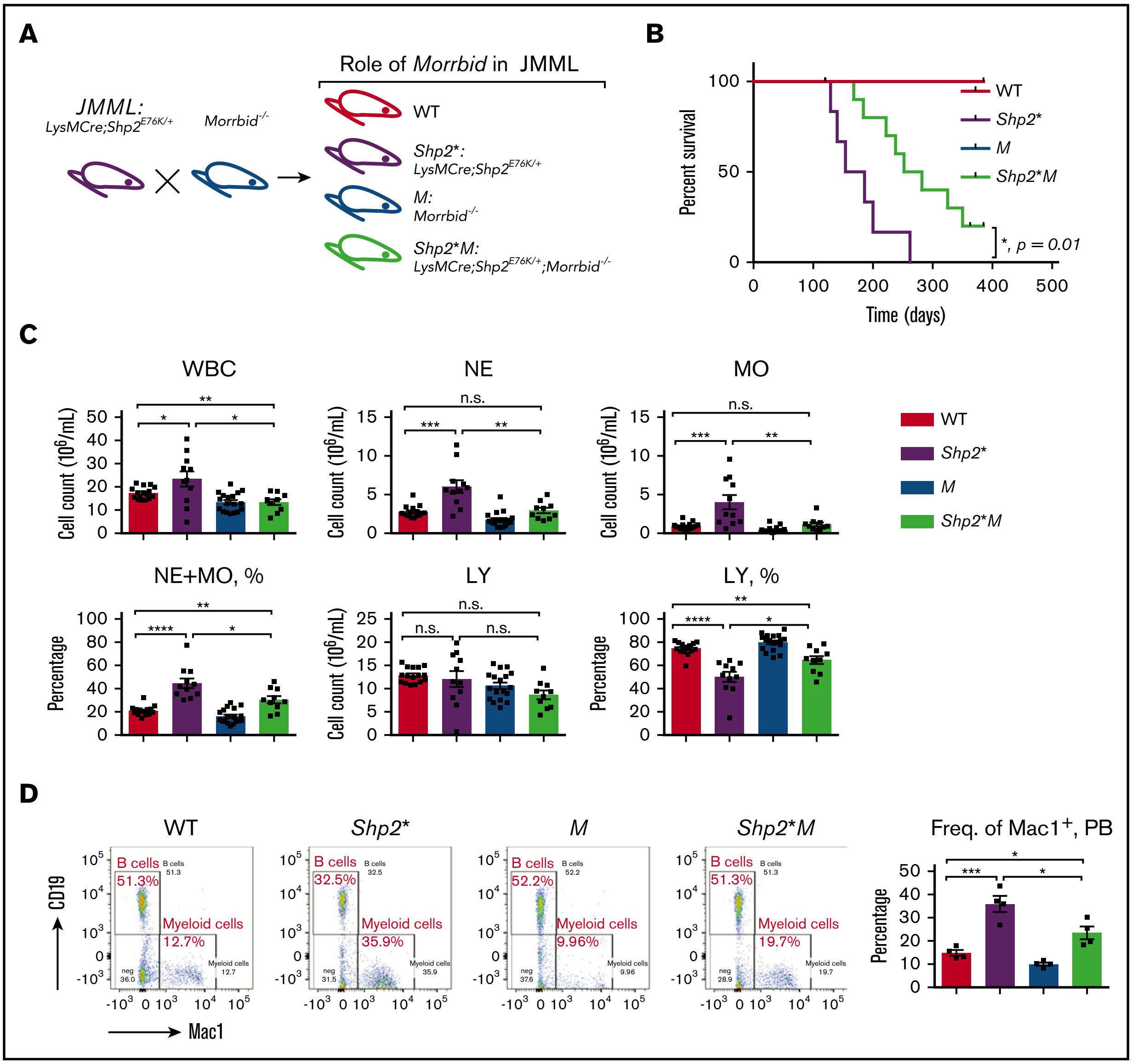

Figure 1. Loss of *Morrbid* enhances the survival of *Shp2*^{E76K}-driven JMML mice. (A) Generation of JMML mice (*Shp2**: *LysMCre; Shp2*$^{E76K/+}$) and JMML mice lacking *Morrbid* (*Shp2*M*: *LysMCre; Shp2*$^{E76K/+}$; *Morrbid*$^{-/-}$) along with WT control and *Morrbid*$^{-/-}$ (*M*). (B) Kaplan-Meier survival curve of *Shp2** and *Shp2*M* mice and control animals. (C) Analysis of hematopoietic cells in PB by the automatic blood cell counter Element HT5 Analyzer. (D) Analysis of WBCs from PB by flow cytometry. Mac1$^+$ staining for myeloid cells and CD19$^+$ staining for B cells (n = 4-18 mice). *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$. See supplemental Figure 1 for improvement of the anemia phenotype in *Shp2*M* mice compared with *Shp2** mice. LY, lymphocytes; n.s., not significant.

Despite the discovery of transcripts derived from the noncoding genome, how these noncoding RNAs, including long noncoding RNAs (lncRNAs), regulate MPN initiation or development is poorly understood. Although only a few lncRNAs related to myeloid malignancies have been described, their function and the targets that they regulate is largely unknown.[10] We have recently shown that, under physiologic conditions, the evolutionarily conserved novel lncRNA *Morrbid* is uniquely expressed in myeloid cells and represses the expression of the proapoptotic gene *Bim* to regulate the lifespan of these cells.[11] Using murine *Shp2*^{E76K}-driven JMML models and a JMML patient database, we studied the role of *MORRBID* in disease initiation and progression. Although genetic loss of *Morrbid* partially mitigates signatures of JMML, our results show that *Morrbid* plays a role in the onset and progression of the disease.

Methods

All the animal experiments were approved by the Laboratory Animal Resource Center at Indiana University School of Medicine. All strains were on a C57/B6 background, and wild-type (WT) mice were procured from the Indiana University School of Medicine Core Facility. JMML model mice (*LysMCre; Ptpn11*$^{E76K/+}$) and *Morrbid*-deficient mice (*Morrbid*$^{-/-}$) have been described[11,12] and were bred to generate the compound mutants. Analysis involving mouse experiments (ie, flow cytometry strategies and intracellular staining

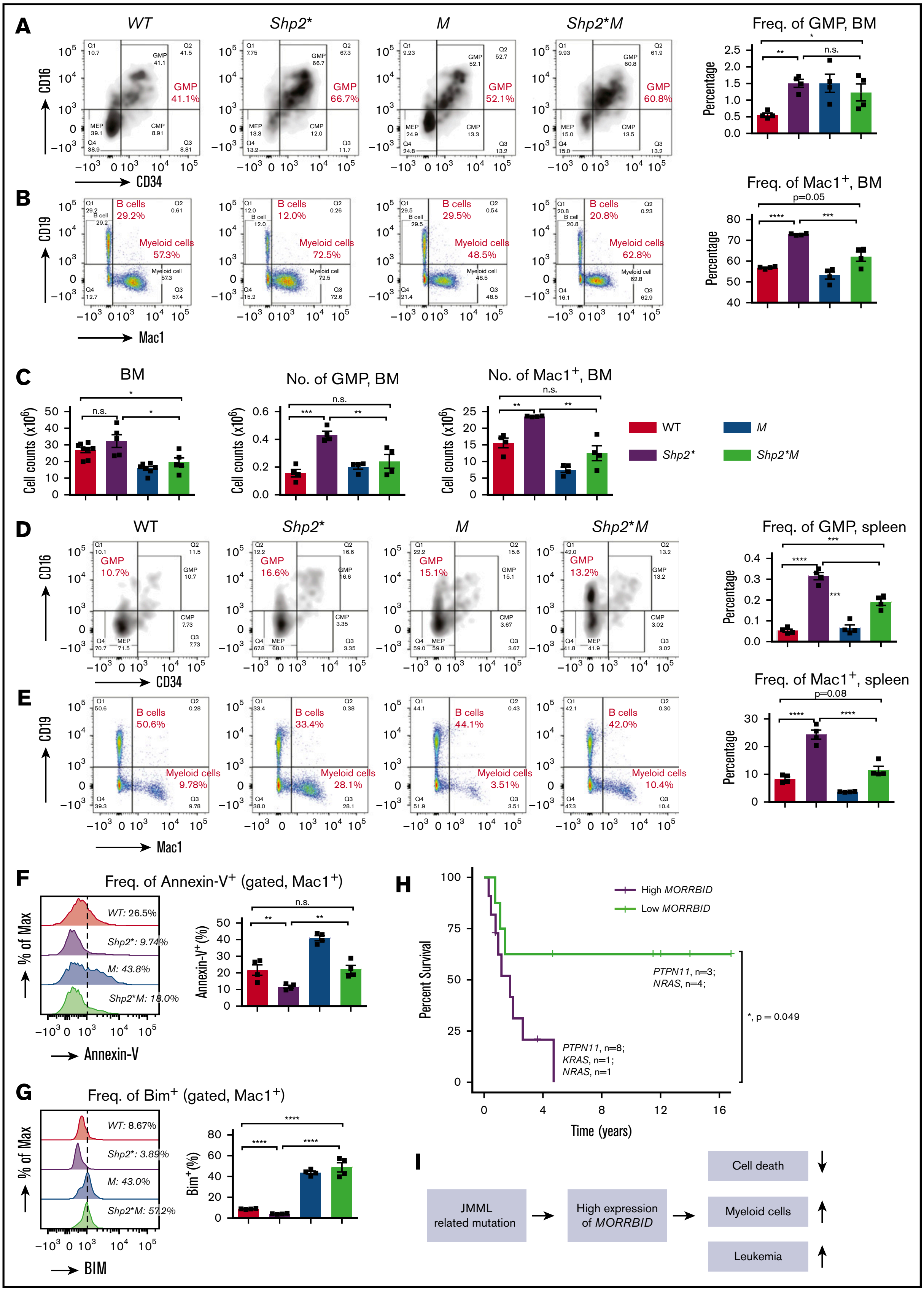
A
WT
Shp2*
M
Shp2*M
CD16
CD34
GMP 41.1%
GMP 66.7%
GMP 52.1%
GMP 60.8%
Freq. of GMP, BM
Percentage
**
n.s.
*
B
CD19
Mac1
B cells 29.2%
Myeloid cells 57.3%
B cells 12.0%
Myeloid cells 72.5%
B cells 29.5%
Myeloid cells 48.5%
B cells 20.8%
Myeloid cells 62.8%
Freq. of Mac1+, BM
p=0.05
****
***
C
BM
No. of GMP, BM
No. of Mac1+, BM
Cell counts (x10^6)
WT
M
Shp2*
Shp2*M
D
Freq. of GMP, spleen
GMP 10.7%
GMP 16.6%
GMP 15.1%
GMP 13.2%
E
Freq. of Mac1+, spleen
p=0.08
B cells 50.6%
Myeloid cells 9.78%
B cells 33.4%
Myeloid cells 28.1%
B cells 44.1%
Myeloid cells 3.51%
B cells 42.0%
Myeloid cells 10.4%
F
Freq. of Annexin-V+ (gated, Mac1+)
% of Max
WT: 26.5%
Shp2*: 9.74%
M: 43.8%
Shp2*M: 18.0%
Annexin-V
Annexin-V+ (%)
G
Freq. of Bim+ (gated, Mac1+)
WT: 8.67%
Shp2*: 3.89%
M: 43.0%
Shp2*M: 57.2%
BIM
Bim+ (%)
H
High MORRBID
Low MORRBID
Percent Survival
Time (years)
PTPN11, n=3;
NRAS, n=4;
PTPN11, n=8;
KRAS, n=1;
NRAS, n=1
*, p = 0.049
I
JMJL related mutation
High expression of MORRBID
Cell death ↓
Myeloid cells ↑
Leukemia ↑

Figure 2.

for annexin-V and anti-Bim) have been described.[13] Unless stated otherwise, statistical analysis in animal experiments was performed with GraphPad Prism 6 for Kaplan-Meier survival curve comparison or using Student *t* test or 1-way analysis of variance for group comparison.

The bioinformatics analysis of *MORRBID* in human JMML was conducted as follows. To identify which lncRNAs potentially associate with JMML, we downloaded raw microarray data for JMML samples, with or without *PTPN11*, *KRAS*, and *NRAS* mutation from the GEO database (GSE71935),[14] along with the pertinent clinical data on the patients. The downloaded data were normalized by the MAS5 method. Survival analysis was performed over the expression of lncRNA *MORRBID* and clinical data, using the log-rank test encoded in the survival R package (https://github.com/therneau/survival). Considering that the data of very young patients (<18 months) were not representative of the disease progression, only older patients with JMML (>18 months at the last follow-up) were included for survival analysis in the study.

Results and discussion

Shp2 is indispensable for hematopoiesis.[15,16] Hematopoietic cells expressing the gain-of-function isoform of Shp2, *Shp2*^{E76K}, which mimics the human *PTPN11* mutation in JMML, confer cell survival advantage and alterations in cell proliferation and differentiation.[12] The murine model bearing *Shp2*^{E76K} (*Ptpn11*$^{E76K/+}$) driven by lysosome-cre (*LysMCre; Ptpn11*$^{E76K/+}$, indicated as *Shp2** mice hereafter) is often used to study JMML, because it induces acute alterations in hematopoiesis similar to human JMML.[12,15] We have shown that, in inflammatory conditions, the expression of lncRNA *Morrbid* is elevated, whereas the level of Bim is downregulated.[11] Consistently, we have shown that *Morrbid* is upregulated, and *Bim* is downregulated in myeloid cells derived from *Shp2** mice.[13] To test the functional requirement of *Morrbid* in the development of JMML, we generated a mouse model of JMML that lacked *Morrbid*: *LysMCre; Shp2*$^{E76K/+}$*; Morrbid*$^{-/-}$ (indicated herein as *Shp2*M*). We compared the disease progression between *Shp2** and *Shp2*M* mice, along with WT and *Morrbid*$^{-/-}$ (*M*) controls (Figure 1A).

We assessed overall survival of *Shp2*M* mice relative to *Shp2** controls up to a year (Figure 1A). Although both WT and *M* mice showed a normal lifespan, the median survival for *Shp2** mice was 170 days, which was extended to 270 days for *Shp2*M* mice (n = 4-10; **P* = .01), indicating that *Shp2*M* mice show an improved overall lifespan (Figure 1B). We next compared the hematological parameters in peripheral blood (PB), bone marrow (BM), and spleen of 4 experimental groups. At the moribund or semimoribund stage (typically, 5-6 months for *Shp2** and 8-9 months for *Shp2*M*), both *Shp2** and *Shp2*M* mice showed comparable high overall white blood cell (WBC) counts and neutrophil (NE) and monocyte (MO) counts in PB (~10-fold higher than WT; data not shown). At 1 to 4 months of age, compared with WT mice, *Shp2** mice developed JMML-like symptoms, including higher WBC, NE, and MO counts, which were noted in animals as young as 3 to 4 weeks. At the same age, however, *Shp2*M* mice showed significantly lower WBC, NE, and MO counts compared with *Shp2** mice (Figure 1C). Furthermore, the percentage of NEs and MOs in WBCs was also reduced in *Shp2*M* mice compared with that in *Shp2** mice, but was still higher than that in WT (Figure 1C). Consistent with these findings, flow cytometry showed a significant correction in the frequency of Mac-1^{+} cells in *Shp2*M* mice compared with *Shp2** mice (Figure 1D).

To determine whether the loss of *Morrbid* alters the hematopoietic abnormalities associated with *Shp2** mice in the BM and spleen, we again analyzed all 4 groups of mice at 3 to 4 months of age. A significant difference in the frequency of granulocyte-macrophage progenitors (GMPs) was observed in the BM of WT and *Shp2** mice (*Shp2** vs WT, *P* < .01; Figure 2A). In contrast, although the increased frequency of GMPs was not modulated in *Shp2*M* mice, the BM cellularity in *Shp2*M* mice was significantly reduced compared with that in *Shp2** mice (*Shp2** vs *Shp2**M, *P* < .05; Figure 2C). Thus, the absolute number of GMPs in *Shp2*M* mice was significantly reduced compared with the number in *Shp2** mice (*Shp2** vs *Shp2*M*, *P* < .01; Figure 2C). With regard to mature myeloid cells in the BM, both the frequency and the absolute number of Mac1^{+} cells were significantly reduced in *Shp2*M* mice compared with *Shp2** mice (Figure 2B-C). Consistent with previous reports, *Shp2** mice manifested an obvious splenomegaly compared with WT mice as young as 1 month. Although, spleen weight and size were comparable between *Shp2** and *Shp2*M* mice at all time points examined (data not shown), *Shp2*M* mice showed a correction in the frequency of both GMPs and Mac1^{+} cells compared with *Shp2** mice (Figure 2D-E). Furthermore, although *Shp2*M* mice rapidly developed anemia at the moribund stage, similar to *Shp2** mice, the onset of the anemia was much later and milder in young *Shp2*M* mice than in *Shp2** mice. At 2 to 4 months of age, *Shp2*M* mice showed normal frequency of erythroid progenitors, red blood cell counts, and hemoglobin concentration, similar to WT mice, whereas *Shp2** mice showed impaired maturation of erythroid progenitors (supplemental Figure 1).

Given the correction in the frequency and absolute number of Mac-1^{+} cells in *Shp2*M* mice compared with *Shp2** mice, we

Figure 2. **Loss of *Morrbid* rescues mature and immature myeloid cells in the BM and spleen of *Shp2** mice.** Mononuclear cells were collected from the BM (A-C) and spleens (D-E) of the 4 experimental groups (WT, *Shp2**, *M*, and *Shp2*M*; age, 3-4 months) for analyzing hematopoiesis. Changes in myeloid progenitors, GMPs, and mature myeloid cells were analyzed. Lin^{-}Sca1^{-}cKit^{+} cells were pregated for GMP flow cytometry profiles (CD34^{+}CD16^{+}). In BM, both the frequency and number of GMPs and mature myeloid cells were quantified. In spleen, only the frequency of GMPs and mature myeloid cells was quantified. (F) The level of apoptosis in mature myeloid cells was determined by flow cytometry using annexin-V staining, a marker for proapoptosis. (G) The level of Bim expression in mature myeloid cells was determined by intracellular flow cytometry with an anti-Bim antibody. (H) Kaplan-Meier survival plots of JMML patients with high or low expression of *MORRBID*. The purple line indicates the survival curve of children with JMML who had high expression of *MORRBID* (n = 10 patient samples: 8 with mutations in *PTPN11*, 1 in *KRAS*, and 1 in *NRAS;* age >18 months), and the green line indicates that of JMML children with low expression of *MORRBID* (n = 7 patient samples; 3 with mutations in *PTPN11* and 4 in *NRAS;* age >18 months). The high expression of *MORRBID* was associated with poor overall survival of these patients (*P* = 0.049). See supplemental Figure 2 for the survival curve of JMML patients without mutations in *PTPN11*, *KRAS*, and *NRAS*. (I) Model for *Morrbid* regulation of myeloid cell survival in JMML. For mouse samples, n = 4 mice; for human samples, n = 7-10. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

sought to determine the extent to which loss of *Morrbid* in these cells impacts their survival. Reduced apoptosis observed in Mac-1^+ *Shp2** cells was normalized to WT levels in *Shp2*M* Mac-1^+ cells (Figure 2F-G), which was associated with increased expression of Bim compared with controls (Figure 2G). These results show that targeting *Morrbid* in *Shp2** Mac-1^+ cells induces apoptosis and significantly corrects the absolute number of mature myeloid cells in the BM, spleen, and PB (Figure 2I).

To assess whether *MORRBID* also plays a role in human JMML, we conducted data mining studies to assess the correlation between expression of *MORRBID* and JMML prognosis. In a limited number of human JMML samples with *PTPN11*, *KRAS*, and *NRAS* mutations, we found that children with JMML who showed increased expression of *MORRBID* were associated with poor overall survival compared with patients who had low levels of *MORRBID* (Figure 2H; $n = 7\text{-}10$; $P = .049$; patients aged >18 months were included for survival analysis, whereas those aged <18 months were excluded). These data are consistent with our survival studies using the murine model of JMML shown in Figure 1B. Moreover, although it was not statistically significant because of the small sample size, we observed a similar trend between *MORRBID* expression and survival in patients with JMML lacking mutations in *PTPN11*, *KRAS*, and *NRAS* ($n = 9$ for each subgroup; $P = .227$; supplemental Figure 2).

In summary, our results demonstrate an essential role for *Morrbid* in regulating the pathogenesis of *Shp2*^{E76K} mice, including hematologic abnormalities and mortality seen in this model of human JMML. Future studies will determine how inhibition of *MORRBID* in JMML patient-derived cells affects their growth and engraftment in mice. In addition, it would also be prudent to assess the impact of *Morrbid* deletion in a model of *Shp2*^{E76K} in which Shp2^{E76K} is active in hematopoietic stem cells. Although *Morrbid* is expressed in myeloid cells, use of a conditional knockout model of *Morrbid* to assess its function specifically in hematopoietic stem cells would shed new light on how *Morrbid* contributes to the JMML phenotypes driven by Shp2^{E76K} in stem cells.

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Authorship

Contribution: Z.C. and R.K. conceived and designed the experiments, analyzed the data, and wrote the manuscript; Z.C. performed most of the experiments and acquired the data; C.Z. performed the bioinformatics analysis; and J.J.K., A.W., and J.H.-M. contributed reagents.

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