JCI The Journal of Clinical Investigation

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J Clin Invest. 2020. https://doi.org/10.1172/JCI140707.

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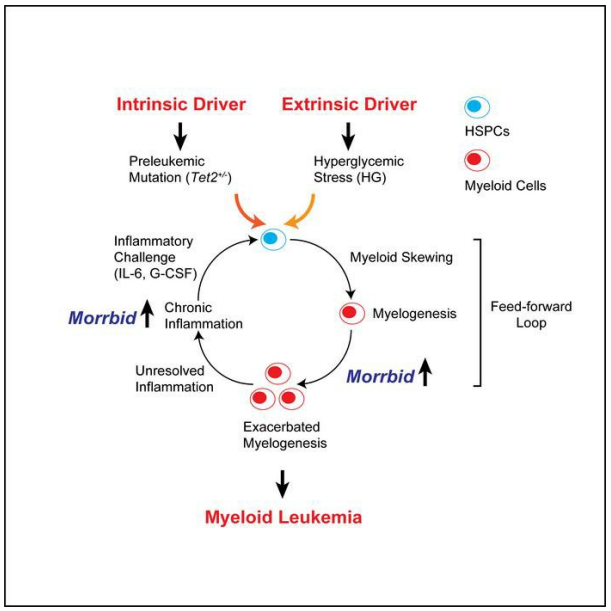

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Concise Communication

Hyperglycemia cooperates with *Tet2* heterozygosity to induce leukemia driven by pro-inflammatory cytokine induced lncRNA *Morrbid*

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KEY WORDS: Leukemia, Hyperglycemic stress, *Tet2*, *Morrbid*, Inflammation

RUNNING TITLE: Hyperglycemia cooperates with *Tet2* mutation to induce leukemia

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ABSTRACT

Diabetes mellitus (DM) is a risk factor for cancer. The role of DM-induced hyperglycemic stress (HG) in blood cancer is poorly understood. Epidemiologic studies show that individuals with DM are more likely to possess higher rate of mutations in genes found in pre-leukemic stem and progenitor cells (pre-LHSC/Ps) including *TET2*. *TET2*-mutant pre-LHSC/Ps require additional hits to evolve into a full-blown leukemia and/or aggressive myeloproliferative neoplasm (MPN). Intrinsic mutations have been shown to cooperate with *Tet2* to promote leukemic transformation. However, extrinsic factors are poorly understood. Utilizing a mouse model bearing haploinsufficiency of *Tet2*, to mimic the human pre-LHSC/P condition and HG stress, in the form of an $Ins2^{Akita/+}$ mutation, which induces HG, we show that the compound mutant mice develop a lethal form of MPN and/or acute myeloid leukemia (AML). RNA-seq revealed that this is in part due to upregulation of pro-inflammatory pathways, thereby generating a feed-forward loop, including the expression of an anti-apoptotic lncRNA *Morrbid*. *Morrbid* loss in the compound mutants rescued the lethality and mitigated MPN/AML. We describe a mouse model for age-dependent AML/MPN and suggest that HG acts as an environmental driver for myeloid neoplasm, which could be prevented by reducing the expression of inflammation-related lncRNA *Morrbid*.

INTRODUCTION

Cancer and Diabetes are prevalent diseases that share multiple common risk factors such as metabolic dysfunction, ageing and chronic inflammation. It has been hypothesized that Diabetes is a risk factor for some types of solid cancers. More importantly and relevant to this study are strong epidemiological findings showing a link between diabetes and leukemia. Studies have shown an increased risk of leukemia development in both patients with Type-1 or Type-2 Diabetes (1) (2) . Given the large number of diabetic individuals worldwide, this has now become a tremendous public health concern. The unaddressed question in the field has to do with if hyperglycemia (HG), contributes to leukemia and/or MPN initiation/development and what are the possible mechanisms by which this occurs. While epidemiologic findings clearly suggest a correlation between DM and blood cancers, no animal models have been described to interrogate their causative relationship, which will be critical to the understanding of the mechanisms of HG induced leukemogenesis and consequently for the development of appropriate therapies.

In an effort to directly examine the correlation between DM and blood cancers, a recent meta-study utilizing exome-sequencing on peripheral blood (PB) cells from individuals with DM and normal individuals revealed that people at an older age or with DM have higher rate of gene mutation in blood cancer genes compared to controls (3). Meta-study also revealed that genes encoding two epigenetic regulators *TET2* and *DNMT3A* are among the top genes mutated in elderly and DM individuals, consistent with the association of *TET2* mutations in age-related clonal hematopoiesis (ARCH) in elderly normal people (3) (4). *TET2* mutations are not only prevalent in healthy normal elderly individuals manifesting ARCH but is also one of the most highly mutated gene in MPNs, chronic myelomonocytic leukemia (CMML), myelodysplasia syndrome (MDS), secondary AML and de novo AML (3, 4).

Murine models for studying the biological function of Tet2 have been established. These mice show: 1) increased HSC self-renewal; 2) myeloid lineage skewing; and 3) age dependent leukocytosis but not AML development (5). This manifestation is mostly seen with close to full penetrance, when both alleles of Tet2 are deleted (*Tet2*$^{-/-}$) but not when a single allele of Tet2 is deleted (*Tet2*$^{+/-}$). In majority of AML, CMML and MPN patients, with *TET2* mutations, only one allele of *TET2* is mutated. These findings suggest that secondary hits in *Tet2*$^{+/-}$ HSC/Ps are necessary for the development of AML and/or full blow MPN. To this end, little is known about the impact of extrinsic factors in the modulation of Tet2 deficient cells and on overall disease pathogenesis. We and others have recently shown that lipopolysaccharide (LPS) or bacterial challenge of Tet2 deficient mice induces a strong inflammatory response (6-8). Based on these findings, we hypothesized that chronic inflammation due to hyperglycemic stress (high glucose exposure) may potentiate and exacerbate the *Tet2*$^{+/-}$ pre-LHSC/P phenotype to result in the development of AML and/or severe MPN. Utilizing a mouse model bearing haploinsufficiency of *Tet2 (Tet2*$^{+/-}$), to mimic the human pre-LHSC/P condition and HG stress, in the form of an *Ins2*$^{Akita/+}$ mutation, which induces HG and Type-1 DM in mice, we show that the compound mutant mice develop a lethal form of MPN/AML driven in part by upregulation of pro-inflammatory pathways, including the expression of an anti-apoptotic lncRNA *Morrbid*.

RESULTS and DISCUSSION

Ins2$^{Akita/+}$ induced HG stress and *Tet2* heterozygosity cooperate to drive aggressive MPN/AML in a mouse model.

Ins2$^{Akita/+}$ mice carry a point mutation in the insulin-encoding gene *Insulin 2* (*Ins2*), which leads to insufficient insulin production and apoptosis in pancreatic cells. This strain is widely used as a genetic murine model for Type-1 Diabetes (T1DM) research and is pathologically comparable

but more stable compared to the streptozotocin (STZ) induced chemical diabetic model (9). *Ins2*$^{Akita/+}$ mice (both males and females) were bred to *Tet2*$^{+/-}$ (males and females) mice to obtain *Ins2*$^{Akita/+}$;*Tet2*$^{+/-}$ compound mutants mice and controls (**Figure 1A**). The compound mutant mice showed increased blood glucose levels and reduced body weight similar to *Ins2*$^{Akita/+}$ mutants (two typical physiological characteristics of T1DM, **Figure 1E**). We did not observe any hematological abnormalities in any of the experimental groups until 6 months of age, when the mice from *Tet2*$^{+/-}$*;Ins2*$^{Akita/+}$ group started to succumb. All of the *Tet2*$^{+/-}$*;Ins2*$^{Akita/+}$ mice died between 6-9 month of age with a median survival time of 221 days while all other control mice survived over 300 days (**Figure 1B**).

In addition to the typical features of myeloid neoplasia including splenomegaly and pale bones, a myeloid sarcoma-like aberrant tissue growth was observed in all of the *Tet2*$^{+/-}$*;Ins2*$^{Akita/+}$ moribund mice (**Figure 1C** and **D**). Hematologic analysis on peripheral blood (PB) samples demonstrated that the moribund *Tet2*$^{+/-}$*;Ins2*$^{Akita/+}$ mice manifest an emergency-granulopoiesis or severe MPN-like phenotype, including increased absolute counts and percentages of neutrophils associated with a significant reduction in red blood cells (**Figure 1F**). Flow cytometry analysis of PB and bone marrow (BM) from these mice demonstrated a significant increase in the frequency of Mac-1$^+$ mature myeloid cells and a profound reduction in CD19$^+$ B cells as well as a significant increase in the frequency of AML blasts, characterized by the expression of cKit$^+$/Mac-1$^+$ cells and Ly6G$^+$/Mac1$^+$ cells (**Figure 1G** to **I; Supplemental Figure 1A** and **B**). Furthermore, in the BM, both the frequency of lineage negative, Sca-1$^+$ and cKit$^+$ (LSK) cells, hematopoietic stem cells (HSCs; LSK/CD48$^-$/CD150$^+$), granulocyte macrophage progenitors (GMPs; LSK/CD16$^+$/CD34$^+$) as well as more mature myeloid cells (Mac-1$^+$) was significantly increased in the compound mutants compared to other genotypes, while B cell development was repressed (**Figure 1I**; **Supplemental Figure 1A**). The cells in the myeloid sarcoma are dominated by Mac1$^+$/Ly6G$^+$ (data not shown). Taken together, these results demonstrate that

Ins2$^{Akita/+}$ induced HG stress cooperates with *Tet2* heterozygosity to induce a severe MPN/AML-like disease between 6 to 9 months of age.

The presence of severe MPN/AML-like phenotype in compound mutants is largely driven by progressive inflammation and inflammatory cytokine induced lncRNA *Morrbid*

To characterize the pathology and potential driver(s) of mortality in *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ compound mutant mice, we performed histological analysis of various tissues, serological analysis of cytokines and RNA-seq analysis on Lin$^-$ hematopoietic progenitors. Except for more blast-like cells in peripheral blood of the compound mutants, no obvious alterations were found in histology of other tissues (**Supplemental Figure 1D** and data not shown). Serum cytokine analysis revealed significant increase in the presence of pro-inflammatory cytokines such as IL-6 as well as in the levels of cytokines involved in driving emergency granulopoiesis such as G-CSF (**Figure 2A**). Furthermore, bioinformatics analysis of the RNA-Seq data revealed significant new information with regard to global unbiased changes in gene expression, which were quite dramatic in Lin$^-$ cells derived from *Ins2*$^{Akita/+}$;*Tet2*$^{+/-}$ mice relative to controls. Gene set enrichment analysis (GSEA) revealed that pathways involved in innate immune reactome, IL-6 mediated signaling and TLR4-NFκB signaling were significantly altered in *Ins2*$^{Akita/+}$;*Tet2*$^{+/-}$ mice compared to controls ($p^* < 0.0025$, **Figure 2C**). Such significant changes were not observed between *Tet2*$^{+/-}$ and WT or between *Ins2*$^{Akita/+}$ and WT at similar time point (data not shown). Although, not statistically significant, the JAK-STAT pathway also appeared to be upregulated in *Ins2*$^{Akita/+}$;*Tet2*$^{+/-}$ mice relative to controls ($p=0.0699$, **Figure 2C**).

We also examined the expression of individual genes by plotting their normalized RPKM values (Reads Per Kilobase of transcript, per Million mapped reads; normalized to WT controls). We confirmed *Tet2* expression was reduced to about 50% level of wild type in *Tet2*$^{+/-}$ and

Ins2$^{Akita/+}$;*Tet2*$^{+/-}$ mice, which was verified by qRT-PCR analysis (**Figure 2B** and **D**). Consistent with the overall evaluation by GSEAs shown in **Figure 2C**, a profound upregulation in production of pro-inflammation cytokines including *Csf3*, *Il1b*, *Cxcl1* and *Cxcl10* and the components associated with innate immune response including the expression of ligands for the toll like receptor (TLR), S100A family of ligands was detected in the *Ins2*$^{Akita/+}$;*Tet2*$^{+/-}$ mice relative to controls (**Figure 2D**). An increase in the expression of downstream innate immune signaling molecules from TLRs such as *Nfkbiz* and *Myd88* was also observed in these mutants and verified by qRT-PCR (**Figure 2D; Supplemental Figure 1E**). Furthermore, consistent with the observed reduction in the frequency of CD19^{+} B cells in the BM, the expression of B cell specific transcription factor Pax5 (encoded by *Pax5*). was also significantly reduced in compound mutants (**Figure 2D**). The increase in myeloid cells correlated with increased in myeloid specific transcription factor PU.1 (encoded by *Spi1*). Importantly, *Gata-2* locus has recently been shown to be impacted in *Tet2*$^{-/-}$*;Flt3*^{ITD} mice, which develop AML associated with increased presence of cKit+/Mac-1+ blasts (10). We too observed a reduction in the expression of *Gata-2* in *Tet2*$^{+/-}$*;Ins2*$^{Akita/+}$ compound mutant mice (**Figure 2D**). Interestingly, in addition to the usual suspects, noted above, RNA-seq analysis revealed a significant upregulation in the expression of an anti-apototic lncRNA, *Morrbid* in *Tet2*$^{+/-}$*;Ins2*$^{Akita/+}$ compound mutant mice (**Figure 2D**), which was verified by qRT-PCR analysis (**Figure 2B**). We have recently demonstrated increased expression of *MORRBID* in human AML cells, which is associated with poor patient survival (11).

Morrbid is specifically expressed in myeloid cells and uniquely represses the expression of the pro-apoptotic gene *Bim* via DNA loop *in cis* to regulate the lifespan of myeloid cells (12). *Morrbid*$^{-/-}$ mice manifest inefficient production of innate immune cells (~50% of wild type level) but have normal activity and lifespan (12). Since *Morrbid* is specifically expressed in myeloid cells (i.e. neutrophils, monocytes and eosinophils) but not in other cell types under physiologic

conditions, the uniqueness of its expression pattern and function offer a great advantage and opportunity to test if it is required for the onset and progression of aggressive MPN/AML-like phenotype in *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ compound mutant mice.

Given the presence of elevated IL-6 and increased *Morrbid* expression in *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ compound mutants, combined with the manifestation of a hyper-inflammatory condition, we hypothesized that inflammation-induced *Morrbid* expression significantly contributes to the mortality and the manifestation of the severe MPN/AML phenotype driven via the cooperative interaction between *Tet2*$^{+/-}$ and *Ins2*$^{Akita/+}$ in *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ mice. To test this, we generated *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$;*Morrbid*$^{-/-}$ triple mutant mice along with controls (**Figure 2E**). Indeed, we observed that *Morrbid* loss completely reversed the early lethality in *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ mice (**Figure 2F**). Although no significant difference in the content of BM progenitors was observed between *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ and *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$;*Morrbid*$^{-/-}$ mice at one time point, a significant correction in splenomegaly, anemia and in the content of mature myeloid cells in the PB and BM was observed in *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$;*Morrbid*$^{-/-}$ mice relative *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ mice (6~8 month of age, **Figure 2G** and **H**). Furthermore, although slightly higher than the levels in the WT mice, serum levels of G-CSF and IL-6 were dramatically decreased in *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$;*Morrbid*$^{-/-}$ mice compared to *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ mice (6~8 month of age, **Figure 2I**). Taken together, “rescue experiment” via *Morrbid* loss strongly suggests that aberrant inflammation contributed to the early onset mortality and severe MPN/AML-like phenotype observed in the aged *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ mice.

A model is described in **Figure 3** to summarize current findings. We suggest that *Tet2* mutation (an intrinsic factor) cooperates with hyperglycemic stress (HG, an extrinsic factor) to induce a higher grade of inflammation, perpetuating a feed-forward loop, resulting in leukemogenesis overtime. Myeloid-specific survival regulator lncRNA *Morrbid* contributes to two critical steps

induced by cooperation of *Tet2* mutation and HG: (1) exacerbated myelogenesis and (2) chronic inflammation. Loss of *Morrbid* effectively breaks the feed-forward loop. In line with this model, we have recently demonstrated that high expression of *MORRBID* is associated with *TET2* mutation in human AML and is associated with poor prognosis (11). It will be intriguing to test if such correlation also exists in AML patients who have diabetes. Additionally, although the present study suggests that inflammation plays an important role in hyperglycemia-induced leukemia development, it does not rule out the possibility that hyperglycemia can directly induce epigenetic changes and promote leukemogenesis, as suggested by a recent study demonstrating that hyperglycemia can alter the stability of TET2 protein via AMPK signaling pathway (13). Furture studies are required to test if hyperglycemia changes TET protein levels and/or activity in the hematopoietic system. Of note, although the survival is greatly improved in *Tet2*$^{+/-}$*;Ins2*$^{Akita/+}$*;Morrbid*$^{-/-}$ mice, the two deaths out of nine manifested splenomegaly, indicating the development of leukemia in these two moribund mice. In our recent study, a similar scenario was observed in a model of juvenile myelomonocytic leukemia (JMML) driven by Shp2^{E76K}; suggesting that loss of *Morrbid* only mitigates or delays leukemia development rather than completely prevents it (14).

In conclusion, the present study not only describes an important animal model to study the relationship between hematologic malignancies and diabetes, but also provides a strong role for inflammation in driving early onset mortality in this model. Thus, the *Tet2*$^{+/-}$*;Ins2*$^{Akita/+}$ and *Tet2*$^{+/-}$*;Ins2*$^{Akita/+}$*;Morrbid*$^{-/-}$ mice can function as a tremendous resource to study the relationship between diabetes, inflammation and myeloid neoplasia.

METHODS

Mice:

C57/B6 mice (CD45.2^{+}) were procured from IUSM core facility and used as wildtype controls. *Tet2*$^{+/-}$ and *Morrbid*$^{-/-}$ mice have been described (5, 12) and *Ins2*$^{Akita/+}$ were obtained from Jackson Laboratory (Cat#003548). Flow cytometry analysis involving mouse experiments have been described in detail in our recent study (6). Measurement of blood glucose was performed in Indiana University Diabetes Research Center.

RNA-seq and GSEA:

Total RNA from Lin-negative cells was purified by Qiagen RNeasy Mini Kit (Cat No./ID: 74104) and used for RNA-seq analysis by Indiana University Center for Medical Genomics. This data has been deposited in public database (GSE158351). For gene set enrichment analysis (GSEA), differential gene expression was computed by using DESeq2. Pathway enrichment analysis was computed by using against Msigdb v6 canonical pathway with $p<0.005$ (or any other cutoff) as a significant cutoff. Log fold change of the expression level of each gene was utilized for the GSEA analysis. For RPKM plotting, individual gene's RPKM of subject groups was normalized to that of WT group.

Statistical analysis:

All experimental procedures on mice were run in parallel with proper controls (sex and age matched littermate controls when possible) for observing experiment variabilities. Analysis of grouped data was not blinded and no samples were excluded. Error bars indicate the standard error of mean (s.e.m.). Except GSEA, statistical anayisis was performed by software Prism 6 (Ordinary one-way ANOVA for multiple comparisions; Kaplan-Meier survival package for survival comparisions).

Study Approval:

Animal experiments were approved by the institutional IACUC (Protocol #11424) and maintained by Laboratory Animal Resource Center at Indiana University School of Medicine.

Author contributions: ZC, LH and RK conceived and designed the experiments, analyzed data and wrote the manuscript. ZC performed most of the experiments and acquired the data. XL, CZ, FF and NK performed the RNA-seq raw data collection and pathway analysis. SN, FA, BR and AH assisted with certain experiments and acquired part of the data. JK, AW and JHM contributed reagents.

Acknowledgments: We thank our colleagues for technical support, for critically reading our manuscript and their suggestions to improve the manuscript. This work was supported in part by grants from National Institutes of Health (R01-CA134777, R01-HL140961, R01-HL146137, R01-CA173852 to RK) and by funds to RK from the Riley Children's Foundation. ZC is supported by fund from T32HL007910. We would also like to thank Ms. Tracy Winkle for her administrative support.

Conflict of interest: The authors have declared that no conflict of interest exists.

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FIGURE LEGENDS

Figure 1. An age-dependent AML/MPN model driven by hyperglycemic stress and preleukemic mutation. (A) Schematic for generating compound mutant *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ mice along with WT, *Tet2*$^{+/-}$ and *Ins2*$^{Akita/+}$ controls. **(B)** Kaplan-Meier survival curve of compound mutant *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ mice along with controls. Only compound mutant *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ mice show early onset of mortality between 6 to 9 months of age with 100% penetration. The median survival day of compound mutant mice is about 7 months (221 days) while all controls survive over the 300-day period after which the experiment was discontinued. **(C)** At the succumb stage (6-9 month old), all the compound mutant *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ mice display visible myeloid sarcoma under the skin around ocular area on one or both sides (red arrow), pale bones (blue arrow) and splenomegaly. **(D)** Quantitation of spleen weight at 6~9-months of age when the compound mutant mice turn moribund. **(E)** Mutant *Ins2*$^{Akita/+}$ and *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ mice manifest increased blood glucose and decreased body weight compared to WT and *Tet2*$^{+/-}$ mice throughout all stages. The plots show data at 5 months of age. **(F)** Hematologic analysis on peripheral blood

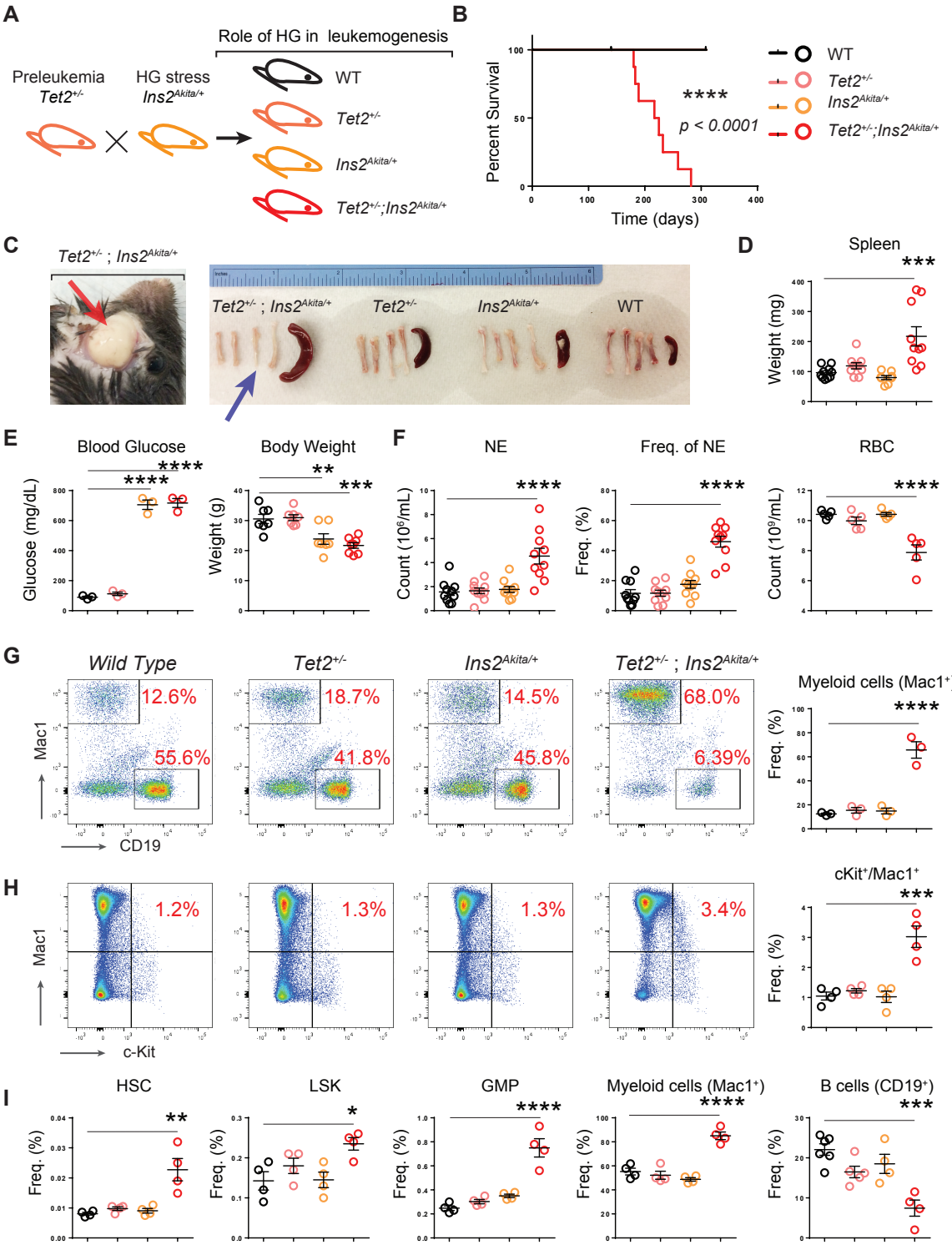
(PB) by automatic blood cell counter Element HT5. NE, neutrophil. RBC, Red blood cells. **(G)** Analysis of myeloid cell compartment by flow cytometry stained by Ly6G and Mac1 antibodies. **(H)** Analysis of bone marrow (BM) mononuclear cells by flow cytometry using antibodies that recognize cKit and Mac1. **(I)** Quantification analysis of frequencies of hematopoietic stem and progenitor cells and mature cells in the BM. n = 4~10 mice. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$. ****, $p < 0.0001$. In Prism 6, Kaplan-Meier survival package for **B** and one-way ANOVA for **D** to **I**.

Figure 2. The MPN/AML-like phenotype in compound mutants is largely driven by progressive inflammation and inflammatory cytokine induced lncRNA *Morrbid*. (A) Serological analysis on PB samples from various genotypes show an increase in G-CSF and IL-6 in compound mutant mice. **(B)** qRT-PCR analysis to detect the expression of *Tet2* and *Morrbid* in lineage-negative cells from various genotypes. **(C)** Gene set enrichment analysis (GSEA) derived from RNA-seq dataset on Lineage-negative cells of BM from 4 groups of mice (6-8 month of age). Comparisons between $Tet2^{+/-};Ins2^{Akita/+}$ and WT reveal significant changes in the innate immune pathway, IL-6 mediated signaling pathway and TLR4-NFkB signaling pathway. In addition, JAK-STAT pathway demonstrated a trend of upregulation in $Tet2^{+/-};Ins2^{Akita/+}$ mice compared to WT. (**D**) Representative quantitative graphs of various innate immune response related genes derived from the RNA-seq dataset, including a *Bim*-targeting lncRNA *Morrbid*, are shown in the right panels. **(E)** Schematic for generating triple mutant $Tet2^{+/-};Ins2^{Akita/+};Morrbid^{-/-}$ mice along with WT, $Tet2^{+/-};Ins2^{Akita/+}$ and $Morrbid^{-/-}$ controls. **(F)** Loss of *Morrbid* in compound mutants rescues the lethality associated with them compared to controls. Nine mice were analyzed in the $Tet2^{+/-};Ins2^{Akita/+};Morrbid^{-/-}$ cohort and the number of mice in the other cohorts were ten. **(G)** Hematological comparison between $Tet2^{+/-};Ins2^{Akita/+};Morrbid^{-/-}$ and $Tet2^{+/-};Ins2^{Akita/+}$ mice. **(H)** Splenomegaly and aberrant myeloid cell/B cell imbalance in compound heterozygous mutants is reversed in $Tet2^{+/-};Ins2^{Akita/+};Morrbid^{-}$

$^{/-}$ mice. **(I)** Serum levels of G-CSF and IL-6. Age of the mice in G to I is ~ 6-8 months old. n = 2~13 mice. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$. ****, $p < 0.0001$. In Prism 6, Kaplan-Meier survival package for **F**, one-way ANOVA for for **A, B, D** and **G-I.**

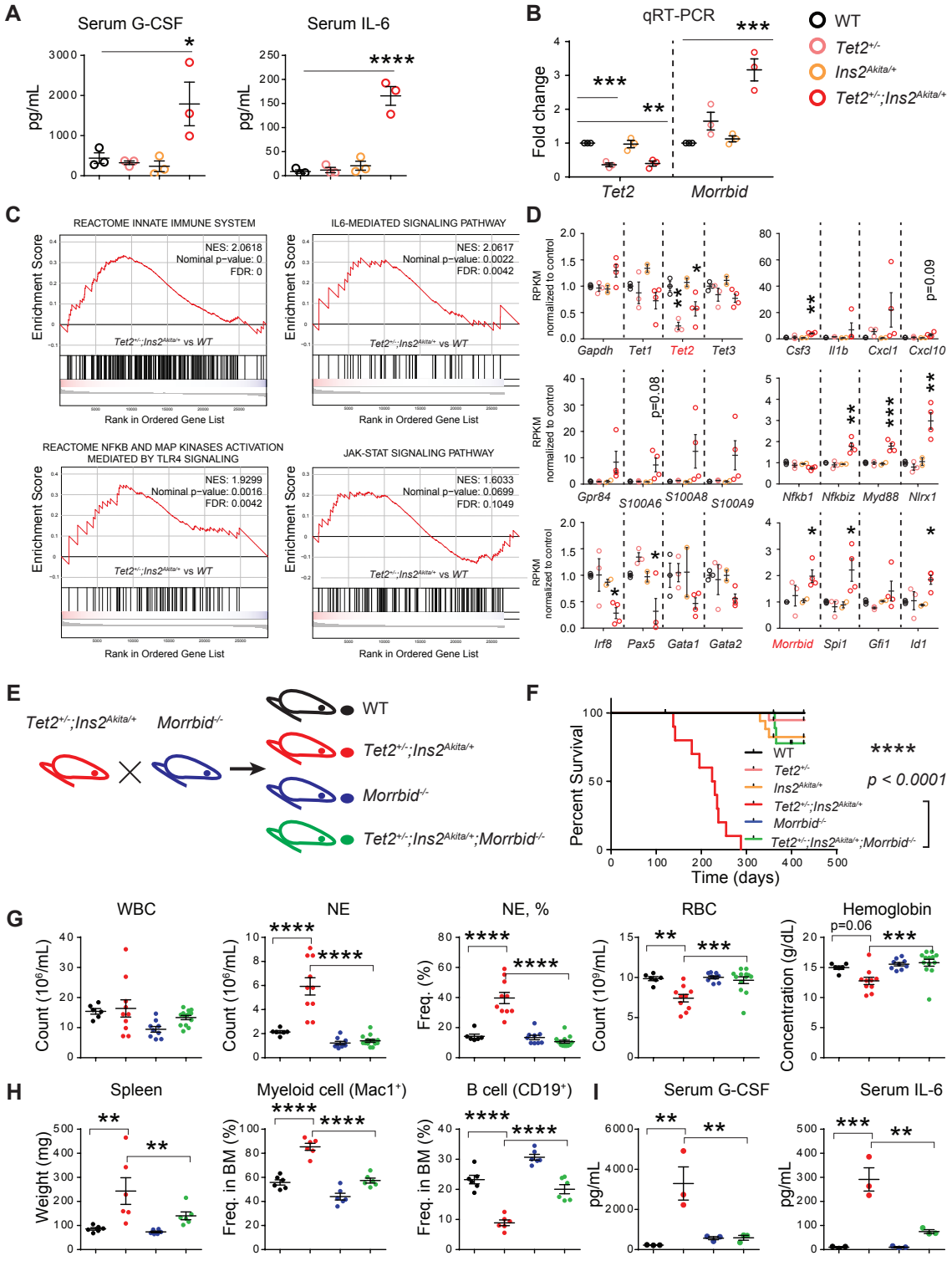
Figure 3. A "feed-forward loop" model for cooperation between *Tet2*$^{+/-}$ preleukemic mutation and hyperglycemic stress (HG). *Tet2* heterozygosity (*Tet2*$^{+/-}$) defines a preleukemic condition in hematopoietic stem cells (HSCs) and intrinsically promotes HSC self-renewal and myeloid skewing under normal physiological conditions. Consequently, the *Tet2*$^{+/-}$ mice develop a low-grade MPN. Extrinsic stress induced by HG exacerbates systematic inflammation and cooperates with *Tet2* heterozygosity to perpetuate myeloid skewing/myelogenesis, chronic inflammation and production of pro-inflammatory cytokines such as IL-6 and G-CSF. Myeloid-specific survival regulator lncRNA *Morrbid* regulates myelogenesis, chronic inflammation, and also functions as a downstream effector of IL-6 and G-CSF. Consequently, loss of *Morrbid* mitigates the feed-forward loop observed in compound mutant mice.

Supplemental Figure 1. *Tet2*$^{+/-}$*;Ins2*$^{Akita/+}$ mice develop inflammation and leukemia over time. (A) Quantification of B cell frequency (CD19^{+}) in peripheral blood. **(B)** Representative flow cytometry profiles of mature myeloid cells (Mac1^{+}/Ly6G^{+}) in peripheral blood. **(C)** Representative flow cytometry profiles of hematopoietic progenitors including LSK cells, HSCs and GMPs in bone marrow. **(D)** Representative H & E staining of blood cells. **(E)** Expression of two genes in TLR4-NFκB pathway, *Myd88* and *Nfkbiz*, re-assessed by qRT-PCR. n = 3~4 mice (6~8 month old). ***, $p < 0.001$. ****, $p < 0.0001$. In Prism 6, one-way ANOVA for for **A** and **E.**



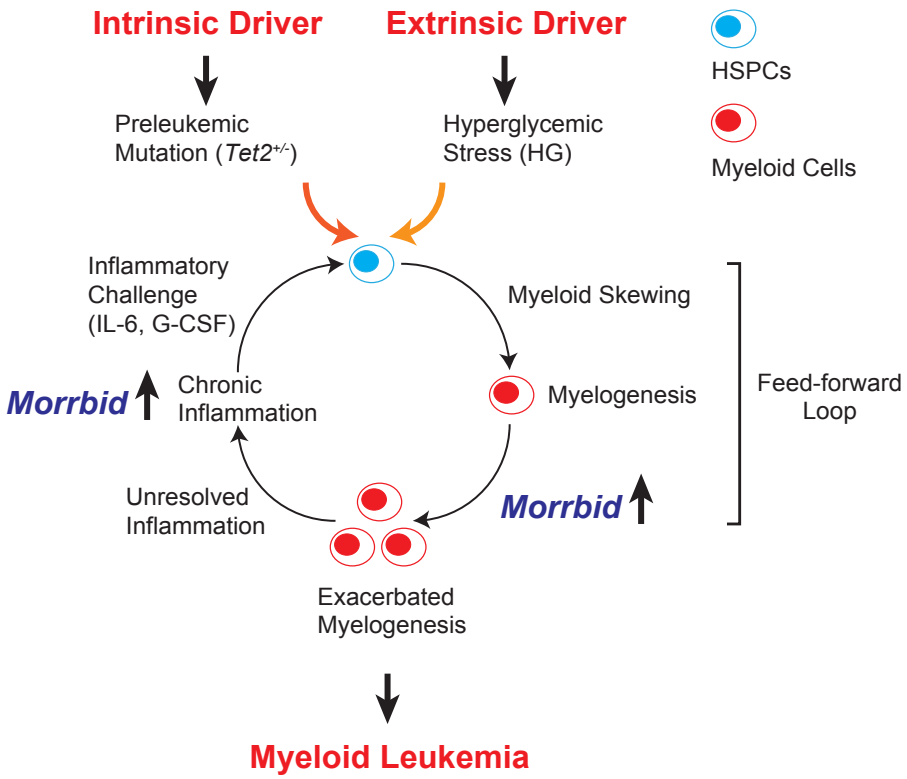
Cai *et al.* Figure 1

Figure 1. An age-dependent AML/MPN model driven by hyperglycemic stress and preleukemic mutation. (A) Schematic for generating compound mutant *Tet2*$^{+/-}$*;Ins2* mice along with WT, T*et2*$^{+/-}$ and *Ins2*$^{Akita/+}$ controls. **(B)** Kaplan-Meier survival curve of compound mutant *Tet2*$^{+/-}$*;Ins2*$^{Akita/+}$ mice along with controls. Only compound mutant *Tet2*$^{+/-}$*;Ins2*$^{Akita/+}$ mice show early onset of mortality between 6 to 9 months of age with 100% penetration. The median survival day of compound mutant mice is about 7 months (221 days) while all controls survive over the 300-day period after which the experiment was discontinued. **(C)** At the succumb stage (6-9 month old), all the compound mutant *Tet2*$^{+/-}$*;Ins2*$^{Akita/+}$ mice display visible myeloid sarcoma under the skin around ocular area on one or both sides (red arrow), pale bones (blue arrow) and splenomegaly. **(D)** Quantitation of spleen weight at 6~9-months of age when the compound mutant mice turn moribund. **(E)** Mutant *Ins2*$^{Akita/+}$ and *Tet2*$^{+/-}$*;Ins2*$^{Akita/+}$ mice manifest increased blood glucose and decreased body weight compared to WT and *Tet2*$^{+/-}$ mice throughout all stages. The plots show data at 5 months of age. **(F)** Hematologic analysis on peripheral blood (PB) by automatic blood cell counter Element HT5. NE, neutrophil. RBC, Red blood cells. **(G)** Analysis of myeloid cell compartment by flow cytometry stained by Ly6G and Mac1 antibodies.**(H)** Analysis of bone marrow (BM) mononuclear cells by flow cytometry using antibodies that recognize cKit and Mac1. **(I)** Quantification analysis of frequencies of hematopoietic stem and progenitor cells and mature cells in the BM. n = 4~10 mice. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$. ****, $p < 0.0001$. In Prism 6, Kaplan-Meier survival package for B and one-way ANOVA for D to I.



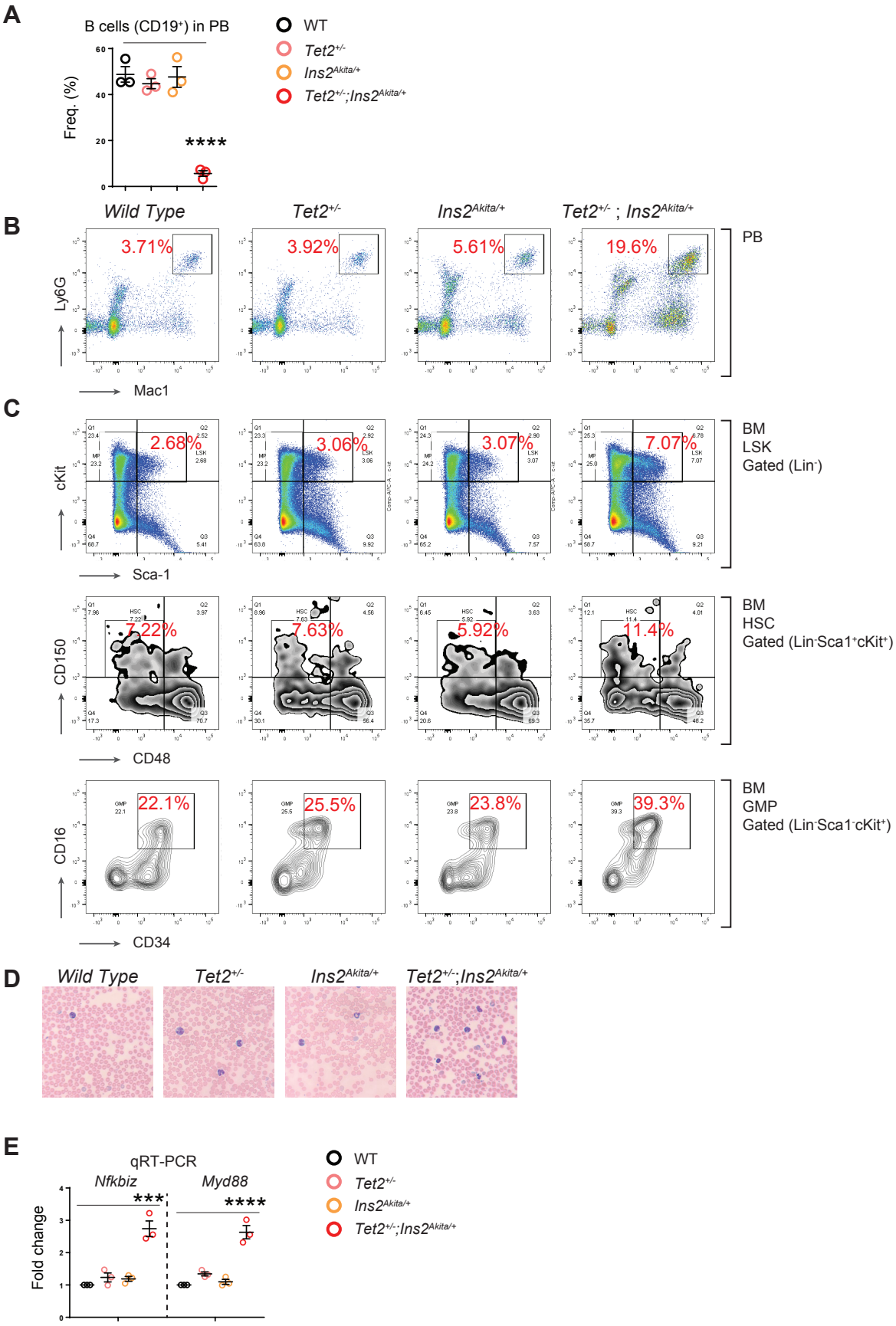
Cai et al. Figure 2

Figure 2. The MPN/AML-like phenotype in compound mutants is largely driven by progressive inflammation and inflammatory cytokine induced lncRNA *Morrbid*. (A) Serological analysis on PB samples from various genotypes show an increase in G-CSF and IL-6 in compound mutant mice. **(B)** qRT-PCR analysis to detect the expression of *Tet2* and *Morrbid* in lineage-negative cells from various genotypes. **(C)** Gene set enrichment analysis (GSEA) derived from RNA-seq dataset on Lineage-negative cells of BM from 4 groups of mice (6-8 month of age). Comparisons between *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ and WT reveal significant changes in the innate immune pathway, IL-6 mediated signaling pathway and TLR4-NFkB signaling pathway. In addition, JAK-STAT pathway demonstrated a trend of upregulation in *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ mice compared to WT. **(D)** Representative quantitative graphs of various innate immune response related genes derived from the RNA-seq dataset, including a *Bim*-targeting lncRNA *Morrbid*, are shown in the right panels. **(E)** Schematic for generating triple mutant *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$;*Morrbid*$^{-/-}$ mice along with WT, *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ and *Morrbid*$^{-/-}$ controls. **(F)** Loss of *Morrbid* in compound mutants rescues the lethality associated with them compared to controls. Nine mice were analyzed in the *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$;*Morrbid*$^{-/-}$ cohort and the number of mice in the other cohorts were ten. **(G)** Hematological comparison between *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$;*Morrbid*$^{-/-}$and *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$ mice. **(H)** Splenomegaly and aberrant myeloid cell/B cell imbalance in compound heterozygous mutants is reversed in *Tet2*$^{+/-}$;*Ins2*$^{Akita/+}$;*Morrbid*$^{-/-}$mice. **(I)** Serum levels of G-CSF and IL-6. Age of the mice in G to I is ~ 6-8 months old. n =2~13 mice. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$. ****, $p < 0.0001$. In Prism 6, Kaplan-Meier survival package for F, one-way ANOVA for for A, B, D and G-I.



Cai et al. Figure 3

Figure 3. A "feed-forward loop" model for cooperation between *Tet2*$^{+/-}$ preleukemic mutation and hyperglycemic stress (HG). *Tet2* heterozygosity (*Tet2*$^{+/-}$) defines a preleukemic condition in hematopoietic stem cells (HSCs) and intrinsically promotes HSC self-renewal and myeloid skewing under normal physiological conditions. Consequently, the *Tet2*$^{+/-}$ mice develop a low-grade MPN. Extrinsic stress induced by HG exacerbates systematic inflammation and cooperates with *Tet2* heterozygosity to perpetuate myeloid skewing/myelogenesis, chronic inflammation and production of pro-inflammatory cytokines such as IL-6 and G-CSF. Myeloid-specific survival regulator lncRNA *Morrbid* regulates myelogenesis, chronic inflammation, and also functions as a downstream effector of IL-6 and G-CSF. Consequently, loss of *Morrbid* mitigates the feed-forward loop observed in compound mutant mice.



Cai *et al.* Supplemental Figure 1

Supplemental Figure 1. *Tet2*$^{+/-}$*;Ins2*$^{Akita/+}$ mice develop inflammation and leukemia over time. (A) Quantification of B cell frequency (CD19+) in peripheral blood. **(B)** Representative flow cytometry profiles of mature myeloid cells (Mac1^{+}/Ly6G^{+}) in peripheral blood. **(C)** Representative flow cytometry profiles of hematopoietic progenitors including LSK cells, HSCs and GMPs in bone marrow. **(D)** Representative H & E staining of blood cells. **(E)** Expression of two genes in TLR4-NFκB pathway, *Myd88* and *Nfkbiz*, re-assessed by qRT-PCR. n = 3~4 mice (6~8 month old). ***, $p < 0.001$. ****, $p < 0.0001$. In Prism 6, one-way ANOVA for for A and E.