Acute megakaryocytic leukemia (AMKL) is a clinically heterogeneous subtype of acute myeloid leukemia characterized by unrestricted megakaryoblast proliferation and poor prognosis. Thrombopoietin receptor c-Mpl is a primary regulator of megakaryopoiesis and a potent mitogenic receptor. aberrant c-Mpl signaling has been implicated in a myriad of myeloid proliferative disorders, some of which can lead to AMKL, however, the role of c-Mpl in AMKL progression remains largely unexplored. Here, we identified increased expression of a c-Mpl alternative splicing isoform, c-Mpl-del, in AMKL patients. We found that c-Mpl-del expression was associated with enhanced AMKL cell proliferation and chemoresistance, and decreased survival in xenografted mice, while c-Mpl-del knockdown attenuated proliferation and restored apoptosis. Interestingly, we observed that c-Mpl-del exhibits preferential utilization of phosphorylated c-Mpl-del C-terminus Y607 and biased activation of PI3K/AKT pathway, which culminated in upregulation of GATA1 and downregulation of DDIT3-related apoptotic responses conducive to AMKL chemoresistance and proliferation. Thus, this study elucidates the critical roles of c-Mpl alternative splicing in AMKL progression and drug resistance, which may have important diagnostic and therapeutic implications for leukemia accelerated by c-Mpl-del overexpression.

INTRODUCTION
Acute megakaryocytic leukemia (AMKL) is a heterogenous subtype of acute myeloid leukemia (AML) characterized by unrestricted proliferation of immature megakaryocytes (megakaryoblasts), and extensive myelofibrosis [1, 2]. Clinically, the disease is bimodally distributed with peaks in children between the ages 1 and 3 and adults ages 50 and 60 [3, 4]. AMKL in children is often strongly correlated with a genetic basis, with a better prognosis, particularly, in Down syndrome patients [5, 6]. In adults, the cytogenetic profile is diverse and the genetic link diffuse, as such the disease is less well understood [7, 8]. Importantly the prognosis is extremely poor and is considered an independent adverse prognostic factor for overall survival in AML [8, 9]. Leukemogenesis in AMKL can be de novo or secondary to myeloproliferative disorders, with a large proportion of patients possessing a normal karyotype, suggesting potential aberrancy at the molecular or protein level [10–12]. Therefore, mechanisms beyond cytogenetic abnormalities may constitute a significant basis for AMKL progression and thus warrants further research.

Fei Li1,11, Yuanyan Xiong1,11, Mo Yang2,11, Peiling Chen1, Jingkai Zhang1, Qiong Wang1,3, Miao Xu4,5, Yiming Wang4,5,6, Zuyong He1, Xin Zhao1, Junyu Huang1, Xiaojiong Gu2, Li Zhang2, Rui Sun6, Xunsha Sun6, Jingyao Li1, Jinxin Ou1, Ting Xu1, Xueying Huang1, Yange Cao1, Xiaohong Ruby Xu4,5, Danielle Karakas4,5, June Li4,5,6, Heyu Ni4,5,10 and Qing Zhang1,3,5

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State Key Laboratory of Bioscience, Sun Yat-sen University, Guangzhou, China. 2Institute of Sun Yat-sen University in Shenzhen, Shenzhen, China. 3Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada. 4Department of Laboratory Medicine, Keenan Research Centre for Biomedical Science of St. Michael’s Hospital, and Toronto Platelet Immunobiology Group, University of Toronto, Toronto, Canada. 5Canadian Blood Services Centre for Innovation, Toronto, Canada. 6Department of Blood Transfusion, Clinical Biological Resource Bank and Clinical Lab, Guangzhou Institute of Pediatrics, Guangzhou Women and Children’s Medical Center, Guangzhou Medical University, Guangzhou, China. 7State Key Laboratory of Oncology in South China, Sun Yat-sen University Cancer Center, Guangzhou, China. 8National Key Clinical Department and Key Discipline of Neurology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China. 9Department of Physiology, University of Toronto, Toronto, Canada. 10Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada. 11These authors contributed equally: Fei Li, Yuanyan Xiong, Mo Yang.

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receptors develop the ability to transduce mitogenic signals [20, 21]. One regulatory mechanism of c-Mpl function is through alternatively spliced isoforms [22, 23]. In humans, four isoforms of c-Mpl have been identified including c-Mpl-p, c-Mpl-k, c-Mpl-tr, and c-Mpl-del, of which c-Mpl-p encodes the full-length 635 amino acid functional receptor [24, 25]. c-Mpl-k possesses an alternate intracellular domain and c-Mpl-tr is a prematurely truncated isoform, with potentially both acting as dominant-negative isoforms of the receptor [23, 26]. Previous studies also have shown that the c-Mpl-del isoform which encompasses a 24 amino acid deletion between exons 8 and 9 in the extracellular region was detected in CD34+ cells, megakaryocytes, and
platelets isolated from healthy donors and essential thrombocythemia (ET) patients as well as megakaryocytic leukemia cells [27–29]. The presence of c-Mpl-del might reflect the stable adaptive regulation for megakaryocyte development and abnormal expansion of hematopoietic cells by having different roles in physiological and pathological functions. However, the relative expression levels of c-Mpl-del in megakaryoblastic leukemia cells are unknown partly due to the rarity of AMKL cases and the lack of quantitative and specific detection to distinguish other c-Mpl isoforms. This highlights the need for a renewed and precise approach to the prognosis and treatment of AMKL.

In this study, we observed a significant increase of c-Mpl-del in AMKL patients and identified a critical role for c-Mpl-del in AMKL progression, survival, and resistance to chemotherapy. These findings not only improve our understanding of AMKL progression but also uncover potential novel therapeutic targets and biomarkers in AMKL.

RESULTS

**c-Mpl-del, but not wild-type c-Mpl-p, is highly expressed in AMKL cells**

c-Mpl-del expression originates from the alternative splicing of 72 bp between exons 8 and 9 in the extracellular region of c-Mpl [29]. To discover the expression of c-Mpl-del isoform and whether c-Mpl-del expression is correlated with the clinical-pathologic characteristics of AMKL patients, we firstly analyzed c-Mpl-del and c-Mpl-p expression in TCGA-LAML and GTEx-blood datasets and distinguished the c-Mpl-del (c-Mpl-del expressed samples) and c-Mpl-p (only c-Mpl-p expression, without c-Mpl-del expression) groups by extracting the splice junction reads in each BAM file (Fig. S1A). Notably, a total of 36 c-Mpl-del expressed AMKL patients (24%) exhibited significantly high expression of c-Mpl compared to the 115 AML patients without c-Mpl-del expression (Fig. 1A). However, only 4 of the 348 (1 percent) blood samples from healthy people showed c-Mpl-del expression (Fig. 1A), suggesting that c-Mpl-del likely has an AML-biased expression. Meanwhile, AML patients with c-Mpl-del expression had shorter survival times than those without c-Mpl-del expression (Fig. 1B). Gene ontology (GO) annotation analysis of the differentially expressed genes (DEGs) between c-Mpl-del and c-Mpl-p samples for TCGA-LAML dataset also demonstrated that the c-Mpl-del expression involved in the regulation of multiple haematological physio-pathological processes (Fig. S1B). In our previous work, we had observed that the c-Mpl-del appears in human AMKL Dami cells, a subtype of AML, by targeting c-Mpl sequence, which could bind TPO and activate ERK1/2 signaling in c-Mpl-del-transfected NIH3T3 cells [27]. To identify the c-Mpl-del isoform within AMKL patients which were confirmed to contain blast hypercellularity (Fig. S1C), we performed qRT-PCR utilizing primers to specifically detect the expression of wild-type c-Mpl-p and splicing mutant c-Mpl-del (Fig. 1C). Amplification of cDNA yielded the full-length wild-type and truncated spliced variant isoform, corresponding to the c-Mpl-p and c-Mpl-del transcripts respectively, in bone marrow cells of AMKL patients (Fig. 1D). Importantly, c-Mpl-del but not c-Mpl-p exhibited significantly increased expression in AMKL patients compared to healthy controls at both the transcript and protein level (Fig. 1E, F). Notably, high expression of c-Mpl on the bone marrow cell surface of AMKL patients as determined by flow cytometry might imply that c-Mpl-del could be expressed on the cell membrane and mediate signal transduction (Fig. 1G). RNA fluorescence in situ hybridization (FISH) using splicing region-specific probes for c-Mpl-del and PCR amplification of cDNA corroborated that c-Mpl-del expressed in AMKL cell lines (Meg-01, UT-7, and Dami) (Fig. 1H, I). This was further verified with melting curve analysis and quantitative PCR that c-Mpl-del was elevated in AMKL cells Meg-01, UT-7, and Dami (Fig. 1J and Fig. S1D, E). Together, these data identify an increased expression of c-Mpl-del in AMKL patients and may potentially contribute to AMKL progression.

**Increased expression of c-Mpl-del enhances AMKL cells proliferation**

to determine the functional role of c-Mpl-del expression in response to TPO simulation in AMKL, we overexpressed c-Mpl-p or c-Mpl-del in Dami and UT-7 cells that were termed Dami-P/UT-7-P, Dami-DEL/UT-7-DEL (Fig. S1E). We observed a dose-dependent proliferative response to TPO in both c-Mpl-p and c-Mpl-del transfected cell lines. However, interestingly, Dami-DEL exhibited higher proliferative potential compared to Dami-P, despite having similar levels of c-Mpl-del and c-Mpl-p overexpression (Fig. 2A, Fig. S1E). Conversely, c-Mpl-del shRNA targeted knockdown (Dami-shDEL), but not c-Mpl-p knockdown (Dami-shP), exhibited inhibited proliferation compared to control GFP-transfected cells (Dami-GFP) after c-Mpl-del or c-Mpl-p expression was successfully silenced by transfecting Dami cells with lentivirus-mediated shRNAs (Fig. 2A, Fig. S2A, B). Similar results were observed in an analogous AMKL cell line UT-7 (Fig. S2C). Meanwhile, although the rescue of c-Mpl-p expression following knockdown of the endogenously expressed c-Mpl restored the proliferative activity of Dami and UT-7 cells to some extent, the rescue of c-Mpl-del expression was more able to promote the proliferation of Dami and UT-7 cells under the same amount of rescue of c-Mpl-del and c-Mpl-p (Fig. 2B, Fig. S2D, E), indicating that c-Mpl-del has a stronger proliferative activity and TPO sensitivity in AMKL cells than c-Mpl-p. Further indicators of enhanced Dami-DEL proliferation included upregulation of cell cycle regulators cyclin D1 and cyclin D2 (Fig. 2C), complimentary with greater proportions of cells in active S and G2/M versus G0/G1 phases (Fig. 2D). In parallel, ex vivo colony formation in presence of TPO was markedly
increased in Dami-DEL cells but was attenuated in Dami-shDEL cells (Fig. 2E). Interestingly, increased expression of the stem cell markers CD34 and CD38 was positively correlated with c-Mpl-del levels in Dami cells (Fig. S2F) suggesting that c-Mpl-del directly supports self-renewal of AMKL cells. Indeed, although human primary CD34+ cells endogenously express lower c-Mpl-del than c-Mpl-p (Fig. S1E), stable transfection of c-Mpl-del resulted in significantly higher proliferation in a TPO dose-dependent manner (Fig. 2F). This was consistent with proliferation marker Ki-67 upregulation and tumor-suppressor gene PTEN downregulation (Fig. 2G). Taken together these results demonstrate that c-Mpl-del confers an enhanced proliferative response of AMKL cells to TPO and putatively contributes to the maintenance of blast self-renewal and leukemogenesis.

**c-Mpl-del overexpression promotes chemotherapeutic drug resistance**

Similar to other AML subtypes, multidrug resistance and relapse remain the major obstacles in conventional AMKL treatment [3, 30]. Ara-c and ATRA are common cytotoxic chemotherapeutics in AML, however, a significant portion of patients do not respond well [18, 31]. We thus investigated the contribution of c-Mpl-del...
on AMKL chemotherapeutic drug resistance. We found increased apoptotic resistance and cell viability in c-Mpl-del overexpressing AMKL cells across various doses of Ara-c and ATRA (Fig. 3A, Fig. S3A). Conversely, c-Mpl-del knockdown reduced the viabilities of AMKL cells compared to control vector-transfected cells (Fig. 3A).

In addition, in puromycin (PM) selected drug-resistant prone Dami cell lines, Ara-c and ATRA maintained increased apoptosis induction by two to three-fold in control Dami-PM but was completely abrogated in Dami-DEL when c-Mpl-del was over-expressed (Fig. 3B). Concomitantly distinct inverse levels of

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Cell Death and Disease (2022) 13:869
upregulated pro-survival Bcl-2 and downregulated pro-apoptotic Bax was not altered by Ara-c or ATRA treatment in Dami-DEL cells. In contrast, Ara-c treatment decreased Bcl-2 and increased Bax in control AMKL cells, with the greatest response seen in c-Mpl-del shRNA knockeddown (Dami-shDEL) (Fig. 3C, Fig. S3B). Moreover, in Ara-c enriched resistant AMKL cells, c-Mpl-del expression significantly increased (Fig. 3D), which suggests a feed-forward response in c-Mpl-del mediated AMKL drug resistance.

We then performed high throughput RNA-seq from Ara-c-treated Dami-DEL and Dami-GFP cells to identify in-depth the differential genes or critical pathways that are activated. The majority of anti-apoptotic genes (such as Bcl-2 and Bcl-xl) were upregulated in Dami-DEL cells. In contrast, the expression of pro-apoptotic gene (such as Bax and Bad) were significantly downregulated by the overexpression of c-Mpl-del. We also saw a shift towards pro-apoptotic gene sets in Ara-c-treated control Dami-GFP cells, including the upregulation of Bax and Bad, which was reversed in Ara-c-treated Dami-DEL cells (Fig. 3E, F and Fig. S3C). Notably, gene set enrichment analysis (GSEA) revealed a highly significant enrichment of negatively correlated DNA damage-inducible transcript 3 protein (DDIT3) genes sets and positively correlated GATA1 in Dami-DEL cells compared to Dami-GFP cells (Fig. 3G, Fig. S3D), both of which have been reported to be involved in the regulation of hematopoiesis and pathogenesis of leukemia, respectively [32, 33]. The qRT-PCR analysis further confirmed the downregulation of DDIT3 and the upregulation of GATA1 in Dami-DEL and UT-7-DEL cells, which was reversed by the transfection of c-Mpl-del-specific shRNAs but not c-Mpl-p-specific shRNAs. Meanwhile, under Ara-c treatment, DDIT3 was upregulated and GATA1 was downregulated in Dami-GFP and UT-7-GFP cells, but this regulation was counteracted by the overexpression of c-Mpl-del (Fig. 3H, I, and Fig. S3E, F), indicating that c-Mpl-del could antagonize Ara-c and downregulate DDIT3 and upregulate GATA1 in AMKL cells. Using CentriMo for motif enrichment analysis of GATA1 from JASPAR database, we also found that GATA1 was significantly positively correlated with c-Mpl-del expression in AML patients (Fig. S3G). In addition, using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, several major signal transduction pathways, including downstream TPO signaling genes, and c-Mpl transcriptional targets, and PI3K/AKT pathways, were modulated by Ara-c in control AMKL cells but blunted in cells with c-Mpl-del overexpression (Table S1, S2). Given that our datamining efforts identified DDIT3 and GATA1 as putative transcriptional factors in response to Ara-c, we next tested the ability of DDIT3 to directly bind to the promoter region of known survival-associated genes including the Bax, Bcl-2, and PTEN by chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR). We found that the DDIT3 protein was able to bind the upstream promoter of Bax and Bcl-2 except for PTEN (Fig. 3J). This supports the notion that the apoptosis-related gene expression was controlled by the transcription factor DDIT3. Thus, these findings suggest TPO-stimulation drives a transcriptional program by which the downregulation of DDIT3 and upregulation of GATA1, skew towards survival and proliferation of c-Mpl-del-expressing AMKL cells.

**TPO/c-Mpl-del-activated PI3K/AKT pathway promotes anti-apoptotic responses**

To further investigate the mechanism by which c-Mpl-del signaling regulates and promotes AMKL cell survival and proliferation, we systematically generated and expressed tyrosine substitution mutants (Y591F/Y567F, Y626F/Y662F, and Y631F/Y607F) within the c-Mpl-p and c-Mpl-del cytoplasmic domain in HEK293T cells. We found lower levels of recovered tyrosine mutants after immunoprecipitation of phosphorylated JAK2 indicating that all Y591/Y567, Y626/Y662 and Y631/Y607 residues in both c-Mpl-p and c-Mpl-del participated in TPO mediated signaling (Fig. 4A). However, we found in c-Mpl-del Y607F mutation TPO-stimulated tyrosine phosphorylation was particularly attenuated, indicating that Y607 is a key tyrosine residue in JAK2-mediated TPO/c-Mpl-del signaling (Fig. 4A). Phosphorylation analysis of downstream signaling molecules in overexpressing c-Mpl-p or c-Mpl-del Dami cells treated with TPO revealed subtle differences and signaling biases, with increased p-AKT in Dami-DEL and increased p-STAT5 and p-JAK2 in Dami-P (Fig. 4B), which was further confirmed by the KEGG analysis of c-Mpl-del-expressed samples for TCGA-LAML dataset (Fig. 4C). Thus, while c-Mpl-p-mediated TPO signaling activate JAK2/STAT5 pathway, c-Mpl-del-mediated TPO signaling may incline towards the PI3K/AKT pathway potentially through preferential phosphorylation of Y607 residue.

We next inhibited TPO/c-Mpl-del signaling pathways in Dami-DEL cells with small-molecule inhibitors of JAK2 (AZD1480), STAT3 (SH-4-54), ERK1/2 (SCH772984) and AKT (AZD5363) to assess its effect on AMKL viability. As expected, AZD1480 and AZD5363, but not SH-4-54 and SCH772984, decreased Bcl-2 expression and upregulated Bax and DDIT3 expression in Dami-DEL cells (Fig. 4D), indicating that the TPO/c-Mpl-del signaling primarily regulates apoptosis-related proteins and transcription factors via the JAK2/PI3K/AKT pathway. Notably, although Dami-DEL cells were resistant to Ara-c and ATRA alone, in the presence of AZD1480 or AZD5363, the pro-apoptotic effect was rescued with decreased Bcl-2 and increased Bax (Fig. 4E). Lentiviral shRNAs transfection also confirmed that c-Mpl-del-mediated Bcl-2 upregulation and Bax downregulation in the presence of Ara-c and ATRA were abolished by JAK2 or AKT knockdown in Dami-DEL cells (Fig. 4F and Fig. S4A). Meanwhile, both Bcl-2 knockdown and Bax overexpression counteracted the antiapoptotic effect of Dami-DEL cells under the treatment of Ara-c and ATRA (Fig. 4G), indicating that c-Mpl-del-mediated anti-apoptotic responses in AMKL cells through JAK2/PI3K/AKT pathway is fully dependent by increased Bcl-2 and decreased Bax.

**c-Mpl-del leads to increased malignant AMKL proliferation in xenograft mice**

Lastly, to establish the in vivo significance of c-Mpl-del expressing AMKL cells in tumorigenesis and metastasis, we established both
an AMKL-ascites and intravenous metastasis mouse model by injection of stable GFP/PM- and luciferase-expressing Dami/Dami-DEL cells into the abdominal cavity or tail vein of NOD/SCID mice (Fig. 5A). In AMKL tumorigenesis model, cells harvested from Dami-DEL established ascites were larger with poly-lobulated nuclei and less apoptotic as evidenced by increased Bcl-2, decreased Bax and DDIT3 compared to control Dami-GFP/Dami-PM (Fig. 5B, C and Fig. S5A).

We further addressed the dynamic in vivo effects of c-Mpl-del on AMKL proliferation and infiltration in a metastatic model.
Overall, mice injected with Dami-DEL cells had increased mortality compared with Dami-GFP injected mice (Fig. 5D). Moreover, measurement of fluorescence intensity in circulation over the course of one month following IV injection demonstrated Dami-DEL-GFP cells underwent increased proliferation (Fig. S5B, C). Histological analysis verified that c-Mpl-del-overexpressing AMKL cells had significantly infiltrated the bone marrow, in addition to causing a complete loss of the pulmonary and hepatic architecture, including vascular structures and germinal centers (Fig. 5E). Similar findings were also observed in the spleen, wherein AMKL cell infiltration was observed, resulting in damage to the spleen architecture with marked splenomegaly and increases in megakaryocyte numbers (Fig. 5E, F).

In vivo imaging of mice confirms increased splenic migration and tumor establishment at 14 days post IV injection of Dami-DEL (Fig. 5G). Altogether, these in vivo findings demonstrate that higher c-Mpl-del expression is significantly correlated with rapid tumor growth, increased AMKL metastasis, and shorter survival which underscore the importance of c-Mpl-del-regulation of atypical megakaryoblasts in leukemogenesis.

**DISCUSSION**

AMKL is clinically challenging subtype of AML with poor prognosis and a not well understood pathogenesis, particularly in adults [2, 3, 34]. Here we elucidated a role of c-Mpl in supporting in AMKL malignancy, where we identified a novel function of a c-Mpl isoform c-Mpl-del that was found to be highly expressed in AMKL cells and bone marrow cells from AMKL patients. Such that, c-Mpl-del confers enhanced AMKL proliferation, survival and chemotherapeutic resistance by mediating TPO signaling, leading to a more aggressive disease in vivo.

The c-Mpl-del isoform arises from a 72 bp deletion which corresponds to a lack of 24 amino acids within the second cytokine receptor motif near the transmembrane domain [29]. Previous studies show during normal hematopoiesis, c-Mpl-del expression is limited to hematopoietic cells of the megakaryocyte lineage with relative expression level to c-Mpl-p increased during late megakaryopoiesis with high expression in platelets [28]. Here, we performed a comprehensive analysis of the expression levels of c-Mpl-del and c-Mpl-p, by analyzing 499 RNA sequencing samples from TCGA AML and GTEx normal blood. We found that the expression pattern of c-Mpl-del and c-Mpl-p were significantly different in cancer and normal, showing tumor-specific high expression, and c-Mpl-del showed tumor-specific expression (Fig. 1A). We further identified and experimentally validated that c-Mpl-del expression levels were significantly correlated with the prognostic value. Our finding of increased ratio of c-Mpl-del to c-Mpl-p in AMKL also reveals a previously unidentified aspect of c-Mpl-del in the regulation of proliferation, viability and chemotherapeutic resistance suggesting the abnormal expression of c-Mpl-del may contribute to neoplastic events. Notably, in the analysis of TCGA-LAML data, although AML patients with c-Mpl-del expression had shorter survival times than those without c-Mpl-del expression, the patients expressing low c-Mpl-del in AML appeared to have a lower survival than those with high (Fig. 1B).

While the difference does not reach statistical significance, it implies that there may be some AML subtype with a shorter survival period in the analysis of total AML, leading to the emergence of lower c-Mpl-del-expressed patients in total AML have a lower survival. In the future, further refinement of the analysis of AML subtypes to reveal the impact of c-Mpl-del on single AML subtypes other than AMKL may have important significance for exploring the pathogenesis of c-Mpl-del-related diseases.

It is not clear why there is an increased utilization of alternative splice sites in AMKL, leading to increased ratio of c-Mpl-del to c-Mpl-p. Although previously, RNA binding motif protein 15 (RBMI15), first identified in translocation t(1:22)(p13;q13) AMKL, was reported to be an important regulator in maintaining full length c-Mpl-p expression through epigenetic regulation and direct mRNA spliceosome recruitment [23, 35, 36]. RBM15 knockdown resulted in increased generation of c-Mpl-tr splice variant isoform in HSCs and c-Mpl-del in Meg-01 cells [23, 36]. Whether there is similar dysregulation of spliceosome function and the potential mechanisms of RBM15 involved in regulating the splicing of c-Mpl-del may be worthwhile to be addressed in the future. In addition, by comparing the c-Mpl-del group with c-Mpl-p group in TCGA-LAML dataset, we found that the high expression of c-Mpl was accompanied by a high level of RUNX1T1 in AML patients (Fig. S6). RUNX1T1 is a RUNX1 partner transcriptional co-repressor 1 and fusion with RUNX1 to jointly regulate the mutation and abnormal expression of a large number of genes in AML, including RBM15 and c-Mpl [19, 37, 38]. This implies that the aberrant expression of RUNX1T1 may be one of the causes of RBM15 mutation and c-Mpl deregulation in AML, which further lead to the high formation of c-Mpl-del.

We observed biased activation of the pro-survival PI3K/AKT pathway particularly at lower levels of TPO (5 ng/mL) (Fig. 4B). It has been previously noted that there is a biphasic pro-survival and proliferation response within the c-Mpl-TPO axis, with a lower threshold of c-Mpl signaling preferentially activating the pro-survival PI3K/AKT followed by a stronger signal induction of proliferative RAS/ERK pathway [39]. Functionally, this is consistent with our observation of a lower proliferative response at lower doses of TPO (Fig. 2A). However, at higher doses of TPO, the switch to enhanced proliferation led to sustained TPO signaling and stronger PI3K/AKT signaling magnitude. As previously reported, the activated PI3K/AKT pathway induced GATA1 upregulation and DDIT3 downregulation [40, 41], and the PI3K/AKT pathway activated by TPO/c-Mpl-del also resulted in the upregulation of proliferation-promoting transcription factor GATA1 and the downregulation of DDIT3, leading to the upregulation of proliferative genes as well as antiapoptotic proteins in AMKL. A more in-depth study on the difference and mechanisms of c-Mpl-p and c-Mpl-del in the preferential activation of signal pathways may be of great significance to analyze the difference between the proliferation and apoptosis in AMKL caused by specific isoforms.

With regards to the downstream of c-Mpl-del signaling pathways, we have determined that downregulation of DDIT3 is required for AMKL progression by c-Mpl-del engagement controls apoptosis both in vitro and in AMKL tumorigenesis NOD/SCID.
mode (Figs. 3 and 5). This finding is in keeping with the previous reports that DDIT3 helps induce apoptosis in chemosensitive AML, and is suppressed by the loss of RUNX1 methylation identified in familial leukemia [42, 43]. Interestingly, the upregulation of GATA1 and down-regulation of DDIT3 may have overlapping functions resulting in PI3K/AKT-related culminating survival signaling based on gene set enrichment analysis in response to Ara-c (Fig. 3G, Fig. S3D). The previous finding is that AKT collaborates with GATA1 to dysregulate megakaryopoiesis, locking megakaryocytes in an undifferentiation state and promoting AMKL [44]. Although
GATA1 has also been associated with decreased sensitivity to Ara-c treatment [45, 46], further studies were warranted to determine whether c-Mpl-del upregulation of GATA1-associated genes could also increase resistance to a new class of AMKL therapeutics such as Aurora Kinase A that induces megakaryocyte polyploidization and differentiation [1]. Moreover, by investigating the downstream effector molecules differentially and mutually controlled by DDIT3 and GATA1, we may determine molecular events broadly important for AMKL malignancy and chemoresistance.

Lastly, when we recapitulated the enhanced proliferative and anti-apoptotic effects of c-Mpl-del on AMKL in an in vivo murine model of AMKL tumorigenesis and metastasis, we observed increased mortality and malignancy (Fig. 5). Although this model could not capture the leukemogenesis potential of c-Mpl-del expression, in our in vivo data demonstrate overexpression of c-Mpl-del in human CD34+ stem cells increase proliferation and decreases apoptosis (Fig. 2F, Fig. S1E). As it has been previously demonstrated that increased c-Mpl expression of CD34+ cells increases leukemia potential [18, 47, 48], it remains to be determined in future clinical studies if the increased c-Mpl is predominantly of the c-Mel-del isoform.

In summary, this study is the first to identify an abnormal increase of c-Mpl-del expression in AMKL patients, which was further elucidated and found to confer increased malignancy and chemotherapeutic resistance to AMKL cells. Although it is clear that c-Mpl is a key contributor to AMKL progression, broad therapeutic targeting of c-Mpl may not be a desirable approach as it may disrupt the hemostatic HSC niche [49]. Thus, our identification of c-Mpl-del and associated downstream signaling pathways may be exploited as distinct therapeutic targets specific for megakaryocytic leukemia cells. Future studies will be required to assess the prognostic value of c-Mpl-del in AMKL patients and whether its expression level can be further correlated with refractoriness or relapse.

**MATERIALS AND METHODS**

**Cell lines and AMKL samples**

Human Meg-01, Dami, UT-7, and HEK293T cells were obtained from ATCC and were cultured in Iscove’s Modified Dulbecco Medium (HyClone, USA) and Dulbecco’s Modified Eagle’s Medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), respectively. The identities of all four cell lines were validated by short tandem repeat profiling analysis or karyotype analysis. All clinical samples were obtained with informed consent from Sun Yat-sen University Cancer Center and approved by the Hospital Protection of Human Subjects Committee. The clinicopathological characteristics of the patients were summarized in Table S3. Detailed methods of human sample preparation are found in supplementary methods.

**RNA-seq dataset analysis**

RNA-sequencing data of TCGA-LAML cohort were achieved through the Cancer Genome Atlas portal (https://portal.gdc.cancer.gov/). GE2x blood RNA-seq raw reads and sample information were downloaded from the dbGaP database (phs000424.v6.p1). Clinical data downloaded by TCGAbiolinks [50] including the survival status, survival time, stages, histology subtype, gender, and race. To distinguish the c-Mpl-p and c-Mpl-del groups in LAML, the BAM file was extracted by samtools and the reads with MAPQ (mapping quality) ≥30 were reserved [51]. For each BAM file, the junction reads spanning exons 8 and 9 of c-Mpl gene were extracted. The number of c-Mpl-del specific isoform reads was obtained according to the CIGAR value (c-Mpl-del junction reads, c-Mpl-del contains 1980N, c-Mpl-p contains 1908N). The samples with the number of c-Mpl-del junction reads >0 were divided into c-Mpl-del group, and the other samples without this specific splicing isoform were divided into c-Mpl-p group. The data were analyzed with the R (version 4.1) and R Biocorector packages. To identify DEGs in c-Mpl-del group against c-Mpl-p group for TCGA-LAML dataset, R package DESeq2 was applied to determine DEGs [52]. The significance criteria for determining DEGs was set as log2FC>1 and p<0.05. The list of identified DEGs was summarized in Table S4, GO and KEGG annotation was performed by R package ClusterProfiler [53].

**Cell transfection and lentivirus-mediated gene knockdown**

Lipofectamine 3000 reagent (Invitrogen, USA) was utilized for the transfection of HEK293T cells according to the manufacturer’s protocol. Stable cell lines were generated by transfecting Dami and UT-7 cells with control or c-Mpl-del/c-Mpl-p expressing lentivirus generated with empty vectors or pCDH-CMV-MVLdel-Mpl-p-EF1-copGFP/pCDH-CMV-Mpl-del/Mpl-p-EF1-puro lentiviral expression vectors with pPAX2 and pMD2.G in HEK293T cells as previously reported [54, 55]. The knockdown lentiviruses expressing negative control shRNA (shNC), shRNA targeting c-Mpl, shRNAs specifically targeting the alternative splicing region of c-Mpl-del (shMpl-del) and c-Mpl-p (shMpl-p), and shRNAs targeting JAK2 (shJAK2), ACT (shAKT), and Bcl-2 (shBcl-2) were constructed using the pLKO.1. The sense sequences of the oligonucleotide for all shRNAs were listed in Table S5. To confirm target knockdown, cells were collected for qRT-PCR or Western blot analysis. Detailed methods of in vitro assays are found in supplementary methods.

**Animal models**

Five-week-old NOD/SCID mice were maintained in the Laboratory Animal Center of Sun Yat-sen University and procedures were performed according to the institutional ethical guidelines for animal experiments. The animal studies were authorized by the Institutional Animal Care and Use Committee of Sun Yat-sen University. The mice were randomized into different groups and used to generate AMKL xenograft mice by injecting 1 × 10^6 stable GFP or PM-expressing Dami/Dami-DEL cells into the abdominal cavity of NOD/SCID mice, or injecting 1 × 10^6 stable luciferase-expressing Dami/Dami-DEL cells into the tail vein as previously reported standard techniques [56]. Detailed methods of animal model assays are found in supplementary methods.

**Statistical analysis**

All data were reported as the mean±SD of three independent experiments. To compare two independent groups, two-tailed student’s t test or Wilcoxon signed-rank tests were performed per parametric and non-parametric independent metrics, respectively. P was set at 0.05 for rejecting null hypotheses. To compare more than two groups, all data were first analyzed to determine whether they adhered to a normal distribution and then subjected to one-way analysis of variance followed by Tukey’s post hoc test. Survival analysis for TCGA-LAML data was performed using R package survminer and a log-rank test was used to determine the statistical significance of differences. For mouse Kaplan–Meier survival curves, P values were produced using a log-rank (Mantel–Cox) test. *P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant.
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F. Li et al.

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AUTHOR CONTRIBUTIONS
FL designed and carried out experiments, analyzed data, and prepared manuscripts. YX and MY performed experiments, analyzed data, and prepared the manuscript. PC, JZ, QW, MX, YW, ZH, JL, JO, TX, and YC performed the research and analyzed the data. XG, LZ, RS, and XS collected and analyzed clinical data. XRX, DK, JL, and HN designed experiments, analyzed data, and wrote the manuscript. QZ supervised the research, carried out experiments, analyzed data, and wrote the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Qing Zhang.

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