DNAJC10 maintains survival and self-renewal of leukemia stem cells through PERK branch of the unfolded protein response

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Abstract

Leukemia stem cells (LSC) require frequent adaptation to maintain their self-renewal ability in the face of longer exposure to cell-intrinsic and cell-extrinsic stresses. However, the mechanisms by which LSC maintain their leukemogenic activities, and how individual LSC respond to stress, remain poorly understood. Here, we found that DNAJC10, a member of HSP40 family, was frequently up-regulated in various types of acute myeloid leukemia (AML) and in LSC-enriched cells. Deficiency of *DNAJC10* leads to a dramatic increase in the apoptosis of both human leukemia cell lines and LSC-enriched populations. Although DNAJC10 is not required for normal hematopoiesis, deficiency of *Dnajc10* significantly abrogated AML development and suppressed self-renewal of LSC in the MLL-AF9-induced murine leukemia model. Mechanistically, inhibition of DNAJC10 specifically induces endoplasmic reticulum stress and promotes activation of PERK-EIF2α-ATF4 branch of unfolded protein response (UPR). Blocking PERK by GSK2606414 (PERKi) or shRNA rescued the loss of function of DNAJC10 both *in vitro* and *in vivo*. Importantly, deficiency of *DNAJC10* increased sensitivity of AML cells to daunorubicin (DNR) and cytarabine (Ara-C). These data revealed that DNAJC10 functions as an oncogene in MLL-AF9-induced AML via regulation of the PERK branch of the UPR. DNAJC10 may be an ideal therapeutic target for eliminating LSC, and improving the effectiveness of DNR and Ara-C.

Introduction

Acute myeloid leukemia (AML) is characterized by uncontrolled clonal expansion and differentiation block of immature myeloid cells. It is the most frequent type of acute leukemia in adults with mortality and relapse rates of approximately 65% and 50%, respectively.[1,2] There is a small population of leukemia stem cells (LSC) which are thought to be responsible for the initiation, development, and relapse of leukemia. LSC are also resistant to traditional chemotherapy treatments due to their capacity for self-renewal and differentiation block. Therefore, unraveling the underlying molecular mechanisms critical for driving LSC self-renewal improved prognostic capability and identified novel targets for treating AML subsets.

Recent studies have demonstrated that various specific surface molecules, including tyrosine kinase receptors,[3] cytokine receptors,[4] adhesion molecules,[5] and immune checkpoint molecules,[6] are required for the maintenance of LSC stemness and may be attractive targets for eliminating LSC. Despite these elegant observations, clinical outcomes of AML patients remain poor. Simultaneously, LSC are long-lived due to their capacity for self-renewal. A consequence of longevity is exposure to cell-intrinsic and cell-extrinsic stresses, including dysregulated proliferation, DNA damage, hypoxia, reactive oxygen species, nutrient deprivation, and changes in Ph levels.[7,8] LSC require frequent adaptation to maintain their self-renewal ability in the face of these challenges. Nevertheless, the mechanism by which LSC maintain their leukemogenic activities, and how individual LSC respond to stress, remain poorly understood.

The endoplasmic reticulum (ER) regulates the adaptive capacity of stimulated cells via activating the stress-induced signal transduction pathway called the unfolded protein response (UPR), which attempts to alleviate ER workload and restore cellular homeostasis. The UPR is governed by

three integral ER membrane sensors: protein kinase RNA-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6).[9] UPR sensors could be activated in the presence of unfolded/misfolded proteins after the release of the chaperone GRP78. Mechanistically, activated PERK phosphorylates eukaryotic initiation factor 2 α subunit (EIF2α), leading to the attenuation of global protein translation. Paradoxically, EIF2α phosphorylation promotes the expression of activating transcription factor 4 (ATF4), a member of the ATF/CREB family harboring alternative open reading frames. ATF4 further promotes expression of the pro-apoptotic C/EBP-homologous protein (*CHOP*) via binding to the promoter region of this gene.[10] ATF4-CHOP cascade can induce the apoptotic pathway following prolonged ER stress,[9] whereas IRE1 splices *XBP1* mRNA to produce the mature form encoding spliced *XBP1* (*XBP1s*) and ATF6 activated by proteolytic cleavage (cATF6), promoting transcription of chaperones and proteins involved in ER biogenesis.[10]

Recent studies revealed that UPR are highly induced in leukemia cells, and are closely associated with cell apoptosis and progression of hematologic malignancies.[11-13] Here, we found that up-regulated expression of DNAJC10, an ER co-chaperone, emerged as an independent predictor for poor survival of AML patients. Previous studies have revealed that DNAJC10 acts as a component of the ER-associated degradation (ERAD) complex involved in recognizing and degrading misfolded proteins.[14-16] Recently, DNAJC10 has been found to be associated with neuroblastoma,[17,18] melanoma,[18] and lung adenocarcinoma.[19] However, the clinical significance of DNAJC10 in hematologic malignancy has not been explored. In this study, we aim to investigate the effect of DNAJC10 on development of AML and self-renewal of LSC, and explore the underlying molecular mechanism.

Methods

Mice

DNAJC10-KO mice (C57BL/6-Dnajc10^{tm1cyagen}) were obtained from Cyagen Biosciences (Cyagen Biosciences (Suzhou) Inc., Jiangsu, China). NSG mice (NOD-Prkdcscid Il2rgem1/Smoc) were purchased from Shanghai Model Organisms (Shanghai Model Organisms Inc., Shanghai, China). All the mice were maintained in the standard pathogen-free animal house. Studies were performed according to the guidelines and approval of the Ethical Committee of Binzhou Medical University (approval N. 2020-087).

Establishment and analysis of the murine MLL-AF9 model

A transplantable MLL-AF9-inducible murine AML model was established as previously described.[6,20] Briefly, Lin$^-$ cells were isolated from the bone marrow (BM) of wild-type (WT) or *Dnajc10*-knockout (KO) mice, and infected with MSCV-MLL-AF9-IRES-YFP-expressing retrovirus. Infected cells (2-3x10^5) were transplanted into lethally irradiated (10 Gy) C57BL/6 recipient mice by retro-orbital injection. For serial transplantation, 3000 or 5000 purified YFP$^+$ BM leukemia cells or 2000 BM YFP$^+$Mac-1$^+$c-Kit$^+$ cells or 1000 L-GMP (Lin$^-$ IL7R$^-$ Sca-1$^-$ c-Kit$^+$ CD34$^+$ CD16/32$^+$) cells from primary or secondary recipient mice together with 3x10^5 normal BM cells were transplanted into lethally-irradiated recipients. Mice were monitored for MLL-AF9 AML development. For the limiting dilution analysis (LDA), the indicated numbers of YFP$^+$ WT or *Dnajc10*-KO MLL-AF9 YFP$^+$ BM cells collected from secondary recipients were co-transplanted with 3x10^5 competitor cells into lethally-irradiated recipient mice. The survival times were recorded and the frequencies of LSC were calculated according to Poisson statistics using ELDA software.

Flow cytometry

For flow cytometry analyses of mouse or human AML cells, cells were stained with anti-Mac-1-APC (M1/70), anti-Gr-1-PE (RB6-8C5), anti-CD3-APC (17A2), anti-B220-PE (RA3-6B2), anti-c-Kit-PE/Cy7 (ACK2), anti-c-Kit-PE (2B8), anti-Ter-119-APC (Ter-119), anti-Gr-1-APC (RB6-8C5), anti-CD3ε-APC (145-2C11), anti-Gr-1-APC (RB6-8C5), anti-B220-APC (RA3-6B2), anti-Mac-1-PerCP/Cyanine5.5 (M1/70), anti-Sca-1-PE/Cyanine7 (E13-161.7), anti-Sca-1-APC (D7), anti-IL-7Rα-APC (SB/199), anti-CD34-BV421 (SA376A4), and anti-CD16/32-APC/Cy7 (93) or anti-hCD45-PE (#555483, BD) monoclonal antibodies. For analysis of cell apoptosis, AML cells were stained with PE-conjugated anti-Annexin V and 7-AAD (BD Pharmingen, China), according to the manufacturer's instructions. Data were analyzed using FlowJo software.

Colony-forming unit assays

For *in vitro* colony-forming unit (CFU) assays, 500 YFP$^+$Mac-1$^+$c-Kit$^+$ LSC-enriched fractions from the BM of secondary MLL-AF9 recipient mice were plated in methylcellulose-based medium (M3534; Stem Cell Technologies, Shanghai, China) according to the manufacturer's protocol. The 1000 cells collected from three dishes of the primary plating were seeded for the secondary plating. The numbers of colonies and total cell numbers were counted at 10 days post transplantation.

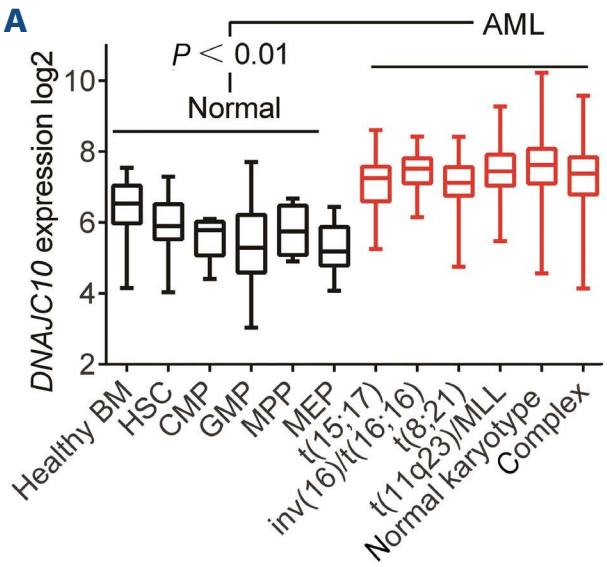
Results

DNAJC10 facilitates cell viability and inhibits apoptosis of human acute myeloid leukemia cells

To determine the function of DNAJC10 in hematopoietic malignancies, we performed an *in silico* analysis of *DNAJC10* mRNA expression in several leukemia microarray dataset. Notably, *DNAJC10* expression was significantly higher in a variety of leukemia cells compared with that in healthy BM and hematopoietic stem/progenitor cells, including hematopoietic stem cells (HSC), common myeloid progenitors (CMP), granulocyte-monocyte progenitors (GMP), multipo-

tent progenitors (MPP), and megakaryocyte-erythrocyte progenitors (MEP) (Figure 1A, *Online Supplementary Figure S1A, B*). Moreover, Kaplan-Meier results showed that the higher expression of *DNAJC10* is associated with inferior overall survival (OS) (Figure 1B, *Online Supplementary Figure S1C-F*). As indicated by Cox regression analyses, DNAJC10 is an independent indicator of poor OS in AML patients (Figure 1C, *Online Supplementary Figure S2*).

A



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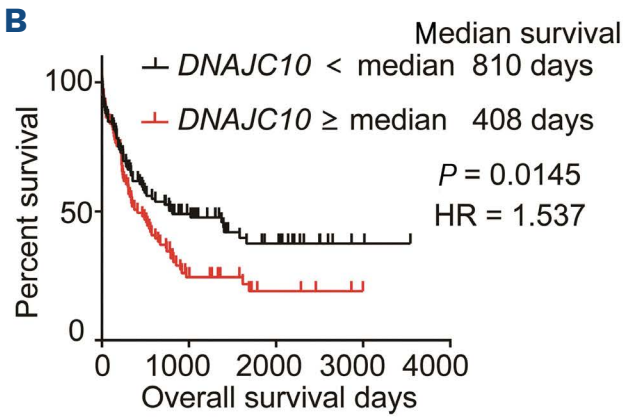
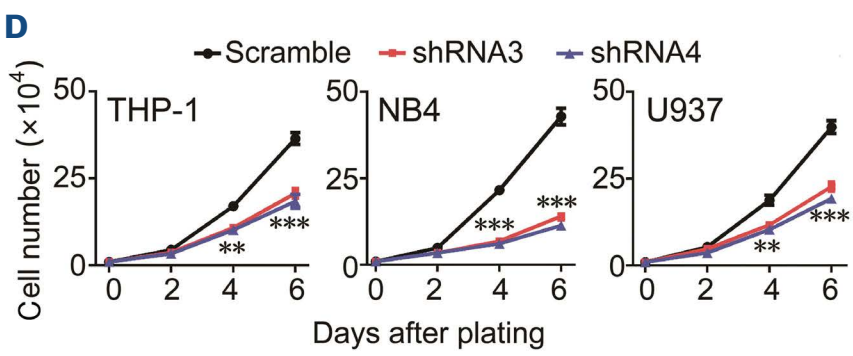


C Univariate and multivariate Cox regression analysis of OS in AML patients

Variable	Value	Univariate		Multivariate	
		HR (95% CI)	*P*	HR (95% CI)	*P*
DNAJC10 expression	< Median	Reference		Reference	
	⩾ Median	1.573(1.009-2.307)	0.0145	1.900(1.216-2.907)	0.005
Gender	Male	Reference			
	Female	1.103(0.7846-1.554)	0.5706		
Age	< 60	Reference		Reference	
	⩾ 60	2.942(2.423-5.046)	0.0001	2.390(1.516-3.766)	0.0001
WBC	< Median	Reference			
	⩾ Median	1.249 (0.890-1.760)	0.1979		
PB blast	< Median	Reference			
	⩾ Median	1.061(0.7553-1.491)	0.7333		
BM blast	< Median	Reference			
	⩾ Median	1.047(0.7455-1.472)	0.7900		
FAB classification	M0	Reference		Reference	
	M1	0.880(0.4598-1.671)	0.6892	0.9342(0.3765-1.5644)	0.7665
	M2	1.102(0.5788-2.108)	0.7638	1.093(0.6231-1.8900)	0.9322
	M3	0.2639(0.099-0.602)	0.0027	0.5687(0.4123-1.1754)	0.1214
	M4	1.080(0.5644-2.065)	0.8129	1.1231(0.4324-2.1543)	0.9066
	M5-M7	1.299(0.6708-2.533)	0.4405	1.180(0.7344-2.6603)	0.5641
Cytogenetics risk	Good	Reference		Reference	
	Intermediate	3.359(1.660-3.912)	0.0001	2.061(1.058-4.016)	0.034
	Poor	4.174(2.338-7.161)	0.0001	3.782(2.233-5.574)	0.002

D



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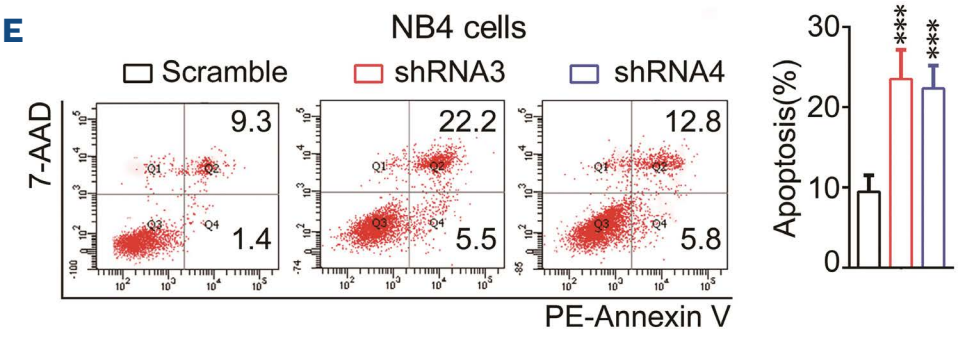

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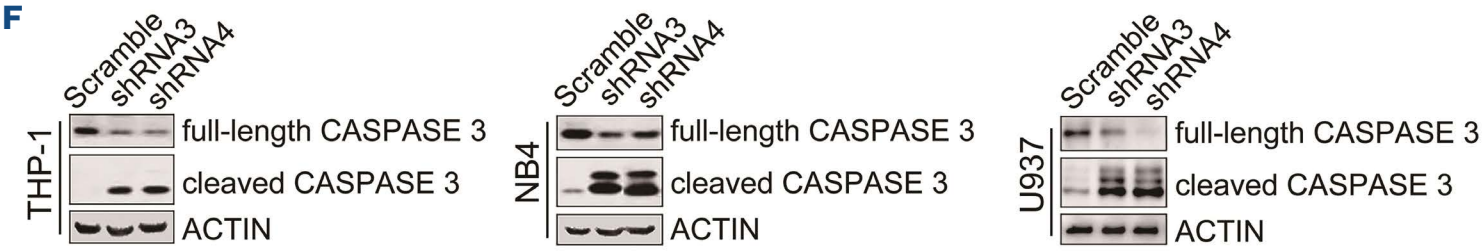

Figure 1. Knockdown of DNAJC10 inhibits growth and promotes apoptosis of acute myeloid leukemia cells. (A) Relative transcription of *DNAJC10* in different hematopoietic / myeloid compartments obtained from the online BloodSpot database. (B) Kaplan-Meier analysis of overall survival (OS) of acute myeloid leukemia (AML) patients relative to *DNAJC10* mRNA expression levels above or below the 50th percentile in the TCGA AML cohort. (C) Univariate and multivariate Cox proportional hazard regression models were used to identify independent prognostic factors and calculate Hazard Ratio (HR) in the TCGA AML cohort. (D) Cell numbers at indicated days after infection were plotted. N=4. (E) Apoptosis was determined in NB4 cells infected with the shRNA indicated. Representative flow cytometric plots (left) and data summary (right) are shown. N=4. ***P<0.0001 compared to Scramble group. (F) Expression of cleaved CASPASE 3 was determined by immunoblotting. CI: Confidence Interval; HSC: hematopoietic stem cells; CMP: common myeloid progenitors; GMP: granulocyte-monocyte progenitors; MPP: multipotent progenitors; MEP: megakaryocyte-erythrocyte progenitors; WBC: white blood cells; PB: peripheral blood; BM: bone marrow; FAB: French-American-British. **P<0.005; ***P<0.001.

To further explore its potential role, we examined expression of DNAJC10 in human AML cell lines. Results showed that DNAJC10 was highly expressed in U937, THP-1, NB4 and HL-60 both at transcription and translation levels, but not in MV4-11 and T-lymphocyte leukemia cell line Jurkat (*Online Supplementary Figure S3A, B*). Next, we knocked down expression of *DNAJC10* by the lentivirus-encoded shRNA (*Online Supplementary Figure S3C-E*). Remarkably, knockdown of *DNAJC10* expression decreased the viability of each of those leukemia cell lines with higher DNAJC10 expression. In contrast, both shRNA3 and shRNA4 had no effect on DNAJC10 negative MV4-11 and Jurkat cells, suggesting the specificity of the shRNA tested (*Online Supplementary Figure S3F*). Knockdown of *DNAJC10* expression consistently induced visible cell growth inhibition in THP-1 cells (*Online Supplementary Figure S3G*) and reduced cell growth in a time-dependent manner (Figure 1D). The decreased cell viability may have resulted from increased apoptosis or cell cycle arrest. Interestingly, knockdown of *DNAJC10* did not change the cell cycle distribution; however, it did significantly increase cell apoptosis rate (Figure 1E, *Online Supplementary Figure S3H*). Moreover, knockdown of *DNAJC10* significantly increased cleaved CASPASE 3 expression (Figure 1F). Together, these results suggest that DNAJC10 facilitates cell viability and inhibits apoptosis of AML cells.

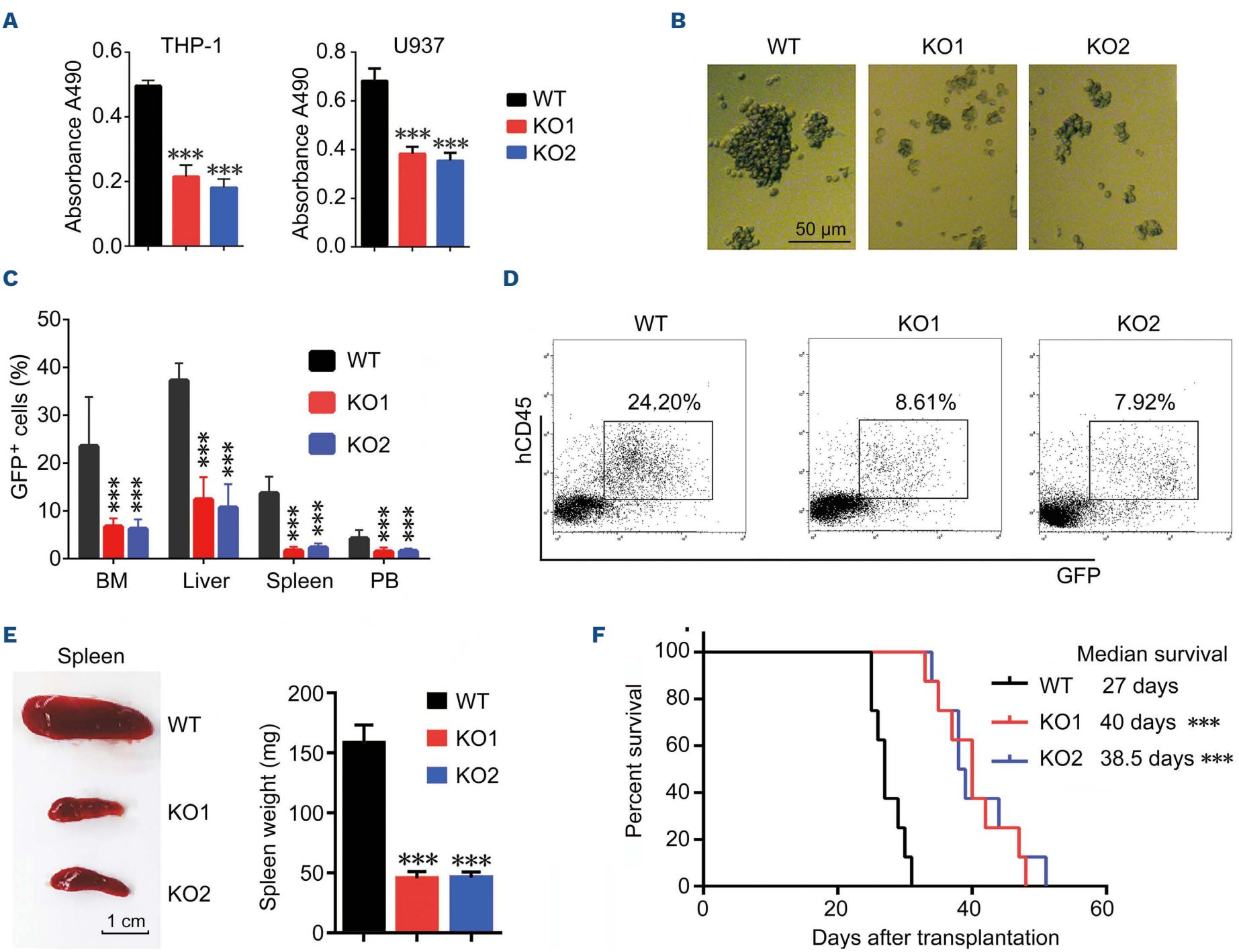

Figure 2. Knockout of DNAJC10 expression blocks xenograft of human leukemia cells. (A) The viability of *DNAJC10*-wild-type (WT) or knockout (KO) cells was determined by CCK-8 assay. N=5. (B) Representative images of *DNAJC10*-WT or KO THP-1 cells at 4 days post culture. (C-F) *DNAJC10*-WT or KO THP-1 cells were transfected into NSG mice. Mice were sacrificed at 28 days post transplantation for analysis. (C) Percentages of GFP⁺ cells in bone marrow (BM), liver, spleen, and peripheral blood (PB) at 28 days post transplantation. N=4 mice for each group. (D) BM cells were collected and stained with anti-human CD45 antibodies. Representative flow cytometry plots are shown. (E) Representative spleen image (left) and spleen weight summary (right). N=4 mice for each group. ***P<0.0001 compared to Scramble group. (F) Kaplan-Meier survival curve of xenografted mice. N=8 for each group. ***P<0.0001.

DNAJC10 is required for the development of human acute myeloid leukemia xenograft

CRISPR/Cas9-mediated *DNAJC10* knockout cell lines were generated to further observe the functions of DNAJC10 *in vivo* (*Online Supplementary Figure S3 I, J*). Notably, deficiency of *DNAJC10* significantly reduced cell growth (Figure 2A, B), consistent with the results in *DNAJC10* knockdown cells mediated by shRNA. Next, we investigated the effect of *DNAJC10* deficiency in transplanted NSG mice. Mice transplanted with *DNAJC10*-KO cells displayed significantly decreased leukemia burden as demonstrated by GFP^+ cells in BM, spleen, liver, and peripheral blood (PB) compared with the WT control (Figure 2C, D). Meanwhile, mice receiving *DNAJC10*-deficient cells displayed a notable reduction in spleen size and weight compared with mice receiving WT cells (Figure 2E). Moreover, *DNAJC10* knockout significantly prolonged the survival of the xenografted mice (Figure 2F). Together, these results indicate that deficiency of *DNAJC10* delayed human AML progression in xenograft recipient mice.

DNAJC10 deficiency impairs development of MLL-AF9-induced leukemia

To gain a deeper understanding of the function of *Dnajc10* on AML initiation and development, a targeted deletion of *Dnajc10* in C57BL/6 mice was created and confirmed by Sanger sequencing, genotyping, and immunoblotting (IB) (*Online Supplementary Figure S4*). Interestingly, the *Dnajc10*-KO mice exhibited normal development, and no obvious defects were observed in their growth or lifespan. Moreover, the *Dnajc10*-KO mice have normal hematopoiesis in PB and normal $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ percentages in BM (*Online Supplementary Figure S5*), suggesting that *Dnajc10* is not required for normal hematopoiesis function, which is consistent with a previous report.[21] Given that *DNAJC10* was higher in patients with MLL-rearrangement (*Online Supplementary Figure S1B*), *Dnajc10*-WT or KO MLL-AF9-induced AML murine models were established using MSCV-MLL-AF9-IRES-YFP retroviruses (*Online Supplementary Figure S6A*). Consistent with a previous report,[22] these AML cells only expressed myeloid cell markers Mac-1 and Gr-1, but not lymphoid cell markers CD3 and B220 (*Online Supplementary Figure S6B, C*).

Remarkably, loss of *Dnajc10* had no effect on the percentage of YFP^+ leukemia cells in PB on primary transplantation (*Online Supplementary Figure S6D*). Mice receiving MLL-AF9-transduced *Dnajc10*-KO cells also had comparable survival time with those receiving *Dnajc10* WT cells (*Online Supplementary Figure S6E*). These results suggest that Dnajc10 is not essential for the initiation of the MLL-AF9-induced murine model.

Next, we examined the function of Dnajc10 on AML development during serial transplantation. The recipient mice receiving MLL-AF9-transduced *Dnajc10*-KO leukemia cells had significantly delayed survival times compared with WT controls (Figure 3A). Consistently, *Dnajc10*-KO remarkably decreased white blood cell (WBC) counts in the PB (*Online Supplementary Figure S6F*), and decreased YFP^+ leukemia cells in PB, BM, and spleen (Figure 3B, C, *Online Supplementary Figure S7*) at second transplantation. The delayed development of the *Dnajc10*-KO AML was also evident from the significantly decreased size and weight of livers and spleens (Figure 3D, E). Moreover, Hematoxylin&Eosin staining displayed a significantly lower frequency of the infiltrated *Dnajc10*-KO leukemia cells in livers and spleens than the WT controls (Figure 3F). These results suggest that Dnajc10 promotes development of MLL-AF9-induced AML.

DNAJC10 is critical for the maintenance of leukemia stem cells in MLL-AF9 leukemia

Leukemia stem cells are significantly enriched in $CD34^+CD38^-$ / low or L-GMP fractions,[23] which contribute to the malignancy of leukemia. Notably, $CD34^+CD38^-$ cells from AML patient blasts expressed higher *DNAJC10* than normal HSC-enriched cells (*Online Supplementary Figure S8A*). L-GMP derived from MLL-AF9 or Hoxa9/Meis1a transduced mouse also expressed higher *Dnajc10* than normal HSC, CMP and GMP, further confirming frequent overexpression of *Dnajc10* in AML LSC-enriched cells (*Online Supplementary Figure S8B*). Therefore, we further investigated whether Dnajc10 affects the role of LSC. Previous studies reported that LSC are enriched in $YFP^+Mac\text{-}1^+c\text{-}Kit^+$ cells in the MLL-AF9 model.[5,22,24,25] Intriguingly, we found that primary transplants with *Dnajc10*-KO cells did not reduce $YFP^+Mac1^+c\text{-}Kit^+$ frequency compared with WT controls (*Online Supplementary Figure S8C, D*). However, frequencies of $YFP^+Mac\text{-}1^+c\text{-}Kit^+$ cells in *Dnajc10*-KO models were simultaneously reduced in BM, spleen, and PB on secondary transplantation (*Online Supplementary Figure S8E-G*). Moreover, both the secondary and tertiary recipient mice receiving *Dnajc10*-KO $YFP^+Mac\text{-}1^+c\text{-}Kit^+$ cells showed longer median leukemia latency (*Online Supplementary Figure S8H, I*). Since the L-GMP population was suggested to be another, more stringent way to determine LSC, we detected the L-GMP frequency and showed that the percentage of *Dnajc10*-KO L-GMP cells was significantly reduced compared with the WT fraction (Figure 4A). In line with this, both the secondary and tertiary recipient mice receiving *Dnajc10*-KO L-GMP cells showed longer median leukemia latency (Figure 4B). Interestingly, the serial plating CFU assay *in vitro* demonstrated that *Dnajc10* deficiency abolished clonogenic potential of $YFP^+Mac\text{-}1^+c\text{-}Kit^+$ cells, as indicated by the dramatic decrease in the colony and total cell numbers (Figure 4C, D). Taken together, these results indicate that Dnajc10 is essential for LSC self-renewal.

Notably, *Dnajc10* deficiency markedly increased apoptosis of $YFP^+Mac\text{-}1^+c\text{-}Kit^+$ LSC-enriched populations in mice receiving a second transplant, as indicated by the increased Annexin-V^+ percentage and up-regulated expression of cleaved Caspase-3 (Figure 4E, *Online Supplementary Figure S8J*). This result suggests that increased apoptosis may be

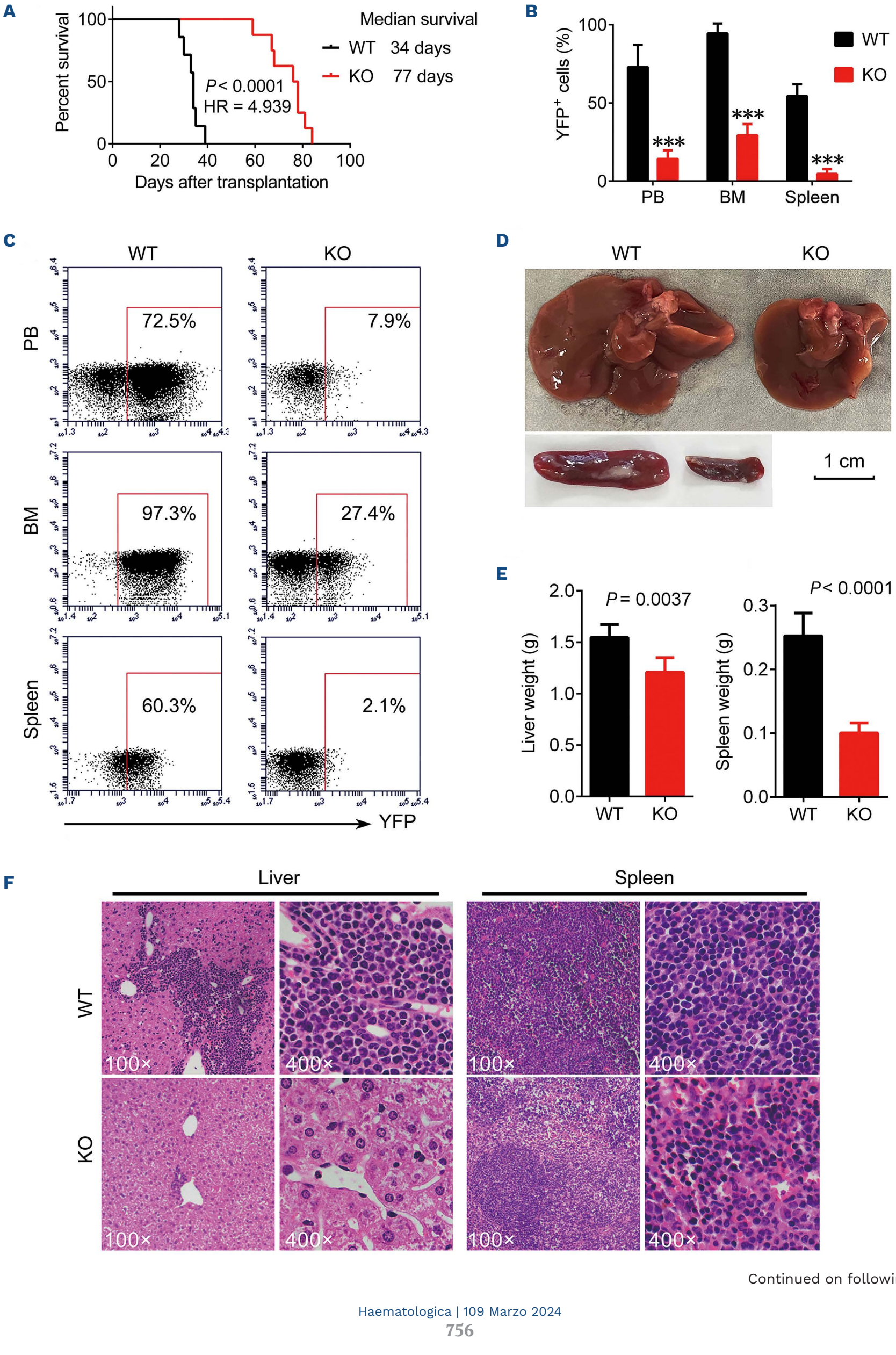

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Figure 3. DNAJC10 is required for the development of MLL-AF9-induced murine acute myeloid leukemia. (A) Survival analysis for recipient mice receiving *Dnajc10* wild-type (WT) or knockout (KO) MLL-AF9 YFP$^+$ bone marrow (BM) cells on the second transplantation. (B) Data summary of percentages of YFP$^+$ leukemia cells in peripheral blood (PB), BM, and spleen of secondary recipient mice receiving *Dnajc10* WT or KO MLL-AF9 YFP$^+$ BM cells. (C) Representative flow cytometry plots for (B). (D) Comparison of size of spleens and livers of secondary recipient mice. (E) Quantification of weight of livers and spleens in (D) (N=5). (F) Hematoxylin&Eosin staining analysis of acute myeloid leukemia infiltration in livers and spleens of secondary recipient mice.

responsible for *Dnajc10* deficiency-induced reduction of LSC frequency. To confirm that *Dnajc10* deficiency reduces frequency of the LSC, LDA was performed in *Dnajc10* WT and KO leukemia cells of secondary recipient mice. Strikingly, the frequency of functional LSC in *Dnajc10*-KO MLL-AF9 model mice was only 1/12 (WT=1/67 *vs*. KO=1/554) of that in WT control mice (Figure 4F, *Online Supplementary Figure S8K*). Taken together, these results suggest that DNAJC10 supports LSC activity.

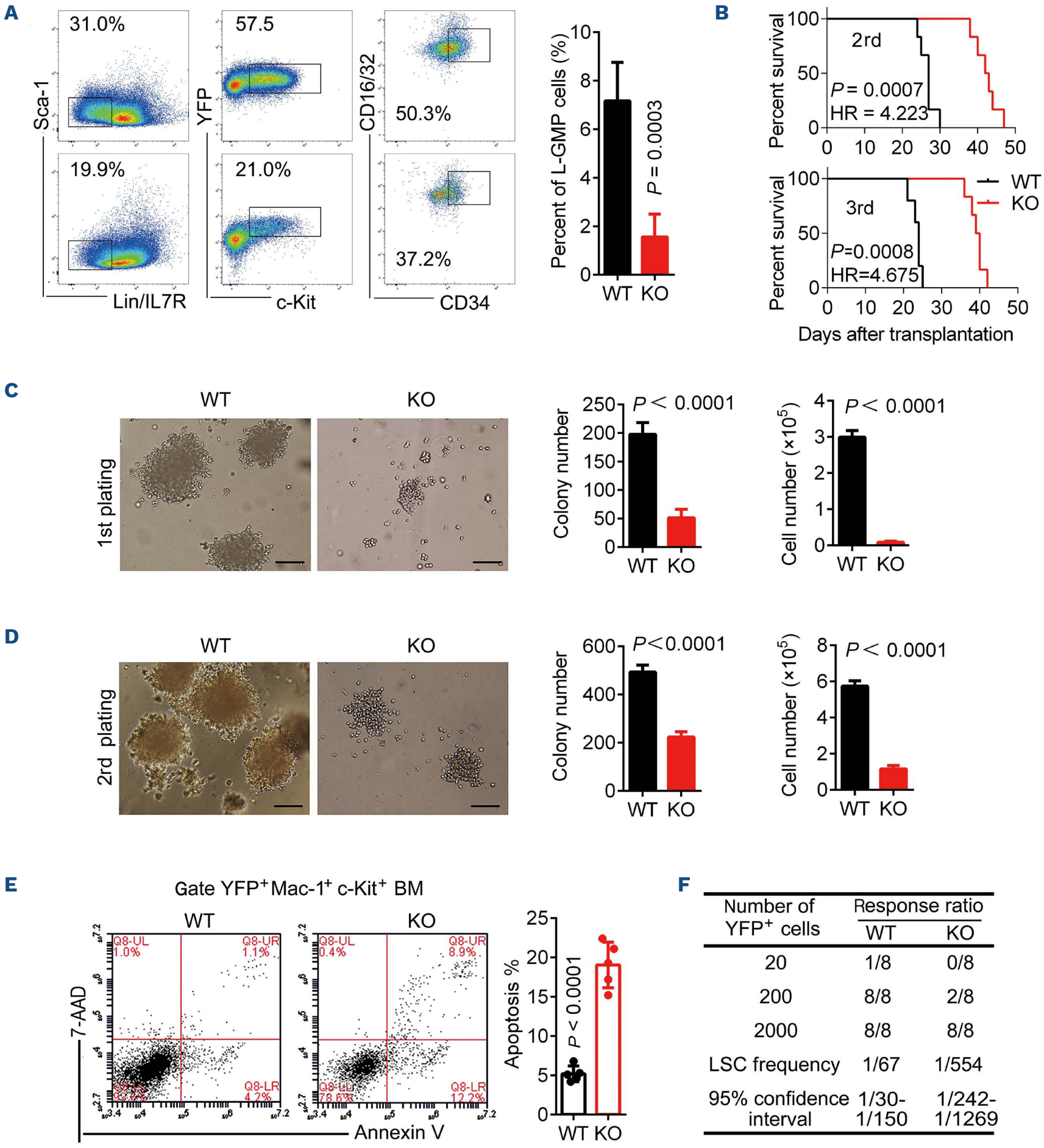
DNAJC10 mediates its effects in acute myeloid leukemia cells through regulation of PERK-EIF2α-CHOP cascade

To understand the molecular mechanisms by which DNAJC10 promotes survival of AML, 958 *DNAJC10*-co-expression genes (*Online Supplementary Table S3, Online Supplementary Figure S9A*) were used for further Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. The top 20 significantly enriched GO terms are shown in *Online Supplementary Figure S9B-D*. Studies have reported that DNAJC10 is involved in quality control of proteins in the ER by recognizing and degrading unfolded/misfolded proteins.[14-16] In agreement with this, we observed that the most significantly enriched KEGG pathways of *DNAJC10* co-expression genes were involved in protein processing in the ER and ubiquitin-mediated proteolysis (Figure 5A). Moreover, transmission electron microscopy (TEM) assay was performed and demonstrated that *DNAJC10*-deficient cells displayed extensively distended and dilated ER compared with control cells (Figure 5B). Moreover, protein levels of GRP78 and GRP94, the sentinel markers of ER stress, were significantly increased in *DNAJC10*-deficient cells (Figure 5C), further suggesting that knockdown of DNAJC10 expression triggers ER stress in AML cells.

Given that UPR is activated through three parallel signaling pathways (PERK, ATF6, and IRE1) (*Online Supplementary Figure S10A*),[8,26] we further analyzed the activation of these effectors of UPR. Interestingly, knockdown of *DNAJC10* did not induce activation of ATF6 and the IRE1/XBP1-mediated branch, whereas it notably induced phosphorylation of PERK and downstream EIF2α (Figure 5C). As previously reported, the PERK branch mainly triggers death or apoptosis of stressed cells.[26] Therefore, the activation of the PERK-EIF2α branch might, to some extent, explain why *DNAJC10* deletion induced apoptosis of AML cells. Furthermore, the crucial players of the PERK branch including GADD34, CHOP, and BAX, which triggers ER stress-induced apoptosis, were significantly up-regulated on knockdown of *DNAJC10* (Figure 5D); vice versa, the anti-apoptotic protein BCL-2 was decreased in *DNAJC10*-deficient cells (Figure 5D). Moreover, PERKi treatment rescued expression of CHOP and BAX (Figure 5E), partially compromised cell viability inhibition (Figure 5F), and attenuated apoptosis (*Online Supplementary Figure S10B*), which were all induced by knockdown of *DNAJC10*. Together, these results suggest that *DNAJC10* knockdown-induced apoptosis and inhibition of cell viability are linked to preferential PERK pathway activation.

PERKi treatment reverses the DNAJC10 deletion-mediated elimination of leukemia stem cells in MLL-AF9 mice

In line with this, we found that the Perk branch was activated in *Dnajc10*-KO leukemic cells *in vivo*, as determined by up-regulated expression of phosphorylated Eif2α, Gadd34, Chop, and Bax (Figure 6A). Therefore, PERKi was used in a rescue experiment performed in *Dnajc10*-KO or WT MLL-AF9 mice (*Online Supplementary Figure S10C*). Interestingly, PERKi treatment could reverse the delayed leukemic progression induced by Dnajc10 deficiency. We observed significant increases in the total numbers of WBC in PB (*Online Supplementary Figure S10D*), in the percentage of leukemic YFP$^+$ cells in BM (Figure 6B, *Online Supplementary Figure S10E*), and a reduced OS (Figure 6C) in PERKi-treated *Dnajc10*-KO MLL-AF9 mice. Next, we examined the role played by Perk in the *Dnajc10* deficiency-induced elimination of AML LSC. In agreement with this, PERKi treatment could significantly reverse the decreased YFP$^+$Mac1$^+$c-Kit$^+$ frequency in the BM of MLL-AF9 mice induced by *Dnajc10* deficiency (Figure 6D). The *in vitro* CFU assays also showed that PERKi treatment significantly compromised *Dnajc10* deficiency-induced self-renewal inhibition, as determined by increased colony and total cell numbers (*Online Supplementary Figure S10F-H*). Furthermore, PERKi-treated *Dnajc10*-KO colonies showed much higher frequency of YFP$^+$Mac1$^+$c-Kit$^+$ cells at third and fourth plating compared to that in PERKi-free *Dnajc10*-KO colonies (Figure 6E). In addition, we confirmed that PERKi treatment rescued Dnajc10 deficiency-induced activation of Perk cascade in YFP$^+$Mac1$^+$c-Kit$^+$ cells, as determined by reversed expression of phosphorylated Eif2α, Gadd34, Chop, Bax, and cleaved Caspase-3 (Figure 6F). Consistent with PERKi, knockdown of *Perk* expression by shRNA could also significantly reverse *Dnajc10* deficiency-induced self-renewal inhibition, as determined by increased colony and total cell numbers in the secondary plating (*Online Supplementary Figure S11A-D*). Collectively, these results indicate that *Dnajc10* deficien-



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Number of YFP+ cells	Response ratio	
	WT	KO
20	1/8	0/8
200	8/8	2/8
2000	8/8	8/8
LSC frequency	1/67	1/554
95% confidence interval	1/30-1/150	1/242-1/1269

Figure 4. DNAJC10 regulates the frequency of leukemia stem cells in MLL-AF9-induced leukemia. (A) Representative flow cytometric analysis for wild-type (WT) and *Dnajc10*-knockout (KO) L-GMP cells in the bone marrow (BM) of the secondary recipients (left) and L-GMP percentage summary (right) (WT: N=5; KO: N=6). The experiment was repeated three times with similar results. (B) Survival (%) for mice receiving WT or *Dnajc10* KO L-GMP cells upon the 2nd and 3rd transplantation. N=6 mice for each group. (C and D) Representative images of colony formation of WT and *Dnajc10*-KO YFP⁺Mac-1⁺c-Kit⁺ leukemia stem cell (LSC)-enriched cells of the secondary recipients in the 1st (C) or 2nd (D) plating (left). Summary of colony numbers and cell numbers (right). Scale bars 10μM. The experiment was repeated three times with similar results. (E) Representative flow cytometric analysis of apoptosis for WT or *Dnajc*10-KO YFP⁺Mac-1⁺c-Kit⁺ LSC-enriched cells from secondary recipients (left). Summary of apoptosis percentage (right) (N=5). The experiment was repeated four times with similar results. (F) Limiting dilution assays (LDA) estimating the frequency of LSC in WT and *Dnajc10*-KO MLL-AF9 leukemia cells. The frequencies were calculated by ELDA software. Table shows the number of recipients that developed leukemia and total number of recipients transplanted per cell dose. The experiment was repeated three times with similar results. Results of the repeated experiements are summarized in *Online Supplementary Table S1*. HR: Hazards Ratio.

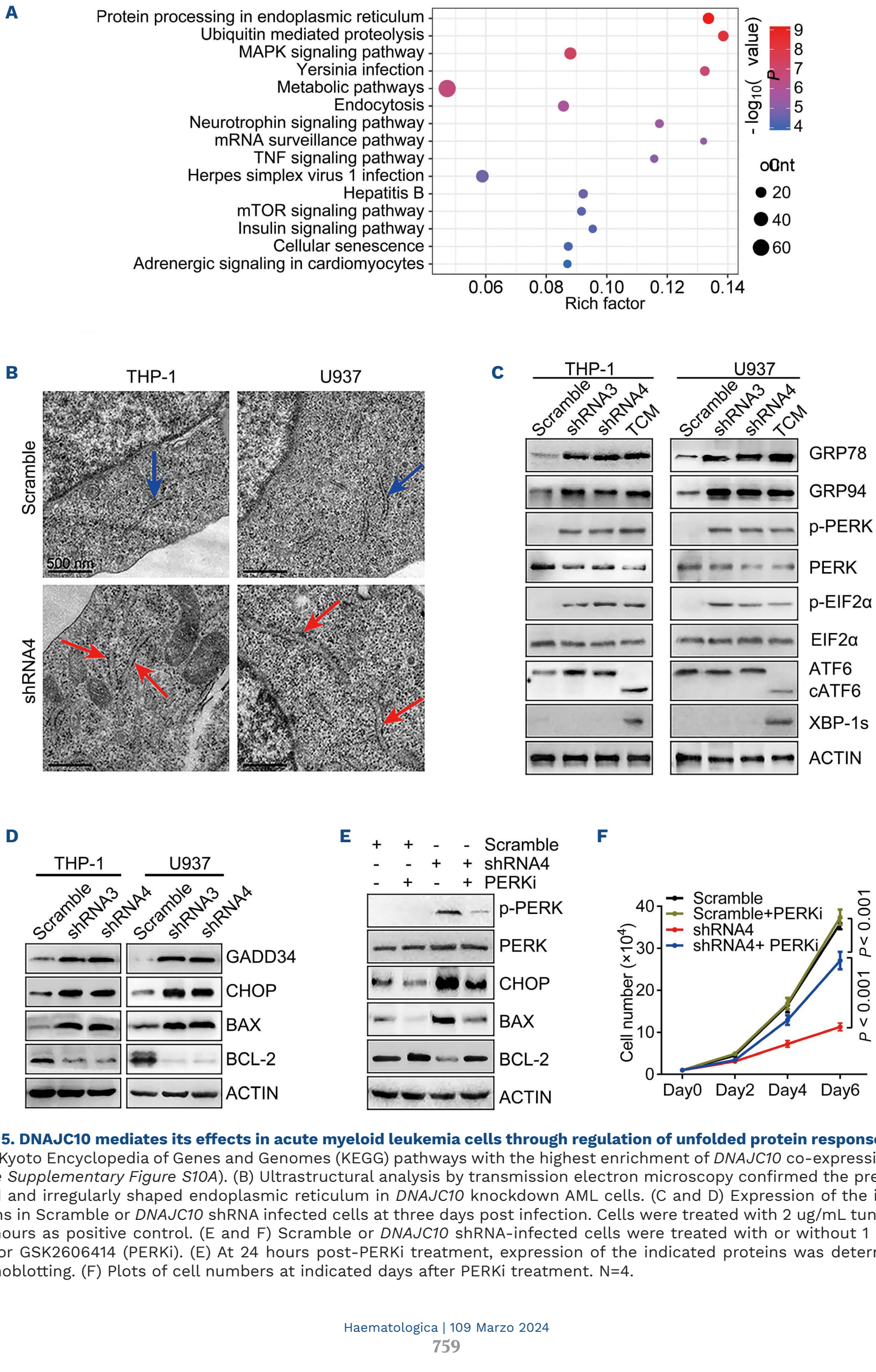

Figure 5. DNAJC10 mediates its effects in acute myeloid leukemia cells through regulation of unfolded protein response. (A) The top 15 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with the highest enrichment of *DNAJC10* co-expression genes (*Online Supplementary Figure S10A*). (B) Ultrastructural analysis by transmission electron microscopy confirmed the presence of dilated and irregularly shaped endoplasmic reticulum in *DNAJC10* knockdown AML cells. (C and D) Expression of the indicated proteins in Scramble or *DNAJC10* shRNA infected cells at three days post infection. Cells were treated with 2 ug/mL tunicamycin for 8 hours as positive control. (E and F) Scramble or *DNAJC10* shRNA-infected cells were treated with or without 1 nM PERK inhibitor GSK2606414 (PERKi). (E) At 24 hours post-PERKi treatment, expression of the indicated proteins was determined by immunoblotting. (F) Plots of cell numbers at indicated days after PERKi treatment. N=4.

cy-mediated reduction in LSC in AML mice is dependent on activation of the Perk-Eif2α branch of UPR.

Knockout of DNAJC10 sensitized acute myeloid leukemia cells to daunorubicin and cytarabine

Importantly, accumulation of unfolded/misfolded proteins that exceed the folding capacity is implicated in sensitization to multiple chemotherapy treatments as it leads to the induction of the pro-apoptotic branch of the UPR.[11,27] Here, we further investigated the association between DNAJC10 and daunorubicin (DNR) / cytarabine (Ara-C) resistance. *In silico* analysis indicated that "DNR + Ara-C" treatment significantly induced *DNAJC10* expression in AML patients (Figure 7A). Moreover, Ara-C or DNR treatment significantly

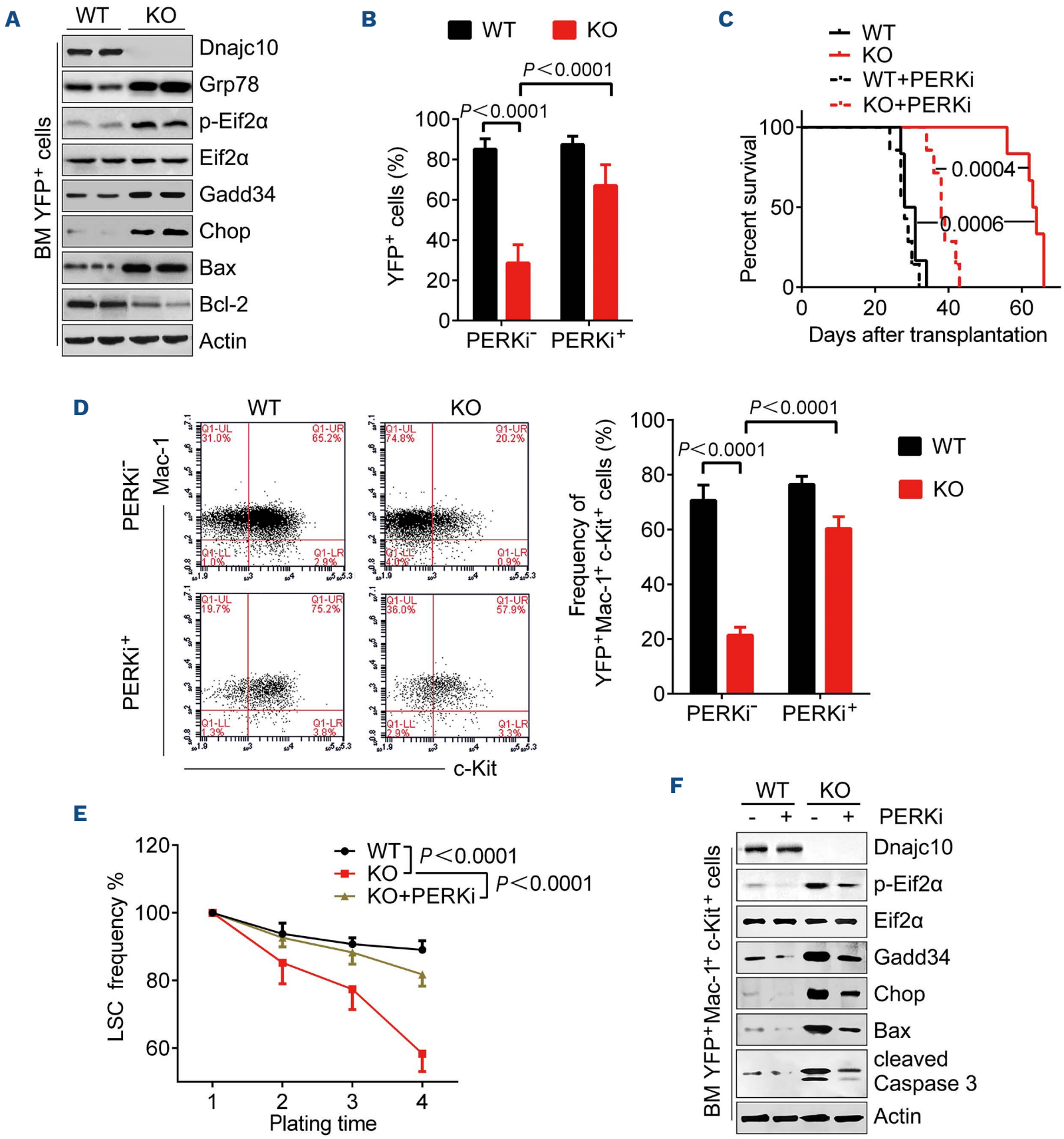

Figure 6. PERKi treatment can partially rescue phenotypes of *Dnajc10* deletion in MLL-AF9 mice. (A) Expression of the indicated proteins in *Dnajc10*-knockout (KO) or wild-type (WT) YFP+ bone marrow (BM) cells of the secondary transplanted mice were determined by immunoblotting (IB). (B) Data summary of percentages of YFP+ leukemia cells in BM at day 22 post transplantation. (C) Survival analysis of *Dnajc10* WT or KO MLL-AF9 mice on 2nd transplantation of rescue experiments. (D) Representative flow cytometry plots for WT and *Dnajc10*-KO YFP+Mac-1+c-Kit+ cells in the rescue experiments (left) and the data summary (right) (N=4-6). (E) Summary of YFP+Mac-1+c-Kit+ cell frequency of the colonies from 1st to 4th plating. (F) Expression of the indicated proteins in *Dnajc10*-KO or WT YFP+Mac-1+c-Kit+ BM cells were determined by IB in the rescue experiments.

induced DNAJC10 and GRP78 up-regulated expression in a dose-dependent manner (Figure 7B). Notably, AML patients with high DNAJC10 expression level tended to have a shorter disease-free survival (DFS) after "DNR + Ara-C" treatment (Figure 7C), suggesting that UPR activation and up-regulated *DNAJC10* expression tend to cause AML cell resistance to Ara-C and DNR.

Therefore, we hypothesized that blocking *DNAJC10* might sensitize AML cells to DNR and Ara-C through activating the pro-apoptotic PERK branch of UPR. As expected, DNAJC10-KO cells exhibit a significantly reduced cell viability compared to the *DNAJC10*-WT cells in ER stress conditions induced by FBS-free starvation or heat-shock (*Online Supplementary Figure S12A, B*). Furthermore, we demonstrated that the DNAJC10 KO cells have decreased IC_{50} of Ara-C and DNR compared to DNAJC10 WT cells (Figure 7D, E, *Online Supplementary Figure S12C, D*). In addition, the IC_{50} of 5-Fluorouracil (F-Fu) for *DNAJC10*-WT THP-1 and U937

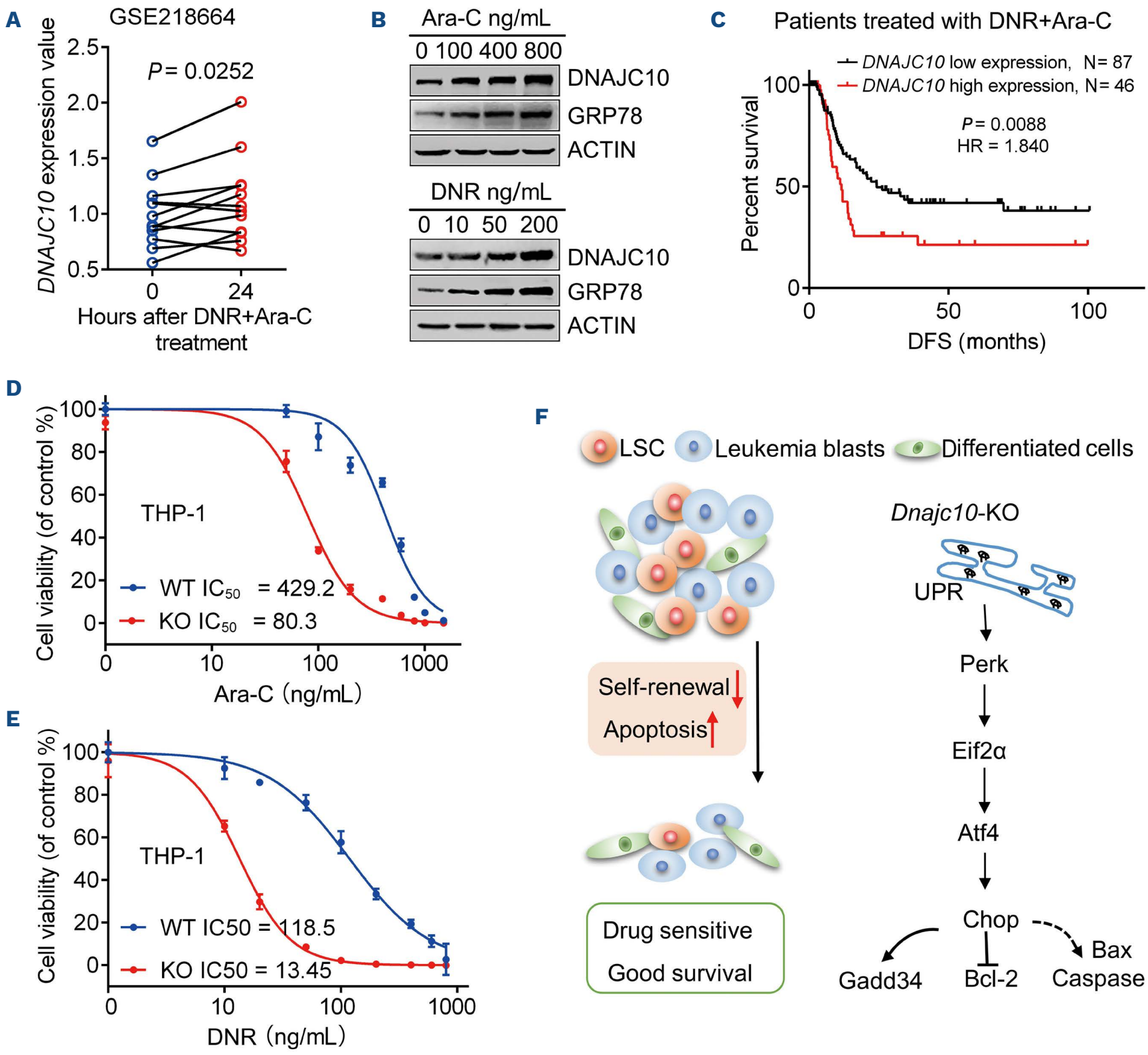

Figure 7. DNAJC10-deficiency sensitizes acute myeloid leukemia cells to daunorubicin and cytarabine. (A) Peripheral blood mononuclear cells (PBMC) from acute myeloid leukemia (AML) patients were pre-treated with daunorubicin (DNR) + cytarabine (Ara-C) for 24 hours (h). Relative transcription of *DNAJC10* was shown (GSE218664). (B) THP-1 cells were treated with Ara-c or DNR at the indicated concentration for 48 h, followed by immunoblotting analysis expression of *DNAJC10* and GRP78. (C) Disease-free survival (DFS) was analyzed in AML patients who received Ara-C+DNR treatment based on *DNAJC10* expression level. (D, E) DNAJC10-WT or KO AML cells were treated with different concentrations of Ara-C, DNR or 5-Fu for 96 h, then cell viability was quantified by CCK-8 assay. IC_{50} was calculated using non-linear regression method. (F) Proposed working model of how DNAJC10 regulates AML LSC and leukemic cells.

cells was almost 8.5 and 5.5-fold that for *DNAJC10*-KO cells (*Online Supplementary Figure S12E, F*).

Finally, to determine whether the activation of PERK is responsible for *DNAJC10* deficiency sensitization of AML cells to DNR and Ara-C, *DNAJC10*-WT or KO THP-1 cells were infected with Scramble or *PERK* shRNA lentivirus, respectively. We found that knockdown of *PERK* partially but significantly reverses sensitization of THP-1 cells to Ara-C or DNR induced by *DNAJC10*-deficiency, as determined by the IC_{50} value (*Online Supplementary Figure S12G-I*). Taken together, these results revealed that the sensitivity of AML cells to DNR and Ara-C significantly increased through DNAJC10 silencing and PERK activation.

Discussion

DNAJC10, an ER co-chaperone, is part of the ERAD complex involved in recognizing, refolding or degrading unfolded/misfolded proteins.[14,15] DNAJC10 had been reported to be important in several cancers but its potential function in AML is unknown. In this study, we systematically analyzed the potential role of DNAJC10 in LSC self-renewal and AML development. Based on this study, we proposed that DNAJC10 enhances ER protein folding, activates the pro-survival branch of UPR, and maintains ER homeostasis, thus increasing LSC repopulation capacity, and leads to chemotherapy resistance. Conversely, *DNAJC10* deficiency leads to accumulation of unfolded/misfolded proteins, activates the pro-apoptotic PERK branch of UPR, and eventually causes apoptosis of LSC and leukemic cells (Figure 7F).

Here, our study revealed that DNAJC10 maintains LSC activity and promotes the development of AML. The positive roles of DNAJC10 in AML are supported by a variety of evidence. 1) High expression of *DNAJC10* is an independent poor prognostic indicator for AML. 2) Deficiency of *DNAJC10* promotes apoptosis of human AML cells both *in vitro* and *in vivo*. 3) Dnajc10 supports the development of AML in the MLL-AF9 mouse model. 4) Dnajc10 is over-expressed in LSC-enriched cells and supports LSC self-renewal. Consistent with our results, a recent report showed that targeting the CENPU-DNAJC10 axis significantly inhibits proliferation and metastasis in lung adenocarcinoma.[19] However, DNAJC10 was found to reduce neuroblastoma cell survival by down-regulating the UPR, suggesting its anti-tumor function. These contradictory results indicated that the function of DNAJC10 in cancer suppression or progression appear to be dependent on different types of cancers and the presence of the variants. Remarkably, DNAJ domain-containing proteins are suggested to stabilize and assist in the transport of p53 to the mitochondria.[28] Furthermore, the stability and transport to the mitochondria of wild-type p53 or mutant p53 are crucial for their tumor suppressive and oncogenic action.[29] Therefore, further studies are needed to explore p53 regulation or DNAJC10 activity in different types of cancers.

Targeting LSC is a promising approach for blocking leukemogenesis and improving outcomes of AML patients. However, it has been a challenge to specifically target LSC while sparing self-renewing normal HSC to protect normal hematopoiesis, because these both appear to use the same self-renewal signaling pathways. Interestingly, the present study revealed that DNAJC10 was specifically highly expressed in LSC-enriched cells but not in normal HSC and hematopoietic progenitor cells (HPC), consistent with a previous report that DNAJ proteins including DNAJA1, DNAJB1, DNAJC9, DNAJC10, and DNAJC12 were all up-regulated in cancer stem cell populations relative to normal cell populations.[30] Importantly, *Dnajc10* deficiency significantly reduced the frequency of functional LSC in MLL-AF9 model mice. Meanwhile, *Dnajc10*-KO mice have normal hematopoiesis and normal $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ percentages in the BM. In agreement with this, a previous report also showed that mice lacking DNAJC10 were viable, healthy, fertile, and displayed a normal life span.[21] Mechanistically, we found that deficiency of DNAJC10 tends to activate the PERK-EIF2α-CHOP axis and promotes LSC apoptosis. However, ER stress stimulation activates both IRE1-XBP-1 and PERK pathways in HSC. Moreover, the HSC pool tend to maintain clonal integrity by clearance of individual HSC after stress to prevent propagation of damaged stem cells.[7] These results suggest that DNAJC10 regulates normal HSC and LSC by distinct signaling axes, and DNAJC10 has different functions in different cell types. Taken together, these data suggest that DNAJC10 is required to maintain the self-renewal ability of LSC, but not HSC, which indicates that DNAJC10 may be an ideal target for eliminating LSC.

Emerging evidence shows that DNAJC10 has a reductase activity, cleaves the disulfide bonds of misfolded proteins, and accelerates ERAD through its physical and functional associations with EDEM (ER degradation-enhancing a-mannosidase-like protein) and GRP78.[14] In line with this, DNAJC10 has been reported to regulate ER stress-associated apoptosis in several solid tumors.[18,31,32] However, the precise mechanism underlying DNAJC10 regulating ER stress and leukemia propagation has not been fully elucidated. Our observations in AML cell lines and MLL-AF9 LSC showed that DNAJC10 deficiency specifically activates the PERK-EIF2α branch of UPR, but not the ATF6 and IRE1/XBP1-mediated branch. More importantly, blocking the PERK branch by PERKi or shRNA significantly compromised DNAJC10 deficiency-induced cell apoptosis and inhibited LSC self-renewal, suggesting that PERK is not a by-stander, but a contributor to DNAJC10 deletion-induced delayed leukemic progression. Remarkably, we showed that DNAJC10 deficiency also selectively enhances the transcription factor ATF4 and, subsequently, downstream effectors such as GADD34, CHOP, and BAX. This is in agreement with previous findings that PERK induce cell death or apoptosis through the PERK/CHOP/BCL2 axis instead of through PERK-medi-

ated translation inhibition or the ATF6-mediated UPR.[11,13,33] These results demonstrate that targeting DNAJC10 might be a novel approach to selectively activate the PERK-EIF2α branch of UPR to induce cancer cell apoptosis.

The combination of DNR for three days and Ara-C as a continuous infusion for seven days (known as '3+7') remains the standard induction regimen for AML patients worldwide. However, resistance to "DNR+Ara-C" therapy is frequently encountered in the clinic.[34] Moreover, the underlying mechanisms of DNR or Ara-C resistance and sensitivity are still unclear. Here, we prove that up-regulated DNAJC10 expression was closely related to Ara-C and DNR resistance in AML patients. DNAJC10 deficiency remarkably enhances DNR and Ara-C sensitivity in AML cells. These findings suggest that DNAJC10 may be a novel therapeutic target for DNR and/or Ara-C resistant AML. Although the basal UPR represents a major cytoprotective mechanism for cancer cells by supporting their rapid proliferation in an unfavorable microenvironment, prolonged or unalleviated ER stress may activate multiple pro-apoptotic signaling pathways to induce cell apoptosis. Therefore, activation of pro-apoptotic elements of the UPR, such as CHOP and GADD34, could be useful to potentiate the effects of drugs targeting other pathways that also activate the UPR as a protective response.[8] Interestingly, we found that DNAJC10 deficiency activates the PERK-EIF2α-CHOP axis and pro-apoptotic GADD34 and BAX, which were reported to be involved in regulating chemotherapy resistance in multiple types of cancers.[35-37] Thus, deficiency of *DNAJC10* may enhance the sensitivity of DNR and Ara-C by activating the pro-apoptotic PERK-EIF2α-CHOP branch of the UPR. Overall, our results suggest that inhibition of DNAJC10 appear to be a good approach to enhance sensitivity and decrease toxicity of DNR or Ara-C, thus obtaining satisfactory clinical outcomes among the so-called poor-prognosis AML subsets.

In summary, we demonstrate the expression pattern, prognostic value, and potential regulatory mechanisms of DNAJC10 in AML. We found that blocked expression of *DNAJC10* decreased cell viability through activating the pro-apoptotic PERK-EIF2α branch. Interestingly, Dnajc10 is not required for normal hematopoiesis; however, *Dnajc10* deficiency significantly slowed AML development and reduced LSC activity in an MLL-AF9 model. Remarkably, blocked expression of *DNAJC10* significantly increased sensitivity of AML cells to DNR and Ara-C. Taken together, our findings support the view that DNAJC10 may serve as a novel prognostic indicator and a therapeutic target for AML treatment and chemotherapy sensitization.

Disclosures

No conflicts of interest to disclose.

Contributions

ML, CJ and YYi contributed to the experimental design and writing of the manuscript. ML, XW, MC and SH prepared Figures 3-6. YYu, XL, EZ and MX prepared Figures 1 and 2. ZY, ZW and NX prepared Figure 7. All authors reviewed the manuscript for publication.

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Data-sharing statement

Data for this study are available from the corresponding authors on reasonable request. Data from the publicly available datasets used in this study can be accessed at: CbioPortal (http://www.cbioportal.org/), GEPIA (http://gepia.cancer-pku.cn/), GEO (GSE13159, GSE218664, GSE30377, and GSE20377) (https://www.ncbi.nlm.nih.gov/geo/), and BloodSpot (https://servers.binf.ku.dk/bloodspot/).

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