

Aging-dependent reduction of KAT7/HBO1 activity impairs imMKCL-based platelet production by promoting immune properties

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SUMMARY

The master cell bank (MCB) system is essential for regenerative cell therapy. We have developed induced pluripotent stem cell (iPSC)-based immortalized megakaryocyte progenitor cell lines (imMKCLs) as an MCB for iPSC-derived platelet (iPSC-PLT) transfusion. However, imMKCLs exhibit both thrombopoietic and immune-skewed properties, with enhanced immune activity impairing platelet production. The link between immune properties and thrombopoietic efficiency remains unclear. Here, we demonstrate that proliferating imMKCLs in G1 and G2/M interphases contribute to platelet generation, while lysine acetyltransferase 7 (KAT7) suppresses immune-biased dominance to maintain these interphases. KAT7 inhibition with WM3835 increases G0 cells, mimicking imMKCL aging, and induces cGAS-STING activation, chromatin instability, and the secretion of tumor necrosis factor (TNF)- α , interferon (IFN)- β , and other pro-inflammatory cytokines. Additionally, TNF- α treatment recapitulates the transition to G0 seen with KAT7 loss. These findings identify KAT7 as a key regulator of imMKCL proliferation by preventing immune-skewed properties, highlighting its potential as a quality control marker in iPSC-PLT manufacturing.

INTRODUCTION

Platelets (PLTs) are generated from megakaryocytes (MKs) via various shedding mechanisms of their cytoplasm (Tilburg et al., 2021). We previously established an *ex vivo* PLT manufacturing system using immortalized MK progenitor cell lines (imMKCLs) as a master cell bank (MCB) for induced pluripotent stem cell (iPSC)-derived PLT (iPSC-PLT) production (Nakamura et al., 2014) and turbulence-dependent bioreactors (Ito et al., 2018). imMKCLs exhibit sustained proliferation in the presence of doxycycline (Dox-ON) and produce iPSC-PLTs after doxycycline depletion in the medium (Dox-OFF). The combination of these technological advancements led to the first-in-human clinical trial, iPLAT1, using patient-derived autologous imMKCLs (Sugimoto et al., 2022). Notably, when focusing on the relationship between imMKCL quality estimated by expandability and iPSC-PLT producibility, we noticed that a rapid proliferation rate in the Dox-ON stage correlated with iPSC-PLT producibility in the latter Dox-OFF stage; a slow rate conversely reduces PLT numbers (Sone et al., 2021). Of note, based on the novel concept that an immune-biased subpopulation emerges during MK development *in vivo* (Li et al., 2024; Sun et al., 2021; Wang et al., 2021a), we recently discovered that an imMKCL subpopulation similarly possesses immune-biased properties, ultimately influencing iPSC-PLT producibility (Chen et al.,

2024). This subpopulation highly expresses immune MK genes, such as platelet factor 4 (PF4 also known as chemokine [C-X-C motif] ligand 4 CXCL4), pro-platelet basic protein (PPBP also known as CXCL7), interleukin-8 (IL-8; also known as CXCL8), and interferon-related genes, which deteriorate the overall MCB quality of imMKCLs. Despite their importance, the molecular mechanisms underlying immune-biased dominance and how the immune MK subpopulation within imMKCLs affects quality control in iPSC-PLT production remain largely unexplored.

Lysine acetyltransferase 7 (KAT7, also known as HBO1 and MYST2) is a critical member of the MYST family of histone acetyltransferases, crucial for chromatin modification and gene regulation. It functions to acetylate histone H3 at lysine 14 and histone H4 at lysines 5, 8, and 12 by forming complexes with MEAF6 (MYST/Esa1-associated factor 6) and scaffold proteins, including ING4/5 (inhibitor of growth 4/5) and JADE (gene for apoptosis and differentiation) or BRPF (bromodomain plant homeodomain finger) (Yokoyama et al., 2024). Such chromatin modifications are essential for regulating gene transcription, DNA replication, and DNA repair processes (Iizuka et al., 2006; Miotto and Struhl, 2008, 2010). In addition, by acetylating H3K14, KAT7 facilitates H3 turnover and CENP-A incorporation, thus antagonizing Suv39h1-mediated inactivation and promoting centromere assembly for accurate chromosome segregation and genomic stability during





cell division (Ohzeki et al., 2016). For instance, in the blood system, KAT7 is required for self-renewal of hematopoietic stem cells (Yang et al., 2022), T cell expansion (Newman et al., 2017), fetal liver erythropoiesis (Mishima et al., 2011), and myeloid leukemia cells (MacPherson et al., 2020). By contrast, it has been reported in Werner syndrome (WS), an autosomal recessive disorder associated with progeria, that KAT7 induces cellular senescence of mesenchymal stem cells (Wang et al., 2021a, 2021b). However, whether the opposing roles of KAT7 in cellular senescence and proliferation are context dependent, varying across cell types and physiological conditions, remains controversial.

Here, we sought to clarify the state of cell cycle transition in imMKCLs and found that KAT7 plays a critical role in suppressing the immune-biased properties of imMKCLs, which is associated with retention in the G1 and G2/M phases of the cell cycle, thus resulting in enhanced PLT production.

RESULTS

Relationship between cell cycle interphase at the proliferation stage and PLT producibility at the MK maturation stage

While we have demonstrated that the rapid proliferation rate during the Dox-ON stage is closely associated with enhanced PLT production in the subsequent Dox-OFF maturation stage by examining different clones of several imMKCLs (Sone et al., 2021), it has been elusive how cell cycle regulation affects the final PLT shedding event. To gain insights into this, we prepared short-term cultured (ST-C) (less than 1 month) and long-term cultured (LT-C) (over 4 months) imMKCLs from the same clone and sought to validate our previous results. Since aberrant glycosylation has been implicated as a marker of aging in MKs and PLTs (Falet et al., 2022), we first confirmed LT-C imMKCLs to have acquired features of aged MKs by lectin staining with *Ricinus communis* agglutinin I (RCA-1) (Figure S1A).

Compared to ST-C imMKCLs, LT-C imMKCLs exhibited slightly larger cell sizes with decreased proliferation capability and PLT producibility caused by impaired polyploidization and proplatelet formation (Figures 1A, 1B, and S1B–S1E). Next, we investigated the impact of cell cycle status on PLT producibility from imMKCLs using the FUCCI reporter system (Figure S1F) (Sakaue-Sawano et al., 2008; Tomura et al., 2013). Notably, whereas most ST-C imMKCLs were in the G1 or G2/M phase, LT-C imMKCLs were mostly in the G0 phase (Figure 1C). Next, we collected cells from each cell cycle stage under the Dox-ON condition and initiated Dox-OFF culture to induce PLT production. Overall, cells from the G2/M phase were the most pro-

ductive for both ST-C and LT-C imMKCLs (Figure 1D). Accordingly, while >8N polyploid cells were dominant in G2/M phase-sorted imMKCLs, 2N cells were in G0 phase-sorted imMKCLs (Figure S1G). Notably, ST-C, but not LT-C, imMKCLs in the G1 phase also contributed substantially to PLT production (Figure 1D) and showed higher ploidy (Figure S1G).

Interestingly, upon Dox-ON culture for 3 days after sorting, all three sorted ST-C imMKCLs transitioned to the pre-sorting pattern, dominated by cells in G1, followed by G2M. In contrast, most G0 phase-sorted LT-C imMKCLs stayed in the G0 phase, and the other two sorted subpopulations showed higher increases of cells in G0 compared to ST-C imMKCLs (Figure S1H). We further investigated the cell cycle status during the Dox-OFF culture immediately after sorting. In maturing Dox-OFF imMKCLs, most G0 phase-sorted cells, regardless of ST-C and LT-C origin, stayed in the G0 phase. Meanwhile, G1 or G2/M phase-sorted populations maintained or progressed to the G1, S, and G2/M phases despite more LT-C imMKCLs going into the G0 phase (Figure S1I). These findings collectively indicate that while both low proliferation and reduced PLT producibility in aging imMKCLs may be due to G0 cell-cycle arrest, young imMKCLs maintain transition to G1 or G2/M phases to generate PLTs.

LT-C imMKCLs have reduced KAT7 and H3K14ac levels

Cell-cycle arrest is also an indicator of cellular senescence (Kumari and Jat, 2021; Mohamad Kamal et al., 2020). Furthermore, we previously reported that clones with low or intermediate PLT producibility show a senescence signature (Sone et al., 2021). Given that KAT7 has been suggested as a novel regulator of cellular senescence (Wang et al., 2021b), we sought to examine whether KAT7 levels in imMKCLs are associated with proliferation rate and subsequent PLT production. Notably, western blot analysis revealed that both LT-C imMKCLs and WS imMKCLs exhibited lower levels of KAT7 and downstream H3K14ac modification compared to ST-C imMK (Figures 2A and 2B). In particular, WS imMKCLs showed diminished cellular division (proliferation rate), resulting in lowered PLT production compared to ST-C imMKCLs (Figures 2C and 2D). These results suggest that KAT7 is a key regulator of imMKCL proliferation, whereby aging-dependent KAT7 levels might influence cell cycle progression.

Pharmacological inhibition of KAT7 causes cell-cycle arrest, impairing imMKCL proliferation and maturation

To define the role of KAT7 in imMKCLs, we evaluated the KAT6/KAT7 inhibitors, WM3835 and WM1119, for their ability to suppress H3K14 acetylation. WM3835 (5 μ M) significantly reduced H3K14ac levels compared to

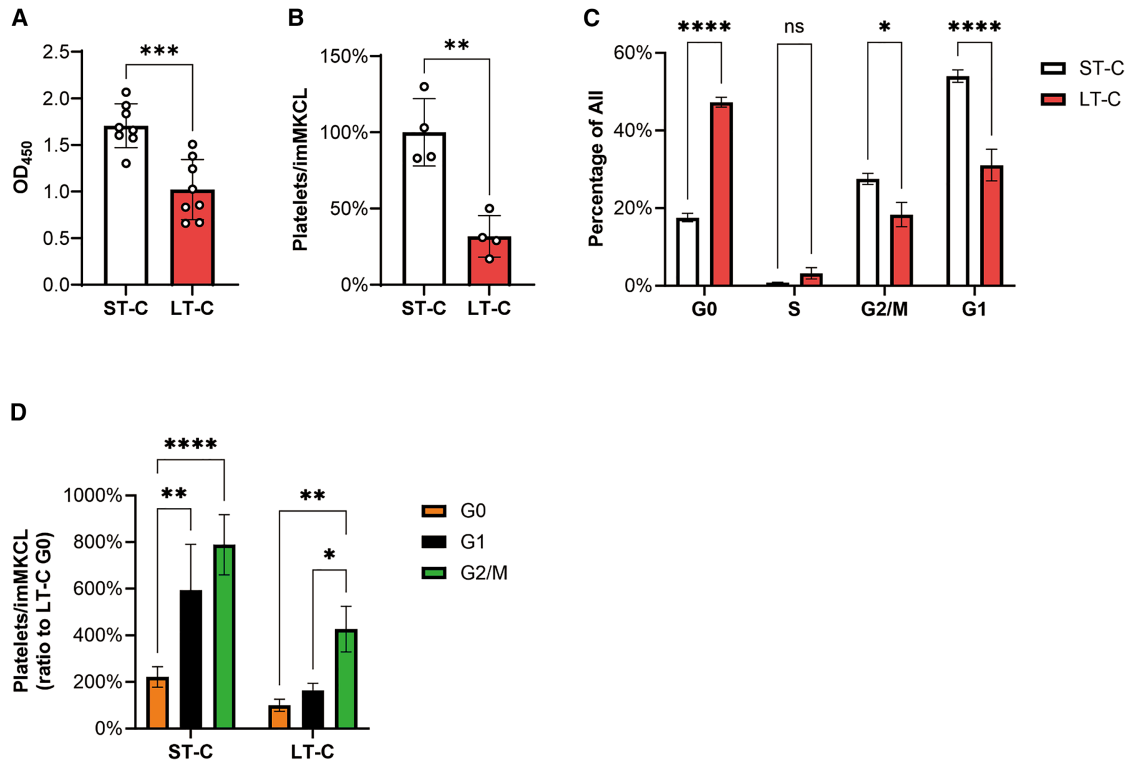


Figure 1. imMKCL-derived platelet producibility determined during the proliferation stage by cell cycle regulation

(A) Cell proliferation as measured by the CCK8 assay for ST-C and LT-C clones during the 3-day Dox-ON culture period. (B) Platelet producibility of ST-C and LT-C clones after the Dox-OFF maturation period. (C) Cell cycle status analyzed using the FUCCI system for ST-C and LT-C clones. (D) Platelet producibility for ST-C and LT-C clones in each cell cycle phase, normalized to LT-C G0 phase. Data are presented as the mean $\pm$ SEM from more than three independent experiments. Statistical significance was assessed using unpaired two-tailed Student's *t* tests or two-way ANOVA. Asterisks indicate statistical significance as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

WM1119 (5 μ M; Figure 3A) and significantly decreased KAT7 protein expression in ST-C imMKCLs (Figure 3B), supporting its suitability as a selective KAT7 inhibitor for this study (MacPherson et al., 2020). WM3835 administration during days 1–5 of the Dox-OFF stage did not influence PLT production, suggesting that KAT7 does not function during the maturation phase (Figure 3C). By contrast, pre-treatment with WM3835 for 9 days, but not 3 or 6 days, during the Dox-ON stage, followed by doxycycline removal and PLT maturation for another 6 days, significantly reduced PLT yield (Figures 3D, 3E, and S2A). Using the FUCCI sensor, we confirmed that KAT7 inhibition increased the number of cells in G0, while decreasing those in G1 and G2/M phases (Figure 3F).

To avoid possible off-target effects by WM3835, we also performed experiments using short hairpin RNA (shRNA)-mediated KAT7 knockdown (shKAT7), which significantly reduced KAT7 mRNA expression, as confirmed by RT-qPCR (Figure S2B). Both cell proliferation and PLT producibility were markedly reduced in shKAT7 imMKCLs

(Figures S2C and S2D). Additionally, the proportion of cells in the G0 phase increased 2-fold in shKAT7 imMKCLs, indicating G0 cell-cycle arrest following KAT7 knockdown (Figure S2E). These results demonstrate a similar phenotype to that observed with WM3835 treatment.

Conversely, KAT7 overexpression did not alter proliferation rate, PLT production, or cell cycle status (Figures S2F–S2J). These results indicate that KAT7 is essential for maintaining the cell cycle in imMKCLs during the Dox-ON stage, emphasizing its role in sustaining imMKCL proliferative potential and influencing PLT yield in the subsequent maturation phase.

Gene expression changes post-KAT7 inhibition activate coagulation and immune-related pathways in imMKCLs

To clarify the underlying mechanism of cell-cycle arrest and declined PLT production caused by KAT7 inhibition, we conducted RNA sequencing (RNA-seq). A differential gene expression analysis revealed many genes with altered

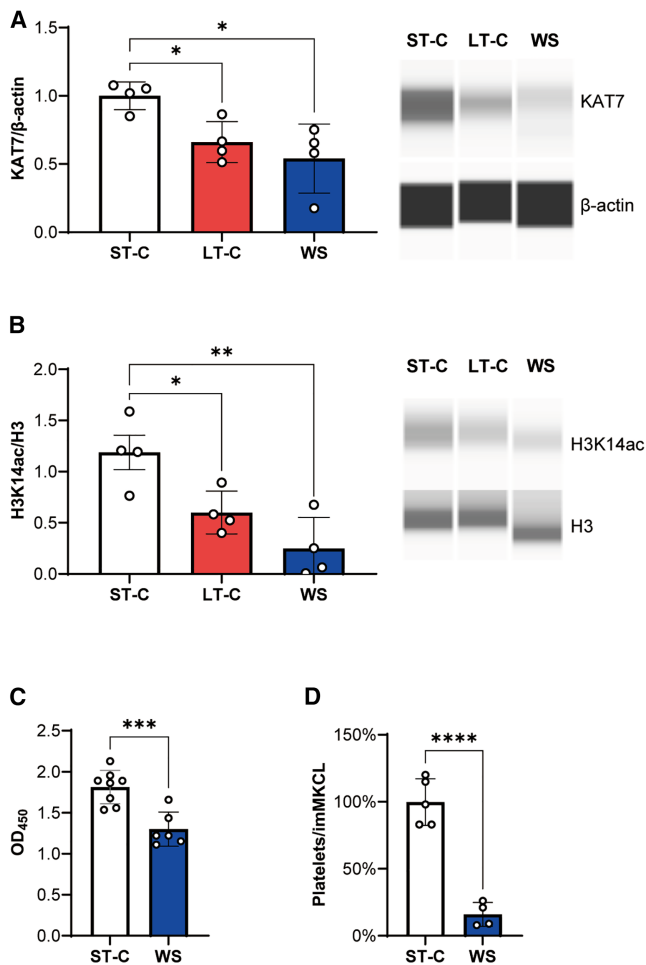


Figure 2. Reduced KAT7 and H3K14ac levels in LT-C imMKCLs
 (A) KAT7 protein levels as measured by the Wes Simple Western system for ST-C, LT-C, and WS clones.
 (B) H3K14ac protein levels as measured by the Wes Simple Western system for ST-C, LT-C, and WS clones.
 (C) Cell proliferation as measured by the CCK8 assay for ST-C and WS clones during the 3-day Dox-ON culture period.
 (D) Platelet producibility for ST-C and WS clones after Dox-OFF maturation period. Data are presented as the mean $\pm$ SEM from more than three independent experiments. Statistical significance was assessed using unpaired two-tailed Student's *t* tests or one-way ANOVA.
 Asterisks indicate statistical significance as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

expression profiles (1,539 upregulated and 1,258 downregulated genes) in imMKCLs upon WM3835 treatment. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment indicated that KAT7 inhibition accelerates PLT activation, complement, coagulation cascades, and immune-related pathways, such as tumor necrosis factor (TNF) signaling pathway, nuclear factor (NF)- κ B signaling pathway, inflammatory response, response

to cytokine, and response to type I interferon (Figure 4A), and that TNF- α , PPBP, CXCL family genes, including PF4 and interferon-related genes, are prominent (Figure 4B), thus indicating a consistent multi-layered immune response leading to the secretion of these molecules by human MKs (Cunin and Nigrovic, 2019). Indeed, RNA-seq reads and RT-qPCR analysis confirmed that RAS-like proto-oncogene B (RALB) expression was upregulated in both WM3835-treated (Figure 4C) and shKAT7 imMKCLs (Figure S3A), suggesting that KAT7 inhibition induces an immune-activated state in imMKCLs, characterized by the elevation of immune-related genes regulated by RALB, including *IRF7* and *ISG15* (Chen et al., 2024). Consistent with this immune-skewed transcriptional profile, KAT7 inhibition also resulted in increased surface expression of immune-related markers, including CD53, CD54/ICAM-1, CD32, and HLA-A/B/C (Figure S3B) (Pariser et al., 2021; Sun et al., 2021), thereby providing direct evidence at the protein level that the loss of KAT7 expands an immune-skewed MK population.

To further validate the immune response observed by RNA-seq, we performed RT-qPCR to quantify the expression of genes involved in type I interferon and NF- κ B signaling pathways. Consistently, imMKCLs displayed higher expression levels of *IFI27*, *IFIT1*, *IRF7*, *ISG15*, and *IFNB1* (type I interferon- β) post-WM3835 treatment (Figure 4D). Meanwhile, upregulated *RELB* expression, rather than *RELA* and *NFKBIA* (Figure 4E), strongly suggests non-canonical NF- κ B signaling pathway activation (Sun, 2011). Similarly, the shKAT7 clone exhibited upregulation of type I interferon and NF- κ B pathways, as evidenced by increased expression of *IFI27*, *IFNB1*, *NFKBIA*, *IL8*, and *TNF* (Figure 4F). We also quantified the levels of pro-inflammatory cytokines in the culture supernatant of imMKCLs by ELISA. Consistent with the RNA-seq data, WM3835 treatment significantly increased protein levels of TNF- α , interleukin (IL)-8, and PF4 (Figure 4G), suggesting that activation of immune-biased imMKCLs might be the source of inflammatory cytokines that negatively affect MK maturation (Emadi et al., 2005; Scheuerer et al., 2000). Moreover, TNF-driven inflammation has been suggested to induce PLT hyperreactivity (Davizon-Castillo et al., 2019), which may be associated with a report of COVID-19 patients revealing TNF-dependent increases in immune MKs as a possible cause of cytokine storms (Ren et al., 2021). These results strongly suggested that KAT7 inhibition drives an immune-skewed phenotype in imMKCLs.

Loss of KAT7 causes centromere dysfunction and triggers imMKCL immune phenotypes via the cGAS-STING pathway

We have recently demonstrated that immune-MK dominance is regulated by RALB (Chen et al., 2024). In that

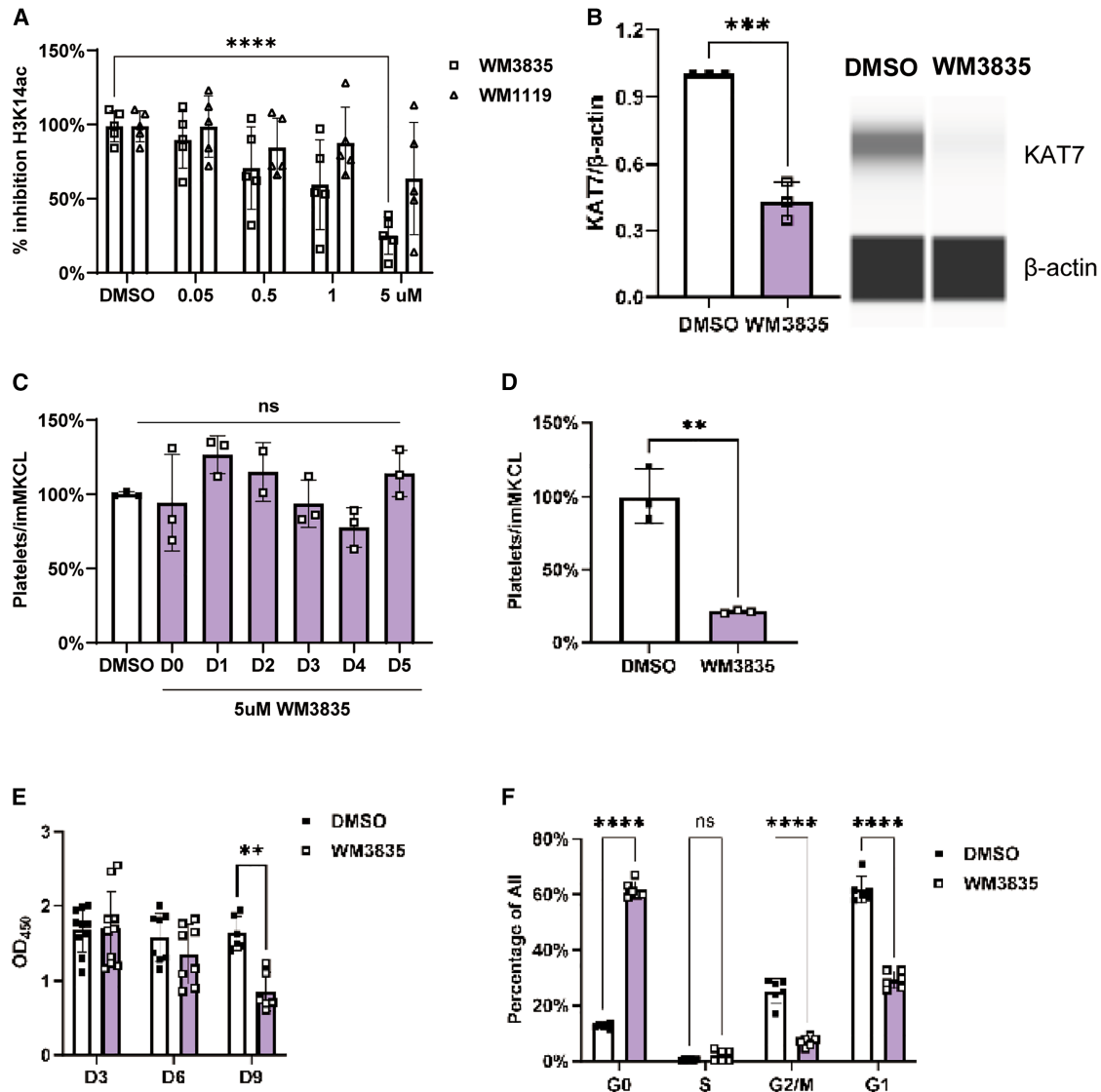
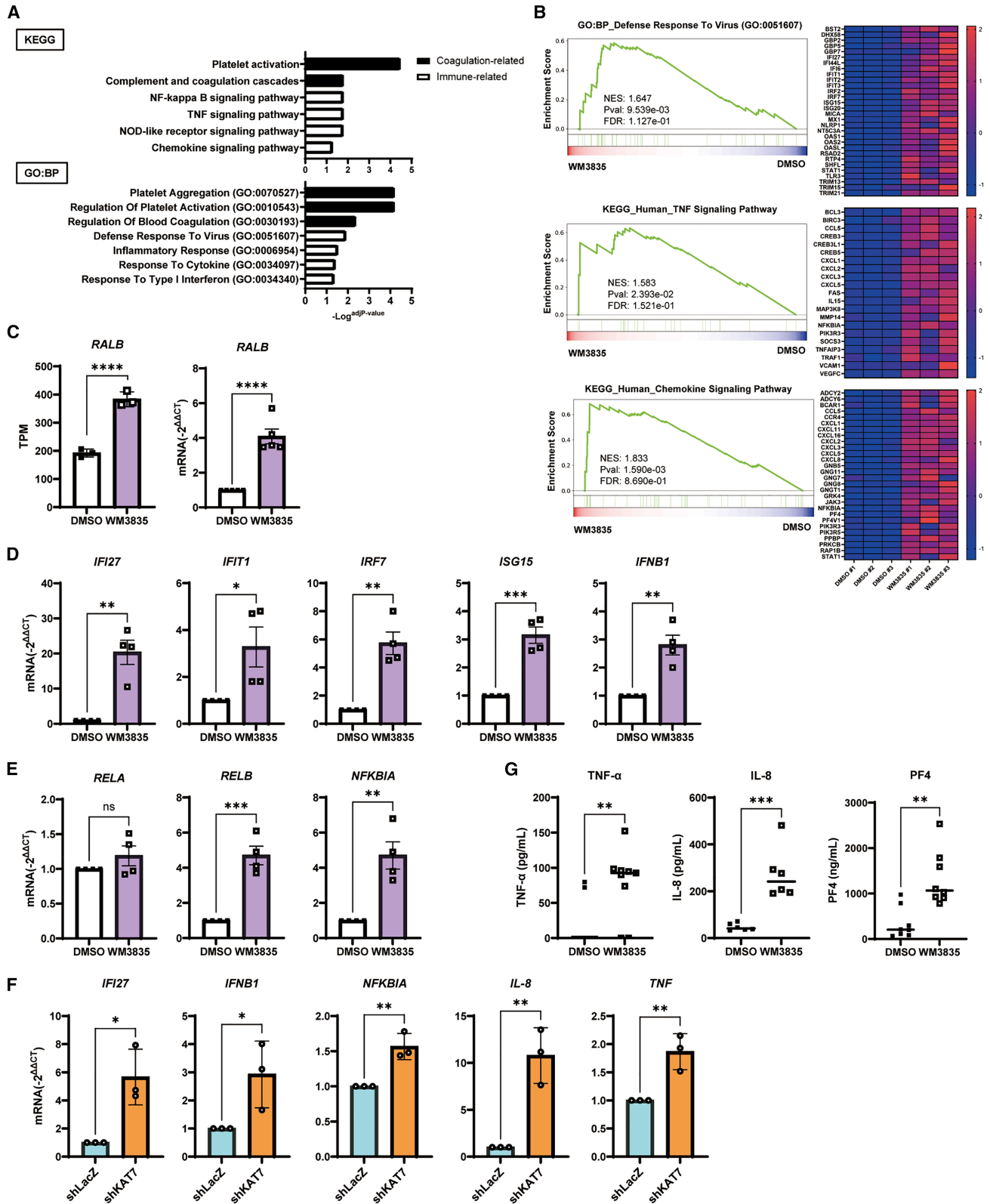


Figure 3. WM3835 impaired imMKCL proliferation and maturation by inducing cell-cycle arrest

(A) WM3835 (5 μ M) effectively inhibited KAT7 signaling compared to WM1119.
 (B) KAT7 protein levels measured by the Wes Simple Western system for DMSO or WM3835-treated ST-C clones.
 (C) Platelet producibility of ST-C clones after WM3835 treatment on the indicated day during the maturation period.
 (D) Reduced platelet production in ST-C clones following WM3835 treatment during the proliferation period.
 (E) Cell proliferation as measured by CCK8 assay for ST-C clones during the 3-, 6-, and 9-day Dox-ON culture periods with WM3835 treatment.
 (F) Cell cycle analyzed using the FUCCI system for ST-C following WM3835 treatment. Data are presented as the mean $\pm$ SEM from more than three independent experiments. Statistical significance was assessed using unpaired two-tailed Student's *t* tests or two-way ANOVA. Asterisks indicate statistical significance as follows: *p* < 0.05 (*), *p* < 0.01 (**), *p* < 0.001 (***), and *p* < 0.0001 (****).

work, we revealed that immune-MKs are associated with downregulated CBX5 (also known as HP1 α), essential for heterochromatin organization, binding to the H3K9me3 histone mark to promote chromatin compaction (Van Wijnen et al., 2021). CBX5 deficiency has been reported to induce chromosomal instability, resulting in defective

chromosome segregation by micronuclei formation and compromised centromere functionality (Kajio et al., 2021; Prieto et al., 2021). Additionally, KAT7 orchestrates centromere assembly, ensuring accurate chromosome segregation and maintaining genomic stability during cell division by antagonizing Suv39h1-mediated



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inactivation. The loss of Suv39h1 disrupts mammalian heterochromatin structure and compromises genome stability (Peters et al., 2001). As expected, pharmacological inhibition and KAT7 knockdown reduced both *CBX5* and *Suv39h1* (Figures 5A and 5B), thereby diminishing genes responsible for regulating core centromeric structures (Figure 5C). Furthermore, immunohistochemistry analysis showed increased micronuclei in imMKCL post-KAT7 inhibition (Figure 5D). To provide direct evidence of chromatin instability, we performed intracellular γ H2AX staining to assess DNA damage. ST-C imMKCLs exhibited significantly elevated γ H2AX levels following KAT7 inhibition, even in the absence of exogenous DNA damage, indicating that KAT7 inhibition results in spontaneous DNA damage and may impair DNA repair capacity following mitomycin C-induced damage, even though this difference did not reach statistical significance ($p = 0.08$) (Figure 5E).

Given the established link we found between chromosomal instability and type I interferon via cyclic guanosine monophosphate-AMP synthase (cGAS)-stimulator of interferon genes (STING) activation (Huang et al., 2023; Li and Chen, 2018), we hypothesized that KAT7 loss engages this pathway. Consistent with this, KAT7 inhibition increased STING phosphorylation (Figure 5F). To determine whether the type I interferon and NF- κ B cascade triggered by KAT7 inhibition specifically depend on cGAS-STING activation, we treated cells with H-151, a selective STING inhibitor (Neufeldt et al., 2022). The results showed that H-151 partially suppressed the KAT7 inhibition-dependent activation of type I interferon signaling (Figure 5G) and NF- κ B pathway-related genes, *RELB*, *NFKB1A*, and *RELB* targets, such as *IL-8* and *TNF* (Figure 5H) (Willemsen et al., 2021). Altogether, our results suggest that KAT7 plays an essential role in cell cycle regulation by suppressing the cGAS-STING pathway, which is associated with the immune-biased MK properties of imMKCLs.

TNF- α induced G0 cell-cycle arrest in imMKCLs

Finally, we examined the direct effects of cytokines released from immune-biased imMKCLs on cell cycle regulation. While administration of TNF- α resulted in a dose-dependent reduction in proliferation, IFN- β did not exhibit such effects (Figure 6A). TNF- α treatment also led to an increase and a corresponding decrease in cells in the G0 interphase and the G1 and G2/M phases, respectively, as confirmed using the FUCCI system (Figures 6B–6D). Furthermore, both TNF- α and IFN- β treatments significantly reduced PLT producibility after doxycycline removal (Figure 6E). These results suggest that KAT7 is a critical regulator for optimal cell division and efficient PLT production by the G1/G2/M subpopulations via suppression of the immune MK program in imMKCLs.

DISCUSSION

We previously established imMKCLs as a robust platform for *ex vivo* iPSC-PLT manufacturing (Nakamura et al., 2014; Sugimoto et al., 2022). Considering the large-scale manufacturing of final cell products from MCB stocks, controlling the proliferation rate after thawing frozen MCB stocks or derived working cell stocks is crucial. The proliferation phase by imMKCL cell division during the Dox-ON stage is indispensable for obtaining adequate cell numbers with optimized properties for efficient PLT production during the subsequent Dox-OFF stage (Ito et al., 2018; Nakamura et al., 2014; Sugimoto et al., 2022). Although we have recognized that reduced proliferation leads to impaired PLT production (Sone et al., 2021), how cell proliferation ultimately determines PLT producibility remained a mystery. Here, we demonstrate a critical mechanism whereby KAT7-mediated chromosomal stability during the proliferation phase governs subsequent PLT production capacity by suppressing immune-biased properties in

Figure 4. KAT7 inhibition activates coagulation and immune-related pathways in ST-C imMKCLs

Pathway analysis and GSEA of upregulated genes as determined by RNA-seq using the GSEAPy package.

(A) Both KEGG and GO: biological process analyses indicate coagulation and immune-related pathway annotation for ST-C following WM3835 treatment.

(B) GSEA plots showing the enrichment of immune-related gene sets in the proliferation phase. Representative enrichment plots from each group are displayed with the normalized enrichment score (NES), the determined nominal (non-adjusted) p value, and the false discovery rate (FDR). Heatmaps of the top differentially expressed genes.

(C) Expression of *RALB*, a novel biomarker for immune-skewed megakaryocytes, for ST-C clones following WM3835 treatment as determined by RNA-seq and real-time qPCR.

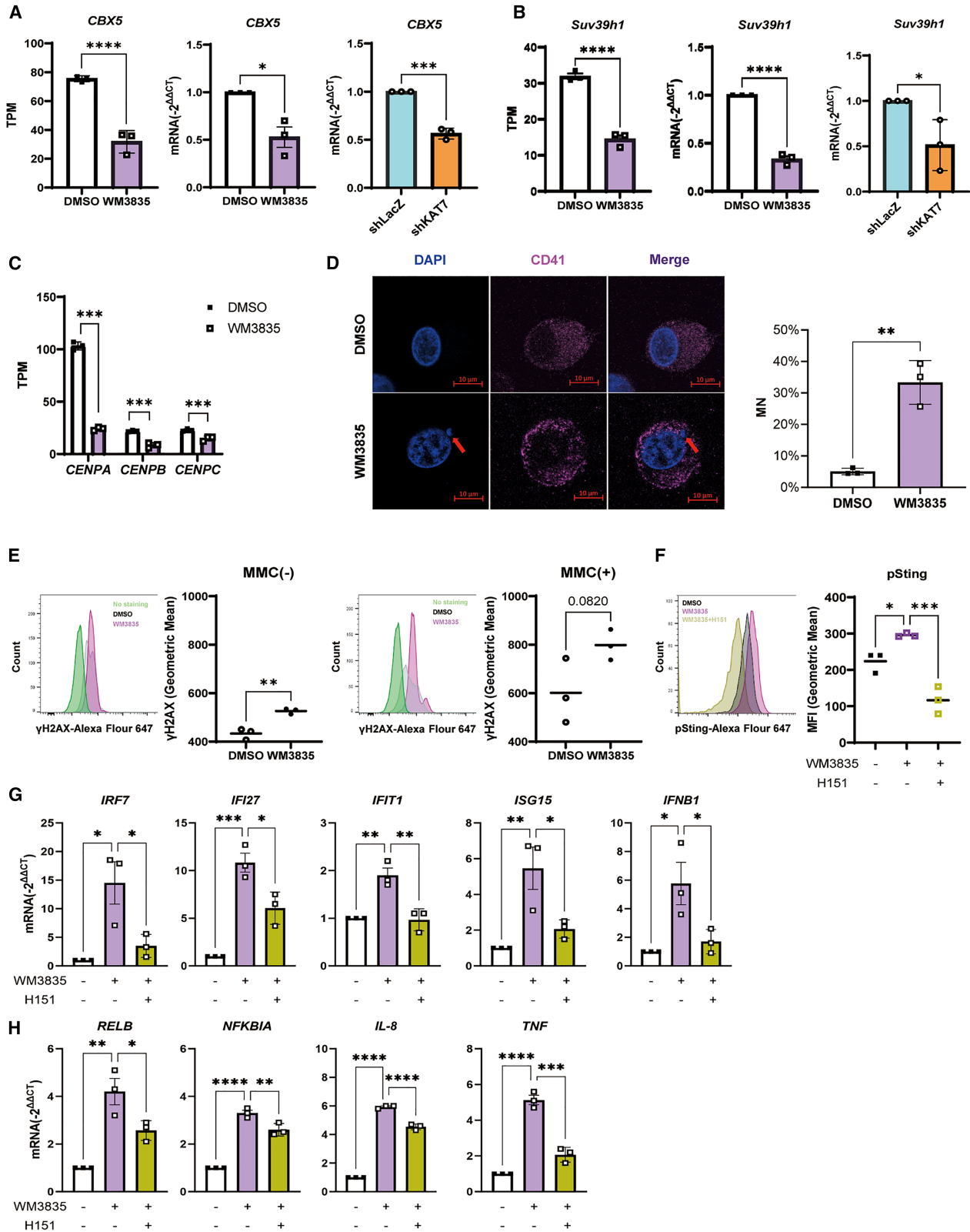
(D) Validation of interferon-related genes by real-time qPCR for ST-C clone following WM3835 treatment.

(E) Validation of TNF signaling-related genes by real-time qPCR for ST-C clones following WM3835 treatment.

(F) Validation of interferon- and TNF signaling-related genes by real-time qPCR for shKAT7 clones.

(G) Inflammatory cytokine levels as measured by ELISA. Data are presented as the mean $\pm$ SEM from more than three independent experiments. Statistical significance was assessed using unpaired two-tailed Student's t tests.

Asterisks indicate statistical significance as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).



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imMKCLs. This mechanism is especially significant considering our recent findings highlighting the heterogeneity of imMKCLs, where the dysregulation of immune-biased imMKCLs results in arrested proliferation and impaired PLT production (Chen et al., 2024).

A key finding of the present study is that cell cycle dynamics during the proliferation stage tightly regulates PLT production. Specifically, cells in G1 and G2/M phases during the Dox-ON stage are critical for thrombopoiesis, whereas increased G0 arrest, particularly in aged imMKCLs, impairs this process. Becker et al. demonstrated that PLT production from mouse fetal liver-derived MKs depends on centrosome clustering during the G1 phase to initiate proplatelet formation (Becker et al., 2024). Our study builds upon this knowledge and focuses on earlier stages of MK development, revealing how maintaining cells in G1 and G2/M phases prepares MKs for maturation, thereby expanding this framework to include a broader contribution by cells in both G1 and G2/M phases to higher PLT yields using imMKCLs.

The molecular mechanism underlying this regulation centers on the role of KAT7 in maintaining chromosomal stability. During normal cell proliferation and division, KAT7 regulates chromosomal stability by facilitating CENP-A deposition at centromeres and counteracting Suv39h1-mediated heterochromatin spread (Ohzeki et al., 2016). This balance between KAT7 and Suv39h1 is critical for maintaining centromeric integrity and accurate chromosome segregation. Indeed, KAT7 deficiency reduces *Suv39h1* and *CBX5* expression, resulting in chromosomal instability and subsequent activation of the cGAS-STING pathway (Fang et al., 2023a). Such effects, in turn, link DNA damage to innate immune responses, thus promoting the production of pro-inflammatory factors, such as IL-6, IL-8, and TNF- α (Figures 4 and 5) (Hong et al., 2019; Li and Chen, 2018). Among those factors, TNF- α directly triggers a transition from G1 or G2/M interphase into G0, potentially establishing a detrimental feedback loop that

further compromises the PLT producibility of imMKCLs. However, in our experimental settings (Figure 6), TNF- α concentrations $\geq 10,000$ pg/mL were required to elicit clear effects on cell cycle distribution and proliferation, substantially higher than endogenous TNF- α levels (~ 100 pg/mL) detected following KAT7 inhibition (Figure 4G). This discrepancy suggests that TNF- α alone at physiological levels cannot fully explain the observed phenotypes. Given that KAT7 inhibition induces multiple cytokines, we hypothesized that synergistic interactions among these factors contribute to cell-cycle arrest and impaired PLT producibility. To test this, we formulated two cytokine combinations (C1 and C2; Figure S4) designed to mimic the inflammatory milieu induced by KAT7 inhibition. Despite the individual cytokine concentrations in these cocktails being below 100 pg/mL, both C1 and C2 significantly reduced proliferation and PLT production, supporting the notion that synergy between cytokines drives the immune-biased, proliferation-deficient phenotype.

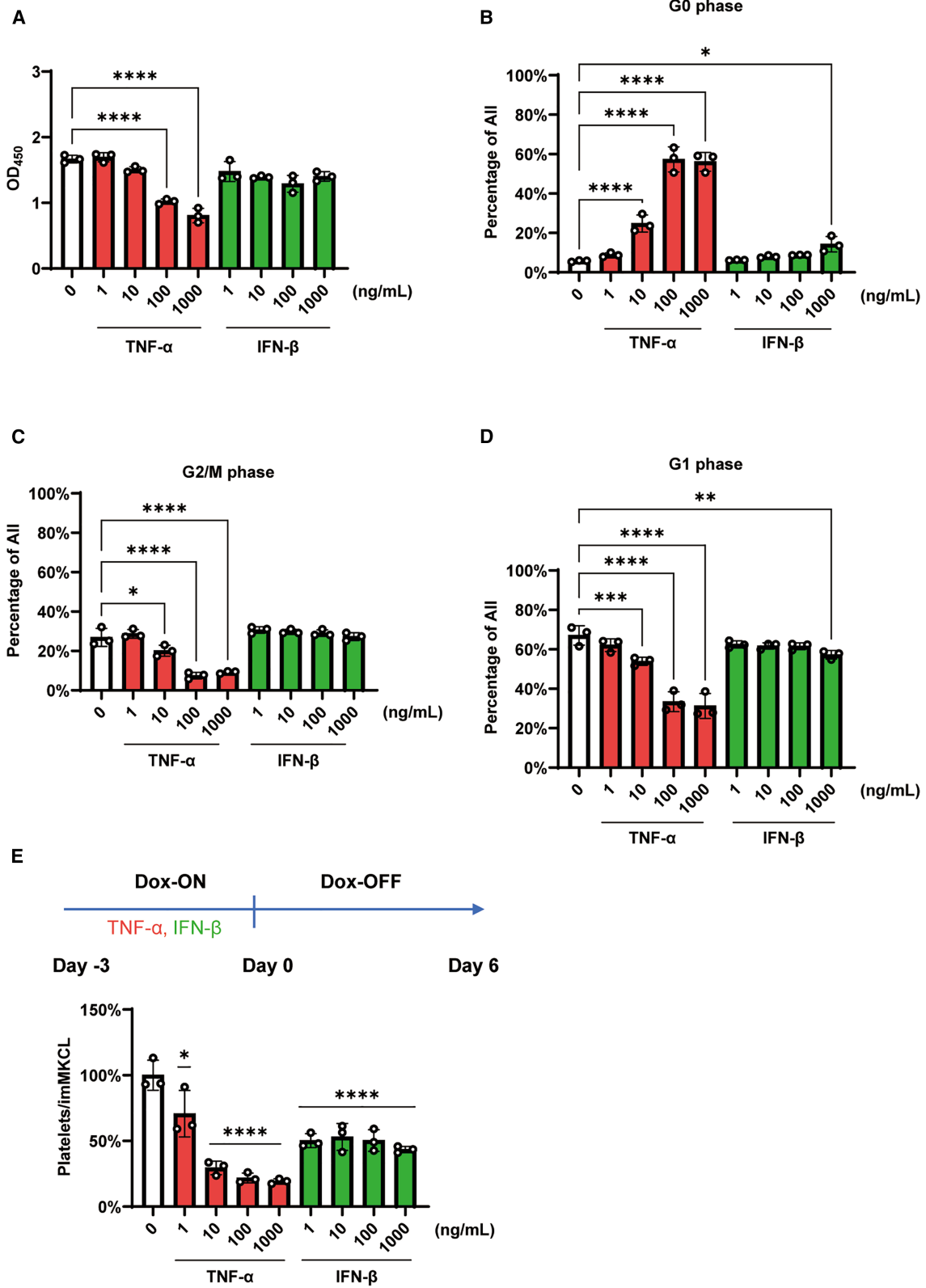
Although we did not compare the heterogeneous nature of native MKs and imMKCLs derived from iPSCs in detail, our findings provide important insights into such MK heterogeneity. Recent evidence based on single-cell RNA-seq identified distinct MK subpopulations, including those with immune-biased properties (Li et al., 2024; Sun et al., 2021; Wang et al., 2021a). Consistently, we have shown that imMKCLs also display heterogeneity, with the dysregulation of immune-biased subpopulations impairing iPSC-PLT production (Chen et al., 2024). In that context, KAT7 may function as a molecular switch regulating the balance between PLT-producing and immune-biased MKs. The aging-dependent reduction in KAT7 levels might shift this balance toward immune-biased subsets by activating inflammatory pathways, reminiscent of the let-7/RALB axis regulating immune-biased subpopulations in imMKCLs (Chen et al., 2024).

In conclusion, our study establishes KAT7 as a critical regulator linking cell cycle dynamics to *in vitro/ex vivo*

Figure 5. Loss of KAT7 causes centromere dysfunction and triggers imMKCL immune-biased phenotypes via cGAS-STING pathway activation

- (A) *CBX5* expression in ST-C clones followed by WM3835 treatment or KAT7 knockdown, as determined by RNA-seq and real-time qPCR.
- (B) *SUV39H1* expression in ST-C clones followed by WM3835 treatment or KAT7 knockdown, as determined by RNA-seq and real-time qPCR.
- (C) Expression of core centromeric structural proteins as determined by RNA-seq.
- (D) Micronuclei detected by immunofluorescence staining of ST-C clones ($N = 3$, more than 50 cells counted for each experiment).
- (E) Chromatin instability was measured by γ H2AX levels via intracellular staining and evaluated by fluorescence-activated cell sorting (FACS), with or without 5 μ g/mL mitomycin C.
- (F) Phosphorylation of STING protein as measured by intracellular staining and evaluated by FACS following WM3835 treatment, with or without 1 μ M H151 treatment.
- (G) Expression of type 1 interferon-related genes after H-151 treatment as determined by real-time qPCR.
- (H) Expression of NF- κ B pathway-related genes after H-151 treatment as determined by real-time qPCR. Data are presented as the mean $\pm$ SEM from more than three independent experiments. Statistical significance was assessed using unpaired two-tailed Student's *t* tests or one-way ANOVA.

Asterisks indicate statistical significance as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).



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thrombopoietic potential through its role in maintaining chromosomal stability. These findings not only explain the correlation between proliferation and PLT production but also suggest novel strategies for monitoring quality control of iPSC-PLT production. Interventions aimed at sustaining KAT7 activity or mitigating the downstream effects of its deficiency hold promise for enhancing the efficiency and clinical applicability of iPSC-PLT manufacturing.

METHODS

Cells and reagents

Original imMKCLs, clone 7, were used in this study (Chen et al., 2024; Ito et al., 2018). ST-C and LT-C of clone 7 imMKCLs were maintained through cell division for 1 and 4 months, respectively, in the presence of doxycycline. WS imMKCLs were generated from iPSCs derived from a WS patient using a Dox-inducible system with defined factors, as previously published (Nakamura et al., 2014; Paul et al., 2024). Doxycycline was employed to regulate the proliferation and differentiation phases, and additional reagents WM3835 (CAS: 2229025-70-9), M1119 (CAS: 2055397-28-7), and H-151 (CAS: 941987-60-6) were obtained from Sigma-Aldrich Co. LLC. All cell line experiments were conducted with approval from the ethics committees of Kyoto University and Chiba University.

Cell proliferation measurement

Cell proliferation was evaluated using the CCK-8 kit (Dojindo), following instructions provided by the manufacturer. imMKCLs cultured for 3 days in the Dox-ON stage were plated in 96-well plates at a density of 5×10^3 cells/100 μ L per well. Absorbance at 450 nm was measured using a microplate reader (Envision 2104, PerkinElmer).

Platelet count measurement

PLT counts were determined through flow cytometric analysis using a FACS Lyric (BD Biosciences) as described previously (Nakamura et al., 2014). Both antibodies used in this analysis, anti-hCD41-APC and anti-hCD42b-PE, were sourced from BioLegend Inc.

Antibody-based quantitative protein analysis (simple Wes)

Cell lysates were prepared using the EpiQuik Total Histone Extraction Kit (Epigentek). Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Lysates were adjusted to a final concentration of 0.5 or 1 μ g/ μ L and analyzed using the Wes automated western blotting system (ProteinSimple). The following antibodies were utilized: mouse anti- β -actin (1:500, Sigma), rabbit anti-Histone H3 (1:50, Cell Signaling Technology), rabbit anti-acetyl-Histone H3 (Lys14) (D4B9) (1:25, Cell Signaling Technology), and rabbit anti-MYST2 (D4N3F) (1:50, Cell Signaling Technology).

Flow cytometry and cell sorting

Cells were suspended in staining medium and incubated for 30 min with appropriate antibodies in the dark. The following antibodies were used for flow cytometry: anti-hCD53-APC, anti-hCD54-APC, anti-hCD32-APC, and anti-hHLA/A/B/C-APC were sourced from BioLegend Inc. and FITC-RCA-1 or biotin-RCA-1 were sourced from VECTOR Laboratories. imMKCLs were sorted using a FACS Symphony S6 (BD Biosciences) equipped with a 100- μ m nozzle and operated with the DIVA software package (v. 8.0.2).

Reverse transcription and real-time PCR

Total RNA was extracted using the miRNeasy Mini Kit (QIAGEN) following the manufacturer's protocol. cDNA synthesis was performed with the ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO). Real-time PCR was conducted using the THUNDERBIRD Next SYBR qPCR Mix (TOYOBO) on a QuantStudio 3 system (Applied Biosystems). *GAPDH* served as the internal control. Primer sequences used are provided in Table S1.

Lentiviral production

Viral vectors were used with approval from the relevant committees at Kyoto University. For overexpression studies, the full-length coding sequence of human *KAT7* was cloned into the CS2-Ubic-IB lentiviral vector plasmid. For *KAT7* knockdown, shRNA sequences targeting *KAT7*

Figure 6. TNF- α and interferon- β reduce imMKCL quality and platelet producibility during Dox-ON maintenance

(A) Cell proliferation as measured by the CCK8 assay for ST-C clones treated with TNF- α or interferon- β during the 3-day Dox-ON culture period.

(B) Changes in cells in G0 interphase after TNF- α or interferon- β treatment.

(C) Changes in cells in G2/M interphase after TNF- α or interferon- β treatment.

(D) Changes in cells in G1 interphase after TNF- α or interferon- β treatment.

(E) Platelet producibility for ST-C clones with TNF- α or interferon- β pre-treatment before the Dox-OFF maturation period. Data are presented as the mean $\pm$ SEM from more than three independent experiments. Statistical significance was assessed using one-way ANOVA. Asterisks indicate statistical significance as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).



or LacZ (control) were inserted into the FG12-HYG lentiviral vector plasmid. The FUCCI vector was obtained from TAKARA Bio. Lentiviral production was carried out using the 293T system (Takayama et al., 2010). The oligonucleotide sequence for shKAT7 was as follows: GCCCTTCCTGT TCTATGTTAT.

Immunofluorescence confocal microscopy

Cells were fixed with 4% paraformaldehyde (Wako) for 10 min and permeabilized using 0.1% Triton X-100 (Sigma) for 5 min. Samples were blocked with 10% goat serum (Sigma) and incubated with mouse anti-CD41a antibodies (eBioscience) for 1 h. Subsequently, cells were treated with Alexa Fluor 647-conjugated secondary antibodies (Thermo Fisher Scientific) for 30 min. Nuclear staining was performed using DAPI (Vector). Images were acquired using a Zeiss LSM900 confocal microscope with a 63×/1.40 numerical aperture oil-immersion objective.

Intracellular staining flow cytometry

imMKCL cells were fixed and stained using the PerFix-nc kit (Beckman Coulter) according to the manufacturer's instructions. Acetyl-Histone H3 (Lys14) Rabbit antibody, Phospho-Histone H2A.X (Ser139) Antibody, and Phospho-STING (Ser366) Rabbit mAb from Cell Signaling Technology were used as primary antibodies, followed by Alexa Fluor 647-conjugated goat secondary antibodies (Thermo Fisher Scientific). Samples were analyzed using a FACSLyric instrument (BD Biosciences).

Analysis of cytokine secretion by imMKCLs

Cytokine levels in culture supernatants were quantified using the Human Inflammatory Cytokine Cytometric Bead Array Kit (BD Biosciences) in accordance with the manufacturer's instructions. In brief, bead populations with distinct fluorescence intensities, coated with specific capture antibodies, were incubated with phycoerythrin-conjugated detection antibodies and either recombinant standards or test samples, forming sandwich complexes. Data acquisition was performed via flow cytometry, and cytokine concentrations were calculated using FCAP Array software (BD Biosciences).

Bulk RNA-sequencing analysis

Total RNA was extracted using the miRNeasy Micro Kit following the manufacturer's protocol. RNA-seq libraries were prepared from approximately 10 ng of total RNA using the SMART-Seq v4 Ultra Low Input RNA Kit (Takara Bio). cDNA was fragmented with an S220 Focused-ultrasonicator (Covaris) and used to generate libraries with the NEBNext Ultra DNA Library Prep Kit for Illumina (New England BioLabs). Sequencing was performed on an Illumina NextSeq 550. Fastq files were generated using

bcl2fastq-2.20. Adapter sequences and low-quality bases were trimmed from raw reads using cutadapt v4.1. Trimmed reads were mapped to human reference genome sequences (hg38) using STAR v. 2.7.10a with the GENCODE (release 32 (GRCh38.p13) gtf file. Raw read counts for each gene were calculated using htseq-count v2.0.2 with the GENCODE gtf file. Gene expression was calculated as transcripts per kilobase million mapped sequence reads.

Gene sets enrichment analysis

Gene set enrichment analysis (GSEA) was performed to identify pathways enriched in bulk RNA-seq data from WM3835- and DMSO-treated imMKCLs using the GSEAPy package in Python (Fang et al., 2023b) and the MsigDB Hallmarks library. A normalized gene expression matrix was used as input, and pathways with false discovery rate and p values <0.05 were considered statistically significant.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Unless otherwise specified, statistical significance was assessed using an unpaired two-tailed Student's t test or ANOVA. Data are presented as mean $\pm$ SEM, as specified in figure legends. Statistical significance thresholds were set as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources, reagents, and data for reanalysis should be directed to Koji Eto (eto.koji.8a@kyoto-u.ac.jp).

Materials availability

Materials are available upon reasonable request to the lead contact e-mail.

Data and code availability

RNA-seq data have been deposited in the NCBI under accession numbers GSE291894.

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AUTHOR CONTRIBUTIONS

W.-Y.Q. designed and performed most experiments, analyzed the data, interpreted the results, prepared figures, and wrote the manuscript. S.N. provided guidance on experimental design and data interpretation. S.K.P. generated WS imMKCLs. T.Y. contributed to RNA-seq bioinformatics analysis. N.T. and N.S. provided intellectual contributions and edited the manuscript. S.J.C. contributed to data interpretation and edited the manuscript. K.E. managed the overall project, proposed ideas, contributed to the experimental design and data interpretation, and wrote the manuscript.

DECLARATION OF INTERESTS

K.E. was a founder of Megakaryon Co. Ltd., without any stock and relationship currently (note that occupational licensing of imMKCLs belongs to Megakaryon Co. Ltd.). All interests of K.E. were reviewed and managed by Kyoto University in accordance with its competing interest policies. S.J.C. and K.E. have a patent application related to this work.

SUPPLEMENTAL INFORMATION

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Supplemental Information

Aging-dependent reduction of KAT7/HBO1 activity impairs imMKCL-based platelet production by promoting immune properties

Wei-Yin Qiu, Sou Nakamura, Sudip Kumar Paul, Takuya Yamamoto, Naoya Takayama, Naoshi Sugimoto, Si Jing Chen, and Koji Eto

1 **Supplementary Information**

2

3 **Aging-dependent reduction of KAT7/HBO1 activity impairs imMKCL-based platelet production**

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7

8 **Supplementary Table 1.** Primer pairs used in RT-qPCR

Gene symbol	Primer	Sequences (5'-3')
<i>RALB</i>	Forward	GACTACGCAGCCATTCGAGA
	Reverse	CCGCTCCTCTAGGTCAGACT
<i>IRF7</i>	Forward	CCCCACGCTATACCATCTAC
	Reverse	CAGCTCCATAAGGAAGCAC
<i>ISG15</i>	Forward	GAGAGGCAGCGAACTCATCT
	Reverse	CTTCAGCTCTGACACCGACA
<i>IFIT1</i>	Forward	GTGAGGAGTCTGGTGACCTG
	Reverse	AGCGGAAGGGATTTGAAAGC
<i>IFI27</i>	Forward	TCACCTCATCAGCAGTGACC
	Reverse	GCCATGGCCACAACCTCT
<i>IFNB1</i>	Forward	ACCAACAAGTGTCTCCTCC
	Reverse	ATGTCAAAGTTCATCCTGTCC
<i>KAT7</i>	Forward	TCAGGATGCCCACTGTATCATAAC
	Reverse	ATCTGCTTATCCCGGCTCTGT
<i>CBX5</i>	Forward	CAATTTCTCAAACAGTGCCGA
	Reverse	CACCACAGGAATCTGTTGCC
<i>GAPDH</i>	Forward	GTTGCCATCAATGACCCCTTC

	Reverse	CATTGATGACAAGCTTCCCG
<i>RELA</i>	Forward	ACCAACAACAACCCCTTCC
	Reverse	CGTGAAATACACCTCAATGTCC
<i>RELB</i>	Forward	AGTGTGTGAGGAAGAAGGAG
	Reverse	TGTCGCAGAGCAAGTAGAG
<i>NFKBIA</i>	Forward	CTGAAGAAGGAGCGGCTACT
	Reverse	ATGGCCAAGTGCAGGAACGA
<i>IL8</i>	Forward	AGACAGCAGAGCACACAAGC
	Reverse	ATGGTTCCTTCCGGTGGT
<i>TNF</i>	Forward	ACCTCTCTCTAATCAGCCCTC
	Reverse	TTATCTCTCAGCTCCACGCC
<i>SUV39H1</i>	Forward	TAGGCCCGAATGTCGTTAGC
	Reverse	TCCTGTTCGCGGATCTTCTT

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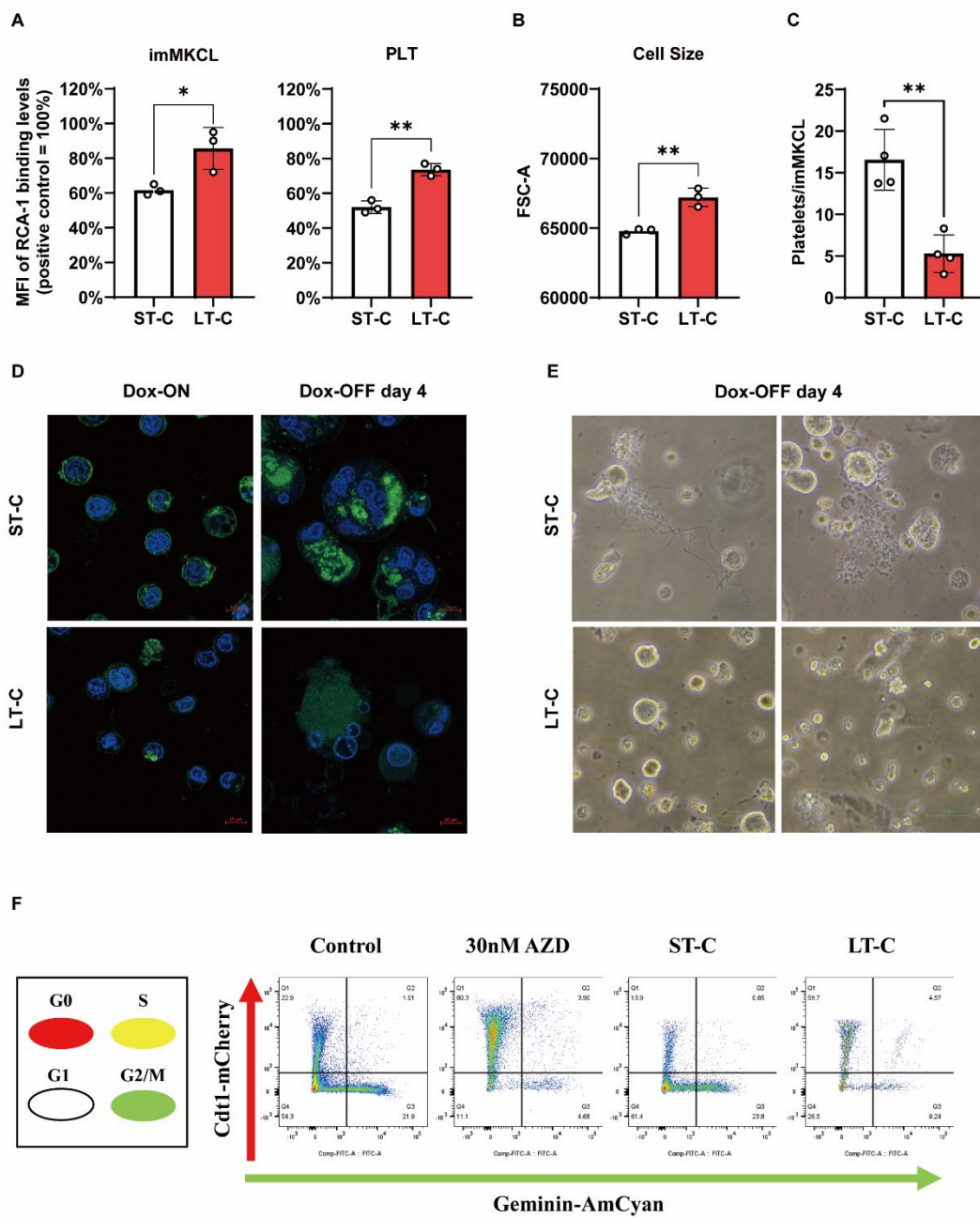
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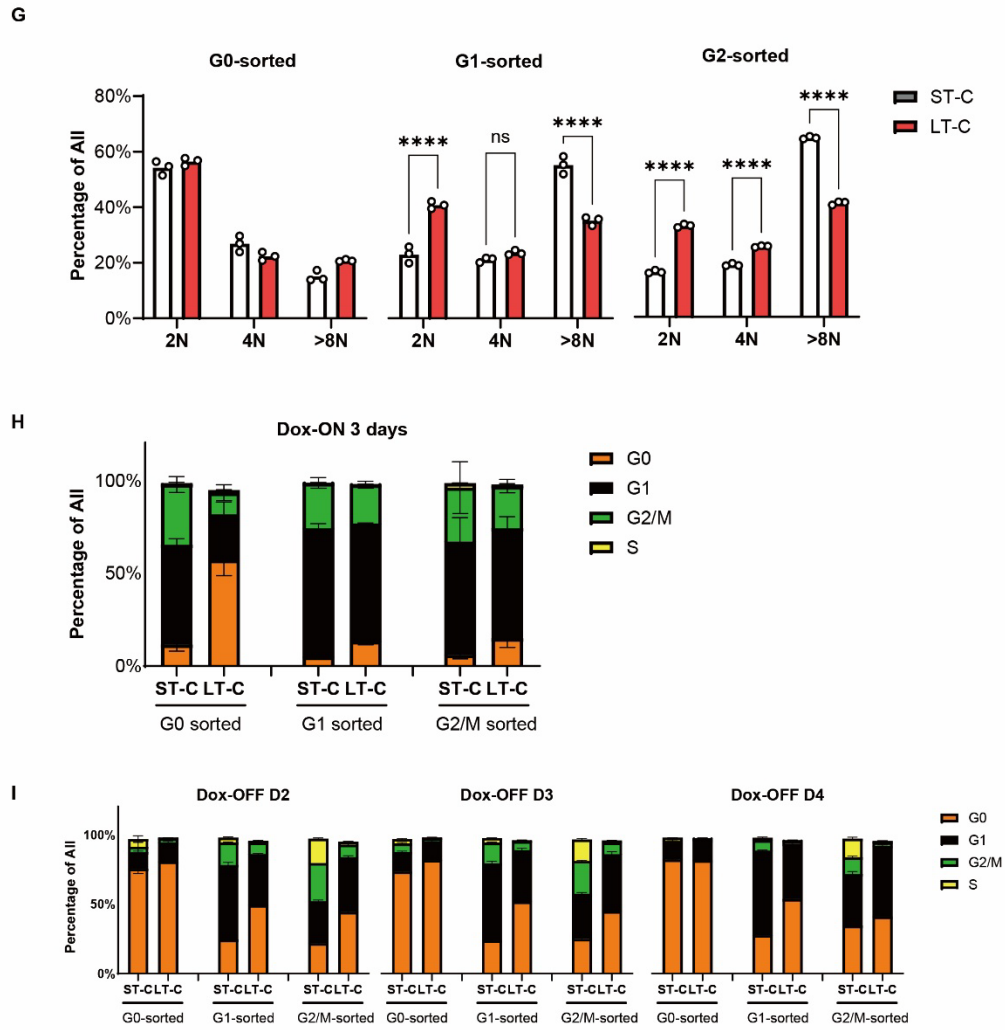


Figure S1 related to Figure 1

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20 **Figure S1, related to Figure 1. A.** Relative RCA-1 levels in both ST-C and LT-C imMKCLs and their

21 derived platelets (PLTs). **B.** cell size and **C.** Absolute platelet productivity of ST-C and LT-C imMKCLs.

22 **D.** Confocal microscopy images of ST-C and LT-C imMKCLs under Dox-ON and Dox-OFF

23 day 4 conditions. Plasma membranes were labeled with PlasMemBright Green (green), and

24 nuclei were counterstained with Hoechst 33342 (blue). **E.** Bright-field images showing

25 representative proplatelet formation morphology. **F.** Representative FACS plots of control, ST-

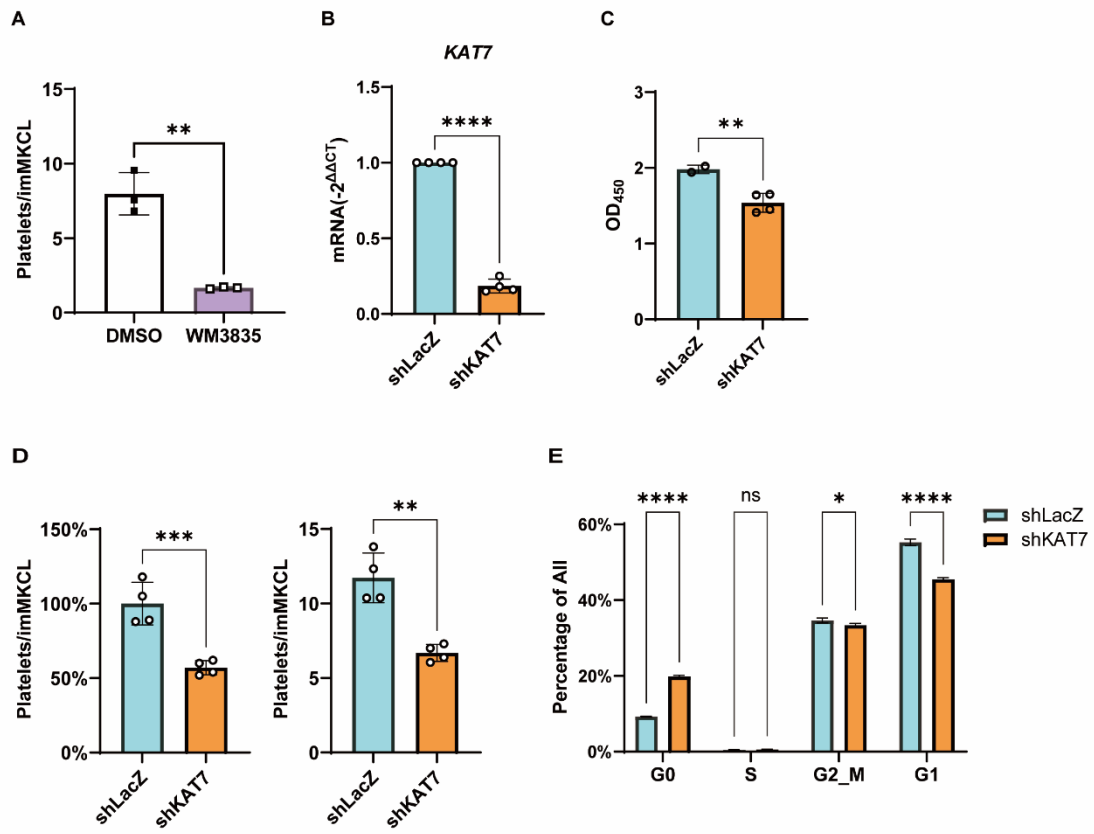
26 C, and LT-C imMKCLs using the Fucci reporter system, showing two-color fluorescence
27 detection of Fucci cell-cycle marker with gates for G0, G1, S, and G2/M phases. **G.** Polyploidy
28 in each interphase subpopulation during the maturation stage. **H.** Cell cycle analysis of each interphase
29 subpopulation after 3 days of proliferation. **I.** Cell cycle analysis of each interphase subpopulation during
30 the maturation stage. Data are expressed as the mean $\pm$ SEM from three independent experiments.
31 Unpaired two-tailed Student's *t*-tests were used to assess statistical significance.

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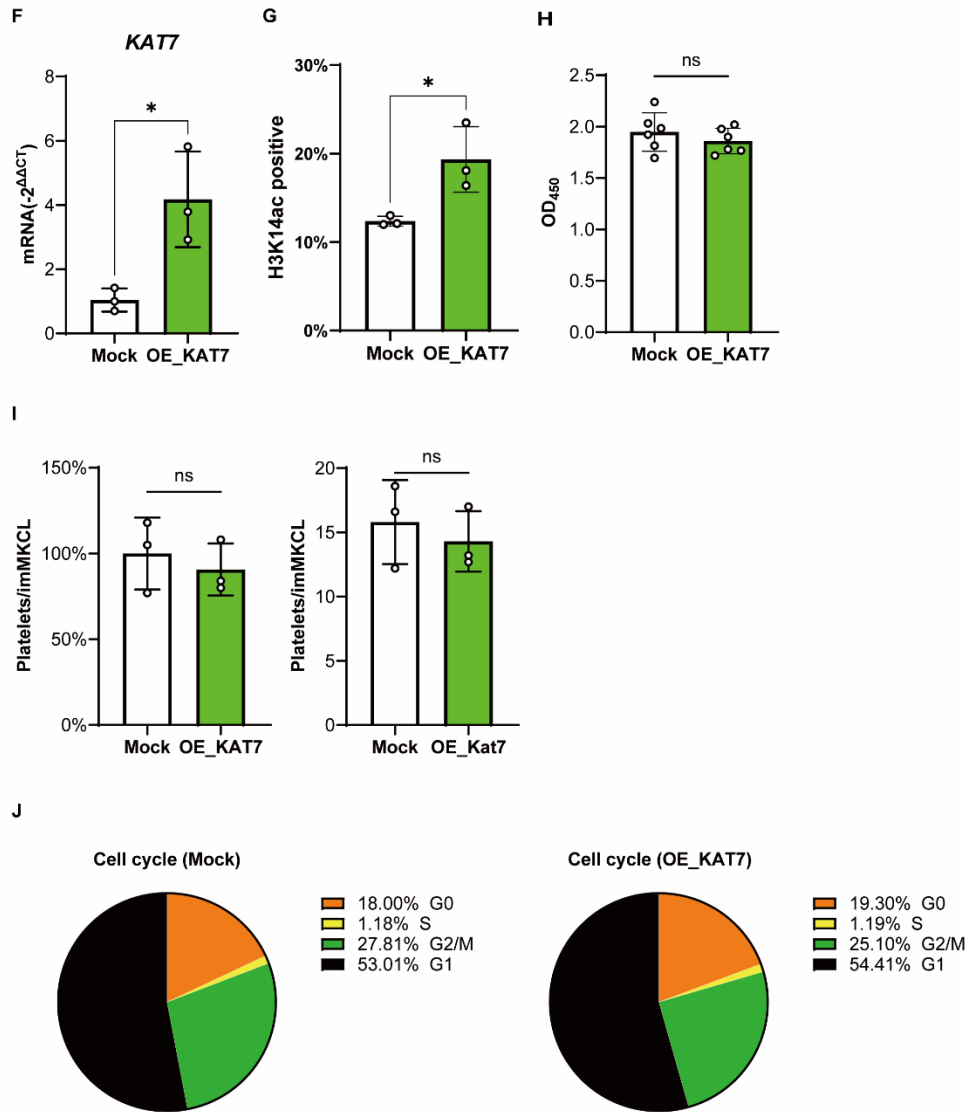


Figure S2 related to Figure 3

Figure S2, related to Figure 3. *KAT7* loss- or gain-of-function analysis. A lentiviral vector encoding shKAT7 or a control vector (shLacZ), and a lentiviral vector containing the *KAT7* CDS sequence or a control vector (Mock), were transduced into imMKCLs. Hygromycin B or blasticidin was used for selection. **A.** Platelet productivity of ST-C after DMSO or WM38535 treatment. **B.** *KAT7* expression determined by real-time qPCR in shLacZ and shKAT7 cells. **C.** Cell proliferation as measured by the CCK8 assay for shLacZ and shKAT7 cells. **D.** Platelet productivity of shLacZ and shKAT7 cells. **E.** Cell

44 cycle analysis during the proliferation stage. **F.** *KAT7* expression determined by real-time qPCR in mock
45 and *KAT7* overexpressed cells. **G.** H3K14ac expression determined by intracellular flow cytometry. **H.**
46 Cell proliferation measured by the CCK8 assay during a 3-day DOX-ON culture period. **I.** Platelet
47 productivity in mock and *KAT7* overexpressed cells. **J.** Cell cycle analysis during the proliferation stage.
48 Data are presented as the mean $\pm$ SEM from more than three independent experiments. Statistical
49 significance was assessed using unpaired two-tailed Student's *t*-tests.

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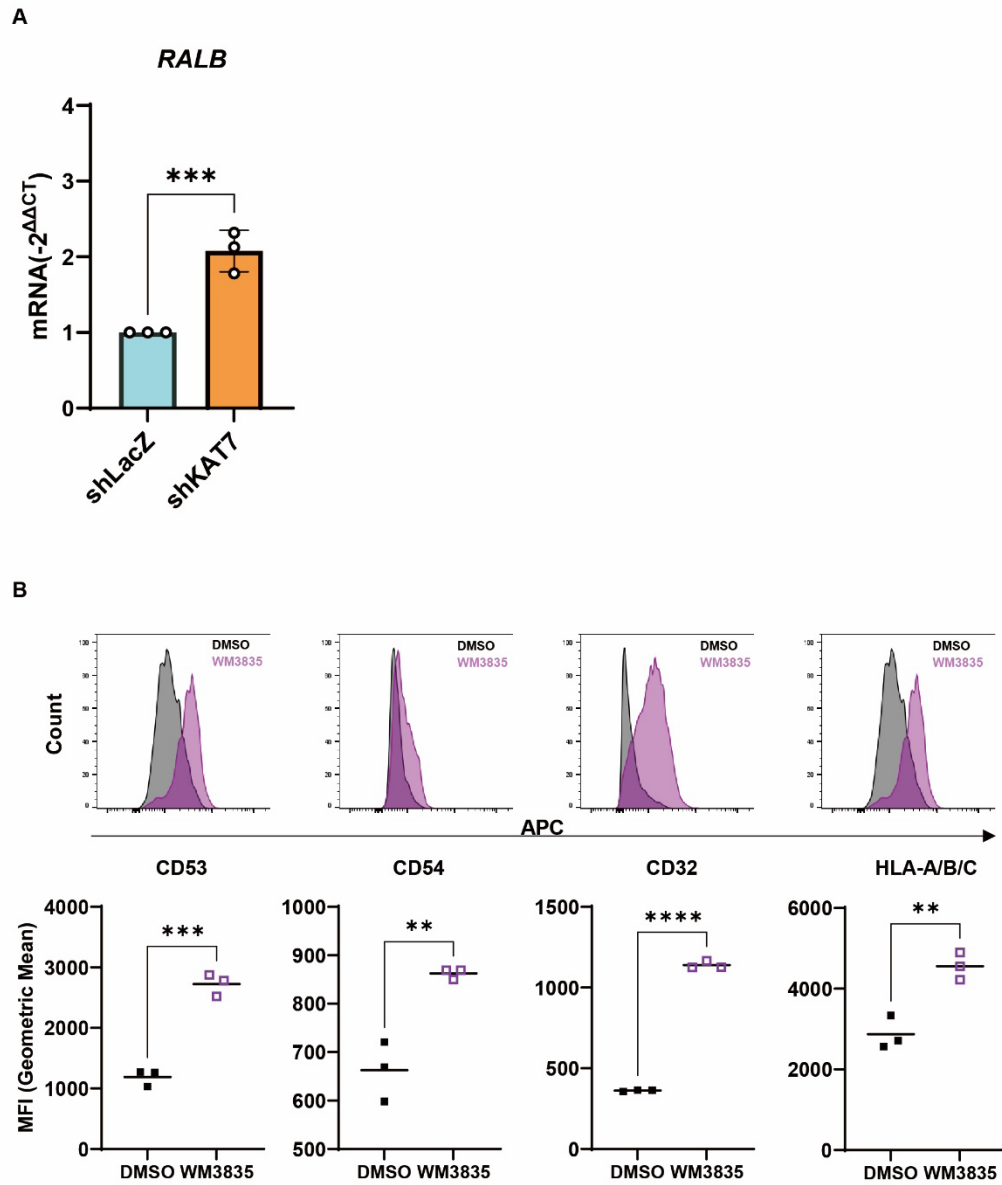


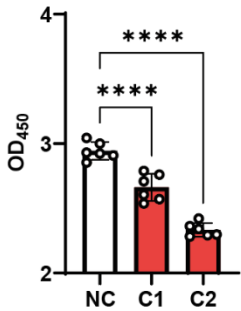
Figure S3 related to Figure 4

Figure S3, related to Figure 4. **A.** *RALB* expression as determined by real-time qPCR for shKAT7 clones. **B.** Flow cytometry analysis of candidate immune-related surface markers in ST-C cells following KAT7 inhibition. Data are presented as the mean $\pm$ SEM from more than three independent experiments. Statistical significance was assessed using unpaired two-tailed Student's *t*-tests.

A

(pg/mL)	TNF- α	IFN- β	IL-8	PF4	CXCL7
C1	10	10	20	100	10
C2	100	100	200	1000	100

B



C

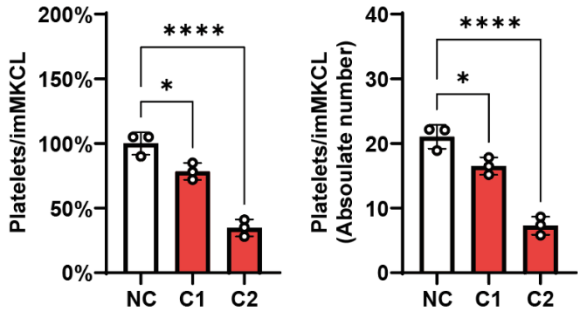


Figure S4 related to Figure 6

Figure S4, related to Figure 6. **A.** The compositions of two inflammatory cytokine cocktails (C1 and C2). **B.** Cell proliferation as measured by the CCK8 assay during a 3-day DOX-ON culture period. **C.** Platelet productivity of imMKCLs treated with negative control (NC) or C1 or C2, respectively. Data are presented as the mean $\pm$ SEM from more than three independent experiments. Statistical significance was assessed using unpaired two-tailed Student's *t*-tests.

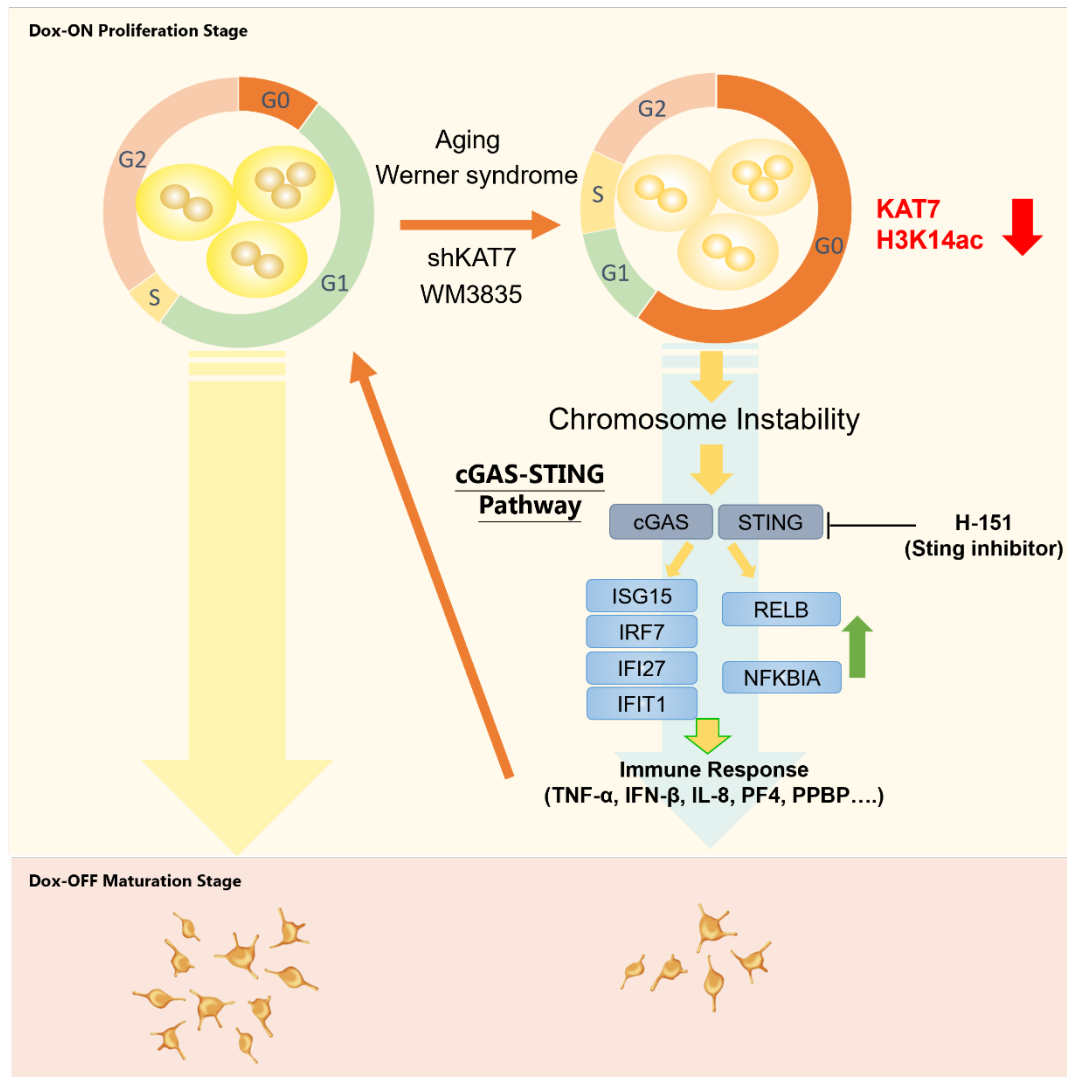


Figure S5. Schematic overview illustrating how the loss of KAT7 function, whether due to aging or exogenous KAT7 inhibition, leads to G0 cell cycle arrest and impaired platelet maturation capacity in imMKCLs. This process switches the cells toward an immune-biased phenotype through cGAS-STING pathway activation driven by chromosome instability. Released immune cytokines further exacerbate the condition by negatively affecting normal imMKCLs.