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KCNN4-mediated potassium ion efflux maintains mitochondrial functions leading to platelet biogenesis

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26 **ABSTRACT**

27 **Background:** Potassium ions (K^+) are essential for platelet function, yet their role in
28 thrombopoiesis—particularly through specific K^+ channels—remains poorly understood. This
29 gap is especially relevant in the context of *in vitro* platelet production from induced
30 pluripotent stem cell (iPSC)-derived immortalized megakaryocyte progenitor cell lines
31 (imMKCLs), which we have developed for clinical-grade platelet manufacturing.

32 **Objective:** We aimed to elucidate how K^+ channels contribute to platelet biogenesis, focusing
33 specifically on the calcium ion (Ca^{2+})-activated K^+ channel KCNN4 (also known as $K_{Ca3.1}$).

34 **Methods:** Using imMKCLs and human cord blood-derived megakaryocytes (CB-
35 megakaryocytes), we analyzed intracellular cation dynamics during platelet production. RNA
36 sequencing profiling was conducted to identify K^+ channel gene expression changes, focusing
37 on *KCNN4*. Its role in proplatelet formation and platelet release was examined using
38 pharmacological inhibitors and gene knockdown (KD). We further investigated the link
39 between KCNN4 and microtubule organization, mitochondrial function, and reactive oxygen
40 species (ROS) levels.

41 **Results:** A progressive decline in intracellular K^+ concentration ($[K^+]_i$) was observed during
42 the six-day maturation period of imMKCLs. KCNN4 was upregulated at onset of platelet
43 generation, and its inhibition or KD led to impaired proplatelet formation and reduced platelet
44 yield in both imMKCLs and CB-megakaryocytes. These effects were accompanied by
45 decreased $[K^+]_i$, reduced mitochondrial membrane potential, and increased ROS
46 accumulation.

47 **Conclusions:** Our findings reveal that the KCNN4-mediated reduction in $[K^+]_i$ is a crucial
48 mechanism linking cytoskeletal reorganization, mitochondrial function, and ROS homeostasis
49 to effective thrombopoiesis. This study provides new insights into platelet biogenesis and
50 offers potential avenues to optimize *ex vivo* platelet production.

51

52 **Keywords:** megakaryocyte, platelet, potassium, mitochondria, ROS

1. INTRODUCTION

Donor-derived red blood cells and platelets are routinely employed in transfusion therapy. However, aging populations in developed countries have raised growing concerns over persistent blood shortages. Compounding this issue, platelet products stored at room temperature exhibit limited efficacy and safety, restricting their shelf life to just 4 days in Japan and up to 7 days in the United States [1, 2]. As such, the ex vivo generation of red blood cells or platelets presents a promising avenue to develop safer and more sustainable alternatives to conventional donor-derived blood products [3–5].

Frequent platelet transfusions also elevate the risk of alloimmune platelet transfusion refractoriness (allo-PTR), a condition driven by alloantibodies targeting human leukocyte antigen class I (HLA-I) or human platelet antigens (HPAs) [6]. This presents a significant clinical challenge, particularly for patients with rare HLA-I or HPA types who require highly compatible donor platelets. In response, we initiated the iPLAT1 study—the first-in-human clinical trial involving ex vivo-generated platelets derived from human induced pluripotent stem cells (iPSCs). This autologous transfusion was administered to a Japanese female patient suffering from allo-PTR caused by HPA-1a alloimmunization, for whom no compatible HPA-1b/1b donor could be identified in Japan [7, 8]. To enable large-scale, clinical-grade production of iPSC-derived platelets (iPSC-platelets), we established a master cell stock of megakaryocytes using imMKCLs—a series of immortalized megakaryocyte progenitor cell lines. These were generated by sequential overexpression of c-MYC, BMI1, and BCL-XL in CD34⁺ hematopoietic progenitor cells differentiated from iPSCs. The proliferation of imMKCLs is maintained under doxycycline-induced transgene expression (Dox-ON),

75 whereas maturation and platelet shedding are promoted upon doxycycline withdrawal (Dox-
76 OFF) [9].

77 Although studies in megakaryopoiesis and thrombopoiesis underscore the importance of
78 adhesion-dependent mechanisms [10], we successfully established adhesion-free, liquid
79 culture conditions supporting both Dox-ON and Dox-OFF stage. This was achieved using a
80 defined cocktail comprising a thrombopoietin (TPO)-mimetic peptide (TA-316), an aryl
81 hydrocarbon receptor (AhR) antagonist, and a Rho/Rho-associated protein kinase (ROCK)
82 inhibitor [11]. Nevertheless, the molecular mechanisms governing platelet production upon
83 toggling the transgene expression of c-MYC, BMI1, and BCL-XL remain poorly understood
84 [11]. Intriguingly, c-MYC has been implicated in modulating the activity of various ion
85 channels [12, 13].

86 In this study, we sought to elucidate the role of ion channels in platelet biogenesis by
87 megakaryocytes, with a particular focus on potassium ion (K^+) channels, prompted by the
88 distinct and continuous decline of intracellular K^+ concentrations ($[K^+]_i$) during
89 megakaryocyte maturation. Among the candidate K^+ channels, we identified KCNN4
90 ($K_{Ca3.1}$), a calcium ion (Ca^{2+})-activated K^+ -selective ion channel, as a key regulator of platelet
91 production in both imMKCLs and human cord blood-derived megakaryocytes (CB-
92 megakaryocytes). Functional inhibition or gene silencing of KCNN4 disrupted microtubule
93 organization, impaired proplatelet formation, reduced mitochondrial membrane potential
94 (MMP), and elevated reactive oxygen species (ROS) in maturing imMKCLs.

2. MATERIAL AND METHODS

Ethics approval

The use and collection of cord blood cells from healthy volunteers with informed consent was approved by the ethics committees of Kyoto University. All experiments using human samples were conducted in accordance with the Declaration of Helsinki.

2.1. Cells

An immortalized megakaryocyte progenitor cell line (imMKCL, clone 7) was established from human iPSCs by introducing doxycycline-inducible defined factors and employed in previous studies [9, 11]. Human cord blood-derived CD34⁺ hematopoietic stem cells (HSCs) from full-term deliveries were purchased from Takara Bio or purified from cord blood obtained at Nakabe Ladies Clinic (Kyoto, Japan) or the Japanese Red Cross Kinki Cord Blood Bank. For purification, mononuclear cells were isolated using Lymphoprep (STEMCELL Technologies), and HSCs were positively selected using a CD34⁺ Cell Isolation Kit (Miltenyi Biotec). HSCs were frozen in 90% fetal bovine serum (FBS; Cell Culture Bioscience) and 10% dimethyl sulfoxide in liquid nitrogen.

2.1.1. imMKCL culture

2.1.1.1. Dox-ON proliferation culture

imMKCLs were cultured in a medium containing doxycycline to induce proliferation through the expression of three doxycycline-regulated transgenes: c-MYC, BMI1, and BCL-XL [9].

Cells were maintained at 37°C in a humidified incubator with 5% CO₂ and passaged to

maintain a cell density between 1.0×10^5 and 2.0×10^6 cells/mL. The base medium, supplemented with 15% fetal bovine serum (FBS; Nichirei) and Cocktail A, was used for culturing. The base medium consisted of Iscove's Modified Dulbecco's Medium (IMDM; Sigma-Aldrich) supplemented with L-glutamine (Thermo Fisher Scientific), insulin-transferrin-selenium (Thermo Fisher Scientific), 50 μ g/mL ascorbic acid (Sigma-Aldrich), and 450 μ M 1-thioglycerol (Sigma-Aldrich). Cocktail A included 50 ng/mL recombinant human kit ligand protein (Cusabio), 200 ng/mL TA-316 (Nissan Chemical), and 1 μ g/mL doxycycline (Clontech), as previously described [11].

2.1.1.2. Dox-OFF differentiation culture

The maturation of imMKCLs was conducted in a humidified incubator at 37°C with 5% CO₂. Cells were maintained at a density of 1.0×10^5 cells/mL for six days. The base medium was supplemented with 15% FBS and Cocktail B. Cocktail B comprised 50 ng/mL recombinant human kit ligand protein, 200 ng/mL TA-316, 15 μ M KP-457 (Kaken Pharmaceutical), 0.75 μ M SR-1 (Calbiochem), and 10 μ M Y-27632 (Wako), as previously described [11].

2.1.2. *in vitro* differentiation of CB-megakaryocytes

CB-megakaryocytes were differentiated using a modified version of a previously published protocol [14, 15]. Briefly, human cord blood-derived CD34⁺ HSCs were cultured in X-VIVO 10 medium (Lonza) supplemented with 1% bovine serum albumin (BSA; StemCell Technologies), 100 ng/mL human stem cell factor (SCF; R&D Systems), 100 ng/mL human Flt-3 ligand (PeproTech EC Ltd.), 50 ng/mL human thrombopoietin (TPO; R&D Systems), 10

ng/mL human interleukin-6 (IL-6; PeproTech EC Ltd.), and 10 ng/mL human interleukin-3 (IL-3; PeproTech EC Ltd.). After 9 days, cells were harvested and counted. The medium was replaced every two days during the differentiation process. A total of 1.0×10^5 cells were seeded and further cultured for 11 days in 1 mL of X-VIVO 10 medium supplemented with 1% BSA, 100 ng/mL SCF, 50 ng/mL TPO, and 10 ng/mL IL-6.

2.2. Reagents

In this study, the following drugs were used: tetraethylammonium chloride (TEA, 5 mM; Nacalai Tesque), 3,4-diaminopyridine (3,4-DAP, 1 mM; TCI), 4-aminopyridine (4-AP, 1.5 mM; Nacalai Tesque), chlorpromazine (CPZ, 1, 2, or 5 μ M; Selleck), senicapoc (10, 20, or 30 μ M; Cayman), TRAM-34 (5, 10, or 15 μ M; Cayman), and tert-butyl hydroperoxide (TBHP, 50 μ M; Sigma-Aldrich). For imMKCLs, drug treatments were administered during the Dox-ON stage as well as on days 0 through 5 of the Dox-OFF stage. For cord blood-derived megakaryocytes (CB-megakaryocytes), senicapoc (30 μ M), TRAM-34 (15 μ M), or TBHP (50 μ M) was added starting on day 14 of differentiation.

2.3. Flow cytometric analysis

Flow cytometric analysis was performed using FACSVerse or FACSLytic (BD Biosciences) as previously described [9]. Data analysis was performed using FlowJo software.

2.3.1. Measurement of intracellular and extracellular ion levels

Intracellular potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), and sodium (Na^+) levels ($[K^+]_i$, $[Ca^{2+}]_i$, $[Mg^{2+}]_i$, and $[Na^+]_i$) were quantified using specific dyes: $[K^+]_i$ with ION Potassium Green-2 AM (IPG-2 AM, final concentration 5 μ M; Abcam), $[Ca^{2+}]_i$ with Fluo-4 AM (final concentration 5 μ M; Invitrogen), $[Mg^{2+}]_i$ with Magnesium Green AM (MG AM, final concentration 5 μ M; Invitrogen), and $[Na^+]_i$ with ION NaTRIUM Green-2 AM (ING-2 AM, final concentration 5 μ M; Abcam). imMKCLs were incubated under the Dox-OFF condition from days 0 to 6, with 1.0×10^5 cells collected every day and centrifuged at $400 \times g$ for 5 minutes. Cells were suspended in phosphate buffer saline (PBS) and incubated with specific dyes described previously and an APC-anti-human CD41 antibody (BioLegend) at 37°C for 30 minutes, followed by washing with PBS twice. Dead cells were excluded using propidium iodide (PI). Fluorescence intensities in $CD41^+$ cells were determined by flow cytometry.

Extracellular K^+ levels ($[K^+]_e$) in the culture medium were measured using i-STAT 1, a portable blood analyzer for measuring K^+ levels in blood samples (Abbott Japan LLC, Tokyo, Japan).

2.3.2. Quantification of platelet production and ploidy level of imMKCLs

For the analysis of platelet production, imMKCLs were cultured under the Dox-OFF condition for 6 days under static conditions. Subsequently, the culture medium was stained with APC-anti-human CD41 (BioLegend) and PE-anti-human CD42b (GPIb α ; BioLegend) antibodies at room temperature in the dark for 20 minutes. The production of $CD41^+CD42b^+$ platelets was quantified using Trucount Tubes (BD Biosciences) via flow cytometry. As for

CB-megakaryocytes, the same antibodies were used, and flow cytometry analysis was performed on day 20 to quantify CD41⁺CD42b⁺ platelets. To analyze the ploidy levels of imMKCLs, 1.0×10^5 cells were collected on day 4 of the Dox-OFF stage and centrifuged at $400 \times g$ for 5 minutes. Cells were fixed in 0.5% paraformaldehyde (Wako) for 15 minutes at room temperature. After rinsing with PBS, cells were treated with ice-cold methanol for 5 minutes. Following another rinse with PBS, cells were permeabilized using 0.01% Triton X-100 (Sigma-Aldrich) with 50 µg/mL PI and 0.1% RNase for 5 minutes. PI fluorescence intensities were measured using flow cytometry.

2.4. RNA sequencing

For imMKCLs, total RNA was isolated using an RNeasy mini kit (Qiagen). RNA integrity was assessed with a 2100 Bioanalyzer (Agilent Technologies). RNA library preparation was performed using a TruSeq Stranded mRNA Sample Prep Kit (Illumina) according to the manufacturer's instructions. Sequencing was performed on an Illumina NextSeq500. FASTQ files were generated using bcl2fastq-2.20. Adapter sequences and low-quality bases were trimmed from the raw sequenced reads using cutadapt ver v3.4 [16]. Trimmed reads were mapped to human reference genome sequences (hg38) using STAR ver 2.7.9a [17] with GENCODE (release 32, GRCh38.p13) [18]. GTF file. Uniquely mapped reads were summarized at the gene level using htseq-count ver. 0.13.5 [19] with the GENCODE GTF file. For CB-megakaryocytes, total RNA was extracted as previously published [20]. The sequencing procedures used were similar to our recent report [20]. The expression of each

gene was calculated as fragments per kilobase of transcript per million mapped reads (FPKM).

2.5. Lentiviral production and gene knockdown (KD)

To construct shRNA lentiviral vectors targeting *KCNH2*, *KCNN4*, and *lacZ* (control), target sense and antisense sequences were designed using the BLOCK-iT RNAi Designer website (rnaidesigner.thermofisher.com/rnaiexpress/). Specific details are available in Supplementary Table S1. The use of viral vectors received approval from committees at Kyoto University. The methods for shRNA cloning and lentiviral production using the 293T system have been described previously [21].

Lentiviral vectors containing shRNA constructs (sh*KCNH2*, sh*KCNN4*, and sh*lacZ*) were introduced into imMKCLs during the Dox-ON stage to construct gene KD of *KCNH2*, *KCNN4*, and *lacZ*, followed by selection with 500 µg/mL hygromycin B to ensure that only transduced cells remained.

2.6. Quantitative RT-PCR

RNA was extracted using a miRNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. cDNAs were synthesized using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO). Real-time PCR was performed using SYBR Premix Ex Taq II (TLi RNaseH Plus) and ROX plus (TaKaRa) according to the manufacturer's instructions. Signals were detected using the StepOnePlus Real-Time PCR System (Applied Biosystems).

Primer sequences for *GAPDH*, *KCNH2*, and *KCNN4* are provided in Supplementary Table S2.

2.7. Western blot

Protein detection was performed using the Simple Western system (Wes; ProteinSimple), an automated capillary-based immunoassay platform. imMKCLs lysates were prepared and quantified using the BCA assay. Samples were diluted to a final concentration of 0.2 µg/µL in sample buffer and denatured at 95°C for 5 minutes. Primary antibodies against KCNN4 (Rabbit, Anti-KCNN4 antibody, 1:100, Abcam, ab75956) and GAPDH (Rabbit, 1:50, D16H11, XP® Rabbit mAb, Cell Signaling #5174), and secondary HRP-conjugated antibodies were used according to the manufacturer's instructions. Data acquisition and analysis were conducted using Compass software.

2.8. Proplatelet formation

After 6 days of culture under the Dox-OFF condition, proplatelet formation in imMKCLs was observed using an inverted microscope (ECLIPSE TS100, Nikon) equipped with a 40x/0.55 magnification lens. Proplatelet-forming megakaryocytes were identified by the presence of long filamentous structures terminating in platelet-sized tips.

2.9. Immunocytochemistry (ICC) analysis

For ICC analysis, 1.0×10^5 imMKCLs at day 4 of the Dox-OFF stage were collected and fixed in 4% paraformaldehyde (Wako) for 10 minutes, then permeabilized with 0.1% Triton

X-100 (Sigma-Aldrich) for 5 minutes. Samples were subsequently blocked in 10% goat serum (Sigma-Aldrich) and incubated with primary antibodies for 1 hour: mouse anti- α -tubulin (1:1000; Abcam) and rabbit anti- β -tubulin (1:1000; Abcam). Samples were incubated with secondary antibodies for 30 minutes: Alexa Fluor 488 goat anti-rabbit IgG (1:1000; Invitrogen), Alexa Fluor 555 goat anti-mouse IgG (1:1000; Invitrogen), Alexa Fluor 647 goat anti-rabbit IgG (1:1000; Invitrogen), or with Alexa Fluor 647 phalloidin (1:40; Invitrogen), or Alexa Fluor 488 anti-TOMM20 antibody (1:1000; Abcam). Coverslips were mounted on glass slides using ibidi Mounting Medium with DAPI (ibidi), and cells were imaged using a confocal microscope (Zeiss LSM900) with a 63x/1.40 numerical aperture oil immersion objective.

2.10. Live-cell imaging (LCI)

Glass bottom dishes were coated with a 0.001% Poly-L-lysine solution and incubated at 37°C for at least 30 minutes. After 4 days of culture under the Dox-OFF condition, 1.0×10^5 imMKCLs were collected and centrifuged at $400 \times g$ for 5 minutes. Cells were resuspended in PBS and incubated with SPY650-tubulin (1:100; Cytoskeleton) for 30 minutes, followed by co-incubation with MitoBright LT Green (1:100; Dojindo) for an additional 30 minutes, on poly-L-lysine-coated glass bottom dishes. Subsequently, cell nuclei were stained with Hoechst 33342, and samples were imaged using a confocal microscope (LSM900, Zeiss) equipped with a 63x/1.40 numerical aperture oil immersion objective.

2.11. Measurement of ROS levels

268 imMKCLs were incubated under Dox-OFF conditions for four days, after which
269 1.0×10^5 cells were collected and centrifuged at $400 \times g$ for five minutes. The cells were
270 resuspended in PBS and incubated with $1 \mu\text{M}$ of CellROX Deep Red Reagent (Invitrogen)
271 and FITC-anti-human CD41 antibody (BioLegend) at 37°C for 30 minutes. Dead cells were
272 excluded using PI. The fluorescence intensities of CellROX in CD41^+ cells were measured
273 using flow cytometry.

274

275 **2.12. Measurement of MMP levels**

276 After incubating imMKCLs under Dox-OFF conditions for four days, 1.0×10^5 cells were
277 collected and centrifuged at $400 \times g$ for five minutes. The cells were resuspended in PBS and
278 incubated with 100 nM of MitoTracker Deep Red (Invitrogen) and FITC-anti-human CD41
279 antibody (BioLegend) at 37°C for 30 minutes. Dead cells were excluded using PI. The
280 fluorescence intensities of MitoTracker in CD41^+ cells were measured using flow cytometry.

3. RESULTS

3.1. $[K^+]_i$ continuously decline during imMKCL maturation

We first sought to assess dynamic cation changes during imMKCL maturation. To achieve this, we applied specific dyes to detect $[K^+]_i$, $[Ca^{2+}]_i$, $[Mg^{2+}]_i$, and $[Na^+]_i$ in imMKCLs. Preliminary tests identified 5 μ M as the optimal concentration of each dye for imMKCLs (Supplementary Figure S1A–H). Fluorescence intensities were measured by flow cytometry over six days during the Dox-OFF maturation stage (Figure 1A–D). Given that the mean fluorescent intensity (MFI) of baseline fluorescence (F_0) in imMKCLs gradually increased, likely due to cell enlargement (Supplementary Figure S1I), we normalized the MFI after indicator dye loading by calculating the relative fluorescence level (F/F_0), as done in other studies [22]. Notably, only $[K^+]_i$ progressively decreased over the same period (Figure 1E–H). Additionally, we measured extracellular potassium levels ($[K^+]_e$) in the culture medium using i-STAT 1, which revealed that $[K^+]_e$ gradually increased during the Dox-OFF stage (Supplementary Figure S1J). In contrast, under Dox-ON conditions, $[K^+]_i$ remained relatively stable over five days (Supplementary Figure S1K).

3.2. Non-selective K^+ -related channel inhibition revealed its importance during early imMKCL maturation

To assess whether the reduction in intracellular potassium concentration ($[K^+]_i$) is related to imMKCL maturation, we next examined the effects of non-specific pan K^+ channel inhibitor tetraethylammonium chloride (TEA) [23], voltage-gated K^+ channel inhibitors, 3,4-diaminopyridine (3,4-DAP) [24], and 4-aminopyridine (4-AP) [25]. The proliferation and

viability of imMKCLs were unaffected by any of these inhibitors during the five-day Dox-ON culture (Supplementary Figures S2A–E).

When inhibitors were administered from day 0 of the Dox-OFF stage, a significant reduction in platelet yield was observed by day 6 (Figure 2A-C), with no effect on ploidy levels (Figure 2D). Furthermore, TEA treatment did not significantly alter the expression of differentiation markers CD41 and CD42b on day 4 of the Dox-OFF stage, suggesting a limited impact on imMKCL differentiation (Supplementary Figure S2F). Interestingly, when drug administration was applied from days 0 to 5, encompassing a full 6-day Dox-OFF culture period (Supplementary Figure S2G), inhibitory effects were only evident when inhibitors were added between days 0 and 2. This finding indicates that K⁺ channels play a critical role in the early stages of imMKCL maturation (Figure 2E-G).

3.3. Similar K⁺ channel expression between imMKCLs and CB-megakaryocytes

To identify the corresponding K⁺-related channel vital to human platelet biogenesis, we referred to the gene expression profiles of several K⁺-related channels of imMKCLs (Dox-ON on day 0 and Dox-OFF on days 1 to 4) and CB-megakaryocytes (CD34⁺CD41⁺ on day 10, CD41⁺ on day 17, and CD41⁺CD42b⁺ on day 22) (Figures 3A, B and Supplementary Figure S3A, B). Based on the suggested importance of K⁺-related channels during early time points (Figure 2E-G), we focused on *KCNH2* (HERG, known as a causal gene of long QT syndrome) [26, 27] and *KCNN4* (K_{Ca}3.1, Ca²⁺-activated K⁺ channel) [28]. Notably, the expression of *KCNH2* and *KCNN4* decreased gradually from day 0 to day 6 (Figure 3A, B).

3.4. KCNN4 inhibition decreased platelet production in imMKCLs and CB-

megakaryocytes

Three different concentrations of the KCNH2-specific inhibitor chlorpromazine (CPZ) [29] or direct KD of *KCNH2* did not affect platelet production (Supplementary Figure S4A-C). In contrast, the administration of KCNN4-specific inhibitors, senicapoc [30] or TRAM-34 [31], led to a dose-dependent decrease in platelet production (Figure 4A, B). Importantly, this decrease in platelet production was not attributed to drug toxicity or induced cell death, as indicated by Annexin V/PI staining (Supplementary Figure S4D-F). The inhibitory effects of these two drugs were independent of ploidy levels (Figure 4C, D; Supplementary Figure S4G, H). Similar to TEA, senicapoc treatment did not significantly alter CD41 and CD42b expression at day 4 of the Dox-OFF stage (Supplementary Figure S4I). *KCNN4* KD also reduced platelet production (Figure 4E-H), which was not due to toxicity-induced cell death (Supplementary Figure S4J), and did not affect ploidy (Figure 4I; Supplementary Figure S4K). We confirmed that *KCNN4* KD significantly impaired the decline of $[K^+]_i$ without influencing $[Ca^{2+}]_i$ (Figure 4J, K). To determine if KCNN4 dependency in the thrombopoietic process also applies to human naïve megakaryocytes, we tested the effects of senicapoc or TRAM-34 on CB-megakaryocytes (Supplementary Figure S4L). The results suggest that KCNN4 is similarly crucial for platelet production from human naïve megakaryocytes (Figure 4L, M).

3.5. KCNN4 inhibition or KD showed impaired proplatelet formation and tubulin

localization in maturing imMKCLs

To gain insights into how KCNN4 inhibition or KD interferes with the maturation process, we performed microscopic analyses of imMKCLs. In line with the absence of any changes in ploidy, neither senicapoc administration nor *KCNN4* KD altered cell enlargement. However, both treatments led to a reduction in proplatelet formation (Figure 5A, B). Microtubule remodeling is structurally essential for cytoplasmic extension and the subsequent formation of proplatelets in maturing megakaryocytes [32]. To understand why KCNN4 inhibition or KD diminished proplatelet formation, we conducted ICC of β -tubulin and F-actin (phalloidin) after cell fixation. Supplementary Figure S5A and Figure 5C show the highly polarized and asymmetric accumulation of microtubules— α -tubulin and β -tubulin in senicapoc-treated imMKCLs, and β -tubulin in *KCNN4* KD (*shKCNN4*) imMKCLs—but not F-actin. This accumulation was quantified by fluorescence intensity in the horizontal plane (Figure 5D) and shows a significant difference (Supplementary Figure S5B and Figure 5E), thus indicating dysregulated microtubule remodeling.

3.6. Excessive ROS levels generated by *KCNN4* KD may reduce platelet production in imMKCLs and CB-megakaryocytes

The KCNN4-specific inhibitor, TRAM-34, has previously been reported to increase intracellular ROS levels in the HepG2 hepatocellular carcinoma cell line [33]. Elevated ROS levels are, in turn, known to interfere with the assembly of free tubulin and disrupt microtubule dynamics [34]. To investigate whether similar effects occur in imMKCLs, we examined the temporal changes in ROS levels during maturation in both mock (*shlacZ*) and *shKCNN4* imMKCLs. We observed significantly higher ROS levels in the *shKCNN4* cells

throughout the 6-day maturation period (Figure 6A, B), suggesting that the polarized and asymmetric accumulation or aggregation of tubulin may result from the elevated ROS levels in these cells. To confirm whether excessive ROS levels contribute to the diminished platelet production observed in imMKCLs and CB-megakaryocytes, we treated cells with the ROS inducer tert-butyl hydroperoxide (TBHP) [35]. The viability of imMKCLs was unaffected by 50 μ M TBHP treatment during the five days of Dox-ON culture (Supplementary Figures S6A, B). As expected, TBHP treatment elevated ROS levels in imMKCLs (Figure 6C) and led to a reduction in platelet production in both imMKCLs (Figure 6D) and CB-megakaryocytes (Figure 6E). Importantly, the inhibitory action of TBHP was independent of ploidy levels in imMKCLs (Supplementary Figures S6C).

3.7. Decreased MMP in maturing *shKCNN4* imMKCLs

Studies have demonstrated that excessively high or low MMP are associated with dysregulated ROS production [36–38]. Interestingly, our confocal imaging studies revealed the colocalization of dysregulated tubulin and mitochondria in *shKCNN4* imMKCLs through ICC analysis of fixed cells and LCI (Figure 7A, B). Sequential MMP measurements revealed that *KCNN4* KD decreased MMP from day 0 (Figure 7C, D), suggesting that damaged mitochondria colocalizing with dysregulated tubulin caused the excessive levels of ROS in imMKCLs with *KCNN4* KD. A similar phenomenon was also observed in TBHP-treated imMKCLs (Supplementary Figure S7A).

4. Discussion

We previously established the imMKCL system for the ex vivo production of iPSC-derived platelets and successfully completed the first-in-human clinical trial [7]. However, the molecular mechanisms by which imMKCLs transition from a proliferative state—induced by transient overexpression of c-MYC, BCL-XL, and BMI1 during the doxycycline-induced (Dox-ON) stage—to a maturation state conducive to platelet generation during the Dox-OFF stage remain incompletely understood. In the present study, whereas we observed a gradual and sustained decline in $[K^+]_i$ from day 0 to day 6 during imMKCL maturation, $[Ca^{2+}]_i$, $[Mg^{2+}]_i$, and $[Na^+]_i$ remained relatively stable throughout the same period (Figure 1). Notably, pharmacological inhibition of K^+ channels at early culture time points (days 0, 1, and 2)—corresponding to the transition between proliferation and maturation—critically impairs subsequent platelet biogenesis (Figure 2). Among several K^+ channel candidates, KCNN4 ($K_{Ca3.1}$), a Ca^{2+} -activated K^+ channel, was highly expressed during this critical transition stage in imMKCLs (Figure 3A). Functional disruption of KCNN4 led to a significant reduction in platelet production (Figure 4), primarily due to impaired proplatelet formation (Figure 5). Further investigation revealed that KCNN4 plays a pivotal role in preserving proper tubulin architecture, maintaining MMP, and modulating ROS levels—all shown to be essential for efficient proplatelet formation (Figures 5–7). A similar role for KCNN4 is suggested in CB-megakaryocytes, as *KCNN4* expression was elevated during early maturation stages (Figure 3B), and its inhibition similarly suppressed platelet production (Figure 4). In excitable cells such as neurons and muscle cells, K^+ efflux through potassium channels is a key mechanism for hyperpolarization following depolarization mediated by Na^+ and Ca^{2+}

influx. In human platelets, the functional role of KCNN4 has been characterized by whole-cell patch-clamp techniques [28, 39] and implicated in enhancing procoagulant activity through K^+ efflux [40]. However, the temporal dynamics and regulatory function of KCNN4 during platelet biogenesis have remained largely unexplored in megakaryocytes, which are typically used as surrogate models for platelet studies [41]. In this study, we systematically monitored daily fluctuations in $[K^+]_i$, $[Ca^{2+}]_i$, $[Mg^{2+}]_i$, and $[Na^+]_i$ during megakaryocyte maturation.

Another K^+ channel, KCNMA1 ($K_{Ca1.1}$), is highly expressed in megakaryocytes and platelets [42]. However, our RNA sequencing revealed the absence of *KCNMA1* in imMKCLs and negligible expression in CB-megakaryocytes (Figure 3A, B). Conversely, KCNA3 ($K_v1.3$), previously described as a key regulator of resting membrane potential in platelets [43], showed increasing expression during CB-megakaryocyte maturation (Supplementary Figure S3B), which aligns with earlier findings [43]. Yet, its expression remained consistently low in imMKCLs (Supplementary Figure S3A), supporting the notion that KCNN4 may be the predominant K^+ channel in this system to drive platelet biogenesis.

KCNN4 has also been implicated in cell cycle regulation, particularly in promoting a prolonged G1 phase followed by increased G1/S and G2/M transitions in various cellular contexts [44–49]. In parallel, a recent study by Italiano and colleagues demonstrated that megakaryocytes initiate proplatelet formation during the G1 phase via tubulin-mediated centrosome clustering, facilitating efficient platelet production [50]. Our work has shown that rapid proliferation during the Dox-ON stage in imMKCLs is tightly coupled with enhanced platelet output during the subsequent Dox-OFF maturation stage [51]. Hence, altogether,

these findings suggest that KCNN4 may contribute to a prolonged G1 phase in imMKCLs, thereby promoting proplatelet formation and subsequent platelet generation. Nonetheless, the mechanistic link between G1 phase dynamics, K^+ efflux, and $[K^+]_i$ during the proliferation-to-maturation transition remains to be elucidated.

Interestingly, KCNN4 has been reported to localize to mitochondria in various cell types [52–54], and its suppression has been associated with elevated superoxide levels and increased intracellular ROS [33, 55], suggesting a potential mitochondrial role in megakaryocytes as well—an avenue we aim to explore in future studies.

Tubulin, composed of α - and β -subunits, contains 12 and 8 cysteine residues, respectively, rendering it susceptible to oxidative modifications by both endogenous and exogenous ROS [56–58]. Superoxide can react with nitric oxide (NO) to generate peroxynitrite ($ONOO^-$), which modifies tubulin cysteines [58, 59]. While physiological ROS levels facilitate tubulin polymerization, excessive ROS may disrupt normal microtubule dynamics [60]. Prior studies have demonstrated that KCNN4 inhibition leads to abnormal hyperpolarization, mitochondrial dysfunction, reduced MMP, and elevated ROS production [33, 55, 61]. These findings parallel our observations in *shKCNN4* imMKCLs, where we noted impaired proplatelet formation (Figure 5), disrupted tubulin organization (Figure 5), elevated ROS (Figure 6), and diminished MMP (Figure 7). Moreover, tubulin has been shown to regulate MMP via its interaction with mitochondrial ion channels [62], underscoring the interconnected roles of ROS, mitochondria, and cytoskeletal dynamics in platelet biogenesis.

In summary, our findings demonstrate that the disruption of KCNN4 function in maturing megakaryocytes hinders the physiological decline of $[K^+]_i$, leading to mitochondrial

455 dysfunction, excessive ROS generation, and impaired microtubule reorganization necessary
456 for proplatelet formation—ultimately resulting in reduced platelet production (see lower
457 panel, Figure 8). These insights illuminate a previously underappreciated molecular pathway
458 in platelet biogenesis, with potential implications for optimizing ex vivo platelet production
459 and understanding the pathogenesis of thrombocytopenic disorders.

460 **Materials availability**

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

462

463 **Data and code availability**

464 RNA sequencing data have been deposited in the NCBI Gene Expression Omnibus (GEO)
465 database under accession number GSE295199 and GSE294634.

466

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479

480 **Author contributions**

481 Q.C. designed and conducted most of the experiments and analyzed the results. S.N. designed
482 and supervised the project and analyzed the results. T.Y. and N.T. conducted the RNA
483 sequencing and analyzed the results. N.S. supervised the project and edited the manuscript.
484 K.E. supervised the project and wrote the manuscript.

485

486 **Declarations of competing interests**

487 K.E. is a founder of Megakaryon Co. Ltd. and currently has no stock. K.E. previously
488 received the collaborating research budget from Megakaryon Co. Ltd. and Otsuka
489 Pharmaceutical Co. Ltd. The interests of K.E. have been reviewed and are managed by Kyoto
490 University in accordance with its conflict-of-interest policies.

Figure Legends (main figures)

FIGURE 1 Intracellular Ion Dynamics in Immortalized Megakaryocyte Progenitor Cell Lines (imMKCLs) During Maturation, Assessed by Flow Cytometry

(A–D) Representative flow cytometry histograms showing intracellular potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), and sodium ion (Na^+) levels ($[K^+]_i$, $[Ca^{2+}]_i$, $[Mg^{2+}]_i$, and $[Na^+]_i$) in imMKCLs. Cells were stained with the following ion-sensitive fluorescent probes: **(A)** ION Potassium Green-2 AM (IPG-2 AM, 5 μ M), **(B)** Fluo-4 AM (5 μ M), **(C)** Magnesium Green AM (MG AM, 5 μ M), and **(D)** ION NaTRIUM Green-2 AM (ING-2 AM, 5 μ M), vehicle control using dimethyl sulfoxide (DMSO). **(E–H)** The quantification of intracellular ion concentrations during the Dox-OFF maturation stage (days 0 to 6). Relative fluorescence intensities (F/F_0) were used as a proxy for **(E)** $[K^+]_i$, **(F)** $[Ca^{2+}]_i$, **(G)** $[Mg^{2+}]_i$, and **(H)** $[Na^+]_i$, as measured using the respective indicators. Fluorescence values on day 0 of Dox-OFF were normalized to 100%. $n \geq 3$; data represent independent experiments and are expressed as mean $\pm$ standard deviation (SD).

FIGURE 2 Effects of Ion Channel Inhibitors on Platelet Production and Ploidy in imMKCLs

(A) Representative flow cytometric dot plots showing expression of CD41 and CD42b in platelets derived from imMKCLs on day 6 of the Dox-OFF stage, following treatment with either double-distilled water (DDW) as vehicle control or 5 mM tetraethylammonium chloride (TEA), a non-selective potassium channel inhibitor. Quantification of CD41⁺CD42b⁺ platelet counts is shown, with TEA-treated samples compared to DDW controls. $n > 3$; data represent

independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by unpaired *t*-test; ****P* < 0.001. **(B, C)** Quantification of CD41⁺CD42b⁺ platelets on day 6 following treatment with **(B)** 1 mM 3,4-diaminopyridine (3,4-DAP) or vehicle control (DMSO), and **(C)** 1.5 mM 4-aminopyridine (4-AP) or vehicle control (DDW). *n* $\geq$ 3; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by unpaired *t*-test; ***P* < 0.01, *****P* < 0.0001. **(D)** Representative histograms depicting DNA ploidy levels of imMKCLs on day 4 of the Dox-OFF stage after treatment with either 5 mM TEA or DDW. Quantification of ploidy distributions is included. *n* > 3; data represent independent experiments and are expressed as mean $\pm$ SD. **(E–G)** Time-course analyses of platelet production from imMKCLs treated with **(E)** 5 mM TEA, **(F)** 1 mM 3,4-DAP, or **(G)** 1.5 mM 4-AP during the Dox-OFF stage. Each compound was administered once at the indicated time point (days 0 to 5), and CD41⁺CD42b⁺ platelet output was evaluated on day 6 to determine the critical window for inhibition. *n* $\geq$ 3; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by ordinary one-way ANOVA; **P* < 0.05, ***P* < 0.01, *****P* < 0.0001.

FIGURE 3 Representative RNA Expression Profiles of imMKCLs During Dox-OFF

Maturation and Human Cord Blood-Derived Megakaryocytes (CB-megakaryocytes)

(A) Transcriptomic analysis of imMKCLs during the Dox-OFF stage (days 0 to 4). Total RNA was extracted at each time point, and gene expression levels for selected targets are shown as fragments per kilobase of transcript per million mapped reads (FPKM). **(B)** Gene

expression profiles of megakaryocytes differentiated from human cord blood CD34⁺ hematopoietic stem cells. RNA was isolated at three distinct stages of in vitro differentiation: day 10 (CD34⁺CD41⁺), day 17 (CD41⁺), and day 22 (CD41⁺CD42b⁺). Expression levels of selected genes are displayed as FPKM values.

FIGURE 4 Effects of KCNN4 Inhibition and Knockdown (KD) on Platelet Production, Ploidy, and Ion Homeostasis in imMKCLs and CB-Megakaryocytes

(A, B) Quantification of CD41⁺CD42b⁺ platelets derived from imMKCLs on day 6 of the Dox-OFF stage following treatment with **(A)** 10, 20, or 30 μ M senicapoc or vehicle control (DMSO), and **(B)** 5, 10, or 15 μ M TRAM-34 or vehicle control (DMSO). $n \geq 3$; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by ordinary one-way ANOVA; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. **(C, D)** DNA ploidy profiles of imMKCLs on Dox-OFF day 4 following treatment with 30 μ M senicapoc or 15 μ M TRAM-34 compared to DMSO. $n \geq 3$; data represent independent experiments and are expressed as mean $\pm$ SD. **(E)** *KCNN4* mRNA expression in imMKCLs transduced with either control shRNA (*shlacZ*) or *KCNN4*-targeting shRNA (*shKCNN4*) during the Dox-ON stage. Expression in *shlacZ* cells was normalized to 1. $n > 3$; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by unpaired *t*-test; **** $P < 0.0001$. **(F)** Representative Simple Western immunoassay of KCNN4 and GAPDH in *shlacZ* and *shKCNN4* imMKCLs on Dox-OFF day 4. **(G)** Quantification of KCNN4 protein expression normalized to GAPDH. $n > 3$; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance

was determined by unpaired *t*-test; **P* < 0.05. **(H)** Quantification of CD41⁺CD42b⁺ platelet output from *shlacZ* and *shKCNN4* imMKCLs on Dox-OFF day 6. *n* > 3; data represent independent experiments and are expressed as mean ± SD. Statistical significance was determined by unpaired *t*-test; *****P* < 0.0001. **(I)** Corresponding DNA ploidy profiles on Dox-OFF day 4. *n* > 3; data represent independent experiments and are expressed as mean ± SD. **(J, K)** Temporal analysis of intracellular ion levels in *shlacZ* and *shKCNN4* imMKCLs during Dox-OFF maturation (days 0–6), as measured by **(J)** IPG-2 AM (5 μM) for [K⁺]_i and **(K)** Fluo-4 AM (5 μM) for [Ca²⁺]_i. Fluorescence intensities were normalized to day 0 (set as 100%). *n* > 3; data represent independent experiments and are expressed as mean ± SD. Statistical significance was determined by two-way ANOVA; not significant (ns), **P* < 0.05, ***P* < 0.01, ****P* < 0.001. **(L, M)** CD41⁺CD42b⁺ platelet production from CB-megakaryocytes on day 20 following treatment with **(L)** 30 μM senicapoc or vehicle control (DMSO), and **(M)** 15 μM TRAM-34 or vehicle control (DMSO). *n* > 3; data represent independent experiments and are expressed as mean ± SD. Statistical significance was determined by unpaired *t*-test; **P* < 0.05, ***P* < 0.01.

FIGURE 5 Impaired Proplatelet Formation and Cytoskeletal Disruption in *KCNN4* KD

imMKCLs

(A, B) Representative phase-contrast images of proplatelet formation in imMKCLs on day 6 of the Dox-OFF stage, captured using an inverted microscope. **(A)** Cells treated with 30 μM senicapoc or DMSO. White arrowheads indicate proplatelet formation in DMSO. **(B)** *shKCNN4* and *shlacZ* imMKCLs. White arrowheads indicate proplatelet formation in *shlacZ*

imMKCLs. Scale bar: 50 μ m. Quantification of proplatelet-forming cells is shown for each condition. More than 30 fields of view were analyzed per group. $n \geq 3$; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by unpaired *t*-test; ***P* < 0.01, ****P* < 0.001. (C) Representative immunocytochemistry (ICC) images of *shlacZ* and *shKCNN4* imMKCLs on Dox-OFF day 4. Cells were stained with rabbit anti- β -tubulin antibody (green), Alexa Fluor 647-conjugated phalloidin (purple), and DAPI (blue). Scale bar: 10 μ m. White arrowheads indicate the biased localization of β -tubulin in *shKCNN4* imMKCLs. (D) Quantification of β -tubulin fluorescence intensity in *shlacZ* and *shKCNN4* imMKCLs. (E) The proportion of cells exhibiting polarized and asymmetric β -tubulin distribution on Dox-OFF day 4. More than 20 fields of view were analyzed per sample. $n = 3$; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by unpaired *t*-test; ***P* < 0.01.

FIGURE 6 Intracellular Reactive Oxygen Species (ROS) Accumulation and Its Effects on Platelet Production in imMKCLs and CB-Megakaryocytes

(A) Representative flow cytometric histograms showing intracellular ROS levels in *shlacZ* and *shKCNN4* imMKCLs. Cells were stained with 1 μ M CellROX Deep Red on Dox-OFF day 4. (B) Temporal analysis of ROS levels in *shlacZ* and *shKCNN4* imMKCLs from day 0 to day 6 of the Dox-OFF stage. ROS levels are presented as mean fluorescence intensity (MFI). $n = 3$; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by two-way ANOVA; **P* < 0.05, ***P* < 0.01. (C) Time-course

of ROS levels in imMKCLs treated with 50 μ M tert-butyl hydroperoxide (TBHP) or vehicle control (DMSO) during Dox-OFF maturation (days 0–6), assessed by MFI of CellROX Deep Red. $n > 3$; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by two-way ANOVA; * $P < 0.05$, ** $P < 0.01$. **(D)** Quantification of CD41⁺CD42b⁺ platelets derived from imMKCLs on day 6 following treatment with 50 μ M TBHP or DMSO. $n > 3$; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by unpaired *t*-test; **** $P < 0.0001$. **(E)** CD41⁺CD42b⁺ platelet output from CB-derived megakaryocytes on day 20 following exposure to 50 μ M TBHP or DMSO. $n > 3$; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by unpaired *t*-test; ** $P < 0.01$.

FIGURE 7 Disrupted Mitochondrial Membrane Potential (MMP) and Altered Microtubule Architecture in *KCNN4* KD imMKCLs

(A) Representative ICC images of *shlacZ* and *shKCNN4* imMKCLs on day 4 of the Dox-OFF stage. Cells were stained with mouse anti- α -tubulin (red), rabbit anti- β -tubulin (green), Alexa Fluor 488-conjugated anti-TOMM20 (purple), and DAPI (blue). Scale bar: 10 μ m. White arrowheads indicate the biased localization of α -tubulin, β -tubulin, or TOMM20 in *shKCNN4* imMKCLs. **(B)** Representative live-cell imaging (LCI) images of *shlacZ* and *shKCNN4* imMKCLs on Dox-OFF day 4, stained with SPY650-tubulin (green), MitoBright LT Green (purple), and Hoechst 33342 (blue). Scale bar: 10 μ m. White arrowheads indicate the biased localization of β -tubulin or mitochondria in *shKCNN4* imMKCLs. **(C)** Representative

histograms from flow cytometric analysis of MMP in *shlacZ* and *shKCNN4* imMKCLs stained with 100 nM MitoTracker Deep Red. **(D)** Temporal quantification of MMP presented as MFI in *shlacZ* and *shKCNN4* imMKCLs throughout the Dox-OFF stage (days 0 to 6). n = 3; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by two-way ANOVA; *P < 0.05, **P < 0.01.

FIGURE 8 Schematic Overview Showing How KCNN4 Inhibition and KD Affect

Platelet Production in imMKCLs

During the Dox-OFF stage, imMKCLs gradually mature into proplatelets that eventually differentiate into platelets. This maturation process is accompanied by a decline in $[K^+]_i$. Inhibition or gene KD of KCNN4 impedes K^+ efflux, disrupts mitochondrial function, leads to elevated ROS levels, and causes abnormal tubulin accumulation. These disturbances ultimately result in impaired proplatelet formation and reduced platelet production.

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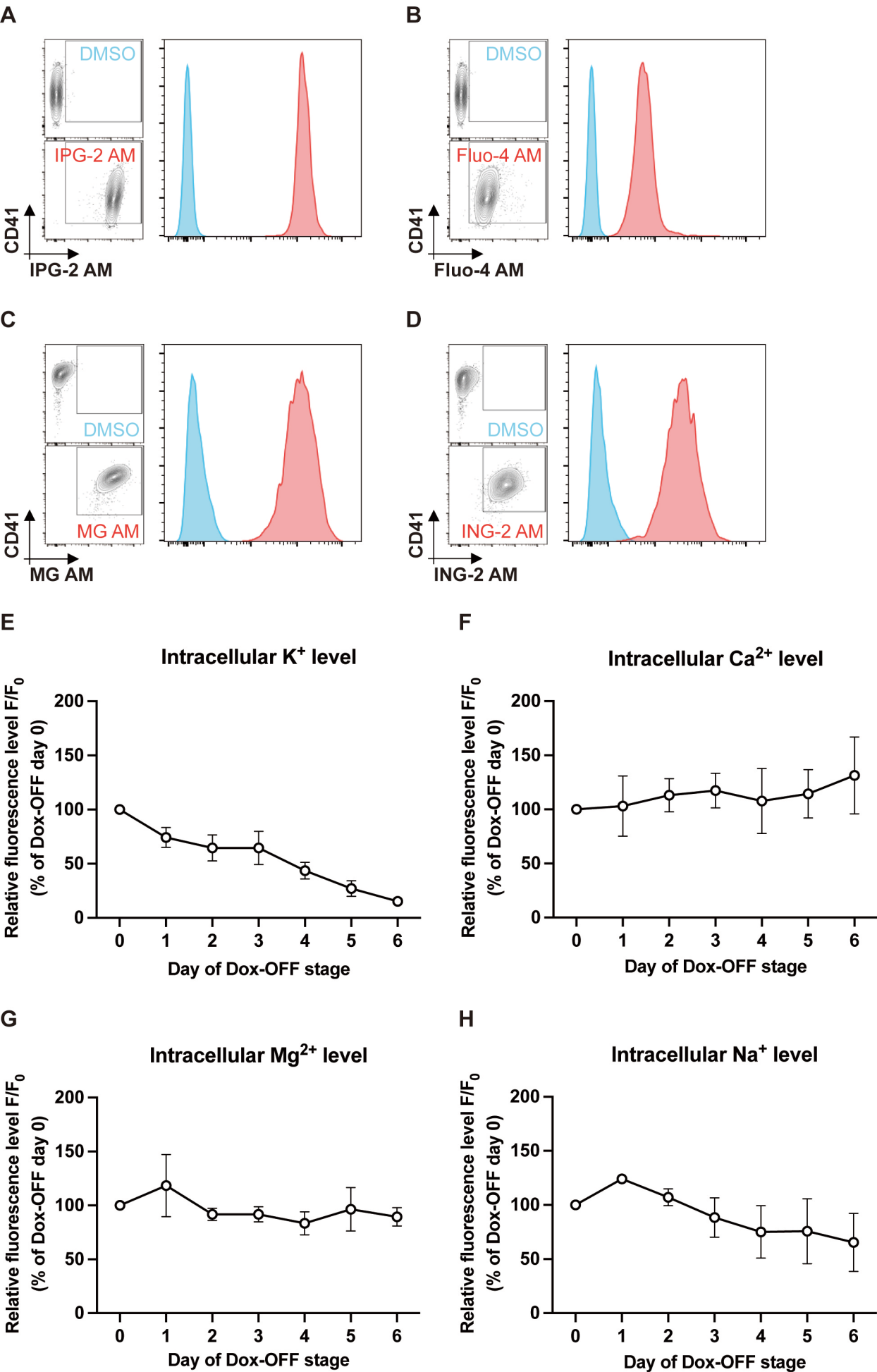
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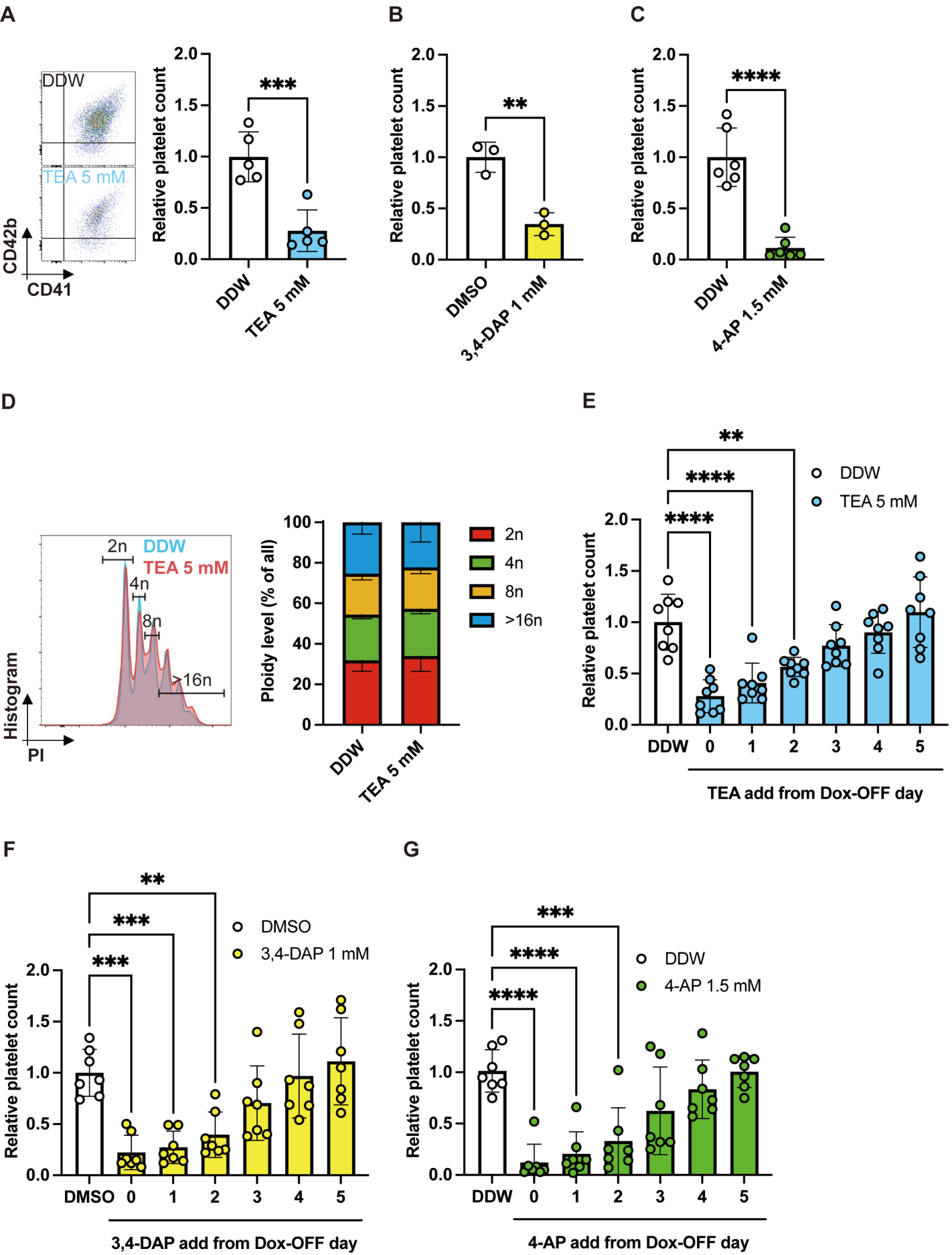
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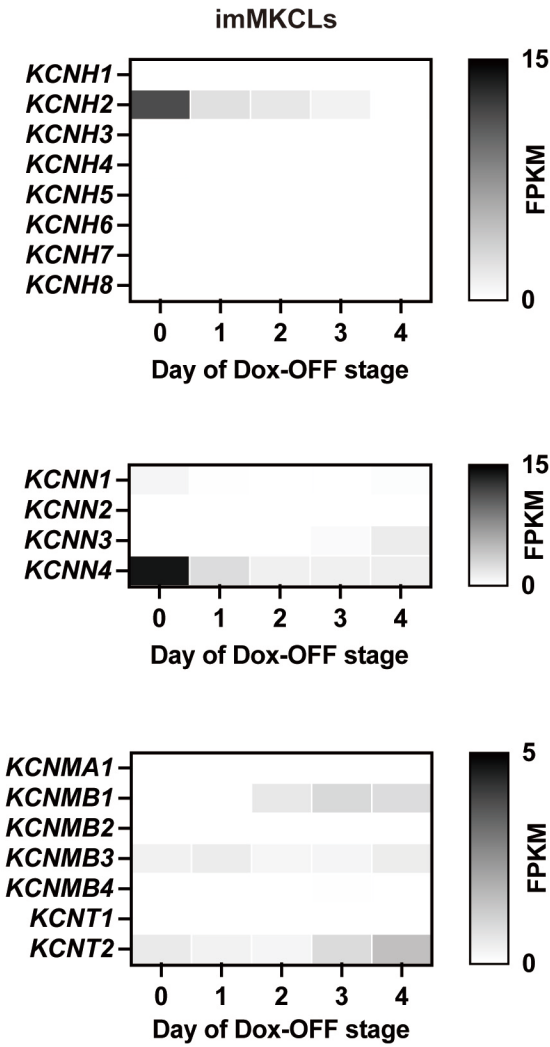


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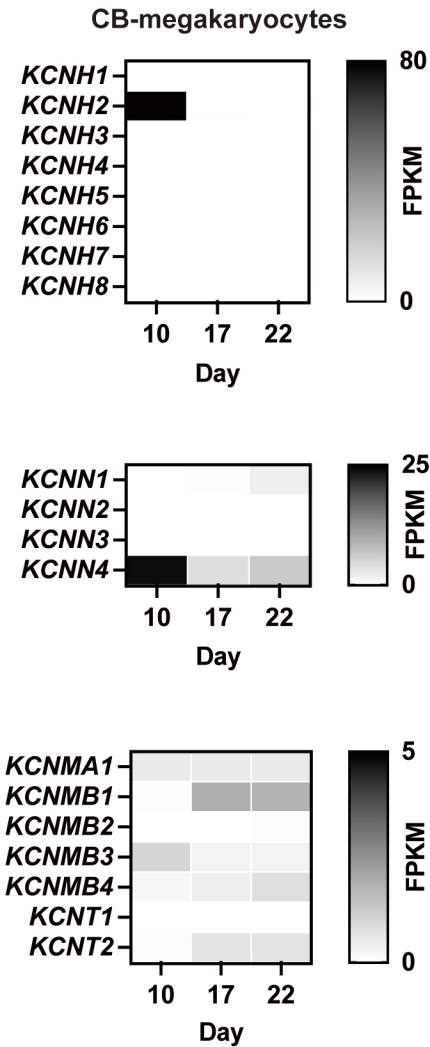


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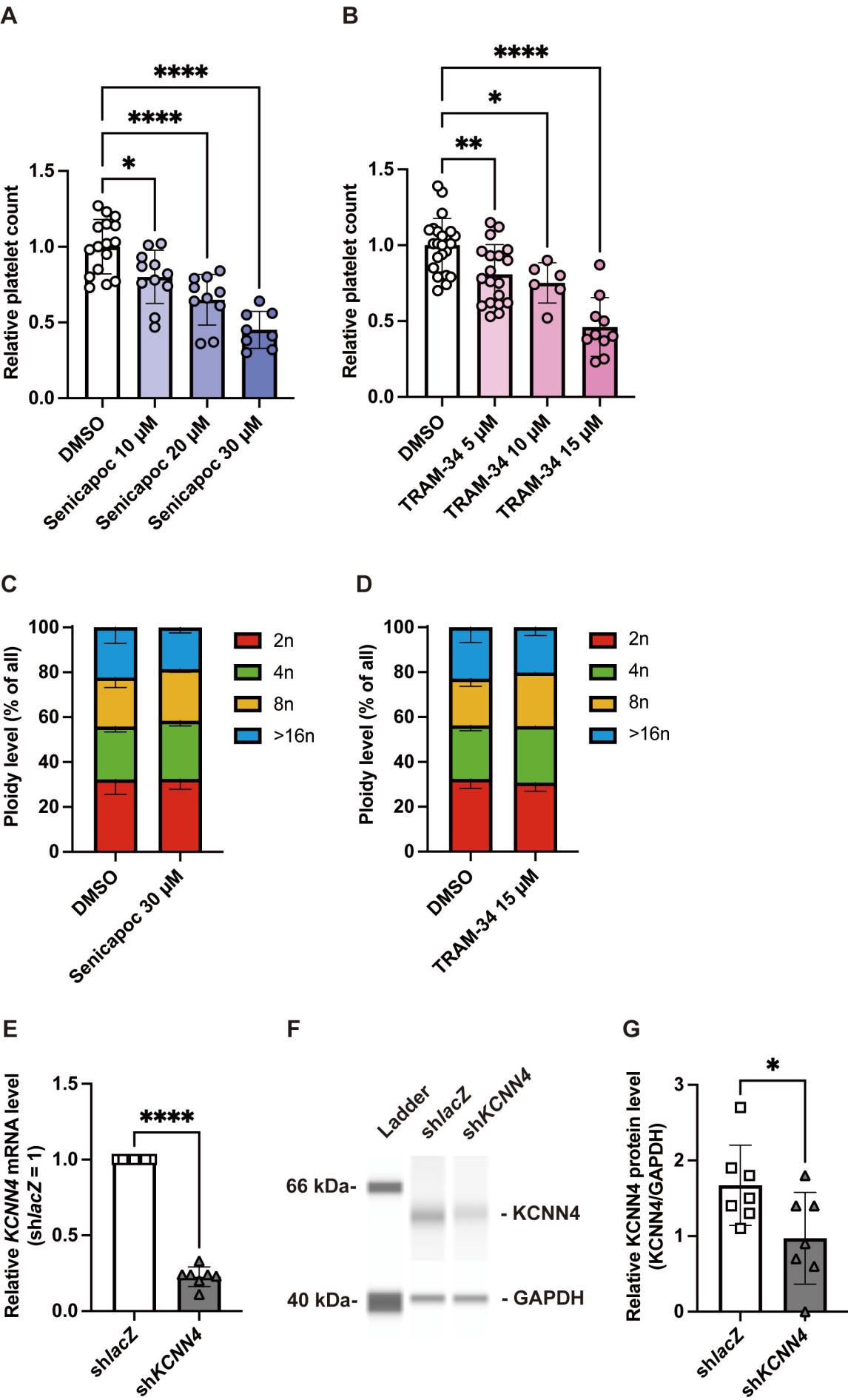
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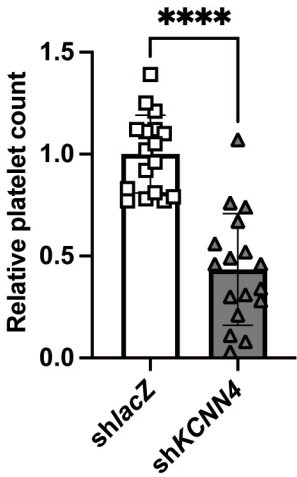
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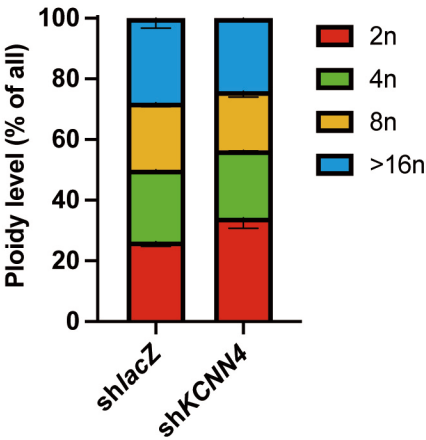
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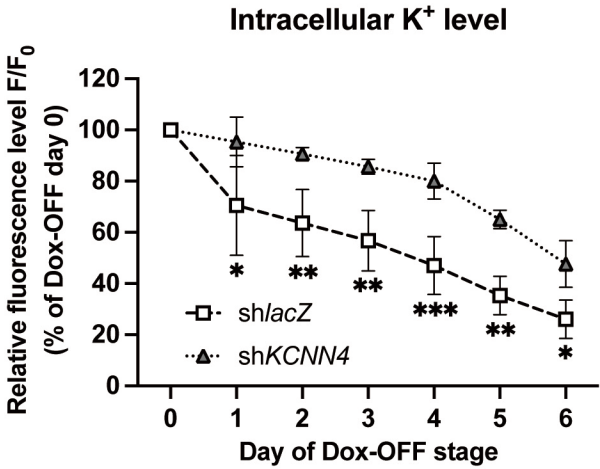
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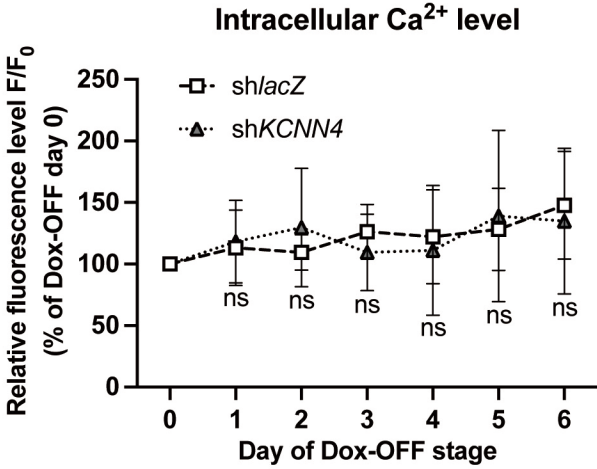
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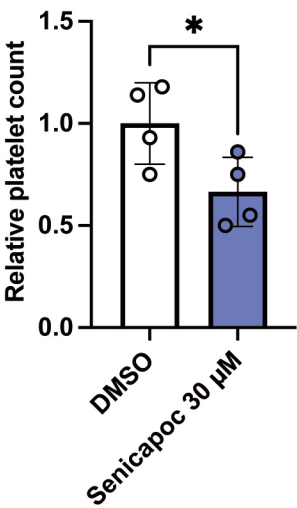
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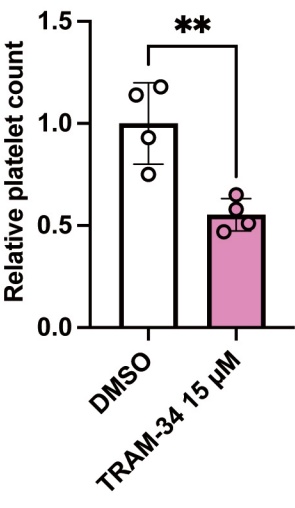
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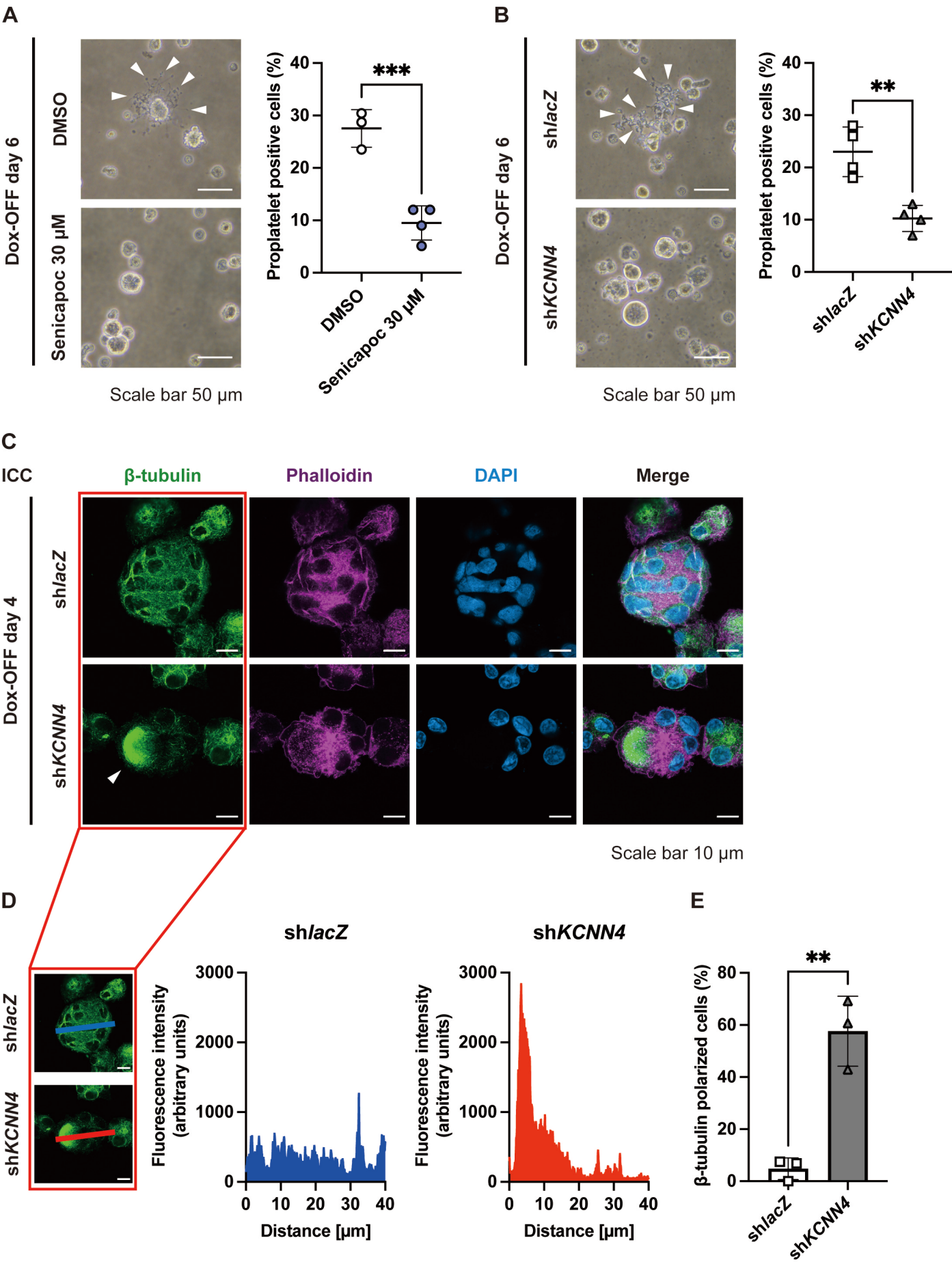


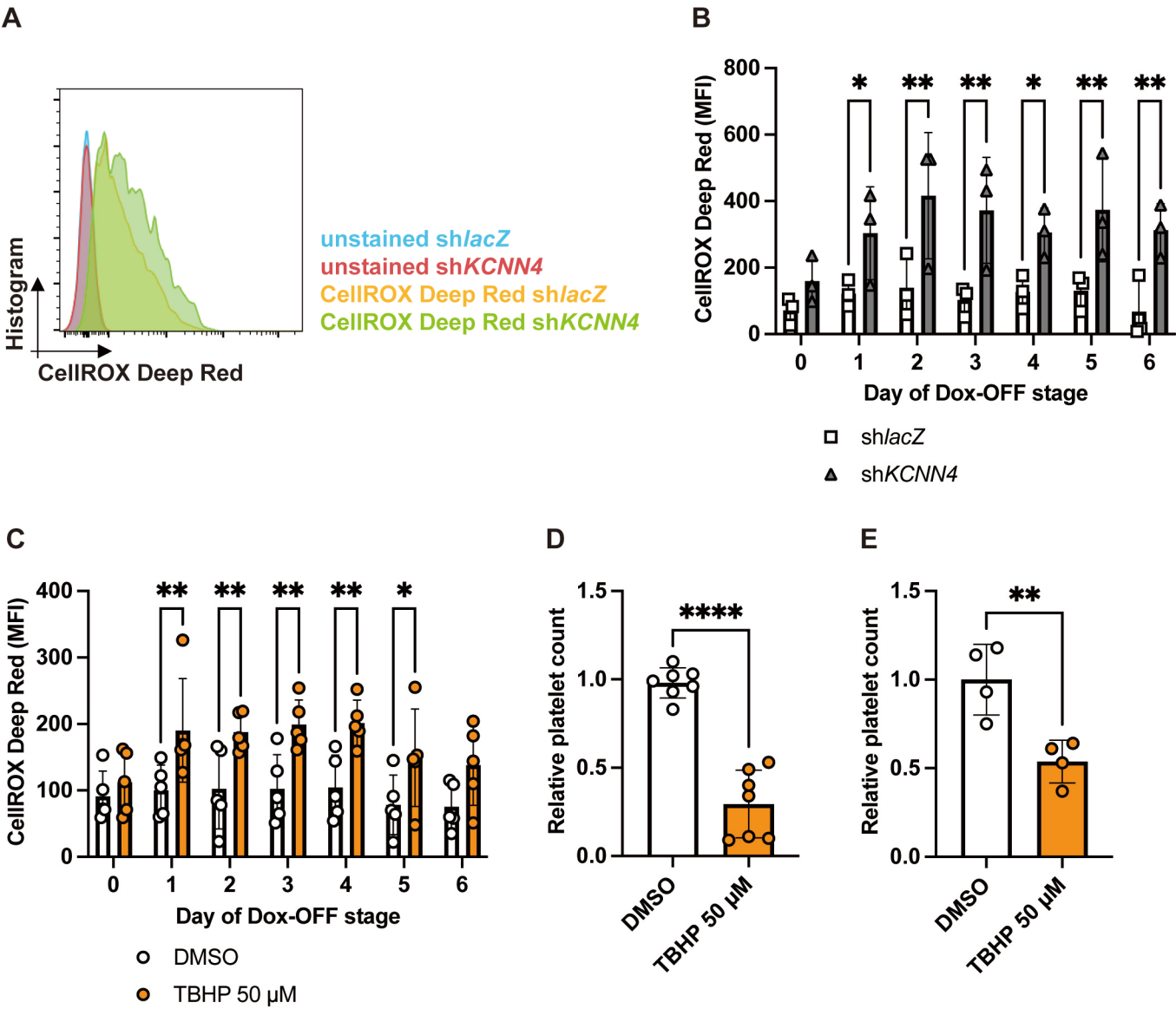
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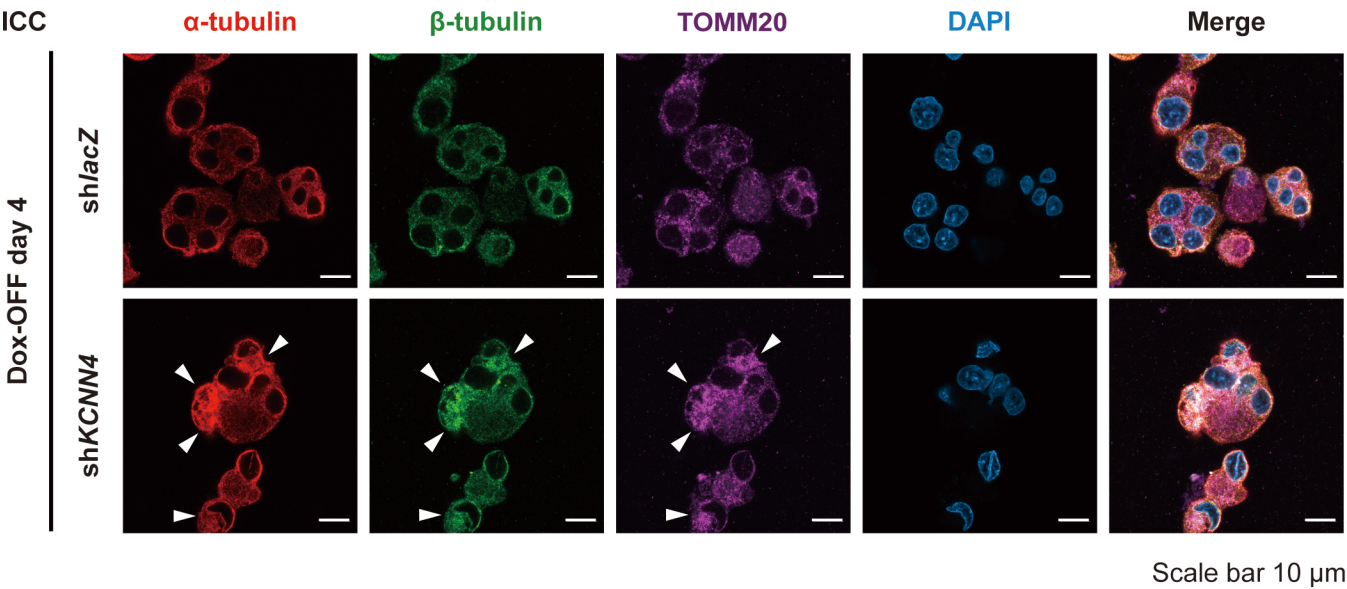




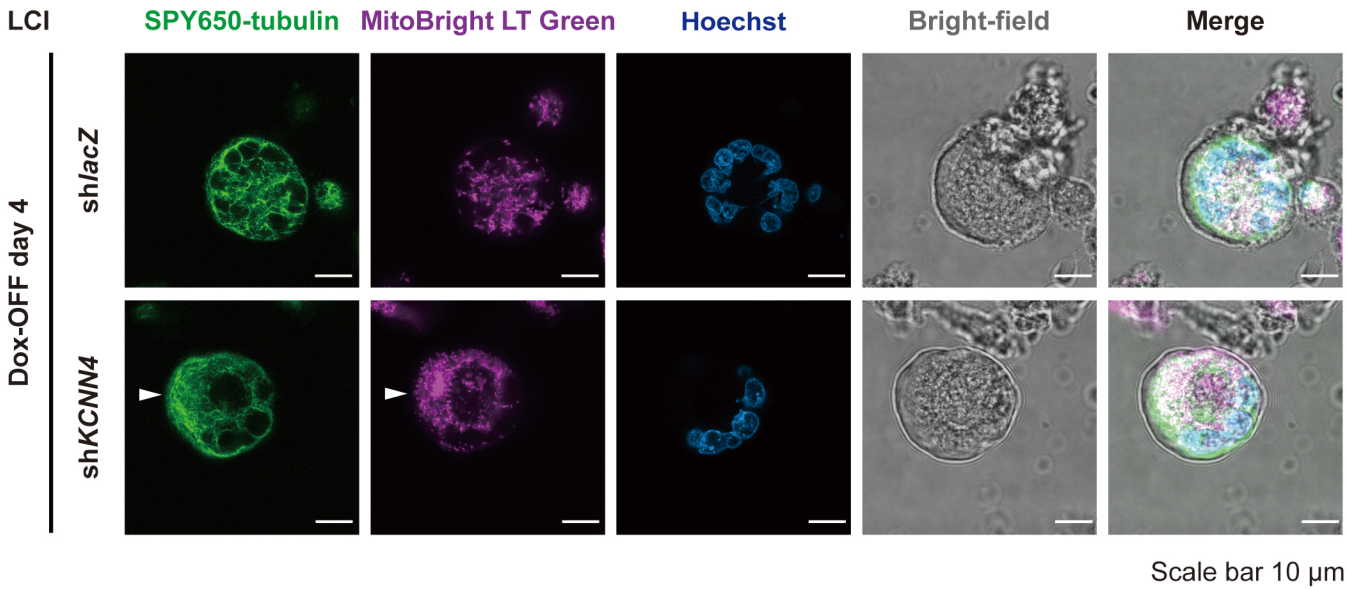


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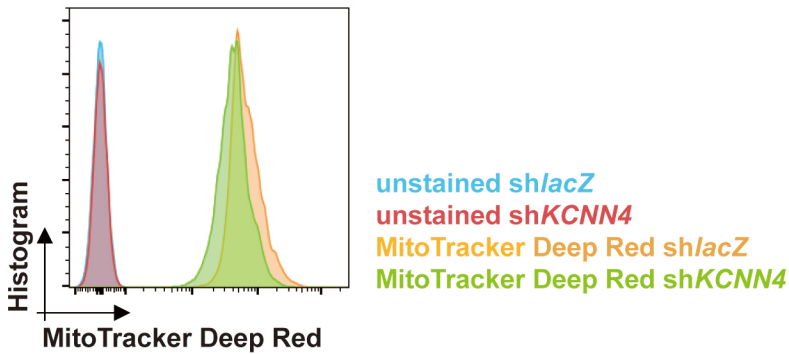
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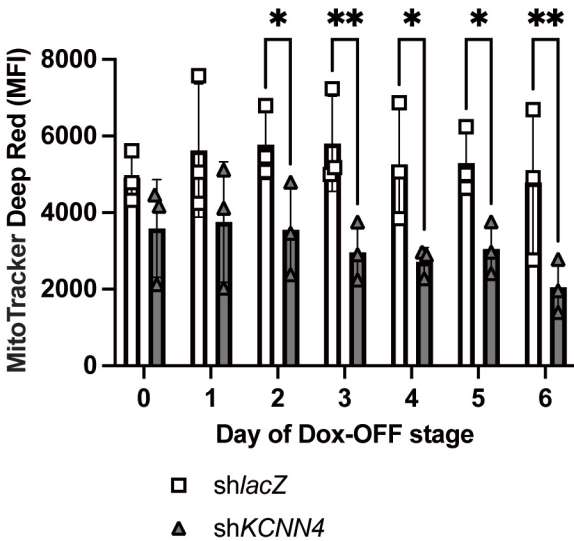
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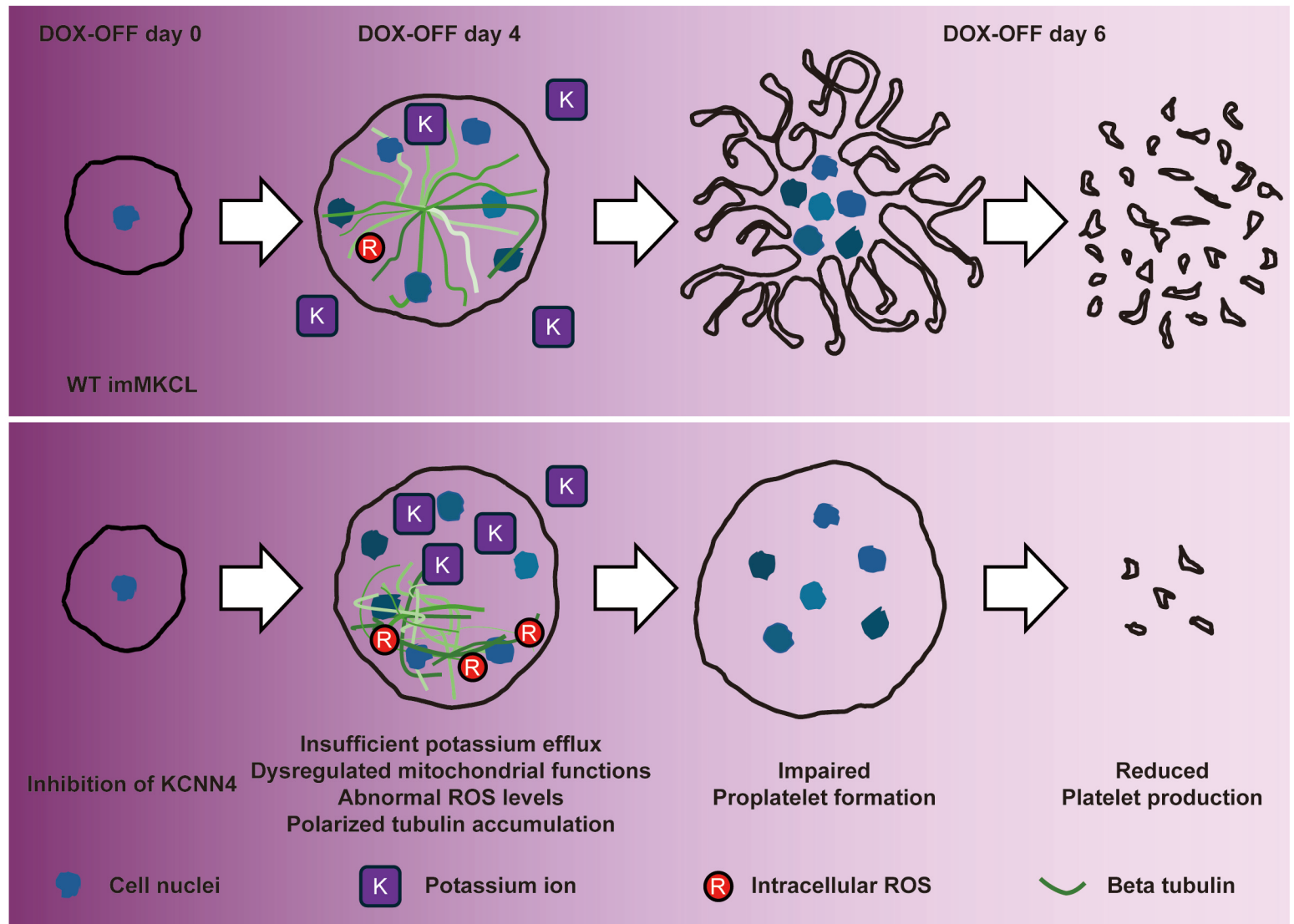
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Chen et al, Figure 8



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KCNN4-mediated potassium ion efflux maintains mitochondrial functions leading to platelet biogenesis

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26 **Supplementary Information**

27 **Supplementary FIGURE S1 Optimization of Ion-Sensitive Dye Concentrations and**
28 **Intracellular Ion Level Assessment in Immortalized Megakaryocyte Progenitor Cell**
29 **Lines (imMKCLs)**

30 **(A)** Representative flow cytometry histograms showing mean fluorescent intensity (MFI) in
31 imMKCLs stained with ION Potassium Green-2 AM (IPG-2 AM) at concentrations of 50 nM,
32 500 nM, 5 μ M, and 50 μ M, vehicle control using dimethyl sulfoxide (DMSO). The bar graph
33 represents the relative fluorescence level F/F_0 . $n = 3$; data represent independent experiments
34 and are expressed as mean $\pm$ standard deviation (SD). Statistical significance was determined
35 by ordinary one-way ANOVA; ns, not significant; **** $P < 0.0001$. **(B)** The proportion of
36 dead imMKCLs treated with IPG-2 AM at the indicated concentrations was assessed by
37 propidium iodide (PI) staining. $n = 3$; data represent independent experiments and are
38 expressed as mean $\pm$ SD. Statistical significance was determined by ordinary one-way
39 ANOVA; ns, not significant; **** $P < 0.0001$. **(C)** Relative fluorescence levels F/F_0 in
40 imMKCLs stained with Fluo-4 AM at concentrations of 50 nM, 500 nM, 5 μ M, 50 μ M, or
41 DMSO. $n \geq 3$; data represent independent experiments and are expressed as mean $\pm$ SD.
42 Statistical significance was determined by ordinary one-way ANOVA; ns, not significant; * P
43 < 0.05 , **** $P < 0.0001$. **(D)** The proportion of dead imMKCLs treated with Fluo-4 AM at the
44 indicated concentrations was assessed by PI staining. $n \geq 3$; data represent independent
45 experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by
46 ordinary one-way ANOVA; ns, not significant; * $P < 0.05$. **(E)** Relative fluorescence levels
47 F/F_0 in imMKCLs stained with Magnesium Green AM (MG AM) at concentrations of 50 nM,

48 500 nM, 5 μ M, 10 μ M, or DMSO. $n \geq 3$; data represent independent experiments and are
 49 expressed as mean $\pm$ SD. Statistical significance was determined by ordinary one-way
 50 ANOVA; ns, not significant; *** $P < 0.001$, **** $P < 0.0001$. **(F)** The proportion of dead
 51 imMKCLs treated with MG AM at the indicated concentrations was assessed by PI staining. n
 52 ≥ 3 ; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical
 53 significance was determined by ordinary one-way ANOVA; ns, not significant; * $P < 0.05$,
 54 *** $P < 0.001$. **(G)** Relative fluorescence levels F/F_0 in imMKCLs stained with ION
 55 NaTRIUM Green-2 AM (ING-2 AM) at 50 nM, 500 nM, 5 μ M, and 50 μ M, or DMSO. $n \geq 3$;
 56 data represent independent experiments and are expressed as mean $\pm$ SD. Statistical
 57 significance was determined by ordinary one-way ANOVA; ns, not significant; *** $P < 0.001$.
 58 **(H)** The proportion of dead imMKCLs treated with ING-2 AM at the indicated concentrations
 59 was assessed by PI staining. $n \geq 3$; data represent independent experiments and are expressed
 60 as mean $\pm$ SD. Statistical significance was determined by ordinary one-way ANOVA; ns, not
 61 significant; * $P < 0.05$. **(I)** MFI representing baseline fluorescence F_0 in imMKCLs during the
 62 Dox-OFF stage (days 0 to 6). $n > 3$; data represent independent experiments and are
 63 expressed as mean $\pm$ SD. **(J)** Extracellular potassium ion (K^+) level ($[K^+]_e$) in the culture
 64 medium of imMKCLs during the Dox-OFF stage, measured using an i-STAT 1 device
 65 (cartridge using EG6+; Abbott). $n > 3$; data represent independent experiments and are
 66 expressed as mean $\pm$ SD. **(K)** Intracellular K^+ level ($[K^+]_i$) in imMKCLs over the 5-day Dox-
 67 ON stage, shown as relative fluorescence level F/F_0 . $n > 3$; data represent independent
 68 experiments and are expressed as mean $\pm$ SD.

69

Supplementary FIGURE S2 Experimental Design and Cell Proliferation Analysis in imMKCLs Treated with Ion Channel Inhibitors

(A) Schematic overview of the cytotoxicity assay using potassium channel inhibitors: 5 mM tetraethylammonium chloride (TEA), 1 mM 3,4-diaminopyridine (3,4-DAP), and 1.5 mM 4-aminopyridine (4-AP). **(B, D)** Cell proliferation rates of imMKCLs during the Dox-ON stage in the presence or absence of **(B)** 5 mM TEA or double distilled water (DDW) as vehicle control, or **(D)** 1 mM 3,4-DAP or dimethyl sulfoxide (DMSO) as vehicle control. $n > 3$; data represent independent experiments and are expressed as mean $\pm$ SD. **(C, E)** Cytotoxicity analysis via Annexin V and PI staining. Apoptotic and dead cell ratios in imMKCLs treated with **(C)** 5 mM TEA or DDW, **(E)** 1 mM 3,4-DAP or DMSO. $n = 3$; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by unpaired t-test; ns, not significant. **(F)** Percentage of CD41⁺CD42b⁺ cells on day 4 of the Dox-OFF stage after treatment with 5 mM TEA or DDW. $n = 3$; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by unpaired t-test; ns, not significant. **(G)** Experimental design for daily treatment of imMKCLs with 5 mM TEA or DDW during the Dox-OFF stage (days 0 to 5).

Supplementary FIGURE S3 Representative RNA Expression Profiles of Dox-OFF Stage in imMKCLs and Human Cord Blood-Derived Megakaryocytes (CB-megakaryocytes)

(A) Transcriptomic analysis of imMKCLs during the Dox-OFF stage (days 0 to 4). Total RNA was extracted at each time point, and gene expression levels for selected targets are shown as fragments per kilobase of transcript per million mapped reads (FPKM). **(B)** Gene

expression profiles of megakaryocytes differentiated from human cord blood CD34⁺ hematopoietic stem cells. RNA was isolated at three distinct stages of in vitro differentiation: day 10 (CD34⁺CD41⁺), day 17 (CD41⁺), and day 22 (CD41⁺CD42b⁺). Expression levels of selected genes are displayed as FPKM values.

Supplementary FIGURE S4 Effects of Chlorpromazine (CPZ) and KCNN4 Inhibition on Platelet Production, Cytotoxicity, and Gene Expression in imMKCLs

(A) Quantification of CD41⁺CD42b⁺ platelets derived from imMKCLs on day 6 of the Dox-OFF stage following treatment with 1, 2, and 5 μ M CPZ or vehicle control (DMSO). $n > 3$; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by ordinary one-way ANOVA; not significant (ns). **(B)** *KCNH2* mRNA expression in imMKCLs transduced with either control shRNA (*shlacZ*) or *KCNH2*-targeting shRNA (*shKCNH2*) during the Dox-ON stage. Expression in *shlacZ* cells was normalized to 1. $n > 3$; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by unpaired t-test; **** $P < 0.0001$. **(C)**

Quantification of CD41⁺CD42b⁺ platelet output from *shlacZ* and *shKCNH2* imMKCLs on Dox-OFF day 6. $n > 3$; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by unpaired t-test; not significant (ns). **(D)**

Schematic overview of the cytotoxicity assay using KCNN4 inhibitors: 30 μ M senicapoc and 15 μ M TRAM-34. **(E, F)** Cytotoxicity analysis via Annexin V and PI staining. Apoptotic and dead cell ratios in imMKCLs treated with **(E)** 30 μ M senicapoc or vehicle control (DMSO), and **(F)** 15 μ M TRAM-34 or vehicle control (DMSO). $n \geq 3$; data represent independent

experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by unpaired t-test; not significant (ns). **(G, H)** Representative ploidy histograms from flow cytometric analysis of **(G)** 30 μ M senicapoc or DMSO, and **(H)** 15 μ M TRAM-34 or DMSO. **(I)** Percentage of CD41⁺CD42b⁺ cells on Dox-OFF stage day 4 after treatment with 30 μ M senicapoc or DMSO. n = 3; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by unpaired t-test; not significant (ns). **(J)** Cytotoxicity analysis by Annexin V and PI staining in *shlacZ* and *KCNN4* KD (*shKCNN4*) imMKCLs. n > 3; data represent independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined by unpaired t-test; not significant (ns). **(K)** Representative ploidy histograms from flow cytometric analysis of *shlacZ* and *shKCNN4* imMKCLs. **(L)** Schematic overview of CB-megakaryocytes.

Supplementary FIGURE S5 Effects of Senicapoc Treatment and *KCNN4* KD on Tubulin Organization in imMKCLs

(A) Representative immunocytochemistry (ICC) images of imMKCLs on Dox-OFF day 4 following treatment with 30 μ M senicapoc or DMSO. Cells were stained with mouse anti- α -tubulin (red), rabbit anti- β -tubulin antibody (green), Alexa Fluor 647 phalloidin (purple), and DAPI (blue). Scale bar: 10 μ m. **(B)** Representative ICC images of *shlacZ* and *shKCNN4* imMKCLs on Dox-OFF day 4, stained with rabbit anti- β -tubulin antibody (green), Alexa Fluor 647 phalloidin (purple), and DAPI (blue). Scale bar: 10 μ m. White arrowheads indicate the biased localization of tubulin (α -tubulin and β -tubulin) in senicapoc-treated imMKCLs or *shKCNN4* imMKCLs.

136

137 **Supplementary FIGURE S6 Reactive Oxygen Species (ROS) Inducer tert-butyl**
138 **hydroperoxide (TBHP)**

139 **(A)** Schematic overview of the cytotoxicity assay using 50 μ M TBHP as a ROS inducer. **(B)**
140 Cytotoxicity analysis by Annexin V and PI staining, showing apoptotic and dead cell ratios in
141 imMKCLs treated with 50 μ M TBHP or vehicle control (DMSO). n = 3; data represent
142 independent experiments and are expressed as mean $\pm$ SD. Statistical significance was
143 determined by unpaired t-test; not significant (ns). **(C)** Ploidy levels of imMKCLs on Dox-
144 OFF day 4 after treatment with 50 μ M TBHP or DMSO. n = 3; data represent independent
145 experiments and are expressed as mean $\pm$ SD.

146

147 **Supplementary FIGURE S7 Morphological Assessment of imMKCLs Treated with**
148 **TBHP**

149 **(A)** Representative ICC images of imMKCLs treated with 50 μ M TBHP or DMSO on Dox-
150 OFF day 4. Cells were stained with mouse anti- α -tubulin (red), rabbit anti- β -tubulin antibody
151 (green), TOMM20 (purple), and DAPI (blue). Scale bar: 10 μ m. White arrowheads indicate
152 the biased localization of tubulin (α -tubulin and β -tubulin) in TBHP-treated imMKCLs.

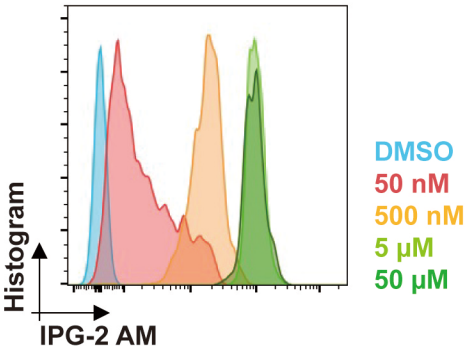
Supplementary TABLE S1

Genes	Inserted oligonucleotide sequences
<i>lacZ</i>	gatcccc CGCTAAATACTGGCAGGCGTT ttcaagaga AACGCCTGCCAGTATTTAGCG ttttgaaaa
<i>KCNH2</i>	gatcccc GGACTCGCTTTCTCAGGTTTC ttcaagaga GAAACCTGAGAAAGCGAGTCC ttttgaaaa
<i>KCNN4</i>	gatcccc GAACTGGCATTGGACTCATGG ttcaagaga CCATGAGTCCAATGCCAGTTC ttttgaaaa

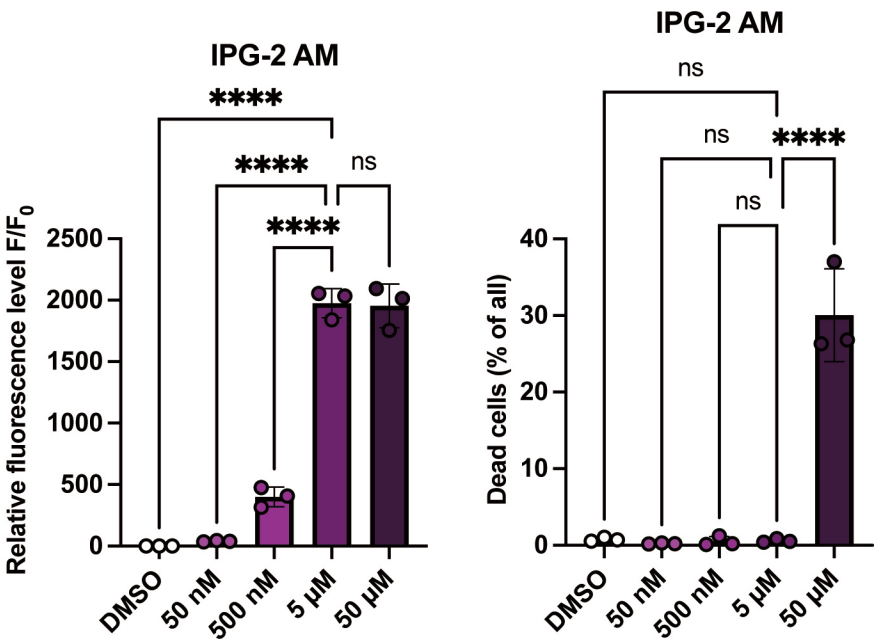
Supplementary TABLE S2

Genes	RT-PCR primer sequences	
<i>GAPDH</i>	Forward	GCATCTTCTTTTGCCTCGCC
	Reverse	GGCGCCCAATACGACCAAAT
<i>KCNH2</i>	Forward	CAACCTGGGCGACCAGATAG
	Reverse	GGTGTTGGGAGAGACGTTGC
<i>KCNN4</i>	Forward	TGGTTCGTGGCCAAGCTTTACA
	Reverse	TGCCCAGTGGCATTAAACAGCCT

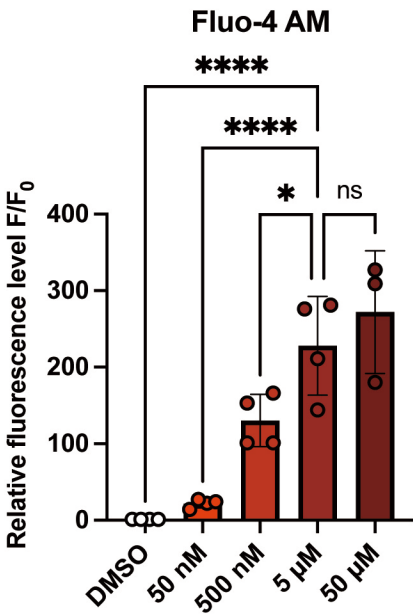
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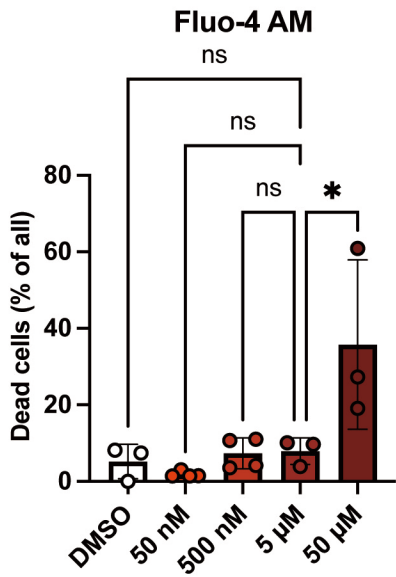
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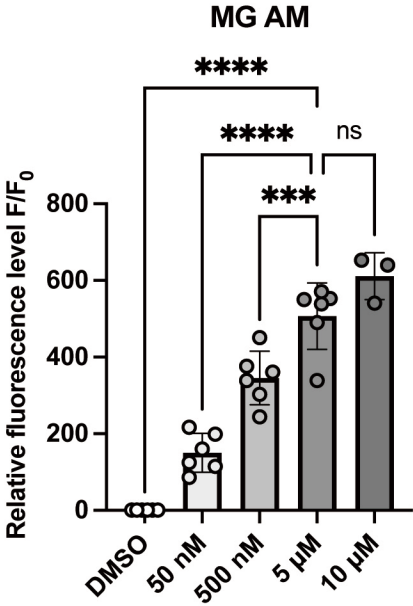
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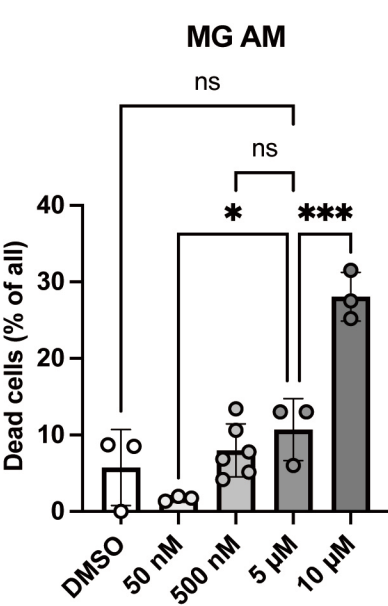
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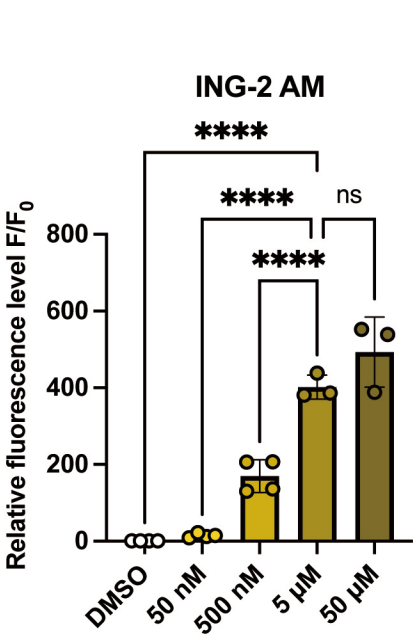
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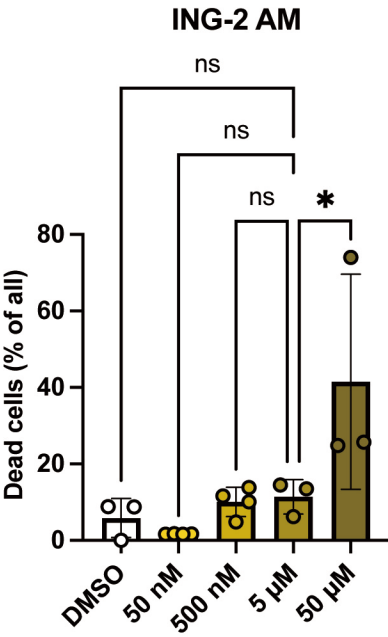
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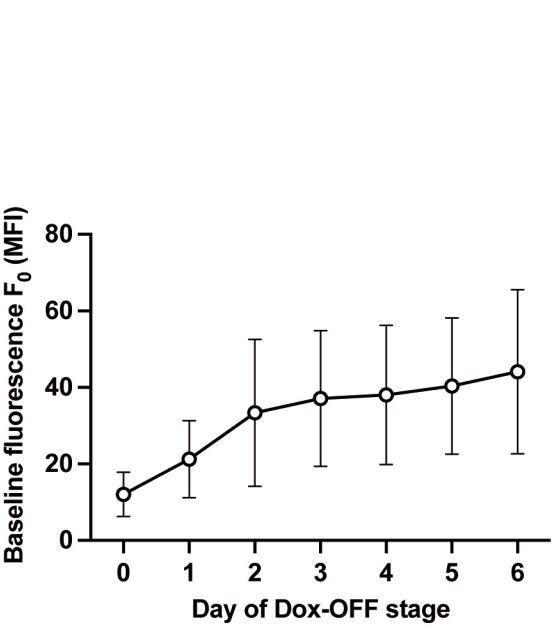
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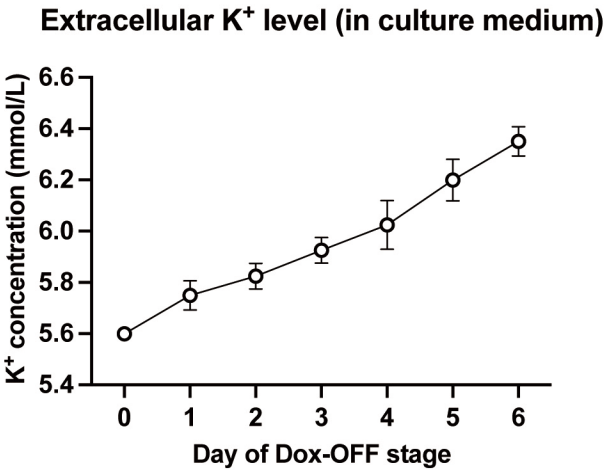
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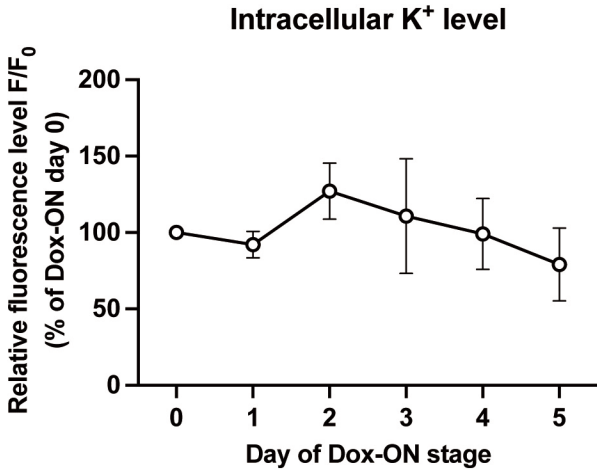
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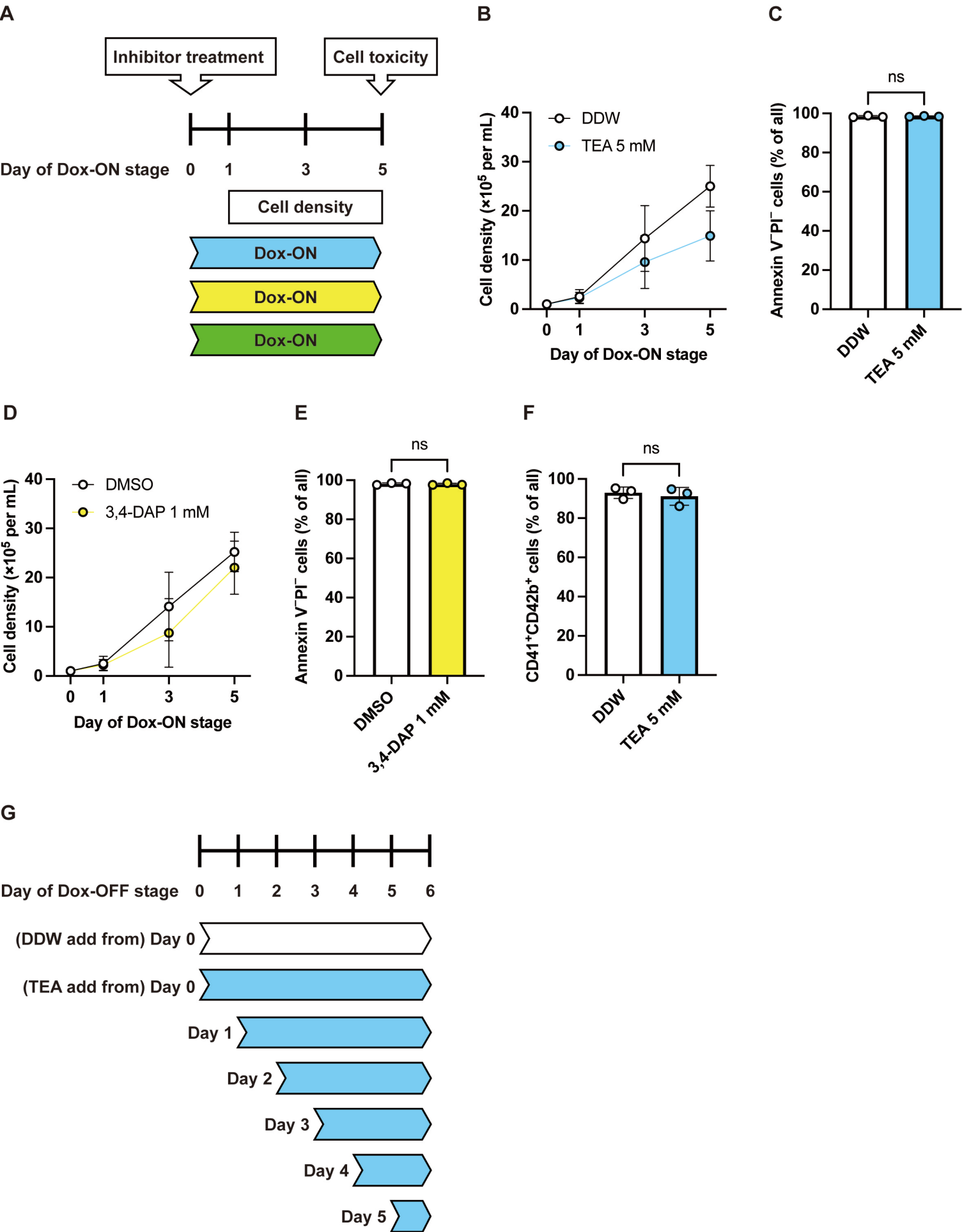
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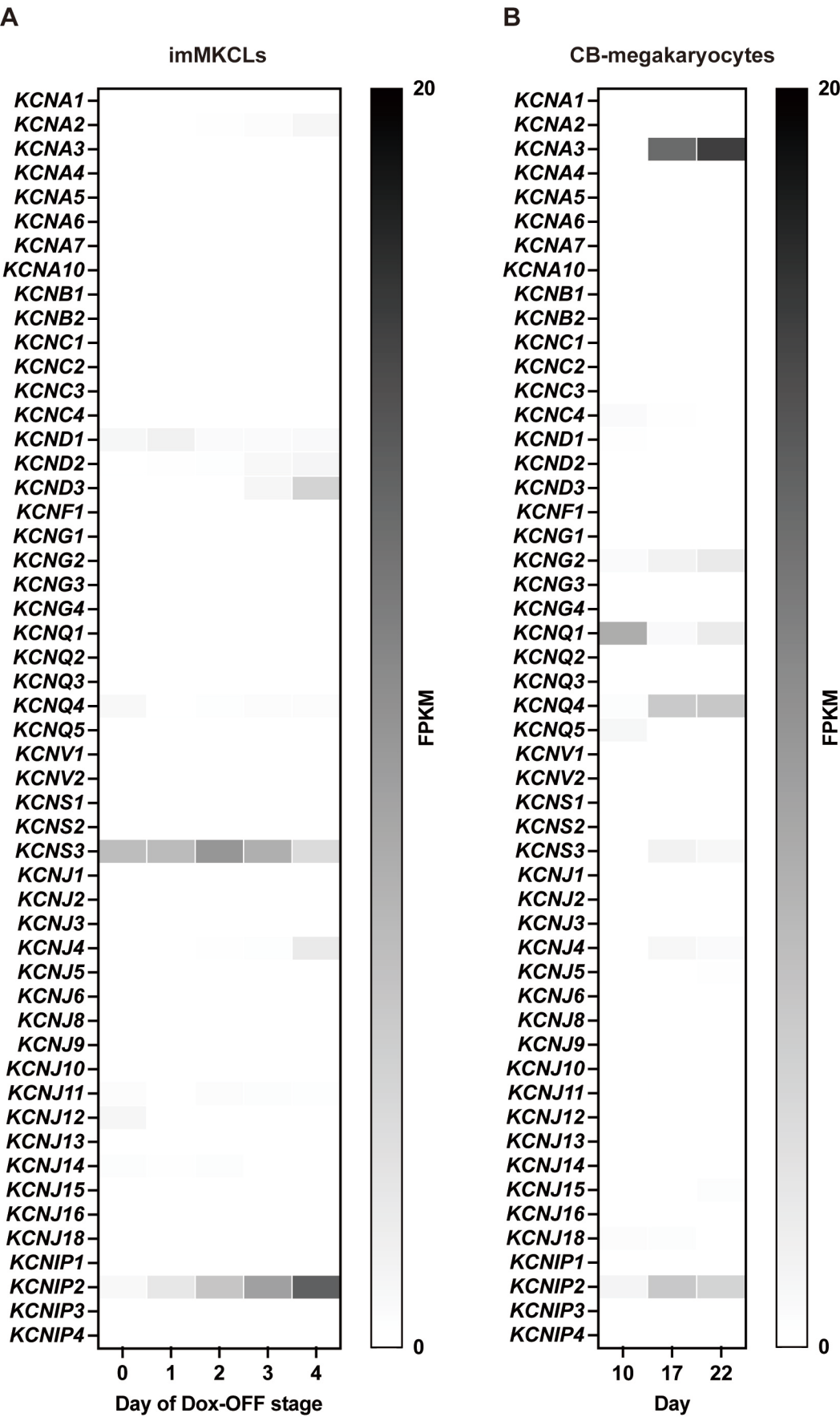


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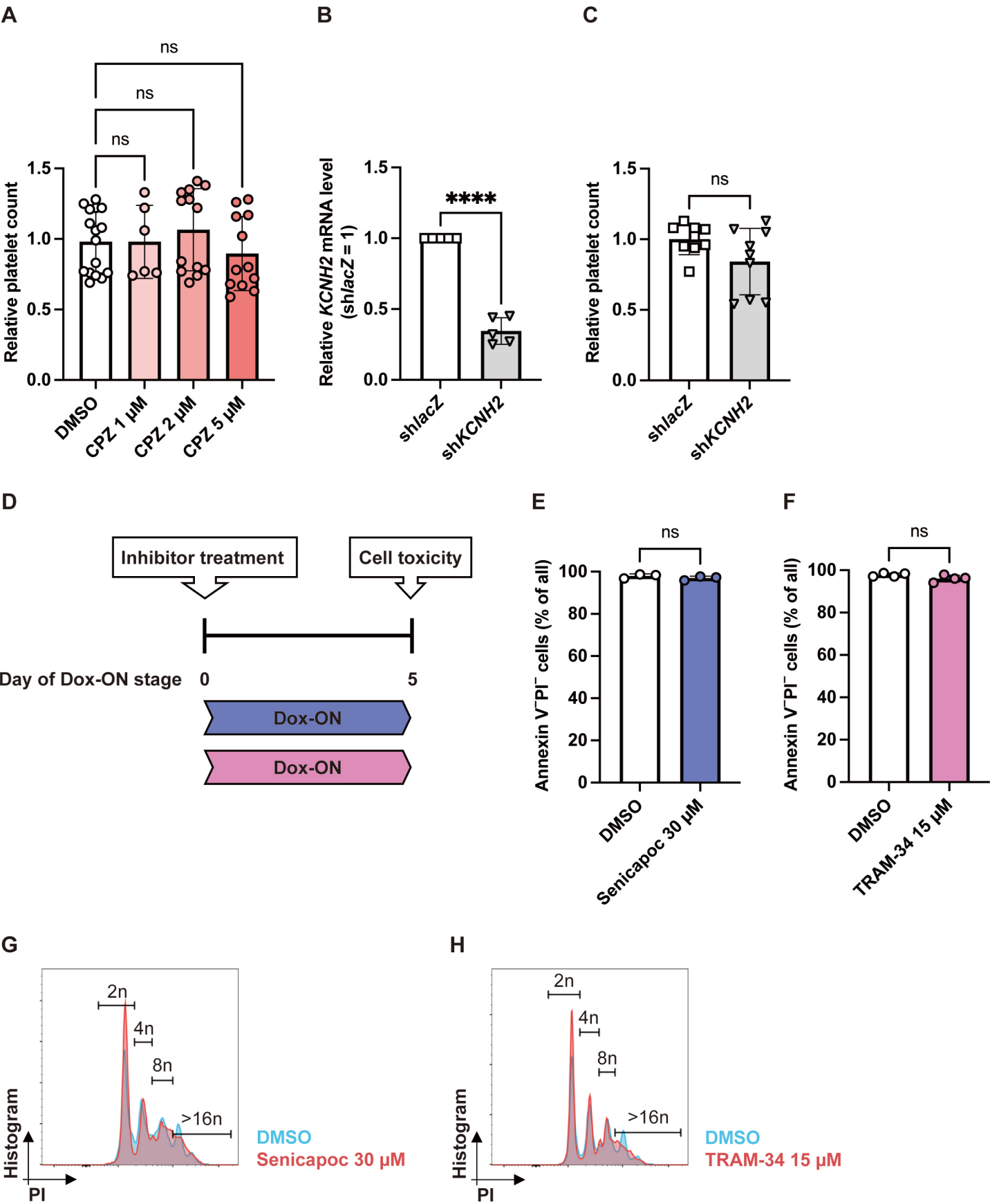


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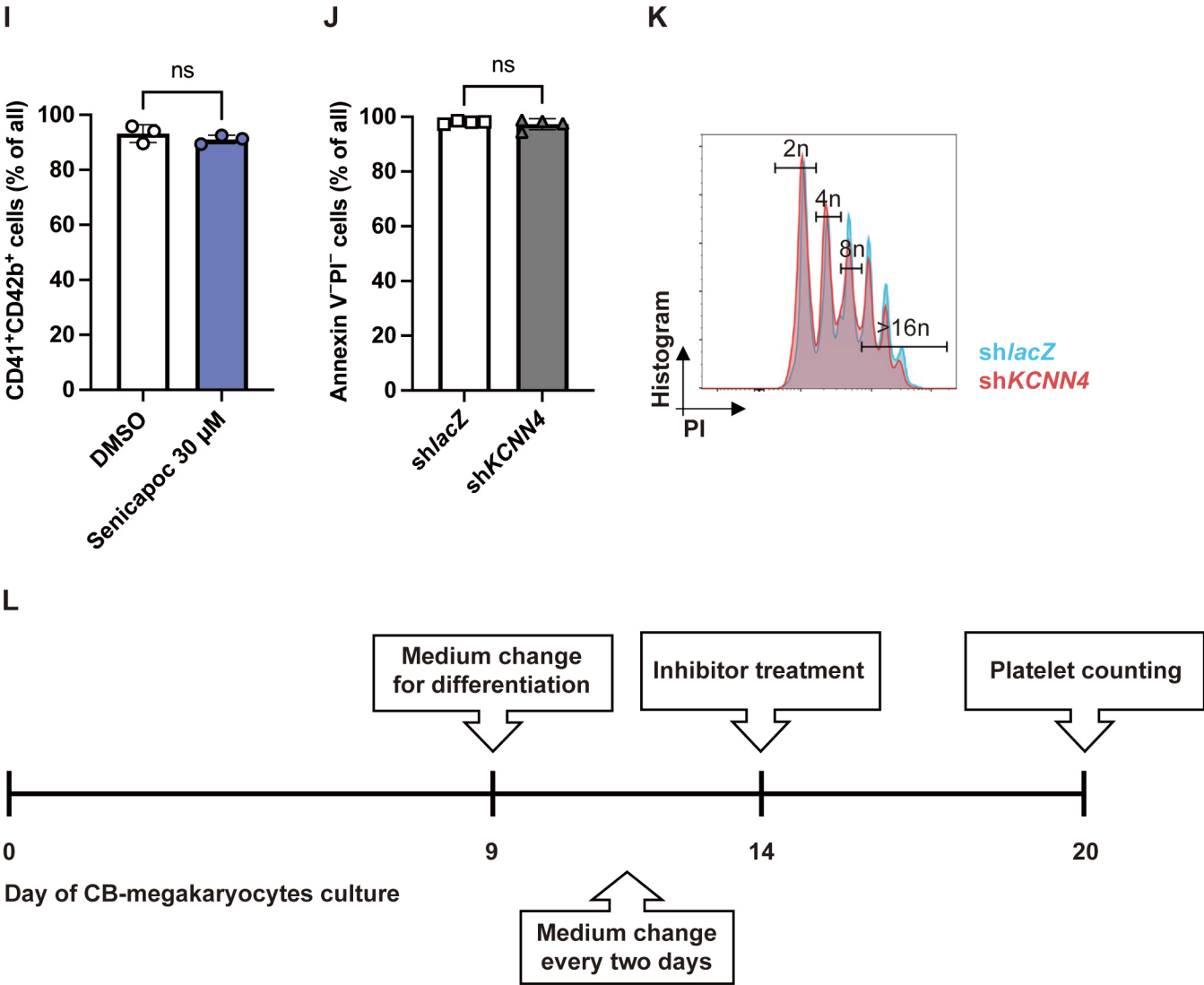


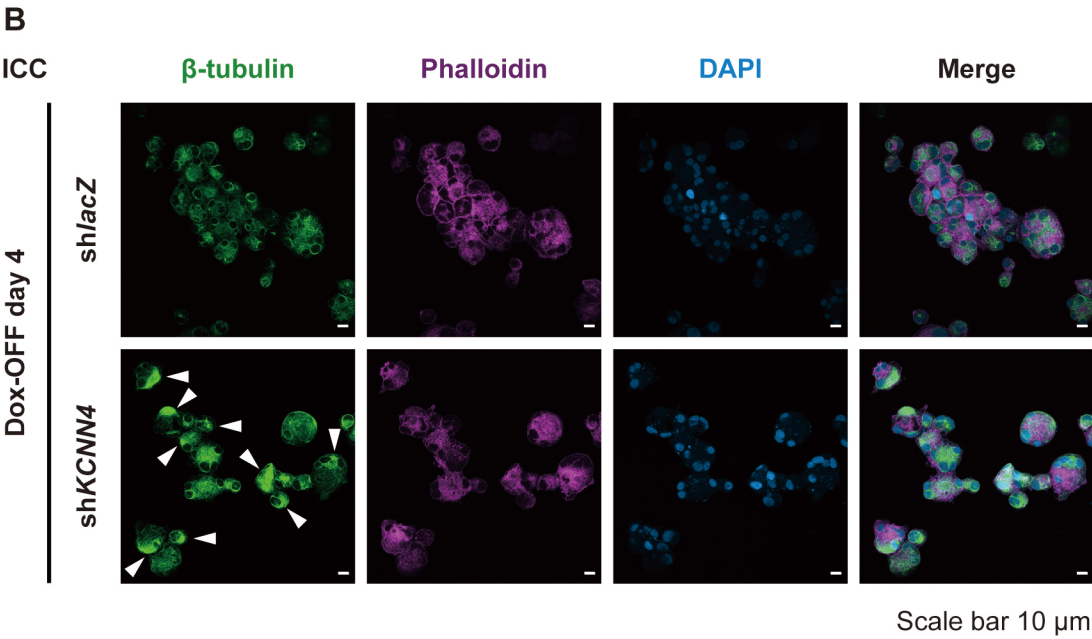
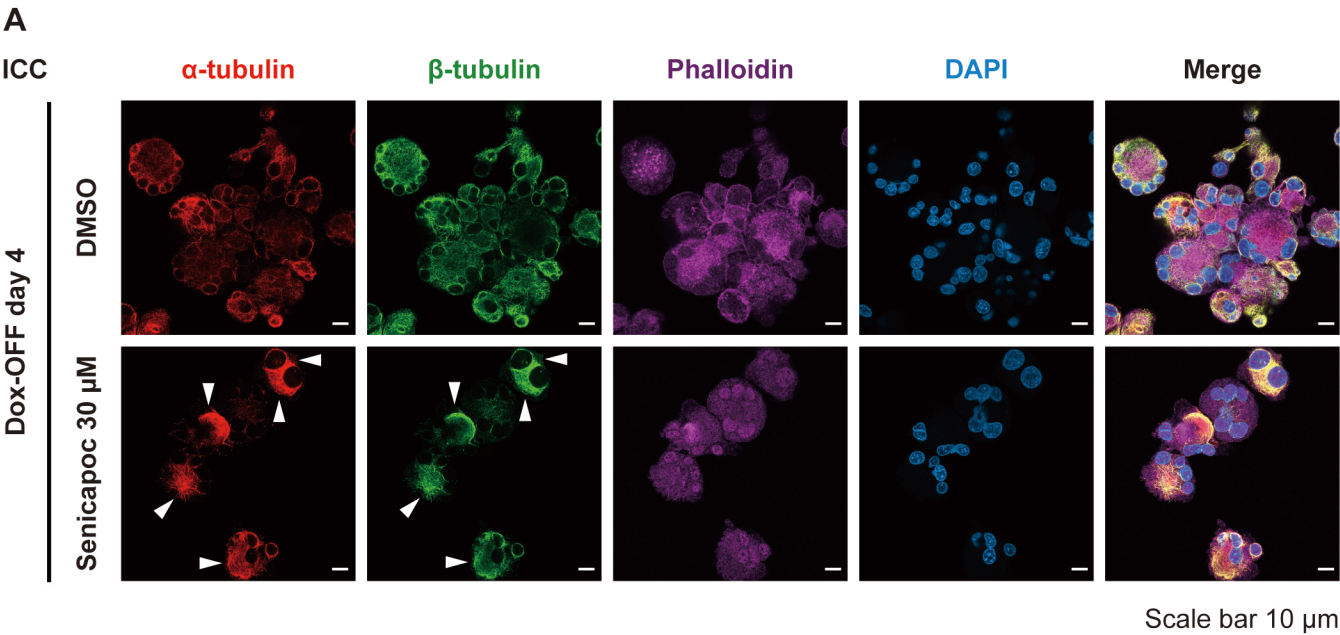


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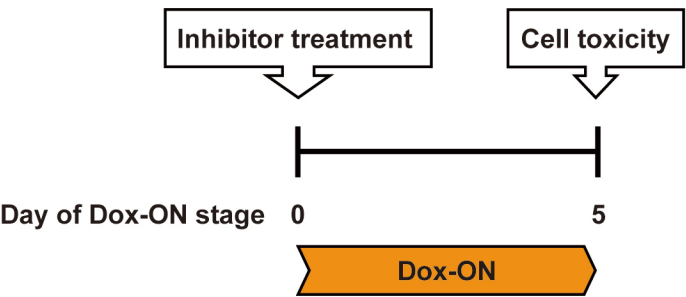
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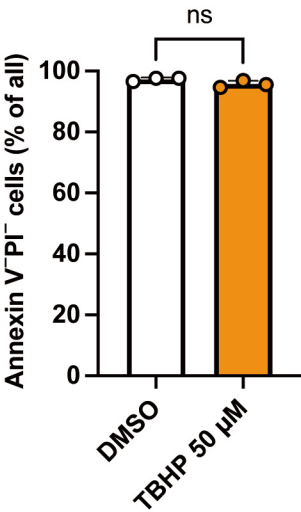


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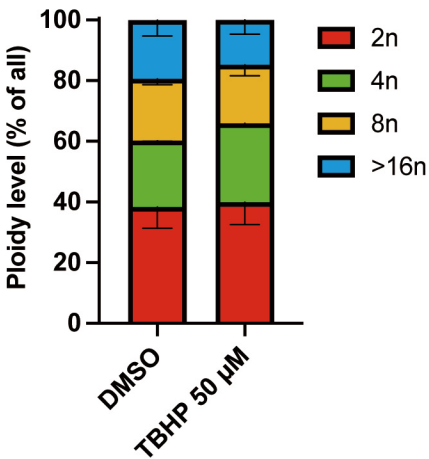
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A

