Megakaryocyte maturation involves activation of the adaptive unfolded protein response

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Abstract

Endoplasmic reticulum stress triggers the unfolded protein response (UPR) to promote cell survival or apoptosis. Transient endoplasmic reticulum stress activation has been reported to trigger megakaryocyte production, and UPR activation has been reported as a feature of megakaryocytic cancers. However, the role of UPR signaling in megakaryocyte biology is not fully understood. We studied the involvement of UPR in human megakaryocytic differentiation using PMA (phorbol 12-myristate 13-acetate)-induced maturation of megakaryoblastic cell lines and thrombopoietin-induced differentiation of human peripheral blood-derived progenitors. Our results demonstrate that an adaptive UPR is a feature of megakaryocytic differentiation, and that this response is not associated with ER stress-induced apoptosis. Differentiation did not alter the response to the canonical endoplasmic reticulum stressors DTT or thapsigargin. However, thapsigargin, but not DTT, inhibited differentiation, consistent with the involvement of Ca^{2+} signaling in megakaryocyte differentiation.

Introduction

Megakaryocytes are bone marrow cells that produce platelets. The differentiation of hematopoietic stem cells (HSC) to megakaryocytes (megakaryopoiesis) and subsequent platelet release (thrombopoiesis) is controlled by a number of transcription factors and cytokines. HSC undergo multiple rounds of endomitosis to produce large polyploid mature megakaryocytes with an extensive internal membrane reservoir for platelet formation. Recent reports suggest that activation of the endoplasmic reticulum (ER) stress response is a feature of both normal megakaryopoiesis and thrombopoiesis (Kovuru et al., 2020; Lopez et al., 2013; Morishima & Nakanishi, 2016), and abnormal megakaryopoiesis in *CALR*-mutated myeloproliferative neoplasms (El-Khoury et al., 2020; Fosselteder et al., 2023; Jutzi et al., 2023; Nam et al., 2019; Prins et al., 2020).

The ER is involved in folding and post-translational modification of secreted and transmembrane proteins, storage of calcium, and lipid membrane biogenesis, with disruptions of these processes leading to ER stress (Braakman & Hebert, 2013; Clapham, 2007; Fagone & Jackowski, 2009). In response to ER stress, eukaryotic cells trigger the unfolded protein response (UPR) that initially helps cells to overcome the stress by upregulating the expression of chaperones to promote folding, repressing mRNA translation to temporarily inhibit global protein synthesis, and initiating ER-associated degradation (ERAD) pathways (adaptive UPR) (Feral et al., 2021; Hetz et al., 2020). If the adaptive UPR is unable to restore protein homeostasis, UPR signaling events may cause apoptosis (terminal UPR). There are three key UPR signal activators in mammals: IRE1α (inositol-requiring enzyme one alpha), PERK (protein kinase R-like ER kinase), and ATF6 (activating transcription factor 6). Activation of the endonuclease activity of IRE1α catalyzes removal of an intron from *XBP1* mRNA, resulting in production of Xbp1s, a transcription factor that drives the expression of genes involved in restoring protein homeostasis, localization and lipid synthesis (Adams et al., 2019). IRE1α also triggers regulated IRE1-dependent decay (RIDD), leading to degradation of RNAs in a cell and stimulus specific manner to regulate functions including apoptosis, metabolism and inflammation (Abdullah & Ravanan, 2018). In the PERK pathway, PERK phosphorylates eIF2α to inhibit global translation and at the same time promote translation of the ATF4 transcription factor. In the ATF6 pathway, ATF6 translocates to the Golgi network where is it cleaved by site 1 protease (S1P) to release the

68 ATF6p50 transcription factor. ATF4 and ATF6p50 control the expression of an overlapping set
69 of genes, including ones encoding proteins that contribute to protein folding in the ER.
70 Activation of these pathways was initially ascribed to dissociation of BiP/GRP78 from each
71 sensor, however it is now apparent that other ER chaperones are involved in a cell- and tissue-
72 specific manner (Kettel & Karagöz, 2024).

73 UPR signaling events, and in particular the Ire1α pathway, are involved in the differentiation of
74 many cell types, including myoblasts (He et al., 2021), fibroblasts (Baek et al., 2012), osteoblasts
75 (Saito et al., 2011), neutrophils (Tanimura et al., 2018), plasma cells (Reimold et al., 2001),
76 mammary epithelial cells (Tsuchiya et al., 2017), eosinophils (Bettigole et al., 2015), dendritic
77 cells and macrophages (Bettigole & Glimcher, 2016). The UPR also has a vital role in sustaining
78 the activity of specialized secretory cells such as plasma cells and pancreatic β cells (Hetz &
79 Papa, 2018).

80 The role of UPR signaling in megakaryopoiesis and thrombopoiesis is unclear. It has been
81 proposed that ER stress activation triggers apoptotic-like caspase activation during
82 megakaryocytic differentiation of human megakaryoblastic cell lines, and may drive proplatelet
83 formation (Kovuru et al., 2020; Lopez et al., 2013; Morishima & Nakanishi, 2016). However,
84 while early reports suggested that localized caspase activation is required for megakaryopoiesis
85 (Clarke et al., 2003; De Botton et al., 2002), the role of caspase activation in megakaryopoiesis is
86 uncertain. Indeed genetic studies in mice convincingly showing that intrinsic apoptosis must be
87 suppressed for successful differentiation (Josefsson et al., 2014; Josefsson et al., 2020)
88 (McArthur et al., 2018). The studies suggesting a role for ER stress-induced apoptosis in
89 megakaryopoiesis have mostly used PMA-induced maturation of various cell lines. However, the
90 PMA concentrations used to induce maturation and draw conclusions about the role of UPR
91 signaling also resulted in extensive cell death (Kovuru et al., 2020; Lopez et al., 2013;
92 Morishima & Nakanishi, 2016). Therefore, it is unclear whether UPR activation in these studies
93 was a specific feature of megakaryocytic maturation or a generalized response to cell stress. Here
94 we investigate the involvement of UPR signaling pathways during in vitro maturation of
95 megakaryocytic cell lines and human peripheral blood (PB) derived progenitors under conditions
96 that induce maturation in the absence of cell death.

97 **Results**

98 **The unfolded protein response is activated during megakaryocytic maturation of K-562**
99 **and MEG-01 cells**

100 The K-562 and MEG-01 cell lines undergo megakaryocytic maturation upon PMA treatment
101 (Herrera et al., 1998; Kamal et al., 2018). Previous studies of the ER stress response in various
102 megakaryocytic cell lines have used concentrations of PMA (10-40 nM) that cause high levels
103 (>30%) of cell death (Kovuru et al., 2020; Lopez et al., 2013). We confirmed that treatment with
104 0.5 nM PMA stimulated maturation as assessed by an increase in the expression of the
105 transcription factor *GATA1* (Figure S1A&B) and the cell surface protein CD61 (Figure S1C).
106 Importantly there was no change in cell viability (Figure S1D) or caspase activation (Table S1)
107 during maturation, providing an experimental system in which we could study the role of the
108 UPR in megakaryocytic maturation in the absence of confounding cell death/apoptosis.

109 To determine whether the UPR is activated during PMA-induced maturation of K-562 and
110 MEG-01 cells we examined the expression of UPR target genes (Figure 1A and B). Expression
111 of the IRE1α target *ERdj4* (*DNAJB9*) and the ATF6 targets *HERPUD1* and *XBP1* increased in
112 response to PMA. The expression of *CHOP* (*DDIT3*), which is induced in response to all three
113 UPR pathways also increased, whereas the expression of the PERK targets *GADD34* and *ATF4*
114 increased in K-562 but not Meg-01 cells. The protein expression of BiP/GRP78, a UPR sensor
115 and a UPR target downstream of ATF6, was unchanged at day 3 (Figure 1C). These results
116 suggest that the activation of UPR signaling pathways occurs during megakaryocytic maturation.
117 Since these changes in expression occurred without a loss of cell viability, this most likely
118 represents involvement of the adaptive rather than terminal UPR. To determine whether this
119 result was a feature of PMA-induced maturation of the cell lines, or also occurred during TPO-
120 induced megakaryocyte differentiation, we analyzed the expression of *ERdj4*, *HERPUD1* and
121 *CHOP* at day 11 (sample 1) or day 15 (sample 2) following TPO treatment of $CD45^+$ cells
122 isolated from human peripheral blood (Figure 1D). All three genes showed increased expression
123 compared to day 0, but insufficient samples were available to determine statistical significance.

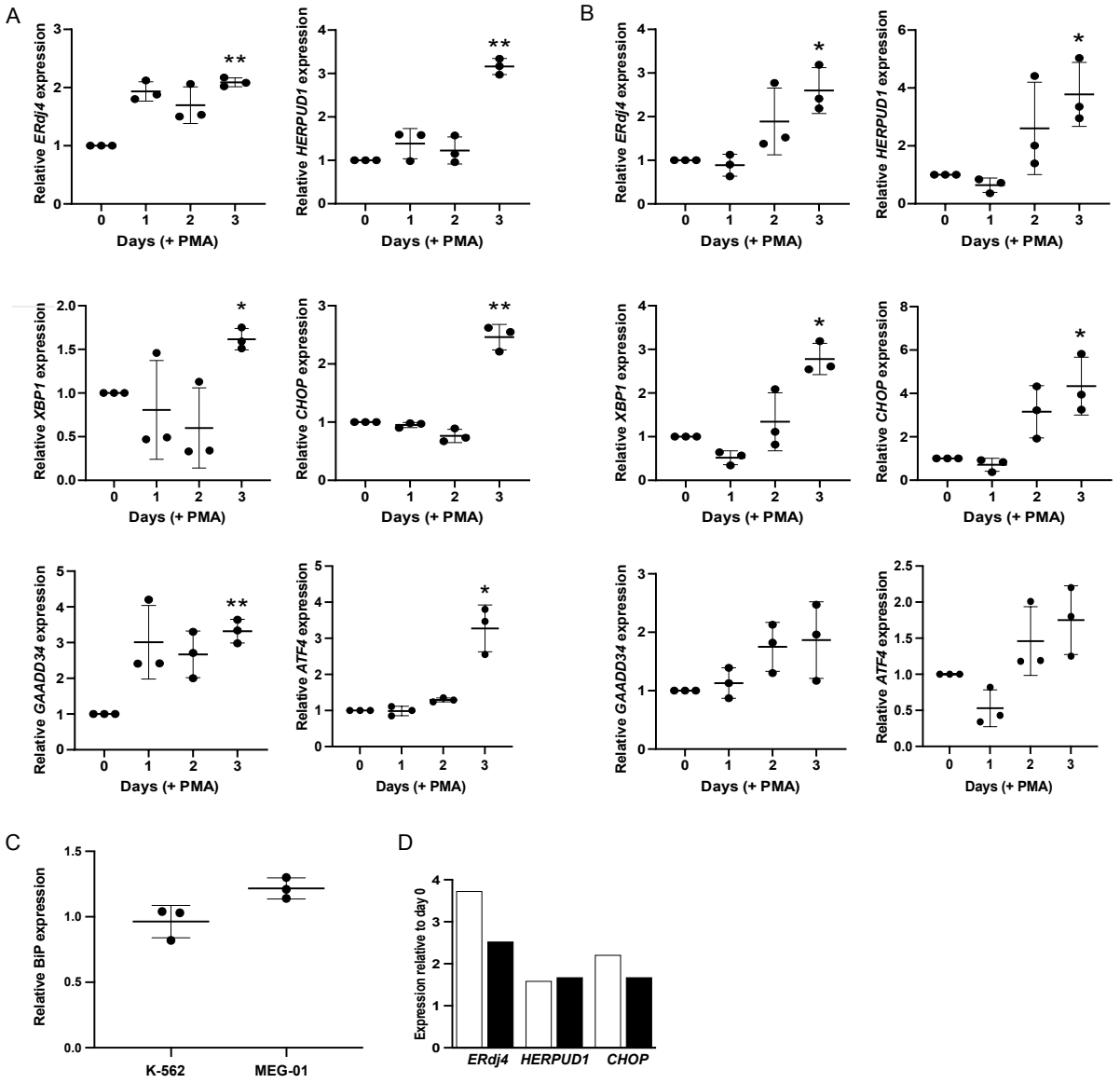

Figure 1. UPR target gene expression is upregulated during PMA-induced maturation of K-562 and MEG-01 cells into megakaryocytes. K-562 (A) or MEG-01 (B) cells were treated with 0.5 nM PMA and expression of *ERdj4, HERPUD1*, *XBP1, CHOP*, *GADD34* and ATF4 mRNA at days 0, 1, 2 and 3 was determined by RT-qPCR. Relative expression was calculated using the value at day 0 as 1. N=3 ± SD. *$P < 0.05$, **$P < 0.01$ compared to day 0 (one sample t-test). (C) K-562 or MEG-01 cells were treated with 0.5 nM PMA and BiP protein expression was determined by western blotting at day 3. Expression is relative to untreated cells. N=3 ± SD. (D) Expression (relative to day 0) of *ERdj4, HERPUD1* and *CHOP* following TPO-induced differentiation of $CD45^+$ peripheral blood cells. White bars = sample 1 at day 11 of culture, black bars = sample 2 at day 15 of culture.

Inhibition of IRE1α endonuclease activity, but not PERK or ATF6, inhibits megakaryocytic maturation

137 We next used inhibitors of the three UPR signaling pathways to determine whether activation of
138 one or more contributes to megakaryocytic maturation. SN34221 inhibits the endonuclease
139 activity of IRE1α (Patterson & Lonergan, 2008), AMG PERK 44 inhibits PERK (Smith et al.,
140 2015), and PF-429242 inhibits the site-1 protease that cleaves ATF6 to release ATF6p50 (Hay et
141 al., 2007). The inhibitors showed the expected specificity with SN34221 decreasing expression
142 of *ERdj4*, AMG PERK 44 decreasing expression of *CHOP*, *HERPUD1* and possibly *GADD34*,
143 and PF-429242 decreasing expression of *CHOP* and *HERPUD1* at day 3 of PMA-induced
144 megakaryocytic maturation of K-562 cells (Figure S2). Inhibition of IRE1α had a negative
145 impact on maturation, with 25% lower CD61 expression and 40% lower *GATA1* expression at
146 day 3 at the highest concentration of the inhibitor (Figure 2A&D). Treatment with 20 and 50 μM
147 SN34221 was associated with a small decrease in cell viability at day 3 (Figure S2D). In contrast
148 when PERK was inhibited with AMG PERK 44, expression of CD61 increased, suggesting an
149 enhancement of maturation, not *GATA1* expression was unchanged, suggesting no impact on
150 maturation (Figure 2B&D). These conflicting results may have occurred because AMG PERK
151 44 also led to up to 50% loss in cell viability (Figure S2E), and while CD61 expression was
152 assessed only in the live cells, *GATA1* expression was assessed in the total cell population. Thus
153 the PERK pathway may be important in both preventing apoptosis of the maturing cells and in
154 the maturation process. Finally, inhibition of the ATF6 pathway had no impact on maturation
155 (Figure 2C&D) or cell viability (Figure S2F).

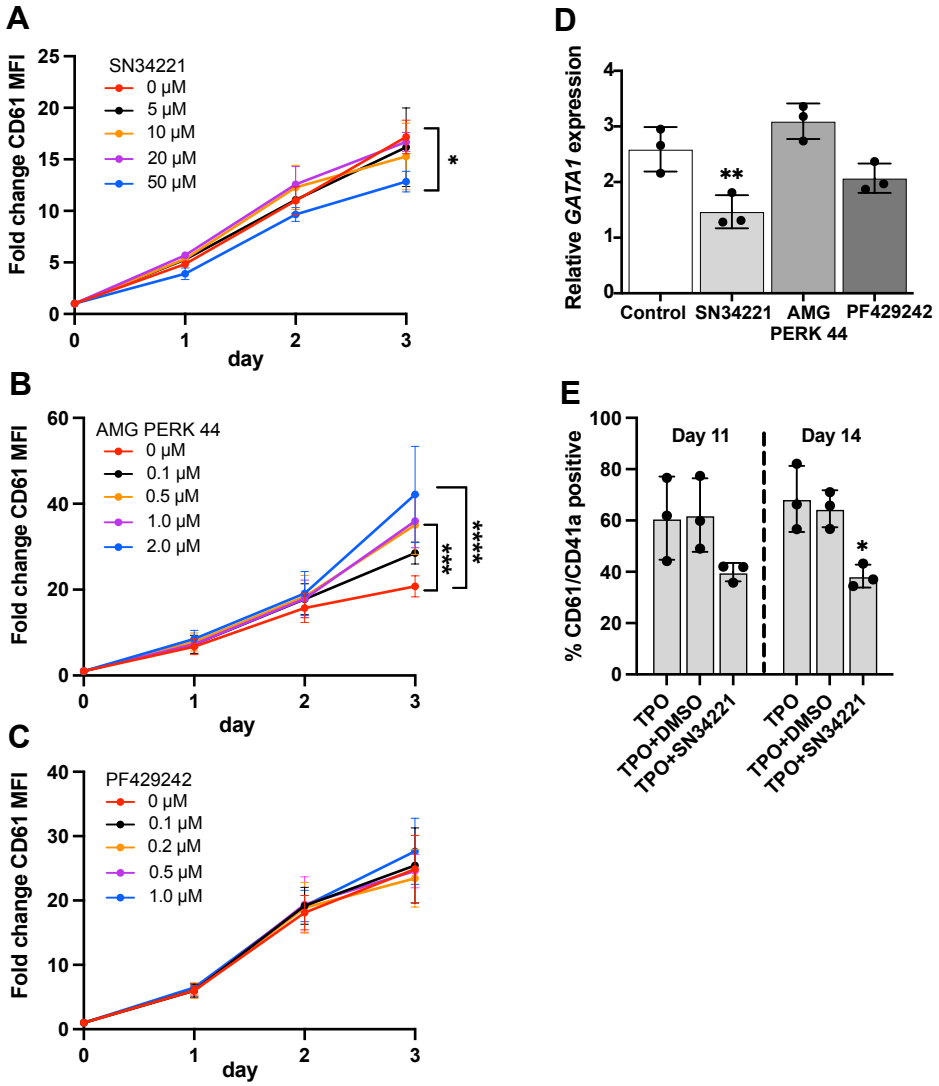

Figure 2. The effect of pharmacological inhibition of UPR on K-562 maturation and peripheral blood CD45$^+$ cell differentiation. A - C. K-562 cells were treated with 0.5 nM PMA in the presence of SN34221 (A), AMG PERK 44 (B) of PF429242 (C) and expression of CD61determined (fold change MFI compared to day 0). N=3 ± SD *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$ (two-way ANOVA). **D.** K-562 cells were treated with 0.5 nM PMA in the presence (white bars) or absence (grey bars) of 50 μM SN34221, 2 μM AMG PERK 44 or 1 μM PF429242 and relative *GATA1* expression measured at day 3. N=3 ± SD. **$P < 0.01$, (one-way ANOVA). **E.** % CD61$^+$/CD41a$^+$ cells at days 11 and 14 of TPO-induced megakaryocyte differentiation of CD45$^+$ peripheral blood cells in the presence of DMSO or 50 μM SN34221. N=3 ± SD *$P < 0.05$ compared to TPO + DMSO (one-way ANOVA).

167 Having observed the potential involvement of the IRE1α UPR pathway in megakaryocytic
168 maturation of K-562 cells, we next determined the impact of IRE1α inhibition on TPO-induced
169 megakaryocyte differentiation of $CD45^+$ cells isolated from human peripheral blood. The IRE1α
170 endonuclease inhibitor SN34221 significantly decreased the percentage of $CD61^+/CD41a^+$
171 megakaryocytes in the live cell population at days 11 and 14 (Figure 2E). Consistent with this
172 result, when cultures were examined by bright field microscopy the cells treated with SN34221
173 were generally smaller and there was no evidence of proplatelet formation or membrane
174 blebbing (Figure S3C). Furthermore, we did not see an increase in the percentage of
175 $CD61^+/CD41a^+$ megakaryocytes in the non-viable (Zombie stained) cells in the cultures. These
176 results suggest that the IRE1α signaling branch of UPR may be involved in PMA-induced
177 megakaryocytic maturation of K-562 cells and TPO-induced megakaryocyte differentiation of
178 primary cells.

179 **Activation of UPR does not enhance PMA-induced maturation of K-562 cells**

180 Our data suggests that activation of the UPR, in particular the IRE1α and PERK pathways, may
181 be involved in megakaryocyte maturation. We next determined whether activation of UPR
182 signaling by an exogenous ER stress enhanced megakaryocyte maturation. We confirmed that
183 the ER stressors DTT, a small-molecule redox reagent for reducing protein disulfide bonds, and
184 thapsigargin, a sarco/endoplasmic reticulum ATPase (SERCA) inhibitor, induced UPR as shown
185 by the splicing of the IRE1α target *XBP1* (Figure S4). Treatment with DTT had no impact on
186 megakaryocytic maturation (Figure 3A) or viability (Figure 3B) of K-562 cells. In contrast,
187 treatment with thapsigargin caused a dose-dependent reduction in PMA-induced maturation of
188 K-562 cells (Figure 3C) with no change in cell viability (Figure 3D). Thapsigargin also inhibited
189 TPO-induced differentiation of $CD45^+$ peripheral blood cells (Figure 3E) and subsequent
190 budding and proplatelet production (Figure S3D). The difference in effect of the two ER
191 stressors suggests that the exogenous UPR activation does not enhance megakaryocyte
192 maturation, whereas alteration of ER Ca^{2+} homeostasis inhibits megakaryocyte differentiation.

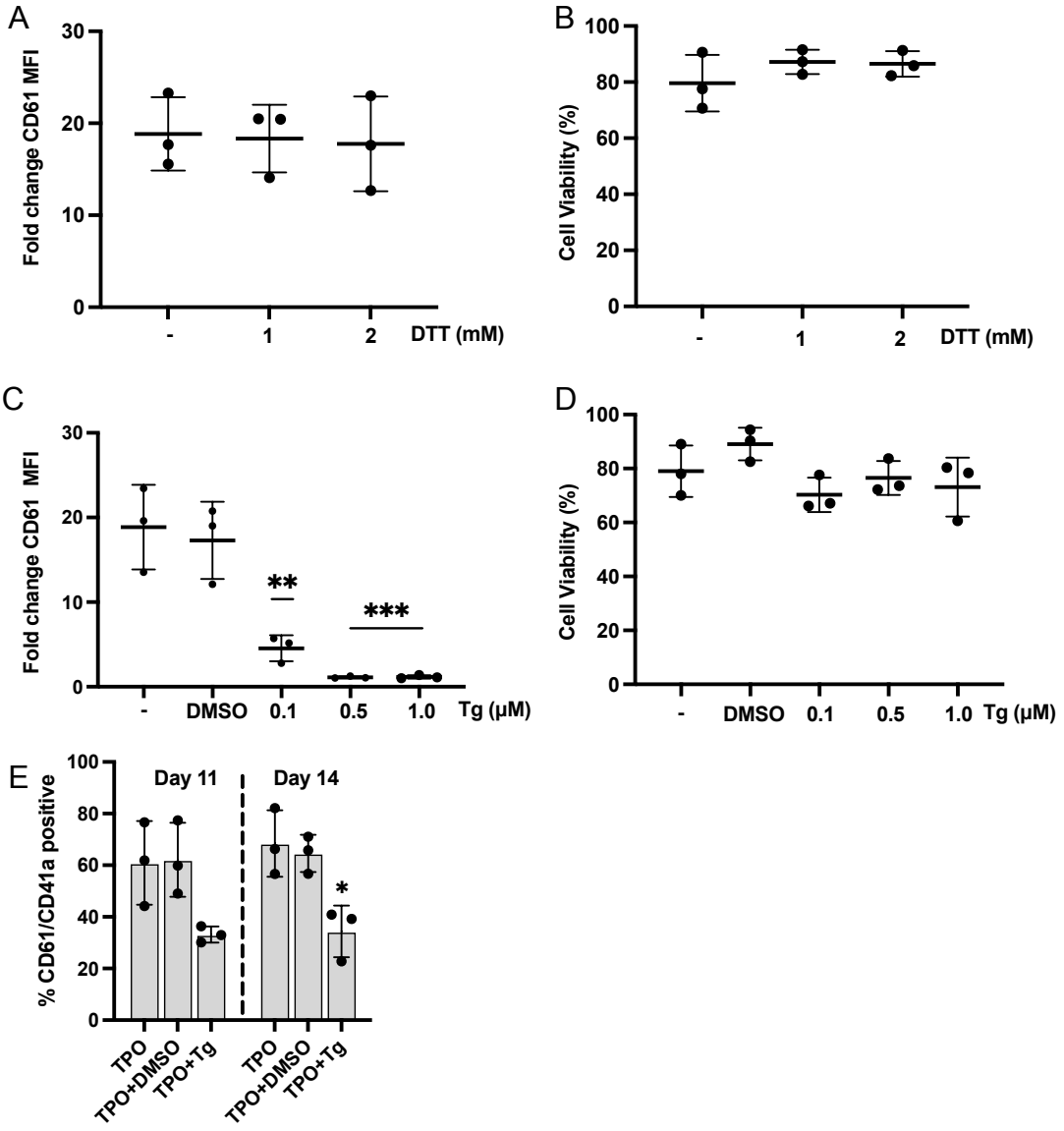

Figure 3. The effect of UPR activation on K-562 maturation and peripheral blood CD45$^+$ cell. A - D. K-562 cells were treated with 0.5 nM PMA for 2 days in the presence of DTT (A and B) or Tg (C and D) and analyzed for expression of CD61 (fold change MFI compared to day 0) (A and C) or cell viability (B and D). N=3 ± SD. *$P < 0.05$, **$P < 0.01$ (one way ANOVA). **E.** % CD61$^+$/CD41a$^+$ cells at days 11 and 14 of TPO-induced megakaryocyte differentiation of CD45$^+$ peripheral blood cells in the presence of DMSO or 1 μM Tg. N=3 ± SD *$P < 0.05$ compared to TPO + DMSO (one-way ANOVA).

Megakaryocytic maturation of K-562 or MEG-01 cells does not alter the response to exogenous ER stressors

203 Finally, we examined whether maturation alters the ability of the K-562 or MEG-01 cells to
204 respond to exogenous ER stressors. K-562 cells or MEG-01 cells were treated with DTT or
205 thapsigargin before or 3 days after PMA treatment, and the expression of UPR target genes was
206 quantified by RT-qPCR. DTT treatment activated the UPR as indicated by a strong induction of
207 *XBP1* splicing, and *CHOP* and *BIP* expression, in immature (untreated) and mature (PMA
208 treated) K-562 cells (Figure 4A). Similarly, thapsigargin treatment induced *XBP1* splicing and
209 increased *CHOP, BIP* and *GADD34* expression in immature and mature K-562 cells (Figure 4B).
210 Similar results were obtained with MEG-01 cells where both ER stressors activated UPR
211 signaling in immature and mature cells (Figure S5). These results indicate that even though UPR
212 pathways are activated during PMA-induced maturation, this does not alter the ability of the cells
213 to further activate UPR signaling in response to exogenous ER stressors.

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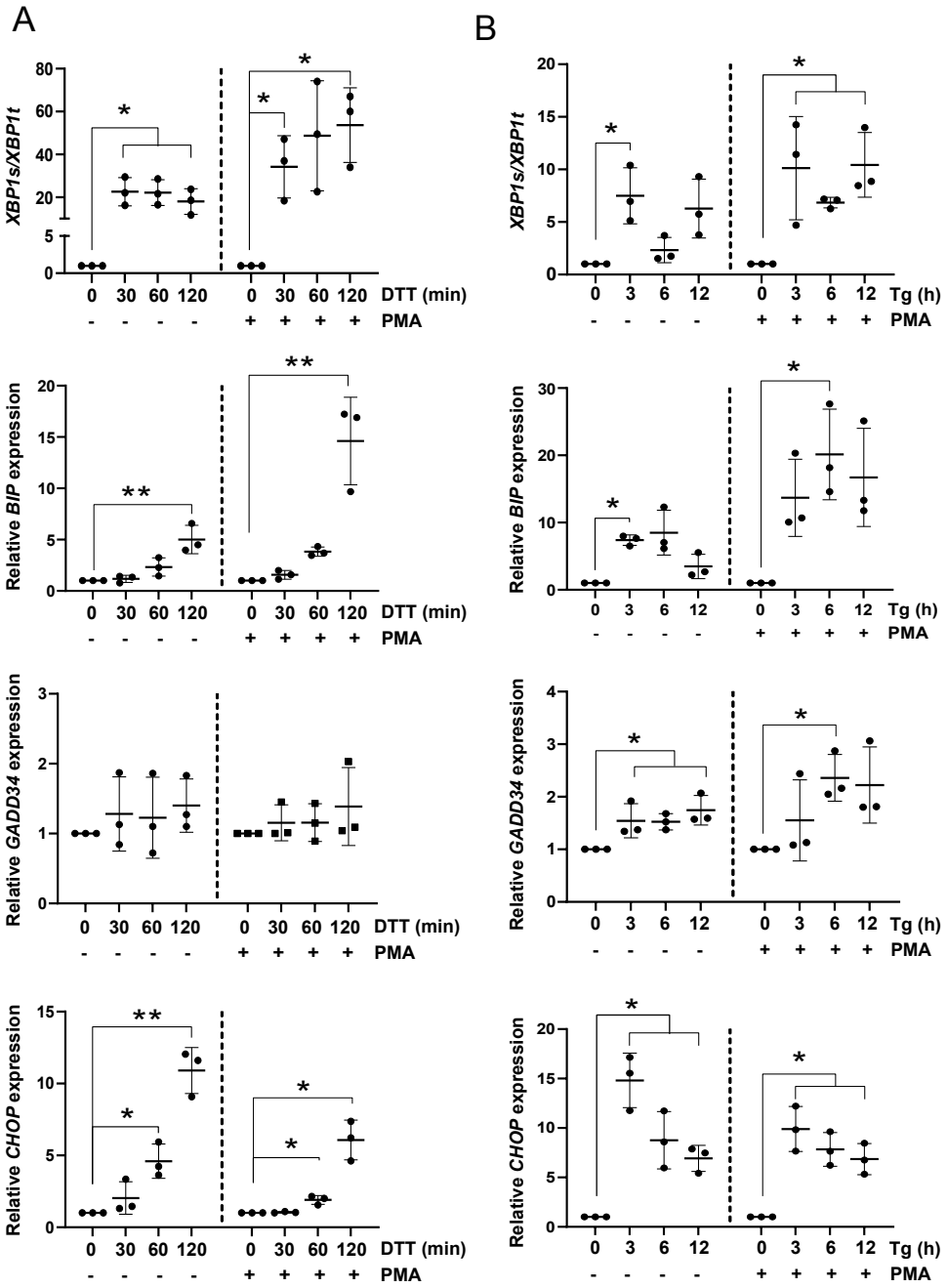

Figure 4. PMA treated and untreated K-562 cells are similarly responsive to ER stress induced by DTT or thapsigargin (Tg). Immature (no PMA) or mature (3 days after PMA treatment) K-562 cells were treated with 10 mM DTT for 0-2 h (A) or 0.1 μM Tg for 0-12 h (B). Expression of *XBP1*s and *XBP1*t, *BIP, CHOP* and *GADD34* mRNA was quantified by RT-qPCR. Expression is relative to 0 h. N=3 ± SD. **P* < 0.05, ***P* < 0.01 (one sample t-test).

Discussion

223 In the current study we used a low concentration of PMA that induced megakaryocytic
224 maturation of K-562 and MEG-01 cell lines in the absence of cell death. Under these conditions
225 maturation was accompanied by increased transcription of UPR target genes *ERDj4, HERPUD1*,
226 *XBP1*, *CHOP*, and *GADD34,* indicating activation of all three UPR signaling pathways. These
227 results are consistent with previous studies reporting an increase in expression of UPR target
228 genes in various models of megakaryocytic maturation (Kovuru et al., 2020; Lopez et al., 2013;
229 Morishima & Nakanishi, 2016). However, in contrast to these earlier studies, increased
230 expression of UPR target genes occurred in the absence of cell death, demonstrating that UPR-
231 induced apoptosis is not required for megakaryocytic maturation. Increased expression of *ERdj4*,
232 *HERPUD* and *CHOP* also occurred during TPO-induced differentiation of $CD45^+$ cells, and an
233 increase in BiP/GRP78 in differentiating $CD34^+$ cells has been reported (Lopez et al., 2013). The
234 terminal stages of megakaryopoiesis involve endomitosis and polyploidization with an
235 accompanying high demand for synthesis of proteins and membrane expansion and remodeling
236 prior to proplatelet formation and platelet release (de Jonckheere et al., 2023; Mazzi et al., 2018).
237 Therefore, it is unsurprising that the adaptive UPR is activated in maturing megakaryocytes to
238 assist terminal megakaryopoiesis by increasing the biosynthesis of chaperone proteins,
239 increasing membrane synthesis, and boosting the protein folding capacity of the ER.

240 To determine whether activation of a specific arm of the adaptive UPR was particularly
241 important for megakaryopoiesis, we inhibited IRE1α endonuclease activity (SN34221), PERK
242 autophosphorylation (AMG PERK 44), and ATF6 cleavage (PF-429242). The IRE1α inhibitor
243 SN34221 partially inhibited PMA-induced maturation of K-562 cells and TPO-induced
244 differentiation of peripheral blood $CD45^+$ cells, implicating the IRE1α UPR pathway in
245 supporting megakaryocytic maturation. Whether *XBP1* splicing and/or RIDD activation is
246 relevant for human megakaryopoiesis remains an open question and requires further
247 investigation. XBP1s is involved in maturation of other hematopoietic cell types (Bettigole &
248 Glimcher, 2016; Reimold et al., 2001), and is an important regulator of specialist secretory cells
249 which require a high level of protein synthesis (Lee et al., 2005; Reimold et al., 2001). XBP1s
250 regulates gene expression by forming heterodimers with other transcription factors including
251 MIST1, an important regulator of differentiation (Acosta-Alvear et al., 2007; Hetz & Papa,
252 2018). A recent characterization of mice with megakaryocyte/platelet-specific deletion of *XBP1*
253 found normal platelet counts but a higher percentage of circulating reticulated (newly released)

254 platelets (Jain et al., 2022) suggesting possible alteration in megakaryopoiesis and/or
255 thrombopoiesis. RIDD has also been implicated in controlling differentiation-associated
256 processes (He et al., 2021; Levi-Ferber et al., 2021) but has not been directly studied in the
257 context of megakaryocyte biology. In contrast, inhibition of the PERK pathway enhanced
258 maturation but also increased cell death, suggesting that this pathway is critical for preventing
259 megakaryocyte apoptosis, but may act to restrain megakaryocyte maturation. This result is
260 consistent with inhibition of PMA-induced megakaryocytic maturation by salubrinal, a drug
261 which potentiates eIF2α phosphorylation (Boyce et al., 2005; Lopez et al., 2013), but in contrast
262 to the decreased platelet count seen in mice with megakaryocyte/platelet-specific deletion of
263 *PERK* (Jain et al., 2022). However, the high level of cell death we saw with PERK inhibition
264 may explain the decreased platelet count observed by Jain et al (2022).

265 Our observations that both immature and mature K-562 and MEG-01cells show similar
266 responses to the exogenous ER stressors DTT and thapsigargin, and that DTT has no impact on
267 megakaryocytic maturation, suggest that the mechanisms of action of the IRE1α and PERK
268 pathways are distinct from a canonical ER stress response. Further studies, including with
269 additional inhibitors, are needed to tease out which downstream targets of XBP1s/RIDD/ATF4
270 are involved in human megakaryopoiesis and thrombopoiesis, and to delineate the role of the
271 UPR pathways in preventing cell death versus facilitating differentiation. It would also be
272 interesting to determine the impact of tunicamycin, which in platelets specifically activates the
273 IRE1α pathway (Jain et al., 2022), on TPO-induced $CD45^+$ or $CD34^+$ differentiation.

274 Treatment with thapsigargin inhibited both PMA-induced maturation of K-562 cells and TPO-
275 induced differentiation of peripheral blood-derived $CD45^+$ cells. Thapsigargin blocks SERCA,
276 inducing protein misfolding and the UPR, and altering Ca^{2+} homeostasis (Carreras-Sureda et al.,
277 2018; Jaskulska et al., 2020; Sehgal et al., 2017). Since DTT had no impact on megakaryocytic
278 maturation, it is likely that dysregulation of Ca^{2+} homeostasis underlies inhibition of maturation
279 by thapsigargin. This is consistent with the known roles of intracellular Ca^{2+} in
280 megakaryopoiesis. Activation of phospholipase C generates inositol trisphosphate (IP3)
281 triggering release of Ca^{2+} from the ER via the IP3 receptor. Subsequent store-operated calcium
282 entry (SOCE) induces expression of nuclear factor of activated T cells (NFAT), a transcriptional
283 regulator implicated in megakaryopoiesis (Hogan et al., 2003; Zaslavsky et al., 2013; Zhu et al.,

284 2018). Additionally, Ca^{2+}entry through a membranous Ca^{2+} ion channel N-methyl-D-aspartate
285 receptor supports megakaryocyte differentiation (Hearn et al., 2020; Hearn et al., 2022). Ca^{2+}-
286 dependent activation of Ca^{2+}/calmodulin-dependent protein kinase (CaMK) induces transcription
287 of CREB (c-AMP response element-binding protein) to activate the genes with CRE elements,
288 including megakaryocyte-lineage specific genes such as *FLI1*, *HOXC6*, *MXD3*, *PRDM16*,
289 *FOXB1*, *TBX6*, *HDAC11* and *NPAS1* (Hogan et al., 2003; Zhang et al., 2005; Zhu et al., 2018).
290 Finally, changes in intracellular Ca^{2+} homeostasis occur in myeloproliferative neoplasms and
291 may contribute to disease pathogenesis through ER stress (Di Buduo et al., 2020; Immanuel et
292 al., 2022; Pietra et al., 2016; Seetharam et al., 2023). Thus, our data provide further evidence for
293 the importance of calcium signaling in megakaryopoiesis.

294 Megakaryopoiesis and thrombopoiesis are complex process that require the activation of many
295 synergistic pathways. Overall, our findings indicate that the adaptive UPR, rather than terminal
296 UPR and associated apoptosis, is a feature of megakaryocyte differentiation. Our data also
297 provide preliminary evidence that activation of the IRE1α pathway enhances maturation,
298 whereas the PERK pathway restrains maturation. We speculate that the two pathways may be
299 active at different times to support different phases of differentiation. To understand the interplay
300 between activation of these two pathways, future work should focus on determining the nature of
301 the initiating events (e.g. is BiP and/or another chaperone important), and the timing of IRE1α
302 phosphorylation and PERK phosphorylation, during differentiation.

303 **Experimental Procedures**

304 *Cell culture and differentiation*

305 Human myeloid leukemic cells, MEG-01 (ATCC CRL-2021) (Ogura et al., 1985) and K-562
306 (ATCC CRL-243) (Tabilio et al., 1983), were maintained in RPMI-1640 (Gibco; 3180002)
307 medium supplemented with 10% fetal bovine serum (FBS), sodium bicarbonate (2 g/L),
308 penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a 5% CO_2 humidified
309 atmosphere. The identity of the cell lines was confirmed by STR analysis (DNA Diagnostics,
310 NZ) and the cells were confirmed to be mycoplasma free. In vitro megakaryocytic maturation
311 was induced by treating K-562 or MEG-01 cells (2.5 x 10^5 cells/mL) in a 6-well plate with 0.5
312 nM phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich).

313 $CD45^+$ cells were isolated and purified from peripheral blood mononuclear cells from individual
314 donors as previously described (Balduini et al., 2009; Ong et al., 2017). To induce progenitor
315 differentiation into megakaryocytes, isolated $CD45^+$ cells (1 x 10^5 cells/ml) were cultured in
316 StemSpan™ medium (StemCell Technologies, Vancouver, Canada) supplemented with 10 ng/ml
317 thrombopoietin (TPO), interleukin (IL) 6 and IL 11 (StemCell Technologies). Cultures were
318 maintained at 37°C in a 5% CO_2 humidified atmosphere for 14 days with media changes on days
319 5, 8, and 11.

320 *ER stress modulators*

321 The conventional ER stressors thapsigargin (Tg) (T9033; Sigma-Aldrich) or dithiothreitol (DTT)
322 (D0632; Sigma-Aldrich) were used at the indicated concentrations and time points. The
323 following compounds were used as UPR inhibitors: PF429242-dihydrochloride (SML0667;
324 Sigma-Aldrich), an ATF6 inhibitor (Hay et al., 2007), AMG-PERK-44 (HY-12661A,
325 MedChemExpress), a PERK inhibitor (Smith et al., 2015), and SN34221 (Supplementary data),
326 an IRE-1α endonuclease inhibitor (Patterson & Lonergan, 2008). The chemicals were dissolved
327 in DMSO or distilled water, and DMSO used as a control when appropriate.

328 *Analysis of megakaryocytic maturation by flow cytometry*

329 Cells were harvested, washed with Dulbecco's PBS, resuspended in 100 µL of Dulbecco's PBS,
330 and stained for 30 min in the dark at room temperature. Antibodies used were anti-CD61
331 phycoerythrin (PE) (1:20; 1075384; BD Biosciences), mouse IgG1 PE (isotype control;
332 7223589; BD Biosciences), anti-CD41a V450 (1:20; 1011309; BD Biosciences), V450 IgG1
333 (isotype control; 7128864; BD Biosciences). For K-562 and MEG-01, live cells were determined
334 with Zombie NIR (1:100; B312060; BioLegend) or Zombie Green (1:100; B334686; BioLegend).
335 After incubation, the cells were washed with Dulbecco's PBS or wash buffer (1X Dulbecco's
336 PBS, 1 mM EDTA, 3% FBS), resuspended in FACS buffer (0.01% sodium azide, 0.1% BSA in
337 Dulbecco's PBS) analyzed using the Guava® easyCyte 5 HPL benchtop flow cytometer
338 (MerckMillipore) or BD LSRFortessa™ cell analyzer (BD Biosciences). Data analysis was
339 performed using FlowJo version 10 (FlowJo, Ashland, USA). Data reported are geometric mean
340 fluorescence intensity (MFI) of the Zombie negative population.

341 *Caspase assay*

342 K-562 cells were seeded into 96-well plates at 2.5 x 10^5 cells/mL in 6-well plate and treated with
343 0.5 nM PMA or 10 mM DTT as a positive control. At 1, 2 or 3 days (PMA) or 22 h (DTT) cells
344 were washed with PBS, harvested and resuspended in100 µL caspase assay buffer (50 mM
345 Hepes pH 7.25, 10% sucrose, 5 mM DTT, 0.1 % CHAPS). Following the addition of 50 µM Ac-
346 DEVD-AMC the production of AMC (λ_{EM} = 390 nm, λ_{EX} = 460 nm) was assayed over 60 min at
347 37 °C in a CLARIOstar™ microplate reader (BMG LABTECH). The results are expressed as fold
348 increase in activity compared to untreated.

349 *RNA isolation and RT-qPCR*

350 Total RNA was isolated from K-562, MEG-01 and primary cells using TRIzol (Invitrogen). The
351 concentration of the RNA was measured using Qubit™ RNA HS Assay Kit (ThermoFisher
352 Scientific). First-strand cDNA synthesis was performed with 1 µg of total RNA in 20 µL
353 reaction volume using SuperScript™ IV VILO™ (SSIV VILO) master mix. Real-time
354 quantitative RT-PCR reactions were run on LightCycler™ 480 Instrument II System (Roche Life
355 Science) using PowerUp™ SYBER™ Green Master Mix (Thermo Fisher Scientific). All the
356 procedures were followed as recommended by the manufacturer. The qPCR primer sequences
357 are shown in Table S2. RT-qPCR was performed in triplicate, and relative expression was
358 quantified using the Pfaffl method (Pfaffl, 2001). Data were normalized to *HPRT* and *GAPDH*
359 (K-562 and MEG-01 cells) or *RPL27* (primary cells) using basket normalization (Vandesompele
360 et al., 2002).

361 *Semi-quantitative PCR analysis for XBP1 splicing detection*

362 To detect *XBP1* splicing, *XBP1* mRNA was amplified (forward primer: 5′
363 CCTTGTAGTTGAGAACCAGG 3′; reverse primer 5′ AGGGGCTTGGTATATATGTGG 3′)
364 (Fribley et al., 2011). Briefly, first-strand cDNA was synthesized with 2 µg of total RNA in 20
365 µL reaction volume. To amplify *XBP1* 5 µL of undiluted cDNA was used as a template in a 50
366 µL reaction mixture with an initial denaturation step for 3 min at 94 °C, followed by 30 cycles of
367 denaturation for 30 s at 94 °C, annealing for 30 s at 54 °C, and extension for 60 s at 72 °C, and a
368 final extension step for 10 min at 72 °C. PCR products were resolved on a 2% (w/v) agarose gel,
369 stained with 0.5 µg/mL ethidium bromide, and visualized using the Gel Doc™ XR+ Imaging
370 System (Bio-Rad).

371 *Protein extraction and western blotting*

372 Cells were lysed in RIPA (radio-immunoprecipitation assay) buffer (50 mM Tris-HCl pH 7.4,
373 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate) containing protease and
374 phosphatase inhibitor cocktail (Sigma-Aldrich; MSSAFE). Total protein (20 µg) was resolved by
375 SDS PAGE under reducing conditions and transferred to nitrocellulose membrane. Membranes
376 were stained with REVERT™ 700 total protein stain (LI-COR Biosciences), washed and imaged
377 on the Odyssey Fc Imaging System (LI-COR Biosciences). The membrane was then blocked
378 with Intercept® Blocking Buffer (LI-COR Biosciences) for 1 h at room temperature and
379 incubated overnight with 1:500 anti-GRp78 (BiP) antibody (sc-1050; Santa Cruz) at 4 ºC.
380 IRDye® 680-LT donkey anti-goat (C60127-06, LI-COR Biosciences) was used as a secondary
381 antibody, and the blots were visualized in the Odyssey Fc Imaging System. Band intensities were
382 quantified with Image Studio Lite version 5.2 (LI-COR Biosciences). Briefly, signals from the
383 total protein stain and target protein were measured, the lane normalization factor (LNF) was
384 calculated for total protein in each sample, and the target protein band intensities were
385 normalized using the LNF.

386 *Statistical analysis*

387 Statistical analyses were performed using GraphPad Prism software version 8.0 (GraphPad
388 Software, San Diego, CA, USA). The comparison of means between different groups was
389 performed using one sample t-test, one-way or two-way ANOVA with Dunnett's multiple
390 comparisons test, or Mann-Whitney. $P < 0.05$ was considered significant. For ANOVA,
391 normality of residuals was confirmed using the D'Agostino, Anderson-Darling, Shapiro-Wilk
392 and Kolmogorov-Smirnov tests. For all experiments the number of biological replicates is
393 indicated.

394

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628 **Supplementary Materials**

629 The supplementary data file contains the following:
630 Synthesis of SN34221
631 Figure S1. PMA induces megakaryocyte maturation of K-562 and MEG-01 cells
632 Figure S2. Impact of UPR inhibitors on cell viability and maturation of K-562 cells
633 Figure S3. Impact of SN34221 or thapsigargin on proplatelet production
634 Figure S4. Thapsigargin and DTT induce *XBP1* splicing in K-562 cells
635 Figure S5. UPR activation in immature and mature MEG-01 cells in response to DTT or
636 thapsigargin
637 Figure S6. SN34221 inhibits *XBP1* splicing.
638 Table S1. Caspase activation during PMA-induced maturation of K-562 cells
639 Table S2. RT-qPCR primers used in the study
640

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