N-Methyl-D-Aspartate Receptor Hypofunction in Meg-01 Cells Reveals a Role for Intracellular Calcium Homeostasis in Balancing Megakaryocytic-Erythroid Differentiation

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Abstract

The release of calcium ions (Ca2+) from the endoplasmic reticulum (ER) and related store-operated calcium entry (SOCE) regulate maturation of normal megakaryocytes. The N-methyl-D-aspartate (NMDA) receptor (NMDAR) provides an additional mechanism for Ca2+ influx in megakaryocytic cells, but its role remains unclear. We created a model of NMDAR hypofunction in Meg-01 cells using CRISPR-Cas9 mediated knockout of the GRIN1 gene, which encodes an obligate, GluN1 subunit of the NMDAR. We found that compared with unmodified Meg-01 cells, Meg-01-GRIN1−/− cells underwent atypical differentiation biased toward erythropoiesis, associated with increased basal ER stress and cell death. Resting cytoplasmic Ca2+ levels were higher in Meg-01-GRIN1−/− cells, but ER Ca2+ release and SOCE were lower after activation. Lysosome-related organelles accumulated including immature dense granules that may have contributed an alternative source of intracellular Ca2+. Microarray analysis revealed that Meg-01-GRIN1−/− cells had deregulated expression of transcripts involved in Ca2+ metabolism, together with a shift in the pattern of hematopoietic transcription factors toward erythropoiesis. In keeping with the observed pro-cell death phenotype induced by GRIN1 deletion, memantine (NMDAR inhibitor) increased cytotoxic effects of cytarabine in unmodified Meg-01 cells. In conclusion, NMDARs comprise an integral component of the Ca2+ regulatory network in Meg-01 cells that help balance ER stress and megakaryocytic-erythroid differentiation. We also provide the first evidence that megakaryocytic NMDARs regulate biogenesis of lysosome-related organelles, including dense granules. Our results argue that intracellular Ca2+ homeostasis may be more important for normal megakaryocytic and erythroid differentiation than currently recognized; thus, modulation may offer therapeutic opportunities.
Introduction

Calcium (Ca$^{2+}$) is an ubiquitous but versatile cytosolic second messenger, oscillations of which regulate gene transcription, including in megakaryocytes (MKs).1,2 Resting cells maintain cytosolic Ca$^{2+}$ concentrations at very low levels to inhibit apoptosis. This is achieved through the transport of cytosolic Ca$^{2+}$ into the extracellular space or sequestration of Ca$^{2+}$ into intracellular stores, of which endoplasmic reticulum (ER) is the main site. Molecules that maintain intracellular Ca$^{2+}$ homeostasis include diverse Ca$^{2+}$ channels, pumps, exchangers and binding proteins, collectively known as the Ca$^{2+}$ signaling „toolkit.” On the background of normal Ca$^{2+}$ homeostasis, oscillations in cytosolic Ca$^{2+}$ levels that vary in amplitude, frequency and duration translate into specific cellular effects.1

The principles of intracellular Ca$^{2+}$ homeostasis in MKs are similar to those in other cells. MK surface receptors activate phospholipase C (PLC) that generates inositol 1,4,5-trisphosphate (IP3).2,3 IP3 binds to IP3 receptors (IP3Rs) located on the ER membrane, triggering the release of Ca$^{2+}$ from the ER. Depleted ER Ca$^{2+}$ stores are refilled from the extracellular space through the process called store-operated calcium entry (SOCE), facilitated by stromal interaction molecule 1 (STIM1). STIM1 recruits ORAI1 channels in facilitated by stromal inter-5

The extracellular space through the process called store-operated calcium entry (SOCE), facilitated by stromal interaction molecule 1 (STIM1). STIM1 recruits ORAI1 channels in the plasma membrane that refill ER Ca$^{2+}$ stores. High levels of cytosolic Ca$^{2+}$ that arise during cell activation are normalized by two main types of Ca$^{2+}$ pumps that either transport Ca$^{2+}$ back to the extracellular space (plasma membrane Ca$^{2+}$ ATPases [PMCA]) or to the ER (sarco-/endo-plasmic reticulum Ca$^{2+}$ ATPases [SERCA]).4

Both ER Ca$^{2+}$ release and SOCE are known to regulate MK development and maturation. In megakaryocytic progenitors, sustained SOCE activates the calcineurin-nuclear factor of activated T cells (NFAT) pathway that inhibits cell proliferation.5 In mature MKs, SOCE supports MK migration, and ER Ca$^{2+}$ release triggers MK adhesion and proplatelet formation.6 SOCE represents the main pathway for Ca$^{2+}$ entry in most cells, but MKs also express other Ca$^{2+}$ channels located in the plasma membrane, including transient receptor potential cation (TRPC) and N-methyl-D-aspartate (NMDA) receptors (NMDARs), the roles of which are much less understood.

NMDARs are glutamate gated, nonspecific cation channels with high Ca$^{2+}$ permeability.7 The first evidence that NMDARs operate as ion channels in MKs was obtained by Genever et al, who demonstrated that tritiated MK-801 injected into mice intracardially bound to MKs in the bone marrow examined 15 minutes later.8 Because MK-801 can only bind within an open NMDAR pore,5 its labeling of MKs was consistent with the NMDAR function as ion channel in these cells. Later, we showed that glutamate, NMDA and glycine induce Ca$^{2+}$ fluxes in Meg-01 cells, and NMDAR blockers (memantine and MK-801) counteract this effect.10,11 Others and we also found that memantine and MK-801 inhibit differentiation of normal mouse and human MKs ex vivo but induce differentiation of leukemic Meg-01 cells in vitro.8,10–12 Further characterization of NMDAR effects using chemical modulators was restricted by toxic, likely off-target effects. Thus, we undertook a gene knockout approach in Meg-01 cells.

We hypothesized that NMDAR-mediated Ca$^{2+}$ influx contributes to intracellular Ca$^{2+}$ homeostasis in megakaryocytic cells, which impacts the transcriptional program of cell differentiation. Using CRISPR-Cas9, we attenuated NMDAR function in a Meg-01 cell line as a model of megakaryocytic-erythroid progenitors and examined subsequent effects on cell phenotype. Our results suggest an important role of intracellular Ca$^{2+}$ homeostasis in balancing megakaryocytic-erythroid differentiation.

Methods

Cell Culture

Meg-01 cells (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were used as models of human megakaryocytic-erythroid progenitors. Meg-01 cell line is derived from acute megakaryoblastic leukemia transformed from chronic myeloid leukemia, but cells undergo megakaryocytic differentiation.13–15 Meg-01 and Meg-01-GRIN1 $^{1/-}$ cells were maintained at 37°C, 5% CO2, in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% foetal bovine serum (FBS; all from Thermo Fisher Scientific, Waltham, Massachusetts, United States), as described previously.16 To induce differentiation, cells were cultured in the presence of phorbol-12-myristate-13-acetate (10 nM; PMA; Sigma–Aldrich, Saint Louis, Missouri, United States) for 72 hours. TrypLE (Thermo Fisher Scientific) was used to collect adherent cells for analysis. Cultures were confirmed to be free from mycoplasma infection using LookOut Mycoplasma PCR Detection Kit (Sigma–Aldrich).

CRISPR-Cas9 Plasmid Design and Transfection

The CRISPR-Cas9 system was applied using a single guide RNA (gRNA) to induce an insertion/deletion (INDEL) causing a frameshift in the GRIN1 gene. The genomic target sequence was positioned in exon 1 of GRIN1, downstream of all known start codons for the gene. The sequence 5’-CAAGATCGTCAA-CATTGGCG-3’ was cloned into a modified pMIG plasmid containing an orange fluorescent protein (OFP) reporter sequence (pMIG-Alpha was a gift from William Hahn, Addgene plasmid #9044; http://n2t.net/addgene:9044). To induce differentiation, cells were cultured for 48 hours, single, high OFP expressing cells were sorted into 96-well plates using the FACSAria II (Becton Dickson, Franklin Lakes, New Jersey, United States). Cells were cultured in RPMI-1640 supplemented with 2 mM Glutamax, 25 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid), 1 mM sodium pyruvate and 40% FBS for 2 weeks. The DNA from the clones was amplified using primers (forward: 5’-CTCGACACA-CACGCCTAC-3’, reverse: 5’-ATAGGCGAGCCAGACAC-3’) targeting the gRNA target site, and amplicons were sequenced for INDELS by Sanger sequencing.
Transfection of Short Interfering RNA
Meg-01 cells were plated at $6 \times 10^5$ cells per well in a six-well plate and allowed to adhere for 4 hours. Endoribonuclease-prepared short interfering RNA (esiRNA) targeting GRIN1 (esiGRIN1; EHU157091, Sigma–Aldrich) were used to transiently knockdown GRIN1 in Meg-01 cells. Transfections were done in serum-free OptiMEM media assisted by Lipofectamine RNAiMAX (both from Thermo Fisher Scientific). OptiMEM was replaced with complete culture media 12 hours after transfections, and cells were harvested for analysis 60 hours later.\(^\text{17}\)

\[^{3}\text{H}]\text{MK-801 Binding Assay}\]
\[^{3}\text{H}]\text{MK-801} was used to label open (i.e. active) NMDARs. Cells were plated at $5 \times 10^5$ cells per well in 24-well plates and allowed to adhere for 4 hours. \[^{3}\text{H}]\text{MK-801} (5 nM, 1 μCi L\(^{-1}\)) (Perkin Elmer, Waltham, Massachusetts, United States) and glutamate 500 μM (NMDAR agonist; Sigma–Aldrich) were added and incubated with cells for 1 hour. Media was removed, cells were washed with serum-free media, and solubilized with 1 N NaOH. \(\beta\)-particle emission was recorded as counts per second using a Wallac Microbeta 1450–021 TriLux Luminometer Liquid Scintillation Counter (LabEquip, Markham, Canada) as described previously.\(^\text{17}\)

Cell Viability, Proliferation and Cell Death Assays
Cell viability and proliferation assays were done as previously described.\(^\text{1,13}\) Briefly, cells were seeded at $1 \times 10^4$ cells per well in 96-well plates and cultured for 72 hours prior to testing using an MTT kit (Thermo Fisher Scientific). Cell proliferation was examined using the Cell Proliferation ELISA BrdU kit (Roche, Basel, Switzerland) after incubation with bromodeoxyuridine (BrdU) for 6 hours. Cytotoxicity was measured using the Cytotoxicity Detection KitPLUS (lactate dehydrogenase [LDH] release assay; Roche). Selected cell survival assays used the following chemicals: NMDA (synthetic NMDAR agonist, 100 μM), L-glutamate (main NMDAR agonist, 500 μM; both from Sigma–Aldrich), glycine (NMDAR co-agonist, 300 μM; VWR International, Radnor, Pennsylvania, United States), memantine (NMDAR antagonist, 100 μM; Sigma–Aldrich), and cytarabine (0.1 μM; Cayman Chemical, Ann Arbor, Michigan, United States).

Recordings of Intracellular Calcium Responses
Intracellular Ca\(^{2+}\) responses were monitored using the Fura-2 Calcium Assay Kit (Abcam, Cambridge, United Kingdom). Cells were plated in poly-D-lysine (Sigma–Aldrich) treated glass-bottom, black, 96-well plates at $9 \times 10^4$ cells per well. Cells were allowed to adhere for 4 hours, then washed with 1× Hank’s Balanced Salt Solution (HBSS), and loaded with Fura-2-AM at 37°C for 1 hour in the dark. Fluorescence was measured using a 510 nm emission filter with 340 and 380 nm excitation filters from the bottom of the plate with a Tecan Spark Multiplate Reader (Tecan, Männedorf, Switzerland) at 37°C, 5% CO\(_2\). Signals were acquired every second for 30 seconds to establish a baseline and then again every second for a further 120 seconds after the addition of NMDA (100 μM) with glycine (300 μM) or glutamate (500 μM) with glycine (300 μM), both with or without BAPTA (5 mM; 1,2-bis(o-aminophenoxy)ethylane-N,N,N′,N′-tetraacetic acid, cell-impermeant calcium chelator; Thermo Fisher Scientific). Other experiments used thapsigargin (2 μM) and ionomycin (5 μg mL\(^{-1}\); both from Sigma–Aldrich).

To measure SOCE fluxes, cells were seeded at $2 \times 10^4$ cells per well and cultured for 3 days. Cells were washed with 1× physiological saline prior to loading with Fura-2–AM as above. Ca\(^{2+}\) signals were measured every 30 seconds for 5 minutes to establish a baseline. Media was then changed to Ca\(^{2+}\)–free physiological saline, supplemented with cyclopiazonic acid (10 μM; a SERCA inhibitor that depletes ER Ca\(^{2+}\) stores) and EGTA (500 μM; ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, a cell-impermeant calcium chelator; both from Sigma–Aldrich), and signals were recorded every 30 seconds for 7.5 minutes. Media was then changed to physiological saline, and monitoring continued every 30 seconds for a further 12.5 minutes. Fluorescence was measured using a 510 nm emission filter with 340 and 380 nm excitation filters as above. Relative intracellular Ca\(^{2+}\) levels were determined, based on the measurement of a fluorescent 340/380 nm ratio.

Flow Cytometry
Nuclear ploidy analysis was performed by staining cells (0.5–1 × 10\(^5\) per tube) with propidium iodide 50 μg mL\(^{-1}\) in a hypotonic sodium citrate buffer (0.1%) for 35 minutes on ice. Cells were washed, resuspended in RPMI-1640, and treated with RNase 50 μg mL\(^{-1}\) at room temperature (RT) for 30 minutes.

The following antibodies (catalog number) were used to characterize myeloid antigen expression: CD13 (561698), CD33 (561816), CD41 (555466), CD42a (558819), CD42b (555473), CD61 (555754), CD71 (347513) (all from BD Biosciences, San Jose, California, United States) and CD235a (IM2212; from Beckman Coulter, Be, California, United States). Cells (0.5–1 × 10\(^5\)) were incubated with the antibodies for 15 minutes at RT, washed with wash buffer (1× phosphate buffered saline [PBS]), 2% PBS, 0.02% sodium azide, and fixed with 0.5% paraformaldehyde (PFA) in 1× PBS.

ER-Tracker Red (10 μM) and LysoTracker Red DNA-99 (50 nM) (both from Thermo Fisher Scientific) were incubated with cells (0.5–1 × 10\(^5\)) at 37°C, 5% CO\(_2\) for 45 and 60 minutes, respectively. Cells were washed with wash buffer and fixed with 0.5% PFA in 1× PBS. All flow cytometry data were acquired on the BD LSRII flow cytometer and analyzed using BD FACSDiVa software v6.1.1.

Microscopy Examination and Ultrastructure
For immunofluorescence, cells were fixed in 4% PFA in 1× PBS for 15 to 20 minutes and permeabilized for 5 minutes with 0.1% Triton X-100 in PBS. Cells were blocked for 30 minutes with 5% goat serum and then incubated with primary antibodies (CD63 1:200, ab59479, Abcam; GluN1 1:500, MAB363, Merk & Co, Kenilworth, New Jersey, United States or Calnexin 1:1000, ab22595, Abcam) at 4°C overnight. After washing, cells were incubated with 2.5 μg mL\(^{-1}\) Dylight 488/594-conjugated secondary antibodies (ab96931 and ab96885, respectively; both from Abcam) for 3 hours. Giemsa staining was performed.
using the Cytopro autostainer (ELITech, Paris, France). Bright-field and immunofluorescence microscopy was conducted using an Eclipse Ni-E microscope (Nikon, Tokyo, Japan). Hoffman and phase contrast images were taken on an Eclipse Ti microscope (Nikon).

Transmission electron microscopy was done as previously described.\(^\text{18}\) Briefly, cells were fixed with 0.2% glutaraldehyde and 2% PFA in White’s saline. Sections were counterstained with uranyl acetate and examined with a Tecnai G2 Spirit Twin transmission electron microscope (FEI Company, Hillsboro, Oregon, United States).

**Western Blotting**

Cells were lysed in radio-immunoprecipitation assay buffer (50mM Tris pH 4.7, 150mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, and 1mM EDTA) with protease and phosphatase inhibitors (Sigma–Aldrich). Cell lysates were quantified using the Pierce BCA protein assay kit (Thermo Fisher Scientific), and proteins were resolved on a 4 to 15% SDS-PAGE gradient gel. Separated proteins were electrophoretically transferred onto polyvinylidene difluoro membranes for subsequent probing with antibodies (CD63 1:1000, ab59479, Abcam; LC3-II 1:1000, CTE4108S, Thermo Fisher Scientific; pan-Actin 1:10000, MAB1501, Abcam). Membranes were washed and incubated with horseradish peroxidase conjugated secondary antibodies (111–035–003, Jackson ImmunoResearch, Pennsylvania, United States). Clarity Western ECL (Bio-Rad, Hercules, California, United States) was used for signal detection using Chemidoc Touch (Bio-Rad). Membranes were stained with Coomassie Blue to observe total protein. Relative protein quantitation was performed by band densitometry using ImageLab 5.2.1 (Bio-Rad; LC3-II was quantified relative to actin, and CD63 relative to total protein after Coomassie staining.

**Gene Expression Profiling and Gene Ontology Analysis**

RNA was isolated as described above. Gene expression profiling was performed using the Clariom S microarray assay (Thermo Fisher Scientific). Data were normalized by gene level Robust Multi-array Average method.\(^\text{23}\) Unmodified Meg-01 and Meg-01-GRIN1\(^{-/-}\) cells were assayed in triplicate. Differentially expressed (DE) genes were identified using eBays one-way analysis of variance (ANOVA) with a Benjamini-Hochberg false discovery rate (FDR) of 0.05; analysis was done using Transcriptome Analysis Console 4.0 (Thermo Fisher Scientific).

The overrepresentation enrichment analysis was performed on DE genes with a high fold change (\(\geq 2.0\)) to identify whether any gene ontology (GO) defined biological processes occurred more than chance would dictate. The GO–Slim set-up was selected to reduce the overlap between the GO processes. The over-representation analysis was performed using the PANTHER V14.1 online tool (http://pantherdb.org; accessed May 2019). A more relaxed cutoff (fold change \(\geq 1.5\); FDR \(\leq 0.05\)) was applied to interrogate expression of genes within the deregulated biological processes, in particular genes coding for the Ca\(^{2+}\) toolkit and hematopoietic transcription factors. Statistical enrichment was determined via Fisher’s exact test; a conservative Bonferroni correction was applied to all nominal \(p\)-values.

**Statistical Analysis**

Data are expressed as mean \(\pm\) standard error of the mean (SEM). Statistical analyses were conducted using GraphPad Prism 8.0 (San Diego, California, United States). Differences in group means were compared with either a Student’s \(t\)-test (two-tailed) or one-way ANOVA with Dunnett post hoc for continuous variables, as indicated in figure legends. An \(\alpha\) of 0.05 was considered statistically significant.

**Results**

**Generating a Model of Reduced NMDAR Expression in Megakaryocytic Cells**

Using CRISPR-Cas9, we knocked out the expression of the GRIN1 gene in Meg-01 cells; GRIN1 encodes an obligate, GluN1 subunit of the NMDAR. A pMIG plasmid was modified to carry gRNA targeting exon 1 of GRIN1. Forty-eight hours after transfection, Meg-01 cells showing high expression of the GFP reporter were sorted by flow cytometry and grown into single cell colonies. Candidate cell clones were screened by Sanger sequencing of the modified genetic region. A clone was identified with a 59 bp deletion in both GRIN1 alleles, predicted to cause a frame-shift and premature stop codons in exons 2 and 3 of GRIN1 (Meg-01-GRIN1\(^{-/-}\) cells; \(\text{Fig. 1A}\)).

Examination by RT-qPCR demonstrated a 91 \(\pm\) 3% knockdown of GRIN1 mRNA compared with unmodified Meg-01 cells (\(\text{Fig. 1Bii}\)). Binding of \([\text{H}]\text{MK-801, use}-\text{dependent NMDAR antagonist, was reduced by 72} \pm\) 16\%, indicating low numbers of remaining functional NMDARs (\(\text{Fig. 1Bii}\)).
Fig. 1 Validation of the GRIN1 knockout in Meg-01 cells (Meg-01-GRIN1/C0/C0 cells). (A) Sequence alignment of Sanger sequences obtained from unmodified Meg-01 cells and Meg-01-GRIN1/C0/C0 cells to the GRIN1 reference sequence (National Center for Biotechnology Information accession number NG_011507.1) for nucleotides 373–449 (exon 1). The dashed line indicates the region of gene deletion (59 bp) generated by CRISPR-Cas9. The relative positions of the guide RNA (gRNA) target and protospacer adjacent motif (PAM) sequences are shown (yellow arrows). (B) Bar graphs showing relative levels of GRIN1 mRNA expression examined by RT-qPCR (i) and [3H]MK-801 binding (ii) in Meg-01-GRIN1/C0/C0 cells, calculated relative to unmodified Meg-01 cells. Bars are mean ± standard error of the mean from four independent experiments for RT-qPCR and two for [3H]MK-801 binding, each in triplicate. “Cold” Meg-01 indicates [3H]MK-801 binding by Meg-01 cells after incubation with nonradioactive (“cold”) MK-801 (specificity control). (C) Representative images showing GluN1 immunofluorescence staining in Meg-01 and Meg-01-GRIN1/C0/C0 cells. Scale bar, 50 μM for both. (D, E) NMDAR-mediated intracellular calcium responses were measured relative to baseline set at (1.0) in Meg-01 and Meg-01-GRIN1/C0/C0 cells. Cells were loaded with Fura-2-AM and stimulated with NMDAR agonists in the presence of extracellular Ca2+: NMDA 100 μM without and with BAPTA 10 mM (i, ii, iii and iv, respectively); and glutamate 500 μM without and with BAPTA 10 mM E (i, ii, iii and iv, respectively). Line graphs in D and E show the mean fluorescent 340/380 nm ratio of Fura-2 recorded over 120 seconds corresponding bar graphs show the peak level (mean ± standard error of the mean) of the fluorescent 340/380 nm ratio recorded during the observation period. Buffer indicates control. Each experimental condition was repeated five times, in triplicate. Statistical significance is shown (Student’s t-test for B and one-way ANOVA with Dunnett post hoc for D and E; *p < 0.05, **p < 0.01, ***p < 0.001). BAPTA, 1,2-bis(o-aminophenoxy) ethane-N,N,N,N-tetraacetic acid; gRNA, guide RNA; NMDAR, N-methyl-D-aspartate receptor; PAM, protospacer adjacent motif; RT-qPCR, quantitative reverse transcriptase-polymerase chain reaction.
Immunofluorescence demonstrated minimal staining for the GluN1 protein. The virtual loss of Ca\(^{2+}\) influx through NMDAR was confirmed by the examination of Ca\(^{2+}\) fluxes in Fura-2-AM loaded cells. No Ca\(^{2+}\) influx was recorded in Meg-01-GRIN1\(^{-/-}\) cells in response to 100\(\mu\)M NMDA (synthetic but specific NMDAR agonist). Peak Ca\(^{2+}\) responses to 500\(\mu\)M glutamate (endogenous but nonspecific NMDAR agonist) were 54% lower in Meg-01-GRIN1\(^{-/-}\) cells compared with unmodified Meg-01 cells, implying contribution from other glutamate receptors. The effect of GRIN1 deletion on NMDAR-evoked Ca\(^{2+}\) influx resembled that of memantine (NMDAR blocker), supporting that Meg-01-GRIN1\(^{-/-}\) cells provide a valid model of reduced NMDAR-mediated Ca\(^{2+}\) entry in Meg-01 cells.

**Loss of N-Methyl-D-Aspartate Receptor Function Has an Antiproliferative and Proapoptotic Effect**

 Morphologically, Meg-01-GRIN1\(^{-/-}\) cells were larger, more adherent, and multiplied visibly slower, compared with unmodified Meg-01 cells. Concomitantly, MTX activity and BrdU incorporation were lower, confirming reduced cell numbers and proliferation respectively. When culture media was supplemented with NMBA (100\(\mu\)M) or glutamate (500\(\mu\)M), proliferation of unmodified Meg-01 cells increased but not of Meg-01-GRIN1\(^{-/-}\) cells. Providing additional evidence that the GRIN1 knockout reduced NMDAR function, LDH release was higher for Meg-01-GRIN1\(^{-/-}\) cells during normal culture, implying an increased level of basal cell death.

**Video 1**

Growth pattern of Meg-01 cells in culture over 24-hour period. Time-lapse microscopy was performed using a Nikon TE2000E inverted microscope equipped with an automated stage, a 20X 0.25 numerical aperture Hoffman modulation contrast objective, and a Solent incubation system (37°C, 5% CO2; Solent Scientific Limited, Portsmouth, United Kingdom). Cells were grown in RPMI-1640 supplemented with 10% FBS. Images were acquired every 10 minutes over 24 hours and videos were assembled using NIS-Elements (Nikon).


**Video 2**


Flow cytometric measurement of DNA content after staining with propidium iodide showed increased ploidy in Meg-01-GRIN1\(^{-/-}\) cells, suggesting megakaryocytic differentiation. However, unexpectedly, expression of megakaryocytic markers (CD41a, CD61; Fig. 2F and Gii–iv; Supplementary Fig. S2, available in the online version) and CD42a and CD42b (Supplementary Figs S3 and S4, available in the online version) were lower. Instead, erythroid markers (CD235a and CD71) were higher, implying increased differentiation toward the erythroid lineage (Fig. 2F and Gii–iv; Supplementary Figs S5 and S6, available in the online version).

Other distinctive features of Meg-01-GRIN1\(^{-/-}\) cells included progressive accumulation of cytoplasmic vacuoles and granules (Fig. 3). Some vacuoles were small, located mostly in the perinuclear location (Fig. 3A and B; black arrowheads), others were large, distributed throughout the cytoplasm (Fig. 3A and B; black arrows). Transmission electron microscopy was performed to clarify the cytoplasmic content, which revealed that some cells were filled with vacuolar-like structures, including frequent immature dense granules (Fig. 3C; blue arrowheads). Staining with CD63 and LysoTracker was increased, confirming accumulation of lysosome-related organelles (Fig. 4A–C; Supplementary Fig. S7A and B, available in the online version); in Meg-01 cells these are known to include both lysosomes and developing dense granules. The lysosomal accumulation raised the possibility of increased autophagy, the induction of which was confirmed by higher lipidation of microtubule associated protein 1 light chain 3 (LC3) compared with unmodified Meg-01 cells (Fig. 4D; Supplementary Fig. S8, available in the online version). The ER-Tracker staining was increased in Meg-01-GRIN1\(^{-/-}\) cells when tested by flow cytometry suggesting ER expansion (Fig. 4E; Supplementary Fig. S7C, available in the online version), corroborated by Calnexin immunofluorescence (Supplementary Fig. S9, available in the online version). The ER expansion suggested ER stress, which was confirmed by RT-qPCR of selected ER stress markers (Fig. 4F). In contrast to the increased presence of dense granules in Meg-01-GRIN1\(^{-/-}\) cells, there was no evidence that α-granules accumulated, as expression of P-selectin (CD62P) and von Willebrand factor remained low (Supplementary Fig. S10, available in the online version).

**Intracellular Calcium Homeostasis is Disturbed in Meg-01-GRIN1\(^{-/-}\) Cells**

Intracellular Ca\(^{2+}\) transients were measured in cells loaded with Fura 2-AM (Fig. 5). We found that compared with unmodified Meg-01 cells, Meg-01-GRIN1\(^{-/-}\) cells had elevated cytosolic Ca\(^{2+}\) levels at baseline (Fig. 5Ai and Aii). In contrast, an effect of ER Ca\(^{2+}\) release tested in the presence of cyclopiazonic acid (an inhibitor of SERCA) was reduced (Fig. 5Ai and Aii). Similarly, the SOCE effect, measured after re-addition of extracellular Ca\(^{2+}\), was lower (Fig. 5Ai and Aiv). Application of thapsigargin (another SERCA inhibitor) and ionomycin (Ca\(^{2+}\) ionophore) confirmed reduced contribution of ER Ca\(^{2+}\) in Meg-01-GRIN1\(^{-/-}\) cells (Fig. 5B and C, respectively).
Considering that little is known about NMDARs in megakaryocytic cells, we profiled transcriptomic effects of GRIN1 deletion using Clariom S microarrays (Fig. 6). DE genes were first determined as probe-sets that showed at least a twofold change compared with unmodified Meg-01 cells, with an FDR adjusted p-value ≤ 0.05. The GO analysis identified 248 genes that were upregulated and 187 genes that were downregulated, with four differentially regulated biological processes, of which “Regulation of developmental process” (GO:0050793) and “Cellular calcium ion homeostasis” (GO:0006874) were the
most deregulated (Fig. 6A; Supplementary Microarray Excel Data File, available in the online version). Then we analyzed expression of 82 core transcripts of the Ca\(^{2+}\) toolkit, using a list of genes studied in cancer cells before. Supplementary Table S2 (available in the online version) provides data on the expression of all Ca\(^{2+}\) toolkit genes we analyzed; here, we summarize the most prominent changes (Table 1).

Meg-01-GRIN1\(^{−/−}\) cells showed reduced expression of TRPC6 and CACNA1A (coding for TRPC6 and Cav2.1, respectively), while MCOLN3 (coding for TRP mucolipin 3, TRPML3) was increased (Table 1). TRPC6 contributes to SOCE in MKs. The role of Cav2.1 in MKs is unclear, but in erythrocytes Cav2.1 is regulated by TRPC6 and NMDAR. We also found reduced expression of two genes encoding Ca\(^{2+}\) pumps, ATP2B4 (coding for PMCA4) and ATP2A3 (coding for SERCA3), as well as SLC24A3 (coding for K\(^+\)-dependent Na\(^+\)/Ca\(^{2+}\) exchanger 3, NCKX3; Table 1). The notable changes affecting Ca\(^{2+}\) binding proteins included downregulation of CALN1 (encoding the ER protein, calneuron 1) and upregulation of CALB1 and SCIN (encoding cytosolic calbindin 1 and scinderin, respectively; Table 1).

Selected microarray data were validated using RT-qPCR in independent passages of Meg-01-GRIN1\(^{−/−}\) cells (Fig. 6B,

**Fig. 3** Cytological effects of GRIN1 knockout in Meg-01 cells. Representative images of cellular morphology of Meg-01 and Meg-01-GRIN1\(^{−/−}\) cells visualized by phase contrast microscopy (A), bright field microscopy after Wright–Giemsa staining (B), and transmission electron microscopy (C). Examples of the following morphological features are pointed to in Meg-01-GRIN1\(^{−/−}\) cells: small, perinuclear cytoplasmic vacuoles (black arrowheads); larger cytoplasmic vacuoles (black arrows); atypical cytoplasmic granules (yellow arrowheads); immature dense granules (blue arrowheads). Frames in i and ii mark regions enlarged in iii and iv, respectively. Scale bars are shown.
pink bars), and in unmodified Meg-01 cells after transient knockdown of GRIN1 using esiGRIN1 (Fig. 6B, white bars), or pharmacologic NMDAR inhibition using memantine (Supplementary Fig. S11, available in the online version). Both esiGRIN1 and memantine recreated the pattern of changes seen in Meg-01-GRIN1−/− cells, although esiGRIN1 effects were weaker (Fig. 6B, white bars), and memantine did not change CALN1 expression (Supplementary Fig. S11, available in the online version). The effects of memantine argued that altered expression of Ca²⁺ channels and pumps detected in Meg-01-GRIN1−/− cells developed due to reduced Ca²⁺ influx; however, lower expression of CALN1 may have been secondary to a longer term deregulation in intracellular Ca²⁺ handling induced by GRIN1 deletion. Collectively, our data highlight significant disturbance in the Ca²⁺ regulatory genes in Meg-01-GRIN1−/− cells, which underscores the important role of NMDAR in Ca²⁺ homeostasis in the parent cell line.
Transcriptomic Features of Increased Erythroid Differentiation in Meg-01-GRIN1−/− Cells

Transcriptome analysis provided further valuable insights into the state of differentiation in Meg-01-GRIN1−/− cells. The highest expressed transcription factors were HEY1, ZEB1 and JUN (Table 2). Transcripts of Krueppel-like factors, including KLF1, a master regulator of erythropoiesis were also increased (Table 2). In contrast, transcription factors favoring megakaryopoiesis (in particular RUNX1, FLI1, ERG and MEIS1) were reduced (Table 2). This pattern aligns with reduced megakaryocytic and increased erythroid differentiation we observed in Meg-01-GRIN1−/− cells. In keeping with the levels of transcriptional regulators, transcripts of molecules associated with megakaryocytic differentiation (e.g. platelet-associated glycoproteins) were lower, but erythroid transcripts (e.g. embryonic haemoglobins and red cell
membrane proteins) were higher in Meg-01-GRIN1/C0/C0 cells compared with unmodified Meg-01 cells (►Supplementary Table S3, available in the online version).

Selected changes in transcription factors were confirmed using RT-qPCR (►Fig. 6C, pink bars). Effects of esiGRIN1 were also tested (►Fig. 6C, white bars), which showed that...
similar to Meg-01-GRIN1−/− cells, transcript levels of HEY1 and KLF3 were increased 3 days after transfections, and FLI1 transcripts were reduced. However, RUNX1 and ERG levels were higher, and JUN levels increased only slightly upon esiGRIN1 treatment, suggesting that changes in Meg-01-GRIN1−/− cells were time dependent (►Fig. 6C). The small upregulation of JUN in short-term experiments with esiGRIN1 appeared consistent with the known, secondary role of JUN after ER stress that we saw in Meg-01-GRIN1−/− cells.

The dominant expression of the erythroid transcription factor, KLF3 persisted in Meg-01-GRIN1−/− cells after culture with PMA (►Supplementary Fig. S12A, available in the online version). Megakaryocytic differentiation also increased, as it is known to occur in unmodified Meg-01 cells in the presence of PMA (►Supplementary Figs S12B, S13 and S14, available in the online version). PKC is known to impact Ca2+ signaling and interact with Ca2+ pathways to induce its full transcriptional effect. Thus, PMA effects appeared in keeping with a cross-talk between PKC and Ca2+ pathways during megakaryocytic-erythroid differentiation.

**Table 1** Differential expression of the Ca2+ toolkit molecules in Meg-01-GRIN1−/− cells compared with unmodified Meg-01 cells determined from Clariom S microarrays

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Calcium toolkit molecule</th>
<th>Fold change</th>
<th>p-Value</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCIN</td>
<td>Scinderin</td>
<td>4.85</td>
<td>1.64E-07</td>
<td>2.41E-05</td>
</tr>
<tr>
<td>CALB1</td>
<td>Calbindin 1</td>
<td>3.73</td>
<td>5.26E-08</td>
<td>1.05E-05</td>
</tr>
<tr>
<td>MCOLN3</td>
<td>Transient receptor potential mucolipin 3 channel</td>
<td>2.64</td>
<td>0.0003</td>
<td>0.0056</td>
</tr>
<tr>
<td>ATP2B4</td>
<td>Plasma membrane Ca2+ ATPase 4</td>
<td>−1.78</td>
<td>0.0004</td>
<td>0.0066</td>
</tr>
<tr>
<td>ATP2A3</td>
<td>Sarco-/endoplasmic reticulum Ca2+ ATPase 3</td>
<td>−1.99</td>
<td>1.04E-07</td>
<td>1.71E-05</td>
</tr>
<tr>
<td>CACNA1A</td>
<td>Voltage-gated calcium channel 2.1</td>
<td>−2.26</td>
<td>0.0007</td>
<td>0.0107</td>
</tr>
<tr>
<td>SLC24A3</td>
<td>K+-dependent Na+/Ca2+ exchanger 3</td>
<td>−2.97</td>
<td>1.42E-08</td>
<td>4.29E-06</td>
</tr>
<tr>
<td>TRPC6</td>
<td>Transient receptor potential channel 6</td>
<td>−6.61</td>
<td>9.69E-10</td>
<td>6.31E-07</td>
</tr>
<tr>
<td>CALN1</td>
<td>Calneuron 1</td>
<td>−8.38</td>
<td>1.24E-10</td>
<td>1.84E-07</td>
</tr>
</tbody>
</table>

Abbreviation: FDR, false discovery rate.

**Table 2** Differential expression of transcriptional regulators in Meg-01-GRIN1−/− cells compared with unmodified Meg-01 cells determined from Clariom S microarrays

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Transcription factor/regulator</th>
<th>Fold change</th>
<th>p-Value</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEY1</td>
<td>Hes related family basic helix-loop-helix transcription factor with YRPW motif 1</td>
<td>16.65</td>
<td>1.36E-11</td>
<td>4.84E-08</td>
</tr>
<tr>
<td>ZEB1</td>
<td>Zinc finger E-box binding homebox 1</td>
<td>16.42</td>
<td>5.24E-12</td>
<td>4.84E-08</td>
</tr>
<tr>
<td>JUN</td>
<td>Jun proto-oncogene</td>
<td>3.47</td>
<td>5.21E-06</td>
<td>3.00E-04</td>
</tr>
<tr>
<td>ID1</td>
<td>Inhibitor of DNA binding 1</td>
<td>3.16</td>
<td>7.88E-09</td>
<td>3.02E-06</td>
</tr>
<tr>
<td>ID3</td>
<td>Inhibitor of DNA binding 2</td>
<td>3.03</td>
<td>2.29E-05</td>
<td>9.00E-04</td>
</tr>
<tr>
<td>EGR1</td>
<td>Early growth response 1</td>
<td>2.67</td>
<td>4.80E-07</td>
<td>5.32E-05</td>
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<tr>
<td>HEY2</td>
<td>Hes related family BHLH transcription factor with YRPW motif 2</td>
<td>2.27</td>
<td>7.69E-07</td>
<td>7.63E-05</td>
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<tr>
<td>PAX6</td>
<td>Paired box 6</td>
<td>2.28</td>
<td>0.0141</td>
<td>0.0926</td>
</tr>
<tr>
<td>KLF3</td>
<td>Kruppel like factor 3</td>
<td>1.93</td>
<td>4.24E-05</td>
<td>1.40E-03</td>
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<tr>
<td>KLF6</td>
<td>Kruppel like factor 6</td>
<td>1.72</td>
<td>2.47E-05</td>
<td>9.00E-04</td>
</tr>
<tr>
<td>CREB3RF</td>
<td>CREB3 regulatory factor</td>
<td>1.68</td>
<td>7.96E-05</td>
<td>0.0023</td>
</tr>
<tr>
<td>NFATC1</td>
<td>Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1</td>
<td>1.7</td>
<td>6.76E-05</td>
<td>0.0020</td>
</tr>
<tr>
<td>KLF1</td>
<td>Kruppel like factor 1</td>
<td>1.57</td>
<td>9.12E-05</td>
<td>2.50E-03</td>
</tr>
<tr>
<td>KLF10</td>
<td>Kruppel like factor 10</td>
<td>1.56</td>
<td>1.00E-04</td>
<td>2.80E-03</td>
</tr>
<tr>
<td>ERG</td>
<td>ETS transcription factor ERG</td>
<td>−1.67</td>
<td>2.70E-06</td>
<td>2.00E-04</td>
</tr>
<tr>
<td>FLI1</td>
<td>FLI-1 proto-oncogene</td>
<td>−1.83</td>
<td>9.98E-07</td>
<td>9.27E-05</td>
</tr>
<tr>
<td>RUNX1</td>
<td>Runt related transcription factor 1</td>
<td>−1.85</td>
<td>1.57E-06</td>
<td>1.00E-04</td>
</tr>
<tr>
<td>MEIS1</td>
<td>Meis homeobox 1</td>
<td>−1.98</td>
<td>3.43E-06</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

Abbreviation: FDR, false discovery rate.
Memantine Increases Cytarabine-Mediated Cell Killing of Meg-01 Cells

The increased levels of cytoplasmic Ca\(^{2+}\), ER stress response, autophagy induction, and higher LDH release suggested that Meg-01-GRIN1\(^{-/-}\) cells had a lower threshold for cell death. We hypothesized this pro-death state would make cells more vulnerable to additional toxic insults, and tested if NMDAR inhibition would increase cell killing by cytarabine (currently, a cornerstone of antileukemia treatment). Memantine was employed in these experiments, as it is an approved drug used in neurological patients. Meg-01 cells were pretreated with 100 µM memantine for 1 hour, followed by 3 days with 0.1 µM (low dose) cytarabine; effects on cell numbers were tested using an MTT assay (Fig. 7). Even this brief exposure to memantine resulted in 3.1-times more cell killing compared with cytarabine alone (Fig. 7).

Discussion

This study provides the first evidence that NMDARs comprise an integral component of the Ca\(^{2+}\) toolkit in Meg-01 cells, with NMDAR function required to prevent cell stress and support megakaryocytic over erythroid differentiation.

CRISPR-Cas9-mediated NMDAR hypofunction caused marked changes in Ca\(^{2+}\) homeostasis in Meg-01 cells, resulting in atypical differentiation, basal ER stress and cell death. Meg-01-GRIN1\(^{-/-}\) cells accumulated lysosome-related organelles, suggesting abnormalities in membrane trafficking. Resting cytosolic Ca\(^{2+}\) levels were higher in Meg-01-GRIN1\(^{-/-}\) cells, but ER Ca\(^{2+}\) release and SOCE were lower after activation. GRIN1 deletion affected the transcription of the following Ca\(^{2+}\) toolkit genes: TRPC6, CACNA1A, MCOLN3, ATP2A3, ATP2B4, SLC24A3, CALN1, CALB1 and SCIN; most of which also changed in response to memantine. Increased levels of JUN, DDIT3, ATF4, PPP1R15A and XBP1 spliced transcripts indicated ER stress, and a shift in megakaryocytic-erythroid transcription factors explained features of erythroid differentiation. Finally, pharmacologic NMDAR inhibition using memantine increased cell killing by cytarabine.

Despite increasing evidence that pharmacologic NMDAR inhibitors disturb megakaryocytic maturation,\(^{8,10-12}\) NMDAR roles in MKs remained speculative. Our work adds solid evidence that NMDARs operate as important components of the Ca\(^{2+}\) toolkit in Meg-01 cells; hence, further analysis in normal and malignant MKs may uncover meaningful NMDAR roles. Similar to the NMDAR, several other components of the Ca\(^{2+}\) toolkit found differentially expressed in Meg-01-GRIN1\(^{-/-}\) cells (e.g. TRPC6, CACNA1A, MCOLN3, CALN1 and CALB1) are best known for their neuronal functions. Our results suggest that these molecules have previously unappreciated roles in megakaryocytic and erythroid differentiation.

Recent work provided computational support for the NMDAR involvement in human erythropoiesis. The GRIN3B gene, encoding the GluN3B subunit of NMDAR, has been linked with signaling through ErbB4 (epidermal growth factor receptor Erb-B2 receptor tyrosine kinase 4), which balances erythropoiesis against other myeloid lineages (in particular megakaryopoiesis) in multiple in vivo and ex vivo models.\(^{31}\) Our results are consistent with these data. Using Meg-01 cell line that carries dual, megakaryocytic-erythroid differentiation potential, we provide the first experimental evidence that NMDARs and intracellular Ca\(^{2+}\) homeostasis balance megakaryocytic and erythroid cell fates.

Differentiation of megakaryocytic and erythroid lineages are closely connected and regulated by a set of transcription factors that include RUNX1, ERG, FLI1, GATA and KLF family members.\(^{32}\) RUNX1 increases megakaryocytic and represses erythroid differentiation by antagonising the erythroid master regulator KLF1.\(^{33}\) The converse is also true, as KLF1 inhibits megakaryopoiesis.\(^{34}\) We found that KLF1, KLF3, KLF6 and KLF10 were expressed to higher levels in Meg-01-GRIN1\(^{-/-}\) cells, GATA1 and GATA2 were unchanged, and RUNX1, FLI1, ERG and MEIS1 were expressed to lower levels compared with control Meg-01 cells, consistent with the phenotypic bias toward erythropoiesis detected in Meg-01-GRIN1\(^{-/-}\) cells. Based on CD41 and CD235a expression, Meg-01-GRIN1\(^{-/-}\) cells were heterogeneous (including erythroid, megakaryocytic, and double-positive cells; Supplementary Fig. S12Bi, available in the online version) that may explain the discrepancy between their higher ploidy and lower CD41/CD61 expression for the overall population.
We do not know what mechanisms were responsible for transcription factor alterations in \( \text{Meg-01-} \cdot \text{GRIN1}^{-/-} \) cells. Microarray analysis showed NFATC1 transcript levels were higher ( \( \cdot \text{Table 2} \) ), but there was no transcriptional signature of enhanced NFAT activity (data not shown). Other pathways through which NMDARs regulate transcription include calcium / calmodulin-dependent protein kinases (CaMK). Expression of CAMKIV was reduced in \( \text{Meg-01-} \cdot \text{GRIN1}^{-/-} \) cells (FC -1.91; \( \cdot \text{Supplementary Microarray Excel Data File} \), available in the online version), raising the possibility that this pathway is functional in MKs, but functional validation is required.

This is not the first study to report that a membranous Ca\(^{2+}\) channel affects the balance of megakaryocytic-erythroid differentiation. Overexpression of TRPA1 (ankyrin 1; a TRPC family channel that contributes to SOCE in \( \text{Meg-01} \) cells\(^\text{35}\)) was shown to suppress erythroid but enhance megakaryocytic differentiation in K-562 and HEL cell lines; however, the mechanism of TRPA1 action was not examined in that study.\(^\text{32}\) Our findings are in agreement, showing the reciprocal effect, as reduced NMDAR function enhances erythroid differentiation. Therefore, similar to TRPA1, normal NMDAR activity favors megakaryocytic differentiation. Previous authors suggested therapeutic opportunities involving modulation of TRPA1 in disorders associated with anemia and thrombocytopenia.\(^\text{37}\) Our results suggest that NMDAR (and possibly other components of the Ca\(^{2+}\) toolkit) may provide similar opportunities. As a proof of principle, we show that memantine, a drug used to treat neurological patients, increases \( \text{Meg-01} \) cell killing by cytarabine, suggesting a drug combination for further testing in primary cells.

Based on what is known about normal functions of the Ca\(^{2+}\) toolkit genes, we propose the following changes compensated for the NMDAR hypofunction in \( \text{Meg-01} \) cells ( \( \cdot \text{Fig. 8} \) ). Reduced expression of TRPC6 and CANA1A restricted Ca\(^{2+}\) influx across the plasma membrane. Lower levels of \( \text{ATP2A3} \) and \( \text{CALN1} \) transcripts reduced ER Ca\(^{2+}\) stores,\(^\text{38}\) implying that overall \( \text{Meg-01-} \cdot \text{GRIN1}^{-/-} \) cells experienced a form of Ca\(^{2+}\) “starvation.” However, resting Ca\(^{2+}\) levels were higher in \( \text{Meg-01-} \cdot \text{GRIN1}^{-/-} \) cells, suggesting an alternative mechanism to increase cytosolic Ca\(^{2+}\) levels. Higher expression of \( \text{MCOLN3} \) may contribute Ca\(^{2+}\) efflux from immature lysosomal organelles that accumulated in \( \text{Meg-01-} \cdot \text{GRIN1}^{-/-} \) cells.\(^\text{39}\) In support, glutamate recruits lysosomal Ca\(^{2+}\) stores in neurons and glia.\(^\text{40}\) In addition, lower levels of \( \text{ATP2A3} \), \( \text{ATP2B4} \) and \( \text{SLC2A4A3} \) transcripts may reduce exclusion of cytosolic Ca\(^{2+}\) into the ER and the extracellular compartment, respectively. Increased cytosolic Ca\(^{2+}\) levels observed in \( \text{Meg-01-} \cdot \text{GRIN1}^{-/-} \) cells were likely required to preserve signaling, but also implied a stressed, pro-apoptotic cell state. It is possible that higher expression of \( \text{CALB1} \) rescued \( \text{Meg-01-} \cdot \text{GRIN1}^{-/-} \) cells from apoptosis,\(^\text{41,42}\) and \( \text{SCIN} \) contributed to differentiation.\(^\text{43}\) Overall, the range of transcriptomic changes we found in \( \text{Meg-01-} \cdot \text{GRIN1}^{-/-} \) cells indicate that NMDARs work together with other Ca\(^{2+}\) channels, such as TRPC6, Cav2.1 and TRPML3 located in the plasma and lysosomal membranes respectively, to support Ca\(^{2+}\) signaling in \( \text{Meg-01} \) cells ( \( \cdot \text{Fig. 8} \) ).

The increase in lysosomal organelles and associated \( \text{MCOLN3} \) upregulation in \( \text{Meg-01-} \cdot \text{GRIN1}^{-/-} \) cells are intriguing. Lysosomes and platelet dense granules share

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**Fig. 8** Schematic summary of the changes caused by \( \text{GRIN1} \) deletion in \( \text{Meg-01} \) cells. \( \text{GRIN1} \) deletion in \( \text{Meg-01} \) cells induced NMDAR hypofunction that led to the significant remodeling of the Ca\(^{2+}\) regulatory network associated with atypical differentiation showing erythroid features. Reduced expression of \( \text{TRPC6} \) and \( \text{CAGN1A1} \) had the potential to reduce Ca\(^{2+}\) entry from the extracellular environment. Lower levels of \( \text{ATP2A3} \) and \( \text{CALN1} \) could reduce ER Ca\(^{2+}\) stores, leading to ER stress. Lower levels of \( \text{ATP2A3} \), \( \text{ATP2B4} \) and \( \text{SLC2A4A3} \), and higher levels of \( \text{MCOLN3} \) had the potential to increase cytoplasmic Ca\(^{2+}\) levels, buffered by increased expression of \( \text{CALB1} \). Higher levels of \( \text{SCIN} \) could assist differentiation. \( \text{GRIN1} \) deletion also altered expression of selected hematopoietic transcription factors, demonstrating a decrease in megakaryocytic but an increase in erythroid regulators, in keeping with increased erythroid differentiation of \( \text{Meg-01-} \cdot \text{GRIN1}^{-/-} \) cells. The subcellular sites of expression indicated on the schematic apply to the proteins encoded by the genes shown. ER, endoplasmic reticulum; Ery, erythroid; MK, megakaryocytic; PM, plasma membrane; TF, transcription factor.
biogenesis and secretion mechanisms. TRPML3 is expressed in early lysosomes where it regulates Ca\(^{2+}\) efflux and related membrane trafficking. Our evidence for increased lysosomal and ER accumulation in Meg-01-GRIN1\(^{-/-}\) cells corroborates previous findings by our group and others of increased cytoplasmic vacuolation arising in megakaryocytic cells in the presence of NMDAR inhibitors. To our knowledge, this is the first study to report potential NMDAR contribution to lysosomal biogenesis in megakaryocytic cells.

Our study has several limitations. The phenotype of Meg-01-GRIN1\(^{-/-}\) cells may be contributed by other mechanisms that occurred downstream or independently of reduced Ca\(^{2+}\) influx through the NMDAR. We did not dissect the roles of individual molecular changes we found, and cannot depict NMDAR pathways in megakaryocytic cells. Our results raise the possibility that NMDARs regulate Ca\(^{2+}\) storage/release from the lysosomal organelles, but no measurements of lysosomal Ca\(^{2+}\) were performed. Our conclusions are derived from studies in one, one cell line. Meg-01 cells were chosen based on our previous findings showing that they are better suited than K-562 and Set-2 cells to study NMDAR function. However, more detailed characterization of the Ca\(^{2+}\) toolkit is required using other models of megakaryocytic and erythroid differentiation. Examination of the Ca\(^{2+}\) toolkit may also be rewarding in myeloproliferative neoplasms, as CALR gene mutations are predicted to reduce Ca\(^{2+}\) binding in the ER, which could trigger ER stress, recently revealed in patient cells.

In summary, CRISPR-Cas9-mediated GRIN1 deletion disturbed Ca\(^{2+}\) homeostasis in Meg-01 cells and shifted differentiation toward the erythroid lineage. The downstream effects of the reduced NMDAR function involved changes in ER Ca\(^{2+}\) release, lysosome-related organelles, Ca\(^{2+}\) toolkit molecules, and megakaryocytic-erythroid transcription factors. Our findings strengthen the evidence for the importance of NMDAR and intracellular Ca\(^{2+}\) homeostasis in megakaryocytic cell function, including balancing of ER stress and megakaryocytic-erythroid differentiation.

**What is known about this topic?**

- Intracellular signaling by calcium ions (Ca\(^{2+}\)) supports differentiation of normal megakaryocytes.
- N-methyl-D-aspartate receptors (NMDARs) appear to operate as Ca\(^{2+}\) channels in megakaryocytic cells.

**What does this paper add?**

- We provide the first genetic evidence that NMDARs are an integral component of the Ca\(^{2+}\) toolkit in megakaryocytic cells.
- NMDARs favor megakaryocytic over erythroid differentiation, support dense granule biogenesis, and contribute to Ca\(^{2+}\) homeostasis in the endoplasmic reticulum.
- Modulation of Ca\(^{2+}\) homeostasis offers potential to inhibit proliferation of malignant megakaryocytes and increase erythroid differentiation.

**Authors’ Contributions**

M.L.K.Z designed the study. J.I.H., T.N.G., M.C., Y.N.S.N., L.L. and M.L.K.Z generated and analysed data. N.C., C.B., R.C.P., D.C.S., S.K.B. and M.L.K.Z provided methodology advice, supervision and mentorship. J.I.H., T.N.G. and M.L.K.Z wrote the manuscript. All authors edited the manuscript and approved the final version for submission.

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**Conflict of Interest**

None declared.

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