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CALR frameshift mutations in MPN patient-derived iPSCs accelerate maturation of megakaryocytes

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SUMMARY

Calreticulin (CALR) mutations are driver mutations in myeloproliferative neoplasms (MPNs), leading to activation of the thrombopoietin receptor and causing abnormal megakaryopoiesis. Here, we generated patient-derived *CALR*ins5- or *CALR*del52-positive induced pluripotent stem cells (iPSCs) to establish an MPN disease model for molecular and mechanistic studies. We demonstrated myeloperoxidase deficiency in granulocytic cells derived from homozygous *CALR* mutant iPSCs, rescued by repairing the mutation using CRISPR/Cas9. iPSC-derived megakaryocytes showed characteristics of primary megakaryocytes such as formation of demarcation membrane system and cytoplasmic pro-platelet protrusions. Importantly, *CALR* mutations led to enhanced megakaryopoiesis and accelerated megakaryocytic development in a thrombopoietin-independent manner. Mechanistically, our study identified differentially regulated pathways in mutated versus unmutated megakaryocytes, such as hypoxia signaling, which represents a potential target for therapeutic intervention. Altogether, we demonstrate key aspects of mutated *CALR*-driven pathogenesis dependent on its zygosity, and found novel therapeutic targets, making our model a valuable tool for clinical drug screening in MPNs.

INTRODUCTION

Myeloproliferative neoplasms (MPNs) are a group of clonal hematopoietic disorders including polycythemia vera, essential thrombocythemia (ET), and primary myelofibrosis (PMF), characterized by their excessive increase of granulomonocytic cells, erythroid cells, and/or platelets as well as different degrees of splenomegaly and bone marrow (BM) fibrosis (Campo et al., 2011). Somatic calreticulin (CALR) mutations were discovered in patients with ET and PMF and have been shown to be mutually exclusive with Janus kinase 2 (JAK2) and thrombopoietin (TPO) receptor (*MPL*) mutations (Klampfl et al., 2013; Nangalia et al., 2013). The most common types of *CALR* mutations are 52-bp deletions (del52; type 1) and 5-bp insertions (ins5; type 2), all leading to a +1 frameshift and a novel C terminus of the CALR mutant protein. Although most *CALR* mutations are heterozygous (het), homozygous (hom) mutations have been shown to be derived from a copy-neutral loss of heterozygosity of chromosome 19p (Klampfl et al., 2013; Stengel et al., 2019; Theocharides et al., 2016).

The oncogenic functions of CALR mutant proteins rely on its binding to the TPO receptor. This leads to the constitutive activation of JAK2 downstream targets such as STAT5, ERK1/2, and AKT, thus causing cellular transformation and abnormal megakaryopoiesis, a hallmark of CALR mutation-positive MPNs (Araki et al., 2016; Chachoua et al., 2016; Han et al., 2016; Kollmann et al., 2017; Marty et al., 2016). Megakaryocytes (MKs) are platelet-releasing cells, which are known to undergo different stages of maturation (Ru et al., 2015), but the impact of *CALR* mutations on megakaryocytic differentiation and function is still incompletely understood.

It has been reported that patients carrying hom *CALR*ins5 exhibit myeloperoxidase (MPO) deficiency as a result of a post-transcriptional mechanism, most likely due to defective CALR chaperone function (Nauseef et al., 1995; Theocharides et al., 2016). Additionally, a *CALR* knockin mouse model demonstrated that hom *CALR*del52

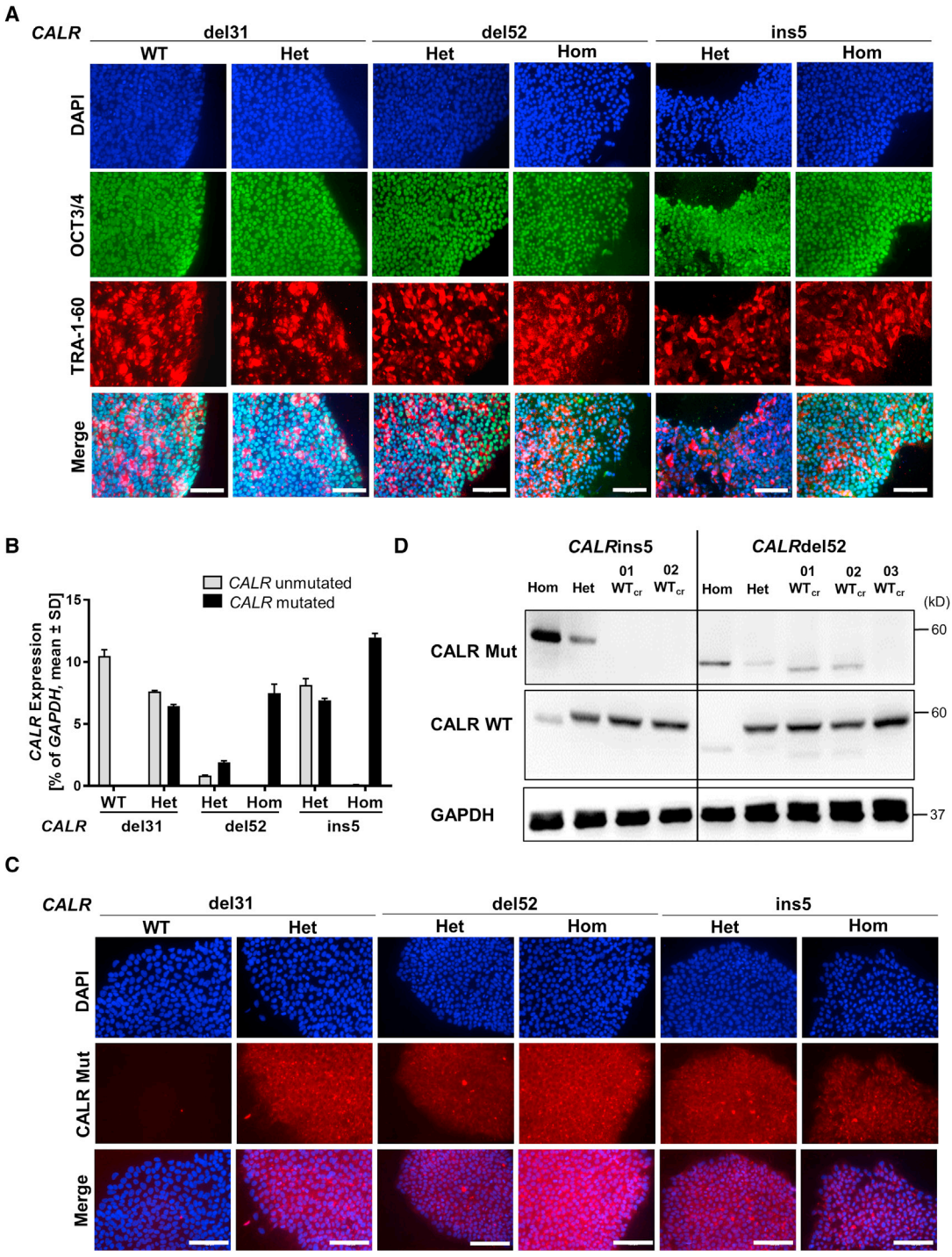

Figure 1. MPN-related iPSC clones exhibit pluripotency state and harbor *CALR* mutations

(A) Pluripotency assessment of indicated iPSC clones by immunofluorescence. Merge represents overlay of DAPI (blue), OCT3/4 (green), and TRA-1-60 (red). Scale bars, 100 μm.

(B) *CALR* mRNA expression of indicated iPSC clones was assessed by *CALR* mutant allele-specific qRT-PCR. Gene expression is depicted as percentage of *GAPDH*. Mean value ± SD of representative clones with indicated *CALR* genotypes, n = 2 independent experiments.

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expression resulted in severe thrombocytosis and myelofibrosis (MF) phenotype (Li et al., 2018). However, the pathogenetic impact of *CALR* mutant zygosity on hematopoietic and megakaryocytic differentiation has not yet been fully addressed.

Several *CALR* mutant disease models have been used in order to uncover its molecular and cellular mechanisms in the pathogenesis of MPN. *CALR* mutant overexpressing cell line models are widely used (Araki et al., 2016; Chachoua et al., 2016; Elf et al., 2016; Han et al., 2016). However, their capacity to differentiate into hematopoietic lineages is limited. MARIMO cells, the only human cell line to date harboring mutated *CALR* (Kollmann et al., 2015), was reported to carry an additional *NRAS* Q61K mutation, which is responsible for transformation independent from mutant *CALR* (Han et al., 2018). Therefore, the validity of this cell line as a model for *CALR* mutation analysis is questionable. CALR expressing mouse models were generated showing MPN-like phenotypes *in vivo* (Benlabiod et al., 2020; Li et al., 2018; Marty et al., 2016). However, the physiological and genetic differences between species might hamper the translation of human disease phenotypes. For these reasons, *CALR* mutant MPN patient samples are desirable for analyzing biological processes. However, the clonal heterogeneity of hematopoietic stem and progenitor cell (HSPC) populations and technical limitations to isolate single clones from patients present major challenges to determining the impact of *CALR* mutant zygosity on clonal composition and diversity in MPN.

Human induced pluripotent stem cells (iPSCs) are primary cell lines with self-renewal capacity and have the potential to differentiate into any cell type of the three germ layers (Takahashi et al., 2007). Patient-derived iPSCs can be further genetically engineered with CRISPR/Cas9 technology to repair or introduce disease-related mutations in the patient's genetic background. This approach provides a valuable tool to study genotype-phenotype correlations at the clonal level.

Thus, to overcome the aforementioned limitations, we generated MPN patient-specific iPSC clones carrying hom or het *CALR* mutations or its isogenic unmutated counterpart. *CALR* mutations accelerated megakaryopoiesis independent of TPO, especially in hom mutant clones, and repair of the mutation using CRISPR/Cas9 reversed the effect. RNA sequencing (RNA-seq) of *CALR*-mutated versus non-mutated MKs demonstrated increase in hypoxia signaling and leptin expression, providing a better understanding of disease biology in ET and PMF and potential novel therapeutic targets.

RESULTS

Generation of MPN patient-derived iPSC clones and repair of hom *CALR*ins5 and *CALR*del52 mutations

iPSCs were established by reprogramming of peripheral blood mononuclear cells (PBMCs) from three MPN patients carrying *CALR*del52, *CALR*ins5, or *CALR*del31 mutations (Table S1) and two healthy donors (HDs). We obtained hom and het CALRdel52 and CALRins5 clones derived from a PMF patient and a post ET-MF patient, respectively. Additionally, het and wild-type (WT) clones were generated from a PMF patient carrying a *CALR*del31 mutation. Pluripotency of iPSCs was confirmed by positive staining for OCT3/4 and TRA-1-60 pluripotency markers (Figure 1A). Normal karyotype of MPN patient-specific iPSC clones was shown by GTG banding (Figure S1A). Expression of mutant *CALR* was confirmed by *CALR* allele-specific qRT-PCR and immunofluorescence (Figures 1B and 1C).

Reprogramming of *CALR*ins5 and *CALR*del52 PBMCs only resulted in hom and het clones but not *CALR* unmutated clones. Thus, *CALR*ins5 and *CALR*del52 mutations were repaired by CRISPR/Cas9 editing to obtain unmutated *CALR* iPSCs. Specific guide RNAs and CALR WT donor templates with homology arms were designed (Tables S2 and S3). Successful correction of *CALR* mutation in hom clones was confirmed by PCR and subsequent Sanger sequencing (Figures S1B and S1C). No off-target effects introduced by CRISPR/Cas9 were found (Figures S1D and S1E). Loss of CALR mutant protein was verified by western blotting in the CRISPR-engineered WT (WT_{cr}) clones (Figure 1D). However, clones 01 WT_{cr} and 02 WT_{cr} of the *CALR*del52 CRISPR approach showed unspecific bands when incubating with CALR-mutated antibody. To preclude that truncated versions of CALR protein resulted after the CRISPR repair, we performed standard amplification of the respective genomic region of the *CALR* gene, Sanger sequencing, and next-generation sequencing (NGS), whereby the absence of mutated genomic *CALR* sequence was confirmed (Table S4).

Using a defined NGS panel of MPN target genes (Kirschner et al., 2018), besides the *CALR* mutations no other clinically relevant MPN-related mutations were found in the generated patient-specific and HD control iPSC clones (Table S4).

(C) Mutant CALR expression (red) was confirmed by immunofluorescence staining with a CALR mutant specific antibody. Unmutated CALR iPSCs served as negative control. Nuclei were stained with DAPI (blue). Scale bars, 100 μm.

(D) Representative western blot analysis of CALR WT and CALR mutant (Mut) protein in iPSC clones after CRISPR repair of hom *CALR*ins5 and hom *CALR*del52 mutations. CALR WT protein was assessed on the same membrane as CALR mutant without stripping of the CALR Mut antibody explaining residual CALR mutant bands. GAPDH was used as loading control.

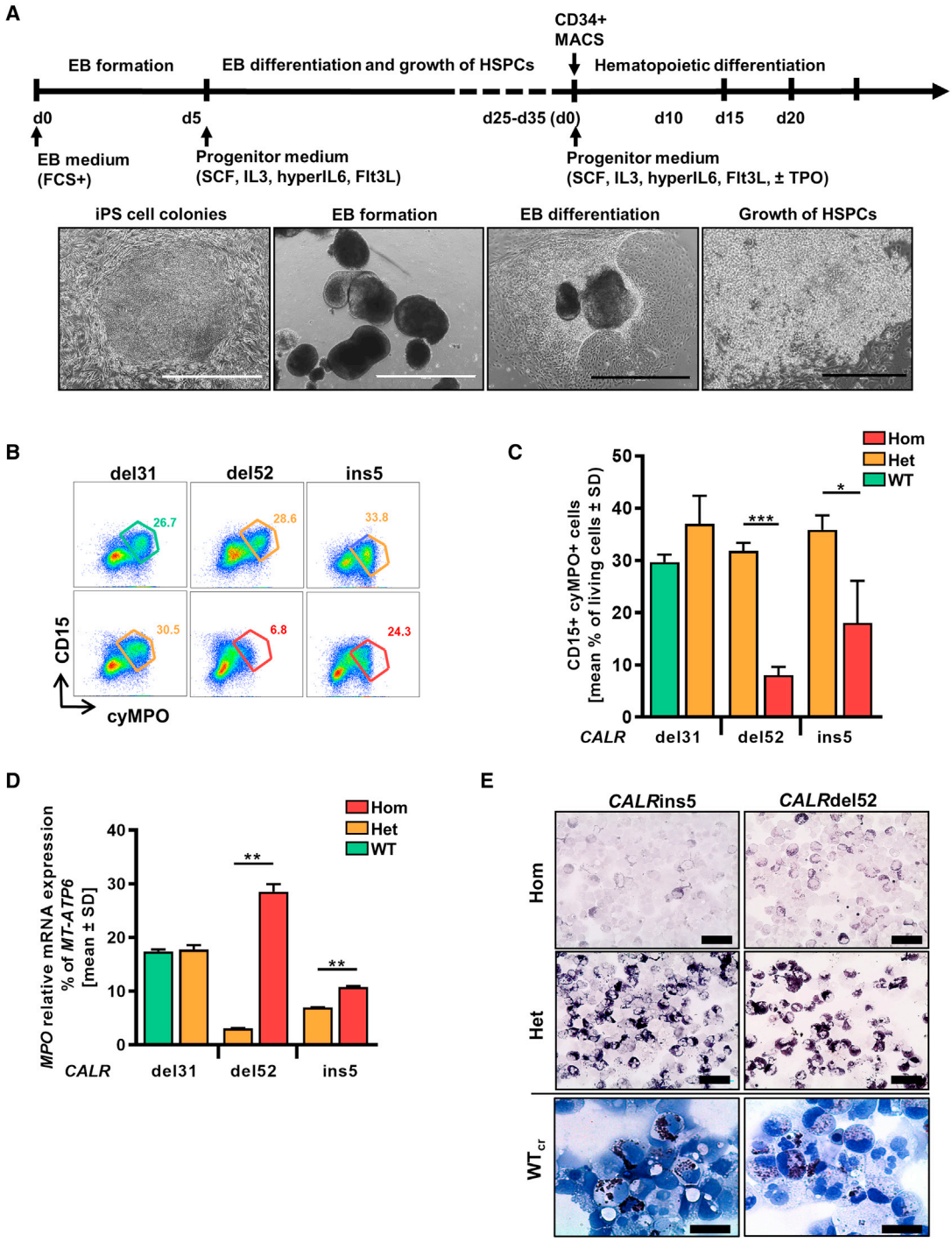

Figure 2. Restoration of MPO activity in MPN-specific iPSCs

(A) Schematic representation of an "EB-based" differentiation protocol of iPSCs toward hematopoietic progenitors. Representative images of the differentiation procedure from EB formation to HSPC production are shown. Typical morphology of iPSC colonies on day 0, embryoid bodies on day 5, mesoderm commitment and differentiated hemogenic endothelial layers on day 8, and HSPC production on day 25. Scale bars, 1,000 μm (white) and 400 μm (black).

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MPO deficiency is detected uniquely in hom *CALR*-mutated iPSC clones and is restored in repaired *CALR* clones

Patient-derived iPSCs harboring the disease-driving mutation(s) allow disease modeling in the culture dish. Hom *CALR*ins5 lead to MPO deficiency in patients (Theocharides et al., 2016), and we recapitulated this feature *in vitro* by differentiating our iPSCs toward hematopoietic cells in a modified embryoid body (EB)-based protocol (Figure 2A) (Kovarova and Koller, 2012). We observed MPO deficiency in iPSC-derived $CD15^+$ cells harboring hom *CALR*ins5 or *CALR*del52 mutations. A significantly smaller $CD15^+cyMPO^+$ (cytoplasmic MPO) population was found in cells carrying hom *CALR*del52 and *CALR*ins5 mutations (Figures 2B and 2C). Meanwhile, relative *MPO* mRNA expression in hom *CALR* cells was upregulated (Figure 2D), confirming the post-transcriptional defect (Theocharides et al., 2016). Furthermore, a severe reduction of MPO activity in the hom *CALR*-mutated cells was confirmed by cytochemical staining (Figure 2E). Most importantly, MPO activity was rescued by the correction of hom *CALR* mutations as shown for the WT_{cr} clones, proving that the hom CALR mutation is responsible for this defect.

iPSCs harboring *CALR* mutations give rise to multiple myeloid cell lineages with stronger proliferative capacity

Next, we aimed at obtaining further mechanistical insights into CALR mutations affecting hematopoietic cells. Therefore, iPSCs that carry *CALR*del52 or *CALR*ins5 were differentiated toward the myeloid lineage with a modified differentiation protocol designated here as "spin-EB" differentiation (Figure 3A) (Liu et al., 2015). From day 8 onward, hematopoietic cells were released from the EBs into suspension, with an increase of released cells until day 14, as shown in representative images of the differentiation. Hom *CALR*ins5 and *CALR*del52 mutant clones as well as cells bearing a het *CALR*del52 mutation produced a significantly higher number of suspension cells compared with WT_{cr} cells on day 14 (Figure 3B). These data demonstrate that *CALR*ins5 and *CALR*del52 mutations enhance hematopoietic proliferation.

Flow cytometry analysis of suspension cells on days 10, 12, and 14 confirmed generation of $CD34^+CD45^+$ HSPCs (Figures S2A–S2C). To study the myeloid progenitor potential of iPSC-derived HSPCs, we performed a colony-forming unit (CFU) assay using day-14 $CD34^+$ cells, previously cultured in the presence of TPO. The het mutant *CALR*ins5 $CD34^+$ cells produced more colonies compared with WT_{cr} cells (Figure S2D). The same trend was observed for the hom *CALR*ins5 and *CALR*del52 cells compared with control ($p = 0.0898$ and $p = 0.0851$, respectively). To examine whether *CALR* mutation impacts on the expansion of $CD34^+$ cells, we evaluated the expression of the proliferative marker *Ki67* in qRT-PCR (Figure S2E). We found that HSPCs harboring a het *CALR*ins5 mutation showed higher *Ki67* expression (although not statistically significant, $p = 0.1411$) compared with WT_{cr} HSPCs. Together, these data confirmed a stronger amplification of cells in the *CALR*-mutated background, as described above. Identities of CFU-E (erythrocytic cells), CFU-M (macrophages), CFU-G (granulocytes), and CFU-GM (granulocytes/macrophages) were confirmed by morphology and Quik-Diff staining (Figure 3C). HSPCs from all analyzed clones gave rise to CFU-E, CFU-M, CFU-G, and CFU-GM colonies. While *CALR*del52 WT_{cr} HSPCs differentiated into the highest number of CFU-M, they showed less CFU-E. An increased number of erythrocytic colonies were found in het *CALR*ins5 clones.

In summary, CALR mutants accelerate hematopoietic cell proliferation, and *CALR*-mutated iPSCs show constant production of HSPCs.

CALR-mutated iPSCs exhibit enhanced TPO-independent megakaryopoiesis and accelerated maturation

Aberrant megakaryopoiesis is the major hallmark of *CALR*-mutated MPN patients as well as in established mouse models. Therefore, we studied the impact of *CALR* mutant zygosity on megakaryopoiesis in our patient-specific iPSC model in our "spin-EB" differentiation (Figure 3A) (Liu et al., 2015). We found that iPSCs carrying hom or het *CALR*ins5 and *CALR*del52 mutation gave rise to significantly more $CD41^+CD42b^+$ mature MKs compared with their WT_{cr} counterparts (Figure 4A). Moreover, no difference in

(B) Flow cytometry gating strategy to identify $cyMPO^+$ neutrophils on day 15 of "EB-based" differentiation. Exemplarily shown for iPSC-derived hematopoietic cells carrying het (orange) or hom (red) *CALR* mutation or unmutated *CALR* (green). Numbers represent frequencies of $CD15^+cyMPO^+$ populations in percentage of living single cells.

(C) Flow cytometry data for cell surface CD15 and intracellular MPO expression of iPSC-derived HSPCs on day 15 of "EB-based" differentiation. Data are shown as mean values ± SD; $^*p < 0.05$, $^{***}p < 0.001$, n = 3 independent experiments.

(D) Relative *MPO* mRNA expression was confirmed by qRT-PCR. Gene expression is depicted as percentage of *MT-ATP6*. Mean value ± SD of representative clones carrying indicated *CALR* genotypes are shown; $^{**}p < 0.01$, n = 2 independent experiments.

(E) MPO functional activity was assessed by cytochemical staining. Representative images of hematopoietic cells harboring indicated *CALR* genotypes are shown. The intensity of the black-brown dye indicates the peroxidase activity. Scale bars, 50 μm.

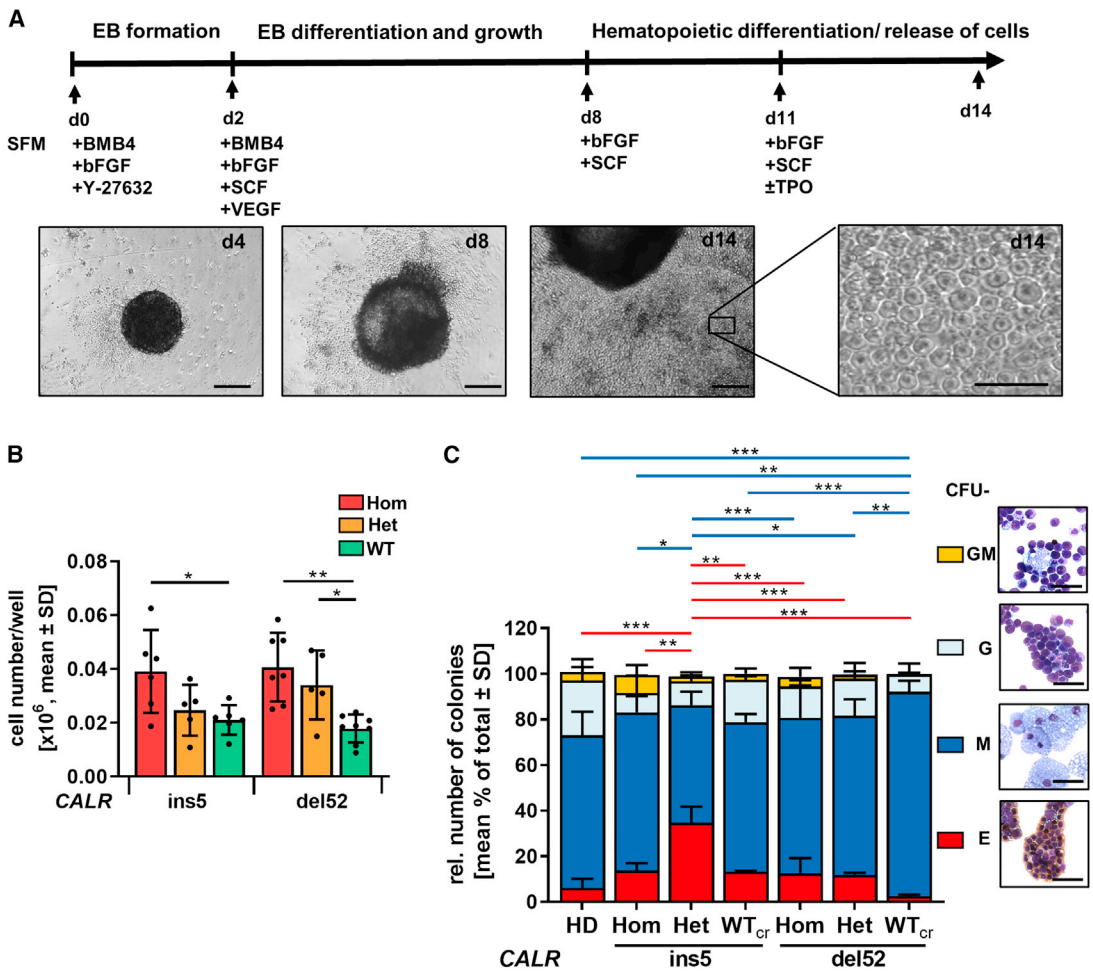


Figure 3. Hematopoietic differentiation potential of iPSC-derived CD34$^+$ cells

(A) Schematic representation of a "spin-EB" protocol to differentiate iPSCs toward hematopoietic stem cells and MKs. Cell culture medium was continuously supplemented with cytokines as indicated. On day 14, suspension cells were harvested for further analysis. Representative cell culture images of indicated days are shown. Scale bars, 50 μm.

(B) Cell number on day 14 of "spin-EB" differentiation calculated for harvested suspension cells/well of 96-well plate. Each data point represents an independent experiment for indicated *CALR* mutation and genotype shown as mean values ± SD; *p > 0.05, **p > 0.01, n = 5–8 independent differentiation experiments.

(C) Colony-forming unit (CFU) assay of purified iPSC-derived CD34$^+$ cells on day 14 of "spin-EB" differentiation. Red and blue lines refer to significant differences in CFU-E and CFU-M, respectively. Data are shown as mean values ± SD; *p > 0.05, **p > 0.01, ***p > 0.001, n = 3 independent experiments per genotype. Morphological appearance of different colony types was assessed by cytospin preparation and Diff-Quik staining. Scale bars, 50 μm.

MK numbers obtained from HD or WT_{cr} iPSCs was observed, demonstrating that the enhanced megakaryopoiesis is due to the *CALR* mutation. Mature megakaryocytes with typical multi-lobular nuclei and slight basophilic cytoplasm were detected in cytospins generated on day 14 of differentiation (Figure 4B). Of note, MKs carrying hom CALR mutation presented variable sizes, indicative of rapid maturation of aberrant MKs.

Mutant CALR binds to and activates the TPO receptor. To further study the impact of TPO on MK development in our iPSC model, we differentiated the cells with and without supplementation of TPO (Figure 4C). In HD control and WT_{cr} clones, we found a significantly lower number of MKs in the absence of TPO. In contrast, the number of iPSC-derived MKs harboring a *CALR* mutation was equal regardless of zygosity and the presence or absence of TPO. Thus, our *CALR*-mutated iPSC model recapitulates a key pathological feature of TPO-independent megakaryopoiesis.

By following *CALR*-mutated megakaryopoiesis *in vitro*, we observed a striking increase in immature CD41$^+$CD61$^+$

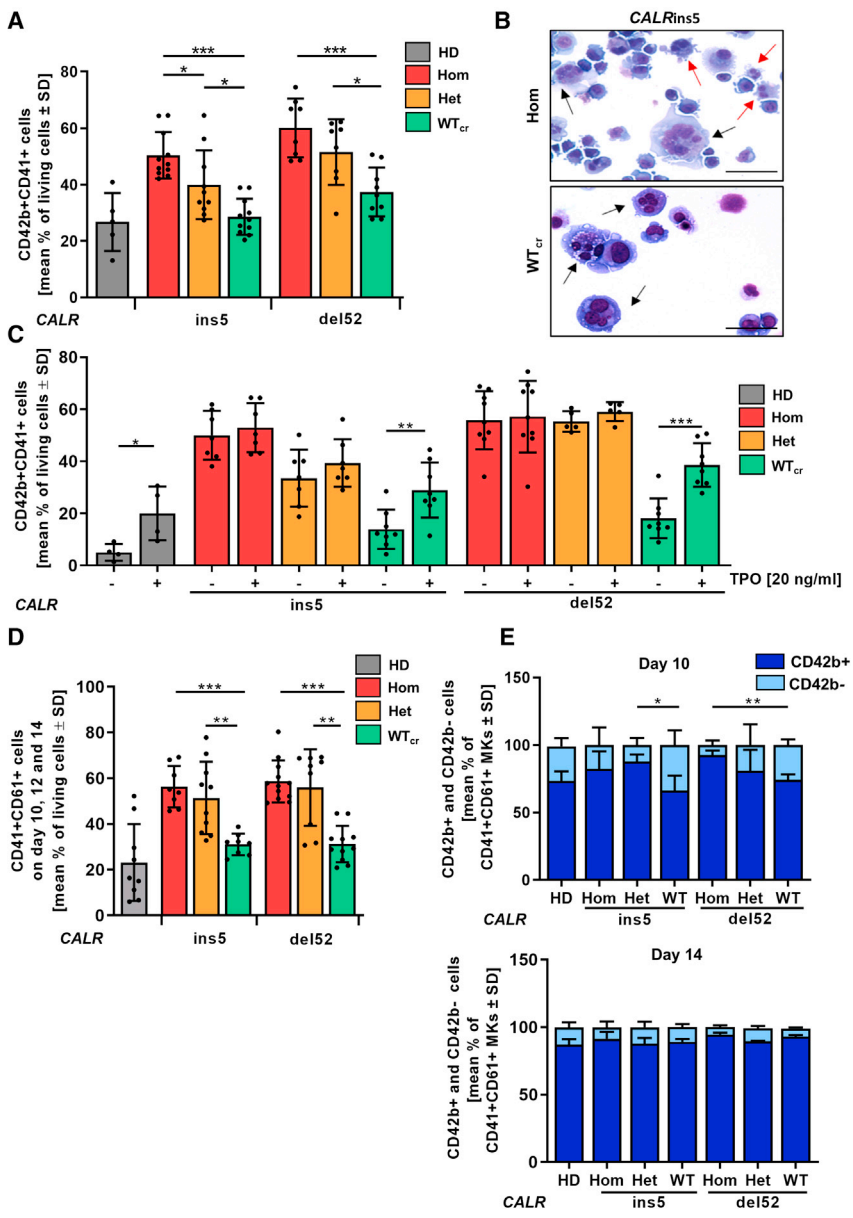


Figure 4. Megakaryocytic differentiation of *CALR*ins5 and *CALR*del52 iPSCs

(A) Percentage of $CD42b^+CD41^+$ matured MKs determined by flow cytometry on day 14 of "spin-EB" differentiation. Numbers of independent experiments performed for each *CALR* genotype and HD control refer to number of data points shown, n = 5–11 independent differentiation experiments. Data are presented as mean ± SD; *p < 0.05, **p < 0.01, ***p < 0.001.

(B) Representative morphology of MKs harvested on day 14 of "spin-EB" differentiation exemplarily shown for *CALR*ins5 hom clone and repaired WT clone (WT_{cr}) stained with Diff-Quik solutions after cytospin preparation. Typical MKs and aberrant smaller MKs are indicated by black and red arrows, respectively. Scale bars, 50 μm.

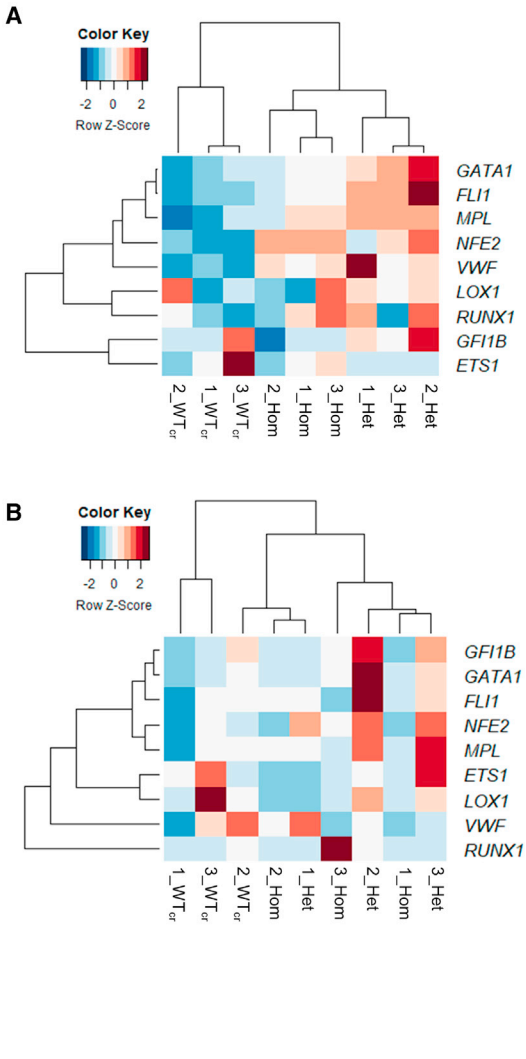
(C) Impact of TPO on MK development analyzed by flow cytometry on day 14 of "spin-EB" differentiation. Percentage of $CD42b^+CD41^+$ MKs is shown for cells treated with (+) or without (−) TPO from day 11 of differentiation onwards. Numbers of independent experiments for each *CALR* genotype and HD control refer to number of data points shown, n = 4–9 independent experiments. Data are presented as mean ± SD; *p < 0.05, **p < 0.01, ***p < 0.001.

(D) Percentage of $CD61^+CD41^+$ immature MKs determined by flow cytometry. Data of day 10, day 12, and day 14 of three independent experiments are combined for each *CALR* genotype and HD controls. Data are shown as mean values ± SD; **p < 0.01, ***p < 0.001, n = 3 independent differentiation experiments.

(E) $CD42b^+$ and $CD42b^-$ cells in $CD61^+CD41^+$ MKs on day 10 and day 14 of "spin-EB" differentiation analyzed by flow cytometry. Statistical analysis compares number of $CD42b^+$ cells of hom clones to corresponding het and WT_{cr} clones. Data are shown as mean values ± SD; **p < 0.01, n = 3 independent differentiation experiments.

MKs in comparison with unmutated cells (Figure 4D). We further analyzed the maturation kinetics in the $CD41^+CD61^+$ cell population to study differences in the MK maturity level along the differentiation, and observed less mature $CD42b^+CD41^+CD61^+$ MKs from *CALR*del52 WT_{cr} iPSCs when compared with their hom counterparts on day 10 of differentiation (Figure 4E). The same was observed for the het *CALR*ins5 clone and with a tendency (p = 0.1794) also for hom *CALR*ins5-mutated MKs. However, on day 14 all genotypes showed the same MK maturity. Altogether, these data demonstrate an accelerated MK maturation process in *CALR*-mutated cells.

MKs arise from progenitors shared with the erythrocytic lineage, the megakaryocyte-erythrocytic progenitors. Therefore, we analyzed the proportion of erythrocytic cells ($CD235a^+CD45^-$) of the day-14 population (Figure S3A). *CALR*ins5-mutated cells showed an increase in the erythrocytic population with a remarkable increase in the WT_{cr}



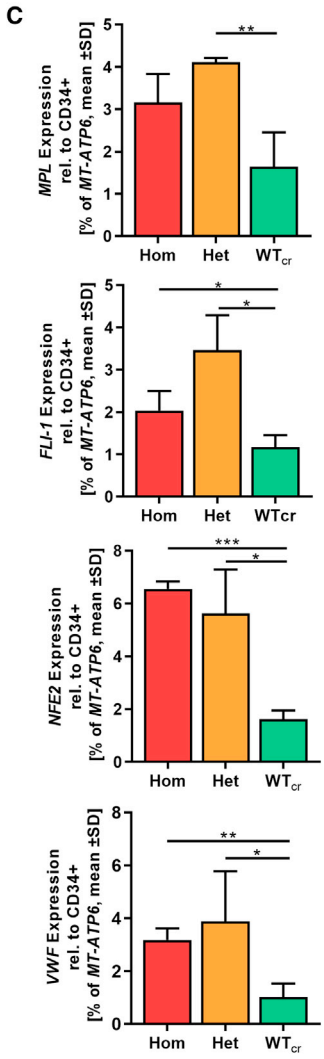


Figure 5. Gene expression profile of iPSC-derived MKs harboring *CALR* mutations

(A and B) Heatmaps for unsupervised hierarchical linkage clustering of selected MK-related genes analyzed by qRT-PCR of $CD61^+$ cells. $CD61^+$ cells were isolated by immunogenic bead selection (MACS) on day 14 of "spin-EB" differentiation. Data are normalized to gene expression of purified $CD34^+$ cells of the same experiment. Calculated *Z* score is shown for individual experiment of indicated *CALR* genotype (ins5 [A], del52 [B], with red and blue indicating high and low expression, respectively); n = 3 independent differentiation experiments for each genotype.

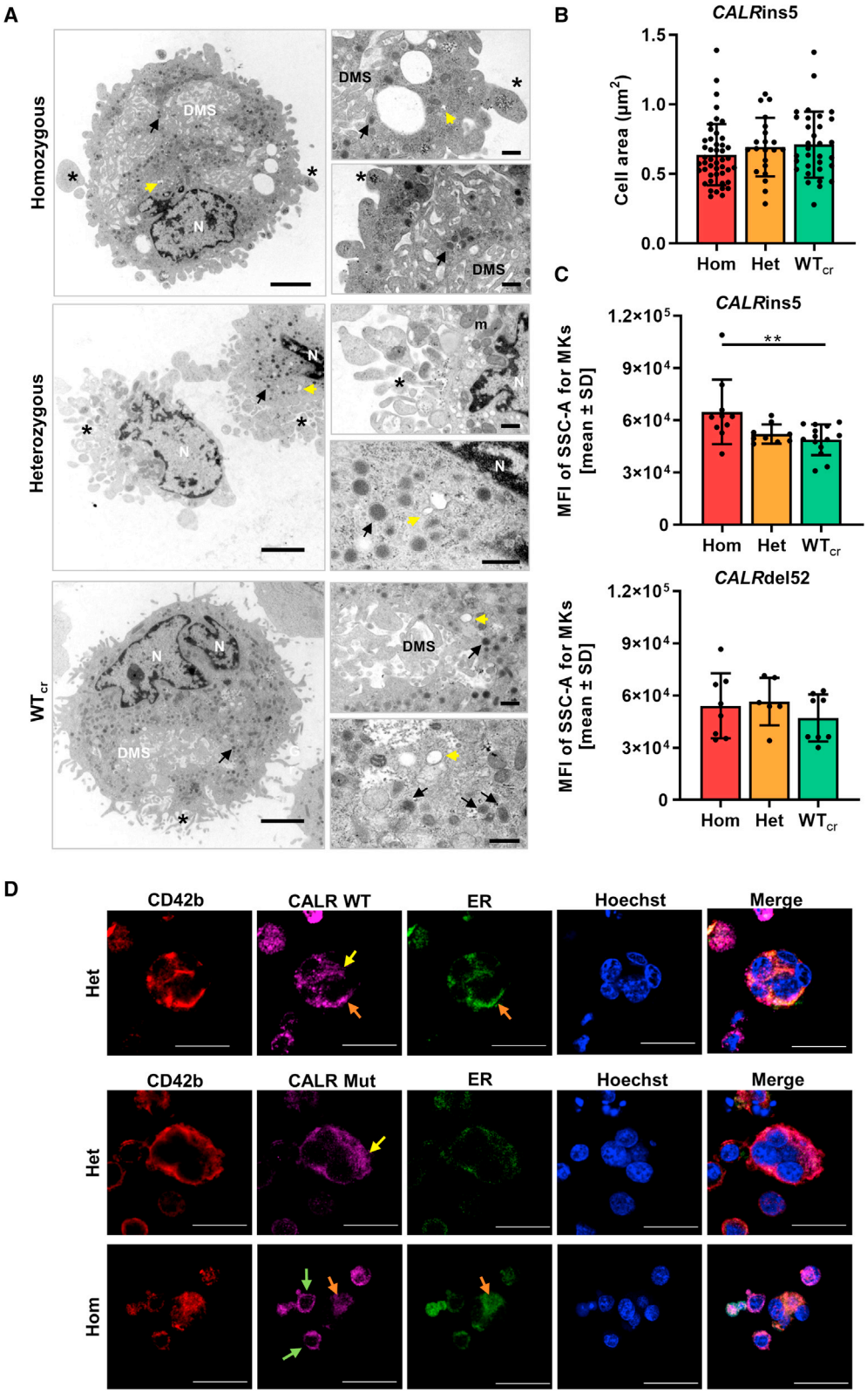
(C) Gene expression profile of $CD61^+$ MKs for indicated genes. Expression was normalized to $CD34^+$ cells from the same experiment. Statistical analysis compares the expression of hom and het *CALR*-mutated MKs with WT_{cr} MKs. Data are presented as mean ± SD; *p < 0.05, **p < 0.01, ***p < 0.001, n = 3 independent differentiation experiments for each genotype.

cells, different to CALRdel52 and HD control iPSCs. These data demonstrate that the repair of the *CALR* mutation in the hom *CALR*ins5-mutated cells led to enhanced erythropoiesis. Based on this, we hypothesized that differentiation properties switch upon the repair of the *CALR* mutation from the megakaryocytic to the erythrocytic lineage, at least in the patient-specific background of our *CALR*ins5-repaired clones.

CALR mutation causes upregulation of MK-related genes in iPSC-derived MKs

MK development is subjected to a highly coordinated process of MK-specific gene expression and simultaneous prevention of erythrocytic development. Our clonal approach using iPSC-derived CALR mutant MKs and their repaired counterparts are especially suitable for the analysis of solely mutated CALR-related changes. To examine whether *CALR*-mutated and -unmutated MKs show differences at the transcriptional level, we analyzed gene expression of typical megakaryocytic genes and related transcription factors.

The megakaryocytic subpopulation was purified by magnetic activated cell sorting on day 14 of "spin-EB" differentiation. We compared the differential expression signatures of MKs within the group of *CALR*ins5 MKs and *CALR*del52 MKs in a heatmap with unsupervised clustering (Figures 5A and 5B). We found that gene expression profiles (GEPs) of MKs with het or hom *CALR*ins5 mutation built a well-defined cluster compared with unmutated MKs. Of note, het *CALR*ins5-mutated MKs showed a stronger upregulation of MK-related genes compared with the hom *CALR*ins5-mutated MKs. Focusing on genes known to be



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involved in megakaryocytic development, we found that the expression of MPL was slightly enhanced in *CALR*ins5 het clones and hom clones ($p = 0.0669$, Figure 5C) compared with WT_{cr} MKs. Moreover, the early transcription factor *FLI1* was highly upregulated in *CALR*ins5 het and hom MKs compared with WT_{cr} MKs ($p = 0.0106$ and $p = 0.051$, respectively). In addition, *NFE2*, an essential marker for terminal MK maturation involved in platelet release and expression of *VWF*, was strongly expressed in both hom and het *CALR*ins5-mutated MKs. Hence, hom *CALR*ins5-mutated MKs showed an upregulation of mature MK markers (*NFE2* and *VWF*), suggesting enhanced megakaryopoiesis of hom mutant cells. However, equal expression of *Ki67* in both mutated *CALR*ins5 and *CALR*del52 and unmutated MKs was observed (Figure S3B).

Unsupervised clustering of MKs with *CALR*del52 mutation did not result in clear clusters of each zygosity as given for *CALR*ins5-mutated MKs (Figure 5B). Nonetheless, gene expression of mutated MKs, both het and hom, clustered closer together than WT_{cr} MKs. In line with the expression data of *CALR*ins5-mutated MKs, the strongest upregulation of MK-related genes was found in het clones, suggesting an underlying mechanism including both mutated and WT protein, which enhances transcription of megakaryocytic genes, likely involving CALR chaperone function.

CALR-mutated MKs exhibit higher granularity

During megakaryopoiesis MKs increase in size, produce alpha and dense granules, and develop a demarcation membrane system (DMS), the plasma membrane for future platelets (Ru et al., 2015). Terminally differentiated MKs form cytoplasmic protrusions, called pro-platelets, which further mature into platelets with open canalicular systems (OCSs) (Escolar and White, 1991).

To study in more detail whether structural features of MK maturation are present in our iPSC-derived MKs, we captured ultrastructural transmission electron microscopy (TEM) images of $CD61^+$ MKs on day14 of "spin-EB" differentiation for each *CALR*ins5 genotype. TEM images of MKs with different genetic background revealed typical megakaryocytic structures including granules, DMS, pro-platelet protrusions, and OCS (Figure 6A). Additionally, we determined the area of each MK from the TEM images (Figure 6B). WT_{cr} MKs showed a slight increase ($p = 0.1679$) in size compared with hom CALR mutant MKs. This supported our previous observation that some small MKs were found in the population of hom mutant MKs in cytospin images (Figure 4B). It is reported that *in vitro* generated MKs undergo different stages of maturation, identifiable by the granularity of the cells defined by side scatter (SSC) in flow cytometry (Sim et al., 2017). Therefore, iPSC-derived $CD42b^+CD41^+CD61^+$ MKs were analyzed for their mean fluorescence intensity of SSC. We found that the granularity of MKs is significantly enhanced in the hom *CALR*ins5 MKs compared with the unmutated counterparts (Figure 6C), confirming that hom *CALR*ins5-mutated MKs showed a higher level of maturation compared with the unmutated MKs. No differences in the granularity of CALR-del52-mutated MKs was observed, again suggesting that type 1 and type 2 *CALR* mutations may not induce identical functional changes, as is also suggested by differences in their clinical profile.

In the following, we analyzed the distribution of CALR WT and mutant protein in our iPSC-derived MKs by immunofluorescence and confocal microscopy (Figures 6D and S4A). CD42b-positive MKs were identified with pronounced polylobulated nuclei in mutated and unmutated MKs, highlighted in z-stacks of hom *CALR*ins5 and WT MKs (Figure S4B). WT and mutated CALR protein were not differentially distributed in the MKs and showed a more diffuse distribution in bigger MKs (yellow arrows). However, smaller MKs with less pronounced cytoplasm showed a clustered localization of mutated CALR at the cell surface (green arrows). In some MKs, we were able to verify a co-localization of CALR protein and the ER (orange arrows).

Figure 6. Structural morphology analysis of iPSC-derived MKs and CALR distribution in MKs

(A) Transmission electron microscopy (TEM) images of iPSC-derived MKs of indicated *CALR*ins5 genotype to evaluate cell morphology. Representative images are shown. N, nucleus; DMS, demarcation membrane system; m, mitochondria. Asterisks indicate pro-platelet protrusions. Black arrows point to granules and yellow arrows indicate open canalicular system. Scale bars, 2.5 μm (large panels) and 500 nm (small panels).

(B) Calculated cell area of MKs in TEM images for indicated *CALR*ins5 genotypes. Each data point represents a single cell, n = 1 differentiation experiment. Data are presented as mean ± SD.

(C) Mean fluorescence intensity (MFI) calculated for the side scatter (SSC-A) of $CD42b^+CD41^+CD61^+$ MKs on day 14 of "spin-EB" differentiation. Indicated data points represent independent experiments for each *CALR*ins5 (n = 10–14) and *CALR*del52 (n = 6–8) genotype. Values are shown as mean ± SD; $^{**}p < 0.01$.

(D) Representative immunofluorescence images of iPSC-derived MKs stained for the ER and WT CALR or mutated (Mut) CALR after 14 days of differentiation for indicated *CALR*ins5 iPSC clones. To identify MKs, samples were additionally stained for CD42b. Hoechst was added for nuclear staining. Diffuse CALR distribution, clustered localization of CALR at the cell surface, and co-localization of CALR and ER are indicated by yellow, green, and orange arrows, respectively. Scale bars, 50 μm.

CALR mutation causes an upregulation of hypoxia-related pathways in MKs

Finally, to further unveil differences between RNA expression dependent on the zygosity of the *CALR* mutation, we performed RNA-seq analysis of day-14 CD61$^+$ iPSC-derived MKs. Three independent differentiation experiments were performed using either WT_{cr}, het, or hom *CALR*ins5-mutated iPSCs. RNA-seq data were normalized using TMM normalization (Robinson and Oshlack, 2010) and subsequently voom transformed (Law et al., 2014).

Unsupervised clustering of differentially expressed genes (DEGs) was performed and depicted in a heatmap (Figure 7A). Samples efficiently clustered according to their *CALR* mutation zygosity, and higher similarity in the GEP of mutated MKs was observed. Unsupervised subclustering of the heatmap provided cluster 1 with mainly upregulated genes and cluster 2 with downregulated DEGs in hom mutant MKs. Gene ontology (GO) analysis of those clusters showed that MKs with hom *CALR* mutation upregulated interferon signaling and the response to hypoxia, while extracellular matrix (ECM) organization was downregulated compared with WT_{cr} MKs as exemplarily shown for *FN1* (Figures 7B and S5). Of note, GO terms including genes related to the ER were found to be affected in het and hom CALR mutant MKs. The observed upregulation of hypoxia signaling was further validated by analysis of pathway responsive genes (PROGENy) (Figure 7C) (Schubert et al., 2018). Moreover, the known hypoxia-related gene *NDRG1* was significantly upregulated in both het and hom *CALR*ins5-mutated MKs compared with WT (Figure 7D). Interestingly, PROGENy analysis showed that phosphatidylinositol 3-kinase signaling was significantly downregulated in both het and hom mutant MKs compared with WT, similar to transforming growth factor β (TGFβ) and epidermal growth factor receptor pathways, which were downregulated in the hom mutant MKs compared with control MKs. In addition, a high number of DEGs was found by comparing different genotypes (Figure S6). Genes found to be significantly upregulated in both het and hom mutant MKs compared with WT_{cr} MKs included *H3C3*, *CTSF*, and leptin. Partial validation of depicted genes was performed by qRT-PCR (Figures 7D and S6D).

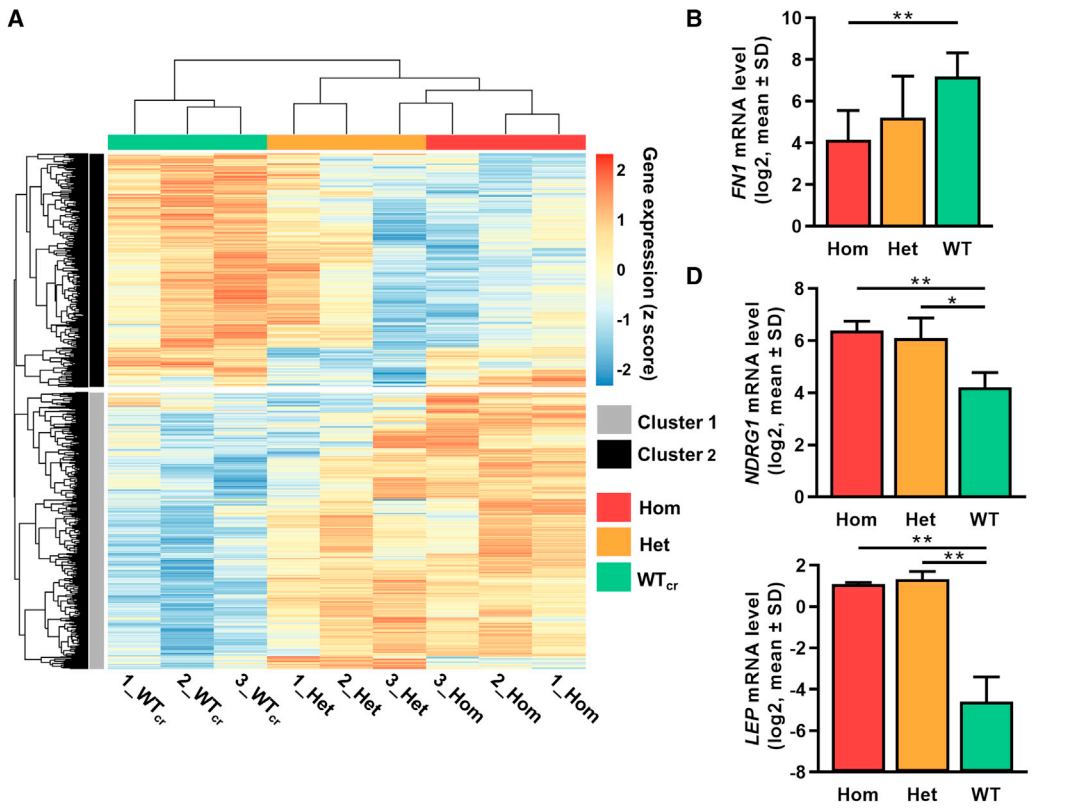
DISCUSSION

In the present study, we generated patient-specific iPSC lines harboring *CALR*del52, *CALR*ins5, or *CALR*del31 mutations, and the iPSC-based model was applied to study the impact of *CALR* mutant zygosity on hematopoietic and more precisely on MK differentiation at the clonal level. Patient-derived iPSCs carrying het *CALR* mutations have been recently described, but detailed comparisons of type 1 and type 2 *CALR* mutations with focus on their zygosity and detailed characterization of derived disease-driving MKs are missing (Gomez Limia et al., 2017, 2018; Secardin et al., 2021; Takei et al., 2018). To obtain isogenic *CALR*-repaired clones for *CALR*ins5 and *CALR*del52-mutated iPSCs, we used CRISPR/Cas9 technology to correct the mutations. Until now, the repair of *CALR*ins5 mutations has been reported only as a conference abstract (Wang et al., 2018), and a CRISPR/Cas9 approach has been used to model *CALR* mutations in murine cell lines (Abdelfattah and Mullally, 2018). To our knowledge, the repair of *CALR*del52 mutations in MPN-specific iPSCs has not been reported. The absence of further MPN-related mutations of clinical significance was confirmed by NGS in our *CALR*-mutated iPSC clones. Hence, our findings can be solely attributed to the *CALR* mutations.

MPO deficiency was demonstrated in hom *CALR*ins5 iPSC-derived CD15$^+$ cells and, for the first time, also in hom *CALR*del52 cells. Loss of MPO activity in MPN patients carrying hom CALR mutations was described by Theocharides et al. (2016), and we were able to restore MPO activity by CRISPR/Cas9 gene repair, demonstrating the recovery of the chaperone function of CALR. The rescue of CALR WT characteristics was underlined by the fact that WT_{cr} and HD control iPSCs generated equal levels of MKs. Importantly, the iPSC-derived MKs exhibited structural features of primary MKs such as formation of DMS, granules, multilobulated nuclei, and pro-platelets.

Megakaryocytic hyperplasia was reported for all types of MPNs and represents a diagnostic criterion of the World Health Organization (Barbui et al., 2018; Swerdlow et al., 2008; Zingariello et al., 2020). Our data demonstrate that both *CALR*ins5- and *CALR*del52-mutated iPSCs gave rise to more MKs than WT_{cr} and HD control iPSCs. In particular, hom mutant clones produced higher numbers of MKs followed by het clones. Thus, the phenotype demonstrated in our study recapitulates the phenotype found in MPN patients. Furthermore, knockin mice expressing hom *CALR*ins5 or *CALR*del52 mutations exhibited stronger ET- and PMF-related phenotypes over het CALR mutant mice, demonstrating that zygosity of the *CALR* mutation has an impact on disease development (Benlabiod et al., 2020). Araki et al. (2019) described mutant CALR protein homomultimers that are presumed to bind and activate the TPO receptor. We can assume that mutant CALR homomultimers are formed more excessively in hom clones than in het clones, since our data confirm a strong bias toward the MK lineage observed for hom *CALR*-mutated iPSCs.

Importantly, we demonstrated in our human iPSC-based model that the *CALR* mutation led to amplification of the MK lineage in a TPO-independent manner while WT and HD control cells showed pronounced MK population only in the presence of TPO. Of note, additional treatment



Comparison \ Pathway	Androgen	EGFR	Estrogen	Hypoxia	JAK-STAT	MAPK	NFkB	p53	PI3K	TGFb	TNFa	Trail	VEGF	WNT
Hom vs WTcr	0,154	0,021	0,435	0,012	0,194	0,114	0,435	0,662	0,021	0,021	0,277	0,561	0,951	0,277
Het vs WTcr	0,380	0,177	0,300	0,177	0,380	0,177	0,380	0,551	0,032	0,330	0,380	0,300	0,551	0,300
Hom vs Het	0,932	0,898	0,932	0,898	0,067	0,932	0,898	0,932	0,898	0,898	0,932	0,898	0,932	0,932

Figure 7. RNA-seq experiments of iPSC-derived MKs of *CALR*ins5-mutated clones

(A) Heatmap for unsupervised hierarchical clustering of RNA-seq data of iPSC-derived MKs with hom or het *CALR*ins5 mutation or WT_{cr} MKs. Numbers represent independent experiments. Unsupervised clustering was performed. n = 3 independent differentiation experiments.

(B) Gene expression profile of *FN1* in iPSC-derived MKs of RNA-seq analysis. Data are presented as mean ± SD; **p < 0.01, n = 3 independent differentiation experiments.

(C) Pathway responsive genes (PROGENy) of multiple comparisons of hom, het, and WT_{cr} iPSC-derived MKs. Upregulated and downregulated pathways are shown in red and blue, respectively. Adjusted p values from multiple comparisons are shown in the table. Significant values ($p_{adjusted}$ < 0.05) are highlighted in red, n = 3 independent differentiation experiments.

(D) Gene expression of *NDRG1* and *LEP* in iPSC-derived MKs of RNA-seq analysis. Data are shown as mean ± SD; *p < 0.05, **p < 0.01, n = 3 independent differentiation experiments.

of TPO did not further increase MK generation of *CALR*-mutated cells. This might be due to the reported oncogenic interaction of the TPO receptor and mutant CALR before reaching the cell surface (Masubuchi et al., 2020; Pecquet et al., 2019), already sufficiently occupying the TPO binding site. However, Secardin et al. (2021) observed an increase of CFU-MKs, generated from iPSC-derived CALR-mutated $CD34^+$ $CD43^+$ progenitors, in the presence of TPO. These differences may be clone dependent or may be due to differences between liquid cultures and collagen/fibrin-based medium.

We showed that hom and het *CALR* mutations induced an accelerated maturation of MKs. Surface expression of CD42b, which is a marker for megakaryocytic maturation (Sim et al., 2017), was increased on mutated MKs in comparison with WT_{cr} and HD control MKs at an early time point of differentiation. Furthermore, higher expression of *NFE2* in hom and het *CALR*ins5 MKs compared with WT_{cr} MKs was observed. Moreover, we found that hom *CALR*ins5-mutated MKs exhibited a higher level of cytoplasmic granularity compared with cells harboring unmutated or het *CALR*ins5. Taken together, our data demonstrate that *CALR* mutations cause an accelerated megakaryopoiesis in iPSC-derived MKs. In future work, our model can be used to study the relationship of accelerated and enhanced megakaryopoiesis with platelet production/release in *CALR*-mutated MKs.

We confirmed the upregulation of typical megakaryocytic genes such as *NFE2*, *FLI1*, and *VWF* in our iPSC-derived MKs by qRT-PCR and proceeded to an in-depth analysis of the global GEP by RNA-seq. In contrast to reported MPN-related transcriptome analysis using primary material (El-Khoury et al., 2020; Psaila et al., 2020), our model allows to study differences of the megakaryocytic transcriptome according to the underlying *CALR* genotype by analyzing a clonal cell population with a defined mutational background to elucidate aberrant megakaryopoiesis in MPN. Our RNA-seq data identified elevated *leptin* gene expression in *CALR*-mutated MKs. Leptin functions as a hormone and adipokine predominantly expressed by adipocytes (Cava and Matarese, 2004). In liver fibrosis, leptin promotes the proliferation and secretion of ECM molecules in myofibroblasts (activated hepatic stellate cells) via the leptin receptor (Vivoli et al., 2016). Also, in BM fibrosis, leptin receptor-positive mesenchymal stromal cells were identified to be responsible for collagen fiber generation and deposition (Decker et al., 2017). Hence, it is conceivable that MK-produced leptin may be implicated in myofibroblast formation in MPN and may contribute to the inflammatory environment in the BM niche.

Of note, the expression of ECM proteins was downregulated in *CALR*-mutated MKs compared with control. The composition of ECM in the BM is essential for MK differentiation and maturation (Noetzli et al., 2019). Synthesis of ECM components in MKs was described to be regulated via autocrine TGFβ1 signaling (Abbonante et al., 2016). Our PROGENy analysis revealed that TGFβ signaling was significantly downregulated in hom *CALR* mutant MKs, which may explain the downregulation of ECM genes.

In addition, the hypoxia signaling pathway was strongly upregulated in *CALR*-mutated versus -unmutated MKs. In solid tumors, it is well established that hypoxia represents a key feature of tumor survival and development (Thomlinson and Gray, 1955), and the BM stem cell niche represents an environment of relatively low oxygen level (Irigoyen et al., 2017). The major regulator of cellular response to hypoxia is the hypoxia-inducible factor 1 (HIF-1a) (Semenza, 2009). Importantly, HIF-1a promotes survival of *JAK2*V617F-positive cells and was discovered to be a promising therapeutic target (Baumeister et al., 2020). Moreover, elevated protein levels of HIF-1a were recently described in blast phase MPN samples, suggesting a role of HIF-1a in MPN disease progression (Marinaccio et al., 2021). Hence, our data support the notion that hypoxia-related factors may be promising targets in *CALR*-mutated MPNs.

Collectively, our study describes a comprehensive iPSC system for evaluating mutant *CALR*-induced phenotypes in MPN with a clonal genetic background, which is an invaluable new tool for clinical drug screening and development of personalized therapies. We identified accelerated megakaryopoiesis and the upregulation of leptin expression as well as hypoxia signaling pathways as potential pathogenic mechanisms in *CALR*-mutated MKs.

EXPERIMENTAL PROCEDURES

Ethics approval and consent to participate

PBMCs were obtained from MPN patients carrying *CALR*del52 or *CALR*ins5 mutation at the Department of Hematology at Zurich University, and from a *CALR*del31-mutated MPN patient at the centralized Biomaterial Bank in RWTH Aachen University Hospital. Healthy donor PBMCs were provided from the Transfusion Medicine in the University Hospital, RWTH Aachen. All PBMCs were donated after written informed consent, as approved by the ethics committees in Zurich and the Medical Faculty of RWTH Aachen (EK127/12, EK 206/09, and EK099/14).

Generation of MPN patient-specific and healthy donor iPSCs

PBMCs were reprogrammed using a CytoTune iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. After reprogramming, iPSCs were seeded as single cells and individual colonies were picked and screened for *CALR* genotypes by PCR or allele-specific PCR.

Differentiation of iPSCs into hematopoietic stem cells and myeloid subsets with the "spin-EB" protocol

Feeder-free iPSC culture was maintained on Matrigel-coated 6-well plates and routinely passaged with Accutase or 0.5 mM EDTA (both Thermo Fisher Scientific). Human iPSCs were differentiated into HSPCs, MKs, and erythrocytic cells adapted from the differentiation protocol by Liu et al. (2015). Details can be found in supplemental experimental procedures.

Statistical analysis

Graphical display and statistical analysis were performed with Prism 9 (GraphPad, San Diego, CSA, USA). In every experiment two different clones for each *CALR* genotype were used, except for the het *CALR*del52 clone. Comparisons among two groups were performed by using unpaired Student's t tests. For multiple group comparisons, ANOVA with Bonferroni post test was performed. p values of <0.05 were considered statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ in figures).

Additional methods

NGS, undirected hematopoietic differentiation, magnetic activated cell sorting of HSPCs and MKs, CFU assay, SDS-PAGE and western blot, CRISPR/Cas9-mediated *CALR* mutation repair, TEM, immunofluorescence staining, cytogenic analysis, flow cytometry, cell morphology analysis, MPO cytochemical staining, RNA isolation, preparation of samples for RNA-seq, and qRT-PCR including list of primers, antibodies, and media compositions are described in supplemental information.

Data availability

RNA-seq data are available at the Gene Expression Omnibus under accession number GEO: GSE182479.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2021.09.019.

AUTHOR CONTRIBUTIONS

Conceptualization, K.O., L.H., M.A.S.d.T., N.C., and S.K.; data curation, J.B., M.G., I.G.C., and A.M.; formal analysis, K.O., L.H., M.A.S.d.T., M.G., I.G.C., A.M., and J.B.; investigation, K.O., L.H., M.A.S.d.T., J.B., A.M., H.M.S., E.M.B., K.P., S.G., and P.B.; methodology, K.O., L.H., M.A.S.d.T., and J.B.; resources, A.T., M.K., D.G., and M.Z.; supervision, M.A.S.d.T., N.C., and S.K.; visualization, K.O., L.H., M.A.S.d.T., and M.G.; writing – original draft, K.O., and L.H.; writing – review & editing, K.O., L.H., M.A.S.d.T., M.G., I.G.C., A.M., M.K., P.B., T.H.B., M.Z., N.C., and S.K.

CONFLICT OF INTERESTS

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