

Human SEC23B-deficient hematopoietic stem cell model of CDAll recapitulates erythroid and cell-cycle defects

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1 **Title page**

2 **Human *SEC23B*-deficient hematopoietic stem cell model of CDAll recapitulates**
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Abstract

Background: Congenital dyserythropoietic anemia type II (CDAII) is an inherited disorder affecting red blood cell development. It is caused by mutations in the *SEC23B* gene, which causes protein hypoglycosylation and morphological alterations in erythroid cells, such as the presence of binucleated erythroblasts in the patient's bone marrow. Patients with CDAII suffer from anemia of varying degrees and jaundice, frequently associated with splenomegaly and/or hepatomegaly. CDAII patients have a liver iron overload that must be monitored and treated because otherwise, it would lead to fatal clinical complications. A more comprehensive understanding of CDAII is hindered by the limited availability of human disease models that accurately recapitulate its pathophysiology.

Results: To address this limitation, we developed a human CDAII model by knocking-out *SEC23B* gene in hematopoietic stem and progenitor cells (HSPCs) using gene editing. Edited cells exhibited impaired erythroid differentiation both *in vitro* and *in vivo*, characterized by a reduced number of mature erythroid cells and an increase in bi/multinucleated cells. Furthermore, transcriptome analysis confirmed impaired erythroid differentiation, increased apoptosis, and identified key genes whose alterations explain the decoupling between the cell-cycle and erythroid differentiation in CDAII.

Conclusions: This human cellular model successfully recapitulates key features of CDAII and provides a platform for studying disease mechanisms. It also offers a valuable tool for the identification and evaluation of novel therapeutic strategies for CDAII.

Keywords

Congenital dyserythropoietic anemia type II; CDAll; *SEC23B*; Erythropoiesis; Hematopoietic stem and progenitor cells; Gene editing; Rare disease model; Erythroid differentiation

Introduction

Congenital dyserythropoietic anemias (CDAs) are inherited disorders characterized by ineffective erythropoiesis and distinctive morphological abnormalities in erythroid precursors. They are classified into four main types, CDAI, II, III, and IV, based on genetic, clinical, and cytomorphological features. A hallmark across all CDA types is the presence of binucleated or multinucleated erythroblasts, reflecting defective nuclear maturation and impaired coordination between cell division and erythroid differentiation (1,2).

CDAll (OMIM #224100) is the most common form, with an estimated prevalence of 0.7 cases per million, caused by mutations in the *SEC23B* gene (3–5). *SEC23B*, a component of the COPII complex, mediates endoplasmic reticulum–Golgi protein trafficking. Specific characteristics of the disease include more than 10% mature binucleated erythroblasts in the bone marrow (BM) and hypoglycosylation of erythroid membrane proteins (3,6–10). *SEC23B* expression decreases during late erythroid differentiation, explaining the lineage-specific phenotype (11–13).

CDAll patients show variable degrees of anemia, often accompanied by jaundice and splenomegaly, with 20% of them being transfusion dependent. Current treatments involve blood transfusions, iron chelation, and splenectomy only in severe cases (4,14,15). The development of new therapeutic approaches is limited by the availability of patients' hematopoietic stem and progenitor cells (HSPCs) and by the limited

availability of a feasible CDAll model fully recapitulating the human disease (16–18). Although *SEC23B*-deficient HUDEP-2 erythroid cell lines and murine models generated through combinatorial deletion of *Sec23* paralogs have been described (19), these systems either rely on immortalized human cells, which may not accurately recapitulate CDAll erythropoiesis *in vivo*, or require loss of multiple *Sec23* alleles in mice to generate an erythroid phenotype, reflecting species-specific compensation not observed in humans. In contrast, biallelic *SEC23B* mutations alone are sufficient to cause CDAll, underscoring the need for a primary human model that more faithfully captures disease pathophysiology. In this context, recent studies using *SEC23B*-deficient human HSPC-derived erythroid cells have demonstrated that pharmacological inhibition or genetic targeting of Lysine-Specific Demethylase 1 (LSD1) restores *SEC23A* expression and rescues the erythroid defect (20), highlighting the value of these primary human models both for elucidating disease mechanisms and for the development of novel therapeutic approaches.

In the present work, we aimed to develop a CDAll model in primary human cells. We disrupted the *SEC23B* gene in human HSPCs using CRISPR/Cas9 gene editing. *SEC23B*^{KO} HSPCs exhibited impaired erythropoiesis *in vitro* and *in vivo*, along with an increased frequency of binucleated erythroblasts, recapitulating a hallmark feature of CDAll. This human cell model also revealed transcriptomic alterations in pathways related to erythroid differentiation, apoptosis, and cell-cycle regulation, highlighting a link between abnormal *SEC23B* and defects in chromosome segregation. Furthermore, we demonstrate that defective protein glycosylation, whether caused by *SEC23B* loss or induced pharmacologically, disrupts erythroid maturation, alters cell-cycle progression, and compromises cell survival. In summary, we have generated a CDAll model in human

116 HSPCs that can be used to study CDAll functions deeply and to develop new therapeutic
117 strategies for this disease.

118 **Methods**

119 *Human stem and progenitor cells.*

120 Mononuclear cells from cord blood (CB) or peripheral blood (PB) were isolated by Ficoll-
121 Hypaque density centrifugation. CD34⁺ HSPCs were purified using MicroBeads or flow
122 sorting. Cells were cultured in SFEM II with cytokines, UM171, and SR1 (see
123 Supplemental Methods).

124 *Nucleofection of gene editing tools.*

125 Cas9 RNPs were assembled by incubating HiFi Cas9 with sgRNAs for 10 min. Single or
126 pooled sgRNAs (sgKO1+2+3) were used. Nucleofection was performed with the
127 4D-Nucleofector, and cells were cultured at 2.5×10⁵/mL. Parameters and sequences are
128 provided in Supplemental Methods.

129 *In vitro erythroid differentiation.*

130 Twenty-four hours after editing, CD34⁺ cells were differentiated for 14 days using a
131 three-stage erythroid protocol with specific cytokine supplementation. The complete
132 protocol is described in Supplemental Methods.

133 *Human hematopoiesis in immunodeficient mice.*

134 Female NBSGW mice (6–8 weeks) were sublethally irradiated (1 Gy) and transplanted
135 with human CD34⁺ cells. Engraftment was analyzed 60 days later by flow cytometry (see
136 Supplemental Methods).

137 *RNA-Seq and differential expression analyses.*

138 RNA from day 9 and 14 erythroid cells was extracted, processed for mRNA libraries, and
139 sequenced on NovaSeq. Reads were aligned to hg38, and expression (RPKM) computed.

140 Differential expression used MeV, and GO enrichment ToppFun. Data are available at
141 GEO GSE230875.

142 *Statistics.*

143 Analyses were performed in GraphPad Prism 9. As most data were non-normal, Mann–
144 Whitney tests were used for two groups and Kruskal–Wallis with Dunn’s test for multiple
145 groups. Engraftment was analyzed assuming lognormal distribution. P values are shown
146 in figures; additional details in Supplemental Methods.

147

Results

SEC23B-deficient hematopoietic progenitors show impaired in vitro erythroid differentiation

To characterize the erythroid defects associated with CDAll and to evaluate whether CRISPR/Cas9-mediated SEC23B loss recreates these abnormalities, we analyzed *in vitro* erythropoietic differentiation in parallel in patient-derived and SEC23B^{KO} HSPCs.

In CDAll patients (Supplemental Table 1), peripheral blood (PB) showed reduced red blood cell (RBC) counts and hemoglobin levels together with increased reticulocytes (Supplemental Figure 1A). Consistently, a trend toward increased intermediate erythroid populations (CD71⁺/CD235a⁺) was observed (Supplemental Figure 1B), supporting a defect in terminal erythroid differentiation. At the molecular level, SEC23B protein levels were reduced and protein glycosylation was impaired (Supplemental Figure 1C–D), as previously reported (21). White blood cell (WBC) counts were increased, mainly monocytes and lymphocytes (Supplemental Figure 1E), while myeloid and lymphoid subset frequencies remained comparable to healthy donors (HDs) (Supplemental Figure 1F), indicating that the defect predominantly affects the erythroid lineage.

To directly compare these alterations with an experimental model, we generated SEC23B-deficient HSPCs using CRISPR/Cas9 targeting strategies (Supplemental Figure 2A). Gene editing efficiency reached >60–75%, with the majority of indels corresponding to frameshift mutations (Supplemental Figure 2D), resulting in a marked reduction in SEC23B protein levels and impaired glycosylation (Supplemental Figure 2E–F), thereby faithfully reproducing the molecular defect observed in CDAll patient samples (Supplemental Figure 1C–D).

To assess erythroid potential, we purified CD34⁺ HSPCs from CDAll and HD PB samples and compared them with *SEC23B*^{KO} HSPCs. Although CD34⁺ frequencies were similar across conditions (Supplemental Figure 1G), both CDAll and *SEC23B*^{KO} HSPCs showed a trend toward reduced erythroid progenitor potential compared to their respective controls (Figure 1A). During erythroid differentiation, CDAll-derived cells exhibited a progressive decline in cell numbers, a phenotype that was recapitulated in *SEC23B*^{KO} HSPCs, which showed modest proliferation defects, particularly with sgKO1+2+3 (Figure 1B). Consequently, total erythroid output was reduced in both CDAll and *SEC23B*^{KO} conditions.

Analysis of erythroid populations further supported this parallel phenotype. In patients, accumulation of CD71⁺/CD235a⁺ intermediate populations (Supplemental Figure 1B) and reduced RBC output suggested impaired terminal maturation. Consistently, *in vitro* differentiation revealed that the CD71⁻/CD235a⁺ mature erythroid population was the most affected in CDAll samples and was also reduced in *SEC23B*^{KO} cultures at day 14 (Figure 1C and Supplemental Figures 1H and 2G).

At the morphological level, CDAll erythroid cells exhibited a significant increase in binucleated erythroblasts, a hallmark feature of the disease (Figure 1D). This phenotype was closely reproduced in *SEC23B*^{KO} erythroid cells, which showed a nearly threefold increase in bi/multinucleated cells, further supporting the robustness of the model.

Notably, longitudinal analysis of edited *SEC23B*^{KO} cells during differentiation revealed a progressive decrease in frameshift alleles and enrichment of wild-type alleles (Supplemental Figure 2H), indicating a selective advantage of *SEC23B*-expressing cells.

This observation further reinforces the functional relevance of *SEC23B* in erythropoiesis and mirrors the ineffective erythropoiesis observed in CDAll patients.

Overall, *SEC23B*-deficient human HSPCs, either from CDAll patients or generated via CRISPR/Cas9, recapitulate key CDAll features, including impaired terminal maturation, defective glycosylation, and increased bi/multinucleated erythroblasts. These findings validate *SEC23B*^{KO} HSPCs as a physiologically relevant model that closely mirrors patient-derived phenotypes and provide a robust platform for studying CDAll pathophysiology.

SEC23B-deficient hematopoietic stem and progenitor cells recapitulate CDAll-associated hematopoiesis in vivo.

To assess *SEC23B* deficiency *in vivo* in a more physiological model of CDAll, sgKO3- and sgKO1+2+3-edited HSPCs were transplanted into sublethally irradiated NBSGW mice. Human engraftment was comparable across groups, with slightly higher levels in sgKO1+2+3 cells (Figure 2A; Supplemental Figure 3A). Lymphoid and progenitor compartments were similar, though myeloid cells increased in the sgKO1+2+3 group (Figure 2B; Supplemental Figure 3A). Furthermore, analysis of human erythroid populations (hCD71⁺/hCD235a⁻, hCD71⁺/hCD235a⁺ and hCD71⁻/hCD235a⁺) showed significant differences (Figure 2C and Supplemental Figure 3B). The most mature erythroid population (hCD71⁻/hCD235a⁺) almost disappeared in the mice transplanted with sgKO1+2+3-gene edited hCD34⁺ cells.

May-Grümwald-Giemsa staining of sorted human erythroid subsets revealed a higher presence of bi/multinucleated cells in the hCD71⁺/hCD235a⁻ population from mice transplanted with *SEC23B*^{KO} cells (Figure 2D). An increased frequency of

bi-/multinucleated cells was also observed in the hCD71⁺/hCD235a⁺ population of animals engrafted with sgKO3-gene edited HSPCs (Figure 2E). Due to limited cell numbers, we were unable to analyze this terminal erythroid population in the sgKO1+2+3 group.

Consistent with the *in vitro* findings, analysis of total human BM cells revealed the highest gene editing frequency in the sgKO1+2+3 group (Supplemental Figure 3C), with the majority of indels disrupting the *SEC23B* open reading frame. Indel frequencies remained stable across myeloid, lymphoid, and progenitor compartments, with a higher proportion of frameshift mutations observed in the sgKO1+2+3 group compared to sgKO3. Notably, in both edited groups, the frequency of frameshift mutations declined by approximately 50% during erythroid differentiation (Supplemental Figure 3D), suggesting a selective advantage of wild-type cells during erythropoiesis *in vivo*, as previously observed *in vitro*.

Together, these results demonstrate that *SEC23B*^{KO} human HSPCs exhibit defective erythroid differentiation *in vivo*, characterized by reduced terminal erythroid output and an accumulation of bi-/multinucleated cells. These phenotypes closely resemble the hematopoietic defects observed in CDAll patients, confirming the utility of this model for studying disease mechanisms.

SEC23B deficiency alters erythroid transcriptional programs and promotes cellular stress

To define the molecular basis of impaired erythropoiesis in *SEC23B*-deficient cells, we performed bulk transcriptome analysis of *in vitro* erythroid differentiation cultures derived from HD and *SEC23B*^{KO} CB-CD34⁺ cells. Importantly, samples were collected at

239 fixed differentiation time points (day 9 and day 14; Supplemental Figure 4A,
240 GSE230875), rather than from stage-matched erythroid subsets, a limitation that should
241 be considered when interpreting the results.

242 Two-way ANOVA analysis identified significant transcriptional changes, with 233 genes
243 upregulated (cluster c1) and 55 genes downregulated (cluster c2) in *SEC23B*^{KO} cells
244 relative to wild-type controls (Figure 3A).

245 Gene Ontology Biological Process (GO-BP) enrichment of cluster c1 revealed pathways
246 associated with chemotaxis, leukocyte activation, cell-cell adhesion, and cellular
247 component movement (Figure 3B). Notably, several of the upregulated genes, including
248 *HLA-DPB1*, *ITGAE*, *HLA-DRA*, and *CXCR4*, are normally downregulated during erythroid
249 maturation (Haemosphere) (22). This pattern suggests a relative enrichment of
250 immature or non-erythroid populations in *SEC23B*-deficient cultures, consistent with
251 delayed erythroid differentiation. Supporting this interpretation, *CXCR4* and *CD235a*
252 expression were mutually exclusive at day 9, with an increased *CXCR4*⁺ fraction in
253 *SEC23B*^{KO} cells (Supplemental Figure 4B).

254 In addition, cluster c1 included genes associated with apoptosis and cellular stress,
255 including *BASP1*, *PYCARD*, *BCL2*, and *NOTCH1*, indicating activation of stress-related
256 programs.

257 In contrast, cluster c2 comprised genes downregulated in *SEC23B*^{KO} cells and enriched
258 for functions critical to terminal erythroid differentiation, including gas transport and
259 hypotonic response (CA1, CA2, CA3) (Figure 3B), consistent with the reduced mature
260 erythroid compartment observed at the phenotypic level. This cluster also included
261 *AHSP* (23) and *SHARPIN* (24,25), together with key regulators of cell-cycle progression

and mitotic integrity, such as *PTTG1* (26), *CDKN3* (27), *KIF22* (28), and *NUF2* (29), suggesting defects in proliferation and cytokinesis.

Together, these transcriptional changes indicate that *SEC23B* deficiency impairs erythroid maturation while promoting cellular stress responses and disrupting cell-cycle-associated programs.

Finally, temporal transcriptomic analysis revealed distinct clusters reflecting altered differentiation dynamics (Figure 3C).

Cluster 3 (c3) comprised genes that are normally upregulated during terminal differentiation in wild-type cells but fail to be induced in *SEC23B*^{KO} cells, including *FZD10*, *SLC12A8*, *NANOG*, and *FCER2*, consistent with defective activation of late erythroid programs.

Cluster 4 (c4) included genes upregulated during differentiation in both wild-type and *SEC23B*^{KO} conditions, such as erythroid-associated genes *HBQ1*, *GYPB*, *HAGH*, and *BLVRB*, reflecting shared differentiation pathways.

In contrast, cluster 5 (c5) contained genes aberrantly maintained or upregulated at later stages in *SEC23B*^{KO} cells, including early erythroid regulators such as *GATA3*, *BMP2*, *HAP1*, and *HOXC8*, indicating persistence of immature transcriptional programs.

These data further support that *SEC23B* deficiency disrupts the balance between early and late erythroid transcriptional programs, resulting in defective terminal differentiation.

283 *Disruption of cell-cycle regulators and COPII components functionally impairs*
284 *erythropoiesis*

285 To determine whether the transcriptional alterations identified above have functional
286 consequences, we disrupted selected cell-cycle regulators (*PTTG1*, *CDKN3*, *KIF22*, and
287 *NUF2*) in wild-type HSPCs using CRISPR/Cas9. In parallel, we targeted *SEC31A* and *SEC13*,
288 given their roles within the COPII complex, to assess whether the phenotype observed
289 in *SEC23B* deficiency reflects broader defects in vesicular trafficking.

290 Knockout efficiency declined during erythroid differentiation for *SEC13*, *NUF2*, and
291 *PTTG1* (Supplemental Figure 4C), suggesting a selective disadvantage for cells lacking
292 these genes. Consistently, disruption of these targets altered erythroid CFU output,
293 particularly affecting erythroid progenitor potential (Figure 3D).

294 During *in vitro* erythroid differentiation, *SEC13*^{KO} HSPCs exhibited the most pronounced
295 phenotype, with reduced erythroid output and increased apoptosis compared to control
296 conditions (Figure 3E–F and Supplemental Figure 4D–E). This is consistent with the stress-
297 associated transcriptional programs identified in *SEC23B*-deficient cells.

298 Morphological analysis further revealed dysplastic erythroid features across several
299 perturbations, including *SEC23B*^{KO} and COPII-related edits, indicative of impaired
300 erythroid maturation (Figure 3G Supplemental Figure 4F).

301 In addition, cell-cycle analysis demonstrated an increased proportion of cells in S-phase
302 in *SEC13*-edited cells, together with more subtle alterations across additional targets
303 (Figure 3H and Supplemental Figure 4G), supporting a defect in cell-cycle progression
304 consistent with the downregulation of mitotic regulators observed at the transcriptional
305 level.

These functional findings (Figure 3D–H) are consistent with the transcriptional alterations identified in clusters c1 and c2 (Figure 3A–B).

Overall, these functional studies establish a direct link between transcriptional dysregulation and defective erythropoiesis. Moreover, the partial phenocopy observed upon *SEC13* disruption supports the notion that impaired COPII-mediated trafficking contributes to the CDAll phenotype.

Inhibition of glycosylation impairs erythroid differentiation, mimicking defects observed in SEC23B-deficient cells

Given that SEC23B is a core component of the COPII complex involved in endoplasmic reticulum–to–Golgi trafficking, and considering that our transcriptomic and functional analyses revealed defects in erythroid maturation, cellular stress, and cell-cycle progression, we next investigated whether impaired protein glycosylation could mechanistically account for the phenotype observed in *SEC23B*-deficient erythropoiesis.

To disrupt glycosylation during erythropoiesis, we treated HSPCs with different inhibitors targeting distinct glycosylation pathways: luteolin (an O-glycosylation inhibitor), benzyl 2-acetamido-2-deoxy- α -D-galactopyranoside (benzyl; an inhibitor of GalNAc-type O-glycosylation), and swainsonine (an N-glycosylation inhibitor).

We first optimized inhibitor concentrations (Supplemental Figure 5A). Treatment with 15 μ M luteolin and 4 mM benzyl most effectively reduced protein glycosylation and impaired cell growth, whereas alternative doses and swainsonine had limited effects on glycosylation (Supplemental Figure 5A–B).

328 To assess the impact on erythropoiesis, healthy CB-CD34⁺ cells were treated with luteolin
329 (7.5 or 15 μ M), benzyl (2 or 4 mM), or swainsonine (0.5 μ g/mL) on days 6 and 9 of
330 differentiation. Both 15 μ M luteolin and 4 mM benzyl markedly reduced erythroid cell
331 output, exceeding the effect observed in the *SEC23B*^{KO} condition (Figure 4A and
332 Supplemental Figure 5C).

333 Apoptosis analysis revealed a strong induction with 15 μ M luteolin, whereas benzyl
334 induced a milder increase, similar to *SEC23B*^{KO} and insufficient to fully explain the
335 marked reduction in erythroid output (Figure 4B; Supplemental Figure 5D). All inhibitors
336 increased the frequency of bi-/multinucleated erythroblasts, with benzyl closely
337 recapitulating the *SEC23B*^{KO} phenotype and luteolin producing a more pronounced
338 effect (Figure 4C and Supplemental Figure 5E).

339 Cell-cycle alterations were also evident. Treatment with glycosylation inhibitors resulted
340 in increased G1 and M-phase fractions together with reduced G2, suggesting
341 perturbation of the G2–M transition (Figure 4D and Supplemental Figure 5F).

342 To determine whether these effects preferentially impact late erythroid differentiation,
343 inhibitors were added only at day 9. As expected, combined day 6 + day 9 treatment
344 caused broader defects than those observed in *SEC23B* deficiency. In contrast, day 9-
345 only treatment (Figure 4E–H) resulted in milder phenotypes closely resembling the
346 sgKO1+2+3 condition, particularly at lower doses.

347 Under these conditions, both inhibitors similarly reduced erythroid output (Figure 4E)
348 and induced apoptosis patterns consistent with *SEC23B* deficiency, especially with
349 15 μ M luteolin (Figure 4F). The frequency of bi-/multinucleated erythroblasts was
350 comparable to that of *SEC23B*^{KO} (Figure 4G). Cell-cycle analysis revealed modest

351 reductions in G2 together with slight increases in M-phase, indicating perturbation—
352 rather than arrest—at the G2–M transition (Figure 4H).

353 Together, these findings demonstrate that inhibition of protein glycosylation
354 recapitulates the key cellular features of *SEC23B* deficiency, including impaired erythroid
355 maturation, increased apoptosis, multinucleation, and altered cell-cycle progression.
356 These results support a model in which defective glycosylation represents a key
357 downstream mechanism linking impaired COPII function to ineffective erythropoiesis in
358 CD41.

359

Discussion

CDAII is caused by mutations in the *SEC23B* gene, a core component of the COPII complex required for protein trafficking from the endoplasmic reticulum to the Golgi apparatus. While the role of *SEC23B* in vesicular transport is well established, how its deficiency leads to ineffective erythropoiesis and nuclear abnormalities remains incompletely understood. Here, we developed a human gene-edited HSPC model that faithfully recapitulates the main features of CDAII, including impaired erythroid differentiation, hypoglycosylation, and increased bi-/multinucleated erythroblasts, both *in vitro* and *in vivo*. Our findings identify *SEC23B* as a critical regulator of erythroid maturation and reveal a previously underappreciated link between COPII function, protein glycosylation, and cell-cycle control.

Using primary human HSPCs, we show that *SEC23B* deficiency reproduces key disease hallmarks observed in CDAII patients, including defective terminal erythroid differentiation and the accumulation of bi-/multinucleated erythroid cells. These findings are consistent with previous reports (6) but extend them by demonstrating that a CRISPR/Cas9-edited primary human model closely mirrors patient-derived phenotypes. Importantly, the phenotype was also recapitulated *in vivo* following xenotransplantation, where *SEC23B*-deficient cells showed a marked reduction in terminal erythroid output together with increased multinuclearity. These results establish this model as a robust platform to study CDAII pathophysiology under physiologically relevant conditions.

Transcriptomic analysis further revealed that *SEC23B* deficiency disrupts erythroid differentiation programs while promoting cellular stress responses and altering the

expression of key cell-cycle regulators. Upregulation of genes typically associated with immature or non-erythroid populations, together with downregulation of genes involved in terminal erythroid function, confirms a delay in erythroid maturation. However, given that transcriptomic profiling was performed on bulk cultures collected at fixed time points, we cannot fully distinguish whether these changes reflect direct transcriptional effects of *SEC23B* loss or shifts in cellular composition due to delayed differentiation. Addressing this question will require future studies using single-cell approaches and stage-matched populations.

A key finding of our study is the identification of altered expression of genes involved in mitotic control, including *PTTG1*, *CDKN3*, *KIF22*, and *NUF2*. Functional disruption of these genes, as well as COPII components such as *SEC13*, partially recapitulated defects in erythroid output, survival, and cell-cycle dynamics, supporting a causal link between altered cell-cycle regulation and ineffective erythropoiesis. These results suggest that *SEC23B* deficiency disrupts the coordination between cell division and erythroid maturation, a hallmark feature of CDAll and other CDAs.

Mechanistically, our data support a model in which defective COPII-mediated trafficking leads to impaired protein glycosylation, which in turn contributes to erythroid dysfunction. Pharmacological inhibition of glycosylation reproduced the key features of *SEC23B* deficiency, including reduced erythroid output, increased apoptosis, multinuclearity, and alterations in cell-cycle progression, particularly affecting the G2–M transition. Of note, late-stage inhibition produced a phenotype that closely resembled *SEC23B*-deficient cells, suggesting that glycosylation defects play a critical role during the final steps of erythroid differentiation.

Several lines of evidence suggest that altered glycosylation may directly impact mitotic regulation. For example, glycosylation-dependent regulation of proteins such as NuMA and APC/C components has been shown to influence spindle organization and cell-cycle progression (30,31). Although these processes have not been fully explored in erythropoiesis, our findings raise the possibility that hypoglycosylation in CDAll contributes to mitotic abnormalities and the generation of bi-/multinucleated erythroblasts, potentially through mechanisms such as mitotic slippage.

Interestingly, different CDA subtypes are associated with mutations in genes involved in cell-cycle control, including *CDAN1*, *KIF23* (32), *RACGAP1* (33), *KLF1* (34), and *GATA1* (35). This convergence further supports the notion that defective coordination between cell-cycle progression and erythroid differentiation is a central feature of these diseases.

Finally, our findings have potential therapeutic implications. Recent studies have shown that pharmacological inhibition or genetic targeting of LSD1 can restore *SEC23A* expression and rescue erythroid defects in *SEC23B*-deficient human HSPCs(20). In the context of our results, which identify disrupted transcriptional programs and cell-cycle imbalance as key drivers of the phenotype, these approaches might act by compensating for the loss of *SEC23B* function. Thus, combining mechanistic insights from our model with targeted epigenetic interventions could provide a promising strategy for the treatment of CDAll.

Despite these advances, several questions remain unresolved. It is still unclear how *SEC23B* deficiency leads to the downregulation of mitotic regulators such as *NUF2* and *PTTG1*, and whether these changes are direct or secondary to impaired differentiation. In addition, the specific glycosylation targets that regulate cell-cycle progression in

erythroid cells remain to be identified. Future studies combining proteomics and single-cell approaches will be essential to dissect these mechanisms.

In summary, our study demonstrates that *SEC23B* deficiency disrupts erythropoiesis through combined effects on protein glycosylation, transcriptional regulation, and cell-cycle control. These findings provide new insight into CDAll pathogenesis and establish a human HSPC-based platform for mechanistic studies and therapeutic development.

Conclusions

In summary, our human cellular model of *SEC23B* deficiency faithfully recapitulates the key features of CDAll and provides mechanistic insight into its pathogenesis. Our data support a model in which impaired COPII-mediated trafficking leads to defective protein glycosylation, resulting in dysregulation of erythroid transcriptional programs, disruption of cell-cycle progression, and ineffective erythropoiesis.

These findings identify a functional link between *SEC23B*, protein glycosylation, and mitotic integrity, suggesting that defective coordination between cell-cycle progression and erythroid differentiation underlies the multinucleated phenotype characteristic of CDAll.

Overall, our study establishes a robust human platform to investigate CDAll pathophysiology and provides a framework for the development and evaluation of novel therapeutic strategies aimed at restoring effective erythropoiesis.

451 **Declarations**

452 ***Ethics approval and consent to participate***

453 Umbilical CB and PB samples from healthy donors and CDAll patients were collected with
454 written informed consent and approved by institutional review boards (Centro de
455 Transfusión de la Comunidad de Madrid, Universidad Internacional de Catalunya, and
456 Fundación Jiménez Díaz). All procedures followed the Declaration of Helsinki. Animal
457 studies were conducted in compliance with European (Directive 2009/41/CE) and
458 Spanish (Law 9/2003; RD 178/2004) regulations, with protocols approved by the CIEMAT
459 Animal Ethics Committee and the “Consejería de Medio Ambiente y Ordenación del
460 Territorio” (PROEX 283.5/20).

461 ***Consent for publication***

462 Not applicable.

463 ***Availability of data and materials***

464 For original data or additional information, please contact oscar.quintana@ciemat.es,
465 jc.segovia@ciemat.es, and mercedesdetsy95@gmail.com. Data from RNA-Seq and
466 differential expression analyses are available at GEO repository under accession number
467 GSE230875. All remaining data are included in this published article and its
468 supplementary information files.

469 ***Competing interests***

470 The authors declare that they have no competing interests.

471

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480 ***Authors’ contributions***

481 MDR, JCS and OQB conceived and designed the experiments. MDR, SFB, RS, OA, R G-E
482 and OQB conducted the experiments. MMM, VV, SP, MPRA, MM, AOC, LGF, MVP, LS,
483 AIPV, AG, CT, GH, PB and MS provided reagents and patient samples. SP, PB and MS
484 contributed with ideas. MDR, JCS and OQB wrote the manuscript. Funding
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Figure legends

Figure 1. *In vitro* erythroid potential of *SEC23B*-deficient hematopoietic stem and progenitor cells (HSPCs). A) Number of hematopoietic progenitors expressed in colony-forming units (CFUs) per 1000 cells, referring to myeloid CFUs (including CFU-GMs [granulocyte-macrophage progenitors], erythroid progenitors (burst-forming unit-erythroid [BFU-E] and colony-forming unit-erythroid [CFU-E]) and CFU-GEMMs [granulocyte erythroid macrophage megakaryocyte colony forming unit]. Left panel: PB-CD34⁺ cells from healthy donors (HDs) (n=5) and CDAll patients (n=5). Right panel: CB-CD34⁺ cells nucleofected only with Cas9 (Mock) (n=10), with sgKO3 (n=5) or sgKO1+2+3 (n=9). **B)** Cell proliferation analysis during *in vitro* erythroid differentiation in liquid culture of PB-CD34⁺ cells from HDs (n=14) and CDAll patients (n=9) (left panel), and Mock (n=12), sgKO3 (n=7), and sgKO1+2+3 (n=12) (right panel). **C)** Erythroid phenotype analysis by flow cytometry at the end of the *in vitro* erythroid differentiation (day 14), derived from PB-CD34⁺ cells of from HDs (n=14) and CDAll patients (n=9) (left panel), and CB-CD34⁺ cells nucleofected only with Cas9 (Mock) (n=9), with sgKO3 (n=4) or sgKO1+2+3 (n=8) (right panel). Erythroid populations were identified using CD71 and CD235a. **D)** May-Grünwald Giemsa staining at the end of the *in vitro* erythroid differentiation (day 14). Upper images: representative cytopins from HDs and CDAll samples. Lower images: representative cytopins from Mock, sgKO3, or sgKO1+2+3 *SEC23B*^{KO} HSPC-derived erythroid cells. Black arrows highlight bi/multinucleated erythroblasts. Graphs: quantification of bi/multinucleated cells of erythroid cells derived from HD (n=6) and CDAll (n=7) PB-HSPCs (left graph), and derived from CB-CD34⁺ cells nucleofected only with Cas9 (Mock) (n=7), with sgKO3 (n=3) or sgKO1+2+3 (n=7) (right graph). Percentages were determined from 10 different micrographs per sample. Non-

parametric unpaired Mann-Whitney tests or unpaired Kruskal-Wallis tests with Dunn's post-hoc correction were used; graphs show mean±SD (A, B and C) or median and interquartile range (D), and corresponding p-value.

Figure 2. Impact of *SEC23B*^{KO} in human hematopoietic progenitors (CB-CD34⁺) during

***in vivo* erythroid differentiation. A)** Human hematopoietic engraftment (hCD45⁺)

assessed by flow cytometry in mouse bone marrow (BM) 2 months post-transplant (mpt)

with wild type CB-CD34⁺ cells (Mock) (n=9) and *SEC23B*^{KO} CB-CD34⁺ cells nucleofected

with sgKO3 (n=8) and with sgKO1+2+3 (n=7). **B)** Frequency of hematopoietic lineages

(myeloid cells [CD33⁺], lymphoid cells [CD19⁺], and progenitor subset [CD34⁺]) assessed

by flow cytometry within the human hematopoietic engraftment in mouse BM 2mpt

with wild type CB-CD34⁺ cells (Mock) (n=9) and *SEC23B*^{KO} CB-CD34⁺ cells nucleofected

with sgKO3 (n=7) and with sgKO1+2+3 (n=7). **C)** Frequency analysis of the different

erythroid differentiation stages (hCD71⁺/hCD235a⁻, hCD71⁺/hCD235a⁺ and hCD71⁻

/hCD235a⁺) assessed by flow cytometry in mouse BM 2 mpt with wild type CB-CD34⁺

cells (Mock) (n=9) and *SEC23B*^{KO} CB-CD34⁺ cells nucleofected with sgKO3 (n=8) and with

sgKO1+2+3 (n=7). **D)** May-Grünwald Giemsa staining and quantification of bi-

/multinucleated cells of sorted hCD71⁺/hCD235a⁻ erythroid population from the bone

marrow of the mice 2 mpt with wild type CB-CD34⁺ cells (Mock) (n=6) and *SEC23B*^{KO} CB-

CD34⁺ cells nucleofected with sgKO3 (n=6) and with sgKO1+2+3 (n=3). Left,

representative images. Right, graph obtained from the quantification of

bi-/multinucleated cells. **E)** May-Grünwald Giemsa staining and quantification of bi-

/multinucleated cells of sorted hCD71⁻/CD235a⁺ erythroid population from the bone

marrow of the mouse 2 mpt wild type CB-CD34⁺ cells (Mock) (n=6) and *SEC23B*^{KO} CB-

CD34⁺ cells nucleofected with sgKO3 (n=6). Left, representative images. Right, graph

676 obtained from the quantification of bi-/multinucleated cells. Black arrows highlight bi-
677 /multinucleated cells. ND, non-determined. Each dot represents a single animal. Data
678 were collected from two independent experiments, each using at least three different
679 cord blood samples. Lognormal ANOVA with Tukey's multiple comparison tests for
680 experiments involving human hematopoietic engraftment, and ordinary ANOVA with
681 Tukey's multiple comparison tests for analyzing bi-/multinucleated cell frequency; graphs
682 show median and interquartile range, and the p-value. When p-value is not indicated, it
683 is $p > 0.9999$.

684 **Figure 3. Transcriptome analysis of erythroid cells derived from *SEC23B*^{KO} human**
685 **hematopoietic stem and progenitor cells. A)** RNA-Seq analysis at day 9 of *in vitro*
686 erythropoiesis of wild-type (Mock, n=3) and *SEC23B*^{KO} (n=3) CB-CD34⁺ cells. Cluster 1 (c1,
687 red square) contains genes upregulated in *SEC23B*^{KO} cells, and cluster 2 (c2, blue square)
688 genes downregulated (see GSE230875), analysed using two-way ANOVA. **B)** GO-BP in c1
689 (upper panel) and c2 (bottom panel). **C)** RNA-Seq analysis at days 9 and 14 of *in vitro*
690 erythropoiesis cells derived from wild type (Mock) (n=3) and *SEC23B*^{KO} CB-CD34⁺ cells
691 (n=3). Cluster 3 (c3, black square) represented genes upregulated during differentiation
692 in Mock cells but downregulated in *SEC23B*^{KO} cells. Cluster 4 (c4, green square)
693 comprised genes upregulated at day 14 in both conditions, while cluster 5 (c5, blue
694 square) contained genes oppositely regulated between Mock and *SEC23B*^{KO} cells (see
695 GSE230875). **D)** Number of hematopoietic progenitors expressed in CFUs per 1000 cells,
696 referring to myeloid CFUs (CFU-GMs, BFU-E and CFU-E) and CFU-GEMMs, of CB-CD34⁺
697 cells under different conditions. **E)** Quantification of different erythroid populations
698 (CD71⁺/CD235a⁻, CD71⁺/CD235a⁺ and CD71⁻/CD235a⁺) at the end of the *in vitro*
699 erythropoiesis (day 14) of CB-CD34⁺ cells under different conditions. **F)** Apoptosis

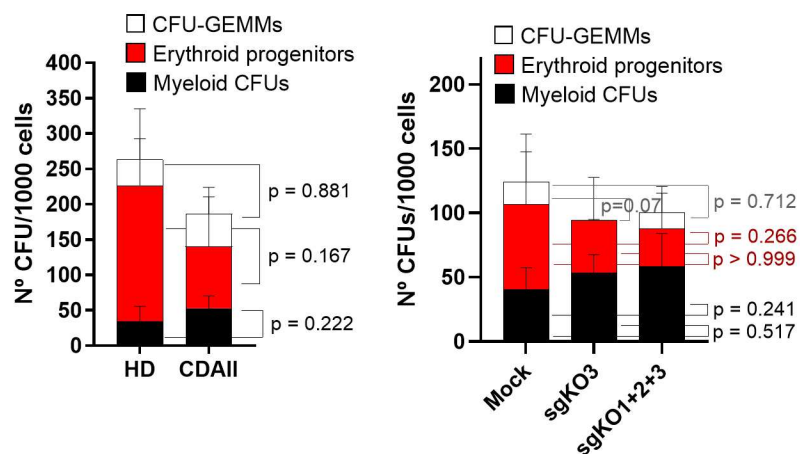
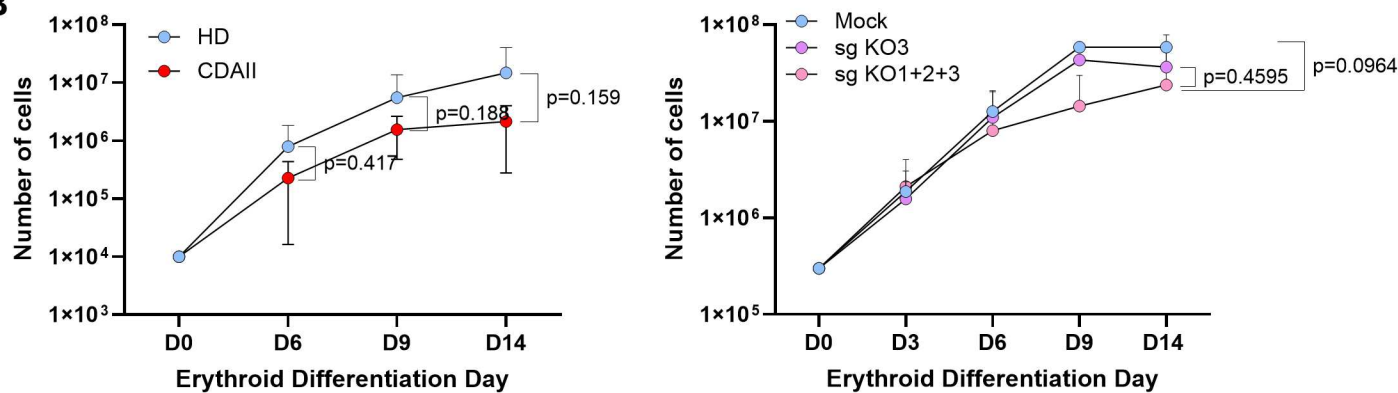
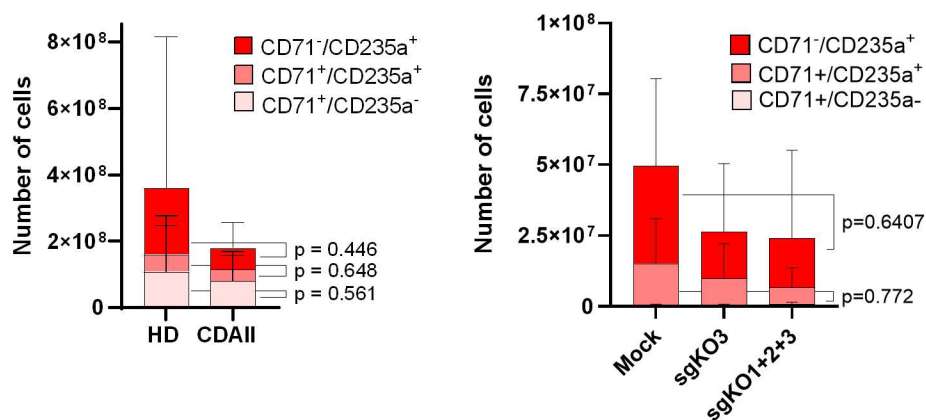
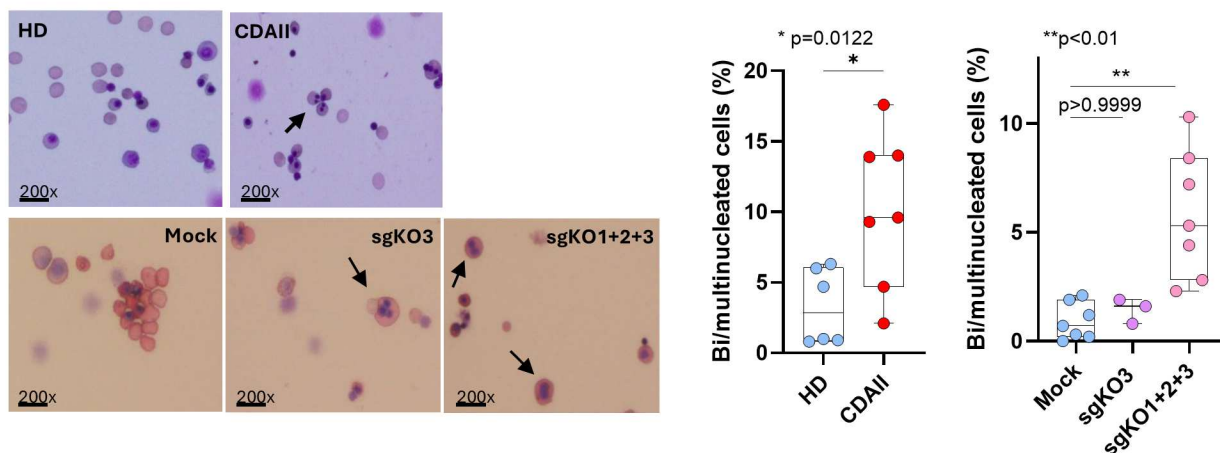
analysis on day 9 of *in vitro* erythropoiesis of CB-CD34⁺ cells under different conditions.

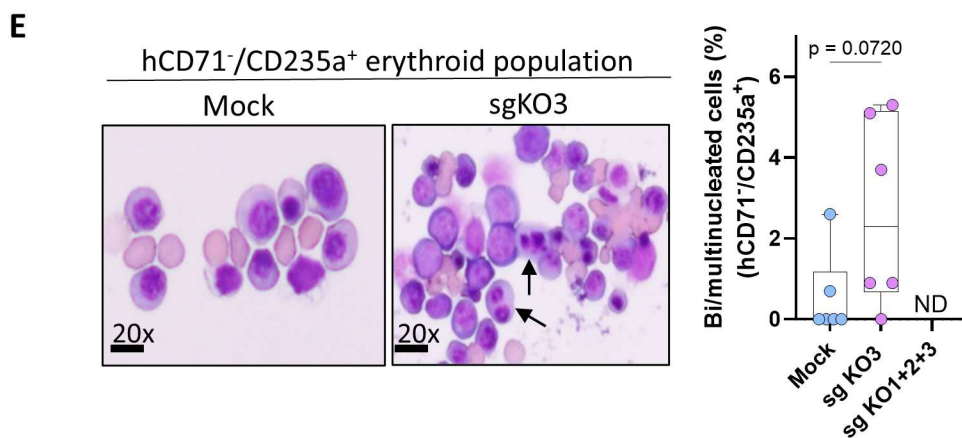
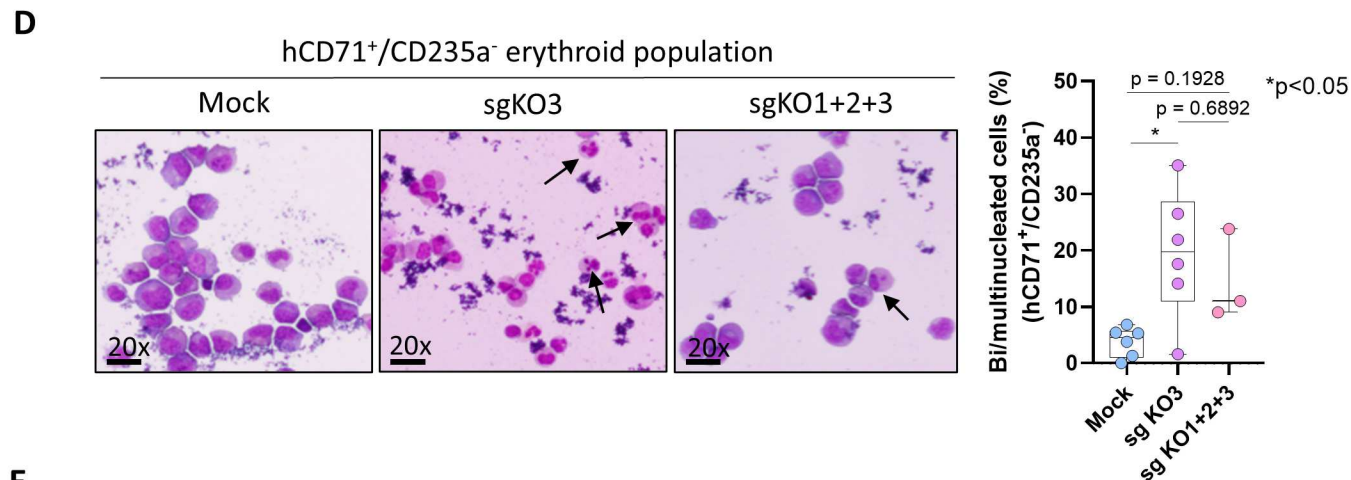
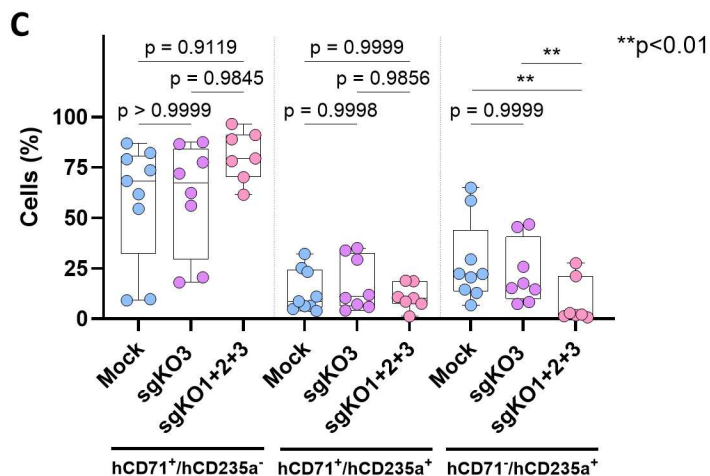
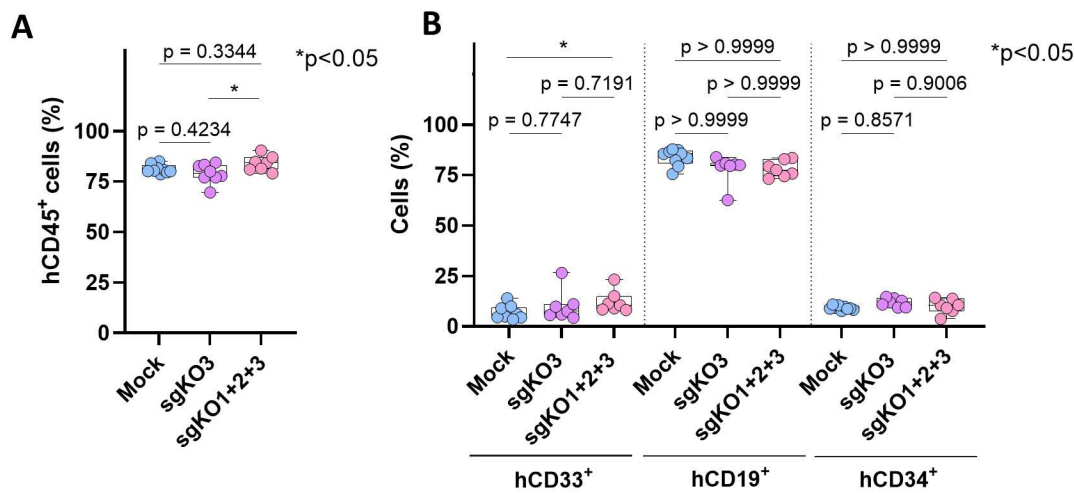
G) Quantification of bi/multinucleated cells of CB-CD34⁺ cells under different conditions at the end of the *in vitro* erythropoiesis (day 14). Percentages were estimated from 10 different micrographs per sample. **H)** Percentage of cell-cycle phases at day 9 of *in vitro* erythropoiesis of CB-CD34⁺ cells under different conditions. All experiments are biological replicates with different cord blood samples. N=4 for all different conditions in Figures C, D, E, F and G. Statistical analyses included ANOVA with Tukey's multiple comparison test and Kruskal–Wallis with Dunn's test. Data are presented as mean ± SD; when no p value is indicated, p > 0.9999.

Figure 4. Glycosylation inhibition of erythroid cells derived from human hematopoietic

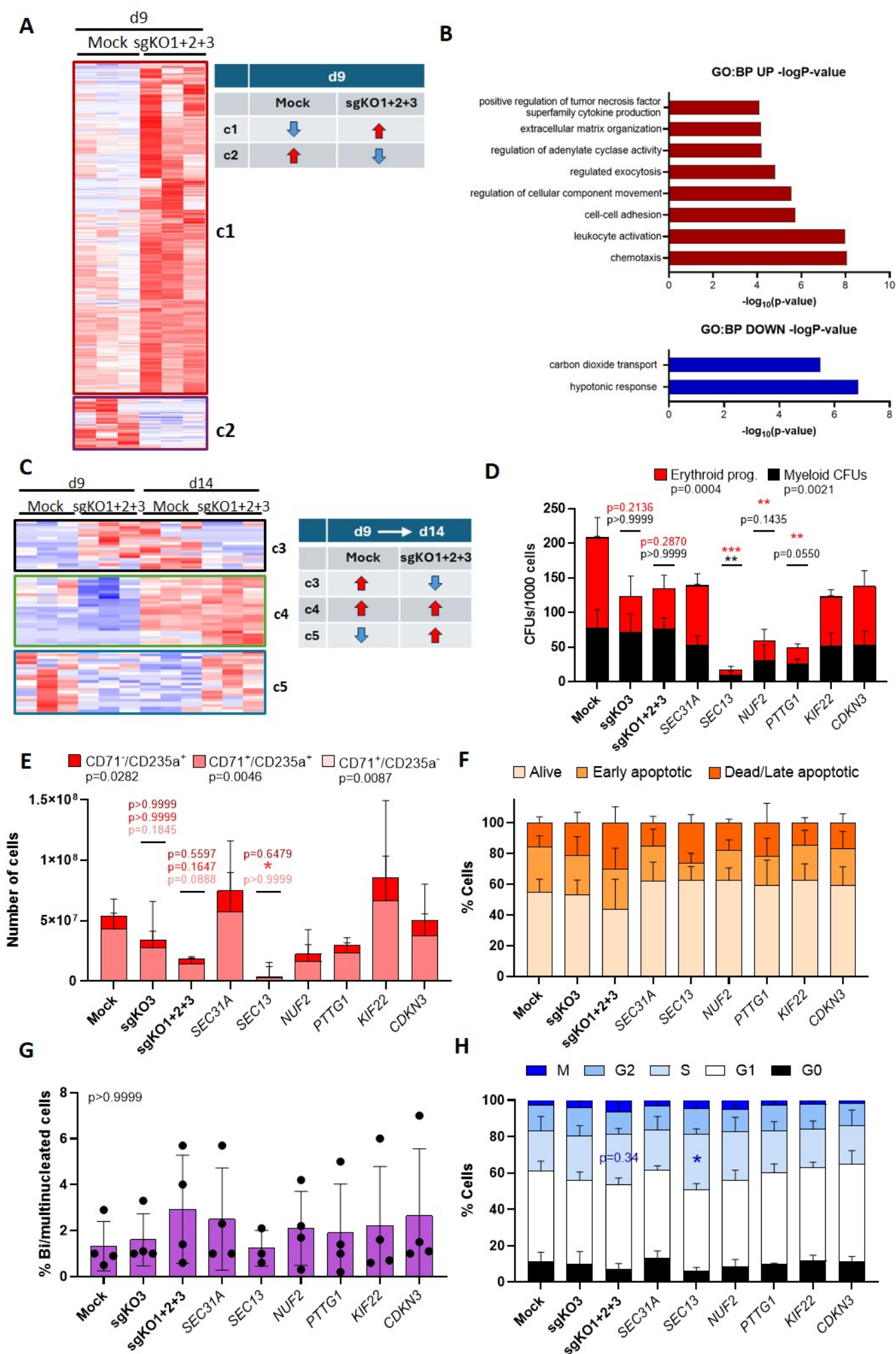
stem and progenitor cells. A) Erythroid phenotype (CD71⁺/CD235a⁻, CD71⁺/CD235a⁺ and CD71⁻/CD235a⁺) at the end of *in vitro* erythropoiesis (day 14) of CB-CD34⁺ cells nucleofected with sgKO1+2+3 or treated with the different glycosylation inhibitors added at days 6 and 9 (n from 3 to 6). **B)** Apoptosis analysis at the end of *in vitro* erythropoiesis (day 14) under the same conditions as in A (n from 3 to 6). **C)** Quantification of bi/multinucleated cells at the end of *in vitro* erythropoiesis (day 14) under the same conditions as in A. Percentages were estimated in 10 different micrographs per sample. **D)** Percentage of the different cell-cycle phases at the end of *in vitro* erythropoiesis (day 14) under the different conditions as in A (n from 3 to 6). **E)** Erythroid phenotype at the end of *in vitro* erythropoiesis (day 14) of CB-CD34⁺ cells nucleofected with sgKO1+2+3 or treated with the different glycosylation inhibitors added at day 9. **F)** Apoptosis analysis at the end of *in vitro* erythropoiesis (day 14) under the different conditions as in E. **G)** Quantification of bi/multinucleated cells at the end of *in vitro* erythropoiesis (day 14) under the different conditions as in E. Percentages were

estimated in 10 different micrographs per sample. **H)** Percentage of the different cell-cycle phases at the end of *in vitro* erythropoiesis (day 14) under the different conditions as in E. All experiments were performed using biological replicates from independent cord blood donors (n = 3). Data were analyzed using a Kruskal–Wallis test. Post hoc pairwise comparisons were performed with Dunn’s test, restricted to comparisons versus the mock group. P values from the Kruskal–Wallis test are reported in the graph legends, highlighting those that reached statistical significance. Significant pairwise differences from Dunn’s test are indicated on the bars as follows: * p < 0.05, ** p < 0.01. ND means non-detectable.

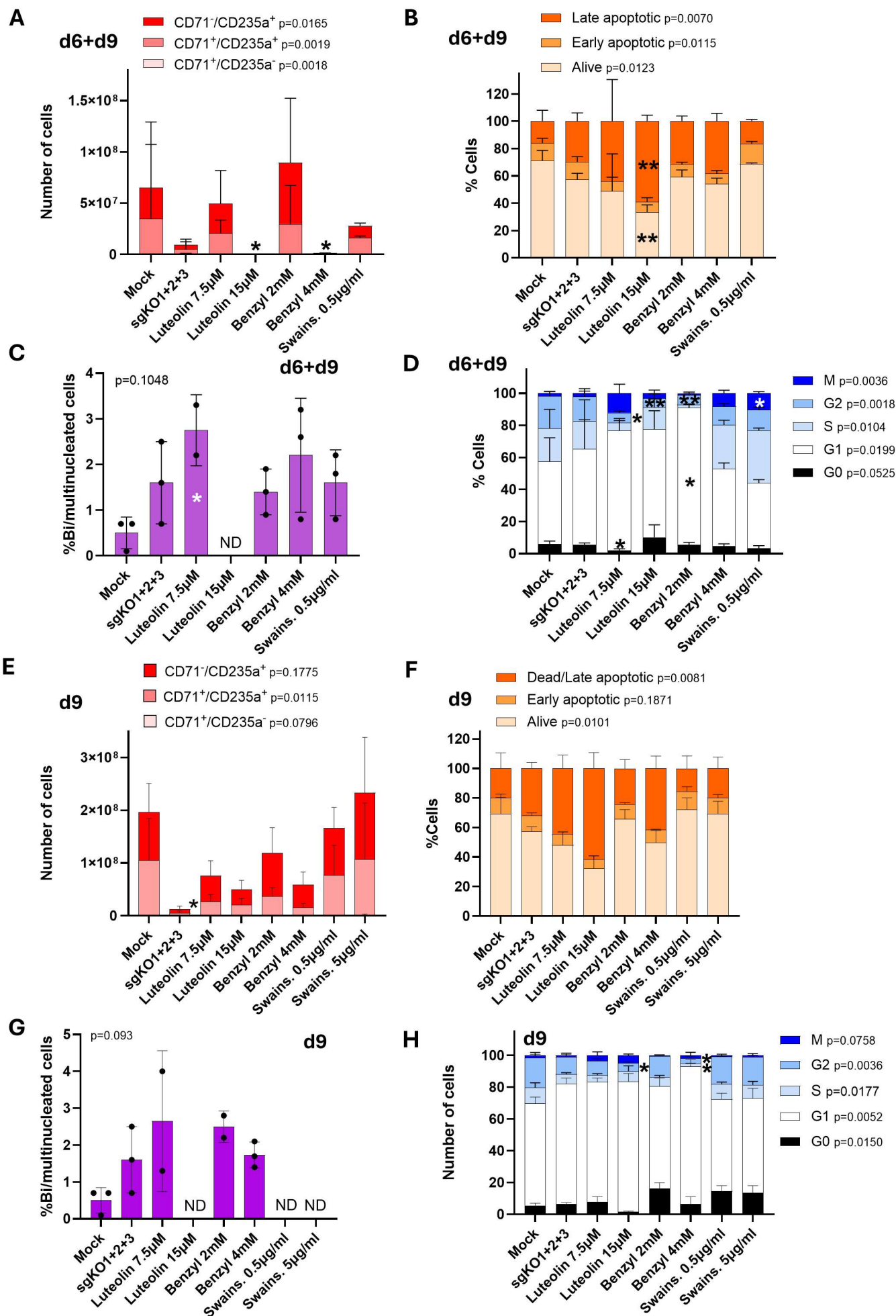
A**B****C****D**



Dessy et al. Figure 2.



Dessy et al. Figure 3.



Dessy et al. Figure 4.

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