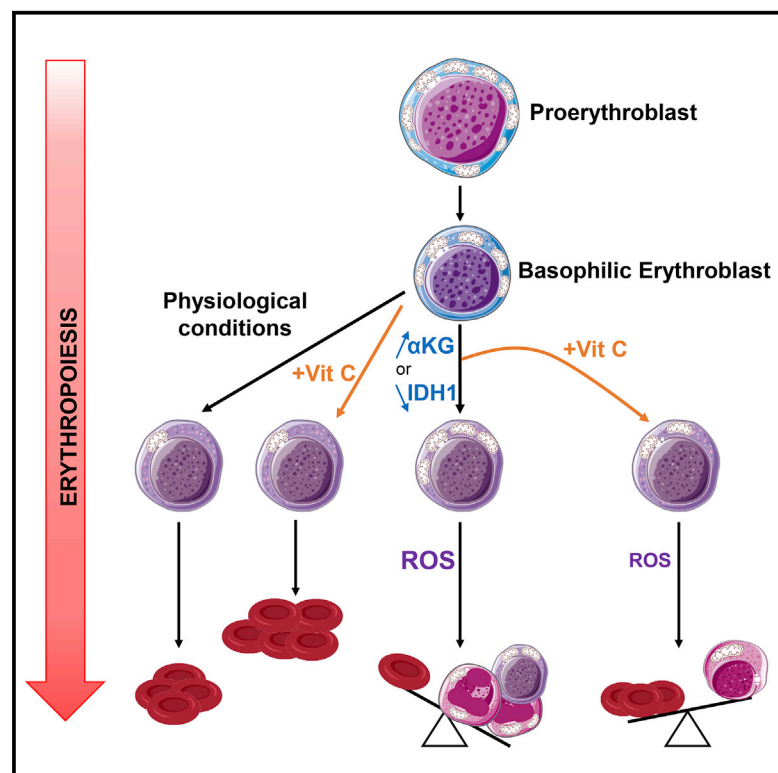


An IDH1-vitamin C crosstalk drives human erythroid development by inhibiting pro-oxidant mitochondrial metabolism

Graphical Abstract



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In Brief

Gonzalez-Menendez et al. show that terminal erythroid differentiation is dependent on the suppression of mitochondrial metabolism. Disturbances in levels of the α -ketoglutarate and IDH1 TCA-cycle regulators result in ineffective erythropoiesis, with generation of morphologically abnormal erythroblasts. Vitamin C restores redox homeostasis and rescues late-stage erythropoiesis by scavenging reactive oxygen species.

Highlights

- Glutamine-dependent OXPHOS drives early erythroid differentiation
- OXPHOS-induced ROS inhibit erythroblast enucleation
- IDH1 downregulation augments ROS, leading to pathological erythroid differentiation
- Vitamin C rescues erythroid differentiation under conditions of oxidative stress



Article

An IDH1-vitamin C crosstalk drives human erythroid development by inhibiting pro-oxidant mitochondrial metabolism

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SUMMARY

The metabolic changes controlling the stepwise differentiation of hematopoietic stem and progenitor cells (HSPCs) to mature erythrocytes are poorly understood. Here, we show that HSPC development to an erythroid-committed proerythroblast results in augmented glutaminolysis, generating alpha-ketoglutarate (α KG) and driving mitochondrial oxidative phosphorylation (OXPHOS). However, sequential late-stage erythropoiesis is dependent on decreasing α KG-driven OXPHOS, and we find that isocitrate dehydrogenase 1 (IDH1) plays a central role in this process. IDH1 downregulation augments mitochondrial oxidation of α KG and inhibits reticulocyte generation. Furthermore, IDH1 knockdown results in the generation of multinucleated erythroblasts, a morphological abnormality characteristic of myelodysplastic syndrome and congenital dyserythropoietic anemia. We identify vitamin C homeostasis as a critical regulator of ineffective erythropoiesis; oxidized ascorbate increases mitochondrial superoxide and significantly exacerbates the abnormal erythroblast phenotype of IDH1-downregulated progenitors, whereas vitamin C, scavenging reactive oxygen species (ROS) and reprogramming mitochondrial metabolism, rescues erythropoiesis. Thus, an IDH1-vitamin C crosstalk controls terminal steps of human erythroid differentiation.

INTRODUCTION

Hematopoietic stem cell (HSC) numbers as well as their differentiation state are tightly regulated throughout the lifetime of an individual, allowing the sustained production of all mature blood lineages under physiological conditions. Circulating mature erythrocytes are a terminally differentiated product of HSCs that have undergone a series of lineage-fate engagements that gradually restrict their potential, resulting in a commitment to the erythroid lineage. Commitment defines the beginning of erythropoiesis; a three-stage process characterized by early erythropoiesis, terminal erythroid differentiation, and reticulocyte maturation. Early erythropoiesis is characterized by commitment of multilineage progenitor cells into erythroid progenitor cells, with proliferation and development into erythroid

burst-forming unit cells, followed by erythroid colony-forming unit cells and proerythroblasts. Terminal erythroid differentiation begins with proerythroblasts differentiating in a stepwise manner to early then late basophilic erythroblasts, polychromatic erythroblasts, and orthochromatic erythroblasts that enucleate to become reticulocytes.

Erythropoiesis is a metabolically daunting process when evaluated at the level of cell numbers. In healthy adults, committed erythroid progenitors support the production of 2.4 million erythrocytes per second via a synchronized regulation of iron, glucose, fatty acid, and amino acid metabolism. Iron is indispensable for heme biosynthesis in erythroblasts (Oburoglu et al., 2016). The utilization of both glutamine and glucose in *de novo* nucleotide biosynthesis is a *sine qua non* for erythroid differentiation (Oburoglu et al., 2014), and glutamine-derived



production of succinyl-coenzyme A (succinyl-CoA) is also required for the production of heme (Burch et al., 2018). Furthermore, amino acids regulate mTOR signaling (Chung et al., 2015) as well as lipid metabolism (Huang et al., 2018) during erythropoiesis. The critical nature of metabolism in erythroid differentiation is further highlighted by the recent identification of “metabolic regulators” as an erythropoietin (EPO)-induced phosphorylation target set (Held et al., 2020).

It is important though to note that terminal erythroid differentiation is a distinctive process; each mitosis results in the production of daughter cells that differ, morphologically and functionally, from their parent cells. This sequential maturation is tightly regulated at each stage of erythroid differentiation, associated with decreased cell size, enhanced chromatin condensation, progressive hemoglobinization, and changes in membrane organization and transcriptome specificities (An et al., 2014; Hu et al., 2013; Li et al., 2014; Ludwig et al., 2019; Schulz et al., 2019). Moreover, during the late stages of mammalian terminal erythroid differentiation, erythroblasts expel their nuclei and lose all organelles, including mitochondria (Moras et al., 2017). Thus, the constraints of a late-stage terminally differentiating erythroblast differ significantly from that of an erythroid-committed progenitor early in erythroid development. Indeed, in mice, early-stage erythroid progenitors have been found to require mTORC1-mediated mitochondrial biogenesis and reactive oxygen species (ROS) production (Liu et al., 2017; Luo et al., 2017; Zhao et al., 2016), while terminal erythropoiesis requires that cells be protected from oxidative stress (Case et al., 2013; Filippi and Ghaffari, 2019; Hyde et al., 2012; Xu et al., 2019; Zhao et al., 2016).

Significant differences in murine versus human erythroid development have been documented, especially as relates to the diversity of erythroid progenitor subpopulations in human bone marrow (BM) (Gautier et al., 2020; Schulz et al., 2019; Yan et al., 2018). Furthermore, regarding oxidative stress, it is important to note that murine and human erythropoiesis differ dramatically as a function of vitamin C/ascorbate plasma concentrations and expression of the SLC2A1/GLUT1 transporter, shuttling dehydroascorbic acid (DHA) across the membrane and rapidly reducing it to ascorbate (reviewed by May et al., 2001). Indeed, humans exhibit a deficiency in vitamin C production, due to inactivation of L-gulonolactone oxidase (GLO), the enzyme that catalyzes the terminal step of L-ascorbic acid (AA) biosynthesis (Burns, 1957). While human erythrocytes express the highest level of the GLUT1 transporter, harboring greater than 200,000 molecules per cell (Helgersson and Caruthers, 1987; Mueckler, 1994), we found that erythroid GLUT1 expression is a specific feature of mammals that have lost the ability to synthesize AA from glucose (Montel-Hagen et al., 2008a, 2008b). This potential evolutionary compensation, and subsequent alterations in the ability of plasma vitamin C to scavenge ROS/reactive nitrogen species (RNS), make it critical to evaluate the stepwise changes that regulate the progression of human erythropoiesis.

Here, we demonstrate that during the early stages of human red blood cell development, erythroid progenitors exhibit increased oxidative phosphorylation (OXPHOS) activity. This correlated with the increased generation of the alpha-ketogluta-

rate (α KG) TCA-cycle intermediate, generated by the anaplerotic utilization of glutamine, and a directly augmented OXPHOS. However, upon terminal differentiation of erythroblasts, mitochondrial biomass, OXPHOS, mitochondrial ROS, and superoxide production were all markedly decreased. Supraphysiological levels of α KG markedly attenuated terminal erythroid differentiation and enucleation. The impact of α KG was directly coupled to its role in mitochondrial metabolism, as enucleation was similarly inhibited by mitochondrial ROS and superoxide, induced by MitoParaquat (MitoPQ) or DHA. Moreover, enucleation was rescued by ROS scavengers, including vitamin C, glutathione (GSH), N-acetylcysteine (NAC), and vitamin E.

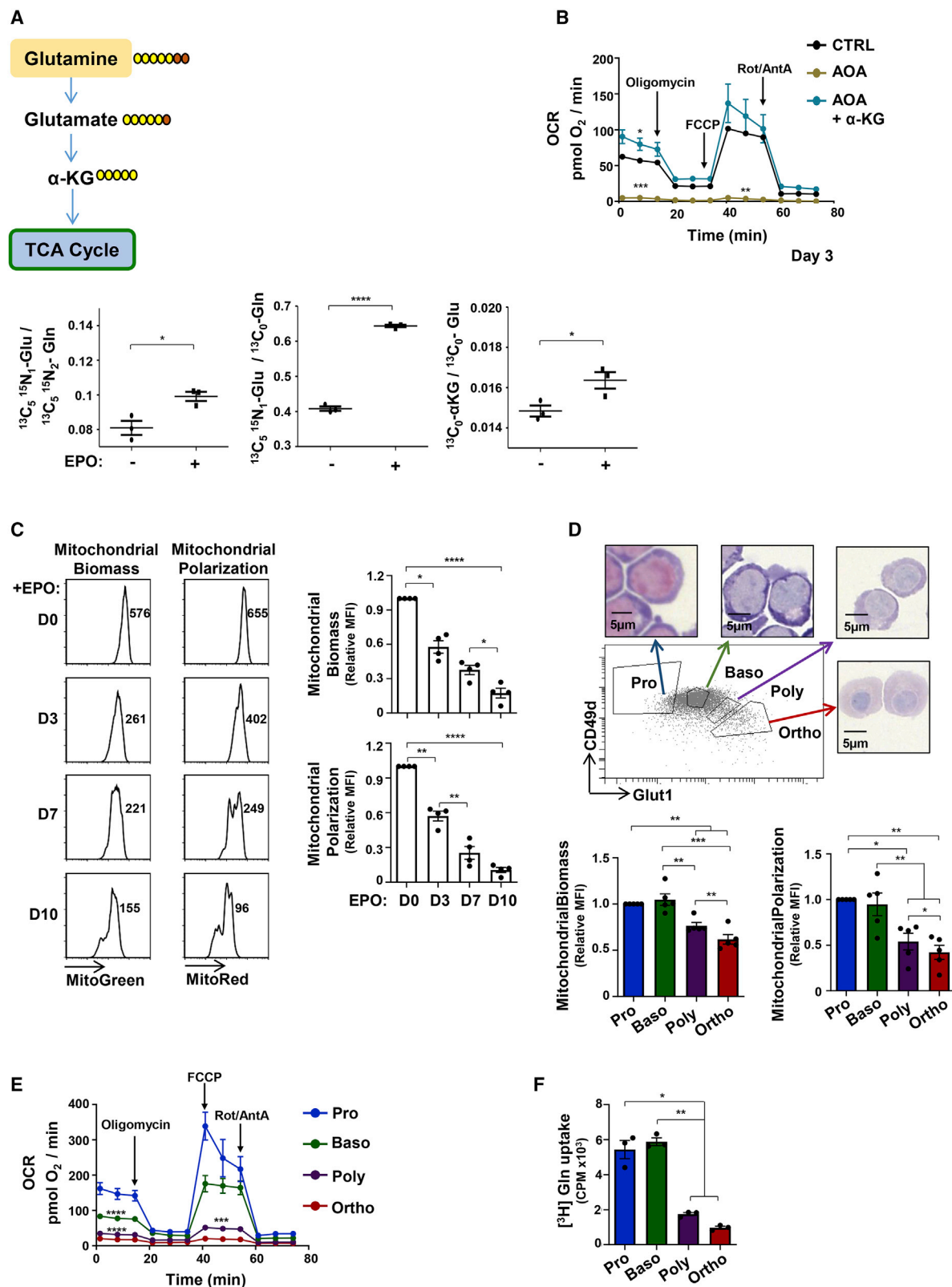
We identified the cytoplasmic isocitrate dehydrogenase 1 (IDH1) enzyme, catalyzing the interconversion between α KG and isocitrate, as a critical enzyme in this process. Lentiviral-mediated downregulation of IDH1, by significantly increasing mitochondrial metabolism and mitochondrial superoxide production, dramatically inhibited enucleation and resulted in the generation of abnormal multinucleated erythroblasts. Notably, both α KG and DHA markedly exacerbated the negative impact of IDH1 downregulation, while the vitamin C antioxidant rescued erythroid differentiation. Indeed, the vitamin-C-mediated quenching of ROS in erythroblasts accelerated human erythroid differentiation. This effect was specific to an IDH1-vitamin C axis; inhibition of TET2 in late-stage erythroblasts did not attenuate enucleation. Thus, our data identify IDH1 as a critical regulator of redox homeostasis, promoting late-stage erythropoiesis. Furthermore, these results highlight the therapeutic potential of vitamin C in human erythropoiesis, especially under pathological conditions where ROS are increased and vitamin C levels are not sufficient to combat oxidative damage.

RESULTS

Early erythropoiesis is associated with augmented glutamine utilization and OXPHOS

As glutaminolysis is required for erythroid lineage specification (Oburoglu et al., 2014), we evaluated the utilization of glutamine during early stages of human erythropoiesis at day 4 following recombinant erythropoietin (rEPO) stimulation, a time point at which the erythroid markers CD36, CD71 (transferrin receptor), and glycophorin A (GlyA) are upregulated (Figure S1A). The anaplerotic contribution of glutamine can be evaluated by tracing glutamine labeled with heavy stable carbons and nitrogens ($^{13}\text{C}_5^{15}\text{N}_2$). As shown in Figure 1A, the portion of glutamate derived from $^{13}\text{C}_5^{15}\text{N}_2$ glutamine, a measure of glutaminase activity, increased significantly following erythropoietin-induced erythroid differentiation of human CD34⁺ progenitors ($p < 0.05$; Figure 1A). Additionally, the contribution of labeled glutamine to the pool of glutamate ($^{13}\text{C}_5^{15}\text{N}_1$ glutamate/ $^{13}\text{C}_0$ glutamate) as well as the portion of $^{13}\text{C}_5$ labeled α KG increased significantly upon erythroid differentiation. This glutamate-derived generation of α KG was critical for OXPHOS in erythroid progenitors, as the oxygen consumption rate (OCR) was abrogated by the aminooxyacetic acid (AOA) transaminase inhibitor, blocking conversion of glutamate to α KG, and was completely restored by α KG supplementation (Figure 1B).

These data show that glutamine supports the tricarboxylic acid (TCA) cycle and downstream OXPHOS via the cataplerotic



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utilization of α KG. As each stage of terminal erythroid differentiation is defined by a decreased cell size with the final stage of erythropoiesis resulting in enucleation and reticulocyte maturation, we assessed whether mitochondrial biomass and transmembrane polarization decrease following commitment of progenitors to an erythroid lineage fate. Indeed, both mitochondrial biomass and polarization, evaluated by MitoGreen and MitoRed staining, respectively, decreased massively upon erythroid differentiation, monitored as a function of day of differentiation (days 0–10) as well as on erythroblast subsets fluorescence-activated cell sorting (FACS) sorted on the basis of their CD49d/GLUT1 staining profiles ($p < 0.05$ to $p < 0.0001$; Figures 1C, 1D, and S1B).

These changes in mitochondrial biomass and polarization had functional consequences on terminal erythroid differentiation (Figure 1D). Erythroblasts at distinct stages of differentiation exhibited significant differences in their levels of OXPHOS, as estimated by their OCRs; basal OCR decreased from 146.9 ± 15.5 to 17.5 ± 0.6 pmol O_2 /min between the proerythroblast and orthochromatic erythroblast stages of differentiation ($p < 0.0001$; Figure 1E). Notably, the respiratory capacities of both polychromatic and orthochromatic subsets were minimal; neither of these terminal subsets exhibited a spare respiratory capacity, as measured by the ability of the cell to increase its respiration in response to an Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP)-mediated disruption of mitochondrial membrane potential (Figure 1E). Similarly, these differences were also detected with increasing time after EPO induction (Figure S1C). Consistent with a critical role for glutamine in the oxidative potential of the erythroblast, glutamine uptake was significantly higher in proerythroblasts and basophilic erythroblasts than in polychromatic/orthochromatic erythroblasts ($p < 0.01$; Figure 1F). Thus, in marked contrast with the high-energy state of early erythroid progenitors, terminal maturation results in a significant attenuation of glutamine uptake and an associated decrease in OXPHOS.

α KG increases mitochondrial function while negatively regulating terminal erythroid differentiation and enucleation

As decreased mitochondrial function of terminally differentiated erythroblasts was associated with decreased glutamine uptake,

we evaluated whether α KG, the anaplerotic product of glutamine that enters into the TCA cycle, directly contributed to the erythroblast's respiratory capacity. Importantly, we found that α KG directly regulates the metabolic state of progenitors, as the injection of a cell-permeable α KG immediately augmented the maximal respiratory capacity of erythroblasts at both day 3 and day 7 of EPO induction ($p < 0.001$ and $p < 0.0001$; Figure 2A). This increase in OXPHOS did not alter the generation of CD34⁺CD36⁺ committed erythroid colony-forming unit (CFU-E) progenitors or GlyA⁺ erythroblasts or the upregulation of CD36 or CD71 erythroid markers (Figures 2B and S2A). While α KG did not alter mitochondrial biomass or polarization at early stages of development (day 3; Figure S2B), it substantially affected these parameters when it was added only at later stages (from days 7 to 10). Mitochondrial biomass and polarization were significantly higher in α KG-supplemented late-stage erythroblasts than in control erythroblasts ($p < 0.01$ and $p < 0.001$; Figure 2C). Furthermore, progression from the CD49d⁺GLUT1⁺ basophilic erythroblast to the CD49d⁺GLUT1⁺ orthochromatic stage was significantly inhibited by α KG ($p < 0.05$ and $p < 0.01$; Figure 2D). This phenomenon was also associated with a significantly attenuated level of enucleation, decreasing by $32\% \pm 7\%$ and $52\% \pm 7\%$ in the presence of α KG at days 10 and 14 of erythroid differentiation, respectively ($p < 0.001$ and $p < 0.01$; Figure 2D). Notably, this effect was specific to α KG and was not recapitulated by other TCA-cycle intermediates such as succinate or citrate (Figure S2C).

Augmented mitochondrial superoxide generation at the basophilic erythroblast stage abrogates terminal erythroid differentiation and enucleation

Generation of ROS within mitochondria is associated with the oxidative function of these organelles (Adam-Vizi and Tretter, 2013; Murphy, 2009), and α KG can directly contribute to ROS generation through the catalytic activity of α KG dehydrogenase (α KGDH) (Starkov et al., 2004; Tretter and Adam-Vizi, 2004). NADH produced by α KGDH also stimulates superoxide production by complex I of the electron transport chain (ETC; Figure 3A) (Adam-Vizi and Tretter, 2013; Murphy, 2009). We therefore evaluated whether addition of α KG, the substrate of α KGDH, alters

Figure 1. Early erythropoiesis results in increased metabolism followed by decreased mitochondrial activity during terminal erythroid differentiation

(A) Schematic representation of glutamine conversion to α -ketoglutarate (α KG), showing the metabolism of [$^{13}C_5^{15}N_2$] heavy-labeled glutamine (Gln) carbons (yellow) and nitrogens (orange; top). Peak area ratios of heavy atoms from [$^{13}C_5^{15}N_2$]Gln into glutamate ([$^{13}C_5^{15}N_1$]Glu), the relative level of [$^{13}C_5^{15}N_1$]Glu, and the portion of [$^{13}C_5$] α KG at day 4 of differentiation in the absence or presence of rEPO are shown (\pm SEM). Horizontal lines represent mean levels. Each dot represents an individual technical replicate, representative of one of two independent experiments.

(B) Oxygen consumption rates (OCRs), a measure of OXPHOS, were monitored by sequential injection of oligomycin, FCCP, and rotenone/antimycin A (Rot/AntA) as indicated. Mean basal oxygen consumption, maximal consumption following FCCP, and minimal levels following inhibition of the electron transport chain (ETC) Rot/AntA (bottom panel) are presented at day 3 of erythroid differentiation in control conditions (CTRL), in the presence of the AOA transaminase inhibitor (1 mM) or AOA + α KG (3.5 mM). Means \pm SEM are presented ($n = 3$).

(C) Mitochondrial biomass and polarization were evaluated by MitoTracker Green and MitoTracker Red staining, respectively, at days 0, 3, 7, and 10 of differentiation ($n = 4$).

(D) Pro-erythroblasts (Pro) and basophilic (Baso), polychromatic (Poly), and orthochromatic (Ortho) erythroblasts were sorted as a function of their GLUT1/CD49d profile (top panel). H&E staining of sorted erythroblasts is shown. Mitochondrial biomass and polarization were evaluated as in (C), and means \pm SEM are presented ($n = 5$).

(E) OCR was monitored 15 h post-sorting and means \pm SEM are presented ($n = 6$).

(F) Glutamine uptake was monitored 18 h following sorting using L-[3,4- 3H]glutamine (0.5 μ Ci) for 10 min at room temperature (RT). Uptake is expressed as mean counts per minute (CPM) for triplicate samples \pm SEM.

Statistical significance was determined by a one-way ANOVA with Tukey's post hoc test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

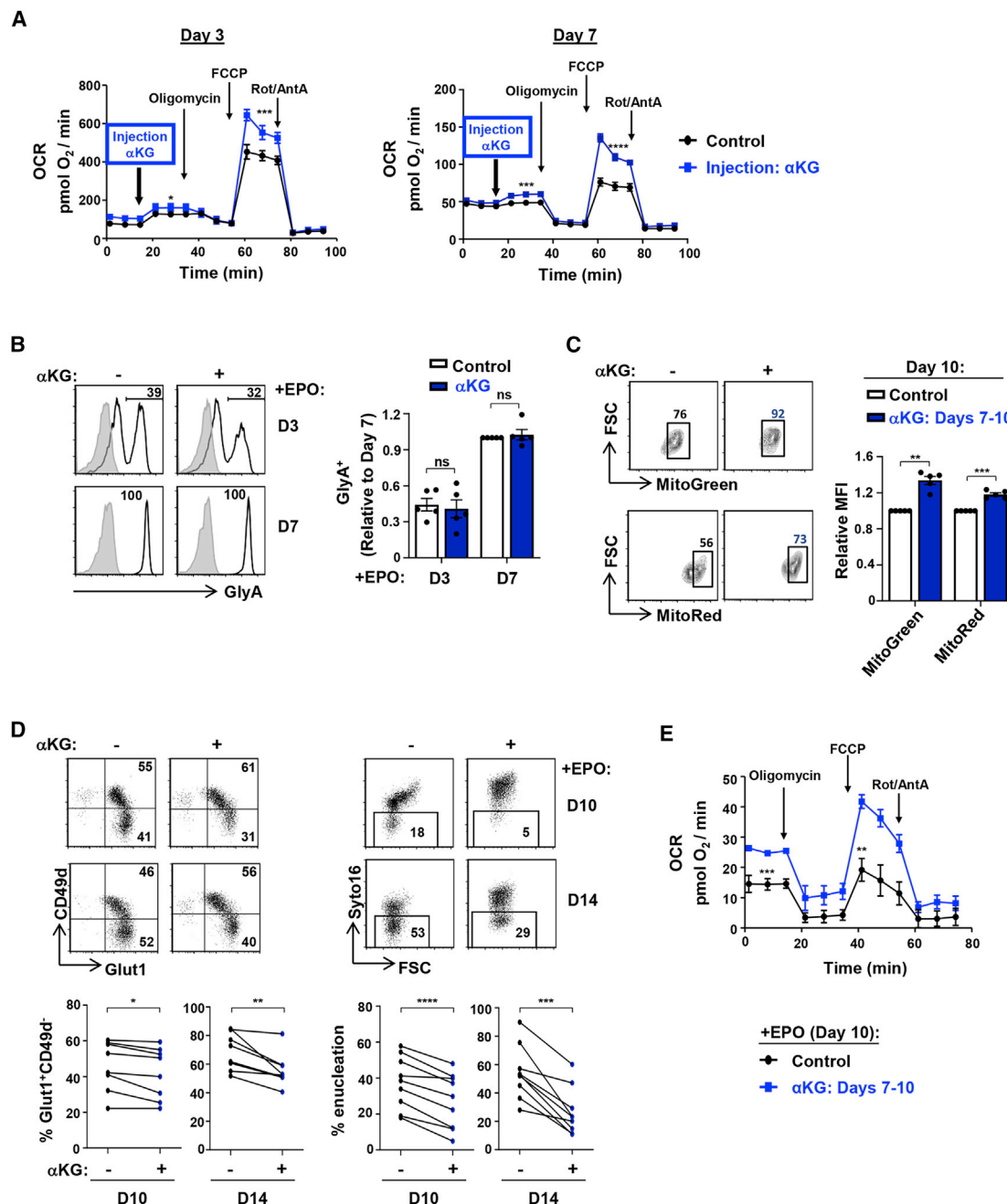


Figure 2. αKG increases mitochondrial function while significantly inhibiting terminal erythroid differentiation and enucleation

(A) CD34⁺ HSPCs were differentiated with EPO, and OXPHOS was evaluated at days 3 (left) and 7 (right) directly following injection of cell-permeable αKG (3.5 mM, blue line) into the Seahorse analyzer. Mean OCR ± SEM are presented for the indicated conditions (n = 5–6 per time point).

(B) Progenitors were stimulated with rEPO in the absence (–) or presence (+) of αKG. Expression of GlyA was monitored at days 3 and 7, and representative histograms (left) are shown (gray histograms, isotype controls; black lines, specific staining). Mean levels of GlyA⁺ cells were quantified in the different conditions (right), with levels at day 7 arbitrarily set at 1 (means ± SEM of five independent experiments; ns, nonsignificant).

(C) Mitochondrial biomass and mitochondrial membrane potential (MMP) were monitored following erythroid differentiation in the absence (–) or presence (+) of αKG (between days 7 and 10). Representative dot plots showing the percentages of cells with high mitochondrial biomass and MMP at day 10 are presented (left). Mean biomass and MMP (±SEM) are presented (right; n = 5).

(D) CD49d/Glut1 profiles of GlyA⁺ erythroblasts differentiated in the absence or presence of αKG (starting at day 7) were monitored at days 10 and 14 of differentiation, and representative dot plots are shown (top left). Percentages of Glut1⁺CD49d[–] orthochromatic erythroblasts are shown for eight individual donors (bottom left). Erythroblast enucleation was evaluated as a function of Syto16 nucleic acid staining, and representative dot plots indicating the percentages of Syto16[–] enucleated cells are presented (top right). Percentage enucleation in nine individual donors is presented (bottom right).

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the redox state of the cell. Direct measurement of mitochondrial superoxide with the fluorescent MitoSOX indicator showed that α KG induced a 1.3-fold increase in superoxide ($p < 0.01$) as well as an overall increase of 1.3-fold in all mitochondrial ROS ($p < 0.001$), monitored as a function of DHR123 fluorescence (MitoROS) (Figure 3B).

These data suggested that the negative impact of α KG on erythroid maturation was linked to its role in altering the erythroblast redox state. We therefore assessed whether directly increasing mitochondrial superoxide would inhibit erythroid maturation. Erythroblasts were treated with MitoPQ, a newly synthesized paraquat compound that is targeted to the mitochondria by conjugation to the lipophilic tri-phenylphosphonium cation (Robb et al., 2015). This compound selectively increases superoxide production within mitochondria at 1,000-fold higher efficiency than untargeted paraquat (PQ) (Robb et al., 2015); within the mitochondria, MitoPQ^{2+} is reduced to the radical monocation at the flavin site of complex I and the monocation then reacts with O_2 to generate superoxide (O_2^-) (Figure 3C). MitoPQ dramatically increased mitochondrial superoxide (MitoSOX), MitoROS, and mitochondrial biomass (MitoGreen) in differentiating erythroblasts ($p < 0.01$ and $p < 0.05$; Figure 3D). The impact of both MitoPQ and α KG was specific to mitochondrial ROS, as total intracellular ROS levels were not significantly changed (Figures S3A and S3B). Notably, short-term MitoPQ treatment significantly altered late erythropoiesis, with a $25\% \pm 5\%$ decrease in erythroblast enucleation ($p < 0.01$; Figure 3E).

To assess whether mitochondrial superoxide production has similar effects on erythroblasts at different stages of differentiation, we FACS-sorted early basophilic and late orthochromatic erythroblasts on the basis of their GLUT1/CD49d profiles. Representative cytopins are shown in Figure 3F. Notably, MitoPQ dramatically decreased the ability of basophilic erythroblasts to differentiate to reticulocytes, attenuating enucleation by $94\% \pm 3\%$ ($p < 0.001$; Figure 3F). In contrast, the maturation of orthochromatic erythroblasts was not affected by MitoPQ treatment (Figure 3F). These data show that a cell type that exhibits only very minimal OXPHOS potential, such as an orthochromatic erythroblast (Figure 1E), is not sensitive to a drug whose impact is dependent on mitochondrial function. In contrast, selectively increasing superoxide production in the mitochondrial matrix of an early erythroblast significantly inhibits erythroid maturation, revealing the critical role of this pathway in the proper progression of erythroid differentiation.

DHA and vitamin C have opposing effects on erythroid maturation

As we found that oxidative stress, monitored as a function of mitochondrial superoxide production and OXPHOS, was associated with attenuated late-stage erythroid differentiation and enucleation, it was of interest to determine whether these parameters were directly responsible for these observed effects. Vitamin C has the potential to promote a redox environment,

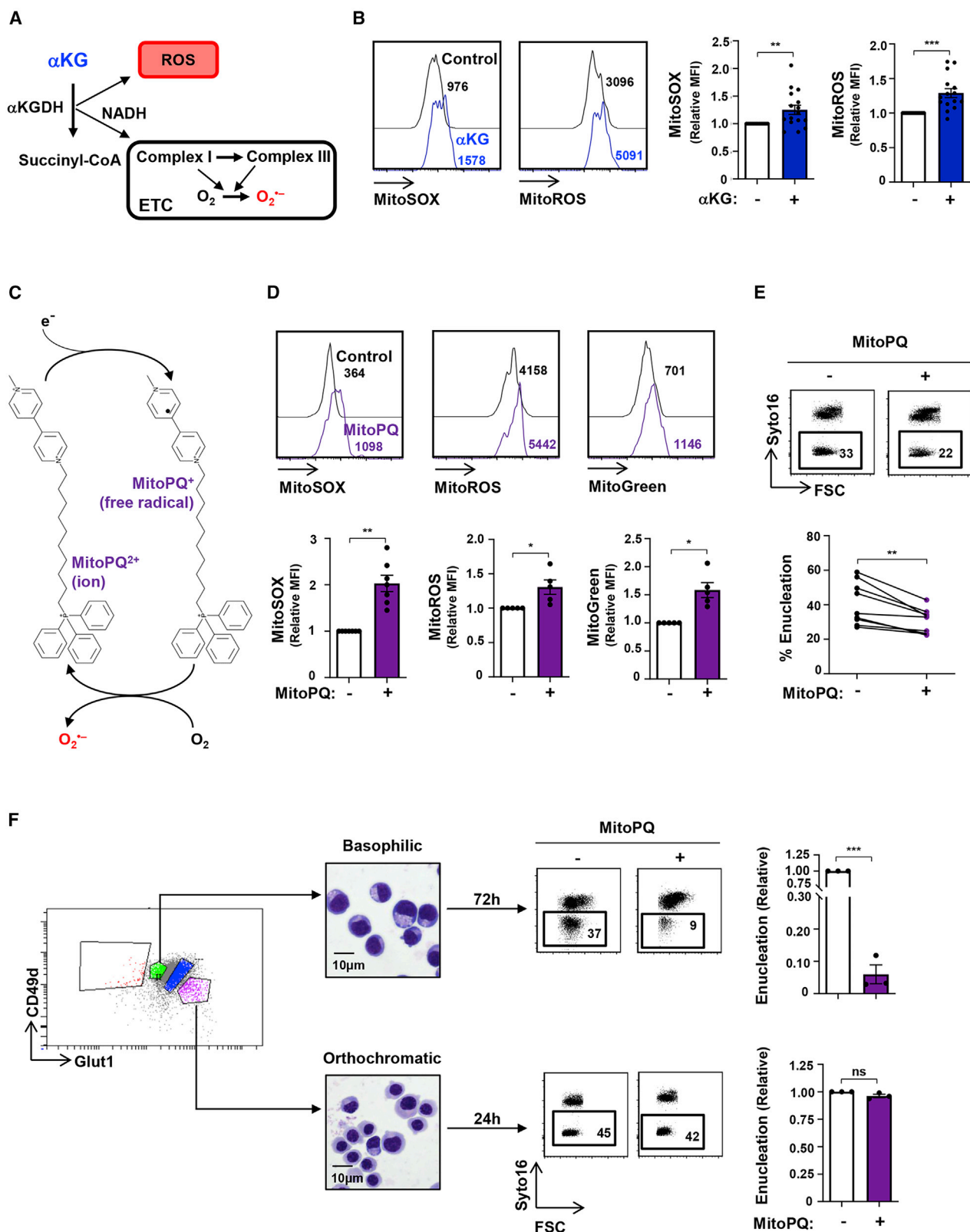
but its activity is countered by the oxidized form of vitamin C, DHA. The rapid reduction of intracellular DHA to vitamin C (Figure 4A) results in concomitant increases in endogenous ROS in tumor cell lines (Kc et al., 2005; Yun et al., 2015), but its role in erythroid cells is not known. Of note, DHA is transported via the GLUT1 glucose transporter, the most highly expressed transporter on human erythrocytes (Bianchi and Rose, 1986; Helgersson and Carruthers, 1987; May, 1998; Montel-Hagen et al., 2008b, 2009). We found that the addition of DHA significantly increased mitochondrial biomass and superoxide production in differentiating erythroblasts (Figure 4B). The DHA-induced increase in superoxide production was associated with a significant decrease in the enucleation of late-stage erythroblasts (from a mean of $40\% \pm 3\%$ to $24\% \pm 3\%$; $p < 0.0001$; Figure 4C). Thus, under all tested conditions of superoxide production, enucleation of late-stage erythroblasts was severely attenuated.

Based on these data, it was important to evaluate whether vitamin C would have opposing effects on the redox state of erythroblasts and, subsequently, on erythroid maturation. As vitamin C is extremely labile, undergoing oxidation to DHA in culture media with a half-life of ~ 70 min (Yun et al., 2015), we evaluated the effect of AA-2-phosphate, which is resistant to spontaneous oxidation (Frikke-Schmidt and Lykkesfeldt, 2010; Hata and Senoo, 1989; Manning et al., 2013). This phosphorylated vitamin C molecule significantly enhanced the antioxidant capacity of the developing erythroblast, with a $23\% \pm 3\%$ decrease in mitochondrial superoxide production ($p < 0.0001$; Figures 4D and S3C). To evaluate whether vitamin C can alter an imbalanced high oxidative state in erythroblasts, we tested its impact on α KG-induced metabolic changes in differentiating erythroblasts. Vitamin C significantly diminished mitochondrial superoxide production as well as mitochondrial biomass and polarization ($p < 0.0001$, $p < 0.01$, and $p < 0.05$; Figures 4E and S3D). Moreover, we found that while both α KG and MitoPQ significantly increased the percentages of erythroblasts in the S-G2/M phases of the cell cycle (with cell death remaining $< 15\%$), vitamin C decreased cell-cycle progression to control levels ($p < 0.05$ and $p < 0.01$; Figures 4F, S3E, and S3F).

Most importantly, vitamin C rescued the enucleation defect observed in these erythroblasts ($p < 0.0001$; Figure 4G). This effect was due to the reduced form of vitamin C; as only the long-acting derivative and not the labile form of vitamin C promoted enucleation (Figure S3G). Moreover, other ROS scavengers, including NAC (a thiol-containing antioxidant), GSH (the most abundant antioxidant), and Trolox (a water-soluble analog of vitamin E lacking the phytyl chain), recapitulated the actions of vitamin C, significantly augmenting terminal enucleation in the presence of α KG (Figure S4A). Similarly to the data shown above, highlighting the regulation of erythroid differentiation by mitochondrial metabolism at early erythroblast stages (Figure 3F), vitamin C significantly increased enucleation of sorted basophilic erythroblasts, but not orthochromatic erythroblasts (Figure S4B).

(E) OCR was evaluated on day 10 erythroid progenitors in control conditions (black line) and following addition of α KG at day 7 (blue line). Mean levels \pm SEM are presented ($n = 5-6$).

ns, nonsignificant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.



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The ensemble of these data strongly suggests that the impact of vitamin C is due to its alteration of the redox state of the erythroblast. However, as autophagy is also a critical regulator of enucleation (Keerthivasan et al., 2011), we evaluated the impact of vitamin C following Spautin1-mediated inhibition of autophagy (Liu et al., 2011). As expected, Spautin significantly inhibited enucleation ($p < 0.01$), but vitamin C did not rescue this defect (Figure S5A), highlighting an autophagy-independent role of vitamin C in erythroid differentiation. Iron and its uptake as diferrous transferrin by the transferrin receptor CD71 are also critical for erythroid differentiation (Kautz and Nemeth, 2014), and we therefore evaluated a potential crosstalk between iron and vitamin C. Interestingly, in the absence of transferrin, vitamin C did not increase enucleation in α KG-treated erythroblasts, but its presence (50 or 200 μ g/mL) positively affected enucleation (Figure S5B). Thus, vitamin C and other ROS scavengers decrease the redox state of erythroblasts, promoting enucleation under conditions of α KG-induced oxidative stress in a transferrin-dependent manner.

Vitamin C accelerates the erythroid differentiation of cord blood, peripheral blood, and BM progenitor cells

The ability of vitamin C to rescue erythroid differentiation under conditions of augmented oxidative stress led us to evaluate its potential to directly modulate erythroid differentiation of CD34⁺ hematopoietic stem and progenitor cells (HSPCs) from different sources. CD34⁺ progenitors from cord blood (CB), adult peripheral blood (PB), and BM were directly differentiated with rEPO. While BM-derived progenitors differentiated much more rapidly than CB- and PB-derived progenitors to an erythroid-committed CFU-E (CD34⁺CD36⁺), vitamin C did not alter the erythroid progenitor continuum (Figure S5C). In agreement with previous data (Yan et al., 2018), CB progenitors exhibited a delayed generation of GlyA⁺ erythroblasts compared to PB and BM progenitors (Figure 5A). Notably, vitamin C significantly increased the percentages of GlyA⁺ cells ($p < 0.05$) and increased maturation to a GlyA⁺CD105⁺ state by 2.1- \pm 0.5-fold ($p < 0.01$, Figure 5A), without altering cell growth (Figure S5D). Furthermore, vitamin C resulted in an enhanced enucleation of erythroid progenitors generated from these three sources (Figure 5B). Thus, vitamin C significantly accelerates erythroid differentiation and enucleation of human CD34⁺ progenitors, irrespective of their source of origin.

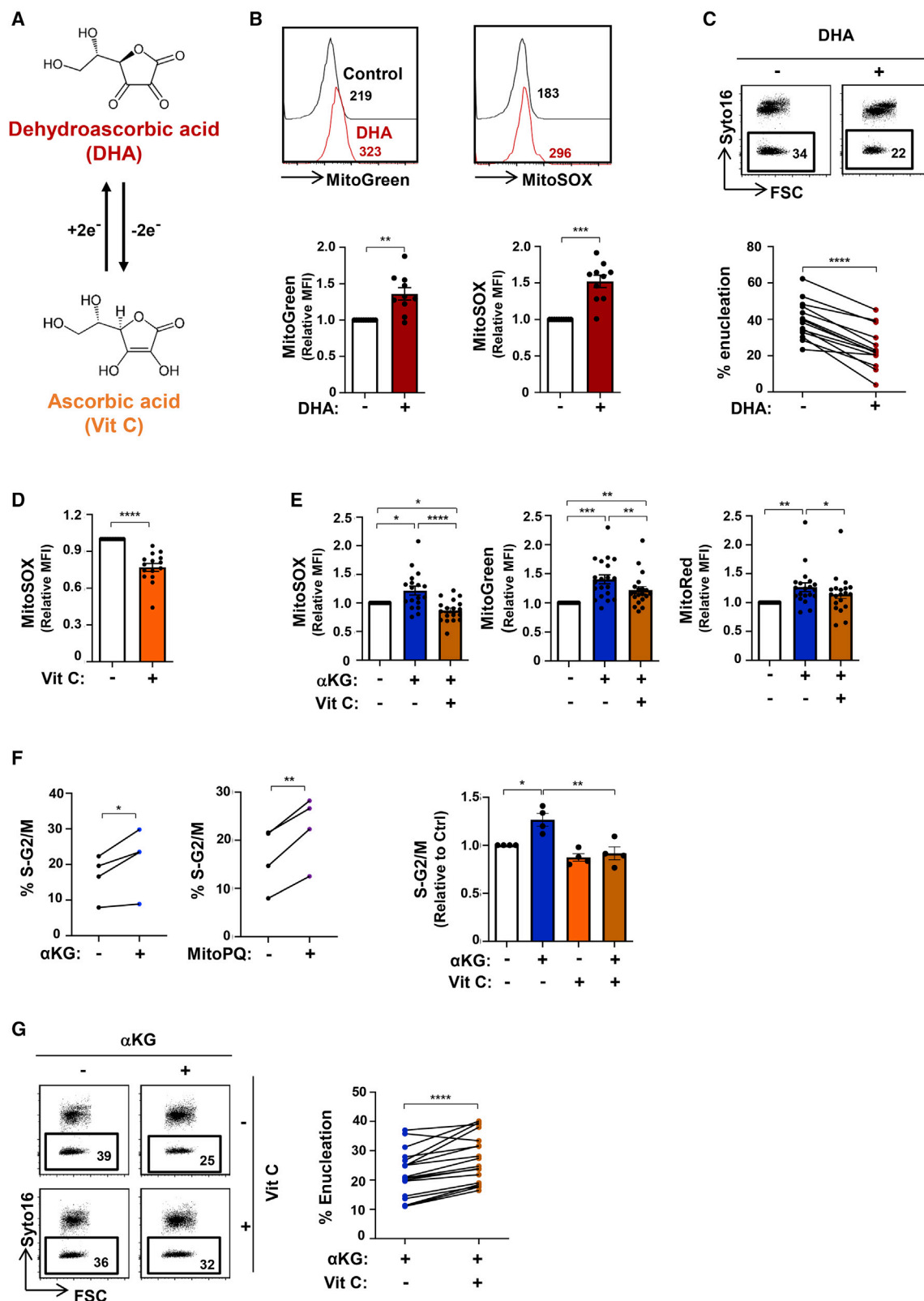
IDH1 activity regulates mitochondrial activity in differentiating erythroblasts

The data presented above demonstrated that conditions that increase mitochondrial activity in human erythroblasts, including ectopic α KG and MitoPQ, inhibit terminal erythroid differentiation. Under physiological conditions, α KG is mobilized in the mitochondria, where it can be converted to succinyl-CoA by α KGDH, producing ROS and the NADH that provides electrons for the ETC (Figure 6A). α KG can be generated from isocitrate in the mitochondria by IDH2/IDH3 and interconverted in the cytoplasm by IDH1. Interestingly, transcripts of all three enzymes are significantly reduced during erythroid differentiation, potentially suggesting a negative role for these pathways in late-stage erythroblasts (Figure S6A). As mitochondrial biomass is markedly decreased during erythroid differentiation, we evaluated the role of the cytoplasmic IDH enzyme, IDH1, in erythroid differentiation by an shIDH1-mediated approach. Using two independent short hairpin RNAs (shRNAs), shIDH1-1 and shIDH1-2, we detected a 36% \pm 7% and 54% \pm 5% reduction, respectively, and as such used shIDH1-2 in the differentiation experiments (Figure S6B).

Importantly, IDH1 knockdown did not inhibit erythroid differentiation, as monitored by the generation of CFU-E, GlyA⁺ erythroblasts or upregulation of the CD36 or CD71 erythroid markers (Figures 6B, S6C, and S6D). However, downregulating IDH1 resulted in a decreased expansion (Figure S6E) and significantly increased mitochondrial superoxide production, mitochondrial biomass, and polarization ($p < 0.01$; Figures 6C and S6F). In accord with these data, IDH1 knockdown augmented the OXPHOS capacity of the cells, with a 3.0- and 3.7-fold increase in basal and maximal OCR levels, respectively (shIDH1-2 construct) (Figure 6D). Notably, the function of IDH1 appears to be substantially more critical for the differentiation of erythroid as compared to non-erythroid progenitors. The percentages of progenitors transduced with an scramble shRNA (shControl) or shIDH1 lentiviral vector harboring the GFP reporter gene remained approximately stable over a 6-day period, with 47%–57% GFP⁺ cells, in the absence of rEPO. These cells had a myeloid phenotype as shown by a CD11b⁺, CD33⁺, CD13⁺ phenotype (Figure S6G). However, following rEPO-mediated erythroid induction, the percentages of shControl cells decreased by 24%, while the percentages of shIDH1-transduced cells decreased by 56% (Figure 6E).

Figure 3. Mitochondrial superoxide production impedes erythroid enucleation

(A) Schematic of the generation of mitochondrial reactive oxygen species (ROS) via the conversion of α KG to succinate, of which superoxide is a major source. (B) The impact of α KG (3.5 mM) on mitochondrial superoxide and mitochondrial ROS production in erythroblasts was evaluated at day 10 of erythroid differentiation as a function of MitoSOX and MitoROS, respectively. Representative histograms are shown (left). Staining in the presence of α KG was normalized to control conditions, and relative median fluorescence intensities (MFIs) are presented ($n = 15$ –16, right). (C) Schematic of MitoParaquat (MitoPQ) cycling within the mitochondria; MitoPQ²⁺ is reduced to the radical monocation at the flavin site of complex I, and the monocation then reacts with O₂ to generate superoxide (O₂^{•−}) (Robb et al., 2015). (D) Differentiating erythroblasts were treated with MitoPQ (50 μ M) at day 7 of differentiation, and representative histograms of mitochondrial superoxide, ROS, and biomass at day 10 are presented (top). MFIs are presented relative to control conditions ($n = 5$ –6, bottom). (E) The impact of MitoPQ on enucleation was evaluated at day 10 by Syto16 staining, and representative dot plots are presented (top). Percent enucleation in nine independent experiments is shown (bottom). (F) The impact of MitoPQ on early basophilic erythroblasts as compared to late orthochromatic erythroblasts was determined by FACS sorting erythroblast populations as a function of their GLUT1/CD49d profiles (left), and representative cytospins of the sorted subsets are shown (middle). Early basophilic and late orthochromatic subsets were then cultured for 72 h or 24 h, respectively, in the absence (−) or presence (+) of MitoPQ, and enucleation was evaluated (right). Enucleation was quantified relative to control conditions ($n = 3$). ns, nonsignificant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



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To better understand the negative selection of erythroid progenitors with downregulated IDH1, we performed metabolomic and flux experiments following IDH1 knockdown. First, we found that at day 4 of IDH1 downregulation (~50%; Figure S6B), there was a mean decrease of α KG and citrate of 43% and 21%, respectively (Figure 6F). These data highlight the importance of the IDH1 enzyme in the generation of α KG and its interconversion to citrate. Furthermore, they demonstrate the role of glutamine metabolism in the generation of these intermediates in early erythroid progenitors (day 4); a mean of 75.1% and 69.0% of α KG and citrate harbored ^{13}C carbons from glutamine, respectively (Figure 6G). Several groups have also found that the relative ratio of α KG to citrate regulates differentiation, either by inhibiting (Carey et al., 2015; Vardhana et al., 2019) or priming (TeSlaa et al., 2016; Tischler et al., 2019) stem cell differentiation. In this regard, it is interesting to note that erythroid differentiation resulted in the loss of both α KG and citrate between days 4 and 7 of differentiation, with a mean loss of 87% and 68%, respectively ($n = 2$ biological experiments performed in triplicate; Figure 6G). These data point to a greater loss of α KG than citrate, consistent with a negative role of high α KG levels in late erythroid differentiation (Figure 2). Furthermore, following IDH1 downregulation, TCA intermediates were maintained at a higher level, with decreases in α KG and citrate of only 31% and 36%, respectively (Figure 6G). Thus, the premature inhibition of IDH1 alters the physiological loss of TCA-cycle intermediates upon erythroid differentiation.

Vitamin C rescues terminal erythroid differentiation in the absence of IDH1

Under conditions of IDH1 downregulation, mitochondrial metabolism was augmented, as shown by increased superoxide production and OXPHOS (Figures 6C and 6D). On the basis of these data, we hypothesized that IDH1 knockdown would attenuate late-stage erythroid differentiation and enucleation, and this was indeed the case. Following transduction of CD34⁺ HSPCs with a vector harboring an shIDH1 RNA, the emergence of Glut1⁺CD49⁺ orthochromatic erythroblasts was significantly decreased (shIDH1-2 construct, $p < 0.01$; Figure 7A). Furthermore, enucleation was decreased by $59\% \pm 2\%$ (shIDH1-2 construct, $p < 0.0001$; Figure 7B), and this effect was detected even when

IDH1 was downregulated at day 4 post-rEPO-induced differentiation (Figure S7A). Consistent with an impact of IDH1 on the redox state of the cell, both α KG and DHA exacerbated the effect of IDH1 knockdown on erythroblast enucleation, from $47\% \pm 8\%$ to $27\% \pm 8\%$ and $24\% \pm 4\%$, respectively ($p < 0.01$ and $p < 0.05$; Figure 7C). Most importantly, vitamin C significantly increased enucleation under these conditions by $35\% \pm 13\%$ ($p < 0.05$, Figure 7D). Intriguingly, other ROS scavengers, including NAC, GSH, and Trolox, did not increase enucleation, even though they were present throughout the differentiation period (Figure S7B). Importantly, vitamin C, but not NAC, GSH, or Trolox, decreased mitochondrial superoxide generation ($p < 0.05$; Figure S7C). Thus, collectively, these data show that IDH1-induced decreases in mitochondrial OXPHOS are required for the later stages of terminal erythroid differentiation and enucleation. However, under conditions of decreased IDH1 expression, this negative effect can be counteracted by a vitamin-C-mediated modulation of the intracellular redox balance.

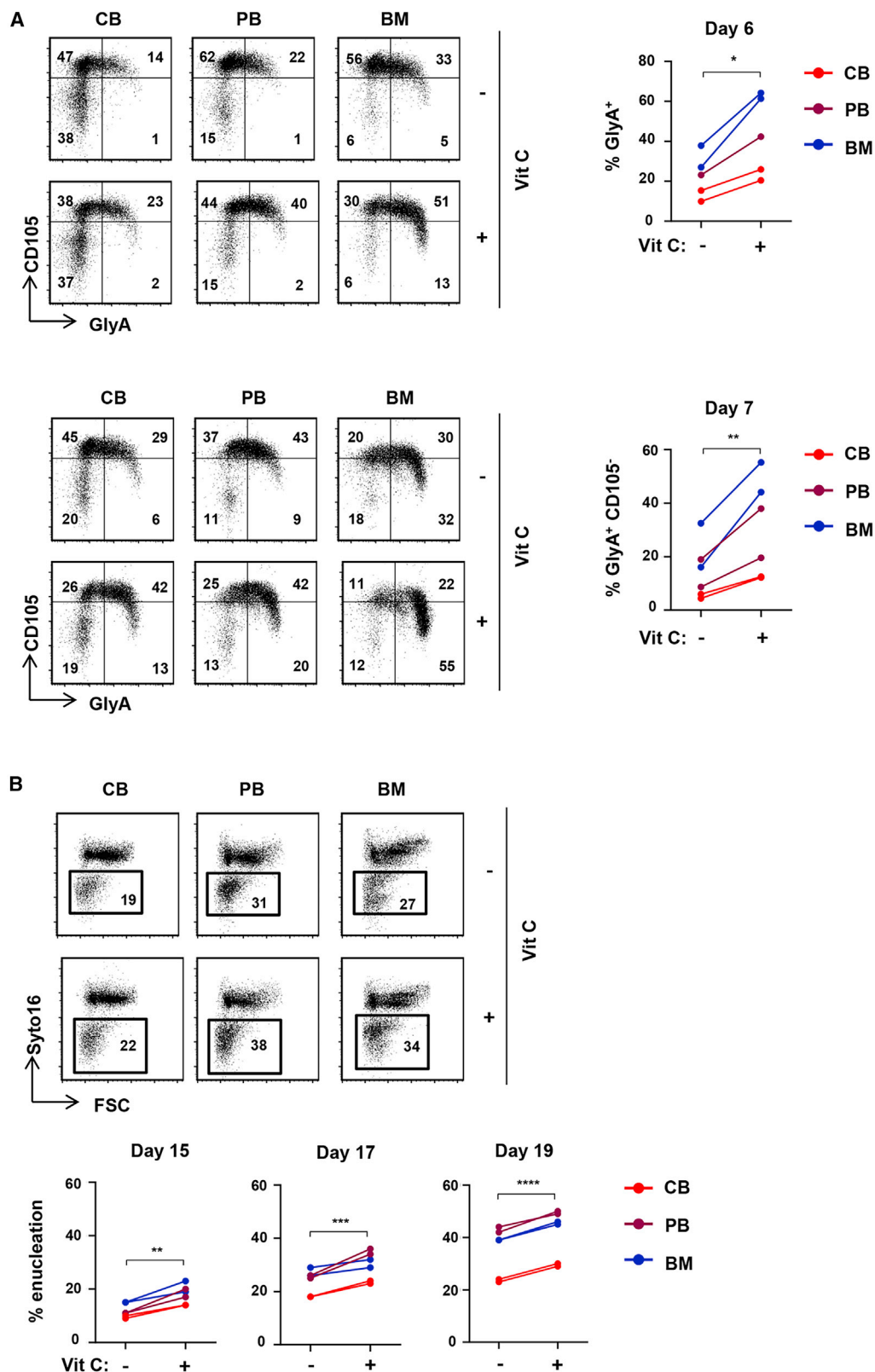
As α KG and vitamin C act as cofactors in supporting the dioxygenase activity of TET2, it was important to assess whether the effects that we detected here were coupled to their impact on TET2 activation (Inoue et al., 2016). However, if α KG served only to support the dioxygenase activity of TET2, then its presence would be expected to augment erythroid differentiation, while our data showed the opposite effect (Figure 2). To directly address this point and evaluate the role of an α KG/TET2 as compared to an α KG/IDH1 axis, we downregulated TET2 by an shRNA approach (Yan et al., 2017). TET2 levels decrease with differentiation ($p < 0.0001$; Figure S7D), but mRNA levels were further diminished by a mean of 55% by shRNA-mediated knockdown (Figure S7E). While this downregulation resulted in a significant delay in the generation of GlyA⁺ terminal erythroblasts ($p < 0.0001$; Figure S7F), it did not delay enucleation when the shRNA-mediated knockdown of TET2 was performed at day 4 of differentiation (Figure 7E). Moreover, enucleation in shTET2-treated erythroid progenitors was not augmented by vitamin C (Figure 7E). These data strongly imply distinct roles for IDH1 and TET2 in regulating enucleation of late-stage erythroid progenitors.

Mutations in IDH1/IDH2 are strongly associated with myelodysplastic syndromes (MDSs) that are characterized by

Figure 4. Antagonistic roles of dehydroascorbic acid and vitamin C on mitochondrial oxidative stress and erythroblast enucleation

- (A) Schematic of the interconversion between dehydroascorbic acid (DHA) and ascorbic acid (vitamin C) via a two-electron reduction/oxidation process, respectively.
- (B) The impact of DHA (1 mM) on mitochondrial superoxide production and biomass in late erythroblasts was evaluated between days 7 and 10 after EPO induction, and representative histograms of MitoGreen and MitoSOX staining, respectively, are shown (top). Quantification relative to control conditions is shown ($n = 10$, bottom).
- (C) Enucleation was evaluated at day 10 after EPO induction in the absence (–) or presence (+) of DHA (days 7–10), and representative dot plots are shown (top). Percent enucleation in 14 independent experiments is presented (bottom).
- (D) The impact of the stable L-ascorbic acid (AA) 2-phosphate derivative of vitamin C (100 μM) was evaluated on mitochondrial superoxide generation (days 7–10), and MFIs were quantified relative to control conditions ($n = 15$, bottom).
- (E) The impact of vitamin C on α KG-mediated increases in MitoSOX, MitoGreen, and MitoRed staining was evaluated by flow cytometry, and relative MFIs are presented ($n = 18$ –20).
- (F) The percentages of cells in S-G2/M of the cell cycle were evaluated in the indicated conditions by phosphatidylinositol (PI) staining ($n = 4$, left). The percentages of erythroid progenitors in S-G2/M were evaluated relative to control conditions, and means \pm SEM are presented (right).
- (G) Enucleation was evaluated in the presence or absence of vitamin C and α KG. Representative histograms (left) as well as changes in percent enucleation are presented ($n = 18$, right).

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.



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ineffective erythropoiesis (reviewed by [Bejar and Steensma, 2014](#); [Fenaux et al., 2014](#)). The anemia that dominates the early course of MDS is associated with an aberrant erythroid differentiation marked by the generation of multinucleated erythroblasts ([Abdel-Wahab and Levine, 2013](#)). Notably, we found that *IDH1* knockdown increased the percentages of erythroblasts with abnormal multinucleated erythroblasts from $10\% \pm 1\%$ to $23\% \pm 2\%$ ($p < 0.05$; [Figure 7F](#)). Moreover, vitamin C markedly decreased the generation of these abnormal erythroblasts, not only in *IDH1*-knockdown erythroblasts but also in normal erythroblasts, to $4\% \pm 0.4\%$ and $4\% \pm 0.2\%$, respectively ($p < 0.05$ and $p < 0.01$; [Figure 7F](#)). These data suggest that the multinucleated erythroblasts detected in MDS patients may be associated with an increased mitochondrial metabolism. Vitamin C, a redox scavenger, significantly attenuated the generation of these abnormal cells.

DISCUSSION

The generation of ROS and its intricate relationship with cellular redox homeostasis is a critical regulator of stem cell homeostasis. Notably, low ROS levels, associated with low mitochondrial biogenesis and activity, are necessary for HSC quiescence and self-renewal ([Filippi and Ghaffari, 2019](#); [Jang and Sharkis, 2007](#); [Qian et al., 2016](#); [Suda et al., 2011](#); [Takubo et al., 2013](#); [Tan and Suda, 2018](#); [Vannini et al., 2016](#); [Yu et al., 2013](#)). Conversely, HSC division and differentiation occur under conditions that promote mitochondrial biogenesis and OXPHOS ([Chen et al., 2008](#); [Mantel et al., 2010](#); [Maryanovich et al., 2015](#); [Yu et al., 2013](#)). In the context of erythropoiesis, it has been shown that early erythroid development requires induction of mitochondrial ROS, a consequence of OXPHOS ([Liu et al., 2017](#); [Zhao et al., 2016](#)). Here, we show that glutamine metabolism, via the anaplerotic contribution of α KG into the TCA cycle, directly increases OXPHOS in human erythroid precursors. This increased OXPHOS promoted erythroid commitment and early erythropoiesis, but terminal erythroid differentiation was dependent on the suppression of TCA-cycle-linked mitochondrial networks. Elevated intracellular α KG levels, with associated increased OXPHOS and ROS, inhibited late-stage erythroid maturation and enucleation.

Mechanistically, we identify the catalysis of α KG by the cytoplasmic *IDH1* enzyme as a critical step in erythropoiesis; the negative repercussions of *IDH1* downregulation in terminal erythroid differentiation was further exacerbated by elevated α KG levels. Furthermore, the impact of a mitochondria-targeted redox cyler, MitoPQ ([Robb et al., 2015](#)), highlights the importance of mitochondrial superoxide as a specific inhibitor of erythroblast maturation and enucleation. Interestingly, all tested ROS

scavengers, including NAC, GSH, vitamin E, and vitamin C, rescued the α KG-mediated decrease in enucleation, but only vitamin C rescued enucleation following *IDH1* downregulation. This is critical in the context of human erythroid differentiation, where the absence of ascorbate synthesis, resulting in 1 mg/kg recommended exogenous supplements for humans ([Pauling, 1970](#)), is significantly lower than the hepatic production rate of 200 mg/kg/day in vitamin-C-synthesizing animals ([Chatterjee, 1973](#); [Stone, 1979](#)). Thus, scavenging by vitamin C is likely to be suboptimal. Indeed, we found that a stable vitamin C analog rescued human terminal erythroid differentiation and enucleation in all tested conditions of oxidative stress.

Ascorbate, transported by the SVCT1/SLC23A1 and SVCT2/SLC23A2 solute carriers, regulates whole-body homeostasis of vitamin C and protection to oxidative stress, respectively ([Savini et al., 2008](#); [Tsukaguchi et al., 1999](#); [Wilson, 2005](#)). While vitamin C has been shown to enhance erythropoiesis in hemodialysis patients with refractory anemia ([Attallah et al., 2006](#); [Gastaldello et al., 1995](#); [Seibert et al., 2017](#); [Sirover et al., 2008](#)), other studies have found that ascorbate induces hemolysis of mature erythrocytes ([Zhang et al., 2016](#)). These apparent discrepancies are likely due to the oxidation state of vitamin C. The loss of a single electron from ascorbate results in the generation of an unstable ascorbate radical, which can then lose a second electron to generate DHA ([Padayatty and Levine, 2016](#)). Unlike vitamin C, the two-electron oxidized DHA intermediate is transported by the GLUT1 glucose transporter ([Bianchi and Rose, 1986](#); [McNulty et al., 2005](#); [Rumsey et al., 1997, 2000](#); [Vera et al., 1993](#)). Thus, cellular uptake of the reduced and oxidized forms of vitamin C is regulated, at least in part, by the expression of their specific transporters. SVCT2 is expressed at very high levels on HSCs, with levels decreasing as a function of erythroid development and differentiation ([Agathocleous et al., 2017](#)). Conversely, GLUT1 is upregulated during human terminal erythroid differentiation ([Montel-Hagen et al., 2008b](#); [Mueckler, 1994](#)), promoting DHA transport ([May, 1998](#); [Montel-Hagen et al., 2008b, 2009](#)). Thus, during intermediate steps of HSPC development, both ascorbate and DHA can potentially be transported into the cell.

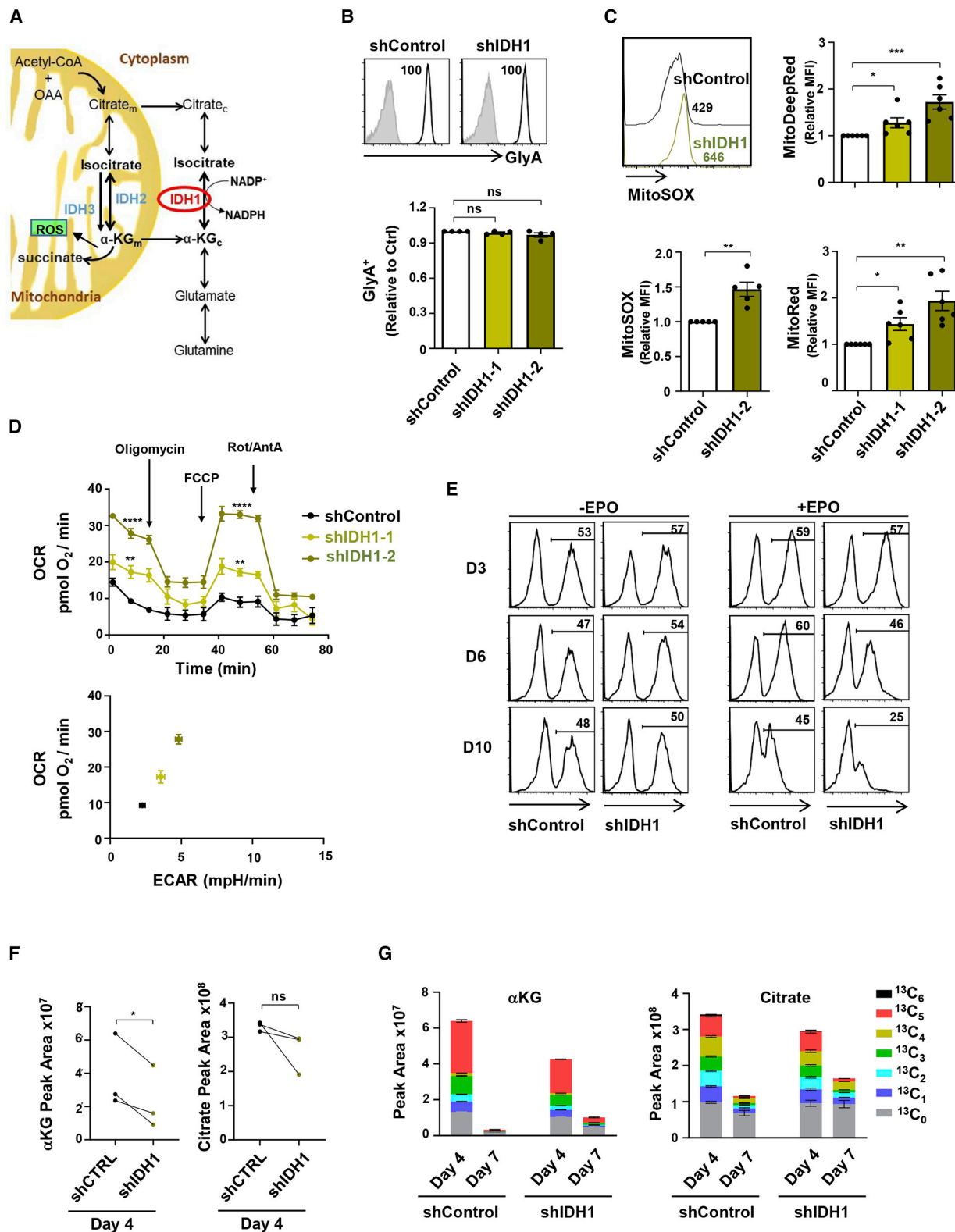
One of the difficulties in studying ascorbate function is its stability. Previous research showed that freshly prepared minimum essential medium α (α MEM) culture media, but not liquid α MEM, supported T cell differentiation from hematopoietic progenitors; the difference between these formulations was due to the lability of vitamin C in the latter ([Manning et al., 2013](#)). Furthermore, several groups found that the negative impact of vitamin C on the growth of cancer cells was actually due to the GLUT1-mediated uptake of DHA, resulting in the accumulation of ROS and subsequent cell death ([Yun et al., 2015](#); [Zhang et al., 2016](#)).

Figure 5. Vitamin C promotes erythroid commitment of EPO-stimulated progenitors from CB, BM, and PB and significantly accelerates erythroid maturation

(A) CD34⁺ HSPCs were isolated from CB, PB, and BM. Progenitors were differentiated with EPO in the absence or presence of vitamin C (100 μ M) and erythropoiesis monitored as a function of CD105/GlyA profiles. Representative profiles at days 6 (top left) and 7 (bottom left) of differentiation are presented. Quantification of GlyA⁺ cells at day 6 ($n = 5$, top right) and GlyA⁺CD105⁺ cells at day 7 ($n = 5$, bottom right) is presented.

(B) The impact of vitamin C on enucleation was evaluated in CB, PB, and BM HSPCs, and representative plots at day 17 are presented (top). Quantification of enucleation at days 15, 17, and 19 is presented ($n = 6$, bottom).

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.



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Indeed, we similarly found that the standard labile form of L-ascorbate markedly decreased erythroblast maturation and enucleation, even in the absence of ascorbate oxidase. However, under conditions where erythroid differentiation was performed in the presence of the stable ascorbate 2-phosphate analog (Hata and Senoo, 1989; Frikke-Schmidt and Lykkesfeldt, 2010; Takamizawa et al., 2004), erythroid differentiation was enhanced. Thus, care must be taken in evaluating studies using the conventional labile ascorbate, as it may not be maintained in a reduced state.

Ascorbic acid has also gained prominence as an anti-cancer therapeutic modality because of its ability to alter the activity of the Tet2 demethylase (Shenoy et al., 2018). Loss of function mutations in *TET2* are one of the most frequent events in myeloid dysplasia and leukemias (Bejar et al., 2011; Busque et al., 2012; Delhommeau et al., 2009; Kosmider et al., 2011). The initiation of demethylation by the TET family of dioxygenases occurs via the introduction of the intermediate mark 5-hydroxymethylcytosine (5hmC), and this function is dependent on several cofactors, including ascorbate, α KG, ferrous iron, and oxygen (Chen et al., 2013; Dang et al., 2009). Indeed, recent studies have shown that ascorbate is able to compensate for TET2 deficiency and suppress leukemogenesis, albeit via both TET2-dependent and TET2-independent mechanisms (Agathocleous et al., 2017; Cimmino et al., 2017). A crosstalk between ascorbate and TET2 in the metabolic status of a hematopoietic progenitor is suggested by the finding that wild-type TET2 protects murine erythroblasts from oxidative stress (Guo et al., 2017). Moreover, downregulation of TET2 in human erythroid progenitors negatively regulated human erythropoiesis in a manner that was independent of any detectable alterations in 5-methylcytosine (5mC) levels (Qu et al., 2018; Yan et al., 2017). These data are in agreement with independent studies showing that terminal erythroid differentiation requires that erythroblasts be protected from oxidative stress (Case et al., 2013; Filippi and Ghaffari, 2019; Hyde et al., 2012; Xu et al., 2019; Zhao et al., 2016).

Our study highlights the importance of ascorbate in regulating the redox state of human erythroid progenitors, especially under

conditions of altered oxidative metabolism. Increases in intracellular α KG and decreases in cytoplasmic IDH1 both augmented oxidative stress and diminished erythroid maturation. IDH1, like Tet2, is mutated in a high percentage of MDS patients (Kosmider et al., 2010; Wang et al., 2017), with anemia dominating the early course of disease. Moreover, recent studies have highlighted the potential for vitamin C to promote the differentiation of IDH1-mutated AML progenitors (Mingay et al., 2018). Our data show that even in the absence of mutation, low levels of wild-type IDH1 suppress late erythroid differentiation. While the specific role of IDH1 in OXPHOS has not yet been fully delineated, it is interesting to note that IDH1 downregulation in neuronal cells resulted in an increase in ROS (Calvert et al., 2017). Furthermore, mutation of IDH1, but not IDH2, has been found to increase OXPHOS in transformed cells (Grassian et al., 2014), and wild-type IDH1 regulates the reductive carboxylation of α KG for *de novo* lipogenesis (Calvert et al., 2017; Metallo et al., 2011).

IDH-regulated α KG production has also been shown to regulate cell fate and differentiation by modulating epigenetic marks via Tet2-induced changes in global histone and DNA demethylation (Carey et al., 2015; TeSlaa et al., 2016; Tischler et al., 2019; Xiao et al., 2012). Notably, under the conditions of erythroid differentiation studied here, α KG levels attenuated terminal erythroid differentiation and late-stage erythropoiesis, while a second TET2 cofactor, ascorbate, exhibited an opposing effect, enhancing and accelerating terminal differentiation. Other agents, such as oxidized ascorbate and MitoPQ, attenuated erythroid differentiation by inducing oxidative stress. Furthermore, while TET2 downregulation delayed early erythroblast differentiation, it did not negatively impact terminal differentiation and enucleation. Together, these data strongly suggest complementary roles for TET2 and IDH1, with the latter playing a critical role in late-stage human erythropoiesis via its impact on redox homeostasis.

These data may promote mechanistic insights into inherited hematologic disease. Like MDS patients, a feature that characterizes patients with congenital dyserythropoietic anemia is the presence of multinucleated or multi-lobulated erythroblasts

Figure 6. IDH activity regulates mitochondrial superoxide production and OXPHOS in differentiation erythroblasts

(A) Schematic representation of the catalytic reactions regulating glutamine catabolism. Glutamine is interconverted to glutamate and cytosolic α KG (α KG_c). Isocitrate dehydrogenase 1 (IDH1), a cytosolic enzyme, catalyzes the production of isocitrate from α KG, promoting both forward and reverse reactions. Conversion of mitochondrial α KG (α KG_m) to succinate results in the generation of ROS.

(B) The expression of GlyA was monitored in shControl- and shIDH1-transduced cells at day 7 day of differentiation, and representative histograms (gray, isotype control; black, specific staining) are shown (top). GlyA expression in cells transduced with one of two different shIDH1 clones (shIDH1-1 and shIDH1-2) was evaluated, and relative levels are presented (n = 4, bottom).

(C) The impact of IDH1 downregulation on MitoSOX was evaluated at day 10. Representative histograms (top left) and quantification of relative MFIs (n = 5) are presented (bottom left). MitoTracker Deep Red (top right) and MitoTracker Red (bottom right) staining in shIDH1-transduced erythroblasts relative to control conditions, are presented (n = 6).

(D) OCR was determined in FACS-sorted shControl- as well as shIDH1-transduced erythroblasts at day 10 of differentiation as indicated (n = 6, top). An OCR/extracellular acidification rate (ECAR) energy map is presented (bottom).

(E) The evolution of shControl- and sh-IDH1-transduced progenitors was monitored as a function of GFP expression at days 3, 6, and 10 of differentiation in the absence (–) or presence (+) of EPO (n = 3).

(F) Total levels of α KG and citrate were evaluated by high-performance liquid chromatography-mass spectrometry (HPLC-MS) at day 4 of erythroid differentiation in shControl- and shIDH1-transduced progenitors, and mean levels are shown (n = 3 independent experiments, with statistical difference evaluated by a one-tailed t test).

(G) The isotopologues of α KG and citrate derived from [¹³C₅¹⁵N₂]Gln are presented from one experiment with three replicates for day 4 and two replicates for day 7.

ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

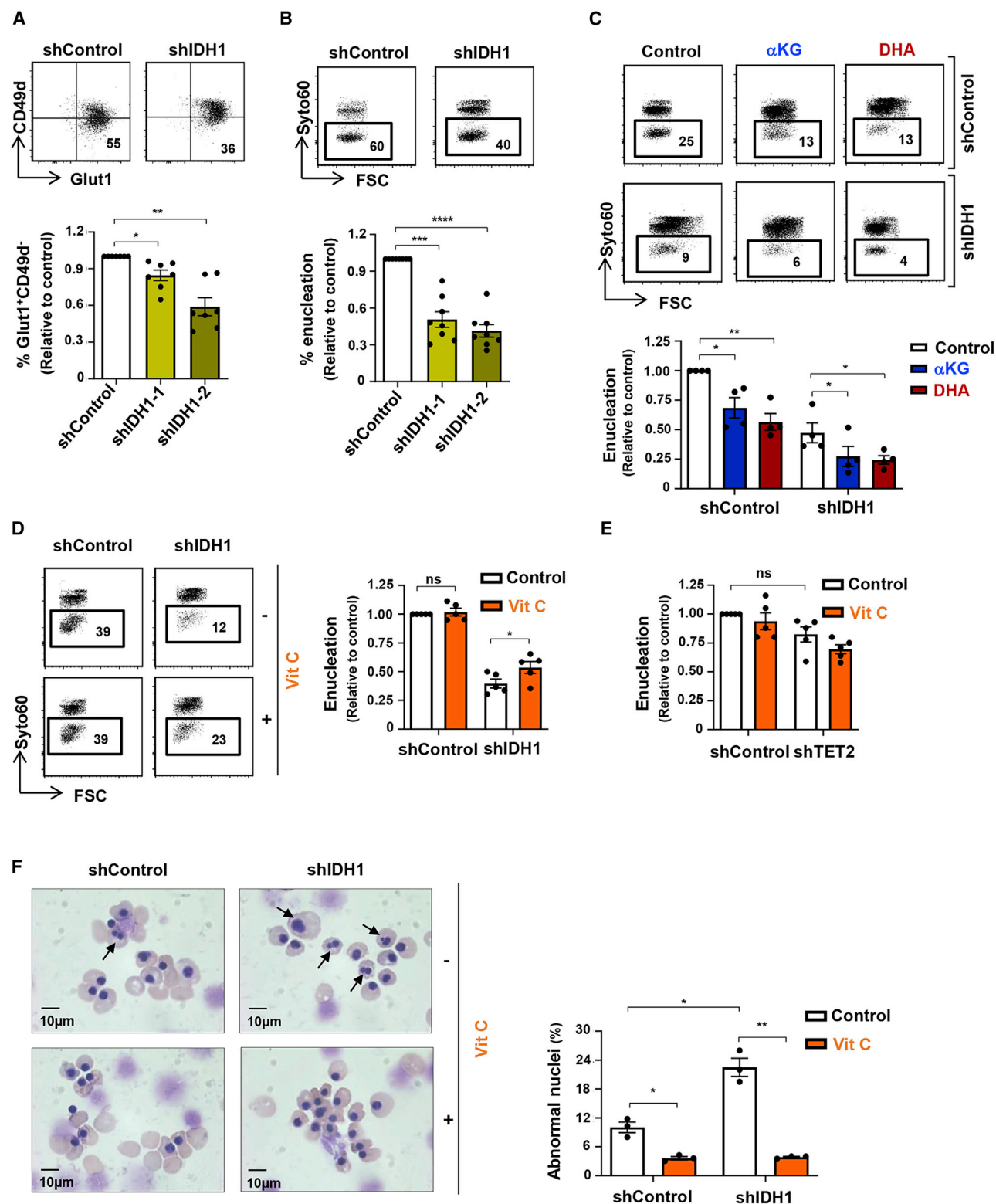


Figure 7. Vitamin C rescues dyserythropoiesis due to IDH1 downregulation

(A) Late-stage erythroblast differentiation was monitored as a GLUT1⁺CD49d⁻ phenotype, and representative dot plots are presented (top). The percentages of GLUT1⁺CD49d⁻ erythroblasts were evaluated following transduction with shIDH1-1 or shIDH1-2 vectors, and mean levels \pm SEM relative to control cells are presented ($n = 7$, bottom).

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(Crookston et al., 1969; Goasguen et al., 2018; Iolascon et al., 1996). IDH1 knockdown resulted in a significant augmentation in the percentage of abnormal multinucleated orthochromatic erythroblasts, and even more importantly, ascorbate reduced these levels back to baseline. Thus, these data provide the first evidence that the abnormal erythroblast morphology observed in diverse dyserythropoietic diseases may be coupled to oxidative stress. Furthermore, our data suggest that antioxidant treatment of these disorders in humans, a species with suboptimal redox capacity (Johnson et al., 2008), with stable ascorbate analogs could potentially restore redox homeostasis, quenching ROS and subsequently rescuing defective late-stage erythropoiesis.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2021.108723>.

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AUTHOR CONTRIBUTIONS

P.G.-M., M.R., S.K., and N.T. conceived the study; P.G.-M., M.R., H.Y., R.D., J.P., L.O., S.T., V.D., C.M., M.S., V.S.Z., L.B., N.M., S.K., and N.T. were involved in study design; and P.G.-M., M.R., H.Y., R.D., J.P., L.O., S.T., C.M., M.D., A.-S.D., I.P., X.Q., P.-N.B., and S.K. performed experiments. All authors participated in data analysis. P.-G.M., S.K., and N.T. wrote the manuscript with important critical input from M.R., H.Y., L.O., S.T., X.A., V.D., M.S., V.S.Z., P.G.G., L.B., and N.M.

DECLARATION OF INTERESTS

M.S. and S.K. are inventors on a patent describing the use of a ligand for evaluation of GLUT1 expression (N.T. gave up her rights). M.S. is a co-founder of METAFORA Biosystems, focusing on metabolite transporters under physiological and pathological conditions, and is head of the scientific board.

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(B) Enucleation of shControl- and shIDH1-transduced erythroblasts was assessed on GFP⁺ cells at day 14, and representative histograms are presented (top). Enucleation levels, relative to control cells, were quantified (n = 8, bottom).

(C) The impact of α KG and DHA on erythroblast enucleation following IDH1 knockdown was evaluated between days 7 and 10, and representative histograms are presented (top). Enucleation relative to control conditions was quantified (n = 4, bottom).

(D) The impact of vitamin C on enucleation of control and IDH1-downregulated erythroblasts was evaluated between days 7 and 10. Representative histograms (top) and quantifications (bottom) are presented (n = 5). Changes in mitochondrial superoxide were evaluated by MitoSOX staining, and MFIs are presented (n = 4).

(E) TET2 was downregulated in erythroid progenitors by shTET2 lentivirus (day 4), and enucleation was monitored at day 10 in the presence or absence of vitamin C. Means \pm SEM are presented (n = 5, one-way ANOVA with Tukey's post hoc test).

(F) Nuclear morphology of orthochromatic erythroblasts differentiated from shControl- and shIDH1-transduced progenitors in the absence or presence of vitamin C was monitored. Representative cytopins are shown (left), and the quantification of orthochromatic erythroblasts with abnormal nuclei was determined by counting 500–1,000 cells (n = 3, right).

ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-hCD11b-PE (clone Bear1)	Beckman Coulter	Cat#IM2581U; RRID: AB_131334
Anti-hCD13-PC7 (clone Immuno103.44)	Beckman Coulter	Cat#A46528
Anti-hCD33 (clone D3HL60.251)	Beckman Coulter	Cat#A07775
Anti-hCD34-FITC (clone 581)	Becton Dickinson	Cat#55582; RRID:AB_396150
Anti-hCD34-APC (clone 581)	Becton Dickinson	Cat#555824; RRID:AB_398614
Anti-hCD34-PE (clone 81)	Beckman Coulter	Cat#A07776
Anti-hCD36-APC (clone FA6.152)	Beckman Coulter	Cat#A87786
Anti-hCD36-FITC (clone FA6.152)	Beckman Coulter	Cat#B49201; RRID:AB_2848117
Anti-hCD36-PB (clone FA6.152)	Beckman Coulter	Cat#B43302
Anti-hCD49d-APC (clone HP2/1)	Beckman Coulter	Cat#B01682
Anti-hCD71-APC (clone MA-A712)	Becton Dickinson	Cat#551374; RRID:AB_398500
Anti-hCD71-APC-AF750 (clone MA-A712)	Beckman Coulter	Cat#A89313; RRID:AB_2800452
Anti-hCD105- PECE594 (clone 266)	Becton Dickinson	Cat#562380; RRID:AB_11154054
Anti-hGlyA-PC7 (clone 11E4B-7-6)	Beckman Coulter	Cat#A71564; RRID:AB_2800449
Anti-hGlyA-APC (clone GA-R2 (HIR2))	eBioscience/ThermoFisher	Cat#17-9987-42; RRID:AB_2043823
Anti-hGlyA- BV421 (clone GA-R2 (HIR2))	Becton Dickinson	Cat#562938
Anti-hIL3R-PC7 (clone 6H6)	eBioscience/ThermoFisher	Cat#25-1239-42; RRID:AB_1257136
Goat anti-Rabbit IgG Alexa Fluor 405	Molecular Probes/ThermoFisher	Cat#A-31556; RRID:AB_221605
GLUT1 RBD-GFP	Metafora Biosystems	Cat#GLUT1_G100
GLUT1 RBD-rFc	Metafora Biosystems	Cat#GLUT1_R100
Bacterial and virus strains		
Top 10 Compotent cells	ThermoFisher	Cat#C404010
Biological samples		
Human umbilical cord blood	Unité De Thérapie Cellulaire de Montpellier	N/A
Human peripheral blood	New York Blood Center	N/A
Human bone marrow	Northwell Health	N/A
Chemicals, peptides, and recombinant proteins		
2-Phospho-L-ascorbic acid	Sigma-Aldrich	Cat#A8960
tetramethylchromane-2-carboxylic acid (TROLOX)	Sigma-Aldrich	Cat#238813
N-Acetylcysteine	Sigma-Aldrich	Cat#A9165
Ascorbate Oxidase from Cucurbita sp.	Sigma-Aldrich	Cat#A0157
L-Ascorbic acid	Sigma-Aldrich	Cat#A92902
O-(Carboxymethyl)hydroxylamine hemihydrochloride (AOA)	Sigma-Aldrich	Cat#C13408
Diethylsuccinate	Sigma-Aldrich	Cat#112402
Dimethyl α -ketoglutarate	Sigma-Aldrich	Cat#349631
L-Glutathione reduced	Sigma-Aldrich	Cat#G4251
MitoParaquat	Abcam	Cat#ab146819
Spautin-1	Sigma-Aldrich	Cat#SML0440
Tri-sodium Citrate dihydrate	Sigma-Aldrich	Cat#S4641
C13-GlutamineL-Glutamine (13C5,15N2)	Cambridge Isotope Laboratories	Cat#CNLM-1275-H-PK

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
L-[3,4- ³ H(N)]-Glutamine	Perkin Elmer	Cat#NET551250UC
Oligomycin	Sigma-Aldrich	Cat#O4876
FCCP	Sigma-Aldrich	Cat#C2920
Rotenone	Sigma-Aldrich	Cat#R8875
Antimycin A	Sigma-Aldrich	Cat#A8674
DMSO	Sigma-Aldrich	Cat#D2650
Human recombinant IL-3	R&D Systems	Cat#203-IL-010
Human recombinant IL-6	R&D Systems	Cat#206-IL-010
rhuSCF	Amgen	N/A
Stem Spam CC100	Stem Cell	Cat#02690
EPREX	Janssen	Cat#3400936992368
Human holo-transferrin	Sigma-Aldrich	Cat#T0665
Human insulin solution	Sigma-Aldrich	Cat#9278
Heparin	Sanofi	Cat#3400930484500
Bovine serum albumin	Sigma-Aldrich	Cat#A8806
IMDM	Life Technologies/ThermoFisher	Cat#21980032
StemSpam SFEM	Stem Cell	Cat#09650
StemSpam H3000	Stem Cell	Cat#09850
RPMI w/o glutamine and glucose	Pan Biotech	Cat#P04-17550
Seahorse XF Assay Medium	Agilent Technologies	102365-100
Seahorse XF Calibrant Solution	Agilent Technologies	100840-000
Ultima Gold liquid scintillation cocktail	Perkin Elmer	Cat#6013329
Critical commercial assays		
Anti-hCD34 MicroBead kit	Miltenyi Biotec	Cat#130-046-702; RRID:AB_2848167
RNeasy Mini kit	QIAGEN	Cat# 74104
QuantiTect™ Reverse Transcription Kit	QIAGEN	Cat# 205311
Experimental models: cell lines		
Jurkat E6-1	ATCC	TIB-152
293 T cells	ATCC	CRL-3216
Oligonucleotides		
IDH1 forward: 5'-GGGTTGGCCTTTGTATCTGA-3'	IDT	Custom order
IDH1 reverse: 5'-TTTACAGGCCAGATGAAGC-3'	IDT	Custom order
IDH2 forward: 5'-TGGCTCAGGTCCTCAAGTCT-3'	IDT	Custom order
IDH2 reverse: 5'-CTCAGCCTCAATCGTCTTCC-3'	IDT	Custom order
IDH3a forward: 5'-GCGCAAACATTTGACCTTT-3'	IDT	Custom order
IDH3a reverse: 5'-TGCACGACTCCATCAACAAT-3'	IDT	Custom order
TET2 forward: 5'-AGCAGCAGCCAATAGGACAT-3'	IDT	Custom order
TET2 reverse: 5'-AGGTTCTGTCTGGCAAATGG-3'	IDT	Custom order
βactin forward: 5'-GTCTTCCCCTCCATCGTG-3'	IDT	Custom order
βactin reverse: 5'-TTCTCCATGTCGTCCAG-3'	IDT	Custom order

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
TRC2pLKO sh Tet2	Sigma Aldrich	Cat# TRCN0000418976
TRC2pLKO sh IDH1	Sigma Aldrich	Cat# TRCN0000312463
TRC1pLKO sh IDH1	Sigma Aldrich	Cat# TRCN0000027284
TRC1pLKO sh non-mammalian control	Sigma Aldrich	Cat# SHC002
Software and algorithms		
FlowJo (version 10)	BD Biosciences	https://www.flowjo.com/
Prism (version 8)	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Wave (version 2)	Agilent Technologies	https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-software/seahorse-wave-desktop-software-740897
Xcalibur 2.2	ThermoFisher	http://www.casco-documentation.com/content/dam/tfs/ATG/CMD/cmd-documents/oper/oper/ms/lc-ms/soft/Man-XCALI-97209-Xcalibur-22-Acquisition-ManXCALI97209-D-EN.pdf
LCquan 2.7	ThermoFisher	https://assets.thermofisher.com/TFS-Assets/CMD/manuals/LCquan-Admin-2-7.pdf
TraceFinder 4.1	ThermoFisher	https://www.thermofisher.com/us/en/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-software/lc-ms-data-acquisition-software/tracefinder-software.html
Other		
CM-H2DCFDA	Molecular Probes/Thermofisher	Cat#C6827
Dihydrorhodamine 123	Molecular Probes/Thermofisher	Cat#D23806
Mitotracker Deep Red FM	Molecular Probes/Thermofisher	Cat#M22426
MitoTracker Green FM	Molecular Probes/Thermofisher	Cat#M7514
MitoTracker Red CMXRos	Molecular Probes/Thermofisher	Cat#M7512
MitoSox Red	Molecular Probes/Thermofisher	Cat#M36008
Propidium Iodide	Sigma-Aldrich	Cat#P4170
Syto16	Molecular Probes/Thermofisher	Cat#S7578
Syto60	Molecular Probes/Thermofisher	Cat#S11342

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Naomi Taylor (taylorn4@mail.nih.gov).

Materials availability

This study did not generate new unique reagents. All lentiviral vectors generated in this study are available on request.

Data and code availability

This study did not generate datasets or code.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

CD34⁺ cell isolation and ex vivo differentiation assays.

All studies involving human samples were conducted in accordance with the declaration of Helsinki. CD34⁺ cells were isolated from umbilical cord blood within 24h of vaginal delivery of full-term infants after informed consent and approval by the “Committee for the Protection of Persons” (IRB) of the University Hospital of Montpellier. Peripheral blood from leukoreduction filters and bone marrow were obtained from deidentified donors at the New York Blood Center and the North Shore-LIJ Health System, respectively, after informed consent and approval from the respective IRBs. Approximately 60 UCB, 10 PB and 6 BM samples were used in this study. Samples did not contain any identifiers including sex, race, or ethnic origin. All samples were split such that they were used in all experimental groups. As such, there was no need to use any specific criteria to allocate biological samples to experimental groups. For the vast majority of experiments, at least 3 biological samples were used. For experiments presented in [Figures 1A, 6F, and 6G](#), 2 biological samples were employed and technical triplicates were evaluated for both samples.

CD34⁺ cells (1×10^5 cells/ml) were expanded in StemSpan H3000 media (StemCell Technologies Inc) supplemented with StemSpan CC100 (StemCell Technologies Inc) at 37°C with a humidified 5% CO₂ atmosphere. Cells were plated in 24-well *Nunc* Delta surface plates (*Nunc, Thermo Fisher Scientific*). After 4 days of expansion, cells were differentiated in IMDM medium (Invitrogen) supplemented with human holo-transferrin (200 mg/L, Sigma-Aldrich), human insulin (10 mg/L, Sigma-Aldrich), rhuSCF (10 ng/ml, Amgen), rhIL-3 (1 ng/ml, R&D Systems). Erythropoiesis was induced by addition of 3 U/ml recombinant human erythropoietin (rEPO; Eprex, Janssen-Cilag). In some experiments, CD34⁺ cells were not expanded and erythropoiesis was directly induced after selection (data in [Figure 5](#)). Cytokines were supplemented every 3–4 days at the same time that media was renewed. Cells were centrifuged at 300 g for 10 min at RT and then resuspended in fresh media to maintain cell concentrations between 0.1 and 1.5×10^6 cells/ml. In some experiments, progenitors were differentiated in the presence of 3.5 mM cell permeable dimethyl α -ketoglutarate (DMK, Sigma-Aldrich), 5 mM diethylsuccinate (Sigma Aldrich), 8 mM citrate (Sigma-Aldrich), 1 mM aminooxyacetate (AOA, Sigma-Aldrich), 50 μ M MitoParaquat (MitoPQ, Abcam), 1 mM L-ascorbic acid (AA, Sigma-Aldrich), 100 μ M 2-phospho-L-ascorbic acid (Vit C, Sigma-Aldrich), 100 μ M N-acetylcysteine (NAC, Sigma-Aldrich), 100 μ M reduced glutathione (GSH, Sigma-Aldrich), 100 μ M of the water-soluble analog of vitamin E (TROLOX, Sigma Aldrich), or 10 μ M of specific and potent autophagy inhibitor-1 (Spautin-1, Sigma-Aldrich). Ascorbate oxidase (1 U/mL, Sigma-Aldrich) was used to completely oxidize L-ascorbic acid to DHA in culture medium before treating the cells. Drugs were freshly added after every media change. In experiments where cells were co-cultured with α -KG (DMK, 3.5 mM) in the presence of antioxidants, α -KG was administered 2 hours after the addition of the corresponding antioxidant. As MitoPQ and TROLOX were dissolved in DMSO, control conditions included cultures using the same concentration of DMSO (0.05 and 0.1%, respectively).

METHODS DETAIL

Cytospin preparation

Cytospins were prepared on glass slides (5×10^4 cells in 200 μ L of PBS), using the Thermo Scientific Shandon 4 Cytospin (300 g for 5 min). Slides were stained with May-Grünwald solution (Sigma-Aldrich) for 5 min, rinsed in 40 mM Tris buffer for 90 s, and subsequently stained with Giemsa solution (Sigma-Aldrich) for 15 minutes. Cells were imaged using a Leica DM2000 inverted microscope under 100 \times objective magnification.

Flow cytometry

Expression of the CD34, CD36, CD49d, CD71, CD105 ([Ashley et al., 2020](#)), Glycophorin A, IL3R, CD11b, CD13, and CD33 surface markers was monitored on $1\text{--}2 \times 10^5$ cells using the appropriate fluorochrome-conjugated monoclonal antibodies at a 1:100 dilution (mAb), except for CD36-APC and CD71-AF750 mAbs that were used at a 1:200 and 1:500 dilution, respectively (Beckman Coulter, Becton Dickinson, eBiosciences), in a total volume of 100 μ L as previously described ([Hu et al., 2013](#); [Schulz et al., 2019](#)). Cells were incubated in the dark for 20 minutes in PBS containing 2% FBS at 4°C and then washed once in the same medium at 300 g for 10 min prior to evaluation. GLUT1 expression was monitored by binding to its retroviral envelope ligand (RBD) fused to eGFP (1:25 dilution in 50 μ L; Metafora biosystems) or to the RBD-rFc fusion protein (1:25 dilution in 50 μ L) for 30 minutes in PBS containing 2% FBS at 37°C followed by staining with an Alexa Fluor 405-coupled anti-rabbit IgG antibody (1:100 dilution in 100 μ L) at 4°C as previously reported ([Kim et al., 2004](#); [Kinet et al., 2007](#); [Manel et al., 2003](#); [Swainson et al., 2005](#)). Mitochondrial biomass was assessed in a total volume of 50 μ L by MitoTrackerGreen or MitoTrackerDeepRed staining (20 nM; Invitrogen, Molecular Probes), while mitochondrial transmembrane potential levels were monitored by staining with MitoTrackerRed (50 nM; Invitrogen, Molecular Probes). Mitochondrial superoxide production, mitochondrial ROS levels and total ROS levels were assessed by staining with MitoSOX, Dihydrorodamine 123 and CM-H2DCFDA, respectively (5 μ M; Invitrogen, Molecular Probes). Incubations were performed in the dark for 20 minutes in PBS + 2% FBS at RT. Enucleation was evaluated by staining with the SYTO16 or SYTO60 nucleic acid stain (1 μ M and 0.5 μ M, Invitrogen, Molecular Probes) for 15 minutes in PBS + 2% FBS at RT. Cell cycle analysis was performed by staining for DNA using 50 μ g/mL propidium iodide (PI; Sigma-Aldrich) following permeabilization in 100 μ L of 0.1% Triton X-100. To assess cell death, cells were incubated with PI (10 μ g/ml). Cell sorting was performed on a FACSARIA high-speed cell sorter (BD

Biosciences) and analyses were performed on a FACS-Canto II (BD Biosciences) cytometers. A minimum of 10,000 events were recorded for each staining. Data analyses were performed using FlowJo software (Tree Star, Ashland, OR).

Virus production and transduction of CD34⁺ progenitor cells

Lentiviral pLKO.1 plasmids harboring shRNAs directed against *IDH1* (shRNA IDH1-1: Clone ID: TRCN0000027284 and shRNA IDH1-2: ID: TRCN0000312463) and lentiviral TRC2-pLKO plasmid harboring shRNA against TET2 (ID: TRCN0000418976) (Yan et al., 2017) were obtained from Sigma-Aldrich. Cells were transduced with the shRNA IDH1-1 lentiviral plasmid, the shRNA IDH1-2 plasmid or the shRNA TET2 plasmid where the 714bp sequence encoding for EGFP was inserted in place of the puromycin gene at the unique BamHI and KpnI restriction sites. Virions were generated by transient transfection of 293T cells (2–3 × 10⁶ cells/100mm plate in 7 mL DMEM) with these vectors (10 mg) together with the Gag-Pol packaging construct PsPax2 (5 mg) and a plasmid encoding the VSV-G envelope, pCMV-VSV-G (2.5 mg), as described previously (Loisel-Meyer et al., 2012). Cells were transfected for at least 18 hours, and transfection efficiency was verified by monitoring GFP fluorescence by microscopy. Viral supernatant was harvested 24 hours post-transfection and virions were concentrated by overnight centrifugation at 4°C at 1,500 g (with no break). Virions were resuspended in approximately 20 μL of RPMI with 10% FBS (per plate of cells), aliquoted and stored at –80°C. Titers were determined by serial dilutions of virus preparations on Jurkat cells and are expressed as Jurkat transducing units (TU/ml).

Prior to transduction of CD34⁺ hematopoietic stem and progenitor cells (HSPCs), cells were expanded for 3 days in StemSpan medium (Stem cell Technologies Inc) supplemented with 5% fetal bovine serum (FBS), 25 ng/ml rhuSCF (Amgen), 10 ng/ml rhIL-3 and 10 ng/ml rhIL-6 (R&D) at 37°C with a humidified 5% CO₂ atmosphere. 5 × 10⁵ cells were then exposed to viral supernatants containing 4.8 × 10⁵ TU to 9.9 × 10⁵ TU (representing a multiplicity of infection of 1–2). After 72h of transduction, CD34⁺ cells (1 × 10⁵ cells/ml) were cultured in presence of rEPO (3 U/ml) to induce erythropoiesis. Transduction efficiency was monitored as a function of GFP expression or after puromycin selection at 72h post-transduction, as per the lentiviral vector. In some experiments, cells were transduced at day 4 of erythroid differentiation (i.e., at day 4 following addition of EPO) under the same conditions as described above, and transduction efficiency was monitored at day 7 of differentiation.

Quantitative real time PCR

Total RNA was isolated at the indicated time points and cDNA was synthesized using the RNeasy Mini kit and the QuantiTectTM Reverse Transcription Kit (QIAGEN) as per the manufacturer's instructions. Quantitative PCR of cDNAs was performed using the Quantitect SYBR green PCR Master mix (Roche) with 10 ng of cDNA (by NanoDrop) and 0.5 μM primers in a final volume of 10 μL. Primer sequences are as follows: *IDH1*: 5'-GGGTGGCCTTTGTATCTGA-3' (forward) / 5'-TTTACAGGCCAGATGAAGC –3' (reverse); *IDH2*: 5'-TGGCTCAGGTCCTCAAGTCT-3' (forward) / 5'-CTCAGCCTCAATCGTCTTCC-3' (reverse); *IDH3a*: 5'-GCGCAAAA CATTTGACCTTT-3' (forward) / 5'-TGCACGACTCCATCAACAAT-3' (reverse); *TET2*: 5'-AGCAGCAGCCAATAGGACAT-3' (forward) / 5'-AGGTTCTGTCTGGCAAATGG-3' (reverse); and β -actin: 5'-GTCTTCCCCTCCATCGTG-3' (forward) / 5'-TTCTCCATGTCGTCCC AG-3' (reverse). Amplification of cDNAs was performed using the LightCycler 480 (Roche). Cycling conditions included a denaturation step for 5 min at 95°C, followed by 40 cycles of denaturation (95°C for 10 s), annealing (63°C for 10 s) and extension (72°C for 10 s). After amplification, melting curve analysis was performed with denaturation at 95°C for 5 s and continuous fluorescence measurement from 65°C to 97°C at 0.1°C/s. Each sample was amplified in triplicate. Data were analyzed by LightCycler[®] 480 Software (Version 1.5) and Microsoft Excel. Relative expression was calculated by normalization to β -actin as indicated (delta-Ct). Real-time PCR CT values were analyzed using the 2(-Delta Delta C(T)) method to calculate fold expression (ddCt).

Mass spectrometry (LC-MS)

For profiling of intracellular metabolites, 10⁶ cells were incubated in IMDM medium without glutamine and were supplemented with 2mM uniformly labeled ¹³C₅-¹⁵N₂ glutamine (Cambridge Isotope Laboratories) for 6 hours (Figure 1A) or 18 hours (Figures 6F and 6G). Cells were rapidly washed in ice-cold PBS, centrifuged twice (400 g for 4 min), and metabolites were extracted in a solution of 50% methanol, 30% acetonitrile, 20% water after shaking for 20 min at 4°C. The solutions were then recentrifuged for 10 min at 16,000 g at 4°C. The volume of the extraction solution was adjusted to achieve a constant ratio of 2 × 10⁶ cells/ml. Alternatively, the peak areas resulting from the intracellular samples were normalized to the number of cells obtained at the end of the incubation period in parallel wells. Supernatants were stored at –20°C until analysis. Samples were analyzed by HPLC-MS as described previously (Vande Voorde et al., 2019). Data were analyzed with Xcalibur 2.2, LCQuan 2.7 and TraceFinder 4.1 software (Thermo scientific). Samples were performed in triplicate, and at least 2 different biological samples were evaluated for each condition.

Seahorse analysis

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured using the XFe-96 Extracellular Flux Analyzer (Seahorse Bioscience, Agilent). The calibration plate was filled with 200 μL Milli-Q water per well and kept at 37°C overnight. At least 2 hours prior to the assay, water was replaced by 200 μL XF calibrant (Agilent)/ well. Culture plates were treated with 20 μL/well of 0.1mg/mL of Poly-D-Lysine for at least 1 hour at RT. Poly-D-Lysine (Sigma-Aldrich) solution was then removed and plates was washed twice with 200 μL Milli-Q water/well, dried, and kept at 4°C until the assay was performed. Cells (2.5 × 10⁵) were placed in 50 μL XF medium (non-buffered DMEM containing 10 mM glucose and 2 mM GlutaMax, pH 7.3–7.4, Agilent), centrifuged in a carrier tray (300 g for 3min with no break), and incubated for 30 min at 37°C. An additional 130 μL XF medium was added during the

calibration time (20–30 min at 37°C). Cells were monitored in basal conditions and in response to oligomycin (1 μ M; Sigma-Aldrich), FCCP (1 μ M; Sigma-Aldrich), rotenone (100 nM, Sigma-Aldrich) and antimycin A (1 μ M; Sigma-Aldrich). In some experiments, α KG (DMK, final concentration of 3.5 mM) was injected directly into the well before the sequential addition of oligomycin, FCCP, rotenone and antimycin A during the Mito Stress Test. Wave software (Agilent) was employed for running the Seahorse assay and analysis. Samples were performed at least in triplicate and generally 5–6 points per experiment and at least 3 independent experiments.

Glutamine uptake assays

Cells (5×10^5) were washed twice in 1.5 mL of PBS containing 2%FBS at 400 g for 4 min at RT, and starved for glutamine by incubation at 37°C in 30 μ L glutamine-free RPMI for 30 min. Radiolabeled glutamine-L-[3,4- 3 H(N)] (Perkin Elmer) was added to a final concentration of 0.5 μ M (0.5 μ Ci/mL in a total volume of 50 μ L). Cells were incubated for 10 min at room temperature, washed twice in cold PBS containing 2%FBS (1000 g, 3 min), and solubilized in 500 μ L of a 0.1% SDS solution. Radioactivity was measured in 4.5 mL liquid scintillation (Perkin Elmer) using a Hidex 300 SL counter. Each independent experiment was performed in triplicate.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are represented as individual values or means. Error bars represent the standard error of the mean (SEM). Data were analyzed with GraphPad Software (GraphPad Prism), and *P*-values were determined by one-way ANOVA (Tukey's post hoc test) or *t* tests, as indicated in the corresponding text and figure legends. Two-tailed *t* tests were used in all figures except for Figure 6F where a one-tailed *t* test was used. Tests were paired or unpaired as indicated and a normal distribution was used in all experiments. All statistical details of experiments can be found in the figure legends. Significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ as evaluated by GraphPad. The graphical abstract was created using BioRender and Servier Medical Art.