ARTICLE

Glutathione peroxidase 4 and vitamin E control reticulocyte maturation, stress erythropoiesis and iron homeostasis

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ABSTRACT

Glutathione peroxidase 4 (GPX4) is unique as it is the only enzyme that can prevent detrimental lipid peroxidation *in vivo* by reducing lipid peroxides to the respective alcohols thereby stabilizing oxidation products of unsaturated fatty acids. During reticulocyte maturation, lipid peroxidation mediated by 15-lipoxygenase in humans and rabbits and by 12/15-lipoxygenase (ALOX15) in mice was considered the initiating event for the elimination of mitochondria but is now known to occur through mitophagy. Yet, genetic ablation of the *Alox15* gene in mice failed to provide evidence for this hypothesis. We designed a different genetic approach to tackle this open conundrum. Since either other lipoxygenases or non-enzymatic autooxidative mechanisms may compensate for the loss of *Alox15*, we asked whether ablation of *Gpx4* in the hematopoietic system would result in the perturbation of reticulocyte maturation. Quantitative assessment of erythropoiesis indices in the blood, bone marrow (BM) and spleen of chimeric mice with *Gpx4* ablated in hematopoietic cells revealed anemia with an increase in the fraction of erythroid precursor cells and reticulocytes. Additional dietary vitamin E depletion strongly aggravated the anemic phenotype. Despite strong extramedullary erythropoiesis reticulocytes failed to mature and accumulated large autophagosomes with engulfed mitochondria. *Gpx4*-deficiency in hematopoietic cells led to systemic hepatic iron overload and simultaneous severe iron demand in the erythroid system. Despite extremely high erythropoietin and erythroferrone levels in the plasma, hepcidin expression remained unchanged. Conclusively, perturbed reticulocyte maturation in response to *Gpx4* loss in hematopoietic cells thus causes ineffective erythropoiesis, a phenotype partially masked by dietary vitamin E supplementation.

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Introduction

Glutathione peroxidase 4 (GPX4) is unique in its ability to reduce lipid peroxidation products in biological membranes *in vivo*. Lipid peroxidation is brought about either enzymatically by lipoxygenases at specific sites or unselectively by non-enzymatic mechanisms, usually through Fe^{2+}-driven Fenton chemistry.[3] Lipoxygenases are dioxygenases that catalyze the incorporation of molecular oxygen into polyunsaturated fatty acids (PUFA) in a site- and stereospecific manner thus yielding the respective hydroxyperoxides. Lipoxygenases become activated by low concentrations of peroxides (the so called "peroxide tone") that oxidize Fe^{2+} to Fe^{3+} in the catalytic site.[4] The peroxide tone and the activity of lipoxygenases is under the control of GPX4. 12/15-Lipoxygenase and GPX4 act antagonistically as far as oxidation (lipoxygenases) and reduction (GPX4) of substrates, and induction of cell death and cell survival are concerned. 12/15-Lipoxygenase induces cell death in murine fibroblasts, whereas GPX4 rescues cells from lipoxygenase-induced cell death.[5] But in other settings, lipoxygenases and GPX4 biochemically cooperate: lipoxygenases generate highly reactive peroxidation products of unsaturated fatty acids (P-O-O-H) that are prone to further uncontrolled lipid membrane peroxidation. GPX4 reduces these peroxides to stable hydroxyl-derivatives (P-O-H).[6-9] Thereby, 15-lipoxygenase in humans or 12/15-lipoxygenase, its functional homolog in mice, and GPX4 constitute a pair of enzymes whose activities are tightly interconnected. In the interplay of lipoxygenases and GPX4 the role of vitamin E also has to be considered. Vitamin E intercalates into membranes, acts as a chain breaker of lipid peroxidation through its high affinity for unpaired electrons and thus antagonizes peroxide production.

GPX4 is 1 of 24 (25 in man) selenoproteins in mammals[10] and is positioned at the top of the hierarchy of selenoproteins, *i.e. Gpx4* expression is maintained even under severe selenium-deficiency when the synthesis of most other selenoproteins has ceased.[11] GPX4 has evolved to carry the 21st amino acid selenocysteine rather than its functional counterpart cysteine in the active site which renders the enzyme highly resistant to irreversible overoxidation through peroxides.[12] Dietary selenium is known to be required for stress erythropoiesis in mice and blockage of the synthesis of all selenoproteins in hematopoietic cells by selective deletion of the selenocysteine-specific t-RNA Trsp in the BM of chimeric mice severely impairs stress erythropoiesis.[13]

Mitochondria are removed from reticulocytes by a particular form of autophagy, called mitophagy, and oxidized lipids are considered to play a crucial role in triggering autophagy in various cell types. Furthermore, macrophages from *12/15-lipoxygenase* knockout (k.o.) mice exhibit abnormal mitochondria, cytoplasmic vacuoles and an altered phospholipidomics pattern indicative of impaired autophagy.[19] In addition, the 12/15-lipoxygenase oxidation product 12-hydroxyeicosatetraenoic acid-phosphatidylethanolamine (12-HETE-PE) was shown to be a better substrate for yeast Atg8 than native PE, whereas native as well as oxidized PE were both effective substrates for LC3 lipidation. With regard to reticulocyte maturation current data suggest that elimination of mitochondria through mitophagy may be stimulated by oxidation products of polyunsaturated membrane phospholipids.

15-lipoxygenase is highly expressed in reticulocytes and was reported to be involved in the elimination of mitochondria by Rapoport and his coworkers.[20-23] The initial work of the Rapoport group was confirmed and extended by van Leyen *et al*. who reported a similar 12/15-lipoxygenase-driven mechanism that degrades organelles in the eye lens.[24,25] Yet, the great interest in this early work decreased with the findings that erythrocyte and reticulocyte counts were normal in *12/15-lipoxygenase* ko mice.[26] Definitive genetic proof for a role of lipid oxidation during reticulocyte maturation is thus still lacking. Several reasons may account for the fact that *12/15-lipoxygenase* k.o. mice exhibit normal red blood and reticulocyte counts: (i) lipoxygenases other than *12/15-lipoxygenase* may compensate for the targeted loss of reticulocyte 12/15-lipoxygenase; (ii) lipoxygenases may become dispensable if non-enzymatic mechanisms of lipid oxidation prevail;[27-29] or (iii) lipid oxidation events are dispensable during reticulocyte maturation.

Our present work was conceived to definitively answer if lipid oxidation is indeed critically involved in mitophagy in reticulocytes using a well-defined genetic approach. Whereas many different enzymatic and non-enzymatic mechanisms may account for the initial lipid oxidation step, GPX4 stands out as the only enzyme that effectively prevents detrimental lipid peroxidation and allows lipid oxidation to proceed in a highly controlled manner. Thus, if lipid oxidation is an essential step in the elimination of mitochondria in reticulocytes, ablation of *Gpx4* should result in uncontrolled lipid peroxidation and perturbation of reticulocyte maturation. Since GPX4 is essential for early embryonic development and the survival of adult mice,[30,31] *Gpx4* had to be deleted specifically in hematopoietic cells. To this end, we took advantage of the Tamoxifen-inducible Cre/lox system,[32] which is of invaluable help when the side effects of Cre[33-37] and Cre activators[38] or inducers[39] are properly controlled. We show that GPX4 is required for stress erythropoiesis. Deletion of *Gpx4* in adult mice causes anemia and ineffective erythropoiesis due to impaired reticulocyte maturation, a phenotype dramatically aggravated by depleting vitamin E from the diet. As a consequence hepatic iron overload develops despite the continuous iron demand for red blood cell production.

Methods

Mice

Mice were bred under SPF conditions. *Gpx4$^{fl/fl}$;CreERT2* mice were backcrossed for at least ten generations onto C57BL/6J mice (Taconic Biosciences, Köln, Germany). All animal experiments were performed according to the institutional guidelines and were approved by the local ethic committees on animal experimentation and by the Government of Upper Bavaria and Kantonales Veterinäramt in Zürich, respectively.

Generation of chimeric mice with *Gpx4*-proficient and *Gpx4*-deficient hematopoietic cells and analysis of blood parameters

Female wild-type (wt) recipient mice of 10 to 12 weeks (Taconic Biosciences, Köln) were lethally irradiated with 850 cGy and reconstituted with 10^6 BM cells from *Gpx4$^{fl/fl}$;CreERT2* or *Gpx4$^{wt/wt}$;CreERT2* donor mice. BM cells had been collected by flushing the leg bones and crushing the pelvic bone.

Gpx4$^{fl/fl}$;*CreERT2* and *Gpx4*$^{wt/wt}$;*CreERT2* mice have been described.[5,31,32] 150 mg/kg 5-fluorouracil (FU) had been administered to donor mice by intraperitoneal (i.p.) injection 24 hours prior to collecting BM cells from donor mice. After hematopoietic reconstitution mice were allowed to recover for 25 weeks. Mice were fed a tamoxifen citrate containing diet for three weeks following the protocol of Kiermayer *et al.*[40] to delete *Gpx4*. Ethylenediamine tetraacetic acid (EDTA)-blood was collected from the tail vein before, at the last day of and at different time points after tamoxifen administration, and subjected to the analy-

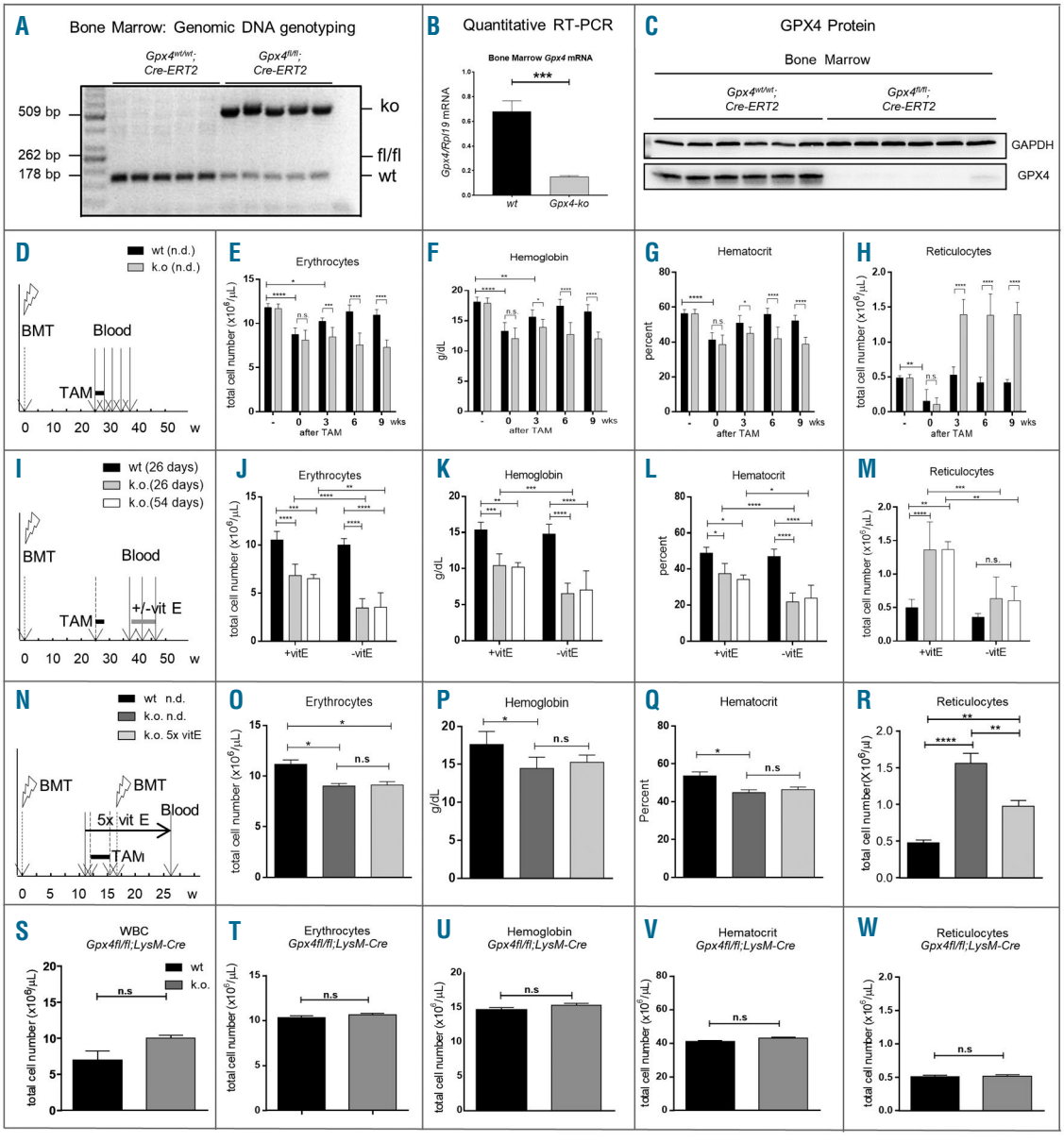

Figure 1. (A-H) *Gpx4* is required for stress erythropoiesis in the recovery phase of anemia. Deletion of *Gpx4* in the bone marrow (BM) was monitored by PCR using two primers pairs. One detects the deleted allele (509 bp), the other discriminates between the floxed and wild-type (wt) allele. Absence of the floxed allele indicates that deletion was complete. (A) Presence of the wt band indicates that BM cells contain a small proportion of cells of non-hematopoietic origin. (B) Quantification of *Gpx4* mRNA in the bone marrow by quantitative RT-PCR. (C) Detection of GPX4 protein by Western blot analysis. (D) Temporal scheme of bone marrow transplantation (BMT), tamoxifen treatment (TAM) and determination of red blood cell (RBC) parameters. Lethally irradiated mice were reconstituted with 10^6 BM cells of *Gpx4*$^{wt/wt}$; *Cre ERT2* (designated wt, black columns, n=10) and *Gpx4*$^{fl/fl}$;*Cre ERT2* mice (designated k.o., grey columns, n=19). Mice were fed a tamoxifen citrate containing diet for three weeks. Blood was drawn before (left columns, - TAM), at the last day of (0), and 3, 6, and 9 weeks after tamoxifen administration (+TAM), and (E) erythrocyte counts, (F) hemoglobin, (G) hematocrit , and (H) reticulocyte counts were determined. (I-M) Vitamin E depletion in the diet severely aggravates the anemia caused by *Gpx4*-deficiency in hematopoietic cells. (I) Temporal scheme of BMT, tamoxifen administration, vitamin E depletion and determination of red blood parameters. Lethally irradiated mice were reconstituted with BM cells of *Gpx4*$^{wt/wt}$;*Cre-ERT2* (designated wt, black columns) and *Gpx4*$^{fl/fl}$;*Cre ERT2* mice (designated k.o., grey and white columns). After tamoxifen administration for three weeks, mice were allowed to recover for 12 weeks before the vitamin E-depleted diet was started (- vitE, n=7 for wt, n=7 for k.o.) or the normal diet continued (+ vitE, n=3 for wt, n=7 for k.o.). Blood was drawn before vitamin E depletion (time point 9 weeks in Figure 1D-H), and 26 (black and grey columns) and 54 days [white columns], n=3) after starting the vitamin E-depleted diet for the determination of (J) erythrocyte counts, (K) hemoglobin, (L) hematocrit levels, and (M) reticulocyte counts. Red blood parameters of blood taken 54 days after starting the vitamin E-depleted diet were unaltered as compared to the earlier time point. N-R) Administration of a vitamin E-enriched diet. (N) Temporal scheme of feeding the mice a vitamin E-enriched diet. (R) A 5-fold increase of α-tocopherol in the diet reduced the degree of reticulocytosis but had not impact on (O) erythrocyte counts, (P) hemoglobin and (Q) hematocrit levels. S-W) White blood counts and red blood parameters and in *Gpx4*$^{fl/fl}$;*LysMCre* and control mice. There is no difference in (S) white blood cell (WBC) counts, (T) erythrocytes, (U) hemoglobin, (V) hematocrit, and (W) reticulocyte counts between *Gpx4*$^{fl/fl}$;*LysMCre* and control mice.

sis of blood parameters using a Sysmex XT-2000iV apparatus as described.[41]

Vitamin E depletion and repletion

Mice were deprived from vitamin E by feeding a vitamin E-depleted diet (SSNIFF, Soest, Germany) containing 7ppm α-tocopherol as compared to 55ppm in the normal diet. In initial experiments the conditions for ruling out side effects of Cre activation[33] with and without vitamin E-depletion using *Gpx4*$^{wt/wt}$*;CreERT2* mice as controls were defined (*Online Supplementary Figure S1*). This allowed to apply a simplified protocol with C57BL/6J mice as controls and tamoxifen-treated, lethally irradiated wt mice reconstituted with BM cells of *Gpx4*$^{fl/fl}$*;CreERT2* mice receiving a normal or a vitamin E-depleted diet as experimental groups (Figure 2-5 and *Online Supplementary Figure S5*). The vitamin E-repleted diet (5x vitamin E) contained 275ppm α-tocopherol (SSNIFF, Soest, Germany).

Staining of erythroid precursor cells from BM and spleen

Spleens were smashed and washed and the four leg bones flushed with 5 mL PBS containing 1mM EDTA and 2% fetal calf

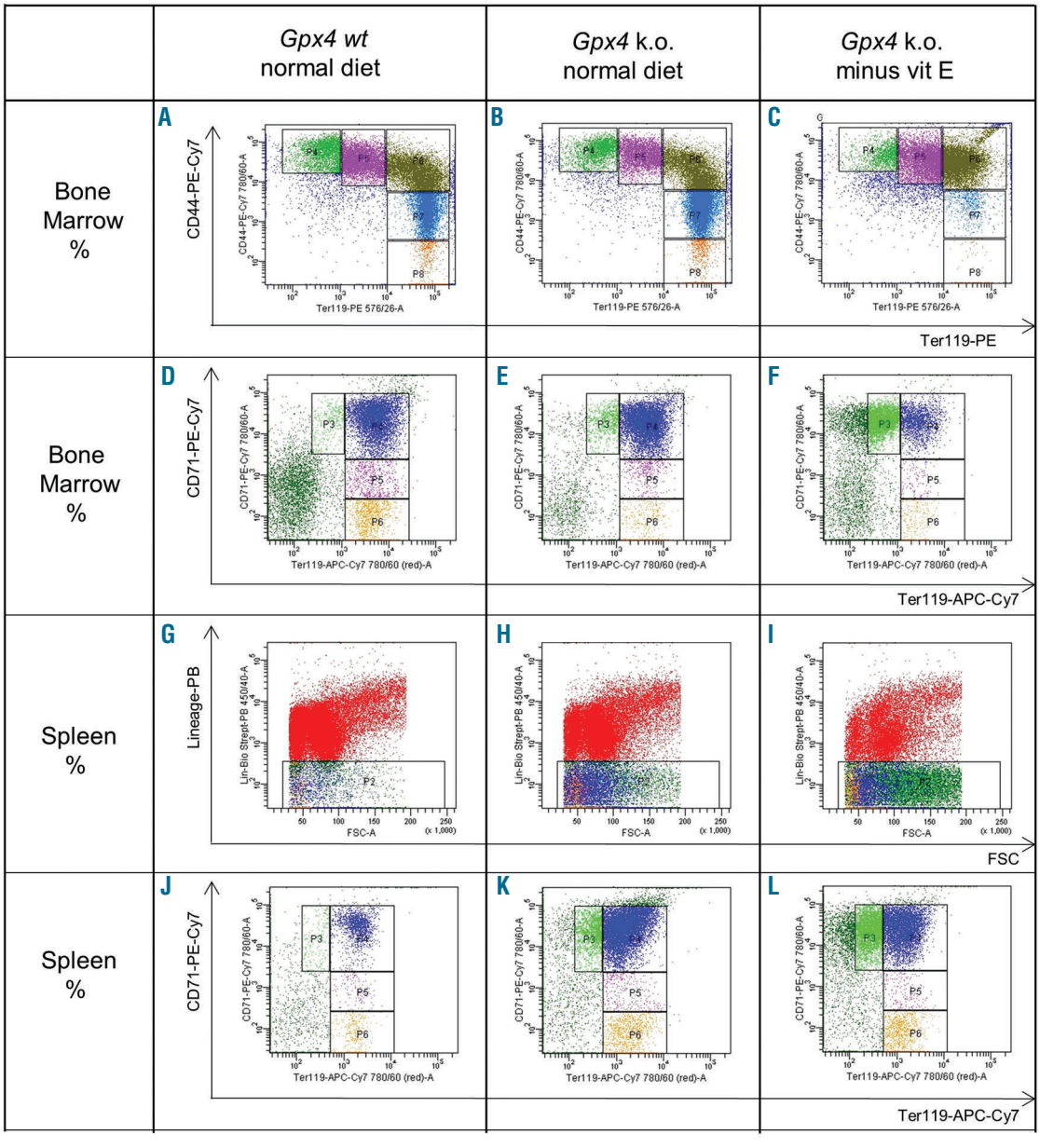

Figure 2. Relative increase in immature erythroid precursor cells in the bone marrow and spleen of mice with *Gpx4*-deficiency in the hematopoetic system. Representative fluorescence-activated cell sorting (FACS) staining of (A-F) BM and (G-L) spleen cells with CD44-PE-Cy7 and Ter119-PE (A-C) as well as with CD71-PE-Cy7 and Ter119-PE antibodies (D-F, J-L). The gate set by forward sideward scatter (FSC) and lineage marker-negative cells (G-I) illustrates the increase in extramedullary erythropoiesis in the spleen of mice with *Gpx4*-deficient hematopoietic cells kept on a (H) normal or (I) on a vitamin E-depleted diet as compared to (G) *Gpx4* wt mice kept on a normal diet. The shift towards immature erythroid precursor cells is strongly increased under combined *Gpx4*- and vitamin E-deficiency (C, F, L).

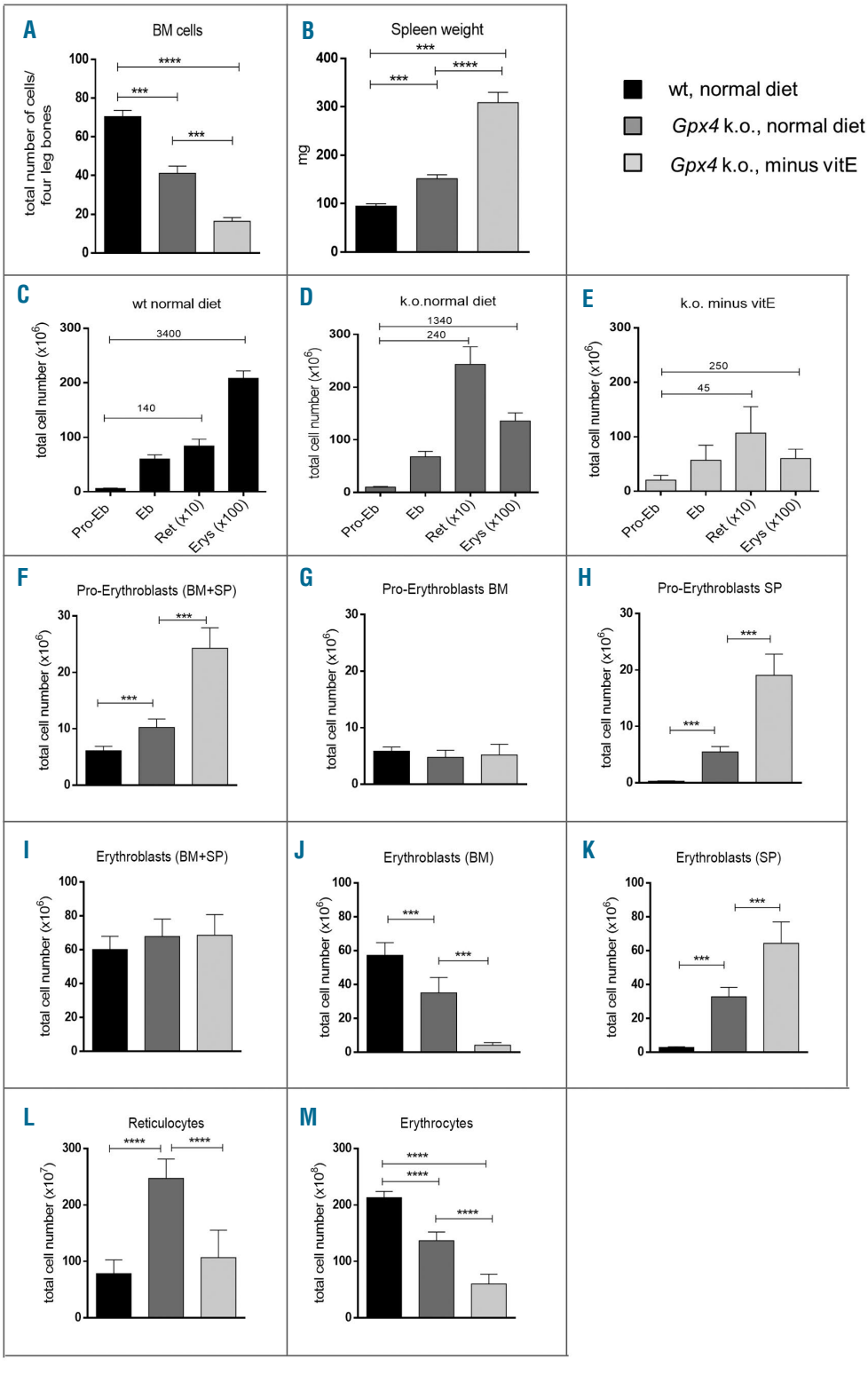

Figure 3. Ineffective erythropoiesis in mice with *Gpx4*-deficiency in the hematopoietic system and severe aggravation by dietary vitamin E deficiency. Total numbers of bone marrow (BM) cells collected from (A) two femura and two tibiae and (B) spleen weights of wild-type (wt) mice (n=8) and of mice with *Gpx4*-deficient hematopoiesis maintained either on a normal (n=8) or a vitamin E-depleted diet (n=9). The total numbers of proerythroblasts and erythroblasts in the BM and spleen, and of reticulocytes and erythrocytes in the blood were assessed as described in the *Online Supplementary Materials and Methods*. Comparative quantification of the total numbers of (F-H) proerythroblasts (n=8 for each condition), of (I-K) erythroblasts (n=8 for each condition), (L) reticulocytes and (M) erythrocytes (both n=42 wt; n=23 k.o., normal diet; n=16 k.o. minus vitE). Proerythroblasts in the BM and spleen were quantified separately in G and H, and erythroblasts in J and K, respectively. The significance was calculated by the Mann-Whitney test. The erythropenia caused by *Gpx4*-deficiency in hematopoietic cells is compensated to a large extent by increased extramedullary erythropoiesis and strongly elevated reticulocyte counts. Under combined *Gpx4*- and vitamin E-deficiency, the number or erythrocytes is strongly decreased, but the number of reticulocytes is not significantly higher than in wt mice. This points to a loss of erythroid progenitor cells at the proerythroblast and/or erythroblast stage in addition to the reticulocyte maturation defect under these conditions.

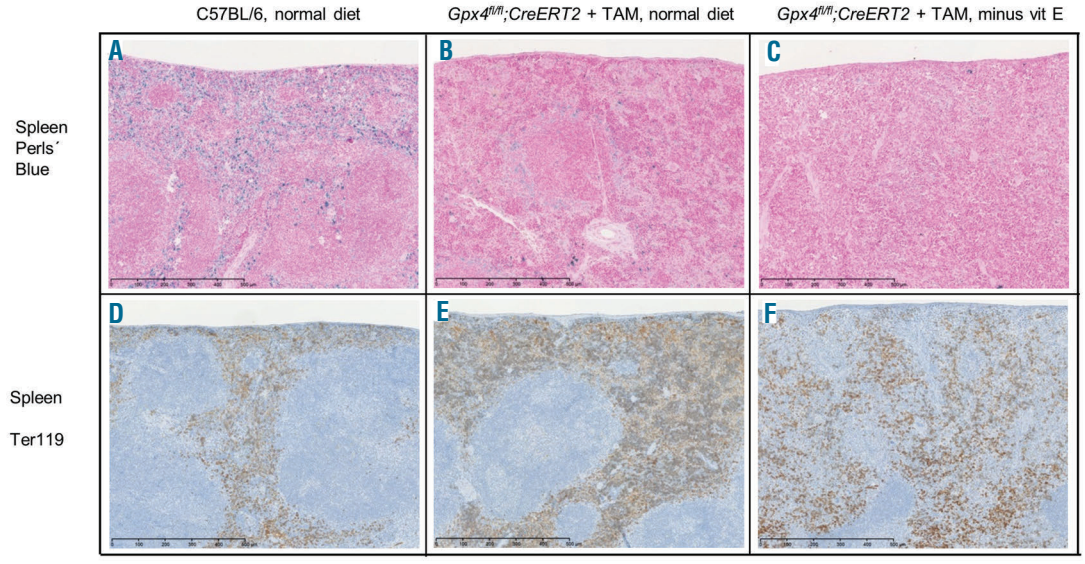

Figure 4. Extramedullary hematopoiesis in lethally irradiated wild-type (wt) mice reconstituted with *Gpx4*-deficient BM cells. (A-F) Histological sections of the spleen of (A,D) *Gpx4* wt mice kept on a normal diet and (B,E) of mice with *Gpx4*-deficient hematopoiesis maintained either on a (C) normal or on a (F) vitamin E-depleted diet, stained with Perls´ blue stain (A-C) or by immunohistochemistry with anti-Ter119 antibody (D-F). The splenic red pulp is increased in mice with *Gpx4*-deficient hematopoiesis (B,E) and the white pulp is almost completely dissipated when vitamin E is additionally depleted (C,F). (A) Iron deposits derived from erythrocyte turnover are clearly visible in the red pulp of wt mice kept on a normal diet, but (B,C) are only faintly visible in the periphery of follicles in the white pulp of mice with *Gpx4*-deficient hematopoiesis. Iron deposits are decreased rather than increased in the spleen of severely anemic mice arguing against increased hemolysis as the cause of anemia.

serum. Cells were individualized, filtered through a 40 μm cell strainer (BD Falcon) and incubated with 0.5 μg anti-CD16/32 antibody in 50 μL

PBS for 30min on ice. BM cells were stained with 0.3 μg CD44-PE-Cy7 (or CD71-PE-Cy7), 0.3 μg Ter119-PE or Ter119-APC-Cy7 antibodies. Spleen cells were stained with the following lineage mix: each 0.5 μg biotinylated CD3e (clone 145-2C11), CD11b (clone M1/70), CD19 (clone 1D3), B220 (clone RA3-6B2) and Gr-1 (clone RB6-8C5) antibodies. Cells were washed and stained with Streptavidin-Pacific Blue (Thermo Fisher) and 0.3 μg CD71-PE-Cy7 and 0.3 μg Ter119-PE (or Ter119-APC-Cy7) antibodies. Lineage-negative cells were gated and plotted as CD71-PE-Cy7-versus Ter119-APC-Cy7-positive cells. Cells were analysed on a FACSAriaI (Becton Dickinson).

Antibodies and primers

See *Online Supplementary Materials and Methods*.

Results

Gpx4-deficiency in hematopoietic cells impairs stress erythropoiesis

To study the role of GPX4 in hematopoiesis, chimeric mice were generated by reconstitution of lethally irradiated wt mice with *Gpx4*$^{fl/fl}$*;Cre ERT2* or *Gpx4*$^{wt/wt}$*;Cre-ERT2* BM cells. *Gpx4* deletion in the hematopoietic system was induced by feeding tamoxifen citrate for three weeks.40 At the last day of feeding tamoxifen red blood cell (RBC) parameters and white blood cell (WBC) counts were significantly decreased and platelet indices increased in the experimental as well as in the control group revealing apparent Cre-mediated side effects[33] (*Online Supplementary Figure S1*). Lymphocyte and monocyte counts were further decreased by simultaneous deletion of *Gpx4* (*Online supplementary Figure S1N-O*).

Thus, the inducible activation of Cre-ERT2 by tamoxifen caused an aplastic anemia with a moderate decrease in red blood cell RBC counts presumably through induction of a DNA damage response regardless of the presence or absence of *Gpx4* in hematopoietic cells. To separate a phenotype caused by *Gpx4*-deficiency from that of Cre side effects, tamoxifen-treated mice harboring *Gpx4*$^{fl/fl}$*;Cre ERT2* and *Gpx4*$^{wt/wt}$*;Cre ERT2* BM cells were allowed to recover and blood was drawn every three weeks (Figure 1). Mice with *Gpx4*-deficient BM cells remained anemic with strongly elevated reticulocyte counts, whereas mice harboring *Gpx4*$^{wt/wt}$*;Cre ERT2* BM normalized their RBC parameters within three to six weeks (Figure 1E-H, additional RBC parameters in the *Online Supplementary Figure S2B-F*). Since recovery from anemia involves stress erythropoiesis,[42] *Gpx4*-deficiency in the hematopoietic compartment thus impairs stress erythropoiesis. The erythropenic phenotype could be serially transplanted into lethally irradiated wt mice in two further rounds using *Gpx4*-deficient BM donor cells. *Gpx4*$^{fl/fl}$*;Cre-ERT2* mice served as controls except that tamoxifen feeding was omitted after the first transplantation (*Online Supplementary Figure S3*). WBC and platelet parameters remained normal except for single outliers that were not reproduced during serial transplantation. The stable transmission of the erythropenic phenotype by serial transplantation is consistent with a marked defect in stress erythropoiesis, as hematopoietic reconstitution after lethal irradiation is driven by stress hematopoiesis.[43]

Gpx4-deficient macrophages do not contribute to the development of erythropenia induced by deletion of *Gpx4* in the hematopoietic system

In our experimental strategy *Gpx4* was deleted in all hematopoietic cells including cells of the myeloid lineage. As macrophages are known to contribute to erythro-

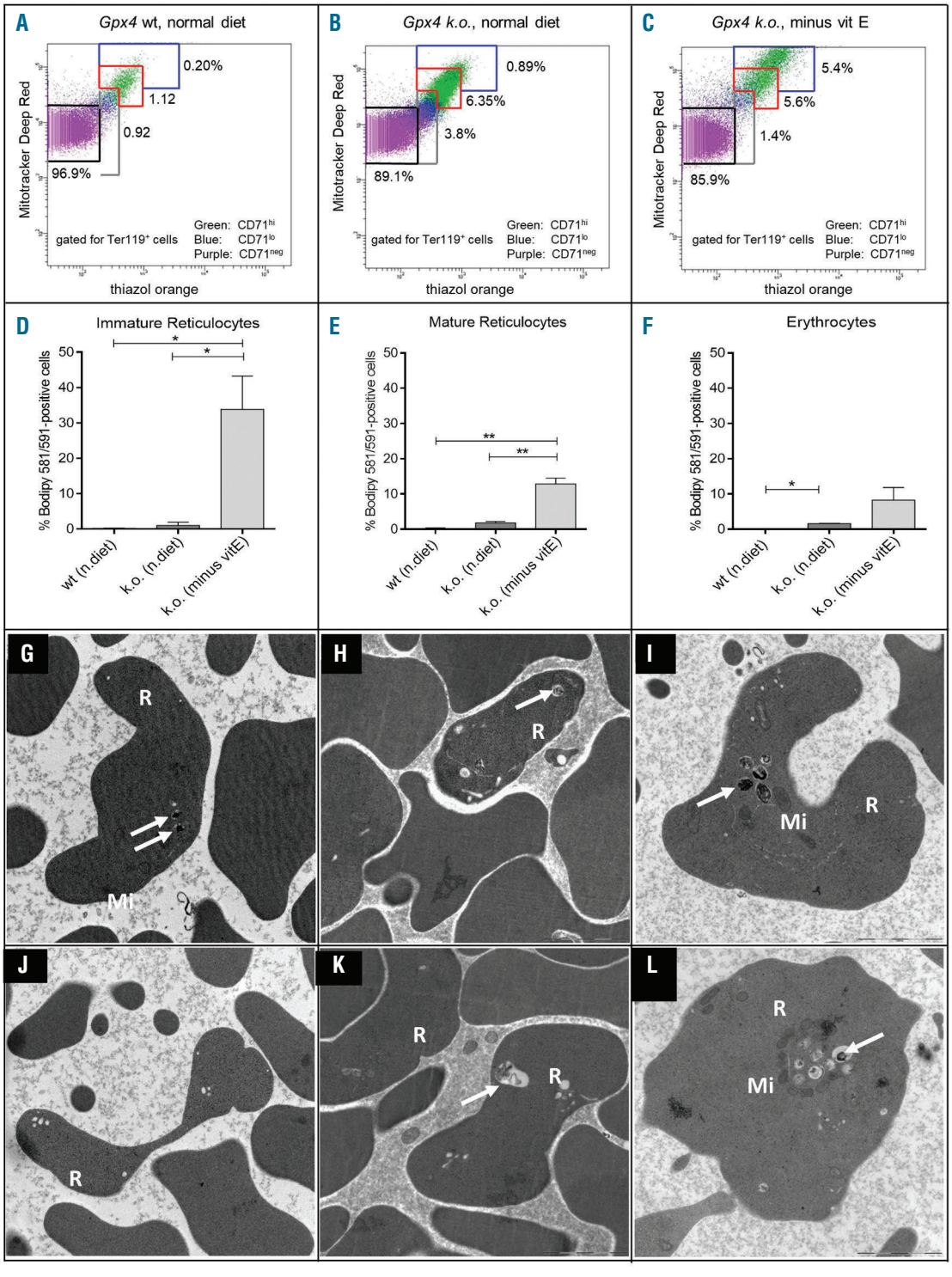

Figure 5. (A-C) *Gpx4*-deficiency in hematopoietic cells causes a reticulocyte maturation defect that is to a large extent compensated by vitamin E *in vivo*. Representative fluorescence-activated cell sorting (FACS) stainings of peripheral blood cells of (A) *Gpx4* wt mice kept on a normal diet and of mice with *Gpx4*-deficient hematopoiesis maintained either on (B) a normal or on (C) a vitamin E-depleted diet (C), stained with Mitotracker Deep Red (MTDR), thiazol orange (TO), and CD71-PE-Cy7 and Ter119-PE antibodies. The experiment is described and quantitatively evaluated in the *Online Supplementary Figure 5*. Ter119-positive cells were gated and plotted as shown in A-C. Immature reticulocytes ($CD71^{high}$) are shown in green, mature reticulocytes ($CD71^{low}$) in blue and erythrocytes ($CD71^{neg}/Ter119^{+}$) in purple. $CD71^{high}$ cells were subdivided into immature and highly immature reticulocytes based on MTDR and TO staining. (C) Under combined *Gpx4*- and vitamin E-deficiency the fraction of highly immature reticulocytes was strongly increased. D-F) Lipid peroxidation is increased in *Gpx4*-deficient reticulocytes and erythrocytes. Peripheral blood cells of the mice shown in A-C mice were stained with anti-CD71-PE-Cy7- and anti-Ter119-APC-Cy7-antibodies and with 2 μM C11-Bodipy(581/591). Ter119-positive cells were gated and the increase in % green fluorescence-positive cells was measured in $CD71^{high}$ cells (immature reticulocytes, D), in $CD71^{low}$ cells (mature reticulocytes, E) and in CD71neg cells (erythrocytes, F) upon excitation at 488 nm. Wild-type, normal diet, (n=2); k.o., normal diet (n=2); k.o., minus vitamin E (n=3). The significance was calculated using an unpaired T-Test. Note the high degree of lipid peroxidation in *Gpx4*-deficient immature reticulocytes upon feeding a vitamin E-depleted diet. G-L) Ultrastructural analysis of red blood cells from the mice shown in A-C. Remnants of mitochondria (Mi) are marked by white arrows. Under *Gpx4*-deficiency (H and K), and more so under combined *Gpx4*- and vitamin E-deficiency (I and L) large unphagocytosed vesicles containing mitochondria accumulated in reticulocytes (R). Blood pellets were processed for transmission electron microscopy as described in the *Online Supplementary Materials and Methods*.

poiesis by forming erythropoietic islands in the BM,[44] it was important to discriminate whether erythropenia is brought about by a cell-autonomous effect of GPX4 on the erythroid lineage or by a cell-non-autonomous effect of GPX4 on macrophages. Liao *et al.* recently described that loss of all selenoproteins in hematopoietic cells by deletion of selenocysteine-specific t-RNA impairs stress erythropoiesis and have attributed this to the loss of selenoprotein W in macrophages.[13] Despite the high position of GPX4 in the hierarchy of selenoproteins, a cell-autonomous contribution of GPX4 to stress erythropoiesis was not considered by the authors.

To address a potential contribution of GPX4 in macrophages on stress erythropoiesis, we used *Gpx4*$^{fl/fl}$*;LysM-Cre* mice that delete Gpx4 in macrophages and neutrophils[45] and analyzed BM erythroblastic island

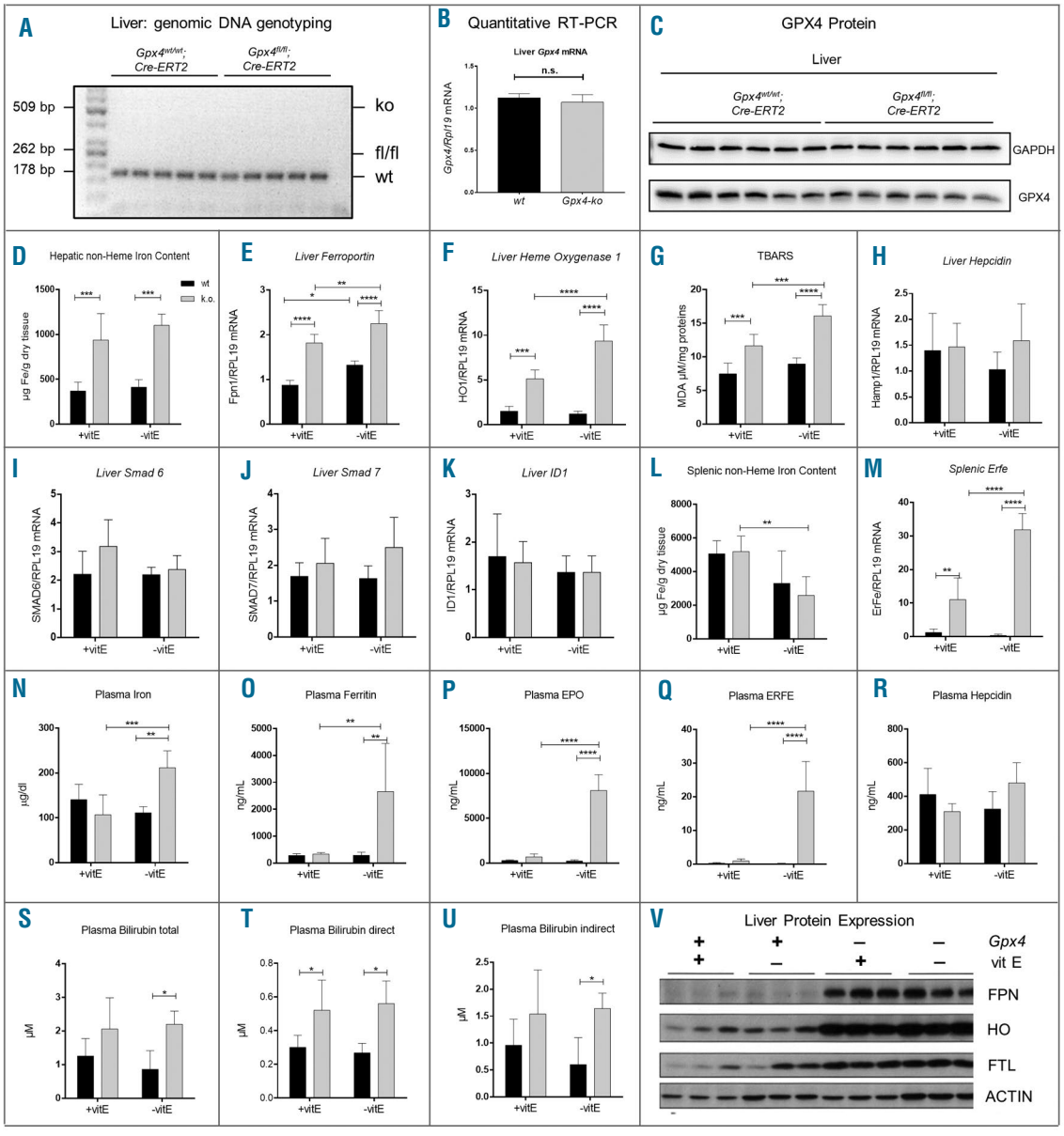

Figure 6. Iron overload in the liver and simultaneous iron demand for erythropoiesis in mice with a *Gpx4*-deficient hematopoietic system. In the liver of mice with *Gpx4*-proficient and *Gpx4*-deficient hematopoiesis only the wild-type (wt) allele of *Gpx4* is detected (A), and the same levels of *Gpx4* mRNA (B) and protein (C) are expressed. (D-K, V) Parameters of iron metabolism in the liver: Non-heme hepatic iron (D), *ferroportin* mRNA (E), *heme oxygenase-1* mRNA (F), thiobarbituric acid reactive substances (TBARS)(G), *hepcidin* mRNA (H), *Smad6* mRNA (I), *Smad7* mRNA (J), and *ID1* mRNA (K). The proteins ferroportin (FPN), heme oxygenase (HO-1), and ferritine light chain (FTL) are expressed at higher level in mice with a *Gpx4*-deficient hematopoietic system (V). Parameters of splenic and peripheral iron metabolism: non-heme splenic iron (L), *splenic Erfe* mRNA (M), plasma iron (N), plasma ferritine (O), plasma EPO (P), plasma ERFE (Q), plasma hepcidin (R), plasma bilirubin total (S), plasma bilirubin direct (T), plasma bilirubin indirect (U). Increased hepatic non-heme iron and TBARS as well as elevated ferroportin and heme oxygenase-1 expression point to hepatic iron overload, whereas highly increased EPO and ERFE levels in the plasma and elevated *Erfe* mRNA expression levels are strong indicators of severe iron demand in the erythropoietic system. Note that hepcidin expression is not down regulated despite the strong erythropoietic iron demand. For HO-1/actin ratio in the liver and duodenal ferroportin expression, see *Online Supplementary Figure S7A-B*.

macrophages (BMEIM) and spleen red pulp macrophages (RPM) by flow cytometry. The number of BMEIM was slightly reduced, while the number of RPM remained unaffected in the absence of *Gpx4* (*Online Supplementary Figure S4A-C*). Importantly, erythrocyte and reticulocyte counts were unaltered as well as hemoglobin and hematocrit values (Figure 1T-W) indicating that GPX4 in macrophages plays a minor if any role in the development of the erythropenia. This strongly argues for a cell-autonomous action of GPX4 in the erythroid system.

Vitamin E-deficiency severely aggravates erythropenia caused by *Gpx4*-deficiency

In cell culture and in certain tissues (endothelium, T cells, hepatocytes) the phenotype of *Gpx4*-deficiency can be partially or completely masked by vitamin E *in vivo*. Vitamin E thus acts as a full or partial backup system for GPX4 in some cell types *in vitro* and *in vivo*. To exclude unintended side effects of Cre and Cre inducers or activators with vitamin E deficiency, *Gpx4* was deleted prior to vitamin E deprivation. Feeding the vitamin E-depleted diet for three to four weeks to mice with *Gpx4*-deficiency severely aggravated erythropenia. Despite the strong decrease in RBC count as well as hemoglobin and hematocrit levels, reticulocyte counts were increased only to a small and non-significant extent (Figure 1I-M). This indicates that the anemia caused by *Gpx4*-deficiency could no longer be compensated by increased production of reticulocytes when the dietary vitamin E level was reduced. In wt control mice, dietary vitamin E deprivation had no impact on RBC parameters (*Online Supplementary Figure S5*). An increase of the vitamin E (i.e. α-tocopherol) concentration to the 5-fold level in the diet had no impact on erythrocyte counts, hemoglobin and hematocrit values but led to a significant decrease in reticulocyte counts (Figure 1N-R) corroborating the fact that reticulocyte counts respond more sensitively to external factors with impact on erythropoiesis than do other RBC parameters.

Ineffective erythropoiesis in mice with *Gpx4*-deficient hematopoietic cells

To get deeper insight into the dynamics of *Gpx4*-deficient erythropoiesis, we quantified the percentage of erythroid precursor cells in the BM and spleen by fluorescence-activated cell sorting (FACS) analysis (Figure 2) and calculated the total numbers of erythroid precursors in these organs (Figure 3A-B). Cell suspensions of BM and spleen were stained with antibodies for CD44 or CD71 and Ter119. Erythroid precursor cells were classified into proerythroblasts based on the expression level of CD44 or CD71 and Ter119. These analyses revealed a marked increase in extramedullary erythropoiesis in the spleen of mice with *Gpx4*-deficient hematopoiesis (Figure 2H,K), and a pronounced shift towards immature precursors cells in the BM and spleen when vitamin E was additionally depleted (Figure 2 F,L). The dynamic changes became more apparent when the total numbers of erythroid precursors were calculated under the different conditions. In mice with *Gpx4*-deficient hematopoietic cells the total number of proerythroblasts increased by a factor of 1.7-fold under a normal diet, and to about 4-fold when vitamin E was additionally depleted (Figure 3F). This increase was due to an increase of proerythroblasts in the spleen (Figure 3H). The number of erythroblasts in the spleen of these mice also increased significantly, and again to a much higher extent when vitamin E was depleted (Figure 3K). Under both conditions of *Gpx4*-deficiency (normal versus vitamin E-depleted diet) the number of erythroblasts in the spleen increased at the expense of erythroblasts in the BM leaving the total number of erythroblasts virtually unchanged (Figure 3 I-K).

The ratio of erythrocytes to proerythroblasts illustrates the efficacy of erythropoiesis. It decreased from about 3400 in wt mice to 1340 in mice with *Gpx4*-deficient hematopoiesis (Figure 3C-D). While the total number of reticulocytes increased by almost 3-fold and the ratio of reticulocytes to total proerythroblasts increased from 140 in wt mice to 240 in mice with *Gpx4*-deficient hematopoiesis, RBC counts decreased to 65% (Figure 3L-M) indicating that maturation of reticulocytes to erythrocytes is defective under *Gpx4*-deficiency.

Under combined *Gpx4*- and vitamin E-deficiency, the ratio of erythrocytes to proerythroblasts decreased to about 250 (Figure 3E). Erythrocyte counts dropped to less than 30% (compared to wt) (Figure 3M), while the number of reticulocyte counts increased only by about 1.3 fold (Figure 3L). The only moderate and non-significant increase in reticulocyte counts is in stark contrast to the severe anemia under these conditions. The ratio of reticulocytes to total proerythroblasts decreased from 240 in mice with *Gpx4*-deficient hematopoiesis to 45 in mice with combined *Gpx4*- and vitamin E-deficiency suggesting that erythroid progenitor cells were lost during differentiation from proerythroblasts to reticulocytes, in addition to the reticulocyte maturation defect observed under these conditions. Mice with *Gpx4*-deficiency in hematopoietic cells (on a normal diet) showed an increase in the size of the red pulp and the number of Ter119-positive cells (Figure 4B,E) corroborating data showing ineffective erythropoiesis. Yet, iron deposits in the red pulp stemming from physiological RBC turnover diminished (Figure 4B), arguing against hemolysis as the cause of anemia. Upon *Gpx4*-deficiency and vitamin E depletion, the white pulp dispersed to a large extent and was intermingled with Ter119-positive erythroid cells (Figure 4C,F).

Impaired reticulocyte maturation and increased lipid peroxidation in *Gpx4*-deficient erythroid cells and aggravation of the phenotype by vitamin E-depletion

Next, we attempted to define the maturation state of the reticulocytes in the anemic mice. To this end, peripheral blood cells were stained with CD71, Ter119, Mitotracker Deep Red, and thiazol orange. Representative examples of mice are depicted in Figure 5A-C (quantification of total numbers of mature, immature and highly immature reticulocytes in the *Online Supplementary Figure S6A-F*). Deletion of *Gpx4* increased not only the total number of reticulocytes, but also that of each fraction: 2.1-fold of mature, 3.5-fold of immature, and 4.2-fold of highly immature reticulocytes (*Online Supplementary Figure S6D-F*). The differentially higher increase in more immature reticulocytes led to a general shift towards more immature reticulocytes. Under combined *Gpx4* and vitamin E deficiency, the number of highly immature reticulocytes increased 7-fold, while the total number of mature reticulocytes decreased by 20%. The failure of reticulocytes to undergo maturation was associated with an increase in lipid peroxidation of reticulocytes and erythrocytes as revealed by Bodipy 581/591-C11-staining. Lipid peroxidation was particularly pronounced in immature

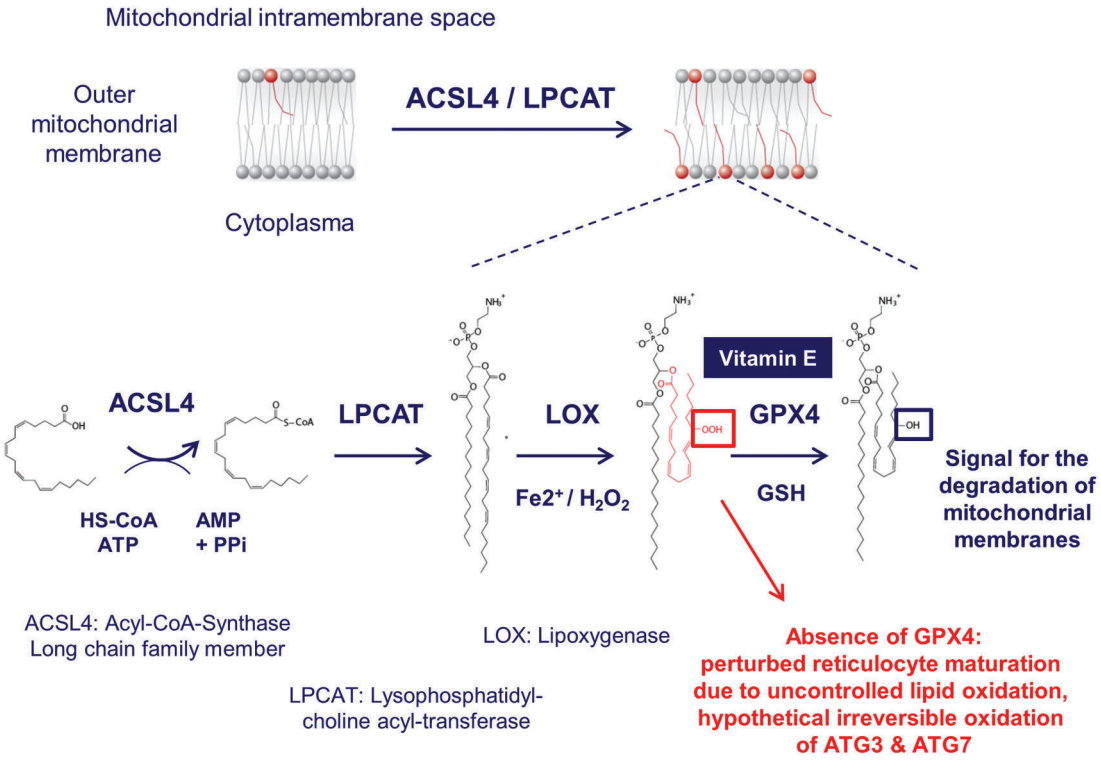

Figure 7. Proposed model for the role of 12/15-lipoxygenase, GPX4 and vitamin E during reticulocyte maturation.

reticulocytes when vitamin E was additionally depleted (Figure 5D).

To further study potential subcellular alterations in response to *Gpx4* loss, erythrocytes and reticulocytes of the peripheral blood were subjected to ultrastructural analysis by electron microscopy (Figure 5G-L). Reticulocytes in blood of wt mice and in blood of mice lacking *Gpx4* in hematopoietic cells were characterized by remnants of ribosomes (fine granular structure) and mitochondria. An accumulation of unphagocytosed mitochondria in large vacuoles was evident when vitamin E was depleted in mice with *Gpx4*-deficient hematopoiesis in vivo (white arrows in Figure 5G-L) indicating that GPX4 and vitamin E physiologically contribute to mitochondrial clearance during reticulocyte maturation.

Iron overload and oxidative stress in the liver of mice with *Gpx4*-deficient hematopoiesis

As shown above, *Gpx4*-deficiency in the hematopoietic system is characterized by ineffective erythropoiesis that is severely aggravated by additional vitamin E deprivation. Ineffective erythropoiesis is also a hallmark of β-thalassemia, and β-thalassemia is associated with iron overload that is known to sustain and to aggravate the anemia in mouse models of β-thalassemia.[51] To address whether similar mechanisms are operating in *Gpx4*-deficient erythropoiesis, iron-related parameters were analyzed in the liver, spleen, and plasma of mice with *Gpx4*-deficient hematopoiesis. Non-heme iron content in the liver was significantly increased upon deletion of *Gpx4* (Figure 6D). At the molecular level iron overload in the liver was confirmed by elevated levels of the iron storage protein Ferritin L (FTL) (Figure 6V). An excess of free iron triggers the formation of reactive oxygen species (ROS) and lipid peroxidation *via* the Fenton reaction.[3] Consistently, markers of oxidative stress such as increased lipid peroxidation products (as revealed by TBARS production) (Figure 6G) and elevated heme oxygenase-1 mRNA and protein levels (Figure 6F,V) were detected in the liver. Combined *Gpx4*- and vitamin E-deficiency further increased heme oxygenase-1 mRNA and protein levels as well as TBARS production in the liver.

Unaltered hepcidin levels despite higher erythropoietic iron demand

Systemic iron homeostasis is maintained by the hepcidin/ferroportin regulatory system. Hepcidin regulates the amount of iron exported into systemic circulation by modulating cell surface expression of the iron exporter ferroportin on iron exporting cells. Remarkably, despite increased liver non-heme iron levels in mice with *Gpx4*-deficient hematopoietic cells, hepatic hepcidin mRNA expression was not affected, regardless whether mice were kept on a normal or on a vitamin E-depleted diet (Figure 6H). Likewise, target genes of the iron-sensing BMP/SMAD signaling pathway in the liver (SMAD6, SMAD7, and ID1) were not significantly altered by *Gpx4*- and vitamin E-deficiency (Figure 6I-K). Hepatic ferroportin mRNA and protein levels were increased upon *Gpx4* ablation with or without vitamin E-deficiency (Figure 6E,V), a finding explained by the oxidative stress that occurs in the liver (Figure 6G). Ferroportin expression in the duodenum was unaltered (*Online Supplementary Figure S7*).

We next measured iron-related plasma parameters. Plasma iron levels were not increased upon *Gpx4* deletion, but were increased when *Gpx4* was deleted and vitamin E simultaneously depleted (Figure 6N). Despite this, hepcidin levels in the plasma were not altered (Figure 6R). Bilirubin levels appeared to increase with the ablation of *Gpx4* under both conditions (± vitamin E), but the standard deviation (SD) was high so that differences became barely significant (Figure 6S-U). Consistent with elevated liver iron levels, plasma ferritin was highly increased under combined *Gpx4*- and vitamin E-deficiency (Figure 6O).

In mouse models with β-thalassemia plasma erythropoietin (EPO) levels are increased. EPO stimulates the expression of the blood hormone erythroferrone (ERFE) in erythroid precursor cells, which then down regulates hepcidin levels. Similarly, EPO and ERFE levels appeared to be increased in mice with *Gpx4*-deficient hematopoiesis, but only EPO levels reached significance. Under combined *Gpx4*- and vitamin E-deficiency plasma EPO and ERFE levels increased to very high and highly significant levels (Figure 6P,Q), apparently mirroring the severity of erythropenia under combined *Gpx4*- and vitamin E-deficiency.[52] Taken together, the defect in reticulocyte maturation by loss of the *Gpx4* gene causes ineffective erythropoiesis with simultaneous iron overload in the liver and iron-deficiency in the hematopoietic system and this phenotype is to a large part masked by vitamin E in the normal diet.

Discussion

Based on biochemical studies Schewe *et al*. reported in 1975 that mitochondrial clearance during reticulocyte maturation is initiated by enzymatic lipid peroxidation.[20] These authors purified the enzyme 15-lipoxygenase from rabbit reticulocytes and showed that it oxidizes polyunsaturated fatty acids in mitochondrial membranes as an initiating event in the disposal of mitochondria. Even though the work has been confirmed and extended by others, these studies fell into oblivion due to the fact that erythropoiesis including erythrocyte and reticulocyte counts appeared to be normal in mice lacking *12/15-lipoxygenase*.[26]

However, for a few years the work of the Rapoport group has been regaining attention due to several important novel findings: (i) GPX4 was shown to be an antagonist of a novel 12/15-lipoxygenase-induced, non-apoptotic cell death pathway in murine fibroblasts,[5] (ii) this mode of cell death initiated by ablation of *Gpx4* has been shown to be driven by iron-induced lipid peroxidation and is now known as ferroptosis, (iii) distinct phospholipid hydroperoxide species have been identified that act as death signals and inducers of ferroptosis in various cell types,[54] (iv) vitamin E synergizes with GPX4 in antagonizing the action of lipid hydroxyperoxides *in vitro* and *in vivo*, (v) 12/15-lipoxygenase and its hydroxylated oxidation products are involved in the regulation of autophagy in murine macrophages,[19] (vi) autophagy is inhibited by oxidation of enzymes like ATG3 and ATG7 that catalyze ATG8/LC3 lipidation,[56] (vii) lipoxygenases are not necessarily the decisive source of lipid hydroperoxides, they may rather sensitize cells to iron-mediated non-enzymatic autoxidation.[29]

It was the aim of our study to identify a missing link between membrane lipid oxidation and reticulocyte maturation by genetic means. Based on the finding that GPX4 is unique in antagonizing lipid membrane hydroperoxides and stabilizing the hydroxylated oxidation products, we hypothesized that deletion of *Gpx4* in hematopoietic cells would perturb mitophagy and thus reticulocyte maturation.

The role of GPX4 in hematopoietic cells had already been studied by Canli *et al*. using *Gpx4*$^{fl/fl}$*;Mx1-Cre* mice.[55] These authors primarily focused, however, on the mode of cell death in hematopoietic precursor cells and not on a comprehensive view on *Gpx4*-deficient erythropoiesis at a quantitative level.

As an inducible k.o. model for *Gpx4* exclusively in hematopoietic cells, we have reconstituted lethally irradiated wt mice with *Gpx4*$^{fl/fl}$*;Cre-ERT2* or *Gpx4*$^{wt/wt}$*;CreERT2* BM cells. We now show that activation of Cre by feeding a tamoxifen-containing diet induces an aplastic anemia affecting RBC as well as WBC not only in mice reconstituted with *Gpx4*-deficient, but also in control mice with *Gpx4*$^{wt/wt}$*;CreERT2* BM cells thus confirming the acute toxic effect of Cre activation on the hematopoietic system described by Higashi *et al*.[33] There is, however, a clear difference between mice harboring or lacking the *Gpx4* gene in hematopoietic cells: mice reconstituted with *Gpx4*$^{wt/wt}$*;CreERT2* BM cells recovered within three to six weeks whereas mice reconstituted with cells lacking the *Gpx4* gene failed to fully recover and remained partially anemic. The erythropenic phenotype could be stably transmitted into lethally irradiated wt mice by two consecutive rounds of transplantation. It is noteworthy that Cre activation by tamoxifen and *Gpx4* ablation cause different types of anemia. Cre activation leads to an aplastic anemia with cessation of RBC (*Online Supplementary Figure S1F*) and WBC formation (*Online Supplementary Figure S1L-P*), whereas ablation of *Gpx4* in the hematopoietic system causes ineffective erythropoiesis with increased formation of reticulocytes (Figure 1H). As recovery from anemia as well as from hematopoietic reconstitution is driven by stress erythropoiesis,[43] stress erythropoiesis is perturbed by *Gpx4*-deficiency in hematopoietic cells. Depletion of vitamin E in the diet strongly aggravated the anemic phenotype. To exclude unintended side effects of vitamin E depletion with the well-known toxicity of Cre and Cre inducers and activators, the *Gpx4* gene was deleted prior to vitamin E depletion. The dynamics of erythropoiesis under the various conditions including the maturation state of reticulocytes was assessed by FACS staining of proerythroblasts and erythroblasts in the BM and spleen and of reticulocytes in the peripheral blood. A more comprehensive picture emerged (i) by calculating the total number of proerythroblasts and erythroblasts in the BM and spleen, (ii) by extrapolating to the total number of reticulocytes and erythrocytes per mouse, and (iii) by quantifying the fraction of mature, immature and highly immature reticulocytes.

In mice with *Gpx4*-deficient hematopoiesis the total number of proerythroblasts was increased, mainly due to an increase in extramedullary erythropoiesis. Total numbers of mature, immature as well as highly immature reticulocytes were also increased. The differentially higher increase in immature and highly immature reticulocytes led to a shift towards immature reticulocytes. This indicates that under hematopoietic *Gpx4*-deficiency the rate

of reticulocyte production exceeded the rate of reticulocyte maturation, a disequilibrium that resulted in ineffective erythropoiesis.

Under combined *Gpx4*- and vitamin E-deficiency the number of proerythroblasts in the spleen was further strongly increased. Within the reticulocyte fraction, there was a pronounced shift towards highly immature reticulocytes. Yet, production of reticulocytes did not keep up with the production of proerythroblasts as total reticulocyte counts were not significantly higher than in wt mice. This suggests that, in addition to the severe reticulocyte maturation defect, erythroid progenitor cells were lost during differentiation from the proerythroblast to the reticulocyte stage. Highest levels of lipid peroxidation, as assessed by C11-Bodipy(581/591)-staining, were observed in immature reticulocytes under combined *Gpx4*- and vitamin E-deficiency which correlates with the severity of the phenotype. Ultrastructural analysis revealed remnants of mitochondria and ribosomes in reticulocytes of wt mice and mice with *Gpx4*-deficient hematopoiesis, and a pronounced accumulation of large vacuoles containing unphagocytosed mitochondria, when dietary vitamin E was lowered. The data support the model that mitophagy is triggered by lipid oxidation that is kept in check by GPX4. Hence, loss of *Gpx4*, especially under vitamin E-restricted conditions, leads to uncontrolled lipid peroxidation and as a consequence, to severely perturbed mitophagy (Figure 7).

The anemia caused by hematopoietic *Gpx4*-deficiency shares a number of features with β-thalassemia, for which the term ineffective erythropoiesis has been coined: decreased RBC, elevated reticulocyte counts, overactive extramedullary erythropoiesis, elevated erythroid progenitors, absence of hemolysis, and systemic iron overload linked to severe iron demand for the erythroid system.[51] This prompted us to study iron metabolism in mice with *Gpx4*-deficient hematopoiesis. Hematopoietic *Gpx4*-deficiency caused liver iron overload and oxidative stress, elevated plasma ferritin and iron levels, parameters aggravated by vitamin E depletion. Despite signs of iron overload in the liver and plasma, there was continuous demand for iron in the erythroid system. As a consequence of the anemia, plasma EPO and ERFE levels were elevated. Likewise, *Erfe* splenic mRNA expression was increased, particularly when *Gpx4* deletion and vitamin E-deficiency were combined. Since ERFE mirrors the erythropoietic activity and there is virtually no background of extramedullary erythropoiesis in the spleen of wt mice under steady state conditions, spleen *Erfe* mRNA emerged as the most sensitive parameter of iron demand.

Despite the dramatic increase in ERFE production in mice with combined *Gpx4*- and vitamin E-deficiency, hepcidin expression in the liver plasma was unchanged. This is reminiscent to what has been observed in β-thalassemic $Th^{3/+}$ mice older than six weeks.[57] Suppression of hepcidin expression is only seen when the mice are young and still loading their livers with iron. Once enough iron has been loaded, hepcidin begins to rise, driven by hepatic iron stores, despite high erythroferrone, so that older mice have high liver iron but normal hepcidin which slows down further iron loading. Thus, hypoxia and elevated iron demand for erythropoiesis decrease hepcidin expression, whereas high plasma and hepatic iron levels counteract the response to ERFE in the liver. An alternative explanation is that a yet-unrecognized factor induced in response to oxidative stress or a direct oxidative modification of ERFE counteracts the action of ERFE on hepcidin expression.

The pronounced similarity between anemia caused by hematopoietic *Gpx4*-deficiency and β-thalassemia raises the question whether underlying pathogenic principles are shared between both conditions. A common denominator is by no doubt the involvement of oxygen radicals. In case of *Gpx4*-deficiency, they arise from increased lipid peroxidation, in case of β-thalassemia from inappropriate folding of globin chains and hemoglobin assembly. This liberates oxygen, heme and iron thus favoring the production of oxygen radicals through non-enzymatic autoxidation. There is ample evidence in the literature that increased lipid peroxidation in RBC and decreased lipid-soluble antioxidant levels in the plasma as well as in erythrocytes are consistent features of β-thalassemia.[59-63] Administration of vitamin E normalized the plasma oxidant/antioxidant balance and vitamin E content of erythrocytes, yet, the clinical benefit of vitamin E administration remained limited due to the persistence of iron-overload in affected patients.[62-65] Taken together, perturbed reticulocyte maturation through uncontrolled lipid peroxidation may be the underlying cause of ineffective erythropoiesis in both conditions.

It still remains unclear to which extent lipoxygenase-mediated enzymatic and/or non-enzymatic mechanisms contribute to lipid peroxidation in the anemia described here. Lipoxygenases lower the threshold for non-enzymatic autoxidation[29] and may be required for initiating lipid oxidation in mitochondrial membranes. In the absence of *12/15-lipoxygenase* other lipoxygenases may functionally compensate for the loss of the missing enzyme. A scenario of non-enzymatic lipid peroxidation is, however, also conceivable. Once lipid peroxidation is triggered, the process may be self-sustaining due to the high concentration of iron, heme compounds and oxygen rendering enzymatic lipid peroxidation dispensable.

Another critical determinant of physiological reticulocyte maturation is the cholesterol concentration in the reticulocyte membrane as well as in the plasma. Holm *et al*. have shown in an elegant study that mice lacking *apolipoprotein E* and *high-density lipid protein receptor I (SR-BI)* are unable to expel autophagocytosed organelles and accumulate autophagolysosomes in their reticulocytes.[66] These mice lack mature erythrocytes and their gas transport relies exclusively on reticulocytes. Remarkably, the block in terminal reticulocyte maturation is cell-non-autonomous in these mice and reversible: transfusion of these reticulocytes into wt mice or hematopoietic reconstitution of lethally irradiated wt mice with *apoliporotein E*- and *SR-BI*-deficient BM cells completely normalized reticulocyte maturation. The phenotype could also be reversed through sequestration of free cholesterol: administration of cyclodextrin led to immediate expulsion of stored autophagolysosomes in vivo and in vitro and normalized the phenotype. Contrary to the apolipoprotein E- and SR-BI-model of Holm *et al*., we are dealing in our model with a cell-autonomous effect of *Gpx4*-deficiency to which cell-nonautonomous factors like the plasma concentration of vitamin E also contribute to a significant extent. In both conditions, the precise molecular underpinnings underlying the defects in reticulocyte maturation await to be elucidated.

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