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Original Contribution

The novel role of peroxiredoxin-2 in red cell membrane protein homeostasis and senescence

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ABSTRACT

Peroxiredoxin-2 (Prx2), a typical two-cysteine peroxiredoxin, is the third most abundant protein in red cells. Although progress has been made in the functional characterization of Prx2, its role in red cell membrane protein homeostasis is still under investigation. Here, we studied Prx2$^{-/-}$ mouse red cells. The absence of Prx2 promotes (i) activation of the oxidative-induced Syk pathway; (ii) increased band 3 Tyr phosphorylation, with clustered band 3; and (iii) increased heat shock protein (HSP27 and HSP70) membrane translocation. This was associated with enhanced in vitro erythrophagocytosis of Prx2$^{-/-}$ red cells and reduced Prx2$^{-/-}$ red cell survival, indicating the possible role of Prx2 membrane recruitment in red cell aging and in the clearance of oxidized hemoglobin and damaged proteins through microparticles. Indeed, we observed an increased release of microparticles from Prx2$^{-/-}$ mouse red cells. The mass spectrometric analysis of erythroid microparticles found hemoglobin chains, membrane proteins, and HSPs. To test these findings, we treated Prx2$^{-/-}$ mice with antioxidants in vivo. We observed that *N*-acetylcysteine reduced (i) Syk activation, (ii) band 3 clusterization, (iii) HSP27 membrane association, and (iv) erythroid microparticle release, resulting in increased Prx2$^{-/-}$ mouse red cell survival. Thus, we propose that Prx2 may play a cytoprotective role in red cell membrane protein homeostasis and senescence.

Peroxiredoxin-2 (Prx2)[1] is a typical two-cysteine peroxiredoxin and is the third most abundant protein in red cells [1]. The importance of Prx2 in red cells is supported by the hemolytic phenotype of Prx2-knockout mice (Prx2$^{-/-}$), which show (i) increased intracellular pro-oxidant environment, (ii) Heinz bodies associated with the membrane, (iii) decreased cell deformation, and (iv) increased oxidation of membrane proteins containing cysteine residues [2–5]. Although these data indicate the presence of a high pro-oxidant environment in Prx2$^{-/-}$ mouse erythrocytes, the role of Prx2 in red cell membrane homeostasis is only partially known.

Studies in normal and pathological erythrocytes have recently shown that Prx2 translocates to the membrane in response to oxidative changes, indicating that Prx2 might be a key protein in the red cell response to redox state [1,6–8]. Recently, we reported that one of the membrane docking sites for Prx2 is band 3 [7], an integral membrane protein playing a crucial role in membrane mechanical stability as part of the multiprotein complexes bridging the membrane to the skeletal network [9–11]. It is of interest to note that band 3 is also the major interactive partner for denatured hemoglobin (Hb) and hemichrome, bringing potent pro-oxidants in close proximity to the membrane [12]. This requires the presence of very efficient local antioxidant systems, such as Prx2.

In red cells membrane oxidative stress is also associated with increased tyrosine (Tyr) phosphorylation of red cell membrane proteins through the activation of Syk, a Src family-related Tyr kinase [10,13–15]. The major functional target of Syk on the red

Abbreviations: SCD, sickle cell disease; WT, wild type; CFSE, carboxyfluorescein succinimidyl ester; Prx2, peroxiredoxin 2; HSP, heat shock protein; MP, microparticle; NAC, *N*-acetylcysteine; HMC, hemichrome; PLB, phosphate lysis buffer; NEM, *N*-ethylmaleimide; FACS, fluorescence-activated cell sorter; Syk, Src-related tyrosine kinase; B3, band 3

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cell membrane is band 3. The highly Tyr-phosphorylated band 3 generates band 3 clusters, promoting red cell removal through the macrophage system and favoring red cell vesiculation with the release of microparticles (MPs) [16]. The proteomic analysis of red cell MPs from β-thalassemia intermedia patients shows that Prx2 is present together with HSP70 and HSP90 in MPs from red cells, suggesting the possible involvement of Prx2 in MP generation. MPs from erythrocytes are important players in severe clinical manifestations of hereditary red cell disorders such as β-thalassemia [16,17].

Here, we evaluated the impact of Prx2 on the red cell membrane stress response machinery in mice genetically lacking Prx2. In $Prx2^{-/-}$ mouse red cells we observed activation of the redox-related signaling pathway through Syk kinase and clusterization of the red cell membrane integral protein band 3, indicating premature red cell senescence. In agreement with these findings, $Prx2^{-/-}$ red cell survival was reduced and the release of MPs to remove oxidized Hb and damaged proteins was increased compared to wild-type mice. Finally, we showed that antioxidant treatment with *N*-acetylcysteine (NAC) reverses the $Prx2^{-/-}$ abnormal red cell features. Our results suggest a novel role for Prx2 as a part of the stress response machinery involved in red cell membrane protein homeostasis and in red cell aging.

Materials and methods

Drugs and chemicals

Details are reported in the supplementary materials and methods.

Mouse models

The study was carried out in accordance with the Scientific Committee for Animal Experimentation (CIRSAL, University of Verona). C57BL6/2J mice, wild-type controls (WT), and $Prx2^{-/-}$ mice (the colony progenitors were kindly gifted by Professor F. Kuypers, Children's Hospital Oakland Research Institute, Oakland, CA, USA) were used [2]. Age- and sex-matched mice 4 months of age (weighing 20–25 g) were studied. Where indicated, both mouse strains were treated with either quercetin (50 mg/kg, in NaCl 0.9%, NaOH 36 mM, pH 9.4; ip) [18,19] or NAC (100 mg/kg, in NaCl 0.9%, NaOH 36 mM, pH 9.4; ip) or vehicle only for 3 weeks [20].

Red cell membrane ghost preparation

Blood was centrifuged at 3000*g* for 5 min at 4 °C to remove plasma. It was then passed through cotton to remove white cells and washed three times with choline wash solution (175 mM choline, 1 mM $MgCl_2$, 10 mM Tris–Mops, pH 7.4 at 4 °C, 320–340 mOsm) [21]. Packed red cells were lysed in phosphate lysis buffer (PLB; 5 mM Na_2HPO_4, pH 8.0, in the presence of a protease inhibitor cocktail tablet, 3 mM benzamidine, 1 mM Na_3VO_4 final concentration) and washed in PLB five times to obtain almost white ghosts. Whenever Prx2 was evaluated in SDS–PAGE analysis, 100 mM NEM was added to the PLB to avoid possible artifacts due to Prx2 oxidation after cell lysis [7].

In vitro treatment of red cells and crossover experiments

Red cells underwent treatment with diamide (2 mM) or with H_2O_2 based on previously reported data from mouse erythrocytes [6]. Where indicated, red cells were pretreated with the Syk inhibitors II and IV (10 μM) for 1 h before incubation with the oxidant [16]. For crossover experiments WT and $Prx2^{-/-}$ red cell ghosts were incubated with the indicated red cell cytoplasmic fractions as we previously reported [6].

Immunoblot analysis of red cell membranes

Monodimensional electrophoresis of protein from the ghosts was carried out as previously described [6,7]. Details are reported in the supplementary materials and methods. Immunoprecipitation of anti-phospho-Tyr proteins from red cell ghosts was also carried out as previously reported [22]. Densitometric analysis of band intensities was done using Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

Measurement of band 3 clusterization, phagocytic index, and membrane-associated hemichromes

Measurement of band 3 clusters and phagocytic assay were carried out as previously reported [16]. For the measurement of band 3 clusters, anti-band-3 immunoblots were performed and the oxidized high-molecular-weight band 3 fraction was quantified by densitometric analysis and expressed as a percentage of total band 3 [16]. Hemichromes (HMCs) bound to the membrane were quantified by measuring (i) heme absorbance in red cell ghosts at 560, 577, and 630 nm as previously reported [16] and (ii) hemichrome autofluorescence via flow cytometric analysis using a FACS Canto flow cytometer (BD Biosciences, Mountain View, CA, USA).

Isolation and protein analysis of erythroid microparticles

MPs were isolated in vitro from red cells incubated in phosphate-buffered saline (Hct 30%) as previously described [16]. MP proteins were separated by electrophoresis. The gels underwent image analysis after colloidal Coomassie staining with Quantity One analysis software (Bio-Rad). Protein bands were then excised and analyzed by mass spectrometry [16]. Details are reported in the supplementary materials and methods.

Measurement of red cell survival

Red cell survival assay was carried out using CFSE (10 μM; Molecular Probes, Invitrogen) as previously described [8]. Details are reported in the supplementary materials and methods.

Statistical analysis

The two-way ANOVA algorithm for repeated measures was used for data analysis. Differences of $P < 0.05$ were considered significant.

Results

Mouse red cells genetically lacking Prx2 show increased band 3 Tyr phosphorylation and Syk activation

Previous studies have shown that the absence of Prx2 is associated with red cell membrane oxidative damage [2,4]. Owing to the high reactivity of critical cysteine residues, Prx2 is a very effective noncatalytic scavenger of low concentrations of hydrogen peroxide. In red cells, the high efficiency in metabolizing hydrogen peroxide is supported by the limited oxidative damage of red cells due to hydrogen peroxide in the absence of catalase inhibitors [23]. Here, we evaluated the presence of pro-oxidants associated with $Prx2^{-/-}$ mouse red cell membrane such as HMCs, which might amplify the membrane oxidative stress similar to the levels

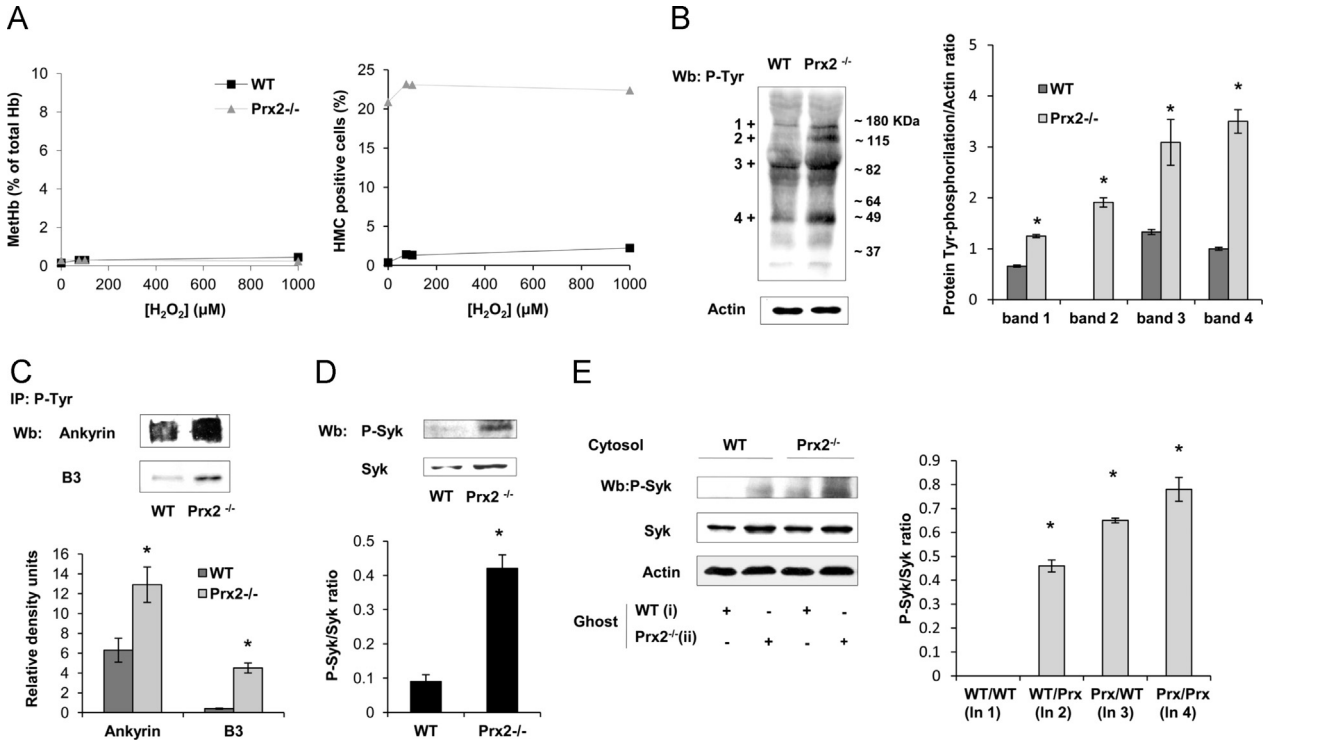

Fig. 1. The absence of Prx2 affects the tyrosine phosphorylation pattern of red cell membrane proteins and induces Syk activation. (A) Levels of MetHb (left) and hemichromes (HMCs, right) bound to the membrane in red cells from wild-type (WT) and Prx2$^{-/-}$ mice exposed to H_2O_2 0, 75, 100, or 1000 μM (2 h at 37 °C). Data are presented as median values ($n = 3$). (B) Left: Western blot (Wb) analysis with specific anti-phospho-tyrosine (P-Tyr) antibodies of red cell membrane proteins from WT and Prx2$^{-/-}$ mice separated by monodimensional electrophoresis. The numbers from 1 to 4 indicate the protein identified by mass spectrometry. Actin was used as a loading control protein. One representative gel of six with similar results is presented; see also Supplementary Fig. 1SA for protein loading control. Right: densitometric analysis of anti-phospho-Tyr immunoblots; data are presented as the protein Tyr phosphorylation/actin ratio and are presented as means ± SD ($n = 6$; $^*P < 0.05$ compared to WT mice). (C) Top: red cell ghosts from WT and Prx2$^{-/-}$ mice underwent immunoprecipitation with specific anti-phospho-Tyr antibodies (IP: P-Tyr) and then were used for Wb analysis with specific anti-ankyrin or anti-band 3 (B3) antibodies. One representative gel of three with similar results is presented (see also Supplementary Fig. 1SB). Bottom: relative quantification of immunoreactivity for ankyrin and band 3 proteins from anti-phospho-Tyr immunoprecipitations from WT or Prx2$^{-/-}$ mouse red cells. Data are presented as means ± SD ($n = 3$; $^*P < 0.05$ compared to WT). (D) Top: Wb analysis with specific anti-phospho-Syk (P-Syk) or Syk antibodies of red cell membrane proteins from WT and Prx2$^{-/-}$ mice. One representative gel of six with similar results is presented. Bottom: densitometric analysis of anti-P-Syk or Syk immunoblots; data are presented as means ± SD ($n = 6$; $^*P < 0.05$ compared to WT mice; see also Supplementary Fig. 1SD for protein loading control). (E) Left: Wb analysis with specific anti-P-Syk or Syk antibodies of (i) isolated red cell membranes from wild-type red cells incubated with the cytoplasm fraction of either control or Prx2$^{-/-}$ mouse red cells and (ii) isolated red cell membranes from Prx2$^{-/-}$ mouse red cells incubated with the cytoplasm fraction of either control or Prx2$^{-/-}$ mouse red cells. Actin was used as a loading control protein. Also shown is a representative experiment of two performed with similar results. Right: densitometric analysis of the immunoblots was carried out for relative quantification of immunoreactivity for P-Syk and Syk. Data are presented as the phospho-Syk/Syk ratio and shown as means ± SD ($n = 3$; $^*P < 0.05$ compared to WT).

observed in β-thalassemic erythrocytes [17]. In Prx2$^{-/-}$ mouse red cells, we found a large amount of HMCs compared to wild-type mice (wild-type HMCs, 2.10 ± 0.15 pmol/10^9 cells, vs Prx2$^{-/-}$ HMCs, 55.72 ± 2.05 pmol/10^9 cells; $P < 0.05$).

To establish whether the low-concentration hydrogen peroxide that can accumulate in Prx2$^{-/-}$ mouse red cells may account for the increase in HMCs, we treated both wild-type and Prx2$^{-/-}$ mouse erythrocytes with hydrogen peroxide (from 0.050 to 1 mM; Fig. 1A). Even prolonged exposure to hydrogen peroxide (3 h at 37 °C) did not induce either MetHb or HMC formation, indicating that catalase, glutathione (GSH) peroxidase, and ultimately MetHb reductase are able to prevent hemoglobin denaturation in the presence of the low concentration of hydrogen peroxide that may accumulate in the absence of Prx2 [1]. These findings are in agreement with the observations of the preserved ability of Prx2$^{-/-}$ mouse red cells to counteract exogenous hydrogen peroxide-mediated stress similar to wild-type erythrocytes [3].

Because the integral membrane protein band 3 is the docking site for HMCs and the oxidized band 3 is targeted by Syk, a Tyr kinase involved in the redox signaling pathway [6,7,16], we analyzed the protein Tyr-phosphorylation profile of red cell membrane from Prx2$^{-/-}$ and wild-type mice. As shown in Fig. 1B, we found an increased Tyr-phosphorylation state of membrane proteins from Prx2$^{-/-}$ mouse red cells compared to wild type (see Fig. 1B, right, for band densitometric analysis). These data are also supported by the absence of reactivity when the membranes were treated with λ-phosphatase, which was used to remove phosphate groups from blotted proteins (Supplementary Fig. 1SB). The differentially Tyr-phosphorylated bands were identified by mass spectrometry (Supplementary Table 1S). Because the same band might contain more than one protein, we validated these results by anti-phospho-Tyr immunoprecipitation from red cell membrane proteins, followed by immunoblotting analysis with specific antibodies. This confirmed the increased Tyr phosphorylation of band 3 in Prx2$^{-/-}$ mouse erythrocytes (Fig. 1C and Supplementary Fig. 1SB).

We then evaluated the redox-signaling pathway through Syk targeting of band 3 in Prx2$^{-/-}$ mouse red cells [10,14,24]. As shown in Fig. 1D, we found increased active Syk (P-Syk) associated with Prx2$^{-/-}$ mouse red cell membrane compared to wild-type mice (see also Supplementary Fig. 1SC). To better evaluate Syk function in Prx2$^{-/-}$ mouse red cells, we carried out crossover experiments using native Syk from wild-type or Prx2$^{-/-}$ cytosol incubated with red cell membranes from either wild-type or Prx2$^{-/-}$ mice. As shown in Fig. 1E, Syk from wild-type cytoplasm did not translocate to wild-type membrane (lane 1), but it bound to the oxidized membrane from Prx2$^{-/-}$ mice (lane 2). Syk from the cytoplasm of Prx2$^{-/-}$ mouse red cells translocated to the

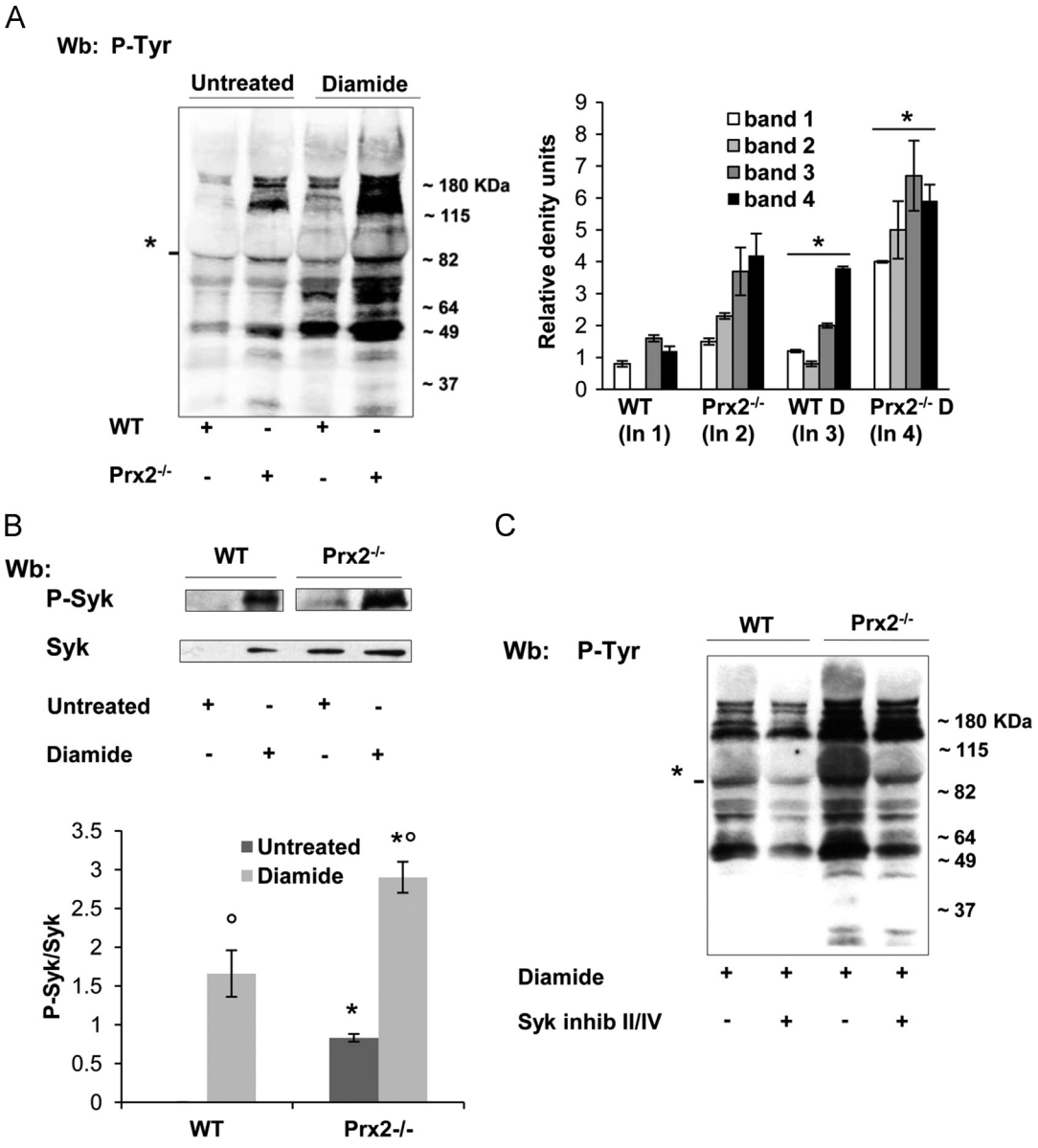

Fig. 2. (A) Left: Western blot (Wb) analysis with specific anti-phospho-tyrosine (P-Tyr) antibodies of red cell membrane from wild-type (WT) and Prx2$^{-/-}$ erythrocytes in the presence or absence of diamide (1 mM). The asterisk indicates band 3 as identified by mass spectrometric analysis (B3, matching peptide, 10; % of coverage, 33). One representative gel of six with similar results is presented (see also Supplementary Fig. 2SA for protein loading control). Right: densitometric analysis of anti-phospho-Tyr immunoblots. In the densitometric analysis we considered only changes in bands 1 to 4 identified in Fig. 1A. (B) Top: Wb analysis with anti-phospho-Syk (P-Syk) or Syk antibodies of red cell membrane from WT and Prx2$^{-/-}$ mice in the presence or in the absence of diamide (1 mM). Also shown is a representative immunoblot of three. Vertical lines have been inserted to indicate a repositioned gel lane. Bottom: densitometric analysis of the immunoblots was carried out for relative quantification of immunoreactivity for P-Syk and Syk. Data are presented as the phospho-Syk/Syk ratio and shown as means ± SD ($n = 3$; *$P < 0.05$ compared to WT). (C) Wb analysis with specific anti-P-Tyr antibodies of red cell membranes from WT and Prx2$^{-/-}$ mice treated with diamide in the presence or absence of Syk inhibitors II/IV (10 μM). The asterisk indicates band 3 as identified by mass spectrometric analysis (B3, matching peptide, 9; % of coverage, 28). One representative gel of six with similar results is presented; see also Supplementary Fig. 2SC for protein loading control.

Prx2$^{-/-}$ oxidized membrane (lane 4) and to a minor extent also to wild-type membranes (lane 3). These data suggest that Syk from Prx2$^{-/-}$ mouse red cells is already activated in the cytosol of Prx2$^{-/-}$ mouse red cells as confirmed by immunoblot analysis of the cytosolic fraction from red cells of both mouse strains (data not shown).

Because diamide has been reported to cause the conversion of reduced thiols to disulfides, to oxidize band 3, and to activate the Syk pathway [6,7], we treated Prx2$^{-/-}$ mouse red cells with diamide. As shown in Fig. 2A, the Tyr-phosphorylation state of several membrane proteins was markedly increased in diamide-treated Prx2$^{-/-}$ mouse red cells compared to diamide-treated wild-type erythrocytes, which, however, displayed a higher Tyr-phosphorylation state compared to untreated wild-type mice (see also Supplementary Fig. 2SA). The Tyr-phosphorylation profile of diamide-treated Prx2$^{-/-}$ and wild-type mouse red cells was compatible with increased band 3 Tyr phosphorylation as confirmed by mass spectrometric analysis (see asterisk on Fig. 2A). In the same samples, we also analyzed Syk activation. We found a higher activation of Syk in diamide-treated Prx2$^{-/-}$ mouse red cells compared to wild-type diamide-treated erythrocytes (Fig. 2B, Supplementary Fig. 2SB). The diamide-induced Tyr phosphorylation of membrane proteins, and in particular of band 3 (see asterisk on Fig. 2C), was reduced in the presence of the specific Syk inhibitors (Fig. 2C, Supplementary Fig. 2SC), which also caused a reduction in Syk membrane association (Supplementary Fig. 3SA). It is of interest

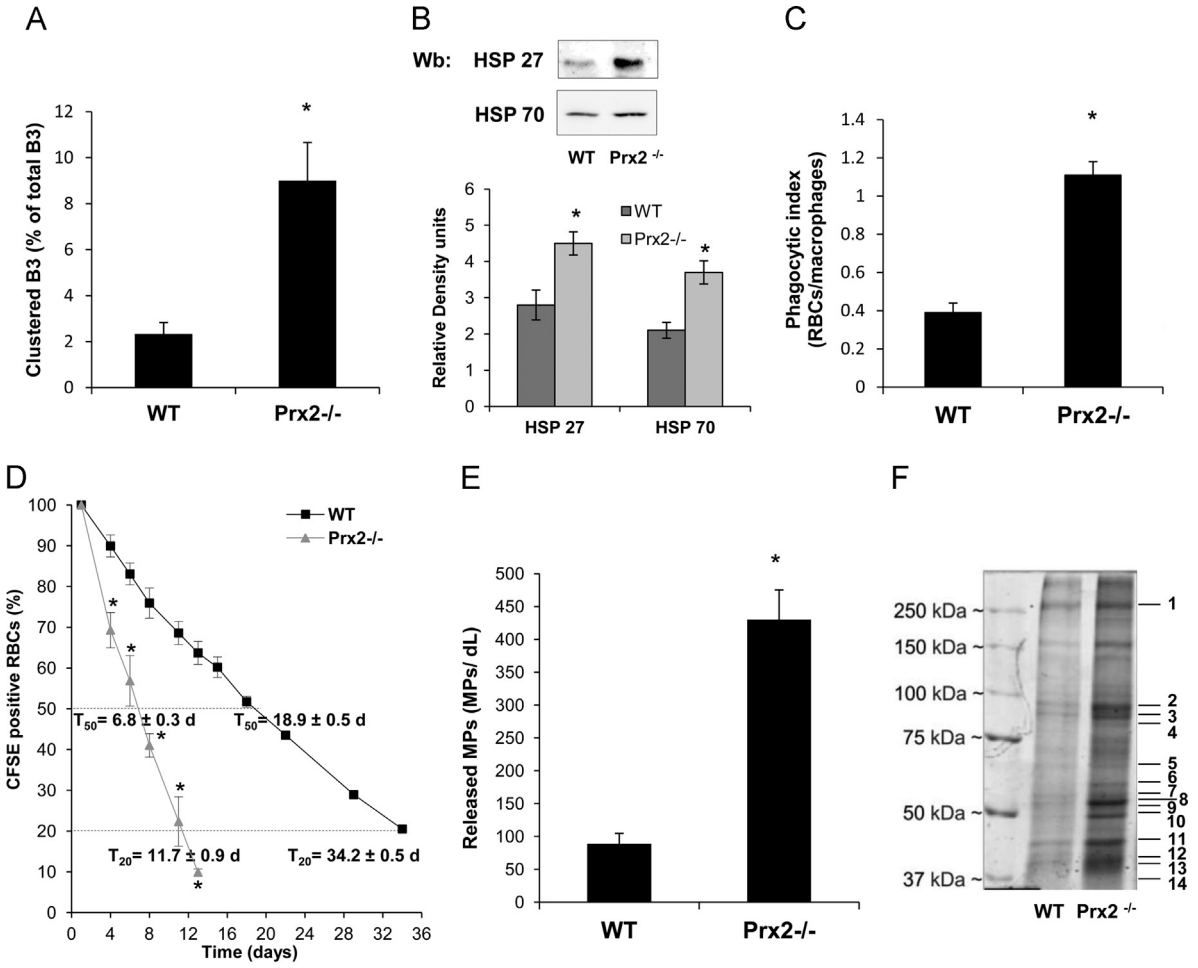

Fig. 3. Prx2$^{-/-}$ mouse red cells show increased band 3 clusters, membrane-associated HSPs, in vitro phagocytic index, decreased in vivo red cell survival, and increased release of erythroid microparticles. (A) Band 3 clusters in wild-type (WT) and Prx2$^{-/-}$ mouse red cells. Data are presented as means ± SD ($n = 5$; $^*P < 0.05$ compared to WT). (B) Top: Western blot (Wb) analysis with specific anti-HSP27 or HSP70 antibody of red cell membrane proteins from WT and Prx2$^{-/-}$ mice separated by monodimensional electrophoresis. One representative gel of five with similar results is presented; see also Supplementary Fig. 3SB for protein loading control. Bottom: densitometric analysis of the immunoblots for HSP27 and HSP70 was carried out. Data are expressed as means ± SD ($n = 5$; $^*P < 0.05$ compared to WT). (C) Red cell phagocytic index for erythrocyte from WT and Prx2$^{-/-}$ mice. Data are presented as means ± SD ($n = 6$; $^*P < 0.05$ compared to WT). (D) Red cell survival of CFSE-labeled red cells from WT and Prx2$^{-/-}$ mice. Data are presented as means ± SD ($n = 4$) from each group; $^*P < 0.05$ compared to wild-type mice. (E) Quantification of induced MPs from red cells of WT and Prx2$^{-/-}$ mice. Data are presented as means ± SD ($n = 6$) from each group; $^*P < 0.05$ compared to WT mice. (F) Proteins from MPs were separated by monodimensional electrophoresis. Proteins identified by mass spectrometry are indicated by numbers from 1 to 14 (see also Supplementary Table 2S for protein identification). One gel is shown as a representative of six with similar results.

to note that a residual Tyr phosphorylation of a few bands was still detectable in diamide-treated red cells in both mouse strains accompanying the presence of Syk inhibition. This is in agreement with our previous observation showing the activation of Tyr kinases of the Src family that target membrane proteins differently from band 3 in response to oxidative stress [22,25].

Prx2$^{-/-}$ mouse red cells show band 3 clusterization, increased erythrophagocytosis, and reduced survival

Previous studies have shown that redox Syk activation correlates with oxidized and abnormally clustered band 3 [13,26]. In Prx2$^{-/-}$ mouse erythrocytes we observed an increase in the clusters of band 3 compared to wild-type mice (Fig. 3A). Because we have recently reported increased membrane association of Prx2 and HSP27 and HSP70 in response to oxidative stress mediated by pathological free iron or hypoxia in a mouse model of SCD [27], we evaluated the amount of HSPs 27 and 70 associated with the membrane in response to membrane oxidative stress in Prx2$^{-/-}$ mouse red cells. We observed higher membrane translocation of both HSP27 and HSP70 in Prx2$^{-/-}$ mouse red cells compared to wild-type mice (Fig. 3B and Supplementary Fig. 3SB). To address the question of whether the increased membrane association of HSPs and the abnormally clustered band 3 might be part of the processes involved in the early removal of damaged red cells through the macrophage systems [19,28], we measured the index of red cell phagocytosis in vitro. This was significantly increased in Prx2$^{-/-}$ mouse red cells compared to wild-type mice (Fig. 3C). In agreement with these data, we found a significant reduction in Prx2$^{-/-}$ red cell survival compared to those of wild-type mice (Fig. 3D). These results suggest that Prx2 plays a key role in the membrane antioxidant system, collaborating with known chaperones in membrane protein homeostasis in response to oxidation-induced damage.

The absence of Prx2 is associated with the increased release of erythroid microparticles

The functional consequence of band 3 clusters is the reduction of red cell membrane mechanical stability, which might result in the generation of MPs to clear damaged hemoglobin and proteins [16,17]. In Prx2$^{-/-}$ mouse red cells, we observed the increased

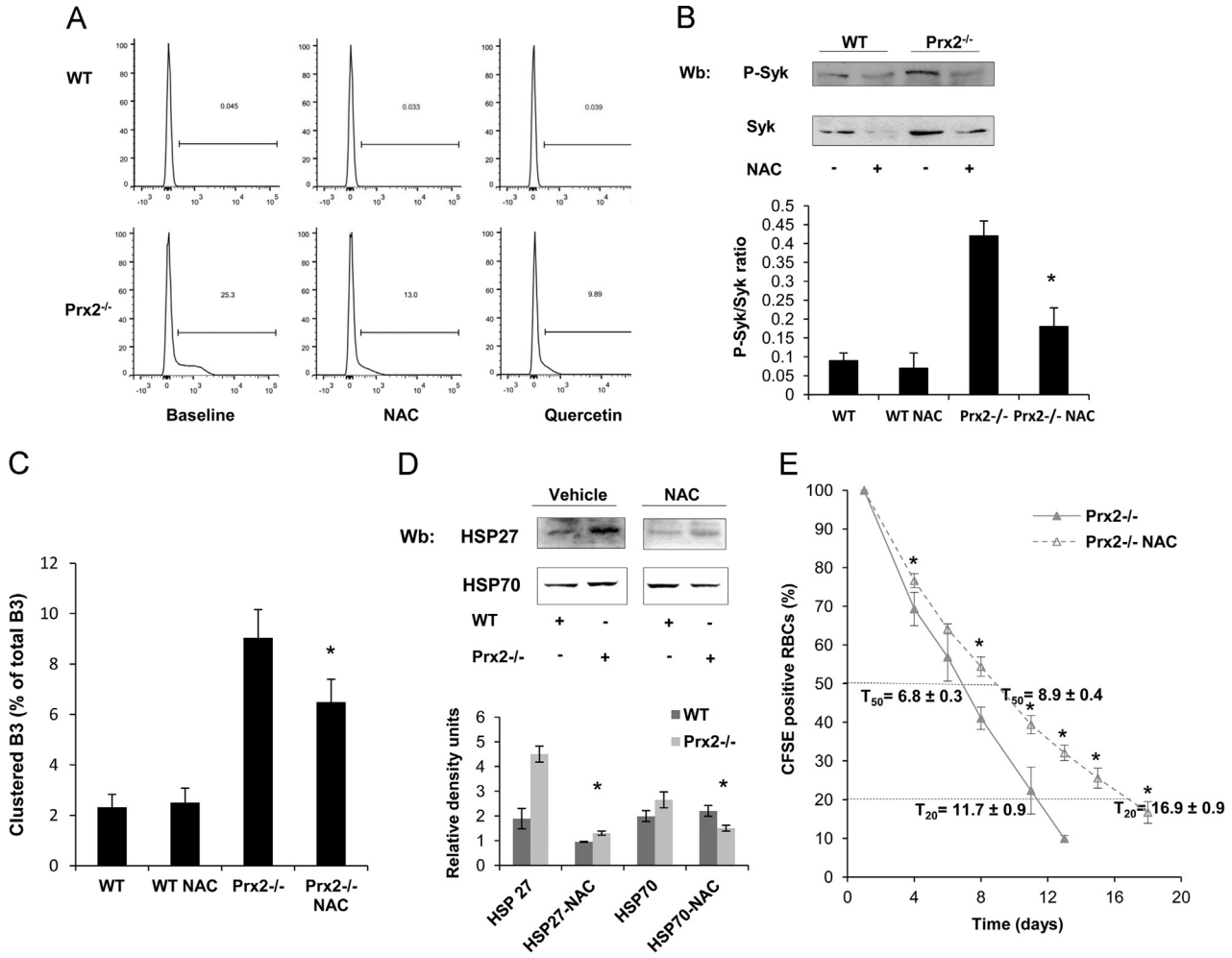

Fig. 4. Antioxidant treatment with either quercetin or NAC improves Prx2$^{-/-}$ red cell features; reduces Syk activation, HSP27 and HSP70 membrane translocation, and band 3 clusterization; and ameliorates Prx2$^{-/-}$ red cell survival. (A) Cytofluorimetric quantification of hemichromes bound to the membrane in wild-type (WT) and Prx2$^{-/-}$ mice treated with vehicle only, *N*-acetylcysteine (NAC), or quercetin. One representative image of six for each mouse group is shown, the relative bar graph is shown in Supplementary Fig. 4SA. (B) Top: Western blot (Wb) analysis with specific anti-phospho Syk (P-Syk) or Syk antibody of red cell membrane proteins from WT and Prx2$^{-/-}$ mice treated in vivo with NAC. One representative gel of six with similar results is presented (see also Supplementary Fig. 4SB for protein loading control). Bottom: densitometric analysis of the immunoblots was carried out. Data are presented as the phospho-Syk/Syk ratio and shown as means ± SD ($n = 3$; $^*P < 0.05$ compared to WT). (C) Percentage of band 3 clusters in erythrocytes from WT and Prx2$^{-/-}$ mice treated with either vehicle only or NAC. Data are presented as means ± SD ($n = 6$; $^*P < 0.05$ compared to vehicle-treated Prx2$^{-/-}$ mice). (D) Top: Wb analysis with specific anti-HSP27 or -HSP70 antibody of red cell membrane proteins from WT and Prx2$^{-/-}$ mice treated with either NAC or vehicle. One representative gel of six with similar results is presented (see also Supplementary Fig. 4SC for protein loading control). Vertical lines have been inserted to indicate a repositioned gel lane. Bottom: densitometric analysis of the immunoblots was carried out for relative quantification of immunoreactivity for HSP27 or HSP70. Data are shown as means ± SD ($n = 3$; $^*P < 0.05$ compared to NAC-treated WT). (E) Red cell survival of CFSE-labeled red cells from WT and Prx2$^{-/-}$ mice. Data are presented as means ± SD ($n = 4$) from each group; $^*P < 0.05$ compared to wild-type mice.

release of in vitro-generated erythroid MPs, compared to wild-type mice (Fig. 3E and F). The bands most expressed in Prx2$^{-/-}$ mouse red cells were then analyzed by mass spectrometry. In MPs from Prx2$^{-/-}$ mouse red cells, we found hemoglobin and proteins from the membrane skeletal network such as band 3, β-spectrin, or actin, but also proteins involved in membrane protein homeostasis such as HSPs or antioxidant systems such as catalase (Supplementary Table 2S).

As a proof of concept that Prx2 is part of the stress response machinery involved in red cell membrane protein homeostasis and red cell aging, we treated Prx2$^{-/-}$ mice in vivo with either quercetin or NAC as an exogenous antioxidant [29,30].

In vivo antioxidant treatment with NAC reduces Syk activation and band 3 clusterization, increases red cell survival, and normalizes MP protein composition in Prx2$^{-/-}$ mice

In Prx2$^{-/-}$ mice treated in vivo with either quercetin or NAC, we observed a significant reduction in the amount of HMCs associated with the membrane, as determined by two different methodological approaches (Fig. 4A, Supplementary Fig. 4SA, left and right). We further studied the effects of in vivo NAC treatment on Syk activation in Prx2$^{-/-}$ mouse red cells. We found a significant reduction in Syk activation and in band 3 clusters in NAC-treated Prx2$^{-/-}$ mice compared to vehicle-treated mice (Fig. 4B and C, Supplementary Fig. 4SB). These findings paralleled the decreased amount of HSPs associated with red cell membrane (Fig. 4D, see also Supplementary Fig. 4SC) and the increased survival of red cells in NAC-treated Prx2$^{-/-}$ mice compared to Prx2$^{-/-}$ vehicle-treated mice (Fig. 4E). Finally, we evaluated the impact of NAC treatment on MP generation from Prx2$^{-/-}$ mouse red cells, based on the reported observation that NAC can prevent in vitro MP release from red cells exposed to oxidative stress [31]. In Prx2$^{-/-}$ mice, NAC significantly reduced the release of erythroid MP, which displayed a protein profile similar to that of wild-type mice (Fig. 5A and B). These data suggest that Prx2 is a critical component of the membrane antioxidant system, counteracting irreversible red cell membrane oxidation-induced damage and participating in red cell membrane protein homeostasis.

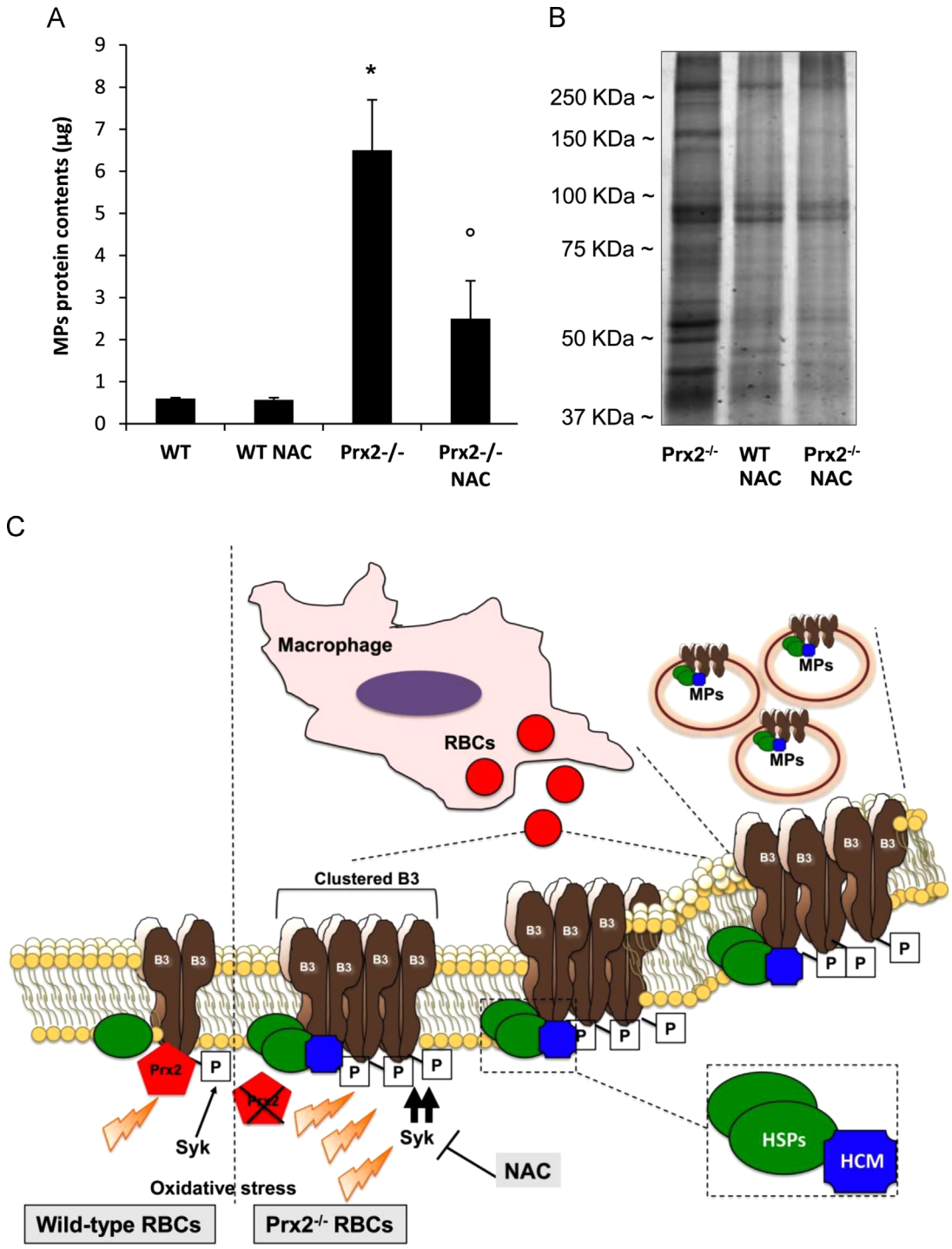

Fig. 5. NAC reduces the release of microparticles from Prx2$^{-/-}$ red cells. (A) Quantification of microparticles (MPs) from red cells of either vehicle- or NAC-treated wild-type (WT) and Prx2$^{-/-}$ mice. Data are presented as means ± SD ($n = 6$) from each group; $^{*}P < 0.05$ compared to WT mice; $^{\circ}P < 0.05$ compared to vehicle-treated Prx2$^{-/-}$ mice. (B) Proteins from erythroid MPs from either vehicle- or NAC-treated mice were separated by monodimensional electrophoresis, and gels were stained with colloidal Coomassie. One gel is shown as a representative of six with similar results. (C) Schematic diagram of the role of Prx2 in the redox response to maintain red cell membrane proteostasis. In wild-type mice, oxidative stress induces translocation to the membrane of heat shock proteins and peroxiredoxin-2, which limits the red cell membrane protein damage and the activation of Syk kinase with Tyr phosphorylation of band 3. In Prx2$^{-/-}$ mice, the absence of Prx2 promotes red cell membrane oxidative damage and translocation to the membrane of a high amount of HSPs, which are unable to counteract band 3 oxidation and clusterization favored by the high activation of Syk. This results in fast removal of prematurely damaged Prx2$^{-/-}$ mouse red cells by the macrophage system (erythrophagocytosis) and increases the release of erythroid microparticles that clear oxidized hemoglobin and damaged proteins. Prx2, peroxiredoxin 2; RBCs, red blood cells; B3, band 3; Syk, Src-related tyrosine kinase; P, phosphorylation of Tyr residues on band 3; HCM, hemichromes; HSPs, heat shock proteins; MPs, microparticles; NAC, *N*-acetylcysteine.

Discussion

Although progress has been made in the understanding of red cell membrane protein function in the past decade, the maintenance of erythrocyte membrane protein homeostasis in response to oxidative stress is still under investigation.

Here, we show that the absence of Prx2 promotes (i) HMC–membrane association and (ii) activation of the Syk redox-related

signaling pathway (Fig. 1). This allows an increase in the Tyr-phosphorylation state of oxidized band 3 and the generation of band 3 aggregates in Prx2$^{-/-}$ mouse red cells (Fig. 3). The increased red cell membrane translocation of HSPs 27 and 70 in Prx2$^{-/-}$ mice suggests the cellular attempt to reduce the red cell membrane protein oxidative damage. Previous studies in red cells have shown the presence of different HSPs involved in cellular responses to various stresses [27,32,33]. Because red cells have no nucleus and cannot newly synthesize damaged proteins, the roles of HSPs and local antioxidant systems are crucial in the homeostasis of red cell membrane proteins during the erythrocyte life span. In Prx2$^{-/-}$ mouse red cells, the increased membrane translocation of HSPs was insufficient to prevent the generation of band 3 clusters (Fig. 3). The functional consequences of the presence of these band 3 aggregates is the rapid removal of prematurely damaged red cells from the peripheral circulation by the macrophage system [16]. In fact, the in vitro erythrophagocytosis of Prx2$^{-/-}$ red cells was increased and paralleled by the in vivo reduction of Prx2$^{-/-}$ mouse erythrocyte survival compared to wild-type mice (Fig. 3). These data suggest a role for Prx2 in counteracting red cell senescence, which is in agreement with the reported increase in the membrane association of Prx2 in red cells stored under blood bank conditions, used as model of in vitro red cell aging [34], or in hereditary red cell disorders characterized by severe membrane oxidative damage, such as hemoglobinopathies [8,27,35]. The membrane association of HMCs is one part of the events involved in red cell senescence [16,28]. However, the mechanism that induces HMC formation in Prx2$^{-/-}$ mouse red cells is still unknown [1]. Here, we observed that a high concentration of hydrogen peroxide (1 mM) was not capable of generating further HMCs in either Prx2$^{-/-}$ or wild-type mouse red cells compared to baseline levels (Fig. 1A). In view of this result, Prx2, in addition to its noncatalytic peroxidase activity, seems to have additional protective functions. Several additional observations support this hypothesis: (i) Prx2's selective binding to band 3 [7], (ii) its translocation to the red cell membrane in response to thermal stress [36], (iii) its chaperone activity [37], and (iv) its association with disulfide isomerases [38]. Thus, our data indicate the important role of Prx2 in the red cell antioxidant machinery with possible action on HMC formation and as a potential protector from membrane oxidative stress [6,7], although the molecular details are still under investigation [1].

The functional relevance of Prx2 in red cell membrane protein homeostasis to prevent accelerated red cell aging is also revealed by the increased vesiculation of Prx2$^{-/-}$ mouse red cells, clearing oxidized hemoglobin and damaged proteins (Fig. 3E and F). The mass spectrometric analyses of MPs confirmed the presence of hemoglobin chains and membrane proteins such as band 3. It is of interest to note that HSPs and catalase were also identified in MPs from Prx2$^{-/-}$ mouse red cells, similar to those isolated from β-thalassemia intermedia red cells [16].

The beneficial effects of the antioxidant treatments on Prx2$^{-/-}$ mouse red cell features represent the proof of concept of the functional relevance of Prx2 in red cell membrane protein homeostasis. Here, we used quercetin and NAC as antioxidants with two different mechanisms of action. Quercetin is a plant-derived flavanoid, which exerts its action by combining the sequestration of free radicals and the chelation of pathological free iron [30,39]. NAC, on the other hand, acts as an exogenous donor of thiols, promoting the restoration of intracellular thiols coupled with the reduction of red cell oxidative damage [20,29,40]. In Prx2$^{-/-}$ mice both antioxidants significantly reduced the amount of HMCs bound to the Prx2$^{-/-}$ red cell membrane (Fig. 4A, Supplementary Fig. 4SA). Because previous studies have shown that NAC efficiently (i) increases GSH in normal red cells exposed to in vitro oxidative stress or in β-thalassemic erythrocytes [29,41], (ii) reduces in vivo red cell oxidative damage in a mouse model of hemolytic anemia [20], (iii) inhibits Syk activation in a cell line [42], and (iv) reduces the hemin-induced erythroid MP release [31], we further studied the effects of in vivo NAC treatment on Prx2$^{-/-}$ red cells. We found that in Prx2$^{-/-}$ mouse red cells, NAC decreased Syk activation with related reduction in band 3 clusterization, indicating an amelioration of the Prx2$^{-/-}$ mouse red cell membrane environment, as also supported by the decreased membrane translocation of HSPs 27 and 70, the increased survival of Prx2$^{-/-}$ red cells, and the reduction in erythroid MP release. These findings are particularly important in the context of hereditary hemolytic disorders such as β-thalassemia or SCD, which are characterized by red cell membrane oxidative damage and increased release of erythroid MPs that contribute to the procoagulant phenotype of these disorders [16,17,43,44].

Thus, we propose that Prx2 participates in the redox response involved in red cell membrane protein homeostasis and red cell aging events (Fig. 5C). The absence of Prx2 allows HMC formation and membrane association, bringing high pro-oxidants close to the red cell membrane network. This results in the activation of the Syk pathway and the clusterization of band 3, which promotes both early removal of damaged Prx2$^{-/-}$ red cells by erythrophagocytosis and the generation of erythroid MPs that clear irreversibly damaged proteins and oxidized Hb (Fig. 5C). NAC treatment breaks this vicious self-maintaining circle, resulting in the amelioration of Prx2$^{-/-}$ red cell features and the improvement of Prx2$^{-/-}$ red cell survival.

In conclusion, our data clearly show the key role of Prx2 in membrane-related redox response and shed light on red cell membrane protein homeostasis.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2014.08.004.

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