Gain-of-function Lyn induces anemia: appropriate Lyn activity is essential for normal erythropoiesis and Epo receptor signaling

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Key Points

- Gain-of-function Lyn mice develop hemolytic anemia with acanthocyte red blood cells and display compensatory extramedullary erythropoiesis.
- Hyperactive Lyn notably alters Epo receptor signaling, particularly an Akt-FoxO3 pathway, enhancing viability and delaying differentiation.

Introduction

The primary regulator of committed erythroid progenitors is erythropoietin (Epo) through its engagement of the Epo receptor and subsequent activation of intracellular signaling cascades, including the Janus kinase/signal transducer and activator of transcription (JAK/STAT), Rat sarcoma/rapidly accelerated fibrosarcoma/mitogen activated protein (ras/raf/MAP)-kinase, and Phosphatidylinositol 3 (PI3) kinase/Akt pathways. ¹⁻⁴ JAK2 is recognized as the kinase involved in initiation of Epo receptor signaling,¹ whereas Lyn, a member of the Src family of tyrosine kinases (SFKs), has been implicated as a key secondary kinase.⁵⁻¹⁰

In vitro studies established a role for Lyn in Epo receptor signaling ⁵,¹¹ and demonstrated that Epo signaling in immortalized J2E-derived cells appeared identical to primary erythroid cells.⁵,⁹,¹¹ Subsequent studies using Lyn⁻/⁻ mice consolidated the importance of this SFK for early erythroid cell expansion and late-stage maturation.¹⁰ Removal, or inhibition, of Lyn attenuates the ability of erythroid cells to differentiate in response to Epo.⁶,⁹⁻¹⁰

On Epo receptor ligation, Lyn is activated ⁶,¹¹ and intersects numerous downstream signaling events, including phosphorylation of STAT5.⁶,⁷ Reduced Lyn in erythroid cell lines diminishes erythropoietin transcription factor (GATA-1 [GATA-binding factor 1], EKLF [Erythroid Kruppel-like Factor]) levels and reduces their ability to differentiate; conversely, transient overexpression of Lyn enhances differentiation.⁶,¹¹ In addition, Lyn stimulates pathways that lead to down-regulation of Epo receptor signaling.¹²,¹³

Mice deficient in Epo, Epo receptor, and JAK2 fail to develop a definitive erythroid compartment.¹⁴⁻¹⁶ Although definitive erythropoiesis occurs in Lyn⁻/⁻ mice, an underlying erythroid defect results in activation of compensatory stress erythropoiesis.⁸,¹⁰ Although the absence of Lyn clearly perturbs erythropoiesis and affects Epo receptor signaling,⁹,¹⁰ the pathways influenced on Lyn activation and their biological roles remain unclear. To address this important question, we examined knock-in mice expressing a constitutively active form of Lyn (Lyn⁺/+), such that constitutively active Lyn would be expressed in a temporally and spatially appropriate manner.¹⁵

In this article, we demonstrate that Lyn⁺/+ mice displayed a hemolytic anemia and had markedly elevated erythroid progenitors and precursors primarily in spleen, indicating active extramedullary...
erythropoiesis. Importantly, constitutively active Lyn had a major impact on Epo receptor signaling, most notably affecting the JAK2-STAT5, GRB2-associated-binding protein 2 (GAB2), and Akt–forkhead box O3 (FoxO3) pathways, suggesting that regulation of Lyn is crucial for normal erythropoiesis. Failure to control Lyn activity, as exemplified by Lyn deficiency or its overactivity, interferes with Epo receptor signaling and is deleterious for erythroid homeostasis.

Materials and methods

Mice, cell morphology, and anemia induction
Lyn−/−, Lyn+/-, Lyn−/−, and Lyn−/- mice, on a C57BL/6 background, were analyzed at days 12.5 to 13.5 of embryonic development, as 8- to 15-week-old adults, and as aged (70-85 weeks old) animals. The Lyn−/- allele is a knock-in activating point mutation of Tyr-to-Phe at residue 508 (the C-terminal regulatory Tyr) of the Lyn gene, generating a constitutively active kinase. All experiments were performed in accordance with National Health and Medical Research Council guidelines for animal experimentation, with approval from the Animal Ethics Committees of the Baker IDI Heart and Diabetes Institute (Melbourne, Australia), the Animal Resource Centre, (Murdoch, Australia), and the Royal Perth Hospital (Perth, Australia). Heparinized pediatric tubes were used for blood collection and blood parameter determination on an Advia 120 (Siemens, Deerfield, IL). Blood smears, bone marrow, and fetal liver and splenic cell morphology were examined microscopically following staining.18 Anemia was induced by injection of phenylhydrazine (PHZ; 60 mg/kg body weight) on 2 consecutive days, followed by blood and erythroid cell analysis 3 and 5 days after initial PHZ injection as previously described.19 Primary CD71 spleen erythroblasts were isolated from day 5 PHZ-treated mice using fluorescein isothiocyanate-CD71 (BD Biosciences, San Jose, CA) and EasySep magnetic FITC Selection Kit (StemCell Technologies, Vancouver, BC, Canada) essentially as previously described.10

Erythroid progenitor assays and flow cytometry
Single cell suspensions of bone marrow and spleen were prepared and assayed for erythroid burst-forming units (BFU-E) and colony-forming units (CFU-E) using methylcellulose cultures, as described.2 Flow cytometry was used to assess erythroid populations in bone marrow and spleen as detailed previously10 using a FACS Aria II flow cytometer (Beckman-Coulter, Palo Alto, CA).

Erythroid cell lines
Immortalized erythroid cell lines were generated by exposing fetal liver progenitors (day E12.5 embryos) to J2 retrovirus as described.22 Individual clones (12 per genotype) were isolated and characterized for viability and hemoglobin production as described.3 Cells were maintained in Iscove’s modified Dulbecco’s medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum. Short-term Epo inductions (5 units/mL, Epoetin alfa; Jansen-Cilag) were carried out on cells after 2-hour serum starvation. Differentiation experiments were performed on cells cultured in thyroid hormone–depleted fetal bovine serum in the presence or absence of Epo (5 units/mL).21

Immunoblotting and immunoprecipitation
Cells were lysed in raft buffer (150 mm NaCl, 1% octylphenoxypolyethox-yethanol (IGEPAL CA-630) [Sigma-Aldrich, St. Louis, MO], 0.5% n-dodecyl-β-D-maltoside [Sigma-Aldrich], 0.2% octyl-o-glucoside [Sigma-Aldrich], 20 mm Tris, pH 8.0, 1% Complete protease inhibitor cocktail [Roche, Mannheim, Germany], 2 mM benzamidine, 2 mM vanadate, 1 mM EDTA, 1 mM EGTA, and 10 mM β-glycerol phosphate). Signaling studies were undertaken by immunoprecipitation and immunoblotting as previously described using antibodies as described in the supplemental Materials and methods on the Blood website.21

Results

Gain-of-function Lyn induces hemolytic anemia

Young adult and aged Lyn−/- mice displayed overt signs of anemia, with hematocrits, hemoglobin content, and circulating red blood cells all significantly reduced (Table 1). Interestingly, young Lyn−/- mice displayed an intermediate phenotype; however, with age, they developed more severe symptoms of anemia, and the decrease in all red cell parameters became statistically significant (Table 1). Blood smears revealed that Lyn−/- mice contained significant numbers of abnormal red blood cells in circulation (Figure 1A). The presence of numerous circulating acanthocytes (Lyn−/-, 0.1 ± 0.0%; Lyn−/-, 14 ± 9.3%; P = .002) and spherocyte-like cells (Lyn−/-, 0.1 ± 0.0%; Lyn−/-, 10.3 ± 12.9%; P = .04) suggested the animals had a hemolytic anemia. These data demonstrate that expression of either 1 (Lyn−/-) or 2 (Lyn−/-) hyperactive Lyn alleles leads to anemia and red blood cell abnormalities.

Altered erythropoiesis in Lyn−/- and Lyn−/- mice

The bone marrow of Lyn−/- mice displayed an altered ratio of erythroid to granulocytic precursors, with proportionally more granulocytic precursors (myeloid:erythroid ratio: Lyn−/-, 41: ± 2.0; Lyn−/-, 7:1 ± 1.9; P = .03; Figure 1B), reflecting their elevated peripheral neutrophil counts.13 Lyn−/- spleen cell preparations showed reduced lymphocytes (lymphocytes: Lyn−/-, 81 ± 6%; Lyn−/-, 31 ± 7%; P < .01) and increased erythroid precursors, blast/progenitor-like cells, and abnormal large polychromatophilic erythroblasts (Figure 1C). Interestingly, the fetal livers of 12.5-day-old Lyn−/- embryos displayed numerous small nucleated (definitive) erythroblasts, whereas control animals possessed mostly large nucleated (primitive) erythroblasts that are normally found at this embryonic stage (nucleated erythroblasts: Lyn−/-, 5.6 ± 9.7%; Lyn−/-, 47.8 ± 18.7%; P < .001; Figure 1D). These morphological analyses show a significant effect of elevated Lyn activity on the erythroid compartment during embryonic development and in adult tissues.

Progenitors in the bone marrow of young adult Lyn−/- and Lyn−/- mice were raised: BFU-E numbers were significantly higher, whereas CFU-E were elevated by ~50% in Lyn−/- and ~20% in Lyn−/- mice (Figure 2A). In spleens, changes were even more dramatic, with BFU-E elevated 2-fold in Lyn−/- and >5-fold in Lyn−/-, whereas CFU-E was increased >10-fold (Figure 2B). The significant extramedullary erythropoiesis was not accompanied by splenomegaly, unlike in Lyn−/- mice (Figure 2C); in fact, the spleens were statistically smaller in Lyn−/- and Lyn−/- mice, due to a major B-cell deficit.22 Taking into account the reduced cellularity of Lyn−/- and Lyn−/- spleens, absolute numbers of BFU-E and CFU-E were still markedly elevated four- and eightfold, respectively.

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<th>Table 1. Red blood cell parameters of Lyn+/+, Lyn+/−, and Lyn−/− mice</th>
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HCT, hematocrit; HGB, hemoglobin; ns, not significant; RBC, red blood cell. *P < .05.
The maturation status of the erythroid compartment was then investigated using flow cytometry. An elevation in the most immature R1 (Lyn+/−, 0.12 ± 0.06%; Lyn+/up, 0.76 ± 0.05%; P < .05) precursor population (CD71high/Ter119low) was observed in the bone marrow of young adult Lyn+/up mice (Figure 2D), commensurate with the rise in BFU-E and CFU-E (Figure 2A). In the spleens of Lyn+/up mice, both R1 (Lyn+/−, 0.06 ± 0.05%; Lyn+/up, 1.01 ± 0.12%; P < .01) and R2 (Lyn+/−, 1.96 ± 0.42%; Lyn+/up, 9.81 ± 1.30%; P < .01) (CD71high/Ter119high) populations were markedly elevated (Figure 2E), consistent with the increase in splenic progenitors (Figure 2B). Lyn+/up mice also displayed a similar increase in R1 and R2 precursors (supplemental Figure 1). Together, these data suggest that hyperactive Lyn induces a red cell membrane defect causing hemolysis and anemia, leading to activation of extramedullary erythropoiesis to maintain homeostasis.

Erythroid progenitors and precursors were then examined in older mice. In contrast to young adult mice, numbers of bone marrow BFU-E were not significantly different in Lyn+/up but were decreased by ~30% in Lyn+/up, suggesting bone marrow exhaustion with age in Lyn+/up mice (Figure 3A). However, CFU-E numbers remained elevated for both Lyn+/− and Lyn+/up, indicating that later-stage expansion capacity persists (Figure 3A). A change in the precursor profile was also detected with a marked increase in the most mature R4 (CD71low/Ter119high) population (Figure 3B). These observations indicate that changes have occurred in the erythroid compartment with age in an attempt to maintain red cell levels.

Extramedullary erythropoiesis was also a feature of older Lyn+/up and Lyn+/up mice, and splenic progenitors remained markedly elevated: BFU-E levels were raised 3-fold, whereas CFU-E were either 7- (Lyn+/up) or 15-fold (Lyn+/up) higher (Figure 3C). Although the R2 population remained high in spleens of aged Lyn+/up mice, an increase in the R4 population was also observed (Figure 3D). These data suggest that compensatory mechanisms to alleviate the anemia caused by hyperactive Lyn are dynamic and that erythroid progenitor and precursor components alter with age in an attempt to produce more erythrocytes.

**Altered response to anemia by Lyn+/up mice**

As Lyn+/up mice display a mild erythroid phenotype, we speculated that stressing their erythroid compartment might reveal more significant underlying perturbations. Accordingly, the ability of Lyn+/up mice to respond to acute anemia induced by PHZ was examined. Both control and Lyn+/up mice displayed comparable hematocrit reduction at 3 and 5 days after PHZ treatment (Figure 4A-B). Although no differences in BFU-E recovery were detected between control and Lyn+/up mice (Figure 4C), the CFU-E expansion at both days 3 and 5 after PHZ treatment was significantly elevated in Lyn+/up mice (Figure 4D). Flow cytometric analyses of erythroid cells 3 and 5 days after PHZ treatment also revealed significant differences in the R0 and maturing R2 and R3 populations (Figure 4E-F). Together, these data show that following acute anemic insult, Lyn+/up mice display an altered erythroid expansion response to recover their hematocrit.

**Lyn+/up erythroid cell lines have greater viability but delayed differentiation in response to Epo**

To determine the consequences of hyperactive Lyn on Epo-initiated signaling cascades, several independent cell lines were generated
from control and Lyn\(^{+/+}\) mice by immortalization of erythroid fetal liver cells with the J2 retrovirus.\(^{20}\) The morphology of the J2-Lyn\(^{+/+}\) cell lines was similar to the lines generated from control mice (J2-WT; Figure 5A). In addition, flow cytometric analyses revealed that these cell lines had similar cell surface marker profiles (Figure 5B), as well as growth and clonogenicity (data not shown). Together, these results suggest that the cell lines from control and Lyn\(^{+/+}\) mice were immortalized at similar stages of maturation.

J2-Lyn\(^{+/+}\) cells exhibited enhanced viability compared with J2-WT cells in the absence of Epo and had a delayed differentiation response to Epo (Figure 5C). Dose-response analyses confirmed the enhanced viability of J2-Lyn\(^{+/+}\) cells with low doses of Epo (Figure 5D), whereas differentiation was Epo dependent for both J2-WT and J2-Lyn\(^{+/+}\) lines (Figure 5D).

Epo receptor signaling in Lyn\(^{+/+}\) cell lines and primary Lyn\(^{+/+}\) erythroblasts: activation of JAK2 and Akt pathways and inhibition of GAB2

To examine the effects of hyperactive Lyn on Epo signaling, biochemical studies were performed (Figure 6A). Anti-phosphotyrosine immunoblots revealed that 2 proteins (pp39 and pp90) were heavily phosphorylated in J2-Lyn\(^{+/+}\) cells before exposure to Epo, suggesting that they are direct Lyn target proteins. Interestingly, although the phosphorylation of both pp39 and pp90 increased following Epo stimulation in J2-WT cells, in J2-Lyn\(^{+/+}\) cells, pp90 underwent a transient dephosphorylation, whereas the phosphorylation of pp39 remained stable. As expected, Lyn (pp53/56) phosphorylation and Epo receptor (pp68/70) phosphorylation increased in Epo-stimulated J2-WT cells, whereas in J2-Lyn\(^{+/+}\) cells, Lyn underwent a transient dephosphorylation, and very little phospho-EpoR was detected. These data indicate that hyperactive Lyn can influence intracellular signaling in the absence of Epo, as well as the kinetics of protein phosphorylation following exposure to Epo.

Previously we showed that Lyn\(^{+/+}\) erythrocyte cell lines have reduced GATA-1 and EKLF.\(^{9}\) Here we demonstrate that J2-Lyn\(^{+/+}\) cells have the same level of GATA-1 expression as J2-WT cells, but is also seen in bone marrow–derived macrophages and primary B cells purified from Lyn\(^{+/+}\) mice.\(^{13,22}\) Interestingly, the kinetics of Lyn inactivation (pLyn-Y508) was also disrupted
Alternatively, although JAK2 is phosphorylated in the absence of Epo receptors, as indicated by the elevated levels of truncated Epo-R, appear to increase basal Epo receptor phosphorylation; one explanation can also stimulate STAT1 and STAT3 phosphorylation,27 but independent of constitutive JAK2 phosphorylation. Epo-R signaling can significantly alter the degree of STAT5 phosphorylation of the receptor was detected in J2-Lyn WT and J2-Lyn up cells (Figure 6B). However, limited phosphorylation of the receptor was detected in J2-Lyn WT cells, whereas J2-Lyn up cells displayed typical JAK2 phosphorylation in response to Epo stimulation, whereas J2-Lyn 1/4 cells displayed elevated JAK2 phosphorylation prior to Epo stimulation and then underwent a transient decrease after exposure to Epo (Figure 6B). Surprisingly, constitutively activated JAK2 in J2-Lyn 1/4 cells did not appear to increase basal Epo receptor phosphorylation; one explanation for this observation could be enhanced turnover of activated Epo receptors, as indicated by the elevated levels of truncated Epo-R. Alternatively, although JAK2 is phosphorylated in the absence of Epo in J2-Lyn 1/4 cells, the Epo-R still requires the presence of Epo to induce an appropriate reorientation that mediates receptor phosphorylation and subsequent downstream pathway activation.24 Further, the constitutive JAK2 phosphorylation was diminished in the presence of the JAK inhibitor, as well as the Lyn/SFK inhibitor PP2 (supplemental Figure 2A).

Both JAK2 and Lyn have been shown to phosphorylate STATs.6-9,25-26 Interestingly, a marked increase in STAT5 phosphorylation occurred after exposure of J2-Lyn 1/4 cells to Epo (Figure 6B), which was markedly reduced by JAK2 and Lyn inhibition (supplemental Figure 2B). This observation indicates that hyperactive Lyn significantly alters the degree of STAT5 phosphorylation. Moreover, STAT5 phosphorylation is Epo dependent, but independent of constitutive JAK2 phosphorylation. Epo-R signaling can also stimulate STAT1 and STAT3 phosphorylation,27 and we show that J2-Lyn 1/4 cells have enhanced Epo-induced STAT3 phosphorylation, whereas STAT1 phosphorylation is reduced (Figure 6B).

The regulation of downstream pathways from the Epo-R is controlled by recruitment and phosphorylation of not only the receptor itself but also several adaptors/scaffolds and phosphatases. Unexpectedly, Epo-induced tyrosine phosphorylation of the adapter Gab2 (at Y452 and total pY levels) was virtually absent in J2-Lyn 1/4 cells, whereas its serine phosphorylation (S159) was markedly elevated, and intriguingly, substantial serine phosphorylation was evident prior to Epo stimulation, which was further enhanced after Epo treatment (Figure 6C). This serine phosphorylation of Gab2 was sensitive to both JAK2 and Lyn inhibition (supplemental Figure 2B). Gab2 is known to be a Lyn substrate involved in the recruitment of the regulatory p85 subunit of PI3 kinase and the phosphatase Src homology region 2 domain-containing phosphatase 2 (SHP-2).28,30 Because Akt-mediated serine phosphorylation of Gab2 prevents its tyrosine phosphorylation, this may explain the lack of appreciable tyrosine phosphorylation of Gab2 in the J2-Lyn 1/4 cells.31 SHP-1 and SHP-2 are involved in modulation of Epo-R phosphorylation,32 Lyn activation,29,33 and regulation of downstream pathways.3,30 Interestingly, marginal Epo-induced phosphorylation of SHP-1 was observed in both J2-WT and J2-Lyn 1/4 cells, whereas robust SHP-2 phosphorylation occurred in both cell types, although this response was reduced considerably in Lyn 1/4 cells. This may be explained by reduced recruitment of SHP-2 to tyrosine phosphorylation sites on Gab2.

Other downstream signaling events were also monitored in J2-Lyn 1/4 cells. Markedly elevated phosphorylation of Akt and its downstream target FoxO3 were detected in J2-Lyn 1/4 cells before, and especially after, Epo induction (Figure 6C). The Epo-induced elevated pAkt in J2-Lyn 1/4 cells was dependent on both JAK2 and Lyn, because inhibition of either almost completely abrogated pAkt levels (supplemental Figure 2B). The increased Akt activity is unlikely to be via Gab2 recruitment of PI3K, because this is likely diminished in J2-Lyn 1/4 cells but could be direct or via intermediate molecules, as SFKs can directly phosphorylate and activate Akt,34 as well as the Akt activator PDK1.35 In contrast, the activation

Figure 3. Altered bone marrow erythropoiesis and elevated spleen erythropoiesis in aged Lyn-/- and Lyn+/- mice. (A) Bone marrow BFU-E and CFU-E are reduced, whereas CFU-E numbers are increased in aged Lyn-/- and Lyn+/- mice. Erythroid colony assays (BFU-E and CFU-E) of Lyn +/-, Lyn-, and Lyn+ mice marrow from mice 70 to 85 weeks of age (n = 3, *P < .05, ns, not significant). (B) Comparison of maturing bone marrow erythroid cells from aged Lyn-/- and Lyn+/- mice. Representative flow cytometric analysis of Lyn +/- (WT) and Lyn+/- bone marrow cells of mice 70 to 85 weeks of age and enumerated as in Figure 2D. (C) Extramedullary erythropoiesis in spleen of aged Lyn-/- and Lyn+/- mice. Erythroid colony assays (BFU-E and CFU-E) of Lyn +/-, Lyn-/-, and Lyn+/- spleens from mice 70 to 85 weeks of age (n = 3, ***P < .01). (D) Elevated mature erythroid cells in the spleen of aged Lyn+/- mice. Representative flow cytometric analysis of spleen cells of Lyn+/- (WT) and Lyn+/- mice 70 to 85 weeks of age and enumerated as in B. Mean ± standard deviation is shown. Statistically significant (2-way ANOVA) differences are indicated (*P < .05).
dynamics of Erk1/2 appeared similar in J2-WT and J2-Lyn$^{+/+}$ cells; although phosphorylation of p38MAPK was elevated in J2-Lyn$^{+/+}$ cells, pJNK levels were not altered (Figure 6C). The protein products of genes classically activated by the Epo receptor were also elevated in J2-Lyn$^{+/+}$ cells, ie, B-cell lymphoma-extra large (Bcl XL), cytokine-inducible SH2-containing protein (CIS), suppressor of cytokine signaling (SOCS)1, and SOCS3 (Figure 6D). Elevated levels of the antiapoptotic molecule BclXL may contribute to the Epo-independent viability$^{36}$ of J2-Lyn$^{+/+}$ (Figure 5C-E), whereas increased CIS, SOCS1, and SOCS3 may represent an attempt to down-regulate intracellular signaling cascades. Collectively, these data demonstrate that constitutively active Lyn has a significant impact on Epo-independent and -dependent intracellular signaling. Because J2E cells are immortalized at the proerythroblast stage,$^{20}$ these data suggest that Lyn plays a major role in transmitting survival signals via STAT5 and Akt in immature erythroid cells.
To investigate this further, signaling molecules were then examined over an extended time frame in high differentiation capacity media. In the presence or absence of Epo, Akt was highly active in J2-Lyn<sup>+/up</sup> cells after 48 hours of culture, whereas STAT5 phosphorylation was Epo dependent in both control and J2-Lyn<sup>+/up</sup> cell lines (Figure 7A). Pathways downstream of Akt were then investigated, revealing significantly enhanced phosphorylation of FoxO3 and reduced tyrosine but elevated inhibitory serine phosphorylation of GAB2 in J2-Lyn<sup>+/up</sup> cells. A modest increase in phosphorylated Bcl-2-associated death promoter (BAD) was seen, whereas phosphorylated GSK3β was decreased. These data suggest a viability pathway involving Akt-FoxO3 is markedly activated in J2-Lyn<sup>+/up</sup> cells, thereby mediating elevated cell survival (Figure 5C-E).

To demonstrate that changes in signaling observed in cell lines reflected effects seen in vivo, we examined signaling in primary CD71<sup>+</sup> erythroid cells isolated from the spleens of PHZ-treated mice. These studies revealed that the alterations to GAB2 serine and tyrosine phosphorylation and the Akt/FoxO3 pathways that we originally observed in the immortalized cells were recapitulated in these primary cells (Figure 7B).

**Discussion**

In previous studies, we established that Lyn is a critical signaling molecule involved in erythroid homeostasis. To further and to unravel the signaling pathways in erythroid cells controlled by Lyn, we examined mice expressing constitutively active Lyn. We demonstrate that expression of gain-of-function Lyn leads to the development of anemia in adult mice and alters erythropoiesis during embryonic development. The presence of dysmorphic spherocyte-like cells and acanthocytes in the peripheral blood of Lyn<sup>+/up</sup> mice suggests that hyperactive Lyn affects the membrane/cytoskeleton of red blood cells, indicating an intrinsic defect that results in hemolytic anemia. Lyn is the most active SFK in erythrocytes and serves to phosphorylate erythrocyte Band 3, a cytoskeletal-linked anion exchanger. Significantly, erythrocyte membrane changes in the erythroid condition chorea-acanthocytosis results from increased Lyn activity altering the phosphorylation status and organization of cytoskeletal proteins. Consequently, this animal model of elevated Lyn activity resembles a human red cell disorder and suggests that targeting Lyn may be a possible therapy. The presence of high numbers of enucleated erythrocytes in the fetal livers of Lyn<sup>+/up</sup> mice may reflect an accelerated transition to definitive erythropoiesis in these mice due to premature maturation of primitive erythroid cells, which is known to be mediated by SFKs. Thus, hyperactive Lyn has a profound impact on the morphology of fetal and adult erythroid cells resulting in a hemolytic anemia.

Within adult Lyn<sup>+/up</sup> mice, there is a compensatory expansion of bone marrow erythropoiesis, as well as a significant expansion of emergency erythropoiesis in the spleen, and Lyn<sup>+/up</sup> mice display a milder intermediate phenotype compared with Lyn<sup>+/up</sup> animals, showing that expression of 1 gain-of-function Lyn allele is sufficient to induce significant alterations to the erythroid compartment. However, this elevated erythropoiesis is not sufficient to alleviate the appearance of anemia in these mice. Although this phenotype is similar to that of Lyn<sup>−/−</sup> mice, the underlying mechanism is likely to be different. A lack of Lyn elicits a stem/progenitor-intrinsic defect
that reduces progenitor expansion and late-stage development, resulting in age-dependent anemia. However, for Lyn 
expression of hyperactive Lyn instigates a red blood cell defect that is only partially compensated by an expansion of erythropoiesis in the bone marrow and spleen and suggests that regulation of Lyn activity during erythropoiesis is critical for maintenance of erythroid homeostasis.

Interestingly, immortalized erythroid lines from Lyn 
animals exhibited enhanced viability in the absence of Epo, albeit delayed differentiation in the presence of this cytokine. Our biochemical data demonstrate that constitutively elevated Lyn activity significantly alters Epo receptor phosphorylation, the JAK/STAT pathway, the signaling adaptor GAB2, and the Akt/FoxO3 cascade, leading to expression of the antiapoptotic protein BclXL. Importantly, alterations to GAB2 and Akt/FoxO3 were recapitulated in primary Lyn 
erythroblasts. Interestingly, the activated Akt in Lyn 
cells fed into a FoxO3 pathway but produced little or no GSK3β activation, whereas BclXL and BAD are located in the cytoplasm. Potentially, this may be mediated by differential GAB2 phosphorylation, which is known to be a link between the Epo-R and PI3 kinase/Akt, because in Lyn 
cells, there is a near absence of Lyn 
phosphorylation, whereas phosphoserine-GAB2 is substantially elevated prior to Epo stimulation. GAB2 also mediate differential PI3 kinase/Akt signaling toward proliferation/viability or differentiation outcomes. Elevation of Akt activity is used in a negative feedback loop to regulate GAB2 tyrosine phosphorylation and its interactions with other proteins. The lack of GAB2 phosphorylation seems counterintuitive, as GAB2 is a substrate of Lyn. GAB2 can also mediate differential PI3 kinase/Akt signaling toward proliferation/viability or differentiation outcomes.

Figure 6. Altered Epo receptor signaling in erythroid Lyn 
cells compared with Lyn 
cells. (A) Altered phosphotyrosine dynamics during Epo induction and elevated EKLF, BclXL, CIS, SOCS1, and SOCS3 in J2-Lyn 
cells. Time course of Epo-induced total phospho-tyrosine (pY) changes in J2-WT and J2-Lyn 
cells. Prominent changes in phospho-proteins between the cell lines are indicated (arrows, and approximate kilodaltons). (B) Altered Lyn and proximal Epo receptor signaling dynamics during Epo induction in J2-Lyn 
cells. Immunoblot analysis of J2-WT and J2-Lyn 
cells for the signaling molecules indicated before and after 10 and 30 minutes of Epo stimulation. Total cell lysates were used for analysis except for pEpo receptor, where immunoprecipitates of the Epo receptor were blotted with anti-pY antibodies. (C) Altered downstream Epo receptor signaling dynamics during Epo induction in J2-Lyn 
cells. Immunoblot analysis of total cell lysates of J2-WT and J2-Lyn 
cells, 0, 10, and 30 minutes after Epo stimulation for the signaling molecules indicated and GAB2 immunoprecipitates probed for total phosphotyrosine (pY) and GAB2. Immunoblot analysis was performed on 2 independent experiments producing equivalent results. (D) Increased levels of Epo receptor downstream targets in J2-Lyn 
cells. Immunoblot analysis of total cell lysates of J2-WT and J2-Lyn 
cells, 0, 10, and 30 minutes after Epo stimulation for BclXL, SOCS1, SOCS3, and CIS.

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A lack of Lyn reduced Epo-mediated STAT5 activation,9 which is consistent with the elevated STAT5 phosphorylation seen in J2-Lyn WT cells. However, elevated Akt activity was observed when Lyn was ablated.8 It may be that Lyn mediates both positive and negative regulation of Akt, consistent with its known roles in both activating and inhibiting signaling pathways.46-48 We propose that because of this altered intracellular signaling caused by a gain-of-function Lyn, through hyperactivation of Akt and reduced tyrosine phosphorylation of GAB2, the balance between viability, expansion, and differentiation of Epo-responsive cells in vivo is severely perturbed (Figure 7C). As a consequence, erythroid progenitor and precursor levels in mice harboring either 1 or 2 Lyn alleles are disrupted, and the animals become increasingly anemic.

We showed previously that loss of Lyn severely impacts erythropoiesis.9,10 Here, we demonstrated that constitutively active Lyn affects signaling in erythroid cells, influences cell viability, and leads to major compensatory changes in the erythroid compartment. Together, these results illustrate that regulation of Lyn activity and gene dosage are crucial for normal erythropoiesis. Insufficient Lyn or inappropriately activated Lyn are similarly deleterious to the erythroid compartment.

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