Published in final edited form as:

Exp Hematol. 2017 May ; 49: 48–55.e5. doi:10.1016/j.exphem.2016.12.010.

Governing Roles for Trib3 Pseudokinase During Stress Erythropoiesis

Arvind Dev¹, Ruth Asch¹, Edward Jachimowicz¹, Nicole Rainville¹, Ashley Johnson¹, Emily Greenfest-Allen², and Don M Wojchowski^{1,3,4}

¹Molecular Medicine Division, Maine Medical Center Research Institute, 81 Research Drive, Scarborough, ME 04074

²Computational Biology and Informatics Laboratory, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA

³Department of Medicine, Tufts University Medical Center, 145 Harrison Ave, Boston, MA 02111

Abstract

In response to anemia, the heightened production of erythropoietin (EPO) can sharply promote erythroid progenitor cell (EPC) formation. Specific mediators of such EPO- accelerated erythropoiesis, however, are not well understood. Presently, we first report that the expression of *Trib3* in adult bone marrow EPCs in vivo is nominal at steady state, but strongly activated upon EPO challenge. In a knockout mouse model, *Trib3* disruption modestly increased steady-state erythrocyte numbers, and decreased mean corpuscular volume. Following 5-fluorouracil myeloablation, however, rebound RBC production and hemoglobin levels were substantially (and selectively) compromised in *Trib3*^{-/-} mice vs *Trib3*^{+/+} congenic controls. Erythrocytes from 5-fluorouracil treated *Trib3*^{-/-} mice additionally were more prone to lysis, and exhibited elevated peroxide-induced reactive oxygen species. Ex vivo, the development of CD71^{pos}Ter119^{pos} erythroblasts from *Trib3*^{-/-} bone marrow progenitors was attenuated, and this was associated with heightened EPO-dependent Erk1/2 activation and moderately increased Akt activation. For developmentally staged EPCs, gene profiling provided further initial insight into candidate mediators of EPO-induced *Trib3* gene expression, including *Cebp-beta*, *Atf4*, *Egr-1*, and *Nab1*. Overall, Trib3 is indicated to act as a novel EPC-intrinsic governor of stress erythropoiesis.

⁴Corresponding author: Don M. Wojchowski, Ph.D., Director, Center of Biomedical Research Excellence in Stem Cell Biology and Regenerative Medicine Maine Medical, Center Research Institute, 81 Research Drive, Scarborough, ME 04074 USA, Phone: (207) 396-8258, Fax: (207) 396-8778, wojchd@mmc.org.

AUTHORSHIP CONTRIBUTIONS: AD and DMW played lead roles in designing and executing the experiments integral to this overall study. AD initially conceptualized these studies and established *Trib3*^{flox/flox} and *Trib3*^{-/-} mouse colonies. RA, NR, AJ, EJ, AD and DMW each contributed to ex vivo EPC investigations. In EPO-regulated transcriptome studies, EG-A and DMW were lead contributors. RT-PCR was performed by RA, NR and EJ, and western blotting was undertaken by NR, RA and AJ. DMW directed these investigations and assumed a lead role in manuscript construction.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Trib3; Erythropoiesis; Erythropoietin; Anemia

Introduction

During anemia, red blood cell production dynamically increases to protect against hypoxic tissue damage. Such accelerated erythropoiesis depends in part upon the heightened effects of glucocorticoids, stem cell factor (SCF) and BMP4 on early erythroid progenitor cell (EPC) expansion [1]. Anemia-associated hypoxia also increases the production (by renal interstitial fibroblasts) of the proerythropoietic hormone EPO up to 1000-fold [2]. Notably, heightened EPO signaling can induce the expression in EPCs of select factors that exert effects predominantly during accelerated and/or stress erythropoiesis. Examples include the intracellular serpin Spi2a that limits erythroblast damage by leached lysosomal cathepsins [3], and Erythroferrone as a novel EPO-induced suppressor of hepcidin [4]. Previously, we initially reported on *Trib3* as a novel EPO target gene in EPC's ex vivo [5]. Presently, we report on in vivo induction of *Trib3* expression in EPCs, and on novel effects of this pseudokinase on red cell formation during accelerated erythropoiesis. In a knockout mouse model, *Trib3* disruption markedly compromised recovery from 5-fluorouracil-induced anemia, potentiated RBC lysis, and heightened ROS. Ex vivo, *Trib3*-KO attenuated Ter119^{pos} erythroblast formation, while escalating EPO-induced Erk1/2 and Akt activation. In addition, gene profiling of EPCs served to provide initial insight into candidate mediators of EPO-induced *Trib3* expression. Studies overall implicate Trib3 as a novel regulator of stress erythropoiesis.

Materials and Methods

Mouse models

The *Trib3* locus in C57BL/6 embryonic stem cells was targeted by loxP flanking of exon 3. *Trib3*^{+/flox} mice were prepared via ES cell injections into diploid blastocysts, chimeric mouse generation, and breeding with C57BL/6NTacFlp mice. Crosses with Gt(Rosa)26Sor(cre/Esr1)Arte mice (Taconic) yielded *Trib3*^{-/-} mice (for genotyping details, see supplemental methods). To model accelerated erythropoiesis, *Trib3*^{-/-} and *Trib3*^{+/+} mice (age 2–10 months) were challenged with rhEPO (Epoetin-alfa, Amgen, 1800U/kg, d0, d1), or with 5-fluorouracil (300mg/kg) [6]. Blood cell counts and additional parameters were determined by ProCyte analysis (IDEXX). In EPO challenge experiments Ter119^{pos} erythroblasts were isolated by magnetic-activated cell sorting (Stemcell Technologies) for use in RT-PCR analyses. In assays of erythrocyte hemolysis, RBCs were prepared in 20mM glucose, dPBS. At 12h of incubation (37°C), hemoglobin release was assayed (A-540nm). All procedures were IACUC approved (Maine Medical Center).

Flow cytometry

EPCs (10⁶) were stained in 0.2mL of phosphate buffered saline containing 0.075% bovine serum albumin with FITC-anti-CD71 (0.5µg), PE-anti-Ter119 (1.2µg) and APC-anti-Kit (0.3µg) (BD Biosciences). ROS was assayed using DCFDA (Abcam). Analyses utilized

FACSARIA IIu (BD Biosciences) or MACSQuant (Miltenyi) cytometers, and FlowJo software (V10, Tree Star).

EPC formation ex vivo, RT-PCR and western blotting

Bone marrow derived EPC development ex vivo utilized an optimized SP34ex culture system [7, 8]. Developmentally staged EPCs were purified by fluorescence activated cell sorting (FACSARIA IIu). RNA isolation, RT and qPCR were performed as previously described [5, 8] using target specific primer pairs from SA-Bioscience. For in silico analyses of erythroid transcriptomes, please see Supplemental Methods. Western blotting procedures (lysates, SDS-PAGE, blotting, ECL) were performed as previously defined [9].

Statistical analysis

Unpaired T-tests with Welch's correction were performed using Prism-6 (GraphPad).

Transcriptome profiling

Please see Supplemental Methods.

Results

Trib3 expression is heightened during accelerated erythropoiesis

In hematopoietic contexts, *Trib3* was initially identified as an EPO response gene in murine EPCs ex vivo. To assess modulated *Trib3* expression in vivo, mice were dosed with EPO (or saline) and early stage Ter119^{pos} EPCs were isolated from bone marrow (FIG-S1). As analyzed by RT-PCR, EPO dosing increased *Trib3* levels 38.4-fold (FIG-1A). As positive controls, *Fam-132b* [4] and *Osm* [10] (known EPO response factors) were induced 10.4-fold and 3.8-fold, respectively (FIG-1A). By comparison, EPO only marginally affected *Trib1* levels, and did not modulate *Trib2* (or *Bcl-x*) expression. Database analyses further showed that during steady-state erythropoiesis, *Trib3* expression is nominal in definitive EPCs, but is activated during an accelerated period of primitive erythropoiesis (FIG-1B) [11]. Additional in silico analyses revealed elevated EPC expression of *Trib3* in the stress contexts of *Rb* disruption (GDS2757:161067_at) or zinc-finger protein *Zfp36l2* disruption (GDS3574:1456225_x_at), each of which act as proerythropoietic factors [12, 13].

Trib3 is required for efficient rebound erythropoiesis during 5-fluorouracil induced anemia

To assess *Trib3*'s functional roles, *Trib3*^{-/-} mice were generated via exon 3 deletion, and insertion of a frame-shift and premature stop codon in exon 4 (FIG-1C). *Trib3*^{-/-}, *Trib3*^{+/-} and *Trib3*^{+/+} alleles were identified by PCR using allele-specific primers. To confirm precise deletion *Trib3* transcripts were also analyzed, and as predicted, *Trib3* cDNA from *Trib3*^{-/-} EPCs was truncated by 300bp (cDNA sequencing further confirmed precise targeting, not shown). Consistent with limited *Trib3* expression in EPCs at steady state, *Trib3*^{-/-} mice exhibited normal blood cell parameters and body composition (Supplemental Table-1) with the exceptions of modestly increased RBC number, and decreased MCVs (FIG-1D).

To assess possible effects of *Trib3*-KO on accelerated erythropoiesis, induced 5-fluorouracil myeloablation was used. Subsequent to the onset of sharp anemia at d8, rebound

erythropoiesis in *Trib3*^{-/-} mice was compromised. Specifically, RBCs and hemoglobin in *Trib3*^{+/-} mice began to rebound at d12, but remained at a nadir in *Trib3*^{-/-} mice (FIG-1E). At d14, anemia in *Trib3*^{+/-} mice further resolved, but remained severe in *Trib3*^{-/-} mice. Recoveries of monocytes and lymphocytes, in contrast, were unaffected by *Trib3* disruption (FIG-S2), suggesting erythroid selective roles for *Trib3*. Despite the persistent anemia generated in *Trib3*-KO mice, no consistent effects on splenic hypertrophy were apparent (FIG-1F). Interestingly, reticulocyte levels in 5-FU *Trib3*^{-/-} mice were not substantially affected (FIG-1F), and this implicated possibly compromised erythrocyte integrity. RBCs from 5-fluorouracil treated *Trib3*^{-/-} mice proved to be prone to spontaneous lysis, and exhibited increased levels of peroxide induced ROS as compared to RBCs from 5-FU treated *Trib3*^{+/-} controls (FIG-1G). These findings suggest effects of induced *Trib3* expression on late stage EPCs (and possibly RBCs per se). In related studies, effects of *Trib3*-KO on RBC (and Hb) recovery from anemia due to limited dietary iron or blood loss also were assessed. Certain trends towards compromised recovery were observed, but did not achieve statistical significance (negative results, data not shown).

In *Trib3*^{-/-} EPCs, late-stage erythroblast formation is attenuated, and EPO-dependent Erk1/2 and Akt activation is heightened

To gain insight into implicated hematopoietic cell-intrinsic actions of *Trib3*, primary bone marrow EPC cultures were employed. In EPCs expanded ex vivo, *Trib3* expression increased during the course of erythroid development (FIG-2A, left panel). In silico analyses further demonstrated similar *Trib3* expression profiles during human EPC development ex vivo (FIG-2A, right panel).

In analyses of murine bone marrow EPC development, frequencies of CD71^{pos}Ter119^{pos} late erythroblast formation from *Trib3*^{-/-} progenitors proved to be attenuated (FIG-2B). However, this developmental shift occurred in the absence of significant effects on survival or proliferation (possibly due to effects of dexamethasone and/or serum-free medium components). To investigate candidate molecular mechanisms associated with altered *Trib3*^{-/-} EPC formation, possible effects on the central EPO/EPOR signaling modules MAPK, PI3K/Akt and Stat5 were assessed. In *Trib3*^{-/-} EPCs, EPO-dependent Erk1/2 activation was substantially heightened (FIG-2C). EPO-induced Akt activation also was moderately increased. Stat5 activation, in contrast, was unaltered. These results point to *Trib3* as a novel governor of EPO- dependent Erk1/2 and Akt activation.

Developmentally specific EPO induction of *Trib3* expression

To gain further insight into candidate mediators of EPO-induced *Trib3* expression, developmentally staged EPCs were isolated from ex vivo cultures as Kit^{high}CD71^{high}Ter119^{neg} CFUe-like “stage-E1” EPCs; Kit^{neg}CD71^{high}Ter119^{neg} “stage-E2” pro-erythroblasts; and Kit^{neg}CD71^{high}Ter119^{pos} “stage-E3” erythroblasts (see ref [8]). Following HGF withdrawal (6 hr) and +/-EPO challenge (90 min), transcriptomes were profiled with a focus on EPO target genes, and specifically those implicated in *Trib3* gene regulation (FIG-3A,B). At stage E1, EPO induced the expression of three defined inducers of *Trib3* as *Cebp-beta* [14], *Atf4* [15] and *Egr-1* [CHEA databases] [16]. *Nab1*, an *Egr-1* inhibitor [17], also was induced. At E2 and E3 stages, neither *Atf4* nor *Cebp-beta* were

EPO-induced, while *Nab1* induction persisted. Consistent with this pattern, high-level EPO-induction of *Trib3* occurred selectively at stage E1. These results are summarized in FIG-3A,B together with basic lists of top-25 EPO-modulated target genes at stages E1–E3 (Supplemental Table-2).

Discussion and Conclusions

Trib3 has been studied predominantly as a modulator of insulin sensitivity [18], and the *TRIB3* polymorphism Q84R is also a diabetic risk factor [19]. *Trib3-KO* mice, however, are unaltered in insulin or glucose homeostasis [20]. Observed EPO regulation of *Trib3* [5] therefore prompted studies in hematopoietic contexts. For *Trib3* expression, EPO per se is a strong inducer. This was observed especially in stage-E1 EPCs, and EPO-response gene profiling further implicated *Cebp-beta* [14], *Atf4* [15] and *Egr1* [16] as candidate mediators (and novel EPO targets). *Atf4* notably is also a signal transducer of iron overload in EPCs [21], and of ROS production [22]. In future studies, possible roles of *Trib3* expression (and action) during oxidative hemoglobinopathies such as thalassemia therefore may be of significant interest to define.

At steady-state, *Trib3-KO* modestly but selectively compromised RBC levels. Beyond this, during recovery from 5-fluorouracil induced anemia *Trib3-KO* substantially and selectively compromised rebound erythropoiesis. The latter could involve *Trib3* effects on various stages of stress erythropoiesis (and perhaps by-stander cells). Ex vivo experiments implicated *Trib3-KO* effects in part on proerythroblasts based on their attenuated development, and on ungoverning of *Erk1/2* and *Akt* activation (intrinsic to EPCs). Previously, dynamically regulated *Erk1/2* signaling has been shown to be important for balanced EPC formation in studies of truncated *EPOR* alleles [23], *PTPN11* [24], and the actions of *MASL-1* (malignant fibrous histiocytoma amplified sequences with leucine-rich tandem repeats-1) [25]. If reinforced, *Trib3*'s governing of *Erk1/2* activation may have therapeutic potential in the contexts of the ineffective erythropoiesis of β -thalassemia [26], *SCD* [27] and/or juvenile myelomonocytic leukemia [28]. In addition, the *Trib3* mutation Q84R recently has been associated with increased risk for chronic kidney disease [29]. As *CKD* progresses towards end stages, possible correlations of this Q84R mutation with anemia severity, and/or EPO-responsiveness should be of interest to assess.

Trib3, *Trib2* and *Trib1* can each also bind the ubiquitin ligase *COP1* [30]. *Trib1* and *Trib2* (but not *Trib3*) are myeloleukemogenic when overexpressed [31, 32] and act by promoting the ubiquitin-mediated degradation of the myeloid differentiation factor *C/EBP α* [32]. In adipocytes, however, *Trib3* can promote *COP1*-mediated ubiquitination and degradation of acetyl-coenzyme A carboxylase-1, and attenuate adipocyte differentiation [30, 33]. Whether *Trib3* might additionally act as an important E3 ubiquitin ligase adaptor within EPCs during accelerated erythropoiesis is therefore meaningful to consider, with such actions perhaps contributing to *Trib3*'s presently observed effects on erythrocyte integrity, and ROS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research Support: National Institutes of Health grant R01 DK089439 (DMW). Maine Medical Center Research Institute's NIH/NIGMS Center for Biomedical Research Excellence (COBRE) in Stem and Progenitor Cell Biology and Regenerative Medicine (NIH P30 GM106391) (DMW, PI).

The authors thank Dr. Chris Stoeckert for expert guidance with ErythronDB analyses. We also thank Dr. Narla Mohandas and Dr. Xiuli An (New York Blood Center) for generously providing human EPC RNAseq data. The authors also wish to most gratefully acknowledge strong additional contributions by Dr. David Kuhrt (EnviroLogix, Portland, ME) in several technical and conceptual aspects of this work. This study was supported by National Institutes of Health grant R01 DK089439 (DMW). Core facility support for flow cytometry and Procyte analyses (Progenitor Cell Analysis Core, Physiology Core) was provided by Maine Medical Center Research Institute's NIH/NIGMS Center for Biomedical Research Excellence (COBRE) in Stem and Progenitor Cell Biology and Regenerative Medicine (NIH P30 GM106391)(DMW, PI).

References

1. Paulson RF, Shi L, Wu DC. Stress erythropoiesis: new signals and new stress progenitor cells. *Current opinion in hematology*. 2011; 18:139–145. [PubMed: 21372709]
2. Becker V, Schilling M, Bachmann J, et al. Covering a broad dynamic range: information processing at the erythropoietin receptor. *Science*. 2010; 328:1404–1408. [PubMed: 20488988]
3. Dev A, Byrne SM, Verma R, Ashton-Rickardt PG, Wojchowski DM. Erythropoietin-directed erythropoiesis depends on serpin inhibition of erythroblast lysosomal cathepsins. *The Journal of experimental medicine*. 2013; 210:225–232. [PubMed: 23319700]
4. Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T. Identification of erythroferrone as an erythroid regulator of iron metabolism. *Nature genetics*. 2014; 46:678–684. [PubMed: 24880340]
5. Sathyanarayana P, Dev A, Fang J, et al. EPO receptor circuits for primary erythroblast survival. *Blood*. 2008; 111:5390–5399. [PubMed: 18349318]
6. Agosti V, Karur V, Sathyanarayana P, Besmer P, Wojchowski DM. A KIT juxtamembrane PY567 - directed pathway provides nonredundant signals for erythroid progenitor cell development and stress erythropoiesis. *Exp Hematol*. 2009; 37:159–171. [PubMed: 19100679]
7. Menon MP, Karur V, Bogacheva O, Bogachev O, Cuetara B, Wojchowski DM. Signals for stress erythropoiesis are integrated via an erythropoietin receptor-phosphotyrosine-343-Stat5 axis. *The Journal of clinical investigation*. 2006; 116:683–694. [PubMed: 16511603]
8. Sathyanarayana P, Dev A, Pradeep A, Ufkin M, Licht JD, Wojchowski DM. Spry1 as a novel regulator of erythropoiesis, EPO/EPOR target, and suppressor of JAK2. *Blood*. 2012; 119:5522–5531. [PubMed: 22508938]
9. Singh S, Verma R, Pradeep A, et al. Dynamic ligand modulation of EPO receptor pools, and dysregulation by polycythemia-associated EPOR alleles. *PloS one*. 2012; 7:e29064. [PubMed: 22253704]
10. Yoshimura A, Ichihara M, Kinjyo I, et al. Mouse oncostatin M: an immediate early gene induced by multiple cytokines through the JAK-STAT5 pathway. *The EMBO journal*. 1996; 15:1055–1063. [PubMed: 8605875]
11. Kingsley PD, Greenfest-Allen E, Frame JM, et al. Ontogeny of erythroid gene expression. *Blood*. 2013; 121:e5–e13. [PubMed: 23243273]
12. Spike BT, Dibling BC, Macleod KF. Hypoxic stress underlies defects in erythroblast islands in the Rb-null mouse. *Blood*. 2007; 110:2173–2181. [PubMed: 17557897]
13. Stumpo DJ, Broxmeyer HE, Ward T, et al. Targeted disruption of Zfp3612, encoding a CCCH tandem zinc finger RNA-binding protein, results in defective hematopoiesis. *Blood*. 2009; 114:2401–2410. [PubMed: 19633199]
14. Du K, Ding J. Insulin regulates TRB3 and other stress-responsive gene expression through induction of C/EBPbeta. *Molecular endocrinology*. 2009; 23:475–485. [PubMed: 19164449]
15. Liew CW, Bochenski J, Kawamori D, et al. The pseudokinase tribbles homolog 3 interacts with ATF4 to negatively regulate insulin exocytosis in human and mouse beta cells. *The Journal of clinical investigation*. 2010; 120:2876–2888. [PubMed: 20592469]

16. Rouillard AD, Gundersen GW, Fernandez NF, et al. The harmonizome: a collection of processed datasets gathered to serve and mine knowledge about genes and proteins. Database: the journal of biological databases and curation. 2016
17. Swirnoff AH, Apel ED, Svaren J, et al. Nab1, a corepressor of NGFI-A (Egr-1), contains an active transcriptional repression domain. Molecular and cellular biology. 1998; 18:512–524. [PubMed: 9418898]
18. Prudente S, Sesti G, Pandolfi A, Andreozzi F, Consoli A, Trischitta V. The mammalian tribbles homolog TRIB3, glucose homeostasis, and cardiovascular diseases. Endocrine reviews. 2012; 33:526–546. [PubMed: 22577090]
19. Prudente S, Trischitta V. The TRIB3 Q84R polymorphism, insulin resistance and related metabolic alterations. Biochemical Society transactions. 2015; 43:1108–1111. [PubMed: 26517932]
20. Okamoto H, Latres E, Liu R, et al. Genetic deletion of Trb3, the mammalian Drosophila tribbles homolog, displays normal hepatic insulin signaling and glucose homeostasis. Diabetes. 2007; 56:1350–1356. [PubMed: 17303803]
21. Suragani RN, Zachariah RS, Velazquez JG, et al. Heme-regulated eIF2alpha kinase activated Atf4 signaling pathway in oxidative stress and erythropoiesis. Blood. 2012; 119:5276–5284. [PubMed: 22498744]
22. Dey S, Sayers CM, Verginadis II, et al. ATF4-dependent induction of heme oxygenase 1 prevents anoikis and promotes metastasis. The Journal of clinical investigation. 2015; 125:2592–2608. [PubMed: 26011642]
23. Menon MP, Fang J, Wojchowski DM. Core erythropoietin receptor signals for late erythroblast development. Blood. 2006; 107:2662–2672. [PubMed: 16332976]
24. Usenko T, Chan G, Torlakovic E, Klingmuller U, Neel BG. Leukemogenic Ptpn11 allele causes defective erythropoiesis in mice. PLoS One. 2014; 9:e109682. [PubMed: 25289670]
25. Kumkhaek C, Aerbajinai W, Liu W, et al. MASL1 induces erythroid differentiation in human erythropoietin-dependent CD34+ cells through the Raf/MEK/ERK pathway. Blood. 2013; 121:3216–3227. [PubMed: 23327923]
26. Wannatung T, Lithanatudom P, Leecharoenkiat A, Svasti S, Fuchareon S, Smith DR. Increased erythropoiesis of beta-thalassaemia/Hb E proerythroblasts is mediated by high basal levels of ERK1/2 activation. Br J Haematol. 2009; 146:557–568. [PubMed: 19594742]
27. Zennadi R, Whalen EJ, Soderblom EJ, et al. Erythrocyte plasma membrane-bound ERK1/2 activation promotes ICAM-4-mediated sickle red cell adhesion to endothelium. Blood. 2012; 119:1217–1227. [PubMed: 22147898]
28. Lyubynska N, Gorman MF, Lauchle JO, et al. A MEK inhibitor abrogates myeloproliferative disease in Kras mutant mice. Science translational medicine. 2011; 3:76ra27.
29. De Cosmo S, Prudente S, Andreozzi F, et al. Glutamine to arginine substitution at amino acid 84 of mammalian tribbles homolog TRIB3 and CKD in whites with type 2 diabetes. Am J Kidney Dis. 2007; 50:688–689. [PubMed: 17900474]
30. Sakai S, Miyajima C, Uchida C, Itoh Y, Hayashi H, Inoue Y. Tribbles-Related Protein Family Members as Regulators or Substrates of the Ubiquitin-Proteasome System in Cancer Development. Current cancer drug targets. 2016; 16:147–156. [PubMed: 26560117]
31. Yokoyama T, Kanno Y, Yamazaki Y, Takahara T, Miyata S, Nakamura T. Trib1 links the MEK1/ERK pathway in myeloid leukemogenesis. Blood. 2010; 116:2768–2775. [PubMed: 20610816]
32. Dedhia PH, Keeshan K, Uljon S, et al. Differential ability of Tribbles family members to promote degradation of C/EBPalpha and induce acute myelogenous leukemia. Blood. 2010; 116:1321–1328. [PubMed: 20410507]
33. Qi L, Heredia JE, Altarejos JY, et al. TRB3 links the E3 ubiquitin ligase COP1 to lipid metabolism. Science. 2006; 312:1763–1766. [PubMed: 16794074]

- In erythroid progenitors *Trib3* is induced by EPO, and stress erythropoiesis
- LOF studies reveal specific roles for Trib3 during rebound erythropoiesis
- Trib3-KO leads to RBC instability, and elevated ROS
- Mechanistically, Trib3 attenuates EPO- dependent activation of Erk1/2 and Akt
- Trib3 exerts novel governing effects on EPO signaling and stress erythropoiesis

Figure 1. EPO induction of *Trib3* expression in bone marrow EPCs in vivo, and compromised rebound erythropoiesis due to *Trib3*-KO

(A) EPO upregulates *Trib3* expression in bone marrow erythroblasts in vivo: C57BL/6J mice were dosed with EPO (1800U/kg) or PBS. At 48h, bone marrow Ter119^{POS} erythroblasts were isolated by magnetic-activated cell sorting. Total RNA was isolated, and quantitative RT-PCR was used to determine expression levels of *Trib3*, *Osm*, *Bcl-x*, *Trib1* and *Trib2* (relative to β -Actin) (mean $\pm$ SE, n=4)(* = p < 0.05). (B) *Trib3* expression in EPCs is activated during primitive erythropoiesis, but repressed during steady-state definitive erythropoiesis: Analyses are in silico [11] for purified primitive and adult bone marrow

EPCs. Expression was determined as the average mRNA expression-level across cell-stage replicates for probe 1456225_x_at from Array Express E-MTAB-1035. Abbreviations are P, proerythroblasts; B, basophilic erythroblasts; O, orthochromatic erythroblasts; R, reticulocytes. **(C) Construction of *Trib3*^{-/-} mice:** The *Trib3* locus in C57BL/6 embryonic stem cells was disrupted using a targeting vector that introduced LoxP sites flanking exon 3. In *Trib3*^{+/-} and *Trib3*^{-/-} mice, Cre-mediated excision of exon 3 generated a 5' truncation of exon 4 via insertion of a translational frame-shift and stop-codon. PCR-based genotyping using primers specific for wild-type or disrupted *Trib3* alleles is illustrated for gDNA from *Trib3*^{-/-}, *Trib3*^{+/-} and *Trib3*^{+/+} mice. *Trib3* disruption was also confirmed via RT-PCR using RNA isolated from *Trib3*^{+/+} and *Trib3*^{-/-} bone marrow EPCs. **(D) Effects of *Trib3* deletion on peripheral red blood cells at steady state:** Among *Trib3*^{-/-} mice, significant increases were observed in RBC numbers (mean ± SE, n=10) (* = p < 0.05), in parallel with decreases in mean corpuscular volume (MCV). **(E) Following 5-fluorouracil challenge, *Trib3*^{-/-} mice exhibit compromised rebound erythropoiesis:** 5-fluorouracil was administered to *Trib3*^{-/-} and control *Trib3*^{+/+} mice. At the indicated time-points, RBC numbers and hemoglobin levels were determined (mean ± SE, n=7). At d12 and d14, note the deficit RBC and hemoglobin levels among *Trib3*^{-/-} mice. **(F) Spleen weights post 5-fluorouracil dosing at d12, and a time course for reticulocyte levels also were determined (mean ± SE, n=7).** **(G) Erythrocytes produced during 5-fluorouracil induced accelerated erythropoiesis are more susceptible to lysis, and exhibit heightened peroxide-induced ROS levels:** Left panel- Erythrocytes isolated at d16 following 5-fluorouracil challenge were washed and transferred to 20mM glucose in PBS. At 12h, lysis was determined based on hemoglobin release (A-540nm) (mean ± SE, n=4). Right panel- Erythrocytes were briefly exposed to peroxide (50μM H₂O₂, 20 minutes) and ROS levels were then determined by flow cytometry based on dichlorofluorescein diacetate (DCFDA) fluorescence. Mean fluorescence intensities ± SE (n=4) were analyzed by two-way ANOVA.

Figure 2. *Trib3* expression is activated during erythroid expansion ex vivo, and *Trib3* deletion attenuates bone marrow Ter119^{pos} erythroblast formation

(A) *Trib3* expression is activated in EPCs during mouse and human erythroid development ex vivo: Left panel- Using quantitative RT-PCR, *Trib3* (and *Alas2*) expression levels were analyzed in proerythroblasts (pro-E), basophilic erythroblasts (baso-E), polychromatic erythroblasts (poly-E) and orthochromatic erythroblasts (ortho-E) isolated by fluorescence activated cell sorting from SP34ex primary murine bone marrow EPC cultures (mean $\pm$ SE, n=3). Right panel- RNAseq analyses of FACS-isolated developmentally staged human EPCs formed ex vivo from CD34^{pos} cord blood progenitors (mean $\pm$ SE, n=3). EPCs analyzed

include pro-E, early baso-E, late baso-E, poly-E and ortho-E. **(B)** *Trib3* disruption attenuates *CD71^{pos}Ter119^{pos}* erythroblast formation: Hematopoietic progenitor cells from *Trib3^{-/-}* and control *Trib3^{+/+}* mouse bone marrow were cultured ex vivo (SP34ex system). At d4, *Ter119^{pos}CD71^{pos}* cell formation (** = $p < 0.005$) and apoptosis were analyzed via flow cytometry. Cell number was also assessed. **(C)** *Trib3* disruption increases EPO-dependent activation of *Erk1/2* and *Akt* within bone marrow erythroid progenitor cells: Bone marrow EPCs from *Trib3^{-/-}* and control *Trib3^{+/+}* mice were expanded ex vivo. At d3, EPCs were cultured for 6 hours in the absence of hematopoietic growth factors, and then challenged with EPO (3U/mL). At the indicated intervals, lysates were prepared and were assayed by western blotting for levels of activated (and total) *Erk1/2*, *Akt* and *Stat5* (left panels). In quantitative analyses of band intensities (right panels), values are normalized to total *Erk1/2*, total *Akt* or total *Stat5* and represent the integrated signals for p*Erk1/2*, p*Akt* and p*Stat5* activation (means $\pm$ SE) for two independent experiments (** = $p < 0.005$).

Figure 3. Working models for EPO induction of *Trib3*, and for modulation of erythroid cell formation during stress erythropoiesis
(A,B) *Candidate mediators of EPO-induced *Trib3* expression in developing EPCs:* To gain initial insight into mediators of EPO-induced *Trib3* expression, EPO response genes were profiled in developmentally staged E1, E2 and E3 EPCs (as propagated ex vivo), with a focus on EPO-regulated transcriptional regulation known to affect *Trib3*. (For top 25 EPO-modulated gene lists in stage E1–E3 EPCs, please see Supplemental Table-2). A: *Trib3*, and the positive regulators *Cebp-beta*, *Egr-1* and *Atf4* were defined as stage E1 specific novel EPO response genes. By comparison, Nab1, an inhibitor of Egr1, was induced by EPO in

stage E2 and E3 EPCs. **B:** Cis elements for these *Trib3* regulators within proximal *Trib3* gene regions are diagrammed, and their regulation by EPO in stage E1–E3 EPCs is summarized. **(C)** During anemia, as EPO levels increase *Trib3* transcript expression is induced. Trib3 then acts in EPCs to attenuate Erk1/2 and Akt signaling. Trib3 expression also leads to a governing of the potential for ROS formation in erythrocytes generated following damage to the erythron.