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To cite this version:
Zahra Kadri, Ritsuko Shimizu, Osamu Ohneda, Leila Maouche-Chretien, Sylvie Gisselbrecht, et al.. Direct binding of pRb/E2F-2 to GATA-1 regulates maturation and terminal cell division during erythropoiesis.. PLoS Biology, Public Library of Science, 2009, 7 (6), pp.e1000123. 10.1371/journal.pbio.1000123. inserm-00707647

HAL Id: inserm-00707647
https://www.hal.inserm.fr/inserm-00707647
Submitted on 13 Jun 2012

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Direct Binding of pRb/E2F-2 to GATA-1 Regulates Maturation and Terminal Cell Division during Erythropoiesis

Zahra Kadri¹,²,³, Ritsuko Shimizu⁴, Osamu Ohneda⁴, Leila Maouche-Chretien¹,²,³, Sylvie Gisselbrecht³, Masayuki Yamamoto⁴, Paul-Henri Romeo³, Philippe Leboulch¹,²,⁵, Stany Chretien¹,²,³,⁵

1 CEA, Institute of Emerging Diseases and Innovative Therapies, Fontenay-aux-Roses, France, 2 UMR INSERM U.962, University Paris XI, CEA, Fontenay-aux-Roses, France, 3 Département d’Hématologie, INSERM U567, CNRS UMR 8104, Institute Cochin and University Paris V René Descartes, Paris, France, 4 Department of Molecular and Developmental Biology, Center for TARA, ERATO Environmental Response Project, University of Tsukuba, Tsukuba, Japan, 5 Genetics Division, Department of Medicine, Brigham & Women’s Hospital and Harvard Medical School, Boston, Massachusetts, United States of America

Abstract

How cell proliferation subsides as cells terminally differentiate remains largely enigmatic, although this phenomenon is central to the existence of multicellular organisms. Here, we show that GATA-1, the master transcription factor of erythropoiesis, forms a tricomplex with the retinoblastoma protein (pRb) and E2F-2. This interaction requires a LXCXE motif that is evolutionarily conserved among GATA-1 orthologs yet absent from the other GATA family members. GATA-1/pRb/E2F-2 complex formation stalls cell proliferation and steers erythroid precursors towards terminal differentiation. This process can be disrupted in vitro by FOG-1, which displaces pRb/E2F-2 from GATA-1. A GATA-1 mutant unable to bind pRb fails to inhibit cell proliferation and results in mouse embryonic lethality by anemia. These findings clarify the previously suspected cell-autonomous role of pRb during erythropoiesis and may provide a unifying molecular mechanism for several mouse phenotypes and human diseases associated with GATA-1 mutations.

Introduction

With more than 100 billion red blood cells generated every day, the erythroid lineage has the largest quantitative output of cell production in adult mammals. This impressive capability requires a pattern of cell proliferation closely related to that of embryonic cells followed by ultimate inhibition of cell division, when terminal erythroid differentiation is completed. Yet, the putative molecular pathways that coordinate cell proliferation and erythroid differentiation remain obscure. The transcription factor GATA-1 is essential for erythroid differentiation as it transactivates all the known erythroid-specific genes upon binding to specific DNA motifs [1,2]. GATA-1 also exerts a repressive action on a subset of genes [3], and its overexpression inhibits cell proliferation [4,5]. The cofactor Friend-of-GATA-1 (FOG-1) binds to GATA-1 [6] and modulates its activity on given target genes, and mice deficient in either GATA-1 [7,8] or FOG-1 [9] die from severe anemia. Perturbation of the cell proliferation machinery also commonly results in lethal fetal anemia, as seen in mice defective in pRb [10–12], the three cyclins D together [13], more than one E2F members or Cdk4/6 [14,15]. With respect to the role of pRb in erythropoiesis during development, conflicting views persist as to its cell-autonomous (intrinsic) or nonautonomous (extrinsic) nature, the latter involving the accessory contribution of macrophages [16] or even the placenta [17] as the primary cause for embryonic lethality. Yet, other studies support the existence of a cell-autonomous component for pRb in erythropoiesis, although the underlying molecular pathways remain unknown [18–21]. Particularly puzzling is the phenotypic paradox of mutations of the GATA-1 gene that result in the synthesis of an N-terminally truncated GATA-1 protein (GATA-1s) [22]. In a family of patients with an inherited syndrome (trisomy 21) are prone to cellular selection of acquired somatic GATA-1 mutations that produce GATA-1s and result in preleukemic myeloproliferative disorders [24]. Here, we provide evidence of a direct physical interaction between pRb/E2F-2 and GATA-1 and document its physiological significance. We have also uncovered a potentially novel function for FOG-1 as a regulator of pRb for the control of cell proliferation. This direct interplay between GATA-1, FOG-1, pRb, and E2F sheds a new light on a constellation of mouse phenotypes and human syndromes that implicate mutations of the Rb or GATA-1 genes.
Author Summary

Red blood cell production, or erythropoiesis, proceeds by a tight coupling of proliferation and differentiation. The earliest erythroid progenitor identifiable possesses remnant stem cell characteristics as it both self-renews and differentiates. Each progenitor gives rise to more than 10,000 cells, including secondary progenitors. Yet, during the next stage of differentiation, much of this renewal capability is lost, and terminal erythroid differentiation progresses in a stepwise manner through several stages separated by a single mitosis. The transcription factor GATA-1 is essential for erythroid differentiation because it induces the expression of all the known erythroid-specific genes. Here, we show that GATA-1 directly interacts with proteins that are central to the process of cell division: the retinoblastoma protein pRb and the transcription factor E2F. Specifically, E2F becomes inactivate after engaging in a GATA-1/pRb/E2F tricomplex. Another erythroid transcription factor, termed FOGERG-1, is able to displace pRb/E2F from this complex in vitro upon binding to GATA-1. We hypothesize that the liberated pRb/E2F can then be the target of subsequent regulation to ultimately release free E2F, which triggers cell division. The physiological role of this new pathway is evidenced by transgenic mouse experiments with GATA-1 mutants unable to bind pRb/E2F, which result in embryonic lethality by anemia.

Results

Direct Interaction between GATA-1 and pRb Requires the Integrity of a LXCXE Motif

By examining the coding sequence of human GATA-1 (hGATA-1), we have identified an LNCME motif located at amino acid positions 81 to 83. This sequence exactly matches the consensus LXCXE motif present in many cellular or viral pRb-binding proteins [25,26]. Peptidic alignment of all known GATA-1 orthologs shows the presence of the LXCXE or its variant LXXXE in all species (Figure 1A). Serine is structurally similar to cysteine but contains a hydroxyl (-OH) group in place of the thiol (-SH) group. Neither of these two motifs (LXCXE and LXXXE) is present in any of the other members of the GATA family (i.e., GATA-2 to -6; unpublished data). To probe for the functionality of this putative Rb-binding domain in hGATA-1 in vivo, NIH-3T3 cells, which do not express GATA-1, were transduced with retroviral vectors that encode either mGATA-1 or hGATA1. Cross communoprecipitation (co-IP) analysis with GATA-1 and pRb (p110) specific antibodies (Abs) confirmed the hypothesis that pRb is associated with h/mGATA-1 in this reconstituted model (Figure S1A), thus suggesting that both LXCXE and LXXXE motifs are functional within the GATA-1 proteins for pRb binding. To demonstrate that pRb/hGATA-1 interaction is specifically dependent upon the LNCME motif, NIH-3T3 cells were transduced with retroviral vectors that express either (1) hGATA-1, (2) a naturally occurring N-terminally truncated form of hGATA-1, referred to as hGATA-1s ("s" for "short") [24], which is initiated at methionine 84 within the LNCME motif, or (3) hGATA-1 bearing two amino acid substitutions within the LNCME motif (LNCME to LNGMK; referred to as hGATA-1Rb) (Figure 1A). Cross co-IP with GATA-1– and pRb-specific Abs showed that only wild-type (wt) hGATA-1 interacts with pRb, whereas hGATA-1Rb and hGATA-1s do not (Figure 1B), thus establishing that the LNCME motif in hGATA-1 is required for direct association of pRb to GATA-1. In all the aforementioned co-IP experiments, potential immunoglobulin (Ig) artifacts were ruled out by analyzing IgG isotype controls for all the Abs used and for each GATA-1 variant expressed, under the same experimental conditions (Figure S1B).

Molecular Composition of the GATA-1/pRb Complex: Presence of E2F-2 and Absence of FOGERG-1

Because pRb activity is regulated by phosphorylation, we analyzed the pRb phosphorylation status within the GATA-1/pRb complex in erythroid (UT7) and nonerythroid (NIH3T3) cells. There are 16 possible CDK phosphorylation sites (Ser/Thr-Pro motifs) in pRb. By western blot analysis, pRb proteins segregate into two distinctly clustered groups of migrating bands: the more slowly migrating correspond to the so-called “hyperphosphorylated” forms, whereas the faster migrating are the “hypophosphorylated” forms, without defined species phosphorylated on specific residues among the 16 always present. Co-IP with an anti-GATA-1 Ab followed by western blot analysis with an anti-pRb Ab indicated that only the hypophosphorylated (“p”) forms of pRb were involved in the GATA-1/pRb complex, while one could detect both hypophosphorylated and (“pp”) hyperphosphorylated forms of pRb by IP of the original sample with an anti-pRb antibody (Figure 1B). Because available pRb antibodies are notoriously fastidious at differentiating hypophosphorylated pRb with optimal clarity, an additional experiment was performed by means of antibodies to phospho-pRb. Co-IP was first performed with specific phospho-pRb antibodies (PpeRb107, PpeRb907911, or PpeRb790) before resolution by western blot analysis reacted with a GATA-1 antibody. We found that GATA-1 was not precipitated under these conditions (Figure 1C).

Because hypophosphorylated pRb is known to bind members of the E2F family and actively suppress their transcriptional activity, whereas it has been recently reported that only E2F-2—among all the E2F members present in end-stage (CD71+, TER119+) fetal liver erythroid cells—directly interacts with pRb (p110) [20], we investigated whether E2F-2 is present within the GATA-1/pRb complex. To this end, we purified CD71+ erythroid cells (>90% TER119+) from embryonic day E12.5 mouse fetal liver. We first established that all Abs used were efficient and specific by immunoprecipitation (IP) and western blot analysis and that signals were not Ig artifacts (IgG isotype controls), especially for GATA-1 proteins that migrate at levels similar to heavy Ig chains (50 kDa) (Figure S2B). Results of co-IP experiments demonstrated that GATA-1, pRb, and E2F-2 do exist as a tricomplex in nuclear extracts from purified E12.5 mouse fetal liver erythroid cells (Figure S2C and S2D).

To further characterize the molecular composition of the GATA-1/pRb/E2F-2 tricomplex, we performed a co-IP assay with an Ab specific for E2F-2 followed by western blot analysis reacted with Abs specific for either pRb, GATA-1, or FOGERG-1, the known partner of GATA-1. Whereas GATA-1 and pRb proteins could be identified within the E2F-2 immunoprecipitate, FOGERG-1 was not found (Figure 1D). As we previously demonstrated that GATA-1 is directly associated with pRb and that GATA-1 is not known to bind directly to E2F-2, we can thus surmise that two pRb complexes can possibly coexist in nuclear extracts from fetal liver erythroid cells: pRb/E2F-2 and GATA-1/pRb/E2F-2. To probe whether the presence of E2F-2 is required for GATA-1/pRb complex formation, we first performed an immunodepletion of E2F-2 followed by western blot analysis of the unbound fraction with GATA-1 or pRb specific Abs (Figure S2E). Results indicate that both GATA-1 and pRb could be detected in the unbound fraction (Figure 1D and S2D). The E2F-2 immunodepleted fraction was then submitted to secondary co-IPs with Abs specific for either pRb, GATA-1, or FOGERG-1. Secondary IP precipitates
were resolved by western blot analysis with Abs specific for either E2F-2, pRb, GATA-1 or FOG-1. An association between pRb and GATA-1 could not be detected in the E2F-2 unbound fraction (Figure 1D).

From these series of experiments, we can conclude that (1) a GATA-1/pRb/E2F-2 tricomplex, in which pRb is hypophosphorylated, is present in late-stage erythroid cells from mouse fetal livers, (2) the GATA-1 LXCXE motif is required for direct association of pRb/E2F-2 to GATA-1, (3) GATA-1 and pRb do not form a bicomplex in the absence of E2F-2, (4) only E2F-2, but no other form of E2F present in these erythroid cells, allows measurable GATA-1/pRb/E2F tricomplex formation, and (5) FOG-1 is excluded from the GATA-1/pRb/E2F-2 tricomplex.

FOG-1 Interferes with the GATA-1/pRb/E2F-2 Association and Displaces pRb from GATA-1 by Direct Protein–Protein Interaction

Before addressing the physiological relevance of these findings, we first probed the apparent paradox that FOG-1 is known to be a key cofactor of GATA-1 in erythroid cells, whereas we have now established that FOG-1 is excluded from the GATA-1/pRb/E2F-2 tricomplex. Although the amino acid residues of GATA-1 known to
interact with FOG-1 lie within a zinc finger domain located outside the LNCME motif [27,28], we investigated whether FOG-1 could, nevertheless, regulate the formation of the tricomplex. To this end, we generated pure populations of NIH3T3 cells that express hGATA-1 after retroviral transfer, in which we subsequently transiently transfected various amounts of a plasmid that expresses human FOG-1 (hFOG-1). We chose NIH3T3 cells because they constitutively express E2F proteins, including E2F-2, although they do not naturally express GATA-1 or FOG-1 in the absence of transfection. Co-IP assays showed that hFOG-1 was able, in a dose-dependent manner, to prevent hGATA-1 from forming a complex with pRb (Figure 2A). To assess whether a direct protein–protein interaction between GATA-1 and FOG-1 is required to dissociate pRb from GATA-1, we expressed relevant mutants of hGATA-1 and hFOG-1 in NIH3T3 cells following an identical experimental approach. We made use of the hGATA-1V205G mutant, which comprises a point mutation that substitutes a glycine for a valine at codon 205. This mutation, naturally found in patients and previously studied in mice, is associated with severe dyserythropoietic anemia and thrombocytopenia [28,29]. It was previously established that this mutation causes the disruption of GATA-1 binding to FOG-1 and, as a consequence, a failure of expression of genes dependent on GATA-1 for their transactivation as well as the lack of repression of the c-Myc and GATA-2 genes [30]. We thus expressed hGATA-1, hGATA-1Rb−, or hGATA-1V205G, in NIH3T3 cells by retroviral transfer followed by transient transfection of a plasmid that expresses hFOG-1. Co-IP revealed that hFOG-1 was unable to dissociate hGATA-1V205G from pRb in contrast to hGATA-1 (Figure 2B). As a corroborating demonstration, we used a compensatory hFOG-1 mutant (hFOG-1S706R) that is able to bind to hGATA-1V205G and rescue the erythroid maturation of GATA-1−/− cells expressing hGATA-1V205G [30]. Co-IP assays showed that hFOG-1S706R was able to trigger a partial dissociation of hGATA-1V205G from pRb (Figure 2B). The residual association of PLoS Biology | www.plosbiology.org 4 June 2009 | Volume 7 | Issue 6 | e1000123

Figure 2. FOG-1 interferes with GATA-1/pRb association. (A) Co-IPs, by means of an anti(α)-GATA-1 Ab, of nuclear extracts from retrovirally transduced NIH-3T3 cells expressing hGATA-1 or hGATA-1Rb− and from the same cells after subsequent transient transfection with increasing amounts (1- to 5-fold, with 1-fold = 0.1 μg) of a hFOG-1 expression plasmid. We verified that >90% cells were successfully transduced by each of the retroviral vectors on the basis of coexpression of eGFP from an internal ribosome entry site (IRES). Immunoprecipitates were then resolved by western blot analysis with either anti(α)-GATA-1 or anti(α)-pRb antibodies, under identical experimental conditions as for Figure 1B and S1. The amount of hFOG-1 expressed after transient transfection was assessed in parallel on total nuclear extracts with an anti(α)-FOG-1 Ab (left column). (B) Nuclear extracts were made from retrovirally transduced NIH-3T3 cells expressing hGATA-1, or hGATA-1V205G, which cannot interact with hFOG-1, or 3 d after transient transfection with a plasmid expressing hFOG-1 or an identical amount of an expression plasmid encoding hFOG-1S706R, which can interact with hGATA-1V205G. We verified that >90% cells were successfully transduced by each of the retroviral vectors on the basis of coexpression of eGFP from an IRES. Co-IP assays were performed with these nuclear extracts by means of the anti(α)-GATA-1 and anti(α)-FOG-1 Abs, followed by western blot analysis with either anti(α)-GATA-1, anti(α)-FOG-1, or anti(α)-pRb Abs. A schematic representation of the various combinations of FOG and GATA variants expressed (lines 1 to 3) is indicated at the bottom of the figure. (C) A proposed protein equilibrium model. doi:10.1371/journal.pbio.1000123.g002
hGATA-1V205G to pRb in the presence of hFOG-1S706R is likely to result from the lower affinity of hFOG-1S706R for hGATA-1V205G compared to that of hFOG-1 for hGATA-1 (Figure 2B and [30]).

Altogether, we thus conclude that FOG-1 is capable of displacing pRb/E2F from GATA-1 in vitro by direct GATA-1/FOG-1 interaction. An equilibrium model between GATA-1, pRb, E2F-2, and FOG-1 is proposed (Figure 2C).

Cell Proliferation Is Controlled by Differential Association of GATA-1 to FOG-1 or pRb

As GATA-1 overexpression is known to inhibit cell growth [4,5], we then set out to study the putative role of the GATA-1/pRb/E2F complex on cell proliferation. Because erythroid cells naturally express both GATA-1 and FOI-G1, thus making it difficult to dissect the respective contribution of each of the factors and to decouple their effects on proliferation per se versus terminal erythroid cell differentiation, we first chose to establish a reconstituted cellular model after forced expression of wt and mutant GATA-1 proteins together with their corresponding FOG-1 interacting partners in the nonerythroid NIH3T3 cell line. We monitored, every day for 4 d, the growth of NIH-3T3 cells transduced with retroviral vectors that express either hGATA-1 or hGATA-1Rb”. Because hGATA-1 and hGATA-1Rb proteins were expressed at similar levels in transduced cells (Figure 1B) with undistinguishable transcriptional activity (Figure S3), this assay becomes relevant. Whereas hGATA-1 expression impaired NIH-3T3 cell proliferation, as previously reported [5], we found that hGATA-1Rb” had no effect on it (Figures 3A and S4). Because pRb/E2F has been shown to be necessary to the G1/S transition [25,26], we investigated whether the cellular distribution of phases of the cell cycle would be altered by the GATA-1/pRb/E2F association. NIH3T3 cells were transduced with retroviral vectors constitutively expressing either hGATA-1 or hGATA-1Rb”. Transduced NIH3T3 cells (eGFP-positive) were then starved in 1% serum (cell synchronization) for 72 h, and cell cycle progression was studied after stimulation in 10% serum at Day 2. As previously described [5], expression of GATA-1 blocked the cell cycle at the G1/S transition, whereas hGATA-1Rb” expression did not (Figure 3B).

To clarify the role of pRb in the observed GATA-1-dependent impairment of cell growth, we knocked down pRb expression in the transduced NIH-3T3 cells by transient transfection of a small interfering RNA (siRNA) directed against pRb. Consistent with our expectation, GATA-1-mediated inhibition of cell growth was abrogated for 2 d before cells ultimately stopped proliferation as the inhibitory effect of the siRNA vanished (Figure S5A). pRb knock-down by siRNA was concurrently assessed by western blot analysis, which indicated that complete inhibition of pRb expression occurred only during the first 2 d after siRNA transfection (Figure S5B) without interference with the expression of p107 (Figure S5C). When the same experiment was performed with the human osteogenic sarcoma SAOS-2 cell line, which does not express endogenous GATA-1 or pRb, neither retrovirologically expressed hGATA-1 nor hGATA-1Rb” had an effect on cell proliferation (Figure S5D). These results together establish that direct interaction of pRb to GATA-1 through its LXXCXE motif is central to the antiproliferative effect of GATA-1.

We then focused on the putative role of FOG-1 in this process. NIH-3T3 cells constitutively expressing hGATA-1 after retroviral transduction were transiently transfected with increasing amounts of a hFOG-1 expression plasmid and cell proliferation monitored for 5 d. hFOG-1 relieved the growth inhibitory effect of hGATA-1 for NIH-3T3 cells in a dose-dependent manner (Figure 3C). When the same experiment was performed with the non-FOG-1-interacting mutant hGATA-1V205G instead of hGATA-1, hFOG-1 was unable to counteract the inhibition of NIH-3T3 cell proliferation mediated by hGATA-1V205G, at the displacing dose of FOG-1 established for hGATA-1 (Figure 3D). When hFOG-1S706R was combined with hGATA-1V205G in a similar experiment, rescue of cell proliferation was observed albeit only partially (Figure 3D), consistent with the lower affinity of hFOG-1S706R for hGATA-1V205G compared to that of hFOG-1 for hGATA-1 (Figure 2B and [30]).

To establish the relevance of these findings for erythroid cells, we made use of the erythroid cell line G1E, which is blocked at the proerythroblast stage and derives from mouse embryonic stem (ES) cells with complete biallelic inactivation of the endogenous GATA-1 genes [GATA-1-/-] [31]. G1E and NIH3T3 cells were transduced with retroviral vector constitutively expressing either hGATA1, hGATA1-Rb”, hGATA1V205G, or hGATA1V205G-Rb”. The hGATA1V205G mutant and the double hGATA1V205G-Rb” mutant were included in this study in an effort to decouple effects on cell proliferation per se versus terminal erythroid cell differentiation, which is known to be dependent upon GATA-1/FOG-1 interaction and would here confuse data interpretation since G1E cells naturally express FOI-1. In agreement with the conclusion we reached in reconstituted NIH3T3 cells, the various GATA-1 forms behaved similarly in G1E and NIH3T3 cells (i.e., inhibition of cell proliferation with hGATA1 and hGATA1V205G as opposed to maintenance of cell proliferation with hGATA1-Rb” and hGATA1V205G-Rb”) (Figure 4A and 4B). However, the rate of cell proliferation was lower in the G1E cell population in the presence of the hGATA1-Rb” mutant as compared to G1E cells expressing the double hGATA1V205G-Rb” mutant and NIH3T3 cells expressing either of these single or double mutants, in agreement with findings discussed in the subsequent section that the hGATA1-Rb” mutant induces partial terminal erythroid differentiation of the G1E cell population through GATA-1/FOG-1 complex formation. Taken together, these findings establish a novel function for FOI-1 and point to the role of the GATA-1/FOG-1 association as a potential molecular rheostat to regulate the inhibition of cell proliferation induced by GATA-1. However, G1E cells transduced with a retroviral vector that expresses GATA-1s hyperproliferate (Figure 4C). This suggests that hGATA1-Rb”, and hGATA1s are not functionally equivalent.

Forced Expression of hGATA1-Rb” Fails to Induce Proper Terminal Erythroid Differentiation of GATA-1/-/- Cells

Since pRb has been implicated in the differentiation processes of several cell types [16,26], in addition to its role in cell cycle control, we then investigated whether the interaction between pRb and GATA-1 is involved in the differentiation of erythroid cells. To this end, we returned to the erythroid cell line G1E. Retrovirus-mediated expression of hGATA1 in G1E cells induced terminal erythroid differentiation of cells positive for coexpression of vector-encoded eGFP, as evidenced by May-Grünwald-Giemsa and benzidine staining for identification and scoring of the various red blood cell precursors (Figure 5A and 5B). In contrast, forced expression of hGATA1-Rb” dramatically altered the distribution of erythroid precursors towards the more immature elements, although the initiation of erythroid differentiation was not impaired (Figure 5A and 5B). The same results were obtained with another erythroid cell line, referred to as GAK14 (M. Yamamoto, unpublished data), which was independently developed from mouse GATA-1.05 ES cells (Figure S6A). To determine more precisely the cell composition of G1E-transduced (eGFP positive) cells, erythroidic maturation was assessed 6 d after transduction by flowcytometry for c-Kit, CD71, and TER119 expression.
Although the complete loss of c-Kit expression in both hGATA-1- and hGATA-1Rb–transduced G1E cells indicated that erythroid differentiation was initiated normally in either case, quantification of CD71hiTER119 (41% vs. 0%) and CD71hiTER119 (2% vs. 72%), respectively, showed that terminal erythroid differentiation was impaired in hGATA-1Rb–G1E cells (Figure 5C). hGATA-1Rb–G1E cells were able to progress to an intermediate (CD71hiTER119lo) stage like hGATA-1 G1E cells (25% vs. 16%, respectively, Figure 5C), but hGATA-1 G1E cells differentiated further. A similar conclusion was reached by morphological identification and scoring of the various red blood cell precursors of the retrovirally transduced GAK14 cell line (Figure S6B).

To study the potential effects of the interplay between GATA-1, pRb/E2F, and FOG-1 upon the differentiation of erythroid cells, we analyzed by flow cytometry the phenotypes of G1E cells expressing hGATA-1, hGATA-1V205G, or hGATA-1V205GRb after retroviral vector transduction (Figure 5C). Whereas hGATA-1–expressing cells became c-Kit+, CD71+, and TER119+, both hGATA-1V205G- and hGATA-1V205GRb–expressing cells failed to express TER119 and continued to express c-Kit. These data indicate that it is the GATA-1/FOG-1 association, but not the GATA-1/pRb/E2F complex, which is required for the down-expression of c-Kit during the early stage of proerythroblast differentiation.
Direct Interaction of GATA-1 with pRb Is Necessary for Terminal Erythropoiesis In Vivo

To assess the putative physiological effects of the protein–protein interaction between GATA-1 and pRb in a whole animal, we performed a transgenic complementation rescue assay [32]. Because the GATA-1 gene is located on the X chromosome, which is randomly inactivated in every cell, neither homozygous GATA-1+/− females nor hemizygous GATA-1−/− males are viable. We thus made use of mice that carry a mutant GATA-1 allele, referred to as GATA-1.05, which expresses only 5% of GATA-1 wt levels. As previously shown, male GATA-1.05/Y mice die in utero from impaired hematopoiesis, whereas heterozygous female mice (GATA-1.05/X) spontaneously recover shortly after birth from embryonic/fetal and neonatal anemia [32]. In preparation for crossing experiments with these female mice, we generated transgenic (Tg) lines of mice that express either hGATA-1Rb− or its wt counterpart hGATA-1 under the control of transcriptional regulatory sequences referred to as GATA-1 hematopoietic regulatory domain (HRD). HRD-driven transcription is known to recapitulate the endogenous GATA-1 gene expression pattern in both primitive and definitive erythroid lineages of Tg mice [1]. We focused on two of the hGATA-1Rb− expressing lines (Tg Lines 2 and 5), because expression of the hGATA-1Rb− transgene was at a level equivalent to that of the endogenous mouse GATA-1 gene (Figure 6A). Mouse Tg line 8 was also analyzed as an example of supraphysiologic expression of the hGATA-1Rb− transgene (level 280% that of the endogenous mouse GATA-1 gene). Males of these Tg mice were then mated with heterozygous GATA-1.05/X females, and their male progeny, referred to as 1.05/Y×hGATA-1Rb− and 1.05/Y×hGATA-1, studied. The expected and the observed pup numbers of XGATA-1.05/Y mice harboring the transgene are indicated in Figure 6B. The first observation we made is that the control hGATA-1 transgene could fully rescue GATA-1.05/Y male mice from lethality. Although some of the 1.05/Y×hGATA-1 male embryos showed a slightly anemic appearance at E13.5, most were indistinguishable from wt littermate embryos (Figure 6C). Importantly, their erythropoiesis caught up during late gestation, and viable pups were born with the expected Mendelian distribution (95% eGFP positive). To increase legibility, curves are numbered 1 to 6 as well as represented with different colors. (B) Same as in (A), but using the G1E cell line. Because of the lower retroviral transduction efficiency of G1E cells (~30%), we first sorted eGFP-positive cells for each of the retroviral vectors used on the basis of coexpression of eGFP from an IRES and then verified that the sorted population was >95% eGFP positive. To increase legibility, curves are numbered 1 to 6 as well as represented with different colors. (C) Same as in (B), but G1E cells were transduced with a retroviral vector (Migr) that expresses GATA-1s, which comprises a deletion of the first 83 amino acids.

Discussion

We have shown here that (1) direct protein–protein interaction between GATA-1 and pRb is required for normal terminal erythroid differentiation in vitro and in vivo, (2) in late-stage erythroid cells from mouse fetal liver, GATA-1 associates, through
Figure 5. GATA-1, FOG-1, and pRb/E2F-2 interplay regulates the differentiation of G1E cells. (A) GATA-1–deficient G1E cells were transduced with either an “empty” retroviral vector (Migr) or one that encodes hGATA-1 or hGATA-1Rb. Transduced G1E cells were first sorted for eGFP expression. We verified that the sorted population was >95% eGFP positive. Cell differentiation was examined on Day 4 posttransduction by May-Grünwald-Giemsa (MGG) (top) and benzidine (bottom) staining. Proerythroblast, intermediaries, mature erythroblast, and benzidine-positive cells are indicated as P, I, M, and B, respectively. (B) Graphic representation of benzidine-positive G1E cells after retroviral transduction (eGFP-sorted population). The percentage of benzidine-positive cells was monitored for 5 d. (C) Erythroid differentiation of G1E cells transduced with an empty
a LXCXE motif, with hypophosphorylated pRb engaged with E2F-2 to form a GATA-1/pRb/E2F-2 tri-complex, (3) GATA-1 and pRb do not form a bicomplex in the absence of E2F-2, (4) the GATA-1/pRb/E2F-2 tricomplex inhibits the proliferation of erythroid precursors (5) the association of GATA-1 to pRb/E2F alters G1-to-S phase progression and (6) FOG-1 is capable of displacing pRb/E2F-2 in vitro from the GATA-1/pRb/E2F-2 tricomplex. The physiological relevance of this latter observation needs to be further investigated. A mechanism by which GATA-1/pRb/E2F-2 tricomplex induces cell cycle arrest during erythroid differentiation is likely to be, at least in part, by sequestering E2F within a complex in which pRb is in its hypophosphorylated form. FOG-1 is able to displace GATA-1 away from the complex, and this may then allow

Figure 6. GATA-1/pRb interaction is necessary for terminal erythropoiesis in vivo. (A) Comparative levels of GATA-1 proteins in fetal livers of E12.5 wt or transgene-expressing mouse embryos. Top, fetal livers from one wt male (X/Y) and one wt female (X/X) E12.5 embryos as well as from two males GATA-1.05/Y (referred to as “1.05/Y” in the figure) transgenic for either hGATA-1 (referred to as “wt” in the figure) or hGATA-1Rb (referred to as “Rb” in the figure). Nuclear extracts were subjected to western blot analysis using an α-GATA-1 Ab and reprobed with an anti–α-actin (α-Actin) Ab (AC-15 clone; Sigma). Bottom, GATA-1 protein levels were quantified by chemiluminescence (Intelligent Dark box, LAS-3000, Fujifilm) and normalized for α-actin protein levels (n=3). Results are expressed as means±standard deviation (SD) of three independent experiments. (B) Crossbreeding of XGATA-1.05/X female mice with transgenic male mice expressing hGATA-1 or the mutant hGATA-1Rb proteins results in four possible genotypes with or without the transgene. Since the GATA-1 gene is located on the X chromosome, hemizygous XGATA-1.05/Y mice die in utero. The expected (if the transgene was resulting in complete rescue) and the observed pup number of XGATA-1.05/Y mice harboring the transgenes are indicated. (C) Top, fetal livers from E13.5 GATA-1.05/Y+hGATA-1wt control embryos (referred to as “1.05/Y+hGATA-1wt” in the figure) show an indistinguishable appearance from their wt littermates (Wt), and viable pups were born with the expected Mendelian distribution. Bottom, in contrast, GATA-1.05/Y+hGATA-1Rb− embryos in lane 5 (referred to as “1.05/Y+hGATA-1Rb−” in the figure) are pale comparatively to their wt littermates (Wt) and die in utero.
E2F-2 to be liberated upon subsequent phosphorylation of pRb. In parallel, the GATA-1/FOG-1 complex exerts its transcriptional effects as part of the program of erythroid differentiation, as exemplified by the extinction of c-Kit expression. Fine-tuning by exogenous inducers is likely to operate, as pRb and GATA-1 are regulated by phosphorylation [34]. This study does not yet address the possibility that the GATA-1/pRb/E2F-2 tricomplex possesses specific transcriptional activity in addition to the mere sequestration of E2F-2. For instance, GATA-1/pRb/E2F-2 may activate or inhibit E2F target genes transcription by (1) displacing other pRb-associated LXCXE proteins, (2) increasing gene transactivation through the GATA-1 transactivator domain, (3) acting as a protein platform for other associated GATA-1 proteins (e.g., acetyltransferase P300/CBP, PU.1, EKLF). Preliminary chromatin immunoprecipitation (ChIP) assays, using a GATA-1–specific Ab for ChIP, indicate that known E2F targets genes such as Cdc6 [35] can be detected (unpublished data). Transcriptional studies and cDNA microarrays analysis would sort through these hypotheses, as previously conducted for other Rb-associated proteins that include Id2 [36].
For the past several years, conflicting reports have sparked an active debate as to whether the fatal anemia observed in Rb<sup>−/−</sup> mice was of extrinsic (cell-nonautonomous) [16,17] and/or intrinsic (cell-autonomous) [18,19] origin. During the revision of this manuscript, two studies have added to our understanding of the role of pRb during erythropoiesis. The Walkley and Orkin laboratories have gathered convincing evidence for a cell-type–intrinsic and cell-autonomous role of pRb in erythropoiesis, using conditional Rb inactivation restricted to the erythroid lineage [21]. Another study from the Macleod laboratory has shed light on the interplay between pRb and E2F-2 during erythropoiesis by showing that the concurrent loss of E2F-2 and pRb is surprisingly capable of rescuing terminal erythroid maturation to Rb null red cells [20]. With the double mutants, erythroid precursors resume normal cycle cell arrest in S phase and concurrent terminal erythroid maturation, although the compensatory mechanisms remain unknown [20]. Our study brings a molecular basis for the existence of an intrinsic component by establishing a direct physical link between pRb/E2F-2 and the master transcription factor of erythroid differentiation GATA-1.

Our findings also provide an explanation for the lack of full rescue of GATA-1-deficient mice by the related factors GATA-2 and GATA-3 when expressed in lieu of GATA-1, since GATA-2 and GATA-3, which share extensive functional similarities with GATA-1 [37], do not contain an LXCXE motif. The variable degree of severity of the observed phenotypes between various studies is likely to reflect the levels of expression obtained for the GATA-2 and GATA-3 factors according to the transgenic approach used, ranging from embryonic lethality by lack of erythropoiesis to viability with anemia at the adult stage [32,37]. A similar observation has been reported with other GATA-1 family of transcription factors of erythroid differentiation GATA-1.

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In humans, a severe anemia has been recently described in a family of patients with an inherited splicing mutation of the GATA-1 gene that results in exon skipping and expression of an N-terminally deleted GATA-1 protein [GATA-1s] [23]. GATA-1s is translated from a downstream ATG initiation codon that encodes Met<sup>84</sup> in the full-length GATA-1. Here, we show that this mutation disrupts the association of GATA-1s to pRb because Met<sup>84</sup> is located within the LNCM<sup>84E</sup> motif, thus deleting it from the GATA-1s form. However, our data also suggest that GATA-1s and hGATA-1pRb<sup>−/−</sup> are not functionally equivalent and, thus, that it is likely that the N-terminal moiety of GATA-1 has an additional Rb-independent role. hGATA-1s may thus result in a phenotype less severe than hGATA-1pRb<sup>−/−</sup> possibly because a double loss of function may elicit a syndrome of lesser severity than a single loss, as observed with the double-null mice E2F-2<sup>−/−</sup> pRb<sup>−/−</sup> [20] or Id2<sup>−/−</sup> pRb<sup>−/−</sup> [16]. A caveat to the interpretation of these experiments is that the mutation in the LXCXE motif may concurrently disrupt some other critical yet unknown function of GATA-1. These observations may also help understand the specific pre-leukemic syndrome associated with acquired somatic GATA-1 mutations, which also result in GATA-1s expression, in patients with the Down syndrome [trisomy 21] [24]. The interplay uncovered here between factors that regulate cell cycle and transcription factors key to the differentiation of the red blood cell lineage should be considered for the interpretation of pRb, GATA-1, or FOG-1 mutant mice and corresponding mutations associated with specific human syndromes. These findings may also form the basis for the discovery of similar pathways in other tissues during both ontogenesis and homeostatic cell production throughout life.

Materials and Methods

Plasmid Construction and Retroviral Transduction

Human GATA-1-Rb<sup>−/−</sup> cDNA was generated by PCR and sequenced. This cDNA was then subcloned in the retroviral vector MIGR. Human FOG-1 cDNA was subcloned in pSV-DESTA3 (Invitrogen), GATA-1<sup>V205G</sup>, GATA-1<sup>V205G/Rb<sup>−/−</sup></sup>, and FOG-1<sup>5706R</sup> mutants were obtained using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) with respective, previously described, plasmids as templates. Construct sequences were confirmed by DNA sequencing. Retroviral production and cell transduction were performed as previously described [34]. Two days after transduction, GFP-positive cells were sorted by flow cytometry (Epic Astra; Beckman Coulter).

Cell Culture and Transfection

NIH-3T3 were maintained at low population doubling and density. NIH-3T3 and SAOS-2 cells were transfected using lipofectamine 2000 transfection reagent (Invitrogen). Upilbe reagent (Interchim) was used for cell proliferation assays with fluorometric excitation at 560 nm and reading at 590 nm (Typhoon 9400; Amersham Bioscience). G1E cells were grown as previously described [34]. Cytospin samples were stained with May-Grünwald-Giemsa to assess and score the various stages of erythroid differentiation. Hemoglobinization was evidenced by benzidine staining. Erythroid cells from mouse fetal livers were obtained from E12.5 C57BL/C embryos.

Protein Analysis

For nuclear extract preparation, cells were washed once with PBS and incubated for 10 min at 4°C in buffer A (10 mM HEPES [pH 7.6], 3 mM MgCl<sub>2</sub>, 10 mM KCl, 5% glycerol, 0.5% NP-40) containing phosphotyrosine phosphatase inhibitors (1 mM Na<sub>2</sub>VO<sub>4</sub>), phosphoserine/threonine phosphatase inhibitors (20 mM NaF, 1 mM sodium pyrophosphate, 25 mM β-glycerophosphate), and proteinase inhibitors. After centrifugation, nuclear pellets were resuspended in buffer A containing 300 mM KCl and incubated for 30 min at 4°C. After centrifugation, nuclear extracts were quantified by BCA staining (Pierce), half-diluted with buffer A and subjected to IP using the appropriate Abs: hGATA-1 C-terminal region epitope, M-20 (sc-1234; Santa Cruz Biotechnology), pRb (Cat. N° 9300; Cell Signaling Technology), pRb<sup>ser780</sup>, pRb<sup>ser870/811</sup>, and pRb<sup>ser795</sup> PhosphoPlus Rb antibody Kit Cat. N° 9300; Cell Signaling Technology).
Generation of Transgenic Mice

Wt and mutant human GATA-1 cDNAs were cloned 3′ to the mouse GATA-1 HRD [32]. DNA fragments were purified from vector sequences and transgenic mice generated by DNA microinjection into fertilized BDF1 eggs using standard procedures. The GATA-1.05 allele was monitored by PCR using primers corresponding to the neomycin-resistance gene in the original GATA-1.05 targeting vector.

PCNA Staining

Whole embryos were fixed in 4% formaldehyde solution at 4°C for 16 h followed by embedding in paraffin. PCNA staining was performed using a kit from Zymed Laboratories.

Flow Cytometry

Phycocerythrin-conjugated anti-mouse TER119 (TER119-PE; BD Pharmingen, cat N° 553673), APC-conjugated anti-mouse CD117 (c-Kit; BD Pharmingen, cat N° 553536), biotin-conjugated anti-mouse CD71 (BD Pharmingen, cat N° 557416) and streptavidin-PE-PC5 secondary antibodies (BD Pharmingen) were used for surface labeling of cells. Flow cytometry was performed using FACSCalibur, and data were analyzed with the Cell Quest Pro software.

Analysis of Cell Cycle Distribution

Cells were pelleted, fixed in 70% ethanol, and then resuspended at a concentration of 10⁶ cells per milliliter in PBS containing 5 mM EDTA and 5 μg/ml Hoechst 33342 (Molecular Probes–Invitrogen). The cells were then analyzed with a LSR II cytometer (BD Biosciences) equipped with both the DIVA and the Flowjo 8.8.3 software using the Dean-Jett-Fox algorithm.

Supporting Information

Figure S1 GATA-1 interaction with endogenous pRb and antibody controls. (A) Human and murine GATA-1 proteins interact with endogenous murine pRb. NIH-3T3 cells were transduced with the “empty” retroviral vector (Mig) or with retroviral vectors encoding either human (h) or murine (m) GATA-1. We verified that >90% cells were successfully transduced by each of the retroviral vectors on the basis of coexpression of eGFP from an internal ribosome entry site (IRES). IPs of GATA-1 (5 μg, α-GATA-1: M-20, sc-1234; Santa Cruz Biotechnology) or pRb (5 μg, α-pRb, n° 554136; BD Pharmingen) were performed with 50 μg of nuclear extract. IPs using 5 μg of nonimmune IgG isotype of the corresponding species (normal goat IgG sc-2028 for GATA-1 and normal mouse IgG sc-2025 for pRb; Santa Cruz Biotechnology) were used for each transfection as negative controls for the specificity of the precipitation and coprecipitation obtained with each immune antibody used in the IP and co-IP experiments. Nuclear extracts before IP (input 5%) and bound proteins were resolved by western blot analysis using the antibody against GATA-1 (α-GATA-1 M-20, sc-1234; Santa Cruz Biotechnology) or pRb (α-pRb, n° 554136; BD Pharmingen) as indicated. The horseradish peroxidase-conjugated secondary Ab used (AffiniPure goat anti-rat IgG light chain specific, 112-035-175; AffiniPure goat anti-mouse IgG light chain specific, 115-035-175) was provided from Jackson ImmunoResearch and did not recognize reduced denaturated IgG heavy chains that comigrate at 50 kDa near GATA-1. (http://www.jacksonimmuno.com/catalog/CatPages/rbic.asp). These secondary Abs were used in all the blotting assays of this paper. This experiment indicates that both human and murine GATA-1 interact with endogenous murine pRb with the same efficiency. (B) Control of the specificity of the Abs utilized. NIH-3T3 cells were transfected with the “empty” retroviral vector (Mig) or with retroviral vectors encoding for either IgGATA-1, hGATA-1-Rb−, or hGATA-1s. IP of GATA-1 (α-GATA-1 M-20, sc-1234; Santa Cruz Biotechnology) or pRb (α-pRb; BD Pharmingen) were performed on 500 μg of nuclear extract. Nonimmune IgG (mice for pRb and goat for GATA-1; Santa Cruz Biotechnology) were used as controls for each transfection (lanes 2, 7, 12, 17, and 4, 9, 14, 19, respectively). Nuclear extracts before IP (input 5%, lane 1, 6, 11, and 16) and bound proteins were resolved by western blot analysis using the Ab against GATA-1 (α-GATA-1 N-1 sc-266; Santa Cruz Biotechnology) or pRb (phospho-specific pRbs: pRbPser780, pRbPser870/811, and pRbPser795 PhosphoPlus Rb antibody Kit Cat. N° 9300; Cell Signaling Technology) as indicated. We thus conclude that only wt GATA-1 can interact with pRb as shown by the complex coprecipitated by GATA-1 antibody as well as the pRb antibody. This interaction is specific as no complex was found in the IgG control lane. Neither GATA-1-Rb− nor GATA-1s can interact with pRb (even with a large amount of protein), indicating that the LNCME motif is required for the interaction.

Found at: doi:10.1371/journal.pbio.1000067.s001 (2.13 MB TIF)

Figure S2 GATA-1 forms a complex with pRb and E2F-2 in primary erythroid cells. (A) Experimental procedures used for (B–D). Fetal livers were dissected from C57BL/6 embryos at embryonic day 12.5 (E12.5) and disaggregated in α-MEM supplemented with phosphotyrosine phosphatase inhibitors (1 mM Na2VO4; Sigma), phosphoserine/threonine phosphatase inhibitors (20 mM NaF, 1 mM sodium pyrophosphate, 25 mM β-glycerolphosphate; Sigma), and proteinase inhibitors (Roche) to a single-cell suspension by serial passage through a 23-ga needle, followed by a 27-ga needle. Viability of cells was assessed by Trypan blue staining (<0.5% of positive cells), and erythroid differentiation stages were checked by cell surface labeling using the following Abs: phycocerythrin-conjugated anti-mouse TER119 (TER119-PE; BD Pharmingen, cat N° 553673), APC-conjugated anti-mouse CD117 (c-Kit; BD Pharmingen, cat N° 553536), biotin-conjugated anti-mouse CD71 (BD Pharmingen, cat N° 557416), and streptavidin-PE-PC5 secondary antibodies (BD Pharmingen). Flow cytometry was performed on an aliquot of 10⁵ cells using FACScan, and data were analyzed with the DIVA software. Eighty-five percent to 90% of fetal liver cells were CD71+TER119+ (E12.5), showing a majority of cells at the late stage of erythroid differentiation. Nuclear extract were prepared as described in Materials and Methods except that the glycerol concentration was 10% instead of 5% to allow for an improved recovery of nuclear pellets. IPs were then performed on equal amounts of nuclear extract (500 μg per point) using specific Abs or nonimmune-related (control) Abs. Immunoprecipitated proteins (bound, lanes 2 and 4) were analyzed by western blot (WB) as well as a 5% fraction of nuclear extract before (input, lane 1) and after (supernatant, lane 3 and 5) each IP. Nucleophosmin (Nph), which does not bind to GATA-1 or pRb complexes, was used as a negative control of the specificity of coprecipitation (lane 6). (B) Efficiency and specificity of immunoprecipitating Abs. IPs were performed using nuclear lysates from E12.5 fetal livers using specific Abs against GATA-1 (sc-1233; Santa Cruz Biotechnology), pRb (554136; BD Pharmingen), E2F-2 (sc-633; Santa Cruz Biotechnology), FOG-1 (sc-9360; Santa Cruz Biotechnology), or with nonimmune corresponding (control) Abs, respectively, normal goat IgG (sc-2028), normal mouse IgG (sc-2025), and normal rabbit IgG (sc-2027; Santa Cruz Biotechnology). Proteins were analyzed by western blot using the same Abs (except for GATA-1, where the sc-266 from Santa Cruz Biotechnology was used). As
expected, all the proteins were totally immunodepleted from the nuclear extracts, and no signal was detected in the supernatants after each IP (lane 4 versus 5). This depletion thus demonstrates specific antigen–Ab interactions, since no protein is immunoprecipitated by the related control Ab (lane 2) or by a nonspecific Ab (Nph, lane 6). (C) Identification of GATA-1 complexes in fetal liver erythroid cells and relevant Ab controls in co-IP assays. Co-IPs were performed using nuclear lysates from E12.5 fetal livers, as described in (A). The first step of IP was performed with a specific Ab directed against GATA-1 (sc-1233; Santa Cruz Biotechnology), a nonimmune corresponding Ab (control Ab: normal goat IgG sc-2028), or nonspecific anti-Nph Ab. Coprecipitated proteins were then analyzed by western blot using the same Abs as in (B), referred to the left-hand side of the figure. None of the proteins studied is unspecifically coprecipitated with the nonimmune idiotype (lane 2) or a nonspecific antibody (lane 6) even in the presence of a large amount of proteins. As expected, FOG-1, a known partner of GATA-1, is coprecipitated and serves as a positive control (lane 4). Endogenous pRb and E2F2 are also copurified with GATA-1 in a specific manner (lanes 4b and 4c). Note that a small portion of each fraction remains unassociated with GATA-1, corresponding to free proteins or to distinct complexes that exclude GATA-1 (lane 5). (D) Identification of E2F2 complexes in fetal liver erythroid cells and relevant Ab controls in co-IP assays. Same as in (C), except that the first step of IP was performed with either a specific Ab directed against E2F2 (sc-633; Santa Cruz Biotechnology), a nonimmune corresponding Ab (control Ab: normal rabbit IgG sc-2027), or nonspecific anti-Nph Ab. As obtained in (C), pRb, the E2F-2 known partner, is copurified by E2F-2 immunodepletion (lane 4b), and we show that GATA-1 is also coprecipitated (lane 4a). These observations allow us to propose the existence of a ternary complex containing pRb, GATA-1, and E2F-2. This complex excludes FOG-1: no FOG-1 protein is observed in lane 4d, and the totality of FOG-1 is recovered in the supernatant (lane 5d versus lane 3d). As a faint amount of GATA-1 and pRb remain in the supernatant (lane 5a), this experiment cannot in and of itself exclude the possibility that GATA-1 and pRb can form a binary complex without E2F-2. The convincing evidence that GATA-1 and pRb do not form a bicomplex in the absence of E2F-2 is provided in Figure 1D. (E) Experimental procedure of Figure 1D.

Figure S3 hGATA-1 and hGATA-1Rb proteins have undistinguishable transcriptional activity. To assess the respective transcriptional activities of hGATA-1, hGATA-1Rb, and hGATA-1s, we performed transient transfection assays in NIH-3T3 cells using a luciferase reporter gene driven by several minimal erythroid-specific promoters that included (1) the erythroid porphobilinogen deaminase promoter (nucleotides −714 to +78 PBGD) [42], (2) the glycoporphin-B promoter (−95GpB) [43], (3) the erythropoietin receptor promoter (−58, +43 Epo-R) [44], (4) the 3XGATA-TK synthetic promoter [45], and (5) the GATA-1 proximal promoter (IE) together with its upstream enhancer element (IE3.9nt) [46]. These different plasmids were cotransfected with the “empty” Migr plasmid or with Migr-derived plasmids expressing hGATA-1, hGATA-1Rb, or hGATA-1s. Firefly luciferase activity was measured according to the manufacturer’s instructions (Dual Luciferase Reporter Assay System; Promega), and individual transfections were normalized by quantification of Renilla luciferase activity (pRL-TK; Promega). The total amount of DNA was kept constant at 700 ng in each transfection (500 ng of expression plasmid, 100 ng of reporter plasmid, and 100 ng of pRL-TK plasmid per well of a 24-well plate). Luciferase activity was determined 24 h after transfection. Data are expressed as relative luciferase activity (RLu). Results are the means±standard error of the mean (SEM) of three independent experiments. No difference in the transcriptional activity of hGATA-1 and hGATA-1Rb was detected with these promoters. With respect to hGATA-1s, the Epo-R minimal erythroid (pEpoR) and the GATA-1 (IE3.9nt) promoters were activated at a lower level (approximately 50%), whereas the erythroporphobilinogene deaminase promoter was not activated, and both the glycoporphin-B (−95GpB) and the synthetic 3XGATA-TK promoters were equally transactivated.

Found at: doi:10.1371/journal.pbio.1000067.s002 (3.13 MB TIF)
Figure S6 GATA-1/pRb interaction is necessary for GATA-1–mediated terminal erythroid differentiation of GAK14. (A) The erythroid cell line GAK14 cells, which is defective in GATA-1 as it derives from GATA-1−/− ES cells (M. Yamamoto, unpublished data) were grown on OP9 stromal cells in the presence of erythropoietin and stem cell factor. GAK14 cells were transduced with retroviral vectors expressing hGATA-1 or hGATA-1Rb. Differentiation of the cells was studied at Day 7 posttransduction by May-Grünwald-Giemsa staining. (B) Retrovirally transduced GAK14 cells were analyzed by microscopy and the distribution of cells at distinct stages of erythroid differentiation scored. More than 300 cells were examined for each sample. Expression of hGATA-1Rb did not impair the initiation of erythroid differentiation but dramatically alter the distribution of erythroid precursors towards the more immature elements. These results indicate that GATA-1/pRb interaction modulates the

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propagation of the various erythroid precursors. Baso, basophilic erythroblasts I and II; Ortho, orthochromatophilic erythroblasts; Poly, polychromatophilic erythroblasts; Pro, proerythroblasts.

Acknowledgments

We thank E. Kobayashi for assistance. We are grateful to T. Andrieu for his help in flow cytometry analysis.

Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: ZK RS OO SC. Performed the experiments: ZK RS OO SC. Analyzed the data: ZK LM-C SG MY P-HR PL SC. Wrote the paper: PL SC. Originator of the central hypothesis: SC.
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