ISG15 Modulates Development of the Erythroid Lineage

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Abstract

Activation of erythropoietin receptor allows erythroblasts to generate erythrocytes. In a search for genes that are up-regulated during this differentiation process, we have identified ISG15 as being induced during late erythroid differentiation. ISG15 belongs to the ubiquitin-like protein family and is covalently linked to target proteins by the enzymes of the ISGylation machinery. Using both in vivo and in vitro differentiating erythroblasts, we show that expression of ISG15 as well as the ISGylation process related enzymes Ube1L, UbcM8 and Herc6 are induced during erythroid differentiation. Loss of ISG15 in mice results in decreased number of BFU-E/CFU-E in bone marrow, concomitant with an increased number of these cells in the spleen of these animals. ISG15⁺ bone marrow and spleen-derived erythroblasts show a less differentiated phenotype both in vivo and in vitro, and over-expression of ISG15 in erythroblasts is found to facilitate erythroid differentiation. Furthermore, we have shown that important players of erythroid development, such as STAT5, Globin, PLC γ and ERK2 are ISGylated in erythroid cells. This establishes a new role for ISG15, besides its well-characterized anti-viral functions, during erythroid differentiation.

Introduction

Erythropoiesis is a tightly regulated process that allows the daily production of large numbers of circulating red blood cells. In adults, it mainly occurs in bone marrow but upon anemia induction, erythropoiesis can also shift to the spleen as “stress erythropoiesis” [1]. The earliest characterized erythroid progenitor is the BFU-E that can be identified by its ability to form large multifocal colonies in semi-solid medium. This progenitor then matures into more differentiated progenitors, CFU-E. From this stage, erythroblasts undergo further maturation that can be identified notably by morphological changes, sequentially giving rise to pro-erythroblasts, basophilic, polychromatic and orthochromatic erythroblasts that finally enucleate to generate reticulocytes. Several signaling pathways have been shown to regulate the balance between proliferation/differentiation and apoptosis of erythroid cells. In vivo and in vitro studies have highlighted the essential role of the erythropoietin receptor (EpoR) during terminal differentiation. In vivo, knockout of either the Epo or EpoR genes lead to embryonic death around E13.5, associated with anemia [2]. This phenotype is linked to the inability of committed CFU-E progenitors to terminally differentiate in erythrocytes. At the molecular level, EpoR is pre-associated with the protein tyrosine kinase JAK2, which becomes activated after Epo binding to its receptor. This results in the phosphorylation of tyrosine residues of the cytoplasmic tail of EpoR leading to the subsequent activation of intracellular signaling pathways including PI3K, ERK1/2 and STAT transcription factors [3]. Several STATs can be activated by EpoR which includes STAT1, STAT3 and STAT5 depending upon the experimental setting. Studies of genetically modified mice for either STAT1 or STAT5 have shown that these factors play an important role during erythropoiesis. Indeed, STAT5 a/b knockout mice suffer from anemia and die around birth [4,5,6]. Lack of STAT1 results in a general reduction of erythroid progenitors that are delayed in their terminal differentiation, accompanied by increased splenic stress erythropoiesis [7].

In a search for new candidates which could be of importance to regulate erythroid differentiation, we have identified ISG15 as an induced gene during this process. ISG15 is one of the earliest genes induced upon interferon (IFN) type I (α/β) stimulation [8]. Study of its promoter has allowed to characterize the ISRE (Interferon-Stimulated Response Element), on which several transcription factors can bind, in particular the ISGF3 complex composed by STAT1, STAT2 and IRF9 [9]. ISG15 belongs to the ubiquitin-like protein family that also includes SUMO, Nedd8 and Fat10. Akin to the ubiquitylation process, ISG15 can be covalently linked to lysine residues of target proteins following a cascade of enzymatic reactions that involves an E1 activating enzyme (Ubc1L), an E2 conjugating enzyme (UbcM8) and several E3 ligases (Herc6, EFP and HHARI) in a process named ISGylation. This process can be reversed and several proteases able to remove ISG15 have been identified, among them Usp18 [10]. Recently, ISGylation was shown to broadly target newly synthesized proteins...
in IFN-I-stimulated cells [11], yet for only a few number of proteins, the consequences of ISGylation have been elucidated [12,13]. ISG15 knockout mice are born viable and fertile without major developmental defect under steady state conditions [14]. However, and in line with the fact that ISG15 is an interferon-inducible gene, these mice show an increased susceptibility to a variety of viruses [10].

We report here that: (i) ISG15 expression and protein ISGylation are induced during erythroid differentiation; (ii) ISG15 induction is mostly independent of IFN signaling and partially dependent upon activation of EpoR signaling; (iii) ISG15+/− erythroblasts have an intrinsic differentiation defect in vivo; (iv) mice lacking ISG15 show impaired erythropoiesis in vivo; (v) important players of erythroid development, including STAT5, Globin, PLCγ and ERK2 are ISGylated in erythroid cells.

Results

ISG15 expression and protein ISGylation are induced during erythroid differentiation

To determine if ISG15 expression and protein ISGylation are induced during in vivo erythroid differentiation and if so, at which stage, mouse bone marrow (BM) cells were sorted according to cell surface expression of the transferrin receptor (CD71), glycoporphin A-associated protein (Ter119) and size (FSC-H) (Figure 1A). These markers are conventionally used to sort immature pro-erythroblasts (Pro-E) from their progeny, namely basophilic erythroblasts (Erya), polychromatic erythroblasts (EryB) and orthochromatic erythroblasts (Eryc)/reticulocytes (Eryc) [15]. RNA extracted from each sorted population was subjected to semi-quantitative RT-PCR analyses. ISG15 transcript was expressed at low levels in ProE, increased in EryA and EryB and reduced in EryC (Figure 1B), thus presenting a kinetic of expression similar to that of β-Globin, a gene strongly induced upon erythroid differentiation. Expression of the ISG15 conjugation enzyme Ube1L and the E3 ligase Herc6 was strongly induced in EryB, their expression profile resembling that of Bel-X, another gene known to be expressed late during erythroid differentiation [16–18]. Figure 1C shows that the expression of these genes is significantly up-regulated comparing ProE and EryB developmental stages, except for Ube1L. At the protein level, ISG15 and protein ISGylation were high in EryB and in EryC, as compared to ProE and EryA progenitors (Figure 1D and quantification of ISG15 expression in Figure 1E). ISG15 expression remained high in circulating red blood cells (RBC) (figure 1D). These results show that ISG15 protein expression and cellular protein ISGylation are up-regulated at the late stages of erythroid differentiation.

Because ISG15 and the genes encoding the enzymes involved in protein ISGylation were first characterized as IFN regulated genes [9], we next investigated whether IFN signaling was responsible for the induction of ISG15 expression and protein ISGylation during terminal erythroid differentiation. For this, we compared the expression of both ISG15 and of the components of the ISGylation machinery in differentiating wild-type and IFNAR−/− primary erythroblasts in which IFNα/β signaling is abrogated [19]. Primary cultures of proliferating erythroblasts can be expanded from bone marrow cells in the presence of Epo, Stem Cell Factor (SCF) and Dexamethasone (Dex) and can be induced to terminally differentiate within 3 days upon SCF and Dex removal and in the continuous presence of Epo [20]. Under these conditions, cells undergo 3 to 4 divisions accompanied with G1 phase shortening which ultimately results in cell size reduction, hemoglobin accumulation and finally enucleation. In the absence of Epo these erythroblasts rapidly die by apoptosis [20]. We found that ISG15, Ube1L, UbcM8 and Herc6 transcripts were up-regulated at the late stages of Epo-induced differentiation of primary wild-type erythroblasts (Figure 2A, WT panel and Figure 2B for quantification and statistical analyses). Their induction profile resembles that of Bel-X and β-Major-Globin (Figure 2A). At the protein level, up-regulation of ISG15 expression was accompanied by protein ISGylation, as evidenced by the accumulation of ISG15 adducts to high molecular weight proteins (Figure 2C, WT panel). Similar results were obtained using a p53−/− erythroid cell line [21] (data not shown and see thereafter). The induction kinetics of ISG15, Ube1L, UbcM8 and Herc6 was found unchanged in differentiating IFNAR−/− erythroblasts (Figure 2A) as compared to wild-type cells, although dampening of the overall expression level of these genes was observed (Figure 2A and 2B). In contrast, expression of Ifnγ, a bona fide IFNα/β responsive gene, was abrogated in IFNAR1−/− differentiating erythroblasts (Figure 2A). At the protein level, ISG15 and protein ISGylation were also found induced in differentiating IFNAR−/− erythroblasts, although at somewhat reduced levels as compared to wild-type cells (Figure 2C and 2D). Similar results were found using primary erythroblasts from IFNAR/IFNGR double-deficient mice (data not shown).

These data show that ISG15 upregulation and ensuing protein ISGylation are mostly independent of IFNα/β and IFNγ signaling in differentiating erythroblasts.

EpoR signaling is an absolute prerequisite for the differentiation of CFU-E cells [2]. We thus analyzed whether ISG15 expression could be regulated through this signaling pathway. Since erythroblasts rapidly die by apoptosis when deprived from Epo, we relied upon previous observations that enforced expression of anti-apoptotic members of the BCL-2 family can rescue this cell death phenotype and allows their terminal differentiation in absence of Epo [22,23]. In the absence of Epo, exogenously expressed hBCL-XL allowed survival and terminal differentiation of erythroblasts as analyzed by flow cytometry (Figure 2E), morphological analysis (Figure 2F) and western blot analysis of globin accumulation (Figure 2G). Under this condition, expression of ISG15 was slightly induced but was clearly weaker as compared to parallel culture of hBCL-xL-expressing erythroblasts differentiated in the presence of Epo (Figure 2G). This suggests that activation of the EpoR signaling pathway may participate to ISG15 induction. Furthermore, as shown in Figure 2H, the level of ISG15 was also found induced in proliferating, undifferentiated erythroblasts expressing a constitutively activated form of STAT5 (STAT5S710F), an essential effector of EpoR signaling in erythroid development [4–6]. Although acute Epo stimulation does not immediately induce ISG15 expression (our unpublished observations), these data suggest that ISG15 expression during erythroid terminal differentiation is at least partially dependent upon EpoR signaling.

ISG15−/− erythroblasts show an impaired ability to differentiate ex vivo

To address the importance of ISG15 expression for erythroid differentiation, we compared expansion and differentiation of erythroblast primary cultures derived from wild-type and ISG15−/− mice. We observed no major difference in expansion kinetics between WT and ISG15−/− erythroblasts cultures (Figure 3A, left panel). However, when these cultures were switched to differentiation conditions, cumulative cell numbers observed 2 and 3 days after differentiation induction were lower in ISG15−/− cultures as compared to wild-type erythroblasts (Figure 3A, right panel). This was accompanied by a lower level of hemoglobin accumulation in ISG15−/− erythroblasts, as analyzed by a colorimetric staining for Hb (Figure 3B) and by the reduced proportion of hemoglobin-
positive cells in cytocentrifugation analyses (Figure 3C and 3D). We also observed the subsistence of immature erythroblasts in ISG15−/− cultures 2 days after differentiation induction (Figure 3C and quantification in Figure 3D). These results thus show that ISG15 deficiency intrinsically interferes with terminal erythroid differentiation.

In the reverse experiment, the p53−/− erythroid cell line was transduced with a retroviral vector expressing Flag-tagged ISG15.
Figure 2. ISG15 expression during erythroid differentiation is independent of IFN signaling and partially dependent upon Epo signaling. (A) Semi-quantitative RT-PCR analyses of ISG15, Ube1L, UbcM8, Herc6, Irf7, Bcl-xL, and ß-Maj-Globin mRNA expression in WT or IFNAR−/− differentiating primary erythroblasts. Bone marrow erythroblasts of each genotype were maintained in proliferation conditions (SCF, Epo and Dex) for one week and next induced to differentiate in response to Epo alone for three days. Cells were collected every 24 hours as indicated and RNA extracted. The experiment was normalized to ß-Actin mRNA expression. (B) Statistical analysis of the induction of the expression of ß-Maj-Globin, Bcl-XL, ISG15, Ube1L, UbcM8 and Herc6 mRNA in differentiating WT and IFNAR−/− erythroblasts. Quantification was performed as described in Mat & Met. Note the unchanged expression of a second housekeeping gene HPRT. au = arbitrary unit. (C) Whole cell protein extracts were prepared from WT or IFNAR−/− erythroblasts maintained as in A and analyzed on a 10% acrylamide gel for ISG15 expression using an anti-ISG15 antibody. Anti-ß-Actin and anti-GAPDH were used as loading controls. (D) Statistical analysis of the induction of ISG15 during erythroid differentiation as normalized to ß-Actin. Quantification was performed as described in Mat & Met. au = arbitrary unit. (E and F) A p53−/− erythroid cell line expressing exogenous hBcl-XL was switched from proliferation conditions (Epo, SCF, Dex) to differentiating medium in the presence or absence of Epo. Cells were collected every 24 hours as indicated and analyzed for their ability to (e) survive as measured by propidium iodide staining in flow cytometry analyses; (f) differentiate as shown by analysis of their morphology after benzidine/May-Grunwald staining. Note the significant induction of cell death in control cells maintained in absence of Epo; in contrast, hBCL-xL erythroblasts are strongly protected from apoptosis. (G) Cells were lysed according to cell
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number and volume. ISG15 expression was analyzed on a 15% acrylamide gel using anti-ISG15 antibody, activation of the EpoR/STAT5 signaling pathway was monitored using anti-P-STAT5 antibody, differentiation was monitored using anti-Globin antibody and loading control was performed using anti-β-Actin antibody. (H) Mock and mscv-puro-STAT5G15F transduced p53-/− erythroid cell line maintained under proliferation conditions were lysed and analyzed for ISG15 expression using anti-ISG15 antibody on a 10% acrylamide gel (Top panel). P-STAT5 was detected at a higher level in mscv-puro-STAT5G15F transduced cells while only a modest increase in the total amount of STAT5 can be noted. Anti-β-Actin was used as a loading control.

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(Figure 4A). We observed that expression of ISG15 in p53-/− erythroid cell line was accompanied by an increase in protein ISGylation even under proliferative conditions (Figure 4A). When switched to differentiation conditions, ISG15 expressing erythroblasts showed an improved ability to differentiate as evidenced by a higher hemoglobin accumulation per live cell (Figure 4B), an increased proportion of hemoglobinized, benzidine-positive cells (Figure 4C), and a higher level of Ter119 expression as compared to controls (Figure 4D). Taken together, these data show that ISG15, the expression of which is increased during erythroid differentiation, facilitates the transition of differentiating cells through the late stages of erythroid maturation.

**ISG15G15F mice show an altered distribution of erythroid progenitors in bone marrow and spleen**

To investigate the importance of ISG15 in erythropoiesis in vivo, we compared erythroblasts maturation in bone marrow and spleen of wild-type and ISG15G15F mice. We observed a decreased proportion of EryC, accompanied by an increased proportion of EryB in ISG15G15F bone marrow cells as compared to wild-type mice (Table 1B, bone marrow). This reduction of EryC population in ISG15G15F erythroblasts did not result from impaired survival of these cells or their progenitors as no increase in annexin V-positive cells was observed in any of the erythroblasts subpopulations from ISG15G15F bone marrow cells. Rather, ISG15G15F erythroblasts showed a reduction in apoptosis as compared to matched wild-type cells (data not shown). These results rather favor the notion of a requirement for ISG15 for the transition of polychromatophilic erythroblasts to the orthochromatophilic/reticulocyte stage.

Although blood erythrocytes numbers and hematocrit level were similar in ISG15G15F and wild-type mice (Table 1A), a slight modification of erythrocytes properties (cell volume and width distribution, Table 1A) was observed. Moreover the reticulocyte numbers were doubled in ISG15G15F mice. This suggested that ISG15G15F mice may develop compensatory stress erythropoiesis. To address this question, we compared the number of BFU-E, and CFU-E from wild-type and ISG15G15F mice in both bone marrow and spleen. ISG15G15F bone marrow contained a reduced number of BFU-E and CFU-E as compared to wild-type bone marrow (Table 1C). This decrease in bone marrow BFU-E/CFU-E was associated with an increase in splenic BFU-E/CFU-E (Table 1C, spleen) and a slight but significant increase of ISG15G15F spleen weight as compared to wild-type mice (Table 1C). These results show that early bone marrow erythropoiesis is inhibited in ISG15G15F mice and is accompanied by a compensatory increase in splenic erythropoiesis. Of note, terminal erythroid differentiation was also altered in splenic erythroblasts. Indeed, a significant decrease in the EryC fraction was observed in ISG15G15F splenic cells as compared to wild-type, with a concomitant increase in the proportion of ProE, EryA and EryB erythroblasts (see Table 1B, Spleen). We next analyzed the response of ISG15G15F mice to stress-induced erythropoiesis. Upon phenylhydrazine-induced hemolytic anemia, the percentage of reticulocytes in peripheral blood was increased in ISG15G15F mice 3 days after the start of the treatment, but reached the same level as that observed in wild-type mice afterwards. However, this faster response did not lead to accelerated recovery from anemia in ISG15G15F mice (data not shown), a phenotype reminiscent to that described in STAT1-deficient mice [7]. Finally, as the level of ISG15 protein was high in erythrocytes (Figure 1B), the consequences of its absence on the lifespan of RBC was analyzed. No major difference could be noticed between wild-type and ISG15G15F RBC lifespan (data not shown).

**PLCγ, ERK2, Globin and STAT5 transcription factors are ISGylated in erythroid cells**

We next investigated the nature of cellular proteins ISGylated in erythroblasts. For this, we used the flag-ISG15-expressing p53-/− erythroid cell line. These cells show an expression level of ISG15 comparable to that obtained in response to IFNβ stimulation, a physiological inducer of ISG15 (Figure 5A and quantification in Figure 5B). Besides, this exogenous level of ISG15 was found only 2 fold higher than the level of ISG15 detected in terminally differentiating erythroblasts (Figure 5A and 5B, compare lane 2 to lane 8). ISGylated proteins were purified by a flag immunoprecipitation from either control or flag-ISG15-expressing erythroblasts (Figure 5C, left panel), Western blot analysis of these immunoprecipitates using antibodies specific to ERK2, PLCγ, STAT5 and Globin identified higher molecular weight adducts only in flag-ISG15 precipitates (Figure 5C, arrows). This shows that endogenous ERK2, PLCγ, STAT5 and Globin can be ISGylated in erythroblasts, the proportion of ISGylated protein never exceeding 4 to 5% of total protein for STAT5 for instance. While enforced ISG15 expression could lead to unspecific ISGylation of a broad spectrum of proteins, we found several other proteins not being ISGylated under the same experimental setting (data not shown). Furthermore, when p53-/− erythroid cell line engineered to express a STAT5-flag protein was analyzed for the status of STAT5 ISGylation using the same immunoprecipitation protocol as in Figure 5C, Western blots analyses of the same immunoprecipitates using antibodies specific to ERK2, PLCγ, STAT5 and Globin identified higher molecular weight adducts only in flag-ISG15 precipitates (Figure 5C, arrows). This shows that endogenous ERK2, PLCγ, STAT5 and Globin can be ISGylated in erythroblasts, the proportion of ISGylated protein never exceeding 4 to 5% of total protein for STAT5 for instance. While enforced ISG15 expression could lead to unspecific ISGylation of a broad spectrum of proteins, we found several other proteins not being ISGylated under the same experimental setting (data not shown).

Ubiquitination is increased in ISG15G15F RBC

A cross-talk between ISG15 and ubiquitination has been previously suggested [12,24,25]. We thus sought to investigate whether absence of ISG15 could alter the ubiquitination profile of erythroid cells. For this, RBC from wild-type and ISG15G15F mice were collected and cell lysates performed in the presence of NEM to prevent degradation of ubiquitinated proteins. ISGylation and ubiquitination were examined by Western blot analysis. High levels of ISG15 as well as ISGylated cellular proteins were observed in RBC but not in ISG15G15F RBC (Figure 5E, top panels). In ISG15G15F RBC, the anti-ubiquitin antibody recognized several additional bands that were weaker or absent in wild-type RBC lysates (Figure 5E, middle panels). These results suggest that a mechanism of ISG15 action in erythroid cells could be its interference with the ubiquitination pathway.
Discussion

The results presented in this study demonstrate that the expression of ISG15 is physiologically up-regulated during late stages of erythropoiesis, show a consistent role for ISG15 in vivo and in vitro erythroid differentiation and identify new targets of ISG15 in the erythroid lineage. ISG15 is one of the earliest genes shown to be induced upon type I IFN (α/β) stimulation [8]. In this study we show that ISG15 up-regulation is mostly independent of IFN signaling in the erythroid lineage. Indeed, in both IFNAR1−/− and IFNAR1−/−/IFNGR−/− double deficient erythroblasts, up-regulation of ISG15, Ube1L, UbcM8 and Herc6 transcripts is
maintained during erythroid differentiation, whereas the expression of Ifi27, a bona fide IFN-stimulated gene, is suppressed as expected. This suggests that in differentiating erythroblasts, IFN plays a minor role in the up-regulation of ISG15 and of the

| Table 1. Altered distribution of erythroid progenitors in ISG15−/− bone marrow and splenic cells. |
|----------------------------------|-----------------|--------|------|
|                                    | WT              | ISG15−/− | P-value | Sign    |
| **A. Blood parameters (N = 14)**  |                 |         |       |
| RBC count. M/mm3                  | 10.90 +/- 0.14  | 10.71 +/- 0.13 | 0.338 | ns      |
| HGB level. g/dL                   | 19.94 +/- 0.26  | 19.97 +/- 0.22 | 0.916 | ns      |
| MCH. pg                           | 18.29 +/- 0.13  | 18.66 +/- 0.15 | 0.073 | ns      |
| MCHC. g/dL                        | 39.29 +/- 0.26  | 39.24 +/- 0.21 | 0.889 | ns      |
| HCT. %                            | 50.74 +/- 0.62  | 50.90 +/- 0.58 | 0.849 | ns      |
| MCV. fL                           | 46.58 +/- 0.26  | 47.56 +/- 0.36 | 0.041 | *       |
| RDW. %                            | 16.41 +/- 0.007 | 16.12 +/- 0.008 | 0.014 | *       |
| Retic count. %                   | 2.75 +/- 0.20   | 5.03 +/- 0.49 | 0.0008 | ***     |
| PLT count. m/mm3                  | 972 +/- 61.92   | 830 +/- 88.43 | 0.199 | ns      |
| WBC count m/mm3                   | 12.08 +/- 0.84  | 13.59 +/- 1.03 | 0.265 | ns      |
| MONO. %                           | 2.346 +/- 0.06  | 2.615 +/- 0.13 | 0.074 | ns      |
| LYMP. %                           | 87.83 +/- 0.72  | 84.91 +/- 1.38 | 0.072 | ns      |
| GR. %                             | 9.864 +/- 0.68  | 12.37 +/- 1.26 | 0.091 | ns      |
| **B. Flow cytometry analysis (N = 13)**

| Bone marrow                        |                 |         |       |
| Ter119+                           | 47 +/- 1.1      | 46 +/- 1.1 | 0.6816 | ns      |
| ProE                              | 1.0 +/- 0.008   | 1.3 +/- 0.11 | 0.0475 | *       |
| EryA                              | 16.90 +/- 0.55  | 17.42 +/- 0.66 | 0.5554 | ns      |
| EryB                              | 21.67 +/- 0.62  | 26.89 +/- 1.87 | 0.0141 | *       |
| EryC                              | 57.50 +/- 0.71  | 51.58 +/- 1.94 | 0.0086 | **      |
| **Spleen                           |                 |         |       |
| Ter119+                           | 66 +/- 2.3      | 56 +/- 2.1  | 0.0045 | **      |
| ProE                              | 0.06 +/- 0.01   | 0.15 +/- 0.04 | 0.0427 | *       |
| EryA                              | 0.90 +/- 0.17   | 2.14 +/- 0.36 | 0.0087 | **      |
| EryB                              | 1.70 +/- 0.17   | 2.48 +/- 0.26 | 0.0277 | *       |
| EryC                              | 93.91 +/- 0.50  | 90.52 +/- 0.68 | 0.0008 | ***     |
| **C. Colony assays (N = 11)**     |                 |         |       |

| Bone marrow                        |                 |         |       |
| BFU-E                             | 57.20 +/- 5.28  | 38.70 +/- 5.25 | 0.023 | *       |
| CFU-E                             | 413.4 +/- 26.65 | 320.8 +/− 31.02 | 0.033 | *       |
| **Spleen                           |                 |         |       |
| BFU-E                             | 10.31 +/- 1.68  | 17.62 +/- 2.13 | 0.013 | *       |
| CFU-E                             | 22.54 +/- 2.71  | 61.23 +/- 5.90 | 0.0001 | ***     |
| Spl. Weight. g                    | 0.083 +/- 0.002 | 0.100 +/- 0.003 | 0.0006 | ***     |

(A) Blood was collected from mice at 8–10 weeks of age. Hematologic measurements were performed on a MS9 Hematology Analyzer (MELET SCHLOSSING Laboratoires). The data are mean +/- SEM (N = 14). RBC indicates red blood cells; HGB, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, MCH concentration (calculated); HCT, hematocrit; MCV, mean corpuscular volume; RDW, RBC distribution width, Retic, reticulocytes; PLT, platelets; WBC, white blood cells; MONO, monocytes, LYMP, lymphocytes and GR, granulocytes. (B) Quantitative analysis of the distribution of the different erythroblasts subsets in age-matched WT versus ISG15−/− mice. Flow cytometry analyses using the cell surface markers CD71 and Ter119 of bone marrow and spleen cells isolated from WT or ISG15−/− mice (as described in Figure 1A). Dead cells (7AAD−) were excluded from the analysis. The data are mean +/- SEM (n = 13). (C) 2.105 BM and 2.105 spleen cells from mice at 8–10 weeks of age were used to assay BFU-E and CFU-E numbers in MethoCult M3334 (StemCell Technologies). For BFU-E assays, colonies were counted at day 2 and for BFU-E assay, at day 4. The data are mean +/- SEM (n = 11). doi:10.1371/journal.pone.0026068.t001
ISGylation machinery enzymes and implies that other signaling pathways regulate the expression of these genes in erythroid cells. Using erythroid cell culture models, we show that activation of the EpoR signaling pathway participates to ISG15 and ISGylation up-regulation. Indeed, Bcl-XL expressing erythroblasts, that can differentiate either in the absence or presence of Epo [22], show a

Figure 5. Identification of several ISGylation substrates in erythroid cells and increased ubiquitination in ISG15−/− RBCs. (A) Proliferating erythroblasts were left either untreated (1), or stimulated with IFNß (100 U/ml) for 24 h (3), or induced to differentiate for 72 h (lane 4 to 8). Cells were lysed according to size and number and ISG15/ISGylation level was compared by western blotting to the level of ISG15/ISGylation present in proliferating Flag-ISG15-expressing p53−/− erythroid cell line. The arrow points to ISG15 band (B) Quantification of ISG15 level in independent western blot experiments. (C) Proliferating control and Flag-ISG15-expressing p53−/− erythroid cell line were lysed and ISGylated proteins searched after a Flag immunoprecipitation. Anti-Flag detect ISG15-Flag and ISGylated proteins (arrow heads) in the crude lysates (dashed line) and are found enriched after the immunoprecipitation (plain line bar). Stars point to light and heavy chains of immunoglobulin. For the detection of ISGylated Globins, cells were induced to differentiate for 72 hours in order to induce globin expression. ISGylated proteins are indicated by arrowheads. Normal unmodified molecular weight of the proteins are: ERK2 (42kDa), PLCc (150kDa), STAT5 (90kDa), Globin (13kDa). For ERK2 and STAT5 detection, cell lysates were run on a 10% acrylamide gel, for ISG15 and Globins detection on a 15% acrylamide gel and for PLCc on a 8% gel. (D) Proliferating or 48 hours-differentiating control and STAT5-Flag-expressing p53−/− cell line were lysed and analyzed for exogenous STAT5 expression either using a Flag (upper left panel) or a STAT5 antibody (upper right panel). Note the modest increase in the total amount of STAT5 induced by the expression of STAT5-Flag. ISGylated STAT5 was searched after a Flag immunoprecipitation followed by either a Flag (bottom left panel) or an ISG15 western blot analyses (bottom right panel). Extracts were run on a 7% acrylamide gel. (E) Western blot analysis of RBC extracts from WT and ISG15−/− mice using a 15% acrylamide gel. ISG15 expression and ISGylation were analysed using anti-ISG15 antibody (top panel), ubiquitination was monitored using anti-ubi antibody (intermediate panel) and anti-β-Actin was used as a loading control.

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ISGylation machinery enzymes and implies that other signaling pathways regulate the expression of these genes in erythroid cells. Using erythroid cell culture models, we show that activation of the EpoR signaling pathway participates to ISG15 and ISGylation up-regulation. Indeed, Bcl-XL expressing erythroblasts, that can differentiate either in the absence or presence of Epo [22], show a
stronger up-regulation of ISG15 and protein ISGylation when differentiated in the presence of Epo as compared to cells differentiated in the absence of Epo. Besides, expression of a constitutively active mutant of STAT5 in proliferating, undifferentiated erythroblasts was sufficient to induce ISG15 and ISGylation. It is interesting to note that, although ISG15 is not an immediate early gene induced in response to Epo (our unpublished observation), its expression closely follows that of BCL-2x, another indirect target of the EpoR/STAT5 axis [17,26].

Our studies of ISG15-deficient mouse show that ISG15 plays a facilitating role in erythroid differentiation. Indeed, ISG15+/c primary erythroblasts present an impaired ability to terminally differentiate in vitro as shown by the decreased accumulation of erythrocytes and the persistence of immature cells in ISG15+/c cultures at the late stages the differentiation process, as compared to wild-type cultures. Although we cannot exclude a role of ISG15 on the erythroid stromal microenvironment, these observations indicate that ISG15+/c erythroblasts have an intrinsic defect at the late stages of differentiation. In vivo, ISG15+/c mice show decreased number of bone-marrow-derived BFU-E and CFU-E with a concomitant increase in the number of these progenitors in the spleen. This phenotype is reminiscent of stress erythropoiesis, which could result from the impaired erythroid terminal differentiation observed in ISG15+/c mice. Indeed, a smaller proportion of orthochromatic erythroblasts/reticulocytes accompanied by an increased proportion of polychromatic erythroblasts was observed in ISG15+/c mice as compared to wild-type mice, a phenotype observed in the erythroid compartment of both the bone marrow and spleen of ISG15+/c animals. However, this altered erythroid differentiation is not due to increased apoptosis of ISG15+/c early erythroblasts, as observed in either STAT5- or STAT1-deficient mice [5,7,27]. Instead, a decreased level of apoptosis was observed in all ISG15+/c erythroblast populations (data not shown). This phenotype is reminiscent to that observed, for example, in Nix-deficient mice. Lack of Nix, a pro-apoptotic protein also induced at the last stages of erythroid differentiation [28,29], leads to a decrease in apoptosis and a defect in terminal erythroid differentiation [30]. As the importance of pro-apoptotic proteins in erythroid differentiation has been previously demonstrated [31,32], one can hypothesize that ISG15, which is normally induced by IFN (a known inducer of apoptosis) could participate as a pro-apoptotic protein important for erythroid differentiation.

At the molecular level, it has been shown that ISG15 can act at least by three distinct mechanisms: (i) ISG15 can play a role of a cytokine; (ii) it can modulate the activity of specific target proteins via ISGylation; (iii) it can modulate the activity of proteins via non-covalent interaction. ISG15 has been purified as a RBC-derived cytokine; (ii) it can modulate the activity of specific target proteins, independently of the ISGylation process. For example, ISG15 inhibits virus budding via its ability to interact with the E3 ubiquitin ligase Nedd4 and thus prevent the ubiquitination of viral proteins [12,25].

Additional experimental evidence suggests a cross-talk between ISG15 and the ubiquitination pathway. For instance, the level of polyubiquitinated proteins is increased in response to ISG15 knockdown in ZR-75-1 breast cancer cells [24], which could result from a competition between ISG15 and ubiquitin for common E2 enzymes, such as UbcH8 [38,39], and UbcH6 [40]. As (i) several essential proteins for the erythroid lineage have been shown to be regulated via their ubiquitination [41-43] and (ii) protein degradation via the proteasome is important for reticulocyte maturation [44], interference of ubiquitination by ISG15 could represent an alternative molecular mechanism to modulate erythroid differentiation. In line with this, we have noticed that ISG15+/c RBCs indeed show an increase in some ubiquitinated proteins, thus showing that ISG15 could modulate protein ubiquitination in erythroid cells. Taken together, our results suggest that ISG15 plays an important role in erythroid differentiation, but that the molecular cues underlying this phenotype may rely on ISGylation dependent and independent mechanisms.

Materials and Methods

Mice

ISG15+/c BL6 and their control littermates [14] and IFNAR-/-129Sv mice and their control littermates [19] were maintained in specific-pathogen-free conditions at the animal facility of the Curie Institute (Orsay, France) and Pasteur Institute (Paris, France) respectively. Genotyping of ISG15-/- mice was done by PCR analysis of tail DNA. The primers used were: WT forward 5’GCCCCCATCCAGAGCCGACGTGTT3 and WT/KO reverse 5’AGCCCCGATGGAGTGGTGTG3 and KO forward 5’CGGGAGAAGGCGCCAC AAAGAA3. All experimental procedures were performed in accordance with the recommendations of the European Community (86/609/EEC) and the French National Committee (87/848) for the care and use of laboratory animals. All animal experiments were carried out under the supervision of J.G., who was authorized by the director of the Veterinary Services of the Préfecture de l’Essonne (agreement number 91-7). Animal care and use for this study were specifically approved by the ethics committee of the Curie Institute in compliance with the institutional guidelines.

DNA plasmid constructs

Generation of pMSCV puro-flag-ISG15 was performed by cloning the amplified flag-ISG15 fragment using as template the pFlag-CMV-ISG17 plasmid and the primers: (Forward: 5’CCA GATCTGGCCACCATGAGTACCAAAAGCAGTGACGG3’; Reverse: 5’GCTTGAATTCCTAGGCAATGGTGTTCCCCTGC3’). The PCR product was cloned into the EcoRI/BglII-digested pCR2.1 Topo plasmid (TOPO TA Cloning kit, Invitrogen) and sequenced. The EcoRI/BglII fragment was then cloned into the BglII/EcoRI-digested pMSCV-puro vector.
Cell culture and retroviral transduction

Plate Ectopic packaging cell line [45] were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin and transduced using CaPO4 co-precipitation method with 40 µg/ml of pMSCV-puro-based retroviral constructs (Clonetech). Twenty-four hours after transfection, medium was changed and 24 hours later medium was collected as retroviral stocks. Primary erythroid progenitors and p35−/− immortalized erythroid cell line (derived from fetal liver cells of p35−/− mice) were cultured in serum-free medium (StemPro 34 plus nutrient supplement, Invitrogen) supplemented with 1 U/ml of human recombinant Erythropoietin (Jansen Gilg), 100 ng/ml of murine recombinant SCF (Peprotech) and 1 µM of Dexamethasone (Sigma). For differentiation induction, erythroblasts were washed twice with PBS and cultured in StemPro medium supplemented with 2 U/ml Epo and 0.5 mg/ml human holo-transferrin (Sigma). Cell numbers and size/volume were determined every day using an electronic cell counter (CASY-1, Scharfe-System) and cell density was maintained at 2–4 x 106 cells/ml. Differentiated cells were analysed for hemoglobin content, cell size/volume and morphology as previously described [46].

For quantification of maturing cells, fields of cytospin preparation of three independent cultures were counted (>200 cells). Large, blue and Benzidine-negative cells were counted as erythroblasts, lightly benzidine stained cells as differentiating cells (Benz and Benzidine-negative cells were counted as erythroblasts, lightly

Flow cytometry

Cultured erythroblasts or single cell suspensions of freshly isolated spleen or bone marrow were obtained from 8 to 12 weeks old mice and were stained with fluorocrome-conjugated monoclonal antibodies, all from BD Biosciences. 2.106 cells were washed with PBS, 3% of FBS, 10 mM of sodium azide and incubated for 30 minutes at 4°C with phycoerythrin (PE)- Ter119 antibody and either fluoroscine (FITC)– anti CD71 or biotin-anti CD71. After washing, the cells were incubated with (APC)- streptavidin, 7AAD and FITC-AnnexinV when indicated. Annexin-V staining was performed using FITC-AnnexinV Apoptosis Detection Kit (BD Biosciences), according to the manufacturer’s recommendations.

Cells were analysed on a FACSCalibur cytometer (BD Biosciences) or sorted using a FACS-Aria II cytometer (BD Biosciences). For sorting, DAPI was used instead of 7AAD to exclude dead cells. Data were analysed with FlowJo (Tree Star).

Immunoprecipitation and western blot

Erythroblasts were washed twice in ice-cold PBS and lysed according to cell number and volume (1.106 cells/ml) in RIPA buffer containing 1% aprotinin; 100 µg/ml phenylmethylsulfonyl fluoride; 10 µg/ml leupeptin, 50 mM NaF, 10 mM Na3Pj and 10 mM N-ethylmaleimide (NEM). The lysates were cleared by centrifugation at 16,000 x g for 20 min, at 4°C. Immunoprecipitation were carried out using 20 µl of anti FLAG M2-agarose beads/500 µl cell lysates (Sigma-Aldrich), for 1 to 2 hours on a rotating platform. After 3 washes with RIPA, immunoprecipitates were eluted with Laemmli sample buffer, samples boiled and analyzed by western blotting. Proteins were separated by SDS-PAGE and processed for western-blot analysis using the indicated rabbit polyclonal antibodies: anti-STAT5 C17 (Santa Cruz), anti-ISG15 (a generous gift from D.J. Lenschow, Washington University School of Medicine, St Louis, Missouri 63110, USA), anti-ERK2 (sc-154 Santa Cruz), anti-STAT1 (Cell signaling, 9172), anti- PLCγ (Cell signaling 2822), anti-Globin N19 (Santa Cruz), and monoclonals anti-PY694/699-STAT5 (Upstate Biotechnology), anti-Bcl-XL (Transduction Laboratories), anti-Flag (F3165 Sigma), anti-actin (AC15, Sigma), anti-GAPDH (mAB374, Millipore). For western blot quantification, films were scanned, bands quantified using ImageJ software and raw data normalized to β-actin signals. Statistical analysis was performed using at least three independent experiments with GraphPrism as indicated below.

Statistical analyses

Statistical analyses were performed using GraphPad Prism. The student t test was used to calculate P values (two tailed). P values of 0.05 or less were considered to be one asterisk, P values of 0.01 or less by two asterisks and P values of 0.001 or less by three asterisks. Data were presented as mean values plus or minus SEM.

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Author Contributions

Conceived and designed the experiments: ALM JG CTQ. Performed the experiments: ALM MP HA XC CTQ. Analyzed the data: ALM JG CTQ. Wrote the paper: ALM JG CTQ.
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