Supplemental Materials and Methods

Generation of p53-deficient, Btk-deficient Cell Lines from BM. Btk-deficient mice (1) and p53+/- mice (2), both on a C57/B6 background, were bred to obtain p53+-/-, Btk-/- animals, which were genotyped by PCR (3). BM was isolated from 6-wk-old animals. All clones were strictly dependent on Epo, SCF, and Dex to maintain renewal divisions. When cells were seeded in limiting dilution, and several clones were isolated and examined for their differentiation potential and factor dependence. All clones were expanded comparable to expansion of human erythroid progenitors from BM (4). Progenitors could be expanded for ~14 d before the cells in the p53 wt culture started to differentiate or die.

In the p53-deficient cultures, a part of the progenitors continued to divide as blasts, similar to what was observed previously for erythroid progenitors expanded from p53-deficient mouse fetal liver (5). The expanding erythroid progenitors were seeded in erythroid differentiation medium (StemPro supplemented with 5 U/ml Epo and 1 mg/ml transferrin), all clones differentiated into hemoglobinized, enucleated erythrocytes within 72 h as indicated by cell morphology and hemoglobin measurement. The p53-deficient, Btk wt clones 2B4 and 2C6 and the p53-deficient, Btk-/- clones 3G4 and 3E8 were used for all experiments described.

Erythropoiesis in Btk-deficient Mice Is Not Compromised. Btk-/- and wt mice had similar numbers of circulating erythrocytes (8.6 ± 0.6 × 10^7/µl and 8.2 ± 0.8 × 10^7/µl) and reticulocytes (3.0 ± 0.3% and 3.1 ± 1.0%, n = 4). Serum levels of Epo were below the detection threshold of 20 U/l, corresponding to normal physiological levels as can be expected when circulating red cells are normal. The number of erythroid progenitors (BFU-E and CFU-E) was determined in E12.5 fetal livers. We did not observe consistent differences between BFU-E and CFU-E in three subsequent experiments, using three Btk-deficient and three wt animals in each experiment (Table S1).

Btk-deficient Cells Contribute to Stress Erythropoiesis. To investigate the contribution of Btk-deficient cells to stress erythropoiesis, four Btk+/- males, four 4 Btk-/- males, and six Btk+/- female mice (11–13 wk of age) were injected with 0.4% phenylhydrazine (PHZ; 0.16 ml/20 g). PHZ was injected three times with 12 h intervals, and 72 h after the last injection, the animals were killed. Spleens and BM were harvested, and total cell numbers were determined. As expected, PHZ treatment led to similarly enlarged spleens in all respective mice (unpublished data). The absolute numbers of TER119 positive cells in both BM and spleen from Btk+/- and Btk-/- males were similar (Fig. S1 A). Because in the Btk-deficient mice, the Btk gene was inactivated by a targeted in-frame insertion of a LacZ reporter (1), Btk expression can be monitored using the LacZ gene activity under the control of the endogenous Btk promoter plus regulatory elements. Btk is located on the X chromosome. Therefore, in Btk+/- females, X inactivation either disables the LacZ-targeted allele or the wt Btk allele, which allows in vivo competition experiments between cells expressing or lacking Btk within a single animal.

We determined the number of LacZ+ cells in the TER119+ erythroid compartment in the BM and the anemic spleens by loading cells with fluorescein-di-γ-galactopyranoside substrate (Molecular Probes) as described previously (1). As controls, we analyzed the myeloid (ER-MP20 [Ly-6C]mid) compartment in the BM, in which there is no known selective disadvantage for Btk-/- cells, and the B220+ B cell compartment in the spleen, in which Btk-/- cells are expected to be essentially absent (1). We found that in Btk-/- males, ~90% of myeloid cells in the BM and B cells in the spleen were LacZ+. In Btk+/- mice, the proportions of LacZ+ cells in the myeloid cells were half of those of Btk-/- males (46–49%), and LacZ+/Btk-/- cells did not contribute significantly to the B cell population in the spleen (Fig. S1 B), consistent with our previous findings (1).

Table S1. CFU-E and BFU-E Colonies from Fetal Liver

<table>
<thead>
<tr>
<th></th>
<th>CFU-E</th>
<th>BFU-E (100 ng/ml SCF)</th>
<th>BFU-E (25 ng/ml SCF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Btk-/-</td>
<td>WT</td>
</tr>
<tr>
<td>exp1</td>
<td>701 ± 32</td>
<td>1,848 ± 170</td>
<td>761 ± 32</td>
</tr>
<tr>
<td>exp2</td>
<td>509 ± 86</td>
<td>580 ± 47</td>
<td>386 ± 107</td>
</tr>
<tr>
<td>exp3</td>
<td>521 ± 67</td>
<td>574 ± 48</td>
<td>534 ± 16</td>
</tr>
</tbody>
</table>

Colony numbers are per 10^5 mononuclear cells. All values shown represent mean values and standard deviation from three individual animals.

*The numbers above the graph indicate the ratio of LacZ+ cells in the Btk+/- cells in the Btk+/- mice compared to the Btk-/-/Y mice.
Within the total erythroid population in BM and spleen, the proportions of LacZ+ cells in Btk+/− females were close to half (~46%) of those found in the Btk− males, indicating that Btk is not critical for expansion or differentiation in the erythroid lineage. To quantify the contribution of Btk− cells to subsequent stages of erythropoiesis in detail, we determined the percentage of LacZ+ cells within the TER119 positive population in four size classes, corresponding to increasing maturation of erythroid cells. In Btk− males, the percentage of LacZ+ cells in the TER119+ population decreased with increasing maturity, reflecting an apparent decrease in expression levels of Btk during erythroid maturation. Strikingly, the number of LacZ+ cells decreased significantly in Btk+/− females (from 46 to 24%, Fig. S1 C), indicating that Btk− cells are impaired in expansion or survival when competing with Btk+/− cells during terminal differentiation in vivo. The same phenomenon has been observed in BM from PHZ-treated and untreated mice (unpublished data).

References