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# Expansion of Bcr-Abl-Positive Leukemic Stem Cells Is Dependent on Hedgehog Pathway Activation

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## SUMMARY

**Resistance of Bcr-Abl-positive leukemic stem cells (LSCs) to imatinib treatment in patients with chronic myeloid leukemia (CML) can cause relapse of disease and might be the origin for emerging drug-resistant clones. In this study, we identified Smo as a drug target in Bcr-Abl-positive LSCs. We show that Hedgehog signaling is activated in LSCs through upregulation of Smo. While *Smo*$^{-/-}$ does not impact long-term reconstitution of regular hematopoiesis, the development of retransplantable Bcr-Abl-positive leukemias was abolished in the absence of Smo expression. Pharmacological Smo inhibition reduced LSCs in vivo and enhanced time to relapse after end of treatment. Our results indicate that Smo inhibition might be an effective treatment strategy to reduce the LSC pool in CML.**

## INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal disease that originates from a single transformed hematopoietic stem cell (HSC) or multipotent progenitor cell harboring the Philadelphia translocation t(9;22) (Maguer-Satta et al., 1996; Nowell, 1962). The expression of the resulting gene product, *Bcr-Abl*, induces molecular changes that result in the expansion of malignant hematopoiesis, including the leukemic stem cell (LSC) pool, and the suppression of nonmalignant hematopoiesis (Nowell, 1962; Stam et al., 1987). Myeloid cells (granulocytes, monocytes, megakaryocytes, and erythrocytes) but also B and T cells express Bcr-Abl, pointing toward the multipotent progenitor cell or HSC as the initiating cell of the disease (Fialkow et al., 1978; Takahashi et al., 1998). In contrast to oncogenes causing acute myeloid leukemia, such as *Moz-Tif2* or *Mll-Enl*, *Bcr-Abl* cannot confer self-renewal properties to committed progenitor cells (Cozzio et al., 2003; Huntly et al., 2004) but rather utilizes and enhances the self-renewal properties of existing self-renewing cells. During the course of the disease, the LSC pool expands, and in the final stage, the blast crisis, the majority of CD34+ stem and progenitor cells carry the Philadelphia translocation (Holyoake et al., 1999; Maguer-Satta et al., 1996). Recent publications suggest that developmental pathways like the Wnt signaling pathway or the Polycomb-group protein Bmi1 might be involved in the regulation and expansion of LSCs during CML blast crisis (Hosen et al., 2007; Mohty et al., 2007; Muller-Tidow et al., 2004; Xie et al., 1998). In contrast to blast crisis, we have only limited knowledge about the mechanisms involved in the initial expansion of the Bcr-Abl-positive LSCs during the chronic phase of the disease.

The Hedgehog (Hh) signaling pathway is a developmental pathway that has been shown to play a role in primitive and adult hematopoiesis (Byrd et al., 2002; Chiang et al., 1996; Trowbridge et al., 2006). Hedgehog ligands (Sonic hedgehog [Shh], Indian hedgehog [Ihh], and Desert hedgehog [Dhh]) produced by stroma cells bind to the seven-transmembrane domain receptor Patched (Ptch). Ligand binding to Ptch releases Ptch binding to

## SIGNIFICANCE

The Abl inhibitor imatinib induces hematologic remissions in the majority of chronic myeloid leukemia (CML) patients, but failure to eradicate the leukemic stem cells (LSCs) can induce relapse of disease after end of treatment and forces patients to undergo lifelong therapy. Therefore, development of drugs that specifically target the LSC pool in CML is essential to cure this disease. Our findings indicate that Smo, which is specifically upregulated in Bcr-Abl-positive cells and which is essential for the expansion of the LSC pool, might be one of those targets while sparing normal hematopoietic stem cells. These results indicate that Smo is a specific and druggable target for Bcr-Abl-positive LSCs and that Smo inhibition in CML patients might be a step toward eventually eliminating the disease.

Smo, a second seven-transmembrane domain receptor protein. This results in a conformational change of Smo and subsequent activation of the downstream signaling pathway leading to induction of the Gli transcription factors (Gli1, Gli2, and Gli3) and transcription of target genes like *Gli1*, *Ptch1*, *cyclin D1*, and *Bcl2* (Duman-Scheel et al., 2002; Lee et al., 1997; Marigo and Tabin, 1996).

Hematopoiesis in vertebrate embryos occurs in two waves, including the primitive embryonic first wave resulting in primitive erythrocytes and the second, definitive or adult wave that generates HSCs that maintain hematopoiesis throughout adult life. Primitive and definitive hematopoiesis of the frog embryo stem from different blastomeres of the 32-cell-stage embryo, indicating important differences in molecular programming during embryogenesis (Ciau-Uitz et al., 2000). Murine explants and embryonic stem (ES) cell studies have suggested a role for Hh signaling in primitive hematopoiesis. During early embryogenesis, secretion of Ihh by visceral endoderm has been shown to be essential for the formation of primitive hematopoietic cells in the yolk sac of murine embryos (Dyer et al., 2001), and embryoid bodies differentiated in vitro from ES cells that lack Ihh or Smo fail to form hematopoietic cells (Byrd et al., 2002; Maye et al., 2000). In contrast, zebrafish embryos with defective mutations in Hh pathway members or treated with the Hh inhibitor cyclopamine display defects in adult HSC formation, but not in the generation of primitive hematopoiesis (Gering and Patient, 2005). Finally, targeted deletion of *Shh* (Chiang et al., 1996), *Ihh* (Byrd et al., 2002), or *Dhh* (Bitgood et al., 1996) in mice has failed to provide convincing evidence for a role of Hh in hematopoiesis at all. These conflicting results might be due to functional redundancy of the different Hh proteins in mammals and could not be clarified accordingly due to the embryonic lethality of $Smo^{-/-}$ embryos in midgestation. Furthermore, there are significant functional differences between defined HSC and progenitor populations of different species (mouse, human, and zebrafish), which are probably due to huge variations in life spans and the resulting diverging needs for maintenance of HSCs.

The development of the Abl kinase inhibitor imatinib was a breakthrough in the therapy of chronic-phase CML. In a 5 year follow-up study (Druker et al., 2006), the authors demonstrated complete hematological response in 98% of the treated patients, a complete cytogenetic response in 87%, and a complete molecular response in about 35%. Relapse rates during continuous treatment were 17%, and discontinuation of treatment due to side effects occurred in 4% of the cases. Despite this great success, several reports indicate that discontinuation of imatinib treatment even in patients who have already achieved molecular response induces a relapse of the disease (Rousselot et al., 2007; Breccia et al., 2006; Mauro et al., 2004; Merante et al., 2005; Cortes et al., 2004), and therefore, patients are forced to undergo lifelong therapy. Further studies have demonstrated that imatinib effectively eradicates Bcr-Abl-positive progenitor cells but does not target Bcr-Abl-positive CD34+ LSCs (Graham et al., 2002; Jiang et al., 2007). While new inhibitors (nilotinib and dasatinib) that target primary imatinib-resistant Abl mutants provide treatment options for relapsed patients or patients in blast-crisis CML, these Abl inhibitors also do not target the LSCs in CML (Copland et al., 2006; Jorgensen et al., 2007). Therefore, it is intriguing to postulate that therapies specifically targeting the LSCs will add additional therapeutic options in CML to prevent relapse of disease after discontinuation of treatment and to prevent the expansion of imatinib-resistant LSC clones during continuous treatment.

Therefore, the aim of our study was to examine the role of the Hedgehog signaling pathway in normal and malignant hematopoiesis and to define Smo as a specific drug target for LSCs in CML.

## RESULTS

### The Hedgehog Signaling Pathway Is Activated in Bcr-Abl-Positive Leukemic Stem Cells and Differentiated Hematopoietic Cells via Upregulation of Smo

To evaluate the activation status of the Hedgehog signaling pathway in Bcr-Abl-positive LSCs versus normal HSCs, we compared the transcript levels of two Hh pathway target genes, *Gli1* and *Ptch1*, in human CD34+ cells from healthy donors versus CD34+ cells isolated from patients with CML in chronic phase or blast crisis (see Table S1 available online). In all CML cases, we observed a greater than 4-fold induction of transcript levels for *Gli1* and *Ptch1*, indicating activation of the pathway as early as in chronic phase (Figure 1A). To further evaluate the role of Hh pathway activation in Bcr-Abl-driven leukemia, we induced a CML-like syndrome in mice (Daley et al., 1990; Heisterkamp et al., 1990). Bone marrow infected with a pMSCV/Bcr-Abl/IRES-GFP virus was transplanted into irradiated recipient mice, and mice developed a CML-like syndrome within 2 weeks after transplantation. Bcr-Abl-positive LSCs (Lin−Kit+Sca+GFP+) obtained from diseased mice displayed enhanced *Gli1* and *Ptch1* transcript levels compared to normal mouse HSCs (Lin−Kit+Sca+), reconfirming our findings from human patient samples (Figure 1A). Elevated *Gli1* and *Ptch1* expression in mouse bone marrow infected with a Bcr-Abl retrovirus was not restricted to the stem cell population alone but was present in all Bcr-Abl-overexpressing cells (Figure 1B).

Several mechanisms have been described that lead to the activation of the Hh signaling pathway in tumor cells. Activating point mutations of *Smo* or inactivating point mutations in *Ptch1* or *SUFU* have been detected in sporadic basal cell carcinoma (Aszterbaum et al., 1999; Oro et al., 1997; Xie et al., 1998), medulloblastoma (Goodrich and Scott, 1998; Marino, 2005), and rhabdomyosarcoma (Tostar et al., 2006). We have recently shown that activation of Hh signaling in lymphoma cells occurs via production of Hh ligands from infiltrating dendritic cells and mesenchymal stem cells (Dierks et al., 2007). Other tumors, like pancreatic cancer and small-cell lung cancer, were described to be dependent on Hh signaling via autocrine activation loops (Berman et al., 2003; Watkins et al., 2003). In contrast to the mechanisms described before, we detected an upregulation of the transmembrane receptor Smo in all pMSCV/Bcr-Abl/GFP-positive bone marrow cells compared to regular hematopoiesis in the same mice. The upregulation of Smo in the Bcr-Abl-positive population could be detected by quantitative PCR (Figure S1) and flow cytometry (Figure 1C) as well as immunohistochemistry (IHC). IHC stainings from spleens and bone marrow of diseased mice with a Smo-specific antibody showed a strong induction of Smo expression in the infiltrating Bcr-Abl-positive population (Figure 1D). IHC stainings for Smo and Gli1 in human CML cases also revealed upregulation of both proteins in corresponding

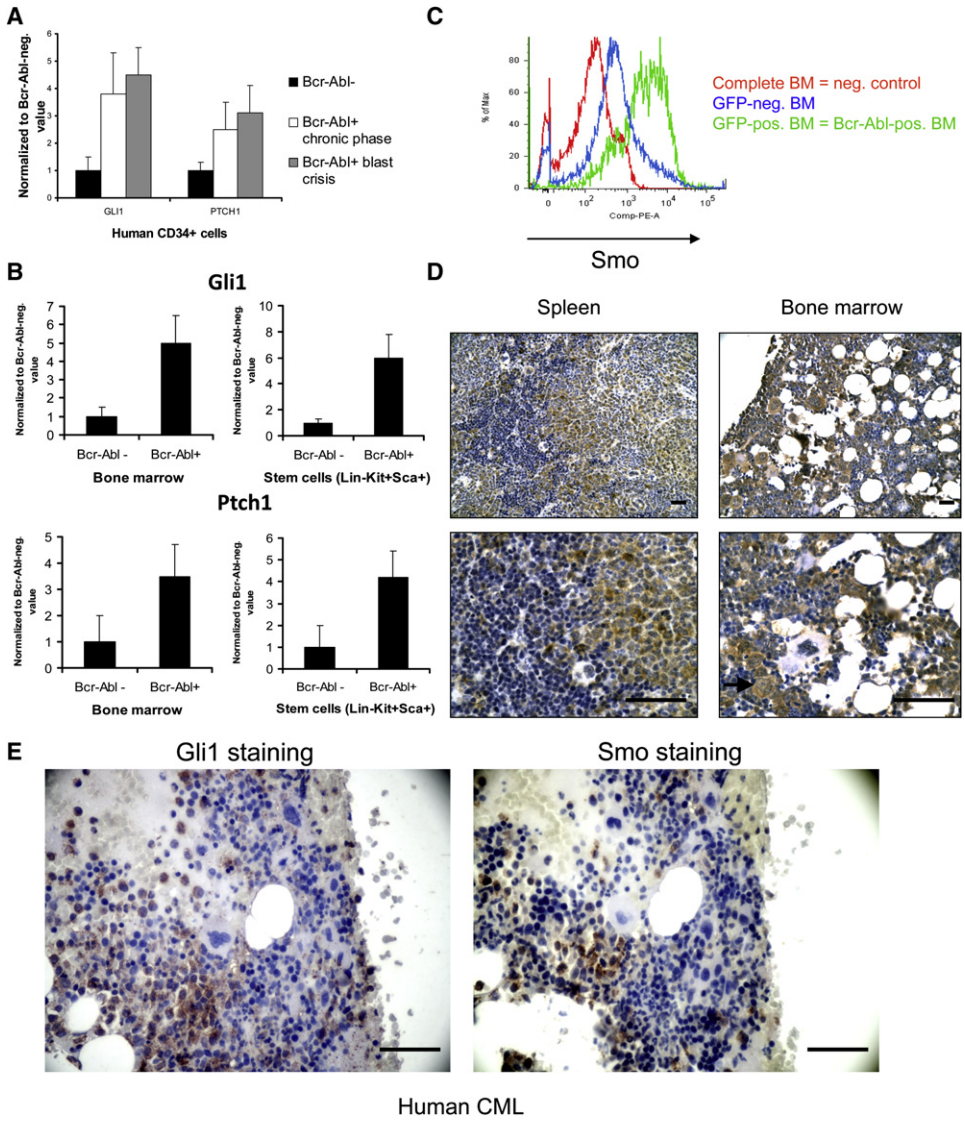


**Figure 1. Bcr-Abl Activates Hedgehog Signaling in Bone Marrow by Upregulation of Smo in Hematopoietic Stem Cells and Differentiated Cells**

(A) Transcript levels for *Gli1* and *Ptch1* in purified CD34+ cells from healthy donors and patients with CML in the chronic or blast crisis phase. Values are normalized to CD34+ cells from healthy donors. n = 3, ±SD.

(B) Expression of *Gli1* and *Ptch1* transcript levels in Bcr-Abl-positive versus -negative whole bone marrow and stem cells in mice. n = 3, ±SD.

(C) Flow cytometric analysis of anti-Smo-PE antibody binding to Bcr-Abl-positive versus -negative bone marrow cells.

(D) Immunohistochemical (IHC) staining for Smo in bone marrow and spleen infiltrated with Bcr-Abl-positive leukemic cells (arrow indicates micromegakaryocytes typical of Bcr-Abl-positive bone marrow). Scale bars = 100 μM.

(E) IHC staining for Smo and Gli1 expression in bone marrow from human CML. Scale bars = 100 μM.

regions of the bone marrow, especially in the blast cell population (Figure 1E). Abl inhibition in murine Bcr-Abl-positive bone marrow cultures using imatinib and nilotinib decreased *Smo* and *Gli1* transcript levels by only 20% (Figure S2), indicating that Smo upregulation is to a significant part independent of Abl kinase activity and that other mechanisms account for the increased expression of Smo in the Bcr-Abl-positive population.

We have recently shown that retroviral expression of *Smo* in lymphoma cells facilitates the growth of Eμ-Myc-positive lymphoma xenografts in nonlymphoid organs like the skin and enhances *Gli1* levels even in the absence of ligand stimulation (Dierks et al., 2007). Therefore, upregulation of Smo in Bcr-Abl-positive bone marrow cells may reveal an additional mechanism of Hh pathway activation in malignancies and can induce constitutive Hh signaling or at least hyperresponsiveness to Hh ligand stimulation in the Bcr-Abl-positive population.

## Inhibition of Hedgehog Signaling In Vitro Induces Apoptosis in Bcr-Abl-Positive Cells and Reduces the Number of Leukemic Stem Cells

To further validate the importance of the Hh pathway in Bcr-Abl-positive bone marrow cells and LSCs in vitro, we inhibited Hh

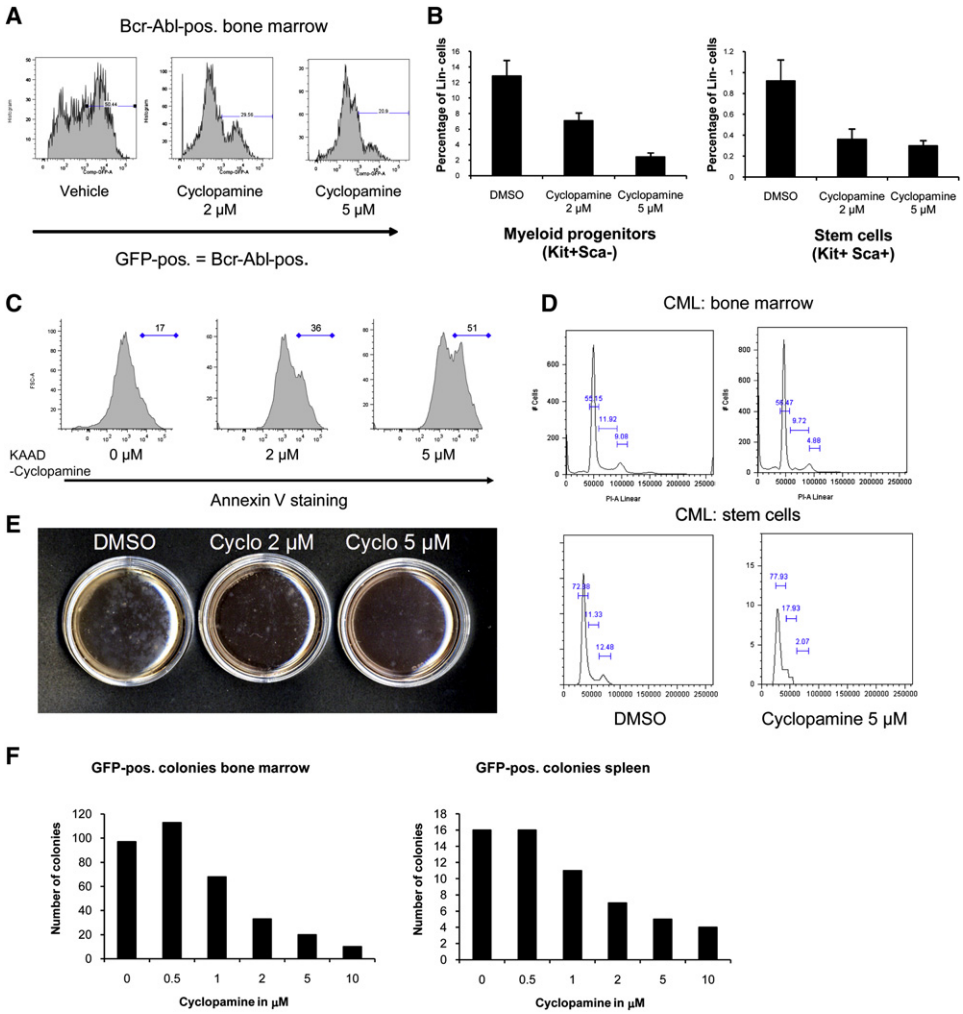


**Figure 2. Inhibition of Hedgehog Signaling In Vitro Induces Apoptosis in Bcr-Abl-Positive Cells and Reduces the Number of Leukemic Stem Cells**

(A) Flow cytometric analysis of GFP-positive cells after in vitro treatment with either DMSO or cyclopamine (2 μM and 5 μM) for 72 hr.

(B) Percentage of Bcr-Abl (GFP)-positive myeloid progenitors (Lin−Kit+Sca−) and leukemic stem cells (Lin−Kit+Sca+) after treatment of mixed bone marrow cultures with cyclopamine for 72 hr in three independent experiments ±SD.

(C) Annexin V staining of Bcr-Abl (GFP)-positive cell cultures after treatment with cyclopamine for 48 hr.

(D) Cell-cycle distribution of gated viable cells after treatment with cyclopamine for 24 hr in Bcr-Abl-positive bone marrow and stem cells.

(E) Colony assays from mixed bone marrow cultures (Bcr-Abl-positive and -negative) in methylcellulose medium without cytokines after 72 hr treatment with cyclopamine.

(F) Total number of colonies counted 10 days after plating of cyclopamine-treated mixed bone marrow cultures.

signaling using KAAD-cyclopamine (Chen et al., 2002), an alkaloid, which locks Smo in its inactive conformation. We used bone marrow from mice with CML-like syndrome that contained about 50% Bcr-Abl/GFP-positive cells versus 50% regular bone marrow cells. Cyclopamine treatment of these mixed bone marrow cultures for 3 days resulted in a dose-dependent reduction of the Bcr-Abl/GFP-positive population compared to the GFP-negative population (Figure 2A). Further characterization of the different cell subsets demonstrated a reduction of the Bcr-Abl-positive myeloid progenitor cells (Lin−Kit+Sca−) by more than 80% and a reduction of the Lin−Kit+Sca+ LSC population by around 70% (Figure 2B). The predominant effect of cyclopamine treatment on Bcr-Abl-positive bone marrow cells was apoptosis induction within 48 hr (Figure 2C) as assessed by annexin V staining. We also detected alterations in the cell cycle, with a relative increase of the G1 phase compared to the S phase and G2 phase in the complete bone marrow (Figure 2D, upper panel). Cell-cycle analysis of the LSC population showed a complete loss of the G2 phase in these cells after Hh pathway inhibition (Figure 2D, lower panel). *Gli1* transcript levels in the bone marrow were reduced after treatment with cyclopamine, verifying the inhibition of the Hh signaling pathway in these cells by the compound (Figure S3).

To further validate the effect of Hh pathway inhibition on the self-renewing progenitor and LSC populations, we treated mixed bone marrow and spleen cultures with different concentrations

**Table 1. Colony Numbers 10 Days after Plating of Fetal Liver Cells**

| Genotype | P1 | P2 | P3 |
|---|---|---|---|
| *Smo*$^{-/-}$ | 130 | 0 | 0 |
| *Smo*$^{+/-}$ | 142 | 0 | 0 |
| *Smo*$^{+/+}$ | 121 | 2 | 0 |
| *Ptch1*$^{+/+}$ | 128 | 3 | 0 |
| *Ptch1*$^{+/-}$ | 136 | 48 | 23 |

P = plating round.

of KAAD-cyclopamine (0, 0.5, 1, 2, 5, and 10 μM) for 48 hr and then plated the cells on methylcellulose plates without supplementary cytokines, so that only Bcr-Abl-positive cells could survive. Colonies were counted 10 days after plating. Bone marrow and spleen cultures pretreated with cyclopamine showed a dose-dependent reduction of Bcr-Abl-positive colonies, indicating that the colony-forming Bcr-Abl-positive cells are dependent on Hh pathway activation (Figures 2E and 2F) (1 out of 3 examples).

## Self-Renewal Capacity of Regular Short-Term Repopulating HSCs and T Cell Development Are Dependent on the Status of Hedgehog Pathway Activation

The role of Hh signaling in normal hematopoiesis is not completely understood, but several publications indicate that Hh signaling plays a role in adult hematopoiesis (Dyer et al., 2001; Gering and Patient, 2005; Trowbridge et al., 2006). Overexpression of Ihh in bone marrow stroma cells enhances hematopoietic regeneration after transplantation (Kobune et al., 2004), and *Ptch1*$^{+/-}$ mice have a higher regeneration potential of short-term repopulating HSCs (Trowbridge et al., 2006). Due to the embryonic lethality of *Smo*$^{-/-}$ mice, the effect of loss of Hh signaling in normal hematopoiesis in mice is not well established, although a distinct knowledge is essential to define risks that could be caused by Smo inhibitors, which are currently being explored for the treatment of solid tumors with mutations in the Hh pathway (medulloblastoma, rhabdomyosarcoma, and basal cell carcinoma).

To bypass this problem, we used fetal HSCs isolated from the liver of embryos at day 14.5 of the gestation period. Fetal liver cells from *Smo*$^{-/-}$, *Smo*$^{+/-}$, *Smo*$^{+/+}$, *Ptch1*$^{+/+}$, and *Ptch1*$^{+/-}$ embryos were analyzed regarding the number of fetal HSCs, number of differentiated hematopoietic cell types, colony-forming capacity, and repopulation potential in a transplantation experiment. We detected no differences in the number of fetal HSCs between the different genotypes and also no significant differences in numbers of B cells (B220), myeloid cells (CD11b), erythroid progenitors (Ter119), or CD3+ T cells (Table S2). Plating of fetal liver cells into methylcellulose agar with supplementary cytokines (IL-3, IL-6, and SCF) did not result in any differences in the number of colonies, in the colony types, or in the percentage of different cell types as measured by flow cytometry 10 days after plating (Table 1 and data not shown). In contrast to the first plating round, we observed significant differences in the colony-forming potentials after replating for a second round. While replated *Ptch1* and *Smo* wild-type (WT) hematopoietic cells showed only very limited colony-forming potential in the second plating round and *Smo*$^{-/-}$ hematopoietic cells had lost colony-forming potential completely, *Ptch1*$^{+/-}$ hematopoietic cells kept their ability to form colonies over more than three plating rounds, indicating that Hh pathway activation enhances the number of regenerating cells and self-renewal properties of *Ptch1*$^{+/-}$ hematopoietic bone marrow (Figure 3C; Table 1).

In a second experiment, we transplanted *Smo*$^{-/-}$, *Smo*$^{+/-}$, *Smo*$^{+/+}$, *Ptch1*$^{+/+}$, and *Ptch1*$^{+/-}$ fetal liver cells (positive for Ly5.2) into sublethally irradiated C57BL/6-Ly5.1-Pep3b mice (B6-Ly5.1 *Smo*$^{-/-}$, B6-Ly5.1 *Smo*$^{+/-}$, B6-Ly5.1 *Smo*$^{+/+}$, B6-Ly5.1 *Ptch1*$^{+/+}$, and B6-Ly5.1 *Ptch1*$^{+/-}$). The regeneration of Ly5.2-positive hematopoiesis in the peripheral blood showed a significant advantage for mice transplanted with *Ptch1*$^{+/-}$ fetal liver cells compared to mice transplanted with fetal liver cells isolated from the other genotypes. The number of *Ptch1*$^{+/-}$ Ly5.2-positive cells in the peripheral blood was about double compared to WT and *Smo*$^{-/-}$ cells over a period of more than 3 months (Figure 3D). The regeneration of *Smo*$^{-/-}$ bone marrow 2 months after transplantation was not significantly different from WT bone marrow, indicating that there are no differences in the long-term regeneration capacity of *Smo*$^{-/-}$ versus *Smo* WT HSCs. However, further analysis of the cell types in the peripheral blood showed differences in the distribution of cells between mice transplanted with *Smo*$^{-/-}$ versus *Smo* WT fetal liver cells. B6-Ly5.1 *Smo*$^{-/-}$ showed a greater than 90% decrease in CD8+ T cells, and the number of CD4+ T cells was decreased by 30%. These results confirm recent findings that Hh signaling is important for T cell development (El Andaloussi et al., 2006; Uhmann et al., 2007), and our results indicate especially that the generation of CD8+ T cells is dependent on intact Hh signaling (Figure 3E).

To further establish the role of Hh signaling in HSCs, we challenged the hematopoietic regeneration capacity of B6-Ly5.1 mice that had been transplanted with Ly5.2-positive *Smo*$^{-/-}$, *Smo*$^{+/-}$, *Smo*$^{+/+}$, *Ptch1*$^{+/+}$, or *Ptch1*$^{+/-}$ fetal liver cells by injecting these mice with 5-fluorouracil (5-FU) (150 mg/kg). Short-term regeneration capacity was significantly reduced in bone marrow lacking Smo. Ten days after 5-FU injection, the number of Ly5.2-positive cells in B6-Ly5.1 *Smo*$^{-/-}$ mice was 70% lower than in B6-Ly5.1 *Smo*$^{+/+}$ mice, indicating a role of the Hh signaling pathway in short-term repopulating HSCs (Figure 3F). As described previously (Trowbridge et al., 2006), B6-Ly5.1 *Ptch1*$^{+/-}$ mice displayed enhanced short-term regeneration potential after 5-FU treatment as compared to B6-Ly5.1 *Ptch1* or *Smo* WT mice, indicating that in regular hematopoiesis, activation of the Hh signaling pathway enhances the regeneration potential of short-term repopulating HSCs. Similar results were obtained in a retransplantation experiment (Figure S4). Interestingly, 40 days after 5-FU treatment, no significant differences remained in the numbers of Ly5.2-positive cells between mice carrying *Smo*$^{-/-}$ versus *Smo* WT bone marrow, indicating that the long-term repopulating HSCs were not affected by the loss of Smo (Figure 3F). Therefore, pharmacological inhibition of Smo might only affect short-term repopulating HSCs in regular hematopoiesis while long-term repopulating HSCs and long-term regeneration of hematopoiesis are not affected.

In contrast to a previous report (Trowbridge et al., 2006), we could not detect an exhaustion of the long-term repopulating cells in mice transplanted with *Ptch1*$^{+/-}$ fetal liver cells. After 5-FU injection, Ly5.2-positive cells remained elevated compared

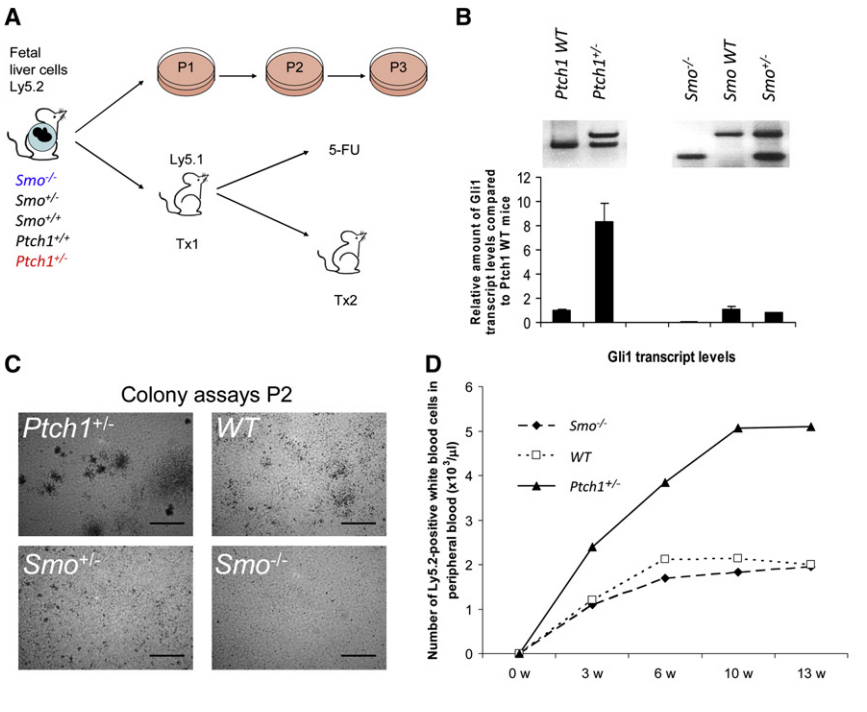


**Figure 3. Self-Renewal Capacity of Short-Term Repopulating Hematopoietic Stem Cells and T Cell Development in Regular Hematopoiesis Are Dependent on the Status of Hedgehog Pathway Activation**

(A) Schematic overview of replating and transplantation experiments with embryonic liver cells.

(B) Genotyping for $Smo^{-/-}$ mouse strain and $Ptch1^{+/-}$ mouse strain. Relative transcript levels for *Gli1* in different mouse strains (mean of n = 3 mice per strain, ±SD) are shown.

(C) Replating assay (P2) with embryonic liver cells. Colonies were photographed 10 days after replating. Scale bars = 1 mm.

(D) Number of Ly5.2-positive white blood cells in peripheral blood after transplantation into irradiated B6-Ly5.1 mice (0–13 weeks after transplantation).

(E) Cell type distribution in peripheral blood within the Ly5.2-positive population 10 weeks after transplantation.

(F) Regeneration of Ly5.2-positive cells in peripheral blood 10 and 40 days after 5-fluorouracil (5-FU) injection (150 mg/kg). Mean of n = 3 mice per group, ±SD.

to all other genotypes for a time period of more than 4 months (data not shown). Also, blood cell counts from 2-year-old $Ptch1^{+/-}$ mice showed no difference in the number of peripheral blood cells compared to *Ptch1* WT mice, indicating that there is no significant lack in the generation of differentiated hematopoietic cells in these mice even after a long period of time (Figure S5).

Since the Hh signaling pathway is activated in Bcr-Abl-positive cells by upregulation of the Smo receptor and not by downregulation of Ptch1, we aimed to investigate the effect of Smo upregulation on regular hematopoiesis. Therefore, we overexpressed a GFP control vector, Smo WT-GFP, or the activated mutant Smo W535E-GFP in the bone marrow of 5-FU-pretreated mice using a pMSCV-based IRES-GFP vector system. Bone marrow cells overexpressing Smo WT or Smo W535E had significantly elevated *Gli1* levels compared to control bone marrow cells (Figure 4A). Irradiated donor mice were transplanted with 10% GFP-positive bone marrow cells mixed with 90% GFP-negative uninfected bone marrow cells, and reconstitution of hematopoiesis was monitored by determining blood cell counts and the fraction of GFP-positive cells in the peripheral blood. The percentage of GFP-positive cells in mice transplanted with bone marrow expressing the GFP control vector remained constant at 10%–12% throughout the experiment. In contrast, recipients of Smo WT- or Smo W535E-infected bone marrow displayed a significant increase in the number of GFP-positive cells for more than 1 year (Figure 4B). The maximum fraction of GFP-positive cells observed in these mice was 30%. There were no significant differences in the

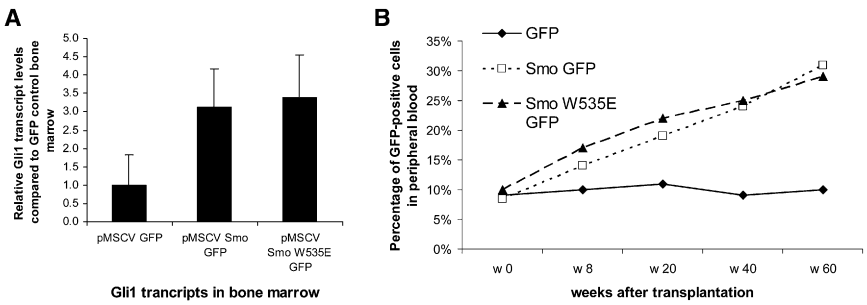


**Figure 4. Overexpression of Smo in Bone Marrow**

(A) Relative *Gli1* transcript levels measured by TaqMan PCR in bone marrow infected with pMSCV control, Smo-GFP, or Smo W535E-GFP vector. n = 3, ±SD.

(B) Percentage of GFP-positive cells in peripheral blood of mice transplanted with bone marrow initially containing 10% GFP-positive cells, 10% Smo-GFP-positive cells, or 10% Smo W535E-GFP-positive cells over a period of 60 weeks.

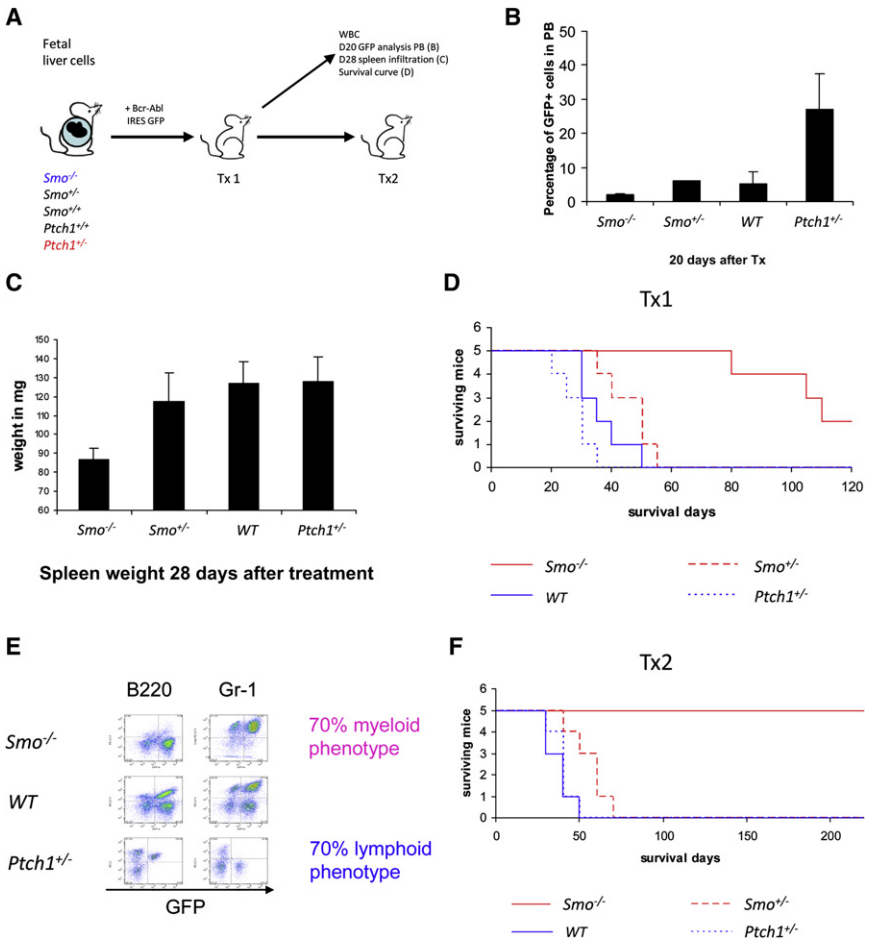


**Figure 5. Loss of Smo Inhibits Expansion of Bcr-Abl-Positive Leukemic Stem Cells in Mice**

(A) Schematic overview of transplantation (Tx) experiments with overexpression of the *Bcr-Abl* oncogene.

(B) Number of Bcr-Abl-positive cells in peripheral blood of transplanted mice 20 days after transplantation. n = 3, ±SD.

(C) Spleen weight of transplanted mice 28 days after transplantation. n = 3, ±SD.

(D) Survival of mice transplanted with Bcr-Abl-infected fetal liver cells.

(E) Flow cytometric analysis of B220 and Gr-1 expression in bone marrow of leukemic mice.

(F) Survival of mice retransplanted with 2 × $10^5$ Bcr-Abl (GFP)-positive bone marrow cells from the indicated genotypes.

fraction of T cells, B cells, or myeloid cells in the GFP-positive population compared to the GFP-negative population, indicating that all lineages were impacted to a similar degree. These data indicate that Smo overexpression, similar to loss of Ptch1, can induce Hh pathway activation and lead to the expansion of the Smo-positive HSC pool. We therefore hypothesized that upregulation of Smo in Bcr-Abl-positive cells might be one of the mechanisms by which Bcr-Abl-positive LSCs expand during the chronic phase of the disease.

## Loss of Smo Inhibits Expansion of Bcr-Abl-Positive Leukemic Stem Cells in Mice and Abrogates Retransplantability of the Disease

To further validate this hypothesis, we overexpressed Bcr-Abl in $Smo^{-/-}$, $Smo^{+/-}$, $Smo^{+/+}$, $Ptch1^{+/+}$, and $Ptch1^{+/-}$ embryonic liver cells using a pMSCV/Bcr-Abl/IRES-GFP retroviral vector. The infection rate was between 3% and 4%, and no difference in the infection rate was observed between fetal liver stem cells isolated from the various mouse strains. Fetal liver cells were then transplanted into lethally irradiated C57BL/6 mice. GFP-positive cells and blood cell counts were measured 20 days after transplantation. Mice transplanted with $Ptch1^{+/-}$/Bcr-Abl/GFP fetal liver cells showed 3-fold higher numbers of Bcr-Abl/GFP-positive cells than mice transplanted with *Ptch1* WT or *Smo* WT fetal liver cells infected with pMSCV/Bcr-Abl/GFP. In contrast, $Smo^{-/-}$/Bcr-Abl/GFP-positive cells had not expanded at all during this time span, and the percentage of the GFP-positive fraction had even dropped below the original infection rate (Figure 5B). Next, spleen weights were compared on day 28 after transplantation (n = 3 mice per group). All mice transplanted with $Ptch1^{+/-}$, *Ptch1* WT, *Smo* WT, or $Smo^{+/-}$/Bcr-Abl/GFP fetal liver cells had a greater than 40% increase in spleen weight, reflecting the onset of a leukemic disease. In contrast, mice transplanted with $Smo^{-/-}$/Bcr-Abl/GFP embryonic liver cells had normal spleen weight (between 90 and 110 mg), indicating that Smo is important for the expansion of Bcr-Abl-positive cells (Figure 5C). Finally, the remaining mice from each group were monitored for onset of disease and disease phenotype. All mice transplanted with $Ptch1^{+/-}$ embryonic liver cells developed a lethal leukemic disease within 38 days after transplantation. Recipients of WT or $Smo^{+/-}$ fetal liver cells (Figure 5D) developed disease with slightly longer latency. Phenotypic analysis of the leukemias developed in diseased mice showed that animals transplanted with $Ptch1^{+/-}$ fetal liver cells were more likely to develop a lymphoid disease phenotype (70%), while mice transplanted with $Smo^{+/-}$ fetal liver cells were more likely to develop a myeloid disease phenotype (70%) (Figure 5E). In sharp contrast, only 60% of the mice transplanted with *Bcr-Abl*-infected $Smo^{-/-}$ fetal liver cells developed any lethal disease. The latency to onset of the disease was increased to more than 3 months, and the disease was characterized by increased spleen weight and infiltration of the bone marrow, but surprisingly, no increase in white blood cell counts was seen in the peripheral blood (data not shown). Remarkably, 40% of the $Smo^{-/-}$/Bcr-Abl/GFP-transplanted mice had not developed any signs of disease even 12 months after transplantation.

To further investigate the importance of activation of the Hh signaling pathway for the LSC population, we collected bone marrow and spleen cells from the diseased mice and retransplanted the cells into lethally irradiated secondary recipients.

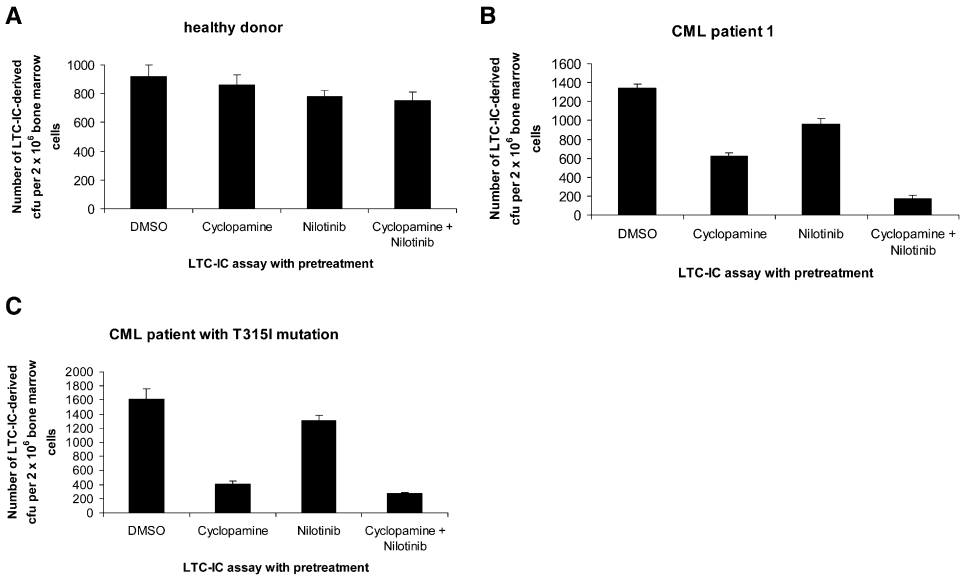


**Figure 6. LTC-IC Assays after Pretreatment with Cyclopamine, Nilotinib, or a Combination of Both**

$2 \times 10^6$ bone marrow cells from one healthy donor and two CML patients were seeded in long-term culture-initiating cell (LTC-IC) medium onto irradiated M2-10B4 cells and treated with DMSO, cyclopamine (5 μM), nilotinib (0.5 μM), or a combination of both. Compound-containing medium was removed after 7 days, and cells were cultured for 4 more weeks in LTC-IC medium before methylcellulose assays were performed. Numbers of colonies were counted after 20 days. Numbers of LTC-IC-derived colony-forming units (cfu) from the healthy donor (A) versus CML patient 1 (B) and a CML patient with T315I mutation (C) are shown. Mean of three independent experiments, ±SD.

All secondary recipients from mice transplanted with $2 \times 10^5$ Bcr-Abl-positive *Ptch1*$^{+/+}$, *Ptch1*$^{+/-}$, *Smo*$^{+/+}$, or *Smo*$^{+/-}$ cells developed leukemias within 2 months after retransplantation, while none of the mice transplanted with Bcr-Abl-positive *Smo*$^{-/-}$ cells developed signs of disease over a 9 month observation period (Figure 5F). Therefore, our results indicate that expansion of Bcr-Abl-positive LSCs and the maintenance of self-renewal properties in this population are dependent on intact and activated Hh signaling.

## Effects of a Combination of Abl and Smo Inhibitors In Vitro and In Vivo

To investigate whether Hh pathway inhibition might be beneficial in the treatment of CML and the elimination of self-renewing LSCs, we examined the effect of a combination of Abl and Smo inhibitors on the maintenance of long-term repopulating cells from bone marrow of CML patients or healthy donors in vitro (Figure 6). Long-term culture-initiating cell (LTC-IC) assays were performed for 5 weeks, including a 1 week treatment of the cultures with DMSO, cyclopamine (5 μM), nilotinib (0.5 μM), or a combination of cyclopamine and nilotinib for the first week. After 5 weeks, adherent and floating cells were plated on methylcellulose, and colonies were counted after 20 days. The number of LTC-IC-derived colony-forming units (cfu) per $2 \times 10^6$ bone marrow cells was increased 1.42-fold in the CML patient compared to the healthy donor (1356 in CML patient versus 958 in healthy donor). Smo inhibition for 1 week in the LTC-IC assay reduced the number of cfu obtained from the CML patient by about 54% (620 cfu) while nilotinib reduced the number of cfu by 29% (951 cfu) (Figure 6B). A combination of both reduced the number of cfu by more than 80%, indicating that the combination of Smo and Abl inhibitors might be an effective combination treatment for the reduction of CML stem cells. Similar results were obtained using bone marrow from two additional CML patients (patient 2 with *Bcr-Abl* WT [Figure S6]; patient 3 with *Bcr-Abl* T315I [Figure 6C]), indicating that LSCs with mutated *Bcr-Abl* are also sensitive to Smo inhibition. The effect of Smo inhibition on the number of LTC-IC-derived colonies was dose dependent (Figure S6). In contrast, cyclopamine and nilotinib treatment of LTC-IC cultures from a healthy donor reduced the number of colonies by only 25% (958 cfu with DMSO/713 with cyclopamine + nilotinib). This effect was mainly due to nilotinib pretreatment (reduction of cfu = 23%; 730 cfu); Smo inhibition alone reduced the colony numbers by less than 10% (870 cfu) (Figure 6A). The effect seen with nilotinib treatment might be due to inhibition of Kit, which besides Abl is a major target of this inhibitor.

To further validate the effect of Smo and Abl inhibitor combinations on the reduction of LSCs in vivo, we induced a CML-like syndrome in mice by transplantation of Bcr-Abl/GFP-expressing bone marrow into irradiated recipient mice. Mice were treated with cyclopamine, nilotinib, a combination of both, or vehicle control starting 7 days after transplantation. Nilotinib was chosen over imatinib due to its 20-fold stronger Abl kinase inhibition (Kantarjian et al., 2006) and its superior pharmacological properties in mice (Weisberg et al., 2005). The compound was dosed at 50 mg/kg/day, a dose shown to lead to sustained inhibition of Abl kinase over 24 hr and therefore assumed to be optimal. Due to the sensitization of mice by irradiation to the side effects of cyclopamine, including diarrhea and seizures, we had to use a lower than optimal dose of 25 mg/kg bid. Importantly, *Gli1* levels in the bone marrow of Bcr-Abl-positive mice dosed with 25 mg/kg/bid of cyclopamine for 7 days were reduced by 50% compared to vehicle-treated mice, but the *Gli1* levels were

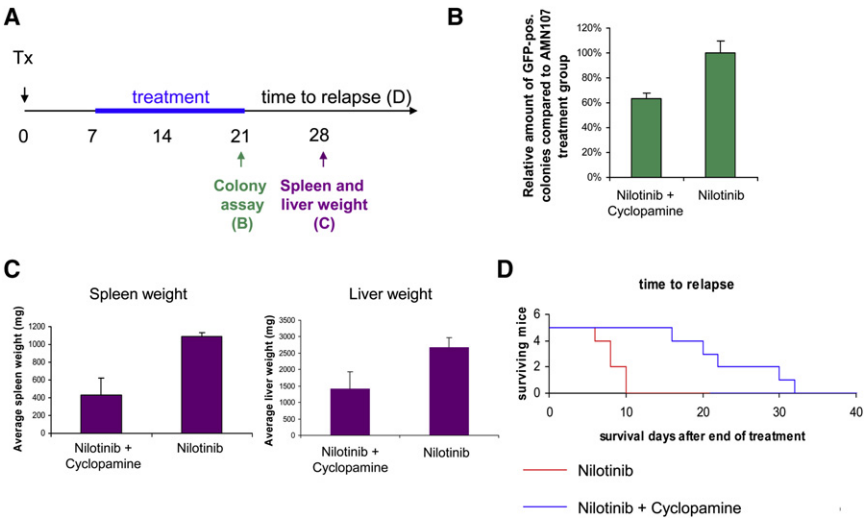


**Figure 7. Combination of Nilotinib and Cyclopamine Reduces the Number of Bcr-Abl-Positive Colony-Forming Cells in Bone Marrow and Enhances Time to Relapse after End of Treatment**

(A) Bone marrow transplantation model with start of treatment 7 days after transplantation with either nilotinib alone (50 mg/kg/day) or a combination of nilotinib (50 mg/kg/day) and cyclopamine (25 mg/kg/bid).

(B) Relative amount of GFP-positive bone marrow colonies in one femur in Bcr-Abl-positive mice treated with either nilotinib alone or a combination of nilotinib and cyclopamine. n = 3 per treatment group, ±SD.

(C) Spleen and liver weights 8 days after end of treatment. n = 3 per treatment group, ±SD.

(D) Survival in days after end of treatment with either nilotinib alone or a combination of nilotinib and cyclopamine.

still elevated about 3-fold compared to mice not expressing Bcr-Abl (Figure S7), indicating that the level of inhibition of the Hh pathway achieved with this dose was incomplete.

After 6 days of treatment, bone marrow was harvested and the percentage of Bcr-Abl/GFP-positive LSCs (Kit+Sca+) in the Lin− population was assessed using flow cytometry. Despite using a suboptimal dose, cyclopamine treatment reduced the number of Bcr-Abl-positive LSCs (Lin−GFP+Sca+Kit+) by about 55%, nilotinib by 44%, and a combination of both by 82%, indicating a combination of both inhibitors as an effective strategy to reduce LSCs in Bcr-Abl-positive mice (Figure S8).

In addition, cyclopamine treatment of Bcr-Abl-positive mice starting at day 7 after transplantation increased survival of the mice by about 4 days (average survival: vehicle 13 days, cyclopamine 17 days) (Figure S7) but did not completely suppress the disease. As indicated by our transplantation experiments using Bcr/Abl-positive *Smo*$^{-/-}$ fetal liver cells, loss of Smo does not necessarily affect all disease-propagating Bcr-Abl-positive progenitor populations but was able to completely suppress retransplantability of the disease, indicating a loss of the LSCs.

In an additional experiment, we attempted to further validate the cooperative effect of Abl and Smo inhibitors on eradication of progenitors and LSCs in vivo. We treated mice carrying Bcr-Abl-transduced bone marrow with either nilotinib alone (50 mg/kg/day) or a combination of nilotinib (50 mg/kg/day) and the Smo antagonist cyclopamine (25 mg/kg bid). Treatment was started 7 days after transplantation and was continued for a total of 14 days. At the end of the treatment, three mice in each group were sacrificed, bone marrow from one femur per mouse was isolated, and cells were plated in methylcellulose without addition of cytokines to detect colony formation of Bcr-Abl-positive cells (Figure 7A). The average number of colonies detected in mice treated with the combination of nilotinib and cyclopamine (N+C) was reduced more than 40% compared to the nilotinib-only (N) treatment group, indicating that addition of cyclopamine leads to an additional reduction of the number of Bcr-Abl-positive cfu (Figure 7B). Peripheral blood cell counts and spleen and liver weights were normal at that time point, and the number of GFP-positive cells was below 5% for both treatment groups. Eight days after the end of treatment, an additional three mice per group were sacrificed and investigated for signs of relapse by comparing liver and spleen weights. We found increased liver and spleen weights in all mice compared to normal mice, but mice treated with N alone had more than double the average spleen size (N, 1090 mg; N+C, 410 mg) and a significantly higher liver weight (N, 2550 mg; N+C, 1430 mg) than mice treated with the N+C combination (Figure 7C). The five remaining mice in each group were monitored for signs of disease and sacrificed when moribund. The average survival after end of treatment was 8 days in the N group versus 24 days in the N+C group (Figure 7D), indicating that the combination of Abl and Smo inhibitors reduced the number of repopulating cells responsible for relapse more than Abl inhibition by itself. This indicates that a combination of Smo and Abl inhibitors might be an effective treatment strategy to eradicate progenitors and LSCs in Bcr-Abl-positive disease.

## DISCUSSION

CML is one of the best understood malignancies due to the fact that a single oncogene, *Bcr-Abl*, is the cause of more than 95% of CMLs. The treatment of these patients with the Abl inhibitor imatinib leads to response rates of greater than 95%, and most patients experience few side effects and can stay on the medication for years (Druker et al., 2006). Despite this great success, there are still drawbacks that need to be addressed. Above all, 17% of patients develop resistance to the therapy partially due to preexisting point mutations in Bcr-Abl-positive LSCs, which expand over time and cause relapse of disease. Furthermore, it is not yet clear whether responsive patients can ever stop taking imatinib, as there is evidence that Bcr-Abl-positive LSCs remain present in the patient's bone marrow even after years of therapy and can cause relapse of disease. Therefore, it is necessary to define targets in Bcr-Abl-positive LSCs that might be candidates for new treatment options.

Our study reveals that one candidate could be Smo. Smo appears to be specifically upregulated in Bcr-Abl-positive hematopoietic cells and LSCs versus in regular hematopoiesis and HSCs. Loss of Smo in regular hematopoiesis had no significant impact on the regeneration of hematopoiesis in general besides

the nearly complete loss of CD8+ T cells and only seems to impact the short-term repopulating HSCs or the acute expansion of stem cells after 5-FU treatment or retransplantation, while long-term reconstitution of the bone marrow was not affected. These findings are of significant importance, as several pharmaceutical companies develop Smo inhibitors for the treatment of solid tumors such as medulloblastomas or basal cell carcinomas. The finding that long-term repopulating cells in the bone marrow are independent of Hedgehog opens new options for the systemic application of these drugs and might allow broader clinical use of Smo inhibitors. On the other hand, the loss of CD8+ T cells in $Smo^{-/-}$ bone marrow indicates that Smo inhibition might cause partial immunosuppression in treated patients, and further studies will be needed to evaluate whether preexisting CD8+ T cells will be affected by Smo inhibition and whether CD8+ T cell suppression is a transient or permanent effect caused by Smo inhibitors. Furthermore, the impact of Smo on the short-term repopulating HSCs indicates that a combination of Smo inhibitors with generally cytotoxic and hematotoxic chemotherapeutics might induce prolonged neutropenias, anemias, and/or thrombocytopenias due to delayed bone marrow regeneration, and it might be more favorable to combine Smo inhibitors with target-specific biological agents or small-molecule inhibitors.

In contrast to regular hematopoiesis, lack of Smo in Bcr-Abl-positive hematopoiesis effectively reduced the development of Bcr-Abl-positive leukemias in mice and abolished retransplantability of the disease, indicating a loss of the Bcr-Abl-positive LSC pool. These findings were corroborated using LTC-IC cultures from CML patients, wherein Smo inhibition significantly reduced the number of long-term repopulating LSCs independent of *Bcr-Abl* mutation status. Furthermore, combination treatment of Bcr-Abl-positive mice with cyclopamine (a Smo inhibitor) and nilotinib (an Abl inhibitor) induced reduction of LSCs and enhanced time to relapse 3-fold after the end of treatment compared to nilotinib treatment alone. Our results indicate that Smo might be a specific target in Bcr-Abl-positive LSCs, while it is not essential for the long-term regeneration potential of regular HSCs. Furthermore, the effect of Smo inhibition on the LSC pool in CML is independent of *Bcr-Abl* mutation status, which indicates that Smo inhibition might help to prevent the expansion of imatinib-resistant LSCs during treatment. Hence, combinations of Abl and Smo inhibitors might offer a new treatment strategy in CML and might help to effectively eliminate the Bcr-Abl-positive LSC pool.

## EXPERIMENTAL PROCEDURES

### Mouse Experiments

$Ptch1^{+/-}$ mice (The Jackson Laboratory), $Smo^{+/-}$ mice (Deltagen), C57BL/6 mice (The Jackson Laboratory), and B6-Pep3b-*Ly5.1* (Pep) mice were maintained and genotyped as described by the vendor. For bone marrow transplantation experiments, C57BL/6 males were injected intraperitoneally with 5-FU (150 mg/kg), and mice were sacrificed 4 days later. Bone marrow mononuclear cells were flushed from the leg bones, red blood cells were lysed with ammonium chloride, and bone marrow cells were then cultivated in DMEM containing 10% FBS, SCF, IL-6, and IL-3.

For compound treatment experiments, cells were infected with a pMSCV/Bcr-Abl/IRES-GFP retrovirus. $5 \times 10^5$ mononuclear cells were transplanted into lethally irradiated C57BL/6 mice. Treatment with nilotinib (50 mg/kg bid orally; Tasigna, Novartis [Basel]) and cyclopamine (25 mg/kg bid subcutaneously; Novartis [Cambridge]) started on day 7 after transplantation for 14 days. At the end of treatment, three mice were sacrificed per group, and bone marrow cells from one femur per mouse were used for colony assays (methylcellulose) without cytokines. Seven days after the end of treatment, another seven mice were sacrificed to compare spleen weights between the treatment groups. The five remaining mice per group were monitored for signs of disease and time to relapse.

For further bone marrow transplantation experiments with pMSCV/IRES-GFP control vector, pMSCV/Smo/IRES-GFP vector, and pMSCV/Smo W535E/IRES-GFP vector, we used the same methods as described above.

For transplantation experiments with fetal liver cells from $Ptch1^{+/-}$, *Ptch1* WT, *Smo* WT, $Smo^{+/-}$, and $Smo^{-/-}$ mice, we used embryos at day 14.5 of the gestation period. Embryos were chilled on ice and decapitated. The embryonic liver was extracted, and liver cells were filtered through a cell strainer (BD Biosciences). Embryonic liver cells were either directly transplanted into sublethally irradiated B6-Pep3b-*Ly5.1* (Pep) mice for repopulation experiments or cultured in stimulation medium and then infected with a pMSCV/Bcr-Abl/IRES-GFP retrovirus. Number of GFP-positive cells was determined 24 hr after infection by flow cytometry, and we tried to keep the infection rate between 4% and 6% to evaluate the expansion of the Bcr-Abl-positive cells. Fetal liver cells were then transplanted into lethally irradiated recipients. Disease development was monitored by weekly weight measurements, biweekly blood cell counts, and detection of GFP-positive cells in the peripheral blood. For retransplantation experiments, we used $2 \times 10^5$ Bcr-Abl/GFP-positive cells or $5 \times 10^5$ Ly5.2-positive bone marrow cells.

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Genomics Institute of the Novartis Research Foundation and were performed in accordance with the US National Institutes of Health Statement of Compliance with Standards for Humane Care and Use of Laboratory Animals.

### Cell Culture Experiments

Bone marrow cells from diseased mice were cultured in DMEM containing 10% FBS (GIBCO), SCF (RDI), IL-3, and IL-6 (R&D Systems). For in vitro treatment experiments, we seeded $4 \times 10^6$ bone marrow or spleen cells into one well of a six-well plate. KAAD-cyclopamine (Toronto Research Chemicals) was dissolved as 1000× stock in DMSO. After 72 hr of treatment, cells were plated in methylcellulose medium containing SCF, IL-6, IL-3, and insulin from StemCell Technologies (M3434) according to the manufacturer's instruction. Colonies were counted 5 days and 10 days after plating. After 12 days, cells were diluted from the plates, washed in PBS, and then either stained for analysis of different cell types or replated into a second or third plating round.

### LTC-IC Assays

$2 \times 10^6$ bone marrow cells from healthy donors or CML patients were cultured with irradiated M2-10B4 fibroblasts in MyeloCult medium (StemCell Technologies H5100) with hydrocortisone according to the manufacturer's instructions. In the first week, cyclopamine (5 μM), nilotinib (0.5 μM), a combination of both, or DMSO was added to the culture. Medium containing the compounds was replaced after 7 days. After 5 weeks of culture, adherent and floating cells were harvested and $5 \times 10^4$ cells were plated into MethoCult medium (StemCell Technologies H4435). Numbers of colonies derived from long-term culture-initiating cells (LTC-ICs) were counted after 20 days.

### Immunohistochemistry

Mouse tissues were fixed for at least 24 hr in formalin, and paraffin-embedded tissues were generated after standard procedures. Human tissues were a kind gift from A. Schmitt-Gräff (University of Freiburg, Germany). Single-color DAB-immunoperoxidase staining was performed on paraffin-embedded sections using antibodies to Gli1 (N-16, Santa Cruz Biotechnology), Smo (H-300, Santa Cruz Biotechnology), and Hh (H-160, Santa Cruz Biotechnology) according to the manufacturer's recommendation.

### RT-PCR and Quantitative PCR

RNA was extracted from CD34+ cells from healthy donors or CML patients in the chronic or blast crisis phase of the disease (Table S1). RNA extraction was performed using a QIAGEN RNA extraction kit according to the manufacturer's recommendation. Quantitative PCR was assessed by TaqMan PCR using

a ThermoScript RT-PCR System for First-Strand cDNA Synthesis kit (Invitrogen). Primers and probes were obtained from Applied Biosystems (see Supplemental Experimental Procedures).

### Cell Staining and Sorting

Flow cytometry stainings for analysis of hematological cell types were performed using the antibodies Sca-PE and Kit-APC and the lineage markers CD3, Gr-1, CD11b, CD19, Ter119 all PE-Cy7-pos., CD4-PE, and CD8-APC from BD Pharmingen according to the manufacturer's instructions. For cell-cycle analysis of stem cells, cells were treated with cyclopamine for 48 hr and then stained with lineage markers, Kit-APC, and Sca-PE. Stained bone marrow was fixed in 2% formalin, and cells were permeabilized with 70% chilled ethanol for at least 1 hr and then treated with propidium iodide (5 mg/ml) for at least 30 min. Cells were analyzed using a flow cytometer (Coulter). Annexin staining was performed after incubation of mixed bone marrow with cyclopamine for 24, 48, and 72 hr. Cells were stained with annexin-PE antibody and 7-AAD (BD Biosciences) according to the manufacturer's instructions.

## SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, eight figures, and two tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/14/3/238/DC1/.

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