

# A role for GPx3 in activity of normal and leukemia stem cells

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**The determinants of normal and leukemic stem cell self-renewal remain poorly characterized. We report that expression of the reactive oxygen species (ROS) scavenger glutathione peroxidase 3 (GPx3) positively correlates with the frequency of leukemia stem cells (LSCs) in *Hoxa9+Meis1*-induced leukemias. Compared with a leukemia with a low frequency of LSCs, a leukemia with a high frequency of LSCs showed hypomethylation of the *Gpx3* promoter region, and expressed high levels of *Gpx3* and low levels of ROS. LSCs and normal hematopoietic stem cells (HSCs) engineered to express *Gpx3* short hairpin RNA (shRNA) were much less competitive *in vivo* than control cells. However, progenitor cell proliferation and differentiation was not affected by *Gpx3* shRNA. Consistent with this, HSCs overexpressing *Gpx3* were significantly more competitive than control cells in long-term repopulation experiments, and overexpression of the self-renewal genes *Prdm16* or *Hoxb4* boosted *Gpx3* expression. In human primary acute myeloid leukemia samples, GPX3 expression level directly correlated with adverse prognostic outcome, revealing a potential novel target for the eradication of LSCs.**

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Abbreviations used: AML, acute myeloid leukemia; CSC, cancer stem cell; FL, fetal liver; H<sub>2</sub>DCFDA, dichlorodihydrofluorescein diacetate; HSC, hematopoietic stem cell; LSC, leukemia stem cell; LTR, long-term repopulating; MDA, malondialdehyde; MFI, mean fluorescence intensity; ROS, reactive oxygen species; shRNA, short hairpin RNA.

The cancer stem cell (CSC) hypothesis postulates that most cancers are heterogeneous in their cellular constituents and that self-renewal potential is restricted to a subset of cells within each tumor. The frequency of CSC appears to vary considerably between tumors and also in time within a given cancer. CSCs are best described in human leukemia in which the leukemia stem cells (LSCs) can be prospectively isolated and transmit the disease when introduced in immuno-compromised mice (Lapidot et al., 1994). A detailed understanding of the molecular bases that regulate LSC self-renewal is lacking. Growing evidence indicates that although certain regulators of self-renewal (e.g., *Bmi1*) are shared between normal cells and LSCs (Lessard and Sauvageau, 2003), others (e.g., NF- $\kappa$ B and Wnt/ $\beta$ -catenin) are more specific to LSC (Guzman et al., 2002; Wang et al., 2010).

Variation in oxidative stress has been associated with changes in self-renewal potential of normal hematopoietic stem cells (HSCs;

Shao et al., 2011). By analogy, oxidative stress may also affect LSC self-renewal but the experimental evidence supporting this possibility remains scarce (Konopleva and Jordan, 2011). Using a series of recently characterized mouse acute myeloid leukemia (AML; Wilhelm et al., 2011) in which the LSC frequency varied from  $\sim$ 1 in 100–350 to 1 in 1.4 cells, we now show that *Gpx3*, a reactive oxygen species (ROS) detoxifying enzyme, determines the activity of normal cells and LSCs, extending the function of oxidative stress to regulation of LSC biology *in vivo*.

## RESULTS AND DISCUSSION

### High LSC frequency leukemia expresses elevated GPx3

We reasoned that comparative mRNA profiling of FLA2 (LSC at 70% or 1 in 1.4 cells;

O. Herault and K.J. Hope contributed equally to this paper.

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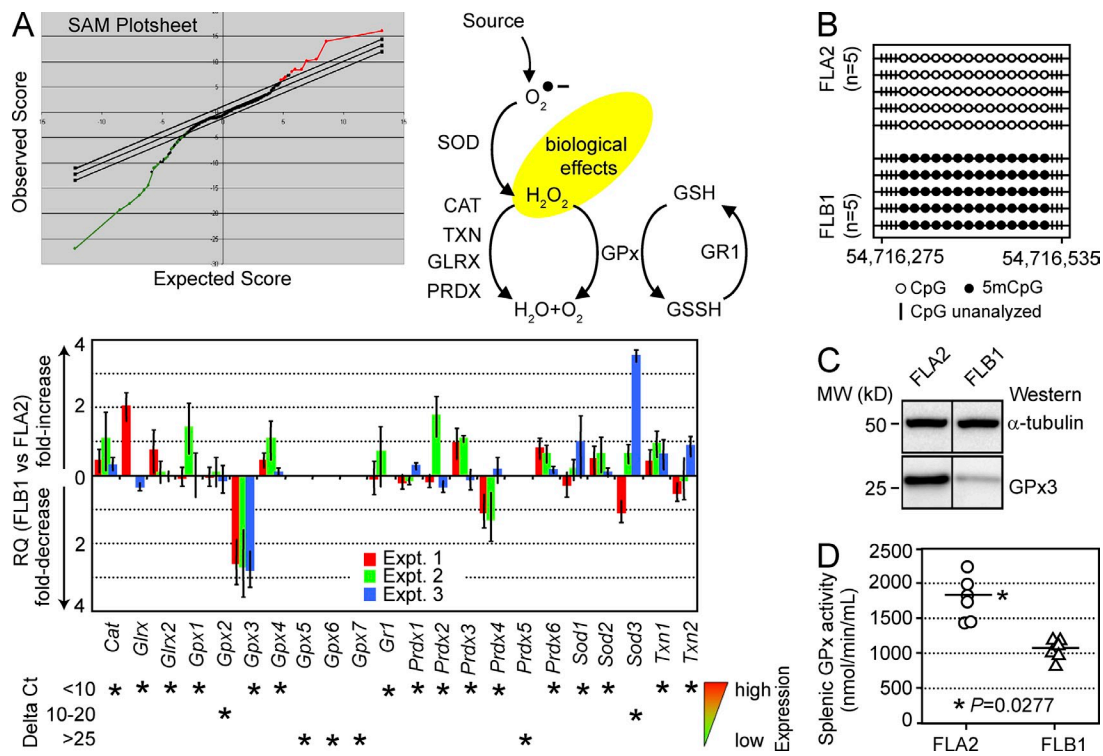
Wilhelm et al., 2011) to the phenotypically similar FLB1 (0.3% LSC) might identify genes uniquely associated with LSC activity (Fig. 1 A). Microarray-based global gene expression profiling identified very few genes—besides some sex chromosome and immunoglobulin light chain (IgK-C) genes—as having differential expression between these two leukemia specimens. After excluding the sex chromosome genes because of the male and female origin of FLA2 and FLB1, only 16 probe sets exhibited significant changes of signal for a false discovery rate of 2.71% (Table S1). In particular, qRT-PCR confirmed a two- to threefold up-regulation of antioxidant enzyme glutathione peroxidase 3 (*Gpx3*) in FLA2 compared with FLB1 (Supplemental Text).

Because redox metabolism has been implicated in normal HSC self-renewal (Ito et al., 2004, 2006; Tothova et al., 2007), we compared the mRNA levels of other antioxidant enzymes implicated in ROS detoxification. In contrast to *Gpx3*, most of these genes are expressed at comparable levels in FLA2 and FLB1 cells (Fig. 1 A), which also express similar

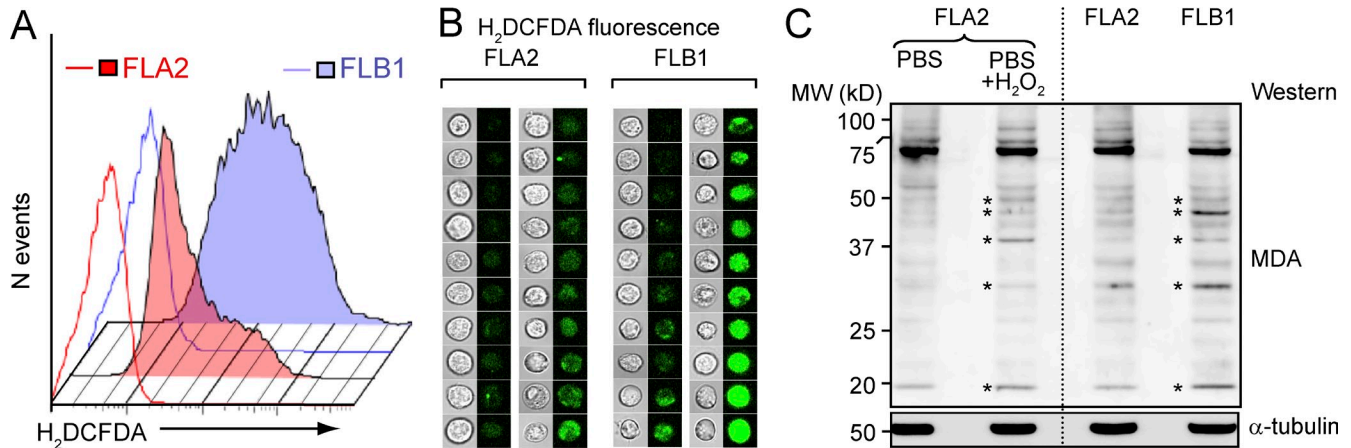
levels of an additional 170 potential regulators of stem cell activity analyzed (Supplemental Text). In accordance with the *Gpx3* overexpression in FLA2, 14/14 tested CpG sequences in the *Gpx3* promoter region were methylated in FLB1 and hypomethylated in FLA2 cells (Fig. 1 B). Higher expression of *Gpx3* in FLA2 compared with FLB1 cells resulted in increased protein levels (Fig. 1 C) and elevated glutathione peroxidase activity ( $1,773 \pm 127$  nmol/min/ml and  $1,077 \pm 60$  nmol/min/ml, respectively; Fig. 1 D).

Flow cytometric comparison of the ROS indicator dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) fluorescence revealed a relative reduction in ROS levels in FLA2 compared with FLB1 leukemia (Fig. 2 A), and confocal analysis confirmed these results (Fig. 2 B). Elevated ROS levels also result in lipid peroxidation and formation of malondialdehyde (MDA) protein adducts, and Western blot analyses demonstrated increase in MDA levels in FLB1 compared with FLA2 cells (Fig. 2 C).

Members of the FoxO subfamily regulate intracellular ROS through transcriptional up-regulation of antioxidative enzymes



**Figure 1. GPx3 is overexpressed in FLA2 cells.** (A) Plot depicting proportions of overexpressed (red points) and underexpressed (green points) genes in FLA2 (high LSC frequency) compared with FLB1 cells (low LSC frequency). RNA isolated from BM cells of leukemic mice ( $n = 3$  per group) was reverse transcribed, and cDNA was hybridized on Affymetrix Mouse Genome 430A microarrays. The stringency of the false discovery rate for the GCRMA-normalized data was adjusted using the SAM software (top left). The antioxidant enzymatic pathway and putative function of each enzyme (top right) is shown. Analyses of antioxidant enzyme expression in FLA2 (high LSC frequency) and FLB1 cells (low LSC frequency) are shown. Results of qRT-PCR assays are presented as FLB1/FLA2 expression ratios (mean  $\pm$  SD,  $n = 3$ ).  $\Delta$ Ct values (versus *Actb*) shown below indicate expression levels of each transcript (bottom). (B) Methylation status of the CpG island in the promoter region of *Gpx3* as determined by sequencing HSO<sub>3</sub>-treated gDNA. Each row represents one leukemic mouse ( $n = 5$  per group). Open circle, nonmethylated CpG; closed circle, methylated CpG. (C) Western blot analysis of GPx3 protein levels in FLA2 and FLB1 cells.  $\alpha$ -Tubulin was used as loading control. Black lines indicate that intervening lanes were spliced out. (D) Quantitation of the GPx (glutathione peroxidase) activity in spleens of FLA2 and FLB1 leukemic mice ( $n = 6$ , two experiments) reveals significant increase ( $P = 0.0277$ ) in splenic GPx activity of FLA2 mice. Spleens of FLA2 and FLB1 leukemic mice had similar weights ( $239 \pm 12$  and  $227 \pm 18$  mg in FLA2 and FLB1 mice, respectively; mean  $\pm$  SD,  $n = 19$ ) and comparable leukemic blast infiltrations (Fig. S1).



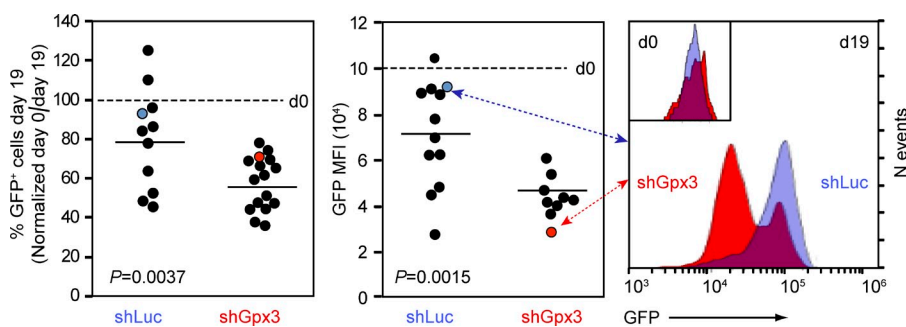
**Figure 2. The ROS level is decreased in FLA2 cells.** (A) Intracellular ROS levels in FLA2 and FLB1 evaluated by flow cytometry using H<sub>2</sub>DCFDA probe immediately after extracting marrow from leukemic mice. Open histograms, DMSO (vehicle control); filled histograms, H<sub>2</sub>DCFDA. (B) Confocal microscopy of FLA2 and FLB1 cells stained with H<sub>2</sub>DCFDA. 20 cells of each leukemia were randomly chosen and classified according to H<sub>2</sub>DCFDA fluorescence. The corresponding phase-contrast pictures are presented. (C) Western blot of MDA-modified protein adducts in FLA2 and FLB1 cells. H<sub>2</sub>O<sub>2</sub>-treated FLA2 cells were used as a positive control and  $\alpha$ -tubulin as a loading control. Asterisks indicate major MDA protein adduct species. The dotted line shows margins of two blots shown in a merged image.

(Kops et al., 2002; Tothova et al., 2007). *FoxO*-deficient HSCs were reported to overexpress several antioxidant enzymes such as *Sod1*, *Sod3*, *Prdx6*, and *Gpx4*, but not *Gpx3* (Tothova et al., 2007). FLA2 and FLB1 leukemias expressed comparable levels of *Foxo3a*, *Foxo4*, and *Foxo6*, whereas *Foxo1* was not expressed (Supplemental Text), indicating that FoxO induction is likely not responsible for differential ROS (Supplemental Text) and suggesting that the only differentially expressed antioxidant enzyme GPx3 may be responsible for the decreased ROS observed in FLA2 leukemia.

**GPx3 promotes LSC in vivo competitiveness**

Correlation between GPx3 levels and LSC frequency could reflect functional dependence of LSC on this enzyme. We exploited retroviruses encoding short hairpin RNAs (shRNAs) and IRES-GFP reporter to explore the in vivo activity of FLA2 cells after GPx3 knockdown (Fig. 3 A). Gene transfer

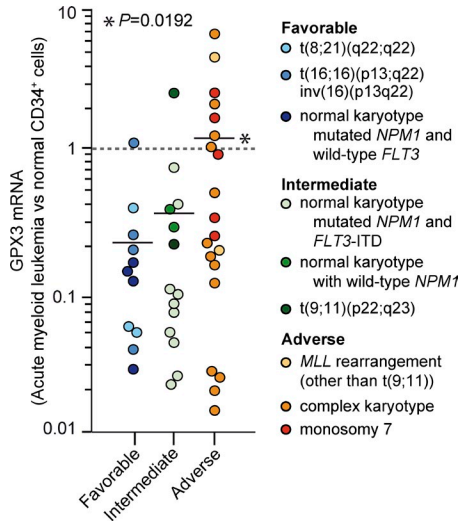
in these experiments ranged from 42 to 66%, and because FLA2 cells poorly survive cell sorting, untransduced leukemia-initiating cells were co-transplanted together with transduced cells. This resulted in competitive repopulation experiments in which both the GFP<sup>+</sup> shRNA (either Luciferase or Gpx3)-transduced and GFP<sup>-</sup> untransduced FLA2 cells contributed to leukemia development in vivo. To assess the effect of shRNA treatment on relative contribution of each cell population to clinical leukemia 19 d later, we compared changes in proportions (Fig. 3 B, left) and mean fluorescence intensity (MFI; Fig. 3 B, middle and right) of GFP<sup>+</sup> FLA2 cells at two different time points (t = 0 and 19 d). The decrease in proportion of GFP<sup>+</sup> FLA2 cells contributing to fulminant leukemia (~19 d) below the values determined for the initial transplant was at least twofold higher after Gpx3 knockdown compared with shLuc control (Fig. 3 B, left). Moreover, the shGpx3-infected FLA2 cells remaining at day



**Figure 3. Gpx3 depletion decreased LSC competitiveness.** Left: effect of the RNAi-mediated depletion of Gpx3 on the competitiveness of FLA2 leukemic cells. 2 × 10<sup>5</sup> fresh FLA2 BM cells were co-cultured with shLuc or shGpx3 retrovirus-producing GP+E-86 cells. After infection (d0), cells were partitioned between flow cytometric analysis of GFP<sup>+</sup> frequency and transplantation of unsorted cells into sublethally irradiated recipients (shLuc, n = 12; shGpx3, n = 16). Contribution of GFP<sup>+</sup> (shRNA-transduced) cells to leukemia was determined on day 19. Results are shown

as normalized values: the GFP<sup>+</sup> frequency in inoculum was considered 100% (dotted line, d0), and the day 19 GFP<sup>+</sup> frequency was calculated as a percentage of that value. Horizontal bars represent mean values determined for each experimental group. Middle: day 19 MFIs of GFP<sup>+</sup> cells in recipients of shLuc- and shGpx3-infected FLA2 leukemic cells. The dotted line represents MFI of GFP<sup>+</sup> cells in shLuc and shGpx3 inocula. Horizontal bars represent mean values determined for each experimental group. Right: GFP fluorescence intensity in representative recipients of shLuc- or shGpx3-infected FLA2 cells. The inset demonstrates comparable MFI GFP fluorescence in shLuc and shGpx3 inocula. \*, P = 0.0037 and P = 0.0015, respectively (Wilcoxon's test).





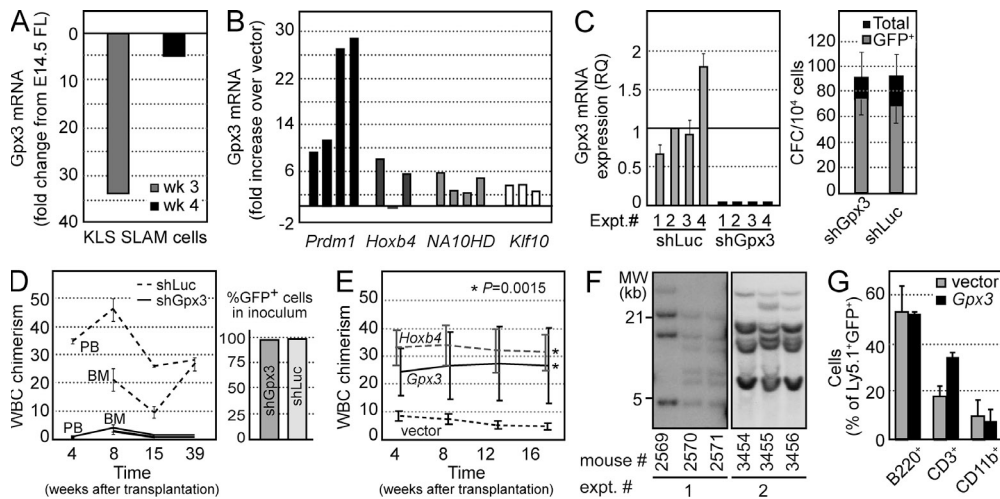
**Figure 4. GPX3 expression in marrow samples from AML patients classified into cytogenetic and molecular risk subgroups.** Expression of *GPX3* was quantified by qRT-PCR ( $\Delta\text{Ct}$  method versus *GAPDH* and *HPRT*) in marrow leukemic cells (leukoblasts > 95%) from AML patients and FACS-sorted marrow  $\text{CD34}^+$  cells from healthy volunteers. Results are presented as relative expression ( $\text{RQ} = 2^{-\Delta\Delta\text{Ct}}$ ) of *GPX3* versus normal marrow  $\text{CD34}^+$  cells (dotted line,  $\Delta\text{Ct} = 10.10 \pm 0.58$ ,  $n = 5$  for normal marrow  $\text{CD34}^+$  cells). Horizontal bars represent mean *GPX3* levels determined for individual risk subgroup. \*,  $P = 0.0192$  (adverse versus favorable risk subgroups; Wilcoxon's test).

19 displayed a significant reduction in MFI of GFP compared with controls (Fig. 3 B, middle and right). These results indicate that  $\text{GFP}^{\text{high}}$  cells were selectively depleted (Fig. 3 B, right, inset), and suggest that *Gpx3* is critical for the competitiveness of LSCs.

We quantified *GPX3* expression in 48 acute myeloid leukemia patients categorized into cytogenetically and molecularly defined risk subgroups designated as favorable, intermediate, and adverse (Mrózek et al., 2007). Within the AML samples, expression of *GPX3* increased with the severity of disease, and the highest expression levels were associated with adverse risk group of patients ( $P = 0.019$ ; Fig. 4). The worst clinical outcomes were thus observed in AML enriched for phenotypically defined LSC (van Rhenen et al., 2005) and high *GPX3* levels, indicating that *GPX3* may be relevant for human leukemia.

**Gpx3 influences normal HSC activity**

Adult BM-derived HSCs are less proliferative than their fetal liver (FL)-derived counterparts (Rebel et al., 1996). Quantitative RT-PCR (qRT-PCR) assays revealed that HSC isolated from BM of 3- and 4-wk-old mice express 39- to 6-fold, respectively, lower *Gpx3* levels than HSC sorted from embryonic day 14.5 (E14.5) FL (Fig. 5.A), suggesting a correlation between *Gpx3* expression and HSC activity. Interestingly, *Gpx3* levels were also elevated in BM HSC/progenitor cells



**Figure 5. Gpx3 regulates the self-renewal capacity of normal HSCs.** (A) *Gpx3* expression in HSC isolated from 3- or 4-wk-old mice (KLS SLAM cells) compared with HSC from E14.5 FL (SLAM  $\text{Sca}^+\text{Mac}^+$ ) cells. Mean  $\pm$  SD,  $n = 2$ . (B) Empty vector ( $n = 3$ ), *Prdm16* ( $n = 4$ ), *Hoxb4* ( $n = 3$ ), *NA10HD* ( $n = 4$ ), and *Klf10* ( $n = 3$ ) were overexpressed in  $\text{Lin}^- \text{CD150}^+ \text{CD48}^-$  sorted BM cells as previously described (Deneault et al., 2009). *Gpx3* mRNA was evaluated by qRT-PCR. (C) 5-FU-treated BM cells were transduced with shLuc or shGpx3. *Gpx3* mRNA was evaluated by qRT-PCR in four experiments (left). CFC output was assessed in two experiments (right; mean  $\pm$  SD;  $n = 8$ ).  $\text{GFP}^+$  cells were shRNA-transduced cells. (D) Evaluation of short-term and LTR ability of KLS  $\text{CD150}^+ \text{CD48}^- \text{Ly5.1}^+$  BM cells expressing shLuc or shGpx3 in competitive transplantations into congenic Ly5.2 mice. Results are presented from both peripheral blood (PB) and BM, and represent mean  $\pm$  SEM at the indicated time points for 8 mice per group. The level of gene transfer was near 100% (right) and *Gpx3* silencing was obtained in all experiments ( $n = 4$ ). Two independent shGpx3 constructs (#5 and #6) were used. (E) *Gpx3*, *Hoxb4*, or empty vector was overexpressed in BM KLS  $\text{CD150}^+ \text{CD48}^-$  sorted cells (as described in Deneault et al., 2009), cells were expanded for 7 d ex vivo, and competitive transplantations in Ly5.2 congenic mice were performed. Results are shown as mean  $\pm$  SEM of peripheral blood reconstitution at the indicated time points for three mice per group in five experiments. \*,  $P = 0.0015$  (Wilcoxon's test). (F) Southern blot analyses of *Gpx3* proviral integrations in DNA isolated from BM from mice reconstituted 20 wk earlier with *Gpx3*-transduced cells.  $n = 2$  experiments, three mice per experiment. (G) Analysis of differentiation potential of vector or *Gpx3*-transduced cells in vivo. Blood cells were collected from recipients transplanted 20 wk earlier and stained with antibodies specific for B, T, and myeloid cells (B220, CD3, and CD11b, respectively). Values are mean  $\pm$  SD (vector:  $n = 5$ ; *Gpx3*,  $n = 2$ ).

engineered to overexpress genes associated with self-renewal such as *Hoxb4*, *NA10HD*, *Klf10*, and *Prdm16* (Sauvageau et al., 1995; Ohta et al., 2007; Deneault et al., 2009). In these transduced cells, levels of *Gpx3* expression increased 3.2- to 19.2-fold compared with controls (Fig. 5 B).

We next infected BM HSC/progenitor cells with retroviruses carrying two different *Gpx3* hairpins with the ability to achieve ~95% *Gpx3* knockdown (Fig. 5 C, left). Clonogenic progenitor cell activity was not affected by this decrease in *Gpx3* expression (Fig. 5 C, right), and there was no increase in apoptosis in the shGpx3 cell population compared with shLuc controls (not depicted). shRNA-mediated *Gpx3* silencing, however, dramatically impeded the ability of transplanted HSC to repopulate the hematopoietic system of recipient mice at early (8 wk) and late (39 wk) time points after transplantation (Fig. 5 D). We also transplanted the shGpx3- and shLuc-transduced 5-FU-derived BM cells and analyzed their ability to home in the BM 48 h after injection. These experiments showed no difference between homing abilities of shGpx3- and shLuc-transduced cells. Together, these results identify *Gpx3* as a critical regulator of short- and long-term repopulating (LTR) HSC.

In reciprocal gain-of-function experiments, BM HSC/progenitor cells engineered to overexpress *Gpx3* showed a marked competitive advantage over controls when transplanted after a 7-d ex vivo culture step (Fig. 5 E). Proviral integration analyses of six recipients confirmed polyclonal hematopoiesis (Fig. 5 F), thus ruling out the possibility of insertional mutagenesis. Moreover, some mice (#2570 and #2571; Fig. 5 F) were reconstituted by the same clones, indicating that self-renewal occurred in vitro before transplantation. GFP<sup>+</sup> *Gpx3*-transduced cells contributed to production of lymphoid and myeloid peripheral blood leukocytes at >30 wk after transplantation, confirming the multipotential nature of the reconstituting clones (Fig. 5 G). These mice were hematologically normal and showed no evidence of myeloproliferation or leukemia. Together, these results indicate that *Gpx3* enhances HSC expansion ex vivo possibly through modulation of self-renewal activity. Unfortunately, such gain-of-function experiments were not possible with our FLB1 leukemia model as we have not yet identified the in vitro conditions that would allow their effective transduction.

In summary, identification of an antioxidant enzyme such as GPx3 acting as a stem cell activity factor in leukemia raises the possibility that other ROS scavenging systems have regulatory importance in other CSS. A low ROS level in breast CSCs has recently been associated with increased expression of *Foxo1* and the glutathione biosynthesis genes *Gss* and *Gdm* (Diehn et al., 2009). In normal hematopoiesis, ROS<sup>low</sup> HSCs have a higher self-renewal potential than ROS<sup>high</sup> HSCs (Jang and Sharkis, 2007), whereas loss of ATM (ataxia telangiectasia mutated) or a FoxO transcription factor correlates with elevated ROS levels and limits self-renewal capacity of HSC during serial transplantations (Ito et al., 2004, 2006; Tothova et al., 2007). ROS can function as signaling molecules in several cell types (Finkel and Holbrook, 2000;

Owusu-Ansah et al., 2008; Takeda et al., 2008) and can modulate the activation of signal transduction pathways involved in cellular proliferation and differentiation (Sundaresan et al., 1995; Sattler et al., 1999; Owusu-Ansah and Banerjee, 2009).

Although the current study highlights the importance of GPx3 in normal and leukemia hematopoiesis, the implication of ROS as the key modulator of GPx3 activity has not been established. This is particularly critical in the context that ROS scavenger (*N*-acetyl-L-cysteine treatment) and p38 inhibition failed to consistently increase LSC frequency of FLB1 leukemia in vivo (unpublished data). Nonetheless, our results indicate that in some conditions (*Hoxb4* or *Prdm16* gain of function) *Gpx3* expression may represent a converging pathway that determines the activity of normal and leukemic stem cells.

## MATERIALS AND METHODS

**Mice.** Donor C57BL/6:Pep3b (Ly5.1) and recipient C57BL/6 (Ly5.2) mice were bred and maintained as previously described (Kroon et al., 1998). Experimental procedures were revised and approved by the University of Montreal animal ethics committee (Comité de Déontologie de l'Expérimentation sur les Animaux de l'Université de Montréal).

**Human cells.** AML tumor samples and normal marrow samples were obtained from the Quebec Leukemia Cell Bank (Montreal, Quebec) and the Orthopedic Surgery Department of the University Hospital of Tours (Tours, France), respectively. All these cells were obtained according to approved protocols of the institutional review boards after informed consent. Patients were classified in three risk groups for cytogenetic and molecular abnormalities (Mrózek et al., 2007).

**Isolation of mouse hematopoietic cell subpopulations.** Cells were first purified for Sca1 using magnetic activated cell sorting as per the manufacturer's instructions (Miltenyi Biotec). Further antibody staining and sorting to purify the c-Kit<sup>+</sup>, lineage<sup>-</sup> (CD45R<sup>-</sup>, CD3<sup>-</sup>, Gr-1<sup>-</sup>, Ter119<sup>-</sup>) population were performed as described previously (Okada et al., 1992). The LTR-HSC from BM (c-Kit<sup>+</sup>Lin<sup>-</sup>Sca1<sup>+</sup>CD150<sup>+</sup>CD48<sup>-</sup>, KLS SLAM) or E14.5 d.p.c. FL (CD150<sup>+</sup>CD48<sup>-</sup>Sca1<sup>+</sup>Mac1<sup>+</sup>Lin<sup>-</sup>, SLAM Sca<sup>+</sup>Mac<sup>+</sup>), and HSC/progenitor cells (CD150<sup>+</sup>CD48<sup>-</sup>Lin<sup>-</sup>) from BM were sorted using FACS-Aria cytometer (BD).

**Retroviral infection and transplantation.** Infection and transplantation procedures were performed as previously described (Thorsteinsdottir et al., 1999). Infection medium consisted of DMEM, 15% FCS, 100 ng/ml IL-11, 50 ng/ml Flt-3 ligand, 100 ng/ml SF, 10 µg/ml ciprofloxacin (Serologicals), and 6 µg/ml polybrene (hexadimethrine bromide; Sigma-Aldrich).

**Methylcellulose cultures.** Progenitor assays were plated and scored as previously described (Kroon et al., 1998).

**Competitive repopulation unit enumeration assays.** Stem cell quantification assays were performed essentially as previously described (Antonchuk et al., 2001).

**Fluorescence studies.** The studies were performed with monoclonal antibodies specific for the following: Sca-1 (D7), Mac1 (M1/70), and Gr-1 (RB6-8C5; BD); kit (2B8), CD48 (HM48-1), and CD45R (RA3-6B2; eBioscience); and CD150 (TC15-12F12.2), B220 (30-F11), CD3 (17A2), CD11b (M1/70), and TER-119 (BioLegend). Staining with H<sub>2</sub>DCFDA was performed according to the manufacturer's instructions (Invitrogen) using fresh, not purified leukemic cells immediately after BM harvest. The peak excitation wavelength for oxidized H<sub>2</sub>DCFDA was 488 nm, and emission was 525 nm. Flow cytometry and confocal analyses were performed

using an LSRII cytometer (BD) and an LSM 510 laser-scanning confocal microscope (Carl Zeiss), respectively.

**DNA and RNA preparation.** Genomic DNA was isolated using DNazol and total cellular RNA with TRIzol, according to the manufacturer's instructions (Invitrogen).

**Southern blot analysis.** All blots were prepared and analyzed as previously described (Kroon et al., 1998).

**Bisulfite conversion and sequencing.** Genomic DNA was converted using the methylSEQR Bisulfite Conversion kit (Applied Biosystems) according to the manufacturer's instructions. The *Gpx3* region was amplified by PCR and sequenced using an M13 tail (BigDye Terminator v3.1; Applied Biosystems). Purified sequencing products were analyzed on a 3730 Genetic Analyzer (Applied Biosystems).

**Hybridization.** RNA samples were sent to the microarray facility at the Ottawa Hospital Research Institute (Ottawa, ON), where the RNA was reverse transcribed, labeled, and hybridized to Affymetrix 430A arrays according to manufacturer's protocols.

**Affymetrix gene expression profiling.** Affymetrix CEL files from biological triplicates for FLA2 and FLB1 were normalized using either RMA or GCRMA, using the LIMMA (Linear Modes for MicroArray data) package for R/Bioconductor. Normalized data were then used to compute a linear model to derive differentially expressed genes, or exported to perform SAM (significance analysis of microarrays) analysis. SAM analysis on both normalized datasets was performed using version 3.01 of the SAM software, with 100 permutations and a twofold minimum change in both cases. Using delta calibration values of 1.236 (GCRMA) and 0.567 (RMA), lists of 33 or 31 genes which showed differential expression (22 genes in common) were generated with false discovery rates of 2.71 and 3.31%, respectively. The ArrayExpress accession no. is E-MEXP-1597.

**qRT-PCR.** qRT-PCR was performed using two strategies: the SYBR green dye and Universal ProbeLibrary assays designed with the ProbeFinder software (Roche) as described using *Hprt*, *Actb*, *GAPDH*, or *HPRT* as endogenous controls. Ct values were determined using the advanced relative quantification algorithm for each target ( $C_{t_{\text{target}}}$ ) and endogenous control reference genes ( $C_{t_{\text{reference}}}$ ). The expression level of the target gene was expressed as  $\Delta C_t$  value ( $\Delta C_t = C_{t_{\text{target}}} - C_{t_{\text{reference}}}$ ). RQ (relative quantities) were calculated as  $RQ = (\Delta C_{t_{\text{test population}}} / \Delta C_{t_{\text{calibrating population}}})$ . Calibrators used for calculation of various RQ values are indicated in the corresponding figure legends. To ensure that our qRT-PCR analyses accurately reflected expression of genes encoding antioxidant enzymes in the LTR-HSC compartment, only sorted HSC populations comprised of ~50% of LTR-HSC as determined by competitive CRU were processed for cDNA synthesis and gene expression studies presented in Fig. 5 A.

**Protein extraction and immunoblots.** Cells were lysed in 10 mmol/liter Hepes, pH 7.9, 10 mmol/liter KCl, 0.1 mmol/liter EDTA, 0.1 mmol/liter EGTA, and 10% (vol/vol) NP-40 containing protease inhibitors. Western blot analyses were done as previously described (Krosl and Sauvageau, 2000) using antibodies against GPx3 (R&D Systems) and MDA (Abcam).

**Glutathione peroxidase assay.** The enzymatic activity of glutathione peroxidase in the spleen of mice was quantified using the Glutathione Peroxidase Assay kit (Cayman Chemical Company) according to the manufacturer's recommendations.

**Construction of shRNA retroviral vectors.** Two shRNAs were selected as single-stranded oligonucleotides also incorporating mir-30 flanking arms using the RNAi Central shRNA psm2 design tool (G. Hannon, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Amplicons were digested with

XhoI-EcoRI (Invitrogen) and subcloned into the LMP vector according to the manufacturer's guidelines (Invitrogen). Confirmatory sequencing was performed on all cloned shRNAs: shGpx3 #5, 5'-TGCTGTTGACAGT-GAGCGCTTCTACACTTTCCTGAAGAAGTAGTGAGCCACA-GATGTAGTTCTTCAGGAAAGTGTAGAATTGCCTACTGCCTC-GGA-3'; shGpx3 #6, 5'-TGCTGTTGACAGT-GAGCGCGCAGTATG-CAGGCAAATATATTAGTGAAGCCACAGATGTAATATATTTGCCT-GCATACTGCTTGCCTACTGCCTCGGA-3'; and shLuc, 5'-TGCT-GTTGACAGT-GAGCGCCGATATGGCTGAATACAAATTAGTGAA-GCCACAGATGTAATTTGTATTAGCCCATATCGTTGCCTACTG-CCTCGGA-3'.

**Apoptosis assay.** Phosphatidylserine externalization was analyzed 4 d after retroviral infection by labeling GFP<sup>+</sup> cells with annexin V-Alexa Fluor 350 and propidium iodide. Flow cytometry analyses were performed using an LSRII cytometer.

**Homings assay.** After retroviral infection, Ly5.1<sup>+</sup> cells were transplanted via tail vein injection into irradiated (1,600 cGy, 137 Cs gamma radiation) C57BL/6J (Ly5.2) mice. A small aliquot of cells was plated at this time in methylcellulose to assess the percentage of transduced CFCs in the inoculum (see Methylcellulose cultures). 4 d after transplant, recipient BM was harvested and the percentage of GFP<sup>+</sup>Ly5.1<sup>+</sup> cells was assessed by flow cytometry with the acquisition of a minimum of 10<sup>6</sup> events (LSRII cytometer). BM was also plated in methylcellulose for later enumeration and scoring of GFP<sup>+</sup> and GFP<sup>-</sup> CFCs (see Methylcellulose cultures).

**Statistical analyses.** Statistical analyses were performed using Wilcoxon's test. The level of significance was set at 0.05.

**Online supplemental material.** Fig. S1 shows representative images of spleens from recipients of FLA2 and FLB1 cells at 19 d after transplantation. Table S1 shows FLA2 gene expression profiling. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20102386/DC1>.

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