Haematopoietic ESL-1 enables stem cell proliferation in the bone marrow by limiting TGFβ availability

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The life-long maintenance of haematopoietic stem and progenitor cells (HSPCs) critically relies on environmental signals produced by cells that constitute the haematopoietic niche. Here we report a cell-intrinsic mechanism whereby haematopoietic cells limit proliferation within the bone marrow, and show that this pathway is repressed by E-selectin ligand 1 (ESL-1). Mice deficient in ESL-1 display aberrant HSPC quiescence, expansion of the immature pool and reduction in niche size. Remarkably, the traits were transplantable and dominant when mutant and wild-type precursors coexisted in the same environment, but were independent of E-selectin, the vascular receptor for ESL-1. Instead, quiescence is generated by unrestrained production of the cytokine TGFβ by mutant HSPC, and in vivo or in vitro blockade of the cytokine completely restores the homeostatic properties of the haematopoietic niche. These findings reveal that haematopoietic cells, including the more primitive compartment, can actively shape their own environment.
Quiescence, an essential feature of haematopoietic stem cells (HSCs), is thought to prevent exhaustion of the most primitive compartment and to ensure protection from environmental stress and DNA-damaging agents. Imaging and computational analyses have revealed that mesenchymal perivascular cells around bone marrow (BM) arterioles promote cycle arrest on HSCs. These arteriolar niches are in turn innervated by nerves ensheathed by Schwann cells, which also contribute to cycle arrest and preservation of HSC in vivo. To preserve a homeostatic balance, however, the environment must also provide HSC with proliferative signals. Among niche cells, endothelial cells promote HSC proliferation through E-selectin. Whether proliferative regulation can also originate from non-stromal cells, including haematopoietic cells that are an integral part of the BM niche, remains an outstanding question.

HSC, which by definition are located within the niche, can produce cytokines that impact their own proliferation and differentiation. Among these cytokines, transforming growth factor β (TGFβ) has been implicated in the regulation of haematopoietic stem and progenitor cell (HSPC) cycling by inhibiting lipid raft clustering and cytokine signalling. The impact of this cytokine for the in vivo maintenance of HSC is highlighted by the loss of both quiescence and function of HSC-lacking the TGFβ receptor II, or by analysis of animals in which TGFβ-producing Schwann cells were eliminated by sympathetic denervation. Defining the mechanisms that regulate TGFβ production is therefore essential to understand how maintenance of HSPC in ensured in vivo.

In this study, while searching for the ligand responsible for relaying the proliferative signals that emerge from vascular-borne E-selectin on HSC, we found that the absence of E-selectin ligand 1 (ESL-1), a major ligand for E-selectin on haematopoietic cells, results in global quiescence in the primitive haematopoietic compartment. Unexpectedly, we show that ESL-1 maintains homeostatic proliferation within the BM not by engaging E-selectin, but by repressing the production of TGFβ by haematopoietic cells, including HSC.

**Results**

**ESL-1-deficiency causes HSPC quiescence and expansion.** E-selectin expressed by vascular cells induces homeostatic proliferation of HSC by signalling through unknown receptor(s) on haematopoietic precursors that are distinct from its canonical ligands PSGL-1 and CD44 (ref. 4). We hypothesized that ESL-1 could be endowed with the capacity to transduce the proliferative signals from the BM vasculature because it is a dominant E-selectin ligand of haematopoietic progenitors, in which it promotes homing to the BM. Indeed, we could detect abundant ESL-1 transcript and protein in phenotypically immature HSC (Supplementary Fig. 1a,b), suggesting that ESL-1 might be functionally relevant in the most primitive haematopoietic compartment.

To test the possibility that ESL-1 promoted HSPC proliferation, we measured cycling of haematopoietic progenitors in mice deficient in ESL-1 (Glg1−/−) and heterozygous littermates using 5-bromodeoxyuridine (BrdU) incorporation assays. Glg1−/− mutants displayed reductions in the frequencies of proliferating Lineage−/−cKit+Scal−/− (LSK) cells and the more primitive LSK CD48+/− and LSK CD48+/−CD150+ precursors (Fig. 1a). We obtained independent confirmation of a reduced frequency of cycling Glg1−/− LSK CD48+/−CD150+ precursors by staining for Ki67, a nuclear marker of cell cycling (Supplementary Fig. 1c).

In parallel to cycle arrest, we found an increase in the frequency of myeloid progenitors (Lineage−/−cKit+Scal−/−; MPs) and LSK cells, as well as in primitive LSK CD135−/−CD90+ cells (LT-HSC) in these mutants (Fig. 1b). Contrasting with the expansion of immature precursors, we did not find increased numbers of mature myeloid cells in the BM of ESL-1-deficient mice, with the exception of resident macrophages which were expanded (Supplementary Fig. 2a). Blood leukocyte counts were slightly elevated but normal for erythrocyte and platelet numbers, and the mice presented a mild splenomegaly (Supplementary Fig. 2b). Collectively, these data indicated that ESL-1 controls HSPC cycling and numbers, suggesting that ESL-1 might be the E-selectin ligand responsible for transducing proliferative signals in vivo.

**Quiescence of Glg1−/− HSPC is transplantable and dominant.** We reasoned that if ESL-1 triggered HSPC proliferation by engaging E-selectin, then reconstitution of Glg1−/− haematopoiesis into WT recipient mice should recapitulate the phenotype of the full mutants. If this was the case, these haematopoietic chimeras would also allow a more thorough examination of whether ESL-1 gives the extremophile low birth rate of Glg1−/− mice (<2% of the 25% expected from heterozygous breeders). WT mice reconstituted with Glg1−/− donor cells recovered well, with blood counts and splenomegaly similar to full mutants, and BM cellularity similar to mice transplanted with WT donors (Supplementary Fig. 2c). As predicted, mice reconstituted with mutant cells reproduced the marked reductions in proliferating LSK and MP cells (Fig. 1c), as well as primitive LSK CD48+/− and LSK CD48+/−CD150+ cells (Fig. 1d); in contrast, mice deficient in a different E-selectin ligand, PSGL-1 (Selplg−/−), only displayed mild reductions in proliferation that were restricted to LSK cells (Fig. 1c). Importantly, mice reconstituted from Glg1−/− donors also displayed increased quiescence as shown by reductions in Ki67 staining in LSK cells (Supplementary Fig. 2d) and increased number of surviving reconstituting HSPC after hydroxyurea treatment (~4-fold increase) that was markedly higher than that seen in untreated mice (Supplementary Fig. 2e). Transplanted mice reproduced the expansion in Glg1−/− mice of primitive progenitors (Fig. 1e), including a ~2.5-fold increase in the number of functional HSC as assessed by long-term reconstitution using limiting dilution assays (Supplementary Fig. 2f). The dramatic increase in Glg1−/− repopulating units in the hydroxyurea suicide assay over the wild-type (WT) group confirmed elevated quiescence of mutant HSPC even if the starting number of progenitors was higher in mice reconstituted with Glg1−/− BM cells. To test the functional consequence of quiescence in Glg1−/− progenitors, we induced haematopoietic exhaustion by repeated administration of the chemotherapeutic agent 5-fluorouracil (5-FU). Although the majority of mice reconstituted from WT donors succumbed by 8 weeks, mice transplanted with Glg1−/− marrow showed a dramatic resistance to exhaustion and death (Fig. 1f). These results provide functional support to the observation that ESL-1 promotes homeostatic proliferation of HSPC.

To delineate whether the phenotype was cell-autonomous as predicted by our model, we co-transplanted WT (expressing the DsRed reporter gene) together with Glg1−/− BM cells into WT recipients, and after 8 weeks analysed HSPC proliferation (Fig. 2a). Unexpectedly, not only ESL-1-deficient progenitors displayed the expected reductions in proliferation, but WT progenitors sharing the same host as Glg1−/− mutants displayed identical reductions, which were not seen when the co-transplanted cells were of WT origin (Fig. 2b,c). These observations indicated that the proliferative phenotype of ESL-1-deficient haematopoietic precursors was transplantable, and that ESL-1 cells transferred their proliferative trait to genetically normal HSPC that share the same microenvironment.
Elevated TGFβ triggers quiescence in the absence of ESL-1. The dominant phenotype of Glg1−/− mutants indicated that E-selectin-derived signals could not fully account for the proliferative arrest. We thus searched for alternative mechanisms for this unexpected phenotype. ESL-1 has been shown to limit the maturation and secretion in chondrocytes of TGBβ8, a cytokine with potent anti-proliferative effects on HSC9,10. We thus tested whether abnormal TGFβ signalling might account for the proliferative arrest observed in the absence of ESL-1. In agreement with this possibility, TGFβ protein levels were elevated in mice reconstituted with Glg1−/− marrow, as well as in full Glg1−/− mutants (Fig. 3a and Supplementary Fig. 3a), and these elevations correlated with enhanced phosphorylation of Smad2/3 in LSK cells (Fig. 3b and Supplementary Fig. 3b). In addition, transcriptomic analyses of WT and mutant HSPC revealed a pattern of expression of cycle inhibitory genes (Fig. 3c) that was fully consistent with the temporal activation of the TGFβ signalling pathway in the context of BM regeneration11. To determine whether elevated TGFβ signalling was responsible for HSPC quiescence in the absence of ESL-1, we treated mice transplanted with mutant or WT BM with an anti-TGFβ antibody that efficiently blocks the cytokine in vivo12. Inhibition of TGFβ for 2 weeks resulted in restoration of HSPC proliferation in Glg1−/− mutants as measured by BrdU incorporation and cell cycle analyses (Fig. 3d,e). Importantly, TGFβ inhibition also restored progenitor numbers (Fig. 3f).
NS, not significant. Each circle represents a mouse. Bar graphs show mean chimeric mice by bone marrow transplantation. (or LinNEG cKit haematopoietic exhaustion upon chronic stress. Interestingly, our findings did not rule out a possible coordination between vascular ESL-1 controls proliferation independently of E-selectin. We therefore examined the distribution of LinNEG CD48NEG CD150 + cells or LinNEG cKit + progenitors in the BM of mice reconstituted with WT or Glg1−/− donors (Fig. 4a,b and Supplementary Fig. 4), and scored the distance between each progenitor and the nearest vascular structure. We found that WT and Glg1−/− progenitors displayed identical distribution within the marrow (Fig. 4a,b and Supplementary Fig. 4), indicating that alterations in progenitor distribution were unlikely to account for the proliferative defects of mutant mice. To further examine whether E-selectin and ESL-1 functioned along the same pathway to prevent quiescence, we generated mixed chimeric mice using WT-DsRed + and Glg1−/− donors that were co-transplanted into WT mice (Fig. 4c). Using this approach, we generated two coexisting populations of HSPC displaying similar proliferative defects (Fig. 2), in which we tested the capacity of TGFβ blockade to restore proliferation. If E-selectin signalled through ESL-1, we predicted that this treatment should rescue proliferation only in WT HSPC. TGFβ blockade, however, restored proliferation of both populations of HSPC (Fig. 4c). In addition, blockade of TGFβ in E-selectin-deficient recipients (Supplementary Fig. 5) or blockade of both E-selectin and TGFβ in WT recipients (Fig. 4c) failed to restore proliferation of WT or Glg1−/− HSPC. These experiments demonstrated that E-selectin and ESL-1 control proliferation through different mechanisms.

Haematopoietic precursors are a relevant source of TGFβ. The transplantation experiments suggested that the elevations in the levels of TGFβ in the Glg1−/− group originated from the haematopoietic compartment. To search for the relevant source of TGFβ among haematopoietic cells, we measured ESL-1 protein in multiple BM populations by western blot. Myeloid leukocytes and immature progenitors displayed the highest levels of ESL-1 (Supplementary Fig. 6a). Because BM-resident macrophages were expanded in Glg1−/− mice (Supplementary Fig. 2a) and are a potential source of TGFβ, we depleted these cells using clodronate liposomes and measured the impact on HSPC proliferation. Despite efficient depletion of macrophages (Supplementary Fig. 6b), this treatment failed to restore HSPC proliferation of Glg1−/− HSPC (Fig. 5a), thus excluding these cells as a relevant source of TGFβ.

We then focused on immature haematopoietic precursors, which also expressed high levels of intracellular ESL-1 (Supplementary Fig. 7a) and produce TGFβ. Given the lack of tools for specific genetic or pharmacological manipulation of HSPC in vivo, we performed ex vivo analyses. We first noticed that Glg1−/− marrow cells grown in semisolid cultures yielded more CFU-C (Fig. 5b) that contained, however, less cells than WT colonies (Supplementary Fig. 7b). Treatment with the TGFβRI inhibitor LY-2157299 reverted the cellularity of Glg1−/− colonies without affecting WT-derived CFU-C (Supplementary Fig. 7b). Glg1−/− cultures also yielded an increase in the frequency of LinNEG cKitHI progenitors after 7 days of culture, which could also be reverted by the TGFβRI inhibitor (one-way analysis of variance with Tukey’s multigroup test; Fig. 5c), and replating assays resulted in elevated numbers of secondary colonies, suggesting a higher self-renewal ability of Glg1−/− progenitors (Fig. 5d). We could reproduce these results with sort-purified LSK cells, whose proliferative capacity was
Figure 3 | ESL-1 controls HSPC proliferation by repressing TGFβ secretion. (a) TGFβ levels in the BM of WT and Glg1−/−-transplanted mice; n = 8. (b) Micrographs of purified WT and Glg1−/− LSK cells stained for pSMAD2/3 (red) and DAPI (blue). Scale bar, 5 μm. Data are from two independent experiments. (c) Expression of p18, p21 and p57 in sort-purified WT and Glg1−/− LSK cells; n = 6–8. (d) Representative contour plots of BrdU incorporation in WT and Glg1−/− LSK cells and MP treated with anti-TGFβ or control antibody. Right, experimental design (top) and bar graphs (bottom) show the percentage of BrdU− cells in the different groups; n = 5. Each circle represents a mouse. (e) Representative plots and quantification of cell cycle analyses of WT and Glg1−/− LSK cells and MP from mice treated with anti-TGFβ or control antibody; n = 6. (f) Absolute numbers of the indicated progenitor populations in mice reconstituted with WT or Glg1−/− marrow, and treated or not with anti-TGFβ antibody; n = 5. (g) Recovery kinetics of leukocytes (WBC) and erythrocytes (RBC) in the blood of mice reconstituted with WT or Glg1−/− BM after treatment with a single dose of 5-FU; n = 10. *P < 0.05; **P < 0.01; ***P < 0.001 determined by Student’s test (a, g). Wilcoxon matched-pairs (c) or one-way analysis of variance with Turkey’s test (d,e,f). Data are show as mean ± s.e.m.

Reduced Glg1−/− cells and this could also be reverted by blocking TGFβ (Fig. 5e and Supplementary Fig. 7c), indicating that early progenitors are an active source of inhibitory TGFβ. Importantly, and agreeing with the role of ESL-1 in limiting posttranslational processing of pro-TGFββ, we did not find changes in Tgfb1 transcript levels in mutant LSK cells (Supplementary Fig. 8a), and in contrast found mild elevations in the levels of latent TGFβ on the surface of Glg1−/− progenitors compared with WT cells (Supplementary Fig. 8b).

Because Glg1−/− cells induced quiescence on neighbouring WT HSPC in vivo (Fig. 2), we sought to reproduce this dominance in vitro using purified LSK cells. Mixed cultures of WT and Glg1−/− LSK cells resulted in reduced proliferation of both types of progenitors, compared with mixtures of WT cells only. Importantly, blockade of TGFβ reversed the proliferative defects of both WT and Glg1−/− cells (Fig. 5f). Altogether, these observations indicated that HSPC are a relevant source of TGFβ, which acts on neighbouring progenitors to block proliferation.

Haematopoietic ESL-1 maintains CXCL12-producing niche cells.
We finally explored whether the repressive signals originating...
from Glg1⁻/⁻ haematopoietic cells could affect the stromal niche. To this end, we transplanted WT or Glg1⁻/⁻ donors into Cxcl12Gfp reporter mice, which allow identification of perivascular cells expressing high levels of the chemokine (CD45<sup>NEG</sup> CD31<sup>NEG</sup> GFP<sup>HIL</sup> CAR cells), as well as endothelial cells (CD45<sup>NEG</sup> CD31<sup>POS</sup> GFP<sup>LO</sup>) and osteoblasts (CD45<sup>NEG</sup> CD31<sup>NEG</sup> GFP<sup>LO</sup>). Imaging of sternal preparations revealed reductions in CXCL12-producing cells in the Glg1⁻/⁻ group (Fig. 6a), and this correlated with strong reductions in CXCL12 protein in the BM (Fig. 6b). Cytometric analyses revealed that these reductions were associated with marked decreases in the number of CAR and endothelial cells, but not osteoblasts (Fig. 6c,d). Importantly, the reductions in CAR and endothelial cells could be corrected by blocking TGFβ (Fig. 6d), and correlated with elevated TGFβ-induced signalling in these two cell types but not on osteoblasts (Supplementary Fig. 9a,b), suggesting that this cytokine produced by ESL-1-deficient haematopoietic cells can directly suppress the stromal niche, as supported by previous reports<sup>16</sup>. Altogether, the data suggested that haematopoietic-borne ESL-1 can control HSPC proliferation directly through cytokine secretion, and/or indirectly through repressive effects on supportive niche cells.

**Discussion**

Although the list of stromal constituents of the niche is being rapidly refined<sup>17</sup>, only a handful of studies have focused on haematopoietic cells as functional niche elements that regulate the fate of the HSC from which they originate. In this study, we show that haematopoietic progenitors have the capacity to regulate the proliferative status of neighbouring HSPC through production of TGFβ and that this function is limited by ESL-1. Our findings reveal that regulation of the niche can originate from haematopoietic cells, including HSPC, and suggest that the partnership of haematopoietic and mesenchymal stem cells that are at the core of the BM niche<sup>18</sup> allows mutual regulation of both partners, as has been evidenced in leukaemic models<sup>19,20</sup>. Besides HSPC, other haematopoietic cells including macrophages<sup>21,22</sup> and megakaryocytes<sup>23,24</sup> may be relevant sources of TGFβ and may thus regulate niche size and HSPC proliferation. Although we have excluded macrophages as a major source, perivascular megakaryocytes, which produce abundant TGFβ<sup>24</sup>, could also have a strong impact on the global levels of the cytokine in the BM in the absence of ESL-1. Nonetheless, we provide evidence that HSPC, which were shown in early in vitro studies to be an autocrine source of TGFβ<sup>25</sup>, can function as regulators of their own environment. This finding is particularly relevant because these cells are by definition the only population unambiguously located within a haematopoietic niche.

An important extension from our study will be to uncover the physiological or pathological scenarios in which the regulatory restraint imposed by ESL-1 becomes inactive. As under steady-state conditions blockade of the TGFβ pathway does not alter HSC proliferation (this study and<sup>11</sup>), we propose two possible scenarios in which loss of this regulation may be relevant: ageing and stress. The finding that Glg1⁻/⁻ mice display a...
myeloid bias and display delayed recovery from stress parallels changes described in the aged haematopoietic system. Interestingly, alterations in TGFβ signalling have been associated with HSC ageing and are likely to underlie the myeloid expansion in old animals. An additional scenario, associated with HSC ageing and are likely to underlie the possibility. Also noteworthy is the finding that subsets of primitive precursors (endothelial and CAR cells) appear repressed in the absence of ESL-1, whereas osteoblasts that are repressed in the absence of ESL-1, whereas osteoblasts that are associated with the lymphoid lineage that expresses little ESL-1 remain largely unaffected, suggesting local regulation of the various haematopoietic environments.

Figure 5 | Haematopoietic precursors are a relevant source of TGFβ. (a) Percentage of BrdU incorporation in WT or G1g1−/− LSK cells of control or macrophage-depleted (Clod) mice; n = 5. (b) Frequency of the different types of CFU-C in mice reconstituted with WT or G1g1−/− donors; n = 6. (c) Contour plots (left) and frequency (right) of Lin−EG cKit+ cells obtained from WT or G1g1−/− marrow grown in the presence or absence of the TGFβRI inhibitor LY-2157299; n = 6. (d) Number of secondary colonies after replating WT or G1g1−/− CFU-C from primary cultures; n = 3 independent experiments. (e) Relative in vitro proliferation of WT or G1g1−/− LSK cells, as measured by BrdU incorporation; n = 3 independent experiments. (f) Experimental design to test the in vitro proliferation of WT and G1g1−/− LSK cells in co-cultures with or without TGFβ blockade. Representative contour plots (right) and quantification of the relative proliferation; n = 3 independent experiments. Data are shown as mean ± s.e.m.; *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant, as determined by Student’s t-test (b,d), or one-way analysis of variance with Turkey’s test (a,c,e and f).

An unexpected finding from our study was that, although ESL-1 has been shown to be a ligand for E-selectin on haematopoietic progenitors, each molecule (ESL-1 and E-selectin) affects HSPC proliferation through independent mechanisms. The predominant expression of ESL-1 inside the cell rather than at the surface (which would be required for selectin binding) is consistent with this independent mechanism. Thus, the identity of the relevant E-selectin ligand(s) on HSPC responsible for the proliferative effects remains unknown, although it is possible that glycosphingolipids, or a combination of various glycoproteins (as shown for the recruitment of neutrophils), cooperate for selectin binding and for cycle arrest. This possibility is sustained by the growing appreciation that a complex array of differentially glycosylated proteins (and lipids) other than PSGL-1 and ESL-1 can function as ligands for E-selectins on haematopoietic cells. This important issue deserves further study. In addition, although it has been speculated that E-selectin might control HSPC by dictating their distribution within the non-uniform BM microenvironment, the mechanism by which this selectin and its ligand(s) ultimately regulate HSPC proliferation remains to be elucidated.

In summary, the identification of an intrinsic pathway controlled by ESL-1 that regulates HSPC proliferation, but can also impact the behaviour of neighbouring stromal cells and HSPC (scheme in Supplementary Fig. 10), yields important insights into how stem cell dynamics are regulated to maintain homeostasis within the BM.
**Methods**

**Mice.** All experiments were performed in 6- to 10-week-old male mice housed in a specific pathogen-free facility. ESL-1 (Glp1r−/−), PSGL-1−/− (Selectin−/−) and E-selectin−/− deficient mice were obtained from B. Lee (Baylor College of Medicine, Houston, TX, USA) and P. Frenette (Einstein College of Medicine, NY, USA), and have already been described. Cxcl12-Gfp knock-in mice were also used as recipients. Mice expressing DsRed under the β-actin promoter were used as donors in some experiments. Wild-type C57BL/6 (CD45.2+), and congenic B6.SJL (CD45.1+/-) mice were used as donors or recipients. All mice were in a pure C57Bl/6 background. Experimental procedures were approved by the Animal Care and Ethics Committee at Fundación CNIC and Comunidad de Madrid.

**Cell isolation.** After euthanasia, femurs were collected and flushed into ice-cold PBS plus 0.5% fetal bovine serum (FBS) for the isolation of BM cells. For estimation of Cxcl12-expressing niche cells, femurs were flushed and incubated with 1 U ml−1 liberase (Roche Applied Science) and 12 mU ml−1 1 DNase I (Sigma) in HBSS for 30 min at 37°C. Cells were sorted on a FACSAria (BD Biosciences) to 95% purity.

**Flow cytometry.** For BM mature myeloid compartment, cells were stained with fluorescein isothiocyanate (FITC)-conjugated Gr-1 (Clone RB6-8C5), PE-conjugated CD115 and Alexa Fluor 647-conjugated F4/80 (Clone CE3-1; purchased from Serotec) antibodies in PBS containing 2 mM EDTA and 0.5% FBS. For LSKs and MPs, cells were stained with biotinylated lineage antibody cocktail (CD3ε, B220, CD11b, Gr1 and Ter119) from BD Bioscience, together with streptavidin conjugated to DyLight-405 and an anti-CD31-APC antibody (Clone: 390; BD Biosciences). Cells were then stained with biotinylated lineage antibody cocktail and anti-CD48 (Clone: HM48-1) together with streptavidin conjugated to Alexa Fluor 405, and antibodies against Sca-1-APC, c-Kit-PE-Cy7 and CD150-PE (Clone: A2F10) and anti-CD90.2-APC (Clone: 53-2.1). For HSC staining using the LSR-Fortessa flow cytometer equipped with DIVA software (BD Biosciences). Data were finally stained with anti-BrdU-APC before cytometric analysis. For detection of Ki67 in LSK or SLAM progenitors, cells were surface stained as above, fixed and permeabilized using the Mouse Foxp3 fixation and permeabilization buffers (BD Biosciences), and then stained with an anti-mouse Ki67 conjugated to Alexa600 (Clone: SolA15; eBioscience) and Hoechst 33342.

To analyse cell cycle on LSK cells and MP, live cells were surface stained with the lineage antibody cocktail together with streptavidin conjugated to APC-Cy7, plus antibodies against Sca-1-APC and c-Kit-APC. Cells were then stained with Hoechst 33342 (Invitrogen) at 37°C for 45 min. Pyronin Y (Sigma-Aldrich) was then added at 1 µg ml−1, and the cells were incubated for another 15 min at 37°C, washed and immediately analysed using a 561-nm excitation laser to prevent bleaching into all other channels used for this staining.

**Generation of BM chimeras by transplantation.** Donor BM cells were harvested from the appropriate genotype (WT DsRed or experimental WT, PSGL-1- or ESL-1-deficient mice) by flushing both tibiae and femora into PBS. Recipient WT C57BL/6 mice were lethally irradiated (6.5 Gy split doses, 3 h apart) before receiving 2 million BM cells by intravenous injection. For mixed chimeras, equal

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**Figure 6 | Haematopoietic ESL-1 preserves the Cxcl12-producing bone marrow stroma.** (a) Whole-mount staining of sternal BM of Cxcl12GFP mice transplanted with WT or Glg1−/− bone marrow cells; n = 4. Scale bar, 50 µm. (b) Cxcl12 protein levels in the BM mice transplanted with WT or Glg1−/− bone marrow cells; n = 5. (c) Representative density plots showing osteoblasts (OB), CAR and endothelial cells (ECs) in the bone marrow of the BM of Cxcl12GFP mice reconstituted with WT or Glg1−/− BM, and treated or not with an anti-TGFβ antibody for 2 weeks, and quantification of their relative numbers (d); n = 5 mice per group. Data are shown as mean ± s.e.m.; *P<0.05; **P<0.01; ***P<0.001 as determined by Student’s t-test (b), or one-way analysis of variance with Turkey’s test (d).
numbers of experimental and WT-DsRed BM cells (10^6 + 10^6) were mixed and injected into the same recipients. Engraftment of recipient animals was assessed at least 8 days after transplantation by flow cytometry for the presence of WT and DsRed^+^ cells in the blood by flow cytometry. This method ensured the exclusion of variability owing to environmental factors and allowed comparing the behaviour of mutant and WT competitor cells within the same physiological environments.

**Competitive repopulation assays.** 5,000, 25,000, 50,000 and 100,000 WT or Gli^+^ BM cells were injected together with 3 x 10^5 BM cells from B6.SJL.CD45.1^+^ mice into lethally irradiated recipients. Lineage chimerism was measured at 16 weeks after transplantation. The number of CD45.2^+^ irradiates B6.SJL recipients. Lineage chimerism was measured at 16 weeks after transplantation, and mice were scored positive when donor contribution was > 1%. The frequency of repopulating cells was calculated using the ELDA software (http://bioinf.wehi.edu.au/software/elda/)^3^, to assess the quiescence of HSC, we treated WT and B6.SJL unirradiated mice with the formula (D^C^ x (10^3(D)/D^C^-)1) to determine the percentage of recipient blood leukocytes that are CD45.2^+^ at the 16-week test bleed, and C the number of competing CD45.1^+^ RUs that were co-injected (C = 3, meaning that 3 x 10^6 competing BM cells were injected). Reconstitution units per femur were then calculated. One RU is defined as the percentage of engrafted CD45.1^+^ B6.SJL untreated mice and transplanted into lethally irradiated B6.SJL recipients. Lineage chimerism was measured at 16 weeks after transplantation. The number of CD45.2^+^ reconstitution units (RUs) injected per reconstituted femur was calculated using the formula (D^C^ x (10^3(D)/D^C^-)1) to determine the frequency of repopulating cells that are CD45.2^+^ at the 16-week test bleed, and C the number of competing CD45.1^+^ RUs that were co-injected (C = 3, meaning that 3 x 10^6 competing BM cells were injected). Reconstitution units per femur were then calculated. One RU is defined as the percentage of HSC.

**In vivo treatments.** For proliferation assays, BrdU (Sigma) was administered at 0.5 mg ml^-1^ in drinking water. For HSC exhaustion experiments, 5-FU (Sigma) was injected at 150 mg per kg body weight intraperitoneally (ip) once or every 10 days. For myelo-suppressive treatments, 5-FU was injected once (150 mg kg^-1^ i.p.). To assess haematological recovery, peripheral blood (50 m. l of liposomes loaded with clodronate 8 days before analysis (C) was injected. Reconstitution units per femur were then calculated. One RU is defined as the percentage of CD45.2^+^ irradiates B6.SJL untreated mice and transplanted into lethally irradiated B6.SJL recipients. Lineage chimerism was measured at 16 weeks after transplantation, and mice were scored positive when donor contribution was > 1%. The frequency of repopulating cells was calculated using the ELDA software (http://bioinf.wehi.edu.au/software/elda/)^3^, to assess the quiescence of HSC, we treated WT and B6.SJL unirradiated mice with the formula (D^C^ x (10^3(D)/D^C^-)1) to determine the percentage of recipient blood leukocytes that are CD45.2^+^ at the 16-week test bleed, and C the number of competing CD45.1^+^ RUs that were co-injected (C = 3, meaning that 3 x 10^6 competing BM cells were injected). Reconstitution units per femur were then calculated. One RU is defined as the percentage of recipient blood leukocytes that are CD45.2^+^ at the 16-week test bleed, and C the number of competing CD45.1^+^ RUs that were co-injected (C = 3, meaning that 3 x 10^6 competing BM cells were injected). Reconstitution units per femur were then calculated. One RU is defined as the percentage of HSC.

**Immunofluorescence.** CXCCL12-expressing niche cells and LSK cells sorted from WT of Gli^1/-^ transplanted mice were cytospin on glass slides, immediately fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, then blocked with 3% BSA in PBS. CXCCL12-expressing cells images were acquired with a Zeiss LSM 700 confocal system (Carl Zeiss MicroImaging). Images were collected using a Leica SP5 multi-line inverted confocal microscope. CXCCL12-expressing cells images were acquired with a Zeiss LSM 700 confocal system (Carl Zeiss Microimaging) and analysed using the Fiji software with custom plugins.

**Western blotting.** For western blot analyses, B and T lymphocytes from BM were stained with biotinylated anti-B220 (Clone: RA3.3A1/6.1) and anti-CD3e-APC (Clone: 145-2C11), respectively. Monocytes, neutrophils, macrophages, MP and LSK were stained as described above. Cells were sorted and lysed in Laemmli’s sample buffer with 50 mM dithiothreitol, and boiled for 5 min. The equivalent of 10^6 cells (for the myeloid compartment) or 1.5 x 10^6 cells (for lymphocytes) was loaded onto each lane of a 7.5% SDS-PAGE gel and after electrophoresis the separated proteins were blotted onto a nitrocellulose membrane (Millipore). Membranes were blocked and incubated with an anti-ESL-1-myc rabbit serum (a gift of Dr M.K. Wild; 1:1,000 diluted) and then washed thrice. The primary antibody was detected by using an sheep-purified donkey-anti-rabbit IgG (GE Healthcare; 1:15,000 diluted). Membranes were incubated with HRP substrate (Luminata Forte Western HRP substrate, Millipore) and luminescence was recorded on an ImageQuant LAS 4000 mini system (GE Healthcare). To obtain an internal load control, the membranes were then stripped and re-blotted with an anti-Jl-actin antibody (Sigma; 1:2,500 diluted).

**Progenitor assays in culture.** BM were collected and 20,000 mononuclear cells were added to semisolid media containing 1.25% methylcellulose (Sigma-Aldrich), 30% FBS (StemCell Technologies), 1% denitrogenated bovine serum albumin, 10^-4^ M 2-mercaptoethanol and conditioned medium (12.7% v/v) from the WEHI3 cell line (containing IL-3, HM-5 cell line (containing GM-CSF and BHK/MKL cell line stably transfected to produce the secreted form of murine Kit Ligand/SCF). In some experiments, cells were plated without KitLigand/SCF (StemCell Technologies) for quantification of different types of progenitors in the presence of irradiated mouse granulocyte-macrophage (CFU-GM) and colony-forming unit granulocyte (CFU-G) burst-forming unit erythroid (BFU-E); colony-forming unit granulocyte, erythroid, macrophage and megakaryocyte (CFU-GEMM). The TGFBR1 inhibitor LY-2157299 (kindly provided by Eli Lilly; Indiana) was added at a concentration of 500 nM. Control cultures were grown in the presence of dimethylsulfoxide (vehicle for the inhibitor). Cultures were plated in duplicates in 35 mm culture dishes (Nunc A/S) and incubated at 37 °C in 5% CO2. CFU-G were scored on day 6 or 7 using an inverted microscope.

**In vitro culture.** Sorted LSK were cultivated overnight in StemSpan medium (StemCell Technologies) supplemented with 100 ng ml^-1^ rmTNF, 100 ng ml^-1^ rIL-3 and 0.5% FBS in the presence of irradiated mouse bone marrow stromal cells at 10 mg ml^-1^. Subsequently, 10^-5^ M BrdU was added to the cultures for 4h, and BrdU incorporation and cell surface phenotypes were analysed.
RNA isolation and real-time quantitative PCR. BM LSks were purified from femurs and tibiae. Staining was performed as described above. Total RNA was prepared with RNA Extraction RNasy Plus Mini- or Micro-kit (Qiagen). RNA was reverse-transcribed with High-Capacity cDNA Reverse Transcription reagents (Applied Biosystems) according to the manufacturer’s protocol. Real-time quantitative PCR (SYBR-green, Applied Biosystems) assays were performed with an Applied Biosystems 7900HT Fast Real-Time PCR System detector. Expression was normalized to Hprt (F-5'-CCTAGATGGCAGGGTGTA-3', R-5'-CCACAGGACTAGAACACTCTGAA-3') and 36b4 (F-5'-ACTGTTCTCA GAGCCGAGAAG-3', R-5'-TCCACCCTTTTGTCCAGTCT-3') expression. The primer sequences for Glk were: F-5'-CAAGATGACGGGCTCATATT-3', R-5'-TGGCAACAGGATCTGCG-3'; for p16: F-5'-TTATGAAATGACA GCCGCTCAATGT-3', R-5'-ACGGACAGCCAACAACTACGG-3'; for p21: F-5'-TGCTGTCACCTGCTTGCTGAAG-3', R-5'-TCTCGGAGAC CAATCTGGC-3'; for p57: F-5'-GGCGGAAAGTCGTTAGTGA-3', R-5'-AGA GTTTCTCCATGCTGCGT-3'; for Tgb1: F-5'-CGGAGCTGCTACTAT-3', R-5'-GTAACGCGCAGAATTG-3'.

Statistical analyses. Data are shown as the mean ± s.e.m. For comparisons between two groups the Student’s t-test was applied. For data with more than two data sets, we used one-way analysis of variance with Turkey’s multigroup test. Log-rank analysis was used for Kaplan–Meier survival curves. For the limiting dilution LT-reconstitution assays, we used Poisson’s statistics as indicated. Analyses were performed with the GraphPad Prism software. P-values <0.05 were deemed significant.

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