All mature blood cells arise from primitive progenitor cells in the BM. These rare and specialized cells, called hematopoietic stem cells (HSCs), exist mostly in a quiescent state (1). Cell division of HSCs results in both their progressive proliferation and differentiation into increasingly lineage-restricted mature blood cells, as well as maintenance of a small pool of HSCs that do not differentiate, but rather carry out hematopoiesis throughout the life of an organism (2). Much work has been done to elucidate the nature of the signals that govern the balance between self-renewal and differentiation of HSCs. The BM microenvironment in which these cells reside, called the HSC niche, is thought to nurture this balance by providing the appropriate signals (3, 4), but the identities and precise coordination of these signals have only recently begun to be understood.

As the entire life of an HSC revolves around the decisions of whether or not and when to divide, it is no surprise that several molecules and signaling pathways that could regulate cell cycle have been implicated in HSC biology (5–10). For example, the proto-oncogene c-Myc has been shown to be essential for regulating both HSC self-renewal and differentiation (11). The effects of c-Myc deletion in the BM are alleviated by simultaneous deletion of the cell cycle inhibitor p21 (12), underscoring the requirement for strict cell cycle regulation to ensure proper HSC function. Both p21 and c-Myc are regulated at the transcriptional level by the Rho GTPase Cdc42, and deletion of Cdc42 from hematopoietic cells results in increased and decreased levels of c-Myc and p21, respectively, and allows quiescent HSCs to enter the cell cycle and differentiate (13).

In addition to c-Myc, the Notch pathway has also been suggested to influence HSC function. Notch1 signaling is essential during hematopoiesis for the development of T lymphocytes (14, 15), but studies of its role in HSCs have yielded a mixture of results and interpretations. For example, inhibition of Notch1 signaling in vivo...
can deplete the HSC compartment, possibly by promoting their
differentiation into more mature, lineage-restricted cells (7).
Also, Stier et al. (16) suggested that Notch pathway activa-
tion increases HSC self-renewal in vivo. On the other hand,
several genetic studies questioned the importance of Notch1
signaling at this stage. Initially, deletion of the central Notch
effector RBP-J (signal binding protein J) caused no significant
HSC or progenitor-specific phenotypes (17). Similarly, no gross
hematopoietic defects were reported when either the Notch
ligand Jagged1 or the Notch regulators Numb and Numblike
were conditionally deleted (18, 19).

Although it is clear that the expression of numerous cell
cycle regulators must be tightly controlled in HSCs to permit
both their self-renewal and differentiation, the molecular
pathways that provide such control are mostly unclear. Notch1
and c-Myc, as well as other cell cycle regulators like cyclin E
and c-Jun, are targeted for proteosomal degradation by the E3
ubiquitin ligase, Fbw7 (20, 21). Fbw7 (also called Fbxw7,
hCDC4, Ago, and SEL-10) is an F-box protein that serves as
a receptor molecule for the Skp1/Cul1/F-box protein (SCF)
ubiquitination complex. Fbw7 binds substrates (e.g., Notch1
and c-Myc) by recognizing a phosphorylated motif (a degron)
via its WD40 repeat domain (22). Fbw7 is simultaneously
bound via its F-box domain to the adaptor protein Skp1,
which in turn is bound to the RING finger protein Rbx1 via
the scaffold protein Cul1, which allows recruitment of target
proteins for poly ubiquitination by an Rbx1-associated ubiqui-
quitin-conjugating enzyme (E2) (23); this marks the target pro-
gain for degradation by the 26S proteasome (24).

Our laboratory and others have recently shown that inac-
tivating mutations in Fbw7 contribute to the accumulation
of Notch1 and c-Myc in T cell–acute lymphoblastic leukae-
mia (T-ALL) (25–27), and several other studies have estab-
lished Fbw7 as an important tumor suppressor in various tissues
(http://www.jem.org/cgi/content/full/jem.20080277/DC1),
Fbw7 mRNA expression was high at the LSK stage and de-
creased as the cells differentiated into the myeloid lineage
(MP: lineage “c-kit+Sca-1-”). B cell progenitors expressed
moderate amounts of Fbw7 message, whereas in the thymus,
the first T cell–committed progenitors (CD44+25+) expressed
high amounts of Fbw7. There was no significant expression
difference during CD4-8- to CD4-8+ transition. However,
single positive (CD4 or CD8) T cells appear to express lower
levels of Fbw7 message (Fig. S1 B). The levels of Cullin 1,
a partner of Fbw7 for the formation of the SCF complex,
follow similar—although not identical—expression patterns.
Finally, to gain further insight in the putative function of
Fbw7 during early hematopoiesis, we have generated PCR
primers specific for the three distinct Fbw7 isoforms. We
were surprised to see that mouse hematopoietic cells express
only the Fbw7α isoform that is exclusively localized in the
nucleolus (Fig. S1 C). These studies suggested a dy-
namic control of Fbw7 expression at different stages of hema-
topoietic differentiation.

**Generation of a conditional Fbw7 allele (Fbw7<sup>fl</sup>)**

As the early lethality of the Fbw7<sup>-/-</sup> embryos precludes any
study of hematopoietic differentiation, we generated an Fbw7-
conditional allele targeting exons 5 and 6, as they encode for
the F-box domain that is essential for SCF assembly (Fig. 1 A).
We have used standard embryonic stem (ES) cell–targeting
techniques and “floxed” these two exons with Loxp sites. We
have also inserted an Frt “floxed” Neo mini-gene that was
later deleted (Fig. 1 B) from the germline of the generated
chimeric progeny (and polyI-polyC)-inducible
Mx1-cre transgenic mouse strain (33) to generate a model of
inducible Fbw7 deletion (Mx-cre<sup>-</sup> Fbw7<sup>fl</sup>). As shown in
Fig. 1 (B and C), three polyI-polyC intraperitoneal injections
are sufficient to induce total Fbw7 locus deletion and effi-
ciently silence Fbw7 expression in several lymphoid tissues,
including the BM, the thymus, and the spleen. The spleen
was also total in the BM LSK fraction (see Fig. 8).

**Deletion of Fbw7 severely affects hematopoietic progenitor
maintenance in the BM**

We focused our initial analysis on the early stages of BM he-
matopoiesis. As early as 2 wk after the end of the polyI-polyC
treatment, there was a significant reduction in the absolute
number of total BM cells (Fig. 2 A). We should note here
that control littermates were either Mx-cre<sup>+</sup> Fbw7<sup>fl</sup>/fl
or Mx-cre<sup>-</sup> Fbw7<sup>fl</sup> mice and were also polyI-polyC injected.
As our goal was to study the potential effects of Fbw7 dele-
tion on HSC homeostasis, we turned our attention to the
lineage<sup>-</sup> compartment. It was immediately obvious that Fbw7
deletion rapidly (at 2 wk after polyI-polyC) and significantly
lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> (LSK) fraction as well as more differen-
tiated myeloid progenitors (MPs; lineage “Sca-1-”c-Kit<sup>-</sup>), B
cell progenitors (B220<sup>+</sup>CD43<sup>+</sup>), and thymic T cell (lineage
CD4<sup>+</sup>CD25<sup>+</sup>) progenitors. As shown in Fig. S1 A (available at
http://www.jem.org/cgi/content/full/jem.20080277/DC1),
Fbw7 mRNA expression was high at the LSK stage and de-
creased as the cells differentiated into the myeloid lineage
(MP: lineage “c-kit+Sca-1”).

**RESULTS**

**Fbw7 expression patterns during early
hematopoietic differentiation**

To study the pattern of Fbw7 expression during early hema-
topoiesis, we have initially purified the HSC-containing

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affected LSK frequency. Most importantly, absolute numbers of LSK cells decreased three- to fivefold (Fig. 2 B). A significant decrease in the numbers of the first MPs (lineage− Sca-1− c-Kit+) was also seen, but there were no significant differences in the relative abundance of common, mega-karyocyte-erythrocyte, and granulocyte-monocyte progenitors (Fig. 2 E). In agreement with these findings, the LSK frequency in the peripheral blood (not depicted) and spleen (Fig. 2 C) was also decreased. Further subdivision of the LSK fraction to long-term HSC (LT-HSC), short-term HSC, and multipotent progenitors (using a combination of CD34 and c-kit staining) did not reveal any subset-specific effects but suggested a more general effect on LSK homeostasis (Fig. 2 D).

As a consequence of the HSC defect, absolute numbers of all BM–mature lineages, including the numbers of immature (B220+ IgM+) and mature B cells, were also significantly decreased (Fig. 2 F). These findings strongly suggested that Fbw7 deletion could affect LSK cell self-renewal and/or differentiation in the BM.

**Deletion of Fbw7 severely affects progenitor maintenance in the thymus**

As LSKs have been shown to represent the major thymus-colonizing population, we analyzed the thymus after Fbw7 deletion and found no significant change in total thymocyte numbers 2 wk after polyI-polyC deletion (Fig. 3 A). However, at later time points (4 wk after polyI-polyC) Mx-cre+ Fbw7fl/fl thymocyte numbers were significantly decreased (Fig. 3 A). Notch1 appeared to be the main Fbw7 substrate in the thymus, as its deletion led to a significant accumulation of intracellular Notch1 protein as detected by two different Notch1-specific antibodies (Fig. S2 A, available at http://www.jem.org/cgi/content/full/jem.20080277/DC1). This finding was consistent with our recent identification of Notch1 as a substrate of Fbw7 in T cells. Supporting this observation, Deltex1 transcription, a reliable Notch1 reporter, was significantly up-regulated in Mx-cre+ Fbw7fl/fl thymi (Fig. S2 B). On the other hand, other putative Fbw7 substrates like c-Myc or cyclin E were not significantly stabilized in total Mx-cre+ Fbw7fl/fl thymocytes at this specific time point (Fig. S2 A).

Deletion of Fbw7 appeared to significantly affect the most immature (CD4–8+) thymocytes as both their frequency and their absolute numbers decreased two- to sixfold (Fig. 3, B and C). As this CD4–8– double negative (DN) population includes the most immature T cell progenitors, we have further subdivided this subset using antibodies against CD25, CD44, and c-kit. This analysis revealed an almost complete absence of both DN2 (CD25+44+) and DN3 (CD25–44+) thymocytes (Fig. 3 F and Fig. S3 A). This could be explained by the later Fbw7 gene deletion in the Lck-cre model (Fig. S3 B). 4-wk-old Lck-cre+ Fbw7fl/fl mice had thymocyte phenotypes dependent on the timing of the deletion mediated by the Mx1 promoter that is active both in thymocytes and their immediate BM progenitors. Indeed, when we deleted Fbw7 only in developing thymocytes using the Lck promoter (34) (Lck-cre+Fbw7fl/fl), none of these DN cell–specific phenotypes were obvious (Fig. 3 F and Fig. S3 A). This could be explained by the later Fbw7 gene deletion in the Lck-cre model (Fig. S3 B). 4-wk-old Lck-cre+Fbw7fl/fl mice had thymocyte numbers similar to Lck-cre+Fbw7wt/wt control littermates, and almost 50% of them developed T cell lymphomas later in life (unpublished data and reference 35).

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**Figure 1.** Generation of a conditional Fbw7 allele. (A) Targeting strategy showing exons 4–7 of the mouse genomic Fbw7 locus before targeting (WT allele) and after homologous recombination in C57BL/6 x 129 ES cells (targeted allele), which introduces an FRT- and LoxP-flanked PGK-Neo cassette between exons 6 and 7, and flanks exons 5 and 6 with LoxP sites. Germline removal of the PGK-Neo cassette before Cre recombinase results in excision of exons 5 and 6 and generates the conditional knockout (CKO) allele. Arrows show locations of primers (A–D) used to genotype resulting mice. (B) Before crossing to the Flpe-deleter strain, PCR using primers A and C were used to confirm transmission of the targeted allele (not depicted). After removal of the PGK-Neo cassette, primers B and C were used to detect both the “floxed” and WT alleles (497 and 315 bp, respectively). After induction of Mx-Cre with polyI-polyC injections, the floxed band was lost from the BM and thymus, and excision of exons 5 and 6 in the BM-mature lineages, including the numbers of immature (B220+ IgM+) and mature B cells, were also significantly decreased (Fig. 2 F). These findings strongly suggested that Fbw7 deletion could affect LSK cell self-renewal and/or differentiation in the BM.
of entering BM stem and progenitor cells, we have quantified the absolute number of the first thymic immigrants (early T cell progenitors [ETPs], defined as lineage-CD25^-c-kit^high cells) in the thymi of control and mutant mice 2 and 4 wk after polyI-polyC injection. As shown in Fig. 3G, ETP numbers were significantly decreased upon Fbw7 deletion at both time points. Our studies demonstrated that deletion of Fbw7 using the Mx-cre^-Fbw7^f/f model severely affects lymphocyte development in the thymus.

**Loss of Fbw7 leads to a cell-autonomous defect of stem cell self-renewal**

As the Mx1 promoter is IFN-α responsive and can drive cre-recombinase expression in nonhematopoietic lineages (e.g., the BM HSC niche), we have tested whether the reported phenotypes were cell autonomous. We used a competitive transplantation assay mixing Mx-cre^-Fbw7^f/f or Mx-cre^-Fbw7^f/f CD45.2^+ BM cells (from polyI-polyC-injected mice) with CD45.1^+ WT cells. At the time of the injection, we had normalized for absolute numbers of LSK cells and injected ~10^5 LSK cells from each genotype. These cells were transplanted in irradiated CD45.1 hosts, and chimerism was studied 5 wk later. We should note here that in this assay we expected <50% CD45.2 chimerism due to the existence of endogenous, host-derived CD45.1^+ cells. The exact CD45.2 chimerism depended on the injection efficiency and in our hands varied from 10 to 45%. Indeed, although Mx-cre^-Fbw7^f/f cells efficiently competed (generating >40%...
chimerism) with CD45.1 BM, Mx-cre<Fbw7<sup>-/-</sup> cells generated only minimal chimerism in the spleen, thymus, and BM of the recipient animals, suggesting a very severe defect in their ability to differentiate and replenish the hematopoietic and immune systems (Fig. 4 A). Interestingly, when we studied chimerism in the LSK compartment we found almost no Fbw7<sup>-/-</sup> LSK cells (Fig. 4 B). Similarly, in the BM, <3% of total BM mononuclear cells derived from Fbw7<sup>-/-</sup> progenitors. Very few of these cells managed to properly differentiate toward the myeloid (not depicted) or B cell lineage (Fig. 4 C). Moreover, <0.1% of total donor-derived thymocytes originated from Fbw7<sup>-/-</sup> BM. Once more, these cells failed to differentiate to the CD4<sup>+</sup><sup>8<sup>+</sup></sup> stage, and the vast majority belonged to the early CD44<sup>+</sup>25<sup>+</sup> DN1 stage (Fig. 4, D and E).

These experiments demonstrated that Fbw7<sup>-/-</sup> stem cells were unable to compete with WT counterparts.

As the previous results could also be explained by altered the homing ability of HSCs upon Fbw7 deletion, we altered our transplantation protocol so that we transplanted 0.5 × 10<sup>6</sup> non-polyI-polyC–injected (Mx-cre<Fbw7<sup>+/</sup> or Mx-cre<Fbw7<sup>+/+</sup>) CD45.2<sup>+</sup> BM cells with an equal number of CD45.1<sup>+</sup> WT cells into irradiated CD45.1 hosts and documented chimerism 3 wk later. Only after transplant did we inject the recipients with polyI-polyC (three times) to delete the Fbw7 locus. 7 wk after polyI-polyC injection, we found no (or very few) mature donor-derived Fbw7<sup>+/+</sup>/H11002<sup>/H11002</sup> cells (thymocytes, B cells, Mac1<sup>+</sup>, NK1.1<sup>+</sup>, and Gr-1<sup>+</sup> cells), including BM LSK (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20080277/DC1).

These combined transplantation results strongly demonstrated that the Fbw7 deletion-induced phenotype is cell autonomous and is not due to the inability of Fbw7 LSK to home to the marrow niche.

Deletion of Fbw7 leads to defective HSC quiescence and self-renewal

To begin understanding the molecular mechanism behind the HSC defect caused by the deletion of Fbw7, we initially studied LSK survival in the BM of polyI-polyC–injected animals (at 2, 3, and 4 wk after injection). No significant differences in the percentage of apoptotic LSK in polyI-polyC–injected littermate mice (Mx-cre<Fbw7<sup>-/-</sup>, Mx-cre<Fbw7<sup>-/-</sup>/LckCre<sup>-/-</sup>, or Mx-cre<Fbw7<sup>-/-</sup>/LckCre<sup>-/-</sup>) were observed at 3 wk after polyI-polyC (Fig. 2).
We then asked whether this loss of quiescence translates to defective HSC self-renewal or differentiation. We used colony-forming cell assays and initially plated total BM from polyI-polyC–treated Mx-cre + Fbw7 wt/wt or Mx-cre + Fbw7 f/f animals and incubated them in semi-solid methylcellulose medium in the presence of a suitable cytokine cocktail. As shown in Fig. 5 C, Mx-cre + Fbw7 f/f cells generated only half the number of colonies compared with Mx-cre + Fbw7 wt/wt progenitors. Also, these colonies were significantly smaller in size (Fig. 5 E).

We then performed similar experiments using highly enriched CD34+/H11002 LT-HSCs. Interestingly, during the initial plating no differences in the absolute numbers of colonies were recorded. However, the replating of colonies that originated from Mx-cre + Fbw7 f/f LT-HSCs showed a complete loss of self-renewal (Fig. 5 D) in agreement with our in vivo competitive reconstitution assays (Fig. 4). These observations clearly showed that Fbw7 deletion promotes aberrant cell cycle entry and loss of self-renewal.

BM Fbw7 deletion leads to c-Myc protein stabilization

As Fbw7 is able to ubiquitinate and degrade several target proteins, we performed an initial protein expression screening of three well-characterized substrates, Notch1, c-Myc, and cyclin E (Fig. 6). We were unable to detect significant expression and stabilization of either Notch1 or cyclin E in total BM cells. This result was not surprising, as Notch1 mRNA is arguing against the involvement of the SCF Fbw7 complex in LSK survival.

To address the effect of Fbw7 deletion on stem cell proliferation, we used Ki67 and DAPI labeling to determine the cell cycle status of LSK cells. The percentage of Mx-cre+ Fbw7 f/f LSK cells that passed the G0-G1 checkpoint and entered cycle was consistently and significantly elevated when compared with Mx-cre+ Fbw7 f/f LSK cells (Fig. 5 A). Interestingly, these effects appear to be LSK specific, as more differentiated cell populations (MPs, T cell progenitors, CD4+8+ thymocytes) do not show similar cell cycle acceleration (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20080277/DC1).

To further support these findings, we have used a short (42-h) pulse of BrdU to mark cells that escape quiescence and enter the cell cycle, and subdivided the LSK compartment into CD34+/H11002 (self-renewing LT-HSC) and CD34− (non-self-renewing short-term-HSC and MPP) subsets. As evident from Fig. 5 B, Fbw7−/− LT-HSCs showed a dramatic loss of quiescence, as almost 80% of them entered cell cycle during the 42 h from the initiation of the BrdU pulse. CD34+ LSK cells also showed a significantly elevated fraction of BrdU+ cells. We should also note here that LSK cycle deregulation was evident even in marrows with less significant alterations in LSK or total cell numbers, suggesting that the functional changes preceded the phenotypic ones (not depicted).
cells from polyI-polyC–injected Mx-cre + Fbw7 f/f mice (and Mx-cre + Fbw7 wt/wt controls). We placed these cells in a liquid culture in the presence of cytokines as shown in Fig. 7. Once more, we have detected no stabilization of either Notch1 or cyclin E (not depicted). On the other hand, c-Myc protein was substantially overexpressed (almost threefold). Also, the accumulated c-Myc protein was phosphorylated on the T58 and S62 residues, in agreement with the idea that phospho species of the c-Myc substrate should accumulate in the absence of proteasomal degradation (Fig. 6 B). Further quantitative RT-PCR experiments have proven that the c-Myc stabilization was due to posttranslational modification (Fig. 6 C). This c-Myc stabilization was persistent between different experiments. These results (Fig. 6 C) suggested that Fbw7 deletion in stem cell and progenitor populations led to a significant stabilization of c-Myc but not Notch1 or cyclin E proteins.

### Dynamic regulation of Fbw7 expression during HSC cycle entry and differentiation

Previously presented results suggest that Fbw7 is an HSC cycle entry “brake” and that its deletion affects HSC quiescence. In agreement with this scenario, Fbw7 expression is significantly down-regulated as LSKs differentiate to become...
MPs (Fig. S1). To further understand the role of Fbw7 during physiological HSC proliferation and differentiation, we purified LSK cells and placed them in cytokine-driven in vitro cultures. Before the cytokine stimulation (day 0), almost 60% of the cells were in G0. Upon stimulation, cells rapidly entered cycle with only 2–3% of cells in G0 at day 2 (Fig. 7 A). Fbw7 expression declined significantly in response to cytokine stimulation, and Cul1 expression followed an identical pattern (Fig. 7 B). Although these results suggested that Fbw7 expression is dynamically regulated at the HSC stage, it is difficult to uncouple proliferation from differentiation-triggered effects. We further quantified cell differentiation by measuring the proportion of cells that lost Sca-1 expression. As shown in Fig. 7 C, at day 1 almost 80% of the cells retained their c-kit ‘Sca-1’ phenotype, whereas Fbw7 expression was decreased three- to fourfold. Although effects of differentiation are not excluded, this observation suggested that Fbw7 message expression is down-regulated as HSCs enter cell cycle.

Fbw7 controls a gene program that correlates with HSC quiescence and self-renewal
To further characterize the molecular mechanism of Fbw7 function in the differentiation and homeostasis of HSCs, we have performed a whole transcriptome analysis. We have used FACs-purified lineage ‘IL7Ra+c-kithighSca-1high stem/multipotential lineage progenitors (LSK)’ and lineage ‘IL7Ra+c-kithighSca-1low MP’ subsets from polyI-polyC-injected Mx-cre+ Fbw7f/f and Mx-cre+Fbw7lov/lov littermate mice, extracted total RNA, and used it for microarray analysis. Both the array analysis (not depicted) and quantitative RT-PCR (Fig. 7 A) showed that deletion of the Fbw7 locus and silencing of its expression in the LSK and MP compartments used for these experiments were complete.

We have performed an analysis of genes that showed a greater than or equal to threefold difference in expression between the control and Fbw7−/− LSK cells. This analysis demonstrated that Fbw7−/− LSK cells were more closely related to the differentiated MP populations than the control LSK subset (not depicted). The combination of published studies (37–40) and our own results (Table S1, available at http://www.jem.org/cgi/content/full/jem.20080277/DC1, and unpublished data) suggested a partial transcriptional “signature” associated with the LT-HSC phenotype. These transcripts are enriched in LT-HSCs and down-regulated upon the loss of self-renewal properties. We thus hypothesized that the transcriptional signature of Fbw7 deletion in HSCs contains the features of the physiological loss of stem cell phenotype associated with the commitment to the transiently amplifying progenitor fate. Such a hypothesis was supported by the identities of genes specifically affected by Fbw7 deletion in the LSK compartment (not depicted), including Evi1, Mpl, p57, Agpt, Eya1/2, Pbx3, and Meis1, which were all previously shown to be enriched in self-renewing HSCs (Table S1). These results were further verified using quantitative RT-PCR analysis of distinct biological samples (Fig. 8 B) and were perfectly in line with the presented phenotypic results, which suggested a loss of HSC quiescence and self-renewal capacity upon Fbw7 deletion. Importantly, the observed global loss of HSC-specific transcriptional signature was not an artifact from contaminating Sca-1low/− cells, as the Ly6A/Sca-1−specific signal was even slightly higher in the Fbw7−/− compared with the control LSK sample (not depicted).

This loss of “LT-HSC–specific” gene expression was accompanied by the activation of a more differentiated gene expression program in cells that lose Fbw7 expression. Indeed, several genes suggesting an erythro-myeloid and lymphoid priming (38) were up-regulated upon Fbw7 loss (i.e., glycoporphin A, CD36, neutrophil elastase, Rag-1, etc.; not depicted). This is consistent with the presented expression analysis data that show that Fbw7 mRNA expression decreases during the transition of undifferentiated LSK cells to the committed progenitor stage, which could be a functional part of the early HSC differentiation program.

Finally, we have searched for genes that could explain the loss of stem cell quiescence upon Fbw7 deletion. As shown in
been previously suggested to be targets of c-Myc, supporting our view that c-Myc overexpression could be an important regulator of cell cycle entry downstream of Fbw7 (41–43).

The presented expression studies provide a very strong mechanistic basis that explains the phenotypic and functional effects of Fbw7 deletion on HSC quiescence and self-renewal.

**DISCUSSION**

In this report we demonstrate that a single E3 ubiquitin ligase, Fbw7, is an essential regulator of HSC quiescence and self-renewal. Fig. 8 C, several cell cycle regulators, including p27, p21, and Ccnd2/3, were unaffected by the deletion of Fbw7. However, the expression of three cell cycle–related genes was altered in response to Fbw7 silencing. The cyclin kinase inhibitor p57kip2, previously implicated in loss of quiescence of stem cells (38), was down-regulated in Mx-cre+ Fbw7+/f LSK cells. On the other hand, the cell cycle regulator E2F2 was significantly up-regulated upon Fbw7 loss. Finally, the D-type cyclin Ccnd1 was up-regulated after Fbw7 gene excision, in agreement with its role in HSC cycle entry. Interestingly, all three genes have been previously suggested to be targets of c-Myc, supporting our view that c-Myc overexpression could be an important regulator of cell cycle entry downstream of Fbw7 (41–43). The presented expression studies provide a very strong mechanistic basis that explains the phenotypic and functional effects of Fbw7 deletion on HSC quiescence and self-renewal.
self-renewal. Fbw7 is expressed in quiescent HSCs, and its expression is down-regulated upon the loss of their self-renewing capacity. These effects are cell autonomous, as Fbw7\\textsuperscript{+/−} LSKs are unable to compete with WT counterparts both in vitro and in vivo. The loss of LSK absolute numbers and self-renewal abilities are also reflected at the early stages of lymphopoiesis, as Mx-cre\\textsuperscript{+/−}Fbw7\\textsuperscript{T393A} mice have lower numbers of B cell, and almost no T cell, progenitors. To mechanistically explain these LSK phenotypes, we have performed comparative gene expression studies using Fbw7\\textsuperscript{+/−} stem cells and MPs. These studies demonstrated that Fbw7 deletion targets specific cell cycle regulators and suppresses the expression of several genes previously suggested to control HSC function. Thus, we have identified a novel function for the proteasome–ubiquitin ligase complex as a genetic “switch” that controls HSC cycle entry and self-renewal.

Our studies are in agreement with a recent report by Matsuoka et al. (44). These investigators also demonstrate that Fbw7 is essential to maintain HSC quiescence and self-renewal, and suggest that c-Myc is a key Fbw7 substrate in HSCs. They also report that Fbw7\\textsuperscript{+/−} animals die by either extreme pancytopenia at 12 wk after polyI-polyC injection or by T-ALL. Development of T-ALL was a later event, as disease appeared only after 16 wk after treatment. Only very few (n = 2) of our polyI-polyC–injected Fbw7\\textsuperscript{+/−} animals were allowed to reach these time points, and all developed pancytopenia. Thus, we cannot directly compare rates of T-ALL induction, as all other polyI-polyC–injected animals presented in this study were analyzed at very early time points. However, both Fbw7–targeted animals developed T-ALL with similar kinetics when crossed to the Lck-cre strain (unpublished data and reference 35). A discrepancy between the two studies exists over the detection of apoptosis in Fbw7\\textsuperscript{−/−} LSK cells. However, Matsuoka et al. (44) reported elevated percentages of apoptosis only in HSCs purified from the marrows of severely cytopenic mice 12 wk after deletion. In the studies presented here, the analysis was performed using LSK cells purified from noncytopenic Fbw7\\textsuperscript{−/−} mice 3 wk after polyI-polyC deletion. At that time point there was a significant alteration of HSC quiescence and a reduction of LSK (and LT-HSC) absolute cell numbers. In agreement with our findings, Matsuoka et al. (44) failed to detect an increased frequency of apoptosis in HSCs purified from the BM of noncytopenic animals, even at 12 wk after deletion.

There are few other examples of stem cell function or differentiation being regulated by specific E3 ubiquitin ligases (30). Initially, the UBR1/2 E3 ligases were shown to be essential for neurogenesis due to their effects on the differentiation and survival of neural stem cells and progenitors (45). Also, in the neural system, the Mindbomb1 ligase regulates expression of Notch ligands and consequently controls differentiation of neural stem cells (46). Finally, DDB1, an E3 ligase member of the cullin4A complex, is important for the viability, genomic integrity, and maintenance of neural stem cells (47). Information on the role of any of the multiple E3 ligases on HSC function is scarce and indirect. Studies using a panel of chemical proteasome inhibitors have shown that suppression of proteasome function induced cell death in human CD34\\textsuperscript{+} hematopoietic progenitors (48). Moreover, primary CD34\\textsuperscript{+} leukemic stem cells also enter apoptosis in response to treatment with the MG-132 proteasome inhibitor (49). However, these were crude experiments that cannot address the complexity of regulation of proteasome function in HSCs by the different ubiquitin ligase complexes. Experiments similar to the ones described in this report, which include E3 ligase expression mapping and gene-targeting experiments, are thus essential for the detailed understanding of the importance of ubiquitination in HSC function.

Multiple protein substrates of Fbw7 have been identified. These include the cell cycle regulator cyclin E and transcription factors such as c-Myc and Notch (50) that could directly or indirectly regulate stem cell function. Our protein expression analyses showed no stabilization of Notch1 in the BM of Mx-cre\\textsuperscript{+/−}Fbw7\\textsuperscript{T393A} mice. In fact, we were unable to detect any Notch1-IC protein expression at all. This was not due to any technical obstacle, as Notch1 was significantly stabilized and overexpressed in the thymus of these mice. Also, we were unable to detect CD4\\textsuperscript{+} cells in the Fbw7\\textsuperscript{−/−} BM (not depicted), usually a reliable and rapid indicator of aberrant Notch activation (51, 52). However, we cannot exclude that Notch stabilization occurs in a small fraction of LSK cells, and this change in protein expression is below the sensitivity of the experimental method used. Indeed, Matsuoka et al. (44) detected Notch1 overexpression using confocal microscopy of individual Fbw7\\textsuperscript{−/−} LSK cells. In agreement with these results, our RT-PCR analysis of Fbw7\\textsuperscript{−/−} LSK cells has revealed a persistent overexpression of Hes-1 and Deltex-1, both well-characterized targets of the Notch pathway (Fig. 8 C).

Similar studies excluded a role for stabilization of another Fbw7 substrate, cyclin E. Indeed, we were unable to detect any significant cyclin E stabilization in response to Fbw7 deletion in either total BM cells or purified stem cell and progenitor populations. These findings are consistent with genetic experiments reported by Loeb et al. (51). These investigators have generated “knock-in” mice that express a mutated cyclin E, cyclin E \textsuperscript{T393A}, T393 falls within a canonical Fbw7 substrate, cyclin E. Indeed, we were unable to detect any significant cyclin E stabilization in response to Fbw7 deletion in either total BM cells or purified stem cell and progenitor populations. These findings are consistent with genetic experiments reported by Loeb et al. (51). These investigators have generated “knock-in” mice that express a mutated cyclin E, cyclin E \textsuperscript{T393A}, T393 falls within a canonical Fbw7 recognition degron, and its phosphorylation allows Fbw7 to bind and ubiquitinate cyclin E. Mutated cyclin E was stabilized and expressed at levels several-fold higher in several tissues, including lymphocytes and thymocytes. Despite this overexpression, cyclin \textsuperscript{T393A}–homozygous mice showed no phenotypic abnormalities in all tissues examined, including the immune system. They also displayed a normal life span with no significant predisposition to autoimmune disease or tumor induction. Together with our protein expression analyses, these elegant genetic studies argue against a role of cyclin E stabilization in HSC function in response to Fbw7 deletion.

On the other hand, our studies suggested that c-Myc could be an attractive target, especially because it was previously shown to be involved in HSC differentiation (11).
Indeed, both total and phosphorylated c-Myc protein was consistently up-regulated in Fbw7+/− BM stem cells and progenitors. These observations were consistent with the confocal microscopy studies reported by Matsuoka et al. (44), strongly suggesting that c-Myc is indeed an important Fbw7 substrate. Putative targets of c-Myc, including p57kip2, E2F2, and Ccnd1, were also up-regulated in Fbw7−/− LSK cells. In agreement with this hypothesis, Wilson et al. (11) have performed experiments in which they have overexpressed c-Myc specifically in LSK cells. Despite proper homing and inhibition of apoptosis, c-Myc–overexpressing HSCs failed to self-renew, a phenotype similar to the one reported here (11). We should add here that although c-Myc appears to represent an important Fbw7 target, it is possible that there are other yet-undefined Fbw7 substrates that are important for the hematopoietic phenotypes reported here.

Our presented studies identify Fbw7 as a novel essential regulator of HSC quiescence and self-renewal. Initially, deletion of Fbw7, which was normally expressed at high levels in noncycling HSCs, affects the expression of key regulators of cell cycle entry, including the D-type cyclin Ccnd1, the cell cycle inhibitor p57kip2, and the downstream transcriptional effector E2F2. The combined action of these regulators could induce exit from quiescence and cell cycle entry, as the BrdU incorporation experiments convincingly demonstrated that upon Fbw7 deletion >80% of LT-HSCs have entered cell cycle. It is tempting to hypothesize that aberrant cell cycle entry forces Fbw7−/− HSCs to exit the quiescent niche and lose their self-renewing ability. Once more, our expression studies provide mechanistic support to this hypothesis. Fbw7 deletion dramatically suppresses the expression of several genes (Mpl, Pbx3, p57, Meis1, Evi1, Eya1/2, Angpt, Thy1, and Ndn) that define a partial “LT-HSC” transcriptional signature (Table S1 and unpublished data).

The identification of Fbw7 as a key regulator of HSC quiescence and self-renewal may have very significant biological repercussions. Initially, Fbw7 function could be important for self-renewal of cancer stem cells with important implications in the therapeutic targeting of their maintenance. In agreement with this notion, Fbw7 deletion suppresses the expression of several genes involved in hematopoietic transformation (Cnd1, Evi1, Pbx3, and Meis1). It is intriguing to mention here that Fbw7 can also function as a tumor suppressor when deleted in T cells using the Lck-cre/Fbw7+/− model (reference 35 and unpublished data), suggesting that Fbw7 function in oncogenesis could differ between uncommitted stem cells, committed progenitors, and mature lymphocytes. For all these reasons, the identification of regulators of Fbw7 activity, including specific deubiquitinating enzymes or substrate–specific priming kinases, could be valuable for both the development of HSC transplantation protocols and the suppression of transformation.

**MATERIALS AND METHODS**

**Generation of the Fbw7flox mice.** An ∼11.5-kb region used to generate the targeting vector was subcloned from a positively identified C57B6 BAC clone using homologous recombination. The region was designed such that the short homology arm ends 2.4 kb 5′ to loxP/FRT-flanked NEO cassette. The long homology arm ends on the 3′ side of the loxP/FRT NEO cassette and was ∼8-kb long. A single lox P site was inserted upstream of exon 5. The target region was 2.1 kb and included exons 5 and 6. The backbone vector was ∼2.1 kb and contained an ampicillin selection cassette. The total size of the targeting construct (including the vector backbone) was ∼16 kb. The targeting vector was linearized using NotI and electroporated into 129 Sv/En × C57BL6 hybrid ES cells. After G418 selection, surviving clones were expanded for PCR analysis to identify recombinant ES cell clones. Oligos for ES cell screening and PCR conditions are available upon request. Five correctly targeted ES cell clones were expanded for microinjection into blastocysts. Strong chimeras were identified and crossed to C57BL6 females to verify germline transmission. The F1 generation was PCR screened and crossed to FLIP recombinase–expressing mice (Jax mice) to delete the Frt-flanked Neo Cassette. Fbw7flx/Neomice were consequently crossed to Mx1-cre and Lck-cre mice.

For our polyol-polyC experiments, we injected 20 μg polyol-polyC per gram of body weight. We initiated injections 2 wk after birth. Mice were injected once every other day for a total of three injections. They were analyzed 1, 2, and 4 wk after injection. All animal experiments were approved by the IACUC of the NYU School of Medicine.

**Transplantation assays.** For the experiment shown in Fig. 4, we have mixed BM from either CD45.2 Mx-cre−/− Fbw7−/− or CD45.2 Mx-cre+/− Fbw7+/− mice (2 wk after the end of polyol-polyC treatment) with WT CD45.1 cells. We normalized for absolute numbers of LSK cells in each marrow such that we injected 1,000 LSK cells from each genotype. The cells were injected in lethally (900 rad) irradiated CD45.1 congenic H57BL6 recipients. We analyzed chimerism 5 wk after transplant.

For the experiment shown in Fig. S4, we mixed (50:50) nonpolyol-polyC–injected whole BM cells (CD45.2 Mx-cre+/− Fbw7+/− or CD45.2 Mx-cre+/− Fbw7+/− with WT CD45.1 cells). After we verified chimerism from both CD45.1/2 donors (using peripheral blood FACS analysis), we began polyol-polyC injections (three). Chimerism was studied 7 wk after the end of the polyol-polyC treatment.

**Genotyping and genomic PCR.** Mouse tails were clipped at 1–2 wk of age and incubated in tail lysis buffer for 12–24 h at 55°C. 6M NaCl was added, insoluble debris was pelleted, and DNA was precipitated with isopropanol. The resulting DNA pellets were washed with 70% ethanol and dissolved in water for PCR analysis. Genomic DNA from BM and thymocytes was prepared by lysing the cells in a solution containing 0.3% NP-40, 0.03% Tween 20, and 7 μg/ml protease K for 30 min at 55°C, followed by 10 min at 100°C. This solution was used directly for PCR. Primers were designed to selectively amplify WT, targeted (Neo+), targeted (Neo−), and CKO Fbw7 alleles as shown in Fig. 2. The sequences are as follows: A F, 5′-GGCTTAGCATATGCATTGAG; B F, 5′-ATTGATACAAACTG- GAGCACGAAG; C R, 5′-ATAGTACCCCCCTGCTTGGC; and D F, 5′-TGGGAGGCCCAGAGGCATTGTGTAGC. McrE and LckCre transgenes were detected using the following primers: Cre F, 5′-GGCGGTCGCTGAGTAAAAACTATC; Cre R, 5′-AAGTGAAGAATATACGTCATTCC; and Lck F, 5′-GGTTGGCCCATACCGAGTG. All PCRs used a Tm = 58°C for 35 cycles, except for Cre PCRs, which used a Tm = 52°C.

**Antibodies and FACS analysis.** Antibody staining and FACS analysis was performed as described previously (11). All antibodies were purchased from BD Biosciences or eBioscence. We used the following antibodies: c-kit (2B8), Sca-1 (D7), Mac-1 (M1/70), Gr-1 (RB6–8C5), NK1.1 (PK136), TER-119, CD3 (145-2C11), CD19 (1D3), IL7R (2B8), Sca-1 (D7), Mac-1 (M1/70), Gr-1 (RB6-8C5), NK1.1 (PK136), and CD25 (PC61), and CD44 (IM7). BM lineage antibody cocktail includes the following: Mac-1, Gr-1, NK1.1, TER-119, CD3, CD4, CD8 (53–67.2), CD25 (PC61), and CD44 (IM7). BM lineage antibody cocktail includes the following: Mac-1, Gr-1, NK1.1, TER-119, CD3, CD4, CD8 (53–67.2), CD25 (PC61), and CD44 (IM7).
Microarray analysis and quantitative PCR validation. For gene expression profiling of primary HSC and progenitor cell subsets, we pooled hind limb BM cells from three mice. Freshly isolated cells were sorted by surface marker expression, and total RNA was extracted using the RNeasy kit (Qiagen). To generate sufficient sample quantities for oligonucleotide gene chip hybridization and quantitative PCR experiments, we used the GeneChip Two-Cycle cDNA Synthesis kit (Affymetrix) for cRNA amplification and labeling. The amplified cRNA was labeled and hybridized to the MOE430 Plus 2 oligonucleotide arrays (Affymetrix) or reverse-transcribed using the First Strand cDNA Synthesis kit (Invitrogen) for quantitative PCR experiments. The Affymetrix gene expression profiling data were normalized using the previously published Robust Multi-array Average algorithm using the GeneSpring 7 software (Agilent Technologies). Messenger RNA expression levels for critical regulators of HSC proliferation and self-renewal were verified by quantitative PCR using the 7900 Real Time PCR system (Applied Biosystems). Primers and probes were used from Applied Biosystems. The relative expression of target genes was calculated using the delta-delta Ct method and normalized to the Hprt1 mRNA content. Two biological duplicates were used for each quantitative RT-PCR experiment. Each duplicate included a mixture of cells (LSK or MP) sorted from two mice with the same genotype for each experiment. Microarray data were deposited in the GEO database under accession numbers GSE11178, GSM281722, GSM281723, GSM281724, and GSM281725.

RT-PCR. Total RNA was isolated using the RNeasy Plus Mini kit (Qiagen) and used to synthesise cDNA with the SuperScript First-Strand Kit (Invitrogen). Real-time quantitative PCR was performed using iQ SYBR Green Supermix and an iCycler (Bio-Rad Laboratories) and Deltex1 R, 5\'-agacctgtagccatcatc-3\' and GSM281725.

In vitro LSK culture. LSK cells were sorted from 4-8-wk-old C57/B6 mice and plated in 96-well plates (5,000 LSK per well) in OPIT-MEM supplemented with 10% fetal bovine serum, 50 ng/ml Flk ligand, 50 ng/ml SCF, 10 ng/ml IL-6, and 10 ng/ml IL-3 (53). Cells were harvested at different time points for cell cycle analysis and quantitative PCR. For cell cycle analysis, cells were fixed and permeabilized according to the manufacturer’s instructions (Fix and Perm; Invitrogen) and stained with PE-conjugated anti-Ki-67 (BD Biosciences) or control anti-IgG antibody. Cells were then resuspended in PBS containing 5 μg/ml RNaseA and 2 μg/ml DAPI, and analyzed by flow cytometry.

Statistical analysis. The means of each dataset were analyzed using the Student’s t test, with a two-tailed distribution and assuming equal sample variance. Data were considered statistically significant when P < 0.05. Significant data are noted in the manuscript as follows: *, P < 0.05; **, P < 0.005.

Online supplemental material. Fig. S1 shows Fbw7 expression in different blood subsets. Fig. S2 shows stabilization of Notch1 in the thymus. Fig. S3 shows the phenotype of Lck-Cre/"Fbw7"/"/thymus. Fig. S4 demonstrates that the Fbw7/"-/-" phenotype is cell autonomous. Fig. S5 shows that the cell cycle defect is specific to HSC. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20080277/DC1.

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