

5. Okamoto, K., Kondo-Okamoto, N. & Ohsumi, Y. Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. *Dev. Cell* **17**, 87–97 (2009).
6. Tanaka, A. *et al.* Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. *J. Cell Biol.* **191**, 1367–1380 (2010).
7. De Duve, C. & Wattiaux, R. Functions of lysosomes. *Annu. Rev. Physiol.* **28**, 435–492 (1966).
8. Narendra, D., Tanaka, A., Suen, D. F. & Youle, R. J. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J. Cell Biol.* **183**, 795–803 (2008).
9. Kitada, T. *et al.* Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* **392**, 605–608 (1998).
10. Valente, E. M. *et al.* Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* **304**, 1158–1160 (2004).
11. Greene, A. W. *et al.* Mitochondrial processing peptidase regulates PINK1 processing, import and Parkin recruitment. *EMBO Rep.* **13**, 378–385 (2012).
12. Jin, S. M. *et al.* Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. *J. Cell Biol.* **191**, 933–942 (2010).
13. Matsuda, N. *et al.* PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. *J. Cell Biol.* **189**, 211–221 (2010).
14. Narendra, D. P. *et al.* PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol.* **8**, e1000298 (2010).
15. Vives-Bauza, C. *et al.* PINK1-dependent recruitment of Parkin to mitochondria in mitophagy. *Proc. Natl Acad. Sci. USA* **107**, 378–383 (2010).
16. Chan, N. C. *et al.* Broad activation of the ubiquitin-proteasome system by Parkin is critical for mitophagy. *Hum. Mol. Genet.* **20**, 1726–1737 (2011).
17. Yoshii, S. R., Kishi, C., Ishihara, N. & Mizushima, N. Parkin mediates proteasome-dependent protein degradation and rupture of the outer mitochondrial membrane. *J. Biol. Chem.* **286**, 19630–19640 (2011).
18. Barik, S. Immunophilins: for the love of proteins. *Cell. Mol. Life Sci.* **63**, 2889–2900 (2006).
19. Hamilton, G. S. & Steiner, J. P. Immunophilins: beyond immunosuppression. *J. Med. Chem.* **41**, 5119–5143 (1998).
20. Lam, E., Martin, M. & Wiederrecht, G. Isolation of a cDNA encoding a novel human FK506-binding protein homolog containing leucine zipper and tetratricopeptide repeat motifs. *Gene* **160**, 297–302 (1995).
21. Shirane, M. & Nakayama, K. I. Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. *Nat. Cell Biol.* **5**, 28–37 (2003).
22. Kang, C. B., Feng, L., Chia, J. & Yoon, H. S. Molecular characterization of FK-506 binding protein 38 and its potential regulatory role on the anti-apoptotic protein Bcl-2. *Biochem. Biophys. Res. Commun.* **337**, 30–38 (2005).
23. Nakagawa, T., Shirane, M., Iemura, S., Natsume, T. & Nakayama, K. I. Anchoring of the 26S proteasome to the organellar membrane by FKBP38. *Genes Cells* **12**, 709–719 (2007).
24. Pawlyk, A. C. *et al.* Novel monoclonal antibodies demonstrate biochemical variation of brain parkin with age. *J. Biol. Chem.* **278**, 48120–48128 (2003).
25. Saita, S., Shirane, M., Natume, T., Iemura, S. & Nakayama, K. I. Promotion of neurite extension by protrudin requires its interaction with vesicle-associated membrane protein-associated protein. *J. Biol. Chem.* **284**, 13766–13777 (2009).
26. Hristova, V. A., Beasley, S. A., Rylett, R. J. & Shaw, G. S. Identification of a novel Zn²⁺-binding domain in the autosomal recessive juvenile Parkinson-related E3 ligase parkin. *J. Biol. Chem.* **284**, 14978–14986 (2009).
27. Horie, C., Suzuki, H., Sakaguchi, M. & Mihara, K. Characterization of signal that directs C-tail-anchored proteins to mammalian mitochondrial outer membrane. *Mol. Biol. Cell* **13**, 1615–1625 (2002).
28. Levine, B., Sinha, S. & Kroemer, G. Bcl-2 family members: dual regulators of apoptosis and autophagy. *Autophagy* **4**, 600–606 (2008).
29. Pattingre, S. *et al.* Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* **122**, 927–939 (2005).
30. Kaufmann, T. *et al.* Characterization of the signal that directs Bcl-x_L, but not Bcl-2, to the mitochondrial outer membrane. *J. Cell Biol.* **160**, 53–64 (2003).
31. Tsutsui, H., Karasawa, S., Shimizu, H., Nukina, N. & Miyawaki, A. Semi-rational engineering of a coral fluorescent protein into an efficient highlighter. *EMBO Rep.* **6**, 233–238 (2005).
32. de Brito, O. M. & Scorrano, L. Mitofusin 2 tethers endoplasmic reticulum to mitochondria. *Nature* **456**, 605–610 (2008).
33. Lee, J. Y., Nagano, Y., Taylor, J. P., Lim, K. L. & Yao, T. P. Disease-causing mutations in parkin impair mitochondrial ubiquitination, aggregation, and HDAC6-dependent mitophagy. *J. Cell Biol.* **189**, 671–679 (2010).
34. Chacinska, A., Koehler, C. M., Milenkovic, D., Lithgow, T. & Pfanner, N. Importing mitochondrial proteins: machineries and mechanisms. *Cell* **138**, 628–644 (2009).
35. Borgese, N. & Fasana, E. Targeting pathways of C-tail-anchored proteins. *Biochim. Biophys. Acta* **1808**, 937–946 (2011).
36. Schmidt, O., Pfanner, N. & Meisinger, C. Mitochondrial protein import: from proteomics to functional mechanisms. *Nat. Rev. Mol. Cell Biol.* **11**, 655–667 (2010).
37. Setoguchi, K., Otera, H. & Mihara, K. Cytosolic factor- and TOM-independent import of C-tail-anchored mitochondrial outer membrane proteins. *EMBO J.* **25**, 5635–5647 (2006).
38. Friedman, J. R., Webster, B. M., Mastrorade, D. N., Verhey, K. J. & Voeltz, G. K. ER sliding dynamics and ER-mitochondrial contacts occur on acetylated microtubules. *J. Cell Biol.* **190**, 363–375 (2010).
39. Nunnari, J. & Suomalainen, A. Mitochondria: in sickness and in health. *Cell* **148**, 1145–1159 (2012).
40. Kornmann, B., Osman, C. & Walter, P. The conserved GTPase Gem1 regulates endoplasmic reticulum-mitochondria connections. *Proc. Natl Acad. Sci. USA* **108**, 14151–14156 (2011).
41. Kornmann, B. *et al.* An ER-mitochondria tethering complex revealed by a synthetic biology screen. *Science* **325**, 477–481 (2009).
42. de Brito, O. M. & Scorrano, L. An intimate liaison: spatial organization of the endoplasmic reticulum-mitochondria relationship. *EMBO J.* **29**, 2715–2723 (2010).
43. Hayashi, T., Rizzuto, R., Hajnoczky, G. & Su, T. P. MAM: more than just a housekeeper. *Trends Cell Biol.* **19**, 81–88 (2009).
44. Simmen, T., Lynes, E. M., Gesson, K. & Thomas, G. Oxidative protein folding in the endoplasmic reticulum: tight links to the mitochondria-associated membrane (MAM). *Biochim. Biophys. Acta* **1798**, 1465–1473 (2010).
45. Kang, J. S. *et al.* Docking of axonal mitochondria by syntaphilin controls their mobility and affects short-term facilitation. *Cell* **132**, 137–148 (2008).
46. Braschi, E. *et al.* Vps35 mediates vesicle transport between the mitochondria and peroxisomes. *Curr. Biol.* **20**, 1310–1315 (2010).
47. Soubannier, V. *et al.* A vesicular transport pathway shuttles cargo from mitochondria to lysosomes. *Curr. Biol.* **22**, 135–141 (2012).
48. Nakayama, K. *et al.* Mice lacking p27^{Kip1} display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* **85**, 707–720 (1996).
49. Nakayama, K. *et al.* Targeted disruption of Skp2 results in accumulation of cyclin E and p27^{Kip1}, polyploidy and centrosome overduplication. *EMBO J.* **19**, 2069–2081 (2000).
50. Kamura, T. *et al.* Cytoplasmic ubiquitin ligase KPC regulates proteolysis of p27^{Kip1} at G1 phase. *Nat. Cell Biol.* **6**, 1229–1235 (2004).

Acknowledgements

We thank T. Kanki, H. Miyoshi, K. Mihara and N. Mizushima for providing vectors or antibodies; A. Tanaka and R. Youle for providing HeLa cells; T. Koshiba and D.C. Chan for *Mfn2*^{+/+} and *Mfn2*^{-/-} MEFs; M. Sasaki and R. Ugawa for performing immunoelectron microscopy; N. Nishimura, K. Tsunematsu, H. Nakatsumi, Y. Hashimoto, T. Ohnishi, and other laboratory members for technical assistance; and A. Ohta for help in preparation of the manuscript. This work was supported in part by a Japan Society for the Promotion of Science (JSPS) Fellowship to S.S.

Author contributions

S.S. planned and performed all experiments. M.S. provided materials and intellectual support. K.I.N. coordinated the study, oversaw the results and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Additional information

Supplementary Information accompanies this paper at <http://www.nature.com/naturecommunications>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Saita, S. *et al.* Selective escape of proteins from the mitochondria during mitophagy. *Nat. Commun.* **4**:1410 doi: 10.1038/ncomms2400 (2013).

Ablation of Fbxw7 Eliminates Leukemia-Initiating Cells by Preventing Quiescence

Shoichiro Takeishi,^{1,2} Akinobu Matsumoto,^{1,2} Ichiro Onoyama,^{1,2} Kazuhito Naka,³ Atsushi Hirao,^{2,3} and Keiichi I. Nakayama^{1,2,*}

¹Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Fukuoka 812-8582, Japan

²CREST (Core Research for Evolutional Science and Technology), Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan

³Division of Molecular Genetics, Center for Cancer and Stem Cell Research, Cancer Research Institute, Kanazawa University, Kakuma-machi, Kanazawa, Ishikawa 920-1192, Japan

*Correspondence: nakayak1@bioreg.kyushu-u.ac.jp
<http://dx.doi.org/10.1016/j.ccr.2013.01.026>

SUMMARY

Imatinib eradicates dividing progenitor cells of chronic myeloid leukemia (CML) but does not effectively target nondividing leukemia-initiating cells (LICs); thus, the disease often relapse after its discontinuation. We now show that Fbxw7 plays a pivotal role in maintenance of quiescence in LICs of CML by reducing the level of c-Myc. Abrogation of quiescence in LICs by Fbxw7 ablation increased their sensitivity to imatinib, and the combination of Fbxw7 ablation with imatinib treatment resulted in a greater depletion of LICs than of normal hematopoietic stem cells in mice. Purging of LICs by targeting Fbxw7 to interrupt their quiescence and subsequent treatment with imatinib may thus provide the basis for a promising therapeutic approach to CML.

INTRODUCTION

Cancer-initiating cells (CICs) are thought to constitute a minor subpopulation of cancer cells that is required for the initiation and maintenance of cancer (Clevers, 2011; Huntly and Gilliland, 2005). This notion is based largely on the characterization of leukemia-initiating cells (LICs), a rare subpopulation of cells that propagates leukemia (Lapidot et al., 1994). LICs were recently shown to share many properties, including self-renewal, pluripotency, and quiescence, with normal hematopoietic stem cells (HSCs) (Clevers, 2011; Huntly and Gilliland, 2005). A fundamental problem in treating leukemia is that the quiescent LIC subpopulation is particularly resistant to conventional chemotherapy and radiation, both of which target cells undergoing DNA replication and are therefore not effective against quiescent

(noncycling) cells (Clevers, 2011; Huntly and Gilliland, 2005). Failure to eradicate quiescent LICs may result in reinitiation of malignancy after a period of latency. The development of therapeutic approaches that target quiescent CICs might therefore be expected to have a profound impact on cancer eradication.

Chronic myeloid leukemia (CML) in humans is characterized by the presence of the Philadelphia chromosome, which is generated by a chromosomal translocation that joins the *BCR* gene on chromosome 22 to the *ABL* gene on chromosome 9 (de Klein et al., 1982; Rowley, 1973). CML is a biphasic myelo-proliferative disorder, which initially assumes a chronic phase before progressing to an accelerated phase and finally to blast crisis. Given that individuals with CML in blast crisis have a poor prognosis associated with a short survival time, it is critical to treat CML patients during the chronic phase. Several lines

Significance

Most cancer-initiating cells (CICs) are quiescent and therefore resistant to anticancer drugs that preferentially target dividing cells. CICs that survive therapy are a potential cause of relapse. Elucidation of the mechanism by which CICs maintain quiescence is thus critical for the elimination of cancer. Here, we show that Fbxw7 plays a pivotal role in maintenance of quiescence in leukemia-initiating cells (LICs) of chronic myeloid leukemia. Our findings reveal that ablation of Fbxw7 in LICs results in deregulated activation of c-Myc and impaired maintenance of quiescence followed by p53-dependent apoptosis and consequent cell exhaustion. Moreover, they provide a rationale for Fbxw7-targeted therapy to sensitize LICs to currently available drugs by interrupting their quiescence, potentially resulting in a substantial survival benefit.

of evidence indicate that LICs of CML emerge as a result of expression of BCR-ABL in normal HSCs (Pear et al., 1998), supporting the notion that CML is a “stem cell disease.”

The BCR-ABL fusion protein possesses constitutive tyrosine kinase activity and triggers molecular events that result in the expansion of malignant hematopoiesis (Deininger et al., 2000). The recent development of the tyrosine kinase inhibitor (TKI) imatinib represented a breakthrough in treatment of the chronic phase of CML, resulting in a marked improvement in the prognosis of CML patients (Druker et al., 2001; Kantarjian et al., 2002). The French CML Intergroup Stop Imatinib study recently found that ~40% of CML patients in complete molecular remission for >2 years while on treatment with imatinib did not relapse within 12 months after discontinuation of imatinib treatment (Mahon et al., 2010). However, this observation suggests that LICs of CML persist in more than half of patients treated with imatinib alone, resulting in relapse after discontinuation of imatinib treatment. Several mechanisms of resistance of CML LICs to imatinib therapy have been suggested, including the maintenance of quiescence (Holtz et al., 2007) and the lack of addiction to BCR-ABL in these cells (Corbin et al., 2011). Although more potent TKIs such as nilotinib and dasatinib have been developed, these drugs also do not target quiescent LICs of CML (Copland et al., 2006; Jørgensen et al., 2007). Therapy with these TKIs thus serves to suppress, not to eliminate, the disease. Moreover, quiescence in CML LICs is thought not only to contribute to TKI resistance but also to be essential for their long-term maintenance. Elucidation of the molecular mechanism by which LICs maintain quiescence is therefore expected to provide a basis for the development of approaches to sensitize CML LICs to TKI therapy, thereby allowing efficient eradication of leukemia cells, prevention of relapse, and increased patient survival. Although several key molecules and signaling pathways have been implicated in LIC maintenance (Ito et al., 2008; Naka et al., 2010; Zhao et al., 2007), the mechanism by which LICs maintain quiescence has been poorly understood.

c-Myc is one of the best characterized proteins found to determine the state of cell proliferation or quiescence (Laurenti et al., 2009). Regulation of the abundance of c-Myc is achieved at several levels, one of which is control of protein stability mediated by posttranslational modification. We and others have shown that the F-box protein Fbxw7 (also known as Fbw7, Sel-10, hCdc4, or hAgo), the substrate-recognition subunit of an SCF-type ubiquitin ligase complex, interacts with and mediates the ubiquitylation of c-Myc (Nakayama and Nakayama, 2006). The ubiquitin-dependent degradation of c-Myc mediated by Fbxw7 has been found to be essential for maintenance of the quiescence and reconstitution capacity of normal HSCs (Matsuoka et al., 2008; Reavie et al., 2010; Thompson et al., 2008). Given that LICs share many properties with normal HSCs, we hypothesized that Fbxw7 might also be required for the maintenance of LICs and that the Fbxw7–c-Myc axis might be a promising target for leukemia therapy.

RESULTS

Fbxw7 Is Required for Maintenance of Quiescence in LICs

To examine whether Fbxw7 expression is modulated during leukemogenesis, we first measured the amount of Fbxw7

mRNA at various stages of the differentiation of leukemic cells in a mouse model of CML caused by the human BCR-ABL fusion protein (Pear et al., 1998). For generation of the model, a c-Kit⁺Sca-1⁺Lin[−] (KSL) fraction of bone marrow cells, which represents immature hematopoietic cells, was infected with a retrovirus encoding both p210^{BCR-ABL} and green fluorescent protein (GFP) and was subsequently transplanted into syngeneic recipients (Figure 1A). Recipient mice developed signs of CML, including decreased activity, weight loss, an increased number of myeloid cells in peripheral blood, and splenomegaly, and all mice died within 4 weeks after transplantation. Bone marrow cells were collected from the recipient mice after they began to show such signs of CML. Reverse transcription (RT) and real-time PCR analysis revealed that Fbxw7 mRNA was highly abundant in the LIC compartment (GFP⁺KSL population), whereas it was present in much smaller amounts in the leukemic progenitor compartment (GFP⁺c-Kit⁺Sca-1[−]Lin[−] population) and its abundance decreased further as cell differentiation progressed (Figure 1B). Similar results were obtained by quantification of the copy number of Fbxw7 mRNA per cell in these various compartments (Figure S1A available online). These data thus suggested that Fbxw7 expression during leukemogenesis is regulated at least in part at the transcriptional level.

We next examined the role of Fbxw7 in the maintenance of LIC quiescence by conditional disruption of the Fbxw7 gene in this CML mouse model. KSL cells from Mx1-Cre;Fbxw7^{+/+} (control) and Mx1-Cre;Fbxw7^{F/F} mice were infected with the retrovirus encoding BCR-ABL and GFP and were then transplanted into syngeneic wild-type mice. The donor mice harbored wild-type (+) or floxed (F) alleles of Fbxw7, as well as a transgene for Cre recombinase under the control of the Mx1 gene promoter. The number of white blood cells in peripheral blood of the recipient mice was determined every 5 days; when it had increased to >20,000/ μ l, GFP⁺KSL cells (2×10^4) were collected from the recipients and transplanted into additional recipient mice (first bone marrow transplantation [BMT]). These recipients were then injected with polyinosinic:polycytidylic acid (plpC) beginning the day after the first BMT to activate the Mx1-Cre transgene and thereby to delete the floxed Fbxw7 allele in Mx1-Cre;Fbxw7^{F/F} leukemia cells (to yield the Fbxw7 ^{Δ/Δ} genotype) (Figure 1C). One week after the final injection of plpC, we confirmed that almost all floxed alleles of Fbxw7 were inactivated in each fraction of the targeted leukemia cells (Figure S1B). Analysis of the cell cycle status of leukemic cells from the recipients of the first BMT by flow cytometry revealed that the frequency of Hoechst^{low}pyronin Y^{low} cells, which represent cells in G₀ phase (quiescence), was significantly smaller in the GFP⁺KSL compartment of Fbxw7 ^{Δ/Δ} leukemia cells than in the corresponding compartment of plpC-treated Mx1-Cre;Fbxw7^{+/+} (control) leukemia cells (Figure 1D). Given that gene ablation induced by plpC is mediated by interferon, which has been shown to act directly on HSCs to induce cell cycle progression (Essers et al., 2009), recipients of control bone marrow cells as well as those of Mx1-Cre;Fbxw7^{F/F} cells were injected with plpC to eliminate any bias attributable to interferon action. In contrast to LICs, most cells in the GFP⁺c-Kit⁺Sca-1[−]Lin[−] compartment were actively cycling, and the proportion of quiescent cells in this compartment did not differ between the two genotypes (Figure 1D), consistent with our observation that

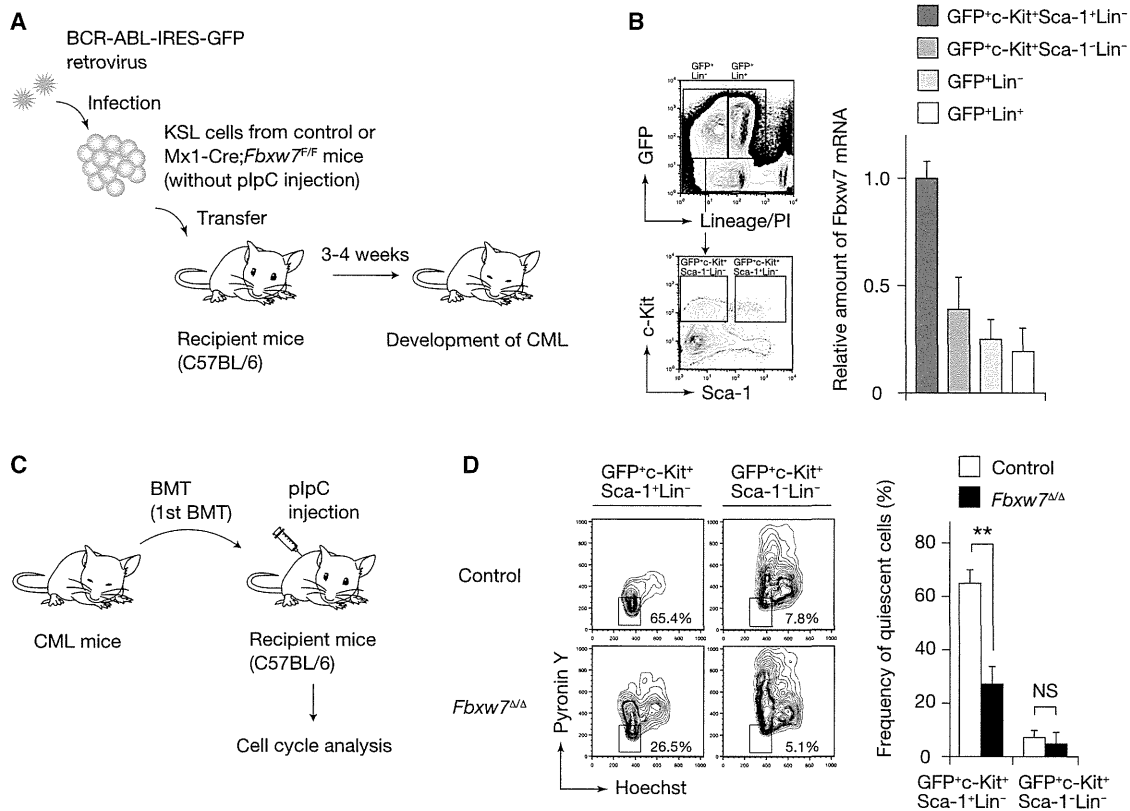


Figure 1. Loss of Fbxw7 in LICs Promotes Cell Cycle Progression

(A) Experimental strategy for generation of a mouse model of CML.

(B) GFP⁺ cells of control CML bone marrow were fractionated by FACS as indicated (left panels; PI, propidium iodide) and then assayed for Fbxw7 mRNA by RT and real-time PCR analysis (right panel; n = 3).

(C) Experimental strategy for deletion of the floxed Fbxw7 allele in leukemic cells.

(D) CML bone marrow cells from recipients of the first BMT were subjected to flow cytometry (left panels) for determination of the percentage of quiescent cells in the indicated fractions (right panel; n = 3).

Data are means ± SD. **p < 0.01; NS, not significant. See also Figure S1.

these cells express Fbxw7 at a low level (Figure 1B). Together, these results suggested that Fbxw7 is expressed predominantly in the LIC fraction of leukemia cells and is required for maintenance of quiescence in LICs.

Fbxw7 Is Required for Leukemic Stemness

The critical role of Fbxw7 in the maintenance of quiescence in LICs and the fact that quiescence is thought to be essential for maintenance of normal HSCs prompted us to examine whether Fbxw7 is essential for LIC maintenance. To test this possibility, we first performed long-term culture-initiating cell assays. Control and Fbxw7^{Δ/Δ} GFP⁺KSL cells were isolated from recipients of the first BMT, cultured on OP-9 stromal cells for 2 or 6 weeks, and then subjected to colony-formation assays (Figure 2A). In these assays, the number of colony-forming cells arising after short-term culture (2 weeks) on OP-9 cells mainly reflects progenitor function, whereas that arising after long-term culture (6 weeks) reflects stem cell function (Matsumoto et al., 2011b). We confirmed that the floxed alleles of Fbxw7 were indeed inactivated efficiently in the Fbxw7^{Δ/Δ} leukemic cells cultured on OP-9 cells (Figure S2A). The number of cells derived from Fbxw7^{Δ/Δ} GFP⁺KSL cells during culture on OP-9 cells was

smaller than that derived from the corresponding control cells (Figure S2B). Furthermore, whereas the number of colonies derived from the cells cultured for the short term (2 weeks) did not differ between the two genotypes (Figure 2B), the number of colonies formed by Fbxw7^{Δ/Δ} GFP⁺KSL cells was significantly smaller than that formed by the control cells after long-term culture (6 weeks), suggesting that increased cycling of Fbxw7^{Δ/Δ} LICs eventually results in their exhaustion. We also performed serial replating assays and confirmed that almost all floxed alleles of Fbxw7 were inactivated in the targeted leukemic cells after serial replating (Figure S2C). Whereas the number of colonies formed did not differ between control and Fbxw7-deficient LICs in the first plating, the number of colonies derived from Fbxw7-deficient LICs was significantly smaller than that derived from control LICs in the second plating (Figure S2D).

To assess the repopulating ability of Fbxw7^{Δ/Δ} LICs in vivo, we performed serial BMT experiments in which we collected control and Fbxw7^{Δ/Δ} GFP⁺KSL cells (2 × 10⁴) from the recipients of the first BMT and transplanted these cells into new recipients (second BMT) (Figure 2C). Almost all floxed alleles of Fbxw7 were inactivated in the targeted leukemic cells isolated from the recipients of the second BMT (Figure S2E). Whereas the

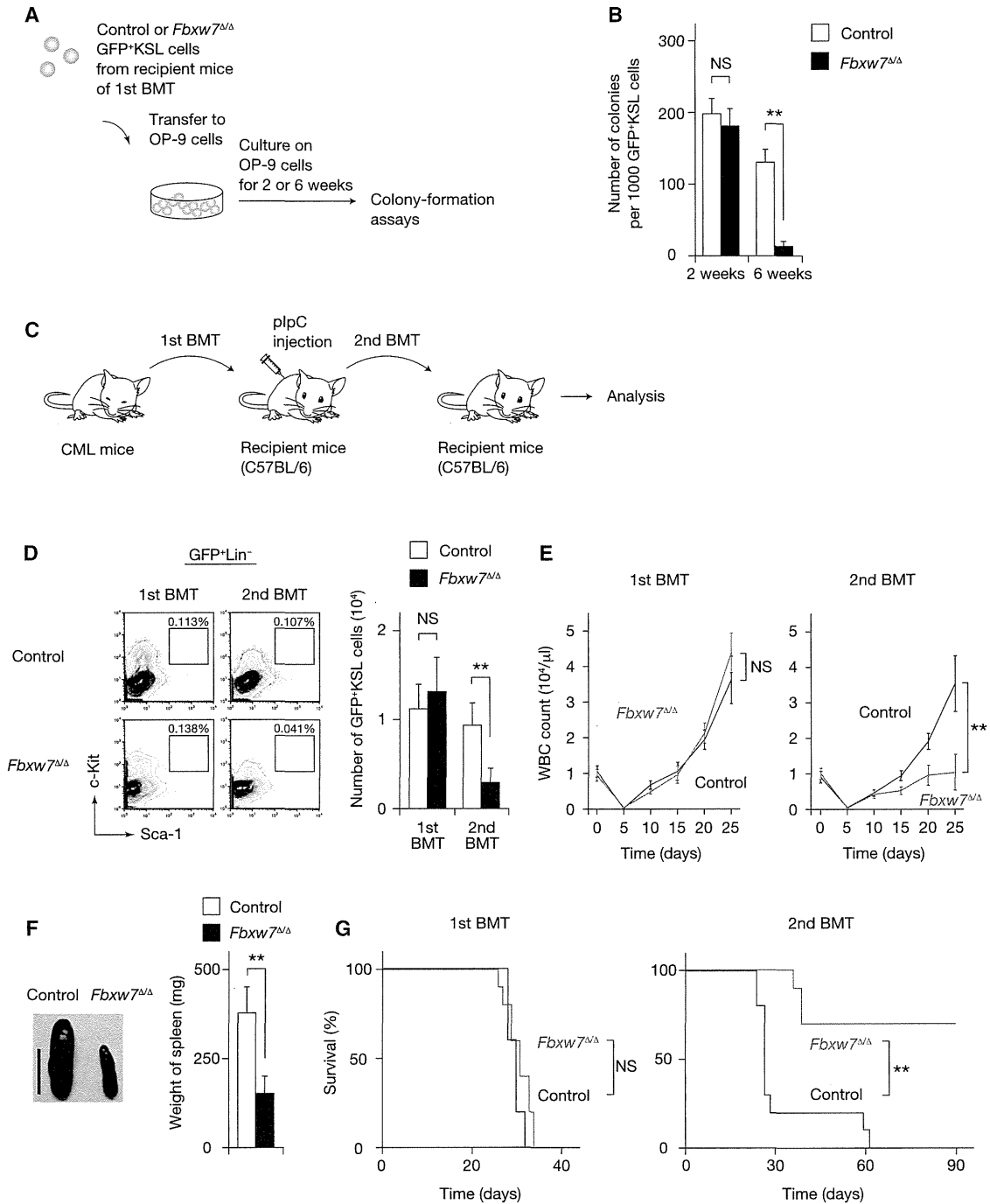


Figure 2. Loss of Fbxw7 in LICs Results in Cell Exhaustion

(A) Experimental strategy for colony formation assays.

(B) Colony formation by BCR-ABL-transduced KSL cells of the indicated genotypes after 2 or 6 weeks of culture on OP-9 cells (n = 3).

(C) Experimental strategy for serial BMT experiments.

(D) Flow-cytometric determination of the absolute number of GFP⁺KSL cells in bone marrow of recipients (n = 5) after the first and second BMTs.

(E) Numbers of white blood cells (WBC) in peripheral blood of recipient mice (n = 10) after the first and second BMTs.

(F) Representative appearance (scale bar, 10 mm) and weight (n = 5) of the spleen in recipients of the second BMT.

(G) Survival of recipient mice (n = 10) after the first and second BMTs.

Data are means ± SD. **p < 0.01; NS, not significant. See also Figure S2.

number of GFP⁺KSL cells isolated from recipients of the first BMT did not differ between the two genotypes, that of GFP⁺KSL cells isolated from recipients of the second BMT was greatly reduced in the case of *Fbxw7*^{Δ/Δ} donor cells compared with that for control donor cells (Figure 2D). These results thus suggested that the loss of *Fbxw7* results in disruption of quiescence, followed by eventual exhaustion of LICs. We also observed that both the proportion and absolute number of *Fbxw7*-deficient leukemic progenitors were significantly smaller than those of control leukemic progenitors in recipients of the second BMT (Figures S2F and S2G). Given that *Fbxw7* deficiency did not affect the cell cycle status of the progenitors (Figure 1D), these latter results were likely attributable to a functional defect in *Fbxw7*-deficient LICs. Consistent with these observations, the timing of CML development did not differ between the two types of recipients of the first BMT (Figure 2E). In contrast, after the second BMT, *Fbxw7* deficiency prevented the propagation of leukemic cells in peripheral blood (Figure 2E) and the spleen (Figure 2F). Furthermore, whereas most recipients of control LICs died of CML at ~30 days after the second BMT, ~70% of mice receiving *Fbxw7*^{Δ/Δ} LICs survived for >120 days (Figure 2G; data not shown). In a similar mouse model of CML, LICs were also shown to give rise to acute lymphocytic leukemia (ALL) with a long latency (Pear et al., 1998). Notably, we did not observe development of ALL or CML in recipients of *Fbxw7*^{Δ/Δ} LICs later than 40 days after the second BMT. Flow cytometric analysis and histological examination revealed the almost complete absence of leukemic cells in peripheral blood and no infiltration of leukemic cells in the spleen, liver, or lungs of the recipients of *Fbxw7*-deficient LICs that survived for >90 days after the second BMT (Figure S2H), suggesting that *Fbxw7*-deficient LICs lose their potential to generate malignancies. *Fbxw7* thus appears to be essential for the long-term maintenance of leukemia-initiating potential.

Accumulation of c-Myc Is Responsible for Loss of Leukemic Stemness

We next investigated the mechanism underlying LIC exhaustion associated with *Fbxw7* deficiency. *Fbxw7* targets many proteins related to HSC maintenance, including c-Myc, Notch1 intracellular domain (NICD1), and mammalian target of rapamycin (mTOR) (Nakayama and Nakayama, 2006). To determine whether these substrates accumulate in *Fbxw7*-deficient LICs, we examined their abundance in LICs isolated from recipients of the first BMT and found that the abundance of c-Myc was increased in *Fbxw7*^{Δ/Δ} GFP⁺KSL cells compared with control GFP⁺KSL cells (Figure 3A). In contrast, the expression levels of NICD1 and mTOR did not differ between control and *Fbxw7*-deficient GFP⁺KSL cells (Figures S3A and S3B).

To determine whether c-Myc accumulation is responsible for the phenotype of *Fbxw7*^{Δ/Δ} LICs, we first cultured control and *Fbxw7*^{Δ/Δ} GFP⁺KSL cells isolated from recipients of the first BMT with the c-Myc inhibitor 10058-F4 (Follis et al., 2009). A colony-formation assay performed after culture of GFP⁺KSL cells for 2 or 6 weeks in the presence of 10058-F4 revealed that the number of colonies did not differ between the two genotypes (Figure 3B). To confirm that 10058-F4 indeed inhibited c-Myc function in these cells, we measured the abundance of mRNAs for cyclin D2 and ornithine decarboxylase 1 (ODC1),

the genes for which are direct targets of c-Myc. The abundance of these mRNAs was increased in *Fbxw7*-deficient LICs compared with that in control cells, and each increase was attenuated by 10058-F4, suggesting that 10058-F4 indeed inhibits c-Myc activity in these cells (Figure 3C). To demonstrate further that c-Myc accumulation contributes to the phenotype of *Fbxw7*-deficient LICs, we next generated *Mx1-Cre;Fbxw7*^{F/F}; *c-Myc*^{+F} mice in order to analyze the cell cycle status and colony-forming ability of *Fbxw7*^{Δ/Δ}; *c-Myc*^{+Δ} LICs. The loss of quiescence and impaired colony-forming ability apparent for *Fbxw7*-deficient LICs were normalized by the additional deletion of one allele of the c-Myc gene (Figures 3D and 3E). In contrast, neither a γ -secretase inhibitor, *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT), which antagonizes Notch signaling, nor rapamycin, which antagonizes mTOR, mimicked the effects of 10058-F4 or c-Myc depletion (Figures S3C and S3D), suggesting that neither Notch nor mTOR contributes to the phenotype of *Fbxw7*-deficient LICs. Collectively, these results thus indeed suggested that the phenotype of *Fbxw7*^{Δ/Δ} LICs is attributable to increased activity of c-Myc.

We next compared the amount of BCR-ABL between control and *Fbxw7*-deficient LICs. Immunoblot analysis revealed no substantial difference in the level of BCR-ABL between these cells (Figure S3E). We further examined whether *Fbxw7* deficiency affects signaling downstream of BCR-ABL in LICs by analyzing the phosphorylation of Stat5 (on Tyr⁶⁹⁴), Crkl (on Tyr²⁰⁷), and Akt (on Ser⁴⁷³). Intracellular flow cytometric analysis revealed that the proportions of cells expressing the phosphorylated forms of Stat5 or Crkl were similar for control and *Fbxw7*-deficient LICs (Figures S3F and S3G). In contrast, the frequency of cells positive for phosphorylated Akt was greater for *Fbxw7*-deficient LICs than for control LICs (Figure S3H). We previously showed that Akt phosphorylation is inhibited by transforming growth factor- β (TGF- β) signaling in CML LICs (Naka et al., 2010), and a recent study indicated that TGF- β is activated by a niche for HSCs (Yamazaki et al., 2011). These observations suggest that *Fbxw7*-deficient LICs might enter the cell cycle and cease to interact with a LIC niche, resulting in a decrease in TGF- β signaling and an increase in Akt phosphorylation. Consistent with this notion, we found that the frequency of cells positive for phosphorylated Smad2/3 was smaller for *Fbxw7*^{Δ/Δ} GFP⁺KSL cells than for control cells (Figure S3I). Moreover, TGF- β 1 treatment reversed the increase in the proportion of *Fbxw7*-deficient LICs positive for phosphorylated Akt (Figure S3J), suggesting that downregulation of TGF- β signaling indeed contributes to this increase. Collectively, our results exclude the possibility that the phenotype of *Fbxw7*-deficient LICs is attributable to downregulation of BCR-ABL itself or of signaling downstream of BCR-ABL.

To further exclude the possibility that *Fbxw7* deficiency in LICs impairs their homing ability, we collected the same number of control and *Fbxw7*^{Δ/Δ} GFP⁺KSL cells from recipients of the first BMT, transplanted the cells into new recipient mice, and determined the proportion of GFP⁺ cells among bone marrow cells by flow cytometry at 12 hr after transplantation. We found that the frequency of GFP⁺ cells did not differ significantly between the two genotypes (Figure S3K), suggesting that the phenotype of mice receiving *Fbxw7*-deficient LICs was not likely a consequence of impaired homing.

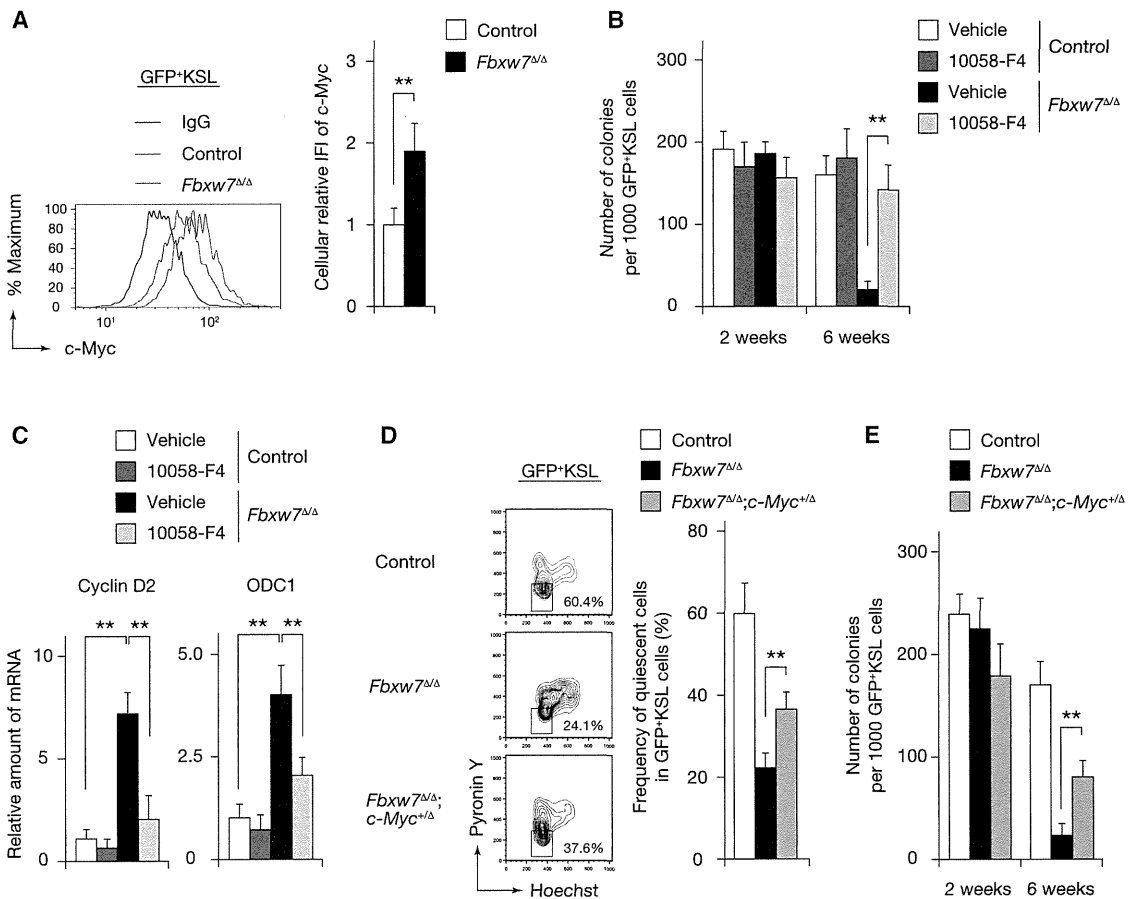


Figure 3. Inhibition of c-Myc Rescues the Phenotype of Fbxw7-Deficient LICs

(A) The abundance of c-Myc in *Fbxw7^{Δ/Δ}* or control GFP+KSL cells from mouse recipients of the first BMT was measured by flow cytometry (left panel). Relative c-Myc immunofluorescence intensity (IFI) was determined (n = 5) (right panel). IgG, immunoglobulin G.

(B) Colony formation by *Fbxw7^{Δ/Δ}* and control LICs after culture for 2 or 6 weeks with or without 10058-F4 (n = 5).

(C) Control and *Fbxw7^{Δ/Δ}* LICs were assayed for cyclin D2 and ODC1 mRNAs by RT and real-time PCR analysis after culture on OP-9 cells for 2 weeks with or without 10058-F4 (n = 5).

(D) The percentage of quiescent cells among GFP+KSL cells was determined for control, *Fbxw7^{Δ/Δ}*, or *Fbxw7^{Δ/Δ}; c-Myc^{+/-Δ}* bone marrow cells from recipients (n = 5) of the first BMT.

(E) Colony formation by BCR-ABL-transduced KSL cells of the indicated genotypes after 2 or 6 weeks of culture on OP-9 cells (n = 3).

Data are means ± SD. **p < 0.01. See also Figure S3.

Loss of Fbxw7 in LICs Induces Apoptosis in a p53-Dependent Manner

Given that deregulation of c-Myc activation often triggers apoptosis in a p53-dependent manner (Matsuoka et al., 2008; Onoyama et al., 2007), we postulated that p53-dependent apoptosis might be induced by accumulation of c-Myc in *Fbxw7*-deficient LICs and contribute to LIC exhaustion. The proportion of apoptotic cells did not differ significantly between control and *Fbxw7*-deficient LICs isolated from recipients of the first BMT. However, the frequency of annexin V⁺ apoptotic cells was markedly greater among *Fbxw7^{Δ/Δ}* GFP+KSL cells than among control GFP+KSL cells from recipients of the second BMT, whereas such a difference was not apparent for leukemic progenitors (Figure 4A). To determine whether this apoptosis in *Fbxw7*-deficient LICs is induced in a p53-dependent manner, we next cultured control or *Fbxw7^{Δ/Δ}* GFP+KSL cells isolated from recipients of the first BMT with the p53 inhibitor pifithrin-α

(PFTα) (Komarov et al., 1999). Analysis of colony formation revealed that the exhaustion apparent in *Fbxw7^{Δ/Δ}* GFP+KSL cells after 6 weeks of culture was efficiently inhibited by treatment of the cells with PFTα (Figure 4B). To confirm that PFTα indeed inhibits p53 function in these cells, we measured the abundance of mRNAs for p21 and Noxa, the genes for which are direct targets of p53. The amounts of these mRNAs were increased in *Fbxw7*-deficient LICs compared with those in control cells, and each increase was attenuated by treatment with PFTα (Figure 4C), suggesting that PFTα indeed inhibits p53 activity in these cells. To further show that *Fbxw7* deficiency in LICs induces apoptosis in a p53-dependent manner, we generated *Mx1-Cre; Fbxw7^{F/F}; p53^{-/-}* mice in order to determine the proportion of apoptotic cells and colony-forming ability for *Fbxw7^{Δ/Δ}; p53^{-/-}* LICs. The increase in the frequency of apoptosis apparent for *Fbxw7*-deficient LICs from recipients of the second BMT was not observed with *Fbxw7^{Δ/Δ}; p53^{-/-}* LICs

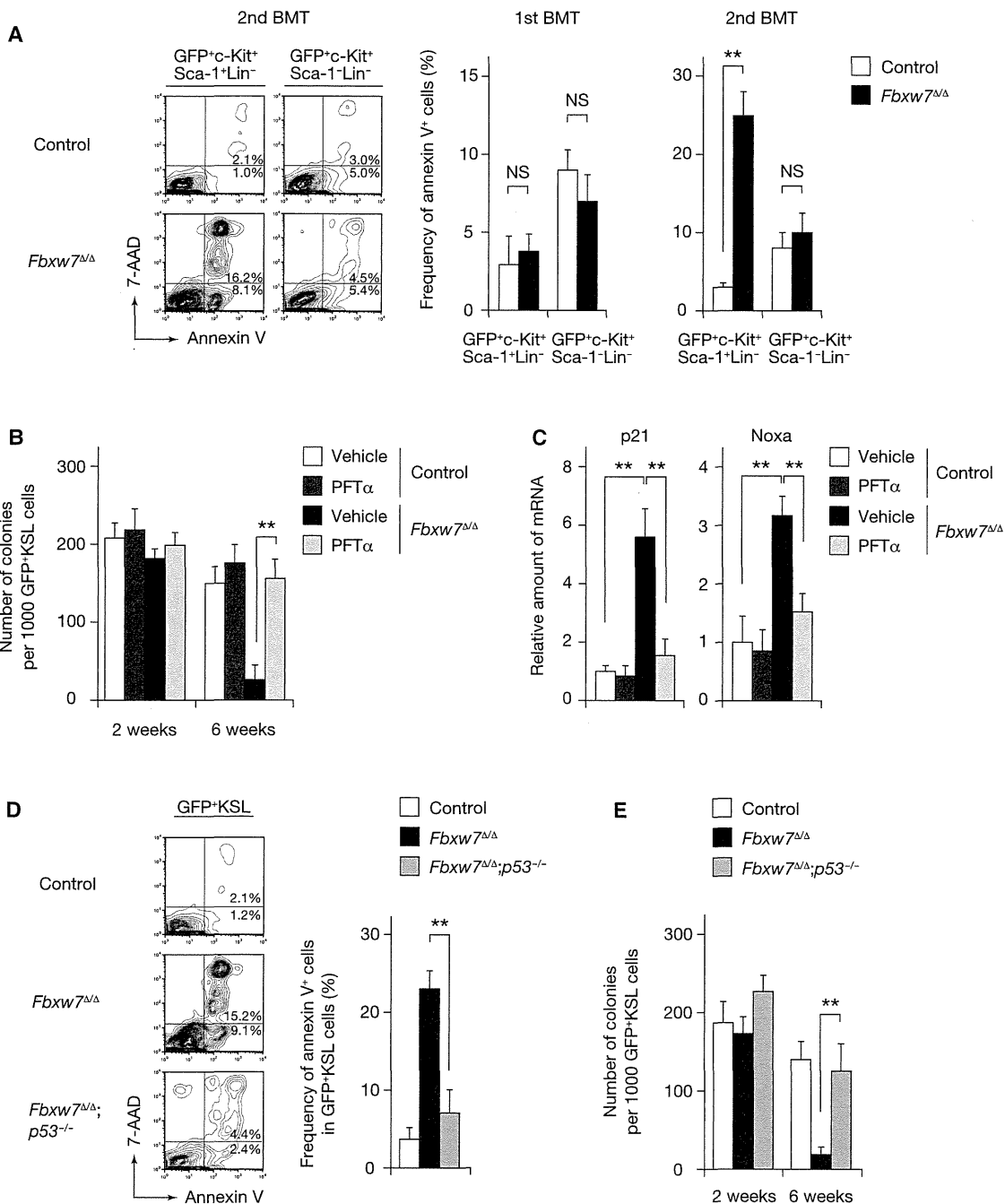


Figure 4. Apoptosis Is Induced in *Fbxw7*-Deficient LICs in a p53-Dependent Manner

(A) The frequency of annexin V⁺ cells among *Fbxw7*^{Δ/Δ} and control cells of the indicated fractions from mouse recipients (n = 3) of the first or second BMT was determined by flow cytometry. 7-AAD, 7-aminoactinomycin D.

(B) Colony formation by *Fbxw7*^{Δ/Δ} and control LICs after culture for 2 or 6 weeks with or without PFTα (n = 5).

(C) Control and *Fbxw7*^{Δ/Δ} LICs were assayed for p21 and Noxa mRNAs by RT and real-time PCR analysis after culture on OP-9 cells for 2 weeks with or without PFTα (n = 5).

(D) The proportion of annexin V⁺ cells among GFP⁺KSL cells was determined by flow cytometry for control, *Fbxw7*^{Δ/Δ}, or *Fbxw7*^{Δ/Δ}; p53^{-/-} bone marrow cells from recipients (n = 3) of the second BMT.

(E) Colony formation by cells of the indicated genotypes after culture for 2 or 6 weeks on OP-9 cells (n = 3).

Data are means ± SD. **p < 0.01; NS, not significant.

(Figure 4D). Consistent with this finding, the decrease in the number of colonies derived from Fbxw7-deficient LICs after 6 weeks of culture on OP-9 cells was reversed by deletion of the p53 gene (Figure 4E). Collectively, these results indicated that Fbxw7 deficiency in LICs results in deregulated c-Myc activation, impaired maintenance of quiescence, subsequent apoptosis as a result of p53 induction, and consequent cell exhaustion.

The Combination of Fbxw7 Ablation and Anticancer Drug Treatment Is Effective for LIC Eradication

Although our results implicated Fbxw7 as a potential target for leukemia therapy, the timing of CML development and the survival rate did not differ between the recipients of the first BMT harboring control or Fbxw7-deficient LICs (Figures 2E and 2G), suggesting that inhibition of Fbxw7 alone would not suffice as an effective therapy for CML. To address this problem, we examined the effects of combining Fbxw7 ablation with imatinib. Recipients of the first BMT were injected with plpC on 7 alternate days beginning the day after transplantation and were then administered imatinib twice a day at 100 mg/kg for 2 weeks beginning 20 days after transplantation (Figure 5A). Determination of the proportion of annexin V⁺ cells among GFP⁺KSL cells revealed that the frequency of apoptosis among imatinib-treated *Fbxw7*^{Δ/Δ} LICs was significantly greater than that among imatinib-treated control LICs (Figure 5B), indicating that Fbxw7-deficient LICs are sensitive to imatinib. In contrast, imatinib was able to efficiently induce apoptosis in both control and Fbxw7-deficient leukemic progenitors and bulk leukemic cells. The number of colonies formed by imatinib-treated *Fbxw7*^{Δ/Δ} GFP⁺KSL cells was smaller than that formed by vehicle-treated *Fbxw7*^{Δ/Δ} GFP⁺KSL cells or by imatinib-treated control GFP⁺KSL cells (Figure 5C).

Analysis of the effects of this combination therapy on CML development *in vivo* revealed that it markedly reduced the number of Fbxw7-deficient LICs (Figure 5D). Consistent with these findings, most mice treated with imatinib alone showed a moderate delay in the onset of disease but developed CML after discontinuation of imatinib (Figure 5E), dying within 60 days after BMT (Figure 5F). In contrast, combination therapy with Fbxw7 ablation and imatinib resulted in a marked attenuation of CML development that remained apparent even after discontinuation of imatinib treatment (Figures 5E and 5F). Furthermore, when LICs isolated from recipients of the first BMT were transplanted into new recipients, we found that, whereas LICs treated with imatinib alone still had the potential to confer disease, an apparently complete cure was achieved in all recipients of LICs treated with the combination therapy of Fbxw7 ablation and imatinib (Figure 5F). These results indicated that the effectiveness of the combination therapy is attributable to disrupted maintenance of LICs. We also combined *Fbxw7* deletion and treatment with the conventional anticancer drug cytosine arabinoside (Ara-C) and obtained similar results (Figures S4A–S4D). Analysis of signaling downstream of BCR-ABL in GFP⁺KSL cells isolated from recipients of the first BMT at 36 days after transplantation revealed no differences in the proportions of cells expressing phosphorylated forms of Stat5, Crkl, or Akt between imatinib-treated control and *Fbxw7*^{Δ/Δ} LICs (Figures S4E–S4G). Collectively, these data thus suggested

that LICs whose quiescence is interrupted by Fbxw7 loss are actively cycling and thus sensitive to imatinib or Ara-C treatment and that these combination therapies of Fbxw7 ablation and anticancer drug administration are able to eradicate LICs and provide a survival advantage compared with currently available treatments.

Fbxw7 Deficiency Affects LICs More than It Does Normal HSCs

Given that Fbxw7 is essential for maintenance of both HSCs and LICs, the targeting of LICs by Fbxw7 ablation combined with anticancer drugs might also be expected to damage HSCs. We thus compared the sensitivity of HSCs and LICs to Fbxw7 ablation. To this end, we infected KSL cells from control or *Mx1-Cre;Fbxw7*^{F/F} mice with the retrovirus encoding the p210^{BCR-ABL} oncoprotein and GFP (to yield LICs) or with a virus encoding GFP alone (to yield HSCs) and then transplanted the cells into recipient mice. GFP⁺KSL cells from these mice were subsequently transferred to new recipients, which were then injected with plpC (Figure 6A). Analysis of GFP⁺KSL cells from these latter recipients revealed that exit from quiescence induced by Fbxw7 ablation was more pronounced for LICs than for HSCs (Figure 6B). Accordingly, whereas the proportion of apoptotic cells among LICs did not differ from that among HSCs isolated from recipients of the first BMT, it was greater for Fbxw7-deficient LICs than for Fbxw7-deficient HSCs isolated from recipients of a second BMT (Figure 6C). Consistent with these results, whereas Fbxw7 deficiency did not affect the number of HSCs or LICs in recipients of the first BMT, it reduced the number of LICs to a greater extent than it did that of HSCs in recipients of the second BMT (Figure 6D).

To examine the mechanism underlying this difference in sensitivity to Fbxw7 deficiency between HSCs and LICs, we first compared the amount of Fbxw7 mRNA in these cells. RT and real-time PCR analysis revealed that the abundance of Fbxw7 mRNA in LICs was more than twice that in HSCs (Figure S5A). Given that such an increase in the amount of Fbxw7 mRNA was not observed in KSL cells expressing a kinase-dead (K1176R) mutant of BCR-ABL (Zhang and Ren, 1998), the upregulation of Fbxw7 mRNA in LICs is likely attributable to BCR-ABL kinase activity. The level of c-Myc mRNA was also markedly increased in LICs compared with that in HSCs or in KSL cells expressing the kinase-dead mutant of BCR-ABL (Figure S5B), and intracellular flow cytometric analysis revealed that the abundance of c-Myc in LICs was about five times that in HSCs or in KSL cells expressing the BCR-ABL mutant (Figure S5C). We also confirmed that these effects of BCR-ABL on KSL cells were reversed by imatinib treatment (Figures S5A–S5C). We further examined whether the difference in sensitivity to Fbxw7 deficiency between HSCs and LICs might be attributable to the difference in the abundance of c-Myc in these cells. Both exit from quiescence and apoptosis induced by *Fbxw7* deletion were more pronounced in HSCs overexpressing c-Myc and were less pronounced in *c-Myc*^{+Δ} HSCs, than in control HSCs (Figures S5D and S5E). Accordingly, the decrease in the number of stem cells induced by Fbxw7 ablation in recipients of the second BMT was greater for HSCs overexpressing c-Myc, and smaller for *c-Myc*^{+Δ} HSCs, than for control HSCs (Figure S5F). We next compared sensitivity to combination therapy with

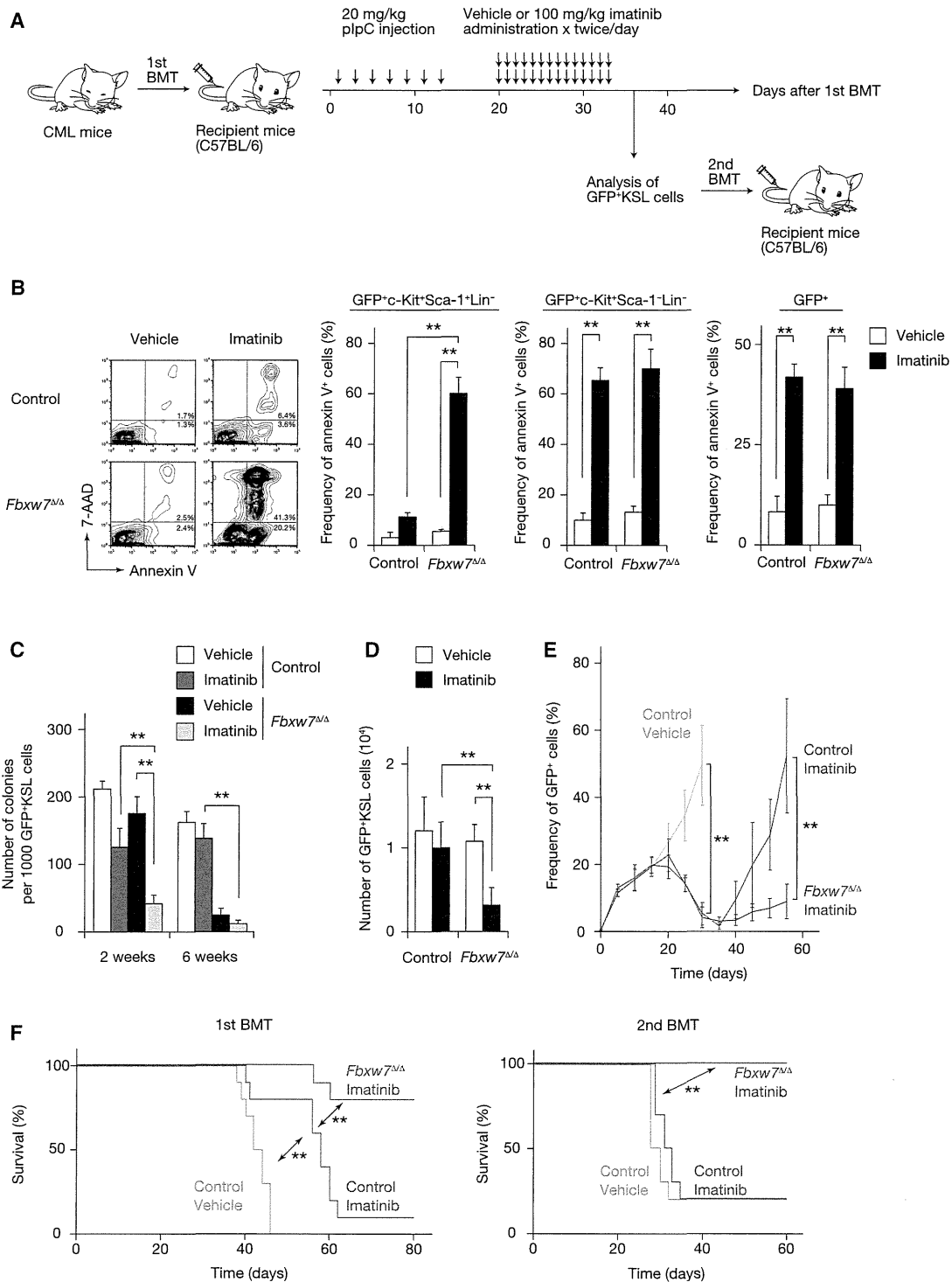


Figure 5. Combination Therapy with Fbxw7 Ablation and Imatinib Eliminates LICs

(A) Experimental strategy for combination therapy with Fbxw7 ablation and imatinib administration.

(B) Frequency of annexin V positivity among *Fbxw7*^{Δ/Δ} or control cells of the indicated fractions isolated from recipients (n = 3) of the first BMT after treatment with imatinib or vehicle.

(C) Colony formation by *Fbxw7*^{Δ/Δ} and control LICs cultured on OP-9 cells with imatinib or vehicle for 2 or 6 weeks (n = 3).

(D) Absolute number of GFP⁺KSL cells in bone marrow from mice (n = 5) treated as in (A).

(E) Frequency of GFP⁺ cells in peripheral blood from mice (n = 10) treated as in (A).

(F) Survival of recipients (n = 10) of the first and second BMT treated as in (A).

Data are means ± SD. **p < 0.01. See also Figure S4.

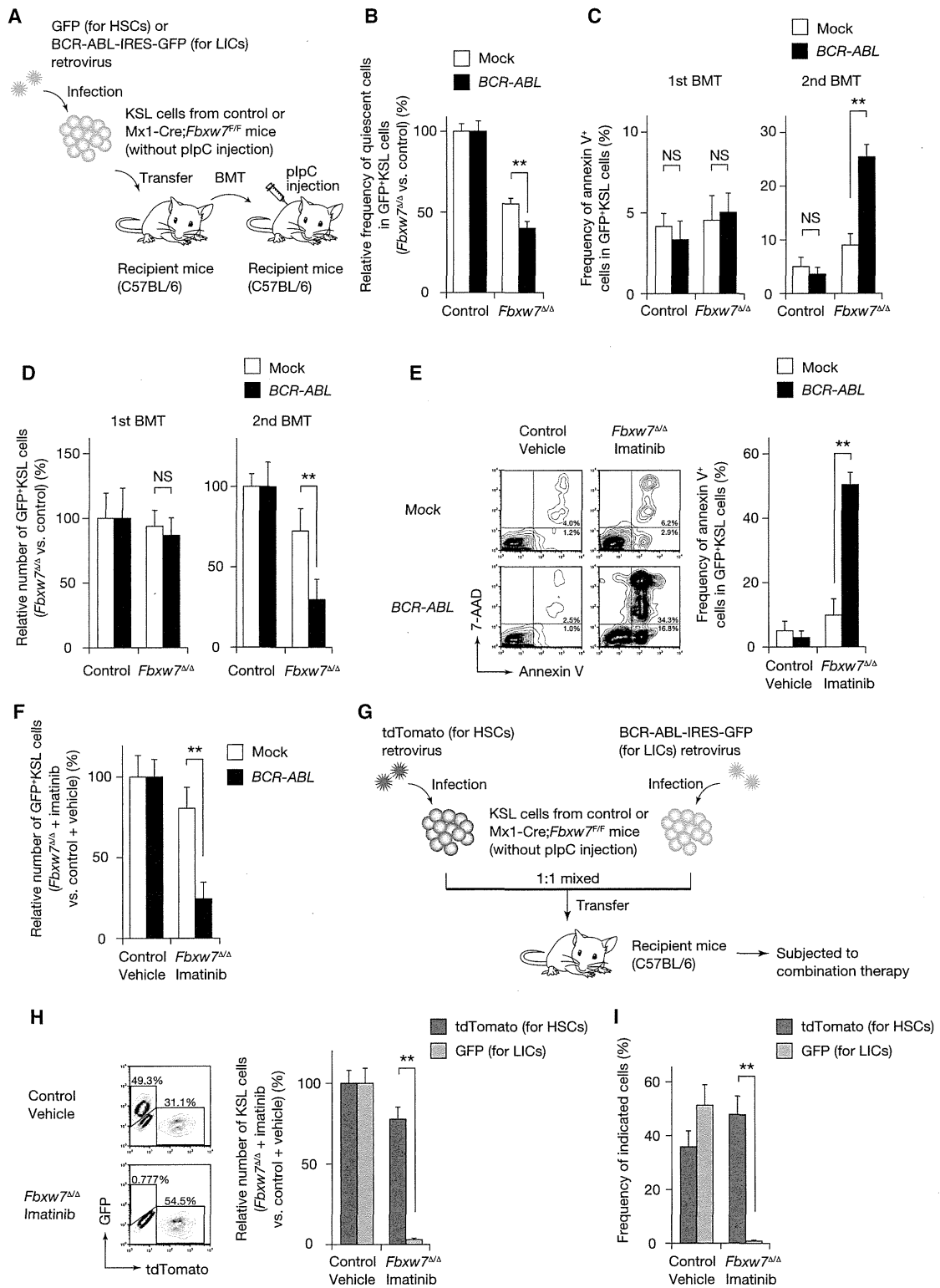


Figure 6. Fbxw7 Deficiency Affects LICs to a Greater Extent than It Does Normal HSCs

(A) Experimental strategy to compare the sensitivity of LICs to Fbxw7 deficiency with that of normal HSCs.

(B) The relative percentage of quiescent cells among GFP⁺KSL cells for Fbxw7^{Δ/Δ} cells compared with that for control cells infected with the corresponding vector was determined for recipients of the first BMT (n = 5).

(C) The frequency of annexin V⁺ cells among GFP⁺KSL cells for Fbxw7^{Δ/Δ} cells compared with that for control cells infected with the corresponding vector was determined for recipients of the first and second BMTs (n = 3).

(legend continued on next page)

Fbxw7 ablation and imatinib administration between HSCs and LICs. The frequency of apoptosis among cells from recipient mice treated with this combination therapy was markedly greater for LICs than for HSCs (Figure 6E). Consistent with these results, the decrease in the number of stem cells was more prominent for LICs than for HSCs after combination therapy (Figure 6F).

Finally, to determine directly whether this combination therapy is able to eradicate LICs while preserving normal HSC function, we transplanted an equal number (1×10^4) of KSL cells infected with a retrovirus encoding the fluorescent marker tdTomato (to yield HSCs) and of KSL cells infected with the virus for BCR-ABL and GFP (to yield LICs) into the same recipient mice and then subjected the animals to combination therapy (Figure 6G). Flow cytometric analysis revealed that this regimen reduced the number of GFP⁺KSL cells (LICs) to a greater extent than it did that of tdTomato⁺KSL cells (HSCs) (Figure S5G). We then collected the same number (1×10^4) of tdTomato⁺KSL cells and GFP⁺KSL cells from these recipients and again transplanted them together into new recipients. Whereas Fbxw7-deficient tdTomato⁺KSL cells (HSCs) persisted in these new recipients, almost all Fbxw7-deficient GFP⁺KSL cells (LICs) were eradicated (Figure 6H). Furthermore, whereas tdTomato⁺ cells (normal progeny of HSCs) were detected in the peripheral blood, virtually no GFP⁺ cells (leukemic progeny of LICs) were detected (Figure 6I). Collectively, these observations thus reveal a difference in sensitivity to the combination therapy between HSCs and LICs, referred to as a “therapeutic window,” and they thus provide a rationale for further development of this potential approach to the treatment of human leukemia.

Downregulation of Fbxw7 Is Effective for Eradication of Human LICs

To examine whether Fbxw7 ablation is indeed effective for eradication of human LICs in CML, we first measured the amount of Fbxw7 mRNA at various stages of the differentiation of bone marrow cells from patients in the chronic phase of CML. Similar to our findings with the mouse model of CML (Figure 1B), Fbxw7 mRNA was abundant in the LIC compartment (CD34⁺CD38⁻Lin⁻ fraction), and its amount decreased markedly as the cells differentiated (Figure 7A). We next transfected bone marrow cells from such patients with small interfering RNAs (siRNAs) specific for Fbxw7 by electroporation, and we confirmed that almost all LICs were successfully transfected without induction of a substantial level of apoptosis by the procedure (Figure S6A) and that Fbxw7 mRNA was depleted efficiently (Figure 7B). Transfection with either of two independent such siRNAs resulted in a decrease in the proportion of quiescent cells in the

LIC compartment (Figure 7C). Depletion of Fbxw7 in human LICs resulted in marked enhancement of the induction of apoptosis (Figure 7D) and the inhibition of colony formation (Figure 7E) by imatinib. Similar results were obtained with human LICs subjected to combination therapy with Fbxw7 depletion and Ara-C (Figures S6B and S6C).

Finally, we compared the sensitivity to Fbxw7 deficiency and combination therapy between human HSCs and LICs. We first measured the amount of Fbxw7 mRNA at various stages of the differentiation of bone marrow cells from healthy volunteers and found that Fbxw7 mRNA was abundant in HSCs and was downregulated during cell differentiation (Figure S6D). Depletion of Fbxw7 induced entry of human HSCs into the cell cycle (Figure S6E), and, as with mouse HSCs and LICs, this effect was greater in LICs than in HSCs (Figure 7F). In addition, induction of apoptosis and inhibition of colony formation by the combination of Fbxw7 depletion and imatinib treatment were more pronounced in LICs than in HSCs (Figures 7G and 7H; Figure S6F). Together, these results suggested that the sensitivity to combination therapy differs markedly between human HSCs and LICs, as was the case with mouse HSCs and LICs and that such therapy is a promising approach to the treatment of human CML.

DISCUSSION

With the use of a mouse model of CML, we have found that Fbxw7 has an indispensable role in maintenance of the quiescence as well as the stemness of LICs. Fbxw7-deficient LICs became exhausted with time and incapable of generating CML in transplanted animals. In contrast to LICs, leukemic progenitors proliferated rapidly, and their cell cycle status was not affected by Fbxw7 deficiency. The abundance of Fbxw7 mRNA was high in LICs and relatively low in leukemic progenitors, suggesting that Fbxw7 expression is regulated at the transcriptional level during leukemogenesis. The difference in cell cycle status between LICs and leukemic progenitors is therefore likely attributable, at least in part, to the difference in the abundance of Fbxw7 in these cells. Although the mechanisms responsible for regulation of Fbxw7 expression remain unknown, given that the HSC niche is thought to maintain HSCs in a quiescent state, it is possible that signals from the LIC niche may control Fbxw7 expression in LICs.

Our data also provide mechanistic insight into the maintenance of LIC quiescence by Fbxw7. We found that c-Myc accumulated in Fbxw7-deficient LICs, and either treatment with a c-Myc inhibitor or deletion of one allele of the c-Myc gene

(D) The relative number of GFP⁺KSL cells among *Fbxw7*^{Δ/Δ} cells compared with that for control cells infected with the corresponding vector was determined for recipients of the first and second BMTs (n = 5).

(E) The proportion of apoptotic cells among GFP⁺KSL cells isolated from recipient mice harboring retrovirus-infected Mx1-Cre;*Fbxw7*^{F/F} or control cells and injected with plpC and then treated with imatinib or vehicle for 14 days was determined (n = 3).

(F) The number of GFP⁺KSL cells in recipient mice processed as in (E) was determined relative to that in corresponding vehicle-treated recipients (n = 5).

(G) Experimental strategy to examine directly the difference in sensitivity to combination therapy between LICs and normal HSCs.

(H) An equal number (1×10^4) of tdTomato⁺KSL cells and GFP⁺KSL cells was collected from the recipients treated as in (G) and transplanted into new recipient mice together with 2×10^5 bone marrow cells from wild-type mice (second BMT). The relative numbers of tdTomato⁺KSL cells and GFP⁺KSL cells in the recipients (n = 5) of the second BMT were determined.

(I) The frequencies of tdTomato⁺ cells and GFP⁺ cells in peripheral blood from the recipients (n = 10) of the second BMT obtained as in (H) were determined. Data are means ± SD. **p < 0.01; NS, not significant. See also Figure S5.

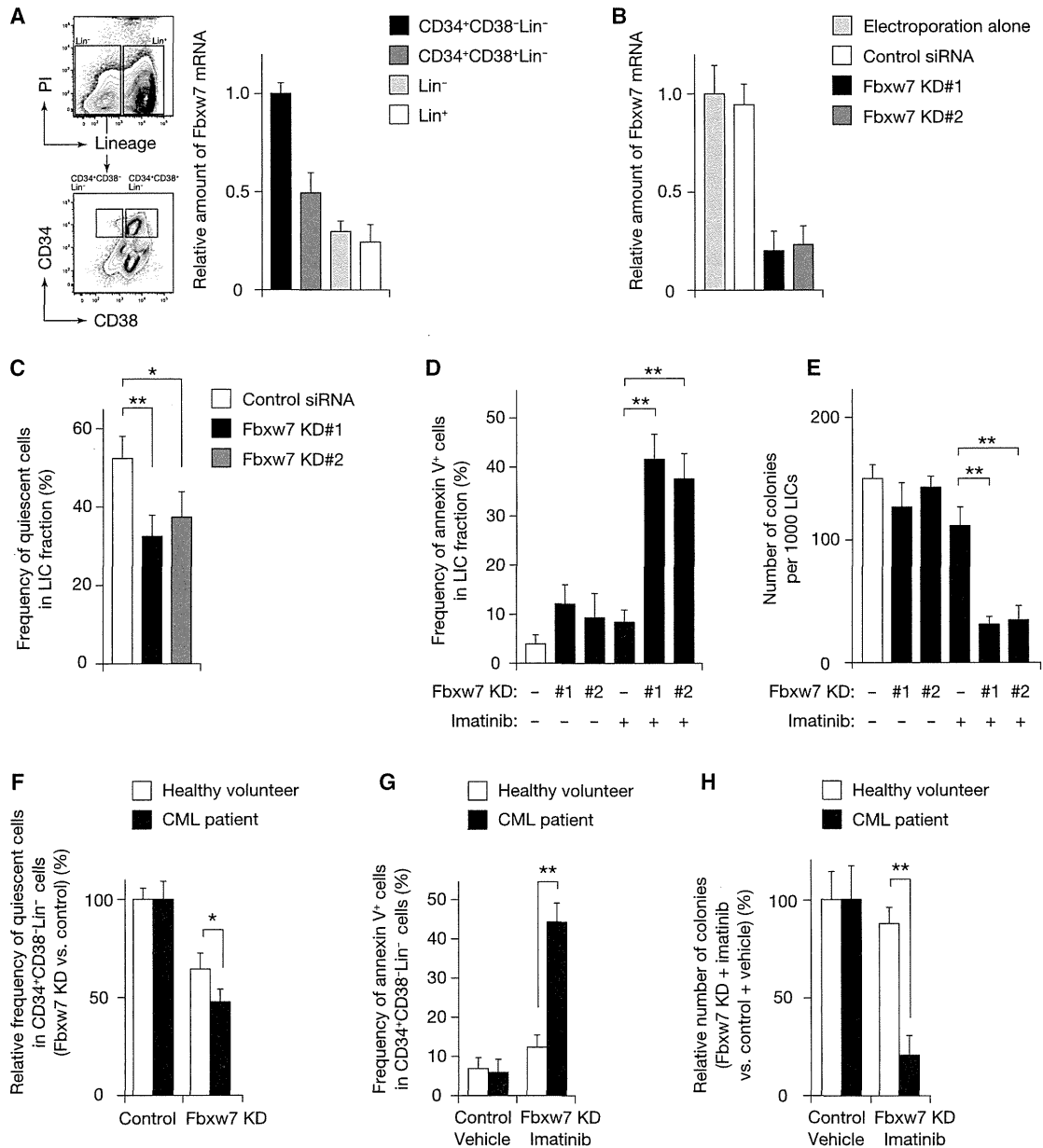


Figure 7. Combination Therapy with Fbxw7 Downregulation and Imatinib Is Effective for Eradication of Human LICs

(A) Bone marrow cells at various stages of differentiation from CML patients were fractionated by FACS and assayed for Fbxw7 mRNA by RT and real-time PCR analysis (n = 3).

(B) Bone marrow cells from patients with CML were transfected with a control siRNA or one of two independent Fbxw7 siRNAs (KD#1 or KD#2), after which the CD34⁺CD38⁻Lin⁻ fraction was cultured on OP-9 cells for 3 days and then assayed for Fbxw7 mRNA (n = 3).

(C) Bone marrow cells from CML patients were transfected with Fbxw7 siRNAs, after which the CD34⁺CD38⁻Lin⁻ fraction was sorted and cultured on OP-9 cells as in (B). The frequency of quiescent cells was then measured by flow cytometry (n = 3).

(D) Cells from CML patients were transfected, sorted, and cultured on OP-9 cells for 7 days. The cells were exposed (or not) to imatinib for the last 4 days of the culture, after which the proportion of apoptotic cells was determined by staining with annexin V (n = 3).

(E) Colony formation by human CML LICs transfected with Fbxw7 siRNAs and treated with imatinib as in (D) (n = 3).

(F) The relative frequencies of quiescent cells among human HSCs and LICs transfected with Fbxw7 or control siRNAs were determined as in (C) (n = 3).

(G) The frequency of annexin V⁺ cells in human HSCs and LICs treated as in (D) (n = 3).

(H) The relative numbers of colonies formed by human HSCs and LICs transfected with Fbxw7 or control siRNAs and treated with vehicle or imatinib as in (D) were determined (n = 3).

Data are means ± SD. *p < 0.05; **p < 0.01. NS, not significant. See also Figure S6.

normalized the phenotype of these cells, including their cell cycle status and colony formation capacity. These results support the notion that c-Myc activation is responsible for cell cycle progression and the resulting exhaustion of Fbxw7-deficient LICs. Previous studies have indicated that an increase in metabolic growth signaling alone, including activation of the mTOR signaling pathway, is sufficient to drive quiescent HSCs into the proliferative state (Yilmaz et al., 2006). Given that mTOR is a candidate substrate of Fbxw7 (Mao et al., 2008), it was possible that increased mTOR activity might be responsible for the phenotype of Fbxw7-deficient LICs. However, we found that mTOR did not accumulate in Fbxw7-deficient LICs, and the mTOR signaling inhibitor rapamycin did not rescue the phenotype of these cells. We therefore conclude that mTOR activity is not related to the phenotype of Fbxw7-deficient LICs.

We also provide evidence that Fbxw7-deficient LICs are sensitive to anticancer drugs and that combination therapy with Fbxw7 ablation and such drugs is effective for LIC eradication, resulting in a survival advantage over treatment with anticancer drugs alone. Although the development of imatinib has substantially improved the prognosis of CML patients (Druker et al., 2001; Kantarjian et al., 2002), CML LICs are resistant to imatinib and residual LICs give rise to relapse after discontinuation of imatinib treatment (Mahon et al., 2010). Several mechanisms underlying resistance to imatinib have been proposed, including quiescence, BCR-ABL mutations such as T315I, and the lack of addiction to BCR-ABL in LICs (Corbin et al., 2011; Gorre et al., 2001; Holtz et al., 2007). Although more potent TKIs such as nilotinib and dasatinib have been developed, these drugs are also not able to overcome these mechanisms of TKI resistance in LICs (Copland et al., 2006; Jørgensen et al., 2007). We now show that combination therapy with Fbxw7 ablation and imatinib markedly reduced the rate of relapse after discontinuation of imatinib. We also obtained results suggesting that such combination therapy is effective for eradication of LICs in human CML.

Although our study suggests that inhibition of Fbxw7 might represent a promising therapeutic approach for CML patients, the application of Fbxw7 inhibition to the treatment of cancer warrants careful consideration. Given that Fbxw7 has been regarded as an oncosuppressor protein because it targets many proto-oncoproteins, growth promoters, and antiapoptotic molecules including cyclin E, c-Myc, Notch, c-Jun (Nakayama and Nakayama, 2006), mTOR (Mao et al., 2008), and Mcl-1 (Inuzuka et al., 2011; Wertz et al., 2011), suppression of Fbxw7 might induce carcinogenesis or promote cancer growth. Nevertheless, our experimental evidence indicates that the combination therapy with Fbxw7 ablation and anticancer drugs is effective for treatment of CML in a mouse model at the animal level as well as for eradication of human LICs.

Another concern about Fbxw7-targeted therapy is whether normal stem cells might be damaged. Fbxw7 also plays a pivotal role in HSC maintenance (Matsuoka et al., 2008; Thompson et al., 2008), and recent studies have shown that Fbxw7 is a key regulator of the viability of neural stem and progenitor cells (Hoeck et al., 2010; Matsumoto et al., 2011a). Although we found that LICs are more sensitive to Fbxw7 deficiency than are HSCs, suggesting that there is a therapeutic window in targeting Fbxw7 for therapy, it remains to be determined to what extent Fbxw7

inhibition might damage stem cells in other tissues. To minimize damage to normal stem cells, we propose that Fbxw7 inhibitors should be used for only a limited period. Our present study suggests that the difference in sensitivity to Fbxw7 inhibition between LICs and HSCs is attributable at least in part to the difference in the abundance of Fbxw7 or c-Myc in these cells and that such therapy may be expandable to other types of human cancer. Fbxw7 is therefore a promising target for the discovery of anticancer drugs with a broad spectrum of activity against many human cancers.

EXPERIMENTAL PROCEDURES

Mice

Generation of *Fbxw7^{F/F}* mice was described previously (Onoyama et al., 2007). They were crossed with Mx1-Cre transgenic mice (kindly provided by K. Rajewsky) to generate Mx1-Cre;*Fbxw7^{F/F}* mice, and Mx1-Cre;*Fbxw7^{+/+}* mice were used as controls. *Fbxw7^{F/F}* mice were also crossed with *c-Myc^{F/F}* mice (kindly provided by I.M. de Alboran) or *p53^{-/-}* mice (Taconic). All these mice were backcrossed with C57BL/6 mice for more than five generations. Expression of Cre recombinase in transplant recipients was induced by intraperitoneal injection of plpC (Merck) at a dose of 20 mg per kilogram of body mass on 7 alternate days. C57BL/6 mice were obtained from The Jackson Laboratory and were used as recipients. All mouse experiments were approved by the Animal Ethics Committee of Kyushu University. For some experiments, mice were injected intraperitoneally with Ara-C (Sigma) at a dose of 150 mg/kg. Imatinib (Novartis) was administered by oral gavage twice a day at a dose of 100 mg/kg.

Generation of CML Model

Immature c-Kit⁺Sca-1⁺Lin⁻ hematopoietic cells (KSL cells) were isolated by fluorescence-activated cell sorting (FACS) from bone marrow of Mx1-Cre;*Fbxw7^{F/F}* and Mx1-Cre;*Fbxw7^{+/+}* mice and were cultured in serum-free S-Clone SF-O3 medium (Sanko Junyaku) supplemented with mouse stem cell factor (Wako) at 100 ng/ml as well as human thrombopoietin (PeproTech) at 100 ng/ml. For generation of the CML mouse model, the KSL cells were infected for 2 days with a retrovirus harboring the MSCV-BCR-ABL-IRES-GFP construct (see Supplemental Experimental Procedures) with the use of CombiMag (OZ Biosciences). GFP⁺ cells (2×10^4) were then isolated by FACS and were injected intravenously into lethally irradiated (9.5 Gy) C57BL/6 mice together with 2×10^5 bone marrow cells from C57BL/6 mice. The efficiency of gene transduction as evaluated on the basis of GFP expression was 30 to 40% in all experiments. When the number of white blood cells had increased to $>20,000/\mu\text{l}$, we collected 2×10^4 GFP⁺KSL cells from the recipient mice and transferred them to new recipients together with 2×10^5 bone marrow cells from C57BL/6 mice (first BMT). These new recipients were injected with plpC beginning the day after BMT as described earlier. For serial transplantation, 1×10^4 or 2×10^4 GFP⁺KSL cells were collected from recipient mice 3 weeks after the first BMT and were transplanted into other recipient mice together with 2×10^5 bone marrow cells from C57BL/6 mice (second BMT).

Colony Formation Assays

Colony formation by mouse cells was examined with the use of Methocult medium (MethoCult GF M3434, StemCell Technologies). Control and *Fbxw7^{Δ/Δ}* GFP⁺KSL cells (1×10^3) were collected from recipients of the first BMT and then cultured under hypoxic (5% O₂) conditions with OP-9 stromal cells (Kodama et al., 1994) (RIKEN Cell Bank) for 2 or 6 weeks in six-well dishes containing α -minimum essential medium (Sigma) supplemented with 12.5% fetal bovine serum (Invitrogen), 12.5% horse serum (Invitrogen), and 1 nM dexamethasone (Wako). The cells (2×10^4) were then collected and transferred to Methocult medium. Colonies were scored 1 week after plating for determination of the number of colonies per 1000 GFP⁺KSL cells. For some experiments, 100 μM 10058-F4 (Merck), 10 μM DAPT (Merck), 25 nM rapamycin (Cell Signaling Technology), 10 μM PFT α (Merck), 100 nM Ara-C, or 5 μM imatinib was added to the culture medium.

Analysis of Primary Human CML Samples

Viable bone marrow mononuclear cells from treatment-naïve patients in the chronic phase of CML and from healthy volunteers who had given informed consent were obtained from AllCells. The use of these purchased samples was considered exempt by the Ethics Committee of Kyushu University. To examine the role of Fbxw7 in the maintenance of LICs for human CML and normal HSCs, we transfected bone marrow mononuclear cells with 300 nM Fbxw7 siRNAs (Stealth Select RNAi siRNA, Invitrogen) by electroporation with the use of an Amaxa Nucleofector II device (Lonza) according to the recommended protocol. The transfected cells were then stained with antibodies to human CD34 (8G12), CD38 (HIT2), CD3 (SK7), CD16 (3G8), CD19 (SJ25C1), CD20 (L27), CD14 (MφP9), and CD56 (NCAM16.2) (BD Biosciences). CD3, CD16, CD19, CD20, CD14, and CD56 were used as lineage markers. CD34⁺CD38⁻Lin⁻ cells (1×10^3) were purified by FACS and cocultured with OP-9 cells for 7 days. For some experiments, 100 nM Ara-C or 5 μ M imatinib was added to the cultures for Days 3 to 7. The human cells were then assayed for apoptosis (see Supplemental Experimental Procedures) or for colony formation; for the latter assay, the cells were transferred to Methocult medium (MethoCult GF H4435, StemCell Technologies), and the number of colonies was counted 1 week after plating.

Statistical Analysis

Quantitative data are presented as means \pm SD and were analyzed by Student's t test, with the exception that survival curves were analyzed by the log-rank nonparametric test. A p value of < 0.05 was considered statistically significant.

Other Experimental Procedures

Flow cytometry, preparation of recombinant retroviruses, cell cycle analysis, detection of apoptosis, RT and real-time PCR analysis, and immunoblot analysis are described in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found in this article online at <http://dx.doi.org/10.1016/j.ccr.2013.01.026>.

ACKNOWLEDGMENTS

We thank K. Rajewsky for Mx1-Cre transgenic mice, I.M. de Alboran for *c-Myc*^{F/F} mice, H. Honda for BCR-ABL cDNA, and T. Kitamura for Plat-E retroviral packaging cells.

Received: June 29, 2011

Revised: April 25, 2012

Accepted: January 22, 2013

Published: March 18, 2013

REFERENCES

- Clevers, H. (2011). The cancer stem cell: premises, promises and challenges. *Nat. Med.* *17*, 313–319.
- Copland, M., Hamilton, A., Elrick, L.J., Baird, J.W., Allan, E.K., Jordanides, N., Barow, M., Mountford, J.C., and Holyoake, T.L. (2006). Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction. *Blood* *107*, 4532–4539.
- Corbin, A.S., Agarwal, A., Loriaux, M., Cortes, J., Deininger, M.W., and Druker, B.J. (2011). Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J. Clin. Invest.* *121*, 396–409.
- de Klein, A., van Kessel, A.G., Grosveld, G., Bartram, C.R., Hagemeijer, A., Bootsma, D., Spurr, N.K., Heisterkamp, N., Groffen, J., and Stephenson, J.R. (1982). A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. *Nature* *300*, 765–767.
- Deininger, M.W., Goldman, J.M., and Melo, J.V. (2000). The molecular biology of chronic myeloid leukemia. *Blood* *96*, 3343–3356.
- Druker, B.J., Talpaz, M., Resta, D.J., Peng, B., Buchdunger, E., Ford, J.M., Lydon, N.B., Kantarjian, H., Capdeville, R., Ohno-Jones, S., and Sawyers, C.L. (2001). Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N. Engl. J. Med.* *344*, 1031–1037.
- Essers, M.A., Offner, S., Blanco-Bose, W.E., Waibler, Z., Kalinke, U., Duchosal, M.A., and Trumpp, A. (2009). IFN α activates dormant haematopoietic stem cells in vivo. *Nature* *458*, 904–908.
- Follis, A.V., Hammoudeh, D.I., Daab, A.T., and Metallo, S.J. (2009). Small-molecule perturbation of competing interactions between c-Myc and Max. *Bioorg. Med. Chem. Lett.* *19*, 807–810.
- Gorre, M.E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P.N., and Sawyers, C.L. (2001). Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* *293*, 876–880.
- Hoeck, J.D., Jandke, A., Blake, S.M., Nye, E., Spencer-Dene, B., Brandner, S., and Behrens, A. (2010). Fbw7 controls neural stem cell differentiation and progenitor apoptosis via Notch and c-Jun. *Nat. Neurosci.* *13*, 1365–1372.
- Holtz, M., Forman, S.J., and Bhatia, R. (2007). Growth factor stimulation reduces residual quiescent chronic myelogenous leukemia progenitors remaining after imatinib treatment. *Cancer Res.* *67*, 1113–1120.
- Huntly, B.J., and Gilliland, D.G. (2005). Leukaemia stem cells and the evolution of cancer-stem-cell research. *Nat. Rev. Cancer* *5*, 311–321.
- Inuzuka, H., Shaik, S., Onoyama, I., Gao, D., Tseng, A., Maser, R.S., Zhai, B., Wan, L., Gutierrez, A., Lau, A.W., et al. (2011). SCF^(Fbw7) regulates cellular apoptosis by targeting MCL1 for ubiquitylation and destruction. *Nature* *471*, 104–109.
- Ito, K., Bernardi, R., Morotti, A., Matsuoka, S., Saglio, G., Ikeda, Y., Rosenblatt, J., Avigan, D.E., Teruya-Feldstein, J., and Pandolfi, P.P. (2008). PML targeting eradicates quiescent leukaemia-initiating cells. *Nature* *453*, 1072–1078.
- Jørgensen, H.G., Allan, E.K., Jordanides, N.E., Mountford, J.C., and Holyoake, T.L. (2007). Nilotinib exerts equipotent antiproliferative effects to imatinib and does not induce apoptosis in CD34⁺ CML cells. *Blood* *109*, 4016–4019.
- Kantarjian, H., Sawyers, C., Hochhaus, A., Guilhot, F., Schiffer, C., Gambacorti-Passerini, C., Niederwieser, D., Resta, D., Capdeville, R., Zoellner, U., et al.; International STI571 CML Study Group. (2002). Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. *N. Engl. J. Med.* *346*, 645–652.
- Kodama, H., Nose, M., Niida, S., Nishikawa, S., and Nishikawa, S. (1994). Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells. *Exp. Hematol.* *22*, 979–984.
- Komarov, P.G., Komarova, E.A., Kondratov, R.V., Christov-Tselkov, K., Coon, J.S., Chernov, M.V., and Gudkov, A.V. (1999). A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* *285*, 1733–1737.
- Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M.A., and Dick, J.E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* *367*, 645–648.
- Laurenti, E., Wilson, A., and Trumpp, A. (2009). Myc's other life: stem cells and beyond. *Curr. Opin. Cell Biol.* *21*, 844–854.
- Mahon, F.X., Réa, D., Guilhot, J., Guilhot, F., Hugué, F., Nicolini, F., Legros, L., Charbonnier, A., Guerci, A., Varet, B., et al.; Intergroupe Français des Leucémies Myéloïdes Chroniques. (2010). Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. *Lancet Oncol.* *11*, 1029–1035.
- Mao, J.H., Kim, I.J., Wu, D., Climent, J., Kang, H.C., DelRosario, R., and Balmain, A. (2008). FBXW7 targets mTOR for degradation and cooperates with PTEN in tumor suppression. *Science* *321*, 1499–1502.
- Matsumoto, A., Onoyama, I., Sunabori, T., Kageyama, R., Okano, H., and Nakayama, K.I. (2011a). Fbxw7-dependent degradation of Notch is required for control of "stemness" and neuronal-glial differentiation in neural stem cells. *J. Biol. Chem.* *286*, 13754–13764.

- Matsumoto, A., Takeishi, S., Kanie, T., Susaki, E., Onoyama, I., Tateishi, Y., Nakayama, K., and Nakayama, K.I. (2011b). p57 is required for quiescence and maintenance of adult hematopoietic stem cells. *Cell Stem Cell* 9, 262–271.
- Matsuoka, S., Oike, Y., Onoyama, I., Iwama, A., Arai, F., Takubo, K., Mashimo, Y., Oguro, H., Nitta, E., Ito, K., et al. (2008). Fbxw7 acts as a critical fail-safe against premature loss of hematopoietic stem cells and development of T-ALL. *Genes Dev.* 22, 986–991.
- Naka, K., Hoshii, T., Muraguchi, T., Tadokoro, Y., Ooshio, T., Kondo, Y., Nakao, S., Motoyama, N., and Hirao, A. (2010). TGF- β -FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. *Nature* 463, 676–680.
- Nakayama, K.I., and Nakayama, K. (2006). Ubiquitin ligases: cell-cycle control and cancer. *Nat. Rev. Cancer* 6, 369–381.
- Onoyama, I., Tsunematsu, R., Matsumoto, A., Kimura, T., de Alborán, I.M., Nakayama, K., and Nakayama, K.I. (2007). Conditional inactivation of Fbxw7 impairs cell-cycle exit during T cell differentiation and results in lymphomagenesis. *J. Exp. Med.* 204, 2875–2888.
- Pear, W.S., Miller, J.P., Xu, L., Pui, J.C., Soffer, B., Quackenbush, R.C., Pendergast, A.M., Bronson, R., Aster, J.C., Scott, M.L., and Baltimore, D. (1998). Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood* 92, 3780–3792.
- Reavie, L., Della Gatta, G., Crusio, K., Aranda-Orgilles, B., Buckley, S.M., Thompson, B., Lee, E., Gao, J., Bredemeyer, A.L., Helmink, B.A., et al. (2010). Regulation of hematopoietic stem cell differentiation by a single ubiquitin ligase-substrate complex. *Nat. Immunol.* 11, 207–215.
- Rowley, J.D. (1973). Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature* 243, 290–293.
- Thompson, B.J., Jankovic, V., Gao, J., Buonamici, S., Vest, A., Lee, J.M., Zavadil, J., Nimer, S.D., and Aifantis, I. (2008). Control of hematopoietic stem cell quiescence by the E3 ubiquitin ligase Fbw7. *J. Exp. Med.* 205, 1395–1408.
- Wertz, I.E., Kusam, S., Lam, C., Okamoto, T., Sandoval, W., Anderson, D.J., Helgason, E., Ernst, J.A., Eby, M., Liu, J., et al. (2011). Sensitivity to antitubulin chemotherapeutics is regulated by MCL1 and FBW7. *Nature* 471, 110–114.
- Yamazaki, S., Ema, H., Karlsson, G., Yamaguchi, T., Miyoshi, H., Shioda, S., Taketo, M.M., Karlsson, S., Iwama, A., and Nakauchi, H. (2011). Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. *Cell* 147, 1146–1158.
- Yilmaz, O.H., Valdez, R., Theisen, B.K., Guo, W., Ferguson, D.O., Wu, H., and Morrison, S.J. (2006). Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature* 441, 475–482.
- Zhang, X., and Ren, R. (1998). Bcr-Abl efficiently induces a myeloproliferative disease and production of excess interleukin-3 and granulocyte-macrophage colony-stimulating factor in mice: a novel model for chronic myelogenous leukemia. *Blood* 92, 3829–3840.
- Zhao, C., Blum, J., Chen, A., Kwon, H.Y., Jung, S.H., Cook, J.M., Lagoo, A., and Reya, T. (2007). Loss of β -catenin impairs the renewal of normal and CML stem cells in vivo. *Cancer Cell* 12, 528–541.

PNAS PLUS

BIOLOGICAL SCIENCES: Neuroscience

**Gating of LTD by $\delta 2$ glutamate receptors—a new mechanism by
coordinated interaction between two AMPA receptor phosphorylation sites**

Kazuhiisa Kohda^{a,b,1}, Wataru Kakegawa^{a,b,1}, Shinji Matsuda^{a,b,c}, Tadashi Yamamoto^d,
Hisashi Hirano^e, Michisuke Yuzaki^{a,b,2}

^aDepartment of Physiology, School of Medicine, Keio University, Shinjuku-ku, Tokyo 160-8582, Japan; ^bCREST, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan; ^cPRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan; ^dCell Signal Unit, Okinawa Institute of Science and Technology Graduate University, Kunigami, Okinawa 904-0495, Japan; ^eDivision of Functional Proteomics, Yokohama City University, Turumi-ku, Yokohama 230-0045, Japan

¹K.K. and W.K. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: myuzaki@a5.keio.jp

Significance Statement

Gating of LTD by α 2 glutamate receptors—a new mechanism by coordinated interaction between two AMPA receptor phosphorylation sites

Kazuhisa Kohda, Wataru Kakegawa, Shinji Matsuda, Tadashi Yamamoto, Hisashi Hirano, Michisuke Yuzaki

Long-term depression (LTD) commonly affects learning and memory in various brain regions. Although LTD in the cerebellum absolutely requires GluD2 glutamate receptors, its underlying mechanisms have remained elusive. LTD is caused by endocytosis of GluA2 glutamate receptors, which is triggered by neuronal activity-induced serine phosphorylation of GluA2. Our work showed that this serine phosphorylation required prior dephosphorylation of the nearby tyrosine residue. By interaction with a tyrosine phosphatase, GluD2 regulated tyrosine dephosphorylation status of GluA2 to gate inducibility of LTD. These findings will provide better understanding of general mechanisms regulating AMPA receptor endocytosis during synaptic plasticity.

Abstract

Long-term depression (LTD) commonly affects learning and memory in various brain regions. While cerebellar LTD absolutely requires the $\delta 2$ glutamate receptor (GluD2) expressed in Purkinje cells, LTD in other brain regions does not; why and how cerebellar LTD is regulated by GluD2 remains elusive. Here, we show that activity-dependent phosphorylation of serine 880 (S880) of GluA2 AMPA receptors, an essential step for AMPA receptor endocytosis during LTD induction, was impaired in *GluD2*-null cerebellum. In contrast, basal-state phosphorylation of tyrosine 876 (Y876) of GluA2 was increased in *GluD2*-null cerebellum. Interestingly, *in vitro* phosphorylation assay revealed that Y876 phosphorylation inhibited subsequent S880 phosphorylation. Conversely, Y876 dephosphorylation was sufficient to restore S880 phosphorylation and LTD induction in *GluD2*-null Purkinje cells. Furthermore, the protein tyrosine phosphatase PTPMEG, which binds to the C-terminus of GluD2, directly dephosphorylated Y876. These data indicate that GluD2 gates LTD by coordinating interaction between two phosphorylation sites of GluA2.

Synaptic plasticity, such as long-term potentiation and long-term depression (LTD), is believed to underlie learning and memory processes *in vivo*. LTD is observed in various brain regions, such as the cerebellum and hippocampus, and is commonly caused by clathrin-dependent endocytosis of postsynaptic AMPA-type glutamate receptors. However, cerebellar LTD is unique in that it absolutely requires the presence of another class of glutamate receptor, the $\delta 2$ glutamate receptor (GluD2) (1), which is predominantly expressed at parallel fiber (PF)–Purkinje cell synapses; *GluD2*-null mice display ataxia and impaired motor learning (2). Although morphological abnormalities at synapses, which may be responsible for certain phenotypes of *GluD2*-null mice, are rescued by expression of a mutant GluD2 transgene lacking the C-terminal seven amino acids, LTD and motor learning remained impaired (3, 4). Furthermore, acute application of an antibody specific for GluD2's extracellular domain to wild-type cerebellum abrogated LTD induction and impaired motor performance (5). These results indicate that GluD2 plays a direct role in LTD induction and motor learning. Nevertheless, the fundamental question about how and why GluD2 regulates cerebellar LTD remains elusive.

AMPA receptor endocytosis is generally regulated by changes in phosphorylation status of AMPA receptor subunits. Activity-dependent phosphorylation of serine 880 (S880) of the GluA2 subunit plays an important role in AMPA receptor endocytosis and LTD induction through the release of glutamate receptor interacting protein 1 (GRIP1), an anchoring protein, from the C terminus of the AMPA receptors at both hippocampal (6, 7) and cerebellar synapses (8-10). However, how this process is regulated and mediates LTD is not completely understood. In addition to phosphorylation of S880, phosphorylation of tyrosine 876 (Y876) of the GluA2 subunit also regulates AMPA