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# Role of Fbxw7 in the maintenance of normal stem cells and cancer-initiating cells

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In addition to the properties of self-renewal and multipotency, stem cells are characterised by their distinct cell cycle status. Somatic stem cells are maintained in a quiescent state but switch reversibly from quiescence to proliferation as needed. On the other hand, embryonic stem cells and induced pluripotent stem cells proliferate rapidly until the induction of differentiation results in inhibition of cell cycle progression. Uncovering the mechanisms underlying cell cycle control in stem cells should thus provide insight into regulation of the balance between self-renewal and differentiation, a key goal of stem cell biology. Recent research has shown that cancer-initiating cells (CICs), a cell population with stem cell-like properties in cancer, are also quiescent, with this characteristic conferring resistance to anticancer therapies that target dividing cells. Elucidation of the mechanisms of CIC quiescence might therefore be expected to provide a basis for the eradication of cancer. This review summarises our current understanding of the role of F-box and WD40 repeat domain-containing 7 (Fbxw7), a key regulator of the cell cycle, in the maintenance of normal stem cells and CICs, as well as attempts to define future challenges in this field.

F-box and WD40 repeat domain-containing 7 (Fbxw7; also known as Fbw7, Sel-10, hCDC4, or hAgo) is an F-box protein that is responsible for substrate recognition by an SCF (Skp1-Cul1-F-box protein)-type ubiquitin ligase complex. The *Fbxw7* gene generates three isoforms (Fbxw7 $\alpha$ , - $\beta$ , and - $\gamma$ ) that differ only at their amino termini, with each isoform possessing the dimerisation domain, the F-box domain that recruits the other components of the ubiquitin ligase complex, and the WD40 repeats that bind substrates (Welcker and Clurman, 2008). Fbxw7 $\alpha$  has been the most extensively studied of these isoforms. Fbxw7 has pivotal roles in cell division, growth, and differentiation by targeting several proteins – including c-Myc, Notch1, Notch4, c-Jun, and cyclin E – for degradation (Nakayama and Nakayama, 2006) (Figure 1A). Fbxw7 binds each of these substrates through a conserved phosphorylated domain known as the Cdc4 phosphodegron. Given that most of these proteins targeted by Fbxw7 for degradation are proto-oncoproteins, Fbxw7 has been thought to function as a tumour suppressor. Indeed, heterozygous mutations in *FBXW7* have been detected in several types of human cancer, including T-cell acute lymphoblastic leukaemia (T-ALL), T-cell lymphoma, and cholangiocarcinoma (Nakayama and Nakayama, 2006; Welcker and Clurman, 2008). Nearly three-quarters of these mutations are point mutations that result in amino-acid

substitutions at key positions in the WD40 repeats and consequent disruption of substrate binding. Most of the remaining mutations are nonsense mutations that result in the production of truncated forms of Fbxw7. These clinical observations thus indicate that Fbxw7 is crucial for preventing carcinogenesis as a result of its role in cell cycle regulation. In this review, we discuss the impact of Fbxw7 on maintenance of both normal stem cells – including somatic stem cells, embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs) – and cancer-initiating cells (CICs), as well as the application of such knowledge to the development of novel anticancer therapies.

## ROLE OF FBXW7 IN THE MAINTENANCE OF NORMAL STEM CELLS

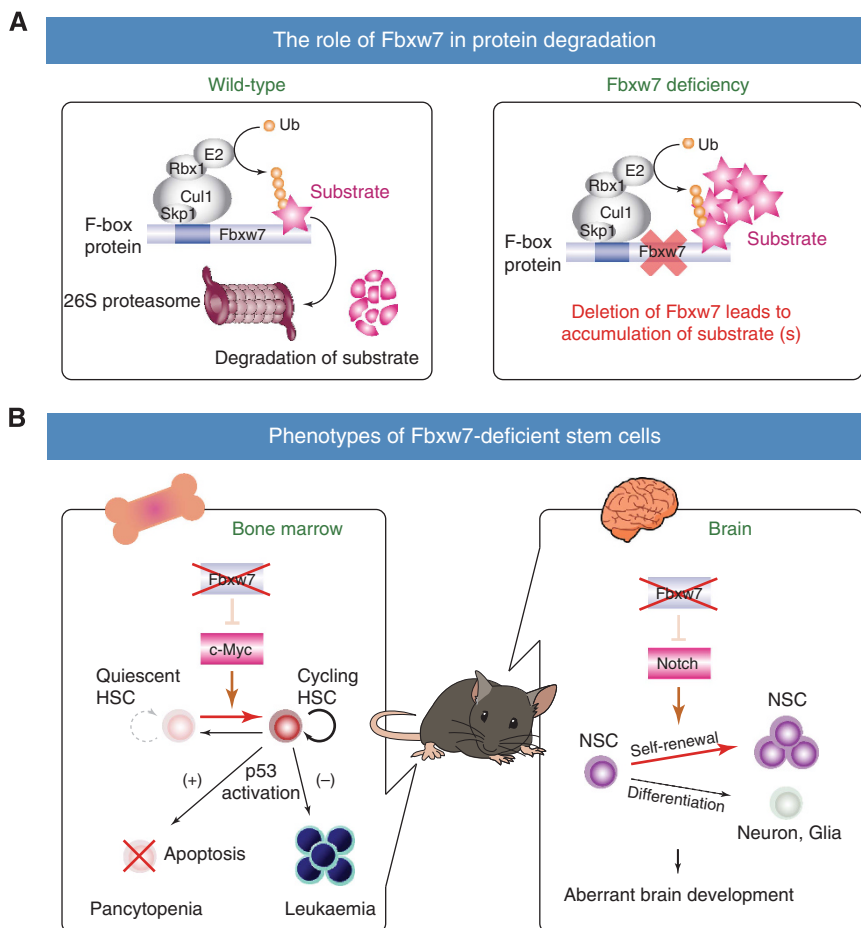
**Haematopoietic stem cells.** Haematopoietic stem cell (HSCs) are among the most well characterised of somatic stem cells and have served as a paradigm for stem cells in other tissues, with the notion that maintenance of quiescence is critical for somatic stem cell function being largely derived from studies of HSCs. Although recent evidence indicates that the entry of HSCs into the cell cycle is triggered by cytokines, including interferon- $\alpha$  and

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**Figure 1.** The F-box protein Fbxw7 is required for maintenance of normal stem cells. **(A)** Fbxw7 is a component of, and responsible for substrate recognition by, an SCF-type ubiquitin ligase. Ubiquitylated substrates are subject to degradation by the proteasome. In the absence of Fbxw7, these substrates accumulate in a manner dependent on cellular context and thereby influence diverse cellular events such as cell cycle progression. **(B)** Fbxw7 ablation in HSCs results in the accumulation of c-Myc and consequent re-entry of the cells into the cell cycle. The cycling HSCs are then subject to apoptosis as a result of p53 activation, leading to the development of pancytopenia. In the absence of p53 induction, the Fbxw7-deficient HSCs eventually give rise to leukaemia. On the other hand, loss of Fbxw7 in NSCs results in Notch accumulation, with the consequent imbalance between self-renewal and differentiation in these cells, leading to aberrant brain development.

granulocyte colony-stimulating factor (Trumpp *et al*, 2010), intrinsic mechanisms underlying direct regulation of the cell cycle in HSCs have only just begun to be unveiled. Consistent with the finding that control of c-Myc abundance is essential for regulation of both self-renewal and differentiation in HSCs (Wilson *et al*, 2004), maintenance of quiescence was found to be defective in HSCs from mice in which Fbxw7 is ablated specifically in the haematopoietic system (Matsuoka *et al*, 2008; Thompson *et al*, 2008; Reavie *et al*, 2010). This phenotype was rescued by the additional deletion of c-Myc, indicating that c-Myc stabilisation resulting from Fbxw7 ablation indeed induced the re-entry of HSCs into the cell cycle (Figure 1B). Fbxw7 was also found to regulate c-Myc abundance in HSCs of fetal mouse liver. In line with the notion that quiescence in stem cells have a pivotal role in sustaining self-renewal capacity throughout the lifetime of an organism as well as in preventing carcinogenesis, ~30% of the mutant mice exhibited a marked decrease in the number of HSCs (referred to as ‘stem cell exhaustion’) and resultant severe pancytopenia, with most of the animals that did not show leukopenia eventually developing T-ALL. Furthermore, whereas a substantial proportion of HSCs in the former animals was found to undergo apoptosis associated with upregulation of p53 expression, this phenotype was not observed in the latter mice. Additional depletion of p53 in the Fbxw7-deficient mice ameliorated anaemia and thrombocytopenia, but led to the development of T-cell

malignancies with a much shorter latency. These findings indicate that p53-dependent apoptosis in Fbxw7-deficient HSCs serves to prevent carcinogenesis in response to c-Myc accumulation at the expense of maintenance of the HSC pool size. Fbxw7 and p53 may thus constitute a dual safety device that ensures HSC integrity. Conversely, forced expression of Fbxw7 in HSCs was shown to suppress c-Myc accumulation and to cause the cells to adopt a more quiescent state (Iriuchishima *et al*, 2011). These Fbxw7-overexpressing HSCs exhibited an increased capacity to reconstitute bone marrow, further supporting the conclusion that Fbxw7 has a central role in the maintenance of HSCs through the regulation of their quiescence.

**Neural stem cells.** The central nervous system is another well-studied tissue that is sustained by stem cells, in this instance designated neural stem cells (NSCs), with the Notch signalling pathway having been found to have a key role in NSC self-renewal (Schuurmans and Guillemot, 2002). Two groups have independently generated mice with brain-specific deletion of Fbxw7 (Hoeck *et al*, 2010; Matsumoto *et al*, 2011) and found that Notch1 accumulates in the Fbxw7-deficient brain at around embryonic day (E) 16.5, as well as in neurospheres derived from Fbxw7-deficient neurons (Figure 1B). Consistent with these findings, the proliferation of NSCs in the ventricular zone of the mutant mice is enhanced at E16.5. In addition to Notch, c-Jun was found to

accumulate in neurospheres derived from Fbxw7-deficient neurons, whereas NSCs deficient in Fbxw7 did not exhibit c-Myc accumulation, unlike Fbxw7-depleted HSCs. Interestingly, a neurosphere assay revealed that, whereas the number of primary neurospheres formed from Fbxw7-deficient neurons was smaller than that formed from control neurons, Fbxw7 deficiency was associated with an increased number of secondary and tertiary neurospheres. This result indicates that loss of Fbxw7 results in an increase in the number of NSCs, although it remains unclear why the effects of Fbxw7 deficiency differ between HSCs and NSCs. The fact that NSCs have not been purified to the same extent as have HSCs and the difference in the substrates that accumulate after Fbxw7 ablation between these two stem cell types may have a role, however. In addition to the impact of Fbxw7 loss on NSC self-renewal, a primary culture assay revealed that Fbxw7-deficient NSCs exhibit impairment of neuronal differentiation and enhanced astroglial differentiation. The phenotypes of Fbxw7-deficient NSCs revealed by both neurosphere and primary culture assays were reversed by treatment with a pharmacological inhibitor of the Notch signalling pathway, indicating that ablation of Fbxw7 in NSCs enhances self-renewal ability and skews differentiation potential by increasing Notch activity. The imbalance between self-renewal and differentiation in Fbxw7-deficient NSCs resulted in morphological abnormalities of the brain, including a dilated and distorted third ventricle with a horizontal sulcus in the mutant mice, as well as their death shortly after birth associated with the absence of suckling behaviour. A recent study showed that Hes5, a key downstream effector of Notch signalling, directly inhibits the production of Fbxw7 $\beta$  mRNA, and that NSCs in Fbxw7 heterozygous knockout mice exhibit impaired differentiation *in vivo* (Sancho *et al*, 2013). Taken together, these observations thus indicate that Fbxw7 is critical for NSC regulation, as well as for HSC maintenance.

**Intestinal stem cells.** The epithelium of the small intestine is organised into crypt–villus units, with each unit being self-renewing and showing a higher self-renewal rate than any other mammalian tissue. Although intestinal stem cells (ISCs) are thought to be responsible for this turnover, their identity and cell cycle status remain to be established (Clevers, 2013). Fbxw7 heterozygous knockout mice were found to manifest impairment of goblet cell differentiation (Sancho *et al*, 2013), and conditional deletion of both alleles of *Fbxw7* in the intestine induced the development of adenomas that accumulate several Fbxw7 substrates, including both c-Myc and Notch (Babaei-Jadidi *et al*, 2011). Whereas Fbxw7 ablation alone did not result in spontaneous intestinal neoplasia, it accelerated intestinal tumourigenesis characterised by accumulation of c-Jun and DEK in the context of adenomatous polyposis coli deficiency. Simultaneous deletion of both Fbxw7 and p53 in the intestine led to the development of aggressive and metastatic intestinal adenocarcinomas (Grim *et al*, 2012). Although this finding indicates that Fbxw7 and p53 cooperatively suppress intestinal cancer, similar to their cooperativity apparent in bone marrow, it remains to be determined whether the phenotypes of the Fbxw7-deficient intestine are attributable to the effects of Fbxw7 ablation on ISCs. Future identification of *bona fide* ISCs should help to decipher the role of Fbxw7 in the maintenance of these cells.

**Embryonic stem cells.** In contrast to adult somatic stem cells, ESCs are characterised by extraordinarily rapid proliferation (Orford and Scadden, 2008). Whereas cyclin E–CDK2 (cyclin-dependent kinase 2) activity is periodic and peaks at the G<sub>1</sub>–S transition of the cell cycle in somatic cells, this complex is constitutively activated in ESCs, resulting in a shortening of the duration of G<sub>1</sub> phase. In line with this characteristic, c-Myc is abundant in self-renewing ESCs, whereas it undergoes down-regulation in response to induction of differentiation. Conversely,

the amount of Fbxw7 is low in self-renewing ESCs and is upregulated on differentiation induction (Reavie *et al*, 2010). Consistent with this finding, ESC-specific knockdown of Fbxw7 did not affect alkaline phosphatase positivity or Nanog expression, indicating that Fbxw7 is dispensable for the self-renewal of ESCs. On the other hand, RNA interference-mediated depletion of Fbxw7 during differentiation induction in ESCs resulted in the retention of colony-like morphology, and these colonies consisted of Nanog- or Oct4-positive ESCs (Buckley *et al*, 2012). Among known substrates of Fbxw7, the abundance of only c-Myc increased in response to Fbxw7 depletion, and additional depletion of c-Myc rescued the phenotypes of the Fbxw7-depleted ESCs. These results indicate that Fbxw7 controls pluripotency of ESCs by regulating c-Myc protein stability. Whereas regulation of ESC pluripotency has been extensively investigated at the level of gene transcription, these studies indicate the importance of posttranslational control in the regulation of ESC function.

**iPSCs.** Induced pluripotent stem cells (iPSCs), which were originally generated by forced expression of Oct3/4, Sox2, Klf4, and c-Myc (OSKM) in fibroblasts (Takahashi and Yamanaka, 2006), are highly similar to ESCs with respect to their morphology, cell cycle status, and developmental potential (Stadtfield and Hochedlinger, 2010). The finding that loss of Fbxw7 results in ‘maintenance’ of pluripotency in ESCs led to the hypothesis that Fbxw7 suppression may also have a role in ‘induction’ of pluripotency, a process referred to as cellular reprogramming. Consistent with this notion, both forced expression of OSKM and knockdown of Fbxw7 in mouse embryonic fibroblasts resulted in the generation of ~60% more alkaline phosphatase-positive colonies compared with forced expression of OSKM alone, showing that silencing of Fbxw7 increases the efficiency of iPSC generation (Buckley *et al*, 2012). Although c-Myc is a key factor responsible for reprogramming efficiency (Takahashi and Yamanaka, 2006), additional depletion of c-Myc did not affect the promotion of iPSC generation induced by loss of Fbxw7, indicating that another Fbxw7 substrate (or substrates) contributes to this effect of Fbxw7 depletion (Okita *et al*, 2012). These results suggest that the molecular mechanisms underlying maintenance and induction of pluripotency may differ, and that identification of Fbxw7 substrates that increase the efficiency of iPSC generation should provide further insight into the reprogramming process and pave the way to improved manipulation of iPSCs.

#### ROLE OF FBXW7 IN THE MAINTENANCE OF CICs AND IMPLICATIONS FOR THE DEVELOPMENT OF NOVEL ANTICANCER THERAPIES

Leukaemia-initiating cells (LICs), a rare sub-population of cells that propagate leukaemia, have contributed to our knowledge of CICs just as HSCs have long served as an important model for delineating generalised mechanisms underlying regulation of normal stem cell function (Huntly and Gilliland, 2005). Leukaemia-initiating cells share many properties, such as self-renewal, multipotency, and quiescence, with normal HSCs, and with quiescence in LICs being thought to contribute to the resistance of leukaemia to conventional chemotherapy (Figure 2A). For instance, although the tyrosine kinase inhibitor (TKI) imatinib has markedly improved the prognosis of individuals with chronic myeloid leukaemia (CML) by targeting cycling leukaemic progenitors (Druker *et al*, 2001), the French CML Intergroup Stop Imatinib (STIM) study recently found that ~60% of CML patients in complete molecular remission for >2 years as a result of imatinib treatment relapsed within 12 months after discontinuation of such treatment (Mahon *et al*, 2010). With the use of a mouse model of CML, we recently showed that genetic ablation of

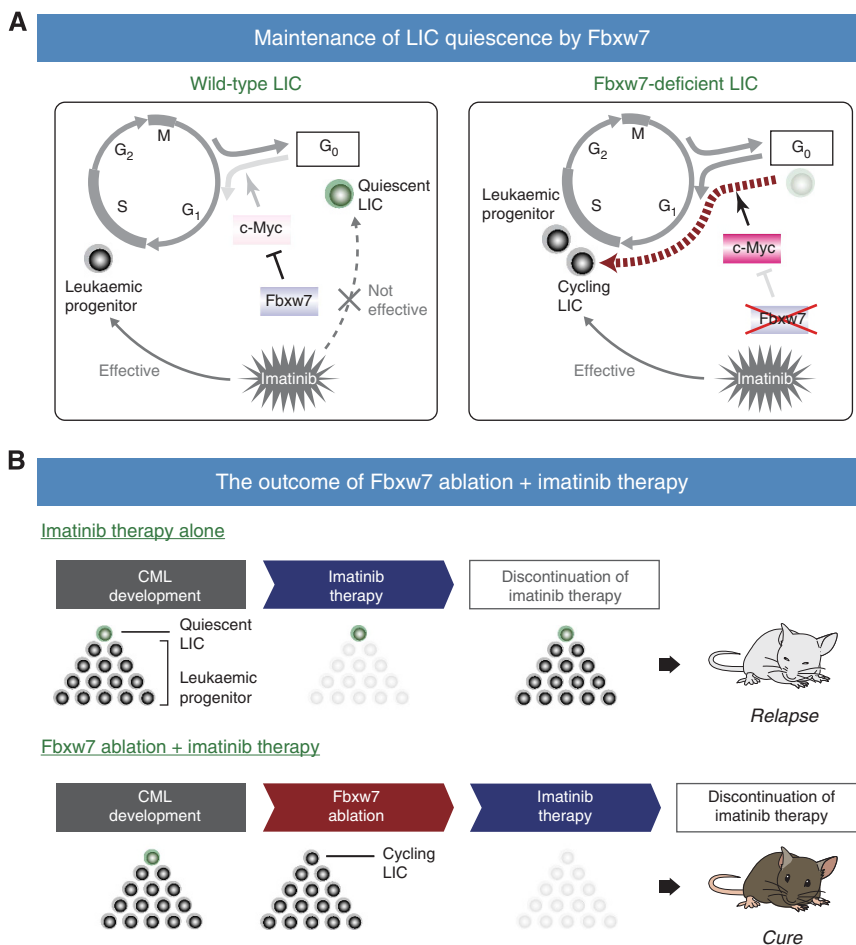


Figure 2. Fbxw7 ablation in LICs for CML sensitises the cells to imatinib by preventing quiescence. (A) Fbxw7 maintains CML LICs in a quiescent state by degrading c-Myc. Ablation of Fbxw7 induces re-entry of LICs into the cell cycle and thereby sensitises them to imatinib. (B) Imatinib therapy alone fails to eliminate quiescent LICs, with the residual LICs being responsible for disease relapse. In contrast, combination therapy with Fbxw7 ablation and imatinib administration is able to target quiescent LICs, thereby resulting in a complete cure.

Fbxw7 induced quiescent LICs to enter the cell cycle by eliciting the accumulation of c-Myc in these cells (Takeishi *et al*, 2013) (Figure 2A). Furthermore, these Fbxw7-deficient LICs were sensitive to imatinib, and the combination of Fbxw7 depletion and imatinib was effective for LIC eradication, leading to a marked decrease in the rate of relapse after discontinuation of imatinib treatment (Figure 2B). Importantly, this combination therapy was also effective for human LICs from CML patients.

Genetic ablation of Fbxw7 in a mouse model of B-cell acute lymphoblastic leukaemia was found to induce apoptosis associated with the expression of p53 target genes in leukaemic cells and to inhibit disease progression (Reavie *et al*, 2013). No increase in c-Myc protein abundance was apparent in the Fbxw7-deficient leukaemic cells, however, raising the possibility that Fbxw7 may target a different substrate (or substrates) in this type of leukaemia.

Whereas most of the findings described so far have been from studies of Fbxw7-knockout mice, recent research has shed light on the effects of missense mutations of Fbxw7 on stem cell function. Biochemical analysis revealed that a heterodimer of wild-type Fbxw7 and Fbxw7 harbouring a mutation in the WD40 repeats (an arginine-to-cysteine change at position 465) interacts with c-Myc to a lesser extent than does a homodimer of wild-type Fbxw7 (King *et al*, 2013). In agreement with this finding, the level of c-Myc in HSCs from mice heterozygous for this Fbxw7 mutation specifically in the haematopoietic system was greater than that in control HSCs but was still lower than that in Fbxw7-deficient HSCs. Surprisingly, this Fbxw7 mutation did not compromise the ability

of HSCs to reconstitute bone marrow, suggesting that the intermediate level of c-Myc accumulation induced by the heterozygous Fbxw7 mutation is tolerated by HSCs. Moreover, unlike Fbxw7 conditional knockout mice, the Fbxw7 mutant mice did not develop leukaemia spontaneously, indicating that mutations in *FBXW7* detected in T-ALL patients are not driver mutations. The Fbxw7 mutation both increased the frequency of LICs and accelerated disease progression in a mouse model of T-ALL induced by overexpression of a truncated form of Notch1, suggesting that Fbxw7 and Notch1 mutations may cooperate in the induction of human T-ALL.

## CONCLUSION

Although the role of Fbxw7 in the maintenance of stem cells has become increasingly clear, many important questions remain unanswered. For instance, whereas we have here discussed the role of the Fbxw7–c-Myc axis in HSC maintenance from the point of view of cell cycle regulation, it is possible that c-Myc accumulation in Fbxw7-deficient HSCs might disrupt HSC maintenance through other mechanisms. Of note, recent research indicates that HSCs contain few mitochondria and utilise anaerobic metabolism, and that this metabolic state directly regulates HSC function (Suda *et al*, 2011). Given that c-Myc regulates the expression of many genes related to mitochondrial biogenesis, the accumulation of



c-Myc in Fbxw7-deficient HSCs might affect the function of these cells through alteration of their metabolic state.

Another mystery is the mechanism underlying the regulation of Fbxw7 function *per se*. At least three mechanisms for regulation of the function of Fbxw7-substrate pairs can be envisioned: regulation of Fbxw7 abundance, of the ubiquitin ligase activity of Fbxw7, or of substrate phosphorylation. Fbxw7 is highly abundant in HSCs and CML LICs (Reavie *et al*, 2010, 2013; Takeishi *et al*, 2013), and our observation that Fbxw7 overexpression in HSCs augments their ability to reconstitute bone marrow (Iriuchishima *et al*, 2011) indicates that the abundance of Fbxw7 is an important determinant of its function. Although several molecules, including microRNAs, have been implicated in the regulation of Fbxw7 abundance in cell lines (Xu *et al*, 2010), it is unclear whether these molecules regulate the level of Fbxw7 in stem cells. A mechanism for control of the ubiquitin ligase activity of Fbxw7 has been described (Min *et al*, 2012), but it also remains to be determined whether this mechanism is operative in stem cells. Most, if not all, substrates of Fbxw7 are phosphorylated by glycogen synthase kinase 3 (GSK3), and GSK3 activity is inhibited by mitogen signalling through the phosphatidylinositol 3-kinase (PI3K)-Akt pathway (Welcker and Clurman, 2008). When the level of mitogenic signalling is low (i.e., GSK3 activity is high), Fbxw7 substrates become phosphorylated, resulting in their degradation and attenuation of cell cycle progression. Given that PTEN (phosphatase and tensin homolog), a negative regulator of the PI3K-Akt pathway, contributes to maintenance of HSC quiescence and function (Orford and Scadden, 2008), it is likely that Fbxw7 function in HSCs is controlled by the PI3K-Akt pathway through its regulation of GSK3 activity. An interesting possibility is that cytokines released from damaged bone marrow inhibit Fbxw7 function through activation of the PI3K-Akt pathway, and thereby induce the re-entry of HSCs into the cell cycle.

Although Fbxw7 appears to be a promising target for eradication of certain types of leukaemia, the application of recent findings to the clinic warrants careful consideration. Given that Fbxw7 has been regarded as an oncosuppressor protein, the attenuation of Fbxw7 function might be expected to induce carcinogenesis or to promote cancer development. However, experimental evidence indicates that combination therapy with Fbxw7 ablation and imatinib administration neither exacerbates CML nor induces T-ALL, suggesting that this approach does not pose a serious risk in this regard, at least in a CML mouse model (Takeishi *et al*, 2013). Although Fbxw7 inhibitors for mammals are currently unavailable, development of such inhibitors would help to determine whether Fbxw7-targeted therapy is indeed safe for human patients. The period of administration for an Fbxw7 inhibitor would need to be as short as possible to minimise any risk.

Another concern regarding Fbxw7-targeted therapy is whether normal stem cells might be damaged, given that Fbxw7 also has a key role in the maintenance of HSCs. Chronic myeloid leukaemia LICs were found to be more sensitive to Fbxw7 deficiency than were HSCs for both mice and humans (Reavie *et al*, 2013; Takeishi *et al*, 2013), however, suggesting that there may be a therapeutic window for the targeting of Fbxw7. Moreover, this difference in sensitivity was shown to be attributable, at least in part, to a difference in the abundance of Fbxw7 and c-Myc between CML LICs and HSCs, with this knowledge being of potential use for the development of Fbxw7 inhibitors with fewer side effects. Although it remains to be determined to what extent Fbxw7-targeted therapy might damage stem cells in other tissues, advances in the purification of other stem cells and in our understanding of the role of Fbxw7 in their maintenance should provide insight into this critical question.

Last, but not least, it will be important to examine whether findings related to Fbxw7 function in HSCs, NSCs, and LICs are

applicable to other stem cells, especially other CICs. Cancer-initiating cells have now been identified in a variety of cancers, including those of the breast, brain, and colon (Clevers, 2011). Recent advances in the generation of mouse models of these cancers, as well as in techniques for xenotransplantation of human cancer into immunodeficient mice, might help to unveil the role of Fbxw7 in other CICs. Such future studies will determine whether Fbxw7 is a promising target for the development of drugs with a broad spectrum of activity against cancers.

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