Knockdown of Fbxo7 reveals its regulatory role in proliferation and differentiation of haematopoietic precursor cells

El Kahina Meziane*, Suzanne J. Randle*, David E. Nelson, Mikhail Lomonosov and Heike Laman‡

University of Cambridge, Department of Pathology, Division of Cellular and Genetic Pathology, Tennis Court Road, Cambridge CB2 1QR, UK

*These authors contributed equally to this work
‡Author for correspondence (hl316@cam.ac.uk)

Accepted 25 February 2011
Journal of Cell Science 124, 2175-2186
© 2011, Published by The Company of Biologists Ltd
doi:10.1242/jcs.080465

Summary
Fbxo7 is an unusual F-box protein because most of its interacting proteins are not substrates for ubiquitin-mediated degradation. Fbxo7 directly binds p27 and Cdk6, enhances the level of cyclin D–Cdk6 complexes, and its overexpression causes Cdk6-dependent transformation of immortalised fibroblasts. Here, we test the ability of Fbxo7 to transform haematopoietic pro-B (Ba/F3) cells which, unexpectedly, it was unable to do despite high levels of Cdk6. Instead, reduction of Fbxo7 expression increased proliferation, decreased cell size and shortened G1 phase. Analysis of cell cycle regulators showed that cells had decreased levels of p27, and increased levels of S phase cyclins and Cdk2 activity. Also, Fbxo7 protein levels correlated inversely with those of CD43, suggesting direct regulation of its expression and, therefore, of B cell maturation. Alterations to Cdk6 protein levels did not affect the cell cycle, indicating that Cdk6 is neither rate-limiting nor essential in Ba/F3 cells; however, decreased expression of Cdk6 also enhanced levels of CD43, indicating that expression of CD43 is independent of cell cycle regulation. The physiological effect of reduced levels of Fbxo7 was assessed by creating a transgenic mouse with a LacZ insertion into the Fbxo7 locus. Homozygous Fbxo7LacZ mice showed significantly increased pro-B cell and pro-erythroblast populations, consistent with Fbxo7 having an anti-proliferative function and/or a role in promoting maturation of precursor cells.

Key words: Fbxo7, Cdk6, Cell cycle, Differentiation, Pro-B cell

Introduction
During the cell cycle, ubiquitin-mediated proteolysis provides a swift and precise means to regulate the abundance of cell cycle regulatory proteins, including cyclins and cyclin-dependent kinase (Cdk) inhibitors. This mechanism for regulating the turnover of proteins is mediated through ubiquitin ligases, which transfer ubiquitin to target proteins, enabling their timely destruction. SCF-type (Skp1–Cullin–F-box) ubiquitin ligases promote the specific attachment of poly-ubiquitin chains, which then triggers proteolysis by the 26S proteasome. SCF-type (Skp1–Cullin–F-box) ubiquitin ligases are multi-subunit complexes that consisting of Skp1, Cullin, Rbx1 and an F-box protein (FBP) (Ang and Wade, 2005; Deshaies, 1999; Jackson and Eldridge, 2002; Nakayama and Nakayama, 2005; Vodermaier, 2004). It is the FBP, which has a crucial role in specifically recruiting the target substrate, usually directed by post-translational modification of the substrate (Cenciarelli et al., 1999; Hermand, 2006; Ho et al., 2008; Jin et al., 2004; Winston et al., 1999). In cell cycle regulation, several FBPs that regulate G1–S phase regulators have been intensively studied. These include the prototypical S-phase kinase-associated protein 2 (Skp2, also known as Fbx11) and the F-box and WD repeat domain containing 7 (Fbxw7) that, respectively, regulate the levels of the Cdk cyclin inhibitor E and cyclin-dependent kinase inhibitor 1B (CDKN1B; also known as and hereafter referred to as p27). In addition, three FBPs – Fbxo4, Fbxw8, Fbxo31 – promote the ubiquitin-mediated degradation of cyclin D1 (Lin et al., 2006; Santra et al., 2009; Okabe et al., 2006).

Another FBP that interacts with G1–S regulatory proteins is F-box protein 7 (Fbxo7). In contrast to the destabilising effects of other FBPs, Fbxo7 acts as a specific assembly factor for cyclin D–Cdk6 complexes. Fbxo7 interacts directly with both Cdk6 and p27, and cooperatively increases cyclin D3 interactions with Cdk6 in vitro (Laman et al., 2005). In vivo, Fbxo7 overexpression in immortalised murine fibroblasts leads to their Cdk6-dependent transformation. These cells had increased levels of cyclin D–Cdk6 complexes and E2F activity, and formed tumours in athymic nude mice (Laman et al., 2005). Many mitogen and cytokine signalling pathways converge on cyclin D–Cdk activity, which – when overstimulated – promotes oncogenesis. Because of its Cdk6-dependent transforming activity, we propose that Fbxo7 functions as an oncogene.

In U2OS and NIH3T3 cells, Fbxo7 has shown selectivity for Cdk6 over Cdk2 and Cdk4. Although the biochemical properties of Cdk4 and Cdk6 are similar, more-recent studies have indicated that differences can be discerned in their selective binding to co-factors (Sugimoto et al., 1999; Laman et al., 2005), preference for phosphorylation sites in pRb in vitro (Takekawa et al., 2005), sensitivities to INK4 family members (Tourigny et al., 2002; Jones et al., 2007) and in vivo partnering with D-type cyclins (Ely et al., 2005). Also, in studies of knockout mice, tissue-specific defects are seen: Cdk4-knockout mice have impaired development of pancreatic β-islet cells (Rane et al., 1999), whereas Cdk6-null mice show deficiencies in haematopoiesis (Malumbres et al., 2004; Hu et al., 2009). In addition, whereas Cdk4–Cdk6 or Cdk2–Cdk4 double-knockout mice are embryonic lethal, Cdk2–Cdk4 double-
knockout mice are viable (Barriere et al., 2007; Malumbres et al., 2004; Berthet et al., 2006). Moreover, there is mounting evidence for a specific role for Cdk6 in differentiation, especially in neuronal and haematopoietic cells (Ericson et al., 2003; Slomiany et al., 2006; Fujimoto et al., 2007; Grossel and Hinds, 2006; Matushansky et al., 2003). These studies support the idea that the G1 Cdk's have overlapping, yet distinct, activities.

Fbxo7 also directly interacts with and stabilizes p27, although Fbxo7 does not require p27 to increase levels of cyclin D-Cdk6 (Laman et al., 2005). p27 was first identified as an inhibitor of cyclin-dependent kinases but it also acts as an assembly factor for cyclin D-Cdk complexes. More recently, p27 has been shown to have roles that extend beyond direct cell cycle regulation to functions in differentiation, motility and migration, and cytoskeletal signalling (reviewed in Borriello et al., 2007). As such, p27 has both tumour suppressor and oncogenic activities – dependent on its subcellular localization and cell type (Besson et al., 2007; Chu et al., 2008).

Because Fbxo7 exhibited a preference for Cdk6, we reasoned that it might have different functions in haematopoietic cells where Cdk6 activity predominates (Laman, 2006). In this study, we tested the activity of Fbxo7 in B cell lines. In contrast to our findings in fibroblasts and osteosarcoma cells, data presented here indicate that Fbxo7 is not a transforming gene in pro-B cells. Instead, the reduction of Fbxo7 protein levels led to an increased rate of proliferation and changed the expression of B cell markers to resemble a less-mature state. In addition, a mouse model presenting decreased Fbxo7 protein levels showed increased populations of precursor pro-B cells and pro-erythroblasts. These data are consistent with a model, in which Fbxo7 has an anti-proliferative role in precursor cells and also a cell-cycle-independent regulatory role in the maturation of haematopoietic cells.

**Results**

**Increased Fbxo7 levels do not transform Ba/F3 cells**

To address whether Fbxo7 is capable of transforming haematopoietic cells, we used the pro-B cell line Ba/F3, which depends on IL-3 for survival, and tested whether exogenous expression of Fbxo7 would alter the proliferation rate or substitute for IL-3 signalling. Ba/F3 cells were infected either with a retrovirus expressing human FBXO7–IRES–GFP or the empty vector, and fluorescence-activated cell sorting (FACS) sorted for GFP+ cells. Immunoblotting of lysates from polyclonal cell lines demonstrated the presence of exogenous Fbxo7 expression, and the increased mobility on SDS-PAGE of human Fbxo7 compared with the endogenous murine Fbxo7 (Fig. 1A). We first tested whether increased Fbxo7 levels would enhance the proliferation rate which was assessed by seeding cells at equal densities and counting live cells at periodic intervals. However, both cell lines proliferated at the same rate (Fig. 1B). In addition, the proportion of live and dead cells was measured, and no significant differences were observed between control and Fbxo7-expressing Ba/F3 cells (Fig. 1C). Next, the ability of Fbxo7 to substitute for IL-3 signalling was tested by withdrawing it from the growth medium and measuring cell viability. As seen in Fig. 1D, vector control and Fbxo7-expressing cells lost viability with essentially the same kinetics. We noticed that 48 hours post withdrawal ~5% more Fbxo7-expressing cells were alive compared with those expressing the vector control; however, this small effect, although statistically significant (P=0.027), was transient, and suggests that Fbxo7 has only a weak ability to provide survival signals to Ba/F3 cells. Thus Fbxo7 expression did not relieve IL-3 dependence in Ba/F3 cells nor was it rate-limiting for their proliferation.

**Decreasing Fbxo7 levels enhanced Ba/F3 cell proliferation**

We next determined whether Fbxo7 is required for proliferation or viability. To achieve this, Ba/F3 cells were infected with retroviruses that encoded miR-30-based short-hairpin RNA targeting the expression of Fbxo7 mRNA or an empty vector. Cell lines were cloned by limiting dilution under antibiotic selection and screened by immunoblotting lysates to test for Fbxo7 expression. Data for two independent clonal lines are shown in Fig. 2A. Expression of short hairpin RNA achieved a significant reduction in Fbxo7 levels, arguing against the idea that high levels of Fbxo7 are required for proliferation or viability. In fact, during cloning, we noticed that

![Fig. 1. Increased Fbxo7 expression in Ba/F3 cells does not affect growth or proliferation.](https://example.com/fig1.png)
cultures with reduced Fbxo7 protein levels proliferated more rapidly and were smaller than control cells. By measuring the rate of proliferation ($P<0.01$ on day 3 and $P<0.001$ on day 17) and the cell volume ($P<0.001$), we confirmed the statistical significance of these observations (Fig. 2B,C).

To investigate the molecular basis for the Ba/F3 cell cycle phenotypes, protein levels of other cell cycle regulators were also determined. Immunoblotting the lysates from control and Fbxo7-knockdown cells showed that the total levels of subunits (cyclin D2, cyclin D3 and Cdk4, Cdk6) were not significantly changed; however, protein expression of the S phase cyclins E and A was markedly increased, whereas p27 protein levels were reduced (Fig. 2A). These alterations suggested that Cdk2 activity was predominantly affected in these cells. To test this, Cdk2 kinase assays were performed. In independent experiments endogenous Cdk2 activity was quantified as being 25% (data not shown) and 52% (Fig. 2D) increased in cells with reduced Fbxo7 as compared with vector control cells. In addition, although we have previously shown in fibroblasts and osteosarcoma cells that Fbxo7 is required for Cdk6 association with D-type cyclins (Laman et al., 2005), in Ba/F3 cells, the amount of Cdk6 associated with endogenous cyclin D2 and D3 was unaffected by the reduction of Fbxo7 levels as tested by in vivo co-immunoprecipitation assays (Fig. 2E). We conclude, therefore, that Cdk6 association with D-type cyclins does not depend on Fbxo7 in this cell type. Together, these data indicate that reducing Fbxo7 protein levels augmented Cdk2 activity, and promoted a rapid G1 to S phase transition and a smaller cell size.

As cells acquire much of their mass during G1 phase, cell cycle profiling was performed. Asynchronous populations of cells were fixed, stained with propidium iodide (PI) and assayed by FACS to determine DNA content ($n=10$). Representative plots shown in Fig. 2F demonstrate that, compared with vector control, Fbxo7-knockdown cells had fewer cells with 2N DNA content in G1.

---

**Fig. 2. Stable reduction of Fbxo7 levels causes an increase in proliferation and a decrease in the size of Ba/F3 cells.** (A) Immunoblot of various cell cycle proteins in lysates from independent clones of Ba/F3 cells infected with retroviruses expressing a miR-30-based hairpin RNA which targets Fbxo7 expression. Actin is used as a loading control. (B) Time course of the proliferation rate of an empty vector control cell line and two independent clonal cell lines that have reduced Fbxo7 protein expression. Cells were seeded at equal densities and counted on the day indicated. (C) Size measurements of cell lines described in B, seeded at equal densities. (D) Kinase assays on immunoprecipitations with antibodies against Cdk2 from cell lysates of Ba/F3 cells with reduced or endogenous levels of Fbxo7. pRb was used as a substrate and the amount of incorporated [$\gamma$-33P]ATP was quantified on a phosphoimager (Cyclone). (E) Immunoblotting of endogenous Cdk6 associated with D cyclins in immunoprecipitations from cell lysates of Ba/F3 cells with reduced or endogenous levels of Fbxo7. (F) Representative FACS plots (from $n=10$) of an asynchronous population of cells, that had been fixed and whose DNA content had been quantified by PI staining, to determine the percentages of cells in the cell cycle phase indicated. (G) Percentages of viable and non-viable cells in an asynchronous culture assessed by PI staining of live cells and by FACS (mean ± s.d., $n=3$). (H) Immunoblotting for pocket proteins in cell lysates of Ba/F3 cells with reduced and endogenous levels of Fbxo7. **$P<0.01$, ***$P<0.001$. 
phase and more cells with >2N DNA content in S and G2–M phases. These data indicate that the more-rapid proliferation and smaller size of the Fbxo7-knockdown cells are due to a shorter G1 phase. The possibility that cells with less Fbxo7 protein resist cell death during culturing, which would account for the increased cell number, was investigated by assaying live cell exclusion of PI; however, the percentages of viable cells were similar for both cell lines (Fig. 2G). The inactivation of pocket proteins (pRb, p107 and p130) is rate-limiting for transition through G1 and passage through the restriction point. Consistent with the observed shortened G1 phase, immunoblotting the pocket proteins in lysates from cells with reduced Fbxo7 levels also showed their levels decreased compared with vector control cells (Fig. 2H).

These data demonstrated that Fbxo7 has an unexpected anti-proliferative role in Ba/F3 cells. To test whether this is specific to Ba/F3 cells, cell size and proliferation rates were tested in other types of cell that have reduced Fbxo7 levels. The human pre-B cell leukemic cell line Nalm-6 was infected with a retrovirus encoding a miR-30-based short hairpin RNA that targets FBXO7 expression. Immunoblotting lysates from FBXO7-knockdown cells showed that levels of Cdk2, Cdk4, Cdk6 and cyclin D3 were unaffected, whereas levels of cyclin A were elevated and those of p27 were reduced when compared with vector control (Fig. 3A). In agreement with the results for the Ba/F3 cells, the reduction of Fbxo7 protein levels also caused a statistically significant increase in the rate of proliferation and a decrease in the volume of these cells (P<0.001) (Fig. 3B,C). Similarly, in the human cervical carcinoma cell line C-33A, reduction of Fbxo7 protein levels by using a different miR-30 short hairpin RNA construct resulted in cells with a faster proliferation rate than in vector control cells (P value <0.05) (Fig. 3D). Cells with less Fbxo7 were also smaller than vector control cells, although the reduction in cell size was more variable and not statistically significant (P=0.052) (Fig. 3E). Immunoblotting showed a reduction in Fbxo7 and a small decrease in p27, but no reduction in the levels of cyclin E in this cell line (Fig. 3F). Thus, in human and mouse B cell lines, and in an adherent human epithelial cell line, reduction of Fbxo7 caused cells to proliferate faster and become smaller in size, indicating that the proliferation of several cell types is regulated by Fbxo7.

Changes to Cdk6 expression did not alter the size or proliferation of Ba/F3 cells

We next wanted to investigate the role of Cdk6 in the cell cycle of Ba/F3 cells. We first tested whether overexpression of Cdk6 affects proliferation rates and cell size. A polyclonal population of cells that overexpress Cdk6 was generated by retroviral infection of viruses that encode CDK6-IREs-GFP, and by using FACS sorting for GFP+ cells. Immunoblotting of lysates from these cells showed that levels of Cdk6 were approximately fivefold increased, whereas the levels of the cell cycle regulators Fbxo7, p27 and cyclin E were the same as for the vector control cells (Fig. 4A). In addition, we used in vivo co-immunoprecipitation assays to test the amount of Cdk6 associated with cyclin D2. Overexpression of Cdk6 alone did not change its association with cyclin D2, arguing that Cdk6 levels are in excess and not limiting its association with cyclin D2 (Fig. 4B). We found that proliferation rate and cell size for cells that overexpress Cdk6 are virtually identical to those of vector control (Fig. 4C,D).

Cdk6 requirement for cell proliferation was tested by using retroviruses that encode short-hairpin RNAs targeting Cdk6 or the empty vector to infect Ba/F3 cells to create stable cell lines. Two independent clonal cell lines were assayed for Cdk6 protein expression and, in both cases, its levels were substantially reduced while levels of Fbxo7 and p27 were unchanged (Fig. 4E). In addition, in vivo co-immunoprecipitation assays showed the lack of Cdk6 associated with cyclin D2 (Fig. 4F). However, when the rate of proliferation and the cell volume were measured, these characteristics were similar between control cells and those with reduced Cdk6 (Fig. 4G,H). Taken together, these data indicate that Cdk6 levels do not influence cell growth or proliferation or impact on the amount of Fbxo7 or p27. Thus Cdk6 is neither rate-limiting nor essential for the proliferation of Ba/F3 cells.

Reduced Fbxo7 levels cannot bypass cell cycle inhibition caused by increased levels of p27 in Ba/F3 cells

Fbxo7 interacted directly with the N-terminus of p27 and stabilized its levels in U2OS cells (Laman et al., 2005) and also in the cell
types tested here (Fig. 2A, Fig. 3A,F). To test directly whether Fbxo7 is required to stabilize levels of p27, we investigated whether Fbxo7-knockdown cells become resistant to the cell cycle inhibitory effects of exogenous p27 expression. For this, a plasmid containing the coding region of p27 fused to dsRED was transfected into Ba/F3 cells with reduced levels of Fbxo7. The dsRED-positive cells were collected by FACS sorting, and DNA content and cell volume were measured. Exogenous expression of p27 approximately doubled the percentage of cells containing 2N DNA (G1 phase) (38% compared with 19.5% in untransfected cells), cells expressing p27-dsRED also showed a 13% increase in cell size (data not shown). These data argue against the idea that Fbxo7 is required for p27 to function as a cell cycle inhibitor, because reduction in Fbxo7 levels did not overcome a p27-induced block to G1–S phase progression.

Decreased p27 levels in Ba/F3 cells increased their proliferation rate and reduced cell size

Because a reduction of Fbxo7 levels led to a decrease in p27 in Ba/F3 cells (Fig. 2A), we next investigated the phenotypic effects of decreasing p27 levels specifically in Ba/F3 cells. Cell lines were created that stably express a miR-30-based short-hairpin RNA targeting p27 expression. Two independent clonal cell lines with reduced p27 protein levels were created (Fig. 5A). When rate of proliferation and cell size were assayed, cells with less p27 protein showed statistically significant increased rates of proliferation ($P<0.001$ from day 4 onwards) (Fig. 5B) and a smaller size ($P=0.005$) (Fig. 5C). The DNA content was also measured by FACS analysis ($n=3$); representative plots demonstrating the decreased percentage of cells in G1 phase and a commensurate increased percentage of cells in S phase are shown in Fig. 5D. Furthermore, when the expression of other cell cycle regulators was assayed, no changes were seen in the expression levels of cyclin D2, Cdk4, Cdk6, Fbxo7 or cyclin E (Fig. 5A). These data demonstrate that, in Ba/F3 cells, reduction of p27 protein alone affects the distribution of cell cycle phases, proliferation rate and cell size, but not the total levels of cell cycle regulators. Thus p27 also has an anti-proliferative role in Ba/F3 cells.

**Ba/F3 cells with reduced levels of Fbxo7 showed increased levels of CD43**

It has been reported that lengthening the G1 phase can increase the probability of differentiation (Johnson et al., 1993; Carroll et al., 1995), whereas shortening the G1 phase decreases the probability of differentiation and favours proliferation (Kato and Sherr, 1993). Reducing the cellular levels of Fbxo7 shortened the duration of G1 phase, so we wanted to investigate any effects Fbxo7 might have on differentiation. Stages of B cell differentiation can be distinguished by the expression of cell surface antigens, so a subset of these common surface markers was assayed, including B220, B7.1, B7.2, CD43, CD25, MHC II(Ia) (Zola et al., 1991). Ba/F3 empty vector control cells and cells with reduced levels of Fbxo7 were stained with fluorescent antibody conjugates and analysed by FACS. Expression of most of the above markers was not changed; however, staining for CD43 showed differences between cells with endogenous or reduced levels of Fbxo7 (Fig. 6A). In separate experiments, Ba/F3 cells with stable Fbxo7 knockdown expressed high levels of CD43.
tested the effect of Fbxo7 overexpression on cell surface marker expression. To achieve this, an expression construct encoding FBXO7–IRES–GFP was transiently transfected into Ba/F3 cells that were then stained for CD43 and CD25; the percentages of CD43hi and CD25hi GFP+ cells were measured by FACS. Consistent with a direct effect of Fbxo7 on CD43 expression, its Fbxo7 overexpression caused a 65.9% decrease in CD43hi expressing cells (n = 3). CD25 expression, however, was also reduced 39.2% when Fbxo7 was overexpressed, suggesting that Fbxo7 expression had an indirect effect on its levels (Fig. 6C). These data suggest that Fbxo7 directly modulates CD43 expression; however, because Fbxo7 overexpression does not increase the proliferation rate, the effect of Fbxo7 on CD43 appears to be independent of its effects on the duration of the cell cycle.

Reduced levels of Cdk6 increased those of CD43
To further investigate the link between cell cycle delay and the expression of these markers of differentiation, the effect of lengthening the cell cycle was tested by transiently transfecting p27–IRES–GFP into Ba/F3 cells and assessing the effect on CD43 and CD25 surface expression. Although a longer G1 phase might...

CD43 was a sialoglycoprotein with a role in cell-cell adhesion and proliferation. During B cell differentiation it is expressed by early precursor cells (pre-pro-B and pro-B cells), and then rapidly downregulated upon progression to pre-B and B cell stages with VDJ rearrangement (Hardy et al., 1991). CD25 encodes the IL-2 receptor α-chain, which steadily increases during these stages of B cell differentiation (Rolink et al., 1994). Thus pro-B cells have CD43hi and low CD25 (CD25lo) expression, whereas small pre-B cells have CD43lo CD25hi expression (Rolink et al., 1994; Zola et al., 1991). The expression of CD25 was also different in these cells. The number of CD25hi cells decreased by 61.7% in Fbxo7-knockdown cells compared with vector control cells (Fig. 6B). These data demonstrate that the reduction of Fbxo7, which increased the proliferation rate, also altered the expression of cell surface markers. Furthermore, the change in their expression levels suggest that cells had reverted to a less-mature state, implying that Fbsx7 normally regulates differentiation at the pro-B cell stage.

As the reduction of Fbsx7 lead to a cell surface marker phenotype that suggested a reversal of differentiation, we also tested the effect of Fbsx7 overexpression on cell surface marker expression. To achieve this, an expression construct encoding FBXO7–IRES–GFP was transiently transfected into Ba/F3 cells that were then stained for CD43 and CD25; the percentages of CD43hi and CD25hi GFP+ cells were measured by FACS. Consistent with a direct effect of Fbsx7 on CD43 expression, its Fbsx7 overexpression caused a 65.9% decrease in CD43hi expressing cells (n = 3). CD25 expression, however, was also reduced 39.2% when Fbsx7 was overexpressed, suggesting that Fbsx7 expression had an indirect effect on its levels (Fig. 6C). These data suggest that Fbsx7 directly modulates CD43 expression; however, because Fbsx7 overexpression does not increase the proliferation rate, the effect of Fbsx7 on CD43 appears to be independent of its effects on the duration of the cell cycle.

Reduced levels of Cdk6 increased those of CD43
To further investigate the link between cell cycle delay and the expression of these markers of differentiation, the effect of lengthening the cell cycle was tested by transiently transfecting p27–IRES–GFP into Ba/F3 cells and assessing the effect on CD43 and CD25 surface expression. Although a longer G1 phase might...
be expected to alter these markers in order to reflect increased differentiation (i.e. decrease of CD43 and increase of CD25 expression), no changes to the expression of either marker were observed in cells transfected with the construct encoding p27 (Fig. 6C). Conversely, the expression of CD43 was assayed in the cell line with reduced p27 levels – which had a shorter G1 phase – also had no significant increase in CD43 staining (Fig. 6D).

In addition to testing the effect of altering the levels of p27, the expression levels of CD43 were measured in cells with reduced levels of Cdk6, because this kinase has a specific role in differentiation (Grossel and Hinds, 2006). Cells with reduced amounts of Cdk6 were also immunostained for CD43 and were found to have an average 2.3-fold increase in CD43hi cells over three independent experiments compared with vector control (Fig. 6E). This was surprising because reducing Cdk6 levels in Ba/F3 cells did not alter the proliferation rate of cells (Fig. 4G), supporting the idea that increased CD43hi expression did not require a more-rapid cell cycle or a shortened G1 phase. Together, these data demonstrate that reducing the expression of Fbxo7 or Cdk6 had the same effect of increasing CD43 expression, despite only Fbxo7-knockdown cells affecting the rate of proliferation. In addition, cells with alterations to p27 levels had unchanged CD43 expression. In sum, these data suggest that Fbxo7 acts as a positive regulator of pro-B cell differentiation because its levels are inversely correlated with CD43 expression, and that the effect of Fbxo7 expression on CD43 is not due to changes in the cell cycle.

Reduction of Fbxo7 levels enhanced erythropoietin-induced differentiation of Ba/F3 cells along the erythroid lineage

Because Fbxo7 appeared to positively regulate pro-B cell differentiation, we next investigated whether differentiation of another haematopoietic cell lineage is also affected by Fbxo7 levels. We exploited the potential of Ba/F3 cells to be differentiated along the erythroid lineage because of expression of erythroid-specific transcriptional factors, including GATA-1, NF-E2 and EKLF (Krosl et al., 1995). Cells will partially differentiate when engineered to express the erythropoietin receptor (Epo-R) and cultured in the presence of erythropoietin (Epo). They cannot, however, terminally differentiate and do not synthesize...
performed to assess β-globin mRNA levels. Samples from cells with reduced Fbxo7 showed a 10-fold increase in the amount of β-globin message as compared with vector (Fig. 7B), suggesting a negative regulatory role for Fbxo7 in this engineered model of erythroid differentiation.

The rate of proliferation of Epo–R–IRES–GFP control cells and cells with reduced Fbxo7 was also determined by a growth curve performed on cells induced to differentiate by growth in Epo. As has been previously reported for Ba/F3–Epo–R cells, a transient slowing of growth was observed when cells were withdrawn from IL-3 and induced to differentiate by the addition of Epo. However, cells with reduced Fbxo7 levels were less delayed, recovered more rapidly, and proliferated faster than vector control cells (Fig. 7C,D). This was especially apparent when growth rates of the cell lines in the first 24 hours were compared. Fbxo7-knockdown cells seeded into Epo showed little delay, proliferating almost as quickly as cells seeded back into IL-3 after starvation (Fig. 7D). By 42 and 48 hours after cytokine withdrawal, the differences in cell number were statistically significant ($P=0.0131$ and $P=0.0158$) (Fig. 7C). These data demonstrate that reduction in Fbxo7 levels results in more-robust differentiation in response to Epo along an erythroid lineage, despite cells proliferating faster than vector control cells. Therefore, these data argue against the requirement of a slower cell cycle to allow erythroid differentiation in Ba/F3–Epo–R cells.

**Mice with reduced levels of Fbxo7 show increased numbers of pro-B cells and pro-erythroblasts**

Our data indicate that Fbxo7 has a cell-type specific anti-proliferative role (Ba/F3, Nalm-6 and C-33A cells) and also positive and negative regulatory roles in the differentiation of pro-B and erythroid cell lineages, respectively. As these in vitro experiments were conducted mainly by using a Ba/F3 cell line model, we also wanted to assess the effect of Fbxo7 on differentiation in a more physiological setting. To achieve this, a transgenic mouse was engineered using targeted ES cells from the International Knockout Mouse Consortium (EUCOMM ID: 23037). These ES cells have an insertion of the LacZ gene with a splice acceptor site between exon 3 and exon 4 of isoform 1 of Fbxo7, and this Fbxo7LacZ allele, which disrupts expression of all Fbxo7 isoforms by splicing in the LacZ-coding sequences. Four chimeric mice were generated by injection of ES cells into blastocysts, two of which showed germline transmission of the Fbxo7LacZ transgene. Heterozygous animals were bred to homozygosity, and lysates from various tissues were immunoblotted for the expression of Fbxo7, showing a gene-dosage-dependent reduction in Fbxo7 expression in the heterozygous and homozygous animals (Fig. 8A). Fbxo7 protein expression was, however, detectable upon very long exposure of immunoblots in the cerebellum demonstrating the production of some wild-type Fbxo7 mRNA (data not shown). Our full phenotype characterisation of these mice will be reported elsewhere (manuscript in preparation).

To address the effect of reduced Fbxo7 expression on B cell development in vivo, splenocytes were harvested from age-matched wild-type ($n=2$) and homozygous ($n=2$) littermates, and stained for B220, CD43 and CD25 to measure the pro-B and pre-B cell populations. Animals homozygous for Fbxo7LacZ showed a statistically significant increase in the percentage of CD43<sup>hi</sup>CD25<sup>lo</sup> pro-B cells as compared with wild-type controls; however, the later pre-B cell stages showed no significant differences (Fig. 8B,C). The increase in the percentage of pro-B cells seen in vivo could be due to increased proliferation of pro-B cells or a block in...
maturation from the pro-B to pre-B cell stages. These data support the results described for Ba/F3 cells and suggest that Fbxo7 usually has an anti-proliferation or a positive role in promoting the differentiation of pro-B cells.

To address the effect of Fbxo7 on erythroid differentiation, the number of erythroid colony-forming units (CFU-Es) was assessed by seeding bone marrow cells into methylcellulose medium containing Epo. Cells from mice homozygous for Fbxo7LacZ formed almost twice as many colonies, indicating a significant increase in the number of CFU-Es (P = 0.0033) in these mice as compared with wild-type animals (Fig. 8D). These data suggest that Fbxo7 normally has an anti-proliferative role in pro-erythroblasts or a role in promoting pro-erythroblast differentiation. Bone marrow from wild-type and homozygous animals was also stained for CD71 and Ter119 protein expression, because together they demarcate the later stages of erythroblast differentiation (Fig. 8E,F). Consistent with data showing an increase in CFU-E colonies containing Epo (n = 2 mice per genotype, each seeded in triplicate). (E) FACS analysis of erythroid populations in bone marrow of WT and homozygous Fbxo7LacZ animals by Ter119 staining on the x-axis and CD71 on the y-axis (n = 2). (F) Percentages of erythroblast populations as indicated. * P < 0.05, ** P < 0.01.

**Discussion**

In a previous study using immortalised fibroblasts, overexpression of Fbxo7 protein increased cyclin D–Cdk6 complexes and caused Cdk6-dependent transformation without affecting the cell cycle or proliferation (Laman et al., 2005). In addition, in osteosarcoma cells, a reduction of Fbxo7 expression led to decreased amounts of cyclin D–Cdk6 complexes, also without altering the cell cycle (Laman et al., 2005), which argues for a direct role of Fbxo7 in promoting the assembly of cyclin D–Cdk6 complexes. We report here that, in an immortalised pro-B cell line, overexpression of Fbxo7 does not transform cells, enhance cell proliferation or viability or affect the levels of cyclin D–Cdk6 complexes. Instead, the reduction of its levels accelerated the rate of proliferation, decreased cell size, and shortened G1 phase, all indicative of an
anti-proliferative function for Fbxo7 in pro-B cells. This effect was not specific to Ba/F3 cells, because reduction of Fbxo7 also increased the proliferation rate of Nalm-6 and C-33A cells. Cell cycle analysis showed that a decrease in Fbxo7 caused a rapid transit through G1 phase. Cells also had higher Cdk2 activity, stemming from increased cyclin E and cyclin A, reduced p27 and an overall reduction in the levels of pocket proteins. One interpretation of these findings is that, in Ba/F3 cells, p27 has a main role in restraining S phase entry, which is supported by the finding that independently decreasing its levels results in smaller cells and a faster rate of proliferation – even without increased cyclin E levels. In addition, changes to Cdk6 levels had no detectable effect on cell cycle parameters, suggesting that, although Cdk6 is abundant in pro-B cells, it is not rate-limiting for cell cycle progression under the experimental conditions used here. Together, these results support the idea that Fbxo7 has different cell cycle activities, and transforming or anti-proliferative properties in a variety of cell types, and we speculate that the ultimate effect of changes to its expression levels depends on the G1 phase circuitry of that cell type or, possibly, to the stage of differentiation of the cell (stem or progenitor cell versus mature cell). We notice, for example, that in differentiating embryonic carcinoma P19 cells, proliferation correlated with the activity of the cyclin-E–cyclin-A–
Cdk2 but not cyclin-D–Cdk4–Cdk6–p27 complexes (Bryja et al., 2008).

Because Fbxo7 affects G1 phase and cell proliferation, we reasoned it might also affect cell differentiation – which is temporally linked to G1. A long-standing correlation exists between a prolonged G1 phase that enhances the differentiation potential and a shortened G1 phase that diminishes it (Kato and Sherr, 1993; Carroll et al., 1995; Johnson et al., 1993). However, there is experimental evidence in a variety of cell types, including lymphocytes (Rush et al., 2002; Lea et al., 2003), which show that these processes can be separated and need not proceed simultaneously (Brown et al., 2003). Experiments here are consistent with the idea that Fbxo7 promotes the differentiation of pro-B cells: overexpression of Fbxo7 decreased CD43, which is downregulated as B cell mature, and reduction of Fbxo7 levels increased its expression. This inverse correlation between Fbxo7 and CD43 expression is suggestive of a direct regulatory relationship, but it is unlikely to be due to cell cycle alterations. Several lines of evidence support this. For example, although Fbxo7 knockdown altered the cell cycle, the overexpression of Fbxo7 did not alter proliferation but did decrease CD43 expression. Moreover, in other experiments where cell cycle length was altered by changes to p27, CD43 expression was not affected. However, Cdk6 knockdown, which had no effect on the cell cycle, increased the frequency of CD43hi-expressing cells. Taken together these data argue that CD43 expression is regulated independently from cell cycle progression. A further implication of this finding is that Fbxo7 also regulates CD43 signalling in other cell types that express this marker, such as activated B cells, macrophages and T cells, a hypothesis that is currently under investigation.

Consistent with the observations in Ba/F3 cells, quantification of pro-B cells (B220+, CD43hi, CD25lo) from spleens showed a statistically significant increase in the proportion of pro-B cells in mice that are deficient for Fbxo7 protein expression compared with wild-type mice. This increase was specific to pro-B cells and could have resulted either from an increase in the proliferation of these precursor cells due to the loss of the anti-proliferation function of Fbxo7 or, alternatively, to a block in the differentiation at the pro-B to pre-B cell stage, due to the inability to downregulate CD43 in the absence of Fbxo7. In the erythroid lineages, a significant increase in the number of CFU-Es and pro-erythroblasts was observed in the bone marrow of homozygous Fbxo7−/− mice. This, again, could reflect either increased proliferation of these lineage-committed precursor cells or a block in the differentiation at this stage in erythropoiesis. In considering these two models we notice that, in the Ba/F3 Epo-R erythroid differentiation model, the reduction in Fbxo7 protein expression enhanced both Epo-induced differentiation and proliferation. This lends support to the model in which increased proliferation, rather than a block in differentiation, explains the in vivo findings. However, a reduction in cell number at the late normoblast stage was also observed and might mean that, when Fbxo7 levels are reduced, erythroid differentiation is inhibited. Alternatively, Fbxo7 might have different pro-proliferation or pro-survival functions later on in erythropoiesis.

How cell cycle regulatory proteins like Fbxo7 and Cdk6 influence differentiation and cell cycle, and at which maturation stage this occurs is an interesting question for future study. There are experimental data that indicate direct binding of Cdk6 to transcription factors that regulate differentiation, suggesting a cell-cycle-independent function (Fujimoto et al., 2007), whereas other studies show that downregulation of Cdk6 protein is crucial for terminal differentiation, presumably by allowing a permanent withdrawal from the cell cycle (Matushansky et al., 2003). In addition, it is possible that these proteins have different regulatory roles, cell cycle and/or transcriptional functions at different times in cell maturation. In summary, Fbxo7 impacts on both cell cycle and differentiation in a cell-type specific manner, which indicates that Fbxo7 has an important and sensitive role in integrating cell signalling and functionality with cell cycle progression during haematopoiesis.

Materials and Methods

Cell culture

Murine Ba/F3 pro-B cells were maintained in complete RPMI-1640 medium supplemented with 10% foetal calf serum (FCS; PAA Laboratories), 10% WEHI-3B conditioned medium, penicillin (50 U/ml), streptomycin (50 U/ml) (Gibco BRL), and 10 ng/ml recombinant murine IL-3 (Fitzgerald Laboratories). Human Nalm-6 pre-B acute lymphoblastic leukaemia cells were maintained in RPMI-1640 medium supplemented with 10% FCS, penicillin (50 U/ml), and streptomycin (50 U/ml). Human C-33A cervical cancer cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS, penicillin (50 U/ml), and streptomycin (50 U/ml). Cells were incubated at 37°C in a humidified 5% CO2 atmosphere.

To reduce the protein levels of human or mouse Fbxo7, or mouse p27, cells were infected with retrovirus that contained either an LMP or PSMP vector and that delivered a miR-30-based short hairpin RNA sequence targeting the genes of interest. For murine Ba/F3 cells, 5′-CGGCCAGTCTGGTGTTTGGAAT (HP_3087), human Fbxo7sh1: 5′-GCGCCAGTCTGGTGTTTGGAAT (HP_3087), human Fbxo7sh2: 5′-GCGCCAGTCTGGTGTTTGGAAT (HP_3087), human Fbxo7sh2: 5′-CGGCATTAGCTAATCTGGAAT-3′ (HP_348428), murine p27: 5′-AGCCATTAGCTAATCTGGAAT-3′ (HP_348428). Either empty LMP or non-silencing vector PSMP (Open Biosystems) were used as negative controls. To decrease Cdk6 protein levels, retrovirus containing PRETROSUPER-based short hairpin constructs were used as previously described (Laman et al., 2005) to interfere with Cdk6 transcription. Independent B cell clones were generated using limiting dilution and grown in the presence of puromycin (2 μg/ml); several clones were analysed. A different short hairpin construct was used to reduce Fbxo7 gene expression in C-33A cells, and polyclonal cell lines were selected in the presence of puromycin (2 μg/ml). To overexpress proteins, cells were infected with retrovirus that encoded M5C-IREX-GFP and expressed the gene of interest. Polyclonal cell lines were detected by FACs for GFP-expressing cells.

Ba/F3 cells expressing Epo-R were generated by retroviral infection with M5C-EpoR-GFP (kindly provided by Tony Green, Department of Hematology, University of Cambridge). Retroviruses encoding the Epo-R-ires-GFP were used to infect
BarF3 cells, which were sorted for GFP expression. Clones were subsequently selected by 10 μM 4-phenyl-butyl pyridinium iodide (PBP) and plated in the presence of 0.05 μM 2-mercaptoethanol (Ep) (Cell Sciences) but lacked IL-3. BarF3-Epo-R cell lines were thereafter maintained in IL-3 during standard sub-culturing.

For transient transfection, 5 μg of DNA encoding p27or Fbxo7, or empty vector plasmid DNA was nuclease treated with 5 x 10⁵ BarF3 cells by using solution V and program X-001, as per manufacturer’s instructions (Lonza). After 72 hours, live GFP™ cells were immunostained (see below) and analysed by flow cytometry, using PI (2.5 μg/ml) as a live–dead discriminator.

To measure the rate of proliferation and the cell volume, cells were seeded in triplicate at a density of 0.3 – 0.5 x 10⁶ cells/ml for (B cells) or 2 × 10⁵ cells per well (C3-A cells), and cell numbers and volumes were assayed every 2 days using a Casy Cell counter (Scharfe System). Three independent experiments were performed.

To measure cell viability, cells were exposed to 50 μM of 7-AAD (AlamarBlue) and counted on a flow cytometer. Three independent experiments were performed.

Dickinson). To quantify DNA content, 1–2 × 10⁶ cells were washed in PBS and fixed in 100% 70% ethanol at 4°C for 30 minutes prior to incubation with primary antibody and addition of protein G-Sepharose beads for 4 hours at 4°C for immunoblotting. Beads were washed three times and co-immunoprecipitated proteins were detected by immunoblotting.

For immunoprecipitations and kinase assays, cells were lysed in 50 mM HEPES pH 7.4, 150 mM NaCl, 10 mM β-glycerophosphate, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail, then sonicated three times and centrifuged at 16,100 × g for 10 minutes at 4°C. Lysates were pre-cleared with 25 μl protein G-Sepharose beads at 4°C for 30 minutes prior to incubation with primary antibody and addition of protein G-Sepharose beads for 4 hours at 4°C for immunoblotting. Beads were washed three times and co-immunoprecipitated proteins were detected by immunoblotting.

For immunoprecipitations and kinase assays, cells were lysed in 50 mM HEPES pH 7.4, 150 mM NaCl, 10 mM β-glycerophosphate, 1 mM DTT, 1 mM PMSF prior to addition of the 0.6 μg of purified plReb substrate (Abcam, ab52670), 1 μl of [γ-32P]ATP and 200 mM ATP and incubating at 30°C for 1 hour. Proteins were resolved using SDS-PAGE and gels fixed in 10% acetic acid/methanol, dried and quantified using a Cyclone Phosphor Imager (PerkinElmer).

To assay B-cell populations, spleen cell suspensions were treated with 200 μg/ml RNAse A and 100 μg/ml propidium iodide (PI) for 30 minutes, and then either analysed immediately or incubated with secondary streptavidin-APC (BD Pharmingen) and then either analysed immediately or incubated with secondary streptavidin-APC (BD Pharmingen) and then either analysed immediately or incubated with secondary streptavidin-APC (BD Pharmingen). Protein samples were prepared by lysing cell pellets in modified RIPA buffer (150 mM NaCl, 1% NP40, 0.1% SDS, 50 mM Tris pH 7.5, protease inhibitor cocktail), incubating on ice for 15 minutes, and centrifuged at 11,200 × g for 10 minutes, 42°C for 60 minutes and 70°C for 10 minutes. cDNA was used in PCR reactions (95°C for 30 seconds, 50°C for 1 minute, 72°C for 1 minute, 25 cycles) using primers to amplify β-globin (forward: 5’-GACCCAGGGATCTTGATGAC-3’, reverse: 5’-TGGAGGCTTGACCA-GTGATCA-3’) and cyclophilin (forward: 5’-CCTGGGCGCGCTCTCCT-3’ and reverse: 5’-CACTGGGCGCGCTCTCCTC-3’) using SYBR Green. Quantification was carried out using a Molecular Imager Gel Documentation System using Quantity One software (BioRad).

Formation of Fbxo7-lacZ mice

Mouse embryonic stem (ES) cells heterozygous for the β-galactosidase gene (lacZ) targeting the Fbxo7 locus between exons 3 and 4 were generated by conditional ‘targeted trapping’ technology employed by the European Conditional Mouse Mutagenesis Program (www.eucomm.org). To generate chimeric mice, two independent Fbxo7-lacZ ES cell lines (EUComm ID: 23007) were obtained, karyotyped and injected into C57BL/6J blastocysts. One clone successfully produced four chimeric male mice, as determined by PCR analysis for the mutant transgene (primers available on request). Two of these males had germline transmission of the transgene and their offspring were bred to homoygosity (Fbxo7-lacZ/Fbxo7-lacZ). Experiments were conducted from heterozygous crosses to enable analysis of homozygous mice with mild-type littermate controls. Spleen and bone marrow were harvested from mice aged 7–12 weeks.

We thank C. Bacon, R. Dickins, A. Green, N. Holmes, S. Lowe and A. Skoulitchi for generously providing reagents, W. Mansfield, W. Khaled and N. Miller for technical advice and assistance, and D. Lagos, N. Holmes, A. Philpott and members of the Laman lab for their critical reading of this manuscript. Funding was provided by the Association for International Cancer Research, the BBSC, Campod, and Cancer Research UK.

References

Ang, X. L. and Wade, H. J. (2005). SCF-mediated protein degradation and cell cycle control. Oncogene 24, 2860-2870.

Barriere, C., Santamaria, D., Cerqueira, A., Galan, J., Martin, A., Ortega, S., Malumbres, M., Dubus, P. and Barbaud, M. (2007). Mice thrive without Cdk4 and Cdk6. Mol. Oncol. 1, 72-83.

Berthet, C., Klarmann, K. D., Hilton, M. B., Suh, H. C., Keller, J. R., Kiyokawa, H. and Kaldis, P. (2006). Combined loss of Cdk2 and Cdk4 results in embryonic lethality and B lymphoproliferation. Dev. Cell. 10, 563-573.

Besson, A., Hwang, H. C., Cicero, S., Donovan, S. L., Gurian-West, M., Johnson, D., Clurman, B. E., Dyer, M. A. and Roberts, J. M. (2007). Discovery of an oncogenic activity in p27kip1 that causes stem cell expansion and a multiple tumor phenotype. Genes Dev. 21, 1731-1746.

Borriello, A., Cucciolla, V., Oliva, A., Zappia, V. and Delta, R. F. (2007). p27kip1 metabolism: a fascinating labyrinth. Cell Cycle 6, 1053-1061.

Brown, G., Hughes, P. J. and Michell, R. H. (2003). Cell differentiation and proliferation-simultaneous but independent? Exp. Cell Res. 291, 282-288.

Bryja, V., Pacherik, N., Vondracke, J., Soucek, K., Cajane, L., Horvath, V., Holubcova, Z., Dvorak, P. and Hampel, A. (2008). Lineage specific composition of cyclin-D-Cdk4/Cdk6-p27 complexes reveals distinct functions of Cdk4, Cdk6 and individual D-type cyclins in differentiating cells of embryonic origin. Cell Proli. 41, 875-893.

Carroll, M., Zhu, Y. and D’Andrea, A. D. (1995). Erythropoietin-induced cellular differentiation requires prolongation of the G1 phase of the cell cycle. Proc. Natl. Acad. Sci. U.S.A. 92, 2869-2873.

Cenciarelli, C., Chiaur, D. S., Guardavaccaro, D., Parks, W., Vidal, M. and Pagano, M. (1999). Identification of a family of human F-box proteins. Curr. Biol. 9, 1177-1179.

Chu, I. M., Hengst, L. and Slingerland, J. M. (2008). Lineage specific composition of cyclin D-CDK4/CDK6-p27 complexes reveals distinct functions of CDK4, CDK6 and individual D-type cyclins in differentiating cells of embryonic origin. Cell Proli. 41, 875-893.

Carroll, M., Zhu, Y. and D’Andrea, A. D. (1995). Erythropoietin-induced cellular differentiation requires prolongation of the G1 phase of the cell cycle. Proc. Natl. Acad. Sci. U.S.A. 92, 2869-2873.

Cenciarelli, C., Chiaur, D. S., Guardavaccaro, D., Parks, W., Vidal, M. and Pagano, M. (1999). Identification of a family of human F-box proteins. Curr. Biol. 9, 1177-1179.

Chu, I. M., Hengst, L. and Slingerland, J. M. (2008). Lineage specific composition of cyclin D-CDK4/CDK6-p27 complexes reveals distinct functions of Cdk4, Cdk6 and individual D-type cyclins in differentiating cells of embryonic origin. Cell Proli. 41, 875-893.

Carroll, M., Zhu, Y. and D’Andrea, A. D. (1995). Erythropoietin-induced cellular differentiation requires prolongation of the G1 phase of the cell cycle. Proc. Natl. Acad. Sci. U.S.A. 92, 2869-2873.

Cenciarelli, C., Chiaur, D. S., Guardavaccaro, D., Parks, W., Vidal, M. and Pagano, M. (1999). Identification of a family of human F-box proteins. Curr. Biol. 9, 1177-1179.
Grossel, M. J. and Hinds, P. W. (2006). Beyond the cell cycle: a new role for Cdk6 in differentiation. J. Cell. Biochem. 97, 485-493.

Hardy, R. R., Carmack, C. E., Shinton, S. A., Kemp, J. D. and Hayakawa, K. (1991). Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J. Exp. Med. 173, 1213-1225.

Hermant, D. (2006). F-box proteins: more than baits for the SCF? Cell Div. 1, 30.

Hershko, A. (1997). Roles of ubiquitin-mediated proteolysis in cell cycle control. Curr. Opin. Cell Biol. 9, 788-799.

Hershko, A. and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. 67, 425-479.

Ho, M. S., Ou, C., Chan, Y. R., Chien, C. T. and Pi, H. (2008). The utility F-box for... (missing text)

Kato, J. Y. and Sherr, C. J. (1993). Inhibition of granulocyte differentiation by G1 cyclins... (missing text)

Krosl, J., Damen, J. E., Krystal G. and Humphries, R. K. (1999). CDK6 blocks differentiation: coupling cell proliferation to the block to differentiation in leukemic cells. Oncogene 22, 4143-4149.

Nakayama, K. I. and Nakayama, K. (2005). Regulation of the cell cycle by SCF-type ubiquitin ligases. Semin. Cell Dev. Biol. 16, 323-333.

Nakayama, K. I. and Nakayama, K. (2006). Ubiquitin ligases: cell-cycle control and cancer. Nat. Rev. Cancer 6, 369-381.

Okabe, H., Lee, S. H., Phuchareon, J., Albertson, D. G., McCormick, F. and Tetsu, O. (2006). A critical role for FBXW8 and MAPK in cyclin D1 degradation and cancer cell proliferation. PLoS ONE 1, e128.

Rane, S. G., Dubus, P., Mettus, R. V., Galbreath, E. J., Boden, G., Reddy, E. P. and Barbacid, M. (1999). Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in beta- islet cell hyperplasia. Nat. Genet. 22, 44-52.

Reed, S. I. (2003). Ratchets and clocks: the cell cycle, ubiquitination and protein turnover. Nat. Rev. Mol. Cell Biol. 4, 855-864.

Reed, S. I. (2006). The ubiquitin-proteasome pathway in cell cycle control. Results Probl. Cell Differ. 42, 147-181.

Rolink, A., Grawunder, U., Winkler, T. H., Karasuyama, H. and Melchers, F. (1994). IL-2 receptor alpha chain (CD25, TAC) expression defines a crucial stage in pre-B cell development. Int. Immunol. 6, 1257-1264.

Russ, J. S., Hasbold, J. and Hodgkin, P. D. (2002). Cross-linking surface Ig delays CD40 ligand- and IL-4-induced B cell Ig class switching and reveals evidence for independent regulation of B cell proliferation and differentiation. J. Immunol. 168, 2676-2682.

Santra, M. K., Wajapeyee, N. and Green, M. R. (2009). F-box protein FBXO31 mediates cyclin D1 degradation to induce G1 arrest after DNA damage. Nature 459, 722-725.

Slomani, P., Baker, T., Eliotti, E. R. and Grossel, M. J. (2006). Changes in motility, gene expression and actin dynamics: Cdk6-induced cytoskeletal changes associated with differentiation in mouse astrocytes. J. Cell. Biochem. 99, 635-646.

Sugimoto, M., Nakamura, T., Ohtani, N., Shosaku, T., Hirai, H. (2006). Changes in motility, gene expression and actin dynamics: Cdk6-induced cytoskeletal changes associated with differentiation in mouse astrocytes. J. Cell. Biochem. 99, 635-646.

Tourigny, M. R., Ursini-Siegel, J., Lee, H., Toellner, K. M., Cunningham, A. F., Franklin, D. S., Ely, S., Chen, M., Qin, X. F., Xiong, Y. et al. (2002). CDK inhibitor p18(Ink4c) is required for the generation of functional plasma cells. Immunity 17, 179-189.

Vodovats, H. C. (2004). APC/C and SCF: controlling each other and the cell cycle. Curr. Biol. 14, R787-R796.

Winston, J. T., Koepp, D. M., Zhu, C., Elledge, S. J. and Harper, J. W. (1999). A family of mammalian F-box proteins. Curr. Biol. 9, 1180-1182.

Zola, H., Macartle, P. J., Flego, L. and Webster, J. (1991). The expression of sub-population markers on B cells: a re-evaluation using high-sensitivity fluorescence flow cytometry. Dis. Markers 9, 103-118.