

Fbxo7 regulates cell cycle progression and differentiation via Cdk6 and p27.

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Abstract (208 words)

Fbxo7 is an exceptional F-box protein in that a number of its interacting proteins are not substrates for ubiquitin-mediated degradation. Fbxo7 directly binds p27 and Cdk6 and enhances cyclin D/Cdk6 complex formation. Fbxo7 over-expression transforms immortalised fibroblasts in a Cdk6-dependent manner. Because Cdk6 is abundant in lymphocytes, the effect of altering Fbxo7 expression in pro-B lymphocytic Ba/F3 cells was analyzed. In this context, reducing Fbxo7 caused increased proliferation and decreased cell size, resulting from a shortened G1 phase. Examination of the cell cycle regulators showed decreased p27 and increased cyclins E and A. Targeted reduction of p27, but not Cdk6, recapitulated cell cycle phenotypes, arguing that Fbxo7 exerts an anti-proliferative effect by stabilising p27. Decreasing Fbxo7 increased the rate of proliferation, and this had the surprising effect of enhancing Ba/F3 differentiation along the erythroid lineage and also increasing the expression of CD43, a marker of B cell differentiation and activation. This latter phenotype was recapitulated when Cdk6, but not p27, was reduced. Our results indicate that differentiation was influenced mainly by Cdk6, and not the duration of G1 phase. We demonstrate that Fbxo7 regulates cell cycle progression and differentiation and show the mechanistic basis for its phenotypic effects on these independent processes are due to p27 and Cdk6.

Keywords and Abbreviations: Fbxo7, Cdk6, p27, cell cycle, differentiation, G1 phase, cell size

F-box protein (FBP); ubiquitin (Ub); cyclin-dependent kinase (Cdk); erythropoietin (Epo)

Introduction

During the cell cycle, ubiquitin (Ub)-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 (CDKI). This mechanism for regulating the turnover of proteins is mediated through Ub ligases which transfer Ub to target proteins, enabling their destruction at particular times (1-5). The ubiquitinating pathway employs an E1 Ub-activating enzyme, E2 Ub-conjugating enzymes, and E3 Ub-ligases. The latter are of particular interest as they catalyse the specific attachment of poly-Ub chains to proteins bringing about proteolysis by the 26S proteasome.

SCF-type (Skp1/Cullin/F-box) E3 Ub ligases are important regulators of cell cycle progression which are quaternary complexes, consisting of Skp1, Cullin, Rbx1/Roc1 and F-box proteins (FBP) (6-10). It is the FBP, which plays a crucial role in specifically recruiting the target substrate, usually dictated by a post-translational modification on the substrate (11-15). Several FBPs that regulate G1/S regulators have been intensively studied. These include the prototypical Skp2 (Fbx11) and Fbxw7, which regulate the abundance of the CDKI, p27, and cyclin E, respectively. In addition, three FBPs, Fbxo4, Fbxw8, Fbxo31, promote the ubiquitin-mediated degradation of cyclin D1 (16-18). Alterations to the levels or activities of FBPs can disrupt normal cell cycle progression and lead to oncogenic changes.

Another FBP that interacts with G1/S regulatory proteins is Fbxo7. In contrast to other FBPs, Fbxo7 acts as an assembly factor for cyclin D/Cdk6 complexes, specifically. Fbxo7 interacted directly with both Cdk6 and p27 and cooperatively increased cyclin D3 interactions with Cdk6 *in vitro* (19). Because of this activity, Fbxo7 may function as an oncogene. In support of this idea, Fbxo7 over-expression in immortalised murine fibroblasts led 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 (19).

Fbxo7 selectively interacts with Cdk6, but not Cdk2 or Cdk4, and although the biochemical properties of Cdk4 and 6 are similar, more recent studies indicate that differences can be discerned in their selective binding to co-factors (19;20), preference for phosphorylation sites in pRb *in vitro* (21), sensitivities to INK4 family members (22;23), and *in vivo* partnering with D-type cyclins (24). Also, in knockout mice studies, tissue specific defects are seen: Cdk4 knockout mice have impaired

pancreatic beta-islet cell development (25) while Cdk6 null mice show deficiencies in haematopoiesis (26;27). In addition, whereas Cdk4/Cdk6 or Cdk2/Cdk4 double knockout mice are embryonic lethal, Cdk2/Cdk6 double knockout mice are viable (26;28;29). These studies support the idea that the G1 Cdk6 have overlapping, but distinct, activities. Interestingly, there is mounting evidence for a particular role for Cdk6 in differentiation, particularly in neuronal and haematopoietic cells (30-34).

Fbxo7 also directly interacts with and stabilizes p27. Although p27 was first identified as an inhibitor of G1 cyclin-dependent kinases, it also acts as an assembly and import factor for cyclin D/Cdk complexes. Interestingly, p27 has also been shown to have roles that extend beyond the direct cell cycle regulation to functions in differentiation, in motility and migration, and in cytoskeletal signaling (reviewed in (35)). Accordingly, p27 has both tumour suppressor and oncogenic activities dependent on its subcellular localization and cell type (36;37).

Because Fbxo7 shows selectivity for Cdk6, it might have enhanced functions in cells where Cdk6 activity is predominant (38). In this study, we tested the role of Fbxo7 in a pro-B lymphocytic cell line, Ba/F3. We report that Fbxo7 is not a rate-limiting factor in cell growth; instead, knockdown of Fbxo7 increased proliferation and decreased cell size, attributable to a shortened G1 phase. These cell cycle phenotypes were similar to the effect of decreasing p27 levels, and could be reversed by re-introducing p27 expression. The role of Fbxo7 in differentiation was tested by engineering the expression of erythropoietin receptor in Ba/F3 cells which will then differentiate in response to erythropoietin. Remarkably, a reduction in Fbxo7 levels increased β -globin, a marker of erythroid differentiation, in response to erythropoietin. In addition, cells with reduced Fbxo7 also up-regulated CD43, a B cell marker of differentiation. Reducing Cdk6, but not p27 levels, also increased CD43 expression, suggesting that Fbxo7 enhancement of Cdk6 activity, rather than G1 delay, affected differentiation. Our data support a model where Fbxo7, through its interactions with p27 and Cdk6, can regulate discretely cell cycle progression and differentiation.

Results

Increasing Fbxo7 does not enhance proliferation or the survival of Ba/F3 cells

Fbxo7 interacts directly with Cdk6 and p27, enhancing the formation of cyclin D/Cdk6 complexes (19). We were interested in determining the effect of altering its expression in cells where Cdk6 is an abundant G1 kinase and chose Ba/F3 cells, a murine pro-B cell lymphoma cell line. Cells were infected either with a retrovirus expressing human Fbxo7-IRES-GFP or the empty vector, and FACS sorted for GFP+ cells. Immunoblotting of lysates from polyclonal cell lines showed the expression of exogenous Fbxo7 compared to the empty vector, and human Fbxo7 can be distinguished from endogenous murine Fbxo7 by its increased mobility by SDS-PAGE (Figure 1A). The rate of proliferation was then assessed by seeding cells at equal densities and counting live cells at periodic intervals using a cell counter. Cells with increased levels of Fbxo7 proliferated at the same rate as vector control cells (Figure 1B). In addition, cell populations were assayed for the proportion of live and dead cells, and no significant differences were observed between the Fbxo7-expressing Ba/F3 cells and the vector control (Figure 1C).

Many mitogenic and cytokine signalling pathways converge on cyclin D/Cdk activity which is an important downstream effector of environmental cues. Ba/F3 cells are dependent on IL-3 for survival, so in order to test whether Fbxo7, as an enhancer of this kinase, could substitute for the survival signals provided by culturing cells in IL-3, the cytokine was withdrawn from the media, and cell viability was measured. As seen in Figure 1D, control and Fbxo7-expressing cells lost viability with the same kinetics. Thus Fbxo7 is not rate-limiting for the proliferation of Ba/F3 cells nor does it provide survival signals in the absence of IL-3 signalling.

Decreasing Fbxo7 enhanced proliferation of Ba/F3 cells

Because Fbxo7 increases Cdk6 activation, we next determined whether Fbxo7 was essential for proliferation or viability. To achieve this, Ba/F3 cells were infected with retroviruses encoding miR30-based short-hairpins, which target the expression of Fbxo7 mRNA or an empty vector as a control. Cell lines were cloned by limiting dilution and screened by immunoblotting lysates for Fbxo7 expression. Short hairpin expression achieved a significant reduction in Fbxo7 levels, and data for two independent lines are shown in Figure 2A. During the cloning process, we observed that cells with

reduced Fbxo7 levels grew noticeably faster than control cells, and were also visibly smaller. The rate of proliferation was determined as in Figure 1B, and the cell volume and diameter were also measured. These measurements confirmed observations that cells with reduced amounts of Fbxo7 proliferated faster and were smaller in size than control cells (Figure 2B, Figure 2C). One possibility which might account for an increase in cell number is that cells with less Fbxo7 might resist cell death during culturing; however, the percentages of viable cells, as assayed by propidium iodide (PI) exclusion, were similar for the control and Fbxo7 short-hairpin cell lines (Figure 2D), arguing against this possibility. Cells acquire much of their mass during G1 phase, so the cell cycle profile of these cell lines was assayed by determining DNA content as an indicator of cell cycle phase. Asynchronous populations of cells were fixed and PI stained and assayed by FACS. An analysis of the DNA content showed that Fbxo7 knock-down cells had fewer cells with 2N DNA content in G1 phase and more cells with >2N DNA content, in S and G2/M phases (Figure 2E). These data suggest that the rapid proliferation and smaller size of the Fbxo7 knockdown cells are due to a shorter G1 phase.

To begin to investigate the mechanistic basis for these cell cycle phenotypes, the levels of other cell cycle regulators were also determined. Immunoblotting of the cell lysates from control and Fbxo7 knock-down cells showed that the levels of components cyclins D2, D3 and Cdk4/6 were not significantly changed (Figure 2A). However, cells with reduced Fbxo7 also had less p27 protein. Moreover, the levels of S phase cyclins E and A were increased (Figure 2A). These data suggest that the cell cycle phenotypes caused by reducing Fbxo7 levels result from changes in the cell cycle regulators that control S phase entry.

The expression of an F box protein lacking its F box domain can act as a dominant negative to stabilise its ubiquitinated targets. We wanted to determine whether the cell cycle phenotypes we observed were due to Fbxo7 ubiquitin ligase activity. If so, then the expression of an F box deletion mutant might mimic the effect of reducing Fbxo7 levels. To test this, Ba/F3 cells were infected with retroviruses encoding wildtype and an F-box deletion mutant, and GFP⁺ cells were collected by FACS sorting to generate polyclonal lines. Cell lysates were assayed by immunoblotting for Fbxo7. The F box deletion results in a smaller protein with faster migration on SDS-PAGE (Supplementary Figure 1A). The proliferation rates (Supplementary Figure 1B) and cell size (Supplementary Figure 1C) were measured, but no differences were seen. These data suggests that the cell cycle phenotypes are not

due to the ubiquitin ligase function of Fbxo7, although, we cannot formally exclude the possibility that the F-box deletion mutant does not act as a dominant negative allele in these assays.

Decreasing p27 enhanced proliferation of Ba/F3 cells

We have previously shown that Fbxo7 directly interacts with the N-terminus of p27 and stabilises its levels (19). Here we demonstrated that reducing the levels of Fbxo7 decreased p27 levels and increased S phase cyclins. In order to dissect the molecular changes that contributed to the cell cycle phenotypes in Ba/F3 cells, cell lines were created which stably expressed mir30-based short-hairpin RNAs, which targeted p27 expression. Two independent clonal cell lines which reduced the levels of p27 were assayed for the expression of cell cycle regulators (Figure 3A). The total level of p27 was reduced, but no changes were seen in the expression of cyclin D2, cyclin E, Cdk4 or Cdk6. Also, the levels of Fbxo7 were unchanged, suggesting that p27 does not stabilise Fbxo7. However, when the rate of proliferation and cell size were assayed, cells with less p27 protein showed increased rates of proliferation (Figure 3B) and smaller cell size (Figure 3C), similar to cells that have reduced amounts of Fbxo7. The DNA content was also measured by FACS analysis to assess the cell cycle profile of these lines. Interestingly, these cells also showed an increase in cells in S phase and a decrease in cells in G1 phase (Figure 3D). However, no increases were observed in G2/M phase as compared to cells with reduced Fbxo7. These data argue that reducing p27 protein alone can affect the cell cycle distribution, proliferative rate and cell size.

To test directly whether increasing p27 levels reversed the cell cycle phenotypes in Fbxo7 knock-down cells, a plasmid encoding p27 fused to dsRED was electroporated into Ba/F3 cells with reduced levels of Fbxo7. DsRED positive cells were collected by FACS sorting and cell volume measured. Cells expressing p27-dsRED showed a 13% increase in cell size (data not shown). In addition, staining of DNA with PI showed exogenous p27 expression approximately doubled the percentage of cells with a 2N DNA content (G1 phase) from 38% compared to 19.5% in untransfected cells (data not shown). Together these data support the idea that the phenotypes observed upon reduction of Fbxo7 levels are due mainly to a decrease in p27 levels, as re-introducing its expression reversed these phenotypes.

Changes to Cdk6 expression do not alter the growth of Ba/F3 cells.

Because Fbxo7 facilitates the formation of D-type cyclins/Cdk6 complexes, we also investigated whether changes to the levels of Cdk6 would affect the growth and proliferation of Ba/F3 cells. First, retroviruses encoding short-hairpin RNAs targeting Cdk6 expression were used to infect Ba/F3 cells to create stable cell lines. Two independent clonal cell lines were assayed for the expression of Cdk6, Fbxo7 and p27. In both instances where Cdk6 levels were substantially reduced, the protein levels of Fbxo7 and p27 were unchanged (Figure 4A). In addition, when the rate of proliferation and the size of the cells with lower levels of Cdk6 were measured, these characteristics were similar to the control (Figure 4B and 4C).

We next tested whether over-expression of Cdk6 would affect the proliferative rate and cell size. A polyclonal line of cells over-expressing Cdk6 was generated by retroviral infection and FACS sorting for GFP⁺ cells. As can be seen in Figure 4D, immunoblotting of lysates from these cells showed Cdk6 was approximately 5 fold increased; however, the levels of Fbxo7, p27, and cyclin E were the same as the vector control cells. Also, the proliferative rate and cell size were virtually identical to the control (Figure 4E and 4F). These data demonstrate that increasing Cdk6 does not alter proliferation or cell size, suggesting that it is not rate-limiting under the conditions assayed here. Taken together, these data indicate that the levels of Cdk6 do not influence the amount of Fbxo7 or p27, nor does this have any phenotypic consequences for cell growth and proliferation.

Ba/F3-Epo-R cells with reduced levels of Fbxo7 differentiate along the erythroid lineage.

The cellular decision to proliferate or terminally differentiate is thought to occur during G1 phase, although the two processes are independently regulated (39). A lengthened G1 phase can increase the probability of differentiation (40;41), while a shortened G1 phase appears to decrease the probability of differentiation and favour proliferation (42). In addition, Cdk6 and p27 have been shown to have a roles in regulating differentiation (33;43;44). Because Fbxo7 regulates these proteins and affected the duration of G1 phase, we wanted to further characterise its activity with regard to differentiation. We exploited the potential of Ba/F3 cells to be differentiated along the erythroid lineage, due to their expression of erythroid-specific transcriptional factors, including GATA-1, NF-E2, and EKLF (45). These cells will partially differentiate when they are engineered to express

erythropoietin-receptor (Epo-R) and are cultured in the presence of erythropoietin (Epo). They cannot, however, terminally differentiate and do not synthesize hemoglobin. Instead the expression of β -globin mRNA is used as a measure of their partial differentiation (41;45;46). To determine directly the effect of Fbxo7 on differentiation, we engineered expression of Epo-R-IRES-GFP in these cells (see materials and methods). To create lines with decreased levels of Fbxo7, Ba/F3-Epo-R cells were infected with retroviruses encoding the miR30 short-hairpin against Fbxo7 or a non-silencing control. Two independent clonal lines were created, and the reduction in Fbxo7 expression was verified by immunoblotting (Figure 5A). Cells were induced to differentiate by culturing them in the presence of Epo, and then total RNA was isolated. Semi-quantitative RT-PCR was performed to assess the levels of β -globin mRNA. Samples from cells with reduced Fbxo7 showed a 10-fold increase in the amount of β -globin message as compared to the vector (Figure 5B). The rate of proliferation of Epo-R-IRES-GFP control cells and cells with reduced Fbxo7 was also determined by performing a growth curve on cells induced to differentiation by growth in Epo. As has been previously reported for Ba/F3-Epo-R cells, we observed a transient slowing of their growth rate when cells differentiate in response to Epo. However, cells with reduced Fbxo7 did not demonstrate this retardation (Figure 5C). These data showed that decreasing the levels of Fbxo7 enhanced Ba/F3 differentiation along an erythroid lineage, and argue against the requirement for a cell cycle delay to promote differentiation.

Ba/F3 cells with reduced levels of Fbxo7 show increased expression of CD43.

Because reducing Fbxo7 levels enhanced the induction of erythroid differentiation in Ba/F3 cells, we reasoned that B cell differentiation might also be affected if Fbxo7 levels were reduced and that this might be reflected in its surface antigens. As Ba/F3 is a pro-B lymphocytic cell line, a subset of common B cell surface markers was assayed. Several different surface markers that are indicative of stages of B cell differentiation were tested, including B220, B7.1, B7.2, CD43, and MHC II/la. Control Ba/F3 cells and cells with reduced levels of Fbxo7 were stained with antibodies conjugated to fluorescent dyes and analysed by FACS. For all but one of the markers tested, there were no differences between the cells with endogenous or reduced levels of Fbxo7 (Figure 6A and data not shown). However, the number of CD43+ cells was on average 2.76 fold and 3.26 fold increased in cells stably knocked down for Fbxo7 compared to the vector control (Figure 6A and data not shown).

CD43 is a sialoglycoprotein with a role in cell-cell adhesion and proliferation. During B cell differentiation it has an 'on-off-on' expression pattern, being expressed by early precursors for B cells (pre-pro-B and pro-B cell), then rapidly down-regulated upon progression to pre-B and B cell stages with VDJ rearrangement, and reappearing at the plasmablast stage of terminal differentiation (47). Thus, the increase in CD43 levels in cells with reduced Fbxo7 suggests that Fbxo7 regulates differentiation and/or functionality within the B cell lineage.

Decreasing Fbxo7 levels in Ba/F3 cells resulted in a concomitant drop in p27 levels, an increase in S phase cyclins and a shorter G1 phase. One possibility is that Fbxo7 affects CD43 expression through its regulation of p27 protein levels, and the effects on cell cycle progression (Figure 3D). In order to directly test whether decreasing p27 protein was sufficient to increase CD43 expression, Ba/F3 cells with reduced levels of p27 (Figure 3A) were assessed for CD43 expression. In two separate experiments, there was an average 1.25 and 1.64 fold increase in CD43 expressing cells in the p27 knockdown cell lines compared to the vector control (Figure 6B). These data demonstrate that reduction of p27 levels alone do not increase CD43 expression to the extent seen with Fbxo7, and argue against the idea that changing the duration of G1 phase alone can lead to differentiation. We also determined whether CD43 levels would be affected by decreasing Cdk6 levels. Ba/F3 cells with reduced levels of Cdk6 were stained for CD43 expression, and an average 1.92 and 3.31 fold increase was observed (Figure 6C). Thus knockdown of Cdk6 protein alone was sufficient to increase CD43 expression to levels similar to those observed with reduction of Fbxo7, and this suggests that Fbxo7 up-regulates CD43 expression through its positive regulation of Cdk6 activity.

Discussion

Fbxo7 is a regulator of cell cycle via p27

In our previous studies, in NIH3T3 immortalised murine fibroblasts where Cdk4 is the main G1 kinase, the over-expression of Fbxo7 resulted in Cdk6-dependent transformation, despite having no effect on cell growth or proliferation (19). However, in Ba/F3 cells, a murine pro-B cell line where Cdk6 is abundant, the over-expression of Fbxo7 did not enhance cell growth, proliferation, viability, or act as a transforming gene and enable cells to survive in the absence of IL-3 signaling. This indicates

that Fbxo7 does not provide a transforming activity in these cells. Instead, we demonstrate that the stable reduction of Fbxo7 led to an accelerated proliferation rate, decreased cell size, and a shorter G1 phase. These data reveal that Fbxo7 can, surprisingly, have an anti-proliferative role in Ba/F3 cells. In our previous studies using U2OS cells, an osteosarcoma cell line, the transient transfection of dsRNA to reduce Fbxo7 led to an approximately 50% reduction in p27, p21 and Cdk6 levels, and a specific decrease in Cdk6 associated with cyclin D1 or D3. However, no changes in the cell cycle profile were seen (19). We speculated that Cdk6 activity was not important or limiting for cell cycle progression in this cell type where Cdk4 activity predominates.

Upon analysing the molecular basis for these anti-proliferative phenotypes in Ba/F3 cells, we observed that reducing Fbxo7 did not alter the level of D-type cyclins or Cdk4/6. Instead, the main effects were on p27 and the S phase cyclins, cyclin E and A. We previously showed that Fbxo7 directly interacts with p27 N-terminus and stabilises it (19), and we support that finding here by showing that reducing Fbxo7 expression decreased p27 levels. However, we also noted an increase in S phase cyclins and functional changes to proliferation rate, size, and cell cycle profile. One implication is that p27 plays a major role in restraining S phase entry and progression in Ba/F3 cells.

We tested directly whether altering Cdk6 levels or decreasing p27 levels would mimic the cell cycle phenotypes observed when Fbxo7 was reduced. We observed that changes to the levels of Cdk6, either through over-expression or reducing its levels, did not change their proliferation or growth. This indicates that Cdk6 protein is not rate limiting for these cell cycle properties. However, targeting p27 levels resulted in a smaller cell size, an increase in proliferation rates, and an altered cell cycle profile. Reducing p27 levels did not change the levels of other cell cycle regulatory proteins, like cyclin E, Fbxo7 or Cdk6. It has been previously shown that p27 delays entry into S phase and cells remain in G1 and accumulate biomass; these results demonstrate that Fbxo7 also regulates size and proliferation via its stabilisation of p27. In support of this idea, the expression of p27-dsRED in cells with reduced Fbxo7 reversed the cell cycle phenotypes, arguing that Fbxo7 exerted control of the cell cycle through p27 regulation.

We hypothesize that Fbxo7 has both transforming and anti-proliferative activities, which are seemingly contradictory properties, in different cell types because of the predominating G1 kinase activity and its ability to interact with and stabilize p27. This heavily regulated and multi-functional

protein has oncogenic and tumour suppressor properties depending on its sub-cellular localization (35-37;48). It acts as a tumour suppressor when nuclear and has oncogenic activity when cytoplasmic. Fbxo7 also shuttles between the cytoplasm and the nucleus, and has shown exclusively cytoplasmic distribution in 10% of colon adenocarcinoma and 65% of lung squamous cell carcinoma cases we analyzed (19). Whether the sub-cellular localization of Fbxo7 can alter its function or determine its tumour suppressing or promoting activities is currently under investigation.

Fbxo7 coordinates cellular functions with cell cycle progression

Differentiation ultimately results in cells that exit the cell cycle, as cells acquire specific functions. The process is temporally linked to the G1 phase, and the duration of G1 influences the likelihood that a cell will undergo terminal differentiation. A prolonged G1 phase enhances differentiation potential, while a shortened G1 phase diminishes it. For this reason, proteins that lengthen G1 phase, like cyclin-dependent kinase inhibitors, are good candidates for being joint regulators of differentiation and proliferation. Indeed there is experimental evidence that proteins, like CDKIs, can regulate both processes (43;49;50). However, there is also substantial experimental evidence in a variety of cell types, including B and T cells (51;52), which show that proliferation and differentiation can be separated, so even though the two processes are co-regulated, there is no requirement for them to proceed simultaneously (39).

Because Fbxo7 interacts with regulators of G1 phase, it might also affect cell differentiation. To test this, Ba/F3 cells were engineered to express the Epo-receptor (EpoR), which enables differentiation along an erythroid lineage in response to erythropoietin (Epo). Proliferative and erythroid-specific differentiation signals are generated simultaneously in the presence of Epo, and previous studies showed that signaling through EpoR causes a transient growth delay and a rapid onset of β -globin mRNA accumulation (45), indicative of the early steps in erythroid differentiation. The authors suggested that cell cycle delay was necessary but not sufficient for the induction of differentiation. Here we show that, when induced to differentiate, EpoR cells with reduced Fbxo7 do not slow their growth and, in fact, show greatly increased levels of β -globin mRNA. This suggests Fbxo7 negatively regulates Epo-induced differentiation.

We also analyzed Ba/F3 cells with reduced Fbxo7 protein for the expression of cell surface markers that denote stages of B cell differentiation and found CD43 expression to be up-regulated. CD43 is a transmembrane glycoprotein and is a ligand for E-selectin, and has been shown to function in cell-cell adhesion and to interfere with an LFA-1/ICAM-1 interaction (53). CD43 expression in B cells is regulated in a sub-lineage or stage-specific manner, being expressed on most lymphocytic and centrocytic B lymphomas, but absent from resting peripheral B cells. Several studies indicate CD43 is advantageous for cell proliferation and survival, and it functions as a co-stimulatory molecule in B cell activation (53;53;54). We find that Fbxo7 negatively regulates CD43 expression, which is an unexpected and novel finding. This relationship could be direct, for example CD43 could be a substrate of Fbxo7 for ubiquitin-mediated degradation, or indirect, and this is currently under investigation.

Our results further distinguish G1 length and the propensity for differentiation. Despite the fact that reducing Fbxo7 levels shortened G1 phase, it also paradoxically appeared to enhance increased the propensity to differentiate. We conclude that the duration of G1 phase is of lesser importance to the signals that drive differentiation in this system. In support of this, we find that in p27 knockdown cells, where cell cycle progression was also affected, CD43 expression is only marginally increased. However, we cannot eliminate the possibility that other changes, like an increase in cyclin E levels in conjunction with p27 knock down, might be necessary to affect CD43 expression. Other evidence supporting the idea that cell cycle progression is less important includes the finding that reducing Cdk6, which did not alter the cell cycle, increased CD43 expression. We infer that the increased CD43 expression seen in Fbxo7 knockdown cells is likely to be due to its effects on Cdk6, and independent of its effect on p27. A specialised role for Cdk6 in differentiation has been reported by several laboratories (reviewed in (33)), and we suggest that tumours where high levels of Cdk6 expression are seen, like lymphomas, glioblastomas, and sarcomas, may have defective differentiation programmes, in addition to deregulation of the pRb pathway.

How Cdk6 influences differentiation is an interesting question for future study, but some experimental data indicates it is directly involved in regulating transcription (32). In some cells, the down-regulation of Cdk6 protein is critical for terminal differentiation (34). We also investigated whether Fbxo7 was similarly down-regulated during terminal differentiation. When we induced

terminal differentiation in U937 and MEL cells, Cdk6 protein was down-regulated but Fbxo7 was not (our unpublished data). Even though Fbxo7 stabilises Cdk6, its removal is not necessary for terminal differentiation to proceed. p27 is also important for cell cycle exit in haematopoietic cells, so Fbxo7 stabilisation of p27 levels may be equally important for terminal differentiation.

Together our data demonstrate that Fbxo7, through its interactions with p27 and Cdk6, regulates cell cycle along with other cellular functions, like cell differentiation or cell adhesion, and propose it has an important and sensitive role in integrating cell decisions and functions in G1 phase.

Materials and methods

Cell culture. Ba/F3 cells were maintained in complete RPMI-1640 medium supplemented with 10% foetal calf serum (PAA Laboratories), penicillin (50U/mL), streptomycin (50U/mL) (Gibco BRL), and supplemented with 10ng/mL recombinant murine IL-3 (Fitzgerald Laboratories). Cells were incubated at 37°C in a 5% humidified CO₂ atmosphere.

To specifically reduce Fbxo7 or p27 protein expression, cells were infected with retroviruses having either an LMP or PSMP vector encoding a miR30-based short-hairpin. Either empty LMP or non-silencing vector PSMP (Open Biosystems) were used as negative controls. To decrease Cdk6 protein, retroviruses containing pRETROSUPER-based short hairpin constructs were used as previously described (19). For every cell line, independent clones were generated by limiting dilution and grown with puromycin selection (2µg/mL), and several clones were analysed. To over-express proteins, cells were infected with retroviruses encoding MSCV-IRES-GFP containing the gene of interest. Polyclonal lines were generated by FACS sorting and collecting GFP expressing cells.

Ba/F3 cells expressing Epo-Receptor were generated by retroviral infection with MSCV-EpoR-GFP (kindly provided by Professor Tony Green, Department of Hematology, University of Cambridge). Retroviruses encoding the Epo-R-IRES-GFP were used to infect Ba/F3 cells, which were sorted for GFP expression. Clones were subsequently selected for Epo-R expression by growing cells in media supplemented with 0.05U/mL erythropoietin (Epo) (Cell Sciences), but lacking IL-3. Ba/F3-Epo-R cell lines were thereafter maintained in IL-3 during standard sub-culturing.

To measure the rate of proliferation and cell volume, cells were seeded in triplicate at a density of 0.3-0.5x10⁶ cells/mL and cell number and volume were assayed every two days using a

Casy cell counter (Scharfe System). Three independent experiments were performed. To measure cell viability, cells were exposed to 50 μ g/mL propidium iodide (Sigma Aldrich) at room temperature for 30mins prior to analysis by FACS (FACS Calibur, Becton Dickinson). To quantify DNA content, 1-2 x10⁶ cells were washed in PBS and fixed in ice cold 70% ethanol at 4°C for 30 min. Cells were washed in PBS and incubated in 50 μ g/mL RNase A and 100 μ g/mL propidium iodide for 30mins and then analysed by FACS.

Immunoblotting. 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 min, and centrifuging at 13000rpm for 10 min at 4°C. Protein concentration was determined using protein assay (Biorad). 50 μ g of total lysate was resolved by SDS-PAGE, and transferred onto PVDF membranes, which were incubated in PBS with 0.05% Tween-20 and 5% dried skimmed milk powder. The following rabbit polyclonal primary antibodies were used: Cdk6 (c-12) (sc-177), Cdk4 (c-22) (sc-260), Cdk2 (M2) (sc-163), cyclin A (H-32) (sc-751), cyclin E (M-20) (sc-481), cyclin D2 (c-17) (sc-181), cyclin D3 (c-16) (sc-182), p27 (c-19) (sc-528) (Santa Cruz Biotechnology Inc.), and actin (Sigma Aldrich). The antibody against Fbxo7 has been previously described (19). Donkey anti-rabbit IgG conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology, Inc.

Differentiation assays. EpoR-expressing Ba/F3 cells, with or without the short-hairpin targeting Fbxo7, knockdown were cultured in the presence of IL-3, Epo (0.05 U/mL or 0.5 U/mL), or both IL-3 and Epo (0.5 U/mL). Cells were harvested at various time points after the addition of Epo, and RNA extracted by lysing cells in Trizol, and centrifugation at 11,000rpm for 10mins at 4°C. A chloroform extraction was carried out before RNA isolation using RNeasy mini kit (Qiagen). RNA concentration was measured using a nanodrop spectrophotometer (Nanodrop Technologies). 1 μ g of total RNA was used for each sample harvested at 3 days post induction of differentiation. 2 μ L of random hexamers (Promega) were added to each sample, which were incubated at 65°C for 5mins and then placed on ice. 7 μ L of a master mix (4 μ L of 5X RT Buffer, 0.5 μ L of RNasin (Promega), 2 μ L of 10mM dNTP, 0.5 μ L of reverse transcriptase (Roche)), was added to each sample and incubated for 25°C for 10mins, 42°C for 60mins, and 70°C for 10mins. cDNA was used in PCR reactions (95°C for 30 sec, 50°C for 1min, 72°C for 1min for 25 cycles) using primers to amplify β -globin (FOR:

GACCCAGCGGTACTTTGATAGC and REV: TGAGGCTGTCCAAGTGATTCA) and cyclophilin (FOR: CCTTGGGCCGCGTCTCCTT and REV: CACCCTGGCACATGAATCCTG) (Invitrogen). Quantification was performed on a Molecular Imager Gel Documentation System using Quantity One software (Biorad).

To assess cell surface markers, 5×10^5 cells were stained with 0.5 μ l CD43 antibody (clone S7) conjugated to PE (BD Pharmingen) in 50 μ l FACS buffer (1% PBS, 0.025% NaN₃, 2.5% FCS) (1:100 dilution) for 30 min at 4°C, washed twice in FACS buffer and analysed by FACS.

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Figure 1. The expression of Fbxo7 in Ba/F3 cells does not affect its growth or proliferation. **(A)** Immunoblot of cell lysates from Ba/F3 cells infected with retroviruses expressing either human Fbxo7 or the empty vector. Actin is used as a control for equivalent loading. **(B)** Graph of a time course of proliferation rate of cells which were seeded at equal densities, and counted on the indicated day. **(C)** Percentages of viable and non-viable cells in an asynchronous culture were assessed by propidium iodide staining of live cells and counted by FACS. **(D)** Plot of the kinetics of survival upon withdrawal of IL-3 from the culture medium. Cells were seeded at equal densities in media without IL-3, and the number of viable cells was determined over time as indicated.

Figure 2. Stable knock-down of Fbxo7 causes an increase in the proliferation and a decrease in the size of Ba/F3 cells. **(A)** Immunoblotting for various cell cycle regulatory proteins of cell lysates from independent clones of Ba/F3 cells infected with retroviruses expressing a miR30-based hairpin which targets Fbxo7 expression. Actin is used as a loading control. **(B)** Graph of a time course of the proliferation rate of an empty vector control cell line and two independent clonal cell lines which have

reduced Fbxo7 expression. Cells were seeded at equal densities and counted on the indicated day. **(C)** Graph of size measurements from cells which were seeded at equal densities. **(D)** Percentages of viable and non-viable cells in an asynchronous culture were assessed by propidium iodide staining of live cells and counted by FACS. **(E)** Graph of DNA content of an asynchronous population of cells, measured by FACS, and used to determine the percentages of cells in various phases.

Figure 3. Reduction of the levels of p27 in Ba/F3 cells increases the rate of proliferation and decreases cell size. **(A)** Immunoblots for various cell cycle regulatory proteins of cell lysates from independent clonal lines stably expressing short-hairpin RNAs targeting p27 expression or the empty vector control. **(B)** Graph of proliferation rates of cell lines with endogenous or reduced levels of p27. **(C)** Graph of cell sizes of cell lines shown in B. **(D)** Graph of the DNA content of asynchronously growing cells lines.

Figure 4. Alternations in the level of Cdk6 do not affect growth or proliferation of Ba/F3 cells. **(A)** Immunoblotting for various cell cycle regulatory proteins of cell lysates from independent clones of Ba/F3 cells infected with retroviruses expressing a pRETROSuper short hairpin targeting Cdk6 expression or the empty vector. Actin is used as a loading control. Graphs of proliferation rates **(B,E)** and cell size **(C,F)** were generated using the same methods as detailed in Figure 2. **(D)** Immunoblotting as in (A) from cell lysates from polyclonal cell lines generated by infecting Ba/F3 cells with retroviruses expressing human Cdk6-IRES-GFP and sorted for GFP expression.

Figure 5. Fbxo7 negatively regulates differentiation. **(A)** Immunoblots for Fbxo7 expression in cell lysates from independent clonal cell lines of Ba/F3 cells expressing the Epo-R and infected with retroviruses expressing a miR30-based hairpin which target Fbxo7 expression. A non-specific band was used as an internal loading control. **(B)** Image of semi-quantitative RT-PCR for beta-globin and cyclophilin from cells which have been induced to differentiate by the addition of Epo. **(C)** Graph of rate of proliferation as cells differentiate in response to Epo.

Figure 6. CD43 expression is upregulated in cells with reduced Fbxo7 or Cdk6. FACS plots for cells with stable reduction of Fbxo7 expression **(A)**, p27 **(B)**, or Cdk6 **(C)** stained with CD43-PE. GFP is on the x axis (FL-1) and CD43-PE is on the y axis (FL-2).

Supplementary Figure 1. The expression of an Fbxo7 mutant lacking its F box domain does not alter the growth or proliferation of Ba/F3 cells. **(A)** Immunoblot for Fbxo7 of cell lysates from Ba/F3 cells infected with retroviruses expressing human Fbxo7, the F box7 deletion mutant (Fbxo7(del Fbox)) or the empty vector control. Actin is used as a control for equivalent loading. **(B)** Time course of proliferation rate of polyclonal cell lines which were seeded at equal densities, and counted on the indicated day. **(C)** Graph of size measurements from cells seeded at equal densities.

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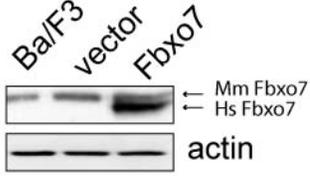
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Figure 1

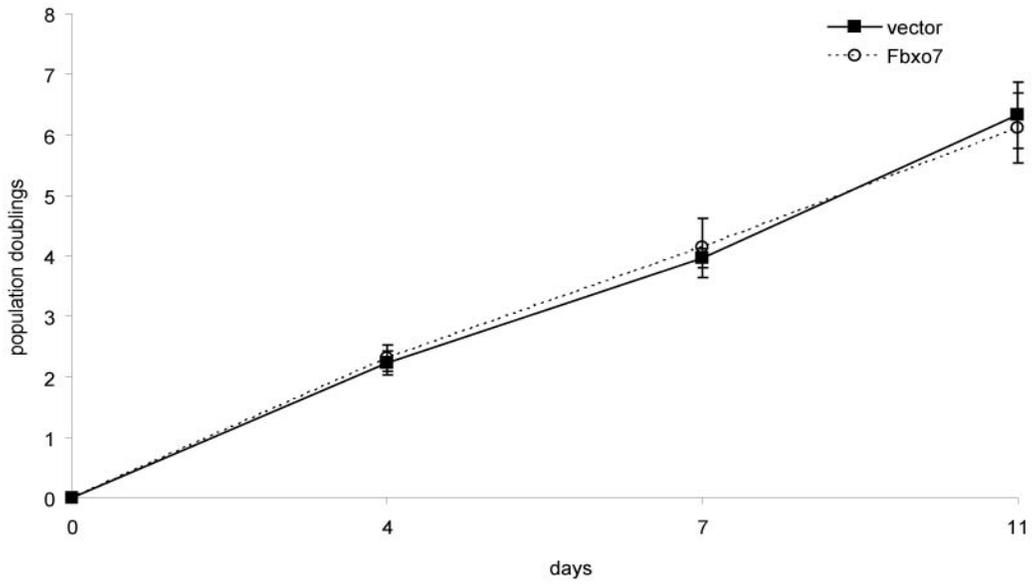
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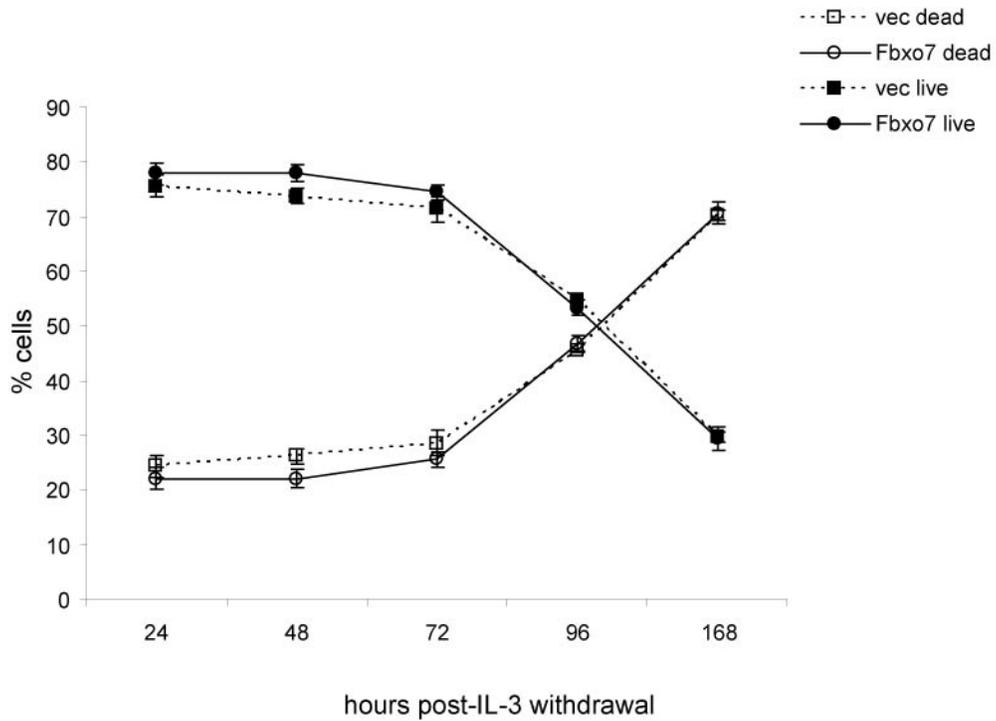
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	% viable	% non-viable
vector	95.75	4.25
Fbxo7	94.14	5.86

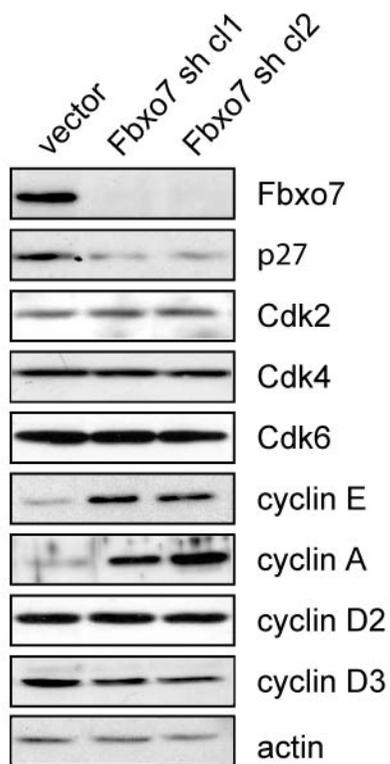
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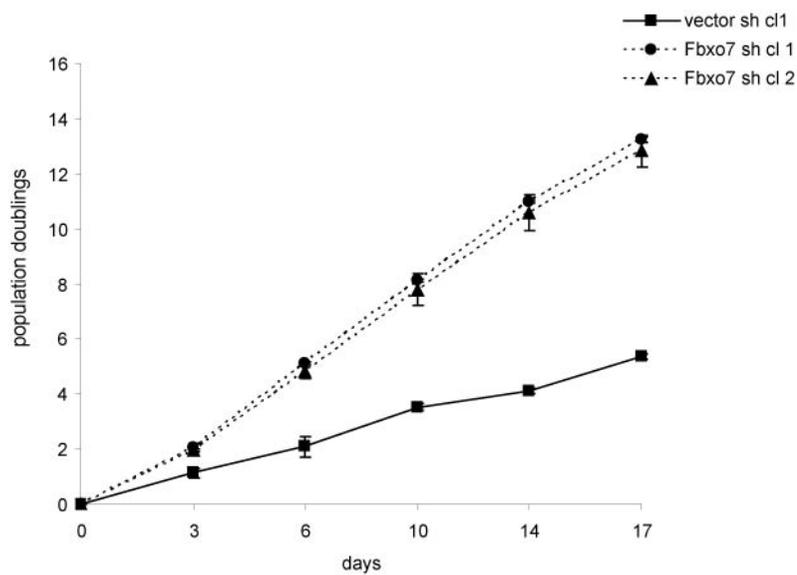
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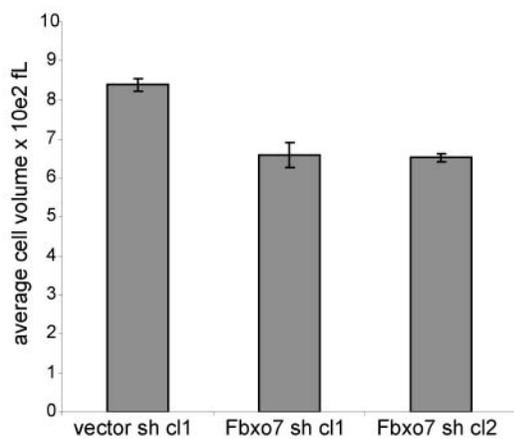
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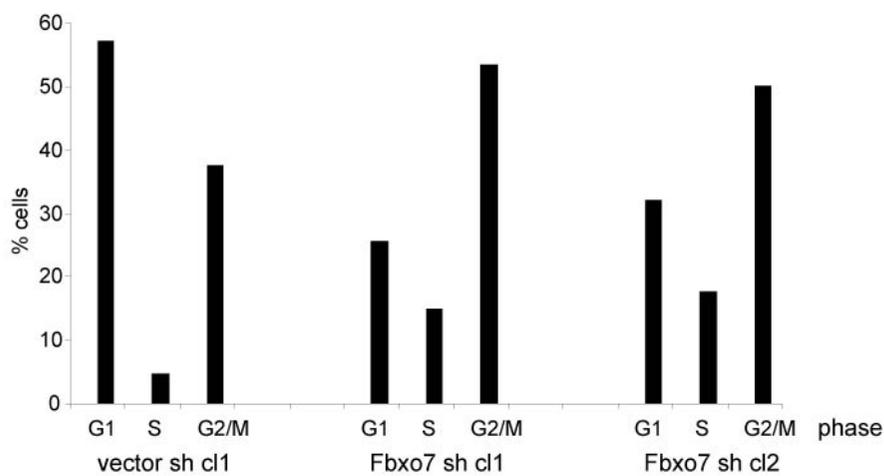
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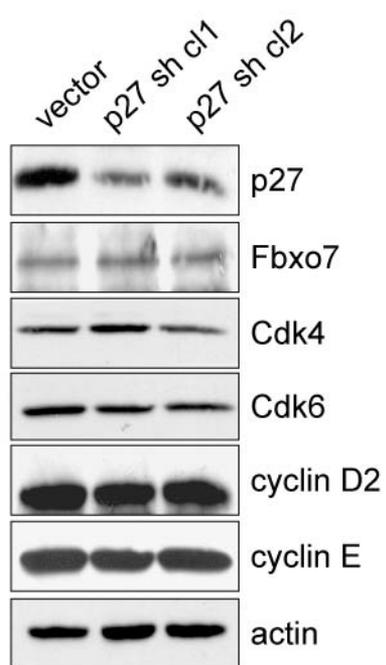
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	<u>% viable</u>	<u>% non-viable</u>
vector	89.96	10.04
Fbxo7 sh cl1	89.54	10.46
Fbxo7 sh cl2	86.14	13.86

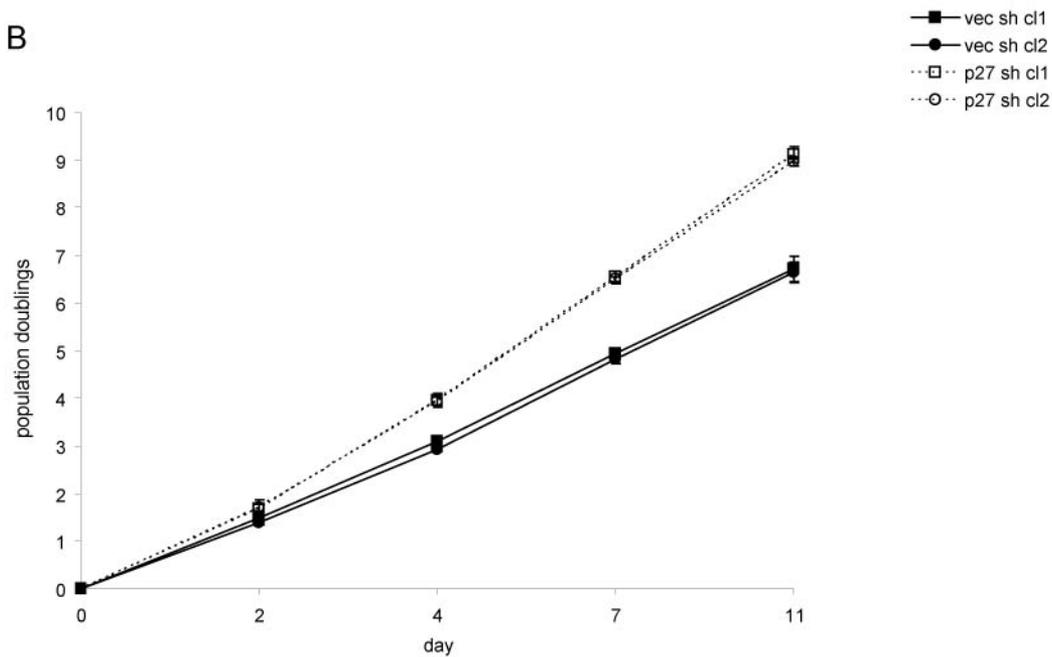
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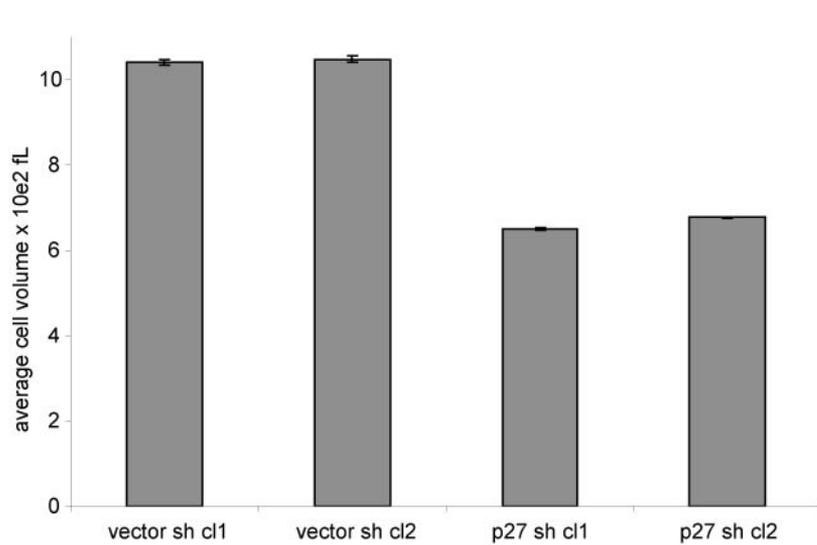
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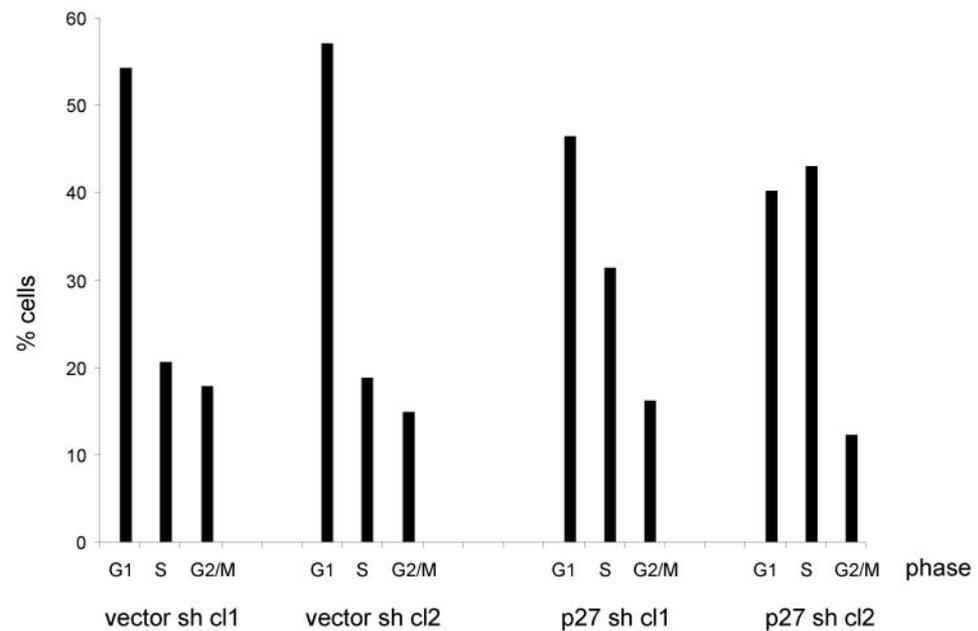
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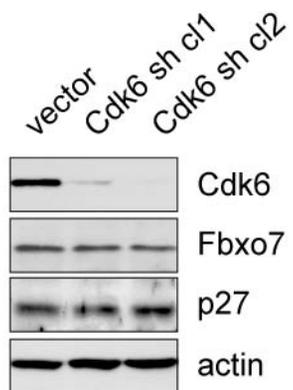
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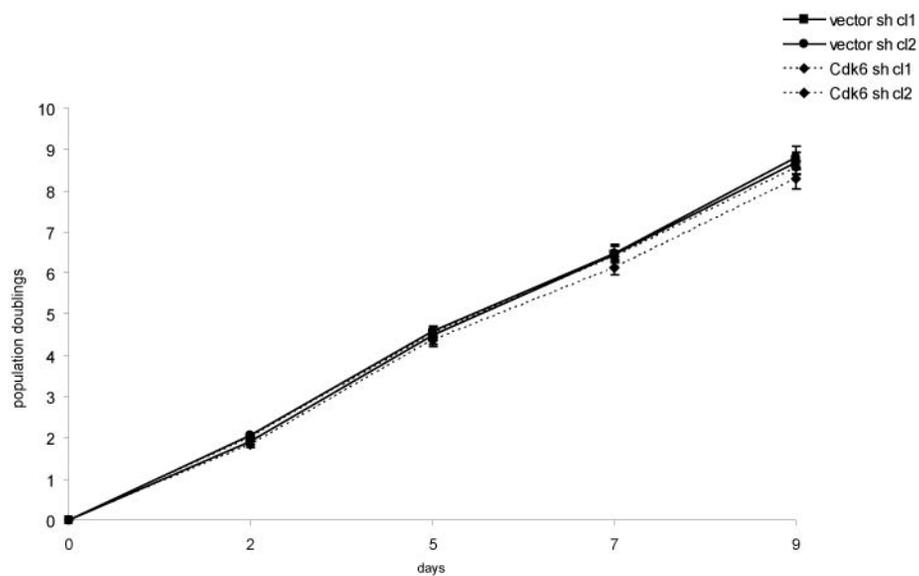
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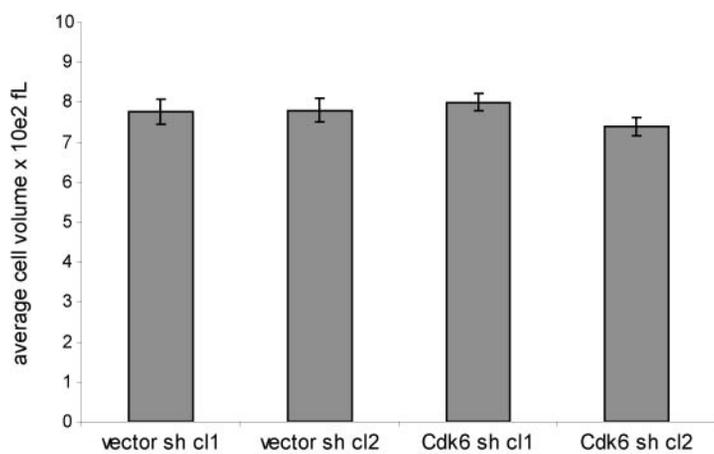
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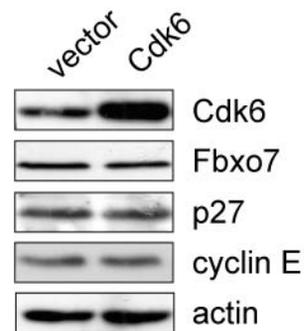
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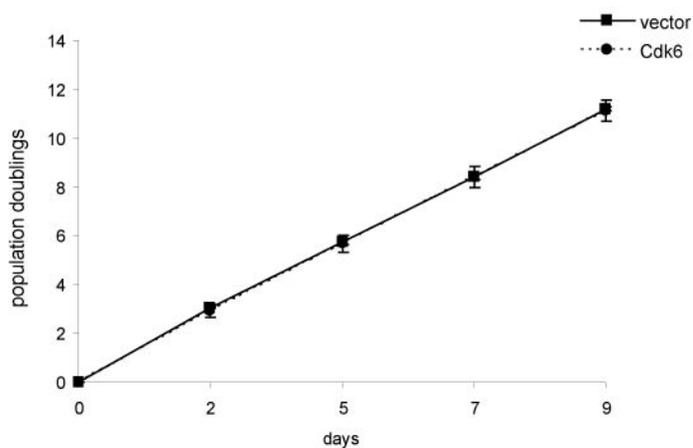
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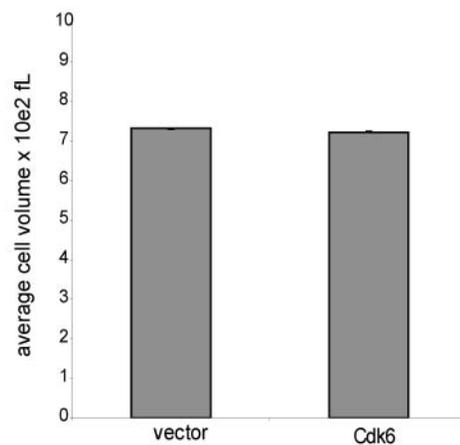
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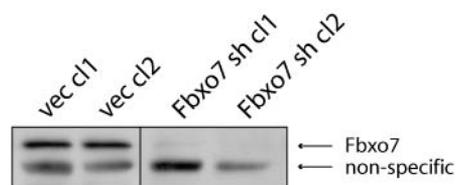
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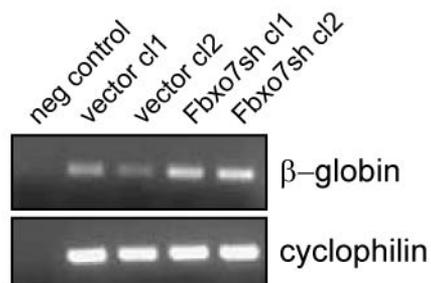
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A



B



C

