

A novel role of proteasomal $\beta 1$ subunit in tumorigenesis

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Synopsis

p27^{Kip1} is a key cell-cycle regulator whose level is primarily regulated by the ubiquitin–proteasome degradation pathway. Its $\beta 1$ subunit is one of seven β subunits that form the β -ring of the 20S proteasome, which is responsible for degradation of ubiquitinated proteins. We report here that the $\beta 1$ subunit is up-regulated in oesophageal cancer tissues and some ovarian cancer cell lines. It promotes cell growth and migration, as well as colony formation. $\beta 1$ binds and degrades p27^{Kip1} directly. Interestingly, the lack of phosphorylation at Ser¹⁵⁸ of the $\beta 1$ subunit promotes degradation of p27^{Kip1}. We therefore propose that the $\beta 1$ subunit plays a novel role in tumorigenesis by degrading p27^{Kip1}.

Key words: degradation, p27^{Kip1}, phosphorylation, tumorigenesis, $\beta 1$ subunit

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INTRODUCTION

There are two main protein degradation machineries in eukaryotic cells, proteasomes and lysosomes [1]. The ubiquitin–26S proteasome-dependent proteolytic pathway plays important roles in many cellular processes by controlling levels of key molecules, which function in cell-cycle progression, antigen presentation, the secretory pathway and signal transduction, etc. [2]. The 26S proteasome is a large multi-subunit complex containing a 20S proteolytic CP (core particle) and a 19S RP (regulatory particle) [3]. The 20S proteasome comprises four heptameric stacked rings ($\alpha_{1-7}/\beta_{1-7}/\beta_{1-7}/\alpha_{1-7}$) and has chymotrypsin-like ($\beta 5$), trypsin-like ($\beta 2$) and caspase-like ($\beta 1$) activities that cleave peptides after hydrophobic, basic and acidic residues, respectively [4]. The free 20S proteasome can mediate ubiquitin-independent degradation of proteins that are naturally unfolded or damaged [5,6].

The $\beta 1$ subunit has caspase-like activity in the constitutively expressed mammalian proteasomal complex and is replaced by the IFN γ (interferon γ)-inducible subunit, $\beta 1i$, in the immunoproteasome [7]. Most β -type subunits are synthesized as pro-proteins, which undergo limited proteolysis during proteasomal

maturation [8–11]. The C-terminal extension of $\beta 7/Pre4$ is required for the post-acidic activity mediated by the $\beta 1/Pre3$ subunit and deletion of the C-terminal tail of $\beta 7/Pre4$ inhibits $\beta 1/Pre3$ propeptide processing and abrogation of post-acidic activity [12,13]. A mutant lacking both BIm10 and the C-terminal extension of $\beta 7/Pre4$ grows extremely poorly, accumulates very high levels of precursor complexes and is impaired in β subunit maturation [14]. The processing of active eukaryotic β subunits is reported to be an ordered two-step mechanism involving autocatalysis [11,15].

Progression through the cell cycle requires the formation and activation of cyclin and CDK (cyclin-dependent kinase) complexes [16,17]. Activation of the G1 phase cyclin-CDK complexes results in the phosphorylation of Rb (retinoblastoma) gene products which oppose cell-cycle progression by controlling gene expression mediated by E2F transcription factors [18]. CDKIs (CDK inhibitors), p21^{cip1}, p27^{Kip1} and p15/p16^{ink4}, regulate this process by inhibiting cyclin/CDK activity and phosphorylation of Rb, resulting in G1 arrest [17,19–21]. p27^{Kip1} is primarily expressed in the G0 phase of the cell cycle and regulates cell-cycle progression [22,23]. p27^{Kip1} specifically inhibits cyclin E/Cdk2 and cyclin A/Cdk2, two kinases necessary for DNA

Abbreviations used: CBB, Coomassie Brilliant Blue; CDK, cyclin-dependent kinase; GST, glutathione transferase; HCC, hepatocellular carcinoma; HEK-293T, HEK-293 cells expressing the large T-antigen of SV40 (simian virus 40); PKA, protein kinase A; Rb, retinoblastoma; Rfp, red fluorescent protein; RP, regulatory particle; shRNA, small hairpin RNA.

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Table 1 Primers used in the present study

| Plasmid | 5' primer oligo | 3' primer oligo | Restriction enzyme sites |
|--|--------------------------------|---------------------------------|--------------------------|
| Wild-type $\beta 1$ | | | |
| p $\beta 1$ -GST | CGCggatccACCACTATCATGGCC | CCGgaattcCGGCGGGTGGTAAAGT | BamHI/EcoRI |
| p $\beta 1$ -His ₆ | CGCggatccACCTTACTAGCTGCT | CCGgaattcGGCGGGTGGTAAAG | BamHI/EcoRI |
| p $\beta 1$ -Rfp | CCGgaattcCACCACTATCATGGCC | CGCggatccGGCGGGTGGTAAAGT | EcoRI/BamHI |
| Point-mutations in $\beta 1$ | | | |
| p $\beta 1$ -His ₆ S158E | TGATGGTAAGGCAGGAATTTGCCATTGG | AGCCTCCAATGGCAAATTCCTGCCTTAC | |
| p $\beta 1$ -His ₆ S158A | TGATGGTAAGGCAGGCCTTTGCCATTGG | AGCCTCCAATGGCAAAGGCCTGCCTTAC | |
| Wild-type p27 ^{Kip1} | | | |
| pp27 ^{Kip1} -GST | CGCggatccATGTCAAACGTGCGAGTGTCT | CCGctcgagTTTACGTTTGACGTCTTCTGAG | BamHI/XhoI |
| pp27 ^{Kip1} -His ₆ | CGCggatccATGTCAAACGTGCGAGTGTCT | CCGctcgagTTTACGTTTGACGTCTTCTGAG | BamHI/XhoI |

replication. When the levels of p27^{Kip1} decrease, Cdk2 is activated and cells enter S phase [24]. Regulation of cellular levels of p27^{Kip1} is therefore one of key points in cell-cycle control. Two post-translational mechanisms were proposed to be involved in p27^{Kip1} breakdown: (a) ubiquitinated p27^{Kip1} is recognized and destroyed by the proteasome [25,26], or (b) the N-terminus of non-ubiquitinated p27^{Kip1} is rapidly cleaved to remove its cyclin-binding domain, a process that is ATP-dependent with high activity in the S phase [27]. In addition, B-lymphoid cells have caspase or caspase-like activities that are inversely regulated with respect to p27^{Kip1} abundance and this activity cleaves a caspase recognition site present in p27^{Kip1} (DPSD139) [28]. Tambyrajah et al. recently used a tetra-peptide substrate, Ac-DPSD-AMC, to mimic a target cleavage site in p27^{Kip1} and traced this activity to the $\beta 1$ subunit of the 20S proteasome [29]. Nevertheless, this tetra-peptide substrate may not adequately represent the p27^{Kip1} protein. Whether $\beta 1$ binds and degrades p27^{Kip1} directly remains unknown.

In the 20S proteasomal phosphoproteome, Ser¹⁵⁷ in murine $\beta 1$ (158 in human) has been suggested to be a PKA (protein kinase A) phosphorylation site [30]. However, the biological significance of this possible phosphorylation is unknown.

We observed that the $\beta 1$ subunit is up-regulated in oesophageal cancer tissues and some ovarian cancer cell lines. It promotes cell growth, colony formation and migration. Interestingly, $\beta 1$ binds and degrades p27^{Kip1} directly and the phosphorylation of $\beta 1$ at Ser¹⁵⁸ plays a key role in the degradation of p27^{Kip1}. We thus present here a novel role of $\beta 1$ subunit in tumorigenesis.

MATERIALS AND METHODS

Construction of $\beta 1$ and p27^{Kip1} expression plasmids

Plasmids were constructed using standard recombinant technique as described previously [31]. The propeptide of $\beta 1$ subunit (34 amino acids in the N-terminal domain of $\beta 1$ zymogen) was deleted and the active sites (Thr³⁵ and Thr³⁶) were therefore exposed [10]. This truncated $\beta 1$ was subcloned into a pGEX 4T2 vector [GST (glutathione transferase)- $\beta 1$] and pDsRed1-C1 vec-

tor (Rfp- $\beta 1$), whereas the full-length $\beta 1$ coding sequence was subcloned into pET 28a vector ($\beta 1$ -His₆). The sequences of primers and the description of plasmids were summarized in Table 1. DNA fragments encoding both $\beta 1$ shRNA (small hairpin RNA) and control shRNA were subcloned into the vector pGenesil-1.0. Both siRNA (small interfering RNA) sequences of *PSM $\beta 6$* (proteasomal subunit β type 6) were used in this study: si $\beta 1$ -1, aatcgagtgactgacaagctg and si $\beta 1$ -2, aatgctctcgtttggccatg, respectively. A plasmid to express RNA without homology to human or mouse sequences was used as a control in silencing experiments [31].

Cell culture

HeLa and HEK-293T [HEK-293 cells expressing the large T-antigen of SV40 (simian virus 40)] cells (obtained from A.T.C.C.) were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) FBS, 100 units/ml penicillin, 100 units/ml streptomycin at 37°C, 5% (v/v) CO₂. Establishment of stably transfected HeLa cells was performed as described previously [32]. Cell proliferation was determined by counting the cell numbers with a haemocytometer at the indicated times after plating cells into 24-well plates for 2 h. Data represent an average of three independent experiments. As previously described [33], HeLa cells were synchronized at G0/G1 by serum starvation for 48 h and then stimulated to re-enter the cell cycle by serum re-addition. Synchronization was monitored by the Coulter EPICS XL cytometer (Beckman Coulter Inc.) using PI (propidium iodide) staining [34].

Crystal violet staining

The plates or dishes were placed on ice and washed twice with ice-cold 1 × PBS. Cells were then fixed with ice-cold 100% (v/v) methanol for 10 min. After aspiration of the methanol, 0.5% (w/v) crystal violet solution (in 25% (v/v) methanol and stored at room temperature (25°C)) was added and incubated at room temperature for 10 min. After rinsing repeatedly with water, the plates were allowed to dry at room temperature and then photographed.

Cell migration assays

Migration assays were performed using Transwells (8- μm pore size, Corning Costar) without MatrigelTM, according to manufacturer's instructions. Cells were allowed to migrate for 12 h at 37°C. The Transwell inserts were fixed in 10% (v/v) formalin, stained with filtered 0.5% (w/v) crystal violet in 10% (v/v) ethanol and then washed in deionized water. The non-migratory cells on the upper surfaces of the membranes were removed using cotton swabs. The membranes were air-dried and mounted for microscopy. For each chamber, the migrating cells in ten randomly chosen fields ($\times 400$) were counted.

Scratch wound assays were performed using a p200 pipette tip to create a 'scratch.' After washing the cells with 1 ml of growth medium, they were recultured with 1 ml of medium and photographed at regular intervals to monitor cell migration.

Human tumour samples

Human oesophageal cancer tissue samples were obtained from Zhongshan Hospital of Xiamen University. Informed consent was obtained from the donors regarding the use of resected tumours for research purposes. The research has been carried out in accordance with the Declaration of Helsinki (2008) of the World Medical Association, that the Ethical Committee of the Institution in which the work was performed has approved it and that the subjects have given informed consent to the work.

Immunohistochemistry

Tissue sections were prepared from formalin-fixed, paraffin-embedded specimens of human cancers. Immunohistochemical analysis was performed as described previously [35] using a mouse monoclonal antibody against $\beta 1$ (#sc-100455) and Rpt3 (Proteintech Group, Inc.).

Preparation of tissue lysates

For whole protein extracts and Western blot analysis of $\beta 1$, tissues was homogenized in lysis buffer (10 mM Tris pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 0.5% (v/v) Triton X-100, 0.02% (w/v) NaN_3 , 0.2 mM PMSF) containing CompleteTM, a protease inhibitor cocktail (# 05892791001, Roche), incubated for 30 min on ice, and then centrifuged for 30 min at 12 000 g. The supernatant was saved for Western blot analysis. Protein concentrations were determined by the Bradford assay with BSA as standard [36].

Analysis of protein–protein interactions

Both GST-tagged $\beta 1$ and p27^{Kip1} and His₆-tagged $\beta 1$ and p27^{Kip1} were overexpressed by IPTG induction in bacteria as described previously [31]. For detection of their interactions, the blot was probed with an anti-His₆ antibody (# sc-803) or an anti-p27^{Kip1} antibody (# sc-1641) and detected by ECL. Immunoprecipitation analysis was performed as described previously [31]. In brief, 5×10^6 cells synchronized at G0/G1 phase were lysed in

lysis buffer [50 mM Hepes-NaOH (pH 7.5), 100 mM NaCl, 0.5% (v/v) Nonidet P40, 2.5 mM EDTA, 10% (v/v) glycerol, 1 mM and 1 mM PMSF]. Samples were precleared by incubation with protein G-plus-agarose beads (#sc2002) at 4°C for 1 h and then received 1 μg of a monoclonal antibody against GFP (#sc-9996), p27^{Kip1} (#sc-1641) or 1 μg of $\beta 1$ (#sc-100455) along with 10 μl of protein G-plus-agarose at 4°C for 4 h. Agarose beads were washed with lysis buffer and suspended in SDS/PAGE sample buffer. After SDS/PAGE, samples were analysed by Western blotting using corresponding antibodies for $\beta 1$ (#sc-100455) and p27^{Kip1} (#sc-1641). 10% of each total lysate was loaded as input and 25% of each bound sample was loaded for each Western blot analysis.

In vitro/in vivo degradation assays

In vitro and *in vivo* degradation assays were partly modified from those described previously [27]. Briefly, His₆-tagged p27^{Kip1} or its mutants and His₆-tagged $\beta 1$ protein or its mutants were expressed in *Escherichia coli* BL21 and purified using Ni SepharoseTM 6 Fast Flow. 1 μg of purified p27^{Kip1}-His₆ or its mutants and 2 μg $\beta 1$ -His₆ or its mutants were mixed together at 37°C in 50 μl of degradation buffer [20 mM Hepes pH 7.2 or pH 6.5, 100 mM NaCl, 10% (w/v) sucrose, 1% (v/v) CHAPS, 10 mM DTT and 1 mM EDTA] plus 2 mM ATP and 5 mM MgCl_2 . The reactions were carried out at 37°C for different periods of time, terminated by adding SDS-gel loading buffer, and each reaction mixture was subjected to SDS/PAGE on a 12% (w/v) gel, followed by immunoblotting with either an anti-p27^{Kip1} or anti- $\beta 1$ antibody.

RESULTS

The expression of $\beta 1$ subunit is up-regulated in human tumour tissues and cells

As the expression of $\beta 1$ subunit was observed to be up-regulated in diethylnitrosamine-treated mouse livers and in human HCC (hepatocellular carcinoma) tissues [37] and the $\beta 1$ subunit has caspase activity that could degrade the key cell-cycle regulator, p27^{Kip1} [29,38], we speculated that the expression of $\beta 1$ is up-regulated in other tumour tissues or cells. We therefore performed immunohistochemical staining of some paraffin-embedded oesophageal cancer tissue specimens using an antibody against $\beta 1$ protein. Like in HCC samples [37], the expression of $\beta 1$ is obviously up-regulated in oesophageal cancer tissues compared with its adjacent normal tissues (Figure 1A). By contrast, expression of Rpt3, a component of the 19S RP of the 26S proteasome, was not up-regulated in oesophageal cancer tissues or in its adjacent normal tissues (Figure 1A). Moreover, expression of $\beta 1$ subunit was increased in several ovarian cancer cell lines (4/5 cell lines) when compared with a normal ovarian cell lines (NOE cell line) (Figure 1B).

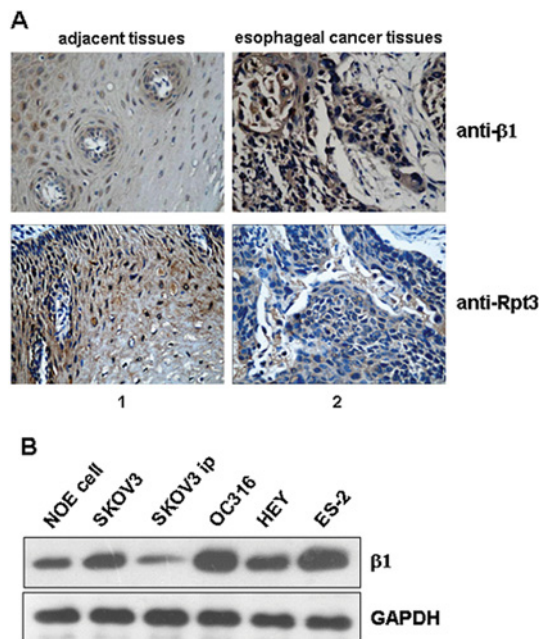


Figure 1 $\beta 1$ subunit is up-regulated in oesophageal cancer tissues and some ovarian cancer cell lines

(A) Immunohistochemical staining of human oesophageal cancer tissues with an anti- $\beta 1$ /Rpt3 antibody. Tissue sections were prepared from formalin-fixed, paraffin-embedded specimens of human cancers. Panel 1 illustrates adjacent normal tissue and panel 2 illustrates tissue of oesophageal cancer ($\times 400$). (B) Levels of endogenous $\beta 1$ in different human ovarian cancer cell lines were detected by Western blot. The level of endogenous GAPDH (glyceraldehydes-3-phosphate dehydrogenase) was used as loading control.

$\beta 1$ promotes cell proliferation

Since the expression of the $\beta 1$ subunit is up-regulated in human tumour tissues and cells, it is of great interest to ask whether $\beta 1$ is a potential onco-protein that promotes cell proliferation and migration. To this end, we established two stably transfected HeLa cell lines expressing the Rfp (red fluorescent protein) or $\beta 1$ tagged with Rfp ($\beta 1$ -Rfp), respectively. As shown in Figure 2(A), cells expressing ectopic $\beta 1$ grew three-time faster than cells expressing only Rfp. Moreover, overexpression of ectopic $\beta 1$ significantly increased colony formation, a hallmark of transformation (Figure 2B). To see whether $\beta 1$ can promote the proliferation of other cell lines, a plasmid expressing $\beta 1$ -Rfp or Rfp was transiently transfected into HepG₂ cells and cells were cultured with Gly⁴¹⁸ at 0.5 mg/ml for 10 days before staining with crystal violet as described under the ‘Materials and methods’ section. As shown in Figure 2(C), $\beta 1$ can also promote colony formation ability of HepG₂ cells (over 60%).

$\beta 1$ promotes cell migration

To test whether $\beta 1$ can promote cell migration, Transwells were used to assay the migration ability of stably transfected HeLa cells. As shown in Figure 3(A), the number of migrated cells expressing ectopic $\beta 1$ was evidently more than twice that of the

control cells. Cell migration was also assessed by overexpressing $\beta 1$ in HEK-293T cells with an *in vitro* scratch assay. Dynamic images of the scratch were acquired and the width of the scratch was measured as a function of time (Figure 3B). These results suggest that $\beta 1$ may be a novel onco-protein, which promotes cell migration.

$\beta 1$ interacts with p27^{Kip1} *in vivo* and *in vitro*

Although a recent study showed that $\beta 1$ has caspase activity and can degrade p27^{Kip1} [29], the mechanism of this degradation is unknown. To investigate this process, we tested possible interactions between $\beta 1$ and p27^{Kip1} using immunoprecipitation and the GST-pull-down protocols. First, we detected the possible interaction of endogenous $\beta 1$ subunit and p27^{Kip1} *in vivo*. As shown in Figure 4(A), endogenous $\beta 1$ and p27^{Kip1} in the precipitated complex were detected by an anti- $\beta 1$ or anti-p27^{Kip1} antibody, respectively, and both precipitated $\beta 1$ and p27^{Kip1} showed a remarkably efficient binding for each other. This suggests that $\beta 1$ interacts with p27^{Kip1} *in vivo*. As the $\alpha 7$ subunit is one of the constituent subunits of the 20S proteasome and it interacts with $\beta 1$ *in vivo* [3,39], we next checked whether $\alpha 7$ was co-precipitated with $\beta 1$. In Figure 4(B), the $\alpha 7$ subunit was found to be precipitated with $\beta 1$. To test whether $\beta 1$ interacts with p27^{Kip1} directly, we used a GST-pull-down assay. As shown in Figure 4(C), His₆-tagged $\beta 1$ can bind with p27^{Kip1}-GST, which was detected by both anti- $\beta 1$ and anti-His₆ antibodies. Interestingly, when the $\beta 1$ -GST fusion protein was incubated with purified p27^{Kip1}-His₆, both anti-p27^{Kip1} and anti-His₆ antibodies were also able to detect p27^{Kip1} (Figure 4D), suggesting that $\beta 1$ and p27^{Kip1} interact with each other directly *in vitro*.

$\beta 1$ degrades p27^{Kip1} directly

We first tested whether overexpression of ectopic $\beta 1$ could reduce the amount of endogenous p27^{Kip1}. HeLa cells stably expressing either $\beta 1$ -Rfp or Rfp were synchronized at G0 phase [33]. After lysis, the amount of endogenous p27^{Kip1} was determined by immunoblotting. As shown in Figure 5(A), the level of p27^{Kip1} in cells expressing $\beta 1$ -Rfp was significantly lower than for cells expressing free Rfp, but the level of $\alpha 7$ subunit was little affected when $\beta 1$ was overexpressed in the stable cell lines. To further confirm the function of $\beta 1$ in degrading p27^{Kip1}, plasmids expressing either control shRNAs or $\beta 1$ shRNAs were expressed in HeLa cells. The amount of p27^{Kip1} in cells expressing $\beta 1$ shRNAs was higher than in cells expressing the control shRNAs (Figure 5B). Interestingly, the level of the $\alpha 7$ subunit was also reduced when $\beta 1$ was down-regulated (Figure 5B). We conclude that some essential subunits of proteasome including $\alpha 7$ subunit could be affected by the down-regulation of $\beta 1$ and that the increased steady-state levels of p27^{Kip1} result from malfunction of proteasome [40] or down-regulating of $\beta 1$.

To learn whether $\beta 1$ alone can degrade p27^{Kip1}, we set up an *in vitro* degradation assay that was partly modified from those described [27]. Briefly, 1 μ g of purified p27^{Kip1}-His₆ and 2 μ g of purified $\beta 1$ -His₆ were mixed in 50 μ l of degradation buffer

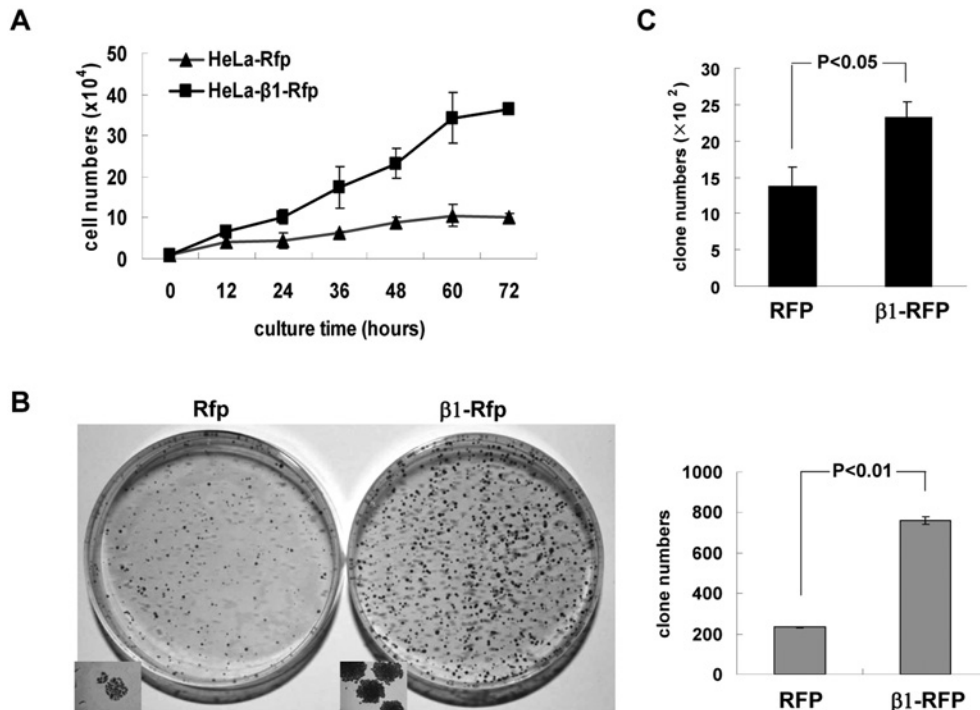


Figure 2 $\beta 1$ promotes cell proliferation

(A) Proliferation of HeLa cells stably expressing Rfp and $\beta 1$ -Rfp was determined by cell counting at the indicated times (h) 2h after cells were plated. Data represent an average of three independent experiments. (B) Colonies formed by HeLa cells (500 cells) stably expressing Rfp and $\beta 1$ -Rfp were stained with crystal violet and counted (left panel). Colonies in the small square boxes were documented by a fluorescence microscope (Nikon, $\times 100$). Numbers of colonies are shown in the right panel. Error bars show S.D. (C) Plasmids expressing $\beta 1$ -Rfp or Rfp were transiently transfected into HepG₂ cells and cells were cultured with Gly⁴¹⁸ at 0.5 mg/ml for 10 days before staining with crystal violet as described under the Materials and Methods section. It was clear that $\beta 1$ can promote colony formation by HepG₂ cells.

at 37°C for increasing periods of time. We observed that the amount of p27^{Kip1} gradually decreased with time: nearly 40% was degraded after 90 min (Figure 5C). Since $\beta 1$ was reported to have caspase-like activity [29,30], it is important to use further optimized conditions to study degradation of p27^{Kip1}. Thus, as shown in Figure 5(D), p27^{Kip1} totally disappeared in incubations at pH 6.5 and a mobility shift of $\beta 1$ band was also observed. Since $\beta 1$ has a propeptide in its N-terminal domain [10], we hypothesized that $\beta 1$ would be activated at pH 6.5. To test this hypothesis, p27^{Kip1}-His₆ and $\beta 1$ -His₆ were mixed in the reaction buffer at pH 6.5 and for increasing periods of time (Figure 5E). The amount of p27^{Kip1} dramatically decreased with time as $\beta 1$ started to self-cleave. Control His₆-tagged p53 showed almost no change under the same conditions change (Figure 5F). The 20S proteasome is a threonine protease and its active sites are located in the N-terminal domain of β subunits. When $\beta 1$ starts to self-cleave, its propeptide (34 amino acids at the N-terminal domain of the zymogen) is deleted and the active sites (Thr³⁵ and Thr³⁶) are therefore exposed. Deletion of the N-terminal threonine or mutating it to alanine led to inactivation of the proteasome [10]. We mutated two threonines into alanines in the N-terminal of $\beta 1$ -His₆ (T35A/T36A) and found that this double mutation significantly weakened not only the interaction between

$\beta 1$ and p27^{Kip1} but also the ability of $\beta 1$ to degrade p27^{Kip1} (Figure 5G). These results suggest that only cleavable and active $\beta 1$ can bind and degrade p27^{Kip1} directly.

Dephosphorylation of $\beta 1$ at Ser¹⁵⁸ enhances its ability to bind and degrade p27^{Kip1}

Ser¹⁵⁷ in murine $\beta 1$ (158 in human) has been suggested to be a PKA phosphorylation site, but its function is largely unknown [30]. Whether PKA phosphorylation of $\beta 1$ regulates its role in degradation of p27^{Kip1} is an open question. To this end, two mutants of $\beta 1$ were constructed: $\beta 1$ S158A, to prevent the phosphorylation of $\beta 1$ at Ser¹⁵⁸, and $\beta 1$ S158E, to mimic phosphorylation of $\beta 1$ at Ser¹⁵⁸ [41–44]. In an interaction assay shown in Figure 6(A), mutation of S158E greatly weakened the interaction between $\beta 1$ and p27^{Kip1}, but mutation of S158A shows an enhanced interaction of $\beta 1$ with p27^{Kip1}. To understand whether phosphorylation of $\beta 1$ at Ser¹⁵⁸ regulates its role in the degradation of p27^{Kip1}, we compared the protein level of endogenous p27^{Kip1} in cells expressing Rfp, $\beta 1$ -Rfp, $\beta 1$ -Rfp S158A or $\beta 1$ -Rfp S158E, respectively and found that the amount of endogenous p27^{Kip1} in cells expressing $\beta 1$ or $\beta 1$ S158A was less than in cells expressing ectopic $\beta 1$ S158E (Figure 6B). These results suggest that

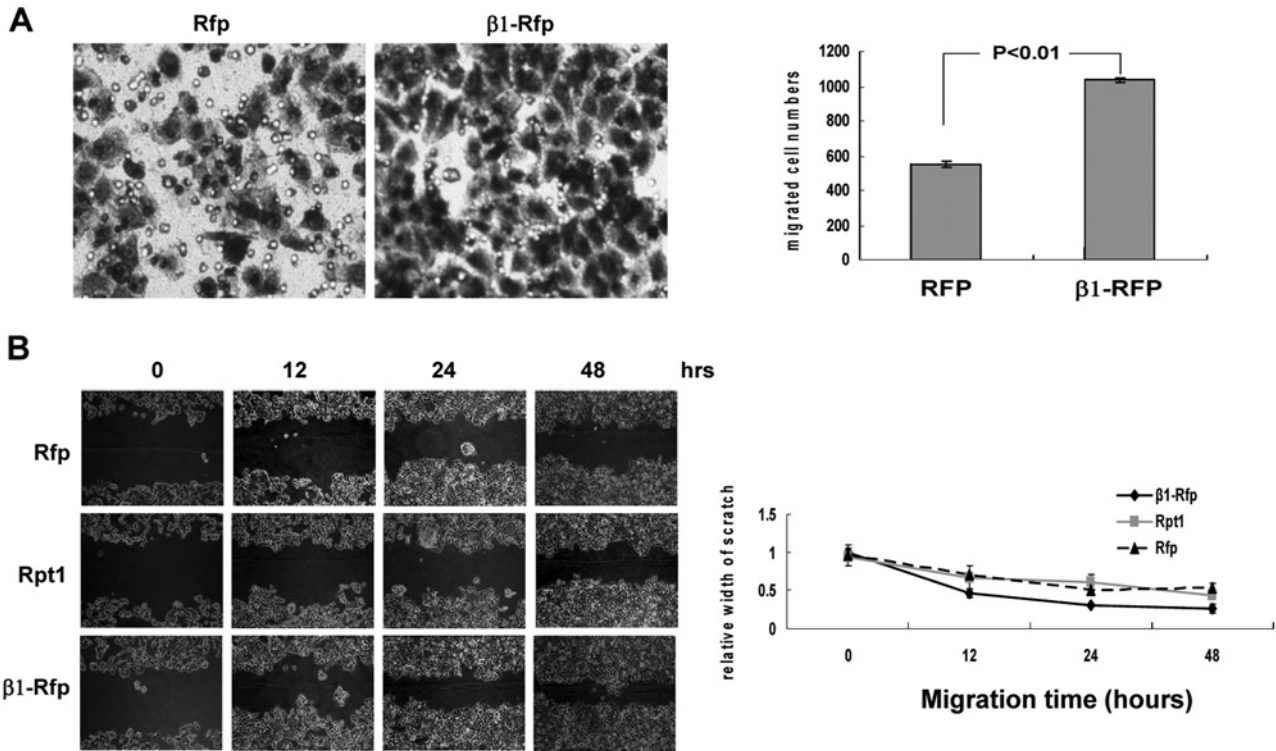


Figure 3 $\beta 1$ promotes cell migration (A) Migration of HeLa cells stably expressing Rfp and $\beta 1$ -Rfp was observed in Transwells (8- μ m pore size, Corning Costar) without MatrigelTM. The membranes were air-dried, stained with crystal violet and then mounted for microscopy (left) (Nikon $\times 200$). For each chamber the migrated cells in ten randomly chosen fields ($\times 100$) were counted (right). Error bars show S.D. (B) HEK-293T cells were transfected with plasmids expressing $\beta 1$ -Rfp, Rpt1 or a control vector. The cell monolayer was then 'scratched' with a p200 pipette tip. The dish was placed under a phase-contrast microscope and images were acquired at 0, 12, 24 and 48 h (upper panel). The width of scratch was measured and used for statistical analysis (lower panel). Error bars show S.D.

dephosphorylation of $\beta 1$ at Ser¹⁵⁸ enhances its ability to degrade p27^{Kip1}.

As p27^{Kip1} regulates cell cycle progression, it is of great interest to ask whether phosphorylation of $\beta 1$ regulates cell proliferation. To this end, the growth of cells expressing $\beta 1$ S158A was compared with the growth of cells expressing $\beta 1$ S158E. As shown in Figure 6(C), cells expressing $\beta 1$ S158A grew faster than cells expressing $\beta 1$ S158E (over 50%).

Taken together, these data suggest that phosphorylation of $\beta 1$ at Ser¹⁵⁸ prevents its binding and degradation of p27^{Kip1}, whereas dephosphorylation of $\beta 1$ at Ser¹⁵⁸ increases its ability to bind and degrade p27^{Kip1}, thus promoting cell proliferation.

DISCUSSION

In vivo measurements show that most of tumours exhibit a significantly acidic pH when compared with normal tissues [45,46]. Increased glycolysis, a trait almost invariably observed in human cancers, confers a selective growth advantage on transformed

cells because it allows them to create an environment that is differentially toxic to normal cells [45]. We found that $\beta 1$ can be activated at pH 6.5 that $\beta 1$ can promote cell proliferation and migration, and that it is up-regulated in oesophageal cancer tissues, HCC tissues [37] and some ovarian cancer cell lines. The weakly acidic environment of tumour tissues and transformed cells may facilitate the activation of the $\beta 1$ proenzyme and therefore increase the ability of $\beta 1$ to degrade its relevant substrates, such as p27^{Kip1}.

CyclinE/Cdk2 activity promotes S phase transition by p27^{Kip1} degradation [47]. CyclinD/Cdk4, 6 promotes cell cycle progression in early G1 to late G1 [48,49]. p27^{Kip1} inhibits the activities of these kinases directly by binding to them negatively regulates cell-cycle progression [24,27], and therefore plays a pivotal role in the control of cell proliferation [50]. The stability of p27^{Kip1} has been of recurrent interest. Several degradation mechanisms have been proposed, including ubiquitination-dependent pathways [26], the ubiquitination-independent pathways [27], a caspase-mediated pathway [51,52] and the Jab1-dependent pathway [53]. In this study, we found that $\beta 1$ has a novel role in promoting cell proliferation by directly binding and degrading p27^{Kip1}. As p27^{Kip1} plays a central role in controlling cell

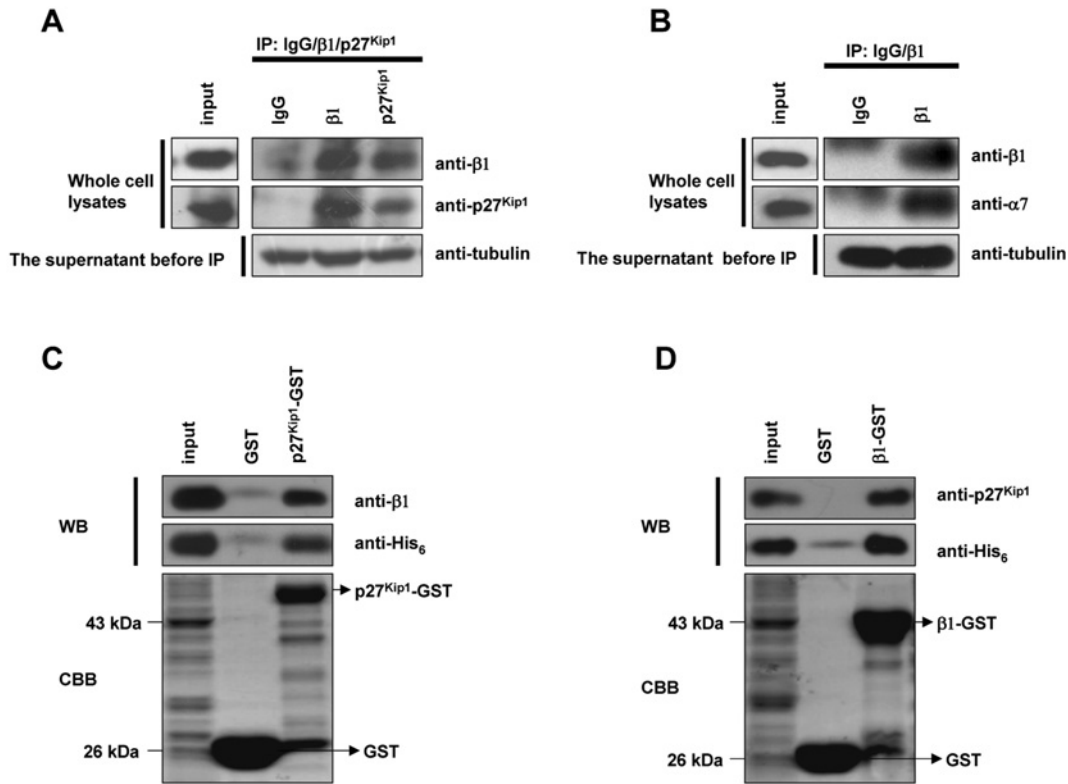


Figure 4 $\beta 1$ interacts with $p27^{Kip1}$ *in vivo* and *in vitro*

(A) Immunoprecipitation analysis of the interactions between $p27^{Kip1}$ and $\beta 1$. Cell lysates from synchronized G0 HeLa cells were divided into three parts and each part was incubated with antibody ($\beta 1$, GFP and $p27^{Kip1}$ mouse monoclonal antibody) overnight and then with protein G-plus-agarose beads at 4°C for 4 h. The beads were washed three times with precipitation buffer and then treated, as above. For detection of interactions, the blot was probed with anti- $\beta 1$ / $p27^{Kip1}$ rabbit polyclonal antibodies, followed by ECL. (B) Immunoprecipitation analysis of the interactions between $\beta 1$ and the $\alpha 7$ subunit. For detection of this interaction, the blot was probed with anti- $\beta 1$ / $\alpha 7$ antibodies, followed by ECL. (C) GST-pull-down analysis of the interactions between $p27^{Kip1}$ and $\beta 1$. $p27^{Kip1}$ -GST fusion proteins were expressed by the addition of IPTG to 0.2 mM at 25°C for 4 h. Bacterial cells were lysed and the supernatants were incubated with the glutathione resin overnight and then with purified $\beta 1$ -His₆ for 4 h at 4°C , followed by washing and elution. To detect interactions, the blots were probed with anti- $\beta 1$ /His₆ antibodies, followed by ECL. The lower panel shows the amounts of bound GST and GST-tagged proteins by CBB (Coomassie Brilliant Blue) staining. (D) $\beta 1$ -GST fusion proteins were incubated with purified $p27^{Kip1}$ -His₆ at 4°C for 4 h and then detected with anti- $p27^{Kip1}$ /His₆ antibody, followed by ECL. The lower panel shows the amounts of bounded GST and GST-tagged proteins by CBB staining.

proliferation [54–56], and is intimately involved in cell death [52,57], it is not surprising that expression of $p27^{Kip1}$ is tightly regulated by multiple mechanisms.

Overexpression of either the $\beta 1$ or $\beta 5$ subunit enhanced proteasomal activity and up-regulates the other proteasomal subunits [58–60]. However, Gaczynska et al. demonstrated that cells transfected with $\beta 1$ and $\beta 5$ subunits have elevated levels of only some proteasomal activities and that the total cellular content of proteasomes does not differ significantly between control and transfected cells [60]. The discrepancy regarding the proteasomal activities and the cellular content of proteasomes in $\beta 1$ - and $\beta 5$ -transfected HeLa and WI38/T cells is possibly caused by different cell lines [59]. In the present study, we observe that the proteasomal $\alpha 7$ subunit does not change significantly when $\beta 1$ was overexpressed in stable HeLa cell lines. As the $\alpha 7$ subunit interacts with $\beta 1$ *in vivo* [3,39], we infer that the structure and

function of the proteasome are not greatly affected by overexpression of $\beta 1$ subunit, but can be impaired upon knock-down of $\beta 1$ expression. Since the relative stoichiometry of proteasomal subunits is controlled by an autoregulatory mechanism that mediates differential poly-ubiquitination and degradation of multiple subunits [61,62], we suggest that overexpression of $\beta 1$ should not increase the integration of $\beta 1$ into proteasomes. Nevertheless, reduction of the level of constitutive subunits (e.g. $\beta 1$) would affect both the structure and the function of the proteasomal complex.

Ubiquitin-dependent degradation of $p27^{Kip1}$ requires the poly-ubiquitination of $p27^{Kip1}$ and other cellular proteins [Skp2 [33], Jab1 [53], etc.] and therefore needs significant time to react to external stimuli. Our data demonstrate that phosphorylation of $\beta 1$ at Ser¹⁵⁸ prevents its direct binding and degradation of $p27^{Kip1}$ while dephosphorylation of $\beta 1$ at Ser¹⁵⁸ increases its ability to bind and degrade $p27^{Kip1}$. PKA may play a key role in regulation

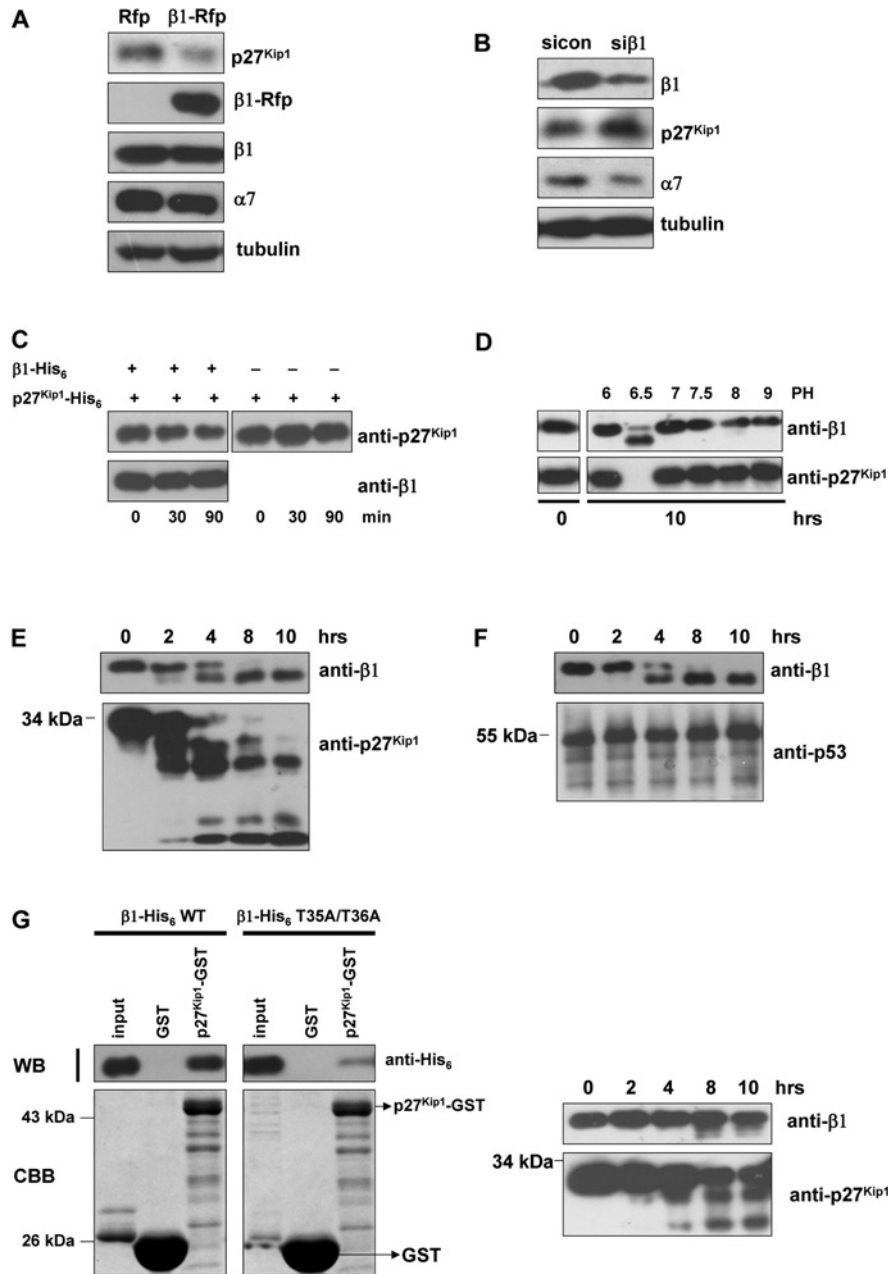


Figure 5 $\beta 1$ is involved in the degradation of $p27^{Kip1}$

(A) Stably transfected HeLa cells were synchronized at G0 phase by serum starvation for 48 h. The levels of $p27^{Kip1}$, $\beta 1$ and $\alpha 7$ were then determined by immunoblotting. (B) shRNA for $\beta 1$ was transiently transfected into HeLa cells for 72 h and the levels of endogenous $p27^{Kip1}$, $\beta 1$ and $\alpha 7$ were detected by Western blot. (C) *In vitro* degradation assay for $p27^{Kip1}$. $p27^{Kip1}$ -His₆ and $\beta 1$ -His₆ were mixed together in degradation buffer. The reactions were carried out for the indicated times and terminated by adding SDS sample buffer. Each reaction mixture was subjected to SDS/PAGE on a 12% (w/v) gel, followed by immunoblotting analysis with the anti- $p27^{Kip1}$ and $\beta 1$ antibodies. (D) $p27^{Kip1}$ -His₆ and $\beta 1$ -His₆ were mixed and the reaction mixtures were incubated in the buffer with the indicated pH for 10 h. Each reaction mixture was then subjected to SDS/PAGE on a 12% (w/v) gel, followed by immunoblotting analysis with anti- $p27^{Kip1}$ and $\beta 1$ antibodies. (E) $p27^{Kip1}$ -His₆ and $\beta 1$ -His₆ were mixed in the reaction buffer at pH 6.5 and then the mixture was incubated for the indicated period of time. The levels of $\beta 1$ and $p27^{Kip1}$ were detected by Western blot. (F) $p53$ -His₆ and $\beta 1$ -His₆ were mixed in reaction buffer at pH 6.5 and this mixture was incubated for the indicated number of h. The levels of $\beta 1$ and $p53$ were also detected by Western blot. (G) Only cleavable and active $\beta 1$ can bind and degrade $p27^{Kip1}$ directly. Left panel, GST-pull-down analysis for interactions between $p27^{Kip1}$ and $\beta 1$ (wide type or mutated form). The lower panel shows the amounts of bound GST and GST-tagged proteins (CBB staining). Right panel, $p27^{Kip1}$ -His₆ and mutated $\beta 1$ -His₆ (T35A/T36A) were mixed in the reaction buffer at pH 6.5 and then this mixture was incubated for the indicated periods of time. The levels of $\beta 1$ and $p27^{Kip1}$ were determined by Western blot.

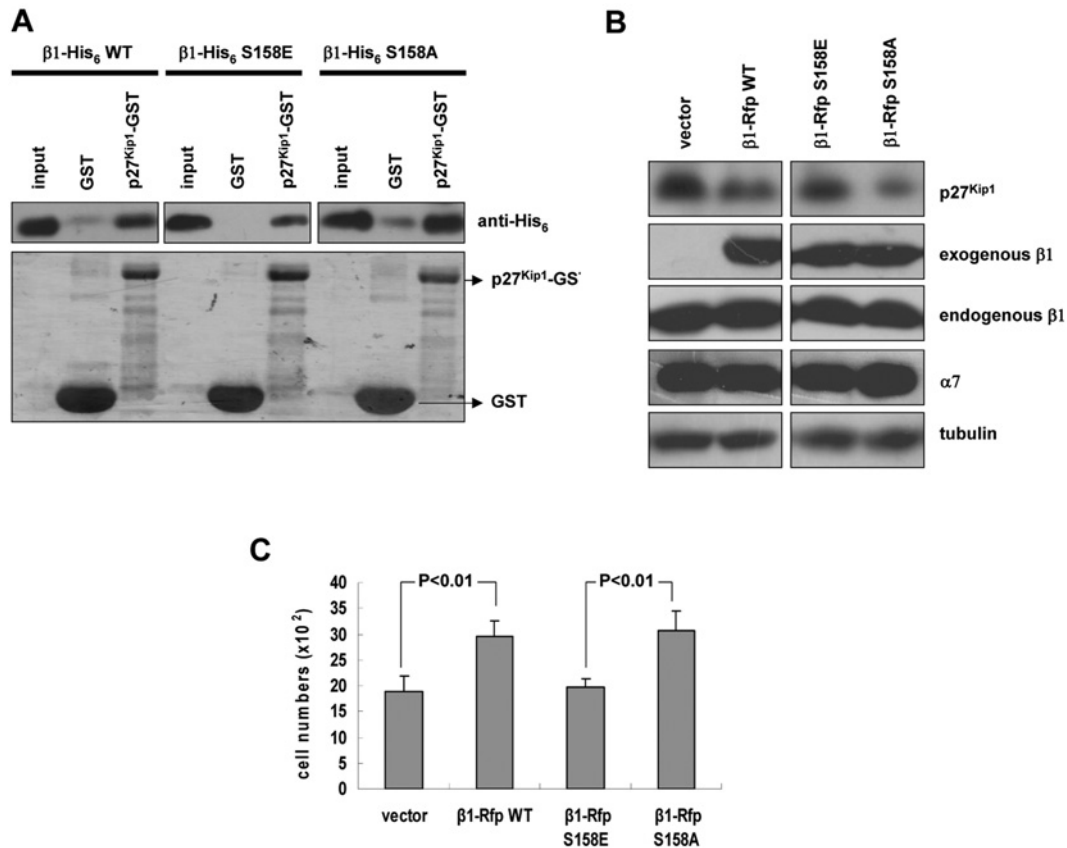


Figure 6 Dephosphorylation of $\beta 1$ at Ser¹⁵⁸ enhances its ability to bind and degrade p27^{Kip1}

(A) Interaction assay for $\beta 1$ (mutated at Ser¹⁵⁸) and p27^{Kip1}. p27^{Kip1}-GST fusion proteins were incubated with purified $\beta 1$ -His₆ wild-type (wt) or its mutant $\beta 1$ -His₆ S158A or $\beta 1$ -His₆ S158E at 4 °C for 4 h and then detected with anti-His₆ antibody. The lower panel shows the amounts of bound GST and GST-tagged p27^{Kip1} (CBB staining). (B) Endogenous p27^{Kip1}, $\alpha 7$ and $\beta 1$ were detected by immunoblotting in cells stably expressing Rfp, $\beta 1$ -Rfp, $\beta 1$ -Rfp S158A and $\beta 1$ -Rfp S158E, using anti-p27^{Kip1} and $\beta 1$ antibodies, respectively. (C) Effects of Rfp-tagged $\beta 1$ and $\beta 1$ mutated at Ser¹⁵⁸ on cell growth *in vivo*. Cell numbers were counted after plating 24 h. The data are the averages of at least three independent experiments. Error bars show S.D.

of $\beta 1$ and p27^{Kip1}. Thus, phosphorylation and dephosphorylation of $\beta 1$ could facilitate rapid adjustment of the level of p27^{Kip1}. Further research will be required to address this possibility.

AUTHOR CONTRIBUTION

Fuqiang Yuan, Tao Tao and Qilin Ma designed the study and wrote the paper. Fuqiang Yuan, Yana Ma, Pan You and Wenbo Lin performed experiments. Haojie Lu, Yinhua Yu, Xiaomin Wang, Jie Jiang and Pengyuan Yang were involved in the conception and design of the project, in the analysis and interpretation of the results and in writing the paper.

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