PTEN regulates the ubiquitin-dependent degradation of the CDK inhibitor $p27^{KIP1}$ through the ubiquitin E3 ligase SCF^{SKP2}

Ramanaiah Mamillapalli*, Nadia Gavrilova*, Valia T. Mihaylova*, Lyuben M. Tsvetkov*, Hong Wu†, Hui Zhang* and Hong Sun*

The PTEN tumor suppressor acts as a phosphatase for phosphatidylinositol-3,4,5-trisphosphate (PIP3) [1, 2]. We have shown previously that PTEN negatively controls the G1/S cell cycle transition and regulates the levels of $p27^{KIP1}$, a CDK inhibitor [3, 4]. Recently, we and others have identified an ubiquitin E3 ligase, the SCF^{SKP2} complex, that mediates p27 ubiquitin-dependent proteolysis [5–7]. Here we report that PTEN and the PI 3-kinase pathway regulate p27 protein stability. PTEN-deficiency in mouse embryonic stem (ES) cells causes a decrease of p27 levels with concomitant increase of SKP2, a key component of the SCF^{SKP2} complex. Conversely, in human glioblastoma cells, ectopic PTEN expression leads to p27 accumulation, which is accompanied by a reduction of SKP2. We found that ectopic expression of SKP2 alone is sufficient to reverse PTEN-induced p27 accumulation, restore the kinase activity of cyclin E/CDK2, and partially overcome the PTEN-induced G1 cell cycle arrest. Consistently, recombinant SCF^{SKP2} complex or SKP2 protein alone can rescue the defect in p27 ubiquitination in extracts prepared from cells treated with a PI 3-kinase inhibitor. Our findings suggest that SKP2 functions as a critical component in the PTEN/PI 3-kinase pathway for the regulation of $p27^{KIP1}$ and cell proliferation.

*Department of Genetics, Yale University School of Medicine, New Haven, Connecticut 06520, USA. †Department of Molecular and Medical Pharmacology, University of California at Los Angeles School of Medicine, Los Angeles, California 90095, USA.

Correspondence: Hong Sun; Hui Zhang
E-mail: hong.sun@yale.edu; hui.zhang@yale.edu

Received: **14 July 2000**
Revised: **30 November 2000**
Accepted: **9 January 2001**

Published: **20 February 2001**

Current Biology 2001, 11:263–267

0960-9822/01/$ – see front matter

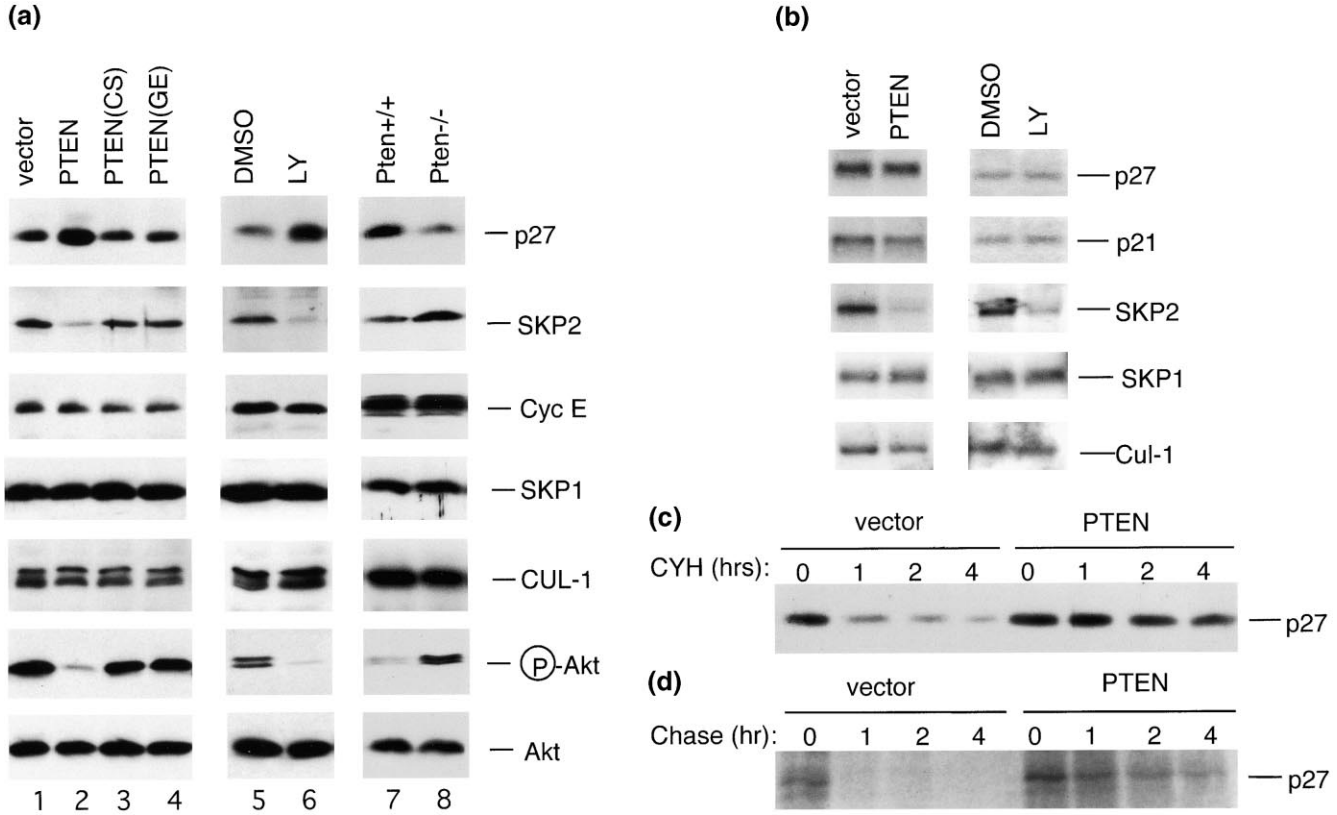
Results and discussion

To determine how PTEN regulates cell cycle progression, we investigated the mechanism by which PTEN controls the levels of $p27^{KIP1}$. To efficiently express PTEN and examine its cell cycle effects, we have reintroduced the *Pten* gene into a PTEN-deficient human glioblastoma cell line, U87MG, by using recombinant retroviruses [3]. Expression of PTEN led to the selective accumulation of p27 [3] (Figure 1a), while the levels of cyclin E were not affected by PTEN status (Figure 1a). Expression of either a PTEN lipid phosphatase–inactive mutant, G129E [8, 9], or the catalytically inactive C124S mutant [3] failed to induce $p27^{KIP1}$ (Figure 1a). The treatment of U87 cells with a specific pharmacologic inhibitor for PI 3-kinase, LY294002, also led to the selective accumulation of p27 (Figure 1a). The specific effect of PTEN on PIP3 was further confirmed by the reduced phosphorylation of Akt, a downstream signaling molecule of the PI 3-kinase pathway (Figure 1a).

Accumulation of p27 could occur at the levels of either mRNA or protein. Northern-blot analysis indicates that PTEN and PI 3-kinase regulate p27 at a posttranscriptional level (Figure 1b). Characterization of p27 levels in the presence of cycloheximide or pulse-chase experiments revealed that p27 protein stability is increased in the cells expressing PTEN (Figure 1c,d). Recent studies have shown that the protein stability of p27 is regulated by the SCF^{SKP2} ubiquitin E3 ligase complex [5–7]. Examination of the levels of SKP1, SKP2, and CUL-1 of the SCF^{SKP2} complex indicates that SKP2, the substrate-targeting subunit of SCF^{SKP2}, was markedly downregulated by the expression of wild-type PTEN or by exposure to the PI 3-kinase inhibitor LY294002 (Figure 1a). Consistently, there was a significant increase of the SKP2 level in $Pten^{-/-}$ mouse ES cells as compared with its isogenic $Pten^{+/+}$ counterpart (Figure 1a). In contrast, the levels of SKP1 or CUL-1 remained unaffected by the PTEN status (Figure 1a). Northern blot analysis further indicated that the mRNA level of SKP2 was greatly reduced in PTEN-expressing U87 cells or in cells treated with LY294002 (Figure 1b). These data suggest that PTEN-mediated accumulation of p27 is likely caused by the specific inhibition of SKP2 expression through downregulation of the PI 3-kinase pathway.

To determine whether SKP2 is a critical target for the PTEN pathway that regulates $p27^{KIP1}$ levels, we tested whether expression of SKP2 in U87 cells is sufficient to restore p27 levels when PTEN is present. As shown in Figure 2a, while PTEN induced high levels of p27, expression of SKP2 together with PTEN completely sup-

Figure 1

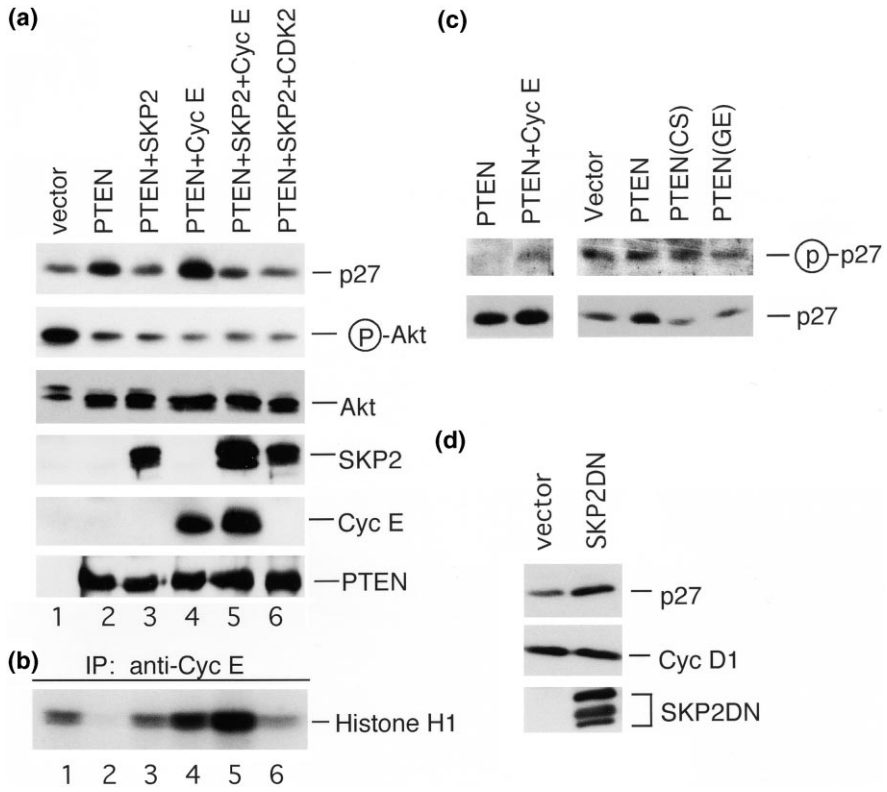


PTEN and PI 3-kinase regulate the levels of p27 and SKP2. **(a)** U87-EcoR cells were infected with retroviruses carrying either an empty vector, wild-type PTEN cDNA, PTEN C124S (CS), or G129E (GE) mutants (lanes 1–4) and were harvested at 48 hr post-infection. Alternatively, U87-EcoR cells were treated with vehicle DMSO or LY294002 (20 μM) for 24 hr (lane 5 and 6). In lanes 7 and 8, lysates were prepared from actively growng *Pten*$^{+/+}$ and *Pten*$^{-/-}$ mouse ES cells. Cell lysates (50 μg each) were examined for the indicated cell cycle regulators by Western blot analysis with the corresponding antibodies. **(b)** U87-EcoR cells were infected with retroviruses carrying an empty vector or PTEN, or they were treated with vehicle DMSO or LY294002 (20 μM). Total RNA (10 μg) from each sample was subjected to Northern blot analysis with the indicated probes. **(c,d)** U87-EcoR cells were infected with vector control viruses or PTEN viruses for 48 hr. (c) Cells were then treated with 50 μg/ml cycloheximide (CYH) for the indicated times, and cell lysates (50 μg each) were examined by Western blot analysis with an anti-p27 antibody. (d) Alternatively, cells were pulse labeled with [^{35}S]methionine for 1 hr and were then chased in the regular medium supplemented with 7.5 μg/ml methionine for 0, 1, 2, or 4 hr. Cell lysates were subjected to immunoprecipitation with an anti-p27 antibody, and the immunoprecipitates were analyzed by SDS-PAGE and fluorography.

pressed the PTEN-induced p27 accumulation. On the other hand, Akt phosphorylation was still inhibited by PTEN, either in the presence or absence of SKP2, and this finding suggests that PIP3 levels remained low. It has been shown that cyclin E/CDK2 can phosphorylate p27 on threonine 187 (Thr 187) [10, 11], and the Thr 187 phosphorylation is essential for the subsequent degradation of p27 [5–7, 10, 11]. In PTEN-expressing cells, p27 accumulation led to the inhibition of cyclin E–associated kinase activity (see below and Figure 2b), while the protein level of cyclin E was not affected by PTEN expression (Figure 1a). We found that ectopic expression of cyclin E failed to downregulate p27 in PTEN-expressing U87 cells (Figure 2a), although the expressed cyclin E resulted in the restoration of the cyclin E/CDK2 kinase activity that was originally inhibited by PTEN (see below and Figure 2b). This result suggests that cyclin E itself, in the absence of SKP2, is not sufficient to trigger the degradation of p27. Expression of CDK2 also failed to rescue the p27 downregulation defect in the PTEN-expressing cells (data not shown). In addition, expression of cyclin E or CDK2 did not further enhance the effect of SKP2 on the downregulation of p27 (Figure 2a). The observation that SKP2, but not cyclin E or CDK2, can reverse the effect of PTEN on p27 accumulation suggests that SKP2 is a rate-limiting factor in p27 ubiquitination and degradation and that PTEN regulates this process.

It is established that the critical function of p27^{KIP1} in the late G1 phase of the cell cycle is to inhibit the kinase activity of cyclin E/CDK2 and thus to prevent premature

Figure 2

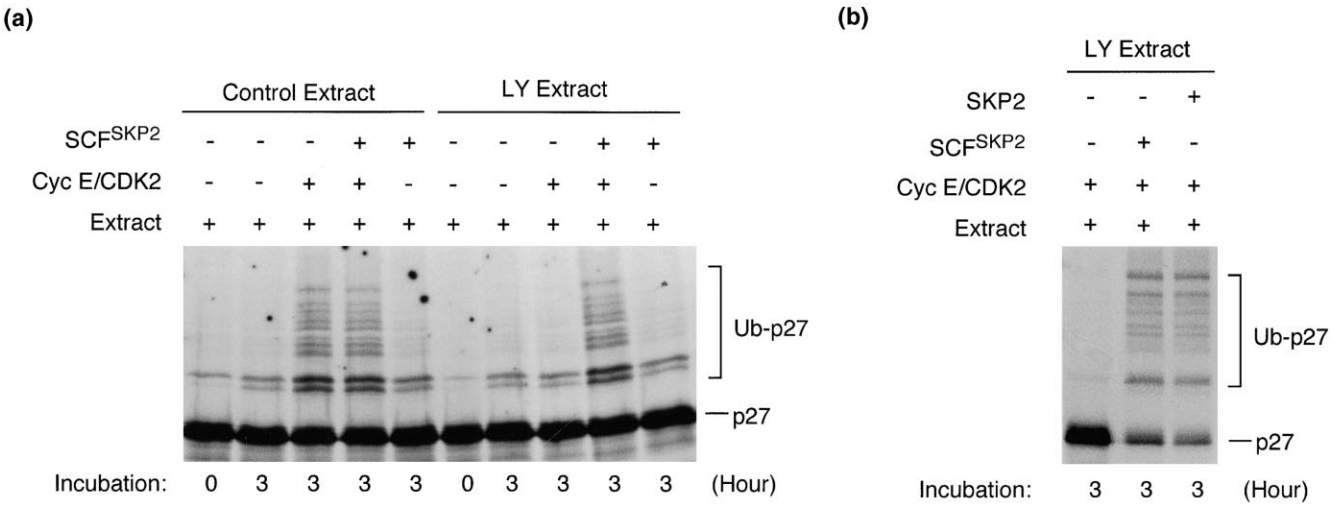


SKP2 suppresses p27 accumulation mediated by PTEN expression. **(a,b)** U87-EcoR cells were infected either alone with vector control viruses or PTEN viruses or together with retroviruses expressing SKP2, cyclin E or CDK2 in combinations as indicated. (a) Forty-eight hours postinfection, cells were harvested, and cell lysates (50 μg each) were subjected to Western blot analysis with the indicated antibodies. (b) Cell lysates (500 μg each) were subjected to immunoprecipitation with anti-cyclin E antibodies. We assayed the immunoprecipitated kinases for activities by using histone H1 as a substrate in the presence of ^{32}P-[γ]-ATP, and we processed them by using SDS-PAGE and autoradiography. **(c)** U87-EcoR cells were infected with retroviruses expressing PTEN or together with retroviruses expressing cyclin E. Alternatively, cells were infected with retroviruses carrying either an empty vector, wild-type PTEN, PTEN C124S (CS), or G129E (GE) mutants. Cell lysates (50 μg each) were examined by Western blot analysis with an antibody specific for the T187-phosphorylated p27 or with a regular anti-p27 antibody. **(d)** U87-EcoR cells were infected with retroviruses carrying either an empty vector or SKP2DN. Cells were harvested 48 hr postinfection, and cell lysates (50 μg each) were subjected to Western blot analysis with the indicated antibodies.

entry into S phase [12]. We examined whether SKP2-mediated rescue of p27 degradation in PTEN-expressing cells is sufficient to restore the cyclin E/CDK2 kinase activity. Indeed, while PTEN expression led to the inhibition of cyclin E/CDK2 kinase activity, coexpression of SKP2 fully relieved such an inhibition (Figure 2b). When cyclin E was coexpressed with PTEN, the cyclin E/CDK kinase activity was higher than that in the vector control cells, as one would expect if the ectopically expressed cyclin E were not completely inhibited by the endogenous p27 (Figure 2b). When SKP2 and cyclin E were coexpressed together with PTEN, a further increase of cyclin E/CDK2 kinase activity was observed, as compared with that of expressing SKP2 or cyclin E alone in the presence of PTEN (Figure 2b). This result suggests that SKP2 can further activate the cyclin E/CDK2 activity, likely through its effect on p27 degradation. In addition, SKP2 can similarly fully suppress p27 accumulation in cells treated with the PI 3-kinase inhibitor LY294002 and can recover cyclin E/CDK2 kinase activity in these cells (See Supplementary material, Figure S1, available with this article on the internet). The ability of SKP2 to fully rescue the cyclin E/CDK2 kinase activity in the PTEN-expressing cells or LY294002-treated cells suggests that SKP2-mediated p27 downregulation in these cells plays an important role in the activation of cyclin E/CDK2.

Previous studies indicate that SCF^{SKP2}-mediated p27 degradation is dependent on the presence of phosphorylated Thr 187 in p27 and that cyclin E/CDK2 can phosphorylate Thr 187 in p27 [5–7, 10, 11]. Our finding that SKP2 expression alone is sufficient to rescue the p27 degradation defect in PTEN-expressing cells suggests that the Thr 187 residue of p27 is still phosphorylated even when the kinase activity of cyclin E/CDK2 is inhibited. To examine the Thr 187 phosphorylation status of p27, we used an antibody that is specific for Thr 187–phosphorylated p27 [7]. To confirm the specificity of this antibody, we coexpressed cyclin E with PTEN. We reasoned that under this condition, Thr 187–phosphorylated p27 should increase as a result of the restored cyclin E/CDK2 kinase activity (Figure 2b). Indeed, we found that there was a higher level of the Thr 187–phosphorylated p27 when cyclin E was coexpressed with PTEN than when PTEN was expressed alone (Figure 2c). Using this antibody, we observed that in the PTEN-expressing cells, the levels of Thr 187–phosphorylated p27 were similar to those in the vector or the PTEN mutant controls (Figure 2c) even though cyclin E/CDK2 kinase activity was nearly completely inhibited when PTEN was present (see Figure 2b). These results raise the possibility that other kinase(s), in addition to cyclin E/CDK2, may be involved in the phosphorylation of p27. The steady-state levels of

Figure 3



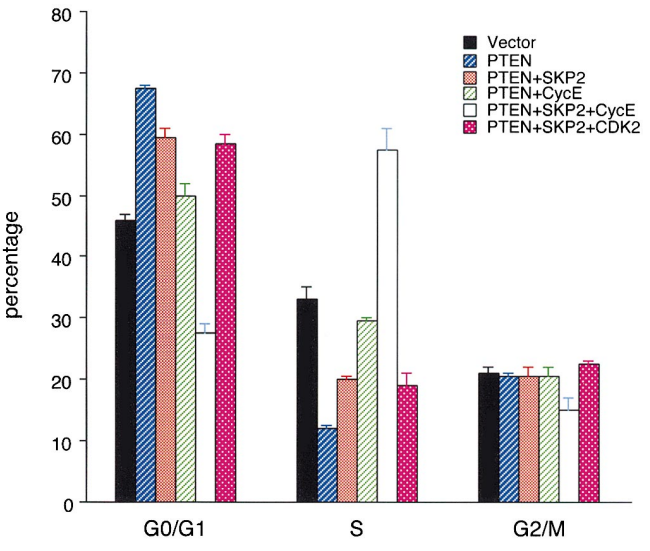
Inhibition of PI 3-kinase affects p27 ubiquitination. Cell extracts were prepared from actively growing U87-EcoR cells (control extract) or cells that had been treated with LY294002 for 24 hr (LY extract). [^{35}S]p27 was incubated with the corresponding extracts at 30°C for 0 or 3 hr in the presence of ATP regeneration system and the ubiquitin analogs ubiquitin aldehyde and methylated ubiquitin. Where indicated, the purified recombinant cyclin E/CDK2 complex, the SCF^{SKP2} complex, or the SKP2 protein itself were also added to the reactions. The reaction products were immunoprecipitated with anti-p27 antibodies and were analyzed by SDS-PAGE and fluorography.

the phosphorylated p27 in cells are thus a combination of the activities of these kinases.

To further determine the function of the endogenous SKP2 in the regulation of p27 in U87 cells, we expressed a dominant negative form of SKP2 (SKP2DN) that contains a deletion in the F box region known to be involved in the binding of SKP2 to the SKP1/Cul-1 complex [7]. Expression of SKP2DN in U87 cells led to accumulation of endogenous p27, while the levels of cyclin D were not affected (Figure 2d). These results further strengthen the notion that downregulation of SKP2 levels by PTEN expression is mechanistically linked to the p27 accumulation in these cells.

To further investigate whether PTEN regulates the ubiquitin-dependent degradation of p27 through the SCF^{SKP2} complex, we examined the consequences of PI 3-kinase inhibition in an in vitro p27 ubiquitination assay [5]. As shown in Figure 3a, while the extracts prepared from actively growing U87 cells (control extract) readily ubiquitinated p27 in the presence of cyclin E/CDK2, the extracts prepared from LY294002-pretreated cells (LY extracts) had very little ubiquitination activity under the same conditions (Figure 3a). The addition of the purified recombinant SCF^{SKP2} complex or SKP2 alone fully restored p27 ubiquitination activity in the LY extracts (Figure 3a,b). These experiments suggest that PTEN and the PI 3-kinase signaling pathway function through SKP2, a key component of the SCF^{SKP2} E3 ligase, to regulate the ubiquitin-dependent proteolysis of p27.

Figure 4



Rescue of the S phase entry defect in PTEN-expressing cells by SKP2. U87-EcoR cells were infected alone with vector control viruses or PTEN viruses or together with retroviruses expressing SKP2, cyclin E, or CDK2 in combinations as indicated. Fifty-seven hours postinfection, cells were pulse labeled with BrdU for 3 hr and were then harvested for the cell cycle profile with the fluoresence-activated cell-sorting analysis. The percentages (vertical axis) of cells in G0/G1, S, or G2/M phases (horizontal axis) in the corresponding samples are presented. The data shown are averages of a duplicate set of experiments, and the standard deviations are indicated.

We have shown previously that ectopic expression of PTEN blocks the G1-to-S-phase cell cycle progression, which is accompanied by the increase of $p27^{KIP1}$ [3]. To determine the contribution of p27 accumulation in PTEN-mediated G1 cell cycle arrest, we examined whether the rescue of the p27 degradation defects by SKP2 is sufficient to allow cells to enter S phase. As shown in Figure 4, PTEN expression in U87 cells reduced the S phase population from 33% to 12%, with a concomitant increase of the G1 phase population. Coexpression of SKP2 and PTEN partially rescued the PTEN-mediated G1 phase arrest, while the S phase population increased to 20%. Coexpression of cyclin E and PTEN rescued the PTEN-mediated G1 arrest and increased the S phase population to 30%, although p27 levels remained high under such conditions (Figure 2). The coexpression of SKP2 and cyclin E has a more profound effect on promoting S phase entry than does the expression of either SKP2 or cyclin E alone, with 58% of cells in the S phase even when PTEN was present. The partial rescue of S phase by SKP2 suggests that the induction of SKP2 by the PI 3-kinase pathway constitutes an important, but not the only, event required for the G1-to-S-phase cell cycle transition. Since high levels of cyclin E/CDK2 kinase activity can effectively promote S phase entry in PTEN-expressing cells, it suggests that there may be additional PI 3-kinase signaling events involved in cyclin E/CDK2 activation. Alternatively, the increased cyclin E/CDK2 kinase activity may substitute for functions of other kinases to promote G1/S transition. In summary, our findings suggest that SKP2 functions as a critical component in the PTEN/PI 3-kinase pathway for the regulation of $p27^{KIP1}$ and cell proliferation.

Supplementary material

Supplementary materials and methods as well as a supplementary figure are available with the electronic version of this article at http://www.current-biology.com/supmatin.htm.

Acknowledgements

We thank Dr. Toshio Kitmura at the University of Tokyo and Dr. Garry Nolan at Stanford University for kindly providing the pMX plasmid vector and Phenix cells, respectively. This work was supported by grants from the National Institute of Cancer, National Institutes of Health, awarded to H. S. (RO1 CA77695) and H. Z. (R01 CA72828). H. W. is an Assistant Investigator of the Howard Hughes Medical Institute. H. S. was a Pew Scholar in Biomedical Sciences.

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