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Skp2-Mediated Degradation of p27 Regulates Progression into Mitosis

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Summary

Although Skp2 has been thought to mediate the degradation of p27 at the G_1-S transition, *Skp2*$^{-/-}$ cells exhibit accumulation of p27 in S-G_2 phase with overreplication. We demonstrate that *Skp2*$^{-/-}$*p27*$^{-/-}$ mice do not exhibit the overreplication phenotype, suggesting that p27 accumulation is required for its development. Hepatocytes of *Skp2*$^{-/-}$ mice entered the endoduplication cycle after mitogenic stimulation, whereas this phenotype was not apparent in *Skp2*$^{-/-}$*p27*$^{-/-}$ mice. Cdc2-associated kinase activity was lower in *Skp2*$^{-/-}$ cells than in wild-type cells, and a reduction in Cdc2 activity was sufficient to induce overreplication. The lack of p27 degradation in G_2 phase in *Skp2*$^{-/-}$ cells may thus result in suppression of Cdc2 activity and consequent inhibition of entry into M phase. These data suggest that p27 proteolysis is necessary for the activation of not only Cdk2 but also Cdc2, and that Skp2 contributes to regulation of G_2-M progression by mediating the degradation of p27.

Introduction

The highly ordered progression of the cell cycle is achieved by a series of elaborate mechanisms that control the periodic expression of many regulatory proteins. One such regulatory protein is the Cdk inhibitor (CKI) p27. In normal cells, the amount of p27 is high during G_0 phase but rapidly decreases on the reentry of cells into G_1-S. Moreover, we and others have demonstrated that *p27*$^{-/-}$ mice are larger than normal mice and exhibit both multiple organ hyperplasia and a predisposition to the development of tumors (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996).

The intracellular concentration of p27 is thought to be regulated predominantly by the ubiquitin-mediated proteolytic pathway (Pagano et al., 1995; Shirane et al., 1999). Degradation of p27 is promoted by its phosphorylation on Thr[187] by the cyclin E-Cdk2 complex (Sheaff et al., 1997; Vlach et al., 1997; Montagnoli et al., 1999). Skp2, an F box protein that functions as the receptor component of an SCF ubiquitin ligase complex, binds to p27 only when Thr[187] of this CKI is phosphorylated; such binding then results in the ubiquitylation and degradation of p27 (Carrano et al., 1999; Sutterluty et al., 1999; Tsvetkov et al., 1999). Skp2 also targets free cyclin E (not that complexed with Cdk2) for ubiquitylation (Nakayama et al., 2000). These biochemical observations are supported by genetic evidence showing that both p27 and free cyclin E accumulate to high levels in the cells of mice that lack Skp2 (Nakayama et al., 2000, 2001). The most obvious cellular phenotype of *Skp2*$^{-/-}$ mice is the presence of markedly enlarged, polyploid nuclei and multiple centrosomes, suggestive of an impairment in the mechanism that prevents endoreplication, in which the genomic DNA content of a cell is increased without cell division. In addition to p27 and free cyclin E, several other substrates have been proposed for Skp2. However, some of these potential substrates were found not to accumulate in cells from *Skp2*$^{-/-}$ mice, suggesting either that they are not bona fide Skp2 substrates or that functional redundancy allows for their ubiquitylation in the absence of Skp2.

Skp2 is almost undetectable or expressed at a low level in G_0 and early to mid-G_1 phase. It begins to accumulate during late G_1 phase, and its abundance is maximal during S and G_2 phases (Hara et al., 2001). The onset of Skp2 expression is unequivocally later than that of the degradation of p27 apparent at G_0-G_1. Moreover, p27 is exported from the nucleus to the cytoplasm at G_0-G_1 (Rodier et al., 2001; Ishida et al., 2002; Connor et al., 2003), whereas Skp2 is restricted to the nucleus (Miura et al., 1999). The discrepancies between the temporal and spatial patterns of p27 expression and those of Skp2 expression suggest the existence of an Skp2-independent pathway for the degradation of p27 at the G_0-G_1 transition. Indeed, the downregulation of p27 at G_0-G_1 occurs normally in *Skp2*$^{-/-}$ cells, but that in S and G_2 phases is impaired (Hara et al., 2001). These findings suggest that the major role of Skp2 might be to reduce the concentration of p27 during S and G_2 phases rather than at late G_1 phase.

To determine whether the accumulation of p27 is essential for the polyploidy and centrosome overduplication in *Skp2*$^{-/-}$ cells, we generated double mutant mice that lack both Skp2 and p27 genes. We now show that, although cyclin E accumulates in the cells of *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice, the markedly enlarged, polyploid

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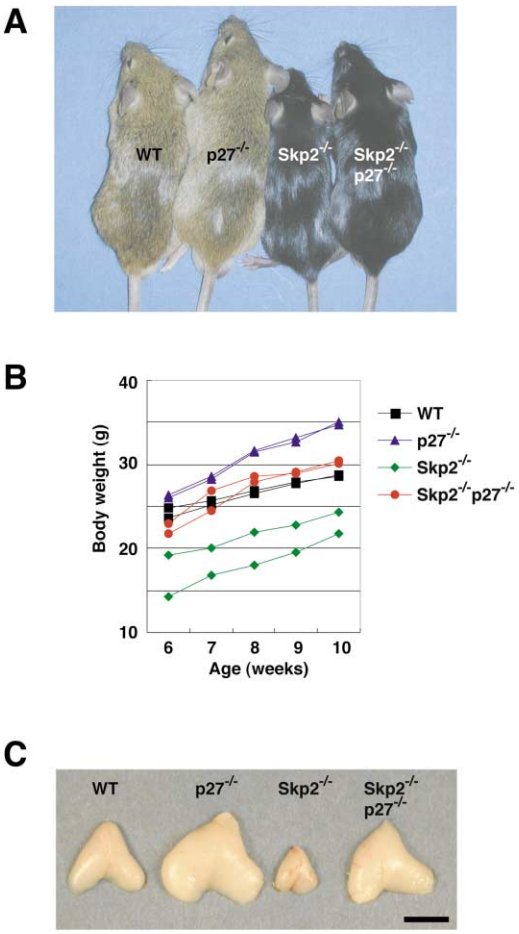

Figure 1. Body and Thymus Size in *Skp2*$^{-/-}$ *p27*$^{-/-}$ Mice

(A) Representative male wild-type (WT), *p27*$^{-/-}$, *Skp2*$^{-/-}$, and *Skp2*$^{-/-}$ *p27*$^{-/-}$ littermates at 8 weeks of age.

(B) Representative growth curves for male wild-type, *p27*$^{-/-}$, *Skp2*$^{-/-}$, and *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice. Similar differences in body weight were apparent for female mice of the various genotypes (data not shown).

(C) Gross appearance of the thymus of male wild-type, *p27*$^{-/-}$, *Skp2*$^{-/-}$, and *Skp2*$^{-/-}$ *p27*$^{-/-}$ littermates at 8 weeks of age. Scale bar, 5 mm.

nuclei and multiple centrosomes associated with Skp2 deficiency are not evident in the double mutant mice. These data suggest that accumulation of p27 is primarily responsible for this cellular phenotype of *Skp2*$^{-/-}$ mice. We also demonstrate that the aberrant increase in p27 expression in *Skp2*$^{-/-}$ cells results in inhibition of the kinase activity of Cdc2 and a consequent block of entry into M phase. Our results thus indicate that Skp2 plays a crucial role in regulation of G_2-M progression by contributing to the ubiquitylation-mediated proteolysis of p27.

Results

Generation of Mice Lacking Both Skp2 and p27

To generate mice lacking Skp2 and p27, we crossed *Skp2*$^{+/-}$ *p27*$^{+/-}$ animals. As we previously described (Nakayama et al., 1996, 2000), the body size of *p27*$^{-/-}$ animals is larger and that of *Skp2*$^{-/-}$ mice is smaller than that of wild-type controls (Figures 1A and 1B). The body size of the *Skp2*$^{-/-}$ *p27*$^{-/-}$ double mutant was slightly larger than that of wild-type controls. We also examined the size of the thymus, which is one of the most affected organs in *Skp2*$^{-/-}$ or *p27*$^{-/-}$ mice; it is hyperplastic in *p27*$^{-/-}$ mice and atrophic in *Skp2*$^{-/-}$ mice (Figure 1C). As with body size, the thymus of *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice was slightly larger than that of wild-type animals. In general, the *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice appeared similar to *p27*$^{-/-}$ mice and exhibited phenotypes opposite to those of *Skp2*$^{-/-}$ mice. The observation that the *Skp2*$^{-/-}$ *p27*$^{-/-}$ double mutant appears similar but not identical to the *p27*$^{-/-}$ single mutant constitutes genetic evidence for the notion that, although p27 is the main target of Skp2, Skp2 may also mediate the ubiquitylation of other substrates.

Absence of Overreplication Phenotype in *Skp2*$^{-/-}$ *p27*$^{-/-}$ Mice

The most obvious cellular phenotype of *Skp2*$^{-/-}$ mice is the presence of markedly enlarged, polyploid nuclei and multiple centrosomes (Nakayama et al., 2000). To determine whether these characteristics are dependent on p27 accumulation, we examined liver, kidney, and lung cells of wild-type, *Skp2*$^{-/-}$, *p27*$^{-/-}$, and *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice (Figures 2A–2L). As previously described, the nuclei of hepatocytes, renal tubule cells, and bronchiolar epithelial cells of *Skp2*$^{-/-}$ mice were substantially larger than those of the corresponding cells in wild-type littermates. Such nuclear enlargement was not apparent in the cells of *p27*$^{-/-}$ or *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice. Flow cytometry also revealed that the DNA content of hepatocytes from *Skp2*$^{-/-}$ mice ranged from 2C to 16C, whereas that of most hepatocytes from wild-type or *p27*$^{-/-}$ animals was 2C or 4C (Figures 2M–2O). The percentage of polyploid cells in *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice was greatly reduced compared with that in *Skp2*$^{-/-}$ mice, although the double mutant did exhibit a small increase in the number of 8C cells relative to that in wild-type animals (Figure 2P).

With the use of fluorescence microscopy, we also examined the nuclear size (as revealed by Hoechst 33258 staining) and centrosome number (as revealed by immunostaining with antibodies to pericentrin) of cultured mouse embryonic fibroblasts (MEFs) derived from the various mice. As with liver, kidney, and lung cells, MEFs derived from *Skp2*$^{-/-}$ mice exhibited a markedly enlarged nucleus, whereas the nuclear size of *Skp2*$^{-/-}$ *p27*$^{-/-}$ MEFs was similar to that of wild-type MEFs (Figures 2Q–2S). The number of centrosomes, which was increased in the *Skp2*$^{-/-}$ MEFs, also appeared normal in the *Skp2*$^{-/-}$ *p27*$^{-/-}$ cells (Figures 2T–2W). These observations indicate that the cellular phenotype of *Skp2*$^{-/-}$ mice is dependent on the presence of an intact p27 gene.

Mice lacking p27 manifest multiple organ hyperplasia, retinal dysplasia, and pituitary tumors (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). The most hyperplastic organs in these animals are the thymus, testis, ovary, adrenal medulla, and intermediate lobe of the pituitary gland. Histopathologic examination revealed that the adrenal medulla of *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice is as hyperplastic as is that of *p27*$^{-/-}$ mice, whereas that of *Skp2*$^{-/-}$ mice appeared hypoplastic (Figures 3A–3D). Similarly, the intermediate lobe of the pituitary, a vestigial structure in adult humans, was hyperplastic in both

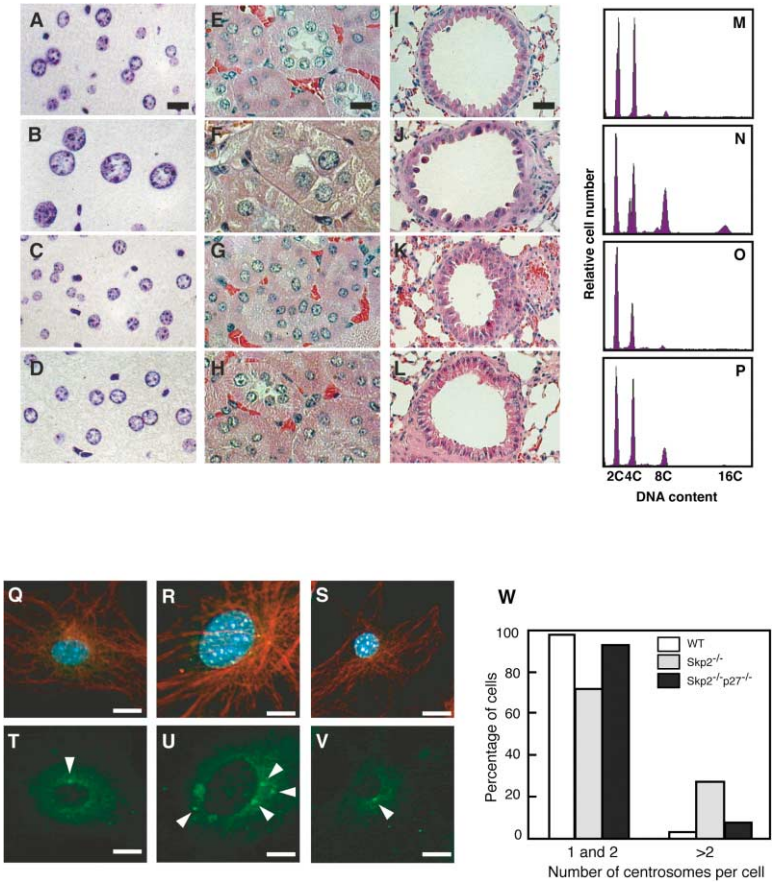

Figure 2. Absence of Nuclear Enlargement and Polyploidy in *Skp2*$^{-/-}$ *p27*$^{-/-}$ Mice

(A–L) Histological analysis of liver (A–D), renal tubules (E–H), and bronchioles (I–L) of adult wild-type (A, E, and I), *Skp2*$^{-/-}$ (B, F, and J), *p27*$^{-/-}$ (C, G, and K), and *Skp2*$^{-/-}$ *p27*$^{-/-}$ (D, H, and L) mice. Sections were stained with Feulgen solution (A–D) or with hematoxylin-eosin (E–L). Scale bars, 25 μm.

(M–P) Flow cytometric analysis of the DNA content of hepatocytes from wild-type (M), *Skp2*$^{-/-}$ (N), *p27*$^{-/-}$ (O), and *Skp2*$^{-/-}$ *p27*$^{-/-}$ (P) mice.

(Q–W) Normal nuclear size and centrosome number of *Skp2*$^{-/-}$ *p27*$^{-/-}$ cells. MEFs derived from wild-type (Q and T), *Skp2*$^{-/-}$ (R and U), and *Skp2*$^{-/-}$ *p27*$^{-/-}$ (S and V) embryos were stained either with both antibodies to β-tubulin and Hoechst 33258 (Q–S) or with antibodies to pericentrin alone (T–V). β-tubulin and pericentrin immune complexes are represented by red and green staining, respectively. Centrosomes are indicated by arrowheads. Blue staining represents Hoechst 33258 labeling of nuclear DNA. Scale bars, 10 μm. The percentages of MEFs either with one or two or with more than two centrosomes were determined by analysis of 400 cells per genotype (W).

p27$^{-/-}$ and *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice (Figures 3E–3H). The intermediate lobe of these animals contained a large number of atypical cells; both *p27*$^{-/-}$ and *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice were thus diagnosed with benign pituitary adenoma. Furthermore, histopathologic examination of the retina of *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice revealed a marked disorganization of the cellular layers in the neural retina, similar to that apparent in *p27*$^{-/-}$ mice (Figures 3I–3L). These observations demonstrate that *Skp2*$^{-/-}$ *p27*$^{-/-}$ double mutant mice preserve the phenotypes of *p27*$^{-/-}$ mice. They therefore constitute genetic evidence in support of the notion that Skp2 is an upstream regulator of p27, although slight differences between *p27*$^{-/-}$ and *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice may reflect other possible functions of Skp2.

Impaired Entry into M Phase in *Skp2*$^{-/-}$ Cells

We hypothesized that the nuclear enlargement, polyploidy, and centrosome overduplication apparent in *Skp2*$^{-/-}$ cells result from reentry of the cells into S phase without passage through M phase. To test this hypothesis, we orally administered the female hormone estriol, which transiently stimulates hepatocyte proliferation (Fujii et al., 1985), to adult wild-type, *Skp2*$^{-/-}$, *p27*$^{-/-}$, and *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice. The giant cells observed in the liver of *Skp2*$^{-/-}$ mice were shown to have entered S phase by their incorporation of 5-bromo-2′-deoxyuridine (BrdU) that was injected intraperitoneally (Figures 4A and 4B). Monitoring of the time-dependent increase in the percentage of cells in S phase revealed no marked difference between wild-type and *Skp2*$^{-/-}$ mice (Figure 4C). Mitotic cells in the liver were evaluated by hematoxylin-eosin staining (data not shown) and immunostaining with antibodies to phosphorylated histone H3 (Figures 4D–4G), which is a marker for cells in M phase. In wild-type mice, cells containing phosphorylated histone H3 were apparent and peaked in number 5 days after estriol administration (Figure 4H). In contrast, no cells that reacted with the antibodies to the phosphorylated histone were detected at any time after estriol treatment in *Skp2*$^{-/-}$ mice. This lack of M phase induction in response to estriol appeared to be attributable to p27 accumulation, given that the liver of *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice responded in a manner similar to that of the liver of wild-type mice (Figure 4G). These results thus suggested that *Skp2*$^{-/-}$ cells are able to enter S phase but not M phase, a characteristic of endoreplication, although a mitotic defect could also account for this abnormality. They also indicate that the inability of *Skp2*$^{-/-}$ cells to enter M phase is due to the abnormal accumulation of p27.

Impairment of Mitotic Entry Induced by a Reduction in Cdc2 Activity

In fission yeast, overexpression of the CKI Rum1 inhibits mitotic cyclin-Cdc2 activity and thereby prevents mitosis (Correa-Bordes and Nurse, 1995). Moreover, a high activity of the mitotic cyclin-Cdc2 complex prevents chromosome replication (Stern and Nurse, 1996). These observations led us to test the hypothesis that accumulation of the CKI p27 during G_2 phase inhibits mitotic cyclin-Cdc2 activity in mammalian *Skp2*$^{-/-}$ cells.

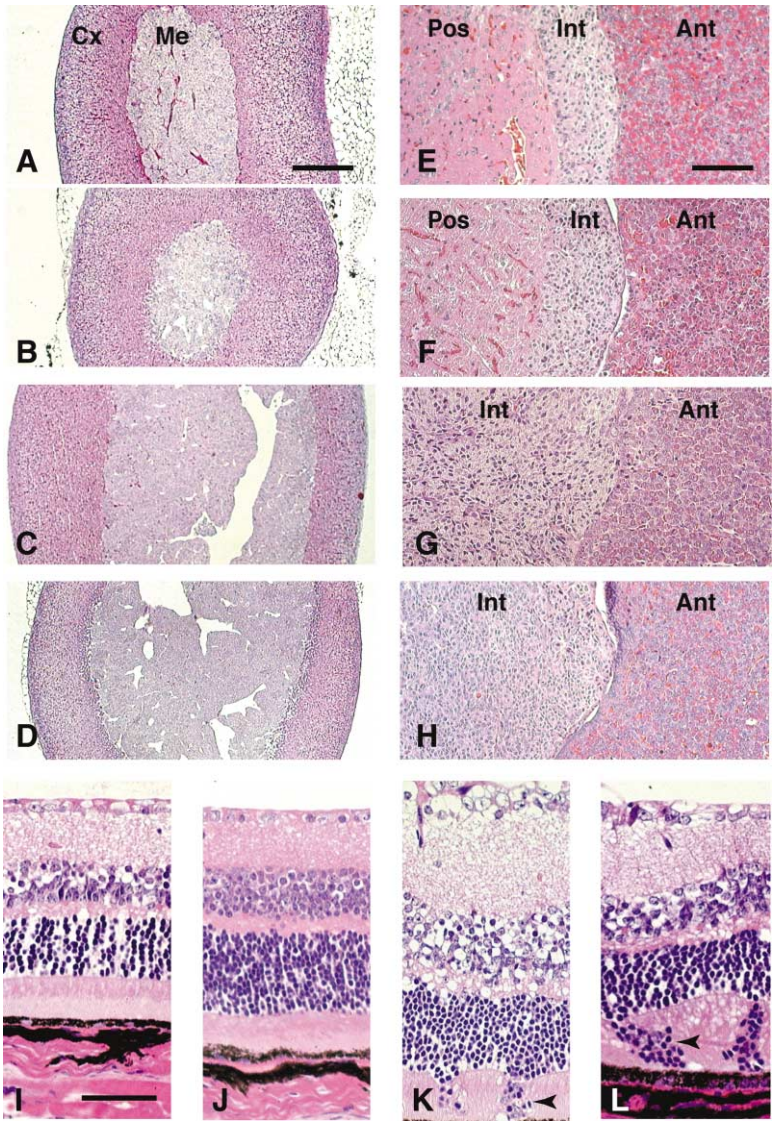

Figure 3. Organ Hyperplasia in *Skp2*$^{-/-}$ *p27*$^{-/-}$ Mice

Histological sections of the adrenal gland (A–D), pituitary gland (E–H), and retina (I–L) of wild-type (A, E, and I), *Skp2*$^{-/-}$ (B, F, and J), *p27*$^{-/-}$ (C, G, and K), and *Skp2*$^{-/-}$ *p27*$^{-/-}$ (D, H, and L) mice are shown. The paraffin-embedded sections were stained with hematoxylin-eosin. Abbreviations: Cx, cortex; Me, medulla; Ant, anterior lobe; Int, intermediate lobe; Pos, posterior lobe. Arrowheads in (K) and (L) indicate the outer granular layer invading the layer of rods and cones beyond the outer limiting membrane of the retina. Scale bars: 400 μm (A–D), 100 μm (E–H), and 25 μm (I–L).

We compared the abundance (Figure 5A) and kinase activities (Figure 5B) of various cell cycle regulators in MEFs derived from wild-type, *Skp2*$^{-/-}$, *p27*$^{-/-}$, and *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice. There were no marked differences in the amounts of cyclin A, cyclin B, Cdk2, or Cdc2 or in the kinase activity associated with Cdk2 among the four types of cells. In contrast to lymphocytes, which express p27 but not p57 (Nagahama et al., 2001), and in which the loss of p27 results in a marked increase in the kinase activity of Cdk2 (Nakayama et al., 1996), *p27*$^{-/-}$ MEFs did not exhibit an increase in such activity, probably because of the presence of p57. Cyclin A-, cyclin B-, or Cdc2-associated kinase activity was substantially reduced in *Skp2*$^{-/-}$ cells compared with that in MEFs of the other three genotypes, however. The observation that *Skp2*$^{-/-}$ *p27*$^{-/-}$ MEFs differed from *Skp2*$^{-/-}$ cells in this regard suggests that accumulation of p27 is responsible for the low kinase activity of cyclin A-Cdc2 or cyclin B-Cdc2 in *Skp2*$^{-/-}$ cells. Cyclin E-associated kinase activity was not increased, even though the abundance of cyclin E was increased, in the *Skp2*$^{-/-}$ cells, suggesting that the accumulated p27 antagonized the kinase activity. Consistent with this notion, the activity of cyclin E-associated kinase activity was increased in *Skp2*$^{-/-}$ *p27*$^{-/-}$ MEFs.

We found that p27 associated with Cdc2, although to only a small extent, in wild-type MEFs (Figure 5C). The amount of Cdc2 bound to endogenous p27 was markedly increased in *Skp2*$^{-/-}$ cells, however. The phosphorylation status of tyrosine-15 and threonine-161 of Cdc2 was similar among the four genotypes of MEFs (Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/6/5/661/DC1), excluding the possibility that the loss of Skp2 or accumulation of p27 affects other molecules that regulate Cdc2 activity directly. The amount of Cdk2 associated with p27 was also increased in *Skp2*$^{-/-}$ cells (Figure 5C). In contrast, the amount of Cdk4 associated with p27 was not affected by the loss of Skp2, which might be explained if the amount of Cdk4 is limited and most of it is bound to p27 even in wild-type cells; the excess p27 that overflows from the Cdk4-bound pool would then be available to bind to Cdk2 or

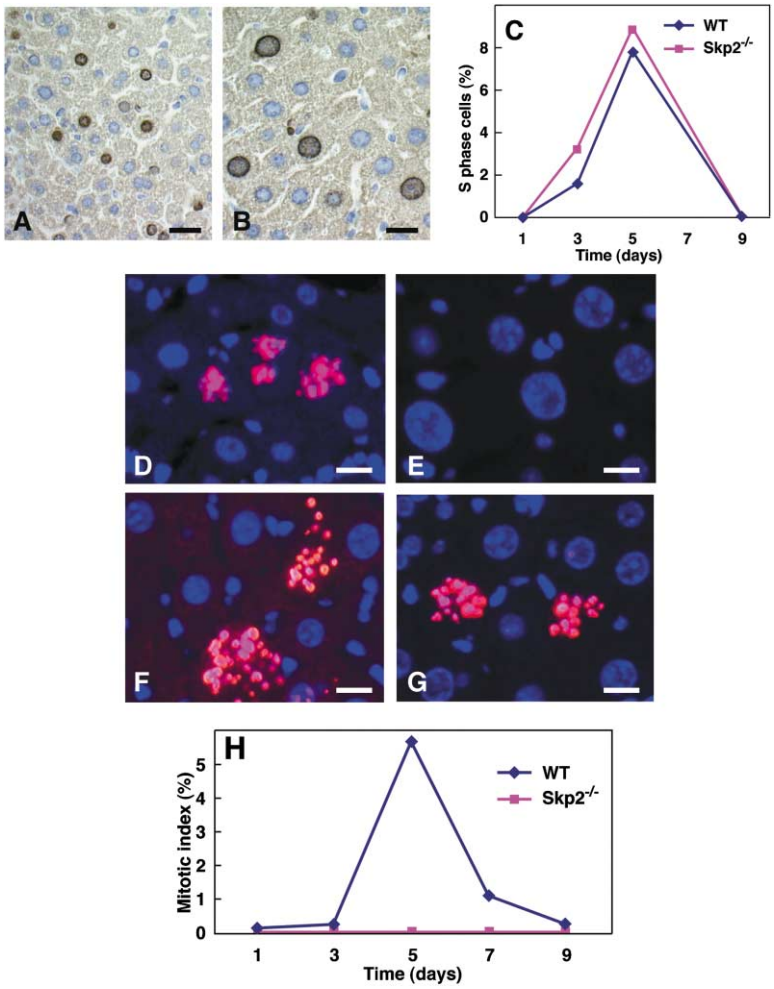

Figure 4. Defective Entry into M Phase of Hepatocytes Exposed to Mitogenic Stimulation in Adult *Skp2*$^{-/-}$ Mice

Adult mice were subjected to a single oral administration of estriol and daily intraperitoneal injection of BrdU. They were killed at various times after estriol administration and sections of the liver were processed for immunostaining.

(A and B) Liver sections prepared from wild-type (A) and *Skp2*$^{-/-}$ (B) mice 5 days after administration of estriol were subjected to immunostaining with antibodies to BrdU (brown).

(C) The percentage of liver cells that stained with the antibodies to BrdU was determined for wild-type and *Skp2*$^{-/-}$ mice at the indicated times after administration of estriol.

(D–G) Liver sections prepared from wild-type (D), *Skp2*$^{-/-}$ (E), *p27*$^{-/-}$ (F), and *Skp2*$^{-/-}$ *p27*$^{-/-}$ (G) mice 5 days after estriol administration were stained with antibodies to phosphorylated histone H3 (red) and Hoechst 33258 (blue).

(H) The percentage of liver cells that stained with the antibodies to phosphorylated histone H3 (mitotic index) was determined for wild-type and *Skp2*$^{-/-}$ mice at the indicated times after administration of estriol.

Data in (C) and (H) are from representative animals. Scale bars: 50 μm (A and B) and 25 μm (D–G).

Cdc2 in *Skp2*$^{-/-}$ MEFs. Immunodepletion of p27 also reduced the amounts of Cdc2 and Cdk2 in *Skp2*$^{-/-}$ cell lysates (data now shown), confirming that a substantial proportion of Cdc2 and Cdk2 is bound to endogenous p27. We also found that FLAG epitope-tagged exogenous p27 interacted with endogenous cyclin A, cyclin B, Cdk2, and Cdc2 as well as inhibited the associated kinase activities in COS-7 cells (Figure 5D). Mass spectrometric analysis of immunoprecipitates prepared from HEK293T cells expressing FLAG-tagged p27 with antibodies to FLAG showed that p27 is complexed with cyclin A2, cyclin B1, cyclin B2, cyclin E1, cyclin E2, Cdc2, Cdk2, Cdk4, and Cdk5 (data not shown).

We next examined whether the observed decrease in the kinase activity of Cdc2 is sufficient to explain the overreplication phenotype of *Skp2*$^{-/-}$ cells. We first treated wild-type MEFs with a potent Cdc2 inhibitor, butyrolactone I (Kitagawa et al., 1993). Exposure of the wild-type MEFs to butyrolactone I resulted in nuclear enlargement and centrosome multiplication (Figures 6A and 6B), characteristics similar to those of *Skp2*$^{-/-}$ MEFs. Flow cytometric analysis revealed that the DNA content of the butyrolactone I-treated cells increased in multiples of 2C (Figure 6C), a characteristic of endoreplication, rather than in a continuous manner, as would be consistent with DNA rereplication. Cell size was also increased by treatment with butyrolactone I (Figure 6D). Given that this compound may affect other types of Cdk, we also examined FT210 cells, which express a temperature-sensitive mutant of Cdc2 (Th'ng et al., 1990). FM210 cells cultured at the restrictive temperature (39°C) exhibited huge nuclei and multiple centrosomes (Figure 6H); neither FM210 cells cultured at the permissive temperature (32°C) (Figure 6G) nor the parental cell line, FM3A, cultured at either temperature (Figures 6E and 6F) exhibited this phenotype. Flow cytometric analysis revealed that FT210 cells cultured at the restrictive temperature, but not those cultured at the permissive temperature, appeared to be arrested at the G_2-M boundary (Figure 6I) with an increase in cell size (Figure 6J). These data thus indicate that a reduction in the kinase activity of Cdc2 results in G_2-M arrest associated with nuclear enlargement and centrosome overduplication in mammalian cells. In *Skp2*$^{-/-}$ mice, the accumulation of p27 due to the lack of its ubiquitylation-mediated proteolysis likely results in such a decrease in Cdc2 activity.

Accumulation of Cyclin E in *Skp2*$^{-/-}$ *p27*$^{-/-}$ Mice

Given that p27 is an inhibitor of the kinase activity of cyclin E-Cdk2 and that the phosphorylation of cyclin E that triggers its degradation is thought to be mediated,

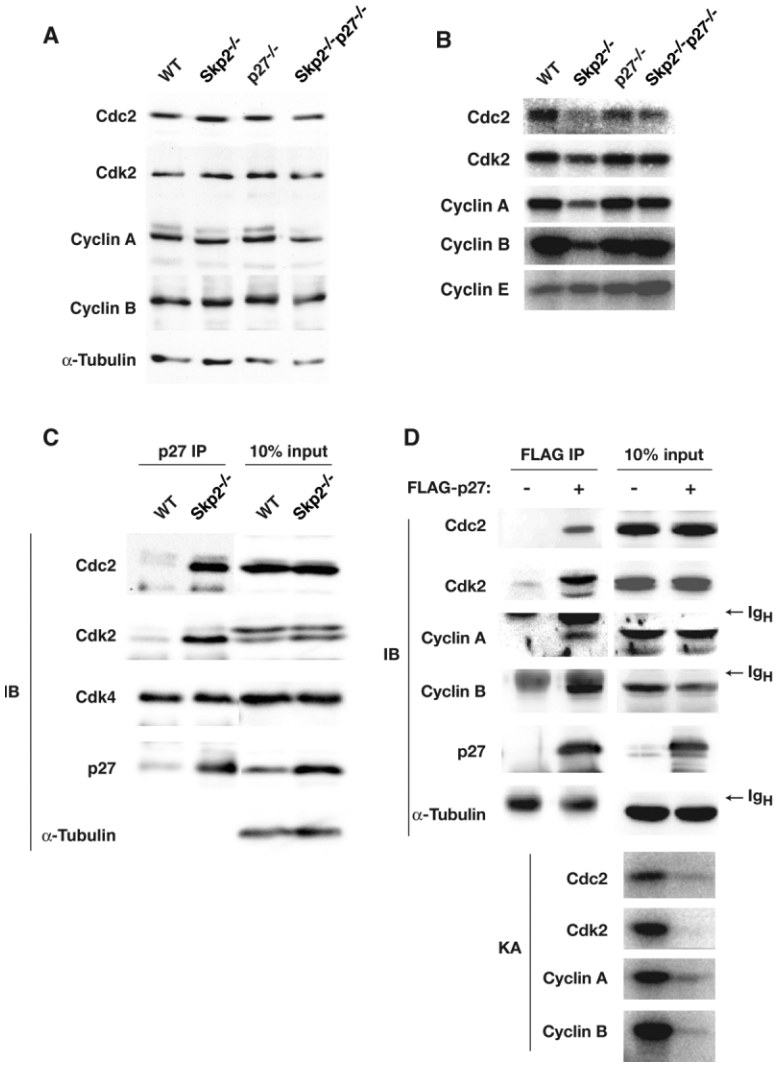

Figure 5. Reduced Cdc2-, Cyclin A-, or Cyclin B-Associated Kinase Activity in *Skp2*$^{-/-}$ Cells and Interaction of p27 with Cdc2, Cdk2, Cyclin A, and Cyclin B

(A and B) Lysates of MEFs from wild-type, *Skp2*$^{-/-}$, *p27*$^{-/-}$, and *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice were subjected either to immunoblot analysis with antibodies to the indicated proteins (A) or to in vitro assays of Cdc2-, Cdk2-, cyclin A-, cyclin B-, or cyclin E-associated kinase activity (B).

(C) Lysates of MEFs from wild-type and *Skp2*$^{-/-}$ mice were subjected to immunoprecipitation (IP) with antibodies to p27, and the resulting precipitates were subjected to immunoblot (IB) analysis with antibodies to the indicated proteins (left panels). A portion (10%) of the input lysates was also subjected directly to immunoblot analysis with the same antibodies (right panels).

(D) COS-7 cells were transfected with an expression vector for FLAG-tagged p27 (or with the empty vector) and with a vector for CD19, and CD19-expressing cells were then isolated with the use of magnetic beads conjugated with antibodies to CD19. Lysates of the CD19-positive cells were subjected to immunoprecipitation with antibodies to FLAG, and the resulting precipitates were subjected to immunoblot analysis with antibodies to the indicated proteins (left panels). Portions (10%) of the input lysates were also subjected directly to immunoblot analysis with the same antibodies (upper right panels) and to in vitro assays of kinase activity (KA) associated with the indicated proteins (lower right panels). Ig_H, immunoglobulin heavy chain.

at least in part, by associated Cdk2, it was possible that the accumulation of cyclin E in the cells of *Skp2*$^{-/-}$ mice was an indirect consequence of p27 accumulation. To investigate this possibility, we examined the amount of cyclin E in MEFs, thymus, testis, and liver of *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice. The abundance of cyclin E in these cells and tissues of the double mutant, however, was markedly greater than that in their wild-type counterparts and similar to that for *Skp2*$^{-/-}$ mice (Figure 7A), suggesting that the accumulation of cyclin E in *Skp2*$^{-/-}$ cells is not dependent on p27 accumulation. The amount of phosphorylated cyclin E in MEFs was similar for the four genotypes (Supplemental Figure S2A at http://www.developmentalcell.com/cgi/content/full/6/5/661/DC1), suggesting that the cyclin E that accumulates in *Skp2*$^{-/-}$ mice is the nonphosphorylated form. The p27-related CKIs p21 and p57, both of which are also targets of Skp2-mediated ubiquitylation (Bornstein et al., 2003; Kamura et al., 2003), were also examined for their binding to cyclin E in wild-type, *Skp2*$^{-/-}$, *p27*$^{-/-}$, and *Skp2*$^{-/-}$ *p27*$^{-/-}$ MEFs. Like p27, more p21 was associated with cyclin E in *Skp2*$^{-/-}$ MEFs than in wild-type MEFs (Supplemental Figure S2B); the increase in the amount of p21 bound to cyclin E was also observed in *Skp2*$^{-/-}$ *p27*$^{-/-}$ MEFs. Association of p57 with cyclin E was not detected in MEFs of any of the four genotypes (data not shown). We therefore are not able to exclude completely the possibility that accumulation of p21 or of other proteins results in inhibition of cyclin E-associated kinase activity and thereby stabilizes cyclin E by blocking its phosphorylation. However, such a possibility seems unlikely because cyclin E-associated kinase activity was not reduced in *Skp2*$^{-/-}$ MEFs (Figure 5B).

We also measured the rate of cyclin E degradation by treatment of MEFs with the protein synthesis inhibitor cycloheximide. In asynchronously cycling cells, cyclin E appeared to be more stable in *Skp2*$^{-/-}$ and *Skp2*$^{-/-}$ *p27*$^{-/-}$ cells than in wild-type or *p27*$^{-/-}$ cells (Figures 7B and 7C). This result was verified by pulse-chase experiments (Supplemental Figure S3). Furthermore, cyclin E was rapidly degraded after the release of wild-type and *p27*$^{-/-}$ MEFs from S phase arrest induced by aphidicolin treatment (Figures 7D and 7E); in contrast, the degradation of cyclin E after release from aphidicolin block was markedly delayed in *Skp2*$^{-/-}$ and *Skp2*$^{-/-}$ *p27*$^{-/-}$ MEFs. In addition, our previous biochemical

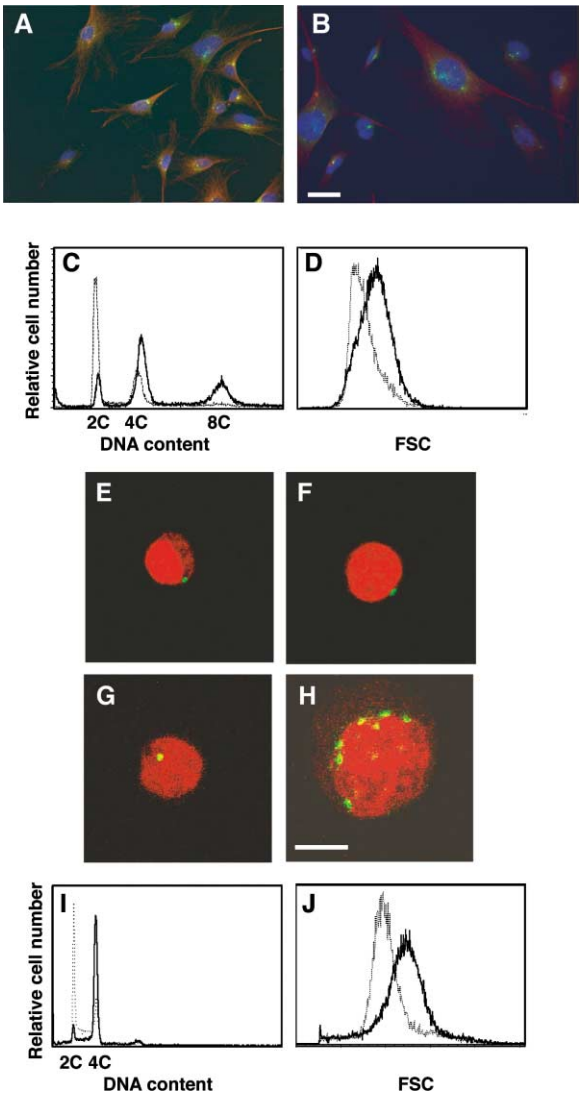

Figure 6. Induction of Nuclear Enlargement by Inhibition of the Kinase Activity of Cdc2

(A–D) Wild-type MEFs were incubated for 72 hr with dimethyl sulfoxide (vehicle control) (A) or with 80 μM butyrolactone I (B), after which they were subjected to immunostaining with antibodies to pericentrin (green) or to β-tubulin (red). Blue staining represents Hoechst 33258 labeling of nuclear DNA. Scale bar, 20 μm. The DNA content (C) and forward light scatter (FSC) (D) of the cells cultured in the absence (dotted lines) or presence (solid lines) of butyrolactone I were also measured by flow cytometry.

(E–J) FT210 cells (G and H), which express a temperature-sensitive mutant of Cdc2, and the parental FM3A cells (E and F) were cultured at 32°C (permissive temperature) (E and G) or 39°C (restrictive temperature) (F and H), after which they were subjected to immunostaining with antibodies to pericentrin (green) and to staining of DNA with propidium iodide (red). The images were taken by laser-scanning confocal microscopy. Scale bar, 10 μm. FT210 cells cultured at the permissive (dotted lines) or restrictive (solid lines) temperature were also subjected to flow cytometric analysis of DNA content (I) or forward light scatter (J).

studies indicated that Skp2 specifically interacts with cyclin E and thereby promotes its ubiquitylation and degradation both in vivo and in vitro (Nakayama et al., 2000). Collectively, these findings suggest that free cyclin E is a candidate substrate of SCF^{Skp2}, and that accumulation of cyclin E alone is not the cause of the cellular abnormalities of *Skp2*$^{-/-}$ mice.

Discussion

p27 Is a Major Physiological Target of SCF^{Skp2}

Protein degradation by the ubiquitin-proteasome pathway plays a fundamental role in determining the abundance of important regulatory molecules. The E3 ubiquitin ligases are thought to determine the substrate specificity of this pathway, and many diverse E3 molecules are therefore thought to exist, although it does not appear to be unusual that several proteins serve as substrates for a given ubiquitin ligase. Skp2, the substrate-recognizing component of an SCF ubiquitin ligase, has thus been shown to recognize p27 (Carrano et al., 1999; Sutterluty et al., 1999; Tsvetkov et al., 1999; Nakayama et al., 2000), the p27-related CKIs p21 (Yu et al., 1998; Bornstein et al., 2003) and p57 (Kamura et al., 2003), cyclin A (Nakayama et al., 2000), cyclin D1 (Yu et al., 1998), free cyclin E (Nakayama et al., 2000), E2F-1 (Marti et al., 1999), p130 (Tedesco et al., 2002; Bhattacharya et al., 2003), ORC1 (Mendez et al., 2002), Cdt1 (Li et al., 2003), Cdk9 (Kiernan et al., 2001), c-Myc (Kim et al., 2003; von der Lehr et al., 2003), and B-Myb (Charrasse et al., 2000). Of these proteins, cyclin A, cyclin D1, E2F-1, and Cdt1 do not accumulate in *Skp2*$^{-/-}$ cells (Nakayama et al., 2000) (our unpublished data), suggesting either that they are not bona fide substrates of Skp2 or that there is redundancy that allows for their ubiquitylation in the absence of Skp2.

Most of the cellular abnormalities apparent in *Skp2*$^{-/-}$ mice are not evident in *Skp2*$^{-/-}$ *p27*$^{-/-}$ double mutant mice. This observation constitutes genetic evidence that the cellular phenotype of nuclear enlargement with polyploidy and overduplication of centrosomes in *Skp2*$^{-/-}$ mice results from the deregulated expression of p27 during the cell cycle, and that p27 is likely the main target of the SCF^{Skp2} ubiquitin ligase. However, the observation that the phenotype of *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice is not completely identical to that of *p27*$^{-/-}$ mice suggests the existence of additional target proteins for Skp2-mediated ubiquitylation.

Role of Skp2 in Prevention of p27 Accumulation during S and G_2 Phases

The concentration of p27 is relatively high in quiescent (G_0) cells, decreases on entry of cells into the cell cycle, and is controlled predominantly by the rate of p27 degradation (Pagano et al., 1995; Shirane et al., 1999) as well as by translational regulation (Hengst and Reed, 1996). This degradation has been thought to require Skp2, which binds to p27 when the latter is phosphorylated on Thr^{187} by the cyclin E–Cdk2 complex (Sheaff et al., 1997; Vlach et al., 1997; Carrano et al., 1999; Montagnoli et al., 1999; Sutterluty et al., 1999; Tsvetkov et al., 1999). The primary function of SCF^{Skp2} might therefore be to render quiescent cells competent to reenter the cell cycle by mediating the degradation of p27. However, there are several inconsistencies with this notion. Skp2 and cyclin E are not expressed until the G_1-S transition of the cell cycle, unequivocally later than the onset of

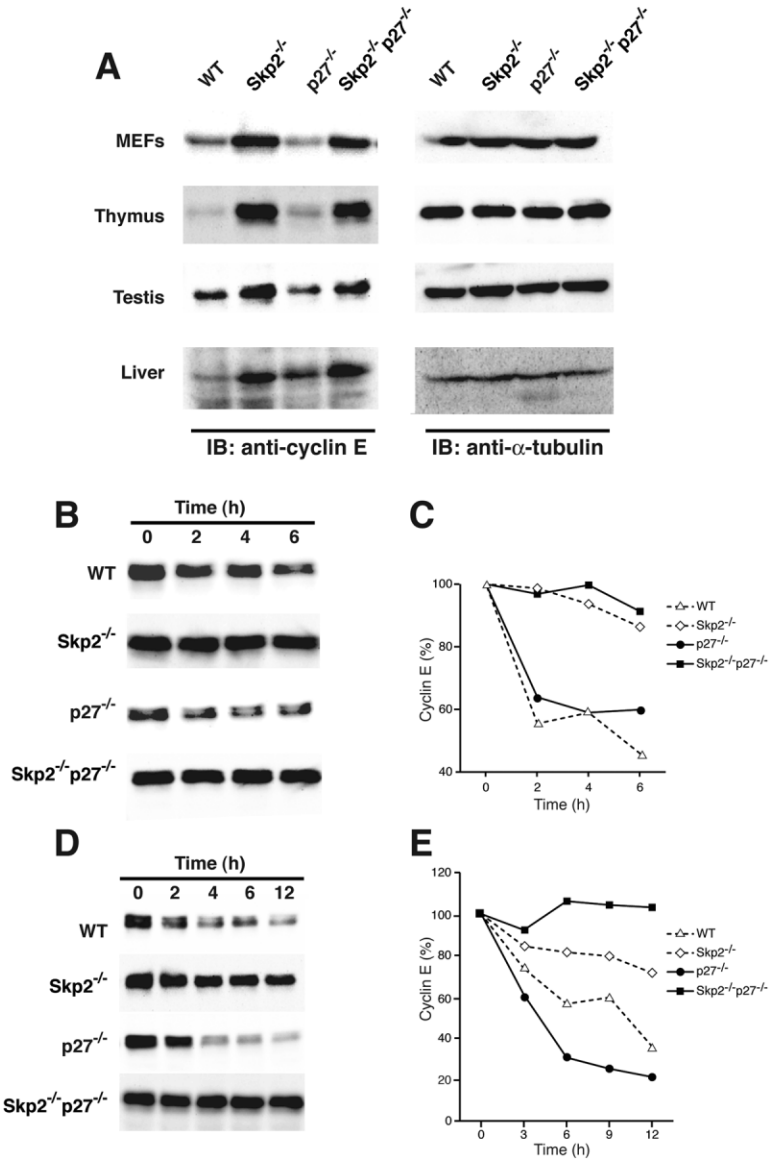

Figure 7. Impaired Degradation of Cyclin E in *Skp2*$^{-/-}$ Mice Is Independent of p27

(A) Immunoblot analysis of cyclin E (left panels) and α-tubulin (control; right panels) in lysates prepared from MEFs, thymus, testis, and liver of wild-type, *Skp2*$^{-/-}$, *p27*$^{-/-}$, and *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice.

(B) Proliferating wild-type, *Skp2*$^{-/-}$, *p27*$^{-/-}$, or *Skp2*$^{-/-}$ *p27*$^{-/-}$ MEFs were incubated in the presence of cycloheximide (50 μg/ml) for the indicated times, after which cell lysates were subjected to immunoblot analysis with antibodies to cyclin E.

(C) The band intensities in (B) were quantitated by image analysis and expressed as a percentage of the corresponding value for time zero.

(D) Wild-type, *Skp2*$^{-/-}$, *p27*$^{-/-}$, or *Skp2*$^{-/-}$ *p27*$^{-/-}$ MEFs were synchronized at S phase by treatment with aphidicolin (1 μg/ml) for 15 hr and then released into aphidicolin-free medium for the indicated times, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to cyclin E.

(E) The band intensities in (D) were quantitated and expressed as described in (C).

p27 degradation apparent at mid-G_1 phase (Hara et al., 2001). Moreover, p27 is exported from the nucleus to the cytoplasm at G_0-G_1 (Tomoda et al., 1999; Rodier et al., 2001; Ishida et al., 2002; Connor et al., 2003), whereas Skp2 is restricted to the nucleus (Miura et al., 1999). The discrepancies between the temporal and spatial patterns of p27 expression and those of Skp2 expression suggest the existence of a Skp2-independent pathway for the degradation of p27. Indeed, the downregulation of p27 at the G_0-G_1 transition occurs normally in *Skp2*$^{-/-}$ cells and is sensitive to proteasome inhibitors (Hara et al., 2001). Biochemical analysis of crude extracts of *Skp2*$^{-/-}$ cells revealed the presence in the cytoplasmic fraction of an Skp2-independent E3 activity that mediates the ubiquitylation of p27 (Hara et al., 2001). This ubiquitylation was not dependent on the phosphorylation of p27 on Thr^{187}, which is a prerequisite for Skp2-mediated ubiquitylation. These data indicate that p27 is degraded at the G_0-G_1 transition by a proteasome-dependent, but Skp2-independent, mechanism.

In contrast, the degradation of p27 during S and G_2 phases is impaired in *Skp2*$^{-/-}$ mice, suggesting that the primary function of Skp2 is to prevent both the accumulation of p27 during S and G_2 phases and the consequent inhibition of mitotic cyclin-Cdc2 activity. Consistent with this idea, experimental inhibition of the kinase activity of Cdc2 in budding yeast, fission yeast, and *Drosophila* forces cells that are normally mitotic to become endoreplicative (Edgar and Orr-Weaver, 2001). In the present study, we show that this is also the case, at least in part, in mammals. Cell cycle synchronization of *Skp2*$^{-/-}$ MEFs also revealed a delay at the G_2-M boundary in comparison with wild-type and *Skp2*$^{-/-}$ *p27*$^{-/-}$ cells (Supplemental Figure S4 at http://www.developmentalcell.com/cgi/content/full/6/5/661/DC1). These data suggest that inactivation of Cdc2 by p27 that accumulates as a result of the lack of Skp2 leads to G_2-M block, although the detailed mechanism of this phenomenon remains to be determined. We thus propose that the major target of p27 at G_1 phase is Cdk2, and that at G_2 phase it may

be Cdc2; Skp2 seems to be important for the regulation of the latter.

Endoreplication of Hepatocytes Induced by Deregulation of p27 Degradation

Polyploidy is apparent in certain tissues of *Skp2*$^{-/-}$ mice including the liver. Maintenance of genome ploidy is a fundamental aspect of cell division. Three possible mechanisms might render cells polyploid. (1) Failure of mitosis. Cells enter mitosis normally but anaphase fails to occur, resulting in the subsequent entry of the cells into interphase with a doubled DNA content. (2) Endoreplication. Cells replicate their genomes in S phase, bypass mitosis, and double their DNA content again in the next S phase. (3) DNA rereplication. Cells arrest in S phase and reinitiate DNA replication continuously. We adopted two criteria to determine which of these three mechanisms gives rise to the polyploidy of *Skp2*$^{-/-}$ hepatocytes. First, we examined whether cells entered mitosis. Immunofluorescence analysis with antibodies to phosphorylated histone H3, a marker of early mitosis, revealed that the number of mitotic cells is reduced in the liver of *Skp2*$^{-/-}$ mice, suggesting that failure of mitosis is not likely responsible for the polyploidy in these animals. Second, we measured the DNA content of *Skp2*$^{-/-}$ hepatocytes after mitogenic stimulation in order to determine whether the increase in DNA content occurred in a stepwise manner. The DNA content of *Skp2*$^{-/-}$ cells was shown to increase in multiples of 2C, a characteristic of endoreplication, rather than in a continuous manner, as would be consistent with DNA rereplication (data not shown). Similar results were also obtained after partial hepatectomy in *Skp2*$^{-/-}$ mice (Minamishima et al., 2002). We thus conclude that the polyploidy of *Skp2*$^{-/-}$ hepatocytes is likely attributable to endoreplication, although the possibility of a mitotic defect cannot be formally ruled out.

The regulation of Cdk activity during endoreplication in mammalian cells, with the exception of megakaryocytes and trophoblasts, is poorly understood (Edgar and Orr-Weaver, 2001). Consistent with the association of Skp2 with Cul1 in the SCF ubiquitin ligase complex, the phenotype of trophoblasts of *Cul1*$^{-/-}$ embryos is similar to that of *Skp2*$^{-/-}$ cells (Dealy et al., 1999; Wang et al., 1999). Unlike most cells, trophoblasts undergo multiple rounds of DNA synthesis without an intervening mitosis, resulting in the formation of giant nuclei (Zybina and Zybina, 1996). The lack of Cul1 or Skp2 appears to augment this process, and our present data indicate that the accumulation of p27 may play an important role in its induction. The abundance of cyclin E remains high and that of cyclin B is reduced in a differentiating trophoblast cell line, and p57, which is structurally and functionally similar to p27, is upregulated during S phase in these cells (Hattori et al., 2000). These characteristics resemble those of *Skp2*$^{-/-}$ cells. However, ectopic expression of a form of p57 with a mutation that stabilizes the protein blocks S phase entry in the trophoblast cell line. The gradual increase in the abundance of p27 in *Skp2*$^{-/-}$ cells may give rise to a window in which p27 inhibits mitosis but not entry into S phase, whereas forced expression of p57 at high levels may block S phase entry immediately.

A fission yeast mutant that lacks the F box protein Pop1, which targets the CKI Rum1 for degradation, also exhibits endoreplication and consequent polyploidy (Kominami and Toda, 1997). Rum1 accumulates to high levels in this mutant. Maintenance of ploidy in fission yeast is controlled by a complex of Cdc2 with the mitotic cyclin Cdc13. Inhibition of the kinase activity of Cdc2-Cdc13 as a result of the increased expression of Rum1 in the *pop1* mutant thus likely leads to polyploidy by promoting the bypass of M phase before the next S phase. A mitotic cyclin-Cdk complex in budding yeast prevents endoreplication through multiple overlapping mechanisms, including phosphorylation of the origin recognition complex (ORC), downregulation of Cdc6 activity, and exclusion from the nucleus of the Mcm2-7 complex (Nguyen et al., 2001). Given the similarities in the phenotypes of the fission yeast *pop1* mutant and mouse *Skp2*$^{-/-}$ cells, the accumulation of p27 in *Skp2*$^{-/-}$ cells may functionally correspond to that of Rum1 in the *pop1* mutant. The prevention of endoreplication through degradation of a CKI mediated by an SCF ubiquitin ligase thus appears to be a mechanism that has been well conserved from yeast to mammals.

Free Cyclin E as a Potential Substrate of SCF^{Skp2}

The marked accumulation of cyclin E in the absence of the antagonizing action of p27 in *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice might have been expected either to compromise tissue organization in the developing embryo, and thereby to result in early embryonic death, or to lead to a high incidence of carcinogenesis. The apparent absence of such outcomes suggests that the deregulated expression of cyclin E might not be a direct cause of carcinogenesis, even though altered expression of cyclin E is apparent in many human cancers. The cyclin E that accumulates in *Skp2*$^{-/-}$ mice, however, appears to be the free form of the protein (Nakayama et al., 2000), not that complexed with Cdk2, and therefore does not contribute to the kinase activity of Cdk2. The nature of the interaction between the pool of free cyclin E and the cyclin E-Cdk2 complex is unclear, but our present data suggest that a simple increase in the abundance of free cyclin E does not directly result in the activation of Cdk2. Another possible interpretation of our data is that the increase in the pool of free cyclin E in *Skp2*$^{-/-}$ cells results in an increase in the activity of the cyclin E-Cdk2 complex, but that this effect is antagonized by the accumulated p27.

Our data do not necessarily imply that SCF^{Skp2} is the main mediator of cyclin E turnover. Rather, the mechanisms for the degradation of cyclin E appear complex. The Skp2-Cul1 complex and Cul3 interact with the free, nonphosphorylated form of cyclin E (Dealy et al., 1999; Singer et al., 1999; Wang et al., 1999; Nakayama et al., 2000), thereby mediating its ubiquitylation-dependent proteolysis. In parallel with these pathways, SCF^{Fbw7} is thought to target phosphorylated cyclin E complexed with Cdk2 (Clurman et al., 1996; Won and Reed, 1996; Koepp et al., 2001; Moberg et al., 2001; Strohmaier et al., 2001). However, mice that lack Fbw7, which die in utero during embryogenesis, show neither accumulation of cyclin E nor an increase in Cdk2 activity (Tsunematsu et al., 2004), whereas *Skp2*$^{-/-}$ mice manifest marked

accumulation of cyclin E in the nucleus. Thus, Fbw7 appears to be dispensable for cyclin E degradation, at least until mid-embryogenesis. In contrast, depletion of Fbw7 by RNA interference results in the accumulation of cyclin E in cultured cells (Koepp et al., 2001) (our unpublished observations), suggesting that Fbw7 might be required for cyclin E turnover in adult tissues. These data render unlikely, however, the possibility that the loss of Skp2 might stabilize other proteins that impair the Fbw7-dependent pathway of protein degradation and are thereby responsible for the accumulation of cyclin E.

Given that p27 accumulates to a high level in *Skp2*$^{-/-}$ cells, it is possible that autophosphorylation of cyclin E complexed with Cdk2 is inhibited by this CKI, resulting in stabilization of cyclin E. The accumulation of cyclin E might thus be secondary to the increase in the abundance of p27. Our present data, however, have shown that cyclin E degradation is also impaired in *Skp2*$^{-/-}$ *p27*$^{-/-}$ mice, providing genetic evidence that the altered expression of cyclin E in *Skp2*$^{-/-}$ cells is independent of p27 accumulation. We have previously shown that Skp2 interacts with free cyclin E and promotes its ubiquitylation both in vitro and in vivo, and that cyclin E degradation is impaired, resulting in loss of periodicity of cyclin E expression, in *Skp2*$^{-/-}$ cells (Nakayama et al., 2000). These biochemical observations are therefore consistent with the genetic evidence that Skp2 directly targets cyclin E. It remains possible that the accumulation of CKIs such as p21, p107, and p130 in *Skp2*$^{-/-}$ mice contributes to the stabilization of cyclin E, although our observation that the kinase activity of Cdk2 is unchanged in *Skp2*$^{-/-}$ cells suggests against this possibility.

Experimental Procedures

Mice

Both Skp2-deficient mice and p27-deficient mice were generated in our laboratory (Nakayama et al., 1996, 2000). We have developed polymerase chain reaction-based protocols (details available on request) to identify wild-type and disrupted alleles of *Skp2* and *p27*.

Histological and Immunofluorescence Analyses

Histological analysis and immunofluorescence analysis of centrosomes and microtubules were performed as described (Nakayama et al., 2000).

Preparation of MEFs

Primary MEFs were isolated from embryos on embryonic day 13.5 and cultured as previously described (Nakayama et al., 1996). In asynchronous culture, there was no substantial difference in cell cycle profiles among wild-type, *Skp2*$^{-/-}$, *p27*$^{-/-}$, and *Skp2*$^{-/-}$ *p27*$^{-/-}$ MEFs (Supplemental Figure S4A at http://www.developmentalcell.com/cgi/content/full/6/5/661/DC1).

Flow Cytometry

Flow cytometric analysis of hepatocytes and MEFs was performed as described previously (Nakayama et al., 1996, 2000).

Immunoblot Analysis

Transfection, immunoprecipitation, and immunoblot analysis were performed as previously described (Hatakeyama et al., 1999; Kitagawa et al., 1999). Antibodies used in this study include those to cyclin A (H-432, Santa Cruz Biotechnology), cyclin B (GNS-1, PharMingen), cyclin E (M-20, Santa Cruz Biotechnology), Cdk2 (M2, Santa Cruz Biotechnology), Cdc2 (17, Santa Cruz Biotechnology), p27 (57, Transduction Laboratories), or α-tubulin (TU-01, Zymed), each at a concentration of 0.2 μg/ml.

In some experiments, subconfluent COS-7 cells grown in four 100 mm dishes were transfected with 5 μg of pcDNA3 or pcDNA-FLAG-p27 and with 2 μg of pCD19 (Tedder and Isaacs, 1989) per dish with the use of the FuGENE6 reagent (Roche Molecular Biochemicals). Twenty-four hours after transfection, the CD19-expressing cells were collected with antibodies to CD19 attached to magnetic beads (Dynal Biotech) and were used for immune-complex kinase assays and immunoprecipitation.

Immune-Complex Kinase Assays

Kinase activity associated with Cdk2, Cdc2, or cyclins A, B, or E was measured with an immune-complex kinase assay as described (Nakayama et al., 1996).

Mitogenic Stimulation of Mouse Hepatocytes by Oral Administration of Estriol

Male mice at 8 weeks of age were subjected to oral administration of 0.4 mg of estriol (Wako) per gram of body mass. They were also subjected to daily intraperitoneal injection of BrdU (100 μg/g) (Sigma). Colchicine (1 μg/g) (Wako) was injected intraperitoneally 6 hr before killing. Sections of the liver were stained with hematoxylin-eosin or were subjected to immunostaining with rabbit polyclonal antibodies to phosphorylated histone H3 (Upstate Biotechnology) followed by Cy3-conjugated goat antibodies to rabbit immunoglobulin G (Chemicon). For detection of BrdU, sections were incubated with a rat monoclonal antibody to BrdU (Harlan Sera-Lab) at a dilution of 1:200 followed by biotinylated secondary antibodies; immune complexes were visualized with the use of a streptavidin-biotin-peroxidase detection kit (Vector) and diaminobenzidine (Wako).

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References

Bhattacharya, S., Garriga, J., Calbo, J., Yong, T., Haines, D.S., and Grana, X. (2003). SKP2 associates with p130 and accelerates p130 ubiquitylation and degradation in human cells. Oncogene *22*, 2443–2451.

Bornstein, G., Bloom, J., Sitry-Shevah, D., Nakayama, K., Pagano, M., and Hershko, A. (2003). Role of the SCF^{Skp2} ubiquitin ligase in the degradation of p21^{Cip1} in S phase. J. Biol. Chem. *278*, 25752–25757.

Carrano, A.C., Eytan, E., Hershko, A., and Pagano, M. (1999). SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat. Cell Biol. *1*, 193–199.

Charrasse, S., Carena, I., Brondani, V., Klempnauer, K.H., and Ferrari, S. (2000). Degradation of B-Myb by ubiquitin-mediated proteolysis: involvement of the Cdc34-SCF(p45^{Skp2}) pathway. Oncogene *19*, 2986–2995.

Clurman, B.E., Sheaff, R.J., Thress, K., Groudine, M., and Roberts, J.M. (1996). Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation. Genes Dev. *10*, 1979–1990.

Connor, M.K., Kotchetkov, R., Cariou, S., Resch, A., Lupetti, R., Beniston, R.G., Melchior, F., Hengst, L., and Slingerland, J.M. (2003). CRM1/Ran-mediated nuclear export of p27(Kip1) involves a nuclear export signal and links p27 export and proteolysis. Mol. Biol. Cell *14*, 201–213.

Correa-Bordes, J., and Nurse, P. (1995). p25rum1 orders S phase

and mitosis by acting as an inhibitor of the p34Cdc2 mitotic kinase. Cell *83*, 1001–1009.

Dealy, M., Nguyen, K.V.T., Lo, J., Gstaiger, M., Krek, W., Elson, D., Arbeit, J., Kipreos, E.T., and Johnson, R.S. (1999). Loss of Cul1 results in early embryonic lethality and dysregulation of cyclin E. Nat. Genet. *23*, 245–248.

Edgar, B.A., and Orr-Weaver, T.L. (2001). Endoreplication cell cycles: more for less. Cell *105*, 297–306.

Fero, M.L., Rivkin, M., Tasch, M., Porter, P., Carow, C.E., Firpo, E., Polyak, K., Tsai, L.H., Broudy, V., Perlmutter, R.M., et al. (1996). A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in $p27^{Kip1}$-deficient mice. Cell *85*, 733–744.

Fujii, H., Hayama, T., and Kotani, M. (1985). Stimulating effect of natural estrogens on proliferation of hepatocytes in adult mice. Acta Anat. *121*, 174–178.

Hara, T., Kamura, T., Nakayama, K., Oshikawa, K., Hatakeyama, S., and Nakayama, K.I. (2001). Degradation of $p27^{Kip1}$ at the G_0-G_1 transition mediated by a Skp2-independent ubiquitination pathway. J. Biol. Chem. *276*, 48937–48943.

Hatakeyama, S., Kitagawa, M., Nakayama, K., Shirane, M., Matsumoto, M., Hattori, K., Higashi, H., Nakano, H., Okumura, K., Onoe, K., et al. (1999). Ubiquitin-dependent degradation of $I\kappa B\alpha$ is mediated by a ubiquitin ligase Skp1/Cul 1/F-box protein FWD1. Proc. Natl. Acad. Sci. USA *96*, 3859–3863.

Hattori, N., Davies, T.C., Anson-Cartwright, L., and Cross, J.C. (2000). Periodic expression of the cyclin-dependent kinase inhibitor $p57^{Kip2}$ in trophoblast giant cells defines a G2-like gap phase of the endocycle. Mol. Biol. Cell *11*, 1037–1045.

Hengst, L., and Reed, S.I. (1996). Translational control of $p27^{Kip1}$ accumulation during the cell cycle. Science *271*, 1861–1864.

Ishida, N., Hara, T., Kamura, T., Yoshida, M., Nakayama, K., and Nakayama, K.I. (2002). Phosphorylation of $p27^{Kip1}$ on serine 10 is required for its binding to CRM1 and nuclear export. J. Biol. Chem. *277*, 14355–14358.

Kamura, T., Hara, T., Kotoshiba, S., Yada, M., Ishida, N., Imaki, H., Hatakeyama, S., Nakayama, K., and Nakayama, K.I. (2003). Degradation of $p57^{Kip2}$ mediated by SCF^{Skp2}-dependent ubiquitylation. Proc. Natl. Acad. Sci. USA *100*, 10231–10236.

Kiernan, R.E., Emiliani, S., Nakayama, K., Castro, A., Labbe, J.C., Lorca, T., Nakayama, K.I., and Benkirane, M. (2001). Interaction between cyclin T1 and SCF^{SKP2} targets CDK9 for ubiquitination and degradation by the proteasome. Mol. Cell. Biol. *21*, 7956–7970.

Kim, S.Y., Herbst, A., Tworkowski, K.A., Salghetti, S.E., and Tansey, W.P. (2003). Skp2 regulates myc protein stability and activity. Mol. Cell *11*, 1177–1188.

Kitagawa, M., Okabe, T., Ogino, H., Matsumoto, H., Suzuki-Takahashi, I., Kokubo, T., Higashi, H., Saitoh, S., Taya, Y., Yasuda, H., et al. (1993). Butyrolactone I, a selective inhibitor of cdk2 and Cdc2 kinase. Oncogene *8*, 2425–2432.

Kitagawa, M., Hatakeyama, S., Shirane, M., Matsumoto, M., Ishida, N., Hattori, K., Nakamichi, I., Kikuchi, A., Nakayama, K.I., and Nakayama, K. (1999). An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of β-catenin. EMBO J. *18*, 2401–2410.

Kiyokawa, H., Kineman, R.D., Manova-Todorova, K.O., Soares, V.C., Hoffman, E.S., Ono, M., Khanam, D., Hayday, A.C., Frohman, L.A., and Koff, A. (1996). Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of $p27^{Kip1}$. Cell *85*, 721–732.

Koepp, D.M., Schaefer, L.K., Ye, X., Keyomarsi, K., Chu, C., Harper, J.W., and Elledge, S.J. (2001). Phosphorylation-dependent ubiquitination of cyclin E by the SCF^{Fbw7} ubiquitin ligase. Science *294*, 173–177.

Kominami, K., and Toda, T. (1997). Fission yeast WD-repeat protein pop1 regulates genome ploidy through ubiquitin-proteasome-mediated degradation of the CDK inhibitor Rum1 and the S-phase initiator Cdc18. Genes Dev. *11*, 1548–1560.

Li, X., Zhao, Q., Liao, R., Sun, P., and Wu, X. (2003). The SCF^{Skp2} ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. J. Biol. Chem. *278*, 30854–30858.

Marti, A., Wirbelauer, C., Scheffner, M., and Krek, W. (1999). Interaction between ubiquitin-protein ligase SCF^{SKP2} and E2F–1 underlies the regulation of E2F–1 degradation. Nat. Cell Biol. *1*, 14–19.

Mendez, J., Zou-Yang, X.H., Kim, S.Y., Hidaka, M., Tansey, W.P., and Stillman, B. (2002). Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. Mol. Cell *9*, 481–491.

Minamishima, Y.A., Nakayama, K., and Nakayama, K.I. (2002). Recovery of liver mass without proliferation of hepatocytes after partial hepatectomy in Skp2-deficient mice. Cancer Res. *62*, 995–999.

Miura, M., Hatakeyama, S., Hattori, K., and Nakayama, K.I. (1999). Structure and expression of the gene encoding mouse F-Box protein, Fwd2. Genomics *62*, 50–58.

Moberg, K.H., Bell, D.W., Wahrer, D.C., Haber, D.A., and Hariharan, I.K. (2001). Archipelago regulates Cyclin E levels in Drosophila and is mutated in human cancer cell lines. Nature *413*, 311–316.

Montagnoli, A., Fiore, F., Eytan, E., Carrano, A.C., Draetta, G.F., Hershko, A., and Pagano, M. (1999). Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. Genes Dev. *13*, 1181–1189.

Nagahama, H., Hatakeyama, S., Nakayama, K., Nagata, M., Tomita, K., and Nakayama, K.I. (2001). Spatial and temporal expression patterns of the cyclin-dependent kinase (CDK) inhibitors $p27^{Kip1}$ and $p57^{Kip2}$ during mouse development. Anat. Embryol. *203*, 77–87.

Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I., Loh, D.Y., and Nakayama, K.I. (1996). Mice lacking $p27^{Kip1}$ display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell *85*, 707–720.

Nakayama, K., Nagahama, H., Minamishima, Y.A., Matsumoto, M., Nakamichi, I., Kitagawa, K., Shirane, M., Tsunematsu, R., Tsukiyama, T., Ishida, N., et al. (2000). Targeted disruption of Skp2 results in accumulation of cyclin E and $p27^{Kip1}$, polyploidy and centrosome overduplication. EMBO J. *19*, 2069–2081.

Nakayama, K.I., Hatakeyama, S., and Nakayama, K. (2001). Regulation of the cell cycle at the G_1-S transition by proteolysis of cyclin E and $p27^{Kip1}$. Biochem. Biophys. Res. Commun. *282*, 853–860.

Nguyen, V.Q., Co, C., and Li, J.J. (2001). Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. Nature *411*, 1068–1073.

Pagano, M., Tam, S.W., Theodoras, A.M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P.R., Draetta, G.F., and Rolfe, M. (1995). Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science *269*, 682–685.

Rodier, G., Montagnoli, A., Marcotullio, D.L., Coulombe, P., Draetta, D.G., Pagano, M., and Meloche, S. (2001). p27 cytoplasmic localization is regulated by phosphorylation on Ser10 and is not a prerequisite for its proteolysis. EMBO J. *20*, 6672–6682.

Sheaff, R.J., Groudine, M., Gordon, M., Roberts, J.M., and Clurman, B.E. (1997). Cyclin E-CDK2 is a regulator of $p27^{Kip1}$. Genes Dev. *11*, 1464–1478.

Shirane, M., Harumiya, Y., Ishida, N., Hirai, A., Miyamoto, C., Hatakeyama, S., Nakayama, K.I., and Kitagawa, M. (1999). Down-regulation of $p27^{Kip1}$ by two mechanisms, ubiquitin-mediated degradation and proteolytic processing. J. Biol. Chem. *274*, 13886–13893.

Singer, J.D., Gurian-West, M., Clurman, B., and Roberts, J.M. (1999). Cullin-3 targets cyclin E for ubiquitination and controls S phase in mammalian cells. Genes Dev. *13*, 2375–2387.

Stern, B., and Nurse, P. (1996). A quantitative model for the Cdc2 control of S phase and mitosis in fission yeast. Trends Genet. *12*, 345–350.

Strohmaier, H., Spruck, C.H., Kaiser, P., Won, K.A., Sangfelt, O., and Reed, S.I. (2001). Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line. Nature *413*, 316–322.

Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M.,

Muller, U., and Krek, W. (1999). $p45^{SKP2}$ promotes $p27^{Kip1}$ degradation and induces S phase in quiescent cells. Nat. Cell Biol. *1*, 207–214.

Tedder, T.F., and Isaacs, C.M. (1989). Isolation of cDNAs encoding the CD19 antigen of human and mouse B lymphocytes. J. Immunol. *143*, 712–717.

Tedesco, D., Lukas, J., and Reed, S.I. (2002). The pRb-related protein p130 is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCF^{Skp2}. Genes Dev. *16*, 2946–2957.

Th'ng, J.P., Wright, P.S., Hamaguchi, J., Lee, M.G., Norbury, C.J., Nurse, P., and Bradbury, E.M. (1990). The FT210 cell line is a mouse G2 phase mutant with a temperature-sensitive CDC2 gene product. Cell *63*, 313–324.

Tomoda, K., Kubota, Y., and Kato, J. (1999). Degradation of the cyclin-dependent-kinase inhibitor $p27^{Kip1}$ is instigated by Jab1. Nature *398*, 160–165.

Tsunematsu, R., Nakayama, K., Oike, Y., Nishiyama, M., Ishida, N., Hatakeyama, S., Bessho, Y., Kageyama, R., Suda, T., and Nakayama, K.I. (2004). Mouse Fbw7/Sel-10/Cdc4 is required for notch degradation during vascular development. J. Biol. Chem. *279*, 9417–9423.

Tsvetkov, L.M., Yeh, K.H., Lee, S.J., Sun, H., and Zhang, H. (1999). $p27^{Kip1}$ ubiquitination and degradation is regulated by the SCF^{Skp2} complex through phosphorylated Thr187 in p27. Curr. Biol. *9*, 661–664.

Vlach, J., Hennecke, S., and Amati, B. (1997). Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. EMBO J. *16*, 5334–5344.

von der Lehr, N., Johansson, S., Wu, S., Bahram, F., Castell, A., Cetinkaya, C., Hydbring, P., Weidung, I., Nakayama, K., Nakayama, K.I., et al. (2003). The F-box protein Skp2 participates in c-Myc proteasomal degradation and acts as a cofactor for c-Myc-regulated transcription. Mol. Cell *11*, 1189–1200.

Wang, Y., Penfold, S., Tang, X., Hattori, N., Riley, P., Harper, J.W., Cross, J.C., and Tyers, M. (1999). Deletion of the Cul1 gene in mice causes arrest in early embryogenesis and accumulation of cyclin E. Curr. Biol. *9*, 1191–1194.

Won, K.A., and Reed, S.I. (1996). Activation of cyclin E/CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. EMBO J. *15*, 4182–4193.

Yu, Z.K., Gervais, J.L., and Zhang, H. (1998). Human CUL-1 associates with the SKP1/SKP2 complex and regulates p21(CIP1/WAF1) and cyclin D proteins. Proc. Natl. Acad. Sci. USA *95*, 11324–11329.

Zybina, E.V., and Zybina, T.G. (1996). Polytene chromosomes in mammalian cells. Int. Rev. Cytol. *165*, 53–119.