p21 Functions to Maintain Quiescence of p27-Deficient Hepatocytes

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A role for p21 in p27-deficient mice

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SUMMARY

Hepatocytes rarely proliferate in the healthy adult liver. We explored the roles of the cyclin kinase inhibitors p21 and p27 in maintaining hepatocyte quiescence. p27 is expressed throughout the wildtype liver, but the related protein p21 was not detected. However, p21 was detected in livers of p27-deficient mice. p21 protein levels did not correlate with an increase in p21 mRNA expression, suggesting that p21 expression was regulated posttranscriptionally. p21 protein levels increased in cultured primary hepatocytes treated with the proteasome inhibitor MG132 and cycloheximide indicating that p21 expression is regulated at the level of protein stability in liver cells. Although increased expression of Cdk4, Cdk2, and PCNA was detected in p27-deficient livers, increased hepatocyte proliferation was detected only in livers of mice deficient for both p21 and p27. In p27-deficient livers, p21 was found in complexes with Cdk2 and Cyclin E and can compensate for the absence of p27. Our data indicate that cyclin kinase inhibitor activity is important for maintaining hepatocyte quiescence in the adult liver. Significant increases in p21 were detected in multiple tissues of mature p27-deficient mice when compared with wildtype mice, suggesting that the ability of p21 to functionally substitute for p27 is not liver specific.
INTRODUCTION

Cell cycle progression is regulated by cyclin dependent kinases (Cdks). Cyclin kinase inhibitors (CKIs), which bind and inhibit the activity of Cdks, play key roles in negatively regulating cell proliferation. p21<sup>(Waf1/Cip1)</sup> is the founding member of the Cip/Kip family of CKIs that also includes p27<sup>(Kip1)</sup> and p57<sup>(Kip2)</sup> (reviewed in 1,2). p21 and p27 bind and inhibit a broad range of cyclin Cdk complexes in vitro, and inhibit the Cyclin A and Cyclin E dependent kinase Cdk2 in vivo (reviewed in 3,4). p21 and p27 have also been shown to facilitate assembly of active Cyclin-Cdk complexes (5,6). In addition to its ability to inhibit Cdk2, p21 can also bind and inhibit the activity of proliferating cell nuclear antigen (PCNA) (reviewed in 7,8).

p21 expression is induced by DNA damage, a variety of cytokines and growth factors, and is often coincident with cell differentiation (reviewed in 9). It plays an essential role in inducing growth arrest following DNA damage (10,11). Aging studies in p21-deficient mice have demonstrated a role for p21 in tumor suppression, albeit a much weaker one than p53 (12). Mice lacking p21 are more susceptible to chemically induced skin carcinoma (13,14). In vivo, p21 appears to play roles in regulating renewal of keratinocytes (13) and hematopoietic cells (15). Female p21-deficient mice have decreased viability and develop a syndrome similar to human lupus due to an increased T-lymphocyte proliferation following prolonged stimulation (16). In the kidney, p21 appears to regulate the balance between hyperplasia and hypoplasia, and its disruption ameliorates progression to chronic renal failure after partial renal ablation (17). In multiple tissues and cell types p21 is induced following different types of challenges and appears to be a general sensor of stress, including CCl<sub>4</sub> toxicity in the liver (18).

Multiple functions for p27 in regulating growth and development have been revealed in p27-deficient mice (19-21). p27-deficient mice grow 20-40% larger than wild-type littermates.
due to alterations in the balance between proliferation and withdrawal from the cell cycle at critical periods of development. These mice develop intermediate lobe pituitary hyperplasia and adenoma, and female homozygous p27-/- mice are infertile. Although ovarian follicles develop, they do not progress to form corpora lutea. p27 has been shown to act as a safeguard against excessive cell proliferation following experimental induction of inflammatory injury in the kidneys of mice (22). p27-deficient and p27+/- mice have been shown to be prone to tumor development in multiple tissues following γ-irradiation and other challenges (23). In general, p27 levels increase as cells become quiescent and decrease when cells are stimulated to reenter the cell cycle (24-26). Posttranscriptional mechanisms are largely responsible for regulation of p27 expression (reviewed in 27,28).

Adult hepatocytes are highly differentiated cells that perform a wide variety of metabolic functions, and these cells rarely divide. Still they can rapidly enter the cell cycle and proliferate following tissue loss due to chemical or physical injury (reviewed in 29,30). Both p21 and p27 have been implicated in regulation of the rapid cell cycle progression that occurs during liver regeneration following injury (18,31,32). However a functional role for these cyclin kinase inhibitors in maintaining quiescence of the mature hepatocyte has not been demonstrated. We examined the expression and functions of p21 and p27 in quiescent hepatocytes in the adult rodent liver, and we detected aberrant hepatocyte proliferation in livers of mice deficient for both p21 and p27. Our data indicate that cyclin kinase inhibitors are required for maintaining hepatocyte quiescence even in the absence of mitogenic signals associated with liver injury and tissue loss.
EXPERIMENTAL PROCEDURES

Animals

Wildtype, p21-/-, p27-/-, and p21/p27-/- male littermates at 8-10 weeks of age were generally used for all experiments except where noted in the results section. Generation of p21-/- and p27-/- mice was previously described (11,19). Female p27-/- mice are infertile. To obtain mice deficient for both p21 and p27, p21-/- females and p27-/- males were crossed resulting in offspring that were double heterozygotes, p21+/--; p27+/-+. Subsequent intercross matings between double-heterozygous males and females produced offspring with the p21/p27-/- genotype. p21-/-;p27+/- males were crossed with p21-/-;p27+/- females to generate additional p21/p27-/- offspring. The genotypes of the mice were confirmed by genomic DNA isolation and PCR. Mice were fed a commercial diet and water ad libitum, and sacrificed in the morning. The left lobe of the liver was fixed in 4% paraformaldehyde and paraffin-embedded for sectioning. Other portions of liver were frozen in liquid nitrogen and kept at -70°C for preparation of protein lysates and total RNA.

Mice were injected intraperitoneally with 50 µg of 5-bromo-2’-deoxyuridine (Sigma, St. Louis, Missouri)/g body weight two hours prior to sacrifice for hepatocyte proliferation studies.

Immunoblotting and Immunoprecipitations

In general, 50 µg of total liver protein was separated on 12 or 15% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore, Bedford, Massachusetts). All membranes were stained with Ponceau S to confirm equal loading and transfer of protein. Immunoblotting was performed using the following primary antibodies obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, California: p21 (SC-397), p27 (SC-528), Cdk2 (SC-163), Cdk4 (SC-260), Cdk6 (SC-177), Cyclin A (SC-596), Cyclin E (SC-481) and PCNA (SC-56). Other antibodies
used included Cyclin D1 (MS-210. NeoMarkers, Fremont, California) and β–actin (A-5441, Sigma, St. Louis, Missouri). Immunoreactive bands were detected using the SuperSignal Substrate (Pierce, Rockford, Illinois).

Immunoprecipitations were performed using the Seize™ Primary Immunoprecipitation Kit (Pierce, Rockford, Illinois) according to the manufacturer's instructions. Twenty-five micrograms of anti-p21 antibody (PC55, Oncogene, Cambridge, Massachusetts) or anti-p27 antibody (SC-528, Santa Cruz Biotechnology, Santa Cruz, California) was used for antibody coupling for each column. Rabbit IgG was used as a control.

**Culture of Primary Hepatocytes and MEFs**

Primary hepatocytes from adult male and female mice (8-10 weeks old) were isolated by a two-step in situ collagenase perfusion procedure as previously described (33). Cell viability was >90% as determined by trypan blue exclusion. After attachment, cells were maintained in hormonally-defined Williams E medium (34). One day after plating, cells were treated with 20 µM MG132 (Biomol Research, Plymouth Meeting, Pennsylvania) and/or cycloheximide 0.5µg/ml (Sigma, St. Louis, Missouri). The vehicle DMSO was added to the medium of untreated control cells. After 5.5 hours, cells were washed with PBS twice, collected in a lysis buffer, incubated for 20 min with agitation at 4°C, and centrifuged for 10 min to collect total cell lysates.

Mouse embryonic fibroblasts (MEFs) were prepared and cultured as previously described (35). MEFs were serum-starved for 72 hours and then restimulated with 10% FBS for 24 hours.

**RT-PCR**

Total liver RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, California) and cDNA was synthesized using 2 µg of total RNA with the SuperScript Preamplification System
(Invitrogen, Carlsbad, California) as previously described (18). For amplification of cDNA, primers for mouse p21 (upstream primer, AGTGTGCCGTTGTCTCTTCG; downstream primer, ACACCAGAGTGCAAGACAGC; annealing temperature 62°C, 30 cycles; product 311 bp) were used. Expression of mouse S16 ribosomal protein was examined as an internal control as previously described (18). For each combination of primers, the kinetics of PCR amplification were studied, the number of cycles corresponding to plateau was determined, and PCR was performed within the exponential range. Amplified products were separated on a 2% agarose gel and visualized with ethidium bromide staining. Quantitation was performed using Kodak 1D Image Analysis Software (Eastman Kodak, Rochester, New York).

**Immunohistochemistry**

Liver sections were pre-treated for antigen recovery and accessibility. For the p21 and p27 antibodies, slides were pretreated by a 5-minute incubation in pepsin solution (AutoZyme, Vector, Burlingame, California). For BrdU, the best signal was obtained subsequent to microwave heating. The DuPont/NEN (Boston, Massachusetts) Tyraramide Signal Amplification (TSA) kit was used to amplify antibody staining. Primary antibody sources and dilutions were anti-p21, PC55 (Oncogene, Cambridge, Massachusetts) 1:150; p27, SC-528 (Santa Cruz Biotechnology, Santa Cruz, California) 1:300; BrdU, #347583 (Becton-Dickenson, San Jose, California) 1:100.
RESULTS

Increased p21 expression in the p27-deficient adult mouse liver

Expression of p21 and p27 protein was examined in livers of wildtype, p21-/- and p27-/- male mice. Total proteins were isolated from four or five individuals of each genotype and subjected to immunoblotting with antibodies against p21, p27 or β–actin. While p27 protein expression was easily detected in the wildtype liver (Fig. 1A), no p21 protein could be detected, even after long exposures (Fig. 1B). However p21 protein was detected in liver lysates prepared from p27-deficient mice (Fig. 1B). These data were confirmed using immunohistochemistry. p27 protein was detected in nuclei throughout the wildtype liver lobule and in bile duct epithelial cells, while no p21 expression was detected in wildtype liver samples (Fig. 2). In the p27-deficient liver, p21 protein expression was detected in hepatocyte nuclei, with highest levels near the central veins (Fig. 2).

p21 expression is frequently regulated at the transcriptional level (9), so we examined levels of p21 mRNA expressed in the wildtype and p27-deficient livers used in the immunoblotting experiments above (Fig. 1B) using semi-quantitative RT-PCR. No increase in p21 mRNA levels was detected in RNA samples from the p27-deficient animals, when compared with the wildtype animals. Expression of the mouse S16 ribosomal protein gene was examined as a control (Fig. 1C and D). The lack of a significant change in p21 mRNA levels suggests that increased p21 protein expression in the p27-deficient mouse liver is regulated at a posttranscriptional level.

To examine possible mechanisms regulating p21 protein expression in the wildtype liver, primary hepatocytes from male and female mice were placed into culture and treated with MG132, an inhibitor of proteasome-dependent proteolysis. It has been shown that MG132
treatment leads to stabilization of p21 protein (36), but not to an increase in p21 mRNA levels (37). An increase in p21 protein levels was detected following treatment of hepatocytes with MG132, suggesting that p21 protein levels are regulated by proteasome-mediated degradation in wildtype hepatocytes (Fig. 3A). To determine if translation of p21 transcripts contributed to the increase in p21 protein levels detected after MG132 treatment, hepatocytes were cultured with MG132 and cycloheximide (CHX), an inhibitor of protein synthesis. Increased p21 protein was detected in the presence of MG132 and cycloheximide providing additional support that p21 is regulated at the level of protein stability in hepatocytes (Fig. 3B and C).

An increase in p21 expression has not been previously reported for p27-/- tissues or cells. We examined p21 protein levels in serum starved and stimulated p27-/- mouse embryonic fibroblasts (MEFs) (Fig. 4A). We could not detect increased p21 expression as observed in the liver, suggesting that increased p21 expression in the absence of p27 might be liver specific. However when we compared p21 protein levels in a variety of differentiated tissues from mature (8 to 19 weeks of age) wildtype and p27 deficient mice, we discovered higher levels of p21 protein in multiple tissues of p27 deficient mice (Fig. 4B). Thus, increased accumulation of p21 may be important for cell cycle control in multiple tissues of the p27-deficient mouse.

**p27-deficient hepatocytes appear primed to replicate, but aberrant proliferation is not detected in animals with an intact p21 gene**

We compared baseline expression levels of a number of cell cycle regulatory proteins including Cdk2, Cdk4, Cdk6, Cyclin A, Cyclin D1, Cyclin E and PCNA in four mice of each genotype (wildtype, p21-/-, p27-/-, and p21/p27-/-) (Fig. 5). β-actin expression levels were examined as a control for protein loading. Increased expression of Cyclin A was detected in all animals deficient for either p21 or p27. Increases in Cdk2, Cdk4 and PCNA expression were
also apparent in livers of p27-/- and p21/p27 -/- mice. Increased expression of proliferation-associated proteins suggested that there might be an increase in hepatic proliferation in mice lacking p27.

To determine if the increase in proliferation-associated protein expression leads to increased cell proliferation in the normally quiescent liver, animals were injected with 5-bromo-2'-deoxyuridine (BrdU) and then sacrificed after two hours (Fig. 6). Significant numbers of BrdU-labeled hepatocytes were detected only in livers of mice deficient for both p21 and p27 (Fig. 6A). While rare proliferating cells could be detected in livers of untreated wildtype mice and mice deficient for either p21 or p27, the majority of these rare cells were not hepatocytes.

The total number of BrdU-labeled cells was counted in twenty random fields in liver sections from each of the four genotypes (Fig. 6B). In p21/p27-/- untreated livers most fields contained two or three BrdU-labeled nuclei. In comparison, most fields were negative in the other three genotypes. Similar results were obtained in separate experiments in which untreated mice were injected with [3H]thymidine one hour prior to sacrifice (data not shown). The few fields from p27-/- liver sections containing more than four labeled cells contained arteries and veins, and the majority of the labeled cells were lymphocytes. The increase in the number of sporadically proliferating hepatocytes in the untreated livers of mice lacking both p21 and p27 suggests that the increase in p21 expression detected in the untreated p27-/- liver can compensate for the absence of p27 and inhibit sporadic proliferation.

**p21 associates with CyclinE/Cdk2 and Cdk4 in p27-deficient livers**

Expression of Cyclins and Cdks was detected in the wildtype mouse liver (Fig. 4), but hepatocyte proliferation was detected only in the absence of both p21 and p27. We were unable to detect consistent increases in Cdk2 activity in the whole tissue extracts from p21/p27-deficient
livers, probably in part because the number of proliferating cells represented only a small fraction of the total cells (data not shown). In addition, evidence suggests that p21 and p27 are also positive regulators of Cyclin/Cdk complexes, facilitating their assembly (reviewed in 3,4). We detected p27 in complexes with Cdk2 and Cdk4 in the livers of wildtype and p21-deficient animals using immunoprecipitation followed by immunoblotting (Fig. 7). In p27-deficient mice, a significant increase in p21 protein levels was detected, and p21 was found in complexes containing Cdk2 and Cdk4 in extracts from p27-deficient liver. Cyclin E was also detected associated with p21 in coimmunoprecipitation experiments (data not shown). While p21 and p27 have been shown to play a positive role in the assembly of Cyclin D-Cdk4 complexes, association of these CKIs with Cdk2 complexes is inhibitory and p21/p27-deficient MEFs have been shown to contain increased levels of Cdk2 activity (6). Our data suggest that increased levels of p21 protein in the p27-deficient liver inhibit proliferation by inhibiting Cdk2.
DISCUSSION

Adult hepatocytes are quiescent and rarely divide under normal conditions. We detected p27 in the nuclei of hepatocytes throughout the adult mouse liver. We determined that p27 expression in quiescent hepatocytes is at least partially responsible for the lack of hepatocyte replication, although disruption of the p27 gene alone did not lead to a significant increase in hepatocyte proliferation. This was because p27-deficient hepatocytes express the related CKI p21, which was undetectable in the wildtype liver. Many sporadically proliferating hepatocytes were detected in animals lacking both p21 and p27, indicating that at least one of these CKIs was required for the maintenance of mature hepatocyte quiescence. An increase in p21 was detected in multiple tissues of mature p27-deficient mice and may represent a general mechanism by which tissues maintain cell cycle control in the absence of p27. We detected p21 protein in Cdk2 and Cdk4 complexes in p27-deficient livers, where it can play an active role in the inhibition of cell cycle progression.

A number of recent reports have emphasized the importance of regulation of p21 protein stability in growth control (38-41). Expression of p21 can be regulated by both ubiquitin-dependent and -independent proteasomal mediated degradation (36,42,43,44). We determined that absence of p21 in wildtype liver may be attributed to proteasomal mediated degradation in liver cells because p21 levels increased in hepatocytes treated with MG132 and cycloheximide. We examined several possible mechanisms that could contribute to changes in p21 protein turnover in the liver, including association of p21 with CCAAT/enhancer binding protein \( \alpha \) (C/EBP\( \alpha \)), and AKT activity (data not shown). C/EBP\( \alpha \) can bind and regulate levels of p21 protein and is expressed at high levels in quiescent hepatocytes (45). However we were unable to detect differences in the expression of C/EBP\( \alpha \) or association of p21 with C/EBP\( \alpha \) between
wildtype and p27-/- animals. Recent reports indicate that AKT activity may regulate p21 protein turnover (41,46,47), but we were unable to detect any changes in AKT activity or AKT association with p21 in p27-deficient livers.

Cooperation between CKIs has been demonstrated in various tissues. p27 and p57 have been shown to act together to control proliferation in lens fiber cells and placental trophoblasts (48), while p21 and p57 play redundant functions in regulating differentiation of skeletal muscle and lung aveoli (49). p19\(^{\text{Ink4d}}\) and p27 cooperate to maintain quiescence of differentiated neurons (50). Functional collaboration between p18\(^{\text{Ink4C}}\) and either p27 or p21 has also been demonstrated in vivo (51). p21 has been reported to be important for maintaining hematopoietic stem cell quiescence (15); while p27 is important for regulating hematopoietic progenitor cell proliferation (52). Here we have shown that increased p21 expression can compensate for the lack of p27 to maintain quiescence of mature hepatocytes.

Recently p27-deficient hepatocytes were shown to more efficiently repopulate diseased mouse livers in transplantation studies, and it has been suggested that p27-deficient hepatocytes may prove to be beneficial for the treatment of human liver diseases (53,54). Our findings indicate that p27 is important for preventing hepatocyte proliferation in the physiological environment of the uninjured liver, and that p21 accumulates in its absence to assume its role. As attempts are made to generate cells useful for human hepatocyte transplantation, it will be important to recognize that either p27 or p21 should be expressed in hepatocytes to prevent aberrant proliferation after repopulation of diseased liver has occurred.

While rarely mutated in cancers, reduced expression of p27 has been correlated with poor survival among patients with breast, prostate, or colorectal carcinomas (reviewed in 27,28). Decreased expression of p27 protein has also been associated with poor prognosis in
hepatocellular carcinomas (55-57). In mouse liver the natural compensatory induction of p21 expression in the liver is sufficient to keep aberrant proliferation in check. Modulation of p27 and/or p21 expression in hepatocytes could have potential therapeutic benefits for patients with liver cancer.
ACKNOWLEDGEMENTS

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REFERENCES


FIGURE LEGENDS

Fig. 1. Expression of p21 and p27 in livers of wildtype mice and mice deficient for p21 or p27.  

A. Immunoblot analyses were performed to examine p27 expression in the livers of wildtype and p21-deficient animals. Expression of β-actin was examined as a control.  

B. Immunoblot analyses were performed to examine p21 expression in livers of wildtype and p27-deficient animals. p21 protein was detected in p27-deficient but not wildtype liver.  

C. p21 mRNA expression in the liver was examined using RT-PCR. Expression of the mouse S16 ribosomal protein gene was examined as an internal control. A typical ethidium bromide stained agarose gel with bands corresponding to p21 and mouse S16 ribosomal protein RNAs from five wildtype and five p27-/- livers is shown.  

D. Relative levels of p21 mRNA expressed in wildtype and p27-/- mouse livers are shown, with the bar representing the mean ± standard deviation, with levels normalized for S16 expression.  

Fig. 2. p27 and p21 protein expression and localization in the adult mouse liver.  

Tyramide-amplified indirect immunostaining was used to examine p27 and p21 expression in livers of adult male mice.  

A. p27 positive hepatocyte nuclei were detected throughout the wildtype liver lobule and in bile duct epithelium.  

B. p21 protein was not detected in the wildtype liver.  

C. p21 protein was detected in hepatocyte nuclei in p27-/- livers. P, portal vein; C, central vein. Bar represents 50 micrometers.  

Fig. 3. Regulation of p21 expression levels by proteasomal mediated degradation in hepatocytes.  

A. Primary hepatocytes were isolated from two male and three female mice, placed in culture and treated with the proteasome inhibitor MG132. Increased p21 levels were detected by immunoblotting following MG132 treatment. β-actin expression was examined as a control.  

B. To determine the contribution of new translation to the increase in p21 protein levels
following treatment with MG132, cultured primary hepatocytes from three male mice were left untreated or treated with MG132 or cycloheximide (CHX), or MG132 + cycloheximide. Increased p21 levels were detected when hepatocytes were treated with both MG132 and cycloheximide indicating that inhibition of proteasomal-mediated degradation results in increased p21 levels in hepatocytes. Expression of β-actin was examined as a control. C. Relative levels of p21 protein normalized to β-actin expression with bars representing the mean ± standard deviation are shown.

Fig. 4. p21 expression in p27-deficient mouse embryonic fibroblasts and tissues. A. Expression of p21, PCNA, and β-actin were examined in wildtype and p27-deficient mouse embryonic fibroblasts and liver extracts by immunoblotting. Increased p21 levels were detected in the p27-deficient liver extract, but not in serum starved (0hr) or stimulated (24hr) p27-deficient MEFs. A longer exposure was required to detect p21 in liver tissue than in MEFs. Increased levels of PCNA were also detected in the p27-deficient liver. B. Protein lysates were prepared from differentiated tissues of wildtype and p27-deficient mice and p21 levels were compared by immunoblotting. Increased p21 protein levels were detected in multiple tissues of mature p27/-/- mice. Expression of p27 and β-actin expression were examined as controls. Membranes were also stained to confirm equal loading of protein as the β-actin antibody does not recognize adult cardiac actin.

Fig. 5. Increased expression of proliferation associated proteins in the livers of p21 and p27-deficient mice. Expression of proliferation-associated proteins Cdk2, Cdk4, Cdk6, Cyclin A, Cyclin D1, Cyclin E, PCNA, and β-actin was examined using immunoblotting and extracts from four mice of each genotype (wildtype, p21/-/-, p27/-/- and p21/p27/-/-). Reproducible
increases in Cdk2, Cdk4, and PCNA protein levels were detected in extracts from livers of mice deficient for p27.

**Fig. 6. Increased proliferation in the livers of mice lacking both p21 and p27.** Animals were injected with BrdU and sacrificed after two hours. **A.** Tyramide-amplified indirect immunostaining of BrdU is shown in livers of wildtype, p21-/-, p27-/- and p21/p27-/- animals. BrdU immunoreactivity is green, while the blue nuclear stain is DAPI. Greatest numbers of replicating cells are observed in livers of p21/p27-deficient mice (arrowheads). **B.** BrdU labeled cells were counted in twenty fields for each genotype. The majority of fields counted in the p21/p27-deficient liver sections contained two or three labeled cells, while no labeled cells could be detected in most fields counted on sections from the other three genotypes.

**Fig. 7. p21 is associated with Cdk2 and Cdk4 in the livers of p27-deficient animals.** Immunoprecipitation of p27 or p21 from wildtype, p21-/- and p27-/- adult mouse liver extracts was followed by immunoblotting with antibodies specific for p27, p21, Cdk 2 and Cdk 4.
Figure 1

A

Wildtype  p21-/-  

p27  

β-actin

B

Wildtype  p27-/-  

p21 (short exposure)  

p21 (long exposure)  

β-actin

C

Wildtype  p27-/-  

p21  

S16

D

![](http://www.jbc.org/)

**p21 mRNA Levels**

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Figure 2
Figure 3

A

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MG132

p21

β-actin

B

Untreated | MG132 | CHX | MG132 + CHX

p21

β-actin

C

p21 mRNA Levels

MG132

Cycloheximide

-    +    -    +
Figure 4

A

B

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Hours post serum

0hr 24hr

p21
PCNA
β-actin

MEF Liver
## Figure 5

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Figure 6

A

Wildtype  p21(-/-)  p27 (-/-)  p21/p27 (-/-)

B

Wildtype  p21(-/-)  p27 (-/-)  p21/p27 (-/-)

# of fields counted

Positive nuclei per field

Positive nuclei per field

Positive nuclei per field

Positive nuclei per field
Figure 7

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**IP:p27**

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p21 functions to maintain Quiescence of p27 deficient hepatocytes
Young Hye Kwon, Aleksandra Jovanovic, Michael S. Serfas, Hiroaki Kiyokawa and
Angela L. Tyner

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