

ORIGINAL ARTICLE

p27^{Kip1} represses the Pitx2-mediated expression of p21^{Cip1} and regulates DNA replication during cell cycle progressionE Gallastegui¹, A Biçer¹, S Orlando¹, A Besson^{2,3,4}, MJ Pujol¹ and O Bachs¹

The tumor suppressor p21 regulates cell cycle progression and peaks at mid/late G₁. However, the mechanisms regulating its expression during cell cycle are poorly understood. We found that embryonic fibroblasts from p27 null mice at early passages progress slowly through the cell cycle. These cells present an elevated basal expression of p21 suggesting that p27 participates to its repression. Mechanistically, we found that p27 represses the expression of Pitx2 (an activator of p21 expression) by associating with the ASE-regulatory region of this gene together with an E2F4 repressive complex. Furthermore, we found that Pitx2 binds to the p21 promoter and induces its transcription. Finally, silencing Pitx2 or p21 in proliferating cells accelerates DNA replication and cell cycle progression. Collectively, these results demonstrate an unprecedented connection between p27, Pitx2 and p21 relevant for the regulation of cell cycle progression and cancer and for understanding human pathologies associated with p27 germline mutations.

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INTRODUCTION

Cell cycle progression is regulated by the sequential activation of members of the cyclin-dependent kinase (Cdk) family.^{1,2} At G₁ phase of the cell cycle, the activated Cdk phosphorylate transcriptional repressor complexes containing E2F4/p130 or E2F1/pRb, associated with different transcriptional co-repressors.³ These complexes repress the expression of genes necessary for G₁ progression and the onset of DNA replication. Sequential Cdk phosphorylation of p130, pRb and other proteins of these complexes during G₁ leads to their disruption, thus allowing the expression of G₁/S genes.⁴

The tumor suppressor p27^{Kip1} (p27) binds to and inhibits the activity of many cyclin-Cdk complexes.^{5,6} Interestingly, in human tumors low levels of p27 are associated with poor prognosis and germline mutations of the p27 gene are involved in the generation of a specific group of human multiple endocrine neoplasia.

Recently, it has been shown that in addition to its classical role as a Cdk-inhibitor, p27 also performs other Cdk-independent functions⁷ among them the regulation of transcription.^{5,8} Specifically, it has been shown that p27 represses transcription by directly associating with p130/E2F4 complexes on the regulatory domains of specific p27-target genes (p27-TGs) that participate in cell cycle progression.⁹ A recent report also revealed a role of p27 in reprogramming mouse fibroblasts into induced pluripotent stem cells by regulating the expression of Sox2 in a p130/E2F4 dependent manner.¹⁰ p27 also regulates transcription by interacting with the transcription factors Ets-1⁹ and Neurogenin 2.¹¹ Thus, p27 participates in cell cycle regulation by modulating the activity of cyclin-Cdk complexes but also by regulating transcription of cell cycle genes.

The transcriptional programs specifically regulated by p27 during cell cycle still remain to be precisely identified. To define

these programs and to determine their functional relevance, it has to be taken into account the levels and expression kinetics of p27 during cell cycle progression.¹² In quiescent cells, levels of p27 are high and after proliferative stimulus it is progressively degraded by the ubiquitin-proteasome pathway.¹³ Thus, it can be hypothesized that during quiescence and at early G₁ phase of the cell cycle the high levels of p27 can participate in the repression of cell cycle genes that would be subsequently de-repressed along cell cycle progression concomitantly with p27 degradation. In addition to its participation in a normal cell cycle by regulating transcription, the role of p27 in tumorigenesis could also be related to its capability to repress gene expression.

In this work, on analyzing the gene expression profiles in quiescent cells lacking p27 we observed that the expression of the tumor suppressor p21^{Cip1} (p21) (a member of the same family of Cdk-inhibitors) is regulated by p27. Specifically, we report here that p27 normally exerts a negative feedback on p21 expression: p27 directly represses the expression of the transcription factor Pitx2 (Pituitary homeobox-2) which in turn maintains decreased p21 levels. Consequently, in cells lacking p27, de-repression of Pitx2 causes the up-regulation of p21. We report here a new mechanism by which p27 regulates cell cycle progression by transcriptionally regulating the expression of Pitx2 and p21.

RESULTS

Differential gene expression in p27^{-/-} vs p27^{WT} MEFs

Expression microarray analyses performed on quiescent mouse embryonic fibroblasts (MEFs) from p27^{-/-} and p27^{WT} animals revealed that 4022 genes were differentially expressed in p27^{-/-} MEFs. Specifically, 1844 genes were up-regulated and 2178 down-regulated (Figure 1a). Maximal up-regulation was for Igfbp2

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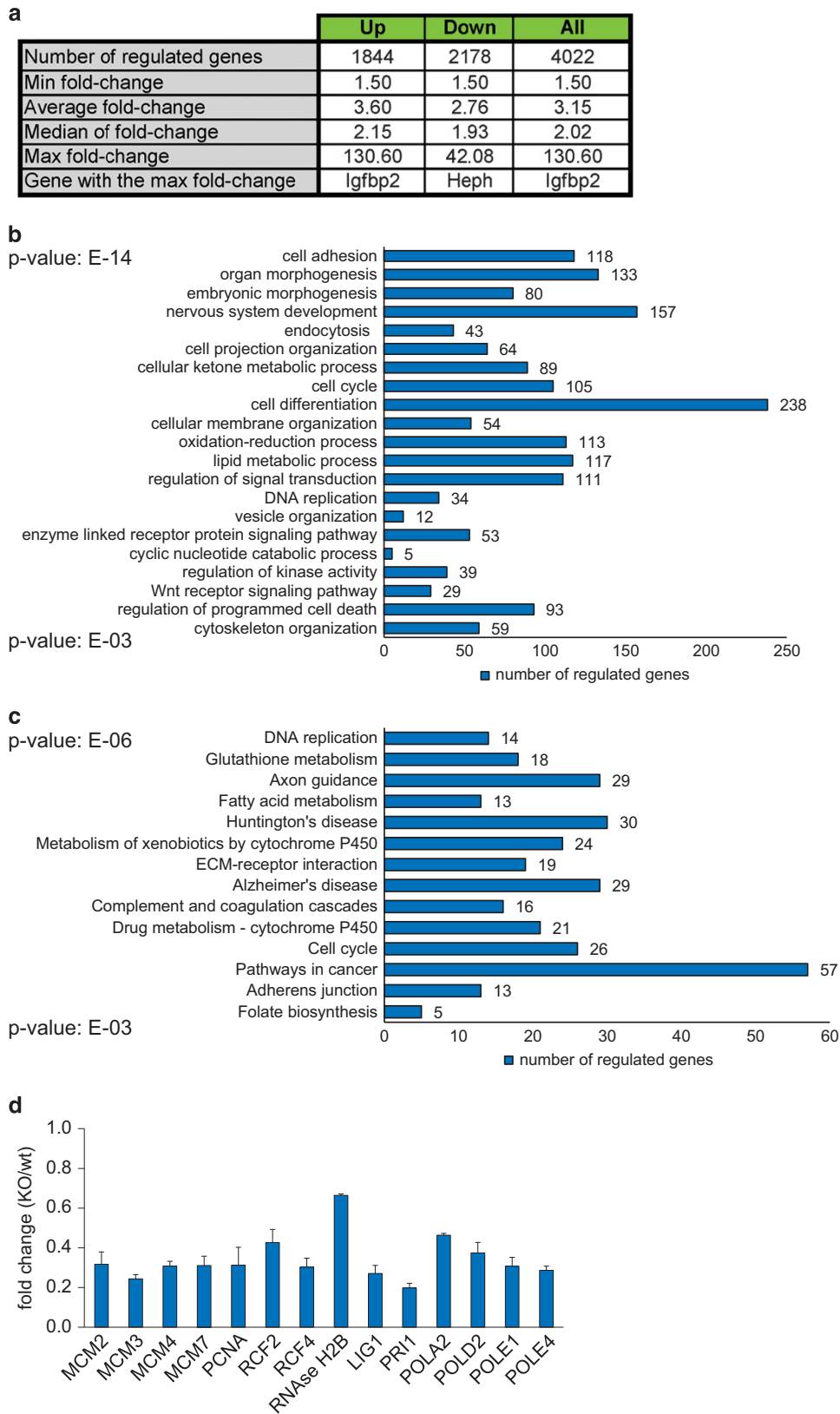


Figure 1. Identification of differentially expressed genes in quiescent $p27^{-/-}$ vs $p27^{WT}$ MEFs. **(a)** Number of genes differentially expressed in $p27^{-/-}$ vs $p27^{WT}$ MEFs and main characteristics of the analysis. **(b)** GO analysis of the differentially expressed genes in $p27^{-/-}$ MEFs. Numbers on the right of the bars indicate the number of differentially expressed genes. **(c)** Pathway analysis (Kegg) of the differentially expressed genes in $p27^{-/-}$ MEFs. Numbers on the right of the bars indicate the number of differentially expressed genes. **(d)** Levels of mRNA of the 14 genes involved in DNA replication differentially expressed in $p27^{-/-}$ MEFs (Kegg analysis) as determined by qPCR. Results are the mean value \pm s.d. of three independent experiments and are expressed as the fold change of KO vs wt.

(130.60-fold change) whereas maximal down-regulation was for Heph (42.08 foldchange).

Despite the fact that p27 associates with a number of gene promoters as observed by ChIP on chip,⁹ the high number of genes up- or down-regulated in p27^{-/-} MEFs suggests that p27 can additionally regulate transcription by associating with other type of regulatory sequences as enhancers or silencers. Moreover, the absence of p27 may directly affect the expression of TFs that subsequently would be responsible for many of the expression changes observed in these cells. In fact, we observed the up-regulation (> 3 foldchange) of 42 TFs (Supplementary Figure S1A) and the down-regulation (> 3 foldchange) of 24 TFs (Supplementary Figure S1B) in p27^{-/-} MEFs. The analysis of this list revealed that TFs regulated by p27 are involved in important biological functions. As examples, seven *Hoxa* genes (*Hoxa1-Hoxa7*) belonging to a cluster involved in embryo development¹⁴ are up-regulated in p27^{-/-} MEFs suggesting a role of p27 in this process. p27 can also participate in patterning and morphogenesis because three of the five Zic homologs (*Zic1, 2* and *5*) are up-regulated in cells lacking p27.¹⁵

Gene ontology (GO) analysis of all the differentially expressed genes showed that the most significant groups were cell adhesion and organ morphogenesis (Figure 1b; Supplementary Figure S2). Other prominent groups were nervous system development,

endocytosis, cell cycle, cell differentiation, oxidation-reduction process, regulation of signal transduction, DNA replication and Wnt receptor signaling pathway, among others (Figure 1b; Supplementary Figure S2).

Pathway analysis (Kegg) of these genes revealed that 44 pathways were altered in p27^{-/-} MEFs and Figure 1c shows the most significant ones. Specifically, in the DNA replication pathway, 14 genes were down-regulated in p27^{-/-} MEFs (Supplementary Figure S3A; S3B) suggesting that progression through cell cycle might be impaired in these cells. This possibility is reinforced by the observation that another significantly altered pathway is cell cycle. In this case, 26 genes of this pathway were differentially expressed in p27^{-/-} MEFs, most of them down-regulated (Figure 1c; Supplementary Figure S3A; S4). The reduced expression of DNA replication genes was validated by quantitative PCR (qPCR) analysis (Figure 1d).

DNA replication is decreased in p27^{-/-} MEFs

To study the effect of the absence of p27 on cell cycle, FACS analysis was performed in primary p27^{WT} and p27^{-/-} MEFs at early passages. Results indicated that in p27^{WT} MEFs, S phase started at 8 h, with a maximal peak of DNA replication at 24 h (Figure 2a; Supplementary Table S2). In contrast, p27 null cells

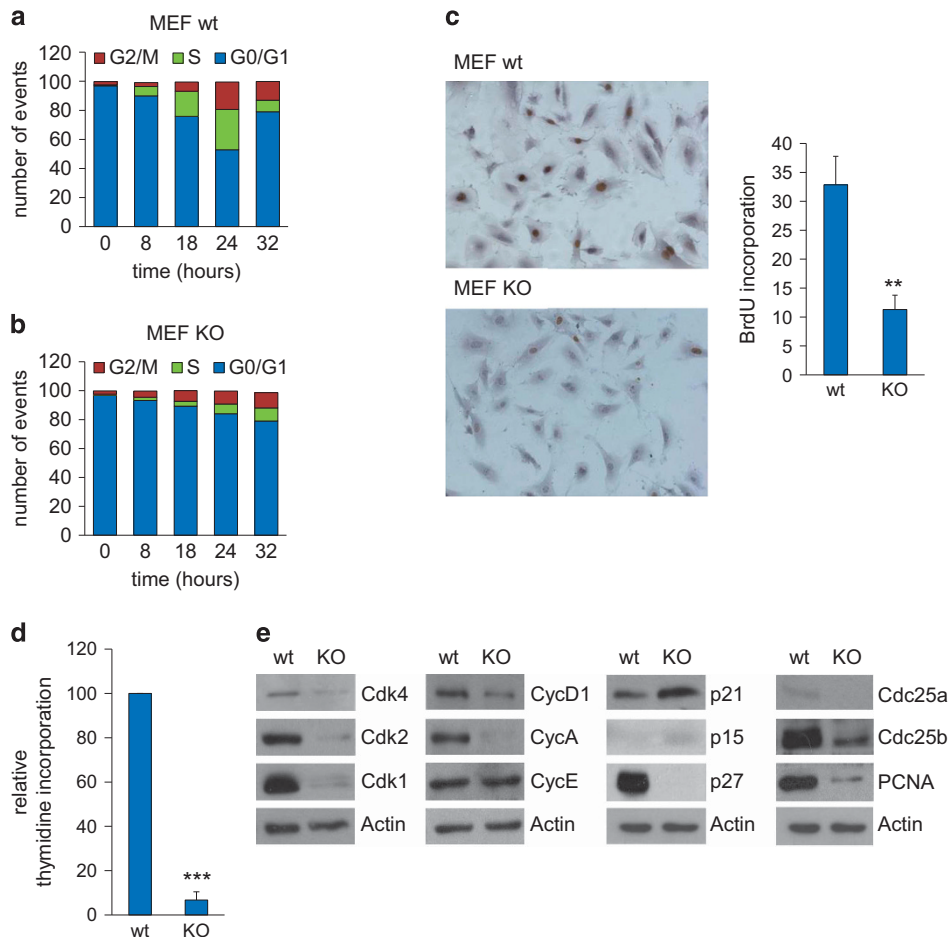


Figure 2. DNA replication in p27^{-/-} and p27^{WT} MEFs. p27^{WT} (a) and p27^{-/-} MEFs (b) were synchronized by serum starvation and released for indicated time intervals. The number of cells at the different cell cycle phases was determined by FACS. (c) (Left) Detection of BrdU incorporation in asynchronously growing p27^{WT} (upper panel) and p27^{-/-} MEFs (bottom panel) by immunostaining using anti-BrdU antibody. (Right) Immunostaining quantification. Results are the mean value ± s.d. of three independent experiments and are expressed as the percentage of BrdU positive cells vs total cells. ***P* < 0.01. (d) Determination of thymidine incorporation in asynchronously growing p27^{WT} and p27^{-/-} MEFs. Results are the mean value ± s.d. of three independent experiments and are expressed as cpm/mg of protein. They are represented as percentage relative to WT. ****P* < 0.001. (e) Levels of different cell cycle proteins determined by WB in asynchronously growing p27^{-/-} and p27^{WT} MEFs. Actin was used as a loading control.

progressed very slowly through cell cycle being the levels of DNA replication at 24 h similar to those at 8 h in control cells (Figure 2b; Supplementary Table S2). These results were confirmed by experiments of BrdU incorporation into the cells. As shown in Figure 2c the amount of BrdU incorporated into p27^{WT} MEFs was higher than in p27^{-/-} MEFs. Finally, thymidine incorporation experiments also confirmed the reduced DNA replication in p27 null cells (Figure 2d).

The down-regulation of genes involved in cell cycle progression in p27^{-/-} MEFs (Supplementary Figure S4) raised the possibility that reduced levels of cell cycle regulatory proteins could participate in the decreased rate of DNA replication observed in these cells. To analyze this possibility, the status of key cell cycle regulatory proteins were analyzed in primary p27^{WT} and p27^{-/-} MEFs at early passages by western blotting (WB). Results showed

that proteins as Cdk4, Cdk2, Cdk1, cyclin D1 and cyclin A were significantly decreased in p27^{-/-} MEFs. In contrast, the levels of the CKIs p15 and p21 were augmented (Figure 2e).

Role of p21 on the decrease of DNA replication in p27^{-/-} MEFs
Cdk2 activity is crucial for the triggering and progression of DNA replication.² Thus, decreased levels of Cdk2 in p27^{-/-} MEFs could be involved in the reduced rate of DNA replication. We first validated this decrease of the Cdk2 mRNA in p27^{-/-} MEFs by qPCR (Supplementary Figure S5A). Then, Cdk2 activity was determined and we observed that it was substantially reduced in cells lacking p27 (Figure 3a and b). Anti-Cdk2 used in the IP to determine Cdk2 activity also immunoprecipitates cyclins A and E (Supplementary Figure S5B). Cdk2 activity associated to cyclin A or

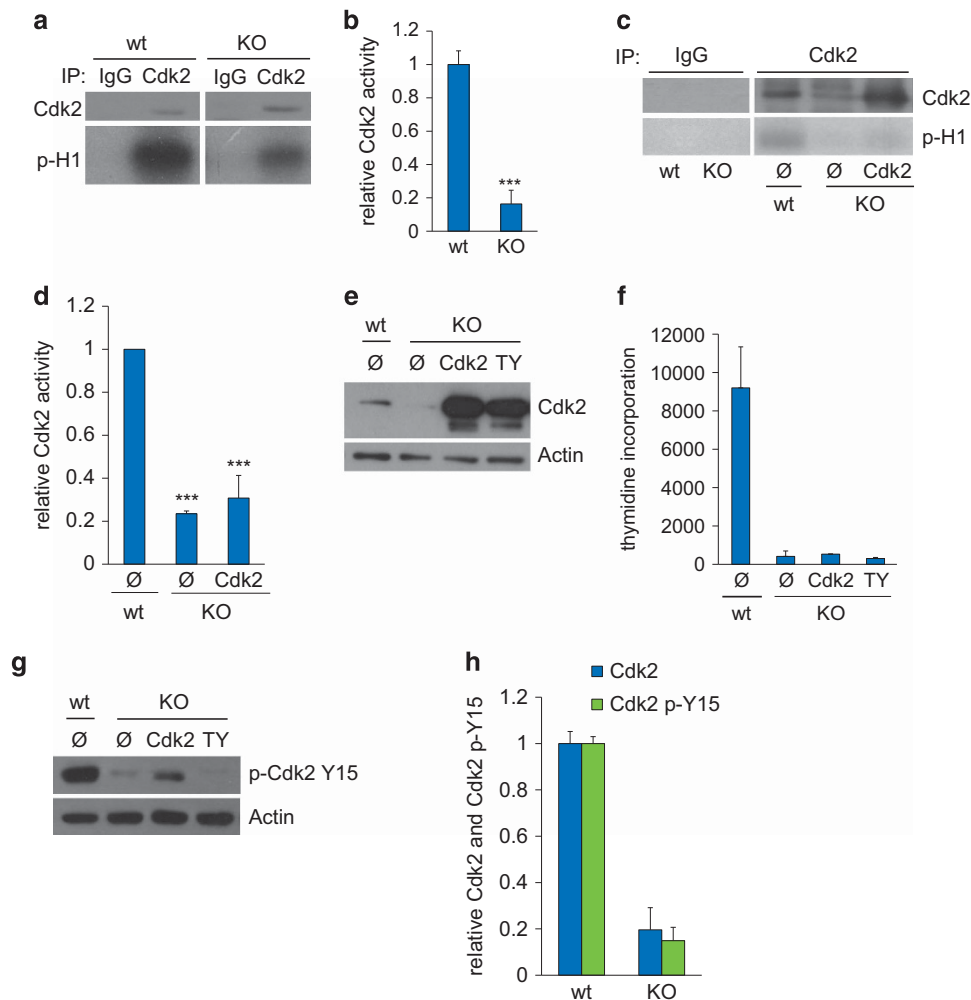


Figure 3. Cdk2 activity in asynchronously growing p27^{-/-} and p27^{WT} MEFs. **(a)** To determine Cdk2 activity in p27^{-/-} and p27^{WT} MEFs cell extracts were immunoprecipitated with anti-Cdk2 or IgG as a control. The immunoprecipitates were subsequently incubated with histone H1 for 30 min in the presence of [³²P]-ATP. Samples were loaded on the same gel and exposed identically. The amount of Cdk2 was determined by WB and radioactive phosphorylated histone H1 by a PhosphorImager. **(b)** Quantification of experiments in **a**. Cdk2 specific activity results are the mean value \pm s.d. of three independent experiments and are expressed as the amount of p-H1 vs Cdk2. They are represented relative to WT *** $P < 0.001$. **(c)** p27^{-/-} MEFs were transfected with Cdk2^{WT} or with an empty vector (Ø). Then Cdk2 activity was determined as in **a**. **(d)** Quantification of experiments in **c**. Cdk2 specific activity results are the mean value \pm s.d. of three independent experiments and are expressed as the amount of p-H1 vs Cdk2. They are represented relative to WT *** $P < 0.001$. **(e)** Cells were transfected with a vector containing Cdk2^{WT}, Cdk2^{TY} or with an empty vector (Ø). Then, the levels of Cdk2 were determined by WB using anti-Cdk2 antibody. Actin was used as a loading control. **(f)** Determination of thymidine incorporation in p27^{-/-} MEFs over-expressing Cdk2^{WT} or Cdk2^{TY}. Results were expressed as cpm/mg of protein and represent the mean value \pm s.d. of three independent experiments. **(g)** The amount of the Y15 phosphorylated form of Cdk2 was determined by WB in p27^{-/-} and p27^{WT} MEFs transfected with Cdk2^{WT}, the Cdk2^{TY} or with an empty vector (Ø) (see **e**). Actin was used as a loading control. **(h)** Quantification of experiments in **g**. Results are the mean value \pm s.d. of three independent experiments and are expressed as the amount of Cdk2 vs p-Y15-Cdk2. They are represented relative to WT.

cyclin E were also decreased in p27^{-/-} cells (Supplementary Figure S5C). We subsequently performed rescue experiments by over-expressing Cdk2^{WT} in p27^{-/-} MEFs and we observed that only a slight increase in its activity (Figure 3c and d) but not in DNA replication (Figure 3e and f) was produced. Similar results were obtained in p27^{-/-} MEFs over-expressing an active Cdk2 mutant (Cdk2^{TY}) that is resistant to inactivation by Wee-1 kinase-regulated phosphorylation at T14 and Y15¹⁶ (Figure 3e and f). Over-expression of cyclin A also gave similar results (data not shown). Furthermore, the combined over-expression of cyclin A and Cdk2 did not significantly increase Cdk2 activity (Supplementary Figure S5D; S5E). In contrast, in wt-MEFs the over-expression of Cdk2^{TY} increased DNA replication (Supplementary Figure S6). All these results suggested the possibility that the reduced Cdk2 activity in p27^{-/-} MEFs could be produced not only by the low Cdk levels but also by an inhibitory phosphorylation or by association to a Cdk inhibitor (CKI). Thus, despite the over-expression of the mutant Cdk2^{TY} in p27^{-/-} cells suggested that an inhibitory phosphorylation should not be the cause of the low Cdk2 activity, we studied the Y15 phosphorylation of Cdk2 in these cells. Results indicated that this phosphorylation was even lower in p27^{-/-} than in control MEFs (Figure 3g and h) indicating that the low Cdk2 activity is not related to its inhibitory phosphorylation at Y15.

The role of CKIs in the low Cdk2 activity observed in p27^{-/-} MEFs was then studied. Figure 2e shows that p21 protein levels are higher in p27^{-/-} than in p27^{WT} MEFs. Thus, we examined the association of p21 with cyclin-Cdk2 complexes by immunoprecipitation (IP) experiments. Results show a two fold increase of p21 associated with Cdk2 complexes in cells lacking p27 vs control cells (Figure 4a; Supplementary Figure S7A). The knock down of p21 (Supplementary Figure S7B) in p27^{-/-} cells induced significant increases in Cdk2 activity (Figure 4b; Supplementary Figure S7C), p130 phosphorylation (Figure 4c), PCNA levels (a protein component of the DNA replication machinery) (Figure 4d) and DNA synthesis (Figure 4e). All these results indicate that the increased amount of p21 in cells lacking p27 is responsible of the low Cdk2 specific activity and, as a consequence, of the decreased rate of DNA replication observed in these cells.

Transcription of p21 is regulated by p27

The increased levels of p21 in p27^{-/-} MEFs suggested that p27 could regulate p21 expression. To this aim, we studied the effect of p27 knock-down on p21 levels. Supplementary Figure S8A

shows that the decrease of p27 induced an elevation of p21 (right panel) similar to that observed in p27^{-/-} MEFs (left panel). Also the levels of p21 mRNA in p27 null cells (Figure 4f) and in cells knocked down for p27 (Figure 4g) were higher than in control cells. These elevations in mRNA were due to increased transcription as observed by qPCR using exon-intron primers to detect the first transcript (Figure 4h). Nevertheless, the expression kinetics of p21 during cell cycle was similar to control cells (Supplementary Figure S8B). Finally, luciferase assays using a 2.3 kb region of the p21 promoter were performed. Results revealed that reduction of p27 induced the expression of p21 (Figure 4i). However, attempts to demonstrate the direct association of p27 with the promoter of p21 were unsuccessful. These results suggested that p27 regulates the expression of p21 probably through an indirect mechanism.

Pitx2 expression is repressed by p27

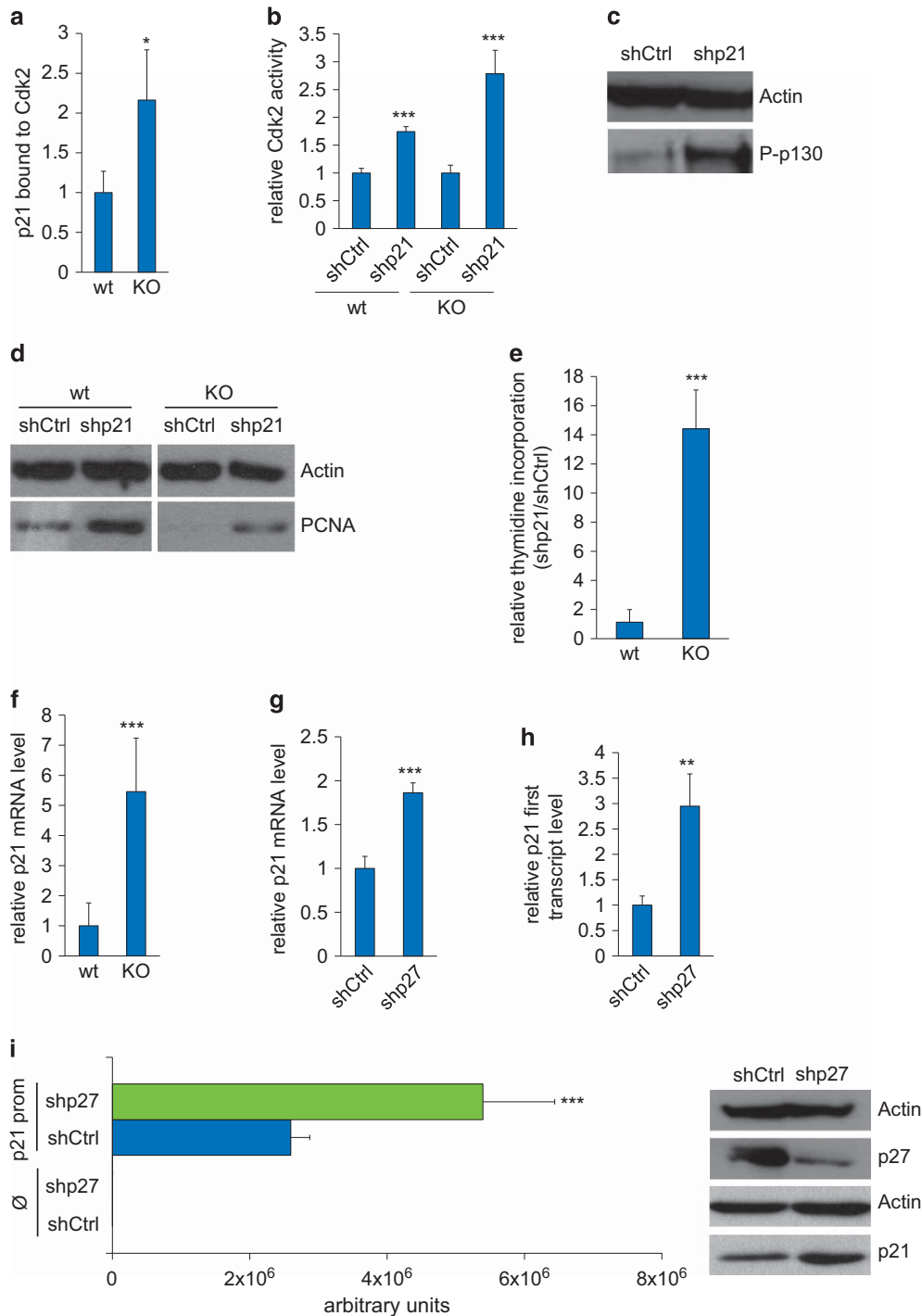
Data from the expression microarray revealed that the transcription factor Pitx2, known to be involved in the regulation of p21 transcription,¹⁷ was elevated in p27^{-/-} MEFs (Supplementary Figure S1). Thus, we analyzed the putative contribution of this protein in p21 expression in p27^{-/-} MEFs. We first validated the increased levels of Pitx2 mRNA in p27^{-/-} MEFs by qPCR (Figure 5a) and then analyzed the protein levels in these cells. As shown in Figure 5b Pitx2 protein was significantly increased in p27^{-/-} MEFs. We also observed that knocking down p27 in control MEFs induced the elevation of Pitx2 mRNA (Figure 5c). The reduction of p27 also induced the transcription of Pitx2 as assessed with luciferase assays with the a vector including a described enhancer of the Pitx2 gene located in a region of intron 5 (ASE region)¹⁸ (Figure 5d). Moreover, ChIP analysis revealed that p27 associated with the ASE region of the Pitx2 gene (Figure 5e). Recent reports showed that p27 can interact with gene promoters in association with the repressive complex p130-E2F4-SIN3A.^{9,10} Thus, we aimed to study whether p27 might be recruited in this manner to the Pitx2-ASE region. In these repressive complexes, E2F4 plays an essential role because it directly associates with the DNA. Thus, we focused our attention on this protein. We first performed ChIP assays using antibodies against E2F4 in quiescent WT-MEFs. Interestingly, we observed the association of E2F4 with the Pitx2-ASE region indicating that this repressive complex associates with this chromatin region (Figure 5f). Next, we studied whether the knock down of E2F4 would result in the de-repression of Pitx2. We observed that the reduction of E2F4 with a specific shRNA resulted in a significant up-regulation of Pitx2 expression

Figure 4. Levels of p21 in p27^{-/-} MEFs and its role in Cdk2 activity and DNA replication. **(a)** Cell extracts from asynchronously growing p27^{-/-} and p27^{WT} MEFs were immunoprecipitated with anti-Cdk2 or IgG as a control. The levels of Cdk2 and p21 in the immunoprecipitates were determined by WB. The amount of p21 associated with Cdk2 was quantified by dividing the amount of p21 vs Cdk2. Results are the mean value ± s.d. of three independent experiments and are relative to WT **P* < 0.05. **(b)** Asynchronously growing p27^{-/-} and p27^{WT} MEFs were infected with shp21 or shCtrl. Then, cell extracts were immunoprecipitated with anti-Cdk2 or IgG, used as a control. The immunoprecipitates were subsequently incubated with histone H1 for 30 min in the presence of [³²P]-ATP. Finally, the amount of Cdk2 was determined by WB and radioactive phosphorylated histone H1 by a PhosphorImager. Cdk2 specific activity results are the mean value ± s.d. of three independent experiments and are expressed as the amount of p-H1 vs Cdk2. They are represented relative to shCtrl ****P* < 0.001. **(c)** Asynchronously growing p27^{-/-} MEFs were infected with shp21 or shCtrl. Then, the levels of phosphorylated p130 were determined by WB. Actin was used as a loading control. **(d)** PCNA levels in asynchronously growing p27^{-/-} and p27^{WT} MEFs infected with shp21 or shCtrl were determined by WB. Actin was used as a loading control. **(e)** Determination of thymidine incorporation in asynchronously growing p27^{-/-} and p27^{WT} MEFs knocked down for p21. Results are the mean value ± s.d. of three independent experiments and are expressed as cpm/mg of protein. They are calculated by dividing the values observed in cells transfected with shp21 vs shCtrl. They are represented as percentage relative to WT ****P* < 0.001. **(f)** Levels of p21 mRNA were determined by qPCR in asynchronously growing p27^{WT} and p27^{-/-} MEFs. Results are the mean value ± s.d. of three independent experiments and are relative to WT ****P* < 0.001. **(g)** Levels of p21 mRNA were determined by qPCR in p27^{WT} MEFs infected with shp27 or shCtrl. Results are the mean value ± s.d. of three independent experiments and are relative to shCtrl ****P* < 0.001. **(h)** Levels of p21 first transcript RNA were determined by qPCR in p27^{WT} MEFs infected with shp27 or shCtrl. The first transcript was determined using a primer of the first exon and another one of the first intron. Results are the mean value ± s.d. of three independent experiments and are relative to shCtrl ***P* < 0.01. **(i)** The effect of knocking down p27 on p21 transcription was determined by luciferase assays using the pGL2 vector including the p21 gene promoter (-2.3 kb) transfected in HEK-293 T cells. Results are the mean value ± s.d. of three independent experiments ****P* < 0.001 (left panel). The levels of p27 and p21 in cells infected with shp27 or with shCtrl were determined by WB (right panel). Actin was used as a loading control.

(Figure 5g). Finally, we studied the expression kinetics of Pitx2 and p21 during G₁ in primary p27^{WT} and p27^{-/-} MEFs at early passages. We observed that Pitx2 mRNA levels were much higher in p27^{-/-} MEFs than in control MEFs (Figure 5a). The amount of Pitx2 mRNA was progressively increasing during G₁ in control cells (Figure 5h). Interestingly, p21 expression followed a similar kinetics (Figure 5i). In contrast, in quiescent p27^{-/-} MEFs, Pitx2 expression kinetics was different, being higher than in control cells at time 0 h, decreasing at 4 h to subsequently increase at 8 h (Figure 5j). Despite of that, p21 mRNA levels progressively increased during G₁ in p27^{-/-} cells (Figure 5k). All these data indicate that p27 represses Pitx2 expression by associating with the regulatory ASE region of this gene.

Pitx2 regulates cell cycle progression by directly inducing the expression of p21

To further investigate the role of Pitx2 on the regulation of p21, the effect of Pitx2 over-expression on p21 transcription was examined using a luciferase reporter gene containing the p21 promoter. As shown in Figure 6a, Pitx2 over-expression clearly induced p21 transcription. Next, by ChIP experiments we found that Pitx2 associated with the p21 promoter (Figure 6b). We next evaluated the effect of silencing Pitx2 with a specific shRNA on p21 transcription in p27^{-/-} MEFs. As shown in Figure 6c knocking down Pitx2 (upper panel) decreased p21 transcription as observed by detecting the first transcript product (bottom panel). Finally,



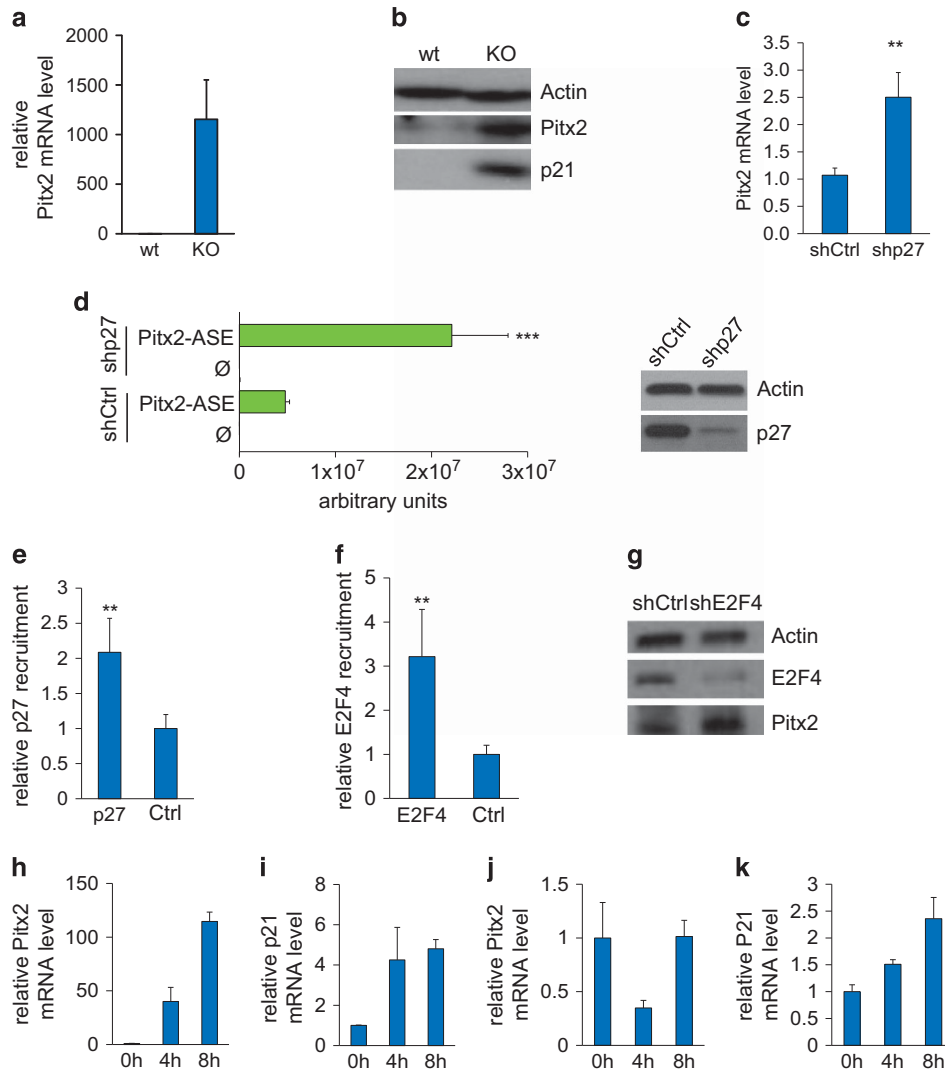


Figure 5. p27 regulates Pitx2 transcription. **(a)** Levels of mRNA for Pitx2 in asynchronously growing p27^{WT} and p27^{-/-} MEFs were determined by qPCR. Results are the mean value \pm s.d. of three independent experiments and are relative to WT. **(b)** Levels of Pitx2 and p21 proteins in asynchronously growing p27^{WT} and p27^{-/-} MEFs were determined by WB. Actin was used as a loading control. **(c)** Levels of Pitx2 mRNA were determined by qPCR in p27^{WT} MEFs infected with shp27 or shCtrl. Results are the mean value \pm s.d. of three independent experiments and are relative to shCtrl $**P < 0.01$. **(d)** The effect of knocking down p27 on Pitx2 transcription was determined by luciferase assays using the pGL2 vector including the regulatory ASE region of the Pitx2 gene transfected in HEK-293 T cells. Results are the mean value \pm s.d. of three independent experiments $***P < 0.001$ (left panel). The levels of p27 protein in cells infected with shp27 or shCtrl were determined by WB (right panel). Actin was used as a loading control. **(e)** The association of p27 with the regulatory ASE-region of the Pitx2 gene was analyzed by ChIP assays using anti-p27 antibodies or without antibodies (Ctrl) as a control. Results are the mean value \pm s.d. of three independent experiments and are relative to the control $**P < 0.01$. **(f)** The association of E2F4 with the regulatory ASE-region of the Pitx2 gene was analyzed by ChIP assays using anti-E2F4 antibodies or without antibodies (Ctrl) as a control. Results are the mean value \pm s.d. of three independent experiments and are relative to the control $**P < 0.01$. **(g)** The levels of Pitx2 and E2F4 proteins, in p27^{WT} MEFs infected with shpE2F4 or shCtrl, were determined by WB. Actin was used as a loading control. **(h)** The levels of Pitx2 mRNA were determined by qPCR at different times after proliferative activation in p27^{WT} MEFs. Results are the mean value \pm s.e. of three independent experiments and are relative to the control. **(i)** The levels of p21 mRNA were determined by qPCR at different times after proliferative activation in p27^{WT} MEFs. Results are the mean value \pm s.e. of three independent experiments and are relative to the control. **(j)** The levels of Pitx2 mRNA were determined by qPCR at different times after proliferative activation in p27^{-/-} MEFs. Results are the mean value \pm s.e. of three independent experiments and are relative to the control. **(k)** The levels of p21 mRNA were determined by qPCR at different times after proliferative activation in p27^{-/-} MEFs. Results are the mean value \pm s.e. of three independent experiments and are relative to the control.

the effect of Pitx2 silencing on p21 transcription was studied using a luciferase reporter gene including the p21 promoter. Figure 6d shows that Pitx2 decrease (bottom panel) results in a diminution of p21 transcription (upper panel). Thus, our data indicate that p21 expression is directly induced by Pitx2. Next we wondered whether the progression of primary p27 null MEFs through the cell cycle could be speeded by the knockdown of Pitx2.

Interestingly, we observed that reducing the level of Pitx2 by specific shRNAs in p27 null MEFs (Figure 6f) increased Cdk2 activity (Figure 6e; Supplementary Figure S9A), p130 phosphorylation (Figure 6f), the levels of the DNA replication marker PCNA (Figure 6g) and DNA synthesis as detected by thymidine incorporation (Figure 6h) and by the increased number of cells in S phase as detected by FACS analysis (Supplementary

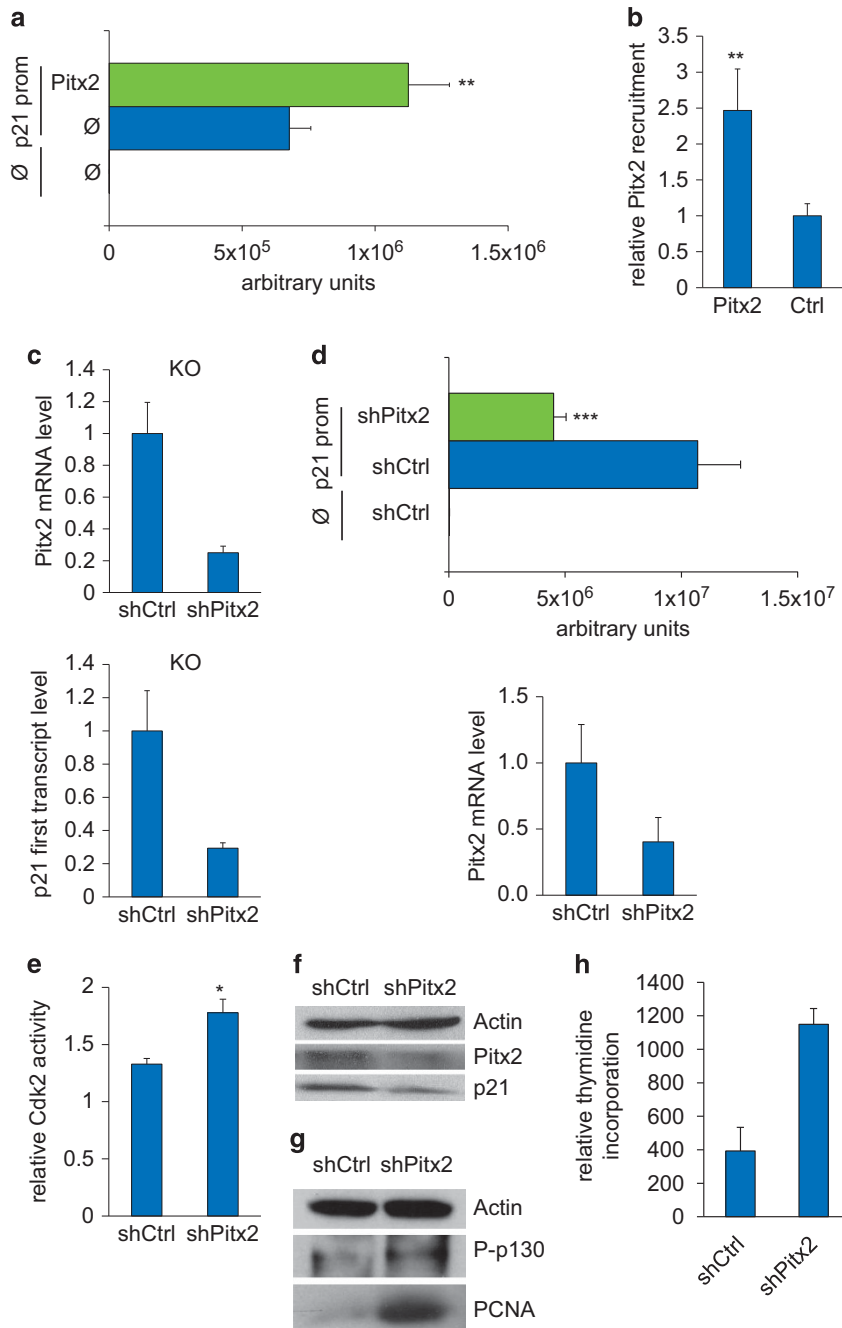


Figure 6. Pitx2 regulates p21 transcription. **(a)** The effect of Pitx2 over-expression on p21 transcription was determined by luciferase assays using a pGL2 vector including the p21 promoter (–2.3 kb). Cells were infected with a Pitx2 containing vector or with an empty vector (∅) that was used as a control. Results are the mean value ± s.d. of three independent experiments $**P < 0.01$. **(b)** The association of Pitx2 with the p21 promoter was analyzed by CHIP assays using anti-Pitx2 antibodies or without antibodies (Ctrl) as a control. Results are the mean value ± s.d. of three independent experiments and are relative to the control $**P < 0.01$. **(c)** Levels of Pitx2 mRNA (upper panel) and p21 first transcript (bottom panel) were determined by qPCR in p27^{-/-} MEFs infected with shPitx2 or shCtrl. Results are the mean value ± s.d. of three independent experiments and are relative to shCtrl. **(d)** The effect of Pitx2 silencing on p21 transcription was determined by luciferase assays using a pGL2 vector including the p21 promoter (–2.3 kb). Cells were infected with shPitx2 or shCtrl. Results are the mean value ± s.d. of three independent experiments $***P < 0.01$ (upper panel). The levels of Pitx2 mRNA were determined by qPCR after infection with shCtrl or shPitx2. Results are the mean value ± s.d. of three independent experiments (bottom panel). **(e)** p27^{-/-} MEFs were infected with shPitx2 or shCtrl. Then, cell extracts were immunoprecipitated with anti-Cdk2 or IgG as a control. The immunoprecipitates were subsequently incubated with histone H1 for 30 min in the presence of [³²P]-ATP. Finally, the amount of Cdk2 was determined by WB and radioactive phosphorylated histone H1 by a PhosphorImager. Cdk2 specific activity results are the mean value ± s.d. of three independent experiments and are expressed as the amount of p-H1 vs Cdk2 $*P < 0.05$. **(f)** p27^{-/-} MEFs were infected with shPitx2 or shCtrl. Then, the levels of Pitx2 and p21 were determined by WB. Actin was used as a loading control. **(g)** p27^{-/-} MEFs were infected with shPitx2 or shCtrl. Then, the levels of phosphorylated p130 and PCNA were determined by WB. Actin was used as a loading control. **(h)** p27^{-/-} MEFs were infected with shPitx2 or shCtrl. Then, thymidine incorporation was determined. Results are the mean value ± s.d. of three independent experiments and are expressed as cpm/mg of protein.

Figure S9B). Collectively, these results provide evidences supporting the concept that p27 is a negative regulator of Pitx2 that in turn is an activator of p21 during cell cycle progression.

DISCUSSION

The identification of p27 as a transcriptional regulator opened a new paradigm for understanding its participation in the regulation of normal cell cycle progression and in tumorigenesis. Thus, its role in these processes can be performed not only by acting as a Cdk modulator but also by regulating specific transcriptional programs. However, these programs still are not well defined. The evidence that quiescent cells have high levels of p27 and that after proliferative stimuli it is progressively degraded suggested a participation of p27 in the repression of cell cycle genes that would be subsequently de-repressed along cell cycle concomitantly with its degradation. In this work we aimed to study the transcriptional programs regulated by p27 in quiescent cells. Specifically, we analyzed the gene expression profiling in quiescent MEFs from p27^{-/-} vs p27^{WT} mice.

GO analysis of differentially expressed genes in p27 null cells revealed that the most significant transcription program regulated by p27 is cell adhesion. Importantly, data also show that p27 regulates the expression of genes belonging to several groups involved in cell proliferation. These groups comprise cell cycle, DNA replication and cell signaling. These results validate the hypothesis of a role of p27 in the transcriptional modulation of cell cycle regulatory genes. Other programs regulated by p27 consist of metabolism, respiration, apoptosis, cytoskeleton organization and endocytosis. Finally, p27 also regulates the expression of genes involved in cell differentiation, morphogenesis and development.

We focused our attention on the role of p27 on the regulation of the DNA replication transcriptional program. The majority of genes involved in DNA replication are negatively regulated by p130/E2F4 complexes¹⁹ and it is known that p27 associates with these complexes.^{9,10} Thus, the down-regulation of genes of this pathway in cells lacking p27 and the observation that primary p27^{-/-} MEFs at early passages progressed very slowly through the cell cycle was completely unexpected. We realized that the slow progression through the cell cycle is only observed in primary p27^{-/-} MEFs at early passages of culture since p27^{-/-} MEFs with more passages proliferate as or faster than control MEFs.

The expression analysis of cell cycle regulatory genes revealed decreases of several cyclins, Cdks and Cdc25 and increases of CKIs, as Cdkn2a and Cdkn2b, in p27^{-/-} MEFs that could explain the slow progression of these cells through cell cycle. WB analysis validated these variations and additionally showed an increase of p21 that was not detected in the microarray analysis perhaps due to low sensitivity of the probes or to the fact that the gene expression profiling was performed in quiescent cells. In agreement with results reported here, elevated levels of p21 were also observed in different tissues from adult p27^{-/-} mice.²⁰ More specifically, authors indicate that in liver from p27^{-/-} mice the amount of p21 is increased, although, in this case, the proposed mechanism is not at the transcriptional level but to a decreased degradation of p21 protein. These different mechanisms might reflect differences in the experimental systems or that diverse regulatory pathways for the p27-dependent p21 expression can exist on depending of the cellular type. Our results are also in agreement with a previous report showing that p21 down-regulates the levels of cdc25A and in such a way modulates Cdk2 activity.²¹ As a consequence of the high levels of p21 in p27^{-/-} cells, its association with cyclin-Cdk2 complexes was higher than in control cells and this led to a decrease of Cdk2 activity. Likely, the reduction of Cdk2 activity is responsible for the down-regulation of DNA replication related genes. This is because Cdk2-dependent phosphorylation of proteins of the p130/E2F4

complexes is necessary to disrupt these complexes and allow gene expression. Decreasing the levels of p21 in p27^{-/-} MEFs rescued the DNA synthesis defect and accelerated progression through the cell cycle, corroborating the role of p21 in the decreased DNA synthesis in p27^{-/-} cells. Since rescue of DNA synthesis was very efficient when decreasing p21 we did not test the putative role of Cdkn2a and Cdkn2b increases in p27^{-/-} cells because they only act on Cdk4/6.

The elevation of p21 observed in p27^{-/-} cells was caused by the loss of p27-mediated repression of p21 transcription via Pitx2. Specifically, we found that p27 together with a repressive complex including E2F4 directly represses Pitx2 expression by associating to a critical regulatory region (ASE) of the Pitx2 gene (Figure 7).

Thus, Pitx2 is increased in p27^{-/-} and p27 knocked down cells and this elevation is ultimately responsible of the induction of p21 transcription in these cells (Figure 7). In concordance with previous reports we found that Pitx2 was able to induce p21 transcription by associating with the p21 gene promoter.^{17,22} The knockdown of Pitx2 increased Cdk2 activity, p130 phosphorylation, PCNA expression and DNA replication demonstrating the key role of Pitx2 in cell cycle progression.

Pitx2 is a TF that plays a role in the development of multiple organs as pituitary, heart and brain but also in craniofacial development and in the late read-out of left/right signaling.²³ Moreover, Pitx2 is over-expressed in different types of tumors.^{24–27} Interestingly, Cyclins A1 and D2 are transcriptional targets of Pitx2 that are also over-expressed in human tumors.^{26,28} Thus, the regulation of Pitx2 by p27 suggests its participation in the mentioned biological functions and in tumor development through this novel mechanism. Because decreased levels of p27 in human tumors are associated with a worse prognosis,²⁹ our data suggest these tumors have to show increased levels of Pitx2 and this could participate in their major malignancy.

Results reported here highlight the role of p27 as a key regulator of cell cycle progression by modulating p21 expression. These results are in agreement with the expression kinetics of p27 and p21 along cell cycle showing that the amount of p21 is low in quiescent cells and it increases at early-mid G₁ concomitantly with the p27 decrease. Interestingly, it has been recently reported that p27 and p21 collaborate in the G₁ transcriptional regulation of genes necessary for DNA replication by recruiting different cyclin-Cdk complexes on the promoters of these genes.³⁰ Thus, p21 appears as a central regulator of Cdk2 activity at mid-late G₁ and that the fine modulation of p21 levels is crucial for progression of cells through G₁ phase. The relevance of the amount of p21 on cell cycle progression has also been emphasized in a recent article showing that the decision at the end of mitosis to either start the next cell cycle or to enter a transient G₀-like state depends on p21 levels.³¹

Our current findings unveil a mechanistic connection among p27, Pitx2 and p21 and thereby contribute to our understanding of the regulation of cell cycle progression but also of the human pathologies associated with the deregulation of these proteins.

MATERIALS AND METHODS

Cell culture and transfection

Primary MEFs were isolated from control and p27^{-/-} mice (C57B6/J background) as previously described.³² All experiments were performed with MEFs at early passages (from two to eight passages). MEFs and HEK-293T cells (from ATCC) were cultured in Dulbecco's modified Eagle Medium supplemented with 10% fetal bovine serum, 5% Glutamine and 5% Penicillin/Streptomycin. Cultures were maintained at 37 °C and 5% CO₂. Plasmids were transfected in HEK-293T cells using lipofectamine 2000 (Invitrogen) following manufacturer's instructions. All cells were free from mycoplasma.

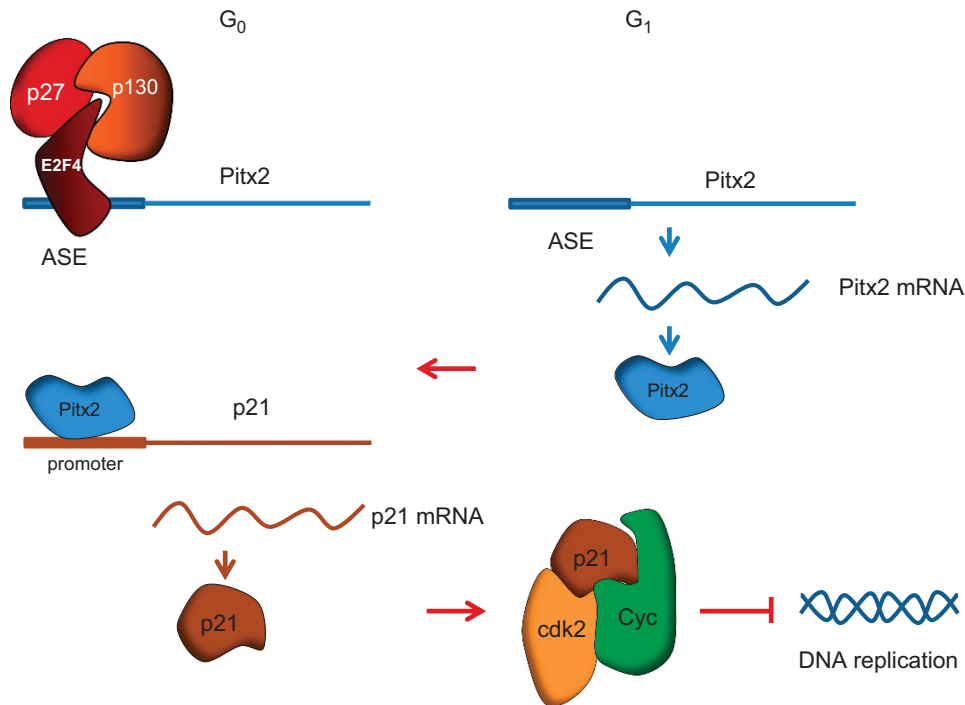


Figure 7. Model of the mechanism of action of p27^{Kip1} in the regulation of Pitx2-mediated expression of p21^{Cip1}, and in the regulation of DNA replication during cell cycle progression. In this model we propose that p27 is associated with E2F4 repressive complexes at the ASE regulatory region of the Pitx2 gene repressing transcription. In the absence of p27 Pitx2 transcription is induced producing and increase of Pitx2 protein. Pitx2 associates to the p21 gene promoter inducing its transcription. The increased levels of p21 inhibit the activity of cyclin-Cdk2 complexes and thus blocking the expression of genes needed for DNA replication (as for instance PCNA).

Cell synchronization

MEFs cells were maintained in serum free medium for 72 h and then activated by serum addition. At different time points after serum re-stimulation, samples were taken and used for the experiments.

Flow cytometry analysis

Cells were fixed with 70% cold ethanol for 2 h at 4 °C, washed with PBS, and finally incubated with 20 µg/ml of propidium iodide and 200 µg/ml RNase for 30 min at RT. Analysis of DNA content was carried out in a Becton Dickinson FACS Calibur. Data were analysed with the WinMDI 2.9 software (Windows, San Diego, CA, USA).

Antibodies

Antibodies against Cd 42k2 (sc-163), Cdk4 (sc-260), Cdk1 (sc-54), p21 (sc-397), CycA (sc-239), CycD1 (sc-246), CycE (sc-377100), Cdc25a (sc-7389), Cdc25b (sc-5619), PCNA (sc-25280) and Pitx2 (sc-8748) were purchased from Santa Cruz. p27 antibody (610242) was from BD Transduction Laboratories. Actin antibody (0869100) was obtained from MP Biomedicals. p15 (C0287) was purchased from Assay Biotech and P-Cdk2 Y15 ([EPR2233Y] ab76146) was obtained from Genetex.

Affymetrix genechip Exon 1.0 ST and GO

Synchronized cells by serum deprivation were washed with PBS and RNA was isolated from three different sets of p27^{WT} and p27^{-/-} cells. RNA quality control was done with Agilent 2100 bioanalyzer. Affymetrix Mouse Exon 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA) were hybridized by GenoSplice technology according the Ambion WT protocol (Life technologies, France) and Affymetrix labelling and hybridization recommendations. Affymetrix Mouse Exon 1.0 ST Array data set analysis and visualization were made using EASANA[®] (GenoSplice technology), which is based on the GenoSplice's FAST DB[®] annotations.^{33,34} Exon Array data were normalized using quantile normalization. Only probes targeting exons annotated from FAST DB transcripts were selected to focus on well-annotated genes whose mRNA sequences are in public databases.³⁴

Kinase assays

Cells were lysed using HAT buffer (50 mM Tris-HCl pH 8, 500 mM NaCl, 0.1 mM EDTA, 5% glycerol, 0.1% NP-40) for 1 h. Then, 500 µg of total protein was immunoprecipitated with 1 µg of Cdk2 antibody (M2, Santa Cruz) or IgG as a control. 40 µg of protein A/G-agarose beads (Pierce) were added for 2 h at 4 °C. Beads were washed three times in HAT buffer and twice in kinase buffer (50 mM Hepes, pH 7.4, 2.5 mM EGTA, 10 mM MgCl₂). Immunoprecipitates were resuspended in a final volume of 30 µl of kinase buffer containing 12.5 µM ATP, 1 µCi of [³²P]-ATP, 2 mM dithiothreitol and 2 µg of histone H1. Then, samples were incubated for 30 min at 30 °C, run in a 12% SDS-polyacrylamide gel, stained with Coomassie Blue and dried. The radioactivity associated to the gels was detected with a PhosphorImager.

BrdU Incorporation. Cells were plated in 24 well plates containing glass coverslips and incubated with BrdU (3 µg/ml) for 2 h. After two washes with PBS, cells were fixed with Ethanol:Acetic Acid (95:5) and washed with PBS again. Glass coverslips were incubated with 40 µl of anti-BrdU (GE Healthcare) for 1 h at RT, washed three times with PBS and then incubated with peroxidase conjugated secondary antibody. After three washes with PBS, glass coverslips were incubated with DAB solution (1:100 DAB (Sigma), 1:1000 H₂O₂ in PBS pH 7.4) for 5 min and cell nuclei were stained with hematoxylin for few seconds. Stained coverslips were mounted using Mowiol (Calbiochem).

[³H]-Thymidine Incorporation. Cells were seeded in a 12 well plate at 70–80% of confluence and incubated with 500 µCi/ml [³H]-thymidine. 2 h later cells were fixed with 70% methanol for 30 min at 4 °C and washed twice with 10% TCA. Cells were solubilized with a buffer containing 1%SDS, 0.3 M NaOH for 30 min at RT. Scintillation solution was added for measurement of ³H-Thymidine and the amount of radioactivity was counted in a scintillation counter.

Immunoprecipitation. Serum starved MEFs were scraped and washed twice with PBS. Pellets were lysed in 1 ml of IP buffer (PBS containing 0.5% Triton X-100, 1 mM EDTA, 100 µM sodium orthovanadate, 0.25 mM PMSF, complete protease inhibitor mixture by Roche Applied Science, and 1/25 vol of DNase I (Sigma)). Cell lysates were incubated on ice for 30 min and

then centrifuged at 3000 rpm at 4 °C for 5 min. After centrifugation, samples were quantified using Lowry method (Lowry et al.³⁵). One milligram of total protein was incubated overnight at 4 °C with 2 µg of anti-Cdk2 (M2, from Santa Cruz). Proteins and antibody were incubated with A-Dynabeads (Invitrogen) for 2 h and the immunocomplexes were extensively washed with IP buffer, subsequently eluted with 0.1 M Citrate pH 2.5 and boiled at 100 °C in Laemmli buffer for WB analysis. As a control, lysates were incubated with irrelevant rabbit IgG.

Chromatin immunoprecipitation

Cells were grown to confluence and synchronized in quiescence or grown in an 80% confluence plate (asynchronous cells). ChIP assay was performed as previously described.³⁶ Briefly, cells were lysed and chromatin from crosslinked cells was sonicated. Chromatin was incubated with 5 µg of anti-p27 (C-19, Santa Cruz) or anti-Pitx2 (C16, Santa Cruz) in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1 mM Na₃VO₄, 0.5 µg/µl aprotinin, 10 µg/µl leupeptin) adding 20 µl of Magna ChIP Protein A magnetic beads (Millipore). Samples were incubated in rotation overnight at 4 °C. Beads were washed with low-salt buffer, high-salt buffer, LiCl buffer and TE buffer. Subsequent elution and purification of the immunoprecipitated DNA-proteins complexes was performed using the IPure kit (Diagenode) according to manufacturer's protocol. Samples were analyzed by qPCR. Primer sequences used for qPCR of Pitx2 and p21 genomic regions are listed in Supplementary Table 1.

RNA extraction, reverse transcription-PCR and qPCR for gene expression analysis

Total RNA from cells was extracted using High Pure RNA Isolation kit (Roche). cDNA was obtained from 1 µg of RNA using SuperScript VILO cDNA synthesis (Invitrogen) according to manufacturer's instructions. Gene expression was analyzed by real-time PCR using LightCycler 480 SYBR green I master mix (Roche), corrected by actin or gapdh expression and expressed as relative units. Primer sequences used for qPCR assessment of p21, Zic1, Pitx2, Sox6 and control genes are listed in Supplementary Table 1.

ShRNA lentiviral infection

MEFs or HEK-293 T cells were infected with MISSION shRNA control vector and specific p27, p21 or Pitx2 shRNA (Sigma Aldrich) with the following sequences: p27: 5'-CCGGGCGCAAGTGGAAATTCGATTCTCGAGAATCGA AATCCACTTGGCCTTTTGG-3'; p21: 5'-CCGGCTACTCACTCAAGCGCAGAT TCTCGAGAATCTGCGCTTGGAGTGATAGTTTTG-3'; Pitx2: 5'-CCGGCCCGGC TATTCGTACAACAATCTCGAGATTGTTGACGAATAGCCGGGTTTTG-3'. The protocol for viral particles production and cell infections has been described elsewhere.³⁷ 24 h after infection, cells were selected with 2 mg/ml of Puromycin (Sigma Aldrich) for 5 days. shRNA-mediated down-regulation was tested by WB with specific antibodies.

β-galactosidase and luciferase assays

Luciferase vector was obtained by cloning a specific region from p21 promoter into a pGL2 Basic vector. Primers for the selected gene were designed by adding *MluI* and *BglII* target sequences at 5' and 3', respectively. The primers used for this amplification were: p21 Forward 5'-CGCAGCGTCAAGCTGTTTTCTC-3', p21 Reverse 5'-CGCAGATCTGCA GTTGGCGTCGAG-3', Amplification of p21 promoter sequence was made by PCR using genomic DNA and cloning the PCR products into pGL2 vector. Pitx2-ASE region luciferase was a kind gift from Dr Hiroshi Hamada. HEK-293T cells expressing shRNA control or shRNA for p27 were co-transfected with CMV-βGal vector and an empty vector, a vector containing p21 promoter or a vector containing Pitx2-ASE region. β-Galactosidase and luciferase assays were performed at 48 h after transfection. β-Galactosidase activity was detected using ONPG (Sigma) and read at 420 nm wavelength. Luciferase assays (Luciferase Assay System; Promega, Madison, WI) were performed as previously described.³⁶ β-Galactosidase/Luciferase ratio was performed and shown as arbitrary units.

Statistical analysis

GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA) was used for data analysis. The Student's *t*-test was applied to determine signi-

ficant differences between groups. In these analyses *P*-values < 0.05 were considered to be significant. At least three independent samples were analyzed in each experiment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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