Genetic Characterization of the Role of the Cip/Kip Family of Proteins as Cyclin-Dependent Kinase Inhibitors and Assembly Factors

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The Cip/Kip family, namely, p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}, are stoichiometric cyclin-dependent kinase inhibitors (CKIs). Paradoxically, they have been proposed to also act as positive regulators of Cdk4/6-cyclin D by stabilizing these heterodimers. Loss of p21^{Cip1} and p27^{Kip1} reduces Cdk4/6-cyclin D complexes, although with limited phenotypic consequences compared to the embryonic lethality of Cdk4/6 or triple cyclin D deficiency. This milder phenotype was attributed to Cdk2 compensatory mechanisms. To address this controversy using a genetic approach, we generated *Cdk2*^{-/-} p21^{-/-} p27^{-/-} mice. Triple-knockout mouse embryonic fibroblasts (MEFs) displayed minimal levels of D-type cyclins and Cdk4/6-cyclin D complexes. p57^{Kip2} down-regulation in the absence of p21^{Cip1} and p27^{Kip1} aggravated this phenotype, yet MEFs lacking all Cip/Kip proteins exhibited increased retinoblastoma phosphorylation, together with enhanced proliferation and transformation capacity. *In vivo*, *Cdk2* ablation induced partial perinatal lethality in p21^{-/-} p27^{-/-} mice, suggesting partial Cdk2-dependent compensation. However, *Cdk2*^{-/-} p21^{-/-} p27^{-/-} survivors displayed all phenotypes described for p27^{-/-} mice, including organomegalia and pituitary tumors. Thus, Cip/Kip deficiency does not impair interphasic Cdk activity even in the absence of Cdk2, suggesting that their Cdk-cyclin assembly function is dispensable for homeostatic control in most cell types.

Extracellular signals that promote cell proliferation are transduced via growth factor receptor-associated protein kinases and a plethora of downstream signaling pathways. A significant fraction of these pathways converges on the regulation of the cell cycle by the cyclin-dependent kinases (Cdks) (1). In a simplified scenario, Cdk activity is controlled not only by cyclin binding but also by interaction with Ink4 and the Cip/Kip family of cyclin-dependent kinase inhibitors (CKIs). However, formation of Cdk4-cyclin D functional heterodimers is largely inefficient, suggesting the requirement of assembly factors for stable complex formation (2). Indeed, Cip/Kip inhibitors have been implicated in the assembly of functional Cdk4/6-cyclin D complexes as well as on their import to the nucleus (3–5). Remarkably, some reports suggest that the Cdk4/6-cyclin D-Cip/Kip complexes remain active, since the majority of the Cdk4/6-cyclin D kinase activity in culture cells is found to be associated with Cip/Kip inhibitors (3, 6). Moreover, Cdk4/6-cyclin D complexes are nearly undetectable in extracts from mouse embryonic fibroblasts (MEFs), liver cells, or thymocytes from mice devoid of p21^{Cip1} and p27^{Kip1} (4). In this scenario, the unopposed activity of Cdk2 has been proposed to compensate for the severe reduction in Cdk4/6-cyclin D levels caused by Cip/Kip deficiency (4, 5). Together, these observations led to the hypothesis that Cip/Kip inhibitors are assembly factors for the formation of Cdk4/6-cyclin D complexes and that a substantial fraction of these ternary complexes retains catalytic activity (7).

However, other reports have described that Cdk4/6-cyclin D complexes, albeit reduced and short-lived, readily form in the absence of p21^{Cip1} and p27^{Kip1} either in asynchronous cultures (8, 9) or during cell cycle reentry from quiescence (5). Even though the levels of Cdk4/6-cyclin D complexes were undetectable by Western blot analysis, cells remained sensitive to p16^{Ink4a}, suggesting the existence of active Cdk4/6-cyclin D complexes (4). In addition, there are different reports regarding the extent of inhibition caused by Cip/Kip binding on the catalytic activity of Cdk4-cyclin

D complexes (3–6, 8, 10, 11). These observations have led to the hypothesis that p21^{Cip1} and p27^{Kip1} are dispensable for the formation of active Cdk4/6-cyclin D complexes and that their binding results in stable but inactive ternary complexes (9).

To evaluate *in vivo* the impact of Cip/Kip deficiency on the formation and stability of Cdk4/6-cyclin D complexes, we set up crosses to eliminate Cdk2 in mice doubly deficient for p21^{Cip1} and p27^{Kip1}. We have analyzed the proliferation and transformation properties of *Cdk2*^{-/-} p21^{-/-} p27^{-/-} MEFs as well as evaluated the impact of Cdk2 elimination in the phenotypes found in p21^{Cip1}- and p27^{Kip1}-deficient mice. The findings reported here support the notion that the Cip/Kip function as pan-Cdk inhibitors prevails over their role as Cdk4/6-cyclin assembly factors. As a consequence p21^{-/-} p27^{-/-} cells and mice display various hyperproliferative phenotypes that are all preserved even in the concomitant absence of Cdk2.

MATERIALS AND METHODS

Mouse strains. *Cdk2*^{-/-}, p21^{-/-}, and p27^{-/-} mice have been previously described (12). Tissue samples were fixed in 10% buffered formalin for 24 h and embedded in paraffin, and 3-μm sections were analyzed after stain-

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FIG 1 Stability of Cdk-cyclin D complexes in the absence of Cip/Kip inhibitors. (a) Early-passage primary MEFs were infected with lentiviral particles expressing either an shRNA against *p57^{Kip2}* (p57) or a scramble control (Ct). Protein extracts were prepared, and the levels of a panel of cell cycle regulators were analyzed by immunoblotting with antibodies elicited against the indicated proteins. Expression of β-actin served as a loading control. Results from two independent MEF cultures are shown for *p21^{-/-} p27^{-/-}* (DKO) and *Cdk2^{-/-} p21^{-/-} p27^{-/-}* (TKO) mice. Cyc, cyclin. (b) Wild-type, *p21^{-/-} p27^{-/-}* (DKO), and *Cdk2^{-/-} p21^{-/-} p27^{-/-}* (TKO) primary MEFs were infected with lentiviral particles expressing either an shRNA against *p57^{Kip2}* (p57) or a scramble control (Ct). Exponentially growing cells were used to prepare whole-cell extracts, and the amount of Cdk4-cyclin D complexes was estimated by coprecipitation of cyclin D1 with anti-Cdk4 antibodies. The levels of Cdk4, used as a loading control, are shown. WB, Western blotting. (c) The amount of starting whole-cell extract from both *p21^{-/-} p27^{-/-}* (DKO) and *Cdk2^{-/-} p21^{-/-} p27^{-/-}* (TKO) MEFs was augmented to equal the cyclin D1 levels in the wild-type controls. The amount of Cdk4 (top) and Cdk6 (middle) that was coimmunoprecipitated with cyclin D1 antibodies was detected by Western blotting. Cyclin D1 levels (bottom) are shown to demonstrate equal estimation of the amount of starting material. Results from two independent MEF cultures are shown. (d) Western blotting of Cdk1 protein levels in whole-cell extracts from wild-type, *p21^{-/-} p27^{-/-}* (DKO), and *Cdk2^{-/-} p21^{-/-} p27^{-/-}* (TKO) MEFs. Expression of β-actin, which served as a loading control, is shown. Results from two independent MEF cultures are shown. (e) *In vitro* kinase activity associated with Cdk1 immunoprecipitates obtained from wild-type, *p21^{-/-} p27^{-/-}* (DKO), and *Cdk2^{-/-} p21^{-/-} p27^{-/-}* (TKO) MEFs. Histone H1 was used as the substrate (S). Results from two independent MEF cultures are shown. Lanes WCE, whole-cell extract at a 1:10 dilution before immunoprecipitation (IP); lanes M, mock immunoprecipitate.

ing with hematoxylin-eosin (H&E). Histopathological analysis of *p21^{-/-} p27^{-/-} Cdk2^{-/-}* embryonic day 18.5 (E18.5) embryos and paired controls was performed by thorough inspection of H&E-stained serially sectioned specimens ($n = 7$). The mice used in this study have been maintained in a mixed 129/SvJ $\times$ C57BL/6J background according to the *Guide for the Care and Use of Laboratory Animals* (13) and in accordance with the Spanish Animal Protection Law (RD1201/2005) and the European Directive (86/609/CEE) established by the European Union to regulate animal care standards. Animal experiments received approval from the CNIO ethical committee (CEUCA code 032-03).

Cell culture assays. MEFs were isolated from E13.5 embryos and propagated according to standard protocols. No less than 10 independent early-passage primary MEF preparations per genotype were used to perform the experiments. Proliferation assays and S-phase entry analysis were carried out with primary MEFs as previously described (14). Knockdown of *p57^{Kip2}* was mediated with lentiviral Mission short hairpin RNA (shRNA) plasmids (Sigma no. SHGLY-NM_009876.2) according to the manufacturer's instructions. After infection, cells were selected with 2 μ g/ml puromycin for 48 h. Focus formation assays were performed as described previously (12).

Protein analysis. Protein lysates were prepared and used for immunoblotting as previously described (12). Antibodies against the following proteins were used: Cdk2 (M2; Santa Cruz), Cdk4 (C22; Santa Cruz), Cdk6 (our own rabbit polyclonal antibodies), cyclin D1 (DCS6; Neo Markers), cyclin D2 (M-20; Santa Cruz), cyclin D3 (DCS22; Cell Signaling), cyclin A2 (H432; Santa Cruz), p21^{Kip1} (C19; Santa Cruz), p27^{Kip1} (catalog no. 610242; BD Transduction Laboratories), p57^{Kip2} (catalog no. P0357; Sigma), β-actin (clone AC15; Sigma), and phospho-specific antibody to phosphorylated residues S807/811 of the retinoblastoma protein (pRb; catalog no. 9308; Cell Signaling). As secondary antibodies, peroxi-

dase-conjugated IgG (Dako) was used, followed by chemiluminescence detection (ECL kit; Amersham).

RESULTS

Formation of Cdk4/6-cyclin D complexes in the absence of Cip/Kip proteins. As illustrated in Fig. 1a, *p21^{-/-} p27^{-/-}* MEFs displayed a significant reduction in the overall cellular levels of the three D-type cyclins and retained 5 to 10% of the cyclin D levels present in wild-type cells. To determine whether the third member of the Cip/Kip family of CKIs, *p57^{Kip2}*, may contribute to the stabilization of active Cdk-cyclin D complexes, we generated lentiviral particles expressing an shRNA against *p57^{Kip2}* and analyzed its impact on cyclin D levels in wild-type and *p21^{-/-} p27^{-/-}* double mutant MEFs. *p57^{Kip2}* depletion in *p21^{-/-} p27^{-/-}* MEFs, irrespective of the presence or absence of Cdk2, caused a further decrease in the levels of expression of cyclins D1 and D2 and a moderate decrease in the case of cyclin D3 (Fig. 1a). Interestingly, we observed a negligible impact in wild-type controls. As expected, the reduction in the overall cellular levels of the D-type cyclins resulted in a similar decrease in the amount of Cdk4-cyclin D complexes, as determined by immunoprecipitation of the kinase subunit (Fig. 1b). These results suggest that *p57^{Kip2}* contributes to the stabilization of Cdk4/6-cyclin D complexes, at least in the absence of *p21^{Cip1}* and *p27^{Kip1}*.

In order to determine whether the Cip/Kip proteins are essential for the formation or stabilization of the Cdk4-cyclin D1 complexes, we matched the total levels of cyclin D1 in the immuno-

precipitation assays by scaling up 9-fold the amount of the starting whole-cell extract from $p21^{-/-}$ $p27^{-/-}$ and $Cdk2^{-/-}$ $p21^{-/-}$ $p27^{-/-}$ MEFs. Technical difficulties owing to the much greater decrease of cyclin D levels upon $p57^{Kip2}$ knockdown prevented us from including this condition in the assay. Nevertheless, under these conditions we compensated for the diminution in the overall levels of cyclin D1 caused by the concomitant absence of $p21^{Cip1}$ and $p27^{Kip1}$, and when immunoprecipitated, it pulled down an equal amount of Cdk4 and Cdk6 in all genotypes (Fig. 1c). This result is in good agreement with those described in a previous report suggesting that free D-type cyclins rapidly associate with their Cdk4/6 cognate catalytic subunits (9), thus suggesting that the kinetics of this association is independent of the Cip/Kip proteins, limiting their role to the stabilization of preformed binary complexes.

Finally, we investigated the expression levels and activity of Cdk1, as it is a likely candidate to compensate for the reduced activity of interphase Cdk. We found that whereas the expression levels of Cdk1 are comparable in MEFs from mice of the three genotypes, its activity is increased in $p21^{-/-}$ $p27^{-/-}$ and $Cdk2^{-/-}$ $p21^{-/-}$ $p27^{-/-}$ cells compared to its activity in the wild-type controls (Fig. 1d and e).

Proliferation properties of $Cdk2^{-/-}$ MEFs devoid of Cip/Kip proteins. To determine the consequences of the reduced levels of Cdk4/6-cyclin D complexes on cell cycle progression in the absence of Cip/Kip proteins, we examined the proliferation properties of primary $p21^{-/-}$ $p27^{-/-}$ MEFs. In parallel, we also analyzed the proliferation of $Cdk2^{-/-}$ $p21^{-/-}$ $p27^{-/-}$ cells since it has been proposed that in the absence of both Cip/Kip inhibitors Cdk2 could play a compensatory role by functionally replacing Cdk4/6-cyclin D complexes (4, 5). As illustrated in Fig. 2a, $p21^{-/-}$ $p27^{-/-}$ cells reproducibly displayed a growth advantage compared to the wild-type controls under standard serum conditions. This growth advantage was even more evident when cells were grown in 2% serum, since under these conditions wild-type cells failed to thrive, whereas MEFs devoid of $p21^{Cip1}$ and $p27^{Kip1}$ managed to proliferate (Fig. 2b). Similar results were obtained in primary MEFs also devoid of Cdk2, indicating that Cdk2 activity is not responsible for driving cell proliferation in the absence of these inhibitors. Finally, we assessed the effect of knocking down the third member of the Cip/Kip family of CKIs, $p57^{Kip2}$. shRNA-mediated depletion of $p57^{Kip2}$ caused a reduction in the proliferative rate compared to that for MEFs infected with control shRNA, particularly under low-serum conditions (Fig. 2). This result, together with the further decrease in the levels of the three D-type cyclins (Fig. 1a), suggests that $p57^{Kip2}$ may function as a Cdk4/6-cyclin D assembly factor in MEFs, at least in the absence of $p21^{Cip1}$ and $p27^{Kip1}$, yet the proliferation rate observed in the concomitant absence of the three CKIs, even in a Cdk2-null background, was consistently higher than that of wild-type MEFs (Fig. 2). Altogether, these experiments suggest that in terms of cell proliferation, the function of the Cip/Kip proteins as Cdk inhibitors prevails over their putative role as Cdk4/6-cyclin D assembly factors in MEFs. The lack of an effect observed upon ablation of Cdk2 expression suggests that the cell cycle inhibitory activity of the Cip/Kip proteins must be mediated through another Cdk, possibly Cdk1, or by a Cdk-independent mechanism.

Next, we focused our attention on cell cycle reentry, a process particularly reliant on Cdk activity (14). As illustrated in Fig. 3a, serum-starved $p21^{-/-}$ $p27^{-/-}$ MEFs entered S phase with a higher

FIG 2 Proliferation of primary MEFs in the absence of Cip/Kip inhibitors. (a) The proliferation of wild-type (Wt), $p21^{-/-}$ $p27^{-/-}$ (DKO), and $Cdk2^{-/-}$ $p21^{-/-}$ $p27^{-/-}$ (TKO) primary MEFs in 10% fetal bovine serum (FBS) was assessed upon infection with lentiviral particles expressing an shRNA against $p57^{Kip2}$ (sh p57) or a scramble control (sh Ctrl). Data are shown as means $\pm$ SDs ($n = 3$). (b) Same as for panel a, but MEFs were maintained under limiting serum conditions (2% fetal bovine serum). arb., arbitrary.

efficiency than wild-type control cells. Similar results were obtained with $Cdk2^{-/-}$ $p21^{-/-}$ $p27^{-/-}$ MEFs (Fig. 3a). Accordingly, the phosphorylation of the Ser807/811 residues of the retinoblastoma protein (Rb) and the appearance of cyclin A2 were detected at earlier time points compared to the times of appearance for the wild-type controls (Fig. 3b). Importantly, when the G_0 exit was assessed in $p57^{Kip2}$ -depleted cells, the higher efficiency observed in both $p21^{-/-}$ $p27^{-/-}$ and $Cdk2^{-/-}$ $p21^{-/-}$ $p27^{-/-}$ MEFs was no longer evident (Fig. 3c), thus adding further evidence that $p57^{Kip2}$ may participate in the assembly of Cdk4/6-cyclin complexes in MEFs. This role of $p57^{Kip2}$, which appears to be negligible in wild-type cells (Fig. 1a), becomes evident in the absence of $p21^{Cip1}$ and $p27^{Kip1}$, particularly under conditions that are more dependent on interphase Cdk activity, such as during proliferation under low-serum conditions or during exit from quiescence.

Cellular transformation in the absence of $p21^{Cip1}$ and $p27^{Kip1}$. To study the role of $p21^{Cip1}$ and $p27^{Kip1}$ in cellular transformation, we infected primary MEFs with retroviral particles expressing combinations of different oncogenes. Transformation was subsequently assessed by focus formation (Fig. 4a). Single $H-Ras^{G12V}$ or $E1a$ oncogenes did not significantly enhance transformation, irrespective of the genotype (data not shown). The maximal efficiency was achieved using concomitant expression of both oncoproteins. In this context, cells lacking $p27^{Kip1}$ expression displayed a minor (15%) increase in their transformation ability compared to that for the wild-type controls. However, in-

FIG 3 Role of Cip/Kip proteins in cell cycle reentry. (a) Percentage of wild-type (Wt), $p21^{-/-}$ $p27^{-/-}$ (DKO), and $Cdk2^{-/-}$ $p21^{-/-}$ $p27^{-/-}$ (TKO) primary MEFs in S phase at the indicated times following serum stimulation from quiescence. Data are shown as means $\pm$ SDs ($n = 3$). (b) Whole-cell extracts from cells for which the results are shown in panel a were prepared at the times (in hours) following serum stimulation from quiescence indicated above each lane. The presence of cyclin D1 and cyclin A2 and the phosphorylation of the Ser807/811 residues of the retinoblastoma protein (pRb) were detected by Western blotting. Expression of β -actin, which served as a loading control, is shown. (c) $p57^{Kip2}$ was depleted in wild-type, $p21^{-/-}$ $p27^{-/-}$ (DKO), and $Cdk2^{-/-}$ $p21^{-/-}$ $p27^{-/-}$ (TKO) primary MEFs by lentivirus-mediated shRNA delivery. Infected MEFs were subsequently made quiescent by serum deprivation. The percentage of S-phase cells at the indicated times (in hours) following serum stimulation from quiescence is shown. Data are shown as means $\pm$ SDs ($n = 3$).

fection of $p21^{-/-}$ MEFs or $p21^{-/-}$ $p27^{-/-}$ double mutant MEFs resulted in a 2-fold increase, indicating that $p21^{Cip1}$ and $p27^{Kip1}$ do not cooperate to prevent malignant transformation, at least *in vitro*. Interestingly, loss of $Cdk2$ expression had no effect on the ability of $p21^{-/-}$ $p27^{-/-}$ MEFs to become transformed by H-Ras^{G12V} and E1a oncoproteins, indicating that $Cdk2$ does not compensate for the low levels of Cdk4/6-cyclin D complexes. Moreover, this also illustrates that $Cdk2$ does not play a role in promoting cell transformation. Finally, we submitted these cells to a standard 3T3 immortalization protocol. As expected, wild-type primary MEFs entered replicative senescence after a few passages, whereas $p21^{-/-}$ $p27^{-/-}$ cells bypassed the crisis period irrespective of their $Cdk2$ status (Fig. 4b).

Phenotypic characterization of $p21^{-/-}$ $p27^{-/-}$ $Cdk2^{-/-}$ mice. In order to determine whether $Cdk2$ might compensate for the reduced formation of Cdk4/6-cyclin D complexes *in vivo*, we crossed $p21^{-/-}$, $p27^{-/-}$, and $Cdk2^{-/-}$ mice and scored the resulting genotypes at weaning ($n = 439$). As shown in Table 1, all the possible genotypes matched the expected Mendelian ratio except for those carrying a single $Cdk2$ allele (17.3% versus 25%; ratio, 0.69) or no $Cdk2$ alleles (5.9% versus 12.5%; ratio, 0.47). Lethality did not appear to occur *in utero*, since all possible genotypes were present at the expected ratios when assayed during late embryogenesis (E18.5) (data not shown). We previously reported that $Cdk2$ ablation in $p27^{-/-}$ mice did not result in increased mortality (15). Thus, the cause of death of $p21^{-/-}$ $p27^{-/-}$ $Cdk2^{-/-}$ neonates must be associated with $p21^{Cip1}$ deficiency. This postnatal lethality appears to result from insufficient overall interphase Cdk activity,

since neonatal mortality was also observed in $p21^{-/-}$ $p27^{-/-}$ $Cdk2^{+/+}$ mice, albeit to a lesser extent (ratio of surviving mice at weaning, 0.69 versus 0.47). Previous studies using Cdk mutant mice have linked interphase Cdk deficiency with embryonic or neonatal lethality. For instance, mice lacking Cdk4 and Cdk2 died at birth due to cardiac abnormalities, and mice deficient for the three interphase Cdk, Cdk4, Cdk6, and Cdk2, died at midgestation primarily due to hematopoietic defects similar to those observed in Cdk4 and Cdk6 double mutant mice. Careful histopathological analysis in serial sections of E18.5 embryos failed to uncover obvious defects that could account for their postnatal lethality, including in those tissues affected by interphase Cdk deficiency, such as fetal hematopoietic organs and heart. Thus, the cause of death of $Cdk2^{-/-}$ $p21^{-/-}$ $p27^{-/-}$ mice before weaning remains unknown.

We next focused our attention on the characterization of adult mice. Ablation of $p27$ alleles results in increased body size, especially in females (12, 16, 17). This and other phenotypic changes associated with $p27^{-/-}$ deficiency were mainly attributed to augmented Cdk activity. Thus, we examined whether the reduced levels of Cdk4/6-cyclin D activity in $p21^{-/-}$ $p27^{-/-}$ mice might revert the phenotypic changes seen in $p27^{-/-}$ animals. Parallel experiments were carried out in $p21^{-/-}$ $p27^{-/-}$ $Cdk2^{-/-}$ mice, which displayed even lower levels of interphase Cdk activity. To this end, we monitored body weight changes from weaning. As represented in Fig. 5, all compound strains devoid of $p27^{Kip1}$ displayed elevated body weights, regardless of their intrinsic Cdk activity. Moreover, all organs with the exception of liver showed

FIG 4 Cip/Kip factors and cellular transformation. (a) Equal amounts of wild-type, $p21^{-/-}$, $p27^{-/-}$, as well as compound mutant $p21^{-/-}$ $p27^{-/-}$ (DKO) and $Cdk2^{-/-}$ $p21^{-/-}$ $p27^{-/-}$ (TKO) MEFs were infected with retroviral particles coexpressing H-Ras and E1a oncogenes. (Left) At 3 weeks after infection, the number of detectable foci was scored for the different genotypes. Data are shown as means $\pm$ SDs ($n = 3$). (Right) A representative image upon crystal violet staining is shown. (b) Immortalization of wild-type, $p21^{-/-}$ and $p27^{-/-}$ single-knockout, $p21^{-/-}$ $p27^{-/-}$ (DKO), and $Cdk2^{-/-}$ $p21^{-/-}$ $p27^{-/-}$ (TKO) primary MEFs following a classical 3T3 protocol. Data are shown as means $\pm$ SDs ($n = 3$).

an increased weight (12, 16–18). Pathological examination of livers from $p21^{-/-}$ $p27^{-/-}$ $Cdk2^{-/-}$ mice showed that they did not display detectable architectural or cytological defects, yet we found that the hepatocyte density was consistently reduced compared to that in age-matched $p21^{-/-}$ $p27^{-/-}$ controls (Fig. 5b and c). Since the livers of newborn $p21^{-/-}$ $p27^{-/-}$ $Cdk2^{-/-}$ mice were phenotypically normal (data not shown), the liver hypoplasia may progressively arise during postnatal development. Taken together, these results suggest that the majority of tissues and cell types in adult mice tolerate the combined deficiency of $p21^{\text{Cip1}}$ and $p27^{\text{Kip1}}$.

Finally, we examined whether these $p21^{-/-}$ $p27^{-/-}$ and/or $p21^{-/-}$ $p27^{-/-}$ $Cdk2^{-/-}$ mutant mice may show defects in the proliferation of insulin-producing pancreatic β -cells, which are exquisitely dependent on the presence of Cdk4 activity for proper proliferation (18–20). All mutant mice were normoglycemic during the course of the study (data not shown). Unlike $Cdk4^{-/-}$ animals, histological analysis of pancreatic sections failed to detect either quantitative or qualitative variations in β -cell islets in any of the tested mice ($n = 21$) (data not shown). These results illustrate that *in vivo*, the decline in Cdk4/6-cyclin D complexes caused by ablation of the $p21^{\text{Cip1}}$ and $p27^{\text{Kip1}}$ alleles is not sufficient to phenocopy the defects caused by the loss of Cdk4 activity.

Tumorigenesis in $p21^{-/-}$ $p27^{-/-}$ mice. Finally, we analyzed the tumor spectrum of $p21^{-/-}$ $p27^{-/-}$ double-knockout mice. As

previously reported, $p21^{\text{Cip1}}$ -null mice developed a variety of spontaneous tumors at an average age of 16 months (21). $p27^{\text{Kip1}}$ deficiency resulted in the appearance of aggressive pituitary tumors, and all mice succumbed by 1 year of age (12, 16, 17). $p21^{\text{Cip1}}$ $p27^{\text{Kip1}}$ -null animals died of pituitary tumors with a timing and pathology indistinguishable from those for the single mutant $p27^{-/-}$ controls, indicating that $p21^{\text{Cip1}}$ does not compensate for the absence of $p27^{\text{Kip1}}$ (Fig. 6). In contrast, the loss of $p27^{\text{Kip1}}$ appears to cooperate with the absence of $p21^{\text{Cip1}}$, at least in accelerating tumor development, since about a quarter of the $p21^{-/-}$ $p27^{-/-}$ mice displayed histiocytic sarcomas at a time (10 months) when none of the $p21^{-/-}$ animals had developed such tumors (21–23) (Table 2). We also recorded the appearance of new pathologies in $p21^{-/-}$ $p27^{-/-}$ double mutant mice that were absent in either single-knockout strain. They included a panel of precancerous lesions, such as gastric hyperplasia, pancreatic intraepithelial neoplasia (PanIN), and myeloproliferation (Table 2). The premature death associated with $p27^{\text{Kip1}}$ deficiency prevented us from studying the evolution of these lesions.

DISCUSSION

In our hands, primary $p21^{-/-}$ $p27^{-/-}$ MEFs displayed enhanced proliferative properties compared to the wild-type controls (Fig. 2). Interestingly, and in agreement with previous reports, these cells retained only 5% of the cellular Cdk4-cyclin D1 complexes found in the wild-type controls. As a side note, this is equivalent to the fraction of $p27^{\text{Kip1}}$ that appears to be tyrosine phosphorylated—and presumably noninhibitory—in asynchronous cells (23), suggesting that a minor proportion of active Cdk4/6-cyclin D complexes suffices to support unperturbed proliferation. Importantly, the enhanced proliferative capacity observed in $p21^{-/-}$ $p27^{-/-}$ MEFs was also present in $Cdk2^{-/-}$ $p21^{-/-}$ $p27^{-/-}$ MEFs (Fig. 2), discarding the possibility that Cdk2 is the putative compensatory mechanism that has been postulated to ameliorate the proliferation of $p21^{-/-}$ $p27^{-/-}$ cells (4, 5). Altogether, these results suggest that in terms of cell proliferation, the Cip/Kip function as pan-Cdk inhibitors is overwhelmingly more relevant than their role as Cdk4/6-cyclin D assembly factors.

It has also been proposed that Cdk4/6-cyclin D complexes perform a relevant noncatalytic role to favor cell proliferation. According to this model, these complexes act as a molecular sink for Cip/Kip proteins, titrating them away from Cdk2 and therefore facilitating its subsequent activation (7). Indeed, no free $p27^{\text{Kip1}}$ can be detected *in vivo* (23). Cdk4/6 double-knockout cells have a lower proliferative capacity than wild-type controls (24). Interest-

TABLE 1 Influence of $p21^{\text{Cip1}}$, $p27^{\text{Kip1}}$, and Cdk2 deficiency on postnatal survival^a

Genotype			Mendelian ratio ^b (%)	
<i>Cdk2</i>	<i>p21</i>	<i>p27</i>	Expected	Observed
+/+	-/-	+/+	12.5	18.7
+/+	-/-	-/-	12.5	11.6
+/+	-/-	+/+	25	31
+/+	-/-	-/-	25	17.3
-/-	-/-	+/+	12.5	15.5
-/-	-/-	-/-	12.5	5.9

^a Data are for 439 mice.

^b The expected and observed Mendelian ratios of the indicated genotypes were scored at weaning (postnatal day 21).

FIG 5 Influence of $p21^{Cip1}$ and $p27^{Kip1}$ deficiency in adult homeostasis. (a) Total body weight and the weight of the indicated individual organs of mice at 11 weeks of age. Values for $p27^{-/-}$, $p21^{-/-} p27^{-/-}$ (DKO), and $Cdk2^{-/-} p21^{-/-} p27^{-/-}$ (TKO) mice normalized to those for wild-type controls of the same age are represented. Data are shown as means $\pm$ SDs ($n = 4$). (b) Representative images of hematoxylin-eosin-stained liver sections from 11-week-old $p21^{-/-} p27^{-/-}$ (DKO) and $Cdk2^{-/-} p21^{-/-} p27^{-/-}$ (TKO) mice. Bars, 20 μ m. (c) Quantification of the total number of hepatocytes in four randomly selected $\times 400$ microscope fields of liver sections from $p21^{-/-} p27^{-/-}$ (DKO) and $Cdk2^{-/-} p21^{-/-} p27^{-/-}$ (TKO) mice. Data are shown as means $\pm$ SDs ($n = 7$).

ingly, $p21^{-/-} p27^{-/-}$ MEFs, which display a severe (20-fold) reduction of Cdk-cyclin D complexes, have the opposite phenotype. This suggests that in wild-type MEFs, the Cip/Kip titration function of Cdk4/6-cyclin D might prevail over their catalytic activity. Indeed, a cyclin D1 mutant that forms kinase-inactive complexes with Cdk4/6 efficiently rescues the developmental defects present in cyclin D1 $^{-/-}$ mice (25). Although this could also be attributed to Cdk-independent functions of cyclin D1, we cannot rule out the possibility that in particular cellular contexts the sequestration

function of Cdk4/6-cyclin D complexes is dominant over their catalytic activity.

We have also shown that $p57^{Kip2}$, the third component of the Cip/Kip family, participates *in vivo* in the stabilization of Cdk4-

FIG 6 Cooperation between $p21^{Cip1}$ and $p27^{Kip1}$ tumor suppressors. Survival curve of $p21^{-/-}$, $p27^{-/-}$, $p21^{-/-} p27^{-/-}$ (DKO), and $Cdk2^{-/-} p21^{-/-} p27^{-/-}$ (TKO) mice.

TABLE 2 Tumor susceptibility in mice lacking $p21^{Cip1}$ and $p27^{Kip1}$			
Lesion type and lesion or pathology	% of mice that developed lesions or pathology ^a		
	$p21^{-/-} p27^{-/-}$ mice ($n = 21$)	$p27^{-/-}$ mice ($n = 11$)	$p21^{-/-}$ mice ($n = 13$)
Hyperplasia/tumor-related lesions			
Gastric neuroendocrine hyperplasia	43	0	0
Gastric mucosa hyperplasia	57	0	0
Histiocytic sarcoma	24	0	0
Myeloproliferation	43	0	0
PanIN	10	0	0
Inflammation/non-tumor-related lesions			
Arteritis	14	0	0
Bone metaplasia	24	0	0
Chronic nephropathy	81	45	23
Chronic pancreatitis	29	0	0
Retinal dysplasia	38	19	0

^a Animals were sacrificed at 10 months of age. Numbers are rounded up to the nearest digit.

cyclin D complexes, at least in the absence of $p21^{Cip1}$ and $p27^{Kip1}$. shRNA-mediated depletion of $p57^{Kip2}$ in $p21^{-/-}$ $p27^{-/-}$ MEFs caused a further reduction of Cdk4-cyclin D1 complexes, reaching levels undetectable by Western blotting (Fig. 1b), yet it is significant that upon $p57^{Kip2}$ depletion, $Cdk2^{-/-}$ $p21^{-/-}$ $p27^{-/-}$ MEFs exited G_0 with kinetics comparable to that of the wild-type controls. In this scenario, the Cdk1-cyclin D complexes that become detectable in the absence of Cdk4 (14) may functionally replace Cdk4/6-cyclin D complexes. This would imply that the assembly of Cdk1-cyclin D complexes does not require Cip/Kip proteins or may be facilitated by alternative assembly factors. In this respect, $p34^{Sei1}$ has been described to be an alternative Cdk-cyclin D assembly factor, particularly during quiescence exit (5). We believe that our $Cdk2^{-/-}$ $p21^{-/-}$ $p27^{-/-}$ mouse model may become a useful tool to elucidate whether indeed other assembly factors exist or if Cdk-cyclin D complexes can form *de novo*.

It is also possible that the required levels of Cdk-cyclin D complexes (and, therefore, the importance of their assembly factors) are cell type specific. Whereas $p21^{-/-}$ $p27^{-/-}$ mice reach adulthood with the expected Mendelian ratio, Cdk4/6 double-knockout (DKO) mice die *in utero* as a consequence of hematopoietic defects (24). This suggests that *in vivo*, redundant mechanisms or the inherent plasticity of the system may bypass the requirement for $p21^{Cip1}$ and $p27^{Kip1}$ proteins as Cdk4/6-cyclin D assembly factors in most cell types. We cannot rule out the possibility that this adaptation was facilitated in part by the use of germ line null alleles. For instance, whereas chronic deficiency of the retinoblastoma protein in MEFs allows adaptation and compensation among other family members, this is largely inefficient upon acute gene ablation (26). In any case, and in support of the functional redundancy model, we show here that $p57^{Kip2}$ stabilizes Cdk4-cyclin D1 in $p21^{-/-}$ $p27^{-/-}$ MEFs but not in wild-type controls (Fig. 1). In addition, others have demonstrated that a knockin of $p27^{Kip1}$ expressed from the $p57^{Kip2}$ promoter can rescue most of the developmental defects present in $p57^{-/-}$ mice, suggesting that they perform partially redundant roles *in vivo* (27). Interestingly, as described above, $p57^{Kip2}$ knockdown in $p21^{-/-}$ $p27^{-/-}$ MEFs caused an additional reduction of Cdk-cyclin complexes and affected their proliferation rate, which, nevertheless, remained higher than that of wild-type cells (Fig. 2b). Moreover, unlike single- and double-knockout strains, 53% of $Cdk2^{-/-}$ $p21^{-/-}$ $p27^{-/-}$ mice died perinatally (Table 1). The fact that this was reduced to 31% by introducing a single $Cdk2$ allele suggests that the lethality is a dose-dependent phenotype associated with insufficient interphase Cdk activity. Nevertheless, 47% of triple-knockout (TKO) animals reached adulthood and displayed a normal life span, suggesting that the attainment of a Cdk activity threshold compatible with life in the relevant target tissue(s) may rely on several overlapping mechanisms yet to be fully characterized.

This also suggests that the cellular context may dictate which of the seemingly opposed roles of Cip/Kip as Cdk-cyclin D assembly factors versus Cdk inhibitors prevail, to an extent that under some circumstances the Cip/Kip tumor suppressors counterintuitively display oncogenic properties. For instance, oligodendrogliomas with elevated $p21^{Cip1}$ result in more aggressive disease (28). Interestingly, in this tumor type the oncogenic function of $p21^{Cip1}$ is a direct consequence of its role as a Cdk4-cyclin D assembly factor and is directly controlled by tyrosine phosphorylation (29, 30). $p27^{Kip1}$ is equally subject to regulation by tyrosine phosphorylation (23, 31, 32). Unfortunately, the only mouse model in which

$p27^{Kip1}$ has been shown to promote tumor development relies on a Cdk-independent function (33), thereby invalidating the option to weigh the importance of the tyrosine phosphorylation control (i.e., its role as an assembly factor) in $p27^{Kip1}$ -mediated cancer promotion.

In sum, and in good agreement with previous observations from different laboratories (34), our results emphasize the notion that Cdk-dependent cell cycle control in multicellular organisms is extremely robust and several overlapping mechanisms facilitate not only the proper execution of cell division but also its timely coordination with developmental processes, even in the concomitant absence of alleged essential factors.

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We declare no conflict of interest.

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