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Somatic cell type specific gene transfer reveals a tumor-promoting function for p21$^{Waf1/Cip1}$

Yuhui Liu[1], Nancy Yeh[1], Xin-Hua Zhu[1], Margaret Leversha[2], Carlos Cordon-Cardo[3], Ronald Ghossein[3], Bhuvanesh Singh[4], Eric Holland[5] and Andrew Koff[1,*]

[1]Department of Molecular Biology, Memorial Sloan-Kettering Cancer Center, New York, NY, USA, [2]Department of Human Genetics, Memorial Sloan-Kettering Cancer Center, New York, NY, USA, [3]Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY, USA, [4]Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, NY, USA and [5]Department of Cancer Biology, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

How proteins participate in tumorigenesis can be obscured by their multifunctional nature. For example, depending on the cellular context, the cdk inhibitors can affect cell proliferation, cell motility, apoptosis, receptor tyrosine kinase signaling, and transcription. Thus, to determine how a protein contributes to tumorigenesis, we need to evaluate which functions are required in the developing tumor. Here we demonstrate that the RCAS/TvA system, originally developed to introduce oncogenes into somatic cells of mice, can be adapted to allow us to define the contribution that different functional domains make to tumor development. Studying the development of growth-factor-induced oligodendroglioma, we identified a critical role for the Cy elements in p21, and we showed that cyclin D1T286A, which accumulates in the nucleus of p21-deficient cells and binds to cdk4, could bypass the requirement for p21 during tumor development. These genetic results suggest that p21 acts through the cyclin D1–cdk4 complex to support tumor growth, and establish the utility of using a somatic cell modeling system for defining the contribution proteins make to tumor development.

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Introduction

Several mouse models recapitulate the cardinal features of human oligodendrogliomas (ODGs) (Holland, 2001a, b, c; Betsholtz, 2004; Shih *et al*, 2004; Salpietro and Holland, 2005). Chronic signaling and overexpression of the ligands and receptors of platelet-derived growth factor (PDGF) signaling pathways are particularly common in human ODG (Guha *et al*, 1995a, b; Louis *et al*, 2001, 2002; Collins, 2004). We have demonstrated that expression of PDGF alone in nestin-positive progenitors is sufficient to induce ODG development (Dai *et al*, 2001).

Alterations that disrupt stem/progenitor cell dynamics and cell cycle arrest may affect the progression of ODG. Cells become committed to undertake a cell cycle when sufficient cyclin-dependent kinase (cdk) activity accumulates during the G1 phase. When cdk activity remains below the threshold level needed to initiate progression through the cell cycle, cells exit the cell cycle, and in some cases differentiate. Thus, interactions between the cyclins and their catalytic partners the cdks are critical in driving the cell cycle forward. Cyclin–cdk complexes are regulated by a group of proteins known as cdk inhibitors (cki). Cki are classified into two subfamilies: the Ink4 subfamily (p15, p16, p18, and p19), which specifically target cdk4 and cdk6 and disrupt the cyclin D–cdk complex; and the Cip/Kip subfamily (p21, p27 and p57), which inhibit cyclin–cdk2 complexes.

Cki are considered tumor suppressors because their binding to cdks generally inhibits cell proliferation; however, cki can also increase cell motility, reduce apoptosis, modulate receptor tyrosine kinase signaling, and alter the activity of a host of transcription factors and chromatin remodeling enzymes (Dotto, 2000; Blagosklonny, 2002; Gartel and Tyner, 2002; Perkins, 2002; Coqueret, 2003; Weiss, 2003; Denicourt and Dowdy, 2004; Gartel, 2005, 2006b). Subcellular location might affect these interactions and roles (Sherr and Roberts, 1999; Coqueret, 2003; Denicourt and Dowdy, 2004; Child and Mann, 2006). For example, cytosolic p27 can modulate rho activity and affect cell migration (McAllister *et al*, 2003; Besson *et al*, 2004; Nguyen *et al*, 2006). p21 and p27 can also bind nuclear cyclin D–cdk4 complexes, preventing their crm1-dependent export into the cytoplasm, where cyclin D1 would be degraded in a ubiquitin-dependent process (Alt *et al*, 2002; Lin *et al*, 2006). Any of these interactions might provide an oncogenic advantage to a proliferating cell; however, because of the lack of suitable genetic systems that allow *in situ* manipulation of the incipient cells that give rise to solid tumors, none of these have been demonstrated to be responsible for the tumor-promoting effects associated with cki expression.

We have used an ODG model where disease is induced by infecting nestin-TvA mice with RCAS vectors expressing PDGF to probe the role that cki play in tumor development (Dai *et al*, 2001). This system takes advantage of the fact that avian retroviruses (RCAS) cannot infect mammalian cells, unless they express the avian retroviral receptor (TvA) (Fisher *et al*, 1999). Transgenic mice expressing TvA under

*Corresponding author. Molecular Biology Program, RRL917D, Box 207, Sloan Kettering Institute, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA. Tel.: +1 212 639 2354; Fax: +1 646 422 2062; E-mail: a-koff@ski.mskcc.org

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the control of the nestin promoter (Ntv-a) allow the infection of oligodendrocyte progenitor cells (OPC) and some earlier neural/glial lineage cell types (Doetsch *et al*, 1997, 2002). Our studies of introducing PDGF into nestin-positive progenitors and driving the development of growth-factor-induced ODG highlighted a critical role for the cyclin-binding domains of p21, and we showed that a mutant of cyclin D1 which accumulates in the nucleus and binds cdk4 in p21-deficient cells could bypass the requirement for p21 during tumor development. Mutants that did not bind cdk4 or did not accumulate in the nucleus did not bypass the requirement. Together, these results establish genetic proof that p21 can act through the cyclin D1–cdk4 complexes to support tumor growth, and establish the utility of using the RCAS/TvA system for delineating the types of contributions that a protein makes to tumor development.

Results

The progression of ODG induced by growth-factor overexpression is affected by the dose of cdk inhibitors

Both a low p27 staining index and a high p21 staining index are associated with poor prognosis in human ODG (Cavalla *et al*, 1999; Miettinen *et al*, 2001). Furthermore, both of these proteins play a role during the development of the oligodendroglial lineage (Casaccia-Bonnefil *et al*, 1997, 1999; Zezula *et al*, 2001; Doetsch *et al*, 2002). Consequently to understand if these cki contributed in a causal manner to the pathogenesis of ODG, we crossed mice with targeted deletion of *p21* or a mutation of p27, $p27^{D51/D51}$, which removes the N-terminal 51 amino acids of the protein preventing cyclin–cdk interaction, onto an Ntv-a background and infected newborn wild-type, heterozygous, and deficient mice with a single intracranial injection of 10^4 DF-1 cells producing RCAS-PDGF-HA (Shih *et al*, 2004). Ntv-a-negative littermate controls did not develop tumors regardless of their genotype. Whereas p27 loss was associated with enhanced progression of tumors, as expected of a tumor suppressor (Wendy See, EH, Marilyn Resh, and AK, manuscript in preparation), the loss of p21 unexpectedly reduced the development of tumors (Table I). There was no evidence of morbidity in $p21^{-/-}$ mice infected with RCAS-PDGF-HA during the 12-week period. All mice were killed at the end of the 12 weeks, and two $p21^{-/-}$ had a tumor visible by gross histology. Because the tumor suppressive properties of p27 are well established, the rest of this report focuses on the role of p21. Our results delineating the tumor suppressor function of p27 will be reported elsewhere.

Similar to gliomas in wild-type animals, tumors in the $p21^{+/-}$ and $p21^{-/-}$ animals were composed of small cells with round nuclei and scant cytoplasm. As shown in the Supplementary Figure 6, we observed intrafascicular queuing in white matter tracts of the corpus callosum, subpial infiltration, and perivascular and perineuronal satellitosis in areas of cortical invasion as described previously. The tumors were positive for oligodendrocyte markers, sox10 and olig2 (Liu *et al*, 2002; Bannykh *et al*, 2006), and negative for a neuronal marker, NeuN (Jin *et al*, 2003). The tumor size did not correlate with genotype; large and small tumors were observed in all genotypes. These tumor characteristics were consistent with a diagnosis of ODG.

Table I Effect of p21 gene dosage on PDGF-induced gliomagenesis

Genotype	Oligodendroglioma[a]
$p21^{+/+}$	21/35
$p21^{+/-}$	5/14
$p21^{-/-}$	2/45

[a]The value is the number of animals that developed a tumor within 12 weeks over the total number of animals infected with RCAS-PDGF-HA.

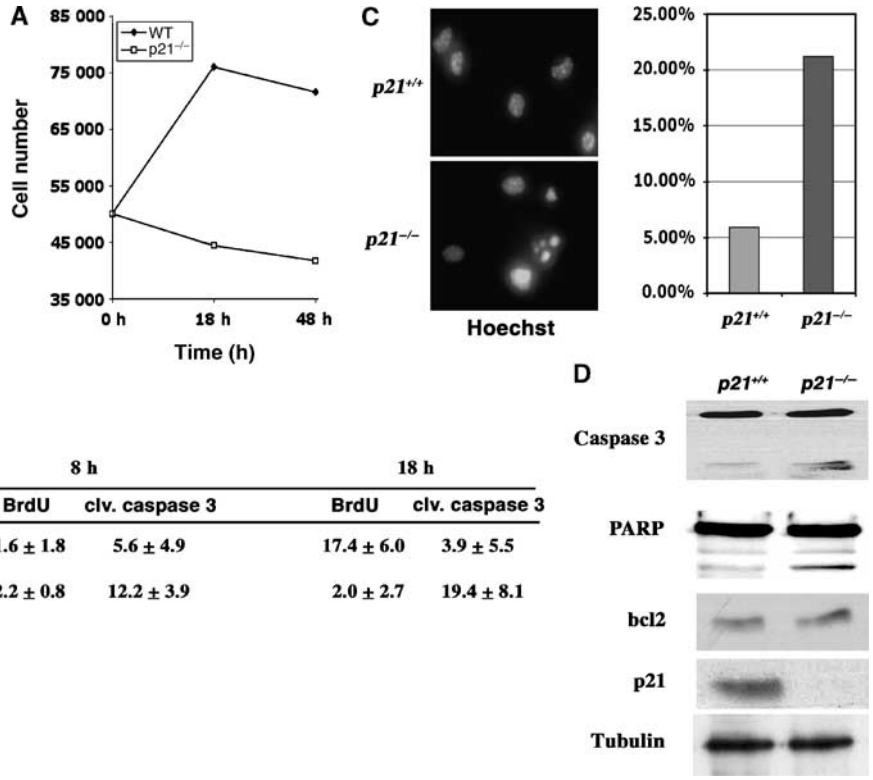
p21 deficiency affects the PDGF-dependent growth of progenitor and incipient tumor cells

We noted that the Ki67 index, a marker for proliferating cells, was approximately two-fold lower in the two tumors arising in the $p21^{-/-}$ mice (per $\times 400$ field: tumor A, 36 ± 25, $P < 0.001$; tumor B, 59 ± 35, $P < 0.05$) compared to the average level seen in five randomly chosen wild-type mice (per $\times 400$ field: 86 ± 53). High standard deviations are typical because of the diffuse nature of ODGs. Furthermore, apoptotic indices, measured by the percentage of cells staining positively for cleaved caspase 3, were approximately 3–4-fold higher in the two tumors that arose in the $p21^{-/-}$ mice (per $\times 400$ field: tumor A, 6 ± 2; tumor B, 8 ± 4) compared to wild-type mice (per $\times 400$ field: 1 ± 1). Thus p21 might support proliferation or reduce apoptosis (or both) during the development of growth-factor-induced ODG.

To ask if these effects of p21 deficiency were seen in the 'progenitor' cells that give rise to the tumors, we isolated OPC, made them quiescent in culture, and subsequently induced them to enter the cell cycle by exposing them to PDGF. p21 status affected the growth, proliferation, and apoptosis of quiescent progenitor cells exposed to PDGF in culture. PDGF induced cell growth when added to purified wild-type cells at a concentration between 10 and 50 ng/ml (Casaccia-Bonnefil *et al*, 1997; Zezula *et al*, 2001), but not when added to $p21^{-/-}$ OPC (Figure 1A). At 18 h after the addition of PDGF, the percent of BrdU-positive cells did not increase in the $p21^{-/-}$ culture, although it did in the wild-type culture (Figure 1B). Additionally, apoptotic indices measured immunohistochemically by cleaved caspase 3 (Figure 1B), by nuclei morphology (Figure 1C), or by immunoblotting extracts for cleaved caspase 3 or cleaved PARP (Figure 1D) were all elevated in the p21-deficient culture. Consequently, p21 levels could affect the growth of progenitors responding to unbalanced PDGF signaling by acting through either the proliferative or apoptotic pathways or both. As shown in the accompanying Supplementary Figure 7, we confirmed that p21 deficiency did not affect the appearance of nestin-positive cells in the brains of neonatal mice or the expression of PDGF from the retroviral vector.

Tumor progression can be restored by re-introducing p21 into somatic cells

Both tumor cell-autonomous and tumor cell-nonautonomous functions of p21 might contribute to tumor development. To determine whether restoring p21 function in glial progenitors was sufficient to support tumor formation, we coinfected animals with an equal number of DF-1 cells expressing RCAS-PDGF-HA and RCAS-3xFp21 and scored for gliomagenesis. RCAS-3xFp21 was constructed by cloning the mouse p21 cDNA with an N-terminal 3xFLAG epitope into an RCAS vector. Employing this methodology results in some cells



B

	8 h		18 h	
	BrdU	clv. caspase 3	BrdU	clv. caspase 3
$p21^{+/+}$	1.6 ± 1.8	5.6 ± 4.9	17.4 ± 6.0	3.9 ± 5.5
$p21^{-/-}$	2.2 ± 0.8	12.2 ± 3.9	2.0 ± 2.7	19.4 ± 8.1

Figure 1 p21 deficiency affects proliferation and apoptosis in primary cells. (**A**) Cell growth. Quiescent OPC were prepared and treated with 10 ng/ml PDGF and cell number counted at the indicated times (*x* axis). This experiment was repeated twice with similar results. (**B**) Proliferation and apoptotic indices were determined at 8 and 18 h by counting positive staining cells in at least five × 400 fields. Note that proliferation did not increase in $p21^{-/-}$ cultures 18 h after PDGF stimulation and that the basal amount of apoptosis was higher in these cultures at both times. This experiment was repeated twice with similar results. The mean and standard deviation are shown. (**C**) At 18 h following PDGF addition, the number of cells with fragmented nuclei is increased. On the left is a representative field of Hoechst-stained cells. On the right, the graph compiles this information from two independent experiments, looking at greater than 300 cells in each. (**D**) Immunoblot: 50 μg of extract was resolved by SDS–PAGE and the amount of caspase 3, PARP, bcl2, and p21 determined in $p21^{+/+}$ and $p21^{-/-}$ OPC treated with PDGF as indicated. Tubulin was a loading control. This experiment was repeated twice with comparable results.

being infected with only RCAS-PDGF-HA, others with only RCAS-3xFp21, and others with both.

Infection with RCAS-3xFp21 alone did not result in tumor formation (0/15). $p21^{-/-}$ animals coinfected with both RCAS-PDGF-HA and RCAS-3xFp21 developed tumors (Figure 2). The majority of dually infected mice (80%) were killed before 4 weeks of age because of symptoms of intracranial pathology such as hydrocephalus and dehydration. All dually infected mice had moderate to high grade anaplastic ODGs, as determined by gross histological examination (Figure 2C). The grading system we used is described in the accompanying Supplementary Figure 6. The three tumors that were selected for additional immunohistochemical analysis were all positive for the oligodendroglial lineage markers, olig2 and sox10, and negative for the neuronal lineage marker, neuN.

Viral infection was also confirmed using interphase fluorescent *in situ* hybridization (FISH) to detect RCAS-DNA. In one animal, 73 of 178 cells from a tumor region were positive, and in the other 67 of 144 cells were positive. Greater than 95% of the cells from the tumor region were positive for a reference marker that specifically reacts with the X-chromosome. In addition, as shown in the Supplementary Figure 8, FLAG-p21 protein accumulated in the nucleus, consistent with its localization in human ODG.

The ability of p21 to promote progression of growth-factor-induced ODG is dependent on the Cy element

Using the RCAS/TvA system allows us the opportunity to introduce multiple gene products into tumor cell progenitors. Thus, we thought it might be suitable for analyzing, at a genetic level, the importance of various protein–protein interaction domains. Specifically, we assessed the need for the carboxyl portion of p21, the amino-terminal domain, and the Cy motifs. All constructs were tagged with a 3xFLAG epitope at the amino terminus. RCAS-Np21 contained the first 32 amino acids (aa) of mouse p21 and was predicted to interact with procaspase 3 (Suzuki *et al*, 1999). RCAS-Cp21 contained aa 33–160 and was predicted to interact with a variety of transcription factors and chromatin remodeling proteins (Dotto, 2000), as well as the apoptotic regulator ask1 (Zhan *et al*, 2007). Both fragments contained a Cy motif (Chen *et al*, 1996). The relative expression of each mutant was assessed by immunoblotting extracts from the viral producer lines (Figure 2A).

Both the RCAS-Cp21 and RCAS-Np21 constructs supported gliomagenesis when introduced with RCAS-PDGF-HA into $p21^{-/-}$*Ntv-a* mice. Tumor latency, incidence and grade were similar to those seen in p21-deficient mice infected with RCAS-3xFp21 and RCAS-PDGF-HA (Figure 2B and C). Mice

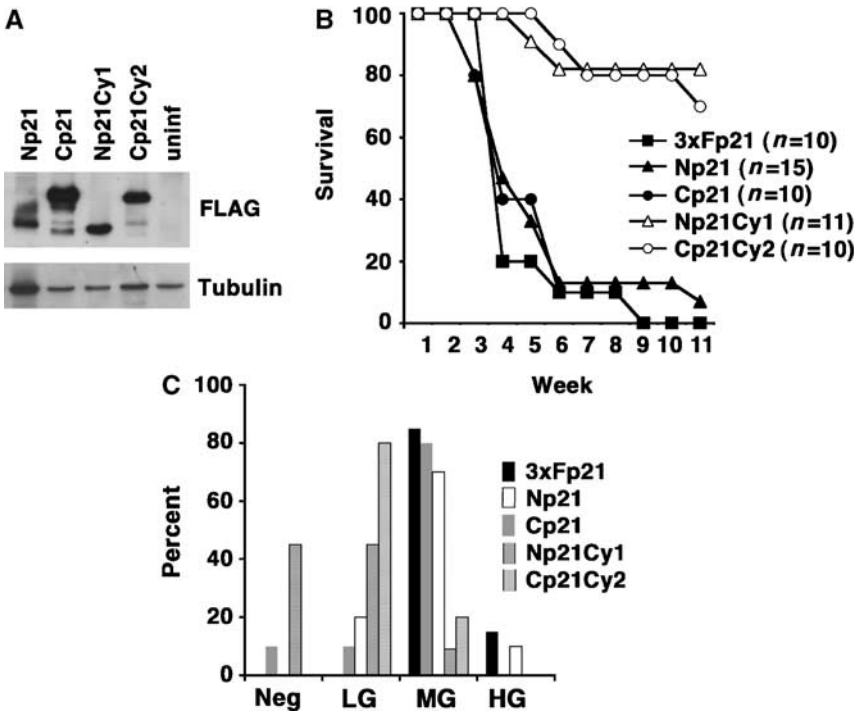


Figure 2 The Cy elements of p21 are required for robust progression of PDGF-induced ODG in mice. (**A**) The expression level of FLAG-tagged Np21, Np21Cy1, Cp21, and Cp21Cy2 mutants were comparable in the DF-1 producer cells injected into the recipient mice. Tubulin served as a loading control. (**B**) Symptom-free survival curve. Mutation of p21 Cy elements (Np21Cy1, Cp21Cy2) slowed disease progression (Np21 versus Np21Cy1, $P=0.0001$; Cp21 versus Cp21Cy2, $P<0.0001$). Animals infected with Np21, Cp21, or 3xFp21 showed a $>50\%$ mortality rate by the fifth week and approached 100% mortality by 11 weeks. Meanwhile, animals infected with Np21Cy1 or Cp21Cy2 showed an $\sim 10\%$ mortality rate by the fifth week and only 20–30% mortality by 11 weeks. The number of mice in each cohort is shown in the figure. (**C**) Tumors were graded histologically as described in the Supplementary Figure 6. Infection with Np21, Cp21, and 3xFp21 was associated with mostly moderate grade tumors, with a few low-grade and high-grade tumors and one tumor-free Cp21-infected mouse. Infection with Np21Cy1 or Cp21Cy2 however resulted in few moderate grade tumors and many low-grade tumors. Moreover, half of the Cp21Cy2-infected mice were tumor free.

lacking the Ntv-a transgene, regardless of p21 status, did not develop disease regardless of which virus or combination of viruses they were infected with. We confirmed expression of nuclear FLAG-tagged Np21 and Cp21 proteins in the tumors by FLAG immunohistochemistry.

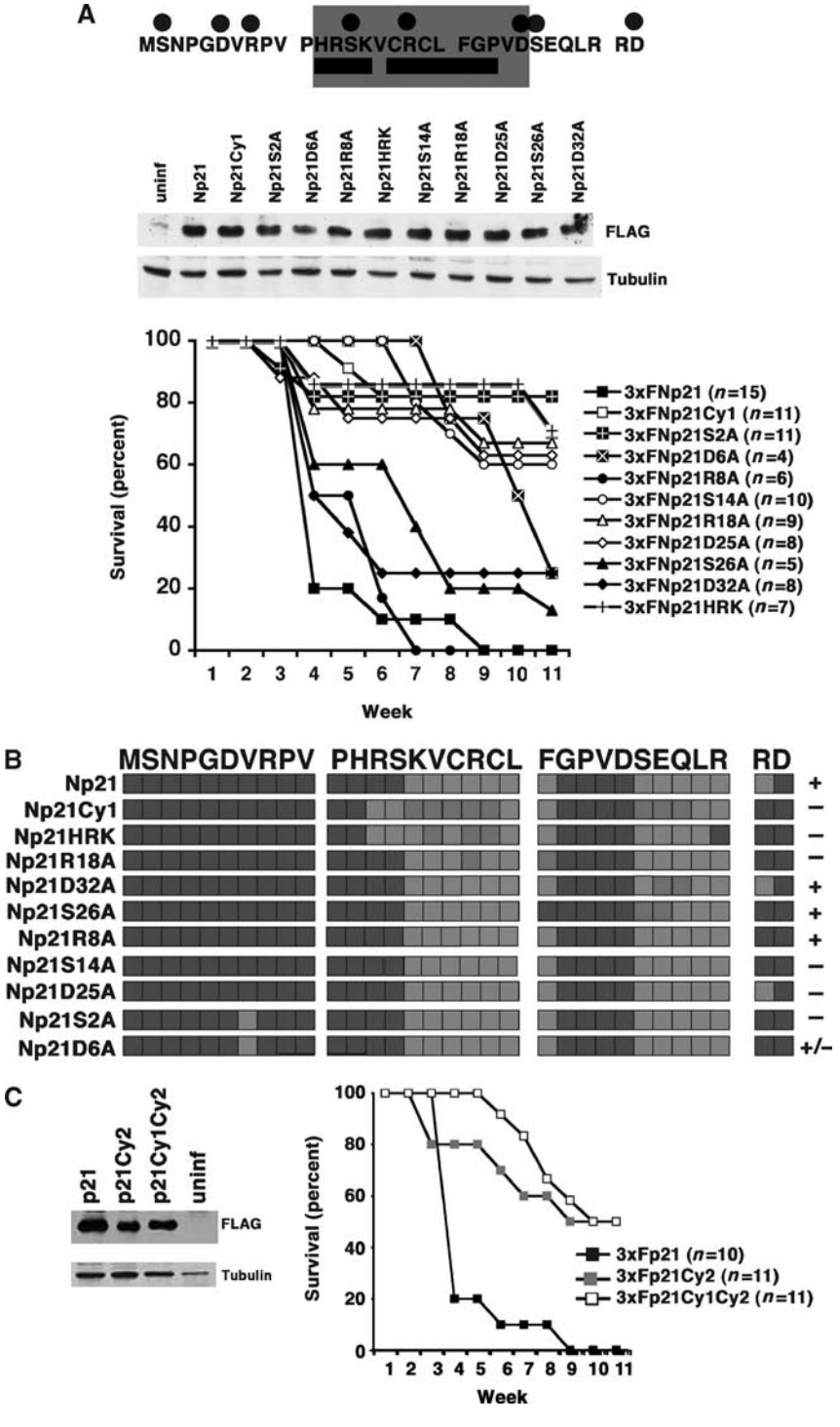
Mutation of the cyclin binding or Cy element, in either fragment, reduced complementation activity (Figure 2B). Tumors that arose in animals infected with constructs containing mutated Cy elements were of lower grade (Figure 2C). Thus, p21 expression facilitates the progression of the disease.

To further test the requirement for the Cy element, we generated eight single amino acid substitution mutants in and around (aa 12–25) in Np21, and introduced these as RCAS vectors into p21-deficient animals with RCAS-PDGF-HA (Figure 3A). Our analysis of these single base alanine sub-

Figure 3 Genetic analysis of the Cy requirement. (**A**) The N-terminal Cy element is shaded. Circles above the sequence indicate the alanine substitution mutations used. The bars below the sequence indicate the multisite mutations used. The expression of the mutants in DF-1 cells is shown below the sequence. Symptom-free survival of the animals coinfected with each mutant and RCAS-PDGF-HA is shown at the bottom. Mutations within the Cy element reduced complementation activity (S14A, $P=0.0001$; R18A, $P=0.001$; D25A, $P=0.005$; HRK, $P=0.001$). In these mutants, at least 70% of the animals were still alive at the end of 7 weeks and no more than 40% of the animals had died by the end of the 11th week. Additionally, the S2A mutant abrogated complementation activity ($P<0.0001$) with two of the 11 mice dying by the fourth week, but no subsequent loss of additional animals. In contrast, most of the other mutations outside the Cy element did not reduce complementation activity (R8A, $P=0.4$; S26A, $P=0.1$; D32A, $P=0.1$). At least 50% of these animals died by the seventh week and fewer than 30% survived to the end of the experiment. The D6A mutation was intermediate, with a significant effect on complementation ($P<0.005$) early, with $\sim 80\%$ of the animals surviving beyond 7 weeks and then a dramatic decline with most animals expiring in the last three weeks. (**B**) *In silico* folding analysis. Proteins were folded using algorithm at Predictprotein.org (Rost *et al*, 2004). The sequence of the N-terminal fragment is shown at the top of the figure. Each row represents one of the proteins analyzed and its ability to complement tumor development is indicated by a (+) or (−) on the right. Blue boxes indicate a high probability ($>70\%$) for folding into a loop, red boxes a high probability ($>70\%$) for folding into a helix, and gray boxes indicate that neither a helix nor loop is of high probability. The Cy1, HRK, and R18A mutations all induce a structural change in the Cy element region bordered by serine 18 and aspartate 25, both of which are required for cyclin–cdk binding. Structural changes in the region encompassing serine 26 to arginine 31 do not correlate with complementation activity. (**C**) Cy elements facilitate the tumor-promoting effect of p21. In the left panel, the expression of FLAG-tagged 3xFp21, 3xFp21Cy1Cy2, and 3xFp21Cy2 mutants were compared in DF1 producer cells injected into recipient mice. Tubulin served as a loading control. In the panel on the right, survival of animals infected with each recombinant p21 virus and RCAS-PDGF-HA is shown. Coinfection with either 3xFp21Cy2 or 3xFp21Cy1Cy2 significantly prolonged survival (3xFp21Cy2, $P=0.001$; 3xFp21Cy1Cy2, $P<0.0001$). Most (8/10) of the control animals injected with 3xFp21 died by week 4, and the two remaining animals died by week 9. Meanwhile, half of the animals infected with either Cy element mutated construct were still living at the conclusion of the experiment. The number of mice in each cohort is shown. A full-color version of this figure is available at the *EMBO Journal* online.

stitutions and another multisite mutation in Cy1 (HRSK-to-AASA) revealed a role for this element in tumor initiation or progression. We also found evidence that there may be additional rate-determining functions in regions outside what we defined as the Cy element. Mutation of D6 slowed tumor development, and mutation of S2 abrogated complementation activity. We think this might reflect a second domain. An *in silico* analysis of protein folding suggests that neither the S2A and D6A mutation converted the flexible linker of the Cy element region, bordered by S14 and D25, to a more rigid helical fold as observed in the other noncomplementing mutants (Figure 3B).

To test if the Cy elements were sufficient to account for the p21 requirement in PDGF-induced ODG, we generated additional RCAS constructs containing full-length p21 harboring either a single mutation at the Cy1 or Cy2 site, or a double mutation at both sites (Figure 3C). The p21Cy2 and p21Cy1Cy2 mutants were expressed at levels similar to full-length p21 (Figure 3C). The expression of the p21Cy1 mutant was much lower than the others and thus we could not analyze its effect (data not shown). Of the 11 p21Cy2-infected animals, we were able to grade 10 tumors (one animal died and that tumor was necrotic which prevents reliable grading). Of the four animals that died within 6 weeks, three had low-



grade tumors and one had a moderate-grade tumor. Although low-grade tumors generally do not affect survival, occasionally these cells infiltrate and destroy critical brain structures leading to morbidity. Of the remaining six animals that lived into the 12th week, three had no tumor, two had low-grade tumors and one had a moderate-grade tumor. We could grade all 11 p21Cy1Cy2-infected animals. Of the five animals that died in the first 9 weeks, three had moderate-grade tumors and two had low-grade tumors. Of the remaining six animals that lived into the 12th week, two were negative and four had low-grade tumors. No mouse, regardless of p21 status, developed tumors when injected with an RCAS vector encoding p21 or any of the p21 mutants used in this study alone. PDGF was required to drive tumor development and p21, through its Cy domains, appears to affect progression.

p21 gene status affects accumulation of nuclear cyclin D and cyclin D-associated kinase activity

Our data implicate the Cy element in the function of p21 during PDGF-induced ODG development. This domain is necessary for the association of p21 with cyclin–cdk complexes (Chen *et al*, 1996). Evidence from our lab and others has suggested that binding of p21 and p27 to cyclin D–cdk4 complexes does not inhibit their kinase activity in proliferating cells (Soos *et al*, 1996; Blain *et al*, 1997; LaBaer *et al*, 1997), but rather can interfere with nuclear export (Alt *et al*, 2002) and cytosolic ubiquitin-dependent protein turnover (Lin *et al*, 2006). In addition to cyclin–cdk interaction, we have found that the Cy element is also required for the interaction of p21 with two proteins involved in receptor and endosome trafficking (YL, Hediye Erdjument-Bromage, Paul Tempst, and AK, unpublished data).

To determine which type of interaction was more likely to account for the p21 requirement during ODG, we looked at both changes in cyclin D and associated kinase activity (see Supplementary data) and PDGF receptor density at the cell surface (see Supplementary data). Cyclin D1 accumulated in the nucleus of tumor cells induced by PDGF in wild-type mice, as it did in any tumor that arose when p21-deficient mice were reconstituted with different alleles of p21 (Supplementary Figure 9A). Furthermore, in PDGF-transformed glial progenitors, p21 was nuclear (Supplementary Figure 9B) and bound to cyclin D–cdk4 (Supplementary Figure 9C). p21 immunoprecipitates contained an Rb kinase activity (Supplementary Figure 9D). In p21-deficient cells, the amount of cyclin D–cdk4 complex and cyclin D-associated kinase activity were reduced (Supplementary Figure 9C and D), even though p27, a related cdk inhibitor, was present (Supplementary Figure 9E), suggesting that there might be a division of function between Kip-family members in this cell type. Furthermore, the amount of the D-type cyclins and cdk6 were reduced in $p21^{-/-}$ PDGF-transformed progenitor cells (Supplementary Figure 9E). The half-life of cyclin D was reduced five-fold, from approximately 60 min in wild-type cells to 12 min in p21-deficient cells.

p21 is an inhibitor of cdk2 activity. While the amount of cyclin E, cyclin A and cdk2 were only modestly affected by p21 deficiency (Supplementary Figure 9E), cdk2- and cyclin A-associated kinase activity was increased approximately 60% in p21-deficient cells (Supplementary Figure 9F). There was no change in cyclin E-associated kinase activity (Supplementary Figure 9F). Thus, in PDGF-transformed glial cells, p21 status clearly affected the accumulation of nuclear cyclin D.

We also looked at the expression of PDGF receptor on the cell surface. The density of PDGF receptors on the cell surface was similar in both wild-type and p21-deficient cells (Supplementary Figure 10). This reduces the likelihood that p21 deficiency is affecting PDGF receptor accumulation at the cell surface by acting in the receptor trafficking or endosome-sorting pathway. Together, these data suggest that the requirement for the Cy element was likely to reflect its role of stabilizing nuclear cyclin D1–cdk4 complexes.

Re-expression of functional cyclin D1 can support PDGF-induced tumor development during oligodendrogliomagenesis

To evaluate the ability of p21 to stabilize the cyclin D–cdk4 complex during PDGF-induced tumor development, we asked if enforcing expression of functional cyclin D1–cdk4 complexes would be sufficient to overcome the effect of p21 deficiency on tumor growth. Simply overexpressing wild-type cyclin D1 did not suffice to drive nuclear accumulation in p21-deficient glial cells; however, two cyclin D1 mutants were previously shown to accumulate in cki-deficient cells, and we confirmed this in our p21-deficient glial cells as well. Mutation of Thr286 to Ala (cycD1T286A) blocks phosphorylation at this residue and prevents nuclear export (Alt *et al*, 2000). Mutation of Thr156 to Ala (cycD1T156A) largely, although not completely, accumulates in the cytosol where it binds cdk4 but fails to activate it (Diehl and Sherr, 1997). Thus, using these two mutants, we could test the relative importance of accumulated cyclin D1–cdk4 complexes.

cDNAs encoding these mutants were cloned into RCAS vectors and their subcellular localization was confirmed by immunofluorescence. Almost all the cycD1T286A protein accumulated in the nucleus, and the cycD1T156A accumulated almost exclusively in the cytosol (Figure 4A). Approximately 15% of the cycD1T156A-expressing cells also had some nuclear staining, consistent with previously published work (Diehl and Sherr, 1997). Although cycD1T286A is a weak oncogene in a lymphoma model (Gladden *et al*, 2006), we did not observe this in our model probably because of the short time frame in which animals were maintained post-infection. Similarly, expression of cycD1T156A alone did not induce tumors in mice.

We subsequently examined the ability of these mutants to promote ODG when introduced into $p21^{-/-}$ mice with RCAS-PDGF-HA. Expression of these mutants in the DF-1 producer cells was approximately equivalent (Figure 4B inset). p21-deficient mice infected with cycD1T286A had reduced survival compared to those infected with cycD1T156A (Figure 4B). Survival of cycD1T286A animals was slightly better, although not statistically significant, than those reconstituted with full-length p21. Greater than 50% of the p21-deficient mice infected with cycD1T286A developed moderate grade ODGs within 5 weeks and most of the mice were dead by 10 weeks with moderate- to high-grade glioma. Consistent with the reduced nuclear accumulation of cycD1T156A, the rate of tumor progression was significantly slower in $p21^{-/-}$ mice infected with the RCAS-cycD1T156A mutant. Of the 10 p21-deficient mice infected with cycD1T156A, only two died within the first 6 weeks and these had low-grade tumors. Four more mice died over the next four weeks, all with low-

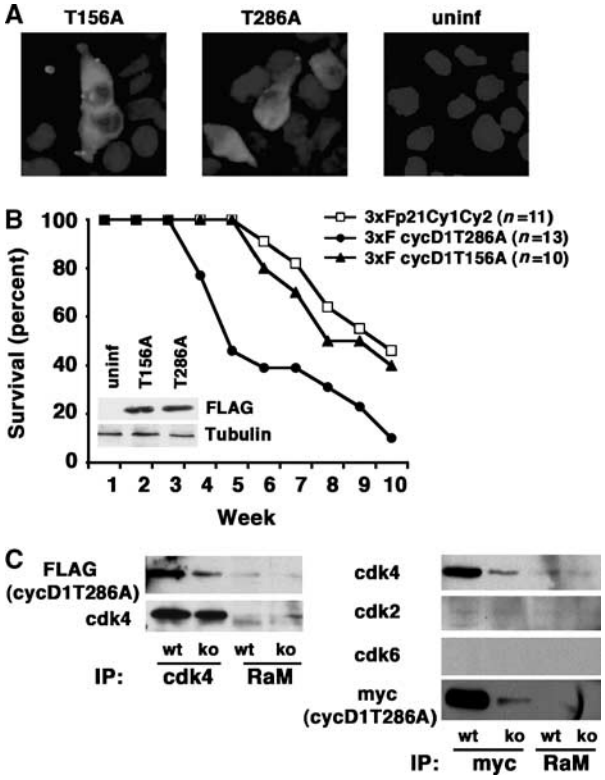


Figure 4 Cyclin D1T286A can complement p21 deficiency for tumor progression. (**A**) Subcellular localization of the cyclin D1 mutants used in this study was determined by immunofluorescence in 293 cells transiently transfected with RCAS-cycD1T156A, RCAS-cycD1T286A, or an empty RCAS vector (uninfected) as indicated above each panel. These cells were incubated with an anti-cyclin D1 antibody (green) and counterstained with DAPI (blue) to identify the nuclei. (**B**) The rate of disease progression was similar in $p21^{-/-}$ mice coinfected with RCAS vectors expressing PDGF-HA and either cycD1T156A, or p21Cy1Cy2, whereas coexpression of cycD1T286A with RCAS-PDGF-HA significantly accelerated the rate of disease progression ($P<0.005$). The number of mice in each cohort is shown. In the inset, immunoblotting shows that the expression levels of cycD1T286A and cycD1T156A were comparable in the DF-1 producer cells injected into the recipient mice. Tubulin served as a loading control. (**C**) CycD1T286A binds to cdk4. PDGF-transformed wild-type and p21-deficient glial cells were transfected with either 3xFLAG-cycD1T286A (left) or myc-cycD1T286A (right) and extracts prepared 48 h later. FLAG-cycD1T286A co-precipitated with cdk4 antibodies. Because the FLAG antibodies did not immunoprecipitate efficiently, we immunoprecipitated myc-tagged cyclin D to look at cdk interactions. Cdk4, but not cdk2 or cdk6 co-precipitated with cycD1T286A. A full-color version of this figure is available at the *EMBO Journal* online.

grade tumors. The tumors that arose in both cycD1T286A- and cycD1T156A-expressing animals were ODGs as judged by histological appearance of the cells, positive staining for olig2 and negative staining for NeuN. cycD1T286A was able to bind to cdk4, and did not bind cdk2 or cdk6 (Figure 4C). This suggests that enforced nuclear accumulation of cyclin D1–cdk4 complexes bypassed the requirement for p21.

The ability of cdk4 to associate with cyclin D is required for complementation activity

Nuclear cyclin D can interact with both Kip-family members and nuclear hormone receptors (Neuman *et al*, 1997; Reutens *et al*, 2001). Association with Kip-family members depends on the ability of the cyclin to bind to cdks, whereas association with nuclear hormone receptors is prevented by cdk binding (Zwijsen *et al*, 1997). Thus, we wanted to determine whether the ability of cycD1T286A to complement the p21 deficiency was also dependent on its ability to bind to cdk4. To accomplish this, we generated an RCAS vector expressing another cyclin D1 mutant, cycD1T286A/K114E, which did not bind to cdk2, 4, or 6 (Figure 5A), but accumulated in the nucleus of all cells (Figure 5B). This cycD1K114E mutant was originally characterized as a non-cdk-binding protein (Hinds *et al*, 1994). We also observed cytosolic accumulation in about 30% of these cells where protein accumulated in the nucleus as well. Alone, when infected into $p21^{-/-}$ mice or wild-type mice, this mutant did not promote tumor development by 9 weeks, when this experiment was ended. However, unlike cycD1T286A, cycD1T286A/K114E did not support the development of ODG induced by RCAS-PDGF (Figure 5C). The one mouse that died during the sixth week was tumor free. Consequently, we conclude that the nuclear cyclin D1–cdk4 is the most likely target of p21 responsible for the tumor-promoting effect of p21.

Discussion

Our understanding of the roles that proteins play in hematological malignancies is further advanced than our understanding in solid tumors. Germline mutations are widely used to study both hematological malignancies and solid tumors; however, in hematological malignancies our ability to isolate stem cells from mice, genetically manipulate these *ex vivo*, and reintroduce them into syngenic animals allows us to determine the role of each protein or pathway in a natural setting. Our understanding of the pathways impacting solid tumor development is commensurately poorer because the cell of origin of many solid tumors remains a mystery and an allograft may not recapitulate the environment in which tumors evolve.

The cki family of proteins was originally identified by their ability to bind to G1 cdks, ultimately inhibiting kinase activity and preventing progression through the G1-S transition. However, we have begun to appreciate that this is only one biochemical activity, and growth suppression is only one role that these proteins have. For example, p21 and p27 play distinct roles in the growth and differentiation of OPC (Casaccia-Bonnefil *et al*, 1997; Zezula *et al*, 2001; Doetsch *et al*, 2002). These proteins are also useful prognostic markers in ODG, albeit in a reciprocal fashion with high p27-staining indices associating with good prognosis, and high p21-staining indices associating with poor prognosis (Cavalla *et al*, 1999; Miettinen *et al*, 2001). In our studies we found that p21 facilitates the development of PDGF-induced ODG in mice. Thus, p21 makes a contribution to tumor progression. It is 'oncogenic.'

How common are tumor-promoting activities? While there is an abundance of examples where cki are growth suppressive, there are a smaller number in which an 'oncogenic' role is consistent with the data. The biochemical activities of cki might reflect the specific cell types or conditions in which they are studied; thus, 'oncogenic' activity might be restricted to certain cell types or carcinogenic insults. In a Pten/Nkx3.1-deficient prostate model (Gao *et al*, 2004), an MMTV-erbB2/neu mammary model (Muraoka *et al*, 2002), and an MMTV-Wnt1 mammary model (Jones *et al*, 1999), the complete absence of p21 or p27 reduces tumor development, suggest-

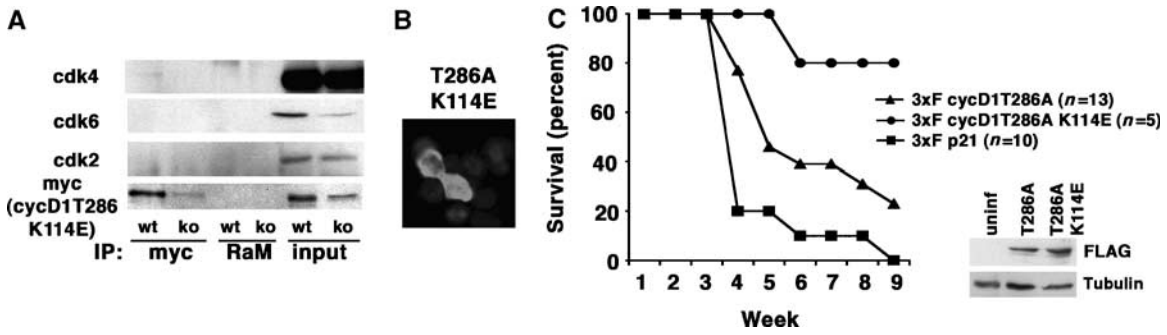


Figure 5 Cdk4 binding is required for cycD1T286A to complement p21 deficiency for tumor progression. (**A**) CycD1T286A/K114E does not bind to cdk4. As described in the legend to Figure 4C, cells were transfected with myc-cycD1T286A/K114E, extracts immunoprecipitated with anti-myc antibodies, and the presence of cdks and myc-cyclin D1 assessed by immunoblotting. (**B**) Subcellular localization of cycD1T286A/K114E was determined by immunofluorescence as described in the legend to Figure 4A. (**C**) The rate of disease progression was diminished in $p21^{-/-}$ mice coinfected with RCAS vectors expressing PDGF-HA and cycD1T286A/K114E ($P<0.005$). The one animal that did develop morbidity had no evidence of a tumor when examined by gross histology. For comparison, we included the survival data of p21-deficient mice reconstituted with either full-length p21 or the cycD1T286A mutant. The number of mice in each cohort is shown. To the right of the Kaplan–Meier curve, immunoblotting showed that the expression levels of cycD1T286A and cycD1T286A/K114E were comparable in the DF-1 producer cells injected into the recipient mice. Tubulin served as a loading control. A full-color version of this figure is available at the *EMBO Journal* online.

ing that at least some level of p21 or p27 might be required for tumor progression under these conditions. In these three studies the cki was nuclear. A more recent study in a p27ck(−) knock-in animal model suggested a cdk-independent function promoting stem cell expansion and tumor development, and p27ck(−) protein was both nuclear and cytoplasmic (Besson *et al*, 2007). In addition to ODG, there are suggestions for p21 'oncogenicity' in other human cancers as well, including prostate (Aaltomaa *et al*, 1999; Baretton *et al*, 1999; Omar *et al*, 2001), cervical (Bae *et al*, 2001; Cheung *et al*, 2001), breast (Ceccarelli *et al*, 2001), squamous cell carcinoma (Sarbia *et al*, 1998), and tall-cell and well-differentiated papillary thyroid cancer (RG, BS and AK, unpublished data). Consequently, a growth- or tumor-promoting role is not unusual, but our understanding of it at the molecular and cellular levels is largely based on inferences drawn from subcellular localization and evaluation of the affect of protein levels on proliferation and apoptosis. Genetic evidence validating such notions has been elusive.

What biochemical activities of p21 and p27 might be important for 'oncogenicity'? cki are found in multiple protein complexes, sometimes operating in distinct subcellular locations (Coqueret, 2003; Denicourt and Dowdy, 2004; Child and Mann, 2006). These features might account for their 'oncogenic' role (McAllister *et al*, 2003; Denicourt and Dowdy, 2004; Wu *et al*, 2006). Some of these interactions occur when the cki are in the cytosol. In neuronal cells and mouse embryo fibroblasts, cytoplasmic p27 interacts with rhoA to affect cell migration (Besson *et al*, 2004; Nguyen *et al*, 2006). Cytoplasmic p27 can also interact with grb2 (Moeller *et al*, 2003). Reducing cytosolic p27 inhibits cancer cell motility and tumorigenicity by affecting rho and akt signaling pathways (Wu *et al*, 2006). Binding of cytosolic p21 to procaspase 3 (Suzuki *et al*, 1998, 1999, 2000a, b; Dotto, 2000; Glaser *et al*, 2001; Weiss, 2003) or ask1 (Asada *et al*, 1999; Zhan *et al*, 2007) can desensitize tumor cells to apoptotic stimuli. Conversely, nuclear roles should be considered. As mentioned previously, nuclear p21 and p27 facilitate tumor development in the Pten/Nkx3.1, MMTV-Wnt1, and MMTV-erbB2/neu models. Nuclear cki can promote the accumulation of cyclin D–cdk4 (Cheng *et al*, 1999; Weiss *et al*, 2000), and p21 can interact with a surfeit of transcription factors and chromatin remodeling proteins (Dotto, 2000; Gartel, 2006a, b). However, establishing that a particular interaction is responsible, *in situ*, in a developing tumor is a considerable challenge. Furthermore, given the cornucopia of possible interactions, it is unlikely that a single mechanism explains its role in all tumors.

In the studies presented here we have shown that p21 accumulates in the nucleus of ODG tumor cells and in glial cells stimulated by PDGF signaling. We have shown that this is associated with the accumulation of nuclear cyclin D1 and formation of cyclin D–cdk4 complexes, and increased proliferation and reduced apoptosis. Most importantly, by using somatic cell engineering, we established that p21 acts cell autonomously to promote tumor development, and this depends on the Cy element. Through this element, p21 interacts with cyclin–cdk complexes, and interacts with components of the receptor trafficking and endosome sorting machinery. Nevertheless, the status of p21 had no effect on the accumulation of PDGF receptors at the cell surface, and we were able to bypass the effect of p21 deficiency by enforcing accumulation of functional cyclin D1. Mutants of cyclin D1 that fail to accumulate in the nucleus but bind cdk4, or that accumulate in the nucleus but fail to bind cdk4 were both unable to support tumor development. All together, this suggests that p21 promotes ODG by stabilizing cyclin D1–cdk4 in the nucleus. Although this mechanism has been suggested before, specifically for p27 in the Pten/Nkx3.1 and MMTV-erbB2 models, and for p21 in the MMTV-Wnt1 model, this is the first time that a genetic proof has been used to assess the veracity of this model.

Nevertheless, our approach to identify protein domains will also benefit from further biochemical refinement. For example, it was surprising that the ability of the p21Cy2 and p21Cy1Cy2 mutants were comparable, albeit there was a 'cy-dose' dependency to the onset of morbidity. We expected that the p21Cy2 mutant, with an intact Cy1 element, would support tumor development, just like Np21. The fact that it does not suggests that its interactions with other proteins in the cell could affect its availability to associate with cyclin D–cdk4, which is unaffected *in vitro* (data not shown).

Additionally, overexpression of the mutants from a heterologous promoter might allow 'weak' alleles to have functional affect.

In the absence of a genetic analysis, suggestions based on knowing where a cki accumulates and the effect of its absence on proliferation and apoptosis might be incorrect. For example, it is difficult to reconcile the suggestion that p21 supports cyclin D–cdk4 accumulation in the MMTV-Wnt1 model, when Yu *et al* (2001) later demonstrated that cyclin D1 was not required in this model. Ultimately, identifying the correct mechanism is critical for providing insight into how to modulate p21 levels for therapeutic gain.

Materials and methods

Cell culture

RCAS vectors were propagated in chicken DF-1 cells (ATCC, CRL-12203), cultured as suggested by ATCC. Only DF-1 cells that had been in culture for less than six passages after transfection with RCAS-viral cDNA were used for infections. PDGF-transformed glial progenitors were generated by infecting whole brain cultures of either $p21^{+/+}$*Ntv-a* or $p21^{-/-}$*Ntv-a* mice with RCAS-PDGF-HA viral supernatants obtained from infected DF-1 cells, and maintained in DMEM supplemented with 10% fetal bovine serum.

Plasmid construction

The FLAG-p21 expression plasmid was constructed by cloning the mouse p21-coding sequence into pcDNA3 (Invitrogen). FLAG-tagged p21 deletion constructs were generated by PCR-based DNA mutagenesis. FLAG- or myc-tagged cyclin D1 expression plasmids were obtained by cloning mouse cyclin D1 into either p3XFLAG-CMV14 (Sigma) or pCMV-myc (CloneTech), respectively. Mutation was carried out using an *in vitro* site-directed mutagenesis kit (CloneTech). All FLAG-tagged cDNAs were subcloned into RCAS vectors using the Gateway *in vitro* recombination system. All mutations were confirmed by sequencing both DNA strands. The RCAS-PDGF-HA expression plasmid employed was described previously (Dai *et al*, 2001).

In vivo infection of TvA-transgenic mice

DF-1 cells producing RCAS viruses were trypsinized, suspended in ~50 μl of media, and placed on ice before injection as described previously (Dai *et al*, 2001). An aliquot of these cells was taken to make an extract to allow the detection of vector expression by anti-FLAG immunoblotting (Shaffer *et al*, 2005).

Proliferation and apoptosis

To measure proliferation, cells were grown on coverslips and incubated for 90 min in medium containing 65 μM BrdU, and subsequently fixed with 4% paraformaldehyde and stained with anti-BrdU antibodies as described previously (Zezula *et al*, 2001; Liu *et al*, 2004). To measure apoptosis, $2 \sim 3 \times 10^5$ cells were analyzed using an Annexin V-FITC apoptosis detection kit according to the manufacturer's instructions (BD Pharmingen).

In tumors, proliferation and apoptotic indices were determined by counting the number of Ki67 or cleaved caspase 3-positive cells in five fields of three random sections for each tumor.

FISH analysis

Brain touch imprints or metaphase spreads on glass slides were air-dried, fixed in 3:1 methanol/glacial acetic acid at −20°C for 20 min, air dried, then stored at −20°C. FISH was performed as described (Shaffer *et al*, 2005). Mouse DX-Was70 was used as reference probe. RCAS probes were labeled with Digoxigenin-dUTP (Roche) and X chromosome probes were nick translation labeled with Spectrum orange-dUTP (Vysis). Two hundred cells were scored for the analysis. Areas of overlapping cells were excluded from analysis.

Statistical analysis

A logrank (Mantel-Cox) test was used to determine significance.

Additional Materials and methods can be found in the Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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