Cdk4 disruption renders primary mouse cells resistant to oncogenic transformation, leading to Arf/p53-independent senescence

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A large number of human cancers display alterations in the Ink4a/cyclin D/Cdk4 genetic pathway, suggesting that activation of Cdk4 is an important role in oncogenesis. Here we report that Cdk4-null mouse embryonic fibroblasts are resistant to transformation in response to Ras activation with dominant-negative (DN) p53 expression or in the Ink4a/Arf-null background, judged by foci formation, anchorage-independent growth, and tumorigenesis in athymic mice. Cdk4-null fibroblasts proliferate at normal rates during early passages. Whereas Cdk4+/Ink4a/Arf−/− cells are immortal in culture, Cdk4−/−Ink4a/Arf−/− cells undergo senescence during continuous culture, as do wild-type cells. Activated Ras also induces premature senescence in Cdk4−/−Ink4a/Arf−/− cells and Cdk4−/− cells with DNp53 expression. Thus, Cdk4 deficiency causes senescence in a unique Arf/p53-independent manner, which accounts for the loss of transformation potential. Cdk4-null cells express high levels of p21Cip1/Waf1 with increased protein stability. Suppression of p21Cip1/Waf1 by small interfering RNA (siRNA), as well as expression of HPV-E7 oncoprotein, restores immortalization and Ras-mediated transformation in Cdk4+/Ink4a/Arf−/− cells and Cdk4−/− cells with DNp53 expression. Therefore, Cdk4 is essential for immortalization and suppression of Cdk4 could be a prospective strategy to recruit cells with inactive Arf/p53 pathway to senescence.

Keywords: Cell cycle; cancer; immortalization; cyclin; Cdk; Ras; Ink4a; p21; stability

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and p27Kip1 without being inactivated [Soos et al. 1996; Blain et al. 1997, Sherr and Roberts 1999]. These Kip/Cip proteins rather promote assembly of cyclin D/Cdk4 [Labaer et al. 1997], suggesting that the physical interaction with cyclin D/Cdk4 titrates p21 and p27 populations available for Cdk2 inhibition. Therefore, Cdk4 plays both catalytic and noncatalytic roles in control of G1 progression.

A large number of human cancers show genetic alterations that deregulate cyclin D/Cdk4 [Hirama and Koeffler 1995; Pestell et al. 1999, Sherr 2000]. Many glioblastomas, gliomas, and sarcomas display Cdk4 overexpression due to gene amplification [Khatib et al. 1993]. Melanoma-prone families have been found to carry germline mutations of Cdk4 at the Arg24 residue that render the kinase refractory to Ink4-dependent inhibition [Wolfe et al. 1995; Zuo et al. 1996]. Various types of cancer show overexpression of D-type cyclins. More frequent cancer-associated alterations are deletions, mutations, and methylation of the Ink4a/Arf locus [Kamb et al. 1994; Sherr 1998; Sharpless and DePinho 1999]. The Ink4a/Arf locus contains two independent genes encoding p16Ink4a and p14Arf [p19Arf in mice], which share exons 2 and 3 on alternative reading frames [Quelle et al. 1995]. Whereas p16Ink4a inhibits Cdk4 and Cdk6, Arf protein interferes with Mdm2-dependent degradation of the tumor suppressor p53, leading to stabilization of p53 [Pomerantz et al. 1998; Stott et al. 1998; Zhang et al. 1998]. Thus, inactivation of the Ink4a/Arf locus results in inappropriate activation of Cdk4 and rapid degradation of p53, both of which could contribute to tumorigenesis in distinct but cooperating manners. Consistent with this notion, mice deficient in both p16Ink4a and p19Arf [Serrano et al. 1996] or mice deficient in p19Arf with intact p16Ink4a [Kamijo et al. 1997] develop spontaneous tumors. Mice lacking p16Ink4a with intact p19Arf are susceptible to tumorigenesis to a lesser extent [Krippenfort et al. 2001; Sharpless et al. 2001]. These data suggest that activation of Cdk4 plays a critical role in tumorigenesis.

To further clarify the role of Cdk4 in cell-cycle control and tumorigenesis, we recently generated mice with targeted disruption of the Cdk4 gene. Cdk4-null mice are viable, and exhibit diabetes mellitus due to degeneration of pancreatic β-cells and growth retardation and infertility associated with severe hypoplasia and dysfunction of the pituitary [Tsutsui et al. 1999; Moons et al. 2002a,b]. Embryonic fibroblasts [MEFs] from Cdk4-null mice proliferate at normal rates, while they display a 4–5-h delay in entry into the cell cycle from quiescence [Tsutsui et al. 1999]. Therefore, Cdk4 is rate-limiting for cell-cycle entry but is dispensable for cell-cycle progression. However, it was unclear whether Cdk4 plays an essential role in immortalization and transformation. In this study, we demonstrate that Cdk4 is required for Ras-mediated transformation of MEFs, and that Cdk4 disruption leads cells to Arf/p53-independent senescence. These findings provide a significant foundation for anticancer therapies that target the cyclin D/Cdk4 pathway.

### Results

Cdk4-null MEFs are resistant to transformation in response to Ras activation and p53 inhibition

To examine the effect of Cdk4 disruption on transformation potential, we prepared Cdk4+/+ and Cdk4−/− MEFs from embryos obtained from intercross breeding of Cdk4+/− mice [Tsutsui et al. 1999]. Cells at early passages (passage 3–4) were infected with a retrovirus for expression of oncogenic H-RasVal12 and a dominant-negative p53 mutant [DNp53], previously described as GSE56 [Ossowskaya et al. 1996]. DNp53 encodes the amino acids 275–368 of p53, and suppresses p53 activity, presumably by interfering with oligomerization of the protein. Under standard culture conditions with 10% fetal bovine serum, Cdk4+/− MEFs proliferated at rates indistinguishable from those of Cdk4+/+ MEFs, as demonstrated previously [Tsutsui et al. 1999]. Following retroviral transduction of H-RasVal12, with or without DNp53, cells were cultured for 21 d without splitting and then stained to visualize transformed foci (Fig. 1).

![Figure 1](image_url)

Figure 1. Cdk4-null mouse embryonic fibroblasts are resistant to transformation induced by expression of H-RasVal12 and dominant-negative p53. (A) Passage 4 mouse embryonic fibroblasts [MEFs] with the indicated genotypes were infected with a retrovirus encoding H-RasVal12 and a dominant-negative p53 (DNp53), previously described as GSE56. MEFs were infected with a Cdk4 retrovirus 48 h prior to H-RasVal12 + DNp53. Cells were then cultured in the medium containing 5% FBS for 21 d. (B) The number of foci per 60-mm dish in the assays are expressed as means ± S.E.M. from three independent MEF preparations.
Strikingly, the numbers of foci developed in Cdk4−/− MEF cultures expressing H-RasVal12 and DNp53 were 95% reduced, relative to those in Cdk4+/+ cultures. Retroviral transduction of H-RasVal12 alone or DNp53 alone did not result in focus formation in either Cdk4−/− or Cdk4+/− MEFs. Immunoblotting confirmed that the levels of Ras expression were comparable in Cdk4−/− and Cdk4+/− cells (data not shown). Retroviral transduction of Cdk4 prior to transduction of H-RasVal12 and DNp53 restored foci formation [Fig. 1B], confirming that the absence of Cdk4 is responsible for the inhibition of foci formation.

We also examined anchorage-independent growth by plating MEFs in soft agar following retroviral transduction [Supplementary Fig. 1]. Whereas Cdk4+/+ MEFs expressing H-RasVal12 and DNp53 efficiently developed colonies in soft agar, Cdk4−/− MEFs did not form detectable colonies under the same conditions. MEFs expressing H-RasVal12 alone or DNp53 alone formed no colonies regardless of the Cdk4 genotype, as expected. These data suggest that Cdk4 disruption inhibits cellular transformation induced by Ras activation and p53 inhibition.

Cdk4−/− Ink4a/Arf−/− MEFs are resistant to Ras-induced transformation

To further examine the effect of Cdk4 deficiency on Ras-mediated transformation, we crossed Cdk4-null mice and mice with deletion of the exons 2 and 3 of the Ink4a/Arf locus (Serrano et al. 1996), and prepared Cdk4−/−Ink4a/Arf−/− and Cdk4+/−Ink4a/Arf−/− MEFs. Cells at early passage were infected with retrovirus for H-RasVal12 or control virus, and then cultured for 17 d without splitting. Cdk4−/−Ink4a/Arf−/− MEFs efficiently developed transformed foci upon retroviral transduction of H-Ras [Fig. 2], as previously demonstrated (Serrano et al. 1996). In contrast, Cdk4−/−Ink4a/Arf−/− MEFs expressing H-RasVal12 poorly formed foci, showing 93% reduction in number. No colonies grew when Cdk4−/−Ink4a/Arf−/− MEFs were inoculated in soft agar following H-RasVal12 transduction, whereas Cdk4+/−Ink4a/Arf−/− MEFs readily developed colonies [data not shown]. These observations suggest that Cdk4 plays a major role in transformation of MEFs induced by Ras activation in the Ink4a/Arf-null background.

Cdk4-null cells isolated from foci are not tumorigenic in vivo

To determine whether Cdk4-null cells that formed foci were tumorigenic in vivo, we injected athymic mice with Cdk4+/+ and Cdk4−/− MEF clones isolated from foci induced by H-RasVal12 and DNp53, as shown in Figure 1. Cdk4+/+ clones exhibited slower proliferation in culture, compared with Cdk4−/− clones [data not shown]. Five independent clones with each genotype were tested [Fig. 3]. At 21 d postinjection, all five Cdk4+/+ clones displayed tumor growth, with diameters of 1.7 ± 0.5 cm [mean ± S.E.M.]. In contrast, none of five Cdk4−/− clones developed detectable tumors in athymic mice during the 6-wk monitoring period. We also examined the in vivo tumorigenericity of Cdk4+/−/Ink4a/Arf−/− and Cdk4−/−/Ink4a/Arf−/− MEF clones isolated and expanded from foci induced by H-RasVal12, as shown in Figure 2. Cdk4−/−/Ink4a/Arf−/− clones did not develop detectable tumors in athymic mice, whereas mice injected with Cdk4+/−/Ink4a/Arf−/− clones readily displayed large tumors [Fig. 3]. These data suggest that Cdk4 disruption abrogates tumorigenericity of MEFs induced by Ras activation with p53 inhibition or Ink4a/Arf disruption.

Cdk4−/− Ink4a/Arf−/− MEFs are resistant to Ras-induced transformation. (A) Passage 4 MEFs were infected with a retrovirus encoding H-RasVal12, or with a control virus with the pBabe-hygro vector. Cells were then cultured in the medium containing 5% FBS for 17 d. (B) The numbers of foci per 60-mm dish in the assays are expressed as means ± S.E.M. from three independent MEF preparations.
examined whether Cdk4−/− Ink4a/Arf−/− MEFs showed an immortal phenotype similar to Cdk4+/+ Ink4a/Arf−/− MEFs. Cells at a late passage (passage 11) were inoculated at a low density (1000 cells per dish) and cultured for 10 d to score colonies derived from isolated cells (Fig. 4A). Cdk4+/+ Ink4a/Arf−/− MEFs formed >200 large colonies, indicating clonogenic proliferation with high plating efficiency. In contrast, Cdk4−/− Ink4a/Arf−/− MEFs exhibited very few colonies. These observations suggest that Cdk4 disruption impairs clonogenic proliferation of Ink4a/Arf-null cells. 

We further assessed the proliferative lifespan of Cdk4+/+ Ink4a/Arf−/− and Cdk4−/− Ink4a/Arf−/− MEFs, monitoring population doublings during continuous culture according to the 3T3 protocol (Fig. 4B). Cdk4+/+ Ink4a/Arf−/− MEFs displayed escape from senescence, as expected. In contrast, Cdk4−/− Ink4a/Arf−/− MEFs underwent growth arrest after 22–24 population doublings, similarly to wild-type MEFs. Cdk4−/− Ink4a/Arf−/− cells at late passages displayed a flat enlarged morphology and senescence-associated β-galactosidase (SAβ-gal) activity (Fig. 4C), which are characteristic of cellular senescence (Dimri et al. 1995). The senescence phenotype was also observed in cells isolated from foci of Cdk4−/− MEFs expressing H-RasVal12 and DNp53, and in cells isolated from foci of Cdk4+/+ Ink4a/Arf−/− MEFs expressing H-RasVal12 (data not shown). These data suggest that the absence of Cdk4 induces senescence even with Ink4a/Arf disruption or p53 inhibition, which could account for the inhibition of oncogenic transformation.

Cdk4-null MEFs express high levels of p21Cip1/Waf1 with increased stability

To further investigate the mechanism of the resistance to Ras-mediated transformation in Cdk4-null cells, we examined the expression of proteins that regulate senescence. In primary mouse and human cells, Ras activation

![Image 1](image1.png)

**Figure 3.** Cdk4-null embryonic fibroblasts isolated from foci are not tumorigenic in athymic mice. (A) Foci were isolated from the confluent cultures at 21 d following retrovirus transduction of H-RasVal12 and DNp53 (see Fig. 1). (B) Foci were isolated from the confluent cultures at 17 d following H-RasVal12 expression (see Fig. 2). Cells were then expanded, and injected into athymic mice (10⁶ cells per site). Mice were examined 21 d after injection.

![Image 2](image2.png)

**Figure 4.** Cdk4 disruption renders cells insensitive to immortalization associated with Ink4a/Arf deficiency. (A) MEFs at passage 11 were plated at a low density (10⁶ cells per 60-mm dish), and cultured for 10 d. Colonies grown from isolated cells were stained with crystal violet. (B) Primary MEFs with indicated genotypes were propagated in culture according to the 3T3 protocol. Accumulated numbers of population doublings are shown. The data represent experiments using three independent MEF preparations for each genotype. (C) Senescence-associated β-galactosidase (SAβ-gal) staining. MEFs at passage 12 were inoculated at 3 x 10⁴ cells per 60-mm dish, and 10 d later, the cells were stained for SAβ-gal, as described in Materials and Methods.
higher in Cdk4−/− MEFs. Similarly, Cdk4−/− Ink4a/Arf−/− MEFs showed higher levels of p21Cip1/Waf1 than Cdk4−/− Ink4a/Arf−/− MEFs. H-RasVal12 did not significantly increase p21Waf1/Cip1 upon Ras activation. H-RasVal12 did not alter the expression of p21Cip1/Waf1. DNp53 or p27Kip1, regardless of the Cdk4 status. To determine whether the increased basal levels of p21Waf1/Cip1 in Cdk4-null cells are associated with p53 activity, we further examined the effect of DNp53 transduction on cellular expression of p21Cip1/Waf1 (Fig. 5B). DNp53 significantly down-regulated p21Cip1/Waf1 expression in both Cdk4−/− and Cdk4−/− MEFs, confirming the role of p53 in p21Cip1/Waf1 transcription. In Cdk4−/− MEFs, which showed higher basal levels of p21Cip1/Waf1 expression, DNp53 transduction decreased p21Cip1/Waf1 only to a level comparable to the basal levels in Cdk4−/− cells, suggesting that Cdk4 deficiency increases p21Cip1/Waf1 in a p53-independent manner. In contrast to the increased protein levels, the cellular amounts of p21Cip1/Waf1 mRNA were unchanged in Cdk4−/− and Cdk4−/− Ink4a/Arf−/− MEFs (Fig. 5C). We further determined that p21Cip1/Waf1 in Cdk4−/− MEFs is significantly more stable than that in Cdk4−/− MEFs, examining the degradation of p21Cip1/Waf1 in cells treated with a protein synthesis inhibitor, cycloheximide (Fig. 5D). Under these conditions, the degradation of p21Cip1/Waf1 was similar in Cdk4−/− and Cdk4−/− Ink4a/Arf−/− MEFs (data not shown). These data suggest that Cdk4 deficiency results in a specific increase in p21Cip1/Waf1, which could play a role in the senescence response.

Suppression of p21Cip1/Waf1 by siRNA restores immortalization and Ras-mediated transformation in Cdk4-null MEFs

To determine whether elevated p21Cip1/Waf1 expression in Cdk4-null MEFs is required for the inhibition of immortalization and transformation, we used small interfering RNA (siRNA) to suppress cellular expression of p21Cip1/Waf1. A 21-mer double-stranded RNA designed specifically from residues 136–156 of the coding region of mouse p21Cip1/Waf1 mRNA was able to suppress cellular p21Cip1/Waf1 expression by more than 90%, suggesting that a majority of cells were successfully transfected (Fig. 6A). The siRNA-based suppression of p21Cip1/Waf1 significantly restored clonogenic proliferation in low-density cultures of Cdk4−/− Ink4a/Arf−/− MEFs (Fig. 6B,C), suggesting that the elevated p21Cip1/Waf1 expression plays a critical role in the limited proliferative lifespan. Moreover, siRNA-mediated suppression of p21Cip1/Waf1 was able to restore foci formation significantly in Cdk4−/− Ink4a/Arf−/− cultures in response to H-RasVal12 transduction (Fig. 6D). The numbers of Ras-induced foci in siRNA-treated Cdk4−/− Ink4a/Arf−/− cultures were about 75% of those in control Cdk4−/− Ink4a/ Arf−/− cultures [24 ± 3 vs. 32 ± 4, mean ± S.E.M., n = 3]. The anti-p21Cip1/Waf1 siRNA treatment increased foci formation modestly (−25%) in Cdk4−/− Ink4a/Arf−/− cul-

Figure 5. Cdk4-null MEFs express high levels of p21Cip1/Waf1 with increased stability regardless of the Arf/p53 status. (A) Cells with indicated genotypes were infected with retrovirus constructed from pBabe-H-RasVal12 or pBabe-hygro control vector. Infected cells were selected for 72 h in the presence of 50 µg/mL hygromycin, and were then analyzed by immunoblotting for the proteins indicated. P, uninfected proliferating cells (no selection); R, cells infected with H-RasVal12 retrovirus; V, cells infected with vector control virus. (B) Cells were infected with retrovirus constructed from LXSN-dominant-negative (DN) p53 or LXSN control vector (V). Infected cells were selected for 72 h in the presence of 2 µg/mL puromycin, and then analyzed by immunoblotting for the expression of p21Cip1/Waf1, a band with nonspecific immunoreactivity. (C) Expression of p21Cip1/Waf1 mRNA is unaltered. Exponentially proliferating cells at passage 4 were analyzed by RT-PCR for the expression of p21Cip1/Waf1 mRNA and GAPDH mRNA. The genotypes of cells: 1, Cdk4−/− (wild-type); 2, Cdk4−/− Ink4a/Arf−/−; 3, Cdk4−/− Ink4a/Arf−/−; 4, Cdk4−/− Ink4a/Arf−/−. (D) p21Cip1/Waf1 is stabilized in Cdk4−/− cells. Exponentially proliferating cells were treated with 40 µg/mL cycloheximide (chx) for the times indicated, and cellular levels of p21Cip1/Waf1 were determined by immunoblotting. These data represent experiments using three independent cell preparations at passage 3 or 4 for each genotype.

or continuous passage in culture induces the expression of p15Ink4b, p16Ink4a, and p21Cip1/Waf1, as well as p19Arf [or p14Arf in human cells, Sherr and DePinho 2000]. Cdk4−/− and Cdk4−/− MEFs displayed similar induction of the expression of p15Ink4b, p16Ink4a, and p19Arf following H-RasVal12 transduction [Fig. 5A]. In contrast, the basal level of p21Cip1/Waf1 expression was significantly higher in Cdk4−/− cells, relative to Cdk4−/− cells, and H-RasVal12 transduction increased p21Waf1/Cip1 expression even
Transfection of the anti-p21Cip1/Waf1 siRNA also restored foci formation significantly in Cdk4−/− MEFs with transduction of H-RasVal12 and DNp53 (data not shown). These data suggest that increased expression of p21Cip1/Waf1 by protein stabilization, which is independent of the Arf/p53 function, plays an essential role in the resistance of Cdk4-null cells to immortalization and Ras-mediated transformation.

The HPV E7 protein fully restores transformation in Cdk4-null MEF

The HPV E7 protein of the human papillomavirus-16 (HPV) inactivates Rb by sequestration and destabilization (Dyson et al. 1989; Boyer et al. 1996). E7 has also been shown to bind to the C terminus of p21Cip1/Waf1 and inactivate its Cdk-inhibitory and replication-inhibitory actions (Funk et al. 1997). Thus, we attempted to determine whether the expression of E7 could restore the transformation potential in Cdk4-null cells [Fig. 7]. E7 was expressed in Cdk4+/−/Ink4a/Arf−/− and Cdk4−/−/Ink4a/Arf−/− MEFs by retroviral transduction, followed by transduction of H-RasVal12 or control vector. Cdk4−/−/Ink4a/Arf−/− MEFs expressing H-RasVal12 and E7 developed a number of transformed foci comparable to Cdk4+/−/Ink4a/Arf−/− MEFs expressing H-RasVal12 with or without E7. Expression of E7 alone did not result in foci formation. The E7 retrovirus also restored foci formation in Cdk4−/− MEFs upon expression of H-RasVal12 and DNp53 almost completely [data not shown]. These data indicate that the HPV E7 oncoprotein fully restores the transformation potential of Cdk4-disrupted cells.

Discussion

In this study we have demonstrated that Cdk4-null MEFs are resistant to Ras-mediated oncogenic transformation. Cdk4-null MEFs proliferate normally under optimal growth-promoting conditions, whereas cell-cycle entry from serum deprivation-induced quiescence is modestly delayed (Tsutsui et al. 1999). Ras activation with p53 inhibition or Ink4a/Arf disruption in Cdk4-null cells results in dramatically reduced foci formation and no detectable proliferation in soft agar. These data suggest that Cdk4 disruption suppresses the two hallmarks of transformed phenotype, lack of contact inhibition and anchorage-independent proliferation (Hartwell and Kastan 1994; Hanahan and Weinberg 2000). More-
over, rare Cdk4-null cells that have apparently lost contact inhibition are not tumorigenic in athymic mice. These observations provide potentially significant insight into prospective therapeutic strategies, implying that genetic or pharmacological suppression of Cdk4 could be an effective approach to render cells insensitive to oncogenic stimuli, without detrimental effects on normal cell-cycle progression. Indeed, we recently demonstrated that Cdk4-null mice display a 97% reduction in susceptibility to carcinogen (DMBA and TPA)-induced skin tumorigenesis (Rodriguez-Puebla et al. 2002). Keratinocytes of Cdk4-null mice exhibit normal proliferation and differentiation, indicating that Cdk4 disruption abrogates transformation potential in vivo without affecting tissue development. Other recent studies demonstrated that cyclin D1-null mice are resistant to skin tumorigenesis induced by the same carcinogens [Robles et al. 1998] and also insensitive to mammary tumorigenesis mediated by MMTV-Ras or Neu [Yu et al. 2001]. However, it has been shown that 3T3-immortalized cyclin D1-null MEF clones are sensitive to Ras-induced transformation [Yu et al. 2001]. This is in contrast to the transformation resistance of Cdk4-null primary MEFs demonstrated in the present study. It is unknown whether cyclin D1 deficiency leads cells to premature senescence. It remains to be determined whether Ras-induced transformation of cyclin D1-null MEFs is related to uncharacterized genetic alterations during 3T3 immortalization, for example, Rb mutations, or activation of Cdk4 by other cyclins, such as cyclins D2 and D3.

The present study provides evidence that Cdk4 disruption inhibits transformation by recruiting cells to senescence under conditions of p53 inhibition or Ink4a/Arf disruption, which normally immortalize cells [Serrano et al. 1996; Kamijo et al. 1997]. This finding is important in the light of an emerging concept that cellular senescence or organismal aging is a major tumor suppressive mechanism in mammals [Sharpless and DePinho 2002]. Expression of activated Ras in primary fibroblasts induces premature senescence phenotypes, such as G1 arrest, the large flat morphology, and SAβ-gal activity, indistinguishable from replicative or "culture shock"-induced senescence [Serrano et al. 1997; Sherr and DePinho 2000]. To induce senescence, p16Ink4a and p19Arf function in parallel yet interacting pathways (Fig. 8; Carnero et al. 2000, Sherr and DePinho 2000). Whereas p16Ink4a inhibits Cdk4 and Cdk6, p19Arf increases p21Cip1/Waf1 transcription by p53 stabilization, consequently inhibiting cyclin E(A)/Cdk2. The inhibition of these G1-Cdks results in G1 arrest with decreased phosphorylation of Rb and other G1/S-specific substrates. Thus, the senescence response upon oncogene activation forms a safeguard mechanism against transformation. MEFs lacking p19Arf or p53 show the alternative response to Ras activation, undergoing transformation instead of senescence (Kamijo et al. 1997). These observations indicate essential roles for the Arf/p53 pathway in senescence-dependent tumor suppression. Coexpression of an "immortalizing oncogene," such as adenovirus E1A or c-myc, with activated Ras can also transform MEFs [de Stanchina et al. 1998; Zindy et al. 1998]. It is speculated that these immortalizing oncogenes trigger apoptotic response via the Arf/p53 checkpoint pathway, and select for emergence of cell variants that have lost Arf or p53 function (Sherr and DePinho 2000). Consistently, Cdk4-null

![Figure 7](https://genesdev.cshlp.org/)

**Figure 7.** Human papillomavirus E7 oncoprotein restores Ras-mediated transformation of Cdk4−/− Ink4a/Arf−/− MEFs. (A) Passage 4 MEFs with indicated genotypes were infected with E7 retrovirus or control virus, followed by infection with H-Rasval-12 retrovirus or control virus at a 24-h interval. Cells were then cultured in the medium containing 5% FBS for 17 d. (B) The numbers of foci per 60-mm dish in the assays are expressed as means ± S.E.M. from three independent MEF preparations.

![Figure 8](https://genesdev.cshlp.org/)

**Figure 8.** Effects of Cdk4 disruption on the pathways controlling senescence and transformation. (A) Senescence response of wild-type cells to Ras activation. (B) Ras-induced transformation with inactivated Ink4a/Arf/p53 pathway. (C) Senescence rendered by Cdk4 disruption.
MEFs are resistant to transformation in response to retroviral transduction of c-Myc and H-RasVal12 [X. Zou and H. Kiyokawa, unpubl.]. Whereas Ras triggers cell cycle-inhibitory changes in the expression of p16Ink4a, p19Arf, p53, and p21Cip1/Waf1, Ras also increases transcription of cyclin D1, which results in activation of Cdk4 [Pestell et al. 1999]. Furthermore, Ras up-regulates Cdk2 activity by destabilizing p27Kip1 [Pruijt and Der 2001]. These cell cycle-promoting actions are important for Ras-mediated oncogenic transformation. Therefore, the Arf/p53 pathway normally determines whether Ras activation results in premature senescence or transformation. Senescence of Cdk4-null MEFs without Ink4a/Arf or p53 function suggests that Cdk4 plays a key role in the oncogenic mechanism that converts the cell fate from senescence to transformation, in response to genetic or epigenetic alterations in the Arf/p53 checkpoint pathway. Cdk4 disruption is a unique approach to activate the senescence-dependent tumor suppressive mechanism in cells even with Ink4a/Arf or p53 inactivated.

Immortalization of Cdk4-null cells restored by anti-p21Cip1/Waf1 siRNA indicates that p21Cip1/Waf1 plays a critical role in the Arf/p53-independent senescence facilitated by Cdk4 deficiency. However, p21Cip1/Waf1-null MEFs with intact Cdk4 senesce normally [Pantoja and Serrano 1999], and MEFs from p21Cip1/Waf1−/−, Cdk4−/− double null mice poorly undergo transformation upon expression of H-RasVal12 and DNp53 [X. Zou and H. Kiyokawa, unpubl.]. These apparently differential effects of p21Cip1/Waf1 inactivation may result from the difference between germline disruption and acute somatic loss of p21Cip1/Waf1−/−, p15Ink4b−/− null MEFs may undergo developmental adaptation to the absence of p21Cip1/Waf1−/−, for which other Kip/Cip inhibitors and possibly p130 [Coats et al. 1999], could account. In contrast, the siRNA-based p21Cip1/Waf1 suppression in MEFs could have more dramatic effects, whereas with intact Arf, the immortalizing effect of anti-p21Cip1/Waf1 siRNA was minimal (Fig. 6C). It is also possible that a cell cycle-inhibitory action of p19Arf independent of p53 or p21Cip1/Waf1 [Ferbeyre et al. 2002] may play a role in inducing senescence in p21Cip1/Waf1−/− MEFs. Cdk4-null MEFs display increased expression of p21Cip1/Waf1 with enhanced protein stability. Cdk4-null MEFs expressing H-RasVal12 display increased amounts of p21Cip1/Waf1/Cdk2 complexes, relative to wild-type controls [X. Zou and H. Kiyokawa, unpubl.]. These observations also suggest that there is a previously undefined regulatory pathway from Cdk4 to the machinery of p21Cip1/Waf1 degradation. The mechanism of p21Cip1/Waf1 stabilization awaits further investigations.

p16Ink4a is a major Cdk4 inhibitor, and plays a role in senescence-dependent tumor suppression. Forced expression of p16Ink4a inhibits Ras-mediated transformation [Serrano et al. 1995]. However, p16Ink4a is dispensable for senescence of primary mouse cells, because p16Ink4a-null MEFs with intact p19Arf senesce as well as wild-type MEFs [Krimpenfort et al. 2001; Sharpless et al. 2001]. Furthermore, the ability of Cdk4−/−/Ink4a/Arf−/− MEFs to undergo senescence suggests that p16Ink4a is not required for senescence. However, an antisense RNA construct directed toward p16Ink4a can induce extended lifespan in primary wild-type MEFs (Carnero et al. 2000). The acute loss of p16Ink4a in this experimental system may significantly activate Cdk4 to a level sufficient for prolonged proliferation in the clonogenic assay used for the study. p15Ink4b also participates in Ras-induced senescence, and p15Ink4b-null MEFs exhibit modestly increased sensitivity to Ras-dependent transformation [Malumbres et al. 2000]. Our preliminary data demonstrate that siRNA-based suppression of p15Ink4b minimally affects Ras-mediated foci formation of Cdk4−/−/Ink4a/Arf−/− MEFs (X. Zou and H. Kiyokawa, unpubl.). Therefore, Cdk4 deficiency facilitates the senescence-mediated tumor-suppressive mechanism specifically in a p21Cip1/Waf1−/− dependent manner, whereas the Ink4 inhibitors are dispensable.

The Rb-family pocket binding proteins, that is, Rb, p107, and p130, are involved in the regulation of senescence and immortalization, especially as substrates of Cdk4. Inactivation of these pocket binding proteins by the papillomavirus E7 oncoprotein, together with telomerase activation, has been shown to immortalize primary human epithelial cells [Kiyono et al. 1998]. Disruption of Rb, p107, and p130 in MEFs results in increased proliferation with shortened G1 phase and immortalization (Dannenberg et al. 2000; Sage et al. 2000). Senescence induced by Rb or, p130 depends on the repressor activity of E2F [Rowland et al. 2002], suggesting the role of the Rb/E2F pathway also as downstream of Arf/p53 in senescence (Fig. 8A). MEFs with targeted Cdk4R24C mutation, which express a constitutively active Cdk4 insensitive to Ink4 inhibitors, exhibit escape from senescence [Rane et al. 2002]. Mice with the Cdk4R24C mutation spontaneously develop various tumors such as endocrine and skin tumors [Sotillo et al. 2001; Rane et al. 2002], supporting the notion that the Cdk4 activity plays a key role in immortalization and transformation. In Cdk4-null MEFs, phosphorylation of Rb, especially Ser780, is markedly diminished [Tsutsui et al. 1999]. The expression of Cdk6 is unchanged in Cdk4-null MEFs, and thus the role of Cdk6 in cell-cycle progression and immortalization of MEFs remains unclear. The complete restoration of Ras-mediated transformation by E7 suggests that the activities of the Rb-family pocket binding proteins are important for the Arf/p53-independent senescence with Cdk4 disruption.

Efforts are ongoing to identify specific chemical inhibitors of cyclin D/Cdk4 and to apply them to clinical trials [Fry et al. 2001; Honma et al. 2001; Soni et al. 2001]. Genetic inactivation of the Ink4a/Arf or p53 locus correlates with poor prognosis in cancer patients, often associated with chemoresistance [Weller 1998; Johnstone et al. 2002]. A recent study showed that the senescence response of cancer cells, dependent on these two genetic loci, contributes significantly to the outcome of chemotherapy in vivo (Schmitt et al. 2002). Continuous investigations should clarify how cyclin D/Cdk4 interacts with Ras and Arf/p53 in the process that determines whether cells undergo senescence or immortaliza-
tion, which should contribute to establishing a solid foundation for therapeutic intervention of the transformation pathways.

Materials and methods

Cells

A targeted null mutation of the Cdk4 gene, Cdk4tm1Kyo, was created in mouse embryonic stem cells, and mice with germline transmission of this mutation were bred in the recombinant C57BL/6 × 129/sv strain background, as described (Tsutsui et al. 1999). MEFs were prepared from day-12.5 mouse embryos and cultured in the Dulbecco’s modified minimum essential medium supplemented with 2 mM glutamine, 100 U/mL penicillin and streptomycin, and 10% fetal bovine serum (FBS; Life Technology), as described (Tsutsui et al. 1999). MEFs dispersed from each embryo using 0.25% trypsin solution containing 0.53 mM EDTA were cultured in a 100-mm culture dish (passage 1). MEFs dispersed and cultured in the Dulbecco’s modified minimum essential medium supplemented with 2 mM glutamine, 100 U/mL penicillin and streptomycin, and 10% fetal bovine serum (FBS; Life Technology) were used for transfection experiments. The Phoenix ecotropic virus packaging cell line, PA317 LXSN 16E7, was obtained from ATCC. Virus-containing supernatants were pooled and filtered through a 0.45-mm membrane. Infections of exponentially growing MEFs were performed with 1.5 mL of various dilutions of virus-containing supernatants, using 10 µg/mL polybrene as a transfection reagent (QIAGEN), and culture supernatants containing infectious retrovirus were harvested 48 h posttransfection. The Phoenix ecotropic virus packaging cell line, PA317 LXSN 16E7, was obtained from ATCC. Virus-containing supernatants were pooled and filtered through a 0.45-mm membrane. Infections of exponentially growing MEFS were performed with 1.5 mL of various dilutions of virus-containing supernatant supplemented with 10 µg/mL polybrene (Sigma) for each 60-mm culture dish. The dilutions of the H-RasVa12 and DNp53+H-RasVa12 were used to determine according to the numbers of transformed foci developed in Cdk4+/−, Ink4a+/− and wild-type MEFS, respectively, in pilot experiments. The E7 retrovirus was used at maximum titer without evidence of mycoplasma contamination.

Retroviral transfection

The Phoenix ecotropic virus packaging cells were obtained from the American Tissue Culture Collection (ATCC) with permission of Gary P. Nolan (Stanford University). The pBabe-hygro vector for expression of H-RasVa12 was described previously (Serrano et al. 1996). The LXSN vector for coexpression of DNp53 (GSE56, Ossovskaya et al. 1996) and H-RasVa12 was constructed using the internal ribosomal entry site. Phoenix cells were transfected with vectors, using the SuperFect transfection reagent (Qiagen), and culture supernatants containing infectious retrovirus were harvested 48 h posttransfection, as described (Pear et al. 1993). The HPV-E7 retrovirus packaging cell line, PA317 LXSN 16E7, was obtained from ATCC. Virus-containing supernatants were pooled and filtered through a 0.45-mm membrane. Infections of exponentially growing MEFS were performed with 1.5 mL of various dilutions of virus-containing supernatant supplemented with 10 µg/mL polybrene (Sigma) for each 60-mm culture dish. The dilutions of the H-RasVa12 and DNp53+H-RasVa12 were used to determine according to the numbers of transformed foci developed in Cdk4+/−, Ink4a+/− and wild-type MEFS, respectively, in pilot experiments. The E7 retrovirus was used at maximum titer without evidence of mycoplasma contamination.

Small interfering RNA (siRNA)

For suppression of cellular p21Cip1/Waf1 mRNA expression, siRNA that specifically targets p21Cip1/Waf1 mRNA was designed according to the manufacturer’s protocol (Dharmacon Research). The sense sequence was 5′-AACGGUGAAUCCCUGACUCUG-3′, corresponding to residues 136–156 of the coding region of mouse p21Cip1/Waf1 mRNA. MEFS were transfected with the anti-p21Cip1/Waf1 siRNA or random 21-mer dsRNA (Dharmacon), using the Oligofectamine reagent (Life Technologies/Invitrogen) according to the instructions of Dharmacon Research. SDS-PAGE and Western transfer, as described (Tsutsui et al. 1999). Antibodies were obtained from Neomarkers for Ras, Cdk6, and p16INK4a; from Santa Cruz Biotechnology for p15INK4b and p21Cip1/Waf1; from Novus Biologicals for p19Arf; and from Sigma for actin. Immunoreactive bands were visualized using peroxidase-conjugated anti-lg antibodies and the Supersignal chemiluminescence reagent (Pierce). Signals on X-ray films were quantified with a GS-700 Imaging Densitometer [Bio-Rad]. For RT–PCR, RNA samples were prepared using TRIZOL reagent (Life Technologies/Invitrogen). RT reactions were performed using Superscript reverse transcriptase (Life Technologies/Invitrogen). The sequences of primers are 5′-TGTCGACTCCGTG GATGTCC-3′ and 5′-TCAGACACCCAGTGCAAGAC-3′ for p21Cip1/Waf1, 5′-CATACGTCCACCCCCAGAAG-3′ and 5′-TGCGTCGAGCAACTTTATTGTG-3′ for GAPDH. PCR reactions were performed at 92°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec with 30 cycles, using a DNA Engine thermal cycler (MJ Research). Semiquantitative conditions for the transcripts were worked out using increasing amounts of RNA.

Focus and colony formation

For transformed focus formation, MEFS were cultured in complete medium with 5% FBS without splitting, for 14–21 d after retrovirus infection. Medium was changed every 3 d. Confluent monolayer cultures with foci were rinsed with phosphate-buffered saline (PBS), and stained with 4% (w/v) crystal violet in 10% methanol. Unstained foci of morphologically transformed cells were picked under a phase microscope (Nikon), subcloned by limited trypsinization, and expanded for the tumorigenicity assay. For colony formation in soft agar, MEFS at 48 h postviral infection were trypsinized, counted, and inoculated at 105 cells per 60-mm dish in 0.3% Noble agar in DMEM supplemented with 10% FBS. Colonies were scored 21–28 d after. When cells isolated from foci were tested for anchorage-independent growth, 2 × 104 cells were inoculated per dish in the Noble agar medium.

Senescence-associated β-galactosidase (SAβ-gal) assay

SAβ-gal activity at pH 6.0 was assayed as described (Dimri et al. 1995; Chang et al. 1999). Cells were washed with PBS supplemented with 1 mM MgCl2 and then stained in X-gal solution [1 mg/mL X-gal, 0.12 mM K3Fe(CN)6, 0.12 mM K4Fe(CN)6, 1 mM MgCl2 in PBS at pH 6.0] overnight at 37°C.

Tumorigenicity assay

For in vivo tumor formation, 105 cells isolated and expanded from foci were injected into flanks of 7-wk-old athymic mice (National Cancer Institute). Two mice were used for each clone. Tumor formation was scored every week, and diameters of palpable tumors were recorded.

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