

Requirement for CDK4 kinase function in breast cancer

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Summary

Cyclin D1 is overexpressed in the majority of human breast cancers. We previously found that mice lacking cyclin D1 are resistant to mammary carcinomas triggered by the *ErbB-2* oncogene. In this study, we investigated which function of cyclin D1 is required for *ErbB-2*-driven mammary oncogenesis. We report that the ability of cyclin D1 to activate cyclin-dependent kinase CDK4 underlies the critical role for cyclin D1 in breast cancer formation. We also found that the continued presence of CDK4-associated kinase activity is required to maintain breast tumorigenesis. We analyzed primary human breast cancers and found high cyclin D1 levels in a subset (~25%) of *ErbB-2*-overexpressing tumors. We propose that this subset of breast cancer patients might benefit from inhibiting CDK4 kinase.

Introduction

Cyclins represent key components of the core cell cycle machinery. Two classes of cyclins are expressed during G1 phase progression in mammalian cells: D-type (cyclins D1, D2, and D3) and E-type (cyclins E1 and E2). In contrast to other cyclins, which are periodically induced during cell cycle progression, the levels of D cyclins are controlled by extracellular mitogenic stimulation. For this reason, D cyclins are regarded as links between the cell environment and the core cell cycle machinery (Sherr and Roberts, 1999).

Several different molecular functions have been ascribed to the D-type cyclins. All three D cyclins were shown to bind cyclin-dependent kinases CDK4 and CDK6 (Bates et al., 1994; Matsushime et al., 1992; Meyerson and Harlow, 1994). The resulting cyclin D-CDK complexes display two distinct functions. In the “kinase-dependent” function, cyclin D-CDK4/6 complexes phosphorylate the retinoblastoma protein pRB, pRB-related proteins p107 and p130, and Smad3 (Matsuura et al., 2004; Sherr and Roberts, 1999). In addition to this “kinase-

dependent” mechanism, cyclin D-CDK complexes titrate p27^{Kip1} and p21^{Cip1} cell cycle inhibitors from cyclin E-CDK2 complexes (which are inhibited by p27^{Kip1} and p21^{Cip1}) to cyclin D-CDK, thereby triggering the activity of the cyclin E-CDK2 holoenzyme (Sherr and Roberts, 1999).

Moreover, D-type cyclins were shown to physically bind and to activate or repress the activity of several transcription factors, possibly through interacting with the associated transcriptional regulators (Coqueret, 2002). Importantly, this function of the D-type cyclins was shown to be independent of the binding to CDKs (Coqueret, 2002). Lastly, D-type cyclins were shown to interact with the components of the basal transcriptional machinery (Adnane et al., 1999).

Consistent with their growth-promoting functions, overexpression of the D-type cyclins is seen in a large fraction of human cancers. The *cyclin D1* gene is rearranged or amplified in squamous cell carcinomas of head and neck or esophagus; mantle cell lymphomas; multiple myelomas; cancers of the lung, uterine cervix, and stomach; and many other malignancies (Cheung et al., 2001; Gao et al., 2004; Hoechtlen-Vollmar et al., 2000;

SIGNIFICANCE

Overexpression of cyclin D1 is seen in all types of human breast cancers. In this study, we demonstrate that, in mice, the catalytic activity of the cyclin D1-associated kinase CDK4 is critically required for mammary neoplasia driven by the *ErbB-2* oncogene. In the accompanying manuscript, Landis et al. demonstrate that knockin mice expressing kinase-deficient cyclin D1-CDK4 complexes develop relatively normally, but they are resistant to mammary carcinomas triggered by *ErbB-2*. These results point to a differential requirement for cyclin D1-CDK4 kinase activity in mammary neoplasia versus in development. These findings suggest that the pharmacological inhibition of CDK4 kinase might preferentially target breast cancer cells. Our findings will hopefully stimulate therapeutic strategies aiming at blocking CDK4 kinase activity in cancer patients.

Lammie et al., 1991; Reissmann et al., 1999; Vandenberghe, 1994). Of all these, the best documented is the frequent involvement of cyclin D1 abnormalities in breast cancer.

Approximately 15%–20% of human breast cancers exhibit amplification of the *cyclin D1* gene (Buckley et al., 1993; Dickson et al., 1995; Lammie et al., 1991), while the majority of human mammary carcinomas overexpress cyclin D1 protein (Bartkova et al., 1994, 1995; Gillett et al., 1994, 1996; McIntosh et al., 1995). Overexpression of cyclin D1 is seen at the earliest stages of breast cancer progression, such as ductal carcinoma in situ (DCIS), but not in premalignant lesions, and it is maintained at all stages of breast cancer progression, including metastatic lesions (Bartkova et al., 1994; Gillett et al., 1996). These observations suggest that cyclin D1 might represent an attractive target for therapeutic intervention in mammary carcinomas.

We previously tested the requirement for cyclin D1 in normal and in oncogenic proliferation by generating mice lacking cyclin D1. These cyclin D1 null mice were viable and displayed very narrow, circumscribed abnormalities, revealing that cyclin D1 is dispensable for normal, nononcogenic proliferation of the vast majority of cell types (Sicinski et al., 1995); similar conclusions were independently reached by Fantl et al. (1995). We next assessed the requirement for cyclin D1 in breast cancer formation by crossing cyclin D1 null mice with mouse mammary tumor virus (MMTV)-oncogene transgenic mouse strains. Female mice of these transgenic strains are highly prone to mammary carcinomas due to overexpression of particular oncogenes in their mammary epithelium (Muller et al., 1988). Cyclin D1 null mice were resistant to breast cancers driven by the *ErbB-2* and *Ras* oncogenes, while being fully susceptible to mammary carcinomas triggered by *Wnt-1* and *c-Myc* (Yu et al., 2001). We concluded that cyclin D1 function is critically required for *ErbB-2*- and *Ras*-driven mammary gland tumorigenesis. However, these studies did not reveal which function of cyclin D1 underlies its critical importance in this process.

Among the many different cyclin D1 interactors listed above, the *CDK4* gene was found to be amplified and the protein overexpressed in a significant fraction of human breast cancers (An et al., 1999; Samady et al., 2004; Takano et al., 1999). We therefore hypothesized that the interaction of cyclin D1 with CDK4 might underlie the critical role for cyclin D1 in *ErbB-2*- and *Ras*-driven breast tumorigenesis. We tested this hypothesis in the studies described below.

Results

Normal development of CDK4 null mammary epithelium

To test the requirement for CDK4 function in breast cancer formation, we used CDK4 null mice (Tsutsui et al., 1999). We first analyzed mammary epithelial development in CDK4-deficient females. Importantly, *CDK4*^{-/-} females are infertile (Rane et al., 1999; Tsutsui et al., 1999), and hence their pregnancy-driven mammary lobuloalveolar development—the most extensive step of mammary epithelial expansion—has not been previously analyzed. We circumvented this developmental block by transplanting mammary epithelium from CDK4 null females into cleared fat pads of wild-type recipients. Mammary epithelium derived from wild-type littermates was transplanted into contralateral fat pads of the same recipients and served as control. When the recipient mice became pregnant, the CDK4 null epithelium underwent extensive lobuloalveolar development that

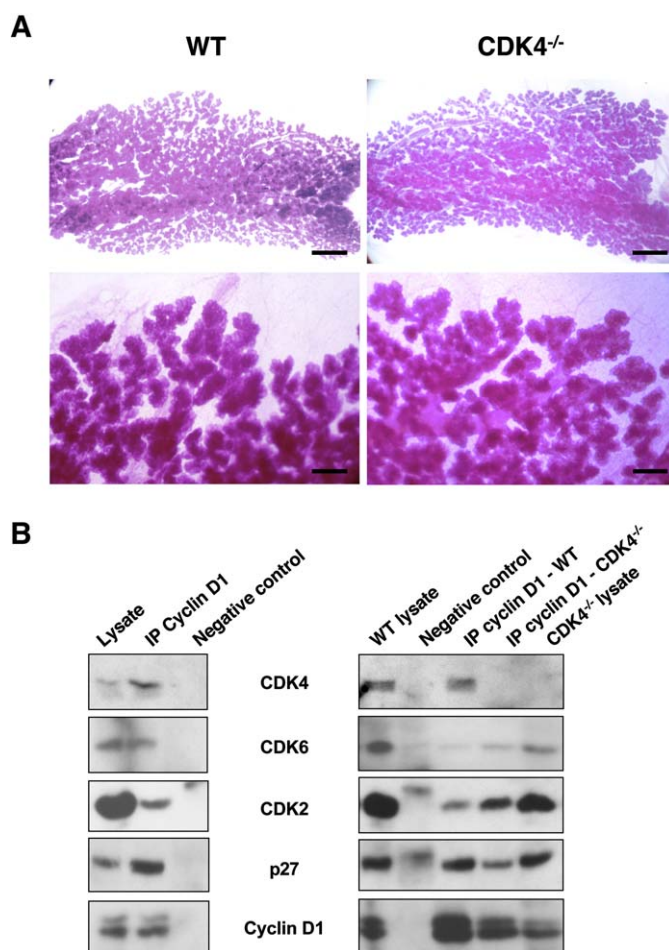


Figure 1. Analyses of mammary glands in CDK4 null females

A: Normal mammary epithelial lobuloalveolar development in CDK4^{-/-} animals. Mammary epithelium was dissected from wild-type (WT) or CDK4^{-/-} females and transplanted into wild-type recipients. The recipient mice were mated, and the mammary glands were collected 1 day postpartum. The mammary epithelium was stained with carmine red. Lower panels depict higher magnification of the whole-mount images. Scale bars correspond to 2 mm (upper panels) or 0.5 mm (lower panels).

B: Composition of cyclin D1-containing complexes. Cyclin D1 was immunoprecipitated (IP Cyclin D1) from mammary glands of day 1 postpartum wild-type mice (left panel) or from mammary glands of wild-type (WT) or CDK4^{-/-} females that were hormonally stimulated to undergo lobuloalveolar development (right panel) (see *Experimental Procedures*), and the immunoblots were probed with the indicated antibodies. Straight lysates were immunoblotted and probed in parallel (Lysate). As a negative control, immunoprecipitation was performed in the absence of primary antibodies (Negative control).

was very similar to that seen in the wild-type counterpart (Figure 1A). These observations indicate that CDK4 is not required for normal mammary epithelial development.

Normal appearance of CDK4 null mammary epithelium (Figure 1A), together with the profound defect seen in cyclin D1-deficient mammary glands (Fantl et al., 1995, 1999; Sicinski et al., 1995), raised the question of which function of cyclin D1 plays a role in driving mammary lobuloalveolar development. Given earlier observations that ablation of p27^{Kip1} rescued the phenotypic manifestations of cyclin D1 deficiency in mammary glands (Geng et al., 2001; Tong and Pollard, 2001), we hypothesized that the major function of cyclin D1 in mammary glands

may be mediated through sequestration of p27^{Kip1} by cyclin D1-CDK complexes. We further hypothesized that, in the absence of CDK4, other CDKs might form complexes with cyclin D1 and allow titration of p27^{Kip1}. To address these possibilities, we tested the composition of cyclin D1-containing complexes in mammary glands of pregnant wild-type mice using immunoprecipitation-Western blotting. We found that, in wild-type glands, cyclin D1 associated with CDK4, CDK6, CDK2, and p27^{Kip1} (Figure 1B). In CDK4 null mammary glands that were stimulated by hormones to undergo lobuloalveolar development, cyclin D1 associated with CDK6, CDK2, and p27^{Kip1} (Figure 1B). These results suggest that, in CDK4 null mammary epithelium, cyclin D1 retains the ability to titrate p27^{Kip1} through its association with other CDKs.

Resistance of *CDK4*^{-/-} mice to breast cancers

We and others previously showed that mice lacking cyclin D1 are resistant to mammary carcinomas triggered by the *ErbB-2* oncogene (Yu et al., 2001; Bowe et al., 2002). In this study, we asked whether cyclin D1's catalytic partner, CDK4, is required for *ErbB-2*-driven oncogenesis. To address this question, we crossed CDK4 null mice with MMTV-*ErbB-2* animals (Muller et al., 1988), and we generated *ErbB-2*;*CDK4*^{+/+} and *ErbB-2*;*CDK4*^{-/-} females. The animals were then observed for tumor occurrence. As expected, all *CDK4*^{+/+} females succumbed to breast cancers and developed a total of 139 tumors. In contrast, none of the *CDK4*^{-/-} females developed mammary carcinomas (Figures 2A and 2B). Upon termination of the experiment, the mammary glands of *ErbB-2*;*CDK4*^{-/-} mice were dissected and extensively examined, and the absence of tumors was confirmed (data not shown). In addition, the hyperplastic changes, seen in *ErbB-2*;*CDK4*^{+/+} mammary glands, were not encountered in CDK4 null counterparts (Figure 2C). Hence, genetic ablation of CDK4, like ablation of cyclin D1, rendered mice resistant to breast tumors triggered by *ErbB-2*. These results suggest that the ability of cyclin D1 to interact with CDK4 may underlie the critical requirement for cyclin D1 in *ErbB-2*-driven breast tumorigenesis.

To distinguish which of the two functions of cyclin D1-CDK4 complexes is required for *ErbB-2*-driven breast tumorigenesis, together with Dr. Hinds' group we generated a "knockin" strain of mice expressing—in place of cyclin D1—a kinase-deficient point mutant of cyclin D1 (see accompanying manuscript of Landis et al., 2006). Importantly, this cyclin D1 mutant retains the ability to bind CDK4 and to titrate p27^{Kip1}, but it is unable to activate CDK4 kinase. Knockin mice expressing kinase-deficient cyclin D1-CDK4 complexes were resistant to breast cancers driven by *ErbB-2*, like mice lacking cyclin D1 or CDK4. Collectively, we interpret these results as an indication that cyclin D1-CDK4 kinase activity is required for *ErbB-2*-driven breast tumorigenesis.

Requirement for CDK4 function in tumor cell proliferation

The experiments described above addressed the requirement for CDK4 kinase in the *initiation* of breast cancers. We next asked whether the continued presence of CDK4 was required to *sustain* tumor cell proliferation. We observed a very strong upregulation of CDK4 and cyclin D1 in *ErbB-2*-driven tumors (Figure 3A). Immunoprecipitation analyses revealed the presence of abundant cyclin D1-CDK4 complexes in *ErbB-2*-driven

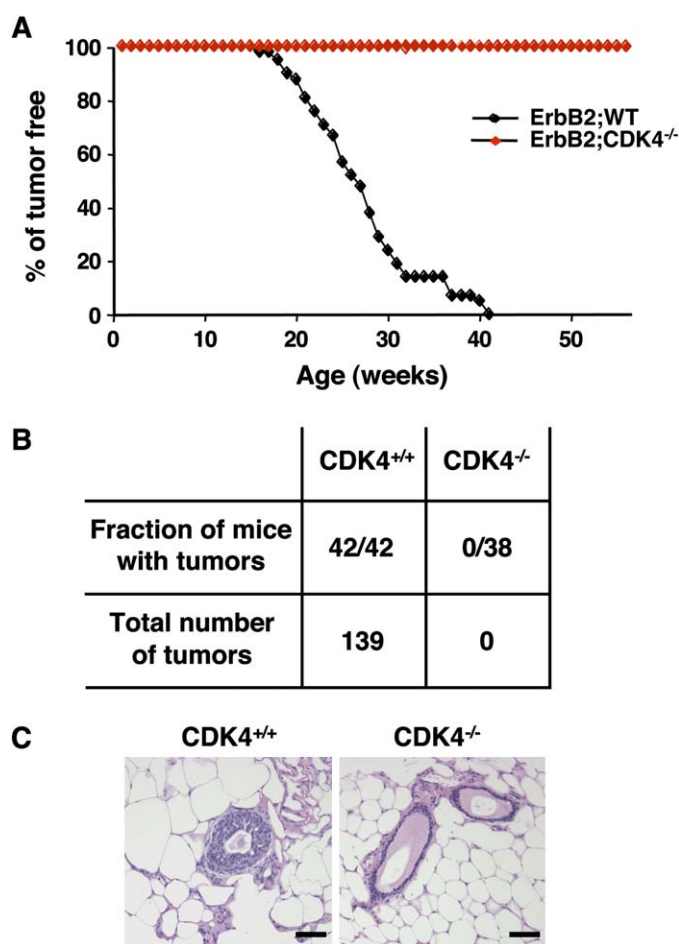


Figure 2. Resistance of *CDK4*^{-/-} mice to *ErbB-2*-driven breast cancers

A: Percentage of tumor-free mice among MMTV-*ErbB-2*;*CDK4*^{+/+} and MMTV-*ErbB-2*;*CDK4*^{-/-} females.

B: The fraction of mice displaying mammary carcinomas at 40 weeks of age, and the total number of tumors encountered in each group throughout the entire observation period.

C: Histological appearance of hyperplastic mammary epithelial ducts encountered in wild-type (WT) but not in *CDK4*^{-/-} females. Formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin. Scale bars correspond to 100 μ m.

mammary carcinomas (Figures 3B and 3C); these tumors had also elevated levels of CDK4- and cyclin D1-associated kinase activity (Figure 3D and Figure S1 in the Supplemental Data available with this article online). It should be noted, however, that the increase in cyclin D1 and CDK4 protein levels (Figure 3A) was much greater than the increase in CDK4- or cyclin D1-associated kinase activity (Figure 3D and Figure S1). These observations are consistent with the notion that the tumor cells may rely on cyclin D1-CDK4 complexes to drive tumor cell proliferation.

In order to rigorously test this possibility, we generated siRNA against CDK4 that efficiently knocked down the levels of CDK4, but not of CDK6 or CDK2 (Figure 4A and data not shown). We isolated cells from mammary carcinomas arising in MMTV-*ErbB-2* mice and cultured tumor cells *in vitro*. We then infected these cells with retroviruses encoding anti-CDK4 siRNA, or with control vectors, and injected the cells into mammary fat pads of recipient mice (Figure 4B). As expected, tumor cells infected with control vectors rapidly formed mammary carcinomas in the

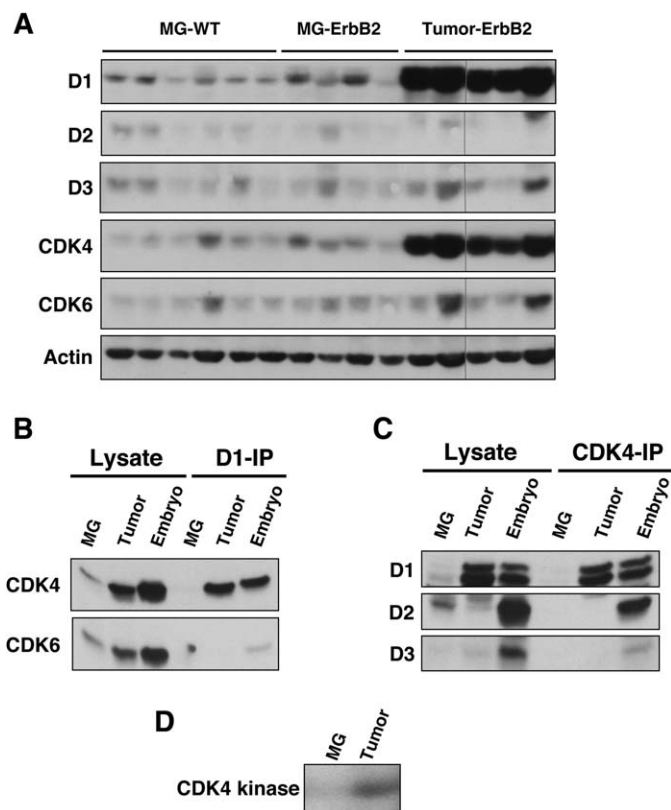


Figure 3. Analyses of ErbB-2-driven mammary carcinomas

A: Western blot analysis of mammary glands derived from wild-type mice (MG-WT), or mammary glands of *MMTV-ErbB-2* mice (MG-ErbB2), or breast tumors arising in *MMTV-ErbB-2* mice (Tumor-ErbB2). Immunoblots were probed with the indicated antibodies. Probing with antibodies against actin was used to ensure equal loading.

B and C: Association of CDK4 with cyclin D1 in breast cancers. Cyclin D1 was immunoprecipitated (D1-IP) (**B**), or CDK4 was immunoprecipitated (CDK4-IP) (**C**) from mammary glands derived from wild-type mice (MG), or from mammary tumors arising in *MMTV-ErbB-2* mice (Tumor), or—for control—from day 13.5 mouse embryos (Embryo), and the immunoblots were probed with the indicated antibodies. Straight lysates were immunoblotted and probed in parallel (Lysate).

D: Increased CDK4-associated kinase activity in breast tumors. CDK4 was immunoprecipitated from mammary glands derived from wild-type mice (MG), or from breast cancers arising in *MMTV-ErbB-2* mice (Tumor), and in vitro kinase assay was performed using the recombinant retinoblastoma protein as a substrate.

recipient mice (Figure 4C). In contrast, knockdown of CDK4 essentially blocked the ability of cells to form tumors (Figure 4C). Consistent with these findings, analyses of in vitro cultured tumor cells revealed that anti-CDK4 siRNA resulted in a decrease in the fraction of cells in the S phase (Figure S2), further underscoring the requirement for CDK4 in tumor cell proliferation.

Occasionally, we observed very small tumors arising from cells infected with viruses encoding anti-CDK4 siRNA (Figure 4C). However, we found that these tumors expressed high levels of CDK4 (Figure 4D), indicating that they arose from the residual uninfected cells, or from cells that escaped knockdown of CDK4. Collectively, these results suggest that CDK4 is critically required both for the *initiation* of tumors, and for *maintenance* of tumor cell proliferation. We verified that this requirement was specific to CDK4, as knockdown of CDK6 using

a specific anti-CDK6 siRNA had little impact on the ability of cells to form tumors (data not shown).

We also tested the requirement for CDK4's regulatory partner, cyclin D1, in tumor cell proliferation. We infected in vitro cultured breast cancer cells with viruses encoding anti-cyclin D1 siRNA (Figure 5A) and inoculated these cells into mammary fat pads. As was the case with knockdown of CDK4, knockdown of cyclin D1 blocked the ability of breast cancer cells to form tumors (Figure 5B); similar results were reported by Lee et al. (2000) using antisense cyclin D1 in MMTV-neuT mammary tumor cell line. We also verified that this requirement was specific to cyclin D1, as knockdown of cyclin D3 using a specific, anti-cyclin D3 siRNA had only marginal impact on the ability of cells to form tumors (data not shown). These observations suggest that cyclin D1-CDK4 complexes are critically required to *maintain* tumor cell proliferation.

Requirement for CDK4 kinase function in tumor cells

We next asked which function of cyclin D1-CDK4 complexes—"kinase-dependent" or "kinase-independent" was required to drive the proliferation of breast cancer cells. As described above, the resistance of knockin mice expressing kinase-deficient cyclin D1-CDK4 complexes to ErbB-2-driven breast tumors revealed that the kinase function of cyclin D1-CDK4 was critically required for the *initiation* of mammary carcinomas. To determine which activity of cyclin D1-CDK4 complexes was needed to *maintain* tumor cell proliferation, we replaced mouse CDK4 in murine breast cancer cells with human wild-type CDK4 or with human kinase-dead CDK4 (van den Heuvel and Harlow, 1993). To this end, ErbB-2-driven breast cancer cells were infected with retroviruses encoding anti-CDK4 siRNA, thereby efficiently knocking down mouse CDK4 (Figure 6A). In the very same cells, we retrovirally expressed human wild-type CDK4, or human kinase-dead CDK4. Importantly, we designed our siRNA against sequences that differ between mouse and human CDK4; therefore, the siRNA was specific for mouse CDK4 but would not affect its human counterpart. The nearly complete knockdown of mouse CDK4 and efficient expression of human CDK4 in tumor cells was verified by the Western blotting (Figure 6A). We next inoculated these cells into mammary fat pads and observed the recipient mice for tumor occurrence. As expected, reexpression of human wild-type CDK4 endowed the cells with the ability to form tumors; however, the tumor size was only 50% of that seen in tumor cells infected with control vectors. In contrast, cells expressing kinase-dead CDK4 were unable to efficiently form tumors (Figure 6B). Hence, the kinase activity of cyclin D1-CDK4 complexes is required both for the initiation of breast cancers and to maintain tumor cell proliferation.

Analyses of human mammary carcinomas

Lastly, we asked whether the ErbB-2 → cyclin D1 connection might also operate in human breast cancers. We collected 22 primary mammary carcinomas containing amplification of the *ErbB-2* gene and examined the levels of cyclin D1 mRNA by microarray analysis. Twenty-three percent of these tumors (5/22) contained high levels of cyclin D1 transcripts (Figure 7A).

It order to confirm these observations at a protein level, we used an independent sample of 70 primary breast cancers with ErbB-2 overexpression. Immunostaining of tumor sections with anti-cyclin D1 antibodies revealed that 19/70 of tumors (27%) contained very high levels of cyclin D1 protein (Figure 7B), which

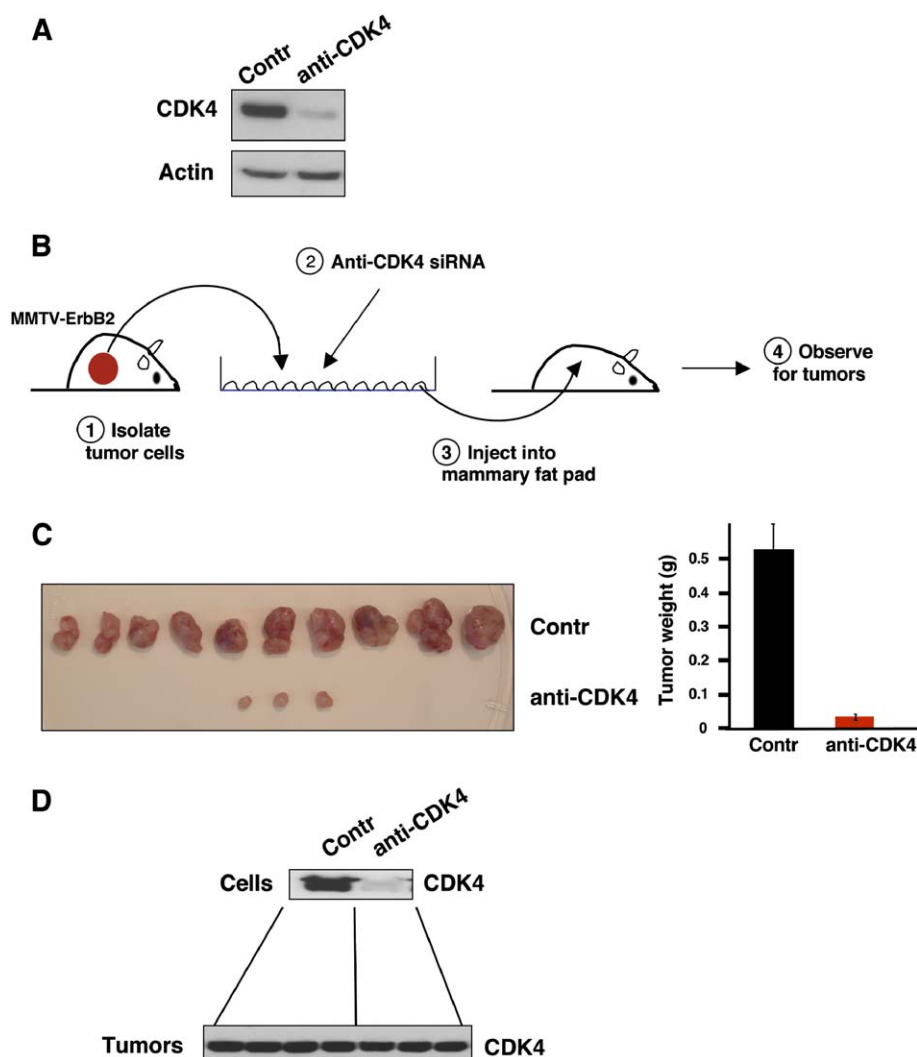


Figure 4. Knockdown of CDK4

A: Characterization of anti-CDK4 siRNA. Western blot analyses of cells infected with retroviruses encoding anti-CDK4 siRNA (anti-CDK4), or infected with control retroviruses (Contr). Immunoblots were probed with an anti-CDK4 antibody, and with an antibody against actin (to ensure equal loading).

B: Experimental outline of the knockdown analyses.

C: (Left) Appearance of tumors deriving from breast cancer cells infected with control retroviruses (Contr), or infected with retroviruses encoding anti-CDK4 siRNA (anti-CDK4). Control cells, and cells that underwent CDK4 knockdown, were inoculated into mammary glands of recipient mice. After 3 weeks, mice were sacrificed and all tumors were dissected. A representative experiment is shown. (Right) Quantification of the tumor weights. Error bars denote SD.

D: Small tumors that occasionally arise from cells infected with viruses encoding anti-CDK4 siRNA reexpress CDK4. Breast cancer cells were infected with retroviruses encoding anti-CDK4 siRNA (anti-CDK4), or with control viruses (Contr). Efficient knockdown of CDK4 in tumor cells was verified by the Western blotting (Cells, CDK4). Cells were injected into mammary fat pads, and the recipient mice were observed for tumor formation. Large tumors arising from cells infected with control viruses, and small tumors that occasionally arise from cells infected with anti-CDK4 siRNA (see **C**) were dissected, and the levels of CDK4 were determined by the Western blotting (Tumors, CDK4). Note that the small tumors expressed high levels of CDK4, indicating that they arose from the residual uninfected cells, or from cells that escaped the knockdown of CDK4.

exceeded the levels seen in the adjacent nontransformed tissue (data not shown). Hence, a significant fraction of human ErbB-2-overexpressing tumors contain elevated levels of cyclin D1. We propose that the patients bearing this subset of mammary carcinomas might benefit from pharmacologically blocking CDK4 kinase activity.

Discussion

In the work described here, we report that—like cyclin D1 null mice—animals lacking cyclin D1's catalytic partner, CDK4, are resistant to mammary carcinomas triggered by the ErbB-2 oncogene. We also found that the continued presence of CDK4 (and of cyclin D1) is critically required to maintain tumor cell proliferation. While cyclin D1-CDK4 complexes have at least two independent functions, we found that the catalytic, “kinase-dependent” function of cyclin D1-CDK4 is needed to sustain the tumorigenic potential of breast cancer cells.

In the accompanying manuscript, Landis et al. in collaboration with us report generation and characterization of a knockin strain of mice in which the wild-type copies of cyclin D1 were replaced by the cyclin D1 K112E “kinase-deficient” point mutant. This mutant version of cyclin D1 can bind the CDKs, but it is

deficient in activating their kinase activities. Importantly, the mutant protein retains the ability of cyclin D1-CDK complexes to titrate cell cycle inhibitors p27^{Kip1} and p21^{Cip1}. Analyses of the homozygous knockin mice expressing kinase-deficient cyclin D1-CDK complexes revealed relatively normal development of cyclin D1-dependent compartments (retinas, mammary glands). These analyses indicate that the kinase activity of cyclin D1-CDK complexes is largely dispensable for development. It seems likely that the major function of cyclin D1-CDK complexes in development is to control cyclin E-CDK2 activation through titration of p27^{Kip1} and p21^{Cip1}. Indeed, ablation of p27^{Kip1} in cyclin D1 null background restored nearly normal development of cyclin D1 null mice (Geng et al., 2001; Tong and Pollard, 2001), while ablation of p27^{Kip1} was shown to partially correct the cell cycle abnormalities seen in CDK4 null cells (Tsutsui et al., 1999). Consistent with this thinking, we observed that titration of p27^{Kip1} by cyclin D1-containing complexes was retained in CDK4 null mammary epithelium, likely explaining normal lobuloalveolar development in the absence of CDK4.

Strikingly, mice lacking cyclin D1, or CDK4, or mice expressing kinase-deficient cyclin D1-CDK complexes were resistant to ErbB-2-driven mammary carcinomas. Collectively, these results point to a differential requirement for cyclin D1-CDK4 kinase

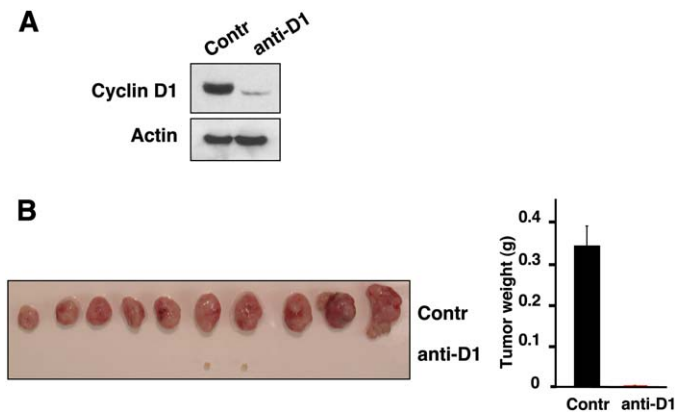


Figure 5. Knockdown of cyclin D1

A: Characterization of anti-cyclin D1 siRNA. Western blot analyses of cells infected with retroviruses encoding anti-cyclin D1 siRNA (anti-D1), or infected with control retroviruses (Contr). Immunoblots were probed with an anti-cyclin D1 antibody, and with an antibody against actin (to ensure equal loading).

B: (Left) Appearance of tumors deriving from cells infected with control retroviruses (Contr), or infected with retroviruses encoding anti-cyclin D1 siRNA (anti-D1). Control cells, and cells that underwent cyclin D1 knockdown, were inoculated into mammary glands of recipient mice. After 3 weeks, mice were sacrificed and all tumors were dissected. A representative experiment is shown. (Right) Quantification of the tumor weights. Error bars denote SD.

activity in mammary neoplasia versus normal mammary development.

Why are breast cancer cells critically dependent on cyclin D1-CDK4 kinase for their proliferation? The expression pattern of the D cyclins and CDKs seen in ErbB-2-driven tumors (Figure 3) may provide a partial answer. We found that these tumor cells express mostly cyclin D1 (but little D2 or D3) and predominantly CDK4. These findings likely explain why knockdown of cyclin D3 or CDK6 has very little impact on tumor cell proliferation. However, it remains unclear why the *kinase* activity of cyclin D1-CDK4 complexes plays such a critical function in tumor cells, but not in developing tissues. One possibility is that, in tumor cells, phosphorylation of the retinoblastoma protein by cyclin D-CDK4 kinase must take place in order to upregulate cyclin E levels (via E2Fs) and to activate cyclin E-CDK complexes. In contrast, in nontransformed cells the noncatalytic function of cyclin D-CDK complexes may suffice to activate cyclin E-associated kinase activity through titration of inhibitors. Alternatively, tumor cells may express a protein (or proteins) that represents a specific target of cyclin D1-CDK4 kinase. However, this requirement for cyclin D1-CDK4 kinase cannot be absolute, as evidenced by our observations that knockin mice expressing cyclin E1 in place of cyclin D1 remained susceptible to ErbB-2-driven breast cancers (Yu et al., 2001). Hence, elevated and/or sustained levels of cyclin E may obviate the requirement for cyclin D1-CDK4 kinase. Indeed, *Bowe et al. (2002)* have reported that a small fraction of *MMTV-ErbB-2;cyclin D1*^{-/-} mice developed mammary carcinomas after very long latency, and that these mammary tumors markedly upregulate cyclin E levels.

Regardless of these questions, our results may have potentially important implications for breast cancers in humans. For this reason, in the current study we extended our analyses to

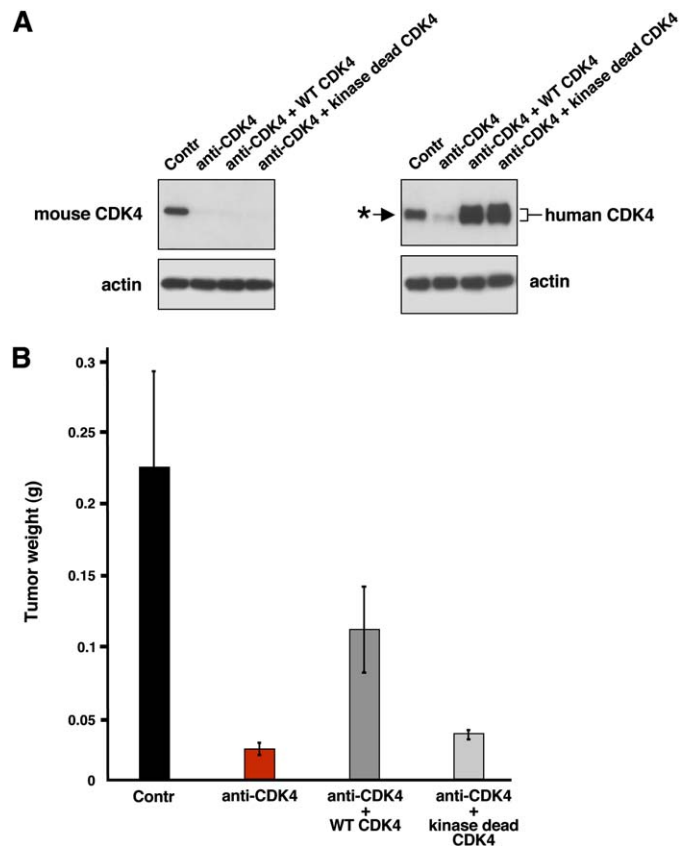


Figure 6. Replacement of mouse CDK4 with human wild-type or kinase-dead CDK4

A: Breast cancer cells were infected with control viruses (Contr), or with viruses encoding anti-mouse CDK4 siRNA (anti-CDK4), or with viruses encoding anti-mouse CDK4 siRNA plus human wild-type CDK4 (anti-CDK4 + WT CDK4), or anti-mouse CDK4 siRNA plus human kinase-dead CDK4 (anti-CDK4 + kinase-dead CDK4). Protein lysates were probed with an antibody recognizing mouse CDK4 (left panel) or human CDK4 (right panel). Note that the anti-human CDK4 antibody also cross-reacts with mouse CDK4 (the cross-reacting band is marked by an asterisk). The blots were also probed with antibodies against actin, to ensure equal loading.

B: Quantification of the tumor weights. Tumor cells were infected with control viruses (Contr), or with viruses encoding anti-mouse CDK4 siRNA (anti-CDK4), or anti-mouse CDK4 siRNA plus human wild-type CDK4 (anti-CDK4 + WT CDK4), or anti-mouse CDK4 siRNA plus human kinase-dead CDK4 (anti-CDK4 + kinase-dead CDK4). Cells were then inoculated into mammary fat pads of the recipient mice. Mice were sacrificed after 3 weeks, and tumors were dissected and weighed. A representative experiment is shown. Error bars denote SD.

human mammary carcinomas. It is very well documented that the majority of human breast cancers overexpress cyclin D1 (Barnes and Gillett, 1998; Musgrove et al., 1996; Zhou et al., 2001), and that up to 40% of mammary carcinomas exhibit amplification and/or overexpression of the *ErbB-2* gene (Andrulis et al., 1998; Bieche and Lidereau, 1995; Slamon et al., 1987). However, it is less clear whether there is any overlap between these two groups. In the current study, we analyzed nearly 100 primary human breast cancer specimens with confirmed ErbB-2 overexpression. We found that approximately 25% of them contained elevated cyclin D1 levels. This is in contrast to MMTV-ErbB-2 transgenic mice, where high cyclin D1 levels are seen in essentially all tumors (Figure 3). We presume that MMTV-ErbB-2 transgenic mice, used in our study, model this

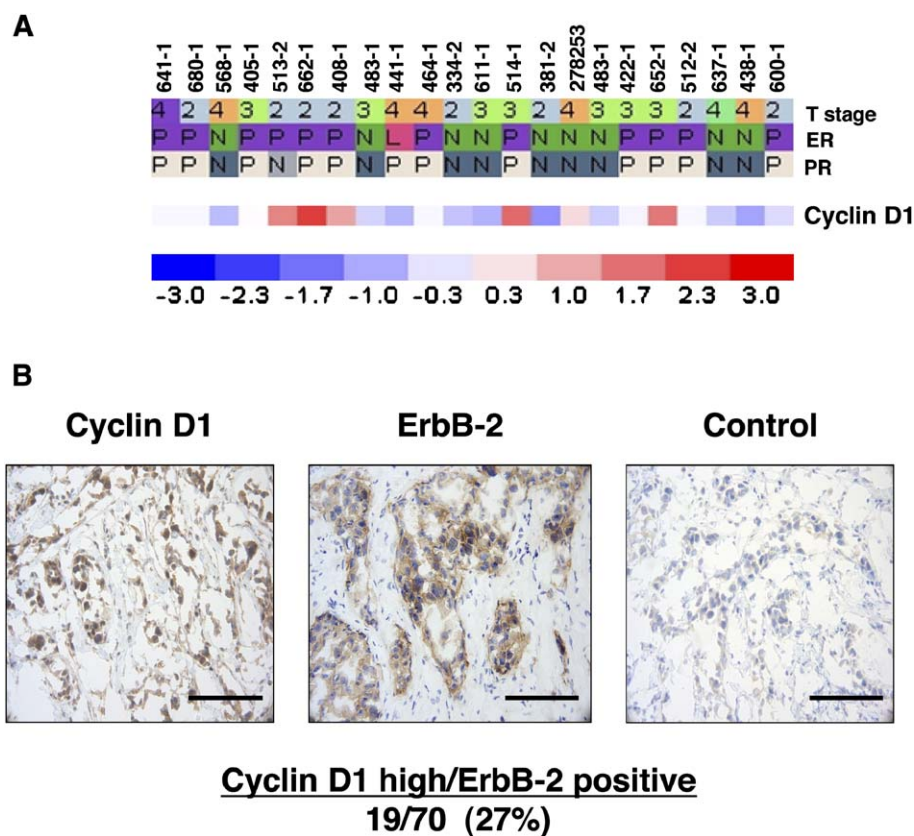


Figure 7. Expression of cyclin D1 in human ErbB-2-positive mammary carcinomas

A: RNA was extracted from pretreatment core biopsy specimens of 22 mammary carcinomas with ErbB-2 gene amplification (as determined by FISH), and with ErbB-2 overexpression ("+++" according to DAKO Herceptest) and hybridized onto Affymetrix Gene Chip. Unsupervised hierarchical clustering was performed using the cyclin D1 probe set. Fold expression of cyclin D1 above and below the mean is depicted below in the red-blue dendrogram. Also shown is the clinical stage of the tumor (AJCC T-stage, 2 to 4) and the estrogen receptor (ER) or progesterone receptor (PR) status (P = positive, N = negative), measured by immunocytochemistry.

B: 238 human primary breast cancer specimens (70 cases of ErbB-2-positive, 168 cases of ErbB-2-negative) were stained with anti-cyclin D1 antibodies and classified into cyclin D1-low, -intermediate, and -high. Among 70 ErbB-2-positive tumors, 19 (27%) scored as cyclin D1 high. An example of an ErbB-2-positive/cyclin D1-high tumor is shown. Sections were stained for cyclin D1 or ErbB-2, or with control antibodies. Scale bars correspond to 100 μ m.

subset of ErbB-2-positive/cyclin D1-overexpressing human mammary carcinomas.

Importantly, patients bearing ErbB-2-positive, cyclin D1-overexpressing tumors have shown particularly poor prognosis, with only 13% of women surviving 7 years (Ahnström et al., 2005). In contrast, patients with ErbB-2-positive/cyclin D1-negative tumors had survival rates of nearly 50% over the same period (Ahnström et al., 2005). Hence, among ErbB-2-positive breast cancers, overexpression of cyclin D1 confers particularly unfavorable prognosis. We propose that the patients bearing this type of breast cancer might benefit from pharmacologically blocking CDK4 kinase activity. We further propose that this therapy might selectively target breast cancer cells, while sparing other tissues where cyclin D1-CDK4 kinase activity seems not to be essential for the normal, nononcogenic proliferation.

The work described in this manuscript focused on mammary carcinomas. However, amplification of the *ErbB-2* gene and overexpression of ErbB-2 protein were reported in several human malignancies including ovarian cancers, pancreatic adenocarcinomas, non-small cell lung cancers, biliary tract cancers, invasive bladder cancers, squamous cell carcinoma of the esophagus, gastric cancers, uterine serous papillary carcinomas, and many others (Berchuck et al., 1990; Eltze et al., 2005; Hansel et al., 2005; Mimura et al., 2005; Nakazawa et al., 2005; Santin et al., 2005; Tanner et al., 2005; Ugocsai et al., 2005). In addition, mutational activation of ErbB-2 was documented in 10% of lung adenocarcinomas (Stephens et al., 2004). Some of these tumor types were also shown to contain elevated levels of cyclin D1 and CDK4 (Cheung et al., 2001; Dong et al., 2001; Igarashi et al., 1999; Masaki et al., 2003). We hypothesize that

these tumors might also benefit from pharmacological inhibition of the CDK4 kinase. Our findings will hopefully stimulate therapeutic strategies aiming at blocking CDK4 kinase activity in cancer patients.

Experimental procedures

Mice

Generation of CDK4-deficient mice was described previously (Tsutsui et al., 1999). MMTV-ErbB-2 mice (strain TG.NK; Muller et al., 1988) were purchased from the Charles River Laboratories. Mating of the two strains yielded the two experimental groups: MMTV-ErbB-2;CDK4^{+/+} (n = 42) and MMTV-ErbB-2;CDK4^{-/-} (n = 38 at 40 weeks, n = 18 at 51 weeks, n = 13 at 55 weeks; CDK4^{-/-} mice show reduced life spans due to diabetes [Tsutsui et al., 1999]). Mice were kept as virgins and were checked biweekly, by palpation, for tumor occurrence. Animals displaying tumors were sacrificed, and the presence of mammary adenocarcinomas was confirmed histologically. Animals were observed for a total of 55–60 weeks. At the end of the observation period, mammary glands were dissected from all tumor-free mice (100% of MMTV-ErbB-2;CDK4^{-/-}, none from the MMTV-ErbB-2;CDK4^{+/+} group) and extensively analyzed for the presence of tumors. All experiments were approved by the Animal Care and Use Committee at the Dana-Farber Cancer Institute and were performed in accordance with relevant institutional and national guidelines and regulations.

Mammary gland analyses

Mammary epithelial transplantations and whole-mount analyses were performed essentially as described (Geng et al., 1999, 2001). Six CDK4^{-/-} and six wild-type female littermates (4 months old) served as donors. Mammary epithelium from each donor mouse was transplanted into at least five to six recipient females. Mice were mated 2 months after the transplantation. For analyses of cyclin D1-containing complexes (Figure 1B), wild-type (n = 6) or CDK4^{-/-} (n = 7) females were implanted with a pellet containing 0.5 mg

of 17 β -estradiol plus 32.5 mg of progesterone (HH-114, Innovative Research of America) for 21 days, and with a pellet (X-999, Innovative Research of America) containing 3.5 mg of prolactin from sheep pituitary (Sigma, L6520) for 14 days. The mammary glands were then collected and processed for immunoprecipitations.

Western blotting, immunoprecipitation, and kinase assays

Protein lysates (25–50 μ g) were resolved using 10% PAGE, transferred to Immobilon-P filters (Millipore), and probed with antibodies against the following proteins: cyclin D1 (Ab-3, Neomarkers), cyclin D2 (M-20, Santa Cruz), cyclin D3 (C-16, Santa Cruz), mouse CDK4 (Ab-5, Neomarkers), human CDK4 (C-22, Santa Cruz), CDK6 (C-21, Santa Cruz), CDK2 (M-2, Santa Cruz), p27^{Kip1} (C-19, Santa Cruz), or actin (MAB1501, Chemicon). For immunoprecipitation, 200 μ g of protein lysates were immunoprecipitated with anti-CDK4 (Ab-1, Neomarkers) or anti-cyclin D1 (72-13G, Santa Cruz) antibodies, resolved on 10% PAGE, and transferred to Immobilon-P filters (Millipore). For in vitro kinase reactions, 200 μ g of protein lysates were immunoprecipitated with antibodies against CDK4 (C-22AC, Santa Cruz), cyclin D1 (17-13G, Santa Cruz), or CDK2 (M-2, Santa Cruz). Immunoprecipitates were incubated with the C-terminal fragment of the retinoblastoma protein (Santa Cruz) or histone H1 protein (Roche) in the presence of γ -³²P-ATP as described (Geng et al., 2001). Labeled proteins were resolved using 10% PAGE and visualized by autoradiography.

siRNA experiments

Mammary carcinomas were dissected from MMTV-ErbB-2 females; the cells were digested with 0.25% trypsin, plated, and cultured in DMEM and 10% fetal bovine serum. Two immortalized cell lines, designated V720 and A249, were derived and used for subsequent experiments, along with the SMF cell line, which was also derived from MMTV-ErbB-2 mammary carcinoma and was kindly provided by Dr. Philip Leder. siRNA sequences against mouse CDK4 (5'-AACATTCTAGTGACAAGTAAT-3') or mouse cyclin D1 (5'-CCACA GATGTGAAGTTTCATTT-3') were cloned into pMKO.1 (Sicinska et al., 2003) retroviral vectors. For control, we used empty pMKO.1 retroviruses or pMKO.1 viruses encoding anti-GFP siRNA (5'-GGCTACGTCCAGGAGCG CACC-3') (kindly provided by Drs. William Hahn and Richard Possemato), anti-mouse CDK6 siRNA (kindly provided by Dr. Phillip W. Hinds), or anti-mouse cyclin D3 siRNA (5'-ATGTATCCTCCATCCATGATC-3'). Tumor cells were infected with pMKO.1 retroviruses and selected with puromycin (6 μ g/ml) for 2–6 days. For replacement of mouse CDK4 with human CDK4, tumor cells were first infected with pLXSN-based retroviruses encoding wild-type human CDK4, or with retroviruses encoding kinase-dead human CDK4 (CDK4^{N158}) containing Asp-to-Asn substitution at amino acid residue 158 (van den Heuvel and Harlow, 1993), or with empty pLXSN vectors, and the cells were selected with 1200 μ g/ml of neomycin for 2 days. Subsequently, cells were infected with pMKO.1 vectors encoding anti-mouse CDK4 siRNA and selected in puromycin as above.

Tumor cells (10⁶) were injected into mammary fat pads of 3- to 4-month-old *Rag1*^{-/-} females (purchased from the Jackson Labs). Mice were monitored by palpation every other day for the presence of tumors. After 3 weeks, mice were sacrificed and tumors were dissected. In each experiment, particular cells were injected into three to five recipient females (six to ten injection sites), and the experiment was repeated at least three times.

Analyses of human breast cancers

For RNA analyses, nucleic acids were extracted from frozen tissue core biopsies of 22 mammary carcinomas using Qiagen nanoprep kit and subjected to two rounds of in vitro transcription; the second round incorporated biotinylated NTPs for labeling. RNA was then hybridized to Affymetrix U113 plus 2.0 Gene Arrays.

For immunocytochemistry, tumors consisted of a subset of 70 ErbB-2-positive breast cancers originating from two previous studies (Ahnström et al., 2005; Stål et al., 2000). The patients were postmenopausal women with the diagnosis of stage II breast cancer (UICC). The ErbB-2 status was assessed by flow cytometry, and 70/238 tumors scored positive. Freshly cut frozen sections were stained for cyclin D1 using a rabbit polyclonal antibody (Neomarkers), followed by a secondary Multilink swine anti-goat/mouse/rabbit antibody conjugated to biotin (Dako, Denmark) and streptavidin-horseradish peroxidase. The immunoreactivity was visualized with 3,3'-diaminobenzidine hydrochloride (DAB), and the cells were counterstained

with hematoxylin. The intensity of cyclin D1 immunostaining was graded as weak, moderate, or strong. For ErbB-2 immunostaining shown in Figure 7B, monoclonal antibody (Ab-2, Oncogene Science) was used, followed by peroxidase-conjugated EnVision™ complexes (Dako, Denmark) and visualization with DAB. Cells were counterstained with hematoxylin.

Supplemental data

The Supplemental Data include two supplemental figures and can be found with this article online at <http://www.cancerres.org/cgi/content/full/65/1/23/DC1>.

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References

- Adnane, J., Shao, Z., and Robbins, P.D. (1999). Cyclin D1 associates with the TBP-associated factor TAF(II)250 to regulate Sp1-mediated transcription. *Oncogene* 18, 239–247.
- Ahnström, M., Nordenskjöld, B., Rutqvist, L.E., Skoog, L., and Stal, O. (2005). Role of cyclin D1 in ErbB2-positive breast cancer and tamoxifen resistance. *Breast Cancer Res. Treat.* 91, 145–151.
- An, H.X., Beckmann, M.W., Reifengerger, G., Bender, H.G., and Niederacher, D. (1999). Gene amplification and overexpression of CDK4 in sporadic breast carcinomas is associated with high tumor cell proliferation. *Am. J. Pathol.* 154, 113–118.
- Andrulis, I.L., Bull, S.B., Blackstein, M.E., Sutherland, D., Mak, C., Sidlofsky, S., Pritzker, K.P., Hartwick, R.W., Hanna, W., Lickley, L., et al. (1998). *neu*/erbB-2 amplification identifies a poor-prognosis group of women with node-negative breast cancer. Toronto Breast Cancer Study Group. *J. Clin. Oncol.* 16, 1340–1349.
- Barnes, D.M., and Gillett, C.E. (1998). Cyclin D1 in breast cancer. *Breast Cancer Res. Treat.* 52, 1–15.
- Bartkova, J., Lukas, J., Muller, H., Luthjohf, D., Strauss, M., and Bartek, J. (1994). Cyclin D1 protein expression and function in human breast cancer. *Int. J. Cancer* 57, 353–361.
- Bartkova, J., Lukas, J., Strauss, M., and Bartek, J. (1995). Cyclin D1 oncoprotein aberrantly accumulates in malignancies of diverse histogenesis. *Oncogene* 10, 775–778.
- Bates, S., Bonetta, L., MacAllan, D., Parry, D., Holder, A., Dickson, C., and Peters, G. (1994). CDK6 (PLSTIRE) and CDK4 (PSK-J3) are a distinct subset of the cyclin-dependent kinases that associate with cyclin D1. *Oncogene* 9, 71–79.
- Berchuck, A., Kamel, A., Whitaker, R., Kerns, B., Olt, G., Kinney, R., Soper, J.T., Dodge, R., Clarke-Pearson, D.L., Marks, P., et al. (1990). Overexpression of HER-2/*neu* is associated with poor survival in advanced epithelial ovarian cancer. *Cancer Res.* 50, 4087–4091.
- Bieche, I., and Lidereau, R. (1995). Genetic alterations in breast cancer. *Genes Chromosomes Cancer* 14, 227–251.
- Bowe, D.B., Kenney, N.J., Adereth, Y., and Maroulakou, I.G. (2002). Suppression of *Neu*-induced mammary tumor growth in cyclin D1 deficient mice is compensated for by cyclin E. *Oncogene* 21, 291–298.

- Buckley, M.F., Sweeney, K.J., Hamilton, J.A., Sini, R.L., Manning, D.L., Nicholson, R.I., de Fazio, A., Watts, C.K., Musgrove, E.A., and Sutherland, R.L. (1993). Expression and amplification of cyclin genes in human breast cancer. *Oncogene* 8, 2127–2133.
- Cheung, T.H., Yu, M.M., Lo, K.W., Yim, S.F., Chung, T.K., and Wong, Y.F. (2001). Alteration of cyclin D1 and CDK4 gene in carcinoma of uterine cervix. *Cancer Lett.* 166, 199–206.
- Coqueret, O. (2002). Linking cyclins to transcriptional control. *Gene* 299, 35–55.
- Dickson, C., Fantl, V., Gillett, C., Brookes, S., Bartek, J., Smith, R., Fisher, C., Barnes, D., and Peters, G. (1995). Amplification of chromosome band 11q13 and a role for cyclin D1 in human breast cancer. *Cancer Lett.* 90, 43–50.
- Dong, Y., Sui, L., Sugimoto, K., Tai, Y., and Tokuda, M. (2001). Cyclin D-CDK4 complex, a possible critical factor for cell proliferation and prognosis in laryngeal squamous cell carcinomas. *Int. J. Cancer* 95, 209–215.
- Eltze, E., Wulfig, C., Von Struensee, D., Piechota, H., Buerger, H., and Hertle, L. (2005). Cox-2 and Her2/neu co-expression in invasive bladder cancer. *Int. J. Oncol.* 26, 1525–1531.
- Fantl, V., Stamp, G., Andrews, A., Rosewell, I., and Dickson, C. (1995). Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. *Genes Dev.* 9, 2364–2372.
- Fantl, V., Edwards, P.A., Steel, J.H., Vonderhaar, B.K., and Dickson, C. (1999). Impaired mammary gland development in Cyl-1(–/–) mice during pregnancy and lactation is epithelial cell autonomous. *Dev. Biol.* 212, 1–11.
- Gao, P., Zhou, G.Y., Liu, Y., Li, J.S., Zhen, J.H., and Yuan, Y.P. (2004). Alteration of cyclin D1 in gastric carcinoma and its clinicopathologic significance. *World J. Gastroenterol.* 10, 2936–2939.
- Geng, Y., Whoriskey, W., Park, M.Y., Bronson, R.T., Medema, R.H., Li, T., Weinberg, R.A., and Sicinski, P. (1999). Rescue of cyclin D1 deficiency by knockin cyclin E. *Cell* 97, 767–777.
- Geng, Y., Yu, Q., Sicinska, E., Das, M., Bronson, R.T., and Sicinski, P. (2001). Deletion of the p27Kip1 gene restores normal development in cyclin D1-deficient mice. *Proc. Natl. Acad. Sci. USA* 98, 194–199.
- Gillett, C., Fantl, V., Smith, R., Fisher, C., Bartek, J., Dickson, C., Barnes, D., and Peters, G. (1994). Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. *Cancer Res.* 54, 1812–1817.
- Gillett, C., Smith, P., Gregory, W., Richards, M., Millis, R., Peters, G., and Barnes, D. (1996). Cyclin D1 and prognosis in human breast cancer. *Int. J. Cancer* 69, 92–99.
- Hansel, D.E., Ashfaq, R., Rahman, A., Wanzer, D., Yeo, C.J., Wilentz, R.E., and Maitra, A. (2005). A subset of pancreatic adenocarcinomas demonstrates coamplification of topoisomerase II α and HER2/neu: use of immunolabeling and multicolor FISH for potential patient screening and treatment. *Am. J. Clin. Pathol.* 123, 28–35.
- Hoechtlen-Vollmar, W., Menzel, G., Bartl, R., Lamerz, R., Wick, M., and Seidel, D. (2000). Amplification of cyclin D1 gene in multiple myeloma: clinical and prognostic relevance. *Br. J. Haematol.* 109, 30–38.
- Igarashi, K., Masaki, T., Shiratori, Y., Rengifo, W., Nagata, T., Hara, K., Oka, T., Nakajima, J., Hisada, T., Hata, E., and Omata, M. (1999). Activation of cyclin D1-related kinase in human lung adenocarcinoma. *Br. J. Cancer* 81, 705–711.
- Lammie, G.A., Fantl, V., Smith, R., Schuurin, E., Brookes, S., Michalides, R., Dickson, C., Arnold, A., and Peters, G. (1991). D11S287, a putative oncogene on chromosome 11q13, is amplified and expressed in squamous cell and mammary carcinomas and linked to BCL-1. *Oncogene* 6, 439–444.
- Landis, M.W., Pawlyk, B.S., Li, T., Sicinski, P., and Hinds, P.W. (2006). Cyclin D1-dependent kinase activity in murine development and mammary tumorigenesis. *Cancer Cell* 9, this issue, 13–22.
- Lee, R.J., Albanese, C., Fu, M., D'Amico, M., Lin, B., Watanabe, G., Haines, G.K., III, Siegel, P.M., Hung, M.C., Yarden, Y., et al. (2000). Cyclin D1 is required for transformation by activated Neu and is induced through an E2F-dependent signaling pathway. *Mol. Cell Biol.* 20, 672–683.
- Masaki, T., Shiratori, Y., Rengifo, W., Igarashi, K., Yamagata, M., Kurokohchi, K., Uchida, N., Miyauchi, Y., Yoshiji, H., Watanabe, S., et al. (2003). Cyclins and cyclin-dependent kinases: comparative study of hepatocellular carcinoma versus cirrhosis. *Hepatology* 37, 534–543.
- Matsushime, H., Ewen, M.E., Strom, D.K., Kato, J.Y., Hanks, S.K., Roussel, M.F., and Sherr, C.J. (1992). Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins. *Cell* 71, 323–334.
- Matsuura, I., Denissova, N.G., Wang, G., He, D., Long, J., and Liu, F. (2004). Cyclin-dependent kinases regulate the antiproliferative function of Smads. *Nature* 430, 226–231.
- McIntosh, G.G., Anderson, J.J., Milton, I., Steward, M., Parr, A.H., Thomas, M.D., Henry, J.A., Angus, B., Lennard, T.W., and Horne, C.H. (1995). Determination of the prognostic value of cyclin D1 overexpression in breast cancer. *Oncogene* 11, 885–891.
- Meyerson, M., and Harlow, E. (1994). Identification of G1 kinase activity for cdk6, a novel cyclin D partner. *Mol. Cell Biol.* 14, 2077–2086.
- Mimura, K., Kono, K., Hanawa, M., Mitsui, F., Sugai, H., Miyagawa, N., Ooi, A., and Fujii, H. (2005). Frequencies of HER-2/neu expression and gene amplification in patients with oesophageal squamous cell carcinoma. *Br. J. Cancer* 92, 1253–1260.
- Muller, W.J., Sinn, E., Pattengale, P.K., Wallace, R., and Leder, P. (1988). Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell* 54, 105–115.
- Musgrove, E.A., Hui, R., Sweeney, K.J., Watts, C.K., and Sutherland, R.L. (1996). Cyclins and breast cancer. *J. Mammary Gland Biol. Neoplasia* 1, 153–162.
- Nakazawa, K., Dobashi, Y., Suzuki, S., Fujii, H., Takeda, Y., and Ooi, A. (2005). Amplification and overexpression of c-erbB-2, epidermal growth factor receptor, and c-met in biliary tract cancers. *J. Pathol.* 206, 356–365.
- Rane, S.G., Dubus, P., Mettus, R.V., Galbreath, E.J., Boden, G., Reddy, E.P., and Barbacid, M. (1999). Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in β -islet cell hyperplasia. *Nat. Genet.* 22, 44–52.
- Reissmann, P.T., Koga, H., Figlin, R.A., Holmes, E.C., and Slamon, D.J. (1999). Amplification and overexpression of the cyclin D1 and epidermal growth factor receptor genes in non-small-cell lung cancer. *Lung Cancer Study Group. J. Cancer Res. Clin. Oncol.* 125, 61–70.
- Samady, L., Dennis, J., Budhram-Mahadeo, V., and Latchman, D.S. (2004). Activation of CDK4 gene expression in human breast cancer cells by the Brn-3b POU family transcription factor. *Cancer Biol. Ther.* 3, 317–323.
- Santin, A.D., Bellone, S., Van Stedum, S., Bushen, W., De Las Casas, L.E., Korourian, S., Tian, E., Roman, J.J., Burnett, A., and Pecorelli, S. (2005). Determination of HER2/neu status in uterine serous papillary carcinoma: Comparative analysis of immunohistochemistry and fluorescence in situ hybridization. *Gynecol. Oncol.* 98, 24–30.
- Sherr, C.J., and Roberts, J.M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* 13, 1501–1512.
- Sicinski, P., Donaher, J.L., Parker, S.B., Li, T., Fazeli, A., Gardner, H., Haslam, S.Z., Bronson, R.T., Elledge, S.J., and Weinberg, R.A. (1995). Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell* 82, 621–630.
- Sicinska, E., Aifantis, I., Le Cam, L., Swat, W., Borowski, C., Yu, Q., Ferrando, A.A., Levin, S.D., Geng, Y., von Boehmer, H., and Sicinski, P. (2003). Requirement for cyclin D3 in lymphocyte development and T cell leukemias. *Cancer Cell* 4, 451–461.
- Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., and McGuire, W.L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 235, 177–182.
- Stål, O., Borg, A., Ferno, M., Kallstrom, A.C., Malmstrom, P., and Nordenskjöld, B. (2000). ErbB2 status and the benefit from two or five years of adjuvant tamoxifen in postmenopausal early stage breast cancer. *Ann. Oncol.* 11, 1545–1550.

- Stephens, P., Hunter, C., Bignell, G., Edkins, S., Davies, H., Teague, J., Stevens, C., O'Meara, S., Smith, R., Parker, A., et al. (2004). Lung cancer: intragenic ERBB2 kinase mutations in tumours. *Nature* **431**, 525–526.
- Takano, Y., Takenaka, H., Kato, Y., Masuda, M., Mikami, T., Saegusa, M., and Okayasu, I. (1999). Cyclin D1 overexpression in invasive breast cancers: correlation with cyclin-dependent kinase 4 and oestrogen receptor overexpression, and lack of correlation with mitotic activity. *J. Cancer Res. Clin. Oncol.* **125**, 505–512.
- Tanner, M., Hollmen, M., Junttila, T.T., Kapanen, A.I., Tammola, S., Soini, Y., Helin, H., Salo, J., Joensuu, H., Sihvo, E., et al. (2005). Amplification of HER-2 in gastric carcinoma: association with Topoisomerase II α gene amplification, intestinal type, poor prognosis and sensitivity to trastuzumab. *Ann. Oncol.* **16**, 273–278.
- Tong, W., and Pollard, J.W. (2001). Genetic evidence for the interactions of cyclin D1 and p27(Kip1) in mice. *Mol. Cell. Biol.* **21**, 1319–1328.
- Tsutsui, T., Hesabi, B., Moons, D.S., Pandolfi, P.P., Hansel, K.S., Koff, A., and Kiyokawa, H. (1999). Targeted disruption of CDK4 delays cell cycle entry with enhanced p27(Kip1) activity. *Mol. Cell. Biol.* **19**, 7011–7019.
- Ugocsai, K., Mandoky, L., Tiszlavicz, L., and Molnar, J. (2005). Investigation of HER2 overexpression in non-small cell lung cancer. *Anticancer Res.* **25**, 3061–3066.
- Vandenberghe, E. (1994). Mantle cell lymphoma. *Blood Rev.* **8**, 79–87.
- van den Heuvel, S., and Harlow, E. (1993). Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* **262**, 2050–2054.
- Yu, Q., Geng, Y., and Sicinski, P. (2001). Specific protection against breast cancers by cyclin D1 ablation. *Nature* **411**, 1017–1021.
- Zhou, Q., Hopp, T., Fuqua, S.A., and Steeg, P.S. (2001). Cyclin D1 in breast premalignancy and early breast cancer: implications for prevention and treatment. *Cancer Lett.* **162**, 3–17.