Cyclin D1-dependent kinase activity in murine development and mammary tumorigenesis

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Summary

Cyclin D1 is a multifunctional protein that activates CDK4 and CDK6, titrates Cip/Kip CDK inhibitors to increase CDK2 activity, and modulates the function of certain transcription factors. To specifically test the importance of cyclin D1-associated kinase activity, we generated "knockin" mice expressing mutant cyclin D1 deficient in activating CDK4/6. The development of several cyclin D1-dependent compartments, including mammary glands, proceeds relatively normally in these animals, demonstrating that cyclin D1-associated kinase activity is largely dispensable for development of these tissues. Strikingly, knockin mice were resistant to breast cancers initiated by ErbB-2. These results demonstrate a differential requirement for cyclin D1-CDK4/6 kinase activity in development versus tumorigenesis and strongly support cyclin D1-dependent kinase activity as a specific therapeutic target in breast cancer.

Introduction

The family of D-type cyclins (D1, D2, and D3) are important regulators of the G_1-S phase transition (Sherr, 1995). Aberrant overexpression of the D cyclins, and cyclin D1 in particular, has been linked to loss of cell cycle control and a wide variety of malignancies. Of notable importance, the cyclin D1 gene is amplified in ~20% of breast cancers, and the cyclin D1 protein is overexpressed in greater than 50% of cases (Donnellan and Chetty, 1998). Genetic models in mice have further emphasized the importance of cyclin D1 in both development and tumorigenesis (Fantl et al., 1995; Sicinski et al., 1995). *cyclin D1*$^{-/-}$ mice are about 50% smaller than their wild-type littermates and show a low frequency of early lethality at about 1 month of age. These mice exhibit a "clasping" phenotype, wherein the mouse clasps its rear limbs together, rather than splaying them outward, when it is lifted by the tail. In addition, a limited number of tissue-specific defects are observed, including profound hypoplasia in the retina and a defect in the ability of the mammary epithelium to proliferate and form lobuloalveolae in response to hormonal signals during pregnancy.

Interestingly, further analysis of the developing neural retina by in situ hybridization indicated that cyclin D1 was the only D-type cyclin whose mRNA was expressed in this tissue at any appreciable level (Fantl et al., 1995; Sicinski et al., 1995). This observation suggested that the lack of cyclin D1 might have a profound effect during retinal development because no other D cyclin is available in this tissue. Extending this line of thought led to the hypothesis that the D cyclins may serve redundant and overlapping roles in most tissues, and that a limited subset of tissues that express only one D-type cyclin may be affected by the loss of that cyclin. Indeed, mice expressing only cyclin D3 have a profound defect in cerebellar development, having a reduced number of folia, decreased cellularity of the inner granular cell layer, and an abnormal Purkinje cell layer (Ciemerych et al., 2002). Interestingly, molecular analysis of the granular neuron precursors showed that the N-myc transcription factor is upstream of cyclins D1 and D2, but not D3, in

SIGNIFICANCE

Normal cell proliferation is driven by complexes of cyclins and cyclin-dependent kinases (CDKs) acting at each cell cycle transition when conditions are appropriate. Altered expression of cyclins can drive aberrant proliferation in cancer. Cyclin D1 is of particular importance, as its expression is increased in many cancers, most notably breast cancer. Mice lacking cyclin D1 fail to develop lactating mammary glands and are protected from ErbB-2-induced tumors. Using a knockin mouse model, we show here that activation of CDKs by cyclin D1 is largely dispensable for development of certain tissues, including mammary gland, but is required for ErbB-2-dependent mammary tumors in mice. These data indicate that inhibition of cyclin D1-dependent CDKs could specifically inhibit mammary tumor cell proliferation.

this tissue. This may explain the cerebellar defect in cyclin D3-only mice: because of dependence on a specific transcription factor that activates only cyclins D1 and D2, the cerebellum is not able to upregulate cyclin D3 to compensate for the loss of cyclins D1 and D2.

In addition to revealing critical functions in development, several models have described the importance of cyclin D1 in mouse tumors. Expression of cyclin D1 in the mouse mammary gland under the control of the mouse mammary tumor virus (MMTV) promoter resulted in deregulated proliferation and the eventual formation of mammary adenocarcinomas that expressed high levels of cyclin D1 protein (Wang et al., 1994). Several other reports highlight the role of cyclin D1 as a critical downstream target of other oncogenes. For example, in three models of oncogenic *ras*-induced skin tumors, deficiency of cyclin D1 led to increased latency and decreased penetrance of tumor formation. These results indicate that cyclin D1 is a preferred downstream effector of oncogenic *ras* signaling in these models (Robles et al., 1998). In addition, cyclin D1 has been shown to be a critical downstream target in mammary tumors induced by MMTV-*ras* and MMTV-*ErbB-2* (Lee et al., 2000; Yu et al., 2001). Whereas *MMTV-ras* and *MMTV-ErbB-2* transgenic mice succumb to mammary gland tumors between 6 months and 1 year with virtually 100% penetrance, their *MMTV-ras; cyclin D1*$^{-/-}$ and *MMTV-ErbB-2;cyclin D1*$^{-/-}$ littermates are completely resistant to tumor formation (Yu et al., 2001). These results indicate that cyclin D1 is a necessary target of the *ras* and *ErbB-2* oncogenes in mammary tumor formation in the mouse.

The critical role of cyclin D1 in development and tumorigenesis may be the result of the ability of D cyclins to bind and activate cyclin-dependent kinases CDK4 and CDK6; these holoenzymes in turn phosphorylate the retinoblastoma protein (pRB) (Bates et al., 1994; Matsushime et al., 1992; Meyerson and Harlow, 1994). Surprisingly, very few additional substrates of cyclin D-CDK4/6 holoenzymes have been identified. However, in addition to activating the kinase activity of CDK4 and CDK6, D cyclins have a number of activities that either do not depend on the catalytic activity of the partner kinase or do not depend at all on the partner kinase. Thus, cyclin D1-CDK4/6 complexes are able to activate cyclin E-CDK2 complexes by titration of the CDK inhibitors p21^{Cip1} and p27^{Kip1} (Cheng et al., 1998; Polyak et al., 1994; Reynisdottir et al., 1995). This kinase-independent function has been postulated by some to represent the major role of cyclin D-CDK4/6 complexes in cell cycle progression (Geng et al., 2001; Tsutsui et al., 1999). In addition, D cyclins can interact with and modulate the activity of a number of transcription factors in a CDK4/6-independent manner (Bienvenu et al., 2001; Fu et al., 2004; Inoue and Sherr, 1998; Knudsen et al., 1999; Neuman et al., 1997; Ratineau et al., 2002; Zwijsen et al., 1997). A major, unresolved issue is the contribution of these various ''kinase-dependent'' and ''kinase-independent'' functions of D cyclins to development and tumorigenesis.

To address this question, we took advantage of a cyclin D1 point mutant, cyclin D1 K112E, which contains a lysine to glutamine substitution at amino acid position 112. This well-characterized mutant has been previously shown to bind to CDK4 and CDK6 with similar affinity to wild-type cyclin D1, but it is unable to activate the catalytic activity of the partner kinase or transform primary rodent cells (Baker et al., 2005; Hinds et al., 1994). Importantly, this ''kinase-deficient'' cyclin D1, when bound to CDK4/6, is expected to normally titrate p21^{Cip1} and p27^{Kip1}, thereby activating cyclin E-CDK2 complexes. Moreover, cyclin D1 K112E and a similarly acting K114E mutant have been shown to normally interact with and modulate the activity of various transcription factors (Bienvenu et al., 2001; Inoue and Sherr, 1998; Knudsen et al., 1999; Neuman et al., 1997; Ratineau et al., 2002; Zwijsen et al., 1997). By introducing K112E into the mouse germline, we show here that activation of CDK4 and/or CDK6 is required for the normal development of some tissues but not others. Notably, retina and mammary gland development are nearly normal in these animals. Nevertheless, like animals lacking cyclin D1 altogether, K112E animals are protected from development of mammary tumors instigated by the ErbB-2 oncogene. Thus, a requirement for CDK activation by cyclin D1 is tumor specific in the mammary gland and could be an excellent therapeutic target.

Results and discussion

Generation of *cyclin D1*$^{KE/KE}$ knockin mice

We used homologous recombination in embryonal stem (ES) cells to replace the wild-type cyclin D1 gene with a point mutant cyclin D1 allele encoding cyclin D1 K112E protein (Figure 1A). Subsequently, homozygous ''knockin'' mice expressing cyclin D1 K112E were generated using standard procedures; these mice will be hereafter referred to as *cyclin D1*$^{KE/KE}$ animals. The tissues of homozygous *cyclin D1*$^{KE/KE}$ mice are expected to express ''kinase-deficient'' cyclin D1-CDK4 and cyclin D1-CDK6 complexes. Importantly, the structure of the recombined allele is expected to preserve normal regulation of expression.

The *cyclin D1*$^{KE/KE}$ mice were born in a normal Mendelian ratio (data not shown). We confirmed the presence of the desired point mutation in the genomes of these mice by DNA sequencing (Figure 1C). To determine whether the KE allele was expressed in a normal manner, we analyzed the levels of cyclin D1 or cyclin D1 KE protein in the tissues of adult *cyclin D1*$^{KE/KE}$ mice and their wild-type littermates. These analyses demonstrated normal levels of the knockin protein in all organs examined (hearts, lungs, spleens, livers, and kidneys) (Figure 1D); we noted, however, that the slower-migrating cyclin D1 species was less abundant in the mutant tissues. Although we have not determined the origin of the two forms of cyclin D1 in these tissues, it has previously been reported that cell cycle-dependent cyclin-CDK complex formation results in phosphorylation of cyclin D1 at T286; this produces a slower migrating form (Matsushime et al., 1994; Diehl et al., 1997). We also verified normal expression levels of cyclins D2 and D3, CDK4, CDK6, and p27^{Kip1} in *cyclin D1*$^{KE/KE}$ animals (Figures 1E and 1F). We conclude that the introduction of a point mutation into the endogenous cyclin D1 locus did not affect overall cyclin D1 levels and did not perturb the expression of other cell cycle regulators. Hence, *cyclin D1*$^{KE/KE}$ mice offer us a tool to address the requirement for cyclin D1-CDK4/6 kinase function in development and in carcinogenesis.

Retinal development and function in *cyclin D1*$^{KE/KE}$ knockin mice

We observed *cyclin D1*$^{KE/KE}$ mice throughout their lives and compared their appearance with that of mice lacking cyclin D1. As reported previously, cyclin D1 null mice display severely

hypoplastic retinas, with all neuronal cell layers (outer nuclear, inner nuclear, and ganglion cell) being profoundly affected (Fantl et al., 1995; Sicinski et al., 1995) (Figure 2A). This indicates that cyclin D1 function is critically required for retinal development. Strikingly, we found that the retinas of the homozygous *cyclin D1$^{KE/KE}$* animals developed relatively normally, being only slightly underdeveloped in comparison to retinas from wild-type littermates (Figure 2A). To obtain a quantitative measure of retinal function in *cyclin D1$^{KE/KE}$* mice, we performed electroretinographic analyses. These studies demonstrated that the retinas of *cyclin D1$^{KE/KE}$* mice responded to light with electroretinographic potentials corresponding to approximately 65% of those seen in wild-type mice. In contrast, cyclin D1 null mice respond at 10% of wild-type levels (Figure 2B). We conclude that, while cyclin D1 function is clearly required for retinal development, kinase activity associated with cyclin D1 is largely dispensable for this process.

Unlike many other tissues, which express two or even all three D-type cyclins, the developing retina expresses mainly cyclin D1. For this reason, we used retinas derived from *cyclin D1$^{KE/KE}$* mice to analyze the properties of the KE protein without interference from other D cyclins. First, we examined the expression of various cell cycle regulators in the lysates of retinas microdissected from 1-day-old pups (Figure 2C). We found that the levels of cyclin D1 KE in the retinas of knockin mice closely resembled the levels of cyclin D1 seen in the retinas of wild-type littermates. As was the case in other tissues (see Figure 1D), we observed that, in the knockin retinas, the faster migrating species of cyclin D1 was more abundant than the slower migrating form (Figure 2C). We also observed slightly decreased levels of CDK4 and p27^{Kip1} in the mutant retinas (Figure 2C).

To verify that cyclin D1 KE-CDK4 complexes retain the ability to titrate p27^{Kip1}, we immunoprecipitated CDK4 from the retinal lysates and probed the immunoblots with antibodies against cyclin D1 and p27^{Kip1}. Of relevance here is data demonstrating that free (uncomplexed with D cyclins) CDK4 is unable to bind p27^{Kip1} (Hall et al., 1995; Kozar et al., 2004), and that the kinase activity of CDK4 is not required for the formation of cyclin D1-CDK4-p27^{Kip1} complexes (Baker et al., 2005). Consistent with these data, we observed that cyclin D1 KE-CDK4 complexes retained the ability to bind p27^{Kip1} (Figure 2D). Although less CDK4 was immunoprecipitated from the *cyclin D1$^{KE/KE}$* lysates, as might be expected due to the decreased CDK4 expression in the knockin retinas, the relative amounts of cyclin D1 KE protein and p27^{Kip1} that coimmunoprecipitated with CDK4 were similar in the wild-type and *cyclin D1$^{KE/KE}$* lysates. As expected, virtually no p27^{Kip1} coimmunoprecipitated with CDK4 from *cyclin D1$^{-/-}$* retinas, confirming the requirement for cyclin D1 in p27^{Kip1} titration (Figure 2D).

To determine whether titration of p27^{Kip1} by cyclin D1 KE-CDK4 complexes resulted in activation of CDK2, we performed in vitro kinase assays to measure CDK2 activity. These analyses demonstrated nearly normal CDK2-associated kinase activity in the retinas of the knockin mice (Figure 2E). We conclude that cyclin D1 KE-CDK4 complexes at least partially retain the ability to activate CDK2 by titrating p27^{Kip1}. In contrast, *cyclin D1$^{-/-}$* retinas lack cyclin D1-CDK4 complexes and are thus unable to titrate p27^{Kip1} (Figure 2D). As a consequence, CDK2-associated kinase activity is not activated in a normal manner in cyclin D1 null retinas (Figure 2E); these retinas are therefore deficient in both CDK4- and CDK2-dependent kinase activity. However, this simple picture is complicated by the observation that KE retina lysates contain elevated levels of cyclin E and slightly reduced levels of the fast-migrating, active form of CDK2. These data suggest that the effects of cyclin D1 KE expression on CDK2 activity are complex. The observed CDK2 activity likely not only results from p27^{Kip1} titration by cyclin D1 KE-CDK4 complexes, but may also depend on reduced steady-state levels of p27^{Kip1}. The mechanisms underlying p27^{Kip1} homeostasis in the retina are currently unclear; however, the above-described multiplicity of effects resulting from alteration of cyclin D1 presumably reflects complex crosstalk among several important cell cycle regulatory proteins.

We next examined the phosphorylation status of the retinoblastoma protein (pRB), which represents a target of cyclin D-CDK4/6, cyclin E-CDK2, and cyclin A-CDK2 complexes. Probing of immunoblots with an antibody recognizing all of the phospho-forms of the retinoblastoma protein demonstrated that the fraction of hyperphosphorylated, slower-migrating pRB was dramatically reduced in the retinas of knockin mice (Figure 2F). The residual levels of pRB phosphorylation seen in *cyclin D1$^{KE/KE}$* retinas are likely contributed by cyclin E-CDK2 kinase. In contrast, the absence of cyclin D1-CDK4 complexes in cyclin D1 null retinas cripples both cyclin D1-CDK4 and cyclin E-CDK2 kinases, leading to even more severe impairment of pRB phosphorylation (Figure 2F).

To carry this analysis further, we utilized a phospho-specific antibody to examine the phosphorylation of pRB at the serine 780 (S780) residue, which is thought to be preferentially phosphorylated by cyclin D-associated kinase activity (Kitagawa et al., 1996), and also less efficiently by CDK2-containing complexes (Kozar et al., 2004) (Figure 2F). We found that the phosphorylation of pRB at S780 was severely compromised in the *cyclin D1$^{KE/KE}$* retinas, as compared to wild-type tissues. The residual phosphorylation at this site in *cyclin D1$^{KE/KE}$* tissues is likely contributed by CDK2-associated kinase. Consistent with this thinking, even lower levels of S780 phosphorylation were observed in cyclin D1 null retinas (Figure 2F). Thus, in agreement with previous studies of the cyclin D1 K112E mutant (Baker et al., 2005), the kinase activity of cyclin D1-CDK4/6 complexes is severely crippled in *cyclin D1$^{KE/KE}$* tissues.

Mammary gland development in *cyclin D1$^{KE/KE}$* mice

We next turned our attention to mammary gland development. A critical requirement for cyclin D1 function in this compartment was previously demonstrated by the observation that mice lacking cyclin D1 fail to undergo the massive mammary epithelial expansion that normally takes place during pregnancy (Fantl et al., 1995; Sicinski et al., 1995). As a consequence, *cyclin D1$^{-/-}$* females are unable to nurse their pups. In striking contrast, analyses of *cyclin D1$^{KE/KE}$* females demonstrated normal mammary epithelial expansion, which was indistinguishable from that seen in wild-type counterparts (Figures 3A and 3B). At the end of pregnancy, *cyclin D1$^{KE/KE}$* females presented fully developed mammary epithelial trees (Figure 3A). In addition, hematoxylin and eosin staining of mammary gland sections demonstrated that mammary glands from *cyclin D1$^{KE/KE}$* females are able to lactate, as indicated by the presence of alveoli containing milk proteins (Figure 3B). These results indicate that, as was the case with retinal development, the kinase activity of cyclin D1-containing complexes is dispensable for breast development.

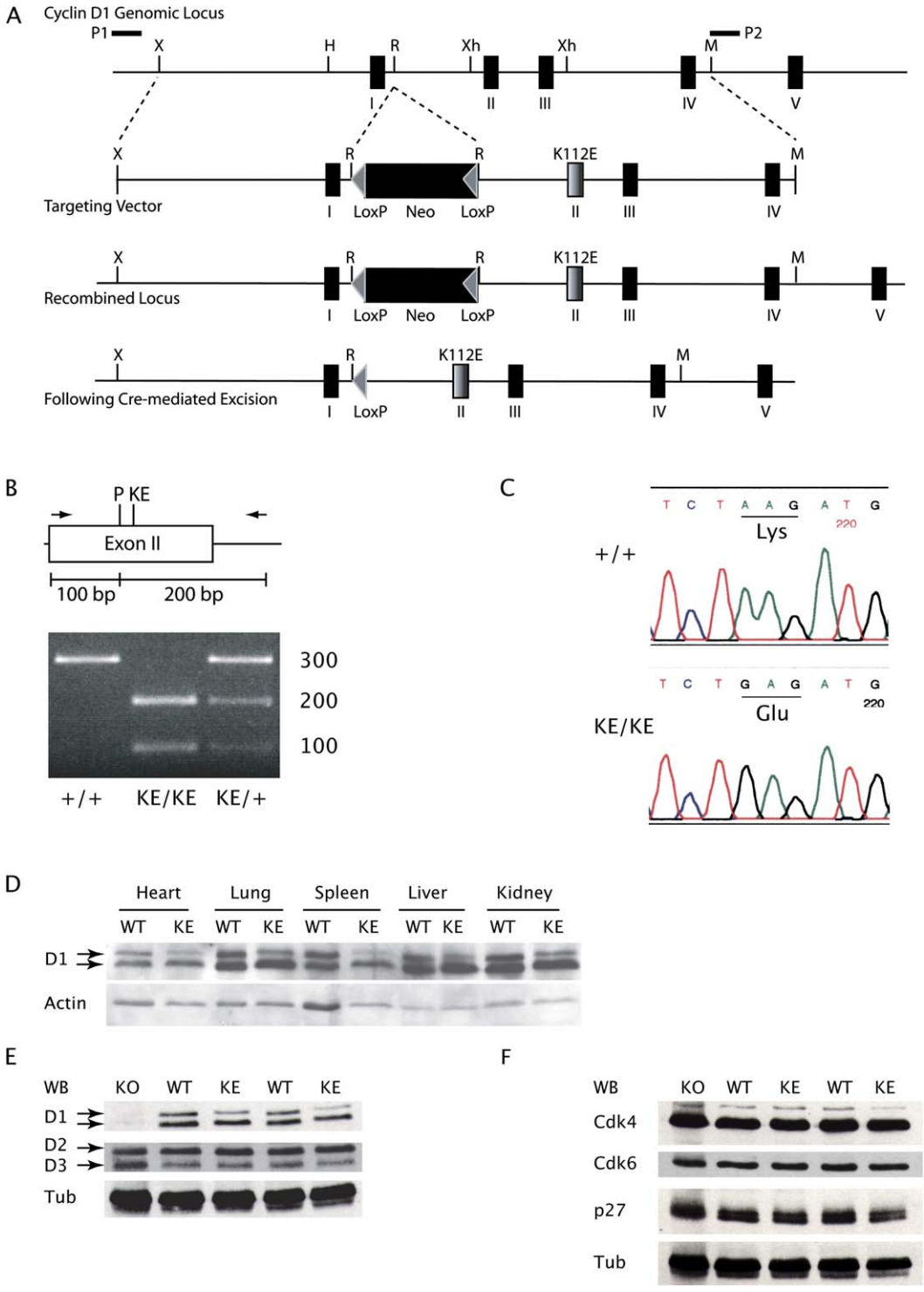

Figure 1. Targeted replacement of the wild-type cyclin D1 gene with the KE allele

A: Schematic diagram of the endogenous cyclin D1 locus and the targeting vector used to engineer embryonal stem (ES) cells by homologous recombination. This vector contains a point mutation in exon II that changes lysine at amino acid position 112 to glutamine (K112E). In addition, a second, closely linked silent mutation was introduced that generates a novel PmlI restriction endonuclease site. The coding exons are marked as black boxes and are numbered. Restriction enzyme sites are also indicated (H, HinDIII; M, MfeI; R, EcoRI; X, XbaI; Xh, XhoI). Probes P1 and P2 were used to screen ES cells for homologous recombination by Southern blotting (data not shown).

B: PCR strategy used to genotype the animals. The region surrounding the K112E mutation was amplified by PCR and digested with PmlI (the PmlI site is indicated as P). An undigested 300 bp fragment is produced from the wild-type allele, while the amplification product from the KE allele is digested into 200 bp and 100 bp fragments. PCR was performed on DNA isolated from tail biopsies; results from wild-type (+/+), *cyclin D1*$^{KE/+}$ (KE/+), and *cyclin D1*$^{KE/KE}$ (KE/KE) animals are shown.

Neurological and growth defects in *cyclin D1$^{KE/KE}$* mice

In addition to these well-defined retinal and mammary epithelial phenotypes, cyclin D1 null mice were reported to show a set of symptoms that suggests the presence of a developmental neurological abnormality (Fantl et al., 1995; Sicinski et al., 1995). Thus, cyclin D1 null mice display growth retardation and a spastic ''leg-clasping'' reflex and show increased premature mortality. Analyses of the knockin mice demonstrated that *cyclin D1$^{KE/KE}$* mice retained some, but not all, of these phenotypic abnormalities. Like cyclin D1 null mice, *cyclin D1$^{KE/KE}$* animals exhibited the pathological ''leg-clasping'' phenotype (Figure 4A). Knockin mice also displayed retarded growth (Figure 4B). The body mass of 18-day-old male *cyclin D1$^{KE/KE}$* mice represented 61% of that seen in their wild-type littermates (Figure 4C). In comparison, cyclin D1 null males had body weights reduced to approximately 50% of normal. Long-term observation of the *cyclin D1$^{KE/KE}$* mice suggests that this growth deficiency persists throughout life. On the other hand, we never observed premature mortality of the ''knockin'' mice, whereas approximately 25% of cyclin D1 null mice died during the first month of life. Altogether, we conclude cyclin D1-associated kinase activity is required for some, but not all, aspects of neurological development. However, the pathological lesions underlying these presumed neurological abnormalities have not been defined; hence, we could not study them further.

One defined compartment in which cyclin D1 is known to play a role in neurological development is represented by the cerebellum. Although cyclin D1-deficient mice were shown to display normal cerebellar development, and this development was very mildly affected in animals lacking cyclin D2, the combined ablation of cyclins D1 and D2 led to a very severe cerebellar hypoplasia (Ciemerych et al., 2002). *cyclin D1$^{-/-}$; cyclin D2$^{-/-}$* cerebella displayed a profound reduction in the number of cells in the inner granular layer, abnormal layering of Purkinje cells, abnormal numbers of folia, and low bromodeoxyuridine incorporation in the proliferative external granular layer (Ciemerych et al., 2002). These observations suggest that cyclins D1 and D2 play redundant, overlapping roles in driving cerebellar development (cyclin D3 is not expressed in this organ).

To test whether cyclin D1-associated kinase activity is required in cerebellar development, we crossed *cyclin D1$^{KE/KE}$* mice with cyclin D2-deficient mice and generated *cyclin D1$^{KE/KE}$;cyclin D2$^{-/-}$* animals. We found that *cyclin D1$^{KE/KE}$;cyclin D2$^{-/-}$* mice displayed severely retarded cerebellar development (Figures 5A and 5B) that resembled the lesions seen in mice lacking cyclins D1 and D2. We interpret these results as an indication that the kinase activity of cyclin D1-containing complexes plays a role in driving cerebellar development.

Collectively, our analyses of *cyclin D1$^{KE/KE}$* mice have demonstrated that the kinase activity of cyclin D1-CDK4/6 is largely dispensable for the development of some cyclin D1-dependent compartments (retinas, mammary glands) but is required for development of selected compartments of the nervous system.

Mammary tumorigenesis in mice expressing ErbB-2

In addition to these developmental abnormalities, cyclin D1-deficient females were observed to be resistant to mammary carcinomas initiated by the activated ErbB-2 oncogene (Yu et al., 2001). This was demonstrated by crossing *cyclin D1$^{-/-}$* mice with a breast cancer-prone transgenic strain expressing the activated ErbB-2 oncogene driven by the MMTV promoter. Having shown that the kinase activity of cyclin D1-CDK4/6 complexes is dispensable for mammary development (Figure 3), we asked whether the kinase activity is required for ErbB-2-driven breast tumorigenesis. To answer this question, we crossed *cyclin D1$^{KE/KE}$* mice with *MMTV-ErbB-2* animals and analyzed the incidence of tumor formation in the resulting *cyclin D1$^{+/+}$; MMTV-ErbB-2* and *cyclin D1$^{KE/KE}$;MMTV-ErbB-2* females. As expected, *cyclin D1$^{+/+}$;MMTV-ErbB-2* mice began to develop mammary tumors at approximately 6 months of age, and virtually all of these mice had developed tumors by 12 months of age, similar to previously reported results (Muller et al., 1988; Yu et al., 2001). In striking contrast, mice expressing kinase-deficient cyclin D1 KE-CDK complexes were resistant to ErbB-2-induced breast cancers (Figure 6A). Importantly, we verified that this resistance was not caused by the absence of cyclin D1 expression (Figure 6B), or by inadequate transgene expression levels (Figure 6C) in the mammary glands of *cyclin D1$^{KE/KE}$; MMTV-ErbB-2* mice. These observations strongly suggest that the kinase activity of cyclin D1-CDK complexes is critically required for ErbB-2-driven breast tumorigenesis.

The work presented here documents a differential requirement for cyclin D1-associated kinase activity in development versus in tumorigenesis. In our analyses, we utilized mice expressing a cyclin D1 mutant that has been previously shown to be deficient in CDK4/6 activation. As is always the case with kinase-deficient mutants, we cannot formally exclude the presence of very low, residual kinase activity of this mutant in vivo. However, even if the cyclin D1 KE protein is not completely deficient in CDK4/6 activation, our results indicate that greatly reduced kinase activity has a dramatically different impact on development versus on tumor formation.

Consistent with this thinking, in the accompanying paper, Yu et al. (2006) demonstrate that mammary glands lacking CDK4, cyclin D1's major catalytic partner in this tissue, develop normally but are completely resistant to mammary carcinomas initiated by ErbB-2. Moreover, the presence of cyclin D1-CDK4 complexes is required not only for the initiation of tumors, but also to sustain tumor cell proliferation. These studies, together with our observations that the kinase activity of cyclin D1-CDK complexes is largely dispensable for development of several compartments, suggests that targeting cyclin D1-associated kinase activity may be a viable and specific strategy for clinical treatment of certain breast tumors.

C: PCR amplification products from wild-type (+/+) and *cyclin D1$^{KE/KE}$* (KE/KE) animals were subcloned and sequenced to demonstrate the presence of the K112E mutation; representative traces are shown.

D: Western blot analysis of cyclin D1 levels in the indicated organs of wild-type (WT) and *cyclin D1$^{KE/KE}$* (KE) mice. Probing for actin was used to ensure equal loading.

E and **F:** Western blot analyses of lysates prepared from wild-type (WT), *cyclin D1$^{KE/KE}$* (KE), or *cyclin D1$^{-/-}$* (KO) day 13.5 embryos. Immunoblots were probed with the indicated antibodies. Probing for tubulin was used to ensure equal loading.

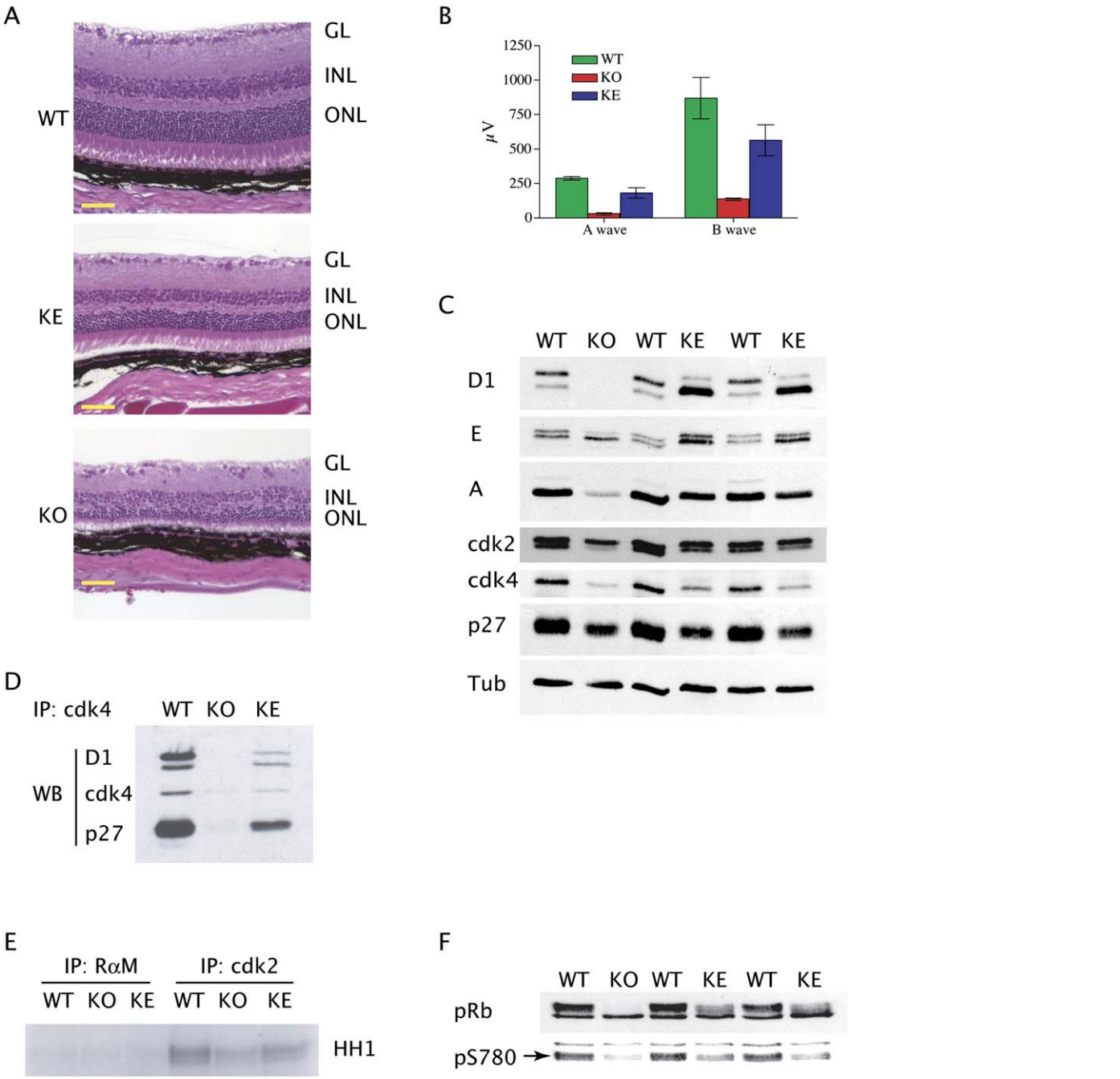

Figure 2. Analyses of the *cyclin D1*$^{KE/KE}$ retinas

A: Appearance of the retinas in 2-month-old wild-type (WT), *cyclin D1*$^{KE/KE}$ (KE), and *cyclin D1*$^{-/-}$ (KO) mice. Histological sections were stained with hematoxylin and eosin. GL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar, 50 μm.

B: Electroretinographic testing of 3-month-old wild-type (WT; n = 3), *cyclin D1*$^{KE/KE}$ (KE; n = 3), and *cyclin D1*$^{-/-}$ (KO; n = 2) mice. The mean amplitudes of the A and B waves generated in response to a flash of light are shown. Error bars represent standard deviation. The results obtained for WT and KO mice are virtually identical to previously reported results (Sicinski et al., 1995).

C: Western blot analyses of retina lysates. Neural retinas were microdissected from 1-day-old pups. Immunoblots were probed with the indicated antibodies.

D: Association of CDK4 with cyclin D1 and p27 in wild-type (WT), *cyclin D1*$^{KE/KE}$ (KE), and *cyclin D1*$^{-/-}$ (KO) retinas. CDK4 was immunoprecipitated from the retina lysates, and the immunoblots were probed with the indicated antibodies. Although less CDK4 was immunoprecipitated from the KE retinas, likely due to reduced expression, the relative amounts of cyclin D1 and p27 bound to CDK4 are very similar to those seen in WT retinas. Note that there is no p27 bound to CDK4 in the absence of cyclin D1 (KO samples).

E: CDK2-associated kinase activity was determined by immunoprecipitating CDK2 from retina lysates of the indicated genotypes and performing in vitro kinase assays using histone H1 (HH1) as a substrate. For control, samples were immunoprecipitated with isotype matched rabbit anti-mouse (RαM) IgG.

F: Phosphorylation of pRB was studied by subjecting retina lysates to immunoblot analyses with an antibody that recognizes all of the phospho-forms of pRB, or an antibody specific to phospho-serine 780 (pS780).

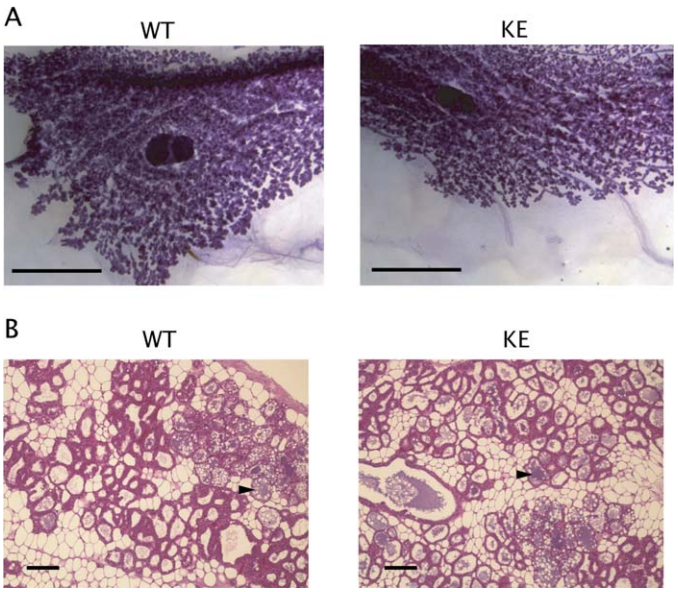

Figure 3. Normal mammary epithelial lobuloalveolar development in *cyclin D1$^{KE/KE}$* females

Whole-mount appearance (**A**) and H&E-stained sections (**B**) of mammary glands from wild-type (WT) and *cyclin D1$^{KE/KE}$* (KE) females on the day of delivery of pups. In **A**, the mammary epithelial tree was visualized by carmine red staining. Arrowheads in **B** indicate alveoli filled with milk proteins. Scale bar in **A**, 0.5 cm. Scale bar in **B**, 100 μm.

Experimental procedures

Engineering of *cyclin D1$^{KE/KE}$* mice

All animal experiments performed herein were approved by and conform to the standards of the Institutional Animal Care and Use Committees at the Dana-Farber Cancer Institute, Harvard Medical School, and Tufts-New England Medical Center.

Genomic fragments of the cyclin D1 locus have been described (Sicinski et al., 1995). A point mutation (5′-TAAGAT-3′ to 5′-TGAGAT-3′) changing K112 to E was generated by PCR and introduced into a targeting vector extending ~4.4 kbp upstream to the XbaI site and ~4.2 kbp downstream to the MfeI site. The vector also contained a closely linked silent mutation (5′-CACCTG-3′ to 5′-CACGTG-3′) creating a novel PmlI site. A LoxP-neo^R-LoxP drug cassette was inserted at the EcoRI site. Linearized targeting vector was electroporated into J1 ES cells, and G418-resistant ES cell clones were selected, expanded, and frozen essentially as described previously (Jacks et al., 1992; Sicinski et al., 1995). DNA from ES clones was screened for homologous recombination from both ends by Southern blot using external probes P1 and P2 (Figure 1A). Chimeric mice were generated essentially as described previously (Jacks et al., 1992; Sicinski et al., 1995). A series of matings to C57BL/6 wild-type, nestin-cre (Trumpp et al., 1999), and C57BL/6 wild-type mice was performed to effect the excision of the drug cassette and remove the cre transgene. Resulting heterozygotes were mated to homozygosity. PCR (forward primer, 5′-TGTGAGGAGCAGAAGTGCGAA-3′; backward primer, 5′-AGATCTGTCCATGGATGGCTG-3′) followed by digestion with PmlI was used to genotype the mice. PCR products from homozygote mutant mice were subcloned (TOPO-TA, Invitrogen) and sequenced to confirm the presence of the mutation.

Western blotting, immunoprecipitation, and in vitro kinase assays

For Western blotting, IP-Westerns, and IP-kinase assays, tissues were dissected and either lysed immediately or snap frozen and stored at −80ºC for later use. Tissues were subjected to Dounce homogenization in ELB, and Western blotting was performed essentially as described previously (Baker et al., 2005). For IP-Westerns and IP-kinase assays, tissues were subjected to Dounce homogenization in D-IP buffer followed by immunoprecipitation and Western blotting or immunoprecipitation followed by in vitro kinase assay essentially as described previously (Baker et al., 2005).

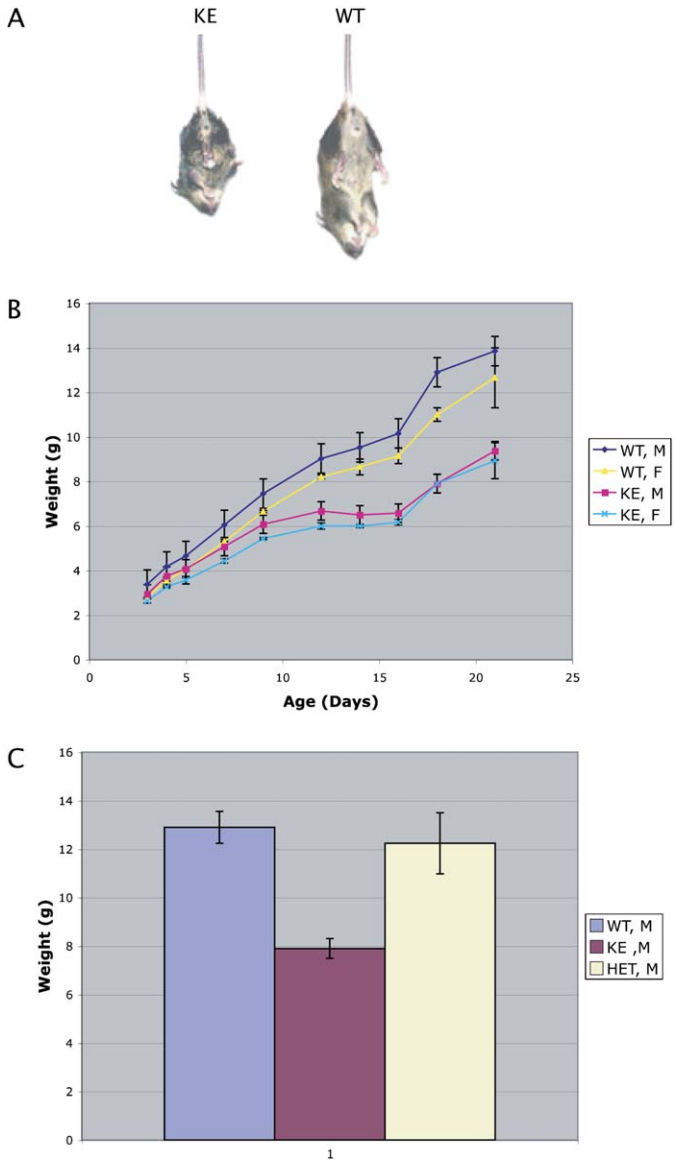

Figure 4. Retarded growth and pathological reflexes in *cyclin D1$^{KE/KE}$* mice

A: Wild-type (WT) and *cyclin D1$^{KE/KE}$* (KE) mice are shown being lifted by their tails. The *cyclin D1$^{KE/KE}$* mouse is smaller and demonstrates a pathological ''leg-clasping'' reflex.

B: Male (M) and female (F) wild-type (WT) and *cyclin D1$^{KE/KE}$* (KE) mice were weighed at the indicated times. Each point represents the average weight of the animals in a given group (n = 4 for WT and KE males, n = 2 for WT females, n = 3 for KE females). Error bars denote standard deviation.

C: Mean body weights of 18-day-old wild-type (WT; n = 4) and *cyclin D1$^{KE/KE}$* (KE; n = 4) male mice. Values for heterozygous *cyclin D1$^{KE/+}$* males (HET; n = 8) are also shown. Error bars denote standard deviation.

Antibodies used in this study included anti-cyclin D1 (72-13G, Santa Cruz), anti-cyclin D2 (M-20, Santa Cruz), anti-cyclin D3 (C-16, Santa Cruz), anti-CDK4 (C-22, Santa Cruz or Ab-2, Neomarkers), anti-CDK6 (C21, Santa Cruz or Ab-3, Neomarkers), anti-cyclin E (M-20, Santa Cruz), anti-CDK2 (M2, Santa Cruz), anti-p27 (anti-Kip1/p27, Transduction Laboratories), anti-cyclin A (CY-A1, Sigma), anti-pRb (Ab-245, Pharmingen), anti-pRb phospho-S780 (Cell Signaling Technologies), anti-actin (C-2, Santa Cruz), and anti-α-tubulin (AB-1, Oncogene Research Products). Immunoprecipitations were carried out using agarose-conjugated anti-CDK4 (C-22, Santa Cruz) and anti-CDK2 (M-2, Santa Cruz) bound to protein A-Sepharose (Amersham Biosciences) for 1 hr before addition of protein lysates. Immunoblot

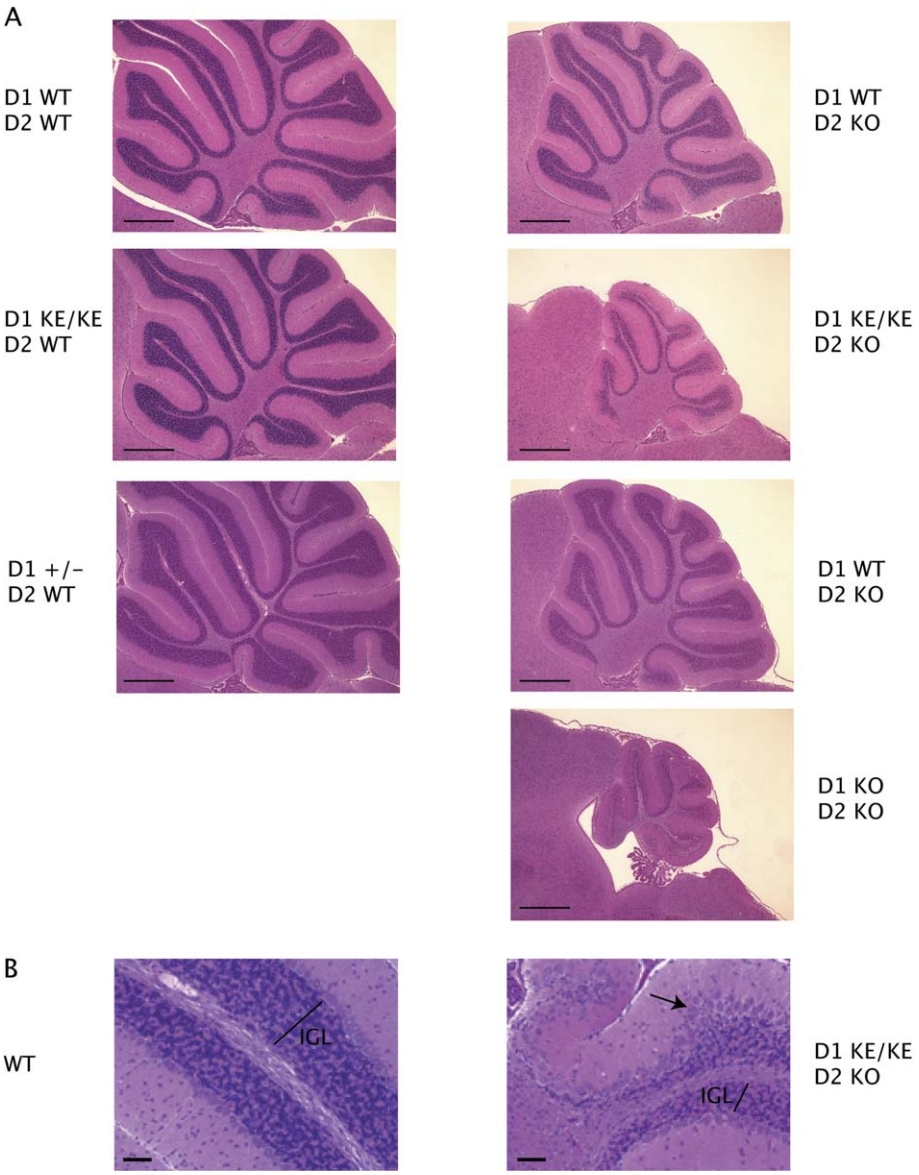

Figure 5. Impaired cerebellar development in *cyclin D1$^{KE/KE}$;cyclin D2$^{-/-}$* mice

A: Abnormal cerebellar development in *cyclin D1$^{KE/KE}$;cyclin D2$^{-/-}$* compound mutant animals. Sagittal sections of cerebella collected from 17-day-old mice were stained with hematoxylin and eosin. The cerebella of *cyclin D1$^{KE/KE}$;cyclin D2$^{-/-}$* animals have reduced numbers of folia and display profoundly reduced cellularity in the inner granular layer (IGL). The cerebella of a *cyclin D1$^{-/-}$;cyclin D2$^{-/-}$* compound mutant animal and its control littermates are shown for comparison. Scale bar, 0.5 mm.

B: Higher magnification of **A** showing abnormal layering of Purkinje cells (arrow) and severely hypoplastic IGL in the *cyclin D1$^{KE/KE}$;cyclin D2$^{-/-}$* cerebellum. Scale bar, 50 μm.

analysis was carried out using horseradish peroxidase-conjugated donkey anti-rabbit or donkey anti-mouse (Jackson ImmunoResearch Laboratories).

Cerebellar, retinal, and mammary gland histology and retinal biochemistry

Tissues were fixed in Bouin's fixative (Sigma) and were processed as described (Sicinski et al., 1995). To obtain neural retinas for biochemical analyses, eyes were dissected from postnatal day 1 pups and placed in PBS. The neural retinas were microdissected away from the lens, snap frozen, and stored at −80ºC until use.

Electroretinographic analyses

Electroretinogram analysis was carried out as described previously (Sicinski et al., 1995).

Mammary gland whole mounts

Inguinal mammary glands were dissected and spread onto glass slides. Staining was performed as described previously (Wang et al., 1990). Briefly, samples were fixed in a 1:3 mixture of glacial acetic acid:ethanol, hydrated, and stained overnight in 0.2% carmine (Sigma) and 0.5% AlK$(SO_4)_2$, dehydrated in graded ethanol solutions, cleared in toluene, and stored in methyl-salicylate.

Tumor experiments

MMTV-ErbB-2 mice (strain TG. NK; Muller et al., 1988) were purchased from the Charles River Laboratories. *cyclin D1$^{KE/+}$* animals were crossed with *MMTV-ErbB-2* animals to eventually generate *cyclin D1$^{KE/KE}$;MMTV-ErbB-2* (n = 31) and *cyclin D1$^{+/+}$;MMTV-ErbB-2* (n = 22) females. Females were monitored biweekly for tumor formation by palpation. Adenocarcinomas arising in *cyclin D1$^{+/+}$;MMTV-ErbB-2* females were excised and confirmed histologically.

RT-PCR

First strand synthesis was carried out using the iScript kit (BioRad) according to the manufacturer's instruction. PCR was carried out using primers specific for the *MMTV-ErbB-2* transgene as described previously (Yu et al., 2001).

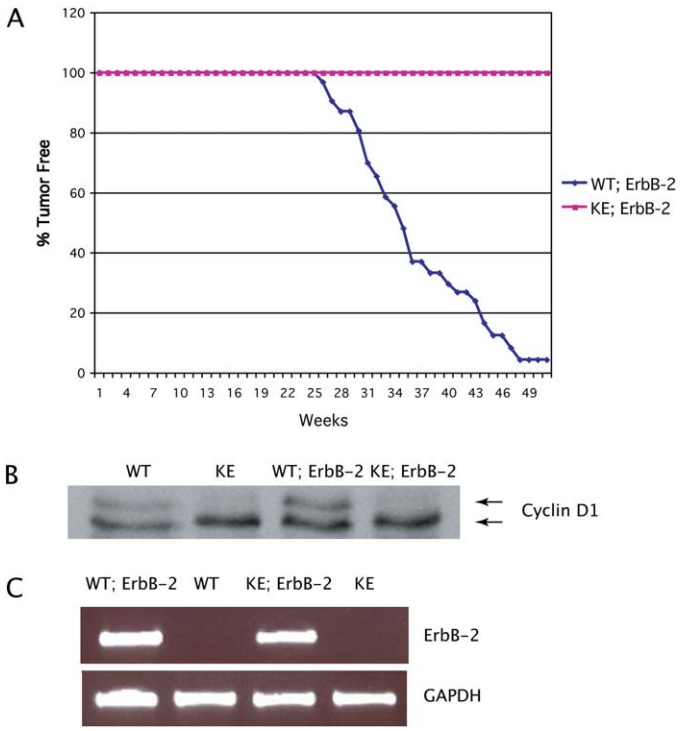

Figure 6. Resistance to mammary carcinomas in *cyclin D1$^{KE/KE}$;MMTV-ErbB-2* animals

A: Percentage of tumor-free mice among *cyclin D1$^{+/+}$;MMTV-ErbB-2* (n = 22) and *cyclin D1$^{KE/KE}$;MMTV-ErbB-2* (n = 31) females.

B: Expression of cyclin D1 in the mammary glands of nontransgenic wild-type (WT) and *cyclin D1$^{KE/KE}$* (KE) females, as well as in transgenic *cyclin D1$^{+/+}$;MMTV-ErbB-2* (WT; ErbB-2) and *cyclin D1$^{KE/KE}$;MMTV-ErbB-2* (KE; ErbB-2) females, was verified by Western blotting.

C: Verification of the *MMTV-ErbB-2* transgene expression. Pooled mammary glands from four or five animals of the indicated genotypes (see **B**) were used as a source of RNA. The expression of the transgene was analyzed by RT-PCR using transgene-specific primers (Yu et al., 2001). GAPDH levels were assessed as an internal control.

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