The LxCxE pRb Interaction Domain of Cyclin D1 Is Dispensable for Murine Development

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Abstract

Cyclin D1 is a multifunctional, tumor-associated protein that interacts with pRb via a conserved LxCxE motif, activates a kinase partner, directs the phosphorylation of pRb, activates cyclin E–cyclin-dependent kinase 2 (cdk2) by titrating Cip/Kip cdk inhibitors, and modulates the activity of a variety of transcription factors. It is thought that some of the proproliferative function of cyclin D1 is exerted by LxCxE-dependent binding to the pRb pocket domain, which might interfere with the ability of pRb to repress transcription by recruiting cellular chromatin remodeling proteins to E2F-dependent promoters. To test the importance of the LxCxE domain in vivo, we have generated a “knock-in” mouse by replacing the wild-type cyclin D1 gene with a mutant allele precisely lacking the nucleotides encoding the LxCxE domain. Analysis of this mouse has shown that the LxCxE protein is biochemically similar to wild-type cyclin D1 in all tested respects. Moreover, we were unable to detect abnormalities in growth, retinal development, mammary gland development, or tumorigenesis, all of which are affected by deleting cyclin D1. Although we cannot exclude the presence of subtle defects, these results suggest that the LxCxE domain of cyclin D1 is not necessary for function despite the absolute conservation of this motif in the D-type cyclins from plants and vertebrates.

Introduction

Cyclin D1 is one group of cyclins expressed during the G1-S phase transition of the cell cycle. The most well understood function of these cyclins (including D1, D2, D3, E1, and E2) is to bind to and activate cyclin-dependent kinase (cdk) partners. The cyclin-cdk complexes phosphorylate cellular targets, including the retinoblastoma protein, pRb, and its family members p107 and p130. Inactivation of pRb by phosphorylation leads to derepression of the E2F family of transcription factors, transcription of S-phase genes, and progression through the cell cycle (1). Furthermore, cyclin D–cdk complexes are able to titrate the cdk inhibitors p21Cip1 and p27Cip1 away from cyclin E–cdk2 complexes, allowing their activation and subsequent phosphorylation of target proteins (2–4).

In addition to these cdk-dependent functions, several kinase independent functions of D-type cyclins have recently been described. In particular, cyclin D1 can interact with and alter the activity of a number of transcription factors, including estrogen receptor, androgen receptor, thyroid hormone receptor, v-Myb, B-Myb, Stat3, DMP-1, BETA2/neuroD, PPARγ, and CAAT/enhancer binding protein (5, 6).

Consistent with its growth promoting functions, cyclin D1 overexpression, due to gene amplification or translocation, has been described in a wide variety of adult cancers (7–10). In particular, amplification of the cyclin D1 gene accounts for 15% to 20% of mammary carcinomas (9, 11, 12) with cyclin D1 protein overexpression found in >50% of cases (13–16).

Mouse models have further emphasized the importance of cyclin D1 during both development and tumorigenesis. Cyclin D1 knockout mice are small, show a low frequency of early lethality, and exhibit a still uncharacterized neurologic impairment manifested by a leg-clasping phenotype. In spite of these overt features, a surprisingly limited number of tissue-specific defects were reported, which include hypoplastic retinas and a profound defect in the ability of the mammary epithelium to proliferate in response to pregnancy (17, 18). Further, cyclin D1 was shown to be a critical downstream target in mammary tumors induced by mouse mammary tumor virus (MMTV)-driven ras and neu oncogenes but not in those tumors originated in MMTV–wnt-1 and MMTV-nergy transgenic mice (19).

In an attempt to separate the kinase-dependent and the kinase-independent functions of cyclin D1, we have recently generated a knock-in mouse, in which a lysine-to-glutamic acid substitution at amino acid position 112 of cyclin D1 was introduced (20). Whereas this mutant can still bind cdk4 and cdk6, it is unable to activate the catalytic activity of the partner kinase or transform primary rodent cells (21, 22). Unlike the conventional knockout models, the development of several cyclin D1–dependent compartments, including the retinas and the mammary glands, proceeds relatively normally in the knock-in animals. In addition, we showed that this “kinase-deficient” cyclin D1, when bound to cdk4, is able to titrate p27Cip1 and thereby activates cyclin E–cdk2 complexes at least in the retinas. Strikingly, knock-in mice were resistant to breast cancer initiated by ErbB-2 (20). These results showed a differential requirement for cyclin D1–cdk4/cdk6 kinase activity during development and tumorigenesis and underscore the need to understand the biology of cyclin D1 and its various functional domains in these processes.

One unique feature of D-type cyclins in vertebrates and plants is the presence of a conserved LxCxE sequence in their N-termini (23, 24). Interestingly, viral oncoproteins from adenovirus (E1A), human papilloma virus (E7), and SV40 (large T antigen) also contain LxCxE domains that mediate the binding and inactivation of pRb. In fact, these oncoproteins depend completely upon the integrity of the LxCxE domain for their function: deletions or point mutations that disrupt the LxCxE domain abolish the transforming function of these oncoproteins, whereas substitutions of the LxCxE motif do not.

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mutations of this domain render the oncoproteins unable to inactivate pRB and transform cells (25–32). It has been thought that the D-type cyclins may exert some of their function by binding to pRB in a manner analogous to the viral oncoproteins. Thus, the viral oncoproteins may have co-opted a method of pRB interaction used by D cyclins to recognize pRB as a substrate for associated kinases. Alternatively, the LxCXE domain of D-type cyclins may compete with other cellular proteins that associate with pRB through the LxCXE binding pocket. Indeed, cyclin D1 can compete with E1A for pRB binding (33). Finally, it is formally possible that the pRB family proteins sequester cyclin D1, limiting its ability to drive proliferation under some circumstances (33).

Initial experiments suggested that despite the extremely high level of conservation, the requirement of the D-type cyclins for an intact LxCXE domain may differ. Point mutations in the LxCXE domains of cyclins D1 (C7G) and D2 (E7K) profoundly diminished their ability to bind pRB in vitro (33, 34). Interestingly, however, cyclin D1 C7G was still able to partially abrogate pRB-induced growth arrest and senescence in a tissue culture model (33). In contrast, cyclin D2 E7K was unable to direct the efficient phosphorylation of pRB or reverse pRB-mediated growth arrest as measured by colony formation, whereas wild-type cyclin D2 efficiently phosphorylated pRB and reversed the pRB arrest, allowing colony growth (34). Unfortunately, these results were difficult to compare because the assays differed and the possibility that the point mutants still retained partial function. More recent work from our laboratory analyzed the function of mutant cyclins D1 and D2 that were exactly lacking the LxCXE domain (ΔLxCXE). These data indicated that both mutant cyclins were able to direct the phosphorylation of pRB in vitro and in transient transfections. However, cyclin D2 ΔLxCXE failed to abrogate pRB function in G1 arrest and senescence assays. In contrast, cyclin D1 ΔLxCXE was able to function in a similar manner to wild-type cyclin D1 in all assays tested (21). Together, these data suggest that the LxCXE domain may be part of the mechanism by which the D-type cyclins recognize their substrates and may explain the proclivity of this class of cyclins to target pRB for phosphorylation. In addition, cyclin D2 may be critically dependent on an intact LxCXE domain, whereas cyclin D1 may not require this domain for biological activity.

In addition to the D-type cyclins and viral oncoproteins, a variety of cellular proteins also bind pRB through LxCXE domains. These cellular proteins include histone deacetylases 1 and 2, the ATP-dependent chromatin remodeling proteins BRG-1 and hBrm, RBP1, CtIP, and a histone H3 methyltransferase, although this interaction is indirect. All of these proteins have been implicated in the ability of pRB to actively repress E2F-mediated transcription (35–41). The large and diverse group of proteins that rely on LxCXE domains to bind pRB would seem to suggest that elimination of this interaction might compromise pRB function. Indeed, it has been reported that pRB lacking the LxCXE-binding domain is unable to establish a permanent cell cycle exit in differentiated cells (42). However, a deleterious effect on the ability of this pRB to enforce G1 cell cycle arrest is less clear. Interestingly, although reports agree that mutation of the LxCXE binding cleft in pRB seems to have little effect on its ability to bind and inactivate E2F, they disagree about whether such mutations compromise its ability to actively repress transcription (43, 44). Given the conservation of LxCXE domains, it seems somewhat surprising that abrogating the function of the binding pocket has such a limited effect on pRB function.

The various proteins that interact with pRB therefore have different requirements for intact LxCXE binding. As the LxCXE domain is so highly conserved in the D-type cyclins, we wanted to determine whether this motif is required for cyclin D1 function. Prior experiments examining cyclin D1 and other pRB-binding proteins have been done in vitro using overexpression systems. Such high-level ectopic expression may drive cell cycle progression in a nonphysiologic manner and thus obviate any requirement for the LxCXE domain of cyclin D1. Furthermore, the previous studies have not addressed the role of cyclin D1's LxCXE domain during differentiation and development, functions that are apparent from studies of the knock out and knockin models of cyclin D1 (17, 18, 20). To assess the requirement for the LxCXE domain of cyclin D1, we have engineered a knock-in mouse that expresses cyclin D1 precisely lacking the LxCXE domain. In contrast to the cyclin D1 knockout mouse, which has several significant tissue-specific defects, the ΔLxCXE mouse is virtually normal in all respects tested, indicating that an intact LxCXE domain does not contribute overtly to cyclin D1 function in vivo.

Materials and Methods

Engineering of cyclin D1ΔLxCXE/ΔLxCXE mice. Genomic fragments of the cyclin D1 locus have been described (18). To generate the targeting vector to knock-in the ΔLxCXE allele, we did PCR using the upstream primer 5’-CCACGGCGCCGCGATGACGTTGGAAGACATCCGCGCGGC-3’, which lacks the 15 nt of exon 1, encoding the LxCXE domain, and the downstream primer 5’-CCCCAGACGCTGACGGCGCTTCTCT-CCCGGG-3’. The resulting ~400 bp PCR product was subcloned using standard methods into an ~9 kbp XbaI to SalI fragment of the cyclin D1 genomic locus in the Litmus 29 vector (NE Biolabs). Exon 1 was sequenced at an intermediate step to ensure that the 15 nt corresponding to the LxCXE domain had been exactly removed. Finally, a LoxP-neo8-LoxP drug resistance cassette (a kind gift of Dr. T.J. Ley) was subcloned into the unique EcoRI site to generate the targeting vector as shown in Fig. 1A. In the gene-targeting construct, the 5’ arm of homology consisted of a 4.4 kbp XbaI-EcoRI fragment, whereas the 3’ arm consisted of a 4.2 kbp EcoRI-MfeI fragment. This vector was linearized with MfeI and subsequently electroporated into J1 ES cells. Homologous recombination of G418-resistant clones was confirmed by Southern blotting after HindIII digestion of genomic DNA and probed with a 900-bp XmnI 3’ external probe (probe B, Fig. 1A). A total of 11 of 48 clones were shown to have undergone homologous recombination at the cyclin D1 locus. Five of these clones were further characterized. DNA from these clones was digested with StuI and Southern blotted using a 600 bp Stul-XbaI 5’ external probe (probe A, Fig. 1A). After karyotype analysis, two clones were injected into C57BL/6 or BALB/c mouse blastocysts, which were implanted into pseudopregnant ICR females. The resulting chimeras were mated to C57BL/6 or BALB/c mice (depending on the type of donor blastocyst), and the progeny were screened for germline transmission of the ΔLxCXE allele by the inheritance of agouti coat color and PCR genotyping. PCR across exon 1 was carried out using the primers 5’-GCGCGACGGAGACACGACGCGG-3’ and 5’-CTTCTCGGAGCTCACGC-3’. Amplifying this region produces a 245-bp product from the wild-type allele and a 230-bp product from the ΔLxCXE allele. To eliminate the neo8 drug resistance cassette, cyclin D1ΔLxCXE-inl-/+ [ΔLxCXE-inl describes a ΔLxCXE allele that contains the LoxP-neo8-LoxP (inl) cassette described below; ΔLxCXE alleles which have undergone Cre-mediated removal of the LoxP-neo8-LoxP cassette will be called Δl] animals were interbred to generate the Δl-/+ animals. Cre animals were mated to C57BL/6 or BALB/c mice. The progeny of this cross were screened for cyclin D1Δl-/+ animals that had inherited the ne-cre transgene. Excision of the LoxP-neo8-LoxP cassette was confirmed in these animals by PCR. The cyclin D1Δl-/+ animals were then interbred to generate...
homzygous cyclin D1ΔLxcE animals. These animals were maintained in a mixed genetic background.

Immunodetection, immunoprecipitation, and in vitro kinase assays.

For Western blotting, immunoprecipitation, and kinase assays, tissues were dissected and either lysed immediately or snap frozen and then stored at \(-80°C\) for later use. Tissues were subjected to Dounce homogenization in ELB [50 mmol/L NaCl, 50 mmol/L HEPES (pH 7.0), 0.1% NP40, 1 μg/mL aprotinin (Sigma), 50 μg/mL Pefabloc (Boehringer Mannheim), 10 μg/mL Leupeptin (Sigma), 1 mmol/L DTT, 100 mmol/L sodium orthovanadate, 10 mmol/L b-glycerophosphate, 10 mmol/L sodium fluoride, and 2 mmol/L sodium PPI] and Western blotting was carried out essentially as described (21). For immunoprecipitation and kinase assays, tissues were subjected to Dounce homogenization in D-IP buffer [50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 2.5 mmol/L EGTA, 0.1% Tween 20, 10% glycerol, 1 μg/mL aprotinin (Sigma), 50 μg/mL Pefabloc (Boehringer Mannheim), 10 μg/mL Leupeptin (Sigma), 1 mmol/L DTT, 100 mmol/L sodium orthovanadate, 10 mmol/L b-glycerophosphate, 10 mmol/L sodium fluoride, and 2 mmol/L sodium PPI], and 150 μg of protein lysate was incubated with bead-conjugated antibody for 1 h at 4°C. After incubation, the beads were allowed to settle by gravity for 15 min and were washed thrice with D-IP buffer. For IP-Westerns, these samples were boiled in SDS sample buffer and separated by SDS-PAGE followed by immunodetection essentially as described (21). For IP-kinase assays, the beads were additionally washed in 1× kinase reaction buffer [50 mmol/L HEPES (pH 7.2), 10 mmol/L MgCl2, 5 mmol/L MnCl2, 1 mmol/L DTT] and incubated in 30 μL of kinase reaction mix [1× kinase reaction buffer, 100 mmol/L ATP, 1 mmol/L DTT, 500 ng histone H1 (H111), 10 μCi γ32P-ATP] for 30 min at 37°C. Samples were boiled in SDS sample buffer and separated by SDS-PAGE. The gels were stained with Coomassie blue, dried, and exposed to film.

Antibodies used in this study included anti-cyclin D1 (72-13G, Santa Cruz; 1:800), anti-cyclin D2 (M-20, Santa Cruz; 1:1,000), anti-cyclin D3 (C-16, Santa Cruz; 1:1,000), anti-cdk4 (C-22, Santa Cruz; 1:2,000), anti-cdk6 (Ab-2, Neomarkers; 1:200), anti-cdk6 (C21, Santa Cruz; 1:1,000), anti-p27 (anti-Kip1/p27, Transduction Laboratories; 1:250), and anti-pRb (Ab-245, PharMingen; 1:100). Immunoprecipitations were carried out using agarose-conjugated anti-cyclin D1, anti-cdk4, and anti-cyclin E (72-13G, C-22, and M-20, respectively; Santa Cruz) and anti-cdk2 (M-2; Santa Cruz) bound to protein A–sepharose (Amer sham Biosciences). Immunoblot analysis was carried out using horse-radish peroxidase–conjugated donkey anti-rabbit or donkey anti-mouse antibodies (Jackson ImmunoResearch Laboratories; 1:2,000).

Cerebellar/retinal histology and retinal biochemistry. Cyclin D1ΔLxcE; cyclin D2ΔLxcE (46) double heterozygous animals were crossed to generate cyclin D1ΔLxcE; cyclin D2ΔLxcE mice. The progeny were sacrificed at 17 days postpartum, and the cerebella were dissected, fixed in Bouin’s fixative (Sigma), paraffin embedded, and stained with H&E.

Similarly, eyes were dissected from 1-month-old animals and subject to the same procedures described above for the cerebella. To obtain retinas for biochemical analysis, eyes were removed from postnatal day 1 pups. The retinas were microdissected, snap frozen, and stored at \(-80°C\) until use.

Mammary gland whole mounts. Inguinal mammary glands were dissected and spread onto glass slides. Staining was done as described previously (18). Briefly, samples were fixed in a 1:3 mixture of glacial acetic acid/ethanol, hydrated, stained overnight in 0.2% carmine (Sigma) and 0.5% AlK(SO4)2, dehydrated in graded ethanol solutions, cleared in toluene, and stored in methylsalicylate.

Results

Targeted replacement of the wild-type cyclin D1 gene. Previous reports using cyclin D1 C7G and cyclin D1 ΔLxcE have shown that mutation or deletion of the LxcE domain of cyclin D1 does not impair its function as a regulator of pRb when overexpressed in tissue culture systems (21, 33). However, these studies may be compromised by the high level and aberrant timing of cyclin D1 expression that results from transient transfection. To assess the requirement for the LxcE domain in vivo, we have genetically engineered a mouse to express cyclin D1 ΔLxcE from...
the endogenous cyclin D1 locus in a manner that is expected to preserve the level and timing of cyclin D1 expression present in wild-type animals. One wild-type allele of the CCND1 gene was replaced in ES cells by homologous recombination using a targeting vector described in the Materials and Methods and shown in Fig. 1A. G418 resistant ES cell clones were screened by Southern blotting (Fig. 1B) and two independent clones were injected into donor blastocysts to generate chimeric cyclin D1<sup>hal</sup> mice. As described in the Materials and Methods, a series of matings was done to eliminate the drug cassette and ultimately resulted in the generation of heterozygous and homozygous animals bearing the ALxCxE allele as depicted in Fig. 1A (bottom).

The genotype of these animals was confirmed by PCR analysis of tail DNA (Fig. 1C), which indicated that all genotypes were obtained at the expected Mendelian ratios.

**Expression of cyclin D1 ΔLxCxE and other cell cycle regulatory proteins.** To assess the expression of the cyclin D1 ΔLxCxE protein, lysates from various tissues of adult mice were prepared and immunoblotted with anti–cyclin D1 antibodies (Fig. 2A). The expression levels of wild-type cyclin D1 protein and the ΔLxCxE protein were virtually indistinguishable in lung, heart, kidney, liver, and spleen. Further, we note that both wild-type and ΔLxCxE cyclin D1 migrate as a doublet. Although the difference between these two species of cyclin D1 is currently unclear, neither is affected by the deletion of the LxCxE motif. Moreover, the electrophoretic mobility of the ΔLxCxE protein is slightly greater than wild-type cyclin D1, indicating its smaller size. In addition, the normal levels of ΔLxCxE expression in these tissues suggest that the genetic engineering at the cyclin D1 locus had very little, if any, effect on the normal expression of the mutant cyclin D1 protein. To examine the overall levels of cell cycle regulators in the developing animal, lysates were prepared from E14.5 and E17.5 embryos and analyzed by immunoblotting (Fig. 2B). Again, cyclin D1 and ΔLxCxE levels were similar in age-matched littermates. Additionally, the levels of cyclins D2 and D3, the partner kinases cdk4 and cdk6, and the cdk inhibitor p27<sup>Kip1</sup> were similar in these embryos. The levels of pRb in wild-type, cyclin D1<sup>−/−</sup>, and cyclin D1<sup>Δ/L</sup> embryos were equivalent. In addition, the abundance of hyperphosphorylated pRb was equivalent in these embryos (Fig. 2C), demonstrating that in whole embryo lysates there was no gross defect in the ability of the D-type cyclins to activate their cdk partners and phosphorylate pRb and presumably other targets. However, as shown by the normal phosphorylation of pRb in cyclin D1<sup>−/−</sup> embryos (Fig. 2C), the majority of this activity may arise from cyclins D2 and D3, rather than from wild-type or ΔLxCxE cyclin D1.

**Appearance of the cyclinD1<sup>Δ/Δ</sup> mice.** Cyclin D1<sup>−/−</sup> animals have several tissue-specific phenotypes, including a significant reduction in size, hypoplastic retina, defective expansion of the mammary epithelium in response to pregnancy, and partially penetrant malocclusion (17, 18). In contrast, the cyclin D1<sup>Δ/L</sup> mice developed normally and showed no overt phenotypes upon necropsy and histopathologic analysis. Indeed, at 3 weeks of age,
the time when the sizes of wild-type and cyclin D1\(^{-/-}\) animals differ the most (18), the weights of wild-type and cyclin D1\(^{\Delta/\Delta}\) littermates are not significantly different (Fig. 3A and B). Figure 3B shows weight measurements from a group of male mice at 3 weeks of age, but similar results were obtained when females of the corresponding genotypes and age were analyzed (not shown). In addition, the premature mortality observed in some cyclin D1\(^{+/+}\) mice (18) was not observed in the cyclin D1\(^{\Delta/\Delta}\) mice. When a cyclin D1\(^{+/+}\) mouse is lifted by the tail, it exhibits a "clasping" phenotype and draws its rear limbs toward its body (18); in contrast, the wild-type reflex is to splay the legs outward. We did not observe the clasping phenotype in the cyclin D1\(^{\Delta/\Delta}\) mice (Fig. 3B). In addition, a significant percentage of cyclin D1\(^{-/-}\) mice exhibit malocclusion, or misalignment of the teeth (17). This defect was never seen in the cyclin D1\(^{\Delta/\Delta}\) mice.

**ΔLxCxE supports normal development of the retina and mammary gland.** It has been reported that cyclin D1 is essential for the normal development of the neural retina. In cyclin D1-deficient animals, the number of cells in each layer of the retina is profoundly reduced, resulting in a thin, hypoplastic retina (17, 18). To determine whether the ΔLxCxE allele was competent to support proper development of the retina, the histologic appearance of retinas from cyclin D1\(^{\Delta/\Delta}\) mice and their wild-type littermates was examined. As shown in Fig. 4A, no differences between wild-type and Δ/Δ retinas were detected, indicating that the loss of the LxCxE domain does not affect normal retinal development.

In addition to its role in retinal development, cyclin D1 also plays a role in the development of the mammary gland. Cyclin D1-deficient animals develop a normal mammary epithelial tree during sexual maturation. However, during pregnancy, cyclin D1-deficient females display a defect in proliferation of the mammary epithelium in response to hormonal signals, leading to reduced lobuloalveolar development (17, 18). To address the requirement for the LxCxE domain of cyclin D1 in this process, mammary glands from virgin and 1-day postpartum cyclin D1\(^{-/-}\) and cyclin D1\(^{\Delta/\Delta}\) females were removed and whole mounted. We found that the mammary glands from cyclin D1\(^{\Delta/\Delta}\) females underwent normal lobuloalveolar development during pregnancy (Fig. 4B).

We have recently reported that despite supporting relatively normal mammary gland development, the K112E mutant of cyclin D1 precludes mammary tumor development in mice bearing the MMTV-ErbB2 oncogene (20). This indicates that subtle changes in cyclin D1 structure can significantly alter tumorigenesis with minimal effect on development. However, our analysis of a small cohort of MMTV-ErbB2; cyclin D1\(^{\Delta/\Delta}\) females showed no differences in number or timing of mammary neoplasms (data not shown), suggesting that the LxCxE domain of cyclin D1 is not required for ErbB2-dependent mammary tumors. Taken together, these results show that the cyclin D1\(^{\Delta/\Delta}\) mice are phenotypically normal in all tested respects.

**Expression and function of G1 cell cycle regulatory proteins in the retina.** The neural retina is one of the few tissues in the mouse that is profoundly affected by the absence of cyclin D1. This phenotype is thought to be a consequence of the almost exclusive expression of cyclin D1 in the retina; little or no expression of cyclins D2 and D3 is observed in retinas of wild-type mice (47). To determine whether there were any effects in the retina due to

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**Figure 4.** Normal development of the retina and mammary gland in knock-in mice. A, histologic examination of H&E stained retinal slices shows little difference between wild-type and knock-in mice; in contrast, the retinas of cyclin D1 knockout mice are profoundly hypoplastic. GL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Note that the pigment layer has become dislodged in the knockout sample. The results were confirmed in at least three animals of the corresponding genotypes. B, mammary glands from virgin and day 1 postpartum wild-type and knock-in females were examined by whole-mount. No significant differences were observed in the ability of the mammary epithelium to undergo proliferation in response to pregnancy-induced signals. The results were confirmed in at least three individual animals of each genotype.
deletion of the LxCxE domain, we examined the expression of various G1-phase regulatory proteins in retina lysates from 1-day-old pups (Fig. 5A). Similar to the situation in whole-embryo lysates, the levels of cyclin D1, cdk4, cdk6, and p27kip1 were nearly identical in wild-type and cyclin D1^Δ/Δ retina lysates. Cyclin D2 was virtually undetectable in these lysates, and there were extremely low levels of cyclin D3. Examination of pRb in cyclin D1^−/− retina lysates showed an overwhelming preponderance of the hypophosphorylated form in retinas from cyclin D1 knock-out mice (Fig. 5B). In contrast, a large proportion of the pRb in the ΔLxCxE and wild-type lysates was present in the hyperphosphorylated, slower-migrating forms (Fig. 5B), suggesting that deletion of the LxCxE domain of cyclin D1 had little or no effect on the ability of the mutant protein to phosphorylate pRb.

To determine the ability of cyclin D1 and ΔLxCxE to form complexes, we immunoprecipitated cyclin D1 or cdk4 from retina lysates and immunoblotted for partner proteins (Fig. 5C). Cyclin D1 and ΔLxCxE were able to bind equivalent amounts of cdk4 and p27kip1. Conversely, cdk4 was able to bind equivalent levels of either cyclin D1 or ΔLxCxE, and p27kip1 (Fig. 5C). Based on these results, we expected that the ability of ΔLxCxE to titrate p27kip1 would be similar to that of wild-type cyclin D1, resulting in normal cyclin E–cdk2 kinase activity. To test this, we immunoprecipitated cyclin E and cdk2 complexes and tested their ability to phosphorylate HH1 in vitro. It has been previously reported that the absence of cyclin D1 led both to a reduction in p27kip1 levels and inhibition of cyclin E–associated kinase activity, consistent with the idea that the remaining p27kip1 binds and inhibits cyclin E–cdk2 complexes (48, 49). In contrast, cyclin E and cdk2 complexes immunoprecipitated from ΔLxCxE and wild-type retina lysates had similar abilities to phosphorylate HH1 (Fig. 5D), demonstrating that the ΔLxCxE protein retains the ability to activate cyclin E–cdk2 complexes via titration of p27kip1. Together with the observed normal morphology of the ΔLxCxE retinas, these results show that the function of the ΔLxCxE protein was no different than the function of wild-type cyclin D1 in the developing mouse eye.

The LxCxE domain of cyclin D1 is not required for cerebellar development. It has been previously shown that the combined ablation of cyclin D1 and cyclin D2 can lead to severely retarded cerebellar development (46). Further, we showed that this impairment could also be observed in cyclin D1^LxE/LxE; cyclin D2^−/− animals (20), indicating that the kinase activity of cyclin D1–containing complexes plays a role in driving cerebellar development. To test the role of the LxCxE domain of cyclin D1 during this process, we crossed cyclin D1^Δ/Δ mice with cyclin D2^−/− mice (46) and generated cyclin D1^Δ/Δ; cyclin D2^−/− animals (Fig. 6). When analyzed at 17 days of age, cerebella of cyclin D1^Δ/Δ; cyclin D2^−/− mice show a mild developmental impairment similar to the one that has been described in cyclin D2^−/− mice (46), indicating that the ΔLxCxE protein can still compensate for cyclin D2 functions in the cerebellum.

Discussion

In vitro experiments using point mutants and, more recently, deletions of the LxCxE domain have indicated that cyclin D1 may not require an intact LxCxE domain for function (21, 33). However, because these experiments were done with overexpressed proteins in nonphysiologic systems, the results may not reflect a physiologic requirement for this domain. To test the function of the LxCxE domain of cyclin D1 in vivo, we have generated a knock-in mouse engineered to express the ΔLxCxE allele from the endogenous Cyclin D1 locus. Our results show that the mutant allele was expressed at normal levels in various tissues and at various times during development. Phenotypically, we were not able to detect any abnormalities in the cyclin D1^Δ/Δ mice. Additionally, no differences in the ability of the ΔLxCxE protein to form complexes with its usual partner proteins were observed in the retina, one tissue that is profoundly affected by loss of cyclin D1. Phosphorylation of pRb in the retina was also normal, indicating that ΔLxCxE is
able to function similarly to wild-type cyclin D1 in terms of pRb inactivation. These results show that, although the LxCxE domain is conserved in all D-type cyclins in plants and vertebrates, its loss does not affect cyclin D1 function in the tissues examined.

It is important to note that the apparent lack of a developmental role for the LxCxE domain does not necessarily preclude a role for this domain in tumorigenesis. Indeed, in DT-40 lymphoma B cells, cyclin D1’s LxCxE domain does play a role in the regulation of the cell cycle. Overexpression of wild-type murine cyclin D1, but not D1 ΔLxCxE, in these cells was able to rescue a cell cycle delay caused by elimination of endogenous cyclin D1 by gene knockout (50). This dependence on LxCxE domain function may be cell-type specific or may perhaps be a consequence of the immortalization process. Further, we have recently shown that the cyclin D1 mutant K112E can direct nearly normal mammary gland development but cannot support ErbB2-dependent tumorigenesis (20). However, our initial studies of a small number of MMTV-ErbB2; cyclin D1ΔLΔA animals showed tumor onset and numbers similar to wild-type littermates, suggesting that the LxCxE domain of cyclin D1 is not required in this transgene-induced tumor system. We have also failed to find spontaneous tumors or other signs of increased proliferation in D1ΔLΔA animals, thus failing to support the suggestion that pRb might negatively regulate cyclin D1 by virtue of the LxCxE-domain interaction (33).

The data reported here certainly do not exclude the possibility of subtle defects not detected by the types of analyses used. Further, the LxCxE domain may function only in cells and tissues that express other D-type cyclins but cannot support ErbB2-dependent tumorigenesis (20). However, our initial studies of a small number of MMTV-ErbB2; cyclin D1ΔLΔA animals showed tumor onset and numbers similar to wild-type littermates, suggesting that the LxCxE domain of cyclin D1 is not required in this transgene-induced tumor system. We have also failed to find spontaneous tumors or other signs of increased proliferation in D1ΔLΔA animals, thus failing to support the suggestion that pRb might negatively regulate cyclin D1 by virtue of the LxCxE-domain interaction (33).

The data reported here certainly do not exclude the possibility of subtle defects not detected by the types of analyses used. Further, the LxCxE domain may function only in cells and tissues that express other D-type cyclins; the functions of the intact LxCxE domains of these cyclins may mask the lack of the domain in cyclin D1. Such a cryptic role for the LxCxE domain might be unmasked by the generation of compound mutant mice expressing the ΔLxCxE cyclin D1 protein in the absence of cyclin D2, cyclin D3, or both. However, at least in the cerebellum, our data suggest that ΔLxCxE domain can support normal development in the absence of cyclin D2 to an extent equivalent to that of the wild-type cyclin D1.

Notably, in vitro evidence suggesting a lack of a role for cyclin D1’s LxCxE domain stands in contrast to data reported using similar cyclin D2 mutants (21, 33, 34) that strongly support a role for the LxCxE domain in cyclin D2 function. Such results further predict that mice bearing a cyclin D2 ΔLxCxE allele would be subject to at least some of the developmental defects observed in mice with a complete loss of cyclin D2. Experiments using such compound mutant animals would also aid in the evaluation of a role for LxCxE-mediated association with pRb in differentiation control and cell-type specific functions of different D-type cyclins that may be masked by functional redundancy in cyclin D1 ΔLxCxE mice. In any case, the data reported here strongly suggest that tissues sensitive to cyclin D1 loss rely, in large part, only on functions of cyclin D1 that are not compromised by LxCxE loss, such as pRb phosphorylation, Cip/Kip inhibitor titration, or transcription factor regulation.

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The research on animals has complied with all relevant federal guidelines and institutional policies.


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