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The Oncoprotein Kinase Chaperone *CDC37* Functions as an Oncogene in Mice and Collaborates with Both c-*myc* and Cyclin D1 in Transformation of Multiple Tissues

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***CDC37* encodes a 50-kDa protein that targets intrinsically unstable oncoprotein kinases including Cdk4, Raf-1, and v-*src* to the molecular chaperone Hsp90, an interaction that is thought to be important for the establishment of signaling pathways. *CDC37* is required for proliferation in budding yeast and is coexpressed with cyclin D1 in proliferative zones during mouse development, a finding consistent with a positive role in cell proliferation. *CDC37* expression may not only be required to support proliferation in cells that are developmentally programmed to proliferate but may also be required in cells that are inappropriately induced to initiate proliferation by oncogenes. Here we report that mouse mammary tumor virus (MMTV)-*CDC37* transgenic mice develop mammary gland tumors at a rate comparable to that observed previously in MMTV-cyclin D1 mice. Moreover, *CDC37* was found to collaborate with MMTV–c-*myc* in the transformation of multiple tissues, including mammary and salivary glands in females and testis in males, and also collaborates with cyclin D1 to transform the female mammary gland. These data indicate that *CDC37* can function as an oncogene in mice and suggests that the establishment of protein kinase pathways mediated by Cdc37-Hsp90 can be a rate-limiting event in epithelial cell transformation.**

Extracellular signals act to coordinate proliferation during the first gap (G_1) phase of the cell division cycle. These signals typically act through receptor tyrosine kinases to activate protein kinase signaling pathways that direct the expression of genes required for proliferation. Recent studies have implicated components of the *ras* pathway in regulating the expression of D-type cyclins, a central component of mitogen-dependent cell cycle entry (1, 41). Ras activation leads to engagement of the Raf/MEK/MAPK pathway (47, 60, 65, 70, 72), and each of these components is necessary and sufficient to induce cyclin D expression (1, 2, 21, 27, 41, 69). D-type cyclins are essential activator subunits of Cdk4 and Cdk6, and holoenzyme complexes of these kinases have been implicated in cell cycle entry through multiple mechanisms. Cyclin D-Cdk4 complexes directly phosphorylate retinoblastoma protein (Rb) and initiate inactivation of its growth suppressor function (9, 12, 20, 34, 36). In addition, cyclin D-Cdk4 complexes may contribute to the activation of cyclin E-Cdk2 by titrating the Cdk inhibitor $p27^{KIP1}$ from Cdk2 complexes (8, 19, 35, 45, 46, 55). Consistent with the central role of cyclin D in *ras*-dependent proliferation is the finding that Cdk4 inhibitors of the p16 class can inhibit *ras*-mediated proliferation in an Rb-dependent manner (30, 37, 41, 52).

The assembly of cyclin D-Cdk4 complexes is complex and appears to involve multiple steps, including a mitogen-dependent step (7, 8, 24, 34, 36). Previously, we cloned a mammalian homolog of the budding yeast and avian *CDC37* gene (4, 15) and demonstrated that $p50^{Cdc37}$ binds to Cdk4 and Cdk6 but not to Cdc2 and Cdk2 (58). In budding yeast, *CDC37* is an essential gene and is required for formation of Cdc28-Cln complexes through an unknown mechanism (14). We and others have demonstrated that mammalian Cdc37 assembles with Cdk4 in high-molecular-weight complexes that also contain the molecular chaperone Hsp90 (11, 25, 58). Molecular analysis revealed that the *CDC37* gene encodes the Hsp90-associated p50 protein (42, 58), previously seen in complexes with v-*src* (5, 6, 18, 66) and Raf (57) but whose identity was unknown. Cdc37 associates with Hsp90 independently of protein kinases and appears to function at least in part as a protein kinase-targeting subunit of Hsp90 (58). Genetic and biochemical data in several systems suggest that particular protein kinases are intrinsically unstable and their association with the Cdc37-Hsp90 chaperone is important for folding and/or activation of the targeted kinase (10, 14, 16, 38, 58, 71). Once Cdk4 is stabilized by the Cdc37 complex, it is released in a step that is not characterized and can then assemble with either inhibitors such as p16 or with cyclin D. Assembly with cyclin D requires a member of the p21 class of Cdk inhibitors, possibly in addition to a mitogen-dependent step (7, 24, 40).

CDC37 is expressed primarily in proliferative zones during embryonic development and in adult tissues, and its pattern of expression closely corresponded to that of cyclin D1 (58). Interestingly, *CDC37* is not expressed in several adult tissues including virgin mammary duct epithelial cells but, like cyclin D1, is induced during pregnancy, consistent with a positive role in proliferation (58). These data, coupled with the fact that *CDC37* is required for proliferation in budding yeast and *Drosophila* cells (10), suggest that *CDC37* expression may be required to support proliferation in those cells that are developmentally programmed to proliferate but may also be required in those cells that are inappropriately induced to initiate pro-

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liferation by oncogenes. If this were the case, then *CDC37* would be predicted to collaborate with transforming oncogenes. Standard tissue culture-based assays that measure oncogenic collaboration employ fibroblasts which already express high levels of Cdc37 (58), suggesting that this approach may not reveal the collaborative potential of Cdc37. Therefore, we sought to examine the effects of Cdc37 in vivo by targeting its expression to cells in the mammary gland and other tissues where it is normally not present in the adult animal. Mouse mammary tumor virus (MMTV)-*CDC37* transgenic mice were found to develop mammary gland tumors at a rate comparable to that observed in MMTV-cyclin D1 mice. Moreover, *CDC37* was found to collaborate with MMTV–c-*myc* in the transformation of multiple tissues, including mammary and salivary glands in females and testis in males, and with cyclin D1 in the mammary gland. In a parallel study (58a), we found that Cdc37 is absent from normal human prostate but is abundant in human prostate cancer. Interestingly, selective expression of *CDC37* in the prostate leads to hyperplasia in transgenic mice (58a). Taken together, these data indicate that Cdc37 can function as an oncogene in mice and suggest that the establishment of protein kinase pathways mediated by Cdc37-Hsp90 can be a rate-limiting event in epithelial cell transformation.

MATERIALS AND METHODS

Generation of transgenic mice. An MMTV-*CDC37* transgene was generated by cloning a *Xho*I fragment containing the 1.6-kb mouse *CDC37* open reading frame (ORF) into a plasmid containing an MMTV promoter, beta-globin splice sequences, and bGH polyadenylation sequences. The 4.63-kb transgene fragment was released from the plasmid by digesting with *Not*I/*Kpn*I and then purified. Transgene DNA was microinjected into male pronuclei of B6D2F1 mouse embryos in the Baylor College of Medicine transgenic core facility. Resulting pups were screened by Southern analysis of genomic DNA isolated from mouse tails digested with *Bam*HI. To establish lines of transgenic mice, founders were continuously mated with ICR mice. Nontransgenic littermates of heterozygous parents were used as controls. MMTV-*CDC37* heterozygous females were mated with MMTV–c-*myc* (Charles River Laboratory) or MMTV-cyclin D1 homozygous transgenic males (64). Both MMTV–c-*myc* and MMTV-cyclin D1 mice were on a inbred FVB genetic background. Resulting progeny carried either both transgenes (c-*myc*+*CDC37* or cyclin D1+*CDC37*) or a single transgene (c-*myc* or cyclin D1). Both groups of animals were monitored for tumor formation for comparison. For nontransgenic controls, MMTV-*CDC37* heterozygous females were crossed with nontransgenic FVB males. The copy number was determined by quantitative Southern blotting of mixtures of tail DNA from nontransgenic and transgenic mice, followed by phosphorimager analysis. This analysis gave 8 and 5 copies for the MMTV-Cdc37.1 and MMTV-Cdc37.2 lines, respectively.

Northern analysis. Total RNA was prepared from mouse tissues, separated on an 1% agarose gel, transferred to Hybond N+ (Amersham) membrane, and blotted with a ^{32}P-labeled *CDC37* cDNA probe to detect endogenous and transgene derived transcripts, or a 5′+3′ probe consisting of rabbit beta-globin splice site sequences and bovine polyadenylation signal DNA, which was used to detect only exogenous *CDC37* transcripts. Blots were stripped and reprobed with a GAPDH (glyceraldehyde-3-phosphate dehydrogenase) probe to control for RNA levels. In some experiments, blots were also probed with a c-*myc* cDNA probe provided by M. Cole.

Histology and immunohistochemistry. For histological analysis, mouse tissues were excised and fixed in 4% formaldehyde–phosphate-buffered saline PBS overnight at 4°C prior to being embedded in paraffin. Embedded tissues were sectioned at a thickness of 5 μm and stained with hematoxylin and eosin (H&E). For immunohistochemistry, 5-μm sections were stained with rabbit polyclonal affinity-purified Cdc37 antibodies or with anti-c-*myc* antibodies (NeoMarkers) as described previously (58).

Western blot analysis. Frozen tumor specimens were used for preparation of protein lysates by homogenization in NP-40 buffer (58), followed by centrifugation and determination of protein concentration by Bradford assays. For Western blotting, 200 μg of extract was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% PAGE gel and then transferred to nitrocellulose. Blotting was performed using polyclonal Cdc37 antibodies (58), Cdk4, Erk1 and Erk2, and c-*myc* antibodies from Santa Cruz or anti-phospho-ERK from New England Biolabs. Detection was accomplished by using horseradish peroxidase-conjugated secondary antibodies in combination with enhanced chemiluminescence (Amersham).

Whole-mount analysis. Inguinal fat pads were excised from the animals, spread on a glass surface and fixed in 10% formalin for 10 to 12 h, and washed in acetone for 48 h, followed by washing in 100 and 95% ethanol (EtOH) for 1 h each. Tissues were stained with hematoxylin for 12 h (0.3% [wt/vol] hematoxylin and 0.34% [wt/vol] FeCl in 0.06 N HCl–80% EtOH). Stained tissues were washed for 1 h in distilled water and increasing concentrations of EtOH (70 to 100%) and finally in xylene. Tissues were stored in glass vials, covered with methyl salicylate.

RESULTS

MMTV-*CDC37* transgenic mice. To assess the possible role of *CDC37* in promoting neoplastic transformation, transgenic mice expressing mouse *CDC37* under the control of the MMTV promoter (Fig. 1A) were generated. Two transgenic founders (Fig. 1B) were produced which transmitted the transgene to their progeny in a Mendelian fashion. Lines of transgenic animals (MMTV-*CDC37*.1 and MMTV-*CDC37*.2) were established by mating each founder with outbred ICR mice. Quantitative analysis of copy number revealed eight and five transgenes, respectively, for MMTV-Cdc37.1 and MMTV-Cdc37.2 strains (see Materials and Methods). The expression of Cdc37 was examined by Northern blotting, immunoblotting, and immunofluorescence, with an emphasis on tissues known to express MMTV-driven transgenes. *CDC37* mRNA was high in the lacrimal, mammary, and salivary glands, uterus, and testis, using both the *CDC37* cDNA (Fig. 1C) and transgene-specific regulatory sequences (5′+3′) (data not shown) as probes, compared to the low levels found in these tissues in nontransgenic animals. The levels of mRNA in the MMTV-*CDC37*.2 strain was ~50% of those in the MMTV-*CDC37*.1 line (data not shown), a finding consistent with the lower copy number. Consistent with this, immunoblot analysis revealed that the Cdc37 protein was undetectable in extracts from normal salivary and virgin mammary glands but was readily detectable in extracts from transgenic mice (Fig. 1D). We previously reported that Cdc37 sometimes migrates as a doublet by SDS-PAGE (58). In normal virgin mammary gland, the more slowly migrating form of Cdc37 is predominant, while the more rapidly migrating form is predominant in salivary tissue. Cdc37 is a phosphoprotein (7), and we have shown that it is phosphorylated by casein kinase in vitro at sites that are also modified in vivo (data not shown). Thus, these isoforms may reflect differential phosphorylation in different tissues. To quantitatively address Cdc37 levels relative to those found normally in cycling cells, we examined Cdc37 protein by immunofluorescence and compared the levels with that found in sites of known Cdc37 expression in vivo. Transgenic Cdc37 was found in the majority of epithelial cells in the salivary gland (Fig. 2A) and Leydig cells in the testis (see Fig. 5) but was not detected in these cell types in nontransgenic animals. In the virgin mammary gland, Cdc37 was present in ~30% of ductal epithelial cells (data not shown). Although *CDC37* mRNA appears to be quite abundant, when examined at the single cell level, the levels of Cdc37 protein in all three tissues examined was similar to that found in proliferative cells in the intestine and in cycling BALB/c fibroblasts in culture (Fig. 2C). It is possible that translational and/or posttranslational events may control the total level of Cdc37 achievable in these tissues.

Ectopic expression of *CDC37* in the mouse breast leads to transformation. MMTV-*CDC37* lines and control littermates were maintained as breeding colonies and monitored for developmental and transformation phenotypes for up to 2 years. Transgenic animals appeared normal at birth, and their growth was indistinguishable from their nontransgenic littermates. Their reproduction, number of pups per litter, and lactation in females were normal, although promiscuous male breast development was detected (see below).

Malignant transformation of the mammary gland or other

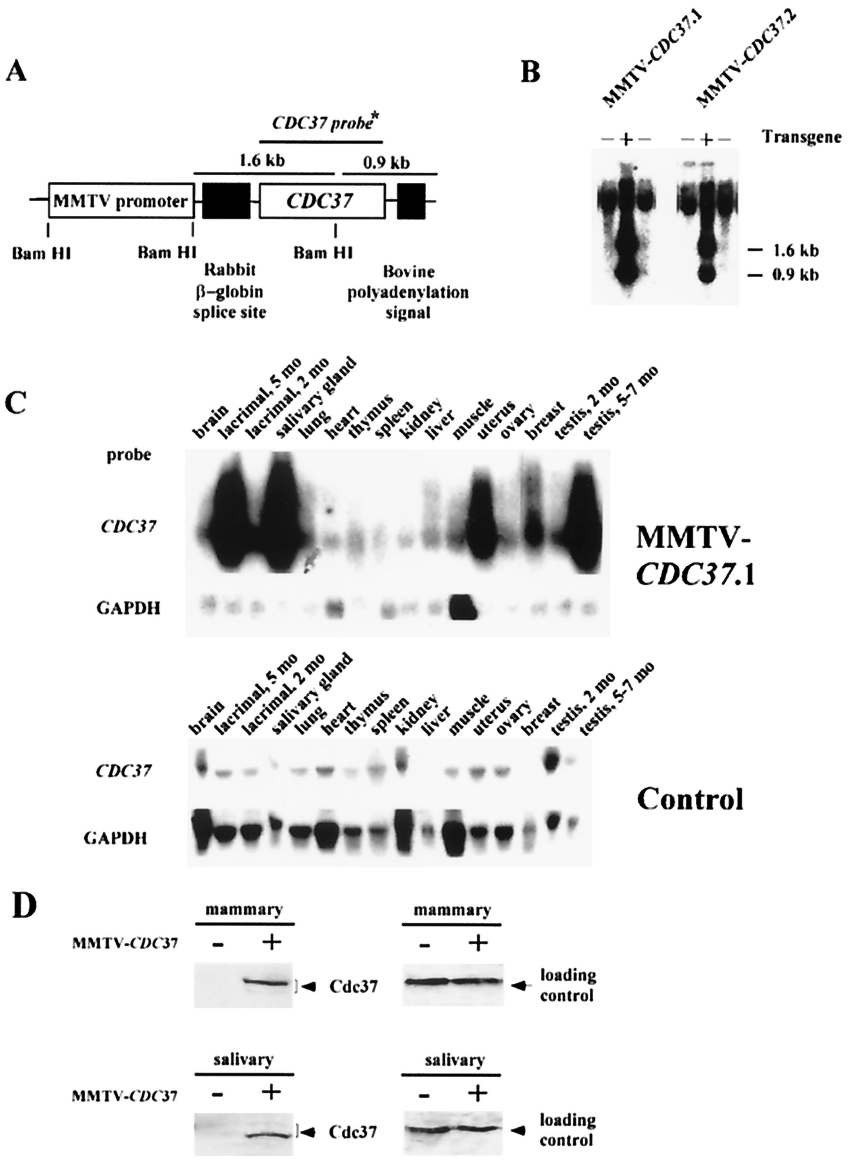


FIG. 1. Characterization of MMTV-*CDC37* transgene expression. (A) Structure of the construct used to generate MMTV-*CDC37* mice (see Materials and Methods for details). (B) Southern blot analysis of MMTV-*CDC37*.1 and MMTV-*CDC37*.2 transgenic lines. Tail DNA was digested with *Bam*HI prior to Southern analysis with the *CDC37* cDNA. The 1.6-kb band corresponds to the construct fragment containing rabbit β-globin splice site, and the 0.9-kb bands represent fragments containing bovine polyadenylation signal (see panel A). +, Mice containing the transgene; −, mice lacking the transgene. (C) Northern blot analysis of *CDC37* expression in tissues derived from transgenic and control animals. Total RNA was hybridized with the *CDC37* cDNA which detects both endogenous *CDC37* and the transgene derived message. The GAPDH probe (GAPDH ORF) is used as a loading control. Muscle tissue has intrinsically higher levels of GAPDH mRNA. (D) Immunoblot analysis of Cdc37 in nontransgenic and MMTV-Cdc37.1 mice. Tissue extracts (100 μg) from the indicated tissues were separated by SDS-PAGE and blotted with affinity-purified anti-Cdc37 antibodies. A nonspecific cross-reacting band observed with monoclonal antibody 9E10 was used as a loading control.

organs was not observed during first 1.5 years of life in *CDC37* transgenic animals. However, as MMTV-*CDC37* animals approached 18 months of age, a significant fraction of animals from both lines developed proliferative disorders, including mammary tumors and lymphomas (Table 1; Fig. 3 and 4A). Histopathological analysis indicated that mammary tumors were adenocarinomas and adenosquamous carcinomas (Fig. 3). By 22 months of age, 100% of MMTV-*CDC37*.1 females had developed tumors in the mammary or lymphoid compartments (Fig. 3A and 4A; Table 1). Mammary tumors arose as singular persistent masses adjacent to normal mammary epithelium. Mitotic figures were rare, indicative of slow-growing carcinomas. Histopathological examination also revealed enlarged nuclei and frequent keratin deposits which are indicative of squamous differentiation (Fig. 4A). Necrotic and apoptotic changes were minimal. Immunohistochemistry revealed Cdc37 protein expression in a large fraction of tumor cells (Fig. 4A). Lymphomas in transgenic females usually manifested themselves as an extreme weakness of the animals and obvious enlargement of the lymph nodes. Two cases of lymphomas were discovered in animals that already had developed mammary adenosquamous carcinomas. All lymphomas exhibited very low mitotic activity (data not shown), which could explain the slow progression of disease. Twenty animals of the MMTV-*CDC37*.2 line were autopsied at 17 months of age. Nine animals displayed evidence of proliferative disorders (Ta-

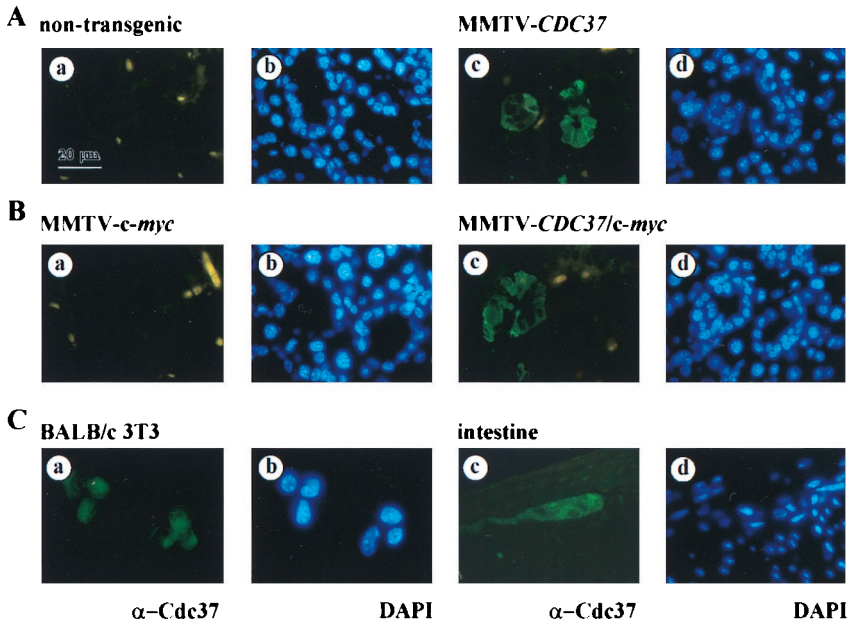


FIG. 2. Analysis of Cdc37 expression by immunofluorescence. (A and B) Salivary gland tissue sections from nontransgenic (Aa and b), MMTV-*CDC37* (Ac and d), MMTV–c-*myc* (Ba and b), and MMTV–*CDC37*/c-*myc* (Bc and d) mice were stained with anti-Cdc37 antibodies and visualized with secondary antibodies labeled with fluorescein isothiocyanate (FITC). Nuclei were visualized with DAPI (4′,6′-diamidino-2-phenylindole). (C) BALB/c 3T3 cells (a and b) or intestinal sections from nontransgenic mice (c and d) were probed with anti-Cdc37 and nuclei identified by DAPI. In the intestine, Cdc37 expression is limited to a narrow band of proliferating cells (58). The same exposures were used for all figures.

ble 1), primarily mammary adenosquamous carcinomas and lymphomas, although one case of sarcoma was found. As in the first line examined, all tumors displayed a low mitotic index with little evidence of apoptosis. Nontransgenic control animals were subjected to a detailed pathological analysis either in parallel with *CDC37* transgenic animals or at 17 to 22 months of age. No evidence of proliferative disturbances was found in nontransgenic animals (Fig. 3A and Table 1).

***CDC37* cooperates with c-*myc* in induction of mammary tumors in breeding females.** *CDC37* is expressed in proliferative zones in adult tissues and is coexpressed with cyclin D1 in several tissues, but it is absent in many differentiated cell types, including many epithelial cell types (58). We therefore hypothesized that *CDC37* expression might be required to support transformation by oncogenic pathways. In this case, we would predict that inappropriate *CDC37* expression might promote proliferative events dependent on oncogenic pathways.

To test this, we crossed MMTV-*CDC37* heterozygous females with MMTV–c-*myc* and MMTV-cyclin D1 homozygous males. To control for differences in genetic backgrounds, we monitored heterozygous c-*myc* and cyclin D1 littermates alongside the double transgenics. Previously, it was shown that multiple rounds of pregnancy and lactation are able to promote expression of the c-*myc* transgene and accelerate tumorigenesis (56). We evaluated the influence of the level of expression of the transgene on tumorigenesis by dividing single and double transgenic females into two groups: one was kept virgin, and the other was kept in the presence of breeder males. Both lines of *CDC37*-expressing animals were used for these experiments. The approximately equal number of double transgenic females carried either MMTV-*CDC37*.1 or MMTV-*CDC37*.2 in combination with c-*myc* transgene. No differences between the two lines were observed in the kinetics of tumor appearance and tumor specificity in either breeding or virgin double transgenic females, and therefore the data for two lines were pooled together (Fig. 3A).

Tumors were observed in breeding MMTV–c-*myc* females as early as 3 months of age and 50% of females had developed tumors by 250 days of age in this genetic background (Fig. 3A and 4B). In contrast, breeding females carrying both c-*myc* and *CDC37* transgenes developed tumors with accelerated kinetics, and 50% of females developed tumors by the age of 115 days (Fig. 3A and 4C). All tumors developed by breeding females were mammary ductal and alveolar adenocarcinomas (Fig. 3B). In addition to the acceleration of tumor incidence, we also observed a dramatic increase in the number of tumors/animal

TABLE 1. Neoplasms found in transgenic females carrying the MMTV-*CDC37* transgene

Pathology	Line	% Mice affected (no./total)
Mammary ductal adenosquamous carcinoma	MMTV-*CDC37*.1	60 (6/10)
	MMTV-*CDC37*.2	20 (4/20)
Lymphoma	MMTV-*CDC37*.1	50 (5/10)
	MMTV-*CDC37*.2	20 (4/20)
Mammary ductal adenocarcinoma	MMTV-*CDC37*.1	10 (1/10)
	MMTV-*CDC37*.2	0 (0/20)
Sarcoma	MMTV-*CDC37*.1	0 (0/10)
	MMTV-*CDC37*.2	5 (1/20)
Total affected	MMTV-*CDC37.1*[a]	100 (10/10)
	MMTV-*CDC37.2*[b]	45 (9/20)
	Nontransgenic[c]	0 (0/30)

[a] Tumors occurred between 18 and 22 months of age.
[b] All animals were sacrificed and analyzed at 17 months of age without outward signs of transformation.
[c] Animals were examined in parallel with MMTV-*CDC37* mice at 17 to 22 months of age.

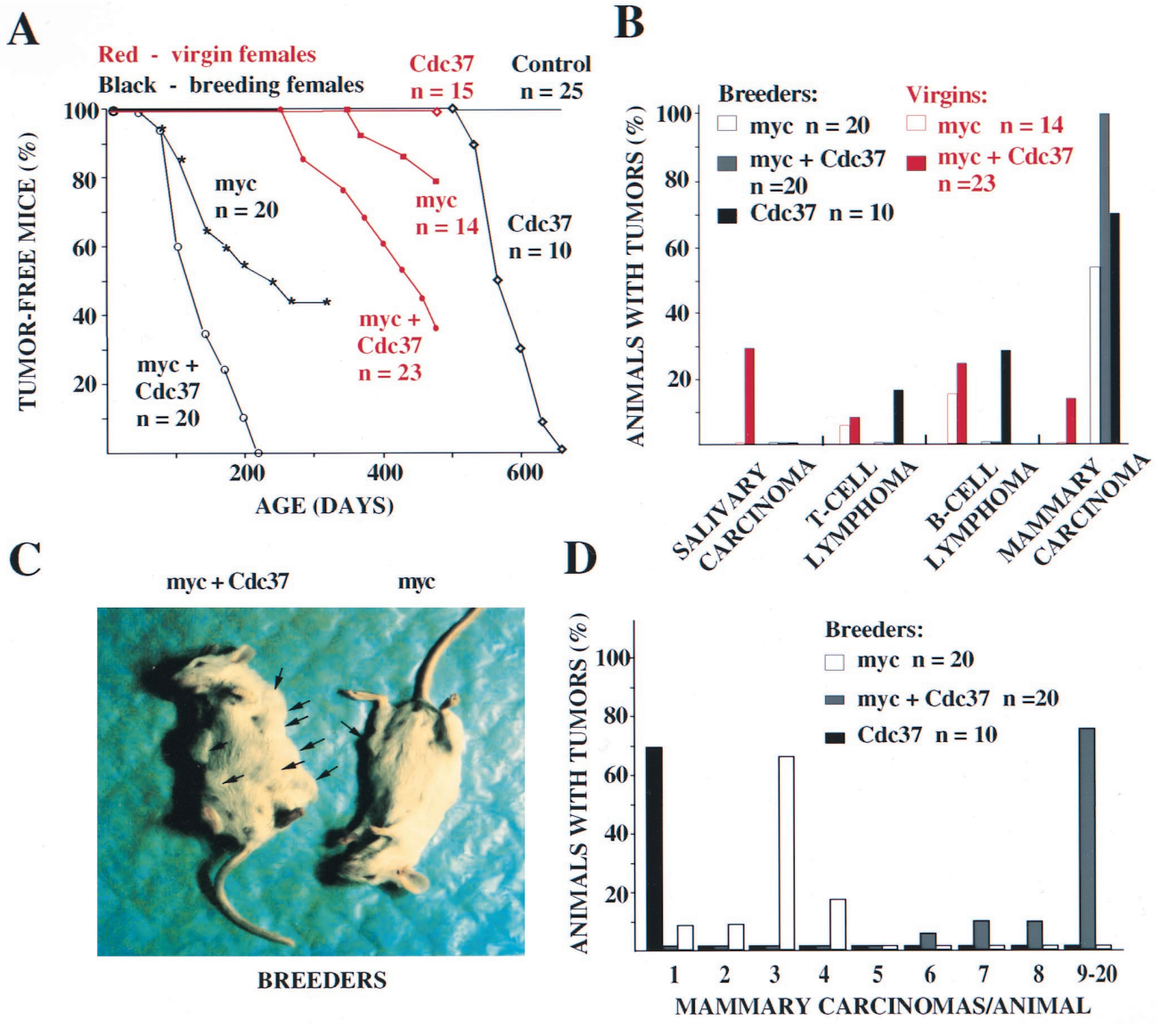


FIG. 3. MMTV-*CDC37* facilitates transformation of the mouse mammary epithelium and collaborates with c-*myc* to transform multiple tissues. (A) Quantitation of incidence of proliferative disorders. Tumor-free animals from breeding females are shown in black, while the tumor incidence in virgin animals is shown in red. n, number of animals in each group. Data shown for Cdc37 mice were from the MMTV-*CDC37*.1 line. Breeding and virgin MMTV–*CDC37*/c-*myc* females bore either MMTV-*CDC37*.1 or MMTV-*CDC37*.2 transgenes. (B) Types of tumors developed by virgin or breeding MMTV-*CDC37*, MMTV–c-*myc*, and double transgenic MMTV–*CDC37*/c-*myc* mice. The percentage of the animals developing each type of tumor from panel A is shown. Some of the animals developed more than one type of malignancy. The ages of breeding animals were as follows: MMTV-*CDC37*, 17 to 22 months; MMTV–c-*myc*, 3 to 12 months; and MMTV–c-*myc*/*CDC37*, 3 to 7 months. The ages of virgin animals were as follows: MMTV–c-*myc*, 12 to 16 months; and MMTV–c-*myc*/*CDC37*, 9 to 16 months. (C) Gross appearance of the breeding females expressing either MMTV–c-*myc* (right) or MMTV–*CDC37*/c-*myc* (left). The double transgenic females develop more tumors per animal than do single c-*myc* transgenics. The additional tumors, which were not visible by gross examination, were detected by detailed histopathological analysis. (D) Quantitation of tumor number per animal. The percentage of animals developing a given number of mammary adenocarcinomas is shown. MMTV-*CDC37* animals developed only one tumor per animal. c-*myc*-expressing animals developed from 1 to 4 tumors/animal, while the majority of the double transgenics had between 9 and 20 tumors/animal. The number of tumors was estimated by counting foci on sections from fixed preparations of all mammary glands. The ages of animals are given in panel B.

(Fig. 3C and D). This included both an increase in the number of glands affected as well as the number of tumors/gland (Fig. 3D). While MMTV–c-*myc* animals rarely had all of the glands affected, virtually all of the double transgenic animals were affected in every gland (Fig. 3C). While MMTV–c-*myc* females had on average three tumors per animal, MMTV–*CDC37*/c-*myc* approached 20 tumors per animal, on average (Fig. 3D). In many cases, the tumor masses were so abundant it prevented an exact determination of the number of tumor foci. On sections of both MMTV–*CDC37*/c-*myc* and MMTV–c-*myc* mammary glands all transitions from normal to transformed epithelium could be seen, including multiple areas of hyperplasia.

Altered tissue specificity of transformation in nonbreeding MMTV–*CDC37*/c-*myc* females. *CDC37* is normally not expressed in virgin mammary epithelium but is induced during pregnancy. c-*myc* has been shown to induce mammary transformation in virgin mice in some genetic backgrounds, although the extent of transformation is much lower than was observed with multiple pregnancies. To examine whether

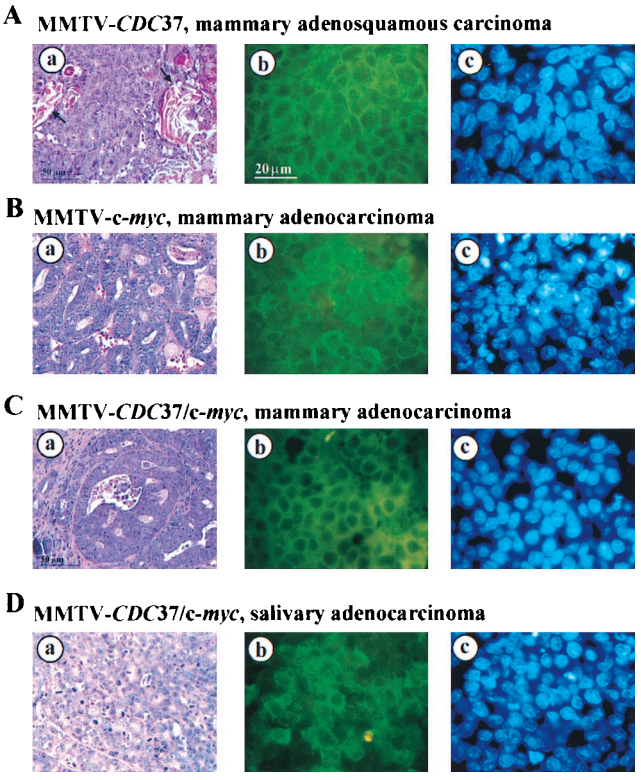


FIG. 4. Phenotypic analysis of tumors developed by MMTV-*CDC37* transgenic mice. (Aa) Ductal adenosquamous carcinoma of the mammary gland derived from an MMTV-*CDC37* mouse was stained with H&E. Arrows indicate squamous differentiation. (Ab and c) Adjacent tumor sections from Aa were stained with anti-Cdc37 antibodies and visualized with FITC (b), while nuclei were visualized with DAPI. H&E, ×400 magnification. Immunofluorescence, ×1,000 magnification. (B) Same as panel A except that the tumor was a mammary adenocarcinoma from an MMTV–c-*myc* mouse. (C) Same as panel A except that the tumor was a mammary adenocarcinoma from an MMTV–*CDC37*/c-*myc* mouse. (D) Same as panel A except that the tumor was a salivary gland adenocarcinoma from an MMTV–*CDC37*/c-*myc* mouse.

CDC37 can collaborate with c-*myc* in the absence of hormonal stimulation, we examined females maintained in the virgin state. In our strain background, MMTV–c-*myc* virgin females typically incurred B-cell lymphomas as opposed to mammary carcinomas (Fig. 3B). The kinetics of tumor development were very slow, and only 25% of females developed tumors by the age 500 days. In contrast, the kinetics of tumor incidence in double transgenics generated from both *CDC37* lines were substantially accelerated (Fig. 3A). Relative to single c-*myc* transgenics, the spectrum of tumors was much wider (Fig. 3B), including both T- and B-cell lymphomas, as well as mammary and salivary gland adenocarcinomas. In double transgenic females, a prevalent tumor type was salivary adenocarcinoma (Fig. 3B). Salivary tumors from MMTV–*CDC37*/c-*myc* animals contain readily detectable Cdc37 (Fig. 4D) and c-*myc* (data not shown). This tumor type has never been reported in c-*myc*-expressing animals, although c-*myc* expression is readily observed in the salivary of phenotypically normal salivary glands in MMTV–c-*myc* mice (see Fig. 6B). Adenocarcinomas found in double transgenics appeared to be fast growing, with many mitotic figures (Fig. 4D). Taken together, these data indicate that MMTV-*CDC37* can alter the rates and extent of transformation in both breeding and nonbreeding MMTV–c-*myc* mice and can also alter the specificity of transformation.

Testicular hyperplasia and transformation in MMTV–*CDC37*/c-*myc* males. MMTV–c-*myc*-expressing males are typically free of proliferative disorders (58). Therefore, we were surprised to find evidence of both overt Leydig cell tumors and testicular hyperplasia in double transgenic males (Fig. 5). Cdc37 is normally not detectable in the testis of an adult mouse but is readily apparent in Leydig cells in MMTV-*CDC37* mice (Fig. 5D). Leydig cell tumors were observed in MMTV–*CDC37*/c-*myc* male mice at as young as 10 months (Fig. 5A, G). One of the four tumor-bearing animals had two distinct Leydig cell tumors, one in each testis. At an age of ~400 days, about two-thirds of all apparently unaffected males were sacrificed, and their testes were subjected to detailed histological analysis. A significant fraction (75%) of double transgenic males displayed Leydig cell hyperplasia (Fig. 5F), a possible precursor to overt transformation. In contrast, only about 20% of MMTV–c-*myc* males displayed modest Leydig cell hyperplasia (Fig. 5B, C and E). Nontransgenic and MMTV-Cdc37 males did not display any hyperplasia.

Biochemical analysis of tumors derived from breeding MMTV–c-*myc* and MMTV–*CDC37*/c-*myc* transgenic females. To begin to address how *CDC37* and c-*myc* collaborate in transformation, we examined the levels of several protein kinases as well as c-*myc* in mammary carcinomas from MMTV–*CDC37*/c-*myc* and MMTV–c-*myc* animals (Fig. 6A). As a control, we also examined the levels of proteins in mammary tumors derived from an MMTV-*ras* mouse (59). As expected, Cdk4 levels were increased in tumors expressing MMTV-*CDC37* (Fig. 6A, lanes 3 to 6), relative to that found with MMTV–c-*myc* alone (lanes 7 to 10), as were the Erk1 levels. We also found that activated Erk levels were higher in MMTV–*CDC37*/c-*myc* mice than in MMTV–c-*myc* mice (Fig. 6A). Unexpectedly, we found that c-*myc* levels were also increased in the presence of MMTV-*CDC37* compared to animals expressing only MMTV–c-*myc* (Fig. 6A). The observed differences in protein levels cannot be explained by the increased number of dividing cells, since no significant difference was observed in the mitotic index of these tumors (data not shown). One explanation for increased c-*myc* abundance is that Cdc37 can affect expression from the MMTV promoter, thereby causing an indirect increase in c-*myc* levels. Analysis of c-*myc* mRNA in tissues derived from MMTV–c-*myc* and MMTV–c-*myc*/*CDC37* mice, however, revealed similar levels of c-*myc* mRNA (Fig. 6B). Thus, Cdc37 does not indirectly influence c-*myc* expression from the MMTV-transgene promoter. An alternative explanation is that Cdc37 expression causes an alteration in the population of cells expressing c-*myc*. To test this, we examined c-*myc* expression in sections containing phenotypically normal tissue from various tissues. c-*myc* staining was not detected in nontransgenic animals (Fig. 6C) but was evident in the cytoplasm of all epithelial cells in the salivary and mammary glands from MMTV–c-*myc* mice (Fig. 6C and data not shown). The presence of Cdc37 had no discernible effect on the levels or extent of c-*myc* expression (Fig. 2B and 6C), ruling out increased numbers of c-*myc*-positive cells as an explanation for the observed increase in c-Myc protein levels. Tumors derived from MMTV-*ras* and MMTV–c-*myc* mice contained primarily the more slowly migrating Cdc37 isoform, while MMTV–*CDC37*/c-*myc* tumors contain both Cdc37 isoforms (Fig. 6A).

***CDC37* cooperates with cyclin D1 in transformation of the mammary epithelium.** To further test the effect of simultaneous expression of *CDC37* with other oncogenes, we created transgenic animals expressing both *CDC37* and cyclin D1 under control of the MMTV promoter. Previous studies have demonstrated that MMTV-cyclin D1 mice develop mammary gland adenocarcinomas with an average age of onset of 534 days (64). In the genetic background of our study, no prolif-

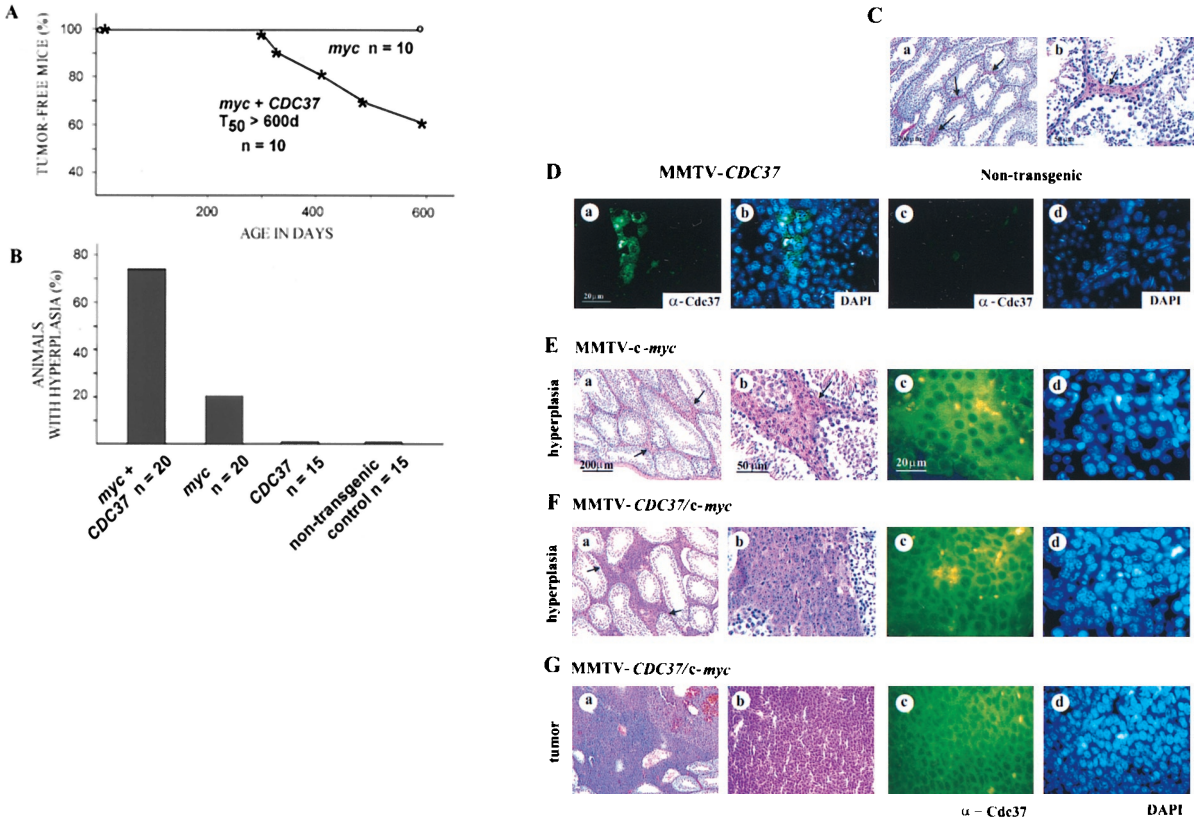


FIG. 5. *CDC37* cooperates with c-*myc* in the induction of the Leydig cell hyperplasia and transformation. (A) MMTV–*CDC37*/c-*myc* double transgenic males develop tumors, while male animals expressing a single transgene are unaffected. A plot of tumor-free mice over time is shown. Three of four tumors were Leydig cell tumors, while the fourth was a lymphoma. (B) MMTV–*CDC37*/c-*myc* double transgenic males display extensive Leydig cell hyperplasia compared to MMTV–c-*myc* and MMTV-*CDC37* animals. Histological sections of testis derived from grossly unaffected males were analyzed at 400 days of age. The number of animals in each group is shown. (C) Tissue section of a normal testis with arrows indicating the positions of Leydig cells located between seminiferous tubules with active spermatogenesis: (a) ×100 magnification, H&E staining; and (b) ×400 magnification, H&E staining to show the usual number and morphology of Leydig cells. (D) Expression of *CDC37* in the testis of MMTV-*CDC37* transgenic (a and b) or nontransgenic (c and d) male mice at ×1,000 magnification: (a and c) *CDC37* expression in the cytoplasm of Leydig cells; and (b and d) DAPI staining to identify nuclei. (E) Mild hyperplasia found in 20% of 400-day-old males expressing MMTV–c-*myc*. (F) High-grade hyperplasia found in 75% of 400-day-old MMTV–*CDC37*/c-*myc* mice. (G) Example of a Leydig cell tumor found in MMTV–*CDC37*/c-*myc* mice: (a) ×100 magnification, H&E staining; (b) ×400 magnification, H&E staining; (c) ×1,000 magnification field stained with anti-Cdc37 antibodies; and (d) ×1,000 magnification, DAPI staining of the same field as panel c to identify nuclei.

erative disturbances were found in MMTV-cyclin D1 mice for up to 650 days (Fig. 7). Similar results have been noted in other mixed genetic backgrounds with MMTV-cyclin D1 mice (E. V. Schmidt and A. Arnold, unpublished data). Animals expressing both cyclin D1 and *CDC37* display evidence of mammary tumors at the age of 13 months, at which time control MMTV-*CDC37* and MMTV-cyclin D1 mice had yet to display a transformation phenotype (Fig. 7A and B).

Tumors developed by double transgenic animals appeared as rapidly dividing single mass adenocarcinomas. The majority of adenocarcinomas were well-differentiated carcinomas with high levels of secretion, although several cases of poorly differentiated adenocarcinomas without apparent secretion were also observed (Fig. 7C). The majority of animals developed one mammary tumor, but frequent cases of metastasis to the lung was observed during pathological analysis (Fig. 7Cd).

Each of the double and single transgenic animals subjected to the detailed pathological analysis also displayed several foci of hyper- and metaplastic mammary epithelia (Fig. 7a). The appearance of hyperplastic areas was reported previously for the single MMTV-cyclin D1 transgenics (64). In our experiment, the frequency of the appearance of the hyper- and metaplastic foci was similar in MMTV-cyclin D1 single and MMTV-*CDC37*/cyclin D1 double transgenic animals at a similar age.

Inappropriate mammary duct development in male MMTV-*CDC37* mice. Phenotypic analysis of mammary glands during development failed to identify significant differences between female MMTV-*CDC37* mice and their wild-type littermates, except for a 2- to 3-day delay in the rate of involution after lactation (data not shown). However, we did observe alterations in the development of male ductal systems, as assessed by whole-mount analysis. The development of rudimentary mammary ducts begins during embryonic development. Sexual dimorphism is already pronounced at embryonic day 14 when the male anlage undergoes significant cell death caused by androgens (22, 50). The degree of breast duct development varies in different mouse strains, ranging from the presence of the initial ductal sprout in some of the fat pads to a relatively well developed branching ductal tree. In the strain background used here, male mice do not develop a significant mammary duct structure, although the fat pad is well developed. In contrast, 60 to 70% of the adult MMTV-*CDC37* male mice have well-formed breast ducts with different degrees of elaboration by the age of 7 months (Fig. 8). In the MMTV-*CDC37.2* line,

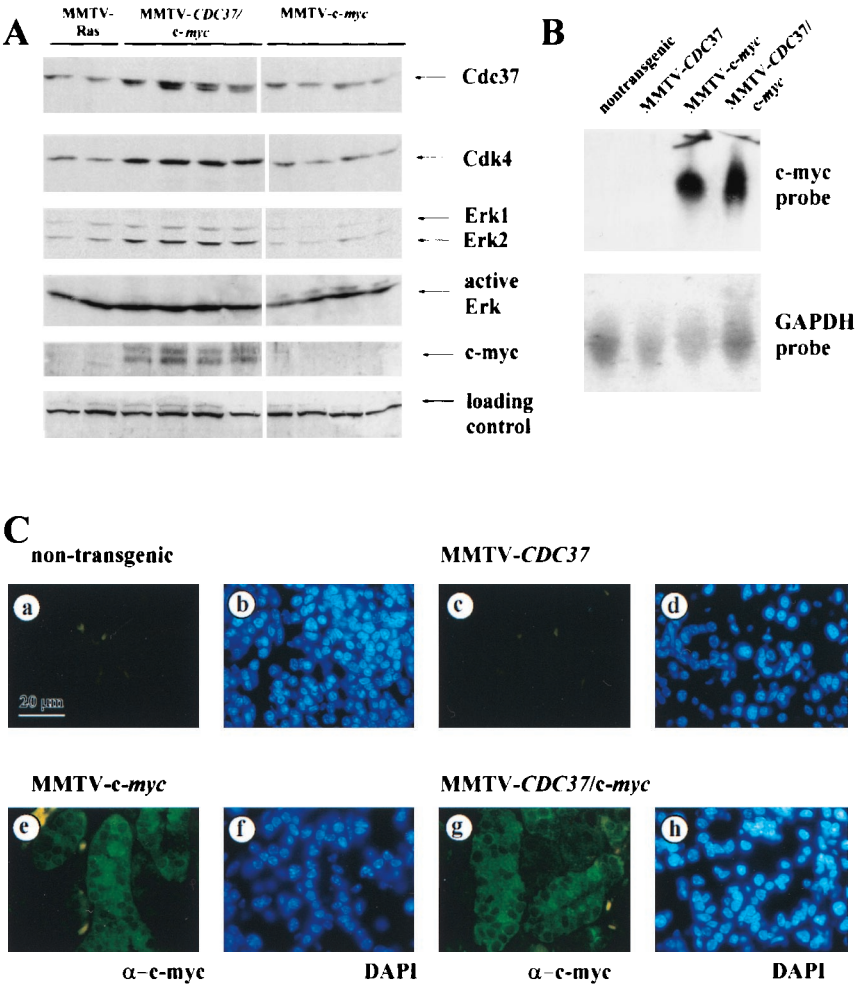


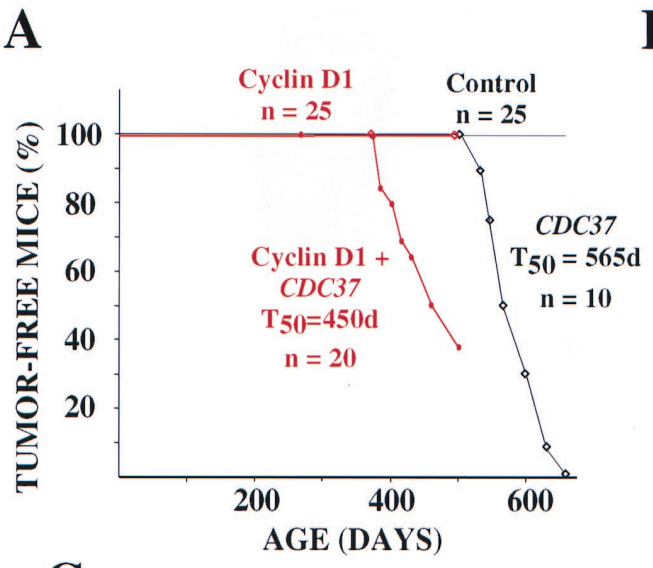
FIG. 6. MMTV–*CDC37*/c-*myc* mammary tumors have higher levels of multiple signaling proteins than tumors from MMTV–c-*myc* animals. (A) Protein extracts (200 μg/lane) from individual tumors derived from the indicated animals were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the indicated antibodies. (B) Northern blot analysis of c-*myc* mRNA in salivary tissue from nontransgenic, MMTV–c-*myc*, and MMTV–*CDC37*/c-*myc* mice. Blots were stripped and reprobed with GAPDH as a loading control. (C) Expression of c-*myc* in phenotypically normal salivary gland tissue from nontransgenic (a and b), MMTV-*CDC37* (c and d), MMTV–c-*myc* (e and f), and MMTV–*CDC37*/c-*myc* (g and h) mice. (a, c, e, and g) Anti-c-*myc*. (b, d, f, and h) DAPI used to visualize nuclei.

which has lower levels of expression, 30 to 40% of male animals developed breast ducts in the inguinal fat pad by the age of 7 months. In control nontransgenic littermates of the similar mixed background, only 10% of adult males have a nonbranching initial sprout structure (Fig. 8B).

To monitor the age dependence of the effect, we performed whole-mount analysis of the male mammary glands at different ages (Fig. 8B). This analysis demonstrated that 70% of 4-week-old MMTV-*CDC37*.1 and control animals have a tiny initial breast sprout which later would give rise to breast ducts. During the first 6 weeks after birth, this ductal sprout regressed in most of the nontransgenic animals, and the fraction that maintained a ductal sprout (10%) did not change for up to 8 weeks and later (Fig. 8B). In contrast, the percentage of MMTV-*CDC37* animals that maintain and elaborate ductal systems remained at ~70%. At 6 weeks of age 70% of transgenic animals have about the same or somewhat better developed initial sprout, and by 8 weeks 70% of transgenic animals have a well-developed branching duct system resembling the structures found in older MMTV-*CDC37* males. There was no significant change in breast duct development between the ages of 8 weeks and 7 months in both transgenic and control groups. The mechanism underlying this developmental alteration is not known at present but could reflect effects of *CDC37* on the androgen receptor, as has been observed in budding yeast cells (13).

DISCUSSION

Proliferation requires the coordinated activation of multiple signaling pathways, which ultimately converge on the cell cycle machinery to promote DNA replication and cell division. Studies in a variety of systems suggest that Cdc37 and Hsp90 are required to establish important signaling pathways through interaction with intrinsically unstable kinases, including the oncoprotein kinases Cdk4 and Raf-1 and *src* family members (16, 58, 62, 71). In this study, we have examined the proliferative role of *CDC37* through analysis of MMTV-*CDC37* transgenic mice. Remarkably, we found that expression of *CDC37* alone promotes neoplastic transformation of both the mammary epithelium and cells of the lymphoid compartment in older females. In this context, *CDC37* functions as a weak oncogene with rates of transformation similar to that observed previously in MMTV-cyclin D1 mice (onset at 18 to 22



B

Neoplasia in MMTV-cyclin D1/*CDC37* females

Mammary tumors/animals[$]	Tumors with metastasis (%)	Mean age at onset (days)
15/12	30	430

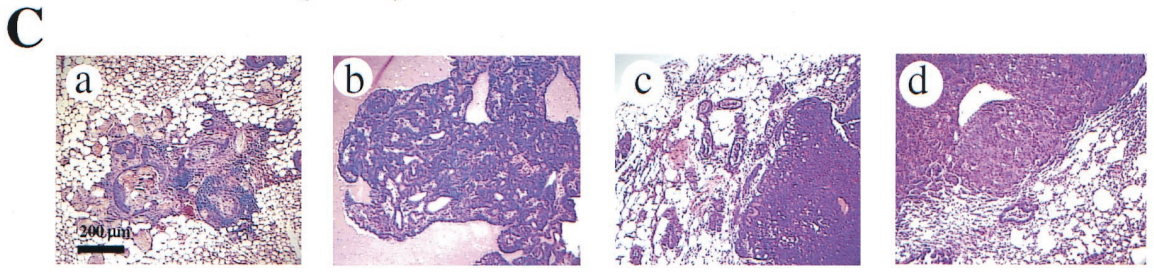


FIG. 7. Cooperation between *CDC37* and cyclin D1 oncogenes in breeding female mice. (A) By 15 months of age, a significant number of MMTV-*CDC37*/cyclin D1 double transgenic breeding females developed mammary adenocarcinomas, while none of the single transgenics developed tumors. A plot of the number of tumor-free animals over the time is shown. (B) Neoplasms developed by MMTV-cyclin D1/*CDC37* breeding females were all mammary adenocarcinomas with frequent methastasis to the lung. [$], number of animals that developed palpable tumors. (C) Histological analysis of proliferative disorders (×100 magnification, H&E staining): (a) metaplastic and hyperplastic changes observed in both single MMTV-cyclin D1 and double MMTV-*CDC37*/cyclin D1 transgenic females; (b) well-differentiated secreting mammary adenocarcinoma, developed by MMTV-*CDC37*/cyclin D1 double transgenic female; (c) poorly differentiated mammary adenocarcinoma, developed by MMTV-*CDC37*/cyclin D1 female; and (d) lung metastasis from a double transgenic mouse.

months) (64). Mammary tumors from these animals displayed low mitotic activity, a finding consistent with their very slow development and growth. Two independent lines of MMTV-*CDC37* mice both displayed transformation in tissues known to be transformed by MMTV-driven oncogenes, although the penetrance of the phenotype is not as severe in the MMTV-*CDC37*.2 strain as in the MMTV-*CDC37*.1 strain (Table 1). Transgenic mice expressing cyclin E, cyclin D, and *ras* also display variability in the extent and tissue specificity of transformation (3, 32, 61, 64). This variability may reflect the site of integration and/or the levels of expression. We consider it likely that the persistent expression of *CDC37* may allow what would otherwise be silent somatic mutations occurring over time in these animals to give rise to transformation. *CDC37* appears to have multiple targets, many of which can promote proliferation in various settings. Thus, it is not clear whether the multiple transformation events we have observed in MMTV-*CDC37* mice reflect mutational activation of a single collaborating pathway or mutations in different pathways in different tumors that occur stochastically.

Because of the link between Raf-1, Cdk4, and Cdc37, we asked whether *CDC37* could cooperate with c-*myc*-dependent transformation by breeding MMTV-*CDC37* and MMTV–c-*myc* mice. In principle, stabilization and/or activation of Raf-1 by ectopic Cdc37, which has been observed in heterologous systems (15), could inappropriately activate the *ras* pathway, and this could be observed as collaboration with c-*myc* in vivo. c-*myc* can collaborate with *ras* to transform a variety of cell types both in vitro and in vivo (26, 56). The ability of *ras* to function as a growth promoter as opposed to a growth inhibitor may rely upon inactivation of the ARF/Mdm2/p53 pathway. In primary fibroblasts, *ras* can induce G_1 arrest and a senescence-like state dependent upon p53 and $p16^{INK4a}$, but this activity is lost with immortalization (29, 53). The selective pressure on c-*myc*-expressing cells to inactivate the ARF-p53 pathway or undergo apoptosis (73), therefore, provides a plausible model for collaboration between *ras* and *myc* in cellular transformation (reviewed in reference 54). c-*myc* may also promote proliferation by controlling Cdk activity. c-*myc* expression can induce Cdk4/cyclin D kinase activity in certain situations (33). There is also evidence that cyclin D1 and Cdk4 are required for the proliferative effects of c-*myc* (17, 48) and that the expression of cyclin D1 and c-*myc* could be interdependent in some systems (48). In addition, c-*myc* expression leads to cyclin E/Cdk2 kinase activation, at least in part through inactivation of p27 (28, 43, 49, 63).

We found that *CDC37* and c-*myc* collaborate to transform multiple tissues in both breeding and nonbreeding females, as well as in males, and both MMTV-*CDC37* lines behaved similarly in this regard. In breeding and virgin females, *CDC37* enhanced both the rate and extent of mammary transformation by c-*myc*. Importantly, the number of tumor foci observed with

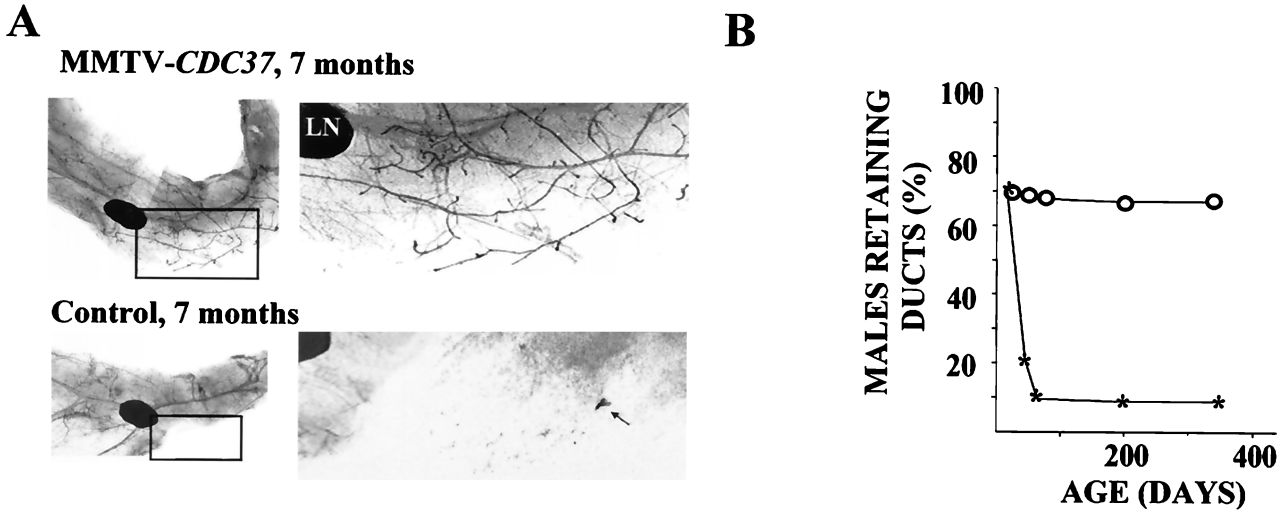


FIG. 8. Inappropriate mammary duct development in MMTV-*CDC37* transgenic males. (A) Whole-mount analysis of the mammary glands from transgenic males and nontransgenic littermates at 7 months of age. Inguinal mammary glands were fixed in formalin, cleared with acetone, and stained with hematoxylin to visualize mammary ducts. By 7 months, a significant number of transgenic males developed an extensive system of breast ducts resembling that of a normal virgin female, while only 10% of the males in the control group had retained an initial sprout. LN, lymph node; black arrow, initial duct sprout in a nontransgenic male. (B) Percentage of transgenic and nontransgenic animals retaining breast structures as a function of age. For each time point, more than 10 inguinal mammary glands were autopsied and analyzed.

c-*myc* in the presence of MMTV-*CDC37* was dramatically increased (from an average of three tumors/animal to an average approaching 20 tumors/animal) in breeding females. This result suggests that in some cell types *CDC37* expression may be rate limiting for transformation. In this regard, we have observed expression of *CDC37* in c-*myc* and *ras* induced mammary tumors, despite the fact that *CDC37* is not expressed in resting mammary epithelium. Interestingly, induction of Cdc37 is not a simple consequence of c-*myc* expression since phenotypically normal tissues expressing abundant c-*myc* lack detectable Cdc37 (Fig. 2). Thus, additional events that give rise to induction of Cdc37 are apparently occurring during the process of c-*myc*-dependent transformation. The increased rates of mammary transformation observed with pregnancy in MMTV–c-*myc* and MMTV-*ras* transgenic mice may reflect the fact that *CDC37* is normally induced during pregnancy (58) and could provide a proliferation-permissive setting that allows for these oncogenes to promote transformation. We expect that other events, including inactivation of the ARF/p53 pathway (reviewed in reference 53), are also involved in c-*myc*-mediated transformation in MMTV-*CDC37* mice.

Unexpected was the finding that Cdc37 expression allowed transformation by c-*myc* in cell types where it is normally not oncogenic. In virgin females, MMTV–*CDC37*/c-*myc* mice developed salivary tumors. Although MMTV-*ras* mice develop salivary tumors (31), MMTV–c-*myc* mice have not been reported to develop salivary tumors. The inability of c-*myc* to transform the salivary epithelium is considered a peculiarity of this oncogene. Our results suggest that the absence of *CDC37* expression in adult salivary glands may contribute to the inability of c-*myc* to transform this tissue.

We also found that expression of *CDC37* allows c-*myc* to transform Leydig cells in the testis. c-*myc* induced a very mild hyperplasia in a small fraction of the animals, but when *CDC37* was coexpressed there was a dramatic increase in the extent and severity of Leydig cell hyperplasia. Moreover, 30% of the double transgenic animals examined displayed evidence of overt Leydig cell neoplasia. The effect of *CDC37* on the extent of proliferation of Leydig cells is possibly due to its effect on Cdk4. Recent studies show that one of the phenotypes of Cdk4 knockout males is the reduction of the number of the Leydig cells and abnormalities in sperm maturation and infertility (44). Moreover, the expression of a mutant form of Cdk4 that cannot bind $p16^{INK4a}$ leads to an increased population of testicular Leydig cells (44). These studies point to the important role of Cdk4 kinase in the proliferation of this cell type. The cooperative behavior of the c-*myc* and *CDC37* in the induction of hyperproliferation and transformation in Leydig cells may therefore be explained by the role of *CDC37* in the stabilization of Cdk4 kinase (58) and c-*myc* in the induction of cyclin D1 expression (48). In contrast to c-*myc*, MMTV-*CDC37* did not affect the rate of mammary transformation induced by MMTV-*neu* in nonbreeding animals, although a severalfold increase in mitotic index was observed (data not shown).

Biochemical data indicate that Cdk4 is a major target of the Cdc37-Hsp90 chaperone complex (11, 58). If ectopic expression could inappropriately stabilize Cdk4, then one might expect to see increased proliferation in response to coexpression of cyclin D1. *CDC37* mRNA is coordinately regulated with cyclin D1 during breast development and in adult tissues, suggesting a functional link (58). Consistent with this, we found that MMTV-*CDC37* can collaborate with MMTV-cyclin D1 in the transformation of mammary epithelial cells.

Although the phenotypic consequences of *CDC37* expression and collaboration with c-*myc* and cyclin D1 are striking, the biochemical mechanisms underlying its action are likely to be complex, possibly involving multiple kinase pathways that function interdependently to promote proliferation. Stabilization and/or activation of Cdk4 or Raf could result in both activation of the *ras* pathway and activation of Cdks. In the latter case, increased Cdk4 levels could simultaneously sequester $p16^{INK4a}$ and promote proliferation via activation by cyclin D. This could, in turn, lead to activation of cyclin E-Cdk2 by both increasing cyclin E expression and by sequestration of p27. We have observed increased levels of both Cdk4 and the Erk1 kinase. Interestingly, we noticed that mammary tumors from MMTV–*CDC37*/c-*myc* animals contained significantly higher levels of c-*myc* than tumors from MMTV–c-*myc* ani-

mals independent of changes in mitotic index. This increase did not reflect effects of Cdc37 expression on MMTV-driven c-*myc* mRNA nor did it reflect an ability of Cdc37 to augment the number of cells expressing c-*myc*. Recent studies suggested that activation of the *ras* pathway stabilizes c-*myc* (51). It is therefore possible that Cdc37, through its interaction with kinases in the *ras* pathway, indirectly stabilizes c-*myc*. Since Cdc37 does not appear to have an effect on c-*myc* levels in phenotypically normal tissues, its effects on c-*myc* levels in tumors may require additional events. Further studies are required to determine whether increased levels of c-*myc* via *CDC37* expression are an important component of the collaborative effects seen in vivo.

In summary, our results suggest that the presence of Cdc37 may be rate limiting for the establishment of oncogenic signaling pathways that promote transformation. Although the effects observed here are in response to Cdc37 expression, recent studies provide correlative data indicative of a role for Cdc37 in human cancer. Normal human prostate epithelium has low to undetectable levels of Cdc37 (58a). However, Cdc37 is highly expressed in human prostatic cancer and is also expressed in preneoplastic lesions in the prostate, a finding consistent with its induction at an early stage of prostate cancer (58a). Similarly, our results indicate that Cdc37 induction occurs during transformation by c-*myc*. The mechanisms responsible for Cdc37 induction during the transformation process remain to be determined. We also note that the role of *CDC37* in transformation suggested by this work may explain the sensitivity of various tumor types to clinically relevant anzamycin derivatives (23, 39, 67, 68), which are known to bind Hsp90 and disrupt *ras*- and cyclin D-dependent signaling pathways.

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