Haploinsufficiency of the cdc2l gene contributes to skin cancer development in mice

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Introduction
Cyclin-dependent kinase 11 (CDK11) (formerly known as PITSLRE) protein is a member of the extended family of p34ck-related kinases. It is becoming increasingly clear that in addition to controlling the cell cycle, cyclin-dependent kinases have other functions within the cell. Recent studies indicate that the p110 isoform of CDK11 (CDK11P110) may be involved in RNA processing or transcription by virtue of the fact that CDK11 co-immunoprecipitates and/or co-purifies with multiple transcriptional elongation factors including ELL1, TFIIF, TFIIS and FACT (1, 2). Cyclin L1 and, more recently, cyclin L2 are regulatory partners of the CDK11 isoforms (3). Cyclin L is an arginine/serine domain protein that may function in pre-mRNA splicing (3). The CDK11P110 localizes to the nucleoplasm and nuclear speckle-staining regions (i.e. splicing factor centers) (1, 2). In addition, CDK11P110 also interacts with the general pre-mRNA splicing factors RNPS1 and 9G8 (both of which are classified as splice-site recognition proteins), RNA polymerase II and casein kinase 2 (1, 5, 6). The major CDK11P110 isoform is encoded by two human genes Cdc2L1 and Cdc2L2. These genes also contain the open reading frame for a smaller isoform, CDK11P46, which is generated by an internal ribosome entry site sequence found in most species. CDK11P46 is a mitosis-specific isoform, which exclusively functions in the G2 and M phases of the cell cycle. By means of RNA interference, it has been demonstrated that CDK11P46, but not the CDK11P110 isoform is required for mitotic spindle formation (7).

In regards to apoptosis, increased expression of CDK11 reduces cell growth due to apoptosis (8). In addition, our group and others have shown that the CDK11P110 isoform and the CDK11P46 isoform are cleaved by caspases to generate a smaller 46–50 kDa protein, CDK11P46, that contains the catalytic portion of the protein (9–11). Generation of this smaller CDK11P46 protein can be triggered by Fas, tumor necrosis factor-α or staurosporine. Caspase inhibitors can modulate the kinase activity of the caspase-processed protein CDK11P46 (9). Over-expression of CDK11P46 can inhibit cell growth and induce apoptosis, whereas tumor cells with abnormal CDK11P46 protein levels appear to be more resistant to Fas and/or staurosporine-induced apoptosis (9, 12).

The human Cdc2L gene locus, encoding CDK11, maps to chromosome band region 1p36, a chromosomal region frequently deleted in many cancers such as childhood sinus tumors, neuroblastomas and lymphomas (13–15) as well as melanoma (16). Reduced mRNA levels of CDK11 have also been reported in expression profile studies of breast cancer, lymphoma, neuroblastoma and melanoma (17, 18). In regards to melanoma, we have documented the deletion or translocation of Cdc2L in melanoma cell lines and reduced protein levels in surgical malignant melanoma specimens (19).

Homozygous deletion of cdc2l gene results in early embryonic lethality in mice due to apoptosis of the blastocyst cells between 3.5 and 4 days of postcoitus whereas heterozygous mice appear to develop normally (20). Molecular analysis of tumors (specifically pheochromocytomas and melanomas) derived from Pten+/− Ink4a/Arf−/− mice revealed loss of CDK11/PITSLRE relative to control DNA (21). In addition, loss of CDK11 expression was associated with concurrent expression of activated H-ras and dominant negative mutant p53 genes in skin tumors derived from H-ras/p53 transgenic mice (22). However, despite these observations suggesting a role of CDK11 in neoplastic transformation and tumor progression, whether or not a deficiency in CDK11 has a direct role in tumorigenesis and progression in vivo has not yet been investigated.

Gene trapping has gained prominence in recent years as a method for inactivating a given gene for in vivo studies (23–25). In the present study, we utilized gene trap technology to generate haploinsufficient cdc2l mice with reduced CDK11 protein levels and a well-characterized mouse model of chemically induced multi-step skin carcinogenesis to explore the role of CDK11 in skin carcinogenesis. Our study shows, for the first time, that reduced gene expression of cdc2l gene in haploinsufficient cdc2lΩGT mice has a significant impact on skin tumor development in a mouse model of chemically induced skin carcinogenesis.

Materials and methods

Generation of cdc2l gene-trapped (cdc2lΩGT) mice
An E14 embryonic stem (ES) cell line, XG836, was selected from the Bay双腿omics Gene Trap Resource Database (http://bay双腿omics.ucsf.edu). This cell line originated from the 129/sv background and contains an insertion of a secretory gene trap vector pGT1.8Ω into intron 12 of the cdc2l gene. The vector contains a splice-acceptor sequence upstream of a selection marker/reporter β-Geo gene (a fusion of β-galactosidase and neomycin phosphotransferase). The insertion of this vector into the mammanal genome generates a fusion transcript containing sequences from the exon 5+ to the insertion site spliced into the β-Geo reporter sequence (Figure 1A). The selected XG836 ES cells were injected into appropriately matured blastocysts from C57BL/6J mice and then transferred to pseudopregnant recipient females. The resulting founder chimeric male (a mixture of C57BL/6J:129/sv strains) was further bred onto C57BL/6J females and males developed benign papillomas (77.75%) as compared with cdc2l+/− mice. There was also an increased frequency of larger papillomas in cdc2lΩGT mice as compared with cdc2l+/− mice. This increased frequency of larger papillomas in cdc2lΩGT mice was further increased when the cdc2lGT mice were subjected to a single topical application of initiation by DMBA and promotion twice a week for 19 weeks with TPA. At 19 weeks, 70% of the cdc2lGT mice and 60% of the cdc2l+/− mice developed benign papillomas. However, there was an overall 3-fold increase in the average number of tumors per mouse observed in cdc2lΩGT mice as compared with cdc2l+/− mice. There was also an increased frequency of larger papillomas in cdc2lΩGT mice. By using the polymerase chain reaction–restriction fragment length polymorphism assay, we found A to T transversion mutations at the 61st codon of H-ras gene in the papilloma tissue of both cdc2lΩGT mice and cdc2l+/− mice. Ki-67 staining revealed increased proliferation in the papillomas in cdc2lΩGT mice and 60% of the cdc2lGT mice were subjected to a single topical application of initiation by DMBA and promotion twice a week for 19 weeks with TPA. At 19 weeks, 70% of the cdc2lGT mice and 60% of the cdc2l+/− mice developed benign papillomas. However, there was an overall 3-fold increase in the average number of tumors per mouse observed in cdc2lΩGT mice as compared with cdc2l+/− mice. There was also an increased frequency of larger papillomas in cdc2lΩGT mice. By using the polymerase chain reaction–restriction fragment length polymorphism assay, we found A to T transversion mutations at the 61st codon of H-ras gene in the papilloma tissue of both cdc2lΩGT mice and cdc2l+/− mice. Ki-67 staining revealed increased proliferation in the papillomas of cdc2lΩGT (77.75%) as compared with cdc2l+/− (30.84%) tumors. These studies are the first to show that loss of one allele of cdc2l gene, encoding CDK11, facilitates DMBA/TPA-induced skin carcinogenesis in vivo.

Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; ES, embryonic stem; PCR, polymerase chain reaction; PI, proliferative index; RT, reverse transcription; TPA, 12-O-tetradecanoylphorbol-13-acetate.

These authors contributed equally to this study.

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one allele harboring the gene trap $cdc2l\beta$-Geo transcriptional fusion and were thus named as $cdc2lGT$ mice (Figure 1A).

**Genotyping of $cdc2lGT$ mice**

All mice were genotyped by polymerase chain reaction (PCR) amplification of genomic DNA using primers that recognize the $\beta$-Geo gene (5' $\text{CAAATGGC-GATTACCGTTGA}$-3' and 5' $\text{TGCCCGTCATAGCCGAATA}$-3'). As an internal control, the $\text{tcrd}$ gene (5' $\text{CAAATGTTGCTTGTCTGGTG}$-3' and 5' $\text{GTCAGTCGAGTGCACAGTTT}$-3') was used. Genomic DNA was isolated from rodent tails using the DNeasy Blood and Tissue Kit from Qiagen (Valencia, CA). The PCR cycling conditions were 95°C for 2.5 min, followed by 30 cycles of 94°C for 1 min, 60°C for 45 s and 72°C for 1 min. Subsequently, a final extension at 72°C for 10 min was conducted. The PCR products were analyzed on a 1% agarose gel.

**Animals and tumor induction protocol**

All experimental procedures on mice were performed as per the regulations of Institutional Animal Care and Use Committees abiding by policies set by University of Arizona. All experimental mice were maintained in a protective environment and handled under sterile conditions in a laminar hood. Mice were shaved on their dorsal part (back) using surgical clippers 2 days prior to the start of the experiment. For the skin carcinogenesis study, four treatment groups were set up each containing 10 $cdc2l^+/+$ and 10 $cdc2lGT$ C57BL/6 mice. Genomic DNA from mouse tail was used in PCR analysis for $\beta$-Geo and $\text{tcrd}$ genes. (C) Real-time RT-PCR analysis comparing CDK11 expression in $cdc2l^+/+$ and $cdc2lGT$ mice. The $cdc2l$ transcript levels are normalized to mouse $\beta$-actin mRNA. (D) CDK11 protein levels from the skin tissues of CDK11 p130 protein was detected by GN1 antibody (dilution 1:1000). Mouse $\alpha$-tubulin is used as loading control.

![Fig. 1. Characterization of $cdc2lGT$ mice. (A) Schematic representation of normal and gene trapped fusion products of $cdc2l$ gene in mouse. The $cdc2l$ gene contains 20 exons resulting in a normal transcript of 2590 bp. Primers P1 and P2 are designed in exons 12 and 13 respectively. Position 1435 on the transcript represents the position at which exons 12 and 13 join after splicing (above). Insertion of the $\beta$-Geo coding sequence within intron 12 of the $cdc2l$ gene results in a $cdc2l\beta$-Geo fusion transcript. The fusion transcript is terminated prematurely at position 1461 (below). Empty and hashed boxes represent exons and $\beta$-Geo coding sequence respectively. Introns are shown as solid lines. (B) Genotyping of $cdc2l^+/+$ and $cdc2lGT$ C57BL/6 mice. Genomic DNA from mouse tail was used in PCR analysis for $\beta$-Geo and $\text{tcrd}$ genes. (C) Real-time RT-PCR analysis comparing CDK11 expression in $cdc2l^+/+$ and $cdc2lGT$ mice. The $cdc2l$ transcript levels are normalized to mouse $\beta$-actin mRNA. (D) CDK11 protein levels from the skin tissues of CDK11 p130 protein was detected by GN1 antibody (dilution 1:1000). Mouse $\alpha$-tubulin is used as loading control.

Lack of $cdc2l$ contributes to skin carcinogenesis
V = length × width × height × (π/6).

Histopathology
The experiment was terminated at week 19. All mice were necropsied. The dorsal skin tissue including tumors were dissected out from euthanized animals and fixed in 10% neutral-buffered formalin (VWR, West Chester, PA) for 24 h and embedded in paraffin. At the same time, frozen tissue sections and solid tumors (wherever possible) were also harvested in liquid nitrogen for various molecular analyses. In addition, 2–3 mm sections from DMBA/TPA (Group IV)-treated skin, as well as cleanly dissected portions of solid tumors, wherever possible, were collected for \( \text{H-ras} \) PCR–restriction fragment length polymorphism analysis. Serial sections (5 μm thick) of paraffin-embedded tissues were prepared and processed in graded ethanol solutions and xylene, purchased from Sigma Chemicals Co. Paraffinized tissue sections were stained with hematoxylin and eosin (Richard Allan Scientific, Kalamazoo, MI) and subjected to review by a pathologist (Dr A.K.Bhattacharyya).

Immunohistochemistry
Cleaved caspase-3 immunohistochemistry. Apoptosis was assessed by cleaved caspase-3 staining as described previously (27). Skin tumors were fixed, paraffin embedded and sectioned as described above. These sections were deparaffinized in xylene, followed by rehydration in graded series of ethanol and ending with water immersion. Antigen retrieval was performed by microwave exposure in sodium citrate buffer (2.1 g/l, pH 6.1). Endogenous peroxidase blocking was performed with 3% \( \text{H}_2\text{O}_2 \) in methanol and sections were blocked with 1.5% normal goat serum (Vector Laboratories, Burlingame, CA). Then sections were incubated with a rabbit polyclonal anti-cleaved caspase-3 antibody.
antibody followed by a biotinylated secondary antibody (Vector Laboratories). Sections were then treated with Vectastain Elite ABC Reagent, used according to the manufacturer’s instructions (Vector Laboratories), diamobenzidine activated with H₂O₂, followed by hematoxylin counterstain. Cleaved caspase-3 expression was evaluated in the tumors directly as well as on adjacent skin. Six mice each of cdc2lGT and cdc2lGT genetic backgrounds, treated with DMBA/TPA (Group IV), were used in this study.

Ki-67 immunohistochemistry. Cell proliferation was assessed by immunohistochemistry staining for Ki-67 antigen using anti-mouse Ki-67 antibody (Novacastro NCL ki-67p raised in rabbit; dilution 1:500) and developed using the biotin–streptavidin complex method. Probing was performed by the Tissue and Cellular/Molecular Analysis Shared Service Core Facility at the Arizona Cancer Center in the Discovery® XT Automated IHC System (Ventana Molecular Diagnosis Systems, Tucson, AZ). To improve antigen detection, sections were subjected to antigen retrieval prior to staining as described above. Proliferation was quantified under a 40× light objective and was expressed as a proliferative index (PI) score that was determined as the mean percentage of nuclei staining positive for Ki-67 antibody in 200 cells at 40× magnification. Two mice each of cdc2lGT and cdc2lGT genetic backgrounds, treated with DMBA/TPA (Group IV), were used in this study. Three to four fields of view for each animal were included in the calculation for PI score.

Western analysis
Tissue protein extracts were prepared from frozen mouse skin tissue minced in 500 μl of tissue lysis buffer [20 mM Tris pH 7.5, 150 mM NaCl, 1 mM ethylendiaminetetraacetic acid, 1% NP-40, 0.5% sodium deoxycholate, 1% protease inhibitor cocktail (Sigma), 1 mM phenyl methyl sulfonyl fluoride, 1 mM sodium orthovanadate]. Following lysis, the samples were centrifuged at 13 000g for 30 min at 4°C and the protein content was estimated using bicinchoninic acid assay (Pierce, Rockland, IL). Equal amounts of protein were resolved by electrophoresis through a 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to polyvinylidene difluoride membrane (Bio-Rad, Philadelphia, PA). The membrane was blocked with 5% non-fat dry milk solution prepared in 1× phosphate-buffered saline and then probed with anti-CDK11 G1 rabbit primary antibody. A secondary probe with horse radish peroxidase-labeled antibody (Sigma Chemicals Co.) was detected by enhanced chemiluminescence (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Anti-mouse γ-tubulin antibody was used as loading control.

Antibodies
Anti-CDK11 G1 rabbit polyclonal antibody recognizes the amino acids 413–414 of CDK11p110 isoform (12). Anti-mouse γ-tubulin monoclonal antibody raised in chicken was purchased from EMD Biosciences (San Diego, CA). Anti-cleaved caspase-3 (asp175) and anti-ki67 antibodies for immunohistochemistry were purchased from Cell Signaling Technology (Danvers, MA) and Novacastro/Vector Laboratories, respectively.

Quantitative real time RT–PCR analysis of cdc2l gene
Total RNA was extracted from frozen skin tissue of mice using RNeasy Mini Kit (Qiagen). Reverse transcription (RT) was performed with 2 μg of total RNA using the cDNA iScript Kit (Bio-Rad). Two microliters of cDNA was used as a template and amplified in triplicates. Threshold cycle (Ct) during the exponential curves were analyzed to determine the specificity of the amplicon. Each sample representing the internal control was standardized to a diameter of 500 μm. Two microliters of tissue lysis buffer [20 mM Tris pH 7.5, 150 mM NaCl, 1 mM ethylendiaminetetraacetic acid, 1% NP-40, 0.5% sodium deoxycholate, 1% protease inhibitor cocktail (Sigma), 1 mM phenyl methyl sulfonyl fluoride, 1 mM sodium orthovanadate]. Following lysis, the samples were centrifuged at 13 000g for 30 min at 4°C and the protein content was estimated using bicinchoninic acid assay (Pierce, Rockland, IL). Equal amounts of protein were resolved by electrophoresis through a 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to polyvinylidene difluoride membrane (Bio-Rad, Philadelphia, PA). The membrane was blocked with 5% non-fat dry milk solution prepared in 1× phosphate-buffered saline and then probed with anti-CDK11 G1N1 rabbit primary antibody. A secondary probe with horse radish peroxidase-labeled antibody (Sigma Chemicals Co.) was detected by enhanced chemiluminescence (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Anti-mouse γ-tubulin antibody was used as loading control.

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Mutation analysis of H-ras gene
Genomic DNA was extracted from skin tissue as well as cleanly dissected portions of solid tumors wherever possible from DMBA/TPA (Group IV)-treated mice using DNeasy Blood and Tissue Kit as per manufacturer’s guidelines (Qiagen). Genomic DNA extracted from tails (for genotyping) was used as a negative control. A 208 bp region from exon 2 of H-ras gene was amplified using primers described by Nagase et al. (28). The amplified product was purified using GFX columns to remove any residual buffer and 4 μg of DNA was further digested using restriction enzyme XbaI at 1 37°C for 3 h and electrophoresed on 4% Nu-sieve 3:1 agarose gel (BioWhittaker Molecular Applications, Rockland, ME) at 80 V for 90 min. Direct DNA sequencing of the PCR product was used to verify H-ras codon 61 mutation in one animal (#105) using primers as described previously.

Epidermal thickness measurements
Serial sections of paraffin-embedded mouse skin tissue were observed under the microscope at 40× magnification. The overall view at 40× magnification was standardized to a diameter of 500 μm. From this, relative skin epidermal thickness measurements were taken as a series of non-overlapping fields of view by spanning the complete skin sections from one end to the other. Four to five complete tissue sections each of three to four mice were used for this study. Supplementary Figure 1 (available at Carcinogenesis Online) shows a typical skin section at 4× magnification with highly varying epidermal thickness. Thickness means ± standard deviations are represented in Table II.

Statistical analyses
Pls from Ki-67 staining and epidermal thickness measurements were analyzed by two sample t-test and considered significant at P < 0.001. Ki-67 immunohistochemistry data were compared in cdc2lGT versus cdc2lGT+ mice. Epidermal thickness measurements were compared between different carcinogen treatment groups as well as in cdc2lGT versus cdc2lGT+ in DMBA/acetone or DMBA/TPA treatment groups. Differences in tumor volumes between cdc2lGT and cdc2lGT+ mice were analyzed by chi-square test and considered significant at P < 0.05. Data for tumor multiplicity were analyzed by the Mann–Whitney U-test for each week of carcinogen treatment and considered significant at P < 0.05.

Results
Genotyping and characterization of gene-trapped cdc2l mice (cdc2lGT+)
Haploinsufficient cdc2l (cdc2lGT) mice were generated by using gene trap technology as described in Materials and methods. Briefly, an ES cell line (XG836), harboring an insertion of β-Geo gene, within the intron 12 of cdc2l gene (as assessed by 5′ RACE on ES cell RNA) was obtained from BayGenomics (data not shown). These ES cells were injected into blastocysts and further implanted onto pseudopregnant C57BL/6J female mice. The resulting founder chimera contained a disruption in the normal message of cdc2l gene due to the formation of a cdc2l-β-Geo fusion transcript (Figure 1A). All mice were genotyped by PCR amplification of genomic tail DNA using primers specific for β-Geo and tcrd genes as described in Materials and methods. Wild-type cdc2l+/- mice generated a single 200 bp product representing the internal control tcrd gene, whereas the gene-trapped (cdc2lGT) mice showed the presence of an additional product (581 bp).
next, we used cdc2lGT trap mutagenesis generated an effective null allele of the mouse cdc2l gene. Hereafter, we use cdc2lGT to denote this insertional mutation. Representing the β-Geo gene insertion (Figure 1B). We verified that this insertion leads to decreased levels of CDK11 mRNA and protein by quantitative real time RT–PCR and Western analyses (Figure 1C and 1D).

To further confirm the insertional gene trap specifically in the context of the cdc2l gene, we intended to amplify the region containing the β-Geo insertion within the processed cdc2l transcript by RT–PCR amplification of cDNA from cdc2lGT mice. We used primers P1 and P2 (designed in exons 12 and 13 of the cdc2l gene, respectively) as well as primers P1 and P3 (primer P3 is designed in the β-Geo coding sequence) to amplify the wild-type and fusion transcripts, respectively, from cDNA synthesized from cdc2lGT mice (Figure 1). Primers P1 and P3 did not yield any detectable product in all cdc2lGT mice (data not shown). Interestingly, primers P1 and P2 yielded a single product representing the normal transcript (125 bp) and not the larger fusion transcript (>500 bp) in all cdc2lGT mice (data not shown). This suggests that cdc2lGT mice contain one functional copy of the cdc2l transcript, whereas the other copy is not functional (Figure 1A). The non-functional cdc2lβ-Geo fusion transcript appears to generate a premature stop codon (UGA) at position 1461 (analyzed in silico at the National Center for Biotechnology Information Open Reading Frame Finder (NCBI ORF Finder). Taken together, PCR genotyping, quantitative real time RT–PCR and Western analyses indicate that the gene trap mutagenesis generated an effective null allele of the mouse cdc2l gene. Hereafter, we use cdc2lGT to denote this insertional mutation.

Disruption of one allele of cdc2l gene results in increased skin papilloma number and growth upon DMBA/TPA-mediated carcinogenesis

Next, we wanted to gain insight into the physiologic role of CDK11 in skin carcinogenesis. For this purpose, we investigated whether loss of CDK11 affected the development of skin papillomas induced by the classical skin carcinogenesis protocol. We studied the susceptibility of wild-type (cdc2l+/+) and gene-trapped (cdc2lGT) mice to chemical carcinogenesis in the DMBA/TPA-induced two-stage skin carcinogenesis model. Four carcinogen treatment groups were set up as detailed in Materials and methods (Figure 2A). The mice were genotyped by PCR analysis prior to the start of the study (supplementary Figure 2A, available at Carcinogenesis Online). Body weights of both cdc2l+/+ and cdc2lGT mice were not significantly different prior to the start of, or at any time during the course of, this study, suggesting that level of DMBA dosing was not toxic to the animals (supplementary Figure 2B, available at Carcinogenesis Online).

Both cdc2l+/+ and cdc2lGT mice started developing papillomas as early as week 10 of DMBA/TPA treatment (Group IV). By week 18 of the study, 50% of cdc2lGT as well as cdc2l+/+ mice developed at least one papilloma (tumor latency). In contrast, no tumors were observed in mice treated with acetone/acetone (Group I) or acetone/TPA (Group III). In the group treated with DMBA/acetone (Group II), a single cdc2lGT mouse developed four papillomas of more than 1 mm3 in size at week 14. Because the tumor burden became excessive in the DMBA/TPA treatment group, we terminated the experiment at week 19. The incidence of papillomas (number of animals with at least one papilloma) after DMBA/TPA treatment for cdc2lGT and cdc2l+/+ mice was similar (70 and 60%, respectively). Starting at week 14, we observed a marked difference in average number of papillomas and the number of papillomas per mouse in cdc2lGT mice relative to cdc2l+/+ mice (P < 0.05). By week 19 of the study, the average number of papillomas and number of papillomas per mouse (tumor multiplicity) in cdc2lGT mice was more than double than that in cdc2l+/+ mice (fold difference cdc2lGT/cdc2l+/+ = 2.7-fold) (Figure 2B). In addition, we also observed a higher frequency of larger tumors (1–5 and >5 mm3) in cdc2lGT mice as compared with cdc2l+/+ mice; P < 0.05 (Table I).

Gene-trapped cdc2lGT mice have reduced CDK11 protein levels

The major p110 isoform of CDK11 protein in humans corresponds to a 130 kDa protein in mice, which we hereby refer to as CDK11p130. By Western analysis, we observed that the protein levels of CDK11p130 were reduced in the skin of cdc2lGT mice as compared with wild-type cdc2l+/+ mice when treated with acetone/acetone (Group I) (Figure 2C, Lanes 1, 2). For DMBA/TPA (Group IV)-treated mice, although CDK11p130 protein was present in the normal skin...
tissue (S) from both $cde2^{+/+}$ (Figure 2C, Lanes 3, 5) and $cde2^G{T}$ (Figure 2C, Lanes 7, 9) mice, the level of CDK11 p130 protein was markedly suppressed in the adjacent papilloma tissue (T) of mice irrespective of their genetic backgrounds compared with vehicle (i.e. acetone/acetone)-treated mice (Figure 2C; $cde2^{+/+}$: Lanes 4, 6; $cde2^G{T}$: Lanes 8, 10). Interestingly, we observed the presence of a prominent smaller isoform of CDK11, 120 kDa in size ($CDK11^{p130}$), in the DMBA/TPA-induced papilloma tissue from wild-type $cde2^{+/+}$ mice in Group IV (Figure 2C, Lanes 4, 6). Taken together, these results suggest that there is a reduction in CDK11 p130 protein levels associated with DMBA/TPA exposure and that the carcinogen treatments led to the generation of a smaller CDK11 p120 isoform in the papilloma tissue of $cde2^{+/+}$ mice.

Harvey ras (H-ras) mutational analysis

Two distinct molecular characteristics associated with DMBA-initiated skin tumorigenesis are (i) oncogenic activation exclusively occurs in the ras family of proto-oncogenes and (ii) the mutations are
almost exclusively an A to T transversion (61CAA to 61CTA) at the middle adenine within codon 61 of the H-ras gene that creates an Xba I restriction site (Figure 3A) (29–31). We amplified the complete 208 bp exon 2 region of H-ras gene by PCR using genomic DNA extracted from papilloma tissue of cdc2l+/+ and cdc2lGT mice (Materials and methods). The primers were designed as described previously (28).

We then performed restriction fragment length polymorphism analysis for Xba I restriction site on these PCR amplified fragments to look for A to T transversion mutations in codon 61 of the H-ras gene (Figure 3B). We observed that the frequency of H-ras codon 61 mutations was high (three out of three cdc2lGT mice; i.e. 100%) in papilloma tissue regardless of the cdc2l allele status. In addition, direct sequencing verified A to T transversion in one of the alleles of the H-ras gene (Figure 3C).

Assessment of tumor growth and proliferation

Histological analysis did not reveal any marked differences between papillomas in cdc2lGT and wild-type mice (Figure 4A, 4B). Benign tumors displayed dyskeratosis, nuclear pleomorphism and an increase in the nuclear to cytoplasmic (N:C) ratio. There was evidence in one tumor (derived from a DMBA/TPA-treated cdc2lGT mouse) of progression toward squamous cell carcinoma. This carcinoma was graded as highly advanced, characterized by the presence of extensive and multiple foci of invasion into the lower dermal layers of the skin (data not shown).

Positive staining for Ki-67 was evident in papillomas as a diffuse nuclear staining with accentuation of the nuclei and chromosomes in proliferating cells. Staining was performed as described in Materials and methods. The PI was significantly higher in papillomas from DMBA/TPA (Group IV)-treated cdc2lGT mice (77.75 ± 3.96%) as compared with papillomas from cdc2l+/+ mice (30.84 ± 7.53%); P < 0.001 (Figure 4C, 4D). Apoptosis was assessed by staining for cleaved caspase-3 as described in Materials and methods. We did not observe any difference in the apoptotic index between cdc2lGT mice and cdc2l+/+ mice (data not shown).

The degree of hyperplasia was analyzed by estimating the epidermal thickness of tissue sections. Epidermal thickness of mice treated with various carcinogens was significantly higher as compared with vehicle-treated mice, irrespective of the genetic status of cdc2l gene (Table II). Interestingly, cdc2lGT mice treated with DMBA/acetone (cdc2lGT: 26.17 ± 24.03 μm versus cdc2l+/+; 10.0 ± 0.00 μm) as well as DMBA/TPA (cdc2lGT: 84.86 ± 42.53 μm versus cdc2l+/+; 62.73 ± 35.97 μm) showed a significant increase in epidermal thickness as compared with cdc2l+/+ mice from the same treatments, suggesting that DMBA treatment may influence cdc2l gene expression directly to affect proliferation (Table II). A representation of skin sections from different carcinogen treatment groups is shown in supplementary Figure 3 (available at Carcinogenesis Online) This result is in accordance with Ki-67 staining indicating higher degree of proliferation in skin tissue sections from cdc2lGT mice as compared with cdc2l+/+ mice (Figure 4E and F). Reference hematoxylin- and eosin-stained sections of the same animals are shown in Figure 4G and H.

Discussion

There is evidence that decreased expression of CDK11 encoded by the cdc2l gene locus is associated with the development of various malignancies in mice (21,22). However, no information is available, to date, to assess whether modulation of the cdc2l expression may directly affect tumorigenesis and/or progression in vivo. Chemically induced skin carcinogenesis studies continue to provide valuable information concerning the impact of genetic alterations on multi-step carcinogenesis and progression in genetically modified mice (32–35).

In the present study, we addressed the role of CDK11 in skin tumor initiation and promotion using haploinsufficient cdc2l mice. These mice express a significantly lower level of CDK11 protein in their skin compared with their wild-type littermates. We now report that in parallel with the decreased CDK11 expression, there was an increase in the multiplicity of benign papilloma lesions in cdc2lGT compared with cdc2l+/+ mice upon topical treatment with the tumor initiator DMBA followed by repeated applications of the tumor promoter TPA (Figure 2B). These results indicate, for the first time, that loss of cdc2l gene expression and reduced CDK11 levels may enhance cutaneous tumor promotion toward malignancy.

Gene trap technology has been invaluable in developing mutant mouse models for inactivating key genes in recent years (23–25). In the present study, we have carefully characterized our gene-trapped cdc2lGT mice. Primers P1 and P2, designed in exons 12 and 13, respectively (Figure 1A), generated a single product of 125 bp in size by RT–PCR analysis of cDNA from cdc2lGT mice. However, the larger transcript representing cdc2lβ-Geo fusion could not be detected. Also, primers P1 and P2, designed to detect a 275 bp product within the fusion region, failed to generate any product. Additionally, the fusion sequence generates a premature stop codon (UGA) at position 1461 suggesting that perhaps the fusion transcript remains undetectable by RT–PCR due to degradation of the null allele. Together with genotyping, real time RT–PCR and Western analyses, our results confirm that the cdc2lGT mice contain only one functional copy of the cdc2l gene. This is in accordance with previous studies showing that homozygote deletion of the cdc2l gene causes early embryonic lethality, whereas heterozygote mice develop normally (20).

We further evaluated the H-ras mutational status in papillomas from cdc2lGT and cdc2l+/+ mice. The present results show high frequency of A to T transversion mutations in codon 61 of H-ras gene in these mice irrespective of their genotype (Figure 3B). This observation is consistent with previous observations on H-ras oncogene activation upon exposure to DMBA/TPA treatments (29,30). These data suggest that the mechanisms by which mutations are introduced into the H-ras gene by DMBA does not differ between cdc2lGT and wild-type mice.

We also investigated the level of CDK11 in adjacent skin tissues and papillomas arising in cdc2lGT and cdc2l+/+ mice after DMBA/TPA treatment. As expected, we observed significantly reduced levels of CDK11 protein in the cdc2lGT mice in both adjacent skin and papillomas after DMBA/TPA treatment as compared with animals without treatment. Interestingly, whereas CDK11 was present in the adjacent skin of wild-type mice, an abundant smaller CDK11P120 isoform was observed in the papillomas derived from these cdc2l+/+ mice treated with DMBA/TPA (Figure 2C). It is plausible that DMBA and subsequent H-ras oncogene activation may lead to alternative promoter usage within the cdc2l gene resulting in the generation of the smaller isoform. Studies by Francone and Mezquita indicate that the presence of alternate CDK11 promoters and exons may play a role in the diversification of CDK11 transcripts during chicken testis development and upon testicular regression by diethylstilbestrol treatment (36). Another possibility is that DMBA may induce mutations within the cdc2l gene in wild-type animals leading to a truncated, smaller CDK11 protein. We are presently pursuing these possibilities.

To gain insight into the mechanism by which loss of cdc2l may contribute to tumor development, we evaluate cell proliferation and apoptosis in DMBA/TPA-induced tumors. We observed an increase in proliferation in tumors derived from the cdc2lGT mice compared with their wild-type littermates (Figure 4). This result is consistent with our observation of increased tumor multiplicity in cdc2lGT mice as compared with cdc2l+/+ mice. We also observed that the thickness of the epidermal skin tissue in cdc2lGT mice treated with DMBA/acetone or DMBA/TPA was higher than that in cdc2l+/+ mice (Table II and supplementary Figure 3, available at Carcinogenesis Online). However, promotion with multiple doses of TPA alone (acetone/TPA) in cdc2lGT and cdc2l+/+ mice showed similar degree of increase in epidermal thickness, suggesting that exposure to DMBA, and not TPA, might be responsible for increase in proliferation of cells in cdc2lGT mice. CDK11 isoforms are implicated in RNA processing and transcription, apoptosis and mitoses; all events that can contribute to
cancer. Exactly which function of CDK11 contributes to tumor promotion will require further study.

In conclusion, this is the first in vivo study using the two-stage skin chemical carcinogenesis protocol to directly address the role of holoenzyme sufficiency of cdc2l gene in tumor development. The loss of CDK11 protein levels markedly increases skin tumor multiplicity. These data suggest that loss of cdc2l, encoding CDK11, plays a role in regulating tumor promotion, possibly by facilitating the proliferation of the initiated cells in the tumor microenvironment and consequently facilitating tumor development.

Supplementary material

Supplementary figures 1–3 can be found at http://carcin.oxfordjournals.org/

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References


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Lack of cdc2l contributes to skin carcinogenesis