The CDK4/6 Inhibitor PD0332991 Reverses Epithelial Dysplasia Associated with Abnormal Activation of the Cyclin-CDK-Rb Pathway

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Abstract
Loss of normal growth control is a hallmark of cancer progression. Therefore, understanding the early mechanisms of normal growth regulation and the changes that occur during preneoplasia may provide insights of both diagnostic and therapeutic importance. Models of dysplasia that help elucidate the mechanisms responsible for disease progression are useful in highlighting potential targets for prevention. An important strategy in cancer prevention treatment programs is to reduce hyperplasia and dysplasia. This study identified abnormal upregulation of cell cycle–related proteins cyclin D1, cyclin-dependent kinase (CDK)4, CDK6, and phosphorylated retinoblastoma protein (pRb) as mechanisms responsible for maintenance of hyperplasia and dysplasia following downregulation of the initiating viral oncprotein Simian virus 40 (SV40) T antigen. Significantly, p53 was not required for successful reversal of hyperplasia and dysplasia. Ligand-induced activation of retinoid X receptor and PPARγ agonists attenuated cyclin D1 and CDK6 but not CDK4 or phosphorylated pRb upregulation with limited reversal of hyperplasia and dysplasia. PD0332991, an orally available CDK4/6 inhibitor, was able to prevent upregulation of cyclin D1 and CDK6 as well as CDK4 and phosphorylated pRb and this correlated with a more profound reversal of hyperplasia and dysplasia. In summary, the study distinguished CDK4 and phosphorylated pRb as targets for chemoprevention regimens targeting reversal of hyperplasia and dysplasia. Cancer Prev Res; 5(6); 810–21. ©2012 AACR.

Introduction
Although human dysplasia is considered to be a potentially reversible lesion, it has been difficult to develop appropriate therapies for this early precancerous stage. Because genetically engineered Tet-op-TAg⁴MMTV-tTA conditional mice show distinct phenotypes at 4 and 7 months of age following T antigen (TAg) downregulation, they can be used to investigate molecular mechanisms responsible for the marked reversal of hyperplasia and dysplasia found at 4 months of age as compared with the 7-month-old mice that exhibit refractory hyperplasia and dysplasia (1–3). As previously published, when TAg is downregulated after 4 months, hyperplasia and dysplasia reverse through a redifferentiation process (2). However, when TAg is downregulated after 7 months, hyperplasia and dysplasia persist (1, 2). If TAg is not downregulated in this model, hyperplasia and dysplasia progress to invasive ductal adenocarcinoma and lung metastases (3) that, like the hyperplasia and dysplasia found at 7 months of age, persist following downregulation of TAg.

Interrupting cancer development by inducing resolution of precancerous lesions is an established cancer prevention goal (4). Mouse models are a tool for investigating the molecular mechanisms of persistent dysplasia and testing candidate chemopreventives (5, 6). This study was initiated to elucidate the molecular mechanisms responsible for maintaining the irreversible stage of epithelial cell dysplasia and to test candidate pharmacologic agents UAB30, rosiglitazone, and PD0332991 for their ability to reverse this dysplasia.

Deregulation of the cell-cycle machinery is a fundamental hallmark of cancer progression and a common feature in carcinogenesis (7). Here, we focused on understanding why cell proliferation would persist when, theoretically, loss of TAg should restore function of retinoblastoma protein (pRb) and associated family members (8). pRb is one of
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the major regulators of proliferation and its phosphorylation is governed by the cell-cycle machinery (9). This machinery is made up of D and E-type cyclins, together with their associated kinases, cyclin-dependent kinase (CDK)4, CDK6, and CDK2 (10). In early and mid-G1, D-type cyclins and their CDK4/6 kinase cohorts are responsible for initiating pRb phosphorylation, leading to its functionally inactive hyperphosphorylated state and consequent passage through the restriction point into S phase (9–11). Cancer progression studies have identified the importance of genes controlling G1 to S phase progression in the cell cycle including, CDK4, CDK6, cyclin D1, its inhibitor p16, pRb, and p53 (7, 12, 13). Deregulation of p16, CDK4, CDK6, and cyclin D activities have been implicated in different preneoplastic conditions (14, 15) and are targets for cancer chemoprevention (4). PD0332991 is a pyridopyrimidine-derived CDK inhibitor with potential antineoplastic activity (16). Early reports documented its in vitro specificity against CDK4/6 and revealed its potent antiproliferative activity against subcutaneous human tumor xenografts (16, 17). An important predictor of response to PD0332991 is the presence of pRb in the targeted cells (18). Here, we used PD0332991 to test whether inhibition of the CDK4/6 pathway would promote regression of “irreversible” dysplasia.

Like pRb, p53 activity is also restored when TAg is downregulated (8). Enhancing p53 activity is one of the mechanisms hypothesized to be responsible for chemopreventive...
Figure 2. Comparison of protein expression patterns when TAg was downregulated in 4-month-old and 7-month-old mice. Representative Western blots illustrate the different expression patterns of cell-cycle regulatory proteins accompanying TAg downregulation in submandibular salivary tissue in 4-month-old (A) and 7-month-old (B) Tet-op-TAg<sub>MMTV-tTA</sub> mice over a 14-day time course. C–G, line graphs illustrate mean relative steady-state protein levels in 4-month-old (dotted lines) and 7-month-old (solid lines) mice before and 2, 4, 6, 10, and 14 days after initiating doxycycline to downregulate TAg. Relative protein expression levels at different time points were compared with Student's t test (2-tailed). C, cyclin D1, CDK6, and CDK4 expression levels were significantly higher in 7-month-old than in 4-month-old mice at the 10-day (cyclin D1, 4-fold (▲, P < 0.02); CDK6, 2-fold (▲, P < 0.01); CDK4, 3-fold (▲, P < 0.01)).
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In this study, we used genetically modified mice with germ line deficiency of p53 (p53−/−; ref. 21) to assess whether p53 was required for reversal of the dysplasia. Rexinoid chemopreventives target the retinoid X receptor (RXR; ref. 22) and may work, at least in part, by down-regulating cyclin D1 expression (23). PPARs form heterodimers with RXR after activation (24), and combination treatment targeting both RXR and PPARs has been reported to be more effective than single-agent therapy (25). PPAR agonists have also been shown to downregulate cyclin D1 expression (26). We tested whether the RXR agonist UIAB30 (27) alone or in combination with PPAR agonist rosiglitazone could promote regression of the "irreversible" hyperplasia.

Results showed that the CDK4/6 inhibitor (PD0332991) was able to reverse previously "irreversible" epithelial dysplasia associated with downregulation of CDK4, CDK6, and profound loss of pRb phosphorylation. In contrast, while the RXR and PPAR ligands reduced levels of CDK6 and phosphorylated pRb, there was no significant impact on CDK4 expression levels and dysplasia was not reversed. p53 was found to be dispensable for the reversal process. The study points to a role for CDK4/6 inhibitors in chemoprevention.

Materials and Methods

Mouse models and pharmaceutical agents

Tet-op-TAp53−/− and tet-op-TAp53−/− mice were identified by PCR (Transnetyx). Tet-op-TAp53−/− mice were generated by breeding Tet-op-TAp53−/− mice (1) with p53−/− mice (21). Male mice were used to control for sexual dimorphic effects in salivary gland development (28). Cohorts of 7-month-old mice were randomized by dividing littermates into intervention or control groups. Submandibular salivary gland tissue was harvested during necropsy and snap frozen or fixed in 10% buffered formalin. Doxycline was administered to downregulate Tag expression either in chow (200 mg/kg; Bio-Serv) or in water (200 μg/ml; Fisher Scientific; ref. 2) either alone or concurrently with the following drugs: UIAB30 (300 mg/kg/chow) alone or with rosiglitazone (400 mg/kg/chow) for 14 or 28 days (provided by University of Alabama), or PD0332991 (150 mg/kg/oral gavage; Selleck Chem) for 14 days (provided by University of Alabama), or PD0332991 (150 mg/kg/oral gavage; Selleck Chem) for 14 days (provided by University of Alabama), or PD0332991 (150 mg/kg/oral gavage; Selleck Chem) for 14 days (provided by University of Alabama).

Real-time RT-PCR

Total RNA was isolated with TRIzol (Invitrogen Life Technologies; ref. 29). TaqMan Gene Expression Assays (ABI Prism 7700) detected Adrp (Mm00445878_m1), Fapb4 (Mm00454587_m1), Pdk4 (Mm00483162_m1), Cdki (Mm00772472_m1), cell division cycle 25 homolog A (Schizosaccharomyces pombe; Cdc25a; Mm00483162_m1), retinoblastoma-like 1 (p107; Rbl1; Mm01250721_m1), and 18s rRNA (Hs99999901_s1) reactions were carried out following manufacturer’s recommendations with ABI Prism 7700 sequence detector and data analyzed with ABI Software (Applied Biosystems). Relative mRNA gene expression normalized against untreated control mice (2ΔΔCt), where ΔCt = Ct (target gene) − Ct (18s rRNA; ref. 30).

Histologic analyses

For statistical analyses, the extent of dysplasia was quantified by determining the percentage of hyperplastic and dysplastic, in-transition, and normal-like ductal structures (n = 1,000 ± 200 ductal structures counted per section) on hematoxylin and eosin (H&E)–stained formalin-fixed sections of the submandibular salivary gland. Normal-like structures were defined as fully striated differentiated ductal epithelial cells with monomorphic small nuclei. In-transition structures showed partially striated ductal epithelial cells. Dysplastic structures were defined as ductal structures that did not contain any differentiated striated ductal cells. Hyperplastic structures were defined as structures with an abnormal increase in the number of ductal cells. An academic board certified pathologist (B.V.S. Kallakury) blinded to the identity of the specimens and interventions identified normal-like, in-transition, hyperplastic, and dysplastic structures in the tissue sections. This analysis confirmed the significant differences in distribution of the 4 different types of structures in the different treatment and intervention groups.

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Western blots and immunohistochemistry

For Western blots (WB), protein samples were quantified (29) and 60 µg fractionated on 45% to 12% gradient Bis-Tris gels (NP0335; Invitrogen Life Technologies, Inc.), electrophoretically transferred onto nitrocellulose membranes (Amersham Biosciences), blocked in 5% nonfat dry milk in Tris-buffered saline and 0.1% Tween (1 hour, room temperature) and exposed to the respective primary antibody overnight at 4°C. Membranes were exposed to the manufacturer-recommended dilution of the appropriate secondary antibody anti-rabbit NA934V (GE Healthcare), anti-goat SC-2768, or anti-mouse SC-2005 (Santa Cruz Biotechnology). Proteins were visualized (ECL Plus Western Blotting Detection kit; Amersham Biosciences), quantified (Adobe Photoshop CS5), and mean relative densities and SEM calculated after normalization to β-actin. Immunohistochemistry (IHC) was carried out with Vectastain ABC or Mouse On Mouse (M.O.M) Peroxidase kits (Vector Laboratories, Inc.) following manufacturer’s recommendations and digital photographs taken (Nikon Eclipse E800M microscope with DMX1200 software; Nikon Instruments, Inc.). Western blot primary antibodies: anti-cyclin D1 (DCS6) 1:2,000; anti-CDK4 (DCS156) 1:10,00; anti-CDK6 (DCS83) 1:1,000; anti-cyclin E (HE12) 1:1,000; anti-pRb-Ser-807/811 (9308) 1:1,000 (Cell Signaling Technology), anti-CDK2 (sc-163) 1:200; anti-pRb (sc-50) 1:200; anti-p21 (sc-6246) 1:200; anti-p27 (sc-528) 1:200; anti-CDK4 (DCS156) 1:200; anti-pRb (sc-50) 1:200; anti-p21 (sc-6246) 1:200; anti-p27 (sc-528) 1:200; anti-CDK2 (sc-163) 1:200; anti-E2F-1 (sc-193) 1:200; and anti-TAg (sc-147) 1:200 (Santa Cruz Technology).
IHC primary antibodies: anti-cyclin D1 (sc-718) 1:50; anti-CDK6 (sc-7961) 1:50; anti-CDK4 (sc-260) 1:200; anti-CDK2 (sc-163) 1:1,500; anti-cyclin E (sc-198) 1:80; anti-p21 (sc-6246) 1:50; anti-pRb (sc-50) 1:25; anti-DP-1 (sc-610) 1:80; anti-E2F-1 (sc-193) 1:40 (Santa Cruz Biotechnology, Inc.), anti–pRb-Ser-807/811 (9308; 1:100; Cell Signaling Technology) and anti-p27 (610241; 1:3,000; BD Biosciences) were used.

Statistical analyses

Differences in relative protein expression levels and percentages of hyperplastic and dysplastic, in-transition and normal-like ductal structures were compared with the Student t tests (2-tailed) and differences in relative RNA expression levels were compared with Mann–Whitney U and one-way ANOVA (GraphPad Prism version 4.03 for Windows, GraphPad Software). Significance was assigned at \( P < 0.05 \).
Treatment with RXRa and PPARγ ligands UAB30 and rosiglitazone did not change percentage of normal-like structures but did alter expression levels of cyclin D1, CDK6, phosphorylated Rb, and E2F1 in 7-month-old mice. Stacked bar graphs comparing distribution of hyperplastic and dysplastic, in-transition and normal-like structures in submandibular salivary glands from untreated control and 14-day UAB30-treated (A) and in untreated control and 28-day UAB30 + ROSI–treated (B) 7-month-old Tet-op-TAgMMTV-tTA/C0 (tTA/TAg) mice in which TAg was coincidentally downregulated by doxycycline.

C, representative Western blots illustrate expression levels of cell-cycle regulatory proteins in submandibular salivary tissue of untreated control and 28-day UAB30 + ROSI–treated 7-month-old Tet-op-TAgMMTV-tTA/C0 mice that coincidentally received doxycycline to downregulate TAg. D, bar graphs comparing relative protein expression levels in submandibular salivary tissue 28 days after TAg downregulation in control untreated and UAB30 + ROSI–treated 7-month-old Tet-op-TAgMMTV-tTA/C0 mice. Expression levels of cyclin D1 were reduced 2-fold ($P < 0.01$), CDK6 were reduced 3-fold ($P < 0.02$), phosphorylated pRb were reduced 3-fold ($P < 0.05$), and E2F-1 were reduced 4-fold ($P < 0.01$) in the UAB30 + ROSI–treated mice. Relative protein expression levels determined by quantification of Western blots using densitometry. Mean and SEM indicated. E, Box plots comparing relative RNA expression levels in submandibular salivary tissue of untreated control and 28-day UAB30 + ROSI–treated 7-month-old Tet-op-TAgMMTV-tTA/C0 mice with Lvl RNA expression levels in control untreated mice. Expression levels of Fabp4 were reduced 1.9-fold ($P < 0.01$), Adrp were reduced 1.8-fold ($P < 0.01$), and Pdk4 were reduced 1.4-fold ($P < 0.01$) in the UAB30 + ROSI–treated mice. Relative RNA expression levels determined by real-time PCR.
Results

Normal histology progressively established following TAg downregulation at 4 but not 7 months of age

TAG expression targeted to the submandibular salivary gland resulted in salivary dysplasia extending throughout most of the gland by 4 months of age in tet-op-TAgMMTV-tTA mice (Fig. 1A). TAg downregulation led to reversal of hyperplasia and dysplasia and establishment of normal-like histology in 4-month-old but not in 7-month-old mice (Fig. 1A–H). The histologic phenotype at both ages consisted of hyperplastic and/or dysplastic (Fig. 1A–C), in-transition (Fig. 1D), or normal-like ductal structures (Fig. 1E) that resembled those of wild-type mice (Fig. 1F). Although the percentages of these structures were similar at 4 and 7 months of age before TAg downregulation, the percentages were significantly different after TAg downregulation. The percentages of hyperplastic or dysplastic [‡, 58% vs. 8%, P < 0.05, Student t test (2-tailed)] and in-transition structures (‡‡, 32% vs. 15%, P < 0.05, Student t test (2-sided)] were significantly higher and the percentage of normal-like structures (‡‡‡, 10% vs. 76%, P < 0.05, Student t test (2-tailed)] were significantly lower in 7-month-old than in 4-month-old mice 14 days after TAg downregulation (Fig. 1G and H).

Activation of the cyclin-CDK-Rb pathway following TAg downregulation correlated with maintenance of dysplasia

Expression levels of cyclin D1, CDK6, and CDK4 were significantly higher in 7-month-old than in 4-month-old mice 10 days after TAg downregulation (all P < 0.05, Student t test (2-tailed)). Expression levels of these proteins initially rose at both ages, but in 7-month-old mice they further increased while they decreased to starting levels in 4-month-old mice (Fig. 2A–C). Phosphorylated pRb, DP-1, and E2F-1 were also expressed at significantly higher levels in the 7-month-old mice by the 14-day time point [all P < 0.05, Student t test (2-tailed); Fig. 2A, B, D, and E)]. There was no significant increase in p21 and p27 expression in the 7-month-old, unlike the 4-month-old mice (Fig. 2A, B, and F). Expression patterns of cyclin E and CDK2 were similar in the 4-month-old and 7-month-old mice with significantly higher levels of cyclin E at the 10-day and CDK2 at the 14-day time point in the 7-month-old mice [both P < 0.05, Student t test (2-tailed; Fig. 2A, B, and G)]. IHC revealed parallel expression changes (Fig. 3A–K). Fourteen days after TAg downregulation, 7-month-old mice showed increased expression of cyclin D1, CDK6, CDK4, CDK2, phosphorylated pRb, DP-1, and E2F-1 with decreased expression of p21 and p27 as compared with 4-month-old mice. Real-time RT-PCR was used to evaluate whether or not these changes in cell-cycle regulatory gene expression altered RNA expression levels of known E2F target genes Cdk1, Rbl1, and Cdc25a in the submandibular salivary tissue (Supplementary Fig. S1). Whereas before TAg downregulation, expression levels were equivalent in 4-month-old and 7-month-old mice, after 14 days of doxycycline expression levels of Cdk1 and Rbl1 were significantly higher in the 7-month-old mice (both P < 0.05, ANOVA). This was due to the more modest reduction in Cdk1 and Rbl1 gene expression seen in the 7-month-old as compared with 4-month-old mice following TAg downregulation. In contrast, Cdc25a expression was equivalently reduced in 4- and 7-month-old mice following TAg downregulation (P < 0.05, ANOVA).

p53 was not required for dysplasia reversal following TAg downregulation in 4-month-old mice

p53 is one regulator of p21 and p27 expression. Theoretically, downregulation of TAG expression would restore p53 function and increase p21 and p27 expression (8, 19, 20). To test whether p53 and associated p21 and p27 upregulation were required for dysplasia reversal, the extent of hyperplasia and dysplasia as well as gene expression changes were compared before and after TAg downregulation in 4-month-old tet-op-TAgMMTV-tTA/p53−/− mice. The percentage of hyperplastic and dysplastic structures was significantly lower 14 days after TAg downregulation [28% vs. 90%, P < 0.01, Student t test (2-tailed)] even though p53 was absent and there was no upregulation of p21 or p27 (Fig. 4A). Expression levels of cyclin D1, CDK6, CDK4, CDK2, phosphorylated pRb, DP-1, and E2F-1 at the 14-day time point were not significantly different compared with 4-month-old tet-op-TAgMMTV-tTA mice [all P > 0.05, Student t test (2-tailed); Fig. 4B)]. A provocative finding was the significantly lower extent of dysplasia at baseline in mice lacking p53 as compared with mice with intact p53 [28% vs. 90%, P = 0.0079, Student t test (2-tailed; Figs. 1G and 4A)].

Treatment with RXR and PPARγ agonists UAB30 and rosiglitazone downregulated expression levels of cyclin D1, CDK6, phosphorylated Rb, and E2F1 but did not change percentage of normal-like structures found in 7-month-old mice following TAg downregulation

To test whether UAB30 or the combination of UAB30 with rosiglitazone could promote restoration of normal-like structures in the submandibular salivary tissue or impact expression of cell-cycle regulatory genes in 7-month-old mice following TAg downregulation, UAB30
Figure 6. CDK4/6 inhibitor, PD0332991, successfully reversed dysplasia in 7-month-old mice. A, stacked bar graphs showing distribution of hyperplastic and dysplastic, in-transition and normal-like structures in submandibular salivary glands from untreated control and 10-day PD0332991-treated 7-month-old Tet-op-TAgMMTV-tTA mice in which TAg was coincidentally downregulated by doxycycline. B, representative Western blots illustrate expression levels of cell-cycle regulatory proteins in submandibular salivary tissue in untreated control and 10-day PD0332991-treated 7-month-old Tet-op-TAgMMTV-tTA mice coincidentally receiving doxycycline to downregulate TAg. Control and PD0332991-treated samples were run on the same blot and cropped to show representative lanes. C, bar graphs comparing relative expression levels of proteins 10 days after TAg downregulation in control untreated and PD0332991-treated 7-month-old mice. Expression levels of cyclin D1 were reduced 3-fold ($P < 0.04$), CDK6 were reduced 5-fold ($P < 0.003$), CDK4 were reduced 11-fold ($P < 0.0003$).
was administered for either 14 or 28 days alone or in combination with rosiglitazone (ROS1) for 28 days. No significant change in the percentage of differentiated normal-like ductal structures were found in the treated mice as compared with untreated controls at the same time point following TAg downregulation (Fig. 5A and B, data not shown for UAB30 treatment alone for 28 days). However, cyclin D1, CDK6, phosphorylated pRb, and E2F-1 were significantly lower in mice exposed to UAB30 and rosiglitazone for 28 days [all $P < 0.05$, Student t test (2-tailed; Fig. 5C and D)]. In contrast, expression levels of CDK4 and DP-1 were not significantly changed (Fig. 5C and D). Expression levels of E2F downstream genes Cdk1 and Rbl1 were reduced but remained significantly higher than those measured in 4-month-old mice treated with doxycycline for 14 days ($P < 0.05$, ANOVA; Supplementary Fig. S1). Downregulation of TAg was confirmed by Western blot (Supplementary Fig. S2A). To test whether drug levels delivered were sufficient to impact expression levels of RXR and PPARγ downstream genes in the submandibular salivary gland and/or reduce liver retinyl palmitate protein levels (31), steady-state RNA expression levels of Fabp4, Adrp, and Pdk4 in salivary and protein levels of retinyl palmitate in liver tissue were compared with untreated controls in all 3 treatment groups. Expression levels of all 3 genes were significantly increased by 14 days of UAB30 treatment with no further increase by extension of UAB30 treatment to 28 days or addition of rosiglitazone in a 28-day regimen ($P < 0.05$ compared with no treatment, Mann--Whitney U test; Fig. 5E). In contrast, liver retinyl palmitate levels were not significantly reduced after 14 days of UAB30 treatment but were significantly reduced after 28 days of treatment with UAB30 alone and UAB30 + ROSI (both $P < 0.02$, Student t test, 2-tailed; Supplementary Fig. S3). These results indicated that drug levels achieved by 28 days of UAB30 and rosiglitazone treatment were sufficient to impact gene expression in the salivary gland as well as alter liver metabolism. A comparison of the expression patterns of cell-cycle regulatory proteins accompanying reversal to normal-like ductal structures in 4-month-old mice to the pattern following treatment with UAB30 and ROSI suggested that significant downregulation of CDK4 and/or DP-1 could be required, perhaps in addition to the changes in other cell-cycle regulatory proteins, to promote the appearance of normal-like ductal structures.

The CDK4/6 inhibitor PD0332991 reversed the "irreversible" dysplasia in 7-month-old mice

CDK4 and DP-1 were identified as potential key targets for downregulation in experiments presented above suggesting that a CDK4/6 inhibitor might be successful in promoting regression of refractory dysplasia. Ten days of exposure to the CDK4/6 inhibitor PD0332991 significantly reduced the percentage of hyperplastic and dysplastic ductal structures [58% vs. 7%; $^*P < 0.01$, Student t test (2-tailed)] and significantly increased the percentage of normal-like structures [9% vs. 62%; $^{**}P < 0.01$, Student t test (2-tailed)] as compared with untreated controls at the same time point following TAg downregulation (Fig. 6A). Cyclin D1, CDK6, CDK4, phosphorylated pRb, DP-1, and E2F-1 were significantly reduced and p21 and p27 significantly increased as compared with untreated controls [all $P < 0.05$, Student t test (2-tailed; Fig. 6B and C)]. Parallel changes were found by immunohistochemistry (Fig. 6D). Expression levels of E2F downstream genes Cdk1 and Rbl1 were not significantly different than those quantified in 4-month-old mice treated with 14 days of doxycycline (Supplementary Fig. S1), illustrating that all the molecular changes measured in the 4-month-old mice receiving doxycycline alone were paralleled in the 7-month-old mice that received PD0332991 with doxycycline. Downregulation of TAg was confirmed by Western blot (Supplementary Fig. S2B).

Discussion

Maintenance of cell-cycle checkpoints is an obligatory element of normal cells to avert malignant transformation through uncontrolled growth. Here, we show that the cyclin-CDK-Rb pathway can be activated in response to oncprotein downregulation in a time-dependent "hit-and-run"--type model and that this activation is sufficient to maintain dysplasia. Perhaps more importantly, our experiments showed that an orally available CDK4/6 inhibitor was able to reverse this dysplasia. Such drugs may be well tolerated as many physiologically mediated growth signals still elicit normal responses even when levels of cell-cycle effector proteins are low (32–35). It is possible that a requirement for upregulated cyclin-CDK-Rb pathway may be limited to cells responding to mitogenic signals leading to malignant transformation (33) and targeting of these cell-cycle proteins might resolve dysplasia while leaving normal cells unaffected (36–38).

The majority of established cancer therapies are cytotoxic agents designed to kill cells (39). In the last decade, cytostatic drugs that inhibit tumor growth in part by decreasing cell proliferation without direct cell killing were developed for their lower toxicity and increased tolerance (40). However, in cancers, single-agent cytostatic drugs have not been as efficacious as required leading to the use of combination therapies with both cytotoxic and cytostatic agents (40). Chemoprevention aims to reverse or halt the progression of...
neoplastic cells to invasive malignancies and cytostatic agents that do so without direct killing have been proposed as potential therapies (20, 41, 42). In this study, a single cytostatic agent, PD0332991, which specifically targets CDK4/6, was sufficient to reverse refractory dysplasia.

Both biologically mediated dysplasia reversal in 4-month-old mice and pharmacologic reversal in 7-month-old mice 14 and 10 days following TAg downregulation were associated with an increased percentage of normal-like structures that was correlated with multifold reductions in levels of CDK6, CDK4, and phosphorylated pRb (4- and 4-fold for CDK6, 22- and 11-fold for CDK4, and 68- and 17-fold for phosphorylated pRb, respectively) and significant reductions in E2F target genes Cdk1 and Rbl1. In contrast, no increased percentage of normal-like structures was found with the more modest 3-fold changes in CDK6 and phosphorylated pRb levels and unchanged CDK4 levels following up to 28 days of treatment with UAB30 and rosiglitazone and expression levels of Cdk1 and Rbl1 were not reduced to the same extent as found following PD0332991 treatment. This suggests that there are thresholds of changes in cell-cycle regulators that are necessary to meet to restore normal-like tissue histology. These parameters of change may serve as molecular measures of therapeutic efficacy in trials of candidate chemopreventive regimens.

Simian virus 40 (SV40) large TAg is a potent oncoprotein that has been shown to transform a variety of cell types. The transformation potential of TAg is largely attributed to its disruption of the p53 and pRb tumor suppressor proteins (43, 44). TAg can bind directly to the specific DNA-binding domain of p53, and it has been hypothesized that upon binding, TAg inactivates the tumor suppressor function of p53 (8). However, the binding of p53 with TAg also increases the half-life and steady-state levels of p53 in cell culture partly due to the entrapment of the p300/mdm2/p53 complex that targets p53 for degradation (45, 46). In vitro tissue culture cell studies have suggested an unexpected role for p53 within the TAg complex by showing that the TAg-p53 complex has growth stimulatory activities that are required for malignant cell growth (47). Depletion of p53 then leads to structural rearrangements of the multiprotein complex resulting in growth arrest (47). The experiments presented here suggest this may also occur in vivo in whole tissue because the salivary glands in our tet-op-TAgMMTV-tTA/ p53−/− mice exhibited less dysplasia than the aged-matched tet-op-TAgMMTV-7TA controls with intact p53. Significantly, the negative cell-cycle regulators p53, p21, and p27 did not seem to play a significant role in dysplasia reversal underscoring the importance of the cyclin-CDK-Rb pathway in maintenance of the dysplasia. Interestingly, the positive cell-cycle regulator Cdk25a may also be disconnected from dysplasia reversal in this model as its expression was repressed with downregulation of TAg regardless of whether or not dysplasia reversal occurred. This direct correlation with TAg expression may be similar to that reported for human papillomavirus E6/E7 protein expression and Cdk25a (48). Like TAg, the papillomavirus E6/E7 proteins also target and inactivate Rb and p53.

Our studies showed that the cyclin-CDK-Rb pathway was activated in a time-dependent manner following oncoprotein downregulation and served to maintain dysplasia in the absence of the initiating oncoprotein. The CDK4/6 specific inhibitor, PD0332991 was used to show that effective targeting of this pathway was associated with reversal of dysplasia and hyperplasia. It is possible that alternative RXR or PPARα agonists or longer regimens not tested here could more effectively target this pathway as an alternative RXR agonist LG100268 was reported to reverse dysplasia in lung epithelium (49). Next steps for the experiments presented here would be to determine how durable the reversal response of a 10-day PD0332991 treatment course is by following mice after treatment and evaluating whether dysplasia returns as well as directly comparing the cancer preventive efficacy of the different regimens investigated here.

In summary, our results indicate a candidate mechanism for reversal of hyperplasia and dysplasia and provide parameters to follow in evaluating potential biomarkers of response.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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