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ORIGINAL ARTICLE

Disruption of cyclin D1 nuclear export and proteolysis accelerates mammary carcinogenesis

DI Lin[1,2], MD Lessie[1,2], AB Gladden[1,2], CH Bassing[1,3], KU Wagner[4] and JA Diehl[1,2]

[1]Abramson Family Cancer Research Institute, University of Pennsylvania, Philadelphia, PA, USA; [2]Department of Cancer Biology, University of Pennsylvania, Philadelphia, PA, USA; [3]Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia, University of Pennsylvania, Philadelphia, PA, USA and [4]Department of Pathology and Microbiology, Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE, USA

Cyclin D1 levels are maintained at steady state by phosphorylation-dependent nuclear export and polyubiquitination by $SCF^{FBX4\text{-}\alpha B\ crystallin}$. Inhibition of cyclin D1 proteolysis has been implicated as a causative factor leading to its overexpression in breast and esophageal carcinomas; however, the contribution of stable cyclin D1 to the genesis of such carcinomas has not been evaluated. We therefore generated transgenic mice wherein expression of either wild-type or a stable cyclin D1 allele (D1T286A) is regulated by MMTV-LTR. MMTV-D1T286A mice developed mammary adenocarcinomas at an increased rate relative to MMTV-D1 mice. Similar to human cancers that overexpress cyclin D1, D1T286A tumors were estrogen receptor-positive and exhibited estrogen-dependent growth. Collectively, these results suggest that temporal control of cyclin D1 subcellular localization and proteolysis is critical for maintenance of homeostasis within the mammary epithelium.
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Introduction

Uncontrolled cellular proliferation is a hallmark of cancer. Cyclin D1, together with its catalytic partner CDK4/6, promotes G_1–S-phase transition via phosphorylation of the retinoblastoma protein and through titration of cell cycle inhibitors $p27^{Kip1}$ and $p21^{Cip1}$. These functions of cyclin D1 ensure efficient initiation of S phase (Cheng *et al*., 1998; Harbour *et al*., 1999). During late G_1 and S phases, cyclin D1 is phosphorylated on Thr-286 (p-286) by GSK3β, which triggers two events (Diehl *et al*., 1998). First, p-286 cyclin D1 is targeted by the nuclear exportin, CRM1, resulting in cyclin D1 nuclear export. Second, once in the cytoplasm, the E3 ubiquitin ligase, $SCF^{FBX4\text{-}\alpha B\ crystallin}$, binds to p-286 and targets cyclin D1 for proteasomal degradation. Subversion of either event results in uncontrolled cellular proliferation (Alt *et al*., 2000; Lin *et al*., 2006).

Cyclin D1 plays an essential role in mammary gland development and carcinogenesis, thereby making it an attractive target for breast cancer. Cyclin D1 null mammary epithelium fails to undergo the proliferative burst associated with pregnancy, resulting in mothers unable to nurse their pups (Sicinski *et al*., 1995). Additionally, cyclin D1 null mice are resistant to mammary tumorigenesis induced by erbB2 and Ras, revealing a requirement for cyclin D1 downstream of these oncogenes (Yu *et al*., 2001). These cyclin D1 functions, together with the demonstration that cyclin D1-dependent tumorigenesis relies on the activation of CDK4 (Landis *et al*., 2006; Yu *et al*., 2006), suggest that pharmacological inhibitors of the cyclin D1-CDK4 kinase could be effective therapeutic agents for human breast cancer. In general, human breast cancers that overexpress cyclin D1 are estrogen receptor-positive, indicating that targeting both cyclin D1-associated kinases and estrogen receptor (ER) may further inhibit tumor growth (Utsumi *et al*., 2000; Butt *et al*., 2005).

Wild-type cyclin D1 is weakly oncogenic, likely reflecting the capacity of cells to efficiently maintain threshold levels of active cyclin D1/CDK4 via cytoplasmic degradation. Indeed, mice overexpressing cyclin D1 in mammary epithelium develop adenocarcinomas with a protracted latency, while overexpression in lymphocytes does not elicit a tumorigenic phenotype (Bodrug *et al*., 1994; Wang *et al*., 1994). In contrast, overexpression of constitutive nuclear, nondegradable cyclin D1 mutants results in transformation of fibroblasts *in vitro* and of lymphocytes *in vivo* (Alt *et al*., 2000; Gladden *et al*., 2006). These studies suggest that nuclear export and cytoplasmic proteolysis reduce the oncogenicity of cyclin D1.

To evaluate the role of constitutively nuclear and nondegradable cyclin D1 in mammary carcinogenesis, we generated transgenic mice expressing wild-type or a

Correspondence: Dr JA Diehl, Abramson Family Cancer Research Institute, University of Pennsylvania, 454 BRB II/III, 421 Curie Blvd., Philadelphia, PA 19104, USA.
E-mail: adiehl@mail.med.upenn.edu
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phosphorylation-deficient cyclin D1 (D1T286A) whose expression is directed by the MMTV-LTR. We demonstrate that cyclin D1 phosphorylation, localization and ubiquitination are regulated events in mammary epithelium *in vivo*. Our data reveal that disruption of normal cyclin D1 phosphorylation, nuclear export and cytoplasmic proteolysis accelerates mammary carcinogenesis, demonstrating that nuclear, nondegradable cyclin D1 is a more potent oncogene than wild-type cyclin D1.

Results

Regulation of cyclin D1 Thr-286 phosphorylation, subcellular localization and ubiquitination in mammary epithelium

We assessed the regulation of cyclin D1 phosphorylation and subcellular localization relative to the proliferative status of mammary epithelium. Immunohistochemical staining revealed intensely nuclear cyclin D1 in epithelial cells during pregnancy, a period of rapid proliferation

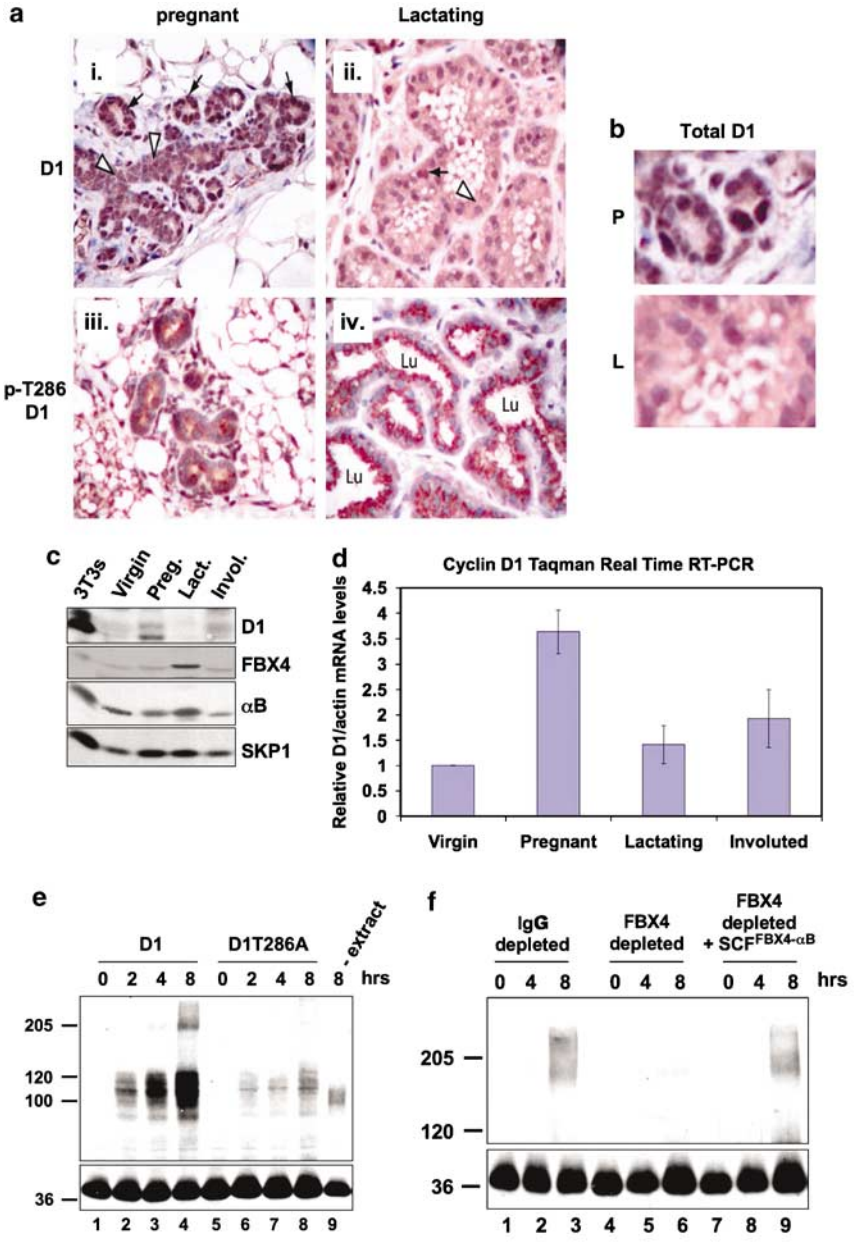

Figure 1 Cyclin D1 Thr-286 phosphorylation, subcellular localization and protein levels are regulated in mammary epithelium *in vivo*. (**a**) Immunohistochemistry for total cyclin D1 during pregnancy (i), lactation (ii) or p-286 cyclin D1 during pregnancy (iii) or lactation (iv). (**b**) High magnification of total D1 during pregnancy (P) and lactation (L). (**c**) Mammary extracts were prepared from age-matched virgin, pregnant (day 10), lactating (day 10) and involuted (day 15) glands from nontransgenic mice and blotted with the indicated antibodies. (**d**) Cyclin D1 mRNA levels during mammary gland development. (**e** and **f**) Cyclin D1 *in vitro* ubiquitination assays with lactating (day 10) mammary extracts.

(Figures 1ai and b, top panel). Tertiary ducts (Figure 1ai, hollow arrows) contained less nuclear cyclin D1 compared to proliferative alveolar cells (Figure 1ai, solid arrows). In contrast, during lactation, a nonproliferative differentiated state, occasional differentiated alveolar cells retained weak nuclear cyclin D1 (Figure 1aii, solid arrow). However, cyclin D1 levels were reduced and cytoplasmic (Figure 1aii, hollow arrow; Figure 1b, bottom panel), suggesting a high rate of phosphorylation-dependent nuclear export and proteolysis during this stage. Indeed, staining with a p-286-specific antibody revealed weak cytoplasmic phospho-cyclin D1 staining during pregnancy (Figure 1aiii) and increased cytoplasmic staining during lactation (Figure 1aiv). This is consistent with *in vitro* analysis demonstrating p-286-dependent cyclin D1 nuclear export (Alt *et al*., 2000).

We confirmed cyclin D1 accumulation during pregnancy and loss during lactation by western analysis (Figure 1c). Cyclin D1 mRNA levels were also high during pregnancy, suggesting that cyclin D1 transcription contributes to its accumulation during pregnancy (Figure 1d).

Strikingly, protein levels of specificity components of the cyclin D1 E3 ubiquitin ligase, FBX4 and αB crystallin, were high in the lactating gland as compared to age- and parity-matched virgin, pregnant and involuted glands (Figure 1c). To directly address the role of FBX4 and p-286 for cyclin D1 ubiquitination in mammary epithelium, we prepared ubiquitination-competent extracts from lactating mammary glands. *In vitro* ubiquitination of cyclin D1 was observed in a time- and p-286-dependent manner (Figure 1e, lanes 1–4 vs 5–8). Ubiquitination was dependent on mammary extracts (Figure 1e, lane 9). Ubiquitination was also temperature-, magnesium- and ATP-dependent (data not shown). Consistent with $SCF^{FBX4\text{-}\alpha B\ crystallin}$ directing p-286-dependent ubiquitination of cyclin D1, depletion of FBX4 (Supplementary Figure S1A) significantly abrogated cyclin D1 ubiquitination (Figure 1f, lanes 1–3 vs 4–6); ubiquitination was restored by reconstitution of depleted extracts with purified, recombinant $SCF^{FBX4\text{-}\alpha B\ crystallin}$ (Figure 1f, lanes 7–9).

Generation of MMTV-D1 and MMTV-D1T286A mice

To determine whether disruption of cyclin D1 phosphorylation contributes to its neoplastic potential in mammary epithelium, we generated transgenic mice harboring either wild-type cyclin D1 or D1T286A, under the control of the MMTV-LTR (Figure 2a). Expression of both transgenes was confirmed by western analysis (Figures 2b–d). Both transgenes were expressed specifically in the mammary gland with slightly greater levels of D1T286A than D1 as expected due to its increased stability. Both D1 and D1T286A assembled with CDK4, $p27^{Kip1}$, $p21^{Cip1}$ and retained kinase activity (Figure 2e and data not shown). During lactation, a period of increased p-286 cyclin D1 (Figure 1a), cyclin D1 exhibited cytoplasmic and nuclear staining (Figure 2f, left panels). In contrast, D1T286A was nuclear during lactation, with increased nuclear intensity relative to transgenic wild-type D1, indicating that p-286 induces cyclin D1 nuclear export and proteolysis in mammary epithelial cells (MECs; Figure 2f, right panels).

Abrogation of cyclin D1 phosphorylation accelerates mammary carcinogenesis

Long-term cohorts were established for assessment of tumor formation. Females underwent two or more pregnancies to maximize transgene expression. Disease manifestation was defined as the development of palpable tumors that were subsequently confirmed to be mammary carcinomas via histological analysis. MMTV-D1T286A developed adenocarcinomas significantly earlier than MMTV-D1 mice (estimated median: 18 months for D1T286A, estimated 95% CI: 16.2–19.8 months vs 22 months for D1, estimated 95% CI: 20.5–23.5 months; log-rank Mantel–Cox test: $P = 0.003$, Figure 3a and Table 1). Both transgenic lines developed tumors with essentially identical penetrance. Because tumor penetrance was less than 100% and estimated medians had to be calculated, as an independent assessment of tumor latency, we also determined the mean age of onset for tumor formation. In contrast to the median, the mean age of onset was 15.8 months for MMTV-D1T286A mice compared to 19.9 months for MMTV-D1 (Student's *t*-test: $P = 0.0004$, Figure 3b).

During our studies, we noted that MMTV-D1T286A mice routinely developed multifocal tumors (Figure 2g). Tumors from a single MMTV-D1T286A mouse generally exhibited distinct histological phenotypes demonstrating that they reflect independent events (Figure 3e and data not shown). To quantify the oncogenic potential of cyclin D1 and D1T286A per mammary gland, we harvested glands from females at 12 months of age and determined hyperplastic foci formation by whole-mount analysis. Abrogation of cyclin D1 phosphorylation increased the number of multifocal hyperplastic lesions from 1.3 to 3.4 per gland (Student's *t*-test: $P = 0.0052$, Figures 3c and d).

Characterization of D1 and D1T286A mammary tumors

Consistent with previous work (Wang *et al*., 1994), our MMTV-D1 mice developed primarily papillary adenocarcinomas (Figure 3e and Table 1). In contrast, MMTV-D1T286A developed mostly secretory glandular adenocarcinomas (Figure 3e and Table 1). Mice of both genotypes developed a small fraction of acinar and solid adenocarcinomas (Cardiff *et al*., 2000; Figure3e and Table 1). In general, tumors of both genotypes appeared aggressive as measured by increased nuclear to cytoplasmic ratio, nuclear pleomorphism, hyperchromatism and layers of disorganized epithelium (Figure 3e). Both D1 and D1T286A lines also developed adenosquamous carcinomas, inflammatory nodules and focal necrotic regions (data not shown).

Most carcinomas of either genotype uniformly expressed cytokeratin 8 (Figure 4a, tumor A) with occasional tumors also expressing cytokeratin 14 (Figure 4a, tumors B and C), revealing that they main-

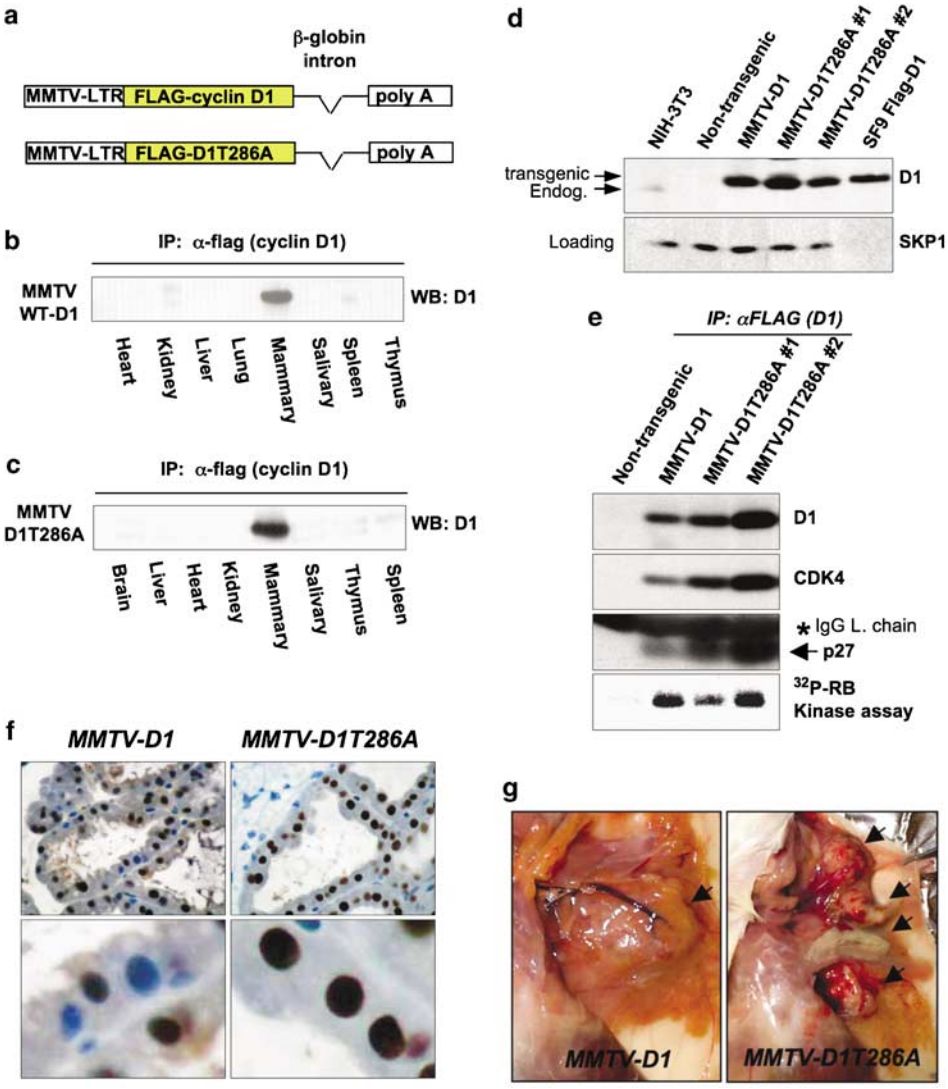

Figure 2 Generation of MMTV-D1 and MMTV-D1T286A transgenic mice. (**a**) Flag-tagged cyclin D1 or D1T286A was placed under the regulation of MMTV-LTR. (**b** and **c**) Lysates were prepared from pregnant mice, precipitated with anti-Flag agarose and blotted for cyclin D1 (**b**, D1 line; **c**, D1T286A line). (**d**) Mammary extracts were prepared from age- and parity-matched lactating mice and probed for D1 or (**e**) were precipitated with anti-Flag agarose, blotted for CDK4 and p27 or assessed for *in vitro* kinase activity against RB. (**f**) Immunohistochemistry for total D1 during lactation (day 10) in MMTV-D1 (left panels) and D1T286A (right panels) mammary glands. (**g**) Photomicrographs of palpable mammary tumors (arrows).

tain primarily luminal and some myoepithelial cell characteristics. Since most human breast carcinomas that overexpress cyclin D1 also express the ER, we determined whether tumors retained expression of ERα. This analysis revealed that 37.5% D1 and 50% D1T286A tumors retained ERα, suggesting that a subset of tumors remained estrogen-dependent (Figure 4a, tumor D). Nuclear staining of uterine epithelial cells and omission of primary antibody served as positive and negative controls for ERα, respectively (Figure 4a and data not shown).

Because inactivation of the RB pathway correlates with genomic instability (Hernando *et al*., 2004; McDermott *et al*., 2006), we determined whether cyclin D1T286A expression is associated with genomic instability. Early passage, tumor-derived MECs were established and analysed by staining metaphase spreads with Giemsa or by spectral karyotyping. MMTV-D1T286A tumor cells exhibited increased aneuploidy relative to cells derived from MMTV-D1 tumors (Figures 4b–d). No chromosomal translocations were detected (Figure 4e) demonstrating that WT-D1 and D1T286A promote aneuploidy through chromosomal gains.

The increase in mitotic index (Supplementary Figure S1B) and aneuploidy observed in MMTV-D1T286A tumors suggested the potential deregulation of DNA replication. We thus determined whether components of the DNA replication machinery were altered in mammary tumors. Analysis of tumor extracts revealed overexpression of DNA replication factors, CDT1 and MCM3 (Figure 5a; MCM3 is shown as a control). Overexpression of CDT1 occurred more frequently in

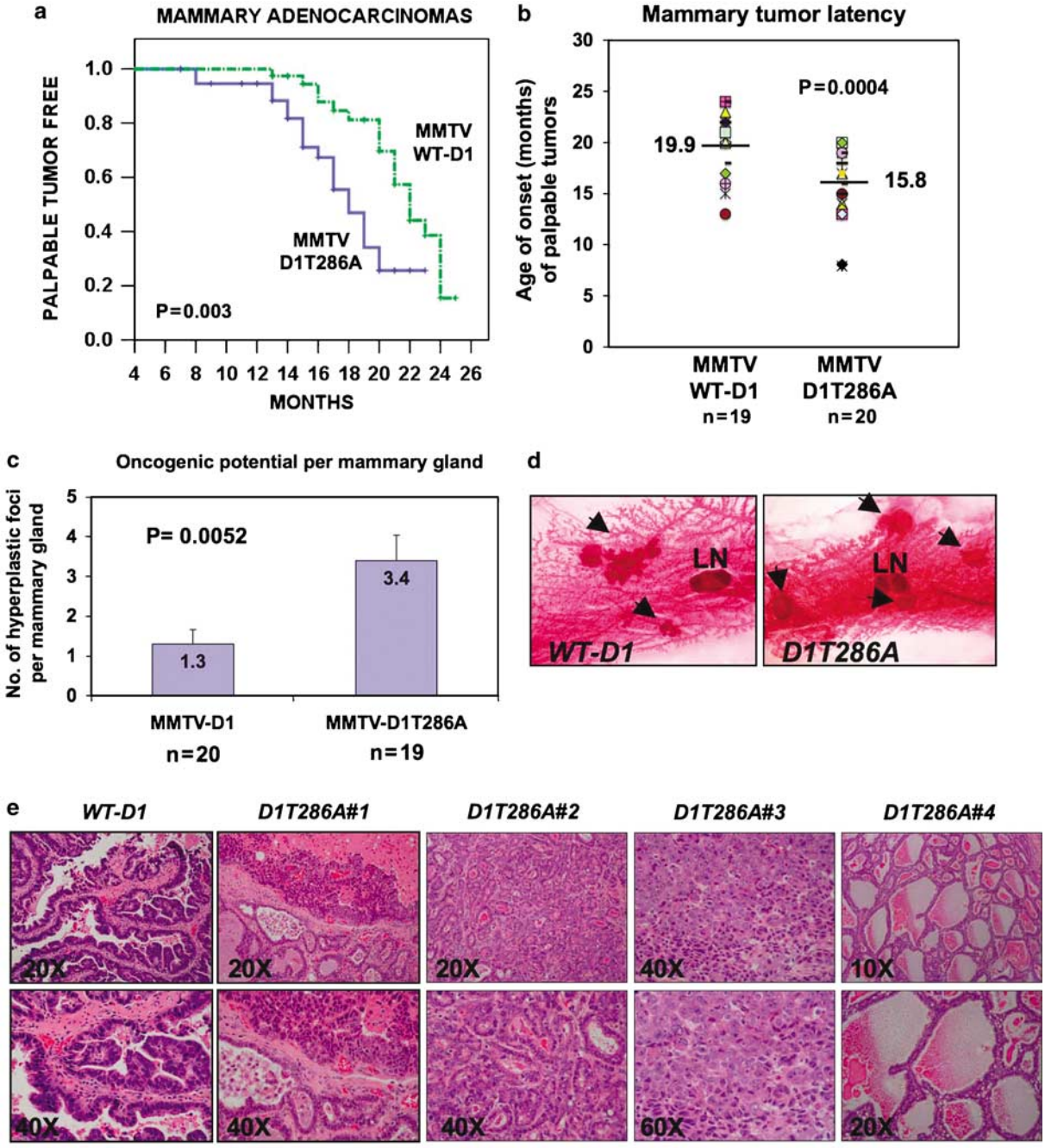

Figure 3 Perturbation of cyclin D1 localization and proteolysis accelerates mammary carcinogenesis. (**a**) Kaplan–Meier curves for the development of palpable mammary adenocarcinomas in MMTV-D1 ($n=40$) and D1T286A ($n=39$) mice. (**b**) Mean age of onset of palpable adenocarcinomas (horizontal bars). Each marker represents the age of onset of the first palpable tumor per mouse. (**c**) One no. 4 gland per mouse was harvested at 12 months of age and assessed for hyperplastic foci formation. Columns represent means. (**d**) Representative whole-mount staining of hyperplastic foci (arrows). The intramammary lymph node (LN) is included as a size reference. (**e**, WT-D1) Papillary adenocarcinoma in MMTV-D1 mice. (**e**, D1T286A no. 1) Adenocarcinoma displaying both glandular and solid histology and focal necrosis in MMTV-D1T286A mice. (**e**, D1T286A no. 2) Glandular adenocarcinoma. (**e**, D1T286A no. 3) Adenocarcinoma, solid type, displaying high nuclear/cytoplasmic ratio, nuclear pleomorphism and hyperchromatism. (**e**, D1T286A no. 4) D1T286A glandular adenocarcinoma, secretory type.

D1T286A-expressing tumors (Figures 5a and b, Supplementary Figure S1C). No changes in CDT1 mRNA levels were observed, indicating that CDT1 overexpression reflects altered post-translation regulation (Figure 5c). Since CDT1 overexpression is associated with DNA damage (Vaziri *et al*., 2003), inhibition of cyclin D1 phosphorylation may enhance genomic instability in part through CDT1 overexpression.

Because CDT1 overexpression can trigger p53 activation (Vaziri *et al*., 2003) and thus might select for p53

Table 1 Characterization of mammary carcinomas in MMTV-D1 and MMTV-D1T286A mice

	MMTV WT-D1	*MMTV D1T286A*
Mammary adenocarcinomas		
Palpable tumors	47.5% (19/40)	51% (20/39)
Mean age of onset	19.9 months	15.8 months
Median (estimated)	22 months	18 months
95% confidence interval of median	20.5–23.5	16.2–19.8
Histological signatures		
Papillary	44% (10/23)	15% (4/27)
Secretory glandular	13% (3/23)	40.5% (11/27)
Acinar	26% (6/23)	26% (7/27)
Solid	4% (1/23)	15% (4/27)
Adenosquamous	13% (3/23)	3.5% (1/27)
ER-positive	37.5% (3/8)	50% (4/8)

loss in tumors, we determined whether the p53 pathway was intact in MMTV-D1T286A tumor cells. At 8 h after 10 Gy irradiation, four out of four D1T286A tumor MECs failed to induce p21 expression (Figure 5d), while two out of four MMTV-D1 tumor MECs were able to induce p21 (Figure 5d, lines 266 and 299). These data demonstrate that the p53 pathway is inactivated in MMTV-D1T286A tumors.

Combined pharmacologic inhibition of CDK4 and ER prevents cancer cell proliferation

As a subset of D1 and D1T286A tumors retained ERα, we determined whether proliferation was estrogen-dependent by treatment of tumor MECs with 4OH-tamoxifen and examination of cell cycle profiles. Treatment of D1T286A tumor-derived cells with increasing concentrations of

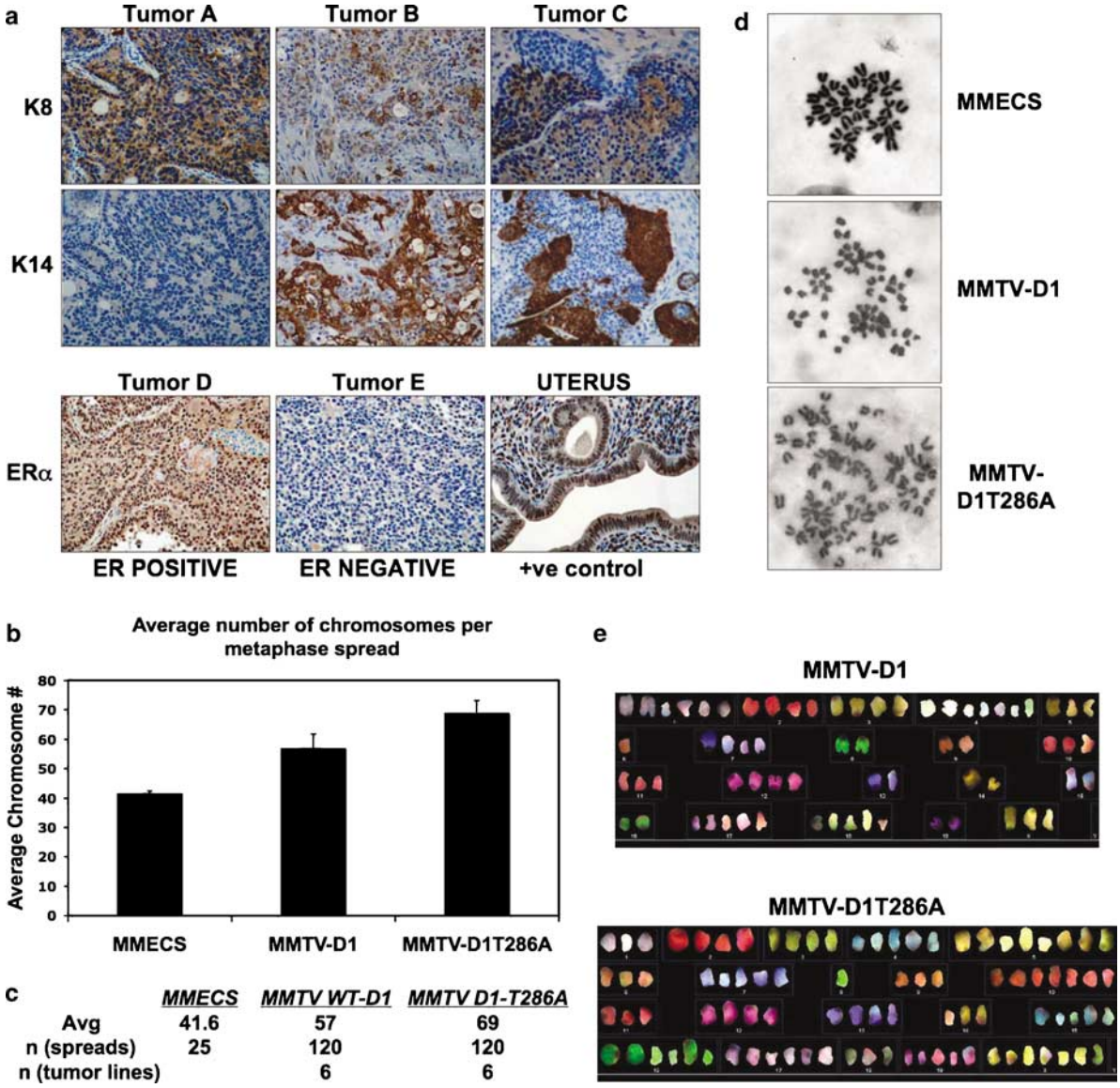

Figure 4 Characterization of MMTV-D1 and MMTV-D1T286A mammary tumors. (**a**) Representative immunohistochemistry for cytokeratin 8 (K8), cytokeratin 14 (K14) and ERα in MMTV-D1 and D1T286A tumors. (**b** and **c**) Average number of chromosomes per metaphase spread in early passage MMTV-D1 and MMTV-D1T286A tumor or normal mouse mammary epithelial cells (MECs). (**d**) Representative metaphase spreads stained with Giemsa or (**e**) SKY. ER, estrogen receptor.

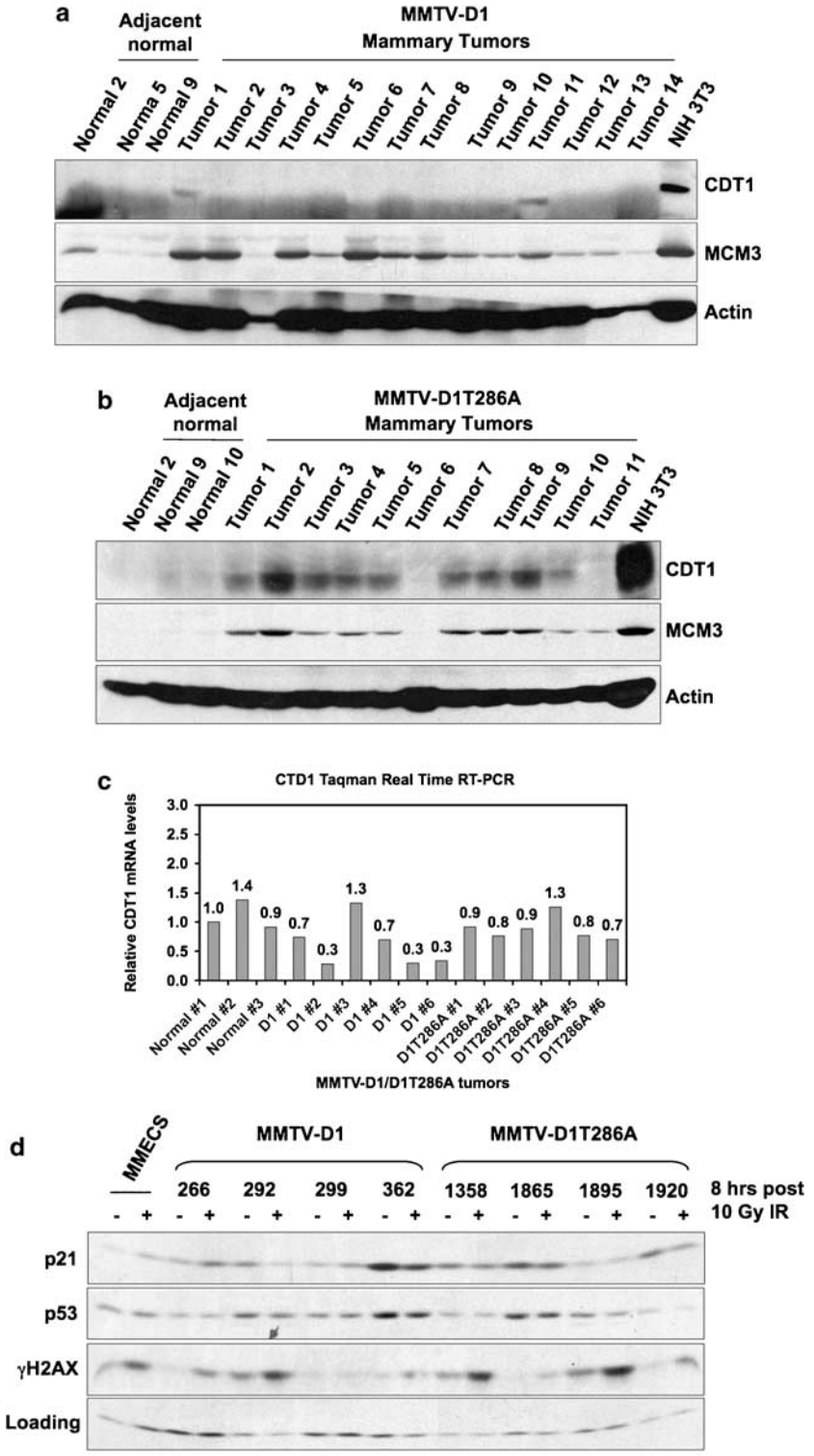

Figure 5 CDT1 is overexpressed and p53 is inactivated in MMTV-D1T286A tumors. (**a**) Lysates were prepared from MMTV-D1 tumors and blotted for CDT1 or MCM3. NIH3T3s served as positive control and actin as a loading control. (**b**) MMTV-D1T286A lysates were blotted as in (**a**). (**c**) RNA was prepared from MMTV-D1/D1T286A tumors and CDT1 mRNA levels were measured by TaqMan qPCR. (**d**) At 8 h after 10 Gy irradiation, tumor mammary epithelial cell (MEC) lysates were prepared and interrogated with the indicated antibodies.

tamoxifen linearly inhibited proliferation (Figure 6a). Similarly, proliferation of tumor MECs was inhibited in media containing charcoal–dextran-stripped serum devoid of any estrogens (data not shown).

MMTV-D1 (data not shown) and MMTV-D1T286A (Figure 6b) cells were also treated with PD0332991, a specific CDK4/6 kinase inhibitor that is currently in phase I clinical trials for cancer (Fry *et al*., 2004).

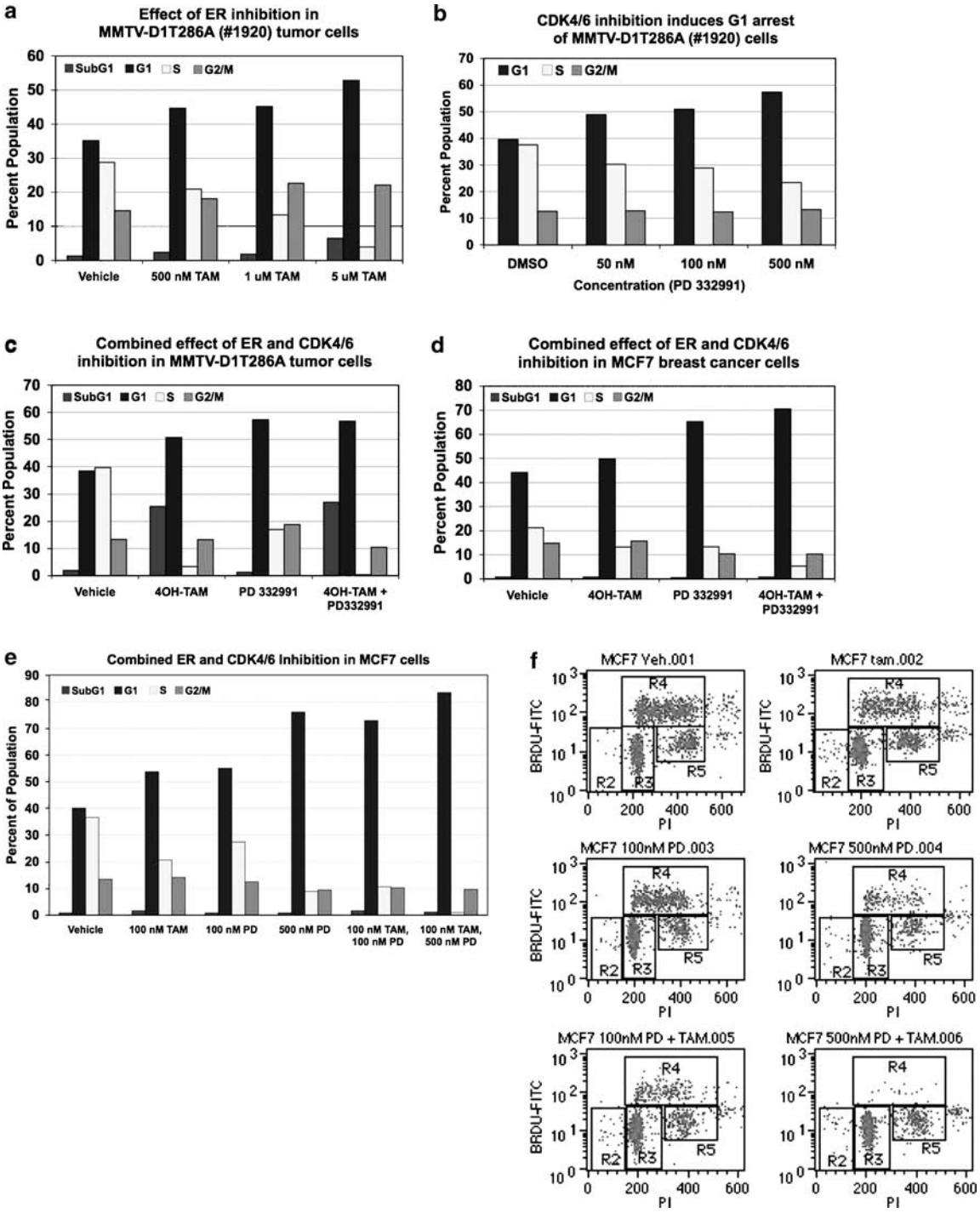

Figure 6 Combined pharmacologic inhibition of CDK4/6 and estrogen receptor (ER) is better than either alone. (**a**) MMTV-D1T286A tumor mammary epithelial cells (MECs) were treated with increasing doses of tamoxifen (TAM) for 48 h and cell cycle profiles were assessed by PI–BrdU fluorescence-activated cell sorting (FACS). (**b**) Cell cycle profiles of MMTV-D1T286A tumor MECs treated with PD0332991 for 48 h. (**c**) Cell cycle profiles of MMTV-D1T286A tumor MECs treated with both 500 nM PD0332991 and 10 μM tamoxifen for 48 h. (**d**) Cell cycle profiles of human ERα-positive MCF7 cancer cells treated with both 500 nM PD0332991 and 100 nM tamoxifen for 48 h. (**e**) Same as in (**d**) except 100 nM PD0332991 was also used. (**f**) Representative FACS plots.

Treatment of D1 or D1T286A tumor MECs with increasing doses of PD0332991 inhibited the percentage of cells in S phase with a concurrent G_1 arrest in five out of six different tumor MECs (Figure 6b and data not shown). Strikingly, treatment of tumor MECs with both tamoxifen and PD0332991 further inhibited proliferation (Figure 6c). Similar results were obtained by treating ER-positive, human MCF7 cancer cells (Figures 6d–f). These results indicate that combined estrogen blockade and CDK4/6 inhibition is more effective than

Table 2 Combined effect of ER and CDK4/6 inhibition in MCF7 cells

	Vehicle	*TAM*	*PD*	*TAM + PD*
Experiment 1 (100 nM TAM, 500 nM PD)				
G_1 (%)	42.1	55.8	64.5	75.8
S (%)	28.7	11.4	13.9	3.9
G_2/M (%)	14.7	15.9	12.2	9.1
Experiment 2 (100 nM TAM, 500 nM PD)				
G_1 (%)	44.1	49.9	75.8	76.4
S (%)	21.2	13.2	3.3	1.5
G_2/M (%)	14.8	15.7	8.3	9.1
Experiment 3 (100 nM TAM, 500 nM PD)				
G_1 (%)	46.1	69.3	79.3	81.6
S (%)	24.6	10.3	3.5	0.4
G_2/M (%)	18.6	14.8	11.7	13.5
Experiment 4 (100 nM TAM, 500 nM PD)				
G_1 (%)	40.0	53.7	76.1	83.4
S (%)	36.6	20.6	8.8	0.9
G_2/M (%)	14.4	14.2	9.4	9.6
Experiment 5 (100 nM TAM, 100 nM PD)				
G_1 (%)	44.1	49.9	65.2	70.6
S (%)	21.2	13.2	13.2	5.3
G_2/M (%)	14.8	15.7	10.4	10.3
Experiment 6 (100 nM TAM, 100 nM PD)				
G_1 (%)	40.0	53.7	55.0	73.9
S (%)	36.6	20.6	27.4	10.6
G_2/M (%)	14.4	14.2	12.4	10.2

Abbreviations: ER, estrogen receptor; TAM, tamoxifen. Paired Student's *t*-test for S phase: $P = 0.002$, combination vs TAM; $P = 0.015$, combination vs PD.

either treatment alone in ER-positive human breast cancers (Table 2; $P < 0.05$).

D1T286A tumors differentially express DNA replication and DNA damage checkpoint genes

To gain additional mechanistic insight, we performed a tumor microarray to compare expression profiles of MMTV-D1/D1T286A tumors. While on a global scale D1 and D1T286A tumors were not significantly different from one another (data not shown), we identified several genes involved in distinct biological processes that were differentially expressed in MMTV-D1T286A tumors compared to MMTV-D1 tumors and normal mammary glands (data not shown). Because of the genetic instability and overexpression of CDT1 seen in D1T286A tumors, we performed cluster analysis of genes involved in DNA replication and DNA damage checkpoints (Figure 7 and data not shown). Differential expression of a subset of these genes, such as claspin, CDC7, E2F8, DNA ligase 1 and Rpa1, in D1T286A tumors was confirmed by TaqMan qPCR (Figure 7). These results suggest that disruption of cyclin D1 phosphorylation induces DNA replication stress and DNA damage during mammary carcinogenesis.

Discussion

Herein, we demonstrate that p-286 directs cyclin D1 nuclear export and ubiquitination by $SCF^{FBX4\text{-}\alpha B\ crystallin}$ in the mammary gland. During stages of high epithelial proliferation, cyclin D1 was nuclear, with weak p-286 staining apparent. In contrast, during lactation, cyclin D1 redistributed to the cytoplasm. Consistent with Thr-286 phosphorylation triggering cyclin D1 proteolysis, p-286 increased during lactation. In contrast, cyclin D1T286A remained nuclear through both developmental stages. Furthermore, cyclin D1 was ubiquitinated in a p-286- and FBX4-dependent manner by mammary extracts. These results support the notion that the weaker tumor phenotype observed with overexpression of wild-type cyclin D1 may reflect the ability of normal epithelium to metabolize cyclin D1 via phosphorylation-dependent nuclear export and FBX4-mediated proteolysis.

Three points of our work support that disrupting cyclin D1 nuclear export and proteolysis enhance the oncogenicity of cyclin D1. First, mice expressing D1T286A develop mammary adenocarcinomas earlier than mice expressing wild-type cyclin D1. Second, abrogation of cyclin D1 phosphorylation increases the number of multifocal lesions per gland. Furthermore, MMTV-D1T286A mice develop mammary tumors with altered tumor histologies, increased mitotic index, greater genetic instability and enhanced expression of genes involved in DNA replication and DNA damage checkpoints.

An important question that remains is whether disruption of cyclin D1 proteolysis alone is sufficient to enhance tumorigenicity. That MMTV-D1 mice develop adenocarcinomas demonstrates that increased cyclin D1 protein levels partly contribute to its neoplastic potential. Indeed, loss of components of the D1 E3 ligase occurs in human breast cancers with simultaneous cyclin D1 stabilization, again implicating impaired D1 proteolysis in breast carcinogenesis (Lin *et al*., 2006). However, disrupting both nuclear export and proteolysis in MMTV-D1T286A mice further accelerates mammary tumor formation, suggesting that altered nuclear export also contributes to the oncogenicity of cyclin D1. Future experiments in which nuclear export and proteolysis are uncoupled through targeted deletion of E3 ligase components should establish the contribution of cyclin D1 localization and proteolysis to mammary tumor formation.

It is important to note that inhibiting phosphorylation may enhance a nuclear function for wild-type D1, which could contribute to the development of additional hits during cellular transformation. In fact, MMTV-D1T286A tumors specifically overexpress CDT1, suggesting that CDT1 stabilization and DNA re-replication may represent nuclear targets. The fact CDT1 mRNA levels were unchanged suggests that the stability of the CDT1 protein is altered by an unknown mechanism.

Cyclin D1 is a mediator of estrogen-dependent proliferation (Buckley *et al*., 1993; Butt *et al*., 2005). Despite initial responses to antiestrogens, breast cancer patients become resistant to antiestrogen therapies. Here, we provide evidence that combined pharmacological inhibition of cyclin D-dependent kinases and antiestrogen therapy may be an effective therapeutic option for ER-positive tumors. In addition, the fact that

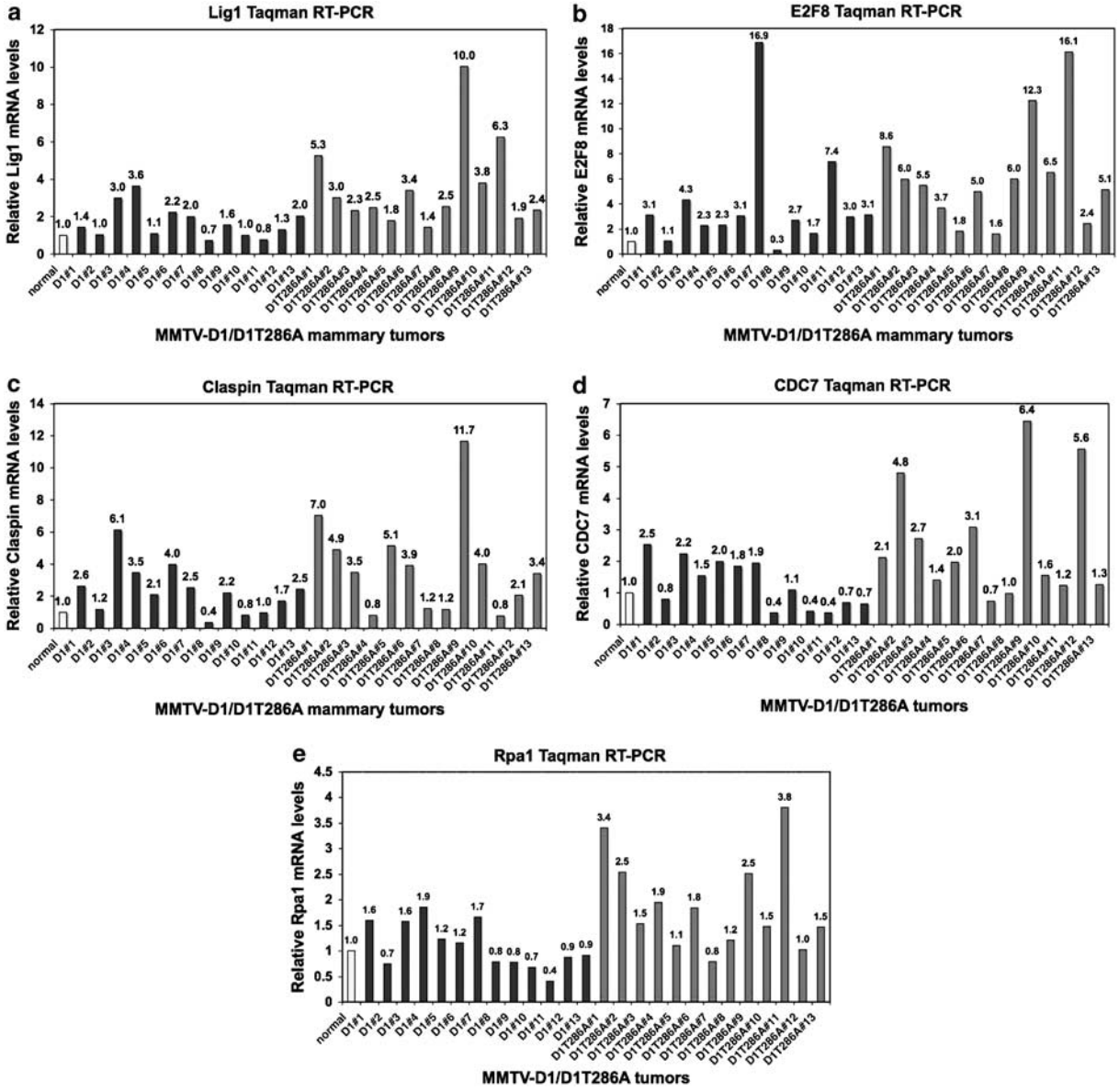

Figure 7 DNA replication and DNA-damage response genes are differentially expressed in D1T286A tumors. TaqMan qPCR analysis of (**a**) DNA ligase 1, (**b**) E2F8, (**c**) claspin, (**d**) CDC7 and (**e**) Rpa1 in 13 MMTV-D1 and MMTV-D1T286A mammary tumors. mRNA levels were normalized to actin levels and normal mammary glands.

up to 50% of MMTV-D1T286A mice develop ER-positive cancers indicates that our mouse model may provide an effective tool to study targeted therapies against both ER- and cyclin D1-dependent kinases.

Cyclin D1 mutations that abrogate p-286 have not been found in breast cancers. However, overexpression of an alternative splice variant of cyclin D1 that lacks Thr-286, cyclin D1b, occurs frequently in breast carcinomas, indicating that nuclear cyclin D1 variants contribute to human breast cancer progression (Lu *et al*., 2003). In conclusion, we demonstrate that disruption of cyclin D1 nuclear export and proteolysis contributes to the oncogenicity of cyclin D1 in mammary epithelium, and we establish a transgenic mouse model to dissect the mechanisms of mammary oncogenesis induced by constitutively nuclear and nondegradable cyclin D1.

Materials and methods

Construction of transgenic mice

cDNA encoding Flag-tagged cyclin D1 and D1T286A were subcloned into a transfer vector containing the MMTV-LTR promoter and a rabbit β-globin intron with its endogenous poly-A (Hennighausen *et al*., 1994; Rowse *et al*., 1998). A *Bam*HI digest released the linearized transgene for injection into FVB zygotes. Founder mice were identified by Southern blot and confirmed by PCR with the following primers: 5′-GGAACAG GAATGCACTTTTGGG-3′ and 5′-CTCACAGACCTCCAG CAT-3′.

Cell purification and culture conditions
Normal and tumor MECs were purified as described (Ip and Asch, 2000). MECs were cultured in DMEM/F12 buffered with HEPES pH 7.6, 10 μg ml^{-1} insulin, 5 ng ml^{-1} EGF, 1 mg ml^{-1} BSA fraction V, 5 μg ml^{-1} linoleic acid–BSA and 2% adult calf donor serum. PD0332991 (Pfizer) was dissolved in dimethyl sulfoxide at 10 mM and 4OH-tamoxifen (Sigma, St Louis, MO, USA) in ethanol. Metaphase spreads were characterized as described (Bassing *et al*., 2003).

Immunoprecipitation, CDK kinase assay and western blotting
Kinase assays were performed as previously described (Benzeno *et al*., 2006; Gladden *et al*., 2006). The following antibodies were used for western blottings: CDK4 (SC-22), p21 (C-19), MCM3 (N-19) (Santa Cruz, Santa Cruz, CA, USA); p27 (BD, clone 57), p53 (PAB421), CDT1 (Anindya Dutta), cyclin D1 (D1-17-13G), FBX4 (Lin *et al*., 2006), αB crystallin (Stressgen, SPA 223, Victoria, BC, Canada), γH2AX (Cell Signaling, Danvers, MA, USA).

In vitro *ubiquitination*
Mammary glands were lysed in EBC buffer (0.5% NP-40, 120 mM NaCl and protease inhibitors). An amount of 50 μg of extracts was incubated with 20 ng of purified D1/CDK4 complexes at 37°C in a 15 μl mix containing 50 mM Tric–HCl pH 8, 5 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM ATP, 60 μg ml^{-1} creatine phosphokinase, 10 mM creatine phosphate and 5 μM ubiquitin. D1/CDK4 complexes were purified from SF9 cells.

Immunohistochemistry
Tissues were fixed in 10% buffered formalin. Antigen was retrieved by microwaving in 10 mM citrate buffer pH 6 (BioGenex, San Ramon, CA, USA). Endogenous peroxidase was quenched with 3% peroxide. Sections were blocked with Power Block (BioGenex). The following primary antibodies were used: cyclin D1 (D1-17-13G), phospho-T286 (Alt *et al*., 2000), cytokeratin 8 (TROMA-I, Developmental Studies Hybridoma Bank, University of Iowa), cytokeratin 14 (Covance, Berkeley, CA, USA), ERα (MC-20, Santa Cruz) and Ki67 (NCL-L-MM1, Novocastra, Newcastle Upon Tyne, UK).

Whole-mount analysis
No. 4 mammary glands were fixed with 60% ethanol, 30% chloroform and 10% acetic acid, washed in 70% ethanol, then washed twice in water and stained with 0.2% carmine and 0.5% aluminum potassium sulfate. Glands were dehydrated in 70, 90, 95 and 100% ethanol, incubated in xylene and mounted.

mRNA microarray and TaqMan qPCR
Total RNA was prepared with TRIzol and RNseasy cleanup. cRNA and hybridization to Affymetrix gene chips were performed by the University of Pennsylvania microarray core facility. TaqMan qPCR was performed by the $\Delta\Delta C_t$ method with primer/probe master mixes purchased from Applied Biosystems (Foster City, CA, USA). Target mRNA levels were normalized relative to β-actin levels.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).