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TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Cooperation between *Dmp1* Loss and Cyclin D1 Overexpression in Breast Cancer

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Cyclin D1 is a component of the core cell-cycle machinery and is frequently overexpressed in breast cancer. It physically interacts with the tumor suppressor Dmp1 that attenuates the oncogenic signals from Ras and HER2 by inducing Arf/p53-dependent cell-cycle arrest. Currently, the biological significance of Dmp1—cyclin D1 interplay in breast cancer has not been determined. Here, we show that cyclin D1 bound to Dmp1 to activate both *Arf* and *Ink4a* promoters and, consequently, induced apoptosis or G2/M cell-cycle delay in normal cells to protect them from neoplastic transformation. The cyclin D1—induced *Ink4a/Arf* gene expression was dependent on Dmp1 because the induction was not detected in *Dmp1*-deficient or *DMP1*-depleted cells. *Arf/Ink4a* expression was increased in pre-malignant mammary glands from *Dmp1*$^{+/+}$;*MMTV-cyclin D1* and *Dmp1*$^{+/+}$;*MMTV-D1T286A* mice but significantly down-regulated in those from *Dmp1*-deficient mice. Selective *Dmp1* deletion was found in 21% of the *MMTV-D1* and *D1T286A* mammary carcinomas, and the *Dmp1* heterozygous status significantly accelerated mouse mammary tumorigenesis with reduced apoptosis and increased metastasis. Overall, our study reveals a pivotal role of combined *Dmp1* loss and cyclin D1 overexpression in breast cancer. *(Am J Pathol 2013, 183: 1339—1350; http://dx.doi.org/10.1016/j.ajpath.2013.06.027)*

As a key sensor and integrator of extracellular signals in early-to-mid G1 phase, cyclin D1 deregulation, including chromosomal translocation, gene amplification, or reduced degradation, results in tumorigenesis and has been linked to breast cancer and other human cancers.[1,2] Cyclin D1 drives cells to enter S phase by binding and activating the Cdk4/6 kinase. The cyclin D1/Cdk4 complex phosphorylates the retinoblastoma protein (pRb), which releases E2F transcriptional factors from pRb constraint. E2Fs then activate the transcription of genes required for cells to enter S phase.[3] Apart from its catalytic function, cyclin D1 also exerts a transcription regulation without the participation of Cdks, even though it is not a transcriptional factor. Cyclin D1 executes the transcriptional regulation through its interaction with transcriptional factors and chromatin modifiers.[4,5] For instance, it directly binds and activates estrogen receptor α (ERα) in breast cancer cell lines in a CDK-independent fashion.[6] By contrast, cyclin D1 interacts and inhibits the transcriptional activity of androgen receptor (AR), Myb-related protein B (B-Myb), E1A-binding protein p300, and NF-κB.[7—10] Overall, these studies suggested that cyclin D1 regulates cell proliferation, growth, and differentiation, and its transcriptional regulatory activity has great potential significance in human cancer progression.[5,11]

Among known cyclin D1—associated transcriptional factors, cyclin D—interacting Myb-like Protein 1 (Dmp1; alias Dmtf1) is a *bona fide* tumor suppressor.[12,13] Dmp1 directly binds and activates *Arf*, thereby inducing Arf-p53—dependent cell-cycle arrest.[14] *Dmp1*-deficient cells can be transformed by oncogenic Ras alone without altering the status of *Arf* and *p53* genes, suggesting that the Arf-p53

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pathway is significantly attenuated in *Dmp1*-deficient cells.[13] Ectopically expressed Dmp1 inhibits the growth of breast cancer cells with wild-type p53, indicating that *p53* is a critical target for Dmp1 to exhibit its biological activity.[15] Both *Dmp1*-null and *Dmp1*-heterozygous mice are prone to develop tumors, and a wild-type *Dmp1* allele is often retained in tumors from *Dmp1*-heterozygous mice. This suggests that Dmp1 is haploinsufficient as a tumor suppressor.[12,13,16] Our recent study in *MMTV-neu* mice showed that mammary carcinogenesis was significantly accelerated in both $Dmp1^{+/-}$ and $Dmp1^{-/-}$ backgrounds, with no difference between groups that lacked one or both *Dmp1* alleles. Hemizygous *Dmp1* deletion was found in >50% of *neu* mammary carcinomas accompanied with significant down-regulation of $p19^{Arf}$ and $p21^{Cip1/WAF1}$, indicating that Dmp1 is a physiological regulator of the Arf-p53 pathway *in vivo*.[17] Consistent with the mouse model studies, the loss of heterozygosity of *DMP1* was found in nearly 45% of human breast carcinomas and mutually exclusive with that of *INK4a/ARF* and *P53*, suggesting primary involvement of *DMP1* in human mammary carcinogenesis.[15]

Cyclin D1 physically interacts with Dmp1, and antagonizes its ability of activating the *CD13/*aminopeptidase N promoters in the absence of functional Cdks.[18] However, by an unknown mechanism, Dmp1 and cyclin D1 collaborate to activate the *Arf* promoter, which suggests that whether cyclin D1 acts as a corepressor or coactivator of Dmp1 is dependent on the context of a target gene.[19] Although Dmp1 and cyclin D1 play vital roles in breast cancer prevention and development, respectively, the biological functions and significance of Dmp1−cyclin D1 interaction in breast cancer remain to be determined. We conducted the current study to elucidate the cooperative role of cyclin D1 and Dmp1 in breast cancer development. We showed that Dmp1 modulated the activation of the *Arf/Ink4a* tumor suppressor pathways induced by cyclin D1 overexpression. We crossed the *MMTV-cyclin D1* and *MMTV-D1T286A* mice with *Dmp1*-deficient mice and found that *Dmp1* heterozygosity significantly accelerated cyclin D1−induced mammary carcinogenesis. We also observed the metastasis of cyclin D1−induced mammary tumors in a *Dmp1*-deficient background.

Materials and Methods

Cell Culture and Luciferase Reporter Assays

Wild-type and *Dmp1*-null murine embryonic fibroblasts (MEFs) were established from 13.5-day-old embryos as previously described.[12] MEF, NIH 3T3, and human mammary epithelial cells were cultured as described previously.[12,15,17] To study the responsiveness of the *Arf/Ink4a* promoters to cyclin D1 and D1T286A, 2×10^5 cells were seeded into 60-mm-diameter culture dishes 24 hours before transfection, and then transfected with 4 μg of luciferase reporter DNA with or without increasing amount of pFLEX1-cyclin D1, pFLEX1 D1T286A,[20] or pFLEX1-cyclin D1Δ[21] and 4 μg of a control plasmid with β-actin promoter−driven secreted alkaline phosphatase (SEAP).[18,19,21] GeneJuice transfection reagent (Novagen; EMD Millipore, Billerica, MA) was used in all transfections.

Chromatin Immunoprecipitation

Tissue and cell chromatin immunoprecipitations (ChIP) were performed as described previously.[19,22−24] Briefly, in tissue ChIP, lysates from $Dmp1^{+/+}$;*MMTV-neu* tumors were precipitated with antibodies against cyclin D1 (SP4; NeoMarkers/Lab Vision, Fremont, CA, and sc-753; Santa Cruz Biotechnology, Santa Cruz, CA) and incubated at 4°C overnight. After reverse cross-linking, the immunoprecipitated DNA was amplified by PCR after being mixed with 1 μCi of [α-^{32}P]dATP (PerkinElmer, Waltham, MA) and separated on a 10% nondenaturing polyacrylamide gel. In cell ChIP, wild-type and *Dmp1*-null MEFs were infected with lentivirus expressing hemagglutinin (HA)-cyclin D1. Forty-eight hours after infection, the cell lysates were precipitated with anti-HA affinity gel (E6779; Sigma-Aldrich, St. Louis, MO). Mouse IgG-agarose (A0919; Sigma-Aldrich) was used as a negative control. For the detection of cyclin D1 on the *Arf* promoter, a sense primer 5′-ACTCGGAGCAAGGGAAACCT-3′ and an anti-sense primer 5′-TAGCAGTAGCTGCGCCCTTT-3′ were used. For the detection of cyclin D1 on the *Ink4a* promoter, a sense primer 5′-GCAAATAGCGCCACCTATGG-3′ and an anti-sense primer 5′-CTGCTCCAGATGGCTCTCCT-3′ were used.

Retrovirus and Lentivirus Production

For retrovirus production, 293T cells were transfected with *pSRαMSV-tkneo* vector, *pSRαMSV-cyclin D1-tkneo*, *pSRαMSV-D1T286A-tkneo*, *pSR* vector, or *pSR*-1131 (*DMP1* shRNA)[16] together with a helper retrovirus plasmid. Viruses were harvested and used to infect cells as previously described.[19] For lentivirus production, 293T cells were transfected with *pSL4-HA*, *pSL4-HA-cyclin D1*, or *pSL4-HA-cyclin D1T286A* with helper lentivirus plasmids including *VSVG*, *RSV-REV*, and *PMDL g/p RRE*. Viruses were harvested 48 hours after transfection. For preparation of doxycycline-inducible cyclin D1, the cyclin D1 cDNA was PCR amplified by *Pfu* DNA polymerase and then subcloned downstream of a 6xTRE minimal promoter in an all-in-one doxycycline-inducible vector that expresses rtTA-advanced protein, generated by our colleagues.

Real-Time PCR

Analysis of *Dmp1* gene copy number and quantification of Dmp1, $p14^{ARF}$, $p19^{Arf}$, $p21^{Cip1/WAF1}$, $p16^{Ink4a}$, and CYCLIN D1 mRNAs were conducted by real-time PCR TaqMan assays on an Applied Biosystems ABI 7500 Real-Time PCR

machine (Life Technologies, Foster City, CA). β-Actin was used as internal control.[22–24]

Western Blot Analysis

Proteins were extracted from cell lysates in ice-cold EBC buffer with proteinase inhibitors.[25] After being separated by gel electrophoresis and transferred to nitrocellulose membranes, proteins were detected by immunoblotting with affinity-purified polyclonal antibodies for Dmp1 [Rabbit Antibody D (RAD)],[22] cyclin D1 (sc-753; Santa Cruz Biotechnology), p53 (sc-6243G; Santa Cruz Biotechnology), Mdm2, or HDM2 (ab16896 [2A10]; Abcam, Cambridge, UK), $p19^{Arf}$ (sc-32748; Santa Cruz Biotechnology), $p14^{ARF}$ (sc-53639; Santa Cruz Biotechnology), cleaved poly(ADP-ribose) polymerase (PARP) (#AF-600-NA; R&D Systems, Minneapolis, MN), cleaved caspase 3 (#9661; Cell Signaling Technology, Danvers, MA), $p16^{Ink4a}$ (sc-74401; Santa Cruz Biotechnology), $p21^{CIP1/WAF1}$ (sc-397G; Santa Cruz Biotechnology), or β-actin (sc-1615, sc-47778; Santa Cruz Biotechnology), followed by incubation with horseradish peroxidase–conjugated secondary antibodies, and visualization using an enhanced chemiluminescence detection kit (PerkinElmer).

Dmp1$^{+/-}$; and *Dmp1*$^{-/-}$; *MMTV-cyclin D1* and *MMTV-D1T286A* Compound Mice

The mouse model studies were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Wake Forest University School of Medicine. *Dmp1*-heterozygous females were backcrossed to the same FVB/NJ males (The Jackson Laboratory, Bar Harbor, ME) for more than eight generations to obtain *Dmp1*$^{+/-}$ mice with >99% FVB/NJ background overall. A male *MMTV-cyclin D1* mouse (provided by Dr. E.V. Schmidt, Harvard Medical School)[26] or *MMTV-D1T286A* mouse (provided by Dr. J.A. Diehl, University of Pennsylvania)[27] in a pure FVB/NJ background was crossed with a *Dmp1*$^{+/-}$ female to obtain *Dmp1*$^{+/-}$;*MMTV-cyclin D1* and *Dmp1*$^{+/-}$;*MMTV-D1T286A* mice. Then *Dmp1*$^{+/-}$;*MMTV-cyclin D1* and *Dmp1*$^{+/-}$;*MMTV-D1T286A* compound transgenic mice were further crossed with *Dmp1*$^{+/-}$ mice to obtain >15 mice with each genetic background. Littermate wild-type mice were used as controls.

Cell-Cycle Analysis

Wild-type and *Dmp1*-null MEFs were infected with lentivirus carrying empty vector or expressing cyclin D1 or D1T286A. Forty-eight hours after infection, cells were trypsinized, washed with PBS, fixed in 70% of ethanol, treated with propidium iodide and ribonuclease A, and then run through a flow cytometer (Accuri; Becton Dickinson, Franklin Lakes, NJ). Cell-cycle distributions were analyzed by ModFit LT software version 3.0 (Verity Software House, Topsham, ME).

Immunohistochemical Staining

Immunohistochemical staining of tissues and tumors were conducted as described previously.[15] The following antibodies were used for immunohistochemistry with formalin-fixed, paraffin-embedded sections: Ki-67 (SP6; NeoMarkers/Lab Vision), cyclin D1 (SP4; NeoMarkers/Lab Vision), cleaved caspase-3 (#9661; Cell Signaling Technology), cytokeratin 8 (ab59400; Abcam), cytokeratin 14 (ab7800; Abcam), and ERα (sc-542; Santa Cruz Biotechnology).

In Vitro Mutagenesis

The murine *Ink4a* promoter Dmp1-binding site deletion/point mutants were generated by use of an *in vitro* mutagenesis kit (Stratagene; Agilent Technologies, Santa Clara, CA). For deletion mutagenesis, the sense primer 5′-CCATCCCTTTCCCCTCCCGTGGGGGGAACAGCA-GTG-3′ and its reverse complementary sequence were used. For point mutagenesis, the sense primer 5′-CCTTTCCCC-TCCCCCTTCCGGAGGTGGGGGGAA-3′ and its reverse complementary sequence were used.

Electrophoretic Mobility Shift Assay

Recombinant DMP1 protein was prepared in Sf9 cells.[25] Electrophoretic mobility shift assays were performed using ^{32}P-labeled oligonucleotide probe covering the DMP1/Ets site on murine or human *INK4a* promoter obtained by annealing oligonucleotide 5′-GGGATCCCTTTCCCCTCCC-CCATCCGGAGGTGGGGGGAACAGCA-3′ (mouse sense strand) or 5′-GGGCTGGGATCAGCTCTCAGCATCCGG-AAGCCTTTGCCTACTAG-3′ (30911 human sense strand) or 5′-GGGATAGACGTGAGCCACC-GCATCCGGACT-TTCCTTTTATGTAA-3′ (33549 human sense strand) (the DMP1/Ets consensus sequence is underlined) with a complementary antisense strand. The probe containing the DMP1/Ets site on murine *Arf* promoter has been described.[14] For competition experiments, a 50-fold excess of unlabeled oligonucleotides were added to reaction mixtures before probe incubation.

Statistical Analysis

Statistical differences of survival in *Dmp1*$^{+/+}$;*MMTV-cyclin D1*, *Dmp1*$^{+/-}$;*MMTV-cyclin D1*, *Dmp1*$^{+/+}$;*MMTV-D1T286A*, and *Dmp1*$^{+/-}$;*MMTV-D1T286A* mice were analyzed by Medcalc software version 12.7.0 (Mariakerke, Belgium). Statistical analyses for all experiments were conducted using unpaired Student's *t*-tests.

Results

Dmp1 Is Essential to Cyclin D1–Mediated Activation of the *Arf* and *Ink4a* Promoters

We previously demonstrated that Dmp1 directly binds and activates the *Arf* promoter.[14,28] To determine whether Dmp1

also regulates *Ink4a* gene expression, we analyzed the murine *Ink4a* promoter and found a candidate Dmp1/Ets consensus sequence (TCCGGATGG: −178 to −166 relative to the transcription initiation site; two mismatches from the originally reported consensus sequences[25] at both ends; the DMP1/Ets consensus sequence is underlined) (Supplemental Figure S1A). In electrophoretic mobility shift assay studies, recombinant Dmp1 directly associated with the oligonucleotide with this sequence at a relatively low affinity compared to its binding to the oligonucleotide with the Dmp1 consensus in the *Arf* promoter (Supplemental Figure S1B). In reporter assays, Dmp1 activated the *Ink4a* promoter by sixfold to eightfold over a control vector, but this effect was not observed in reporter constructs with the Dmp1/Ets site mutated or deleted (Supplemental Figure S1C), suggesting that this consensus was necessary for Dmp1-mediated *Ink4a* transactivation. We also found two consensus sequences of Dmp1 in the human *INK4a* promoter, which was bound and activated by Dmp1 up to threefold over a control vector (Supplemental Figure S1, D and E). Thus, our data support that Dmp1 regulates *Ink4a* expression in both humans and mice.

Cyclin D1 induces cell cycle arrest, senescence, and apoptosis when overexpressed.[29] Thus, we investigated the potential effect of cyclin D1 on the *Arf* and *Ink4a* promoters. Both wild-type cyclin D1 and its constitutively active mutant D1T286A activated the *Arf* and *Ink4a* promoters, although the former showed better response than the latter (sevenfold versus fourfold) (Figure 1A). Cyclin D1−mediated activation of *Arf* and *Ink4a* was also observed in $Dmp1^{+/+}$ MEFs, but this effect was abrogated in $Dmp1^{-/-}$ MEFs (Figure 1B). However, ectopically expressed Dmp1 in $Dmp1^{-/-}$ MEFs restored cyclin D1−induced luciferase activity mediated by the *Arf* and *Ink4a* promoters, suggesting that Dmp1 is indispensable for cyclin D1−activated *Arf* and *Ink4a* expression (Figure 1C). Moreover, a cyclin D1 mutant, D1Δ142−253 that is deficient in Dmp1 binding,[21] failed in activating either the *Arf* or *Ink4a* promoter, suggesting that the effect of cyclin D1 on these promoters depended on cyclin D1−Dmp1 interaction (Figure 1, A and C). Additionally, the transcription of endogenous $p19^{Arf}$ and $p16^{Ink4a}$ was promoted by ectopic cyclin D1 or D1T286A in wild-type MEFs, but this effect was diminished in *Dmp1*-null MEFs (Figure 1D).

Consistently, we observed the recruitment of cyclin D1 on the *Arf* and *Ink4a* promoters in ChIP assays in wild-type MEFs infected with lentivirus expressing HA-tagged cyclin D1, but this binding was not detected in *Dmp1*-null MEFs (Figure 1E). The association of endogenous cyclin D1 with the *Arf* and *Ink4a* promoters was confirmed by ChIP assays using lysates from *MMTV-neu* mammary tumors that expressed high levels of both Dmp1 and cyclin D1 (Figure 1F).[17,30] Collectively, these data suggest that cyclin D1 binds and activates both the *Arf* and *Ink4a* promoters, and this regulation depends on the presence of Dmp1.

Cyclin D1 Induces G2/M Cell-Cycle Delay and Apoptosis Mediated by Dmp1

Both cyclin D1 and D1T286A increased $p19^{Arf}$ protein level when wild-type MEFs were starved in serum-free medium for 48 hours. Those cells underwent apoptosis as indicated by increased levels of cleaved PARP and caspase-3. However, there was no increase of $p19^{Arf}$ or induction of cleaved PARP and caspase-3 in $Dmp1^{-/-}$ MEFs on cyclin D1 or D1T286A overexpression (Figure 2A). We also stained the cells with annexin V and determined apoptotic cells by flow cytometry. Ectopic expression of cyclin D1 or D1T286A resulted in 20% increase of apoptosis in $Dmp1^{+/+}$ MEFs, but this effect was not observed in $Dmp1^{-/-}$ MEFs (data not shown). Consistent with the aforementioned data, ectopic expression of cyclin D1 and D1T286A in wild-type MEFs by lentiviral infection promoted $p19^{Arf}$ expression. We also detected increased expression of $p16^{Ink4a}$ protein in those cells, although neither $p19^{Arf}$ nor $p16^{Ink4a}$ showed this response toward cyclin D1 in $Dmp1^{-/-}$ MEFs (Figure 2B).

We further investigated the potential effect of cyclin D1 on cell-cycle profile. Ectopic expression of cyclin D1 and D1T286A through lentiviral infection led to G2/M phase delay of wild-type MEFs, but they only slightly increased the S phase fraction of $Dmp1^{-/-}$ MEFs (Figure 2, C and D), indicating that cyclin D1−mediated G2/M phase delay of the cell cycle depends on Dmp1.

Similar to the findings in MEFs, both $p14^{ARF}$ and $p16^{INK4a}$ mRNA levels increased in human mammary epithelial cells when ectopic cyclin D1 was expressed by a doxycycline-inducible system (Figure 3A). The protein level of $p14^{ARF}$ was increased accordingly, whereas the $p16^{INK4a}$ level was too low to be detected (Figure 3A). With *DMP1* knockdown by its shRNA[15,16] in human mammary epithelial cells, ectopic cyclin D1 or D1T286A failed in altering $p14^{ARF}$ or $p16^{INK4a}$ transcription (Figure 3B), although the two cyclin D1 proteins were expressed at comparable levels despite Dmp1 expression (Figure 3B). Moreover, human mammary epithelial cells underwent G2/M phase delay on cyclin D1 and D1T286A overexpression, whereas *DMP1* silencing abolished this effect (Figure 3C). Taken together, our data indicate that elevated cyclin D1 expression activates *Arf/Ink4a* genes in both mice and humans, and induces G2/M phase delay or apoptosis in a Dmp1-dependent fashion.

Dmp1 Is Required for the Increased $p19^{Arf}/p16^{Ink4a}$ Expression in Mammary Tumors of *MMTV-D1* and *D1T286A* Mice

Because *Dmp1* deletion was frequently detected (approximately 50%) in mammary tumors of *MMTV-neu* transgenic mice, whereas the *Ink4a/Arf* or *p53* locus deletion was not observed,[17] we next focused on the *Dmp1* gene copy number study in mammary tumors of *MMTV-D1* and

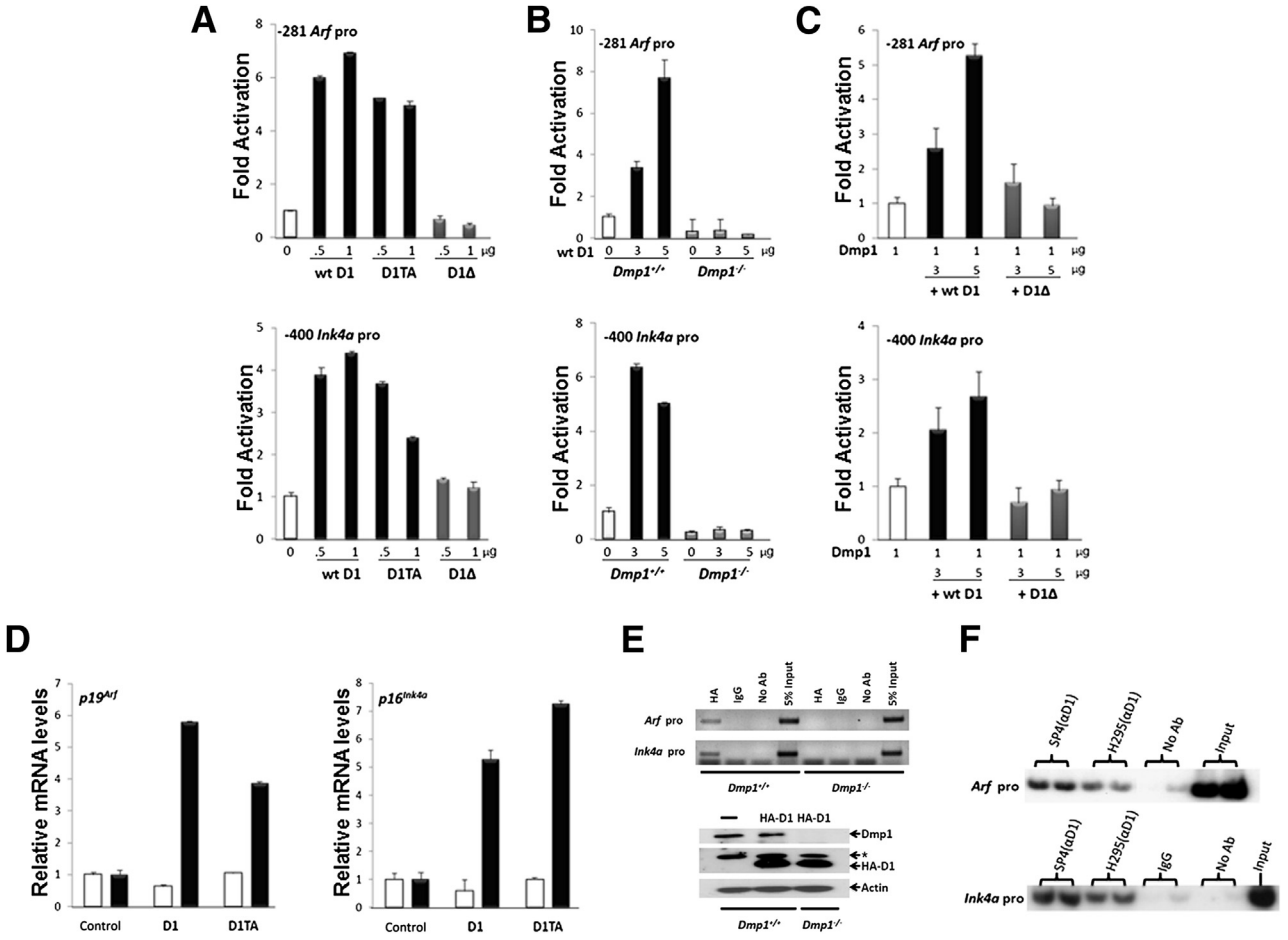

Figure 1 Both $p19^{Arf}$ and $p16^{Ink4a}$ promoters are activated by cyclin D1 and the cyclin D1T286A mutant. **A:** The *Arf* (−281) or *Ink4a* (−400) promoter luciferase construct was cotransfected with indicated amounts of cyclin D1 [wild-type (wt)], D1T286A (D1TA), or D1Δ142−253 (D1Δ)[21] expression vectors in NIH 3T3 cells. The numbers show the fold activation of the luciferase reporter normalized by internal controls of SEAP. **B:** The *Arf* (−281) or *Ink4a* (−400) promoter luciferase construct was cotransfected with increasing amounts of cyclin D1 expression vector in $Dmp1^{+/+}$ or $Dmp1^{-/-}$ MEFs. The numbers show the fold activation of the luciferase reporter normalized by internal controls of SEAP. **C:** Luciferase reporter construct encoding *Arf* (−281) promoter or *Ink4a* (−400) promoter was cotransfected with 1 μg of pFLEX1-Dmp1 together with either 3 or 5 μg of pFLEX1-cyclin D1 or pFLEX1-D1Δ142−253 into *Dmp1*-null MEFs. The numbers show the fold activation of the luciferase reporter normalized by internal controls of SEAP. **D:** Primary $Dmp1^{+/+}$ (black bars) or $Dmp1^{-/-}$ (white bars) MEFs were infected with retrovirus carrying an empty vector or vector expressing cyclin D1 or D1T286A protein. After neomycin selection, cells were harvested for the analysis of Arf and Ink4a mRNA levels by real-time PCR using β-actin as a control. **E:** ChIP analysis of cyclin D1 binding to the *Arf* and *Ink4a* promoters in $Dmp1^{+/+}$ and $Dmp1^{-/-}$ MEFs infected with lentivirus expressing HA-cyclin D1. Lysates derived from $Dmp1^{+/+}$ and $Dmp1^{-/-}$ MEFs were immunoblotted for Dmp1 and HA. β-Actin was used as a loading control. The **asterisk** indicates a nonspecific band. **F:** ChIP analysis for the binding of endogenous cyclin D1 to the *Arf* and *Ink4a* promoters in MMTV-*neu* tumors. Tissue ChIP was conducted with formalin-fixed tumors from the *MMTV-neu* mice with wild-type *Dmp1*. The cyclin D1 protein was precipitated by two different antibodies to cyclin D1 (SP4 and H295). Data are means ± SD from $n = 3$ individual experiments (**A**−**D**). Ab, antibody; pro, promoter.

MMTV-D1T286A mice. We found one *Dmp1* allele deletion in two of nine mammary tumors from *MMTV-D1* mice and 2 of 10 mammary tumors from *MMTV-D1T286A* mice (4 of 19, or 21%) (Supplemental Figure S2A). Significant induction of the $p19^{Arf}$ and $p16^{Ink4a}$ (2-fold to 18-fold) gene transcription was detected in more than half of *Dmp1* wild-type mammary tumors compared to those in normal mammary glands. Markedly, among four tumors with one *Dmp1* allele lost, two tumors showed significant down-regulation of $p19^{Arf}$ (Supplemental Figure S2B), and three exhibited very low $p16^{Ink4a}$ expression (Supplemental Figure S2C). A previous study discovered that the p53 pathway can be inactivated by mutations in *MMTV-D1* and *D1T286A* tumors.[27] To determine whether the increased $p19^{Arf}$ expression was due to p53 inactivation,[28] we sequenced the region of the *p53* gene encoding its DNA-binding domain. One *MMTV-D1* tumor and one *MMTV-D1T286A* tumor had *p53* mutations, whereas others with elevated $p19^{Arf}$ expression maintained wild-type *p53*. As expected, tumors with *p53* mutations showed a dramatically increased level of $p19^{Arf}$ (Supplemental Figure S2B). In summary, these observations indicate that Dmp1 is essential for cyclin D1−activated $p19^{Arf}$/$p16^{Ink4a}$ expression in mammary tumors of $Dmp1^{+/+}$;*MMTV-cyclin D1* and $Dmp1^{+/+}$;*MMTV-D1T286A* mice.

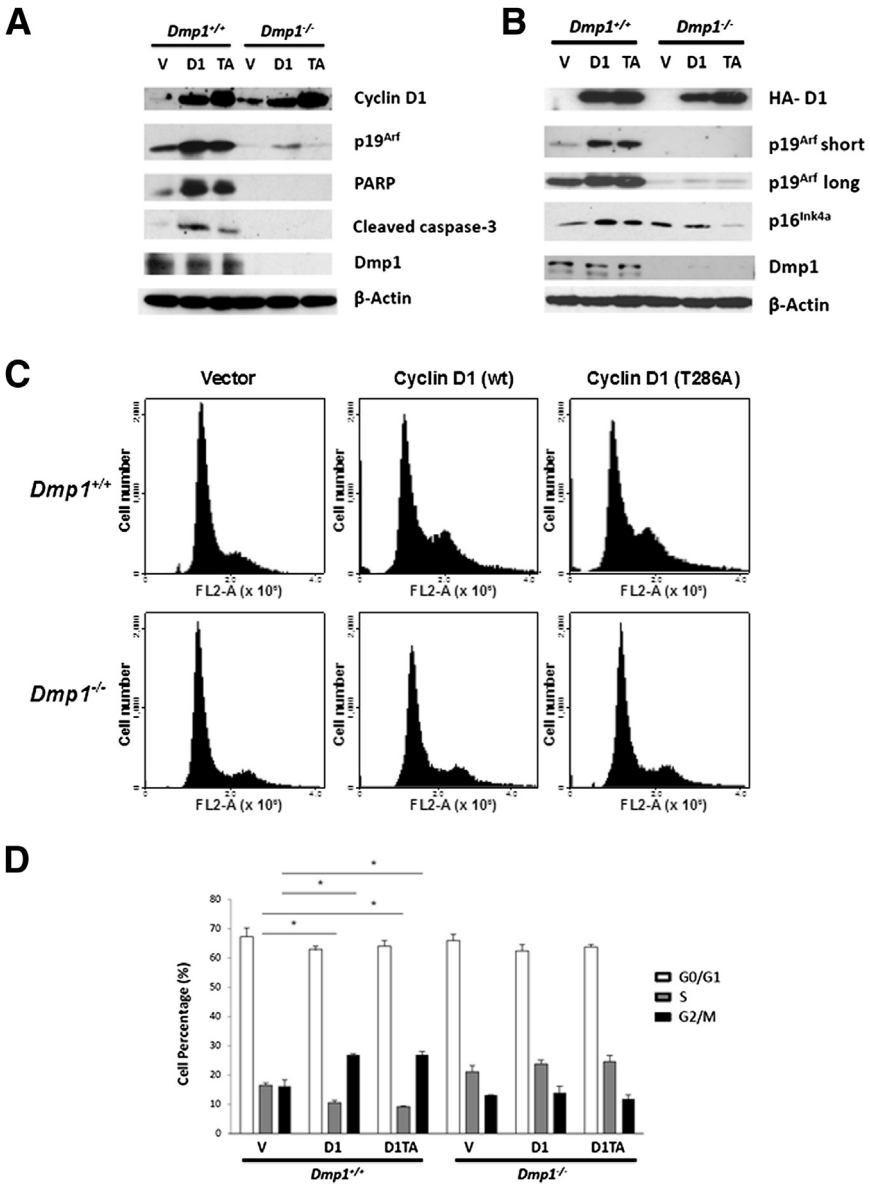

Figure 2 Overexpression of cyclin D1 or D1T286A induces apoptosis and G2/M phase delay in MEFs. **A**: Primary *Dmp1*$^{+/+}$ and *Dmp1*$^{-/-}$ MEFs were infected with control retrovirus (V) or retrovirus expressing cyclin D1 (D1) or D1T286A (TA) protein. Cells were starved for 48 hours in serum-free medium and then harvested for immunoblot analysis of cyclin D1, p19^{Arf}, PARP, cleaved caspase-3, and Dmp1. β-Actin was used as a loading control. **B**: Primary *Dmp1*$^{+/+}$ and *Dmp1*$^{-/-}$ MEFs were infected with control lentivirus or lentivirus expressing HA-tagged cyclin D1 or D1T286A protein. Cells were harvested after 48 hours for immunoblot analysis of HA-cyclin D1, p19^{Arf}, p16^{Ink4a}, and Dmp1. β-Actin was used as a loading control. **C**: Representative images of cell-cycle profiles of *Dmp1*$^{+/+}$ and *Dmp1*$^{-/-}$ MEFs infected by lentivirus carrying an empty vector or expressing cyclin D1 or D1T286A (T286A) and stained with propidium iodide 48 hours after infection. **D**: Quantification of flow cytometric analyses was from three independent experiments. Error bars indicate means ± SD. FL-2A, fluorescent pulse-area; wt, wild-type. *$P < 0.05$.

Cyclin D1– and D1T286A-Induced Mammary Carcinogenesis Is Accelerated in *Dmp1*$^{+/-}$ Mice

To study whether there is any cooperation between *Dmp1* loss and cyclin D1 overexpression *in vivo*, we crossed *MMTV-cyclin D1* and *MMTV-D1T286A* mice with *Dmp1*-null mice to generate the compound mice (Supplemental Figure S3). Consistent with a previous study,[27] both cyclin D1 and D1T286A were highly expressed in mammary glands of the transgenic mice, and D1T286A showed increased nuclear intensity in mammary epithelial cells relative to these in the mice with wild-type cyclin D1 (Supplemental Figure S4A). We did not detect cyclin D1 or D1T286A expression in other organs. Dmp1 showed substantially reduced expression in the mammary glands of *Dmp1*$^{+/-}$ mice compared to these in wild-type mice (Supplemental Figure S4B). We next assessed the proliferation and *p19*^{Arf}/*p16*^{Ink4a} expression in pre-malignant mammary glands from female mice between the ages of 8 and 10 months. The percentages of Ki-67–positive cells were significantly increased in the mammary glands of *Dmp1*$^{+/-}$; *MMTV-cyclin D1* and *Dmp1*$^{+/-}$;*MMTV-D1T286A* mice over these in *Dmp1*$^{+/+}$;*MMTV-cyclin D1* and *Dmp1*$^{+/+}$;*MMTV-D1T286A* mice (Figure 4A). Consistent with our observation in mammary tumors (Supplemental Figure S2, B and C), both p19^{Arf} and p16^{Ink4a} mRNA levels were significantly increased (5-fold to 35-fold for *p19*^{Arf}, 4-fold to 9-fold for *p16*^{Ink4a}) in mammary glands of *Dmp1*$^{+/+}$;*MMTV-cyclin D1* and *Dmp1*$^{+/+}$; *MMTV-D1T286A* mice compared to their counterparts without the *CYCLIN D1* transgene. On the other hand, the p19^{Arf} mRNA levels were significantly decreased in mammary glands from both *Dmp1*$^{+/-}$ and *Dmp1*$^{-/-}$ mice compared to those from *Dmp1*$^{+/+}$ mice (Figure 4, B and C), whereas *p16*^{Ink4a} showed marked reduction only in mammary glands from *Dmp1*$^{-/-}$ mice (Figure 4D). Taken together, these findings suggest that cyclin

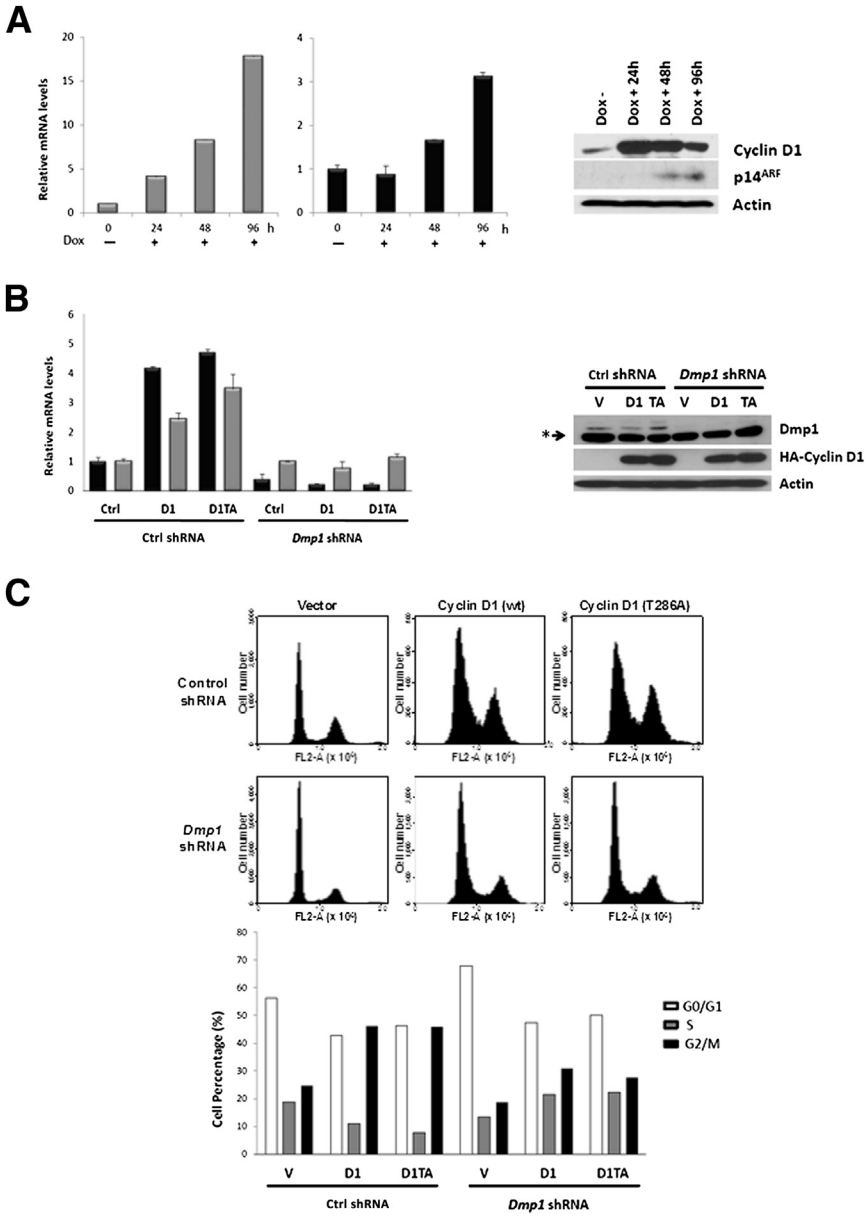

Figure 3 Overexpression of cyclin D1 activates both *p14*^{ARF} and *p16*^{INK4a} in human mammary epithelial cells (HMECs). **A**: HMECs were infected with lentivirus expressing doxycycline (Dox)-induced cyclin D1. After puromycin selection, 0.5 μg/mL Dox was added, and cells were harvested after 0, 24, 48, and 96 hours, respectively. p14^{ARF} (light gray bars) and p16^{INK4a} (dark gray bars) mRNA levels measured by real-time PCR using β-actin as a control. Cyclin D1 and p14^{ARF} expression were analyzed by immunoblots with β-actin as a loading control. **B**: HMECs were manipulated to coexpress control or *DMP1* shRNA (Ctrl) and cyclin D1 (D1) or D1T286A (D1TA) as indicated. Real-time PCR analysis of p14^{ARF} (light gray bars) and p16^{INK4a} (dark gray bars) mRNA levels after normalized against β-actin. Immunoblot analysis of Dmp1 and HA-tagged cyclin D1 expression. β-Actin was used as loading control. The **asterisk** indicates nonspecific bands. **C**: HMEC cells were treated as in **B** and stained with propidium iodide followed by flow cytometric analysis (**top panel**). Quantification of cell-cycle distribution is shown in the **bottom panel**.

D1 overexpression in mammary glands up-regulates both *p19*^{Arf} and *p16*^{Ink4a} in *Dmp1*$^{+/+}$ mice, but this regulation is compromised in *Dmp1*-deficient backgrounds.

We established long-term murine cohorts to assess the effect of cyclin D1 overexpression and *Dmp1* loss on tumor formation in these transgenic mice. We focused on mammary tumor development in *Dmp1*$^{+/-}$ mice because the *Dmp1*$^{-/-}$; *MMTV-cyclin D1* and *Dmp1*$^{-/-}$;*MMTV-D1T286A* mice frequently developed lung carcinomas or had high incidence of deaths in the early months after birth for unknown reasons before they developed mammary tumors. This design was also clinically relevant, because human breast cancers showed hemizygous deletion of human *DMP1* (h*DMP1*) in nearly half of the cases, whereas biallelic deletion of *hDMP1* was rare.[15] The cyclin D1− and D1T286A-induced mammary tumor development was significantly accelerated in *Dmp1*$^{+/-}$ mice, with an estimated median disease-free survival from 810 to 600 days ($P = 0.0238$) for wild-type cyclin D1, and from 730 to 645 days in D1T286A ($P = 0.0284$) (Figure 5A). There were higher percentages of *Dmp1*$^{+/-}$;*MMTV-cyclin D1* and *Dmp1*$^{+/-}$;*MMTV-D1T286A* mice developing mammary tumors, with shorter survival times than those of the *Dmp1* wild-type cohorts (Table 1). In genomic DNA analyses, mammary tumors of *Dmp1*$^{+/-}$ mice retained a wild-type *Dmp1* allele in all of the seven tumors examined (Figure 5A), confirming the haploinsufficiency of *Dmp1* in suppressing cyclin D1−driven tumor formation. *Dmp1*$^{+/+}$; and *Dmp1*$^{+/-}$; *MMTV-cyclin D1* and *MMTV-D1T286A* mice developed mammary ductal adenocarcinomas. The mammary tumors from *Dmp1*$^{+/+}$;*MMTV-cyclin*

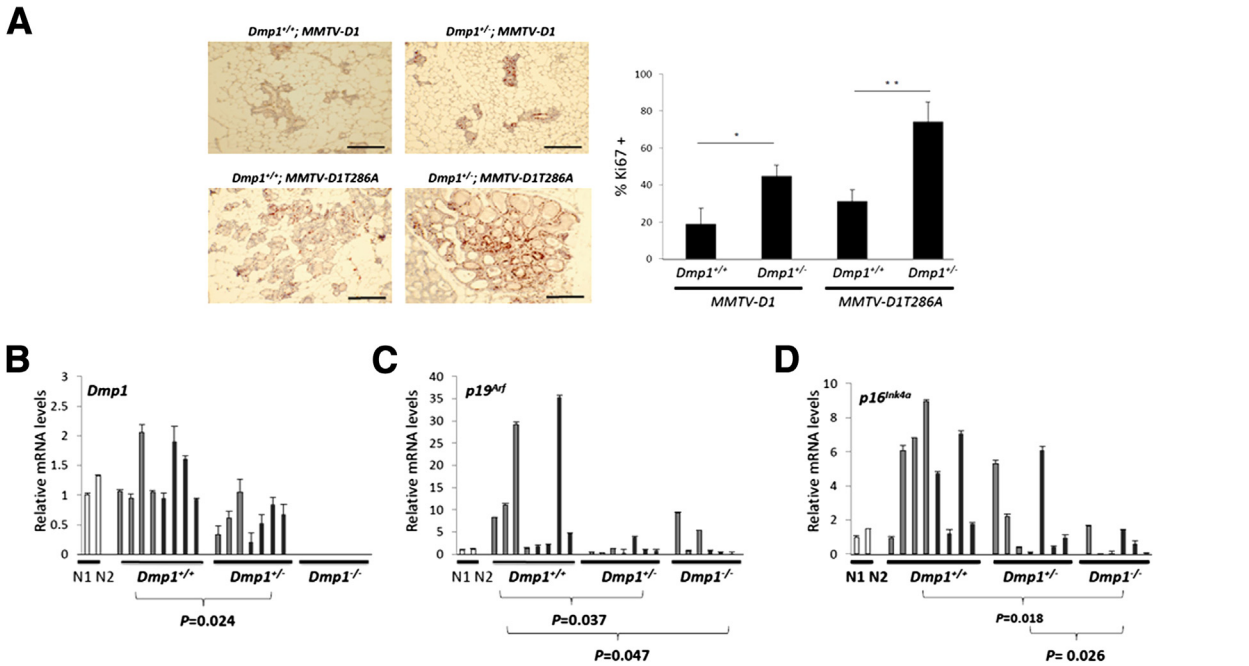

Figure 4 Mammary glands from *Dmp1*$^{+/+}$;*MMTV-cyclin D1* and *Dmp1*$^{+/+}$;*MMTV-D1T286A* mice show lower proliferation and increased *Arf/Ink4a* expression. **A**: Mammary tissues derived from *MMTV-cyclin D1* and *Dmp1*$^{+/+}$;*MMTV-D1T286A* mice showed increased proliferation at *Dmp1* heterozygous background. Representative images of Ki-67 immunostaining (red) in mammary glands from *Dmp1*$^{+/+}$; and *Dmp1*$^{+/-}$; *MMTV-cyclin D1,* and *MMTV-D1T286A* mice. Nuclei were counterstained with hematoxylin (blue). Scale bars: 100 μm. Quantification of Ki-67—positive cells per mammary gland section is shown. Error bars indicate means ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$. **B**—**D**: Real-time PCR analysis of Dmp1, p19^{Arf}, and p16^{Ink4a} mRNA levels in mammary glands from *MMTV-cyclin D1* and *MMTV-D1T286A* mice in *Dmp1*$^{+/+}$, *Dmp1*$^{+/-}$, and *Dmp1*$^{-/-}$ backgrounds. Data were normalized against β-actin mRNA levels. Gray columns represent the samples from *MMTV-cyclin D1* mice and black columns represent the samples from *MMTV-cyclin D1T286A* mice. N, normal mammary gland from nontransgenic mice.

D1 and *Dmp1*$^{+/+}$;*MMTV-D1T286A* mice showed squamous metaplasia and microinvasion as indicated by blurred epithelial—stromal interface in some glands and a corresponding tissue desmoplasia, whereas the tumors from *Dmp1*$^{+/-}$; *MMTV-cyclin D1* and *Dmp1*$^{+/-}$;*MMTV-D1T286A* mice yielded a more aggressive phenotype as measured by an increased nuclear/cytoplasmic ratio, nuclear pleomorphism, and layers of disorganized epithelium (Figure 5B). Moreover, mammary tumors from *Dmp1*$^{+/+}$;*MMTV-cyclin D1* and *Dmp1*$^{+/+}$; *MMTV-D1T286A* mice displayed a significantly higher rate of apoptosis compared to those from *Dmp1*$^{+/-}$;*MMTV-cyclin D1* and *Dmp1*$^{+/-}$;*MMTV-D1T286A* mice (Figure 5C), as indicated by cleaved caspase-3 staining (percentage of cleaved caspase-3—positive cells: 3.83% in *Dmp1*$^{+/+}$ versus 0.11% in *Dmp1*$^{+/-}$, $P < 0.01$).

Most human breast carcinomas with high cyclin D1 levels also express ER, and a large portion of mammary tumors from *MMTV-cyclin D1* and *MMTV-D1T286A* mice with intact *Dmp1* are ER-positive.[27] Thus, we wanted to determine whether cyclin D1—induced mammary tumors on the *Dmp1*$^{+/-}$ background retained ERα expression. Our immunohistochemical analyses revealed >50% of mammary tumors (12 of 21) from *Dmp1*$^{+/-}$;*MMTV-cyclin D1* and *Dmp1*$^{+/-}$;*MMTV-D1T286A* mice were still ERα-positive (Supplemental Figure S5), suggesting that the loss of *Dmp1* did not change the estrogen dependence of cyclin D1—induced mammary tumors.

Frequent Metastasis of Mammary Tumors in *Dmp1*$^{+/-}$; *MMTV-cyclin D1* Mice

Apart from mammary adenocarcinomas, a small number of other tumor types, including lymphoma and sarcoma, were also found in both *Dmp1*$^{+/+}$ and *Dmp1*$^{+/-}$ cohorts (Table 1). Of note, lung carcinomas were only found in *Dmp1*$^{+/-}$ mice, confirming the haploinsufficiency of *Dmp1* in lung cancer suppression.[16,31] Importantly, four of nine mammary tumors from *Dmp1*$^{+/-}$;*MMTV-cyclin D1* (multiparous) mice and one of seven tumors from *Dmp1*$^{+/-}$; *MMTV-D1T286A* (nulliparous) mice metastasized to other organs, including liver, ovary, and uterus (Table 1 and Figure 6A). Intense cyclin D1 staining was detected in the metastatic tumors but not in adjacent normal tissues (Figure 6B). The high expression of human *CYCLIN D1* transgene in both primary mammary and metastatic tumors was confirmed by real-time PCR studies (Figure 6C). Furthermore, both primary and metastatic tumors expressed cytokeratin 8 and 14 (Figure 6D), indicating that they derived from the same epithelial origin. Finally, we still detected low levels of Dmp1 in the metastatic tumors, as in primary tumors, suggesting that they still retained the one *Dmp1* allele (Figure 6D). In summary, these results suggest that Dmp1 reduction due to its heterozygosity promotes the metastasis of cyclin D1— and D1T286A-initiated mammary tumors.

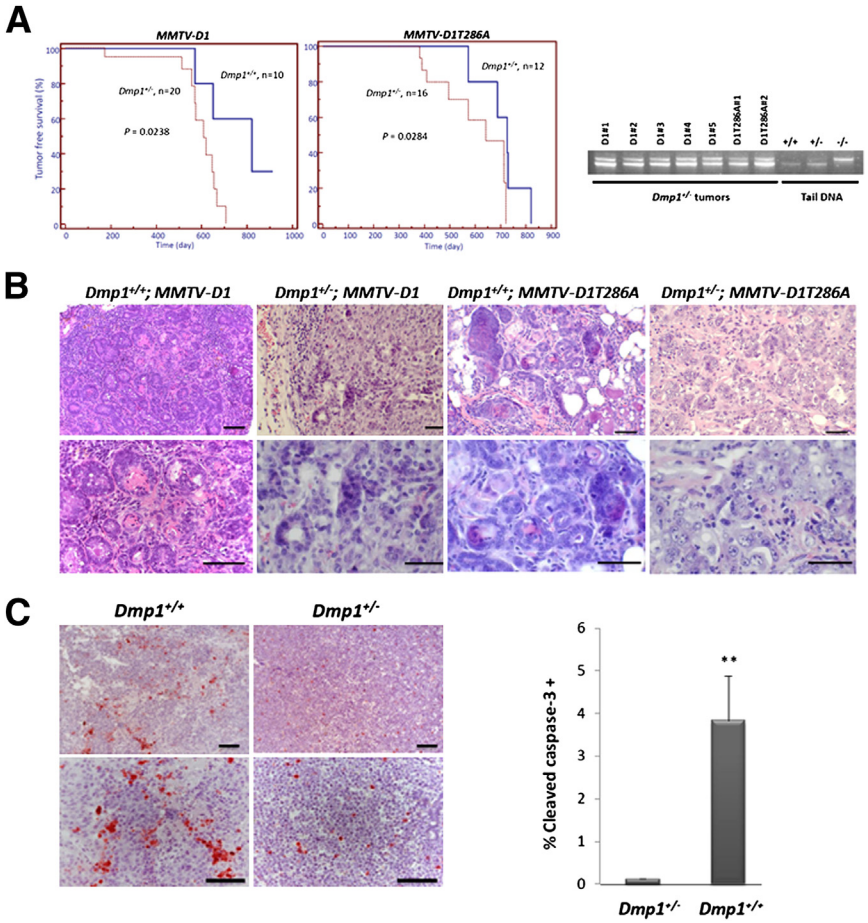

Figure 5 Cyclin D1— and D1T286A-induced mammary carcinogenesis is accelerated in *Dmp1*$^{+/-}$ mice. **A:** Tumor-free survival of *Dmp1*$^{+/+}$;*MMTV-cyclin D1* (blue) and *Dmp1*$^{+/-}$;*MMTV-cyclin D1* (red) compound transgenic mice (**left panel**) and *Dmp1*$^{+/+}$;*MMTV-DT286A* (blue) and *Dmp1*$^{+/-}$;*MMTV-DT286A* (red) compound transgenic mice (**center panel**). Cyclin D1—mediated-tumor development was significantly more accelerated in *Dmp1*$^{+/-}$ than in *Dmp1*$^{+/+}$ genetic background. PCR confirmed retention of the wild-type *Dmp1* locus in mammary carcinomas from *Dmp1*$^{+/-}$;*MMTV-cyclin D1* and *Dmp1*$^{+/-}$;*MMTV-D1T286A* mice (**right panel**). *MMTVD1, MMTV-cyclin D1*. **B:** Representative images of mammary adenocarcinoma from *Dmp1*$^{+/+}$;*MMTV-cyclin D1* and *Dmp1*$^{+/-}$;*MMTV-cyclin D1* mice and *Dmp1*$^{+/+}$;*MMTV-D1T286A* and *Dmp1*$^{+/-}$;*MMTV-D1T286A* mice. Histological sections were stained with H&E. **C:** Representative images of immunostaining for cleaved caspase-3 (red) in mammary tumors from *Dmp1*$^{+/+}$;*MMTV-cyclin D1*, *Dmp1*$^{+/+}$;*MMTV-D1T286A*, *Dmp1*$^{+/-}$;*MMTV-cyclin D1*, and *Dmp1*$^{+/-}$;*MMTV-D1T286A* mice. Nuclei were counterstained with hematoxylin (blue). Scale bars: 100 μm. Quantification of caspase-3—positive area in the tumor sections. For each section, three independent areas were scanned and quantified using Image-Pro Plus software version 6.3.0. Error bars indicate means ± SD. $^{**}P < 0.01$.

Discussion

It has been shown that Dmp1 is a physiological activator of the *Arf* promoter.[13,31] The *Arf* promoter is transactivated by a variety of oncogenic signaling to prevent incipient cells from undergoing full transformation to cancers. It has been reported that Ets transcription factors play essential roles in activating the *p16*^{INK4a} promoter through an Ets consensus in response to oncogenic Ras-Raf-MEK kinase signaling.[32] Here, we showed that Dmp1 receives signals from cyclin D1 overexpression and binds to the Dmp1/Ets site on the *Ink4a* promoter, which is different from the Ets consensus characterized in the previous study. Thus the *Ink4a* gene is transactivated by different oncogenes through distinct Ets consensus sequences.

The Dmp1-binding site is within approximately 200 bp from the transcription initiation site of the murine *Ink4a* gene. Although Dmp1 directly binds to both mouse and human *INK4a* promoters, the affinity was lower than that in the *Arf* promoter. Of note, reduced affinity binding of Dmp1 has been reported on other promoters such as *Areg*,[22] indicating that the extent of Dmp1 involvement in gene regulation is dependent on the sequences of the promoter, where CCCG(G/T)ATG(T/C) (Dmp1/Ets core is underlined) shows high affinity (eg, the *Arf*[14], *CD13*[18] promoters) and XXCG(G/T)ATGX (Dmp1/Ets core is underlined; X could be any nucleotide) has low affinity in Dmp1 binding (eg, the *Ink4a*, *Areg* promoters).

Previous studies from our group showed that Dmp1 regulates the Arf-p53 pathway by activating *Arf*[14] and interacting with p53 to neutralize its antagonism by Mdm2.[33] Although Dmp1 has been isolated as a cyclin D2—binding transcription factor, very little is known about its role in cyclin D—mediated signal transduction and tumorigenesis. Because cyclin D1 does not have a DNA-binding domain, it needs to interact with transcription factors to regulate gene expression. Dmp1 is the critical binding partner of *Ink4a/Arf* transcription. Both *Arf* and *Ink4a* promoters were transactivated by cyclin D1, which was confirmed by mRNA analyses of pre-malignant mammary glands from *cyclin D1* and *D1T286A* transgenic mice. In the process of increased *p19*^{Arf} and *p16*^{Ink4a} expression and induction of cell-cycle arrest or apoptosis, wild-type cyclin D1 and its constitutively active mutant D1T286A had very similar effect, suggesting that high expression of wild-type cyclin D1 is sufficient to induce Arf and Ink4a responses in normal cells to quench potentially oncogenic signals. In the activation of both *Ink4a/Arf* promoters, cyclin D1—Dmp1 interaction was necessary for this process due to lack of DNA binding of cyclin D1. Consistently, the cyclin D1 mutant D1Δ142—253, deficient in interacting with

Table 1 Tumor Characterization in *Dmp1*$^{+/+}$; and *Dmp1*$^{+/-}$; *MMTV-D1* and *MMTV-TA* Mice

	Dmp1$^{+/+}$;*MMTV-D1*	*Dmp1*$^{+/-}$;*MMTV-D1*
Percentage of tumor-bearing mice	40% (4/10)	57.9% (11/19)
Mean survival time (months)	21	19
Tumor spectrum		
Adenocarcinoma of breast	3/4	9/11*
Lymphoma	1/4	1/11
Sarcoma	0/4	1/11
	Dmp1$^{+/+}$;*MMTV-TA*	*Dmp1*$^{+/-}$;*MMTV-TA*
Percentage of tumor-bearing mice	58.3% (7/12)	62.6% (10/16)
Mean survival time (months)	22.71	19.38
Tumor spectrum		
Adenocarcinoma of breast	6/7	7/10†
Sarcoma	1/7	1/10
Lung carcinoma	0/7	2/10

*The tumors metastasized to other organs, including liver, ovary, and uterus, in four of nine *Dmp1*$^{+/-}$;*MMTV-cyclin D1* mice.

†The tumors metastasized to other organs, including liver, ovary, and uterus, in one of seven *Dmp1*$^{+/-}$;*MMTV-D1T286A* mice with mammary adenocarcinomas.

MMTV-D1, *MMTV-cyclin D1*; *MMTV-TA*, *MMTV-D1T286A*.

Dmp1, did not activate these two promoters, and cyclin D1 did not bind to these promoters in *Dmp1*-deficient cells. Thus, we conclude that Dmp1 plays critical roles in *Arf* and *Ink4a* gene activation in response to cyclin D1 overexpression. The fact that the *MMTV-cyclin D1* and *D1T286A* mice eventually developed mammary tumors is possibly due to loss of *Dmp1* or overexpression of *Ink4a/Arf* repressors (such as Bmi1, Tbx2/3, Twist, Pokemon) during tumor development. Further studies are needed to clarify the roles of these repressors in cyclin D1–driven mammary tumorigenesis.

We found that endogenous cyclin D1 bound to endogenous DMP1 and E2F1 in MCF7 breast cancer cells (DMP1 is shown in Supplemental Figure S6; E2F1 data not shown). C/EBPβ is known to bind to cyclin D1,[4] and our data showed that cyclin D1 did not activate the *Arf* promoter with deleted C/EBP-consensus sequence (data not shown). Thus, we cannot exclude the possibility that other transcriptional factors are also involved in cyclin D1–mediated activation of the *Arf* promoter. Recent studies indicated that cyclin D1 interacts with transcription factors including NF-Y, STAT, CREB2, ELK1, ZNF423, and CUX1,[11] and we also identified their consensus sequences in the *Arf/Ink4a* promoters (data not shown). Because our data showed the Dmp1-dependent *Arf/Ink4a* activation, future studies will be needed to determine whether Dmp1 collaborates with C/EBPs or other transcription factors in regulating *Arf/Ink4a* expression.

Our results showed the levels of p21^{Cip1} induction did not change significantly between *Dmp1*$^{+/+}$ and *Dmp1*$^{-/-}$ MEFs, consistent with a previous study that Arf-induced cell-cycle arrest independent of p21^{Cip1}.[34] ARF directly binds to DP1

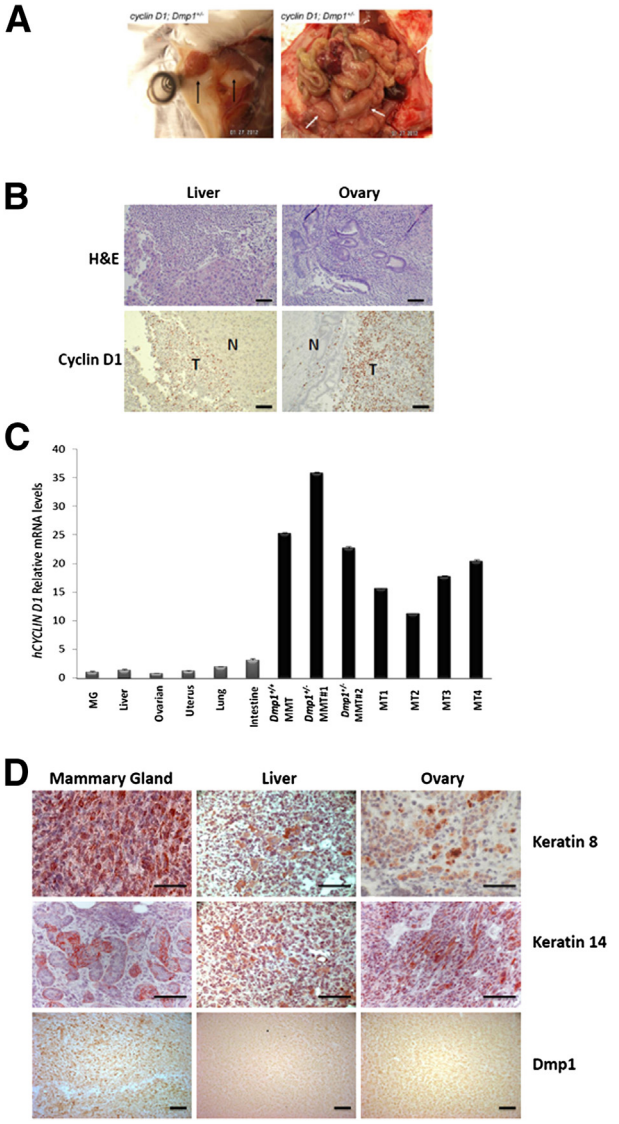

Figure 6 Mammary tumors induced by cyclin D1 and D1T286A metastasize in *Dmp1*$^{+/-}$ mice. **A**: Photomicrographs of mammary and metastatic tumors from one *Dmp1*$^{+/-}$;*MMTV-cyclin D1* mouse; tumor cells disseminated throughout the body to other organs, including the uterus and ovaries. **Left panel**: primary mammary tumor (**arrows**). **Right panel**: metastasis of tumor cells (**arrows**) throughout the abdomen. **B**: Representative images of immunostaining for H&E and cyclin D1 (red) in metastatic tumors in liver and ovary from *Dmp1*$^{+/-}$;*MMTV-cyclin D1* mice. High cyclin D1 expression was detected only in the tumor region but not adjacent normal tissues. Scale bars: 100 μm. N, normal; T, tumor. **C**: Real-time PCR analyses of human cyclin D1 transgene mRNA levels in normal organs of nontransgenic mice and mammary/metastatic tumors in *Dmp1*$^{+/+}$;*MMTV-cyclin D1* and *Dmp1*$^{+/-}$;*MMTV-cyclin D1* mice. Data were normalized against that of β-actin. MG, mammary gland; MMT, mouse mammary tumor; MT, metastatic tumor. **D**: Representative images of immunostaining for keratin 8 (red), keratin 14 (red) and Dmp1 (red) in primary mammary tumors and metastatic tumors from liver and ovary, respectively, of *Dmp1*$^{+/-}$;*MMTV-cyclin D1* mice. Nuclei were counterstained with hematoxylin after keratin 8 and keratin 14 staining. Scale bars: 100 μm.

and inhibits DP1-E2F1 interaction.[35] The activity of E2F1 can also be inhibited by INK4a.[14] Therefore, it would be needed to study the roles of E2F1 and its target genes in cyclin D1 overexpression-induced cell-cycle arrest.

We showed that both cyclin D1− and D1T286A-driven mammary carcinogenesis was accelerated in *Dmp1*$^{+/-}$ mice. Importantly, mammary tumors from *Dmp1*$^{+/-}$ mice exhibited significantly increased Ki-67 in comparison to those from *Dmp1*$^{+/+}$ mice because Ki-67 is one of the target genes for E2Fs,[36] suggesting that the activity of E2Fs stimulated by cyclin D1 is suppressed by Dmp1. Our study showed that the *Dmp1* locus was deleted in 4 of 19 (21%) mammary tumors from *MMTV-cyclin D1* and *MMTV-D1T286A* mice, in which neither *Ink4a/Arf* nor *p53* deletion was observed. Frequent deletion of one *Dmp1* allele was also observed in *MMTV-neu* tumors,[17] suggesting the critical role of *Dmp1*-loss in cyclin D1− and neu-induced mammary tumorigenesis. The current study showed that the *p16*^{Ink4a} was only significantly down-regulated in *Dmp1*$^{-/-}$ mammary glands, but not in *Dmp1*$^{+/-}$ tissues, in comparison to *Dmp1*$^{+/+}$ samples, whereas *p19*^{Arf} was significantly down-regulated in both *Dmp1*$^{+/-}$ and *Dmp1*$^{-/-}$ mammary glands. Similar findings were observed in mammary tumors from *MMTV-neu* mice as well as in the lungs from three different Dmp1 backgrounds.[16,17] The difference of *p16*^{Ink4a} expression in *Dmp1*$^{+/-}$ background from that in *Dmp1*$^{-/-}$ background may explain more accelerated tumor development in *Dmp1*$^{-/-}$ mice than that in *Dmp1*$^{+/-}$ mice although the difference was not statistically significant.[13,16]

Given the link between Ras signaling and Dmp1, it is noteworthy that neither overexpression of cyclin D1 nor hemizygous loss of *Ink4a* and *Arf* accelerated tumorigenesis in *MMTV-ErbB2* mice.[37] The above study is consistent with a previous report that HER2/neu-driven mammary carcinogenesis was not observed in *cyclin D1*−null mice,[38] suggesting that cyclin D1 is a critical target of HER2/neu/ErbB2 in promoting mammary tumorigenesis. Our published study showed that both *Ink4a* and *Arf* inductions in response to HER2/neu overexpression were markedly attenuated (>80%) in mammary tissue from *Dmp1*$^{-/-}$ mice.[17] Our current study also showed the reduced p16^{Ink4a}/p19^{Arf} mRNA levels in pre-malignant mammary tissues from *MMTV-cyclin D1* and *D1T286A* mice in both *Dmp1*$^{+/-}$ and *Dmp1*$^{-/-}$ backgrounds compared to that in *Dmp1* wild-type mice (Figure 4, C and D). The above data explain the difference between no acceleration of cyclin D1−induced mammary tumor developed in *Ink4a/Arf*$^{+/-}$ mice[37] and the significantly accelerated carcinogenesis in *Dmp1*$^{+/-}$ mice (Figure 5A). Meanwhile, the data also suggest that other targets of Dmp1 may be involved in cyclin D1−induced mammary carcinogenesis.

Our study also showed the significantly increased metastasis of keratin-positive cyclin D1−induced mammary tumors in *Dmp1*$^{+/-}$ mice in comparison to *Dmp1*$^{+/+}$ mice. Most cyclin D1 tumors were adenosquamous ductal carcinomas that metastasized to liver, ovary, uterus, and intestines, although we did not see any brain/bone metastasis that is commonly found in human breast cancer patients. Nevertheless, our results imply an important role of Dmp1 in preventing the mammary tumor metastasis induced by cyclin D1 and provide a potential mechanism of breast cancer metastasis. Currently, the frequency of human breast cancer with high cyclin D1 overexpression with or without h*DMP1* deletion, their prognostic values, and correlation with clinical stages of patients are unknown. Thus, it will be necessary to analyze human breast cancer samples for the correlation between cyclin D1/Dmp1 alterations and patient survival data to determine the clinical values of our discovery.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.06.027*.

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