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# Critical roles of DMP1 in HER2/neu-Arf-p53 signaling and breast cancer development

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## Abstract

HER2 overexpression stimulates cell growth in *p53*-mutated cells while it inhibits cell proliferation in those with wild-type *p53*, but the molecular mechanism is unknown. The *Dmp1* promoter was activated by HER2/neu through the PI3K-Akt-NF-κB pathway, which in turn stimulated *Arf* transcription. Binding of p65 and p52 subunits of NF-κB was demonstrated to the *Dmp1* promoter and that of Dmp1 to the *Arf* promoter upon HER2/neu overexpression. Both Dmp1 and p53 were induced in pre-malignant lesions from MMTV-*neu* mice and mammary tumorigenesis was significantly accelerated in both $Dmp1^{+/-}$ and $Dmp1^{-/-}$ mice. Selective deletion of *Dmp1* and/or overexpression of Tbx2/Pokemon was found in >50 % of wild-type HER2/neu carcinomas while the involvement of Arf, Mdm2, or p53 was rare. Tumors from $Dmp1^{+/-}$, $Dmp1^{-/-}$, and wild-type *neu* mice with hemizygous *Dmp1* deletion showed significant downregulation of *Arf* and $p21^{Cip1/WAF1}$, showing p53 inactivity and more aggressive phenotypes than tumors without *Dmp1* deletion. Notably, endogenous h*DMP1* mRNA decreased when *HER2* was depleted in human breast cancer cells. Our study demonstrates the pivotal roles of Dmp1 in HER2/neu-p53 signaling and breast carcinogenesis.

### Keywords

Dmp1 (Dmtf1); HER2/neu; NF-κB; Arf; p53; Tbx2; Pokemon; breast cancer

## Introduction

Breast cancer is one of the largest public health issues in the United States and most of the industrialized world (1–4). Breast cancers that are positive for the estrogen receptor (ER) are usually responsive to adjuvant hormonal therapy with anti-estrogens and/or aromatase inhibitors, and thus have a more favorable prognosis (1). On the other hand, ER-negative breast cancers are often associated with aggressive disease, including amplification of *HER2* or c-*Myc* oncogenes and mutation of the *p53* gene (5). Chemotherapy plus use of the

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humanized monoclonal antibody to HER2 (trastuzumab) is considered the best treatment for hormone-unresponsive or resistant patients, but the prognosis of such patients is poor (5).

HER2/neu encodes a receptor-type tyrosine kinase that belongs to the EGFR family (5–9). It is overexpressed in ~30 % of breast cancer cases, primarily due to gene amplification. HER2/neu overexpression is found in metastatic lesions, and thus is associated with poor prognoses (4–6). Recent studies have stressed the importance of phosphatidylinositol-3'-kinase (PI3K) and serine/threonine kinase Akt/protein kinase B in HER2/neu signaling (10). The PI3K-Akt signaling has also been linked to the induction of NF-κB (11–13). Since transcriptional activation by NF-κB requires its nuclear translocation, degradation of IκB molecules triggered by phosphorylation of serine residues 32/36 by IκB kinases has been considered a key rate-limiting step in NF-κB activation (11). Importantly, both human breast cancer cell lines and clinical specimens often show constitutive activation of NF-κB (14), suggesting oncogenic roles of subsets of NF-κB in breast cancer development.

Dmp1, a cyclin D binding myb-like protein 1 (also called Dmtf1), was originally isolated in a yeast two-hybrid screen of a murine T-lymphocyte library with cyclin D2 as bait (15). Dmp1 shows its activity as a tumor suppressor by directly binding to the *Arf* promoter to activate its gene expression, and thereby induces Arf- and p53-dependent cell cycle arrest (16,17; for Arf reviews, 18,19). *Dmp1*-null cells can easily give rise to immortalized cell lines that retain wild-type $p19^{Arf}$ and functional p53 and are transformed by oncogenic Ras alone, suggesting that the activity of the Arf-p53 pathway is significantly attenuated in *Dmp1*-deficient cells (20,21). The murine *Dmp1* promoter is efficiently activated by oncogenic Ras and is repressed by mitogenic signals mediated by E2Fs and genotoxic signals by NF-κB (22–24; for review ref. 25). Both $Dmp1^{-/-}$ and $Dmp1^{+/-}$ mice are prone to tumor development when neonatally treated with dimethylbenzanthracene or by ionizing radiation (20,21). Tumors induced by the *Eμ-Myc* or *K-Ras* transgene were greatly accelerated in both $Dmp1^{+/-}$ and $Dmp1^{-/-}$ backgrounds with no differences between groups lacking one or two *Dmp1* alleles, suggesting haploid-insufficiency of Dmp1 in tumor suppression (21,26). Moreover, tumors from *Eμ-Myc* or $K\text{-}Ras^{LA}$ mice rarely showed *p53* mutation or *Arf* deletion, indicating that Dmp1 is a physiological regulator of the Arf-p53 pathway in lymphoid and lung epithelial cells (21,26; for reviews refs. 27,28).

The h*DMP1* gene is located on chromosome 7q21, a region often deleted in human breast cancer and hematopoietic malignancies (29–32). We recently found that loss of heterozygosity of h*DMP1* was present in ~35 % of non-small cell lung carcinomas (26). It was reported that HER2 overexpression in *p53* wild-type human ovarian carcinoma cell line became apoptotic shortly after transfection, while HER2 expression was associated with cell proliferation in cells with mutated *p53* (33). However, the signaling pathway that links HER2 overexpression and activation of p53 has never been demonstrated. Moreover, very little is known about the roles of *Dmp1* (or h*DMP1*) in breast cancer development. The current study was conducted to elucidate the roles of Dmp1 and Arf in HER2/neu signaling and breast carcinogenesis. We show that both *Dmp1* and *Arf* promoters are selectively activated by HER2/neu and both Dmp1 and p53 proteins are induced in pre-cancerous mammary glands from MMTV-*neu* mice (34). Of note, the value of MMTV-LTR driven transgenic mice as models for human breast cancer has recently been reconfirmed since the discovery of MMTV *env*-, LTR-like sequences in ~40 % of human breast carcinomas (35,36). We crossed MMTV-*neu* mice with *Dmp1*-deficient mice to observe the latency period for tumorigenesis, and have conducted extensive molecular genetic analyses of mammary tumors. We also demonstrate that human *DMP1* is regulated by endogenous HER2 overexpressed in breast epithelial cells.

## Materials and Methods

### Establishment of *Dmp1*$^{+/-}$, *Dmp1*$^{-/-}$; MMTV-*neu* compound mice

*Dmp1*-heterozygous females were backcrossed to the same FVB/NJ males (Jackson Laboratories, #001800) for more than 8 generations to obtain *Dmp1*$^{+/-}$ mice with >99 % FVB/NJ background overall. One male MMTV-*neu* (mutant) mouse (Jackson Laboratories, #005038) was crossed with two *Dmp1*$^{+/-}$ females to obtain *Dmp1*$^{+/-}$; MMTV-*neu* mice. Then *Dmp1*$^{+/-}$; MMTV-*neu* compound transgenic mice were further crossed with *Dmp1*$^{+/-}$ mice to obtain more than 25 mice with each genetic background. Littermate wild-type mice were used as controls. Mice were maintained in accordance with the Guide for the Care and Use of Laboratory Animals.

### Real-time PCR

Quantitation of *Dmp1*, *p19*$^{Arf}$, *p21*$^{Cip1/WAF1}$, and *p16*$^{Ink4a}$ mRNAs were conducted by real-time PCR Taqman assay by ABI7500 (Applied Biosystems, Foster City, CA) using β-*actin* as an internal control (23,24,37). For *p21*$^{Cip1/WAF1}$, Mm01303209_m1 was used; other assays were custom-designed at ABI. Gene copy number assays for *Dmp1, Arf, p53, grm3*, and *abcb1* were also performed by real-time PCR using β-*actin* as an internal control (26).

### Western blotting

Proteins were extracted with ice-cold EBC buffer (15) with proteinase inhibitors from frozen mammary tumor cells or human breast cancer cell lines. After gel electrophoresis and transfer to nitrocellulose membranes, proteins were visualized by immunoblotting with affinity-purified polyclonal antibodies to Dmp1 (RAX) (23), p53 (sc-6243G, Santa Cruz Biotech), Mdm2 (ab16896 [2A10], Abcam), p19$^{Arf}$ (sc-32748), p14$^{ARF}$ (ab3642, Abcam), p16$^{Ink4a}$ (sc-1207), p21$^{CIP1/WAF1}$ (sc-397G), TBX2 (sc-17880), Pokemon (A300-548A, Bethyl Inc., TX), Twist (sc-15393), or β-Actin (sc-1615, sc-47778), followed by incubation of the filters with HRP-conjugated second antibodies, and reaction with the enhanced ECL detection kit (PerkinElmer).

Cell culture, reporter assays, chromatin immunoprecipitation, *in vitro* mutagenesis of the *Dmp1* promoter, immunohistochemical staining, retroviruses for *HER2* shRNA, and statistical analyses are described in the Supplementary Materials and Methods.

## Results

### Both the *Dmp1* and *Arf* promoters are specifically activated by HER2/neu

We tested whether murine *Dmp1* and *Arf* promoter can be activated by overexpression of HER2 (Fig. S1*A*). Both promoters were activated by HER2 expression in dose-dependent fashion (Fig. 1*A*, left panel). Both human *DMP1* and *p14*$^{ARF}$ promoters were also responsive to HER2 (Supplementary Fig. S1*B*). The specificity of these promoter activations by HER2 was confirmed by reporter assays with *p27*$^{KIP1}$, *p16*$^{Ink4a}$, and *Hdm2* promoters, which were all repressed by HER2 (Supplementary Fig. S1*B*). We then confirmed the results of reporter assays by quantitating the endogenous *Dmp1* and *Arf* mRNA by real-time PCR in wild-type MEFs infected with HER2 virus (Fig. 1*A*, middle). The Dmp1 protein induction by HER2 was confirmed by transiently expressing HER2 in 3T3 cells (Fig. 1*A*, right). The *Dmp1* promoter activation by HER2 was not inhibited by the MEK-ERK inhibitor, but was completely inhibited by the PI3K inhibitor and by the Akt inhibitors (Fig. 1*B*). The *Dmp1* promoter was efficiently inhibited by proteosomal NF-κB inhibitor PS341, or by co-expression of a constitutively-active IκB super repressor (Fig. 1*B*). Consistent with these findings, *in vitro* mutagenesis of either NF-κB site #1 or #2 significantly decreased the responsiveness of the *Dmp1* promoter to HER2 (Fig. 1*C*). We confirmed significant binding

of the endogenous p65/relA and p52 subunits of NF-κB to the *Dmp1* promoter upon HER2 overexpression by chromatin immunoprecipitation (ChIP) (Fig. 1*D*, left). Binding of p65 and p52 to the *Dmp1* promoter was also confirmed by tissue ChIP with lysates from MMTV-*neu* tumors (Fig. 1*D*, right).

We then mapped the HER2-responsive element on the *Arf* promoter. When the Dmp1/Ets site was mutated, the *Arf* promoter was not responsive to HER2 expression, suggesting that the promoter activation was Dmp1/Ets-dependent (Fig. 2*A*, middle). The *Arf* promoter activation by HER2 was dependent on Dmp1 since the promoter was not activated in $Dmp1^{-/-}$ cells (Fig. 2*A*, right). Binding of endogenous Dmp1 to the endogenous *Arf* promoter was confirmed by ChIP with lysates from 4 independent *neu* tumors (Fig. 2*B*). Thus, our data indicate that HER2/neu stimulates the Arf-p53 pathway through activation of the novel PI3K-Akt-NF-κB-Dmp1 signaling (Fig. 2*C*).

## Acceleration of *neu*-induced mammary tumor development in *Dmp1*-knockout mice

MMTV-*neu* females develop multiple mammary tumors (~5 mm in diameter) with a mean latency of 7 months in FVB/N strain. Since the *Dmp1* promoter is selectively activated by HER2/neu, we studied if Dmp1 and p53 proteins were induced in response to active *neu* in pre-malignant lesions (i.e. hyperplastic, non-transformed mammary glands mixed with islands of early stage tumors) isolated from 5.5 months old MMTV-*neu* females. Real-time PCR analysis showed upregulation of the *Dmp1* mRNA in early stage mammary tumors (Fig. 3*A*, left panel). Significant induction of the Dmp1 protein in hyperplastic pre-malignant lesion was confirmed by immunohistochemical staining of mammary glands from a 5.5-month-old female (Fig. 3*A*, 3rd panel) as well as those adjacent to *neu* tumors from a 7-month-old female (Fig. 3*A*, 4th panel, arrows; n=5, $P < 0.001$). The p53 protein was barely detectable in normal mammary glands (Fig. 3*B*, left), but significant amount of p53 was induced in hyperplastic mammary glands from *neu* mice (Fig. 3*B*, middle, arrow, $P < 0.01$). We also observed significant induction of $p21^{Cip1/WAF1}$ in pre-malignant lesions (Fig. 3*B*, right, arrow, $P < 0.01$). On the other hand, the pro-apoptotic p53 target, Puma was not induced in early stage *neu* tumors (data not shown). Our data indicate activation of the Dmp1-p53-$p21^{Cip1/WAF1}$ signaling in pre-malignant hyperplastic mammary glands or early stage mammary tumors in response to oncogenic HER2/neu signaling.

To study the cooperation between *Dmp1*-loss and HER2/neu overexpression/activation *in vivo*, we crossed MMTV-*neu* mice with *Dmp1*-null mice. Mammary glands from $Dmp1^{-/-}$ or $Dmp1^{+/-}$ virgin females are morphologically indistinguishable from those from wild-type females, with nearly the same staining patterns for ER, PR, and Ki67 (Supplemental Figures S2 and S3). HER2/neu-induced mammary tumor development was significantly accelerated from 200 days to 162 days in $Dmp1^{+/-}$ and 154 days in $Dmp1^{-/-}$ mice ($P < 0.0001$), with no statistically significant differences between $Dmp1^{+/-}$ and $Dmp1^{-/-}$ (Fig. 3*C*). Analysis of genomic DNA from mammary tumors of $Dmp1^{+/-}$ mice showed that the wild-type *Dmp1* locus was retained in all the eight tumors examined (Fig. 3*C*). The tumors from $Dmp1^{+/-}$ mice expressed the Dmp1 protein, showing haploid insufficiency (Fig. 3*D*). p53 was barely detectable in the mammary glands or tumors from $Dmp1^{+/-}$ or $Dmp1^{-/-}$ mice, suggesting inactivity of the p53 pathway (Fig. 3*D*). These data indicate that Dmp1 has a critical role both as a mediator of HER2-p53 signaling and in prevention of *neu*-induced mammary tumor development.

## Frequent deletion of *Dmp1* in *neu*-induced mammary tumors

The Dmp1 protein was often downregulated in tumor tissues in comparison to pre-malignant mammary glands in wild-type MMTV-*neu* mice (Fig. 3*A*, 4th panel, T). To investigate the molecular mechanism for this finding, we studied the gene copy numbers of *Dmp1* by real-

time PCR (Fig. 4*A*). One allele of the *Dmp1* gene was deleted in 6 of 10 mammary tumors from single MMTV-*neu* transgenic mice, and 6 of 8 tumors in double *neu* transgenic mice (Fig. 4*A*). Conversely, one allele of *Arf* was lost only in 1 of 10 tumors, and none of the tumors showed *p53* deletion (Supplementary Fig. S4). Specific deletion of *Dmp1* was further confirmed by real-time PCR analyses of *grm3* and *mdr1* (Fig. 4*B*). Hypermethylation of the *Dmp1* promoter was not found in any of the randomly chosen 10 tumor DNAs from wild-type MMTV-*neu* mice (data not shown). The tumors with hemizygous deletion for *Dmp1* (*Dmp1*$^{wt}$HD) expressed significantly lower levels of *Dmp1* mRNA than those without *Dmp1* deletion (*Dmp1*$^{wt}$ND) ($P < 0.0001$, Fig. 4*C*). The *Dmp1* mRNA was significantly downregulated in tumors from *Dmp1*$^{+/-}$ mice than in *Dmp1*$^{wt}$ND ($P < 0.0001$), but the levels were not different from those of *Dmp1*$^{wt}$HD tumors (Fig. 4C). *p19*$^{Arf}$ mRNA expression was lower in mammary tumors from *Dmp1*$^{wt}$HD, *Dmp1*$^{+/-}$, and *Dmp1*$^{-/-}$ mice than in *Dmp1*$^{wt}$ND tumors, but the levels were not significantly different among tumors from *Dmp1*$^{wt}$HD, *Dmp1*$^{+/-}$, and *Dmp1*$^{-/-}$ mice (Fig. 4*D*). Likewise, the p53 target, *p21*$^{Cip1/WAF1}$ mRNA expression was significantly downregulated in mammary tumors from *Dmp1*$^{wt}$HD, *Dmp1*$^{+/-}$, and *Dmp1*$^{-/-}$ mice in comparison to *Dmp1*$^{wt}$ND (Supplementary Fig. S5*A*) while the expression of pro-apoptotic target *Puma* did not change significantly among the three *Dmp1* genotypes (data not shown). The *p16*$^{Ink4a}$ mRNA expression in tumors from *Dmp1*$^{wt}$HD and *Dmp1*$^{+/-}$ tumors was not significantly different from that in *Dmp1*$^{wt}$ND, while it was downregulated in *Dmp1*$^{-/-}$ (Supplementary Fig. S5*B*).

We then studied protein expression involved in the Arf-Mdm2-p53 tumor surveillance pathway and *Ink4a/Arf* modulators in *neu* tumors from the three *Dmp1* genetic backgrounds. The Dmp1 protein expression was 2–10 times higher in wild-type *neu* tumors than in non-transgenic wild-type mammary glands (MMG) from 12-week-old virgin females (Fig. 5*A*), reflecting the promoter activation. As expected, *Dmp1*$^{wt}$ND tumors showed higher levels of Dmp1 than in *Dmp1*$^{wt}$HD tumors. The Dmp1 protein expression was higher than that in normal mammary glands in some *Dmp1*$^{+/-}$; *neu* tumors or at the levels of normal mammary glands in others (Fig. 5*B*). None of the tumors from the three *Dmp1* genotypes overexpressed p19$^{Arf}$ or p53 at the level of the *p53*-mutant cell line (Fig. 5), suggesting that *neu* tumors of the three different *Dmp1* genotypes retained wild-type *p53*. Sequencing of the *p53* cDNAs confirmed that these tumors expressed wild-type *p53* regardless of the *Dmp1* genotype. Mdm2 was not overexpressed in any of the *neu* tumors (Fig. 5). Tbx2 overexpression was found in ~70 % of the tumors from *Dmp1* wild-type mice and in 20–30 % of *Dmp1*$^{+/-}$ and *Dmp1*$^{-/-}$ tumors (Fig. 5). Pokemon overexpression was found in nearly all the tumors from HER2/neu tumors, regardless of the *Dmp1* genotype, while none of the mammary tumors overexpressed Twist (Fig. 5). None of the MMTV-*neu* tumors overexpressed Tbx3 or Bmi1 (data not shown). Together, our molecular genetic analyses of *neu*-induced mouse mammary tumors showed that 1) hemizygous deletion of *Dmp1* is found in ~50 % of wild-type *neu* tumors, 2) mutation/deletion/overexpression of key components of the Arf-Mdm2-p53 pathway is rare in *neu* tumors, 3) both *p19*$^{Arf}$ and *p21*$^{Cip1/WAF1}$ mRNAs are significantly downregulated in *Dmp1*$^{wt}$HD, *Dmp1*$^{+/-}$, and *Dmp1*$^{-/-}$ tumors in comparison to *Dmp1*$^{wt}$ND tumors, showing the inactivity of the p53 pathway and the mechanism of haploid insufficiency of Dmp1, 4) *p16*$^{Ink4a}$ mRNA level is low only in *Dmp1*$^{-/-}$ mammary tumors, and that 5) both Tbx2 and Pokemon proteins are often overexpressed in wild-type *neu* tumors; Tbx2 overexpression is less frequent in *Dmp1*$^{+/-}$ or *Dmp1*$^{-/-}$ tumors while Pokemon overexpression is independent of the *Dmp1* genotype.

## Histopathological features of HER2/neu tumors from *Dmp1*-deficient mice

Macroscopically tumors from *Dmp1*$^{+/-}$ or *Dmp1*$^{-/-}$ mice showed more aggressive phenotypes (i.e. high nuclear grade, local invasion, increased angiogenesis and metastasis) than those from *Dmp1*$^{+/+}$ mice. At sacrifice, the total tumor weight was significantly

increased in $Dmp1^{+/-}$ ($P = 0.039$) and $Dmp1^{-/-}$ ($P = 0.0015$) mice compared to $Dmp1^{+/+}$ mice (Fig. 6*A*, *B*). Metastatic disease was more frequent in $Dmp1^{-/-}$ (4/26, 15.4 %) or $Dmp1^{+/-}$ mice (4/37, 10.8 %) than in $Dmp1^{+/+}$ mice (2/35, 5.7 %). Tumors from MMTV-*neu* mice were then categorized using the published grading criteria (38). Grade A is encapsulated, low grade nodular mammary tumor with uniform nuclear size with low mitotic count (Fig. 6*C*, upper panel, found in $Dmp1^{+/+}$); grade B is intermediate, invasive mammary adenocarcinoma (i.e. tumor cells have broken out the lobule and begun to spread to other areas) with small and large nests of cancer cells infiltrating the mammary stroma (middle panel, arrow, from $Dmp1^{+/-}$); grade C is a high-grade, solid, invasive carcinoma with remarkably high mitotic figure count and central comedo necrosis with high-grade nuclei (lower panel, arrow, from $Dmp1^{-/-}$) (38). Tumors from both $Dmp1^{+/-}$ and $Dmp1^{-/-}$ mice showed significantly increased scores of grade B and C tumors, showing the more aggressive pattern of tumor development, while there was no significant difference in the features between samples deficient in one or two alleles of *Dmp1* (Fig. 6*D*, upper panel). When *Dmp1* wild-type tumors were compared between HD and ND groups, tumors with *Dmp1* deletion showed significantly increased scores for grade B ($P = 0.0033$), but not for grade C (Fig. 6*D*, lower panel). The difference in the invasiveness between $Dmp1^{wt}$HD tumors and $Dmp1^{+/-}$ tumors can be explained by the duration of *Dmp1* deletion, which should be significantly longer in tumors from $Dmp1^{+/-}$ mice. Thus, our data indicate that loss of *Dmp1* contributes to the more invasive and metastatic phenotypes of mammary carcinomas in *neu*-transgenic mice.

### Endogenous HER2 upregulates h*DMP1* mRNA in human breast epithelial cells

To study the whether endogenous HER2 upregulates h*DMP1* in human breast epithelial cells, h*DMP1* levels were quantitated by real-time PCR. We found that h*DMP1* mRNA levels were significantly higher in human breast cancer cell lines with HER2 overexpression than those with low or no HER2 expression ($P = 0.0076$, Supplementary Fig. S6*A*). Downregulation of endogenous *HER2* with two different shRNAs (39) (> 95 %) resulted in significant decrease of the h*DMP1* mRNA in three different *HER2*-amplified human breast cancer cell lines, SK-BR-3, BT-474, and HCC1569 (Supplementary Fig. S6*B*, HCC1569 data not shown). $p14^{ARF}$ mRNA also decreased in BT-474 cells treated with shRNA to *HER2* (data not shown). Inhibition of PI3K, Akt, or NF-κB activity by specific inhibitors downregulated endogenous h*DMP1* levels in these breast cancer cells (Supplementary Fig. S6*C*), and induced cell cycle arrest or apoptosis in SK-BR-3 and BT-474 cells (Supplementary Fig. S6*D*). Together, overexpression of HER2 increases endogenous h*DMP1* through activation of the PI3K-Akt-NF-κB pathway.

## Discussion

In this study, we have characterized the signaling pathway that links HER2/neu overexpression and p53 activation. Although HER2 overexpression activates both Ras-Raf-MEK-ERK-AP1 and PI3K-Akt-NF-κB signaling, our study shows that HER2-Dmp1 signaling is independent of the former signaling cascade. Dependence of the *Dmp1* promoter activation by NF-κB was confirmed by 1) proteosomal inhibitor PS341 treatment, 2) expression of IκBα super repressor, and by 3) mutating the NF-κB sites on the *Dmp1* promoter. Moreover, we confirmed the binding of endogenous p65 and p52 subunits of NF-κB to the endogenous *Dmp1* promoter in HER2 virus-infected cells as well as in mammary tumors from MMTV-*neu* mice by tissue ChIP. It has been reported that phosphorylation of p65 Ser-536 in transactivation domain 1 by IKKα/β/ε or other kinases can stimulate p65 transactivation (40). Conversely, phosphorylation of p65 Thr-505 in transactivation domain 2 by Chk1 results in transcriptional repression of some NF-κB target genes, by increased association of p65 with HDAC1 (41,42). Phosphorylation of p65 at Thr-505 occurs when the

cells are exposed to genotoxic stimuli. Thus, NF-κB plays roles in both activation (HER2/neu, this study) and repression (genotoxic stimuli) (24) of the *Dmp1* promoter dependent on the stress the cells receive.

It was reported that $p19^{Arf}$ inhibits HER2/neu-mediated oncogenic growth by antagonizing Akt-mediated $p27^{Kip1}$ phosphorylation and increasing $p27^{Kip1}$ stability (43). Our study showed that the *Arf* promoter is activated by HER2/neu. Wild-type MMTV-*neu* tumors that retained two alleles of *Dmp1* expressed the *Arf* mRNA at levels 2–40 times higher than that in normal mammary epithelial cells. Induction of $p19^{Arf}$ by HER2/neu is largely dependent on Dmp1 since 1) the *Arf* promoter activation was not found in *Dmp1*-deficient cells, and 2) *Arf* mRNA levels were significantly lower in $Dmp1^{-/-}$, $Dmp1^{+/-}$, and in $Dmp1^{wt}$HD; *neu* tumors than in $Dmp1^{wt}$ND. The critical role of the Dmp1-Arf-p53 pathway in preventing mammary tumorigenesis was demonstrated by immunohistochemical staining of pre-neoplastic regions found in early stage *neu* tumors, where we found significant upregulation of Dmp1, p53, and $p21^{Cip1/WAF1}$ proteins. Although significant induction of *Arf* mRNA was detectable by real-time PCR in $Dmp1^{wt}$ND; *neu* tumors, immunohistochemical demonstration of $p19^{Arf}$ in pre-malignant mammary tissue was technically difficult possibly because the absolute expression levels of $p19^{Arf}$ were very low. It is generally believed that very low levels of *Arf* are enough to show tumor suppressive activity, and that further induction provides the selective pressure for the emergence of tumors that have inactivated the gene (44).

Interestingly, the mouse *Dmp1* gene was hemizygously deleted in ~50 % of *neu* mammary tumors with significant downregulation of the Dmp1 protein. The gene deletion was limited to the *Dmp1* locus in 80 % of the mouse tumors, according to our analysis of neighbor gene deletions by real-time PCR. However, *neu*-induced mammary tumors are different from human breast cancers in that the *Ink4a/Arf* or *p53* locus is not frequently involved. Mdm2 overexpression was not observed in any of the *neu* tumors, regardless of the *Dmp1* genotype. In contrast, we found frequent overexpression of *Ink4a/Arf* repressors, Tbx2 and Pokemon, in HER2/neu tumors. The frequency of Pokemon overexpression did not change significantly in $Dmp1^{+/-}$ and $Dmp1^{-/-}$ tumors, while the frequency of Tbx2 overexpression was decreased from 70 % to 20–30 % in $Dmp1^{+/-}$ and $Dmp1^{-/-}$ tumors. This indicates that *Dmp1* deletion may alleviate the function of Tbx2 overexpression to some extent. It has been reported that *TBX2* is amplified in 8.6–21.6 % of sporadic human breast carcinomas (45), and ectopic expression of Tbx2 results in DNA polyploidy and cisplatin resistance (46). On the other hand, very little is known about the role of Pokemon in human breast cancer (47). Further studies will be required to reveal how these *Ink4a/Arf* repressors collaborate with *Dmp1*-loss in breast (or mammary) carcinoma development.

Our current study clearly demonstrates the haploid insufficiency of Dmp1 in *neu*-induced mammary tumor suppression. Consistent with these findings, the $p19^{Arf}$ and $p21^{Cip1/WAF1}$ mRNA levels were significantly downregulated in both $Dmp1^{+/-}$ and $Dmp1^{-/-}$ tumors, with no differences between the two cohorts. Of note, downregulation of *Arf* and $p21^{Cip1/WAF1}$ was also observed in $Dmp1^{wt}$HD tumors, indicating that naturally occurring hemizygous deletion of *Dmp1* inactivates the Arf-p53 pathway as well. In either case, loss of *Dmp1* was associated with more aggressive disease than $Dmp1^{wt}$ND tumors. In contrast, $p16^{Ink4a}$ mRNA was downregulated only in mammary tumors from $Dmp1^{-/-}$ mice as compared to $Dmp1^{wt}$ND mice, suggesting differential regulation of the *Ink4a* and *Arf* promoters by Dmp1. This can be explained by the fact that the $p16^{Ink4a}$ promoter lacks typical Dmp1 consensus sequences, at least within 500 bps from the transcription initiation site (17,48).

In conclusion, our study demonstrates a novel signaling cascade that links HER2/neu and p53. Since Dmp1 is induced in pre-malignant tissues, activation of Dmp1 by small molecules may be a reasonable approach to prevent breast cancer development.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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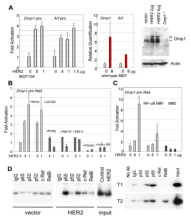

**Figure 1. Both the *Dmp1* and *Arf* promoters are specifically activated by HER2/neu**

*A*, left panel. Murine *Dmp1* (−374 *Nsi*I) (22) and *Arf* promoter (−281 *Bam*HI) (17) luciferase constructs were co-transfected with increasing amounts of HER2 expression vector in MCF10A cells. *A*, middle panel. Wild-type MEFs were infected with a retrovirus expressing HER2 and mRNA for *Dmp1* and *Arf* were quantitated by real-time PCR Taqman assay. *A*, right panel. NIH 3T3 cells were transfected with HER2 expression vectors and the Dmp1 protein was analyzed with specific antibody. *B*, the *Dmp1* promoter activation by HER2 is inhibited by LY294002 (PI3K inhibitor), Akt inhibitor IV/V, PS341 (proteosomal NF-κB inhibitor), and IκBα SR (super repressor) demonstrating critical involvement of the PI3K-Akt-NF-κB pathway. *C*, the *Dmp1* promoter activation by HER2 is dependent on the integrity of the two NF-κB sites. Mutation of the NF-κB site #1 caused partial inhibition while that of NF-κB site #2 nullified the effects of HER2 on *Dmp1* transcription. *D*, left panel. Chromatin immunoprecipitation (ChIP) analysis of NF-κB binding to the *Dmp1* promoter on HER2 expression. Binding of endogenous NF-κB to the *Dmp1* promoter was observed with HER2 virus infection of NIH 3T3 cells. *D*, right panel. Binding of p65 and p52 to the endogenous *Dmp1* promoter in mammary tumors (T1 and T2) from MMTV-*neu* mice.

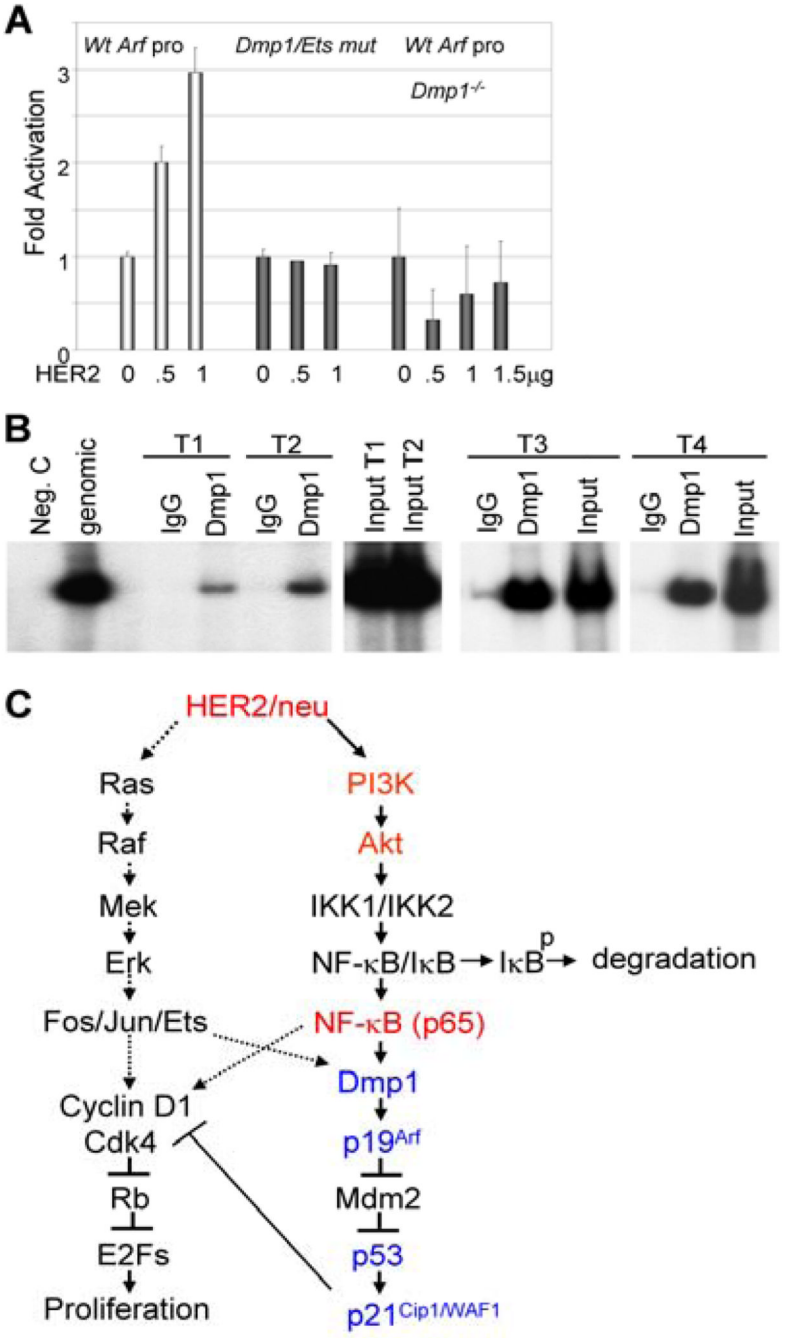


**Figure 2. Activation of the *Arf* promoter by HER2 and the signaling pathways that link HER2 and p53**

*A*, activation of the *Arf* promoter by HER2 is dependent on Dmp1. Reporter assays were conducted in 3T3 cells (left and middle panels) or in *Dmp1*$^{-/-}$ cells (right panel). The baseline (without HER2 expression vector) was set at 1.0. *B*, binding of endogenous Dmp1 to the *Arf* promoter in four different MMTV-*neu* tumors. Tissue ChIP was conducted with formalin-fixed tumors from wild-type MMTV-*neu* mice. The Dmp1 protein was detectable on the *Arf* promoter with two different antibodies to Dmp1 (RAX for T1 and T2, and RAD for T3 and T4). *C*, proposed signaling pathway that links HER2/neu overexpression and p53 activation. Activation of the *Dmp1* promoter is mediated by PI3K-Akt-NF-κB signaling, and

induction of *Arf* by HER2/neu is dependent on Dmp1. This diverts toxic hyperproliferative signaling from HER2/neu to a p53-dependent cell cycle arrest or apoptosis.

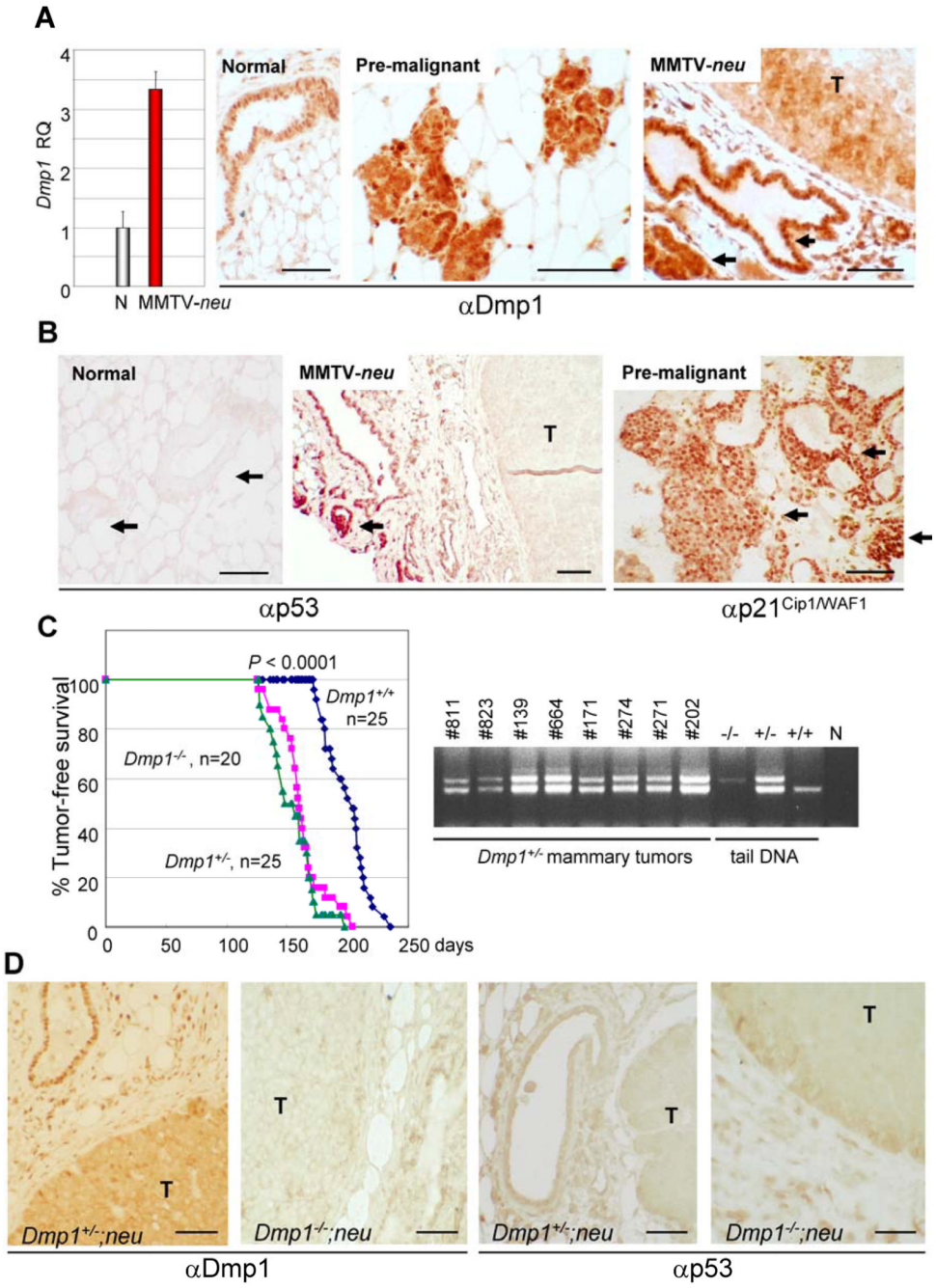


**Figure 3. Induction of Dmp1 and p53 *in vivo* by HER2/neu and acceleration of *neu*-induced mammary carcinogenesis in *Dmp1*-knockout mice**

*A*, left. Real-time PCR analysis of the *Dmp1* mRNA in early stage mammary tumors from MMTV-*neu* mice (5–6 months old, 1–2 mm tumors, n = 3). *A*, right. Detection of the Dmp1 protein in normal mammary gland (2nd panel), hyperplastic, non-transformed mammary glands from a MMTV-*neu* mouse (3rd panel, pre-malignant), and those adjacent to a MMTV-*neu* tumor (4th panel, arrows). Note that once a mouse develop mammary tumor (T), the Dmp1 expression levels go down due to hemizygous gene deletion (Fig. 4*A*). *B*, left. p53 is barely detectable in normal mammary glands (arrows). *B*, middle. Induction of the p53 protein in hyperproliferative mammary glands (arrow) from a MMTV-*neu* mouse. p53

is significantly downregulated in the tumor (T). *B*, right. Induction of the $p21^{Cip1/WAF1}$ protein in pre-malignant lesions (arrows) from a MMTV-*neu* mouse. *C*, left. Tumor-free survival of $Dmp1^{+/+}$ (blue), $Dmp1^{+/-}$ (pink), and $Dmp1^{-/-}$ (green); MMTV-*neu* compound transgenic mice. Tumor development was significantly accelerated on $Dmp1^{-/-}$ and $Dmp1^{+/-}$ genetic backgrounds as compared to wild-type mice (both $P < 0.0001$). *C*, right. Retention of the wild-type *Dmp1* locus in mammary carcinomas from $Dmp1^{+/-}$; *neu* mice. *D*, left. Detection of the Dmp1 protein in $Dmp1^{+/-}$; *neu* tumor and neighboring tissue. Right. Background signals from a mammary carcinoma from a $Dmp1^{-/-}$; *neu* mouse. *D*, right. p53 is barely detectable in *neu*-induced mammary tumors from a $Dmp1^{+/-}$ (left) or a $Dmp1^{-/-}$ mouse (right). Scale bars are 100 μm.

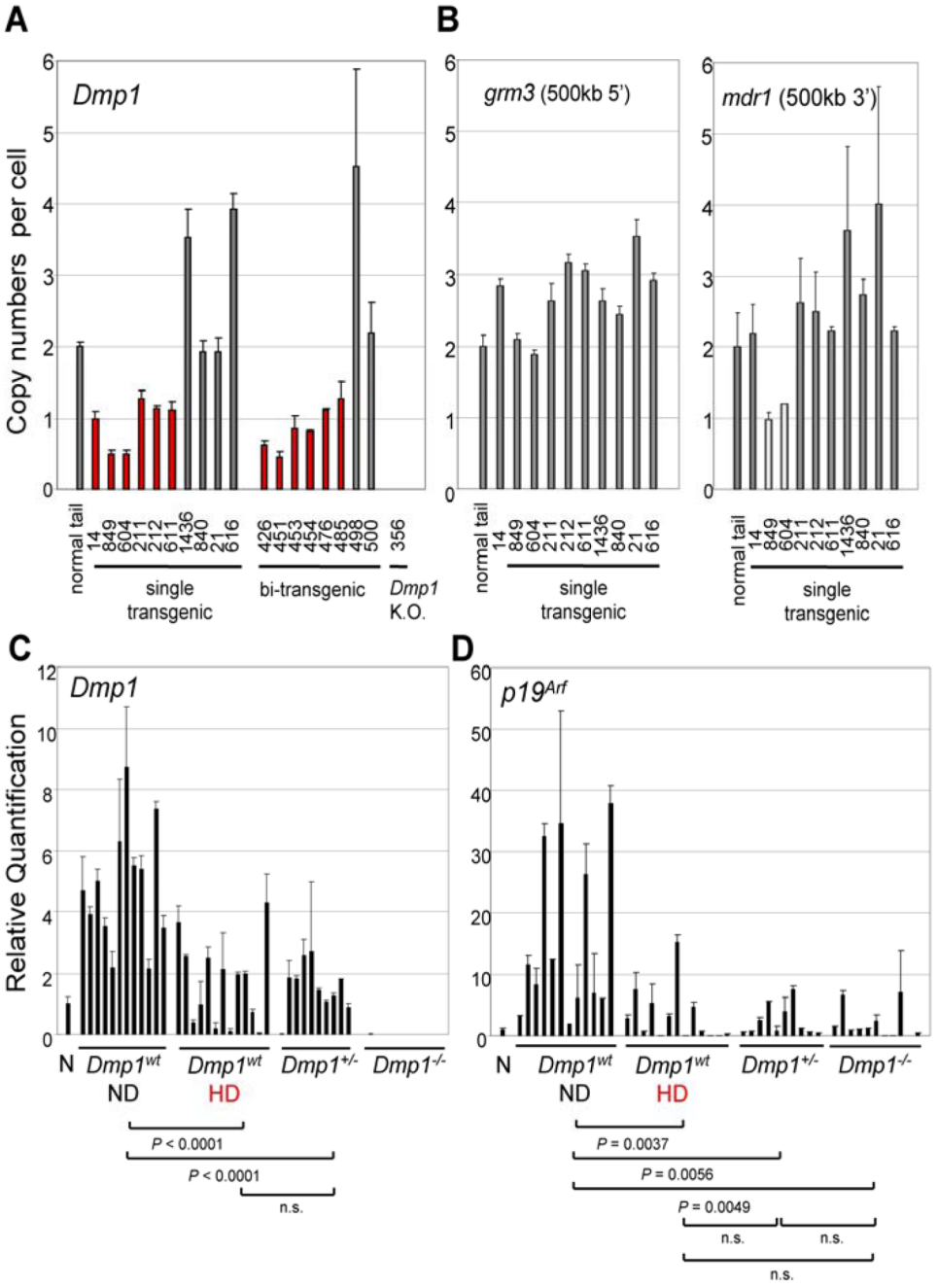


**Figure 4. Specific deletion of *Dmp1* and expression of *Dmp1/Arf* mRNA in *neu*-induced mammary tumors**

*A*, real-time PCR analysis of the *Dmp1* copy numbers in wild-type *neu* tumors showing *Dmp1* deletion in 60 % of single *neu*-transgenic and 75 % of double *neu*-transgenic mice. *B*, the *grm3* (*glutamate receptor 3*) gene, which is located ~500 kb upstream from the *Dmp1* locus, was not deleted in any of the *neu* tumors. The *mdr1* (*multi drug resistance 1*) gene, located ~500 kb downstream from the *Dmp1* locus, was deleted in only 2 of 10 cases examined. *C*, relative expression of the *Dmp1* mRNA in mammary carcinomas from MMTV-*neu* mice. *Dmp1*$^{wt}$ND indicates tumor cells without *Dmp1* deletion; *Dmp1*$^{wt}$HD (red) shows those with hemizygous deletion of *Dmp1*. *D*, relative expression of the *Arf*

mRNA in mammary carcinomas from *neu* mice. The *Arf* expression was significantly downregulated in *Dmp1*$^{wt}$HD, *Dmp1*$^{+/-}$, and *Dmp1*$^{-/-}$ tumors.

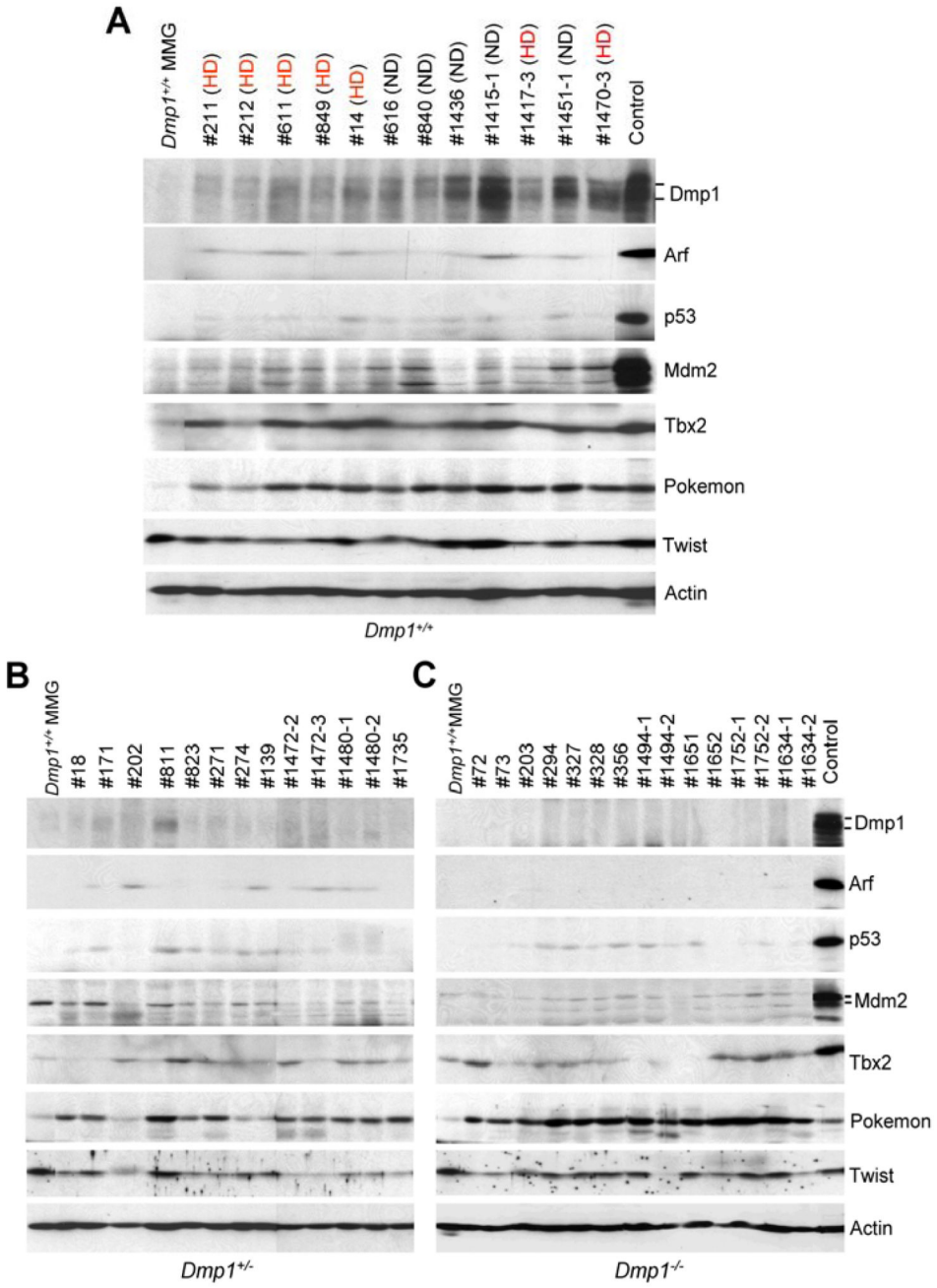


**Figure 5. Analyses the Arf-Mdm2-p53 pathway and *Ink4a/Arf* modulators in HER2/neu tumors**

*A*, analyses of wild-type MMTV-*neu* mammary tumors. The Dmp1 protein expression was 2–16 folds higher in tumors from *Dmp1* wild-type mice than normal mammary glands (*Dmp1*$^{+/+}$ MMG from 15-week-old non-lactating females), reflecting the *Dmp1* promoter activation by HER2/neu. None of the tumors showed *p53* mutation or Mdm2 overexpression. The *Arf* gene was not deleted in any of the mammary tumors. As a positive control, lysates of NIH 3T3 cells were used for Dmp1, Tbx2, Pokemon, and Twist; immortalized MEF with *p53* mutation was used for p19$^{Arf}$ and p53; and dm3T3 cells were used for Mdm2. *B* & *C*, analyses of *Dmp1*$^{+/-}$ and *Dmp1*$^{-/-}$; *neu* mammary tumors. p19$^{Arf}$ or p53 was barely detectable in mammary tumors from *Dmp1*$^{+/-}$ and *Dmp1*$^{-/-}$ mice. The

frequency of Tbx2 overexpression was significantly lower in these tumors than those from wild-type MMTV-*neu* mice while the pattern of Pokemon overexpression did not change in *Dmp1*-knockout tumors. MMG: mammary glands.

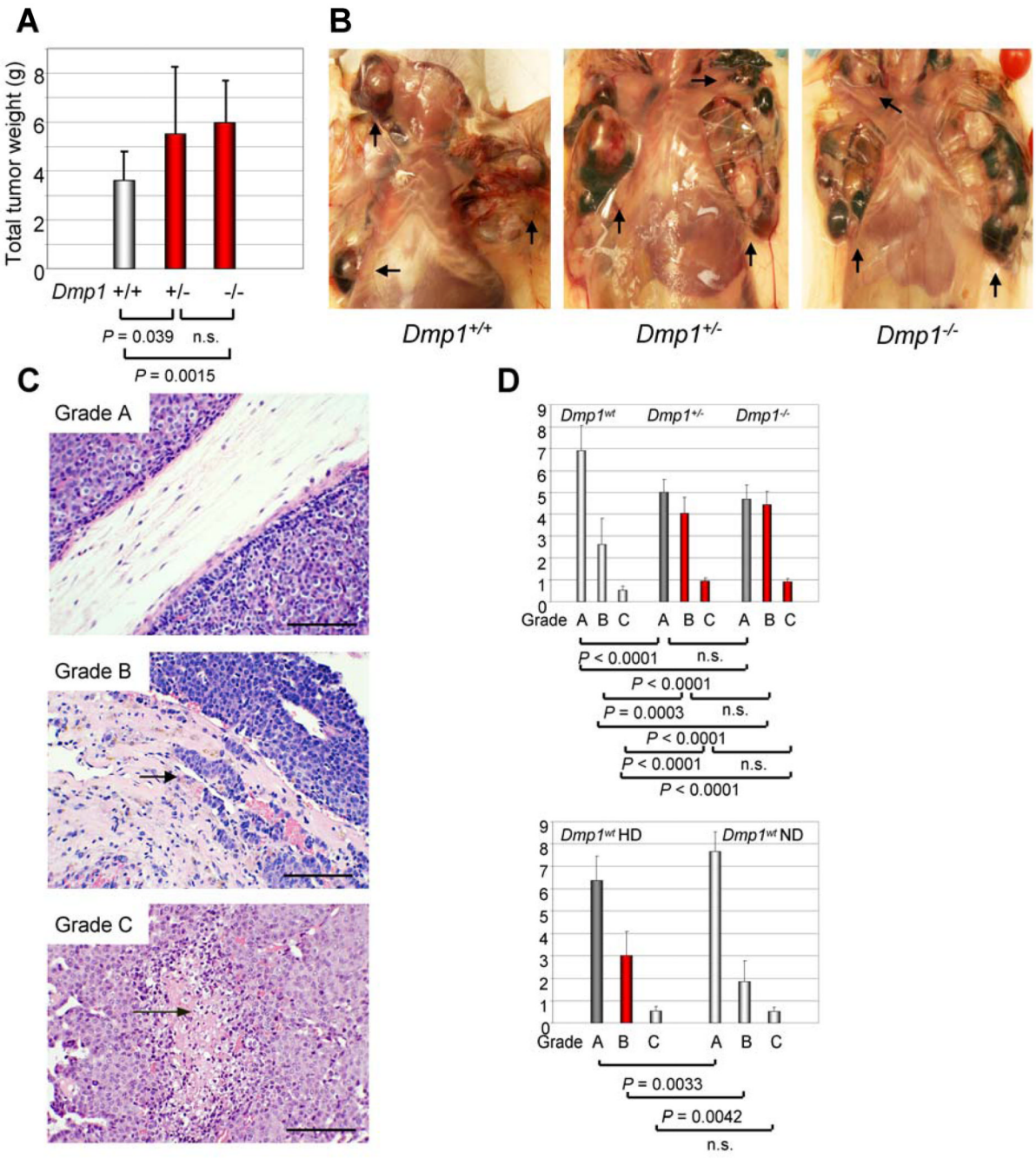


**Figure 6. Histological grading of *neu*-induced mammary tumors dependent on the *Dmp1* genotype**

*A*, total tumor weight per mouse (mean +/− SD). $Dmp1^{+/-}$ and $Dmp1^{-/-}$ tumors were significantly heavier, showing accelerated growth. *B*, pictures of mammary tumors found in $Dmp1^{+/+}$ (left panel), $Dmp1^{+/-}$ (middle panel), and $Dmp1^{-/-}$ (right panel) *neu* mice. Arrows show the location of tumors. *C*, mammary tumors from *neu* transgenic mice were classified into grades A to C (38). Scale bar is 100 μm. *D*, upper panel. Grading of mammary carcinomas from *neu* mice dependent on the *Dmp1* genetic background. *D*, lower panel. Differential grading of wild-type *neu* tumors by deletion of *Dmp1*.