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Supplementary Materials

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

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Supplementary Materials and Methods

Cell culture and reporter assays

Wild-type and *Dmp1*-null MEFs were established from 13.5-day-old embryos and maintained as previously described (20). Human breast carcinoma cell lines were obtained from the institutional core facility or purchased from the American Type Culture Collection. For reporter assays, 2 x 10^5 cells were seeded into 60 mm diameter culture dishes 24 hours before transfection. In order to study the responsiveness of the *Dmp1* and *Arf* promoters to HER2, 4 μg of luciferase reporter DNA was co-transfected with 0.5 to 1 μg of HER2 expression vector (pME18S-c-*ErbB2*, gift from Dr. T. Yamamoto, University of Tokyo) and 4 μg of internal control human β-Actin promoter-secreted endocrine alkaline phosphatase vector (a gift from M. Ostrowski, Ohio State University). The luciferase construct for the human $p14^{ARF}$ promoter was obtained from Dr. P. Jones (Univ. of Sothern California), the human $p27^{KIP1}$ promoter was received from Dr. A.T. Look (Dana Farber Cancer Institute), and the murine $p16^{Ink4a}$ promoter (17) was received from Drs. C. Sherr and M. Roussel (St. Jude Children's Research Hospital). The 1.4 kb genomic DNA for the human Mdm2 (*Hdm2*) promoter containing both P1 and P2 was cloned in our laboratory. Genejuice (Novagen) was used in all transfections. Specific MAP kinase inhibitors, U0126 (for MEK/ERK), LY294002 (for PI3K), and Akt inhibitors IV and V were purchased from EMD Chemicals (Gibbstown, NJ) and were used at the concentration of 5 μM for U0126 and LY294002, 2.2 μM for Akt inhibitor IV, and 8.3 μM for Akt inhibitor V. PS341 (Velcade™) was purchased from Millennium Pharmaceuticals (Cambridge, MA) and was used at 100 nM. The expression vector for IκBα super repressor was received from Dr. A. Thorburn (Univ. of Colorado).

Chromatin immunoprecipitation

Chromatin immunoprecipitations was performed as described previously (22, 24) (see http://genomecenter.ucdavis.edu/farnham/farnham/protocols/tissues.html for tissue ChIP). The lysates were precipitated with specific antibodies to NF-κB family proteins (all from Santa Cruz Biotech; p50: sc-7178x, p52: sc-7386x, p65: sc-372x, RelB: sc-226x, and c-Rel: sc-71x), or with anti-Dmp1 antibody (RAX or RAD) (23, 37). For detection of the endogenous NF-κB family transcription factors on the murine *Dmp1* promoter, sense primer 5'-AAAGCGAGGTCACACTCACG-3' and antisense primer 5'-CCCGACGTCACTTCCGTCT-3' were used (shown as SE#8 and AS#8 in Supplementary Fig. S1*A*). The primers for detection of the endogenous Dmp1 binding to the *Arf* promoter have been reported (22).

***in vitro* mutagenesis**

The murine *Dmp1* promoter deletion/point mutants were generated by use of an *in vitro* mutagenesis kit (Stratagene). The oligonucleotide sequences used for *in vitro* mutagenesis of the *Dmp1* promoter for the NF-κB site #1 was reported (24). For mutagenesis of the NF-κB site #2, 5'-GCGTGCCGACGCAGACACGCGTGAAGTCGGGGGTGGCGCGC-3' and its reverse complementary sequence were used. The mutated NF-κB site is underlined. The murine *Arf* promoter construct with a mutant Dmp1/Ets site has been described (17).

Immunohistochemical staining

Immunohistochemical staining of normal and mammary tumor tissues were conducted as described previously (23, 26). These antibodies were used for immunohistochemistry with formalin-fixed, paraffin-embedded sections: Dmp1 (RAX to amino acids 136 to 150 of Dmp1, RAZ to the amino acids 740-756, and RAD to the full length His-Dmp1) (23, 26, 28,

37), p53 (P4235, Sigma), $p21^{Cip1/WAF1}$ (sc-6246), HER2 (sc-284), ERα (sc-543), PR (sc-538), and Ki67 (SP6, NeoMarkers).

Statistical analyses

Statistical differences of survival in *Dmp1*$^{+/+}$, *Dmp1*$^{+/-}$, and *Dmp1*$^{-/-}$; MMTV-*neu* mice were analyzed by XLSTAT-Life software (Addinsoft). Mann-Whitney test (two-sided) were used to generate the *P values* (significance level, $\alpha = 0.05$). Statistical analyses of gene expression and histopathology of *neu*-induced mammary tumors were conducted by two-sided Chi square tests and unpaired Student's *t*-tests.

Human breast cancer cell lines

Human breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA).

Retroviruses for *HER2* shRNA

Amphotropic retroviruses to knockdown *HER2* were prepared by using the pSUPERretro RNA interference system (Oligoengine, Seattle, WA). The 19-bp target sequences for *HER2* was 5'-GGGGCTGGCTCCGATGTAT-3' for #3386, 5'-TGGGGTCGTCAAAGACGTT-3' for #3682 (39). The effectiveness of downregulation of HER2 by shRNA was confirmed by real-time PCR and Western blotting with specific antibodies.

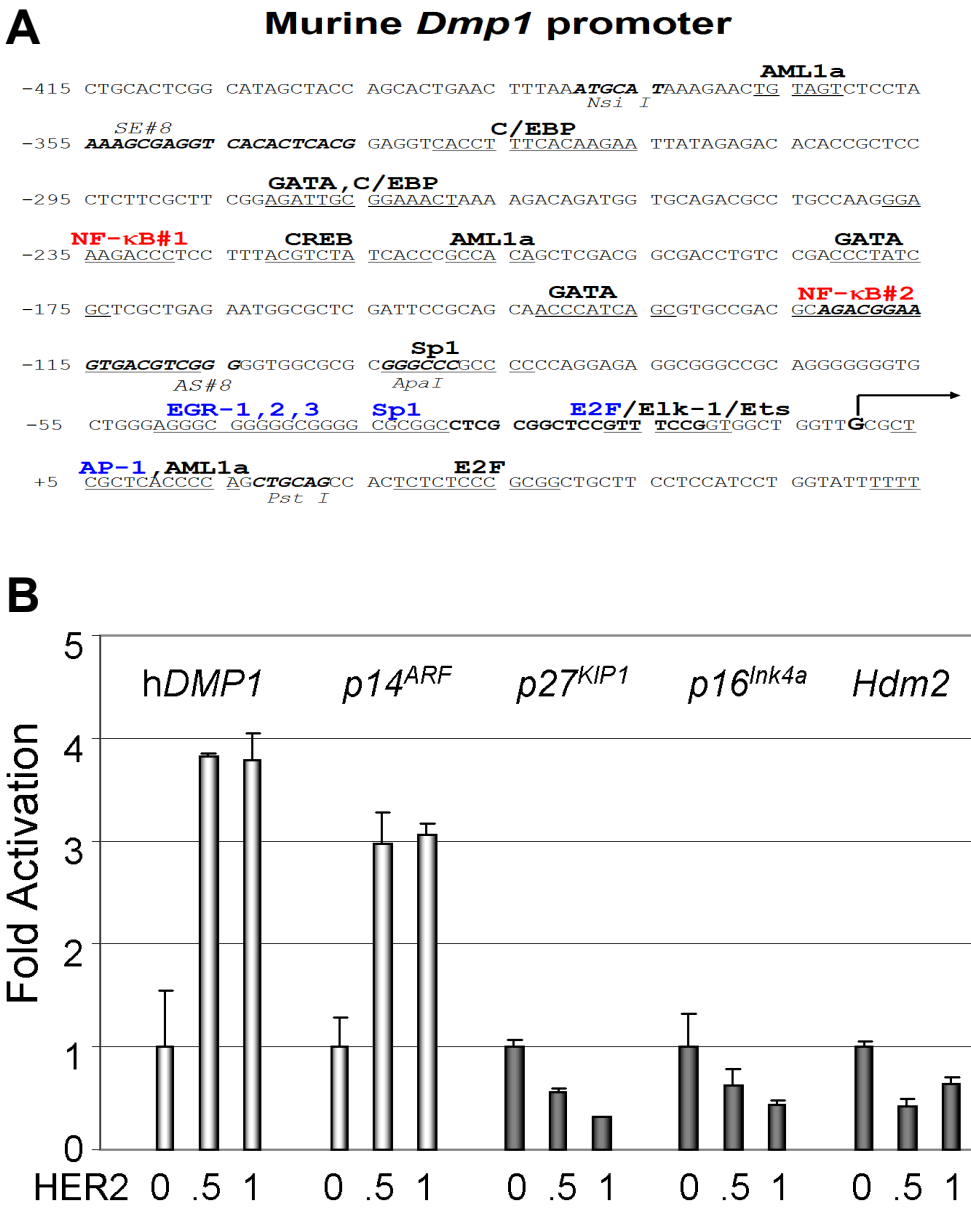


Figure S1. Potential transcription factor-binding sites on the murine *Dmp1* promoter and responsiveness of the h*DMP1*, *p14*^{ARF}, *p27*^{KIP1}, *p16*^{Ink4a}, and *Hdm2* promoters to HER2.

A, potential transcription factor binding sites on the murine *Dmp1* promoter. The *Dmp1* promoter has an AP-1 site responsible for oncogenic Ras (22), E2F sites for cell cycle-dependent expression of *Dmp1* (23), and NF-κB sites for *Dmp1* repression by genotoxic stimuli (24). The two NF-κB consensus sequences on the promoter are shown in red. The transcription initiation site is shown as "**G**" in bold.

B, effects of HER2/neu overexpression on h*DMP1*, *p14*^{ARF}, *p27*^{KIP1}, *p16*^{Ink4a}, and *Hdm2* promoters. Reporter assay was performed in MCF10A cells with increasing amount of the HER2/neu expression vector. The h*DMP1* promoter was efficiently activated while other promoters were repressed by HER2/neu overexpression in mammary epithelial cells.

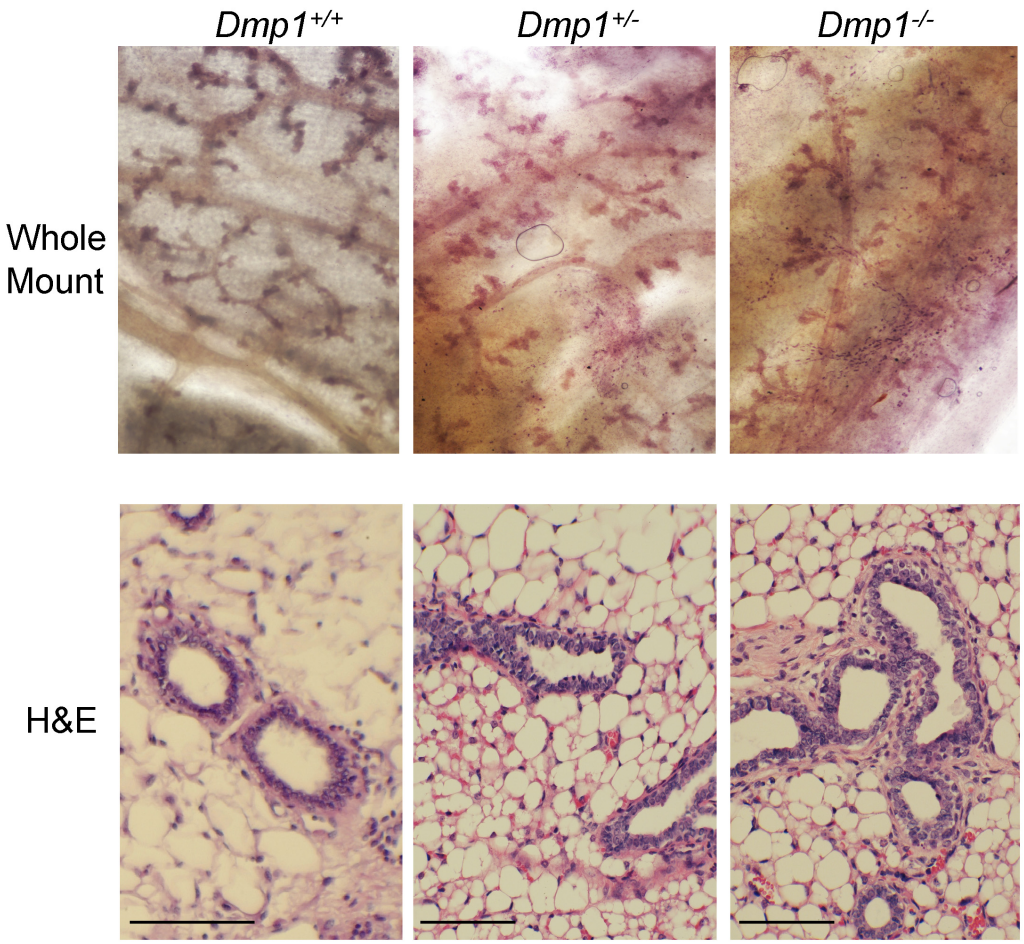


Figure S2. Analysis of mammary glands from *Dmp1*-knockout mice.
Mammary glands were isolated from twelve-week-old virgin females of each genotype, and were studied by whole mount (upper panels) and by H&E staining (lower panels). Mammary gland branching was slightly underdeveloped in *Dmp1*$^{-/-}$ tissue, but was histologically normal by H&E staining. Scale bar is 100 μm.

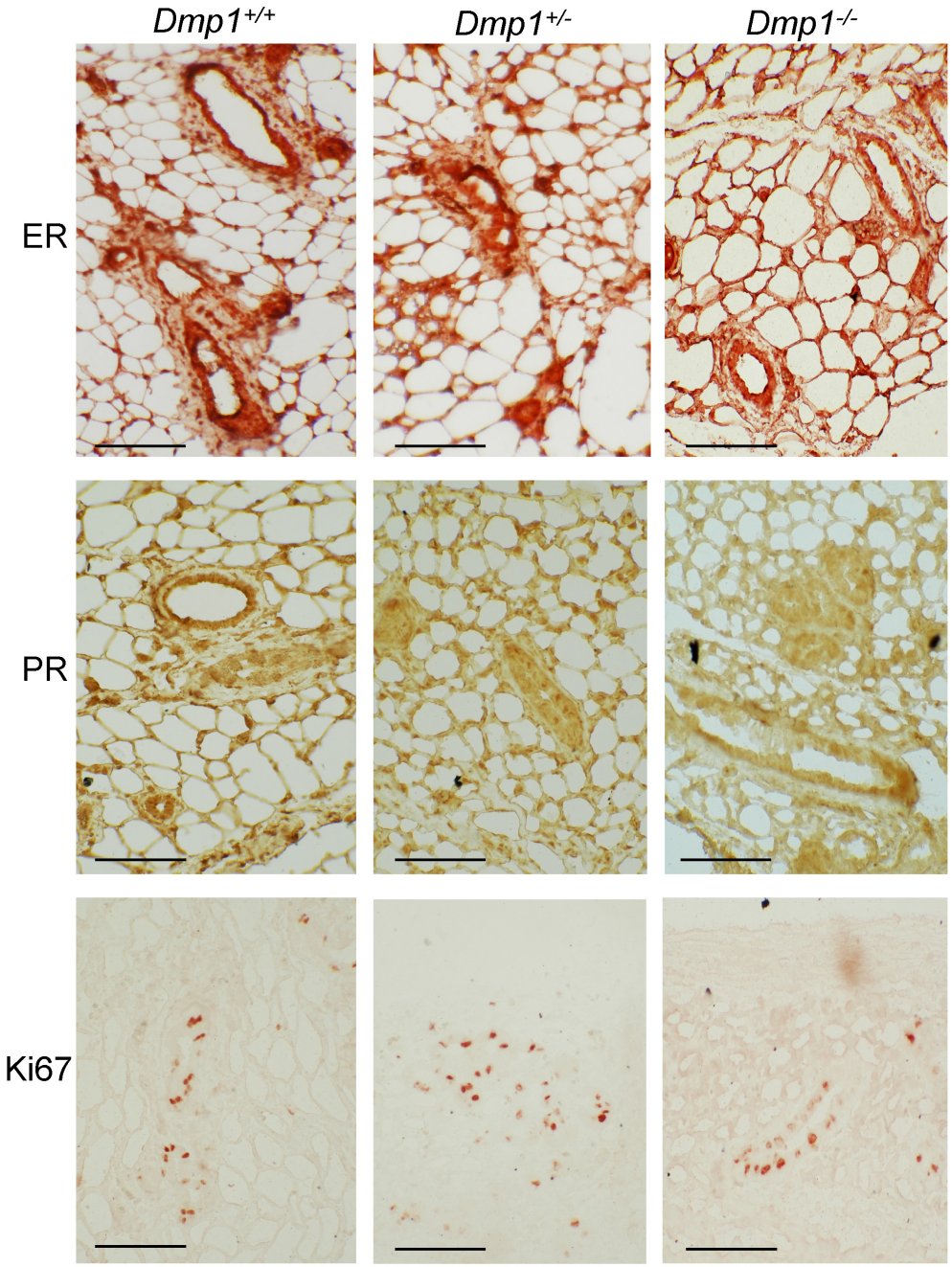


Figure S3. Immunohistochemical analysis of mammary glands from *Dmp1*-knockout mice. Mammary glands were isolated from twelve-week-old virgin females of each *Dmp1* genotype, and were stained for estrogen receptor (ER, sc-543), progesterone receptor (PR, sc-538), or Ki67 (SP6, NeoMarkers). There was no significant difference in the staining pattern for these proteins among the three *Dmp1* genotypes. Scale bar is 100 μm.

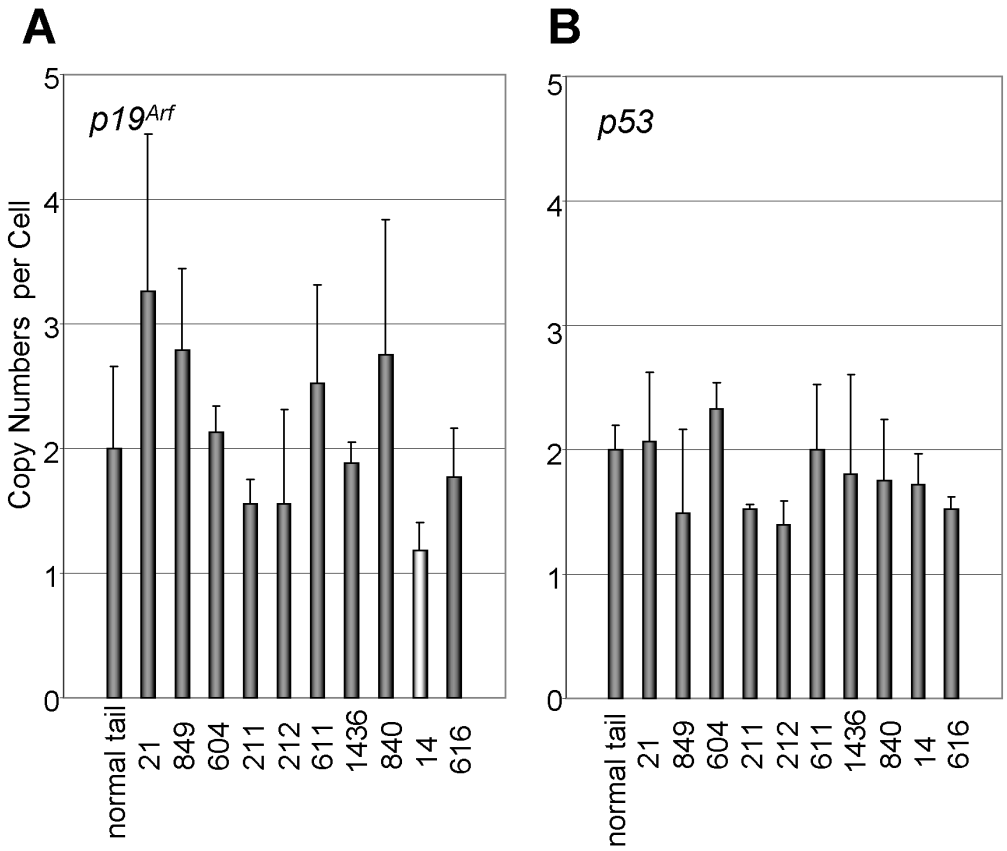


Figure S4. The *p19*^{Arf} and *p53* genes are not frequently deleted in wild-type MMTV-*neu* mammary tumors.

The copy numbers for *p19*^{Arf} and *p53* were quantified by real-time PCR Taqman assay with *β-actin* as an internal control. These tumor suppressor genes were not frequently deleted in mammary tumors from MMTV-*neu* mice, showing the specificity of the deletion of the *Dmp1* locus. The numbers indicate the ID of each mouse.

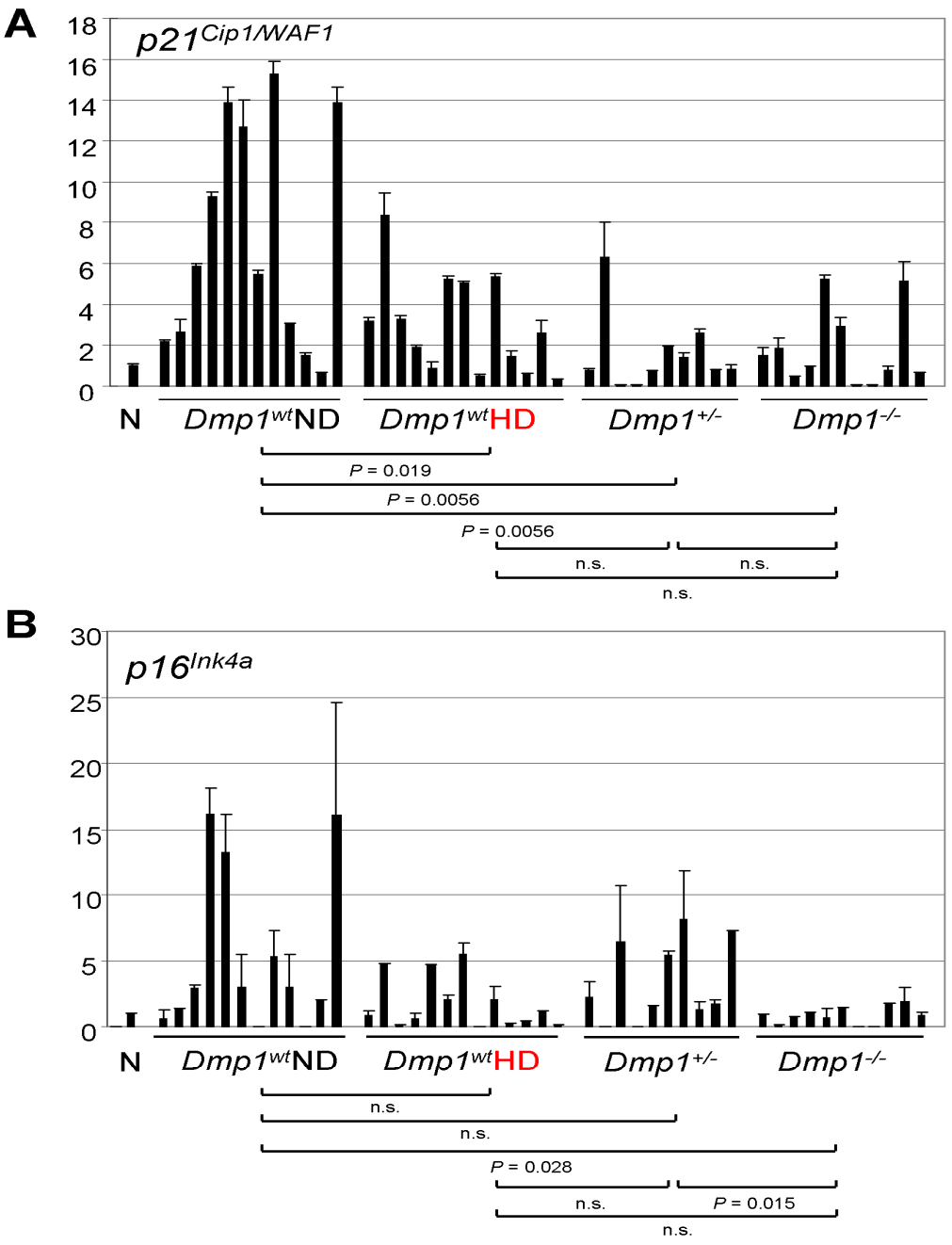
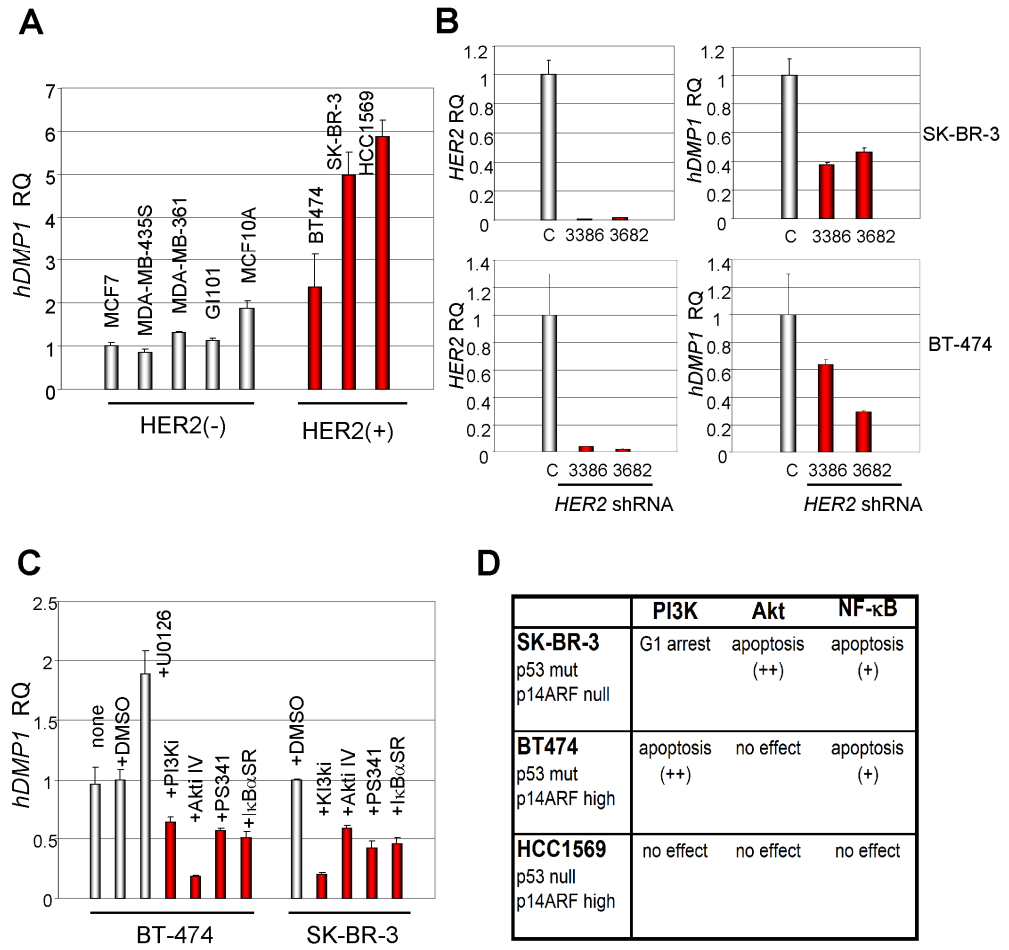


Figure S5. Expression of *p21*$^{Cip1/WAF1}$ (A) and *p16*^{Ink4a} (B) mRNA in tumors from MMTV-*neu* mice.

Mammary tumors from *Dmp1*$^{+/+}$, *Dmp1*$^{+/-}$, and *Dmp1*$^{-/-}$; MMTV-*neu* mice were analyzed for the expression of *p21*$^{Cip1/WAF1}$ and *p16*^{Ink4a} mRNA by real-time Taqman assay with *β-actin* as an internal control. Tumors from wild-type MMTV-*neu* mice were divided into two groups ND (*Dmp1* was not deleted) and HD (*Dmp1* showed hemizygous deletion). N: normal mammary gland from a wild-type mouse. The *p21*$^{Cip1/WAF1}$ mRNA was significantly downregulated in tumors from *Dmp1*$^{+/-}$ and *Dmp1*$^{-/-}$ mice as well as in wild-type tumors that showed hemizygous deletion for *Dmp1*. The *p16*^{Ink4a} mRNA was significantly downregulated only in tumors from *Dmp1*$^{-/-}$ mice. Statistical analyses were conducted by unpaired Student's *t* tests.



	PI3K	Akt	NF-κB
SK-BR-3 p53 mut p14ARF null	G1 arrest	apoptosis (++)	apoptosis (+)
BT474 p53 mut p14ARF high	apoptosis (++)	no effect	apoptosis (+)
HCC1569 p53 null p14ARF high	no effect	no effect	no effect

Figure S6. HER2 upregulates the h*DMP1* mRNA in human breast epithelial cells.
A, real-time PCR Taqman assay of h*DMP1* in human breast cancer cell lines. The h*DMP1* levels are higher in breast cancer cells with HER2 overexpression. *B*, specific shRNAs for *HER2* downregulate endogenous h*DMP1* in human breast cancer cell lines with HER2 amplification. *C*, HER2 increases the h*DMP1* mRNA through the PI3K-Akt-NF-κB pathway. BT-474 and SK-BR-3 cells were treated with specific inhibitors and real-time PCR was conducted for h*DMP1*. *D*, effects of inhibition of PI3K (LY294002), Akt (Akt inhibitor IV), or NF-κB (PS341) on the growth and survival of HER2-overexpressing breast cancer cell lines.