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ORIGINAL REPORT

Deletion of the *PER3* Gene on Chromosome 1p36 in Recurrent ER-Positive Breast Cancer

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ABSTRACT

Purpose
To investigate the role of the *PER3* circadian rhythm gene, located within the commonly deleted region of chromosome 1p36, in human breast cancer development.

Patients and Methods
The frequency of genetic alterations at 1p36 and *PER3* gene copy number status were analyzed in 180 lymph node–negative breast cancers from patients who had received treatment with chemotherapy and/or tamoxifen. The expression levels of *PER3* were also analyzed using published microarray profiles from > 400 breast cancer samples. Finally, the effect of loss of *Per3* on tumor susceptibility was tested using two mouse models of breast cancer.

Results
Deletion of *PER3* is directly related to tumor recurrence in patients with estrogen receptor (ER) –positive breast cancers treated with tamoxifen. Low expression of *PER3* mRNA is associated with poor prognosis, particularly in a subset of tumors that are ER positive, and either luminal A or ERBB2-positive tumors. Mice deficient in *Per3* showed increased susceptibility to breast cancer induced by carcinogen treatment or by overexpression of *Erbb2*.

Conclusion
Disruption of *PER3* function may serve as an indicator of probability of tumor recurrence in patients with ER-positive tumors. Further investigations of this pathway may reveal links between deregulation of sleep homeostasis and breast tumorigenesis.

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INTRODUCTION

Chromosomal region 1p36 is among the most commonly deleted regions in human cancers. Deletion of 1p36 is especially frequent in breast tumors and is associated with progression and lymph node metastasis,[1] poor prognosis,[2] higher rate of recurrence,[3] larger tumor size, and DNA aneuploidy.[4] However, no direct relationship between breast carcinogenesis or prognosis and any specific tumor suppressor gene on 1p36 has been established. Recent elegant studies have identified *CHD5*[5] and more recently *KIF1B*[6] as candidate tumor suppressor genes in this region, but no specific roles for these genes in breast cancer development have been demonstrated.

The human *PER3* gene is located within 1.5 Mb of *CHD5*, and the mouse homolog is a member of the period gene family that controls circadian rhythms.[7,8] Members of the period family of circadian rhythm genes (*Per1* and *Per2*) have been implicated in cell cycle control, DNA damage responses, and tumor progression.[9-12] Although inactivation of *Per3* in the mouse germline has only subtle effects on circadian clock function,[13] it has been shown that *Per3* transcripts exhibit a clear circadian rhythm both in the suprachiasmatic nucleus[7] and in mouse peripheral tissues.[14] Similar data have been shown in human peripheral blood cells, where circadian oscillations were more robust for *PER3* expression than for other clock genes including *PER1* and *PER2*.[15,16] The possible functions of *PER3* in tumor development have not been explored, but links to breast cancer are supported by biochemical studies demonstrating the existence of complexes, including proteins of the PER family together with the estrogen receptor (ER),[17,18] and by reports of association between a polymorphism in the human *PER3* gene and breast cancer susceptibility.[19]

The location of the *PER3* gene within a region that is commonly deleted in breast cancers suggested a possible link to epidemiologic studies[20,21] showing an association between disrupted sleep cycles and higher risk of developing breast cancer. We used a combination of human breast tumor analysis and

mouse models to show that disruption of *PER3* may serve as a prognostic biomarker of tumor recurrence in patients with ER-positive, luminal A, and/or ERBB2-positive tumors.

PATIENTS AND METHODS

Sample Selection

We used three previously published breast cancer data sets that included clinical, gene expression, and/or array comparative genomic hybridization (CGH) data.[22-24] Data on disease-free survival (DFS; defined as the time to a first event) and overall survival (OS) were available for all patients in the three data sets except for one patient in samples from a study by Chin et al.[24]

Copy Number Analysis of PER3

All tumor DNA samples were obtained from frozen breast tumors with > 50% tumor cells.[22] The genomic sequence of *PER3* (GenBank accession NM_016831.1) was used to design a set of primers and probes specific to the *PER3* gene (Primer Express software version 1.0; Applied Biosystems, Foster City, CA; see Data Supplement for detailed description).

PER3 Gene Expression Analysis

We examined *PER3* expression in 413 breast tumor expression arrays taken from studies by van de Vijver et al[23] (n = 295) and Chin et al[24] (n = 118). In each data set, a sample s_i in the set S was labeled as "*PER3* low," "*PER3* normal," or "*PER3* high" using the rule:

If $s_i \leq$ (mean [S] − ½ × standard deviation [S]), assign LOW.

If $s_i \geq$ (mean [S] + ½ × standard deviation [S]), assign HIGH.

Otherwise, assign NORMAL.

This method allowed us to compare relative *PER3* expression levels across both data sets fused as a single group of patients.

Statistical Analysis

The association between *PER3* deletion or *PER3* expression and clinical-pathologic parameters was analyzed using Fisher's exact test. All reported P values were two-tailed. Significant differences in DFS and OS time were calculated using the Cox proportional hazard (log-rank) test. Multivariate Cox regression analysis was used to prove statistical independence of *PER3* from other known prognostic factors. Statistical analysis was performed using SPSS version 12.0 (SPSS, Chicago, IL).

Mice and Tumor Induction

Wild-type ($Per3^{+/+}$) and *Per3* knockout ($Per3^{-/-}$) 129/sv mice (provided by Drs. Y.H. Fu and L.J. Ptáček, University of California, San Francisco [USCF], San Francisco, CA) were bred and treated according to Laboratory Animal Resource Center regulations. Female mice 7 weeks old from the F_2 intercross population ($Per3^{+/+}$, $Per3^{+/-}$, and $Per3^{-/-}$) were treated with six doses of 1 mg of 7,12-dimethylbenz[a]anthracene (DMBA) diluted in corn oil by weekly oral gavage. A second group of mice was treated with corn oil only as a group control. In a second experiment, male $Per3^{-/-}$ mice were crossed with female FVB mice expressing the *Neu* (*ErbB2*) proto-oncogene under control of the mouse mammary tumor virus (MMTV) 3′ long terminal repeats promoter[25] (provided by Dr. Z. Werb, UCSF) to generate F_1 transgenic mice heterozygous for *Per3* (*Neu*/$Per3^{+/-}$). F_1 males and females were intercrossed to produce the F_2 generation consisting of *Neu*/$Per3^{+/+}$, *Neu*/$Per3^{+/-}$, and *Neu*/$Per3^{-/-}$ animals. Identification of animal genotypes is described in the Data Supplement.

In the DMBA gavage experiment, female mice were examined every 3 days for sickness or symptoms of tumor development for up to 19.7 months. MMTV *neu*/*Per3* transgenic female mice were examined weekly by palpation for mammary tumor development for up to 25.8 months. Mice that showed significant weight loss, morbidity, or excessive tumor burden were killed by cervical dislocation after being anesthetized according to the UCSF Animal Care and Use protocol. Tumors and tissues were fixed in 4% neutral buffered paraformaldehyde for histologic examination. Mice found dead were censored from the study.

RESULTS

Deletion of 1p36 and Loss of PER3 Genetic Variants in Breast Cancers

We previously reported genome-wide array CGH profiles of 185 lymph node–negative breast cancers from a Spanish cohort,[22] of

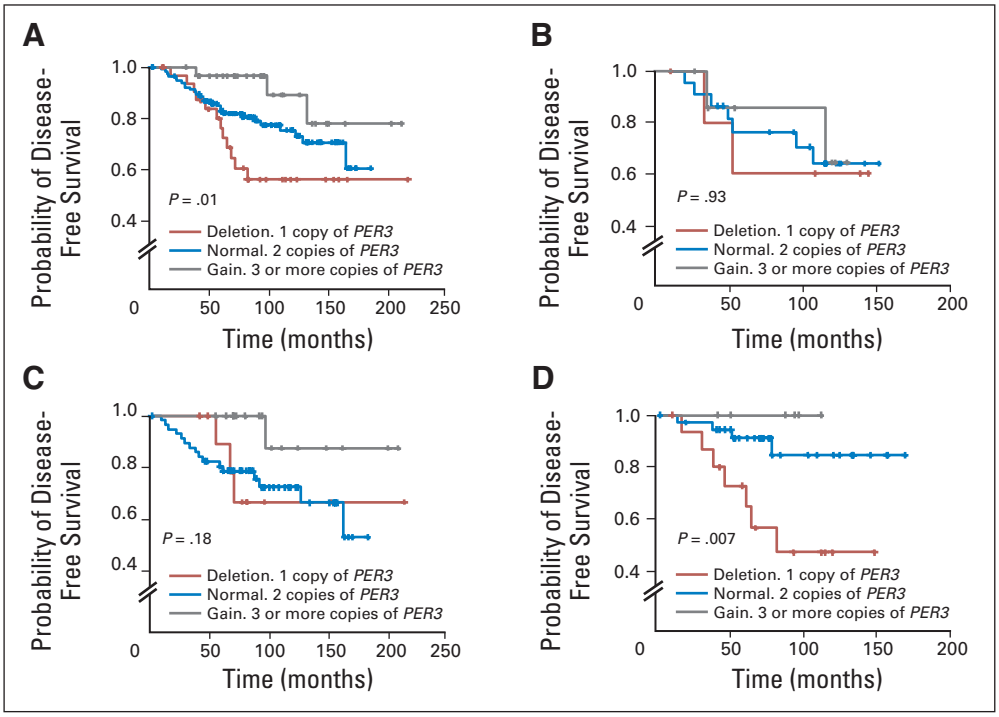

Fig 1. Association between *PER3* deletion and disease-free survival in breast cancer patients. (A) TaqMan copy number analysis of *PER3* in all patients, (B) in patients who received no treatment or (C) were treated with anthracycline chemotherapy, and (D) in a subset of 59 patients who were estrogen receptor–positive and/or progesterone receptor–positive and were treated only with tamoxifen.

whom 85 received anthracycline chemotherapy (chemo group) and 95 received no chemotherapy (non-chemo group). To search for genetic events related to resistance to hormonal (tamoxifen) therapy, we divided the non-chemo group into two subgroups on the basis of whether they had received hormonal treatment. Of the 95 patients in the non-chemo group, 59 patients with ER-positive and/or progesterone-positive tumors received tamoxifen, whereas 36 did not receive any treatment. Analysis of CGH profiles for these patients revealed that deletion of chromosome 1p was associated with recurrence in the subgroup of ER-positive tamoxifen-treated patients ($P < .05$ after multiple testing correction using method of Benjamini and Hoffberg; Fig DS1).[26]

The chromosome 1p36 locus is frequently deleted in many human tumors, but the region of deletion is large. Separate, nonoverlapping chromosome fragments have been implicated,[27-29] suggesting that multiple tumor suppressor genes are involved. We considered *PER3* to be a good candidate for involvement in breast cancer because of its location within one of the minimal deletion regions on 1p36.2,[5,6] as well as the epidemiologic[19] and mechanistic[17] data linking circadian rhythm genes to hormone status and breast cancer. We therefore

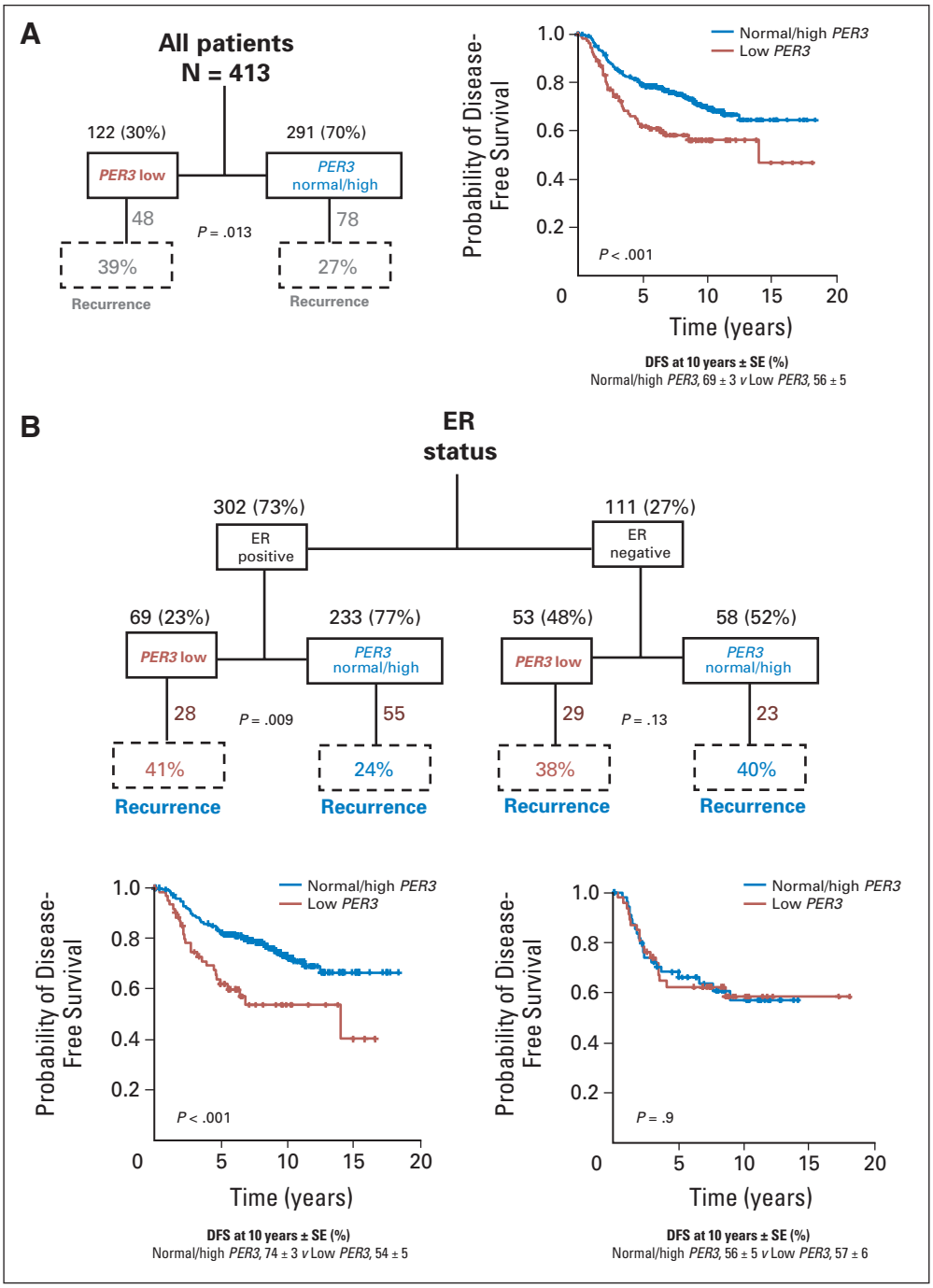

Fig 2. Association between *PER3* gene expression and survival of breast cancer patients. (A) *PER3* low expression (red) versus *PER3* normal/high expression (blue) in all patients. (B) Comparison of *PER3* expression with estrogen receptor (ER) status. DFS, disease-free survival; SE, standard error.

examined the copy number status of *PER3* by quantitative TaqMan analysis (Applied Biosystems) in DNA samples from 180 breast cancer patients. The relationship between the frequency of deletion or copy number gain and clinico-pathologic characteristics of the patients is shown in Table DS1. The number of copies of *PER3* showed a significant gene dosage association with recurrence-free survival at 10 years ($P = .01$; Fig 1A). The proportion of disease-free surviving patients after 10 years was lowest in patients with single-copy *PER3* deletion

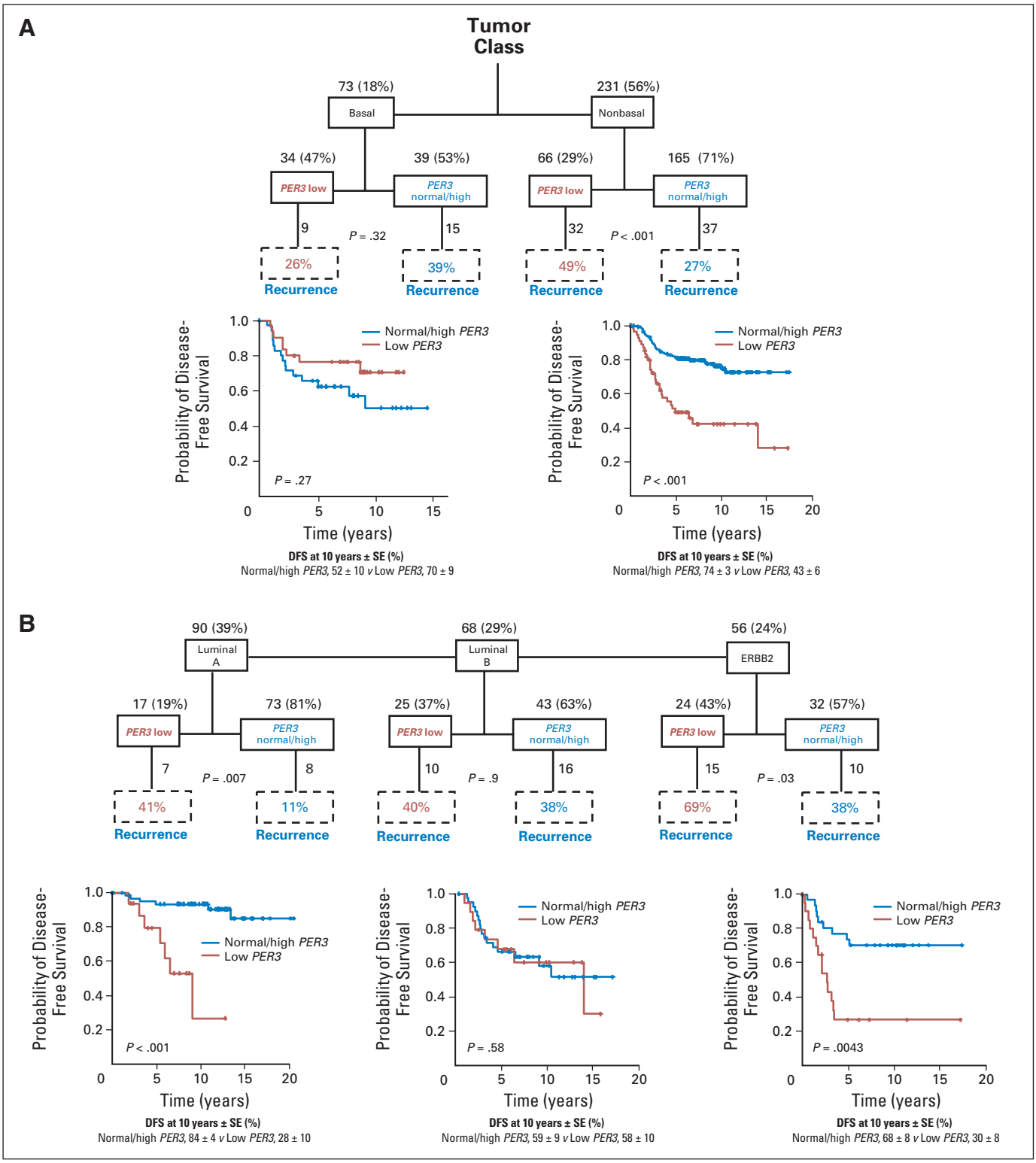

Fig 3. Effect of *PER3* expression levels on survival according to molecular subtypes. Kaplan-Meier estimates of disease-free survival (DFS) among the 413 patients, according to the *PER3* expression. (A) Basal versus non-basal tumors; (B) luminal A versus luminal B and ERBB2-positive subgroups of tumors. SE, standard error.

(56% ± 8.6; red line) compared with those having two (75% ± 4.0; blue line) or more (89% ± 5.6; gray line) copies of the *PER3* gene (Fig 1A). Further analysis showed that the effect of *PER3* deletion was most pronounced in the tamoxifen-treated group, with no significant association in the nontreated or chemotherapy-treated groups (Figs 1B through 1D). Among the 59 patients who received only tamoxifen treatment (Fig 1D), patients with single-copy *PER3* deletions had a significantly lower DFS rate at 10 years (47% ± 12) than those with normal *PER3* (84% ± 6) or copy number gains (100% DFS; $P = .007$). Follow-up for all patients was 82 months (range, 1.5 to 219 months). To look for potential inactivating mutations in *PER3* in breast cancers, we initially sequenced the complete coding region of *PER3* in a panel of 35 breast cancer cell lines. No clear pathogenic (nonsense or missense) mutation was identified. However, many known[30] and some unknown polymorphisms and alternative splicing isoforms were found (see Data Supplement for full detailed description). One of the polymorphic variants identified by sequencing had been associated with breast cancer susceptibility in other studies[19] and also with disruption of sleep homeostasis.[31-33]

Low Expression of PER3 *Is Associated With Reduced Survival*

We next examined *PER3* gene expression in 413 breast tumor expression arrays taken from two publicly available data sets (van de Vijver et al[23] [n = 295] and Chin et al[24] [n = 118]). A full description of the stratification of the patients into different subgroups according to *PER3* expression together with DFS curves for all patients in each subgroup is shown in Figures 2 and 3. Patients with lower *PER3*

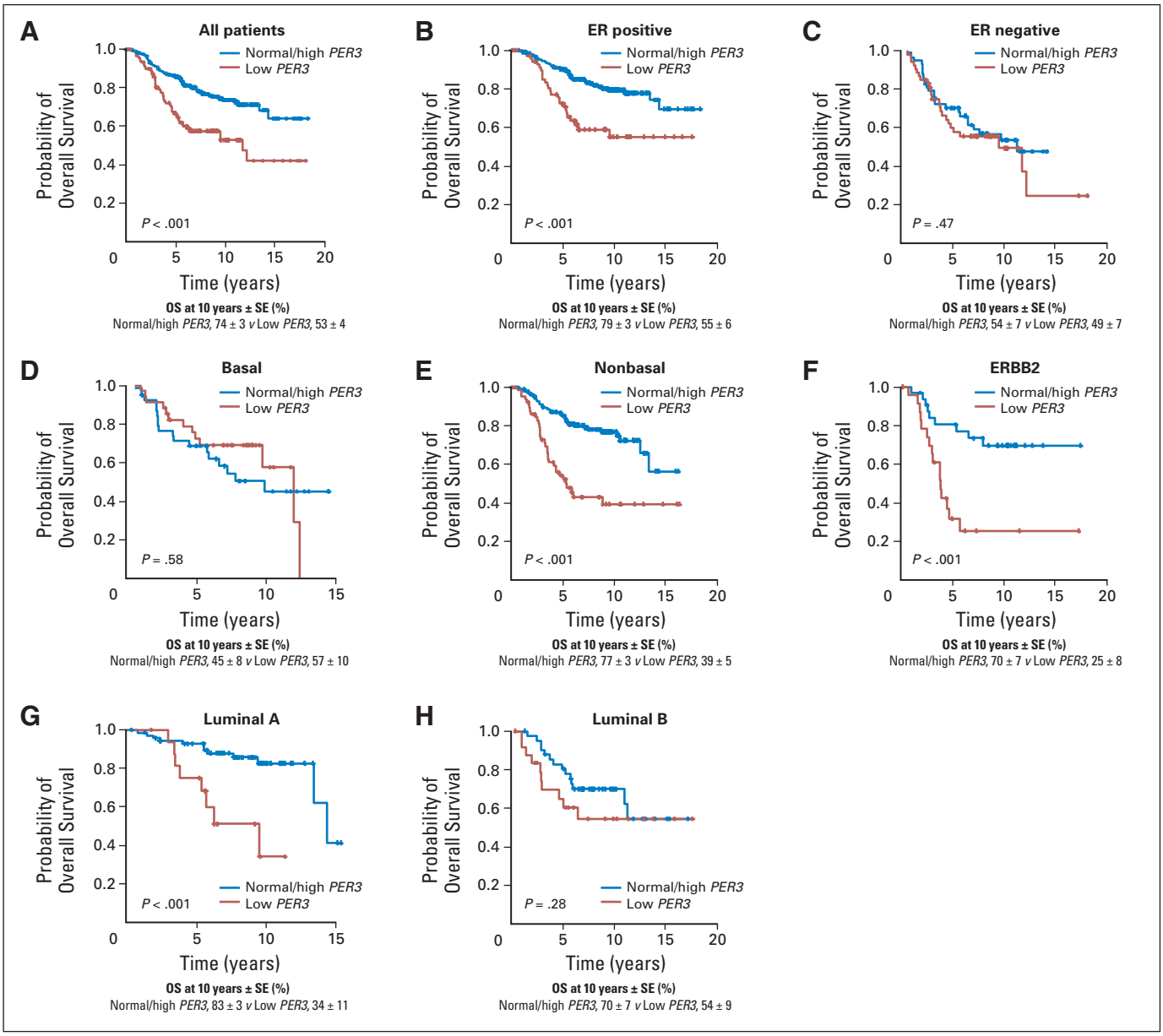

Fig 4. Kaplan-Meier estimates of overall survival (OS). The different expression levels of *PER3* were evaluated in all the patients (A) and the different subgroups of patients based on (B) ER-positive, (C) ER-negative, (D) basal, (E) nonbasal, (F) ERBB2-positive, (G) luminal A, and (H) luminal B tumors. SE, standard error.

expression ("*PER3* low" [n = 122]) were significantly more likely to recur than those with normal or higher expression ("*PER3* normal/high" [n = 291]; $P = .013$; Fig 2A). DFS analysis showed that *PER3* low patients had significantly worse survival rates than *PER3* normal/high patients ($P < .001$). ER status is an important predictor of recurrence and greatly influences treatment regimens.[34,35] If low expression of *PER3* segregates with ER status, any effect of low *PER3* expression could be confounded with the effect of ER status. We therefore performed a subset analysis of *PER3* in ER-positive and ER-negative tumors. Low *PER3* levels were significantly associated with recurrence ($P = .01$) and shorter DFS times ($P < .001$) in patients with ER-positive, but not ER-negative tumors (Fig 2B). We conclude that the association between low *PER3* expression and recurrence in the complete patient sample set was driven by the ER-positive tumors, with no effect being detected in the ER-negative tumors. These data are in agreement with the independent association between deletion of *PER3* and recurrence specifically in the tamoxifen-treated (ER-positive tumors) patients in Figure 1D.

We next asked whether stratifying tumors according to their molecular subtype[36,37] could reveal additional information. The tumors were labeled using a nearest centroid classifier, and a label was assigned only if correlation with a target class was above 0.1.[31,32] This resulted in samples labeled luminal A (n = 90), luminal B (n = 68), ERBB2 (n = 56), normal-like (n = 17), basal (n = 73), or unclassified (n = 109; Fig 3 and Fig DS4). Of these groups, low *PER3* expression had significant association with recurrence only in luminal A–type ($P = .007$) or ERBB2-type tumors ($P = .03$; Fig 3B). None of the patients with ERBB2-positive tumors received anti–ERBB2-targeted therapy. DFS analysis for luminal A–type and ERBB2-type tumors indicated that *PER3* low patients had lower DFS rates at 10 years than patients with *PER3* normal/high (luminal A: 28% ± 10 *v* 84% ± 4; $P < .001$ and ERBB2: 30% ± 8 *v* 68% ± 8; $P = .004$). There was also a striking effect on OS rate at 10 years in all the patients and in the subgroups of ER-positive, luminal A, and ERBB2 patients (Fig 4): The 10-year OS rate for patients with ER-positive tumors and with low *PER3* was 55% ± 6 versus 79% ± 3 for normal/high patients ($P < .001$; Fig 4B). The OS rate was 25% ± 8 for patients with ERBB2 and low *PER3* versus 70% ± 7 for patients with ERBB2 and normal/high *PER3* ($P < .001$; Fig 4F). The OS rate at 10 years in luminal A patients with low *PER3* was 34% ± 11 versus 83% ± 3 for patients with normal/high *PER3* ($P < .001$; Fig 4G). Importantly, multivariate analysis showed that *PER3* expression is independently significant from all the prognostic factors tested both for DFS ($P < .001$) and OS ($P = .001$; Table 1).

The possible links between expression levels and probability of tumor recurrence were evaluated for all 54 annotated genes in the 1p36.31 to 1p36.22 region (chromosome 1: 6,084,440 to 9,512,808 [3.5 Mb]). Gene expression was discretized as described for *PER3*, and log-rank *P* values were calculated using the survival library for R. This analysis showed that *PER3* was the only gene with an uncorrected $P < .05$ in all data sets analyzed. Although chromosome engineering studies have previously identified *CHD5* as a candidate tumor suppressor gene within the minimal deletion region on 1p36.2[5], no association of *CHD5* expression levels with recurrence or survival was found in any of the subgroups of breast cancer patients analyzed (Figs DS5 and DS6). These data do not exclude the possibility that *CHD5* plays an important role as a tumor suppressor in other tumor types.

Inactivation of Per3 *Increases Breast Tumor Susceptibility in Mouse Models*

To investigate a possible causal association between loss of *Per3* function and breast tumor development, we performed two studies involving mouse models of breast cancer (Fig 5 and Table 2). A total of 86 mice carrying normal or inactivated alleles of the *Per3* gene

Table 1. Cox Proportional Hazard Ratio Multivariate Analysis

Variable	Disease-Free Survival			Overall Survival		
	Hazard Ratio	95% CI	*P*	Hazard Ratio	95% CI	*P*
All patients						
PER3	2.13	1.40 to 3.24	< .001	2.04	1.34 to 3.10	.001
Tumor size	1.72	1.13 to 2.63	.012	2.02	1.31 to 3.12	.002
Age < 40 years	0.49	0.32 to 0.74	.001	0.54	0.35 to 0.83	.005
ER	0.75	0.49 to 1.15	.19	0.53	0.35 to 0.80	.003
Lymph node	1.36	0.90 to 2.06	.14	1.85	1.18 to 2.77	.007
Tumor grade						
Good	0.93	0.55 to 1.60	.8	1.05	0.61 to 1.80	.87
Intermediate	1.18	0.74 to 1.89	.48	1.38	0.87 to 2.20	.17
ER-positive patients						
PER3	2.92	1.71 to 4.97	< .001	2.63	1.49 to 4.63	.001
Tumor size	1.62	0.96 to 2.63	.072	1.87	1.05 to 3.32	.03
Age < 40 years	0.58	0.33 to 0.99	.047	0.57	0.32 to 1.04	.06
ER*						
Lymph node	1.40	0.83 to 2.39	.21	2.07	1.18 to 2.77	.02
Tumor grade						
Good	1.14	0.59 to 2.23	.69	1.09	0.54 to 2.24	.8
Intermediate	1.34	0.73 to 2.46	.34	1.32	0.70 to 2.49	.38

NOTE. Risk of distant recurrence or death among patients with breast cancer.
Abbreviation: ER, estrogen receptor.
*All tumors are ER positive.

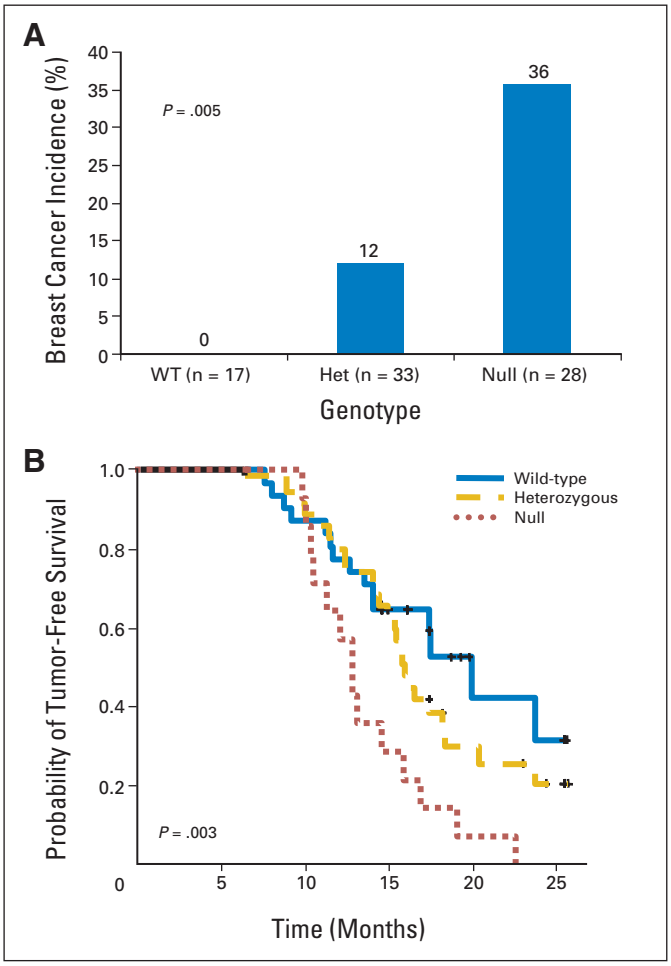

Fig 5. Effect of loss of *Per3* on tumor susceptibility in two different mouse models. (A) Breast cancer incidence in a group of mice treated with 7,12-dimethylbenz[a]anthracene on the basis of the different genotypes (wild type$^{+/+}$[WT], heterozygous$^{+/-}$[Het], and null $^{-/-}$). (B) Kaplan-Meier estimates of probability of tumor-free survival in the group of mouse mammary tumor virus neu-*Per3* mice.

(17 wild-type [*Per3*$^{+/+}$], 35 heterozygous [*Per3*$^{+/-}$], and 34 null [*Per3*$^{-/-}$]) were treated by oral gavage with DMBA, a protocol known to induce breast cancer in sensitive strains of mice.[38] Eight mice (two heterozygous and six null) were found dead before the end point, and no tissues were collected from them. The median follow-up of the remaining 78 mice included in the study was 8.3 months (range, 3.8 to 15.0 months). All of the mice treated with DMBA developed tumors of various kinds, including lymphoma and solid tumors of the lung, ovary, and skin (Table DS5). However, development of breast tumors was specifically associated with *Per3* deficiency. Thirty-six percent of *Per3*$^{-/-}$ mice treated with DMBA developed breast tumors, while 12% of the *Per3*$^{+/-}$ mice developed breast tumors. In striking contrast, none of the control *Per3*$^{+/+}$ mice developed a breast tumor ($P = .005$; Fig 3A). A group of 65 mice (19 wild-type, 25 heterozygous, and 21 null) were used as controls with no DMBA gavage treatment. Two of the *Per3*$^{-/-}$ control mice developed sporadic breast tumors, but none of the remaining mice were found sick or developed any other class of tumor during the time course of this experiment (24 months).

Table 2. Tumor-Free Rate for MMTV-Neu–Positive Mice

No. of Mice	Median Follow–Up (months)	Range (months)	Tumor-Free Rate at 15 Months ± SE (%)
30	16	7.5-26.4	63 ± 6
35	16	6.3-26.5	63 ± 6
14	13	9.8-22.5	21 ± 8

Abbreviations: MMTV, mouse mammary tumor virus; SE, standard error.

The second mouse model was based on the observation that low levels of *PER3* expression were strongly associated with recurrence in ERBB2-type human breast cancers. MMTV-Neu mice overexpress *ErbB2* in the mammary gland and spontaneously develop breast tumors.[26] We generated a total of 79 MMTV-Neu–positive mice, of which 30 (38%) were *Per3*$^{+/+}$, 35 (44%) were *Per3*$^{+/-}$, and 14 (18%) were *Per3*$^{-/-}$. The median follow-up of all mice was 14.9 months (range, 6.3 to 25.8 months). All *Per3*$^{-/-}$ mice developed breast tumors, whereas 25 (71%) of the *Per3*$^{+/-}$ and 14 (47%) of the *Per3*$^{+/+}$ mice developed breast tumors. The proportion of *Per3*$^{-/-}$ null mice free of tumors at 15 months (21% ± 8) was significantly lower than the proportion in the heterozygous and the wild-type mice (63% ± 6 in both *Per3*$^{+/-}$ and *Per3*$^{+/+}$; $P = .003$). Histologic analysis of tumors from both models of breast cancer showed that loss of *Per3* did not affect the tumor class or morphology, since both DMBA-induced and MMTV-Neu–induced tumors in *Per3*$^{-/-}$ mice resembled equivalent tumors from *Per3* wild-type animals (data not shown). We also evaluated the possible loss of the wild-type *Per3* allele in tumors from the *Per3* heterozygous mice. No loss was observed, suggesting that homozygous loss is not essential in this mouse model.

DISCUSSION

Our data indicate that deletion and/or reduced expression of the *PER3* gene on human chromosome 1p36 is associated with breast cancer recurrence, particularly in patients with ER-positive tumors treated with tamoxifen who did not receive chemotherapy. No effect of deletion was seen in patients with basal type ER-negative breast tumors. Within the ER-positive category, the effect was primarily in tumors classified as luminal A–type or ERBB2-type, but not in the luminal B–type, which shares some expression features with basal tumors.[36,37] Direct evidence for a causal role for loss of *PER3*, rather than an alternative gene in this commonly deleted region of the genome,[5,6] comes from analysis of two different mouse models of breast cancer. Both chemically induced and *Neu (ErbB2)* –induced breast cancers are increased in frequency and/or reduced in latency in mice carrying inactivated *Per3* alleles. Although these data do not prove that *Per3* is the only functional tumor suppressor gene in this chromosome interval, they indicate that *Per3* is a bona fide tumor suppressor in these mouse models, with a key role in breast tissue.

While disruption of the mouse period gene family members *Per1* and *Per2* by gene targeting induces biologic clock phenotypes,[39] loss of *Per3* function induces only subtle effects on circadian rhythm.[13,40] Nevertheless, evidence in favor of *PER3* involvement in both sleep disruption and breast cancer comes from studies of a human structural polymorphism in the *PER3* coding sequence that has been associated with delayed sleep phase syndrome, diurnal preference, and

waking performance,[31,41,42] but also with increased breast cancer risk,[19] particularly in premenopausal women.

Although the specific molecular mechanisms remain to be elucidated, increasing evidence points to a role for circadian rhythm genes in cell cycle control and DNA damage responses[11,43] as well as in hormonal control of gene expression.[17,18] *PER2* has been identified as an estrogen-inducible ER corepressor that forms heterodimers with *PER3* to enter the nucleus. Deletion of *PER3* prevents nuclear import and, instead, promotes accumulation of *PER2* in the cytoplasm.[44] Whether coordinated functional deregulation of all period family genes occurs in breast cancers remains to be determined.

There are several clinical implications of these observations. First, the presence of *PER3* deletions in ER-positive tumors may identify patients who do not respond to tamoxifen-based hormone therapy and who may benefit from other therapeutic regimens. Second, previous data from clinical trials of chronotherapy suggest that the timing of cancer treatment during the day may affect individual patient responses.[45,46] Elucidation of the relationship between control of sleep homeostasis and circadian rhythms, *PER* gene expression, and DNA damage responses may help in understanding the epidemiologic data linking sleep disruption to breast cancer susceptibility,[17,20,21] but further detailed studies will be required to elucidate the exact mechanisms involved. Finally, small-molecule drugs that help to restore the balance of the biologic clock in individuals with frequent sleep disruption may have potential as chemopreventive agents for breast and some other cancer types.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

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Data analysis and interpretation: Joan Climent, David A. Quigley, Il-Jin Kim, Kuang-Yu Jen, Jian-Hua Mao, Allan Balmain
Manuscript writing: Joan Climent, David A. Quigley, Allan Balmain
Final approval of manuscript: Joan Climent, Jesus Perez-Losada, David A. Quigley, Il-Jin Kim, Reyno Delrosario, Kuang-Yu Jen, Ana Bosch, Ana Lluch, Jian-Hua Mao, Allan Balmain

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