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SHORT COMMUNICATION

Wwox inactivation enhances mammary tumorigenesis

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Breast cancer is the leading cause of cancer-related death in women worldwide. Expression of the WWOX tumor suppressor is absent or reduced in a large proportion of breast tumors suggesting that loss of WWOX may contribute to breast tumorigenesis. *Wwox*-deficient mice die by 3–4 weeks of age precluding adult tumor analysis. To evaluate the effect of WWOX-altered expression on mammary tumor formation, the *Wwox*-heterozygous allele was back crossed onto the C3H mammary tumor-susceptible genetic background ($Wwox^{C3H}+/-$) and incidence of mammary tumor formation was evaluated. Although 50% of the female $Wwox^{C3H}+/-$ mice developed mammary carcinomas, only 7% of $Wwox^{C3H}+/+$ mice did. Intriguingly, mammary tumors in $Wwox^{C3H}+/-$ mice frequently lost WWOX protein expression suggesting a genetic predisposition toward mammary tumorigenesis. Immunohistochemical staining of hormone receptors revealed loss of estrogen receptor-α (ER) and progesterone receptor in the majority of these tumors. *In vitro*, depletion of WWOX in MCF7 ER-positive cells led to reduced ER expression and reduced sensitivity to tamoxifen and estrogen treatment and was associated with enhanced survival and anchorage-independent growth. Finally, cDNA array analyses of murine normal mammary epithelial cells and mammary tumors identified 163 significantly downreguated and 129 upregulated genes in the tumors. The majority of differentially expressed genes were part of pathways involved in cellular movement, cell-to-cell signaling and interaction, cellular development, cellular growth and proliferation and cell death. These changes in gene expression of mouse mammary tumors in $Wwox^{C3H}+/-$ mice resemble, at least in part, human breast cancer development. Our findings demonstrate the critical role that the WWOX tumor suppressor gene has in preventing tumorigenesis in breast cancer.
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Introduction

Breast cancer is still one of the most common types of tumors, affecting one woman out of eight, and is the second leading cause of cancer death in women in the USA (Jemal *et al*., 2010). The model of breast cancer progression suggests that its development involves defined pathological and clinical stages, starting with ductal hyperproliferation, with subsequent evolution into *in situ* and invasive carcinomas, and finally into metastatic disease (Polyak, 2007). Genome-wide microarray and copy number analyses revealed that breast tumors exhibit a diverse pattern of genetic changes when compared with normal mammary gland epithelial cells (Perou *et al*., 2000). Although enormous advances have been made in understanding breast cancer, the etiology and molecular signaling pathways that contribute to breast cancer formation remain largely elusive.

The *WWOX* gene encoding a protein with tumor suppressor function, a WW domain-containing oxidoreductase (Del Mare *et al*., 2009), was originally identified as a putative tumor suppressor in breast cancer as it lies in a fragile genomic region (FRA16D) that is frequently altered in pre-neoplastic and neoplastic lesions (Bednarek *et al*., 2000; Ried *et al*., 2000). Indeed, expression of WWOX is deregulated in two-thirds of breast cancer cases (Guler *et al*., 2005; Nunez *et al*., 2005). We and others have recently reported that WWOX loss is a frequent event in breast cancers that are negative for estrogen receptor (ER), progesterone receptor (PR) and HER2 (triple negative (Sorlie *et al*., 2001)) and that loss of WWOX expression is associated with nodal metastasis and unfavorable outcome (Aqeilan *et al*., 2007a; Guler *et al*., 2009)). Loss of heterozygosity and hypermethylation affecting WWOX have been reported as main causes of WWOX alterations in breast tumors (Bednarek *et al*., 2000; Iliopoulos *et al*., 2007). Furthermore, ectopic expression of WWOX in WWOX-negative breast cancer cells suppresses tumorigenicity (Iliopoulos *et al*., 2007). These findings suggest that alteration of WWOX expression may have roles in the pathogenesis of breast cancer.

Recently, we generated a mouse carrying a targeted deletion of the *Wwox* gene (Aqeilan *et al*.,

2007c). Spontaneous osteosarcomas were observed in juvenile *Wwox*-null mice before their death, whereas targeted loss of one allele increased the incidence of spontaneous and chemically induced lung and forestomach tumors (Aqeilan *et al*., 2007b, c; Kurek *et al*., 2010) thus confirming *Wwox bona fide* tumor suppressor function. At the biochemical level, cumulative evidence suggests that WWOX has an indispensable role in several signaling pathways (Salah *et al*., 2010), however, the molecular mechanism of WWOX tumor suppressor function remains largely unknown.

In this study, we set out to determine whether targeted ablation of *Wwox* in mice is associated with mammary tumor formation. To this end, we partially transferred the *Wwox*+/− allele into the C3H mammary tumor-susceptible genetic background mice and monitored mammary tumor development. We found that *Wwox*+/− mice are significantly more prone to spontaneous mammary tumor formation as compared with wild-type (*Wwox*+/+) control littermates and that these tumors closely resemble human breast cancers. Our findings provide the first *in vivo* evidence that WWOX has critical roles in the pathogenesis of breast cancer.

Results and discussion

Mammary tumor incidence in C3H-Wwox mice

We previously reported that *Wwox*+/− mice on B6-129 mixed background develop higher incidence of lung tumors as compared with *Wwox*+/+ mice (Aqeilan *et al*., 2007c). We also noted that some of these mice develop mammary tumors though with very low penetrance (data not shown). These data prompted us to examine the effect of WWOX altered expression on mammary tumor formation using the C3H mammary tumor-susceptible genetic background. The *Wwox*-heterozygous allele was back crossed onto the C3H background for three rounds (N3/F1; ~87% purity) and mammary tumor burden compared with wild-type littermate animals was assessed. Monitoring of mice for 18-months revealed that $Wwox^{C3H}+/-$ developed mammary tumors earlier and at a higher frequency than $Wwox^{C3H}+/+$ mice did. In particular, 50% (9/18) of $Wwox^{C3H}+/-$ mice developed mammary tumors compared with 7% (1/14) of $Wwox^{C3H}+/+$ mice (Table 1). The number of tumor-bearing animals among $Wwox^{C3H}+/-$ mice was 7-fold higher ($P=0.019$) than that of wild-type mice (Table 1). The incidence of tumors per mouse in $Wwox^{C3H}+/-$ mice was 11-fold higher than

Table 1 Characterization and incidence of mammary tumors in $Wwox^{C3H}+/-$ and $Wwox^{C3H}+/+$ mice

Mouse #	*Grade*	*Histology*	*WWOX*	*ER*	*PR*	*CK14*
M21	G-III	IDC	Neg	Neg	Neg	Neg
M22	G-III	IDC	Neg	Neg	Neg	Neg
M23	G-III	IDC	Red	Neg	Pos	Neg
M24	G-III	IDC with metaplastic SCC	Neg	Neg	Neg	Pos
M25	G-I/II	IDC	Red	Pos	n/a	Neg
M26-T	G-III	IDC	Red	Neg	Neg	Pos
M26 T-2F	G-III	IDC, +DCIS	Neg	Neg	Neg	Pos
M26-T3	G-I	DCIS with metaplastic SCC	Pos	Pos	Pos	Pos
M27-N		*Normal*	*Pos*	*Pos*	*n/a*	*Pos*
M27-1	G-III	IDC with metaplastic SCC	Neg	Neg	Neg	Pos
M27-T2	G-III	IDC	Neg	Pos	Pos	Pos
M27-T4	G-II	IDC	Neg	Neg	Neg	Pos
M27-T5	G-III	IDC	Neg	Neg	Neg	Pos
M28-N		*Normal*	*Pos*	*Pos*	*Pos*	*Pos*
M-29	G-III	IDC	Red	n/a	n/a	Pos
M30N		*Normal, per-lobular inflammation*	*Pos*	*n/a*	*n/a*	*Pos*
M31 N (WT)		*Normal*	*Pos*	*Pos*	*Pos*	*Pos*
M35 T	G-III	IDC, +DCIS	Red	n/a	n/a	Pos
M35 N		*Normal*	*Pos*	*Pos*	*Pos*	*Pos*

	HET	*WT*	P-*value*
Total no mice	18	14	
Tumor incidence	9/18 (50%)	1/14 (7%)	0.019
Tumor/mouse	14/18 (78%)	1/14 (7%)	<0.0001

Abbreviations: CK14, cytokeratine 14; DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; IDC, invasive ductal carcinomas; n/a, not available; PR, progesterone receptor; SSC, squamous cell carcinoma; WT, wild type.
Three back-crosses of *Wwox*-heterozygous (*Wwox*+/−) mice on B6-129 mixed background with pure C3H/HeJ mice were made generating $Wwox^{C3H}+/-$ N3/F1 (87.50% C3H genetic background). This C3H/HeJ mouse strain purchased from Jackson Laboratory does not carry the mouse mammary tumor virus and females are known to develop spontaneous mammary tumors by age of 6–15 months (http://tumor.informatics.jax.org/mtbwi/tumorFrequencyGrid.do;jsessionid=D9E7769C40B059F342610671F2B3C2B8). To evaluate spontaneous mammary tumors, 32 of N3-C3H female mice (8–18 months of matched age) were killed and autopsies were performed after CO_2 asphyxiation. Tissues were fixed in 10% phosphate-buffered formalin and examined histologically by two pathologists after hematoxylin and eosin staining for the presence of hyperplasia, DCIS and carcinomas. Tumor incidence was analyzed by two-tailed Fisher's test and tumor multiplicity using one-way analysis of variance. *P*-values <0.05 were considered statistically significant. Italic indicates normal tissues.

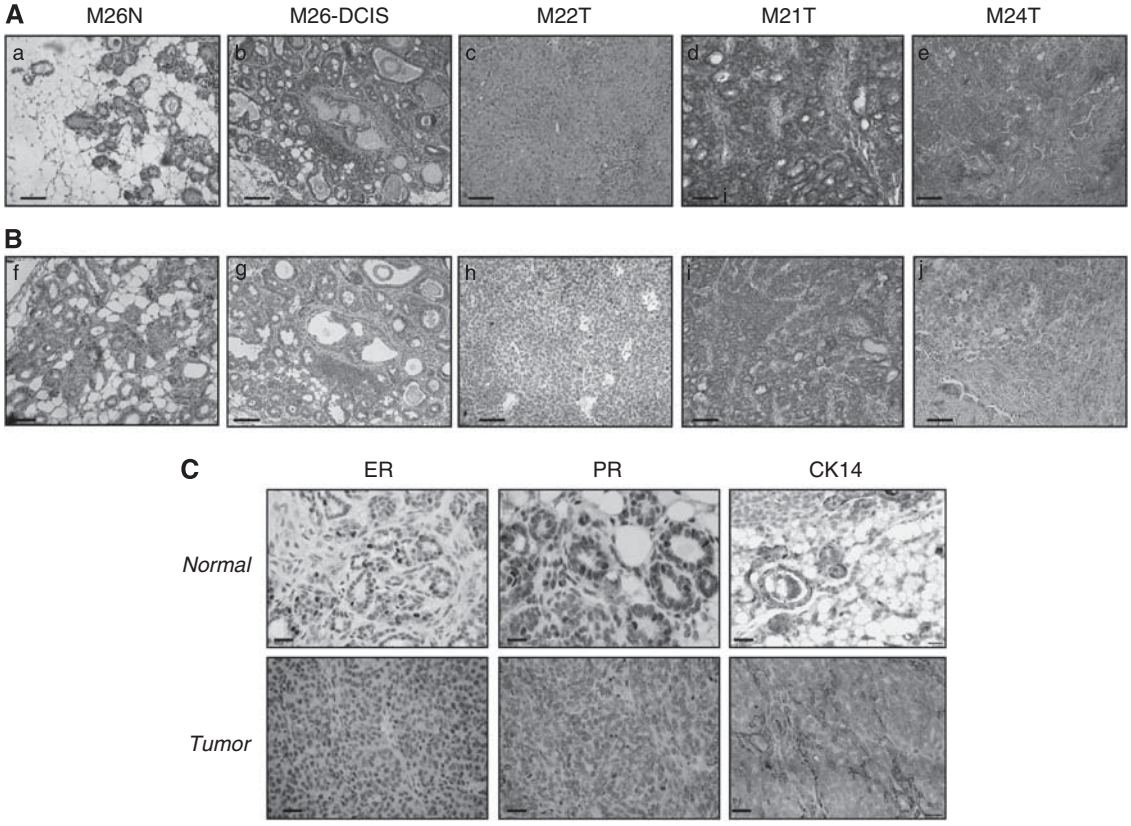

Figure 1 Characterization of mammary tumors in *Wwox*-heterozygous mice. (**A**) hematoxylin and eosin-stained sections of mammary tissues from $Wwox^{C3H}+/-$ female mice (× 20); (a) normal murine mammary gland architecture is characterized by large adipocytes in the stroma surrounding a sparse population of ducts. (b) Ductal hyperplasia and ductal carcinoma *in situ* (DCIS) commonly seen in $Wwox^{C3H}+/-$ female mice. (c–e) Invasive ductal carcinoma (c) with small acinar structures (d) and with epidermoid metaplastic features (e). Magnification bars represents 100 μm. (**B**) Expression of WWOX protein in mammary tissues from $Wwox^{C3H}+/-$ female mice (× 20) as described previously (Kurek *et al*., 2010). Polyclonal anti-WWOX antibody (Guler *et al*., 2009) at a dilution of 1:10 000 was used. Tumors were grouped into three categories (positive, reduced, negative) based on the intensity of cytoplasmic staining in all tumor cells present on the slides. The 'positive' category was assigned when the intensity was equivalent to that found in normal mammary glands from healthy tissues (f). The 'reduced' category was used for tumors with clearly diminished intensity (g, h) and 'negative' category for IDC tumors with no WWOX immunostaining as in i and j. Magnification bars represents 100 μm. (**C**) Expression of specific markers in mammary tissues from $Wwox^{C3H}+/-$ female mice (× 60) was performed as described (Gao *et al*., 2005) in normal (upper) and tumors (lower). Magnification bars represent 20 μm. Antibodies used were: mouse monoclonal anti-ER-α antibody (ER: 6F11 1:25 from Novocastra Lab, Newcastle upon Tyne, UK), rabbit polyclonal anti-PR (PR: A0098 1:250 from Dakocytomation, Carpinteria, CA, USA) and cytokeratine 14 (CK14: LL002, 1:8000 from AbD-Serotec, Raleigh, NC, USA).

in $Wwox^{C3H}+/+$ mice ($P<0.001$). The median age of $Wwox^{C3H}+/-$ mice that developed mammary tumors was 13 months whereas the only wild-type animal that developed tumors was 18 months old. These data suggest that additional genetic alterations that accumulate with time might be required for tumor development. Of note, only a small percentage of wild-type N3/F1-C3H mice develop mammary tumors, perhaps, due to the mixed genetic background of the colony. That such statistical significance was achieved with a relatively small number of mice suggest that targeted deletion of *Wwox* in the C3H background is indeed associated with increased incidence of spontaneous mammary tumor formation, though larger cohort of pure mice would be necessary in the future to further validate these findings.

*Mammary tumor phenotype in C3H-*Wwox$+/-$ *mice*

Although mammary carcinomas were rare in $Wwox^{C3H}+/+$ mice (1/14), they were the most prevalent tumor type in $Wwox^{C3H}+/-$ mice, accounting for half of the mice (Table 1). Mammary tumors originated in both the inguinal and thoracic glands with a latency of 8–18 months. All of the female heterozygous mice that developed mammary abnormalities developed ductal carcinoma *in situ* (Figure 1A-b) and poorly differentiated invasive ductal carcinomas (Figures 1A-c and d). Three cases demonstrated squamous cell carcinomas, invasive ductal carcinoma with epidermoid metaplastic features (Figure 1A-e). Two animals (M26 and M27) developed at least three separate primary mammary tumors in different glands that were of different histological types (Table 1). Epithelial hyperplasia was detected in female $Wwox^{C3H}$ mice that did not develop mammary tumors including wild-type mice. Cumulatively, these data suggest that inactivation of *Wwox* in mice is associated with mammary tumor phenotypes resembling those developing in breast cancer (Guler *et al*., 2005; Nunez *et al*., 2005; Aqeilan *et al*., 2007a).

Table 2 Association of WWOX immunoreactivity with expression level of biomarkers in mammary tumors

	ER	*PR*	*CK14*
WWOX	0.759[a]	0.737[a]	0.239
P value	0.001	0.003	0.324
N	16	14	19

Abbreviations: CK14, cytokeratine 14; ER, estrogen receptor; PR, progesterone receptor.
Pairwise correlations of biomarkers were assessed using Spearman's correlation test.
[a]Correlation is significant at the 0.01 level (two-tailed). Correlations were done on available cases excluding all n/a.

Frequent loss of WWOX, ER and PR in mammary tumors from $Wwox^{C3H}+/-$ *mice*

To analyze the status of the WWOX protein expression in mammary tumors developed in $Wwox^{C3H}+/-$ mice, we next performed immunohistochemical analyses using anti-WWOX antibody. WWOX expression was uniformly strong in the cytoplasm of the normal mammary epithelial glands (Figure 1B-f). The intensity of WWOX in ductal carcinoma *in situ* was attenuated as compared with normal glands (Figure 1B-g). In 79% (11/14) of the mammary tumors, there was partial or complete loss of WWOX (Figures 1B-h–j). These results suggest that the second wild-type allele of WWOX might be lost in mammary tumors from $Wwox^{C3H}+/-$ mice suggesting loss of heterozygosity and that inactivation of both *WWOX* alleles might accelerate mammary tumor formation. Further, our data in mice are consistent with reduced levels of WWOX in more advanced stages (G-III) of human breast tumors (Aqeilan *et al*., 2007a).

To better characterize these tumors, we next evaluated the expression of ER and PR using immunohistochemical staining. We found that in most of the poorly differentiated tumors (grade III), expression of ER and PR was lost (Table 1, Figure 1C). By contrast, ER and PR staining was retained in the well-differentiated (grade I) tumors, as in normal mammary glands (Table 1). We have also examined the expression of cytokeratine 14, a basal cell marker, in these tumors and found that most tumors strongly expressed cytokeratine 14 (Figure 1C). In univariate analysis, WWOX loss was more frequent in ER and PR negative basal-like tumors (Table 2). These data suggest that characteristics of mammary tumors in $Wwox^{C3H}+/-$ mice resemble those of human breast cancers where WWOX loss is associated with triple negative basal-like (CK5/6) breast cancers (Guler *et al*., 2009). This group, accounting for 15% of all breast cancers, is often characterized by *BRCA1* mutations. Being a DNA repair protein, BRCA1 was previously shown to be important in loss of fragile genes, such as WWOX (Turner *et al*., 2002). Therefore, it is likely that inactivation of the BRCA1 pathway in basal/triple negative-cancers may contribute to loss of WWOX expression in these cancers.

Depletion of WWOX in MCF7 cells is associated with enhanced survival and anchorage-independent growth

The preceding observations suggest that WWOX loss might enhance mammary tumor cell growth. Therefore, we next investigated whether loss of WWOX expression in ER-positive MCF7 cells enhances its tumorigenic traits. To do so, we generated MCF7 cells containing a stably integrated WWOX-targeting small hairpin RNA expression vector. Immunoblot analysis confirmed that the expression of WWOX was reduced by more than 80% in two cell clones stably transfected with WWOX small hairpin RNA (MCF7shWWOX), compared with that of control small hairpin RNA-transfected cells (Figures 2a and b). We next examined these cells for their *in vitro* cell growth behavior. Colony formation assay demonstrated that WWOX-depleted MCF7 cells grew more colonies as compared with control cells (Figure 2c). Next, we examined whether knockdown of WWOX in MCF7 cells is associated with anchorage-independent cell growth. We found that reduced WWOX expression strongly enhanced growth in soft agar of MCF7 cells (Figure 2d). These results suggest that depletion of WWOX in MCF7 cells is associated with a tumorigenic phenotype.

Knockdown of WWOX in MCF7 cells is associated with reduced ER expression and function

Our results also suggest that WWOX loss might deprive breast cancer cells of attributes, which when lost are associated with high-grade malignancy, that is, loss of ER expression (Figure 1C). To determine whether knockdown of WWOX in MCF7 resembles loss of WWOX in mammary tumors in $Wwox^{C3H}+/-$ mice, we examined if WWOX-depleted MCF7 cells display loss of ER expression. We found that levels of ER were significantly downregulated in WWOX-depleted MCF7 cells compared with MCF7-EV cells as assessed by western blot and real-time PCR analyses (Figures 2a and e). To determine the functional relevance of this downregulation, we examined the effect of Tamoxifen (Tam) and 17β-estradiol (E_2) treatments on the different cell clones. Tam acts primarily through ER by modulation of gene expression leading to inhibition of proliferation of breast cancer cells (Radmacher and Simon, 2000). To examine the sensitivity of WWOX-depleted MCF7 cells to Tam treatment and E_2, cells were incubated in serum-free RPMI-1640 medium containing 10^{-6} Tam or 10^{-9} E_2 and cell proliferation was determined. We found that although Tam treatment attenuated control cell growth, WWOX-depleted MCF7 cells were significantly less sensitive (Figure 2f). In the presence of E_2, WWOX-depleted MCF7 cells showed reduced proliferation as compared with control MCF7 (Figure 2g). The modest differences in response to either Tam or E_2 could be attributed to the fact that WWOX knockdown leads to downregulation and not to total depletion of ER. Nevertheless, our findings suggest that knockdown of WWOX levels in MCF7 ER-positive cells is associated with tumorigenic traits and that WWOX loss in breast cancer may affect ER signaling

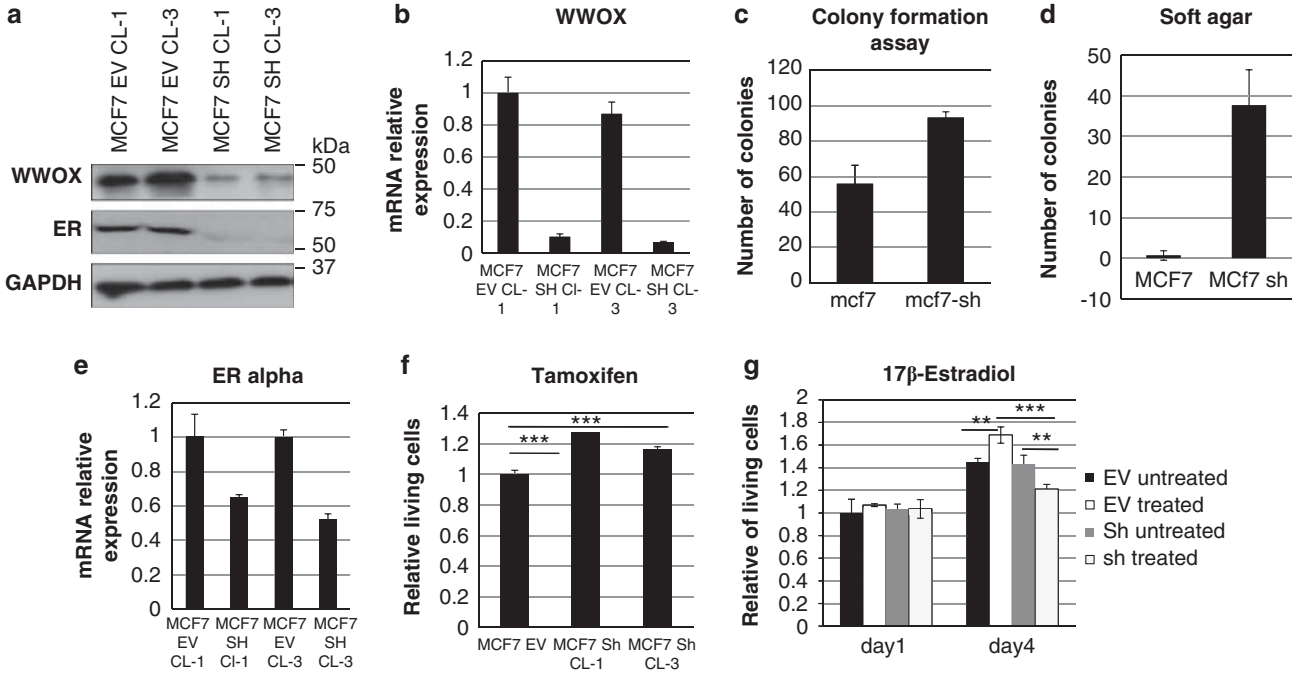

Figure 2 Phenotypes of WWOX depletion in MCF7 cells. (**a**) Establishment of MCF7 stable clones depleted of WWOX using lentiviral vector carrying small hairpin RNA against *WWOX* sequence. As control, empty vector (EV) was used. Cells were lysed using 0.5% NP40, 50 mM Tris–HCL (pH7.5), 150 mM NaCl, 0.5 mM EDTA, 10% glycerol and 1 × protease inhibitor (Calbiochem, Gibbstown, NJ, USA). Western blot analysis of WWOX expression (using goat polyclonal anti-WWOX antibody, 1:1000, sc-20529) in MCF7-shWWOX and -EV cells showing knockdown of >80% of WWOX expression in two clones is shown (CL1 and 3) (upper panel). Middle panel shows expression of ER protein in WWOX-depleted MCF7 cells using anti-ER (H184) antibody (sc-7207). GAPDH served as loading control. (**b**) *WWOX* mRNA relative expression in WWOX-depleted MCF7 cells as determined by real-time reverse transcriptase (RT–PCR) analysis. Total RNA was isolated from the different clones followed by homogenizing in TRI-reagent (Sigma, St Louis, MO, USA)) according to the manufacturer's protocol. The cDNA was synthesized with oligo(dT) primers using the Super-Script first strand synthesis kit (Applied Biosystems (ABI), Foster City, CA, USA) according to the manufacturer's protocol. Gene expression was assessed by quantitative real time PCR (ABI) using primers listed in Supplementary Table 3. *UBC* cDNA served as internal control. *Columns*, mean of triplicate samples from two independent experiments; *bars*, s.d. (**c**) Effect of WWOX depletion on survival, as assessed by colony formation assay. MCF7 cells were plated at a density 5×10^2/well in a six-well plate in triplicate. After 10 days, cells were fixed with 70% ethanol, stained with Giemsa and colonies were counted. *Columns*, mean of triplicates samples; *bars*, s.e.m., ($P<0.001$). (**d**) Effect of WWOX depletion on anchorage-independent growth, as assessed by soft agar assay. Cells were plated between two layers of agarose. The agar base was prepared by melting agar 1% in water at 42 °C and mixed with an equal volume of RPMI 2 × containing 20% fetal calf serum. The plates were kept at 4 °C for at least 30 min. The top layer was prepared by melting agarose 0.7% in water that was mixed with an equal volume of RPMI 2 × and 5000 cells/well in triplicate. This solution was then gently poured on the base layer. The plates were incubated at 37 °C for 14–21 days. Colonies were detected by crystal violet staining and then counted. *Columns*, mean of triplicates samples; *bars*, s.e.m. ($P<0.001$). (**e**) *ESR1* mRNA relative expression in WWOX-depleted MCF7 cells was determined by real-time RT–PCR analysis as in **b**. (**f**, **g**) Effect of Tamoxifen (**f**) and 17β-estradiol (**g**) treatment on MCF7shWWOX growth rate. MCF7 cells were grown for 2 days without E_2 and then incubated in serum-free medium containing ethanol (0.2%), 10^{-6} Tam or 10^{-9} E_2. Proliferating cells were analyzed at the indicated time points using an XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate) proliferation assay according to the manufacturers instruction (Biological Industries, Beit Haemek, Israel). WWOX-depleted cells were less responsive to Tam and E2 treatment in the different clones. Relative living cells ratio represents the ratio between the optical density obtained from treated cells to that obtained from untreated cells. ** represents $P<0.01$ and *** represents $P<0.001$ as analyzed by two tailed Student's *t*-test.

contributing to mammary tumorigenesis. The molecular mechanism underlying the tumor suppressor function of WWOX in breast cancer cells remains to be fully elucidated. Whether WWOX affects ER and PR transactivation function is unknown. Recently, it was reported that the WW domain-binding protein 2 is essential for proper activation of PR and ER (Dhananjayan *et al*., 2006). Intriguingly, WW domain-binding protein 2 contains a PPxY motif that associates with WW domain protein YAP (Sudol *et al*., 1995), leading to ER and PR transactivation. As WWOX is known to interact with PPxY-containing proteins as well (Salah *et al*., 2010), it is possible that WWOX may associate with WW domain-binding protein 2 and regulate ER/PR signaling. Further analysis of WWOX-ER/PR association in breast cancer would be required to better explore WWOX molecular function.

mRNAs profiling using Affymetrix analysis

To further determine the molecular and cellular functions of tumor suppressor WWOX in mammary tumor development, we studied the differential expression of mRNAs in invasive ductal carcinomas tumors developed in $Wwox^{C3H}+/-$ mice compared with control mammary epithelial cells. The mRNAs were analyzed using mouse

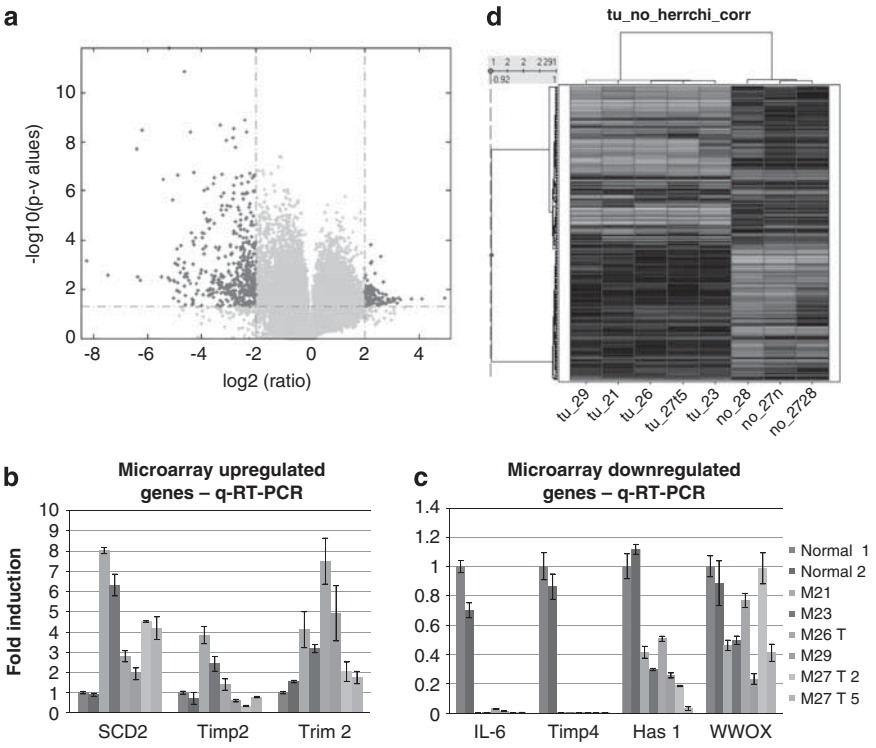

Figure 3 Affymetrix analysis of mammary tumors from *Wwox*-heterozygous mice. Total RNAs were isolated from five mammary tumors from five $Wwox^{C3H}+/-$ mice and three normal mammary epithelial glands from three control $Wwox^{C3H}+/-$ littermate mice. RNAs were hybridized with Affymetrix mouse gene-chip 430 2.0 arrays. (**a**) Volcano plot analysis of the differentially expressed genes (log 2 scale) versus the *P* values of the genes (−log 10 scale). The quality assurance, calibration, data normalization, principal component analysis, and volcano plot for the Affymetrix cell format files were performed by Partek Genomics Suite 6.5 (Partek Genomics, St Louis, MO, USA). Genes with a fourfold expression difference and cutoff *P*-values <0.001 were defined significant. The dots at the left upper side of the plot represent the statistically significant downregulated genes in tumors, whereas those at the right upper side represent the genes that were upregulated in tumors. (**b**, **c**) Real-time PCR validation of six deregulated genes in six mammary tumors and two normal mammary epithelial cells. Total RNA was isolated using Tri-reagent (Sigma). RNA (1 μg) was reversed transcribed using the iScript cDNA synthesis kit (Applied Biosystems). Real-time PCR was done using SYBR Green PCR Master Mix (Applied Biosystems). *Ubc* was used as control. *Columns*, mean of triplicates samples; *bars*, s.d. Supplementary Table 4 shows list of primer sequence used for real-time PCR. (**d**) Heat map of commonly deregulated genes in Wwox +/− mammary tumors relative to normal mammary tissues. Negative fold change (transcripts with decreased expression in tumors) is represented in *green* and positive fold change (transcripts with increased expression in tumors) is represented in *red*.

Affymetrix microarray gene-chip 430 2.0 arrays (Affymetrix, Santa Clara, CA, USA). The analysis revealed numerous genes that exhibited modified expression levels in the mammary tumors (Supplementary Table 1). Principal component analysis of all samples revealed that this gene set effectively separates tumors from normal epithelial cells (Supplementary Figure 1). To challenge the statistical significance of the microarray results, we performed volcano analysis, using a threshold of 4-fold in gene expression with cutoff *P*-value <0.001 (Figure 3a). We identified 163 significantly downregulated and 129 significantly upregulated genes in mammary tumors (Supplementary Table 2). Examples of upregulated transcripts in tumors include *Sox4*, *Akt1*, *Scd2* and *Timp2* whereas *Cxcl1*, *Il6* and *Has1* were downregulated. To validate some of the most significant observations, we performed quantitative reverse transcriptase PCR of various relevant transcripts. We confirmed the increase in expression of several genes in mouse mammary tumors, including *Scd2*, *Timp2* and *Trim2* (Figure 3b). Similarly, a significant decrease in expression of several transcripts including *Il-6*, *Timp4*, *Has1* and *Wwox* was confirmed in $Wwox^{C3H}+/-$ mammary tumors (Figure 3c). Although in some tumors we observed a mixed pattern, likely due to contamination with stromal cells, these data suggest that WWOX loss is associated with differential expression of tumor-related genes.

Hierarchical unsupervised clustering of gene expression revealed the presence of 4 major clusters (Figure 3d). Of these, two clusters included genes that were strongly upregulated or downregulated in tumors (tu) as compared with normal samples (no) (Figure 3d). In order to better understand the biological function of these genes, we used the Ingenuity (IPA) system algorithms. We found multiple pathways that are altered in tumors including cellular movement, cell-to-cell signaling and interaction, cellular development, cellular growth and proliferation and cell death (Supplementary Table 3). Interestingly, several differentially expressed genes identified in tumors of $Wwox^{C3H}+/-$ mice are well documented in human breast cancers and in other

mammary tumor mouse models (Hu *et al*., 2004; Klein *et al*., 2007).

In conclusion, we propose that loss of WWOX expression in mouse mammary tissues is associated with significant genetic and molecular changes that in turn contribute to mammary tumor formation. These tumors were mostly ER/PR-negative, a feature characterizing aggressive tumors and those with worse outcome, resembling loss of WWOX in human breast cancers. Our findings may suggest that $Wwox^{C3H}+/-$ mice might be used as a tool to study inactivation of WWOX in human breast cancer.

Conflict of interest

The authors declare no conflict of interest.

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