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# DHHC-7 and -21 are palmitoyltransferases for sex steroid receptors

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**ABSTRACT** Classical estrogen, progesterone, and androgen receptors (ERs, PRs, and ARs) localize outside the nucleus at the plasma membrane of target cells. From the membrane, the receptors signal to activate kinase cascades that are essential for the modulation of transcription and nongenomic functions in many target cells. ER, PR, and AR trafficking to the membrane requires receptor palmitoylation by palmitoyltransferase (PAT) protein(s). However, the identity of the steroid receptor PAT(s) is unknown. We identified the DHHC-7 and -21 proteins as conserved PATs for the sex steroid receptors. From DHHC-7 and -21 knockdown studies, the PATs are required for endogenous ER, PR, and AR palmitoylation, membrane trafficking, and rapid signal transduction in cancer cells. Thus the DHHC-7 and -21 proteins are novel targets to selectively inhibit membrane sex steroid receptor localization and function.

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## INTRODUCTION

Sex steroids act through their classical receptors in the nucleus to regulate the genes that mediate many actions of these hormones in target cells (O'Malley *et al.*, 1970; Payvar *et al.*, 1981). However, it was demonstrated ~40 yr ago that sex steroids, such as estrogen, signal in seconds to generate calcium flux and cyclic AMP in vitro and in vivo; this signaling occurs through plasma membrane (PM)-localized binding sites (Szego and Davis, 1967; Pietras and Szego, 1977). More recently, it was shown that activation of kinase cascade(s) primarily originates from signaling by PM-localized pools of endogenous classical sex steroid receptors, such as estrogen receptor  $\alpha$  (ER $\alpha$ ) (Razandi *et al.*, 1999; Pedram *et al.*, 2006). There are many

mechanisms by which rapid signaling from PM-localized receptors affects gene transcription, which is modulated by the same sex steroid receptors in the nucleus (Vasudevan *et al.*, 2001; Bjornstrom and Sjoberg, 2005; Harrington *et al.*, 2006; Vicent *et al.*, 2006; Bredfeldt *et al.*, 2010; Guillermo *et al.*, 2010). In addition, rapid signaling by PM-localized steroid receptors affects nongenomic functions, including those that prevent osteoporosis and acute vascular injury in rodent models (Kousteni *et al.*, 2001; Chambliss *et al.*, 2010), rescue neurons from in vivo ischemic insult (Suzuki *et al.*, 2009), and stimulate oocyte meiosis (Finidori-Lepicard *et al.*, 1981). Thus it is important to understand the crucial steps required for sex steroid receptor trafficking to the PM.

Many integral membrane proteins are posttranslationally modified by lipids that promote membrane localization (Mundy, 1995). These lipid modifications include acylation, palmitoylation, and myristylation. Previous studies showed that S-palmitoylation occurs on a conserved cysteine as part of a nine-amino acid motif in the ligand binding domains of all sex steroid receptors (Pedram *et al.*, 2007). Palmitoylation promotes the physical interaction of ER with the caveolin-1 protein (Acconcia *et al.*, 2004), and caveolin-1 transports ER from cytoplasm to caveolae rafts in the PM (Razandi *et al.*, 2003a). At the caveolae, ER activates small G protein  $\alpha$ - and  $\beta\gamma$ -subunit signaling in seconds, generating additional rapid signals (Razandi *et al.*, 1999; Kumar *et al.*, 2007). Similar signaling has been shown for membrane-localized progesterone receptors (PRs; Faivre *et al.*, 2005) and androgen receptors (ARs; Gill and Hammes, 2007). Some of these studies indicate rapid ER signaling from the membrane of breast cancer cells promotes cell proliferation, migration, and survival (Levin and Pietras, 2008). Similarly, rapid signaling from membrane ARs to Src activation in prostate cancer cells stimulates cell proliferation (Migliaccio *et al.*, 2000).

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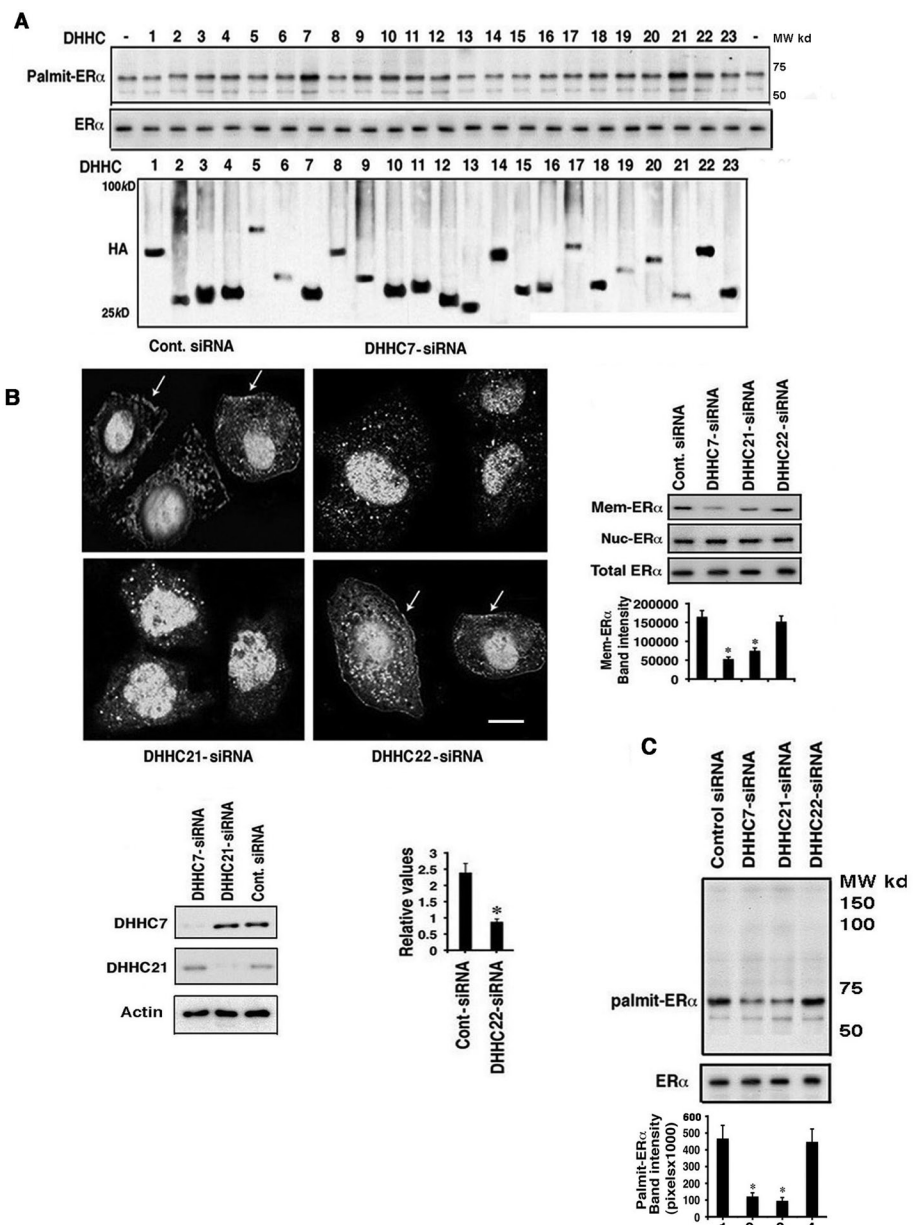
A.P. and M.R. performed the research; R.J.D. and E.L. designed the research and analyzed the data.

The authors report no conflicts of interest and have nothing to disclose.

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Abbreviations used: ANOVA, analysis of variance; AR, androgen receptor; DHHC, Asp-His-His-Cys; DHT, dihydrotestosterone; E, ligand binding; E2, 17- $\beta$ -estradiol; EGF, epidermal growth factor; ER, estrogen receptor; ERE, estrogen-response element; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; GPCR, G protein-coupled receptors; HA, hemagglutinin; NLS, nuclear localization signal; P, progesterone acetate; PAT, palmitoyltransferase; PI3K, phosphatidylinositol 3-kinase; PM, plasma membrane; PMSF, phenylmethylsulfonyl fluoride; PR, progesterone receptor; qRT-PCR, quantitative RT-PCR; RT-PCR, real-time PCR; siRNA, small interfering RNA; WT, wild-type.

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**FIGURE 1:** Identification of ER PATs. (A) Transfection of MCF-7 cells was carried out with each of 23 separate plasmids from a cDNA library in a separate well of cells, which was followed by [ $^3$ H]palmitic acid labeling, immunoprecipitation of endogenous ER $\alpha$  from cell lysates, separation by SDS-PAGE, and fluorography. Immunoblots of ER total protein and HA-tagged DHHC PAT proteins are also shown in this representative experiment ( $n = 3$  experiments). (B) Distribution of ER $\alpha$  in MCF-7 cells. Top, cells were transfected with the various siRNAs and analyzed by immunofluorescence microscopy. Arrows indicate membrane ER $\alpha$ . Bar graph represents the mean  $\pm$  SEM densities of ER $\alpha$  blots in membrane or nuclear cell fractions from three experiments, density-analyzed by ANOVA plus Scheffe's test. \*,  $p < 0.05$  for control vs. DHHC siRNA. Bottom, validation of the siRNA for endogenous DHHC PATs proteins and RT-PCR for the DHHC-22 transcript. (C) DHHC-7 or -21 siRNA(s) significantly reduces [ $^3$ H]palmitate incorporation into endogenous ER $\alpha$  in MCF-7 cells. Bar graph shows combined data from three experiments. \*,  $p < 0.05$  for control siRNA vs. DHHC-7 or -21 siRNA.

Identification of the enzyme(s) that causes this posttranslational modification of the sex steroid receptors is needed to understand the dynamics of receptor palmitoylation and trafficking. Fukata and colleagues identified 23 mammalian proteins as palmitoyltransferases (PATs), and all contain an Asp-His-His-Cys (DHHC) amino acid signature sequence (Fukata *et al.*, 2004; Hou *et al.*, 2009). In

this paper, we report that DHHC-7 and -21 proteins serve as PATs for ER, PR, and AR, driving membrane localization and rapid signaling by these receptors to important functions in hormone-responsive cancer cells.

## RESULTS

### DHHC-7 and -21 are ER PATs

To identify potential PATs for ER $\alpha$ , we expressed single plasmids coding for each of the known 23 mammalian DHHC proteins in MCF-7 breast cancer cells. Only expression of DHHC-7 or -21 significantly enhanced endogenous ER $\alpha$  palmitoylation (Figure 1A). All DHHC proteins were detected using hemagglutinin (HA) antibody, indicating that the unbiased screen probably detected all candidate PATs.

We then used small interfering RNAs (siRNAs) to candidate DHHC PATs endogenously expressed in MCF-7 cells to further support ER $\alpha$  as substrate. Protein knockdown of DHHC-7 and -21 was specific (Figure 1B, left), and DHHC-22 transcripts were determined, since no commercial antibody is available. Endogenous ER $\alpha$ 's at the PM in MCF-7 cells were only decreased from DHHC-7 or -21 knockdowns (Figure 1B, right). We counted 200 cells in each condition and found PM ER $\alpha$  in 92% of the control siRNA-transfected cells (white arrows). Membrane localization of ER was reduced to 24 and 33%, respectively, from DHHC-7 or -21 knockdown, but was insignificantly affected by DHHC-22 knockdown (85%; Table 1). The selective effects for PM localization were confirmed by Western blotting (Figure 1B, far right), also indicating that localization of nuclear ER $\alpha$  was unaffected. The latter result reflects the fact that nuclear ER $\alpha$  is not palmitoylated (Pedram *et al.*, 2007). Knockdown of either DHHC-7 or -21 (but not DHHC-22) significantly decreased endogenous ER $\alpha$  palmitoylation (Figure 1C), and the residual acylation probably reflects incomplete knockdown and redundancy of the function of the two DHHC proteins.

Rapid signaling by 17- $\beta$ -estradiol (E2) occurs through PM and not nuclear ER (Pedram *et al.*, 2006, 2009b). We therefore determined the impact of the PATs on these functions. Only the knockdown of DHHC-7 or -21 significantly impaired the ability of E2 to stimulate extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/AKT kinases (Figure 2A), and to generate cAMP (Figure 2B). In contrast, knockdown of DHHC-22 did not affect E2 activation of ERK and PI3K/AKT (Figure 2C). Importantly, reduction of either putative ER PAT significantly, but incompletely, impaired E2 signaling that enhanced cell proliferation/viability, as shown by the MTT assay (Figure 2D). This finding is consistent with previous work that showed that

both nuclear and PM ER pools contribute to breast cancer cell proliferation (Razandi *et al.*, 2003a). Interestingly, knockdown of both DHHC-7 and -21 was not significantly more potent than the individual PAT protein knockdowns in inhibiting rapid signaling by E2 (Figure 2A). This finding may be due to cooperation between DHHC-7 and -21 proteins to palmitoylate ER, and the knockdown of one protein significantly disrupted the steroid receptor localization and function.

We also determined whether DHHC-7 or -21 influenced epidermal growth factor (EGF) signaling, since transactivation of the EGF receptor is required for membrane ER signaling in hormone-responsive cancers (Razandi *et al.*, 2003b). EGF stimulated ERK and PI3K/AKT activity and increased the viability of MCF-7 cells. These actions were unaffected by DHHC knockdown, indicating specificity of the PATs for ER (Figure 2E). The results also suggest that EGF receptor signaling does not require cross-talk to membrane-localized ER $\alpha$ .

### Structure–function interactions and localization of DHHC proteins and ER $\alpha$

We previously determined that a nine–amino acid palmitoylation motif in the ligand-binding (E) domains of all sex steroid receptors is important for the acylation that drives the receptors to the PM (Pedram *et al.*, 2007). The cysteine at amino acid 451 is the site of palmitic acid attachment to ER $\alpha$ . We therefore expressed wild-type (WT) or a C451A mutant ER $\alpha$  and DHHC-7 or DHHC-21 in ER-null CHO cells and determined protein interactions. Expressed DHHC-7 and -21 proteins each strongly associated with WT ER $\alpha$  but failed to significantly interact with C451A ER $\alpha$  (Figure 3A). The results indicate that an intact palmitoylation site is required for PAT binding to ER $\alpha$ . Importantly, only WT, but not C451A ER $\alpha$ , was robustly palmitoylated upon DHHC-7 or -21 expression (Figure 3B). In contrast, DHHC-22 expression did not stimulate ER palmitoylation beyond the basal palmitoylation seen from endogenous PAT(s) (Figure 3B, lanes 1 and 7). We conclude that the PATs promote palmitoylation of ER $\alpha$  specifically at cysteine 451, consistent with the inability of C451A ER $\alpha$  to traffic to the PM (Pedram *et al.*, 2007).

Palmitoylation of ER $\alpha$  appears to occur by a reversible thioester linkage of palmitic acid at the internal C451 site (Pedram *et al.*, 2007). We examined this by expressing WT ER $\alpha$  in CHO cells with DHHC control vector or DHHC-7 or -21 protein-encoding plasmids. Palmitoylation was determined in the absence or presence of hydroxylamine, which significantly cleaves thioester bonding of fatty acyl groups (Drisdell *et al.*, 2006; Pedram *et al.*, 2007). Hydroxylamine inhibited both basal ER $\alpha$  palmitoylation that resulted from the endogenous PATs and the additional palmitoylation promoted by expressing DHHC-7 or -21 (Figure 3C).

Membrane-bound, but not nuclear, ER $\alpha$  is palmitoylated. Palmitoylation likely occurs at the Golgi (Pedram *et al.*, 2007), where many proteins are palmitoylated by DHHC proteins (Fukata and Fukata, 2010). We determined where endogenous DHHC-7 and -21 proteins colocalize with ER. In MCF-7 cells, ER $\alpha$  is densely localized to the nucleus and is seen at extranuclear sites (Figure 3D). DHHC-7 and -21 proteins were found to be exclusively extranuclear and colocalized with some ER $\alpha$  in cytoplasm. We also determined that endogenous DHHC-7 and -21 colocalize with a Golgi marker (Figure 2E, top panels) and that some ER $\alpha$  also localizes to the Golgi (Figure 3E, bottom panels). These results support the idea that the ER $\alpha$ –PAT protein interaction (Figure 3A) occurs in cytoplasm (Figure 3D), at least in part at the Golgi organelle (Figure 3E).

### Signaling by membrane ER affects epigenetic transcription

Palmitoylation and the resulting trafficking of ER $\alpha$  are relevant only for the membrane-localized pool (Pedram *et al.*, 2006, 2009b). Thus

| Condition     | Cells showing receptor (%) |                         |
|---------------|----------------------------|-------------------------|
|               | Nucleus                    | Plasma membrane         |
| Control siRNA | 100 $\pm$ 2                | 92 $\pm$ 7              |
| DHHC-7 siRNA  | 100 $\pm$ 3                | 24 $\pm$ 3 <sup>a</sup> |
| DHHC-21 siRNA | 100 $\pm$ 1                | 33 $\pm$ 2 <sup>a</sup> |
| DHHC-22 siRNA | 100 $\pm$ 3                | 86 $\pm$ 4              |

Data are mean  $\pm$  SEM from three experiments, derived from counting 200 MCF-7 cells per condition in each of the separate experiments.

<sup>a</sup>  $p < 0.05$  by ANOVA plus Scheffe's test for nucleus vs. plasma membrane.

TABLE 1: Localization of ER $\alpha$  in MCF-7 cells.

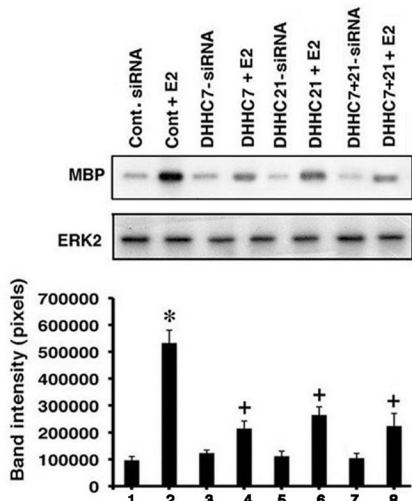
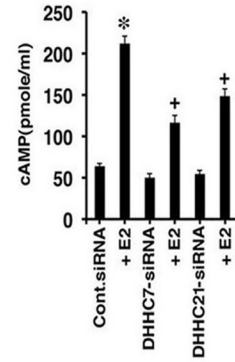
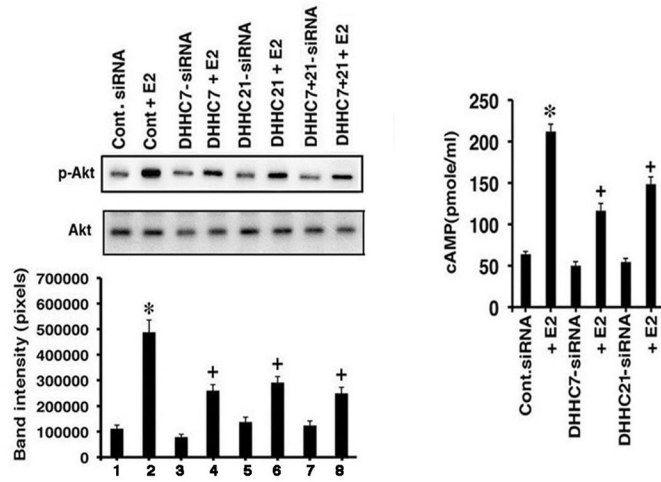
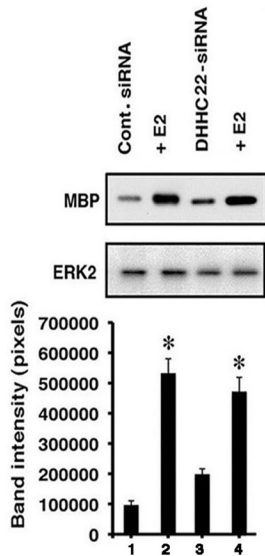
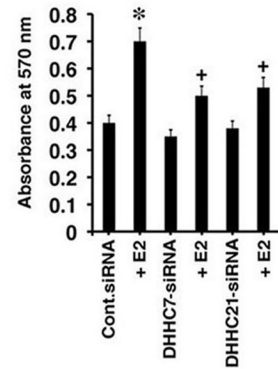
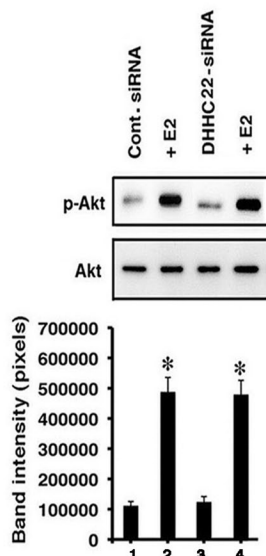
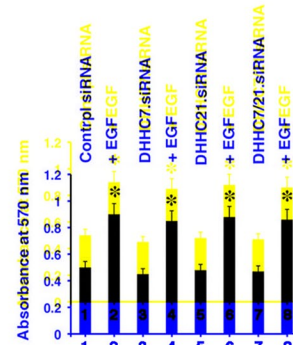
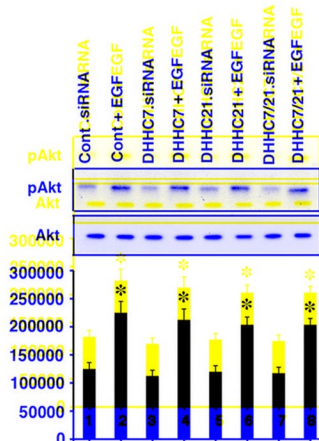
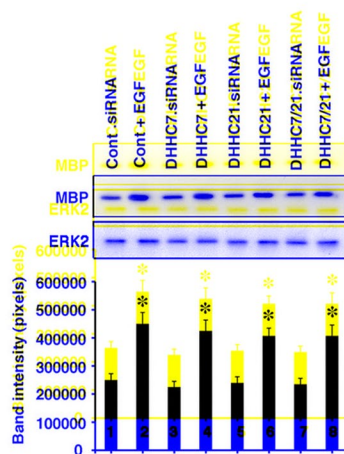
the loss of palmitoylation should not significantly affect the transcription of genes regulated by nuclear ER $\alpha$  binding to promoter/enhancer estrogen-response elements (EREs). To test this, we knocked down DHHC-7 or -21 and determined endogenous pS2 gene abundance in MCF-7 cells. pS2 transcription is ERE-mediated (Harrington *et al.*, 2006), and E2 comparably stimulated endogenous gene expression under control or DHHC-specific siRNA expression conditions (Figure 4A). Thus E2 modulation of the pS2 gene does not require membrane ER $\alpha$  signaling.

In contrast, ERK activation by membrane PR or ER collaborates with nuclear-localized sex steroid receptors to enhance transcription of some genes (Madak-Erdogan *et al.*, 2008; Subtil-Rodriguez *et al.*, 2008). Interestingly, several estrogenic compounds stimulate PR gene expression, in part by signaling through AKT to phosphorylate Ser-21 of EZH2. Phosphorylation of EZH2 at this site inhibits the histone trimethyltransferase activity of this protein (Bredfeldt *et al.*, 2010). EZH2 is the primary histone 3 trimethyltransferase for Lys-27 (repressive mark; Margueron *et al.*, 2008), and the PR gene is epigenetically derepressed as a result of estrogen signaling (Bredfeldt *et al.*, 2010). AKT signaling by ER in the study by Bredfeldt *et al.* presumably occurred through PM-localized receptors, as we have shown in MCF-7 cells (Pedram *et al.*, 2009a).

To investigate the impact of DHHC proteins on this important function, we determined that E2-stimulated AKT activity was diminished from DHHC knockdown or by a soluble inhibitor of PI3K, LY 290042 (Pedram *et al.*, 2009a; Figure 4B, top panel). Inhibition of E2-induced AKT activity by either approach prevented sex steroid-induced phosphorylation of EZH2 at the inhibitory site (Ser-21) (middle panel). E2 also inhibited the H3K27Me3 (trimethylation) repressive mark (bottom panel), which was reversed by DHHC protein knockdown or LY290042. This signaling pathway importantly contributed to E2-stimulated endogenous PR gene expression, since either inhibiting the DHHC proteins or blocking AKT activation significantly reversed this steroid effect (Figure 4C). In this way, DHHC-induced trafficking of ER to the PM results in rapid signaling that induces epigenetic modulation of gene expression.

### DHHC-7 and -21 are PATs for PRs and ARs

All sex steroid receptors must be palmitoylated for localization to the PM (Pedram *et al.*, 2007). We therefore sought to identify the PATs that promote PR and AR trafficking to and functioning at the PM in hormone-responsive cancer cells. Expression in MCF-7 cells of the individual DHHC-encoding plasmids from a cDNA library is seen in Figure 1A, an experiment that allowed us to also probe the endogenous PR palmitoylation shown here. Only expression of DHHC-7 and -21 clearly promoted the enhanced palmitoylation of endogenous PRB (Figure 5A). We previously showed that both PRA

**A****B****C****D****E**

and PRB are found at the PM of MCF-7 cells (Pedram *et al.*, 2007), but PRB is dominant. Therefore we used an antibody that only identifies PRB. Knockdown of DHHC-7 and -21 in the MCF-7 cells significantly prevented endogenous PR palmitoylation (Figure 5B) and PM localization (Figure 5C). As a result, progesterone signaling through ERK and PI3K was inhibited, and the enhanced cell viability/proliferation seen in response to the steroid ligand decreased significantly (Figure 6, A and B).

Similarly, expression of the individual plasmids in C4-2 prostate cancer cells resulted in enhanced palmitoylation of endogenous AR only by the same PATs for palmitoylating ER and PR (Figure 7A). Knockdown of DHHC-7 or -21 with siRNA diminished AR localization only at the PM (Figure 7B) and diminished AR palmitoylation (Figure 7C). PAT knockdown inhibited dihydrotestosterone (DHT) signaling to ERK and PI3K/AKT (Figure 8A) and decreased cell proliferation and viability (Figure 8B). These latter results indicate that membrane AR signaling in prostate cancer cells contributes to the hormone-induced biology. Thus the DHHC-7 and -21 proteins are conserved as PATs for all classes of sex steroid receptors.

## DISCUSSION

Ongoing research continues to reveal the important roles of extranuclear steroid receptors (Chambliss *et al.*, 2010; Wong *et al.*, 2010; Levin, 2011). Rapid signaling from PM-localized ER, PR, and AR affects genomic and nongenomic functions (Faivre *et al.*, 2005), for example, in hormone-responsive cancers (Migliaccio *et al.*, 2000; Hammes and Levin, 2007). Therefore understanding receptor trafficking creates targets to use for selective interference with rapid signaling by sex steroids.

Trafficking of all sex steroid receptors to the PM requires palmitoylation at a highly conserved nine-amino acid motif within the ligand-binding/E domain (Acconcia *et al.*, 2004; Pedram *et al.*, 2007). Palmitoylation promotes the physical association of ER with caveolin-1, thus facilitating ER translocation to the PM (Acconcia *et al.*, 2004; Pedram *et al.*, 2007). Endogenous ER is highly enriched in PM caveolae rafts (Chambliss *et al.*, 2002; Razandi *et al.*, 2002), in which caveolin-1 is a coat protein that scaffolds a localized signaling complex to interact with sex steroid receptors. Interactions of signaling molecules in proximity to membrane ER results in the generation of calcium flux, cyclic nucleotides, and kinase(s) activation (Pedram *et al.*, 2006; Hammes and Levin, 2007). Interestingly, many classical G protein-coupled receptors (GPCRs), including dopaminergic and adrenergic receptors, contain a very similar nine-amino acid sequence (F(X)<sub>6</sub>LL, where "X" is any amino acid) found in conserved intracellular loops (Duvernoy *et al.*, 2006). Although this sequence contributes to the membrane localization of these receptors, there is neither a cysteine in this motif nor does palmitoylation occur on classical GPCRs, so it is unclear how this sequence promotes trafficking to the membrane.

A key to understanding sex steroid receptor PM trafficking is identification of the PAT(s) that cause receptor acylation. In this study, we identify the DHHC-7 and -21 proteins as PATs for ER, PR,

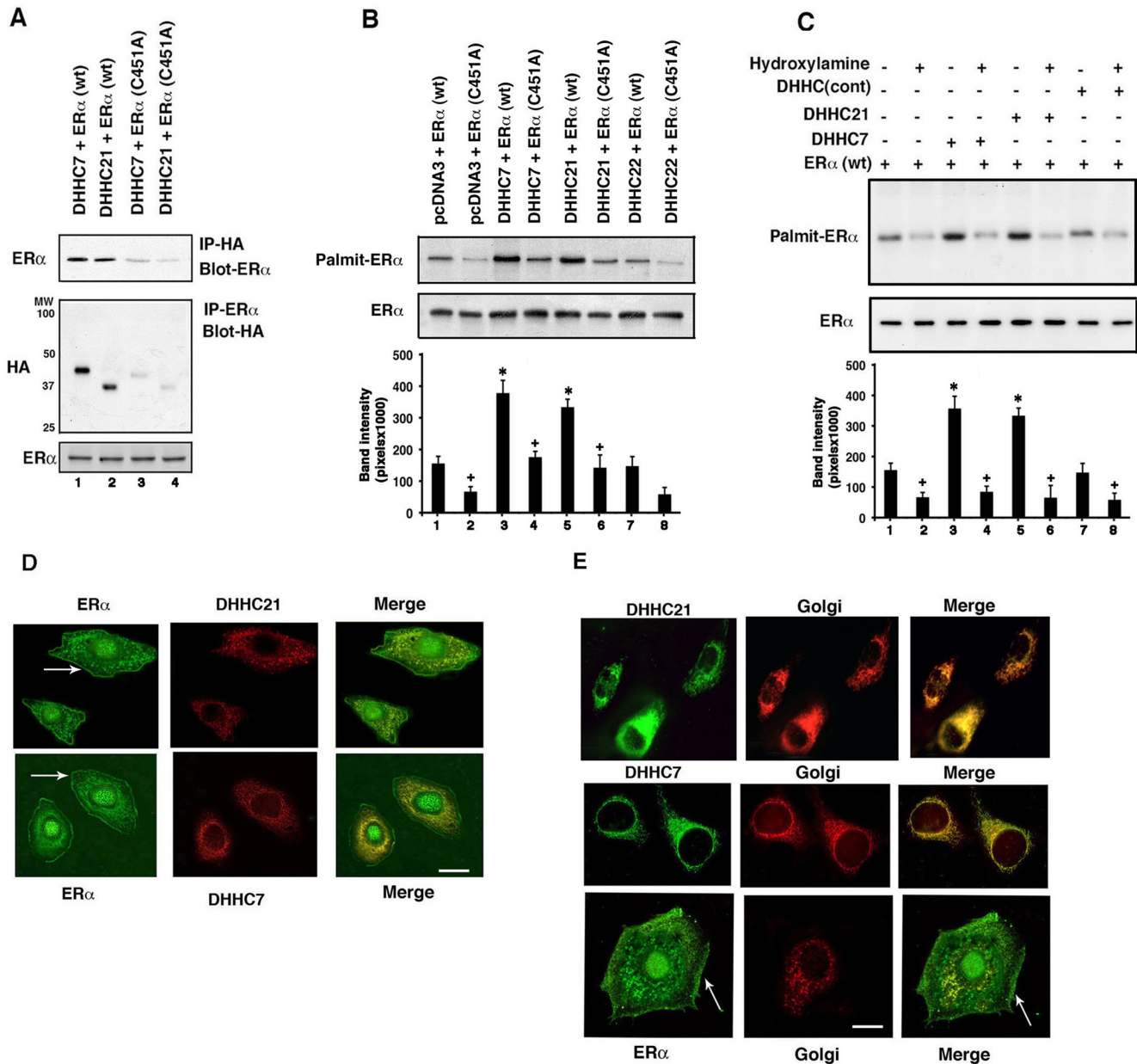
and AR. Selective knockdown of endogenous DHHC-7 and -21 prevents PM localization and rapid signaling, and impairs both nongenomic and genomic functions, including epigenetic up-regulation of the PR gene by ER. These findings indicate that DHHC PATs for ER may be potential targets to limit the epigenetic effects of xenoestrogens (Bredfeldt *et al.*, 2010), environmental sex steroid mimics/disrupters (Portier, 2002), or endogenous sex steroid actions in hormone-responsive cancers. Up-regulation of PR by ER significantly promotes mammary cancer in mice (Pool *et al.*, 2006) and breast cancer from sex hormone replacement after menopause (Rossouw *et al.*, 2009). We suggest that additional rapid signaling by steroid receptors at the PM may affect other epigenetic functions.

The physical complex formed between the DHHC PATs and the palmitoylation motif of ER $\alpha$  creates a functional interaction. Because mutation of the cysteine amino acid at 451 of ER $\alpha$  prevents the association with PATs, the PATs cannot palmitoylate the receptor. As a result, the S451A ER $\alpha$  protein fails to significantly traffic to the PM (Pedram *et al.*, 2007) and does not significantly activate multiple rapid signals. We report that the identified, endogenous PATs colocalize with ER $\alpha$  in cytoplasm, especially in the Golgi. Based upon our previous studies (Pedram *et al.*, 2007) and the current data, we suggest that receptor palmitoylation takes place at this organelle, a common site for plasma membrane-bound protein palmitoylation (Fukata and Fukata, 2010). This idea is supported by the finding that palmitoylation of ER prior to its transport to the PM caveolae is required for its cytoplasmic interaction with caveolin-1 (Acconcia *et al.*, 2004; Pedram *et al.*, 2007).

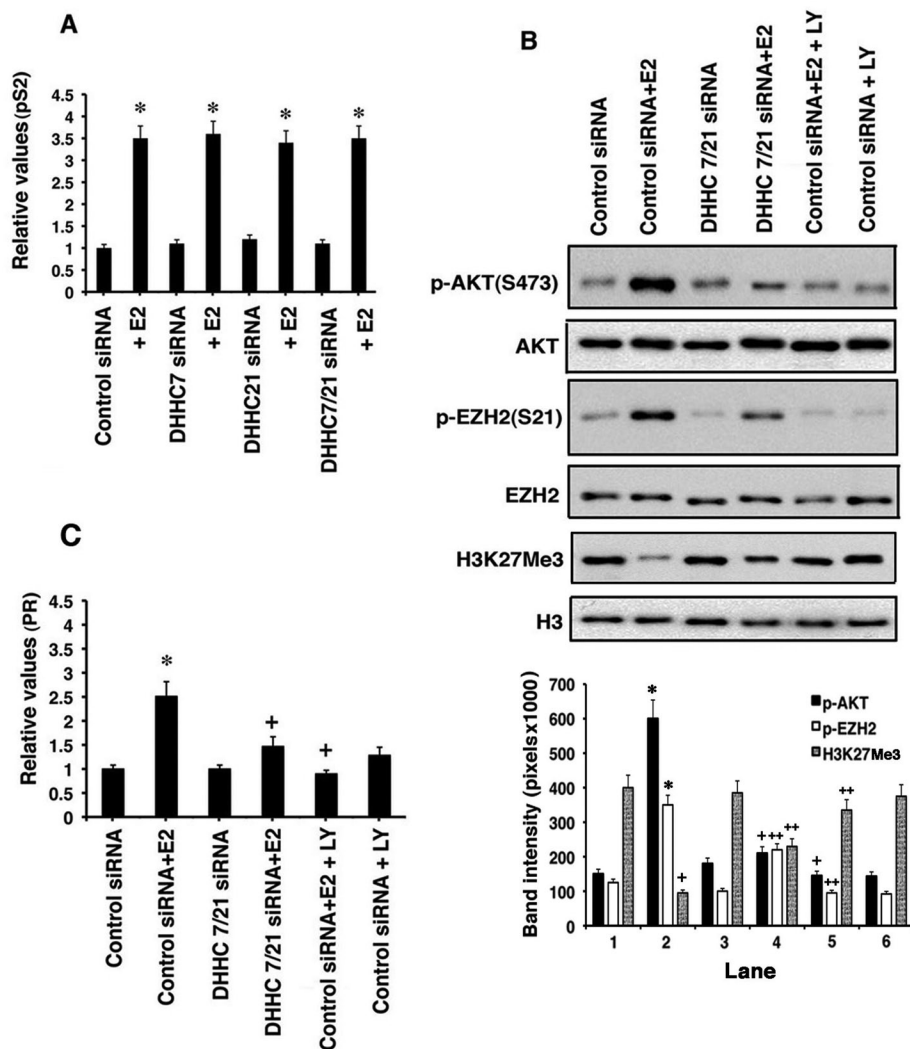
Based on our results, we propose a conserved mechanism of trafficking for all sex steroid receptors. Interestingly, both identified PATs contribute to receptor palmitoylation, suggesting redundancy of function. This is not unusual, since some proteins undergo palmitoylation by several PATs, while individual PATs acylate multiple substrates (Fukata *et al.*, 2004). DHHC-7 palmitoylates the SNAP25 protein that is essential for neuronal exocytosis (Greaves *et al.*, 2010), and both DHHC-7 and -21 PATs palmitoylate the membrane endothelial nitric oxide synthase enzyme that produces nitric oxide in the vasculature (Fernandez-Hernando *et al.*, 2006). Also, cooperation between two PATs to palmitoylate a single substrate site has been demonstrated but is poorly understood (Fukata *et al.*, 2004). This may be applicable to sex steroid receptor palmitoylation.

Receptor localization to discrete sites of the cell is critical for proper function, and redundancy of PATs may ensure proper trafficking. In epithelial cells, mislocalization of tumor suppressor or oncogenic proteins underlies the development of malignancy (Calnan and Brunet, 2008; Jones, 2008; Lee and Kim, 2009; Neufeld, 2009). Aggressive breast cancer or breast cancer that is resistant to endocrine therapy is sometimes associated with significantly increased ER $\alpha$  localization and function outside the nucleus, including at the PM (Kumar *et al.*, 2002; Fan *et al.*, 2007). Our search of the OncoPrint database revealed that the DHHC-21 gene is significantly overexpressed in human breast cancer compared with normal breast

**FIGURE 2:** Signaling activity of ER is diminished by DHHC-7 and -21 protein knockdown. (A) Rapid signaling by E2 to ERK and PI3/AKT kinase activation. Bar graph data are mean  $\pm$  SEM densities from three experiments. \*,  $p < 0.05$  for control vs. E2; +,  $p < 0.05$  for E2 vs. E2 plus either siRNA to DHHC-7, DHHC-21, or both DHHC siRNAs. (B) cAMP generation is prevented by DHHC-7 or -21 siRNA. Bar graph represents the mean  $\pm$  SEM results from three experiments. \*,  $p < 0.05$  for control vs. E2; +,  $p < 0.05$  for E2 vs. E2 plus either DHHC-7 or -21 siRNA. (C) DHHC-22 siRNA does not affect E2 signaling. Bar graphs are from three experiments. \*,  $p < 0.05$  for control or DHHC-22 siRNA vs. either siRNA plus E2. (D) E2-induced cell proliferation/viability determined by the MTT assay ( $n = 3$  experiments). (E) DHHC-7 and -21 have no effect on EGF signaling. MCF-7 cells were incubated with DHHC-7 or -21 siRNAs, then incubated with EGF (10 ng/ml) for 15 min (ERK or PI3K/AKT activation; left) or for 24 h for MMT proliferation/viability studies (right). The studies were repeated three times. \*,  $p < 0.05$  for condition vs. condition plus EGF.



**FIGURE 3:** Structural basis for interaction of ER and DHHC proteins. (A) DHHC-7 and -21 proteins physically interact with WT but not mutant ER $\alpha$ . HA-tagged, plasmids encoding DHHC-7 or -21 were expressed in CHO cells with WT or C451A palmitoylation-site mutant ER $\alpha$ . The cells were lysed and immunoprecipitation (IP) of ER was followed by Western blotting with HA antibody, or by first doing HA immunoprecipitation, which was followed by blotting for ER. A representative study from three experiments is shown. (B) CHO cells were transfected to express either ER construct with DHHC constructs and then labeled with palmitic acid to determine ER palmitoylation. Bar graph is data from three experiments. \*,  $p < 0.05$  for pcDNA3 plus ER WT (lane 1) vs. DHHC-7 (lane 3) or DHHC-21 (lane 5) plus ER WT; +,  $p < 0.05$  for pcDNA3 plus ER WT (lane 1) vs. pcDNA3 plus ER C451A (lane 2), DHHC-7 plus ER WT (lane 3) vs. DHHC-7 plus ER C451A (lane 4), DHHC-21 plus ER WT (lane 5) vs. DHHC-21 plus ER C451A (lane 6). (C) CHO cells were transfected with WT ER, DHHC (control, as empty vector), and/or DHHC-7 or -21, then labeled with palmitic acid and exposed to 10 mM hydroxylamine for 4 h. Bar graph is data from three experiments. \*,  $p < 0.05$  for ER WT (lane 1) vs. DHHC-7 (lane 3) or DHHC-21 (lane 5) plus ER WT; +,  $p < 0.05$  for ER WT (lane 1) vs. ER WT plus hydroxylamine (lane 2) or ER WT plus DHHC control (lane 7) vs. ER WT plus DHHC control plus hydroxylamine (lane 8), DHHC-7 plus ER WT (lane 3) vs. DHHC-7 plus ER WT plus hydroxylamine (lane 4), DHHC-21 plus ER WT (lane 5) vs. DHHC-21 plus ER WT plus hydroxylamine (lane 6). (D) Localization of endogenous ER $\alpha$  and DHHC-7 and -21 proteins in MCF-7 cells. MCF-7 cells were fixed and exposed to ER $\alpha$  rabbit anti-human antibody, which was followed by FITC-conjugated goat-anti rabbit second antibody. For DHHC proteins, cells were incubated with mouse monoclonal antibodies, and then with Texas red-conjugated, anti-mouse IgG; cells were examined by fluorescence microscopy. The study shown was repeated two more times (E) Colocalization of DHHC-7 or -21 and ER $\alpha$  in the Golgi organelle. MCF-7 cells were incubated with primary antibodies for DHHC-7, DHHC-21, or ER $\alpha$ , and with antibody to human Golgi protein, and then with FITC-conjugated secondary antibodies; cells were examined by fluorescence microscopy. White arrows indicate membrane ER $\alpha$ . A representative study of three is shown.



**FIGURE 4:** DHC regulates signaling to the epigenetic modulation of the PR gene. (A) pS2 gene expression (qRT-PCR) induced by E2 is not affected by DHHC-7 or -21 knockdowns. DHHC siRNAs alone had no effects. The bar graph is from three experiments; \*,  $p < 0.05$  for any siRNA alone condition vs. siRNA(s) plus E2. (B) DHHC knockdown inhibits E2 signaling through PI3K/AKT (top panel) to the phosphorylation of EZH2 (middle panel), altering histone 3 trimethylation at Lys-27 (bottom panel). The soluble PI3K inhibitor LY 290042 (10  $\mu$ M) was also used. Total AKT, EZH2, and histone 3 proteins are shown. Bar graphs are from three experiments. \*,  $p < 0.05$  for control siRNA vs. control siRNA plus E2 (p-AKT and p-EZH2); +,  $p < 0.05$  for control siRNA plus E2 vs. DHHC siRNA plus E2, or control siRNA plus E2 plus LY 290442 (p-AKT), or for control siRNA vs. control siRNA plus E2 (H3L27Me3); ++,  $p < 0.05$  for control siRNA plus E2 vs. DHHC-7/21 siRNA plus E2 or control siRNA plus E2 plus LY (p-EZH2, H3K27Me3). (C) PR gene expression is stimulated by E2 through PI3K/AKT in MCF-7 cells but is inhibited by DHHC siRNA. The data are normalized for GAPDH expression in the same samples. Bar graph represents combined data from three experiments. \*,  $p < 0.05$  for control siRNA vs. same plus E2; +,  $p < 0.05$  for control siRNA plus E2 vs. DHHC siRNA plus E2 or control siRNA plus E2 plus LY 290442.

epithelium. It is possible that alterations in steroid receptor PAT abundance or function contributes to increased ER at the PM in some situations.

However, a more important limiting factor for PM trafficking is that E2 rapidly induces the dimerization of ~85% of ER $\alpha$  (Razandi *et al.*, 2004). Since palmitoylation preferentially occurs on ER $\alpha$  monomers (Razandi *et al.*, 2010), the steroid ligand controls the amount of receptor available for palmitoylation by controlling dimerization. Additionally, all ERs contain a nuclear localization signal (NLS) that drives ~80% of the receptors to the nucleus (Pedram *et al.*, 2006). Perhaps

the abundance of the nuclear receptor is required to bind the many promoters and enhancer elements throughout the genome. However, significant pools of endogenous mitochondrial sex steroid receptor (~15% of total ER $\alpha$ ) and PM-localized ER exist. To overcome the strong influence of the NLS in driving receptors exclusively to the nucleus, all sex steroid receptors require palmitoylation for trafficking to the PM (Pedram *et al.*, 2007). In normal cells, the abundance of PATs is likely to be a minor contributing factor, because the amount/availability of enzyme substrate (steroid receptor) is more important. As mentioned above, however, increased PAT abundance may contribute to the enhanced extranuclear ER localization and kinase activation seen in some breast cancers (Kumar *et al.*, 2002; Fan *et al.*, 2007).

Heat-shock protein 27 (Hsp27) facilitates palmitoylation of all sex steroid receptor classes upon binding to their palmitoylation motifs (Razandi *et al.*, 2010). The palmitoylation motif is localized deep within the receptor structure, even as a monomer. We propose that Hsp27 binds the receptor and thereby changes its conformation, thus allowing the now-identified PATs to bind at the same site and palmitoylate the receptors. The dynamics of these protein interactions will require complicated nuclear magnetic resonance structural studies.

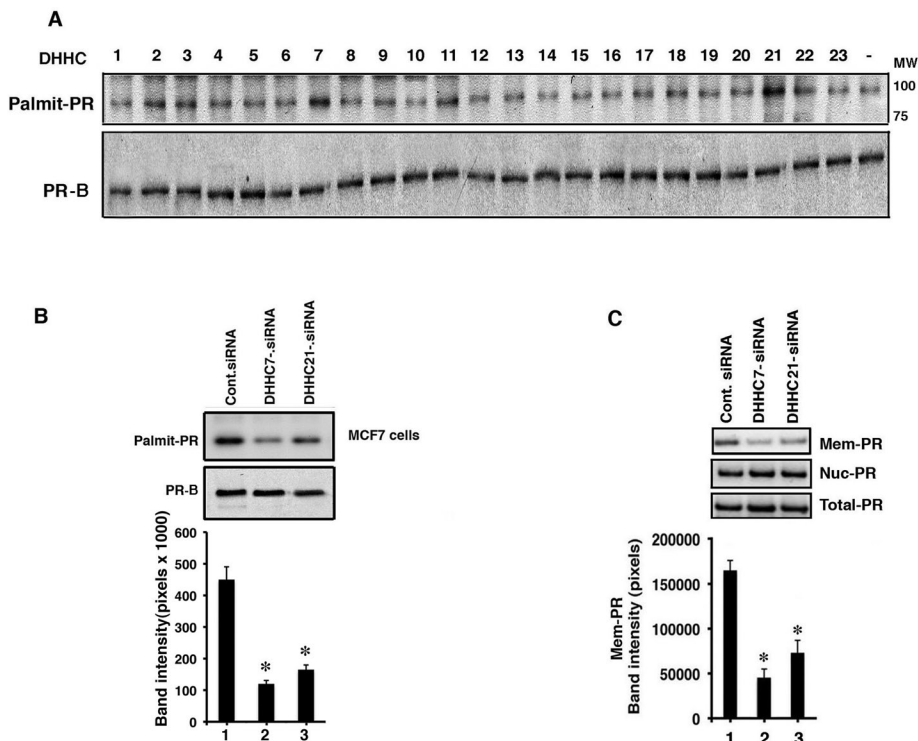
Finally, diminishing DHHC function inhibits both ER and PR localization at the PM of breast cancer cells. Preventing PM localization of DHHC substrates, such as Ras and Src kinases, by PAT knockdown may further contribute to the loss of rapid signal transduction in these settings. Hence, DHHC PATs may be a therapeutic target in aggressive breast cancers that require ER, PR, and growth factor tyrosine kinase receptor signal transduction for proliferation and survival (Dowsett *et al.*, 2006). Targeting PATs may also enable repression of AR function at the membrane of aggressive prostate cancers. Selective antagonists for membrane sex steroid receptors and enhanced small-interfering RNA (siRNA) delivery techniques directed against PATs *in vivo* may be useful to inhibit multiple signaling pathways in these tumors.

## MATERIALS AND METHODS

### Cell culture and reagents

MCF-7 and CHO-K cells were from ATCC and C4-2 prostate cancer cells (AR positive) were a gift from Ganesh Raj (University of Texas, Southwestern, Dallas, TX). Cells were cultured in phenol red-free DMEM-F12, 10% charcoal-stripped fetal bovine serum (FBS), 1% antibiotic-antimycotic (15240-062; Life Technologies, Carlsbad, CA) at 37°C to 75% confluency in 100-mm dishes or six-well plates and exposed to 10 nM E2, 100 nM progesterone acetate (P) or 10 nM DHT (Steraloids, Newport, RI) for the times





**FIGURE 5:** DHHC-7 and -21 are progesterone receptor PATs. (A) Transfection of individual plasmids into MCF-7 cells encoding one DHHC protein each from a cDNA library of 23 plasmids was followed by [<sup>3</sup>H]palmitic acid labeling, immunoprecipitation of endogenous PRB from cell lysates, and separation by SDS-PAGE and fluorography. Immunoblots for PR total protein are shown. A representative experiment from three experiments is shown. DHHC protein expression is from the same experiment(s) that is also used for ER PAT screening and is shown in Figure 1A. (B) DHHC-7 or -21 siRNA significantly reduced [<sup>3</sup>H]palmitate incorporation into endogenous PR. Total PR protein serves as loading/normalization control from a representative experiment of three experiments total. Bar graphs are mean ± SEM densities from three experiments. \*, *p* < 0.05 for control siRNA vs. DHHC-7 or -21 siRNA. (C) Distribution of PR in MCF-7 cells. Cells were transfected with siRNAs and Western blots of cell fractions were performed. Bar graph is membrane PR density data from three experiments. \*, *p* < 0.05 for siRNA control vs. DHHC siRNA.

indicated in the experiments. ER $\alpha$  (MC-20), PRB (C-19), and AR (C-19) antibodies (all polyclonal, raised in rabbits); green fluorescent protein antibody (GFP; polyclonal, rabbit); and HA antibodies (monoclonal, mouse) were from Santa Cruz Biotechnology (Santa Cruz, CA). Golgi marker antibody (AE-6, mouse monoclonal) from Santa Cruz is directed against the isolated Golgi cisternae functional regions composed of various enzymes and is derived from a human lymphoma cell line, per the manufacturer. Antibodies to DHHC-7 and -21 (monoclonal, mouse), phospho-EZH2 Ser-21, histone H3, and H3K27Me3 (polyclonal, rabbit) antibodies were from Abcam (Cambridge, MA), and were used for immunoblots in subcellular fractions and for immunofluorescence microscopy. We also used a DHHC-7 polyclonal antibody raised in rabbits (Novus Biologicals, Littleton, CO) for the Golgi colocalization study. Phospho-AKT Ser-473 antibodies (monoclonal, rabbit) were from Cell Signaling Biotechnology (Danvers, MA). Double-stranded control and RNAs for the DHHC-7, -21 and -22 proteins (two siRNAs each) were obtained from Santa Cruz. Each siRNA was used for all key experiments, and the data shown are from experiments using either of the two siRNAs. Construction and transfection/expression of all ER, AR, PR, and GFP (pEGFP vector; Clontech, Mountain View, CA) plasmids were previously described (Pedram *et al.*, 2007). The DHHC cDNA plasmid li-

brary used the pEF-BOS-HA backbone vector and was provided by Y. Fukata and M. Fukata (Fukata *et al.*, 2004). PD 98059, a MEK inhibitor, was a gift from Alan Saltiel (Parke-Davis, Ann Arbor, MI), and the LY290042 (PI3K inhibitor) was from Calbiochem (San Diego, CA).

### Cell fraction isolation

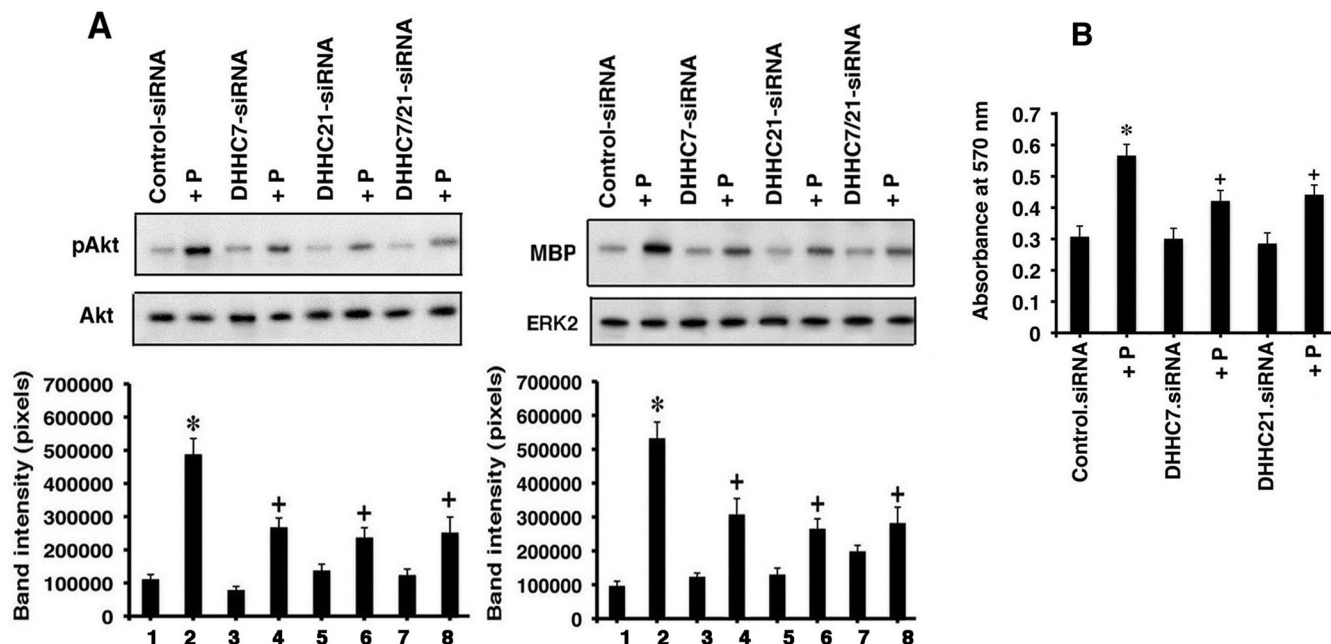
Cells were homogenized using 15–20 strokes of a tight-fitting Dounce homogenizer until ~ 90% of the cells were broken. Homogenates were centrifuged at 4000 rpm for 10 min to pellet the nuclear fractions. Supernatants were layered onto a discontinuous sucrose gradient (1.0–2.5 M) made up in buffer containing 10 mM Tris (pH 7.6), 2 mM EDTA, 2 mM dithiothreitol, protease inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The mixtures were centrifuged at 2000 rpm for 30 min at 4°C to produce a top layer (cytosol). Cytosolic fractions were centrifuged at 100,000 × *g* for 1 h. The pellet was centrifuged again to isolate the membrane fraction, which was separated by SDS-PAGE on a 10% gel, transferred to nitrocellulose, and then immunoblotted with the indicated antibodies (Santa Cruz), which were detected using the ECL kit (Amersham/GE Healthcare, Waukesha, WI). Western blots were also used to normalize total receptor or other proteins.

### Steroid receptor palmitoylation

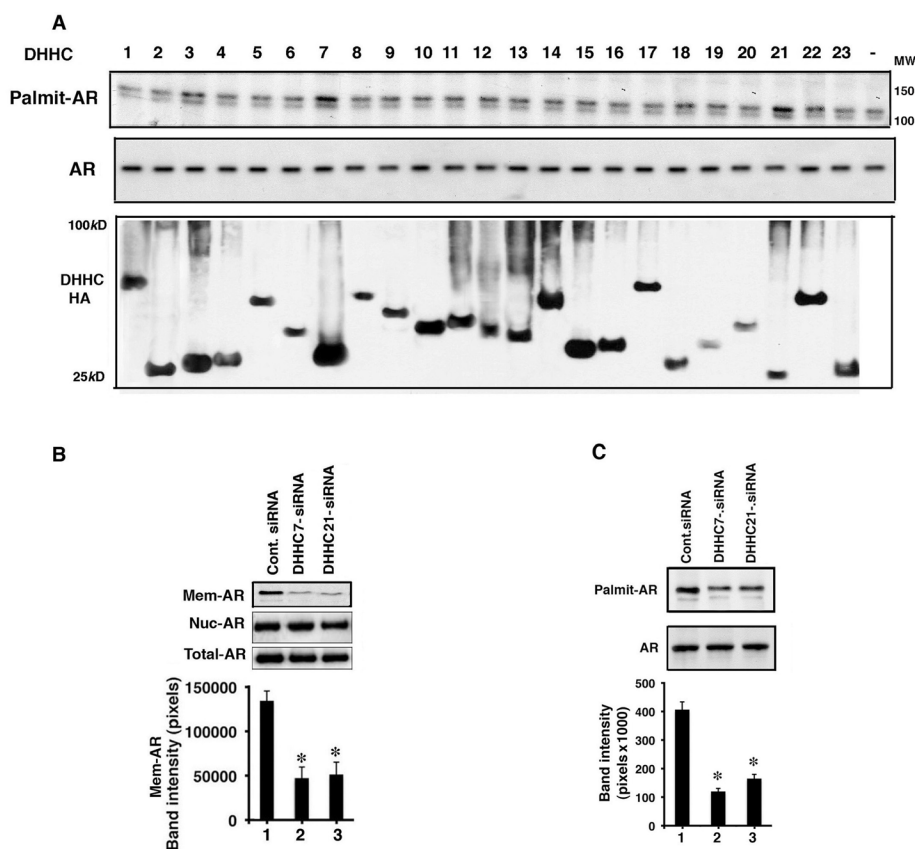
Palmitoylation of sex steroid receptors was carried out as previously described (Pedram *et al.*, 2007). MCF-7, CHO, or C4-2 cells were labeled with [<sup>3</sup>H]palmitic acid (0.5 mCi/ml) for 4 h in the absence of sex steroids. Cytoplasmic extracts were immunoprecipitated with antibodies to ER $\alpha$ , PR, or AR, each of which was conjugated to protein A Sepharose. Separation of samples by SDS-PAGE was followed by autoradiography with emulsifying enhancer.

### ERK and PI3K/AKT activity

ERK was immunoprecipitated from lysates of MCF-7 cells exposed to various steroids or EGF (10 ng/ml). Equal ERK protein amounts from each experimental condition were added to separate tubes containing [<sup>32</sup>P]ATP and substrate peptide (myelin basic protein; Sigma-Aldrich, St. Louis, MO) in buffer to determine kinase activity. Activity was determined by autoradiography of phosphorylation of substrate protein. (Pedram *et al.*, 2006). PI3K activity was determined as phosphorylation of AKT at Ser-473 (p-AKT) by Western blotting (Pedram *et al.*, 2006) after 15 min of exposure of the synchronized cells to steroid or EGF. Total ERK or AKT protein, as identified by Western blotting, was used for sample activity normalization. In control DHHC signaling experiments, MCF-7 cells were incubated with DHHC-22 siRNA, recovered overnight in DMEM-F12 medium without serum or phenol red, and then incubated with 10 nM E2 for 15 min, with ERK or PI3K/AKT activation determined at that time.



**FIGURE 6:** Signaling functions of PR are decreased by DHH-7 and -21 protein knockdown. (A) Rapid signaling by P to ERK and PI3/AKT kinase activation is prevented by DHH-7 or -21 siRNA. Bar graphs represent the mean  $\pm$  SEM from data combined from three experiments. \*,  $p < 0.05$  for control vs. P; +,  $p < 0.05$  for P vs. P plus DHH-7, DHH-21, or both siRNA(s). (B) E2-induced cell viability is decreased upon DHH-7 or -21 knockdown. Viability is determined by the MTT assay. The bar graph represents data combined from three experiments. \*,  $p < 0.05$  for control vs. P; +,  $p < 0.05$  for P vs. P plus DHH-7 or DHH-21 siRNA.



**FIGURE 7:** ARs are substrates for DHH-7 and -21. (A) Transfection of individual DHH-encoding plasmids into C4-2 prostate cancer cells was followed by [ $^3$ H]palmitic acid labeling. Immunoblots for AR total protein and HA-tagged DHH PAT proteins are shown. A representative experiment repeated two more times is shown. (B) Distribution of AR in C4-2

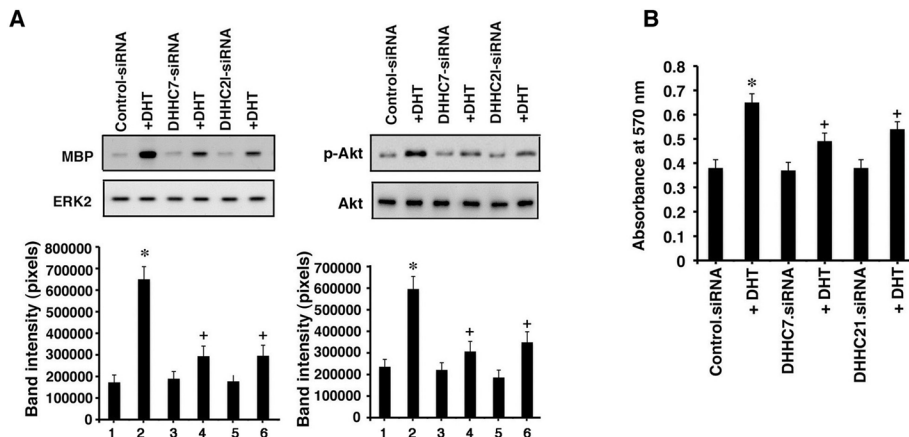
#### cAMP generation

Adenylate cyclase activity in the membrane suspension was determined by measuring cAMP generation in the presence of IBMX (phosphodiesterase inhibitor) after cells were incubated for 5 min with 10 nM E2 in DMEM-F12 medium. After incubation, the cells were washed and 0.1N HCl was added to the cell monolayers for 20 min at room temperature to extract cAMP. The supernatants were collected and recentrifuged for use in the cAMP radioimmunoassay according to the manufacturer's protocol (Perkin Elmer-Cetus, Waltham, MA; Pedram et al., 2006).

#### MTT assay

Cell proliferation/viability was quantified by a colorimetric assay according to the

cells. Left, cells were transfected with the siRNAs and Western blots of cell fractions were performed. Knockdown of DHH-7 or -21 decreased membrane, but not nuclear, localization of the steroid receptor. Bar graphs are combined mean  $\pm$  SEM membrane AR densities from three experiments. \*,  $p < 0.05$  for control siRNA vs. DHH siRNA. (C) DHH-7 or -21 siRNA significantly reduced [ $^3$ H]palmitate incorporation into endogenous AR. Total AR protein is shown. Bar graphs are data from three experiments. \*,  $p < 0.05$  for control siRNA vs. DHH siRNA.



**FIGURE 8:** Signaling functions of AR are decreased by DHHC-7 and -21 protein knockdown. (A) Rapid signaling by DHT to ERK and PI3/AKT kinase activation is inhibited by DHHC-7 or -21 knockdown. Bar graph data represent the combined mean  $\pm$  SEM from three experiments. \*,  $p < 0.05$  for control vs. DHT; +,  $p < 0.05$  for DHT vs. DHT plus DHHC-7 or -21 siRNA. (B) DHT-induced cell viability is decreased upon DHHC-7 or -21 knockdown and is determined by the MTT assay. The bar graph represents combined data from three experiments. \*,  $p < 0.05$  for control vs. DHT; +,  $p < 0.05$  for DHT vs. DHT plus DHHC-7 or -21 siRNA.

manufacturer's instructions (Sigma Chemical Corporation). The kit measures mitochondrial dehydrogenase activity in viable cells based on the conversion of the MTT to MTT-formazan crystal by mitochondrial enzyme. In brief, synchronized MCF-7 and C4-2 cells were exposed to various treatments in DMEM-F12 medium for 24 h. At the end of incubation, the cells were washed and then incubated with medium containing 1 mg/ml MTT for 4 h at 37°C. MTT-formazan was extracted by overnight incubation at 37°C with 100  $\mu$ l extraction buffer (20% SDS, 50% formamide adjusted to pH 4.7 with 0.02% acetic acid, and 0.025 N HCl). Optical densities at 570 nm were measured using extraction buffer as a blank.

### Cell localization of steroid receptor proteins

Steroid receptor localization was determined by immunofluorescent cell microscopy (Drisdell *et al.*, 2006). After MCF-7 or C4-2 cells were cultured on glass bottom dishes (MatTek, Ashland, MA), the cells underwent siRNA transfection or other treatments, and then they were recovered over 24 h, fixed with 3.5% paraformaldehyde, permeabilized with 0.2% Triton X-100, and then incubated with sex steroid receptor antibody overnight at 4°C. Nonspecific immunoglobulin G (IgG) antibody served as control. Incubation with the first antibody was followed by incubation with fluorescein isothiocyanate (FITC)-conjugated second antibody (green color) or IgG antibody (no visualization) for 2 h at room temperature. For colocalization studies, human-specific antibodies to Golgi markers (Santa Cruz Biotechnology) were used for separate red and green fluorescence. A similar method was used for fluorescence colocalization of endogenous ER $\alpha$  with DHHC proteins or Golgi in MCF-7 cells using monoclonal antibodies against each protein. Identically treated MCF-7 cells were also used for plasma membrane and nuclear fraction isolation. The fractions were separated by SDS-PAGE on a 10% gel, transferred to nitrocellulose, immunoblotted with the indicated antibodies (Santa Cruz Biotechnology), and detected using the ECL kit.

### Epigenetic modulation studies

MCF-7 cells were cultured in DMEM-F12 medium and transfected with DHHC siRNAs (3  $\mu$ g/well of cells in six-well plates), which was followed by 24 h of recovery and overnight synchronization. The

cells were incubated with 10 nM E2  $\pm$  10  $\mu$ M LY294002 for 30 min to determine AKT and EZH2 phosphorylation, and for 24 h to determine PR gene expression using real-time PCR (RT-PCR). MCF-7 cell monolayers were washed in ice-cold phosphate-buffered saline supplemented with 5 mM sodium butyrate to retain histone acetylation. After centrifugation at 2000 rpm for 5 min in the cold, cell pellets were resuspended in lysis buffer (10 mM Tris-HCl, pH 7–7.4, 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, and complete protease inhibitor cocktail) with 0.5% NP-40 on ice. Cell lysates were centrifuged for 5 min at 6000 rpm at 4°C. Phospho-AKT (Ser-473) was determined from supernatants by immunoblotting as readout for PI3K activity (Pedram *et al.*, 2006). The activity-inhibiting phosphorylation at Ser-21 of the histone methyltransferase EZH2 was also investigated. Total AKT and EZH2 served as loading controls. Pellets were resuspended in media with 5 mM MgCl<sub>2</sub> and 0.8M HCl, sonicated at low speed for 20 s, and incubated on ice for 1 h. The solution of histone proteins was centrifuged at 14,000 rpm for 10 min at 4°C. Supernatants were transferred to a new tube, and histones were precipitated with a 50% solution of trichloroacetic acid dissolved in ddH<sub>2</sub>O. Precipitated histones were collected by centrifugation for 20 min at 14,000 rpm at 4°C. The resultant pellet was washed with ice-cold acetone, dried, and subsequently resuspended with ddH<sub>2</sub>O (150  $\mu$ l) and 1.5 M Tris-HCl (pH 8.8; 2  $\mu$ l). Histone proteins (10  $\mu$ l) were resolved by SDS-PAGE on a 10–20% Tris-tricine gel (Bio-Rad, Hercules, CA), and changes in trimethylation of histone 3 at Lys-27 relative to total histone 3 were investigated by immunoblotting with antibodies from Abcam.

### Quantitative RT-PCR

MCF7 cells were grown in phenol red-free DMEM with 10% charcoal dextran-treated FBS for greater than 24 h before transfection. Cells were transiently transfected with control or DHHC siRNAs plasmids for 24 h, then washed and incubated for 24 h in either 10 nM E2  $\pm$  LY290042 or an ethanol vehicle. Total RNA was extracted using the RNeasy kit from Qiagen (Valencia, CA). cDNA was synthesized using the Improm-II reverse transcription system (Promega, Madison, WI). Quantitative RT-PCR (qRT-PCR) was used to determine pS2 and PR gene expression, and RT-PCR was used to determine DHHC-22 expression, normalized to the housekeeping gene, GAPDH. Primers were designed with Primer3 (<http://frodo.wi.mit.edu>) and were blasted for specificity.

pS2: F5'-ATACCATCGACGTCCCTCCA-3', R5'-AAGCGTGTCTGAGGTGTCCTG-3'; PR: F5'-CGCGCTCTACCCTGCACTC-3', R5'-TGAATCCGGCCTCAGGTAGTT-3'; ZHDHC22: F5'-GCCTACATCTCCGCTGCTCTT-3', R5'ATGGCGAACCAGAGGTAGAGCA-3'; GAPDH: F5'-CCACAGTCCATGCCATCA-3', R5'-GGATGACCTTGCCACAG-3' primers were designed for annealing temperature of 60°C and to amplify regions of ~95–120 base pairs. PCR amplicon sizes were confirmed by agarose gel electrophoresis prior to qRT-PCR analysis. For qRT-PCR, 500 ng of cDNA was used in a 50- $\mu$ l reaction consisting of 25  $\mu$ l of SYBR Green, qPCR Supermix (Invitrogen, Carlsbad, CA), 1  $\mu$ l of 10  $\mu$ M forward/reverse primer stocks, and nuclease-free water. Thermocycling was carried out using the iCycler (Bio-Rad)

with a melting-curve temperature of 60°C. Relative mRNA levels were calculated using the  $C_t$  method.

### Image acquisition

The microscopic images were obtained using a Nikon Eclipse TE-200 microscope with magnification from 200–400 $\times$ , at room temperature. Imaging medium (Vectashield) with antifade properties was from Vector Laboratories (Burlingame, CA). A Nikon Diagnostic Instruments camera (Model 3.2.0) was used in conjunction with Spot Advance software (Sterling Heights, MI) to capture and transfer images to the computer. Rhodamine-conjugated (red) and FITC-conjugated (green) secondary antibodies (Vector Laboratories) were used for fluorescence visualization.

### Data analysis

Data from multiple experiments were used to calculate a mean  $\pm$  SEM and were analyzed by analysis of variance (ANOVA) plus Scheffe's test at a  $p < 0.05$  level of significance.

### ACKNOWLEDGMENTS

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