



Published in final edited form as:

Oncogene. 2011 April 7; 30(14): 1706–1715. doi:10.1038/onc.2010.543.

PTPH1 cooperates with vitamin D receptor to stimulate breast cancer growth through their mutual stabilization

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Abstract

Tyrosine phosphorylation is tightly regulated by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), and plays a critical role in malignant transformation and progression. While PTKs have a well-established role in regulating breast cancer growth, contribution of PTPs remains mostly unknown. Here, we report that the tyrosine phosphatase PTPH1 stimulates breast cancer growth through regulating vitamin D receptor (VDR) expression. PTPH1 was shown to be over-expressed in 49% of primary breast cancer and levels of its protein expression positively correlate with the clinic metastasis, suggesting its oncogenic activity. Indeed, PTPH1 promotes breast cancer growth by a mechanism independent of its phosphatase activity but dependent of its stimulatory effect on the nuclear receptor VDR protein expression and depletion of induced VDR abolishes the PTPH1 oncogenic activity. Additional analyses showed that PTPH1 binds VDR and increases its cytoplasmic accumulation leading to their mutual stabilization and stable expression of a nuclear localization deficient VDR abolishes the growth-inhibitory activity of the receptor independent of 1, 25-dihydroxyvitamin D₃ (vitamin D₃). These results reveal a new paradigm in which a protein tyrosine phosphatase may stimulate breast cancer growth through increasing cytoplasmic translocation of a nuclear receptor leading to their mutual stabilization.

Keywords

PTPH1; VDR; VDR cytoplasmic translocation; mutual stabilization; breast cancer

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Introduction

Reversible tyrosine phosphorylation is controlled by protein tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs), which together regulates important signal transduction pathways in control of cell growth, invasion and transformation (Ostman et al., 2006). Abnormal tyrosine phosphorylation can lead to various human diseases including cancers and many oncogenes have in fact been found to be hyperactively mutated or overexpressed PTKs. In breast cancers, for example, the human epidermal growth factor receptor 2 (Her-2), a membrane PTK, is over-expressed in about 25% of the diseases and its expression levels are strongly associated with the poor prognosis (Slamon et al., 1987). The therapeutic intervention against the Her-2 signaling has consequently become an important strategy to control breast cancer progression (Yu and Huang, 2000). Roles of PTPs in human breast cancer, on the other hand, remain mostly unexplored. The phosphatase SHP1, for example, is over-expressed in 58% of primary breast cancer (Yip et al., 2000) but whether SHP1 can promote human breast cancer growth has not been established (Ostman et al., 2006). Studies of PTPs for their roles in regulating breast cancer growth are therefore highly warranted.

PTPH1 (also called *PTPN3*) is a 120-kDa protein that belongs to the non-transmembrane PTP super-family (Yang and Tonks, 1991). Previous genetic analysis showed that PTPH1 and its several family members are mutated in human colon cancer but the functional consequence of these mutations remains un-established (Wang et al., 2004). Our recent studies showed that p38 γ , a p38 MAPK family member, increases Ras oncogenesis independent of phosphorylation (Tang et al., 2005) and PTPH1 dephosphorylates and cooperates with p38 γ to promote Ras oncogenesis through a complex formation (Hou et al., 2010). Importantly, PTPH1 was found to be over-expressed in primary human colon cancer and its depletion inhibits colon cancer growth (Hou et al., 2010). In this report, we tested the hypothesis that PTPH1 may also positively regulate breast cancer growth. Our results showed that PTPH1 is over-expressed in about 49% of primary human breast cancer and its expression levels positively correlate the clinic metastasis. PTPH1 was further shown to increase breast cancer growth by a mechanism independent of phosphatase activity but dependent of its stimulatory effect on vitamin D receptor (VDR) protein expression. Additional experiments revealed that PTPH1 binds VDR and increases its cytoplasmic accumulation leading to their mutual stabilization and stable expression of a nuclear localization deficient VDR abolishes its growth-inhibitory activity independent of 1, 25-dihydroxyvitamin D3 (vitamin D3). These results indicate that the tyrosine phosphatase PTPH1 may stimulate breast cancer growth through a positive feedback loop involving stimulating VDR protein expression and increasing its cytoplasmic accumulation leading to their mutual stabilization via a complex formation. Targeting PTPH1 expression and/or regulating VDR localizations may be a novel approach to control human breast cancer progression.

Results

PTPH1 is over-expressed in primary human breast cancer and its protein expression levels positively correlate with the lymph node metastasis

To investigate roles of PTPH1 in breast cancer, a group of primary breast cancer tissues were analyzed by immunohistochemistry (IHC) for PTPH1 protein expression with a specific antibody (Hou et al., 2010). Results in Figure 1 showed that PTPH1 protein is over-expressed in breast cancer specimens over their matched normal tissues with about 50% of tumor samples having an increased PTPH1 expression. In addition, levels of increased PTPH1 protein expression are significantly higher in lymph node metastatic tumors, indicating that PTPH1 may play a promoting role in human breast cancer growth and progression. Moreover, PTPH1 expression appears to be higher in invasive ductal over lobular carcinomas (Figures 1a and b), *albeit* a statistically significant difference was not reached due to the limited number of specimens analyzed. Since there are distinct clinical profiles and gene-expression pathways in these two types of breast cancers (Korkola et al., 2003) and patients with estrogen receptor negative (ER-) ductal carcinoma are associated with a worse survival than their counterparts with lobular tumor (Mhuircheartaigh et al., 2006), PTPH1 may play a particular role in more malignant ductal carcinomas. These results together indicate a role of PTPH1 in clinical breast cancer metastasis and progression.

PTPH1 signals independent of p38_γ in breast cancer but specifically regulates the nuclear receptor VDR protein expression

Our recent studies showed that both Ras and p38_γ induce PTPH1 protein expression (Hou et al., 2010) and we therefore next examined if p38_γ stimulates PTPH1 expression as compared to its family member p38_α through adenovirus-mediated gene over-expression. To study effects of PTPH1 on p38 MAPK signaling, breast cancer cells were engineered to express tetracycline-inducible (Tet-on) PTPH1 (Qi et al., 2006) and its effects on endogenous as well as ectopically expressed p38s were examined by Western blot (WB). Results in Figure 2a showed that in contrast to rat epithelial intestinal IEC-6 cells (Hou et al., 2010), p38_γ overexpression has no substantial effects on endogenous PTPH1 expression. In a similar manner, Tet inducible PTPH1 in ER- 231 and ER+ MCF-7 cells does not significantly impact p38_γ expression. These results indicate that PTPH1 may signal independent of p38_γ in these breast cancer cells under the current experimental conditions.

Nuclear receptors play an important role in regulating breast cancer growth via ligand-dependent and -independent pathways, with ER α (ER) and progesterone receptor (PR) generally considered proliferative and VDR anti-proliferative (Conzen, 2008). We therefore next examined if PTPH1 may affect protein expression of nuclear receptors by WB analyses. Results in Figures 2b–e, 3d, 4b, 5a and b showed that PTPH1-forced expression up-regulates VDR whereas its depletion decreases levels of VDR protein expression without consistent effects on ER or PR in this group of cell lines. The signaling specificity between PTPH1 and VDR was further demonstrated in 293T cells through co-expressions (Figure 2d) in which only the ectopically expressed VDR (but not ER or PR-B) is elevated by co-transfected PTPH1 but not Wip1, a serine/threonine phosphatase previously found to play a role in breast cancer growth (Bulavin et al., 2004). Additional experiments showed that

PTPH1 increases VDR protein but not RNA expression (Figures 2e and f). These results together indicate that PTPH1 positively regulates VDR protein (but not RNA) expression, which may play an important role in regulating breast cancer growth.

PTPH1 increase of breast cancer growth is independent of phosphatase activity but coupling with its regulatory effects on VDR protein expression

The tyrosine phosphatases PTP1B and SHP1 are over-expressed in human breast cancer (Wiener et al., 1994) (Yip et al., 2000) and PTP1B was recently further shown to be required for Her-2 induced transformation in vitro (Arias-Romero et al., 2009) and mammary tumor formation in vivo (Bentires-Alj and Neel, 2007). However, thus far, there have been no functional studies about their roles in regulating human breast cancer growth. We therefore examined if PTPH1 may promote human breast cancer growth. Results in Figure 3a showed that stable expression of PTPH1 and its phosphatase-deficient mutant PTPH1/DA (Zhang et al., 1999) in ER- 231 cells increases colony formation, indicating a promoting role of PTPH1 in breast cancer growth independent of phosphatase activity. A similar growth-stimulatory effect was further demonstrated in ER+ T47D cells by lentiviral-mediated PTPH1 overexpression (Figure 3b) and in ER+ MCF-7 and ER- 231 cells by Tet-induced PTPH1 expression (Figure 3c), suggesting that PTPH1 increases breast cancer growth independent of ER expression. Furthermore, stable PTPH1 depletion by lentiviral-mediated shRNA delivery inhibits the colony formation in T47D and MCF-7 cells, which couples with a decreased VDR protein expression (Figure 3d). These results together indicate that PTPH1 stimulates breast growth independent of phosphatase activity likely through a mechanism affecting VDR protein expression.

VDR is required for PTPH1 to stimulate breast cancer growth

A positive regulation of VDR protein expression by PTPH1 suggests that VDR may be required for PTPH1 oncogenic activity. To examine this possibility, VDR was depleted from PTPH1 stably expressed T47D cells by siRNA and its effect on colony formation was examined. Results in Figures 4a and b (left panel) showed that silencing VDR gene expression alone increases the colony formation and decreases endogenous PTPH1 expression, indicating that endogenous VDR is growth-inhibitory and may also be required for endogenous PTPH1 expression. Importantly, reducing VDR protein expression to a level similar to the vector control completely blocks PTPH1-induced growth stimulation (lane 4 vs. 1 from left in Figures 4b, left panel and a). These results indicate a required role of induced VDR in PTPH1 stimulation of breast cancer growth.

To further demonstrate the role of VDR in PTPH1 stimulating breast cancer growth, mouse VDR knockout (VDR^{-/-}) mammary tumor cells and the wild-type counterparts (VDR^{+/+}) (Zinser et al., 2003) were next stably expressed with PTPH1 by lentiviral infection and analyzed for cell growth. Because these cells failed to form colonies (Zinser et al., 2003), the growth was estimated by the cyquant[®] NF cell proliferation assay. Of great interest, the PTPH1-forced expression only increases the growth of VDR^{+/+} but not VDR^{-/-} cells (Figure 4c), further indicating the role of endogenous VDR in PTPH1 promoting breast cancer growth. However, WB analyses from these cells showed that endogenous PTPH1 protein expression was undetectable in VDR^{-/-} cells compared to the WT counterparts and

the PTPH1 stable expression failed to increase VDR protein expression in both lines (Figure 4b, right panel). These results further indicate that endogenous VDR is required for PTPH1 protein expression, and suggest that PTPH1 induction of VDR protein expression may be species-specific. Nevertheless, results from both human and mouse breast cancer cells are consistent with the conclusion that PTPH1 requires VDR to stimulate breast cancer growth.

PTPH1 binds VDR and increases its cytoplasmic expression leading to their mutual stabilization

VDR expression and activation in human cancer generally lead to a growth-inhibitory response (Campbell et al., 2000; Maruyama et al., 2006; Qi et al., 2002). Although classically considered as a nuclear receptor, VDR has been shown to shuttle between the cytoplasm and the nucleus through interacting with other proteins (Prufer and Barsony, 2002; Yasmin et al., 2005). VDR activations by ligand vitamin D3 (Racz and Barsony, 1999) and its partner retinoid X receptor (RXR) (Prufer et al., 2000) are also known to facilitate its nuclear localization and a decreased VDR nuclear localization can lead to a suppressed VDR transcription activity and a resistance to vitamin D-induced growth inhibition (Garay et al., 2007; Yang et al., 2001). To understand why PTPH1 increases breast cancer growth and concomitantly stimulates expression of the growth-inhibitory protein VDR, cellular VDR distributions were examined in response to PTPH1 depletion and expression by cell fractionation analyses. Results in Figure 5a showed that endogenous PTPH1 is exclusively cytoplasmic and there was a decreased VDR protein expression in cytoplasmic but not nuclear compartments following the PTPH1 depletion. In addition, the ectopically expressed PTPH1 also only presents in the cytoplasm, which again led to an increased VDR protein expression in the cytosolic fraction without affecting its nuclear concentration (Figure 5b). Similar PTPH1-regulatory effects on cytoplasmic VDR protein expression were also observed after cells were cultured with vitamin D3 (Figures 5a and b, bottom). These results together indicate that PTPH1 is exclusively cytoplasmic and only cytoplasmic VDR is sensitive to PTPH1 regulations independent of ligand.

Previous studies showed that increases of VDR cytoplasmic accumulation by mutations of its nuclear localization signal inhibit VDR transcriptional activity (Prufer et al., 2000). We sought next to determine if the PTPH1 resultant increase in cytoplasmic VDR also regulates VDR transcriptional activity. In this case, PTPH1 stably expressed T47D cells were transiently transfected with a VDRE-Luc (Li et al., 2007; Ward et al., 2001) by including an ERE-Luc (Obrero et al., 2002; Qi et al., 2004) for comparison and luciferase activity was determined two days later. Results in Figure S1a showed that PTPH1 significantly inhibits the VDRE activity without affecting the ERE-dependent transcription. The VDRE suppressive role was further consolidated by a decreased RNA expression of CYP24, a VDR target gene, in PTPH1 expressed cells (Figure 5d). Incubation of cells with vitamin D3, however, switched the VDRE-suppressive effect of PTPH1 into a stimulatory response without significant impacts on its inhibition of CYP24 expression (Figures 5c and d). This difference may be in part due to the fact that the VDRE-Luc is an artificial luciferase reporter driven by two VDRE repeats from osteopontin (*opn*) gene (Ward et al., 2001) and vitamin D3 can even distinctively regulate histone-acetylation on endogenous *opn* and *cyp24* promoters (Kim et al., 2005). These results indicate that increased PTPH1 expression and

resultant cytoplasmic VDR accumulation may together lead to a decreased VDR transcriptional activity in the absence of vitamin D3.

To explore if PTPH1 may increase cytoplasmic VDR expression through a complex formation, V5-tagged VDR was co-expressed with PTPH1 and its DA mutant, and V5 precipitates were examined for PTPH1 protein expression. Results in Figure 6a showed that both PTPH1 and PTPH1/DA bind VDR proteins. Additional experiments revealed that VDR also binds PTPH1 in vitro and incubation of cells with vitamin D3 does not significantly affect their binding (Figures S1c and S2). To demonstrate if a complex formation regulates their stability, VDR and PTPH1 protein stability were analyzed with and without PTPH1 over-expression in T47D and VDR^{+/+} and VDR^{-/-} breast cancer cells as described (Loesch et al., 2010). Results in Figure 6b showed that the PTPH1 expression increases VDR protein stability, indicating that PTPH1 may up-regulate VDR by increasing its protein stability. More interestingly, the ectopically expressed PTPH1 is more stable in VDR^{+/+} cells than in its knockout counterparts (Figure 6c), suggesting that endogenous VDR also stabilizes PTPH1 protein. Similar results were also obtained in the presence of vitamin D3 (Figures S1d and e). These results together indicate that the cytoplasmic PTPH1 may bind and stabilize VDR independent of vitamin D3, leading to their mutual stabilization and enhanced breast cancer growth by a positive feedback (Figure 7e).

VDR requires its nuclear localization to inhibit breast cancer growth

The coupling of PTPH1 stimulating breast cancer growth with its increases of cytoplasmic VDR accumulation promoted us next to examine if VDR alone regulates cell growth dependent of its cellular localization. In this regard, a mutant VDR (VDR/mNLS) was generated by changing three amino acids on its nuclear localization signal (NLS) as previously described (Prufer et al., 2000) and stably expressed in T47D cells through antibiotic selection together with a wild-type VDR for comparison. Cell fractionation and immunostaining analyses showed that in contrast to the wild-type receptor this mutant is mostly localized in the cytoplasm and much less responsive to vitamin D3-induced nuclear translocation (Figures 7b and S3) as previously described (Prufer et al., 2000). Consistent with the notion that cytoplasmic VDR may cooperate with PTPH1 to promote breast cancer growth through a complex formation (Figure 7e), the NLS mutated receptor appears to have a stronger affinity in binding endogenous PTPH1 compared to the wild-type protein (Figure 7a). Most importantly, the mutant expressed cells grew significantly faster than those expressing the wild-type VDR by short-term proliferation and long-term colony formation assays (Figures 7c and d). An addition of vitamin D3 did not significantly affect their growth-regulatory activities (Figure S1b). These results indicate that VDR requires its nuclear localization to inhibit breast cancer growth independent of vitamin D3 and thereby further consolidate our conclusion that cytoplasmic VDR may cooperate with PTPH1 to promote breast cancer growth through their mutual stabilization.

Discussion

The role of protein tyrosine kinases such as Her2 in breast cancer growth has been well-established (Yu and Huang, 2000), whereas the contribution of protein tyrosine

phosphatases has been mostly unexplored. In this report, we showed that the tyrosine phosphatase PTPH1 is over-expressed in primary human breast cancer and its increased protein expression further correlates with the clinical metastasis. Furthermore, PTPH1 was shown to stimulate breast cancer growth independent of phosphatase activity likely through increasing cytoplasmic VDR protein expression via a complex formation thereby forming a positive feedback loop leading to their mutual stabilization. This was demonstrated by the following: 1) PTPH1 stimulates breast cancer growth and increases VDR protein expression in several breast cancer cell lines; 2) depletion of induced VDR abolishes the PTPH1 oncogenic activity and PTPH1 also loses its growth stimulatory activity in VDR^{-/-} cells; 3) PTPH1 is exclusively cytoplasmic and only regulates cytoplasmic VDR protein expression; 4) PTPH1 binds and stabilizes VDR protein; 5) PTPH1 protein expression is also decreased in VDR depleted or knockout cells and there is a reduced PTPH1 protein stability in VDR^{-/-} cells compared to their ^{+/+} counterparts, indicating their mutual stabilization effect; and 6) a nuclear-localization deficient VDR loses its growth inhibitory activity. Thus, up-regulated cytoplasmic VDR may cooperate with PTPH1 to stimulate breast cancer growth through their complex formation and mutual stabilization (Figure 7e). These results together reveal a new role of a protein tyrosine phosphatase in regulating breast cancer growth through signaling cross-talk with a nuclear receptor.

So far only few PTPs have been reported to be involved in regulating breast cancer growth. A tumor-suppressor function, for example, has been proposed for the protein tyrosine phosphatase alpha (PTP_α) that inhibits in vitro breast cancer growth but is paradoxically over-expressed in 29% of primary breast cancer (Ardini et al., 2000). The phosphatase PTP1B, on the other hand, is hyperexpressed in primary breast tumors (Wiener et al., 1994) and required for Her-2 induced breast epithelial cell transformation in vitro (Arias-Romero et al., 2009) and for Her-2 resultant mammary tumor development in mice (Bentires-Alj and Neel, 2007). However, PTP1B inhibits the transformation in fibroblasts by Her-2 (Brown-Shimer et al., 1992) and Ras (Liu and Chernoff, 1998) and no studies so far have shown a role of PTP1B in regulating human breast cancer growth. PTPH1, on the other hand, is induced by Ras and overexpressed in primary colon (Hou et al., 2010) and breast cancers (Figure 1), and stimulates the malignant growth in both ER⁺ and ER⁻ breast cancer (Figure 3). To our knowledge, this may be the first report showing that an over-expressed PTP in primary breast tumors acutely promotes human breast cancer growth. Further experiments are needed to investigate if PTPH1 promotes breast cancer growth in vivo and whether PTPH1 signals through Ras and/or Her-2 to increase breast cancer development and progression.

Our result that a nuclear-localization deficient VDR loses its growth-inhibitory activity is consistent with the conclusion that PTPH1 may increase breast cancer growth through increasing cytoplasmic VDR expression and resultant mutual stabilization. Although previous studies implicate that only nuclear VDR is transcriptional active (Prufer et al., 2000; Prufer et al., 2002) and/or growth inhibitory (Garay et al., 2007; Yang et al., 2001), no studies reported thus far have directly demonstrated a functional role of the NLS in VDR regulating malignant growth. While VDR/mNLS expressed cells tend to be more proliferative than the vector control, its enforced expression alone failed to significantly increase breast cancer cell growth (Figures 7c and d), indicating that the cytoplasmic

receptor may only act to assist other associated oncoproteins, such as PTPH1, to promote the malignant growth. In supporting this speculation, two recent clinical studies showed that increased cytoplasmic VDR protein expression in primary lung cancer predicts an advanced stage of the disease (Menezes et al., 2008) and in primary colon cancer correlates with PIK3CA and K-Ras mutations (Kure et al., 2009). It would be of great interest to investigate further if increased PTPH1 expression in primary breast cancer couples with elevated cytoplasmic VDR expression and whether such combined up-regulations predict a poor clinic outcome. Studies of the signaling cross-talk between PTPH1 and VDR may reveal a novel strategy for cancer therapeutic targeting by regulating PTPH1 expression and/or VDR localizations.

Materials and Methods

Cell lines, constructs, shRNAs and reagents

MCF-7, T47D and 231 (MDA-MB-231) human breast cancer cells were purchased from ATCC and maintained as previously described (Qi et al., 2004; Qi et al., 2006). Mouse mammary tumor cell lines from VDR wild-type (+/+) and knockout (-/-) mice have been previously described (Zinser et al., 2003). HA-tagged wild-type PTPH1 and its phosphatase-deficient mutant (PTPH1/DA) expression constructs were kindly provided by Dr. N. K. Tonks (Yang and Tonks, 1991; Zhang et al., 1999). Wip1 expression plasmid was a gift from Dr. L. A. Donehower (Lu et al., 2005), while PR-B expression construct was kindly provided by Dr. C. R. Lange (Lange et al., 2000). VDR and ER expression plasmids as well as luciferase expression constructs driven by vitamin D response element (VDRE-Luc) or estrogen response element (ERE-Luc) have been previously described (Li et al., 2007; Obrero et al., 2002; Qi et al., 2006; Ward et al., 2001). A nuclear localization defective VDR (mNLS) was generated by PCR as previously described (Prufer et al., 2000) (primers: forward, 5'- CTTCTTCAGGCGAAGCATGCAGGGCGAGGCACTATTCACCTGCCC- 3'; Reverse: 5'- GGGCAGGTGAATAGTGCCTCGCCCTGCATGCTTCGCCTGAAGAAG- 3'). Lenti-virus systems were purchased from Invitrogen and used to deliver PTPH1 shRNA as described (Hou et al., 2010). The siRNA oligos against human VDR were purchased from Santa Cruz (sc-45920A) and used as a pool (Strand A: AUCCGUAGUCCCCUGAAA; Strand B: CACGUUCCUUACUGCAGAA; Strand C: GGAACUCCUGGAAAUAUCA) by using a non-target oligo as a control. MEM medium, serum and other cell culture materials were supplied by Gibco and chemicals by Sigma. 1, 25-dihydroxyvitamin D3 {1,25(OH)2D3 or vitamin D3} was supplied by Hoffmann-La Roche (Nutley, NL). Antibodies used in this study include V5 from Invitrogen, HA from Immunology Consultants Lab (Oregon), p38 γ from R&D Systems, and Flag (sc-807), PTPH1 (sc-9789), and GAPDH (sc-47724) were from Santa Cruz. A mouse anti-PTPH1 monoclonal antibody was a gift from Dr. Nicholas Tonks.

Gene expression and silencing

The inducible expression system (T-Rex) was purchased from Invitrogen and used to express a full-length human PTPH1 in MCF-7 and MDA-MB-231 cells as previously described (Qi et al., 2006). For stable PTPH1 protein expression, cells were either transfected with PTPH1 and PTPH1/DA in a pcDNA3 vector (Qi et al., 2004) or infected

with lentiviral PTPH1 (Hou et al., 2010), followed by antibiotic selection, whereas Fugene6 was used for transiently expressing PTPH1 in VDR^{+/+} and VDR^{-/-} cells. To silence PTPH1, the shLuc or shPTPH1 containing lenti-viruses were generated in packaging cells and used to infect target cells through antibiotic selection (Hou et al., 2010). To express PTPH1 in Tet-on system, cells were incubated with and without Tet overnight and then subjected to various analyses (Qi et al., 2006).

Colony formation and cyquant[®] NF cell proliferation assays

To determine the long-term effect of PTPH1 on cell growth, colony formation assays were performed as described (Chen and Waxman, 1994). Briefly, 500 cells were plated per well in 6-well plates and colonies formed were stained, photographed and manually counted about two weeks later. To assess proliferation of VDR^{+/+} and VDR^{-/-} mouse breast tumor and PTPH1 expressed human breast cancer T47D cells, 1500 cells were seeded per well in a 96-well plate and cell growth was assessed using the cyquant[®] NF cell proliferation assay kit according to the manufacturer's instructions. Resultant fluorescence signals were measured in a plate reader (Invitrogen, Sunnyvale, CA) with excitation at 485 nm and emission detection at 530 nm. To assess effects of ligand, vitamin D3 was added into the culture at a final concentration of 10 nM with a proper solvent control throughout the entire period.

Human breast cancer specimens and immunohistochemistry (IHC)

All human breast cancer tissues were collected by Department of Pathology, Medical College of Wisconsin with informed consent. The IHC analyses were conducted in accordance with Institutional Review Board approval from Medical College of Wisconsin. Briefly, sections of formalin-fixed and paraffin-embedded blocks were subjected to immuno-staining as described (Hou et al., 2010). A mouse anti-PTPH1 (1:600) was used as primary antibodies. Staining intensity and extent were scored independently by two observers and a consensus score was reached and assigned to each case, based on the criteria: 0, negative, 1 to 4, weak; 5 to 8, moderate, and 9 to 12, strong staining as described (Hou et al., 2010).

Cell fractionation, Immunoprecipitation (IP) and Western blot analyses

To prepare cell fractions, cells were lysed in a buffer containing 20 mM HEPES, pH 7.4, 5 mM KCl, 0.137 mM NaCl, 5.5 mM glucose, 10 μ M EDTA, 0.05% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin, followed by incubation at 4°C for 20 min. Lysates were then centrifuged at 1000 rpm to separate cytoplasmic fraction from nuclei (Qi et al., 2006). Resultant pellets were washed and re-suspended in 200 μ l of buffer containing 1% Triton X-100, 400 mM KCl, 10 mM Tris-HCl, pH 7.5, 0.1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, and 1 μ g/ml leupeptin. For IP analyses, cells were washed with cold PBS and lysed in modified RIPA buffer, which were then incubated with a primary antibody overnight. For cell fractionation, protein concentration in each preparation was measured and the same amount of protein was analyzed by WB, while for direct WB, cells were lysed in 1x loading buffer and separated by SDS-PAGE (Qi et al., 2006).

Luciferase reporter assays and qRT-PCR

For luciferase assay, cells were transiently transfected with VDRE-Luc (Li et al., 2007) or ERE-Luc (Qi et al., 2004) and assayed for luciferase activity using a dual luciferase kit (Promega) in a TD-20/20 Luminometer (Turner Designs). To measure VDR and CYP24 expression, total RNAs were prepared by TRIzol and subjected to quantitative real-time reverse transcription PCR analyses (qRT-PCR) using iScript™ One-Step RT-PCR approach with SYBR® Green kit (Bio-Rad Laboratories) as described (Loesch et al., 2010). Primers for VDR are: forward, 5'-CTTCAGGCGAAGCATGAAGC-3'; reverse, 5'-CCTTCATCATGCCGATGTCC-3' and for CYP24: forward, 5'-GTG GCT CCA GCC AGA CCC TA-3', reverse, 5'-GGC GAG GTT GGT ACG AGG TG-3' and for GAPDH, forward, 5'-GGT GGT CTC CTC TGA CTT CAA CA-3', reverse, 5'-GTT GCT GTA GCC AAA TTC GTT GT-3'. Results are normalized to GAPDH and expressed as relative to the vector control.

Statistical analysis

Colony numbers, VDRE activity and CYP24 RNA expression were analyzed by Student's t test or ANOVA for statistical difference. A Kruskal-Wallis test was used to examine the correlation of PTPH1 protein expression with other pathological parameters in primary breast cancers. Increased protein expression (PTPH1) in breast cancer tissues versus matched normal tissues was analyzed by a paired t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

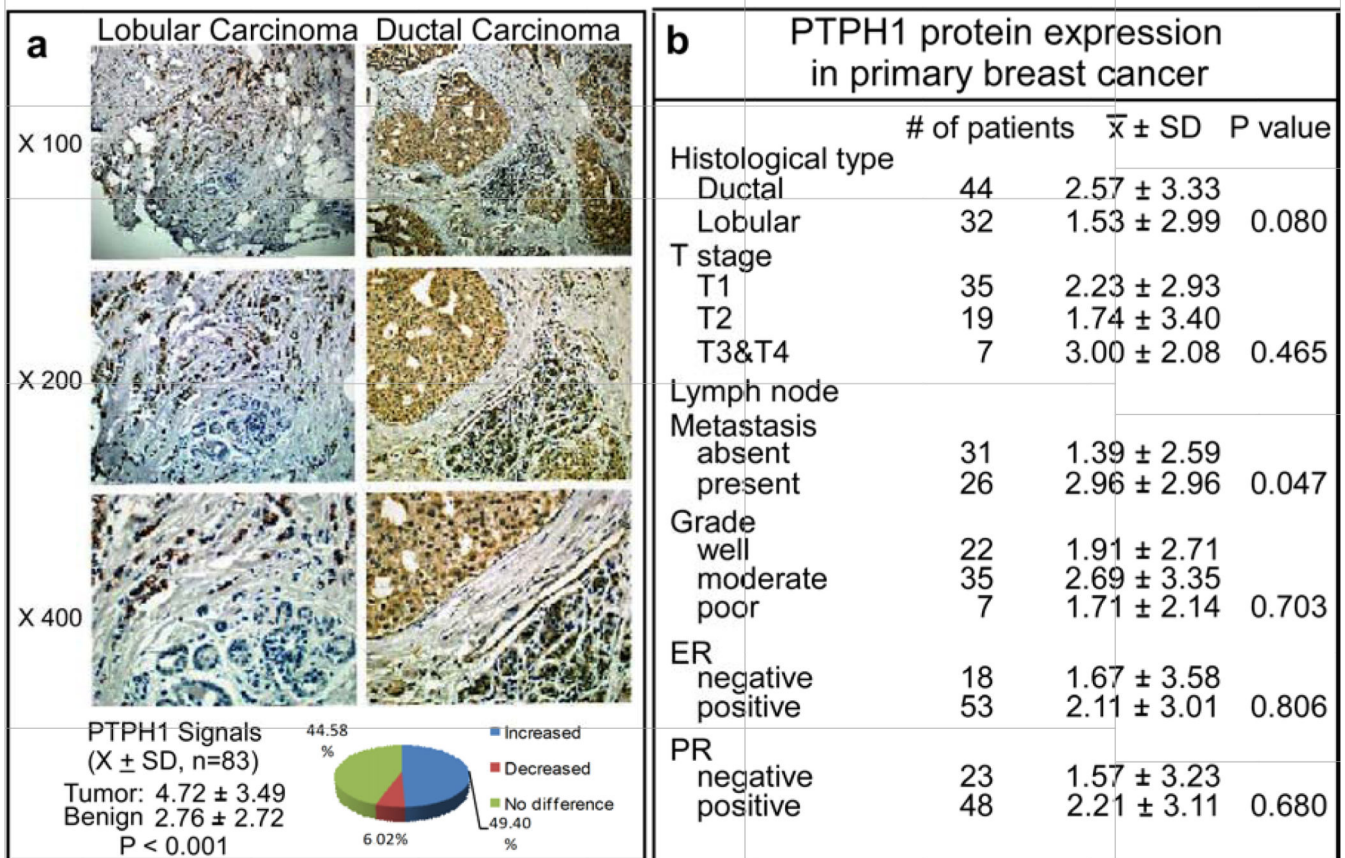
This study was supported by grants from National Institutes of Health (2R01 CA91576), Department of Veterans Affairs (Merit Review), and Cancer Center of Medical College of Wisconsin (G.C.). We would like to thank Drs Nicholas K. Tonks, JoEllen Welsh, Jiahui Han, Leonard Freedman, David Shapiro, Lawrence A. Donehower and Carol E. Lange for various reagents that made this work possible. We also appreciate Justin Reitsman and Dr. Xiaomei Qi for the technical support and Chen's lab members for suggestions and discussions.

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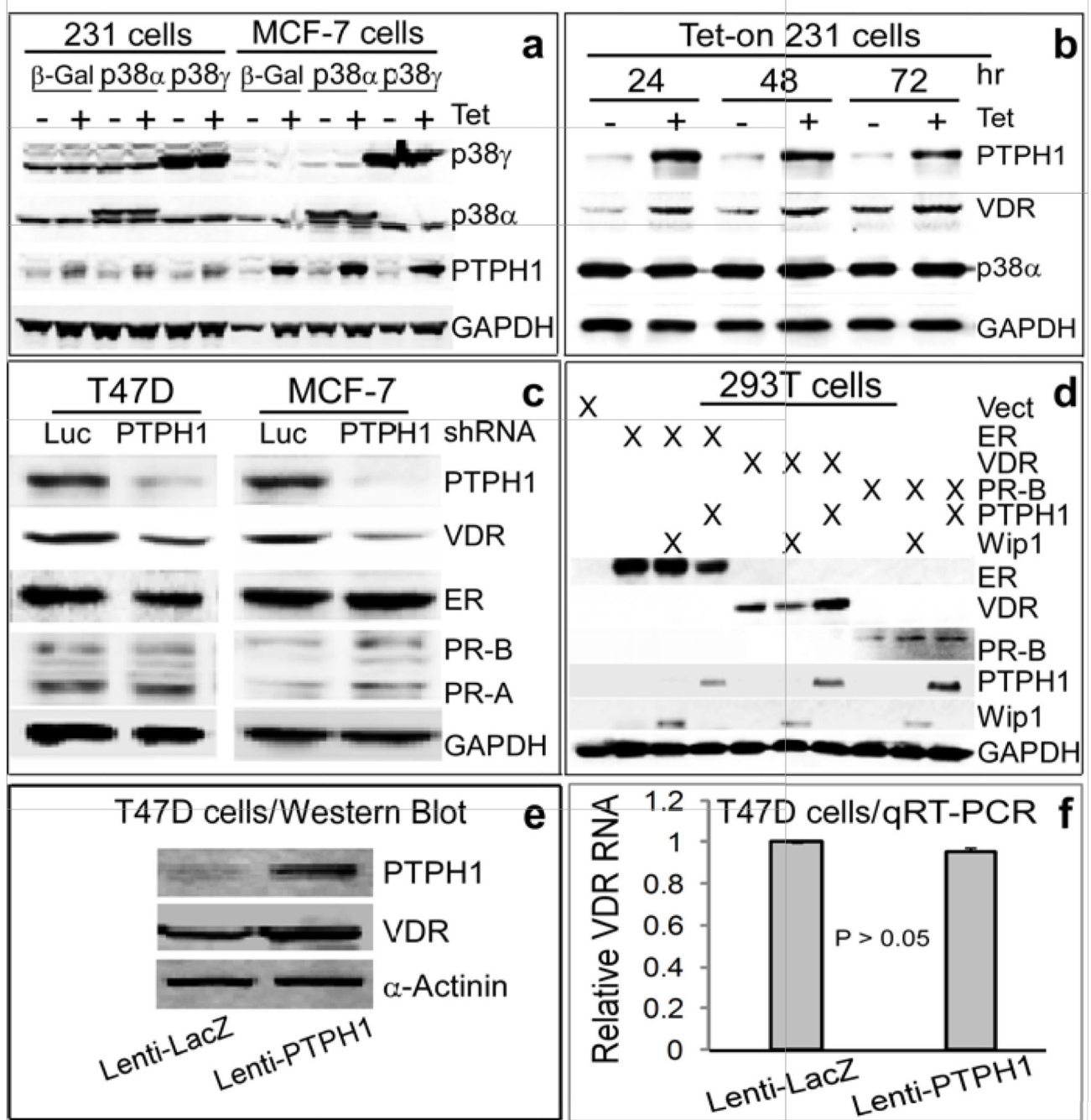
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**Figure 1.**

PTPH1 is overexpressed in primary human breast cancer and levels of its protein expression significantly correlate with the clinical metastasis. **(a)** Representative pictures showing increased PTPH1 protein expression in ductal and lobular carcinomas with the bottom panel summarizing PTPH1 signal increases in this group of breast cancer tissues (tumor) over their matched normal controls (benign). **(b)** Pathological parameters for PTPH1 protein expression in breast cancer patients. Please note that the case number for the individual parameters varies due to the incomplete clinic information. The P values were calculated by Kruskal-Wallis test.

**Figure 2.**

PTPH1 increases VDR but not ER or PR protein expression. (a) p38_γ fails to increase PTPH1 expression in breast cancer cells. Tet-on PTPH1 cells were infected with adenoviruses expressing β-galactosidase (Gal) or p38_{α/γ} and examined for protein expression by WB (no phosphorylated p38_γ and p38_α were detected under this condition). (b) PTPH1 increases VDR protein expression. Cells were cultured with and without Tet for indicated time and examined for protein expression. (c) PTPH1 depletion specifically decreases VDR expression. Cells were stably depleted of PTPH1 protein expression by

lentiviral infection and examined for protein expression. **(d)** A specific stimulation of VDR protein expression by co-expressed PTPH1 in 293T cells. Cells were transiently transfected with indicated constructs and assessed for protein expression 24 hr later. **(e, f)** PTPH1 increases VDR protein but not RNA expression. T47D cells were stably expressed with PTPH1 by lenti-viral infection, which were then examined for VDR protein expression by Western blot **(e)** and for VDR RNA expression by qRT-PCR **(f)**.

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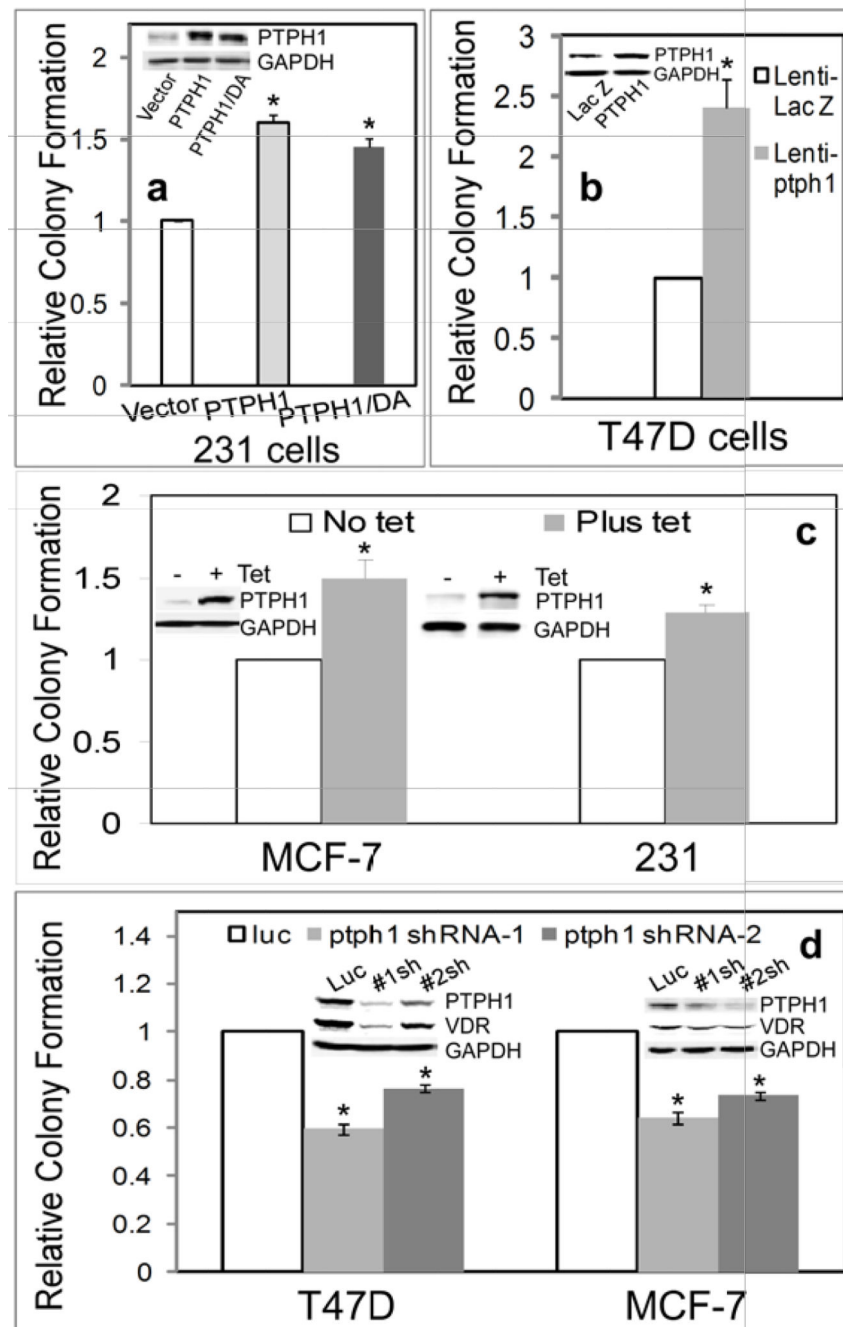


Figure 3. PTPH1 stimulates breast cancer growth. (a, b, c) PTPH1-forced expression increases colony formation. PTPH1 and/or its phosphatase-deficient mutant was expressed in breast cancer cells via stable transfection or infection or Tet-on system as indicated and modified cells were examined for protein expression (inserts) and colony formation (mean of at least three separate experiments, bars, SE, the same for all other colony formation and proliferation assays, * $P < 0.05$ vs. vector or Lac-Z or no Tet control). (d) PTPH1 depletion decreases VDR expression and inhibits breast cancer growth. Cells were stably depleted of PTPH1 by

lentiviral shRNA and assessed for protein expression (inserts) and colony formation (* P < 0.05 vs. respective control).

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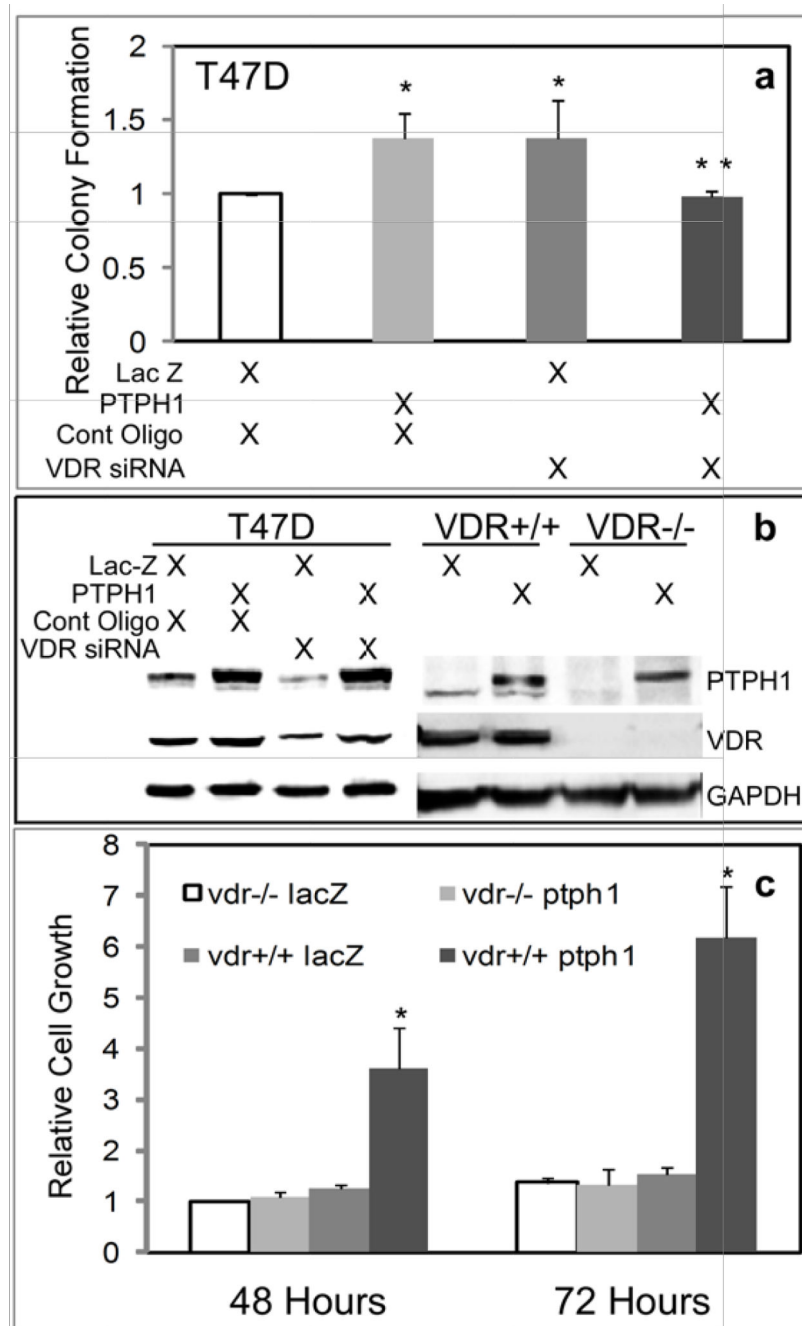


Figure 4. PTPH1 requires VDR to increase breast cancer growth. **(a, b, left)** VDR depletion blocks PTPH1 stimulation of human breast cancer growth. The vector and PTPH1 stably transfected cells were incubated with control and VDR siRNA oligos for 48 hr, which were then analyzed for protein expression and colony formation (* $P < 0.05$ vs. Lac-Z plus control oligo; ** $P < 0.05$ vs. PTPH1 or VDR siRNA and $P > 0.05$ vs. Lac-Z plus control oligo). **(b, right, c)** PTPH1 only increases proliferation in VDR expressed mouse breast cancer cells. VDR^{+/+} and VDR^{-/-} cells were stably expressed with PTPH1 by lentiviral infection and

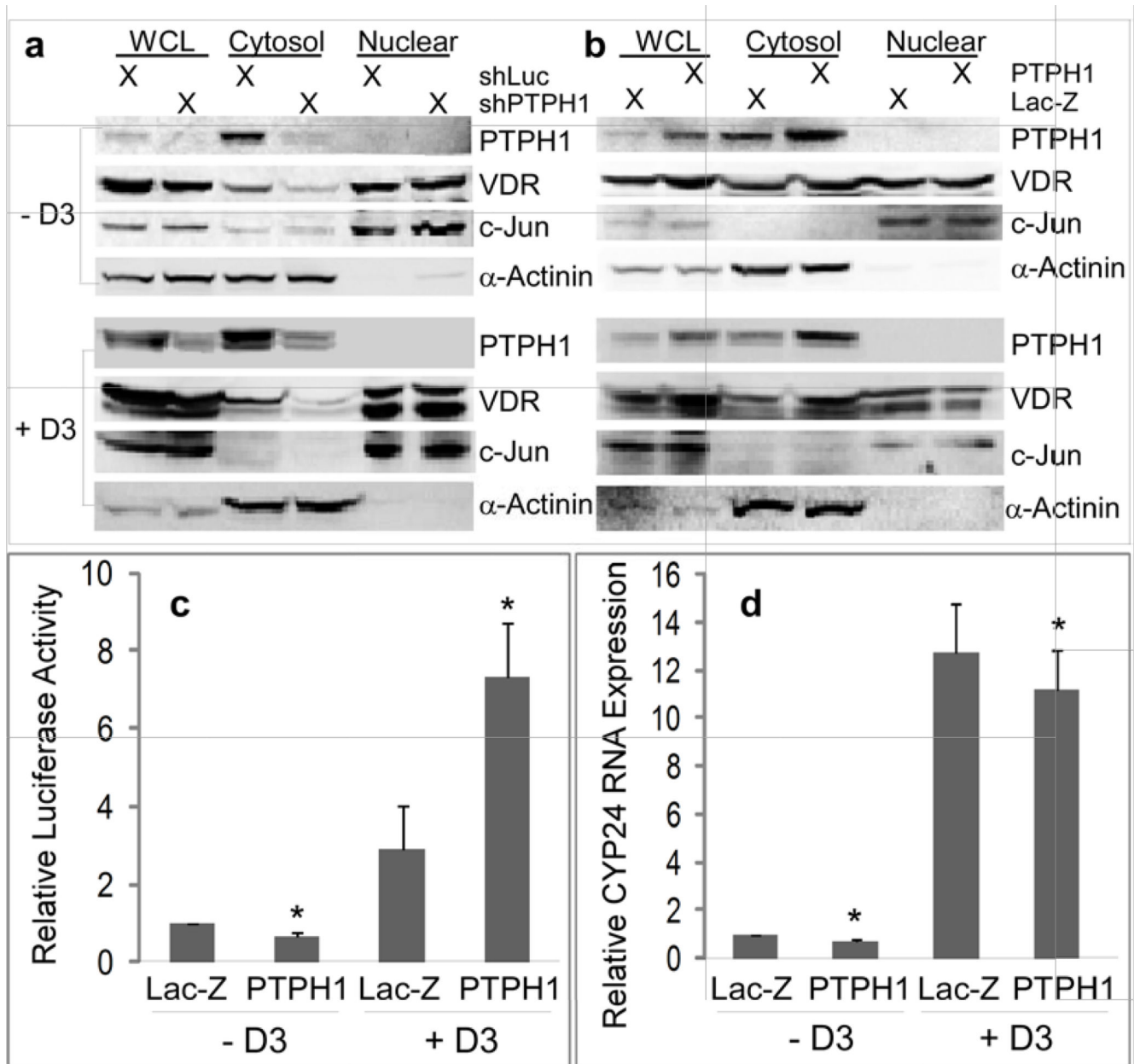
analyzed for protein expression and cell proliferation 48 and 72 hr after plating by a fluorescence plate reader (* P < 0.05 vs. Lac-Z control).

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**Figure 5.**

PTPH1 increases cytoplasmic VDR protein expression and inhibits VDR transcriptional activity. (a, b) PTPH1 expression increases and its depletion decreases cytoplasmic VDR protein expression. T47D breast cancer cells stably engineered for PTPH1 overexpression or depletion were subjected to cell fractionation and the same amount of proteins analyzed for protein expression by including the whole cell lysates (WCL) collected in 1 X loading buffer for comparison {top two panels: - D3 (without vitamin D3)}. A separate set of experiments was performed by incubation of cells with 10 nM vitamin D3 for 24 h before analyses {bottom two panels: + D3 (plus vitamin D3)}. (c) PTPH1 inhibits VDRE-Luc activity only in the absence of vitamin D3. PTPH1 or vector stably expressed T47D cells were transiently

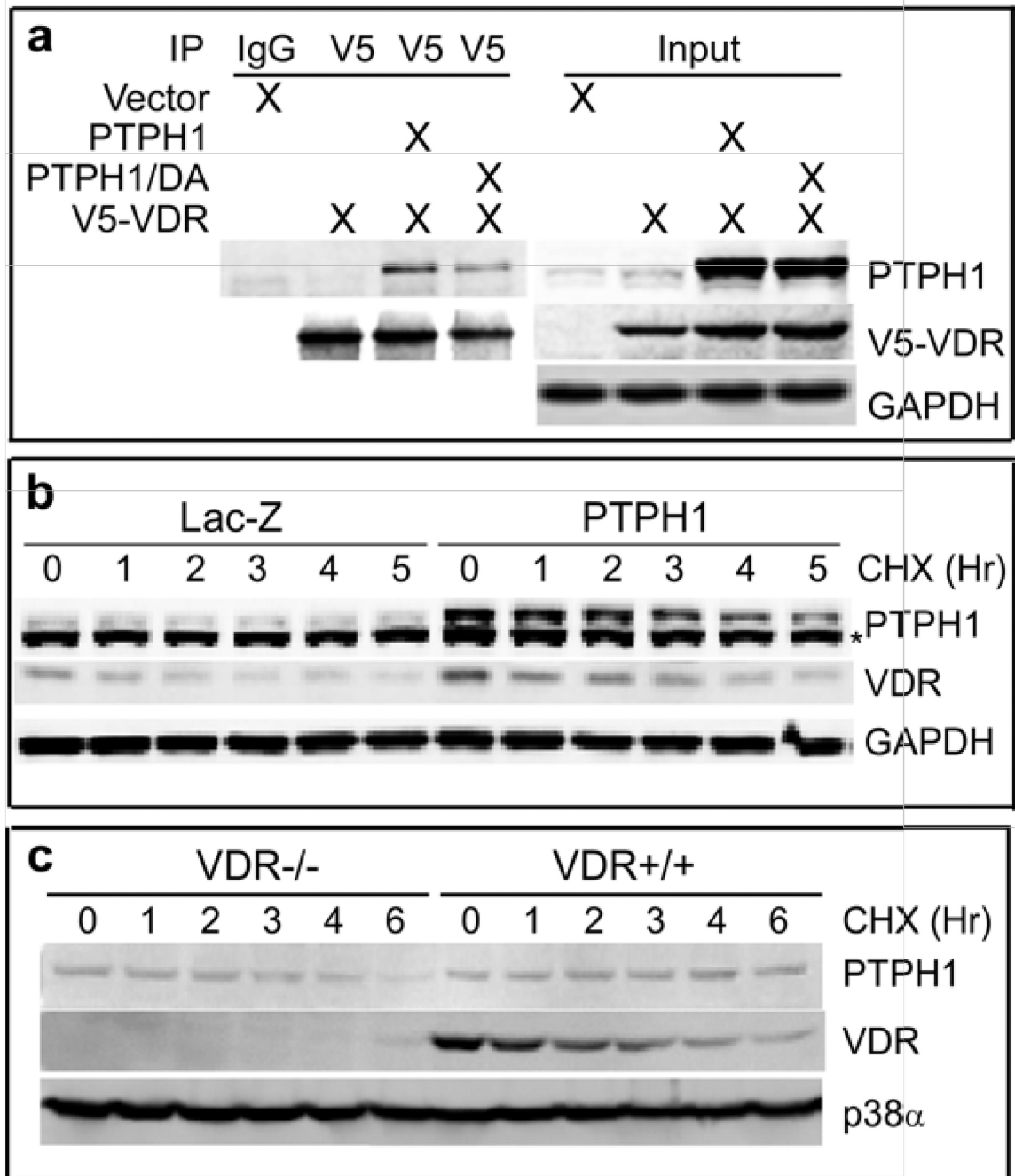
transfected with a VDRE-Luc and luciferase activity assessed 48 hr later following treatment with and without 10 nM vitamin D3 for the last 24 h (* P < 0.05). (d) PTPH1 decreases the VDR target gene CYP24 expression independent of vitamin D3. Indicated T47D cells were cultured with 10 nM vitamin D3 or solvent control (ethanol) and analyzed for CYP24 RNA expression by qRT-PCR (* P < 0.05).

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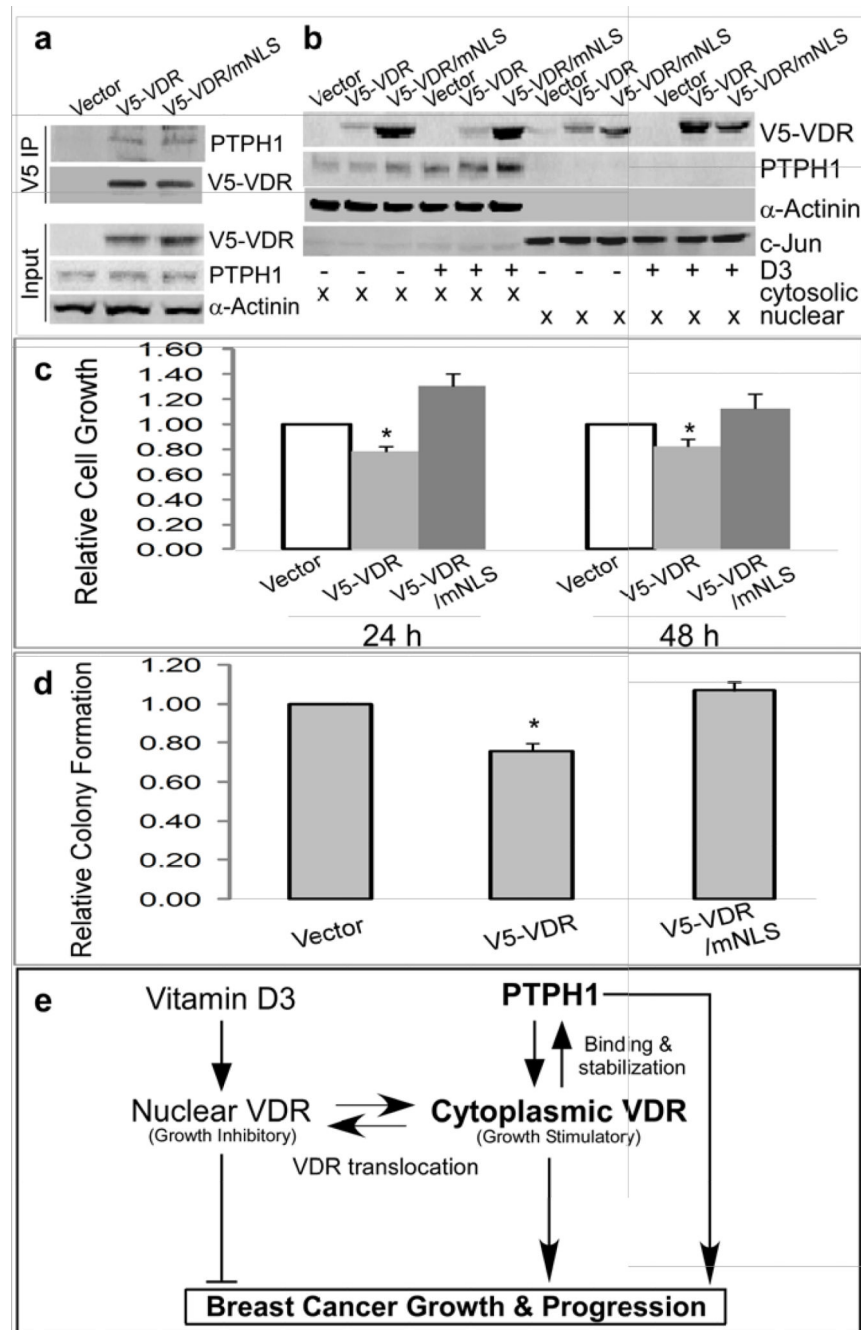
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**Figure 6.**

PTPH1 forms a complex with VDR leading to their mutual stabilization. (a) PTPH1 and its mutant bind VDR protein in 293T cells. (b) PTPH1 increases VDR protein stability in T47D cells. The vector and PTPH1 stably expressed cells were incubated with cycloheximide (CHX, 100 μ g/ml) for the time as indicated and analyzed for protein expression (* indicates a non-specific band). (c) VDR increases PTPH1 stability. Cells were transiently expressed with HA-PTPH1 and analyzed for protein expression 48 hr later as described above.

**Figure 7.**

A nuclear localization-deficient VDR loses its growth-inhibitory activity in breast cancer cells. (a) VDR binds PTPH1 in breast cancer cells. T47D cells stably expressed with VDR or its mutant were analyzed for protein complex formation by V5 IP and WB analyses. (b) Regulatory effects of vitamin D3 on VDR and its mutant nuclear translocation. Cells were cultured in the absence and presence of 10 nM vitamin D3 for 24 h and collected for cell fractionation analyses. (c, d) Indicated cells were assessed for proliferation (c) or colony formation (d) (* P < 0.05 vs. vector or VDR expressed cells) as described in Figures 4c and

3, respectively. (e) PTPH1 stimulates breast cancer growth through increasing cytoplasmic VDR expression. Nuclear VDR is known to be growth inhibitory, whereas our results presented here suggest that cytoplasmic VDR loses this inhibitory function. Since PTPH1 promotes breast cancer growth by a mechanism that couples with its activity to bind and stabilize VDR protein and to increase cytoplasmic VDR expression, our results of a VDR-dependent growth-stimulation by PTPH1 together with a decreased PTPH1 protein expression / stability in VDR depleted cells suggest that PTPH1 may increase breast cancer growth through cooperation with cytoplasmic VDR via mutual stabilization. This model suggests that regulation of PTPH1 expression and/or VDR localization may be a new approach to control breast cancer growth and progression.