Cell Reports
Article

Identification of PHRF1 as a Tumor Suppressor that Promotes the TGF-β Cytostatic Program through Selective Release of TGIF-Driven PML Inactivation

Asma Ettahar,[1,7] Olivier Ferrigno,[1,7] Ming-Zhu Zhang,[2] Mutsuko Ohnishi,[2] Nathalie Ferrand,[1] Céline Prunier,[1] Laurence Levy,[1] Marie-Françoise Bourgeade,[3] Ivan Bieche,[4] Damian G. Romero,[5] Frédéric Colland,[6] and Azeddine Atfi[1,2,*]

[1]Laboratory of Cell Signaling and Carcinogenesis, INSERM UMRS938, 184 Rue du Faubourg St-Antoine, 75571 Paris, France
[2]Harvard School of Dental Medicine, 188 Longwood Avenue, Boston, MA 02115, USA
[3]INSERM U785, 12 Avenue Paul Vaillant Couturier, 94807 Villejuif, France
[4]INSERM U735, 35 Rue Dailly, 92211 St-Cloud, France
[5]Department of Biochemistry, University of Mississippi Medical Center, 2500 N. State Street, Jackson, MS 39216, USA
[6]Hybrigenics, 3-5 impasse Reille, 75014 Paris, France
[7]These authors contributed equally to this work
*Correspondence: azeddine.atfi@inserm.fr

http://dx.doi.org/10.1016/j.celrep.2013.07.009

SUMMARY

The homeodomain protein TGIF (TG-interacting factor) restricts TGF-β/Smad cytostatic signaling by interfering with the nucleocytoplasmic transit of the tumor suppressor cPML. Here, we identify PHRF1 as a ubiquitin ligase that enforces TGIF decay by driving its ubiquitination at lysine 130. In so doing, PHRF1 ensures redistribution of cPML into the cytoplasm, where it associates with SARA and coordinates activation of Smad2 by the TGF-β receptor. The *PHRF1* gene resides within the tumor suppressor locus 11p15.5, which displays frequent loss in a wide variety of malignancies, including breast cancer. Remarkably, we found that the *PHRF1* gene is deleted or silenced in a high proportion of human breast cancer samples and cancer cell lines. Reconstitution of PHRF1 into deficient cells impeded their propensity to form tumors in vivo, most likely because of the reemergence of TGF-β responsiveness. These findings unveil a paradigm behind inactivation of the cPML tumor suppressor network in human malignancies.

INTRODUCTION

TGIF (TG-interacting factor) belongs to the superfamily of homeodomain proteins that regulate a vast array of biological processes, including embryonic development, proliferation, and differentiation. TGIF was originally identified by virtue of its ability to compete with retinoid receptors for binding to their cognate promoters and thereby suppresses retinoic acid (RA) signaling (Bertolino et al., 1995). Besides retinoic signaling, TGIF was shown to function as a suppressor of TGF-β (transforming growth factor β) signaling, which regulates cell fate in a variety of physiological contexts ranging from embryonic development to adult tissue homeostasis (Feng and Derynck, 2005; Whitman, 1998). TGF-β initiates responses by contacting two types of transmembrane Ser/Thr kinases called type I (TβRI) and type II (TβRII) receptors, promoting phosphorylation and activation of TβRI by the TβRII kinase (Massagué et al., 2005). In the canonical pathway, the activated TβRI propagates signals by phosphorylating Smad2 and Smad3 (Smad2/Smad3), a process coordinated by the adaptor protein SARA (Smad anchor for receptor activation) (Tsukazaki et al., 1998). The phosphorylation of Smad2/Smad3 also depends on their interaction with the cytoplasmic variant of the promyelocytic leukemia protein (cPML), whose functions are first to bridge together Smad2/Smad3 and SARA and then to bring that complex within the proximity of the TGF-β receptor (Lin et al., 2004; Seo et al., 2006). Phosphorylation of Smad2/Smad3 induces dissociation from SARA and cPML with concomitant association with Smad4 and translocation to the nucleus, where they induce expression of TGF-β target genes through cooperative interaction with general coactivators, prominent among them p300/CBP (Massagué et al., 2005).

Although TGIF was initially thought to function in the nucleus as a Smad transcriptional corepressor because of its ability to associate with histone deacetylases (HDACs), recent advances made in our laboratory argue that the TGIF inhibitory action mainly proceeds via mechanisms unrelated to transcriptional repression. For instance, we have demonstrated that TGIF can facilitate degradation of active Smad2/Smad3 through recruitment of the E3 ubiquitin ligase WWP1/Tiul1, although this function appears to be restricted to terminating TGF-β signaling (Seo et al., 2004). Moreover, we found that TGIF can function at early steps to constrain initiation of Smad signaling, presumably by shifting the nucleocytoplasmic shuttling of cPML toward

the nucleus, in turn precluding the assembly of cPML-SARA complex that is instrumental to Smad2/Smad3 phosphorylation by TβRI (Faresse et al., 2008; Lin et al., 2004; Seo et al., 2006).

The *PML* gene encodes a tumor suppressor that was initially identified in patients with acute promyelocytic leukemia (APL), where it is fused to RARα as a consequence of reciprocal t(15;17) chromosomal translocations (Salomoni and Pandolfi, 2002; Scaglioni and Pandolfi, 2007). In transgenic mice, expression of PML-RARα in the myeloid lineage causes leukemia with features of APL. Functionally, PML controls a variety of processes, such as growth suppression, apoptosis, or senescence (Salomoni and Pandolfi, 2002; Scaglioni and Pandolfi, 2007). The ability of PML to achieve such tumor suppressor responses underscores the possibility that its recurrent inactivation may also culminate in the pathogenesis of nonhematological malignancies. Unequivocal support to this concept came with the demonstration that PML-deficient mice are more susceptible to chemical- or oncogene-induced carcinogenesis in multiple tissues (Salomoni and Pandolfi, 2002; Scaglioni and Pandolfi, 2007). From a clinical point of view, PML inactivation has been noted in a large portion of human cancers, where it correlates with poor prognosis (Gurrieri et al., 2004). Notwithstanding these intriguing hints highlighting the vulnerability of PML to alterations that are inherent to cancer development, at this point, it is becoming increasingly obvious that additional efforts should be put into the discovery of mechanistic paradigms of PML inactivation, given that they remain, for the most part, elusive. The present study sheds light on these persistent concerns because we identify and characterize PHRF1 as a TGIF ubiquitin ligase whose deficiency culminates in impairment of the cPML tumor suppressor network that empowers TGF-β-induced cytostatic responses. We provide proof-of-principle experiments that the *PHRF1* gene is somatically altered in a high proportion of breast cancers, findings that could have a profound impact on our ability to predict breast cancer rates in large numbers of patients and may even influence screening, follow-up, and treatment options.

RESULTS

Interaction of PHRF1 with TGIF

To identify potential modifiers of the TGIF/cPML interplay that could be prone to disruption in cancer, we undertook a yeast two-hybrid approach using TGIF as bait. Screening of a human universal cDNA library yielded recovery of a gene with unknown functions, named in databases as PHRF1 (PHD and RING finger 1). PHRF1 possesses an N-terminal PHD/bromodomain followed by a RING finger and a large C-terminal domain of unique sequence (see Figure 2G). To determine whether PHRF1 binds to TGIF in mammalian cells, we conducted coimmunoprecipitation experiments using transfected MDCK cells. As shown in Figure 1A, PHRF1 interacts robustly with TGIF at steady state, and exposure of cells to TGF-β induced a marked decrease in this interaction, which became apparent only at 2 hr poststimulation and persisted for at least 6 hr. This decrease depends on protein neosynthesis because cotreatment of cells with the transcription inhibitor actinomycin D blocked TGF-β-induced disassembly of the PHRF1-TGIF complex (Figure S1C). The interaction of PHRF1 with TGIF is specific because PHRF1 failed to interact with c-Ski (Figure 1B), which shares with TGIF many biological properties related to suppression of TGF-β signaling (Massagué et al., 2005). Likewise, we were unable to detect an interaction between TGIF and Ectodermin (Figure 1C), another PHD/RING finger-containing protein operating in the TGF-β signaling pathway (Dupont et al., 2009).

To analyze in more detail the PHRF1-TGIF interaction, we sought to define the domains that mediate their interaction. Employing various TGIF deletion mutants, we mapped the PHRF1-interacting region to the middle region of TGIF between amino acids 148 and 164 (Figures 1D and S1A). Reciprocally, we tested the interaction of a series of PHRF1 deletion mutants with TGIF and found that TGIF bound to the N-terminal domain spanning the PHD/RING finger motif (Figure 1E).

Next, we raised a highly specific antibody for PHRF1, as gauged by immunoblotting of extracts from cells either overexpressing or depleted of PHRF1 by small hairpin RNA (shRNA) (Figure S1B). Using two TGF-β-sensitive cell lines, HepG2 and HaCat, we could clearly detect a strong interaction between endogenous PHRF1 and TGIF, but this was decreased in response to TGF-β, becoming manifest 2–4 hr poststimulation (Figure 1F). Together, these results demonstrate that PHRF1 and TGIF can form a physical complex whose stoichiometry appears to decline upon prolonged activation of TGF-β signaling.

PHRF1 Contributes to TGF-β Signaling

We next wondered whether expression of PHRF1 could influence critical aspects of TGF-β signaling, such as transcriptional and cytostatic responses. Notably, overexpression of PHRF1 enhanced the ability of TGF-β to induce transcription from two distinct reporters, $CAGA_9$-Lux and ARE_3-Lux, which are readouts of Smad3 and Smad2, respectively (Figure S2A). This finding was further substantiated by using MDCK cells stably overexpressing PHRF1, which showed that ectopic expression of PHRF1 was effective in enhancing the sensitivity of cells to TGF-β-induced expression of endogenous PAI-1 and growth arrest (Figures 2A and 2B). Such ability of PHRF1 to foster TGF-β signaling likely represents a widespread phenomenon because HepG2 or HaCat cells stably overexpressing PHRF1 also displayed enhanced sensitivity to TGF-β-induced expression of PAI-1 as well as growth arrest (Figures S2B and S2C). Of note, TGF-β stimulation did not induce apoptosis or senescence in these cells (data not shown), suggesting that PHRF1 may contribute to the ability of TGF-β to block DNA synthesis and progression through the cell cycle.

We also investigated whether PHRF1 deficiency could affect TGF-β transcriptional and growth inhibitory responses. In transient transfection experiments, expression of two independent specific siRNAs both induced a marked decrease in TGF-β-induced $CAGA_9$-Lux activity (Figure S2D). To further demonstrate that depletion of PHRF1 disrupts TGF-β signaling while further ruling out off-target effects, we initially generated MDCK cells stably expressing a shRNA targeting a third region of PHRF1 (sh-PHRF1). As anticipated, depletion of PHRF1 in MDCK cells prevented TGF-β-induced expression of endogenous PAI-1 and growth arrest (Figures 2C and 2D). We also generated HepG2 or HaCat cells stably expressing two independent PHRF1 shRNAs and again found that PHRF1 knockdown

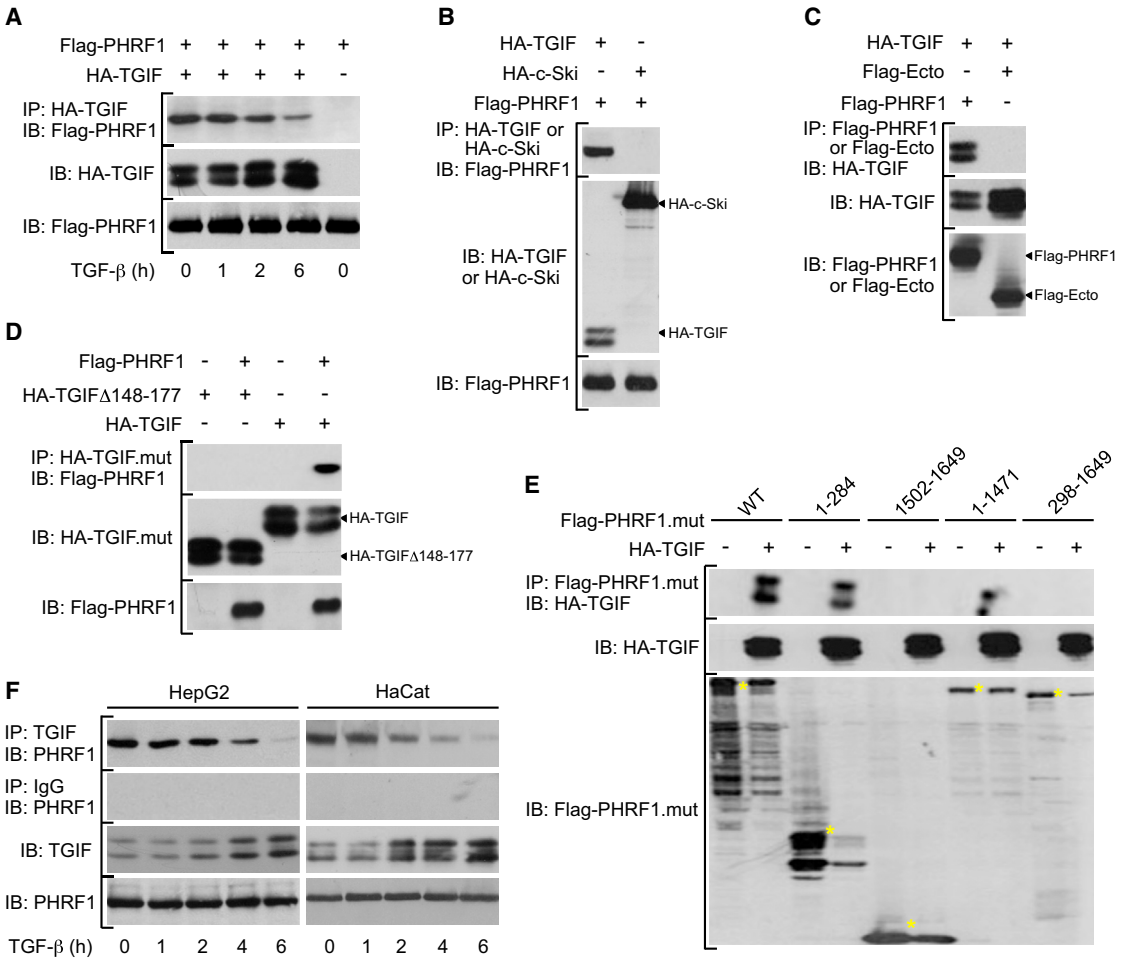

Figure 1. PHRF1 Interacts with TGIF

(A) MDCK cells were transfected with Flag-PHRF1 in the absence or presence of HA-TGIF and treated with TGF-β for increasing times. Cell lysates were subjected to anti-HA immunoprecipitation (IP) followed by immunoblotting (IB) with anti-Flag. In this and all the following experiments, the expression of proteins under investigation (input) was determined by direct immunoblotting.

(B and C) 293 cells were transfected with the indicated combinations of HA-TGIF, Flag-PHRF1, HA-c-Ski, and Flag-Ecto. Cell lysates were subjected to anti-HA (B) or anti-Flag (C) immunoprecipitation followed by immunoblotting with anti-Flag (B) or anti-HA (C).

(D and E) Mapping the regions of TGIF and PHRF1 that mediate their interaction using extracts from 293 cells transfected with the indicated combinations of TGIF and PHRF1 deletion mutants is shown. WT, wild-type.

(F) HepG2 or HaCat cells were left untreated or treated with TGF-β for increasing times, and extracts were immunoprecipitated with anti-TGIF or IgG. Immune complexes were detected by immunoblotting with anti-PHRF1.

See also Figure S1.

was able to block TGF-β-induced expression of PAI-1 as well as expression of two other endogenous TGF-β/Smad target genes, Smad7 and JunB (Figures 2E and S2E). Depletion of PHRF1 in HaCat or HepG2 cells also blunted the growth inhibitory effect of TGF-β (Figure 2F), in line with the idea that PHRF1 functions as a bona fide component of the TGF-β signaling pathway in multiple cell systems. In another strategy, taking advantage of our discovery of cancer cell lines deficient in PHRF1 (e.g., MCF7-TR and MDA-MB435), we found that restoration of PHRF1 expression rendered cells responsive to TGF-β-induced expression of PAI-1 and growth arrest (see Figures 6F, 6G, S6F, and S6G). Collectively, these results argue that PHRF1 is a critical determinant of TGF-β responses, and they further raise the interesting possibility that loss of PHRF1 may attenuate TGF-β cytostatic signaling in cancer cells.

To gain initial insights into the mode of action of PHRF1, we attempted to rescue the TGF-β transcriptional response in MCF7-TR using our panel of PHRF1 deletion mutants. Interestingly, expression of either PHRF1.mutA or PHRF1.mutC, which includes the RING finger and retains their TGIF binding properties, rescued TGF-β transcriptional responses (Figures 1E and 2G). However, none of the other two mutants (i.e., mutB and mutD) that we tested restored TGF-β responsiveness. Because none of the later mutants binds to TGIF, we concluded that the physical interaction between PHRF1 and TGIF is essential for the ability of PHRF1 to promote TGF-β signaling.

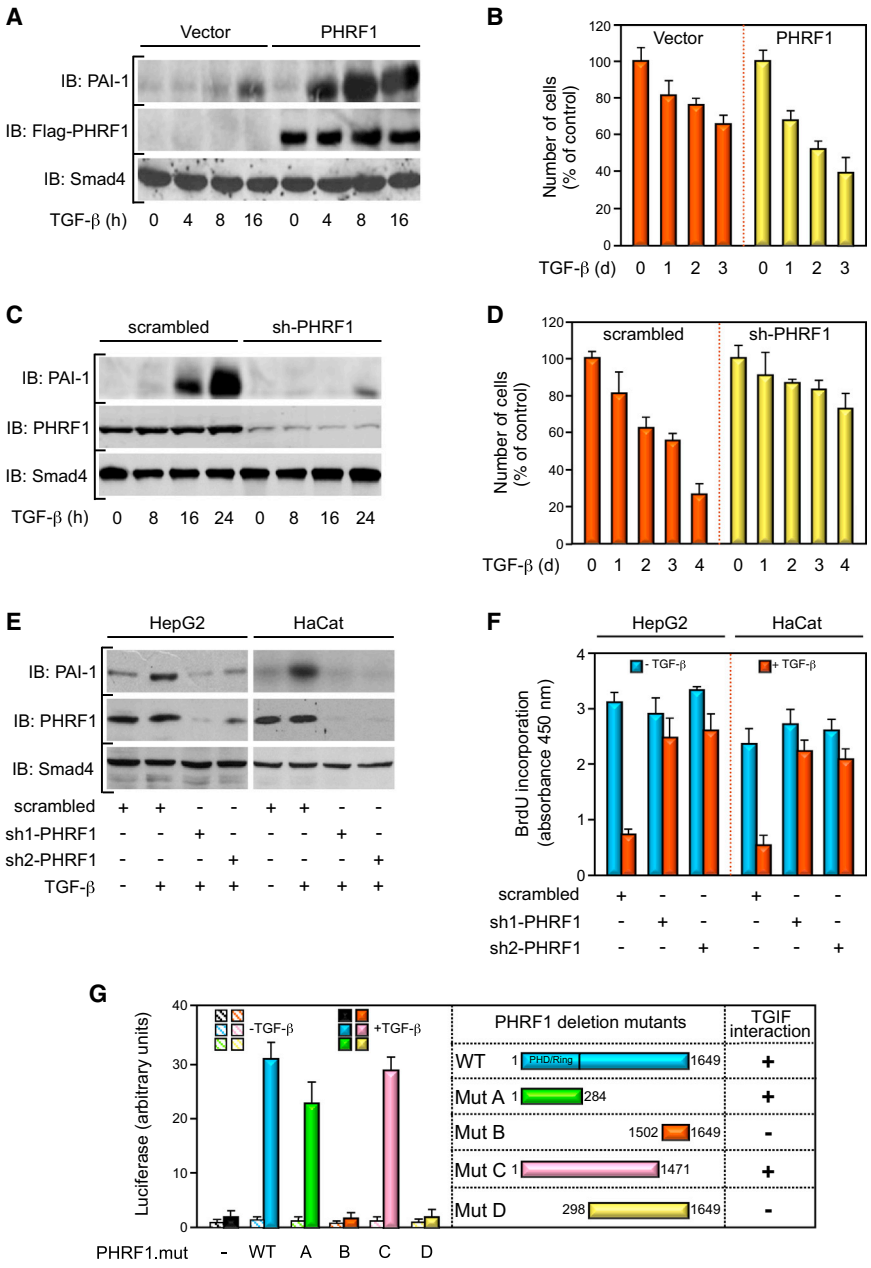

Figure 2. PHRF1 Promotes TGF-β Signaling

(A and B) MDCK cells stably expressing empty vector or PHRF1 were treated with 200 pM TGF-β for increasing times. (A) Cell lysates were analyzed by immunoblotting using antibodies against PAI-1, Flag, or Smad4 as a loading control. (B) Cells were counted automatically using a cell counter, and the results (mean ± SD of triplicates) were expressed as the percentage of cells cultured in the absence of TGF-β.

(C and D) MDCK cells stably expressing scrambled or sh-PHRF1 were treated with 200 pM TGF-β for increasing times. Then, TGF-β-induced expression of PAI-1 (C) or growth arrest (D) was determined as described in (A) and (B), respectively.

(E and F) HaCat or HepG2 cells were stably transfected with scrambled or two different shRNAs (sh1-PHRF1 and sh2-PHRF1) and treated with or without TGF-β for 16 hr (E) or 24 hr (F). (E) Expression of PAI-1 was determined by immunoblotting. (F) Cell proliferation was determined by the incorporation of BrdU, and the results (absorbance at 450 nm) were expressed as mean ± SD of triplicates from a representative experiment performed three times.

(G) MCF7-TR cells were transfected with $CAGA_9$-Lux and PHRF1 deletion mutants, treated with or without TGF-β, and analyzed for luciferase activity. Data are expressed as mean ± SD (n = 3).

See also Figure S2.

PHRF1 Induces TGIF Degradation

Quite unexpectedly, we noticed during our earlier analyses of the PHRF1/TGIF interaction that overexpression of PHRF1 resulted in decreased expression of the TGIF protein in a manner dependent on their association (Figure 1D). We also noticed that activation of TGF-β signaling induced accumulation of the TGIF protein, concurring with decreased association of PHRF1 and TGIF (Figures 1A and 1F). Crucially, under the same experimental conditions, TGF-β did not affect the abundance of TGIF mRNA (Figure S3A). Together, these findings provide initial hints that PHRF1 might affect TGIF stability. We at first attempted to corroborate this notion by demonstrating that, although depletion of PHRF1 dramatically increased the abundance of the endogenous TGIF protein, it failed to affect the abundance of TGIF mRNA (Figures 3A and S3B). Next, we reasoned that if PHRF1 indeed compromises TGIF stability, then expression of PHRF1 should modulate biological processes other than TGF-β signaling that are subject to regulation by TGIF, such as repression of RA signaling. In fact, overexpression of PHRF1 enhanced RA-induced transcription with efficiency similar to that elicited by depletion of TGIF (Figure S3C). As a specificity control, expression of PHRF1 did not influence the activity of a Notch reporter, similar to depletion of TGIF (Figure S3D). Hence, PHRF1 and TGIF appear to regulate the same cellular functions but with opposite outcomes, which is in consonance with the hypothesis that PHRF1 may hamper TGIF stability.

Having discovered that PHRF1 affects the abundance of the TGIF protein, we next investigated the possibility that PHRF1 might function as a TGIF ubiquitin ligase. As shown in Figure 3B, treatment of cells with the proteasome inhibitor MG132 prevented PHRF1-induced TGIF degradation. In alternative experimental approaches, we found that overexpression of PHRF1 not only provoked a striking increase in ubiquitin-conjugated TGIF protein but also enhanced its turnover (Figures 3C and 3D). To directly demonstrate that PHRF1 functions as a TGIF ubiquitin ligase, we introduced a point mutation in the RING finger

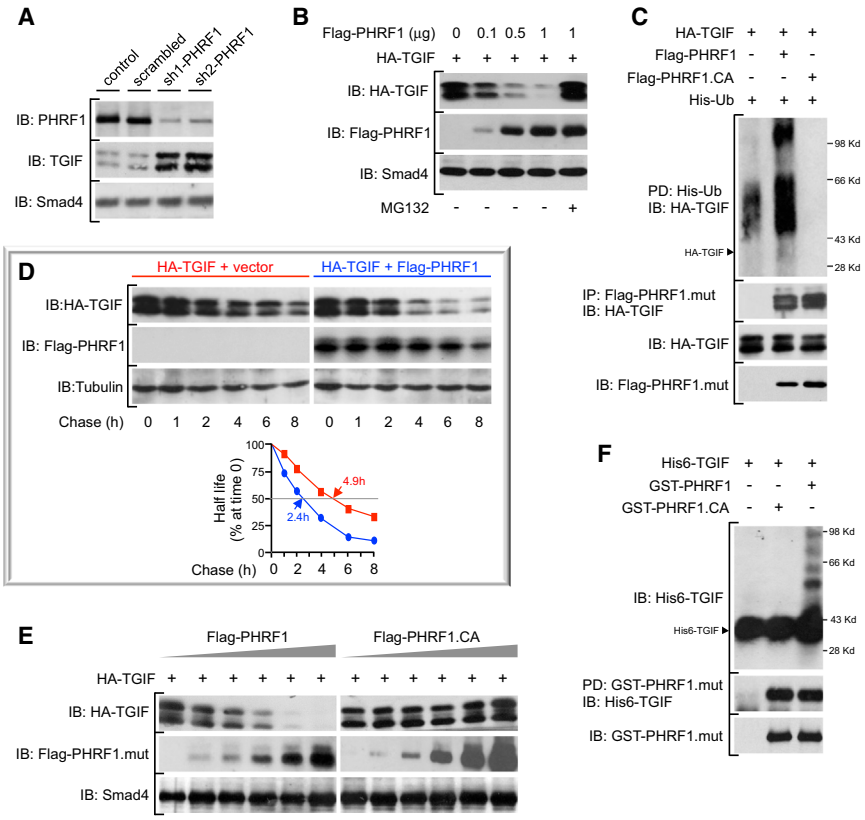

Figure 3. PHRF1 Triggers TGIF Degradation

(A) Lysates of MDCK cells stably transfected with scrambled or two different PHRF1 shRNAs (sh1-PHRF1 and sh2-PHRF2) were analyzed by immunoblotting using antibodies against PHRF1, TGIF, or Smad4 as a loading control.

(B–E) 293 cells were transfected with the indicated combination of HA-TGIF, Flag-PHRF1, Flag-PHRF1.CA, and His-Ub. (B) Cells were treated with vehicle or MG132 for 6 hr and analyzed by immunoblotting. (C) Cells were incubated with MG132 for 6 hr, and lysates were pulled down (PD) with nickel-Sepharose and immunoblotted with anti-HA. To detect the interaction of TGIF with PHRF1, extracts were immunoprecipitated with anti-Flag and immunoblotted with anti-HA. (D) Cells were incubated with cycloheximide (CHX) for various time periods before being analyzed by immunoblotting. The expression levels of TGIF were determined by scanning laser densitometry, and the results were expressed as the percentage of control at time 0. The half-life of TGIF is indicated.

(E) Expression of TGIF or PHRF1 was determined by immunoblotting.

(F) In vitro ubiquitination assay was performed using purified His6-TGIF, GST-PHRF1, or GST-PHRF1.CA, and recombinant ubiquitin, E1, and UbcH5a. To detect ubiquitinated TGIF, samples were subject to immunoblotting with anti-His. To detect the association of TGIF with PHRF1, samples were pulled down with glutathione-Sepharose and immunoblotted with anti-His.

See also Figure S3.

(Cys108Ala), creating an inactive mutant (PHRF1.CA) that retains normal TGIF binding capability. In contrast to wild-type PHRF1, PHRF1.CA behaved as a dominant-negative mutant to boost the steady-state level of TGIF while decreasing its polyubiquitination (Figures 3C and 3E). An in vitro ubiquitination assay using purified proteins confirmed that PHRF1 was effective in catalyzing TGIF polyubiquitination, whereas PHRF1.CA was void of any enzymatic activity (Figure 3F).

PHRF1 Promotes TGF-β Signaling by Inducing Ubiquitination of TGIF at Lysine 130

To investigate in more detail the mechanism by which PHRF1 promotes TGIF degradation, we individually substituted all of the Lys by Arg (except Lys2, Lys4, Lys5, Lys100, and Lys103 that were processed simultaneously, given their close proximity in TGIF) and tested for their destabilization by PHRF1. The result indicated that only mutation of Lys130 (TGIF.K130R), which lies close to the PHRF1-interacting motif, made TGIF resistant to PHRF1 (Figure 4A). To corroborate this result, we designed several experiments to compare the ubiquitin-dependent degradation of TGIF.K130R and wild-type TGIF. Initially, we employed a FlpIn-dependent recombination system to achieve a single copy integration of TGIF mutants at the same genomic locus under the control of a Dox-inducible promoter, thereby avoiding any fluctuations that could arise from transient transfection or integration of multiple copies. As expected, these isogenic cell lines expressed similar levels of TGIF and TGIF.K130R mRNAs following induction with Dox (Figure S4A). However, immunoblotting revealed higher expression of TGIF.K130R, a difference that was abolished by MG132, which is indicative of increased stability of TGIF.K130R (Figure S4B). Next, we used the FlpIn cell system to compare the decay rate of TGIF and TGIF.K130R following Dox relaxation and found that TGIF.K130R displayed decreased turnover (Figure 4B). Finally, we found that TGIF.K130R was less polyubiquitinated than TGIF (Figure 4C). Of note, we used TGIF.K159R as an alternative control and found its ubiquitination status to be similar to that of wild-type TGIF (Figure 4C). These results indicate that a physiological function of PHRF1 is to trigger TGIF polyubiquitination at Lys130, with subsequent clearance through the proteasome pathway.

These preceding data at least suggest that targeting TGIF for degradation may be one of the possible mechanisms by which PHRF1 promotes TGF-β signaling. Consistent with this view, enforced expression of PHRF1.CA exerted a dominant-negative effect on TGF-β transcriptional responses, yet this was blunted by TGIF depletion (Figure 4D). To exclude the possibility that PHRF1 might deploy other mechanisms to foster TGF-β signaling, we sought to determine the extent to which enforced expression of PHRF1 affects the ability of TGIF.K.130R versus wild-type TGIF to suppress TGF-β-induced transcription, with the assumption that TGIF.K130R would preserve its inhibitory activity owing to escape from degradation by PHRF1. In contrast to wild-type TGIF, the inhibitory effect of TGIF.K130R was insensitive to coexpression of PHRF1 (Figure 4D). In a control sample, expression of PHRF1.CA was ineffective in relieving the inhibitory effect of TGIF (Figure 4D), lending further support to the

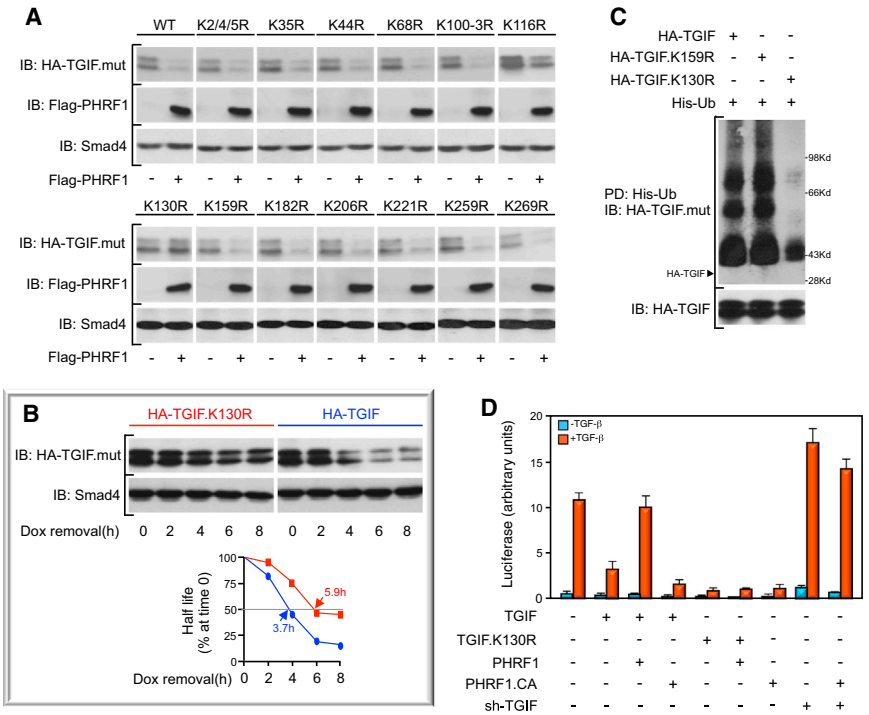

Figure 4. PHRF1 Promotes Ubiquitination of TGIF at Lys 130

(A) Effects of Flag-PHRF1 on the abundance of HA-TGIF Lys mutants were determined by immunoblotting analysis of lysates from transfected 293 cells.

(B) 293 FlpIn cells stably expressing wild-type HA-TGIF or HA-TGIF.K130R were treated with Dox for 24 hr. Then, cells were incubated in regular media for increasing times and subjected to immunoblotting. The half-life of each TGIF mutant is indicated.

(C) 293 cells were transfected with His-Ub together with HA-TGIF, HA-TGIF.K130R, or HA-TGIF.K159R, and cells were treated with MG132 for 6 hr. Then, cell lysates were pulled down with nickel-Sepharose and immunoblotted with anti-HA.

(D) HepG2 cells were transfected with $CAGA_9$-Lux and the indicated combinations of TGIF, TGIF.K130R, PHRF1, PHRF1.CA, and sh-TGIF, treated with or without TGF-β, and processed for luciferase activity. Data are expressed as mean ± SD (n = 3).

See also Figure S4.

hypothesis that the biological activity of PHRF1 in TGF-β signaling stems primarily from its ubiquitin ligase activity toward TGIF, rather than other mechanisms.

PHRF1 Facilitates cPML Function in TGF-β Signaling

The possibility raised by the foregoing observation, that PHRF1 promotes TGF-β signaling by opposing TGIF, was further investigated by evaluating another process affected by TGIF, namely phosphorylation of Smad2 (Faresse et al., 2008; Seo et al., 2006). As anticipated, TGF-β stimulation induced a transient increase in Smad2 phosphorylation, which peaked at 1 hr and gradually declined in the presence of continuous TGF-β, inversely correlating with changes in TGIF abundance (Figure S1D). Remarkably, depletion of PHRF1 in MDCK cells blocked TGF-β-induced phosphorylation of endogenous Smad2, a phenomenon that can be further supported by the impaired assembly of the endogenous Smad2/Smad4 complex, the formation of which depends on Smad2 phosphorylation (Figures 5A and 5B). On the other hand, we detected a marked increase in the sensitivity of cells to TGF-β-mediated phosphorylation of endogenous Smad2 in MDCK, HepG2, or HaCat cells stably overexpressing PHRF1 (Figures S5A and S5B).

Next, we carried out experiments to examine whether PHRF1 facilitates Smad2 phosphorylation by a mechanism that is dependent on TGIF. As shown in Figure S5C, overexpression of PHRF1 was able to counteract TGIF-suppressed Smad2 phosphorylation. In an alternative strategy, depletion of PHRF1 in control cells blocked Smad2 phosphorylation, whereas depletion of PHRF1 in cells that were simultaneously depleted of TGIF had little or no effect on Smad2 phosphorylation (Figure 5C), strengthening the hypothesis that PHRF1 may promote Smad2 phosphorylation by a mechanism that relies upon TGIF degradation.

One major mechanism by which TGIF suppresses Smad2 phosphorylation depends on its ability to sequester cPML in the nucleus (Faresse et al., 2008; Seo et al., 2006). Owing to its capability to destabilize TGIF, PHRF1 could promote Smad2 phosphorylation by facilitating relocalization of cPML into the cytoplasm. In an initial effort to approach this question, we found that depleting PHRF1 in MDCK cells prevented assembly of the endogenous cPML/SARA complex (Figure 5D), which is known to occur in the cytoplasm in the absence of TGF-β signaling (Faresse et al., 2008; Lin et al., 2004). Conversely, stable expression of PHRF1 in MDCK cells resulted in a marked increase in the association of endogenous cPML and SARA (Figure 5E). To extend these results, we carried out immunofluorescence experiments to determine whether depletion of PHRF1 could affect the subcellular distribution of endogenous cPML. Analysis of control cells showed that some of the PML staining localized to distinctive nuclear bodies, although strong diffuse cytoplasmic staining, which is reminiscent of cPML distribution, could also be detected (Figure 5F), as previously reported by Faresse et al. (2008), Lin et al. (2004), and Seo et al. (2006). Interestingly, depletion of PHRF1 resulted in the disappearance of the immunofluorescence staining in the cytoplasm, which likely occurred as a consequence of increased accumulation of the total TGIF pool in the nucleus (Figure S5D). A similar result was obtained by means of a cell fraction approach (Figure S5E). Thus, by fostering TGIF clearance, PHRF1 promotes cPML movement into the cytoplasm, where it functions in conjunction with SARA to facilitate Smad2 phosphorylation.

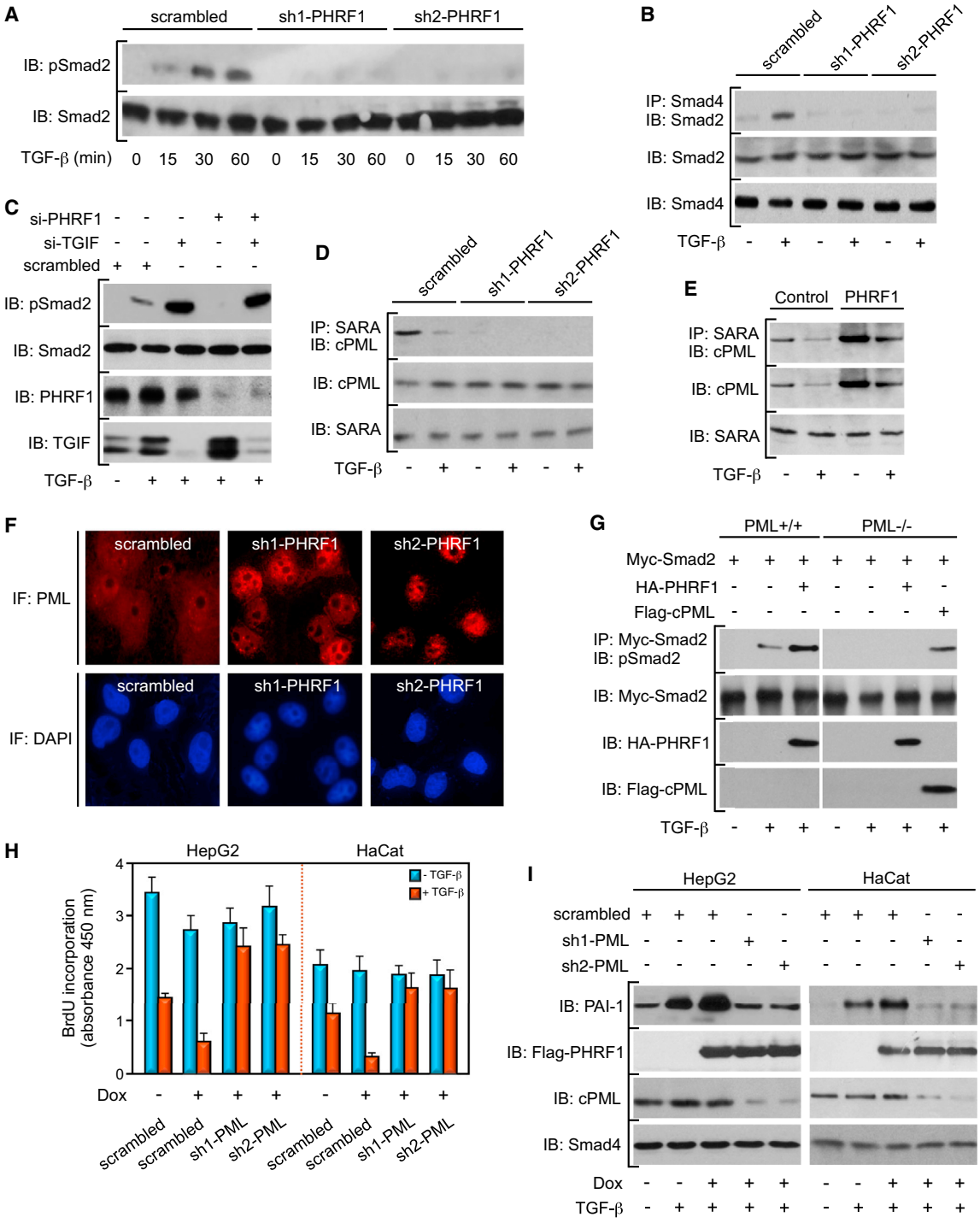

Figure 5. PHRF1 Facilitates cPML Function in TGF-β Signaling

(A and B) MDCK-scrambled, MDCK-sh1-PHRF1, or MDCK-sh2-PHRF1 cells were treated with or without TGF-β for increasing times (A) or 1 hr (B). (A) The phosphorylation of Smad2 was assessed by immunoblotting with anti-pSmad2. (B) The association of Smad2 with Smad4 was analyzed by blotting anti-Smad4 immunoprecipitates with anti-Smad2.

(C) 293 cells were transfected with the indicated combinations of scrambled, siTGIF, and siPHRF1, and cultured with or without TGF-β for 1 hr. Then, the phosphorylation of Smad2 was assessed by immunoblotting.

(D and E) MDCK cells stably depleted for (D) or overexpressing (E) PHRF1 were treated with or without TGF-β for 1 hr, and the association of endogenous cPML with SARA was visualized by blotting anti-SARA immunoprecipitates with anti-PML.

(F) MDCK-scrambled, MDCK-shPHRF1, or MDCK-shPHRF2 cells were immunostained with anti-PML (red) or DAPI (blue).

(legend continued on next page)

To directly demonstrate that PHRF1 promotes Smad2 phosphorylation by facilitating relocalization of cPML into the cytoplasm, we conducted comparative experiments using wild-type and PML$^{-/-}$ MEFs. We surmised that PML deficiency would compromise PHRF1-induced Smad2 phosphorylation if this merely proceeds through the release of cPML from nuclear retention constraint due to TGIF destabilization. In fact, overexpression of PHRF1 enhanced TGF-β-induced phosphorylation of Smad2 in wild-type MEFs but failed to do so in PML$^{-/-}$ MEFs (Figure 5G), indicating that PHRF1 and cPML function in the same biochemical circuit that governs Smad2 phosphorylation. In a control sample, add back of cPML into PML$^{-/-}$ MEFs restored TGF-β-induced Smad2 phosphorylation to the level detected in wild-type MEFs.

The data described so far suggest that cPML is epistatic to PHRF1 and strengthen the notion that PHRF1 might function to facilitate relocalization of cPML to the cytoplasm, where it coordinates phosphorylation of Smad2. If this prediction holds true, then cPML deficiency should impinge on PHRF's ability to promote TGF-β signaling. To probe this possibility, we chose to deplete cPML in HaCat or HepG2 cell lines we engineered to express PHRF1 under the control of a Dox-inducible promoter. As expected, induction of PHRF1 expression enhanced TGF-β-induced growth arrest and expression of PAI-1, Smad7, and JunB. Crucially, depletion of cPML severely blunted those effects (Figures 5H, 5I, and S5F). To validate this finding, we devised a reciprocal strategy aimed at investigating whether PHRF1 deficiency could impinge on cPML's ability to promote TGF-β signaling. As expected, stable reconstitution of PML$^{-/-}$ MEFs with cPML rescued the sensitivity of cells to TGF-β-induced growth arrest and expression of PAI-1, Smad7, and JunB (Figures S5G and S5H). Remarkably, depletion of PHRF1 rendered cPML-reconstituted PML$^{-/-}$ MEFs again resistant to TGF-β, further underscoring the epistatic relationship between PHRF1 and cPML.

Deregulated Expression of PHRF1 in Human Breast Cancer

Interrogation of databases revealed that the *PHRF1* gene maps to 11p15.5, a strong candidate tumor suppressor locus displaying frequent loss of heterozygosity (LOH) in several human solid cancers, including breast cancer (Ali et al., 1987; Jonas and Kimonis, 2001; Karnik et al., 1998; Winqvist et al., 1995). It is also worth mentioning that two recent independent studies reported two somatic mutations in the *PHRF1* gene in breast cancer: one of them is a missense mutation, whereas the other is located within an intron (Ellis et al., 2012; Nik-Zainal et al., 2012). Moreover, we found a deletion and several point mutations in the PHRF1-coding region in other types of cancer (http://www.sanger.ac.uk), further suggesting that PHRF1 may display the prominent hallmarks of a tumor suppressor gene. These facts, together with our preceding findings revealing PHRF1 as a key player in the TGF-β cytostatic program, prompted us to assess the clinical relevance of PHRF1 in human cancers. Accordingly, we performed qRT-PCR experiments using 106 breast tumor samples representing a progressive spectrum of neoplasia, ranging from benign breast tumors to advanced metastasis. When compared with adjacent normal tissue, a marked decrease in PHRF1 mRNA (cutoff value of more than a 2-fold decrease) was detected, even at early stages of neoplasia, although the frequency of such alteration tends to increase in more advanced stages, reaching 57% of samples from patients with metastasis (Figures 6A and 6B). We cross-validated this finding by gene expression profiles (up to 28% decrease) derived from two data sets summarizing 786 breast cancers: E-MTAB-365 and GSE4922 (Guedj et al., 2012; Ivshina et al., 2006). During the course of these analyses, we also found a robust correlation (correlation coefficient, 0.74; $p < 0.001$) between expression of PHRF1 and probable activation of TGF-β signaling, using combined expression of p21, p15, PAI-1, and JunB as a TGF-β signature.

Next, we extended our analysis to genomic DNA, seeking possible alterations in the *PHRF1* gene. To this end, we used high-resolution array CGH to quantify DNA copy number of the *PHRF1* gene in a series of 37 primary breast tumors. The result indicated that eight samples (21.6%) carried deletion of the *PHRF1* gene (Figures S6A and S6B). This deletion appears to be specific because many neighboring genes were not affected or even amplified. Consistent with our findings, recent analyses (http://www.broadinstitute.org/tcga/home) indicated that PHRF1 is significantly deleted across the entire data set of the 4,404 tumors analyzed at the Broad Institute, including breast cancer (20.9%) (Figures S6A and S6C). Comparative analysis indicated that deletion of PHRF1 occurs with a frequency approaching that of BRCA1 (31%) or BRCA2 (26%), the most prominent tumor suppressor genes of breast cancer. Together, these observations strongly suggest that PHRF1 is frequently deleted or mutated in breast cancer and that somatic alterations in the *PHRF1* gene might account, at least in part, for the loss of PHRF1 mRNA expression.

To begin dissecting the impact of PHRF1 loss on breast cancer pathogenesis, we screened a number of human breast cancer cell lines for PHRF1 deficiency. Analysis of PHRF1 mRNA revealed that two out of eight (25%) of the cell lines displayed a near-complete extinction of PHRF1 expression (Figures 6C and 6D), which is in excellent agreement with the frequency of PHRF1 loss in tumor samples. Here again, we cross-validated the loss of PHRF1 in breast cancer cell lines by researching public databases (http://www.sanger.ac.uk). Our efforts revealed that 19 out of 53 (30%) exhibit LOH at the PHRF1 locus.

(G) PML$^{+/+}$ or PML$^{-/-}$ MEFs were transfected with the indicated combinations of Myc-Smad2, HA-PHRF1, and Flag-cPML, and treated with or without TGF-β for 1 hr. The phosphorylation of Smad2 was visualized by blotting anti-Myc immunoprecipitates with anti-pSmad2.

(H and I) HepG2 or HaCat cells stably expressing Dox-inducible PHRF1 were infected with lentiviruses encoding scrambled or two different shRNAs targeting PML (sh1-PML and sh2-PML). Cells were left untreated or treated with Dox for 24 hr before being treated with or without TGF-β for 24 hr (H) or 16 hr (I). (H) Cell proliferation was determined by the BrdU method, and data are expressed as mean ± SD (n = 3). (I) Cell lysates were immunoblotted with antibodies against PAI-1, Flag, PML, or Smad4 as a loading control.

See also Figure S5.

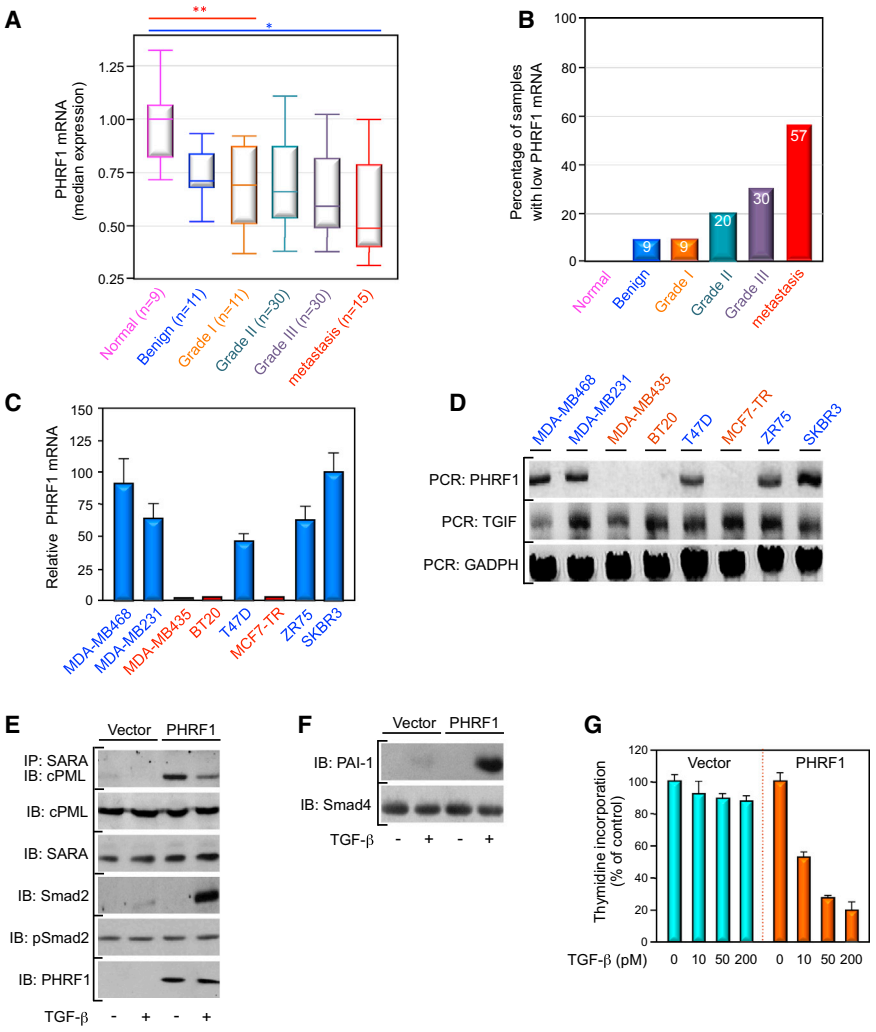

Figure 6. Deregulation of PHRF1 in Breast Cancer

(A and B) Expression of PHRF1 mRNA in human breast cancer samples was determined by qRT-PCR, and the results were normalized on the basis of TBP expression. (A) The results are represented as the median of normalized expression of PHRF1 mRNA in each sample during different stages of tumor progression (*$p < 0.05$, **$p < 0.01$). (B) Percentage of samples with low expression of PHRF1 mRNA in each stage of tumor progression (cutoff value of more than 2-fold decrease) is shown.

(C and D) Expression of PHRF1 mRNA in human cancer cell lines was determined by qRT-PCR, and the results were normalized on the basis of TBP expression and expressed as mean ± SD (n = 3) (C). Portions of the qRT-PCR products were analyzed by agarose gel (D).

(E and F) MCF7-TR cells stably transfected with empty vector or PHRF1 were treated with or without TGF-β for 1 hr (E) or 16 hr (F). Then, the association of endogenous cPML and SARA (E), phosphorylation of Smad2 (E), or expression of PAI-1 (F) was examined.

(G) MCF7-TR cells stably transfected with empty vector or PHRF1 were treated with increasing doses of TGF-β for 24 hr, and cell proliferation was determined by the thymidine incorporation method. Data (mean ± SD of triplicates) are expressed as percentage of the radioactivity incorporated by cells in the absence of TGF-β.

See also Figure S6.

To ascertain whether any causal relationship between PHRF1 loss and tumor pathogenesis could exist, we first carried out a set of experiments to establish whether restoring expression of PHRF1 in the PHRF1-defective cancer cell line MCF7-TR could rescue TGF-β cytostatic signaling. We found these cells to be impaired in their capacity to support the assembly of the cPML/SARA complex, a defect that was corrected by restoration of PHRF1 expression (Figure 6E). The ability of PHRF1 to rescue the assembly of the cPML/SARA complex is physiologically relevant because expression of PHRF1 also rendered these cells sensitive to TGF-β-induced phosphorylation of Smad2, expression of PAI-1, and growth arrest (Figures 6F and 6G). It should be noted that restoration of PHRF1 expression in BT20 cells failed to restore TGF-β signaling, suggesting that these cells might accumulate alterations in other components of this pathway (data not shown). Regardless of the mechanism behind the loss of TGF-β signaling in BT20, the data outlined so far suggest that the PHRF1 level may exert profound influence on TGF-β signaling in breast cancer cells. However, this concept is not limited to breast cancer cells because we have found by serendipity that the melanoma cell line MDA-MB435 (used in this study because it was initially thought to be a breast cancer cell line) is deficient in PHRF1 expression (Figures 6C and 6D). Here again, restoration of PHRF1 expression in MDA-MB435 was able to rescue TGF-β responses (Figures S6D–S6G).

Dysfunction of the TGF-β cytostatic program is a prominent hallmark of human cancers (Derynck et al., 2001; Dumont and Arteaga, 2003; Massagué, 2008). Because PHRF1 appears to exert a tight control over this aspect of TGF-β signaling, we were curious if PHRF1 deficiency could provide a selective growth advantage to breast tumor cells. We chose to tackle this question by determining whether restoring expression of PHRF1 in MCF7-TR cells could affect their propensity to form tumors. Analysis of tumor growth in mice revealed that restoration of PHRF1 expression did indeed suppress tumor growth (Figures 7A–7C and S7A–S7C). This effect could be attributable to restoration of autocrine TGF-β cytostatic signaling because MCF7-TR cells were found to secrete TGF-β, and more crucially, depletion of TβRII blocked the ability of PHRF1 to suppress tumor formation in vivo (Figures 7A–7C). Similar results were obtained when tumor growth was analyzed using an in vitro surrogate assay for tumorigenicity: the soft agar colony-forming assay (Figure 7D). We also investigated whether PHRF1 restricts tumor formation by a mechanism dependent on TGIF degradation. The

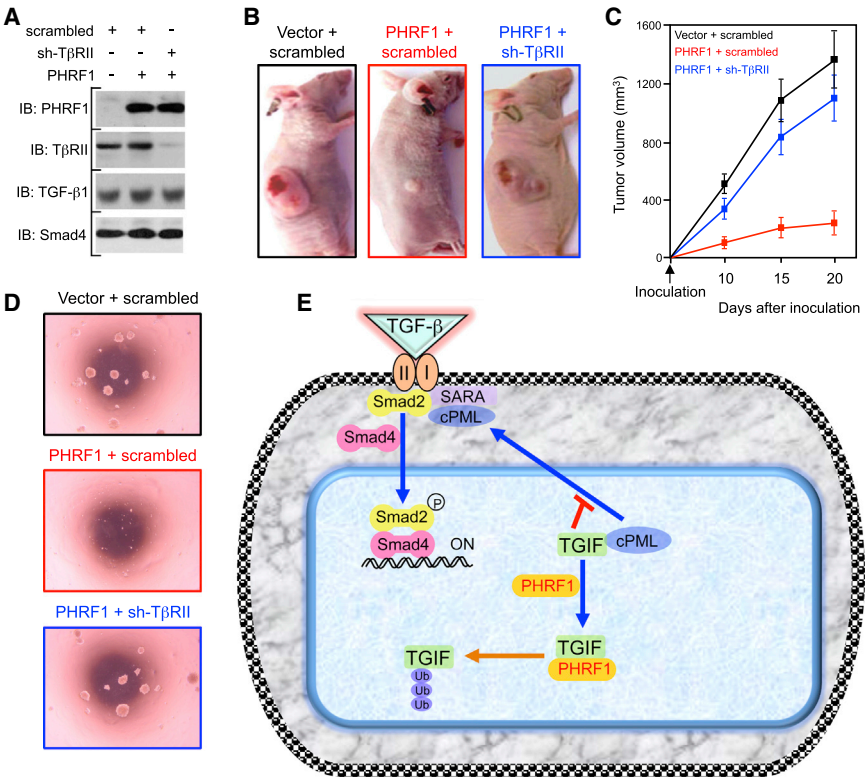

Figure 7. PHRF1 Suppresses Tumor Formation

(A) Expression of PHRF1, TβRII, and TGF-β1 (secreted into media) in MCF7-TR cells stably transfected with the indicated combinations of scrambled, PHRF1, and sh-TβRII.

(B and C) Examination of the ability of the different cell lines generated in (A) to form tumors in athymic nude mice. (B) Representative photographs of tumors were taken at day 20 after inoculation. (C) Tumor volumes were measured, and the results were expressed as mean ± SD of measurements obtained with six animals in each group.

(D) The anchorage-independent growth of the different cell lines generated in (A) was determined using soft agar prepared with media containing phenol red. Color photographs of live colonies were taken by a phase-contrast microscope.

(E) A model depicting the link of PHRF1 to the TGIF/PML-RARα/cPML signaling axis is shown. See also Figure S7.

result revealed that expression of PHRF1 was ineffective in suppressing the growth of MCF7-TR cells coexpressing the degradation-resistant mutant, TGIF.K130R (Figures S7A–S7C). Moreover, expression of the catalytic-inactive mutant PHRF1.CA was unable to suppress the growth of MCF7-TR cells in mice and soft agar (Figures S7A–S7C). During the course of these analyses, we also found that restoration of PHRF1 expression in MDA-MB435 cells suppressed tumor formation, whereas PHRF1.CA was ineffective (Figures S7D–S7G). Together, these findings strongly suggest that PHRF1 may exert a tumor-suppressive function.

DISCUSSION

In the present work, we report on the identification of PHRF1 as a ubiquitin ligase that targets TGIF for degradation. We propose a model in which PHRF1 contributes to Smad signaling by destabilizing TGIF and thereby facilitating the relocalization of cPML into the cytoplasm, where it coordinates phosphorylation of Smad2 by the activated TβRI (Figure 7E). Based on the location of the *PHRF1* gene within the tumor suppressor locus 11p15.5, we went on to show that human breast cancer biopsies and breast cancer cell lines display frequent loss of PHRF1 and that restoration of PHRF1 activity in PHRF1-deficient cancer cells is sufficient to hamper their mitogenic behavior. Thus, our findings on the mode of action of PHRF1 provide critical insights into the role of a key component of the TGF-β tumor suppressor network in malignant transformation.

Although TGIF is well established as a critical negative regulator of the TGF-β cytostatic program in many cell systems, our knowledge of the regulation of its expression or posttranslational modification remains limited. The findings outlined in the present study provide molecular evidence that PHRF1 functions as a ubiquitin ligase to promote TGIF degradation through the proteasome pathway. We identified Lys130 as the major residue that is targeted by PHRF1 and showed that its mutation led to increased TGIF stability. Thus, by eliciting a highly stringent control over the availability of TGIF, PHRF1 fulfills its function in an important regulatory position in the TGF-β signaling pathway. This would provide a mechanism to enable cells to reach a suitable level of TGF-β stimulation that ensures proper maintenance of cell fate and tissue homeostasis. Because PHRF1 dissociates from TGIF upon prolonged activation of TGF-β signaling, another alternative possibility is that PHRF1 functions to restrict a negative feedback loop, thereby maintaining low abundance of the TGIF protein, which would allow cells to respond efficiently to a new acute activation of TGF-β signaling.

Of particular interest, our findings place cPML downstream of PHRF1 in the TGF-β signaling pathway, enlightening the requirement of intact PHRF1 function in activation of cPML. Inactivation of cPML appeared to play a crucial role in the pathogenesis of many types of human cancers, although the basis of its inactivation remains to be fully elucidated. Therefore, our demonstration that PHRF1 and cPML functions are interconnected in a tightly regulated signaling circuit that integrates TGF-β cytostatic signals, together with the fact that cPML function is impaired in cells deficient in PHRF1, opens avenues for improving our understanding of the role of the tumor suppressor cPML in neoplastic transformation.

The mapping of the *PHRF1* gene to the tumor suppressor locus 11p15.5 motivated our attempts to search for possible alterations of PHRF1 function in human cancers. Our attention turned particularly to breast cancer, which displays a high frequency of LOH at this locus (Ali et al., 1987; Karnik et al., 1998;

Winqvist et al., 1995). It is now well established that breast cancers progress through accumulation of genomic and epigenomic aberrations that often disable the cytostatic function of TGF-β, endowing malignant cells with the capability to proliferate and metastasize when exposed to TGF-β (Dumont and Arteaga, 2003; Massagué, 2008). The molecular basis for the loss of TGF-β cytostatic responses has been elusive in most instances. Because TGF-β receptors or downstream Smad signal transducers are rarely inactivated in human breast cancer (Derynck et al., 2001; Dumont and Arteaga, 2003), it is conceivable that alterations in the expression or activity of uncharacterized components that operate downstream of the core TGF-β signaling pathway disable TGF-β's tumor-suppressive activities. Our study suggests that PHRF1 could represent a potential candidate for such a component because the loss of PHRF1 in breast cancer cells is associated with the loss of TGF-β signaling, and restoration of its expression not only rescued TGF-β responsiveness but also blocked their capability to form tumors. Thus, we anticipate that unveiling PHRF1 as an essential component of the TGF-β cytostatic program, and validating the link of PHRF1 to the cPML tumor suppressor network in the mammary tissue, will hold tantalizing insights for meaningful progress in understanding the complex etiology of this overwhelming disease.

Allelic loss at 11p15.5 occurs in a high proportion of breast cancer (up to 65%) and other solid tumors, suggesting the presence of tumor suppressor gene(s) within this region (Ali et al., 1987; Jonas and Kimonis, 2001; Karnik et al., 1998; Winqvist et al., 1995). However, positional cloning efforts to identify the tumor suppressor genes(s) have been complicated by the high frequency and complexity of LOH at 11p15.5 (Karnik et al., 1998). Thus, by showing hat PHRF1 is deleted or silenced in a high proportion of breast cancers, our study provides compelling evidence that PHRF1 could represent a strong candidate for a tumor suppressor gene at 11p15.5. This finding together with the recent demonstration that PHRF1 is frequently deleted across a large number of tumors (4,404 tumors) or somatically mutated in breast cancer and other types of malignancies strongly suggests that PHRF1 might fulfill a tumor suppressor function. Whether PHRF1 also functions as a tumor promoter at late stages of carcinogenesis, where TGF-β paradoxically acts as a potent prometastatic factor, remains to be established (Derynck et al., 2001). Besides alterations of PHRF1 in cancers, the *PHRF1* gene was also found to be frequently mutated in systemic lupus erythematosus (SLE), an autoimmune disease that sometimes develops alongside breast cancer in patients (Harley et al., 2008; Kontos and Fentiman, 2008). Given the connection of PHRF1 to the pathogenesis of breast cancers, it will be relevant to investigate the clinical importance of these somatic alterations in large cohorts of patients displaying both SLE and breast cancer, which could reveal whether PHRF1 deficiency represents a common basis for SLE and breast cancers.

In conclusion, our identification of PHRF1 as a potential tumor suppressor gene constitutes an appreciable advance in unraveling mechanistic paradigms of breast cancer and other malignancies, allowing the perception of the PHRF1 → TGIF → cPML signaling module as an important target for developing novel therapies for these diseases.

EXPERIMENTAL PROCEDURES

Cell Culture

All cell lines were cultured in DMEM supplemented with 10% fetal calf serum (FCS). For all experiments involving TGF-β, cells were cultured in medium containing 0.5% FCS for 24 hr before being treated with 200 pM TGF-β1 (Sigma-Aldrich), unless the concentrations are specified in the figures.

Immunoprecipitation and Immunoblotting

Cell lysates were prepared using TNMG buffer as previously described by Demange et al. (2009). Cells extracts were cleared by centrifugation and incubated with the appropriate antibody for 2 hr, followed by adsorption to Sepharose-coupled protein G for 1 hr. Immune complexes were washed five times with TNMG buffer, separated by SDS-PAGE, and analyzed by immunoblotting.

Immunofluorescence Analysis

Cells were fixed in 4% paraformaldehyde for 30 min and permeabilized in 0.1% Triton X-100. Cells were then incubated with anti-PML or anti-TGIF antibodies for 2 hr at room temperature and washed three times with PBS before being incubated with the secondary antibody conjugated to Texas red or FITC for 1 hr at room temperature. After washing three times with PBS, the nuclei were stained with DAPI, washed three times with PBS, and the coverslips were mounted in PBS containing 50% glycerol and viewed on a fluorescence microscope.

Real-Time PCR

Poly(A)$^+$ RNA was prepared using the RNeasy Mini Kit (QIAGEN). cDNA was synthesized using 1 μg of total RNA and SuperScript 2 according to the manufacturer's instructions (Invitrogen). A total of 50 ng of cDNA was mixed with 12.5 μl iQ SYBR Green Supermix (Bio-Rad), 10.5 μl of water, and 0.5 μl of 10 μM sense and antisense primers, denatured at 95°C for 3 min, and amplified by 40 cycles of 95°C/57°C/72°C for 30 s each using an iCycler (Bio-Rad). All samples were normalized to GAPDH expression.

Analysis of DNA Synthesis by BrdU

The evaluation of cell proliferation by the incorporation of bromodeoxyuridine (BrdU) was performed using a BrdU cell proliferation assay kit following the manufacturer's instructions (Cell Signaling Technology). Briefly, cells were plated, and 24 hr later, they were treated with or without TGF-β for 24 hr before being incubated with BrdU for the last 16 hr at 37°C. After removing BrdU, the cells were incubated with the fixing/denaturing solution for 30 min at room temperature and then with peroxidase-conjugated anti-BrdU antibodies at room temperature for 30 min. The substrate for peroxidase was added at room temperature for 30 min, and the reaction was terminated by the addition of the stop solution. The absorbance of each sample at 450 nm was measured by a standard spectrophotometer-based procedure.

Tumor Growth Assays

Base layers consisting of growth medium (with phenol red) containing 0.5% agarose were poured onto p60 dishes and allowed to solidify. A total of 5,000 cells were plated in top layers consisting of growth medium containing 0.25% agarose, and colonies were visualized after 2–3 weeks.

For xenograft studies, 2×10^6 cells were inoculated subcutaneously into 4-week-old athymic mice. Tumor formation was monitored visually, and volumes of tumors were measured every 5 days. The INSERM Animal Care approved all of the animal studies. See the Extended Experimental Procedures for more information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.07.009.

ACKNOWLEDGMENTS

We thank Drs. S. Piccolo, C. Brou, D. Wotton, and P.P. Pandolfi for providing cell lines and plasmids. We thank Drs. W.C. Horne, Y.Y. Mo, and K. Watabe for their critical review of the manuscript. This work was supported by INSERM, CNRS, Association pour la Recherche sur le Cancer, La Ligue Comité de Paris, and the Mississippi (P20RR016476) funded by the National Institutes of Health.

Received: November 27, 2012
Revised: April 17, 2013
Accepted: July 8, 2013
Published: August 1, 2013

REFERENCES

Ali, I.U., Lidereau, R., Theillet, C., and Callahan, R. (1987). Reduction to homozygosity of genes on chromosome 11 in human breast neoplasia. Science *238*, 185–188.

Bertolino, E., Reimund, B., Wildt-Perinic, D., and Clerc, R.G. (1995). A novel homeobox protein which recognizes a TGT core and functionally interferes with a retinoid-responsive motif. J. Biol. Chem. *270*, 31178–31188.

Demange, C., Ferrand, N., Prunier, C., Bourgeade, M.F., and Atfi, A. (2009). A model of partnership co-opted by the homeodomain protein TGIF and the Itch/AIP4 ubiquitin ligase for effective execution of TNF-α cytotoxicity. Mol. Cell *36*, 1073–1085.

Derynck, R., Akhurst, R.J., and Balmain, A. (2001). TGF-β signaling in tumor suppression and cancer progression. Nat. Genet. *29*, 117–129.

Dumont, N., and Arteaga, C.L. (2003). Targeting the TGF β signaling network in human neoplasia. Cancer Cell *3*, 531–536.

Dupont, S., Mamidi, A., Cordenonsi, M., Montagner, M., Zacchigna, L., Adorno, M., Martello, G., Stinchfield, M.J., Soligo, S., Morsut, L., et al. (2009). FAM/USP9x, a deubiquitinating enzyme essential for TGFbeta signaling, controls Smad4 monoubiquitination. Cell *136*, 123–135.

Ellis, M.J., Ding, L., Shen, D., Luo, J., Suman, V.J., Wallis, J.W., Van Tine, B.A., Hoog, J., Goiffon, R.J., Goldstein, T.C., et al. (2012). Whole-genome analysis informs breast cancer response to aromatase inhibition. Nature *486*, 353–360.

Faresse, N., Colland, F., Ferrand, N., Prunier, C., Bourgeade, M.F., and Atfi, A. (2008). Identification of PCTA, a TGIF antagonist that promotes PML function in TGF-β signalling. EMBO J. *27*, 1804–1815.

Feng, X.H., and Derynck, R. (2005). Specificity and versatility in tgf-β signaling through Smads. Annu. Rev. Cell Dev. Biol. *21*, 659–693.

Guedj, M., Marisa, L., de Reynies, A., Orsetti, B., Schiappa, R., Bibeau, F., MacGrogan, G., Lerebours, F., Finetti, P., Longy, M., et al. (2012). A refined molecular taxonomy of breast cancer. Oncogene *31*, 1196–1206.

Gurrieri, C., Capodieci, P., Bernardi, R., Scaglioni, P.P., Nafa, K., Rush, L.J., Verbel, D.A., Cordon-Cardo, C., and Pandolfi, P.P. (2004). Loss of the tumor suppressor PML in human cancers of multiple histologic origins. J. Natl. Cancer Inst. *96*, 269–279.

Harley, J.B., Alarcón-Riquelme, M.E., Criswell, L.A., Jacob, C.O., Kimberly, R.P., Moser, K.L., Tsao, B.P., Vyse, T.J., Langefeld, C.D., Nath, S.K., et al.; International Consortium for Systemic Lupus Erythematosus Genetics (SLEGEN). (2008). Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. Nat. Genet. *40*, 204–210.

Ivshina, A.V., George, J., Senko, O., Mow, B., Putti, T.C., Smeds, J., Lindahl, T., Pawitan, Y., Hall, P., Nordgren, H., et al. (2006). Genetic reclassification of histologic grade delineates new clinical subtypes of breast cancer. Cancer Res. *66*, 10292–10301.

Jonas, R.E., and Kimonis, V.E. (2001). Chest wall hamartoma with Wiedemann-Beckwith syndrome: clinical report and brief review of chromosome 11p15.5-related tumors. Am. J. Med. Genet. *101*, 221–225.

Karnik, P., Paris, M., Williams, B.R., Casey, G., Crowe, J., and Chen, P. (1998). Two distinct tumor suppressor loci within chromosome 11p15 implicated in breast cancer progression and metastasis. Hum. Mol. Genet. *7*, 895–903.

Kontos, M., and Fentiman, I.S. (2008). Systemic lupus erythematosus and breast cancer. Breast J. *14*, 81–86.

Lin, H.K., Bergmann, S., and Pandolfi, P.P. (2004). Cytoplasmic PML function in TGF-β signalling. Nature *431*, 205–211.

Massagué, J. (2008). TGFbeta in Cancer. Cell *134*, 215–230.

Massagué, J., Seoane, J., and Wotton, D. (2005). Smad transcription factors. Genes Dev. *19*, 2783–2810.

Nik-Zainal, S., Alexandrov, L.B., Wedge, D.C., Van Loo, P., Greenman, C.D., Raine, K., Jones, D., Hinton, J., Marshall, J., Stebbings, L.A., et al.; Breast Cancer Working Group of the International Cancer Genome Consortium. (2012). Mutational processes molding the genomes of 21 breast cancers. Cell *149*, 979–993.

Salomoni, P., and Pandolfi, P.P. (2002). The role of PML in tumor suppression. Cell *108*, 165–170.

Scaglioni, P.P., and Pandolfi, P.P. (2007). The theory of APL revisited. Curr. Top. Microbiol. Immunol. *313*, 85–100.

Seo, S.R., Lallemand, F., Ferrand, N., Pessah, M., L'Hoste, S., Camonis, J., and Atfi, A. (2004). The novel E3 ubiquitin ligase Tiul1 associates with TGIF to target Smad2 for degradation. EMBO J. *23*, 3780–3792.

Seo, S.R., Ferrand, N., Faresse, N., Prunier, C., Abécassis, L., Pessah, M., Bourgeade, M.F., and Atfi, A. (2006). Nuclear retention of the tumor suppressor cPML by the homeodomain protein TGIF restricts TGF-β signaling. Mol. Cell *23*, 547–559.

Tsukazaki, T., Chiang, T.A., Davison, A.F., Attisano, L., and Wrana, J.L. (1998). SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell *95*, 779–791.

Whitman, M. (1998). Smads and early developmental signaling by the TGFbeta superfamily. Genes Dev. *12*, 2445–2462.

Winqvist, R., Hampton, G.M., Mannermaa, A., Blanco, G., Alavaikko, M., Kiviniemi, H., Taskinen, P.J., Evans, G.A., Wright, F.A., Newsham, I., et al. (1995). Loss of heterozygosity for chromosome 11 in primary human breast tumors is associated with poor survival after metastasis. Cancer Res. *55*, 2660–2664.