Pleckstrin homology domain leucine-rich repeat protein phosphatases set the amplitude of receptor tyrosine kinase output

Gloria Reyes[a,1], Matt Niederst[a,b,1], Ksenya Cohen-Katsenelson[a], Joshua D. Stender[c], Maya T. Kunkel[a], Muhan Chen[d], John Brognard[a,2], Emma Sierecki[a,3], Tianyan Gao[e], Dawid G. Nowak[c], Lloyd C. Trotman[d], Christopher K. Glass[c], and Alexandra C. Newton[a,4]

Departments of [a]Pharmacology and [c]Cellular and Molecular Medicine and [b]Biomedical Sciences Graduate Program, University of California, San Diego, La Jolla, CA 92093; [d]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724; and [e]Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY 40536

Edited by Tony Hunter, The Salk Institute for Biological Studies, La Jolla, CA, and approved August 14, 2014 (received for review March 6, 2014)

Growth factor receptor levels are aberrantly high in diverse cancers, driving the proliferation and survival of tumor cells. Understanding the molecular basis for this aberrant elevation has profound clinical implications. Here we show that the pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) suppresses receptor tyrosine kinase (RTK) signaling output by a previously unidentified epigenetic mechanism unrelated to its previously described function as the hydrophobic motif phosphatase for the protein kinase AKT, protein kinase C, and S6 kinase. Specifically, we show that nuclear-localized PHLPP suppresses histone phosphorylation and acetylation, in turn suppressing the transcription of diverse growth factor receptors, including the EGF receptor. These data uncover a much broader role for PHLPP in regulation of growth factor signaling beyond its direct inactivation of AKT: By suppressing RTK levels, PHLPP dampens the downstream signaling output of two major oncogenic pathways, the PI3 kinase/AKT and the Rat sarcoma (RAS)/ERK pathways. Our data are consistent with a model in which PHLPP modifies the histone code to control the transcription of RTKs.

Binding of growth factors to receptor tyrosine kinases (RTKs) initiates a multitude of key cellular processes, including growth, proliferation, and survival (1). Two of the major growth factor-activated pathways downstream of RTKs are the Rat sarcoma (RAS)/ERK and phosphatidylinositol-3 kinase (PI3 kinase)/protein kinase AKT pathways. Dysregulation of either pathway leads to uncontrolled cell proliferation and evasion of apoptosis, both hallmarks of cancer (2). Amplified signaling by RTKs is associated with diverse human cancers, as a result of somatic gain-of-function mutations of the RTKs, gene amplification, or epigenetic changes that cause increased expression of these receptors (3). Underscoring the prevalence of increased RTK levels in cancers, amplified expression of the EGF receptor (EGFR) family member human epidermal growth factor receptor 2 (HER2) is present in up to 30% of human breast cancers (4), a disease which accounts for a striking 30% of all new cancer cases in the United States each year (5). Similarly, 30% of prostate cancers have been reported to have elevated expression of EGFR without evidence of gene amplification (6). This increased expression of RTKs correlates with poor disease prognosis (7, 8).

The regulation of protein expression by epigenetic mechanisms is reversible and thus is a particularly attractive target for cancer therapy (9, 10). Covalent modifications of histones, including acetylation, phosphorylation, methylation, and ubiquitination, form a dynamic and complex "histone code" that is "written" and "erased" by histone modifiers and "read" by chromatin-remodeling complexes and transcriptional coregulators to control gene transcription (11–14). Small-molecule inhibitors of chromatin remodelers show potential as effective chemotherapeutic targets (15). Most notably, histone deacetylases (HDACs) are of significant interest as chemotherapeutic targets (16, 17). Phosphorylation is gaining increasing recognition as a key symbol in the histone code (18). Collaboration between phosphorylation and acetylation/methylation on histone tails influences a multitude of cellular processes, including transcription of target genes. For example, multiple lines of evidence support synergism between histone acetylation and phosphorylation in the induction of immediate-early genes (such as *c-jun*, *c-fos*, and *c-myc*) after mitogenic stimulation. Furthermore, having one modification can increase the efficiency of an enzyme catalyzing a second modification; for example, phosphorylation of histone H3 on Ser10 (H3S10) promotes Lys acetylation by the GCN5 acetyltransferase (18–21). H3S28 phosphorylation at gene promoters induces demethylation and acetylation of Lys27 (K27), thereby activating transcription of these genes (22). Thus, phosphorylation of histones is a critical component of the histone code. Much emphasis has been placed on the kinases that modify this code (12, 18, 23), but much less is known about the phosphatases that modify the histone code.

The pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) family of Ser/Thr phosphatases was discovered in a search to identify a phosphatase that could directly dephosphorylate and inactivate the prosurvival kinase AKT (24). PHLPP phosphatases specifically dephosphorylate the hydrophobic motif of AKT (Ser473 in Akt1) but not the activation loop site (Thr308 in Akt1, a site phosphorylated by PDK1), and its genetic depletion, overexpression, or inhibition by small molecule inhibitors

Significance

This work unveils a previously unidentified function of the tumor suppressor pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) in inhibiting oncogenic signaling by suppressing the steady-state levels of receptor tyrosine kinases such as the EGF receptor. Specifically, PHLPP modifies the histone code to control the transcription of receptor tyrosine kinases. This epigenetic function can account for the upregulation of receptor tyrosine kinases in the multiple cancer types where PHLPP function is compromised.

Author contributions: G.R., M.N., K.C.-K., J.D.S., M.T.K., M.C., L.C.T., C.K.G., and A.C.N. designed research; G.R., M.N., K.C.-K., J.D.S., M.T.K., M.C., J.B., E.S., T.G., and D.G.N. performed research; E.S. contributed new reagents/analytic tools; G.R., M.N., K.C.-K., J.D.S., M.T.K., M.C., C.K.G., and A.C.N. analyzed data; and A.C.N. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]G.R. and M.N. contributed equally to this work.

[2]Present address: Signalling Networks in Cancer Group, Cancer Research UK Manchester Institute, University of Manchester, Manchester M20 4BX, United Kingdom.

[3]Present address: Institute for Molecular Bioscience, University of Queensland, St Lucia, QLD 4072, Australia.

[4]To whom correspondence should be addressed. Email: anewton@ucsd.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1404221111/-/DCSupplemental.

(25) has been shown to regulate the phosphorylation state of Ser473 in numerous studies (e.g., refs. 26–30). The family comprises two genes: *PHLPP1*, which is alternatively spliced to yield *PHLPP1α* and *PHLPP1β*, and *PHLPP2* (24, 31). The expression of both PHLPP1 and PHLPP2 is commonly decreased in a large number of diverse cancers (reviewed in ref. 32), and genetic deletion of one isoform, PHLPP1, is sufficient to cause prostate tumors in a mouse model (33). Their down-regulation is associated with hypoxia-induced resistance to chemotherapy (34), further underscoring their role in cancer. Consistent with their tumor-suppressive function, PHLPP1 and PHLPP2 are on chromosomal loci (18q21.33 and 16q22.3, respectively) that frequently are deleted in cancer (33). The PHLPP2 locus is one of the most frequently deleted in breast cancer (35), and that of PHLPP1 is one of the most highly deleted in colon cancer (36). Recent studies have established that PHLPP1 and PHLPP2 suppress oncogenic signaling by at least two mechanisms (reviewed in ref. 37): (*i*) direct dephosphorylation and inactivation of the prosurvival kinase AKT, PKC (38), and S6 kinase (39), and (*ii*) direct dephosphorylation and activation of the proapoptotic kinase Mst1 (40). Whether additional mechanisms account for the tumor-suppressive function of PHLPP is largely unexplored.

Here we report that PHLPP controls the amplitude of growth factor signaling by a previously unidentified mechanism that is independent of its direct dephosphorylation of AKT, PKC, or S6 kinase: It suppresses the steady-state levels of RTKs such as the EGFR by suppressing histone phosphorylation and acetylation and thus receptor transcription. These data reveal that PHLPP isozymes play a much broader role in blunting the cell's ability to respond to EGF and activate downstream signaling cascades: In addition to regulating individual signaling pathways (e.g., AKT and PKC), PHLPP isozymes set the amplitude of RTK signaling by serving as regulators of RTK transcription.

Results

Analysis of immortalized mouse embryonic fibroblasts (MEFs) from *Phlpp1*$^{-/-}$ mice (in which both *PHLPP1α* and *PHLPP1β* are deleted) revealed that levels of EGFR protein are highly elevated compared with those in wild-type MEFs. Fig. 1*A* shows a robust (5.9 ± 0.7-fold) increase in steady-state levels of EGFR protein in *Phlpp1*$^{-/-}$ MEFs (lane 2) compared with wild-type MEFs (lane 1). This increase could be partially rescued by reintroduction of full-length PHLPP1β into the *Phlpp1*$^{-/-}$ MEFs (lane 3), but not a construct lacking catalytic activity (41) (lane 4) or one lacking the LRR segment (lane 6). This latter construct increased the EGFR levels even further, suggesting that it functions as a dominant negative to the remaining PHLPP isozyme, PHLPP2. In contrast, constructs lacking the PH domain (lane 5) or PSD-95, disheveled, and ZO1 (PDZ) ligand (lane 7) suppressed EGFR levels similar to those in wild-type PHLPP1. Note that all deletion constructs of PHLPP retain catalytic activity. As a control for the

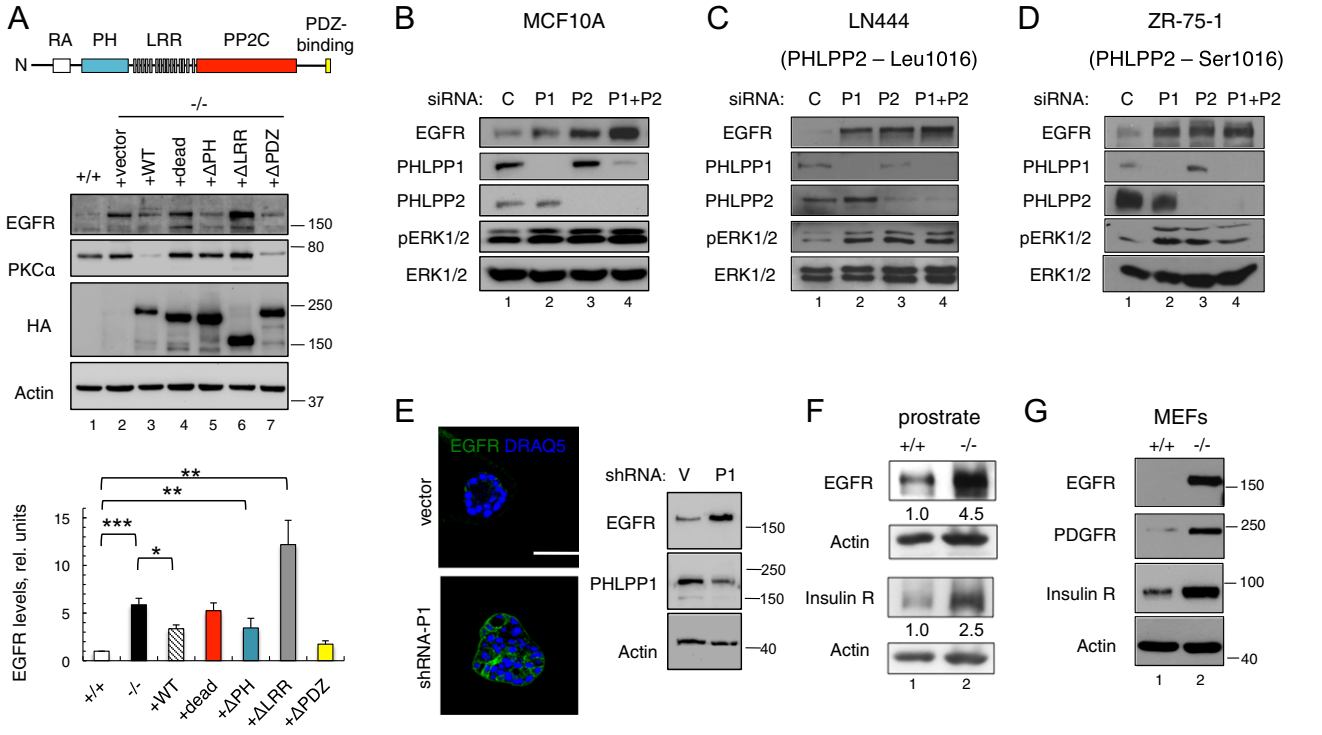

Fig. 1. PHLPP1 restricts EGFR expression and signaling. (*A*) Western blots (*Middle*) of *Phlpp1*$^{+/+}$ (+/+), *Phlpp1*$^{-/-}$ (–/–), and *Phlpp1*$^{-/-}$ cells reconstituted with different PHLPP1β constructs showing EGFR and PKCα total levels. The HA signal indicates the expression of wild-type PHLPP1β (WT), catalytically inactive PHLPP1β (dead), PHLPP1β without the PH domain (ΔPH), PHLPP1β without the LLRs (ΔLRR), or PHLPP1β from which the PDZ binding motif was deleted (ΔPDZ). Domain structure of PHLPP1β (*Top*), which also includes a putative Ras association (RA) domain. The graph (*Bottom*) represents the quantification of three independent reconstitution experiments. ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ by Student *t* test. (*B*) EGFR levels in the MCF10A breast cell line treated with control (C), PHLPP1 (P1), PHLPP2 (P2), or PHLPP1 and PHLPP2 (P1+P2) siRNA. (*C*) EGFR levels in an LN444 glioblastoma cancer cell line treated with control (C), PHLPP1 (P1), PHLPP2 (P2), or PHLPP1 and PHLPP2 (P1+P2) siRNA; these cells have the more common and active variant of PHLPP2 with Leu at position 1016. (*D*) EGFR levels in the ZR-75-1 breast cancer cell line treated with control (C), PHLPP1 (P1), PHLPP2 (P2), or PHLPP1 and PHLPP2 (P1+P2) siRNA; these cells have the L1016S polymorphic variant of PHLPP2 that is inactive toward Akt and PKC. Western blots in *B–D* also probed for total and phosphorylated Erk (pT202/pY204) and are representative of three independent experiments. (*E*, *Left*) MCF10A cells transduced with shRNA against PHLPP1 were grown on Matrigel for 20 d to form 3D structures. The structures then were fixed and stained with anti-EGFR antibody, and the nuclei were counterstained with DRAQ5. (Scale bar: 50 μm.) (*Right*) The blots show the level of EGFR and the knockdown of PHLPP1 in these structures. (*F*) EGFR and insulin receptor (INSR) levels in *Phlpp1*$^{+/+}$ (+/+) and *Phlpp1*$^{-/-}$ (–/–) mouse prostate tissue. (*G*) Western blots probing for EGF, PDGF, or INSR levels in whole-cell lysates from *Phlpp1*$^{+/+}$ (+/+) and *Phlpp1*$^{-/-}$ (–/–) MEFs.

function of PHLPP, we confirmed that lack of PHLPP1 resulted in increased steady-state levels of PKCα (PKCα panel; see ref. 38, which shows that phosphorylation increases the stability of PKC isozymes); reintroduction of PHLPP1β decreased the steady-state levels of PKCα in a manner that depended on the catalytic activity and presence of the PH domain (which is required for PHLPP to recognize PKC in cells) but not in the presence of the PDZ ligand (which is not required for PHLPP to recognize PKC in cells) (38). These data reveal that PHLPP1 suppresses the steady-state levels of the EGFR by a mechanism depending on its catalytic activity and an intact LRR segment.

To ask whether PHLPP2 also controls RTK levels, we depleted PHLPP1, PHLPP2, or both in a number of normal and cancer cell lines. Depletion of either PHLPP1 or PHLPP2 by siRNA resulted in an increase in EGFR levels in the normal breast cell line MCF10A (Fig. 1*B*), the glioblastoma cell line LN444 (Fig. 1*C*), and the breast cancer cell line ZR-75-1 (Fig. 1*D*). Similar results were observed in HeLa cells and A549 lung adenocarcinoma cells. Knockdown of both PHLPP isozymes resulted in an even greater increase in EGFR levels, with the magnitude of the increase varying with cell type. Previously we have shown that the ZR-75-1 cells contain the Leu1016Ser polymorphic variant of PHLPP2 that does not regulate AKT or PKC: Knockdown of PHLPP2 in these cells has no effect on the phosphorylation state of AKT or levels of PKC (42). Importantly, knockdown of PHLPP2 in the ZR-75-1 cells causes a robust increase in EGFR levels and downstream ERK activation (Fig. 1*D*, lane 3). Thus, the Leu1016Ser polymorphic variant maintains function toward regulation of EGFR levels. These data reveal that knockdown of either PHLPP1 or PHLPP2 causes a robust increase in the steady-state levels of EGFR and that, at least for PHLPP2, this effect is independent of the ability of PHLPP to dephosphorylate AKT directly. EGFR levels were elevated in MCF10A cells treated with shRNA against PHLPP1 compared with control, as assessed by both Western blot and immunocytochemistry of MCF10A acinar structures (Fig. 1*E*).

Previously we have shown that the loss of *Phlpp1* triggers neoplasia in prostate, consistent with its frequent alteration in human prostate cancer (33). Western blot analysis revealed that steady-state levels of the EGFR were elevated in prostate samples from *Phlpp1*$^{-/-}$ mice as compared with wild-type mice, suggesting that PHLPP regulation of EGFR levels may be integral to its tumor-suppressive function in this context (Fig. 1*F* (33). Levels of the INSR were elevated in prostates of *Phlpp1*$^{-/-}$ mice (Fig. 1*F*) and *Phlpp1*$^{-/-}$ MEFs (Fig. 1*G*); PDGF receptor (PDGFR) also was elevated in *Phlpp1*$^{-/-}$ MEFs (Fig. 1*G*). These data are consistent with PHLPP's suppressing the levels of multiple oncogenic RTKs.

To assess whether the increased EGFR protein was localized at the plasma membrane where the EGFR signals, wild-type or *Phlpp1*$^{-/-}$ MEFs were surface-labeled with biotin, and the amount of EGFR present in pulldowns using the biotin-reacting compound streptavidin was assessed (Fig. 2*A*). The amount of EGFR pulled down by streptavidin (and hence surface-biotinylated) was an order of magnitude higher in *Phlpp1*$^{-/-}$ MEFs than in wild-type MEFs (Fig. 2*A*, *Upper*, compare lanes 3 and 4). As a control, streptavidin pulled down comparable levels of the most readily accessible surface proteins [compare the intensities of major streptavidin-labeled bands in Fig. 2*A*, *Right* and similar levels of transferrin receptor in wild-type (lane 3) and *Phlpp1*$^{-/-}$ (lane 4) MEFs].

To explore whether the elevated EGFR protein resulting from the depletion of PHLPP is signaling competent, we took advantage of a genetically encoded FRET-based EGFR kinase activity sensor (43) to monitor the basal (serum-starved) and agonist-dependent EGFR kinase activity in real time, in live

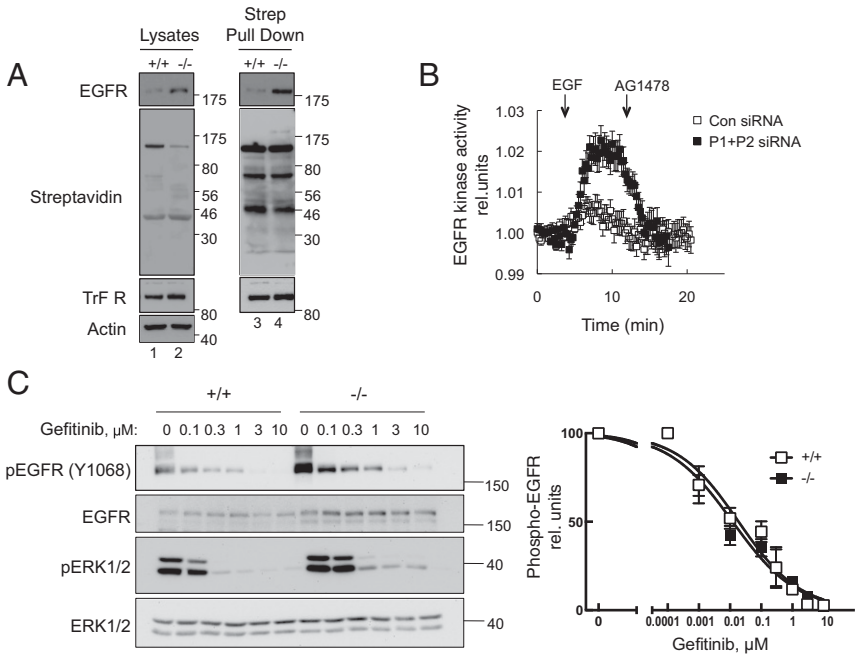

Fig. 2. EGFR activity in PHLPP1-knockdown and -knockout cells. (*A*) Surface proteins of *Phlpp1*$^{+/+}$ (+/+) and *Phlpp1*$^{-/-}$ (–/–) fibroblasts were biotinylated and pulled down using streptavidin beads. EGFR levels were detected using EGFR antibody. Transferrin receptor (TrF R), streptavidin, and actin were used as controls. (*B*) HeLa cells transfected with control (empty boxes) or with PHLPP1 and PHLPP2 (filled boxes) siRNA and an EGFR kinase activity reporter were starved overnight and treated with 50 ng/mL of EGF followed by 100 nM of the EGFR inhibitor AG1478. Data represent the ratio of yellow (FRET) emission to cyan emission, which was monitored over time as a measure of EGFR kinase activity. (*C*) *Phlpp1*$^{+/+}$ (+/+) and *Phlpp1*$^{-/-}$ (–/–) MEFs were treated with 0–10 μM of the EGFR kinase inhibitor gefitinib for 4 h and then were treated with EGF (10 ng/mL) for 5 min. (*Left*) The levels of total EGFR and phosphorylated EGFR r(pY1068) were detected by Western blot. A representative experiment is shown. (*Right*) The graph represents the dose-dependent activity, as measured by relative EGFR phosphorylation at Y1068. Data shown represent the mean ± SEM of EGFR in three independent experiments.

cells. The amplitude of EGF-stimulated EGFR tyrosine kinase activity was significantly higher in HeLa cells in which both PHLPP1 and PHLPP2 were depleted by siRNA than in control cells (Fig. 2*B*). This EGF-dependent activity was reversed by the EGFR tyrosine kinase inhibitor AG1478. Because activity was reversed to the starting basal (serum-starved) level, these data suggest that there is no significant basal EGFR kinase activity, regardless of PHLPP depletion. Rather, PHLPP suppresses agonist-dependent receptor activity.

We next addressed whether PHLPP altered the pharmacology of the EGFR. Treatment of either wild-type or *Phlpp1*$^{-/-}$ MEFs with gefitinib, an active-site inhibitor of the tyrosine kinase activity of the EGFR and an effective therapy in nonsmall cell lung carcinoma (44), resulted in identical dose–response curves (Fig. 2*C*). Thus, PHLPP alters the levels but not the pharmacological profile of the EGFR.

The increased EGFR levels and activity conferred a proliferation advantage to cells: Fig. 3*A* shows that immortalized MEFs isolated from *Phlpp1*$^{-/-}$ mice (filled squares) proliferated at a considerably faster rate than those isolated from wild-type mice (open squares). These immortalized MEFs likely have lost the ability to activate p53, because we previously have shown that loss of p53 is required for *Phlpp1*$^{-/-}$ MEFs to proliferate at a higher rate than control MEFs (33). This increased proliferation rate resulted from lack of PHLPP1 catalytic activity, because stable reintroduction of wild-type PHLPP1β (gray triangles), but not a catalytically-inactive construct of PHLPP1β (red triangles), into *Phlpp1*$^{-/-}$ MEFs reduced the rate of proliferation to that observed in the wild-type MEFs. Pharmacological inhibitors revealed that this increased proliferation resulted in part from increased PI3 kinase signaling and in part from increased ERK signaling: The reduction in the G1/S ratio (reflecting cells entering the cell cycle at a faster rate) observed in cells depleted of both PHLPP isozymes compared with wild-type cells was decreased by approximately half in cells treated with either LY294002 to inhibit PI3 kinase/AKT or U0126 to inhibit MEK/ERK but was abolished in cells treated with both inhibitors (Fig. 3*B*). In summary, these data reveal that loss of PHLPP results in an elevation of signaling-competent EGFR, which leads to amplified signaling by two downstream pathways, PI3 kinase and ERK, in turn increasing cell proliferation.

Reasoning that the increase in EGFR levels would increase downstream signaling, we focused on assessing the impact of PHLPP knockdown on the ERK pathway. Both basal and EGF-dependent ERK phosphorylation was markedly enhanced in the glioblastoma cell line LN444 and the breast cancer cell line ZR-75-1 depleted of PHLPP1, PHLPP2, or both isozymes (Fig. 3 *C* and *D*; see also Fig. 1 *C* and *D*). Analysis of the kinetics of agonist-evoked ERK phosphorylation revealed a robust increase in the amplitude of ERK phosphorylation both in LN444 cells depleted of both PHLPP1 and PHLPP2 (Fig. 3*E*) and in *Phlpp1*$^{-/-}$ MEFs (Fig. 3*F*). However, the rate of subsequent dephosphorylation of ERK was not significantly different in cells with or without PHLPP ($t_{1/2}$ for dephosphorylation was 50 ± 7 and 44 ± 2 min, respectively, in LN444 cells and 26 ± 13 and 28 ± 3 min, respectively, in MEFs). Thus, PHLPP suppresses the agonist-dependent phosphorylation of ERK but does not control the postactivation dephosphorylation of ERK. These data reveal that PHLPP1 and PHLPP2 suppress the activation of ERK in response to EGF, a logical consequence of their role in suppressing EGFR levels.

We next addressed whether PHLPP suppresses the steady-state levels of EGFR by (*i*) inhibiting its biosynthesis or (*ii*) promoting its degradation. To examine the rate of biosynthesis, wild-type or

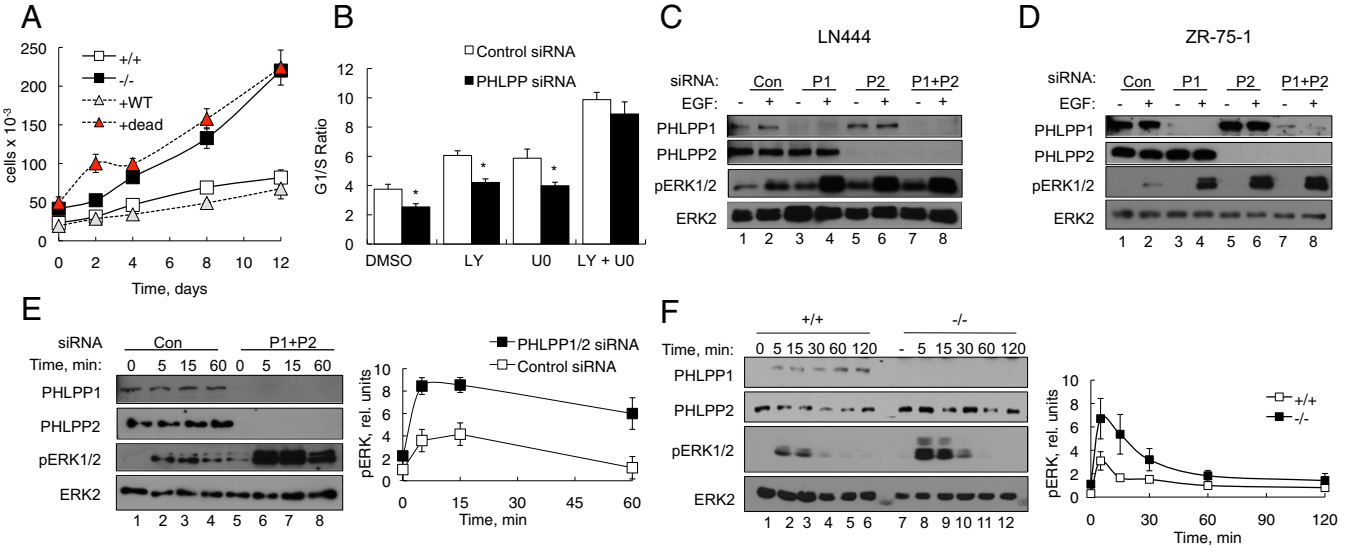

Fig. 3. PHLPP suppresses proliferation through Akt and ERK signaling. (*A*) Proliferation curves of *Phlpp1*$^{+/+}$ (+/+) and *Phlpp1*$^{-/-}$ (–/–) fibroblasts and *Phlpp1*$^{-/-}$ cells reconstituted with wild-type HA-PHLPP1β (+WT) or the catalytically inactive HA-PHLPP1β (+dead). Data represent the average of three independent experiments; error bars represent the SEM. (*B*) Graph depicting the G1/S ratio of LN444 cells transfected with control (empty bars) or PHLPP1 and PHLPP2 siRNA (filled bars) and then treated with vehicle (DMSO), LY294002 (LY; 20 μM), U0126 (U0; 20 μM), or both LY294002 and U0126 (LY+U0; 20 μM each) for 24 h. Data represent the average of three independent experiments in which assays were completed in triplicate; error bars indicate the SEM. Statistical significance was determined by Student *t* test; *$P < 0.01$. (*C* and *D*) Increased ERK signaling in PHLPP1 and PHLPP2 knockdown as well as in *Phlpp1*$^{-/-}$ cells. Control (Con), PHLPP1 (P1), PHLPP2 (P2), or PHLPP1 and PHLPP2 (P1+P2) siRNA-transfected cells were starved overnight in 0.1% FBS and then were treated with EGF (10 ng/mL) for 15 min. Two cell lines were chosen: LN444 with a normal PHLPP2 isoform (*C*), and ZR-75-5 with a polymorphic variant of PHLPP2 (L1016S) (*D*). Erk phosphorylation was detected by Western blot using phospho-specific antibody (pT202/pY204). (*E*, *Left*) Time course of ERK phosphorylation in LN444 cells treated with siRNA control (Con) or siRNA against PHLPP1 and PHLPP2 (P1+P2) upon stimulation with 10 ng/mL EGF for the indicated times. (*Right*) The graph shows the average of three independent experiments; error bars indicate the SEM. (*F*, *Left*) Time course of ERK phosphorylation in *Phlpp1*$^{+/+}$ (+/+) and *Phlpp1*$^{-/-}$ (–/–) fibroblasts starved overnight and treated with EGF (10 ng/mL) for the indicated times. (*Right*) The graph shows the average ERK phosphorylation (pERK pT202/pY204) from three independent experiments in wild-type (empty squares) and *Phlpp1*$^{-/-}$ (filled squares) cells; error bars indicate the SEM.

Phlpp1$^{-/-}$ MEFs were incubated with medium containing ^{35}S-Met/Cys, and the incorporation of radioactivity into immunoprecipitated EGFR was monitored as a function of time. Quantitative analysis of incorporated radioactivity at a band comigrating with the immunoprecipitated EGFR revealed a 2.5-fold increase in the rate of biosynthesis in cells lacking PHLPP1 (252 ± 50 cpm incorporated per minute) compared with wild-type cells (104 ± 10 cpm incorporated per minute) (Fig. 4*A*). In contrast to the rate of biosynthesis, the rate of EGF-stimulated degradation of the EGFR was the same in wild-type (30 ± 17 min) and *Phlpp1*$^{-/-}$ MEFs (30 ± 15 min) (Fig. 4*B*). Similarly, the basal rate of turnover of the receptor, assessed after treatment of cells with cycloheximide to prevent protein synthesis, was not significantly different in wild-type cells (8 ± 4 h) and cells lacking PHLPP1 (11 ± 4 h) (Fig. 4*C*). Given the increased rate of biosynthesis, we reasoned that mRNA levels of the EGFR might be elevated in cells lacking PHLPP1. Indeed, quantitative RT-PCR (qRT-PCR) analysis revealed a 20 ± 5-fold increase in the levels of EGFR mRNA in *Phlpp1*$^{-/-}$ MEFs compared with wild-type MEFs; this increase was reduced twofold upon reintroduction of PHLPP1β into *Phlpp1*$^{-/-}$ MEFs (Fig. 4*D*). Importantly, EGFR mRNA was elevated in the *Phlpp1*$^{-/-}$ mouse: EGFR mRNA was approximately twofold higher in prostates from 6-mo-old *Phlpp1*$^{-/-}$ mice than in prostates from wild-type littermates (Fig. 4*E*). Last, we examined whether the mRNA levels were elevated because of increased stability. The addition of actinomycin D to prevent mRNA transcription revealed that the rate of decay of existing mRNA was the same in wild-type and *Phlpp1*$^{-/-}$ MEFs (Fig. 4*F*). Thus, loss of PHLPP1 resulted in increased mRNA levels and an increased rate of protein translation of the EGFR, resulting in increased steady-state levels. [Note that there is not a linear correlation between mRNA and protein translation rates (45)]. Taken together, these data show that PHLPP suppresses the transcription of the EGFR.

We next used a pharmacological approach to identify targets of PHLPP that could control the transcription of RTKs and thus their steady-state levels. Pharmacological inhibitors of known PHLPP targets (including the EGFR itself) had no significant effect on the ability of PHLPP depletion to increase EGFR expression (Fig. S1). Inhibition of PI3 kinase signaling with LY294002 (LY) (lane 3), ERK signaling with U0126 (UO) (lane 4), PKC signaling with Gö6983 (Gö) (lane 5), or EGFR tyrosine kinase signaling with AG1478 (AG) (lane 6) had no significant effect on the increased levels of EGFR in PHLPP-knockdown cells relative to untreated controls (lane 2).

The finding that PHLPP controls the transcription of the EGFR led us to ask whether nuclear localization of PHLPP is necessary for this function. Specifically, we examined whether addition of a nuclear localization signal (NLS) onto the ΔLRR construct of PHLPP, which loses the ability to regulate the EGFR, could restore regulation of the EGFR. The data in Fig. 5*A* show that reconstitution of *Phlpp1*$^{-/-}$ MEFs with wild-type PHLPP1β (lane 2) reduced the EGFR level compared with vector control (lane 1), whereas reconstitution with the ΔLRR construct increased EGFR levels (lanes 3 and 4). However, a construct in which an NLS was fused to the ΔLRR effectively reduced EGFR levels (lanes 5 and 6). These findings are consistent with the LRR segment of PHLPP driving or retaining PHLPP in the nucleus, where it regulates EGFR transcription.

Given that PHLPP loss up-regulated several RTKs, we hypothesized that PHLPP could modify the histone code so as to suppress transcription. Thus, we explored the effect of PHLPP deletion on histone acetylation and histone phosphorylation. Analysis of histone modifications revealed a selective increase in

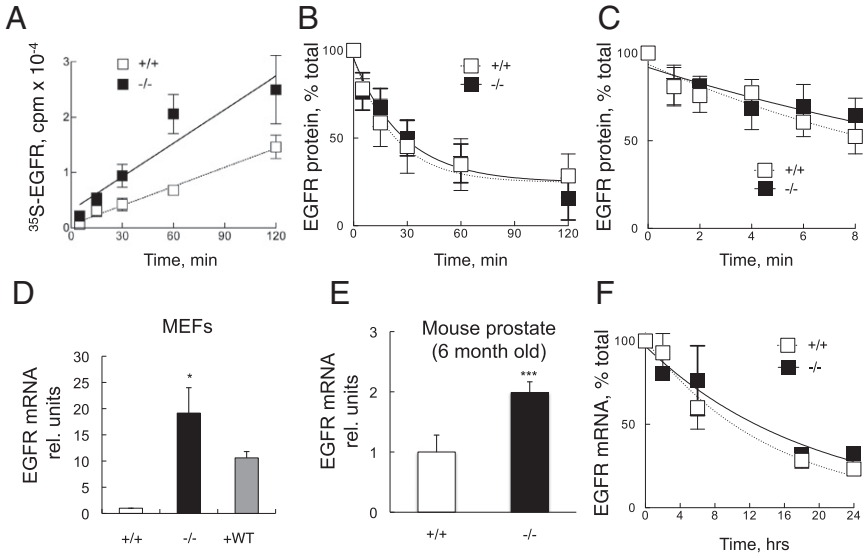

Fig. 4. PHLPP1 regulates synthesis, not degradation, of the EGFR. (*A*) *Phlpp1*$^{+/+}$ (+/+) or *Phlpp1*$^{-/-}$ (–/–) MEFs were labeled with [^{35}S]Met/Cys for the indicated times, and total EGFR was immunoprecipitated from the lysates. The graph shows the quantification of radiolabeled EGFR of three independent experiments; the error bars indicate the SEM. (*B*) Degradation curve of the EGFR in *Phlpp1*$^{+/+}$ (+/+) and *Phlpp1*$^{-/-}$ (–/–) cells upon EGF stimulation (10 ng/mL) for the indicated times. EGFR levels were analyzed by Western blot analysis of whole-cell lysates. Each point represents the percentage of EGFR normalized to time 0 (no EGF). The graphs show the quantification of three independent experiments. Error bars indicate the SEM. (*C*) Degradation curve of the EGFR in *Phlpp1*$^{+/+}$ (+/+) and *Phlpp1*$^{-/-}$ (–/–) cells under steady-state conditions. Cells were treated with cycloheximide (5 μM) for the indicated times. Total EGFR levels were analyzed by Western blot with an anti-EGFR antibody. Each point represents the percentage of EGFR normalized to control (without cycloheximide). The graph shows the quantification of three independent experiments. Error bars indicate SEM. (*D*) qRT-PCR of EGFR in *Phlpp1*$^{+/+}$ (+/+), *Phlpp1*$^{-/-}$ (–/–), or *Phlpp1*$^{-/-}$ cells reconstituted with wild-type HA-PHLPP1β (+WT). Each point was normalized to TATA box-binding protein as an internal control and then to wild type. Error bars represent the SEM of three independent experiments. (*E*) qRT-PCR of EGFR (mean ± SEM) from 6-mo-old *Phlpp1*$^{+/+}$ (+/+) and *Phlpp1*$^{-/-}$ (–/–) mouse prostate tissue. ***$P < 0.001$ by Student *t* test. (*F*) EGFR mRNA levels in *Phlpp1*$^{+/+}$ (+/+) and *Phlpp1*$^{-/-}$ (–/–) cells treated with actinomycin D (5 μg/mL) for the indicated times. Each point was normalized to an internal control (GAPDH) and then to control (without actinomycin D). Error bars indicate the SEM of three independent experiments.

the acetylation of specific histones at specific sites in *Phlpp1*$^{-/-}$ MEFs compared with wild-type MEFs (Fig. 5*B*). Specifically, acetylation was increased on K27 but not K9 on H3; phosphorylation was not significantly altered on S10 and S28; there was a robust increase in the double modification of S10 and K9 but not of S10 and K14. Lys acetylation was increased in H2A and H2B, but not H4; S139 phosphorylation was increased in H2A; no change in phosphorylation was noted on S14 in H2B. The increases in histone acetylation were reversed in *Phlpp1*$^{-/-}$ MEFs in which PHLPP1β had been reintroduced (Fig. S2). These data reveal a selective modification of the histone code by PHLPP1.

We next asked whether acetylated histones preferentially associated with the EGFR, INSR, and PDGFR promoters. ChIP assays revealed a significant increase in H3K27ac at the EGFR, PDGFR, and INSR promoters but not at the promoters of two genes whose mRNA levels are not affected by PHLPP1, *Cxcl10* or *Ccl4*, in samples from *Phlpp1*$^{-/-}$ MEFs as compared with wild-type MEFs (Fig. 6*A*). In contrast, there was no significant difference in the H4K5ac at the EGFR promoter or control promoters, but there was increased H4K5ac at the INSR and PDGFR promoters in *Phlpp1*$^{-/-}$ vs. wild-type MEFs. Although not detected on Western blot, there was a modest increase in H3K9ac at the EGFR promoter in *Phlpp1*$^{-/-}$ compared with wild-type MEFs. These data are consistent with PHLPP suppressing the transcription of RTKs by regulating histone acetylation at their receptor promoters.

If PHLPP controls the transcription of RTKs, we reasoned that loss of PHLPP would result in an increase of actively transcribed RTK mRNAs. We performed a global run-on experiment, which measures actively transcribing mRNAs (46), to show that the rate of transcription of EGFR, INSR, and PDGFR, but not the control genes *36B4*, *CCL4*, or *CXCl10*, was increased significantly in *Phlpp1*$^{-/-}$ vs. wild-type MEFs (Fig. 6*B*).

Last, we asked whether PHLPP1 binds to the RTK promoters. ChIP using antibodies to endogenous PHLPP1 revealed robust recruitment of PHLPP1 to the promoters of the EGFR, PDGFR, and INSR but not to the two control promoters (Fig. 6*C*). Note that the data are presented as the binding observed in the wild-type cells over the *Phlpp1*$^{-/-}$ MEFs to control for any nonspecific binding. These data demonstrate that PHLPP is actively recruited to the RTK promoters.

Discussion

The foregoing data unveil a previously unidentified mechanism by which PHLPP suppresses growth factor signaling: suppression of the steady-state levels of RTKs, notably the EGFR, by inhibiting their transcription (Fig. 7, *Left*). This mechanism is distinct from the previously characterized direct dephosphorylation of

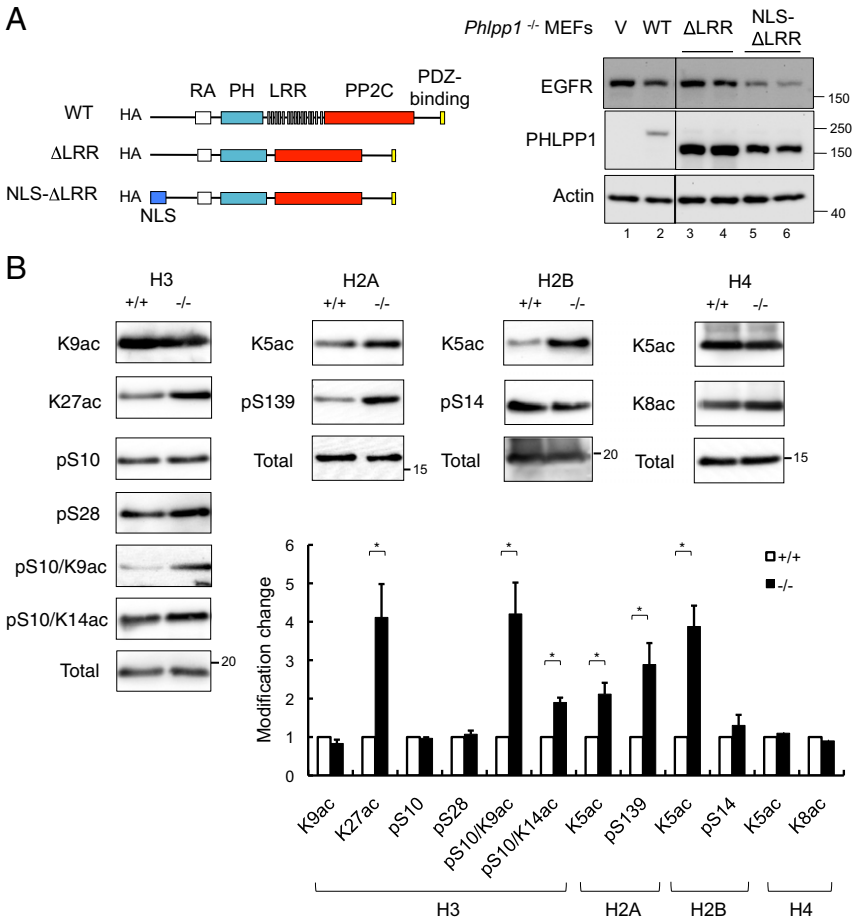

Fig. 5. Nuclear PHLPP1 regulates EGFR transcription. (*A*) Cartoon (*Left*) of wild-type PHLPP1β (WT), construct lacking LRR (ΔLRR), and construct lacking LRR with a nuclear localization signal (NLS- ΔLRR). Western blots of *Phlpp1*$^{-/-}$ or *Phlpp1*$^{-/-}$ cells reconstituted with different PHLPP1β constructs showing EGFR and PHLPP1 levels. Cells were reconstituted with an empty vector (V), wild-type PHLPP1β (WT), the ΔLRR construct, or the NLS-ΔLRR construct. (*B*) Western blot analysis of histone extracts from wild-type (+/+) or *Phlpp1*$^{-/-}$ (–/–) MEFs probed for the indicated acetylation and phosphorylation sites on the four core histones. The graph shows the mean ± SEM of quantified acetylation or phosphorylation normalized to total histone and to wild-type control from three independent experiments; *$P < 0.01$ by Student *t* test.

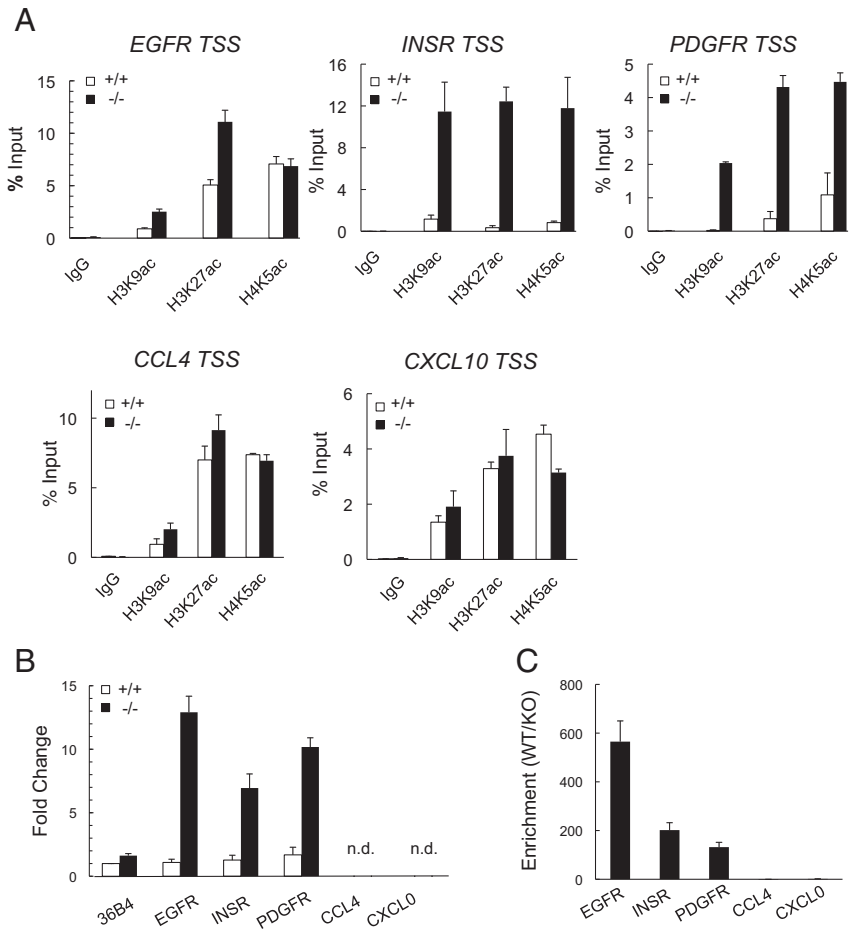

Fig. 6. PHLPP1 binds RTK promoters and controls RTK transcription. (*A*) ChIP assays assessing the levels of H3K9ac, H3K27ac, and H4K5ac at the transcription start sites (TSS) for the EGFR, INSR, PDGFR, Cxcl10, and Ccl4 genes in wild-type (+/+) and *Phlpp1*$^{-/-}$ (–/–) MEFs. (*B*) Quantification of nuclear run-on for 36B4, EGFR, INSR, PDGFR, Cxcl10, and CCL4 mRNA in wild-type (+/+) and *Phlpp1*$^{-/-}$ (–/–) MEFs. (*C*) ChIP assays assessing the recruitment of PHLPP1 at the transcription start sites (TSS) for the EGFR, INSR, PDGFR, CCl4, and CXCL10 genes in wild-type and *Phlpp1*$^{-/-}$ (KO) MEFs. Data shown represent the enrichment of the signal from the wild-type MEFs vs. signal from the *Phlpp1*$^{-/-}$ MEFs.

AKT (Fig. 7, *Right*) or PKC, which is selective for the hydrophobic motif, does not occur with the Leu1016Ser polymorphic variant of PHLPP2, requires an intact PDZ ligand (for AKT) or PH domain (for PKC), and dominates under basal conditions (24, 31, 42). In contrast, the mechanism regulating EGFR transcription is upstream of AKT and thus affects the phosphorylation state of both the hydrophobic motif and activation loop of AKT, remains intact with the Leu1016Ser polymorphic variant of PHLPP2, does not depend on the PDZ ligand or PH domain, requires an intact LRR and nuclear localization, and dominates under agonist-evoked conditions. Our data reveal an additional tumor-suppressive role for PHLPP as a regulator of RTK levels, thus broadly controlling multiple oncogenic pathways downstream of RTKs.

PHLPP Suppresses Histone Acetylation and RTK Transcription. Analysis of tissue or MEFs from *Phlpp1*$^{-/-}$ mice reveals that loss of PHLPP1 results in a robust increase in the steady-state levels of multiple RTKs, including the EGFR. Depletion of either PHLPP1 or PHLPP2 in both normal and cancer cell lines reveals that both PHLPP isozymes suppress RTK levels. Pharmacological analysis showed that the suppression of RTK levels by PHLPP is unlikely to be controlled by any of the known PHLPP substrates: Chronic inhibition of the three pathways (AKT, ERK, and PKC) currently known to be suppressed by PHLPP yielded no significant change in EGFR levels. Rather, RTK levels are regulated by a previously unidentified nuclear function of PHLPP in suppression of the acetylation of specific histones at specific sites. Rescue experiments reveal that the catalytic activity of PHLPP is required, as is the LRR domain; however the requirement for the LRR can be bypassed by forcing PHLPP into the nucleus by fusing an NLS on the protein. This nuclear localization is supported by previous fractionation studies showing PHLPP1 accumulation in the nucleus (47) and the ability to detect PHLPP1 at RTK promoters by ChIP assay. This nuclear-localized PHLPP suppresses acetylation of specific sites on the N-terminal tails of histones H2A, H2B, H3, and H4. At this point the mechanism for the PHLPP1-dependent increase in acetylation is unclear, but it is intriguing to note that these N-terminal tails are modified by phosphorylation. Thus, it will be of interest to determine whether PHLPP1 directly targets the phosphorylation of specific histone residues or influences the activity of histone acetyltransferases.

PHLPP as a Modifier of the Histone Code. Our data support a key role of PHLPP in modifying the histone code. Loss of PHLPP results in robust increases in phosphorylation and/or acetylation at specific sites on each of the four core histones. Whether these changes in the histone code result from direct dephosphorylation of residues such as S10 and S28 on H3 and S139 on H2A, in turn influencing acetylation at adjacent sites, remains to be elucidated. Indeed, molecular crosstalk is known to occur between histone phosphorylation and acetylation (18). Notably, phosphorylation at S10 and T11 of H3 leads to an increase in acetylation at

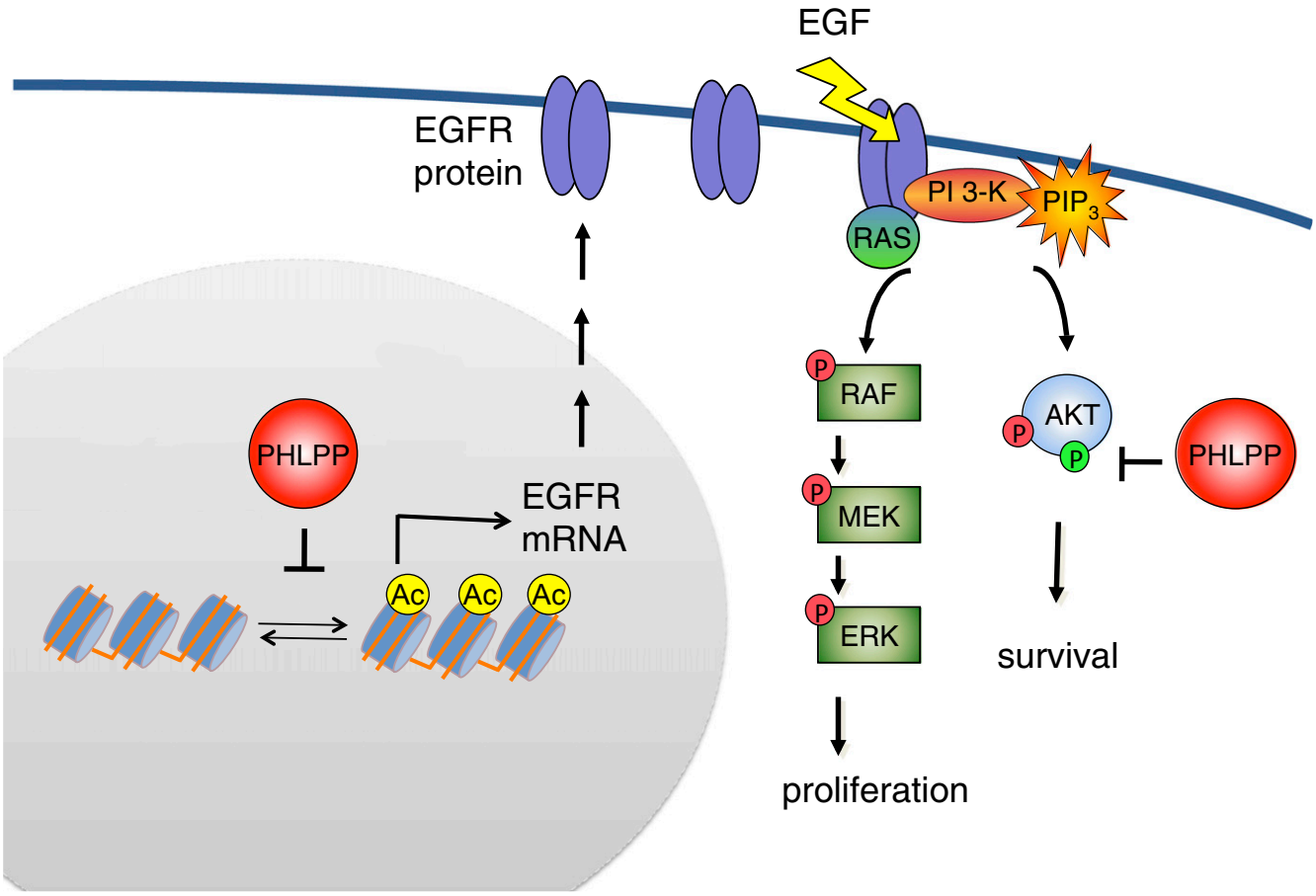

Fig. 7. Proposed model for PHLPP-dependent suppression of EGFR signaling. On the left side of the figure, nuclear-localized PHLPP suppresses histone acetylation and thus represses the transcription of EGF and other RTKs, acting as a master regulator of growth factor signaling. Reduced levels of EGFR result in a lower amplitude of agonist-evoked signaling by both RAS/ERK and PI3 kinase/AKT oncogenic pathways. This function of PHLPP is distinct from the previously characterized direct dephosphorylation of Akt on the hydrophobic motif (green circle on Akt) shown on right side of figure. Also shown are the activation loop phosphorylations on Raf, MEK, ERK, and AKT (red circles), which are not reported to be direct targets of PHLPP. Note that these two mechanisms can be differentiated by a number of criteria, as described in the text.

K9 and K14 (19, 48, 49), as is consistent with our observation of coincident increases in phosphorylation on S10 and acetylation on K9 and K14 in cells lacking PHLPP1. The finding that PHLPP1 localizes at promoters whose transcription is suppressed by PHLPP but not at promoters whose transcription is not sensitive to PHLPP supports the possibility that PHLPP directly modifies histones on these promoters. Alternatively, PHLPP could dephosphorylate the writers or erasers that modify the code, thus modifying their function. Arguing against this notion is the finding that the effects of PHLPP are selective for certain promoters; one might have predicted less selectivity in the transcriptional target genes if PHLPP broadly regulated HDACs or histone acetyltransferases.

Our data are consistent with a model in which PHLPP serves as an eraser of the histone code by dephosphorylating specific histone residues, in turn suppressing acetylation, and thus repressing transcription. Interestingly, it has been shown that stimulation with growth factors increases phosphorylation of histone H3 on S10, a modification that correlates with the transcriptional activation of immediate-early genes that lead to proto-oncogenic induction (50). Our data suggest that PHLPP opposes this mechanism, contributing to its strong tumor-suppressive phenotype.

Tumor-Suppressive Function of PHLPP. Because RTKs are up-regulated in diverse cancers, receptor levels often serve as markers for tumor progression (51, 52), and designing drugs that inhibit the EGFR has become a major focus of cancer therapeutics in recent years (53, 54). The identification of PHLPP as a master switch to control RTK levels is likely to be clinically relevant. Gene-expression studies have revealed that PHLPP1 and PHLPP2 expression is reduced in glioblastoma, providing a possible mechanism for the overexpression of EGFR in the absence of gene amplification (55). Another EGFR family member, HER2, is frequently overexpressed in later-stage, more metastatic breast cancers in which PHLPP1 expression is reduced (56, 57). The expression of the PHLPP isozymes is reduced in many tumor types and, in some cases, decreases as tumorigenesis progresses (57–59). Consistent with this reduction, the ectopic expression of PHLPP in a glioblastoma and in a colon cancer cell line significantly reduced tumor growth in vivo in xenograft mouse models (24, 58). Whether these tumor-suppressive effects of PHLPP are mediated directly by its regulation of growth factor receptor levels remains to be explored. Importantly, the dramatic effects of PHLPP on EGFR signaling place PHLPP at center stage as a global suppressor of oncogenic pathways.

Conclusion

The foregoing data show an even broader role for PHLPP as a tumor suppressor, beyond its role as a direct phosphatase for AKT, PKC, S6K, and Mst1, by suppressing the steady-state levels of RTKs, such as the EGFR, via its control of histone phosphorylation/acetylation and hence transcription. Given the high up-regulation of multiple RTKs in cancers (3), PHLPP may provide a pharmacological

target to suppress levels of these oncogenic proteins effectively, particularly because the pharmacological profile of the up-regulated receptor is unchanged. PHLPP is a more tenable pharmacological target than most other phosphatases because of its distinct structural composition with regulatory/targeting moieties encoded in the same polypeptide as the phosphatase domain.

Materials and Methods

Phlpp1$^{+/\Delta}$ heterozygous mice were intercrossed to isolate embryos and were used for *Phlpp1*$^{+/+}$ and *Phlpp1*$^{-/-}$ fibroblast production, as described previously (60). EGFR levels were analyzed in immortalized MEFs stably expressing various PHLPP constructs, mammalian cells overexpressing PHLPP1 and/or PHLPP2, or mammalian cells in which PHLPP1 and/or PHLPP2 were silenced by siRNA. A full description of biochemical and cell biological assays to probe EGFR function, stability, and turnover, as well as the methods used to detect mRNA levels and perform ChIP assays, can be found in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Roger Tsien for the gift of the EGFR kinase activity reporter construct and Dr. Frank Furnari for the pcL10A vector for retroviral packaging. M.N. was supported in part by the University of California at San Diego Graduate Training Program in Cellular and Molecular Pharmacology through NIGMS, National Institutes of Health (NIH) Institutional Training Grant T32 GM007752. This work was supported by NIH Grants CA137050 (to L.C.T.) and GM067946 (to A.C.N.).

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