Pla2g16 phospholipase mediates gain-of-function activities of mutant p53

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***p53*$^{R172H/+}$ mice inherit a p53 mutation found in Li-Fraumeni syndrome and develop metastatic tumors at much higher frequency than *p53*$^{+/-}$ mice. To explore the mutant p53 metastatic phenotype, we used expression arrays to compare primary osteosarcomas from *p53*$^{R172H/+}$ mice with metastasis to osteosarcomas from *p53*$^{+/-}$ mice lacking metastasis. For this study, 213 genes were differentially expressed with a *P* value <0.05. Of particular interest, *Pla2g16*, which encodes a phospholipase that catalyzes phosphatidic acid into lysophosphatidic acid and free fatty acid (both implicated in metastasis), was increased in *p53*$^{R172H/+}$ osteosarcomas. Functional analyses showed that *Pla2g16* knockdown decreased migration and invasion in mutant p53-expressing cells, and vice versa: overexpression of *Pla2g16* increased the invasion of *p53*-null cells. Furthermore, Pla2g16 levels were increased upon expression of mutant p53 in both mouse and human osteosarcoma cell lines, indicating that Pla2g16 is a downstream target of the mutant p53 protein. ChIP analysis revealed that several mutant p53 proteins bind the *Pla2g16* promoter at E26 transformation-specific (ETS) binding motifs and knockdown of ETS2 suppressed mutant p53 induction of Pla2g16. Thus, our study identifies a phospholipase as a transcriptional target of mutant p53 that is required for metastasis.**

mammary tumor | fatty acid metabolism

The p53 tumor suppressor pathway is inactivated in ∼50% of human cancers (http://p53.iarc.fr). Missense mutations in particular account for 80% of *p53* alterations, suggesting that mutant p53 proteins provide additional advantages for tumor cell growth (1). Li-Fraumeni syndrome patients with p53 missense mutations have a higher cancer incidence and an earlier age of tumor onset than individuals with truncating or splicing mutations (2). *p53* knockin mice show a gain-of-function (GOF) phenotype in vivo, with high metastatic capacity compared with mice inheriting a *p53*-null allele (3, 4). GOF activities of mutant p53 are mediated by suppression of the p53 family members, p63 and p73 (3–6). Other mechanisms of mutant p53 GOF include mutant-p53 complexes with Smad that fuel TGF-β–induced metastasis (7) and integrin recycling (8). Additionally, mutant p53 interacts with the vitamin D receptor and converts vitamin D into an antiapoptotic agent (9–14). More recently, mutant p53 was reported to form transcriptional complexes on promoters of genes encoding several enzymes of the Mevalonate pathway, which increases metastasis of breast cancer cells (9). These data suggest multiple pathways contribute to the GOF phenotypes of cells with mutant p53. Although mutant p53 lacks sequence-specific DNA binding activity, its interaction with other transcriptional factors or the components of basic transcriptional machinery allow it to modulate gene expression (15). ChIP-on-chip and ChIP-sequencing techniques show that mutant p53 affects transcription of many genes (9, 13, 16, 17).

In this study, expression array analyses identified gene differences between *p53*$^{R172H/+}$ metastatic osteosarcoma samples and *p53*$^{+/-}$ osteosarcomas that lack metastatic potential (3, 18). We focused on *Pla2g16* because it was present at high levels in p53 mutant tumors and it encodes an A2 group 16 phospholipase with reported roles in tumor metastasis.

Pla2g16 is also called H-REV-107, HRASLS3 (Ha-RAS like suppressor 3), and AdPLA (adipose specific PLA2) (19–21) and was first identified as a class II tumor suppressor, because it suppressed Ras-mediated transformation in cultured cells, and its overexpression led to proliferation inhibition and apoptosis (19, 22–24). However, Pla2g16 was also labeled an oncogene because it increases proliferation of nonsmall-cell lung cancer cells and its overexpression correlates with a poor prognosis (25). Functionally, Pla2g16 is a member of the phospholipase family of small lipases that exhibit diverse functions, including digestion of dietary phospholipids and cell signaling (Fig. S1) (21, 26, 27). More importantly, Pla2g16 generates lysophosphatidic acid and free fatty acid from phosphatidic acid, both of which increase proliferation, migration, and metastasis (26, 28–30). *Pla2g16*-null mice are lean when fed a high-fat diet and crosses with an obese mouse model, *Ob*/*Ob*, resulted in double-null mice being significantly leaner than *Ob*/*Ob* mice (31). Because obesity contributes to tumor progression and poor prognosis (32, 33), these studies suggest that Pla2g16 plays an important role in fat metabolism, which may contribute to more aggressive tumor phenotypes.

Pla2g16 shRNA knockdown or overexpression in osteosarcoma and mammary tumor effects cell proliferation, migration, and invasion. Our results further demonstrate that mutant p53 binds E26

Significance

Mutations of p53 occur in approximately 50% of human cancer. p53 missense mutations exhibit gain-of-function activities. In this study, we discovered a previously unidentified mechanism of mutant p53 gain-of-function in osteosarcoma and mammary tumors. Our data indicate that mutant p53 binds to E26 transformation-specific motifs in the *Pla2g16* phospholipase promoter to induce its expression, which leads to more aggressive and metastatic phenotypes. Thus, the study implicates mutant p53 regulation of lipid metabolism in cancer cells to confer its gain-of-function. The study suggests new therapeutic options for patients with mutant p53.

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transformation-specific (ETS) sequences in the *Pla2g16* promoter indirectly through ETS2, revealing a previously unidentified mechanism of mutant p53 GOF. Thus, Pla2g16 may be a therapeutic target for metastatic osteosarcomas and mammary tumors.

Materials and Methods

Mice and Tumor Analysis. All mouse experiments were performed in compliance with the M. D. Anderson Cancer Center (MDACC) Institutional Animal Care and Use Committee. Tumors from $p53^{+/-}$ and $p53^{R172H/+}$ mice in a C57BL/6 background were used for the array analysis. $p53^{+/-}$ mice in a BALBc/J background were purchased from the Jackson Laboratory; $p53^{R172H/+}$ breeders were backcrossed into BALBc/J background until 99% BALBc/J as determined by polymorphic allele analysis by the Research Animal Support Facility–Smithville, Genetic Services. For radiation treatment, 4-wk-old female mice were irradiated, as previously described (34).

Affymetrix Array Analysis. Total RNA was extracted from $p53^{+/-}$ and $p53^{R172H/+}$ osteosarcoma tumors using the RNAeasy kit (Qiagen). Affymetrix Genechips (U430, 2.0 plus; Affymetrix) were used for the analysis by the MDACC Microarray Core Facility. Data analyses were performed using dChip software v2010 as previously reported (35). The raw data were normalized against a default baseline array by the Invariant Set Normalization method (36).

shRNA Knockdown and Overexpression of *Pla2g16* in Cells. Tumor cell lines from $p53^{R172H/+}$ osteosarcoma (H76) and from $p53^{+/-}$ osteosarcomas (026-3, 222) were generated. All of these cell lines had lost the wild-type *p53* allele. The lentivirus plasmids for *Pla2g16,* human *p53*, and control *EGFP* knockdown were purchased from Sigma, and mouse p63 and p73 lentivirus plasmids were obtained from the MDACC shRNA Core Facility. The primers used for real-time quantitative PCR are: Mouse Pla2g16: forward primer GCTCCTCCAAGTGAAATCGC; reverse primer AGCAGACATGATGCTGGCTG. Human Pla2g16: forward primer CCAGGTCAACAACAAACATGATG; reverse primer CCCGCTGGATGATTTTGC. *GAPDH* or *RPLP0* genes were used as quantitative RT-PCR (qRT-PCR) internal controls (37). The murine *p53* knockdown plasmid was reported previously (38). Flag-tagged mouse *Pla2g16*, mouse *p53R172H*, or human *p53R175H* cDNAs were cloned in pBabe-puro vector and transfected into Phoenix cells. pWZL-BlastGFP, R175H, H179R, G245S, R248Q, and R273H plasmids were used to overexpress human p53 mutants in Saos2 cells. The overexpression cells were selected with puromycin or blasticidin for 1 wk.

Western Blotting and Immunohistochemical Analyses. Antibodies used were Pla2g16 (Cayman Chemical), mouse p53 (CM5) (Vector), human p53 (DO-1), and Flag (M2), β-actin (AC-15), vinculin, and γ-tubulin (GTU-88) (Sigma). Immunohistochemical staining was performed with 1:200 dilution of Pla2g16 antibody on human tissue microarrays (OS804, slide no.14) from US Biomax.

Migration, Invasion, Cell Proliferation, and Colony Formation Assays. Migration and invasion assays were modified from previous studies (39). H76 and H318 cells were incubated for 16 and 40 h, respectively, and 4T1 and LM7 cells were incubated for 5 h and 16 h for migration and invasion, respectively. Two-thousand cells for H318-1, LM7, 4T1 and 6,000 cells for H76, Saos2 and MDA-MB231 were used in cell proliferation and colony formation assays. Methyl thiazolyl tetrazolium (MTT) at 5 μg/mL was incubated in 24-well plates for 3 h, and the optical density at 550 nm was measured daily. For colony formation assays, H76, H318-1, and LM7 cells were cultured for 14 d. MDA-MB231, Saos2-R175H, and 4T1 cells were cultured for 8 d.

Xenograft Tumor Growth Experiment. Three-million H318-1 cells with control or Pla2g16 shRNA were injected subcutaneously into wild-type mice (50% C57BL/6J and 50% 129/J), and primary tumors were measured using digital calipers 3 mo after injection.

ChIP Assay and siRNA Knockdown. ChIP was performed as previously described and according to the protocol for the p53 ExactaChiP kit (R&D Biosystems) (16). The primers used for PCR analysis of ChIP samples were BS1 forward: GAAACAGTGGATTTGAACTT and BS1 reverse: ATTCAGAGGATGGGATTT, and BS2 forward: GGATTTATTGTCATTAACAGG and BS2 reverse: GCGAGAAAGTTGTTAAAGG. Saos2-R273H cells were transfected with either control, ETS1 or ETS2 siRNA, and processed for Western blot or real-time RT-PCR analysis, as previously described (16).

Results

Overexpression of *Pla2g16* in $p53^{R172H/+}$ Osteosarcomas with Metastasis. The observations that mutant p53 contributes to the GOF metastatic phenotype and that the N-terminal transcriptional activation domain is required for its GOF activities (40) led us to explore the changes in gene expression that accompany mutant p53 expression in vivo. Because osteosarcomas are common in Li-Fraumeni syndrome patients and osteosarcomas commonly metastasize in the $p53^{R172H/+}$ mouse model, we performed expression arrays to identify differences between metastatic osteosarcomas from eight $p53^{R172H/+}$ mice versus three osteosarcomas that lacked metastatic potential from $p53^{+/-}$ mice. Next, 213 genes were identified that had a P value < 0.05 and an average fold-difference larger than 1.5 (Fig. 1*A* and Dataset S1). Because *Pla2g16* was reported to be involved in metastasis, and it was up-regulated on average 3.9-fold ($P = 0.04$) in $p53^{R172H/+}$ osteosarcomas compared with osteosarcomas from $p53^{+/-}$ mice, we next validated its expression in a larger group of tumors. The expression of *Pla2g16* was significantly higher by 3.3-fold ($P = 0.0197$) in 11 primary osteosarcoma samples from $p53^{R172H/+}$ mice with lung or liver metastases compared with 9 nonmetastatic primary osteosarcomas from $p53^{+/-}$ mice (Fig. 1*B*). Immunohistochemical analysis of Pla2g16 on available osteosarcoma samples showed 75% (six of eight) of tumors from $p53^{R172H/+}$ mice were positive, but only 22% (two of nine) of tumors from the $p53^{+/-}$ mice showed positive staining (Fig. 1*C*). These data indicated that higher expression of *Pla2g16* may be associated with metastatic $p53^{R172H/+}$ osteosarcoma tumors.

Pla2g16 Contributes to Osteosarcoma Progression and Metastasis. To examine the functional consequences of Pla2g16 expression, we performed *Pla2g16* shRNA knockdown in H76 and H318-1 primary cell lines generated from $p53^{R172H/+}$ osteosarcomas that

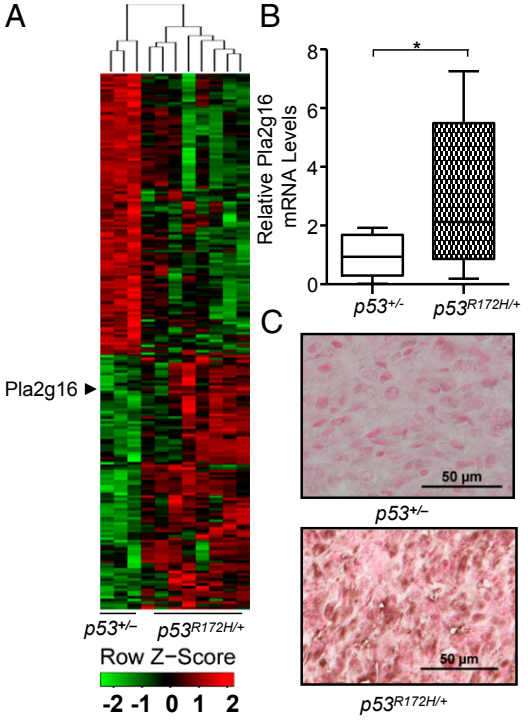

Fig. 1. Increased Pla2g16 expression in $p53^{R172H/+}$ osteosarcomas. (*A*) Heat map of Affymetrix array. (*B*) Validation of *Pla2g16* expression in osteosarcoma tumors was determined by real-time quantitative PCR (*$P < 0.05$). (*C*) Representative immunohistochemical staining of Pla2g16 in $p53^{+/-}$ and $p53^{R172H/+}$ primary and metastatic osteosarcoma samples. (Scale bar, 50 μm.) Tumors were considered to be positive when 10% or more of dysplastic/tumor cells were stained.

had lost the wild-type *p53* allele (3). Pla2g16 knockdown significantly decreased expression of *Pla2g16* by more than 80% by qRT-PCR and Western blot analyses (Fig. 2*A*). MTT assays showed that H76 and H318-1 cells with *Pla2g16* knockdown clearly grew more slowly (Fig. S2*A*). To test the specificity of *Pla2g16* knockdown, we next examined the effects of inhibition of Pla2g16 enzymatic activity by a previously reported chemical inhibitor, MAFP (methyl arachidonyl fluorophosphate) (21). MAFP showed strong inhibition of H76 and H318-1 proliferation by MTT assays, indicating that Pla2g16 activity is important for osteosarcoma cell proliferation (Fig. S2*B*). Cell migration and invasion were determined by Boyden chamber assays. H76 and H318-1 cells treated with *Pla2g16* shRNA had a 4.3- and 10.8-fold reduction of migration, and 5.6- and 6.6-fold reduction of invasion compared with the control EGFP knockdown cells, respectively (Fig. 2 *B* and *C*). Importantly, the proliferation of *Pla2g16* shRNA-treated cells did not vary significantly during the time frame of the migration and invasion assays (16 and 40 h, respectively) (Fig. S2*A*), indicating that Pla2g16 contributed to the metastatic potential of these cells. A measure of low-density colony formation using the shRNA-treated cells to test individual cell survival and proliferation also showed less colonies in *Pla2g16* knockdown cells compared with control cells (Fig. 2*D*), demonstrating that Pla2g16 expression is also important for clonogenic ability of osteosarcoma cells. Finally, we injected pools of *Pla2g16* knockdown H318-1 cells subcutaneously into wild-type mice. All six mice injected with control shRNA had significantly larger primary tumors with metastasis to the lungs, whereas injections of cells with knockdown of *Pla2g16* yielded only one tumor that metastasized to the lung (Fig. 2*E* and Fig. S2*C*). This one outlier had an expression level of Pla2g16, similar to that of mice that received control shRNA injections, suggesting that the outlying tumor was derived from cells that had escaped *Pla2g16* knockdown. The expression levels of *Pla2g16* in metastatic lung lesions from these mice were similar to the tumors that arose at the injection sites (Fig. S2*D*). To validate the specificity of shRNA knockdown, we generated a shRNA-resistant cDNA by using an alternative amino acid codon and transfected it back into the H318-1 shRNA-treated cells. Cell proliferation and colony formation were increased with restored *Pla2g16* expression (Fig. S3), which demonstrated that the phenotypes affected by shRNA knockdown were specifically caused by Pla2g16. To determine whether we could make nonmetastatic cells adopt metastatic properties by simply overexpressing *Pla2g16*, we generated stable overexpression of Flag-tagged *Pla2g16* in two *p53*-null osteosarcoma cell lines (222 and 026-3) that lacked metastatic potential. These cells showed, on average, a sixfold increase in invasion in Boyden chamber assays (Fig. 2*F*). These data clearly demonstrated the importance of Pla2g16 in osteosarcoma cell proliferation, migration and metastasis.

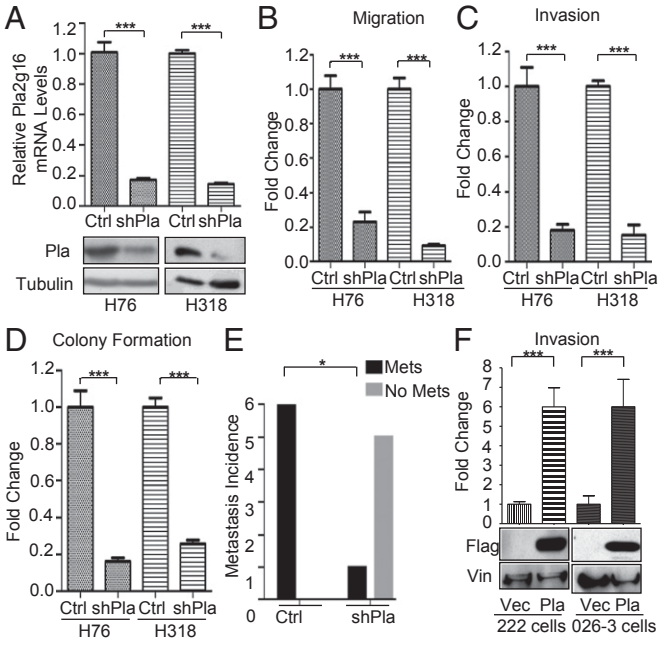

Fig. 2. Pla2g16 contributes to the metastatic potential of $p53^{R172H/+}$ osteosarcomas. (*A*) shRNA knockdown of Pla2g16 in $p53^{R172H/+}$ osteosarcoma cell lines H76 and H318-1 was determined by qRT-PCR (*Upper*) and Western blot analysis (*Lower*). Migration (*B*) and invasion (*C*) assays were performed using *Pla2g16* knockdown cells for 16 h and 40 h, respectively. (*D*) Colony formation assays were performed for *Pla2g16* knockdown cells. (*E*) Number of mice with or without metastasis after injection with control and *Pla2g16* knockdown cells. (*F*) Overexpression of *Pla2g16* in $p53^{-/o}$ osteosarcoma cells increased invasion. Pla, Pla2g16; Flag, Flag-tag; Vin, vinculin; Ctrl, shRNA of EGFP; shPla, shRNA of Pla2g16; vec, pBabe-puro; $^{*}P < 0.05$ and $^{***}P < 0.0005$.

To investigate the role of Pla2g16 in human osteosarcoma metastasis, we first examined the expression levels of Pla2g16 in a pair of osteosarcoma cell lines: Saos2 (parental) and LM7 (a metastatic subline developed by seven rounds of repetitive tail-vein injections) (41). Interestingly, Pla2g16 expression in LM7 cells was 2.4- and 2.0-fold higher than in Saos2 cells, as measured by qRT-PCR and Western blotting, respectively (Fig. 3*A* and Fig. S4*A*). Because these cells have a *p53* deletion, the data suggest that p53-independent mechanisms can also contribute to increased Pla2g16 levels. Next, we knocked down *Pla2g16* expression in LM7 cells to examine whether decreased Pla2g16 expression affected their metastatic potential. Two independent human shRNAs (shRNA1 and shRNA2) reduced *Pla2g16* expression to 40% and 71%, respectively (Fig. 3*B*). These two *Pla2g16* shRNAs clearly reduced cell proliferation as measured by MTT assays (Fig. S4*B*), decreased migration by 2.6- and 2.1-fold, respectively (Fig. 3*C*), and also inhibited invasion by 3.5- and 2.6-fold, respectively (Fig. 3*D*). Furthermore, low-density colony formation also decreased by 3.6- and 1.7-fold in these two Pla2g16 knockdown cells, respectively (Fig. 3*E*). The efficiency of shRNA1 knockdown was better than shRNA2 (Fig. 3*B*) and this correlated with a higher decrease in proliferation, migration, invasion, and colony formation with shRNA1 than shRNA2, implicating dosage dependency of *Pla2g16*. Conversely, when we overexpressed *Pla2g16* in Saos2 cells (Fig. 3*G*), cell proliferation and colony formation were increased (Fig. 3*F* and Fig. S4*C*). Taken together, these data strongly implicate the importance of *Pla2g16* expression in cell proliferation and metastatic potential in both mouse and human osteosarcoma.

To further explore the role of Pla2g16 in human osteosarcoma patients, we examined the levels of Pla2g16 in a commercial human osteosarcoma tumor microarray by immunohistochemical staining. Among 40 human osteosarcoma samples, 21 tumors (52.5%) had positive Pla2g16 staining (with 10% or more positive tumor cells), indicating that high levels of Pla2g16 was a common event in human osteosarcoma (Fig. 3*H*). The *p53* status of these samples is unknown.

Pla2g16 Is Regulated by Mutant p53. The above studies demonstrated that increased Pla2g16 expression contributed to the metastatic potential of osteosarcoma cell lines. We next investigated whether the induction of Pla2g16 was dependent on p53R172H levels. Knockdown of *p53R172H* expression in H318-1 osteosarcoma cells showed a decrease in Pla2g16 phospholipase levels (Fig. 4*A*). Conversely, when p53R172H was overexpressed in $p53^{-/-}$ mouse embryonic fibroblasts or $p53^{-/0}$ osteosarcoma cells (222), Pla2g16 mRNA and protein were increased (Fig. 4*B* and Fig. S5 *A* and *B*), indicating the Pla2g16 expression was directly induced by p53R172H.

To examine the relationship between Pla2g16 expression and human mutant p53, *p53R175H* (equivalent to mouse p53R172H) was introduced into *p53*-null Saos2 cells. Pla2g16 levels were increased by *p53R175H* overexpression at both mRNA (Fig. S5*C*) and protein levels (Fig. 4*C*), indicating that increased

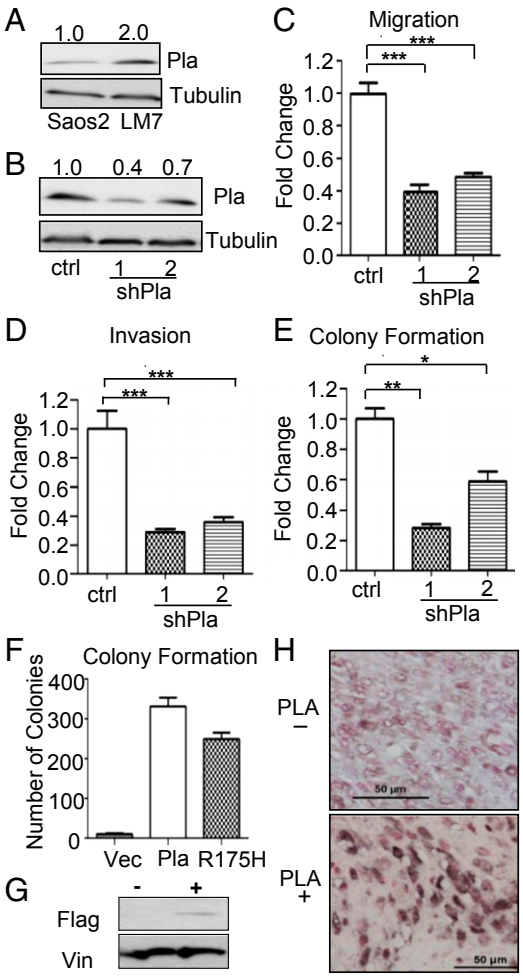

Fig. 3. Higher expression of Pla2g16 in human osteosarcoma cells contributes to higher metastatic potential. (*A*) Expression of Pla2g16 in LM7 osteosarcoma cells was determined by Western blot analysis. (*B*) Two shRNAs were used to knock down Pla2g16 in LM7 cells which showed reduced migration (*C*), invasion (*D*), and colony formation (*E*). (*F*) Western blot of Pla2g16 overexpression in Saos2 cells affects colony formation (*G*). (*H*) Representative immunohistochemical staining of human osteosarcoma samples with a Pla2g16 antibody. (Scale bar, 50 μm.) $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$.

Pla2g16 expression was mediated by p53R175H in human osteosarcoma cells. As expected, the expression of *p53R175H* dramatically increased cell proliferation and colony formation as previous studies have shown (Fig. 4*D* and Fig. S5*D*) (42, 43). We then knocked down *Pla2g16* expression in the *p53R175H* expressing Saos2 cells with two different shRNAs (Fig. 4*C*). Strikingly, decreased Pla2g16 expression in the *p53R175H*-expressing Saos2 cells strongly inhibited proliferation and colony formation (Fig. 4*D* and Fig. S5*D*), indicating these GOF phenotypes clearly required expression of *Pla2g16*. Taken together, these data demonstrate that increased Pla2g16 levels by p53R172H or p53R175H are important in osteosarcoma progression and metastasis.

To determine if the effect of p53R175H on Pla2g16 expression was more broadly applicable to other p53 mutants, we tested the following human mutants: p53H179R, p53G245S, p53R248Q, and p53R273H. Expression in Saos2 cells showed the levels of Pla2g16 were clearly increased by all mutants (Fig. 5*A*), suggesting that expression of Pla2g16 could be important for the GOF activities of other p53 mutants as well. The levels of mutant p53 expressed are much lower in comparison with human tumor cell lines (Fig. S5*E*).

Mutant p53 Interacts with ETS2 to Regulate Pla2g16 Expression. Previous studies show p53R172H exerts its GOF through suppression of p53 family members p63 and p73 (3, 4, 7, 8). However, comparison of Pla2g16 expression in $p63^{-/-}$ or $p73^{-/-}$ mouse embryonic fibroblasts (MEFs) to wild-type MEFs showed no significant difference in Pla2g16, suggesting that *Pla2g16* is not a transcriptional target of p63 or p73 (Fig. S6 *A* and *B*). In addition, when p63 or p73 was overexpressed in H318-1 cells, the expression of Pla2g16 also did not change (Fig. S6*C*). Thus, our data indicated that p53R172H-mediated Pla2g16 overexpression likely occurs through p63- and p73-independent pathways.

Because some GOF activities are mediated by mutant p53 interaction with other transcriptional factors in the promoters of multiple genes, ChIP assays were performed to determine if mutant p53 was present on the *Pla2g16* promoter. Previously, we reported a genome-wide analysis of mutant p53 binding, and we mined these datasets to determine if mutant p53 was associated with the *Pla2g16* promoter (16). We identified two putative binding sites (BS1 and BS2) for mutant p53 in the *Pla2g16* promoter and we confirmed that it bound to these sites using an independent ChIP (Fig. 5*B*). Furthermore, we found four other p53 mutants associated with the *Pla2g16* promoter (Fig. 5*B*). Interestingly, both of these sites contained ETS binding motifs, suggesting that mutant p53 may up-regulate Pla2g16 by interacting with ETS family members. To determine if this was the case, we assessed the impact of knocking down either ETS1 or ETS2 using previously characterized siRNAs (16) on *Pla2g16* expression. Whereas knockdown of ETS1 had no effect, ETS2 knockdown significantly reduced *Pla2g16* expression, suggesting that ETS2 was required for the recruitment of mutant p53 to the *Pla2g16* promoter (Fig. 5*C*). We then performed invasion assays using ETS knockdown cells. Invasion was reduced by both ETS1 and ETS2 knockdown, suggesting that ETS proteins regulate invasion by Pla2g16-dependent and -independent mechanisms (Fig. S7). A comparison of the ChIP-seq data from Do et al. (16) and the 213 probe sets that were differentially expressed in this study showed that 54% of the genes overlap. Additional

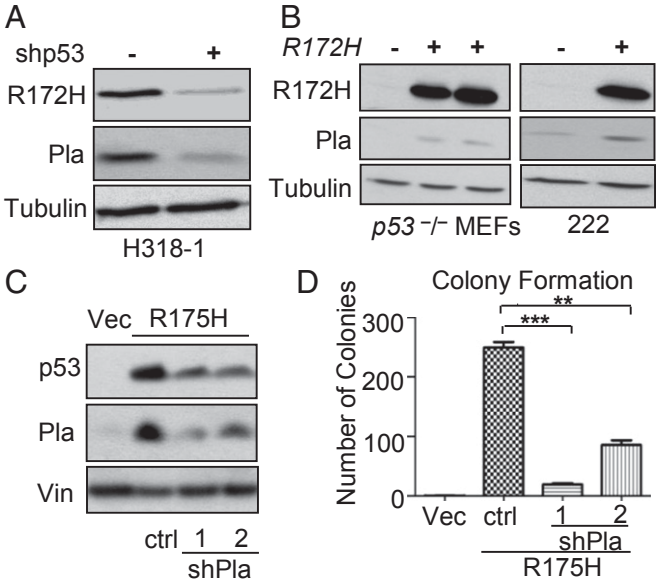

Fig. 4. Up-regulation of Pla2g16 by p53 mutants. (*A*) Western blots of H318-1 cells with and without p53R172H knockdown. (*B*) Western blots of $p53^{-/-}$ MEFs and $p53^{-/o}$ osteosarcoma cell line (#222) with addition of p53R172H. (*C*) Western blots for *p53R175H* overexpression in Saos2 cells with Pla2g16 shRNA knockdown. (*D*) shRNA knockdown of Pla2g16 in *p53R175H* expressing Saos2 cells inhibited colony formation. shp53, shRNA for p53; shpla, shRNA for Pla2g16; Vec, *pBabe* vector for overexpression of *p53R175H*. $**P < 0.005$, $***P < 0.0005$.

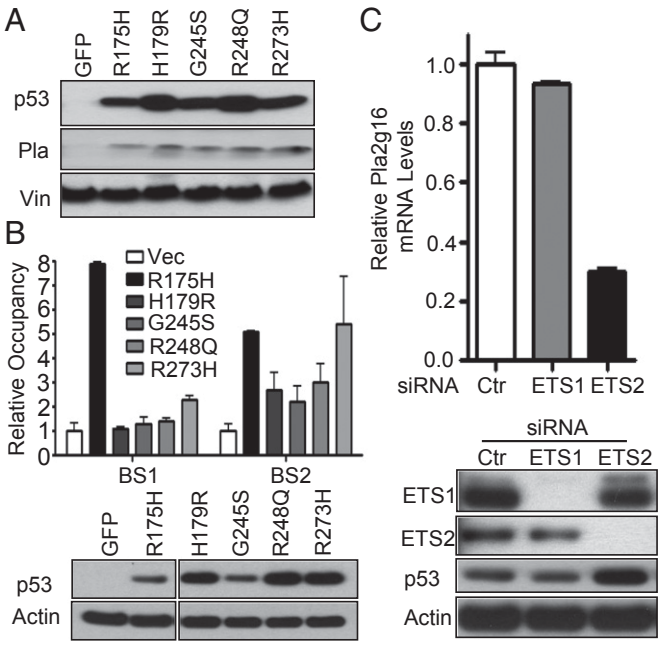

Fig. 5. ETS2 mediated up-regulation of Pla2g16 by mutant p53. (*A*) Western blot of Saos2 cells and (*B*) ChIP assays in Saos2 cells engineered to express different p53 mutants. (*Upper*) Relative occupancy of binding site 1 (BS1) and binding site 2 (BS2) in the Pla2g16 promoter; (*Lower*) levels of p53 mutants from the same extracts used in ChIP assays loaded on one gel. (*C*) Knockdown of ETS2 reduced *Pla2g16* expression in mutant p53 overexpressing cells. (*Upper*) Relative expression levels of Pla2g16 in siRNA knockdown cells determined by qRT-PCR; (*Lower*) Western blots of ETS1 and ETS2 in siRNA knockdown cells. GFP: pWZL vector for overexpression of GFP and p53 mutations, R175H, H179R, G245S, R248Q, R273H. Ctr, Control.

experiments will be needed to determine if mutant p53 binds all of these genes through the same motif.

***Pla2g16* Overexpression Contributes to Metastatic Potential of Mammary Tumors.** To investigate whether increased expression of *Pla2g16* contributes to progression and metastasis in other types of tumors, we generated $p53^{R172H/+}$ mice in a BALB/cJ background. BALB/cJ mice are inherently susceptible to mammary tumors and the absence of *p53* increases tumor incidence (44). We also treated these mice with a sublethal dose of radiation to decrease the latency of tumorigenesis, as previously described (34). Clearly, radiation accelerated the tumorigenesis of $p53^{R172H/+}$ mice with median survival time of 303 d compared with 423 d (Fig. 6*A*). In addition, radiation treatment increased metastasis of mammary tumors from 23% to 34% (Table 1). Expression of *Pla2g16* was 6.7- and 5.5-fold higher (P value < 0.05) in nonirradiated and irradiated $p53^{R172H/+}$ mammary tumors with lung metastasis than in $p53^{+/-}$ tumors without metastasis, respectively (Fig. 6*B*). To further explore the role of *Pla2g16* in mammary tumor progression and metastasis, we chose a highly metastatic mouse mammary tumor cell line, 4T1, and knocked down *Pla2g16* expression by using the same shRNA plasmid used in H318-1 cells. The shRNA knockdown reduced Pla2g16 expression by 80% in the 4T1 cells as measured by qRT-PCR and Western blotting (Fig. 6 *C* and *D*). Pla2g16 knockdown clearly inhibited cell proliferation as measured by MTT assays (Fig. S8*A*). Migration and invasion were reduced by 2.5- and 4.3-fold, respectively (Fig. 6 *E* and *F*), and colony formation was suppressed by 1.9-fold (Fig. S8*B*). Next, we examined the effect of knocking down *Pla2g16* in metastatic human breast cancer cell lines with various p53 mutations: SKBr3 (p53R175H), MDA-MB231 (p53R280K), Au565 (p53R175H), and BT549 (p53R249S). Mutant p53 knockdown caused a decrease in Pla2g16 levels in SKBr3 and Au565 cell lines but had no effect in MDA-MB231 or BT549 (Fig. 6*G*). Because the MDA-MB231 cell line had low levels of Pla2g16 (comparable to that of SKBr3 cells knocked down for mutant p53), we overexpressed Flag-tagged Pla2g16 in MDA-MB231 human mammary tumor cells. Overexpression of Flag-tagged Pla2g16 showed increased cell proliferation and colony formation (Fig. 6 *H–J*), suggesting that increased levels of Pla2g16 may also contribute to human breast cancer progression. Taken together, these data indicated that *Pla2g16* expression is also important in mammary tumor progression and metastasis. In addition, some mammary cell lines express Pla2g16 by mechanisms independent of mutant p53 levels.

Discussion

Expression array comparisons of osteosarcomas from $p53^{R172H/+}$ mice with metastasis and $p53^{+/-}$ mice without metastasis led to the identification of numerous gene differences, including *Pla2g16*, which encodes a phospholipase that metabolizes phospholipids. Pla2g16 in particular removes a fatty acyl chain from phosphatidic acid to generate lysophosphatidic acid and free fatty acid. Lysophosphatidic acid stimulates cell migration and plays a role in tumor metastasis through G protein-coupled signaling pathways (28, 30). Our data indicate that the increase in Pla2g16 levels by several p53 mutants in osteosarcomas and mammary tumor cell lines is a novel mechanism that contributes to the mutant p53 GOF phenotype. Importantly, not all p53 mutants regulate Pla2g16 levels.

Pla2g16-null mice are lean in the context of the *Ob*/*Ob* obese background, suggesting this lipase is important for regulating fat metabolism (31). Obesity increases the risk of many types of cancer (45–48). Thus, these data support a role of Pla2g16 in tumorigenesis in vivo. Although *Pla2g16*-null mice or mice with mutations in other phospholipases are not cancer-prone, many of these mice decrease tumor progression when combined with other oncogenic

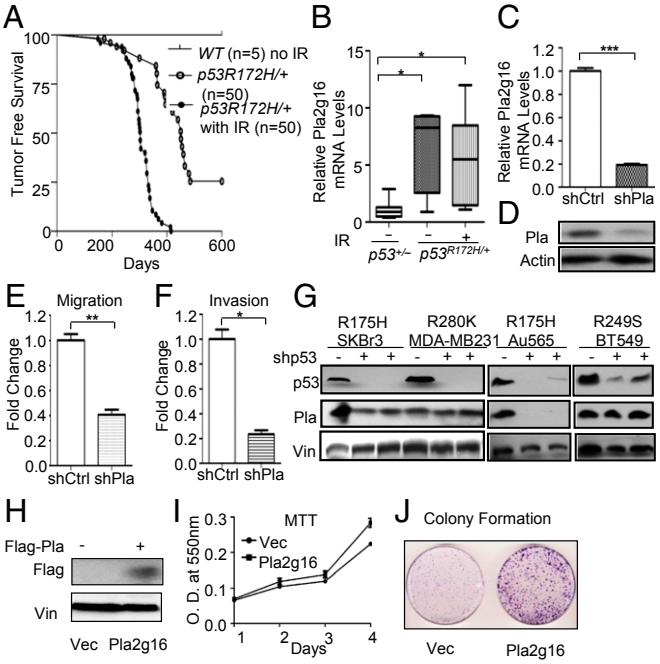

Fig. 6. Pla2g16 contributes to $p53^{R172H/+}$ mammary tumor metastatic potential. (*A*) Kaplan–Meier tumor-free survival curves for $p53^{R172H/+}$ mice with or without radiation. (*B*) *Pla2g16* expression levels in $p53^{R172H/+}$ metastatic and nonmetastatic $p53^{+/-}$ primary mammary tumors by qRT-PCR. shRNA knockdown of Pla2g16 in the metastatic mammary tumor cell line 4T1 was measured by qRT-PCR (*C*) and Western blot analysis (*D*). *Pla2g16* shRNA treated 4T1 cells had reduced migration (*E*), and invasion (*F*). (*G*) Western blots with mutant p53 knockdown in breast cancer cells. (*H*) Flag-tagged Pla2g16 overexpression in human MDA-MB231 breast cancer cells was measured by Western blotting. Proliferation (*I*) and colony formation (*J*) assays were performed in Pla2g16 overexpressing cells. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$.

Table 1. Tumor spectra in $p53^{R172H/+}$ Balbc/J mice

	Non-IR (n = 50)		IR (n = 50)	
Tumor types	Primary	Metastasis	Primary	Metastasis
Sarcoma	9 (18%)	1 (11%)	3 (6%)	0
Mammary carcinoma	22 (44%)	5 (23%)	32 (64%)	11 (34%)
Lymphoma	19 (38%)	NA	15 (30%)	NA

IR, irradiated; NA, not applicable.

defects (49). Crosses of *Pla2g16* mice with *p53* mutant mice will be invaluable in deciphering the in vivo role of Pla2g16 in metastasis.

The role of p53 in metabolism also contributes to its tumor-suppressive function (50, 51). Loss of p53 increases the Warburg effect, resulting in high rates of glycolysis and compromised oxidative phosphorylation (52, 53). The $p53^{3KR}$ knockin mice, which cannot induce p53-dependent cell cycle arrest, apoptosis, and senescence, show a delayed tumor phenotype by regulating energy metabolism (54). The metabolic activities of mutant p53 are also mediated through the Mevalonate pathway (9). In contrast, it is worth noting that deletion of the p53 target TIGAR (Tp53-induced glycosis and apoptosis regulator) in mice actually showed reduced tumorigenesis in an *APC*-deficient intestinal adenoma model (55). The opposing role of metabolism here may be context-specific. Thus, metabolic changes mediated by *p53* loss or mutation contribute to tumorigenesis.

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