Truncating PREX2 mutations activate its GEF activity and alter gene expression regulation in NRAS-mutant melanoma

Yonathan Lissanu Deribe[a,1,2], Yanxia Shi[a,b,1], Kunal Rai[a], Luigi Nezi[a], Samir B. Amin[a], Chia-Chin Wu[a], Kadir C. Akdemir[a], Mozhdeh Mahdavi[a], Qian Peng[a], Qing Edward Chang[c], Kirsti Hornigold[d], Stefan T. Arold[e], Heidi C. E. Welch[d], Levi A. Garraway[f,g], and Lynda Chin[a,c,2]

[a]Department of Genomic Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX 77030; [b]Sun Yat-Sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborate Center for Cancer Medicine, Guangzhou 510060, China; [c]Institute for Applied Cancer Science (IACS), The University of Texas MD Anderson Cancer Center, Houston, TX 77030; [d]The Babraham Institute, Babraham Research Campus, Cambridge CB22 3AT, United Kingdom; [e]Division of Biological and Environmental Sciences and Engineering, King Abdullah University of Science and Technology, Thuwal, 23955-6900 Saudi Arabia; [f]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02215; and [g]Broad Institute of MIT and Harvard, Boston, MA 02141

Edited by Dennis A. Carson, University of California at San Diego, La Jolla, CA, and approved January 26, 2016 (received for review July 14, 2015)

PREX2 (phosphatidylinositol-3,4,5-triphosphate-dependent Rac-exchange factor 2) is a PTEN (phosphatase and tensin homolog deleted on chromosome 10) binding protein that is significantly mutated in cutaneous melanoma and pancreatic ductal adenocarcinoma. Here, genetic and biochemical analyses were conducted to elucidate the nature and mechanistic basis of PREX2 mutation in melanoma development. By generating an inducible transgenic mouse model we showed an oncogenic role for a truncating PREX2 mutation (PREX2^{E824*}) in vivo in the context of mutant NRAS. Using integrative cross-species gene expression analysis, we identified deregulated cell cycle and cytoskeleton organization as significantly perturbed biological pathways in PREX2 mutant tumors. Mechanistically, truncation of PREX2 activated its Rac1 guanine nucleotide exchange factor activity, abolished binding to PTEN and activated the PI3K (phosphatidyl inositol 3 kinase)/Akt signaling pathway. We further showed that PREX2 truncating mutations or PTEN deletion induces down-regulation of the tumor suppressor and cell cycle regulator CDKN1C (also known as p57^{KIP2}). This down-regulation occurs, at least partially, through DNA hypomethylation of a differentially methylated region in chromosome 11 that is a known regulatory region for expression of the CDKN1C gene. Together, these findings identify PREX2 as a mediator of NRAS-mutant melanoma development that acts through the PI3K/PTEN/Akt pathway to regulate gene expression of a cell cycle regulator.

PREX2 | melanoma | Rac1 | PI3K/Akt | mouse models of cancer

Recent large-scale multidimensional genomic analyses of many cancers have established a framework in which biological functions and genetic interactions of established and novel cancer genes can be explored (1, 2). We initially identified PREX2 (phosphatidylinositol-3,4,5-triphosphate-dependent Rac-exchange factor 2) as being significantly mutated in human melanomas (3), an observation that was corroborated by the recently completed TCGA melanoma study (4). PREX2 is a guanine nucleotide exchanger (GEF) for Rac1 (5, 6) and is known to bind to the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) (7). PREX2 has recently been shown to regulate insulin signaling and glucose homeostasis through modulation of the PI3K (phosphatidyl inositol 3 kinase) pathway, and also to regulate Rac1 mediated cellular invasion in a manner that cross-talks with PTEN signaling (8). Further expanding the significance of genetic perturbations of PREX2 in cancer, a recent report by the International Cancer Genome Consortium (ICGC) described the identification PREX2 as a significantly mutated gene in pancreatic ductal adenocarcinoma (PDAC) (9). Interestingly, PREX2 harbors a wide spectrum of mutations including missense and truncating mutations in PDAC, similar to observations in melanoma (3, 9).

To date, the most obvious connection between PREX2 and cancer relevant pathways is through its physical interaction with PTEN (7). PTEN catalyzes the conversion of phosphatidylinositol-3,4,5-trisphosphate to phosphatidylinositol-4,5 bisphosphate. PTEN acts as a tumor suppressor and plays important roles in multiple cellular processes primarily by antagonizing PI3-kinase-AKT signaling (10–13). Pathologically, PTEN is inactivated via multiple mechanisms in about a third of melanoma tumors resulting in activation of the downstream PI3K/Akt signaling pathway (14, 15). Despite these connections to cancer signaling pathways, the exact mechanism of tumorigenesis by PREX2 mutations remains unknown. Here, we elucidated a previously unidentified mechanism of action of PREX2 mutations in melanoma pathogenesis.

To study PREX2 mutations in vivo, we generated an inducible transgenic mouse model that expresses one of the truncating PREX2 mutants observed in melanoma patients, and showed that melanoma development was accelerated in this genetic context. Using integrated gene expression analysis, we identified several cellular

Significance

Mutations in the PI3K/PTEN/Akt signaling pathway occur frequently across multiple tumor types. These mutations primarily serve to activate PI-3 and Akt kinases. PREX2 is a guanine nucleotide exchanger for Rac1 that is significantly mutated in melanoma and pancreatic ductal adenocarcinoma. Here we report that a mouse model of a truncating PREX2 mutation shows accelerated melanoma development in the context of mutant NRAS. Truncating PREX2 mutations have increased Rac1 guanine nucleotide exchange factor activity, and tumors harboring these mutations have elevated PI3K/Akt pathway activation and reduced expression of critical negative cell cycle regulators leading to increased cell proliferation. This work provides evidence for a previously unidentified mechanism of activating Rac1, the PI3K pathway, and regulation of cell cycle progression in melanoma.

Author contributions: Y.L.D., L.A.G., and L.C. designed research; Y.L.D., Y.S., K.R., L.N., M.M., Q.P., and K.H. performed research; Y.L.D., S.B.A., C.-C.W., K.C.A., Q.E.C., K.H., S.T.A., and H.C.E.W. analyzed data; and Y.L.D. and L.C. wrote the paper.

The authors declare no conflict of interest.

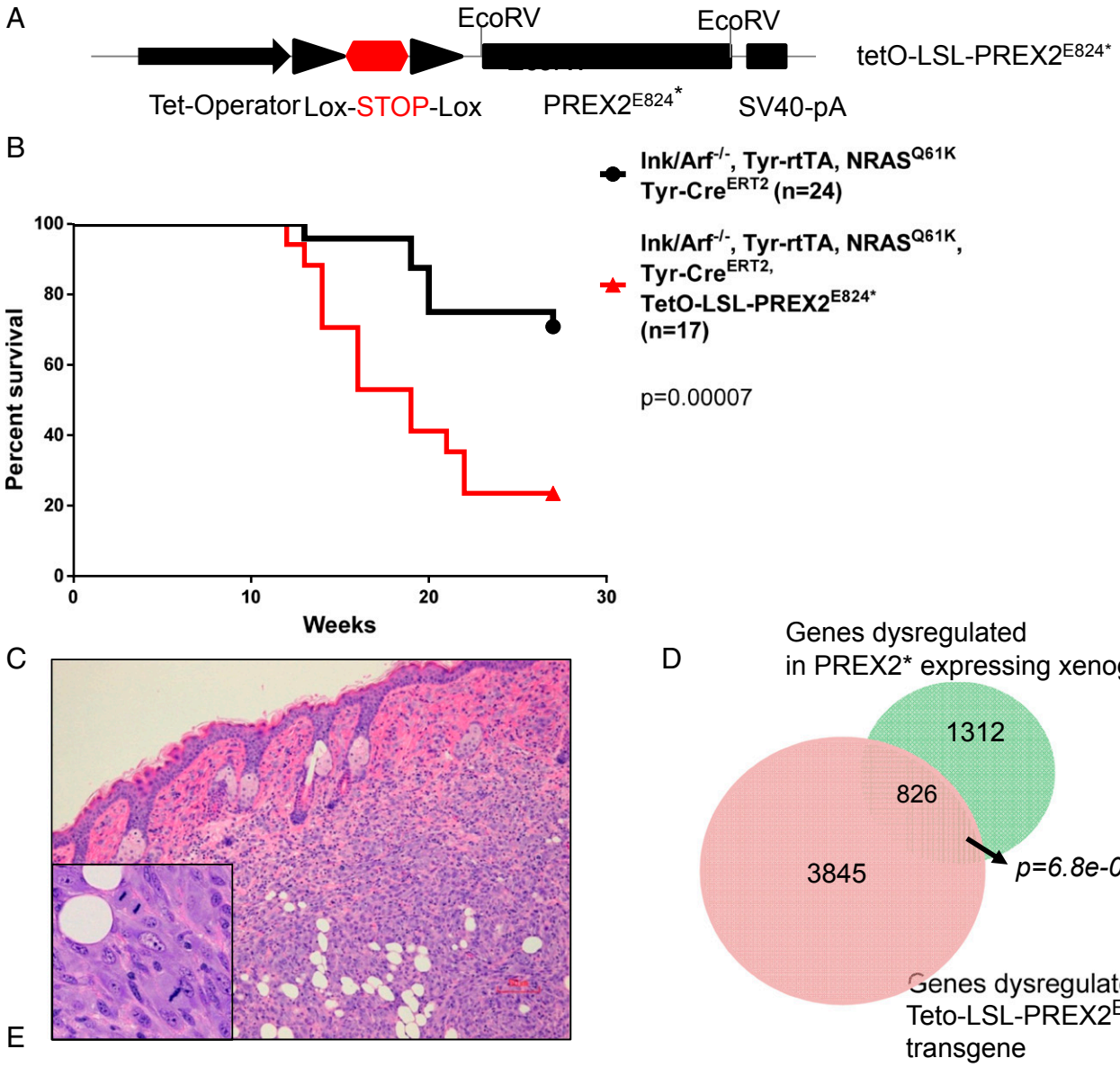
This article is a PNAS Direct Submission.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE57311).

[1]Y.L.D. and Y.S. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: ylissanu@mdanderson.org or lchin@mdanderson.org.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1513801113/-/DCSupplemental.



NAME	SIZE	ES	NOM p-val
NEGATIVE_REGULATION_OF_CELL_ADHESION	16	0.67218393	0
CELL_CYCLE_ARREST_GO_0007050	50	0.48414493	0
KEGG_RIBOSOME	47	0.47214276	0
RNA_PROCESSING	144	0.42866966	0
MITOCHONDRION_ORGANIZATION_AND_BIOGEN	40	0.4741075	0.003006012
KEGG_ADHERENS_JUNCTION	64	0.45136175	0.001
MITOTIC_CELL_CYCLE_CHECKPOINT	19	0.51834327	0.013157895
LIPID_TRANSPORT	23	0.50737894	0.010111224
KEGG_RENAL_CELL_CARCINOMA	65	0.44745925	0
ACTIN_FILAMENT_ORGANIZATION	24	0.4998334	0.007007007

Fig. 1. Inducible truncating PREX2 transgene is oncogenic in vivo. (*A*) Schematic representation of the construct used to generate transgenic mice consisting of a doxycycline responsive promoter and a stopper cassette flanked by loxp sites for tighter regulation of a truncated PREX2 mutant (E824*) fused to FLAG epitope tag. (*B*) Kaplan–Meier curve of tumor free survival of indicated genotypes. Cohort size of each group is indicated. To induce the expression of the transgene, mice were injected with tamoxifen (activates CreERT2) and fed with doxycycline-containing water at 3 wk of age to induce expression of transgenes from the TetO promoter. (*C*) Hematoxylin and eosin (H&E) staining of a tumor from mice harboring *TetO-LSL-PREX2*^{E824*} transgene. (*D*) Plot showing number of overlapped genes whose expression is significantly changed in *TetO-LSL-PREX2*^{E824*} transgenic tumors and xenograft tumors overexpressing PREX2^{E824*}. (*E*) Gene set enrichment analysis. After identifying genes whose expression change overlapped between xenograft and transgenic lines as in *D*, gene set enrichment was performed. Top 10 significantly altered cellular signaling pathways between tumors harboring a truncating PREX2 mutant and those that do not have a PREX2 mutation.

pathways including cell cycle regulation and cytoskeletal organization to be deregulated in PREX2 mutant tumors. Biochemically, we showed that truncating PREX2 mutations increase its Rac1 guanine nucleotide exchange (GEF) activity, abolish binding to the tumor suppressor PTEN and activate the PI3K/Akt signaling pathway. Finally, we showed that PREX2 mutation or PTEN deletion induces DNA hypomethylation and down-regulation of expression of CDKN1C ($p57^{KIP2}$), a critical cell cycle regulator. In conclusion, this study demonstrates the oncogenic capability of PREX2 truncations in vivo and identifies a mechanistic link to an established oncogenic signaling pathway and downstream regulation of a tumor suppressor to impact melanoma pathogenesis.

Results

A Truncating PREX2 Mutation ($PREX2^{E824*}$) Cooperates with NRAS to Accelerate Melanoma Development. Melanomas harbor both missense and truncating mutations in PREX2 (3, 4). We have previously shown that, although missense PREX2 mutations produce a mixed oncogenic phenotype, all truncating PREX2 mutations consistently accelerated xenograft tumor growth in vivo (3). Hence, to understand the basic biology behind PREX2 mutations in melanoma, we genetically modeled one of the representative truncating mutations, E824*, in mice. To control the expression of this truncating PREX2 mutant in a tissue and time specific manner, we generated a conditional $PREX2^{E824*}$ transgene under the control of a tet-operator with a lox-STOP-lox cassette inserted between the promoter and the start codon of the $PREX2^{E824*}$ ORF, designated as TetO-LSL- $PREX2^{E824*}$ (Fig. 1*A*). We crossed these mice to *Tyr-Cre*^{ERT2} and *Tyr-rtTA* mice to express $PREX2^{E824*}$ only in melanocytes in response to doxycycline and Cre activation by tamoxifen (16, 17). Subsequently, we crossed these mice into an established mouse model of melanoma, termed iNRAS (consisting of the alleles: *Ink/Arf*$^{-/-}$, *TetO-NRAS*^{Q61K}, *Tyr-rtTA*) (18) and generated two cohorts of mice with or without the inducible *TetO-LSL-PREX2*^{E824*} allele. We chose these particular genotypes because we observed that truncating PREX2 mutations cooperated with mutant NRAS in driving xenograft tumor growth, but not BRAF mutations (3) (*SI Appendix*, Fig. S1 *A* and *B*). Mice carrying the *TetO-LSL-PREX2*^{E824*} transgene exhibited accelerated melanoma development with increased penetrance compared with the iNRAS model alone (Fig. 1 *B* and *C*). Together with our previous work in a xenograft model system (3), these in vivo transgenic mouse studies demonstrate that truncating PREX2 mutations observed in melanoma cooperate with mutant NRAS to drive tumor formation.

Multiple Cellular Pathways Are Deregulated in PREX2 Mutant Tumors. To elucidate the molecular basis of their oncogenic activity, we profiled the transcriptomes of tumors with truncating PREX2 mutants in transgenic and xenograft model systems. Specifically, we profiled melanomas from the iNRAS transgenic mouse model, and xenograft tumors derived from isogenic primary immortalized human melanocytes (19) expressing wild type PREX2 or one of three truncating mutations (K278*, E824*, and Q1430*). Cross-species comparison revealed statistically significant overlap between the genes altered in mouse tumors expressing the *TetO-LSL-PREX2*^{E824*} transgene and in xenograft tumors derived from truncating PREX2 overexpressing human melanocytes (Fig. 1*D*).

Gene set enrichment analysis of the genes concordantly up-regulated and down-regulated in both model systems revealed cell adhesion and actin filament organization, cell cycle and mitotic checkpoint regulation, and ribosomal and mitochondrial biogenesis pathways as top significantly altered pathways (Fig. 1*E* and *SI Appendix*, Fig. S2). Pathway alterations in cell adhesion and actin filament organization may reflect PREX2's role as a bona fide guanine nucleotide exchange factor for Rac GTPases (5, 6), and changes in the ribosomal and mitochondrial biogenesis likely relate to its intimate link to PTEN and PI3 kinase pathways (7). However, the direct molecular links of PREX2 to cell cycle regulation and mitotic checkpoint have not been reported previously and were investigated further.

PREX2 Mutant Tumors Have Markedly Increased Cell Proliferation. Next, we performed Ki67 staining to assess the extent of cellular proliferation in tumors derived from mice with activated *TetO-LSL-PREX2*^{E824*} transgene or controls. Consistent with the gene expression profiling experiment (Fig. 1*E*), we saw statistically significant increase in Ki67-positive staining in PREX2 transgene-containing tumors compared with the control (Fig. 2*A*, *Upper* and *Lower*). In line with the histology and pathway analysis implicating cell cycle regulation, the most consistently down-regulated gene in xenografts expressing PREX2 truncations was the known cell cycle regulator CDKN1C (also known as $p57^{KIP2}$). This down-regulation was verified by orthogonal methods, including expression profiling by qRT-PCR (Fig. 2 *B* and *C*) and Western blotting on total tumor lysates (Fig. 2*D*). Similar down-regulation of CDKN1B is observed in transgenic tumors expressing activated *TetO-LSL-PREX2*^{E824*} allele (Fig. 2*E*). Furthermore, we observed reduced expression of p21 and p27 on Western blots of tumors harboring *TetO-LSL-PREX2*^{E824*} allele (*SI Appendix*, Fig. S3*A*). Interestingly, IGF2, which is found in a genomic locus that is epigenetically coregulated with CDKN1C, is highly expressed in truncating PREX2 expressing xenograft tumors compared with control tumors (*SI Appendix*, Fig. S3*B*).

Oncogenic Truncating PREX2 Mutations Increase its Rac1 GEF Activity and Abolish Binding to PTEN. To define a direct biochemical link between the observed biological changes and PREX2 mutations, we investigated how PREX2 mutations affect its known GEF enzymatic activity and protein-protein interactions. Due to their consistent oncogenic activity in xenograft models, we focused our study on the truncating PREX2 mutations observed in our initial sequencing project (*SI Appendix*, Fig. S4*A*) (3). It is important to note that additional PREX2 truncating mutations have been recently described (4, 20) (*SI Appendix*, Fig. S4*B*).

To test whether PREX2 truncations have any direct effect on its GEF activity, we purified recombinant full length PREX2 and an N-terminal fragment consisting of Dbl homology (DH) and pleckstrin homology (PH) domains (Fig. 3*A*, *Left*). We used these purified proteins in an in vitro GEF assay using Rac1 as a substrate. We observed that PREX2 N-terminal fragment has significantly higher GEF activity than full length PREX2 (Fig. 3*A*, *Right*). Next we asked whether truncating PREX2 mutants also have increased GEF activity in cells. To answer this question, we performed a pulldown experiment using Rac/Cdc42 (p21) binding domain (PBD) from p21 activated kinase 1 (PAK-PBD) that binds to Rac1 only when loaded with GTP (21–23). Interestingly, we observed a robust increase in Rac1 loaded with GTP in primary immortalized melanocytes expressing PREX2 truncating mutants (Fig. 3*B*). Similarly, increased GEF activity as inferred by increased GTP loaded Rac1 was seen in mouse xenograft tumor-derived cells harboring PREX2 truncating mutants, compared with GFP control or various full-length PREX2 mutants (Fig. 3*C*). Taken together, these experiments show that truncated PREX2 proteins possess an increased GEF activity.

To explore the molecular mechanisms underlying the increase in GEF activity in PREX2 truncations, we produced a structural homology model of the Rac1 GTPase bound to the PREX2 N terminus, based on the recent X-ray crystal structure of the Rac1:PREX1_DH-PH complex (24). The DH-PH regions of PREX1 and PREX2 are 71% identical in sequence, resulting in a high-confidence Rac1:PREX2_DH-PH structural model (Fig. 3*D*). Model analysis showed that the Rac1 interface on the PREX1 DH domain is strictly conserved in PREX2. In agreement with the PREX1:Rac1 study, the model does not support a direct molecular contribution from the PH domain to activating the Rac1 GTPase

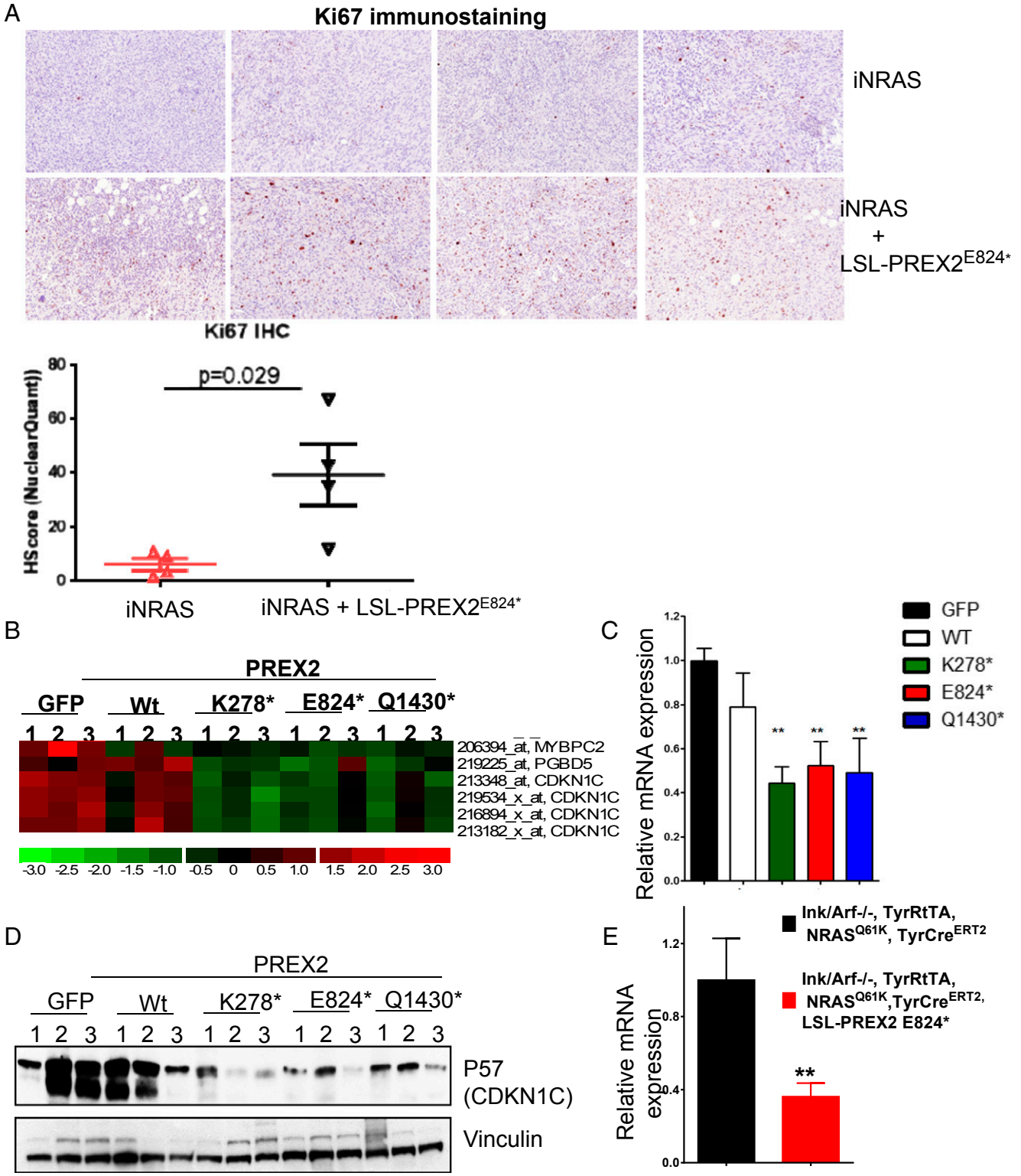


Fig. 2. Tumors expressing a PREX2 truncating mutant show increased proliferation and down-regulation of inhibitory cell cycle regulators. (*A*, *Upper*) Ki67 staining of formalin fixed paraffin embedded sections of four representative tumors derived from each GEM model of the iNRAS genotype or iNRAS+ *TetO-LSL-PREX2*^{E824*} alleles. (*Lower*) Tumors from each genotype were stained for Ki67 and nuclear staining was quantified and plotted by a trained pathologist using NuclearQuant 1.15.1 software from 3DHISTECH. (*B*) Heatmap of most consistently down-regulated genes in PREX2 mutants compared with GFP or PREX2 expressing xenografts. Shown are down-regulated probe sets in PREX2 mutants compared with control GFP and wild-type PREX2 expressing xenografts showing at least 1.5-fold change and *P* value less than 0.05. (*C*) Gene expression of CDKN1C from xenograft tumors derived from primary melanocytes harboring the indicated genotypes. $^{**}P < 0.05$ compared with GFP controls by Student's *t* test. (*D*) Western blot of xenograft whole tumor lysates expressing either GFP, PREX2 wild type, or truncating mutants showing CDKN1C (p57) expression. Three independent tumor lysates per experimental condition are shown. (*E*) Comparison of mRNA expression of CDKN1B in tumors derived from transgenic mice with or without *TetO-LSL-PREX2*^{E824*} transgene. $^{***}P < 0.001$ by Student's *t* test.

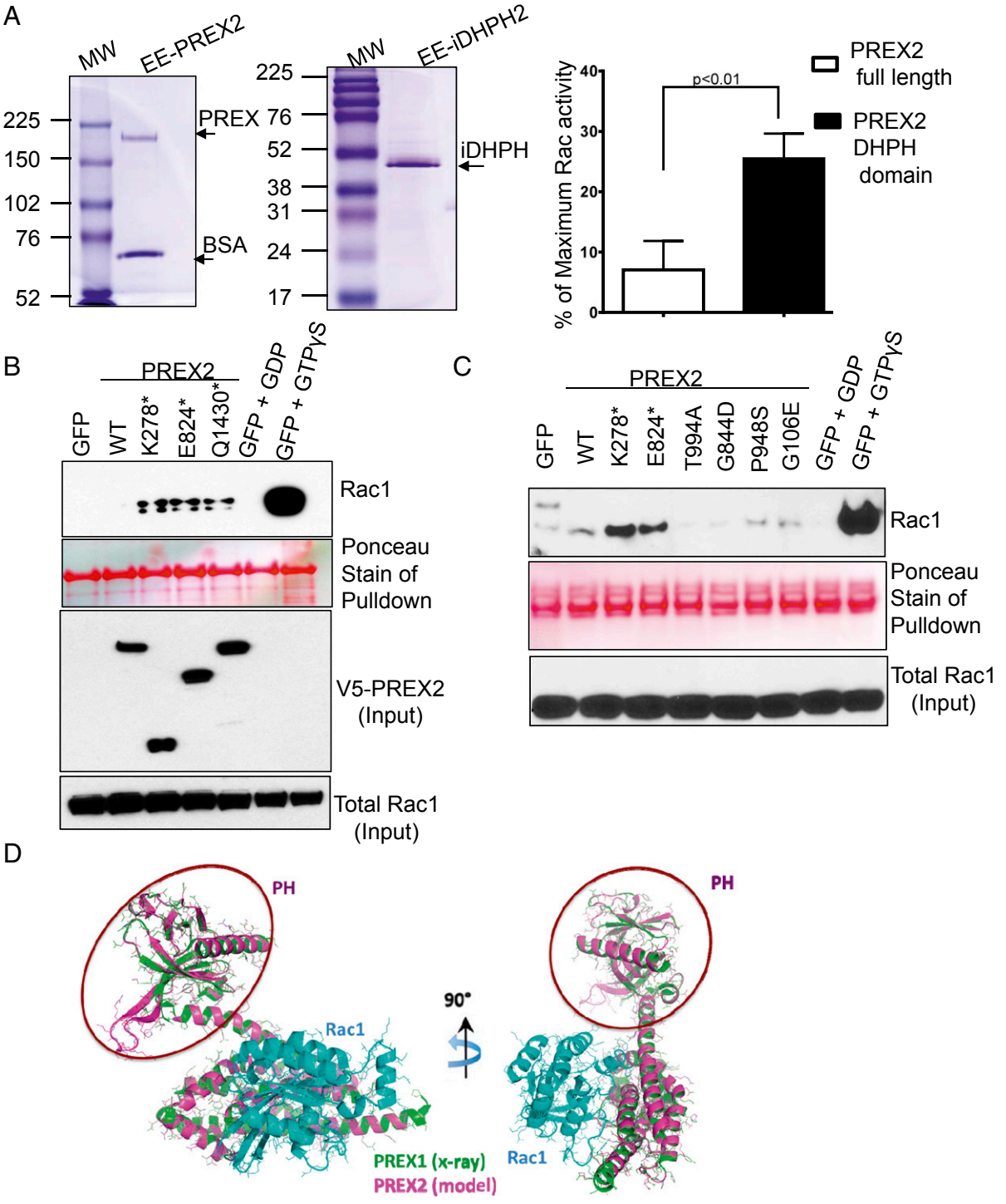


Fig. 3. PREX2 truncating mutations have increased Rac1 guanine nucleotide exchange factor (GEF) activity. (*A*) Coomassie Blue staining of Sf9 cell purified recombinant human full length PREX2 and an N-terminal fragment of PREX2 containing the DH-PH domains used in GEF activity assay (*Left*). (*Right*) Result of in vitro GEF activity assays of PREX2. We measured the amount of GTP loaded by PREX2 on the substrate, Rac1. Radioactive GTP loaded on Rac1 was quantified and plotted as a percentage of theoretical maximum GTP loading activity induced by loading Rac1 with a nonhydrolyzable analog of GTP (GTPγS). Result shown is a composite of four independent experiments. (*B*) In vivo Rac1 activation assay using PAK-PBD pulldown. PMEL-NRAS cells expressing GFP, PREX2 WT, or indicated truncating mutants were grown to maximum of 30% confluency, serum deprived for 5 h and lysed using assay buffer. PAK-PBD pulldown was done to detect GTP loaded Rac1, which was immunoblotted. Cellular lysates incubated with a nonhydrolyzable analog of GTP (GTPγS) serves as a positive control and cell lysates incubated with an excess of GDP as negative control. Ponceau stain shows equal amounts of PAK-PBD used. Rac1 and V5-PREX2 were probed using indicated antibodies from the input cell lysates. (*C*) In vivo Rac1 activation assay using PAK-PBD. Xenograft tumor-derived cell lines were grown to 30% confluency, serum deprived for 5 h, and PAK-PBD pulldown performed and immunoblotted as indicated. Positive and negative controls as in *B*. (*D*) Schematics of structural model of Rac1:PREX2 DH-PH domain. A high confidence Rac1:PREX2 DH-PH domain structural model was generated using sequence homology of PREX1:Rac1 X-ray structure (Protein Data Bank ID 4YON). PREX2 (magenta) DH domain makes extensive contacts with Rac1 (teal). PREX2 PH domain makes no molecular contributions to this interaction as described for PREX1 (green). On the right, the structure is rotated 90° on the *y* axis to show the interface between Rac1 and the GEFs.

in PREX2. Given that the DH-PH architecture and Rac1 binding sites appear completely conserved in PREX2, the mechanism of GEF activity is also expected to be conserved. Importantly, Lucato et al. suggested that the C-terminal domains of PREX1 autoinhibit the GEF function of PREX1 through intramolecular interactions with the DH-PH domains (24). Such an autoinhibitory mechanism is supported by another study that revealed a C-terminal intramolecular inhibition of GEF activity where PH, DEP, and PDZ domains hold the DH domain in a low-activity state in absence of stimuli (25). Moreover, the C-terminal inositol polyphosphate 4-phosphatase homology (IP4P) domain is needed for keeping PREX1 correctly structured and stable through its intramolecular interactions with the DEP and PDZ domains (26). Importantly, deletion of the C-terminal 34 residues was sufficient to abolish binding of IP4P to other parts of PREX1 (25, 26). We therefore propose that truncating PREX2 mutations remove the autoinhibitory capacity of the C terminus, thus increasing the GEF activity toward Rac1.

Interestingly, it has been recently reported that the GEF activity of PREX2 is suppressed by PTEN and that certain PREX2 missense mutations affect its interaction with PTEN (27). Hence we investigated the binding of truncated PREX2 proteins to PTEN. All three PREX2 truncating mutants failed to bind endogenous PTEN in two independent melanocyte lines (*SI Appendix*, Fig. S4 *C* and *D*). Additionally, we assessed the binding of ectopically expressed PREX2 mutants to PTEN in HEK293T cells. Again, the truncating PREX2 mutants lost the ability to bind to PTEN (*SI Appendix*, Fig. S4*E*). Further protein–protein interaction studies revealed that the PREX2-PTEN interaction is independent of PTEN's catalytic activity but dependent on its C-terminal PDZ binding domain (*SI Appendix*, Fig. S5). In conclusion, the lack of an autoinhibitory C terminus and loss of binding of a negative GEF regulator, PTEN, can explain the increased GEF activity of PREX2 truncating mutants.

Oncogenic Truncating PREX2 Mutations Activate Phosphoinositide-3/Akt Signaling. Regulation of the PI3K/Akt pathway is the best documented role of PTEN (28). Hence, we looked at phosphorylation of Akt as a surrogate for activation of this pathway in immortalized primary melanocytes (PMEL-NRAS) expressing GFP, wild-type PREX2, or various truncating PREX2 constructs (*SI Appendix*, Fig. S6). Cells expressing PREX2 truncations had consistently elevated levels of phosphorylated Akt both on Ser473 and Thr308 residues, indicating full activation of the kinase (Fig. 4*A*). Additionally, we performed reverse-phase protein array (RPPA) on total protein lysates of xenograft tumors derived from GFP, wild-type PREX2, or PREX2 truncation expressing melanocytes (*SI Appendix*, Table S1). Similar to results using immortalized melanocytes, we observed increased levels of phosphorylated Akt in tumor lysates with PREX2 truncations compared with GFP or wild-type PREX2 controls (Fig. 4*B*). Next, we performed immunoblotting on tumor lysates derived from two mouse models, iNRAS and iNRAS with *TetO-LSL-PREX2*^{E824*}, which demonstrated a similar increase in phosphorylation of Ser473 and Thr308 residues in Akt (Fig. 4*C*).

We hypothesized that the increased GEF activity of PREX2 mutants and resulting activation of Rac1 contributes to this activation of Akt based on several reports that have shown Rac1 can activate PI3K/Akt signaling by directly binding to PI3K (29–31). To test this hypothesis in our model system, we expressed a constitutively active Rac1 construct, Q61L, in primary melanocytes. We observed an impressive increase in Akt phosphorylation both under serum deprivation and regular serum growth conditions (Fig. 4*D*). Importantly, we asked whether Akt phosphorylation mediated by truncating PREX2 could be modulated by suppression of the Rac1 pathway. Using the Rac1 inhibitor EHT 1864 (32–34), we observed greatly abrogated levels of phosphorylated Akt (Fig. 4*E*), suggesting Akt activation by PREX2 mutation is dependent, at least partially, on Rac1.

Taken together, our data suggest that the mechanism of activation of Akt by PREX2 mutations is multifactorial and at least partially mediated by Rac1. One compounding factor as shown in our expression analysis is that PREX2 mutant tumors have high levels of IGF2, which is known to activate the PI3K pathway (35, 36). Indeed addition of IGF2 to serum deprived immortalized melanocytes potently activated Akt phosphorylation, suggesting this as a possible additional mechanism of Akt activation by PREX2 mutation that deserves further study (*SI Appendix*, Fig. S7*A*).

Mechanism of Downstream Gene Expression Dysregulation. Finally, to provide a direct mechanism for the observed increase in proliferative index in tumors expressing truncated PREX2, we investigated the impact of PREX2 mutation on the expression of a known cell cycle regulator, CDKN1C, as a model. We chose to study CDKN1C because it was the most consistently down-regulated gene in all PREX2 mutants compared with GFP and wild-type PREX2 controls (Fig. 2 *B*–*D*). Functionally, CDKN1C (also known as p57^{KIP2}) is a well-known cell cycle regulator and tumor suppressor whose loss of expression is a cause of Beckwith-Wiedemann syndrome (BWS) (37–41). Additionally, CDKN1C is a classic imprinted gene located in an imprinting cluster on chromosome 11 (42). Molecularly, in addition to mutation and chromosomal rearrangement of the CDKN1C gene, epigenetic alterations at the imprint control region, differentially methylated region (DMR), such as loss of methylation at the DMR, are known to affect CDKN1C expression leading to BWS. However, the mechanism(s) regulating methylation at the DMR has not been elucidated. Hence, we first asked whether down-regulation of CDKN1C in PREX2 mutant cells is associated with changes in the methylation status of the DMR (43, 44). Accordingly, DNA methylation of the DMR as assessed by methylated DNA immunoprecipitation and qPCR confirmed a marked DNA hypomethylation at the imprint control region of CDKN1C in PREX2 mutant cells (Fig. 5*A*), with consequent reduction in CDKN1C expression (44) (summarized in *SI Appendix*, Fig. S8). Additionally, we also observed consistent down-regulation of CDKN1C in multiple independently derived MEFs upon acute depletion of PTEN (Fig. 5*B*), phenocopying cells with PREX2 truncating mutants. Interestingly, in agreement with a prior report (45), we observed that IGF2 can induce reduction in expression of CDKN1C (*SI Appendix*, Fig. S7*B*). Taken together, our data showed that PREX2 truncating mutants down-regulate CDKN1C tumor suppressor through hypomethylation of its DMR, although the precise molecular link is not yet identified.

Human Melanoma with PTEN Deletion Have Decreased CDKN1C Expression and Impaired DNA Methylation at the DMR. To demonstrate the human relevance of our findings, we interrogated the multidimensional epi/genomic data set generated by TCGA to seek evidence of dysregulation of CDKN1C expression and altered methylation of its DMR in human melanoma samples (4). Because the number of samples with PREX2 truncations is small and inadequate for statistical tests of significance, we compared CDKN1C expression and CpG island methylation at DMR in PTEN homozygously deleted versus PTEN wild-type samples. As expected, both CDKN1C expression and DMR CpG methylation were significantly lower in PTEN deleted samples compared with PTEN wild-type cases, (Fig. 5 *C* and *D*; P value = 0.04, and $P = 0.004$, $P = 0.001$ for two independent CpG probes, respectively). This correlative finding supports our thesis that PREX2 mutation that loses binding to PTEN, or direct loss of PTEN, results in down-regulation of known tumor suppressor CDKN1C through hypomethylation of the DMR CpG and consequent promotion of melanoma development.

Discussion

We have generated a genetically engineered mouse (GEM) model with inducible expression of a truncating PREX2 mutant

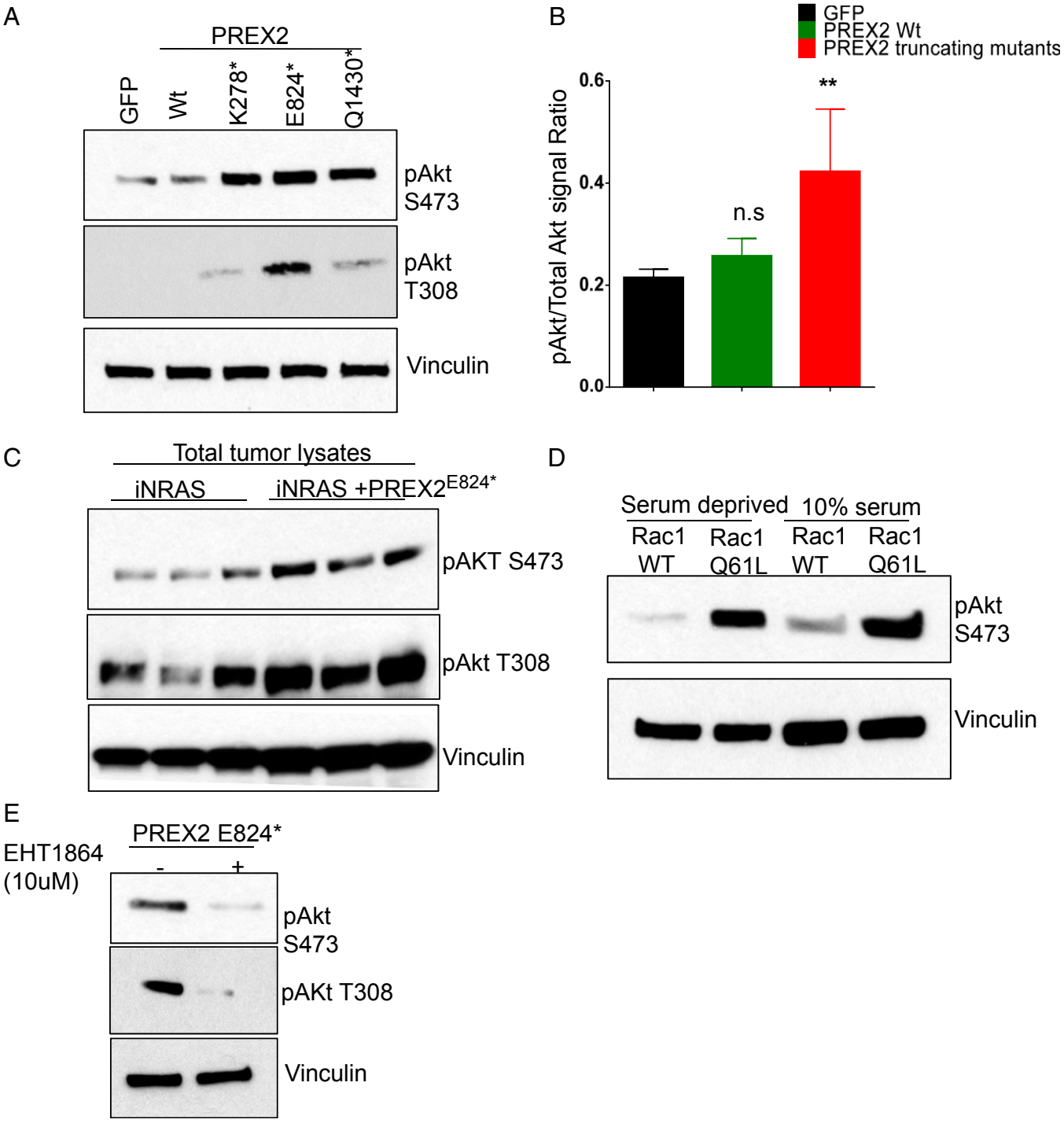


Fig. 4. PREX2 truncating mutants induce elevated PI3K/Akt activation. (*A*) PMEL-NRAS cells expressing GFP, wild-type PREX2, or various truncating mutants were grown to 30–50% confluence, serum deprived for 2 h, lysed in RIPA buffer, and immunoblotted using the indicated antibodies. (*B*) Three xenograft tumors derived from each of GFP, wild-type PREX2, or truncating mutant expressing PMEL-NRAS cells were subjected to total tumor lysis and probed using the RPPA platform. Phospho-Akt T308/Total Akt signal was calculated from median centered values and plotted. The depicted *P* values are derived from Student's *t* test performed to compare mutants and wild-type PREX2 against GFP control tumors. (*C*) Three independent tumors each from iNRAS and iNRAS+TetO-LSL-PREX2^{E824*} were lysed as in *B* and phosphorylation of Akt detected using both phospho-S473 and phospho-T308 antibodies. (*D*) PMEL-NRAS cells were transduced with lentivirus encoding the constitutively active Rac1 Q61K mutant or wild-type Rac1. Seventy-two hours later, cells were either deprived of serum or grown in regular culture media. Cells were then lysed and immunoblotted with indicated antibodies. (*E*) PMEL-NRAS cells expressing PREX2 E824* truncating mutant were treated with DMSO or 10 μM of the Rac1 inhibitor EHT1864 for 2 h, lysed, and immunoblotted with indicated antibodies.

(PREX2^{E824*}) in a tissue- and time-dependent manner. We showed that selective expression of this transgene in melanocytes in a tumor sensitizing background results in accelerated melanoma development with increased prevalence in mice. This analysis confirmed our previous observation that truncating PREX2 mutants cooperate with NRAS mutation in a xenograft model

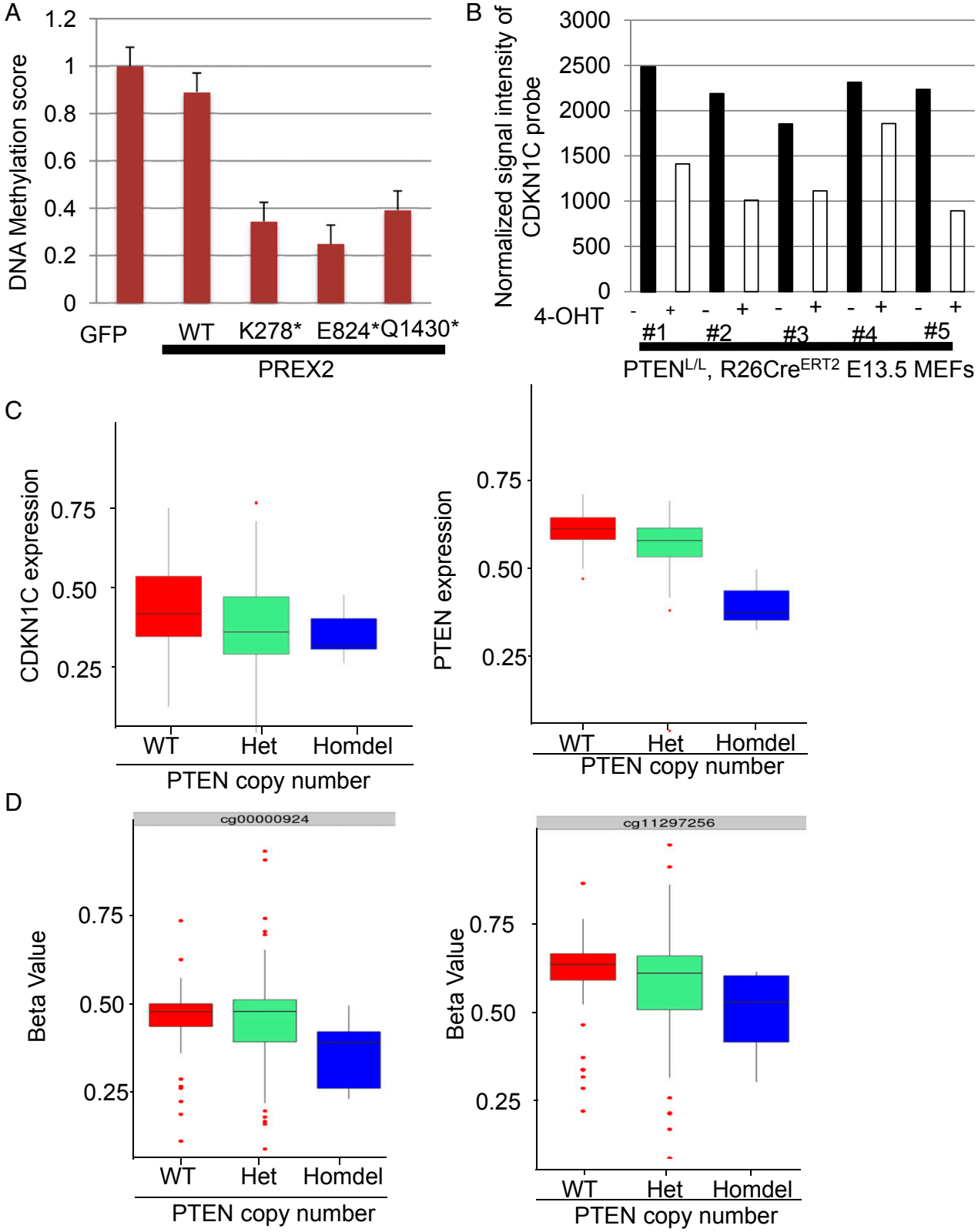


Fig. 5. Reduction of DNA methylation in CDKN1C regulatory region is induced by PREX2 mutations or PTEN deletion. (*A*) DNA methylation detected by methylated DNA immunoprecipitation (MeDIP) followed by qPCR to amplify a region in the differentially methylated region that serves as the imprint control and regulatory region of CDKN1C located in Chromosome 11. (*B*) PTEN depletion phenocopies truncating PREX2 mutation in MEFs with respect to CDKN1C expression. Expression of CDKN1C in early passage primary fibroblasts derived from five independent $PTEN^{L/L}$, Rosa26-Cre^{ERT2} E13.5 embryos treated with vehicle or 4-OHT to induce Cre^{ERT2} mediated recombination and depletion of PTEN. Plot shows raw signal intensity of CDKN1C probe after normalization. (*C* and *D*) TCGA data interrogation, boxplots showing reduced expression of CDKN1C (*P* value = 0.04) (*C*), and beta values of methylation probes at two CpGs (*P* value of 0.004 and 0.001 for probe cg00000924 and cg11297256, respectively) (*D*) in PTEN homozygously deleted melanoma tumor samples.

system (3). Based on the recent ICGC data on the prevalence of PREX2 mutations in PDAC (9), our PREX2 transgenic mice could also be used to model PDAC in combination with KRAS mutation.

Combined gene expression profiling in the GEM and xenograft model systems revealed dysregulation of cell adhesion, cell cycle regulators, and ribosomal biogenesis. These alterations are consistent with known biological roles of PREX2 in regulating the Rac1 GTPase (5, 6) and affecting the actin cytoskeleton, as well as its connection to the PTEN-PI3K signaling pathway, and influencing protein synthesis and growth (7, 8). In this study, we have elucidated a previously unidentified role for PREX2 in cell cycle regulation through down-regulating expression of CDKN1C (p57), p21, and p27. Furthermore, increased expression of the growth factor IGF2 in PREX2 mutant tumors is expected to contribute to these phenotypes.

Biochemically, we showed that truncating PREX2 mutations increase its Rac1 GEF activity both in vitro and in vivo. Structural homology modeling revealed a highly conserved mechanism of interaction with Rac1. We propose that the GEF domain in PREX2 truncating mutants is activated because of the removal of the autoinhibitory C terminus, as shown for other GEFs including the highly similar PREX1 (24, 25, 46). Interestingly, Rac1 is recurrently mutated and constitutively activated in cutaneous melanoma (47, 48). Hence, overactivation of Rac1 signaling pathway either through direct mutational activation or through its upstream regulators (e.g., PREX2) appears to be an emerging theme in melanoma biology. Truncating PREX2 mutations also abolish its interaction with PTEN. We showed that truncating PREX2 mutations activate downstream PI3K/Akt signaling pathway by activating Rac1. Further, we have shown that Akt activation by PREX2 is partially dependent on Rac1 using a Rac1 inhibitor. As pharmacologic agents can have nonspecific effects, future studies using genetic approaches such as dominant negative Rac1 mutants will be helpful to strengthen our conclusion. This observation expands on the role of Rac1 in activating the PI3K pathway as documented recently (29–31). Additionally, as a well-known modulator of growth signaling, the increase in IGF2 expression in PREX2 mutants is also expected to contribute to the activation of the PI3K/Akt pathway. Importantly, the direct biochemical consequences of the lack of PREX2/PTEN interaction due to PREX2 truncating mutations is still not full resolved. Interestingly, a recent study demonstrated that PTEN has a suppressive effect on the GEF activity of PREX2 and that certain missense mutations can affect its interaction with PTEN (27). Hence we propose that the lack of binding of PTEN to truncating PREX2 mutations contributes to the activation of PREX2 GEF activity.

Additionally, this study identified a previously unidentified molecular mechanism controlling expression of an important tumor suppressor, CDKN1C, downstream of PREX2 mutations or inactivation of PTEN, thus showing the intricacies of regulation of oncogenes and tumor suppressor. We showed deficiency of DNA methylation at the imprint control region of CDKN1C in PREX2 mutant and PTEN deleted samples explaining, at least partially, why CDKN1C is down-regulated in such samples. Furthermore, the dysregulated expression of IGF2, which is found in the genomic locus that is coregulated with CDKN1C, via genomic imprinting in PREX2 mutant tumors adds further evidence to the involvement of an epigenetic mechanism downstream of PREX2 mutations. However, the direct molecular link between the PREX2/PI3K/Akt/PTEN signaling axis and the epigenetic machinery regulating DNA methylation is still unclear.

In conclusion, this study expands on the pleiotropic oncogenic mechanisms associated with small GTPases and PI3K pathway activation (28, 49). Our study also supports the thesis that oncogenic PREX2 mutations couple PI3K/Akt signaling to gene expression regulatory machinery involving, among others, cell cycle control in melanoma development.

Materials and Methods

To generate the *tetO-Lox-Stop-Lox-PREX2^{E824*}* transgene, a fragment containing *PREX2^{E824*}* and a FLAG epitope sequence followed by a stop codon was inserted into a previously described transgenic vector (50) by EcoRV digestion. *TetO-LSL-PREX2^{E824*}* mice were produced by injecting a linearized transgenic fragment into FVB/N pronuclei according to standard protocols. Transgenic founders were screened by PCR-based approach. TyrCreERT2 mice and the iNRAS mouse melanoma model have been described (16, 18). Mice were interbred and kept on a FVB/C57Bl6 hybrid background in specific pathogen free environment at MD Anderson Cancer Center mouse facility. For induction of expression of transgenes, mice were fed with doxycycline water (2 g/L, sucrose 20 g/L). Tamoxifen was administered by i.p. injection in sterile corn oil carrier (100 μL of 20 mg/mL stock solution). All animal manipulations were performed with University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee approval.

All remaining materials and methods are described in detail in *SI Appendix, SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Timothy Heffernan and Trang Tieu of the Institute for Applied Cancer Science for help with various plasmids, Jim Horner and Erin Paul of the MD Anderson GEM facility for pronuclear injection of PREX2 transgene, and Chang-Gong Liu of MD Anderson Sequencing and Non-coding RNA Core Facility for microarray profiling and Denise Spring for critical reading of the manuscript. L.C is a recipient of the Cancer Prevention and Research Institute of Texas (CPRIT) Established Investigator Recruitment Award. S.T.A. was supported by the King Abdullah University of Science and Technology.

1. Garraway LA, Lander ES (2013) Lessons from the cancer genome. *Cell* 153(1):17–37.
2. Vogelstein B, et al. (2013) Cancer genome landscapes. *Science* 339(6127):1546–1558.
3. Berger MF, et al. (2012) Melanoma genome sequencing reveals frequent PREX2 mutations. *Nature* 485(7399):502–506.
4. Cancer Genome Atlas Network (2015) Genomic classification of cutaneous melanoma. *Cell* 161(7):1681–1696.
5. Donald S, et al. (2004) P-Rex2, a new guanine-nucleotide exchange factor for Rac. *FEBS Lett* 572(1-3):172–176.
6. Welch HC, et al. (2002) P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac. *Cell* 108(6):809–821.
7. Fine B, et al. (2009) Activation of the PI3K pathway in cancer through inhibition of PTEN by exchange factor P-REX2a. *Science* 325(5945):1261–1265.
8. Hodakoski C, et al. (2014) Regulation of PTEN inhibition by the pleckstrin homology domain of P-REX2 during insulin signaling and glucose homeostasis. *Proc Natl Acad Sci USA* 111(1):155–160.
9. Waddell N, et al.; Australian Pancreatic Cancer Genome Initiative (2015) Whole genomes redefine the mutational landscape of pancreatic cancer. *Nature* 518(7540):495–501.
10. Maehama T, Dixon JE (1998) The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 273(22):13375–13378.
11. Leevers SJ, Vanhaesebroeck B, Waterfield MD (1999) Signalling through phosphoinositide 3-kinases: The lipids take centre stage. *Curr Opin Cell Biol* 11(2):219–225.
12. Di Cristofano A, Pesce B, Cordon-Cardo C, Pandolfi PP (1998) Pten is essential for embryonic development and tumour suppression. *Nat Genet* 19(4):348–355.
13. Furnari FB, Lin H, Huang HS, Cavenee WK (1997) Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain. *Proc Natl Acad Sci USA* 94(23):12479–12484.
14. Conde-Perez A, Larue L (2012) PTEN and melanomagenesis. *Future Oncol* 8(9):1109–1120.
15. Robertson GP, et al. (1998) In vitro loss of heterozygosity targets the PTEN/MMAC1 gene in melanoma. *Proc Natl Acad Sci USA* 95(16):9418–9423.
16. Bosenberg M, et al. (2006) Characterization of melanocyte-specific inducible Cre recombinase transgenic mice. *Genesis* 44(5):262–267.
17. Chin L, et al. (1999) Essential role for oncogenic Ras in tumour maintenance. *Nature* 400(6743):468–472.
18. Kwong LN, et al. (2012) Oncogenic NRAS signaling differentially regulates survival and proliferation in melanoma. *Nat Med* 18(10):1503–1510.
19. Garraway LA, et al. (2005) Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. *Nature* 436(7047):117–122.
20. Hodis E, et al. (2012) A landscape of driver mutations in melanoma. *Cell* 150(2):251–263.
21. Burbelo PD, Drechsel D, Hall A (1995) A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J Biol Chem* 270(49):29071–29074.

22. Leonard DA, et al. (1997) Use of a fluorescence spectroscopic readout to characterize the interactions of Cdc42Hs with its target/effector, mPAK-3. *Biochemistry* 36(5): 1173–1180.
23. Zhang B, Chernoff J, Zheng Y (1998) Interaction of Rac1 with GTPase-activating proteins and putative effectors. A comparison with Cdc42 and RhoA. *J Biol Chem* 273(15):8776–8782.
24. Lucato CM, et al. (2015) The Phosphatidylinositol (3,4,5)-Trisphosphate-dependent Rac Exchanger 1·Ras-related C3 Botulinum Toxin Substrate 1 (P-Rex1·Rac1) Complex Reveals the Basis of Rac1 Activation in Breast Cancer Cells. *J Biol Chem* 290(34): 20827–20840.
25. Hill K, et al. (2005) Regulation of P-Rex1 by phosphatidylinositol (3,4,5)-trisphosphate and Gbetagamma subunits. *J Biol Chem* 280(6):4166–4173.
26. Urano D, Nakata A, Mizuno N, Tago K, Itoh H (2008) Domain-domain interaction of P-Rex1 is essential for the activation and inhibition by G protein betagamma subunits and PKA. *Cell Signal* 20(8):1545–1554.
27. Mense SM, et al. (2015) PTEN inhibits PREX2-catalyzed activation of RAC1 to restrain tumor cell invasion. *Sci Signal* 8(370):ra32.
28. Song MS, Salmena L, Pandolfi PP (2012) The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol* 13(5):283–296.
29. Dillon LM, et al. (2015) P-REX1 creates a positive feedback loop to activate growth factor receptor, PI3K/AKT and MEK/ERK signaling in breast cancer. *Oncogene* 34(30): 3968–3976.
30. Fritsch R, et al. (2013) RAS and RHO families of GTPases directly regulate distinct phosphoinositide 3-kinase isoforms. *Cell* 153(5):1050–1063.
31. Yang HW, et al. (2012) Cooperative activation of PI3K by Ras and Rho family small GTPases. *Mol Cell* 47(2):281–290.
32. Désiré L, et al. (2005) RAC1 inhibition targets amyloid precursor protein processing by gamma-secretase and decreases Abeta production in vitro and in vivo. *J Biol Chem* 280(45):37516–37525.
33. Raynaud F, et al. (2014) Rho-GTPase-activating protein interacting with Cdc-42-interacting protein 4 homolog 2 (Rich2): A new Ras-related C3 botulinum toxin substrate 1 (Rac1) GTPase-activating protein that controls dendritic spine morphogenesis. *J Biol Chem* 289(5):2600–2609.
34. Shutes A, et al. (2007) Specificity and mechanism of action of EHT 1864, a novel small molecule inhibitor of Rac family small GTPases. *J Biol Chem* 282(49): 35666–35678.
35. Soroceanu L, et al. (2007) Identification of IGF2 signaling through phosphoinositide-3-kinase regulatory subunit 3 as a growth-promoting axis in glioblastoma. *Proc Natl Acad Sci USA* 104(9):3466–3471.
36. Danielsen SA, et al. (2015) Portrait of the PI3K/AKT pathway in colorectal cancer. *Biochim Biophys Acta* 1855(1):104–121.
37. Hatada I, et al. (1996) An imprinted gene p57KIP2 is mutated in Beckwith-Wiedemann syndrome. *Nat Genet* 14(2):171–173.
38. Lee MH, Reynisdóttir I, Massagué J (1995) Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev* 9(6): 639–649.
39. Matsuoka S, et al. (1995) p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes Dev* 9(6):650–662.
40. Weksberg R, Shuman C, Beckwith JB (2010) Beckwith-Wiedemann syndrome. *Eur J Hum Genet* 18(1):8–14.
41. Zhang P, et al. (1997) Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. *Nature* 387(6629): 151–158.
42. Matsuoka S, et al. (1996) Imprinting of the gene encoding a human cyclin-dependent kinase inhibitor, p57KIP2, on chromosome 11p15. *Proc Natl Acad Sci USA* 93(7): 3026–3030.
43. Kavanagh E, Joseph B (2011) The hallmarks of CDKN1C (p57, KIP2) in cancer. *Biochim Biophys Acta* 1816(1):50–56.
44. Shin JY, Fitzpatrick GV, Higgins MJ (2008) Two distinct mechanisms of silencing by the KvDMR1 imprinting control region. *EMBO J* 27(1):168–178.
45. Grandjean V, O'Neill L, Sado T, Turner B, Ferguson-Smith A (2001) Relationship between DNA methylation, histone H4 acetylation and gene expression in the mouse imprinted Igf2-H19 domain. *FEBS Lett* 488(3):165–169.
46. Aghazadeh B, Lowry WE, Huang XY, Rosen MK (2000) Structural basis for relief of autoinhibition of the Dbl homology domain of proto-oncogene Vav by tyrosine phosphorylation. *Cell* 102(5):625–633.
47. Krauthammer M, et al. (2012) Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. *Nat Genet* 44(9):1006–1014.
48. Davis MJ, et al. (2013) RAC1P29S is a spontaneously activating cancer-associated GTPase. *Proc Natl Acad Sci USA* 110(3):912–917.
49. Shen WH, et al. (2007) Essential role for nuclear PTEN in maintaining chromosomal integrity. *Cell* 128(1):157–170.
50. Ying H, et al. (2012) Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. *Cell* 149(3):656–670.