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

Yonathan Lissansu Deribe1,2, Yanxia Shi3,6,1, Kunal Rai3, Luigi Neri4, Samir B. Amin5, Chia-Chin Wu4, Kadri C. Akdemir3, Mozdeh Mahdavi5, Qian Peng5, Qing Edward Chang6, Kirsti Hornigold7, Stefan T. Arold8, Heidi C. E. Welch8, Levi A. Garraway1,9, and Lynda Chin8,9,2

1Department of Genomic Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX 77030; 2Sun Yat-Sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborate Center for Cancer Medicine, Guangzhou 510060, China; 3Institute for Applied Cancer Science (IACS), The University of Texas MD Anderson Cancer Center, Houston, TX 77030; 4The Babraham Institute, Babraham Research Campus, Cambridge CB22 3AT, United Kingdom; 5Division of Biological and Environmental Sciences and Engineering, King Abdullah University of Science and Technology, Thuwal, 23955-6900 Saudi Arabia; 6Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02215; and 7Broad 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 (PREX2E584X) 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 (phosphatidylinositol-3,4,5-trisphosphate to phosphatidylinositol-3,4,5-bisphosphate) 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 p57KIP2). 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 expression of a cell cycle regulator.

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

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-3,4-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 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.


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).

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

2To 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.

www.pnas.org/cgi/doi/10.1073/pnas.1513801113
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 lox 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-PREX2E824* transgene. (D) Plot showing number of overlapped genes whose expression is significantly changed in TetO-LSL-PREX2E824* transgenic tumors and xenograft tumors overexpressing PREX2E824*. (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 (p57KIP2), 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 (PREX2E824*) 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 the truncating PREX2 mutant in a tissue and time specific manner, we generated a conditional PREX2 mutant in a tissue and time specific manner, we cross-transgenic mice carrying the PREX2E824* ORF, designated as TetO-LSL-PREX2E824* (Fig. 1A). We crossed these mice to Ty-CreERT2 and Tyr-rTA mice to express PREX2E824* 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+/−, TetO-NRASQ61K, Tyr-rTA) (18) and generated two cohorts of mice with or without the inducible TetO-LSL-PREX2E824* 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-PREX2E824* transgene exhibited accelerated melanoma development with increased penetrance compared with the iNRAS model alone (Fig. 1B 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 mutations 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 Q1480*). Cross-species comparison revealed statistically significant overlap between the genes altered in mouse tumors expressing the TetO-LSL-PREX2E824* transgene and in xenograft tumors derived from truncating PREX2 overexpressing human melanocytes (Fig. 1D).

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. 1E 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-PREX2E824* transgene or controls. Consistent with the gene expression profiling experiment (Fig. 1E), we saw statistically significant increase in Ki67-positive staining in PREX2 transgene-containing tumors compared with the control (Fig. 24, 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 p57KIP2). 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. 2D). Similar down-regulation of CDKN1B is observed in transgenic tumors expressing activated TetO-LSL-PREX2E824* allele (Fig. 2E). Furthermore, we observed reduced expression of p21 and p27 on Western blots of tumors harboring TetO-LSL-PREX2E824* allele (SI Appendix, Fig. S34). 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. S3B).

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. S44) (3). It is important to note that additional PREX2 truncating mutations have been recently described (4, 20) (SI Appendix, Fig. S4B).

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. 3A, 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. 3A, 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. 3B). 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. 3C). 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. 3D). 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 HEK293 cells. Again, the truncating PREX2 mutants lost the ability to bind to PTEN (SI Appendix, Fig. S4E). 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. 4A). 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. 4B). Next, we performed immunoblotting on tumor lysates derived from two mouse models, iNRAS and iNRAS with controls (Fig. 4C). Functionally, CDKN1C (also known as p57kip2) 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). Moleculally, 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. 5A), 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 deletion of PTEN (Fig. 5B), 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. S7A). 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 epigenomic 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...
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-PREX2E824+ 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.

(PREX2E824+) 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<sup>L/L</sup>, Rosa26-Cre<sup>ERT2</sup> E13.5 embryos treated with vehicle or 4-OHT to induce Cre<sup>ERT2</sup> 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-P13K 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 non-specific 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 truncating mutations also abolish its interaction with PTEN. We showed 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 contribute to the activation of PREX2 GEF activity.

Materials and Methods

To generate the teto-Stop-Stop-PREX2Δ504-Δ24, a fragment containing PREX2Δ504-Δ24 and a FLAG epitope sequence followed by a stop codon was inserted into a previously described transgenic vector (50) by EcoRV digestion. Teto-LSL-PREX2Δ504-Δ24 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/C57Bl/6 background in specific pathogen free environment at MD Anderson Cancer Center mouse facility. For induction of expression of transgenes, mice were fed with dosycycline 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.


