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TITLE: The Role of PRIP in Breast Cancer

PRINCIPAL INVESTIGATOR: Yijun Zhu, M.D.
Janardan K. Reddy, M.D.
Sunil S. Badve, M.D.

CONTRACTING ORGANIZATION: Northwestern University
Evanston, Illinois 60208-1110

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Estrogen plays an important role in the normal breast and breast cancer development. Estrogens exert their cellular effects through ER that is a member of nuclear receptor superfamily. PRIP (Peroxisome proliferator receptor interacting protein) is a nuclear receptor coactivator that is amplified and overexpressed in breast cancer. The proposal was to investigate how the PRIP dysregulation contributes to abnormal growth and neoplastic development of breast. During the second year of the award, we disrupted PRIP gene in mice by homologous recombination. Mice nullizygous for PRIP died between embryonic day 11.5 and 12.5 (post coitum), indicating that PRIP like PBP, CBP, and p300 is an essential and nonredundant coactivator. We have created heterozygous mice with conditional inactivated PRIP gene which will finally help to reveal the function of this important coactivator in mammary gland.

We found that PRIP overexpression occurs in about 60% and gene amplification occurs 10% of the breast cancers, suggesting that this coactivator plays an important role in the breast cancer development.
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INTRODUCTION

Estrogens play an important role in the normal breast and breast cancer development. Estrogens exert their cellular effects through ER which is a member of nuclear receptor superfamily. Nuclear receptors achieve transcriptional activation through the participation of additional factors known as nuclear receptor coactivators. PRIP (Peroxisome proliferator receptor interacting protein) is a nuclear receptor coactivator amplified and overexpressed in some breast cancers. The proposal was to investigate how PRIP dysregulation contributes to abnormal growth and neoplastic development of breast. Specifically, transgenic animal models overexpressing PRIP would be developed to determine if overexpression of PRIP is sufficient to cause abnormal growth of mammary glands and if such growth ultimately leads to the development of breast cancers. PRIP transgenic mouse and PRIP null mutated mouse would be used to examine the role of coactivator PRIP in determining the susceptibility of breast cancer development induced by estrogen and chemical carcinogen. The expression of estrogen responsive genes would be examined to test the role of PRIP as an estrogen receptor coactivator in vivo and identify the estrogen responsive genes that are specifically affected by PRIP overexpression and null mutation. The prevalence of PRIP upregulation and amplification in breast cancers would be examined in breast cancers. The potential polymorphisms in PRIP genes would be identified and the linkage between the identified polymorphisms and breast cancer risk would be elucidated by examining normal female and breast cancer patients. The proposed studies would generate novel information regarding the role of nuclear receptor coactivator in breast cancer development and the importance of the variation of nuclear receptor coactivator in the etiology of breast cancer, and most likely provide new insights regarding prevention and treatment strategies.
Task 1a. Generation of transgenic animal models overexpressing PRIP to determine if overexpression of PRIP is sufficient to cause abnormal growth of mammary glands and if such growth ultimately leads to the development of breast tumors.

We made a construct MMTV-PRIP to overexpress PRIP in mammary gland. We then used this construct to develop transgenic mice. Transgenic founders were identified by PCR with primers recognizing the promoter region of the transcription unit. 7 transgenic mouse lineages were identified. The founder mice were bred with C57BL/6 mice to generate F1 progeny. The expression of PRIP transgene in mammary glands from F1 progeny was examined by RNAse protection assay. The expression level of PRIP transgene was about half of that from the endogenous PRIP, which was not high enough for further studies. More transgenic mice will be developed to obtain the mice overexpressing PRIP.

Task 1b. Investigation of the role of coactivator PRIP in the susceptibility for breast cancer development to estrogen and chemical carcinogen using PRIP transgenic mouse and PRIP null mutation mouse.

We disrupted PRIP gene in mice by homologous recombination. Mice nullizygous for PRIP died between embryonic day 11.5 and 12.5 (post coitum) due in most part to defects in the development of placenta, heart, liver, nervous system and retardation of embryonic growth(1). Transient transfection assays using fibroblasts isolated from PRIP'''' embryos revealed a significant decrease in the capacity for ligand-dependent transcriptional activation of RXRα and to a lesser effect on PPARγ transcriptional activity. These observations indicate that PRIP like PBP, CBP, and p300 is an essential and nonredundant coactivator.

As PRIP null mutation is embryonic lethal, we will mate LoxP-PRIP mouse with MMTV-Cre transgenic mice to obtain mouse with conditional PRIP mutation in mammary gland so that the function of PRIP in mammary gland development and its response to estrogen and carcinogen treatment can be studied.

Task 1c. Examination of the expression of estrogen responsive genes and identification of the estrogen responsive genes that are specifically affected by PRIP overexpression and null mutation.

Mammary glands were isolated from ovariectomized animals treated with 17β-estradiol or ICI 182,780 for three days. Poly(A)^+ RNA was isolated from the mammary glands. cDNA probe was made from poly A^+ RNA for hybridization to Mouse cDNA microarray filters. The filters were then exposed to a phosphor imaging system and analyzed using software to obtain the relative expression level of each gene. We found that estrogen treatment increased the mRNA levels of about fifty genes. GAS6 was picked up for further studies. The induction of GAS6 mRNA expression by estrogen was confirmed by RNAse protection assay. The mammary gland from mice deficient for the GAS6 receptors was examined and found to have increased budding and decreased branching.

Task 2. Determination of the prevalence of PRIP upregulation and amplification in breast cancers.
We have collected about 100 breast cancer specimen from the Department of Pathology, Robert Lurie cancer center. RNAse protection assay revealed that 62% of the breast cancers have PRIP overexpression. Southern blot studies showed that 10% of the breast cancers have PRIP gene amplification.


PCR were performed to cover all exons and 500 bp promoter region of PRIP gene. The PCR products were analyzed by SSCP (single strand conformational polymorphism) and the potential polymorphisms were determined by sequencing. We found polymorphism A to G at nucleotide 289, G to A at nucleotide 2408. We are currently examining these polymorphisms in breast cancer patients and normal females.
KEY RESEARCH ACCOMPLISHMENTS

* PRIP gene was disrupted in mice by homologous recombination. Mice nullizygous for PRIP was found to die between embryonic day 11.5 and 12.5.
* About fifty estrogen-inducible genes including GAS6 were identified. The mammary gland from mice deficient for the GAS6 receptors was found to have increased budding and decreased branching.
* PRIP overexpression and amplification were found in about 60% and 10% of breast tumors, respectively.
REPORTABLE OUTCOMES

CONCLUSIONS

We disrupted PRIP gene in mice by homologous recombination. Mice nullizygous for PRIP died between embryonic day 11.5 and 12.5 (post coitum), indicating that PRIP like PBP, CBP, and p300 is an essential and nonredundant coactivator. We have created heterozygous mice with conditional inactivated PRIP gene which will finally help to reveal the function of this important coactivator in mammary gland. We found that PRIP overexpression occurs in about 60% and gene amplification occurs 10% of the breast cancers, suggesting that this coactivator plays an important role in the breast cancer development.
REFERENCES

APPENDICES

One article is attached.
Coactivator PRIP, the Peroxisome Proliferator-activated Receptor-interacting Protein, Is a Modulator of Placental, Cardiac, Hepatic, and Embryonic Development*

Yi-Jun Zhu, Susan E. Crawford, Veronica Stellmach, Rama S. Dwivedi, M. Sambasiva Rao, Frank J. Gonzalez, Chao Qi, and Janardan K. Reddy

From the Departments of Pathology and Pediatrics, Northwestern University, the Feinberg School of Medicine, Chicago, Illinois 60611-3008 and The Laboratory of Metabolism, NCI, National Institutes of Health, Bethesda, Maryland 20892

Nuclear receptor coactivator PRIP (peroxisome proliferator-activated receptor (PPAR)-γ-interacting protein) and PRIP-interacting protein with methyltransferase activity, designated PIMT, appear to serve as linkers between cAMP response element-binding protein (CBP)/p300-anchored and PBP (PPARγ-binding protein)-anchored coactivator complexes involved in the transcriptional activity of nuclear receptors. To assess the biological significance of PRIP, we disrupted the PRIP gene in mice by homologous recombination. Mice nullizygous for PRIP died between embryonic day 11.5 and 12.5 (postcoitum) due in most part to defects in the development of placenta, heart, liver, nervous system, and retardation of embryonic growth. Transient transfection assays using fibroblasts isolated from PRIP−/− embryos revealed a significant decrease in the capacity for ligand-dependent transcriptional activation of retinoid X receptor α and to a lesser extent on PPARγ transcriptional activity. These observations indicate that PRIP like PBP, CBP, and p300 is an essential and nonredundant coactivator.

Our understanding of the mechanisms underlying transcriptional activation by nuclear receptors has been advanced by the identification of nuclear receptor coactivators or coregulators that appear to influence embryonic development, cell proliferation, and differentiation (1). These include p160SRC-1 (steroid receptor coactivator-1) family with three members (SRC-1, TIF/GRIP/JSRC-2, and p65/IP/160ACTR/RAC3/TARML/SRC3) (2-6), CREB-binding protein (CBP) (7), adenovirus E1A-binding protein p300 (8), peroxisome proliferator-activated receptor-γ (PPARγ)-binding protein (PBP) (9), PPAR-interacting protein (PRIP/ASC-2/RAP250/TRBP/NRC) (10-14) and PPARγ coactivator-1 (PGC-1) (15), among others. Nuclear receptor coactivators contain one or more conserved LXXLL (where L is leucine and X any amino acid) signature motif, which has been found to be necessary and sufficient for ligand-dependent interactions with the activation function-2 domain present in the C-terminal hormone-binding region of the nuclear receptors (1-6). It is generally held that coactivators play a central role in mediating nuclear receptor transcriptional activity by functioning as at least two large multiprotein complexes formed either sequentially or combinatorially (1). The first complex anchored by CBP/p300 and containing p160 cofactors/SRC-1 cofactors exhibits histone acetyltransferase activity necessary for remodeling chromatin (1, 4, 7, 16), while the second multiprotein complex, variously referred to as TRAP/DRIP/ARC mediator complex, which is anchored by PBP (17-19), facilitates interaction with RNA polymerase II complexes of the basal transcription machinery (1). Deletion of CBP/p300 and PBP genes in the mouse results in embryonic lethality around E11.5 days, indicating that disruption of these pivotal anchoring coactivators affects the integrity of the cofactor complexes, thus altering the function of many nuclear receptors and most likely of other transcription factors (20-24).

Of interest is that the recently identified coactivator designated PRIP/ASC2/RAP250/NRC/TRBP has also been shown to interact with several nuclear receptors and with CBP/p300 and TRAP130 of the TRAP/DRIP/ARC complex (10-14). Thus, PRIP appears to serve as a bridge between the first complex anchored by CBP/p300 and the downstream TRAP/DRIP/ARC mediator complex anchored by PBP. Furthermore, the recently isolated PRIP-interacting protein with RNA methyltransferase activity, designated PIMT (25), forms a complex with CBP, p300, and PBP (26), further attesting to the possibility that two major multiprotein cofactor complexes anchored by CBP/p300 and PBP, respectively, merge into one megacomplex on DNA template (28). Since PRIP and PBP-binding protein PIMT appear to link the two cofactor complexes under in vitro conditions, we have found it necessary to explore the biological function of PRIP by generating mice with PRIP null phenotype. We now demonstrate that PRIP is critical for the embryonic development, since disruption of the PRIP gene in the mouse leads to embryonic lethality around E11.5 to E12.5 days, implying that PRIP (like CBP/p300 and PBP) is also critical for embryonic development and survival.

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1015 From the Departments of Pathology and Pediatrics, Northwestern University, the Feinberg School of Medicine, Chicago, Illinois 60611-3008 and The Laboratory of Metabolism, NCI, National Institutes of Health, Bethesda, Maryland 20892.
Construction of Targeting Vector—The genomic DNA fragment containing the full-length PRIP gene was isolated from a mouse 129/Sv P1 bacteriophage library (Genome Systems, St. Louis, MO), using polymerase chain reaction with primers 5'-CAATGCAGCTGTCTCGT-3' and 5'-GCTGCTGCACTACATCACTGAAA-3' designed from mouse PRIP cDNA sequence (10). A Cre-LoxP system was employed to delete exon 7 from the PRIP gene. For this purpose, we constructed a triple-LoxP PRIP targeting vector to generate a "floxed" mouse PRIP-targeted locus.

Generation of PRIP Null Mice—The targeting vector was linearized and electroporated into ES embryonic stem (ES) cells (28). Transfected ES cells were selected in the medium with G418 (200 μg/ml), and the surviving colonies were screened for homologous recombination by PCR with primers P1 5'-CCTACAGCTGCAAGGCAATGATC-3' and P2 5'-TATACGAAATTTGCGGGCTG-3'. ES cells with the appropriate PRIP floxed targeted locus were further confirmed by Southern blot analysis (Fig. 1B), and the euploid selected ES cells were used for injection into 3.5-day-old blastocysts derived from C57/B6 mouse by the Northwestern University Targeted Mutagenesis Facility to generate chimeric mice. Chimera male mice were bred with wild type C57/B6 female to produce heterozygous mice, which were then crossed with EIIa-Cre transgenic mice to delete the DNA fragment between LoxP1 and LoxP3. EIIa-Cre-mediated recombination occurs early in development (2-8 cells), and mice carrying the allele with deletion were crossed with wild type C57/B6 to achieve germ line transmission. The heterozygous mice with expected deletion were interbred to generate homozygous mutants.

Genotyping of Mice and Embryos—DNA extracted from the tail tips of mice and from the yolk sac of embryos was used for genotyping by PCR. The mice carrying the recombination between LoxP1 and LoxP3 on one allele were identified by PCR with primers P1/P2 and P3 (5'-GCCTGCCGATAACCTCTGATT-3') and Giemsa stain was done using the standard protocol.

RT-PCR and Western Blots—for RT-PCR, total RNA was extracted from embryos with Trisol reagent (Invitrogen). Primers 5'-CTCATAGCCAGGAAACG-3' and 5'-CAGAAAGCTGTGATACGA-3' were used to amplify the region between exon 6 and exon 8 from the PRIP homozygous mutant. To detect PRIP protein in embryos, whole embryonic fibroblasts (MEF) were isolated from 3.6-day-old embryos by the Northwest University Targeted Mutagenesis Facility to generate chimeric mice. Chimeric male mice were bred with wild type C57/B6 female to produce heterozygous mice (2-8 cell stage), and it induces the recombination between the two loxP sites with the same orientation (29). We detected the expected, all types of recombinants among the offspring (29) (see above) (Fig. 1C).

Histological Analysis and Immunohistochemistry—Age-matched embryos were fixed in paraformaldehyde or 10% of buffered formalin, embedded in paraffin, serially sectioned at 5-μm thickness in sagittal or transverse planes, and stained with hematoxylin and eosin. Immunohistochemical staining for the localization of proliferating cell nuclear antigen (PCNA) was performed using an avidin-biotin-peroxidase complex protocol as described previously (24). Giemsa stain was done using the standard protocol.

Isolation of Fibroblasts from Embryos and Transfection of Primary Fibroblasts—Mouse embryonic fibroblasts (MEF) were isolated from E11.5 embryos and cultured in Dulbecco's modified Eagle's medium with 10% of fetal bovine serum as described (22). For transfection, 2 × 10⁶ of MEFs were plated in six-well plates for overnight culture. The transfections were carried out with LipofectAMINE-2000 Plus reagent according to manufacturer's instruction (Invitrogen). Plasmids pCMV-PFPα, pFRE-TK-LUC, pCMV-RXR, pFRE-TK-LUC, pCMV-RAR and pFRE-TK-LUC were described previously (10, 25, 26). β-Galactosidase assay vector pCMVβ was used as a cotransfectant, which served as control for transfection efficiency. Cell extracts were prepared 36 h after transfection and were assayed for luciferase and β-galactosidase activities.

RESULTS

Disruption of PRIP Gene in Mice—We constructed a conditional knockout allele of PRIP by using the Cre/LoxP recombination system according to the strategy in Fig. 1A. To generate conventional PRIP knockout mice (in which the gene is permanently inactivated at the germ cell stage), it was necessary to induce recombination between loxP sites. To achieve heterozygosity, PRIP-targeted mice were bred with homozygous Cre transgenic mouse line, EII-cre (29). The EII-cre mice carry the Cre transgene under the control of the adenovirus EIIa promoter and express Cre recombinase only in early mouse embryos (2-8 cell stage), and it induces the recombination between the two loxP sites with the same orientation (29). We detected the expected, all types of recombinants among the offspring (29). The recombination between loxP1 and loxP3 resulted in the deletion of PRIP exon 7 and a reading frame shift to create a stop codon right after the fusion between exon 6 and exon 8. The chimeras were crossed to wild type mice to produce heterozygous mice carrying one recombinant PRIP allele, and the homozygous mice were obtained from heterozygous mating. By sequencing the RT-PCR products, exon 7 was not found in mRNA transcribed from the recombinant PRIP allele, and the reading frameshift was introduced by the deletion leading to a premature stop codon (data not shown). As a result, only 488 amino acids at the N terminus containing no LXXLL motif can possibly be translated from the mRNA, but no PRIP protein was detected by Western blot analysis (data not shown).

Embryonic Lethality and Growth Retardation of PRIP Null Mice—Among 26 newborn pups, and 54 mice that were 3 weeks old generated from intercrosses between heterozygous PRIP mutant mice, no homozygous mutants were detected. Genotyping the embryos at different stages of gestation showed that no PRIP null embryos survived beyond E13.5 (13.5 days postcoitum). However, heart beating was observed among the majority of viable PRIP null embryos recovered between E11.5 and E12.5 and few were moribund or dead. These observations indicate lethality occurred in a relatively narrow window of time at no viable PRIP null embryos were seen at E13.5. The viable PRIPnull embryos recovered at E11.5 and E12.5 exhibited clear evidence of growth retardation compared with the wild type and heterozygous littermates. PRIPnull embryos appeared strikingly different at the gross level at E12.5, they were pale and smaller in size than their PRIP+/− and PRIPnull littermates (Fig. 2). Normally, extraembryonic mesoderm of the yolk sac gives rise to blood and endothelial cells, which form blood islands. The extraembryonic membrane covering PRIP null embryos contained fewer vessels in contrast to wild type yolk sac with its well developed blood vessels (Fig. 2B). In addition, superficial vasculature was less obvious in
The development of heart requires coordinated differentiation of several embryonic lineages, including the myocytes of myocardium, the endothelial cells of the endocardium, and the cells of the neural crest that form the outflow tract. At E12.5, the heart of PRIP"" embryos exhibited defects involving all three of the neural crest that form the outflow tract. At E12.5, the cardium, the endothelial cells of the endocardium, and the cells of the neural crest were detected in PRIP"" mutants (not illustrated). Lack of Organized Spongiotrophoblast Layer in PRIP Mutant Placenta—In wild type placenta at E12.5 contains three distinct trophoblast cell structures: the innermost labyrinthine layer, the intermediate spongiotrophoblast layer, and the outermost trophoblast giant cell layer. The labyrinthine zone formed by the fusion of chorion with allantois is composed of extensively branched fetal blood vessels and maternal blood sinususes among strands of diploid trophoblast cells that separate the maternal blood sinususes from fetal blood vessels. In PRIP null placenta, no compact layer of spongiotrophoblast cells was observed between labyrinth zone and trophoblast giant cell layer. Instead, islands of spongiotrophoblast-like cells dissociated from trophoblast giant cell layer and migrating into the labyrinth zone were common occurrence in PRIP"" placenta (Fig. 2B). In wild type placenta blood sinususes are filled with maternal blood cells throughout the labyrinthine layer (Fig. 3, A, C, and F), whereas most of the tortuous vessels in the PRIP"" placenta were enlarged, ruptured, and generally empty (Fig. 3, B, D, and F). While the choioallantoic fusion appeared to occur in PRIP mutant placenta, chorionic trophoblast cells clustered in labyrinth had multiple nuclei, and these clusters showed insufficient blood vessel branching. These changes are reminiscent of some of the placental defects observed in PBP null mutants (22, 24). However, changes in PRIP"" placenta appeared less profound when compared with PBP"" placenta (24). Nucleated fetal erythroblasts in PRIP"" placenta had irregular shaped nuclei with very little cytoplasm (Fig. 3F). Trophoblast cell proliferation in PRIP null placenta as assessed by PCNA immunostaining was significantly lower than that observed in PRIP"" placenta (data not shown).

PRIP"" Embryos Manifest Cardiac Defects—Inefficient pumping by the heart leading to circulation failure is one of the major causes of embryonic lethality during middle gestation. The development of heart requires coordinated differentiation of several embryonic lineages, including the myocytes of myocardium, the endothelial cells of the endocardium, and the cells of the neural crest that form the outflow tract. At E12.5, the heart of PRIP"" embryos exhibited defects involving all three lineages (Fig. 4, A and B). In PRIP"" heart, the epicardium, consisting of a single layer of mesothelial cells lining against the compact layer of myocardium, appeared to separate from underlying myocardium and this space is filled with blood cells (Fig. 4B). Pericardial space surrounding the heart in mutant was much smaller than their wild type littermate. Unlike the ventricles in wild type heart, which consisted of multicell thick compact layer with well developed trabeculae, the ventricles in PRIP"" heart only contained one or two cell layers of myocardium (Fig. 4). Cell-cell adhesion among cardiocytes appeared to
be defective, which possibly contributes to leakage of blood cells into epicardial spaces. Cell proliferation in PRIP⁻/⁻ myocardium was markedly reduced as compared with PRIP⁺/+ myocardium (Fig. 4, C and D). The failure of ventricular myocardium of PRIP⁻/⁻ embryos to stratify into the multilayer compact structure required to sustain adequate cardiac function appears similar to that noted in FBP null and PPARγ null mutants (22, 24, 30).

Defective Hepatopoiesis and Hepatic Hematopoiesis in PRIP⁻/⁻ Mutants—Liver of PRIP null mutants appeared considerably smaller in size when compared with their littermates (Fig. 5, A and B). The function of the liver at E12.5 days is to become the major site of hematopoiesis so as to gradually replace yolk sac based hematopoiesis. Histological examination of PRIP⁻/⁻ liver revealed reduction in hepatocyte population and an increase in hepatocyte apoptosis (Fig. 5D). A marked decrease in the number of erythroid progenitors was also evident, and these cells had large nuclei with scant minimally hemoglobinized cytoplasm. Liver exhibited large numbers of megakaryocytes.

Differential Reduction of Transactivation by Nuclear Receptors in PRIP⁻/⁻ Primary Fibroblasts—To assess the impact of loss of PRIP on transcriptional activities of nuclear receptor, we isolated MEFs from PRIP⁻/⁻ and PRIP⁺/+ embryos. They were used for assaying the transcriptional activities of PPARγ, RXR, and RAR. In wild type MEFs transfected with a RXR expressing vector and RXR-responsive element-linked reporter, the addition of RXR-ligand 9-cis-retinoic acid induced marked increase (~76-fold) in the transcription (Fig. 6A). In PRIP⁻/⁻ MEFs, the induction of ligand-mediated RXR transcription was markedly reduced (~3-fold). Transcription assays with PPARγ (Fig. 6B) and RARα (data not shown) showed that the influence of PRIP was only modest. These results demonstrated that nuclear receptors require PRIP to achieve their full transcriptional potential, although the contribution of PRIP to the activities of the three nuclear receptors examined here differed somewhat in that PRIP seemed to influence RXR maximally.

DISCUSSION

The nuclear hormone receptors comprise a superfamily of transcription factors that regulates coordinated expression of gene networks involved in developmental, physiological, and metabolic processes (1). Notable among this nuclear receptor superfamily is PPAR subfamily comprising of three isoforms, PPARα, PPARγ, and PPARβ/δ, since these receptors have emerged in recent years as a critical player in regulating energy metabolism. In an effort to understand the factors controlling cell and gene specific transcriptional events initiated by nuclear receptors, the ligand-binding domain of nuclear receptors was used in the yeast two-hybrid screen to identify receptor interacting proteins (2-7, 31). During the past 7 years, more than 26 nuclear receptor coactivators have been cloned raising the issue of redundancy, since these coactivators generally appear promiscuous in their coactivation potential.

To fully appreciate the in vivo biological functions of these coactivators, molecular genetic approaches are being increasingly employed. Previous studies have demonstrated that mice lacking SRC-1 or p/CIP/SRC-3 are viable and manifest either partial or full redundancy for certain nuclear receptor actions (32-36). In contrast, deletion of more general coactivators such as CBP/p300 and PBP in mice leads to embryonic lethality implying that these are essential coactivators (20-24). Thus, there appear to be at least two broad classes of coactivators: essential and redundant. Our observations reported here now add coactivator PRIP to the class of essential coactivators because of the embryonic growth retardation, defects in placental, cardiac and hepatic development, and embryonic lethality. Embryonic lethality was noted between E11.5 and E12.5 days with no viable embryos at E13.5. Defects were noted in heart (reduced amount of myocardium and noncompaction), liver (small liver with reduction in hepatocyte population, and hepatocyte apoptosis), and defects in erythropoiesis (reduced hemoglobinization) and placenta (maturity block of trophoblast with vascularization defect). The placental defects, although not as pronounced as those encountered in PBP⁻/⁻ placenta (22, 24),
It should be noted that we isolated both PRIP and PBP using PPARγ as bait in the yeast two-hybrid screen and identified them as nuclear receptor coactivators (9, 10). PBP has since been emerged as a central piece in large TRAP-DRIP-ARC-PRIC complexes (11), suggesting that both PBP and PRIP by virtue of their coactivating function may augment cell proliferation and promote growth factors, and oxygen, which may contribute to embryonic growth retardation. The death of embryos at about mid-gestation has been reported in a number of null mutants induced by gene targeting, including PPARγ (30), RXRa (37), PBP (22–24), and N-myc (38).

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