Ppp1r15 gene knockout reveals an essential role for translation initiation factor 2 alpha (eIF2α) dephosphorylation in mammalian development

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Diverse cellular stress responses are linked to phosphorylation of serine 51 on the alpha subunit of translation initiation factor 2. The resultant attenuation of protein synthesis and activation of gene expression figure heavily in the adaptive response to stress, but dephosphorylation of eIF2α (P) has been less well understood. GADD34 and CREP, the products of the related mammalian genes Ppp1r15a and Ppp1r15b, can recruit phosphatase catalytic subunits of the PPP1 family to eIF2α, but the significance of their contribution to this dephosphorylation has not been explored systematically. Here we report that unlike Ppp1r15a mutant mice, which are superficially indistinguishable from wild type, Ppp1r15b−/− mouse embryos survive gestation but exhibit severe growth retardation and impaired erythropoiesis, and loss of both Ppp1r15 genes leads to early embryonic lethality. These loss-of-function phenotypes are rescued by a mutation, GADD34sS74, that prevents regulated phosphorylation of eIF2α. These findings reveal that the essential process of eIF2α (P) dephosphorylation is the predominant role of Ppp1r15 proteins in mammalian development.

Regulated phosphorylation of serine 51 of the alpha subunit of translation initiation factor 2 (eIF2α) attenuates rates of translation initiation and thereby protein synthesis in response to diverse stressful conditions (1). The protein kinases, PERK, GCN2, PKR, and HRI, respectively, couple the stress of protein misfolding in the endoplasmic reticulum (ER) stress, amino acid deprivation, viral infection, and heme deficiency to eIF2α phosphorylation (2). The phenotypes associated with loss of these kinases are well characterized and indicate that the ability to downregulate protein synthesis favors survival of cells experiencing ER stress (3), amino acid starvation (4), or heme deficiency (5) and the survival of the host during viral invasion (6).

Dephosphorylation of eIF2α (P) is less well studied. Somatic cell genetic screens have led to the identification of 2 related genes, Ppp1r15a and Ppp1r15b, encoding the proteins GADD34 and CREP, whose overexpression promotes eIF2α (P) dephosphorylation (7, 8). Both proteins use their related C-terminal domain (of ~200 aa) to recruit a catalytic subunit from one of several related protein phosphatase I (PPP1) isozymes to form a holophosphatase complex that can dephosphorylate eIF2α (P) in vitro (7–10). GADD34 levels are low in unstressed cells, but the Ppp1r15a/ GADD34 gene is transcriptionally induced by rising levels of eIF2α (P) (7, 11, 12). GADD34 induction then correlates with the declining phase of eIF2α (P) later in the recovery phase of the stress response. Consequently, cells lacking Ppp1r15a/GADD34 phosphatase activity exhibit impaired recovery of protein synthesis (13, 14). Remarkably, basal levels of eIF2α (P) are little affected by the mutation, and apart from a measure of resistance to the lethal affects of ER stress, the Ppp1r15a/GADD34 mutant mice are superficially indistinguishable from the wild type (12). CREP, in contrast to GADD34, is constitutively expressed, and knockout of Ppp1r15b/CREP (by siRNA) led to a mild defect in basal levels of eIF2α (P) dephosphorylation in cultured cells (8). However, until now, the significance of Ppp1r15b/CREP to mammalian physiology remained unexplored.

Although an important adaptation to a variety of stressful conditions, sustained elevation of eIF2α (P) is poorly tolerated (1, 15, 16). However, the role of dephosphorylation in protecting against the consequences of deregulated elevation in eIF2α (P) has not been studied. Though it is clear that the Ppp1r15 family members GADD34 and CREP can promote eIF2α (P) dephosphorylation, neither their contribution to this process in vivo nor the potential existence of other, redundant mechanisms to control levels of eIF2α (P) have been fully explored. Furthermore, the functional importance of other activities of Ppp1r15 proteins has not been addressed experimentally. Here we report on a phenotypic analysis of mice with induced mutations in Ppp1r15a and Ppp1r15b that lack functional GADD34 or CREP and compound mice lacking both genes. Our findings indicate that inadequate eIF2α (P) dephosphorylation dominates the phenotype of the mutants and that eIF2α (P) dephosphorylation is the essential function provided by the Ppp1r15 family.

Results

A deletion encompassing 768 bases of the promoter and the portion of exon 1 encoding the N-terminal 417 aa of the Ppp1r15b gene (which includes all of CREP’s AUG codons) was created by homologous recombination in mouse embryonic stem cells, and the mutant allele was transmitted through the germline of chimeric mice (Fig. 1A). Mouse embryo fibroblasts (MEFs) derived from homozygous mutant embryos had no CREP protein detectable by Western analysis using a phospho-specific antiserum, with a phospho-specific antiserum, were slightly higher in the Ppp1r15b−/− compared with wild-type cells and increased transiently in cells of both genotypes after exposure to thapsigargin, an agent that promotes ER stress and activates the eIF2α kinase PERK. In both genotypes, the declining phase of eIF2α phosphorylation coincided with the induction of GADD34 protein (Fig. 1C Upper), as previously described (7). MEFs derived from embryos

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homozygous for a Pprr15a mutation that deletes the C-terminal PPI1c-interacting domain of GADD34 (Pprr15a<sup>AC</sup>) also showed a slight increase in basal levels of phosphorylated eIF2α. Unlike the wild-type and Pprr15b<sup>−/−</sup> MEFS, the Pprr15a<sup>AC/AC</sup> MEFS exhibited sustained increase in phosphorylated eIF2α throughout the stress response (Fig. 1C Lower), as previously noted (13, 14). A single copy of functional Pprr15a was sufficient to promote the decline in levels of phosphorylated eIF2α at later time points of the stress response even in cells lacking CREP, indicating that feedback regulation of eIF2α phosphorylation in the unfolded protein response is maintained by GADD34 (Fig. 1C Lower).

Pprr15b<sup>−/−</sup> embryos were recovered at the expected ratio up to the moment of birth; however, homozygous mutant newborns were about half the size of their wild-type littermates, notably pale (see next paragraph), and failed to nurse, and none survived the first day of postnatal life (Fig. 2 A–C). Embryos heterozygous for the Pprr15b<sup>−/−</sup> mutation were indistinguishable from wild type. Consistent with the observations made in cultured MEFS, levels of phosphorylated eIF2α were only modestly elevated in tissues of Pprr15b<sup>−/−</sup> embryos (data not shown), but in some tissues, such as liver, constitutively elevated levels of GADD34 protein may have compensated for CREP deficiency (Fig. 2D).

The paller of the Pprr15b<sup>−/−</sup> embryos seemed well explained by low hematocrit and red blood cell count (Fig. 3A). These quantitative abnormalities in red cell mass were associated with significant qualitative abnormalities in red cell size and shape (Fig. 3B and C), and histological examination of the liver was consistent with compensatory proliferation of blood precursors (Fig. 3C). These findings were further supported by FACS analysis, which showed a reduced percent of Ter119-positive, CD71-negative late erythroid precursors in the mutant liver (17, 18) (Fig. 3D). A similar but much milder defect had been reported previously in homozygous Pprr15a<sup>−/−</sup> mutant mice (19), suggesting that an activity common to both of these homologous proteins was important to fetal erythropoiesis.

To examine the role of unmitigated eIF2α phosphorylation in the phenotype of the Pprr15b<sup>−/−</sup> embryos, we crossed Pprr15b<sup>−/−</sup> mice with mice carrying a mutant allele of Eif2as<sup>S51A</sup> that replaces serine 51 with alanine. Although homozygosity for the Eif2as<sup>S51A</sup> allele abolishes all regulation of protein synthesis by eIF2α phosphorylation and markedly sensitizes cells to a variety of stresses, homozygous mutant Eif2as<sup>S51A</sup> embryos survive gestation and are superficially indistinguishable at birth from wild type (20). At embryonic day 18.5, Pprr15b<sup>−/−</sup>; Eif2as<sup>S51A/S51A</sup> progeny of the transheterozygous cross were significantly larger (P = 7.9 × 10<sup>−8</sup>, one-sided t test) than Pprr15b<sup>−/−</sup>; Eif2as<sup>S51A/S51A</sup> progeny of the heterozygous cross were significantly larger (P = 7.9 × 10<sup>−8</sup>, one-sided t test) than Pprr15b<sup>−/−</sup>; Eif2as<sup>S51A/S51A</sup> progeny of the transheterozygous cross were significantly larger (P = 7.9 × 10<sup>−8</sup>, one-sided t test) than Pprr15b<sup>−/−</sup>; Eif2as<sup>S51A/S51A</sup> progeny of the transheterozygous cross were significantly larger (P = 7.9 × 10<sup>−8</sup>, one-sided t test) than Pprr15b<sup>−/−</sup>; Eif2as<sup>S51A/S51A</sup> progeny of the transheterozygous cross were significantly larger (P = 7.9 × 10<sup>−8</sup>, one-sided t test) than Pprr15b<sup>−/−</sup>; Eif2as<sup>S51A/S51A</sup> progeny of the transheterozygous cross were significantly larger (P = 7.9 × 10<sup>−8</sup>, one-sided t test) than Pprr15b<sup>−/−</sup>; Eif2as<sup>S51A/S51A</sup> progeny of the transheterozygous cross were significantly larger (P = 7.9 × 10<sup>−8</sup>, one-sided t test). The latter were noted to have a trend...
tional allele of a PPP1R15 gene displayed phenotypes similar to the Ppp1r15a all PPP1R15 function (Fig. 5).

the dephosphorylation accounts for the conspicuous growth defect of the Ppp1r15b−/− embryo and supports a role for regulated levels of eIF2α in erythropoiesis. However, reports have linked the GADD34 (the better-studied member of the 2-membered family) to other cellular functions (e.g., signaling by the TGFβ and TSC/TOR pathways) (21–23). Therefore, we wished to exploit the conspicuous embryonic phenotype of the mutation to gain further insight into the relative importance of defective eIF2α dephosphorylation versus other proposed functions of PPP1R15 family members.

Crosses of Pppr15a+Δ/Δ; Pppr15b−/− transheterozygotes yielded no compound homozygous mutant embryos at e13.5 (n = 97, P = 5.78 × 10−20), consistent with early lethality of mice lacking all PPP1R15 function (Fig. 5A). Mutants carrying a single functional allele of a PPP1R15 gene displayed phenotypes similar to the single homozygous mutants such that Pppr15a+Δ/Δ; Pppr15b−/− embryos perish in the perinatal period and Pppr15aΔ/Δ; Pppr15b−/− pups survive to adulthood and are fertile. The Pppr15aΔ/Δ; Pppr15b−/− mice were intercrossed to examine the timing of embryonic lethality. Preimplantation embryos were isolated from uteri on e3.5 and cultured for 2.5 days in ES cell medium.

PCR genotyping revealed that all 25 embryos that hatched were positive for the wild-type Pppr15b allele (P = 8.9 × 10−10), indicating that embryos lacking all PPP1R15 function failed to form a blastocyst cavity, grow, or hatch from the zona pellucida, and do not develop past the preimplantation period (Fig. 5B).

To estimate the role of defective eIF2α dephosphorylation in this lethal phenotype, we intercrossed Pppr15aΔ/Δ; Pppr15b−/−; Eif2aS51A/+ mice and scored the genotypes in embryos late in gestation (at e17.5). With the Eif2aS51A allele in the background, compound homozygous mutant Pppr15aΔ/Δ; Pppr15b−/− individuals were recovered. Remarkably, all 7 Pppr15aΔ/Δ; Pppr15b−/− embryos identified were of the Eif2aS51A/Δ genotype (P = 6.1 × 10−65), indicating that the lethal phenotype of loss of PPP1R15 function can be rescued by a block in eIF2α phosphorylation (Fig. 6A). Furthermore, the compound mutant Pppr15aΔ/Δ; Pppr15b−/−; Eif2aS51A/+ embryos were indistinguishable in size and appearance from their littersmates carrying at least one wild-type PPP1R15 allele (Fig. 6B), indicating that a mutation that prevents regulated phosphorylation of eIF2α can bypass those functions of PPP1R15 proteins that are critical to mouse embryogenesis.

To confirm the previous observations and determine if the dephosphorylation of eIF2α is an important cell-autonomous function of the PPP1R15 proteins, we procured MEFs from compound Pppr15b−/−; Pppr15aΔ/Δ; Eif2aS51A/+ mutant embryos, control Pppr15b−/+; Pppr15aΔ/Δ; Eif2aS51A/+; and wild-type Pppr15b−/+; Pppr15aΔ/Δ; Eif2a−/+ embryos and transduced each with either a wild-type Eif2a+ or mutant
Discussion

GADD34 (PPP1R15a) and CREP (PPP1R15b), which share the ability to associate with the catalytic phosphatase PPP1 subunit and repress eIF2α phosphorylation when overexpressed, have less than 22% overall identity at the amino acid level. This study proves that despite their relatively weak homology, both proteins have important overlapping functions in mouse embryogenesis, as loss of CREP renders GADD34 essential and vice versa. Furthermore, the critical function of GADD34 and CREP can be rescued by a mutation (Eif2αS51A) that prevents eIF2α phosphorylation. Together, these observations prove that eIF2α(p) dephosphorylation is the common critical function of the PPP1R15 family of phosphatase regulatory subunits in mouse embryogenesis.

The arrest of embryos lacking both PPP1R15 genes at the preimplantation stage is consistent with a role for the encoded proteins in eIF2α dephosphorylation, as failure of this process would plausibly frustrate the marked increase in protein synthesis that normally occurs at the early 2- to 8-cell stage (24). This phase of embryonic development entails changes in expression of genes affecting translation initiation, which include activation of genes that normally occur at the early 2- to 8-cell stage (24). This phase of embryonic development entails changes in expression of genes affecting translation initiation, which include activation of genes that normally occur at the early 2- to 8-cell stage (24). The latter cell-autonomous phenotype nicely explains the deleterious, dominant affect of expression of a wild-type (phosphorylatable) eIF2α allele in cells lacking both PPP1R15 genes.

Both inadequate signaling by eIF2α(p)—exemplified by the deficiency in ATF4, a translationally induced target of eIF2α phosphorylation (28), or by homozygosity for eIF2αS51A allele (Fig. 4C)—and its converse, excessive signaling by eIF2α(p) of the...
Together, these findings call attention to the fact that eIF2α (from intercrosses of Ppp1r15a−/− and Ppp1r15b−/−) embryos by deletion of HRI, the predominant eIF2α kinase of the adult liver parenchyma (data not shown), suggesting that the erythropoietic defect, too, might be imposed by more than one kinase.

Previous studies implicated GADD34 (the better-studied member of the PPP1R15 family) in TGFβ signaling and in regulating the activity of the tuberous sclerosis complex (TSC) (21, 23). However, the near-complete rescue of the combined GADD34 and CREP deficiency by the Eif2aS51A mutation argues against a prominent role for the PPP1R15 proteins in regulating the activity of these pathways, as the severe perturbation of mammalian development associated with deregulated TGFβ (32) or TSC activity (33–35) would not be rescued by the Eif2aS51A mutation. Furthermore, we detected no differences in the activity of S6 kinase, a downstream target of the TSC complex, between wild-type cells and those lacking both PPP1R15 genes (Fig. S1).

Though a subtle role for PPP1R15 proteins in regulating processes other than levels of eIF2α (αP) could have been missed in a study reliant on detecting perturbation to mouse embryonic development, the evidence at hand does not support the previously published hints for pleiotropy in PPP1R15 protein action. In this regard, the proteins involved in eIF2α (αP) dephosphorylation are similar to the known eIF2α kinases in their commitment to a simple linear pathway with a single integrating node: the phosphorylation of eIF2α on serine 51.

Though this study highlights the untoward consequences of a complete loss of the ability to dephosphorylate eIF2α (αP), other experiments have indicated that more-modest increases in the levels of phosphorylated eIF2α (αP) and in the activity of the downstream gene expression program may promote resistance to various stressful conditions (36, 37). Indeed, both genetic and pharmacological interventions that modestly reduce the activity of PPP1R15 family members protect cells against stress (12, 38). This study indicates that the salubrious features of partial inhibition of GADD34 and CREP are indeed mediated by their effects on levels of eIF2α (αP) and not some other function. Furthermore, the evidence that the PPP1R15 proteins contribute mainly to a linear signaling pathway that hinges on levels of eIF2α (αP) suggests that specific inhibitors of this class of phosphatase regulatory subunits may have narrow and predictable consequences on animals’ physiology that may be cautiously exploited to useful ends.

Materials and Methods

Gene Targeting and Mouse Breeding. The murine Ppp1r15b (CREP) gene was targeted in ES cells with a positive-negative selection vector based on pNT5 in which a weak PGK:neo cassette on the antisense strand replaced a 2,013-bp genomic region encompassing the proximal promoter and the part of exon 1 encoding amino acids 1–417 (which include all of the in-frame methionines of CREP). Once homologous recombination was confirmed, a short-range PCR strategy was used to detect a wild-type 322-bp fragment, derived by PCR with Neo.255R (5′ GGAAACATACCTCTCGGATGAC 3′) and CREP.24AS (5′ CAGAGCTGGCTTCCAAGTC 3′) and a mutant 235-bp fragment, derived by PCR with Neo.255R (5′ GCCCTACCGGGATGTCGAACTGC 3′) and a mutant 235-bp fragment, derived by PCR with Neo.255R (5′ GCCCTACCGGGATGTCGAACTGC 3′) and CREP.24AS. Germine transmission was obtained following injection of the heterozygous Ppp1r15b−/+ targeted ES cell (G7, Ppp1r15a knockout (GADD34−/−), HRI−/−, and eIF2αS51A mice have been described previously (13, 20, 39). All experiments in mice were approved by the New York University Institutional Animal Care and Use Committee.

Analysis of Embryonic Phenotypes. Postnatal day 18.5 embryos were rinsed in PBS, dabbed dry, and weighed. Blood was isolated from the carotid artery using heparinized capillary tubes diluted 1:10 and analyzed on a Cell-Dyn 4000 (Abbott Labs). Blood smears were stained with the Wright Stain Kit from Fisher Scientific. Manual red blood cell counts were done on heparinized capillary tubes diluted 1:10 and analyzed on a Cell-Dyn 4000. Hemoglobin concentration was measured on a HemoCue analyzer (HemoCue). Manual red blood cell counts were done on heparinized samples diluted 1:100 using a hemocytometer. Histological analysis was performed on paraffin-embedded tissues using standard methods.

Embryos were isolated from intercrossed Ppp1r15alox/+, Ppp1r15b−/− mice by flushing the uteri of superovulated females 3.5 days postcoitus (40). The isolated embryos were scored as having normal multicellular blastocyst morphology (i.e., a blastocoe) (25), abnormal multicellular morphology (no blastocoe) (14), erythroblasts, or by deletion of PERK, the predominant eIF2α kinase of the adult liver parenchyma (data not shown), suggesting that the erythropoietic defect, too, might be imposed by more than one kinase.
or the granular/pebbled appearance of unfertilized eggs (29). No genotypes were obtainable from the last category, and they were discarded. The 39 multicellular embryos were cultured in ES cell medium for an additional 2.5 days and scored for hatching from the zona pellucida. All 25 blastocysts (of normal morphology at isolation) hatched, whereas none of the 14 embryos of abnormal morphology hatched. PCR genotyping revealed that all 25 embryos that hatched were positive for isolation) hatched, whereas none of the 14 embryos of abnormal morphology hatched.

Cell Culture and Analysis of Cellular Phenotypes. Cell lines from Ppp1r15b−/−, Ppp1r15b−/−, Ppp1r15b−/−, eIF2αS51A, eIF2αS51A, and combined genotypes were obtained by serial passage of SV40 T-antigen transfected MEFs. The cells were cultured in DMEM supplemented with 10% FetalClone II serum (HyClone), 1× MEM nonessential amino acids, 55 μM β-mercaptoethanol, penicillin-streptomycin, and glutamine. For immunoblot analysis, cytoplasmic proteins were isolated from cell lines using detergent lysis as previously described (41).

Puromycin-resistant (Puro) retroviruses encoding wild-type and SS1A mutant human eIF2α were constructed in the pBABEPuro vector and packaged in 293T cells as phosphate 1 and inhibitor 1. Mol Cell Biol 21:6841–6850.

Liver extracts were made by homogenization of fresh tissue in a Teflon-glass homogenizer in 4 volumes of extract buffer (20 mM Tris-HCl [pH 7.5], 300 mM KCl, 10 mM MgCl2, 1 mM DTT, 1 μg/ml peptatin and aprotinin, and 1 mM PMSF) followed by clearing at 14,000 rpm. CReP from 2 mg of total protein was immunopurified using 2 μl of antiserum and protein A Sepharose. Washed immunoprecipitates or 50 μg of total proteins were separated by PAGE and transferred to nitrocellulose and probed with previously described (CReP, GADD34, total eIF2α) and/or commercially available eIF2α (BioSource/Invtigron) antiseras.

Statistical Analysis. All numerical data are displayed as mean ± SD or graphed as mean ± SD. Differences in the mean values between groups (Figs. 2 A and 3A) were determined by paired two-tailed Student’s t test. Evaluation of the significance of the rescue of the CReP phenotype by the eIF2αS51A/SS1A genotype was evaluated by paired one-tailed Student’s t test (Fig. 4 B and C). Calculated probabilities (P) of allele distribution among progeny (Figs. 2 A, 5 A, and 6 A) assume Mendelian segregation of unlinked loci.

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