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A critical role for PKR complexes with TRBP in immunometabolic regulation and eIF2α phosphorylation in obesity

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SUMMARY

Aberrant stress and inflammatory responses are key factors in the pathogenesis of obesity and metabolic dysfunction, and the double-stranded RNA-dependent kinase (PKR) has been proposed to play an important role in integrating these pathways. Here, we report the formation of a complex between PKR and TAR RNA-binding protein (TRBP) during metabolic and obesity-induced stress, which is critical for the regulation of eukaryotic translation initiation factor 2 alpha (eIF2α) phosphorylation and c-Jun N-terminal kinase (JNK) activation. We show that TRBP phosphorylation is induced in the setting of metabolic stress, leading to PKR activation. Suppression of hepatic TRBP reduced inflammation, JNK activity, and eIF2α phosphorylation and improved systemic insulin resistance and glucose metabolism, while TRBP overexpression exacerbated the impairment in glucose homeostasis in obese mice. These data indicate that the

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AUTHOR CONTRIBUTIONS

T.N. developed the hypothesis, designed and performed experiments, and analyzed data. R.C.K. designed and performed experiments, and analyzed data. C.Z., T.K., C.L.Y., B.B., and Y.N. performed experiments and analyzed data. S.P.G. supervised proteomics experiments and analyzed data. G.S.H. developed the hypothesis, designed, and supervised experiments. T.N., R.C.K., and G.S.H. wrote the manuscript. All authors discussed the results and commented on the manuscript.

association between PKR and TRBP integrates metabolism with translational control and inflammatory signaling, and plays important roles in metabolic homeostasis and disease.

INTRODUCTION

It is established that obesity coexists with a state of metabolic inflammation, also called metaflammation (Hotamisligil, 2006), in which excess nutrients trigger metaflammation and stress responses, including recruitment of immune cells into metabolic tissues, activation of IκB kinase (IKK) and c-Jun N-terminal kinase (JNK) pathways, and elevated production of an array of immune mediators (Donath and Shoelson, 2011; Gregor and Hotamisligil, 2011; Lumeng and Saltiel, 2011; Olefsky and Glass, 2010; Pedersen and Febbraio, 2010; Sabio and Davis, 2010). Inflammation is a central adaptive and repair mechanism essential for tissue homeostasis and survival (Wernstedt Asterholm et al., 2014). However, chronic and systemic activation of these inflammatory and stress signaling pathways underlies metabolic pathologies such as insulin resistance, type 2 diabetes, and atherosclerosis (Glass and Olefsky, 2012; Jin et al., 2013). A critical mechanism for metabolic dysfunction in obesity also involves endoplasmic reticulum (ER) stress and dysfunction through direct actions on both insulin signaling and pathways controlling glucose, lipid, and protein metabolism and in crosstalk with inflammatory signaling pathways (Vallerie and Hotamisligil, 2010). However, it remains unclear how these diverse stress pathways are activated, integrated, and coupled to the regulation of protein translation and metabolic homeostasis under conditions of metabolic challenge.

The unfolded protein response (UPR) is engaged during ER stress as an adaptive response and an attempt to restore homeostasis by suppression of global protein synthesis, stimulation of protein degradation, and synthesis of components of the folding machinery (Hetz, 2012; Malhi and Kaufman, 2011; Ron and Walter, 2007). However, chronic ER stress and the resulting dysfunctional UPR, in a manner similar to a prolonged inflammatory response, also contribute to pathology, including metabolic deterioration and disease (Fonseca et al., 2011; Malhi and Kaufman, 2011; Vallerie and Hotamisligil, 2010). One common strategy of cells to control translation during stress is the induction of eIF2α phosphorylation mediated by at least four kinases: double-stranded RNA dependent kinase (PKR), PKR-like endoplasmic reticulum kinase (PERK), heme-regulated inhibitor kinase (HRI) and general control nonrepressed 2 (GCN2) (Holcik and Sonenberg, 2005). This pathway is ubiquitously engaged in metabolic tissues such as liver in both obese humans and experimental models, resulting in increased eIF2α phosphorylation and downregulation of global protein synthesis accompanied by reduced polyribosome activity (Fu et al., 2011; Gregor et al., 2009; Karinch et al., 2008; Sharma et al., 2008). Similarly, JNK activation (Bluher et al., 2009; Fernandez-Veledo et al., 2009; Hirosumi et al., 2002; Sourris et al., 2009; Vallerie and Hotamisligil, 2010) and metaflammation (Gregor and Hotamisligil, 2011; Odegaard and Chawla, 2013) is essentially universal to experimental and human obesity and critical for metabolic deterioration. Despite the importance of these signaling events in metabolic homeostasis, the molecular mechanisms by which these stress signaling pathways converge in obesity remain unknown.

We have recently shown that PKR, a pathogen-sensing protein, is activated by excess nutrients and that its aberrant activity plays a key role in metabolic abnormalities under stress (Nakamura et al., 2014; Nakamura et al., 2010). Similar observations were made in an independently-derived strain of PKR deficient mice (Carvalho-Filho et al., 2012). Importantly, PKR is involved in eIF2α phosphorylation in high-fat diet fed mice and is a critical component of inflammasome activation (Lu et al., 2012; Nakamura et al., 2010), raising the possibility that PKR may function as a common node that responds to both pathogen- and nutrient-induced inflammatory and stress signals in order to suppress insulin action and regulate protein translation under metabolic stress conditions. Exposure of cells and tissues to lipotoxicity also leads to PKR activation and PKR-dependent JNK activation, raising the possibility that PKR serves as a sensor for metabolic stress signals. Indeed, PKR activity and JNK phosphorylation are elevated in multiple tissues in obese humans (Boden et al., 2008; Carvalho et al., 2013; Gregor et al., 2009; Sourris et al., 2009), strongly suggesting that this role of PKR is conserved and relevant to human disease. However, the mechanism that underlies the assembly of an active PKR complex, and how this complex is coupled to metaflammation, translational suppression, and metabolic dysregulation in obesity remain unknown. Here, we show that metabolic stress triggers the formation of a PKR complex that features RNA–induced silencing complex (RISC)-loading complex (RLC) proteins and TRBP, resulting in PKR activation and metabolically-induced eIF2α phosphorylation, JNK activation, and related inflammatory responses.

RESULTS

Identification of the components of the PKR protein complex in liver

We and others previously reported that PKR is activated in mouse and human tissues in obesity (Carvalho et al., 2013; Carvalho-Filho et al., 2012; Nakamura et al., 2010), however, the molecular mechanism that underlies this activation remains to be elucidated. We hypothesized that a unique PKR-containing protein complex forms in the obese condition and that the complex component(s) may impact PKR activity, inflammatory responses, and protein translation in obesity. To address this question, we performed biochemical purification and identification of the PKR protein complexes from the liver tissues of lean and obese (*ob/ob*) mice. We utilized an adenovirus-mediated gene transfer method to deliver HA-tagged PKR into the liver and purified PKR-containing complexes from these compartments using an agarose-conjugated anti-HA monoclonal antibody (Figure 1A). Then, we determined the PKR-associated proteins by exhaustive rounds of ‘shotgun’ liquid chromatography and tandem mass spectrometry (LC-MS/MS) for protein identification and quantification (Figures 1B and 1C). These experiments were performed as three independent biological replicates, and statistical analysis of fold change values (*t*-tests) was used to distinguish PKR-interacting proteins from background signals with high confidence (red and grey dots, respectively, Figures 1B, 1C, Table S1 and S2).

Among the high confidence PKR interaction molecules, we detected known partners of PKR such as eIF2α also referred to ase IF2S1, validating our experimental system. PKR has been shown to be a pathogen sensor through its recognition of pathogen-derived double-stranded RNA (dsRNA) (Robertson and Mathews, 1996). Given that PKR interacts with various

proteins involved in RNA processing and biology in the liver, we hypothesized that PKR might be closely integrated with endogenous RNA processing components, particularly under metabolic stress conditions. To test this hypothesis, we analyzed the list of high confidence PKR interactors in lean and obese liver tissue through enriched gene ontology (GO) in biological processes and molecular function categories (Figure 1D). Enriched GO terms common to lean and obese samples included RNA processing, RNA binding, and protein translation. Interestingly, the functional clades enriched only in the lean samples were found to be associated with metabolism and cellular maintenance while many of the GO terms enriched only in the obese samples were related to RNA splicing and mRNA processing (Figure 1D). These results support the assembly of distinct protein complexes in lean and obese liver tissue and suggest the possibility that PKR complexes detected in liver tissue of obese animals may have specialized metabolic functions.

In an additional analytical approach, we also considered all PKR-exclusive proteins, *i.e.* proteins categorized with a $\log_2$(PKR/GFP) ratio over 35, with the postulate that some of the PKR-exclusive proteins may also be of unique functional interest. This analysis yielded additional proteins involved in the regulation of RNA splicing and mRNA metabolism in the samples from obese animals including Dicer, a known component of the RLC (Czech and Hannon, 2011; Kim et al., 2009), which was observed as a PKR-interacting protein preferentially in obesity (Table S1 and S2). To confirm the interaction, we performed co-immunoprecipitation experiments and found that the PKR-Dicer interaction was enhanced in the liver in the context of obesity (Figure 1E). Mammalian Dicer also interacts with two closely related proteins, TAR RNA-binding protein (TRBP) and PACT (Chendrimada et al., 2005; Kim et al., 2009; Lee et al., 2006), which in turn regulate Dicer stability and contribute to RLC formation (Kok et al., 2007). In addition, both PACT and TRBP have been shown to interact with PKR, although their significance in inflammatory responses and metabolic regulation is not known and no studies have been conducted to explore their function in obesity (Clerzius et al., 2011). We found that PKR interacted with TRBP in liver upon exogenous PKR expression, and that this interaction was enhanced in obese animals (Figure 1E). To evaluate the formation of endogenous complex, we pulled-down TRBP from liver lysate and performed western blot analyses with anti-PKR and Dicer antibodies. In this setting, TRBP interaction with PKR was similarly enhanced in the setting of obesity (Figure S1A).

PKR activation promotes formation of a functional complex

Given that the interactions between PKR, Dicer, and TRBP are enhanced in obesity, a condition in which PKR is also highly activated, we hypothesized that these interactions may be required for or dependent on PKR activity. To investigate these possibilities, we next examined whether treatment with polyinosinic-polycytidylic acid (PolyI:C), which mimics virus-derived dsRNA and is one of the strongest known PKR activators (Garcia et al., 2006), leads to enhanced association between PKR and RLC components. As shown in Figure 2A, PolyI:C treatment resulted in enhanced interaction between PKR, Dicer, and TRBP in mouse embryonic fibroblasts (MEFs). Similarly, Dicer interaction with PKR was enhanced when PKR was activated by PolyI:C in hepatocytes (Figure S1B).

To further investigate the mechanisms and consequences of the PKR-TRBP and Dicer interactions, we used PKR-deficient MEFs reconstituted with flag-tagged PKR. The exogenous PKR expression level in *Pkr*$^{-/-}$ MEFs was comparable to endogenous PKR in*Pkr*$^{+/+}$ MEFs and was unaffected by PolyI:C treatment (Figure 2B). PolyI:C treatment enhanced the interaction between PKR and endogenous Dicer and TRBP, and formation of this ternary complex was further strengthened in the presence of exogenous TRBP protein (Figure 2C). Since PKR's N-terminal region, which contains two dsRNA-binding domains, mediates intermolecular interactions (Cosentino et al., 1995), we asked whether RNA-binding activity of PKR was critical for the interaction between PKR and RLC. We reconstituted *Pkr*$^{-/-}$ MEFs with WT and RNA-binding defective PKR (RD), which has point mutations on lysines 64 and 154 in the RNA-binding motifs. A kinase dead (KD) mutant, which has a point mutation on its lysine 296 residue in the kinase domain, was used as an additional control. The RD mutant lost the ability to interact with TRBP and Dicer, both at baseline and following PolyI:C stimulation (Figure 2D), suggesting that PKR RNA binding capacity may be required for formation of a ternary complex with Dicer and TRBP. Although the interaction between KD-PKR, Dicer and TRBP was weaker than that of WT-PKR, this mutant still retained some residual ability to interact with Dicer and TRBP in the presence of PolyI:C (Figure 2D). Importantly, although the RD mutant has an intrinsically intact kinase activity *in vitro* (data not shown), it did not exhibit PolyI:C-induced PKR activation (Figure 2E), suggesting that formation of a complex with TRBP and Dicer may be important for induction of PKR activity and its downstream functions in this context, including translational and metabolic regulation through eIF2α phosphorylation.

To determine the potential impact of TRBP on eIF2α phosphorylation, we performed immunoprecipitation experiments to pull down TRBP from MEFs expressing HA-tagged TRBP in the presence or absence of PKR. As shown in Figure 2F, TRBP interacted with PKR only when the cells were stimulated by PolyI:C. Importantly, stimulated interaction of TRBP with PKR did not affect the intensity of interaction between TRBP and Dicer. In this setting, as above, PolyI:C stimulation of complex formation resulted in robust eIF2α phosphorylation (Figure 2G). These data implicate the potential involvement of TRBP in translational regulatory pathways through PKR-mediated induction of eIF2α phosphorylation.

TRBP is involved in palmitate-induced PKR activation

We previously reported that PKR is activated by palmitate exposure and that the RNA-binding activity of PKR is required for palmitate-induced PKR activation (Nakamura et al., 2010). Thus, we asked whether lipid exposure also triggers the interaction between PKR and the RLC to link this potential mechanism to metabolic context. As shown in Figure 3A, palmitate treatment resulted in increased interaction between PKR and RLC components, TRBP and Dicer. These interactions also required PKR RNA-binding activity, as they were lost in cells expressing the RD-PKR mutant (Figure 3B). Thus the interaction between PKR and RLC may also be responsive to nutrient-induced metabolic stress and PKR activation levels.

We also examined whether other fatty acids could affect PKR activity. To address this question, we treated *Pkr*$^{-/-}$ MEFs expressing WT-PKR with four different saturated fatty acids (laurate, myristate, palmitate, and stearate) and an unsaturated fatty acid (oleate), followed by PKR kinase assay. As shown in Figure S2A, PKR activity was induced by relatively longer fatty acids palmitate and stearate. Importantly, although the number of carbon atoms is the same between stearate and oleate, oleate was a less effective PKR activator than stearate. As longer saturated fatty acids are intrinsically prone to induce metabolic inflammation compared to unsaturated fatty acids, these data indicate that PKR activity may be a reflection of the level of metabolic stress within cells. Indeed, we observed that eicosapentaenoic acid (EPA) and palmitoleate, unsaturated fatty acids known to lower inflammation and improve metabolic homeostasis (Cao et al., 2008; Li et al., 2013; Spann et al., 2012), did not activate PKR and diminished palmitate-induced PKR activation (Figures S2B and S2C). Consistent with these observations, palmitate-enhanced interaction between PKR and TRBP was also blocked in the presence of these unsaturated fatty acids (Figure 3C).

Given that PKR interacts with the RLC in response to metabolic stress, we next examined whether RLC components are critical for palmitate-induced PKR activation. As shown in Figure 3D, palmitate-induced PKR activation was intact in Dicer-deficient MEFs, although the magnitude was moderate. However, palmitate treatment failed to activate PKR in the absence of TRBP (Figure 3E). This is an unexpected result as TRBP can act as an inhibitor of PKR (Clerzius et al., 2011; Park et al., 1994). Numerous experiments have indicated that PKR is also required for JNK activation *in vitro* and *in vivo* (Carvalho-Filho et al., 2012; Goh et al., 2000; Nakamura et al., 2010; Takada et al., 2007). Consistent with a role for TRBP in PKR activation, palmitate-induced JNK activation, which is also moderate in these cells, as well as eIF2α phosphorylation were diminished in TRBP-deficient fibroblasts (Figure 3F). Taken together, these data suggest that although TRBP has been previously considered as an inhibitor of PKR in certain contexts (Clerzius et al., 2011; Park et al., 1994), its function appears to be more diversified and is regulated by metabolic stress. Furthermore, these observations indicate a requirement for PKR-TRBP complex formation in the activation of PKR in response to nutrient signals and key downstream events such as JNK activation and eIF2α phosphorylation.

TRBP phosphorylation links metabolic stress to PKR activation

In the context of interferon-induced PKR activation, it has been proposed that TRBP inhibits PKR function both by dsRNA sequestration and by direct protein-protein interaction (Benkirane et al., 1997; Park et al., 1994). In contrast, we describe above that metabolic stress induces interaction between TRBP and PKR, leading to PKR activation and suggesting a dual regulatory role for TRBP as has been reported for some other components of the RLC (Clerzius et al., 2013; Diederichs and Haber, 2007). In an attempt to gain mechanistic insights into TRBP function, we asked whether a post-translational modification of TRBP might alter its interaction with PKR and differentially regulate its activity. Recently, Paroo et al. reported that four serine residues of TRBP are phosphorylated by the mitogen-activated protein kinase ERK (Figure 4A)(Paroo et al., 2009). In agreement with this report, we found that expression of constitutively active JNK (MKK7-JNK1) also

induced phosphorylation of TRBP, and determined that this modification occurred predominantly on two of the previously identified residues, S142 and S152 (Figure 4B). Remarkably, phospho-mimetic (serine-to-aspartate [SD]) variants of TRBP robustly interacted with PKR in the absence of additional stimuli (Figure 4C). Conversely, the interaction of the 4SD mutant with the RD-PKR mutant was much weaker than that with WT-PKR (Figure 4C), suggesting that PKR RNA binding activity may be critical for the interaction between PKR and TRBP.

Next, we examined the effect of TRBP phosphorylation on PKR kinase activity. In reconstituted *Pkr*-deficient MEFs, expression of the 4SA TRBP mutant moderately suppressed PKR activity, while the 4SD mutant TRBP lacked this activity (Fig. 4D). These data suggest that TRBP phosphorylation in this context may be important not only for interaction with PKR but also for PKR activity. Next, we generated a phospho-specific TRBP antibody that specifically detects TRBP's phospho-serine 152 residue, and confirmed specificity by mutational analysis (Figure S3A). Using this antibody, we determined TRBP phosphorylation status upon induction of metabolic stress. As shown in Figure 4E, the level of TRBP serine 152 phosphorylation was increased by palmitate exposure, and TRBP phosphorylation was also observed in MEFs treated with PolyI:C (Figure S3B). We then asked whether TRBP phosphorylation was present in the setting of obesity, a context in which PKR is activated (Nakamura et al., 2010). Indeed, we observed TRBP serine 152 phosphorylation in the liver of obese mice (Figure 4F). These data suggest that TRBP phosphorylation occurs in response to metabolic stress and is associated with PKR activity.

Metabolic functions of the PKR-TRBP axis in obese liver

Independent studies previously showed that PKR-deficient mice fed a high-fat diet demonstrate reduced JNK activation and eIF2α phosphorylation and are protected against obesity and insulin resistance (Carvalho-Filho et al., 2012; Nakamura et al., 2010). Direct regulation of insulin sensitivity and JNK activation by PKR was also shown using a short-term lipid infusion model (Nakamura et al., 2010). In an attempt to generate a new model to test PKR's effects on JNK activation, eIF2α phosphorylation, and glucose metabolism, we intercrossed PKR-deficient mice with the *ob/ob* model, which displays severe obesity with insulin resistance and hyperglycemia. Importantly, in the C57BL/6J-*ob/ob* genetic background, $Pkr^{-/-}$ and $Pkr^{+/+}$ mice exhibited similar body weights (Figure 5A) with only a minor difference in adiposity (data not shown). However, in glucose tolerance tests (GTT), $Pkr^{-/-}$*ob/ob* mice showed significantly improved glucose clearance after a glucose challenge compared to controls at 7 weeks of age (Figure 5B). Similarly, during insulin tolerance tests (ITT), 9-week-old $Pkr^{-/-}$*ob/ob* mice were more insulin sensitive than $Pkr^{+/+}$*ob/ob* (Figure 5C). Consistent with these observations, fasting blood glucose levels of *ob/ob* mice lacking PKR were significantly lower than those of $Pkr^{+/+}$*ob/ob* controls, while serum insulin levels were comparable between the groups (Figures 5D and 5E). In addition, levels of circulating triglyceride, cholesterol, and very-low-density lipoprotein (VLDL) in $Pkr^{-/-}$*ob/ob* mice were significantly reduced compared to those in $Pkr^{+/+}$*ob/ob* controls (Figure S4A, S4B, and S4C).

We next assessed the contribution of PKR to obesity-induced JNK activity and eIF2α phosphorylation in this setting. As shown in Figure 5F, JNK activation was highly induced in liver of *Pkr*$^{+/+}$*ob/ob* compared to that of lean mice, as expected (Hirosumi et al., 2002; Sreejayan et al., 2008). In contrast, activation levels of JNK in liver of *Pkr*$^{-/-}$ *ob/ob* mice were significantly lower than those detected in *Pkr*$^{+/+}$*ob/ob* controls. Similarly, the increased eIF2α phosphorylation seen in the liver of *ob/ob* mice in numerous independent studies (Ozcan et al., 2004; Sreejayan et al., 2008; Tsutsumi et al., 2011; Yang et al., 2010) was significantly reduced to levels close to lean controls upon loss of PKR (Figure 5G). Of note, activation of PERK, the ER resident eIF2α kinase, remained elevated in liver of *Pkr*$^{-/-}$*ob/ob* mice (Figure S4D). These data indicate that PKR is the dominant mediator of hepatic JNK activation and eIF2α phosphorylation in obesity. In addition, consistent with previous observations (Nakamura et al., 2014; Nakamura et al., 2010), we also confirmed that obesity-induced IRS1 serine phosphorylation levels were decreased in the absence of PKR in this setting (Figure 5H).

We next utilized the *ob/ob* model to investigate the role of TRBP in obesity using adenovirus-mediated gene transfer to exogenously express TRBP in liver and examine the effects on glucose metabolism, JNK activation, and eIF2α phosphorylation. The *ob/ob* mice overexpressing TRBP in liver exhibited similar weight gain and liver weight compared to a control group (Figure 6A and 6B). However, enhanced-expression of TRBP in liver of *ob/ob* mice resulted in a small but significant increase in fasting glucose levels (Figure 6C) and a further deterioration of glucose tolerance as determined by GTT compared to control mice (Figure 6D). Importantly, we observed that TRBP overexpression in liver led to further induction of JNK activation and a further increase in hepatic eIF2α phosphorylation compared to the controls (Figure 6E). Next, we asked whether the TRBP-induced JNK activation and eIF2α phosphorylation were dependent on PKR function using adenoviral expression systems. As shown in Figure 6F, PKR-deficient *ob/ob* mice showed decreased levels of JNK activation and eIF2α phosphorylation compared to a PKR-intact control group virally expressing a control gene (*Gus*). When TRBP was exogenously expressed in liver of PKR-deficient *ob/ob* mice, the levels of phosphorylated JNK were partially recovered, however, eIF2α phosphorylation levels remained low. These data suggest that TRBP requires PKR for induction of eIF2α phosphorylation but may mediate JNK activation in both PKR dependent and independent manners.

Physiological roles of TRBP in obese liver

Taken together, our observations demonstrate that the complex formation between TRBP and PKR may promote PKR's adverse metabolic functions. To further explore the physiological relevance of the PKR-TRBP axis in obesity, we designed and established a short hairpin RNA (shRNA)-mediated TRBP knockdown system in which TRBP expression levels in cells were reduced approximately 85% (data not shown). Palmitate-induced PKR activation was diminished by shRNA-mediated suppression of TRBP (Figure 7A), consistent with our observations in TRBP-deficient fibroblasts (Figure 3E). To investigate the consequence of reduced TRBP in obesity, we used an adenoviral system to express shRNAs against TRBP in the liver of *ob/ob* mice, which reduced TRBP protein expression up to 90% (Figure 7B). In this setting, suppression of hepatic TRBP led to reduced steatosis

accompanied by decreased liver weight (Figure 7C), although body weight remained similar to that of control mice (Figure 7D). Suppression of hepatic TRBP also resulted in a significant reduction in fasting blood glucose levels and improved glucose tolerance indicating improved glucose metabolism (Figure 7E, 7F). This improvement was observed with two independent shRNA constructs, which achieved similar knockdown efficiency. Consistent with these metabolic outcomes, TRBP suppression resulted in significant downregulation of genes involved in hepatic glucose production (G6Pase and PEPCK) as well as lipogenesis (FAS and SCD1) (Figure 7G). Importantly, expression levels of inflammatory cytokines (TNFα and IL-1β) and ER stress markers (Grp78 and CHOP) were also significantly reduced following the suppression of hepatic TRBP, while expression levels of β-oxidation-related genes (CPT1 and PGC1α) remained unaffected (Figure 7G).

As inflammatory responses, which were assessed by PKR activity and inflammatory cytokine expression, were downregulated upon suppression of hepatic TRBP expression in *ob/ob* mice, we assessed the effects of hepatic TRBP knockdown on JNK activation. Consistent with results obtained from PKR-deficient *ob/ob* mice, suppression of hepatic TRBP significantly reduced JNK activity assessed by phosphorylation levels of JNK (Figure 7H). In addition, reduced expression of hepatic TRBP resulted in a significant reduction of eIF2α phosphorylation in liver of *ob/ob* mice (Figure 7H), similar to what was observed in the PKR-deficient *ob/ob* mice (Figure 5F and 5G). We also examined the impact of these alterations on IRS-1 phosphorylation and insulin action in liver. TRBP suppression in obese mice resulted in a significant reduction of IRS1 serine phosphorylation levels (Figure 7I) and restoration of insulin-induced AKT phosphorylation in liver tissue (Figure 7J).

DISCUSSION

Until recently, the main role of PKR has been believed to be the suppression of global protein translation of host cells through induction of eIF2α phosphorylation upon pathogen infection (Garcia et al., 2006). It is now clear that PKR is also a critical regulator of metabolic homeostasis, placing this enzyme at a critical juncture in immunometabolic pathways (Carvalho-Filho et al., 2012; Nakamura et al., 2010). Here, we demonstrate that PKR can interact with RLC components to integrate metabolic signals with eIF2α phosphorylation and inflammatory responses. This is not only consistent with the need of host cells to have more efficient and diverse intrinsic strategies to prevent amplification of pathogens but also provides a mechanism by which intrinsic or extrinsic metabolic exposures and stress are linked to immune outputs. As in the case of pathogen exposure, PKR coordinates the eIF2α phosphorylation-mediated suppression of protein translation with the induction of inflammatory responses upon exposure to excess nutrients and energy.

Phosphorylation of eIF2α is considered an important protective event to restore homeostasis upon ER malfunction induced by stresses such as virus infection, accumulation of unfolded or misfolded proteins, lipid overloading, and inflammation (Fu et al., 2011; Holcik and Sonenberg, 2005; Ron and Walter, 2007; Vallerie and Hotamisligil, 2010). This in turn is believed to lead to a reduction of the ER burden and conservation of cellular energy sources needed to sustain vital processes. If the cellular stress levels are beyond a threshold, cells activate apoptotic or maladaptive pathways such as JNK and p38 signaling (Wagner and

Nebreda, 2009; Williams, 2001). Under obesity-induced chronic metabolic stress, these protective and maladaptive mechanisms co-exist, and thus opposing cellular stress responses compromise the molecular networks that affect regulation of systemic metabolism (Hirosumi et al., 2002; Liu and Cao, 2009). Therefore, disordered signaling events may occur and be amplified in obesity, leading to disruption of metabolic homeostasis. It is of critical importance to understand the key regulatory events maintaining chronic stress signaling, and our observations position PKR as a potential regulator of this metastable state.

Signaling through eIF2α is involved in a variety of molecular networks including regulation of development, organelle function, inflammation, and metabolic homeostasis (Holcik and Sonenberg, 2005; Malhi and Kaufman, 2011; Vallerie and Hotamisligil, 2010). Sustained phosphorylation of eIF2α has been observed as a feature of obesity and insulin resistance in both experimental systems and humans (Gregor et al., 2009; Oyadomari et al., 2008; Ozcan et al., 2004; Ozcan et al., 2006; Sharma et al., 2008; Sreejayan et al., 2008; Tsutsumi et al., 2011; Wang et al., 2009; Zhou et al., 2009) and this has previously been attributed exclusively to ER stress-related activation of PERK function (Birkenfeld et al., 2011; Cummings et al., 2011; Delibegovic et al., 2009; Gregor et al., 2009; Oyadomari et al., 2008; Ozcan et al., 2004; Sharma et al., 2008; Sreejayan et al., 2008). However, our findings demonstrate that PKR dominates this regulation in obese liver. Despite the fact that molecular networks of ER stress responses including PERK remain active in *Pkr*$^{-/-}$*ob/ob* mice, eIF2α phosphorylation levels were significantly diminished without a change in body weight. These findings imply that 1) PERK may be engaged in other molecular regulations beyond eIF2α phosphorylation in obese tissues, 2) obesity causes complex cellular stresses, namely metaflammation, and that PKR integrates these inputs into induction of eIF2α phosphorylation and metabolic outcomes, 3) regulatory factors involved in PKR activation play critical roles as interfaces linking translational deregulation to metabolic dysfunction in obesity. In this regard, identification of Dicer and TRBP as components of the activated PKR complex strongly indicates the possibility of involvement of endogenous double-stranded RNA species, in addition to potential microbe-derived ligands, in PKR activation in obesity and possibly other metabolic stresses, and identifying these ligands will be an interesting opportunity to pursue in future studies. Integrating the TRBP-RLC and PKR-eIF2α axes could be an efficient strategy that allows cells to respond collectively to environmental and metabolic perturbations. Our data raise the possibility that in certain stress conditions, TRBP and RLC components are involved in the regulation of certain RNA species that induce PKR activation leading to eIF2α phosphorylation. In addition, as protein synthesis is tightly linked to cellular energy consumption, this mechanism may serve to modulate energy utilization in response to metabolic stress in obesity.

TRBP is a unique dsRNA-binding protein that has at least two molecular functions; enhancing miRNA maturation processes as a component of the RLC and regulating inflammatory responses by modifying PKR activity. Importantly, these TRBP functions are modulated by phosphorylation induced by MAPK such as ERK and JNK, which are activated in the liver in obesity and involved in pathogenesis of obesity-induced metabolic diseases (Hirosumi et al., 2002; Jiao et al., 2013). In the setting of metaflammation, TRBP leads to PKR activation as well as enhancement of general miRNA maturation processing

(Paroo et al., 2009). Co-existence of positive and negative regulatory capacity in RNA binding molecules such as PACT has been reported before and attributed to the stoichiometry of the intermolecular interactions (Clerzius et al., 2013). Here we provide evidence that this dual function of TRBP, at least in part, is also regulated by JNK-MAPK-mediated phosphorylation. It is worth noting that obesity constitutes a condition of chronic JNK activation, which through TRBP phosphorylation and other mechanisms contribute to sustained PKR activity and potentially other outcomes such as increased general miRNA expression (Kornfeld et al., 2013), which are all observed simultaneously. Our finding that TRBP suppression results in alleviation of obesity-induced hepatic steatosis and glucose intolerance suggests a direct role for TRBP in the pathogenesis of obesity and chronic metabolic complications. Recent research suggests that miRNA biogenesis is critical for protecting cells from ER stress-induced cell death (Cawley et al., 2013), therefore by acting in this pathway as well by mediating JNK activation and eIF2α phosphorylation, TRBP may modulate cellular resistance to metaflammation or its resolution. In chronic metabolic stress conditions, these TRBP-mediated events may contribute to exacerbation of inflammatory responses and deteriorate metabolic homeostasis beyond their adaptive effects.

Notably, PKR activity is highly correlated with JNK activation, and in turn JNK may phosphorylate TRBP, indicating a regulatory loop between these kinases via TRBP, which may be key to their activation in metabolic context. As the assembly of PKR with RLC is highly associated with PKR and JNK activation, and PKR can also participate in inflammatory activity (Carvalho-Filho et al., 2012; Lu et al., 2012; Nakamura et al., 2010), TRBP phosphorylation may be a core component of a signaling node representing the metabolic inflammasome (metaflammasome). Finally, our findings indicate that the inhibition of PKR and TRBP function may result in simultaneous relief from multiple stress responses and may provide new opportunities for clinical applications against chronic stress-related diseases including obesity-induced metabolic disease and cancer.

EXPERIMENTAL PROCEDURES

Detailed procedures can be found in the Supplemental Methods Section.

Mice

Animal care and experimental procedures were performed with approval from animal care committees of Harvard University. Two different types of targeted mutations of PKR have been established and reported in mice, RNA-binding domain defective and kinase-domain defective models (Abraham et al., 1999; Baltzis et al., 2002). In this study, the kinase-domain defective PKR-deficient mice were used, after breeding into the C57Bl/6J genetic background and validation by genome-wide SNP analysis. PKR-deficient mice were then intercrossed with heterozygote animals in the *ob* (leptin) locus in order to generate PKR-deficient *ob/ob* mice. GTTs were performed by intraperitoneal glucose injection (0.5 g/kg) following an overnight food withdrawal. ITTs were performed by intraperitoneal insulin injection (3 IU/kg) following a 6-hour daytime food withdrawal. After 10 weeks, these mice were sacrificed and tissues were collected for further analysis. Total body fat mass was assessed by dual energy X-ray absorptiometry (DEXA; PIXImus). Adenovirus carrying HA-

tagged PKR, GFP, LacZ-shRNA, or TRBP-shRNA was delivered into the *ob/ob* or lean mice intravenously at a titer of 3×10^{11} virus particles/mice. Serum insulin was measured by an ELISA purchased from Alpco (Salem, NH). Serum triglyceride, cholesterol, and very-low-density lipoprotein levels were measured with Piccolo-lipid panel plus, purchased from Abaxis (Union City, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Metabolic stress triggers interaction between PKR and RISC-loading complex components.
- TRBP phosphorylation is induced in metabolic stress and leads to PKR activation.
- The TRBP-PKR interaction is critical for JNK activation and eIF2a phosphorylation.
- Experimental modulation of TRBP in liver impacts glucose metabolism in obese mice.

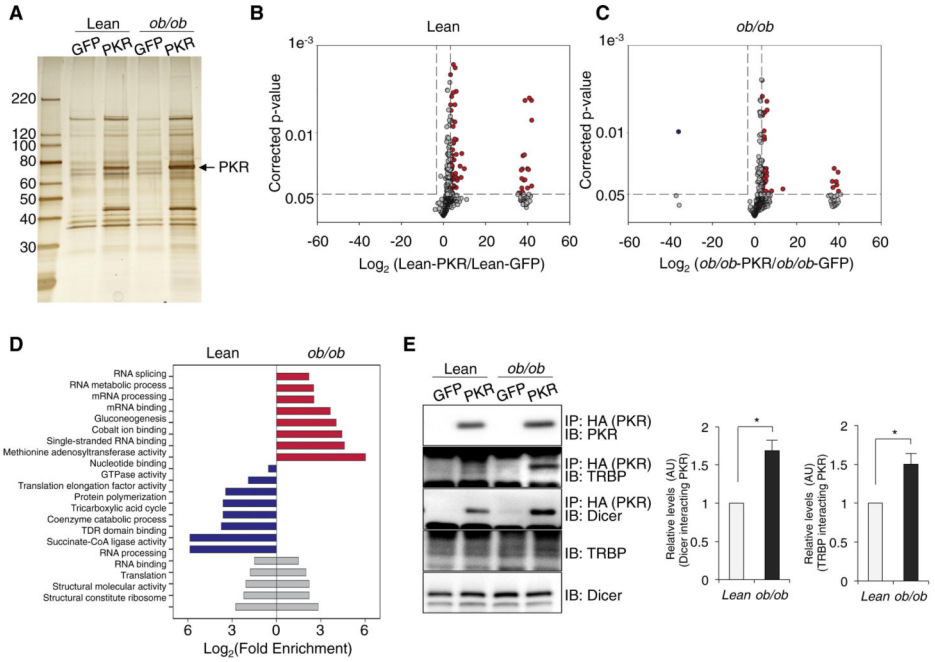

Figure 1. Identification of hepatic PKR protein complex in lean and obese mice

(A) Silver staining of PKR-associated proteins following immunoprecipitation from lean and obese liver. (B and C) Results of mass-spectrometry analyses for identification of PKR protein complexes in lean and obese (*ob/ob*) mice. Vertical hashed lines indicate a fold change of 10 (or -10), horizontal hashed lines indicate a corrected *p*-value of 0.05. Proteins are distributed into two distinct populations based on exclusivity, *i.e.*, molecules found only in the PKR but not in the control samples (or vice versa) center at Log2(PKR/GFP) ratio of >35. See also Table S1 and S2. (D) Gene ontology analysis of statistically significant PKR interactors in liver of lean and obese mice. (E) Physical interaction between PKR and components of RLC, TRBP and Dicer, in liver of lean and obese mice. The graph on the right shows the quantification of the TRBP-PKR interaction and PKR-Dicer interaction. Data are shown as the mean ± SEM (n=3).

*p<0.05. See also Figure S1A.

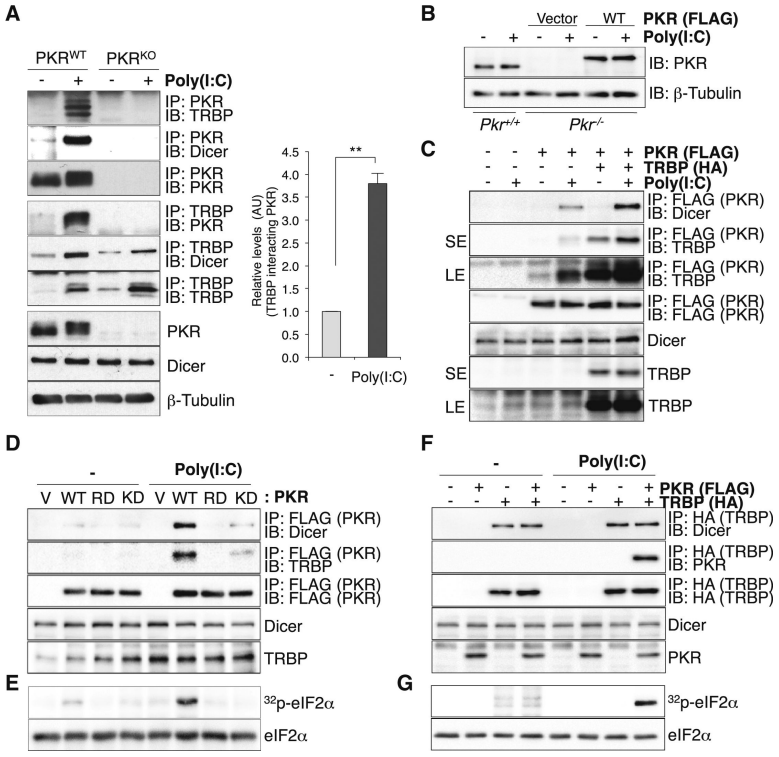

Figure 2. The role of RLC in regulation of PKR kinase activity and induction of eIF2α phosphorylation

(A) Physical interaction between endogenous PKR and Dicer in MEFs in the presence of PolyI:C. *Pkr*$^{+/+}$ and *Pkr*$^{-/-}$ MEFs were treated with 1 g/ml of PolyI:C for one hour, followed by immunoprecipitation and western blots with antibodies as indicated. The graph on the right shows the quantification of the TRBP-PKR interaction. Data are shown as the mean ± SEM (n=3). ***p*<0.01. See also Figure S1B. (B) Expression level PKR in MEFs. Flag-tagged PKR was introduced by retrovirus-mediated gene transfer in *Pkr*$^{-/-}$ MEFs. (C) Physical interaction between PKR, Dicer, and TRBP in MEF cells. Cell lysates were prepared from *Pkr*$^{-/-}$ MEFs reconstituted with retrovirally expressed Flag-tagged wild type (WT) PKR and HA-tagged TRBP in the absence or presence of 1 g/ml of PolyI:C. SE: Short exposure. LE: Long exposure. (D) Effects of mutations in PKR's RNA-binding domains and kinase domain on formation with the PKR-RLC. Cell lysates were prepared from *Pkr*$^{-/-}$ MEFs reconstituted with vector (V), Flag-tagged WT PKR (WT), RNA-binding defective PKR (RD), and kinase-dead mutant (KD) in the absence or presence of 1 g/ml of PolyI:C for one hour. (E) Effects of mutations of PKR on PKR kinase activity. PKR activity was assessed with cell lysates used in Figure 2D using recombinant eIF2α protein. (F) Interaction of RLC with PKR. Cell lysates were prepared from *Pkr*$^{-/-}$ MEFs reconstituted with WT PKR and HA-tagged TRBP in the absence or presence of 1 g/ml of PolyI:C for one hour. G) Ability of TRBP to induce eIF2α phosphorylation through recruitment of PKR. *In vitro* kinase assay was performed with cell lysates used in Figure 2F using recombinant eIF2α protein.

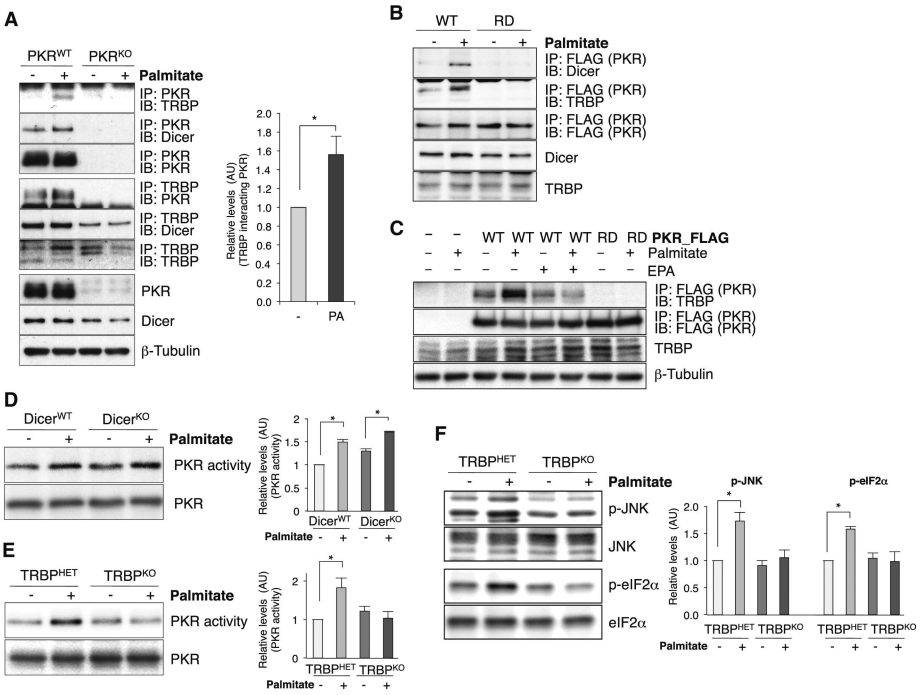

Figure 3. Association of TRBP with PKR in palmitate-induced metabolic stress

(A) Physical interaction between endogenous PKR and Dicer in MEFs in the presence of palmitate. *Pkr*$^{+/+}$ and *Pkr*$^{-/-}$ MEFs were treated with 100 μM of palmitate for one hour, followed by immunoprecipitation and immunoblotting with antibodies as indicated. The graph on the right shows the quantification of PKR-TRBP interaction. Data are shown as the mean ± SEM (n=4). *p<0.05. (B) Physical interaction between PKR, Dicer, and TRBP in MEF cells after palmitate exposure. *Pkr*$^{-/-}$ MEFs reconstituted with retrovirally expressed Flag-tagged WT or RD PKR were treated with 100 μM palmitate, followed by immunoprecipitation with anti-Flag antibody. (C) Palmitate-induced physical interaction between PKR and TRBP in MEF cells pretreated with 100 μM EPA for 14 hours. *Pkr*$^{-/-}$ MEFs reconstituted with Flag-tagged WT PKR (WT) were cultured in the presence of 100 μM EPA for 14 hours. These cells were treated with 100 μM palmitate for 2 hours at the end of EPA culture period. See also Figure S2A, S2B, and S2C. (D and E) Effects of Dicer or TRBP deficiency on palmitate-induced PKR activation. Dicer (D) and TRBP (E) knockout fibroblasts and their control cells were treated with 100 μM palmitate for 2 hours, followed by *in vitro* PKR kinase assay. Blots shown are representative of three independent experiments; graphs are mean ± SEM (n=3). *p<0.05. (F) TRBP deficiency results in reduced palmitate-induced JNK and eIF2α phosphorylation levels. TRBP knockout fibroblasts and control cells were treated with 100 μM palmitate for 2 hours, followed by western blot analysis. Blots shown are representative of four independent experiments; graphs are mean ± SEM (n=4). *p<0.05.

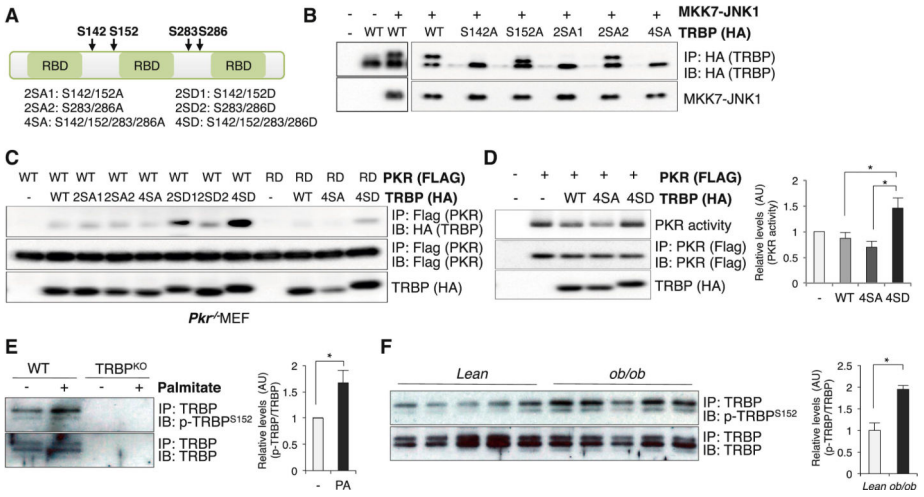

Figure 4. Role of TRBP phosphorylation on PKR activity

(A) Schematic domain structure of TRBP with arrows indicating the positions of four serine (S) phosphorylation residues as shown previously (Paroo et al., 2009). Abbreviations of TRBP mutants we use in this proposal are indicated below. (B) Effect of co-expression of constitutively active JNK1 (MKK7-JNK1) with WT or TRBP SA mutants on their mobility shifts detected by anti-TRBP antibody. (C) Interaction between PKR and TRBP variants in *Pkr*$^{-/-}$ MEFs. PKR and TRBP variants were examined by immunoprecipitaion followed by immunoblotting. (D) PKR activity in *Pkr*$^{-/-}$ MEFs reconstituted with Flag-tagged WT PKR retrovirally expressing WT, 4SA, or 4SD TRBP. The graph on the right shows the quantification of the results. Data are shown as the mean ± SEM (n=3). *p<0.05. (E) Palmitate-induced TRBP phosphorylation levels. WT MEFs and TRBP-deficient fibroblasts and were treated with 100 μM palmitate for 2 hours, followed by immunoprecipitation with anti-TRBP and antibody and western blot analysis with anti-phospho TRBP (ser152) antibody. Blots shown are representative of three independent experiments; graphs are mean ± SEM (n=3). *p<0.05. See also Figure S3A and S3B. (F) TRBP phosphorylation levels in liver of WT and leptin-deficient obese mice. The graph on the right shows the quantification of the results. Data are shown as the mean ± SEM (n=5). *p<0.05.

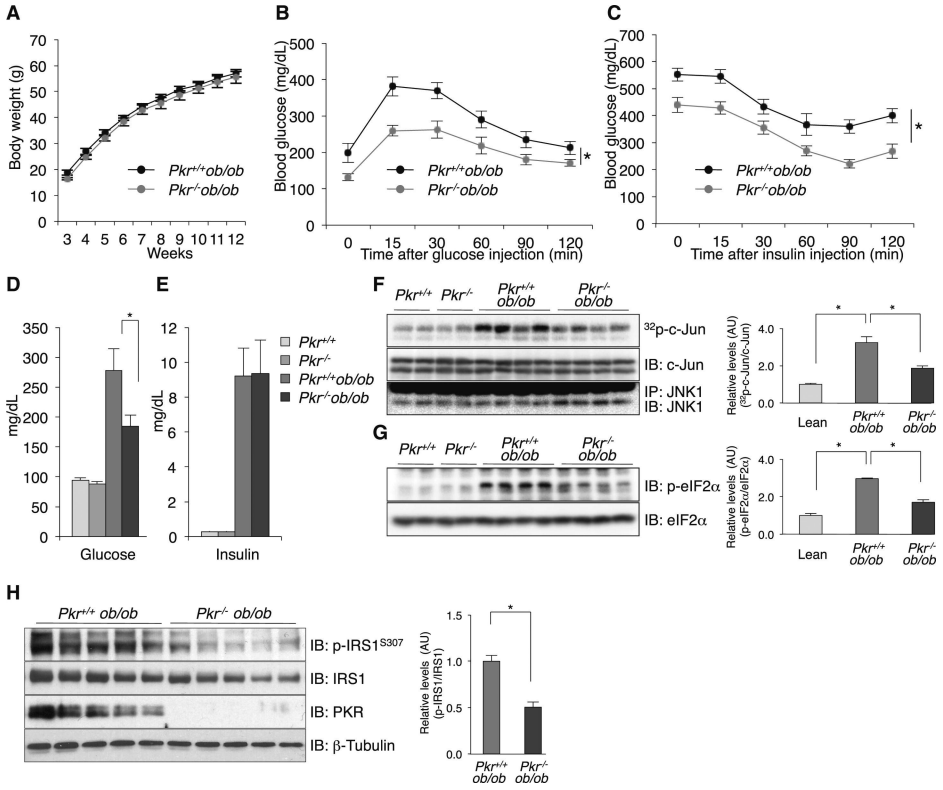

Figure 5. Improved glucose metabolism and insulin sensitivity in *Pkr*-deficient *ob/ob* mice

(A) Effects of Pkr-deficiency on body weight in *ob/ob* mice. (B) Glucose tolerance tests, performed in $Pkr^{+/+}ob/ob$ (*n*=12) and $Pkr^{-/-}ob/ob$ (*n*=7) at 7 weeks of age. Data are shown as the mean ± SEM (n=5). **p*<0.05. (C) Insulin tolerance tests, performed on $Pkr^{+/+}ob/ob$ (*n*=12) and $Pkr^{-/-}ob/ob$ (*n*=7) at 9 weeks of age. Data are shown as the mean ± SEM (n=5). **p*<0.05. (D and E) Blood glucose (D) and serum insulin (E) levels after 14 hours food withdrawal in $Pkr^{+/+}$ (*n*=8), $Pkr^{-/-}$ (*n*=8), $Pkr^{+/+}ob/ob$ (*n*=8) and $Pkr^{-/-}ob/ob$ (*n*=8) at 10 weeks of age. See also Figure S4A, S4B, and S4C. (F and G) Phosphorylation level of eIF2α on serine 52 (F), which was detected by anti-phospho-eIF2α antibody, and JNK1 kinase activity (G), which was measured by a kinase assay using immunopurified JNK1, ATP $[\gamma\text{-}^{32}P]$ and recombinant c-Jun protein as substrate, in liver of $Pkr^{+/+}$, $Pkr^{-/-}$, $Pkr^{+/+}ob/ob$, and $Pkr^{-/-}ob/ob$ mice at 10 weeks of age. The graph on the right shows the quantification of the results. Data are shown as the mean ± SEM. **p*<0.05. See also Figure S4D. (H) Phosphorylation levels of IRS1 on serine 307 detected by anti-phospho IRS1 antibody. The graph on the right shows the quantification of the results. Data are shown as the mean ± SEM. **p*<0.05.

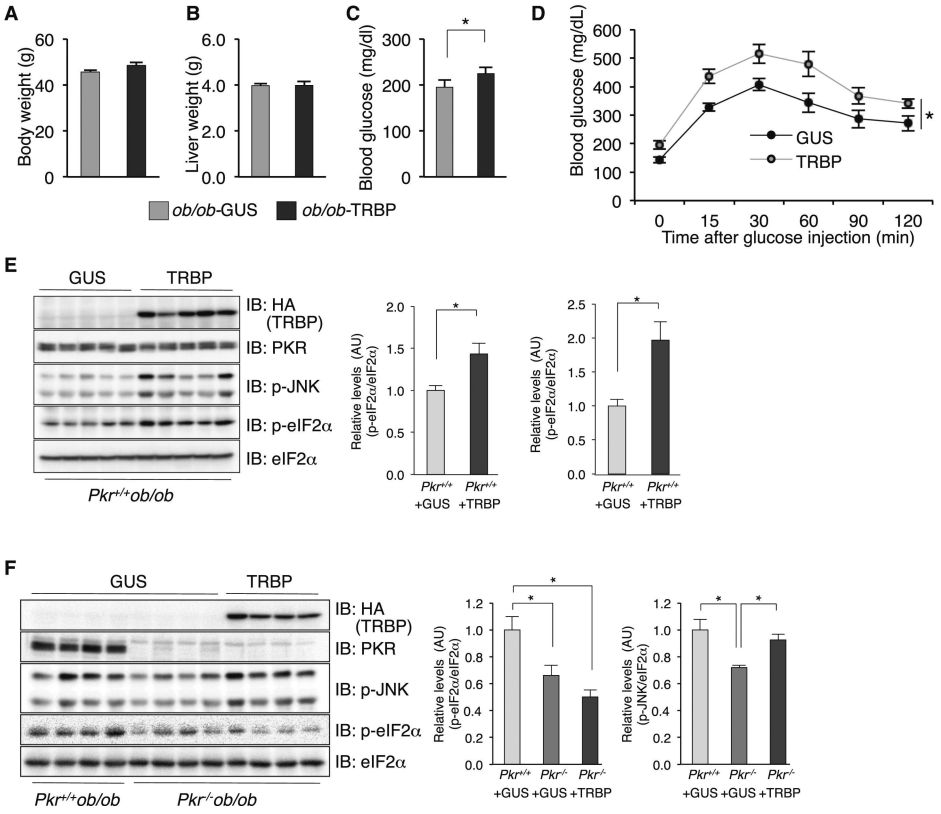

Figure 6. Effects of TRBP expression on glucose metabolism and eIF2α phosphorylation in a genetic model of obesity

(A and B) Effects of hepatic TRBP expression in *ob/ob* mice on body weight (A) and liver weight (B). Data are shown as the mean ± SEM (*n*=8). (C) Effect of hepatic TRBP expression in *ob/ob* mice on fasting blood glucose. Data are shown as the mean ± SEM(*n*=8). **p*<0.05. (D) Effects of hepatic TRBP expression on systemic glucose metabolism assessed by glucose tolerance tests. Data are shown as the mean ± SEM (*n*=8). **p*<0.05 (E and F) Effects of hepatic TRBP expression on JNK and eIF2α phosphorylation. Phosphorylation levels of eIF2α and JNK were detected by anti-phospho-antibodies in liver lysates of $Pkr^{+/+}ob/ob$ (E) and $Pkr^{-/-}ob/ob$ (F) mice exogenously expressing a control gene (GUS) or TRBP. The graph on the right shows the quantification of the results. Data are shown as the mean ± SEM. **p*<0.05.

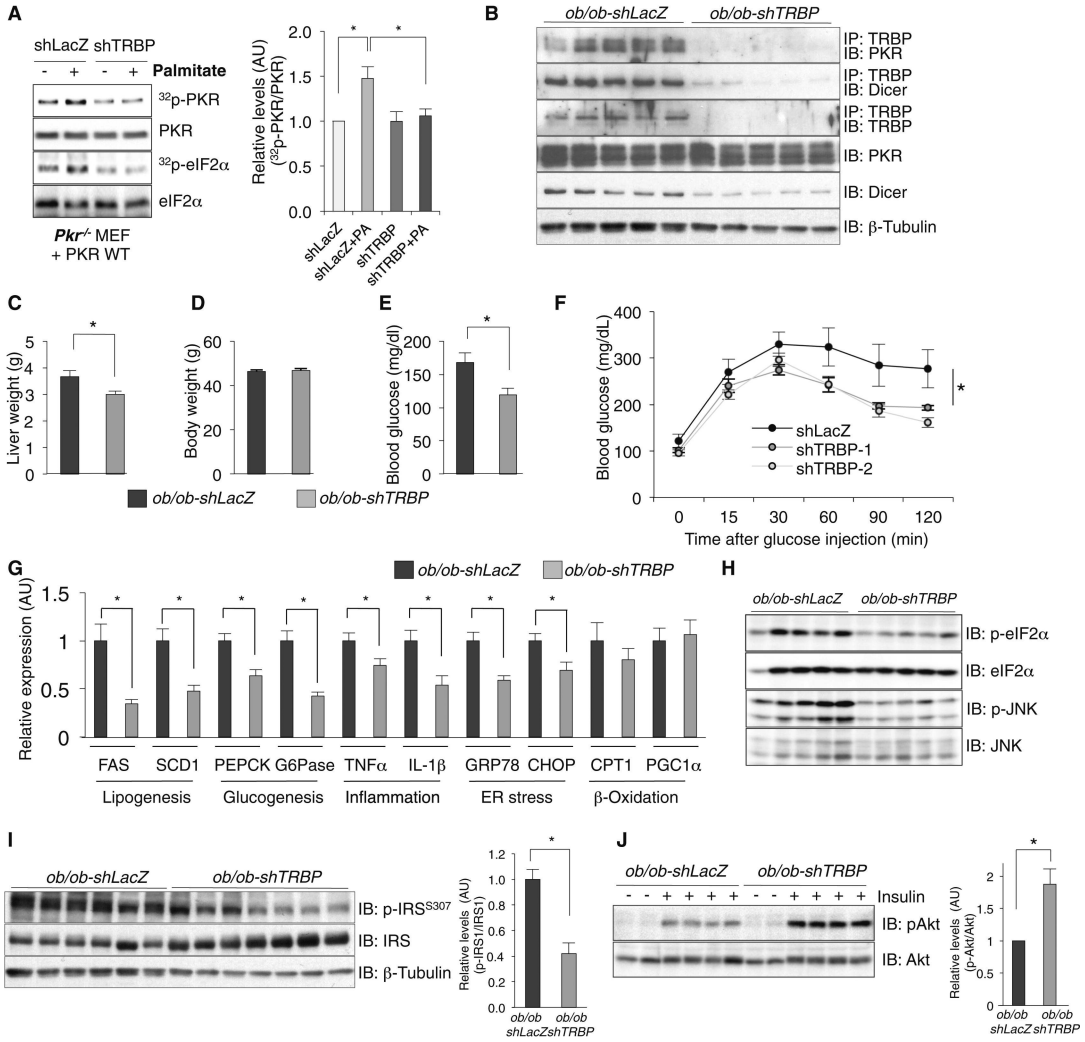

Figure 7. Physiological roles of hepatic TRBP in obesity

(A) Effects of TRBP suppression on palmitate-induced PKR activation. TRBP expression in *Pkr*$^{-/-}$ MEFs reconstituted with Flag-tagged WT PKR were reduced by adenovirus-mediated shRNAs. These cells were treated with 100 mM palmitate for 2 hours, followed by immunoprecipitation with anti-Flag antibody, and *in vitro* kinase assay with recombinant eIF2α protein. The graph on the right shows the quantification of the results. Data are shown as the mean ± SEM. **p*<0.05. See also Table S3. (B) Effects of hepatic TRBP suppression in *ob/ob* mice on TRBP, PKR, and Dicer expression following transduction of adenovirus-mediated control shRNA to LacZ. Liver tissue lysates were analyzed by immunoblotting with antibodies as indicated. To analyze the levels of PKR and Dicer interacting TRBP, the tissue lysates were immunoprecipitated with anti-TRBP antibody, followed by immunoblotting with antibodies as indicated. (C and D) Effects of hepatic TRBP reduction in *ob/ob* mice on liver weight (C) and body weight (D). Data are shown as the mean ± SEM (*n*=8). **p*<0.05. (E) Effect of hepatic TRBP knockdown in *ob/ob* mice on fasting blood glucose. Data are shown as the mean ± SEM (*n*=8). **p*<0.05. (F) Effects of hepatic TRBP suppression on systemic glucose metabolism assessed by glucose tolerance tests. Hepatic

TRBP was reduced using two different shRNA constructs introduced by adenovirus-mediated gene transfer in *ob/ob* liver. Data are shown as the mean ± SEM (n=8). *p<0.05. (G) Effects of hepatic TRBP suppression in *ob/ob* mice on expression of key regulators of liver function. Expression levels of the genes were detected by quantitative PCR method. Data are shown as the mean ± SEM (n=8). *p<0.05. See also Table S4. (H) Hepatic TRBP knockdown results in reduced PKR expression, eIF2α phosphorylation, and JNK phosphorylation levels in obese liver. (I) Serine phosphorylation level of IRS1 was detected by an anti-phospho IRS1 antibody recognizing residue 307. The graph on the right shows the quantification of the results. Data are shown as the mean ± SEM. *p<0.05. (J) Enhanced insulin signaling, which was assessed by anti-phospho specific AKT antibody, observed in TRBP knocked-down liver of *ob/ob* mice. The graph on the right shows the quantification of insulin-induced phosphorylation levels of Akt. Data are shown as the mean ± SEM. *p<0.05.