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Missense Mutation in the Second RNA Binding Domain Reveals a Role for Prkra (PACT/RAX) during Skull Development

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Abstract

Random chemical mutagenesis of the mouse genome can causally connect genes to specific phenotypes. Using this approach, reduced pinna (rep) or microtia, a defect in ear development, was mapped to a small region of mouse chromosome 2. Sequencing of this region established co-segregation of the phenotype (rep) with a mutation in the Prkra gene, which encodes the protein PACT/RAX. Mice homozygous for the mutant Prkra allele had defects not only in ear development but also growth, craniofacial development and ovarian structure. The rep mutation was identified as a missense mutation (Serine 130 to Proline) that did not affect mRNA expression, however the steady state level of RAX protein was significantly lower in the brains of rep mice. The mutant protein, while normal in most biochemical functions, was unable to bind dsRNA. In addition, rep mice altered morphology of the skull that was consistent with a targeted deletion of Prkra showing a contribution of the gene to craniofacial development. These observations identified a specific mutation that reduces steady-state levels of RAX protein and disrupts the dsRNA binding function of the protein, demonstrating the importance of the Prkra gene in various aspects of mouse development.

Introduction

The Prkra gene encodes a double-stranded RNA binding protein, which was identified and named independently as Protein Activator of PKR (PACT) in human [1], and PKR-associated protein X (RAX) in mouse [2]. PACT and RAX are almost identical in their amino acid sequences; only 6 out of 313 residues are different with 4 substitutions being with similar residues. Initial studies on this protein were focused on its ability to induce autophosphorylation of and activate interferon inducible, double-stranded RNA dependent protein kinase (PKR) (encoded by the Eif2ak2 gene) in response to various stresses such as ceramide [3], arsenite [24], tumor necrosis factor a (TNFa) [5], ethanol [6], low dose actinomycin D [7], growth factor withdrawal [2,4], chemotherapeutics [8], endoplasmic reticulum (ER) stress [9,10], or peroxide [2,4]. Activation of PKR results in phosphorylation of eukaryotic initiation factor 2a (eIF2a) leading to inhibition of protein synthesis [11,12]. In addition to PACT/RAX, PKR is modulated by another dsRNA binding protein, TAR (trans-activating region) RNA-binding protein (TRBP in human, PRBP in mouse) [encoded by the Tarbp2 gene] [13,14,15]. In contrast to PACT/RAX, TRBP/PRBP inhibits PKR activation [14]. Aside from binding PKR, PACT and TRBP have also been shown to heterodimerize through interaction of their N-terminal dsRNA binding motifs, as well as through their C-terminal Merlin-Dicer-PACT liaison (Medipal) domain [16,17].

Upon appropriate stimulation, PACT is phosphorylated on serine 246 and serine 287 [18], while RAX is phosphorylated on serine 18 [19]. Phosphorylation causes PACT/TRBP heterodimers to dissociate [20,21], freeing PACT to bind PKR through its two amino-terminal double stranded RNA binding domains [7]. This leads to conformational change facilitating interaction of PACT’s carboxy-terminal domain with the kinase domain of PKR (residues 328–335) leading to PKR activation [16,22,23] and subsequent eIF2a activation.

Studies in mice in which the Prkra gene was disrupted (Prkra+/-/-/Cre/mice) produced unexpected results. In contrast to mice in which the Eif2ak2 gene has been disrupted (Eif2ak2+/-/-/Cre/mice), which had no discernable developmental phenotype [24], Prkra+/-/-/Cre/mice showed defects in ear and craniofacial development, growth and fertility [25]. Further investigation revealed that Prkra+/-/-/Cre/mice developed hypoplastic anterior pituitaries resulting from reduced cell proliferation in this tissue [26]. As the anterior pituitary contains
cells which secrete hormones required for growth and sexual development, this likely accounts for some of the developmental anomalies observed in the mouse [26].

In addition to its ability to activate PKR, PACT has also been shown to have a role in production of small RNAs involved in RNA silencing. PACT (as well as TRBP) interacts with Dicer, which processes small RNAs from their precursor to mature forms, and is a component of the RNA Induced Silencing Complex (RISC) whose key components include Dicer and Argonaute proteins [27]. While not essential for cleavage of pre-miRNAs to their mature form by Dicer, PACT may be required for RISC assembly, as depleting PACT led to reduced levels of mature miRNAs in vitro [27]. While knockout mice for Dicer have been generated, these are embryonically lethal [28]. The relevant tissue specific knockouts for Dicer, however [29,30] show similar reproductive defects to the Prkra-null mice. This observation supports the idea that at least some of the developmental defects seen in the Prkra deficient mouse might result from defects in miRNA processing.

In humans, mutations in Prkra are associated with Dyt16, an autosomal recessive young onset dystonia-parkinsonism disorder [31,32]. Dyt16 patients show retarded speech learning in infancy and involuntary muscle contraction starting during teenage years. The mutation was induced by ENU on the C57BL/6J background and was further established by backcrossing on the C3HeB/FeJ genetic background. We used this backcross to precisely map the position of the mutation between rs13476586 and rs13476589 on mouse chromosome 2 (Table 1). The weight difference was also found in younger individuals starting at 7 days post partum, with no major difference between sexes in homozygous mice (Figure 2). Second we noticed a defect in fertility when we crossed the Prkra mutant mice with wild-type (wt) or heterozygous mice. No progeny were obtained by breeding 7 homozygous females with wt males over a 2 month period.

Histopathological analysis of the ovaries showed all stages of folliculogenesis from primordial to preovulatory follicles and corpus luteum formation were present in mutant mice (Figure 3A, B). No abnormalities were observed in the histology of testes from male mutants (Figure 3C, D). We also observed differences in the number of germ cells which secrete hormones required for growth and sexual development, this likely accounts for some of the developmental anomalies observed in the mouse [26].

Results

Characterization of the reduced pinna recessive mutation affecting the Prkra gene

In the course of the Phenotype Homozygote Mutants program [34], the rep mutant mouse line which displays microtia (Figure 1A) and growth retardation (Figure 2, Table 1) was isolated. The mutation was induced by ENU on the C57BL/6J background and was further established by backcrossing on the C3HeB/FeJ genetic background. We used this backcross to precisely map the position of the mutation using a panel of markers already described [34,35]. The rep mutation was located between rs13476586 and rs13476589 on mouse chromosome 2 (Figure 1B). Looking at candidate genes in this region, we identified the Prkra gene for which a knock-out displaying similar dysmorphology was previously described [25]. We sequenced the Prkra coding sequence and exon/intron borders. We found a point mutation T→C in exon 4 affecting codon 130 and introducing the missense mutation S130P (Figure 1C). Thus we conclude that the rep mutation affected the Prkra gene so we named the mutation Prkra*rep. We verified that this change was not found in either C57BL/6J or C3HeB/FeJ background, or in other mouse strains including 129S2, BALB/c and DBA2/J (data not shown). Performing a more detailed phenotypic analysis we noticed two major changes in Prkra*rep homozygous mice. First, adult homozygous mice were smaller compared to their control littermates (Table 1). The weight difference was also found in younger individuals starting at 7 days post partum, with no major difference between sexes in homozygous mice (Figure 2). Second we noticed a defect in fertility when we crossed the Prkra*rep females with wild-type (wt) or heterozygous mice. No progeny were obtained by breeding 7 homozygous females with wt males over a 2 month period.

Presence of RAX mRNA in the brains of rep mice

To understand the physiological defects seen in rep mice it was important to determine whether Prkra mRNA was present at wild-type levels in these animals. It was possible that the S130P mutation would alter the production, processing or stability of Prkra mRNA. RT-PCR was used to determine whether Prkra mRNA was produced in brain. Primer sequences targeting the 5’ region (exons 2 and 3) and 3’ region (exon 8) were used in separate reactions to determine whether the entire mRNA or simply the portion 5’ of the T-C substitution leading to the S130P mutation was generated. Both 5’ and 3’ portions of Prkra mRNA were present in rep mice at levels comparable to those seen in wt mice, indicating the S130P mutation does not impair production of full length Prkra mRNA (Figure 5).

RAX (S130P) dimerizes and activates PKR in response to stress, but is unable to bind dsRNA

To investigate the biochemical basis for the rep phenotype, the S130P mutation was introduced in RAX for expression in mouse
and bacterial cells. The mutant protein was characterized for its ability to bind and activate PKR, bind dsRNA and homodimerize.

The ability of RAX (S130P) to bind dsRNA was determined by electrophoretic mobility shift assays using radiolabeled dsRNA and bacterially-expressed, purified WT and mutant His-RAX. There was a clear shift in electrophoretic mobility of the dsRNA probe in the presence of 1 mM WT His-RAX which could be competed out by unlabeled synthetic dsRNA, poly(I:C), demonstrating dsRNA specific binding activity of WT RAX. The mutant protein however could not bind dsRNA, as measured by this assay (Figure 6A). To further examine this observation, a different dsRNA-binding assay was used. In this assay, His-RAX was incubated with radiolabeled dsRNA and then purified using Ni-NTA agarose; the amount of dsRNA probe, bound to RAX, was quantified by scintillation counting. Again, there was clear dsRNA binding to the WT protein, which could be competed out with the addition of unlabeled poly(I:C), while the mutant protein was unable to bind the dsRNA probe (Figure 6B). These results demonstrate that the S130P mutation disrupts the dsRNA binding capacity of RAX.

Dimerization was examined by incubating purified WT or S130P His-RAX with lysates from L929 cell lines expressing similar levels of WT or S130P FLAG- RAX, as generated by lentiviral transduction (Figure 6C, middle panels). Both WT and mutant RAX homodimerized (Figure 6C, top panel) demonstrating that the S130P mutation disrupts the dsRNA binding capacity of RAX.

Table 1. Weights of adult rep mice compared to those of WT controls.

| Sex     | WT         | rep/rep     | P Value |
|---------|------------|-------------|---------|
| Male    | 24.9±2.1 g | 19.2±2.4 g  | 0.0013  |
| Female  | 21.8±1.1 g | 17.5±0.5 g  | 0.0005  |

Weights of adult rep and WT littermates (male age 54 days+/−1.4 days; female age 54.3+/−1.3 days) and the P-values of the corresponding Student T-test. doi:10.1371/journal.pone.0028537.t001
ing that the mutation does not interfere with dimerization of RAX.

To assess the ability of the S130P mutant protein to bind PKR, cell lysates from WT or S130P FLAG-RAX expressing L929 cells treated with sodium arsenite (to induce RAX phosphorylation) were immunoprecipitated using a FLAG antibody, followed by western blot for endogenous PKR. There were clear interactions between PKR and WT or S130P RAX proteins, independent of the arsenite treatment (Figure 6D), demonstrating that the S130P mutation does not interfere with PKR interaction of RAX.

For testing the ability of RAX (S130P) to activate PKR, we generated L929 cells in which expression of RAX had been ablated by a shRNA that targets the 3' UTR of RAX mRNA. In this cell line, WT or mutant RAX was ectopically expressed using lentiviral vectors encoding the corresponding RAX mRNAs without the UTRs (Figure 6E, bottom panel). These cells were treated with sodium arsenite to activate RAX whose ability to activate PKR was monitored by measuring eIF2α phosphorylation; there were comparable stress-induced increases in phospho-eIF2α in cells expressing WT or mutant RAX (Figure 6E, top panel) demonstrating that the S130P mutation does not impair the stress-induced PKR activation function of RAX.

Reduced steady-state levels of ectopically expressed mutant RAX

In L929 cells, ectopic expression levels of FLAG-RAX (S130P) were consistently lower than those of WT FLAG-RAX (data not shown). To determine whether the observed difference was operative at the transcription or the translation level, we measured the levels of RAX protein by western blot and mRNA by realtime RT-PCR, in several cell lines that we generated. In cells infected with RAX-expressing lentivirus at moi of 1, as compared to WT, there was a slightly reduced level of the mutant mRNA (Figure 7A) but an almost undetectable level of the mutant protein (Figure 7B). However, when the mutant-expressing virus was used at a moi of 3, a comparable level of the mutant protein was expressed from more than twice the level of the mutant mRNA, indicating a defect in the synthesis or turnover of the mutant protein. To distinguish between the two possibilities, we measured the stability of the mutant protein in cells expressing equal levels of WT and mutant proteins. New protein synthesis was inhibited by cyclohexamide treatment, and the rate of decay of existing RAX was monitored by western blot analysis at different time points (Figure 7C). Data were quantified as FLAG signals relative to actin signals (Figure 7D). We did not observe any significant difference in the rates of WT and mutant protein decay. These results indicate that the lower steady-state level of
the mutant protein is probably due a defect in its synthesis, but not due to an accelerated decay.

Significantly reduced levels of mutant RAX in the brains of rep mice

To extend our observations in cell lines to mice, we measured RAX protein levels in brains of WT and rep mice; we chose the brain as a representative tissue because RAX is highly expressed there. Proteins precipitated from the trizol extracts of the brains of the mice used to measure RAX mRNA levels (Figure 5) was used to detect RAX protein levels by western blot, revealing significantly reduced levels in mutant mice compared with WT mouse (Figure 8A). To rule out the possibility that the reduced protein levels were an artifact of precipitation from the trizol fractionation, we prepared a third rep brain, along with WT and tm1Gsc (RAX2/2) brains, using conventional detergent lysis. These additional samples showed reduced mutant RAX protein in the rep mouse and no RAX in the RAX2/2 mouse brains (Figure 8B). These results, combined with those shown in Figure 5, indicate that in rep mice much less RAX is present because of a defect in the synthesis of the mutant protein.

Discussion

In this report we described the characterization of a new ENU-induced missense mutation (S130P) in the coding sequence of the Prkra gene. The new allele of the Prkra gene (rep) induces several alterations in growth, and the development of the ear and skull of the mutant mice. During this analysis we further characterized in rep homozygous mutants, the craniofacial defects previously observed in tm1Gsc mice. Rep mice were found to have a very short nasal bone and a reduced coronoid process and mandibular condyle. Parallel analysis carried out for the Prkratm1Gsc mutation revealed similar mandibular alteration but major changes of the skull shape with a lack of fusion of the frontal and parietal bones. These series of data elaborate upon the role of Prkra in controlling cranio-facial development.
Figure 6. Functional characterization of the RAX (S130P) mutant. **A** DsRNA electrophoretic mobility shift assay: Purified His-RAX or His-RAX (S130P) was incubated at the indicated concentration with 5' end labelled dsRNA. In the indicated lanes, poly(I:C) was added to the reaction as a competitor to demonstrate dsRNA-binding specificity. **B** DsRNA-pull-down assay: Purified His-RAX or His-RAX (S130P) was incubated with 5' end labelled dsRNA. His-RAX was pulled-down from the reactions with Ni-NTA agarose and bound dsRNA was measured by liquid scintillation (expressed in counts per minute). CPM bound to BSA control has been subtracted from all lanes as background. **C** Dimerization assay: Purified His-RAX or His-
The S130P mutation is located in the second dsRNA binding domain of the protein and disrupts dsRNA binding without affecting RAX dimerization or its ability to activate PKR. The mutation also leads to a decrease in steady-state levels of RAX protein, in tissue such as the brain, resulting in the phenotypes observed in the rep mutant which are similar to those of a mouse with a targeted disruption in the Prkra gene and as such the phenotype is likely a result of the significantly reduced steady-state protein level. At this time, we can not discern whether this is connected to the inability of the mutant protein to bind dsRNA. Another factor, that may play a role, is TAR RNA binding protein (TRBP); its binding to PACT is partly mediated by the second domain, the site of the S130P mutation [17].

Human TRBP and PACT directly interact with each other and associate with Dicer to facilitate the production of small interfering RNA [27,37]. Recently, Zehir et al. (2010) demonstrated that controlled inactivation of Dicer in neural crest cells (which function in skull development) results in craniofacial malformation [38]. The phenotypes we have observed in Prkra mutant mice may be reminiscent of hypomorphic phenotypes of Dicer indicating that defects in the miRNA pathway might contribute to the craniofacial malformations we observed. As the rep mutation disrupts dsRNA binding by RAX, there may be an effect on the miRNA pathway in the rep mutant.

The rep mutant is, to our knowledge, the third allele of Prkra. Rowe et al. (2006) published the Prkratm1Gsc mutant for Prkra with a targeted disruption of the 3′9 end of the gene that was used here for comparison. Almost all the phenotypes present in the tm1Gsc mouse were recapitulated to some extent in the rep mutant. Interestingly the two mutations respectively affect the second and the third RNA binding domains of the protein. In contrast, deletion of the entire gene, Prkratm1Wsmay, engineered by Bennett

![Figure 7. Reduced ectopic expression of RAX (S130P) in L929 cells.](Image)

A RAX mRNA levels: Realtime RT-PCR analyses were used to determine the levels of FLAG-RAX mRNA relative to 18S rRNA using RNA samples isolated from the indicated cells. B RAX protein levels: FLAG western blot of L929 cells infected with empty lentivirus, or lentivirus encoding a provirus to ectopically express FLAG-RAX or FLAG-RAX (S130P). Three times more virus was required for FLAG-RAX (S130P) to achieve protein levels comparable to WT. C RAX turnover analyses: Cells were treated with cycloheximide to inhibit de novo protein synthesis, cells lysates were prepared at the indicated time points and protein levels were measured by Odyssey quantitative western blot of FLAG-RAX and FLAG-RAX (S130P) using actin as the internal control. D Normalized levels of RAX: FLAG-RAX signal was normalized to that of actin and plotted at the indicated times following cycloheximide treatment.

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et al. (2008), induces an early developmental lethality with no embryos detected after 3.5 days post conception. The less severe phenotype seen in the Prkratm1Gsc and Prkrap loaded with creatine kinase as a loading control, and the absence of RAX protein in Prkratm1Wsm suggests that a minimal level of expression of the RAX protein, sufficient to rescue the early embryonic phenotypes observed in Prkratm1Wsm homozygote animal, is necessary and theoretically partial loss of function mutants have been studied for other purposes but both models should be explored further for traits observed in human patients to be validated as a model for Dystonia 16.

Materials and Methods

Mice

The rep mutation was isolated from PhenHomut a genome wide recessive mutagenesis program for phenotyping homozygote mutants previously described [34,35]. The screening was oriented toward recessive mutations affecting morphology, the cardiovascular system, metabolism and the immune response. For rep; F1 males, derived from the first progeny of ENU-treated C57BL/6J (B6) males, were mated with wild-type females C3HeB/FeJ (C3H) to generate G2 individuals. G3 individuals were derived from the backcross of G2 females with the F1 males, and were screened following a hierarchical and standardized phenotyping analysis. We also took advantage of the backcross for the genetic mapping and phenotypic analysis as described previously [34,35]. For analysis of RAX expression, brains were dissected, snap-frozen and stored in liquid nitrogen prior to isolation of RNA and/or protein. All experiments were performed within the guidelines of the French Ministry of Agriculture for experiments with laboratory animals or in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee of Cleveland Clinic (Approval Number ARC 08738) or the Ethical Committee for the Region Centre to Y Herault (law 87 848; VH accreditation 45-31). All efforts were made to maximize animal welfare.

Phenotypic analysis of mice

Cohorts of age and sex-matched mutant and wild-type mice were tested for a variety of phenotypic parameters according to the standard operating procedures of the EUmorphia network [34,35]. Mice were weighed daily to generate growth curves. For histological analysis of gonads, tissues were fixed in Bouin’s and stained with hematoxylin. For X-ray analysis skulls were preserved in 95% ethanol at 4°C prior to analysis.

To examine variations in skull and mandible shape, we used a 3D morphometric method based on landmark comparisons adapted from Hallgrimsson et al. 2004 [39]. Landmarks were taken on virtual reconstructions of the specimens’ skulls and mandibles obtained by X-ray microtomography. The resulting voxel size varied among specimens between 10 and 24 μm. The skull and mandible surfaces were extracted using VGStudioMax software. Twenty-two landmarks were defined using Landmark software on each mandible and forty-nine on each skull (Figure S1). Specimen size was normalised and landmarks from different specimens superimposed using the Procrustes method. Principal Component Analyses was performed using Morphologika v2.4 software using the full tangent space projection. Statistical tests (MANOVA) were performed using Statistica software.

Plasmids, cell Lines and reagents

Mouse L929 and human HT1080 and HEK293T cell lines were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Atlanta Biologicals) supplemented with glucose (4.5 g/L), penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (2 mM) and sodium pyruvate (1 mM). Sodium arsenite was obtained from Sigma. Trizol reagent was obtained from Invitrogen. The plasmids pLVX-IRES-ZsGreen1 and pLKO.1-puro were obtained from Addgene, pET15b was obtained from Novagen, pGL3-pDNA3 was obtained from Invitrogen, pLKO.1-puro was obtained from Sigma.

Figure 8. Lower levels of RAX protein in the brains of rep mice. A RAX levels in proteins recovered from the same samples used for RAX mRNA measurements: Protein re-precipitated from trizol extracts of the same brain preparations shown in Figure 5 was analyzed by western blot for RAX and actin expression. B RAX levels in conventional protein extracts of brains: RAX western blot from brains homogenized and lysed in detergent-lysing buffer (see materials and methods). RAX expression was analyzed in wild-type, Prkratm1Gsc (denoted –/–) and rep (denoted 3 mice). doi:10.1371/journal.pone.0028537.g008
Cloning of FLAG-RAX

The oligonucleotides AGC TTG GAT CCT ATG TCC CAT AGC AGG C and 3' AGT AGT TTC TCG TTG TAG TCT TTG GAT CCT ATG TTT AAA T were annealed and ligated into pLVX-IRES-ZsGreen1 (Clontech) with EcoRI (New England Biolabs) using Rapid DNA Ligation Kit (Roche), this clone was designated pLVX-FLAG-RAX-IRES-ZsGreen1. The oligonucleotides AGC TTG GTA CCA TTA CAG GAA TTA GCA ATT CAC CAT G and 3' AAT CTA GAG GAT CCC TAC TTT CTT TCT GCT ATT ATC TTT AAA T with Expand High Fidelity PCR System (Roche), the PCR product was digested with XhoI and XbaI (New England Biolabs) and ligated into pLVX-IRES-ZsGreen1 (Clontech) and Rapid DNA Ligation Kit (Roche) to generate pLVX-FLAG-RAX-IRES-ZsGreen1.

Mutation of FLAG-RAX

The S130P mutation was introduced into pLVX-FLAG-RAX-IRES-ZsGreen1 by primer overlap mutagenesis, briefly one PCR reaction was performed using the primers: 5' AAT CTA TCA CAT GGA TCA CTA CAA GG and 3' AAT CTA GAG GAT CCC TAC TTT CTT TCT GCT ATT ATC TTT AAA T while a second PCR reaction was performed using the primers: 5' GCG CCA CTA TTA GAG CAA TTA GCA ATT CAC CAT G and 3' AAT CTA GAG GAT CCC TAC TTT CTT TCT GCT ATT ATC TTT AAA T, both reactions were performed using Expand High Fidelity PCR System (Roche). The resulting overlapping PCR products were then used as template for a second round PCR using the primers: 5' AAT CTA GAG GAT CCC CCA GAA TTA GCA ATT CAC CAT G and 3' AAT CTA GAG GAT CCC TAC TTT CTT TCT GCT ATT ATC TTT AAA T, both reactions were digested with XhoI and XbaI (New England Biolabs) and ligated into pLVX-IREZs-ZsGreen1 (Clontech) with Rapid DNA Ligation Kit (Roche) to generate pLVX-FLAG-RAX-IRES-ZsGreen1.

Construction of the RAX shRNA expression clone

The DNA oligonucleotides CCG GCC GTC AAC TTT CCA GAT TTC TCG AGA AAT CTG GAA AGT TGA CGG TTT TTG and AAT CTA AAA CCC GTC AAC TTT CCTA GCA ATT CAC CAT G were annealed and ligated into pLVX-IRES-ZsGreen1 (Clontech) with AgeI and EcoRI (New England Biolabs) using Rapid DNA Ligation Kit (Roche) to generate pLVX-FLAG-RAX-IRES-ZsGreen1 or pLVX-FLAG-RAX-IRES-ZsGreen1. The lentiviral plasmids pLVX-FLAG-RAX-IRES-ZsGreen1, pLVX-FLAG-RAX-IRES-ZsGreen1, pLVX-FLAG-RAX (S130P)-IRES-ZsGreen1 or pLVX-FLAG-RAX (1199)-puromycin were co-transfected with the packaging plasmids pCMV-dR8.74 and the pseudotyping plasmid pVS-G by calcium phosphate into HEK293T cells. Lentivirus-containing supernatants were harvested three times every 12–16 hours, the collections were pooled, and the lentivirus titered on HT1080 cells by estimating percentage ZsGreen1 positive cells microscopically. L929 cells were infected with recombinant lentivirus in complete DMEM containing 10% FBS, penicillin and streptomycin (containing 8 μg/ml polybrene (hexadimethrine bromide) for 24 hours before splitting into fresh media without polybrene and passaging as a stable line. For RAX knockdown with pLVX-FLAG-RAX (1199)-puromycin, after 48 hours of recovery in complete DMEM, puromycin was added at 5 μg/ml; after selection the cells were grown in puromycin containing media as a stable line.

Bacterial expression and purification of recombinant RAX

pLVX-FLAG-RAX-IRES-ZsGreen1 was used as a template in a PCR using the primers: 5' AAA AGC TTG CAT CCT ATG TCC CAT AGC AGG CAG CAT CG and 3' AAT CTA GAG GAT CCC TAC TTT CTT TCT GCT ATT ATC TTT AAA T using Expand High Fidelity PCR System (Roche), the product was digested with BamHI (New England Biolabs) and ligated into pET15b (Novagen) using Rapid DNA Ligation Kit (Roche) to generate pET15b-RAX. pLVX-FLAG-RAX-IRES-ZsGreen1 was used as a template in a PCR using the primers: 5' AAA AGC TTG CAT CCT ATG TCC CAT AGC AGG AGG C and 3' AAT CTA GAG GAT CCC TAC TTT CTT TCT GCT ATT ATC TTT AAA T using Expand High Fidelity PCR System (Roche), the PCR product was digested with XhoI and XbaI (New England Biolabs) and ligated into pLVX-IREZs-ZsGreen1 (Clontech) with Rapid DNA Ligation Kit (Roche) to generate pLVX-FLAG-RAX-IRES-ZsGreen1.

RNA isolation, reverse transcription and RT-PCR

RNA was isolated from brains of wild-type, tnfgc or rep mice using TRIZOL (Invitrogen) according to the manufacturer’s instructions. RNA was isolated from L929 cell lines using TRIZOL according to the manufacturer’s instructions. Following RNA isolation, residual genomic DNA contamination was removed by DNase I treatment using DNA-free (Ambion). RNA was reverse transcribed using the SuperScript III cDNA First Strand Synthesis Kit (Invitrogen) according to manufacturer’s
instructions using random hexamer primers. RT-PCR using Clontech Advantage 2 Taq was performed using 18S rRNA primers on DNase treated RNA (without reverse transcription) and cDNA samples to determine the efficacy of DNase treatment. RT-PCR using this same protocol was used to amplify sequences in the 5’ and 3’ regions of RAX, cDNA from 100 ng RNA was used per amplification reaction, and the reaction cycle number titrated for each primer set to determine the logarithmic range for product growth. Cycling was as follows: 1’ 95° C, followed by cycles of 1’ 95° C; 30” Tm, 1’ 68° C, finishing with 5’ 68° C. Primer sequences, melting temperatures and cycle numbers were as follows: RAX Exons 2 and 3: 31 cycles, Tm = 52° C. 5’ primer TAA GCC TGG GAA AAC ACC, 3’ primer CCA GCT TCT TAC TGC TAG CCT CTT. RAX Exon 8: 30 cycles, Tm = 60° C. 5’ primer TCT CTT CAG ATT CGG TCA ACT TTC, 3’ primer ACA TTC ATC ACA AGC CTC AAC AC. 18S rRNA: 25 cycles, Tm = 55° C. 5’ primer ATT GAG GGA AGG GCA CCA CCC G, 3’ primer CAA ATC GCT CCA CCA ACT AAG AAC G.

Real-time PCR was performed using SYBR Green Core reagents (Ambion) using 18S rRNA primers from RT-PCR method and FLAG-PACT/RAX primers: 5’ primer CTA CAA GGA CGA TGA CGA TAA GC, 3’ primer CAG CTT CTT ACT TGT ACC AGG G. The reactions were run in a Roche Lightcycler 480, cycling was as follows: 3’ 95° C followed by 50 cycles of 30’ 95° C, 1’ 52° C, 30’ 72° C (SYBR green signal was acquired during the 72°C incubation per cycle).

DsRNA electrophoretic mobility shift assay

The RNA oligonucleotide GGG AAC AAA AGC UGG GUA CCG GCC CCC CCC was 5’ end labelled using T4 polynucleotide kinase (Promega) according to the manufacturer’s instructions. The oligonucleotide GGG GCC GGG GCC UAC CCA GCU UUU GUU GCC CCC was annealed to the radiolabelled oligonucleotide and ethanol precipitated. His-RAX or His-RAX (S130P) was added to binding buffer (20 mM Tris pH 7.5, 50 mM KCl, 2 mM MgCl2, 2 mM MnCl2, 5% glycerol) to the indicated concentration along with 30000 cpm (25 fmol) labelled dsRNA probe; for poly(UC) competition, poly(UC) was added to 10 ng/μl and incubated on ice for 10 minutes prior to adding labelled probe. Binding reactions were incubated at room temperature for 15 minutes, prior to loading on a gel of 0.25X TBE, 5% Acrylamide (37.5:1 acrylamide:biacrylamide) which was run in cold buffer in a cold room.

DsRNA pull-down assay

Labelled dsRNA was incubated with His-RAX in binding buffer (20 mM Tris pH 7.5, 50 mM KCl, 2 mM MgCl2, 2 mM MnCl2, 5% glycerol), the protein was pulled-down by Ni-NTA agarose, washed and bound radioactivity was measured by scintillation counting. Incubation with BSA was used to measure non-specific binding of the probe and unlabelled poly(UC) was used as a competitor, where indicated.

Protein isolation and western blot

Protein was isolated from tissue by re-precipitating the organic phase generated during TRIZOL (Invitrogen) RNA isolation according to the manufacturer’s instructions. Briefly this involved re-precipitation of protein using isopropanol followed by washing with guanidine hydrochloride. Protein was isolated from cultured cells and whole brain by washing in cold PBS followed by lysis in Triton X-100 lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol, supplemented with Complete protease inhibitor tablet and PhoSTOP tablet (Roche). Where indicated, sodium arsenite dissolved in water to 100 mM was added to culture media to a final concentration of 100 μM for one hour prior to harvesting cells. Protein was separated using SDS-PAGE and transferred to PVDF membrane for western blotting.

Antibodies

We used commercial antibodies to PACT/RAX [40], β-actin (Clone AC-15, Sigma-Aldrich, A1978), FLAG (Clone M2, Sigma-Aldrich, Catalog # F1804), c-eIF2α (Ser52) (Invitrogen, Catalog # 44-728), cIF2α (Cell Signalling, Catalog # 9722), His Probe (H-15, Santa Cruz Biotechnology, Catalog # sc-803), PKR (D-20, Santa Cruz Biotechnology, Catalog # sc-708).

Immunoprecipitation and pulldown

FLAG-RAX was immunoprecipitated from cell lysates with anti-FLAG M2 affinity gel (Sigma). Immunoprecipitation was performed in Triton X-100 lysis buffer. Immunoprecipitated samples were washed twice with lysis buffer then washed once with 1X micrococcal nuclease buffer, resuspended in 50 μl 1X micrococcal nuclease buffer containing 2000 gel units micrococcal nuclease (New England Biolabs), and incubated at 37°C for 30 minutes. Samples were washed an additional two times before separation by SDS-PAGE, and subsequent western blotting. Recombinant 6xHis-tagged WT or mutant RAX (2 μg) were incubated with 200 ng empty vector, FLAG-RAX or FLAG-RAX (S130P) transduced L299 lysate in binding buffer (20 mM Tris-HCl, 150 mM NaCl, 10 mM imidazole, 10% glycerol, supplemented with complete protease inhibitor tablet and PhoSTOP tablet (Roche)), the samples were then pulled-down using Ni-NTA agarose (Qiagen). Two washes were performed with binding buffer, then washed once in 1X micrococcal nuclease buffer, resuspended in 50 μl 1X micrococcal nuclease buffer containing 2000 gel units micrococcal nuclease (New England Biolabs), and incubated at 37°C for 30 minutes. Samples were washed an additional four times with binding buffer before separation by SDS-PAGE, and subsequent western blotting.

FLAG-RAX decay kinetics

L299 cells expressing empty provirus, FLAG-RAX or FLAG-RAX (S130P) were split into replicate plates and treated with cycloheximide (100 μg/ml). Cells were lysed in Triton X-100 lysis buffer at 0, 2, 4, 8, 12 or 24 hours following cycloheximide treatment. Lysates were analyzed for FLAG-RAX by quantitative western blot using the Odyssey infrared detection system (Lico; IRDye 680 goat anti-rabbit for actin detection and IRDye 800 goat anti-mouse for FLAG detection). FLAG-RAX signal was normalized to actin and the normalized signal was plotted as a function of time.

Supporting Information

Figure S1 Landmarks used in morphological analysis of the skull (shown only for left side). A Landmarks of the skull: 1. nasale; 2. nasion; 3. bregma; 4. parietal-occipital junction; 5 midline of the interparietal-occipital junction; 6. dorsal midpoint of the foramen magnum; 7. antero-lateral corner of the nasale; 8. parietal-premaxillary-maxillary junction; 9. anterior-most point of the zygomatic spine; 10. posterior-most point of the frontal-maxillary dorsal junction; 11. Anterior-most point of the squamosal-parietal junction; 12. anterior-most point of the forehead.
temporal-zygomatic junction; 13. ventral-most point of the incisor alveoli; 14. anterior-most point of the anterior palatine foramen; 15. Ventral-most point of the premaxilla, maxilla and anterior palatine foramen junction; 16. posterior-most point of the anterior palatine foramen; 17. medial-most point of the first upper molar cervix; 18. Point of greatest curvature of the V-shaped margin of molar process; 19. distal-most point of the third upper molar cervix; 20. Posterior-most point of the zygomatic faceta on the zygomatic process of the squamosal; 21. posterior-most point of the zygomatic/squamosal junction; 22. Antero-medial projection of ectotympanic in basicranial, 23. junction of basioccipital, ectotympanic and basioccipito. 24. Posterior edge of ectotympanic along its margin with basioccipital; 25. anterior process of auditory bulla; 26. ventral midpoint of the foramen magnum; 27. Anterior-most point of the nasal/premaxillary junction; 28. dorsal-auditory bulla; 29. tip of the post-tympanic hook.

**B Landmarks of the mandible:** 1. tip of the coronoid process; 2. distal-most point of the third lower molar cervix; 3. mesial-most point of the first lower molar cervix; 4. dorsal-most point of the incisor alveoli; 5. inferior-most point of the incisor alveoli; 6. inferior-most point of the mandibular symphysis; 7. Posterior-most point of the mandibular symphysis; 8. distal-most point of the third lower molar cervix; 9. distal-most point of the third upper molar cervix; 10. Posterior-most point of the incisor alveoli; 11. anterior-most point of the anterior palatine foramen.

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