Published in final edited form as:
Mech Dev. 2008 ; 125(9-10): 777–785. doi:10.1016/j.mod.2008.06.009.

RAX is required for fly neuronal development and mouse embryogenesis

Richard L. Bennett[1], **William L. Blalock**[2], **Eun-Jung Choi**[3], **Young J. Lee**[3], **Yanping Zhang**[4], **Lei Zhou**[4], **S. Paul Oh**[3], and **W. Stratford May**[1,*]

1 *University of Florida, Shands Cancer Center, Kevin Cameron laboratory, University of Bologna, Italy*

2 *Cell Signaling Laboratory, Department of Human Anatomical Sciences, University of Bologna, Italy*

3 *University of Florida, Department of Physiology and Functional Genomics*

4 *University of Florida, Department of Molecular Genetics and Microbiology*

Abstract

RAX was originally discovered as the unique cellular activator for the dsRNA-dependent, interferon-inducible protein kinase PKR. Recent findings indicate that RAX is also a critical component of the RNA-induced silencing complex and a regulator of transcription. Here we report novel phenotypes for both fruit flies carrying a transposon insertion in the 5' UTR of *dRax* (independently identified as *loqs*/R3D1) and mice with a deletion of the entire *Rax* gene. In *Drosophila* we observe a high level of *dRax* expression in the developing nerve cord. Mutant fly embryos homozygous for the insertion dRax[f00791] display highly abnormal commissural axon structure of the CNS and 70% of the flies homozygous for the mutant allele die prior to adulthood. Surviving male flies have reduced fertility and female flies are sterile. Furthermore, these flies appear to have a severe defect in nervous system coordination or neuromuscular function resulting in significantly reduced locomotion. Mice were also generated that are heterozygous for a deletion of the entire *Rax* gene (exons 1–8). While mice that are heterozygous for the mutant allele are viable and appear normal, we are unable to obtain mice homozygous for this mutant allele. Furthermore, we have not observed any embryo obtained by mating heterozygous mice at either E3.5, 7, or 14 that is nullizygous for the *Rax* gene. Since *Rax* is expressed in preimplantation blastocysts, these data indicate that deletion of the entire *Rax* gene is embryonic lethal in mice at a preimplantation stage of development. Collectively, these findings in two different species illustrate the importance of RAX for embryonic development.

Keywords

RAX; PKR; dsRNA; fly; mouse; embryogenesis; knockout

1. Introduction

RAX and its human ortholog PACT were independently discovered as the cellular activator for the interferon-inducible, dsRNA-dependent protein kinase PKR (Ito et al., 1999a; Ito et al.,

*Corresponding author: W. Stratford May, University of Florida, Shands Cancer Center, 1376 Mowry Rd. Box 103633 Gainesville, FL 32610-3633 Tel.: 352-273-8010; Fax: 352-273-8285 email: smay@ufl.edu.

1999b; Patel et al., 2000; Patel and Sen, 1998). In this role, cellular stresses such as IL-3 withdrawal from factor-dependent hematopoietic cells, treatment with inflammatory cytokines or viral infection induce RAX phosphorylation to promote activation of PKR and inhibition of eIF2α-dependent protein synthesis (Bennett et al., 2006; Bennett et al., 2004; Ito et al., 1994; Ito et al., 1999b). While PKR has been primarily studied for its anti-viral and anti-proliferation effects via its capacity to inhibit protein synthesis, it has also been reported to regulate several transcription factors, including p53, NF-κB and STAT1 (Bonnet et al., 2000; Cuddihy et al., 1999a; Cuddihy et al., 1999b; D'Acquisto and Ghosh, 2001; Fremont et al., 2006; Gil et al., 2000; Wong et al., 1997; Yeung and Lau, 1998; Zamanian-Daryoush et al., 2000). Thus, the RAX-PKR stress-signaling pathway is necessary for the cellular response to a broad range of apoptosis-inducing stress by functioning as a regulator of both transcription and translation.

RAX and PACT are 98% identical in amino acid structure and consist of three conserved dsRNA binding domains (Figure 1A). The N-terminal first and second domains bind dsRNA and are necessary for RAX/PACT association with PKR, whereas the third C-terminal domain is apparently not necessary for dsRNA binding or interaction with PKR but is required for activating PKR's kinase activity (Huang et al., 2002; Peters et al., 2001). It has been demonstrated that RAX/PACT can efficiently bind to and activate PKR *in vitro* (Ito et al., 1999b; Patel and Sen, 1998). However, *in vivo* RAX/PACT mediated PKR activation is dependent on a stress application to cells (Ito et al., 1999b; Patel et al., 2000). Although both RAX/PACT and PKR are dsRNA-binding proteins, PKR activation may not require dsRNA binding since *in vitro* activation by RAX/PACT does not require dsRNA.

In addition to its role as an activator of PKR, RAX has recently been reported to be an integral component of the RNA-induced silencing complex (RISC) (Kok et al., 2007; Lee et al., 2006). RAX associates with a ~500 kDa complex containing Tar binding protein (TRBP), Dicer, and Argonuate-2 that is capable of pre-miRNA processing and target cleavage (Lee et al., 2006). Furthermore, depletion of RAX from HeLa cells inhibits the siRNA-mediated silencing of a reporter gene and prevents the accumulation of mature miRNA (Lee et al., 2006). Interestingly, the third C-terminal dsRNA-binding domain of RAX interacts with the N-terminal helicase motif of Dicer and it has been proposed that RAX has a function in RISC assembly (Lee et al., 2006). The *D. melanogaster* ortholog of RAX (also named *loqs* or R3D1) is required for normal pre-miRNA processing and interacts with Dicer-1 (Forstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005). It has been reported that female flies homozygous for the insertion of a transposon in the first exon of *dRax* are sterile due to a defect in ovary germline stem cell maintenance (Forstemann et al., 2005; Jiang et al., 2005). Furthermore, *dRax* is required for silencing of the endogenous *Stellate* locus by *Suppressor of Stellate* in the testes of male flies (Forstemann et al., 2005).

Recently, it has been reported that disruption of the mouse *Rax* gene causes developmental defects in the otic system and mice are smaller in size (Rowe et al., 2006). This *Rax* targeted mouse was generated by replacing the last exon of the Rax gene, exon 8, with a neomycin resistance cassette in a strategy designed to delete all of the coding sequence for the C-terminal, third dsRNA binding domain of RAX (Rowe et al., 2006). Thus, mice homozygous for this mutant allele produce an aberrant transcript containing the first seven exons of the *Rax* ORF but apparently no protein product is observed that corresponds to the C-terminal truncation mutant (Rowe et al., 2006).

Concurrent with the aforementioned studies using the *Drosophila Rax* mutant strain and a mouse with a deletion of *Rax* exon 8, our laboratory initiated studies of the *dRax* fly mutant and was in the process of generating a *Rax* knockout mouse to ascertain the physiologic function of RAX. Here we report that flies homozygous for dRax[f00791] not only have reduced fertility

as observed by others but also display defects in central nervous system development. Furthermore, we find that deletion of exons 1 – 8, comprising the entire ORF of the murine *Rax* allele, is embryonic lethal at the preimplantation stage. These findings demonstrate the importance of RAX for embryogenesis.

2. Results

2.1. Drosophila ortholog of Rax (dRax) is required for central nervous system development

A *Drosophila* gene, CG6866, was identified as the ortholog of *Rax/Pact* based on sequence comparison. Like RAX, the dRax protein has three predicted double strand RNA binding domains (dsRBDs, Figure 1A). The same gene was identified independently by Forstemann et al (Forstemann et al., 2005) and Jiang et al (Jiang et al., 2005), whose work reveals that dRax (loqs) is indeed a dsRNA binding protein required for miRNA maturation and germ cell development. The *Drosophila* RAX ortholog has similar amino acid homology to either the mammalian proteins RAX/PACT or Tar binding protein, TRBP (Figure 1A). Significantly, the dsRNA binding domains of these proteins share considerable (52 – 77%) homology (Figure 1B).

Using a cRNA probe, we performed *in situ* hybridization to monitor the expression of *dRax* during embryogenesis. *dRax* mRNA is deposited in the eggs prior to the onset of zygotic expression. At stage 9–10, high level of *dRax* expression is observed in the developing nerve cord (Figure 2A). This stage corresponds to formation of the early nervous system, when the neuroblasts delaminate from the ectoderm to form the nerve cord. Thus, nerve cord specific expression of *dRax* suggests that dRax function may play a role in nervous system differentiation. The strong *dRax* mRNA signal in the central nervous system (CNS) persists through embryogenesis even after the early differentiation stage. The CNS and some neuroganglia are the only tissues with a strong *dRax* signal at the end of embryogenesis (Figure 2B).

Correspondingly, we found that *dRax* is required for proper nervous system development during embryogenesis. In mutant embryos homozygous for the insertion dRax[f00791], both the longitudinal and commissural axon structures of the CNS are highly abnormal (Figure 2C vs. D), reflecting the disorganization of the nervous system. The mutant is a PBac insertion in the first exon of *dRax* directly upstream of the translation start site that creates a hypomorphic mutant allele by attenuating its transcription (Forstemann et al., 2005). *In situ* hybridization analysis of the dRax[f00791] mutant embryos demonstrates a significant decrease of *dRax* expression in the developing nerve cord (data not shown). However, this is not a null mutant since there are detectable levels of *dRax* expression in the nerve cord of the mutant embryos (data not shown). Nonetheless, about 70% of homozygous animals die before adulthood while surviving males have reduced fertility and females are sterile. The CNS abnormality observed for mutant embryos manifested at the end of embryogenesis. A general, albeit subtler, abnormality in the CNS could also be detected using markers of specific neurons, such as Futsch (22C10) and Elav (data not shown). However, of the markers tested, BP102 staining that labels all axons in the CNS revealed the most striking phenotype (Figure 2C and D). Interestingly, we observe that the surviving homozygous flies are often stuck and unable to escape from food, which indicates a severe defect in nervous system coordination or neuromuscular function. Furthermore, using a Random Locomotion Test we observed that male homozygous dRax mutants collected 3 days after eclosion have a 69% decrease in locomotor activity compared to heterozygous control flies ($P < 0.01$, Figure 2E). Taken together these results point to the requirement of dRax for nervous system development.

2.2. Deletion of the Rax gene in mice results in preimplantational embryonic lethality

In order to investigate the function of RAX in mice, we generated a novel *Rax* null allele in which the entire RAX coding sequence is deleted. A 2.2 Kb genomic fragment corresponding to the *Rax* promoter and the first 130 bps of *Rax* exon 1 (just prior to the *Rax* AUG start codon), and a 7 Kb genomic region corresponding to the last 300 bps of *Rax* exon 8 (non-coding) and 6.7 Kb of intergenic sequence were used as 5′ and 3′ homology arms of the knockout vector, respectively (Figure 3A). This strategy avoids a "partial" or incomplete knockout of *Rax* that might produce a hypomorph and complicate data interpretation. In addition, there are no known/predicted genes overlapping the *Rax* locus that might be affected by removal of the targeted sequence.

The *Rax* targeting vector was linearized and electroporated into mouse embryonic stem (ES) cells. DNA was isolated from ES cell clones and analyzed by Southern blotting using a 5′ probe to detect proper insertion by a shift in the size of the genomic DNA *EcoR V* fragment from 15 Kb to 7 Kb. Two ES cell clones positive for correct targeting into the *Rax* locus (Figure 3B) were injected into C57BL/6J blastocysts, and implanted into pseudo-pregnant females. Offspring coat color was examined to determine chimerism. Five chimeric mice with significant brown agouti coat color were generated (3 male, 2 female). These chimeric offspring were further mated with C57BL/6J wild type mice and maintained in a 129/B6 hybrid background. Germline transmission of the *Rax* knockout allele was confirmed by Southern blot analysis (Figure 3C). Mice heterozygous for the *Rax* mutant allele ($Rax^{+/-}$) were intercrossed to produce *Rax*-null ($Rax^{-/-}$) mice. Significantly, among a total of 76 mice from $Rax^{+/-}$ crosses (12 litters; average litter size of 6 – 7 mice) at weaning age, 17 and 59 mice were identified as $Rax^{+/+}$ and $Rax^{+/-}$ mice, respectively, but no $Rax^{-/-}$ mice were recovered ($P < 0.05$, Figure 3D and Table I). We have not observed any postnatal lethality of the $Rax^{+/-}$ mice. Furthermore, mating heterozygous $Rax^{+/-}$ mice of either sex with wild type mice results in an approximately 1:1 ratio of wild type to heterozygous offspring, demonstrating that there is no defect in production and/or transmission of the zygote carrying the mutant allele (data not shown). Taken together, these results indicate that deletion of the entire *Rax* gene is embryonic lethal.

In order to determine the embryonic stage at which lethality occurred for $Rax^{-/-}$ mice, embryos derived by $Rax^{+/-}$ crosses were isolated at various gestational stages, and their genotypes were determined by both Southern blotting and PCR analyses (Figure 3E and F). Both methods of genotyping yielded the same result. No embryos were identified as $Rax^{-/-}$ at either embryonic day (E)13.5 or E7.5 (Table I). Significantly, upon examination of 33 blastocysts (E3.5 embryos) isolated from 6 litters of $Rax^{+/-}$ crosses by PCR, we detected 9 $Rax^{+/+}$, 24 $Rax^{+/-}$ and 0 $Rax^{-/-}$ embryos ($P < 0.05$, Table I and Figure 3F). Taken together, these results suggest that deletion of the *Rax* gene is embryonic lethal at a preimplantation stage of development.

In an effort to generate a *Rax* null embryonic stem (ES) cell line, we used the targeted ES cells heterozygous for the *Rax* mutant allele and high G418 concentration (2mg/ml) to select, by gene conversion, ES cells devoid of both *Rax* alleles. However, after three separate attempts, we were unable to select any ES cell clone with both alleles carrying the *Rax* deletion mutant (data not shown). Since Rax expression can be detected in wild type ES cells by RT-PCR (data not shown), these data suggest that RAX may be required for ES cell growth.

Rax is ubiquitously expressed throughout the mouse (UniProtKB/SwissProt entry Q9WTX2). To demonstrate that mice with the *Rax*-knockout allele produce less *Rax* mRNA and protein than $Rax^{+/+}$ littermates, we compared lung and brain tissues, two tissues that express high levels of RAX, from $Rax^{+/+}$ or $Rax^{+/-}$ mice by quantitative real-time PCR and by Western blotting. Significantly, an average of 15% fewer Rax transcripts are detected in the brains and lungs from $Rax^{+/-}$ mice than those from $Rax^{+/+}$ mice (Figure 4A, $P = 0.05$). In addition, lysates

generated from the brains of $Rax^{+/-}$ mice have an average of 33% less RAX protein compared to lysates from $Rax^{+/+}$ brains as determined by densitometry of western blots (Figure 4B and C, P = 0.04). These data indicate that the *Rax* knockout allele does not produce RAX-coding transcripts.

Finally, by RT-PCR with primers specific for *Rax* mRNA, we have detected *Rax* gene expression in wild type mouse blastocysts and ES cells (data not shown). Thus, *Rax* is expressed at a pre-implantation stage of embryogenesis.

3. Discussion

The results of these studies establish the requirement for the dsRNA-binding protein RAX during early development and embryogenesis of both the fly and mouse. Significantly, our analysis of the *Drosophila* ortholog of *Rax*, dRax (loqs, R3D1), indicates that *dRax* is expressed during nervous system development and required for nervous system organization. Interestingly, flies homozygous for the dRax[f00791] transposon insertion are unable to escape after having landed on food and have a severe reduction in locomotion activity, indicating either a neurological or neuromuscular defect. Similar to reports from other laboratories, we observed that the homozygous dRax[f00791] mutant is female sterile, a phenotype previously attributed to dRax's role in processing miRNAs specific for germ cell maturation during normal oogenesis (Czech et al., 2008; Forstemann et al., 2005; Jiang et al., 2005; Park et al., 2007). Thus, our findings confirm those of others and extend them by indicating that dRax is also required for maturation of the drosophila nervous system. Future work may now include detailed analysis of the nervous system abnormalities in the dRax mutant flies to determine whether the mechanism for this defect is dependent on nervous system specific miRNA processing, cell proliferation and/or apoptosis.

The *Rax* gene is expressed throughout all tissues of the adult mouse and mRNA is detectable even at the preimplantation, blastocyst stage of embryogenesis. To investigate the function of murine *Rax*, we generated mice carrying a mutant *Rax* allele where exons 1 through 8, comprising the entire *Rax* ORF, have been deleted. Heterozygous $Rax^{+/-}$ mice appear normal in size, display no overt abnormal behavior and there is no statistically significant difference between the littersize of embryos and live born mice. Importantly, when $Rax^{+/-}$ mice are mated with wild type mice the expected 1:1 distribution of wild type to heterozygous offspring is observed, confirming that there is not a defect in transmission of the mutant allele. While the $Rax^{+/-}$ heterozygous mice are viable, we were unable to obtain any viable $Rax^{-/-}$ homozygous mice or embryos even at the blastocyst stage, indicating that deletion of the entire *Rax* gene is crucial for early embryonic development. Taken together, these results demonstrate that RAX is required for normal mouse embryonic development.

Significantly, tissues from $Rax^{+/-}$ mice produce more than half of the *Rax* transcripts and protein level present in the $Rax^{+/+}$ tissues. In addition, we observed that the level of RAX protein varied from mouse to mouse relative to control proteins. These findings suggest that $Rax^{+/-}$ mice may have increased expression of the remaining wild type *Rax* allele by some uncharacterized autoregulatory feedback mechanism.

In contrast to the findings presented here, it was recently reported that mice homozygous for a deletion in the last exon of the *Rax* gene (exon 8) are viable but display a developmental defect of the ear, auditory deficiency and are smaller in size than normal littermates (Rowe et al., 2006). While these null mice produce a RAX transcript encoding a C-terminal deletion mutant of RAX that would be missing the third dsRNA binding domain, the authors did not detect any protein product by Western blotting (Rowe et al., 2006). However, it remains a formal possibility that a partially functional mutant RAX protein, which remains able to bind

dsRNA and/or other proteins, could be produced to cause a hypomorphic phenotype. Such a RAX mutant product may not be detectable with the antibody used and could account for any discrepancy with results reported here.

Since *Rax* is ubiquitously expressed even during early mouse embryogenesis and there is no other homolog that may compensate any loss of expression, it is not surprising that complete deletion of *Rax* is early embryonic lethal in the mouse. In addition to functioning as an activator of PKR, recent evidence reveals that RAX is also a component of the RISC (Kok et al., 2007; Lee et al., 2006). Thus, while the Rowe *et al* mutant *Rax* allele may not produce a detectable protein product capable of activating PKR, any truncated RAX produced may remain able to perform other less well-characterized signal transduction and/or RNA interference pathway functions. In support of this notion, *Pkr* knockout mice are viable and do not display an overt phenotype, suggesting that RAX may function as more than just an activator of PKR (Yang et al., 1995). In addition, our laboratory and others have observed that RAX and TRBP can from a heterodimer that is dependent on their first and second dsRNA binding domains (data not shown; Kok et al., 2007; Laraki et al., 2008). It has been suggested that this RAX-TRBP heterodimer may not function to regulate PKR activity, but instead interacts with dicer to contribute to the RNA interference pathway (Kok et al., 2007; Laraki et al., 2008). Significantly, mice deficient for several other dsRNA binding proteins involved in RNA interference have also been reported to display catastrophic developmental phenotypes similar to what we observe for the *dRax* mutant fly and the *Rax* knockout mouse (Bernstein et al., 2003; Zhong et al., 1999). For example, deletion of the mouse ortholog of TRBP yields viable born mice that either die immediately after weaning or, in the case of any surviving males, have a defect in sperm maturation (Zhong et al., 1999). Moreover, Dicer$^{-/-}$ null mice are early embryonic lethal (Bernstein et al., 2003). Interestingly, *Rax* is more highly evolutionarily conserved than *Pkr* since a PKR ortholog has not been identified in *Drosophila* (Murphy et al., 2008). Collectively these results support the importance of RNA interference during embryogenesis and may indicate that RAX is a necessary regulator of RNA interference as well as PKR activation. In addition, mammalian RAX may be a functional link between any miRNA-mediated silencing and PKR-dependent inhibition of protein synthesis that occurs during cellular stress and/or development. In support of this notion, it has been recently reported that members of the RISC can associate with translation initiation proteins present in the ribosome to inhibit protein synthesis (Chendrimada et al., 2007; Thermann and Hentze, 2007). Future work will now be required to determine whether and how RAX-dependent PKR activation is regulated by miRNA produced during cellular stress to inhibit protein synthesis.

In summary, our findings illustrate the importance of the *Rax* gene to fly and mouse development. In addition, our data suggest that the previously reported *Rax*-knockout mouse allele may be hypomorphic. Unfortunately, we are unable to further assess the role of RAX as a regulator of RNA interference or an activator of PKR during early development due to the very early embryonic lethality of our *Rax*-null mice. Future work may now include generation of tissue-specific or inducible Rax-knockout systems to further elucidate the *in vivo* function of RAX.

4. Experimental Procedures

4.1. Drosophila work

The fly strain, PBac{WH}[f00791]/Cyo, was obtained from the Bloomington stock center. The insertion site was verified via In Verse PCR and sequencing. cDNA for *dRax* was isolated from an embryonic cDNA library by PCR, verified by sequencing, subcloned into pBluescript, and used as template for synthesizing the cRNA probe. *In situ* hybridization and histological analysis using anti-102 (visualizing the axon structure) were performed as previously described

(Zhou et al., 1997). To perform the Random Locomotion Test male dRax homozygous mutants and their control littermates of genotype dRax[f00791]/Cyo were collected daily after eclosion. At day 3 they were subjected to the random locomotion test as described (Kane et al., 1997). F test, and subsequently Welch's t-test were performed using R.

4.2. Generation of the Rax knockout mouse

The targeting vector for the *Rax* knockout was constructed using pTK-loxNeo as a vector backbone with the neomycin resistance (*neo*^{r}) cassette flanked by fragments of the *Rax* gene. To isolate a portion of the 3′ end of the *Rax* gene, a mouse 129/SV genomic DNA BAC library was screened by Genome Systems to obtain a BAC clone containing *Rax* genomic DNA. DNA sequencing of this BAC clone revealed that it contained about a 15Kb genomic fragment corresponding to exons 4 through 8 of the *Rax* genomic sequence. A 7 Kb *Hind III* fragment consisting of approximately the last 300 bps of *Rax* exon 8 (non-coding) and 6.7 Kb of non-transcribed and non-coding sequence downstream of the *Rax* gene was inserted into the *Hind* III site of pTK-loxNeo (downstream of the *neo*^{r} cassette) to create the vector pTK-3'RAX. To generate a DNA fragment for the 5′ end of the *Rax* gene, BAC clone RPCI-22 68D6 (Invitrogen) containing the entire *Rax* gene (Rowe and Sen, 2001), was used as a template to amplify a 2.2 Kb portion of the *Rax* gene corresponding to the *Rax* promoter and the first 120 bps of *Rax* exon 1 (just prior to the *Rax* AUG start codon) by PCR. This 5′ flanking fragment was inserted into the *Cla I* site of pTK-3′RAX between the human thymidine kinase gene (hTK; for positive selection) and the *neo*^{r} cassette to generate the final targeting vector, pKO-RAX.

60μg of the targeting vector pKO-RAX was linearized with *Xho I* and electroporated into J1 ES cells. Cells were grown in ES medium (DMEM, 20% FBS, 1% nonessential amino acids, 0.5% Penicillin-Streptomycin, 10^5 U/ml LIF) containing 400 mg/ml G418 and 250 nM FIAU. DNA was isolated from the resulting clones, digested with *EcoR V*, and homologous insertion was determined by Southern blotting using a ^{32}P-labeled 520 bp probe with sequence corresponding to a region 5′ to the site of homologous insertion (chr2: 76490155 – 76490676). Two out of 360 ES cell clones were identified as positive for the RAX knockout allele, injected into C57BL/6J blastocysts, and implanted into psuedopregnant females. Chimeric offspring were then mated with C57BL/6J wild type mice (Charles River laboratories) and germline transmission of the *Rax* mutant was confirmed by Southern blot analysis using the 5′ probe (Figure 2).

In addition, PCR was performed on tail or embryo genomic DNA to confirm mouse genotype using primers specific to *Rax* wild type and mutant alleles, (Figure 2). Primer sequences used for genotyping are as follows (5′ – 3′): RAX1-FWD, TCTGAGAAGCTCTGGGAACCCAACTAG; RAX1-REV, GATGCCTGCTATGGGACATGGCGAGAAG; NeoF, ATGAACTGCAGGACGAGGCAGCG; NeoR, GGCGATAGAAGGCGATGCGCTG.

4.3. PCR and western blotting with mouse tissues

Lung and Brain tissues were isolated from mice, flash frozen in liquid nitrogen and stored at -80^o C. Tissues were disrupted by mortar and pestle and homogenized using a QIAshredder (Qiagen, Valencia CA). DNA, RNA, and protein extracts were prepared from whole tissues using the Allprep DNA/RNA/Protein isolation kit (Qiagen, Valencia CA). RNA integrity and concentration were determined by nanodrop and bioanalyzer QC analysis. Quantitative real-time PCR was performed by the University of Florida, real-time PCR core facility using TaqMan gene expression assay Mm00478737_m1 for *PRKRA* (RAX) and mouse β-actin as endogenous control (Applied Biosystems, Foster City CA). Western blotting was performed using rabbit polyclonal antibody to RAX as described (Bennett et al., 2004). Individual bands

in Western blots were quantified using Imagequant 5.0 (GE healthcare) and statistical significance determined using ANOVA data analysis in Microsoft Excel.

In addition, mRNA from 20 pooled C57BL/6J blastocysts was isolated using a Dynabeads mRNA DIRECT Micro kit (Invitrogen, Carlsbad, CA) and cDNA was prepared using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City CA). The primers used to detect RAX mRNA were: RAX2-3FWD: 5′-TGCACAGGTGAAGGTACGAG - 3′ and RAX6-7REV 5′ – CACGTTCGTTAGAGAAATGTGG – 3′.

Acknowledgements

This work was supported by NIH grant 5R01HL054083 and Bankhead-Coley award 06BB-14. We wish to thank Dr. Peter Ruvolo, Dr. Edward Scott and Gary Brown for their assistance in designing the RAX knockout mouse strategy, Zhigang Jiang for technical assistance during targeting of the mouse ES cells. Dr. Zhou is supported by CA95542.

References

Bennett RL, Blalock WL, Abtahi DM, Pan Y, Moyer SA, May WS. RAX, the PKR activator, sensitizes cells to inflammatory cytokines, serum withdrawal, chemotherapy and viral infection. Blood. 2006

Bennett RL, Blalock WL, May WS. Serine 18 phosphorylation of RAX, the PKR activator, is required for PKR activation and consequent translation inhibition. J Biol Chem 2004;279:42687–93. [PubMed: 15299031]

Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ. Dicer is essential for mouse development. Nat Genet 2003;35:215–217. [PubMed: 14528307]

Bonnet MC, Weil R, Dam E, Hovanessian AG, Meurs EF. PKR stimulates NF-kappaB irrespective of its kinase function by interacting with the IkappaB kinase complex. Mol Cell Biol 2000;20:4532–42. [PubMed: 10848580]

Chendrimada TP, Finn KJ, Ji X, Baillat D, Gregory RI, Liebhaber SA, Pasquinelli AE, Shiekhattar R. MicroRNA silencing through RISC recruitment of eIF6. Nature 2007;447:823–8. [PubMed: 17507929]

Cuddihy AR, Li S, Tam NW, Wong AH, Taya Y, Abraham N, Bell JC, Koromilas AE. Double-stranded-RNA-activated protein kinase PKR enhances transcriptional activation by tumor suppressor p53. Mol Cell Biol 1999a;19:2475–84. [PubMed: 10082513]

Cuddihy AR, Wong AH, Tam NW, Li S, Koromilas AE. The double-stranded RNA activated protein kinase PKR physically associates with the tumor suppressor p53 protein and phosphorylates human p53 on serine 392 in vitro. Oncogene 1999b;18:2690–702. [PubMed: 10348343]

Czech B, Malone CD, Zhou R, Stark A, Schlingeheyde C, Dus M, Perrimon N, Kellis M, Wohlschlegel JA, Sachidanandam R, Hannon GJ, Brennecke J. An endogenous small interfering RNA pathway in Drosophila. Nature. 2008

D'Acquisto F, Ghosh S. PACT and PKR: turning on NF-kappa B in the absence of virus. Sci STKE 2001;2001:RE1. [PubMed: 11752660]

Forstemann K, Tomari Y, Du T, Vagin VV, Denli AM, Bratu DP, Klattenhoff C, Theurkauf WE, Zamore PD. Normal microRNA maturation and germline stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. PLoS Biol 2005;3:e236. [PubMed: 15918770]

Fremont M, Vaeyens F, Herst CV, De Meirleir KL, Englebienne P. Double-stranded RNA-dependent protein kinase (PKR) is a stress-responsive kinase that induces NFkappaB-mediated resistance against mercury cytotoxicity. Life Sci 2006;78:1845–56. [PubMed: 16324719]

Gil J, Alcami J, Esteban M. Activation of NF-kappa B by the dsRNA-dependent protein kinase, PKR involves the I kappa B kinase complex. Oncogene 2000;19:1369–78. [PubMed: 10723127]

Huang X, Hutchins B, Patel RC. The C-terminal, third conserved motif of the protein activator PACT plays an essential role in the activation of double-stranded-RNA-dependent protein kinase (PKR). Biochem J 2002;366:175–86. [PubMed: 11985496]

Ito T, Jagus R, May WS. Interleukin 3 stimulates protein synthesis by regulating double-stranded RNA-dependent protein kinase. Proc Natl Acad Sci U S A 1994;91:7455–9. [PubMed: 7519779]

Ito T, Warnken SP, May WS. Protein synthesis inhibition by flavonoids: roles of eukaryotic initiation factor 2alpha kinases. Biochem Biophys Res Commun 1999a;265:589–94. [PubMed: 10558914]

Ito T, Yang M, May WS. RAX, a cellular activator for double-stranded RNA-dependent protein kinase during stress signaling. J Biol Chem 1999b;274:15427–32. [PubMed: 10336432]

Jiang F, Ye X, Liu X, Fincher L, McKearin D, Liu Q. Dicer-1 and R3D1-L catalyze microRNA maturation in Drosophila. Genes Dev 2005;19:1674–9. [PubMed: 15985611]

Kane NS, Robichon A, Dickinson JA, Greenspan RJ. Learning without performance in PKC-deficient Drosophila. Neuron 1997;18:307–14. [PubMed: 9052800]

Kok KH, Ng MH, Ching YP, Jin DY. Human TRBP and PACT directly interact with each other and associate with dicer to facilitate the production of small interfering RNA. J Biol Chem 2007;282:17649–57. [PubMed: 17452327]

Laraki G, Clerzius G, Daher A, Melendez-Pena C, Daniels S, Gatignol A. Interactions between the double-stranded RNA-binding proteins TRBP and PACT define the Medipal domain that mediates protein-protein interactions. RNA Biol 2008:5. [PubMed: 18388484]

Lee Y, Hur I, Park SY, Kim YK, Suh MR, Kim VN. The role of PACT in the RNA silencing pathway. Embo J 2006;25:522–32. [PubMed: 16424907]

Murphy D, Dancis B, Brown JR. The evolution of core proteins involved in microRNA biogenesis. BMC Evol Biol 2008;8:92. [PubMed: 18366743]

Park JK, Liu X, Strauss TJ, McKearin DM, Liu Q. The miRNA pathway intrinsically controls self-renewal of Drosophila germline stem cells. Curr Biol 2007;17:533–8. [PubMed: 17320391]

Patel CV, Handy I, Goldsmith T, Patel RC. PACT, a stress-modulated cellular activator of interferon-induced double-stranded RNA-activated protein kinase, PKR. J Biol Chem 2000;275:37993–8. [PubMed: 10988289]

Patel RC, Sen GC. PACT, a protein activator of the interferon-induced protein kinase, PKR. Embo J 1998;17:4379–90. [PubMed: 9687506]

Peters GA, Hartmann R, Qin J, Sen GC. Modular structure of PACT: distinct domains for binding and activating PKR. Mol Cell Biol 2001;21:1908–20. [PubMed: 11238927]

Rowe TM, Rizzi M, Hirose K, Peters GA, Sen GC. A role of the double-stranded RNA-binding protein PACT in mouse ear development and hearing. Proc Natl Acad Sci U S A 2006;103:5823–8. [PubMed: 16571658]

Rowe TM, Sen GC. Organizations and promoter analyses of the human and the mouse genes for PACT, the protein-activator of the interferon-induced protein kinase, PKR. Gene 2001;273:215–25. [PubMed: 11595168]

Saito K, Ishizuka A, Siomi H, Siomi MC. Processing of pre-microRNAs by the Dicer-1-Loquacious complex in Drosophila cells. PLoS Biol 2005;3:e235. [PubMed: 15918769]

Thermann R, Hentze MW. Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation. Nature 2007;447:875–8. [PubMed: 17507927]

Wong AH, Tam NW, Yang YL, Cuddihy AR, Li S, Kirchhoff S, Hauser H, Decker T, Koromilas AE. Physical association between STAT1 and the interferon-inducible protein kinase PKR and implications for interferon and double-stranded RNA signaling pathways. Embo J 1997;16:1291–304. [PubMed: 9135145]

Yang YL, Reis LF, Pavlovic J, Aguzzi A, Schafer R, Kumar A, Williams BR, Aguet M, Weissmann C. Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. Embo J 1995;14:6095–106. [PubMed: 8557029]

Yeung MC, Lau AS. Tumor suppressor p53 as a component of the tumor necrosis factor-induced, protein kinase PKR-mediated apoptotic pathway in human promonocytic U937 cells. J Biol Chem 1998;273:25198–202. [PubMed: 9737981]

Zamanian-Daryoush M, Mogensen TH, DiDonato JA, Williams BR. NF-kappaB activation by double-stranded-RNA-activated protein kinase (PKR) is mediated through NF-kappaB-inducing kinase and IkappaB kinase. Mol Cell Biol 2000;20:1278–90. [PubMed: 10648614]

Zhong J, Peters AH, Lee K, Braun RE. A double-stranded RNA binding protein required for activation of repressed messages in mammalian germ cells. Nat Genet 1999;22:171–4. [PubMed: 10369260]

Zhou L, Schnitzler A, Agapite J, Schwartz LM, Steller H, Nambu JR. Cooperative functions of the reaper and head involution defective genes in the programmed cell death of Drosophila central nervous system midline cells. Proc Natl Acad Sci U S A 1997;94:5131–6. [PubMed: 9144202]

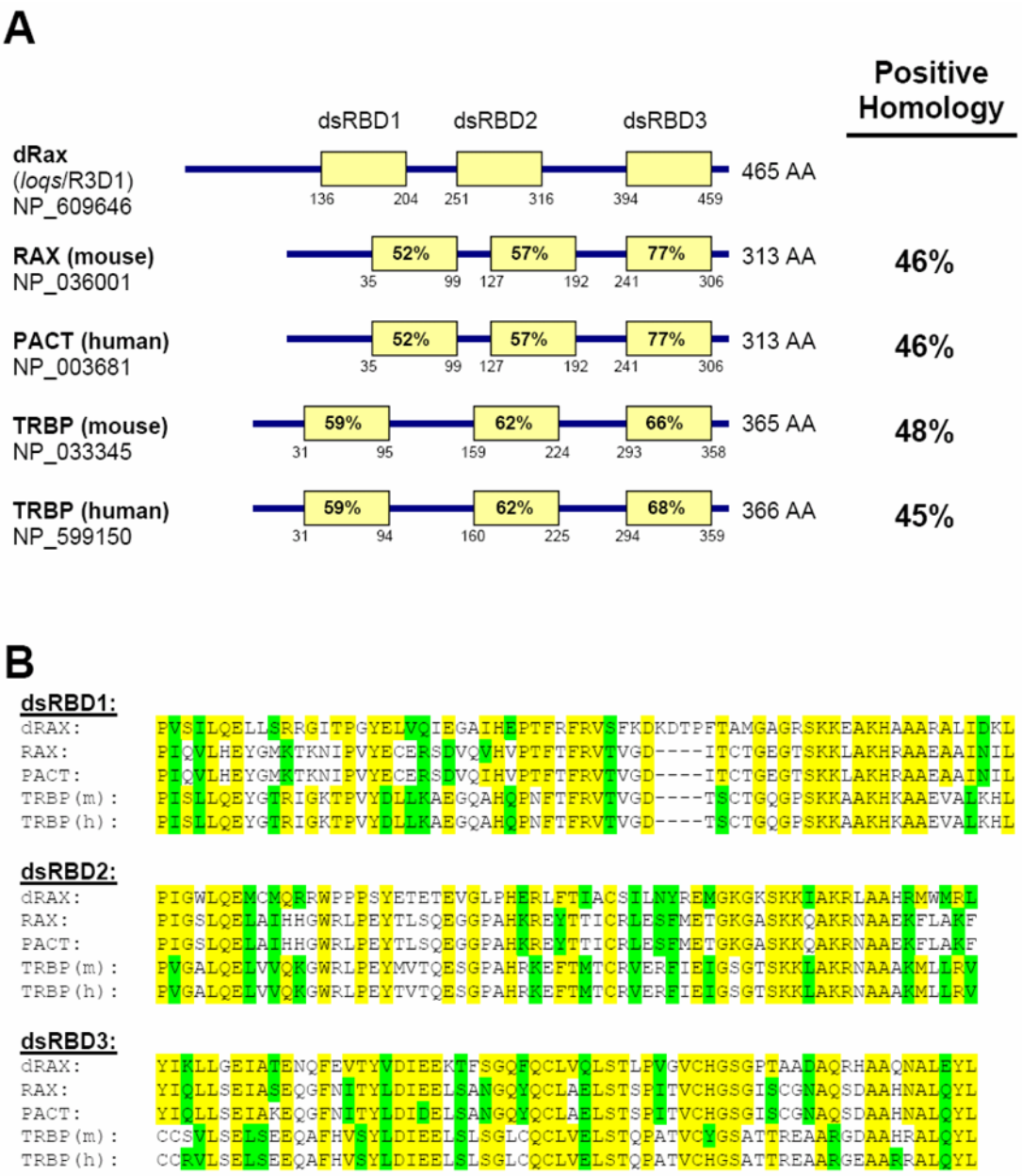

Figure 1. CG6866 is a *Drosophila* ortholog to either mammalian RAX/PACT or TRBP

A. Comparison of the domain structures of dRax protein to RAX, PACT and TRBP proteins. The length of each protein is given in amino acids (AA) and the position of each dsRNA-binding domain (dsRBD) is indicated below the corresponding box. The dsRBDs were predicted using ScanProsite. Positive homology (identical and conserved residues) to dRax was determined using BLASTp. Homology between each mammalian dsRBD and its fly counterpart is indicated within each dsRBD box. B. Amino acid sequence alignment between dsRNA binding domains of the fly and mammalian orthologs. Residues identical to dRax are highlighted in yellow while conserved homology is highlighted in green.

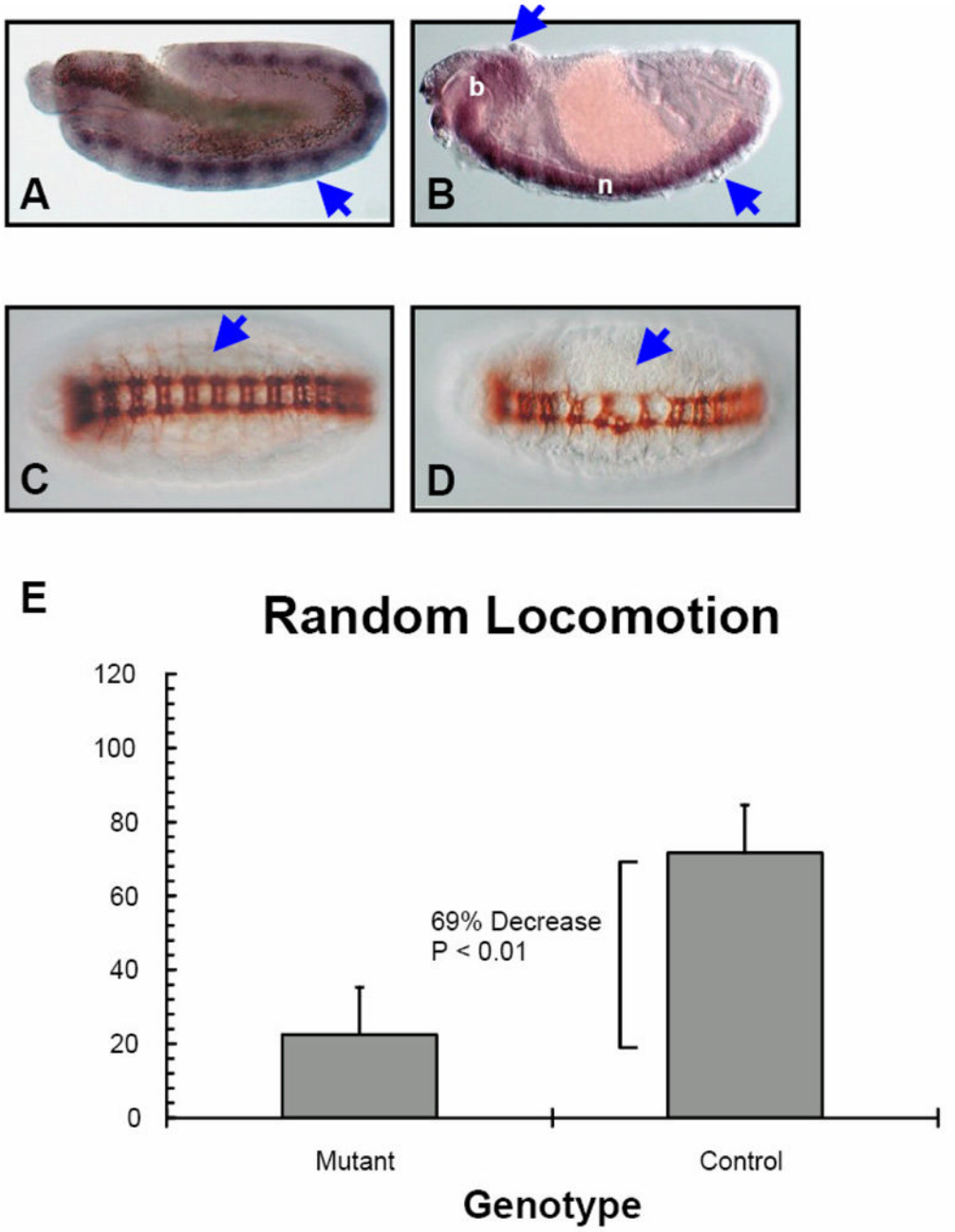

Figure 2. *Drosophila* ortholog of Rax (*dRax*) is required for nervous system development

A. Stage 9–10 wild type embryo, sagittal view. dRax is specifically expressed in the early differentiating nerve cord (indicated by arrowhead). B. Stage 15 wild type embryo, sagittal view. In later stage embryos, dRax expression persists in the differentiating/differentiated central nervous system (arrowheads indicate elevated dRAX expression: b, brain; n, nerve cord). C. Wild type embryo stained with anti-102 to visualize the commissural axon structure (arrowhead). D. The commissural axon structure is disrupted in homozygous dRax mutant embryos (arrowhead). E. Compared to heterozygous (dRax[f00791]/cyo) control flies, homozygous dRax mutant flies have significantly reduced locomotor activity (data represented as Avg.+Stdev.; n=12).

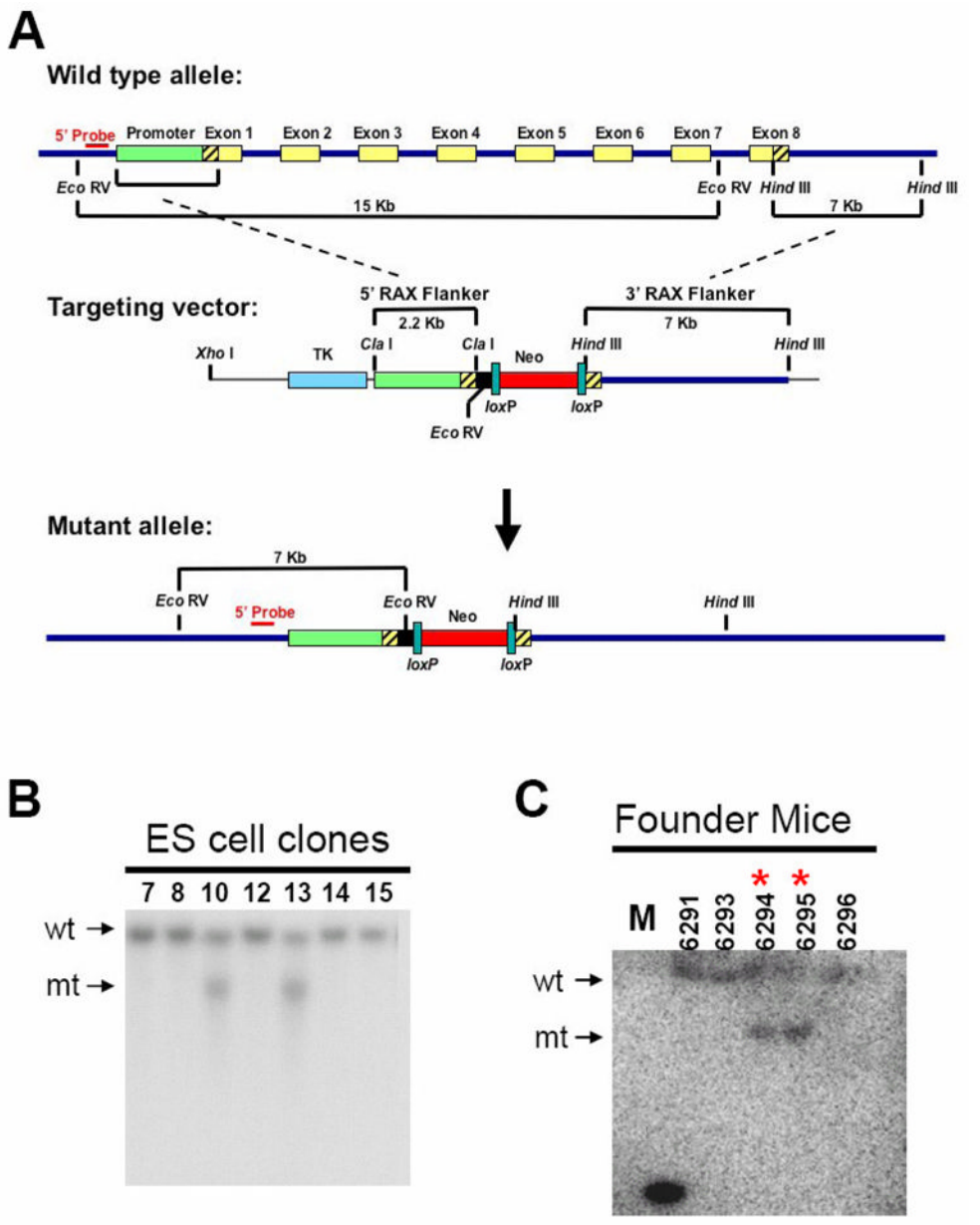

Figure 3. Targeted disruption of the mouse *Rax* gene by homologous recombination

A. (top) The wild type *Rax* mouse allele contains a 15 Kb *EcoR V* fragment and a 7 Kb *Hind III* fragment. Exons are indicated by yellow boxes while noncoding regions of exon 1 and 8 are indicated by the diagonal lines. (middle) The *Rax* targeting vector was constructed using the 2.2 Kb *Rax* promoter region and the 7 Kb *Hind III Rax* fragment. (bottom) The mutant allele generated after homologous recombination and the diagnostic *EcoR V* restriction fragments are indicated. B. Southern blot analysis of *EcoR V*-cleaved genomic DNA isolated from targeted and selected ES cell clones using a ^{32}P-labeled probe 5′ to the region of homologous insertion. wt, 15 Kb EcoR V fragment from wild type allele. mt, 7 Kb restriction fragment from mutant allele. C. Analysis of tail DNA from progeny of chimeric mice crossed with wild type mice demonstrates germline transmission of the disrupted *Rax* allele. M, marker. * indicates $Rax^{+/-}$ mice. D. Genotypes of offspring generated from mating of $Rax^{+/-}$ mice were determined by southern blotting. E. Southern blot using DNA isolated from E13.5

embryos generated from mating *Rax*$^{+/-}$ mice. F. Genotypes of E3.5 embryos generated from mating *Rax*$^{+/-}$ mice as determined by PCR. mt: mutant allele, wt: wild type allele

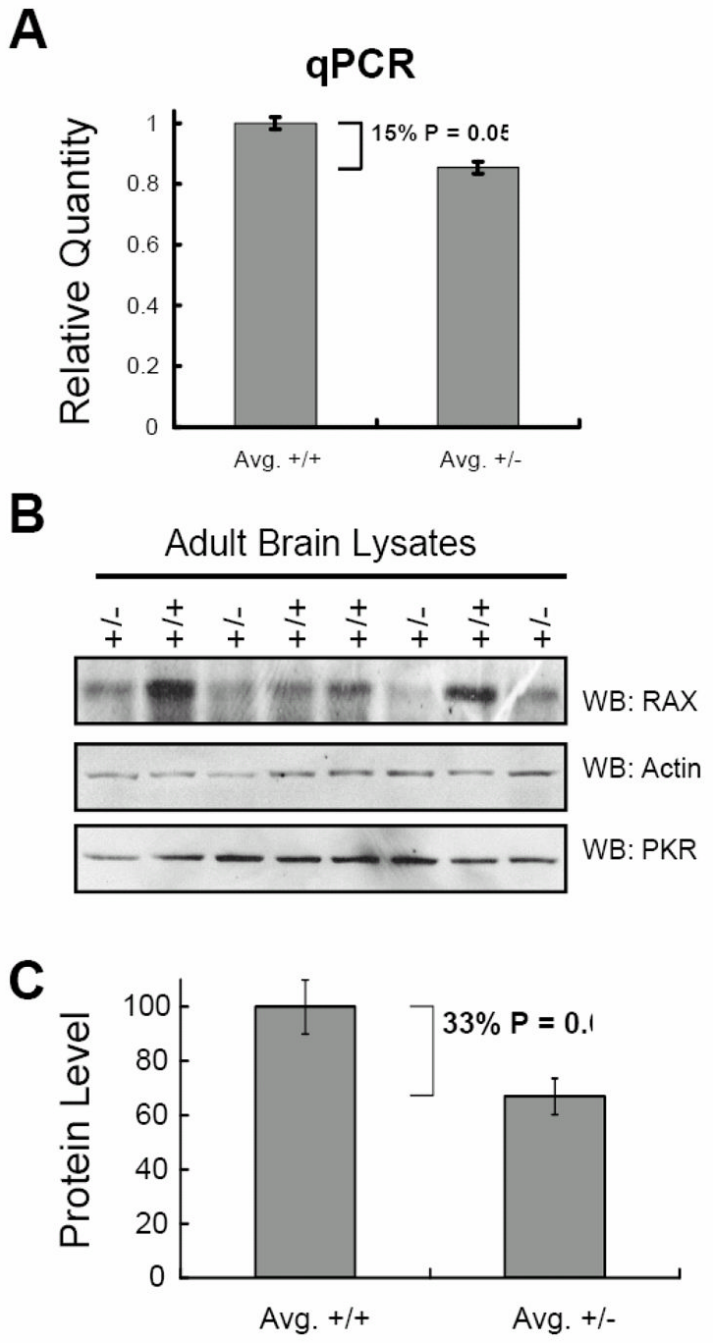

Figure 4. *Rax*$^{+/-}$ mice have reduced *Rax* gene expression and protein level

A. Quantitative real-time PCR was performed using RNA isolated from the lungs and brains of 5 mice heterozygous for the *Rax* knockout allele and of 5 *Rax*$^{+/+}$ littermate mice. The average relative quantity of RAX mRNA is compared between normal (Avg. +/+) vs. heterozygous (Avg. +/−) mice using β-actin as an endogenous reference. B. Western blots of lysates generated from adult mouse brain tissue using antibody specific for RAX, actin, and PKR. Normal littermate (+/+) and heterozygous (+/−) lysates are indicated. C. The level of RAX and actin proteins were quantified by densitometry analysis of Western blots using lysates from the lungs and brains of 5 mice heterozygous for the *Rax* knockout allele and of 5 *Rax*$^{+/+}$ littermate mice.

The average level of RAX protein relative to actin is depicted for both normal (Avg. +/+) and heterozygous (Avg. +/-) tissue lysates. Statistical analysis was performed using ANOVA.

Table I

Genotypes of progeny from ***Rax***$^{+/-}$ X ***Rax***$^{+/-}$ matings

Total mice	No. of Litters	Avg. Litter size	***Rax***$^{+/+}$	**Rax**$^{+/-}$	***Rax***$^{-/-}$
76 viable adults[a]	12	6.3	17	59	0
23E13.5[a]	3	7.6	2	21	0
36 E7.5[a]	5	7.2	13	23	0
33 E3.5[b]	6	5.5	9	24	0

[a] Genotype determined by both PCR and southern blotting of genomic DNA

[b] Genotype determined by PCR of genonnic DMA