RESEARCH ARTICLE

The Human dsRNA binding protein PACT is unable to functionally substitute for the Drosophila dsRNA binding protein R2D2 [version 2; peer review: 2 approved]

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Abstract
The dsRNA binding protein (dsRBP) PACT was first described as an activator of the dsRNA dependent protein kinase PKR in response to stress signals. Additionally, it has been identified as a component of the small RNA processing pathway. A role for PACT in this pathway represents an important interplay between two modes of post-transcriptional gene regulation. The function of PACT in this context is poorly understood. Thus, additional approaches are required to clarify the mechanism by which PACT functions. In this study, the genetic utility of Drosophila melanogaster was employed to identify dsRNA-binding proteins that are functionally orthologous to PACT. Transgenic Drosophila expressing human PACT were generated to determine whether PACT is capable of functionally substituting for the Drosophila dsRBP R2D2, which has a well-defined role in small RNA biogenesis. Results presented here indicate that PACT is unable to substitute for R2D2 at the whole organism level.

Open Peer Review

Approval Status  

1 2

version 2 (revision)
28 Mar 2014

version 1
18 Oct 2013

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Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Ganes C Sen (seng@ccf.org)
Competing interests: No competing interests were disclosed.
Grant information: This work was supported by NIH grant CA062220, awarded to G. Sen. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
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How to cite this article: Dickerman BK, McDonald JA and Sen GC. The Human dsRNA binding protein PACT is unable to functionally substitute for the Drosophila dsRNA binding protein R2D2 [version 2; peer review: 2 approved] F1000Research 2014, 2:220
https://doi.org/10.12688/f1000research.2-220.v2

First published: 18 Oct 2013, 2:220 https://doi.org/10.12688/f1000research.2-220.v1
A mature guide RNA, Dicer, one or more accessory dsRBPs, and one of the four members of the Argonaute protein family (Agol–4) which function as the effector enzymes in the terminal silencing step.

The RNA silencing pathways of metazoans have largely been investigated in the model organisms Caenorhabditis elegans and Drosophila melanogaster. In both systems, it was discovered that the endonucleases required for cleavage of pri-miRNAs (Drosha) and pre-miRNAs/siRNA precursors (Dicer) form complexes with specific dsRBPs. These proteins, like PACT, are comprised of tandem dsRBDS with no other identifiable domains (Drosophila complexes with Pasha/DGCR8(15), C. elegans Dicer binds RDE-4(16), D. melanogaster Dicer-1 binds Loquacious (LOQS) and D. melanogaster Dicer-2 binds R2D2(18)). Furthermore, these dsRBPs are required for the processing functions of the complexes. In mammalian systems, processing of both siRNA and miRNA is performed by a common Dicer enzyme. Both PACT and a similar dsRBP Tat Trans-activation RNA binding protein (TRBP) in human/Proctamine-1 RNA binding protein (PRBP) in mouse have been demonstrated to bind Dicer through either their N-terminal dsRNA binding motifs or their C-terminal Merlin-Dicer-PACT Liaison (Medipal) domain (which contains the PKR activation domain of PACT)(9,10,11,12). The binding of PACT, TRBP or both with Dicer enhances the ability of Dicer to process dsRNA into siRNAs in vitro(6). These findings suggest a functional significance to the PACT/TRBP/Dicer interactions, although the mechanism by which this occurs is unclear. These reports suggest that in addition to the regulating PKR activation, PACT may affect post-transcriptional control via small RNA pathways.

In Drosophila, processing of small RNA precursors is accomplished by separate enzymes, Dicer-1 for miRNA and Dicer-2 for siRNA(19). The dsRNA binding proteins LOQS(17) and R2D2(18) bind to Dicer-1 and Dicer-2, respectively. LOQS and R2D2 sort precursor RNA molecules based on the presence or absence of mismatches in the stemloop of the hairpin or dsRNA precursor to the appropriate Dicer processing pathway(20) and facilitate RISC loading(21,22). LOQS encodes four isoforms; LOQS-PA and LOQS-PB contain three tandem dsRBDS whereas both LOQS-PC and LOQS-PD contain the third C-terminal dsRBD. R2D2 encodes a single isoform that is comprised of two dsRBDS(19) (Figure 1). Within the dsRBDS of these proteins, there is a high degree of sequence similarity (71–78% similarity, see Table 1) between PACT and LOQS PA and PB (which have all three dsRBDS in common). This sequence similarity coupled with the tandem dsRBD architecture (see Figure 1 and Table 1) indicates potential functional orthology between PACT and LOQS. Although R2D2 is significantly less similar to PACT (46% sequence similarity within dsRBD2, with no detectable similarity within dsRBD1), it has nonetheless been demonstrated to bind Dicer(23) and possesses a tandem dsRBD architecture similar to PACT and LOQS and thus may also function orthologously to PACT/RAX.

Previous studies have produced mutant D. melanogaster lines lacking either of the dsRBPs that interact with Dicer-1 and Dicer-2. Flies deficient in all four isoforms of LOQS display development lethality, and do not survive past the pupal stage(24). In contrast,
Figure 1. Schematic representation of PACT and Drosophila dsRBPs. Schematic diagram of the PACT, LOQS and R2D2 proteins depicting the organization of their dsRBD domains. Dashed lines designate dsRBD1, 2 or 3.

Table 1. Amino acid sequence similarity between PACT and Drosophila dsRBPs. Amino acid sequence identity and similarity (positives) between dsRNA binding domains of PACT and the Drosophila dsRNA binding proteins determined by BLAST (blastp) alignment; ~ indicates no detectable similarity. The three dsRBDs shown for LOQS correspond to the shared dsRBDs of PA and PB isoforms; the PC and PD isoforms share the first and second but lack the third dsRBD.

| Protein  | dsRBD1 | dsRBD2 | dsRBD3 |
|----------|--------|--------|--------|
| LOQS     | 55%    | 71%    | ~      | | |
|          | 62%    | 78%    | ~      | | |
|          | ~      | ~      | 57%    | 76% |
| R2D2     | ~      | ~      | 26%    | 46% | ~ |
|          | ~      | ~      | ~      | ~  |

flies homozygous mutant for a null allele of r2d2 are semi-lethal but the surviving flies have severe fertility defects. The fertility phenotype results from defective oogenesis. Specifically, loss of R2D2 disrupts formation of the stalk cells, which normally separate individual follicles from each other. This phenotype is enhanced by a dicer-1 mutant, indicating an unexpected interaction between R2D2 and Dicer-1 rather than Dicer-2.

We hypothesized that one or more dsRNA binding proteins with known function in D. melanogaster are functionally orthologous to PACT, and that identification of the functional ortholog(s) would provide insight into the small RNA processing function of PACT. We sought to identify proteins orthologous to PACT by transgenically expressing human PACT in the well-characterized Drosophila genetic model system. We report here our efforts to determine the degree to which human PACT is a functional ortholog of Drosophila R2D2.

Materials and methods

Cell lines, plasmids and antibodies
HeLa cells (ATCC CCL-2) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing glucose (4.5g/L), penicillin (50U/ml), streptomycin (50g/ml), L-glutamine (2mM) and sodium pyruvate (1mM) (Cleveland Clinic Lerner Research Institute Media Preparation Core) supplemented with 10% fetal bovine serum (Atlanta Biologicals) at 37°C in 5% CO₂. The pUASP vector was obtained from Drosophila Genomics Resource Center. A polyclonal rabbit antibody raised against full length PACT (produced in-house) was used at 1:8000 and was detected using 1:10000 horseradish peroxidase (HRP) conjugated polyclonal goat anti-rabbit secondary (Rockland Immunochemicals # 611-103-122).

Generation of pUASP plasmids
To obtain PACT cDNA, total RNA was extracted from HeLa cells using Trizol (Invitrogen) according to the manufacturer’s instructions,
followed by DNase treatment using the DNA-free kit (Ambion). Extracted, DNase-treated RNA was then reverse transcribed with the Superscript III system (Invitrogen) using random hexamers. The resulting cDNA was used to amplify the coding sequence of PACT by PCR using the primers 5’BamHI-PACT(UAS) (AAG GAT CCA AAC ATG TCC TCC CAG AGC CCC C) and 3’XbaI-PACT (GGC GGA TCC TTA CCT TTC TTT GGC TAT TAT TAT TAA ATA C). The resulting product was digested with BamHI (New England Biolabs Inc. #R0136) and XbaI (New England Biolabs Inc. #R0145) and ligated into pUASP to generate pUASP-PACT. The full-length Drosophila r2d2 cDNA was amplified by PCR from a plasmid obtained from Drosophila Genome Research Center (cDNA #LD06392) using 5’BamHI-R2D2(UAS) (AAG GAT CCA AAC ATG GAT AAC AAG TCA GCC GTA TC) and 3’XbaI-R2D2 (AAA TCT AGA TTA CTT TCT TTC TGC TAT TAT CTT TAA ATA C). The resulting product was digested with TAT TAT ATG G). The resulting product was digested with BamHI and XbaI and ligated into pUASP to generate pUASP-R2D2. Final cloned plasmids were sequence verified using the primers listed above by the Cleveland Clinic Lerner Research Institute Genomics Core.

Protein isolation and western blot
Protein was isolated by homogenizing individual adult flies in Triton X-100 lysis buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 1% Triton X-100, 1mM EDTA, 5mM 2-mercaptoethanol, 10% glycerol, supplemented with Complete protease inhibitor and PhoSTOP (Roche)). Protein was separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon, Millipore) for western blot analysis. Western blots were visualized using enhanced chemiluminescence (ECL) detection reagents (GE Healthcare Lifesciences #RPN2106).

Drosophila strains and genetics
Flies were maintained and crossed at 25°C according to standard protocols16. Fly food was prepared according to the standard recipe from Bloomington Drosophila Stock Center with minor modifications (83.3ml/L molasses, 33.75g/L yeast, 97.58g/L cornmeal, 31g/L agar, 15.67ml/L Tegosept, all purchased from Genesee Sciences (83.3ml/L molasses, 33.75g/L yeast, 97.58g/L cornmeal, 31g/L agar, 15.67ml/L Tegosept, all purchased from Genesee Sciences (#RPN2106)). Protein was separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon, Millipore) for western blot analysis. Western blots were visualized using enhanced chemiluminescence (ECL) detection reagents (GE Healthcare Lifesciences #RPN2106).

Egg collection and hatch rate determination
Females of the genotype tub-GAL4; r2d2/DF(2L)BSC142; +/UAS-x (where x is PACT or r2d2) were outcrossed with w1118 males for fertility tests. Crosses to test fertility were established in vials of food for 24 hours prior to transferring flies to an egg collection chamber. Females were allowed to lay eggs on grape juice agar plates (Genesee Scientific, #47–102) for 1 hr prior to collecting the plate, and incubating at 25°C for 24 hours to allow eggs to hatch. The total number of eggs laid was determined by counting the number of larvae and the number of unhatched eggs. Hatch rate was calculated as the number of larvae divided by the number of total eggs laid. Student’s t-test of two independent experiments (defining \( P < 0.05 \) as statistically significant) was performed using Graphpad Prism version 6.02 software package (Graphpad Software).

Results
Generation of human PACT-expressing transgenic Drosophila melanogaster
We reasoned that the genetically tractable Drosophila model system could be used to study the in vivo functions of PACT. Because there is no known PKR ortholog in Drosophila, this also allows the role of PACT in small RNA processing to be investigated independent of PKR activation. Moreover, the well-characterized siRNA and miRNA processing pathways and availability of \\textit{logos} and r2d2 mutant alleles make this an attractive system to study the role of PACT in the small RNA pathways. Various human proteins have been used to rescue Drosophila mutant phenotypes in order to identify homologous and orthologous proteins17–24, therefore to identify proteins orthologous to PACT, a human PACT transgene was constructed and introduced into Drosophila. This transgenic line was used to determine whether PACT can substitute for Drosophila dsRNA binding proteins that regulate small RNA processing (see below).

Human PACT-expressing D. melanogaster were created using the Gal4-UAS bipartite expression system25. This system allows the generation of flies containing transgenes of interest under the transcriptional control of the UAS element. These lines then can be crossed to a number of Gal4-driver flies to tailor transgene expression to the needs of a specific experiment. The full-length coding region of the Human PACT gene was cloned into the pUASP vector under the transcriptional control of the UASp element, a modified form of UAS that permits expression in both somatic and germline tissues26. Several UAS-PACT transformants were obtained and their chromosomal insertions mapped. A stock that carried an insert on chromosome 3 was retained and crossed to the ubiquitous tub-GAL4 driver.

We next tested whether Human PACT was expressed at detectable levels in vivo. Flies expressing tub-Gal4 alone, containing the UAS-PACT transgene alone, and flies with tub-Gal4 driving UAS-PACT were analyzed by western blot for PACT expression in whole flies. Using an antibody that recognizes Human PACT28, we detected a protein band of the expected size (~34 kDa) in tub-GAL4; UAS-PACT flies but not in the individual parental lines (Figure 2). Importantly, because we obtained viable tub-GAL4; UAS-PACT adult flies, this experiment also demonstrates that ubiquitous transgenic expression of PACT does not impair Drosophila development.

Expression of human PACT is insufficient to overcome the Drosophila R2D2 deficient phenotype
The focus of the work presented here is on PACT rescue of the r2d2 loss-of-function mutant phenotype. Investigation of the functional substitution between human PACT and Drosophila LOQS
was investigated in a separate project in our lab and will be reported elsewhere.

Flies mutant for r2d2 have reduced fertility\textsuperscript{27}. Therefore, we sought to determine the extent to which PACT can suppress r2d2 mutant fertility defects. We performed fertility tests using a standard egg-laying and hatching assay. Crosses were performed to obtain female progeny that carried the r2d2\textsuperscript{1} allele over a deficiency that removes the r2d2 genetic locus (i.e. r2d2\textsuperscript{1}/–), and simultaneously carried tub\textsuperscript{-}GAL4 driving either UAS\textsuperscript{-}PACT or UAS\textsuperscript{-}r2d2 (Figure 3A; see Materials and methods for details). These female F1 progeny were then outcrossed to w\textsuperscript{1118} males to test for fertility; a cross of w\textsuperscript{1118} females to w\textsuperscript{1118} males was used as the wild-type control. Eggs resulting from these outcrosses were collected on grape juice agar plates, and allowed to hatch for 24 hours at 25°C. The total number of eggs produced and the number of hatched larvae were counted for each cross and hatch rate was calculated as the number of hatched larvae divided by the total number of eggs (Figure 3B & 3C). Flies heterozygous for r2d2 (either r2d2\textsuperscript{1}/+ or Df(2L)BSC142/+ ) produced eggs and hatch rates equivalent to the wild-type control. Notably, expression of either UAS\textsuperscript{-}PACT or UAS\textsuperscript{-}r2d2 in r2d2 heterozygous flies did not influence fertility or hatch rates. Consistent with a previous report\textsuperscript{27}, flies homozygous mutant for r2d2 had a strong reduction in both the total number of eggs laid and the hatch rate compared to wild-type (Figure 3). We observed a rescue of both total number of eggs (p=0.0512) and hatch rate (p=0.0671) in r2d2 mutant flies by ubiquitous (tub\textsuperscript{-}GAL4) transgenic expression of UAS\textsuperscript{-}r2d2 that is approaching statistical significance and consistent with previous reports\textsuperscript{27}. In contrast, ubiquitous expression of UAS\textsuperscript{-}PACT did not suppress the homozygous r2d2 mutant fertility defects; the hatch rate and number of eggs laid was equivalent to the r2d2 mutants alone (Figure 3). Despite a documented interaction between PACT and the mammalian small RNA processing machinery and similar domain architecture between these proteins, these results demonstrate that Human PACT is not sufficient to rescue the R2D2 deficient phenotype.

Discussion

Identification of PACT as a component of the small RNA processing pathway implicates this protein in multiple pathways of post-transcriptional gene regulation through general inhibition of translation by PKR and target-specific regulation by siRNA/miRNA. As such, it is important to determine the precise nature of PACT’s involvement in this pathway in order to understand the overall function of PACT in post-transcriptional gene regulation, and the physiological effects of the protein. Here, we investigated the role of PACT in RNA silencing by attempting to identify orthologous proteins in D. melanogaster. Human PACT was introduced into Drosophila to determine whether it could substitute for R2D2, which has a well-characterized function in RNA silencing. Expression of PACT was unable to rescue the phenotypes of flies lacking R2D2. These results suggest that the Dicer-associated dsRBPs PACT and R2D2 are not functionally orthologous.

While our study demonstrates that Human PACT cannot substitute for R2D2 function in flies, there are a number of important caveats to the results described here. Despite the lack of rescue, it is nonetheless possible that PACT plays the same (or a similar) role in mammalian cells as R2D2 plays in Drosophila cells. One possibility could be that PACT protein is sufficiently different from R2D2 that it cannot interact in vivo with Drosophila partner proteins that are necessary for R2D2 function. Alternatively, R2D2 may be required to bind specific RNA precursor molecules that PACT is unable to bind (or has a lower affinity for). Investigation of biochemical interactions between PACT and Drosophila RNA silencing proteins such as Dicer-1 and Dicer-2 and a biochemical comparison of the specific RNA binding functions of these proteins would begin to address these issues.

In addition to functional discrepancies between these proteins, there may be other potential explanations for these results that would not rule out orthologous function between PACT and R2D2. Although less likely, the lack of PACT rescue of the r2d2 mutant may be due to inherent difficulty in controlling the tissue-specificity and relative levels of transgene expression in vivo. In the case of R2D2, expression in the stalk and follicle cells of the ovary is required for egg development\textsuperscript{1}. Expression of UAS\textsuperscript{-}PACT by tub\textsuperscript{-}GAL4 in whole flies was validated by western blot; however, this does not provide information about transgenic expression of PACT in the appropriate tissues. Thus, even though PACT expression was driven by a ubiquitous GAL4 driver we cannot rule out insufficiently high expression of PACT in the relevant cell types. Expression of
Figure 3. Rescue of the R2D2 deficient fertility defect by transgenic expression of R2D2 but not human PACT. (A) Schematic representation of the experimental cross used to test fertility. UAS-x refers to the transgene shown in (B) and (C); for control crosses with no transgene the genotype Df(2L)BSC142/CyO; UAS-x was replaced with DF(2L)BSC142/CyO. (B) Calculated hatch rate of eggs produced by wild-type (w¹¹¹º) flies, flies with one copy of r2d2 (+/-; either r2d2/CyO or Df(2L)BSC142/CyO) and flies lacking both copies of r2d2 (-/-, r2d2/Df(2L)BSC142) and either expressing no UAS-transgene or expressing UAS-PACT or UAS-r2d2; these flies were outcrossed to w¹¹¹º males for the fertility tests. (C) Total number of eggs laid from the crosses depicted in (B). Graphs show mean ± SEM of two independent experiments, p-values were calculated by Student's t-test.
UAS-R2D2 using the same tub-GAL4 driver however rescued the r2d2 loss-of-function fertility defect. Although this result provides some degree of confidence in the expression levels, the genomic context of the P-element insertion and stability of transgenically-produced RNA and protein can all contribute to expression variability in vivo. As such, it still remains a possibility that PACT was not expressed as highly as R2D2 using the same transgenic expression system, and thus PACT may not have rescued fertility due to insufficient expression rather than a lack of orthologous function. This issue could be addressed in future experiments by expressing epitope-tagged R2D2 or PACT in r2d2 mutant flies to correlate rescue of the r2d2 deficient phenotype with a direct comparison of protein expression level\(^7\). Additional experiments investigating tissue-specific expression levels of these proteins as well as using tissue-specific GAL4 drivers would begin to address these concerns.

While the complementation experiments described here indicate that PACT is unable to functionally substitute for R2D2, there are complications to the interpretation of these experiments. Further technical refinements will be necessary to definitively demonstrate or rule out functional orthology between PACT and R2D2. It also remains a distinct possibility that PACT is instead orthologous to LOQS, a topic that we are interested in investigating further in the future.

**Supplementary materials**

**Table S1. Hatch rate and egg count raw data replicate 1.**

|            | Wild type | No transgene | UAS-R2D2 | UAS-PACT |
|------------|-----------|--------------|----------|----------|
|            | W1118     | +/-          | +/-      | +/-      |
| Larvae     | 884       | 743          | 153      | 969      | 403      | 728      | 145      |
| Eggs laid  | 983       | 1042         | 355      | 1112     | 631      | 1021     | 380      |
| % Hatched  | 89.92879  | 71.30518     | 43.09859 | 87.14029 | 63.86688 | 71.30264 | 38.15789 |

**Table S2. Hatch rate and egg count raw data replicate 2.**

|            | Wild type | No transgene | UAS-R2D2 | UAS-PACT |
|------------|-----------|--------------|----------|----------|
|            | W1118     | +/-          | +/-      | +/-      |
| Larvae     | 663       | 651          | 71       | 763      | 579      | 809      | 131      |
| Eggs laid  | 718       | 785          | 187      | 870      | 721      | 948      | 361      |
| % Hatched  | 92.33983  | 82.92994     | 37.96791 | 87.70115 | 80.30513 | 85.33755 | 36.28809 |

**Author contributions**

BKD contributed to the conception, design, execution, analysis and interpretation of the experiments detailed in this manuscript, as well as drafting and revising the manuscript. JAM contributed to the conception, design, analysis and interpretation of the experiments detailed in this manuscript, as well as revising the manuscript. GCS contributed to the conception, design, analysis and interpretation of the experiments detailed in this manuscript, as well as revising the manuscript.

**Competing interests**

No competing interests were disclosed.

**Grant information**

This work was supported by NIH grant CA062220, awarded to G. Sen.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Acknowledgments**

We wish to thank George Aranjuez for technical assistance with Drosophila work.
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Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 09 May 2014

https://doi.org/10.5256/f1000research.4070.r4717

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Anthony Sadler
Monash Institute for Medical Research, Monash University, Melbourne, Australia

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 18 February 2014

https://doi.org/10.5256/f1000research.2593.r2387

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Anita Corbett
Emory University, Atlanta, GA, USA

The manuscript by Dickerman et al. describes an attempt to study the functional conservation of RNA binding proteins implicated in processing of small RNAs and activation of dsRNA-dependent protein kinase (PKR). The work exploits a Drosophila model where PKR is not relevant thus allowing focus on the small RNA processing pathways. The approach is logical to try to rescue a fertility phenotype observed for the Drosophila protein (R2D2) with transgenic expression of a related human protein, PACT. Unfortunately, the authors were not able to demonstrate rescue and thus are left with a negative result.
The data presented do support the conclusions drawn, that no rescue could be detected. There are a number of caveats for this result as the authors describe in the Discussion.

This is a reasonable contribution to the literature in that it would prevent other researchers from wasting time undertaking the identical experiment. The data presented is robust and convincing.

I would suggest some modifications to the text for clarification:

1. The authors begin by describing both related human proteins, PACT and the isoforms of LOQS. While it is logical to describe the family of proteins, this study actually only analyzes the PACT protein. This point should be clearly communicated earlier in the Introduction to the study not just as the final sentence of the Introduction.

2. The section in the Introduction describing Figure 1 is very convoluted and really could be re-written to be clearer. An example with grammatical errors is the second sentence of this paragraph, which reads: “The dsRNA binding proteins LOQS and R2D2 bind Dicer-1 to Dicer-2 respectively.” I think what the authors mean to say is: “The dsRNA binding proteins, LOQS and R2D2, bind TO Dicer-1 AND Dicer-2, respectively.”

   The description of the proteins really needs to be rewritten for clarification.

3. If the authors could perform an additional experiment to address one of the caveats that they mention, ideally they could compare the expression levels for the transgenic human and fly proteins. Such a direct comparison would really only be possible if the proteins are tagged with an epitope tag.

Given the number of caveats for the study presented here, the real value as described above is simply to communicate that these experiments have been attempted using this approach.

**Competing Interests:** No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

**Author Response 03 Apr 2014**

**Benjamin Dickerman,** Cleveland Clinic, Cleveland, OH, USA

We have addressed the comments presented in a revised manuscript. A sentence reading “To this end, we investigated the functional orthology between PACT and the Drosophila melanogaster dsRBP R2D2.” has been added to the end of the first paragraph of the introduction, to highlight the focus of this manuscript before describing the family of dsRBPs. The paragraph describing figure 1 has been reordered and edited for clarity, and to address the specific grammatical error mentioned by the reviewer. A sentence reading “This issue could be addressed in future experiments by expressing epitope-tagged R2D2 or PACT in r2d2 mutant flies to correlate rescue of the r2d2 deficient phenotype with a direct comparison of protein expression level.” has been added to the relevant paragraph of the discussion section.
suggesting this experiment as part of future studies. This has been referenced as a comment by the reviewer to credit the suggestion.

**Competing Interests:** No competing interests were disclosed.

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**Reviewer Report 06 December 2013**

https://doi.org/10.5256/f1000research.2593.r2676

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Anthony Sadler
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This is a useful study that is well executed and that makes a valuable contribution. The results are clearly described. An attempt to address the acknowledged uncertainties in this study, by measuring the levels of PACT in the critical tissues, comparison of the levels of expression of PACT to the transgenic R2D2, detecting an association between human PACT and Drosophila Dicer-2, and/or otherwise confirming function of the PACT transgene would have increased confidence in the conclusion. These measures would be less germane if the parallel experiment to rescue the function of Drosophila LOQS, which is the more likely homolog for PACT, had demonstrated the functionality of the PACT transgene. Perhaps a sentence could be introduced into the discussion that these separate experiments validated the PACT transgene functioned in Drosophila - if this was the case.

I have a number of very minor criticisms that might improve the manuscript.

The statement in the abstract that the primary function of PACT is to activate PKR is not accurate. As the authors themselves state the relative physiological consequence of PACT, in small RNA biogenesis or protein translation, is uncertain. This sentence might be better expressed by, for instance, writing PACT was first described to function by activating PKR.

The sentence in the abstract stating that additional models are required to clarify the function of PACT does not best justify the study approach. Alternatively, describing the utility of Drosophila to decipher small RNA biogenesis would seem more appropriate.

It might improve the clarity of the text if the authors selected a simpler abbreviation, rather than PACT/RAX, such as either PACT or RAX.

**Competing Interests:** No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of
expertise to confirm that it is of an acceptable scientific standard.

Author Response 03 Apr 2014

Benjamin Dickerman, Cleveland Clinic, Cleveland, OH, USA

We agree that inclusion of LOQS rescue data would strengthen the arguments presented in this manuscript. Despite repeated efforts, and likely due to technical limitations, *Drosophila* LOQS (UAS-LOQS-PB) was unable to rescue *loqs* loss-of-function mutations in our hands. This important control has been previously reported (*Park et al., 2007*) to be sufficient to rescue the *loqs* loss-of-function mutation that was used in this study. Because these rescue studies were inconclusive, we are currently unable to confirm that LOQS and PACT are functional orthologous.

We have addressed the remaining comments presented here in a revised manuscript. The abstract has been revised to clarify stress-induced PKR activation as the first described rather than the primary function of PACT, as well as to highlight the genetic utility of Drosophila as a model system, rather than merely suggesting that additional model systems are required. To address the final comment regarding PACT nomenclature, the manuscript has been revised for clarity by changing PACT/RAX to PACT unless specifically referring to the mouse protein RAX.

**Competing Interests:** No competing interests were disclosed.