THE EMBO JOURNAL

Manuscript EMBO-2011-78283

An Extended dsRBD with a Novel Zinc-Binding Motif Mediates Nuclear Retention of Fission Yeast Dicer

Pierre Barraud, Stephan Emmerth, Yukiko Shimada, Hans-Rudolf Hotz, Frédéric H.-T. Allain, and Marc Bühler

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Review timeline:

Submission date:	26 May 2011
Editorial Decision:	24 June 2011
Revision received:	18 July 2011
Editorial Decision:	20 July 2011
Accepted:	22 July 2011

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 24 June 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by three referees and I enclose their reports below. As you will see from their comments they are positive about the study and recommend publication after a few issues have been clariifed and controls have been added to the current manuscript. They also suggest some re-organisation of the figures. Given the interest in the study, should you be able to address the concerns we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor

The EMBO Journal

Referee #1

Dicer enzymes generate small regulatory RNAs that function in RNA interference (RNAi) pathways in eukaryotes. Structural insights into Dicer function remain limited, due in part to the large size of Dicer and its multi-domain composition. This manuscript describes the solution structure of the C-terminal domain of Schizosaccharomyces pombe Dicer (Dcr1). In this structure, a predicted double-stranded RNA binding domain (dsRBD) fold is found to include a zinc-binding motif that is conserved among Dicer enzymes in yeast. Although the C-terminal domain of Dcr1 binds to nucleic acids, this property is not necessary for Dcr1 function. However, disruption of zinc coordination limits Dcr1localization to the cytoplasm, leading to changes in gene expression and loss of heterochromatin assembly. These findings illuminate the mechanism of nuclear retention of Dcr1 and suggest that this type of dsRBD may function more generally in nucleo-cytoplasmic trafficking. The authors also suggest that as a unique regulatory feature of fungal Dicers, the C-terminal domain could be a potential target for therapeutic intervention. Overall this is an excellent piece of work, and the authors have been very thorough in their analysis. Although the mechanism of nuclear retention is not entirely clear, the results provide convincing evidence that the structural interactions in the C-terminal dsRBD, and in particular the coordination of a single Zn ion, are critical. I have just two minor comments for the authors' attention:

1. Of the nine figures presented in the main text, two or three could probably be combined or moved to supplemental panels. For example, I suggest combining Figs. 1 and 3 and moving Figs. 2 and 9 to supplemental material.

2. I could not find a reference to Figure 6B in the text.

Referee #2

This manuscript describes a structural and functional study of the role of the C-terminal dsRBD domain from the S. pombe protein Dcr1, which is one of the key components of RNAi pathways in pombe. It was known previously that this dsRBD is unusual in that it is extended at its C-terminus by a short conserved motif called C33, and the present authors had already shown that C33 is involved in preventing dsRBD-mediated export of Dcr1, resulting in its accumulation in the nucleus. In the present paper, the authors show that C33 forms part of a novel zinc finger motif that extends the structure of the dsRBD; two zinc-binding ligands are contributed by C33 and the other two by the dsRBD. Structural integrity of this zinc finger is required for Dcr1 localisation and function, while, surprisingly, RNA binding by the dsRBD is not. The authors speculate that dicer dsRBDs may generally play a role in nuclear localisation rather than in substrate binding. I think this work is likely to be of widespread interest, and suitable for publication in EMBO Journal after attention to the following points:

1) I would much prefer to have seen the co-ordination of the zinc by the Nε atom of His 1312, as opposed to the Nδ atom, established using a long-range 15N-1H correlation experiment, as published by Legge et al., J.M.B. (2004) 343, 1081-1093. Establishing the connectivity by measuring distances in preliminary structures, as was done in this case, can work but can also sometimes be unreliable, since the differences between corresponding distances present in the two alternative structures can be quite small. Also, I think the authors implication that co-ordination through Nε is intrinsically the more likely for a novel zinc-binding domain is quite unsafe - many zinc fingers use Nδ (e.g. some GATA-type fingers and some PHD fingers), and some even use both Nδ and Nε from different histidines binding the same metal (e.g. Legge et al., J.M.B. (2004) 343, 1081-1093). I would not insist on this for publication, but if there is some reason why the long-range correlation experiment could not be used this should be stated. If there is no such reason I would strongly suggest the authors still consider carrying it out, if only for their peace of mind. At the least they should present (in Supplementary Material) the data that allowed them to make the choice of co-ordinating atom.

2) In several places the authors refer to 11 dihedral angle constraints that are applied around the zinc to specify tetrahedral co-ordination geometry. All but one of these are in fact bond angles (specified by 3 atoms), not dihedral angles (specified by 4 atoms).

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5) The authors show that the dsRBD binds not only to A-form dsRNA but also, somewhat unexpectedly, to B-form dsDNA. Did they check whether the mutants in helix 1 and loops 2 and 4 that abolish RNA binding have any effect on DNA binding? If the authors have data on this it would be good to include it in the paper (or Supplementary Material).

6) The authors mention that adding the residues of the C33 motif to the C-terminus of the DICER1 protein, the human homolog of Dcr1, did not bring about nuclear localisation of DICER1 (which unlike Dcr1 is normally cytoplasmic). They state that this previous result can now be rationalised by the fact that structural integrity of the zinc finger in Dcr1 requires not only the presence of the residues of C33, but also the presence of the two zinc-binding residues in the dsRBD; these are present in Dcr1 but absent in DICER1. Did the authors consider mutating these zinc-binding residues into the dsRBD of DICER1? Again, if there is data on this it should be included.

Referee #3

The report by Barraud et al on the structure and function of the S. pombe C-terminal dsRBD domain is most interesting. It sheds a completely new light on the dsRBD of Dcr1 and of other Dicer proteins in general. It builds on previously published findings from the Buhler lab and now provides strong structural support for these findings, that are further tested through mutational analysis guided by the structural data. This will be of great general interest.
Below some suggestions that would further strengthen the manuscript.

1) The authors say the SHSS mutant is insiluble and hence conclude that it is likely misfolded. This sheds anotehr light on the in vivo data, since the authors may be looking at a protein without any dsRBD function. Further in the manuscript the authors suggest that R1311 may be involved in compensating the net -1 charge of the CHCC-Zn module. My suggestion would thus be to mutate R1311 into a hydrophobic residue to see whether this would solubilise the SHSS mutant. If so, this would be a more informative mutant to study in vivo (and by NMR).

2) The authors did not address whether the RNA binding mutants lost DNA binding. This is required to fully appreciate the effects of these mutations.

3) The N1344A, Y1384A and S1349A mutants shown in figure 7 seem to display significantly more nuclear Dcr1 than the C33 deletion or the SHSS mutants. Is this indeed the case? If so the authors should discuss this finding.

4) With regard to the small RNA data displayed in figure 5C: please give some more information. What's their length profiles? Are they Dicer products? Are the remaining cen reads true cen siRNAs?

1st Revision - authors' response 18 July 2011

Response to the referees' comments

We thank all three referees for their positive feedback on our manuscript. We are confident that we could address all the referees' comments to their satisfaction and we are happy to submit a revised version of our manuscript.
Below we address each of the referees' comments:

Referee #1

Dicer enzymes generate small regulatory RNAs that function in RNA interference (RNAi) pathways in eukaryotes. Structural insights into Dicer function remain limited, due in part to the large size of Dicer and its multi-domain composition. This manuscript describes the solution structure of the C-terminal domain of Schizosaccharomyces pombe Dicer (Dcr1). In this structure, a predicted double-stranded RNA binding domain (dsRBD) fold is found to include a zinc-binding motif that is conserved among Dicer enzymes in yeast. Although the C-terminal domain of Dcr1 binds to nucleic acids, this property is not necessary for Dcr1 function. However, disruption of zinc coordination limits Dcr1 localization to the cytoplasm, leading to changes in gene expression and loss of heterochromatin assembly. These findings illuminate the mechanism of nuclear retention of Dcr1 and suggest that this type of dsRBD may function more generally in nucleo-cytoplasmic trafficking. The authors also suggest that as a unique regulatory feature of fungal Dicers, the C-terminal domain could be a potential target for therapeutic intervention. Overall this is an excellent piece of work, and the authors have been very thorough in their analysis. Although the mechanism of nuclear retention is not entirely clear, the results provide convincing evidence that the structural interactions in the C-terminal dsRBD, and in particular the coordination of a single Zn ion, are critical. I have just two minor comments for the authors' attention:

1. Of the nine figures presented in the main text, two or three could probably be combined or moved to supplemental panels. For example, I suggest combining Figs. 1 and 3 and moving Figs. 2 and 9 to supplemental material.

As suggested, we have combined Figs. 1 and 3 and moved Fig. 2 to the supplemental material. We kept Fig. 9 (new numbering: Fig. 7) in the paper as the comparison with other dsRBD structures is an important point of this study.

2. I could not find a reference to Figure 6B in the text.

The specified Figure is now cited in the text as Figure 4B.

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This manuscript describes a structural and functional study of the role of the C-terminal dsRBD domain from the S. pombe protein Dcr1, which is one of the key components of RNAi pathways in pombe. It was known previously that this dsRBD is unusual in that it is extended at its C-terminus by a short conserved motif called C33, and the present authors had already shown that C33 is involved in preventing dsRBD-mediated export of Dcr1, resulting in its accumulation in the nucleus. In the present paper, the authors show that C33 forms part of a novel zinc finger motif that extends the structure of the dsRBD; two zinc-binding ligands are contributed by C33 and the other two by the dsRBD. Structural integrity of this zinc finger is required for Dcr1 localisation and function, while, surprisingly, RNA binding by the dsRBD is not. The authors speculate that dicer dsRBDs may generally play a role in nuclear localisation rather than in substrate binding. I think this work is likely to be of widespread interest, and suitable for publication in EMBO Journal after attention

to the following points:

1) I would much prefer to have seen the co-ordination of the zinc by the Nε atom of His 1312, as opposed to the Nδ atom, established using a long-range 15N-1H correlation experiment, as published by Legge et al., J.M.B. (2004) 343, 1081-1093. Establishing the connectivity by measuring distances in preliminary structures, as was done in this case, can work but can also sometimes be unreliable, since the differences between corresponding distances present in the two alternative structures can be quite small. Also, I think the authors implication that co-ordination through Nε is intrinsically the more likely for a novel zinc-binding domain is quite unsafe - many zinc fingers use Nδ (e.g. some GATA-type fingers and some PHD fingers), and some even use both Nδ and Nε from different histidines binding the same metal (e.g. Legge et al., J.M.B. (2004) 343, 1081-1093). I would not insist on this for publication, but if there is some reason why the long-range correlation experiment could not be used this should be stated. If there is no such reason I would strongly suggest the authors still consider carrying it out, if only for their peace of mind. At the least they should present (in Supplementary Material) the data that allowed them to make the choice of co-ordinating atom.

We measured a long-range ^{1}H-^{15}N HSQC spectrum to confirm the coordination mode of H1312. This data shows a typical pattern of cross peaks that is uniquely compatible with coordination through the Nε2 atom. This spectra has been included in supplemental material and the sentence on page 6 “In addition, preliminary structure calculations allowed us to unambiguously identify H1312 as coordinating the zinc ion via its Nε, as commonly found in zinc-binding domains.” has been replaced by “In addition, long-range ^{1}H-^{15}N HSQC shows a characteristic pattern of cross peaks that allowed us to unambiguously identify H1312 as coordinating the zinc ion via its Nε (Supplementary Figure 4)”. We also make reference to Pelton et al. (1993) Protein Sci. 2, 543–558 and to Legge et al. (2004) J. Mol. Biol. 343, 1081-1093.

2) In several places the authors refer to 11 dihedral angle constraints that are applied around the zinc to specify tetrahedral co-ordination geometry. All but one of these are in fact bond angles (specified by 3 atoms), not dihedral angles (specified by 4 atoms).

The denomination of the constraints defining the tetrahedral coordination around the zinc ion have been corrected in both the text and Table II. The tetrahedral coordination was indeed defined with 4 bond restraints, 10 bond angles restraints and 1 dihedral angle restraint.

3) Is it necessary to show the mass spectroscopic data that establishes zinc binding both in Figure 2 and in Table 1, both in the main paper? I would have thought one of these display items should go into Supplementary Material.

Figure 2 as been moved to Supplementary Material as Supplementary Figure 1.

4) In Table 2, what is meant by a "consistent" violation of the NOE constraints? Does this entry mean that there is no violation greater than 0.3 Å that is consistently present in every structure (but which could still be present in most)? In my view that would not be a very helpful statistic. In any event, the meaning should be defined.

By “consistent violation > 0.3 Å”, we meant that a particular NOE violation > 0.3 Å is found in more than 3 structures of the bundle. There is no such a violation in our structure. We have replaced this statistic in Table II by the number of NOE violations > 0.2 Å (mean and standard deviation). We also added the maximum NOE violation (mean and standard deviation).

5) The authors show that the dsRBD binds not only to A-form dsRNA but also, somewhat unexpectedly, to B-form dsDNA. Did they check whether the mutants in helix 1 and loops 2 and 4 that abolish RNA binding have any effect on DNA binding? If the authors have data on this it would be good to include it in the paper (or Supplementary Material).

Binding to dsDNA has been investigated for the mutants that abolish binding to dsRNA. Mutants K1265A in helix 1, Δloop 2 and R1322A in loop 4 have also completely lost their ability to bind to dsDNA. This result has been added to Supplementary Figure 7 and reference is made in the text.

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This would have been indeed a neat experiment. Unfortunately, the two dsRBDs are differing too much, which prevented us from a rational design of such mutations. In particular, the position of helix 1 relative to the rest of the domain (Figure 7) is very different in Dcr1's dsRBD compared to canonical dsRBDs. Therefore, a prediction of an equivalent residue to the first coordinating residue C1275 is impracticable.

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As suggested, we tested the solubility of a zinc-binding mutant SHSS in which R1311 has been mutated to a Valine (as in many other dsRBD sequences – Figure 6). Unfortunately, this mutant also express entirely in the insoluble fraction of *E. coli* and as for the SHSS mutant, we didn't succeed in refolding this recombinant purified protein.

2) The authors did not address whether the RNA binding mutants lost DNA binding. This is required to fully appreciate the effects of these mutations.

As suggested, binding to dsDNA has been investigated for the mutants that abolish binding to dsRNA. Mutants K1265A in helix 1, Δloop 2 and R1322A in loop 4 have also completely lost their ability to bind to dsDNA. This result has been added to Supplementary Figure 7 and reference is made in the text.

3) The N1344A, Y1384A and S1349A mutants shown in figure 7 seem to display significantly more nuclear Dcr1 than the C33 deletion or the SHSS mutants. Is this indeed the case? If so the authors should discuss this finding.

Indeed, the three single amino acid mutants (N1344A, Y1384A and S1349A) display more nuclear Dcr1 than the C33 deletion or the SHSS mutant. Importantly, when combined in one strain (Dcr1-triple mutant), the loss of nuclear localization of Dcr1 resembles very much what we observe for the C33 deletion or the SHSS mutants. This further strengthens our hypothesis that these residues are part of a protein-protein interaction surface that is important for nuclear retention of Dcr1. This new result has been added to Figure 5 and is now mentioned in the text.

4) With regard to the small RNA data displayed in figure 5C: please give some more information. What's their length profiles? Are they Dicer products? Are the remaining cen reads true cen siRNAs?

Additional information regarding size distribution, 5' nucleotide identity, and abundance of small RNA reads matching individual elements in the respective mutants is now shown in Supplementary Figure 6.

2nd Editorial Decision 20 July 2011

Thank you for resubmitting your revised manuscript to EMBO J. I have looked through your responses to the referees concerns and find that you have satisfactorily addressed all the initial issues raised. I am happy to accept the study for publication in The EMBO Journal. You will receive the official acceptance letter in the next couple of days.

Yours sincerely,

Editor
The EMBO Journal

