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# hDbr1 is a nucleocytoplasmic shuttling protein with a protein phosphatase-like motif essential for debranching activity

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SUBJECT AREAS:  
RNA SPLICING  
RNA DECAY  
ENZYME MECHANISMS  
HYDROLASES

Received  
13 September 2012

Accepted  
27 December 2012

Published  
21 January 2013

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In higher eukaryotes most genes contain multiple introns. Introns are excised from pre-mRNAs by splicing and eventually degraded in the nucleus. It is likely that rapid intron turnover in the nucleus is important in higher eukaryotes, but this pathway is poorly understood. In order to gain insights into this pathway, we analyzed the human lariar RNA debranching enzyme1 (hDbr1) protein that catalyzes debranching of lariar-intron RNAs. Transfection experiments demonstrate that hDbr1 is localized in a nucleoplasm of HeLa cells through a bipartite type nuclear localization signal near carboxyl-terminus. The conserved GNHE motif, originally identified in protein phosphatase protein family, is critical for hDbr1 to dissolve lariar structure *in vitro*. Furthermore, heterokaryon experiments show that hDbr1 is a nucleocytoplasmic shuttling protein, suggesting novel role(s) of hDbr1 in the cytoplasm.

In higher eukaryotes splicing of precursor of mRNA (pre-mRNA) is a critical step for gene expression, since most genes encoded in the nucleus contain introns<sup>1–3</sup>. Splicing comprises two catalytic steps. As the first step, the cleavage at the 5' splice site occurs and concurrently the 5' end of the intron is joined to the branch nucleotide by forming a 2'–5' phosphodiester bond. This results in the production of 5' exon and a lariar intermediate RNA that consists of a lariar-form intron and 3' exon. These intermediates are subject to the second step reaction in which the 3' splice site is cleaved and two exons are ligated to produce mRNA. The excised lariar introns are supposed to be retained and degraded in the nucleus.

Splicing takes place in a large protein–RNA complex, termed spliceosome<sup>1,4</sup>. More than a hundred of protein and RNA factors were identified as components of spliceosome<sup>4</sup>. In contrast, the intron turnover pathway after splicing remained largely unclear. Only several factors for the intron turnover pathway in the nucleus have been identified almost exclusively by analyses of budding yeast mutants. Upon the completion of the splicing reaction, mRNA is released from the spliceosome, and the remaining post-splicing RNPs containing the lariar introns are disassembled by two members of the ATP-dependent DExH box RNA helicase family, Prp22 and Prp43<sup>5,6</sup>. Prp22 is required for the release of the spliced RNA<sup>5</sup>, while Prp43 is necessary for the disassembly of the remaining intron-containing RNP<sup>7,8</sup>. It has been also shown that two splicing factors, Ntr1 and Ntr2, in the form of a hetero dimer, associate with Prp43. The resultant trimeric complex is called as the 'NTR complex'<sup>9,10</sup>. The NTR complex is functional in catalyzing the disassembly of the spliceosome<sup>9</sup>. After dissociation of U snRNPs and other splicing factors, the lariar intron is linearized by a lariar intron debranching enzyme, Dbr1, before being degraded<sup>11</sup>.

The RNA-lariar debranching enzyme 1 protein, Dbr1, specifically hydrolyzes the 2'–5' phosphodiester bond in the intron, which converts lariar form introns into linear molecules<sup>12</sup>. The linearized intron is likely to be degraded by either Xrn1, a 5'–3' exonuclease and/or the exosome that has a 3'–5' exonuclease activity<sup>11,13,14</sup>. Dbr1 was originally identified and cloned from budding yeast (*Saccharomyces cerevisiae*) by a genetic screening to identify host cellular factors involved in retrotransposon Ty1 element transposition<sup>15</sup>. The *Dbr1* mutant of *S. cerevisiae* has reduced Ty1 transposition frequency and shows the high level accumulation of lariar introns in the cell<sup>15</sup>. This result indicates that debranching has to occur prior to intron degradation. Thus debranching is one of the key steps for intron turnover.

*Dbr1* gene is not essential for cell viability in *Saccharomyces cerevisiae*, although *Dbr1* mutant shows accumulation of lariar RNAs<sup>15</sup>. In fission yeast, *Schizosaccharomyces pombe*, *Dbr1* is also unessential for its viability<sup>16</sup>.



However, *Dbr1* null mutant cells of *S.pombe* show severe growth defect and elongated cell shape in addition to the accumulation of RNA lariat<sup>16</sup>. One possible explanation for the difference of phenotypes between *S. cerevisiae* and *S. pombe* is the percentage of genes that contain introns. Only about 2.5% of genes contain introns in *S. cerevisiae*, while 40% in *S. pombe*<sup>17–19</sup>. The cDNAs of *Dbr1* have been isolated from many species, such as *Caenorhabditis elegans*, mouse and human<sup>16,20,21</sup>. These homologues can complement *S.pombe Dbr1* null mutant, indicating that the function and sequence of *Dbr1* is conserved among many species. The *Dbr1* cDNA was also isolated from a plant, *Arabidopsis thaliana*, and *A. thaliana Dbr1* mutant was turned out to be embryonic lethal<sup>22</sup>. Taken together, it is likely that rapid intron turnover including debranching is important for higher eukaryotes that contain many introns.

In human, almost all genes encoded in the nucleus are separated by multiple introns that occupy, in sum, about 95% of the primary transcripts. It is therefore highly expected that the pathway for rapid intron turnover in the nucleus is critical in human. Although the homologs of the factors described above in yeast have been identified in mammals<sup>16,20,21,23–26</sup>, this pathway was not well understood. We have been analyzing this pathway in human by using *in vitro* splicing assay system. We found that introns are degraded through the formation of two complexes, IL (Intron Large) and IS (Intron Small) complexes<sup>27</sup>. IL complex is a 40S complex, and it contains U2, U5 and U6 snRNPs and hPrp19 complex proteins, while IS complex, which is a 20 S form, does not contain those snRNPs and protein factors<sup>27</sup>. We have also identified the TFIP11, a human homolog of yeast Ntr1 protein, in the IL complex and demonstrated that TFIP11 recruits the hPrp43 protein to the IL complex through interactions mediated by its N-terminal G-patch region, which is required for the transition from IL complex to IS complex<sup>27</sup>. Furthermore, we also showed that hDbr1 is accessible to IS complex, but not to IL complex<sup>27</sup>, suggesting that hDbr1 is involved in disassembly of IS complex prior to degradation of linear introns. To get further insight into the intron turnover mechanism, we decided to analyze human *Dbr1* (hDbr1) protein. The hDbr1 cDNA was previously identified in the human Expressed Sequence Tag (EST) database and isolated by RT-PCR<sup>20</sup>. It was demonstrated that hDbr1 was functional in interspecies complementation experiments and that the corresponding recombinant hDbr1 protein had a debranching activity *in vitro*<sup>20</sup>. However the precise structural domain analysis has not been performed. Here we report the functional domain analysis of hDbr1. The hDbr1 protein is localized in the nucleoplasm of HeLa cells through a bipartite classical type nuclear localization signal (NLS) near its carboxyl-terminus. The protein phosphatase 1 like motif, point mutations of which were shown to inactivate yeast *Dbr1p in vitro* and *in vivo*<sup>28,29</sup>, is also critical for debranching activity of hDbr1 *in vitro*. Furthermore, in spite of its nuclear localization at steady state, hDbr1 protein has a capacity to shuttle between the nucleus and the cytoplasm. This result strongly suggests that hDbr1 has novel function(s) in the cytoplasm.

## Results

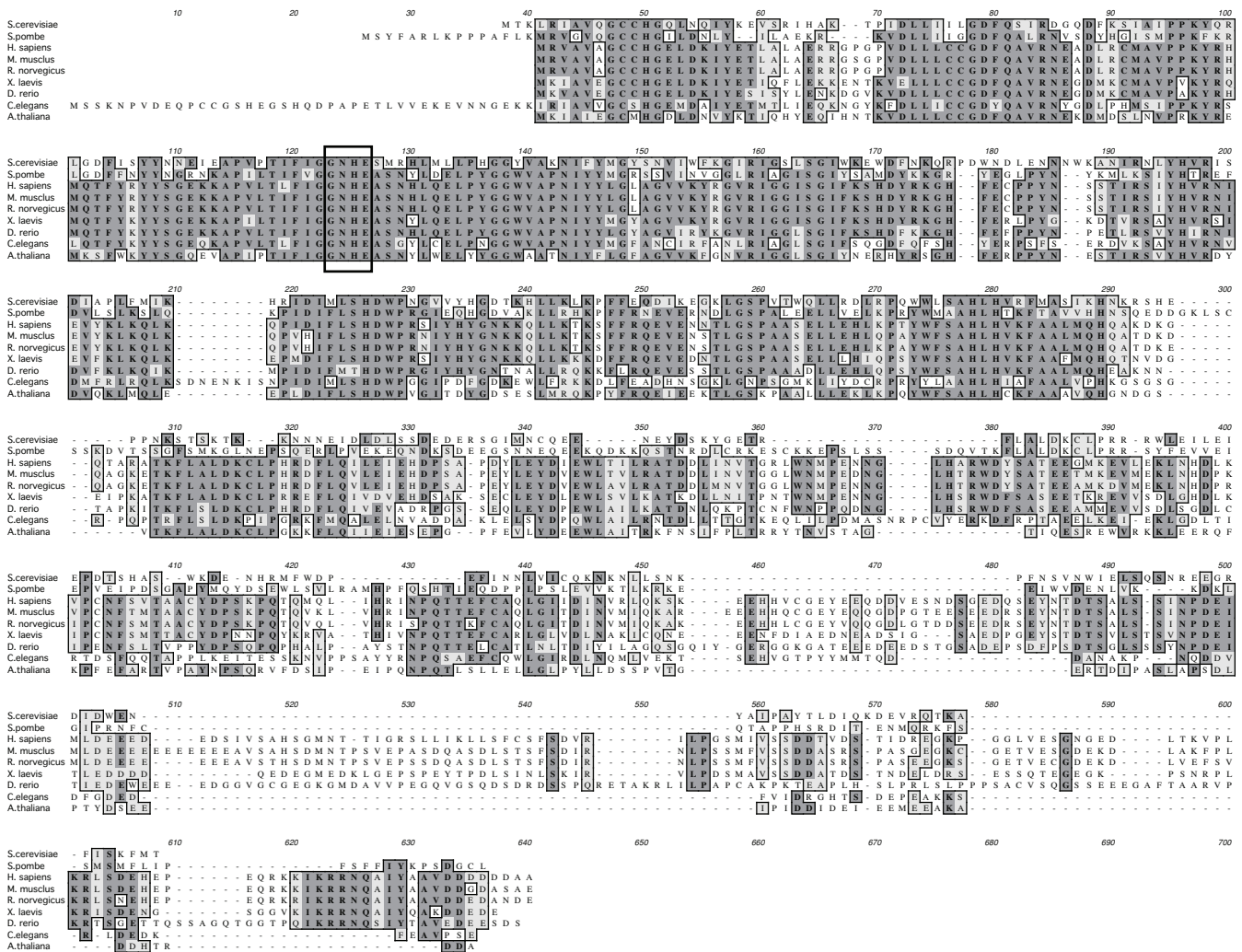
The homologues of *Dbr1* protein from many species were isolated<sup>16,20–22</sup> and the amino acid sequence of this protein is well conserved (Figure 1). The amino terminus of *Dbr1* protein especially shows high similarity, while the carboxyl terminus is relatively diverse (Figure 1). In order to analyze the function of human *Dbr1* protein, we first determined the subcellular localization of human *Dbr1* protein by transfection experiments. The full-length hDbr1 protein, fused to either myc or Flag tag was transiently expressed in HeLa cells. The immunofluorescence experiments shown in Figure 2 revealed that both myc- and Flag- tagged hDbr1 proteins were localized in the nucleoplasm of HeLa cells (Figure 2, panel FITC). This localization is consistent with the function of hDbr1,

because hDbr1 catalyzes debranching reaction of lariat introns, which is supposed to occur in the nucleus.

The nuclear localization of exogenously expressed hDbr1 protein predicted that hDbr1 protein has a nuclear localization signal (NLS). In order to identify the hDbr1 NLS, we fused several domains of hDbr1 to the reporter protein and tested the subcellular localization of the corresponding fusion proteins in HeLa cells. As a reporter protein, we used the myc-tagged chicken Pyruvate Kinase (myc-PK), which is exclusively localized in the cytoplasm (Figure 3A)<sup>30,31</sup>. If the domain from hDbr1 has a NLS activity, the fusion protein should be localized to the nucleus. When the full-length hDbr1 was fused to myc-PK, the chimeric protein was localized in the nucleus (Figure 3A, PK-hDbr1(1-544)). We first separated hDbr1 into two parts and fused to myc-PK. We found that the fusion of N-terminal 240 aa of hDbr1 to PK (PK-hDbr1(1-240)) produced cytoplasmic protein, while PK fused to the C-terminal 304 aa of hDbr1 protein (PK-hDbr1(241-544)) showed exclusively nuclear staining (Figure 3A). These results indicate that C-terminal region of hDbr1 confers its nuclear localization. We further deleted C-terminal region of hDbr1 and found that the fusion protein of C-terminal 37 aa of hDbr1 to PK (PK-hDbr1(508-544)) is localized in the nucleus (Figure 3A). Since PK fused to the rest of hDbr1 showed exclusive cytoplasmic staining (Figure 3A, PK-hDbr1(1-507)), it was strongly suggested that hDbr1 has one NLS in its C-terminal 37 aa. region.

In the last 37 amino acids of hDbr1, we could find two basic residue stretches that were good candidates for classical type nuclear localization signal (Figure 3B). To test whether these regions are critical for nuclear localization or not, we expressed the mutant proteins that had deletion in either the first or the second basic stretch from myc-tagged PK with 37 amino acids of hDbr1 protein (PK-hDbr1(508–544Δ1) and PK-hDbr1(508–544Δ2), Figure 3B). As shown in Figure 3, neither of the deletion mutants were localized in the nucleus. The deletion of the last 37 amino acids from full-length hDbr1 protein fused to myc-PK resulted in the cytoplasmic localization (Figure 3A). These results indicate that hDbr1 has a bipartite type NLS that is located in the last 37 amino acid region of hDbr1.

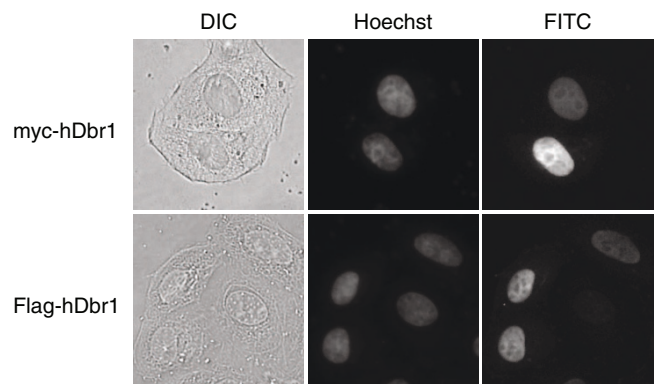
*Dbr1* protein has a GNHE motif, which is one of the signatures of metallophosphoesterase superfamily protein<sup>23,32</sup>. This protein superfamily consists of  $\lambda$  phosphoprotein phosphatase, protein phosphatases 1 and 2, Mre11 and calcineurin and this motif is demonstrated to be important for protein phosphatase activity by binding to metal ion<sup>24–26</sup>. This GNHE motif is conserved among *Dbr1* homologues from many species (Figure 1), suggesting that this motif is also important for *Dbr1* protein function. Indeed, it is demonstrated that this GNHE motif is critical for debranching activity of yeast *Dbr1p*<sup>28,29</sup>. In order to test whether GNHE motif also has a vital role in debranching activity of hDbr1, we decided to prepare several mutants that have a point mutation in either 84<sup>th</sup> Asparagine (N) residue or 85<sup>th</sup> Histidine (H) residue according to the mutations used in the previous study with both protein phosphatase 1<sup>26</sup> and yeast *Dbr1p*<sup>28,29</sup>. These residues were substituted by either Alanine (A) or Serine (S), and the resultant mutants are called as N84A, H85S and H85A. It is already known that the corresponding mutations cause the loss of activity in the case of protein phosphatase 1<sup>26</sup>, and it was also demonstrated that these residues are critical in yeast *Dbr1p* for both debranching activity and Ty1 transposition<sup>28,29</sup>. These mutant proteins with Flag-tag were transiently expressed to determine their subcellular localization. In HeLa cells all the mutant proteins as well as the wild type protein were localized in the nucleoplasm (Supplementary Figure 1). To obtain both wild type and mutant proteins, we transfected HEK293T cells with the plasmids carrying cDNA of either wild type or mutant proteins with Flag-tag and prepared the whole cell extracts from transfected cells<sup>33</sup>. To test the effect of these mutations on debranching activity of hDbr1, we carried out *in vitro* debranching assays using purified Flag-tagged proteins from the



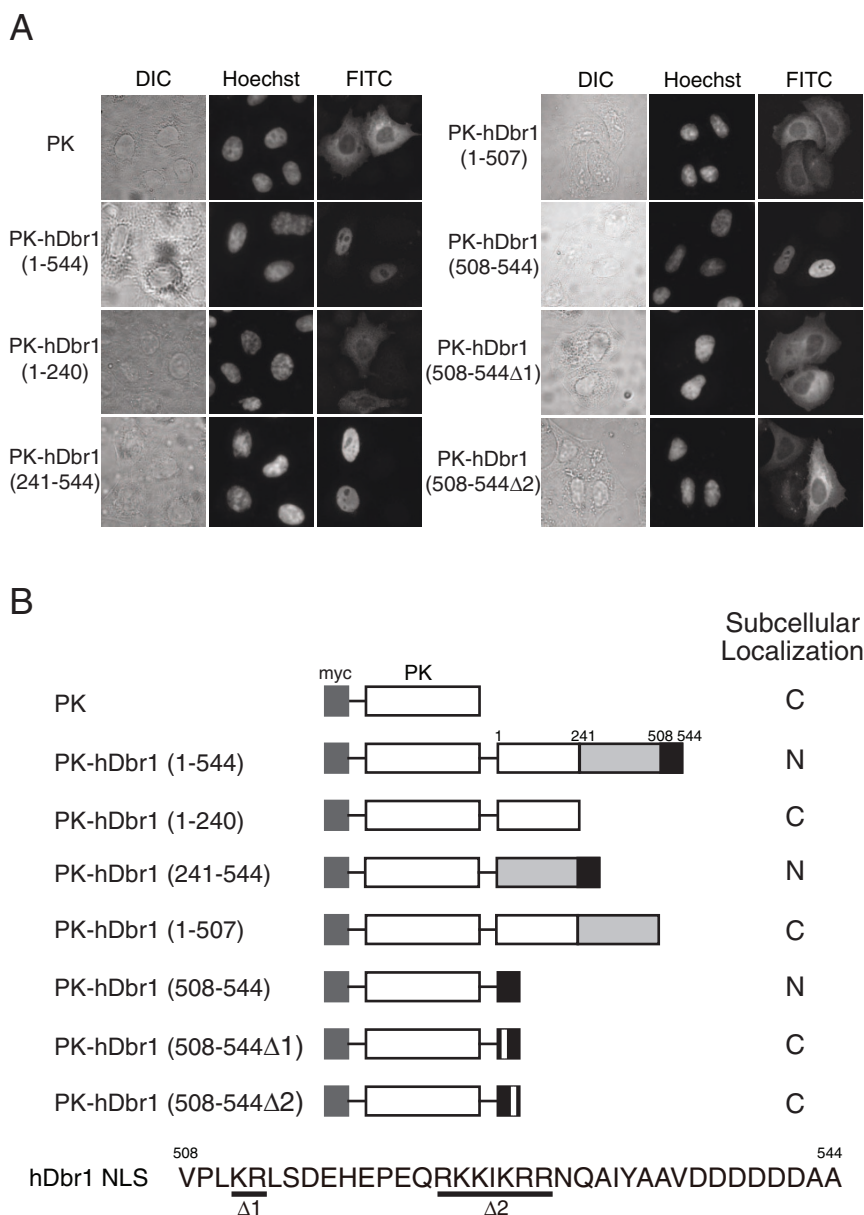
**Figure 1 | Amino acid sequence alignment of Dbr1 from various organisms.** Dbr1 proteins from *Saccharomyces cerevisiae* (NP\_012773.1), *Schizosaccharomyces pombe* (NP\_593470.2), *Homo sapiens* (NP\_057300.2), *Mus musculus* (NP\_113580.2), *Rattus norvegicus* (NP\_001102907.1), *Xenopus laevis* (NP\_001080368.1), *Danio rerio* (NP\_955947.1), *Caenorhabditis elegans* (NP\_491868.2) and *Arabidopsis thaliana* (NP\_567881.1) were aligned using ClustalW alignment program (<http://www.genome.jp/tools/clustalw/>). Identical residues are indicated by dark shading and similar residues are shown by light shading. An open box indicates the position of a conserved GNHE motif that is also found in Protein Phosphatase 1 (PP1) as a catalytic center.

whole cell extracts by using anti-Flag M2 resin<sup>34</sup>. After elution with 3X Flag peptides, the eluates were analyzed by SDS-PAGE. As shown in Figure 4A, almost equal amount of proteins could be recovered from the extracts. These proteins were incubated with <sup>32</sup>P-labeled lariat intron RNAs derived from Adeno virus immediate early gene pre-mRNA, Ad2, which had been gel-purified from *in vitro* splicing reaction products. The results in Figure 4B demonstrated that the wild type hDbr1 protein could debranch the lariat intron (lane WT). However H85A, H85S and N84A mutants had a greatly reduced debranching activity (Figure 4B, lanes H85A, H85S and N84A). Essentially the same results were obtained with the lariat RNA from chicken  $\delta$ -crystallin pre-mRNA (Figure 4B). The results shown in Figure 4 indicate that the GNHE motif, which is also found in protein phosphatases, is critical for debranching activity of hDbr1.

Dbr1p was originally isolated as a host gene product critical for yeast retrotransposon element Ty1<sup>15</sup>. During its life cycle, Ty1 RNA is synthesized in yeast nucleus and exported to the cytoplasm to serve as a template for the translation of the encoding proteins. Ty1 RNAs are incorporated into virus like particles (VLPs) with Ty1 encoding proteins including reverse transcriptase and integrase, and these particles are subsequently imported to the nucleus<sup>35,36</sup>. The synthesis of Ty1 cDNA by reverse transcription is thought to occur in VLPs in the cytoplasm. In *Dbr1* mutants, the accumulation of Ty1 cDNAs is



**Figure 2 | hDbr1 localizes to the nucleoplasm by immunofluorescence.** Expression vectors encoding either myc-tagged (upper panels) or Flag-tagged hDbr1 (lower panels) were transfected into HeLa cells. Twenty-four hours after transfection, the cells were stained with either anti-myc (MC045, Nacalai Tesque, Japan, panel FITC, upper right) or anti-Flag (M2, SIGMA, panel FITC, lower right). The cells were also stained with Hoechst 33342 (SIGMA, panel Hoechst, middle) to label the nuclei. Differential interference contrast (DIC) images of the cells are also shown as DIC panels at the left.



**Figure 3 | Identification of the nuclear localization signal (NLS) of hDbr1.** A) HeLa cells were transfected with expression vectors encoding myc-tagged PK fusion proteins. The numbers above each panel correspond to the amino acids within hDbr1 that were fused to PK. Δ1 and Δ2 indicate that the corresponding regions shown at the bottom of B by underlines were deleted. The subcellular localization of each protein was determined by indirect immunofluorescence using anti-myc monoclonal antibody, MC045. DIC images and Hoechst staining pattern of the cells are also presented as DIC and Hoechst, respectively. B) Schematic drawing of the PK fusion proteins and summary of their intracellular localization as displayed in (A). Also the bipartite NLS of hDbr1 is shown at the bottom marked with underlines. The Δ1 mutant has a deletion of amino-acid residues, KR, whereas the Δ2 mutant contains a deletion of RKKIKRR residues.

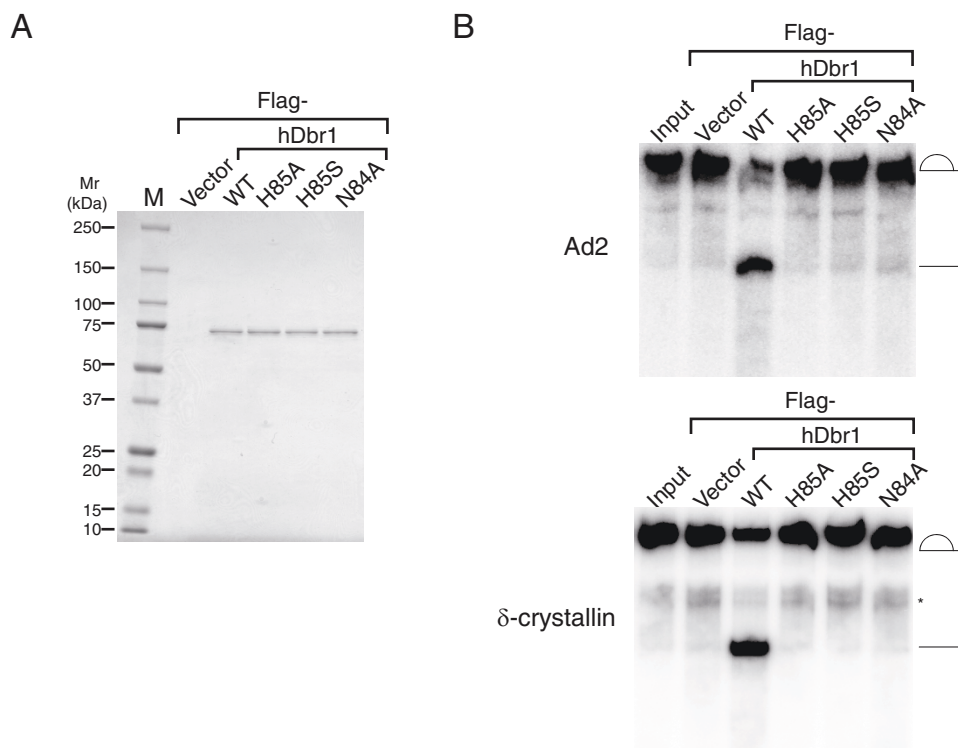
reduced, whereas the protein synthesis from Ty1 mRNA is at the wild-type level<sup>37</sup>. These results suggest that DBR1 protein is required for reverse transcription and/or the stability of Ty1 cDNA. Since Ty1 cDNA reverse transcription is supposed to occur in the cytoplasm, it was possible that DBR1 shuttles between the nucleus and the cytoplasm, although it is nuclear at the steady state.

To determine if hDbr1 has a shuttling activity, we carried out a heterokaryon assay (Figure 5). Heterokaryons were formed by Polyethylen Glycol (PEG)-mediated fusions of HeLa cells that had been previously transfected with the indicated constructs and mouse NIH3T3 cells. Staining of the cells with the DNA dye, Hoechst, allows us to distinguish between the HeLa and the NIH3T3 nuclei. Fusions and subsequent incubations were carried out in the presence of

cycloheximide to ensure that the observed signals result from proteins in the HeLa nucleus and not from newly synthesized proteins in the cytoplasm<sup>30,38–41</sup>. A construct containing the non-shuttling hnRNP C1 protein served as a negative control, while the rapid shuttling protein hnRNP A1 as a positive control. The results presented in Figure 5 show that hDbr1 shuttles between the nucleus and the cytoplasm, since the hDbr1 signal was clearly seen in both HeLa and NIH3T3 nuclei. This result strongly suggests that hDbr1 has novel cytoplasmic function(s).

## Discussion

In this paper we analyzed the several domains of hDbr1 protein, which is involved in intron turnover with its debranching activity.



**Figure 4 | The conserved GNHE motif homologous to protein phosphatase 1 is essential for debranching activity of hDbr1 *in vitro*.** A) Equal amount (0.1  $\mu$ g each) of recombinant proteins purified from either Flag-vector- or the hDbr1 cDNA-transfected HEK293T cells on a Coomassie Brilliant Blue-stained 5–20% gradient SDS-polyacrylamide gel. The plasmids used for transfection are indicated above the panel as follows; Vector : Flag-pCDNA3 vector, WT : Flag-wild type hDbr1, H85A : Flag-hDbr1 containing histidine to alanine mutation at amino-acid position 85, H85S : Flag-hDbr1 harboring histidine to serine change at amino-acid position 85, N84A : Flag-hDbr1 carrying asparagine to alanine mutation at amino-acid position 84. Input lanes contain the lariat RNAs used for the assays. B) *In vitro* debranching assays with purified proteins shown in A). Lariat intron RNAs derived from either Ad2 (upper panel) and  $\delta$ -crystallin (lower panel) pre-mRNA were incubated with recombinant proteins and analyzed by 6% denaturing polyacrylamide gel electrophoresis. The structure of RNAs corresponding to each band is demonstrated schematically. The asterisk shows the contaminated pre-mRNAs.

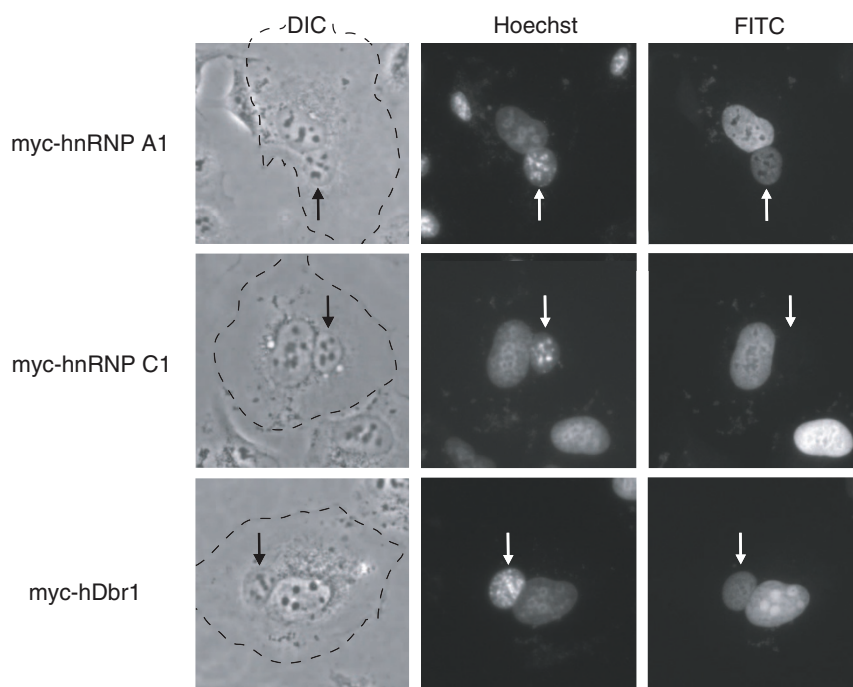
The hDbr1 is a nuclear protein that has a bipartite classical type NLS near its Carboxyl terminus (Figure 2,3). The similar NLS-like sequence can be found in mouse, rat, *Xenopus*, zebra fish proteins, but not in other organisms (Figure 1). Dbr1 proteins show high similarity especially in the region of first 200 amino acids. However, carboxyl terminal half is relatively diverse among species (Figure 1). The budding yeast Dbr1p is a 406 amino acid protein, while hDbr1 is 544 amino acids long with a longer carboxyl terminus. It is reported that Green Fluorescent Protein (GFP) tagged-yeast Dbr1p is localized both in the nucleus and cytoplasm<sup>42</sup>, and its NLS has not been identified. It may have a different type NLS, rather than a classical type NLS. Further delineation of yeast Dbr1 NLS remains to be performed.

The hDbr1 protein has a conserved GNHE motif, which is a signature of metallophosphatase superfamily. This motif is known to bind to metal ion in metallophosphatases and the point mutation in asparagine (N) or histidine (H) in this motif abolishes their phosphatase activity<sup>26</sup>. The GNHE motif is also demonstrated to be important for debranching activity of yeast Dbr1p both *in vitro* and *in vivo*<sup>28,29</sup>. The motif is perfectly conserved among Dbr1 proteins from many species, suggesting that this GNHE signature is catalytically important in Dbr1 proteins from other species. As expected, the mutations of asparagine and histidine in this motif of hDbr1 abolished its debranching activity *in vitro* (Figure 4). These two amino-acid residues are proposed to contact to the 2'-5' phosphodiester of the RNA by analogy to the Mre11-dAMP complex<sup>29</sup>. The yeast Dbr1 protein was demonstrated to prefer manganese to magnesium as the metal cofactor for debranching<sup>29</sup>. It is of great

interest to dissolve crystal structure of Dbr1 protein with manganese and branched RNA.

The heterokaryon assay revealed that hDbr1 is a nucleocytoplasmic shuttling protein (Figure 5). In this respect, the cytoplasmic function(s) of hDbr1 is proposed as a novel feature of hDbr1. One possible function is to debranch introns in the cytoplasm. It was demonstrated that the introns in the homeobox gene *Pem* could be detected in the cytoplasmic fraction<sup>43</sup>. In addition, it was shown that the splice-defective splicing intermediates (lariat introns with 3' exon) are debranched by Dbr1p and degraded by the cytoplasmic exonuclease Xrn1p and cytoplasmic exosome<sup>44</sup>. These results strongly suggest that the intron RNAs that have a lariat structure can be exported to the cytoplasm possibly by accident and hDbr1 debranches them in the cytoplasm. It is possible that hDbr1 monitors the presence of lariat-introns in the cytoplasm and rapidly debranches them to degrade. In order to test these possibilities, further experiments are required to detect lariat RNAs from the cytoplasm when hDbr1 protein level is reduced by siRNA or shRNA.

Another possible function of hDbr1 in the cytoplasm is to be involved in the cDNA synthesis by reverse transcription. The *Dbr1* gene was originally isolated by a genetic screening to identify host factors required for Ty1 retrotransposon transposition<sup>15</sup>. The *Dbr1* mutant strains exhibit low level of Ty1 cDNA, suggesting that Dbr1p is involved in reverse transcription of Ty1 mRNA. It was reported that Ty1 RNA forms a 2'-5' branch structure characteristic of a lariat intron and this lariat structure is an intermediate of Ty1 cDNA synthesis<sup>45</sup>. This branch structure was suggested to facilitate the minus-strand transfer from the upstream to the downstream of



**Figure 5 | The hDbr1 protein shuttles between the nucleus and the cytoplasm.** Expression vectors encoding hnRNP A1, C1, and hDbr1 with myc tag were transfected into HeLa cells. After expression of the transfected cDNAs, the cells were fused with mouse NIH3T3 cells to form heterokaryones and incubated in media containing 100  $\mu\text{g}/\text{ml}$  cycloheximide for 2 hours. The cells were then fixed and stained for immunofluorescence microscopy with anti-myc tag antibody (panel FITC) to localize the proteins, and Hoechst 33342 (SIGMA, panel Hoechst), which differentiates the human and mouse nuclei within the heterokaryon. The arrows identify the mouse nuclei. The panels marked DIC show the phase-contrast image of the heterokaryons and the cytoplasmic edge is highlighted by a broken line.

Ty1 RNA during cDNA synthesis. The structural similarity of Ty1 to the animal retroviruses suggests that retrovirus genomic RNA forms a branch structure as well. It was also reported that reduction of hDbr1 mRNA level by siRNA led to the inhibition of Human Immunodeficiency Virus-1 (HIV-1) replication by reducing viral cDNA and protein production<sup>46</sup>. This paper suggests that debranching activity of hDbr1 is required for reverse transcription completion. However, it is still controversial whether Ty1 RNA has a branch structure or not, since the lariat structure of Ty1 RNA could not be detected in many different methods<sup>47,48</sup>. Therefore the molecular basis for the involvement of Dbr1p in Ty1 transposition is still unclear. Further analysis of hDbr1 including the identification of specific interactors are required to uncover the bona fide cytoplasmic function(s) of hDbr1.

## Methods

**Plasmid construction.** Full-length hDbr1 cDNA was amplified by PCR reaction from Marathon ready HeLa cDNA library (Clontech) and cloned between *Bam*HI and *Xho*I sites of myc- (myc-hDbr1) and Flag-pCDNA3 (Flag-hDbr1)<sup>41,49</sup>. For myc-chicken Pyruvate Kinase (PK)-hDbr1 fusion constructs, myc-hDbr1 was used as a series of PCR reactions. Primers were designed which included an *Kpn*I site in the 5' partner and an *Xho*I site in the 3' partner to amplify fragments of the hDbr1 coding sequences corresponding to amino acids 1–544 (Full length), 1–240, 241–544, 241–517, 1–507 and 508–544. These individual fragments were then digested with *Kpn*I and *Xho*I and subcloned into similarly digested myc-PK<sup>30</sup>. For the series of NLS deletion constructs, by using the plasmid encoding myc-PK fused with amino acids 508–544 of hDbr1 as a template, the entire region of the template plasmid except the portion that has to be deleted was amplified by PCR reaction. The hDbr1 mutant cDNAs that have a point mutation were also prepared by PCR-based mutagenesis by using Flag-hDbr1 plasmid as a template with a QuikChange site-directed mutagenesis kit (STRATAGENE) as the manufacturer recommends.

**Cell culture and transfection.** HeLa and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Nacalai tesque, Japan) supplemented with 10% fetal bovine serum (EQUITECH-BIO, Texas) and 1% penicillin-streptomycin (Gibco-BRL) in an incubator adjusted to 37°C with 5% CO<sub>2</sub>.

Transfection of cultured cells was carried out by using Lipofectamine2000 (Invitrogen) as a manufacturer recommends.

**Protein expression in HEK 293T cells and purification.** Both wild type and mutant hDbr1 proteins with Flag-tag were transiently expressed in HEK293T cells by transfection. Whole cell lysates were prepared from those transfected cells as previously described<sup>33</sup>. Flag-tagged proteins were purified from whole cell lysates by using anti Flag-M2 resin (SIGMA) as described previously<sup>34</sup>.

**Immunofluorescence.** Transfected HeLa cells were fixed and stained for immunofluorescence microscopy with either anti-myc (MC045, Nakalai tesque, Japan) or anti-Flag M2 antibody (SIGMA) as described previously<sup>50</sup>. Hoechst 33342 (SIGMA) was included at 5  $\mu\text{g}/\text{ml}$  during the secondary antibody incubations.

**In vitro splicing and debranching assays.** HeLa cell nuclear extracts were obtained from CILBIOTECH (Belgium). Lariat-intron was prepared by *in vitro* splicing reaction. *In vitro* splicing reaction was carried out as described previously<sup>33,41,51</sup>. Briefly, the splicing reaction mixture was supplied onto 6% denaturing acrylamide gel and the band corresponding to lariat-intron RNA was excised and recovered from the gel. About 0.1 pmol of the lariat-intron RNA was incubated with 50 ng (approximately 0.7 pmol) of purified Flag-tagged proteins in the debranching reaction mixture (20 mM Hepes-KOH, pH7.9, 40 mM KCl, 3 mM MgCl<sub>2</sub>, 4% Glycerol) 30 minutes at 30°C in 20  $\mu\text{l}$  reaction mixture. After incubation RNA was recovered by phenol extraction and ethanol precipitation, followed by analysis on 6% denaturing polyacrylamide gel electrophoresis.

**Heterokaryon assay.** Heterokaryon assay was performed as described previously<sup>30,31,38–41</sup>. Briefly, HeLa cells grown in 100 mm dishes were transfected as described. Twenty-four hours later the cells were trypsinized and transferred to 6 hole plates containing 18 mm<sup>2</sup> glass coverslips. Next day an equal number of NIH3T3 cells that had been cultured in the presence of 75  $\mu\text{g}/\text{ml}$  cycloheximide 30 minutes were seeded on the coverslips. Cells were fused as described previously<sup>30,31,38–41</sup> and cultured in the medium containing 100  $\mu\text{g}/\text{ml}$  cycloheximide for another 2 hours prior to fixation for immunofluorescence.

- Hastings, M. L. & Krainer, A. R. Pre-mRNA splicing in the new millennium. *Curr Opin Cell Biol* **13**, 302–309 (2001).
- Maniatis, T. & Reed, R. An extensive network of coupling among gene expression machines. *Nature* **416**, 499–506 (2002).



3. Dreyfuss, G., Kim, V. N. & Kataoka, N. Messenger-RNA-binding proteins and the messages they carry. *Nat Rev Mol Cell Biol* **3**, 195–205 (2002).
4. Jurica, M. S. & Moore, M. J. Pre-mRNA splicing: awash in a sea of proteins. *Mol Cell* **12**, 5–14 (2003).
5. Company, M., Arenas, J. & Abelson, J. Requirement of the RNA helicase-like protein PRP22 for release of messenger RNA from spliceosomes. *Nature* **349**, 487–493 (1991).
6. Staley, J. P. & Guthrie, C. Mechanical devices of the spliceosome: motors, clocks, springs, and things. *Cell* **92**, 315–326 (1998).
7. Martin, A., Schneider, S. & Schwer, B. Prp43 is an essential RNA-dependent ATPase required for release of lariat-intron from the spliceosome. *J Biol Chem* **277**, 17743–17750 (2002).
8. Arenas, J. E. & Abelson, J. N. Prp43: An RNA helicase-like factor involved in spliceosome disassembly. *Proc Natl Acad Sci U S A* **94**, 11798–11802 (1997).
9. Tsai, R. T. *et al.* Spliceosome disassembly catalyzed by Prp43 and its associated components Ntr1 and Ntr2. *Genes Dev* **19**, 2991–3003 (2005).
10. Tanaka, N., Aronova, A. & Schwer, B. Ntr1 activates the Prp43 helicase to trigger release of lariat-intron from the spliceosome. *Genes Dev* **21**, 2312–2325 (2007).
11. Moore, M. J. Nuclear RNA turnover. *Cell* **108**, 431–434 (2002).
12. Nam, K. *et al.* Yeast lariat debranching enzyme. Substrate and sequence specificity. *J Biol Chem* **269**, 20613–20621 (1994).
13. Mitchell, P. & Tollervey, D. mRNA turnover. *Curr Opin Cell Biol* **13**, 320–325 (2001).
14. Coller, J. & Parker, R. Eukaryotic mRNA decapping. *Annu Rev Biochem* **73**, 861–890 (2004).
15. Chapman, K. B. & Boeke, J. D. Isolation and characterization of the gene encoding yeast debranching enzyme. *Cell* **65**, 483–492 (1991).
16. Nam, K., Lee, G., Trambley, J., Devine, S. E. & Boeke, J. D. Severe growth defect in a *Schizosaccharomyces pombe* mutant defective in intron lariat degradation. *Mol Cell Biol* **17**, 809–818 (1997).
17. Prabhala, G., Rosenberg, G. H. & Kaufer, N. F. Architectural features of pre-mRNA introns in the fission yeast *Schizosaccharomyces pombe*. *Yeast* **8**, 171–182 (1992).
18. Rodriguez-Medina, J. R. & Rymond, B. C. Prevalence and distribution of introns in non-ribosomal protein genes of yeast. *Mol Gen Genet* **243**, 532–539 (1994).
19. Kalogeropoulos, A. Automatic intron detection in nuclear DNA sequences of *Saccharomyces cerevisiae*. *Yeast* **11**, 555–565 (1995).
20. Kim, J. W. *et al.* Human RNA lariat debranching enzyme cDNA complements the phenotypes of *Saccharomyces cerevisiae* *dbl1* and *Schizosaccharomyces pombe* *dbl1* mutants. *Nucleic Acids Res* **28**, 3666–3673 (2000).
21. Kim, H. C., Kim, G. M., Yang, J. M. & Ki, J. W. Cloning, expression, and complementation test of the RNA lariat debranching enzyme cDNA from mouse. *Mol Cells* **11**, 198–203 (2001).
22. Wang, H., Hill, K. & Perry, S. E. An Arabidopsis RNA lariat debranching enzyme is essential for embryogenesis. *J Biol Chem* **279**, 1468–1473 (2004).
23. Zhuo, S., Clemens, J. C., Stone, R. L. & Dixon, J. E. Mutational analysis of a Ser/Thr phosphatase. Identification of residues important in phosphoesterase substrate binding and catalysis. *J Biol Chem* **269**, 26234–26238 (1994).
24. Egloff, M. P., Cohen, P. T., Reinemer, P. & Barford, D. Crystal structure of the catalytic subunit of human protein phosphatase 1 and its complex with tungstate. *J Mol Biol* **254**, 942–959 (1995).
25. Goldberg, J. *et al.* Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. *Nature* **376**, 745–753 (1995).
26. Zhang, J., Zhang, Z., Brew, K. & Lee, E. Y. Mutational analysis of the catalytic subunit of muscle protein phosphatase-1. *Biochemistry* **35**, 6276–6282 (1996).
27. Yoshimoto, R., Kataoka, N., Okawa, K. & Ohno, M. Isolation and characterization of post-splicing lariat-intron complexes. *Nucleic Acids Res* **37**, 891–902 (2009).
28. Salem, L. A., Boucher, C. L. & Menees, T. M. Relationship between RNA lariat debranching and Ty1 element retrotransposition. *J Virol* **77**, 12795–12806 (2003).
29. Khalid, M. F., Damha, M. J., Shuman, S. & Schwer, B. Structure-function analysis of yeast RNA debranching enzyme (Dbr1), a manganese-dependent phosphodiesterase. *Nucleic Acids Res* **33**, 6349–6360 (2005).
30. Michael, W. M., Eder, P. S. & Dreyfuss, G. The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein. *Embo J* **16**, 3587–3598 (1997).
31. Kataoka, N. *et al.* Specific Y14 domains mediate its nucleo-cytoplasmic shuttling and association with spliced mRNA. *Scientific reports* **1**, 92 (2011).
32. Koonin, E. V. Conserved sequence pattern in a wide variety of phosphoesterases. *Protein Sci* **3**, 356–358 (1994).
33. Kataoka, N. & Dreyfuss, G. A simple whole cell lysate system for in vitro splicing reveals a stepwise assembly of the exon-exon junction complex. *J Biol Chem* **279**, 7009–7013 (2004).
34. Pellizzoni, L., Baccon, J., Rappsilber, J., Mann, M. & Dreyfuss, G. Purification of native survival of motor neurons complexes and identification of Gemin6 as a novel component. *J Biol Chem* **277**, 7540–7545 (2002).
35. Roth, J. F. The yeast Ty virus-like particles. *Yeast* **16**, 785–795 (2000).
36. Wilhelm, F. X., Wilhelm, M. & Gabriel, A. Reverse transcriptase and integrase of the *Saccharomyces cerevisiae* Ty1 element. *Cytogenet Genome Res* **110**, 269–287 (2005).
37. Karst, S. M., Rutz, M. L. & Menees, T. M. The yeast retrotransposons Ty1 and Ty3 require the RNA lariat debranching enzyme, Dbr1p, for efficient accumulation of reverse transcripts. *Biochem Biophys Res Commun* **268**, 112–117 (2000).
38. Pinol-Roma, S. & Dreyfuss, G. Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. *Nature* **355**, 730–732 (1992).
39. Michael, W. M., Choi, M. & Dreyfuss, G. A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. *Cell* **83**, 415–422 (1995).
40. Nakielyny, S. & Dreyfuss, G. The hnRNP C proteins contain a nuclear retention sequence that can override nuclear export signals. *J Cell Biol* **134**, 1365–1373 (1996).
41. Kataoka, N. *et al.* Pre-mRNA splicing imprints mRNA in the nucleus with a novel RNA-binding protein that persists in the cytoplasm. *Mol Cell* **6**, 673–682 (2000).
42. Kumar, A. *et al.* Subcellular localization of the yeast proteome. *Genes Dev* **16**, 707–719 (2002).
43. Clement, J. Q., Maiti, S. & Wilkinson, M. F. Localization and stability of introns spliced from the *Pem* homeobox gene. *J Biol Chem* **276**, 16919–16930 (2001).
44. Hilleren, P. J. & Parker, R. Cytoplasmic degradation of splice-defective pre-mRNAs and intermediates. *Mol Cell* **12**, 1453–1465 (2003).
45. Cheng, Z. & Menees, T. M. RNA branching and debranching in the yeast retrovirus-like element Ty1. *Science* **303**, 240–243 (2004).
46. Ye, Y., De Leon, J., Yokoyama, N., Naidu, Y. & Camerini, D. DBR1 siRNA inhibition of HIV-1 replication. *Retrovirology* **2**, 63 (2005).
47. Coombes, C. E. & Boeke, J. D. An evaluation of detection methods for large lariat RNAs. *Rna* **11**, 323–331 (2005).
48. Pratico, E. D. & Silverman, S. K. Ty1 reverse transcriptase does not read through the proposed 2',5'-branched retrotransposition intermediate in vitro. *Rna* **13**, 1528–1536 (2007).
49. Kim, V. N., Kataoka, N. & Dreyfuss, G. Role of the nonsense-mediated decay factor hUpf3 in the splicing-dependent exon-exon junction complex. *Science* **293**, 1832–1836 (2001).
50. Kataoka, N., Diem, M. D., Kim, V. N., Yong, J. & Dreyfuss, G. Magoh, a human homolog of *Drosophila mago nashi* protein, is a component of the splicing-dependent exon-exon junction complex. *Embo J* **20**, 6424–6433 (2001).
51. Pellizzoni, L., Kataoka, N., Charroux, B. & Dreyfuss, G. A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing. *Cell* **95**, 615–624 (1998).

## Acknowledgements

We are grateful to Dr. Gideon Dreyfuss for the plasmids. We also thank members of both Ohno laboratory and Hagiwara laboratory for discussions. This work is supported by Grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. N.K. was supported by Program for Improvement of Research Environment for Young Researchers from Special Coordination Funds for Promoting Science and Technology (SCF) commissioned by the MEXT of Japan.

## Author contributions

N.K., M.H. and M.O. designed experiments and N.K. and I.D. performed them. N.K. and M.O. analyzed the data and N.K., M.H. and M.O. wrote the manuscript.

## Additional information

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

**Competing financial interests:** The authors declare no competing financial interests.

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**How to cite this article:** Kataoka, N., Dobashi, I., Hagiwara, M. & Ohno, M. hDbr1 is a nucleocytoplasmic shuttling protein with a protein phosphatase-like motif essential for debranching activity. *Sci. Rep.* **3**, 1090; DOI:10.1038/srep01090 (2013).