THE EMBO JOURNAL

Manuscript EMBO-2010-76362

RNA-Seq analysis in mutant zebrafish reveals role of U1C protein in alternative splicing regulation

Tanja Dorothe Rösel, Lee-Hsueh Hung, Jan Medenbach, Katrin Donce, Stefan Starke, Vladimir Benes, Gunnar Rätsch and Albrecht Bindereif

Corresponding author: Albrecht Bindereif, University of Giessen

Review timeline:

Submission date:	28 October 2010
Editorial Decision:	26 November 2010
Revision received:	24 February 2011
Editorial Decision:	10 March 2011
Accepted:	10 March 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 26 November 2010

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 the referees express interest in role of U1C in regulating alternative splicing, but they ask for some further experimental analysis to make the manuscript suitable for The EMBO Journal.

While the referees all comment on the high quality of the data in the study, one major issue raised by referee #2 and #3 remains to be resolved, which surrounds if the effect of loss of U1C is a direct effect on alternative splicing, this should be addressed by depletion of other U1 snRNP components and in vitro studies. Referee #2 also asks for further analysis if U1C also plays a role in alternative slicing in HeLa cells. Given the interest in the study should you be able to address these two issues, we would be happy to consider a revised manuscript.

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 COMMENTS

Referee #1 (Remarks to the Author):

This manuscript addresses the role of U1C, one of only 3 specific protein components of the U1 snRNP, in alternative pre-mRNA splicing in the context of a null mutation in zebrafish. Remarkably, U1C deletion is not lethal until 5 days post fertilization (5dpf). Instead, this study utilizing RNAseq reports discrete changes in alternative splicing of a limited number of transcripts analyzed at 3dpf, before the onset of major defects in the embryo. This study is beautifully carried out and controlled in the biologically relevant context of a living organism; the data is of an extraordinary high quality. The finding that U1C is essentially an alternative splicing factor is a fascinating contribution to the field, where increasing examples of gene-specific splicing defects can be attributed to what were previously considered core spliceosome components. I believe that the study will be of high interest for EMBO J readers working in splicing as well as the regulation of gene expression in general. I find the paper immediately acceptable for publication, though I have a few suggestions for improvements that I hope the authors will consider:

The main suggestion would be to carry out the analysis shown in Figure 6, using RT-qPCR, in order to permit assignment of statistical significance to the various manipulations. The current quantitation is not very convincing, because some of the bands seem saturated and there is no variance assessed among replicates. This should be straight forward to carry out in the HeLa cell model where mutagenesis of minigenes is carried out. I do not think this is important for the embryo samples.

It would be helpful to label the figures with the appropriate embryonic timepoint (dpf) at which the experiments were performed (eg Fig 1B, and on the photos in Fig 5A). In the legend of Figure 2, it would be helpful to mention the % gels used for running the RNA samples. I initially assumed A&B must be different to account for why the lower band of U1 is not seen in A. I now realize this is not the case.

On page 6, it is speculated that U1C protein and mRNA is maternally contributed. This could be checked against the abundant literature on maternal gene expression (papers by Mathavan lab) and referenced.

In the second paragraph of the discussion, I feel it is important to emphasize that the phenotype of the U1C null is clearly not consistent with an overall block of splicing since morpholinos that block splicing lead to embryo death at 4 hpf (König et al, 2007) and even a 50% reduction in overall snRNP levels in embryos leads to death within 24 hours (Strzelecka et al., 2010). Therefore, loss of U1C very surprisingly has more subtle effects that those expected by crippling the splicing machinery in general.

Discussion bottom of page 13 where disease is discussed, it seems strange that non-zebrafish papers are referenced. I would recommend instead citing published studies in zebrafish (eg papers from the Beattie and Utz labs, which have established SMA models in zebrafish.) also the recently published paper in retinitis pigmentosis in zebrafish (Linder et al 2010) would be an apt addition.

In the supplement, it is stated that the Benjamini-Hochberg method was used to control the FDR. Please explain the basic assumptions of this method and provide a reference.

Referee #2 (Remarks to the Author):

The spliceosomal U1snRNP particle is well known for its role in 5' splice site recognition but it is largely unknown if and how individual proteins of U1 affect splice site selection. In this manuscript, the Bindereif group uses genome wide in vivo approaches to investigate the role of U1C in splicing

regulation. Using an RNAseq analysis of a zebrafish mutant null for U1C they uncover a large set of transcripts that changed their alternative splicing pattern. A bioinformatic analysis of the affected transcripts further revealed an association of U1C-dependent splicing with an intronic U-rich sequence element. These in vivo results hence reveal a thus far unknown role of U1C in alternative splicing regulation of a specific set of target proteins. The experiments shown are very clean and most of the results presented are convincing. Nevertheless, a number of issues need to be addressed to fully support the conclusions drawn by the authors.

Major points:

#1: It is clear that U1C as an integral component of the U1snRNP fulfills a general function in the splicing reaction. As a consequence, the reduced expression is very likely to compromise pre-mRNA processing of a large group of mRNAs to some extend. However, when dealing with such a "housekeeping factor" the important question arises whether the RNAseq analysis indeed identifies those transcripts that are direct targets of U1C deficiency. It is likewise possible that a primary target encodes a splicing factor that is in turn responsible for the observed effects in alternative splicing. To exclude such indirect effects, the direct influence of U1C on the splicing of the supposed targets needs to be shown, for example by in vitro analysis.

#2: The bioinformatics analysis of the single-exon skipping targets nicely shows that there is a U-rich sequence motif in downstream-introns of target-mRNAs. While this indeed might be a cis-element for U1C mediated splicing regulation, also other scenarios might apply. For example, short introns can differ in their splicing mode (exon-defined versus intron-defined), which is in part dependent on their pyrimidine content. If U1C deficiency would predominantly affect mRNAs belonging to this group, such sequence elements would be enriched in the affected introns without being directly connected to U1C.

#3: The authors claim a "functional conservation of the effects observed" in the abstract. However, in the HeLa knockdown experiments that were performed to show the role of the U-rich sequence motif, zebrafish minigene constructs were used. To convincingly show a conserved role of U1C in alternative splicing, this heterologous system is not sufficient. Instead, HeLa transcripts with a similar U-rich sequence motif should be identified and tested for U1C regulation. This could be performed for endogenous or minigene transcripts. This would also strengthen the point that the U-rich sequence element mediates the altered splicing patterns observed for U1C deficiency.

Minor:

#1: The strategy used to identify the "specific set of U1C-dependent splicing events" from the RNAseq data needs to be explained in more detail in the manuscript. Figure 3 and a reference to the supplementary information is not sufficient here.

#2: The rescue analysis shown is really impressive but it would be important to show the level of C-protein expression in the rescued fish. Also the phenotyping of the zebrafish in this experiment is very "soft" and requires more solid data (i.e. gain of pigmentation, loss of deformations etc.)
#3: The term "knockout" zebrafish is not accurate, as a viral insertion is described and used.

Referee #3 (Remarks to the Author):

Several lines of evidence suggest that individual pre-mRNA substrates have distinct requirements for "core" spliceosome components, however the details of this have only been investigated for a handful of proteins. Here Bindereif and coworker utilize the experimental tractability of the zebrafish system to investigate the splicing phenotype caused by U1C depletion. The RNA-seq data is analyzed rigorously, as indicated by the exceptionally high validation rate, and their methodology is carefully laid out in a manner that can be easily followed by others. Moreover, the use of the Hela system allows for further direct confirmation of the relevance of U1C to specific alternative splicing patterns. The conclusion of the work is that depletion of U1C causes highly gene-specific changes in splicing, that are consistent with both a role in 5'ss selection and a functional interaction with TIA-1. This conclusion provides significant insight into the functional significance of U1C in normal and

regulated splicing events. However, there is one major question which needs to be addressed with regards to the conclusion, and several minor points that would further strengthen the study.

Major point:

1) The one major concern is regarding the conclusion that the changes in splicing are due to specific loss of U1C as opposed to decreased activity of the U1 snRNP. This is based on the fact that the U1 snRNP does not seem to have fallen completely apart in cells lacking U1C, but this does not mean that the snRNP is functional. The missing important control is whether similar changes in splicing are observed upon knock-down of an additional U1 component such as U1A, U170K or the U1 snRNA itself. This should be straightforward to test in the Hela system with the minigenes. If the splicing effects observed here are due to loss of snRNP activity this is no less interesting than attributing the splicing defects specifically to U1C, however it does change the resulting models and so must be determined.

Minor points:

1) the mutation of the U rich stretch in the 2 minigenes is not overly convincing as the basal splicing or expression level is changed sufficiently by the mutations that this could abrogate any effect by the U1C mutation. Mutation of the U sequences in additional constructs would significantly strengthen the argument that these sequences are a determinant in U1C responsiveness. Alternatively, can adding a U-rich stretch to a 5'ss confer U1C responsiveness?

2) In the discussion the authors state that the fact U rich elements correlate with alternative 5' splice sites suggests additional trans-acting factors (other than TIA1) might be involved. The rationale for this statement is unclear.

3) Given the recent paper describing CLIP targets for TIA1 is there any direct evidence for TIA1 binding to the U elements identified in these genes - or could the authors look for such by directed crosslinking (in vivo or in vitro) or splicing effects upon knockdown of TIA1.

4) The notion in figure 2 that U1C protects the U1 snRNA from degradation is highly speculative without additional data. However, this is not an important part of the paper so is best simply stated less strongly

1st Revision - authors' response 24 February 2011

Referee #1:

This manuscript addresses the role of U1C, one of only 3 specific protein components of the U1 snRNP, in alternative pre-mRNA splicing in the context of a null mutation in zebrafish. Remarkably, U1C deletion is not lethal until 5 days post fertilization (5dpf). Instead, this study utilizing RNAseq reports discrete changes in alternative splicing of a limited number of transcripts analyzed at 3dpf, before the onset of major defects in the embryo. This study is beautifully carried out and controlled in the biologically relevant context of a living organism; the data is of an extraordinary high quality. The finding that U1C is essentially an alternative splicing factor is a fascinating contribution to the field, where increasing examples of gene-specific splicing defects can be attributed to what were previously considered core spliceosome component . I believe that the study will be of high interest for EMBO J readers working in splicing as well as the regulation of gene expression in general. I find the paper immediately acceptable for publication, though I have a few suggestions for improvements that I hope the authors will consider:

The main suggestion would be to carry out the analysis shown in Figure 6, using RT-qPCR, in order to permit assignment of statistical significance to the various manipulations. The current quantitation is not very convincing, because some of the bands seem saturated and there is no variance assessed among replicates. This should be straight forward to carry out in the HeLa cell model where mutagenesis of minigenes is carried out. I do not think this is important for the embryo samples.

We actually did several repeats of these heterologous experiments in the HeLa cell system, and representative examples are shown in Figure 6. The new **Supplementary Figure S3** (the former Figure S3 is now Figure S4) summarizes several additional biological replicates including quantification, to demonstrate that each of the replicate experiments gives the same conclusion, although the exact values differ between individual experiments. This may be due to the fact that the U1C knockdown (as determined by Western blot) differs between experiments. Therefore we decided not to show standard deviations in Figure 6, but rather only one representative experiment for each gene tested.

We refer to this in the Results part, p.10, section "U1C-dependent 5' splice sites are ...", line 5: "(**Figure 6**; for biological replicates, see **Supplementary Figure S3**; ....)"

It would be helpful to label the figures with the appropriate embryonic timepoint (dpf) at which the experiments were performed (eg Fig 1B, and on the photos in Fig 5A). In the legend of Figure 2, it would be helpful to mention the % gels used for running the RNA samples. I initially assumed A&B must be different to account for why the lower band of U1 is not seen in A. I now realize this is not the
case.

We have added time points to Figure 1B ("3 dpf"), to the legend of Figure 2 (p.21; panels A and B: "… at 3 dpf …"), and to Figure 5A; in Figure 1A this was included already before.

The percentage of denaturing polyacrylamide gels is now stated in Materials and methods, section "Embryo lysates, glycerol gradient centrifugation, and Northern blotting" (p.17): "… analyzed by 10% denaturing PAGE and …".

On page 6, it is speculated that U1C protein and mRNA is maternally contributed. This could be checked against the abundant literature on maternal gene expression (papers by Mathavan lab) and referenced.

According to the literature the U1C gene is transcribed and accumulates at the blastula stage during zebrafish embryonic development (Mathavan et al, 2005). This coincides with the maternal-to-zygotic transition with the onset of zygotic transcription at around 2.5 hpf. Our data indicate that the U1C hi1371 allele cannot produce a functional gene product; hence we believe that the small amounts of U1C protein detectable in mutant animals stem from oocyte development and maternal contribution. We do not claim that the mRNA or the protein are maternally loaded into the developing oocyte, we merely indicate that some of the mRNA/protein persists during early development of the embryo.

To include this, we have added in the Results section on p.6, end of second paragraph:

"….maternal contribution, supported by the microarray-based transcriptome analysis of zebrafish embryogenesis (Mathavan *et al*, 2005)".
New reference added (p.28): Mathavan et al, 2005

In the second paragraph of the discussion, I feel it is important to emphasize that the phenotype of the U1C null is clearly not consistent with an overall block of splicing since morpholinos that block splicing lead to embryo death at 4 hpf (König et al, 2007) and even a 50% reduction in overall snRNP levels in embryos leads to death within 24 hours (Strzelecka et al., 2010). Therefore, loss of U1C very surprisingly has more subtle effects that those expected by crippling the splicing machinery in general.

As the reviewer suggests, we have added in the Discussion, second paragraph, line 3 (p.13), this sentence, as well as the two references on p.28/29):
"….; however, this does not reflect a general splicing block, which causes a

developmental arrest as early as 4.5 hours-post-fertilization (hpf) (König *et al*, 2007); even a 50% reduction of the major spliceosomal snRNPs is lethal within 24 hpf (Strzelecka *et al*, 2010)."

Discussion bottom of page 13 where disease is discussed, it seems strange that non-zebrafish papers are referenced. I would recommend instead citing published studies in zebrafish (eg papers from the Beattie and Utz labs, which have established SMA models in zebrafish.) also the recently published paper in retinitis pigmentosis in zebrafish (Linder et al 2010) would be an apt addition.

As suggested, we have added four references here (Discussion, p.15 top; References, p.26/28/30) that refer to using zebrafish as a model system for human disease:

McWhorter *et al*, 2003;
Winkler *et al*, 2005;
Boon *et al*, 2009;
Linder et al, 2011

In the supplement, it is stated that the Benjamini-Hochberg method was used to control the FDR. Please explain the basic assumptions of this method and provide a reference.

In the *Supplementary Methods* (p.4, bottom paragraph) we have added a brief description of the Benjamini-Hochberg method, including the reference (Benjamini and Hochberg 1995).

Referee #2:

The spliceosomal U1snRNP particle is well known for its role in 5' splice site recognition but it is largely unknown if and how individual proteins of U1 affect splice site selection. In this manuscript, the Bindereif group uses genome wide in vivo approaches to investigate the role of U1C in splicing regulation. Using an RNAseq analysis of a zebrafish mutant null for U1C they uncover a large set of transcripts that changed their alternative splicing pattern. A bioinformatic analysis of the affected transcripts further revealed an association of U1C-dependent splicing with an intronic U-rich sequence element. These in vivo results hence reveal a thus far unknown role of U1C in alternative splicing regulation of a specific set of target proteins. The experiments shown are very clean and most of the results presented are convincing. Nevertheless, a number of issues need to be addressed to fully support the conclusions drawn by the authors.

Major points:

#1: It is clear that U1C as an integral component of the U1snRNP fulfills a general function in the splicing reaction. As a consequence, the reduced expression is very likely to compromise pre-mRNA processing of a large group of mRNAs to some extend. However, when dealing with such a "housekeeping factor" the important question arises whether the RNAseq analysis indeed identifies those transcripts that are direct targets of U1C deficiency. It is likewise possible that a primary target encodes a splicing factor that is in turn responsible for the observed effects in alternative splicing. To exclude such indirect effects, the direct influence of U1C on the splicing of the supposed targets needs to be shown, for example by in vitro analysis.

That we see the same effect in the ZfU1C mutant and after U1C knockdown in HeLa cells, argues for a conserved function of the U1C protein and that we are looking at direct U1C effects (see also comments to Reviewer 3, major point #1). The reviewer suggests additional *in vitro* analysis to support this notion. However, several attempts to immunodeplete U1C from HeLa nuclear extract, using two different antibodies (4H12, Santa Cruz; scFV-hU1C, Hoet *et al*, 1998), were at best 50% effective, even when done at 750 mM KCl; at this level of depletion we observed no effect on alternative splicing. In addition, we have depleted U1 snRNPs with a biotinylated antisense-U1 snRNA oligonucleotide and streptavidin agarose; however, complementation with recombinant U1C protein was not sufficient to rescue splicing (data not shown).

Hoet RM, Raats JM, de Wildt R, Dumortier H, Muller S, van den Hoogen F, van Venrooij WJ. (1998) Human monoclonal autoantibody fragments from combinatorial antibody libraries directed to the U1snRNP associated U1C protein; epitope mapping, immunolocalization and V-gene usage. *Mol Immunol.* **35:** 1045-1055.

#2: The bioinformatics analysis of the single-exon skipping targets nicely shows that there is a U-rich sequence motif in downstream-introns of target-mRNAs. While this indeed might be a cis-element for U1C mediated splicing regulation, also other scenarios might apply. For example, short introns can differ in their splicing mode (exon-defined versus intron-defined), which is in part dependent on their pyrimidine content. If U1C deficiency would predominantly affect mRNAs belonging to this group, such sequence elements would be enriched in the affected introns without being directly connected to U1C.

We considered the possibility that the enrichment of U-stretches may reflect a bias towards short introns in the set of targets, and this had been included already in the *Supplementary Methods*, section 5 (p.15, top paragraph), on "Sequence motif analysis of single-exon skipping targets": Analyzing 176 target exons of the total of 230, that is all single-exon skipping targets with GU 5' splice sites and a minimal length of 135 bp for the downstream intron, we found their length distribution (mean: 2,764 bp; median: 1,743 bp) does not strongly deviate from that of all 94,604 annotated refSeq gene introns (mean: 3278 bp; median: 1,374 bp) (data not shown). Therefore this explanation was ruled out.

Accordingly we added the following in the Results, p.11, second paragraph, line 3:

"We focussed on the 230 predicted single-exon skipping targets, from which 176 target exons were selected for further analysis that have GU 5' splice sites and a downstream intron with a minimal length of 135 bp. This length requirement ruled out the possibility that the introns selected may be biased towards short introns."

#3: The authors claim a "functional conservation of the effects observed" in the abstract. However, in the HeLa knockdown experiments that were performed to show the role of the U-rich sequence motif, zebrafish minigene constructs were used. To convincingly show a conserved role of U1C in alternative splicing, this heterologous system is not sufficient. Instead, HeLa transcripts with a similar U-rich sequence motif should be identified and tested for U1C regulation. This could be performed for endogenous or minigene transcripts. This would also strengthen the point that the U-rich sequence element mediates the altered splicing patterns observed for U1C deficiency.

We admit that the notion of "functional conservation of the effects observed" in the Abstract is not very precise and may even be misleading. To clarify this point, we would like to stress that our heterologous experiments with zebrafish target minigenes in HeLa cells where U1C had been downregulated indicate "that the functional role of U1C in alternative splicing regulation is conserved" (added in Discussion, p.15, middle paragraph, line 5 from bottom). However, as we have stated next in the Discussion, "the conservation does not extend to target gene specificity: We tested several human orthologs of our zebrafish-specific exon skipping targets for their splicing pattern after U1C knockdown in HeLa cells; no U1C-dependent effects on alternative splicing were observed (**Supplementary Figure S6**)."
We concluded this based on the following evidence: We had identified human orthologs of our zebrafish-specific exon skipping targets (NCBI HomologGene database), using these stringent criteria: first, there has to be unique homology between zebrafish and human genes; second, the exon-intron structure in the targetexon region should be conserved between human and zebrafish; third, the gene has to be expressed in HeLa cells, based on microarray expression data (BioGPS, NC160 on U133A Affymetrix genechip; http://biogps.gnf.org); fourth, evidence is required for alternative splicing in human gene, based on UCSC Genome Browser

information (http://genome.ucsc.edu/cgi-bin/hgGateway; hg19, mRNA and EST tracks). As a result, 9 targets were found and tested for their splicing pattern after U1C knockdown in HeLa cells; however, we observed no U1C-dependent effects on alternative splicing (see **Supplementary Figure S6**).

In sum, our results demonstrate that the functional activity of the U1C protein as an alternative splicing regulator is conserved. However, which target genes are U1C-regulated, appears to be species-specific.

Minor:

#1: The strategy used to identify the "specific set of U1C-dependent splicing events" from the RNAseq data needs to be explained in more detail in the manuscript. Figure 3 and a reference to the supplementary information is not sufficient here.

In response we have significantly expanded this paragraph, which summarizes the bioinformatic procedure of how we identified U1C-dependent splicing events (Results, p.7, section "Global RNA-Seq analysis identifies a specific set of U1C-dependent splicing events in zebrafish"). It now reads as following: "We looked for alternative splicing changes between these two samples in the following six modes: single- and multiple-exon skipping, intron retention, alternative 5' and 3' splice site usage, and mutually exclusive exons. A data analysis procedure was developed to predict U1C-dependent alternative splicing targets, consisting of the following five stages (for details, see **Supplementary Methods**):

1) alignment (both junction and non-junction) and mapping of sequence reads to the annotated zebrafish refSeq genes,
2) calculating the read-density (that is sequence-read coverage) of exonic and intronic regions as mRNA expression index,
3) measuring junction-count (number of sequence reads spanning a specific splice junction) to predict the alternative splicing mode,
4) calculating the ratio of the read-density of each exon or intron and the junction-count of each splice junction between the two samples,
5) defining two information groups for each of alternative splicing modes (for example, for exon inclusion and skipping information), and quantitating these values as index of expression changes between the alternative isoforms, thereby defining parameters of reciprocal effects for target prediction."

#2: The rescue analysis shown is really impressive but it would be important to show the level of Cprotein expression in the rescued fish. Also the phenotyping of the zebrafish in this experiment is very "soft" and requires more solid data (i.e. gain of pigmentation, loss of deformations etc.)

We were ourselves surprised by the efficiency of the rescue, and we agree with the reviewer that the phenotyping procedure described in the manuscript appears to be very soft. However, sorting by a "soft" criterium such as e.g. reduced diameter of the eyes (scoring for microphthalmia) still allows reliable separation of wildtype from U1C mutant siblings, which we convincingly demonstrate by the biochemical and genetic analysis of pooled (**Figure 1**) and individual (**Figure 5**) animals. We admit that phenotypic inspection and the use of soft criteria can easily bias experimental results. To control for this we have analyzed rescued embryos thoroughly on a biochemical level (see **Figure 5**). These data clearly and convincingly back up our results from the visual inspection, revealing that a rescue of U1C mutant animals monitored on the level of splicing results in an aphenotypic appearance judged by the size of the eyes and the proper orientation of the body axis. Still we would like to apologize for the inadequate phrasing of the respective section in the original manuscript. To better describe the phenotypic appearance of the mutant embryos, we have now replaced the critical paragraph by a more precise phrasing (p.9, section *ZfU1C cRNA rescues wildtype phenotype and restores splicing of target genes*, line 7-10):

"At this stage the mutant phenotype is characterized by microphthalmia, a dorsally bent body axis, pericardic edema, and reduced pigmentation, which we did not or only weakly observe in individuals after rescue (for a phenotypic description, see also Amsterdam *et al*, 2004)."

The reviewer furthermore asks for a quantification of U1C protein levels in rescued animals. Due to the limited amount of material we unfortunately could not detect U1C protein expression from individual embryos after isolating both genomic DNA and total RNA for analyzing their genetic background and alternative splicing. For detection of the U1C protein the genetic analysis of individual embryos is a prerequisite, since the injected RNA codes for a protein, which is indistinguishable from the wildtype one by Western blot. Therefore –instead of protein analysis- we had included analysis of U1C mRNA in **Figure 5**. Here a discrimination of wildtype mRNA from the injected one is easily possible due to differences in the 3' UTRs. In response to the reviewer's suggestion, we performed additional Western blot analysis of single embryos, from which we only isolated DNA for the genotyping PCR to save as much material as possible for the U1C-protein quantification (see **Figure 1 for the reviewer)** (not included in this Review Process file).

#3: The term "knockout" zebrafish is not accurate, as a viral insertion is described and used.

We agree with the reviewer that the use of the word "knockout" is not entirely correct, since the U1C hi1371 allele is caused by a viral insertion instead of a genomic ablation (as described in Results, p.6, first paragraph). However, our data clearly indicate a functional knockout of the U1C protein, as we are neither able to detect any embryonic U1C mRNA nor protein expression. In most of the manuscript we therefore changed the wording to "U1C mutant" zebrafish instead of using the term "knockout".

_ Abstract, p.2, "knockout" taken out twice;
_ Introduction, p.4, line 3 from bottom (now "U1C mutant zebrafish");
_ Results, subheading, p.6 (now "U1C hi1371 mutant"); "knockout" taken out in these places: p.6, line 4 from bottom; p.8, third paragraph, line 7; p.8, line 5 from bottom; p.9, bottom line; p.10, second paragraph, line 3 (now "…independently of the loss of U1C …"); p.10, line 2 from bottom ("U1C mutant and wildtype zebrafish");
_ Discussion, p.13, second paragraph, first line („… U1C mutant zebrafish, *hi1371*, …"), and line 6 ("U1C mutant embryos"), and bottom paragraph, line 2 ("U1C mutant zebrafish"); p.14, second paragraph, line 5 from bottom ("zebrafish mutants");
_ Materials and methods, p.17, section "Zebrafish culture" ("homozygous U1C mutant embryos"); p.19, line 5 ("uninjected U1C mutant individuals");
_ Figure legends: Figure 1 legend, p.22, title ("in U1C mutant zebrafish embryos"), and taken out twice in legend text; same in Figure 2A legend, p.22; same in Figure 3B legend, p.23; same in Figure 4 (A-D) legend, p.23.

Referee #3:

Several lines of evidence suggest that individual pre-mRNA substrates have distinct requirements for "core" spliceosome components, however the details of this have only been investigated for a handful of proteins. Here Bindereif and coworker utilize the experimental tractability of the zebrafish system to investigate the splicing phenotype caused by U1C depletion. The RNA-seq data is analyzed rigorously, as indicated by the exceptionally high validation rate, and their methodology is carefully laid out in a manner that can be easily followed by others. Moreover, the use of the Hela system allows for further direct confirmation of the relevance of U1C to specific alternative splicing patterns. The conclusion of the work is that depletion of U1C causes highly gene-specific changes in splicing, that are consistent with both a role in 5'ss selection and a functional interaction with TIA-1. This conclusion provides significant insight into the functional significance of U1C in normal and regulated splicing events. However, there is one major question which needs to be addressed with

regards to the conclusion, and several minor points that would further strengthen the study.

Major point:

1) The one major concern is regarding the conclusion that the changes in splicing are due to specific loss of U1C as opposed to decreased activity of the U1 snRNP. This is based on the fact that the U1 snRNP does not seem to have fallen completely apart in cells lacking U1C, but this does not mean that the snRNP is functional. The missing important control is whether similar changes in splicing are observed upon knock-down of an additional U1 component such as U1A, U170K or the U1 snRNA itself. This should be straightforward to test in the Hela system with the minigenes. If the splicing effects observed here are due to loss of snRNP activity this is no less interesting than attributing the splicing defects specifically to U1C, however it does change the resulting models and so must be determined.

We have addressed the important issue of U1C specificity by two approaches:

First, as described already in our manuscript, we were successful in reproducing specific splicing defects that we initially found in the U1C mutant zebrafish also in the heterologous HeLa cell system after RNAi knockdown. This supports the idea that we are looking at a U1C-specific and U1C-dependent alternative splicing changes (see also comment to Reviewer 2, point #3).

Second, as requested by this reviewer, we have carefully analyzed the effects of knockdown of other U1 snRNP components, using the HeLa cell system and comparing to the effects described for U1C knockdown. Standard RNAi knockdown were attempted for human U1A, U1-70K, using at least two different siRNAs each, as well as a morpholino-type knockdown of the U1 snRNA. This latter strategy was most successful. Following U1 snRNA morpholino knockdown, the same zebrafish minigenes as used after U1C knockdown were tested for their alternative splicing pattern. As shown in the new **Supplementary Figure S5**, we were able to inactivate *in vivo* this way at least 90% of the U1 snRNA (see silver staining after RNase H protection assays, upper panels), but alternative splicing of zebrafish *zgc:112089* and *c2orf24* (exon skipping targets) as well as *zgc:162329* and *ilf3* (alternative 5' splice site targets) was not significantly affected (lower panels). We have summarized this in the Results section, p.14, end of middle paragraph, as following:
"In addition, a functional knockdown of the U1 snRNP with an antisense morpholino blocking the 5' end of the U1 snRNA (Kaida *et al.* 2010) did not show any significant changes in the alternative splicing patterns of the minigene constructs (**Supplementary Figure S5**).Taken together, those results clearly validate the splicing defects as primary, U1C-linked and -specific events."

Third, regarding U1-70K knockdown in HeLa cells, we found that effects on alternative splicing are similar to those seen after U1C knockdown (see **Figure 2 for the reviewer**; compare with Figure 6) (not included in this Review Process file); this is based on the RT-PCR analysis of two representative zebrafish minigenes, *c2orf24* (exon skipping target) and *zgc:162329* (alternative 5' splice site target). This is plausible, considering that U1C interacts with U1-70K within the U1 snRNP (Pomeranz Krummel et al, 2009; Nelissen et al, 1994); accordingly, U1-70K depletion would be expected to result in dissociation of U1C from the U1 snRNP and thereby in the same U1C-dependent alternative splicing pattern as after U1C knockdown.

Pomeranz Krummel DA, Oubridge C, Leung AK, Li J, Nagai K. (2009) Crystal structure of human spliceosomal U1 snRNP at 5.5 A resolution. *Nature* **458:** 475-80.
Nelissen RL, Will CL, van Venrooij WJ, Lührmann R. (1994) The association of the U1-specific 70K and C proteins with U1 snRNPs is mediated in part by common UsnRNP proteins. *EMBO J* **13:** 4113-25.

Minor points:

1) the mutation of the U rich stretch in the 2 minigenes is not overly convincing as the basal splicing or expression level is changed sufficiently by the mutations that this could abrogate any effect by the U1C mutation. Mutation of the U sequences in additional constructs would significantly strengthen the argument that these sequences are a determinant in U1C responsiveness. Alternatively, can adding a U-rich stretch to a 5'ss confer U1C responsiveness?

We agree that the RT-PCR analysis of the two constructs of *c2orf24* originally presented in **Figure 6B** gives the impression that the expression levels might be very different between the wildtype and the mutant construct; therefore we replaced this RT-PCR gel by another replicate. All three biological replicates we now show in **Figure 6** and **Supplementary Figure S3A** demonstrate that the expression levels are not changed by the U-stretch mutation. Accordingly, we adjusted the description in the results section, p.11, third paragraph, lines 3-6:

"**Figure 6B** demonstrates that this substitution reduced the effect of the U1C knockdown on exon skipping in comparison to the wildtype sequence (6.6 % and 25.2% for the wildtype versus 8.5% and 19.8% for the mutant; compare lanes 8 and 9 with 10 and 11, respectively), …"

Furthermore, in the new **Supplementary Figure S3**, which shows several replicate experiments of our *in vivo* splicing analysis of the zebrafish-derived minigene constructs, we included an additional U-stretch mutant for the *zgc:112089* construct (**panel A**, lanes labeled with *mut*). Similarly as in the *c2orf24* or *ilf3* mutant constructs a U-rich sequence element located 28 bp downstream of exon 2 of *zgc:112089* was substituted by a C-rich element (see schematic in **Supplementary Figure S3A**, top). RT-PCR analysis demonstrates that the alternative splicing pattern of this mutant did not significantly change upon knockdown of U1C, which further supports our notion that the U-stretches are functionally relevant for U1C-dependent splicing regulation.

We refer to this additional mutant in the results section, p. 11, end of second paragraph:

"To test whether this U-rich elements…, mutated versions of the *c2orf24*, *ilf3* and *zgc:112089 (*see **Supplementary Figure S3A**) minigene constructs were generated…"

2) In the discussion the authors state that the fact U rich elements correlate with alternative 5' splice sites suggests additional trans-acting factors (other than TIA1) might be involved. The rationale for this statement is unclear.

Discussion, p.16, end of top paragraph: Since this is just a speculative point, and an obvious possibility, we have taken out the last part of this sentence ("…; this raises the possibility that additional *trans*-acting factors might be involved in U1Cdependent regulation of 5' splice site choice.").

3) Given the recent paper describing CLIP targets for TIA1 is there any direct evidence for TIA1 binding to the U elements identified in these genes - or could the authors look for such by directed crosslinking (in vivo or in vitro) or splicing effects upon knockdown of TIA1.

To search for TIA CLIP targets (as recently published in Wang et al, 2010) in the orthologous human genes, seems at first an attractive approach. However, it is only the functional role of U1C in splicing regulation that is conserved between zebrafish und human, but NOT the actual target genes (see also response to Reviewer 2, major point #3). Therefore we unfortunately cannot combine the published human TIA CLIP data with our zebrafish U1C alternative splicing targets. That leaves only the experimental route: obtaining TIA CLIP data in the zebrafish system, or doing both RNA-Seq and CLIP approaches in the human system. We are

pursuing the latter option, but this is ongoing and a project in itself.

Wang Z, Kayikci M, Briese M, Zarnack K, Luscombe NM, Rot G, Zupan B, Curk T, Ule J. (2010) iCLIP predicts the dual splicing effects of TIA-RNA interactions. *PLoS Biol.* **8:** e1000530.

4) The notion in figure 2 that U1C protects the U1 snRNA from degradation is highly speculative without additional data. However, this is not an important part of the paper so is best simply stated less strongly.

Results, p.7, end of top paragraph: Since we have shown by RNA analysis that the 5' end of U1 snRNA is truncated in the U1C mutant zebrafish, this is consistent with a protective role of U1C in the U1 snRNP. To tone down this point, we have replaced "argues for" by "suggests".

2nd Editorial Decision 10 March 2011

Your revised manuscript has been reviewed once more by two of the original referees who find that you have satisfactorily addressed the original concerns raised. I am therefore, happy to accept the manuscript for publication in The EMBO Journal. You will receive the official acceptance letter in the next day or so.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2

In their revised manuscript, Bindereif and coworkers have addressed most points raised by the referees in a satisfactory manner.

The only major point that could not be addressed experimentally and which was also raised by referee 3 is whether the observed effect on certain classes of introns is direct rather than a secondary effect.

I appreciate the authors attempt to immunodeplete U1-C from splicing active extracts to test whether this would also affect splicing in a selective manner. As these experiments are technically not feasible, this point remains open. However, the point of the authors that the function of U1-C is conserved in different organisms. This indeed argues for a direct role of U1-C in alternative splicing. Hence I consider this study as very interesting and important and support publication in the EMBO Journal.

Referee #3

The authors have addressed my concerns. In particular, supplementary Figure 5 strengths the primary conclusions of the study. I do not find many of the issues raised by reviewer #1 to be a major concern, and do feel the authors do a good job of addressing these. I do agree the quality of some of the gels in Figure 6 is not ideal - and the quantification is not immediately apparent by eye. However, I trust the authors are reporting the correct values here and these assays are sufficiently difficult that the data shown is reasonable and further replicates would not appreciably add value.

