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The intronic splicing code: multiple factors involved in ATM pseudoexon definition

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1st Editorial Decision 27 August 2009

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three experts and their comments to the authors are provided below.

As you can see, the referees express an interest in the findings, however it is also clear that different issues have to be resolved before publication in the EMBO Journal can be considered. While the referees appreciate the experimental approach used, concerns are raised regarding the interpretations of some of the data, in particular with the conclusion that U2 snRNP recruitment is impaired at the wt ATM pseudoexon. Also, important controls are missing and better quantitation of the data is needed. Should you be able to address the concerns raised in full then we would consider a revised manuscript. Acceptance of your paper will be dependent upon persuading the referees that you have provided a sufficient amount of new data to answer all their criticisms. I should add that it is EMBO Journal policy to allow a single round of revision only and it is therefore important to address the points raised if you wish the manuscript ultimately to be accepted.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Dhir et al follow up their previous observation that a four nucleotides deletion in intron 20 of the ATM gene leads to activation of a pseudoexon in an ataxia-telengiectasia patient, an effect attributed to the loss of a 5' splice site-like sequence which normally silences gthe pseudoexon by recruiting U1 snRNP. The authors recapitulate these effects in vitro and show that replacing the U1 binding site by a U11 binding site, repression is largely maintained. Complex assembly analysis indicate that A-like complexes assemble on the wt and mutant substrates but the former complexes are less stable, progress less efficiently into B-like complexes and contain lower levels of U2 snRNA. Proteomic and functional analysis implicate the SR protein SF2/ASF in activation of the pseudoexon and a putative RNA secondary structure in the silencing of this activity by U1 snRNP. These are interesting results that provide useful information regarding the pathogenic mechanism of an intronic mutation as well as the mechanisms of silencing splice site-like sequences which occur very frequently in introns.

In my opinion the manuscript would benefit from the following revisions:

1. Figure 1: the difference between ATMΔ and ATMΔ U11 could be very well due to the suppressive effects of U11 binding, as argued by the authors, but can we exclude that the sequence introduced is not an exonic silencer motif mediated by factors other than U11? Or that upon deletion of the U1 motif, the joining of the flanking sequences does not generate an exonic enhancer?

2. Several issues concerning Figure 2B need to be clarified. First, the experiment needs a control that not any RNA added to the sample displays similar effects. Second, it could be interesting to know what the stability of the complexes formed on the two RNAs in the absence of any other added RNA (or in the presence of cold RNA ATM competitors) over a longer time course. Third, adding a 5' splice site RNA oligo to the samples has two possible effects in the context of ATM transcripts: in addition to promoting B complex formation as observed by Konarska et al, the 5' ss oligo could also compete with the regulatory ISPE sequence and potentially de-repress the use of the exon by squelching U1 snRNP. Such an effect obviously would not happen in the mutant. How can the authors distinguish between these possibilities, i.e. evaluate the relative contributions of each to the observed effects?

3. Page 7: from Figure 2 the authors conclude that "... the ATM WT exon definition complex is defective primarily at the level of the A-like complex formation." This may be misleading because a complex A-like is formed, while the transition from A-like to B-like induced by the 5' ss oligo in trans does not occur. The authors should also be careful with their assessment of the presence of U2 snRNP: they state that the amounts of U2 snRNA detected in the A-like complex are "negligible", while Figure 3 shows a reduction in U2 snRNA associated with the ATM RNA, but the levels are detectable fairly well and may not be decreased by more than two fold. (By the way, please explain FT, W and E in the main text and in the figure legend 3).) Or are the authors claiming that the A and A-like complexes of Figure 2 do not contain U2 snRNP? Once again, the claims for reduced stability (page 8) need to be substantiated by chase competition experiments to distinguish between A complex stability and reduced ability to support U4/5/6 recruitment. This possible misinterpretation permeates the whole paper, including one of the main conclusions from the abstract.

4. Fig 4B: the authors state that the comparison between polypeptides showed "not many differences...except for the additional U1 associated proteins". There are in fact several clear differences in addition to the bands labeled as U1 70K and U1A (by the way, how was the identity of these bands determined?). For example, the mutated region 1/45Δ shows a dramatic decrease in the association of two bands of approximately 34-35 kDa, among others (e.g around 55 kDa). These species could be relevant for splicing repression or activation. For instance, the authors speculate on the potential role of hnRNP A1 (Fig. 7B), is there a difference in binding of this hnRNP between wt and the mutant?

5. Figure 5C: it would be good to document the degree of SF2/ASF depletion as well as the specificity (e.g. by western blot with a specific antibody and an antibody that recognizes other SR proteins -e.g. mab 104- as a control. Can the addition of recombinant ASF/SF2 to the depleted

extract restore pseudoexon inclusion?

6. The authors indicate -as data not shown- that an excess of SF2/ASF does not have any effect on the wild type substrate. Does it decrease U1 snRNP association ?

7. The model proposed by the authors makes the straightforward prediction that decreasing SF2/ASF levels in patient cells (or at least in other cells transfected with the mutant minigene) should revert the effects of the ataxia mutation in cell culture. Such result would not only confirm the involvement of this SR protein in vivo, but also suggest possibilities for future therapies applicable to similar cases.

8. Figure 7: the rationale for the experiments is unclear, the results are confusing and depend critically on secondary structure predictions that remain hypothetical, at least for the mutants.

9. Figure 8: does titration of U1 with the U1AS oligo increase pseudoexon inclusion on wt ATM ?

10. The writing of the paper is at times rough and inaccurate and in my opinion the authors should make an effort to be not only grammatically correct but also be precise in their statements. Picking just examples from the Title and the Abstract: the first part of the title implies a general code, but generalization from this example is unlikely. Abstract: "...the lack of active pseudoexons...": can pseudoexons be active/inactive or included/skipped, recognized/unrecognized?; "interaction of SF2/ASF with its motif seems to be dependent on RNA structure and U1snRNP interaction." should be "...this motif..." ; "Our results suggest a complex combinatorial interplay of RNA structure and trans-acting factors in determining the splicing outcome and present the "intronic"splicing code for ATM pseudoexon", "...represent..."?

Referee #2 (Remarks to the Author):

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Overall this is an interesting manuscript and the data support most of the author's conclusions. However there are some major and minor points that need to be addressed that will clarify and improve the manuscript.
Minor Comments
ABSTRACT page 2. The last sentence "...and present the intronic splicing code for ATM pseudoexon." should be changed to "... and contribute to understanding the intronic splicing code for ATM pseudoexon."

INTRODUCTION page 4. Mammalian introns can be >100,000 nts in length so "several thousand nucleotides" should be modified to reflect this.

Pages were not numbered so this makes it more difficult for reviewers to describe their critique.

Major Points.

Point 1. Figure 1A. The U11 is fine as an experiment, however since it gave a negative result it leaves one with only ambiguous conclusions and the authors were correct to state U11 is 100x less abundant than U1. Why were suppressor U1 snRNA (5' end modified U1 snRNA) experiments not done as the level of such suppressor U1s can be controlled ranging from a few percent up to 40% of endogenous U1. This should be done in stable cell lines. This would allow the role of U1 stem loop 1, 2 and 3 in ATM pseudoexon to be tested and would indicate whether just basepairing of a U1 is enough or whether any U1 snRNP proteins are involved. Perhaps such work has been done in earlier work such as Pagani et al. 2002 or Lewandowska et al. 2005. If such work has been done then this should be cited and described in the manuscript. If it has not been done then doing it would greatly strengthen the manuscript.

Point 2. Figure 1B. Quantitation of the ratio is needed and should be shown below the picture.

Point 3. Figures 2 and 3 are fine but in Figure 3 what is the experiment for the autoradiogram shown at the bottom of the panel and what is the band that is shown? If this band is the pre-mRNA then the

input lane should be shown or alternatively the efficiency of recovery in the eluted sample should be provided.

Point 4. Figure 4 is not adequate for 2 reasons. First, the arrows indicating the sizes of the U1-70K and U1-A and SF2 polypeptides appear shifted downwards and the U1A band is not visible. Second, Mass Spec is not adequate because it is not quantitative. Antibodies for Western blot are available for U1-70K, U1-A and SF2/ASF and so the affinity pull-down samples should be probed with these antibodies so as to quantitate (as best quantitation as can be done with Western) changes in efficiency of binding.

Point 5. Figure 5B. Although the SF2/ASF signal is clearly reduced in the +56 lane, the experiment was not well explained and may even be problematic. From what I can tell Fig. 5B was done with HeLa Nuclear Extract. If this is the case then I would expect the DNA oligos to cause RNase H-mediated degradation of the RNA substrate thereby confounding the experiment. If this is not the case then I do not understand this experiment/ Perhaps the authors are using recombinant SF2/ASF?

Point 6. Bottom of Page 9 - Top of Page 10 describing Figure 5D. The authors state that there is a dose response in splicing for lanes 2 to 3 but the picture does not show this. A better, more convincing image is needed. Also quantitation + error bars are needed for Figures 5C-D.

Point 7. Bottom of Page 10. "complete inhibition" should be changed to "strong inhibition" as there is still partial activity. Also the statement that the "ATM Δ40-60 Del mutant displayed pseudoexon inclusion with an even greater efficiency than ATM Δ ..." is not supported by the picture in Figure 7C. As with point 6, a better, more convincing image is needed. Figure 7C needs quantitation and error bars.

Point 8. Figure 8. The "U1AS" experiment is not adequately explained and needs better controls and a follow up experiment. The U1AS sample presumably has the 5' end of U1 snRNA removed but this was not clear. We need to know how efficient this removal was, that is what percent of U1 is full length and what percent is truncated. An anti-U1 Northern blot should be done that has probing of another snRNA (eg. U2) so as to show a specific effect on U1. A follow up experiment is that the U1AS sample should be used in pull down assays with the ATM WT substrate (like the one in Figure 3) as we would predict U2 levels should increase relative to U1.

Referee #3 (Remarks to the Author):

In this manuscript Dhir et al further explore the mechanisms of pseudoexon repression. The authors build on previous studies from the Baralle lab investigating the pathological ATM pseudoexon whose inhibition is mediated by U1 snRNP binding to an intronic splicing processing element (ISPE). The new work focused on characterizing in more detail why the pseudoexon is not used in its native context. The authors use a series of spliceosomal complex assays to demonstrate that A complex formation is inhibited because of a U2 snRNP recruitment block. A second set of experiments correlates the activities of the splicing activator SF2/ASF with the formation of RNA secondary structures within the pseudoexon. The authors propose a model whereby the unique RNA secondary structure of the pseudoexon permits the recruitment of U1 within the exon, thus sterically hindering U2 snRNP binding to the 3' splice site. As a consequence, the pseudoexon is not recognized as exonic. Overall, this manuscript provides support for the notion that multiple factors combine to establish the regulation of exon inclusion. While this result is of interest, several questions regarding the data interpretation need to be addressed.

The authors claim that the presence of U1 snRNP within the pseudoexon leads to reduced U2 snRNP binding, presumably through steric hindrance (model). What is striking from the data provided is the fact that within wt context no pseudoexon inclusion is detected (Fig. 1 and previous pubs). However, when A complex assembly is evaluated, the authors show even with the wt construct A complex is formed (Fig. 2B). At the 5 min time point, it appears that equal efficiencies are observed between wt and the deletion mutant. It is hard to gauge whether the later time points do show a difference between wt and mutant because it seems that the overall stability of the wt construct is significantly reduced when compared to the mutant (see H complex intensities). Clearly, more careful quantitation of this experiment is in need. Fig 2D also shows significant A complex

formation for the wt construct while no spliced product is observed. Furthermore, Fig 3 clearly shows that U2 snRNP is present, perhaps at a somewhat reduced affinity. Based on these observations, this reviewer doe not agree with the conclusion that U2 snRNP binding is significantly inhibited. Even if it were to the degree Fig 2B, A complex bands alone, attempts to indicate, it does not explain why no spliced products are observed. Rather than an A complex inhibition, it appears that later spliceosomal complexes are interfered with.

In light of the proposed RNA secondary model that covers essentially the whole pseudoexon (Fig. 6), performing a proteomic comparison on split pseudoexons does not seem to be the most promising approach to identify wt specific interaction partners. This experiment should have been carried out with the entire pseudoexon.

In Fig 6 the authors introduce a mutation that reduces SF2/ASF binding, yet presumably leaves the RNA structure intact. This mutation also knocks out a SC35 binding site. Have the authors investigated whether SC35 involvement is modulated in this switch?

How did the authors quantitate their western blots in Figs 6-8? Was this done through scanning autorads or through direct measurement of light emission (the much preferred approach to obtain quantitative information)? What are the error bars for these experiments and the statistical significance of the differences observed?

All new secondary structures provided are Mfold derived. Experimental verification of these structures, such as was done for the patient mutant (Buratti, NAR 2007) would significantly raise confidence.

1st Revision - authors' response 17 November 2009

Referee #1 (Remarks to the Author):

Dhir et al follow up their previous observation that a four nucleotides deletion in intron 20 of the ATM gene leads to activation of a pseudoexon in an ataxia-telengiectasia patient, an effect attributed to the loss of a 5' splice site-like sequence which normally silences gthe pseudoexon by recruiting U1 snRNP. The authors recapitulate these effects in vitro and show that replacing the U1 binding site by a U11 binding site, repression is largely maintained. Complex assembly analysis indicate that A-like complexes assemble on the wt and mutant substrates but the former complexes are less stable, progress less efficiently into B-like complexes and contain lower levels of U2 snRNA. Proteomic and functional analysis implicate the SR protein SF2/ASF in activation of the pseudoexon and a putative RNA secondary structure in the silencing of this activity by U1 snRNP. These are interesting results that provide useful information regarding the pathogenic mechanism of an intronic mutation as well as the mechanisms of silencing splice site-like sequences which occur very frequently in introns.

In my opinion the manuscript would benefit from the following revisions:

1. Figure 1: the difference between ATMΔ and ATMΔ U11 could be very well due to the suppressive effects of U11 binding, as argued by the authors, but can we exclude that the sequence introduced is not an exonic silencer motif mediated by factors other than U11? Or that upon deletion of the U1 motif, the joining of the flanking sequences does not generate an exonic enhancer?

With regards this experiment, the sequence introduced 'ATATCCTTT' is a consensus U11 5' splice site as cited in the literature. In keeping with this, we were able to obtain pulldown of U11-35K specifically with ATMΔ U11 RNA as detected with U11-35K antibody (data not shown). Furthermore, an analysis using common bioinformatics tools to detect the presence of enhancer or silencer sequences (ESEfinder, PESE, Rescue-ESE etc.) failed to detect the creation/abolishment of particular trans-acting factor binding sites. Taken together, therefore, although we cannot rule out the binding of additional silencer/enhancer elements to the U11 sequence we think that this

possibility is reduced to a minimun.

Secondly, to rule out the possibility that deletion of U1 motif GTAA doesnít generate new enhancer motifs via joining of the flanking sequence we have analyzed both WT and Δ sequence across this region through ESE finder, RESCUE-ESE, PESX. No difference for predicted ESE/ESS motifs could be observed. In addition, this conclusion is strengthened by our previous data (Pagani et al 2002), that just a two nucleotide substitution in the ISPE (GTAA-GTCT , underlined nts are substituted) result in two mismatches with endogenous U1snRNA and lead to the activation of the ATM pseudoexon (which can be repressed by in vivo cotransfection with a modified U1-CT that can compensate for the presence of the mismatches). In our opinion, these results clearly demonstrate that no enhancer motif has been created.

2. Several issues concerning Figure 2B need to be clarified. First, the experiment needs a control that not any RNA added to the sample displays similar effects.

This control is now shown in supplementary Fig.S1A.

Second, it could be interesting to know what the stability of the complexes formed on the two RNAs in the absence of any other added RNA (or in the presence of cold RNA ATM competitors) over a longer time course.

To address this issue, in the absence of any other added RNA the amount of incubation time was increased up to 30 minutes. Even after this amount of time it can be observed that A-like complex formation on the Δ substrate remains stable whilst on WT RNA gradually disappears (the data are shown in supplementary Fig.S1B). Quantification of the A-like complex plotted as relative intensity show an average difference of 2 fold between Δ and WT (added to Fig.S1B).

Third, adding a 5' splice site RNA oligo to the samples has two possible effects in the context of ATM transcripts: in addition to promoting B complex formation as observed by Konarska et al, the 5' ss oligo could also compete with the regulatory ISPE sequence and potentially de-repress the use of the exon by squelching U1 snRNP. Such an effect obviously would not happen in the mutant. How can the authors distinguish between these possibilities, i.e. evaluate the relative contributions of each to the observed effects?

The sequence of the 5'ss oligo used in these experiments presents several mismatches with regards to the ATM sequence (5'-cuguucagguaaguau-3', mismatches are underlined). Previous splicing analysis using the ATM WT and Δ substrates using recombinant U1snRNP molecules have shown that even a single mismatch at the level of the U1snRNA-ISPE base pairing can be sufficient to inactivate the functioning of this element. Moreover, if this was the case then we would have expected a better efficiency of A-like and subsequent B-like complex formation in WT on addition of 5íss oligo, which rather doesnít seem to be the case. For these reasons, we believe that the 5'ss oligo would not be able to derepress use of the exon by squelching the natural U1snRNP.

3. Page 7: from Figure 2 the authors conclude that "... the ATM WT exon definition complex is defective primarily at the level of the A-like complex formation." This may be misleading because a complex A-like is formed, while the transition from A-like to B-like induced by the 5' ss oligo in trans does not occur.

In the revised text we now provide a better explanation of our reasoning. It is true, in fact, that the A-like complex is formed across both WT and Δ with almost similar extent at 5 mins time point. However, their similarity ends here because snRNP composition especially with respect to the U2 presence is very low in WT as compared to Δ (Fig.3). In addition, the relative stability of the A-like complex is very different than WT, being far less stable at longer time points as compared to Δ (Fig.2E and Fig.S1B). Taken together, these observations suggest a clear qualitative difference between WT and Δ A-like complex formation. Finally, this conclusion is also substantiated by a clearly observable difference in the extent of spliceosomal A complex formation in biexonic ATM WT RNA as compared to ATM Δ at 5 mins time point (Fig.2B).

The authors should also be careful with their assessment of the presence of U2 snRNP: they state that the amounts of U2 snRNA detected in the A-like complex are "negligible", while Figure 3

shows a reduction in U2 snRNA associated with the ATM RNA, but the levels are detectable fairly well and may not be decreased by more than two fold. (By the way, please explain FT, W and E in the main text and in the figure legend 3). Or are the authors claiming that the A and A-like complexes of Figure 2 do not contain U2 snRNP? Once again, the claims for reduced stability (page 8) need to be substantiated by chase competition experiments to distinguish between A complex stability and reduced ability to support U4/5/6 recruitment. This possible misinterpretation permeates the whole paper, including one of the main conclusions from the abstract.

FT, W, E lanes are now better explained in the revised figure legend 3 and 'Negligible' signal is replaced with 'substantially reduced' signal for U2 snRNA. More importantly, we don't claim that the A/A-like complex of Fig. 2 don't contain U2 snRNP. It's rather the differences in the extent of A/A-like complex formation (which reflects stable binding of U2 snRNP on to the 3'ss region) between WT and Δ as observed in Fig.2B (or reduced stability of the A-like complex in WT as seen in Fig.2E) that we infer that less stable U2 snRNP recruitment in case of WT A-like complex takes place, as also evident from the UsnRNP composition in Fig.3. Taken together, these observations suggest an unproductive U2 snRNP recruitment in ATM WT case. For these reasons, we believe that the reduced ability of A-like to B-like progression observed in wild-type is the consequence of an unproductive U2 recruitment in A-like complex as evident from the presence of reduced yet visible levels of B-like complex formation in wild type (Fig.2E).

4. Fig 4B: the authors state that the comparison between polypeptides showed "not many differences...except for the additional U1 associated proteins". There are in fact several clear differences in addition to the bands labeled as U1 70K and U1A (by the way, how was the identity of these bands determined?). For example, the mutated region 1/45Δ; shows a dramatic decrease in the association of two bands of approximately 34-35 kDa, among others (e.g around 55 kDa). These species could be relevant for splicing repression or activation. For instance, the authors speculate on the potential role of hnRNP A1 (Fig. 7B), is there a difference in binding of this hnRNP between wt and the mutant?

We understand the reviewer's concerns with this figure. As a result, the entire issue of this initial pulldown analysis has been completely revised in the new manuscript. First of all, we have now added a new panel to this figure containing the pulldown profile of full length ATM WT and ATM constructs. As shown in the new figure Fig.4B, left panel, clear differences between the factors pulled down by both RNAs could only be detected (as determined by mass-spec analysis) at the level of the U1snRNP subunits. These differences were confirmed by western blot analysis (Fig.4B, right panel). At the same time, we also probed the gel for the "usual suspects" (ie. hnRNP A1 and SF2/ASF). In these cases, no binding differences could be seen for hnRNP A1 but they were detected for SF2/ASF. Interestingly, SF2/ASF was also the only SR protein that could bind to the ATM Δ construct, as determined using immunoprecipitation analysis (now shown Supplementary Fig.S2). In order to better map the SF2/ASF binding site we then performed the analysis using shorter RNA sequences (former Fig.4B now kept as Fig.4C).

5. Figure 5C: it would be good to document the degree of SF2/ASF depletion as well as the specificity (e.g. by western blot with a specific antibody and an antibody that recognizes other SR proteins -e.g. mab 104- as a control. Can the addition of recombinant ASF/SF2 to the depleted extract restore pseudoexon inclusion?

A Western blot that shows the level of SF2/ASF depletion using our pulldown affinity procedure has now been added to Fig.5C together with a tubulin control. As the sequence used to achieve this depletion (GAAGAAGA) can immunoprecipitate all types of SR proteins (Buratti et al., 2004, MCB, 24:1387-1400). Addition of recombinant SF2/ASF mix resulted in a significant, but small, restoration of pseudoexon inclusion. It is for this reason that, in order to be more convinced, we used the strategy outlined in Fig.5D.

6. The authors indicate -as data not shown- that an excess of SF2/ASF does not have any effect on the wild type substrate. Does it decrease U1 snRNP association?

The fact that excess SF2/ASF does not lead to pseudoexon inclusion suggests that U1snRNP binding has not been significantly affected. This probably reflects the better efficiency of RNA-RNA interaction in the case of the U1 binding to the ISPE with respect to the protein-RNA interaction of

SF2/ASF binding to the enhancer motif.

7. The model proposed by the authors makes the straightforward prediction that decreasing SF2/ASF levels in patient cells (or at least in other cells transfected with the mutant minigene) should revert the effects of the ataxia mutation in cell culture. Such result would not only confirm the involvement of this SR protein in vivo, but also suggest possibilities for future therapies applicable to similar cases.

We agree with the reviewer and, as a result, this possibility has now been briefly discussed in the Discussion section.

8. Figure 7: the rationale for the experiments is unclear, the results are confusing and depend critically on secondary structure predictions that remain hypothetical, at least for the mutants.

We have now tested in vitro the RNA structure of these two mutants using RNAse mapping experiments. As shown in Figs. S3A and S3B, the cleavage profiles are largely consistent with the mFold predictions.

9. Figure 8: does titration of U1 with the U1AS oligo increase pseudoexon inclusion on wt ATM?

This is an interesting experiment. However, it should be kept in mind that the effect of the U1AS oligo on the cellular U1 snRNP would not only affect the pseudoexon inclusion event but, with very high probability, also affect in an unpredictable manner the recognition of the splice sites in the flanking exons. For this reason, any result would be of very difficult interpretation.

10. The writing of the paper is at times rough and inaccurate and in my opinion the authors should make an effort to be not only grammatically correct but also be precise in their statements. Picking just examples from the Title and the Abstract: the first part of the title implies a general code, but generalization from this example is unlikely. Abstract: "...the lack of active pseudoexons...": can pseudoexons be active/inactive or included/skipped, recognized/unrecognized?; "interaction of SF2/ASF with its motif seems to be dependent on RNA structure and U1snRNP interaction." should be "...this motif..." ; "Our results suggest a complex combinatorial interplay of RNA structure and trans-acting factors in determining the splicing outcome and present the "intronic"splicing code for ATM pseudoexon", "...represent..."?

In the revised version we have now made an effort to improve the writing of the paper (see comments above). We are aware that several of the technicalities of the ATM system will certainly not apply to other examples. However, with the term "intronic code" we meant to convey the concept that splicing regulation within intronic sequences (that in theory have not been optimized in this regard by evolution) can be just as complex as some exon regulatory mechanisms.

Referee #2 (Remarks to the Author):

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Overall this is an interesting manuscript and the data support most of the author's conclusions. However there are some major and minor points that need to be addressed that will clarify and improve the manuscript.

Minor Comments

ABSTRACT page 2. The last sentence "...and present the intronic splicing code for ATM pseudoexon." should be changed to "... and contribute to understanding the intronic splicing code for ATM pseudoexon."

Done.

INTRODUCTION page 4. Mammalian introns can be >100,000 nts in length so "several thousand nucleotides" should be modified to reflect this.

Done

Pages were not numbered so this makes it more difficult for reviewers to describe their critique.

We apologize for this inconvenience. In this revised version the pages have been numbered.

Major Points.

Point 1. Figure 1A. The U11 is fine as an experiment, however since it gave a negative result it leaves one with only ambiguous conclusions and the authors were correct to state U11 is 100x less abundant than U1. Why were suppressor U1 snRNA (5' end modified U1 snRNA) experiments not done as the level of such suppressor U1s can be controlled ranging from a few percent up to 40% of endogenous U1. This should be done in stable cell lines. This would allow the role of U1 stem loop 1, 2 and 3 in ATM pseudoexon to be tested and would indicate whether just basepairing of a U1 is enough or whether any U1 snRNP proteins are involved. Perhaps such work has been done in earlier work such as Pagani et al. 2002 or Lewandowska et al. 2005. If such work has been done then this should be cited and described in the manuscript. If it has not been done then doing it would greatly strengthen the manuscript.

Several of our earlier and most recent works on ATM pseudoexon inclusion have addressed the issue of suppressor U1snRNAs. In Pagani et al., 2002, we have used suppressor U1snRNAs to recover splicing inhibition in the ATM Δ substrate. This work was further extended in Lewandowska et al., 2005 where we used this technique to better map the minimal U1snRNP binding distance from the 3'ss required to obtain pseudoexon inclusion inhibition. Finally, the recent work by Pastor et al., has shown that in the ATM WT context the insertion of spacer regions between the ISPE and the 3'ss can result in pseudoexon activation. All these observations are consistent with a view that U1snRNP is acting at the level of sterical hindrance. At the moment, we do not know what is the relative importance of individual U1snRNP proteins in the inhibitory role. However, the fact that U11snRNP can functionally substitute for U1snRNP suggests that the role of associate proteins is simply to increase the size of the molecular complex assembled on the ISPE.

Point 2. Figure 1B. Quantitation of the ratio is needed and should be shown below the picture.

Quantification has now been added to the bottom of the figure.

Point 3. Figures 2 and 3 are fine but in Figure 3 what is the experiment for the autoradiogram shown at the bottom of the panel and what is the band that is shown? If this band is the pre-mRNA then the input lane should be shown or alternatively the efficiency of recovery in the eluted sample should be provided.

Autoradiogram at the bottom of the panel corresponds to pre-mRNA. Also, the efficiency of recovery in the eluted sample which is 46% is provided. This has been calculated on the bases of cpm counts measured before and after the elution. This has now been mentioned in the revised Figure 3 legend.

Point 4. Figure 4 is not adequate for 2 reasons. First, the arrows indicating the sizes of the U1-70K and U1-A and SF2 polypeptides appear shifted downwards and the U1A band is not visible. Second, Mass Spec is not adequate because it is not quantitative. Antibodies for Western blot are available for U1-70K, U1-A and SF2/ASF and so the affinity pull-down samples should be probed with these antibodies so as to quantitate (as best quantitation as can be done with Western) changes in efficiency of binding.

In Fig.4B we are now providing a Western blot analysis of the U1-70K, U1-A, and SF2/ASF proteins as suggested by the reviewer. In any case, as also reviewer #1 asked several changes and clarifications, the entire issue of this initial pulldown analysis has been completely revised in the new manuscript. First of all, we have now added a new panel to this figure containing the pulldown profile of full length ATM WT and ATM Δ constructs. As shown in the new figure Fig.4B, left panel, clear differences between the factors pulled down by both RNAs could only be detected (as determined by mass-spec analysis) at the level of the U1snRNP subunits. These differences were confirmed by western blot analysis (Fig.4B, right panel). At the same time, we also probed the gel for the "usual suspects" (ie. hnRNP A1 and SF2/ASF). For these last two proteins, no binding

differences could be seen for hnRNP A1 but they were clearly detected for SF2/ASF. Interestingly, SF2/ASF was also the only SR protein that could bind to the ATM Δ construct, as determined in immunprecipitation analysis (now shown Supplementary Fig.S2). In order to better map the SF2/ASF binding site we then performed the analysis using shorter RNA sequences (former Fig.4B now kept as Fig.4C).

Point 5. Figure 5B. Although the SF2/ASF signal is clearly reduced in the +56 lane, the experiment was not well explained and may even be problematic. From what I can tell Fig. 5B was done with HeLa Nuclear Extract. If this is the case then I would expect the DNA oligos to cause RNase H-mediated degradation of the RNA substrate thereby confounding the experiment. If this is not the case then I do not understand this experiment/ Perhaps the authors are using recombinant SF2/ASF?

The amount of oligo used and the incubation time used in our IP assay were designed to minimize degradation to a minimum. An internal control that degradation did not occur is represented by the observation that all oligos, with the exception of 56, give an IP signal which is comparable to the control (oligo free) lane. Furthermore, a recent paper by Vorechovski I (Hum Genet, 2009, in press) has identified (G)AA(G) enhancer motifs to be particularly represented in pseudoexons derived from transposable elements. This result is totally consistent with the region covered by the 56 oligo and has been briefly mentioned in the revised discussion.

Point 6. Bottom of Page 9 - Top of Page 10 describing Figure 5D. The authors state that there is a dose response in splicing for lanes 2 to 3 but the picture does not show this. A better, more convincing image is needed. Also quantitation + error bars are needed for Figures 5C-D.

We have now repeated the experiment to provide a better, more convincing picture (new Fig.5D). This experiment, together with the one shown in Fig.5C, has also been quantitated and the standard errors reported refer to three independent experiments.

Point 7. Bottom of Page 10. "complete inhibition" should be changed to "strong inhibition" as there is still partial activity.

Done.

Also the statement that the "ATMΔ 40-60 Del mutant displayed pseudoexon inclusion with an even greater efficiency than ATMΔ ..." is not supported by the picture in Figure 7C. As with point 6, a better, more convincing image is needed. Figure 7C needs quantitation and error bars.

In the revised version we have now provided a more convincing 7C picture of this experiment. In addition, quantification has also been performed and standard deviations values from three independent experiments have been included.

Point 8. Figure 8. The "U1AS" experiment is not adequately explained and needs better controls and a follow up experiment. The U1AS sample presumably has the 5' end of U1 snRNA removed but this was not clear. We need to know how efficient this removal was, that is what percent of U1 is full length and what percent is truncated. An anti-U1 Northern blot should be done that has probing of another snRNA (eg. U2) so as to show a specific effect on U1.

Efficiency of U1 snRNA cleavage at 30 minutes (RT) with different concentrations of U1AS is now shown in Fig. S4. In our analysis,we used 100 ng of U1AS oligo (5ng/μl) to make sure that all 5'end of U1 was cleaved.

A follow up experiment is that the U1AS sample should be used in pull down assays with the ATM WT substrate (like the one in Figure 3) as we would predict U2 levels should increase relative to U1.

We agree that this might represent an interesting experiment. However, the effect of the U1AS oligo on the cellular U1 snRNP (to obtain the U1AS sample) might not only affect the pseudoexon inclusion event but, with very high probability, also affect in an unpredictable manner the recognition of the 5' splice site (5'gc) in the substrates used for the experiment in Fig.3. Hence, the overall complex formation on the wild-type and delta substrates might be different from the one observed with untreated nuclear extract.

Referee #3 (Remarks to the Author):

In this manuscript Dhir et al further explore the mechanisms of pseudoexon repression. The authors build on previous studies from the Baralle lab investigating the pathological ATM pseudoexon whose inhibition is mediated by U1 snRNP binding to an intronic splicing processing element (ISPE). The new work focused on characterizing in more detail why the pseudoexon is not used in its native context. The authors use a series of spliceosomal complex assays to demonstrate that A complex formation is inhibited because of a U2 snRNP recruitment block. A second set of experiments correlates the activities of the splicing activator SF2/ASF with the formation of RNA secondary structures within the pseudoexon. The authors propose a model whereby the unique RNA secondary structure of the pseudoexon permits the recruitment of U1 within the exon, thus sterically hindering U2 snRNP binding to the 3' splice site. As a consequence, the pseudoexon is not recognized as exonic. Overall, this
manuscript provides support for the notion that multiple factors combine to establish the regulation of exon inclusion. While this result is of interest, several questions regarding the data interpretation need to be addressed.

The authors claim that the presence of U1 snRNP within the pseudoexon leads to reduced U2 snRNP binding, presumably through steric hindrance (model). What is striking from the data provided is the fact that within wt context no pseudoexon inclusion is detected (Fig. 1 and previous pubs). However, when A complex assembly is evaluated, the authors show even with the wt construct A complex is formed (Fig. 2B). At the 5 min time point, it appears that equal efficiencies are observed between wt and the deletion mutant. It is hard to gauge whether the later time points do show a difference between wt and mutant because it seems that the overall stability of the wt construct is significantly reduced when compared to the mutant (see H complex intensities). Clearly, more careful quantitation of this experiment is in need.

We have now quantified the levels of A-like complex formation in revised Fig. 2E (see below). As the reviewer points out, the results show that whilst formation of A-like complex on the ATM WT and substrates is very similar in the beginning it then drops rapidly at later time points. To overcome the influence of the overall drop in the intensities of WT complex, we have now taken into consideration the ratio of A-like to H complex. As observed, assembly of A-like complex occurred with similar efficiency (calculated as the ratio of A/H complex) at 5 mins for both WT and but rapidly dissociate at 10 mins in the WT substrate as compared to (see attached picture). This dissociation is not due to probe degradation as in Fig. 2B no sign of specific H complex degradation in WT is observed.

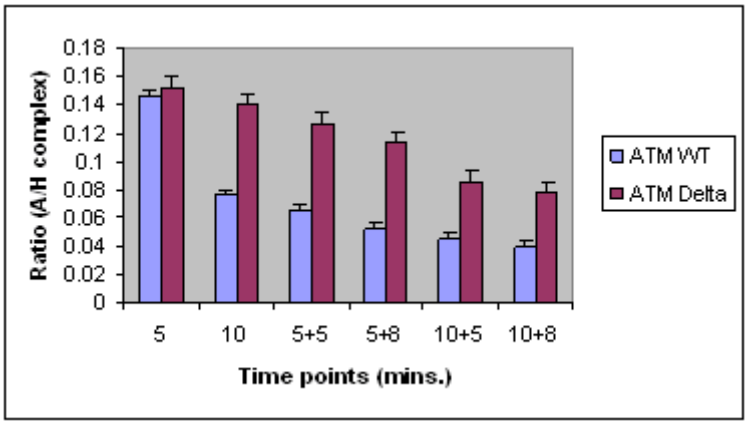
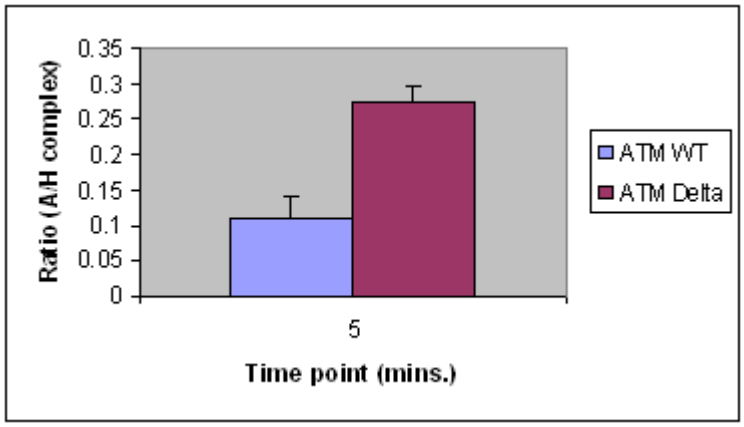


Fig 2D also shows significant A complex formation for the wt construct while no spliced product is observed. Furthermore, Fig 3 clearly shows that U2 snRNP is present, perhaps at a somewhat reduced affinity. Based on these observations, this reviewer does not agree with the conclusion that U2 snRNP binding is significantly inhibited. Even if it were to the degree Fig 2B, A complex bands alone, attempts to indicate, it does not explain why no spliced products are observed. Rather than an A complex inhibition, it appears that later spliceosomal complexes are interfered with.

We have now quantified the efficiency of A complex formation as the ratio of A/H complex. The difference in the efficiency of A complex formation (A/H ratios) between WT and Δ is approx. 2.5-3 fold (see figure below). More importantly, we donít claim that in the A/A-like complex of Fig. 2 U2snRNP is inhibited. Itís rather from the differences in the extent of A/A-like complex formation (which reflects stable binding of U2 snRNP on to the 3íss region) between WT and Δ as observed in Fig.2B (or reduced stability of the A-like complex in wt as seen in Fig.2E) that we infer that less stable U2 snRNP recruitment in case of WT A-like complex takes place, as also evident from the U snRNP composition in Fig.3. Taken together, these observations suggest an unproductive U2 snRNP recruitment in the ATM WT case. For these reasons, we believe that the reduced ability of A-like to B-like progression observed in wild-type is the consequence of an unproductive U2 recruitment in A-like complex as evident from the presence of reduced yet visible levels of B-like complex formation in wild type (Fig.2E).



In light of the proposed RNA secondary model that covers essentially the whole pseudoexon (Fig. 6), performing a proteomic comparison on split pseudoexons does not seem to be the most promising approach to identify wt specific interaction partners. This experiment should have been carried out with the entire pseudoexon.

As this was a common concern to all reviewers, the entire issue of this initial pulldown/proteomic analysis has been completely revised in the new manuscript. First of all, we have now added a new panel to this figure containing the pulldown profile of full length ATM WT and ATM Δ constructs. As shown in the new figure Fig.4B, left panel, clear differences between the factors pulled down by both RNAs could only be detected (as determined by mass-spec analysis) at the level of the U1snRNP subunits. These differences were confirmed by western blot analysis (Fig.4B, right panel). At the same time, we also probed th gel for the "usual suspects" (ie. hnRNP A1 and SF2/ASF). In these cases, no binding differences could be seen for hnRNP A1 but they were detected for SF2/ASF. Interestingly, SF2/ASF was also the only SR protein that could bind to the ATM Δ construct, as determined using immunprecipitation analysis (now shown Supplementary Fig.S2). In order to better map the SF2/ASF binding site we then performed the analysis using shorter RNA sequences (former Fig.4B now kept as Fig.4C).

In Fig 6 the authors introduce a mutation that reduces SF2/ASF binding, yet presumably leaves the RNA structure intact. This mutation also knocks out a SC35 binding site. Have the authors investigated whether SC35 involvement is modulated in this switch?

Based on the results now presented in Fig.S2 we have ruled out any direct interaction with SC35 and the ATM Δ mutant.

How did the authors quantitate their western blots in Figs 6-8? Was this done through scanning autorads or through direct measurement of light emission (the much preferred approach to obtain quantitative information)? What are the error bars for these experiments and the statistical significance of the differences observed?

To perform quantifications, we have used a Ultro Scan XL, Pharmacia LKB - laser densitometer at

633nM wavelength according to manufacturer's instructions. The results from these analyses have now been added as additional panels to all the relevant panels in Figs.6 to 8.

All new secondary structures provided are Mfold derived. Experimental verification of these structures, such as was done for the patient mutant (Buratti, NAR 2007) would significantly raise confidence.

We have now tested in vitro the RNA structure of these two mutants using RNAse mapping experiments. As shown in Figs. S3A and S3B, the cleavage profiles are largely consistent with the mFold predictions.

2nd Editorial Decision 08 December 2009

Thank you for submitting your revised manuscript to the EMBO journal. Your manuscript has now been seen by the original referees #1 and 2 and their comments to the authors are provided below. As you can see, both referees appreciate the introduced changes and both support publication in the EMBO journal. I am therefore pleased to proceed with the acceptance of the manuscript for publication here. You will receive the formal acceptance letter shortly.

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The authors have addressed several of the issues raised in my previous report. They chose not to carry out chase competition experiments to better document differential stability of A and A-like complexes and not to reduce the levels of SF2/ASF in patient cells as a proof of principle for potential therapies, revisions which I believe would have strengthened the paper. Nevertheless, the manuscript is substantially improved and makes a very relevant point regarding mechanisms of pseudoexon activation, an important issue in RNA processing with significant implications in genetic disease. Therefore I support publication in EMBOJ.

Referee #2:
The authors have done suitable, in fact a very good job responding to my critiques.

