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Alternative splicing coupled mRNA decay shapes the temperature-dependent transcriptome

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Responses to Reviewer Comments

Reviewer 1

Comments for the Authors

The authors recently published a very interesting study in Mol Cell, where they described that the activity of CLK kinases is extremely sensitive to physiological temperature changes. This is important since these kinases phosphorylate SR proteins and this temperature-mediated regulation of CLK activity has profound implications for the regulation of Alternative study (AS). In this interesting study Neumann and colleagues focus on the role of temperature in controlling Alternative splicing coupled to Nonsense-mediated decay (AS-NMD). First, the authors carried out a thorough analysis of temperature dependent AS-NMD events in mouse hepatocytes. For this, they analyze splicing changes which are stabilized by translation inhibition through CHX upon temperature shifts. They find that many RNA-binding proteins (RBPs) and SR proteins known to regulate their own expression through AS-NMD can be regulated further by temperature which results in their altered expression. Next, Neumann and co-authors show that temperature-dependent AS-NMD regulation is evolutionary conserved across endotherms. In an interesting experiment, the authors show that temperature-dependent AS-NMD regulation is essential for rhythmic gene expression. They clearly show this by using genome editing to create cell lines that lack either Srsf2 or Srsf1 NMD inducing exons, which represent heat-induced and cold-induced NMD respectively. They clearly show that removing these exons results in the abrogation of rhythms to these cell lines. These studies were extended to Arabidopsis thaliana, where they found that temperature-controlled AS-NMD also operates on pre-mRNAs encoding SR proteins in plants. Finally, the authors show that AS-NMD generates Gene expression rhythms in vivo, as inferred from studies in mouse liver. In summary, this an interesting study that clearly shows that AS-NMD is controlled by temperature and this has important implications for rhythmic gene expression by providing a mechanism that operates independently of circadian clocks. This manuscript is very well-written, it presents the experiments in a very clear manner and the conclusions are strongly supported by the data.

Specific comments

- On Figures 1 and S1, have the authors used any other way of inhibiting NMD other than using CHX, since this not only inhibits NMD but also affect other translation dependent quality control mechanism such as No-Go decay and more drastically shuts off translation of proteins. It seems necessary that the authors confirm true AS-NMD targets by depleting core NMD factors such as UPF1 in mouse hepatocytes and measure the half-live changes.

To obtain a quantitative measure to which extent the CHX-stabilized isoforms are targeted via NMD we followed two approaches: First, we have performed analysis of SMG6/SMG7 double KD (NMD specific factors) RNA-seq data from HeLa cells (Colombo *et al*., RNA 2017), as there was no data for mouse available that was suitable for alternative splicing analysis (the data from Hurt *et al*., Genome Res 2013 are not deep enough and the reads are too short for proper AS analysis). We used the LiftOver tool to convert the coordinates of our proposed AS-NMD events from mouse to human. We then analyzed the HeLa data for alternative splicing using Whippet. We queried the Whippet results for the regions obtained by LiftOver. 28% of AS events in the matched regions showed a significant regulation upon SMG6/SMG7 KD. From the 19 examples we show, we identified SMG-dependent events in 16 genes. These data are included in Sashimi plots for SRSF2 and SRSF10 (new Figure S3D) and in Table S1 for all targets.

Second, we performed analysis to check for premature termination codons in the CHX-dependent isoforms and find them clearly enriched in exons predicted to be poison exons according to our CHX data (see below). Furthermore, all examples shown in the manuscript contain PTCs or lead to the generation of PTCs, thereby making it very likely that they are degraded by NMD.

We overall feel confident that the examples we show are true AS-NMD events. However, we agree with the reviewer that we cannot clearly distinguish between the different decay pathways within our data. We therefore have reduced the usage of the term 'NMD' and now write more generally about mRNA decay. We are sure that this new concept of temperature-regulated AS that leads to decay and thus differential gene expression is of great interest for the scientific community, independent of the exact decay mechanism. Accordingly, we changed the title of our manuscript to: *Alternative splicing coupled mRNA decay shapes the temperature-dependent transcriptome*.

We added the following paragraph to the manuscript (see also next point):

To quantitatively estimate the percentage of potential NMD targets, we tested for the generation of PTCs. Stop codons are only rarely present in exons whose exclusion form is stabilized by CHX-treatment or which exhibit no CHX-dependent change in AS (6.3% and 8.9% of exons, respectively). Notably, the rate of stop codons in exons with a CHX-stabilized inclusion isoform is strongly increased (to 27.7%, Figure S1D), suggesting the NMD to be a prominent – but not the only – pathway through which these isoforms can be degraded. This number likely underestimates the true impact of the NMD pathway, as it does not account for PTCs that occur in later exons due to generation of frameshifts. Overall, these numbers are consistent with published data showing that about one third of all genes regulated upon CHX-treatment are also regulated upon NMD factor knock-down and we expect these isoforms to be degraded by the NMD pathway (Hurt *et al.*, 2013). Finally, we find that about 30% of the CHX-sensitive events were also SMG6/SMG7-dependent using RNA-seq data from HeLa cells where these NMD-specific factors (Chakrabarti *et al*, 2014) were knocked down (see Table S1 and methods section; data from (Colombo *et al*, 2017)). These data further validate the impact of NMD in degrading temperature-controlled isoforms and highlight evolutionary conservation of this mechanism. However, despite the important impact of NMD we are not able to clearly distinguish between different degradation pathways in our data and we therefore refer more generally to mRNA decay in the following.

- Could the authors clarify how many of the CHX stabilized events result in generating a PTC containing transcript compared to the non-CHX responsive group?

We performed this analysis for skipped exon events and conclude that a considerable proportion (almost 30%) of exons which are included upon CHX-treatment contain a novel stop codon. This number is below 10% in the case of all temperature-regulated alternative exons. We added these data as new Figure S1D.

- On Fig. 1B, do the authors find other splicing changes apart from cassette exons? How predominant is this category among their total splicing changes identified with Whippet? It is represented in Table S1, but it would be easier to see it plotted.

We added a new Figure S1B with this information. All types of AS are present in the results, with transcription start sites, transcription ends and cassette exons being the major contributors. We have however, mainly focused on cassette exons, as in our experience, the other types of alternative splicing are more difficult to validate.

- On Fig. 1D, do NMD events in this graph correspond to NMD genes in Figure S1?

The NMD event genes in these figures are the same, the numbers in Figure S1 were not updated to represent the more stringent gene list we implemented during manuscript preparation. We corrected the numbers, thank you for pointing this out. The % overlap does not change with this correction (22% before, 23% after).

- Figure 2, does the observation of altered gene expression result in detectable changes in SR protein levels?

We used a reporter system to address this question and show that temperature-regulated AS-decay can lead to altered gene and protein expression (new Figures 2D-G and S2B). We added the following paragraph to the manuscript:

To provide evidence that temperature regulated AS-decay can also result in altered protein levels, we generated a splicing sensitive GFP reporter. Here, exon inclusion results in an interrupted or non-functional GFP variant while exon skipping results in functional GFP (Figure 2D and S2B). Both an exon encoding a stop codon from *Ythdf3* and an exon that interrupts the GFP-coding frame from human *Synerg* (Haltenhof *et al.*, 2020) show exon inclusion almost exclusively in cold (Figure 2E). In both examples, exon skipping in warm clearly correlates with higher GFP expression (Figure 2F). Finally, temperature shifts antagonistically regulate GFP expression within ~4 hours (Figure 2G) thus highlighting how temperature-controlled non-productive splicing (i.e. AS-decay) can generate rhythms in gene and protein expression.

- Figure 3, some of the RBPs changes can be observed already independently of CHX. Could the temperature shift only amplify already existing auto-feedback mechanism? The authors could test whether other forms of stress also can affect AS-NMD regulation (for example starvation, genotoxic stress, ER stress) and gene expression of RBPs.

In all presented examples, as well as globally, the direction of PSI changes in DMSO correlate with PSI changes in CHX, confirming that these variants are present also in DMSO but are stabilized after CHX treatment (Figure S1I).

We tested other stresses using publicly available RNA-seq data, namely ER-stressed cells (via knock-down of the ER stress molecule Creb3l1, PRJNA622706) and embryonic stem cells in which a diapause (dormant) state was induced (PRJNA600822, Hussein *et al*., Dev Cell 2020). In these data, we can identify the presence of certain amounts of the poison isoforms, but no general trend of higher or lower decay induction. We do not see a regulation in any of the examples that we present in the manuscript upon stress induction.

- Fig. 3A, please clarify: For Srsf10 the PTC is upstream of exon 3 in exon 2. Exon inclusion or exclusion generates an NMD target. Are both variants stabilized through CHX and only exon 3 inclusion is a temperature sensitive event?

The splicing event in Srsf10 is indeed very complex involving competing 5'splice sites which are either recognized by the major or the minor spliceosome (see a detail analysis in Meinke *et al.*, 2020, eLife). Exon 2 contains two 5' splice sites, the upstream 5' splice site is recognized by the minor spliceosome and can therefore only splice to the minor 3' splice site of exon 4, resulting in functional Srsf10 mRNA and protein. In contrast, the downstream 5' splice-site in exon 2 is recognized by the major spliceosome and therefore exclusively spliced to the major 3' splice site of exon 3. Therefore, generation of the PTC encoding isoform (usage of the downstream major 5' splice site in exon 2) is always coupled to exon 3 inclusion (as the downstream major 5' splice site cannot splice to the minor 3' splice site of exon 4). By removing exon 3 via CRISPR-Cas9 (Figure 4) we therefore observe exclusively the usage of the upstream minor 5' splice site coupled to exon 4 inclusion. This is also shown by our radioactive RT-PCR in Figure S4B, which would be able to separate the different 5' splice sites, but shows exclusive formation of E2up5'ss-E4.

Upon addition of CHX we find stabilization only of the E2dn5'ss-E3 isoform. We however also note that this stabilization is much more pronounced in hepatocytes when compared to Hek293 cells. This is mentioned in the manuscript (see below). We have now also investigated whether the short mRNA resulting from the usage of a polyA site in exon 3 could result in a functional protein variant. Cloning of this variant however resulted in a very instable/undetectable protein, indicating that this protein is not functionally important (recently published in Meinke *et al.*, eLife 2020*).*

To increase clarity, we have now included a detailed paragraph describing the complex alternative splicing of Srsf10 in the revised version of our manuscript:

The splicing event in *Srsf10* represents an exciting example for autoregulatory control of two competing 5' splice sites (Meinke *et al*, 2020). The upstream 5' splice site in exon 2 is recognized by the minor spliceosome and splices exclusively to exon 4, allowing the formation of productive *Srsf10* mRNA. In contrast, the downstream 5' splice site is recognized by the major spliceosome and coupled to exon 3 inclusion thereby introducing a PTC encoded by the additional sequence from exon 2. The strength of this PTC depends on the usage of an alternative polyadenylation signal in exon 3. In hepatocytes, the exon 3 isoform is strongly stabilized by CHX, correlating with splicing from exon 3 to exon 4. In Hek293 cells, the polyadenylation site in exon 3 is used resulting in only mild stabilization of the respective isoform via CHX (see Figure 3B).

- If both splice variants use the same PTC where does the size difference of the protein in Figure 6 originates from?

We apologize for not clarifying this. Alternative processing of SRSF10 is very complex, it also contains two alternative last exons 7a and 7b. These encode for two protein variants with either a long (7a, SRSF10-fl) or a short (7b, SRSF10-2) RS domain. Both proteins are functionally similar with SRSF10-fl being slightly more active (Meinke *et al.*, eLife 2020). This is now also mentioned in the respective Figure legend:

SRSF10-fl and SRSF10-2 are the result of differential last exon usage.

- Fig 3D, the yellow line for AS-NMD exons is hardly visible. It looks like temperature-controlled AS-NMD and AS-NMD exons are equally well conserved, the authors should therefore state this more carefully.

We have updated the Figure to increase clarity and now state in the text that temperature-dependent AS-decay variants are as conserved as other AS-decay variants:

Consistent with Thomas *et al*, 2020, this conservation is present in all AS-decay exons and is not specific for temperature-dependent events.

- Fig. 4, please include the depletion of NMD factors (UPF1, UPF2) as a control to prove the AS-NMD events for SRSF2 and SRSF10 Crispr lines (Figure S4)

SR proteins are classic AS-NMD targets as described e.g. in (Lareau *et al.*, Nature 2007). We additionally confirm the target exons as clearly NMD-inducing using SMG6/SMG7 double KD data (see above; new Figure S3D).

- Fig. 5, the authors analyzed published data for plants. Could they overlap their plant temperature sensitive AS-NMD with targets identified using NMD factor depletion or mutants in plants.

In the manuscript, we used RNA-seq data of CHX-treated cells to identify AS-decay events in general. We then checked AS of these events in response to temperature changes. Splicing patterns of control plants change in a similar manner when treating with CHX and when dysfunctional Upf1/Upf3 mutations are introduced (see Figure R1). We have not included these data in the manuscript (but would of course be open to the reviewer's or editor's suggestion).

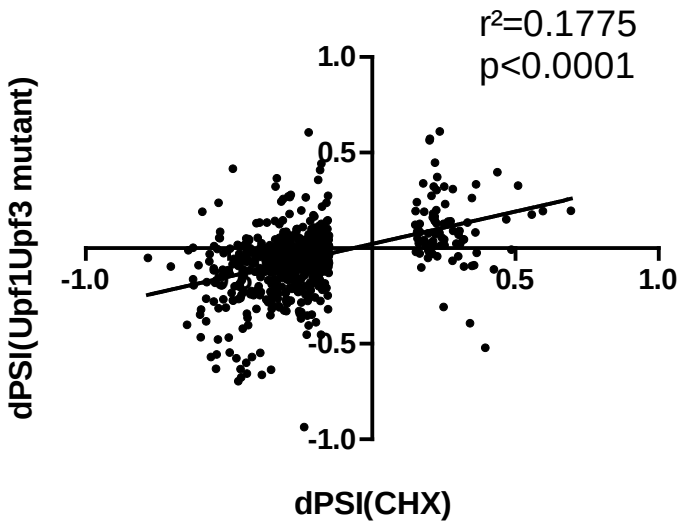

Figure R1: Correlation of dPSI(CHX vs ctrl) and dPSI(Upf1+Upf3 mutant vs control). Shown are CHX sensitive cassette exons.

We also used the data from Upf1/Upf3 mutant RNA-seq to prepare a similar correlation plot between temperature-dependent poison isoform inclusion and target gene expression (compare Figure 5E). We observe a clear correlation between increased poison isoform and decreased gene expression and the effect is even slightly stronger compared to the one shown with CHX (see Figure R2).

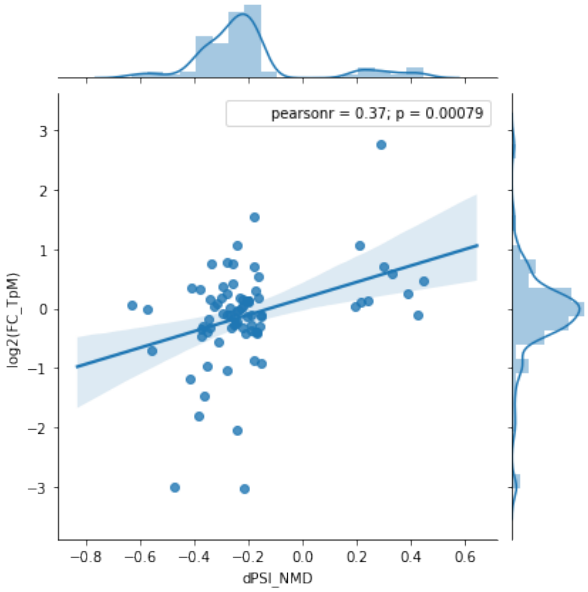

Figure R2: Correlation of poison isoform inclusion and GE levels for temperature-dependent poison skipped exon events. Shown are the log2 fold change (FC) in GE versus the dPSI of the poison isoform. Targets were identified by using Upf1/Upf3 mutant plant data. R^2 and P (deviation from zero slope) are indicated. Compare to Figure 5E.

Minor

- A few typos should be corrected throughout the manuscript, e.g. On page 4 "Here, we investigated to which extent (and NOT extend) - The Haltenhof et al (2020) reference has to be updated

We have updated the citation and carefully looked for typos.

Reviewer 2

Comments for the Authors

In the manuscript "Alternative splicing coupled to nonsense-mediated decay shapes the temperature-dependent transcriptome" Florian Heyd, Marco Preußner and co-workers report that many genes produce temperature-dependent mRNA splice variants that are degraded by nonsense-mediated decay. Nonsense-mediated mRNA decay (NMD) is a well-studied quality control pathway that degrades mRNAs containing premature translation termination codons (PTCs). NMD substrates do not only result from genomic nonsense mutations, but can also be produced by alternative splicing (AS) events that incorporate sequences into the mRNA containing PTCs or leading to premature translation termination by frameshifting. Several RNA-binding proteins, particularly SR proteins, have been previously shown to regulate their own expression by nonproductive alternative splicing, which involves alternatively spliced poison exons containing PTCs. If a poison exon is spliced into an mRNA, the resulting mRNA will eventually be degraded by NMD. The lab of Florian Heyd has previously demonstrated that the circadian clock can be regulated by temperature-dependent AS. Very recently, they have shown that temperature-dependent AS is mediated by alternative phosphorylation of SR proteins, which serve as molecular thermometers for the circadian clock. In the manuscript "Alternative splicing coupled to nonsense-mediated decay shapes the temperature-dependent transcriptome" Florian Heyd, Marco Preußner and co-workers report that many genes produce temperature-dependent mRNA splice variants that are degraded by nonsense-mediated decay. Nonsense-mediated mRNA decay (NMD) is a well-studied quality control pathway that degrades mRNAs containing premature translation termination codons (PTCs). NMD substrates do not only result from genomic nonsense mutations, but can also be produced by alternative splicing (AS) events that incorporate sequences into the mRNA containing PTCs or leading to premature translation termination by frameshifting. Several RNA-binding proteins, particularly SR proteins, have been previously shown to regulate their own expression by nonproductive alternative splicing, which involves alternatively spliced poison exons containing PTCs. If a poison exon is spliced into an mRNA, the resulting mRNA will eventually be degraded by NMD. In this manuscript they continue their work on AS and the circadian clock. They show that AS coupled to NMD can occur in a temperature-dependent manner and can also lead to rhythmic gene expression. By analyzing transcriptome-wide AS in hepatocytes grown at different temperature with or without inhibited NMD, they identify several thousand AS events that differentially affect gene expression at different temperature. The AS events can be classified into cold- or heat-induced NMD and examples for these categories are shown in more detail. Different amounts of NMD isoforms are produced at different temperatures. Since the NMD-targeted isoforms are degraded, temperature-dependent AS determines the expression levels of the mRNA encoding the respective full-length protein. Finally, this SR protein-dependent mechanism is conserved in different animals and plants.

This manuscript reports a solid piece of work, which was carried out at a high technical level. However, the role of the NMD on the circadian clock has only been insufficiently investigated and the temperature-dependent characteristics of the AS events are not described in sufficient detail.
It is also worth mentioning that the phenomenon of AS-NMD has been known for some time. This manuscript mainly adds the specialty of temperature dependent splicing to this process.

Specific major concerns:

1. One of my main concerns is that the function of NMD on the circadian clock is not directly demonstrated in the manuscript. It has to be noted that many NMD-targeted isoforms (i.e. including poison exons) of SR proteins (with the exception of SRSF2) do not encode a functional protein. Therefore, it is not clear if the splicing event, producing an mRNA encoding a truncated protein, or the activity of NMD, removing the PTC-containing isoform, is more important in this context. A direct confirmation that NMD activity affects the circadian rhythm would be required. This could, for example, be investigated using hepatocytes in which a non-essential NMD factor has been removed. Although there is not much data available on NMD factor function in vivo, knockouts of SMG8 or SMG9 could be used for this purpose, since many of the central NMD factors have been reported to be essential.

We agree with the reviewer that the connection of mRNA decay, in particular NMD, and the circadian clock is worth further and more detailed investigation. We do show that rhythms in gene expression can be generated by rhythmic generation of a splicing isoform, which is then target for a decay pathway (e.g. NMD). As the reviewer points out, even if these PTC-containing isoforms escape degradation, in most cases this would still lead to rhythmic expression of the functional protein. For example in some SR proteins, the PTC-containing exons would prevent translation of the C-terminal RS domain, which is essential for functionality (this would actually have an even stronger rhythmic effect, as such a truncated SR protein could act in a dominant negative manner). Therefore, independent of what happens with the PTC-containing transcript, body-temperature controlled alternative splicing can generate rhythmic protein expression, which is one of the important points of our manuscript (and not so much the specific involvement of the NMD pathway). To take emphasis away from the NMD pathway and to focus more on the generation of rhythmic gene expression we changed the title of the manuscript to:

Alternative splicing coupled mRNA decay shapes the temperature-dependent transcriptome.

We would also like to point out that the impact on circadian behavior through mutations of central NMD factors in different organisms has been investigated by others. These studies are now also mentioned in the introduction: In flies and fungi NMD mutants with altered circadian rhythms were described (Ri *et al*, 2019; Wu *et al*, 2017), thus suggesting a direct function of the NMD pathway in modulating the circadian clock (Mateos *et al*, 2018).

2. Unfortunately, the study does not include an analysis of NMD-dependent targets downstream of SRSF2 and SRSF10. A transcriptome-wide analysis of AS events in the AS-NMD insensitive Srsf2 and Srsf10 cells (Figure 4, cells lacking the poison exons) would help to delineate the consequences and importance of the NMD-dependent regulation at different temperatures. This is also important with respect to the general role of NMD for the circadian clock (see comment 1).

We fully agree that such an analysis would be intriguing. However, we think a complete transcriptome-wide study of these cell lines would be the basis for a follow-up project that would go beyond the scope of the present manuscript. We did however investigate the expression of central clock components and the synchronization of these cells by temperature cycles. Cells lacking the SRSF10 poison exon show a clear defect in both expression and rhythmicity of core clock factors, confirming a direct impact of SRSF10 AS-NMD on the circadian clock. The following data and text have been added to the manuscript:

To investigate a potential function of AS-NMD controlled SR protein expression on temperature entrainment of the circadian clock, we investigated temperature-controlled expression of the core-clock components *Bmal1* and *Per1* via RT-qPCR in control cells and in our cell line lacking SRSF10 exon 3. Temperature cycles induce 24-hour rhythmic gene expression of *Bmal1* in both cell lines, but basal expression is strongly altered in the cell line lacking the SRSF10 NMD exon (Figure 4D). Consistent with temperature entrainment data from Liu *et al.*, 2013, in WT cells *Bmal1* expression is lowest at the beginning of the cold phase when *Per1* expression is highest (Figures 4D and 4E). In contrast, in the edited cell line we find highest expression of both *Bmal1* and *Per1* in the middle of the cold phase. There is an additional reduction of Per1 expression in the middle of the warm phase in both cell lines (Figure 4E), which might be due to the short pre-entrainment

of only 48 hours. Together, these data strongly argue for a direct function of SRSF10 – and the NMD exon – in controlling temperature entrainment of the circadian clock.

3. It would also be important to know what determines whether a given AS event is heat- or cold-induced. Are there specific sequences associated with heat- or cold-dependent splicing? Is it possible to change the temperature response of an mRNA encoding an SR protein by changing the sequences around the alternatively spliced exons? Would this affect the circadian clock and change the rhythm in response to temperature changes?

In a previous paper, we identified Srsf2 and Srsf7 as factors that control temperature-dependent alternative splicing in U2af26 (Preußner *et al*., 2017, Mol Cell). We additionally investigated the mechanism of temperature-controlled AS regarding *cis*-acting elements in a later publication (Goldammer *et al*., 2018, RNA Biol), where we identified two enhancer elements that interact with Srsf2/Srsf7 and are required for AS regulation of U2af26 exon 6. Using RNA-seq and iCLIP data, we then could show a global regulation of temperature-dependent splicing events by SR proteins. Since we already published these findings, we focused on the process of AS-decay in this manuscript.

We now used the AME tool to identify further RNA motifs enriched in the exonic regions of temperature-dependent decay targets. To this end, exons were split into 4 categories (heat-included decay, cold-included decay, heat-excluded decay, cold-excluded decay). Temperature-dependent cassette exons that did not change splicing upon CHX treatment were used as background control. We identified the following factors:

Enriched motifs	Induced inclusion	Induced exclusion
Cold induced decay	RBM45, RBP1-LIKE, HNRNPA1, B52 (SRSF6)	None
Heat induced decay	None	CG14718

See below for a detailed description of the obtained motifs:

Logo	**Motif ID**	**Gene name**	**p-value**	**E-value**
	**RNCMPT00241**	**RBM45**	**1.66e-3**	**4.05e-1**
	**RNCMPT00127**	**RBP1-LIKE**	**2.85e-3**	**6.95e-1**
	**RNCMPT00022**	**HNRNPA1**	**3.79e-2**	**9.24e0**
	**RNCMPT00134**	**B52 (SRSF6)**	**3.99e-2**	**9.73e0**

	**RNCMPT00006**	**CG14718**	**1.24e-3**	**3.03e-1**

E-value threshold for reporting results was set to 10 (suggested standard setting).

We however feel that further investigation of the exact mechanism would be better suited for a separate project. Changing SR protein exons (for example between Srsf10 and Srsf2) and examining the consequences is a project we are currently planning. As a proof of principle that this approach can alter expression levels, we have included a reporter system that shows either cold- or heat-induced exon inclusion thereby changing expression of a GFP-reporter Figure 2D-G (also see reviewer 1, comment on Figure 2).

Minor comments

4. It has been reported in the literature that some cells (e.g. neurons) have a lower NMD activity. Does this imply, that these cells do not have a stable circadian clock?

The observation that the abundance of NMD factors is often decreased upon differentiation and is low e.g. in differentiated neurons is indeed very exciting. At this point we can only speculate how this would affect cycles in SR protein expression or the circadian clock. Our two examples SRSF2 and SRSF10 actually present two different cases, and it would be interesting to investigate how their rhythms are effected by a reduced NMD activity: In SRSF2 the NMD inducing exon lies after the canonical stop codon and therefore both isoforms encode the same protein. In such an example the absence of the NMD pathway should abolish rhythms in mRNA (and protein) expression. In contrast, for SRSF10 we have shown that the NMD-inducing isoform is encoding an instable/undetectable protein. If this mRNA variant is not degraded, mRNA expression cycles should be abolished but rhythms in SRSF10 protein expression should remain. It is an exciting question how this would affect rhythms in gene expression and the circadian clock. It has been reported that NMD mutants affect the circadian clock in diverse organisms (see point 1), which could suggest that also within the same organism different levels of NMD in different cell types could control the circadian clock in a tissue specific manner.

5. Figure 3A: The position of the PTC in Srsf2 is incorrect. According to ENSEMBL it is located at the end of exon 2, not in exon 3.

(https://www.ensembl.org/Mus_musculus/Transcript/Summary?db=core;g=ENSMUSG00000034120;r=11:116849901-116853094;t=ENSMUST00000136914).

This is correct. We apologize for the mistake. We have updated the Figure with the correct position of the PTC at the end of exon 2. As described by the reviewer, SRSF2 protein is encoded by exons 1 and 2. Upon inclusion of the poison exon 3, a second exon junction complex is deposited downstream on this Stop codon. The canonical Stop codon therefore becomes a PTC and results in NMD-induced degradation of the mRNA. This is now also clarified in the text:

For *Srsf2* we observed and validated strong heat-induced inclusion of exon 3 (46% DPSI in CHX, Figures 3A, left and S3B). Inclusion of exon 3 generates a second exon junction complex downstream of the canonical stop, which therefore becomes a PTC (Lejeune & Maquat, 2005; Sureau *et al.*, 2001).

Ref#2 referee cross-commenting

After reading the comment of reviewer 1 about the position of the PTC in SRSF10 (Figure 3A) I realized a serious inaccuracy in the analysis of SRSF10. As mentioned by reviewer 1, the PTC in SRSF10 is annotated to be in exon2 and not in exon3 (Figure 3A). However, exon3 of SRSF10 is removed in the experiment shown in Figure 4. I agree with reviewer 1 that this does not seem to be logical. The ENSEMBL database shows a very complex splicing situation for SRSF10. In summary, SRSF10 shows a very complex splicing behaviour that is carried out by different spliceosomes. Furthermore, the effects of the removal of exon 3 on the overall splicing behavior are unclear and have not been investigated in this work (or are not shown). Therefore, I am afraid I have to conclude that SRSF10 is not suitable for the analyses shown in the manuscript and that the data generated with SRSF10 can only be used with great caution.

The splicing event in Srsf10 is indeed very complex involving competing 5'ss which are either recognized by the major or the minor spliceosome, which is why we decided to analyze this in detail in a separate manuscript (Meinke *et al.*, eLife 2020). For a more detailed answer, see comment to Figure 3A, Reviewer #1.

Reviewer 3

Comments for the Authors

In the manuscript entitled "Alternative splicing coupled nonsense-mediated decay shapes the temperature dependent transcriptome", Neumann et al investigate the potential involvement of alternative splicing - Non sense mediated decay (AS-NMD) for the generation of circadian gene expression in response to small temperature changes. Interestingly, the authors find a similar mechanism operating in plants which suggest evolutionary conservation. Briefly, the authors first determine the gene expression profile of hepatocytes at 34 or 38C in presence (or not) of CHX. Their rationale is that transcripts under NMD regulation will be substantially stabilized. By doing so, they identify a large number of RNAs (enriched for RNA binding proteins, RBPs) that contains exons with Premature Stop Codons (PTCs) which are differentially included or excluded as a function of temperature. The authors observe similar results in human, hamster, rabbit and chicken cells. Moreover, the authors found that this regulation might be important for the regulation of gene expression by temperature of the alternatively spliced genes. Last but not least the authors show that a similar regulation exists in plants. This is an interesting manuscript that addresses an important and timely issue in circadian biology by potentially identifying new mechanisms by which 24hs gene expression rhythms are modulated by temperature. However, while the results are interesting, the experiments fail to deliver a message important enough for publication in EMBO J.

Major Points:

1. Temperature Range and indirect measurement of alternative splicing and NMD. The whole manuscript is based on identifying RNAs which are changing between 34 and 38 C. As the authors state, the circadian clock senses and responds to much smaller changes in temperature, so the range utilized is not relevant from the physiological point of view. In addition, the authors utilized steady state RNAseq to determine changes in alternative splicing. Moreover, to uncover RNAs that have been degraded by NMD, they incubate the cells with the translational inhibitor CHX. There are several problems with these approaches. First, treatment with CHX will increase the levels of all translated RNAs and the stabilization might be proportional to the translation levels. In this sense, some of the effect might be due to changes in RNA stability due to processes other than NMD (in particular for those RNAs without PTCs). Second, the experiment is really indirect, especially to determine the ratio of AS but also to identify NMD as the regulation responsible for this effect. There are easy experiments that can do this univocally (like looking at nascent RNA splicing upon changes in temperature and total RNAseq experiments upon more specific inhibition of the NMD pathway).

While we do understand the general criticism to some degree, we would like to point out what our initial idea was and how we succeeded with our experimental approach. Posttranscriptional generation of 24-hour rhythms in gene expression is known since several years but a mechanistic explanation remained enigmatic. Having identified alternative splicing as an extremely temperature sensitive mechanism (Preussner *et al.*, Mol Cell 2017; Haltenhof *et al.*, Mol Cell 2020) we hypothesized that alternative splicing coupled to mRNA decay could generate rhythms in gene expression. We are confident that our approach indeed reveals hundreds of such splicing events (we agree that other degradation pathways than NMD are likely involved) and thus uncovered a mechanism for the generation of rhythmic gene expression. To furthermore emphasize this point, we have now included a GFP reporter confirming that temperature-controlled splicing can generate rhythmic protein expression (new Figure 2D and S2B).

To address the concerns more specifically:

- We use a temperature differential of 4°C in our study to slightly facilitate the bioinformatics identification of temperature dependent AS. We however have previously shown that differentials of 1°C are sufficient to yield alternative splicing changes even in total RNA, that can be detected via RT-PCR, and therefore belief that our approach is viable

(Preussner *et al.*, Mol. Cell 2017). Especially, since temperature-dependent differences in splicing and gene expression are also observable under natural body temperature cycles *in vivo* (e.g. within SRSF10, Figure 6). Additionally, we would like to point out that comparable temperature differentials were and are used in the circadian field and are termed 'simulated body temperature cycles' (Brown *et al.*, Curr Biol. 2002).

- We agree that our approach using CHX does not specifically identify NMD targets and therefore changed the title of our manuscript to: *Alternative splicing coupled mRNA decay shapes the temperature-dependent transcriptome*. We have investigated the potential role of NMD further by predicting PTC creation, which happens in ~30% of proposed poison exons. We added these data as new Figure S1D. Furthermore, about 30% of the proposed AS-NMD events and 16 of the 19 presented examples are dependent on the NMD-specific factors SMG6 and SMG7 (see reviewer #1, comment on figure 1) demonstrating the strong impact of the NMD pathway. However, we carefully rephrased parts of the manuscript and now more generally mention mRNA decay instead of NMD (unless there is direct evidence for degradation via NMD).
- Using nascent RNA is indeed a good idea, which we will consider for future experiments. For the present manuscript, isoforms designated for decay should be absent in the control samples and strongly enriched after inhibition of RNA decay using CHX. We are therefore confident that these isoforms are easily detectable in total RNA, and we think that our approach is valid for the question we have addressed here.

2. It is not surprising that SR proteins regulate their own levels and it is known that splicing efficiency is altered by temperature. So, the fact that temperature has a wide effect on splicing, and that it is dependent on SR proteins is not surprising. What is not clear is what is the physiological/cellular importance of this regulation. Is this important for temperature compensation or adaptation of the circadian clock? What happens with the molecular rhythms of those cell lines when this regulation is abolished? Without a physiological context the results are anecdotical.

To investigate a potential function of AS-NMD controlled SR protein expression on the circadian clock, we investigated temperature-controlled expression of the core-clock components Bmal1 and Per1 via RT-qPCR in control cells and in our cell line lacking SRSF10 exons 3 (new Figures 4D and 4E). These experiments clearly show differences in Bmal1 abundance and in-phase rhythms in Bmal1 and Per1 in the edited cell line, thus indicating a direct effect of SRSF10 AS-NMD in temperature entrainment of the core clock.

3. It is not clear what is the conservation the authors hypothesize. More relevant than a comparison with plants will be a comparison with insects which have a very similar circadian clock than mammals.

Our idea was to investigate whether temperature dependent expression of SR proteins is more widely conserved. We find it striking that both in mammals and in plants we find most SR proteins regulated by temperature. For us this indicates that SR proteins in general are sensitive to temperature and that groups of SR proteins are antagonistically affected (which at least indicates an active regulation and points to an ancient mechanism/function). This function is not necessarily related to the circadian clock but may rather be involved in temperature adaptation. The wide evolutionary conservation suggest an ancient and important function of the mechanism we describe here. To obtain further evidence, we have – as suggested by the reviewer – analyzed temperature-dependent SR protein gene expression and alternative splicing in RNA-Seq data from Drosophila. We have used publicly available RNA-Seq data from drosophila ovaries, which

were maintained at either 18°C (120 hours) or 29°C (48 hours) after hatching. In line with an ancient mechanism, we find most SR proteins affected by temperature. As in vertebrates, SRSF2 (SC-35) shows higher expression in cold (to our knowledge, there is no SRSF10 homolog in Drosophila). As in other species, we find examples for low-abundance splicing isoforms which anti-correlate with the total mRNA abundance. We have included these data as new supplementary Figures S3D and S3E.

Minor Points

1. The authors make many overclaims, including: a. The authors claim that "splicing-regulation is reverted already within 4-8 hours at one temperature, which is indicative for an autoregulatory feedback loop". This is at least highly speculative. To show a feedback loop the authors should show that additional amount or KD of the factor changes the splicing of the endogenous gene.

We agree with the reviewer, that an autoregulatory feedback loop is not addressed in our manuscript and apologize for this and other overstatements. We have erased the respective sentence and in general controlled for precise wording of our conclusions.

We were intrigued by the dynamic regulation of the splicing event in SRSF2, which is strongly promoted in the first 8 hours at a higher temperature and then suddenly starts to decrease. Autoregulatory control of SRSF2 exon 3 inclusion was actually one of the first described examples for AS-NMD (Sureau *et al.*, EMBOJ 2001), which is why we did not further investigate it here. For us, our data together with the published documentation of an autoregulatory feedback loop, indicated that SRSF2 activity is first induced by higher temperature (probably through altered phosphorylation) but then starts to decrease with prolonged time at the higher temperature (probably through inclusion of exon 3, which results in decreased SRSF2 expression level).

To show that an autoregulatory feedback loop is also involved in controlling temperature-dependent alternative splicing of SRSF10, we also performed the suggested experiments for SRSF10. This experiments clearly demonstrated that SRSF10 strongly autoregulates inclusion of its own exon 3. However, as this splicing event is very complex involving competing 5'ss which are either recognized by the major or the minor spliceosome (see also comments to reviewers #1 + #2), these analyses are part of a separate manuscript (Meinke *et al.*, eLife 2020).

b. The authors say: "we find that temperature-dependent NMD exon inclusion in SR proteins anticorrelates with SR protein expression and is highly evolutionary conserved indicating an essential function in regulating temperature dependent GE" This is really another example of a clear overstatement.

As we do not show a function of temperature dependent SR protein expression we changed the sentence to: In summary, we find that temperature-dependent poison exon inclusion in SR proteins is evolutionary conserved and anticorrelates with SR protein expression.

2. The authors mention sickness or other sudden changes in temperature. But this system is based on co-transcriptional responses, is then quick enough? Wouldn't this depend on the half life of the RNAs?

Our data and previous publications show that temperature-dependent AS changes within a few hours of shifting the temperature (even on total RNA level we can detect changes within 30 minutes; Preussner *et al.*, Mol. Cell 2017). We therefore are positive that these changes are fast enough to play a role in disease (in fact, fever generally lasts several days) or in an unusually warm winter day in the case of plants. While it is true that this depends on the half-life of the RNA

(which is now also mentioned in our manuscript), our investigation of rhythmic Srsf2 and Srsf10 expression clearly shows that this mechanism can change gene expression within hours. In contrast, the classical circadian clock is rather robust to environmental changes e.g. upon jet-lag the clock adapts to the new environment only by one hour per day.

1st Editorial Decision 15th Sep 2020

Dear Dr. Preußner

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees. Referee 3 still has a few suggestions,
and referee 1 agrees that these should be addressed; please see the cross-comments below. I would thus like to ask you to address the remaining points so that we can proceed with the official acceptance of your manuscript.

In addition, a few formal changes will be required:

Appendix table S1 needs to be called either Dataset EV1 or Table EV1 given that it is a complex excel file. The supplementary figures need either to be EV Figures (that expand when clicked in the html version of your manuscript) or need to be moved into a single Appendix pdf file. We can only offer a maximum of 5 EV figures, but you could may be combine 2 to reduce the total number to 5. You can find more information about our file types in our guide to authors online:
https://www.embopress.org/page/journal/14693178/authorguide#expandedview

Appendix Table S2 could be renamed to Table EV2 (or Table EV1, if there is no Table EV1) so that no Appendix file will be required.

Please upload all main and EV figures as individual files.

Figure 2E seems to contain a splice, please send us a higher resolution image and the source data (labeled and uncropped gels) for this figure panel.

Please remove the statement from the Data Availability Section (DAS) regarding the accession numbers of previously published datasets. It is fine to include these in the Expanded View Table or Dataset. The DAS should only contain links to deposited data that were generated in this manuscript.

The statement DATA NOT SHOWN on page 9 is not allowed per journal policy, please remove.

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Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors have satisfactorily addressed my comments. Therefore, the manuscript is suitable for publication in EMBO Reports.

Referee #2:

I acted as a reviewer for the original submission to the EMBO J. I raised a few concerns that I thought should be addressed in a revision. The authors have now performed an acceptable revision and have addressed the concerns that were raised during the first round of review. As such, it is my view that the revised manuscript is improved and that this paper is now acceptable for publication in EMBO reports.

Referee #3:

In the revised version of their manuscript "Alternative splicing coupled mRNA decay shapes the temperature-dependent transcriptome", Neumann et al. added a significant amount of work and effort in order to address the concerns raised by the reviewers. The authors have answered most of my concerns (and looks like they have also done a good job answering to the other reviewers), excepts for the most important one. While I might be able to live with the experiments being performed at 34 and 38C (instead of 36.5-37.5), in my opinion, the manuscript cannot be published without showing the effects are due to changes on splicing as well as clearly determining the existence of NMD. These two concerns can be addressed by performing qPCR from nascent RNA. In addition, if the authors want to claim that the process is evolutionary conserved, they need to show that also exists in a more related organism (in which the circadian system is similar like flies). However, I don't believe the latter is essential for accepting the manuscript, but if the authors don't expand their consideration to a more similar system they should exclude the conservation analysis altogether.
Major points:
1. My main criticism to the manuscript was that the measurement of NMD was indirect and that the changes of 4C in temperature were too big compared to the small fluctuations observed in vivo. In particular the authors propose that NMD might mediates oscillations in vivo. In this sense I find the explanation unsatisfactory, if the authors show that the changes observed in vivo can produce circadian oscillations (or even enhance their amplitude) then the manuscript has a novelty but if the authors can't show this I am not sure why this is important. Performing the nascent-qPCR experiments is very easy and straightforward and will clearly show the existence of NMD, it is difficult to understand why the authors chose not to perform it.

2. I also have concerns regarding the evolutionary conservation of the process and I suggested the authors examine the conservation with a more related system (e.g. insects). In their answer the authors try to go around instead of providing a rational explanation or by analyzing available relevant data. The data from fly ovaries is certainly not relevant for circadian studies and I believe there is data available from fly heads or brains from recent papers that could easily be tested for this.

Cross-comments on referee 3's report by referee 1:

One of these points ("showing the effects are due to changes on splicing as well as clearly determining the existence of NMD") caught my attention as well, although I am not sure if I mentioned it in my report. I think it would be good if this issue could be addressed with a simple assay such as qPCR on nascent RNA. The second point (conservation) does not seem to be as important, which is also noted by the reviewer him/herself.

Cross-comments on referee 3's report by referee 2:

I would ignore comments of Referee 3 and publish the paper. He/she raises some interesing issues, but it looks to me none of this is essential.

Responses to Reviewer Comments

We thank the reviewers for their insightful and mostly positive assessment of our work. We have addressed the remaining points as follows:

Referee #3:

In the revised version of their manuscript "Alternative splicing coupled mRNA decay shapes the temperature-dependent transcriptome", Neumann et al. added a significant amount of work and effort in order to address the concerns raised by the reviewers. The authors have answered most of my concerns (and looks like they have also done a good job answering to the other reviewers), excepts for the most important one. While I might be able to live with the experiments being performed at 34 and 38C (instead of 36.5-37.5), in my opinion, the manuscript cannot be published without showing the effects are due to changes on splicing as well as clearly determining the existence of NMD. These two concerns can be addressed by performing qPCR from nascent RNA. In addition, if the authors want to claim that the process is evolutionary conserved, they need to show that also exists in a more related organism (in which the circadian system is similar like flies). However, I don't believe the latter is essential for accepting the manuscript, but if the authors don't expand their consideration to a more similar system they should exclude the conservation analysis altogether.

Major points:

1. My main criticism to the manuscript was that the measurement of NMD was indirect and that the changes of 4C in temperature were too big compared to the small fluctuations observed in vivo. In particular, the authors propose that NMD might mediates oscillations in vivo. In this sense I find the explanation unsatisfactory, if the authors show that the changes observed in vivo can produce circadian oscillations (or even enhance their amplitude) then the manuscript has a novelty but if the authors can't show this I am not sure why this is important. Performing the nascent-qPCR experiments is very easy and straightforward and will clearly show the existence of NMD, it is difficult to understand why the authors chose not to perform it.

In our manuscript we propose that temperature-dependent alternative splicing coupled to nonsense mediated decay (or a different type of decay pathway) is capable of producing rhythmic gene expression. Therefore, the question, whether temperature-induced differences in gene expression are indeed a result of alternative splicing coupled to decay is absolutely crucial, but we think that it is already addressed in our manuscript in several lines of evidence:

First, we show that removing the respective exons using CRISPR/Cas9 abolishes temperature-dependent gene expression in Srsf10 and Srsf2, thus confirming that the presence of these exons is necessary for temperature dependent expression (Figure 4). Our reporter assays with temperature dependent exons additionally indicate that such exons are also sufficient for temperature dependent gene expression (Figures 2D-G). In our opinion these data demonstrate a proof-of-principle: Temperature dependent exons can generate temperature-dependent (rhythmic) gene expression.

Second, the examples in SR proteins are textbook examples of alternative splicing mediated generation of a premature stop codon (the classical signal for nonsense mediated decay) and many publications from different labs have linked this to NMD. To confirm this again, we have provided additional data showing an almost exclusive detection of the exon 3 containing isoforms of Srsf2 and Srsf10 after depletion of the NMD-specific factors SMG6/7 (Figure EV3D). We think that this leaves no doubt that these isoforms are NMD targets in the wt situation. Additionally, the temperature-dependent detection of these isoforms (actually also in DMSO, e.g.

Figure 3A) antagonizing gene expression strongly argues for these splicing isoforms being the cause of temperature dependent expression.

Additionally, we have now – following the suggestion – performed a Srsf10 qPCR with chromatin associated RNA as template. Therefore, Hek293 cells were pre-entrained to 34 or 38°C and shifted after 12 hours. 2 hours later we isolated RNA from the cytoplasm and the chromatin fraction and quantified the Srsf10 variants in qPCRs (Figure 1). We indeed find the NMD-inducing isoform of Srsf10 (E3) strongly enriched in chromatin-associated RNA. Additionally, we find this isoform to be induced already after 2 hours at colder temperature (specifically in the chromatin fraction), consistent with our model of temperature-controlled generation of an NMD-inducing isoform.

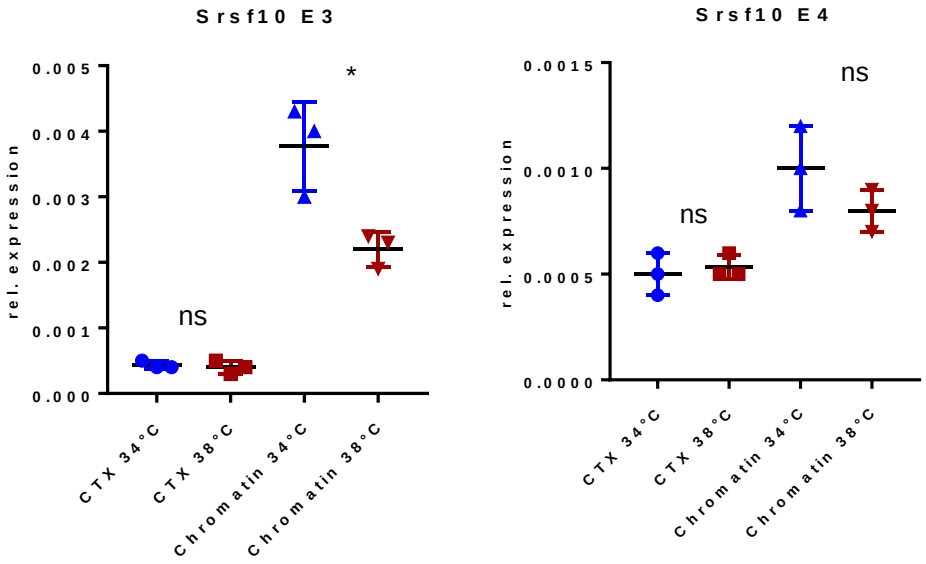

Fig 1: Quantifying SRSF10 isoform expression in chromatin associated RNA. The amount of SRSF10 E3 (left) and E4 (right) is shown relative to GAPDH. Specifically, the NMD-inducing E3 variant is enriched in the chromatin fraction and additionally more abundant already after 2 hours at 34°C (blue). N=3, statistical significance was determined by an unpaired t-test; *p<0.05.

The question, how much the mechanism of temperature-dependent AS-NMD contributes to rhythmic gene expression in vivo is certainly valid, but we believe we have already provided quite some evidence that it actually does. First, we find many temperature-dependent genes from hepatocytes also 24-hour rhythmic in sequencing data from liver (Figure 6). Second, using the example of *U2af26* we previously demonstrated that body temperature cycles are indeed sufficient to generate rhythmic alternative splicing patterns (Preussner et al., Mol. Cell 2014 and 2017). Third, the cold-induced RNA binding protein (Cirbp) is one of the first examples for a body temperature driven rhythmic gene (remains rhythmic in the absence of a local circadian oscillator). The presence of a heat-induced NMD isoform within Cirbp (Figure 2, see also Haltenhof et al., Mol. Cell 2020) indicates a contribution of the here described mechanism in this expression pattern. Finally, the example of SRSF10 showing lower mRNA and protein expression anti-correlating with more of the E3 isoform during the ‘cold’ day (Figure 6) is in agreement with our model of AS-NMD induced temperature dependent gene expression – also in vivo under natural body temperature cycles.

2. I also have concerns regarding the evolutionary conservation of the process and I suggested the authors examine the conservation with a more related system (e.g. insects). In their answer the authors try to go around instead of providing a rational explanation or by analyzing available relevant data. The data from fly ovaries is certainly not relevant for circadian studies and I believe there is data available from fly heads or brains from recent papers that could easily be tested for this.

In our manuscript we describe temperature dependent alternative splicing of NMD-inducing exons in RNA binding proteins including many SR proteins. These exons and their surrounding introns show an extraordinarily high evolutionary conservation across mammals and birds (this is actually how these exons within SR proteins were originally identified). Consistently, we were able to confirm temperature dependent alternative splicing of events identified in mouse hepatocytes also in human cells (Figure 3) – and at least for Srsf2 in additional mammalian species and even in chicken cells. Therefore, the response to temperature is clearly a conserved feature (at least across mammals and birds).

Next, we aimed to investigate whether SR proteins with a different evolutionary origin are also responsive to temperature. Therefore, we screened available RNA sequencing data for samples with a defined temperature treatment. Only in plants we found RNA sequencing data of defined temperatures across a complete circadian day. In our opinion the observation that all SR proteins are regulated by temperature – and many contain promising NMD exon candidates – is remarkable and argues for a general role of these exons in controlling SR protein expression in response to temperature changes (which is furthermore supported by the RNA seq data from fly ovaries from two distinct temperatures). How important these exons are for the circadian clock of ectotherms – experiencing greater differences in temperature – is an exciting question for future research.

Cross-comments on referee 3's report by referee 1:

One of these points ("showing the effects are due to changes on splicing as well as clearly determining the existence of NMD") caught my attention as well, although I am not sure if I mentioned it in my report. I think it would be good if this issue could be addressed with a simple assay such as qPCR on nascent RNA. The second point (conservation) does not seem to be as important, which is also noted by the reviewer him/herself.

Cross-comments on referee 3's report by referee 2:

I would ignore comments of Referee 3 and publish the paper. He/she raises some interesting issues, but it looks to me none of this is essential.

We would like to thank referees #1 and #2 for their cross-comments. We have added a detailed answer to the remaining concerns about temperature-dependent AS-NMD (see Reviewer #3).

1st Revision - Editorial Decision 8th Oct 2020

Marco Preußner
Freie Universität Berlin
Institute of Chemistry and Biochemistry
Takustr. 6
Berlin 14195
Germany

Dear Dr. Preußner,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-51369-T

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A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical calculations were performed to estimate sample size
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Data exclusion was not performed
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
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Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	No test was performed to asure for normal distribution. We used a standard t-test to compare two conditions (unpaired, two-sided) or a 2-way anova to test several conditions
Is there an estimate of variation within each group of data?	Standard deviation is plotted in each figure

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Is the variance similar between the groups that are being statistically compared?	Variance similarity was not tested

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The following antibodies were used for Western blotting: hnRNP L (4D11, Santa Cruz), SRSF10 (T-18, Santa Cruz).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T cell line has been present in the lab for over 5 years and is maintained in liquid nitrogen. Rabbit RK-13, Chinese hamster CHO and chicken enterocyte 8E11 cell lines were a kind gift from Dusan Kunec (FU Berlin). For all cell lines, cell morphology and growth is routinely assessed and corresponds to the expected phenotype. Early passage aliquots are thawed periodically and tested negative for mycoplasm monthly.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All animal experiments were performed with C57BL/6 mice in accordance with institutional and governmental recommendations and laws. Mice were kept under constant 12-hour light-dark conditions.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

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12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	All sequencing data have been deposited to GEO, GSE158882
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	All sequencing data have been deposited to GEO, GSE158882
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA