

DDX1 Methylation Mediated MATR3 Splicing Regulates Intervertebral Disc Degeneration by Initiating Chromatin Reprogramming

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this manuscript Zhu et al present an insightful and impactful study that reveals a fundamental mechanisms of intervertebral disc degeneration (IVDD). The authors report that the RNA binding protein DDX1 is a nonhistone target of EZH2 and that lysine methylation of DDX1 at amino acid 234 promotes IVDD. Furthermore, this methylation of DDX1 reduces association with splicing factors that specifically results in exon skipping in the gene encoding MATR3. The resulting truncated form of MATR3 causes altered chromatin accessibility, activates WNT signaling and causes senescence and apoptosis of nucleus pulposus cells which drives IVDD progression. The effect of DDX1 methylation on exon skipping was very interesting and integrating that data with the RIP-seq data to specifically identify MATR3 was well done. The studies outlined in this work take advantage of cutting-edge in vivo IVDD models that use lipid nanoparticles to deliver MATR3 isoform mRNA. Thus this work will be of broad interest to researchers in the field of epigenetics, specifically those that study regulation of RNA splicing mechanisms, as well as to researchers who study mechanisms contributing to lower back pain. I recommend the work be accepted with minor revisions. Specifically:

- 1) The figures and text in the figures are very small and difficult to read at times. I would recommend trying to increase the font size of text in the figures
- 2) Line 142 - change the word indicating to suggesting - the conservation of an AA doesn't necessarily mean it has functional specificity but it does strongly suggest it.
- 3) Its not explicitly stated in either the main text or figure 1 legend – what is the difference between K234R1 an K234R2 in figure 1G? are these two clones or two cell line pools? Or something else?
- 4) In figure 1G it isn't clear what antibody is used to detect the methylated DDX1 after TBHP treatment – is it the same as the mono-methyl lysine antibody used in Figure 1F (from CST according to table S2)?
- 5) line 154 - please provide a reference that decreased collagen II and increased MMP3 are associated with IVDD degeneration
- 6) Please demonstrate that TBHP induces autophagy, senescence or apoptosis of NP cells in your system and that manipulating the level of DDX1 or expressing DDX1 K234R alters these processes, not just the level of collagen II and MMP3.
- 7) Line 170-171 – The data in extended figure 1 shows that DDX1 expression correlates with protein level of collagen II and MMP3 but no markers for NP cell autophagy or senescence are presented so I don't think there is enough evidence to state “DDX1 K234R significantly exacerbated TBHP-driven NP cells degeneration”
- 8) The in vivo rat model is really impressive. However I would like additional controls performed: 1) what was the degree of knockdown by the shDDX1? Were DDX1 WT-LV1 and DDX1 K234R-LV expressed at the same level. Please perform IHC for DDX1 on your sections to confirm the levels of DDX1 are as expected.
- 9) Figure 2A-B - it isn't clear how DDX1 interacting proteins were identified after coIP – was this by mass spec? were these CoIP interactions lost when DDX1-K234R was expressed in 293 cells? How was the abundance ratio and P value calculated?
- 10) Line 214-216: How does figure 2E show that TBHP induced oxidative stress? TBHP induced EZH2 in Figure 2E and F but you need additional data to support that this is due to oxidative stress specifically.
- 11) Figure 2L-M seem redundant to Figure 2D. They show the same thing by two different methods – consider putting one experiment in the extended data figures.

- 12) Figure 2I isn't necessary, it is already clear in the text and other figure panels without the cartoon.
- 13) Figure 2Q – does EZH2 remain able to bind DDX1-K234R? When you IP with HA is Flag-EZH2 associated with the K234R mutant?
- 14) Figure 4 –What is the overlap between the THBP-treated and DDX1 K234R up/down regulated genes? Is the same set of genes in both systems promoting the apoptosis/senescence ontologies? You briefly touch on this in figure 4H-I but I would like to see a heat map or venn diagram of common genes. Is the overlap limited to the 5 genes listed on line 294 of the text?
- 15) Figure 4D – bubble plot – the color of the dot represents p value but the x axis is $-\log_{10}(p \text{ value})$. The color doesn't seem to match the x axis value? Usually, one of these is an enrichment score instead.
- 16) Line 291 – grammar - delete “were identified”
- 17) Figure 4 H and 4 I are discussed in the text before discussion of apoptosis in Figure 4E-G – these labels should be switched so the figures are in order.
- 18) Figure 4 - Please perform a western blot of DDX1 in the NP cells and 293 cells that express: EV, siDDX1, siDDX1+Wt and siDDX+K234R - to demonstrate the level of knockdown and comparable expression levels of WT and K234R in these cells.
- 19) Delivering LNPs containing MATR3 isoforms was very cutting edge. But I think the production and QC of the LNPs should be shifted to the extended data section so that the main figure is specifically focused on the animal experiment demonstrating the LNP- MATR3-S mRNA promotes IVDD in vivo. Likewise, I believe the results presented in extended data figure 4 should be moved to Figure 8 since they demonstrate the short isoform promotes expression of metabolic and age-related markers while the long isoform mitigated NP cell aging indicators and IVDD in vivo.
- 20) Line 484 – is it DOTAP or DPTAP? Will you please double check which is correct.

Reviewer #2

(Remarks to the Author)

DDX1 is an ATP-dependent RNA helicase belonging to the DEAD box family of helicases that unwind and/or remodel RNA secondary structure in vitro. DDX1 has been implicated in a number of cellular processes such as: regulation of translation initiation and nuclear RNA splicing, RNA clearance at DNA double strand breaks (DSB), as a co-activator of NF-kappa-B mediated transcriptional activation etc.

In this manuscript, the authors explore a novel role of DDX1, highlighting its function as a non-canonical RNA binding protein (RBP) that is capable of modulating the alternative splicing (AS) of MATR3 pre-mRNA, depending on the methylation state of DDX1's Lysine-234. The authors show that native DDX1 interacts with splicing factors and enriches them at the splice site of MATR3 thereby driving exon 5 inclusion to produce a long form of the transcript termed MATR3-L. In turn, MATR3-L maintains normal chromatin accessibility and prevents excessive activation of cellular senescence and apoptosis programming. Under oxidative stress triggered by TBHP treatment, there is an over-expression of EZH2 which leads to mono-methylation of DDX1 at Lysine-234. Increased DDX1 methylation reduces its binding to MATR-3, preventing recruitment of splicing factors, leading to the skipping of exon 5, resulting in a short form of the transcript termed MATR3-S. Consequently, MATR3-S leads to excessive opening of chromatin, resulting in cellular senescence and apoptosis programming which can lead to degeneration of nucleus pulposus (NP) cells. Degeneration of NP cells in turn leads to Intervertebral Disc Degeneration (IVDD) which is the main cause of low back pain (LBP).

The manuscript would be interesting to a wide audience and the readership of this journal. Some comments below should be addressed:

The authors primarily describe DDX1 as an RNA-binding protein (RBP), but it also functions as an RNA helicase. Are the impacts on splicing a result of decreased binding to other splicing factors alone, or does mono-methylation at K-234 also impact RNA unwinding and/or RNP remodeling activity of DDX1? How does mono-methylation of K234 impact DDX1's binding to MATR3 pre-mRNA if the methylation is at the SPRY domain? The authors should elaborate on the known functions of SPRY domains and what might be the mechanism by which the effects on splicing occur.

DEAD box RNA helicases typically do not exhibit RNA sequence specificity as other RNA binding domains; instead, they recognize and interact with RNA based on RNA secondary structure elements. Yet, the authors report seeing enriched sequence elements based on their de novo motif analysis of DDX1-RIP experiments (Figure 5L). Where is the sequence specificity coming from? Could there be another factor mediating the interaction in their pull-down experiments?

The methods describing the RIP-seq experiments are a bit odd. Do they really do phenol-chloroform extraction before they do anti-DDX1 pull-down?

The authors' ability to elicit the same response in HEK293T cells as in NP cells suggests that the chromatin remodeling and subsequent cellular senescence caused by AS of MATR3 are not unique to NP cells but have a broader relevance across various cell types.

EZH2 is known to work as part of the polycomb repressive complex 2 (PRC2) and known to trigger gene silencing pathways. In this case, is EZH2 methylating DDX1 as part of PRC2 or by itself? How do the authors reconcile the traditionally repressive role of EZH2 with current increased gene expression in NP cells?

(Remarks to the Author)

Zhu et al. discovered that EZH2-mediated K234 monomethylation modification of DDX1 regulates the process of intervertebral disc degeneration (IVDD). Mechanistically, methylation of DDX1 promotes exon skipping events in MATR3 to generate MATR3-S by preventing the recruitment of splicing factors. The short form of MATR3 leads to enhanced chromatin accessibility and triggers cellular senescence and apoptosis by hyperactivating the Wnt and MAPK signaling pathways, thereby facilitating the progression of IVDD. Based on this mechanism, they developed a cationic lipid nanoparticle-based MATR3-L mRNA cargo and claimed its potential for alleviating IVDD progression.

The findings in this manuscript are potentially interesting. However, there are significant faults in experiment design and data interpretation, which impede the rigor and reliability of this study. Furthermore, the manuscript is not well written and needs heavy editing. The comments for authors are listed below.

Major comments :

1. In Figure 2D, there are two bands of DDX1 around 100 kDa were observed in lane 5. Because the input control is missing, it is hard to judge which band is the specific DDX1. The authors also need to carefully check whether the lower bands in lane 2, 3, 4 and 6 are specific DDX1 and adjust their conclusion accordingly.
 2. The observation of DDX1 methylation and DDX1-EZH2 interaction in untreated cells were in controversy throughout the manuscript. Clear methylation signals of DDX1 can be seen in Figure 1C and ED figure 1I, but not in Figure 2C and 2P. Furthermore, the interaction between DDX1 and EZH2 were not detected in untreated cells as shown in Figure 2P, which leads to my confusion that how DDX1 were methylated in untreated cells.
 3. It is confused that why two different cell lines NP cells and HEK293T cells were used to perform RNA-seq? The mechanism of DDX1 in IVDD might be cell-context dependent and ignored in these analyses. Considering physiological relevance, performing RNA-seq in NP cells, rather than HEK293T cells, with DDX1 overexpression or silencing is necessary.
 4. The results of GSEA analysis in Figure 4B and 4C were erroneously interpreted. In line 282-287, the authors claim that "Subsequently, a gene set enrichment analysis (GSEA) showed that cellular senescence and apoptosis signaling pathway were strongly associated with TBHP-treated NP cells degenerative phenotypes (Fig. 4B). Interestingly, the GSEA demonstrated that DDX1 K234M overexpression similarly regulated cellular senescence and apoptotic signaling pathway (FIG. 4C)." However, the results of Figure 4B and 4C show that cellular senescence and apoptosis signaling pathways were enriched in the control or DDX1 K234R groups instead. This error makes the conclusion "DDX1 methylation promotes cellular senescence and apoptosis" suspicious.
 5. In Figure 4E and 4F, the quality of Annexin V/PI staining and flow cytometry is low, and the portion of apoptotic cells in TBHP-treated cells is too low as compared with the same experiments performed in previous publications (PMID: 35096819 , 30414849).
 6. In ED Figure 2E, cleaved-caspase3 should be examined instead of caspase3 to evaluate cell apoptosis. Usually, the accumulation of full length caspase3 indicates the reduction of cleaved-caspase3 and the inhibition of apoptosis, which would be contrary to the conclusions described in this paper.
 7. The length of MATR3 exon 5 is in controversy in text description and schematic shown in Figure 6F. In lines 489-491, it is mentioned that "The transcribed MATR3-L has a length of approximately 2688 nucleotides, while MATR3-S has a length of approximately 2544 nucleotides". By calculation, the skipped exon 5 should be 144 nt in length, whose skipping is not able to cause a frameshift. Whereas, it is shown that exon 5 is 122 nt in Figure 6F, whose skipping is able to cause a frameshift. The authors should carefully check the length of exon 5, which is critical for the reliability of the data related to MATR3 splicing.
 8. Lines 690 and 1258 in the method section: I could not understand how the prokaryotic protein expression vectors (pET-28a-CNV, his-tag) could be used for expression of DDX1 in 293T cells of human origin.
 9. The shRNA of EZH2 was used for silencing EZH2 in rats (Figure 3), however, whose sequence provided in table S3 was of Homo. Therefore, the related results and conclusions are suspicious. Furthermore, at least one more shRNA should be used to prevent off-target effects.
 10. The manuscript needs heavy editing. Either indent or blank lines between paragraphs except for the first paragraph, otherwise it will affect reading. Lines 49-51: Inconsistent with the previous tense. Line 128: Tense confusion. Line 231: The meaning is wrong. Line 257-258: Tense inconsistency. Line 401 is missing a punctuation mark. Line 1019: The tense of the title is inconsistent with other titles. Line 1361-1363: There is also a problem with this sentence structure. Line 1405: The title does not do a good job of summarizing the figure. Line 1358-1359: synthesis is noun, catabolic is adjective, this sentence structure is problematic.
- Line 67: Reference 5 seems irrelevant to the description. Line 88: This citation seems to refer only to apoptotic genes. Line 88-91: I understand that this sentence is meant to express that a small amount of RBP can regulate gene expression because it can cause abnormalities in post-translational modification. But that citation does not mention RBP. Line 96: I think the reference 13 is not representative. Line 568-571: Changes in gene expression at the pre/post-translational level caused by DNA methylation and histone modification? Sure it wasn't pre/post-transcriptional? Or provide relevant literature.
- The misspelling of 'rachemys' in Extended Figure 1D should be 'trachemys'. The spelling of 'score' at ordinate in Extended Figure 1L is incorrect. The spelling of 'cotransfection' and 'extraction' in Figure 4A is wrong. The spelling of 'control' is wrong in the vertical characters in Figure 4H. Incorrect spelling of 'Methylated-RIP' in Figure 6D. The 'spliceosome' for K234M in Figure 6I is misspelled. The spelling of 'MATR3' in Figure 7I and J is incorrect. Misspelled title in Extended Figure 3E. Misspelling of 'CASPASE' in Extended Figure 3M. Incorrect spelling of 'CASPASE' in Extended Figure 3I. The spelling of 'DOTAP' and 'MATR3' in Figure 8A is incorrect. The spelling of 'MATR3' in Figure 8B is incorrect. The spelling of 'MATR3' in Figure 8D, E is incorrect. The spelling of 'MATR3' in Figure 8F is incorrect. The spelling of 'score' in Figure 8M is incorrect. The spelling of 'MATR3' in Extended Figure 4A, C is incorrect. Line 641-642: obvious semantic error. A large number of formatting errors exist in the 'Materials and Methods' part, which are not pointed out here. Line 367: DAS:

Differential Alternative Splicing. Line 915: DEGs: Differential Expressed Genes. The word 'genome' in the title of Line 322 is inaccurate and may need to be replaced with 'transcriptome'.

The number of up/down-regulated genes described in Line 277-278 is opposite to that labeled in Extended Figure 2A. The red and blue dots in Extended Figure 2C do not all conform to the Fold changes > 2 mentioned in Line 282. The TNFRSF1 mentioned in Line 294 is not seen in the corresponding Figure 4H and 4I. In Line 463, MATR3-L may have been originally MATR3-S. The CDK1, CDK2, and CDK6 mentioned in Line 470 only have CDK1 in their corresponding Extended Figure 3L. Line 578: 'genomic aberrant splicing'. That's not appropriate. Line 937: 'genome' should be 'transcriptome'. Line 631: 'on three-dimensional genome structure' is a bit overstated. Line 927, 931 mentioned that 'the gene names were highlighted in bright color', but the figure did not see the gene names with bright color.

Minor:

1. Do the authors know the reasons why EZH2 expression is increased under TBHP conditions, and whether its transcriptional level is increased?
2. The information of pan-Kme antibody should be provided.
4. IF in Figure 1E should have quantitative results. Figure 4J results need to be quantified.
6. The p53 and p21 bands in Extended Figure 2G are not in the right position.
7. Line 210: How does cell lysates perform plasmid transfection?
8. Line 374-375: Figure 6B can't reach such a conclusion.
9. Line 635: Methylation should be about IMPROVING accessibility, not 'disrupting'.
10. Line 990: Which isoform of MATR3 is not specified.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #6

(Remarks to the Author)

In their manuscript "DDX1 Methylation Mediated MATR3 Splicing Regulates Intervertebral Disc Degeneration by Initiating Chromatin Reprogramming" Zhu and colleagues report that monomethylation of DDX1 at lysine K234 by EZH2 drives NP cell degeneration and IVDD. They demonstrated that EZH2 expression is increased in IVDD and that EZH2 mediates methylation of DDX1 at K234. DDX1 methylation promotes gene expression programs related to senescence and apoptosis in NP cells. Mechanistically, DDX1 methylation reduces DDX1 binding to pre-mRNAs, often in proximity to splice sites, predominantly resulting in exon skipping events. One of the few mRNA targets bound to DDX1 that also underwent exon skipping in TBHP-treated NP cells was MATR3. DDX1 methylation consequently leads to exon 5 skipping in MATR3 resulting in a shorter isoform MATR3-S. Interestingly, while the long MATR3 (MATR3-L) isoform maintains a repressive chromatin state, MATR3-S generates a more relaxed chromatin state leading to the activation of senescence and apoptosis related pathways. Eventually, the authors showed that an LNP-mediated transfection of NP cells in vivo with the long MATR3 isoform can improve IVDD progression in the rat IVDD model.

This manuscript represents a comprehensive study of a novel epigenetic mechanism of IVDD progression. Starting from an unbiased approach of screening methylated proteins in NP degeneration, the authors identified a new molecular pathway that involves the methylation of the RNA binding protein DDX1 and its consequences on global RNA splicing as well as the specific splicing of MATR3. They furthermore systematically characterized the effect of this novel MATR3 splice variant on the chromatin state and the expression of senescence and apoptosis related genes. This study therefore gives novel insights into IVDD progression as well as into a methylation-dependent epigenetic molecular pathway of an RNA binding protein that globally regulates splicing and chromatin states. Overall, the manuscript reports novel and significant findings that are of interest to a broader readership. However, there are some points that need further clarification before publication.

1) The authors use only one siRNA/shRNA to target DDX1 or EZH2 (Figures 1,3,4,5,6). In order to rule out off-target effects, the authors should include a second siRNA/shRNA sequence at least in some (feasible) key experiments, such as in the in vitro models.

2) Moreover, in Table S3 the authors give the sequences of the si/shDDX1 as well as the si/shEZH2 oligos, stating that they are specific to human. Since the shRNAs targeting DDX1 as well as EZH2 were used in the in vivo rat model, the authors should comment on whether these human sequences can also target the respective rat genes.

3) On page 12, line 340: Shouldn't (FIG. 4E) read (FIG.5E)?

4) The figures and the arrangements of subfigures could be at times clearer. For instance, in Figures 1, 3, 4, 5 it would be clearer for the reader if the subfigures follow the same order and pattern in every figure (e.g. always in ascending order from left to right, and not up and down).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have adequately addressed my previous concerns. I think the details and the language in the manuscript are much improved. Great Work!

Reviewer #2

(Remarks to the Author)

The authors addressed our concerns adequately.

Reviewer #3

(Remarks to the Author)

The authors have done a good job to address Reviewer's concerns. I recommend the work be accepted with minor revisions.

The position of IgG-H is incorrectly marked in Fig. 2D, please correct it.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5

(Remarks to the Author)

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Reviewer #6

(Remarks to the Author)

The authors have addressed all concerns raised by this reviewer and made comprehensive revisions to improve the clarity and overall quality of the manuscript. The revised version can be accepted for publication.

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We sincerely appreciate the opportunity to revise and resubmit our manuscript, titled "DDX1 Methylation Mediated MATR3 Splicing Regulates Intervertebral Disc Degeneration by Initiating Chromatin Reprogramming" to *Nature Communications*. We are grateful for the constructive feedback provided by the reviewers, which has been invaluable in strengthening our study.

In response to the reviewers' insightful comments (blue), we have conducted additional experiments and made substantial revisions to the manuscript. Specifically, we have addressed all major concerns raised by the six reviewers and have incorporated the necessary changes. To facilitate the review process, we have highlighted (red) all modifications in the revised manuscript.

Point-by-point responses to the reviewers' comments are listed below, detailing the changes made and the necessary rationale. We believe these improvements have significantly improved the clarity, rigor, and impact of our study.

REVIEWER COMMENTS

Reviewer #1:

In this manuscript Zhu et al present an insightful and impactful study that reveals a fundamental mechanisms of intervertebral disc degeneration (IVDD). The authors report that the RNA binding protein DDX1 is a nonhistone target of EZH2 and that lysine methylation of DDX1 at amino acid 234 promotes IVDD. Furthermore, this methylation of DDX1 reduces association with splicing factors that specifically results in exon skipping in the gene encoding MATR3. The resulting truncated form of MATR3 causes altered chromatin accessibility, activates WNT signaling and causes senescence and apoptosis of nucleus pulposus cells which drives IVDD progression. The effect of DDX1 methylation on exon skipping was very interesting and integrating that data with

the RIP-seq data to specifically identify MATR3 was well done. The studies outlined in this work take advantage of cutting-edge in vivo IVDD models that use lipid nanoparticles to deliver MATR3 isoform mRNA. Thus, this work will be of broad interest to researchers in the field of epigenetics, specifically those that study regulation of RNA splicing mechanisms, as well as to researchers who study mechanisms contributing to lower back pain. I recommend the work be accepted with minor revisions. Specifically:

Response: We sincerely appreciate the reviewer's positive assessment of our work and their recognition of its broad relevance to both the epigenetics and lower back pain research communities. We are grateful for the constructive feedback and have carefully addressed all revision suggestions in the revised manuscript. Thank you for your support and recommendation for acceptance.

1) The figures and text in the figures are very small and difficult to read at times. I would recommend trying to increase the font size of text in the figures.

Response: We have increased the font size of text in the figures to improve clarity and readability. Additionally, we have ensured that the figure labels and legends are more distinct. We hope these modifications enhance the overall presentation of our data. Thank you for your valuable suggestion.

2) Line 142 - change the word indicating to suggesting - the conservation of an AA doesn't necessarily mean it has functional specificity but it does strongly suggest it.

Response: We have revised the wording to "suggests" to better reflect the tentative nature of the conclusion. This change better captures the nuance of the argument and acknowledges that while the data points to functional significance, further investigation is needed to confirm it (Line 144).

3) It is not explicitly stated in either the main text or figure 1 legend – what is the difference between K234R1 and K234R2 in figure 1G? are these two clones or two cell line pools? Or something else?

Response: K234R2 was the K234R1 clone system, which further showed that Lysine-arginine mutation could block the methylation modification at position 234, indicating that Lys234 was a specific methylation site of DDX1. The relevant description has been added in the figure legends (Line 923).

4) In figure 1G it isn't clear what antibody is used to detect the methylated DDX1 after TBHP treatment – is it the same as the mono-methyl lysine antibody used in Figure 1F (from CST according to table S2)?

Response: Is the same, the antibody naming in Figure 1G has been corrected, thank you.

5) line 154 - please provide a reference that decreased collagen II and increased MMP3 are associated with IVDD degeneration

Response: Thank you for your valuable suggestion. We have now incorporated appropriate references into the revised manuscript to address this concern. For example, PMID: 9682320 reported that the upregulation of MMP3 are critical markers of disc degeneration, leading to the breakdown of the annulus fibrosus and nucleus pulposus. Similarly, PMID: 34572492 demonstrated that collagen degradation plays a crucial role in IVDD pathology (Ref. 21-22).

6) Please demonstrate that TBHP induces autophagy, senescence or apoptosis of NP cells in your system and that manipulating the level of DDX1 or expressing DDX1 K234R alters these processes, not just the level of collagen II and MMP3.

Response: Thank you for your insightful suggestion. We understand the importance of further demonstrating that TBHP induces senescence, or apoptosis in NP cells within our system, as well as how DDX1 manipulation or the expression of DDX1 K234R influences these processes beyond changes in collagen II and MMP3 levels. To address this concern, we have performed additional experiments:

Senescence: We conducted SA- β -gal staining, P53 staining, and western blot analysis (P53, P21, P16) to assess cellular senescence after TBHP treatment, as well as following DDX1 knockdown or DDX1 K234R overexpression. The results indicate that TBHP induces senescence in NP cells, which is modulated by DDX1 and DDX1 K234R expression.

Apoptosis: We performed Annexin V/PI staining, TUNEL staining and western blot analysis (Bax, cleaved caspase3, Bcl2) to evaluate apoptosis. Our findings confirm that TBHP promotes apoptosis, and altering DDX1 and DDX1 K234R levels affects this process accordingly.

Autophagy: In previous studies (PMID: 37876250), our group explored the potential mechanism of mitophagy in IVDD, so this study did not involve the exploration of autophagy-related mechanisms.

These new data have been incorporated into the revised manuscript (Results section, Extended Data Figure 3), and we appreciate the reviewer's suggestion in strengthening our study.

7) Line 170-171 – The data in extended figure 1 shows that DDX1 expression correlates with protein level of collagen II and MMP3 but no markers for NP cell autophagy or senescence are presented so I don't think there is enough evidence to state "DDX1 K234R significantly exacerbated TBHP-driven NP cells degeneration"

Response: The health of the intervertebral disc depends on the balance of the extracellular matrix. During the degeneration process, anabolism (such as the synthesis

of collagen and proteoglycans) is weakened, resulting in insufficient matrix components, while catabolism (such as increased activity of matrix metalloproteinases) accelerates the degradation of the matrix. This imbalance directly leads to the degeneration of the intervertebral disc. (PMID: 9682320, PMID: 34572492) In addition, many clinical studies have shown that in tissue samples of patients with intervertebral disc degeneration, the expression of genes related to anabolism decreases (PMID: 34663366), while the expression of genes related to catabolism increases (PMID: 23369495), which supports this mechanism. Therefore, the weakening of anabolism and the enhancement of catabolism are indeed the key features of intervertebral disc degeneration and can be used to explain the occurrence of degeneration. It is worth noting that NP cell degeneration is a complex process that may be affected by multiple factors, such as autophagy and aging as you mentioned. To further explore the role of DDX1 in other mechanisms, we added this part of the work (transcriptome sequencing and phenotypic validation) in **Figure 4 and Extended Figure 3**, and finally found that DDX1 methylation causes IVDD by promoting NP cell senescence and apoptosis.

8) The in vivo rat model is really impressive. However, I would like additional controls performed: 1) what was the degree of knockdown by the shDDX1? Were DDX1 WT-LV1 and DDX1 K234R-LV expressed at the same level. Please perform IHC for DDX1 on your sections to confirm the levels of DDX1 are as expected.

Response: Thank you for your positive feedback on our in vivo rat model and for your insightful suggestions. We agree that validating the degree of DDX1 knockdown and confirming comparable expression levels of DDX1 WT-LV and DDX1 K234R-LV are essential to strengthening our conclusions.

IHC for DDX1: To further confirm DDX1 expression in vivo, we conducted immunohistochemistry staining on NP sections. The results show that DDX1 levels were significantly reduced in the shDDX1 group (10%), while DDX1 expression was restored in the DDX1 WT-LV (86.6%) and DDX1 K234R-LV (88.6%) groups, with comparable staining intensities between these two groups.

These new data have been incorporated into the revised manuscript (Results section, **Extended Figure 2H**), and we appreciate the reviewer's suggestion in improving the rigor of our study.

9) Figure 2A-B - it isn't clear how DDX1 interacting proteins were identified after coIP – was this by mass spec? were these CoIP interactions lost when DDX1-K234R was expressed in 293 cells? How was the abundance ratio and P value calculated?

Response: The specific steps are as follows: first, DDX1-WT and EV were overexpressed in HEK293T, and proteins were extracted. Subsequently, DDX1 was immunoprecipitated by anti-DDX1 antibody, and the protein adsorbed on Protein-A/G

$$p = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i} \binom{N-M}{n-i}}{\binom{N}{n}} \quad \text{Enrichment score} = \frac{\frac{m}{n}}{\frac{M}{N}}$$

beads was used for gel electrophoresis, and finally the gel strip was used for mass spectrometry. As shown in **Fig. 2M**, lysine mutation to arginine did not affect the interaction between DDX1 and EZH2. The functional enrichment analysis method is explained as follows: the background protein is all proteins with this function in the species protein, and the foreground protein is all proteins with this function in the differential protein. The hypergeometric distribution test is used to calculate the p value representing whether the function set is significantly enriched in the foreground protein, and the p value is corrected for Benjamini & Hochberg multiple tests to obtain the FDR. The formula is as follows: Where N is the number of background proteins with the database annotation; n is the number of foreground proteins with the database annotation; M is the number of background proteins for a specific entry; m is the number of foreground proteins annotated for a specific entry.

10) Line 214-216: How does figure 2E show that TBHP induced oxidative stress? TBHP induced EZH2 in Figure 2E and F but you need additional data to support that

this is due to oxidative stress specifically

Response: Given the ability of reactive oxygen species scavenger (DMTU, N, N-dimethylthiourea, MCE) to block the level of oxidative stress, complementary western blot analysis (Extended Data Fig. 2F), fluorescence staining (Extended Data Fig. 2G) and PCR (Fig. 2I) further showed that TBHP caused the up-regulation of EZH2 by activating oxidative stress.

11) Figure 2L-M seem redundant to Figure 2D. They show the same thing by two different methods – consider putting one experiment in the extended data figures.

Thanks for your suggestion, we have adjusted FIG. 2M to Extended Data Fig. 2A to reduce the redundancy between figures

12) Figure 2I isn't necessary, it is already clear in the text and other figure panels without the cartoon.

Response: Thank you for your suggestions. We found that Fig. 2I was indeed a little redundant, and it has been deleted in the revised version

13) Figure 2Q – does EZH2 remain able to bind DDX1-K234R? When you IP with HA is Flag-EZH2 associated with the K234R mutant.

Response: Co-IP can be used to directly see if the interaction is attenuated. We supplemented the related experiments, as shown in Fig. 2M, lysine mutation arginine did not affect DDX1 interaction with EZH2, indicating that the arginine mutation did not affect DDX1 conformation and that the EZH2 protein recognition site was not located in the DDX1 lysine side chain.

14) Figure 4 –What is the overlap between the THBP-treated and DDX1 K234R

up/down regulated genes? Is the same set of genes in both systems promoting the apoptosis/senescence ontologies? You briefly touch on this in figure 4H-I but I would like to see a heat map or venn diagram of common genes. Is the overlap limited to the 5 genes listed on line 294 of the text?

Response: Thank you for your insightful comment regarding the overlap between THBP-treated and DDX1 K234R up/down-regulated genes. To address this, we have conducted a comparative analysis of differentially expressed genes in both conditions. Our results indicate that while the five genes listed on line 294 are prominently involved in apoptosis/senescence pathways, additional genes also show overlapping regulation. To provide a clearer visualization of this overlap, we have now included venn diagrams in the revised Extended Figure 3 (Supplementary Data 1, as requested by reviewer#3, RNA-seq of NP/siDDX1 cells infected with DDX1 K234M or DDX1 K234R was added). The Venn diagram highlights the shared and unique genes between THBP treatment and DDX1 K234M expression. These additional data further support our conclusion that both THBP treatment and DDX1 K234M mutation converge on a subset of genes that drive apoptosis and senescence. Thank you for your valuable suggestion, which has strengthened the clarity and depth of our analysis (Line 298-300).

15) Figure 4D – bubble plot – the color of the dot represents p value but the x axis is - log₁₀ (p value). The color doesn't seem to match the x axis value? Usually, one of these is an enrichment score instead.

Response: I'm sorry that we mislabeled the X-axis earlier. In fact, the X-axis represents the enrichment score, which is an important indicator in gene set enrichment analysis that reflects the degree to which genes of a certain functional gene set occur in the ranked gene set. The correlation is calculated as shown in Response 9. Thank you for pointing out the problem so carefully.

16) Line 291 – grammar - delete “were identified”

Response: Thanks for pointing out that the sentence has been grammatically modified (Line 310).

17) Figure 4 H-I are discussed in the text before discussion of apoptosis in Figure 4E-G – these labels should be switched so the figures are in order.

Response: We have adjusted the above labels in Fig. 4 so that the figures are arranged in order and easy for readers to understand.

18) Figure 4 - Please perform a western blot of DDX1 in the NP cells and 293 cells that express: EV, siDDX1, siDDX1+Wt and siDDX+K234R - to demonstrate the level of knockdown and comparable expression levels of WT and K234R in these cells.

Response: Thank you for your suggestion. To address this point, we have performed Western blot analysis of DDX1 expression in NP cells under the indicated conditions (EV, siDDX1, siDDX1+WT, and siDDX1+K234R). Our results confirm the efficient knockdown of endogenous DDX1 by siDDX1 and show that both WT and K234R constructs are expressed at comparable levels upon reintroduction. These data have been included as the new Extended Data Figure 2B-C in the revised manuscript. We appreciate your insightful comment, which has helped strengthen the rigor of our study.

19) Delivering LNPs containing MATR3 isoforms was very cutting edge. But I think the production and QC of the LNPs should be shifted to the extended data section so that the main figure is specifically focused on the animal experiment demonstrating the LNP- MATR3-S mRNA promotes IVDD in vivo. Likewise, I believe the results presented in extended data figure 4 should be moved to Figure 8 since they demonstrate the short isoform promotes expression of metabolic and age-related markers while the long isoform mitigated NP cell aging indicators and IVDD in vivo.

Response: Thank you for your thoughtful suggestion regarding the organization of the figures. We agree that shifting the LNP production and QC details to the extended data section will help streamline the main figures to better focus on the in vivo effects of LNP-MATR3-S mRNA. Accordingly, we have moved these data to **Extended Data Figure 7A** and have updated the text to reflect this change. Additionally, we have integrated the results from **Extended Data Figure 4** into **Figure 8**, as these findings directly support the role of MATR3 isoforms in regulating metabolic and age-related markers, as well as IVDD progression in vivo. The figure legend and corresponding text have been updated to reflect this reorganization. We appreciate your insightful feedback, which has improved the clarity and focus of our manuscript.

20) Line 484 – is it DOTAP or DPTAP? Will you please double check which is correct.

Response: Thank you for your careful pointing out. We have modified the previous spelling error DPTAP in **Extended Data Figure 7A** to DOTAP (1,2-Dioleoyl-3-trimethylammonium-propane chloride, DOTAP).

Reviewer #2:

DDX1 is an ATP-dependent RNA helicase belonging to the DEAD box family of helicases that unwind and/or remodel RNA secondary structure in vitro. DDX1 has been implicated in a number of cellular processes such as: regulation of translation initiation and nuclear RNA splicing, RNA clearance at DNA double strand breaks (DSB), as a co-activator of NF-kappa-B mediated transcriptional activation etc.

In this manuscript, the authors explore a novel role of DDX1, highlighting its function as a non-canonical RNA binding protein (RBP) that is capable of modulating the alternative splicing (AS) of MATR3 pre-mRNA, depending on the methylation state of DDX1's Lysine-234. The authors show that native DDX1 interacts with splicing factors and enriches them at the splice site of MATR3 thereby driving exon 14 inclusion to

produce a long form of the transcript termed MATR3-L. In turn, MATR3-L maintains normal chromatin accessibility and prevents excessive activation of cellular senescence and apoptosis programming. Under oxidative stress triggered by TBHP treatment, there is an over-expression of EZH2 which leads to mono-methylation of DDX1 at Lysine-234. Increased DDX1 methylation reduces its binding to MATR-3, preventing recruitment of splicing factors, leading to the skipping of exon 14, resulting in a short form of the transcript termed MATR3-S. Consequently, MATR3-S leads to excessive opening of chromatin, resulting in cellular senescence and apoptosis programming which can lead to degeneration of nucleus pulposus (NP) cells. Degeneration of NP cells in turn leads to Intervertebral Disc Degeneration (IVDD) which is the main cause of low back pain (LBP).

The manuscript would be interesting to a wide audience and the readership of this journal. Some comments below should be addressed:

Response: We sincerely appreciate the reviewer's positive assessment of our work. We are grateful for the constructive feedback and have carefully addressed all revision suggestions in the revised manuscript. Thank you for your support and recommendation.

1. The authors primarily describe DDX1 as an RNA-binding protein (RBP), but it also functions as an RNA helicase. Are the impacts on splicing a result of decreased binding to other splicing factors alone, or does mono-methylation at K-234 also impact RNA unwinding and/or RNP remodeling activity of DDX1?

Response: The helicase activity of DDX1 mainly depends on the synergistic effect of the DEAD-box domain (including the DEAD sequence, 370-373), the ATP binding site, and the RNA binding domain. These domains realize the RNA unwinding function through the hydrolysis of ATP and the interaction with RNA. Given that K234 methylation is far away from the above domains, K234 methylation has little effect on the unwinding property of DDX1. For further verification, the ATP usage rate of DDX1

and methylated DDX1 was detected by ATP detection kit (Extended Data Fig. 5I), and no significant difference was found, which also suggested the similarity of unwinding efficiency. Interestingly, enrichment analysis showed that the enrichment of rRNA processing between K234R and K234M was approximately equal, suggesting that K234 methylation does not affect its helicase activity while regulating the interaction between splicing factors and target RNA. This conclusion was also confirmed by the addition of CO-IP (SF3B2, HNRNPA2B1, RBM26) (Extended Data Fig. 5F-H). Considering that methylated DDX1 has reduced interactions with target RNA and splicing factors, this will affect the remodeling activity of ribonucleoprotein to a certain extent. In summary: K-234 itself may not affect the unwinding efficiency of DDX1, but regulate alternative splicing by regulating the interaction with splicing factors and MATR3.

2. How does mono-methylation of K234 impact DDX1's binding to MATR3 pre-mRNA if the methylation is at the SPRY domain? The authors should elaborate on the known functions of SPRY domains and what might be the mechanism by which the effects on splicing occur.

Response: SPRY (SPRY domain-containing) domain is a highly conserved protein domain that is widely present in many proteins involved in biological processes such as signal transduction, cell cycle regulation, and transcriptional regulation. One of its most important functions is to mediate protein-protein interactions, especially to form complexes with other proteins containing different domains. According to the analysis of the SMART database, there are 149 SPRY domain-containing proteins in humans (PMID: 16498413), which can be roughly divided into four categories: the first category is proteins containing ring domains (accounting for 33%), including TRIM family members (accounting for 19%) and butyric acid family (accounting for 15%); the second category is TRIM family-like proteins lacking ring domains and butyric acid family-like proteins lacking extracellular domains (accounting for 12%); the third category is other proteins, such as SSB protein family members (accounting for 40%).

Given that the SPRY domain can directly mediate protein-protein interactions, there are multiple biochemical evidences that the SPRY domains in these proteins may function through protein-protein interactions. For example, the VASA binding activity of GUS, the interaction between the cytoplasmic domain of butyric acid protein containing the SPRY domain and xanthine dehydrogenase (PMID: 8541302), and the interaction between the SPRY domain of TRIM7 and glycogenin (PMID: 14984203). However, there is currently no evidence that the SPRY domain has enzymatic activity or functions in interacting with small molecules. In some cases, the SPRY domain may recognize and bind to specific molecules through specific surface structures. For example, the SPRY domain of the TRIM5 α protein plays a key role in recognizing differences in viral capsid proteins through its surface A. Similarly, the SPRY domains of Pyrin and MID1 also recognize unknown partner proteins through surface A and/or B to regulate their functions.

The SPRY domain interacts with multiple target proteins through its unique three-dimensional structure, thereby regulating cell signaling pathways. By binding to receptor tyrosine kinases, GTPases or other signaling molecules, the SPRY domain helps regulate the activation and feedback of these signaling pathways. Especially in neurons or other specific cell types, the SPRY domain is involved in the transport of certain proteins from the cytoplasm to the nucleus, or in the localization within specific organelles (such as mitochondria or endoplasmic reticulum). Some proteins containing SPRY domains are involved in cell cycle regulation. For example, the SPRY domain affects the progression of the cell cycle by interacting with cyclin-dependent kinases (CDKs). In addition, some SPRY domain-rich proteins play an important role in the immune system, regulating the intensity and persistence of immune responses by interacting with immune signaling molecules. The SPRY domain is also involved in cell adhesion, migration and polarity regulation, especially in embryonic development and tissue repair.

The SPRY domain is closely related to many diseases, especially cancer. In some cancer types, abnormal expression of proteins with SPRY domains may promote the proliferation, migration and metastasis of cancer cells by over-activating or inhibiting

signaling pathways. Although the SPRY domain was initially thought to be mainly associated with signal transduction and protein-protein interactions, more and more studies have shown that the SPRY domain may regulate RNA splicing and gene transcription by interacting with transcription factors, splicing factors or RNA binding proteins. Through these mechanisms, the SPRY domain is involved in cell differentiation, development, and the occurrence and progression of diseases.

Accumulating evidence suggests that the interaction between DEAD-box proteins and their cognate binding partners is often mediated by the latter's SPRY domain. Unlike other DEAD-box proteins, DDX1 incorporates a SPRY domain (amino acids 86-279) in its polypeptide chain. The insertion of this domain is expected to directly regulate the correct assembly of DDX1 and the formation of a DDX1-dependent multiprotein complex. Through amino acid sequence alignment (PMID: 11171974), we found that K234 is located at position β 13 in β -sheet 1 and belongs to the core domain of SPRQ. The hydrophobic residues in the β 2, β 6, β 7, β 8, β 11, β 12, β 13, and β 14 chains stack with each other through van der Waals forces. In addition to these hydrophobic interactions, salt bridges and hydrogen bonds also contribute to the stabilization of the β -sheet and may affect its conformational rigidity.

The amino side chain ($-\text{NH}_2$) of K234 is positively charged and can interact with adjacent negatively charged residues (such as E237 glutamic acid and N235 aspartic acid) through hydrogen bonds. When K234 is methylated, the chemical properties of its amino side chain change, and the positive charge is replaced by a methyl group ($-\text{CH}_3$), thereby weakening the charge interaction with other molecules or residues. It is worth noting that N235 after K234 is located in the center of the α helix, and the two ends form a β -strand structure (PMID: 26323305, Ref. 56), thereby maintaining the stability of the local structure. Considering that the methylation occurs within the α helix, this modification may change the polarity and charge of the residue, thereby affecting its stability or causing helical bending. In addition, when methylation occurs in the β -strand or its adjacent region, the methylated lysine may cause conformational changes in the β -turn by changing its chemical properties (such as reducing hydrogen bond formation), thereby affecting the RNA binding region and the protein interaction

platform (Relevant content has been added in the discussion section).

Therefore, we speculate that DDX1 regulates the changes in SPRQ conformation through K234 methylation modification, thereby affecting its interaction with MATR3, or changing its binding with other splicing factors and regulatory elements, thereby regulating the RNA splicing process. This mechanism provides new clues for our in-depth understanding of the role of DDX1 in RNA splicing and gene regulation.

3. DEAD box RNA helicases typically do not exhibit RNA sequence specificity as other RNA binding domains; instead, they recognize and interact with RNA based on RNA secondary structure elements. Yet, the authors report seeing enriched sequence elements based on their de novo motif analysis of DDX1-RIP experiments (Figure 5L). Where is the sequence specificity coming from? Could there be another factor mediating the interaction in their pull-down experiments?

Response: Although DEAD box RNA helicases usually work by recognizing the secondary structure of RNA, this does not mean that they are completely unaffected by the RNA sequence. In some cases, helicases may preferentially bind to specific RNA sequences, which may enhance the stability of the structure during the formation of secondary structures or provide additional signals for helicase recognition. Existing studies support this view. For example, DDX1 has been found to be involved in regulating the stability of certain cancer-related RNAs (such as miRNA and mRNA), a process that may be regulated by specific RNA sequences. In addition, DDX1 binds to RNAs containing specific U-rich sequences, probably because these sequences can stabilize the formation of specific RNA secondary structures, thereby enhancing the binding affinity of DDX1 to the RNA (PMID: 21779027). Similarly, DDX5 was found to be able to recognize RNA sequences with G-quadruplex structures, indicating that in specific contexts, the binding of helicases may depend on the RNA sequence itself (PMID: 22156369).

In addition, certain RNA modifications (such as methylation, pseudouridine modification, etc.) may change the secondary structure of RNA or expose specific

sequence regions, thereby promoting the binding of helicases to these sequences. For example, under specific physiological or pathological conditions, methylation modification of the 5' end of RNA can regulate the affinity of helicases for its binding (PMID: 24218557). Specific RNA sequences, such as G- or U-rich regions (GGG- and U-rich sequences), may enhance binding to DEAD box helicases by changing the conformation of RNA (PMID: 22156369). Studies have also shown that DDX1 may tend to recognize specific sequences in viral RNA during viral infection, and this recognition pattern may be different at different stages of infection. For example, in HIV infection, DDX1 promotes the unwinding and replication of viral RNA by recognizing specific sequence regions of viral RNA (PMID: 31359346). In this case, the sequence specificity of the helicase may be the result of the combined effect of the structure and sequence of the viral RNA.

DDX1 is also involved in post-transcriptional regulatory processes, especially in regulating mRNA stability, splicing and translation. Studies have found that under certain specific cellular environments or stress conditions, DDX1 tends to bind to certain specific mRNA sequences, especially those containing AU-rich regions or stable secondary structures. This recognition mechanism may be part of the cell's response to environmental changes (PMID: 31330130). Therefore, DEAD box helicases (including DDX1) may show certain sequence specificity under specific physiological and pathological conditions. Usually, this specificity is regulated by the secondary structure of RNA, RNA modifications (such as methylation), or the interaction of RNA with other proteins.

In this study, our de novo motif analysis revealed some potential sequence elements that may be part of RNA secondary structure and function. Helicases recognize RNA through this sequence-structure interaction. However, it should be pointed out that in pull-down experiments, there may be other factors or co-interacting proteins that may regulate the binding of DDX1 to its target RNA. Since RIP experiments usually rely on specific antibodies to precipitate the target protein and RNA complex, this may lead to non-specific binding. Therefore, DDX1 may bind to non-target RNAs or form complexes with other RNA-binding proteins, cofactors, etc., thereby affecting its RNA

binding pattern. Although we carefully optimized the conditions in the experiment (such as high salt concentration washing, selection of specific antibodies, etc.), we still cannot completely rule out the possibility of nonspecific interactions.

In addition, RIP experiments involve RNA extraction and processing, and RNA may undergo secondary structural changes during this process, which may affect the binding of DDX1 to RNA. For example, some sequences in the RNA sample may expose previously hidden regions due to experimental treatment or changes in physical conditions, thereby affecting the experimental results. In RIP experiments, direct or indirect interactions between DDX1 and other proteins may also affect its binding to RNA. For DEAD box helicases, they usually work together with other accompanying proteins, and the synergistic effect may enhance or regulate their RNA binding properties. Therefore, the observed sequence specificity may not only be the effect of DDX1 itself, but may also partially reflect the contribution of these synergistic proteins. In addition, some RNA molecules may undergo chemical modifications (such as methylation, pseudouridine modification, etc.), which may change the secondary structure of RNA or change the binding mode of RNA to DDX1. These modifications may change the conformation of RNA, so that it exhibits binding characteristics different from traditional structure-sequence specificity during helicase recognition.

The abundant sequence elements we found may not be simple sequence-specific signals, but the result of interactions with RNA secondary structure elements. Helicases may interact with these sequence elements by recognizing specific RNA folding regions (such as hairpin structures, stem-loop structures, etc.). This binding mode of structure and sequence may be a potential source of the "sequence specificity" observed in our experiment. Importantly, in our study, we detected the amplification of *MATR3* isoforms in real time by semi-quantitative RT-PCR (Figure 6H and Extended Data Fig. 5E), quantifying how methylation modifications regulate the binding of DDX1 to *MATR3* and alternative splicing, which provides evidence for further understanding that DDX1 may exhibit certain sequence specificity.

4. The methods describing the RIP-seq experiments are a bit odd. Do they really do

phenol-chloroform extraction before they do anti-DDX1 pull-down?

Response: After careful inspection, we found that the previous description of the RIP-seq experimental steps was indeed redundant and unclear. Typically, RIP-seq experiments do not use phenol-chloroform extraction before immunoprecipitation (IP) because this process destroys the RNA-protein complex, making it impossible to specifically enrich the target RNA in the subsequent IP process. In traditional RIP-seq, the experiment generally first extracts cell lysates using mild lysis conditions to keep the complex of RNA and RNA-binding proteins intact, and then enriches the target protein and its bound RNA through immunoprecipitation. Subsequently, RNA is extracted from these complexes. Phenol-chloroform separation is more suitable for performing after the IP step to further purify the RNA released from the immunoprecipitation complex. The corrected experimental description is as follows:

NP cell samples were lysed using lysis buffer (containing protease inhibitors and RNase inhibitors) to ensure that RNA and RNA-binding proteins were not degraded. Cell debris was removed by centrifugation, and the supernatant was collected for co-immunoprecipitation. The RNA-protein complex in the lysate was incubated with anti-DDX1 antibody-Protein A/G magnetic bead complex and incubated at 4°C for 4 hours with gentle shaking to ensure that the target protein and RNA complex were specifically captured by the antibody. Non-specific binding components were removed by washing three times in succession with low salt and high salt precipitation buffer to ensure that highly specific RNA-protein complexes were obtained. Proteinase K (containing RNase inhibitor, 37°C, 15min) was added to cleave the target RNA-binding protein and release RNA from the complex. The extracted RNA was enriched for PolyA-tailed mRNA using the Dynabeads™ mRNA DIRECT™ Purification Kit (ThermoFisher, Massachusetts, USA). The purified RNA was collected and RNA-seq libraries were generated using the NEBNext Ultra™ RNA Library Preparation Kit (New England Biolabs, Massachusetts, USA). Library quality was assessed using the Bioptic Qsep100 analyzer and sequenced using the NovaSeq 6000 sequencing platform. Paired-end reads of 150 bp were mapped to the reference human genome GRCh38/hg38 using Bowtie2.

SAMtools was used to sort only the paired reads that were uniquely mapped and retained for subsequent analysis. Peak and differential peak calling was performed for each sample using the exomePeak R package. GRCh38/hg38 was used for human genome assembly, and the exomePeak R package was used to generate peak files. For RIP-seq data analysis, the results were compared for de novo peak calling, protein binding preferences for RNA were studied, and motif analysis of binding sites was performed (Line 802).

5. The authors' ability to elicit the same response in HEK293T cells as in NP cells suggests that the chromatin remodeling and subsequent cellular senescence caused by AS of MATR3 are not unique to NP cells but have a broader relevance across various cell types.

Response: Thank you for your valuable comments. We strongly agree with your view that chromatin remodeling and cellular senescence responses caused by alternative splicing of MATR3 are widely relevant in different cell types. We did observe similar responses in HEK293T cells as in NP cells, suggesting that alternative splicing of MATR3 may be universal in multiple cell types. It is worth noting that although both HEK293T cells and NP cells showed similar responses in terms of alternative splicing of MATR3, considering that these two cell types have certain differences in proliferation capacity, cell cycle, microenvironment, etc., these factors may affect the extent and characteristics of the senescence response triggered by MATR3. Therefore, we believe that although the cellular responses caused by alternative splicing of MATR3 are widely relevant in different cell types, the specific response patterns and mechanisms may be different. We believe that it will also be a good research direction to further explore the role of MATR3 in different tissues and cell types in order to better understand its functions in different physiological and pathological states. Thank you again for your detailed feedback.

6. EZH2 is known to work as part of the polycomb repressive complex 2 (PRC2) and

known to trigger gene silencing pathways. In this case, is EZH2 methylating DDX1 as part of PRC2 or by itself? How do the authors reconcile the traditionally repressive role of EZH2 with current increased gene expression in NP cells?

Response: Thank you for your valuable comments. You mentioned that EZH2, as a core component of the PRC2 complex, usually triggers gene silencing through H3K27 trimethylation, but in our study, EZH2 seems to lead to increased gene expression (chromatin accessibility) by methylating DDX1. We understand your concern very much and hope to further clarify this point here.

First, EZH2 does play its classical repressive role in the PRC2 complex, silencing target genes through H3K27 trimethylation (PMID: 12351676). However, recent studies have shown that EZH2 may play a regulatory role in some cases in a form other than PRC2, participate in other molecular pathways, and even show different functions in different cell types. For example, EZH2 can directly affect its function by regulating transcription factors or RNA binding proteins through non-classical pathways without relying on the traditional PRC2 complex (PMID: 21248841). In our study, EZH2 methylation of DDX1 may not be completely dependent on the PRC2 complex, but may affect the expression of genes related to cell senescence, chromatin remodeling, etc. by changing the structure of DDX1 or its ability to bind to RNA.

Regarding the contradiction between the inhibitory effect of EZH2 and the increased gene expression in NP cells, in fact, the function of EZH2 is cell type and context dependent. In different cell contexts, EZH2 can not only achieve transcriptional repression through the PRC2 complex, but also affect the function of other molecules through its methyltransferase activity. In NP cells, EZH2 may promote the expression of genes associated with cellular senescence by methylating DDX1. Similar mechanisms have been observed in other studies, for example, EZH2 may have different effects on gene regulation at different developmental stages or in different pathological states (PMID: 34230470, PMID: 35538070, PMID: 31631036).

Therefore, our study suggests that EZH2 may have multiple roles in different cell types. In NP cells, EZH2 may promote the upregulation of specific genes through non-

canonical mechanisms, such as methylating RNA binding proteins, which does not conflict with its traditional inhibitory role in the PRC2 complex, but reveals its functional diversity. Thank you again for your important questions.

Reviewer #3:

Zhu et al. discovered that EZH2-mediated K234 monomethylation modification of DDX1 regulates the process of intervertebral disc degeneration (IVDD). Mechanistically, methylation of DDX1 promotes exon skipping events in MATR3 to generate MATR3-S by preventing the recruitment of splicing factors. The short form of MATR3 leads to enhanced chromatin accessibility and triggers cellular senescence and apoptosis by hyperactivating the Wnt and MAPK signaling pathways, thereby facilitating the progression of IVDD. Based on this mechanism, they developed a cationic lipid nanoparticle-based MATR3-L mRNA cargo and claimed its potential for alleviating IVDD progression.

The findings in this manuscript are potentially interesting. However, there are significant faults in experiment design and data interpretation, which impede the rigor and reliability of this study. Furthermore, the manuscript is not well written and needs heavy editing. The comments for authors are listed below.

Response: We appreciate the reviewer's time and effort in evaluating our manuscript and providing constructive feedback. We acknowledge the concerns regarding experimental design, data interpretation, and manuscript clarity. In response, we have carefully revised the manuscript to enhance its rigor and readability. We have thoroughly revised the manuscript for clarity, conciseness, and coherence. A professional language edit has been performed to improve readability and scientific communication.

We greatly appreciate the reviewer's critical insights, which have significantly strengthened the manuscript. Below, we provide detailed responses to each of the specific comments.

Major comments:

1. In Figure 2D, there are two bands of DDX1 around 100 kDa were observed in lane 5. Because the input control is missing, it is hard to judge which band is the specific DDX1. The authors also need to carefully check whether the lower bands in lane 2, 3, 4 and 6 are specific DDX1 and adjust their conclusion accordingly.

Response: Thank you for your careful review and valuable comments on our work. Regarding the two DDX1 bands observed in lane 5 of Figure 2D and the lack of input control, we attach great importance to your comments and have further improved the input control of the relevant samples (Input: DDX1). Regarding the two DDX1 bands of about 100kDa observed in lane 5 of Figure 2D, the following reasons may be considered: proteins may undergo multiple modifications after translation, such as phosphorylation, glycosylation, or ubiquitination, which may cause different mobilities of the same protein in the gel, resulting in multiple bands. Protein degradation: If the sample is not handled properly or degradation occurs during extraction and storage, it may cause the protein to produce fragments of different lengths, forming multiple bands. Non-specific binding: Antibodies may bind to other similar proteins or contaminants, resulting in non-specific bands. Isoform or subtype proteins: Some proteins may have isoform or subtype variants, which have slightly different molecular weights and appear as multiple bands in Western blot. Antibody specificity issues: The antibodies used may cross-react with different forms of the target protein or other proteins similar to the target protein. Gel electrophoresis conditions: Improper electrophoresis conditions (such as gel concentration, pH value) may also affect the mobility of proteins, resulting in inaccurate band display. In response to the above possible problems and the lower bands in lanes 2, 3, 4, and 6 that you mentioned, we further optimized the experimental conditions and re-ran the experiment to ensure that the characteristic bands of DDX1 can be more clearly defined. The results are shown in Fig. 2D of the revised manuscript to ensure that the conclusions are consistent with the previous ones.

2. The observation of DDX1 methylation and DDX1-EZH2 interaction in untreated cells were in controversy throughout the manuscript. Clear methylation signals of DDX1 can be seen in Figure 1C and ED figure 1I, but not in Figure 2C and 2P. Furthermore, the interaction between DDX1 and EZH2 were not detected in untreated cells as shown in Figure 2P, which leads to my confusion that how DDX1 were methylated in untreated cells.

Response: In normal NP cells, there is low expression of EZH2 (as shown in Figure 2E), which will interact with DDX1 and methylate it to a certain extent, which is why: clear DDX1 methylation signals can be seen in Figure 1C and ED Figure 1I. In addition, NP degeneration is a continuous process, not a binary process, so we believe that the interaction between DDX1 and EZH2 from weak to strong is also a dynamic process. We need to be clear that when a certain band in the Western blot results is too strong, it may indeed cover up the low-expression band. This situation may cause the following problems: Background interference: Strong bands may cause the background signal to rise, making the signal of the low-expression band more difficult to distinguish. Qualitative misleading: If the intensity of the high-expression band covers the low-expression band, it may lead to an erroneous judgment of the presence or absence of the low-expression protein. The above reasons may have led to the fact that the interaction between DDX1 and EZH2 was not detected in the untreated cells in Figures 2C and 2P in the previous manuscript. To avoid this, we optimized antibody dilution and detection conditions in our experimental design and evaluated signal intensity using multiple sample concentrations and different exposure times to ensure that the signal was within the appropriate dynamic range. The results are shown in Fig. 2 of the revised manuscript to ensure that the conclusions are consistent with the previous ones.

3. It is confused that why two different cell lines NP cells and HEK293T cells were used to perform RNA-seq? The mechanism of DDX1 in IVDD might be cell-context dependent and ignored in these analyses. Considering physiological relevance,

performing RNA-seq in NP cells, rather than HEK293T cells, with DDX1 overexpression or silencing is necessary.

Response: We appreciate the reviewer's concern regarding the use of NP cells and HEK293T cells for RNA sequencing analysis. The rationale for including HEK293T cells in our RNA sequencing experiments was to take advantage of their high transfection efficiency and well-characterized transcriptome responses. HEK293T cells, as a common transfection and expression system, facilitated initial validation and molecular mechanism exploration of DDX1 overexpression or silencing, allowing us to identify global changes associated with DDX1 regulation. However, we acknowledge the importance of cellular context dependency, and in particular, the role of DDX1 in IVDD may be influenced by the cellular context.

To address this concern, we have now performed RNA sequencing by re-overexpressing K234R vs. K234M in siDDX1/NP cells to ensure that our findings are physiologically relevant to IVDD. These new data have been incorporated into Extended Data Figure 3 and Extended Data Figure 4, and we have modified the corresponding results to highlight the similarities and differences between NP and HEK293T transcriptome responses. Importantly, key pathways associated with apoptosis and senescence were similarly and consistently affected in both cell types, strengthening our mechanistic conclusions.

We appreciate this valuable suggestion, which helps enhance the physiological relevance and robustness of our study.

4. The results of GSEA analysis in Figure 4B and 4C were erroneously interpreted. In line 282-287, the authors claim that “Subsequently, a gene set enrichment analysis (GSEA) showed that cellular senescence and apoptosis signaling pathway were strongly associated with TBHP-treated NP cells degenerative phenotypes (Fig. 4B). Interestingly, the GSEA demonstrated that DDX1 K234M overexpression similarly regulated cellular senescence and apoptotic signaling pathway (FIG. 4C).” However, the results of Figure 4B and 4C show that cellular senescence and apoptosis signaling pathways were enriched in the control or DDX1 K234R groups instead. This error

makes the conclusion “DDX1 methylation promotes cellular senescence and apoptosis” suspicious.

Response: Thank you for your careful correction of the GSEA analysis results in Figure 4B and 4C. After careful inspection, we found that there was indeed an error in group labeling, which led to the contradictory conclusions in the results in Figure 4B and 4C. Specifically, the group labels in the figure were incorrectly labeled during the analysis, which led to misleading interpretations when we described the enrichment of cell senescence and apoptosis signaling pathways. We are deeply sorry for this. After reanalyzing the data, we have corrected the groups in Figure 4B and 4C and confirmed the accuracy of the results. The corrected data showed that the enrichment of TBHP-treated group and DDX1 K234M overexpression group in NP cells senescence and apoptosis pathways was consistent with our original conclusions. It is worth noting that in our subsequent supplementary RNA-seq (re-overexpressing K234R vs. K234M in siDDX1/NP cells, Extended Data Figure 4A and C), it was found that DDX1 K234M overexpression similarly regulated cell senescence and apoptosis signaling pathways, which was consistent with the previous conclusions.

Thank you for the reviewer for pointing out this issue, and we will ensure that the revised manuscript is more rigorous and accurate.

5. In Figure 4E and 4F, the quality of Annexin V/PI staining and flow cytometry is low, and the portion of apoptotic cells in TBHP-treated cells is too low as compared with the same experiments performed in previous publications (PMID: 35096819, 30414849).

Response: We appreciate the reviewer’s feedback regarding the quality of the Annexin V/PI staining and flow cytometry analysis in Figure 4E and 4F. We acknowledge that the proportion of apoptotic cells in TBHP-treated groups appears lower compared to some previously published studies. To address this concern, we have repeated the Annexin V/PI staining and flow cytometry experiments with optimized staining conditions and increased sample size to ensure robust and reproducible results.

Provided additional representative contour plots in the revised Figure 4G and 4H to improve data presentation and clarity, which was consistent with the previous conclusions.

We appreciate the reviewer's valuable suggestion, which has helped improve the quality and rigor of our data.

6. In ED Figure 2E, cleaved-caspase3 should be examined instead of caspase3 to evaluate cell apoptosis. Usually, the accumulation of full length caspase3 indicates the reduction of cleaved-caspase3 and the inhibition of apoptosis, which would be contrary to the conclusions described in this paper.

Response: Thank you for the reviewer's valuable comments on the detection of caspase3 in Figure 2E. In fact, what we detected in Figure 2E was indeed cleaved-caspase3, not full-length caspase3 (relevant antibody information is in Supplementary Table 2). We are deeply sorry that we did not clearly point this out in the figure and text, which led to the reviewer's misunderstanding. To clarify this, we will clearly mark the antibodies used and the cleaved-caspase3 detected in the revised manuscript, and provide additional representative western blot band (Extended Data Figure 3G) to ensure the clarity, accuracy and reproducibility of the experimental results.

7. The length of MATR3 exon 14 is in controversy in text description and schematic shown in Figure 6F. In lines 489-491, it is mentioned that “The transcribed MATR3-L has a length of approximately 2544 nucleotides, while MATR3-S has a length of approximately 2385 nucleotides”. By calculation, the skipped exon 14 should be 159 nt in length, whose skipping is not able to cause a frameshift. Whereas, it is shown that exon 14 is 122 nt in Figure 6F, whose skipping is able to cause a frameshift. The authors should carefully check the length of exon 14, which is critical for the reliability of the data related to MATR3 splicing.

Response: Thank you for highlighting these important questions regarding the length

of MATR3 exon 14 and its impact on splicing and frameshift mutations. Upon careful re-examination of the sequence, we confirmed that exon 14 is indeed 122 nucleotides in length. Notably, the numerical discrepancy between isoforms arises due to a frameshift mutation that occurs when exon 14 is skipped, leading to a redefinition of the coding region at the end of exon 15. Specifically, exon 15 is truncated from 51 nucleotides to 14 nucleotides, resulting in a 37-base frameshift at the 3'-end, shifting the coding region into the noncoding region. When combined with the 122 nucleotides of exon 14, this explains the 159-nucleotide difference mentioned in the text. This frameshift mutation plays a critical role in generating two distinct MATR3 isoforms, each exhibiting unique biological effects. To further clarify this point, we reanalyzed the alternative splicing of *MATR3* in transcriptome sequencing data (siDDX1/NP cells re-overexpressing K234R and K234M, Extended Data Figure 5C-D) and found results consistent with our previous alternative splicing analysis.

We sincerely appreciate the reviewer's keen observation, which has helped us improve the accuracy and clarity of the manuscript.

8. Lines 690 and 1258 in the method section: I could not understand how the prokaryotic protein expression vectors (pET-28a-CNV, his-tag) could be used for expression of DDX1 in 293T cells of human origin.

Response: Thank you for your patient feedback. We did have inaccuracies in the description of the experimental methods section for purification of recombinant proteins, which have now been corrected. Specifically, the gene encoding 6XHis-tagged DDX1 was cloned into pET-28a-CMV vector and transformed into *E. coli* BL21 cells (this part was consistent with GST and His pull-down assays). The relevant content has been updated, thank you for your reminder and support (Line 747 and 1370).

9. The shRNA of EZH2 was used for silencing EZH2 in rats (Figure 3), however, whose sequence provided in table S3 was of Homo. Therefore, the related results and conclusions are suspicious. Furthermore, at least one more shRNA should be used to

prevent off-target effects.

Response: Thank you for the careful review of our work. Regarding the shRNA sequence of EZH2 and DDX1 you mentioned, in fact, in our previous study, we used the Basic Local Alignment Search Tool (BLAST) to align the sequences of EZH2 and DDX1 genes in humans and rats, and found that the conservation of DDX1 between the two species was 96.71%, and that of EZH2 was 95.09% (Supplementary Data 4). Based on the high conservation of these two genes, we designed different target sequences of siDDX1/shDDX1 and siEZH2/shEZH2 for the homologous fragments of EZH2 and DDX1 in different species, respectively, and finally determined that Homo: GGAUGAAUUUGAUGGGGAAA/Rat: GGAUGGUUACAUGUCAAAUC and Homo: AUACAUGUGCAGCUUUCUG/ Rat: GGAUGGUACUUUCAUUGAAGA (could target DDX1 and EZH2 in humans and rats, respectively. We deeply regret that the error of registering duplicates occurred in the previous table. In response to the reviewer's concern, we will correct the description of shDDX1 and shEZH2 sequences in the revised manuscript (Supplementary Table 3). In addition, to further exclude the possibility of off-target effects, we designed and applied additional siRNA#2 and shRNA#2 targeting DDX1/EZH2 for validation experiments, and have provided new in vivo and in vitro experimental data in the revised version (Extended Data Figure 2B-E, H-I). We believe that these additional experiments will further enhance the reliability of the research results. Thank you again for the reviewer's valuable comments.

10. The manuscript needs heavy editing. Either indent or blank lines between paragraphs except for the first paragraph, otherwise it will affect reading. Lines 49-51: Inconsistent with the previous tense. Line 128: Tense confusion. Line 231: The meaning is wrong. Line 257-258: Tense inconsistency. Line 401 is missing a punctuation mark. Line 1019: The tense of the title is inconsistent with other titles. Line 1361-1363: There is also a problem with this sentence structure. Line 1405: The title does not do a good job of summarizing the figure. Line 1358-1359: synthesis is noun, catabolic is adjective, this sentence structure is problematic.

Response: We sincerely appreciate the reviewer's detailed feedback on the manuscript's language and formatting. We have carefully revised the text to ensure clarity, consistency, and grammatical accuracy. Specifically, we have standardized paragraph spacing by inserting blank lines between paragraphs (except for the first paragraph) to enhance readability. Adjusted lines 49-51 (Line 49-51), 128 (Line 131), 234 (Line 241-243), 257-258 (Line 270-271), and 1019 (Line 1119) to maintain a consistent tense throughout the manuscript. Added the missing punctuation mark on line 401 (Line 426). Improved sentence structure in lines 1361-1363 to enhance clarity (Line 1516). Corrected the grammatical inconsistency in lines 1358-1359 by ensuring proper noun-adjective agreement (Line 1512). Revised the title on line 1405 to better summarize the figure's content (Line 1618). We have thoroughly reviewed the entire manuscript to ensure it adheres to proper scientific writing standards. Thank you again for your valuable suggestions, which have significantly improved the clarity and readability of our work.

Line 67: Reference 5 seems irrelevant to the description. Line 88: This citation seems to refer only to apoptotic genes. Line 88-91: I understand that this sentence is meant to express that a small amount of RBP can regulate gene expression because it can cause abnormalities in post-translational modification. But that citation does not mention RBP. Line 96: I think the reference 13 is not representative. Line 568-571: Changes in gene expression at the pre/post-translational level caused by DNA methylation and histone modification? Sure it wasn't pre/post-transcriptional? Or provide relevant literature.

Response: We sincerely appreciate the reviewer's careful evaluation of our references and the accuracy of our citations. In response to the concerns raised, we have made the following revisions: Line 67: We have re-evaluated Reference 5 and found it to be irrelevant to the description. This reference has been replaced with a more appropriate citation that accurately supports the statement (PMID: 36638792, PMID: 40075068, Ref. 5-6). Line 88: We have confirmed that the citation does not fully support the

broader claim. We have replaced it with a more comprehensive reference or clarified the text to align with the existing citation (PMID: 34018319, PMID: 36563682, Ref. 11-12). Lines 88-91: Since the current citation does not mention RBPs, we have replaced the reference with two articles that specifically supports the role of RBPs in gene regulation through post-translational modifications (PMID: 38679071, PMID: 34048572, Ref. 13-14). Line 96: We agree that Reference 13 may not be representative of the claim. We have identified a more appropriate and widely recognized reference to replace it (PMID: 38302461, Ref. 16). Lines 568-571: The terminology regarding pre/post-translational vs. pre/post-transcriptional regulation has been carefully reviewed. Given that DNA methylation and histone modifications primarily influence transcriptional regulation, we have corrected the phrasing accordingly (line 605). We appreciate the reviewer's keen observations, which have significantly improved the accuracy and rigor of our manuscript. Thank you for your valuable feedback.

The misspelling of 'rachemys' in Extended Figure 1D should be 'trachemys'. The spelling of 'score' at ordinate in Extended Figure 1L is incorrect. The spelling of 'cotransfection' and 'extraction' in Figure 4A is wrong. The spelling of 'control' is wrong in the vertical characters in Figure 4H. Incorrect spelling of 'Methylated-RIP' in Figure 6D. The 'spliceosome' for K234M in Figure 6I is misspelled. The spelling of 'MATR3' in Figure 7I and J is incorrect. Misspelled title in Extended Figure 3E. Misspelling of 'CASPASE' in Extended Figure 3M. Incorrect spelling of 'CASPASE' in Extended Figure 3I. The spelling of 'DOTAP' and 'MATR3' in Figure 8A is incorrect. The spelling of 'MATR3' in Figure 8B is incorrect. The spelling of 'MATR3' in Figure 8D, E is incorrect. The spelling of 'MATR3' in Figure 8F is incorrect. The spelling of 'score' in Figure 8M is incorrect. The spelling of 'MATR3' in Extended Figure 4A, C is incorrect. Line 641-642: obvious semantic error. A large number of formatting errors exist in the 'Materials and Methods' part, which are not pointed out here. Line 367: DAS: Differential Alternative Splicing. Line 915: DEGs: Differential Expressed Genes. The word 'genome' in the title of Line 322 is inaccurate and may need to be replaced with 'transcriptome'.

Response: We sincerely appreciate the reviewer's meticulous attention to detail in identifying spelling, formatting, and terminology errors throughout the manuscript. We have carefully revised the manuscript to correct these issues, ensuring accuracy and clarity. Specifically, we have made the following corrections: Corrected 'rachemys' to 'trachemys' in Extended Figure 1D. Fixed the spelling of 'score' on the ordinate in Extended Figure 1L (Extended Figure 1M). Corrected misspellings of 'cotransfection' and 'extraction' in Figure 4A. Fixed the spelling of 'control' in the vertical axis labels of Figure 4H (Figure 4E). Corrected the misspelling of 'Methylated-RIP' in Figure 6D. Fixed the spelling of 'spliceosome' for K234M in Figure 6I. Corrected the spelling of 'MATR3' in Figures 7I and 7J. Fixed the misspelled title in Extended Figure 3E. Corrected the spelling of 'CASPASE' in Extended Figures 3M and 3I (Extended Figures 6M and 6I). Fixed the spelling of 'DOTAP' and 'MATR3' in Figure 8A (Extended Figures 7A). Corrected 'MATR3' in Figures 8B, 8D, 8E, and 8F (Figures 8A, 8C, 8D and 8E). Fixed the spelling of 'score' in Figure 8M (Figure 8L). Corrected 'MATR3' in Extended Figures 4A and 4C (Figures 8M and Extended Figures 7B). Lines 641-642: Addressed the semantic error to ensure clarity (line 693-696). Formatting Errors in Materials and Methods: We conducted a thorough review of this section and corrected inconsistencies and errors. Line 367: Corrected DAS as Differential Alternative Splicing (line 390). Line 915: Corrected DEGs as Differentially Expressed Genes (correcting the terminology) (line 999). Line 322: The title was modified to replace 'genome' with 'transcriptome', ensuring accuracy in describing the study's scope (line 344 and 1020). We greatly appreciate the reviewer's effort in identifying these errors, which has allowed us to refine the manuscript further. Thank you for your valuable feedback!

The number of up/down-regulated genes described in Line 277-278 is opposite to that labeled in Extended Figure 2A. The red and blue dots in Extended Figure 2C do not all conform to the Fold changes > 2 mentioned in Line 282. The TNFRSF1 mentioned in Line 294 is not seen in the corresponding Figure 4H and 4I. The CDK1, CDK2, and CDK6 mentioned in Line 470 only have CDK1 in their corresponding Extended Figure

3L. Line 578: 'genomic aberrant splicing'. That's not appropriate. Line 937: 'genome' should be 'transcriptome'. Line 631: 'on three-dimensional genome structure' is a bit overstated. Line 927, 931 mentioned that 'the gene names were highlighted in bright color', but the figure did not see the gene names with bright color.

Response: We sincerely appreciate the reviewer's careful examination of our manuscript and figures. We have thoroughly reviewed the identified discrepancies and made the necessary corrections to ensure consistency and clarity. Specifically, Lines 277-278: The number of up/down-regulated genes has been corrected to match the data presented in Extended Figure 2A (line 291, Extended Figure 3A). Extended Figure 2C: The red and blue dots have been re-evaluated, and the Fold change > 2 threshold mentioned in Line 282 has been checked for accuracy. Any inconsistencies have been corrected either in the figure or in the text (line 295, Extended Figure 3D, Fold change>1.5). Line 294: In fact, TNFRSF10A was incorrectly labeled as TNFRSF1, and the relevant content has been corrected (line 313). Thank you for your careful pointing out. Line 470: The mention of CDK1, CDK2, and CDK6 was inconsistent with Extended Figure 3L, where only CDK1 appears. We have revised the text for accuracy (line 503). Line 578: The term 'genomic aberrant splicing' was deemed inappropriate. We have revised this phrase to more accurately reflect the biological process described (line 612). Line 937: 'Genome' has been replaced with 'transcriptome' for accuracy (line 1020). Line 631: The phrase 'on three-dimensional genome structure' was an overstatement. We have modified this claim to better align with the study's findings (line 681-685). Lines 927 and 931: The article mentioned that the gene name was highlighted in bright colors, which actually refers to orange, and we have revised the text (line 1005 and 1009). We appreciate the reviewer's keen attention to these details, which has helped improve the accuracy and clarity of our manuscript. Thank you for your valuable feedback!

Minor:

1. Do the authors know the reasons why EZH2 expression is increased under TBHP

conditions, and whether its transcriptional level is increased?

Response: Our experimental results showed that the protein level of EZH2 increased in response to TBHP treatment. We hypothesized that this increase may be related to the cellular response to oxidative stress, especially under conditions of oxidative damage, and that EZH2 may act as an important regulator involved in cellular stress response, DNA repair, and transcriptional regulation. However, at present, we have not systematically investigated the specific mechanism of increased EZH2 expression, which may involve its regulation through transcription factors or other stress response pathways. As for EZH2 transcript levels, we have examined them by qRT-PCR, which showed a significant increase in EZH2 mRNA levels after TBHP treatment, suggesting that the upregulation of EZH2 expression may be driven by increased transcript levels. We will further supplement this experimental data in the revised manuscript. Thanks again to the reviewers for their valuable questions (Fig. 2I).

2. The information of pan-Kme antibody should be provided.

Response: We thank the reviewers for their valuable comments. We deeply regret that there is an error in the labeling in Figure 1G. In fact, we used a mono-methyl lysine antibody rather than a pan-Kme antibody. This marker has been corrected in a revised edition and details of the antibody are included in the Supplementary table 2. Thank you for your corrections and we will ensure that accurate information is provided in the revised manuscript

4. IF in Figure 1E should have quantitative results. Figure 4J results need to be quantified.

Response: With respect to the quantitative results in Figure 1E and 4J that you mentioned, we have noted this. Immunofluorescence images should indeed provide quantitative analysis to improve data rigor and reproducibility. Therefore, we provide

quantitative results from immunofluorescence experiments in Figure 1E (**Extended Figure 1G**) and include statistical analysis in a revised version that clearly shows differences between groups. Similarly, for the experimental results in Figure 4J, we carried out quantitative analysis (**Extended Figure 4F**) and provided relevant statistical data, so that the quantitative results of the experiment could be presented more clearly. We thank the reviewers for their meticulous suggestions and believe that these modifications will further enhance the scientific validity of the study and the persuasive power of the data.

6. The p53 and p21 bands in Extended Figure 2G are not in the right position.

Response: Thank you for your valuable feedback. We acknowledge that the p53 and p21 bands in Extended Figure 2G are not in the correct positions. We have carefully reviewed the figure and will provide a corrected version in the revised manuscript to ensure accuracy (**Extended Data Figure 3J**). We appreciate your thorough review and attention to detail.

7. Line 210: How does cell lysates perform plasmid transfection?

Response: We thank the reviewers for their valuable comments. There is indeed some ambiguity regarding the statement on line 210. We hereby clarify that cell lysates in the original article refer to the evaluation of cell lysates from HEK-293T cells transfected with specific plasmids using anti-Flag IP and anti-DDX1 IB techniques, and not to plasmid transfection of cell lysates. We have amended the relevant descriptions in light of this clarification and will present them exactly in the revised manuscript (**Line 221**).

8. Line374-375: Figure 6B can't reach such a conclusion.

Response: With respect to the conclusion of Figure 6B mentioned in lines 374-375, we understand the reviewer's concern and agree that the conclusion may go beyond what

is supported by the data itself. We have revisited the results of Figure 6B in the revised manuscript and revised the relevant conclusions (Line 395).

9. Line 635: Methylation should be about IMPROVING accessibility, not 'disrupting'.

Response: Regarding the statement on line 635, you pointed out that methylation should be described as "improving accessibility" rather than "disrupting", and we will modify this section to ensure that the description is more accurate and in line with existing theory (Line 688).

10. Line 990: Which isoform of MATR3 is not specified.

Response: With regard to the MATR3 isomer issue mentioned in line 990, it is true that in the original text, it is not clear which MATR3 isomer was used. We apologize for that. In the revised manuscript, we will explicitly specify that MATR3 truncated isoforms are used, ensuring accuracy and clarity of information (Line 1073).

Reviewer #4:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: Thank you for your valuable time and effort in reviewing this manuscript.

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: We sincerely thank you for your valuable time and effort to review this manuscript, which has greatly helped us to improve this manuscript.

Reviewer #6:

In their manuscript “DDX1 Methylation Mediated MATR3 Splicing Regulates Intervertebral Disc Degeneration by Initiating Chromatin Reprogramming” Zhu and colleagues report that monomethylation of DDX1 at lysine K234 by EZH2 drives NP cell degeneration and IVDD. They demonstrated that EZH2 expression is increased in IVDD and that EZH2 mediates methylation of DDX1 at K234. DDX1 methylation promotes gene expression programs related to senescence and apoptosis in NP cells. Mechanistically, DDX1 methylation reduces DDX1 binding to pre-mRNAs, often in proximity to splice sites, predominantly resulting in exon skipping events. One of the few mRNA targets bound to DDX1 that also underwent exon skipping in TBHP-treated NP cells was MATR3. DDX1 methylation consequently leads to exon 14 skipping in MATR3 resulting in a shorter isoform MATR3-S. Interestingly, while the long MATR3 (MATR3-L) isoform maintains a repressive chromatin state, MATR3-S generates a more relaxed chromatin state leading to the activation of senescence and apoptosis related pathways. Eventually, the authors showed that an LNP-mediated transfection of NP cells in vivo with the long MATR3 isoform can improve IVDD progression in the rat IVDD model.

This manuscript represents a comprehensive study of a novel epigenetic mechanism of IVDD progression. Starting from an unbiased approach of screening methylated proteins in NP degeneration, the authors identified a new molecular pathway that involves the methylation of the RNA binding protein DDX1 and its consequences on

global RNA splicing as well as the specific splicing of MATR3. They furthermore systematically characterized the effect of this novel MATR3 splice variant on the chromatin state and the expression of senescence and apoptosis related genes. This study therefore gives novel insights into IVDD progression as well as into a methylation-dependent epigenetic molecular pathway of an RNA binding protein that globally regulates splicing and chromatin states. Overall, the manuscript reports novel and significant findings that are of interest to a broader readership. However, there are some points that need further clarification before publication.

Response: Thank you for your thoughtful evaluation of our manuscript and for your valuable feedback. We appreciate your recognition of the novelty and significance of our findings, particularly in relation to IVDD progression and the methylation-dependent epigenetic regulation of RNA-binding proteins. Your insights and constructive comments are invaluable in refining our work. We acknowledge the points that require further clarification and will carefully address each of them to strengthen the manuscript. We truly appreciate your time and effort in reviewing our study and contributing to the peer-review process.

1.) The authors use only one siRNA/shRNA to target DDX1 or EZH2 (Figures 1,3,4,5,6). In order to rule out off-target effects, the authors should include a second siRNA/shRNA sequence at least in some (feasible) key experiments, such as in the in vitro models.

Response: To rule out the possibility of off-target effects, we designed and applied additional siRNA and shRNA targeting DDX1 and EZH2 for validation experiments, and new experimental data have been provided in the revised edition. We believe that these supplementary experiments (Extended Data Figure 2B-E, H-I) will further enhance the reliability of the findings and thank the reviewers again for their valuable comments.

2.) Moreover, in Table S3 the authors give the sequences of the si/shDDX1 as well as the si/shEZH2 oligos, stating that they are specific to human. Since the shRNAs targeting DDX1 as well as EZH2 were used in the in vivo rat model, the authors should comment on whether these human sequences can also target the respective rat genes.

Response: Thank you for the careful review of our work. Regarding the shRNA sequence of EZH2 and DDX1 you mentioned, in fact, in our previous study, we used the Basic Local Alignment Search Tool (BLAST) to align the sequences of EZH2 and DDX1 genes in humans and rats, and found that the conservation of DDX1 between the two species was 96.71%, and that of EZH2 was 95.09% (Supplementary Data 4). Based on the high conservation of these two genes, we designed different target sequences of siDDX1/shDDX1 and siEZH2/shEZH2 for the homologous fragments of DDX1 and EZH2 in different species, respectively, and finally determined that Homo: GGAUGAAUUUGAUGGGAAA/Rat: GGAUGGUACAUGUCAAAUC and Homo: AUACAUGUGCAGCUUUCUG/ Rat: GGAUGGUACUUUCAUUGAAGA (could target DDX1 and EZH2 in humans and rats, respectively. We deeply regret that the error of registering duplicates occurred in the previous table. In response to the reviewer's concern, we will correct the description of shDDX1 and shEZH2 sequences in the revised manuscript (Supplementary Table 3). In addition, to further exclude the possibility of off-target effects, we designed and applied additional siRNA#2 and shRNA#2 targeting DDX1/EZH2 for validation experiments, and have provided new in vivo and in vitro experimental data in the revised version (Extended Data Figure 2B-E, H-I). We believe that these additional experiments will further enhance the reliability of the research results. Thank you again for the reviewer's valuable comments.

3) On page 12, line 340: Shouldn't (FIG. 4E) read (FIG.5E)?

Response: As for the image number error mentioned in line 340, it is true that Figure 4E was mistakenly written as Figure 5E in the original text. We have noted this error

and have corrected it in the revised manuscript to ensure that the figure numbers are consistent with the actual content. We thank the reviewers for their careful review and help us improve the accuracy of the manuscript (Line 362).

4.) The figures and the arrangements of subfigures could be at times clearer. For instance, in Figures 1, 3, 4, 5 it would be clearer for the reader if the subfigures follow the same order and pattern in every figure (e.g. always in ascending order from left to right, and not up and down).

Response: Thank you for the reviewer's valuable suggestions. We fully agree with your opinion that the arrangement of sub-figures in a figure is very important to improve the clarity and readability of the figure. Regarding the order of sub-figures in Figures 1, 3, 4, and 5, we will readjust the arrangement of sub-figures in each figure in the revised manuscript to ensure that the order and pattern of sub-figures are consistent and arranged in a uniform ascending order from left to right. We believe that such a modification will make the figures easier to understand.

Thanks again to the editor and all reviewers for their time and patient consideration of this manuscript. We look forward to your feedback and maintain any further clarification.

Best regards,

Cao Yang

Thank you for your email and for the opportunity to revise our manuscript entitled "DDX1 Methylation Mediated MATR3 Splicing Regulates Intervertebral Disc Degeneration by Initiating Chromatin Reprogramming" for *Nature Communications*. We are very pleased to hear that the journal is happy, in principle, to accept our work.

We have carefully addressed all the editorial requests outlined in the Author Checklist and have thoroughly revised the manuscript to incorporate any remaining reviewer comments, as detailed in the follow point-by-point response. To facilitate the review process, we have highlighted (red) all modifications in the revised manuscript.

REVIEWER COMMENTS

Reviewer #1: The authors have adequately addressed my previous concerns. I think the details and the language in the manuscript are much improved. Great Work!

Response: We truly appreciate your thoughtful review and are grateful for your recognition of our efforts to improve the manuscript. Thank you for your support.

Reviewer #2 The authors addressed our concerns adequately.

Response: We appreciate your positive assessment and are glad that our revisions have resolved the concerns. Thank you again for your valuable input and support.

Reviewer #3: The authors have done a good job to address Reviewer's concerns. I recommend the work be accepted with minor revisions. The position of IgG-H is incorrectly marked in Fig. 2D, please correct it.

Response: We appreciate your careful review and helpful observation. We have

corrected the labeling of IgG-H in Figure 2D as suggested. The revised figure has been updated in the manuscript accordingly. Thank you again for your thoughtful input and support of our work.

Reviewer #4: I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: Thank you for your valuable review and support.

Reviewer #5: I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: Thank you for your valuable review and support.

Reviewer #6: The authors have addressed all concerns raised by this reviewer and made comprehensive revisions to improve the clarity and overall quality of the manuscript. The revised version can be accepted for publication.

Response: We truly appreciate your positive assessment and are grateful for your support and encouragement. Your feedback has been invaluable in strengthening the manuscript.

We appreciate the constructive feedback and support from both the editorial team and reviewers, which have helped us improve the quality of our work. We hope the revised version meets the journal's requirements and look forward to your final decision.

Sincerely,

Cao Yang