nature portfolio

Peer Review File

A male-essential miRNA is key for avian sex chromosome dosage compensation

Corresponding Author: Professor Henrik Kaessmann

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
This very interesting study addresses the phenomenon of dosage compensation in birds. Unlike in therian mammals, it has been known for many years that birds appear not to show dosage compensation of their sex chromosomes. In birds, males have two Z sex chromosomes (ZZ) and females have one Z and one W (ZW). The Z carries about 1,000 genes. The W has very few – about 28. This means that males, ZZ, have , in theory , twice the level of Z-linked gene expression than females. This has been shown at the mRNA many times in chicken tissues, and in other birds. However, there is evidence that a subset of Z-linked genes are dosage sensitive and are up-regulated from the single Z of females to match the two Z expression levels of males.
Yet, the molecular details of this process have been for many years unclear. This paper links the microRNA , miR2954, with chicken Z-linked dosage compensation. The authors report in vivo functional and evolutionary analysis of this microRNA. They find that this Z-linked miRNA has predicted targets that are Z dosage which genes, which are indeed up-regulated when tis miRNA is knocked out in chicken (and caused embryo lethality in males= presumably due to aberrant up-regulation of those Z-linked targets).
They conclude that miR2954 is key to avian sex chromosome dosage compensation, because it regulates dosage sensitive Z-linked target genes. The most interesting finding is that miR-2954 knockout had a sex-specific phenotype, causing embryo lethality in ZZ males. This implies a sex-specific function and the authors propose that it is involved in regulating Z-linked genes as part of the bird dosage compensation system.
The study is very interesting and it advances the field, with many novel aspects. It is very detailed and meticulous, with appropriate controls. The deep conservation of this miRNA among birds is very interesting (esp. in the “primitive” ratites). I believe that the study would be of broad interest to the readers of Nature.
I have the following questions that should be addressed.

1. The system of CRISPR/Cas genome editing of germ cells to delete miR2954 in elegant and has been published previously. Given the embryo lethality of the miR2954 KO (knockout) phenotype, can the authors be sure that they have targeted this locus only? Could there be off-targeting events? Does the repeated out-crossing definitely rule out off-targeting? Could the Z-targeting affected other parts of the Z chromosome, hence the male-specific mortality>

2. The lethality of miR2954 KO only in homozygous males (ZZ) is very striking. Could the authors consider why this is manifest after E7 and not before?

3. Can the authors show the normal endogenous expression pattern of miR2954? For example, using whole mount in situ hybridisation. There are good protocols for this, using LNA probes for small RNAs such as miRs.

4. RNA-seq was used to assess gene transcript abundance in WT, het and homozygous KO embryos (prior to the time of embryo lethality in the affected males). Targetscan was used to predict the potential targets of miR2954 and these were scrutinised for changes in the KO embryos (expected to go up, if miR2954 normally down-regulates them). I found this data very clear and convincing. Were there any genes that showed substantial down-regulation the miR2954 KO males?

5. Could the authors provide more information on the annotations of the dosage sensitive Z-linked genes affected by miR2954. What, exactly, do these genes do? Any common thread?

6. The hypothesis that avian sex chromosome evolution first involved Z “up-regulation” due to gene loss on the W , followed by necessary male-specific down-regulation in ZZ males is interesting but I don’t think the study addresses the first part of this scenario, only the second. It is stated in the Discussion: “We unveiled a scenario where, driven by the decay of the W chromosome, dosage sensitive genes 342 became upregulated on the Z chromosome not only in (ZW) females but, as a secondary consequence, also in (ZZ) males.” I think it is only the last part that the study has clearly demonstrated.

(Remarks on code availability)
None

Referee #2

(Remarks to the Author)
Dosage compensation evolves to balance the expression of hemizygous X or Z chromosome to that of autosomes, after the Y or W chromosome has become degenerated. Its mechanism has been well elucidated in Drosophila, C. elegans and mouse. And chicken, another important model organism representing the largest land vertebrate group was considered to have incomplete or gene-by-gene dosage compensation, as its Z chromosome exhibits a global lower expression level in females than in males, given the 2-fold dosage differences of gene copies between sexes. This work characterized a Z-linked microRNA, miR-2954, which the group identified in a previous work, and found it is responsible for biasedly downregulating many Z-linked dosage sensitive genes relative to autosomal targets. And they provided evidence that this is the consequence of evolution of upregulation of these Z-linked target genes in females. This supports the classic model of evolution of dosage compensation proposed by Ohno, that the first step of dosage compensation is to upregulate the X- or Z-linked gene expression in male or female, where these genes have become hemizygous. And the second step is to resolve the byproduct of the first step, i.e., the overexpression of these genes, by evolving a downregulating strategy. In the case of chicken, the second step seems to be achieved by a Z-linked microRNA.

The major advancement of this work (Figure 1-3) is the knockout of miR-2954, which I understand nowadays in chicken is still not as regular as in mouse. One significance result of the work is that this miR-2954 turns out to be essential (mutants die before embryonic day 7) and is broadly expressed throughout different tissues. This is consistent with its targets that are enriched for important dosage sensitive genes. However, one major issue with the work is that how much new conclusion can the reader find in the rest of the paper (Figure 4-5) compared to authors’ previous work in Genome Res (Warnefors et al. 2017). Thanks to the solid knockout work in the first part, this work has a more accurate list of target genes (248 genes that show upregulation upon knockout) by miR-2954, compared to the predicted target genes (47 genes conditioned on 8mer sites) by Warnefors et al. 2017. The standard of 8mer for screening targets was also used in Figure 4a of this work, and although with an additional requirement of multiple target site in this work, the conclusions is the same to that of Warnefors et al. 2017: that the targets are enriched on the Z. The conclusion of figure 4d of this work is also the same with figure 4d of Warnefors et al. 2017, that ohnologs are overrepresented among the Z-linked targets. And finally, that the miR-2954 is playing a conserved role across bird species, i.e., figure 5 of this work, is similar to figure 4e of Warnefors et al. 2017. In summary, I think Figure 1-3 of this work is experimentally new and confirms the function of miR-2954, but the rest figures 4-5 only exhibit minor improvements to what we have already known about miR-2954.

I also have other comments detailed as below:

1. Line 78, “...have only been partially and/or in part..” this sentence is redundant here, or the authors mean something else?
2. Figure 1c, the heterozygote mutant ZKOZ males seem to have a higher lethality rate at E7-13 compared to the females. Is this significant? How about the survival rate beyond E13? I wonder if the survival rate of heterozygote mutant is impacted at a later stage compared to the homozygote? Are there any organ or other types of defects shown by the homozygous mutant before E7?
3. Figure 2a is very interesting, that over 50% of the predicted Z-linked targets are impacted compared to only 3-5% autosomal predicted targets. It remains unclear when and how miR-2954 achieves such specificity against the Z-linked genes. Either the Z-linked genes increased their sensitivity (as suggested Figure 4a) by changing their sequence seeds, or autosomal targets decreased their sensitivities. It is unclear whether the latter has happened, without aligning the sequences against an avian outgroup. Another possibility is that the autosomal genes evolved secondary compensatory mechanisms to offset the downregulating effect of miR-2954. These possibilities should be tested and discussed, as besides becoming male specific, how miR-2954 achieves its specific regulation of Z-linked vs. autosomal genes is very critical during evolution of its role in dosage compensation.
4. Figure 4f, it is clear that miR-2954 targets are indeed upregulated during evolution, however their expression levels are still significantly lower than those of outgroup species. How to explain this, that it seems suboptimal for dosage compensation not being able to lift the expression level of hemizygous Z to that of autosome or compared to an outgroup? The same question applies below to the dosage sensitive genes on the Z, that also they have been upregulated, they are still male-biased in birds. Would that pose a challenge or deleterious effects?
5. It is very interesting to find that miR-2954 ortholog is also present in other birds. But without proper experimental data, one should be conservative to suggest it plays a similar function in emu, ostrich etc, and call it ‘highly conserved across birds’.

(Remarks on code availability)

Referee #3

(Remarks to the Author)
While sex chromosome dosage compensation mechanisms have been studied extensively in a few model organisms (mice, c. elegans, drosophila), the extent, mechanisms and importance of dosage compensation in birds has remained unclear. Here the manuscript reports a breakthrough discovery, showing that Z-chromosome dosage compensation is essential in birds and that it is mediated by a Z-linked miRNA. The main discovery is that deletion of miR-2954 in chicken results in male-specific lethality, suggesting that Z-dosage compensation is essential also in birds. A series of analyses in chicken with different genotypes are performed to show that miR-2954 preferentially targets Z-chromosomal coding genes and reduces their expression in male ZZ birds. Moreover, sequence conservation and an analysis performed in a different bird species, the zebra finch, suggest that the discovered mechanism is conserved across birds. Although previous studies had shown that miR-2954 is more highly expressed in males compared to females, its functional role in Z-dosage compensation is a major discovery. Overall, the manuscript is a pleasure to read and I mostly only have small suggestions how to improve it. However, given the central importance of the knock-out phenotype for the study, I would suggest to perform an additional experiment to show that miR-2954 can indeed rescue the male-specific lethality.

Major points

1. Although deletion of miR-2954 only affects a small region, it might still have effects of the host gene expression independently of miR-2954 function. Moreover, Cas9 might induce genomic rearrangements that are difficult to detect by genotyping. Given that host gene expression is affected by the deletion, it would be important to provide robust evidence that the phenotype is indeed due to absence of miR-2954. To inequivocally show that indeed miR-2954 is responsible for the observed phenotype, it would be useful to perform a rescue experiment, where miR-2954 is provided in trans.

Minor points

2. Although the expression pattern of miR-2954 has been investigated in previous studies, it would be helpful to report its expression pattern within the paper.

3. On p. 2 it is stated that miR-2954 is a "largely male-specific Z-chromosome-derived microRNA". I would suggest to be more precise here and state how much higher it is expressed in males, since it is not absent in females, as I understand?

4. It remains a bit unclear from the analyses what fraction of Z-linked genes are actually dosage compensated. This might be difficult to estimate given the difficulty to identify miRNA targets in general. However, a short discussion of this question would improve the manuscript.

5. In Fig. 4h, I would suggest to also mark the position where miR-2954 is located.

6. On p. 10 the conservation of miR-2954 across birds is analyses. It would be helpful to state how many bird species are included in the comparison that showed 100% sequence conservation.

7. Since they have identified a central function for miR-2954 the authors might consider giving it a second name that might be more easy to remember.

(Remarks on code availability)
I looked at the code only superficially, and it could use a bit better documentation.

Version 1:

Reviewer comments:

Referee #2

(Remarks to the Author)
This is a revised version of the authors' previous work that identified a male-biased miRNA that is responsible for preferentially downregulating the Z-linked genes in chicken, as a mechanism of dosage compensation. I appreciate the authors' efforts of answering my previous concerns and questions, after performing many additional analyses. My previous main cricitism was about the Figure 4-5, which are difficult to find enough novelty from their previous works of Warnefors et al. During this revision, the authors have mostly addressed my cristicism, although I think the pattern of Z-linked targeted genes still comprises a minor advancement, I found the pattern of female targeted genes interesting. Overall, I think this version represents a significant improvement compared to the previous version, and some minor revions are only needed. My detailed comments are as follows:
1. Let me summersize the novelty based on the answers from the authors to my first question, with some respective questions and comments. These questions are not expected to change the conclusion, but only I hope to refine their conclusions:
1) The Z-linked targeted genes exhibit distinict sequence features compared to autosomal taregets by miR-2954, including longer/more abundant target sites and higher seed-pairing stablity. This explains the specificity of miR-2954 to the Z rather than the autosomes, but it should be made clear that these are all predicted results. As the target genes here are derived from the KO experiment rather than the RNA-binding assays of miR-2954. In addition, when comparing these targets between autosomes and Z chromosomes, are they all direct targets (i.e., with predicted target sites).

2) In both transcription and translation levels, the authors found upregulation of female Z-linked genes relative to the ancestral expression level approximiated by mouse, particularly the dosage sensitive genes. This as far as I understand was not presented in the previous Warnefors et al's work, and is an very important evidence for Ohno's model on how dosage compensation might have evolved.
3) The authors extended evolution analyses to about 300 bird genomes and revealed the precursor sequence of miR-2954 is highly conserved, but absent in the outgroup species of birds. This suggests the origination of this miRNA accompanied that of bird species and the ZW sex system. This is also an important finding, although this only predicts the conserved function, but is sufficient for the authors to make a suggestive claim.
I think authors' claim 'advancing well beyond Warnefors et al. in every section of our paper' is an overstatement, and authors' conclusion at the begnining 'extend and refine the insights' is a more precise description.
2. For the previous question 3, if I understand it correctly, the authors suggested that Z-linked genes aquired sequence contexts that enabled them more frequently being targeted by the miR-2954, without too much changes to the autosome genes, is this correct? If so, one would predict that compared to outgroup species that lacks such a dosage compensation mechanism, target sequence changes more often and more dramatically occurred to between orthologs of avian Z linked genes vs. their reptilian/mammalian couterparts, relative to those autosomal ortholog pairs. Is that so?
3. Again, for question 4, I think the new Ribo-seq analyses comprise a great effort clarifying dosage compensation effects at the transcription vs. translation level, which eventually may achieve a complete compensatory effect. I appreciate the authors' work in doing this.

(Remarks on code availability)

Referee #3

(Remarks to the Author)
The revised version of the paper has comprehensively addressed the previous concerns. I just have a few minor points (see below) and I support acceptance of this article.

1. I really appreciate the effort for orthogonal validation using a knock-down approach. The description in the results section so very vague ("using a specific inhibitor and delivery agent"). It would be helpful to describe the nature of the "inhibitor".

2. Some labels in the extended data figures are very small. A bigger font size would make them more accessible (e.g. EDFig. 5a)
3. In Fig. 2a or in the corresponding part of the text, it would be useful to state how many expressed genes are on the Z chromosome in total, to better understand what fraction of all genes are predicted to be miR-2954 targets, of which then later ~50% are upregulated in the knock-out.

(Remarks on code availability)
The Github repository is missing a readme file.

Referee #4

(Remarks to the Author)
The manuscript describes a novel mechanism of dosage compensation through the evolution of an avian miRNA (miR-2954) that preferentially targets mRNAs on the Z chromosome. This mechanism has several potential advantages, including enhanced specificity (i.e. targeting only dosage-sensitive genes rather than silencing the entire sex chromosome). The authors generate a miR-2954 knockout chicken and describe both gross and molecular phenotypes. miR-2954 is male essential, and homozygous knockout results in completely penetrant male embryonic lethality. Furthermore, this miRNA is expressed at much higher levels in male chickens, enabling its male-specific role. Predicted targets of miR-2954 accumulate in the knockout animal, and Z-linked targets experience a much greater degree of derepression than autosomal targets. Further examination of predicted targets shows that Z-linked targets are predicted to be more strongly repressed by miR-2954. Experimentally supported Z-linked targets are more likely to be dosage sensitive and are more broadly expressed compared to Z-linked nontargets. Evolutionarily, the authors show that Z-linked miR-2954 targets have been upregulated in females at the transcriptional level, highlighting the need for the concurrent evolution of some method of repression in males. Moreover, Z-linked target genes are more similarly expressed between male and female chickens than Z-linked nontarget mRNAs. Finally, the authors show that this mechanism of dosage compensation occurs across bird species but does not extend to other closely related species (such as crocodile). With respect to miRNAs, this work elevates the known importance of miRNA-mediated regulation and describes the most severe phenotype of a recently emergent miRNA. With respect to dosage compensation, the discovery of this essential component of avian dosage compensation fills a critical gap in the field and brings the understanding of dosage compensation in birds closer to the much more intensively studied mechanisms of mammals, flies, and worms. (Although one reviewer is concerned that the idea of miRNA-mediated dosage

compensation and some informatics supporting this idea had been published earlier, the KO phenotype and the molecular analyses enabled by the KO are major advances that will elevate the interest in this study far beyond that of the previous one.)

Minor concerns:
1. Line 99. miR-2954 is expressed from an intron of XPA, but not all miRNAs expressed from introns are mirtrons, and miR-2954 is not a mirtron. For mirtrons, the splicing machinery excises the pre-miRNA in a form that it can be processed by Dicer without need for processing by Drosha. In contrast, miR-2954 appears to be a standard Drosha-dependent miRNA.

2. Extended Data Fig. 1. The authors might mention in the legend that miR-1583 (also shown in the figure) is not in the miRGeneDB database and thus is not a confidently annotated microRNA.

3. Figure 1a. It would be helpful to indicate when the different sex chromosomes emerged. As the figure currently stands, the XY chromosomes of lizard and human appear to be ancestral to the tree, when in fact they presumably emerged independently from different autosomes of their common ancestor.

4. Lines 179-180. As currently phrased, this sentence implies that different miRNAs have different prominent modes of action. In reality, all of the miRNAs seem to have the same prominent mode of action, which is to accelerate poly(A)-tail shortening of most of their targets. In most contexts, this tail shortening causes more rapid decapping and degradation, but in some contexts (most notably, pre-gastrulation embryos) tail shortening lowers translational efficiency. A suggested edit would be to delete the word “most” and just say “… the predominant mode of action of microRNAs in most contexts.”

5. Figure 2A. The authors might consider plotting the data on the left as cumulative distribution functions. The authors should also compare their predicted targets to UTR-length-matched cohorts of nontarget mRNAs instead of comparing to all nontarget mRNAs (as we know predicted miRNA targeting correlates with UTR length.) Additionally, from the legend, it is unclear how the data from E3 and E5 were combined.

6. Figure 2; Figure 4a. When discussing these and other data indicating weaker sites on autosomes, the authors might want to include in their model the idea that autosomal mRNAs have presumably evolved to avoid miR-2954 targeting, with the result that the seed matches that remain in autosomal 3’UTRs are minimally effective. Indeed, this selective avoidance of autosomal targeting could be shown in an expanded version Figure 5a.

7. Figure 4e; Lines 283-290. A brief discussion of interpretation of the Tau score would be helpful.

8. Figure 4f, Extended Data Fig. 5b, c. The description of the grey boxes is missing in the figure legend. More broadly, the implications of the red and grey boxes are confusing. For example, it seems the reader is meant to interpret $p>0.05$ for a log2(Current-Z/Proto-Z) of -1 (red box) and $p<0.05$ for log2(Current-Z/Proto-Z) of 0 or 1 (grey boxes) as log2(Current-Z/Proto-Z) being most similar to -1. This interpretation is incorrect, as $p>0.05$ cannot be used to assert that the value is equal to -1 but rather that it is not likely to be different from -1. The authors might consider making the direct comparison between the Z-nontargets and Z-targets within each tissue. This seems like a more appropriate comparison to demonstrate upregulation specific to Z-linked target genes. Alternatively, the authors could use an explicit equivalence test (ex. TOST test).

9. Figure 4h. Why are there two red vertical lines?

10. Lines 376-377. This sentence might be misread to be saying that the increased translation is global, occurring for mRNAs from all chromosomes, when in fact the increased translation is specific to mRNAs from Z.

11. Figure 5a. Although drawing miRNAs as pairing at both their seed and 3’ regions is not uncommon in these types of figures, it is not an accurate depiction of typical miRNA targeting. All of the targeting in this study was dominated by seed pairing, with little or no contribution of 3’ pairing. Indeed, equal pairing at both the seed and 3’ regions causes degradation of the miRNA, not the mRNA.

12. Figure 5c, 5d. The legend could be clarified. For example, “Proportions of miR-2954 target genes on autosomes (Pre-A) and Z chromosome (Pred-Z) in chicken that are shared between chicken and zebrafinch.”

13. Line 446. This statement should be softened, by for example, adding the word “presumably” before “because.”

14. Lines 448-450. The logic here is a bit mixed up. First, Ref. 15 shows that plenty of single-miRNA knockouts have strong or lethal phenotypes. Secondly, miR-2954 is the only member of its family, and so from this perspective the lethal phenotype is not remarkable. However, what is remarkable is that the miR-2954 KO is (to my knowledge) the most severe knockout phenotype of a miRNA that has emerged since the last common ancestor of amniotes.

(Remarks on code availability)

Referee #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

(Remarks on code availability)

Referee #6

(Remarks to the Author)
Upon request from the editor, I evaluated the resubmission and authors' response to review by a previous reviewer (reviewer 1) who was unavailable for a response. The reviewer raised several constructive questions/comments on the previous version that the authors' addressed extensively in their revision (see below). This lead to refined and additional analyses resulting in improvement of the manuscript and stronger support of the conclusions drawn. The paper is based on good methodology, data quality, and analyses. It is well written and presented. All the comments of Reviewer 1 are sufficiently and well addressed.

Overall, this very interesting manuscript "A male-essential microRNA is key for avian sex chromosome dosage compensation" addresses the phenomenon of dosage compensation mechanisms in birds (ZZ/ZW, male/female sex chromosome system). It convincingly shows that a microRNA, miR2954 with strongly males-biased expression levels is essential for Z-linked dosage compensation. The paper blends functional/mechanistic in vivo studies with an evolutionary analyses.
First, it shows that knock-out of miR-2954 in chicken using Crisp/Cas technology results in very high, male-specific early-stage embryo mortality in knockout homozygotes, and, that this likely results from specific upregulation (disinhibition) of dosage-sensitive Z-linked target genes. Second, an analysis of the phylogenetic distribution of miR-2954 suggests that dosage-sensitive target genes on the female Z-chromosome underwent transcriptional and translational upregulation. From this the authors propose a somewhat speculative scenario in which evolutionary pressures resulting from W-gene loss selected for transcriptional and translational upregulation of dosage-sensitive Z- genes in females; the also upregulated transcription in males produced excess transcripts from their two gene copies (ZZ) which then is cancelled by the degradation of transcripts via miR-2954. This microRNA-mediated sex chromosome dosage compensation mechanism became critical for male embryo survival.

Below I comment on the authors' response to the main comments/questions of previous reviewer 1.

1. The system of CRISPR/Cas genome editing of germ cells to delete miR2954 in elegant and has been published previously. Given the embryo lethality of the miR2954 KO (knockout) phenotype, can the authors be sure that they have targeted this locus only? Could there be off-targeting events? Does the repeated out-crossing definitely rule out off-targeting? Could the Z-targeting affected other parts of the Z chromosome, hence the male-specific mortality>

The authors address this comment quite well. They argue that their repeated outcrossings and absence of a phenotype in heterozygous knockout males and hemizygous knock-out females strongly rule out off-target effects on the autosomes; to rule out off-target effects on closely linked regions on Z, they conducted deep long-read genome sequencing of knockout males and controls. This analyses reveal no evidence for off-target effects and are included in the revision.
The authors point out that their revision also includes new results from an orthogonal in vivo knockdown approach which shows that miR-2954 is essential for male embryo survival supporting the knockout results sex-specific embryo mortality. Moreover, additional new assessment of annotation of the dosage-sensitive Z-linked target genes reveal critical roles in development (and adulthood) that may be functional linked to the male embryo lethality.

2. The lethality of miR2954 KO only in homozygous males (ZZ) is very striking. Could the authors consider why this is manifest after E7 and not before?

In response to this query, the resubmission includes a paragraph/data that address the high male embryo mortality of miR2954 knockouts before ED 7. Reported developmental abnormalities from upregulation of the dosage-sensitive, Z-linked genes include delayed overall growth, small eyes/brains, stunted elongation (Supp. Data 1 and 2) suggesting critical functional roles in early developmental processes (development of e.g. vasculature, allantois, organs) whose disruption is lethal.

3. Can the authors show the normal endogenous expression pattern of miR2954? For example, using whole mount in situ hybridisation. There are good protocols for this, using LNA probes for small RNAs such as miRs.

The authors refer to their statement in the Introduction of published small RNA sequencing and Northern blot work showing elevated miR-2954 expression in adult organs and whole male embryos. In addition, they refer to their RT-qPCR quantification of the miRNA in E12 embryos.

4. RNA-seq was used to assess gene transcript abundance in WT, het and homozygous KO embryos (prior to the time of embryo lethality in the affected males). Targetscan was used to predict the potential targets of miR2954 and these were scrutinised for changes in the KO embryos (expected to go up, if miR2954 normally down-regulates them). I found this data very clear and convincing. Were there any genes that showed substantial down-regulation the miR2954 KO males?

The authors respond that 99+% of the differentially regulated Z-genes in knockdown homozygous miR 2954 males were up-regulated, while this fraction was much lower for autosomal targets. Only 4 genes were down-regulated (reported in Table 4) and that they prefer to not speculate about this small number of genes any further.

5. Could the authors provide more information on the annotations of the dosage sensitive Z-linked genes affected by miR2954. What, exactly, do these genes do? Any common thread?

In response to this query, a new analysis was performed and results are presented on page 10 (Suppl. Table 7). Functionally related target genes appear to play roles in development, multiple essential pathways, stem cell pluripotency, etc.. This response is sufficient and links well to the observed embryo lethality in knockout miR2954 homozygous males.

6. The hypothesis that avian sex chromosome evolution first involved Z “up-regulation” due to gene loss on the W , followed by necessary male-specific down-regulation in ZZ males is interesting but I don’t think the study addresses the first part of this scenario, only the second. It is stated in the Discussion: “We unveiled a scenario where, driven by the decay of the W chromosome, dosage sensitive genes became upregulated on the Z chromosome not only in (ZW) females but, as a secondary consequence, also in (ZZ) males.” I think it is only the last part that the study has clearly demonstrated.

The authors respond extensively to this critique arguing that their work already included some of the support for the first part of their hypothesis but that they now expand their analysis: They argue that 1) their previously published work revealed an overall partial transcription upregulation in females; 2) in the present study they compare present-day Z-chromosome gene upregulation by miR2954 to those of an inferred ancestral state Z-chromosome and find Z-linked target genes of miR2954 being upregulated in female organs; 3) also newly included analyses using ribosome profiling (Extended data Fig. 5) show translational upregulation in females. These findings are presented in lines 365 – 378.
Together, I feel that this provides sufficient support for the authors’ presented 2-step scenario for the evolution of this dosage compensation mechanism in birds.

(Remarks on code availability)
This is beyond my expertise.

We would like to thank the editor and the reviewers for their positive feedback on our work and the very useful and constructive comments, which helped us to extend and refine our original findings and overall improve our manuscript.

Please find below a summary of the major additions to the study and manuscript:

- We performed deep long-read genome resequencing of miR-2954 in male knockouts (KOs) and controls, selectively enriching for Z-chromosomal sequences. This analysis confirmed the miR-2954 deletion in the KOs and revealed no additional structural changes. Together with our repeated outcrossings, these findings rule out off-target edits on both the autosomes or Z chromosome in KO animals.
- We validated the male essentiality of miR-2954 and its mechanism of action using an orthogonal in vivo knockdown (KD) approach. Specifically, male KD embryos showed significantly higher mortality than female KD embryos and negative controls, and we observed upregulation exclusively of miR-2954 target genes and the *XPA* host gene (likely indirectly regulated by miR-2954), mirroring the findings from our KO experiments.
- We assessed in detail the broad, strongly male-biased expression of miR-2954 in developmental tissues using RT-qPCR.
- Leveraging extensive new ribosome-profiling (Ribo-seq) data, we showed that Z-linked miR-2954 target genes in females have undergone upregulation at both the transcriptome and translatome layers of gene expression during evolution. This restoration of ancestral (proto-sex chromosomal) expression levels in females results in more similar—or even fully balanced—expression levels between the sexes.
- Using Ribo-seq data, we also showed that the pronounced transcriptional Z-target upregulation in male KOs is also evident in the translatome, indicating that the loss of miR-2954 in the homozygous KO males is not mitigated by translational buffering and indeed leads to increased Z-linked protein abundances.
- Functional characterization of Z-linked miR-2954 target genes revealed that these genes are not only broadly expressed and overall dosage-sensitive but also play critical roles in both development and adulthood. Combined with our new estimate that miR-2954 regulates ~54% of all developmentally expressed Z-linked genes, these findings provide a compelling explanation for the severe (lethal) phenotype observed in homozygous male KOs.
- We showed that the selective targeting of Z-linked genes is driven not only by longer and more numerous target sites in their 3'-UTRs compared to other genes, but also by factors like increased seed-pairing stability, more favorable local sequence context, and optimal target site positioning.
- We have now extended our miR-2954 conservation analyses to all 363 available bird genomes, which revealed that its mature sequence is identical across birds and its precursor sequence is fully conserved, with the exception of two species, each with a single single-nucleotide substitution. This observation highlights that miR-2954 has been strongly preserved by purifying selection during avian evolution.
- New comparative genome analyses with non-avian reptiles and mammalian outgroups revealed that miR-2954 target sites have accumulated specifically in the UTRs of Z-linked target genes during avian evolution, following the emergence of miR-2954, and have since been preferentially preserved compared to (largely spurious) sites in other genes.

Below, we address each reviewer's comment individually.

Referee #1 (Remarks to the Author):

This very interesting study addresses the phenomenon of dosage compensation in birds. Unlike in therian mammals, it has been known for many years that birds appear not to show dosage compensation of their sex chromosomes. In birds, males have two Z sex chromosomes (ZZ) and females have one Z and one W (ZW). The Z carries about 1,000 genes. The W has very few – about 28. This means that males, ZZ, have , in theory , twice the level of Z-linked gene expression than females. This has been shown at the mRNA many times in chicken tissues, and in other birds. However, there is evidence that a subset of Z-linked genes are dosage sensitive and are up-regulated from the single Z of females to match the two Z expression levels of males.
Yet, the molecular details of this process have been for many years unclear. This paper links the microRNA , miR2954, with chicken Z-linked dosage compensation. The authors report in vivo functional and evolutionary analysis of this

microRNA. They find that this Z-linked miRNA has predicted targets that are Z dosage which genes, which are indeed up-regulated when tis miRNA is knocked out in chicken (and caused embryo lethality in males= presumably due to aberrant up-regulation of those Z-linked targets).
They conclude that miR2954 is key to avian sex chromosome dosage compensation, because it regulates dosage sensitive Z-linked target genes. The most interesting finding is that miR-2954 knockout had a sex-specific phenotype, causing embryo lethality in ZZ males. This implies a sex-specific function and the authors propose that it is involved in regulating Z-linked genes as part of the bird dosage compensation system.
The study is very interesting and it advances the field, with many novel aspects. It is very detailed and meticulous, with appropriate controls. The deep conservation of this miRNA among birds is very interesting (esp. in the "primitive" ratites). I believe that the study would be of broad interest to the readers of Nature.
I have the following questions that should be addressed.

We are very grateful for the referee's positive feedback and enthusiasm regarding our work as well as for their very helpful specific comments, which we address below.

1. The system of CRISPR/Cas genome editing of germ cells to delete miR2954 in elegant and has been published previously. Given the embryo lethality of the miR2954 KO (knockout) phenotype, can the authors be sure that they have targeted this locus only? Could there be off-targeting events? Does the repeated out-crossing definitely rule out off-targeting? Could the Z-targeting affected other parts of the Z chromosome, hence the male-specific mortality>

Our repeated outcrossings – as well as the lack of a phenotype in heterozygous KO males and hemizygous KO females (Page 4, Lines 139-142) – effectively rule out potential off-target edits on the autosomes, but not necessarily for closely linked regions on the Z chromosome.

We therefore performed deep long-read genome resequencing of miR-2954 in male knockouts (KOs) and controls, selectively enriching for Z-chromosomal sequences, yielding ~10-32 fold coverage for this chromosome (Extended Data Fig. 2a,b). Analyses of these data confirmed the miR-2954 deletion in the KOs and revealed no additional structural changes (Extended Data Fig. 2c). Together with our repeated outcrossings and the lack of a phenotype in heterozygous KO males and hemizygous KO females, these findings rule out off-target edits on both the autosomes or Z chromosome in the KO animals. These new data and analyses are detailed in the revised manuscript:

Page 3, Lines 116-124 "Our KO strategy enabled us to compare phenotypic effects among outbred sibling birds of the different genotypes, thereby minimizing variability in the genetic background. Our repeated outcrossing effectively ruled out potential off-target edits on the autosomes, as any such mutation would need to be closely linked to the Z-chromosomal miR-2954 deletion. To test the latter possibility, we performed deep long-read genome resequencing of five homozygous KO males and four wild-type controls using an adaptive sampling procedure[23] that selectively enriched for Z-chromosomal sequences, yielding ~10-32 fold coverage for this chromosome (Extended Data Fig. 2a,b) (Methods). Analyses of these data detected only the intended 36 base pair (bp) miR-2954 deletion in the KO animals, with no additional structural variation between KOs and controls, confirming the absence of off-target edits (Extended Data Fig. 2c) (Methods)."

Notably, motivated by comments from Referee #3, we have now also validated the male essentiality of miR-2954 and its mechanism of action using an orthogonal (non-genome editing) *in vivo* knockdown (KD) approach (Page 8, Lines 236-249). Specifically, male KD embryos showed significantly higher mortality than female KD embryos and negative controls (Extended Data Fig. 7a,b), and we observed upregulation exclusively of miR-2954 target genes and the *XPA* host gene (likely indirectly regulated by miR-2954) (Extended Data Fig. 7c), mirroring the findings from our KO experiments.

Finally, motivated by this reviewer's comment #5 (see details below), we carefully assessed the functional annotations of the dosage-sensitive Z-linked target genes of miR-2954, which revealed that they not only broadly expressed and overall dosage-sensitive but also play critical roles in both development and adulthood (Page 10, Lines 292-300; Supplementary Table 7). Combined with our new estimate (made in response to comment #4 by referee #3) that ~54% of all Z-linked genes may be affected in the miR-2954 KO (Page 10, Lines 302-316; Extended Data Fig. 8, Supplementary Table 3), these findings provide a compelling explanation for the severe (lethal) phenotype observed in homozygous male KOs.

2. The lethality of miR2954 KO only in homozygous males (ZZ) is very striking. Could the authors consider why this is manifest after E7 and not before?

Our analyses show that by embryonic day 5 (E5), over 60% of male KO embryos had died, with complete mortality by E7–E13 (Fig. 1c–e). This finding indicates that depletion of miR-2954, which is highly and broadly expressed (see also response to comment #3 below), has highly deleterious effects early in embryonic development. As noted above (comment #1) and discussed further below (comment #5), the upregulated Z-linked targets of miR-2954 include both broadly and tissue-specifically expressed dosage-sensitive developmental genes. Their upregulation in KOs likely causes cumulative deleterious effects leading to lethality by E5, a crucial period marked by an increase in blood circulation, rapid embryonic growth, and differentiation of essential structures such as the allantois and vasculature. Disrupting these processes—whether cardiovascular development, metabolic regulation, organogenesis timing, or cell differentiation—could individually or collectively account for the observed male KO lethality. Interestingly, female *Xist* KO mice exhibit lethality between E5 and E12 (Marahrens et al., *Genes Dev.* 1997; PubMed ID: 9009199), resembling approximately the developmental window in which miR-2954 KOs die in this study.

We now added a paragraph that summarizes theses notions as well as our microscopic KO phenotypic observations at the end of the section in which we assess the functions of miR-2954 targets:

Page 10-11, Lines 318-328 "Consistent with the broad spatiotemporal expression and diverse developmental functions of miR-2954 targets, $Z^{KO}Z^{KO}$ embryos exhibit variable developmental abnormalities before E7, including delayed growth, small eyes and brains, and stunted axial elongation, with many embryos fully degraded by later stages (Supplementary Data 1 and 2). The upregulation of dosage-sensitive Z-linked developmental genes in KOs likely leads to cumulative deleterious effects, contributing to lethality by E5 – a critical phase involving an increase in blood circulation, rapid growth, and differentiation of key structures such as the allantois and vasculature. Disruptions in these processes, whether affecting cardiovascular development, metabolic regulation, organogenesis timing, or cell differentiation, likely underlie the observed lethality. Notably, the timing and variability of the miR-2954 KO phenotypes resemble those observed in *Xist* knockout mice, where loss of this lncRNA – responsible for secondary dosage compensation in placental mammals – also leads to lethality in a similar developmental window[43] (i.e., between E5 and E12)."

3. Can the authors show the normal endogenous expression pattern of miR2954? For example, using whole mount in situ hybridisation. There are good protocols for this, using LNA probes for small RNAs such as miRs.

We and others previously showed that miR-2954 is approximately 5-10 times more highly expressed across various adult organs and whole embryos using small RNA sequencing (Warnefors et al. *Genome Res.* 2017; Pubmed ID: 29079676) and Northern blots (Zhao et al. Nature 2010; Pubmed ID: 20220842), respectively. We now more clearly state this in the introduction (also motivated by comment #3 of referee #3):

Page 2-3, Lines 86-92 "Notably and consistently, we recently characterized a Z-chromosome-derived microRNA (miR-2954) that is largely male-specific (with expression levels ~5-10 times higher in male than female organs and whole embryos[1,14]) and whose predicted target genes…".

We had originally also assessed its expression, together with that of other microRNAs, using small RNA sequencing in three male tissues at E5 (Page 7, Lines 214-217; Fig. 3a,b).

Additionally, to address this reviewer's comment, we used RT-qPCR to quantify miR-2954 expression across seven organs from both sexes at E12. This analysis revealed 7- to 15-fold higher expression in males than in females, further emphasizing its high, broad and strongly male-biased expression (Fig. 3c). We refer to these new data in manuscript when interpreting the female KO expression patterns compared to those of male KOs:

Page 7, Lines 219-223 "When investigating gene expression in female hemizygous ($Z^{KO}W$) embryos, we again find a specific upregulation of Z-linked target genes compared to wild-type controls, but the pattern is even substantially weaker than that for male heterozygous ($Z^{KO}Z$) embryos (Fig. 2c), in accord with the very low (i.e., 7- to 15-fold lower)

expression of miR-2954 in females when compared to males[1,28] (Fig. 3c; see above) and the absence of a deleterious phenotype in $Z^{KO}W$ female embryos."

4. RNA-seq was used to assess gene transcript abundance in WT, het and homozygous KO embryos (prior to the time of embryo lethality in the affected males). Targetscan was used to predict the potential targets of miR2954 and these were scrutinised for changes in the KO embryos (expected to go up, if miR2954 normally down-regulates them). I found this data very clear and convincing. Were there any genes that showed substantial down-regulation the miR2954 KO males?

As described in the manuscript (Page 5, Lines 181-192), nearly all (~99–100%, depending on the tissue) of the predicted Z-target genes that are significantly differentially expressed in male homozygous KOs are upregulated, whereas this fraction is substantially lower (38–54%) among predicted autosomal targets. The only exceptions among the predicted Z-linked targets are four genes: ENSGALG00010013515, *RBP4* (head); ENSGALG00010012726, *IL7R* (body); ENSGALG00010005790, *MUSK* (heart); and ENSGALG00010016463, *FREM* (heart) (see Supplementary Table 4 for all individual gene expression changes in KOs vs. wild types). One possibility is that these genes are not direct miR-2954 targets but rather indirect targets whose downregulation results from the upregulation of a transcriptional silencer in KOs. However, we prefer to not speculate in the manuscript regarding the reasons for this small number of downregulated cases.

5. Could the authors provide more information on the annotations of the dosage sensitive Z-linked genes affected by miR2954. What, exactly, do these genes do? Any common thread?

Thank you for this comment, which prompted a more in-depth evaluation of the known functions of these genes, yielding valuable insights. Our analysis confirmed that Z-linked miR-2954 target genes are not only broadly expressed and overall dosage-sensitive but also play essential roles in both development and adulthood, including key signaling and transcription factor functions. We present the details of this new analysis in the context of findings that help explain the lethal phenotype of homozygous male miR-2954 KOs:

Page 10, Lines 292-300 "Consistent with the aforementioned findings, functional characterizations revealed that Z-linked target genes are known to play critical developmental roles, being involved in multiple essential biological pathways (e.g., JAK-STAT[35], PI3K-Akt[36], Rap1[37], and stem cell pluripotency signaling pathways; growth hormone synthesis) and key biological processes (nervous system development, cell proliferation, apoptosis regulation, Golgi organization, and cell adhesion) (Supplementary Table 7). Notable examples include dosage-sensitive ohnologs such as *PTCH1*, encoding the sonic hedgehog receptor[38], and the transcription factor gene *KLF9* (ref. [39]) (Supplementary Table 7). The Z-linked targets also comprise broadly expressed genes with fundamental cellular and developmental functions (e.g., *RAD23B*, ref. [40]) and more specialized genes, crucial for the development of specific organs (e.g., *TAL2* for the brain[41]; *ALPK2* for the heart[42])."

As mentioned above in our response to comment #1 by this referee, these findings, combined with our new estimate (made in response to comment #4 by referee #3) that ~54% of all Z-linked genes may be affected in the miR-2954 KO (Page 10, Lines 302-316; Extended Data Fig. 6), provide a compelling explanation for the severe (lethal) phenotype observed in homozygous male KOs.

6. The hypothesis that avian sex chromosome evolution first involved Z "up-regulation" due to gene loss on the W , followed by necessary male-specific down-regulation in ZZ males is interesting but I don't think the study addresses the first part of this scenario, only the second. It is stated in the Discussion: "We unveiled a scenario where, driven by the decay of the W chromosome, dosage sensitive genes 342 became upregulated on the Z chromosome not only in (ZW) females but, as a secondary consequence, also in (ZZ) males." I think it is only the last part that the study has clearly demonstrated.

We did also address the first part of the scenario, but have extended our analyses and findings, as detailed below.

In previous studies, we revealed a signal of an overall partial transcriptional upregulation of genes on the Z chromosome in females (Marin et al. *Genome Res.* 2017; Pubmed ID: 29133310 and Julien et al. *PLoS Biol.* 2012; Pubmed ID: 22615540), but in these studies we didn't identify the genes that drove this pattern.

In this study, we tested whether the observed Z transcriptional upregulation is driven by dosage-sensitive Z-linked genes targeted by miR-2954 by comparing present-day expression levels to those inferred for the ancestral (proto-) Z chromosome. We found that in females, Z-linked miR-2954 targets have indeed undergone significant upregulation across multiple organs, unlike other Z-linked genes (Fig. 4f). Meanwhile, male upregulation is secondarily buffered by miR-2954, resulting in more comparable expression levels for dosage-sensitive Z-target genes between the sexes (Fig. 4g). For details of these analyses and results from the original manuscript, see the "Mechanisms of avian dosage compensation" section (Page 11, Lines 331–390).

Prompted by the reviewer's feedback, comment #4 of referee #2, and our prior finding that the mammalian X chromosome is upregulated at both the transcriptome and translatome layers in response to Y-chromosome decay (Wang et al. *Nature* 2020; Pubmed ID: 33177713), we conducted new analyses using high-quality ribosome-profiling (Ribo-seq) data, which enable the detailed assessment of protein synthesis rates, and matched RNA-seq data, to further investigate Z upregulation. These analyses show that translational upregulation in females can further elevate expression levels of dosage-sensitive Z-linked target genes, potentially achieving complete compensation for W gene loss (i.e., current expression levels are statistically indistinguishable from ancestral ones), as demonstrated for the adult brain (Extended Data Fig. 5b). We have now incorporated this Z translational upregulation analysis into the paragraph on Z transcriptional upregulation (with the new translatome findings highlighted in **bold**).

Page 11, Lines 354-365 "This analysis revealed that, in females, Z-linked miR-2954 target genes have indeed become significantly upregulated during evolution across different organs (i.e., current/ancestral expression output ratio > 0.5 [$\log_2$ ratio > −1]) – contrary to other Z-linked genes (Fig. 4f). In males, current expression levels are similar to ancestral levels (i.e., current/ancestral expression output ratio not significantly different from 1 [$\log_2$ ratio of 0]) for both Z-linked targets and other Z-linked genes, consistent with the maintenance of two Z copies for all genes in males and the repressive effect exerted by miR-2954 that compensates for the upregulation of the targets and is lost in our KO chicken model. **Notably, an analogous analysis based on Ribo-seq data for the brain reveals an even more pronounced upregulation of Z-linked target genes in females, such that present-day expression levels are statistically indistinguishable from ancestral levels (Extended Data Fig. 5b). Collectively, this finding suggests that combined transcriptional and translational upregulation can fully restore ancestral (proto-Z) expression levels in female birds, akin to the scenario we previously uncovered for the X chromosome in therian mammals[6].**"

As a result of this female-biased translational upregulation, we show that – at the translatome layer – (dosage-sensitive) Z-linked target genes exhibit even more similar (embryonic head) expression levels between the sexes than at the transcriptome layer, or even statistically indistinguishable (adult brain) expression levels (Extended Data Fig. 5c,d). We have added this new translatome data analysis alongside the corresponding transcriptome analysis in this section:

Page 11-12, Lines 367-378 "Next, we tested the final expectation of our dosage compensation evolution model: that Z-linked target genes, due to the different compensation mechanisms, should show more similar expression levels in females and males (i.e., female levels should be closer to those of males) than other Z-linked genes. Using an extensive RNA-seq dataset spanning multiple organs and developmental stages[34], we indeed found that female-to-male expression level ratios are significantly higher (i.e., female levels are closer to male levels) for Z-linked targets than for other Z-linked genes across all organs and stages (Fig. 4g). **Furthermore, our analyses of translatome (Ribo-seq) data generated for the adult brain and embryonic head revealed even higher female-to-male ratios than those observed for the transcriptome (RNA-seq) data (Extended Data Fig. 5c), and in the adult brain, this ratio is even statistically indistinguishable from 1 ($\log_2$ ratio = 0]). These findings suggest that translational upregulation in females, reflected by higher translational efficiencies in this sex (Extended Data Fig. 5d; also reported in ref. [45]), combined with transcriptional upregulation in both sexes and miR-2954-mediated mRNA degradation in males, can lead to fully balanced protein abundances between the sexes.**"

Thus, together with our original transcriptome analyses, the new translatome data reveal a striking scenario in females, where upregulation of dosage-sensitive Z-linked miR-2954 target genes at both the transcriptome and translatome layers compensates for W gene loss. In contrast, the resulting transcriptional overabundance in males—due to the non-sex-specific nature of the Z-target transcriptional upregulation mechanism—is secondarily counterbalanced by miR-2954-mediated transcript degradation. We have also added these translatome aspects to the abstract (Page 2, Lines 47-51), our original hypothesis (Page 11, Lines 334-344), summary of this section (Page 12, Lines 386-390), and in the Discussion section (Page 13, Lines 434-438).

Finally, we would like to point out that we now added another new analysis based on Ribo-seq data, to show that the upregulation of Z targets in the KO males also manifests at the translatome layer (Extended Data Fig. 5), indicating that that the loss of miR-2954 in the homozygous KO males is not mitigated by translational buffering and indeed leads to increased Z-linked protein abundances.

Page 5, Lines 170-179 "Notably, using high-quality ribosome-profiling (or ribosome-sequencing, Ribo-seq) data (Methods) (Extended Data Fig. 4), which enable the detailed assessment of protein synthesis rates[26], and matched RNA-seq data, we show that the pronounced transcriptional Z-target upregulation in $Z^{KO}Z^{KO}$ samples is likewise evident in the translatome. Specifically, Z-target genes display similarly elevated expression levels at this expression layer compared with other Z-linked and autosomal genes (Extended Data Fig. 5a), indicating that the loss of miR-2954 in the homozygous KO males is not mitigated by translational buffering and indeed leads to increased Z-linked protein abundances. Furthermore, the comparable extent of upregulation across the transcriptome and translatome layers suggests that miR-2954 primarily reduces target mRNA stability rather than repressing translation, consistent with the predominant mode of action of most microRNAs[14,23,24]."

Referee #1 (Remarks on code availability):

None

We would like to thank the referee again for the appreciation of our work and very helpful comments.

Referee #2 (Remarks to the Author):

Dosage compensation evolves to balance the expression of hemizygous X or Z chromosome to that of autosomes, after the Y or W chromosome has become degenerated. Its mechanism has been well elucidated in Drosophila, C. elegans and mouse. And chicken, another important model organism representing the largest land vertebrate group was considered to have incomplete or gene-by-gene dosage compensation, as its Z chromosome exhibits a global lower expression level in females than in males, given the 2-fold dosage differences of gene copies between sexes. This work characterized a Z-linked microRNA, miR-2954, which the group identified in a previous work, and found it is responsible for biasedly downregulating many Z-linked dosage sensitive genes relative to autosomal targets. And they provided evidence that this is the consequence of evolution of upregulation of these Z-linked target genes in females. This supports the classic model of evolution of dosage compensation proposed by Ohno, that the first step of dosage compensation is to upregulate the X- or Z-linked gene expression in male or female, where these genes have become hemizygous. And the second step is to resolve the byproduct of the first step, i.e., the overexpression of these genes, by evolving a downregulating strategy. In the case of chicken, the second step seems to be achieved by a Z-linked microRNA.

The major advancement of this work (Figure 1-3) is the knockout of miR-2954, which I understand nowadays in chicken is still not as regular as in mouse. One significance result of the work is that this miR-2954 turns out to be essential (mutants die before embryonic day 7) and is broadly expressed throughout different tissues. This is consistent with its targets that are enriched for important dosage sensitive genes. However, one major issue with the work is that how much new conclusion can the reader find in the rest of the paper (Figure 4-5) compared to authors' previous work in Genome Res (Warnefors et al. 2017). Thanks to the solid knockout work in the first part, this work has a more accurate list of target genes (248 genes that show upregulation upon knockout) by miR-2954, compared

to the predicted target genes (47 genes conditioned on 8mer sites) by Warnefors et al. 2017. The standard of 8mer for screening targets was also used in Figure 4a of this work, and although with an additional requirement of multiple target site in this work, the conclusions is the same to that of Warnefors et al. 2017: that the targets are enriched on the Z. The conclusion of figure 4d of this work is also the same with figure 4d of Warnefors et al. 2017, that ohnologs are overrepresented among the Z-linked targets. And finally, that the miR-2954 is playing a conserved role across bird species, i.e., figure 5 of this work, is similar to figure 4e of Warnefors et al. 2017. In summary, I think Figure 1-3 of this work is experimentally new and confirms the function of miR-2954, but the rest figures 4-5 only exhibit minor improvements to what we have already known about miR-2954.

We thank the reviewer for the overall appreciation of our work and the useful specific comments, which we address further below.

The core focus of our work is indeed the miR-2954 knockout and the striking lethality observed in homozygous male KOs. This *in vivo* finding, and our detailed exploration of its molecular basis, underpins the rest of the study, allowing us—for the first time—to unravel the precise nature, phenotypic relevance, and evolutionary origins of avian dosage compensation. Accordingly, the later figures (Figs. 4 and 5), along with the associated Extended Data Figures and Supplementary Data, are novel; we note that they now also contain several new analyses incorporated during the revision (see details below). While certain concepts were initially explored in our earlier Warnefors et al. work, providing the motivation and basis for this study, these new *in vivo* data enabled us to substantially extend and refine those insights with new analyses and interpretations.

For example, our new set of 248 target genes is not only more than five times larger than the previous set from Warnefors et al., but it also represents experimentally validated targets identified through our KO lines, whereas the Warnefors et al. set consisted solely of predicted targets. This large new set of actual target genes then allowed us to, for the first time, show that the selective targeting of Z-linked genes is driven not only by longer and more numerous target sites in their 3'-UTRs compared to other genes (shown in our original manuscript; Fig. 4a), but also by factors like increased seed-pairing stability, more favorable local sequence context, and optimal target site positioning (assessed in the revised work by the context+ score, an analysis prompted by comment #3 of this reviewer – see detailed response below that comment; Fig. 4b) (page 7, lines 229–234). Furthermore, our list of actual targets also enabled us to discover that miR-2954 targets dosage-sensitive Z-linked genes more broadly – beyond ohnologs – based on a novel mammalian triplosensitivity/haploinsufficiency resource (Fig. 4c,d) (Page 10, Lines 276–281). We could thus also further characterize the functions of target genes; in the original manuscript, we found that Z target genes show broader spatiotemporal expression profiles than other Z-linked genes (Fig. 4e), and our revision analyses (prompted by comment #5 of referee #1) revealed that they are known to also play various critical roles in both development and adulthood (Page 10, Lines 283–300; Supplementary Table 7). Together, these analyses therefore enable us to unravel the mechanisms of miR-2954-mediated dosage compensation in birds and explain the male essentiality of this microRNA.

We next used a set of experimental Z target genes to assess the initial (upregulation) mechanism of dosage compensation in birds. That is, we use this list to uncover that among Z-linked genes, only (dosage-sensitive) Z-target genes have been transcriptionally upregulated in females during evolution (a finding not made in Warnefors et al.) (Fig. 4f) (Page 11, Lines 354–360). Notably, prompted by comment #4 of this reviewer, comment #6 of referee #1, and our prior finding that the mammalian X chromosome is upregulated at both the transcriptome and translatome layers in response to Y-chromosome decay (Wang et al. *Nature* 2020; Pubmed ID: 33177713), we conducted new analyses using high-quality ribosome-profiling (Ribo-seq) data, which enable the detailed assessment of protein synthesis rates, and matched RNA-seq data, to further investigate Z upregulation. These analyses show that translational upregulation in females can further elevate expression levels of dosage-sensitive Z-linked target genes, potentially achieving complete compensation for W gene loss (i.e., current expression levels are statistically indistinguishable from ancestral ones), as demonstrated for the adult brain (Extended Data Fig. 5b) (Page 11, Lines 360–365).

As a result of this female-biased translational upregulation, we further show that – at the translatome layer – (dosage-sensitive) Z-linked target genes exhibit even more similar (embryonic head) expression levels between the sexes than at the transcriptome layer (Fig. 4g, Extended Data Fig. 5c,d), or even statistically indistinguishable (adult brain) expression levels (Extended Data Fig. 5c,d) (Page 11, lines 367–375). Altogether, these findings suggest that translational upregulation in females, reflected by higher translational efficiencies in this sex (Extended Data Fig. 5d; also reported in

ref. [45]), combined with transcriptional upregulation in both sexes and miR-2954-mediated mRNA degradation in males, can lead to fully balanced protein abundances between the sexes (Page 11-12, Lines 375–378).

Additionally, the large number of validated miR-2954 targets allowed us, for the first time, to examine their distribution along the Z chromosome, revealing an even distribution (Fig. 4h) and thus not supporting the previously proposed restriction of dosage compensation to specific regions (Page 12, Lines 380–384).

In the final results section ("Origin and evolution of miR-2954-mediated dosage compensation in birds"), we present extensive evolutionary analyses—extended and refined during the revision (also prompted by this reviewer's comments #3 and #5; see our responses for details)—that go well beyond Warnefors et al. We have now expanded our miR-2954 conservation analyses to all 363 available bird genomes, revealing that its mature sequence is identical across birds, with only two species showing a single-nucleotide substitution in the precursor (Fig. 5b, Extended Data Fig. 9, Supplementary Data 3) (Page 12, Lines 393–400). This observation underscores that miR-2954 has been under strong purifying selection throughout avian evolution. Moreover, building on our original manuscript and on findings from Warnefors et al., new comparative genome analyses with non-avian reptiles and mammalian outgroups show that miR-2954 target sites have specifically accumulated in the UTRs of Z-linked target genes following the emergence of miR-2954, and have since been preferentially retained compared to largely spurious sites in other genes (Fig. 5c–e and Extended Data Fig. 10) (Page 12, lines 402–421).

Overall, informed by our KO results (e.g., the extensive list of experimentally validated miR-2954 targets), we elucidate the mechanisms and evolutionary origins of the unique avian dosage compensation system, advancing well beyond Warnefors et al. in every section of our paper.

I also have other comments detailed as below:

1. Line 78, "...have only been partially and/or in part.." this sentence is redundant here, or the authors mean something else?

We intended this phrase to convey that the overall incomplete (median) upregulation signal of Z-linked genes in female birds, as observed in our previous work, could result either from the *partial* upregulation of all (or most) genes or, alternatively, from the full upregulation of only a subset (*in part*) of Z-linked genes. However, we recognize that the wording was unclear and apologize for any confusion. We have now reworded and split this sentence to clarify its message:

Page 2, Lines 79-83 "Previous work revealed an incomplete transcriptional upregulation signal for Z-linked genes in female birds, which could result from the partial upregulation of all or most Z-linked genes and/or the full upregulation of a subset of genes. This incomplete upregulation has rendered their transcript abundances lower than those of their evolutionary precursors on the ancestral autosomes[5,9]."

2. Figure 1c, the heterozygote mutant ZKOZ males seem to have a higher lethality rate at E7-13 compared to the females. Is this significant? How about the survival rate beyond E13? I wonder if the survival rate of heterozygote mutant is impacted at a later stage compared to the homozygote? Are there any organ or other types of defects shown by the homozygous mutant before E7?

Thanks for the comment, which allowed us to clarify this point.

$Z^{KO}Z$ males (alive embryos: 26; total number: 43) do not have a significantly higher lethality rate than $Z^{KO}W$ (alive: 30; total: 39) and ZW wildtype females (alive: 28; total: 39) (*P*-values > 0.15, two-sided χ^2 test) (Supplementary Table 2). By contrast, $Z^{KO}Z^{KO}$ embryos all die before day 7 of embryonic development and thus have a significantly higher lethality rate than all the other genotypes (*P*-values < 10^{-9}, two-sided χ^2 test). We now added this statistical information to the relevant section, with the new information highlighted in **bold** below ("miR-2954 is male-essential"):

Page 3, Lines 127-137 "To assess potential phenotypic consequences of the miR-2954 KO, we first assessed the viability of 297 G2 embryos on embryonic (E) days E3, E4-5, and E7-13 by assessing their morphology and heartbeat under a

stereo microscope (Methods, Supplementary Table 2, and Supplementary Data 1 and 2). We found that wild-type female (ZW) chicken embryos as well as hemizygous female ($Z^{KO}W$) and heterozygous male ($Z^{KO}Z$) KO embryos exhibited similar **and statistically indistinguishable average survival rates of approximately 79-85% (*P*-values > 0.15, two-sided χ^2 test)** (Fig. 1c, d). These observations demonstrate that miR-2954 is not essential for female development, consistent with its very low expression in this sex[1], and that the absence of one copy of the miR-2954 locus in males is not deleterious, implying that this microRNA is haplosufficient in male chicken embryos. In stark contrast to these genotypes, all males with the $Z^{KO}Z^{KO}$ genotype died before day 7 of embryonic development **and thus have a significantly higher lethality rate than the other genotypes (*P*-values < 10^{-9}, two-sided χ^2 test)** (Fig. 1c, d)."

For ethical reasons (i.e., to minimize unnecessary animal breeding and sacrifice) and given the study's focus on the striking early $Z^{KO}Z^{KO}$ lethality, we raised only a limited number of $Z^{KO}Z$ males beyond E13 for outcrossing. These males were fertile and exhibited no overt phenotypes, but the small sample size precluded systematic phenotypic quantification.

In agreement with the broad spatiotemporal expression of miR-2954 and its targets, as well as the diverse functions of targets (now illuminated in more detail – Page 10, Lines 292–300 – as mentioned above), $Z^{KO}Z^{KO}$ embryos show variable abnormal phenotypes at different stages before E7 in our microscopy assessments, with overall delayed development and reduced growth, reflected by small eyes and brains as well as stunted axial growth especially in later stages; many embryos were completely degraded (Supplementary Data 1 and 2). Interestingly, these variable phenotypic patterns and the overall lethality (including its timing) are highly reminiscent of that observed for mouse KO mutants for the *Xist* lncRNA (Marahrens et al., *Genes Dev.* 1997; PubMed ID: 9009199), which carries out an analogous secondary dosage compensation function in placental mammals, as discussed in our manuscript (Introduction and Discussion).

The upregulated Z-linked targets of miR-2954 include both broadly and tissue-specifically expressed dosage-sensitive developmental genes. Their upregulation in KOs likely causes cumulative deleterious effects leading to lethality by E5, a crucial period marked by an increase in blood circulation, rapid embryonic growth, and differentiation of essential structures such as the allantois and vasculature. Disrupting these processes—whether cardiovascular development, metabolic regulation, organogenesis timing, or cell differentiation—could individually or collectively account for the observed male KO lethality.

We now added a paragraph that summarizes these notions at the end of the section in which we assess the functions of miR-2954 targets:

Page 10-11, Lines 318-328 "Consistent with the broad spatiotemporal expression and diverse developmental functions of miR-2954 targets, $Z^{KO}Z^{KO}$ embryos exhibit variable developmental abnormalities before E7, including delayed growth, small eyes and brains, and stunted axial elongation, with many embryos fully degraded by later stages (Supplementary Data 1 and 2). The upregulation of dosage-sensitive Z-linked developmental genes in KOs likely leads to cumulative deleterious effects, contributing to lethality by E5 – a critical phase involving an increase in blood circulation, rapid growth, and differentiation of key structures such as the allantois and vasculature. Disruptions in these processes, whether affecting cardiovascular development, metabolic regulation, organogenesis timing, or cell differentiation, likely underlie the observed lethality. Notably, the timing and variability of the miR-2954 KO phenotypes resemble those observed in *Xist* knockout mice, where loss of this lncRNA – responsible for secondary dosage compensation in placental mammals – also leads to lethality in a similar developmental window[43] (i.e., between E5 and E12)."

3. Figure 2a is very interesting, that over 50% of the predicted Z-linked targets are impacted compared to only 3-5% autosomal predicted targets. It remains unclear when and how miR-2954 achieves such specificity against the Z-linked genes. Either the Z-linked genes increased their sensitivity (as suggested Figure 4a) by changing their sequence seeds, or autosomal targets decreased their sensitivities. It is unclear whether the latter has happened, without aligning the sequences against an avian outgroup. Another possibility is that the autosomal genes evolved secondary compensatory mechanisms to offset the downregulating effect of miR-2954. These possibilities should be tested and discussed, as besides becoming male specific, how miR-2954 achieves its specific regulation of Z-linked vs. autosomal genes is very critical during evolution of its role in dosage compensation.

Thank you for your appreciation of this finding.

In our previous analysis, we demonstrated that the strong preferential targeting of Z-linked genes by miR-2954 is driven by an enrichment of 8-mer binding sites and the presence of multiple target sites (6–8 nt) among predicted Z-linked targets compared to autosomal targets (Fig. 4a). Motivated by the referee's comment, we further investigated this specificity using the context+ score from TargetScan, which accounts for additional factors influencing targeting efficacy, such as seed-pairing stability, local sequence context, and target site positioning within the UTR. This analysis confirmed that predicted Z-linked targets exhibit significantly lower context+ scores than autosomal targets (Fig. 4b), supporting the notion that predicted autosomal targets are largely spurious (i.e., due to spurious sequences resembling – overall short – miR-2954 target sites) and not actually targeted *in vivo*. We have now incorporated this new context+ analysis into the paragraph discussing the specific targeting of Z-linked genes by miR-2954:

Page 7, Lines 225-234 "We then sought to understand why miR-2954 preferentially targets Z-linked genes; that is, why a substantially higher proportion of predicted Z-linked targets compared to predicted autosomal targets become upregulated in KO animals. The efficacy of microRNA-mediated repression depends on the extent of seed–UTR complementarity, with 8-mer target sites providing the strongest repression and multiple sites within a UTR amplifying this effect[14,25]. Additional factors, such as seed-pairing stability, local sequence context, and the positioning of target sites within the UTR further influence targeting efficacy, as assessed by the "context+" TargetScan score[25]. Consistent with these principles and the preferential repression of Z-chromosomal genes by miR-2954, we found Z-linked targets to be significantly enriched for 8-mer binding sites and the presence of multiple target sites (6-8 nt) compared to autosomal targets (Fig. 4a). Furthermore, Z-linked targets exhibit significantly lower context+ scores, indicating enhanced repression sensitivity (Fig. 4b)."

Our new comparative genome analyses with non-avian reptiles and mammalian outgroups also revealed that miR-2954 target sites have accumulated specifically in the 3'-UTRs of Z-linked target genes during avian evolution; that is, they are only enriched in the 3'-UTRs of Z-linked birds, including representatives of the most diverged avian lineages (i.e., chicken, zebra finch, and ostrich), and not in the 3'-UTRs of 1:1 orthologs from the outgroup species (Extended Data Fig. 10). We note that these analyses could not be done directly based on aligned sequences, due to large evolutionary distances of the avian, non-avian reptile, and mammalian lineages, which substantially limit the alignability of UTR sequences. However, we now not only show that Z-linked computationally predicted targets are significantly more likely to also be targets in chicken than autosomal predicted targets (Fig. 5c,d), we also show – based on 3'UTR sequence alignments – that Z-linked targets contain a higher proportion of target sites that are conserved between chicken and zebra finch (Fig. 5e). Together, these analyses show that Z-linked targets/target sites have been preferentially preserved compared to (largely spurious) targets/target sites in autosomal genes (Fig. 5d, e), reinforcing the view notion that predicted autosomal genes are not actual targets *in vivo*.

These analyses are described in detail in the manuscript (section: "Origin and evolution of miR-2954-mediated dosage compensation in birds"):

Page 12, Lines 402-421 "To further assess the conservation of miR-2954-mediated dosage compensation across birds, we compared aspects of this mechanism between chicken and zebra finch, which represent two major avian lineages (Galliformes/Anseriformes and Neoaves, respectively) that diverged ~91 million years ago[47] and mark the deepest split within Neognathae. First, we found that ~78% of the experimental Z targets we identified in chicken are also computationally predicted targets of miR-2954 in zebra finch, a species for which strongly male-biased expression of miR-2954 was previously shown as well[48] (Fig. 5c). Moreover, in zebra finch, Z-linked computationally predicted targets are significantly more likely to also be targets in chicken than autosomal predicted targets (Fig. 5d). Consistently, Z-linked targets contain a higher proportion of target sites that are conserved between chicken and zebra finch (Fig. 5e). Together with our previous finding that predicted Z-linked targets (rather than autosomal predicted targets) and in particular Z-linked ohnologs are predominantly upregulated upon miR-2954 knockdown in a male zebra finch cell line[1], these observations strongly support that the functional role of miR-2954 in zebra finch is similar to that in chicken.

Further analyses revealed an enrichment of miR-2954 target genes also on the Z chromosome of the ostrich, a representative of flightless ratites (i.e., Paleognathae), the sister lineage of all other extant birds (Neognathae), which display less pronounced sex chromosome differentiation compared to other birds[49]. By contrast, autosomal orthologs of Z-linked genes in non-avian reptile and mammalian outgroup species (i.e., crocodile and human) are not enriched for

miR-2954 target sites (Extended Data Fig 10). These findings suggest that miR-2954 target sites started to accumulate on the Z in the common avian ancestor, following the emergence of miR-2954."

4. Figure 4f, it is clear that miR-2954 targets are indeed upregulated during evolution, however their expression levels are still significantly lower than those of outgroup species. How to explain this, that it seems suboptimal for dosage compensation not being able to lift the expression level of hemizygous Z to that of autosome or compared to an outgroup? The same question applies below to the dosage sensitive genes on the Z, that also they have been upregulated, they are still male-biased in birds. Would that pose a challenge or deleterious effects?

Genes vary in their dosage sensitivity, and while miR-2954 targets are generally more dosage-sensitive than other Z-linked genes, there is also considerable variation among the targets themselves—evident in their triplosensitivity and haploinsufficiency score distributions (Fig. 4d). Consequently, the evolutionary pressure for full versus partial upregulation differs from gene to gene: some require only partial upregulation to avoid deleterious effects, whereas others depend on complete restoration of ancestral expression levels. Thus, the median current-to-ancestral expression ratios (Fig. 4f), along with their interquartile ranges, need not align with full upregulation for all Z-linked targets. Instead, they represent an aggregate of varying degrees of upregulation, reflecting the distinct dosage requirements of each target gene.

However, this being said, prompted by the reviewer's feedback, comment #6 of referee #1, and our prior finding that the mammalian X chromosome is upregulated at both the transcriptome and translatome layers in response to Y-chromosome decay (Wang et al. *Nature* 2020; Pubmed ID: 33177713), we conducted new analyses using high-quality ribosome-profiling (Ribo-seq) data, which enable the detailed assessment of protein synthesis rates, and matched RNA-seq data, to further investigate Z upregulation. These analyses show that translational upregulation in females can further elevate expression levels of dosage-sensitive Z-linked target genes, potentially achieving complete compensation for W gene loss (i.e., current expression levels are statistically indistinguishable from ancestral ones), as demonstrated for the adult brain (Extended Data Fig. 5b). We have now incorporated this Z translational upregulation analysis into the paragraph on Z transcriptional upregulation (with the new translatome findings highlighted in **bold**).

Page 11, Lines 354-365 "This analysis revealed that, in females, Z-linked miR-2954 target genes have indeed become significantly upregulated during evolution across different organs (i.e., current/ancestral expression output ratio > 0.5 [$\log_2$ ratio > −1]) – contrary to other Z-linked genes (Fig. 4f). In males, current expression levels are similar to ancestral levels (i.e., current/ancestral expression output ratio not significantly different from 1 [$\log_2$ ratio of 0]) for both Z-linked targets and other Z-linked genes, consistent with the maintenance of two Z copies for all genes in males and the repressive effect exerted by miR-2954 that compensates for the upregulation of the targets and is lost in our KO chicken model. **Notably, an analogous analysis based on Ribo-seq data for the brain reveals an even more pronounced upregulation of Z-linked target genes in females, such that present-day expression levels are statistically indistinguishable from ancestral levels (Extended Data Fig. 5b). Collectively, this finding suggests that combined transcriptional and translational upregulation can fully restore ancestral (proto-Z) expression levels in female birds, akin to the scenario we previously uncovered for the X chromosome in therian mammals[6].**"

As a result of this female-biased translational upregulation, we show that – at the translatome layer – (dosage-sensitive) Z-linked target genes exhibit even more similar (embryonic head) expression levels between the sexes than at the transcriptome layer, or even statistically indistinguishable (adult brain) expression levels (Extended Data Fig. 5c,d). We have added this new translatome data analysis alongside the corresponding transcriptome analysis in this section:

Page 11-12, Lines 367-378 "Next, we tested the final expectation of our dosage compensation evolution model: that Z-linked target genes, due to the different compensation mechanisms, should show more similar expression levels in females and males (i.e., female levels should be closer to those of males) than other Z-linked genes. Using an extensive RNA-seq dataset spanning multiple organs and developmental stages[34], we indeed found that female-to-male expression level ratios are significantly higher (i.e., female levels are closer to male levels) for Z-linked targets than for other Z-linked genes across all organs and stages (Fig. 4g). **Furthermore, our analyses of translatome (Ribo-seq) data generated for the adult brain and embryonic head revealed even higher female-to-male ratios than those observed for the transcriptome (RNA-seq) data (Extended Data Fig. 5c), and in the adult brain, this ratio is even statistically**

indistinguishable from 1 (log_2 ratio = 0]). These findings suggest that translational upregulation in females, reflected by higher translational efficiencies in this sex (Extended Data Fig. 5d; also reported in ref. [45]), combined with transcriptional upregulation in both sexes and miR-2954-mediated mRNA degradation in males, can lead to fully balanced protein abundances between the sexes."

Thus, together with our original transcriptome analyses, the new translatome data reveal a striking scenario in females, where upregulation of dosage-sensitive Z-linked miR-2954 target genes at both the transcriptome and translatome layers compensates for W gene loss. In contrast, the resulting transcriptional overabundance in males—due to the non-sex-specific nature of the Z-target transcriptional upregulation mechanism—is secondarily counterbalanced by miR-2954-mediated transcript degradation. We have also added these translatome aspects to the abstract (Page 2, Lines 47-51), our original hypothesis (Page 11, Lines 334-344), summary of this section (Page 12, Lines 386-390), and in the Discussion section (Page 13, Lines 434-438).

Finally, we would like to point out that we now added another new analysis based on Ribo-seq data, to show that the upregulation of Z targets in the KO males also manifests at the translatome layer (Extended Data Fig. 4), indicating that that the loss of miR-2954 in the homozygous KO males is not mitigated by translational buffering and indeed leads to increased Z-linked protein abundances.

5. It is very interesting to find that miR-2954 ortholog is also present in other birds. But without proper experimental data, one should be conservative to suggest it plays a similar function in emu, ostrich etc, and call it 'highly conserved across birds'.

Thanks for your appreciation of this finding.

We have now extended our miR-2954 conservation analyses to all 363 available bird genomes, which revealed that its mature sequence is identical across birds (including ostrich and emu) and its precursor sequence is fully conserved, with the exception of two species, each with a single single-nucleotide substitution (Supplementary Data 3 and Extended Data Fig. 9). This observation highlights that miR-2954 has been strongly preserved by purifying selection during avian evolution. We have now modified the related paragraph:

Page 12, Lines 393-400 "We finally sought to investigate the origin and evolution of this avian dosage compensation system. To this end, we first screened for the presence of the miR-2954 locus across amniotes (Methods). Notably, miR-2954 is present across bird genomes but not in the genomes of any other species (including crocodiles – the closest living bird relatives), which, however, all contain the host gene of miR-2954 in birds, *XPA* (Fig. 5b and Extended Data Fig. 9). Strikingly, not only the mature sequence (22 nt) but also its precursor sequence (68 nt) of miR-2954 are identical across 351 of the 363 examined species[48]. The exceptions are two species, each with a single nucleotide substitution in the precursor sequence that does not overlap the mature sequence, and ten species with missing sequencing data for this region (Supplementary Data 3)."

As mentioned in our response to comment #3 above, we have also carried out new comparative genome analyses with non-avian reptiles and mammalian outgroups, which revealed that miR-2954 target sites have accumulated specifically in the 3'-UTRs of Z-linked target genes during avian evolution; that is, they are only enriched in the 3'-UTRs of Z-linked birds, including representatives of the most diverged avian lineages (i.e., chicken, zebra finch, and ostrich), and not in the 3'-UTRs of 1:1 orthologs from the outgroup species (Extended Data Fig. 10) (Page 12, lines 415-421). We note that the less pronounced enrichment of target sequences in the ostrich compared to the other birds is consistent with the less pronounced sex chromosome differentiation in this species, but might also reflect the poorer annotation of 3'-UTRs in its genome.

Together with the upregulation analyses, these analyses suggest that the avian sex chromosome dosage compensation system, including the secondary compensatory mechanism mediated by miR-2954, emerged in the common avian ancestor following the differentiation of the ZW sex chromosomes and has since been selectively preserved across birds. However, we agree with the reviewer that confirming its full conservation across all birds would require functional validation of miR-2954 in additional species (e.g., emu and ostrich). Consequently, we have moderated our manuscript's

language to reflect this caveat, refraining from stating that the dosage compensation system is "highly conserved across birds". We thus now end this last result section with the following paragraph:
Page 12-13, Lines 423-426 "Altogether, our evolutionary analyses suggest that the avian sex chromosome dosage compensation system, including the secondary compensatory mechanism mediated by miR-2954, emerged in the avian stem lineage between approximately ~108-245 million years ago[47] (Fig. 5b) upon the emergence of the ZW sex chromosomes and has since been selectively preserved across birds."

And in the Discussion section, we now write:

Page 14, Lines 444-445 "Our findings suggest that the miR-2954 mechanism is present across birds, consistent with its crucial role in avian males".

We would like to thank the referee again for the appreciation of our work and all their comments, which were very useful and helped us to improve the manuscript.

Referee #3 (Remarks to the Author):

While sex chromosome dosage compensation mechanisms have been studied extensively in a few model organisms (mice, c. elegans, drosophila), the extent, mechanisms and importance of dosage compensation in birds has remained unclear. Here the manuscript reports a breakthrough discovery, showing that Z-chromosome dosage compensation is essential in birds and that it is mediated by a Z-linked miRNA. The main discovery is that deletion of miR-2954 in chicken results in male-specific lethality, suggesting that Z-dosage compensation is essential also in birds. A series of analyses in chicken with different genotypes are performed to show that miR-2954 preferentially targets Z-chromosomal coding genes and reduces their expression in male ZZ birds. Moreover, sequence conservation and an analysis performed in a different bird species, the zebra finch, suggest that the discovered mechanism is conserved across birds. Although previous studies had shown that miR-2954 is more highly expressed in males compared to females, its functional role in Z-dosage compensation is a major discovery. Overall, the manuscript is a pleasure to read and I mostly only have small suggestions how to improve it. However, given the central importance of the knock-out phenotype for the study, I would suggest to perform an additional experiment to show that miR-2954 can indeed rescue the male-specific lethality.

We are very grateful for the reviewer's positive feedback and enthusiasm as well as for their very helpful specific comments, which we address below.

Major points

1. Although deletion of miR-2954 only affects a small region, it might still have effects of the host gene expression independently of miR-2954 function. Moreover, Cas9 might induce genomic rearrangements that are difficult to detect by genotyping. Given that host gene expression is affected by the deletion, it would be important to provide robust evidence that the phenotype is indeed due to absence of miR-2954. To inequivocally show that indeed miR-2954 is responsible for the observed phenotype, it would be useful to perform a rescue experiment, where miR-2954 is provided in trans.

We thank the reviewer for this comment, which allowed us to exclude off-target edits/effects, confirm that the *XPA* host gene upregulation is explained by its indirect regulation through miR-2954 (rather than by the 36-bp intronic KO deletion), and thus validate that the observed miR-2954 KO phenotype indeed reflects the male-essential function of this microRNA in sex chromosome dosage compensation.

Specifically, we first sought to fully exclude off-targets edits. Our repeated outcrossings – as well as the lack of a phenotype in heterozygous KO males and hemizygous KO females (Page 4, Lines 139-142) – effectively rule out potential off-target edits on the autosomes, but not necessarily for closely linked regions on the Z chromosome.

We therefore performed deep long-read genome resequencing of miR-2954 in male knockouts (KOs) and controls, selectively enriching for Z-chromosomal sequences, yielding ~10-32 fold coverage for this chromosome (Extended Data Fig. 2a,b). Analyses of these data confirmed the miR-2954 deletion in the KOs and revealed no additional structural

changes (Extended Data Fig. 2c). Together with our repeated outcrossings and the lack of a phenotype in heterozygous KO males and hemizygous KO females, these findings rule out off-target edits on both the autosomes or Z chromosome in the KO animals. These new data and analyses are detailed in the revised manuscript:

Page 3, Lines 116-124 "Our KO strategy enabled us to compare phenotypic effects among outbred sibling birds of the different genotypes, thereby minimizing variability in the genetic background. Our repeated outcrossing effectively ruled out potential off-target edits on the autosomes, as any such mutation would need to be closely linked to the Z-chromosomal miR-2954 deletion. To test the latter possibility, we performed deep long-read genome resequencing of five homozygous KO males and four wild-type controls using an adaptive sampling procedure[23] that selectively enriched for Z-chromosomal sequences, yielding ~10-32 fold coverage for this chromosome (Extended Data Fig. 2a,b) (Methods). Analyses of these data detected only the intended 36 base pair (bp) miR-2954 deletion in the KO animals, with no additional structural variation between KOs and controls, confirming the absence of off-target edits (Extended Data Fig. 2c) (Methods)."

We recognize the reviewer's suggestion of a trans-rescue experiment in chicken; however, this approach is not practically feasible. miR-2954 has a broad yet highly tissue-specific expression pattern (Fig. 3a,b, and new panel 3c), and its targets exhibit similarly complex expression profiles (Fig. 4e). Even if a locus analogous to ROSA26 in mice were available in chicken, recapitulating miR-2954's high and nuanced expression levels would be exceedingly difficult, if not impossible. Delivering large, precise amounts of an miR-2954 mimic across embryonic tissues through repeated injections to rescue the KO phenotype would pose similar challenges. Moreover, implementing the suggested approach would necessitate large-scale rebreeding of the miR-2954 KO lines to generate sufficient numbers of $Z^{KO}Z^{KO}$ males for adequate statistical analyses, inevitably producing many additional genotypes that would go unused. This would violate the 3Rs ethics principles (Replacement, Reduction, and Refinement; https://nc3rs.org.uk/who-we-are/3rs), which are also strongly upheld at the Roslin Institute, where the miR-2954 KO lines were generated and bred. Such a violation would be particularly inappropriate given the slim likelihood of success of this approach and the availability of an alternative approach – as detailed hereafter.

That is, to address the reviewer's concern, we implemented a comprehensive *in vivo* miR-2954 knockdown (KD) experiment in wild-type embryos. This alternative trans approach allowed us to equally validate the male essentiality of miR-2954 and its mechanism of action and effectively rule out the possibility of a technical artifact caused by the genome editing procedure.

Specifically, using this orthogonal trans approach, which involved repeated injections of a miR-2954 inhibitor and negative control into a total of 240 male and female wildtype embryos (Methods, Page 21-22, Lines 832-869), we found that male KD embryos showed substantially (approximately 3-fold) and significantly higher mortality than female KD embryos and negative controls, which showed statistically indistinguishable lethality rates (Extended Data Fig. 7a,b). We note that the fact that the higher lethality rate in KOs versus KDs is obviously expected, given the permanent and tissue-wide miR-2954 elimination in KOs and transient nature and incomplete elimination in the KD approach.

Moreover, using RT-qPCR, we observed upregulation exclusively of miR-2954 target genes and the *XPA* host gene (Extended Data Fig. 7c), mirroring the findings from our KO experiments. The upregulation of *XPA* also in the KD experiment suggests that this gene is indirectly regulated by miR-2954, as various other genes on the Z chromosome, and rules out that the upregulation in the homozygous KO animals is merely due to the 36-bp intronic deletion. We now added a paragraph describing this new work to the main text, at the end of the section "Derepression of genes on the Z chromosome in KO animals":

Page 8, Lines 236-249 "We next sought to validate the male essentiality of miR-2954 and its mechanism of action using an orthogonal knockdown (KD) approach; that is, we knocked down miR-2954 *in vivo* using a specific inhibitor and delivery reagent (Methods). Consecutive injections at E2.5 and E4 resulted in significantly higher mortality in male KD embryos by E12 compared to both female KD embryos (P = 0.0005, two-sided χ^2 test) and the negative controls (P = 0.0006, two-sided χ^2 test) (Extended Data Fig. 7a,b). Additionally, qPCR analysis of eight target genes, eight non-target genes, and the *XPA* host gene in male KD hearts at E5 revealed significant upregulation of five target genes (Benjamini-Hochberg-corrected $P < 0.1$) and *XPA*, but no significant changes in non-target genes (Extended Data Fig. 7c). Furthermore, the target genes exhibited a substantially higher overall fold-change (mean $\log_2$FC = 1.06) compared to

the non-target genes (mean $\log_2$FC = 0.17) within the same set of samples (*P* = 0.011, paired t-test). These findings confirm that the lethal phenotype in male chickens is attributable to the absence of miR-2954 and further rule out off-target effects. The observation that – similarly to KO animals (see above) – the *XPA* gene is significantly upregulated in KD animals (Benjamini-Hochberg-adjusted *P* = 0.03), suggests that this gene is indeed indirectly regulated by miR-2954, for example through an upregulated transcription factor (activator) that is itself a direct target of miR-2954."

Finally, we note that we now carefully assessed the functional annotations of the dosage-sensitive Z-linked target genes of miR-2954, which revealed that they not only broadly expressed and overall dosage-sensitive but also play critical roles in both development and adulthood (Page 10, Lines 292-300; Supplementary Table 7). Combined with our new estimate (made in response to comment #4 by this referee) that ~54% of all Z-linked genes may be affected in the miR-2954 KO (Page 10, Lines 302-316; Extended Data Fig. 6), these findings provide a compelling explanation for the severe (lethal) phenotype observed in homozygous male KOs.

Minor points

2. Although the expression pattern of miR-2954 has been investigated in previous studies, it would be helpful to report its expression pattern within the paper.

Thanks for this suggestion, which together with comment #3 from referee #1, prompted us to elaborate on the expression pattern of miR-2954 in our paper in both sexes.

We and others previously showed that miR-2954 is approximately 5-10 times more highly expressed across various adult organs and whole embryos using small RNA sequencing (Warnefors et al. *Genome Res.* 2017; Pubmed ID: 29079676) and Northern blots (Zhao et al. Nature 2010; Pubmed ID: 20220842), respectively. We now more clearly state this in the introduction (also motivated by comment #3 of referee #3):

Page 2, Lines 82-92 "Notably and consistently, we recently characterized a Z-chromosome-derived microRNA (miR-2954) that is largely male-specific (with expression levels ~5-10 times higher in male than female organs and whole embryos[1,14]) and whose predicted target genes…".

We had originally also assessed its expression, together with that of other microRNAs, using small RNA sequencing in three male tissues at E5 (Page 7, Lines 211-217; Fig. 3a,b).

Additionally, to address the comments of this reviewer and reviewer #1, we used RT-qPCR to quantify miR-2954 expression across seven organs from both sexes at E12. This analysis revealed 7- to 15-fold higher expression in males than in females, further emphasizing its high, broad and strongly male-biased expression (Fig. 3c). We refer to these new data in the manuscript when interpreting the female KO expression patterns compared to those of male KOs:

Page 7, Lines 219-223 "When investigating gene expression in female hemizygous ($Z^{KO}W$) embryos, we again find a specific upregulation of Z-linked target genes compared to wild-type controls, but the pattern is even substantially weaker than that for male heterozygous ($Z^{KO}Z$) embryos (Fig. 2c), in accord with the very low (i.e., 7- to 15-fold lower) expression of miR-2954 in females when compared to males[1,28] (Fig. 3c; see above) and the absence of a deleterious phenotype in $Z^{KO}W$ female embryos."

3. On p. 2 it is stated that miR-2954 is a "largely male-specific Z-chromosome-derived microRNA". I would suggest to be more precise here and state how much higher it is expressed in males, since it is not absent in females, as I understand?

Thanks for the useful suggestion. As also mentioned in our response to the previous comment, we now explicitly state in this sentence how much higher miR-2954 is expressed in males, based on our data (Warnefors et al.) and those from others (Zhao et al.).

Page 2, Lines 86-92 "Notably and consistently, we recently characterized a Z-chromosome-derived microRNA (miR-2954) that is largely male-specific (with expression levels ~5-10 times higher in male than female organs and whole embryos[1,14]) and whose predicted target genes…".

4. It remains a bit unclear from the analyses what fraction of Z-linked genes are actually dosage compensated. This might be difficult to estimate given the difficulty to identify miRNA targets in general. However, a short discussion of this question would improve the manuscript.

Thank you for this suggestion, which led us to estimate the total number of genes potentially regulated—either directly or indirectly—by miR-2954. To do so, we analyzed all Z-linked genes expressed during chicken development (as assessed in our previous extensive RNA-seq resource; Cardoso-Moreira et al., Nature 2019, ref. 34 in the manuscript) and identified those with significantly elevated expression in the KOs. Notably, this analysis detected 311 genes, including 64 not computationally predicted as targets. These non-predicted genes exhibit significantly higher triplosensitivity and haploinsufficiency scores than non-predicted genes that are not differentially expressed (Extended Data Fig. 8, Supplementary Table 3), suggesting that they represent true indirect or previously unrecognized direct targets. When combined with the 247 upregulated predicted targets, this implies that ~54% (311 of 576) of all developmentally expressed Z-linked genes may be influenced by miR-2954 KO. This extensive regulation of dosage-sensitive Z-linked genes further underscores the critical role of miR-2954 and provides a compelling explanation for the severity of the KO phenotype.

We now added this new analysis/estimate in the section "miR-2954 targets dosage sensitive Z-linked genes", after the results/discussions pertaining to the dosage-sensitivity and key functions of miR-2954 targets:

Page 10, Lines 302-316 "Given our evidence of a substantial number of indirectly regulated genes or unpredicted direct targets (see above), and our motivation to comprehensively illuminate the functional importance of miR-2954, we sought to estimate the total number of Z-linked genes affected by the loss of miR-2954. To this end, we identified 576 Z-linked genes expressed during chicken development[34] and assessed their expression in homozygous male KOs, thus extending beyond the predicted differentially expressed targets examined thus far. We found that 311 of these 576 genes are upregulated in at least one tissue in homozygous male KOs (Benjamini-Hochberg-corrected $P < 0.1$ and $\log_2$-fold change > 0.2), suggesting they may represent direct or indirect miR-2954 targets. Notably, these 311 upregulated genes include 64 genes not computationally predicted to be targets, and these non-predicted targets exhibit significantly higher triplosensitivity and haploinsufficiency scores than non-predicted genes that are not differentially expressed (Extended Data Fig. 8 and Supplementary Table 3). This observation suggests that these non-predicted genes reflect true indirect or previously unrecognized direct targets. Combined with the 247 upregulated predicted targets, overall ~54% (311 of 576) of all developmentally expressed Z-linked genes may be influenced by the KO of miR-2954. Such extensive regulation of dosage-sensitive Z-linked genes by miR-2954 underscores its importance and helps explain the severity of the KO phenotype."

5. In Fig. 4h, I would suggest to also mark the position where miR-2954 is located.

Thanks for the useful suggestion – done.

6. On p. 10 the conservation of miR-2954 across birds is analyses. It would be helpful to state how many bird species are included in the comparison that showed 100% sequence conservation.

We have now substantially extended and refined this analysis during the revision. Specifically, we have now expanded our miR-2954 conservation analyses to all 363 available bird genomes, revealing that its mature sequence is identical across birds, with only two species showing a single-nucleotide substitution in the precursor (Fig. 5b, Extended Data Fig. 9, Supplementary Data 3) (Page 12, lines 393–400). This observation underscores that miR-2954 has been under strong purifying selection throughout avian evolution. The paragraph describing this work now reads:

Page 12, Lines 393-400 "We finally sought to investigate the origin and evolution of this avian dosage compensation system. To this end, we first screened for the presence of the miR-2954 locus across amniotes (Methods). Notably, miR-2954 is present across bird genomes but not in the genomes of any other species (including crocodiles – the closest living bird relatives), which, however, all contain the host gene of miR-2954 in birds, *XPA* (Fig. 5b and Extended Data Fig. 9). Strikingly, not only the mature sequence (22 nt) but also its precursor sequence (68 nt) of miR-2954 are identical

across 351 of the 363 examined species[48]. The exceptions are two species, each with a single nucleotide substitution in the precursor sequence that does not overlap the mature sequence, and ten species with missing sequencing data for this region (Supplementary Data 3)."

7. Since they have identified a central function for miR-2954 the authors might consider giving it a second name that might be more easy to remember.

We truly appreciate this thoughtful suggestion; however, strict naming conventions for microRNAs (https://www.mirbase.org/help/#) unfortunately prevent us from assigning miR-2954 a second name.

Referee #3 (Remarks on code availability):

I looked at the code only superficially, and it could use a bit better documentation.

We have sought to optimize the documentation of our code.

We would like to thank the referee again for the positive appreciation of our work and all their comments, which were very useful and helped us to improve the manuscript.

We are thrilled about the editor's and reviewers' highly positive feedback on our work and their final very useful comments and suggestions.

Please find below our responses.

Referee #2 (Remarks to the Author):

This is a revised version of the authors' previous work that identified a male-biased miRNA that is responsible for preferentially downregulating the Z-linked genes in chicken, as a mechanism of dosage compensation. I appreciate the authors' efforts of answering my previous concerns and questions, after performing many additional analyses. My previous main cricitism was about the Figure 4-5, which are difficult to find enough novelty from their previous works of Warnefors et al. During this revision, the authors have mostly addressed my cristicism, although I think the pattern of Z-linked targeted genes still comprises a minor advancement, I found the pattern of female targeted genes interesting. Overall, I think this version represents a significant improvement compared to the previous version, and some minor revions are only needed. My detailed comments are as follows:
We thank the referee for the positive feedback and remaining minor points, which we address below.

1. Let me summersize the novelty based on the answers from the authors to my first question, with some respective questions and comments. These questions are not expected to change the conclusion, but only I hope to refine their conclusions:

1) The Z-linked targeted genes exhibit distinict sequence features compared to autosomal taregets by miR-2954, including longer/more abundant target sites and higher seed-pairing stablity. This explains the specificity of miR-2954 to the Z rather than the autosomes, but it should be made clear that these are all predicted results. As the target genes here are derived from the KO experiment rather than the RNA-binding assays of miR-2954. In addition, when comparing these targets between autosomes and Z chromosomes, are they all direct targets (i.e., with predicted target sites).

We now more carefully describe in the beginning of the relevant section what we mean by "experimentally validated" targets; that is, computationally predicted Z-linked targets that were significantly differentially expressed in at least one tissue of homozygous male KOs, suggesting (but not definitely proving) that represent actual targets of miR-2954 (Page 5, Lines 223-226).

2) In both transcription and translation levels, the authors found upregulation of female Z-linked genes relative to the ancestral expression level approximiated by mouse, particularly the dosage sensitive genes. This as far as I understand was not presented in the previous Warnefors et al's work, and is an very important evidence for Ohno's model on how dosage compensation might have evolved.

Thank you for the appreciation of this finding.

3) The authors extended evolution analyses to about 300 bird genomes and revealed the precursor sequence of miR-2954 is highly conserved, but absent in the outgroup species of birds. This suggests the origination of this miRNA accompanied that of bird species and the ZW sex system. This is also an important finding, although this only predicts the conserved function, but is sufficient for the authors to make a suggestive claim. I think authors' claim 'advancing well beyond Warnefors et al. in every section of our paper' is an overstatement, and authors' conclusion at the begnining 'extend and refine the insights' is a more precise description.

Thank you for the appreciation of our findings. We agree that the phrase 'extend and refine the insights' more accurately characterizes our overall advancement in relation to prior work, including Warnefors et al. We would like to note that we do not make any evaluative statements in terms of novelty in our manuscript but just describe our findings in light of previous work.

2. For the previous question 3, if I understand it correctly, the authors suggested that Z-linked genes aquired sequence

contexts that enabled them more frequently being targeted by the miR-2954, without too much changes to the autosome genes, is this correct? If so, one would predict that compared to outgroup species that lacks such a dosage compensation mechanism, target sequence changes more often and more dramatically occurred to between orthologs of avian Z linked genes vs. their reptilian/mammalian couterparts, relative to those autosomal ortholog pairs. Is that so?

Yes, this is correct. Our results support that Z-linked genes in chickens have acquired sequence features that make them more frequently targeted by miR-2954, whereas autosomal genes have remained largely unchanged in this regard. To further test this notion, we performed a new analysis comparing target site density (normalized by 3' UTR length) between chickens and humans, using human as an outgroup species (Page 8, Lines 367-370). We found that Z-linked chicken genes have significantly higher target site density than their 1:1 human orthologs (Extended Data Fig 10b). In contrast, autosomal genes show no significant difference between chicken and human. These observations support our original notion that Z-linked genes have specifically gained target sites that facilitate effective targeting by miR-2954 during evolution.

3. Again, for question 4, I think the new Ribo-seq analyses comprise a great effort clarifying dosage compensation effects at the transcription vs. translation level, which eventually may achieve a complete compensatory effect. I appreciate the authors' work in doing this.

Many thanks for appreciating our new translatome analyses.

Referee #3 (Remarks to the Author):

The revised version of the paper has comprehensively addressed the previous concerns. I just have a few minor points (see below) and I support acceptance of this article.

We thank the referee for the positive assessment and support for acceptance. We appreciate the valuable feedback throughout the review process and have addressed the remaining minor points as outlined below.

1. I really appreciate the effort for orthogonal validation using a knock-down approach. The description in the results section so very vague ("using a specific inhibitor and delivery agent"). It would be helpful to describe the nature of the "inhibitor".

We now briefly describe the nature of the inhibitor in the main text (Page 5, Lines 206-208: "(i.e., complementary chemically modified oligonucleotides that sequester miR-2954")), leaving details to the Methods section, given the strict space constraints for the main text and to also be consistent with the level of details of other methods used in our work.

2. Some labels in the extended data figures are very small. A bigger font size would make them more accessible (e.g. EDFig. 5a)

We agree and thank the referee for pointing this out. We have increased the font sizes across all relevant figures to improve clarity and accessibility.

3. In Fig. 2a or in the corresponding part of the text, it would be useful to state how many expressed genes are on the Z chromosome in total, to better understand what fraction of all genes are predicted to be miR-2954 targets, of which then later ~50% are upregulated in the knock-out.

Figure 2a is based on the analysis of total number of protein-coding genes. We considered adding the number of developmentally expressed Z-linked genes to the figure but felt it could cause confusion in this context. However, we do explicitly report this in the text: approximately 54% (311 out of 576) of all developmentally expressed Z-linked genes are upregulated in the knockout (Page 6, Lines 274-276), providing the relevant context for interpreting the extent of miR-2954 targeting.

Referee #3 (Remarks on code availability):

The Github repository is missing a readme file.

We have now added a README file to the GitHub repository to provide an overview of the data and codes.

Referee #4 (Remarks to the Author):

The manuscript describes a novel mechanism of dosage compensation through the evolution of an avian miRNA (miR-2954) that preferentially targets mRNAs on the Z chromosome. This mechanism has several potential advantages, including enhanced specificity (i.e. targeting only dosage-sensitive genes rather than silencing the entire sex chromosome). The authors generate a miR-2954 knockout chicken and describe both gross and molecular phenotypes. miR-2954 is male essential, and homozygous knockout results in completely penetrant male embryonic lethality. Furthermore, this miRNA is expressed at much higher levels in male chickens, enabling its male-specific role. Predicted targets of miR-2954 accumulate in the knockout animal, and Z-linked targets experience a much greater degree of derepression than autosomal targets. Further examination of predicted targets shows that Z-linked targets are predicted to be more strongly repressed by miR-2954. Experimentally supported Z-linked targets are more likely to be dosage sensitive and are more broadly expressed compared to Z-linked nontargets. Evolutionarily, the authors show that Z-linked miR-2954 targets have been upregulated in females at the transcriptional level, highlighting the need for the concurrent evolution of some method of repression in males. Moreover, Z-linked target genes are more similarly expressed between male and female chickens than Z-linked nontarget mRNAs. Finally, the authors show that this mechanism of dosage compensation occurs across bird species but does not extend to other closely related species (such as crocodile). With respect to miRNAs, this work elevates the known importance of miRNA-mediated regulation and describes the most severe phenotype of a recently emergent miRNA. With respect to dosage compensation, the discovery of this essential component of avian dosage compensation fills a critical gap in the field and brings the understanding of dosage compensation in birds closer to the much more intensively studied mechanisms of mammals, flies, and worms. (Although one reviewer is concerned that the idea of miRNA-mediated dosage compensation and some informatics supporting this idea had been published earlier, the KO phenotype and the molecular analyses enabled by the KO are major advances that will elevate the interest in this study far beyond that of the previous one.)

We are very grateful for the referee's very positive feedback and expert comments below, which helped us to refine and finalize our manuscript.

Minor concerns:
1. Line 99. miR-2954 is expressed from an intron of XPA, but not all miRNAs expressed from introns are mirtrons, and miR-2954 is not a mirtron. For mirtrons, the splicing machinery excises the pre-miRNA in a form that it can be processed by Dicer without need for processing by Drosha. In contrast, miR-2954 appears to be a standard Drosha-dependent miRNA.

Thank you for this important comment. We have corrected the text to reflect that miR-2954 is intronic but not a mirtron (e.g., Page 3, Line 89).

2. Extended Data Fig. 1. The authors might mention in the legend that miR-1583 (also shown in the figure) is not in the miRGeneDB database and thus is not a confidently annotated microRNA.

Thanks for pointing this out. We have added a note to the legend of Extended Data Fig. 1 clarifying that miR-1583 is not listed in miRGeneDB and is therefore not considered a confidently annotated microRNA.

3. Figure 1a. It would be helpful to indicate when the different sex chromosomes emerged. As the figure currently stands, the XY chromosomes of lizard and human appear to be ancestral to the tree, when in fact they presumably emerged independently from different autosomes of their common ancestor.

We have added a note to the figure legend indicating that the XY sex chromosomes in lizards and humans evolved independently from different ancestral autosomes.

4. Lines 179-180. As currently phrased, this sentence implies that different miRNAs have different prominent modes of action. In reality, all of the miRNAs seem to have the same prominent mode of action, which is to accelerate poly(A)-tail shortening of most of their targets. In most contexts, this tail shortening causes more rapid decapping and degradation, but in some contexts (most notably, pre-gastrulation embryos) tail shortening lowers translational

efficiency. A suggested edit would be to delete the word "most" and just say "… the predominant mode of action of microRNAs in most contexts."

Thanks for the good suggestion. The sentence now reads (Page 4, Lines 153-156): "…consistent with the predominant mode of action of microRNAs in most contexts[23-25]".

5. Figure 2A. The authors might consider plotting the data on the left as cumulative distribution functions. The authors should also compare their predicted targets to UTR-length-matched cohorts of nontarget mRNAs instead of comparing to all nontarget mRNAs (as we know predicted miRNA targeting correlates with UTR length.) Additionally, from the legend, it is unclear how the data from E3 and E5 were combined.

While we retained the boxplots in Figure 2a for consistency and clarity, we have now included cumulative distribution function plots of the same data in Extended Data Fig. 3c (cited on Page 4, Line 146).

To control for the known correlation between 3' UTR length and miRNA target prediction, we performed a new analysis comparing target site density (i.e., number of sites per kb of UTR) between Z-linked and autosomal genes. This normalization accounts for UTR length. The results confirm that Z-linked genes have a significantly higher target site density. We have included this analysis in the revised manuscript (Page 8, Lines 367-375; Extended Data Fig. 10b).

We now clarify in the legend of Figure 2a that expression differences between genotypes were calculated using a statistical model that accounts for embryonic age.

6. Figure 2; Figure 4a. When discussing these and other data indicating weaker sites on autosomes, the authors might want to include in their model the idea that autosomal mRNAs have presumably evolved to avoid miR-2954 targeting, with the result that the seed matches that remain in autosomal 3'UTRs are minimally effective. Indeed, this selective avoidance of autosomal targeting could be shown in an expanded version Figure 5a.

While we agree that there may have been selection against the evolution of target sites in 3'-UTRs of autosomal genes in birds, our previous (Extended Data Fig. 10a) and especially our new (density) analyses of target site evolution (Extended Data Fig. 10b), stimulated by comments from this reviewer (see previous point) and comment #2 from reviewer #2, suggest that there was strong selection for the evolution of efficient target sites in 3'-UTRs of Z-linked genes, whereas the random emergence of (weak) spurious sites could be tolerated (i.e., has overall not had a deleterious impact). That is, our new analyses show that target site densities are similar between human Z orthologs, other human autosomal genes, and chicken autosomal genes, but significantly higher for chicken Z-linked genes (Extended Data Fig. 10b). The new analyses our now outlined in the main text on Page 8, Lines 367-375.

7. Figure 4e; Lines 283-290. A brief discussion of interpretation of the Tau score would be helpful.

We have added a clarification to the legend of Figure 4e stating that "Tau scores range from 0 (broad expression) to 1 (highly tissue- or stage-specific expression)". We also provide information about Tau scores in the Methods section (Page 17, Lines 824-830).

8. Figure 4f, Extended Data Fig. 5b, c. The description of the grey boxes is missing in the figure legend. More broadly, the implications of the red and grey boxes are confusing. For example, it seems the reader is meant to interpret p>0.05 for a log2(Current-Z/Proto-Z) of -1 (red box) and p<0.05 for log2(Current-Z/Proto-Z) of 0 or 1 (grey boxes) as log2(Current-Z/Proto-Z) being most similar to -1. This interpretation is incorrect, as p>0.05 cannot be used to assert that the value is equal to -1 but rather that it is not likely to be different from -1. The authors might consider making the direct comparison between the Z-nontargets and Z-targets within each tissue. This seems like a more appropriate comparison to demonstrate upregulation specific to Z-linked target genes. Alternatively, the authors could use an explicit equivalence test (ex. TOST test).

Thanks for this useful suggestion. We performed equivalence testing using the two one-sided test (TOST) procedure to formally assess whether gene expression levels are statistically equivalent to proto-Z levels. We have updated the Methods section to describe the TOST analysis (Page 18, Lines 867-873), and modified Figure 4f and Extended Data Figure 5b,c accordingly. The figure legends now clarify the interpretation of red and grey boxes, and the updated visualizations reflect the results of the TOST tests. The results of the TOST tests are in full agreement with our previous analyses; for example, the full upregulation of Z-target genes, as assessed by the current Z to proto-Z comparisons, is reflected in that their overall expression output is not significantly different from 0 (old analysis) as well as significantly similar (equivalent) to 0 (new TOST test).

9. Figure 4h. Why are there two red vertical lines?

The two red vertical lines indicate the positions of male hypermethylated regions MHM1 and MHM2, as is stated in the figure legend: "The positions of male hypermethylated regions 1 and 2 (MHM1 and MHM2) and *miR-2954* are indicated (red and green vertical lines)".

10. Lines 376-377. This sentence might be misread to be saying that the increased translation is global, occurring for mRNAs from all chromosomes, when in fact the increased translation is specific to mRNAs from Z.

Thanks for pointing this out. We have refined the text to properly reflect that the increased translation is specific to mRNAs from Z (Page 7, Lines 326-329): "These findings suggest that translational upregulation in females, reflected by higher translational efficiencies of Z-linked mRNAs in this sex (Extended Data Fig. 5d; see also ref. [45]), together with transcriptional upregulation in both sexes and miR-2954-mediated mRNA degradation in males, can lead to full protein-level dosage compensation of Z targets."

11. Figure 5a. Although drawing miRNAs as pairing at both their seed and 3' regions is not uncommon in these types of figures, it is not an accurate depiction of typical miRNA targeting. All of the targeting in this study was dominated by seed pairing, with little or no contribution of 3' pairing. Indeed, equal pairing at both the seed and 3' regions causes degradation of the miRNA, not the mRNA.

Thanks for pointing this out. We have modified Figure 5a to more accurately reflect seed-dominated miRNA targeting.

12. Figure 5c, 5d. The legend could be clarified. For example, "Proportions of miR-2954 target genes on autosomes (Pre-A) and Z chromosome (Pred-Z) in chicken that are shared between chicken and zebrafinch."

We clarified the legend as suggested.

13. Line 446. This statement should be softened, by for example, adding the word "presumably" before "because."

We have added "presumably" before because as suggested.

14. Lines 448-450. The logic here is a bit mixed up. First, Ref. 15 shows that plenty of single-miRNA knockouts have strong or lethal phenotypes. Secondly, miR-2954 is the only member of its family, and so from this perspective the lethal phenotype is not remarkable. However, what is remarkable is that the miR-2954 KO is (to my knowledge) the most severe knockout phenotype of a miRNA that has emerged since the last common ancestor of amniotes.

Thanks for this useful comment and information and appreciating the remarkable phenotype of such a young microRNA. We realized that an evaluative statement at this point in the discussion is not needed, and so removed this sentence altogether.

Referee #5 (Remarks to the Author):

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

Thank you for your efforts.

Referee #6 (Remarks to the Author):
Upon request from the editor, I evaluated the resubmission and authors' response to review by a previous reviewer (reviewer 1) who was unavailable for a response. The reviewer raised several constructive questions/comments on the previous version that the authors' addressed extensively in their revision (see below). This lead to refined and additional analyses resulting in improvement of the manuscript and stronger support of the conclusions drawn. The paper is based on good methodology, data quality, and analyses. It is well written and presented. All the comments of Reviewer 1 are sufficiently and well addressed.
Overall, this very interesting manuscript "A male-essential microRNA is key for avian sex chromosome dosage compensation" addresses the phenomenon of dosage compensation mechanisms in birds (ZZ/ZW, male/female sex chromosome system). It convincingly shows that a microRNA, miR2954 with strongly males-biased expression levels

is essential for Z-linked dosage compensation. The paper blends functional/mechanistic in vivo studies with an evolutionary analyses.
First, it shows that knock-out of miR-2954 in chicken using Crisp/Cas technology results in very high, male-specific early-stage embryo mortality in knockout homozygotes, and, that this likely results from specific upregulation (disinhibition) of dosage-sensitive Z-linked target genes. Second, an analysis of the phylogenetic distribution of miR-2954 suggests that dosage-sensitive target genes on the female Z-chromosome underwent transcriptional and translational upregulation. From this the authors propose a somewhat speculative scenario in which evolutionary pressures resulting from W-gene loss selected for transcriptional and translational upregulation of dosage-sensitive Z- genes in females; the also upregulated transcription in males produced excess transcripts from their two gene copies (ZZ) which then is cancelled by the degradation of transcripts via miR-2954. This microRNA-mediated sex chromosome dosage compensation mechanism became critical for male embryo survival.

Below I comment on the authors' response to the main comments/questions of previous reviewer 1.

We are delighted by the referee's very positive feedback and grateful that they carefully checked and fully approved our revisions made in response to the original referee #1 (marked in **green** here, to facilitate reading).

1. The system of CRISPR/Cas genome editing of germ cells to delete miR2954 in elegant and has been published previously. Given the embryo lethality of the miR2954 KO (knockout) phenotype, can the authors be sure that they have targeted this locus only? Could there be off-targeting events? Does the repeated out-crossing definitely rule out off-targeting? Could the Z-targeting affected other parts of the Z chromosome, hence the male-specific mortality.

The authors address this comment quite well. They argue that their repeated outcrossings and absence of a phenotype in heterozygous knockout males and hemizygous knock-out females strongly rule out off-target effects on the autosomes; to rule out off-target effects on closely linked regions on Z, they conducted deep long-read genome sequencing of knockout males and controls. This analyses reveal no evidence for off-target effects and are included in the revision.

The authors point out that their revision also includes new results from an orthogonal in vivo knockdown approach which shows that miR-2954 is essential for male embryo survival supporting the knockout results sex-specific embryo mortality. Moreover, additional new assessment of annotation of the dosage-sensitive Z-linked target genes reveal critical roles in development (and adulthood) that may be functional linked to the male embryo lethality.

2. The lethality of miR2954 KO only in homozygous males (ZZ) is very striking. Could the authors consider why this is manifest after E7 and not before?

In response to this query, the resubmission includes a paragraph/data that address the high male embryo mortality of miR2954 knockouts before ED7. Reported developmental abnormalities from upregulation of the dosage-sensitive, Z-linked genes include delayed overall growth, small eyes/brains, stunted elongation (Supp. Data 1 and 2) suggesting critical functional roles in early developmental processes (development of e.g. vasculature, allantois, organs) whose disruption is lethal.

3. Can the authors show the normal endogenous expression pattern of miR2954? For example, using whole mount in situ hybridisation. There are good protocols for this, using LNA probes for small RNAs such as miRs.

The authors refer to their statement in the Introduction of published small RNA sequencing and Northern blot work showing elevated miR-2954 expression in adult organs and whole male embryos. In addition, they refer to their RT-qPCR quantification of the miRNA in E12 embryos.

4. RNA-seq was used to assess gene transcript abundance in WT, het and homozygous KO embryos (prior to the time of embryo lethality in the affected males). Targetscan was used to predict the potential targets of miR2954 and these were scrutinised for changes in the KO embryos (expected to go up, if miR2954 normally down-regulates them). I found this data very clear and convincing. Were there any genes that showed substantial down-regulation the miR2954 KO males?

The authors respond that 99+% of the differentially regulated Z-genes in knockdown homozygous miR 2954 males were up-regulated, while this fraction was much lower for autosomal targets. Only 4 genes were down-regulated (reported in Table 4) and that they prefer to not speculate about this small number of genes any further.

5. Could the authors provide more information on the annotations of the dosage sensitive Z-linked genes affected by miR2954. What, exactly, do these genes do? Any common thread?

In response to this query, a new analysis was performed and results are presented on page 10 (Suppl. Table 7). Functionally related target genes appear to play roles in development, multiple essential pathways, stem cell pluripotency, etc. This response is sufficient and links well to the observed embryo lethality in knockout miR2954 homozygous males.

6. The hypothesis that avian sex chromosome evolution first involved Z "up-regulation" due to gene loss on the W , followed by necessary male-specific down-regulation in ZZ males is interesting but I don't think the study addresses the first part of this scenario, only the second. It is stated in the Discussion: "We unveiled a scenario where, driven by the decay of the W chromosome, dosage sensitive genes became upregulated on the Z chromosome not only in (ZW) females but, as a secondary consequence, also in (ZZ) males." I think it is only the last part that the study has clearly demonstrated.

The authors respond extensively to this critique arguing that their work already included some of the support for the first part of their hypothesis but that they now expand their analysis: They argue that 1) their previously published work revealed an overall partial transcription upregulation in females; 2) in the present study they compare present-day Z-chromosome gene upregulation by miR2954 to those of an inferred ancestral state Z-chromosome and find Z-linked target genes of miR2954 being upregulated in female organs; 3) also newly included analyses using ribosome profiling (Extended data Fig. 5) show translational upregulation in females. These findings are presented in lines 365 – 378.
Together, I feel that this provides sufficient support for the authors' presented 2-step scenario for the evolution of this dosage compensation mechanism in birds.

Referee #6 (Remarks on code availability):

This is beyond my expertise.