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Targeted blocking of gene splicing can dysregulate intron-embedded primary microRNAs



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Addressing whether antisense oligonucleotides (ASOs)-based targeting of genes embedded with intronic noncoding RNAs (ncRNAs) affects the expression and function of intronic ncRNAs is crucial to the success of ASOs in clinical trials. While studying zebrafish posterior pituitary development—an important neuroendocrine interface—we found that an ASO targeting the *slit3* splice site, but not the translation start site, disrupts pituitary axonal morphogenesis. In addition to altered *slit3* splicing, we observed increased expression of *slit3*, its intron-embedded microRNA *mir-218a-1*, its longest intron, and the paralogous *slit2* gene. The ASO-induced phenotype does not occur when mature *mir-218a-1* is blocked by an ASO or in *mir-218a-1*^{-/-} mutants, or when the splicing of the exon immediately upstream of the *mir-218a-1* embedded intron is blocked by an ASO. The axonal phenotype also phenocopies in samples injected with *mir-218a* mimic in a dose-dependent manner. Our results indicate that despite the ASO-induced genetic compensation response, intron-retained transcripts can escape the nonsense-mediated decay (NMD) machinery, become stabilized, and lead to increased intronic primary microRNA expression and function. As prematurely terminated intron-retained transcripts can translocate to axons and affect neuronal function, our study warrants further validation for other classes of ncRNAs. Moreover, the idiosyncratic phenotypes observed with translation- versus splice-blocking ASOs can serve as markers to identify the role of intronic microRNAs.

Antisense oligonucleotides (ASOs) are one of the promising therapeutic options for monogenic disorders. Approximately 3000 human protein-coding genes are predicted to be haploinsufficient and a quarter of them are associated with diseases^{1,2}. In addition, splice-modulating ASOs are also under consideration for developing patient-specific ASOs^{3,4}. However, some ASOs can affect mRNA and pre-mRNA levels differently. Premature termination of intron-retained transcripts has even been observed in the axons of vertebrate animal models, as well as in the axons of human amyotrophic lateral sclerosis patients^{5,6}. Generally, when testing the potential of candidate ASOs in vitro and in animal models, the focus is largely on the rescue of the affected protein function and/or the disease phenotype. However, relatively few studies have addressed if the intronic non-coding RNAs (ncRNAs) in

the targeted genes are affected by the host gene-targeting ASOs and the potential role of such ncRNAs in failure of ASOs in clinical trials^{7–10}. Such ncRNAs may reduce ASO efficiency by limiting access, altering RNA structure, or modifying transcript abundance in a direct or indirect manner.

In some tissues such as the brain or liver, intronic RNA can constitute a major proportion of the total cellular RNA^{11–14}. One of the well-studied ncRNA are the miRNAs and approximately 40–50% of all the miRNAs in human, mice and chicken genome are of intronic origin^{15–17}. Some intronic miRNAs can target their host genes and function in a positive- or negative-feedback loop^{18,19}. Several of monogenic human disease-linked genes are embedded with intronic miRNAs^{20,21}. If ASOs targeting splicing of such genes must succeed in clinical trials, it is critical to understand if

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blocking the splicing of host genes affects the intronic miRNA expression and function.

Apart from the potential of ASOs in therapeutics, ASOs were also widely used in developmental biology studies and are still in use. However, despite the fact that several ASOs can phenocopy the mutants, some ASOs can cause non-specific phenotypes that cannot be reproduced by mutants^{22–24}. We observed a similar discrepancy in phenotypes in our results of *pank2* splice-blocking ASO injected zebrafish embryos and *pank2*^{−/−} mutant embryos^{21,25,26}. Generally, the idiosyncrasy in the strong phenotypes arising from ASO-injected embryos versus lack of a phenotype in zebrafish mutants are largely attributed to Tp53 (P53)-based off target effects (15–20% of all ASO experiments)^{23,24,27–29}. In our case, the injection of the *pank2*-specific ASO in *pank2*^{−/−} embryos, did not result in any phenotype, thus excluding this hypothesis²⁵. Alternatively, the discrepancy can be due to genetic compensation or lack of maternal zygotic genetic background^{27,30,31}. However, the fact that off-target phenotypes arising from blocking the splicing of a single gene are dependent on the ASO target sequence suggests additional roles due to the aberrant products of unspliced transcripts²⁷.

Whether ASOs targeting the splicing of genes embedded with intronic miRNA inadvertently affect embedded pri-miRNA transcripts expression and its mature miRNA function is still under debate. We addressed this question in three zebrafish genes *slit3*, *pank2* and *dnm2a* that contain the intronic *mir-218a-1*, *mir-103* and *mir-199-2a*, respectively. In this study, using zebrafish embryos as vertebrate model, we show that the phenotypes due to splice-blocking ASO targeting at least 3 of the intronic miRNA-embedded host genes *slit3*, *pank2* and *dnm2a* can be partially or completely rescued by blocking the intronic miRNAs.

Results

slit3 splice-blocking ASO upregulates *slit3* and its intronic primary miRNA transcript and disrupts pituitary axon development

Gene-embedded miRNAs are also in the introns of zebrafish protein-coding genes. Firstly, we performed a data-mining approach to identify all the zebrafish genes with intronic miRNAs as listed in the NCBI RefSeq annotated database. Out of 27,158 protein-coding genes, 66 genes contain intronic miRNAs and the majority of them contain 1 or 2 intronic miRNAs (Fig. 1A, B and Supplementary Data 1). Gene ontology (GO) term analysis revealed that intronic miRNA-containing genes were associated with functions such as developmental morphogenesis, calcium ion regulation, and other processes (Fig. 1C, D, Supplementary Data 2). During our data-mining analysis, we also found 10 and 65 protein-coding genes with intronic snRNAs and snoRNAs, respectively (Supplementary Fig. 1A–C, Supplementary Data 3 and 4). At least for these three classes of ncRNAs, we did not find any host genes that contain more than one class of ncRNA (data not shown).

Next, we wanted to narrow down few genes in which we could investigate if targeting the gene will affect the intronic miRNA embedded in the gene. Towards this, we compared the 66 miRNA-embedded genes with the zebrafish posterior pituitary glial cell-enriched transcriptome³². We identified 30 intronic miRNA-embedded genes with a minimum normalized expression count of 10 that were enriched in the glial transcriptome genes (Supplementary Data 5). Out of these 30 genes, we focused on *slit3*, an evolutionarily conserved axonal guidance protein-coding gene^{33,34}. *slit3* contains *mir-218a-1* in the intron between exon 14 and 15 that has been previously reported to target Slit receptor-coding *robo* genes³⁵.

To investigate if targeting *slit3* with a splice-blocking ASO will differently affect pituitary axons, we performed *slit3* knockdown experiments in zebrafish embryos with two different ASOs (morpholinos). The first ASO is a well-established *slit3* translation-blocking ASO^{36–38}. The second ASO was designed to block the *slit3* splicing by targeting the exon 9-intron 9 junction that codes for the leucine-rich repeat domain required for Robo receptor binding (Fig. 1E)^{33,39}. Splice-blocking ASOs can trigger exon skipping or intron retention in transcripts of the targeted gene²⁴. As expected, the *slit3* splice-blocking ASO resulted in the retention of intron 9, which will lead to the introduction of premature translation codons in the transcript (Fig. 1F).

In contrast to the control samples, we observed a stronger expression of exon 8-exon 9 amplicons and a reduction of exon 8-exon10 amplicons in the splice-blocked samples. This suggests that intron retention increases *slit3* expression or stabilizes the *slit3* transcripts, and the intron-retained longer transcript spanning exon8-exon10 is too long to be amplified under our polymerase chain reaction conditions.

Next, we used the oxytocin neuron transgenic reporter line Tg(*oxt*:EGFP) to visualize and quantify the number of pituitary-projecting axons (Fig. 1G, H). In comparison to control and translation-blocking ASOs-injected larvae, we observed a reduced number of pituitary-projecting axons (ϵ^2 effect size 0.54, p -value < 0.0001) in the 5 days post fertilization (dpf) larvae that were injected with the splice-blocking ASO at one-cell stage (Fig. 1H, I). This phenotype was not due to oxytocin neuron deficits, as the number of oxytocin neurons in the neurosecretory preoptic area (NPO) was similar between control, translation- and splice-blocking ASO injected larvae (η^2 effect size of 0.03, p value > 0.05) (Supplementary Fig. 1D–F, Supplementary Videos 1 and 2).

Since ASO-based phenotypes in zebrafish larvae can be also due to off-target effects mediated via Tp53, we tested if *tp53* knockdown can suppress the *slit3* splice-blocking ASO-induced phenotype²⁷. In comparison to control and *slit3* splice-blocking ASO injected larvae, co-injection of *tp53* translation-blocking ASO with *slit3* splice-blocking ASO did not result in any rescue in the number of pituitary-projecting axons (Supplementary Fig. 1G, ϵ^2 effect size 0.72, p -value < 0.0001). These results suggest that *slit3* splicing-blocking ASO induced pituitary axonal defects are not due to TP53-based off-target effects that can be encountered in ASO studies.

We next tested if the two *slit3*-targeting ASOs affect the expression of *slit3* and *slit3*-embedded primary *mir-218a-1* transcripts by in situ hybridization technique (Fig. 1J). To observe primary *mir-218a-1* transcript expression, we designed and synthesized a probe that span the entire primary *mir-218a-1* and adjacent sequences⁴⁰. As a control for primary *mir-218a-1* expression, we chose another intronic *pri-mir-206* which has been previously reported to be expressed in the trunk muscles of zebrafish embryos⁴¹. In control samples, unlike *slit3* which has higher expression in forebrain and hindbrain, *pri-mir-218a-1* expression was broader and we did not observe similar enrichment in the forebrain and hindbrain (Fig. 1J). In comparison to control larvae, we observed an increase in expression of *slit3* and *pri-mir-218a-1*-coding transcripts in the splice-blocking ASO-injected samples. Unlike a strong increase in *pri-mir-218a-1* transcripts, we did not observe a similar increase in the expression of *pri-mir-206* in *slit3* splice-blocking ASO injected larvae (Fig. 1J). These results suggest that blocking of *slit3* splicing increases the expression of *slit3* and the *slit3* intron-embedded primary *mir-218a-1* transcripts.

slit3 splice-blocking ASO-induced phenotype is due to intronic *mir-218a-1*

Since primary miRNA transcript levels and mature miRNA levels are not always correlated, we next tested whether the expression of mature *mir-218a-1* is also affected by *slit3* splice-blocking ASOs. We performed in situ hybridization with LNA-based *mir-218a* probes. Compared to control samples, we observed increased expression of *mir-218a* in *slit3* splice-blocking ASO-injected samples ($n = 14/23$; $p < 0.01$ by Fisher's exact test) (Fig. 2A). Since the LNA-based probe can also bind to primary miRNA transcripts, we performed RT-qPCR-based analysis. Although we observed increased variability in expression levels in the injected *slit3* splice-blocked samples, we did not observe a significant increase in *miR-218a* levels (Fig. 2B, C).

Next, we tested whether *mir-218a-1* and *slit3* are involved in a feedback loop and whether *slit3* splice-blocking ASO can regulate such a regulatory mechanism. We injected one-cell stage embryos with *slit3* splice-blocking and *mir-218a*-blocking ASOs, either individually or in combination, and tested for expression changes by RT-qPCR analysis. As expected, in *slit3* splice-blocking ASO-injected samples, we observed a significant increase in *slit3* expression including the exons spanning the *mir-218a-1*-embedded intron (Fig. 2B). Blocking *mir-218a-1*, either individually or in combination

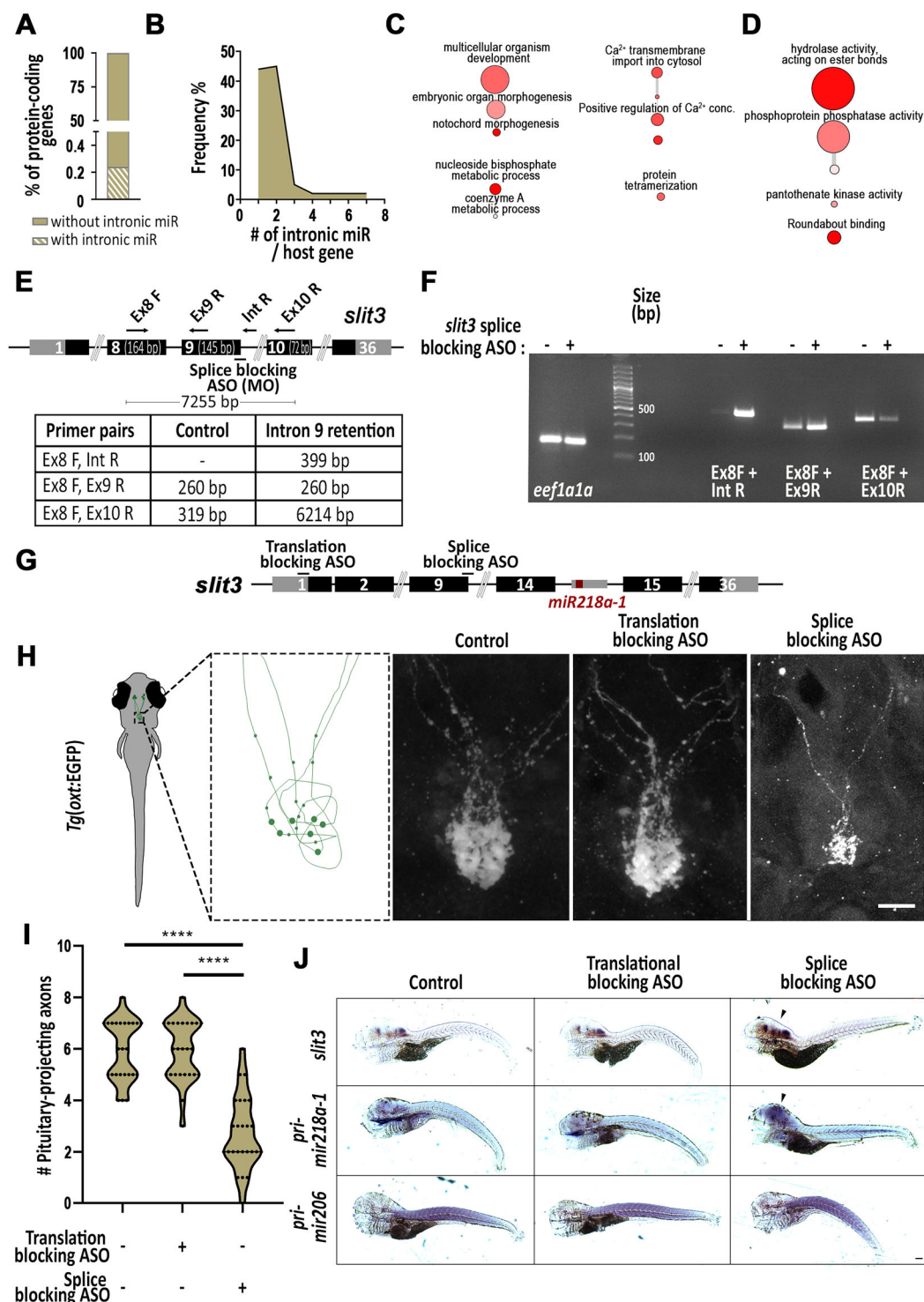


Fig. 1 | ASO targeting *slit3* splicing upregulates *slit3* and intron-embedded *pri-mir-218a-1* and causes axonal defects during pituitary development. **A** Graph showing % of zebrafish protein coding genes with and without intronic miRNA. **B** Graph showing the frequency % and number of miRNA per miRNA-embedded host gene. **C**, **D** Figure showing the enriched biological process (**C**) and molecular function (**D**) GO terms in host genes. The color and size of the bubble correspond to the P value and LogSize value of the GO Term, respectively. **E** Scheme of *slit3* gene and approximate location of splice-blocking ASO target and the oligonucleotide primers. The expected amplicon size upon intron retention event are also mentioned. **F** Gel electrophoresis image showing amplified mRNA splicing products in control embryos and embryos injected with a *slit3* splice-blocking ASOs. Amplicon products of housekeeping gene *eef1a1a* are also shown. **G** Scheme of *slit3* gene. ASOs designed to target *slit3* translation and splicing are denoted by black lines and

labelled. **H** Scheme describing the pituitary axons in transgenic reporter Tg(*ox:t:EGFP*) larvae and representative images showing whole-mount confocal microscope imaging of pituitary of 5 dpf transgenic Tg(*ox:t:EGFP*) zebrafish following immunostaining with anti-EGFP. Images are from control larvae and following microinjection of *slit3* translation- or splice-blocking ASOs. Scale 20 μ m. **I** Violin plot showing the number of pituitary-projecting OXT axons in 5 dpf control ($n = 15$) vs *slit3* translation-blocking ASO injected larvae ($n = 14$) and *slit3* splice-blocking ASO injected larvae (**** $p < 0.0001$; Kruskal-Wallis test). Analysis were performed from 3 independent injections. **J** Representative images showing the expression of *slit3*, *pri-mir-218a-1* and *pri-mir-206* observed by digoxigenin-labeled probes in 5 dpf control larvae and following microinjection of translation- or splice-blocking ASO against *slit3*. Scale 100 μ m.

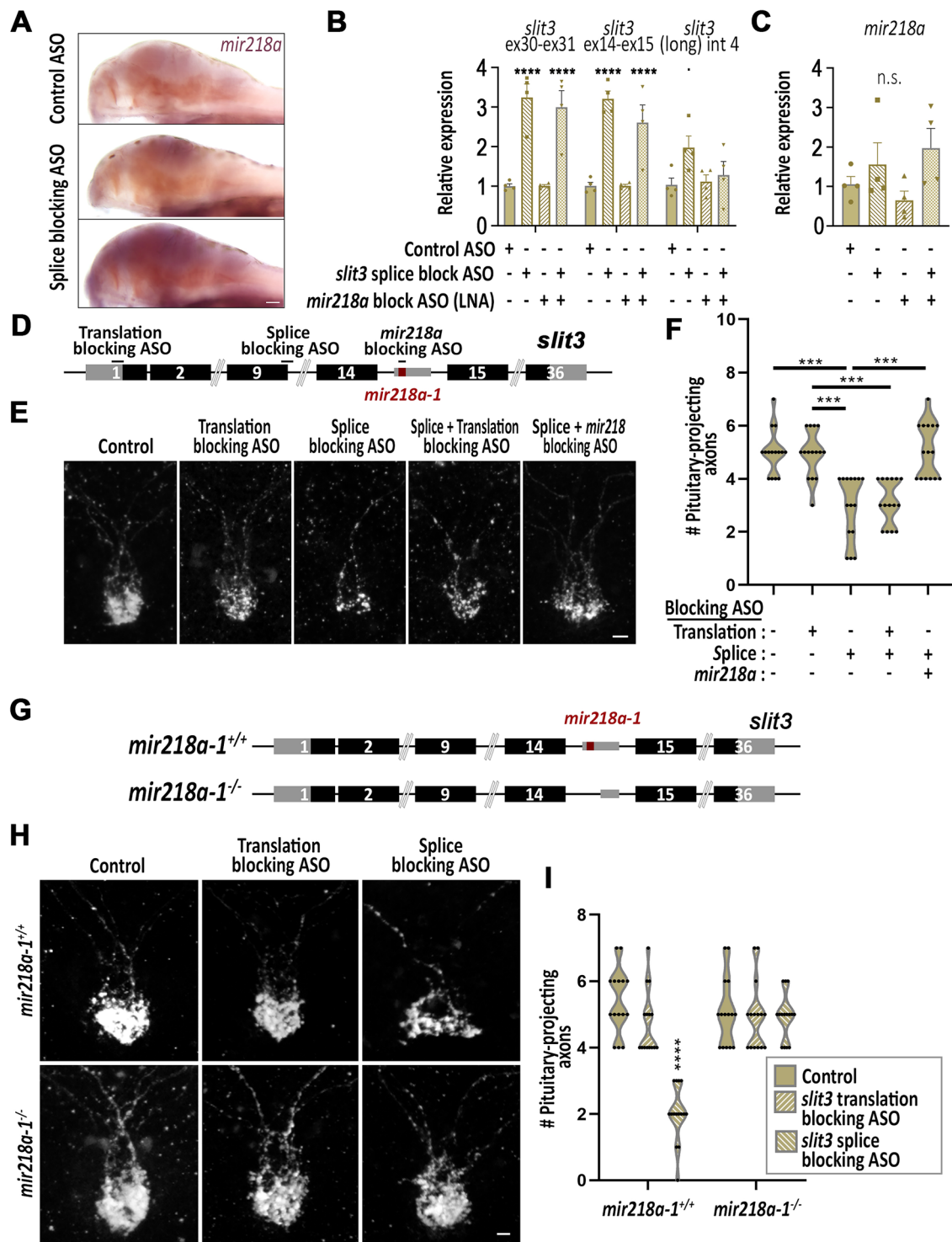


Fig. 2 | Axonal phenotypes due to *slit3* splice-targeting ASO require *mir-218a-1*.

A Representative images showing the expression of *mir-218a* observed by LNA-based digoxigenin-labeled probes in 5 dpf control ASO or *slit3* splice-blocking ASO-injected larvae. Correlation between the high *mir-218a* expression and splice-blocking ASO was determined by Fisher's exact test ($n = 14/23$; $p < 0.01$). Scale 50 μ m. **B** Graph showing the quantification of *slit3* expression by quantitative real-time PCR analyses. (**** $p < 0.0001$; $p < 0.10$; Two-way anova, $N = 4$. Error bars indicate SEM). **C** Graph showing the quantification of *mir-218a* expression normalized to U6 snRNA by quantitative real-time PCR analyses. (One-way anova, $N = 4$. Error bars indicate SEM). **D** Scheme of *slit3* gene. ASO designed to target *slit3* translation, splicing and *mir-218a-1* are denoted by black lines and labelled. **E** Representative images showing whole-mount confocal microscope imaging of pituitary of 5 dpf transgenic Tg(oxl:EGFP) zebrafish larvae following

immunostaining with anti-EGFP. Images from control larvae and following microinjection of individual or co-injected ASOs as listed. Scale 10 μ m. **F** Violin plot showing the number of pituitary-projecting OXT axons in 5-dpf control larvae or larvae injected with individual or co-injected ASOs as listed (*** $p < 0.001$; Kruskal-Wallis test, $n = 15$). **G** Scheme of *slit3* gene in *mir-218a-1* wildtype and knockout zebrafish. *mir218a-1* is denoted by red lines and labelled. **H** Representative images showing whole-mount confocal microscope imaging of pituitary of 5 dpf transgenic Tg(oxl:EGFP) zebrafish larvae following immunostaining with anti-EGFP. Images are from control larvae and following microinjection of *slit3* translation- or splice-blocking ASOs in *mir-218a-1*^{+/+} or *mir-218a-1*^{-/-} embryos at single-cell stage. Scale 10 μ m. **I** Violin plot showing the number of pituitary-projecting OXT axons in 5-dpf *mir-218a-1*^{+/+} vs *mir-218a-1*^{-/-} control larvae or larvae that were injected with *slit3* translation- or splice-blocking ASOs (*** $p < 0.001$; Kruskal-Wallis test, $n = 15$).

with *slit3* splice-blocking ASOs, did not significantly affect *slit3* expression (relative to control ASO) or *mir-218a-1* expression (relative to *slit3* splice-blocking ASO alone) (Fig. 2B, C). We also tested whether *slit3* splice blocking ASO affected the expression of the intron 4, which at approximately 120 kb is the longest intron in *slit3*. We observed an increase in expression of *slit3* intron 4 transcripts expression only in the *slit3* splice-blocking ASO-injected samples (Fig. 2B). Taken together, these results suggest that *mir-218a* does not regulate *slit3* expression and that blocking of *slit3* splicing upregulates *slit3* transcripts, including the long intron-containing primary transcripts.

Since we observed the expression of misspliced *slit3* transcripts in splice-blocking ASO-injected larvae, we next tested whether the axonal phenotype was due to the potential truncated Slit3 protein from misspliced *slit3* transcripts or the primary *mir-218a-1*. Toward this, we first performed ASO co-injection experiments. Specifically, we co-injected the *slit3* splice-blocking ASO with either *slit3* translation site-targeted or LNA-based *mir-218a*-targeted ASO (Fig. 2D). Only in the samples in which *slit3* splice-blocking ASO was co-injected with the *mir-218a*-targeting ASO, we observed a rescue in the number of pituitary-projecting axons with a large effect size of ϵ^2 of 0.53, p -value < 0.0001 (Fig. 2E, F).

Finally, to definitely prove that primary *mir-218a-1* dysregulation is the cause for the axonal morphogenesis phenotype, we undertook two approaches. In the first approach, we blocked *slit3* splicing in embryos of previously characterized *mir-218a-1*^{wz15} knockout fish (Fig. 2G)⁴². Specifically, we injected *slit3* translation- or splice-blocking ASO in *mir-218a-1*^{+/+} and *mir-218a-1*^{-/-} single-cell stage embryos. We observed a decrease in pituitary axons in the *mir-218a-1*^{+/+} larvae and not in *mir-218a-1*^{-/-} larvae with a large effect size of partial η^2 of 0.39 (Fig. 2H, I). Injection of the translation-blocking ASO did not have any effect on the pituitary axonal morphogenesis in neither of the genotype.

In the second approach, we injected an LNA-based mature *mir-218a* mimic. We validated the functionality of the mimic by testing the expression of its target genes, *robo1* and *robo2*^{35,43}. Compared to the control samples, the expression of *robo1* and *robo2* mRNA decreased in the samples injected with the *mir-218a* mimic (Supplementary Fig. S2A). Next, we quantified the pituitary axons in the samples. Compared to the control samples, we observed a dose-dependent decrease in pituitary axons in the samples injected with the *mir-218a* mimic (Supplementary Fig. S2B, ϵ^2 effect size 0.79, p value < 0.0001). These results complement the *mir-218a-1* mutant experiments and collectively suggest that, blocking of *slit3* splicing dysregulate *mir-218a-1* function and pituitary axonal projections.

***slit3* exon 14 splice-blocking ASO lacks an axonal phenotype**

Phenotypic penetrance can depend on the ASO target site. CRISPR gRNA-based targeting of the exons encoding functional domains also shows relatively higher phenotypic penetrance in zebrafish larvae⁴⁴. Since our *slit3* splice-blocking ASO targets the *slit3* exon 9-intron 9 (ex9-int9) junction, whereas the *mir-218a-1* loci is in intron 14, we next tested whether direct blockade of exon 14 splicing has similar effects on the pituitary axonal projections. To this end, we designed a new ASO targeting the *slit3* intron 13-exon14 (int13-ex14) junction and first characterized the efficacy of the ASO. We observed that *slit3* int13-ex14 blocking ASO injected samples exhibited intron 13 retention, but we observed only a relatively small decrease in intron-less exon 13-exon14 amplicons, suggesting that the ASO is not fully efficient in blocking of exon 14 splicing (Fig. 3A, B).

Next, we tested whether the *slit3* int13-ex14 blocking ASO also affected the development of pituitary projecting axons. In comparison to the control ASO-injected samples, and in contrast to the *slit3* ex9-int9 junction targeting ASO, we did not observe any significant effect on the number of pituitary projecting axons in the 5 dpf larvae injected with the *slit3* int13-ex14 junction targeting ASO at the one-cell stage (Fig. 3C, ϵ^2 effect size 0.70, p -value < 0.0001). Next, we tested whether the *slit3* int13-ex14 splice-blocking ASO had any effect on *slit3* or *mir-218a-1* expression or on *slit3* intron 4-containing transcripts. Compared to control samples, *slit3* int13-ex14 splice-blocking ASO injection had no significant effect on *slit3* or *mir-*

218a-1 expression or on *slit3* long intron expression (Fig. 3D, E). Taken together, our results suggest that although we observe intron retention events similar to those observed in exon 9 targeted ASO samples, this does not result in defects in axonal projections or increased *slit3* or *mir-218a-1* expression.

***slit3* exon 9 splice-blocking ASO triggers genetic compensation response**

To test whether the *slit3* exon 9 splice-blocking ASO target site has additional transcriptional regulatory mechanisms, bioinformatic analysis was performed. Specifically, we evaluated the conservation of the *slit3* ex9-int9 junction among teleost *slit3* orthologs in *Ensembl* database. We observed nucleotide-level conservation at the exon, splice junction, and the first few intronic bases (Fig. 3F and S3). While these findings suggest the potential conservation of splice junctions, they do not provide evidence for the conservation of intronic regulatory elements. Therefore, the effect of the *slit3* exon 9 splice-blocking ASO effect is unlikely due to the perturbation of a shared cis-element at this site. However, the potential involvement of lineage-specific or non-sequence-conserved regulatory elements cannot be ruled out.

Intron retention can lead to nonsense mutations that can trigger genetic compensation-based upregulation of paralogous genes^{30,31}. Since both of our *slit3* ex9-int9 and int13-ex14 splice site targeted ASOs led to intron retention, we tested whether they also led to genetic compensation-based expression regulation of *slit3* paralogs, namely *slit1a*, *slit1b*, and *slit2*⁴⁵. Compared to control ASO and *slit3* int13-ex14 splice-blocking ASO-injected samples, we observed a significant increase in the expression of *slit2* expression only in the *slit3* ex9-int9 splice-blocking ASO-injected samples (Fig. 3G, H). We also observed no significant effect on the expression of *slit3* paralogs in samples injected with *mir-218a* blocking ASO. These results suggest that in the case of the relatively efficient ASO targeting the ex9-int9 *slit3* splice site, which encodes the functional leucine-rich domains of Slit3 protein, we observe a genetic compensation response.

***pank2* and *dnm2a* splice-blocking ASOs phenotypes can be rescued by intronic *mir-103* and *mir-199*-blocking ASOs, respectively**

Next, we investigated the generality of the rescue of ASO-induced phenotypes by targeting the intronic miRNA in the host genes. Towards this, we chose the *pank2* and *dnm2a* gene which contain *mir-103* and *mir-199-2a* in its intron, respectively. We previously showed that injection of splice-blocking ASOs targeting *pank2* exon1-intron1 and intron1-exon2 junctions in 1-cell stage embryos leads to hydrocephalus and cardiac edema; however, neither a 4-bp nor a 50-bp deletion mutants of *pank2* recapitulated this phenotype^{21,25,26}.

We hypothesized that the *pank2* splice-blocking-induced phenotype could be partially due to the intronic *mir-103*. To test this, we injected 1-cell stage embryos either with control ASO or *pank2* intron1-exon2 junction targeting splice-blocking ASO. In addition, we also co-injected *pank2* splice-blocking ASO with LNA-based control ASO or *mir-103* blocking ASO (Fig. 4A). Unlike the control ASO-injected embryos, majority of the *pank2* splice block-injected embryos developed hydrocephalus and cardiac edema (Fig. 4B–D, G). Co-injection of *pank2* splice-blocking ASO with *mir-103* block ASO led to a partial rescue of the phenotype, whereas co-injection with a control ASO did not result in a partial rescue (Fig. 4E–G, Cramér's V effect size 0.56, p < 0.0001). The phenotypes were also not due to TP53-based off-target effects as co-injection of *pank2* splice-blocking ASO with *tp53* translation-blocking ASO did not rescue the phenotypes (Supplementary Fig. S4A, Cramér's V effect size 0.55, p -value < 0.001). These results suggest that the phenotypes resulting from use of *pank2* splice-blocking ASO can be partially rescued by injection of intronic *mir-103*-targeting ASOs.

Next we investigated if the previously reported *dnm2a* splice-blocking ASO-induced gross developmental phenotypes are also due to its intronic *mir-199-2a* that contains duplex miRNAs *mir-199-3p* and *mir-199-5p*^{46,47}.

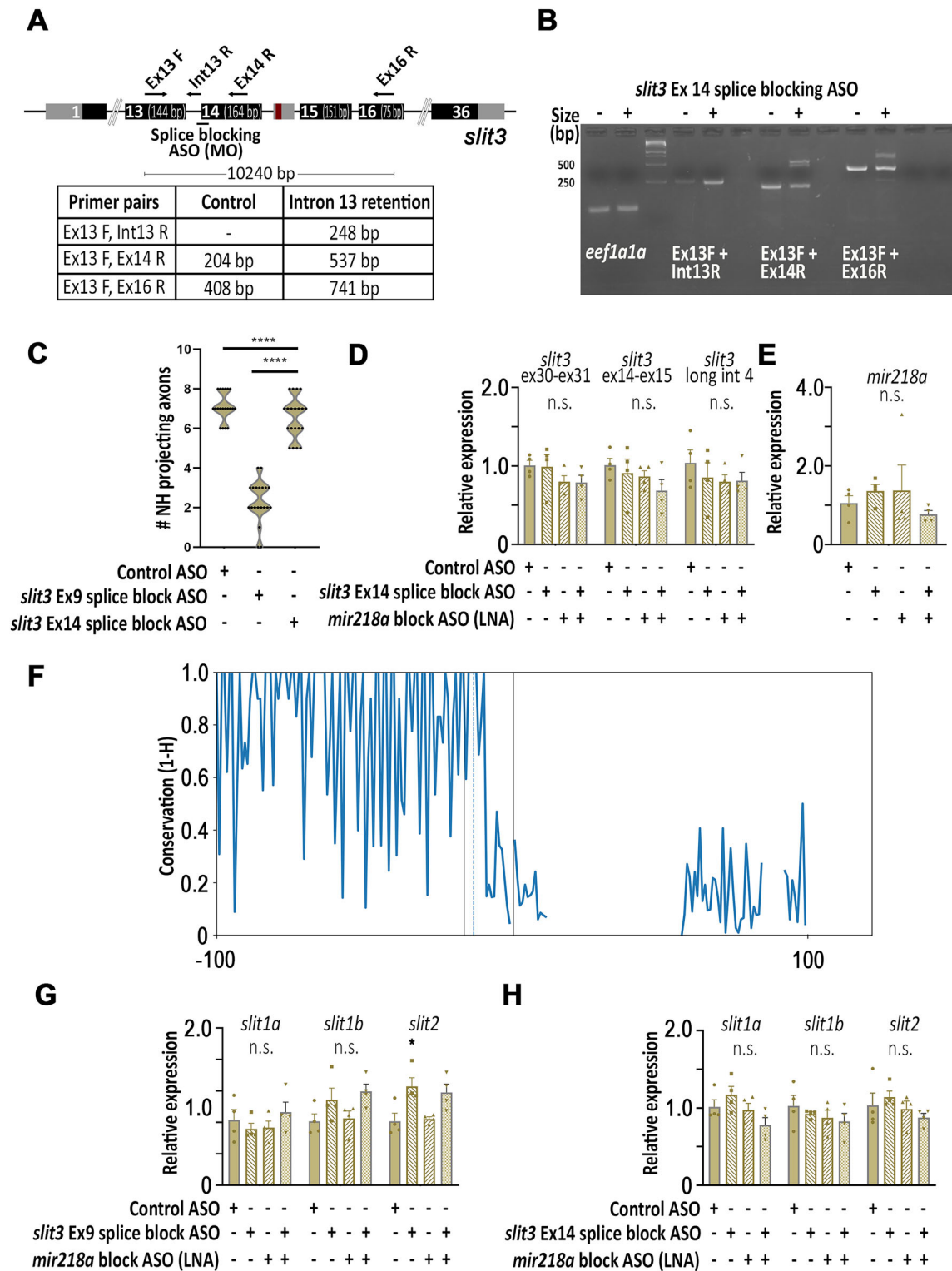


Fig. 3 | *slit3* exon 14 splice site-targeting ASO lacks axonal phenotypes. **A** Scheme of *slit3* gene and approximate location of intron13-exon14 splice-blocking ASO target and the oligonucleotide primers. The expected amplicon size upon intron 13 retention event are also mentioned. **B** Gel electrophoresis image showing amplified mRNA splicing products in control embryos and embryos injected with a *slit3* splice-blocking ASOs. Amplicon products of housekeeping gene *eef1a1a* are also shown. The expected splice products and the size of the amplicon products are described in Supplementary Data 6. **C** Violin plot showing the number of pituitary-projecting OXT axons in 5-dpf larvae that were injected with control ASO, *slit3* ex9-int9 or int13-ex14 splice blocking ASOs at single-cell stage (**** $p < 0.0001$; Kruskal-Wallis test, $n = 20$). **D** Graph showing the quantification of *slit3* expression by quantitative real-time PCR analyses. (Two-way

anova, $N = 4$. Error bars indicate SEM). **E** Graph showing the quantification of *mir-218a* expression normalized to U6 snRNA by quantitative real-time PCR analyses. (One-way anova, $N = 4$. Error bars indicate SEM). **F** Sequence conservation 1-H per alignment column, where H is nucleotide relative entropy. Dashed line marks the exon-intron junction. Black rectangle marks *slit3* ex9 splice block ASO target. **G** Graph showing the quantification of *slit3* paralogue expression by quantitative real-time PCR analyses in larvae that were injected with control ASO, *slit3* ex9-int9 splice blocking or *mir-218a*-blocking ASOs. (* $p < 0.05$; Two-way anova, $N = 4$. Error bars indicate SEM). **H** Graph showing the quantification of *slit3* paralogue expression by quantitative real-time PCR analyses in larvae that were injected with control ASO, *slit3* int13-ex14 splice blocking or *mir-218a*-blocking ASOs. (Two-way anova, $N = 4$. Error bars indicate SEM).

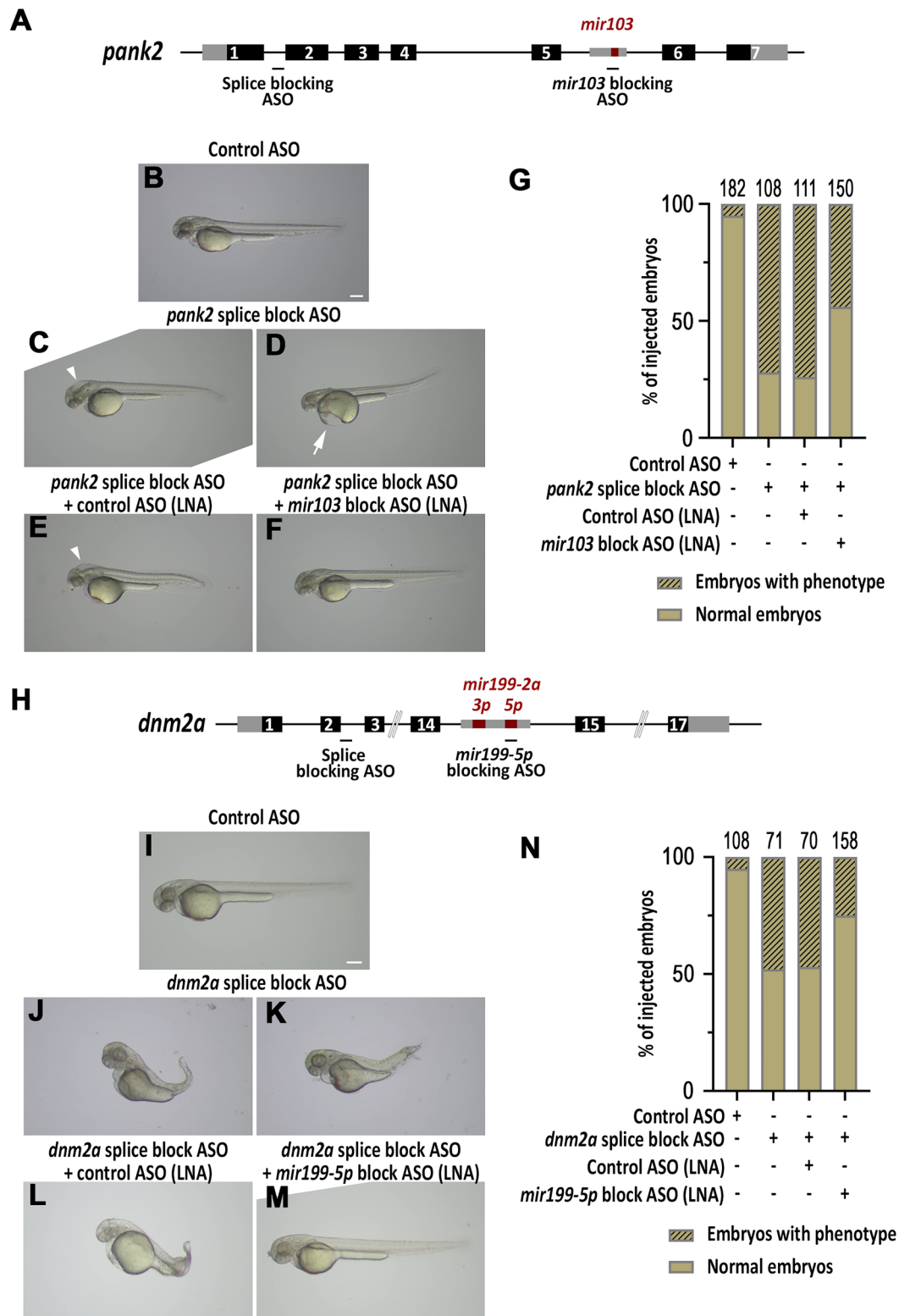
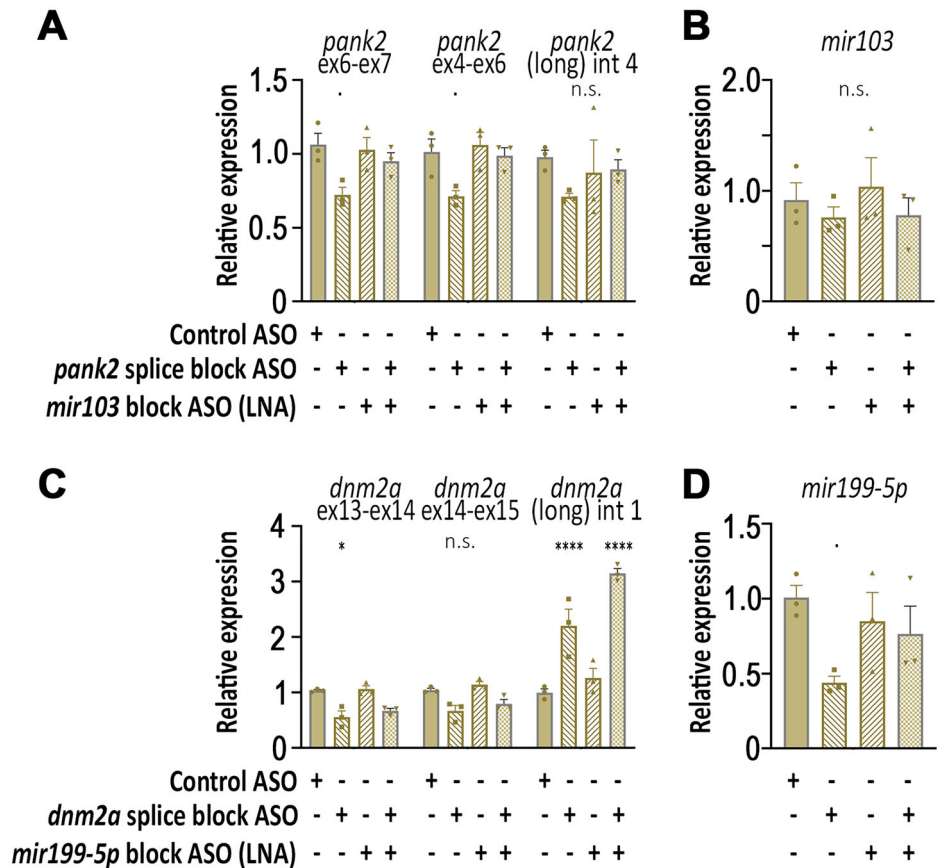


Fig. 4 | Developmental phenotypes due to *pank2* and *dnm2a* splice-blocking ASOs can be partially rescued using *mir-103* and *mir-199-5p* blocking ASOs, respectively. **A** Scheme of *pank2* gene. ASOs designed to target *pank2* splicing and *mir-103* are denoted by black lines and labelled. **B–F** Representative images showing whole-mount light microscope imaging of zebrafish embryos at 2 dpf. Images from embryos following microinjection of individual or co-injected ASOs as listed. White arrow heads indicate hydrocephalus and white arrow indicate cardiac edema. Scale 250 μ m. **G** Stacked bar plot showing the analysis of morphological evaluation of embryos performed at 2-dpf and the number of embryos in which the analysis was performed is listed on the top of each bar. Morphological analysis were performed

from 3 independent injections. $\chi^2 = 124.7$, $p < 0.0001$, $df = 3$. **H** Scheme of *dnm2a* gene. ASOs designed to target *dnm2a* splicing and *mir-199-5p* are denoted by black lines and labelled. **I–M** Representative images showing whole-mount light microscope imaging of zebrafish embryos at 2 dpf. Images from embryos following microinjection of individual or co-injected ASOs as listed. Scale 250 μ m. **N** Stacked bar plot showing the analysis of morphological evaluation of embryos performed at 2-dpf and the number of embryos in which the analysis was performed is listed on the top of each bar. Morphological analysis were performed from 3 independent injections. $\chi^2 = 58.5$, $p < 0.0001$, $df = 3$.

Fig. 5 | *mir-103* and *mir-199-5p* differentially regulate *pank2* and *dnm2a* splice-blocked transcripts. A Graph showing the quantification of *pank2* expression by quantitative real-time PCR analyses. ($p < 0.10$; Two-way anova, Error bars indicate SEM). **B** Graph showing the quantification of *mir-103* expression normalized to U6 snRNA by quantitative real-time PCR analyses. (One-way anova, $N = 3$. Error bars indicate SEM). **C** Graph showing the quantification of *dnm2a* expression by quantitative real-time PCR analyses. (* $p < 0.05$; **** $p < 0.0001$; Two-way anova $N = 3$. Error bars indicate SEM). **D** Graph showing the quantification of *mir-199-5p* expression normalized to U6 snRNA by quantitative real-time PCR analyses. ($p < 0.10$; One-way anova, Error bars indicate SEM).



To test this, we injected 1-cell stage embryos with control ASO or exon2-intron2 targeting splice-blocking ASO. In addition, we also co-injected *dnm2a* splice-blocking ASO with LNA-based control ASO or *mir-199-5p*-blocking ASO (Fig. 4H). We chose to block only *mir-199-5p* because it has been shown to be more abundantly expressed than *mir-199-3p*⁴⁸. Unlike the control ASO injected embryos, majority of the *dnm2a* splice-blocking injected embryos developed shorter body axis, cardiac edema, and an upward tail curvature (Fig. 4I–K, N). Co-injection of *dnm2a* splice-blocking ASO with *mir-199-5p* blocking ASO led to a partial rescue of the phenotype, whereas co-injection with a control ASO did not result in a rescue (Fig. 4L–N, Cramér's V effect size 0.38, $p < 0.0001$). The phenotypes were also not due to TP53-based off target effects as co-injection of *dnm2* splice-blocking ASO with *tp53* translation-blocking ASO did not rescue the phenotypes (Supplementary Fig. S4B, Cramér's V effect size 0.47, p -value < 0.001). These results suggest that the phenotypes resulting from *dnm2a* splice-blocking ASO can also be partially rescued by injection of intronic *mir-199-5p* targeting ASOs.

We next tested whether the rescue of the phenotype upon the injection of *mir-103*- and *mir-199*-targeting ASO was due to rescue of *pank2* and *dnm2* expression or effects on the respective intronic miRNAs. To this end, we injected splice-blocking and *mir*-blocking ASOs into one-cell stage embryos. Compared to control ASO-injected samples, *pank2* and *dnm2a* splice-blocking ASO-injected samples showed a decrease in the expression of *pank2* and *dnm2a/mir199-5p*⁴⁶. We did not observe a significant decrease in *mir-103* levels in *pank2* splice-blocking ASO-injected samples. Blocking *mir-103* and *mir-199-5p* had no effect on *pank2/mir-103* and *dnm2a/mir-199-5p* expression, respectively. However, *mir-103*-blocking ASO and *pank2* splice-blocking ASO co-injected samples showed a rescue in *pank2* expression, whereas we did not observe a similar rescue effect in *dnm2a* expression in *mir-199-5p* and *dnm2a* blocked samples (Fig. 5A–D).

Moreover, when we tested the changes in the expression of the longest intron in *pank2* and *dnm2a*, we observed the increase only in the intron 1 of *dnm2a* and not in intron 4 of *pank2* (Fig. 5A, C). Overall, our results suggest that blocking intronic miRNA can rescue the host gene splice-blocking ASO-induced phenotypes, but the mechanisms seem to differ between host genes.

Discussion

Previous studies using ASO in zebrafish have largely attributed ASO-induced phenotypes that are not reproduced by knockouts to TP53-based off-target effects (15–20% of all ASO experiments)^{23,24,27}. However, our data suggest that there is an additional possible explanation for the observed phenotypes. When *slit3* splicing is specifically blocked at exon 9, which encodes the leucine-rich repeat domains required for Robo receptor binding, our results indicate that although a genetic compensation response occurs, some of the *slit3* transcripts appear to escape the nonsense-mediated decay (NMD) machinery and, in addition, the increased primary *mir-218a-1*-derived from intron 14 causes the axonal phenotype. We were able to reproduce these phenotypes by the injection of *mir-218a* mimics. *mir-218a-1* does not seem to affect *slit3* expression, but it can potentially target and regulate the expression of not only *robo* genes but also any of the conserved target genes (Amin et al., 2021).

Previously, studies in zebrafish and mice have shown that *mir-218a-1*/ *Mir-218-2* embedded in the *slit3/Slit3* introns contain independent cis-regulatory elements that decouple the expression of *pri-mir-218a-1/pri-Mir-218-2* and *slit3/Slit3* transcripts^{43,49}. Our results suggest that blocking of *slit3* splicing potentially leads to override of the cis-regulatory elements-mediated regulation and loss of the decoupling mechanisms that prevents *slit3* and *mir-218a-1* co-expression. Why the *slit3* exon 9 splice-blocking ASO leads to increased *slit3* expression when it is more plausible to expect an

NMD machinery-mediated decrease in *slit3* transcript levels is unclear. Such regulatory mechanisms on the host gene expression have been also reported for other intronic miRNAs and also for lncRNA loci's^{50,51}. However, both computational and experimental studies in mice and zebrafish has shown that *slit3* is not a target of *mir-218a-1*^{43,52}.

In the case of exon9 splice-blocking ASO, the fact that it triggers a genetic compensation response and *slit2* upregulation suggests that the *slit3* transcripts have been subjected to the NMD machinery. Neither *slit3* nor *mir-218a* is involved in a positive feedback loop with *slit2*. However, since *slit3* expression increases in exon 9 splice-blocking ASO-injected samples, it appears that some of the *slit3* transcripts also escape the NMD machinery, perhaps due to stabilization of the transcripts either by the ASO or by the intron 9 sequences or reinitiation of translation events⁵³. The role of the retained intron could also be potentially important. Intron retention could lead to the sequestering of the premature termination codon-containing transcripts in the nucleus, thereby preventing the transcripts from being subjected to the cytoplasmic NMD machinery⁵⁴.

Other possibilities to explain the increase in *slit3* expression include increased transcription of *slit3*, or due to the use of a novel/alternative *slit3* promoter sequence, or due to a lack of coupling mechanism between the spliceosome and the transcriptional machinery^{55–58}. Finally, another possibility is that *slit3* transcripts with the premature termination codon in intron 9 are subjected to NMD but the feedback mechanism involving the COMPASS protein complex, which promotes H3 modification of related compensatory gene promoters, is unable to discriminate between the promoter of *slit3* and the promoter of its paralogs such as *slit2*, which is also upregulated^{30,31}.

It is unclear why the *slit3* int13-ex14 targeted ASO, although causing intron retention that must potentially lead to the nonsense mutation, does not lead to NMD-based genetic compensation response. This could be due to either the relatively low efficiency of the ASO or differential strength of NMD based on the location of premature stop codons (5' > 3') and reinitiation of translation events^{53,59}. One of the closest cis-regulatory enhancer elements of *mir-218a-1* is localized approximately 20 kb upstream to the *slit3* exon 9 splice-blocking ASO target site⁴⁹. It is unclear whether the activity of such enhancers are differentially regulated by exon 9 versus exon 14-targeting ASO.

Similar reports have been made regarding ASO-based targeting of a host gene resulting in intron retention, and increased expression of an intronic microRNA expression in the disease-associated gene *PKD1* and its intronic *miR-1225*⁶⁰. However, this effect largely depends on the cis-regulatory elements adjacent to the *miR-1225* locus. In our experiments, we focused on splice sites rather than sites adjacent to the *mir-218a-1* locus. Additionally, we cannot exclude the possibility that such intronic regulatory sites play a role in the *mir-218a-1* mutant fish, given that approx. 130 bp are deleted at the *mir-218a-1* locus. A similar tiling-based ASO study could potentially identify additional cis-regulatory sites that can regulate *mir-218a-1* in vivo.

Although we observed a similar rescue effect when *pank2* and *dnm2a* splicing and their respective intronic miRNA were blocked, the exact mechanism by which *mir-103* or *mir-199-5p* blocking ASOs rescue the phenotypes is unclear. While in the case of *pank2*, it seems to be a rescue of *pank2* expression, in the case of *dnm2a* no such rescue of *dnm2a* expression is observed. It is possible that the phenotype is due to function of *mir-103* or *mir-199-5p* itself, rather than its expression level. In addition, *dnm2a* long intron coding transcripts regulation is also increased upon *dnm2a* splice-blocking ASO-injected samples suggesting other altered splicing based mechanisms or regulatory elements in the introns. It is unclear why blocking of *mir-103* rescues the *pank2* expression or blocking of *dnm2a* splicing affects long intron expression⁶¹. According to the TargetScanFish v6.2 database, both *mir-103* and *mir-199-5p* do not target the 3' UTRs of the *pank2* and *dnm2a*, respectively⁶².

It is worth to note here that the lack of phenotypes similar to *pank2* splice blocking ASOs in our 4-bp and 50 bp *pank2* mutants embryos could be due to genetic robustness or compensation mechanisms^{25,26,30,63}. And

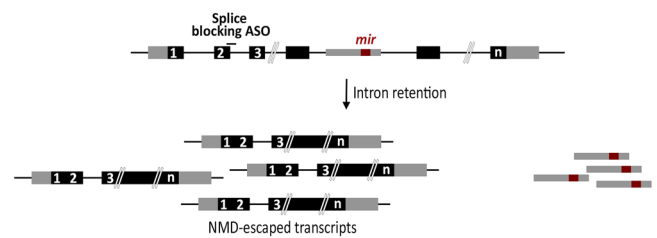


Fig. 6 | Splice-block ASOs can affect intronic primary microRNAs. ASO-based targeting of splicing of genes embedded with intronic miRNA may not only lead to intron retention or exon skipping, but also to stabilization of host gene transcripts, increased expression and function of primary intronic miRNAs.

injection of 1pmol of *pank2* splice-blocking ASO into 50 bp *pank2* mutant embryos do not recapitulate the *pank2* splice-blocking ASO phenotypes²⁵. Hence, our results could be due to *pank2* splice-blocking ASO dose-dependent phenotype. However, it should be noted in ASO-based pre-clinical trials using mammalian models, higher ASO concentrations are used in order to rescue the phenotype.

Nevertheless, our data contradict the generally held view that ASO-induced off-targets are due to Tp53. We are able to rescue the phenotypes (either completely or partially) by blocking the intronic mature miRNA of the targeted gene. Taken together, our work shows that the phenotypes caused by targeted blockade of gene splicing in vivo can be attributed to the intronic primary miRNA transcripts (Fig. 6).

Our in vivo zebrafish studies warrant further analysis of the potential impact of intronic non-coding RNAs when using splice-site targeted ASOs either for therapeutic purposes or for developmental biology studies. Finally, since introns embedded in host genes may contain multiple ncRNAs or classes of ncRNAs, the idiosyncratic phenotypes between translation- and splice-blocking ASOs can potentially be used as a marker to screen for the potential collective role of all the intronic ncRNAs embedded in such host genes. Moreover, with the recent push to move away from animal-based drug safety testing, at least for genes with conserved sequences at the ASO target sites, vertebrate model organisms such as zebrafish embryos must be considered to test the in vivo effects of therapeutically promising splice-blocking ASO candidates⁶⁴. Finally, although ASOs can also trigger NMD-dependent genetic compensation, our results show that some intron-retention causing ASOs can potentially stabilize the transcripts of the targeted gene. Moreover, such transcripts can potentially escape the NMD machinery, and the transcript-embedded intronic ncRNAs may play additional unregulated direct or moonlighting roles^{65,10}.

Materials and Methods

Zebrafish husbandry and maintenance

Zebrafish were maintained in a circulation system (Techniplast, Italy) at 28 °C with a light: dark cycle of 14:10 h at the Faculty of Biology, Adam Mickiewicz University, Poznan. The study received ethical approval from the Lokalna Komisja Etyczna ds. Doświadczeń na Zwierzętach w Poznaniu. Zebrafish were raised and bred according to standard protocols in line with standard procedures and ethical guidelines⁶⁶. AB/TL hybrid line were obtained from the International Institute of Molecular Cell Biology - Zebrafish Core facility, Warsaw. Zebrafish transgenic lines Tg(*oxf:EGFP*)^{wz01} (ZDB-ALT-111103-1)⁶⁷ and *mir-218a-1*^{wz15} (ZDB-ALT-190814-7)⁴² were of TL background and were bred in house. *mir-218a-1*^{wz15} line were genotyped using PCR-based assay⁴². Embryos from hybrid wildtype line AB/TL were used for *pank2* and *dnm2a*-related experiments. Embryos were grown at 28 °C in 0.3X Danieau's medium at constant uncrowded densities (17.4 mM NaCl, 0.21 mM KCl, 0.12 mM MgSO₄, 0.18 mM Ca(NO₃)₂, 1.5 mM HEPES, pH 7.4).

Bioinformatic analyses

To identify protein-coding genes containing intronic miRNAs and other non-coding RNAs (ncRNAs) in *Danio rerio*, the GRCz11 genome annotation

(GCF_000002035.6-RS_2024_08) was analyzed. Specific RNA biotypes, including miRNAs, snoRNAs, and snRNAs, were extracted based on their annotations. Using BEDTools v2.31.1, a sequence of strand-specific intersections was performed to identify ncRNAs overlapping protein-coding genes while excluding those overlapping exonic regions, ensuring only intronic ncRNAs were retained⁶⁸. GO term analysis on host genes were performed using REVIGO web server⁶⁹. Adult zebrafish posterior pituitary glial cells expressed genes (mean AMCA+ count >10) were downloaded from³². *slit3* ex9 splice block ASO target site conservation was evaluated by comparison of sequences across *Ensembl* teleost orthologs. Orthologs, genomic sequences, and exon–intron coordinates were retrieved from *Ensembl*. For each species, *slit3* locus was searched to locate the site most similar to the zebrafish *slit3* ex-9 boundary by local Smith–Waterman alignment. MAFFT alignment was performed using strand-aware ± 100 -bp windows centered on that site. Positional conservation was summarized as 1-H, where H is per-column Shannon entropy over A/C/G/T⁷⁰. To avoid gap artifacts, we analyzed only columns with $\geq 90\%$ nucleotide coverage across species. Transcript annotations were used to overlay exon/intron context in the figure, and exact full-length ASO matches were noted for reference.

Antisense oligonucleotides, primers and RT-qPCR

The sequences of ASOs and primers can be found in Supplementary Data 6. For ASO-based targeted blocking of gene translation or splicing and mature miRNA function, morpholino and LNA (miRCURY LNA Power Inhibitor & *mir-218* mimic) were purchased from Genetools (Philomath, OR, USA) and QIAGEN, respectively. The *slit3*, *pank2*, *dnm2*, and *tp53* morpholino-based ASO stock was prepared by dissolving it in distilled water at 1 mM, and one-cell stage embryos were microinjected at 1.67 ng of ASO/embryo. The anti-*mir-218*, anti-*mir-103* and anti-*mir-199-5p* LNA-based ASO stock was prepared by dissolving it in distilled water at 50 μ M, and one-cell stage embryos were microinjected at approximately 0.08 ng of ASO/embryo. MO-ASO and LNA-ASO were co-injected at 1.67 ng and 0.08 ng per embryo, respectively. The *mir-218* mimic ASO and *mir* negative control mimic ASO stock was prepared by dissolving it in distilled water at 66.7 μ M, and one-cell stage embryos were microinjected at 0.1 and 0.2 ng per embryo.

Total RNA extraction was performed on either at 5-dpf (*slit3/mir-218a*-related samples) or 2-dpf (*pank2/mir-103* and *dnm2a/mir-199-5p*-related samples) larvae using Qiagen Total RNA Extraction kit according to the manufacturer's instructions. RT-qPCR was performed using SYBR-based reagents based on cDNA synthesized from RNA extracted from 15 larvae per group. cDNA synthesis for mRNA and miRNA were performed using Invitrogen Superscript III Reverse transcription and Qiagen LNA miRNA SYBR Green PCR kit (miRCURY RT Enzyme mix), respectively with on-column DNase treatment. Experiments were performed in at least 3 groups. Relative expression of *mir-218a* was quantified with respect to U6 snRNA levels using miRCURY LNA-based primers (Qiagen).

In situ hybridization and immunostaining

In situ and immunostaining were performed on zebrafish larvae were euthanized using tricaine overdose (1 g/L) before fixation in 4% PFA. RNA in situ hybridization was performed as described in ref. 71. For probe preparation, *slit3* (NM_131736.3), *pri-mir-218a-1* (host gene NCBI gene ID 80354) and *pri-mir-206-1* (host gene NCBI gene ID 137488637)-based probes of sizes 346 bp, 525 bp, and 432 bp, respectively were synthesized from zebrafish larval cDNA-based PCR templates amplified using the primers listed in Supplementary Data 6. The purified products served as a template to synthesize digoxigenin-labeled antisense mRNA probes using DIG RNA labeling mix (Roche #11277073910). *mir-218a* in situ hybridization was performed using LNA-based probes (Qiagen) as described in ref. 72.

Immunofluorescence staining was performed as described in ref. 73 and using the rabbit anti-EGFP primary antibody (RRID AB_1074893, Life technologies/Thermo Fisher, Waltham, MA, USA) and secondary antibodies (RRID AB_2338046) were purchased from Jackson

ImmunoResearch Laboratories (West Grove, PA). Briefly, PFA-fixed larvae were washed in PBS (2 $\times$ 10 min), dehydrated using methanol series (25–50–75–100%) and stored at -20°C overnight. The samples were rehydrated from methanol (75–50–25%) to PBS, washed in PBS-Tx (Triton X100, 0.3%; 2 $\times$ 10 min) and blocked in 500 μ L of blocking solution (PBS + 10% goat serum + 1% DMSO + 0.3% Triton X100) for 30 min at room temperature. The solution was then replaced with 200 μ L of fresh blocking solution with primary antibodies at 1:200 concentration and incubated overnight at 4°C . Samples were washed with PBS-Tx (3 $\times$ 30 min) and treated with 200 μ L of secondary antibodies in blocking solution at 1:200 concentration, overnight at 4°C . Then, samples were washed with PBS-Tx (3 $\times$ 30 min), transferred to 75% glycerol (25–50–75%) and the jaws were removed using a pair of hypodermic syringe before mounting the larvae with its ventral side facing the objective.

Image acquisition and analysis

Colorimetric in situ images were obtained using 5X objective on a Leica DM4B microscope (Leica, Germany). Images of whole embryos fluorescently labeled OXT neurons and axons were obtained by using Nikon A1R confocal microscope with Plan Apo VC 20X objective. Confocal images were analyzed using the Fiji/ImageJ application. The number of oxytocin neurons were counted using the Cell Counter plugin.

Statistics and reproducibility

Normality tests was performed using Shapiro-Wilks test. ANOVA or Kruskal–Wallis test were used for comparing multiple groups. All data sets were corrected for multiple comparisons. Tukey's or Dunn's multiple comparisons tests were used as post-hocs. Fisher's exact test was performed to validate correlation between ASO injection and in situ-based expression of *mir-218a*. Effect sizes were calculated using epsilon squared (ϵ^2) for the Kruskal–Wallis test, eta squared (η^2) for the one-way ANOVA, partial eta squared (η^2) for the two-way ANOVA and Cramér's V for Chi-square test. All the experiments were performed on at least 3 different replicates (pooled group of larvae or embryos). Additionally, RT-qPCR analysis had technical replicates.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Numerical source data for graphs can be found in Supplementary Data 7. Uncropped agarose gel images are provided in Supplementary Fig. 5. All other data are available from the corresponding author (or other sources, as applicable) on reasonable request.

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Author contributions

H.A. contributed to study design, performed all the experiments, performed confocal imaging, data analysis and contributed in manuscript preparation. A.R.R. performed RT-qPCR experiments and partially contributed to study design. P.K. performed the bioinformatic analysis, W.K. assisted in confocal microscopy, and S.P. and N.K. assisted in in situ hybridization experiments. N.N. assisted in microinjections and performed mature miRNA in situ hybridization experiments. B.G. and D.F. reviewed the manuscript and obtained independent funding related to *pank2* research. S.A. conceived, designed the study, obtained the funding, and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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