

Antisense oligonucleotides targeting transcription termination windows disrupt mRNA 3' end processing and decrease gene expression

Corresponding Author: Dr Łukasz Kiełpiński

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Winczura et al describe a novel mechanism of ASO-mediated gene knockdown whereby ASO directs RNase H1-mediated cleavage in the transcriptional termination window downstream of polyadenylation sites, resulting in an aberrant transcript that is subsequently degraded by the exosome in the nucleus. This downstream noncoding sequence space has not been previously considered for ASO design or off-target prediction, and therefore this discovery will have a significant impact on the ASO field. The manuscript is very well written, and the conclusions are supported by the data. I would like to ask the authors to discuss how the length of the transcriptional termination windows is defined, what is their length distribution across genes transcribed by RNA Polymerase II, and estimate how much additional sequence space this creates in relation to the pre-mRNA sequence space already considered for ASO designs. Also, I'm curious how the authors arrived at the handful of ASOs shown in the paper – was there a larger screen conducted? How easy is to find active DG-ASOs compared to GB-ASOs? Are there specific sequence or PAS distance requirements?

Reviewer #2

(Remarks to the Author)

In this manuscript, Winczura et al showed that antisense oligoes (ASO) targeting regions downstream of poly(A) site within the termination window can knock down mRNA levels and they provided evidence that this involves RNase H digestion, polyadenylation of the upstream cleaved RNAs, and degradation by the RNA exosome. These results revealed potentially novel targets for ASO, downstream of gene (DG) regions, and characterized the underlying mechanism. The results are interesting, but the authors need to address several issues before the study can be published.

Major points:

1. The authors selected one DG ASO for each of the two example genes. How does the position of the ASO target region affect the efficacy of the ASO? As the key novelty point for this study to show that DG-ASO can be used to inhibit gene expression, it would be important to provide information on how to select ASO targets.
2. The role of RNase H needs to be further substantiated: 1) In addition to knockdown, the authors should rescue RNase H expression with wild-type or a catalytically inactive mutant and examine their effect (Fig. 3C). 2) the western blots in Fig. 3C used cropped images. Please do the western on the same gel and show an uncropped image.

Minor point:

As the authors only tested DG-ASO on four genes in several cell lines, they should tone down their claim of “a universal effect of DG-ASOs on mRNA downregulation” (Fig. 2).

Reviewer #3

(Remarks to the Author)

In this manuscript by Winczura et al., the authors report that the region between polyA site and termination site, aka downstream-of-gene (DG) region, has critical roles for ASO-based gene knockdown. They show that DG-ASOs trigger RNase-H1 cleavage of nascent RNA before cleavage and polyadenylation (CPA) machinery can act at the polyA site, and the resulting 5' and 3' nascent RNAs are degraded by the nuclear RNA exosome (via 3'-5' exonuclease DIS3) and by the

5'-3' exonuclease XRN2, respectively. The latter accelerates Pol II termination. The authors show that DG-ASOs can effectively knock down gene expression. In addition, the DG region can function as off-target sites for ASOs designed for other regions. Overall this work was well carried out. While the ASO-XRN2-termination mechanism was also reported before, most of the findings of this work are informative for using ASOs as therapeutics.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors addressed all my comments, no further revisions needed.

Reviewer #2

(Remarks to the Author)

The authors have addressed my concerns satisfactorily and thus I support publication.

Reviewer #3

(Remarks to the Author)

The authors have addressed all my concerns.

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AUTHORS' RESPONSES TO REVIEWER'S COMMENTS

We appreciate the time the Reviewers spent assessing our work and thank them for their helpful comments. In the revised version of the manuscript, we included the requested experimental approaches or explanations.

We discuss all the points raised by the Reviewers in detail below.

REVIEWER COMMENTS

REVIEWER #1:

Winczura et al describe a novel mechanism of ASO-mediated gene knockdown whereby ASO directs RNase H1-mediated cleavage in the transcriptional termination window downstream of polyadenylation sites, resulting in an aberrant transcript that is subsequently degraded by the exosome in the nucleus. This downstream noncoding sequence space has not been previously considered for ASO design or off-target prediction, and therefore this discovery will have a significant impact on the ASO field.

The manuscript is very well written, and the conclusions are supported by the data. I would like to ask the authors to discuss how the length of the transcriptional termination windows is defined, what is their length distribution across genes transcribed by RNA Polymerase II, and estimate how much additional sequence space this creates in relation to the pre-mRNA sequence space already considered for ASO designs. Also, I'm curious how the authors arrived at the handful of ASOs shown in the paper – was there a larger screen conducted? How easy is to find active DG-ASOs compared to GB-ASOs? Are there specific sequence or PAS distance requirements?

Authors responses to Reviewer's specific comments:

1. Reviewer 1: *This downstream noncoding sequence space has not been previously considered for ASO design or off-target prediction, and therefore this discovery will have a significant impact on the ASO field.*
The manuscript is very well written, and the conclusions are supported by the data.

Authors: We thank the Reviewer for appreciating our work.

2. Reviewer 1: *I would like to ask the authors to discuss how the length of the transcriptional termination windows is defined, what is their length distribution across genes transcribed by RNA Polymerase II and estimate how much additional sequence space this creates in relation to the pre-mRNA sequence space already considered for ASO designs.*

Authors: Transcription termination windows were first precisely mapped in 2016 (Schwalb et al., 2016, *Science* 6290), where the authors developed and used a technique for mapping strand-specific active transcription, called transient transcriptome sequencing (TT-seq). The termination windows in human cells were defined by the TT-seq signal downstream of genes (polyadenylation sites). Their analysis of ~7000 genes (median length 24kb) revealed that the termination windows had a median width of ~3.3 kb and could be up to 10 kb wide.

More recently, a large bioinformatics analysis of various nascent RNA sequencing methods (PRO-seq, POINT-seq, NET-seq) as well as specific RNA Polymerase II phosphorylation marks associated with termination (phosphorylation of Pol II C-terminal domain at threonine 4) confirmed the original findings from 2016 (Kopczynska et al., 2024, *Nucleic Acids Research* 53).

Based on the TT-seq data (Schwalb et al., 2016, *Science* 6290), we concluded that the additional space for ASO design, created by termination windows, increases by ~14% genome wide. However, the expansion of targetable space created by termination windows should be considered for individual genes. For example, this is more pronounced for short genes. In the revised version of the manuscript, we present the case of the widely expressed transcription factor ATF4 which is 2.1 kb long; of this, 1.1 kb are the exons, and 1 kb is the intronic sequence. The termination window of ATF4 in HEK293 cells is about 3.5 kb long, which increases the total sequence available for ASO design by ~2.5 fold and the pre-mRNA targetable sequence by 4.5 fold (Supplementary Fig. 3). Moreover, when exclusive targeting of pre-mRNA, rather than mature mRNA, is required for intronless genes (according to the Intronless Gene Database, ~700 genes in the human genome do not contain introns) ASOs acting within termination windows are the only option.

We have modified the manuscript to make this information more accessible to readers.

3. Reviewer 1: *how the authors arrived at the handful of ASOs shown in the paper – was there a larger screen conducted?*

Authors: The DG-ASOs used for most of the experiments were selected from a larger pool of 20 ASOs covering the first 500 nt of the termination windows of *CHMP1A* or *KPNB1*: 16 DG-ASOs decreased the levels of *CHMP1A* by more than 50%, while in the case of *KPNB1*, 14 DG-ASOs downregulated mRNA by more than 50%. The DG-ASOs selected for further experiments were relatively close to the 3' ends of the genes and displayed representative activity downregulating the accumulation of steady-state levels of the respective mRNAs. We have now included the outcomes of the screen in the manuscript (Supplementary Fig. 1).

4. Reviewer 1: *How easy is to find active DG-ASOs compared to GB-ASOs?*

Authors: Finding active DG-ASOs is straightforward when using standard design algorithms. We applied the same design approach for both GB- and DG-ASOs, and a screen of 20 DG-ASOs targeting the first 0.5 kb of the termination window showed that most candidates successfully downregulated their target mRNAs (Supplementary Fig. 1).

5. Reviewer 1: *Are there specific sequence or PAS distance requirements?*

Authors: The impact of the distance from the PAS and the sequence around the PAS on DG-ASO activity needs to be carefully evaluated experimentally. Our DG-ASO screen, now included in the manuscript, shows that DG-ASOs work well when located within 0.5 kb of termination windows. Moreover, in the case of KPNB1, DG-ASOs were positioned 2 and 2.5 kb downstream of the two major PAS sequences and still decreased mRNA levels. However, we prefer not to make any general claims about distance dependence in DG-ASO design, as this requires more in-depth studies. We also clearly state in the Discussion that the precise distances from polyadenylation sites at which DG-ASOs remain effective may need to be individually established for specific genes, cell types, and tissues. We believe that our future research will provide more specific guidelines regarding DG-ASO design, including sequence or PAS distance requirements.

REVIEWER #2:

In this manuscript, Winczura et al showed that antisense oligoes (ASO) targeting regions downstream of poly(A) site within the termination window can knock down mRNA levels and they provided evidence that this involves RNase H digestion, polyadenylation of the upstream cleaved RNAs, and degradation by the RNA exosome. These results revealed potentially novel targets for ASO, downstream of gene (DG) regions, and characterized the underlying mechanism. The results are interesting, but the authors need to address several issues before the study can be published.

Major points:

1. The authors selected one DG ASO for each of the two example genes. How does the position of the ASO target region affect the efficacy of the ASO? As the key novelty point for this study to show that DG-ASO can be used to inhibit gene expression, it would be important to provide information on how to select ASO targets.

2. The role of RNase H needs to be further substantiated: 1) In addition to knockdown, the authors should rescue RNase H expression with wild-type or a catalytically inactive mutant and examine their effect (Fig. 3C). 2) the western blots in Fig. 3C used cropped images. Please do the western on the same gel and show an uncropped image.

Minor point:

As the authors only tested DG-ASO on four genes in several cell lines, they should

tone down their claim of “a universal effect of DG-ASOs on mRNA downregulation” (Fig. 2).

Authors responses to Reviewer’s comments:

1. Reviewer 2: *The results are interesting, but the authors need to address several issues before the study can be published.*

Authors: We thank the Reviewer for the positive assessment of our work. We have addressed all the points raised by the Reviewer.

2. Reviewer 2: *The authors selected one DG ASO for each of the two example genes. How does the position of the ASO target region affect the efficacy of the ASO? As the key novelty point for this study to show that DG-ASO can be used to inhibit gene expression, it would be important to provide information on how to select ASO targets.*

Authors: We initially performed DG-ASOs screen (included now in the manuscript, Supplementary Figure 1), in which we show DG-ASOs covering 0.5 kb of the termination windows. From this screen, we selected representative DG-ASOs to continue the project, as mechanistic experiments on a large number of ASOs would not be feasible. This is now described in the manuscript.

We agree with the Reviewer that it is important to provide information on how to select DG-ASO targets. In the revised version of the manuscript, we show that the first 500 nt of the *CHMP1A* and *KPNB1* termination windows are suitable for targeting. In the case of *KPNB1*, DG-ASO was positioned 2 and 2.5 kb downstream of the two major PAS sequences and still decreased mRNA levels. However, we would prefer not to make any general claims on the distance-dependence in the DG-ASO design as this requires more in-depth studies using ASOs that fully cover the termination windows of multiple genes. We also clearly state in the *Discussion* that the precise distances from polyadenylation sites at which DG-ASOs remain effective may need to be individually established for specific genes, cell types and tissues. Such comprehensive and complex analyses require dedicated research and are planned in the future projects, therefore they are outside the scope of this manuscript. Since the use of ASOs is tightly connected with therapeutic approaches and drug development, we prefer not to speculate on the guidelines without extensive supporting data. We believe that our future research will provide more specific information regarding DG-ASO design.

3. Reviewer 2: *The role of RNase H needs to be further substantiated: 1) In addition to knockdown, the authors should rescue RNase H expression with wild-type or a catalytically inactive mutant and examine their effect (Fig. 3C). 2) the western blots in Fig. 3C used cropped images. Please do the western on the same gel and show an uncropped image.*

Authors: To rigorously substantiate the role of RNase H1, we used a dominant-negative approach by overexpressing a catalytically inactive mutant, as suggested by the Reviewer. We overexpressed wild-type or mutant (D210N) RNase H1 (constructs used in Tang-Wong et. al, 2019, *Mol Cell* 76) and tested the effects of DG-ASOs on *CHMP1A* and *KPNB1* mRNA levels (Fig. 3d). The introduced mutation allows RNase H1 to bind the DNA:RNA hybrid; however, its cleavage activity is impaired. Under these conditions, overexpressed RNase H1 D210N outcompetes endogenous RNase H1 for hybrid binding, resulting in a loss-of-function phenotype. We now show that expression of catalytically inactive RNase H1 rescued the levels of *CHMP1A* or *KPNB1* in the samples treated with GB- or DG-ASOs, compared with cells transfected with the plasmid overexpressing wild-type RNase H1.

Overall, in the revised version of the manuscript, we present three different and independent experiments (RNase H1 siRNA-dependent knockdown, the dominant-negative effect of RNase H1 catalytic mutant as well as the use of mixmer DG-ASOs with chemistry that prevents RNase H1 cleavage) confirming the role of RNase H1 in the DG-ASO-dependent mRNA degradation.

We also re-ran the Western blot from Fig. 3c to show the samples next to each other. The uncropped raw image is shown in new Supplementary Fig. 4.

4. Reviewer 2: *As the authors only tested DG-ASO on four genes in several cell lines, they should tone down their claim of “a universal effect of DG-ASOs on mRNA downregulation” (Fig. 2).*

Authors: We have toned down the statements as suggested by the Reviewer.

REVIEWER #3:

In this manuscript by Winczura et al., the authors report that the region between polyA site and termination site, aka downstream-of-gene (DG) region, has critical roles for ASO-based gene knockdown. They show that DG-ASOs trigger RNase-H1 cleavage of nascent RNA before cleavage and polyadenylation (CPA) machinery can act at the polyA site, and the resulting 5' and 3' nascent RNAs are degraded by the nuclear RNA exosome (via 3'-5' exonuclease DIS3) and by the 5'-3' exonuclease XRN2, respectively. The latter accelerates Pol II termination. The authors show that DG-ASOs can effectively knock down gene expression. In addition, the DG region can function as off-target sites for ASOs designed for other regions. Overall this work was well carried out. While the ASO-XRN2-termination mechanism was also reported before, most of the findings of this work are informative for using ASOs as therapeutics.

Authors responses to Reviewer's comments:

1. Reviewer 3: *Overall this work was well carried out. While the ASO-XRN2-termination mechanism was also reported before, most of the findings of this work are informative for using ASOs as therapeutics.*

Authors: We thank the Reviewer for appreciating our work. Indeed, the ASO-XRN2-termination mechanism has been reported previously, and we reference all three papers that first described this mechanism. However, in these experiments ASOs were always located within the gene body and therefore ASO-XRN2-termination mechanism did not compete with the cleavage and polyadenylation-dependent termination, as it does in our case. We amended the manuscript to clarify this point and acknowledge the work of other groups:

“...suggesting that co-transcriptional cleavage by RNase H may be followed by XRN2-mediated degradation leading to transcription termination as it has been reported previously for ASOs acting within gene bodies...”