Data in Brief 60 (2025) 111565

Contents lists available at ScienceDirect

Data in Brief

journal homepage: www.elsevier.com/locate/dib

Data Article

Integrating datasets to dissect *NFYC-AS1* RNA- and transcription-dependent functions: comparative transcriptome profiling of knockdown strategies

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ARTICLE INFO

Article history:
Received 15 January 2025
Revised 31 March 2025
Accepted 11 April 2025
Available online 15 April 2025

Dataset link: Silencing of NFYC-AS1 by Gapmer antisense oligonucleotides and CRISPR/Cas9 TSS deletion. (Original data)

Keywords:
Antisense oligonucleotides
CRISPR/Cas9
Antisense RNA
Transcriptomics
Knockdown strategies
RNA sequencing

ABSTRACT

The recent discovery of antisense RNAs (asRNAs) as key regulators of biological processes has highlighted the need to challenge their mechanism(s) of action using complementary approaches. Indeed, asRNAs can act *in cis* on their sense gene or *in trans* on distally located targets, by exploiting either transcription- or RNA-dependent mechanisms. Here we present a comparative transcriptome profiling of cancer cells knocked-down for the asRNA *NFYC-AS1* with two different approaches: i) Gapmer Antisense Oligonucleotides to assess RNA-dependent mechanisms, and ii) CRISPR/Cas9 deletion of the transcription start site to study transcription-dependent mechanisms. We describe in detail the strategies used to silence the asRNA and evaluate the consequences at the transcriptome level by RNA-sequencing. Moreover, we outline the analyses conducted to correctly manage the variability across replicates and the off-target effects of either method. The integration of the obtained datasets revealed commonalities and divergencies of the two approaches, which was fundamental for dissecting *NFYC-AS1* function. The information reported here can help researchers to reuse the data described in the datasets. Finally, the comparative workflow can

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https://doi.org/10.1016/j.dib.2025.111565

be potentially applied to the functional study of any asRNA of interest.

Specifications Table

Subject	Biological Sciences, Molecular Biology
Specific subject area	Cancer Research, Cell Biology, Epigenetics, Molecular Biology, Transcriptomics
Type of data	Raw data: Illumina FASTQ files; Processed data: TPM and counts; Analysed data: Figures, Graphs, and Tables
Data collection	H520 cells at 48 hours after Gapmer antisense oligonucleotide transfection and CRISPR/Cas9 deleted and non-deleted clones were collected. Total RNA was isolated from cellular pellets using QIAzol Lysis Reagent and miRNeasy Mini Kit (QIAGEN, Hilden, Germany). Polyadenylated RNA was purified through oligo-dT-based RNA capturing, randomly fragmented, and transformed into cDNA using random hexamers with NEB library preparation protocol. Expression profiling by high throughput paired-end sequencing was carried out using Illumina NovaSeq 6000 by Novogene UK.
Data source location	University of Milan, Department of Biosciences Milan, Italy
Data accessibility	Repository name: NCBI Gene Expression Omnibus Data identification number: GSE240468 Direct URL to data: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE240468
Related research article	C. Pandini, G. Pagani, M. Tassinari, E. Vitale, E. Bezzecchi, M. K. Saadeldin, V. Doldi, G. Giannuzzi, R. Mantovani, M. Chiara, A. Ciarrocchi, P. Gandellini. The pancancer overexpressed NFYC Antisense 1 controls cell cycle mitotic progression through in cis and in trans modes of action. Cell Death Dis 15, 206 (2024). 10.1038/s41419-024-06576-y

1. Value of the Data

- Our dataset is a unique resource as it is the first transcriptomic dataset where *NFYC-AS1* has been modulated through different knockdown strategies.
- Our dataset can be useful for biomedical researchers interested in studying *NFYC-AS1* functions.
- The comparative workflow described here is broadly applicable to study any asRNA of interest.

2. Background

Antisense RNAs (asRNAs) are non-coding RNAs transcribed from the antisense DNA strand, with respect to protein coding genes, recently recognized as key regulators of biological processes [1]. To exert their functions, asRNAs exploit RNA-dependent mechanisms by interacting with nucleic acids through sequence complementarity [2,3] and with proteins through their secondary structure [4], or transcription-dependent mechanisms where the sole act of their transcription regulates nearby gene(s) [5]. As for most asRNAs, the function of *NFYC-AS1* was poorly characterized with little information about its biological role and mechanism of action [6,7]. Recently, we provided a comprehensive characterization of *NFYC-AS1* in *Pandini & Pagani* et al. [8].

Here, we describe in detail the experimental procedures used for the comparative transcriptome characterization of cells silenced for *NFYC-AS1*. To discriminate between RNA- and transcription-dependent mechanisms, we knocked-down *NFYC-AS1* using Gapmer Antisense Oligonucleotides (ASO) to degrade the target RNA, and CRISPR/Cas9 deletion of transcription

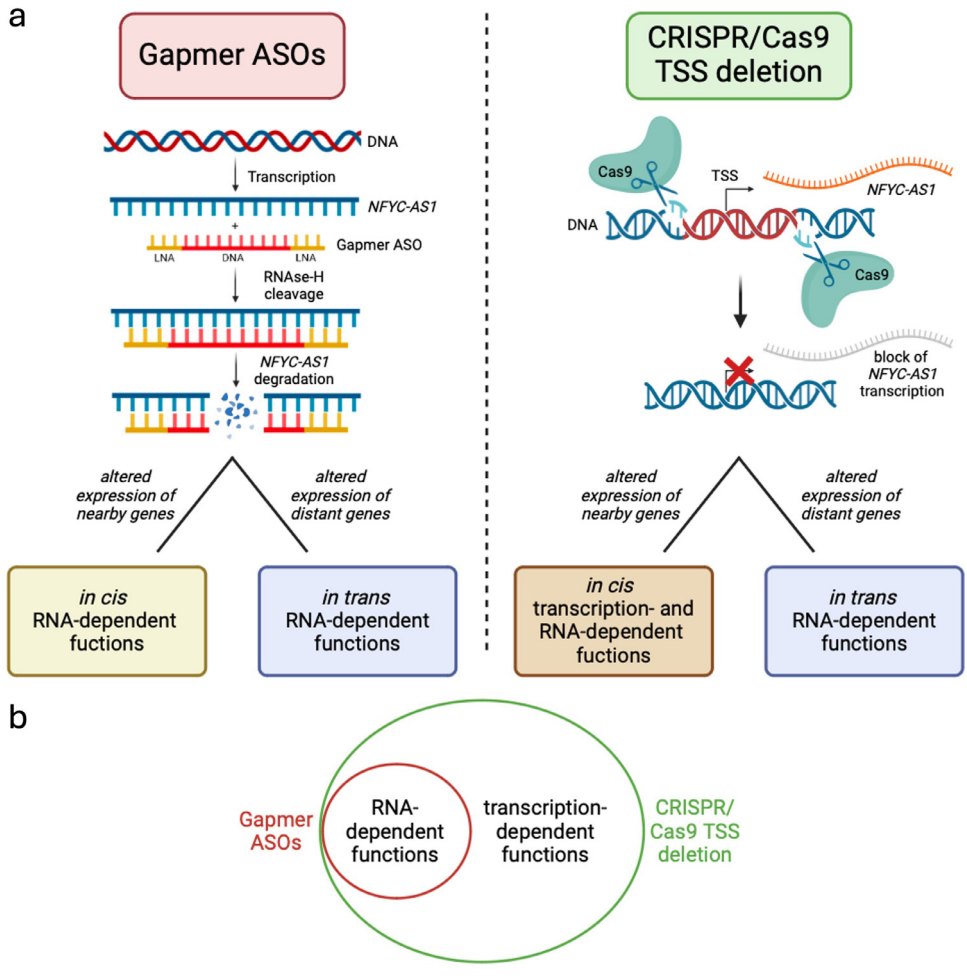

Fig. 1. Design and rationale of the study. (a) Schematic representation of the design and rationale of loss-of-function experiments followed by RNA-sequencing. (b) Intersection representing the commonalities between the effects arising after Gapmer ASO-treatment and CRISPR/Cas9 TSS deletion.

start site (TSS), which prevented its transcription. Starting from the need of finding new technologies and pipelines to study asRNAs, we integrated multiple complementary datasets from different experimental approaches to dissect *NFYC-AS1* function and exclude any potential off-target effects. The comparative workflow described here is broadly applicable to study any asRNA of interest.

3. Data Description

This article describes the dataset (GEO accession number GSE240468) containing raw and processed RNA-seq data of H520 lung squamous carcinoma cells where we have modulated *NFYC-AS1* level through different strategies (Fig. 1). To study RNA-dependent functions, we used Gapmers ASOs (QIAGEN, Hilden, Germany) that target and induce RNase-H mediated cleavage of complementary mature RNAs, in principle without altering transcription [9]. Thus, if ASO-

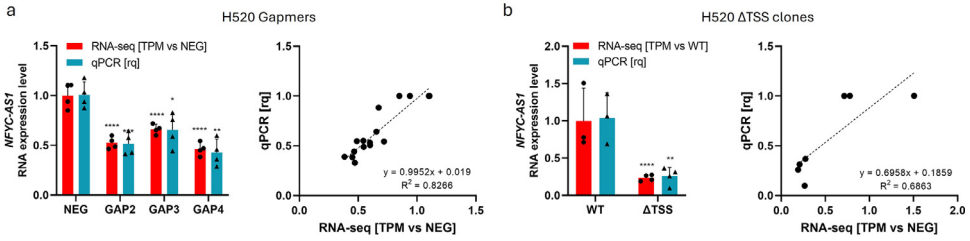

Fig. 2. RNA-sequencing analysis. (a) Left panel: bar plot showing *NFYC-AS1* expression level as from RNA-seq data and measured through qRT-PCR with Pr4 in Gapmer-treated samples. Results are normalized against NEG samples and reported as mean ± sd, as from n = 4 biological replicates. Right panel: correlation between the TPM for *NFYC-AS1* and its expression level measured through qRT-PCR using Pr4 and expressed as relative quantity (rq) in the single Gapmer-treated samples. (b) Left panel: bar plot showing *NFYC-AS1* expression level as from RNA-seq data and measured through qRT-PCR with Pr4 in non-deleted (WT) and deleted clones (ΔTSS). Results are normalized against WT samples and reported as mean ± sd, as from n = 3 WT and n = 4 ΔTSS independent biological replicates. Right panel: correlation between the TPM for *NFYC-AS1* and its expression level measured through qRT-PCR using Pr4 and expressed as relative quantity (rq) in the WT and ΔTSS samples. Analyses were performed using R software and t-test *p*-values are reported; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

mediated knockdown alters the expression of neighboring genes, *NFYC-AS1* is likely to work *in cis* through RNA-dependent mechanisms. Instead, if the expression of distant genes is altered, an RNA-dependent function *in trans* becomes more likely [1] (Fig. 1a). Instead, to study transcription-dependent functions, we employed CRISPR/Cas9-based approach to delete *NFYC-AS1* TSS in H520 cells, abrogating its transcription. In this case, if TSS deletion alters the expression of neighboring genes, *NFYC-AS1* is likely to work *in cis* through transcription- or RNA-dependent mechanisms. Otherwise, if the expression of distant genes is altered, *NFYC-AS1* probably has an RNA-dependent function *in trans* [1] (Fig. 1a). These two strategies of modulation allowed us to discriminate *NFYC-AS1* mechanisms of action. Indeed, TSS deletion is useful to highlight transcription-dependent functions that cannot be evidenced in the ASO experiment. In contrast, the commonly deregulated genes after *NFYC-AS1* modulation by these two methods represents the targets of its RNA-dependent functions (which are abrogated by both approaches) (Fig. 1b).

The GSE240468 dataset includes samples treated with three different Gapmers (GAP2, GAP3, and GAP4) and the negative control (NEG) in quadruplicates, four independent deleted clones (ΔTSS), and three non-deleted clones (WT) (in Supplementary Table S1 sample name and GEO accession number are reported).

The extent of knockdown assessed by qRT-PCR, as described in the reference paper [8], and RNA-sequencing, expressed in transcript per million (TPM), was the same (Fig. 2a, b left panels), with a good correlation between *NFYC-AS1* TPM in the different samples and replicates and the expression level measured through qRT-PCR in the same samples and replicates (Fig. 2a, b right panels). All the three Gapmers effectively downmodulated *NFYC-AS1* in H520 cells with a reduction ranging from about 40 % of GAP3 to about 50 % of GAP2 and GAP4 (Fig. 2a left panel). This is in accordance with the higher level of accessibility obtained for GAP2 and GAP4 and the lower level of accessibility obtained for GAP3 (Supplementary Fig. S1). Instead, the four deleted clones showed a 70–90 % reduction of *NFYC-AS1* compared to the non-deleted clones in qRT-PCR experiments (Fig. 2b left panel). Given that *NFYC-AS1* is a monoexonic transcript, we could not design exon-exon primers to measure its expression, thus this step was crucial to establish that qRT-PCR primers really measured *NFYC-AS1* expression level and not genomic DNA.

Gapmer-treated samples were analyzed both as individual Gapmer versus NEG (Fig. 3a–c), to consider possible off-targets of each Gapmer, and as all Gapmers versus NEG (Fig. 3d-f). The results showed good correlation within each pair of Gapmers (range=0.60–0.79) (Fig. 3a-c) and between each Gapmer and the “all Gapmers versus NEG” comparison (range=0.84–0.90) (Fig. 3d-f). These analyses revealed that, although each Gapmer has its own off-targets, there

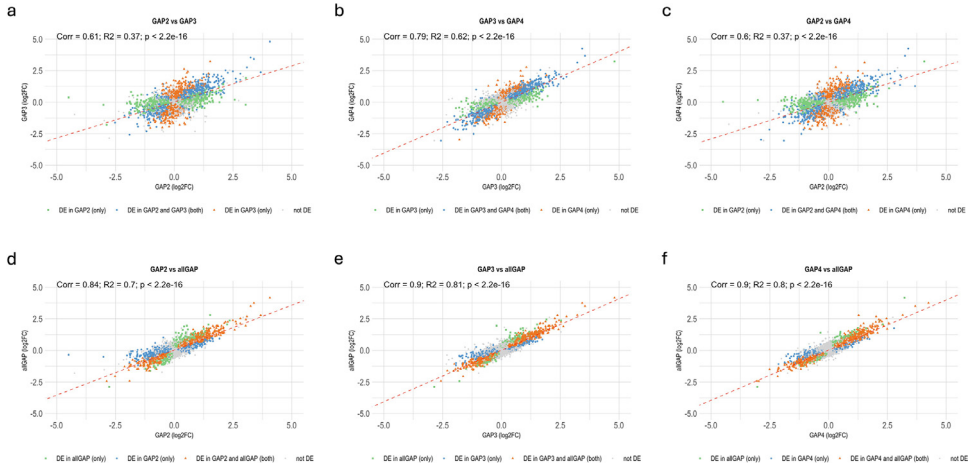

Fig. 3. Gapmer ASO correlation analysis. (a) Scatterplot reporting the correlation between the fold-change (expressed as log_2FC) of significantly differentially expressed (DE) and non-DE genes in GAP2- and GAP3-treated samples, (b) GAP3- and GAP4-treated samples, (c) GAP2- and GAP4-treated samples, (d) GAP2-treated sample and all GAP, (e) GAP3-treated sample and all GAP, and (f) GAP4-treated sample and all GAP.

is a strong concordance between the different Gapmers across both significantly and non-significantly DE genes. Therefore, "all Gapmers versus NEG" comparison was used for additional enrichment analyses in *Pandini & Pagani* et al. [8].

Instead, as expected, only a minor fraction of DE genes is shared and concordant in the two experimental approaches, as shown by the correlation analysis of the gene expression FC (log_2FC) of "all Gapmers versus NEG" and CRISPR experiment (Fig. 4a). Nevertheless, the enrichment analysis performed with Enrichr on DE genes coherently modulated by the two approaches (Fig. 4b, c) recapitulated the pathways found to overlap between the two strategies in the original manuscript [8]. The rationale behind focusing on pathways rather than on individual genes is because RNA-seq was designed to evidence phenotypic effects arising from *NFYC-AS1* knockdown, rather than identifying putative direct targets. It was indeed performed 48h after transient transfection with Gapmers and on unsynchronized independent deleted clone cultures from CRISPR experiment (to account for clonal variations). This highlights the importance of combining different strategies when studying the function of an asRNA, in order to exclude off-targets dependent on the knockdown approach itself and thus focus on genuine effects due to *NFYC-AS1* knockdown.

4. Experimental Design, Materials and Methods

Design and rationale. We performed *NFYC-AS1* knockdown by transfecting three different Gapmers (GAP2, GAP3, and GAP4) in comparison with NEG in H520 cells (Supplementary Figure S2). As for TSS deletion, two sgRNAs targeting *NFYC-AS1* TSS region were designed and cloned into PLV-Cas9-T2A-GFP plasmid (Addgene #53190 - Cambridge, MA, USA) (Supplementary Figure S2). H520 cells were transfected as described in the reference manuscript [8].

RNA isolation and integrity assessment. Total RNA from all the samples was isolated from cellular pellets using miRNeasy Mini kit (QIAGEN, Hilden, Germany). RNA integrity was checked through agarose gel electrophoresis. RNA yield and A260/A280 ratio were monitored with Nabi - UV/VIS Nano Spectrophotometer MicroDigital (TWIN HELIX SRL, Rho, Italy). The ratio of A260/A280 in the range 1.8–2.0 was considered acceptable. Subsequently, RIN for each sample was measured using 4200 TapeStation System (Agilent Technologies, Santa Clara, CA, USA).

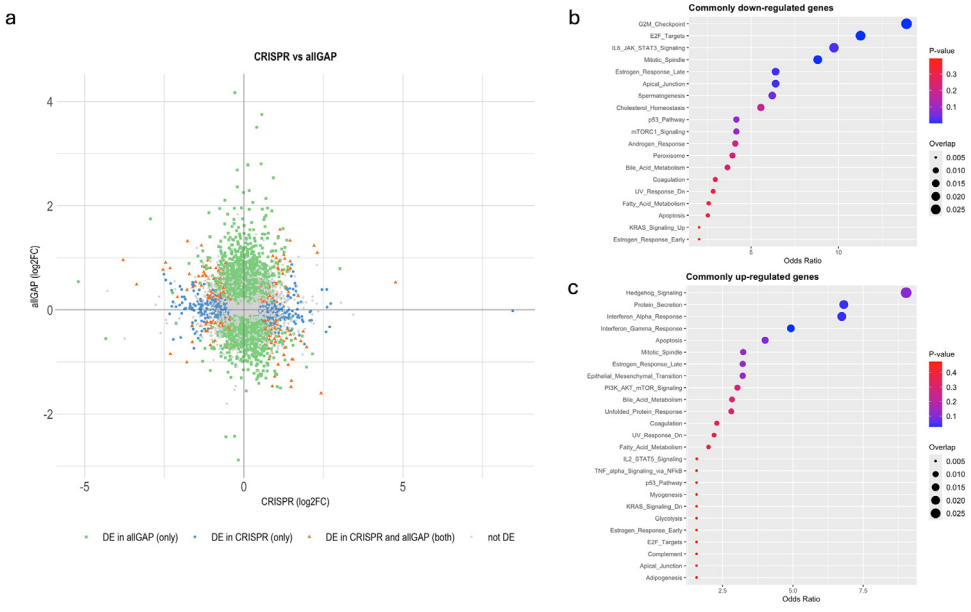

Fig. 4. Comparison of Gapmer-treated and CRISPR samples. (a) Scatterplot reporting the correlation between the fold-change (expressed as $\log_2$FC) of significantly differentially expressed (DE) and non-DE genes between all GAP and CRISPR samples. (b) Dot plot showing the cancer hallmark gene sets enriched in the commonly downregulated genes and (c) in the commonly upregulated genes, performed using Enrichr. The odds ratio represents the degree of enrichment, while the overlap represents the number of enriched genes.

Library preparation and sequencing. RNA sequencing was conducted in quadruplicate on H520 cells treated with GAP2, GAP3, and GAP4, and compared to NEG cells, 48 h post-transfection. Additionally, RNA sequencing was performed on four independent CRISPR/Cas9-deleted clones and compared to three independent non-deleted clones. Polyadenylated RNA was isolated using oligo-dT-based RNA capture, followed by random fragmentation and reverse transcription into cDNA employing random hexamers, as per the NEB library preparation protocol. High-throughput paired-end sequencing for expression profiling was executed on the Illumina NovaSeq 6000 platform. The sequencing was conducted with a read length of 150 base pairs and a depth ranging from 60 million to 100 million reads per sample. The library preparation, paired-end sequencing, and subsequent data quality control were carried out by Novogene, UK.

Quality validation of RNA data. More than 95 % and 93 % of called bases showed a Phred quality score higher than 30, for all the Gapmer-treated samples and deleted clones, respectively. As from Novogene report, data quality summary is reported in Supplementary Table S2.

Preprocessing methods. FASTQ files containing raw sequencing reads were aligned to NCBI RefSeq GRCh38/hg38 genome using STAR and gene expression levels (expressed as transcript per million (TPM)) and raw counts were computed using RSEM-1.3.1 software using NCBI RefSeq (GRCh38/hg38) as a reference. We report below the lines of code used to compute gene intensities and to build the reference genome:

rsem-calculate-expression
-p 6
–paired-end
–STAR
–output-genome-bam
–estimate-rspd
input.1.fastq

input.2.fastq
/hg38_RefSeq_rsem/hg38_RefSeq
rsem-prepare-reference
–gtf GCA_000001405.15_GRCh38_full_analysis_set.refseq_annotation.gtf
–star GCA_000001405.15_GRCh38_full_analysis_set.fna hg38_RefSeq_rsem/hg38_RefSeq

Quality of the alignment. We assessed the percentage of aligned reads using *samtools flagstat* for each sample. More than 96 % of reads were properly mapped to the reference genome, and more than 92 % of reads were properly paired (Supplementary Table S3).
Principal component analysis. Principal component analysis (PCA) of RNA-sequencing samples and replicates was performed using *vst* and *plotPCA* functions from DESeq2 [10] R package. PCA showed that all the quadruplicates for each Gapmer-treated sample were well clustered indicating a good level of reproducibility. In addition, the samples treated with GAP3 and GAP4 showed a higher degree of similarity compared to the samples treated with GAP2 (Supplementary Fig. S3a). This was not ascribable to a difference in silencing efficiency, as we achieved a similar level of silencing with GAP2 and GAP4 as compared to GAP3. As expected, PCA of deleted clones showed a lower level of clustering (Supplementary Fig. S3b). Indeed, it is well known that tumor cell lines are composed of different subpopulations and, subsequently, single clones, which are picked up from pre-existing subclones within the bulk population during the CRISPR/Cas9 editing protocol, can exhibit different features depending merely on the clonality [11]. For this reason, we sequenced four different deleted clones and three different wildtype clones to increase the variability and decrease clonality effects, consequently. This highlights the importance of considering the commonalities between deleted clones compared to non-deleted clones as each clone represents a true biological replicate for *NFYC-AS1* deletion.
Gapmer design quality control. The difference in silencing efficiency with Gapmers may depend on a different accessibility of the portions targeted by the different ASOs. In this regard, we performed a target accessibility analysis through Sfold, which is a web-accessible tool used for the *in silico* prediction of the 2D structure based on thermodynamics starting from the RNA sequence, and it computes the probability of a certain base position to be structured or freely accessible [12]. The estimated accessibility of each Gapmer-targeted region was calculated as the average of the accessibility percentage of each single base belonging to the *NFYC-AS1* Gapmer-targeted portions. As input for this analysis, we used *NFYC-AS1.1* reconstructed isoforms [8]. Higher accessibility was estimated for the GAP2-, followed by GAP4- and GAP3-targeted regions (Supplementary Fig. S1).

Moreover, the difference of GAP2 samples in the PCA may be also due to different Gapmer off-targets, intended as the genes to which a given Gapmer can pair, even partially, and eventually degrade. Gapmer off-targets were detected using the basic local alignment search tool (BLAST) [13] (Supplementary Table S4), considering the BLAST E-value (E-val), which is the number of expected hits of similar quality that could be found just by chance. For instance, E-val of 10 means that up to 10 hits are expected to be found just by chance, given the same size of a random database. Only transcripts complementary to the different Gapmers, which are referred to as minus respect to the Gapmer sequence (Plus/Minus match), were considered as possible Gapmer off-targets. We also considered the corresponding fold-change and adjusted *p*-value calculated in the downstream analysis of the RNA-sequencing data, which allowed us to have an estimation of which predicted off-targets are really downmodulated upon silencing using the three Gapmers. We found that the three Gapmers have different off-targets and some of them resulted to be significantly modulated, with GAP4 having more off-targets (Supplementary Table S4). The fact that the expression of few genes other than *NFYC-AS1* is affected might lead to different effects in differently treated samples, thus possibly explaining the differences in the PCA. This again suggests the importance of using different multiple targeting Gapmers, as recommended by *Stojic* et al. [11]. Indeed, considering only the commonalities between Gapmer-treated samples was crucial to exclude Gapmer-specific off-targets and to focus on genuine effects solely due to *NFYC-AS1* knockdown.

Differential expression and gene set enrichment analyses. Differential expression analysis for each Gapmer compared to NEG and deleted clones versus non-deleted clones were performed using DESeq2 [10] R package. Raw counts were rounded and used as input to compute the differential expression analysis. Genes with less than 10 raw counts in at least four samples were filtered out. We used *DESeqDataSetFromMatrix* function from DESeq2 [10] R package to build the working matrix according to the experimental design (reported in Supplementary Table S5). Differential expression analysis was performed using the working matrix as input for *DESeq* function from DESeq2 [10] R package.

Log2FC, the corresponding adjusted *p*-value, and *stat* value were all reported by *DESeq* function and were used to search for differentially expressed (DE) genes.

Correlation analysis. Correlation analysis was performed between the gene expression FC (log_2FC) of the different Gapmers, and between expression FC (log_2FC) of "all Gapmers versus NEG" and CRISPR experiment. The *cor.test* R function was employed and ggplot2 R package was used to plot the result.

Code Availability. Except for special instructions provided throughout the paper, all tools were used with default parameters. The R code was run and tested using R version 4.1.2. For additional information see the reference manuscript [8].

Data Records. The FASTQ files for the raw data and the normalized gene expression data have been deposited at the NCBI Gene Expression Omnibus (GEO) as a series with the accession number GSE240468. The GEO accession number of each sample was listed in Supplementary Table S5.

Limitations

Not applicable.

Ethics Statement

Authors declare that have read and followed the ethical requirements for publication in Data in Brief and that the current work does not involve human subjects, animal experiments, or any data collected from social media platforms.

CRediT Author Statement

Giulia Pagani: Investigation, Formal analysis, Writing – original draft. **Cecilia Pandini:** Investigation, Formal analysis, Writing – original draft. **Martina Tassinari:** Investigation. **Paolo Gandellini:** Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. All authors read and approved the final manuscript.

Data Availability

Silencing of NFYC-AS1 by Gapmer antisense oligonucleotides and CRISPR/Cas9 TSS deletion. (Original data) (Gene Expression Omnibus GEO)

Acknowledgments

The research leading to these results has received funding from AIRC under IG 2020 - ID. 24325 – P.I. Gandellini Paolo.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.dib.2025.111565.

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