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Mukim, Amey
et al.

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Use of GapmeRs for gene expression knockdowns in human primary resting CD4+ T cells

Hosiana Abewe[1,2,†], **Savitha Deshmukh**[1], **Amey Mukim**[1], **Nadejda Beliakova-Bethell**[1,2,*]

[1]San Diego VA Medical Center and Veterans Medical Research Foundation, San Diego, CA 92161, USA

[2]University of California San Diego, La Jolla, CA 92093, USA

Abstract

Human primary resting CD4+ T cells are difficult to transfect while preserving viability. The present study evaluated gymnotic delivery and RNase H1-dependent gene expression knockdown mediated by antisense oligonucleotides, called GapmeRs. Exposure of primary resting CD4+ T cells to GapmeRs did not cause cell activation or affect cell viability. Gene expression knockdowns were stable at least up to 48 hours after removal of GapmeRs from culture. Exposure to GapmeRs resulted in comparable levels of degradation along the entire transcript, which could be important when studying function of regulatory long non-coding RNAs. Efficiency of transcript degradation was not solely dependent on the dose of GapmeR, RNA target and its localization. When using GapmeRs, some optimization is required, and all targets have to be individually tested; however, using GapmeRs is advantageous in experiments where preservation of the resting state of the human primary CD4+ T cells and targeting nuclear RNAs are desired. In certain cases, combining GapmeR with siRNA for the same target may improve knockdown efficiency.

Keywords

gene expression; knockdown; antisense oligonucleotides; GapmeRs; primary CD4+ T cells; resting CD4+ T cells

[*]Correspondence: University of California San Diego, Stein Clinical Research Building, Rm. 304; 9500 Gilman Drive, #0679; La Jolla, CA 92093-0679, USA; nbeliako@ucsd.edu.

Author contributions

HA conducted all initial GapmeR tests and protocol optimization. HA, SD and AM performed the experiments. NBB supervised the work and analyzed the data. NBB and HA wrote the manuscript. All authors contributed to revision of the manuscript for important intellectual content.

[†]Current affiliation: University of Utah School of Medicine, Salt Lake City, UT 84112, USA.

Conflict of interest

The authors declare no conflict of interest.

1 Introduction

Human primary resting CD4+ T cells are difficult to transfect while preserving viability. Cell activation may improve delivery of knockdown agents into the cells and overcome effect on viability; however, for some applications it is important to preserve cells in a resting state during experimental gene expression manipulations. GapmeRs manufactured by Exiqon Inc. (now Qiagen, Inc., Valencia, CA) are antisense oligonucleotides (ASOs) that can be taken up by cells via unassisted delivery (gymnosis) (Soifer et al., 2012; Stein et al., 2010). GapmeR is a type of ASO comprised of a synthetic single strand containing central block of DNA nucleotides flanked by locked nucleic acids on each side, which increase strand stability. The host RNase H1, a ubiquitous enzyme located in the nucleus as well as in the cytoplasm (Liang et al., 2017), recognizes RNA-DNA hybrids formed after sequence-specific binding of antisense oligonucleotides to their target mRNA and degrades the mRNA. GapmeRs present advantages over other commonly used synthetic molecules for knockdown, such as small interfering RNA (siRNA) due to their small size and stability. In addition, GapmeRs exhibit strand specificity, which reduces off-target activity. Finally, due to ubiquitous cellular localization of RNase H1, GapmeR-mediated RNA targeting is not limited to the cytoplasm.

Initial evaluation of GapmeRs was performed using different cell lines (Soifer et al., 2012; Stein et al., 2010), while the study that investigated the mechanism of GapmeR uptake used primary CD4+ T cells that were activated prior to exposure to GapmeRs (Fazil et al., 2016). The goal of the present study was to assess GapmeR uptake and activity in untouched resting CD4+ T cells. We determined effect of treating resting CD4+ T cells with GapmeRs on cell viability and activation, characterized GapmeR uptake, knockdown efficiency and stability, evaluated efficiencies of GapmeRs designed for cytoplasmic and nuclear targets, investigated GapmeR-induced degradation of long transcripts, and assessed dual knockdowns using GapmeRs in combination with siRNA against the same target messenger RNA. The results from this study will be a valuable resource for researchers who are planning functional studies in primary resting CD4+ T cells that require gene silencing.

2 Materials and methods

2.1. Isolation of primary CD4+ T cells

Peripheral blood from healthy study participants was collected by venipuncture according to institutional review board approved protocols into BD Vacutainer plastic blood collection tubes with sodium heparin. All participants gave written informed consent. CD4+ T cells were isolated from whole blood using negative selection (StemCell Technologies, Inc., Vancouver, Canada). All CD4+ T cell samples had >95% purity and <10% expression of activation marker HLA-DR, as assessed using the Accuri C6 flow cytometer (BD Biosciences, Inc., San Jose, CA, USA). Primary CD4+ T cells were incubated overnight at 37°C, 5% CO_2 in RPMI 1640 medium supplemented with 5% human AB serum, before initiation of knockdown experiments. In some cases CD4+ T cells underwent freezing/thawing procedure, and were cultured overnight after thaw before initiation of the knockdown experiments.

2.2. GapmeRs and siRNAs

GapmeR specific to Metastasis Associated Lung Adenocarcinoma Transcript 1 (*MALAT1*) (sequence: 5' CGTTAACTAGGCTTTA 3') is the positive control GapmeR designed by Exiqon, Inc. (now Qiagen, Inc., Valecia, CA). Control A with non-specific sequence (5' AACACGTCTATACGC 3') was used as a negative control. Custom-designed GapmeRs were used for targeting Nuclear Paraspeckle Assembly Transcript 1 (*NEAT1*) and High Mobility Group AT-Hook 1 (*HMGA1*). These custom-designed GapemRs were obtained from Exiqon, Inc. and had the following sequences: *NEAT1* N1+N2: 5' ATCTGCTGTGGACTTT 3'; *NEAT1* N2: 5' ATGGAGCGTCGTAGAG 3'; *HMGA1* exonic: 5' GTAGTGTGGTGGTGAG 3'; *HMGA1* intronic 1: 5' GATGGTTGAAGCTAGT 3'; *HMGA1* intronic 2: 5' TACGTGCACTGAATCT 3'; *HMGA1* intronic 3: 5' GCCGTAGATAGAATA 3'; *HMGA1* intronic 4: 5' TCGTCTTTACTGCATT 3'. For the measures of cellular uptake efficiency, *MALAT1*, *NEAT1* N1+N2 and Control A GapmeRs were conjugated to 6-fluorescein at 5' end (5' 6-FAM) and formulated as *in vivo* Ready. GapmeRs for the remaining experiments were not conjugated to a fluorophore and were formulated as *in vitro* Standard. SiRNA were obtained from Dharmacon, Inc. (Lafayette, CO, USA) in the Accell format. GapmeRs were diluted to the final concentration of 50 μM with water, and siRNA to the final concentration of 100 μM with the 5X siRNA buffer (catalog number B002-0000-4B-100, Dharmacon, Inc., Lafayette, CO, USA) diluted 1:5 with water. These stocks were then used to treat the cells. Working concentrations of GapmeRs ranged from 100 to 500 nM, and siRNA was used at a concentration of 500 nM.

2.3. Treatment with GapmeRs and siRNA

Cells were plated into 96-well U-bottom plates at a concentration of 300,000–500,000 cells / well in a 200 ul volume. For GapmeR treatment, cells were grown in RPMI 1640 medium supplemented with 5% human AB serum. For siRNA treatment and dual treatments with siRNA and GapmeRs, cells were grown in Accell siRNA Delivery Medium (catalog number B-005000100, Dharmacon, Inc., Lafayette, CO, USA), which does not contain serum. Cells were treated with GapmeRs for 3, 4, or 5 days as indicated in each experiment. Cells were treated with siRNA for 4 days.

2.4. Assessment of cell viability and activation

Cell activation and viability were assessed following treatment with either negative control GapmeR (Control A) or positive control GapmeR (*MALAT1*) at 300 nM and 500 nM concentrations. Cells were washed with phosphate buffered saline + 0.1% bovine serum albumin and stained with Aqua fluorescence reactive dye (Thermo Fisher Scientific, Inc. Waltham, MA, USA) to assess viability, and with the following antibodies to assess activation: CD69-FITC clone L78, CD38-PE-Cy7 clone HB7, and HLA-DR-APC-Cy7 clone L243 (BD Biosciences, Inc., San Jose, CA, USA). Custom-made CD4-APC clone SK3+SK4 (BD Biosciences, Inc., San Jose, CA, USA) was used to stain CD4+ T cells to exclude small percentage of contaminants from the analysis. The data was acquired using FACS Canto II instrument (BD Biosciences, Inc., San Jose, CA, USA) and analyzed using FlowJo v10 software (FlowJo, LLC, Ashland, OR, USA). Gating strategy is shown in Figure S1A.

2.5. Assessment of GapmeR uptake by CD4+ T cells

GapmeR uptake was assessed using Accuri C6 flow cytometer (BD Biosciences, Inc., San Jose, CA, USA). Live CD4+ T cells were gated based on forward versus side scatter profiles, followed by FL1 channel (laser 488 nm, filter options 533/30 or 530/30) to identify cells that took up GapmeRs (Figure S2). GapmeR uptake was calculated as the percentage of 5' 6-FAMpositive live cells of all viable cells (raw data are available in Figure S2). Mean fluorescence intensity (MFI) was assessed in FlowJo v10 software (FlowJo, LLC, Ashland, OR, USA).

2.6. RNA isolation

Following incubation with GapmeRs or siRNAs, cells in each sample were counted, centrifuged and lysed using RLT buffer containing β-mercaptoethanol (Qiagen, Inc.). RNA was isolated using Qiagen RNeasy micro kit (Qiagen, Inc., Valecia, CA). RNA concentration was assessed using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Inc. Waltham, MA, USA).

2.7. Droplet digital (dd) PCR

Twenty nanograms of RNA from each sample was converted to cDNA using qScript (Quanta Bio, Beverly, MA) in a total reaction volume of 20 μl. DdPCR reactions were set up and run as described previously (White et al., 2015). Housekeeping gene Ribosomal Protein L27 (*RPL27*) was used as a normalizer (Beliakova-Bethell et al., 2014; Beliakova-Bethell et al., 2013; White et al., 2015). The assays to measure *MALAT1* (Hs01910177_s1), *HMGA1* (Hs00852949_g1) and *RPL27* (HS03044961_g1) were purchased from Applied Biosystems (now Thermo Fisher Scientific, Inc., Waltham, MA, USA). Six different assays designed to measure both isoforms or only the long isoform of *NEAT1* were as follows: assay 1: forward primer 5' TTCATGGACCGTGGTTTG 3', probe 5' TTCCTCATGGCGAGCAGATGGAAC 3', reverse primer 5' CTGCAATGCTAGGACTCAC3'; assay 2: forward primer 5' CGCAGATTGATGCCTTGTA 3', probe 5' TTTGCCTGCCTTCTTGTGCGTTTC 3', reverse primer 5' AGGCTAAGAACTTCTCCGA 3'; assay 3: Hs03453535_s1; assay 4: forward primer 5' ACGTGTTGCATGGTTTCT3', probe 5' AACAGTAGGGAGATGCCTGGGAGTA 3', reverse primer 5' ATGAGGGCAGTTCTCTGT 3'; assay 5: forward primer 5' TTCTTGTCATCTGTGTGTGAA 3', probe 5' CATGGGCTTAATGCTGACAAGGCC 3', reverse primer 5' CATGATTATATGTCTTGGACCCT 3'; assay 6: forward primer 5' TTGCTTCATCGGCAGGTT 3', probe 5' CCGTGAATGTTTCCTCTCTGCTGCT 3', reverse primer 5' CTTCAGCCTCCAAACACACTA 3'. Assays 1, 2, 4, 5, and 6 were custom designed and ordered from Integrated DNA Technologies, Inc. (Coralville, IA, USA). Assay 3 was purchased from Applied Biosystems (now Thermo Fisher Scientific, Inc., Waltham, MA, USA). To measure highly abundant transcripts (*MALAT1*, both isoforms of *NEAT1*, and *RPL27*), cDNA was diluted 1:10. For measurements of the *NEAT1* long isoform and *HMGA1*, cDNA was used without dilution. DdPCR data is expressed as copies of each measured transcript per number of *RPL27* molecules. When indicated, ddPCR data is presented as percent expression relative to negative Control A.

2.8. Visualization of expression levels of the two *NEAT1* isoforms in primary CD4+ T cells.

To independently assess and visualize relative expression the short and long *NEAT1* isoforms in primary CD4+ T cells, we used RNA-Seq data deposited at Gene Expression Omnibus (GEO) repository, accession number GSE114883. Samples were processed according to the established pipeline (Beliakova-Bethell et al., 2019) and visualized using Integrated Genome Viewer version 2.3.89 (Thorvaldsdóttir et al., 2012).

2.9. Statistical analysis

Flow cytometry data (percentages of cells bearing various activation markers) and cell viability were compared across GapmeR treatment conditions using beta regression modeling available in the *betareg* library (Cribari-Neto and Zeileis, 2010) in the R computing environment. *MALAT1*, *NEAT1* and *HMGA1* expression following treatment with GapmeRs was analyzed by normalizing ddPCR data to housekeeping gene *RPL27*, and then using these values to determine percent expression relative to samples treated with the negative Control A GapmeRs. These relative values were then $\log_2$ transformed. Repeated measures analysis of variance (RM ANOVA) was implemented for multi-group comparisons using library *nlme* in R, followed by post-hoc Tukey test. To compare GapmeR efficiency with different formulations and different RNA targets, $\log_2$ transformed data was analyzed using unpaired *t* tests. Correlation between dose of *MALAT1* GapmeR and knockdown efficiency was assessed using cor.test function in R. Graphs were constructed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

3. Results and discussion

3.1. CD4+ T cell activation, viability, knockdown efficiency and stability following exposure to GapmeRs

Previously, it was demonstrated that exposure of human primary CD4+ T cells to GapmeRs did not induce unwanted immunologic responses, such as secretion of interleukins and interferon gamma (Fazil et al., 2016). Some applications, for example, identification of genes that promote latent viral infection (e.g. human immunodeficiency virus), require that cells remain resting during the exposure to knockdown reagent. This is because T cell activation, not knockdown of the gene of interest, will result in induction of viral replication. Therefore, we first aimed to assess the effect of GapmeRs on CD4+ T cell activation. Cells were exposed to either Control A or *MALAT1* GapmeR at 300 nM or 500 nM for 4 days. In general, none of the activation markers (CD69, CD38 or HLA-DR) were significantly elevated following exposure of cells to GapmeRs (Figure S1B). One potential exception was the early activation marker, CD69, which was elevated in one of the three tested donors with 500 nM of *MALAT1* GapmeR. In the other case where a significant difference between untreated and treated cells was detected, percentage of CD38+ cells in 500 nM *MALAT1* GapmeR-treated sample was lower than that in the untreated sample. There was no decrease of cell viability following exposure to GapmeRs at either concentration (Figure S1B).

To assess the efficiency of GapmeR uptake, GapmeRs labeled with 5’ 6-FAM were used. The assessment was conducted over a range of doses of *MALAT1* GapmeR and 300 nM of *NEAT1* N1+N2 GapmeR following 3 day exposure. *MALAT1* uptake was dose-dependent

and began to plateau at 300 nM (Figure 1A, **circles**). Mean fluorescence intensity (MFI) of 5' 6-FAM increased over the dose curve in a similar fashion, beginning to plateau at 300 nM (Figure 1B, circles). Likewise, the uptake of *NEAT1* GapmeR at 300 nM was very efficient (Figure 1A, **triangles**); cells were overall much brighter compared to cells treated with the same concentration of *MALAT1* GapmeR (Figure 1B, **tirangles**). When cells were treated with the negative Control A GapmeR at 500 nM, less than half the cells were 5' 6-FAM positive in two out of three experiments (Figure 1A, **rhombus**), with a lesser MFI compared to the samples treated with the same concentration of *MALAT1* GapmeR (Figure 1B, **rhombus**). In general, we did not observe a consistent change in the percentage of 5' 6-FAM positive cells when cells were washed prior to flow cytometric assessment (average 76.6% before wash *vs* 76.8% after wash over 10 measurements at different time points and with different concentrations of *MALAT1* GapmeR). However, there was on average a 6-fold reduction in MFI over 10 measurements for *MALAT1* exposure at different conditions. Washing the cells prior to flow cytometric assessment likely rids cells of GapmeRs bound to the surface, but without consistent effect on the estimate of total cells that took up GapmeRs. Overall, these results indicate that GapmeR uptake is very efficient, dose-dependent for individual ASOs, but is highly variable for different ASOs administered at the same dose.

Next, gene expression knockdown was assessed in the cells treated with different doses of *MALAT1* GapmeR. With increasing concentration of GapmeR, *MALAT1* expression decreased (Figure 1C); mean knockdown efficiency of three independent experiments highly correlated with dose (R=0.96, p=0.004). There was a continued decrease in *MALAT1* expression at higher doses when percentage of cells that have taken up GapmeR and MFI plateaued (300–500 nM). Efficiency of *MALAT1* knockdown did not depend on formulation (*In vivo* Ready *vs In vitro* Standard, Figure 1D). Expression of *NEAT1* decreased following exposure to 300 nM *NEAT1* N1+N2 GapmeR; however, efficiency of *NEAT1* knockdown was modest (Figure 1D), despite its high uptake efficiency evidenced by percentage of 5' 6-FAM-positive cells and MFI (Figure 1 A and B). Overall, these results suggest that knockdown efficiency may depend not only on the amount of GapmeR that enters individual cell and the target RNA. Fazil and colleagues previously showed that GapmeR uptake in primary CD4+ T cells occurs via macropinocytosis (Fazil et al., 2016), which represents one of the pharmacologically 'non-productive' pathways for ASOs (Crooke et al., 2017). While our results demonstrate that at high enough concentrations GapmeRs are capable of inducing robust knockdown, depending on the target, sequestration of GapmeRs to endocytic vesicles following macropinocytosis (Crooke et al., 2017; Yoon and Rossi, 2018) may represent one of the limiting factors.

To assess stability of knockdown following removal of GapmeR from culture, a time course experiment with *MALAT1* GapmeR was performed, in which cells were either incubated continuously with the GapmeR for 5 days, or GapmeR was removed on day 3 and cells were followed till day 5. In the continuous presence of GapmeR, knockdown improved from day 3 to day 4 (p<0.001), with no further improvement on day 5 (Figure 1E). Interestingly, knockdown efficiency continued to improve to day 4 even when *MALAT1* GapmeR was washed out (p<0.001), and remained stable to day 5. The observed slight increase in *MALAT1* expression on day 5 when GapmeR was washed out was not statistically

significant either compared to day 4 with the wash or day 5 without the wash (Figure 1E). These results suggest that gene knockdown following exposure to *MALAT1* GapmeR is independent of the presence of GapmeR in the culture and continues to improve as the presence of GapmeR is maintained inside the cells.

3.2. Degradation of a long non-coding transcript following exposure to GapmeRs

When non-coding RNA compete for binding of regulatory microRNAs (Salmena et al., 2011), it might be important that the entire non-coding transcript is degraded. To test whether GapmeRs induced equal degradation along the entire transcript, one of the longest transcripts expressed in CD4+ T cells, long non-coding RNA (lncRNA) *NEAT1* N2 isoform (23 kB), was measured by ddPCR using multiple assays following exposure of cells to *NEAT1* GapmeRs. The other *NEAT1* isoform (N1) is shorter (3.7 kB), shares promoter and overlaps with the long isoform (Kawaguchi and Hirose, 2012) and is expressed at higher levels in primary CD4+ T cells, compared to the long isoform (Figure 2A). GapmeRs that target both short and long isoforms (N1+N2) or long isoform only (N2) were used in this experiment (Figure 2A). Assays were designed directly up- and downstream of each GapmeR, as well as further away from each GapmeR. Two of the designed assays were located close to 5' and 3' of the long isoform (N2) transcript (Figure 2A). Out of 4 experiments, one experiment was an outlier, where incubation of cells in the presence of Control A GapmeR resulted in reduction of *NEAT1* expression. As a result, when expression of *NEAT1* in the samples that were exposed to different *NEAT1* GapmeRs was normalized to *NEAT1* expression in the sample treated with Control A GapmeR, no apparent knockdown could be detected (Figure 2B, **black triangles**). For the remaining three experiments, Control A and *NEAT1* GapmeRs behaved as expected, with Control A GapmeR having negligible effect on *NEAT1* expression. RM ANOVA analysis was performed after excluding the outlier sample. There was no difference in the knockdown efficiencies measured with different assays along both short (Figure 2B, **left**) and long (Figure 2B, **right, assays #4,5,6**) isoforms, suggesting that GapmeRs induce even degradation of *NEAT1* along the entire transcript. Not surprisingly, when the lowly expressed long isoform was targeted (Figure 2B, **right**), *NEAT1* expression measured with assay #3 that maps to both isoforms was significantly higher compared to expression measured with assays #4,5 and 6 that map to the long isoform (Figure 2B, **group of samples highlighted by a box**).

3.3. Targeting a protein coding RNA by GapmeRs

Thus far, we have used GapmeRs that target lncRNAs, *MALAT1* and *NEAT1*, which localize to the nucleus. Next, we aimed to test GapmeR-mediated degradation of a protein coding transcript, *HMGA1*. Custom GapmeRs were designed to target both exons and introns of the gene sequence. Though all tested GapmeRs against nuclear lncRNA targets including the long isoform of *NEAT1* had decent activity, the four GapmeRs with the best scores assigned by the design algorithm that targeted intronic sequences of *HMGA1* were less active than the GapmeR against the exonic *HMGA1* sequence (Figure 3A). RM ANOVA demonstrated significant differences between exonic and each of the intronic GapmeRs ($p<0.0001$), while no remarkable differences could be detected among the four intronic GapmeRs ($p>0.05$). This result indicates that factors other than transcript

localization contribute to efficient GapmeR-mediated degradation. Some possibilities may include unpredicted RNA folding or protein binding sites that encompass sequences complementary to GapmeRs.

Because siRNAs and GapmeRs use different cellular machinery to degrade target RNA, we hypothesized that targeting a cytoplasmic RNA using a combination of a siRNA and GapmeR may result in improved knockdown efficiency as compared to either one reagent alone. GapmeRs and siRNAs targeting *HMGA1* exonic sequences were added to cells at the same time in the Dharmacon siRNA delivery medium. The following conditions were used as controls: 1). *HMGA1* GapmeR and non-target siRNA to measure the knockdown induced by GapmeR alone; 2). Control A GapmeR and *HMGA1* siRNA to measure the knockdown induced by siRNA alone; 3). Control A GapmeR and non-target siRNA for a negative control. Though for this particular gene siRNA-mediated knockdown was more efficient than GapmeR-mediated knockdown, when GapmeR and siRNA were added together, knockdown was more efficient than with either treatment alone ($p<0.0001$) (Figure 3B).

4. Conclusions

In conclusion, GapmeRs are suitable for knockdowns of both lncRNAs and mRNAs in human primary resting CD4+ T cells. Cell viability and resting state are preserved during exposure to GapmeRs. Gene expression knockdowns are stable at least up to 48 hours after removal of GapmeRs from culture. GapmeR-mediated degradation occurs equally along the entirety of long transcripts, which could be important when studying function of regulatory lncRNAs. Efficiency of transcript degradation is not solely dependent on the dose of GapmeR, RNA target and its localization. Some optimization is required, and all targets have to be individually tested; however, using GapmeRs is advantageous in experiments where preservation of the resting state of the human primary CD4+ T cells and targeting nuclear RNAs are desired. In certain cases, combining GapmeR with siRNA for the same target may improve knockdown efficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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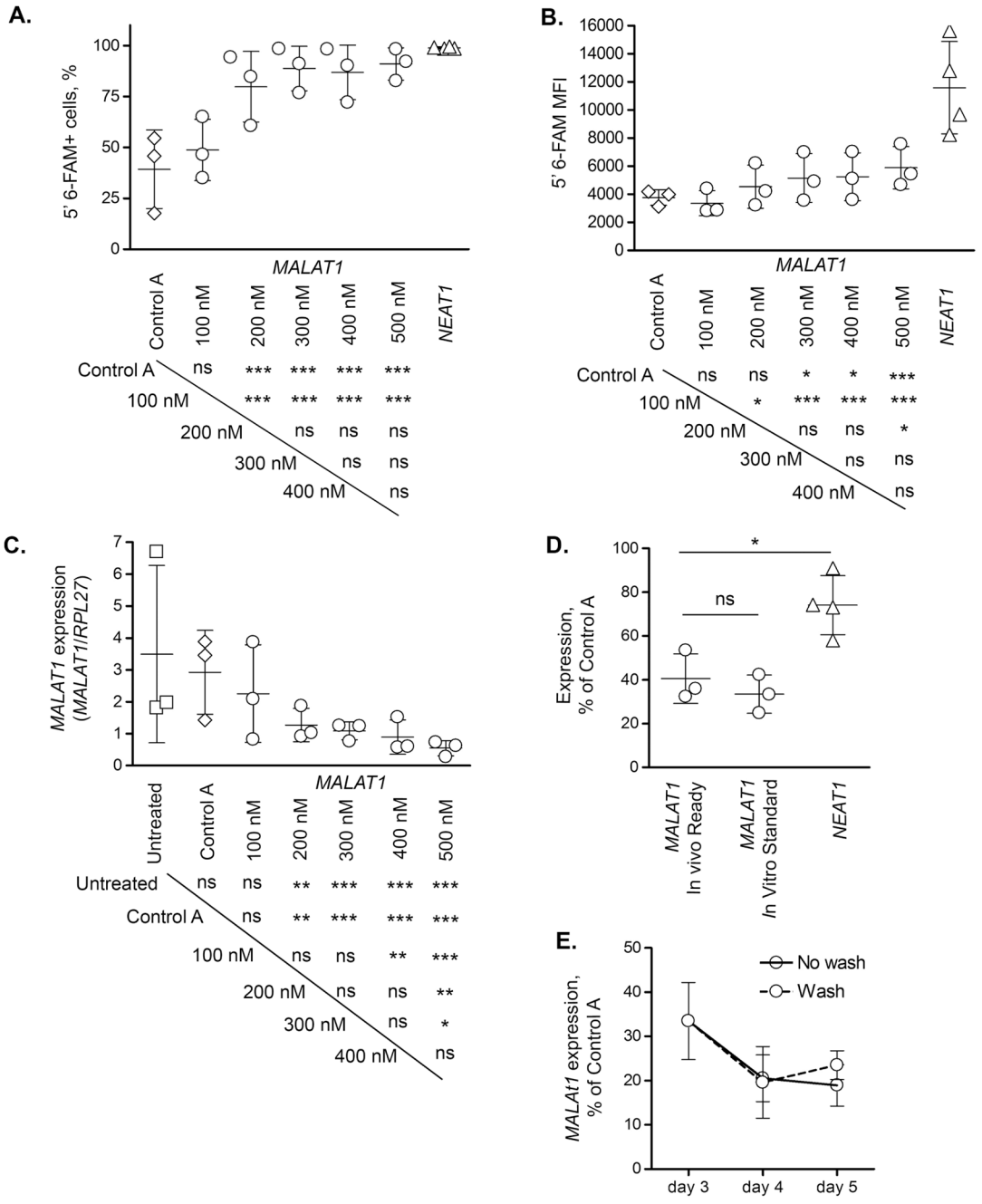

Figure 1. Efficiency of GapmeR uptake and gene expression knockdown in resting primary CD4+ T cells.

A. Cells from 3 different donors were treated with 500 nM non-target GapmeR Control A (rhombus), or *MALAT1*-specific GapmeR (circle) over a dose range 100–500 nM. In a set of separate experiments (N=4), 300 nM *NEAT1* N1+N2 GapmeR uptake was tested (triangle). B. Mean fluorescence intensity (MFI) for the same samples as in (A). C. *MALAT1* expression over the dose response curve was measured by ddPCR and normalized to the expression of the housekeeping gene *RPL27*. D. Comparison of knockdown efficiency for *MALAT1* GapmeRs of *In vivo* Ready and *In vitro* Standard formulations and *NEAT1*

N1+N2 GapmeRs used at 300 nM (lower percent expression relative to samples treated with Control A GapmeR indicates better knockdown efficiency). Note: *NEAT1* dataset for 300 nM concentration is the same as in Figure 2, *NEAT1* N1+N2 GapmeR, assay #3; *MALAT1 In vitro* Standard dataset is the same as in Figure 1E, day 3 time point. E. Cells from 3 different donors were exposed to 300 nM *MALAT1* or Control A GapmeRs for 3, 4, or 5 days (solid line). At day 3, GapmeRs were removed from an aliquot of cells and incubated in parallel till day 4 and 5 in medium in the absence of GapmeRs (dashed line). Expression of *MALAT1* was measured by ddPCR and normalized to expression of host housekeeping gene *RPL27*. Data are presented as percent *MALAT1* expression in each sample relative to corresponding sample treated with Control A GapmeR. Significance matrices under the graphs in (A), (B) and (C) show significance for each comparison at the intersection of the column names (X-axis on the graphs) and row names to the left of the slanted lines. Data in all graphs are presented as individual data points, mean of all values is shown. * $0.01<p<0.05$; ** $0.001<p<0.01$; *** $p<0.001$; *ns*, not significant ($p>0.05$). Error bars represent standard deviation.

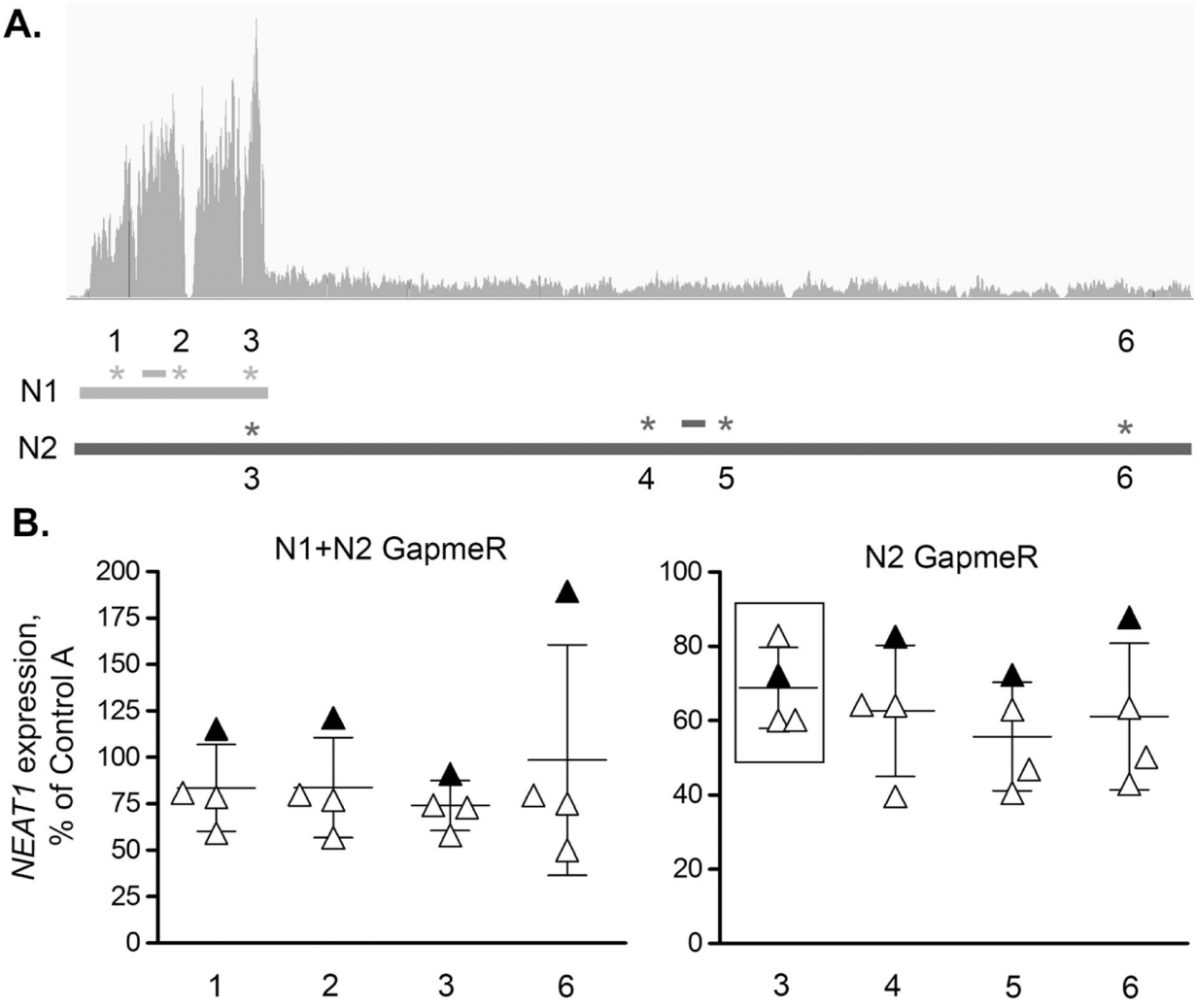

Figure 2. Degradation efficiency of long transcripts by GapmeRs.

NEAT1-specific GapmeRs were used to test whether the entire transcript may be degraded by GapmeR in both directions. A. Expression levels of the two *NEAT1* isoforms, N1 (short) and N2 (long) is illustrated using RNA-sequencing (a sample from Gene Expression Omnibus repository, accession number GSE114883, was processed and imaged using Integrated Genome Viewer version 2.3.89). Higher peaks indicate higher expression levels. A diagram of *NEAT1* showing two isoforms, N1 (light grey) and N2 (dark grey) and mapping of relative locations of N1+N2 and N2-specific GapmeRs (light and dark grey small lines) and ddPCR assays (stars 1–6) to measure expression of *NEAT1* are shown above isoform expression representation. B. Cells were treated with 300 nM of N1+N2 (left) or N2-specific GapmeRs (right) for 3 days. Expression of *NEAT1* was measured by ddPCR and normalized to expression of host housekeeping gene *RPL27*. Data are presented as individual data points for four independent donors as percent *NEAT1* expression relative to Control A GapmeR. The outlier sample in which knockdown did not occur is represented by a black triangle. The results for the experiment with N2 GapmeR where *NEAT1* was measured with an assay mapping to both isoforms (assay #3) are highlighted by a box. Error bars represent standard deviation.

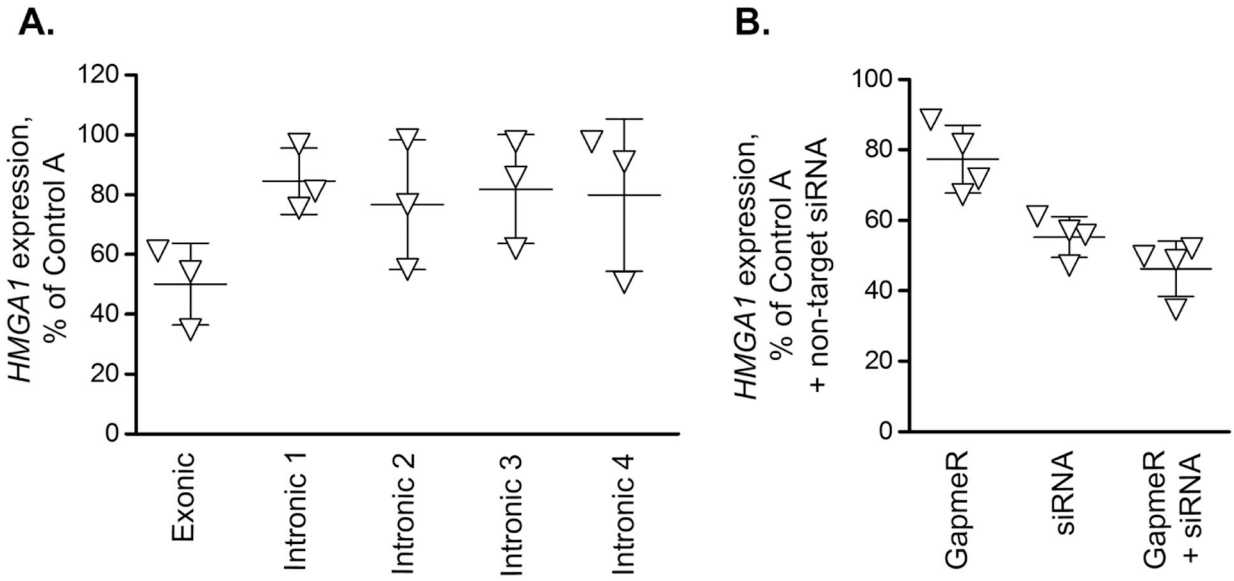

Figure 3. GapmeR-mediated degradation of a protein coding transcript, *HMGA1*.

A. Knockdown efficiency of GapmeRs designed against exonic and intronic (1–4) sequences. Cells were incubated in the presence of 300 nM *HMGA1*-specific GapmeRs for 3 days. Expression of *HMGA1* was measured by ddPCR and normalized to expression of host housekeeping gene *RPL27*. Data are presented as individual data points for three independent donors as percent expression relative to Control A GapmeR. B. Combined treatment with GapmeR and siRNA. Cells were treated with 300 nM *HMGA1*-specific exonic GapmeR (+ non-target siRNA) or 500 nM *HMGA1*-specific siRNA (+ Control A GapmeR), or both *HMGA1*-specific exonic GapmeR and siRNA, for 4 days. Expression of *HMGA1* was measured by ddPCR and normalized to expression of host housekeeping gene *RPL27*. Data are presented as individual data points for three independent donors as percent expression relative to corresponding non-target control (Control A+non-target siRNA). Error bars represent standard deviation.