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How to cite

GALLI, Valentina et al. Caprin-1 Promotes Cellular Uptake of Nucleic Acids with Backbone and Sequence Discrimination. In: Helvetica Chimica Acta, 2020, vol. 103, n° e1900255, p. 1–10. doi: 10.1002/hlca.201900255

This publication URL: https://archive-ouverte.unige.ch/unige:129366

Publication DOI: 10.1002/hlca.201900255

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Caprin-1 Promotes Cellular Uptake of Nucleic Acids with Backbone and Sequence Discrimination

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ABSTRACT: The cellular delivery of oligonucleotides has been a major obstacle in the development of therapeutic antisense agents. PNAs (Peptide Nucleic Acid) are unique in providing a modular peptidic backbone that is amenable to structural and charge modulation. While cationic PNAs have been shown to be taken up by cells more efficiently than neutral PNAs, the generality of uptake across different nucleobase sequences has never been tested. Herein, we quantified the relative uptake of PNAs across a library of 10 000 sequences for two different PNA backbones (cationic and neutral) and identified sequences with high uptake and low uptake. We used the high uptake sequence as a bait for target identification, leading to the discovery that a protein, caprin-1, binds to PNA with backbone and sequence discrimination. We further showed that purified caprin-1 added to cell cultures enhanced the cellular uptake of PNA as well as DNA and RNA.

Introduction

Peptide nucleic acids (PNA) are artificial oligomers in which the phosphoribose backbone of DNA or RNA has been replaced by a peptidic one.[1-3] PNAs hybridize to DNA or RNA according to Watson and Crick base pairing, forming more stable duplex with DNA or RNA than the corresponding homo-DNA or RNA duplex, and are metabolically stable.[4] These remarkable properties inspired significant work using PNA for the regulation of gene expression.[5-8] While this potential has been demonstrated *in cellulo* and to some extent *in vivo*, the poor cellular uptake of unmodified PNA has curtailed their therapeutic potential;[9] successful applications have been leveraged on conjugates,[5, 8] encapsulation[10] or supramolecular assemblies.[11] Several modifications of the original N-(2-aminoethyl) glycine backbone have been reported to be well tolerated or even to enhance hybridization.[12] In particular, the substitution of the glycine for D-Arg in the backbone yields guanidinium substituted PNA (arg-PNA, also known as GPNA) with enhanced cellular uptake (4-6 residue within a sequence are sufficient for cellular uptake).[13-15] Modifications with a chiral γ-substituent (*L*-stereochemistry) generate a steric bias that favors right-handed helicity with base stacking, resulting in enhanced duplex stability with DNA.[16, 17] These γ-modified PNAs have been reported with neutral substituents (alanine,[16] serine[18] or pegylated serine side chain[19]), cationic substituent[20, 21] and anionic substituents.[22, 23] PNAs are interesting because

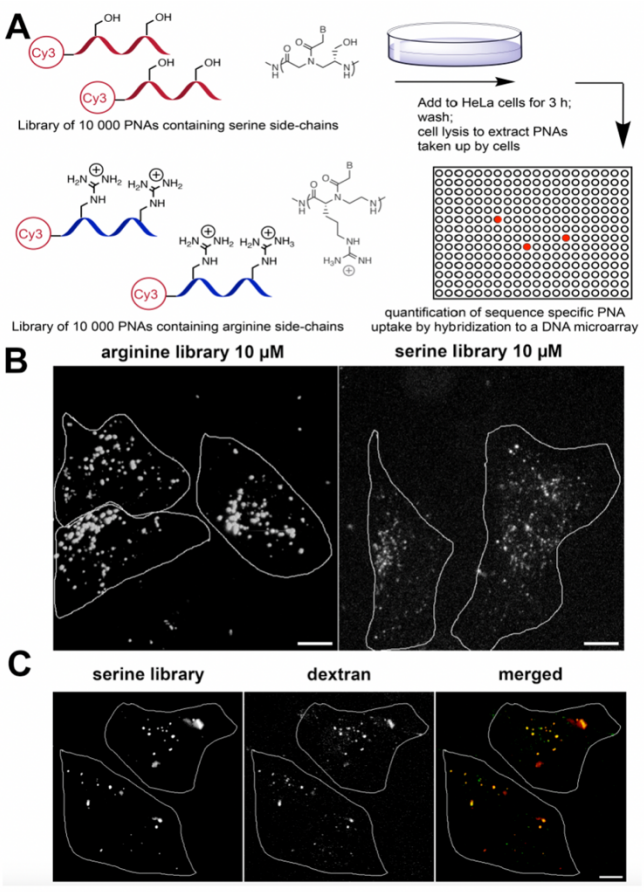

Figure 1: Analysis of the uptake of PNA libraries in cells. **A.** Description of the technical procedure followed for the qualitative analysis of the uptake of Ser-PNA and Arg-PNA libraries in cells (structure of Arg and Ser-PNA monomer shown in grey). **B.** Maximum projection of Z-stack images showing the internalized Cy3 labelled Ser-PNA and GPNA libraries by HeLa cells. **C.** Maximum projection of Z-stack images

they combine the hybridization properties of DNA with the modularity of peptides. Arg-PNAs have been shown to be generically taken up by cell in analogy to poly-Arg peptides,[24] through a mechanism that involves an electrostatic association at the membrane followed by endocytosis. On the other hand, neutral PNAs, including PNAs with a serine side chain at the γ-position, are not taken up efficiently in cultured cells. However, the generality of these assumption across different oligomer sequences has never been tested. For instance, sequence biases in cellular uptake may arise from differential interactions to a binding partner. We hypothesized that these effects would be more pronounced for a neutral PNA backbone with very low intrinsic cellular uptake than for cationic PNA. To test the sequence bias, we set up a screen to identify differential cellular uptake from a library of 10 000 sequences of γ-serine modified PNA (ser-PNA) and compared it to the same library of 10 000 sequence of Arg-PNA as shown in Figure 1.

Results and Discussion

Assessment of cellular uptake in HeLa comparing libraries with two different PNA backbones. Libraries of 10 000 unique 14-mer PNAs were obtained by split and mix combinatorial synthesis as previously reported [25, 26] (Fmoc chemistry for the Arg-PNA library[27] and Mtt chemistry for the ser-PNA library[18]). The N-termini of PNAs were capped with either Cy3 or biotin for quantification of individual sequences within the library through hybridization onto a DNA microarray containing the complementary sequences. The PNA sequences were designed to minimize cross-hybridization while having a balanced G/C content and have been used to tag multiple libraries of small molecules.[28-30] Both libraries were prepared with a D-Arg residue at the beginning and the end of the PNA sequence to ensure good solubility of the library. The Arg-PNA library included a guanidinium-modified residue at every third position (four in total) while the Ser-PNA library includes a modified residue at every other position (seven in total).

To evaluate the overall uptake in HeLa cells, the PNA libraries were separately incubated for 3 hours at 10 µM (concentration of entire library, i.e. 1nM for individual sequences). Then, the cells were thoroughly washed to remove extracellular PNAs and cells were imaged through 3D spinning-disk confocal microscopy. Concurring prior observations, the Arg-PNA library yielded higher cellular uptake compared to the Ser-PNA library (Figure 1B). In both cases, the majority of fluorescence appears as distinct punctae throughout the cytoplasm at 3h post incubation. This localization pattern suggested that the majority of PNAs were in the endosomes at this time point, which are the main organelles receiving endocytosed material. To confirm this, we stained endosomes with FITC-dextran following Cy3 labeled PNA library internalization in cells and we observed a substantial co-localization between Cy3 and FITC signal, confirming PNAs presence in the endosomes and their probable uptake in HeLa cells via endocytosis (Figure 1C).

In order to assess whether a sequence bias was obtained across the PNA libraries, we repeated the experiment and subsequently performed a gentle lysis of the cells in order to recover the cytoplasm (cytosol and organelles, except plasma membrane and nuclei). The cytoplasmic extracts containing the internalized PNAs were analyzed by microarray hybridization to quantify the relative amount of each sequence, using HeLa cells lysates mixed with entire PNA library as a reference (i.e. to control for the biases of hybridization in the presence of crude cell extracts). For the Arg-PNA library, we observed no significant uptake preference (see supplementary Table S1). But we observed the preferential uptake of distinct Ser-PNA sequences; 8 sequences show ≥ 10-fold enrichment while 36 sequences ≥ 2-fold depletion (see supplementary Table S1, we excluded sequences with low detection in the library alone). From this set, one sequence from the high uptake set (HU for High Uptake) and one sequence from the low uptake set (LU; filtered for sequence that do not hybridize efficiently in the library alone) were resynthesized and purified to evaluate their cellular uptake as individual sequences.

Characterization of single PNA sequences uptake. A sequence bias in the cellular uptake could arise from multiple factors such as a sequence specific interaction with biomolecules, a given fold in the PNA that alters its physicochemical properties or fortuitous assemblies of PNAs through partial pairing between different sequences. To investigate if the specific sequence of a PNA had an impact on the uptake by cells in absence of the remaining library, we synthesized the highest (HU) and lowest (LU) internalized sequences found by microarray and labeled them with Cy3 (see figure 2C for exact sequences). For comparison, the same sequences were synthesized with arginine-modified backbone (arg-PNA) and serine-modified backbone (Ser-PNA). For further comparison, we also prepared peptide sequences with the same backbone length and charge as the corresponding PNA (replacing the Arg-PNA monomer by Arg-Gly and a ser-PNA monomer by Gly-Ser).

To assess the internalization of HU sequences and LU sequences by HeLa cells, the ratios between total fluorescent intensity (sum of the intensity of all pixels in a determined area) of the fluorescent signal and the area of the cells were analyzed (see Figure 2 for representative images and quantification). In general, PNAs with

an arginine side chain are better internalized (Figure 2B), as seen for the whole library (Figure 1B). The HU PNA sequence displays a significantly higher uptake than the serine LU sequence (Figure 2B), showing that the PNA sequence itself had a strong impact on the cellular uptake. We next investigated the contribution of the peptide backbone to the preferential uptake. We first observed that even though arginine-based PNAs were always taken up better than the serine-based PNAs, the difference between the two sequences (Arg HU vs. Arg LU, Figure 2B) is maintained, regardless of the backbone. The comparison with the peptides that contain the same number of atoms in the backbone and the same overall charge status (Arg-peptide vs. Arg-PNA and Ser-peptide vs. Ser-PNA) also benchmarks the impact of the nucleobase sequence. While there is no statistically significant difference in the uptake of HU Arg-PNA compared to the Arg-peptide, there is a notable difference with the uptake of LU Arg-PNA. For the Ser-PNA, both HU and LU sequence have lower uptake than the corresponding peptide. Taken together, these results clearly point to a sequence bias in the yield of cellular uptake and hints at specific interactions with one or several proteins present at the surface of the cell, which could trigger internalization of PNAs during their recycling through the endosomes. The difference in uptake could depend on the specific affinity of a given sequence for binding to these proteins. Thus, we next investigated whether a specific binding partner could mediate the enhanced uptake.

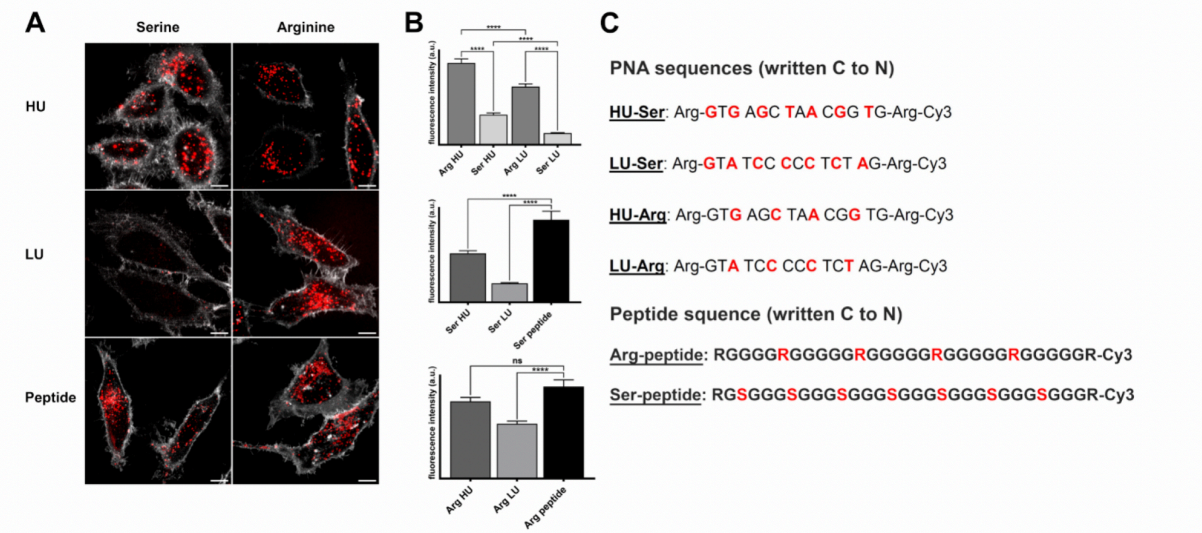

Figure 2. Single sequence specificity uptake of Ser-PNAs in HeLa cells. **A.** Maximum projection of Z-stacks images comparing the internalization of Cy3 labelled HU sequence (high uptake sequence) and LU sequence (low uptake sequence) and Arg/Ser peptides. Cell membrane was stained with CellMask Green Plasma Membrane Stain (Thermo Fisher Scientific, Thermo Fisher Scientific, Waltham, MA, USA). **B.** Plots of Cy3 fluorescent signal quantification in cells. Number of cells analyzed: Ser-HU n=61, Ser-LU n=68, Arg-HU n=70, Arg-LU n=57, Ser-peptide n=19, Arg-peptide n=14 (bars are averages+/- SEM, unpaired *t*-test, p-values < 0.0001). **C.** HU and LU nucleobase sequences. The modified residues (either α-arginine modification or γ-serine modification) are shown in red. Scale bar: 10 µm.

Identification of caprin-1 as a candidate for promoting the preferential uptake of Ser-PNA sequences. In order to identify a possible candidate carrier responsible for sequence-discriminatory PNA uptake in cells, we immobilized biotinylated PNA sequences (HU and LU PNA sequences with either serine or arginine modification) onto streptavidin magnetic beads and we carried out an affinity pull-down assay with HeLa cells lysates. After stringent washes, samples containing proteins bound to PNA beads were analyzed by SDS-PAGE. We identified bands that were not present in the control (beads without PNAs), and stronger in the samples with arginine PNAs (Figure 3A). We analyzed these bands by mass spectrometry, and searched for possible candidates with known affinity for nucleic acids in the cytoplasm and plasma membrane localization. The only pulled-down protein with such characteristics was caprin-1 (Uniprot Q14444). Caprin-1 is known to interact with RNAs and miRNAs in the cytoplasm and was first proposed to be a GPI-anchored membrane protein involved in transcytosis in Caco-2 cells.[31]

To validate this putative PNA- caprin-1 interaction, we assayed the pull down of caprin-1 from HeLa cells lysate using HU and LU PNA sequences by western blot analysis for more sensitive detection threshold. As shown in figure 3B, both HU and LU sequences are pulling down caprin-1 with different efficiencies among the serine PNAs, while the difference in pull down efficiencies between arginine HU and LU is less dramatic. The pull-down assay with the arginine-containing peptide (same length and charge density as HU and LU Arg-PNA) revealed a slight interaction with caprin-1, but no interaction with serine peptide (Figure 3D). This suggested that the nucleobase sequence was an important determinant of the interaction with caprin-1. This is also consistent with the observed sequence-specific uptake. However, the nature of the backbone, most probably its charge, also seemed to be an important factor in caprin-1 affinity.

Since the observed pull-down of caprin-1 from crude lysate could be the result of an interaction with a larger protein complex rather than a caprin-1 specific interaction, we next purified caprin-1. Caprin-1 was cloned in pDEST24 with gateway technology in frame with a c-terminal GST tag and purified from Rosetta strain bacteria. Despite multiple attempts, we could only obtain a substantially degraded (about 30% of full-length) sample of caprin-1 (see Figure 3C). Despite the high level of degradation, most of the remaining fragments (except the smallest ones) were pulled-down by biotinylated PNAs on magnetic streptavidin beads, recapitulating the same affinity differences between HU and LU observed in cell lysate pull-downs (Figure 3C). All fragments of caprin-1-GST strongly interacted with arginine HU and LU sequences but display a significantly stronger interaction with serine HU than with LU sequence. Control pull-down experiments with HU and LU sequences and GST did not show any ability of GST to pull down Ser or Arg PNAs sequences (Figure 3E).

We next measured the binding kinetics of the different PNA sequences for caprin-1 using bio-layer interferometry (BLI) (Figure 3F). These measurements confirmed a much stronger affinity of caprin-1 for arginine PNAs compared to serine PNAs (ca. 10-fold), concurring the results observed with protein pull down.

These results show that caprin-1 binds to PNAs and that the affinities qualitatively correlate to the level of cellular uptakes. This observation is not entirely surprising based on the fact that caprin-1 is known to bind RNA and miRNAs and with, among other functions, a potential role in transcytosis. [31] However, it is surprising that a cationic PNA would be a better substrate than neutral PNA based on the fact that the endogenous substrate is anionic. While the pull-down assays of endogenous caprin-1 correlate directly with cellular uptake efficiency and purified caprin-1 is sufficient for binding, we cannot rule out that caprin-1 acts in concert with other partners or contains an endogenous nucleic acid substrate.

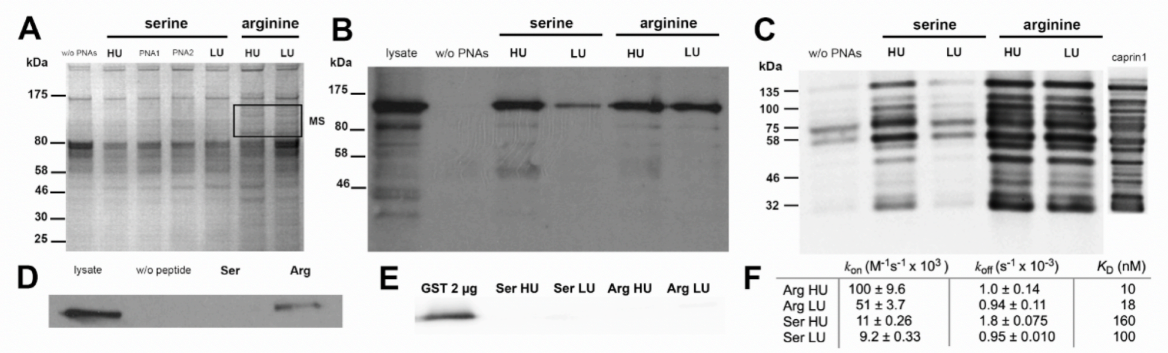

Figure 3. Biochemical analysis of HU and LU PNAs binding to caprin-1. **A.** SDS page showing the proteins pulled down by biotinylated HU and LU PNA sequences coupled with streptavidin magnetic beads from HeLa cell lysate. In the black rectangle: bands analysed by mass spectrometry. **B.** Western blot showing the different recovery of caprin-1 from HeLa cell lysate by biotinylated HU and LU PNA sequences coupled with streptavidin magnetic beads (pull-down assay). **C.** Western blot showing the pull-down assay with Ser or Arg HU and LU coated beads mixed with a solution of 200 nM purified caprin-1-GST (truncated). **D.** Western blot showing the pull-down assay with HeLa cell lysate and biotinylated arginine and serine peptides coupled with streptavidin magnetic beads. **E.** Western blot showing the pull-down assay with Ser or Arg HU and LU coated beads mixed with a solution of 200 nM purified GST (negative control). **F.** Plot of the affinities of Arg and Ser HU and LU PNAs for caprin-1, measured by bio-layer interferometry (the values likely underestimate the affinities since caprin-1 used in the experiment is partially degraded).

Caprin-1 enrichment in cell culture medium increases Ser-PNAs HU and LU uptake in HeLa cells. Next, we studied the effect of caprin-1 addition in the supernatant of HeLa cells on PNA uptake to investigate the aptitude of the purified caprin-1 to increase internalization of serine HU and LU. We incubated 500 nM caprin-1 with 500 nM Ser HU and LU PNAs for 15 minutes before adding the mix to HeLa cells. After 3h incubation we found a significant increase of Ser PNAs uptake: both HU and LU sequences increase their uptake (Figure 4), and, while Ser HU roughly doubles its uptake in presence of purified caprin-1 in the supernatant, the effect on Ser LU is much more dramatic and its internalization increases by 8-fold when caprin-1 is added to the supernatant (Figure 4B). We also tested the effect of caprin-1 on Arg HU and LU sequences uptake (Figure 4): caprin-1 in the supernatant does not influence the internalization of Arg LU, while it slightly decreases Arg HU uptake (Figure 4B).

Taken together, these results show that a higher concentration of caprin-1 outside the cells is sufficient to increase significantly ser-PNA uptake, thus indicating that this protein promotes the

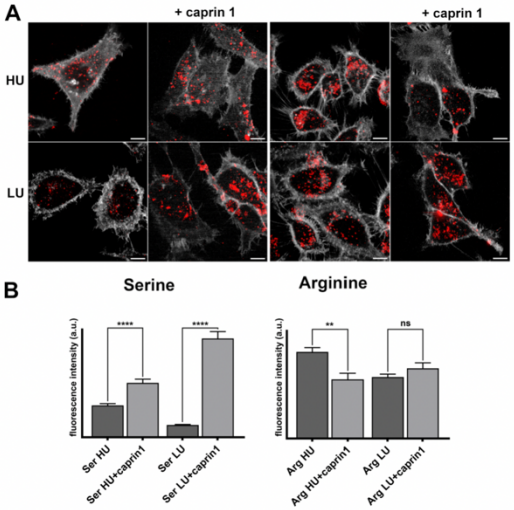

Figure 4: Caprin-1 addition in the medium increases PNAs uptake in HeLa cells. **A.** Maximum projection of Z-stacks images comparing the internalization of Cy3 labelled HU sequence (high uptake sequence) and LU sequence (low uptake sequence) in control HeLa cells and in HeLa cells in medium with purified caprin-1. **B.** Plots of Cy3 fluorescent signal quantification in cells. Number of cells analyzed: Ser-HU n=61, Ser-HU+caprin-1 n=31, Ser-LU n=68, Ser-LU+caprin-1 n= 33, Arg-HU n=70, Arg-HU+caprin-1 n=21, Arg-LU n=53, Arg-LU+caprin-1 n=31 (bars are averages+/- SEM, unpaired *t*-test, p-values < 0.05). Scale bar: 10 µm.

sequence-specific internalization of serine PNA in cells. This effect suggests that caprin-1 functions as a cycling factor between the endosomes and the external medium of the cell for internalization of small nucleic acids. As the affinity of caprin-1 for Ser-PNA sequences is less than Arg-PNA, the addition of caprin-1 with Ser-PNA may help in saturating entry points of the cells, which are already saturated with endogenous caprin-1 in the case of Arg-PNA sequences.

Caprin-1 increases RNA and DNA HU and LU uptake. Our present findings with PNAs show that caprin-1 promotes PNA uptake. We then tested if adding caprin-1 to cell medium would have the same effect on RNA or DNA uptake. We treated HeLa cells with Cy3 labeled DNA and RNA oligomers having the same sequence as HU and LU PNAs. The experiment was carried out both in absence and presence of purified caprin-1 (Figure 5). Addition of the purified protein in the cell medium increased the uptake of HU and LU RNA and DNA sequences compared to cells without purified caprin-1 in the medium (Figure 5). Interestingly, as for Ser-PNA, the LU sequence has a larger gain in cellular uptake following caprin-1 addition. On average, the levels of fluorescent signals were weaker than with PNAs as well as more variable, indicating a less efficient uptake of DNA and RNA sequences compared to PNAs. However, the fact that caprin-1 addition led to an increased uptake imply a general activity of this protein in promoting uptake of nucleotidic sequences in cultured cells, regardless of their backbone.

The observation that caprin-1 enhances oligonucleotide uptake of DNA and RNA sequences points to a physiological function of caprin-1 in intercellular RNA exchange. There is mounting evidence that exosomes mediate the transfer of mRNAs and microRNAs between cells.[32] Recently, it has been reported that PTEN suppression in brain metastatic tumor cells is epigenetically regulated by microRNAs delivered by astrocyte-derived exosome promoting intercellular transfer of PTEN-targeting microRNAs to metastatic tumor cells.[33, 34] Caprin-1 has been associated with the formation of RNA granules and serves as a regulator of post-transcriptional and epigenetic expression.[35] Ectopic caprin-1 expression has been correlated with the promotion of metastasis in human osteosarcoma and resistance to apoptosis,[36, 37] and, small molecules targeting caprin-1 have been shown to exert an antitumor activity.[38, 39] This ectopic caprin-1 expression could be harnessed to promote selective PNA uptake in metastatic areas.

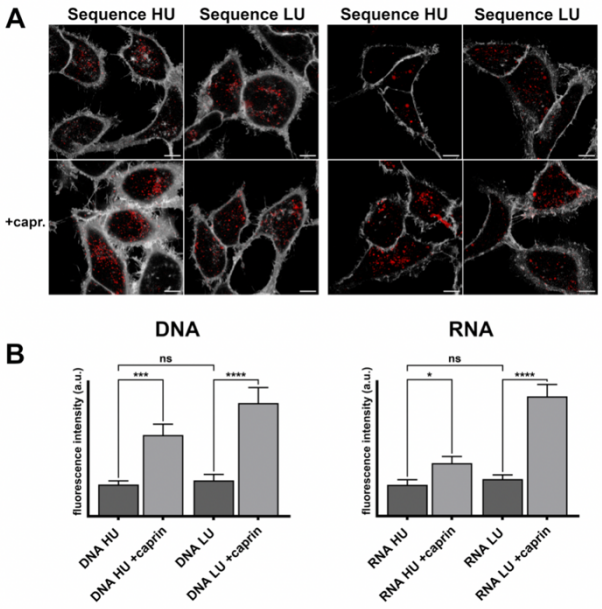

Figure 5: Caprin-1 increases DNA and RNA uptake in HeLa cells. **A.** Maximum projection of Z-stacks images comparing the internalization of Cy3 labelled DNA and RNA HU and LU sequences in HeLa cells in medium with or without addition of purified caprin-1. **B.** Plots of Cy3 fluorescent signal quantification in cells with addition of caprin-1 Number of cells analyzed: DNA-HU n=16, DNA-HU +caprin-1 n=21, DNA-LU n=15, DNA-LU +caprin-1 n=14 RNA-HU n=16, RNA-HU +caprin-1 n=11, RNA-LU n=18, RNA-LU +caprin-1 n=16 (bars are averages+/- SEM, unpaired *t*-test, p-values < 0.05). Scale bar: 10 μm.

Conclusions

The cellular delivery of oligonucleotides for the regulation of gene expression remains a significant hurdle in their therapeutic development. An appealing feature of therapeutic intervention through expression regulation is the notion that a successful technology should be broadly applicable to diverse targets using unique sequences. However, discrepancy of cellular uptake across a wide range of sequence has not been studied. Herein, we investigated the sequence-dependent uptake for a prominent oligonucleotide platform: PNA. We discovered that PNAs interact with caprin-1, which promotes the endocytosis of PNAs. The efficiency of caprin-1 mediated uptake depends on the sequence of nucleobase and the backbone of PNA (arginine-modified PNA have a stronger interaction with caprin-1 than serine-modified PNA). Caprin-1 was also shown to promote the uptake of DNA and RNA in a sequence selective manner. The present findings indicate that caprin-1 could be useful as a vector for oligonucleotide delivery and that endogenous caprin-1 could be harnessed to silence genes involved in specific diseases or in general to transport specific nucleic acids into cells.

Experimental Section

PNA library and single sequence synthesis and labeling (with biotin and Cy3)

PNA synthesis was performed in an automated fashion using an Intavis peptide synthesizer according to previously reported protocols.[18, 27] The library synthesis was performed using a split and mix combinatorial strategy (see SI for full synthetic details and characterization). Briefly, a set of 100 sequences (7-mer) were synthesized by automated solid phase peptide synthesis in microtiter plates. The resin corresponding to the 100 sequences were removed from the plate, mixed and redistributed into 100 wells to give a statistical distribution of each sequence within a well. PNA synthesis was continued with the next set 100 unique sequences (7-mer) to obtain the combinatorial output of 100 x 100 sequences (14-mer). The quality of synthesis for each pool was verified by MALDI analysis of the cleavage product from a resin aliquot. The N-terminus of PNAs was capped with either Cy-3 or biotin for quantification of individual sequences within the library through hybridization onto a DNA microarray containing the complementary sequence.

PNA uptake in HeLa cells

HeLa cells were seeded on glass bottom MatTek plates in order to reach 70-80% confluency the following day. For the treatment, culture media (Minimum Essential Media, MEM, Thermo Fisher Scientific, Waltham, MA, USA) completed with serum was removed and, after washing the plate with PBS, substituted with serum free MEM containing 10 µM PNAs library (1 nM/single PNA) Cy3 labeled or 500nM PNA (Cy3 labeled) single sequences. After 3h of incubation, plates were washed two times with PBS before being replenished with MEM complete with serum and imaged with a spinning-disk microscope (3I Inc., Denver, CO, USA and Nikon, Tokyo, Japan). To confirm endosomal partitioning of PNAs after treatment, cells were incubated with FITC-Dextran 40 µg/ml (Sigma, St. Louis, MO, USA) for 15 minutes, washed and imaged. For the observation of PNA single sequences (Cy3 labeled) uptake, 500 nM PNA single sequences were incubated at room temperature for 15' with 500 nM purified caprin-1-GST tagged before being added to cell plates containing serum free MEM and incubated for 3h before washing and imaging.

Microarray analysis

HeLa cells were seeded on 100 mm Petri dishes in order to reach 70-80% confluency the day after, and treated with 10 µM PNAs library Cy3 labeled as described above. After 3h incubation a mild cytoplasmic extraction to avoid breakage of nuclei was performed. Briefly, cells were gently scraped from the dish and centrifuged at low speed (800 rpm) for 5' before being resuspended with cold buffer (Imidazole 3 mM, 8.5% sucrose) and centrifuged at 2000 rpm for 10'. Cell pellet was resuspended in cold buffer (Imidazole 3 mM, 8.5% sucrose) + complete protease inhibitor (Roche Applied Science, Indianapolis, IN, USA) and the mixture was passed through a 22G ¼ needle using a 1 ml syringe, propping the needle to the sides of the tube while expelling the mix, allowing for shearing of cells. Lysis of the cells and integrity of the nuclei was confirmed by using a bright field microscope. Cell lysates were centrifuged at 2000 rpm for 10' and the supernatant was used for the following microarray analysis. Microarray analysis was performed on Agilent custom arrays (Agilent Earray design 041896) 4x44K slides containing four folds redundancy for each sequence (randomly distributed on the array). The PNA solution was hybridized on the array overnight at 50 °C, in PBS buffer, 40 % formamide using the Agilent incubation chamber and incubation oven with rotor for mixing the microarray solution. The slides were removed from the oven, allowed to cool to room temperature and washed twice with PBS-T and once with milli-Q water to eliminate non-hybridized material. The microarray fluorescence intensities were measured on a Genepix Personal Scanner 4100A using the filter corresponding to Cy3 excitation/emission and the fluorescence signal quantified using GenepixPRO7 software, averaged over the 4 copies of each sequence.

Sequences of PNAs and peptides

Sequences are written C- to N-ter:

1: HU-Ser: Arg-**G**T**G** A**G**C **T**A**A** C**G**G **T**G-Arg-Cy3

2: LU-Ser: Arg-**G**T**A** T**C**C **C**C**C** T**C**T **A**G-Arg-Cy3

3: HU-Arg: Arg-GT**G** AG**C** TA**A** CG**G** TG-Arg-Cy3

4: LU-Arg: Arg-GT**A** TC**C** CC**C** TC**T** AG-Arg-Cy3

underlined bold letters denote a modified PNA residue (either α-arginine modification or γ-serine modification).

Peptide Sequence:

6: RGSGGGSGGGSGGGSGGGSGGGSGGGSGGR-Cy3
7: RGGGGRGGGGGRGGGGGRGGGGGRGGGGGR-Cy3

RNA and DNA sequences

RNA and DNA labeled with Cy3 sequences correspondent to HU (5'-GTG AGCTAACGGTG-3' or 5'-GUGAGCUAACGGUG-3') and LU (5'-GTATCCCCCTCTAG-3' or 5'-GUAUCCCCCUCUAG-3') PNAs were purchased from Microsynth, Balgach (Switzerland).

Mass spectrometry analysis

To isolate proteins binding to PNAs in cells, HeLa cells were lysed using the mild protocol described above and the lysate was incubated 2h at room temperature (rotating) with biotinylated PNAs (Ser HU, SerLU, Arg HU, Arg LU and other 2 PNA sequences resulted with high permeability in the microarray analysis) bound to magnetic streptavidin-Dynabeads (Thermo Fisher Scientific, Waltham, MA, USA). After the incubation, 5 washes with PBS were carried out by pelleting the beads with a magnetic tube stand, and removing the lysate or PBS. The sample were denatured and run on NuPAGE gel (GEL Thermo Fisher Scientific, Waltham, MA, USA) and stained with SimpleBlue (Thermo Fisher Scientific, Waltham, MA, USA). Interesting bands were cut and analyzed by mass spectrometry by Proteomic Core Facility (EPFL, Faculté des Sciences de la Vie, Lausanne, Switzerland).

Purification of caprin-1

Human caprin-1 sequence from the Caprin-1 pEGFP-C1 (kind gift of John W. Schrader, University of British Columbia, Vancouver, Canada) was cloned in pDEST24 with C-terminal GST tag using Gateway technology (Thermo Fisher Scientific, Waltham, MA, USA).
Rosetta strain bacteria were inoculated with the vector and cultured overnight at 37°C (400 ml culture with TB broth). The culture was diluted up to 4 liters and 2h grown at 37°C before being induced with IPTG 0.5 mM overnight at 30°C. Bacteria were then centrifuged and the pellet was resuspended in 100 mM Hepes pH7.4, 20mM NaCl, Triton 1%, protease inhibitor complete tablet (Roche Applied Science, Indianapolis, IN, USA) and sonicated four times, 3' each, before being centrifuged for 30'. 25 ml of lysate were incubated with 12.5 ml Glutatione-Sepharose (GE healthcare, Little Chalfont, UK) for 1h at 4°C and washed with 100 mM Hepes pH7.4, 20mM NaCl before being mounted and run through a column (BIO-RAD, Hercules, CA, USA). Elution was carried out in 100 mM Hepes pH7.4, 20mM NaCl, L-Glu 30 mM.

Pull-down assays

500nM PNA biotinylated single sequences or Arg or Ser peptides in 200 µl PBS pH7.4 were coupled with magnetic streptavidin-Dynabeads (Thermo Fisher Scientific, Waltham, MA, USA) and incubated 30' at room temperature rotating. After the incubation, beads were pelleted and further incubated in 500 µl PBS pH7.4 BSA 3% to block non-specific adsorption sites (30', room temperature, rotating); the beads were subsequently incubated for 2h rotating with HeLa cell lysate or 200 nM purified caprin-1. After removing the lysate, the samples were denaturated and immunoblotted using polyclonal rabbit anti-caprin-1 (SAB1101135, Sigma, St. Louis, MO, USA) and secondary goat antibody against rabbit, and HRP-conjugated (Sigma, St. Louis, MO, USA).

Affinity measurements

Affinity measurements were realized with 10 µM biotinylated PNAs and four concentration of purified caprin-1-GST (1.25, 2.5, 5 and 10 µM) using biolayer interferometry (BLI) on a BLItz apparatus (Pall Life Sciences, Port Washington, NY, USA). The streptavidin sensor was incubated with biositin 100 µM before use to saturate non-specific adsorption sites.

Imaging and analysis

Z-stack images (step-size of 0.5 µm) were obtained using a spinning disk confocal microscope (3I Inc., Denver, CO, USA and Nikon, Tokyo, Japan) with an EM-CCD camera (iXONEM+, Andor Tech. Ltd., Belfast, UK) and maximum projection were obtained with ImageJ. From the maximum projections background was removed by subtracting the average gray mean value of the areas surrounding cells, and cells were manually profiled to avoid inclusion of fluorescent aggregates. The ratio between raw integrated density of the Cy3 signal in profiled cells and the area was used to quantify the level of uptake. Plots and statistics were performed using GraphPad Prism.

Supplementary Material

General synthetic procedures and characterization of the synthetic products (Supplementary Information); Microarray data (Table S1).
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/MS-number.

Raw data associated with experiments has been deposited and is available (https://yareta.unige.ch: 10.26037/yareta:grrtg4elinhphfldoeto5rwhpy).

Acknowledgements

The authors thank John W. Schrader for its kind gift of the caprin-1 plasmid. The authors acknowledge support from the Swiss National Science Foundation, the NCCR chemical biology and the University of Geneva. AR acknowledges funding support from: Human Frontier Science Program (HFSP: RGY0076-2008); the European Research Council (ERC: 311536-MEMFIS).

References

[1] M. Egholm, O. Buchardt, L. Christensen, C. Behrens, S. M. Freier, D. A. Driver, R. H. Berg, S. K. Kim, B. Norden, P. E. Nielsen, 'PNA hybridizes to complementary oligonucleotides obeying the Watson□Crick hydrogen-bonding rules', *Nature* **1993**, *365*, 566-568.

[2] B. Hyrup, P. E. Nielsen, 'Peptide Nucleic Acids (PNA): Synthesis, properties and potential applications', *Bioorg. & Med. Chem.* **1996**, *4*, 5-23.

[3] P. E. Nielsen, 'Peptide Nucleic Acid. A Molecule with Two Identities', *Acc. Chem. Res.* **1999**, *32*, 624-630.

[4] V. V. Demidov, V. N. Potaman, M. D. Frank-Kamenetskil, M. Egholm, O. Buchard, S. H. Sönnichsen, P. E. Nlelsen, 'Stability of peptide nucleic acids in human serum and cellular extracts', *Bioch. Pharm.* **1994**, *48*, 1310-1313.

[5] M. Pooga, U. Soomets, M. Hallbrink, A. Valkna, K. Saar, K. Rezaei, U. Kahl, J.-X. Hao, X.-J. Xu, Z. Wiesenfeld-Hallin, T. Hokfelt, T. Bartfai, U. Langel, 'Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo', *Nat. Biotech.* **1998**, *16*, 857-861.

[6] B. A. Janowski, K. Kaihatsu, K. E. Huffman, J. C. Schwartz, R. Ram, D. Hardy, C. R. Mendelson, D. R. Corey, 'Inhibiting transcription of chromosomal DNA with antigene peptide nucleic acids', *Nat. Chem. Biol.* **2005**, *1*, 210-215.

[7] H. Yin, C. Betts, A. F. Saleh, G. D. Ivanova, H. Lee, Y. Seow, D. Kim, M. J. Gait, M. J. A. Wood, 'Optimization of Peptide Nucleic Acid Antisense Oligonucleotides for Local and Systemic Dystrophin Splice Correction in the mdx Mouse', *Mol. Ther.* **2010**, *18*, 819-827.

[8] C. J. Cheng, R. Bahal, I. A. Babar, Z. Pincus, F. Barrera, C. Liu, A. Svoronos, D. T. Braddock, P. M. Glazer, D. M. Engelman, W. M. Saltzman, F. J. Slack, 'MicroRNA silencing for cancer therapy targeted to the tumour microenvironment', *Nature* **2015**, *518*, 107-110.

[9] J. Saarbach, P. M. Sabale, N. Winssinger, 'Peptide nucleic acid (PNA) and its applications in chemical biology, diagnostics, and therapeutics', *Curr. Opin. Chem. Biol.* **2019**, *52*, 112-124.

[10] I. A. Babar, C. J. Cheng, C. J. Booth, X. P. Liang, J. B. Weidhaas, W. M. Saltzman, F. J. Slack, 'Nanoparticle-based therapy in an in vivo microRNA-155 (miR-155)-dependent mouse model of lymphoma', *P Natl Acad Sci USA* **2012**, *109*, E1695-E1704.

[11] J. Gasparello, A. Manicardi, A. Casnati, R. Corradini, R. Gambari, A. Finotti, F. Sansone, 'Efficient cell penetration and delivery of peptide nucleic acids by an argininocalix[4]arene', *Sci. Rep.* **2019**, *9*, 3036.

[12] S. Barluenga, N. Winssinger, 'PNA as a Biosupramolecular Tag for Programmable Assemblies and Reactions', *Acc. Chem. Res.* **2015**, *48*, 1319-1331.

[13] P. Zhou, M. Wang, L. Du, G. W. Fisher, A. Waggoner, D. H. Ly, 'Novel Binding and Efficient Cellular Uptake of Guanidine-Based Peptide Nucleic Acids (GPNA)', *J. Am. Chem. Soc.* **2003**, *125*, 6878-6879.

[14] A. Dragulescu-Andrasi, P. Zhou, G. He, D. H. Ly, 'Cell-permeable GPNA with appropriate backbone stereochemistry and spacing binds sequence-specifically to RNA', *Chem. Commun.* **2005**, 244-246.

[15] S. M. Thomas, B. Sahu, S. Rapireddy, R. Bahal, S. E. Wheeler, E. M. Procopio, J. Kim, S. C. Joyce, S. Contrucci, Y. Wang, S. I. Chiosea, K. L. Lathrop, S. Watkins, J. R. Grandis, B. A. Armitage, D. H. Ly, 'Antitumor Effects of EGFR Antisense Guanidine-Based Peptide Nucleic Acids in Cancer Models', *ACS Chem. Biol.* **2013**, *8*, 345-352.

[16] A. Dragulescu-Andrasi, S. Rapireddy, B. M. Frezza, C. Gayathri, R. R. Gil, D. H. Ly, 'A Simple γ-Backbone Modification Preorganizes Peptide Nucleic Acid into a Helical Structure', *J. Am. Chem. Soc.* **2006**, *128*, 10258-10267.

[17] R. Bahal, E. Quijano, N. A. McNeer, Y. Liu, D. C. Bhunia, F. López-Giráldez, R. J. Fields, W. M. Saltzman, D. H. Ly, P. M. Glazer, 'Single-Stranded γPNAs for In Vivo Site-Specific Genome Editing via Watson-Crick Recognition', *Curr. Gen. Ther.* **2014**, *14*, 331-342.

[18] D. Chouikhi, M. Ciobanu, C. Zambaldo, V. Duplan, S. Barluenga, N. Winssinger, 'Expanding the Scope of PNA-Encoded Synthesis (PES): Mtt-Protected PNA Fully Orthogonal to Fmoc Chemistry and a Broad Array of Robust Diversity-Generating Reactions', *Chem. Eur. J.* **2012**, *18*, 12698-12704.

[19] B. Sahu, I. Sacui, S. Rapireddy, K. J. Zanotti, R. Bahal, B. A. Armitage, D. H. Ly, 'Synthesis and Characterization of Conformationally Preorganized, (R)-Diethylene Glycol-Containing gamma-Peptide Nucleic Acids with Superior Hybridization Properties and Water Solubility', *J. Org. Chem.* **2011**, *76*, 5614-5627.

[20] R. Mitra, K. N. Ganesh, 'Aminomethylene Peptide Nucleic Acid (am-PNA): Synthesis, Regio-/Stereospecific DNA Binding, And Differential Cell Uptake of (alpha/gamma,R/S)am-PNA Analogues', *J. Org. Chem.* **2012**, *77*, 5696-5704.

[21] P. Gupta, E. E. Rastede, D. H. Appella, 'Multivalent K-L gamma-PNA oligomers bind to a human telomere DNA G-rich sequence to form quadruplexes', *Bioorg. & Med. Chem. Lett.* **2015**, *25*, 4757-4760.

[22] C. Avitabile, L. Moggio, G. Malgieri, D. Capasso, S. Di Gaetano, M. Saviano, C. Pedone, A. Romanelli, 'gamma sulphate PNA (PNA S): Highly Selective DNA Binding Molecule Showing Promising Antigene Activity', *Plos One* **2012**, *7*.

[23] N. Tilani, S. De Costa, J. M. Heemstra, 'Evaluating the Effect of Ionic Strength on Duplex Stability for PNA Having Negatively or Positively Charged Side Chains', *Plos One* **2013**, *8*, 58670.

[24] P. A. Wender, D. J. Mitchell, K. Pattabiraman, E. T. Pelkey, L. Steinman, J. B. Rothbard, 'The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters', *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 13003-13008.

[25] J. L. Harris, N. Winssinger, 'PNA encoding (PNA=peptide nucleic acid): from solution-based libraries to organized microarrays', *Chem. Eur. J.* **2005**, *11*, 6792-6801.

[26] C. Zambaldo, S. Barluenga, N. Winssinger, 'PNA-encoded chemical libraries', *Curr Opin Chem Biol* **2015**, *26*, 8-15.

[27] F. Debaene, J. A. Da Silva, Z. Pianowski, F. J. Duran, N. Winssinger, 'Expanding the scope of PNA-encoded libraries: divergent synthesis of libraries targeting cysteine, serine and metallo-proteases as well as tyrosine phosphatases', *Tetrahedron* **2007**, *63*, 6577-6586.

[28] A. Novoa, T. Machida, S. Barluenga, A. Imberty, N. Winssinger, 'PNA-encoded synthesis (PES) of a 10 000-member hetero-glycoconjugate library and microarray analysis of diverse lectins', *ChemBioChem* **2014**, *15*, 2058-2065.

[29] J. P. Daguer, C. Zambaldo, M. Ciobanu, P. Morieux, S. Barluenga, N. Winssinger, 'DNA display of fragment pairs as a tool for the discovery of novel biologically active small molecules', *Chem. Sci.* **2015**, *6*, 739-744.

[30] S. Barluenga, C. Zambaldo, H. A. Ioannidou, M. Ciobanu, P. Morieux, J. P. Daguer, N. Winssinger, 'Novel PTP1B inhibitors identified by DNA display of fragment pairs', *Bioorg Med Chem Lett* **2016**, *26*, 1080-1085.

[31] J. A. Ellis, J. P. Luzio, 'Identification and Characterization of a Novel Protein (p137) Which Transcytoses Bidirectionally in Caco-2 Cells', *J. Biol. Chem.* **1995**, *270*, 20717-20723.

[32] H. Valadi, K. Ekstrom, A. Bossios, M. Sjostrand, J. J. Lee, J. O. Lotvall, 'Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells', *Nat. Cell Biol.* **2007**, *9*, 654-659.

[33] L. Zhang, S. Zhang, J. Yao, F. J. Lowery, Q. Zhang, W. C. Huang, P. Li, M. Li, X. Wang, C. Zhang, H. Wang, K. Ellis, M. Cheerathodi, J. H. McCarty, D. Palmieri, J. Saunus, S. Lakhani, S. Huang, A. A. Sahin, K. D. Aldape, P. S. Steeg, D. Yu, 'Microenvironment-induced PTEN loss by exosomal microRNA primes brain metastasis outgrowth', *Nature* **2015**, *527*, 100-104.

[34] J. W. Clancy, Y. Zhang, C. Sheehan, C. D'Souza-Schorey, 'An ARF6-Exportin-5 axis delivers pre-miRNA cargo to tumour microvesicles', *Nat. Cell Biol.* **2019**, *21*, 856-866.

[35] P. Anderson, N. Kedersha, 'RNA granules: post-transcriptional and epigenetic modulators of gene expression', *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 430-436.

[36] A. A. Sabile, M. J. Arlt, R. Muff, K. Husmann, D. Hess, J. Bertz, B. Langsam, C. Aemisegger, U. Ziegler, W. Born, B. Fuchs, 'Caprin-1, a novel Cyr61-interacting protein, promotes osteosarcoma tumor growth and lung metastasis in mice', *Biochim. Biophys. Acta* **2013**, *1832*, 1173-1182.

[37] Z. S. Yang, H. Qing, H. Gui, J. Luo, L. J. Dai, B. Wang, 'Role of caprin-1 in carcinogenesis', *Oncol. Lett.* **2019**, *18*, 15-21.

[38] Y.-Q. Qiu, C.-W. Yang, Y.-Z. Lee, R.-B. Yang, C.-H. Lee, H.-Y. Hsu, C.-C. Chang, S.-J. Lee, 'Targeting a ribonucleoprotein complex containing the caprin-1 protein and the c-Myc mRNA suppresses tumor growth in mice: an identification of a novel oncotarget', *Oncotarget* **2014**, 2148-2143.

[39] Y.-Q. Qiu, C.-W. Yang, Y.-Z. Lee, R.-B. Yang, C.-H. Lee, H.-Y. Hsu, C.-C. Chang, S.-J. Lee, 'Tylophorine compounds exert anti-cancer activity by targeting a ribonucleoprotein complex containing caprin-1 and c-Myc mRNA', *FASEB* **2015**, *29*, LB132.

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