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(54) Title: STRUCTURAL DOMAINS OF ANTISENSE RNA MOLECULES UP-REGULATING TRANSLATION

(57) Abstract: There are disclosed functional nucleic acid molecules comprising a target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced; and a regulatory sequence having two-dimensional structures comprising specific stem-loop and internal loop domains and displaying translation enhancing efficiency.

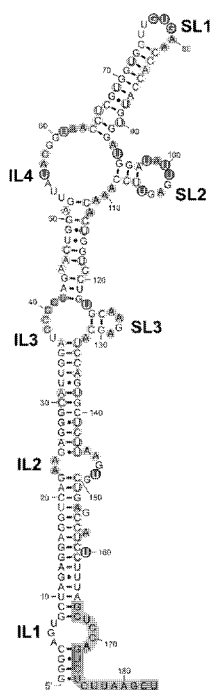


FIG. 2

SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
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**"STRUCTURAL DOMAINS OF ANTISENSE RNA MOLECULES UP-
REGULATING TRANSLATION"**

Cross-Reference To Related Applications

5 This Patent application claims priority from Italian Patent Application No. 102018000002411 filed on February 5, 2018, the disclosure of which is incorporated by reference.

Technical Field of the Invention

10 The present invention relates to functional nucleic acid molecules comprising a target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced; and a regulatory sequence having two-dimensional structures comprising specific stem-loop and internal loop domains
15 and/or specific three-dimensional structures.

Prior Art

Large genomic projects such as ENCODE and FANTOM have shown that the majority of the genome is transcribed, but less than 5% of transcribed RNA encode for proteins. Indeed,
20 long non-coding RNAs (lncRNAs) and transposable elements (TEs) seem to represent the major cellular transcriptional output. In the past ten years, an increasing number of studies are unveiling lncRNA functions. Altogether, it is becoming evident that lncRNAs act primarily as regulatory
25 molecules, in the nucleus and in the cytoplasm, through a wide repertoire of mechanisms. Despite this progress, only a small portion of annotated lncRNAs has been functionally validated.

Recently, a novel functional class of antisense (AS)
30 lncRNAs was identified, which increase translation of partially overlapping sense protein-coding mRNAs. These RNAs are also called SINEUPs, as they are antisense RNAs that contain SINE elements that cause UP-regulation of protein translations.

35 Antisense (AS) Uchl1, a lncRNA antisense to the mouse

orthologue of human Uchl1/PARK5 gene, can be considered the representative member of SINEUPs as it was found to increase Uchl1 protein synthesis acting at a post-transcriptional level. AS Uchl1 activity depends on the combination of two functional domains: at the 5' end, the overlapping region, indicated as "Binding Domain", dictates AS Uchl1 specificity towards Uchl1 mRNA; at the 3' end, the non-overlapping region contains an embedded inverted SINE B2 element, which acts as "Effector Domain" and triggers translation up-regulation of bound target mRNA.

More than 30 antisense lncRNAs promote translation up-regulation of partially overlapping mRNAs. By swapping the Binding Domain, it is possible to re-direct AS Uchl1 activity towards a target mRNA of choice.

These natural and synthetic molecules were named SINEUPs, as they require a SINE B2 element to UP-regulate translation and are disclosed in WO 2012/133947. AS Uchl1 can be considered the representative member of this new class of lncRNAs, where an embedded transposable element is exploited as functional RNA domain to achieve protein up-regulation.

The inverted SINE B2 element is an example of well-defined lncRNA domain. Evolutionary studies have indicated that the primary sequences of SINE elements, like lncRNAs in general, are poorly conserved. Partial conservation within SINE elements is only observed at A and B boxes, that, in non-embedded SINEs, represent internal promoters for RNA polymerase III. Interestingly, in spite of poor sequence identity, at least murine SINE B2 elements display functional conservation as a number of SINE B2 sequences have been shown to act as effector domains in natural and chimeric SINEUPs. Also a human SINE element was identified and proved to work as SINEUP (Schein A. et al., Sci Rep. (2016), 6:33605).

Therefore, domain functionality of embedded SINE has been hypothesized to reside on structural-based rather than

sequence-based motifs.

RNA folding has been hypothesized to provide the functional cues to lncRNAs and embedded TEs. RNA sequences are capable of folding to form complex secondary and tertiary structures that are difficult to predict when only the primary nucleotide sequence is known. Computational methods for systematically identifying lncRNA secondary structure modules are still unsatisfactory. Current algorithms are capable of correctly predicting about 50-70% of known base pairs. Most importantly, a value of 50-70% accuracy in the secondary structure prediction model results from the average between regions where the model is correct and regions of profound inconsistency. Therefore, measuring the actual structure of RNA molecules in solution is the only available method to gain insights into RNA functions. Methods are available based on chemical modification of water-accessible residues (chemical footprinting) and based on the magnetic features of nucleic acids (NMR). More recently, a novel biochemical approach, termed icSHAPE (in vivo click selective 2'-hydroxyl acylation and profiling experiment), has been developed, which enables the first global view of RNA secondary structures in living cells for all four bases.

In view of the above, there is a need to identify whether specific two-dimensional or three-dimensional molecular structures contribute to the function of lncRNAs in order to be able to effectively and efficiently exploit these molecules in further research and in the clinical setting. In particular, lncRNAs represent an ideal tool for a broad range of research activities, from studying gene function to industrial protein production and for in vivo applications, such as treatment of haploinsufficiencies.

Summary of the Invention

It is an object of the present invention to identify the secondary and tertiary structure of the inverted SINE B2 element derived from mouse AS Uchl1, of other mouse SINEs

and of SINEs from evolutionary distant species that are embedded in functional antisense lncRNAs with partial overlap to a protein-coding gene and translation up-regulation activity. The identification of motifs in the secondary and tertiary structures of these molecules and their appropriate combination would allow the design of SINE elements with increased translation up-regulation function.

In addition, the identification of these motifs and their appropriate combination would allow the design of shorter functional sequences. In fact, for therapeutic applications, the delivery of SINEUP RNA is a crucial challenge. RNA molecules are highly unstable in living organisms. Chemical modifications and/or encapsulation in lipid nanoparticles have been widely used to overcome the natural instability of RNAs. However, these delivery methods have been proven inefficient for long RNA molecules. The design of shorter variants of the inverted SINE B2 effector domain retaining only discrete and active structural elements "cleans" the effector domain from "non-essential" sequences to obtain the shortest active SINEUP RNA molecule.

This object is achieved by means of the functional nucleic acid molecule as defined in claim 1 or 2.

Other objects of the present invention are to provide a functional nucleic acid molecule as defined in claims 12, 15, 17, 19, 22 and a composition as defined in claim 26.

Definitions

By the term "miniSINEUP" there is intended a nucleic acid molecule consisting of a binding domain (complementary sequence to target mRNA), a spacer sequence (in the exemplified case a 21 nt spacer), and any SINE or SINE-derived sequence as the effector domain (Zucchelli *et al.*, Front Cell Neurosci., 9: 174, 2015.).

In particular, by the term "miniSINEUP-GFP" there is intended that the binding domain of the miniSINEUP is designed to complementary sequence to EGFP mRNA (Zucchelli

et al., Front Cell Neurosci., 9: 174, 2015.).

By "functional nucleic acid molecule" there is intended generally that the nucleic acid molecule is capable of enhancing the translation of a target mRNA of interest.

5 Brief Description of the Drawings

Figure 1 related to Example 1 shows chemical footprinting of the inverted SINE B2 sequence from natural AS Uchl 1.

10 Figure 2 related to Example 2 shows the secondary structure of the inverted SINEB2 sequence from natural AS Uchl 1.

Figure 3 related to Example 3 shows structures in solution of termini-deleted fragments of inverted SINE B2 RNA.

15 Figure 4 related to Example 4 shows that the first stem-loop domain (SL-1) contributes to AS Uchl1 activity by Western blot and qRT-PCR.

20 Figure 5 related to Example 5 shows that the first stem-loop domain (SL-1) is required for translation up-regulation of AS Uchl1 nucleic acid molecule in human hepatocellular cell line and with overexpressed Uchl1-FLAG by Western blot and qRT-PCR.

25 Figure 6 related to Example 6 shows that the first stem-loop domain (SL-1) contributes to the translation up-regulation activity of antisense lncRNA overlapping with GFP mRNA (SINEUP-GFP) in HEK 293T/17 cells by Western blot and qRT-PCR.

30 Figure 7 related to Example 7 shows determination of structure in solution of the inverted SINE B2 fragment (38 nt) by NMR.

Figure 8 related to Example 8 shows aromatic-anomeric region of a 2D NOESY NMR spectrum ($\tau_m=250$ ms) of invSINEB2/38.

35 Figure 9 related to Example 9 shows the connectivity map of NMR signals used to properly reconstruct the 3D

structure of inverted SINE B2 38nt fragment.

Figure 10 related to Example 10 shows high-resolution 3D structure of the SL-1 region of the inverted SINE B2 effector domain.

5 Figure 11 related to Example 11 shows deleted sequences on the inverted SINE B2 and the effect of these deletions on inverted SINE B2 activity (Western blot and qRT-PCR).

10 Figure 12 related to Example 12 shows mutated sequences on the inverted SINE B2 and the effect of these mutations on inverted SINE B2 activity (Western blot and qRT-PCR).

Figure 13 related to Example 13 shows mutated sequences on a shorter version of the inverted SINE B2 and the effect of these mutations on inverted SINE B2 activity.

15 Figure 14 related to Example 14 shows that SINEB2 elements from different mouse AS lncRNAs can act as Effector Domain in miniSINEUPs and up-regulate GFP protein expression in HEK293T (Western blot).

Figure 15 related to Example 15 shows ClustalW sequence alignment of SINEB2 elements from different mouse AS lncRNAs.

20 Figure 16 related to Example 16 shows SINE sequences from evolutionary distant species acting as effector domain in antisense functional nucleic acid molecules with translation up-regulation activity (Western blot and qRT-PCR).

25 Figure 17 related to Example 17 shows the correlation between more potent translation up-regulation activity of SINE sequences from evolutionary distant species and more complex structures.

30 Figure 18 related to Example 18 shows 2D structure motif analysis of SINE sequences from evolutionary distant species.

35 Figure 19 related to Example 19 shows that the first stem loop domain (SL-1) is sufficient for translation up regulation activity of antisense lncRNA overlapping with GFP mRNA (59-96 SL1 SINEUP-GFP) in HEK 293T/17 cells.

Figure 20 related to Example 20 shows that a structure-based shorter version of the inverted SINE B2 is sufficient for translation up regulation of antisense lncRNA overlapping with GFP mRNA (44-120 SINEUP-GFP) in HEK 293T/17
5 cells by Western blot.

Figure 21 related to Example 21 shows that a structure-based shorter version of the inverted SINE B2 is sufficient for translation up regulation of antisense lncRNA overlapping endogenous DJ-1 mRNA (44-120 SINEUP-DJ-1) in HEK
10 293T/17 cells. The function of two variants with point mutations has also been tested.

Figure 22 shows a schematic overview of the SINEUP-icSHAPE experiment.

Figure 23 shows the secondary (2D) structure of the miniSINEUP-GFP effector domain in HEK293T/17 cells and motifs therein.
15

Figure 24 shows a comparison of 2D structures of AS Uchl1 SINEB2 (A) in long SINEUP-GFP and (B) in miniSINEUP-GFP using ExpaRNA.

Figure 25 shows ExpaRNA exact pattern match result for 2D structures of AS Uxt SINEB2-b (A) in long SINEUP-GFP and (B) in miniSINEUP-GFP.
20

Figure 26 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP and (B) AS Txnip SINEB2 in long SINEUP-GFP.
25

Figure 27 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP and (B) AS Uxt SINEB2-b in long SINEUP-GFP.

Figure 28 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP and (B) AS Uxt SINEB2-b in miniSINEUP-GFP.
30

Figure 29 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP and (B) SINEB2/B3 consensus (reverse complement) in miniSINEUP-GFP.
35

Figure 30 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP and (B) AS Nars2 SINEB2 in miniSINEUP-GFP.

Figure 31 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP and (B) AS Abhd11 SINEB2 in miniSINEUP-GFP.

Figure 32 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP and (B) AS Epb4.9 SINEB2 in miniSINEUP-GFP.

Figure 33 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP and (B) AS Wfdc5 SINEB2 in miniSINEUP-GFP.

Figure 34 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP and (B) AS Pgbd1 SINEB2 in miniSINEUP-GFP.

Figure 35 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP and (B) AS Gsk3 β SINEB2 in miniSINEUP-GFP.

Figure 36 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP and (B) AS Rhod SINEB2-Mm2 in miniSINEUP-GFP.

Figure 37 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP and (B) AS Rhod SINEB2-B3A in miniSINEUP-GFP.

Figure 38 shows ExpaRNA exact pattern match result for 2D structures of (a) AS Uchl1 SINEB2 in miniSINEUP-GFP and (b) AS E4f1 SINEB2-Mm2 in miniSINEUP-GFP.

Figure 39 shows ExpaRNA exact pattern match result for 2D structures of (a) AS Uchl1 SINEB2 in miniSINEUP-GFP and (b) AS E4f1 SINEB2-Mm1t in miniSINEUP-GFP.

Figures 40 and 41 shows the structure dynamics of AS-Uchl1 SINEB2.

Figure 42 shows how synthetic SINEUPs of various ED length and structure motif combinations up-regulate GFP protein expression.

Figure 43 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP (icSHAPE-guided) and (B) miniSINEUP 071 ED (RNAfold-predicted).

5 Figure 44 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP (icSHAPE-guided) and (B) miniSINEUP 072 ED (RNAfold-predicted).

10 Figure 45 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP (icSHAPE-guided) and (B) miniSINEUP 073 ED (RNAfold-predicted).

15 Figure 46 shows ExpaRNA exact pattern match result for 2D structures of (a) AS Uchl1 SINEB2 in miniSINEUP-GFP (icSHAPE-guided) and (b) miniSINEUP 074 ED (RNAfold-predicted).

20 Figure 47 shows ExpaRNA exact pattern match result for 2D structures of (a) AS Uchl1 SINEB2 in miniSINEUP-GFP (icSHAPE-guided) and (b) miniSINEUP 075 ED (RNAfold-predicted).

Figure 48 shows how structure fragments of SINEB2 from AS Uchl1 up-regulate GFP protein expression.

Figure 49 related to Example 48 shows that SINEUP-DJ-1 is active when expressed as circular RNA.

25 Detailed Description of the Invention

In the present invention, there is disclosed - among other things - the secondary and tertiary structure of inverted SINE B2 transposable element derived from AS Uchl1 as representative effector domain of SINEUPs.

30 Deletion mutants are used to show the structural elements required for SINEUP activity.

Further, the structure SINE B2 sequences from antisense lncRNAs with function of translation up-regulation (natural SINEUPs) is determined in living cells.

35 Structure-function relation is also defined based on

biochemical characterization of SINEUP activity and icSHAPE profiles of active and inactive SINEs (the latter not shown).

Structure-based activity motifs of SINE B2 sequences are disclosed.

5 The translation up-regulation activity of functional effector domains of SINE sequences derived from evolutionary distant species whose primary sequence is not conserved and whose secondary structure elements are known is also disclosed.

10 In particular, functional experiments indicate that a short terminal hairpin loop structure, also referred to as the first stem-loop domain (SL-1), is the key structural determinant for the ability of SINEUPs to up-regulate translation of partially overlapping mRNAs and additional
15 structural elements are also important for enhanced activity and discriminate those SINEs that are more active from those that are less active.

 It is proved that the primary sequence of an RNA molecule does not necessarily lead to a predetermined
20 secondary structure and that, depending on the conditions, alternative folding of the molecule can occur. In other words, the secondary structure obtained from a primary sequence of an RNA molecule is by no means predetermined and predictable only from the primary sequence.

25 In addition, several variants of effector domains of SINE sequences are provided which are mutated to strengthen the secondary structure and/or to limit the size of the molecule. These variants have been designed by identifying particularly relevant and conserved motifs in the secondary
30 structure and combining them so as to arrive at the minimal structure required for function and at the optimum sequence and conditions to obtain such a structure.

 Indeed, a functional nucleic acid molecule of the present invention comprises a target binding sequence
35 comprising a sequence reverse complementary to a target mRNA

sequence for which protein translation is to be enhanced, and a regulatory sequence having a two-dimensional structure comprising a first stem-loop domain (SL-1) comprising a stem with 8 to 12 paired nucleotides and a loop comprising 5 to 9 nucleotides. The regulatory sequence has protein translation enhancing efficiency.

According to the invention, it has been shown that, as long as the secondary structure is conserved, the bases of the primary sequence can vary to a large extent.

The target binding sequence needs to have only about 60% similarity with a sequence reverse complementary to the target mRNA. As a matter of fact, the target binding sequence can even display a large number of mismatches and retain activity. Indeed, miRNAs can affect their sense mRNA partners even having a large number of mismatches. This concept has already been shown in WO 2012/133947.

The target binding sequence and the regulatory sequence are preferably connected by a spacer/linker sequence.

Preferably, the stem of the first stem-loop domain (SL-1) is G/C rich, whereby by "G/C rich sequence" there is intended a sequence having more than 50% Gs and Cs, and preferably more than 75% Gs and Cs.

Preferably, the loop of the first stem-loop domain (SL1) is A/U rich, whereby by "A/U rich sequence" there is intended a sequence having more than 50% As and Us, and preferably more than 75% As and Us.

The stem of the first stem-loop domain (SL-1) preferably has 10 paired nucleotides and the loop of the first stem-loop domain (SL-1) preferably has 7 nucleotides.

A functional nucleic acid molecule of the present invention comprises a target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced, and a regulatory sequence having a two-dimensional structure comprising a first stem-loop domain (SL-1) having a sequence with at least

50% identity with SEQ ID NO:1, preferably at least 75% identity with SEQ ID NO:1, more preferably at least 90% identity with SEQ ID NO:1.

In the exemplified inverted SINE B2 transposable element derived from AS Uchl1, the first stem-loop domain (SL-1) comprises SEQ ID NO:1, i.e. nucleotides 64 to 92 of SEQ ID NO:5 (the latter being the complete sequence of the inverted SINE B2 transposable element derived from AS Uchl1).

Preferably, the stem of the first stem-loop domain (SL-1) has an A-type helical three-dimensional structure, as shown by high-resolution NMR.

The two-dimensional structure of the regulatory sequence preferably further comprises a second stem-loop domain (SL-2) and a fourth internal loop domain (IL-4) or a fourth stem-loop domain (SL-4), the second stem-loop domain (SL-2) comprising a stem with 2 to 4 paired nucleotides and a loop comprising 6 to 10 nucleotides and the fourth internal loop domain (IL-4) comprising 14 to 24 nucleotides, and the fourth stem-loop domain (SL-4) comprising a stem with 3 to 5 paired nucleotides and a loop comprising 6 to 10 nucleotides.

The stem of the second stem-loop domain (SL-2) is preferably G/C rich. The loop of the second stem-loop domain (SL-2) and/or the fourth internal loop domain are/is preferably A/U rich.

The stem of the second stem-loop domain (SL-2) preferably has 3 paired nucleotides and the loop of the second stem-loop domain (SL-2) preferably has 8 nucleotides. The fourth internal loop domain (IL-4) preferably comprises 16 to 22 nucleotides.

A functional nucleic acid molecule of the present invention preferably comprises a regulatory sequence having a two-dimensional structure comprising a sequence with at least 50% identity with SEQ ID NO:2, preferably at least 75% identity with SEQ ID NO:2, more preferably at least 90%

identity with SEQ ID NO:2.

In the exemplified inverted SINE B2 transposable element derived from AS Uchl1, the regulatory sequence comprises SEQ ID NO:2, i.e. nucleotides 52 to 112 of SEQ ID NO:5.

The two-dimensional structure of the regulatory sequence preferably further comprises a third internal loop domain (IL-3) comprising 6 to 14 nucleotides, preferably 7 to 13 nucleotides.

The two-dimensional structure of the regulatory sequence preferably further comprises a third stem-loop domain (SL-3) comprising a stem with 1 to 3 paired nucleotides and a loop comprising 3 to 5 nucleotides.

The functional nucleic acid molecule of the present invention preferably comprises a regulatory sequence having a two-dimensional structure comprising a sequence with at least 50% identity with SEQ ID NO:3, preferably at least 75% identity with SEQ ID NO:3, more preferably at least 90% identity with SEQ ID NO:3.

In the exemplified inverted SINE B2 transposable element derived from AS Uchl1, the regulatory sequence comprises SEQ ID NO:3, i.e. nucleotides 36 to 133 of SEQ ID NO:5.

The two-dimensional structure of the regulatory sequence preferably further comprises a second internal loop domain (IL-2) comprising 6 to 12 nucleotides, preferably 7 to 11 nucleotides. The second internal loop domain (IL-2) is preferably asymmetrical and/or A/U rich.

The functional nucleic acid molecule of the present invention preferably comprises a regulatory sequence having a two-dimensional structure comprising a sequence with at least 50% identity with SEQ ID NO:4, preferably at least 75% identity with SEQ ID NO:4, more preferably at least 90% identity with SEQ ID NO:4.

In the exemplified inverted SINE B2 transposable

element derived from AS Uchl1, the regulatory sequence comprises SEQ ID NO:4, i.e. nucleotides 22 to 150 of SEQ ID NO:5.

5 The two-dimensional structure of the regulatory sequence preferably further comprises a first internal loop domain (IL-1) comprising 6 to 12 nucleotides, preferably 7 to 11 nucleotides.

10 The functional nucleic acid molecule of the present invention preferably comprises a regulatory sequence having a two-dimensional structure comprising a sequence with at least 50% identity with SEQ ID NO:5, preferably at least 75% identity with SEQ ID NO:5, more preferably at least 90% identity with SEQ ID NO:5.

15 In the exemplified inverted SINE B2 transposable element derived from AS Uchl1, the regulatory sequence has SEQ ID NO:5.

20 Another functional nucleic acid molecule according to the present invention comprises a target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced; and a regulatory sequence comprising a sequence having at least 50%, preferably at least 75%, even more preferably at least 90% identity with a sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:15, SEQ ID NO:7, SEQ ID NO:22, and SEQ ID NO:23.

30 icSHAPE analysis shows that the number of paired nucleotides in the above disclosed stems and the number of nucleotides in the above disclosed loops can vary beyond the disclosed ranges.

Another functional nucleic acid molecule according to the present invention comprises a target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced; and a regulatory sequence comprising a sequence having at

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least 50%, preferably at least 75%, even more preferably at least 90% identity with a sequence selected from the group consisting of SEQ ID NO:7, SEQ ID NO:21, SEQ ID NO:17, SEQ ID NO:22, SEQ ID NO:65, SEQ ID NO:66 and SEQ ID NO:67.

5 The regulatory sequence preferably comprises SEQ ID NO:7, SEQ ID NO:21, SEQ ID NO:17, SEQ ID NO:22, SEQ ID NO:65, SEQ ID NO:66 and SEQ ID NO:67.

10 In an alternative embodiment the functional nucleic acid comprises a regulatory sequence comprising a sequence having at least 50%, preferably at least 75%, even more preferably at least 90% identity with SEQ ID NO:12, even more preferably the sequence is SEQ ID NO:12.

15 Another functional nucleic acid molecule according to the present invention comprises a target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced; and a regulatory sequence comprising one or more of the motifs formed by an RNA sequence having 50%, preferably 75%, even more preferably 90% identity with GCU (motif 1), AGGGC (motif 3), UGGAU (motif 4), UGGUGG (motif 6), or UGGA (motif 7) and by a hybridised reverse complementary RNA thereof, or the motif formed by an RNA sequence having 50%, preferably 75%, even more preferably 90% identity with AGGAGG (motif 2) and by a hybridised partially complementary reverse RNA thereof, or the motif formed by an RNA sequence having 50%, preferably 75%, even more preferably 90% identity with UGG (motif 5) and by a hybridised partially complementary reverse RNA thereof.

30 Even more preferably the motifs are respectively formed by GCU (motif 1), AGGGC (motif 3), UGGAU (motif 4), UGGUGG (motif 6), and UGGA (motif 7) and by a hybridised reverse complementary RNA thereof, AGGAGG (motif 2) and by a hybridised partially complementary reverse RNA thereof, and UGG (motif 5) and by a hybridised partially complementary reverse RNA thereof.

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The regulatory sequence of the above-said functional nucleic acid molecule preferably comprises at least the motif formed by an RNA sequence having 90% identity with UGGUGG (motif 6) and by a hybridised reverse complementary RNA
5 thereof. More preferably the motif is UGGUGG.

Preferred combinations of the above-said motifs are shown in Figures 24 to 39, 43 to 47 and 48B.

More specifically the above said functional nucleic acid molecule comprises as regulatory sequence a sequence
10 having at least 50%, preferably 75%, even more preferably 90% identity with a sequence selected from the group consisting of SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, and SEQ ID NO:77. Even more preferably, the sequence
15 comprises SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, and SEQ ID NO:77. Among these, the preferred sequence is SEQ ID NO:72, which represents an example of minimal functional sequence, which - despite the lack of several
20 motifs of the full length SINEUP effector domain - maintains the ability of increasing translation of target RNA.

The functional nucleic acid molecule according to the present invention is preferably a circular molecule. This conformation leads to a much more stable molecule that is
25 degraded with greater difficulty within the cell and therefore remains active for a longer time.

The present invention also relates to compositions comprising the above said functional nucleic acid molecules. Any compositions are included allowing to deliver the above
30 said functional nucleic acid molecules by viral vectors (AAV, lentivirus and the like) and non-viral vectors (nanoparticles, lipid particles and the like).

Examples

Example 1

35 This example demonstrates the accessibility to chemical

agents (DMS and CMCT) of each nucleotide in the inverted SINE B2 sequence from natural AS Uchl1. Accessibility to chemical agents is strictly dependent on the local structure of the molecule.

5 Figure 1 shows chemical footprinting of the invSINEB2/183 RNA construct. The left panel shows a fluorescent gel with C and A sequencing lanes with nucleotide numbering. DMS lanes with invSINEB2/183 RNA treated with increasing DMS concentrations with positive (+) and negative
10 (-) controls. The right panel shows a fluorescent gel with G and U sequencing lanes with nucleotide numbering. CMCT lanes with invSINEB2/183 RNA treated with increasing CMCT concentrations with positive (+) and negative (-) controls. Experiments were carried out as described in Tijerina P. et
15 al., (2007), Nature Protocols 2:2608-2623.

Example 2

 This example shows the two-dimensional structure of the inverted SINE B2 effector domain of functional AS Uchl1. The structure is organized in Stem-Loops (SL1, SL2 and SL3),
20 Internal Loops (IL1, IL2, IL3 and IL4) and intervening stem structures. The structure is generated based on data from Example 1.

 Figure 2 shows the secondary structure of the inverted SINE B2 effector domain of AS Uchl1. DMS reactive nucleotide
25 are shown in black characters in circles with grey background and CMCT reactive nucleotides are shown in white characters in circles with black background, respectively. Internal loops and stem-loops are labeled as ILx and SLx, respectively. Non-reactive nucleotides are only circled. The
30 segment shaded in gray corresponds to the DNA primer hybridization site.

Example 3

 This example demonstrates that shorter fragments of the inverted SINE B2 sequence retain the terminal SL1 structure.

35 Figure 3 shows the structures in solution of termini-

deleted fragments of inverted SINE B2 RNA. Figure 3A shows the 2D structure of the invSINEB2/183 effector domain of AS Uchl1. Regions with removed termini are indicated in squares. Figure 3B shows the schematic structure of termini-deleted fragments. Nucleotide positions are indicated relative to the full-length sequence. Figure 3C shows imino proton region of inverted SINE B2 from mouse AS Uchl1. Imino ^1H signals are assigned for each termini-deleted fragment. Fragments #1 (SEQ ID NO:9), #2 (SEQ ID NO:8), #3 (SEQ ID NO:7) and #4 (SEQ ID NO:6) tracks are shown. The SL1 stem structure is observed in all fragments.

Example 4

This example demonstrates that SL1 is required for the activity of the inverted SINE B2 effector domain in functional nucleic acid molecule (AS Uchl1) for translation up-regulation.

Murine neuroblastoma N2a cells (ATCC Cat No. ATCC-CCL-131) were transfected with AS Uchl1 and ΔSL1 mutant constructs. In the ΔSL1 mutant construct, nucleotides 68–77 of embedded inverted SINE B2 are deleted (SEQ ID NO:10). In the ΔSL1 mutant the stem loop structure of SL-1 is destroyed, but the overall inverted SINE B2 assembly is retained. Control cells were transfected with an empty control plasmid. 48 hours after transfection, cells were lysed and processed for protein and RNA levels. Western blot was performed with anti-UCHL1 antibody (Figure 4A). β -actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β -actin and relative to empty control samples. Expressions of Uchl1 mRNA (gray bars) and AS Uchl1 (white bars) were monitored by qRT-PCR using specific primers. Data indicate mean $\pm$ st. dev. Data are representative of N=5 independent replicas. Figure 4B shows a graphical representation of AS Uchl1 and ΔSL1 translation enhancement activity on endogenous Uchl1 mRNA in N2a cells (N=5). *, $p=0.01$; NS, not significant ($p>0.5$).

Experiments were carried out as described in Zucchelli S. et al., (2015), Front Cell Neurosci. 9:174.

Example 5

This example demonstrates that SL-1 is required for translation up-regulation function of AS Uchl1 nucleic acid molecule in human hepatocellular cell line and with overexpressed mRNA. Indeed, the deletion of SL-1 abolishes AS Uchl1 activity when tested with overexpressed Uchl1-FLAG in human HepG2 cells (ATCC Cat No. ATCC-HB-8065).

Human hepatocyte HepG2 cell line were transfected with Uchl1-FLAG construct in combination with AS Uchl1 and Δ SL1 mutant constructs. Control cells were transfected with an empty control plasmid. 48 hours after transfection, cells were lysed and processed for protein quantities by Western blot. Western blot was performed with anti-FLAG antibody to monitor overexpressed protein (Figure 5A). β -actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β -actin and relative to empty control samples. AS Uchl1 translation enhancer activity is highlighted in bold. Cells treated as disclosed above were processed for RNA quantification (Figure 5B). Expressions of Uchl1 mRNA (right) and AS Uchl1 (left) were monitored by qRT-PCR using specific primers. Data indicate mean $\pm$ st. dev.

Example 6

This example demonstrates that SL-1 is required for translation up-regulation function of synthetic functional nucleic acid molecule targeting overexpressed mRNA (SINEUP-GFP). Additional structural elements are preferably also required since StemDM mutant (which destabilize the stem) has diminished, but not abolished effector domain activity.

Figure 6 shows that the SL-1 structural element contributes to SINEUP-GFP activity in HEK 293T/17 cells (ATCC Cat No. ATCC-CRL-11268). Figure 6A is a zoomed-in image of the terminal hairpin structure (SL-1) from the inverted SINE

B2 effector domain. A Δ SL1 mutant was generated by deletion in the SINE B2 effector domain of SINEUP-GFP. Similarly, site-directed mutations (G70A and G73A, circled) were introduced in SINEUP-GFP to generate the StemDM mutant (SEQ ID NO:11). In Figure 6B HEK 293T/17 cells were transfected with eGFP expressing plasmid in combination with an empty vector (-), or SINEUP-GFP full-length (FL), Δ SL1 or StemDM mutants, as indicated. Western blot was performed with anti-GFP antibody to monitor overexpressed protein. β -actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β -actin and relative to empty control samples. SINEUP-GFP translation enhancer activity is highlighted in red. In Figure 6C, cells treated as in Figure 6B were used to prepare RNA. Expressions of GFP mRNA (grey) and SINEUP-GFP (black) were monitored by qRT-PCR using specific primers. Data indicate mean $\pm$ st. dev.

Example 7

This example demonstrates that the inverted SINE B2 fragment (38 nucleotide) covering the SL1 structural region (SEQ ID NO:12) adopts a single structure in solution as determined by NMR. In this experiment, NMR data validate the structure of SL-1 in shorter fragment (as observed by other techniques in Examples 1, 2 and 3).

Figure 7A shows imino and aromatic regions of ^1H NMR spectra of 38 nt RNA in 5% $^2\text{H}_2\text{O}$ /95% H_2O acquired at 0 and 25 $^\circ\text{C}$. Imino ^1H signals are assigned. Figure 7B shows imino region of the ^{15}N HSQC NMR spectra. Signals observed at 25 and 0 $^\circ\text{C}$ are in dark grey and light grey, respectively. Figure 7C shows the secondary structure of the SINEB2/38 construct. Base pairs with imino signals observable at 25 and 0 $^\circ\text{C}$ are shaded in dark grey and light grey, respectively. Experiments were carried out as described in Sybren S. et al., (1998), Prog. Nucl. Magn. Reson. Spectrosc. 32:287-387.

Example 8

This example demonstrates that NMR signals can be assigned in 2D NOESY NMR spectrum to properly reconstruct the 3D structure of inverted SINE B2 38nt fragment.

Figure 8 shows the assignment of relevant resonances. Aromatic-anomeric region of a 2D NOESY NMR spectrum ($\tau_m=250$ ms) of invSINEB2/38. The sequential walk is depicted as a black line.

Example 9

This example demonstrates the connectivity map of NMR signals that is used to properly reconstruct the 3D structure of inverted SINE B2 38nt fragment.

Figure 9 shows a diagram of NOE connectivities. A diagram of strong (bold line) medium (solid line) and weak (dashed line) NOE connectivities for the invSINEB2/38 molecule.

Example 10

This example demonstrates the high-resolution 3D structure of the SL-1 region of the inverted SINE B2 effector domain. The hairpin adopts an A-type helical stem. The loop exhibits dynamic properties in solution.

Figure 10 shows the high-resolution structure of SL1. Figure 10A shows a side view and Figure 10B shows top views of the 10 lowest energy structures of the 29 nt hairpin (SEQ ID NO:1). Figures 10C and 10D are representative structures of loops with orientations of G77, U78 and G79 bases.

Example 11

This example demonstrates that additional structural elements (IL3-SL3 region, IL4 and basal stem structure) contribute to inverted SINE B2 effector domain activity in functional nucleic acid molecule (in particular, SINEUP-GFP).

Figure 11A is a schematic representation of Δ SL1 (SEQ ID NO:10), Δ polIII (SEQ ID NO:13) and Δ Box (SEQ ID NO:14) deleted sequences annotated on inverted SINE B2 secondary structure. In Figure 11B, HEK 293T/17 cells were transfected with eGFP expressing plasmid in combination with an empty

vector (-), or SINEUP-GFP full-length (FL), Δ SL1, Δ polII or Δ Box mutants, as indicated. Western blot was performed with anti-GFP antibody to monitor overexpressed protein. β -actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β -actin and relative to empty control samples. SINEUP-GFP translation enhancer activity is highlighted in bold. The contribution of structural mutants is shown in red. In Figure 11C, cells treated as in Figure 11B were used to prepare RNA. Expressions of GFP mRNA (grey) and SINEUP-GFP (black) were monitored by qRT-PCR using specific primers. Data indicate mean $\pm$ st. dev.

Example 12

This example demonstrates that new synthetic effector domain sequences can be designed based on structural features (intervening stem structure at the base of IL-4, SL-2, SL-1). These sequences can enhance translation up-regulation activity in functional nucleic acid molecule with overexpressed mRNA. Enhanced activity is obtained with different primary nucleotide sequence, but same structure.

In particular, structure-based variants of the inverted SINE B2 display increased translation up-regulation activity as effector domain in SINEUP-GFP. Figure 12A shows a schematic representation of structure-based mutations annotated on inverted SINE B2 secondary structure. In circles: single point mutations; in squares: sequence swapping of structural elements (stem and loop-base). In Figure 12B, HEK 293T/17 cells were transfected with eGFP expressing plasmid in combination with an empty vector (-), or SINEUP-GFP full-length (FL) as positive control. SINEUP-GFP constructs with structure-based mutations were transfected as indicated. Western blot was performed with anti-GFP antibody to monitor overexpressed protein. β -actin was used as loading control. Figure 12C shows the relative increase in GFP protein levels (left panel) and unchanged

GFP mRNA levels (right panel) in N=9 experiments, that have been performed as in B. The expression of each SINEUP-GFP structural mutant (AS GFP) is monitored by qRT-PCR and included in the right panel. All mutants are expressed at the same level.

Example 13

This example demonstrates that new synthetic effector domain sequences can be designed based on structural features (intervening stem structure at the base of IL-4, SL-2, SL-1) and obtain enhanced translation up-regulation activity in functional nucleic acid molecule with overexpressed mRNA. Enhanced activity is obtained with different primary nucleotide sequence, but same structure.

In particular, a shorter and structure-based variant of the inverted SINE B2 display increased translation up-regulation activity as effector domain functional nucleic acid molecule targeting endogenous mRNA.

Figure 13A is a schematic representation of structure-based mutations annotated on inverted SINE B2 secondary structure (27-142 corresponds to SEQ ID NO:21, 44-120 corresponds to SEQ ID NO:7, 44-120 with A45G corresponds to SEQ ID NO:22 and 44-120 A51C corresponds to SEQ ID NO:23). Single point mutations are indicated. Figure 13B shows translation up-regulation activity of functional nucleic acid molecules targeting endogenously expressed mRNA in which the effector domain is a synthetic sequence with improved structural features. Depending on the conditions, alternative folding occurs and may become stronger such as SEQ ID NO:7.

Example 14

This example demonstrates that SINE sequences from natural antisense long non-coding RNAs are effector domains in functional nucleic acid molecules for translation up-regulation of partially overlapping mRNA.

In particular, SINEB2 elements from different mouse AS

lncRNAs can act as Effector Domain in miniSINEUPs and up-regulate GFP protein expression in HEK293T (Riken Brc Cat. No. RCB2202).

Figure 14A shows SINE B2 sequences derived from natural functional antisense lncRNAs, which were cloned into miniSINEUP-GFP to replace the original inverted SINE B2 sequence from AS Uchl1 as Effector Domain. GFP targeting sequence was -40/+4 nucleotides, relative to initiating AUG codon. Sequences are in the order: SEQ ID NO:44, SEQ ID NO:47, SEQ ID NO:50 to SEQ ID NO:62. Figure 14B shows GFP protein fold induction in HEK293T cells after co-transfection with sense EGFP and miniSINEUP-GFP (24 h post-transfection). Western blot images and corresponding GFP band intensities (normalized to ACTB expression level) are calculated using ImageJ software. Empty vector is used as negative control while AS Uchl1 (AS Uchl1 SINEB2 containing miniSINEUP) as positive control. Sample labels indicate names of AS lncRNAs from which SINEB2 sequences were isolated. SINEB2 sequences representing four sub-families (based on RepeatMasker annotation) namely B3, B3A, Mm2, and Mm1t were tested. SINEB2 elements are from B3 sub-family unless specifically stated. AS Uchl1 + AS Uxt-b means AS Uchl1 SINEB2 and second SINEB2 of AS Uxt combined in ED. 3X indicates three repeats of SINEB2 and SINEB2/B3 consensus is the B3 sub-family consensus sequence taken from RepBase database. n = 5; error bars $\pm$ SEM; **** P<0.00005; *** P<0.0005; ** P<0.005; * P<0.05; two-tailed Student's t-test

Figure 14C shows SINEB2 sequences tested as ED in SINEUP-GFP.

Figure 14D shows Western blot images with anti-GFP and anti-ACTB antibodies and corresponding GFP band intensities (normalized to ACTB expression level) calculated using ImageJ software. ACTB is used as loading control. Here, AS Uchl1 SINEB2 represents SINEUP-GFP with -40/+32 BD design with respect to the EGFP start codon (+21 nt spacer

sequence), and AS Uchl1 SINEB2 as ED in addition to the Alu and 3' tail from AS Uchl1. AS Txnip SINEB2 and AS Uxt-SINEB2 b follow the same design except that the AS Uchl1 SINEB2 is replaced by the SINEB2 elements from AS Txnip and AS Uxt (SINEB2-b) respectively. AS Uchl1_Δ5'-28nt is same as AS Uchl1 SINEB2 but with shorter BD (-40/+4 with respect to EGFP start codon). Direct = direct orientation of SINEB2; Δ = deletion. n = 3; error bars ± SEM; ** P<0.005; * P<0.05; two-tailed Student's t-test

10 Example 15

This example demonstrates that active SINE sequences in functional nucleic acid molecules for translation up-regulation function have poor sequence identity. This example suggests structure-dependence of effector domain functionality.

Figure 15 shows ClustalW sequence alignment of SINEB2 elements from different mouse AS lncRNAs. Mouse SINEB2 sequences that were selected for translation up-regulation function in SINEUP-GFP and miniSINEUP-GFP (example 14) are aligned by ClustalW in MEGA5 software. Sequences are in the order: SEQ ID NO:44 to SEQ ID NO:49 and SEQ ID NO:52 to SEQ ID NO:62. Gap in the alignment is indicated by "-". Bases are highlighted by four different shades of grey. A and B-box are denoted by black squares. Alignment is showing sequence variation within the members of same family of SINE.

25 Example 16

This example demonstrates that SINE sequences from evolutionary distant species act as effector domain in antisense functional nucleic acid molecules with translation up-regulation activity.

As shown in Figure 16A, SINE sequences from evolutionary distant species and with defined secondary structures (Sun Fen-Jie, et al., Trends in Genetics, 2011) were selected for the analysis. These sequences display poor sequence identity by alignment analysis. The sequence listing includes RNA

sequences corresponding to the DNA sequences shown in Figure 16A (SEQ ID NO:24 to SEQ ID NO:33). The sequence listing also includes the inverted RNA sequences (SEQ ID NO:34 to SEQ ID NO:43). In Figure 16B, HEK 293T/17 cells were transfected with eGFP expressing plasmid in combination with an empty vector (-), or SINEUP-GFP with original inverted SINE sequence (dark blue, B2) or with the indicated Effector Domain swapping mutants, cloned in direct and inverted orientation, as indicated. GFP autofluorescence was recorded using CeligoS automated imaging system. Data indicate the results of GFP fluorescence normalized to empty vector (pCDNA3.1) transfected cells (N>7 replicas). Data indicate mean $\pm$ stdev. The framed columns show conditions where SINEUP activity is enhanced as compared to SINE B2 positive control. As shown in Figure 16C, qRT-PCR analysis indicate that protein up-regulation occurs with stable GFP mRNA levels (post-transcriptional). After the Celigo S measurement, RNAs were extracted and purified. GFP mRNAs and SINEUP-GFP RNAs were analyzed qRT-PCR with specific primers. N=3, SINE B2 is set to 1.

Example 17

This example demonstrates that more potent translation up-regulation activity of SINE sequences from evolutionary distant species correlates with more complex secondary structures (combinations of stem loops).

In Figure 17A, HEK 293T/17 cells were transfected with eGFP expressing plasmid in combination with an empty vector (-), or SINEUP-GFP with original inverted SINE sequence (SINE B2) or with the indicated Effector Domain swapping mutants. Western blot analysis was performed with anti-GFP antibody to monitor overexpressed protein. β -actin was used as loading control. In Figure 17B, GFP protein quantities were calculated by image analysis, upon normalization to β -actin control. Data from N=5 replicas are represented as mean $\pm$ stdev. Statistical analysis was performed using t-test (p

values shown). Figure 17C shows the most active SINEs from various species, with their relative structure. Overall SINE structures are represented with lines (stems) and circles (hairpin loops). It should be noted that all of the most active SINEs share a conserved apical stem loop structure. Indeed, this stem-loop structure is shown to be a key element for translation up-regulation activity in SINEs of evolutionary distant species.

Example 18

This example demonstrates that structural motifs (motif 1, IL-3/SL-3 and motif 2, SL-1) are shared between the more potent SINE sequences in functional nucleic acid molecules.

Figure 18 shows the 2D structure motif analysis of SINE sequences from evolutionary distant species that have translation up-regulation activity in functional nucleic acid molecules. Figure 18A shows the 2D structure of inverted SINE B2 RNA from example 2 (SEQ ID NO:5). Figure 18B shows the predicted 2D structure of B2 consensus SINE RNA (SEQ ID NO:30) determined by RNA fold WebServer with default settings. Figure 18C shows the 2D structure of SmaI SINE RNA (SEQ ID NO:27) using enzymatic probing. The putative recognition region for LINE RT are boxed by dashed line (H Kawagoe-Takaki et al. Gene 365 (2006), 67-73). Figure 18D shows the stem structure Motif1 (intermediate stem, IL-3, SL-3) from 2D structure of inverted SINE B2 and B2 SINE. Motif 1 structures stem between middle loop structures. Figure 18E shows the stem structure Motif2 (SL-1) from 2D structure of inverted SINE B2, B2 SINE and SmaI SINE. Motif2 structures stem close to the edge loop structure.

Again it is noted that the three structures in Figures 18A, 18B and 18C share a conserved apical stem loop structure.

Example 19

This example demonstrates that new synthetic effector domain sequences can be designed based on structural features

(38 nt, stem loop structure SL1) and that these possess translation up-regulation activity in functional nucleic acid molecule with overexpressed mRNA. Activity is obtained with different primary nucleotide sequence, but same structure. In particular, a shorter and structure-based variant of the inverted SINE B2 displays translation up-regulation activity as effector domain functional nucleic acid molecule targeting endogenous mRNA (59-96 SL1 SINEUP-GFP). Figure 19A is a schematic representation of structure based mutations annotated on inverted SINE B2 secondary structure highlighting SL1 (59-96 nt corresponds to SEQ ID NO:12). Figure 19B shows translation up-regulation activity of functional nucleic acid molecules targeting endogenously expressed GFP mRNA in which the effector domain is a shorter synthetic sequence with conserved structural features (38 nt SL1). HEK 293T/17 cells were transfected with eGFP expressing plasmid in combination with an empty vector (-), or 1-183 SINEUP-GFP (including SEQ ID NO:5), 1-183 SINEUP-GFP polyA (SEQ ID NO:63) and SL1 59-96 polyA SINEUP-GFP (SEQ ID NO:64), as indicated. Western blot was performed with anti-GFP antibody to monitor overexpressed protein. β -actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β -actin and relative to empty control samples. Data from 4 independent experiments are shown in Figure 19C. SL1 SINEUP-GFP translation enhancer activity is indicated in red. Data indicate mean $\pm$ st. dev.

This experiment shows that the first stem loop domain (SL-1) is sufficient for translation up regulation activity of antisense lncRNA overlapping with GFP mRNA (59-96 SL1 SINEUP-GFP) in HEK 293T/17 cells.

Example 20

This example demonstrates that new synthetic effector domain sequences can be designed based on structural features (44-120 nt SINEUP, containing IL4, SL1 and SL2 and corresponding to SEQ ID NO:7) and that they possess

translation up-regulation activity in functional nucleic acid molecule with overexpressed mRNA. Activity is obtained with different primary nucleotide sequence, but same structure. In particular, a shorter and structure-based variant of the inverted SINE B2 displays translation up-regulation activity as effector domain functional nucleic acid molecule targeting endogenous mRNA. Furthermore, the mutation U78G within the SINEUP with the truncated inverted SINE B2 element 44-120 maintains a similar translational up-regulation activity while the triple mutation GUG77-79CCC diminishes considerably the activity proving the functional importance of nucleotides 77-79. Figure 20A is a schematic representation of structure based mutations annotated on the truncated inverted SINE B2 secondary structure highlighting the wild type and mutated sequences (44-120 with U78G corresponds to SEQ ID NO:65; 44-120 with GUG77-79CCC corresponds to SEQ ID NO:66). Figure 20B shows translation up-regulation activity of functional nucleic acid molecules targeting endogenously expressed GFP mRNA in which the effector domain is a shorter synthetic sequence with wt or mutated sequences in 77-79 nucleotides positions. HEK 293T/17 cells were transfected with eGFP expressing plasmid in combination with an empty vector (-), or 44-120 SINEUP-GFP, 44-120 SINEUP-GFP with U78G and 44-120 SINEUP-GFP with GUG77-79CCC. Western blot was performed with anti-GFP antibody to monitor overexpressed protein. β -actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β -actin and relative to empty control samples. 44-120 SINEUP-GFP activity was similar both to the SINEUP-GFP activity with the entire inverted SINE B2 sequence and to the 44-120 SINEUP-GFP with the truncated 44-120 effector domain with the U78G mutation. By comparison, 44-120 SINEUP-GFP with GUG77-79CCC displayed a reduced activity.

This experiment shows that a structure-based shorter

version of the inverted SINE B2 is sufficient for translation up regulation of antisense lncRNA overlapping with GFP mRNA (44-120 SINEUP-GFP) in HEK 293T/17 cells. It also shows that nucleotides 77-79 of the inverted SINE B2 sequences are important for the ability to up-regulate translation. All three constructs are functional and the secondary structure presents in an optimal manner the three nucleotides of the 77-79 loop. Even if these three nucleotides are modified, the molecule retains functions.

10 Example 21

This example demonstrates that new synthetic effector domain sequences can be designed based on structural features (44-120 nt SINEUP, containing IL4, SL1 and SL2 corresponds to SEQ ID NO:7) and that they possess translation up-regulation activity in functional nucleic acid molecule with endogenously expressed mRNA. Activity is obtained with different primary nucleotide sequence, but same structure. In particular, a shorter and structure-based variant of the inverted SINE B2 displays translation up-regulation activity as effector domain functional nucleic acid molecule targeting endogenous mRNA. Furthermore, structure based mutations annotated on the truncated inverted SINE B2 secondary structure can increase the translational activator ability (a 44-120 SINEUP-DJ-1 variant with 6 point mutations: A45G; U48C; U66G; U88C; U90C; U115C in the truncated inverted SINE B2 sequence and called 44-120 SINEUP-DJ-1-Strong corresponding to SEQ ID NO:67) while other structure based mutations annotated on the truncated inverted SINE B2 secondary structure can keep the translational up-regulation ability unchanged or lower (a 44-120 SINEUP-DJ-1 variant with 5 point mutations: G49A; U66A; G68A; G116A; C119A in the truncated inverted SINE B2 sequence and called 44-120 SINEUP-DJ-1-Weak corresponding to SEQ ID NO:68). The rationale behind the design of the two variants is that, according to the structure of the 44-120

SINEUP, in the two stretches of dsRNA (44-50 with 114-120 and 64-74 with 82-92) there are 5 base pairs mismatches (45-119; 48-116; 49-115; 66-90; 68-88). In 44-120 SINEUP Strong, mutant base pairs have been designed to establish G:C pairs. In 44-120 SINEUP Weak, mutant base pairs have been designed to establish U:A pairs. Since G:C pairs are more stable due to, among other factors, the formation of three versus two hydrogen bonds, the mutant 44-120 SINEUP with 5 G:C pairs has been indicated as Strong while the mutant 44-120 SINEUP with 5 U:A pairs has been indicated as weak.

Figure 21A is a schematic representation of structure based mutations annotated on the truncated inverted SINE B2 secondary structure for 44-120 SINEUP-DJ-1-Strong and 44-120 SINEUP-DJ-1-Weak. The positions of mutations are indicated by arrows. Figure 21B shows translation up-regulation activity of functional nucleic acid molecules targeting endogenously expressed DJ-1 mRNA in which the effector domain is a shorter synthetic sequence (44-120 SINEUP-DJ-1) or the same sequence modified with 6 points mutations (44-120 SINEUP-DJ-1-Strong) or 5 point mutations (44-120 SINEUP-DJ-1-Weak). HEK 293T/17 cells were transfected with eGFP expressing plasmid in combination with an empty vector (-), or 44-120 SINEUP-DJ-1, 44-120 SINEUP-DJ-1-Strong and 44-120 SINEUP-DJ-1-Weak. Western blot was performed with anti-DJ-1 antibody to monitor overexpressed protein. β -actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β -actin and relative to empty control samples. Data from 3 independent experiments are shown in Figure 21C. 44-120 SINEUP-DJ-1 activity was similar to 44-120 SINEUP-DJ-1-Weak while 44-120 SINEUP-DJ-1-Strong was higher in a statistically significant manner. Data indicate mean $\pm$ st. dev.

This experiment shows that a structure-based shorter version of the inverted SINE B2 is sufficient for translation

up regulation (44-120 SINEUP-DJ-1). Furthermore, 44-120 SINEUP-DJ-1-Strong presents higher translational activator activity with respect to 44-120 SINEUP-DJ-1. 44-120 SINEUP-DJ-1-Weak presents a translational up-regulation activity similar or lower than 44-120 SINEUP-DJ-1.

Example 22

Figure 23A shows icSHAPE data guided secondary structure of AS Uchl1 SINEB2 in miniSINEUP-GFP. icSHAPE enrichment score data was used as soft constraint in RNAfold tool of ViennaRNA. Linear mapping method was used to derive pairing probabilities and method described by Zarrinhalam et al. (2012) was selected to incorporate guiding pseudo energies into folding algorithm. To draw the secondary structure forna server was used. Dark dot, icSHAPE enrichment score ≥ 0.8 ; light dot, enrichment score 0.5-0.8. Enrichment scores are scaled on 0 to 1 scale where 1 indicates high probability for a nucleotide to be in unpaired state and 0 for tendency of being paired. B-box and A-box regions of SINEB2 are marked with lines (respectively in higher part of the Figure and lower part of the Figure). Sequence and icSHAPE-guided 2D structure of all of the SINEB2s functionally tested in this study were pairwise compared to this structure and sequence of AS Uchl1 SINEB2 using ExpaRNA tool (Heyne, S. et al. Bioinformatics, 2009, 25:2095-2102) which computes the exact matching sequence-structure motifs common to two RNAs. Thus derived motif files from all of the pairwise comparisons were then screened to extract the positions of AS Uchl1 SINEB2 which were present in one or multiple motifs of at least 2mer in length in all of the comparisons. The position of similar sequence and structure motifs shared among all of the SINEB2s are indicated with squares with their corresponding motif number. As 2mers and longer motifs were taken into account to identify the common regions and SINEB2 sequence are highly variable, in most of

the cases only partial match could be found. Figure 23B shows a zoomed in view of common sequence and structure motifs shown in Figure 23A. It is to be noted that motifs 2 and 3, and motifs 4, 5, 6 and 7 are similar and mainly composed of AGG and UGG trinucleotides respectively. The different motif no. is assigned based on their sequence and structure neighborhood and position on SINEB2. Due to their similar sequence composition, sometimes one hit in query sequence matches to multiple motifs, or one motif matches to multiple positions in the query sequence.

Example 23

Figure 24 shows a comparison of 2D structures of AS Uchl1 SINEB2 (A) in SINEUP-GFP and (B) in miniSINEUP-GFP using ExpaRNA. Different shaded regions indicate corresponding exact match of sequence and structure between two RNAs. The AS Uchl1 SINEB2 2D structure in and miniSINEUP-GFP is almost identical which suggests that this structure is highly stable and dominant inside the cell. The only region of structure variation is marked with a black rectangle.

Example 24

Figure 25 shows ExpaRNA exact pattern match result for 2D structures of AS Uxt SINEB2-b (A) in SINEUP-GFP and (B) in miniSINEUP-GFP. Different shaded regions indicate corresponding exact match of sequence and structure between two RNAs. The overall structure is quite different between SINEUPs and miniSINEUPs here, but some regions preserved the structure (eg.- boxed region) which might be crucial for the SINEUP function.

Example 25

Figure 26 shows ExpaRNA exact pattern match and motif analysis result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP and (B) AS Txnip SINEB2 in SINEUP-GFP. Different shaded regions indicate corresponding unique patterns of exact sequence and structure match between two

RNAs. The position of similar sequence and structure motifs shared among all of the SINEB2s are indicated with squares with their respective motif numbers. Due to their similar sequence composition, in some instances motifs 2 and 3 may overlap, and same goes for motifs 4, 5, 6, and 7.

Example 26

Figure 27 shows ExpaRNA exact pattern match and motif analysis result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP and (B) AS Uxt SINEB2-b in SINEUP-GFP. The same as above applies.

Example 27

Figure 28 shows ExpaRNA exact pattern match and motif analysis result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP and (B) AS Uxt SINEB2-b in miniSINEUP-GFP. Different shaded regions indicate corresponding unique patterns of exact sequence and structure match between two RNAs. The same as above applies.

Example 28

Figure 29 shows ExpaRNA exact pattern match and motif analysis result for 2D structures of (A) AS Uchl1 SINEB2 and (B) SINEB2/B3 consensus (reverse complement) in miniSINEUP-GFP. The same as above applies.

Example 29

Figure 30 shows ExpaRNA exact pattern match and motif analysis result for 2D structures of (A) AS Uchl1 SINEB2 and (B) AS Nars2 SINEB2 in miniSINEUP-GFP. The same as above applies.

Example 30

Figure 31 shows ExpaRNA exact pattern match and motif analysis result for 2D structures of (A) AS Uchl1 SINEB2 and (B) AS Abhd11 SINEB2 in miniSINEUP-GFP. The same as above applies.

Example 31

Figure 32 shows ExpaRNA exact pattern match and motif analysis result for 2D structures of (A) AS Uchl1 SINEB2 and

(B) AS Epb4.9 SINEB2 in miniSINEUP-GFP. The same as above applies.

Example 32

Figure 33 shows ExpaRNA exact pattern match and motif
5 analysis result for 2D structures of (A) AS Uchl1 SINEB2 and
(B) AS Wfdc5 SINEB2 in miniSINEUP-GFP. The same as above
applies.

Example 33

Figure 34 shows ExpaRNA exact pattern match and motif
10 analysis result for 2D structures of (A) AS Uchl1 SINEB2 and
(B) AS Pgbd1 SINEB2 in miniSINEUP-GFP. The same as above
applies.

Example 34

Figure 35 shows ExpaRNA exact pattern match and motif
15 analysis result for 2D structures of (A) AS Uchl1 SINEB2 and
(B) AS Gsk3 β SINEB2 in miniSINEUP-GFP. The same as above
applies.

Example 35

Figure 36 shows ExpaRNA exact pattern match and motif
20 analysis result for 2D structures of (A) AS Uchl1 SINEB2 and
(B) AS Rhod SINEB2-Mm2 in miniSINEUP-GFP. The same as above
applies.

Example 36

Figure 37 shows ExpaRNA exact pattern match and motif
25 analysis result for 2D structures of (A) AS Uchl1 SINEB2 and
(B) AS Rhod SINEB2-B3A in miniSINEUP-GFP. The same as above
applies.

Example 37

Figure 38 shows ExpaRNA exact pattern match and motif
30 analysis result for 2D structures of (a) AS Uchl1 SINEB2 and
(b) AS E4f1 SINEB2-Mm2 in miniSINEUP-GFP. The same as above
applies.

Example 38

Figure 39 shows ExpaRNA exact pattern match and motif
35 analysis result for 2D structures of (a) AS Uchl1 SINEB2 and

(b) AS E4f1 SINEB2-Mmlt in miniSINEUP-GFP. The same as above applies.

Example 39

Figure 40 shows the structure dynamics of AS-Uchl1
5 SINEB2. The purpose of dividing the SINEB2 RNA structure
into smaller fragments is that the full-length (FL) is too
big to analyze current NMR magnetic power. The structure
needs to be divided to correctly assign imino proton signals
of 1H NMR spectra. Divided RNAs should form similar structure
10 to FL. 1H NMR spectra of TM and C and MC2 suggest that they
do. In addition, these structures form differently in the
NMR aqueous buffer with respect to chemical foot printing
measurements (Figure 2). The structure dynamics may happen
in the physiological condition, such as the different
15 cellular compartment (Sun et al., bioRxiv 2018.,
<https://doi.org/10.1101/399386>).

Figure 40A shows the computational prediction structure
of FL SINEB2 from AS-Uchl1 and parts of structure of it by
RNA fold program. FL was divided into three structures (TM,
20 C and MC2).

Figure 40B shows the assignment of residues with NMR 1H
spectra. Almost all signal peaks of MC2, C, TM structures
were confirmed to the FL structure. These results suggest
that MC2, C and TM form the same structure to the FL
25 structure.

Figure 40C shows five stem structures (dotted square
marked stem at the left) were assigned at the Nuclear
Overhauser effect spectroscopy (NOESY) (right).

Example 40

30 Figure 41 shows the structure dynamics of AS-Uchl1
SINEB2.

Both computational predictions of FL and C form stem
structure at 46-49 and 58-61 (dotted circle in (A)), which
was not confirmed by chemical foot printing in Figures 1 and
35 2. In addition, the loop at 50-57 and single strand region

at 18-123 were not confirmed by chemical foot printing in Figures 1 and 2. However, ¹H NMR spectra and NOESY confirmed the stem structure at 46-49 and 58-61 (Figures 40B and 40C) as same to computational prediction structures. It was
5 analyzed whether the stem structure of 46-49 and 58-61 is associated with any other unstructured residues at the loop 50-57 and at the single strand region at 18-123 by using A51>C mutant RNA and part of SINEB2 RNAs by ¹H NMR spectra. In other words, it was investigated whether undetermined
10 structure of chemical foot printing measurement simultaneously appeared and associated with each other in the NMR buffer condition.

This example demonstrates

a) computational prediction structures of FL and C
15 domain (34-122) by RNAfold program. C structure was divided into unfolded region of FL structure (118-123). 5 residues (118-120) at C structure made stem with opposite residues.

b) ¹H NMR spectra of three small RNAs (34-120, 34-121 and 34-122) and FL. 34-120 did not show stem of 45-49 and
20 58-61 (peak at vertical lines of U61, G60, G49, U48 and G59) but other three structures (FL, 34-121 and 34-122) showed these peaks. Figure 41B shows that unfolded loop from 118-123 were important to form stem at 46-49 and 58-61. Figure 41B shows ¹H NMR spectra of A51C, which increased protein
25 synthesis (Figure 12). As a result of A51>C mutation, chemical shift happened at peaks of U48 and G59 (vertical lines). Additionally, two unassigned peaks at FL (vertical lines at the left of U61 and U48) disappeared. These peak shifts suggest that mutation at A51 changes the binding
30 position of stems at 46-49 and increase the function of protein synthesis. Over all, loop structure at 50-57 and 118-123 interact with the structure formation of stem at 46-49 and 58-61 in the aqueous condition of NMR buffer (20 mM MES (pH6.0), 50 mM NaCl, 8% D2O, 298K). Figure 41 suggests
35 that SINEB2 structure is not strict but some structures are

dynamic. Circles in A correspond to circles in B.

Example 41

Figure 42 shows how synthetic SINEUPs of various ED length and structure motif combinations up-regulate GFP protein expression.

Average GFP protein fold change in HEK293T/17 cells after co-transfection with sense EGFP and (A) SINEUPs (n=3), and (B) miniSINEUPs (n=4) (24 h post-transfection). Western blot images and corresponding GFP band intensities (normalized to ACTB expression level) calculated using ImageJ software. Empty vector is used as negative control while AS Uchl1 (AS Uchl1 SINEB2 containing SINEUP or miniSINEUP) as positive control. SINEUP IDs are followed by the ED length in parentheses. error bars $\pm$ SD; **** P<0.00005; *** P<0.0005; ** P<0.005; * P<0.05; two-tailed Student's t-test.

Structure motif combinations (based on the motifs described in Figure 23 tested in this experiment are provided below with complete features of the construct. For clarity reasons the SEQ ID NOs only indicate the sequence of the effector domain, which is the same in the SINEUP and in the corresponding miniSINEUP.

SINEUP 071/miniSINEUP 071 (206 nt): From 5' end partial motifs 1, partial motifs 2 and 3 (overlapping), complete motif 6, partial motifs 4 and 7

SINEUP 071

Recipient plasmid: pcDNA3.1(-)

Cloning restriction sites: XhoI-HindIII

Features: BD= -40/+32 (+21 nt spacer)

ED (SEQ ID NO:69)= Between SacII and ClaI restriction sites; 206 nt long sequence derived from consensus 2D structure based on global sequence and structure alignment of AS Uchl1, AS Uxt-b, and AS Txnip SINEB2 elements (+Alu and 3' tail region from AS Uchl1)

SINEUP length= 1380 nt

miniSINEUP 071
Recipient plasmid: pcDNA3.1(-)
Cloning restriction sites: XhoI-HindIII
Features: BD= -40/+4 (+21 nt spacer)
5 ED= Between EcoRI and HindIII restriction sites; (SEQ
ID NO:69)
SINEUP length= 289 nt
SINEUP 072/miniSINEUP 072 (206 nt): From 5' end partial
motif 2, complete motif 1, partial motif 4, complete motif
10 7
SINEUP 072
Recipient plasmid: pcDNA3.1(-)
Cloning restriction sites: XhoI-HindIII
Features: BD= -40/+32 (+21 nt spacer)
15 ED (SEQ ID NO:70)= Between SacII and ClaI restriction
sites; 206 nt long sequence derived from consensus 2D
structure based on global sequence and structure alignment
of AS Uchl1 and AS Uxt-b SINEB2 elements (+Alu and 3' tail
region from AS Uchl1)
20 SINEUP length= 1380 nt
miniSINEUP 072
Recipient plasmid: pcDNA3.1(-)
Cloning restriction sites: XhoI-HindIII
Features: BD= -40/+4 (+21 nt spacer)
25 ED= Between EcoRI and HindIII restriction sites; SEQ ID
NO:70
SINEUP length= 289 nt
SINEUP 073/miniSINEUP 073 (156 nt): From 5' end complete
motif 1, partial motifs 2 and 3 (overlapping), partial motif
30 4, complete motif 6, partial 7
SINEUP 073
Recipient plasmid: pcDNA3.1(-)
Cloning restriction sites: XhoI-HindIII
Features: BD= -40/+32 (+21 nt spacer)
35 ED (SEQ ID NO:71)= Between SacII and ClaI restriction

sites; 156 nt long sequence derived from consensus 2D structure based on local sequence and structure alignment of AS Uchl1 and AS Uxt-b SINEB2 elements (+Alu and 3' tail region from AS Uchl1)

5 SINEUP length= 1330 nt

miniSINEUP 073

Recipient plasmid: pcDNA3.1(-)

Cloning restriction sites: XhoI-HindIII

Features: BD= -40/+4 (+21 nt spacer)

10 ED= Between EcoRI and HindIII restriction sites; SEQ ID
NO:71

SINEUP length= 239 nt

SINEUP 074/miniSINEUP 074 (68 nt): From 5' end partial
1, 7, 6 (overlapping), partial 2

15 SINEUP 074

Recipient plasmid: pcDNA3.1(-)

Cloning restriction sites: XhoI-HindIII

Features: BD= -40/+32 (+21 nt spacer)

20 ED (SEQ ID NO:72)= Between SacII and ClaI restriction
sites; 68 nt long sequence derived from consensus 2D
structure based on local sequence and structure alignment of
AS Uchl1, AS Uxt-b, and AS Txnip SINEB2 elements (+Alu and
3' tail region from AS Uchl1)

SINEUP length= 1242 nt

25 miniSINEUP 074

Recipient plasmid: pcDNA3.1(-)

Cloning restriction sites: XhoI-HindIII

Features: BD= -40/+4 (+21 nt spacer)

30 ED= Between EcoRI and HindIII restriction sites; SEQ ID
NO:72

SINEUP length= 151 nt

SINEUP 075/miniSINEUP 075 (110 nt): From 5' end complete
1, 2, 3, and 6

Recipient plasmid: pcDNA3.1(-)

35 Cloning restriction sites: XhoI-HindIII

Features: BD= -40/+32 (+21 nt spacer)

ED (SEQ ID NO:73)= Between SacII and ClaI restriction sites; 110 nt; Δ 28-32, Δ 42-60, Δ 90-116, Δ 131-135 AS Uchl1 SINEB2 deletion mutant (+Alu and 3' tail region from AS Uchl1)

SINEUP length= 1284 nt

miniSINEUP 075

Recipient plasmid: pcDNA3.1(-)

Cloning restriction sites: XhoI-HindIII

Features: BD= -40/+4 (+21 nt spacer)

ED= Between EcoRI and HindIII restriction sites; SEQ ID NO:73

SINEUP length= 193 nt

AS Uchl1 and AS Uchl1_Δ5'-28nt (167 nt): Positive control, all of the 7 motifs

All of the motif combinations tested in this experiment up-regulate GFP expression, though none could surpass the protein fold change induced by the positive control. This suggests that different motifs have combinatory effect on SINEUP activity. As explained in Example 22, most of the motifs have similar sequence composition that might compensate for any missing motif and ensure a minimal level of SINEUP activity. Optimum SINEUP effect is obtained by all of the 7 motifs in their respective order as evident from positive control (AS Uchl1 SINEB2). Interestingly, SINEUP 074 and miniSINEUP 074, despite the short ED and therefore lack of motifs, increases GFP protein expression and represents an example of minimal functional sequence required for SINEUP activity.

Example 42

Figure 43 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP (icSHAPE-guided) and (B) miniSINEUP 071 ED (RNAfold-predicted). Different shaded regions indicate corresponding unique patterns of exact sequence and structure match between

two RNAs. The position of similar sequence and structure motifs shared among all of the SINEB2s are indicated with squares with their respective motif numbers. Due to their similar sequence composition, in some instances motifs 2 and 3 may overlap, and the same goes for motifs 4, 5, 6, and 7.

Example 43

Figure 44 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP (icSHAPE-guided) and (B) miniSINEUP 072 ED (RNAfold-predicted). The same as above applies.

Example 44

Figure 45 shows ExpaRNA exact pattern match result for 2D structures of (A) AS Uchl1 SINEB2 in miniSINEUP-GFP (icSHAPE-guided) and (B) miniSINEUP 073 ED (RNAfold-predicted). The same as above applies.

Example 45

Figure 46 shows ExpaRNA exact pattern match result for 2D structures of (a) AS Uchl1 SINEB2 in miniSINEUP-GFP (icSHAPE-guided) and (b) miniSINEUP 074 ED (RNAfold-predicted). The same as above applies.

Example 46

Figure 47 shows ExpaRNA exact pattern match result for 2D structures of (a) AS Uchl1 SINEB2 in miniSINEUP-GFP (icSHAPE-guided) and (b) miniSINEUP 075 ED (RNAfold-predicted). The same as above applies.

Example 47

Figure 48 shows how structure fragments of SINEB2 from AS Uchl1 up-regulate GFP protein expression.

(A) Average GFP protein fold change in HEK293T/17 cells after co-transfection with sense EGFP and SINEUPs (n=2) (24 h post-transfection). Western blot images and corresponding GFP band intensities (normalized to ACTB expression level) calculated using ImageJ software. Empty vector is used as negative control.

All of the structure fragments in this experiment up-

regulated GFP expression, though none could surpass the protein fold change induced by the AS Uchl1 SINEB2. These fragments form the same structure as the AS Uchl1 SINEB2 structure (Figure 23).

5 This suggests that structure with different motifs have combinatory effect on SINEUP activity. As explained in Figure 23, most of the motifs have similar sequence composition that might compensate for any missing motif and ensure a minimal level of SINEUP activity. Optimal SINEUP effect is
10 obtained by all of the 7 motifs in their respective order as evident from positive control (AS Uchl1 SINEB2).

 (B) This AS Uchl1 SINEB2 structure is confirmed in the NMR spectroscopy solution as alternative structures. Other fragments keep forming as shorter structure of AS Uchl1
15 SINEB2. Motif combinations correspond to new Figure 23.

 Structure motif combinations (based on the motifs described in Figure 23) are provided below with complete features of the construct. For clarity reasons the SEQ ID Nos only indicate the sequence of the effector domain.

20 AS Uchl1 SINEB2: From 5' end complete motifs 1, partial motifs 2, complete motif 3, partial motif 5, complete motif 6 and complete motifs 7

 24-150 (126 nt): From 5' end complete motif 3, partial motif 4, partial motif 5, complete motif 6 and complete motif
25 7

 Recipient plasmid: pcDNA3.1(-)

 Cloning restriction sites: XhoI-HindIII

 Features: BD= -40/+32 (+21 nt spacer)

 ED (SEQ ID NO:74)= Between SacII and ClaI restriction
30 sites; 126 nt long sequence derived from AS Uchl1 SINEB2 elements (+Alu and 3' tail region from AS Uchl1)

 SINEUP length= 1300 nt

 C_34-122 (89 nt): From 5' end partial motif 4, 5 and complete motif 6 and complete motif 7

35 Recipient plasmid: pcDNA3.1(-)

Cloning restriction sites: XhoI-HindIII

Features: BD= -40/+32 (+21 nt spacer)

ED (SEQ ID NO:75)= Between SacII and ClaI restriction sites; 89 nt long sequence contains in AS Uchl1 SINEB2 elements (+Alu and 3' tail region from AS Uchl1)

SINEUP length= 1263 nt

TM (72 nt): From 5' end complete motif 1, complete motif 2, complete motif 3, complete motif 4 and partial motifs 6 (overlapping)

10 Recipient plasmid: pcDNA3.1(-)

Cloning restriction sites: XhoI-HindIII

Features: BD= -40/+32 (+21 nt spacer)

ED (SEQ ID NO:76)= Between SacII and ClaI restriction sites; 72 nt long sequence contains in AS Uchl1 SINEB2 elements (+Alu and 3' tail region from AS Uchl1)

SINEUP length= 1246 nt

MC2 (94 nt): From 5' end complete motif 3, complete motif 4, partial motif 5 and complete motif 7. Recipient plasmid: pcDNA3.1(-)

20 Cloning restriction sites: XhoI-HindIII

Features: BD= -40/+32 (+21 nt spacer)

ED (SEQ ID NO:77)= Between SacII and ClaI restriction sites; 94 nt long sequence derived from AS Uchl1 SINEB2 elements (+Alu and 3' tail region from AS Uchl1)

25 SINEUP length= 1268 nt

Example 48

Figure 49 shows that SINEUPs are active when expressed as circular RNA. Circular SINEUP-DJ-1 (circSINEUP-DJ-1) is able to increase endogenous DJ-1 protein levels in a Binding Domain-dependent manner with no change in target DJ-1 mRNA level in HEK 293T/17 cells.

This example demonstrates that SINEUP sequences can be circular RNA and that these possess translation up-regulation activity in functional nucleic acid molecules with endogenous mRNA. Activity is obtained with the same

primary nucleotide sequence but with the 5' end and the 3' end of the linear SINEUP-DJ-1 covalently ligated. Figure 49A is a schematic representation of the circular SINEUP-DJ-1 RNA (circSINEUP-DJ-1) that contain both the canonical sequence inverted SINE B2 and the Binding Domain for DJ-1 mRNA. Figure 49B shows translation up-regulation activity of functional nucleic acid molecules targeting endogenously expressed DJ-1 mRNA in which the SINEUP is in the circular form. HEK 293T/17 cells were transfected with an empty vector, or a circular SINEUP-DJ-1 and a circular SINEUP-DJ-1 deltaBD lacking the antisense sequence to DJ-1 mRNA. Western blot was performed with anti-DJ-1 antibody to monitor overexpressed protein. β -actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β -actin and relative to empty control samples. Figure 49C presents real time qPCR analysis to quantify circular SINEUP-DJ-1 RNA and DJ-1 mRNA levels proving the mechanism is post-transcriptional.

Advantages

The identification of SINE B2 structural elements allows a structure-directed strategy for the selection and design of SINEUP variants with increased activity and potential utility in humans. In particular, shorter SINEUP variants are advantageous for a number of reasons, among which in vivo delivery and stability.

CLAIMS

1. A functional nucleic acid molecule comprising:

- a target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced; and
- a regulatory sequence having a two-dimensional structure comprising a first stem-loop domain (SL-1) comprising a stem with 8 to 12 paired nucleotides and a loop comprising 5 to 9 nucleotides.

2. A functional nucleic acid molecule comprising:

- a target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced; and
- a regulatory sequence having a two-dimensional structure comprising a first stem-loop domain (SL-1) having a sequence with at least 50% identity with SEQ ID NO:1.

3. The functional nucleic acid molecule according to claim 2, wherein the first stem-loop domain (SL-1) has a sequence with at least 75% identity with SEQ ID NO:1.

4. The functional nucleic acid molecule according to claim 3, wherein the first stem-loop domain (SL-1) has a sequence with at least 90% identity with SEQ ID NO:1.

5. The functional nucleic acid molecule according to any of claims 1 to 4, wherein the stem of the first stem-loop domain (SL-1) has an A-type helical three-dimensional structure.

6. The functional nucleic acid molecule according to any of claims 1 to 5, wherein the two-dimensional structure of the regulatory sequence further comprises a second stem-loop domain (SL-2) and a fourth internal loop domain (IL-4) or a fourth stem-loop domain (SL-4), the second stem-loop domain (SL-2) comprising a stem with 2 to 4 paired nucleotides and a loop comprising 6 to 10 nucleotides, the fourth internal loop domain (IL-4) comprising 14 to 24 nucleotides, and the fourth stem-loop domain (SL-4)

comprising a stem with 3 to 5 paired nucleotides and a loop comprising 6 to 10 nucleotides.

7. The functional nucleic acid molecule according to claim 6, further comprising a third internal loop domain (IL-3) comprising 6 to 14 nucleotides.

8. The functional nucleic acid molecule according to claim 7, further comprising a third stem-loop domain (SL-3) comprising a stem with 1 to 3 paired nucleotides and a loop comprising 3 to 5 nucleotides.

9. The functional nucleic acid molecule according to claim 8, further comprising a second internal loop domain (IL-2) comprising 6 to 12 nucleotides.

10. The functional nucleic acid molecule according to claim 9, further comprising a first internal loop domain (IL-1) comprising 6 to 12 nucleotides.

11. The functional nucleic acid molecule according to any of claims 1 to 10, wherein the regulatory sequence comprises a sequence having at least 50% identity with SEQ ID NO:2.

12. A functional nucleic acid molecule comprising:
- a target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced; and
- a regulatory sequence comprising a sequence having at least 50% identity with a sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:15, SEQ ID NO:7, SEQ ID NO:22, and SEQ ID NO:23.

13. A functional nucleic acid molecule according to claim 12, wherein the regulatory sequence comprises a sequence having at least 75% identity with a sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:15, SEQ ID NO:7, SEQ ID NO:22, and SEQ ID NO:23.

14. A functional nucleic acid molecule according to claim 13, wherein the regulatory sequence comprises a sequence having at least 90% identity with a sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:15, SEQ ID NO:7, SEQ ID NO:22, and SEQ ID NO:23.

15. A functional nucleic acid molecule comprising:
- a target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced; and
- a regulatory sequence comprising a sequence having at least 90% identity with a sequence selected from the group consisting of SEQ ID NO:7, SEQ ID NO:21, SEQ ID NO:17, SEQ ID NO:22, SEQ ID NO:65, SEQ ID NO:66, and SEQ ID NO:67.

16. A functional nucleic acid molecule according to claim 15, wherein the regulatory sequence comprises a sequence selected from the group consisting of SEQ ID NO:7, SEQ ID NO:21, SEQ ID NO:17, SEQ ID NO:22, SEQ ID NO:65, SEQ ID NO:66 and SEQ ID NO:67.

17. A functional nucleic acid molecule comprising:
- a target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced; and
- a regulatory sequence comprising a sequence having at least 90% identity with SEQ ID NO:12.

18. A functional nucleic acid molecule according to claim 17, wherein the regulatory sequence comprises SEQ ID NO:12.

19. A functional nucleic acid molecule comprising:
- a target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced; and
- a regulatory sequence comprising one or more of:
- the motifs formed by an RNA sequence having 75%

identity with GCU (motif 1), AGGGC (motif 3), UGGAU (motif 4), UGGUGG (motif 6), or UGGA (motif 7) and by a hybridised reverse complementary RNA thereof, or

5 - the motif formed by an RNA sequence having 75% identity with AGGAGG (motif 2) and by a hybridised partially complementary reverse RNA thereof, or

 - the motif formed by an RNA sequence having 75% identity with UGG (motif 5) and by a hybridised partially complementary reverse RNA thereof.

10 **20.** A functional nucleic acid molecule comprising:

 - a target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced; and

 - a regulatory sequence comprising one or more of:

15 - the motifs formed by an RNA sequence having 90% identity with GCU (motif 1), AGGGC (motif 3), UGGAU (motif 4), UGGUGG (motif 6), or UGGA (motif 7) and by a hybridised reverse complementary RNA thereof, or

 - the motif formed by an RNA sequence having 90% identity with AGGAGG (motif 2) and by a hybridised partially complementary reverse RNA thereof, or

 - the motif formed by an RNA sequence having 90% identity with UGG (motif 5) and by a hybridised partially complementary reverse RNA thereof.

25 **21.** The functional nucleic acid molecule according to claim 20, wherein the regulatory sequence comprises at least the motif formed by an RNA sequence having 90% identity with UGGUGG (motif 6) and by a hybridised reverse complementary RNA thereof.

30 **22.** A functional nucleic acid molecule comprising:

 - a target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced; and

35 - a regulatory sequence comprising a sequence having at least 90% identity with a sequence selected from the group

consisting of SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, and SEQ ID NO:77.

5 **23.** The functional nucleic acid molecule according to claim 22, wherein the regulatory sequence comprises a sequence having at least 90% identity with SEQ ID NO:72.

10 **24.** The functional nucleic acid molecule according to claim 22, wherein the regulatory sequence comprises a sequence selected from the group consisting of SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, and SEQ ID NO:77.

25. The functional nucleic acid molecule according to claim 24, wherein the regulatory sequence comprises SEQ ID NO:72.

15 **26.** The functional nucleic acid molecule according to any of the preceding claims, wherein the molecule is circular.

27. A composition comprising a functional nucleic acid molecule according to any of the preceding claims.

20

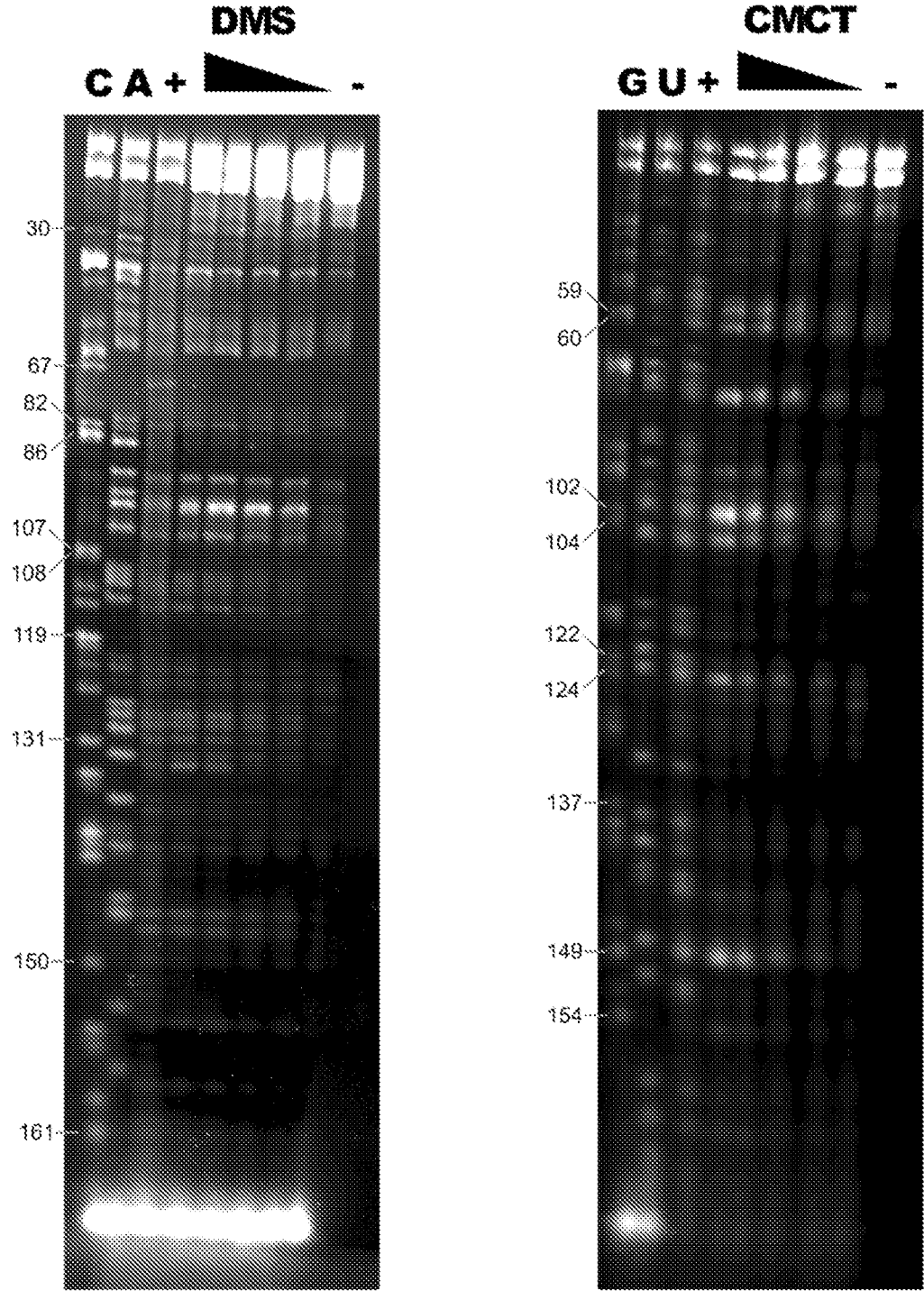


FIG. 1

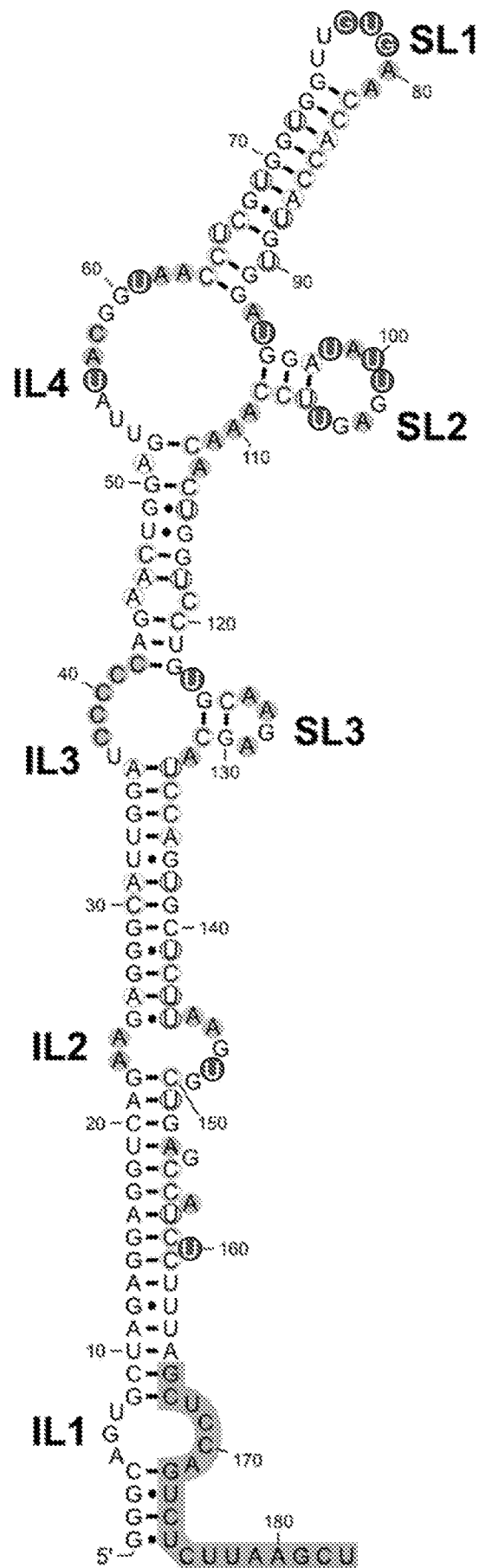


FIG. 2

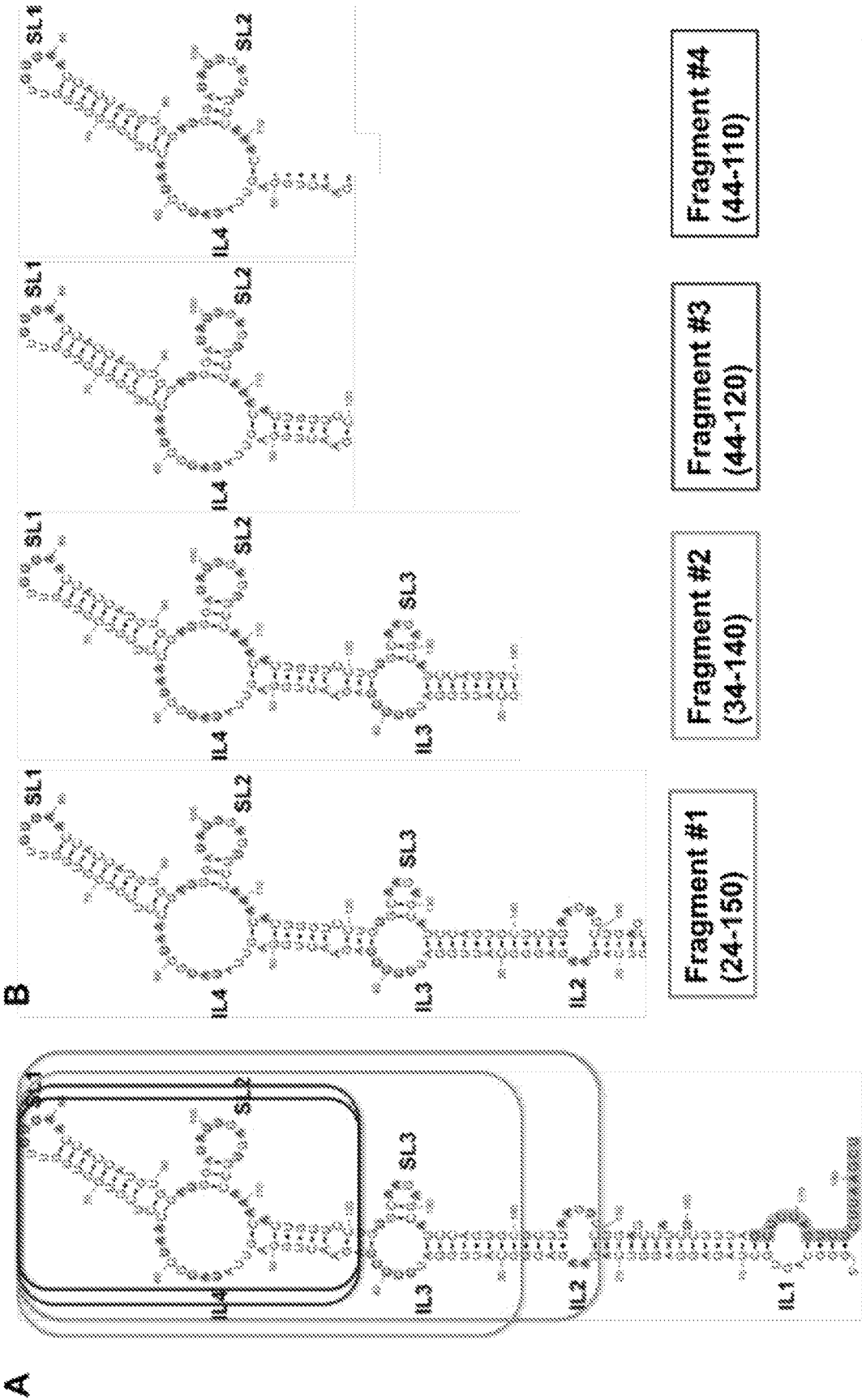


FIG. 3

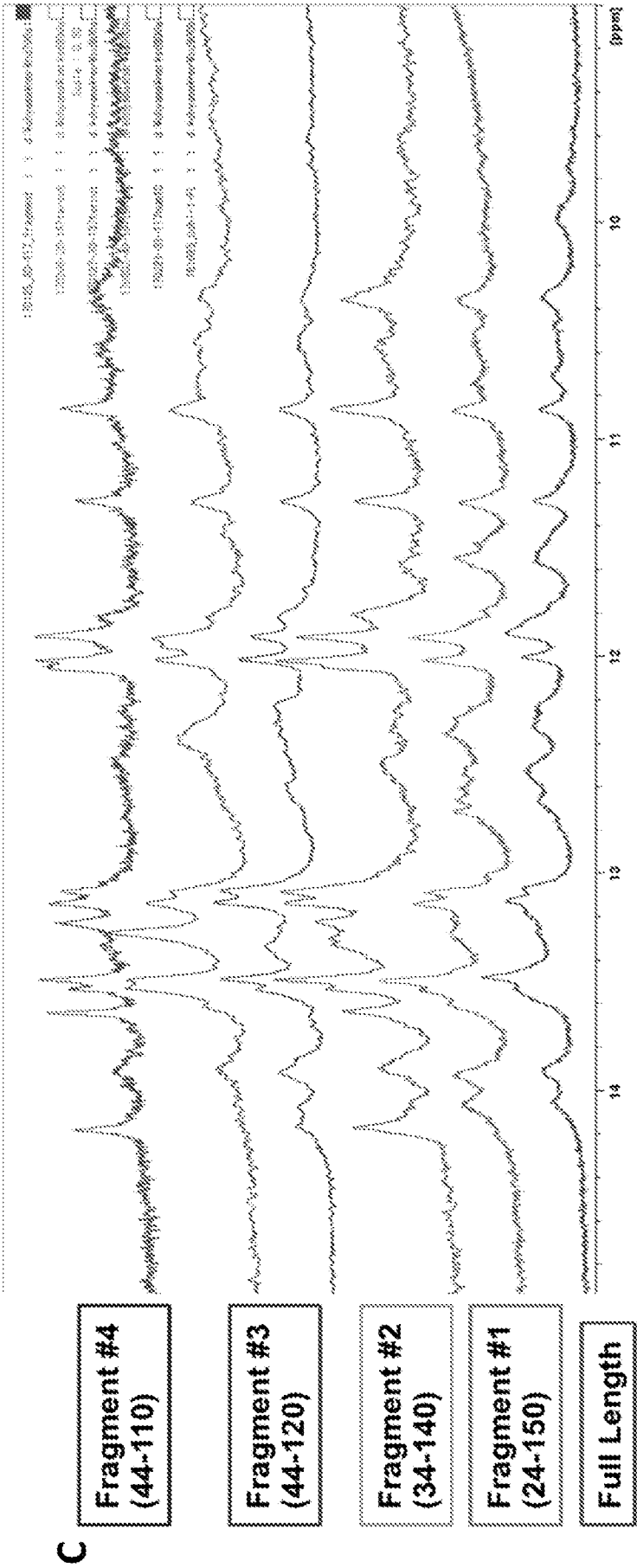


FIG. 3

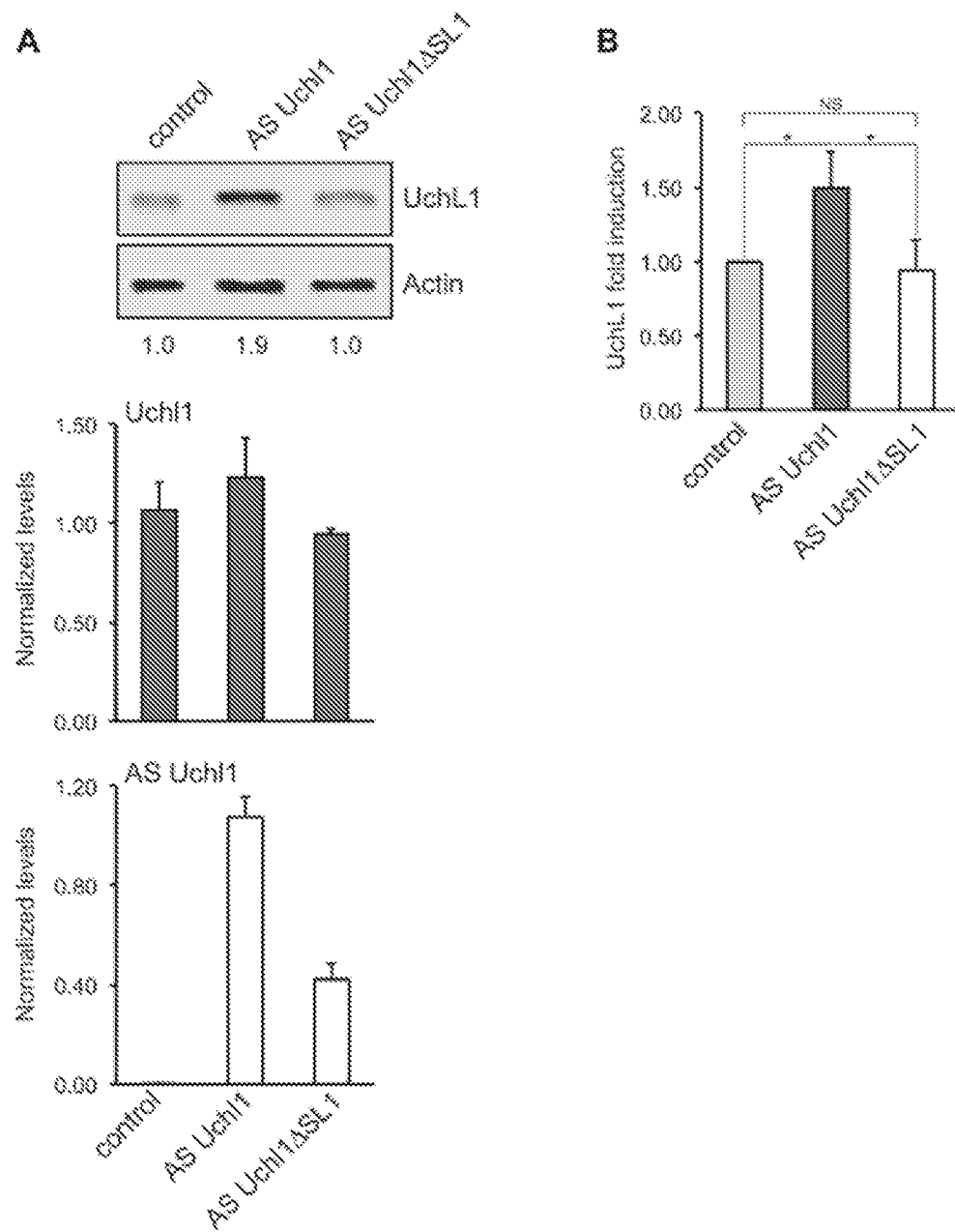


FIG. 4

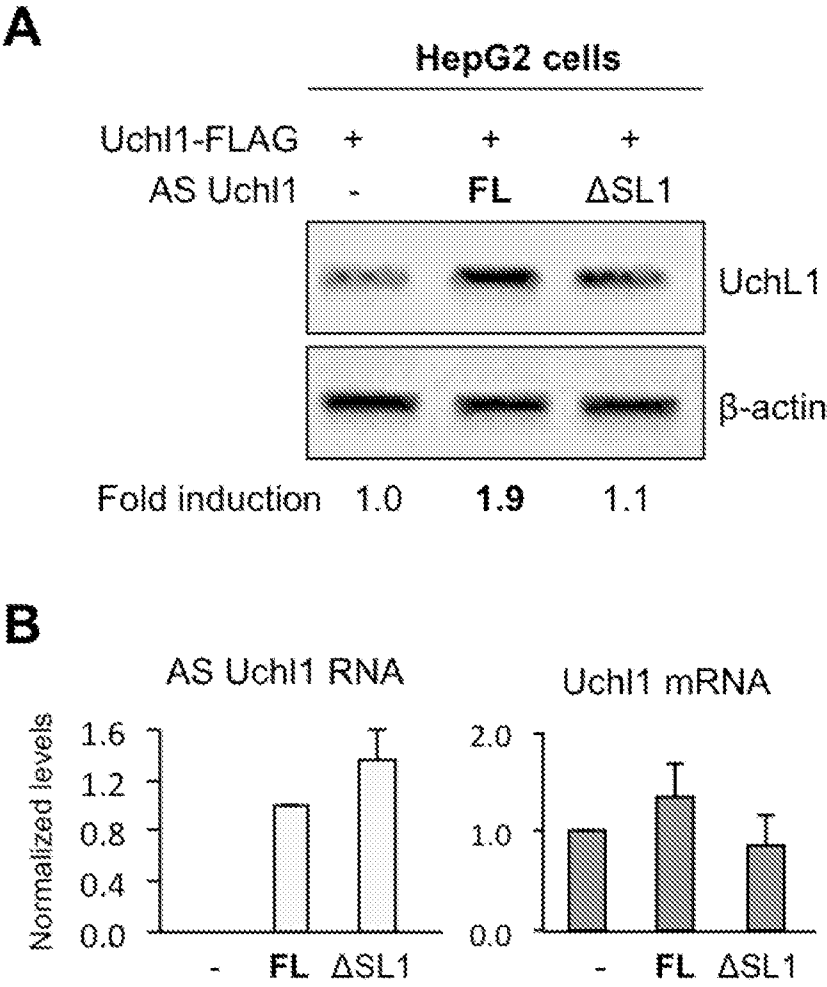


FIG. 5

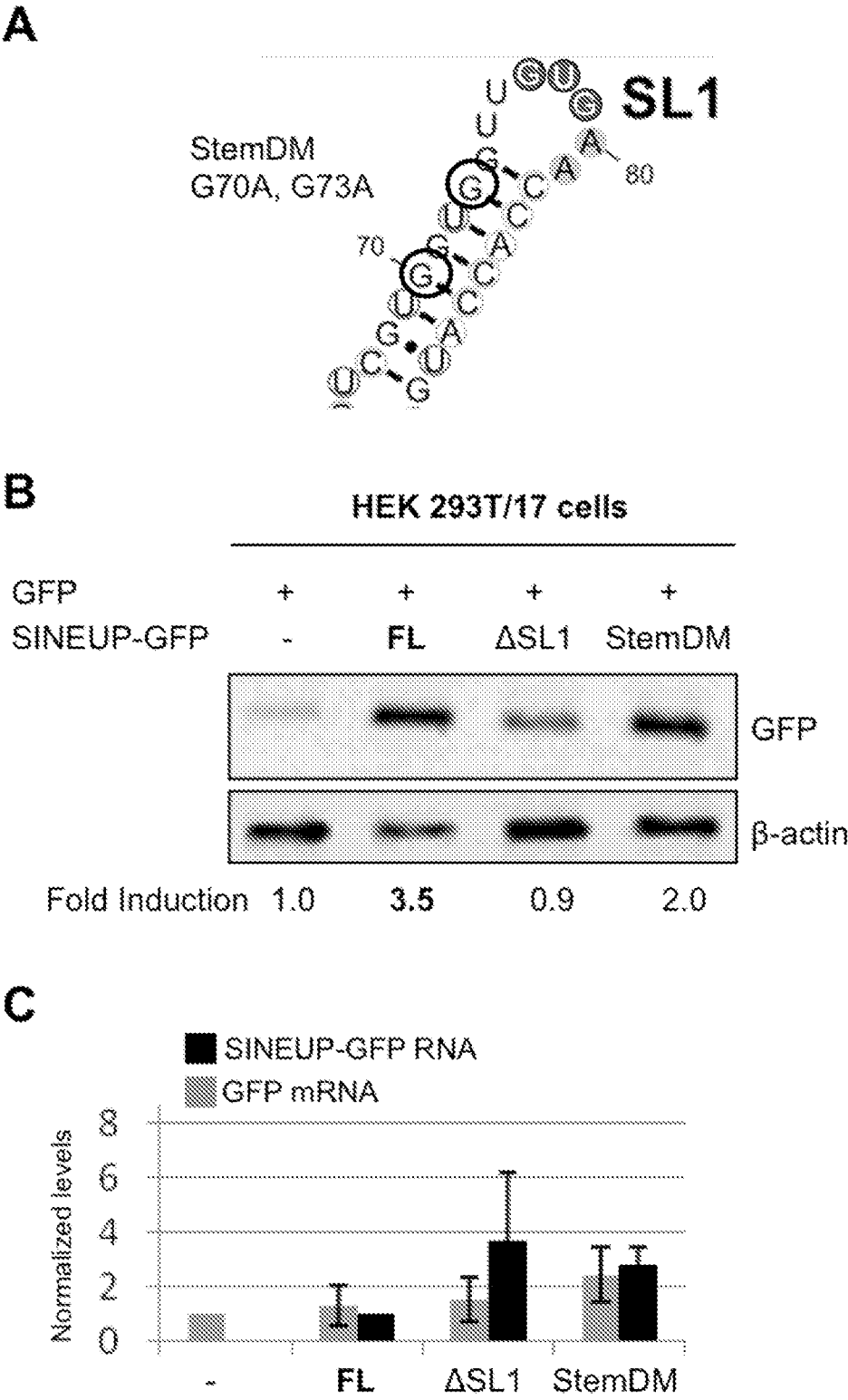


FIG. 6

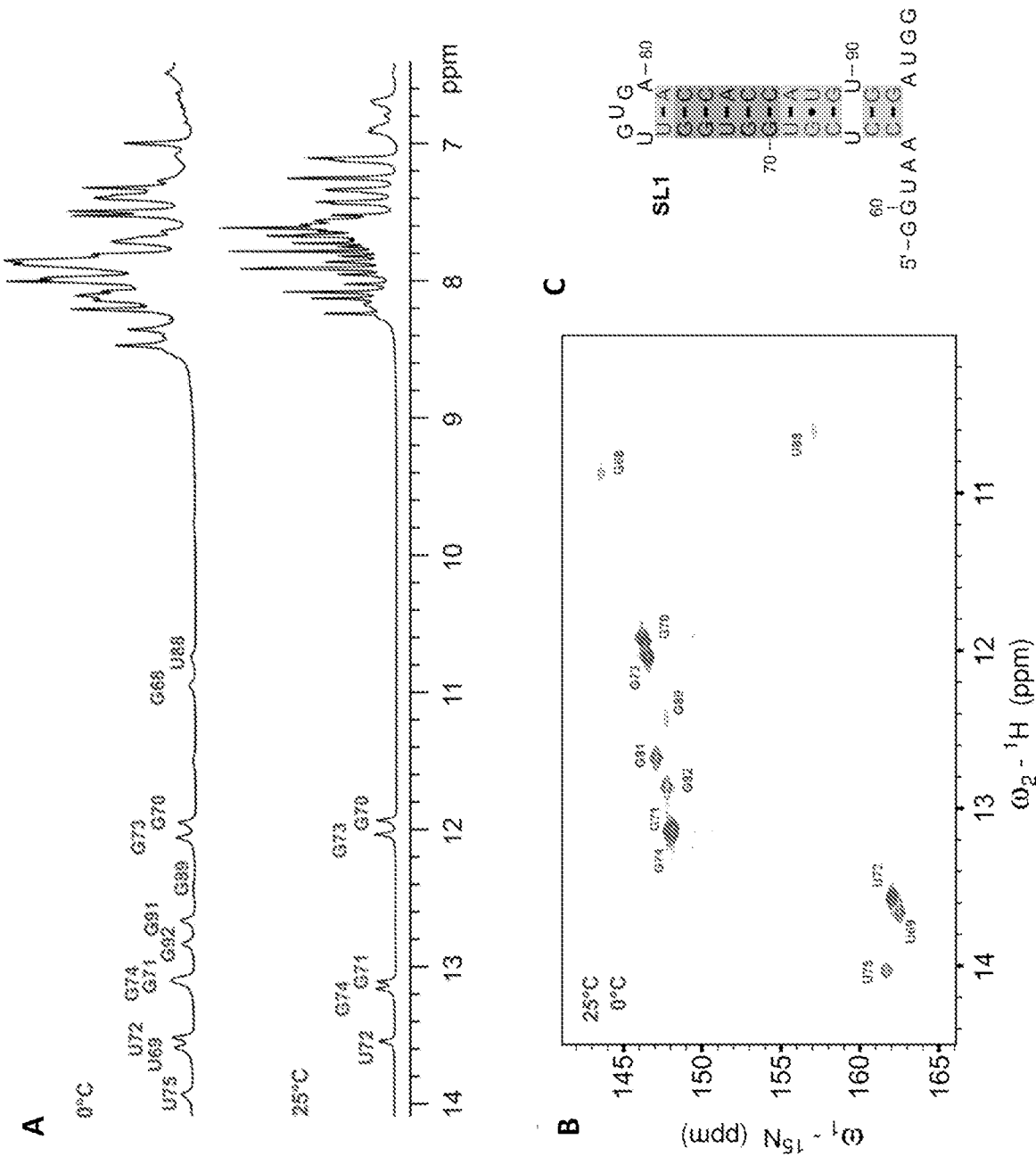
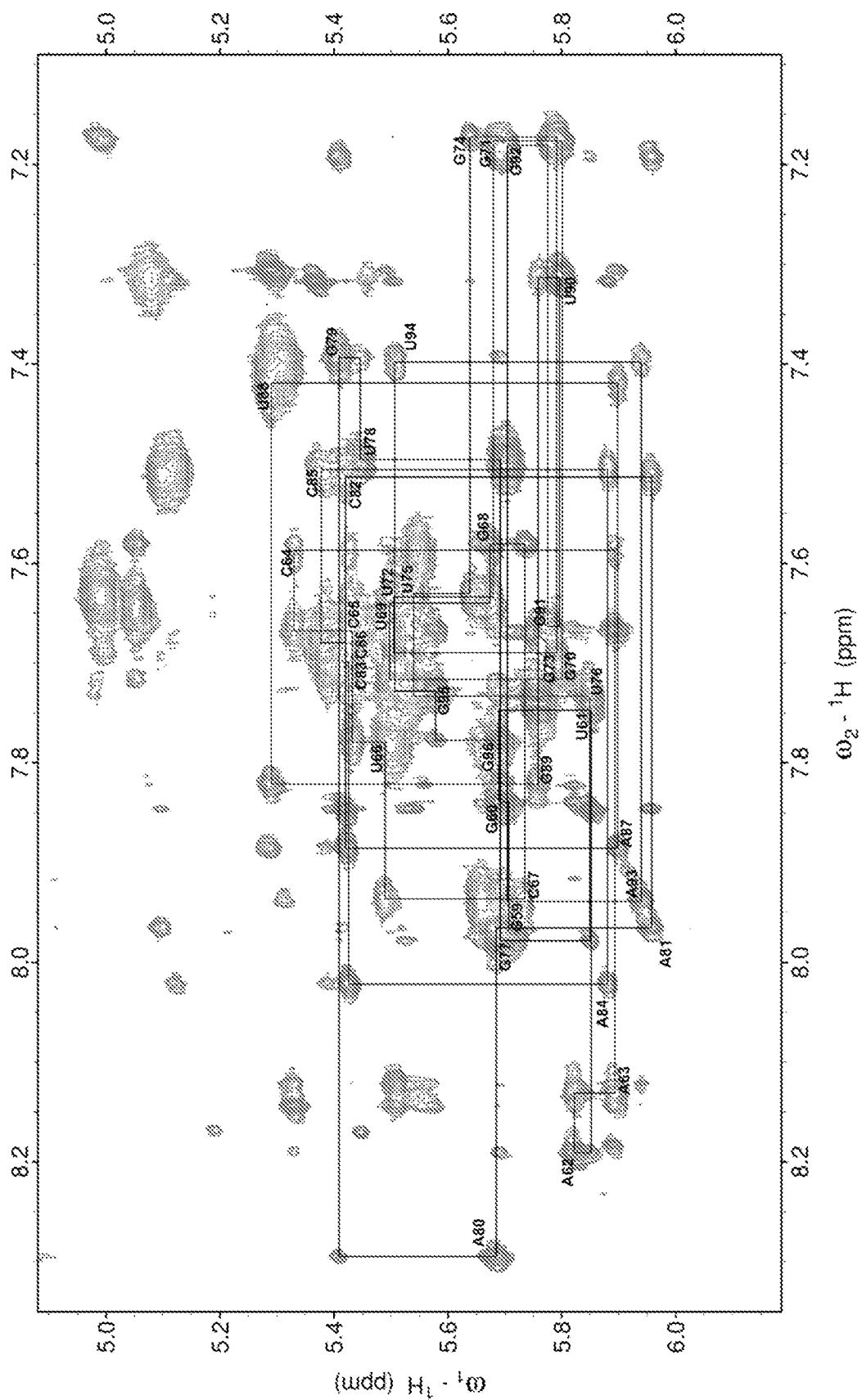


FIG. 7



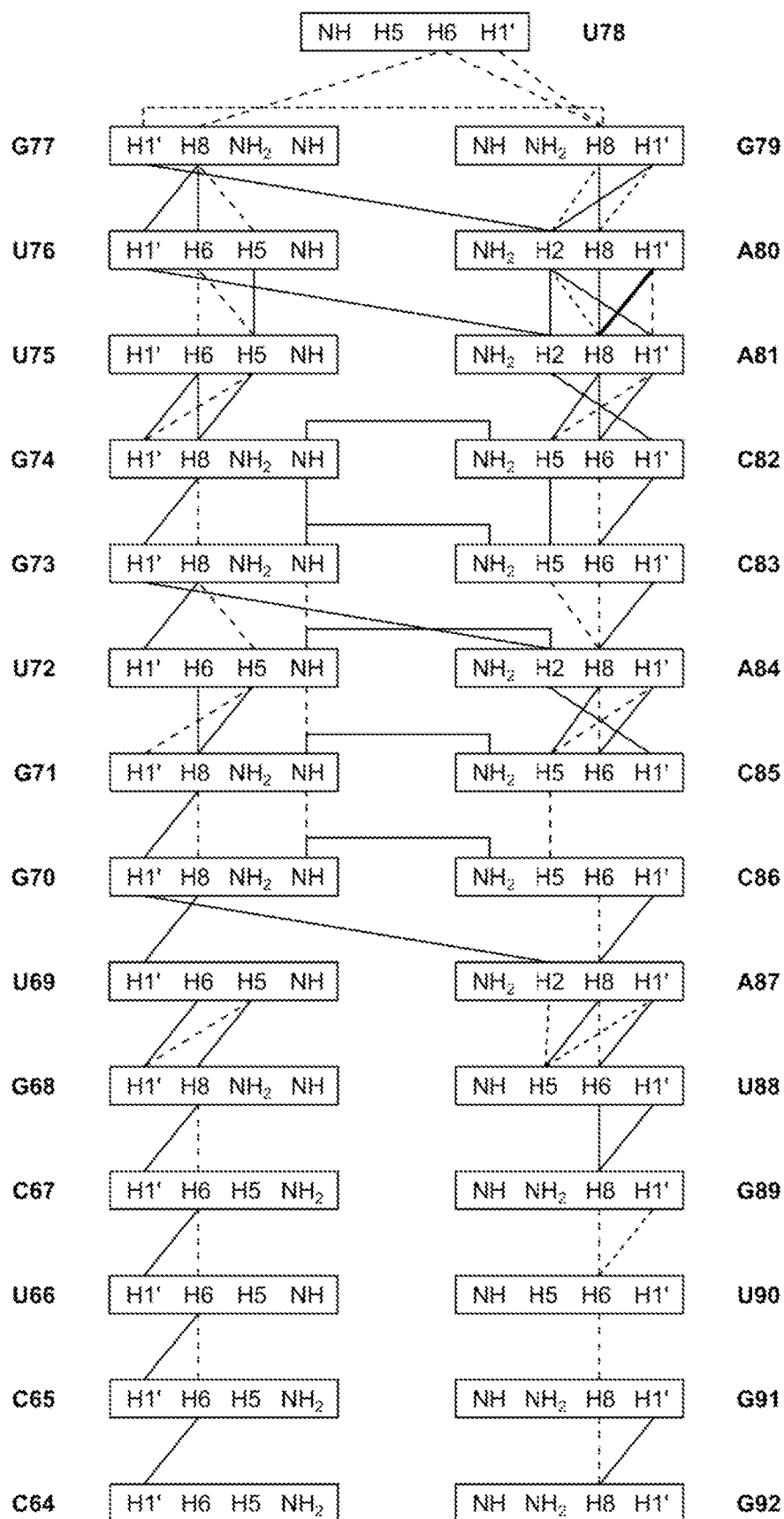


FIG. 9

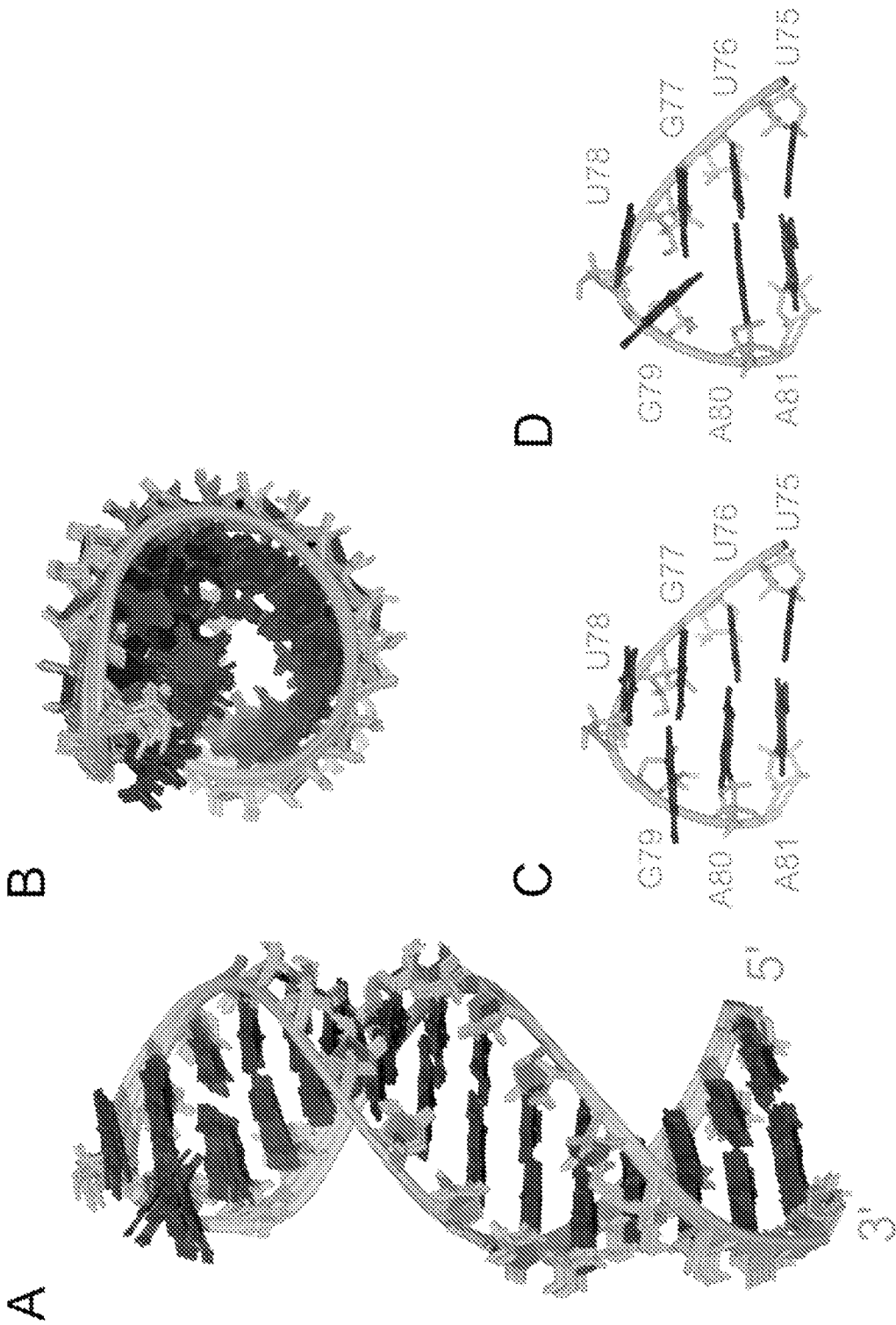


FIG. 10

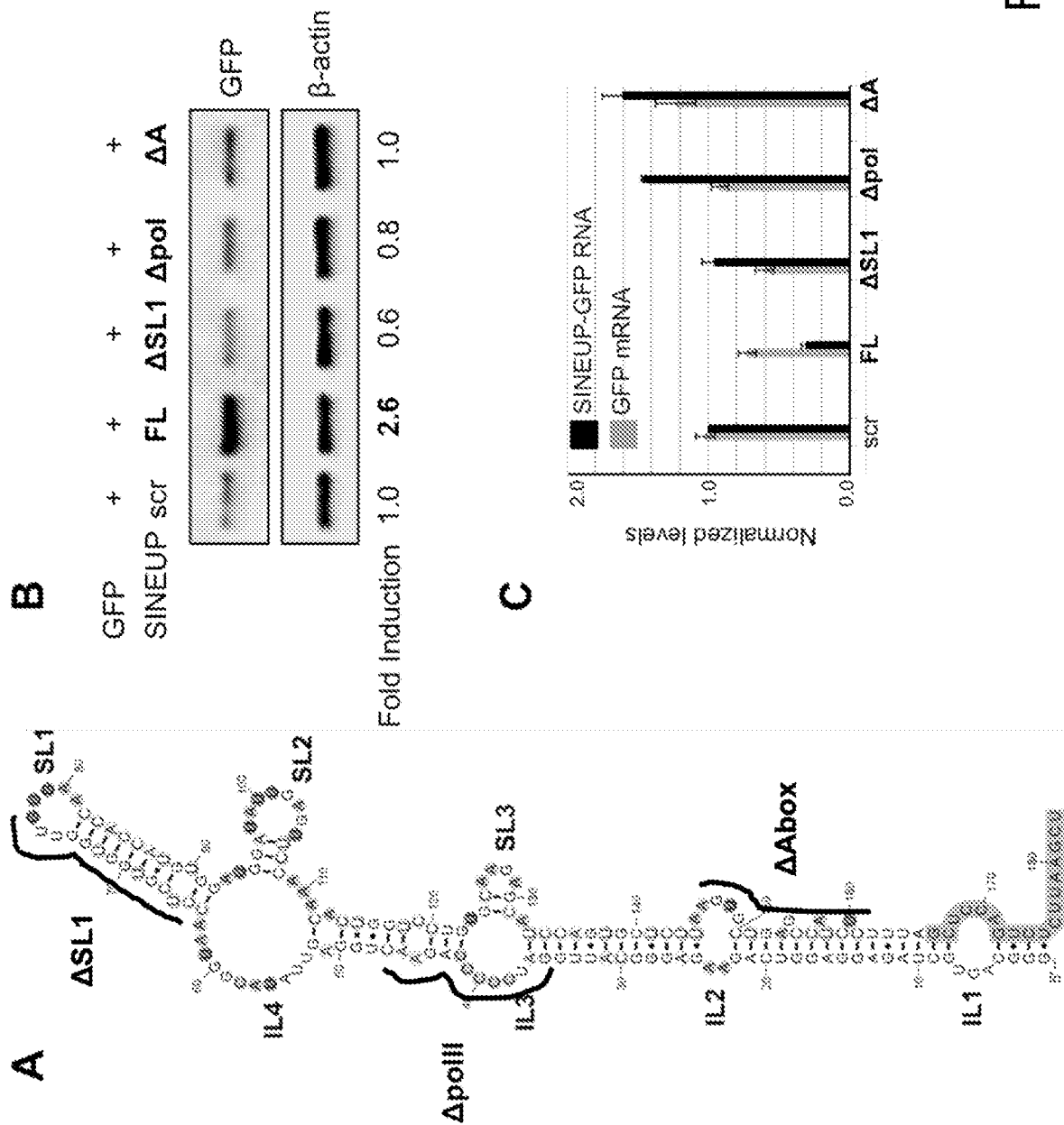
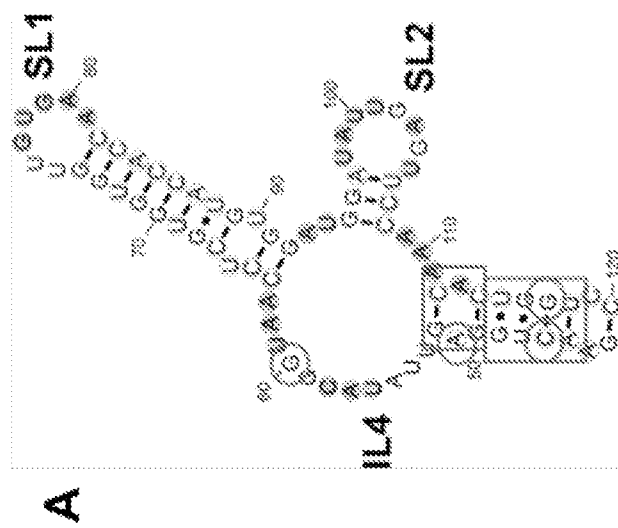


FIG. 11



Structure-based Mutants

#3.3 (G60A)	#3.5 (46-49/115-118) ---stem swap
#3.4 (G60C)	#3.6 (50-53/112-114) ---base swap
#3.1 (A51C)	
#3.2 (C47G,G117C)	

HEK 293T/17 cells									
GFP	+	+	+	+	+	+	+	+	+
SINEUP-GFP	-	FL	#3.1	#3.2	#3.3	#3.4	#3.5	#3.6	

FIG. 12

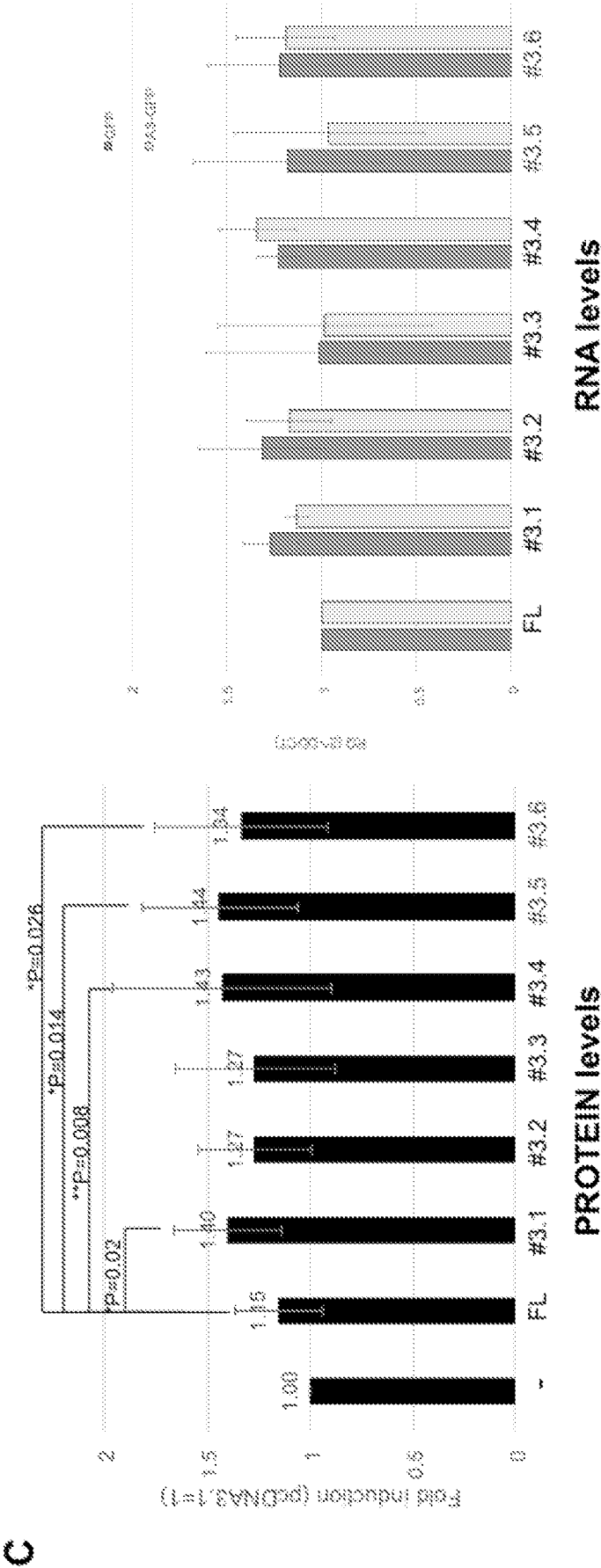


FIG. 12

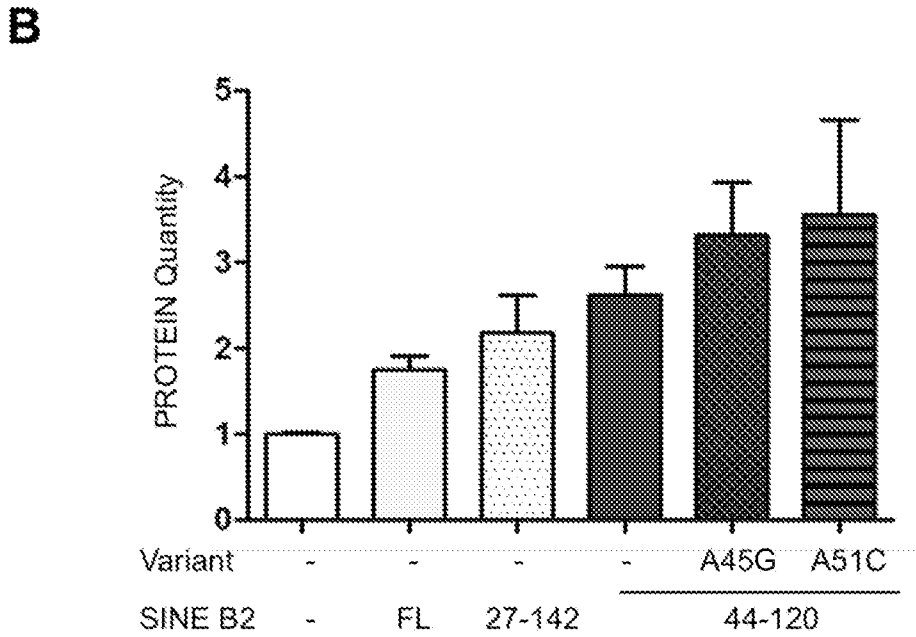
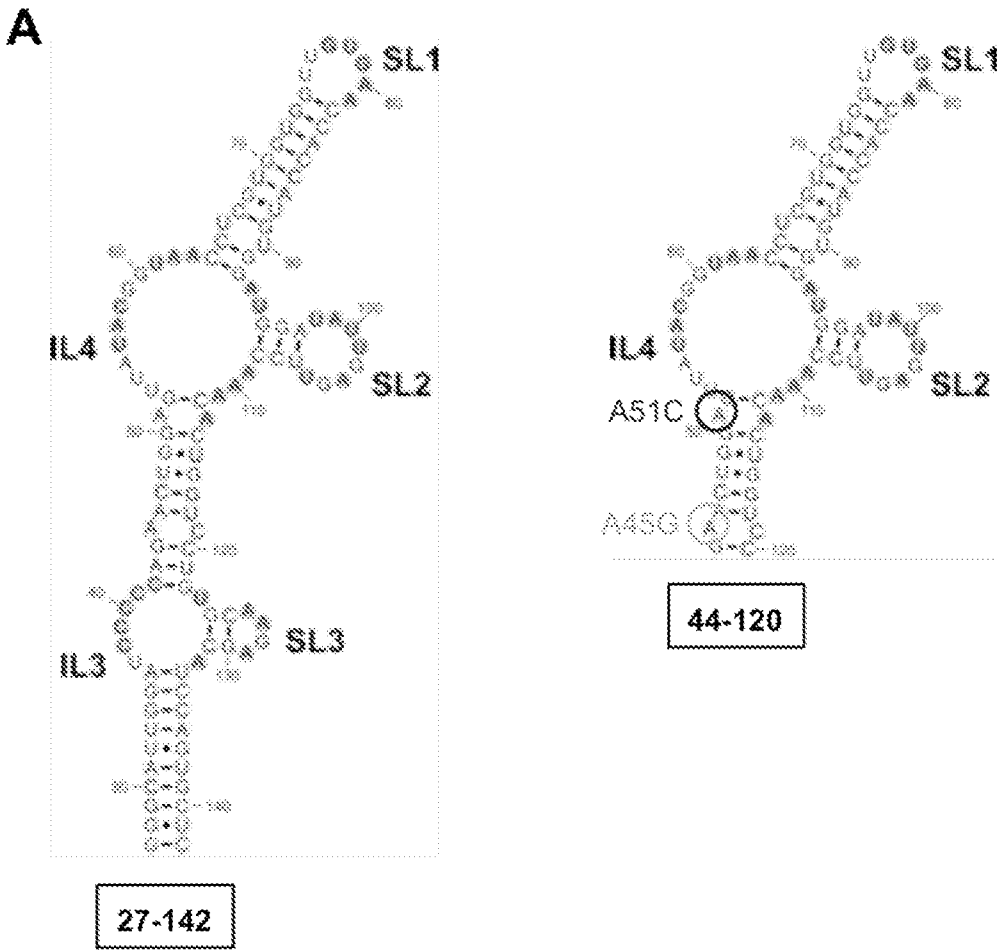


FIG. 13

A

S.No.	Description	Size of ED (bp)
1.	AS Uchl1 SINEB2	167
2.	AS Uchl SINEB2-b	187
3.	AS Uchl1 SINEB2 + AS Uchl SINEB2-b	357
4.	Three repeats of AS Uchl1	532
5.	SINEB2/B3 consensus sequence from RepBase	228

S.No.	SINEB2 from Antisense family	Orientation/ Repeat class or family/ Matching repeat	Size of ED (bp)	Accession No.
6.	AS Nars2	C/SINEB2/B3	214	AK032194
7.	AS Abhd11	C/SINEB2/B3	200	AK041742
8.	AS Egb4.9	C/SINEB2/B3	214	AK046309
9.	AS Wfdc5	C/SINEB2/B3	205	AK042861
10.	AS Pgdh1	C/SINEB2/B3	121	AK034331
11.	AS Gsk3b	C/SINEB2/B3	191	AK145079
12.	AS Rhod	C/SINEB2/B2_Mm2	205	AK044305
13.	AS Rhod	C/SINEB2/B3A	190	AK044205
14.	AS E4f1	C/SINEB2/B2_Mm2	169	AK163105
15.	AS E4f1	C/SINEB2/B2_Mm1f	159	AK163105

B

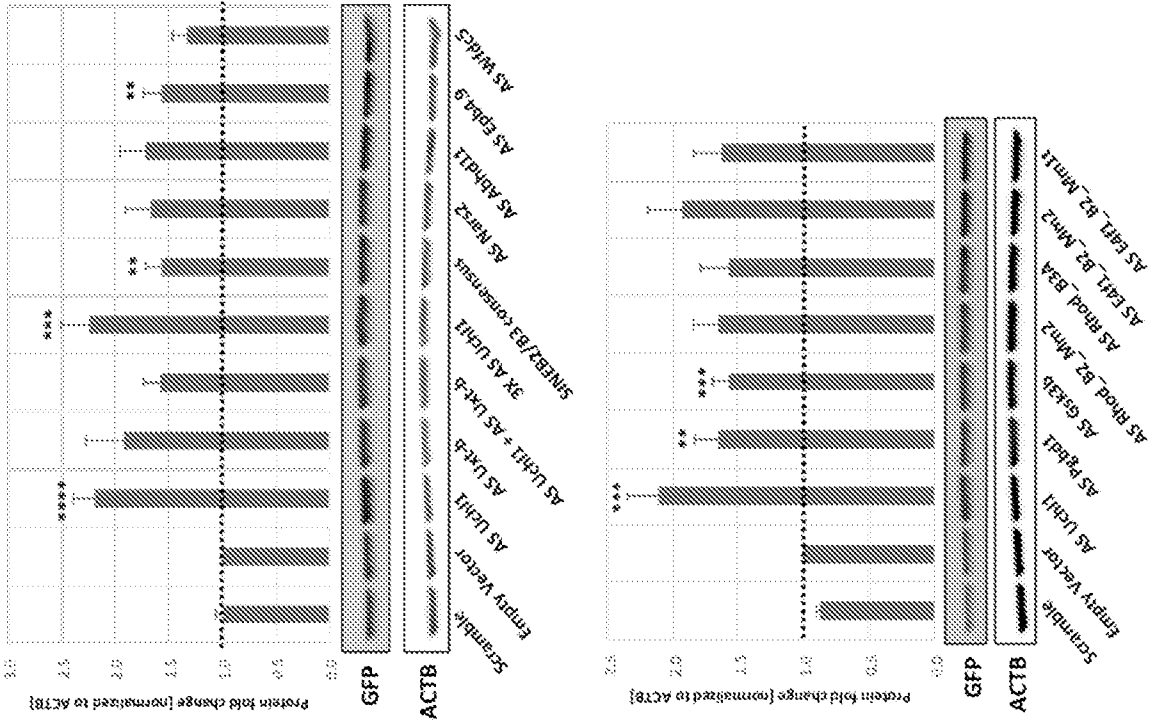


FIG. 14

C

S.No.	SINEB2 from Antisense	Orientation/Repeat class or family/ Matching repeat	Length of SINE (bp)	Accession No.
1.	Uchl1	C/SINEB2/B3	167	AK078321
2.	Uxt	C/SINEB2/B3	SINEB2 b- 187	AK029359
3.	Txnip	C/SINEB2/B3	107	AK143784

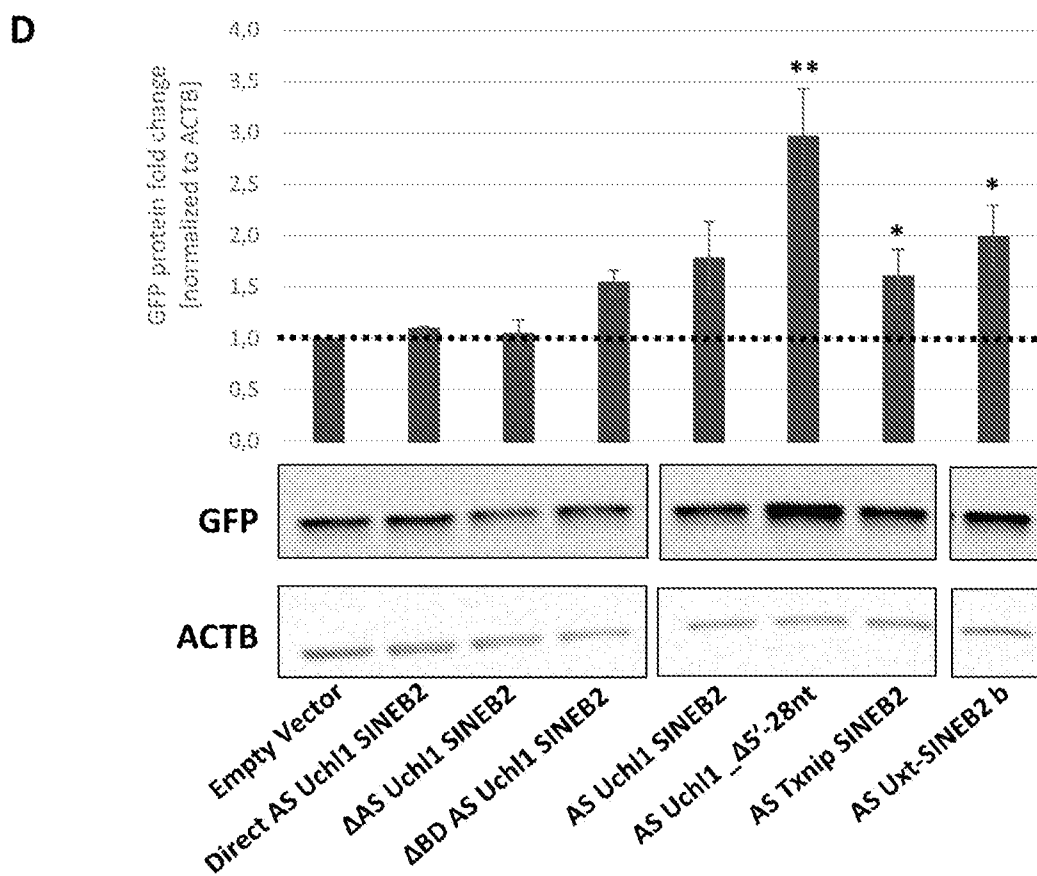
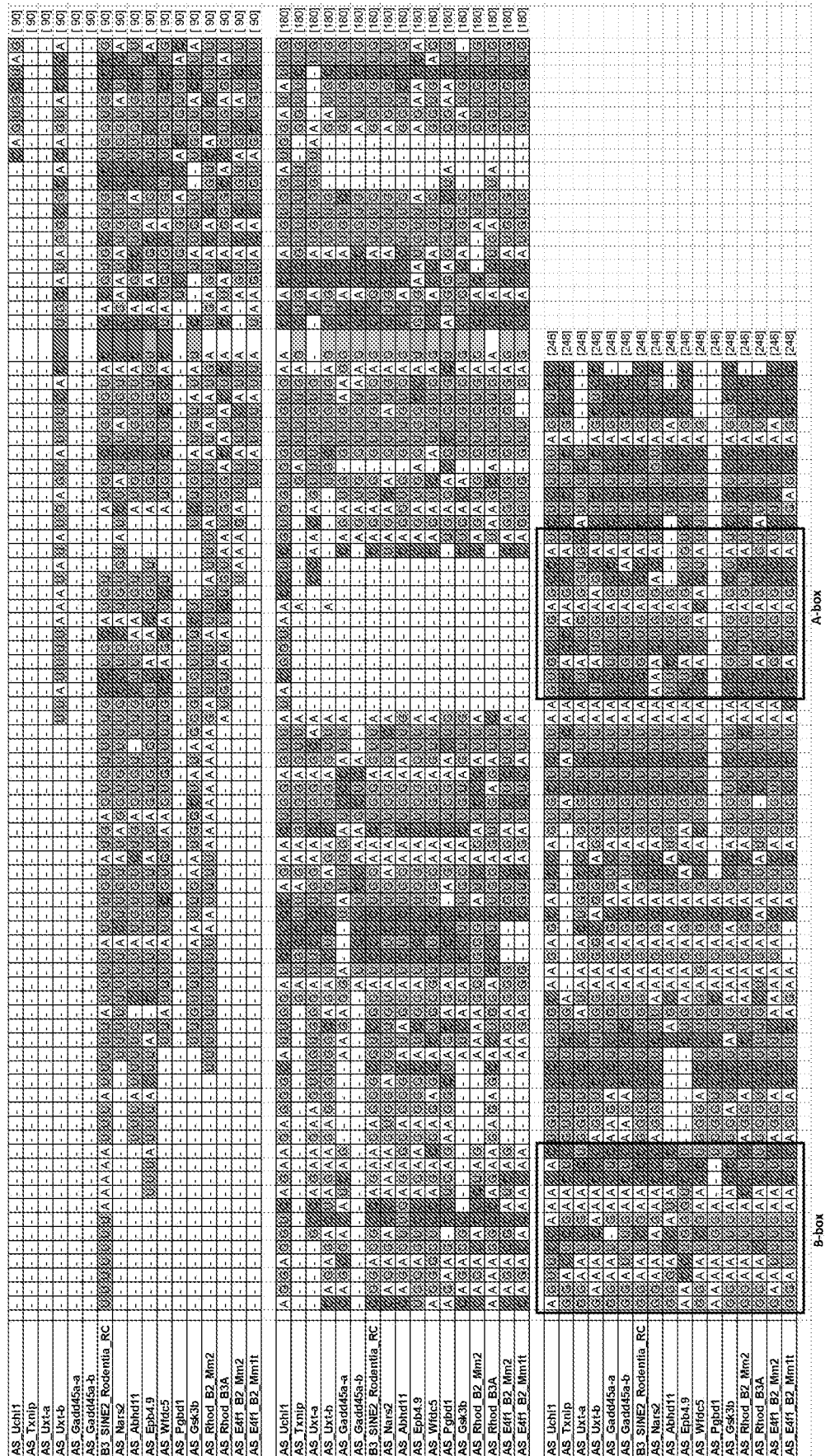


FIG. 14



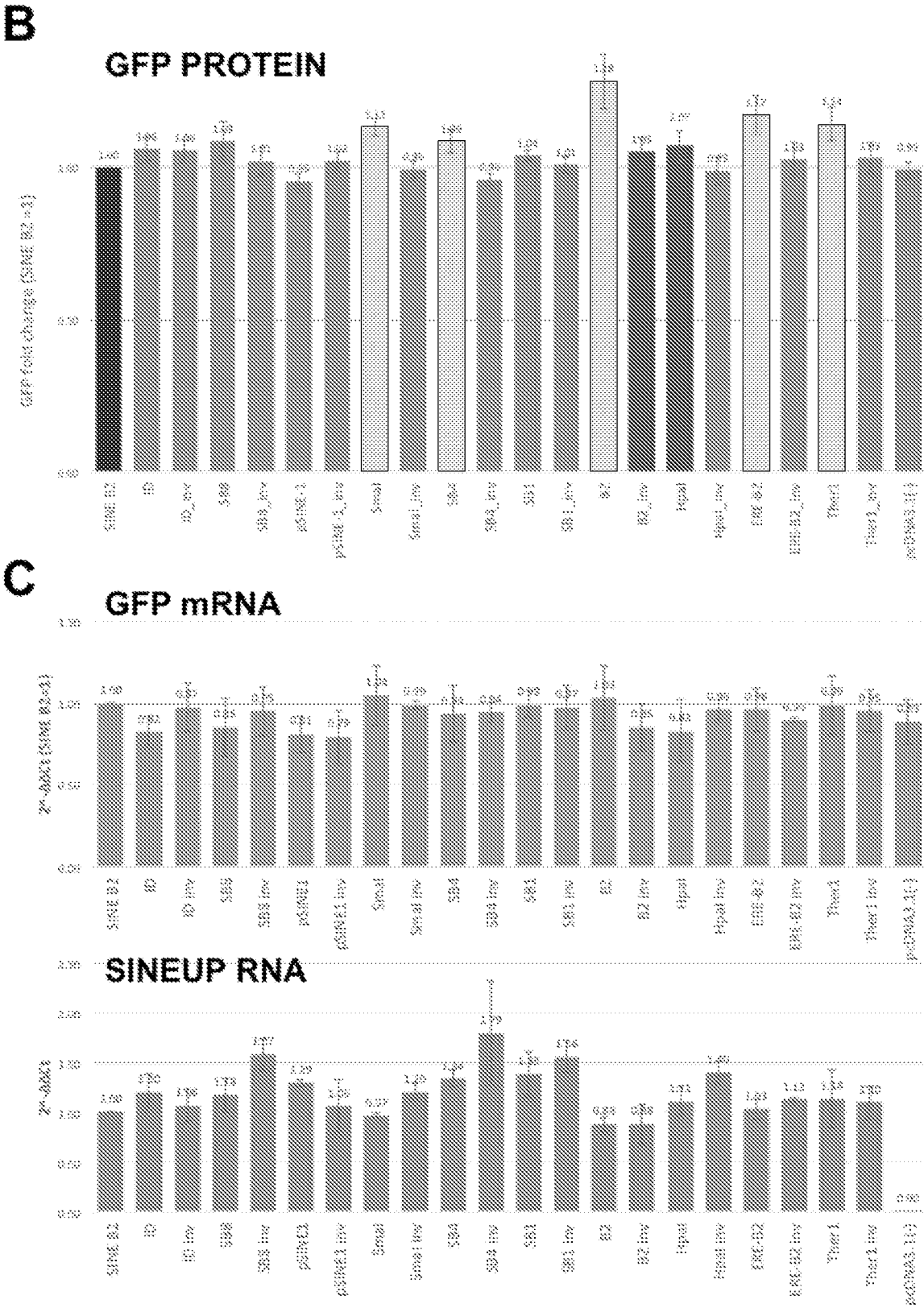


FIG. 16

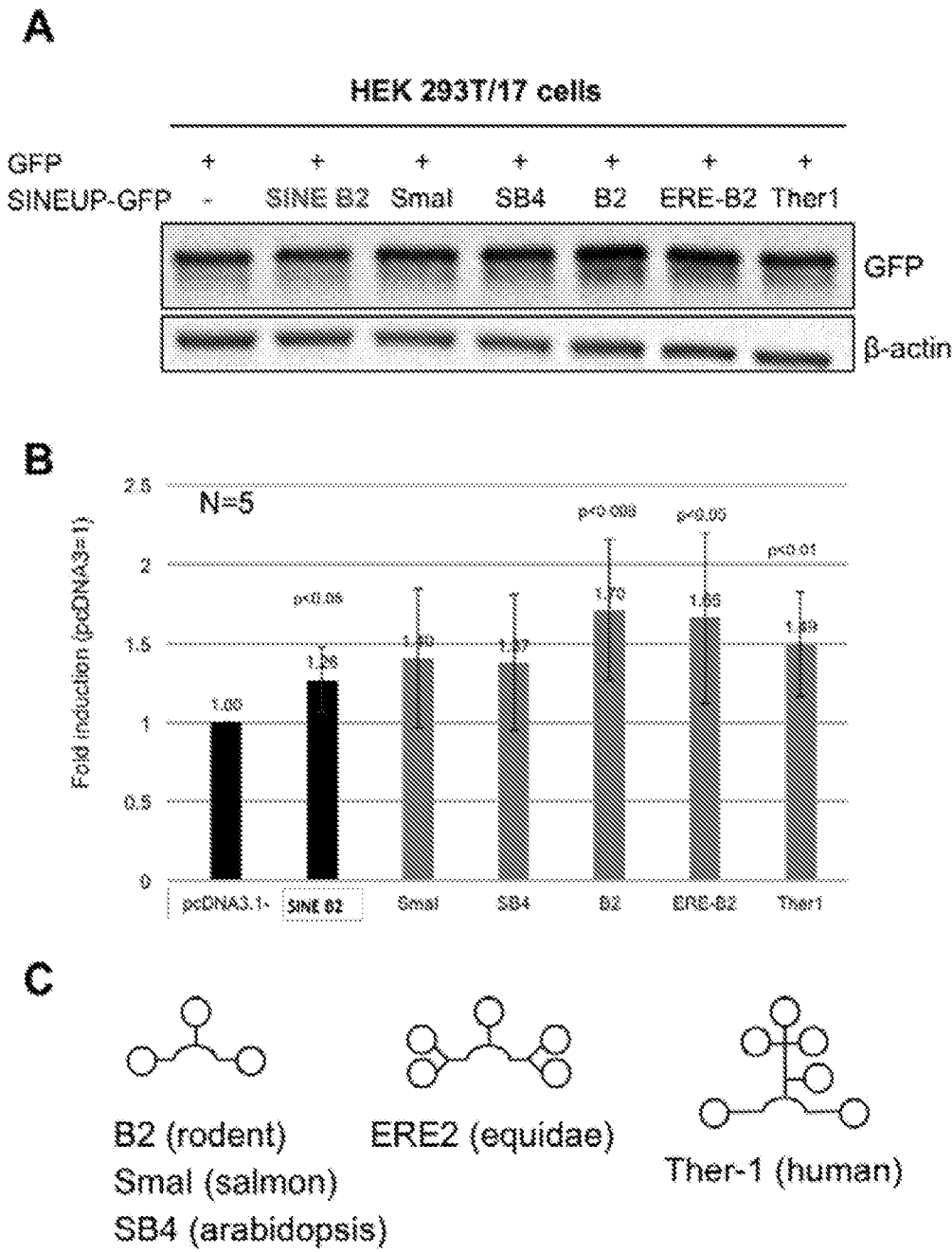


FIG. 17

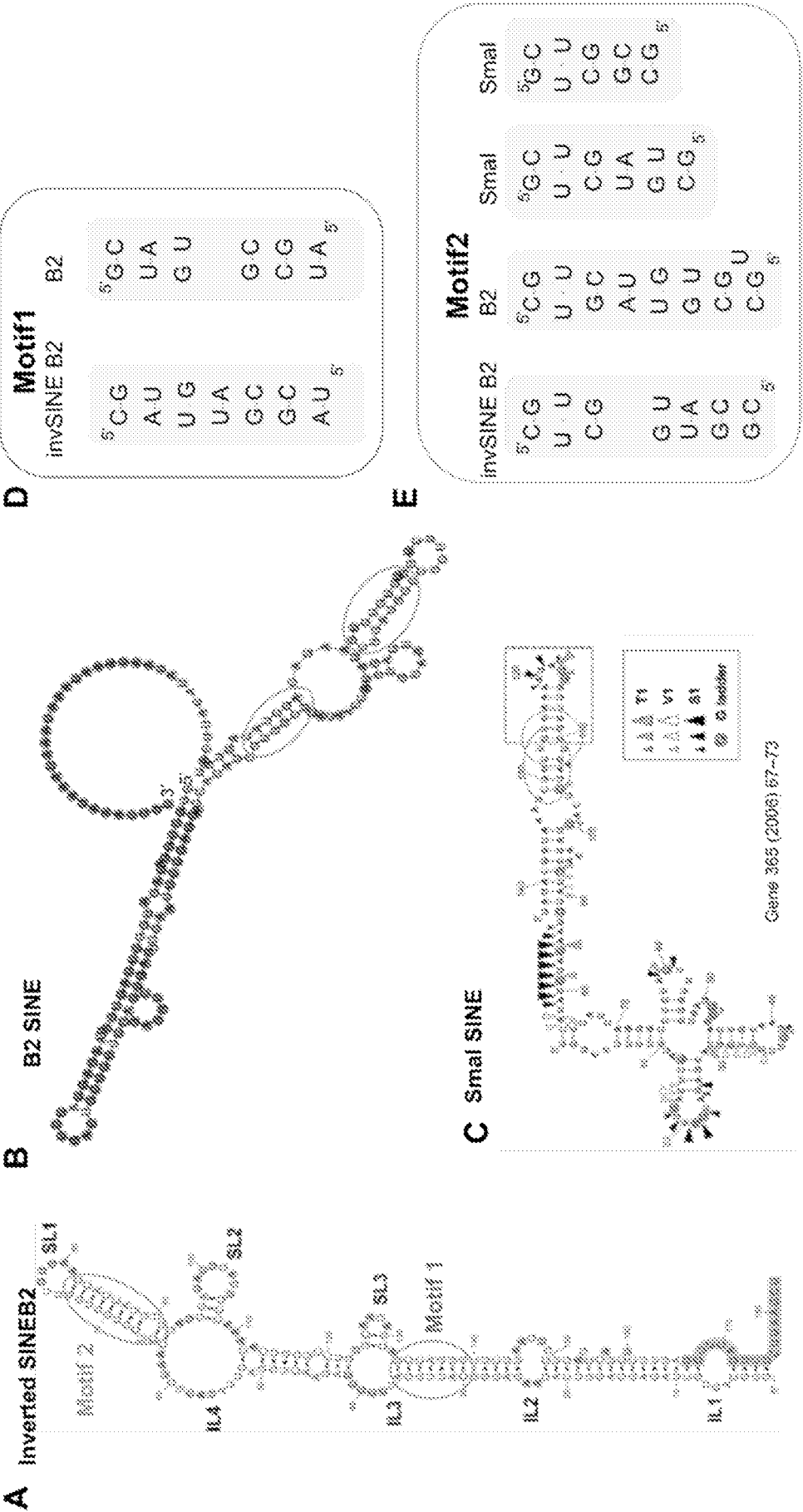


FIG. 18

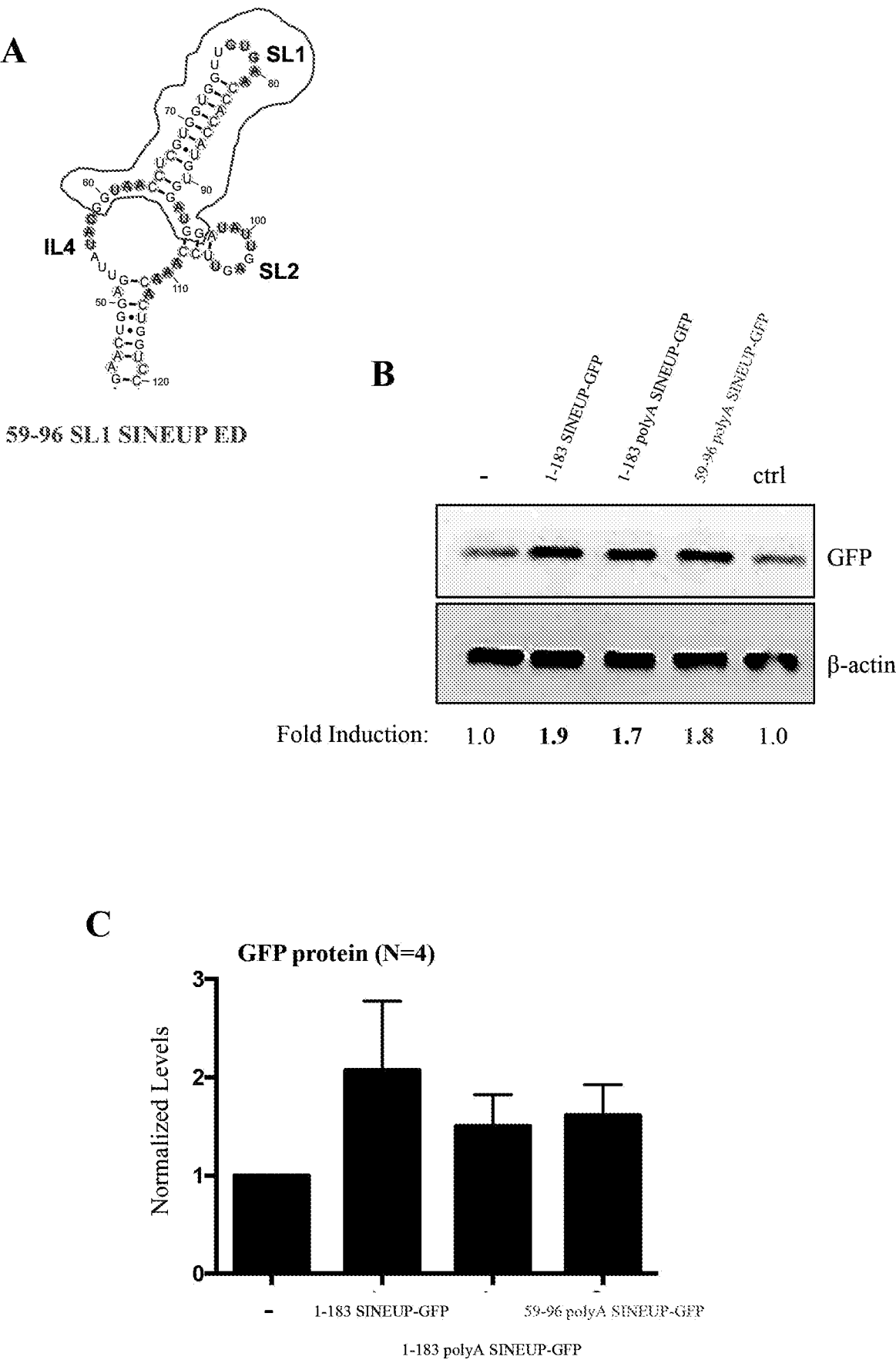
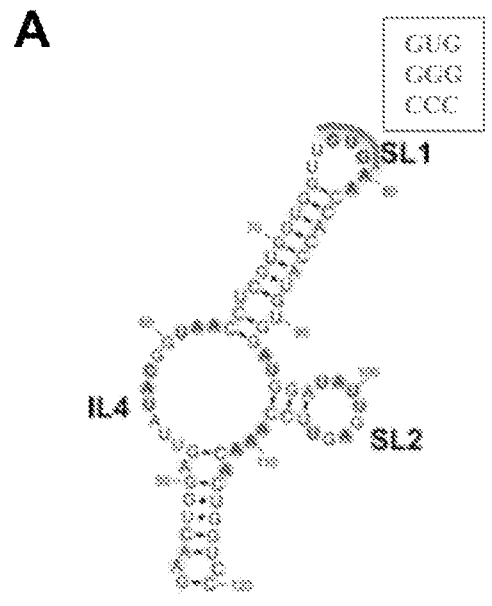


FIG. 19



Negative controls: GFP o/l (without ED)
Positive control: SINEUP-GFP

44-120 SINEUP-GFP:
BD GFP -40/+4
ED invB2(44-120) (wt: 77-79 GUG)

44-120 U78G SINEUP-GFP:
BD GFP -40/+4
ED invB2(44-120) (U78G)

44-120 GUG77-79CCC SINEUP-GFP:
BD GFP -40/+4
ED invB2(44-120) (GUG77-79CCC)

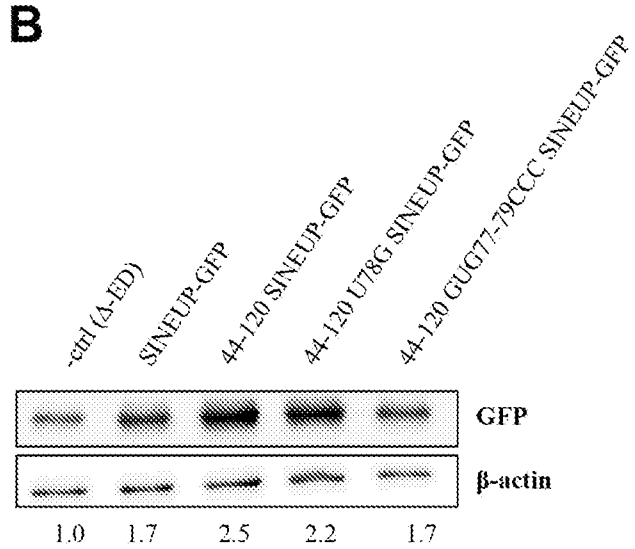


FIG. 20

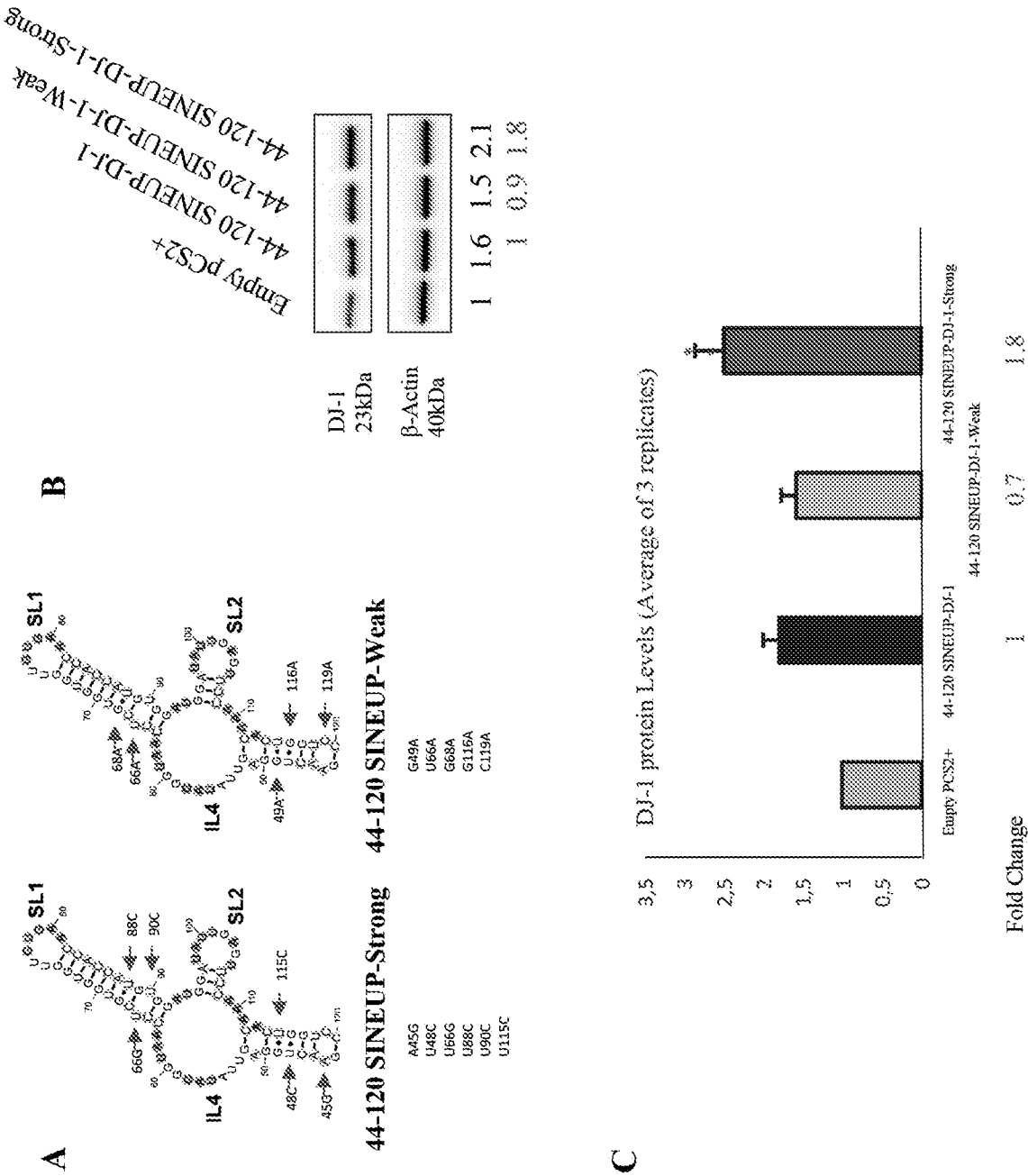


FIG. 21

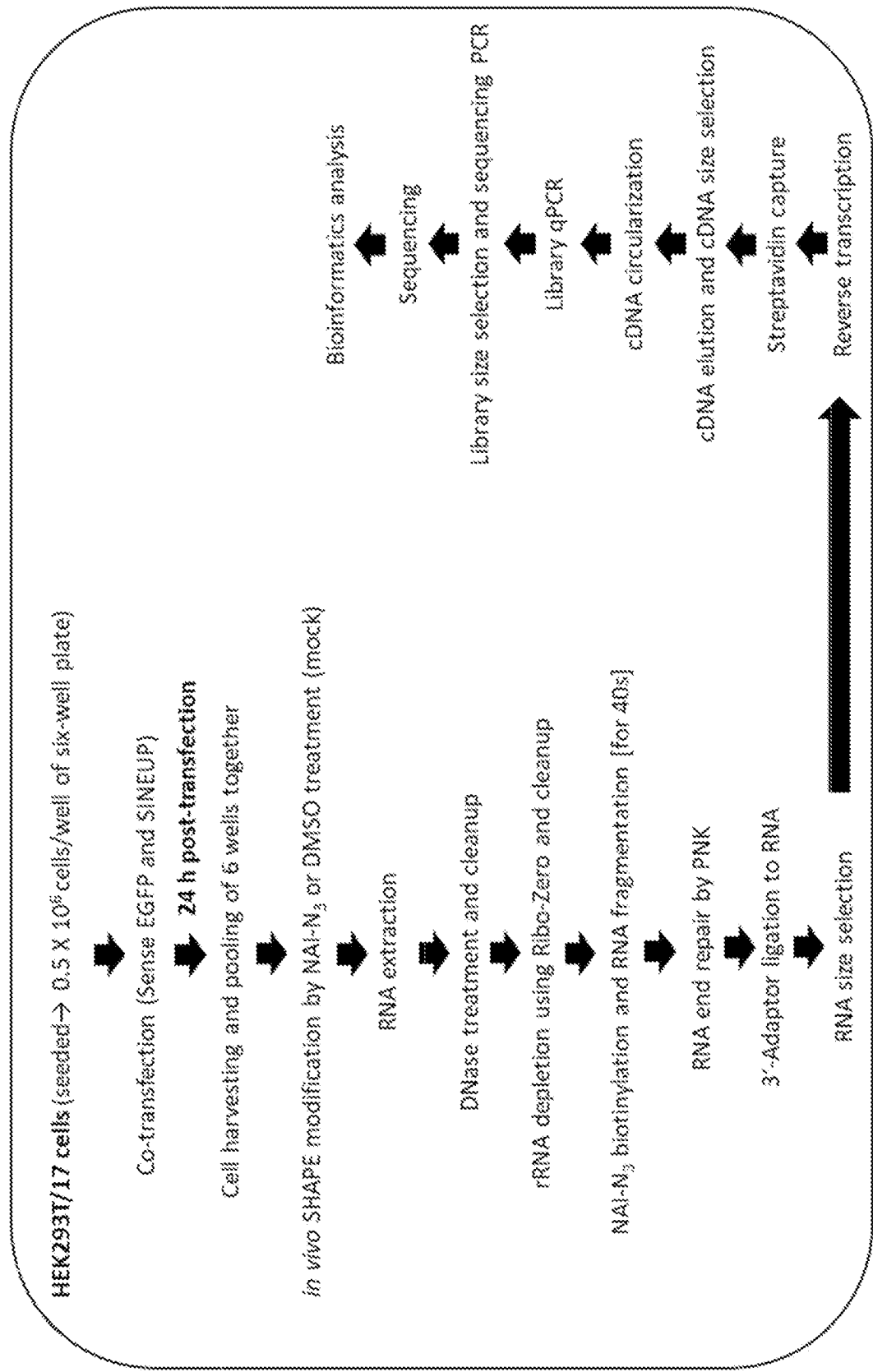
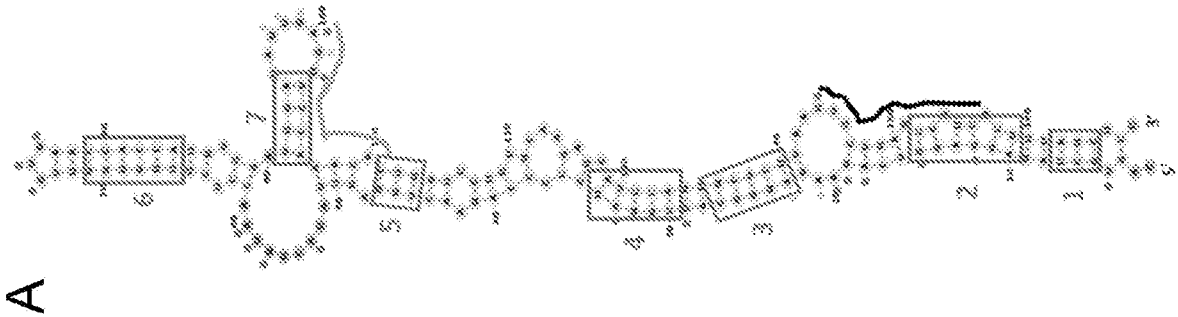


FIG. 22



B

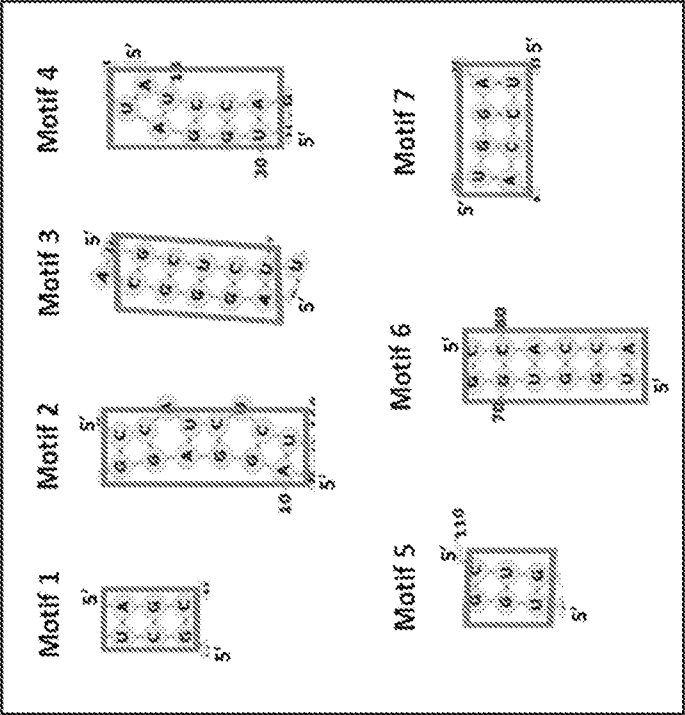


FIG. 23



FIG. 24

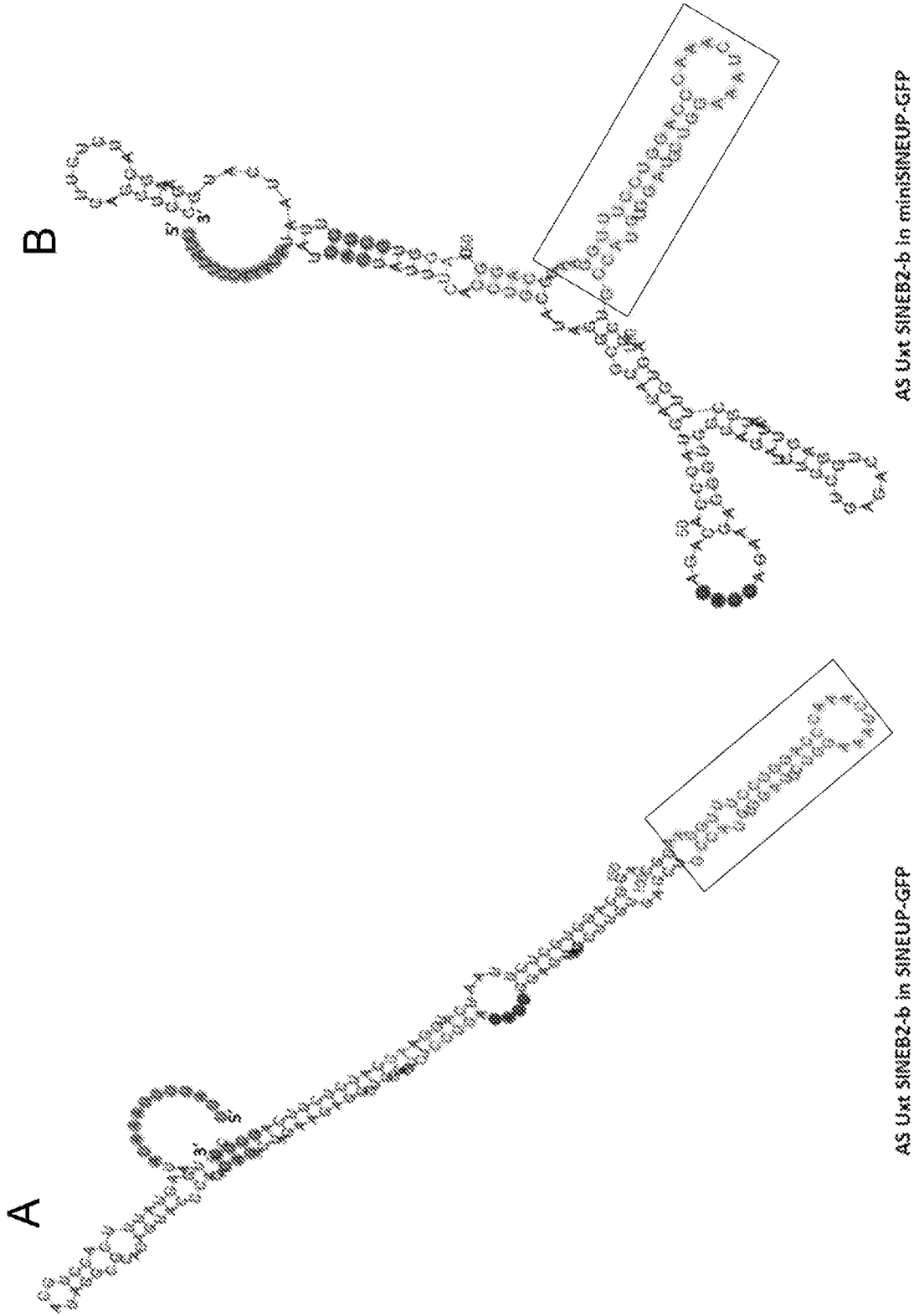


FIG. 25

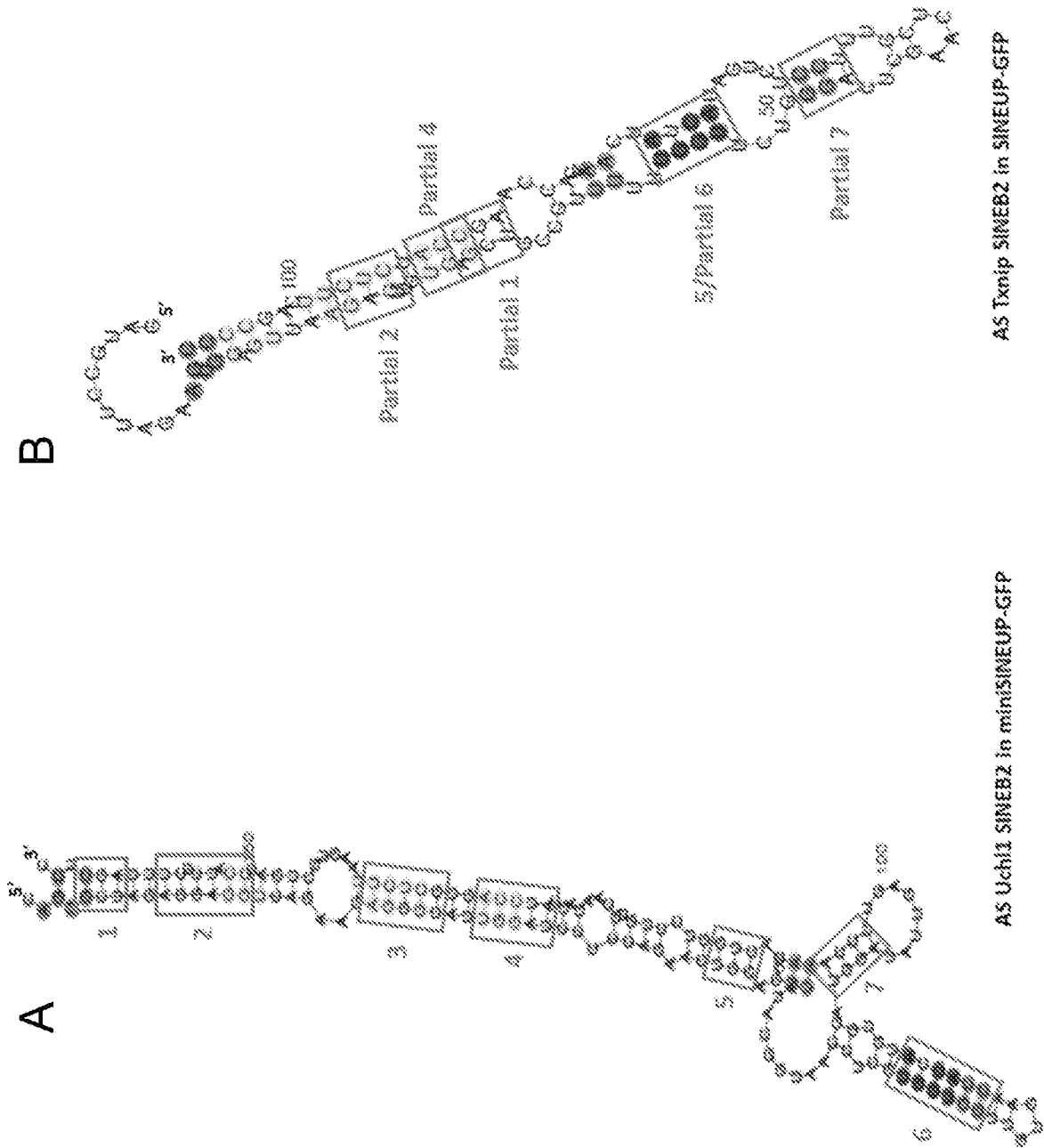
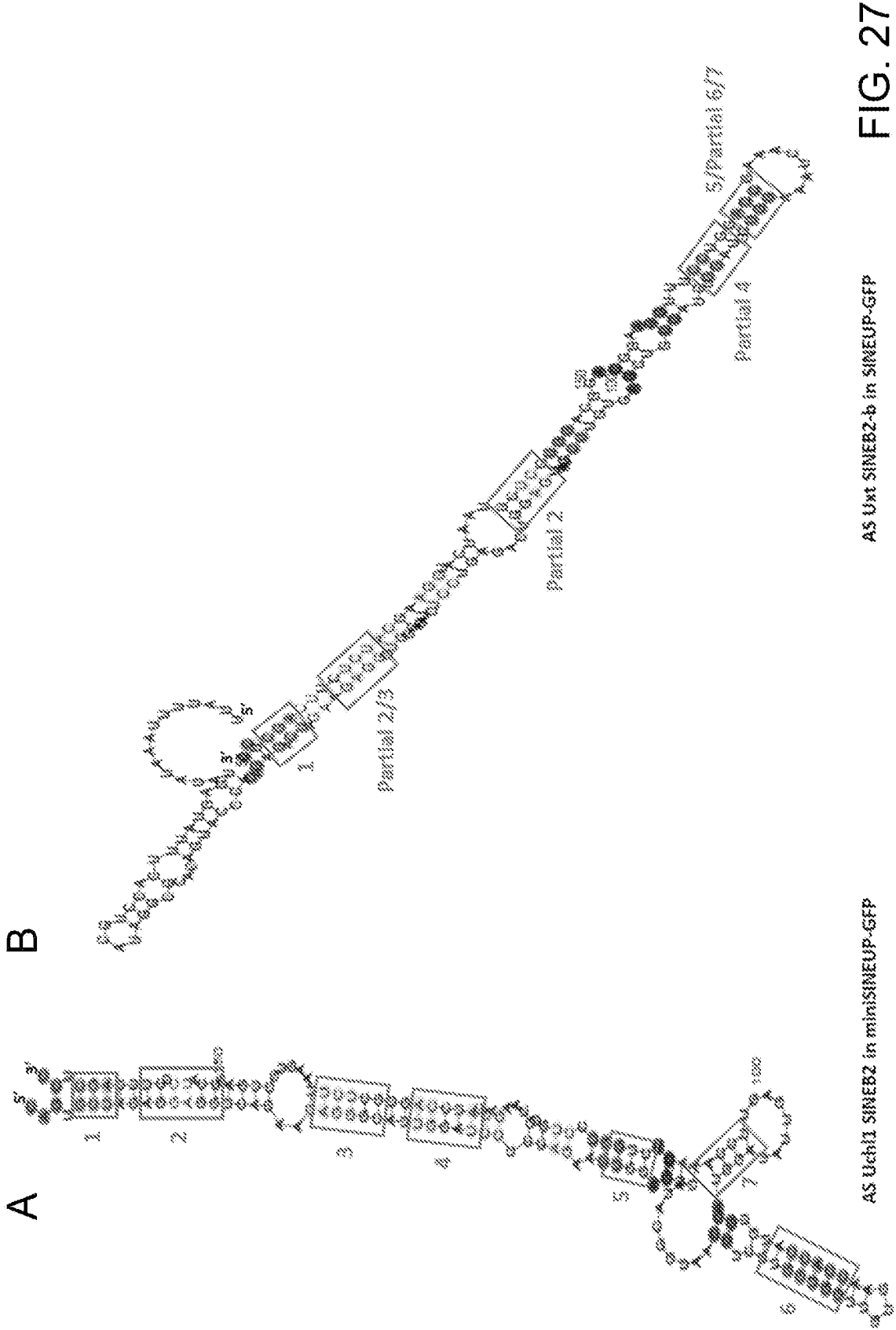


FIG. 26



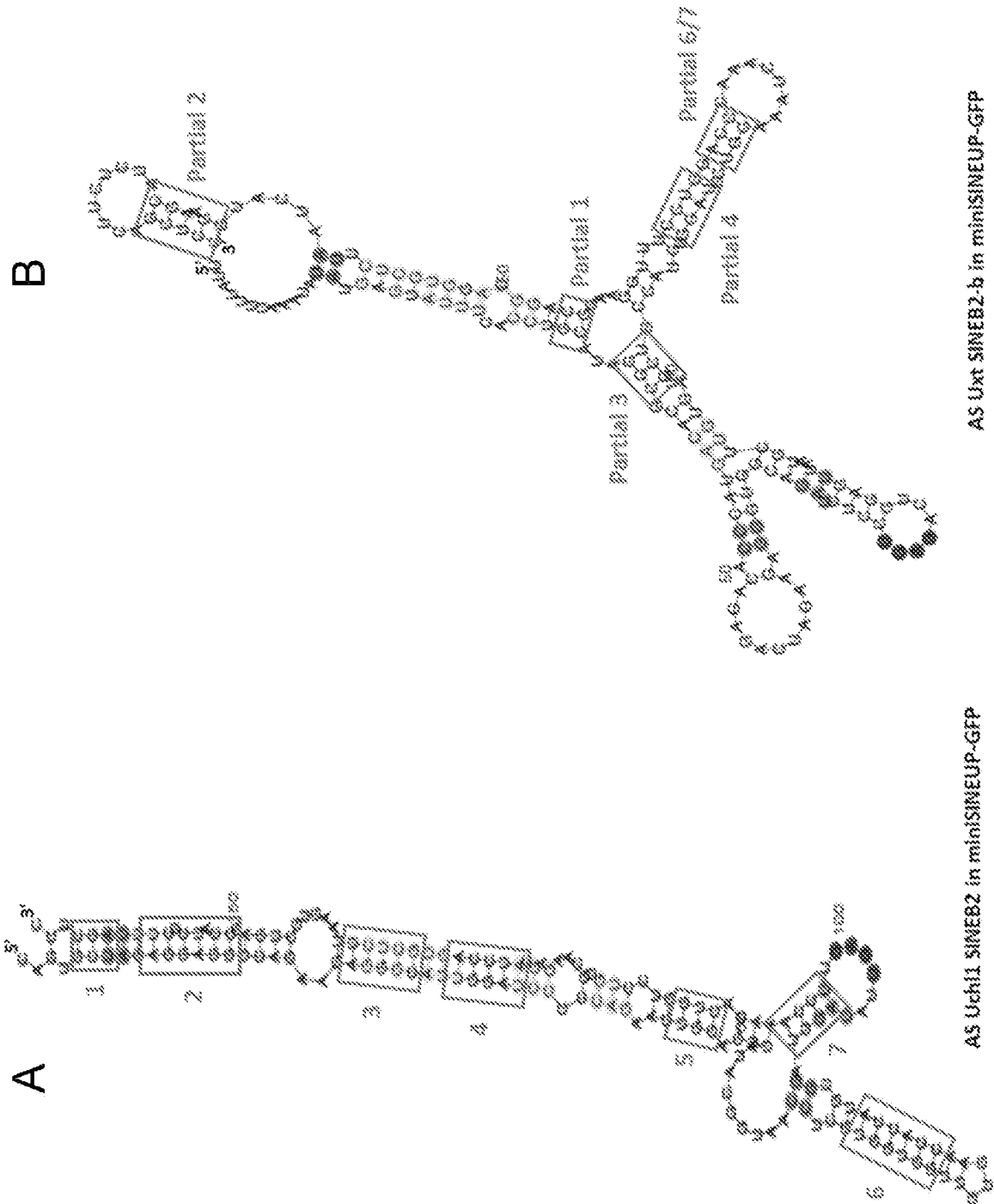


FIG. 28

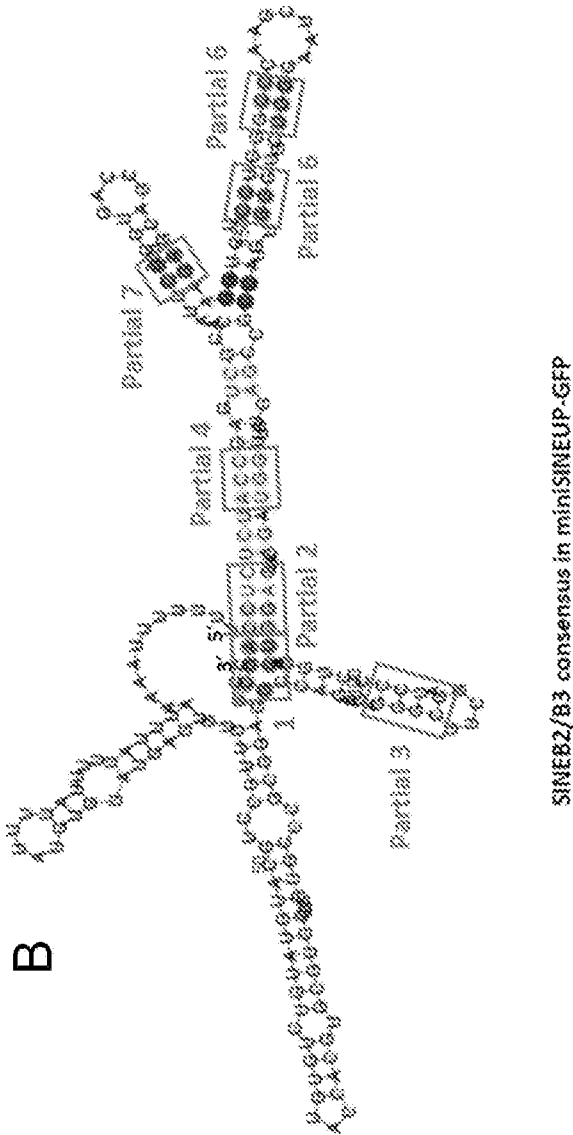
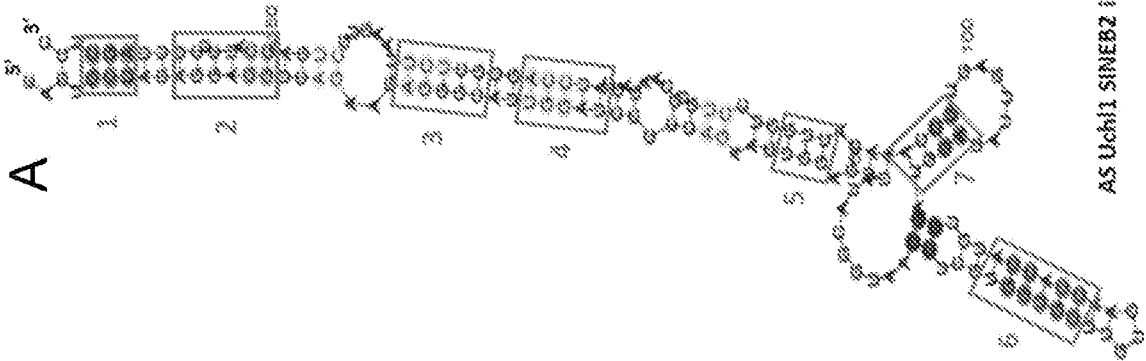


FIG. 29

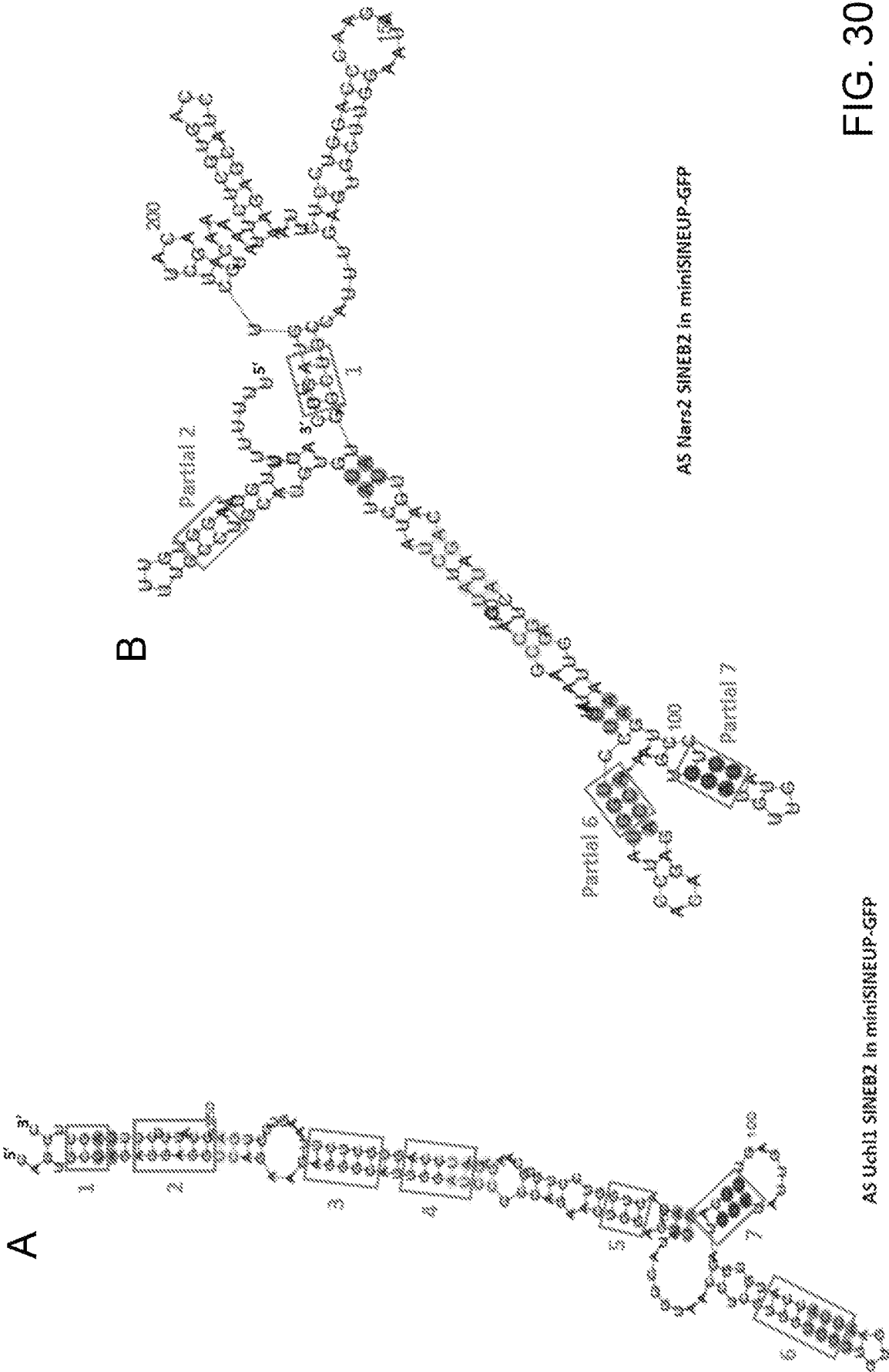


FIG. 30

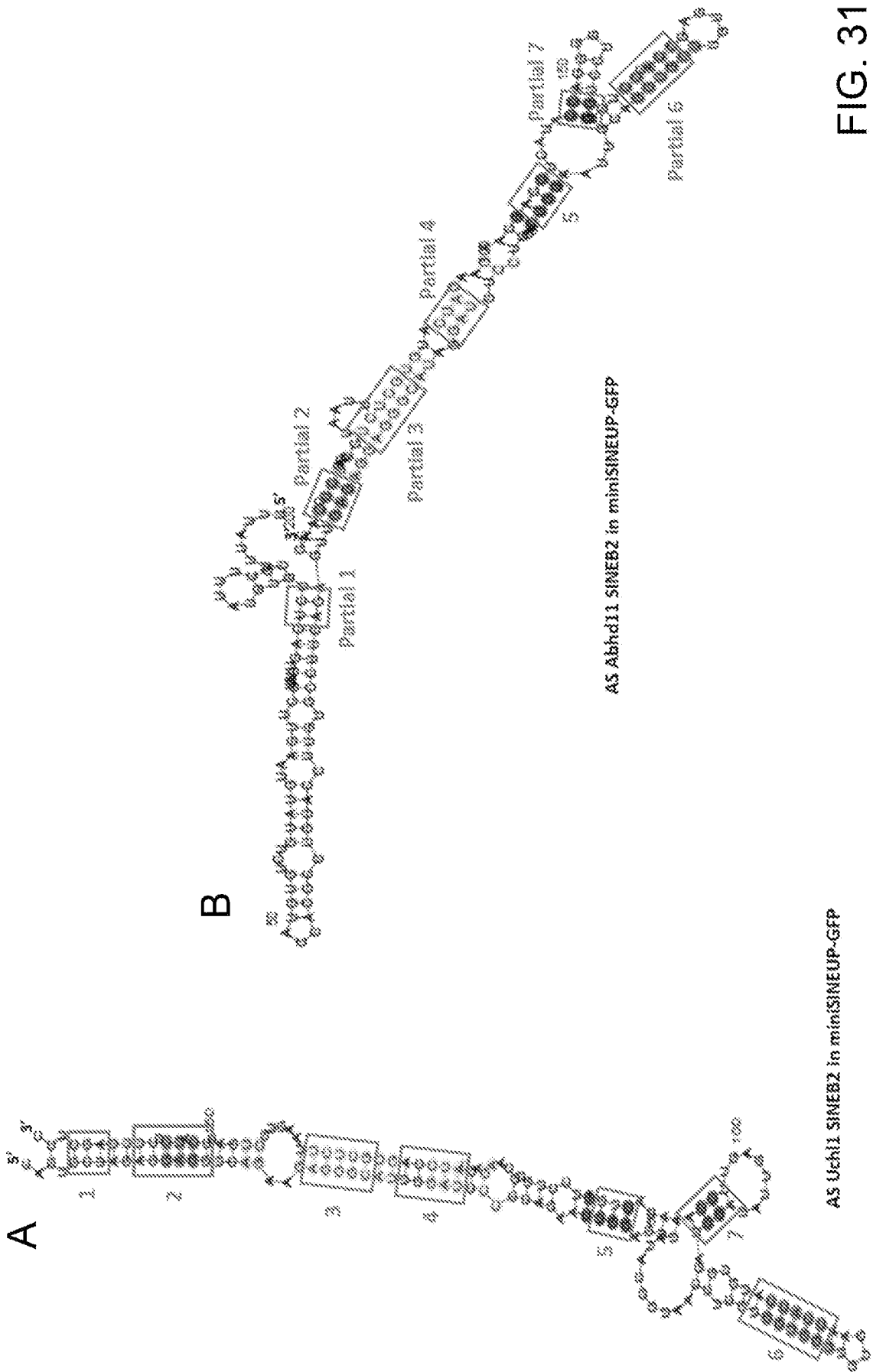
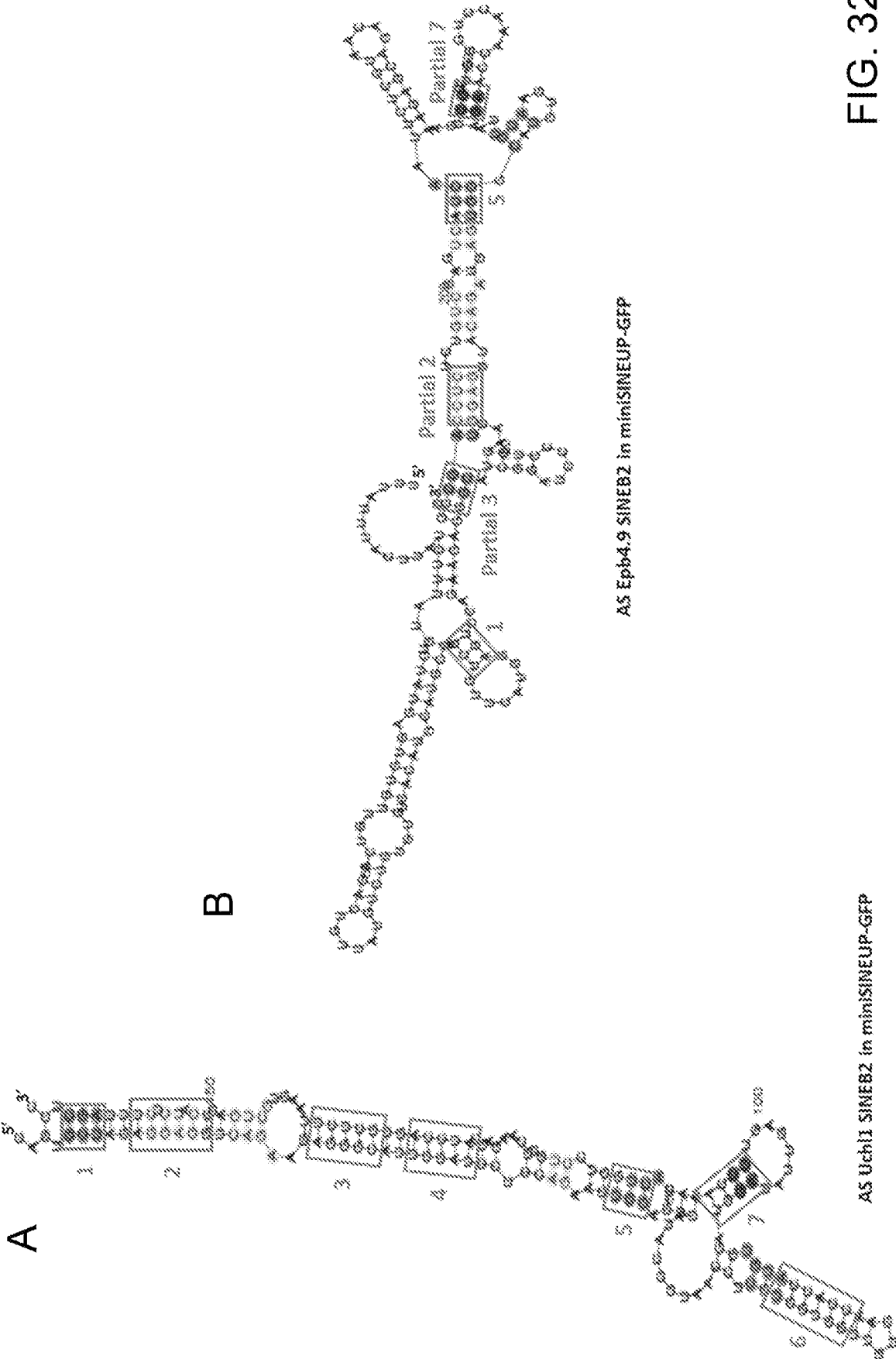


FIG. 31



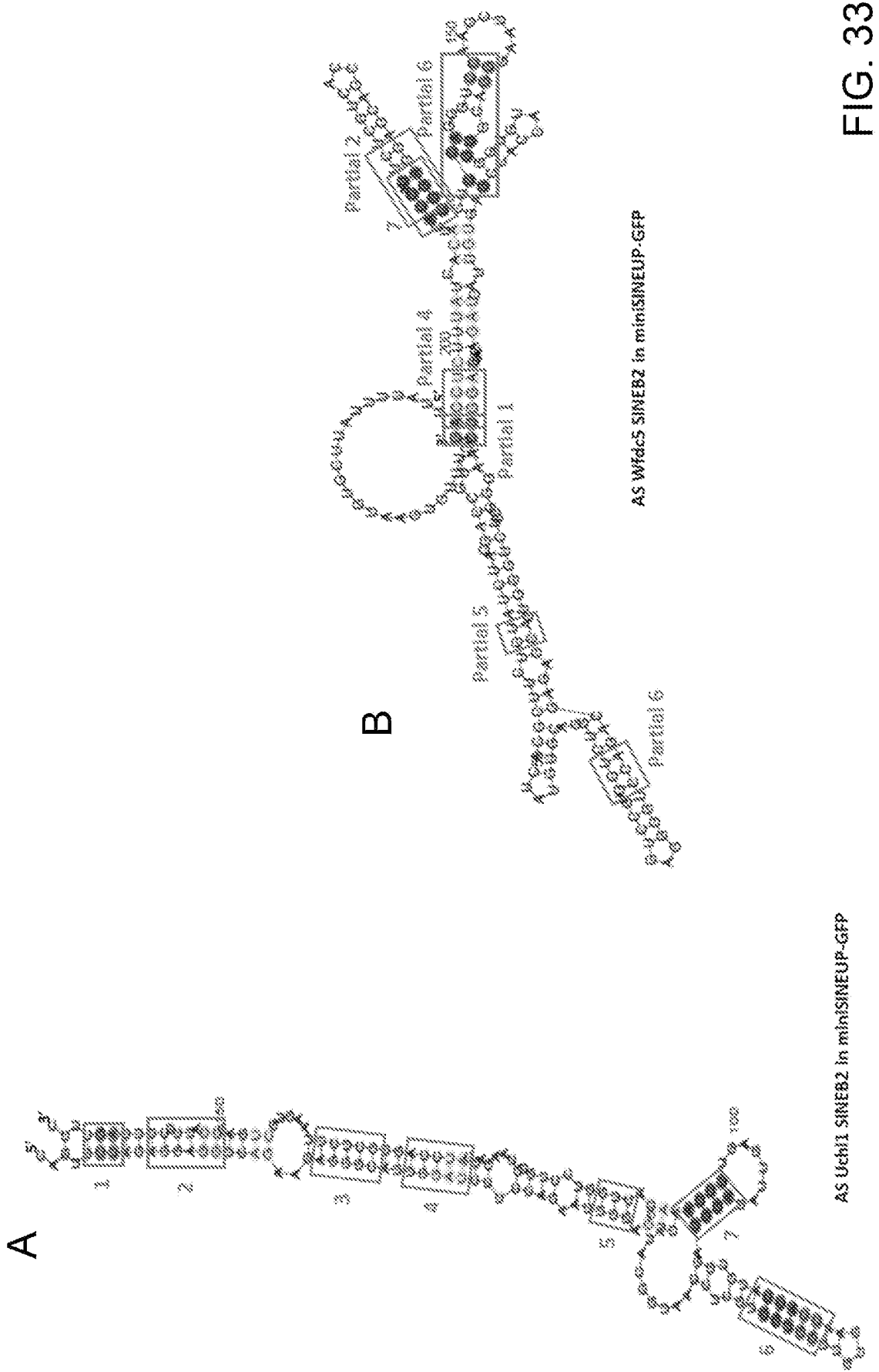
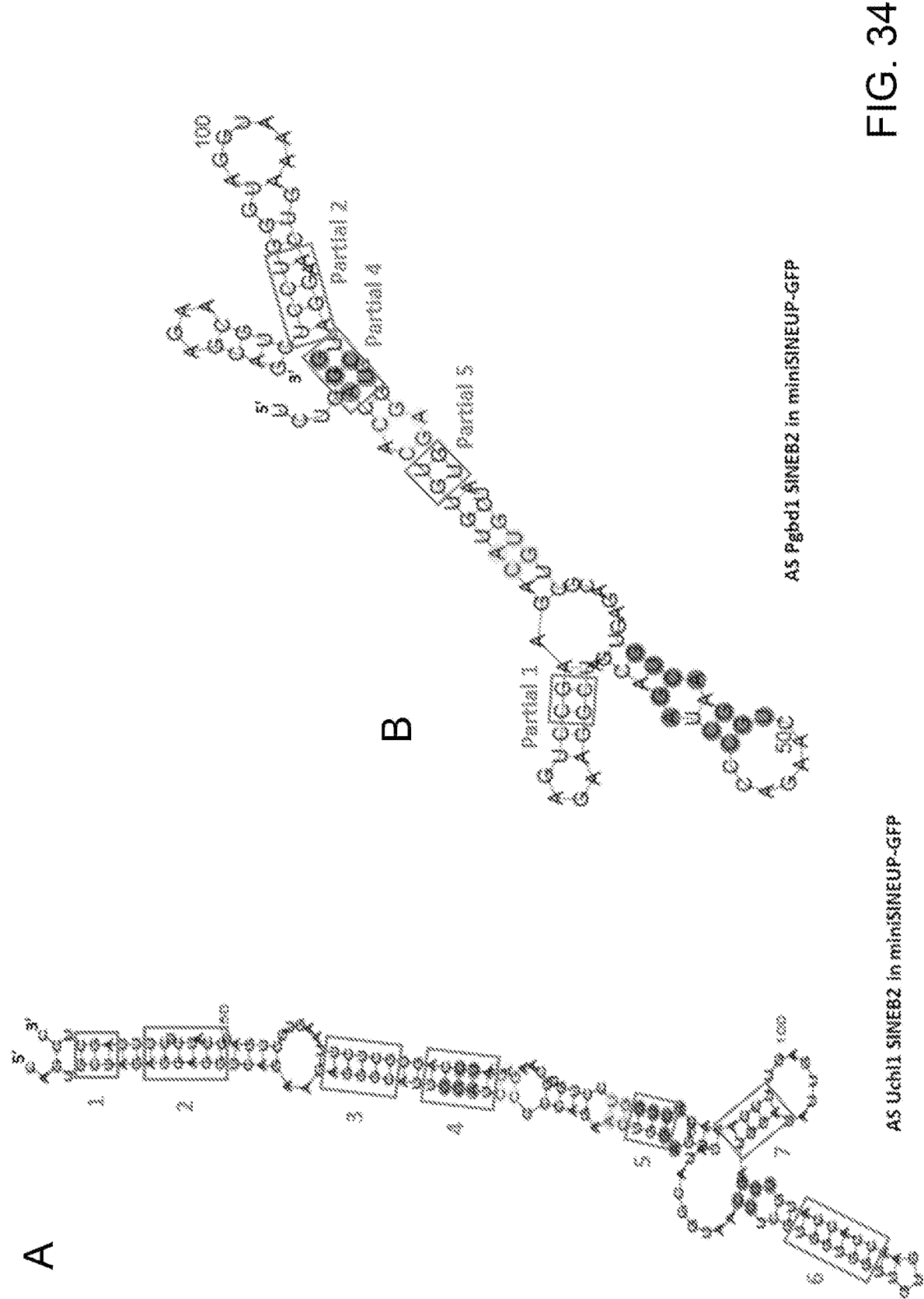


FIG. 33



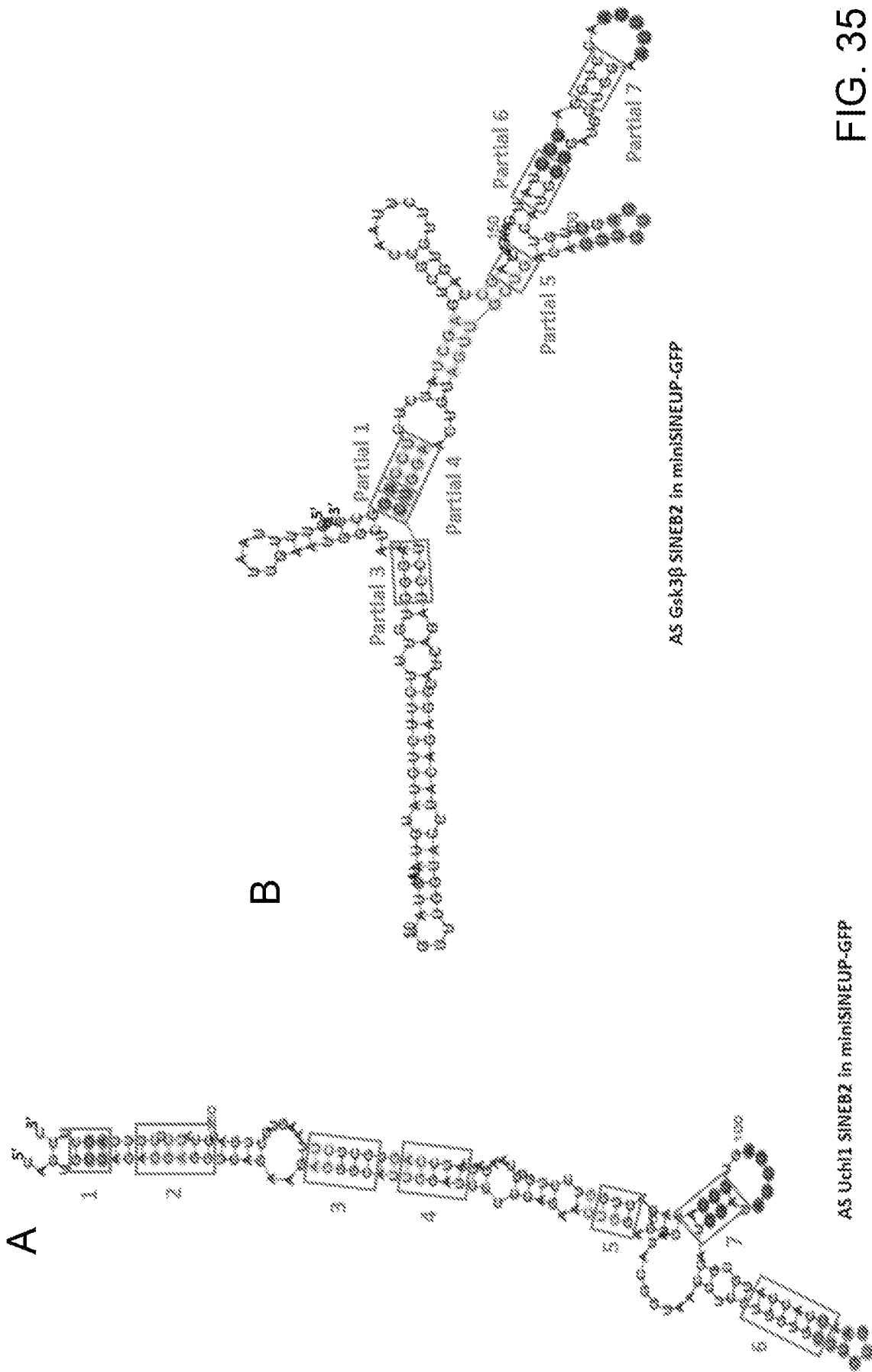
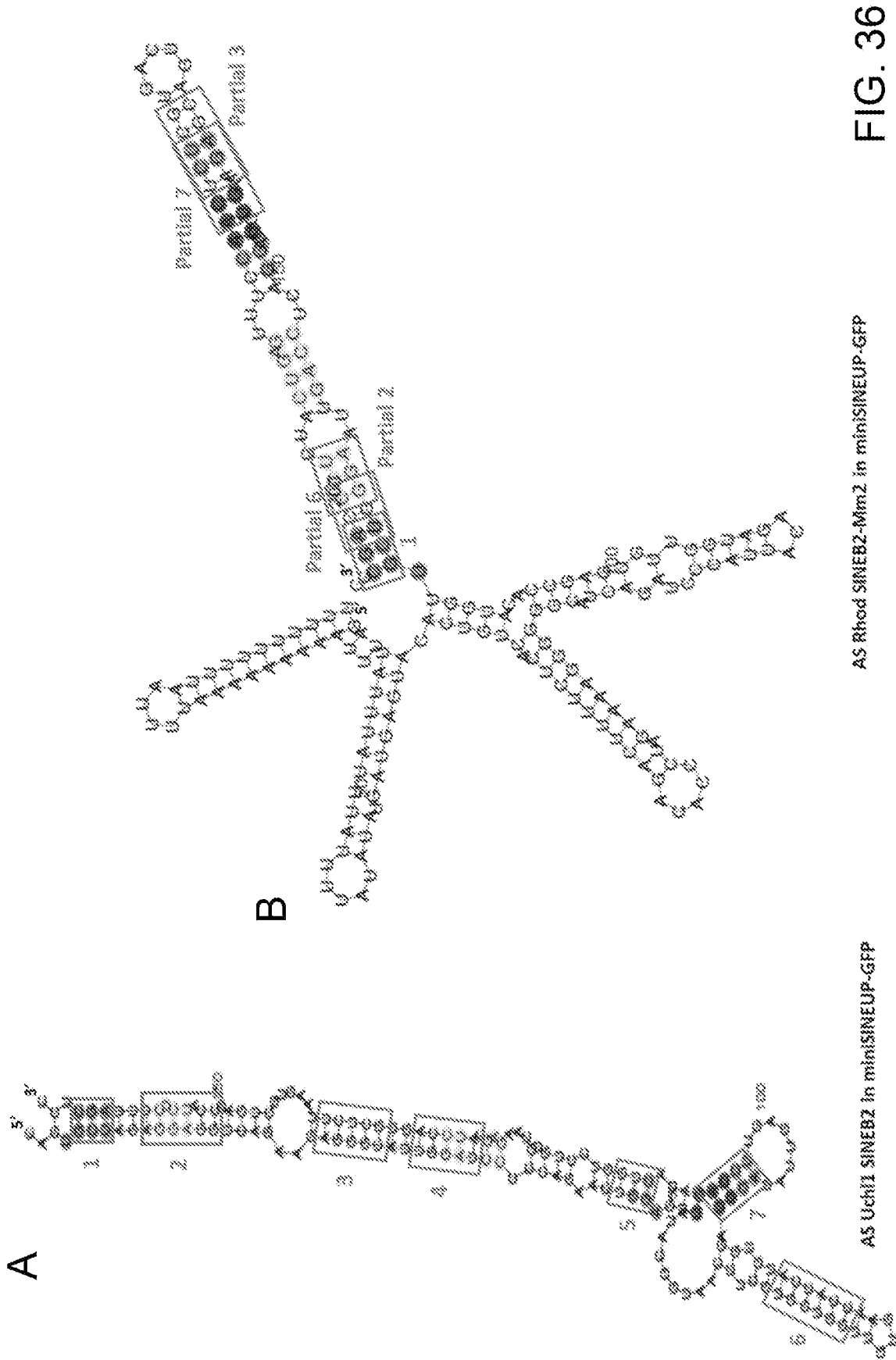


FIG. 35



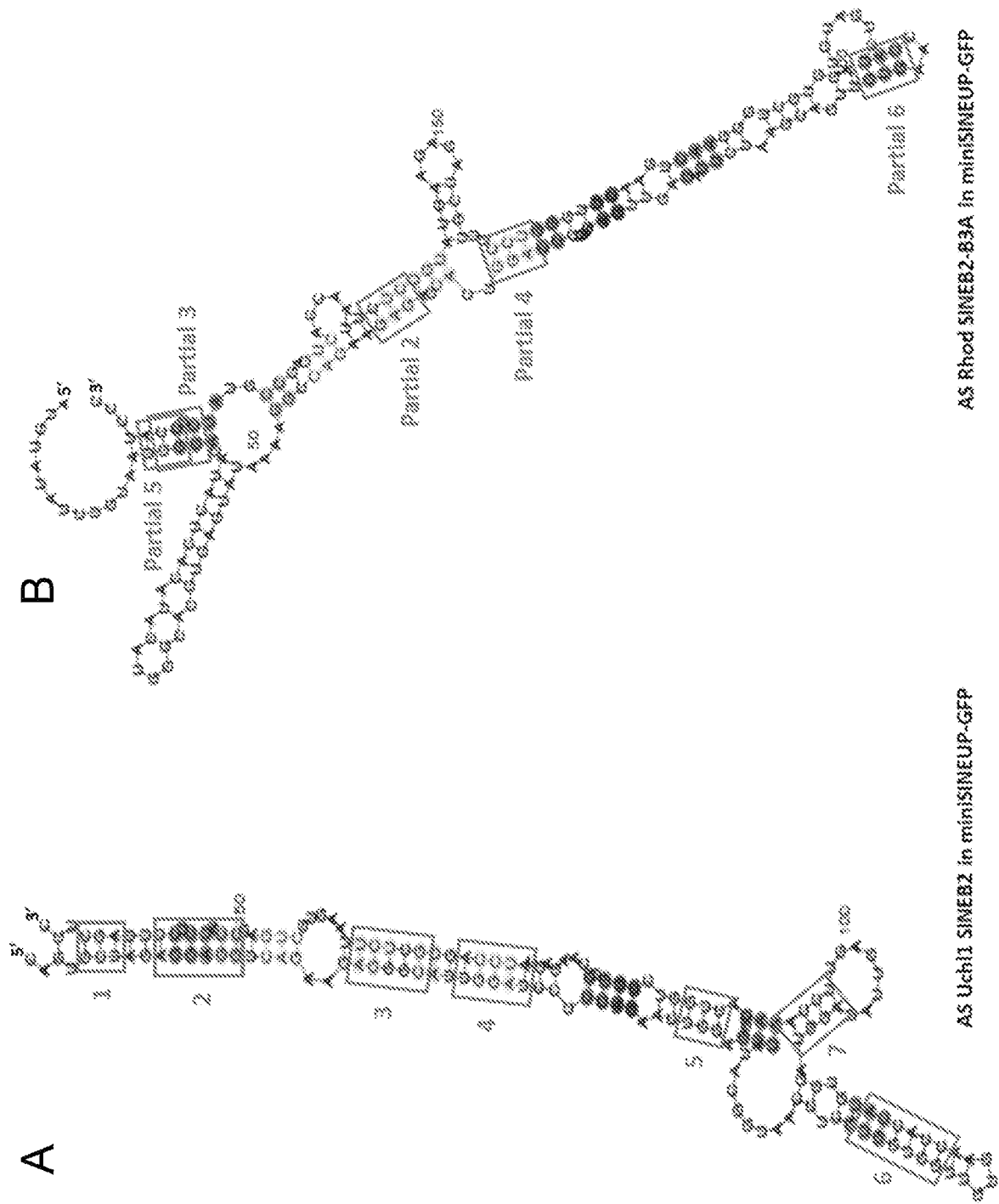
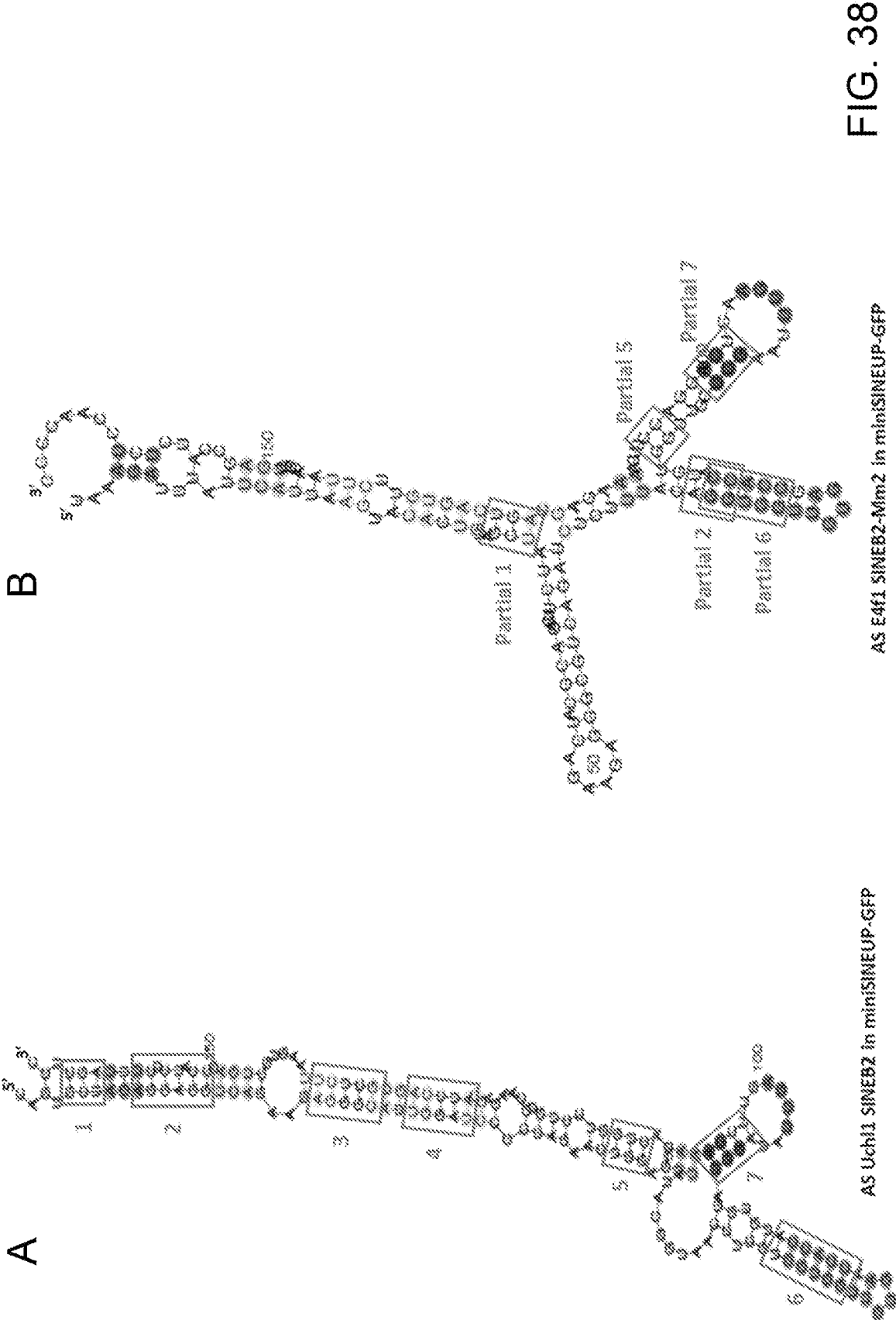


FIG. 37



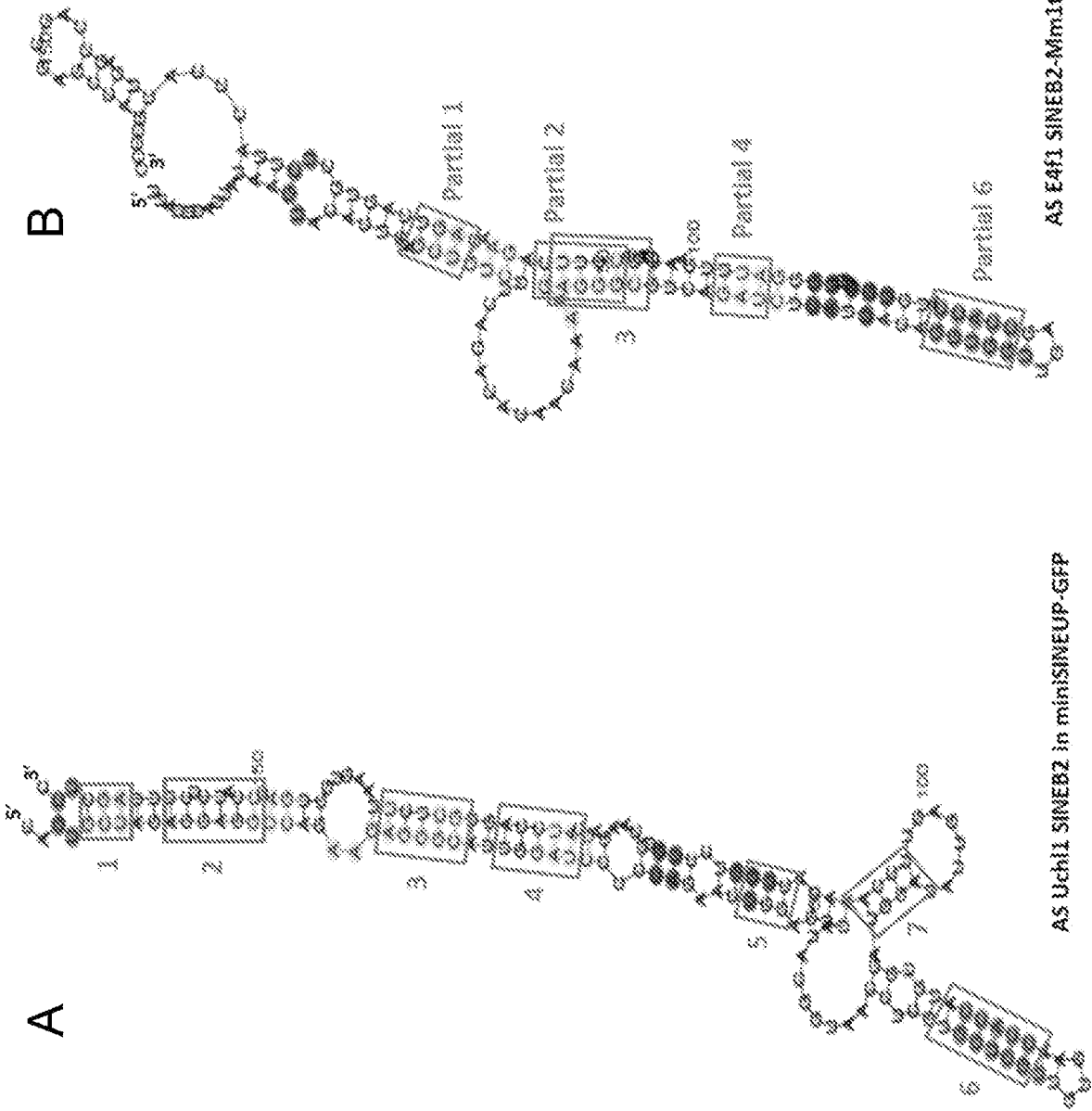


FIG. 39

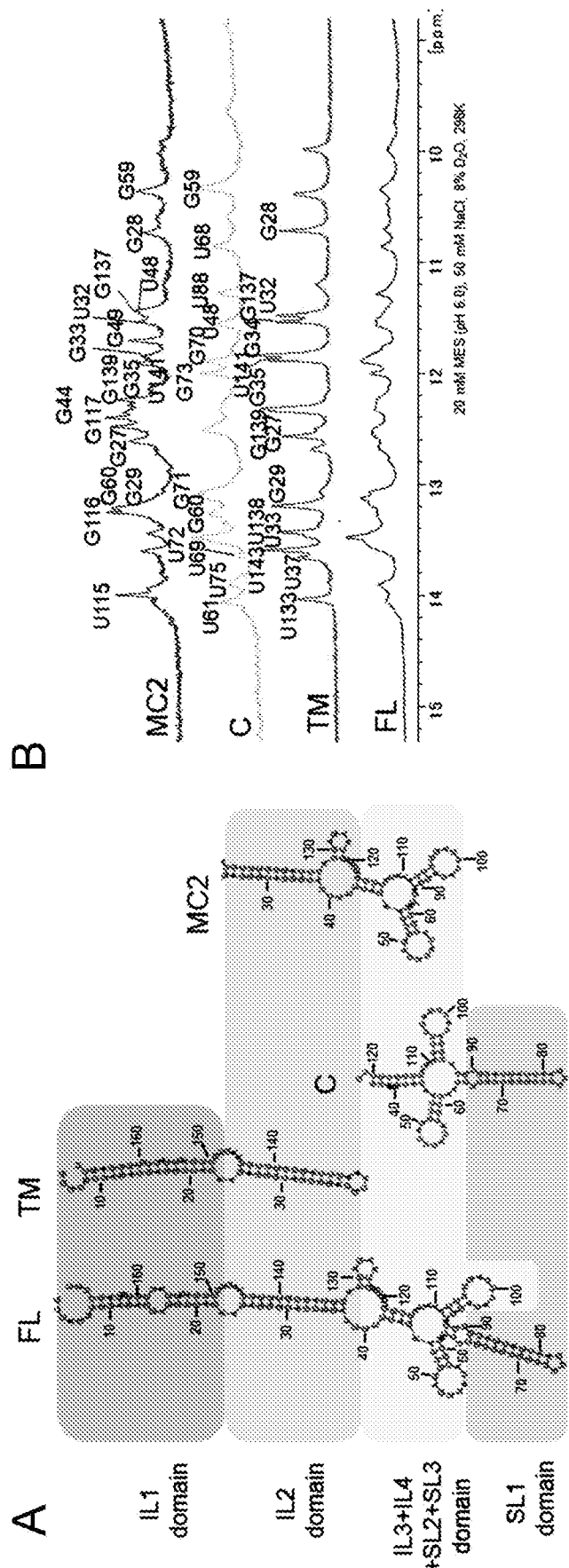


FIG. 40

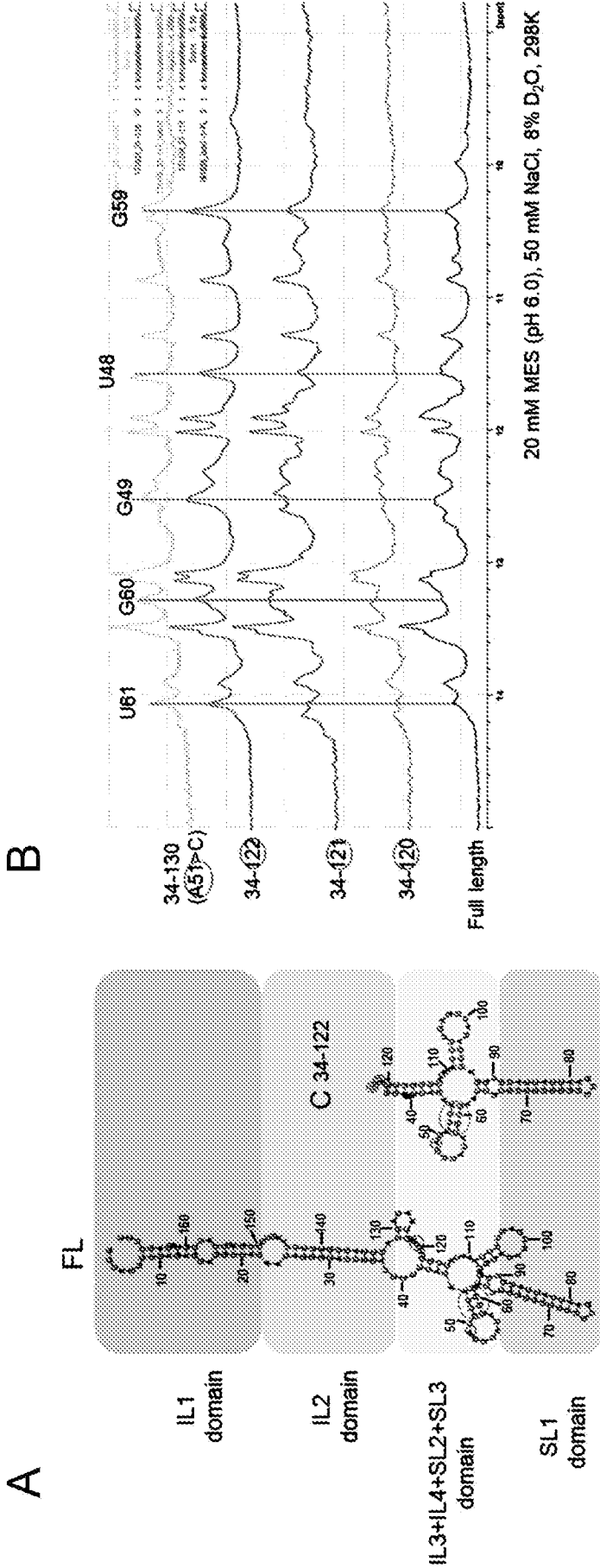


FIG. 41

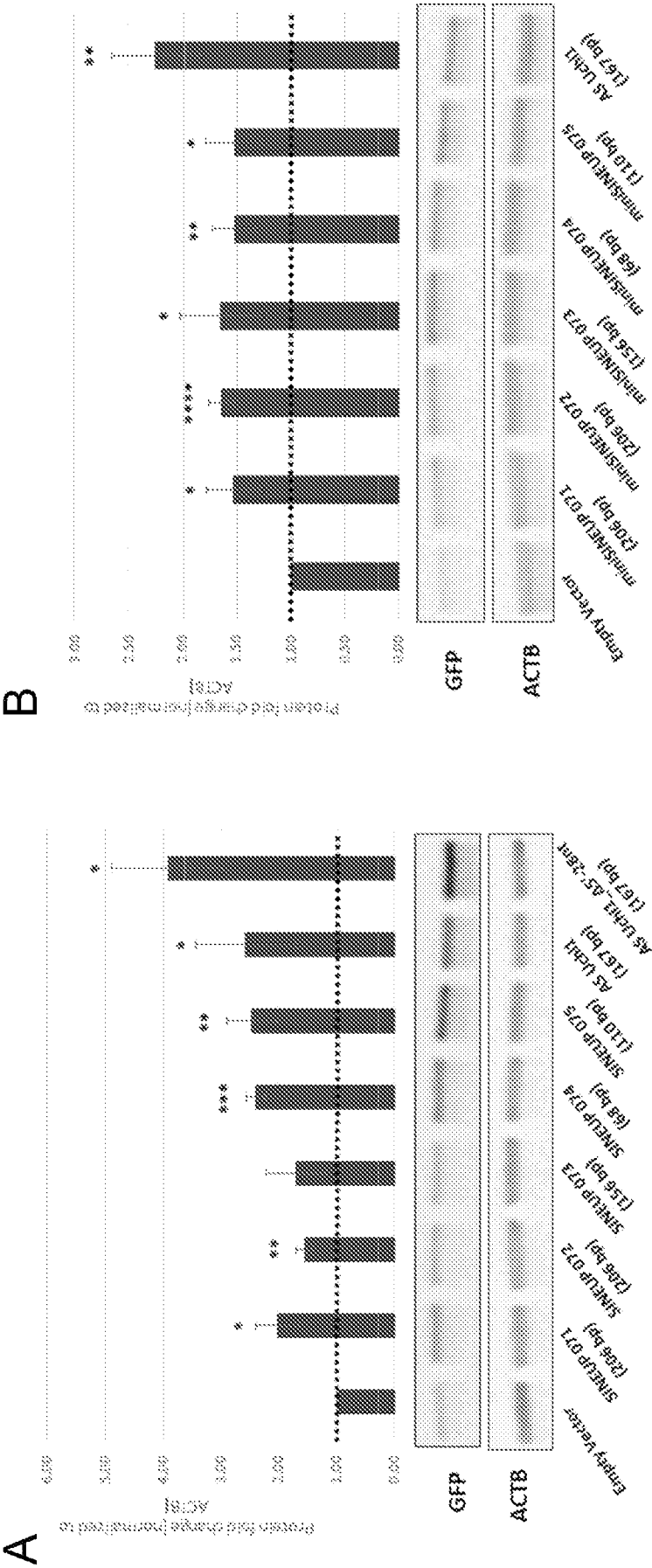
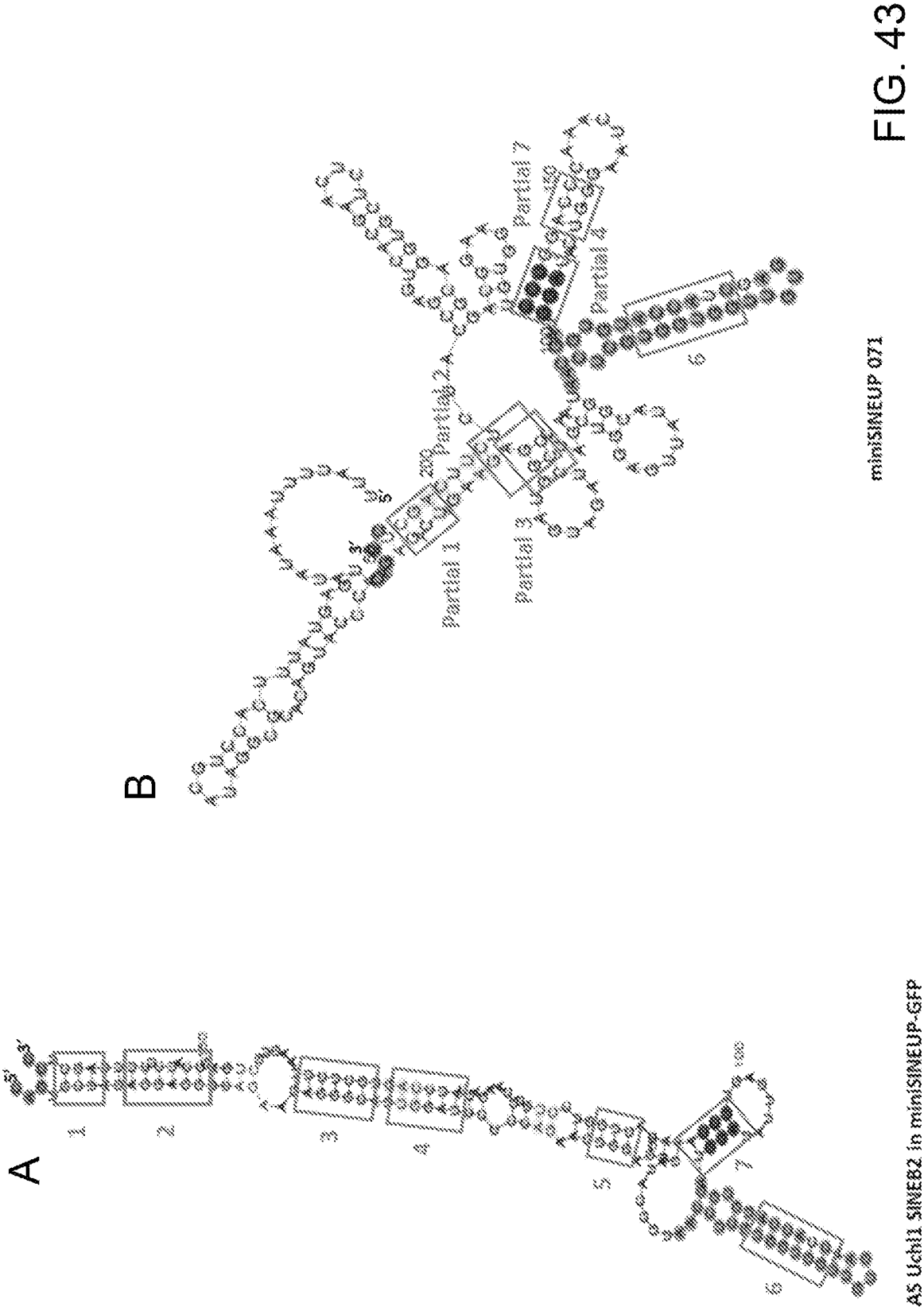


FIG. 42



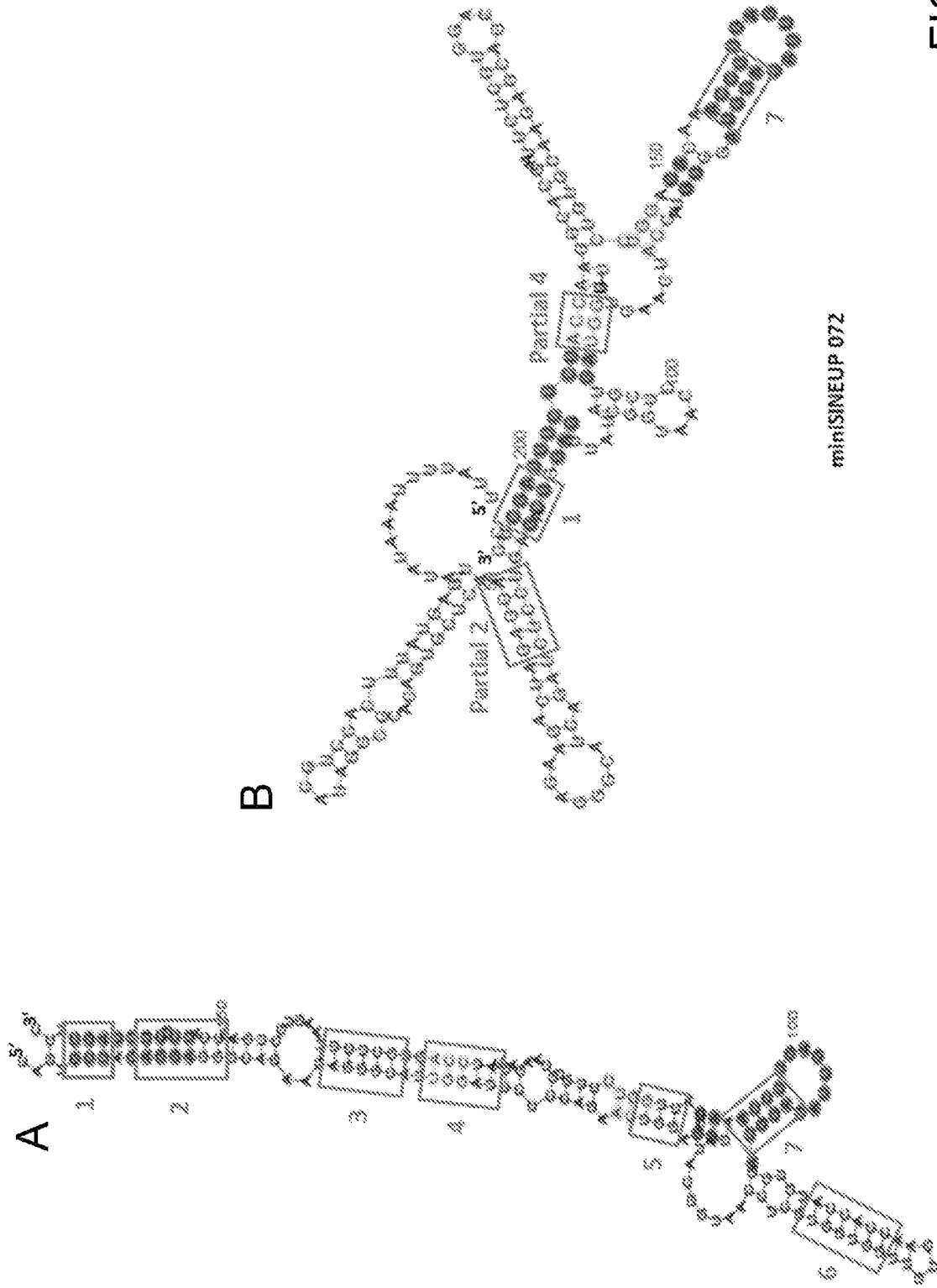


FIG. 44

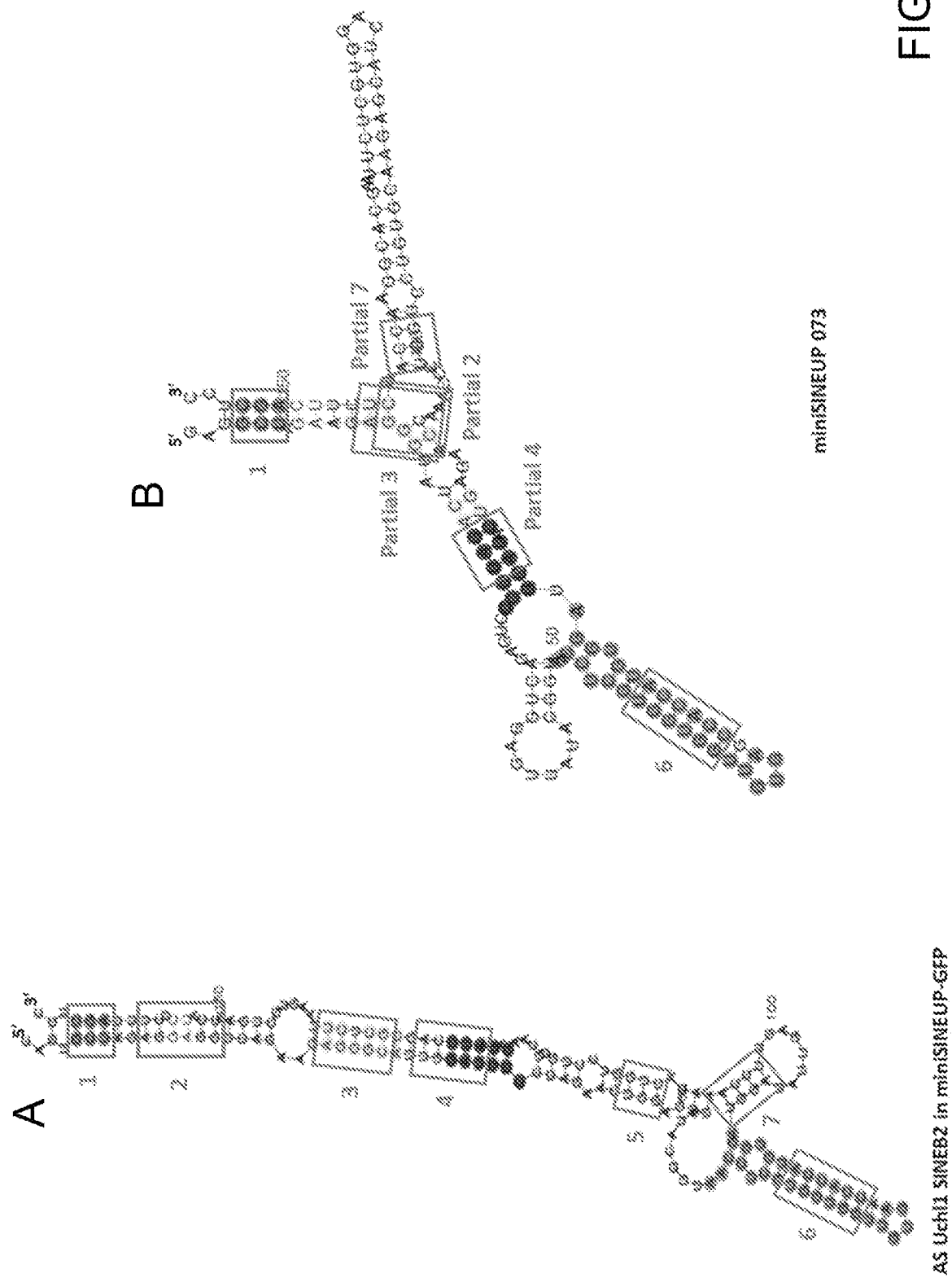


FIG. 45

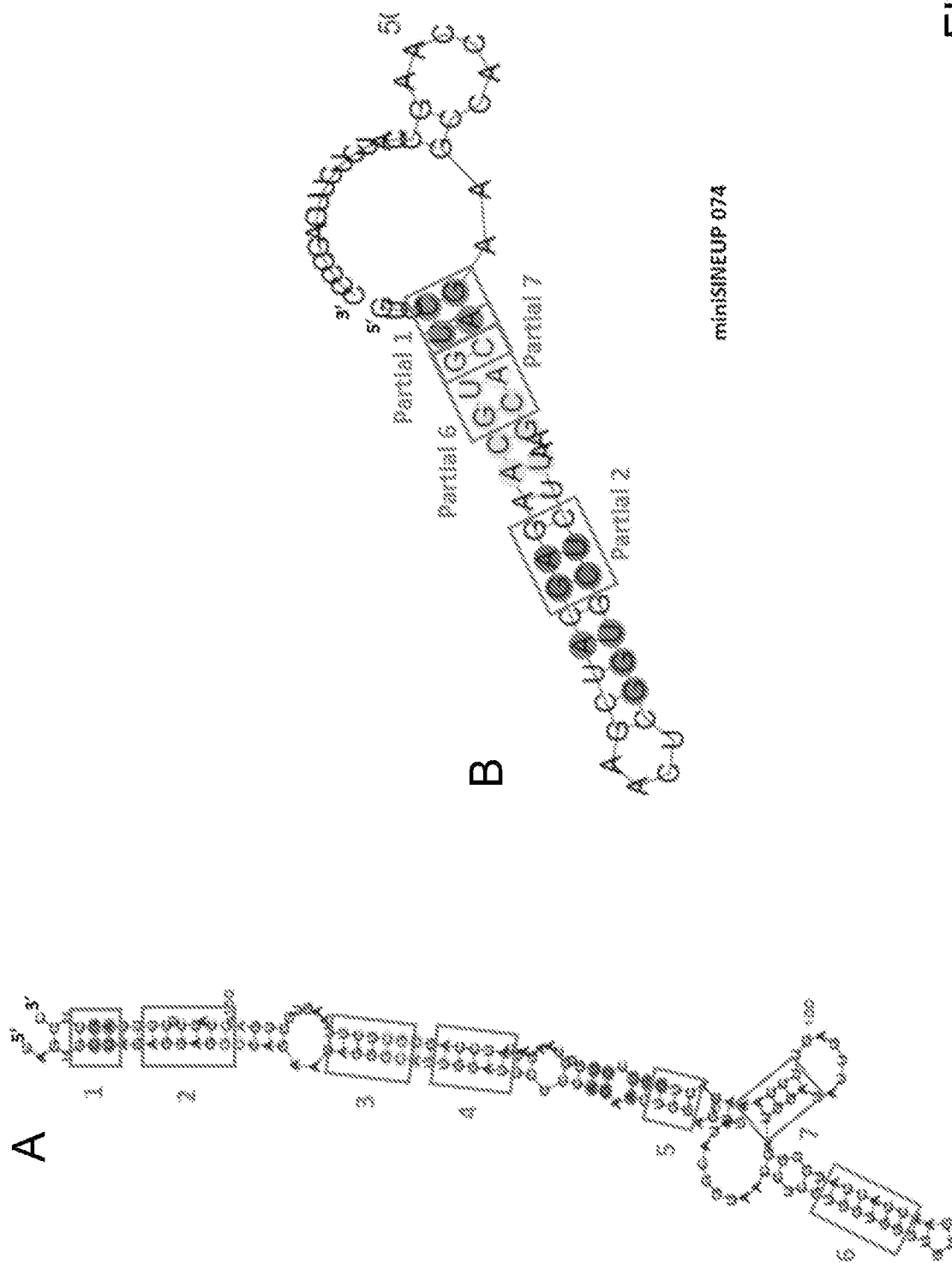


FIG. 46

AS Uch11 SWE82 in miniSWEUP-GFP

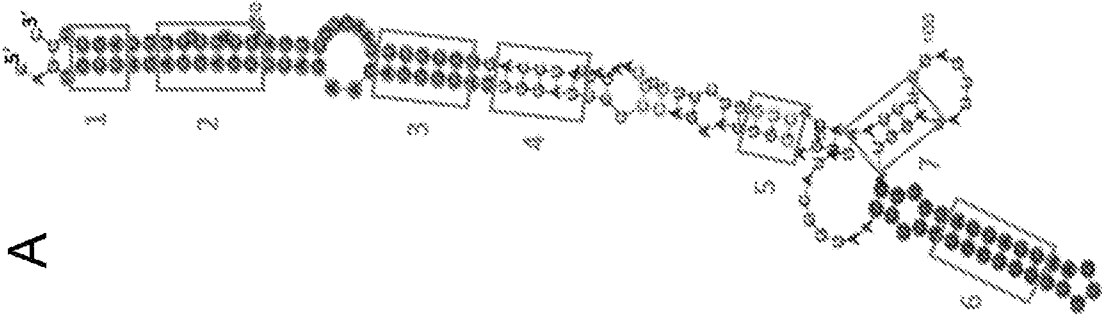
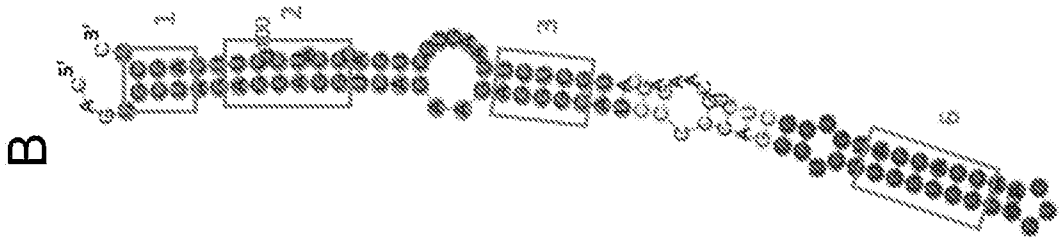


FIG. 47

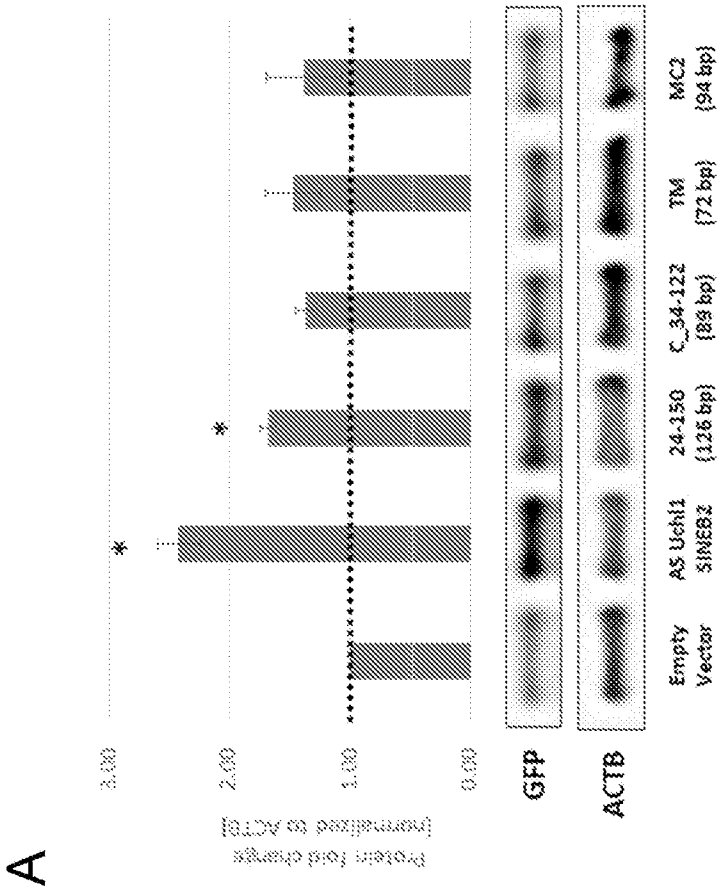


FIG. 48

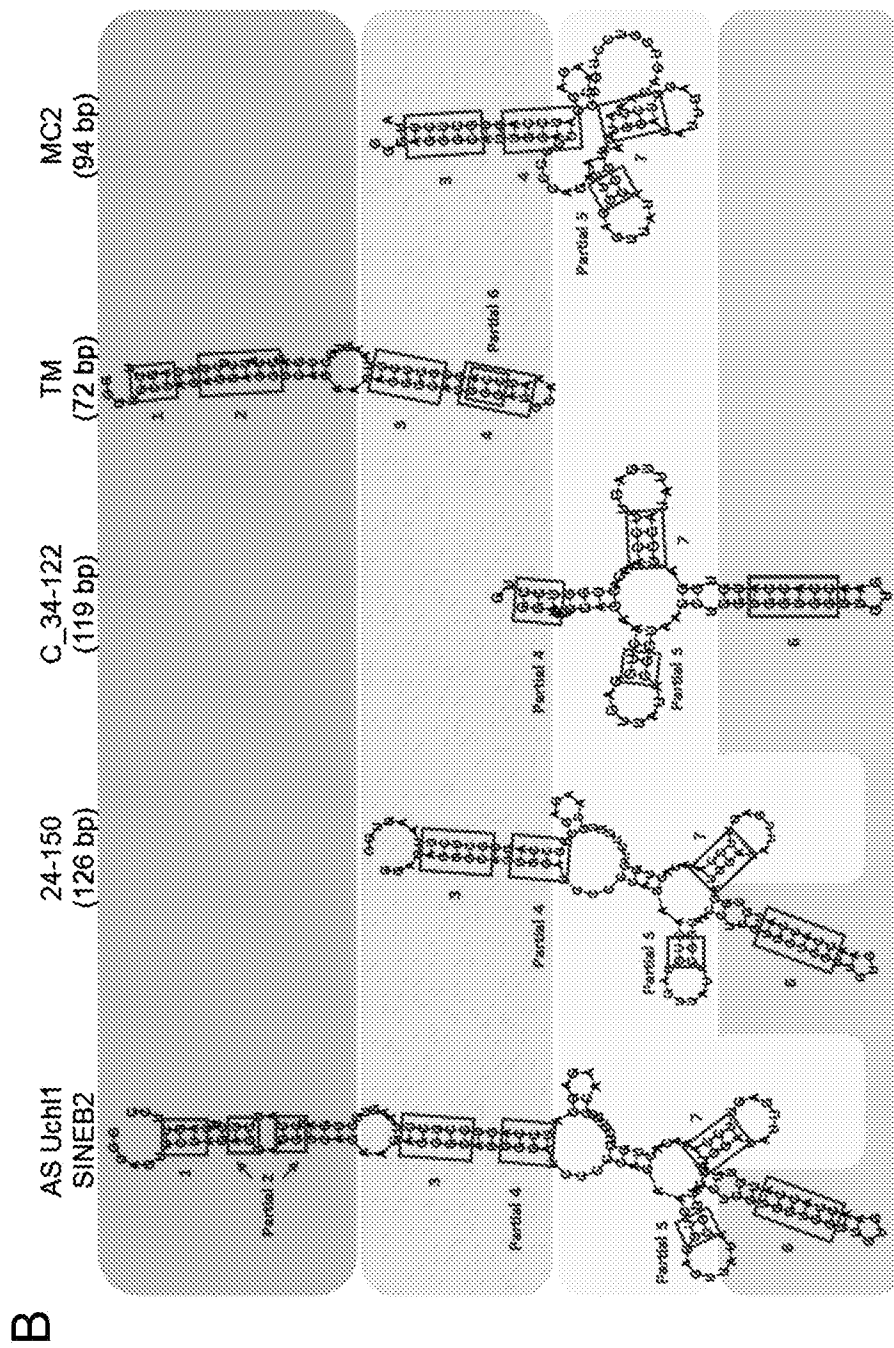


FIG. 48

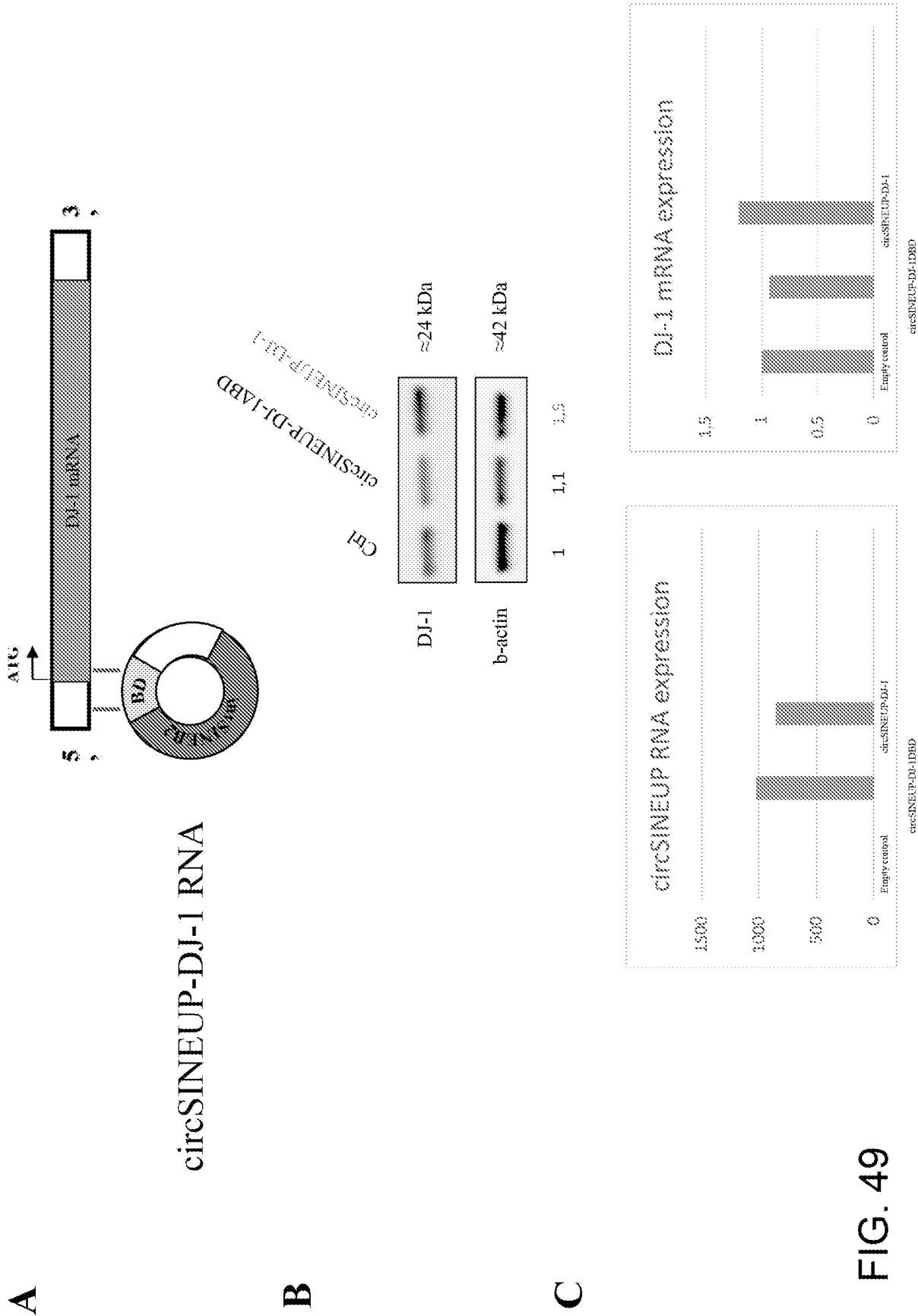


FIG. 49

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2019/050914

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N15/67 C12N15/113
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, COMPENDEX, Sequence Search, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/133947 A1 (RIKEN [JP]; INTERNAT SCHOOL FOR ADVANCED STUDIES [IT]; CARNINCI PIERO) 4 October 2012 (2012-10-04) cited in the application the whole document page 17, lines 12-22; page 5, par. 3 Fig. 2, 3, 7; claims; figures; examples; sequence 1 ----- -/--	1-14, 19-21, 26,27

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

2 April 2019

Date of mailing of the international search report

13/06/2019

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Madruga, Jaime

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2019/050914

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATRUCCO LAURA ET AL: "Engineering mammalian cell factories with SINEUP noncoding RNAs to improve translation of secreted proteins", GENE, ELSEVIER, AMSTERDAM, NL, vol. 569, no. 2, 2 June 2015 (2015-06-02), pages 287-293, XP029247971, ISSN: 0378-1119, DOI: 10.1016/J.GENE.2015.05.070 the whole document -----	1-14, 19-21, 26,27
X	WO 2016/070853 A1 (UNIV TSINGHUA [CN]) 12 May 2016 (2016-05-12) the whole document claim 4; figure 1; sequence 61 -----	1-14, 19-21, 26,27
A	GOSUKE HAYASHI ET AL: "Activation of prokaryotic translation by antisense oligonucleotides binding to coding region of mRNA", BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 429, no. 1-2, 27 October 2012 (2012-10-27), pages 105-110, XP055265704, AMSTERDAM, NL ISSN: 0006-291X, DOI: 10.1016/j.bbrc.2012.10.072 the whole document	1-14, 19-21, 26,27
A	-& Gosuke Hayashi ET AL: "Supplementary Data: Activation of prokaryotic translation by antisense oligonucleotides binding to coding region of mRNA", Biochemical and Biophysical Research Communications, 27 October 2012 (2012-10-27), pages 105-110, XP055483951, United States DOI: 10.1016/j.bbrc.2012.10.072 Retrieved from the Internet: URL:https://ars.els-cdn.com/content/image/1-s2.0-S0006291X1202044X-mmcl.doc [retrieved on 2018-06-13] the whole document ----- -/--	1-14, 19-21, 26,27

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2019/050914

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DEMOOR J M ET AL: "ANTISENSE NUCLEIC ACIDS TARGETED TO THE THYMIDYLATE SYNTHASE (TS) MRNA TRANSLATION START SITE STIMULATE TS GENE TRANSCRIPTION", EXPERIMENTAL CELL RESEARCH, ELSEVIER, AMSTERDAM, NL, vol. 243, 25 August 1998 (1998-08-25), pages 11-21, XP002923758, ISSN: 0014-4827, DOI: 10.1006/EXCR.1998.4059 abstract pages 17-18 the whole document -----	1-14, 19-21, 26,27
A	S ZUCHELLI ET AL: "SINEUPs: A new class of natural and synthetic antisense long non-coding RNAs that activate translation", RNA BIOLOGY, vol. 12, no. 8, 3 August 2015 (2015-08-03), pages 771-779, XP055386212, US ISSN: 1547-6286, DOI: 10.1080/15476286.2015.1060395 the whole document -----	1-14, 19-21, 26,27
A	SILVIA ZUCHELLI ET AL: "SINEUPs are modular antisense long non-coding RNAs that increase synthesis of target proteins in cells", FRONTIERS IN CELLULAR NEUROSCIENCE, vol. 9, 13 May 2015 (2015-05-13), XP055573857, CH ISSN: 1662-5102, DOI: 10.3389/fncel.2015.00174 the whole document -----	1-14, 19-21, 26,27
A	ALEKS SCHEIN ET AL: "Identification of antisense long noncoding RNAs that function as SINEUPs in human cells", SCIENTIFIC REPORTS, vol. 6, no. 1, 20 September 2016 (2016-09-20), XP055573834, DOI: 10.1038/srep33605 the whole document ----- -/--	1-14, 19-21, 26,27

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2019/050914

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	-& ALEKS SCHEIN ET AL: "Supplementary Data: Identification of antisense long noncoding RNAs that function as SINEUPs in human cells", SCIENTIFIC REPORTS, vol. 6, no. 1, 20 September 2016 (2016-09-20), XP055573845, DOI: 10.1038/srep33605 the whole document figures S4, S5	1-14, 19-21, 26,27
X,P	----- PETER PODBEVSEK ET AL: "Structural determinants of the SINE B2 element embedded in the long non-coding RNA activator of translation AS Uchl1", SCIENTIFIC REPORTS, vol. 8, no. 1, 16 February 2018 (2018-02-16), XP055573827, DOI: 10.1038/s41598-017-14908-6 the whole document -----	1-14, 19-21, 26,27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2019/050914

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-14, 19-21, 26, 27(all partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-14, 19-21, 26, 27(all partially)

A functional nucleic acid molecule comprising: (i) a target binding sequence comprising a sequence reverse complementary to a target mRNA sequence for which protein translation is to be enhanced; and (ii) a regulatory sequence, wherein the regulatory sequence has a two-dimensional structure comprising a first stem-loop domain (SL-1) comprising a stem with 8 to 12 paired nucleotides and a loop comprising 5 to 9 nucleotides; A composition comprising such functional nucleic acid molecule; wherein the regulatory sequence comprises a sequence with at least 50% identity to SEQ ID NO: 15.

2-24. claims: 1-27(partially)

As invention 1, but wherein the regulatory sequence comprises a sequence with at least 50% identity to SEQ ID NO: 7, 12, 16-23, 65-77, respectively.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IB2019/050914

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012133947	A1	04-10-2012	EP 2691522 A1 05-02-2014
			JP 2014514921 A 26-06-2014
			JP 2017169573 A 28-09-2017
			US 2014107187 A1 17-04-2014
			WO 2012133947 A1 04-10-2012

WO 2016070853	A1	12-05-2016	CN 105624156 A 01-06-2016
			WO 2016070853 A1 12-05-2016
