Engineering REST-specific synthetic PUF proteins to control neuronal gene expression: a combined experimental and computational study

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SUPPLEMENTARY TABLE AND FIGURE LEGENDS

Figure S1. REST 3'UTR RNA sequence (714 bp, corresponding to bp 3554-4266 of REST mRNA, NCBI #NM_011263.2). *RESTRNA8* is highlighted in red, *RESTRNA16* spans the sequence of *RESTRNA8* and flanking ribonucleotides highlighted in bold, marked by the black rectangle. The sequence of *RESTRNA16-2.0* is in dark blue.

Figure S2. Characterization of the PUF16rest-2.0 construct. (**A**) PUF16rest-2.0 with its own target sequence *RESTRNA16-2.0*. Panel description as in Figure 1. (**B**) HEK293T cells were transfected with a plasmid coding PUF16rest-2.0, and protein expression was analyzed by western blotting using anti-Flag antibodies and antibodies for the housekeeping gene gapdh. (**C**) Confocal images of HEK293T cells transfected with the indicated construct were processed for indirect immunofluorescence using anti-Flag antibodies (red) to detect PUF constructs and Hoechst (blue) to visualize cell nuclei. The overlay image reveals the cytosolic localization of the construct. Scale bar: 10 µm. (**D**) Increasing concentrations of PUF16rest-2.0 were incubated with R16 and R2.0 sequences (0-10 µM).

Figure S3. (**A,B**) Non-bonded interactions energies and distances for the R8-N1 interface in the three eight-repeat constructs. Even though no mutations were involved in this particular interface, the PUF8wt:*RESTRNA8* system showed signs of stress in both analysis. (**C-F**) Distances and non-bonded interactions energies for the mutation sites R3-N14 (C,D), R4-N13 (E,F) and R14-N3 interfaces (G,H) in the three sixteen-repeat PUF proteins. Again, the PUF16wt:*RESTRNA16* systems shows higher instability than the other two systems, particularly in the R4-N13 interface where a base-flipping event is maintained for the major part of the simulation.

Figure S4. Purification of PUF constructs**.** Cytosolic extracts of HEK293T cells transfected with the indicated constructs were incubated with magnetic beads conjugated to anti-Flag antibodies, eluted with 3xFlag peptides, analyzed by SDS-PAGE on a 10% polyacrylamide gel and subsequently silver-stained.

Figure S5. Evaluation of binding specificity of PUF8rest-ns and PUF16rest-ns. (**A**) EMSA analysis of PUF8rest-ns (0-1-2-5-10 µM) incubated with biotinylated *NRE* (N) and *RESTRNA8* (R8) ribonucleotide sequences. (**B**) PUF16rest-ns (0-1-2-5-10 µM) was incubated with biotinylated *2XNRE* (2N), and *RESTRNA16* (R16). No specific RNA-protein complexes were detected, under any of the experimental conditions tested.

Figure S6. Crosslink RNA immunoprecipitation (CLIP) of the REST-PUF constructs mutated in the stacking residues for endogenous Gapdh mRNA**.** (**A**) CLIP was performed with agarose A beads, anti-Flag and IgG antibodies on N2a cell lysates transfected with the indicated constructs. Gapdh values were normalized against the input value and plotted as percent specific precipitation. IP values were not statistically significant vs IgG values, for all samples. Student's *t*-test, $p>0.05$. Data are shown as means ± S.D. of n=3 independent experiments. (**B**) Immunoblot of immunoprecipitated complexes revealed with anti-flag antibodies. Input (INP) represents 20% of the cell extract before immunoprecipitation for all the indicated constructs. NC, negative control: cells transfected with the empty 'Flag' vector.

Figure S7. Modulation of the stability of REST mRNA through fusion of REST-specific PUF proteins and GLD2. (**A**) PUF-GLD2 fusion proteins are expressed in HEK293T cells. HEK293T were transfected with the indicated constructs (n.t: non transfected cells). Protein expression was analyzed by western blotting using anti-Flag antibodies and antibodies for the housekeeping protein β-tubulin. (**B**) PUF8wt-GLD2, but not PUF8rest-sY-GLD2, increases the stability of its target mRNA. HEK293T cells were co-transfected with the indicated constructs and the corresponding reporter plasmid (see Materials and Methods). Forty-eight hrs after transfection, luciferase activity was measured. The

Renilla / Luciferase ratio was first calculated for each sample, and data (means ± sem) were subsequently normalized to the activity of the non-transfected control (n=3 independent experiments). (**C**) PUF8rest-sY does not increase the stability of endogenous REST mRNA. N2a cells were transfected with the indicated constructs. After 48 h, REST mRNA levels were quantified via qRT-PCR analysis. Gapdh was used as control housekeeping gene; data were normalized to REST mRNA levels in cells transfected with the empty Flag vector, set to 1. Data (means ± SD) of n=2 independent experiments.

Supplementary table 1: MicroRNA sites prediction analysis

In order to evaluate the microRNA sites within mouse REST mRNA 3'UTR (NCBI #NM_011263.2) we queried the microRNA database mirdb. The analysis predicted 66 miRNA sites. We analysed 42 microRNA prediction sites out of 66 according to the predicted score > 80, which is considered by the algorithm the most likely to be real. From our study, none of 42 microRNA prediction sites overlapped with PUF target sequences in the REST mRNA.

Target Detail	Target Rank	Target Score	miRNA Name	Gene Symbol	Gene Description
Details	1	99	mmu-miR-8118	Rest	RE1-silencing transcription factor
Details	2	98	mmu-miR-495-3p	Rest	RE1-silencing transcription factor
Details	3	98	mmu-miR-12200-5p	Rest	RE1-silencing transcription factor
Details	4	98	mmu-miR-1192	Rest	RE1-silencing transcription factor
Details	5	97	mmu-miR-883b-3p	Rest	RE1-silencing transcription factor
Details	6	97	mmu-miR-883a-3p	Rest	RE1-silencing transcription factor
Details	7	97	mmu-miR-29b-3p	Rest	RE1-silencing transcription factor
Details	8	97	mmu-miR-29c-3p	Rest	RE1-silencing transcription factor
Details	9	97	mmu-miR-29a-3p	Rest	RE1-silencing transcription factor
Details	10	96	mmu-miR-217-5p	Rest	RE1-silencing transcription factor
Details	11	95	mmu-miR-677-3p	Rest	RE1-silencing transcription factor
Details	12	92	mmu-miR-20a-5p	Rest	RE1-silencing transcription factor
Details	13	92	mmu-miR-6383	Rest	RE1-silencing transcription factor
Details	14	92	mmu-miR-106b-5p	Rest	RE1-silencing transcription factor
Details	15	92	mmu-miR-106a-5p	Rest	RE1-silencing transcription factor
Details	16	92	mmu-miR-93-5p	Rest	RE1-silencing transcription factor
Details	17	92	mmu-miR-20b-5p	Rest	RE1-silencing transcription factor
Details	18	92	mmu-miR-17-5p	Rest	RE1-silencing transcription factor
Details	19	91	mmu-miR-19b-3p	Rest	RE1-silencing transcription factor
Details	20	91	mmu-miR-19a-3p	Rest	RE1-silencing transcription factor
Details	21	90	mmu-miR-291a-3p	Rest	RE1-silencing transcription factor
Details	22	90	mmu-miR-7001-3p	Rest	RE1-silencing transcription factor
Details	23	90	mmu-miR-302d-3p	Rest	RE1-silencing transcription factor
Details	24	90	mmu-miR-294-3p	Rest	RE1-silencing transcription factor
Details	25	90	mmu-miR-669j	Rest	RE1-silencing transcription factor
Details	26	90	mmu-miR-669i	Rest	RE1-silencing transcription factor
Details	27	90	mmu-miR-295-3p	Rest	RE1-silencing transcription factor
Details	28	90	mmu-miR-302b-3p	Rest	RE1-silencing transcription factor
Details	29	90	mmu-miR-302a-3p	Rest	RE1-silencing transcription factor
Details	30	89	mmu-miR-26b-5p	Rest	RE1-silencing transcription factor
Details	31	89	mmu-miR-7649-3p	Rest	RE1-silencing transcription factor
Details	32	89	mmu-miR-3473c	Rest	RE1-silencing transcription factor

Details	33	89	mmu-miR-26a-5p	Rest	RE1-silencing transcription factor
Details	34	88	mmu-miR-448-3p	Rest	RE1-silencing transcription factor
Details	35	87	mmu-miR-6397	Rest	RE1-silencing transcription factor
Details	36	86	mmu-miR-5619-3p	Rest	RE1-silencing transcription factor
Details	37	85	mmu-miR-6896-3p	Rest	RE1-silencing transcription factor
Details	38	84	mmu-miR-153-3p	Rest	RE1-silencing transcription factor
Details	39	83	mmu-miR-186-5p	Rest	RE1-silencing transcription factor
Details	40	82	mmu-miR-130a-5p	Rest	RE1-silencing transcription factor
Details	41	82	mmu-miR-7119-3p	Rest	RE1-silencing transcription factor
Details	42	81	mmu-miR-335-3p	Rest	RE1-silencing transcription factor
Details	43	79	mmu-miR-6343	Rest	RE1-silencing transcription factor
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Details	45	75	mmu-miR-6360	Rest	RE1-silencing transcription factor
Details	46	74	mmu-miR-32-3p	Rest	RE1-silencing transcription factor
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Details	52	64	mmu-miR-7116-3p	Rest	RE1-silencing transcription factor
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Details	63	52	mmu-miR-7b-3p	Rest	RE1-silencing transcription factor
Details	64	51	mmu-miR-6986-3p	Rest	RE1-silencing transcription factor
Details	65	51	mmu-miR-7050-3p	Rest	RE1-silencing transcription factor
Details	66	50	mmu-miR-6371	Rest	RE1-silencing transcription factor

SI Materials and Methods

Primers

REST3'UTR	Fw 5'-GCATAAATCTTAGCAAATCCTCGGGAG-3' Rv 5'-GGCAGACAAGGCAAGTGGTGTG-3'
Gapdh3'UTR	Fw 5'-GAAACCCTGGACCACCCAC-3' Rv 5'-GTGGGTGCAGCGAACTTTATTG-3'
REST CDS	Fw 5'-TTCACATTTATACGGGCGTTC-3' Rv 5'-CCTGCAGCAAGTGCAACTAC-3'
Gapdh CDS	Fw 5'-AGGTCGGTGTGAACGGATTTG-3' Rv 5'-TGTAGACCATGTAGTTGAGGTCA-3'
PUF8rest-ns	Fw 5'-CAAATTTGCAAatAATGTTGTGcAGAAGTGTGTTACTC-3' Rv 5'-GAGTAACACACTTCTgCACAACATTatTTGCAAATTTG-3'
PUF8rest-s	Fw 5'-GCATAAATTTGCCAATtACGTGGTTCAAAAATGTG-3' Rv 5'-CACATTTTTGAACCACGTAATTGGCAAATTTATGC-3'
PUF8rest-sh	Fw 5'- GCATAAATTTGCCAATCACGTGGTTCAAAAATGTG-3' Rv 5'-CACATTTTTGAACCACGTgATTGGCAAATTTATGC-3'
PUF16rest-ns	Fw Rep3 5'-GGCACTGCAAATGTATGGTAATCGTGTTATTCAGAAAGCCCTGG-3' Rv Rep3 5'-CCAGGGCTTTCTGAATAACACGATTACCATACATTTGCAGTGCC-3' Fw Rep45'-GTGTGAAAGATCAGAATGGCTGTCATGTTGTGCAGAAATG-3' Rv Rep4 5'-CATTTCTGCACAACATGACAGCCATTCTGATCTTTCACAC-3 Fw Rep12 5'-CAAATTTGCAAatAATGTTGTGcAGAAGTGTGTTACTC-3' Rv Rep12 5'-GAGTAACACACTTCTgCACAACATTatTTGCAAATTTG-3' Fw Rep14 5'-GTATGGAAGCTATGTGATTCGTCATGTTCTGGAACATG-3' Rv Rep14 5'-CATGTTCCAGAACATGACGAATCACATAGCTTCCATAC-3'
PUF8NotI	Fw 5'-GCATAAATTTGCCAATAACGTGGTTCAAAAATGTG-3'
PUF8BamHI	Rv 5'-CACATTTTTGAACCACGTTATTGGCAAATTTATGC-3'
PUF16NotI	Fw 5'-CATAGCGGCCGCACCATGGGTCGTAGCCGTCTG-3'
PUF16BamHI	Rv 5'-CATAGGATCCGCCCAGGTCCACGCCATTTTTC-3'

GLD2-FLAG	Fw 5’-CCAGGGATCCACCATGCCATCTCCACCTAC-3’ Rv 5’-CCAGGGATCCTTGAGATACATTTGATGATGCCATC-3'
PUF(GLD2)	Fw 5’-CATTGCGGCCGCACCATGGGTCGTAG-3’ Rv 5’- CATTTCTAGACCCGCCCAGGTCCACGCCATTTTTC-3’
RESTRNA8	Sense 5’-TCGAGTTTATATAGC-3’ Antisense 5’-CGCCGCTATATAAA3-
NRE	Sense 5’-TCGAGTGTATATAGC-3’ Antisense 5’-CGCCGCTATATACAC-3’
RESTRNA16	Sense 5’-TCGAGTGCTTTATATAAATTAGC-3’ Antisense 5’-CGCCGTAATTTATATAAAGCAC-3’
2XNRE	Sense 5’-TCGAGTGTTGTATATAATATAGC-3’ Antisense 5’-CGCCGTATATTATATACAACAC-3’

REST mRNA 3'UTR

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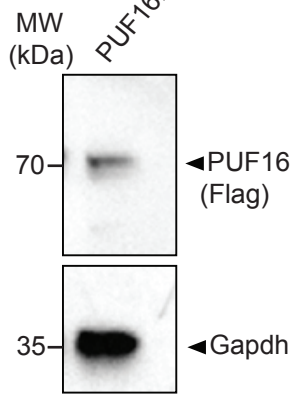
  1  CUGAGCCUCGGCAGAAGCACCGUGCAGACUUUGUGAGCAUGCAAUUUUAA  50
 51  UUUGUAGACAAACGCAAGCUUGCUUUAAUUAGUCUCCAAGGCUGAGUUUU 100
101  CAGUAACAUUCUUUUUCUUAGGACUGUACAUCUAUUUAGUGUUUGUUGCA 150
151  UAAAUCUUAGCAAAUCCUCGGGAGUUAAUGUAAGAGGACAGAUAUGUAAC 200
201  UAGCUCGUGCAGGCAGGUGCAAGGAGAAGGGUAAGAUGGUGGAACACACC 250
251  ACUUGCCUUGUCUGCCUACAACCUGUUGGGUUUUCUUUUCACGGUAGUUC 300
301  CUAAUUUUUAGUUACUUGUUUAGAUCGAUAAAAAUUGGCUUAGUAAAUUA 350
351  CUUGAAGAAUUUGCCUGCUUUAUAUAAAUUAAGUUAGCACUUUACAGUUU 400
401  CUUUAGAGAUGAAAAAAAAGAGAUUUUAAUUGGAGAGAAAUUCUCAACAU 450
451  UGGACAUUGUAUCUGUCCAGGUAAUUGCUUCCUAACUUGCUAUCAAUAUU 500
501  UUGUGUUUAUAUGUUAAUCGUUAUAAAAAGUGAUUUUUGUUUUUUGGGUA 550
551  UUUUUUAUUUUGGUGCUUUUCUGGCUUAAGAUGUUGCACAUGGUUCUUGU 600
601  UUUUGUUUCUUUAACCUAUGCAGUUAAUCUCCCUUCCCCUGAAACAGCGU 650
651  UGUGUUAAAUAGUAACACUAUACAGAUAUAUGCAUGGUUUUUUUUUUUGU 700
701  UUGUUUGUUUGUU                                       713
```

A

R	2XNRE	PUF16wt	RESTRNA16-2.0	PUF16 rest-2.0
16	**U**	**NQ-Y**	**A**	**CQ-R**
15	**G**	**SE-N**	**U**	**NQ-Y**
14	U	NQ-Y	U	
13	**U**	**NQ-Y**	**G**	**SE-N**
12	G	SE-N	G	
11	**U**	**NQ-Y**	**C**	**SR-Y**
10	**A**	**CQ-R**	**U**	**NQ-Y**
9	U	NQ-Y	U	
8	A	CQ-R	A	
7	**U**	**NQ-Y**	**G**	**SE-N**
6	**A**	**CQ-R**	**U**	**NQ-Y**
5	A	CQ-R	A	
4	**U**	**NQ-Y**	**A**	**CQ-R**
3	A	CQ-R	A	
2	U	NQ-Y	U	
1	**A**	**CQ-R**	**U**	**NQ-Y**

B



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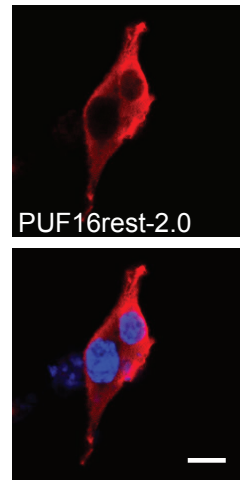

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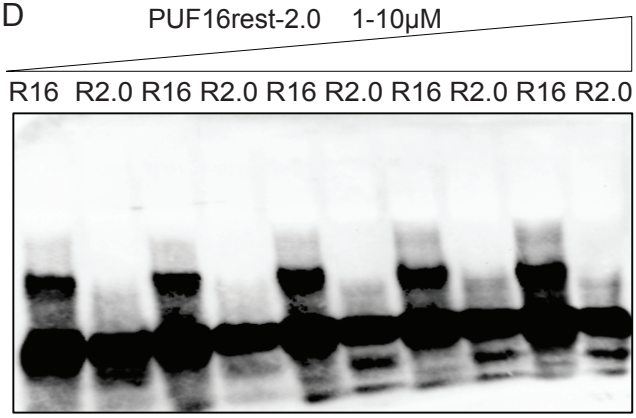

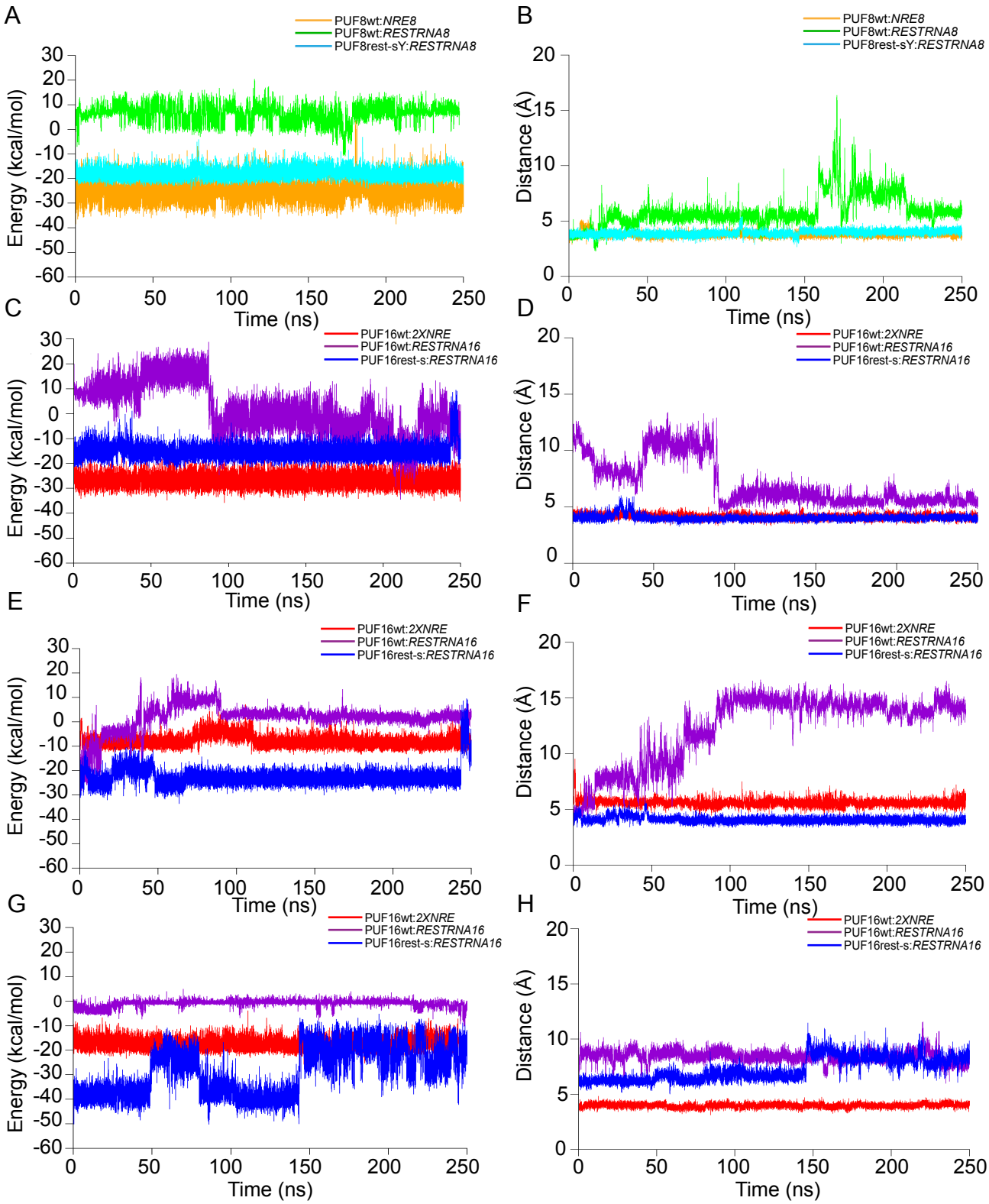
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PUF8wt:RESTRNA8
PUF8rest-sY:RESTRNA8
Energy (kcal/mol)
Time (ns)
B
PUF8wt:NRE8
PUF8wt:RESTRNA8
PUF8rest-sY:RESTRNA8
Distance (Å)
Time (ns)
C
PUF16wt:2XNRE
PUF16wt:RESTRNA16
PUF16rest-s:RESTRNA16
Energy (kcal/mol)
Time (ns)
D
PUF16wt:2XNRE
PUF16wt:RESTRNA16
PUF16rest-s:RESTRNA16
Distance (Å)
Time (ns)
E
PUF16wt:2XNRE
PUF16wt:RESTRNA16
PUF16rest-s:RESTRNA16
Energy (kcal/mol)
Time (ns)
F
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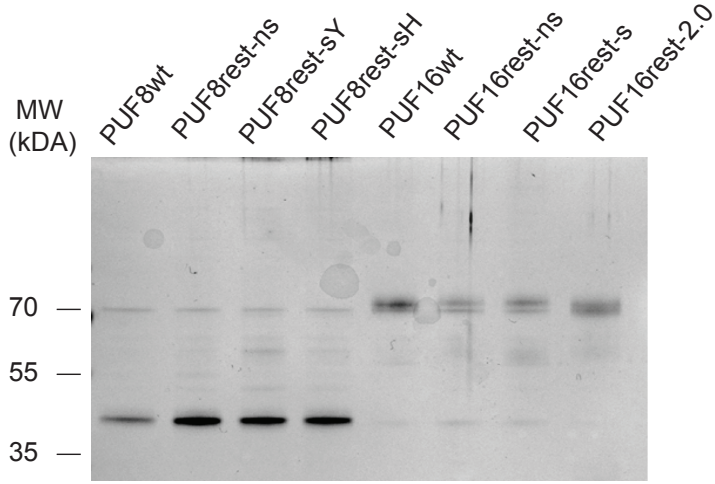
PUF16wt:RESTRNA16
PUF16rest-s:RESTRNA16
Distance (Å)
Time (ns)
G
PUF16wt:2XNRE
PUF16wt:RESTRNA16
PUF16rest-s:RESTRNA16
Energy (kcal/mol)
Time (ns)
H
PUF16wt:2XNRE
PUF16wt:RESTRNA16
PUF16rest-s:RESTRNA16
Distance (Å)
Time (ns)

Criscuolo, Gatti et al. **Figure S4**



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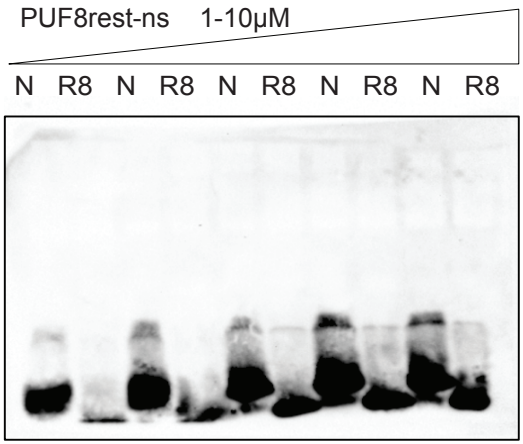
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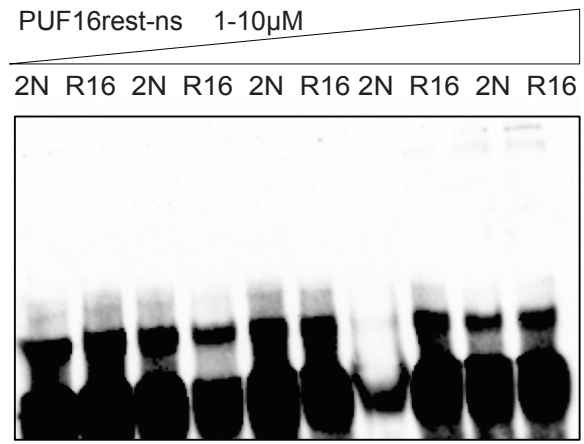
N R8 N R8 N R8 N R8 N R8

B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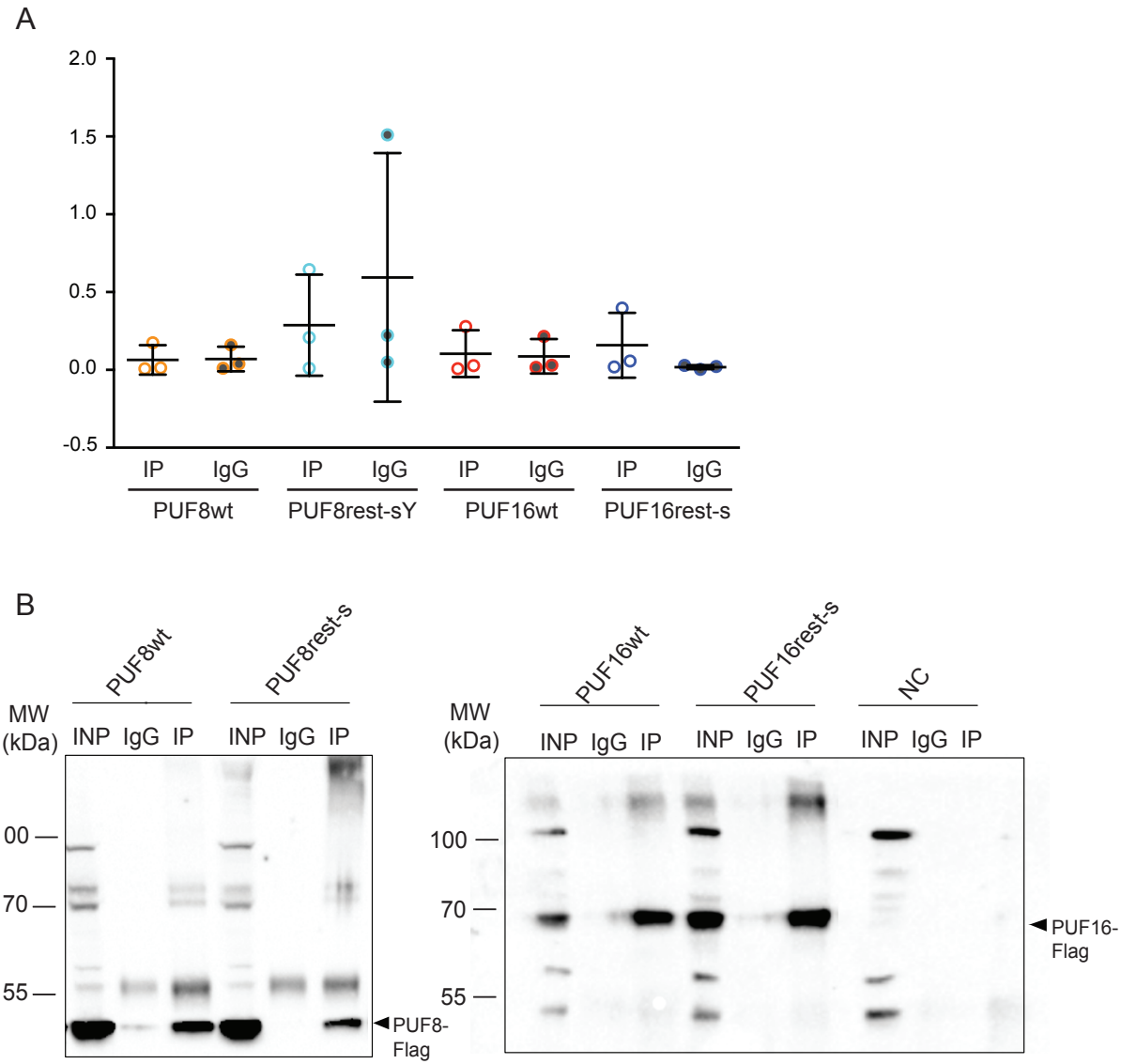
2N R16 2N R16 2N R16 2N R16 2N R16


A
2.0
1.5
1.0
0.5
0.0
-0.5
IP
IgG
IP
IgG
IP
IgG
IP
IgG
PUF8wt
PUF8rest-sY
PUF16wt
PUF16rest-s
B
PUF8wt
PUF8rest-s
MW
(kDa)
INP IgG IP INP IgG IP
100
70
55
PUF8-
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100
70
55
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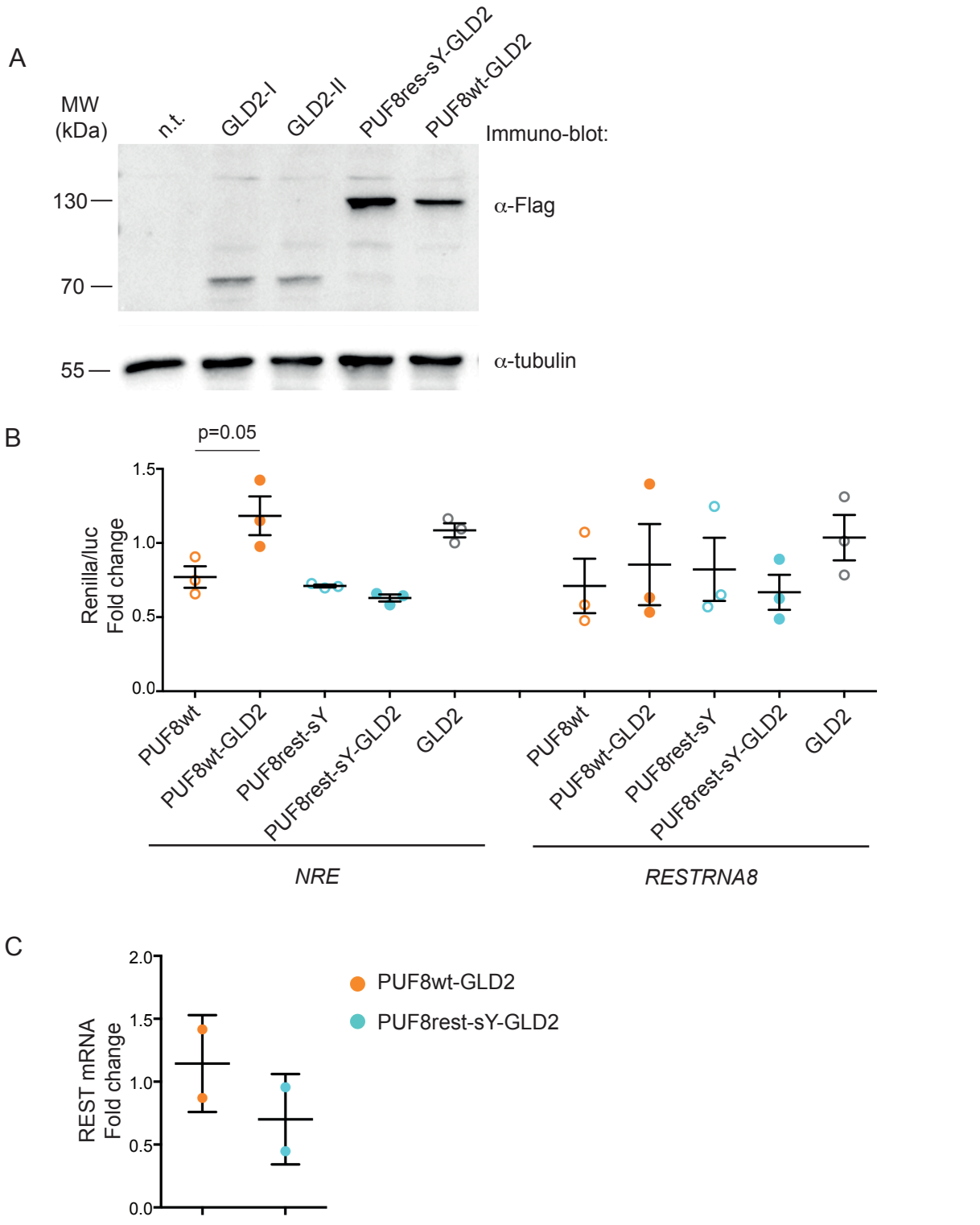
A
MW
(kDa)
n.t.
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GLD2-II
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PUF8wt-GLD2
Immuno-blot:
130
70
55
α-Flag
α-tubulin
B
p=0.05
Renilla/luc
Fold change
1.5
1.0
0.5
0.0
PUF8wt
PUF8wt-GLD2
PUF8rest-sY
PUF8rest-sY-GLD2
GLD2
NRE
RESTRNA8
C
REST mRNA
Fold change
2.0
1.5
1.0
0.5
0.0
PUF8wt-GLD2
PUF8rest-sY-GLD2