Chapter 18

Design of RNA-Binding Proteins: Manipulate Alternative Splicing in Human Cells with Artificial Splicing Factors

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Abstract

The majority of human genes undergo alternative splicing to produce multiple isoforms with distinct functions. The dysregulations of alternative splicing have been found to be closely associated with various human diseases; thus new approaches to modulate disease-associated splicing events will provide great therapeutic potentials. Here we report protocols for constructing novel artificial splicing factors that can be designed to specifically modulate alternative splicing of target genes. By following the method outlined in this protocol, it is possible to design and generate artificial splicing factors with diverse activities in regulating different types of alternative splicing. The artificial splicing factors can be used to change splicing of either minigenes or endogenous genes in cultured human cells, providing a new strategy to study the regulation of alternative splicing and function of alternatively spliced products.

Key words Alternative splicing, Artificial splicing factors, SR proteins, hnRNP proteins, RRM, RS domain, Glycine-rich domain

1 Introduction

The majority of genes in higher eukaryotes are transcribed as pre-mRNAs containing multiple introns that are removed to generate mature mRNA through RNA splicing. As an essential step of gene expression, splicing involves a series of precisely controlled reactions catalyzed by a dynamic ribonucleoprotein complex known as the spliceosome. Most human genes undergo alternative splicing (AS) to produce multiple spliced isoforms [1], providing a major mechanism to regulate gene functions and increase the coding complexity of human genome. The choice of different spliced isoforms is tightly regulated in different tissues and developmental stages, and the disruption of splicing regulations is a common cause of a variety of human diseases [2]. Therefore, modulation of alternative splicing may have broad applications in both basic molecular research and translational research.

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Generally, the splicing process is regulated by multiple *cis*-elements that serve as splicing enhancers or silencers to control core splicing events. Based on the activities and relative locations of these *cis*-elements, they are classified as exonic splicing enhancers (ESEs) or silencers (ESSs), and intronic splicing enhancers (ISEs) or silencers (ISSs). The splicing regulatory *cis*-elements are recognized by various *trans*-acting protein factors (i.e., splicing factors) to either promote or suppress the use of nearby splice sites [3, 4]. Many splicing factors have modular organization, with separate sequence-specific RNA-binding modules to recognize their targets and splicing effector module to change splicing. The best known examples are the members of the Serine/Arginine-rich (SR) protein family, which contain N-terminal RNA recognition motifs (RRMs) that bind to *e*xonic *s*plicing *e*nhancers (ESEs) in pre-mRNAs and C-terminal RS domains that promote exon inclusion [5]. As another example, the hnRNP protein hnRNP A1 binds to *e*xonic *s*plicing *s*ilencers (ESSs) through its RRM domains and inhibits exon inclusion through a C-terminal Glycine-rich domain [6].

Because the majority (~90 %) of all human genes undergo alternative splicing, engineering novel splicing factors to specifically modulate splicing offers tremendous promise for both basic and applied research. This can be achieved in principle by a modularly designed protein factor that contains a target recognition module and a splicing effector module. However, natural splicing factors usually contain RNA recognition motif (RRM) or K homology (KH) domains that recognize short RNA elements with moderate affinities. It is impractical to engineer an RNA recognition module using these domains due to their weak RNA-binding affinity and the absence of a predictive RNA recognition "code." By using the unique RNA recognition module of PUF domains (named for *Drosophila* Pumilio and *C. elegans fem-3* binding factor), we have, for the first time, engineered novel artificial splicing factors that can specifically recognize almost any given pre-mRNA target [7]. These *artificial* factors are engineered by combining different splicing regulatory domains to a modified PUF domain that specifically recognize different RNA sequences. The canonical PUF domain contains eight repeats of three α-helices, each recognizing a single base in an 8-nt RNA target. The amino acids at the certain positions of the second α-helix form specific hydrogen bonds with the edge of RNA base and thus determine the RNA-binding specificity (Fig. 1a). The code for base recognition of PUF repeat is surprisingly simple (Fig. 1a), which can direct the mutagenesis of PUF domain to generate modified PUFs that recognize any possible 8-base (reviewed in Ref. 8). By choosing different functional domains, such *e*ngineered *s*plicing *f*actors (ESFs) can function as either splicing activator or splicing inhibitor and can

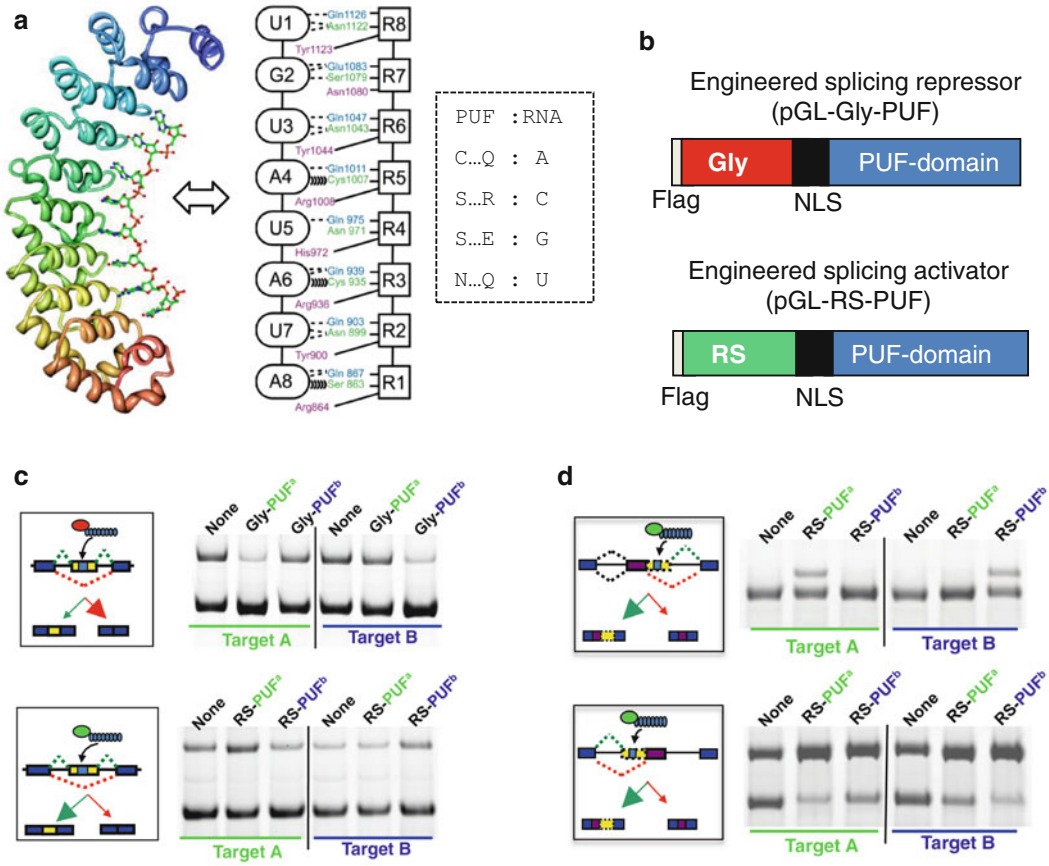

Fig. 1 Design of ESFs and their activity in modulating exon skipping. (**a**) Specific binding between PUF domain and RNA targets is illustrated with RNA-PUF structure and a schematic diagram. The PUF binding code for each of the four RNA bases is shown at *right*, which is used to design PUF mutations. (**b**) Modular domain organization of ESFs. The C-terminal RNA-binding module (PUF domain) is fused with a functional module (Gly-rich domain or RS domain) to produce novel splicing repressors or activators with designed specificity. In addition, a NLS and the FLAG epitope tag are included to facilitate nuclear localization and detection of ESFs. Expression of the ESFs is driven by CMV promoters (*arrow*). (**c**) Gly-PUF ESFs were co-expressed with exon skipping reporters, and splicing pattern was assayed by RT-PCR. The modified PUF^a and PUF^b specifically bind to 8-mer targets A and B respectively (*in same colors*). All combinations were used so the PUF-target pairs of different color serve as controls. The effects of RS-PUF on exon skipping (**c**), competing 5′ or 3′ splice site reporter (**d**) were assayed by methods similar to panel (**c**)

regulate various types of alternative splicing events associated with splicing reporters or endogenous genes.

Here we describe in detail how to design and construct ESFs and how to use them to manipulate alternative splicing. We focus on its application in cultured human cells and the common assay of ESF activities of splicing modulation. Using the Bcl-x gene as example, we also describe how to manipulate the splicing of endogenous genes and analyze the downstream effect of splicing changes.

2 Materials

2.1 Reagents

Information regarding vendors and suppliers provided herein is for convenience rather than a strict requirement. Many commonly used reagents are available from various reliable sources.

1. Plasmids encoding ESFs (pGl-RS-PUF, pGl-Gly-PUF).
2. Splicing reporter plasmid (pGZ3-NRE, pEZ-1B-A6G, pEZ-2F-GU).
3. Lentiviral expression vector pWPXLd-Gly-PUF.
4. QuickChange Site-Directed Mutagenesis kit (Stratagene).
5. Cultured cells (HEK293T cells, MDA-MB-231 cells, A549 cells, HeLa cells).
6. Dulbecco's modified Eagle medium (DMEM; Invitrogen).
7. Opti-MEM I (Invitrogen).
8. Lipofectamine 2000 transfection reagent (Invitrogen).
9. TRIzol reagent (Invitrogen) ! CAUTION TRIzol reagent includes phenol, which can cause burns. Wear gloves when handling.
10. 70 % ethanol.
11. DNase I.
12. Superscript III reverse transcriptase (Invitrogen).
13. Cy5-dCTP (GE Healthcare Life Sciences).
14. Primers used for RT and PCR: *see* Table 1
15. Acrylamide (Sigma) ! CAUTION Neurotoxic; Take precautions to avoid contact and ingestion.

Table 1
Primers used in constructing ESF expression vectors

Primer name	Sequence	Notes
Pum-F1	CACGGATCCTCCCCCCCAAGAAAAAGAGGA AGGTATCTAGAGGCCGCAGCCGCCTTTTG	Encodes NLS between BamHI and XbaI sites
Pum-R1	GTGGTCGACTTACCCTAAGTCAACACC	Encodes a stop codon and SalI site
ASF-RS-F	CACGCTAGCATGGACTACAAGGACGACGATGACA AGGGTCTCGAGAGAAGTCCAAGTTATGGAAG	Encodes an N-terminal FLAG tag after NheI site
ASF-RS-R	CACGGATCCCCGTACGAGAGCGAGATCTG	Contains BamHI site for cloning

Restriction enzyme digestion sites are *underlined*

16. Bis-acrylamide (Fluka) ! CAUTION Neutrotoxic; wear gloves when handling.
17. 10 % 1× TBE PAGE gel (*see* Subheading 2.2).
18. PBS (phosphate balance saline).
19. FITC-conjugated goat anti-mouse IgG.
20. Vector shield's mounting medium with DAPI.
21. SDS-PAGE gel running buffer (*see* Subheading 2.2).
22. 2× SDS-PAGE sample buffer (*see* Subheading 2.2).
23. 12 % SDS-PAGE gel (*see* Subheading 2.2).
24. Antibodies: Caspase-3 (Cell signaling); PARP (Cell Signaling); Bcl-x (BD Bioscience); Actin (Sigma); Alpha-tubulin (Sigma); FLAG M5 (Sigma).
25. Nitrocellulose membrane (Amersham-Pharmacia).
26. ECL Western Blotting Detection Reagents (GE Healthcare).
27. Cell proliferation reagent WST-1 (ROCHE).
28. Cisplatin (Sigma).
29. Paclitaxel (Sigma).
30. TNF-alpha (GenScript).
31. TRAIL (GenWay).

2.2 Reagent Setup

1. *Cultured cells*: The human embryonic kidney cell line 293 T, lung cancer cell line A549, breast cancer cell line MDA-MB-231, and human cervical cancer cell line HeLa were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum.
2. *30 % (wt/vol) Acrylamide (29:1) solution*: Add 29 g of acrylamide and 1 g of bis-acrylamide into water to a final volume of 100 ml.
3. *2× SDS-PAGE sample buffer*: 0.2 % bromophenol blue (wt/vol), 20 % glycerol (vol/vol), 100 mM Tris–HCl, pH 6.8, 4 % SDS (wt/vol), 715 mM 2-mercaptoethanol.
4. *5× SDS-PAGE running buffer*: Adding 15.14 g of Tris, 93.8 g of glycine, and 5 g of SDS to 1 l H_2O. Adjust the pH to 8.3 and store at room temperature.
5. 12 % SDS-PAGE separating gel.

	Separating gel (10 ml)
H_2O (ml)	3.3
30 % Acrylamide (wt/vol) (29:1) (ml)	4.0
1.5 M Tris–HCl (pH 8.8) (ml)	2.5
10 % SDS (wt/vol) (ml)	0.1

	Separating gel (10 ml)
10 % APS (wt/vol) (ml)	0.1
TEMED (ml)	0.004

	Stacking gel (5 ml)
H_2O (ml)	3.4
30 % Acrylamide (wt/vol) (29:1) (ml)	0.83
1 M Tris–HCl (pH 6.8) (ml)	0.63
10 % SDS (wt/vol) (ml)	0.05
10 % APS (wt/vol) (ml)	0.05
TEMED (ml)	0.005

6. 10 % 1× TBE PAGE gel (10 ml).

H_2O (ml)	3.4
30 % Acrylamide (wt/vol) (29:1) (ml)	3.3
5× TBE	2.0
10 % APS (wt/vol) (ml)	0.10
TEMED (ml)	0.004

3 Methods

3.1 Construction of ESF Expressing Plasmids (See Note 1)

Generate ESF expression constructs by using the pCI-neo vector. We started with an expression construct that encodes from the N-terminus to the C-terminus: FLAG epitope, Gly-rich domain of hnRNP A1 (residues 195–320 of NP_002127), and the MS2 coat protein (gift of Dr. R. Breathnach from Institut de Biologie-CHR [6]).

3.1.1 Engineered Splicing Repressor

1. The fragment encoding the MS2 coat protein fragment is removed using *BamH*I/*Sal*I digestion and replaced with a fragment encoding a NLS (PPKKKRKV) and the PUF domain of human Pumilio1, which is generated by PCR amplification using primers Pum-F1 and Pum-R1 (*see* Table 1).
2. The resulting construct expresses a Gly-PUF type ESF (pGL-Gly-PUF) under the control of a CMV promoter (Fig. 1b, top panel).

3.1.2 Engineered Splicing Activator

1. To make an expression construct for an RS-PUF type ESF (pGL-RS-PUF) (Fig. 1b, bottom panel), remove the fragment encoding the FLAG/Gly-rich domain with *Nhe*I/*BamH*I digestion,

and replace it with a fragment that encodes the RS domain of ASF/SF2 protein with an N-terminal FLAG epitope, which is amplified using primers ASF-RS-F and ASF-RS-R (*see* Table 1).

3.1.3 ESF Variants

1. To generate constructs for different ESFs with mutated PUF domains that recognize different RNA targets (pGL-Gly/RS-PUF), we design the PUF mutations according to the binding code of PUF repeat [8] and introduce the point mutations in consecutive steps using a QuikChange Site-Directed Mutagenesis kit [9].
2. The additional functional domains of ESF (RS domains/fragment or Gly-rich domains/fragment) are amplified by PCR (or synthesized oligonucleotides for short fragments), and cloned between XhoI and BamHI sites.
3. The RS domains are residues 123–238 of 9G8 (NP001026854), residues 180–272 of SRp40 (NP008856), residues 117–221 of SC35 (NP003007), and an $(RS)_6$ sequence.
4. The Gly-rich domains are residues 203-353 of hnRNP A2 (NP112533), residues 211–378 of hnRNP A3 (NP919223), and the short peptide of GYGGGGPGYGNQGGGYGGG.

3.2 Construction of Splicing Reporter (See Note 2)

3.2.1 To Assess the Effect of ESFs on Inclusion/Skipping of a Target Exon

1. A modular reporter (pGZ3) is used for insertion of splicing regulatory sequences (each recognized by different ESFs, shown in different colors in Fig. 1c, d) into the test exon (Exon 12 of the human IGF-II mRNA-binding protein 1, IGF2BP1, Ensembl ID ENSG00000159217).
2. The test exon, together with its flanking introns, is inserted between two GFP exons in pEGFP-C1 vectors as described previously [10].
3. To insert target sequences of PUF domains into this reporter vector, synthesize and anneal oligonucleotides containing the candidate sequences (designated by N_8) flanked by *Xho*I and *Apa*I sites.
4. The resulting DNA fragments are digested and ligated into the *Xho*I/*Apa*I digested vector (inside the test exon).
5. The inclusion of the test exon is assayed by body-labeled RT-PCR, as described previously, with primers corresponding to the first and third exon (two GFP exons) of the reporter minigene [11].

3.2.2 To Assess the Effect of ESFs on the Alternative Use of 5′ and 3′ ss

1. Use reporters with competing 5′ (pEZ-1B) and 3′ ss (pEZ-2F) [11].
2. The corresponding target sequences of PUF domains (NRE, A6G, GU) are inserted into these reporters using either *Xho*I/*Apa*I sites (for the competing 3′ ss reporter) or *Xho*I/*Eco*RI sites (for the competing 5′ ss reporter) as described above.

3.3 Construction of Lentiviral Expression Vectors

1. The full-length ESFs are PCR amplified from original expression vectors and integrate into the lentiviral expression vector pWPXLd between MluI and SpeI sites.
2. Lentiviruses are generated by co-transfecting HEK293 cells with packaging vectors pPAX2 and pMD2.G with either pWPXLd-Gly-PUF (531), pWPXLd-Gly-PUF (wt) (Control), or pWPXLd-GFP (mock) using the standard calcium phosphate precipitation method.
3. The titer of lentivirus is determined by infecting HEK293 cells with serial dilutions of the virus preparation.

3.4 Analysis of Exon Inclusion or Alternative Use of Splice Sites of Minigene Reporters

3.4.1 Co-transfection of ESF Plasmids and Splicing Reporter Plasmids

1. Seed 2×10^5 HEK293T cells into each well of a 24-well plate and grow the cells overnight in a humidified incubator at 37°C and 5 % CO_2.
2. Mix Lipofectamine 2000 by gently inverting bottles before use, then dilute 2 μl lipofectamine 2000 in 50 μl Opti-MEM I medium. Mix gently and incubate for 5 min at room temperature.
3. Dilute 0.04 μg of pGL-Gly-PUF expression vectors and 0.2 μg of pGZ3 reporter plasmids or 0.4 μg of pGL-RS-PUF expression vectors and 0.2 μg of pGZ3 reporter plasmids or 0.4 μg of pGL-RS-PUF expression vectors and 0.2 μg of pEZ-1B or pEZ-2F reporter plasmids in 50 μl Opti-MEM I medium in a sterile tube.
4. After 5 min incubation, mix the diluted Lipofectamine 2000 with the diluted plasmids gently and incubate for 20 min at room temperature to allow complex formation to occur.
5. After incubation, add the transfection mixture to each well and incubate for at least 12 h in the incubator.

3.4.2 Isolation of RNA

1. After 12 h incubation, discard medium from each well and wash it with 500 μl PBS per well.
2. Discard PBS and add 200 μl trypsin to each well, incubate in the incubator for 5 min. Then add 1 ml medium to stop the digestion and transfer the cells to a sterile 1.5 ml tube.
3. Centrifuge tubes for 3 min at $5000 \times g$ and discard the medium. Then add 500 μl TRIzol reagent per tube to lyse cells by repetitive pipetting. Incubate the homogenized samples for 5 min.
4. For each TRIzol treated sample, add 0.1 ml of chloroform per 500 μl of TRIzol reagent. Invert tubes for 15 s and incubate for 3 min at room temperature.
5. Centrifuge tubes for 15 min at $12{,}000 \times g$ at 4°C and transfer the aqueous phase to a fresh tube and use 0.25 ml of isopropanol per 0.5 ml of TRIzol reagent used for the initial homogenization.
6. Mix rigorously and incubate at room temperature for 10 min and then centrifuge at $12{,}000 \times g$ for 10 min at 4°C. The RNA precipitate is usually visible after centrifugation on the bottom of the tube.

7. Discard the supernatant and wash the RNA pellet with 0.5 ml of 75 % ethanol per 0.5 ml of TRIzol reagent used before.
8. Mix rigorously and centrifuge at 7500×*g* for 5 min at 4 °C. Remove the supernatant and dry the RNA pellet. Then dissolve RNA in 50 μl RNase-free water.
9. Add 2 μl DNase I, 7 μl buffer, and 11 μl H_2O to each 50 μl RNA solution and incubate at 37 °C for 1 h and then heat inactivate DNase at 70 °C for 15 min.

3.4.3 Detection of Splice Variants by RT-PCR

1. For each sample, add the following components to a 0.2 ml nuclease-free tube:

Primer: Gexon 3r(10 μM) or Oligo dT	1 μl
RNA (2 μg)	5 μl
dNTP (10 mM)	1 μl
H_2O	3 μl

2. Heat mixture to 65 °C for 5 min and incubate on ice for at least 2 min to prevent reformation of the secondary structure, then add the following components to the same tube.

5× Fist strand buffer	4 μl
DTT (0.1 M)	1 μl
Superscript III RT	1 μl
H_2O	4 μl

3. Mix by pipetting gently up and down and incubate at 50 °C for 60 min and then inactivate the reaction by heating at 70 °C for 15 min.
4. For each sample, add the following to a PCR tube to do the body-labeled PCR.

10× PCR buffer	2.5 μl
10 mM dNTP mix	0.5 μl
Gexon 1f (10 μm) or Bcl-x-fwd (10 μm)	1 μl
Gexon 3r (10 μm) or Bcl-x-rev (10 μm)	1 μl
Taq DNA polymerase (5U/μl)	0.25 μl
Cy5-dCTP	0.5 μl
cDNA	2 μl

5. Heat reaction to 94 °C for 2 min to denature and perform 25 cycles of PCR with 94 °C 30 s, 60 °C 30 s, and 72 °C 30 s, then followed by 72 °C 7 min.
6. PCR products were resolved by electrophoresis through a 10 % PAGE gels with 1× TBE buffer and scanned with a Typhoon 9400 scanner. The amount of each splicing isoform is measured with ImageQuant 5.2 software.

3.5 Analysis of Endogenous Bcl-x Splicing (See Note 3) and Measure Its Effects on Apoptosis (See Note 4)

3.5.1 Transfection of ESF Plasmids

1. Plate 2×10^5 HeLa cells to each well of a 24-well plate and then grow cells overnight in an incubator at 37 °C and 5 % CO_2.
2. After 12 h, transfect cells with 2 μg of pGL-Gly-PUF(wt), 0.2, 1, 2 μg of pGL-Gly-PUF(531) respectively.
3. 24 h later, harvest the cells, 1/3 of the cells for the RNA isolation and PCR analysis (*see* Subheadings 3.4.2 and 3.4.3) and 2/3 for the protein isolation.

3.5.2 Western Blot Analysis of Apoptotic Protein Markers

1. For the western blot analysis, the total cell pellets were boiled in 2× SDS-PAGE loading buffer for 10 min and then resolved by 12 % SDS-PAGE gel and transferred the proteins onto a nitrocellulose membrane.
2. Block the membrane with 5 % milk for 1 h at room temperature and incubate the membrane with Caspase-3 (1:1000), PARP (1:1000), beta-actin (1:5000) primary antibodies respectively at 4 °C overnight.
3. Wash the membrane with PBS containing 0.1 % Tween 20 (PBS-T) for 5 min at room temperature three times on a rocking shaker, then incubate the membrane with HRP-linked secondary antibodies for 1 h at room temperature.
4. Wash the membrane with PBS-T three times at room temperature and then develop the membrane using ECL Western Blotting detection reagents.

3.5.3 Detection of the ESFs (See **Note 5**) and DNA Fragmentation (See **Note 6**)

1. For the immunofluorescence assay measuring the apoptosis, 5×10^5 HeLa cells were seeded onto poly-lysine coated glass coverslips in a 6-well plate and transfected with pGL-Gly-PUF(wt) or pGL-Gly-PUF(531) plasmids using Lipofectamine 2000 (*see* **steps 5–9**).
2. 24 h after transfection, fix the cells on coverslips with 1 ml 4 % formaldehyde in 1× PBS for 20 min at room temperature.
3. Gently wash cells on coverslips by adding 2 ml 1× PBS and incubate for 5 min, then remove the PBS with pipettes. Repeat the wash for three times.
4. Permeabilize cells with 0.2 % Triton X-100 in 1× PBS for 10 min and then wash with 1× PBS three times.
5. Block cells with 3 % BSA in 1× PBS for 10 min and wash with 1× PBS three times.
6. Dilute the FLAG antibody to 1:1000 in 3 % BSA/PBS, and pipet 30 μl of the diluted FLAG antibody into a parafilm sheet.
7. Take out the coverslip with cells, dry the excess buffer carefully with Kimwipes, and put it upside down (the cell side down) on the 30 μl primary solution, incubate for 1 h at room temperature.

8. Add about 500 μl of 1× PBS from the side of coverslips incubated with the FLAG antibody to allow it float on top of the solution, then take it back to the 6-well plate with 1× PBS in the well.
9. Wash it again with 1× PBS three times and 5 min for each time.
10. Dilute the anti-mouse secondary antibody (1:500) in 3 % BSA/PBS; again, use 30 μl for each coverslip; put the coverslip upside down on the secondary antibody solution, incubate for 15 min at room temperature.
11. Remove the coverslips from the parafilm as described in **step 8** and put it back to 6-well plate, wash again with 1× PBS three times and 5 min for each time.
12. Mount the coverslips with mounting medium (with DAPI), absorb the excess medium carefully with Kimwipes, and seal the edge with nail polish.
13. Visualize cells using an Olympus fluorescence microscope and photograph using a digital camera.

3.6 Viral Transduction of ESF and the Measurement of Apoptosis by Flow Cytometry

1. To detect the apoptosis with propidium iodide staining, split 2×10^6 HeLa cells, MDA-MB-231 cells, and A549 cells into 60 mm dishes respectively.
2. 24 h later, refresh the medium and prepare the lentivirus.
3. Dilute ten million lentivirus pWPXld-Gly-PUF(wt), pWPXld-Gly-PUF(531), or pWPXld-GFP stocks into 4 ml of fresh medium to make the ratio of virus vs. cell number equal to 5.
4. Change the medium in the plates with 4 ml of virus containing medium prepared in **step 3**. 12 h after the infection, change the medium.
5. After 24 h of infection, collect cells and stain for 5 min in a PBS solution containing a final concentration of 2 μg/ml propidium iodide (PI).
6. Analyze the PI-stained cells with a FACScalibur fluorescence-activated cell sorter (FACS) using CELLQuest software (*see* **Note 7**).

3.7 Determine the Effect of ESFs on Sensitizing Cancer Cells to Anti-tumor Drugs

1. For the cell viability assay, HeLa cells, MDA-MB-231 cells, and A549 cells were infected with lentivirus expressing GFP (pWPXld-GFP), control ESF (pWPXld-Gly-PUF (wt)), or designer ESF (pWPXld-Gly-PUF (531)) (*see* **steps 1–4** in Subheading 3.6).
2. After 72 h of infection, seed 5×10^4 cells per well in 96-well plates and grow cells overnight.
3. The next morning, add cisplatin (5 μM), paclitaxel (10 nM), TNF-alpha (10 nM), TRAIL (2 μg/ml) to each cell line in the 96-well plates, and incubate at 37 °C in the presence of 5 % CO_2 for 24 h.

4. After 24 h incubation, add 10 μl of proliferation reagent WST-1 to each well. The cells are further incubated with WST-1 reagent for 30 min and the absorbance at 450 nm is measured using a Benchmark microplate reader. At least three independent experiments need to be performed for each sample (*see* **Note 8**).

4 Notes

1. We have demonstrated methods for engineering splicing factors construction, splicing reporters construction, and how to use ESFs to modulate the splicing of endogenous gene and provide therapeutic potentials [7].
2. To provide a principle of how the ESFs work, we have included examples derived from our own work. The ESFs not only can be used to promote or inhibit the inclusion of the cassette exon containing a cognate target sequence (Fig. 1c) but also can affect the usage of alternative splice sites in the reporter system (Fig. 1d).
3. The ESFs can also be applied to specifically modulate RNA splicing of endogenous genes. We demonstrate this potential by targeting Bcl-x that produces two antagonistic isoforms using alternative 5′ splice sites. We design a Gly-PUF (531) ESF to recognize an 8-mer between the two alternative 5′ splice sites and "reprogram" splicing code to increase Bcl-xS isoforms (Fig. 2a). When transfected into HeLa cells where Bcl-xL is the predominant form, the Gly-PUF (531) ESF increases splicing of the Bcl-xS isoform in a dose-dependent manner, whereas the control ESF, Gly-PUF(wt), does not affect the Bcl-xS level (Fig. 2b). The increase of Bcl-xS protein level is also confirmed by Western blotting using a Bcl-x antibody (Fig. 2c).

Fig. 2 (continued) PUF(531) was fused with the Gly-rich domain of hnRNP A1 to inhibit use of the downstream 5′ ss (indicated with the *heavy red arrow*). (**b**) Modulation of Bcl-x 5′ ss usage. HeLa cells were transfected with different amounts of the Gly-PUF(531) expression construct. Gly-PUF(wt) was used as a control. Two isoforms of Bcl-x are detected with RT-PCR using primers corresponding to exons 1 and 3 of the Bcl-x gene. The percentage of Bcl-xS isoform is quantified and shown at the bottom. (**c**) Expression levels of Bcl-xL and Bcl-xS in the presence of ESFs. Samples are in the same order as panel (**b**), and all proteins are detected by Western blots. The expression of ESFs is detected with anti-Flag antibody, and the tubulin level is used as control. The blot was exposed for a longer time for Bcl-xS because the available Bcl-x antibody detects Bcl-xL with much higher sensitivity. (**d**) Cleavage of PARP and caspase 3 in HeLa cells transfected with different amounts of ESF expression constructs. Samples are detected by western blot at 24 h after transfection. (**e**) Localization of ESFs in transfected HeLa cells detected using immunofluorescence microscopy with anti-FLAG antibody. The cells were co-stained with DAPI to show nuclei. Some nuclei, especially in cells transfected with Gly-PUF(531), are fragmented due to apoptosis. (**f**) Percentage of cells undergoing apoptosis (i.e., with fragmented nuclear DNA) from pictures of randomly chosen fields. Data are from Ref. 7

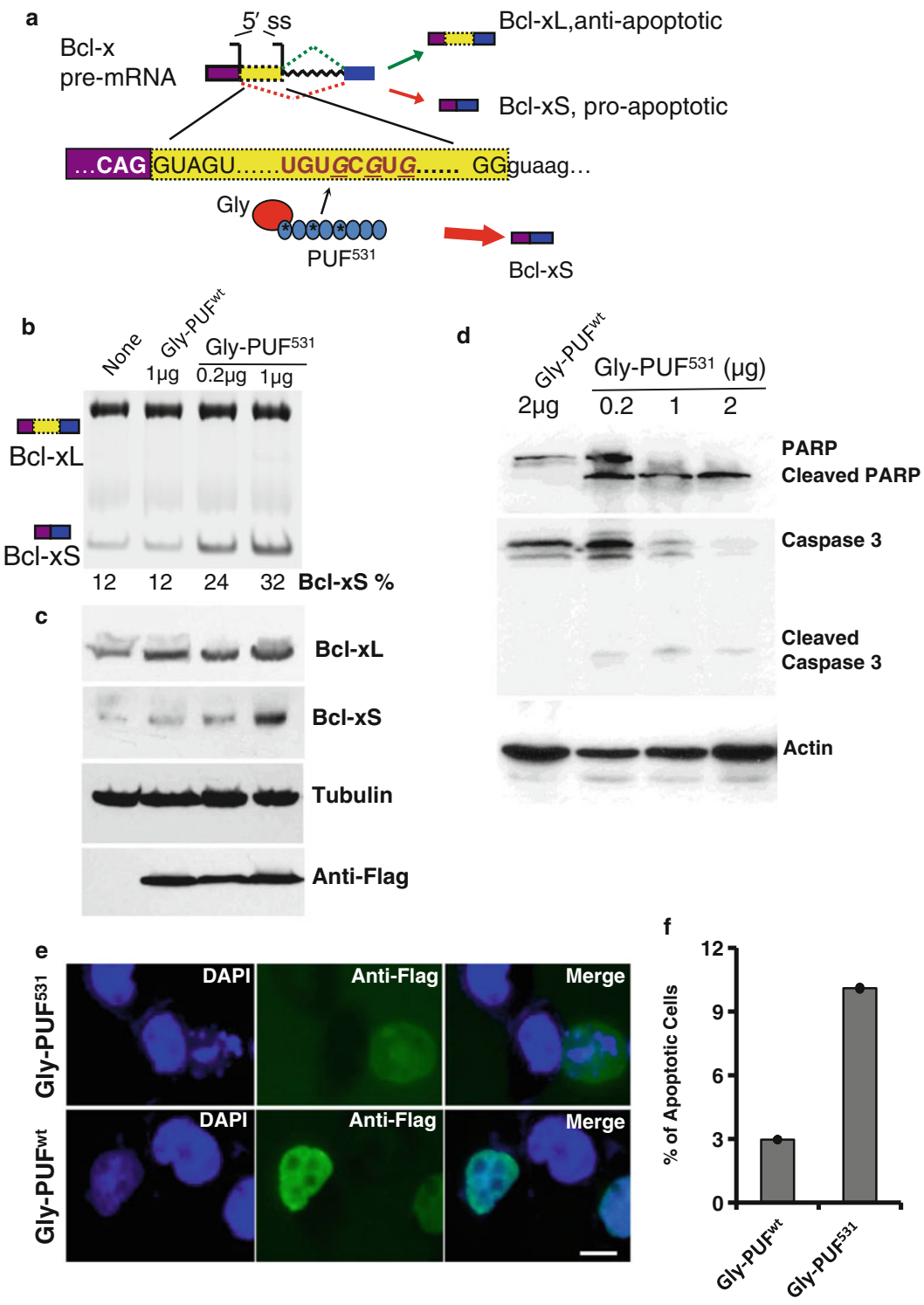

Fig. 2 Use an ESF to modulate splicing of endogenous Bcl-x pre-mRNA. (**a**) Alternative splicing of Bcl-x pre-mRNA. Two alternative 5′ ss in exon 2 of Bcl-x are used to generate Bcl-xL and Bcl-xS. The sequence UGUGCGUG between the two splice sites was chosen as the ESF target, and mutations (*asterisks*) were introduced in wild-type PUF repeats 1, 3, and 5 (Q867E/Q939E/C935S/Q1011E/C1007S) to recognize this sequence. The resulting

4. The increased splicing of the pro-apoptotic Bcl-x isoform can promote cells expressing the designer ESF (Gly-PUF (531)) to undergo apoptosis. The induction of the pro-apoptotic Bcl-xS isoform leads to cleavage of caspase 3 and poly (ADP-ribose) polymerase (PARP), two known molecular markers in the apoptosis pathway (Fig. 2d).
5. Using immunofluorescence microscopy with anti-FLAG antibodies, the ESFs are predominantly localized in the nuclei of the transfected cells (Fig. 2e).
6. Many cells expressing Gly-PUF^{531} have fragmented nuclear DNA, indicating that they are undergoing apoptosis (Fig. 2e). Examination of >200 cells from randomly chosen fields indicates that cells transfected with Gly-PUF(531) have more fragmented nuclear DNA (Fig. 2f).
7. The increase of apoptosis can also be observed with flow cytometry of propidium iodide stained cells (not shown).
8. Different cancer cells stably expressing Gly-PUF(531) also have increased chemosensitivity to the treatment of common antitumor drugs (Fig. 3).

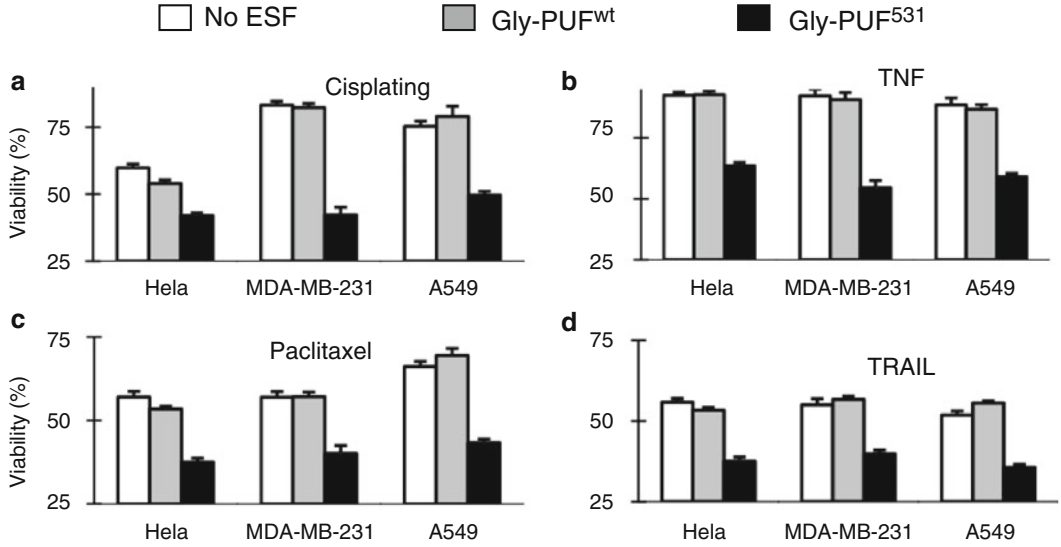

Fig. 3 Effect of ESFs in the chemosensitivities of multiple cultured cancer cell lines. (**a**) Effect of ESFs on cisplatin sensitivity of different cancer cell lines. Cancer cells were infected with lentivirus to express Gly-PUF(531), control ESF (Gly-PUF(wt)), or GFP (as mock infection), and cisplatin was added to a final concentration of 5 μM at 72 h after infection. Cell viability was measured with the WST-1 assay 24 h after drug treatment. All treatments were repeated at least twice, and the means with error bars representing the standard deviation of triplicate samples in one representative experiment are plotted. *White bars* represent cells of mock infection, *grey bars* represent control Gly-PUF(wt) infection, and black bars represent Gly-PUF(531) infection. (**b**, **c**, and **d**) Effect of ESFs on the sensitivities to TNF-alpha, paclitaxel, and TRAIL in different cancer cell lines. Experimental conditions are the same as described for panel (**b**) except final concentrations of 20 ng/ml TNF-alpha (**b**), 10 nM paclitaxel (**c**), or 100 ng/ml TRAIL (**d**) were used. The significant differences ($P < 0.05$, judged by paired *T*-test) of cell viabilities were observed for all drug treatments between the Gly-PUF(531) and Gly-PUF(wt) infected cells. Data are from Ref. 7

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