Supplementary information

EXTENDED DATA FIGURES

SUPPLEMENTARY TABLES

EXTENDED DATA FIGURES

Extended Data Fig. 1. LLPS of EWS-FLI1 alters its DNA binding specificity.

(a) Purified FLI1-related proteins with N-terminal GST tags;

(b) EMSA of FLI1 C-terminal using a pre-mixed equal molarity mixture of specified DNA substrates;

(c) Representative images show SYGQ2-FLI1 droplet fusion at 160 μM concentration and 50 mM NaCl. Fusion indicated by white arrows. Scale bar, 10 μm.

(d) Workflow of the sedimentation assay.

(e) Sedimentation assays for SYGQ2-FLI1 (left) and GST-mCherry control (right) over a NaCl concentration gradient.

(f) Quantification of sedimentation assays for SYGQ2-FLI1 and GST-mCherry, based on three replicates, presented as mean ± SD. *P*-values calculated using an unpaired Student's t-test. "ns" denotes not significant, "*" $p < 0.05$, "**" $p < 0.01$, "***" $p < 0.001$.

(g) Representative images of SYGQ2-FLI1 droplet formation at varying NaCl concentrations. Scale bar: 10 μm. Quantification (right) for SYGQ2-FLI1 droplet formation showing effects of different protein concentrations. Analysis includes the largest 200 droplets per condition. Statistical significance determined by an unpaired Student's t-test, with "***" indicating $p < 0.001$.

(h) Sedimentation assay using SYGQ2-FLI1 with four different DNA substrates from Fig. 2e, analyzed by agarose gel electrophoresis of pellet fractions;

(i) Images of droplet formation by 10 μM EGFP-FUS-ERG. Scale bar, 10 μm.

(j) Sedimentation assay of EGFP-FUS-ERG at 10 μM with 10% 1,6-hexanediol;

(k) Fluorescence microscopy images of EGFP-FUS-ERG droplets with Cy3-labeled DNA, at 2 μM EGFP-FUS-ERG. Scale bar, 10 μm.

(l) EMSA of FUS-ERG with the individual DNA substrates;

(m) Sedimentation assay using FUS-ERG with four different DNA substrates from Fig. 2l, analyzed by agarose gel electrophoresis of pellet fractions.

Extended Data Fig. 2. The binding of free and phase-separated EWS-FLI1 to DNA relies on its DBD specificity.

(a) Domain structures of purified GST-SYGQ2-FLI1, mutated SYGQ2-FLI1 and SYGQ2 domain tagged with N-terminal GST and C-terminal fluorescent protein. Three tyrosine residues (Y332, Y342, Y343 of FLI1) mutated to serine are highlighted with red bars within the DNA binding domain (DBD);

(b) Coomassie blue staining of purified GST-SYGQ2-FLI1-EGFP, GST-SYGQ2-FLI1(3YS)-EGFP, and GST-SYGQ2-mCherry protein;

(c) EMSA using SYGQ2-FLI1, SYGQ2-FLI1(3YS) and SYGQ2 domain and indicated pre-mixed DNA substrates (left) and quantification of the DNA in EMSA (right). The data comprise three independent replicates and are represented as mean ± SD;

(d) Sedimentation assay using SYGQ2-FLI1, SYGQ2-FLI1(3YS), SYGQ2 domain and GST-mCherry and indicated DNA substrates. The supernatant fractions were analyzed using agarose gel electrophoresis (right). The quantification of DNA in these assays (left) is based on data from three individual replicates, and the results are expressed as mean ± SD;

(e) Phase separation of the short fragment EWS-FLI1 is promoted by multi-GGAA DNA substrates. Representative fluorescence microscopy images of EGFP-tagged SYGQ2-FLI1 or SYGQ2-FLI1(3YS) droplet formation in the presence of indicated Cy3-labeled DNA. Scale bar, 10 μm;

Extended Data Fig. 3. LLPS provides additive multivalence interaction between EWS-FLI1 and multi-GGAA.

(a) Sedimentation assay using SYGQ2-FLI1 and a combination of three DNA substrates from Fig. 4f, with supernatant fractions analyzed by agarose gel electrophoresis;

(b) EMSA of SYGQ2-FLI1 with a combination of four DNA substrates from Fig. 4g;

(c) Sedimentation assay using SYGQ2-FLI1 and a combination of three DNA substrates, with supernatant fractions analyzed by agarose gel electrophoresis;

(d) Sedimentation assay using FUS-ERG with a combination of three DNA substrates from Fig. 4g, with

both supernatant (left) and pellet (right) fractions analyzed by agarose gel electrophoresis.
(e) Western blot shows expression of Flag-tagged EWS-FLI1 and EWS(YS)-FLI1 in HEK-293T cells.

Extended Data Fig. 4. DNA occupancy redistribution upon interfering with phase separation of FET-ETS fusion protein.

(a) EMSA with SYGQ2-FLI1 and three DNA templates, performed with (left) and without (right) 10% PEG-8000 supplementation;
(b) EMSA assessing SYGQ2-FLI1 binding to ETS site (left) and multi-GGAA (right) DNA substrates, with and without 10% PEG-8000;
(c) EMSA evaluating His-EGFP-FUS-ERG binding to ETS site (left) and multi-GGAA (right) DNA substrates, with and without 10% 1,6-hexanediol;
(d) EMSA with His-EGFP-EWS-FLI1 and three DNA templates, conducted with (left) and without (right) 10% PEG-8000;
(e) Schematic model showing how IDR-IDR interaction enhances DBD recognition of adjacent binding sites on DNA during FET-ETS fusion protein interaction with multi-GGAA DNA substrate.

Extended Data Fig. 5. Preferential Binding of ETV6 to Short-Fragment GGAA Repeats.

(a) EMSA using full-length ETV6 with specified DNA substrates.
(b) EMSA assessing binding of full-length ETV6 and Tev-treated full-length ETV6 to a mixture of specified DNA substrates.
(c) EMSA evaluating the binding of SYGQ2-FLI1, full-length ETV6, and ETV6 DNA-binding domain (DBD) to various DNA sequence variants.

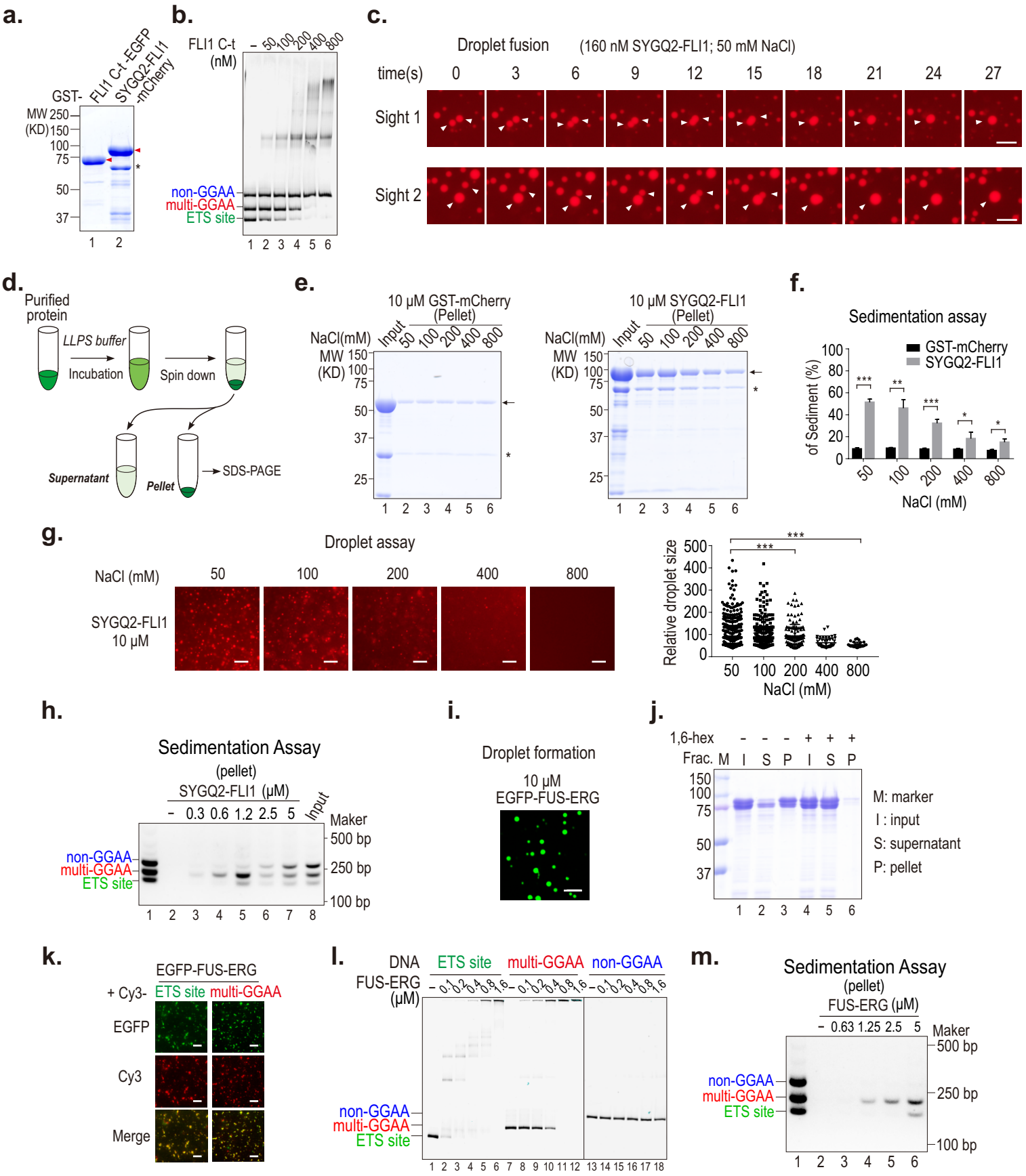
Extended Data Fig. 6. ETV6 oligomerization and preferential binding to GGAA repeat sites.

(a) Outline of the glycerol gradient sedimentation assay.
(b) Glycerol gradient sedimentation was conducted using three protein monomers to determine the molecular weight of ETV6 oligomers as shown in Fig.5a. Protein concentrations in each fraction were

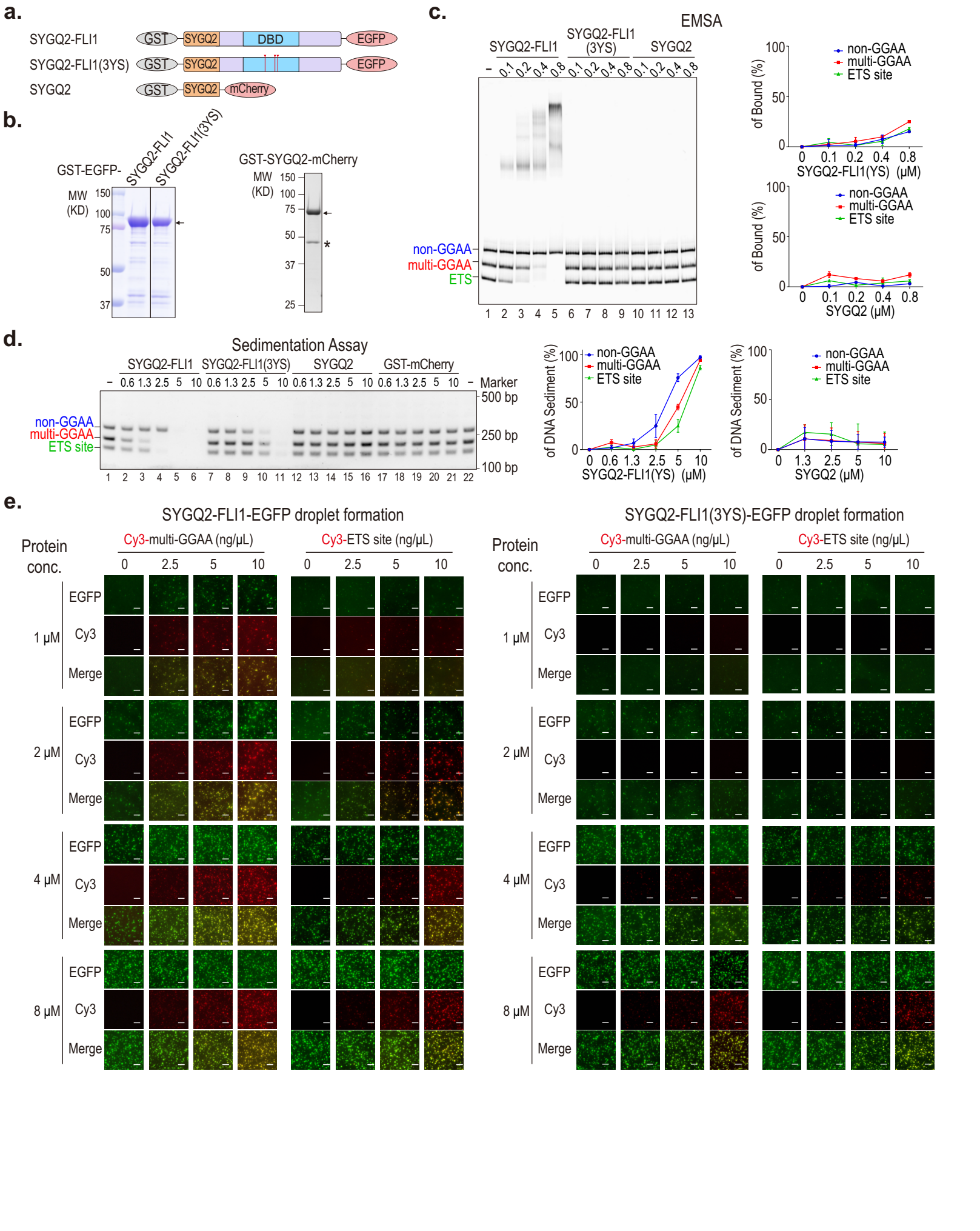
quantified using the Bradford assay.
(c) Sedimentation profiles of specified proteins analyzed by SDS-PAGE and visualized with Coomassie blue staining. The major bands of FUS-ERG or ETV6 are marked with blue or red arrows, respectively.
(d) EMSA of SYGQ2-FLI1 with various DNA substrates (left) and quantification of free DNA substrates (right), based on three independent experiments, presented as mean ± SD.
(e) Diagram illustrating the model where phase-separated EWS-FLI1 and oligomerized ETV6 interact with 5'-GGAA-3' repeat DNA in distinct patterns.

Extended Data Fig. 7. AlphaFold3 Predictions of ETV6 Oligomerization Structures.
AlphaFold3 was used to predict the structures of oligomerized ETV6, both with and without binding to a 99-bp double-stranded DNA containing multiple GGAA sequences. The structure map colors represent the pLDDT scores, indicating prediction confidence. Colors in the PAE (predicted aligned error) plot denote the expected positional errors in the predictions.

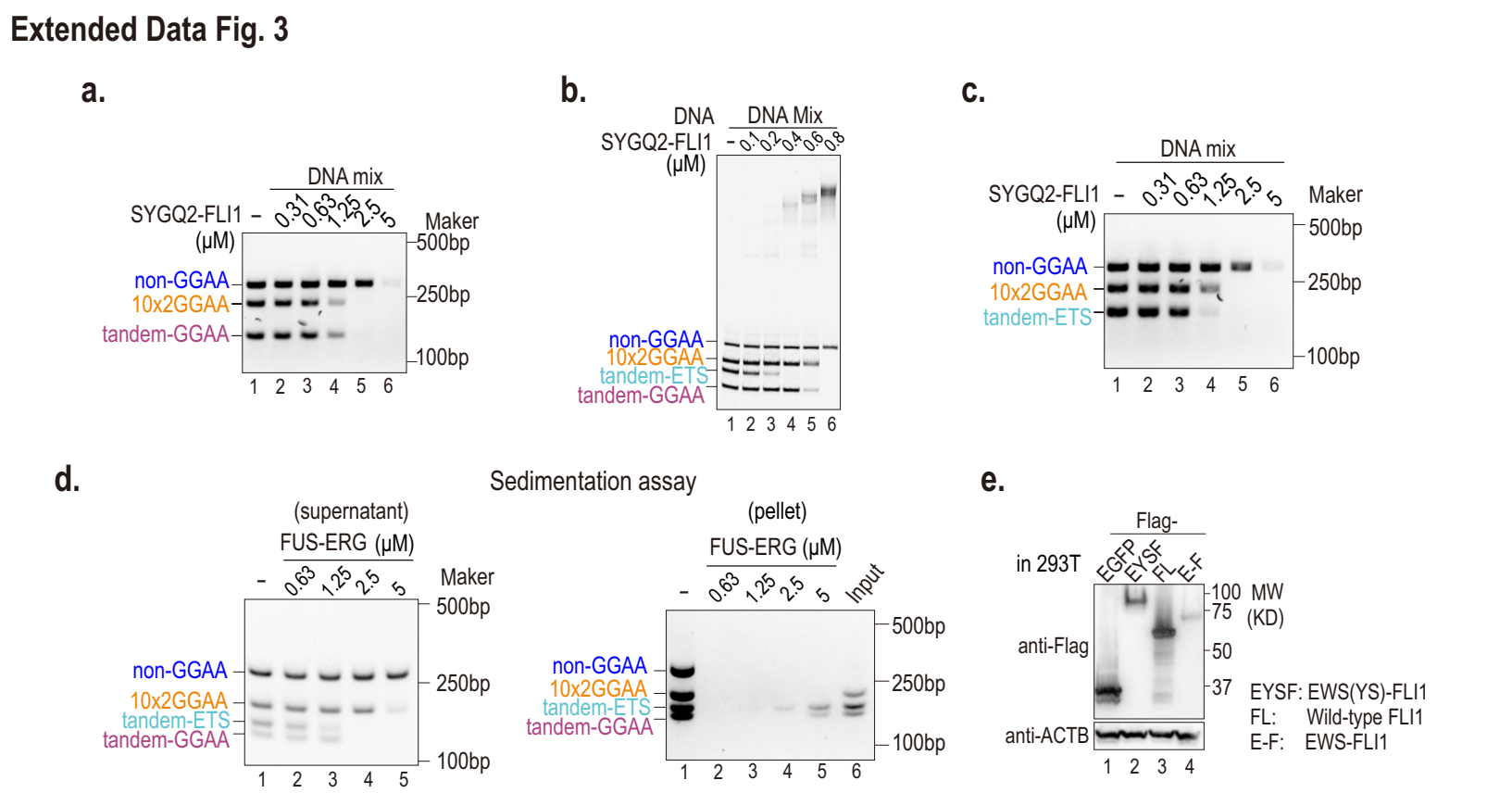
Extended Data Fig. 1



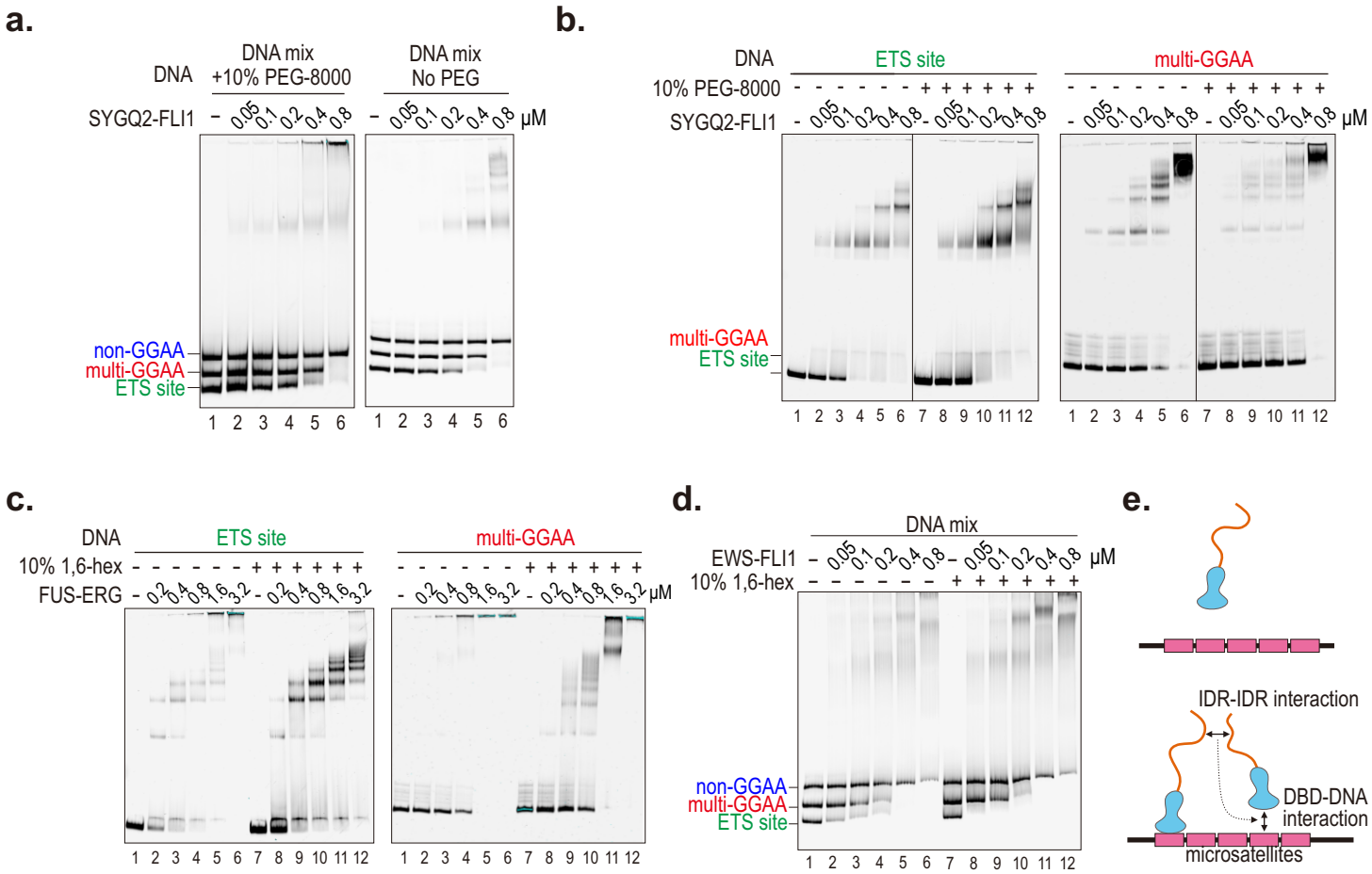
Extended Data Fig. 2



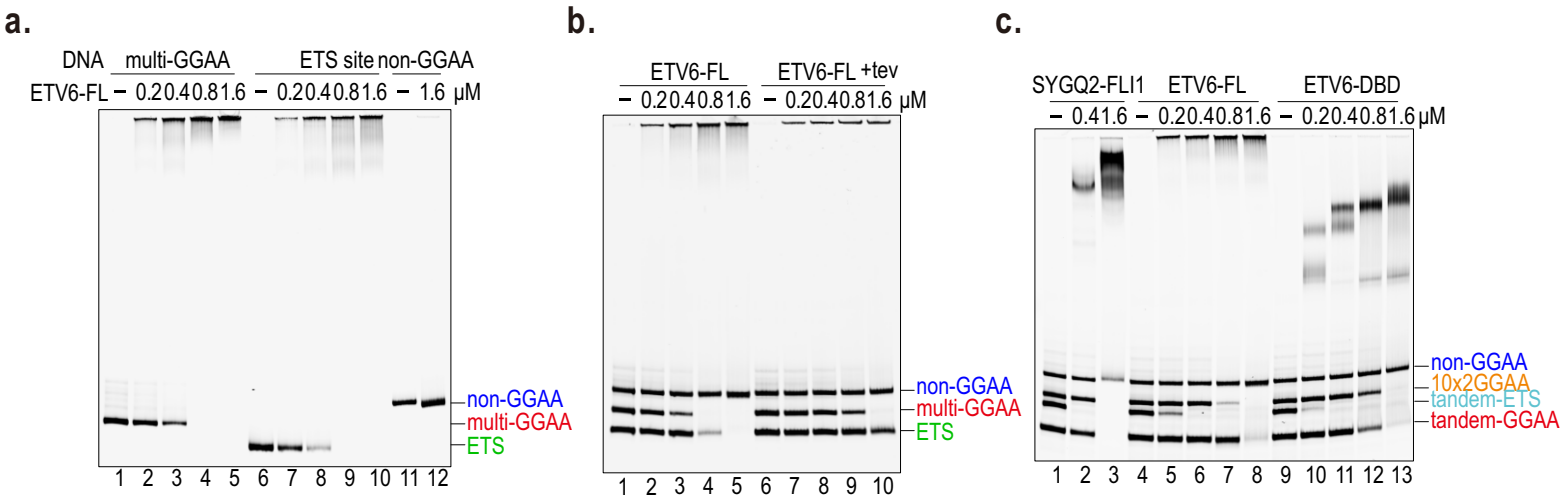
Extended Data Fig. 3



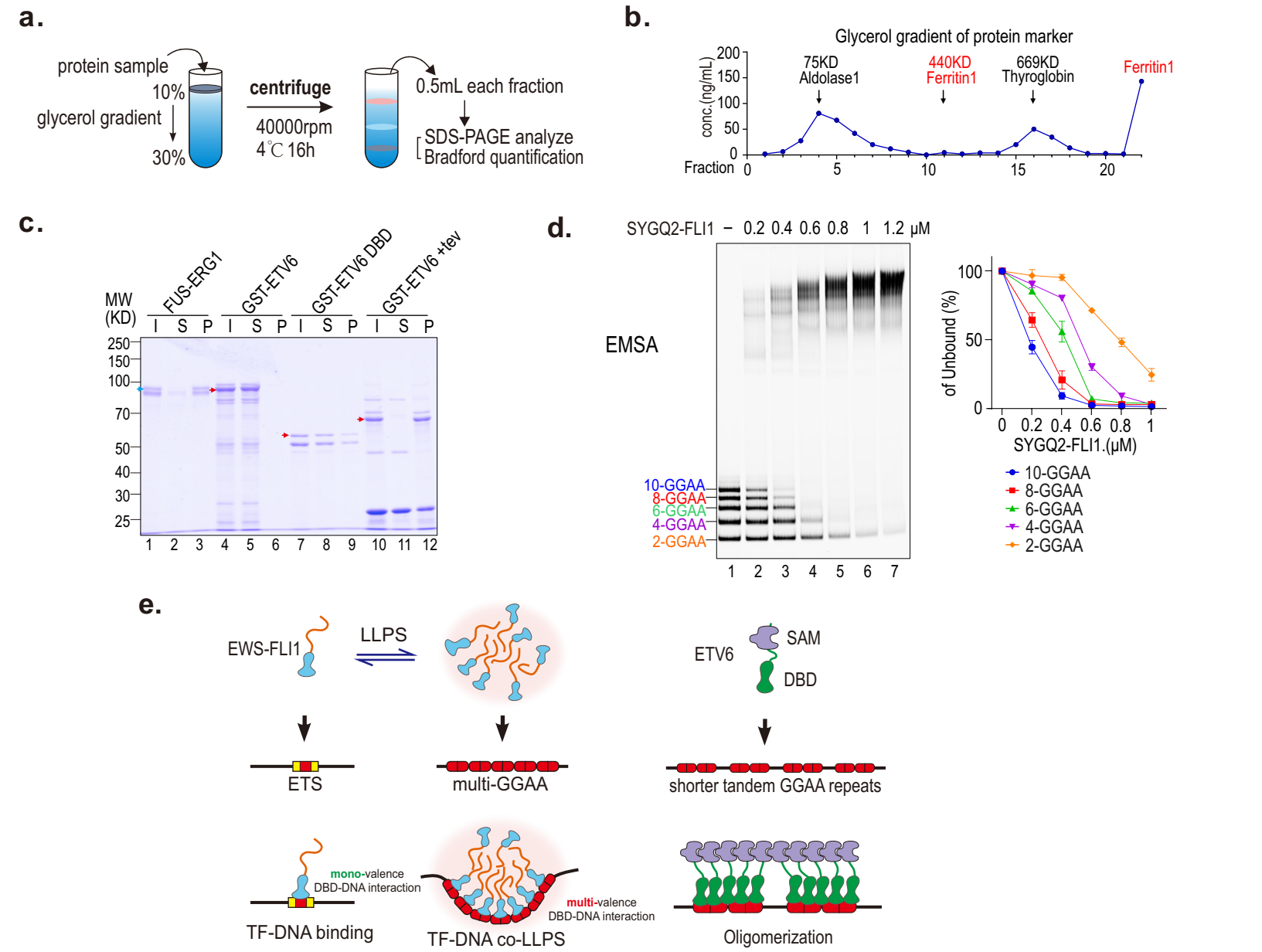
Extended Data Fig. 4



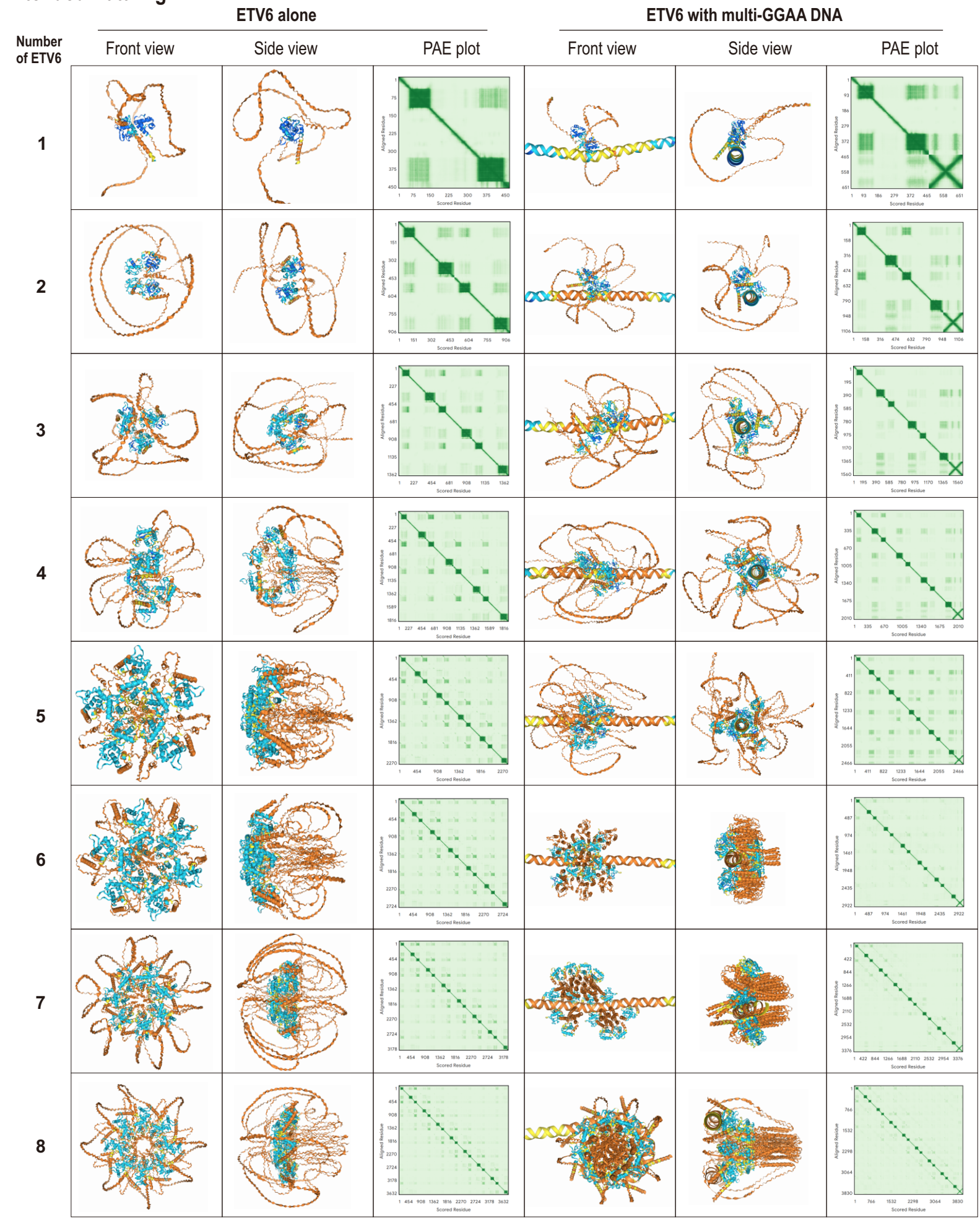
Extended Data Fig. 5



Extended Data Fig. 6



Extended Data Fig. 7



SUPPLEMENTARY TABLES

Supplementary Tables 1. The list of DNA templates used in this study

Supplementary Tables 2. The list of plasmids used in this study

Supplementary Tables 3. The list of primers used in this study

Supplementary Tables 4. The list of reagents or resource used in this study

Supplemental Table1. The list of DNA templates used in this study

DNA templates	Description	label	Product size (bp)	PCR based			Figure Numbers
				Plasmid templates	Primers[a] 1	Primers[a] 2	
probe-01	multi-GGAA	5'-Cy5.5	219	#pMD172	7007*	2402	1c-g; 2d-g; 2k-l; 4c-d; S1b-m; S2c-d; S4a-d; S5a-b
probe-02	ETS site	5'-Cy5.5	181	#pMD160	7007*	3697	1c-g; 2d-g; 2k-l; 4c-d; S1b-m; S2c-d; S4a-d; S5a-b
probe-03	non-specific	5'-Cy5.5	281	#pMD210	7007*	4409	1c-g; 2d-g; 2k-l; 3d-h; 4c-d; S1b-m; S2c-d; S3a-d; S4a-d; S5a-b
probe-04	Cy3-multi-GGAA	5'-Cy3	219	#pMD172	7007**	2402	2h-i; S1k; S2e
probe-05	Cy3-ETS site	5'-Cy3	181	#pMD160	7007**	3697	2h-i; S1k; S2e
probe-06	WT ETS	5'-Cy5.5	236	#pMD221	4151	4152*	3a-b
probe-07	GGAAA	5'-Cy5.5	236	#pMD223	4151	4152*	3a-b
probe-08	GGAAG	5'-Cy5.5	236	#pMD222	4151	4152*	3a-b
probe-09	2x AAGG	5'-Cy5.5	239	#pMD226	4151	4152*	3a-b
probe-10	2x GGAA	5'-Cy5.5	239	#pMD224	4151	4152*	3a-b
probe-11	3x GGAA	5'-Cy5.5	244	#pMD225	4151	4152*	3a-b
probe-12	10x 2GGAA	5'-Cy5.5	223	#pMD271	7007*	4409	34c; 3f-h; 4e-f; S3a-d; S5c
probe-13	interval GGAA	5'-Cy5.5	207	#pMD169	7007*	2977	3c-e
probe-14	10x tandem ETS	5'-Cy5.5	177	#pMD170	7007*	3698	3c-g; S3b-d; S5c
probe-15	10x tandem GGAA	5'-Cy5.5	144	#pMD171	7007*	3294	3c-h; 4e-f; S3a-d; S5c
probe-16	2-GGAA	5'-Cy5.5	161	#pMD365	6895*	6896	5f; S6e
probe-17	4-GGAA	5'-Cy5.5	191	#pMD366	6895*	6897	5f; S6e
probe-18	6-GGAA	5'-Cy5.5	220	#pMD367	6895*	6898	5f; S6e
probe-19	8-GGAA	5'-Cy5.5	252	#pMD368	6895*	6899	5f; S6e
probe-20	10-GGAA	5'-Cy5.5	280	#pMD369	6895*	6900	5f; S6e

a. the serial number for each primer (see Supplemental Table 3)

** indicates a 5'-Cy5.5 flouresence; ** indicates a 5'-Cy3 flouresence.*

123
124

Supplemental Table 2. The list of plamids used in this study

Plasmids	Backbone	Parental	Plasmid description	Source
#pMD001	pRET	/	pRET-GST-EGFP-Flag	This study
#pMD002	pRET	/	pRET-GST-mCherry-Flag	This study
#pMD007	pRSF	/	pRSF-EGFP-FUS-ERG	Pilong Li
#pMD014	pRET	/	pRET-GST-FLI1-Flag	This study
#pMD017	pRSF	/	pRSF-EGFP-EWSR1	This study
#pMD018	pRSF	/	pRSF-EGFP-EWS-FLI1	This study
#pMD019	pRET	**#pMD001**	pRET-GST-SYGQ2-FLI1-EGFP	This study
#pMD020	pRET	**#pMD002**	pRET-GST-SYGQ2-FLI1-mCherry	This study
#pMD023	pRET	**#pMD001**	pRET-GST-FLI1 C-term-EGFP	This study
#pMD070	pRET	**#pMD002**	pRET-GST-SYGQ2-mCherry	This study
#pMD129	pcDNA	/	pcDNA3.1-Flag-EGFP-XN	This study
#pMD160	pEasy-Blunt	/	pEasy-blunt-EME2(160bp)	This study
#pMD169	pEasy-Blunt	/	pEasy-blunt-10X interval GGAA	This study
#pMD170	pEasy-Blunt	/	pEasy-blunt-10X single GGAA binding site	This study
#pMD171	pEasy-Blunt	/	pEasy-blunt-10X tandem GGAA	This study
#pMD172	pEasy-Blunt	/	pEasy-blunt-multi-GGAA(ccnd1 200bp)	This study
#pMD210	pEasy-Blunt	/	pEasy-blunt GFP non-GGAA	This study
#pMD217	pRET	**#pMD001**	pRET-GST-SYGQ2-FLI1(YS)-EGFP	This study
#pMD221	pGEM-T	/	pGEM-FLI1 normal binding site	This study
#pMD222	pGEM-T	/	pGEM-FLI1 GGAAG binding site	This study
#pMD223	pGEM-T	/	pGEM-FLI1 GGAAA binding site	This study
#pMD224	pGEM-T	/	pGEM-FLI1 2XGGAA site	This study
#pMD225	pGEM-T	/	pGEM-FLI1 3XGGAA site	This study
#pMD226	pGEM-T	/	pGEM-FLI1 AAGG site	This study
#pMD271	pUC57	synthetic DNA	10x tandem 2xGGAA	This study
#pMD278	pGL3	/	pGL3-basic	Li Lab
#pMD279	pRL	/	pRL-TK	Li Lab
#pMD280	pGL3	**#pMD286**	pGL3-EME2-miniP	This study
#pMD281	pGL3	**#pMD286**	pGL3-CCND1-miniP	This study
#pMD282	pGL3	**#pMD286**	pGL3-10xETS-miniP	This study
#pMD283	pGL3	**#pMD286**	pGL3-10xGGAA-miniP	This study
#pMD284	pGL3	**#pMD286**	pGL3-interval GGAA-miniP	This study
#pMD285	pGL3	**#pMD286**	pGL3-10x 2GA-miniP	This study
#pMD286	pGL3	**#pMD278**	pGL3-miniP	This study
#pMD287	pcDNA	**#pMD129**	pCDNA3.1-Flag-FLI1	This study
#pMD288	pcDNA	**#pMD129**	pCDNA3.1-Flag-EWS-FLI1	This study

#pMD289	pcDNA	**#pMD129**	pCDNA3.1-Flag-EWS(YS)-FLI1	This study
#pMD327	pGL3	**#pMD286**	pGL3-EBF2-2K	This study
#pMD338	pGL3	**#pMD286**	pGL3-hEBF2-delGGAA	This study
#pMD345	pGL3	**#pMD286**	pGL3-XG-2K	This study
#pMD346	pGL3	**#pMD286**	pGL3-XG-2K-delGGAA	This study
#pMD359	pRET	**#pMD001**	pRET-GST-ETV6 FL-V5	This study
#pMD360	pRET	**#pMD001**	pRET-GST-ETV6 DBD-V5	This study
#pMD365	pRET	**#pMD001**	non-specific-2-GGAA	This study
#pMD366	pRET	**#pMD001**	non-specific-4-GGAA	This study
#pMD367	pRET	**#pMD001**	non-specific-6-GGAA	This study
#pMD368	pRET	**#pMD001**	non-specific-8-GGAA	This study
#pMD369	pRET	**#pMD001**	non-specific-10-GGAA	This study

Supplemental Table 3. The list of primers used in this study

Primers	name	Sequence
2353	EWSR1 1F	tccctcgagatggcgtccacggattacag
2354	EWSR1 1965R	catgcggccgcgtagggccgatctctgcgct
2355	FLI1 1F	tccctcgagATGGACGGGACTATTAAGGA
2356	FLI1 1356R	catgcggccgcGTAGTAGCTGCCTAAGTGTG
2400	CCND1_e_clon_F	ATCTGGACATGCTCTCGCTT
2401	CCND1_e_clon_R	TGGCCTTTACTAAATGGTGTGTA
2402	CCND1_e_prob_F	AGCCTGGCAGCAGAGCGA
2403	CCND1_e_prob_R	TTGTTGACTGCTTGATGGAAA
2434	EWS-FLI1 755F	gtcaatatagccaacagagcagcagctacgggcagcagaGTCCTCCCCTTGGAGGG
2435	EWSR1 793R	tctgctgcccgtagctgctgctctgttggctatattgacttg
2566	FLI1 stop NotI R	catgcggccgcTTAGTAGTAGCTGCCTAAG
2578	EWSR1 601F XhoI	tccctcgagactagttatgatcagagcag
3695	EME2 1kb-F	CACCCGCGTGAGGCGCCAG
3696	EME2 1kb-R	CTCGCTCGGGTCCACCGCAG
3697	EME2 150bp-F	CAGCCGCGGACGCACCTTC
3698	EME2 150bp-R	AACCCGCGCCATGGCCGGA
4409	non-specific GFP-F	atgaaagtgagcaagggcgaggag
4410	non-specific GFP-R	cttgaagaagtcgtgctgcttcatg
4739	GGAAG motif F	ttggCGTGGAAGTCa
4740	GGAAG motif R	gatctGACTTCCACGccaacta
4741	GGAAA motif F	ttggCGTGGAAATCa
4742	GGAAA motif R	gatctGATTTCCACGccaacta
4743	3xGGAA motif F	ttggCGTGGAAGGAAGGAAATCa
4744	3xGGAA motif R	gatctGATTTCCTTCCTTCCACGccaacta
4745	2xGGAA motif F	ttggCGTGGAAGGAAATCa
4746	2xGGAA motif R	gatctGATTTCCTTCCACGccaacta
4747	AAGGAAGG motif F	ttggCGTAAGGAAGGTCa
4748	AAGGAAGG motif R	gatctGACCTTCCTTACGccaacta
4767	miniP-F	gatctAGAGGGTATATAATGGAAGCTCGACTTCCAGa
4768	miniP-R	agcttCTGGAAGTCGAGCTTCCATTATATACCCTCTa
6362	GYG2_2K_KpnI_F	ATGGGTACCcttcgtagctgtgaatatgatgcc
6363	GYG2_2K_BamHI_R	ATGGGATCCgaactttcatactacagacctaag
6364	EBF2_2K_KpnI_F	ATGGGTACCcccactttccctatttgaacaactag
6365	EBF2_2K_BglII_R	ATGAGATCTctccagcaagggcaacaactaagg
6384	EBF2_overlap_F	catcagtaaattctgacctagagcgtcctccagcctttc
6385	EBF2_overlap_R	ggctggaggacgctctaggtcagaatttactgatgac

6386	GYG2_overlap_F	caacagagtgagactctcaaataaatttgattcttttcgccc
6387	GYG2_overlap_R	gggcgaaaagaatcaaatttatttgagagtctcactctgttgc
6394	mRplp0-qPCR-F	GAAACTGCTGCCTCACATCCG
6395	mRplp0-qPCR-R	GCTGGCACAGTGACCTCACACG
6396	mCidea_qPCR_F	GCAGCCTGCAGGAACTTATCAGC
6397	mCidea_qPCR_R	GATCATGAAATGCGTGTTGTCC
6398	mPpara_qPCR_F	GCAGTGCCCTGAACATCGA
6399	mPpara_qPCR_R	CGCCGAAAGAAGCCCTTAC
6400	mPparg_qPCR_F	CCGTAGAAGCCGTGCAAGAG
6401	mPparg_qPCR_R	GGAGGCCAGCATCGTGTAGA
6833	ETV6_SalI_F	ATGGTCGACATGTCTGAGACTCCTGCTCAGTG
6834	ETV6_300_SalI_F	ATGGTCGACGAAGGGAAGCCCATCAACCTCTC
6835	ETV6_XbaI_R	ACCTCTAGAGCATTCATCTTCTTGGTATATTTGTTC
6836	XbaI_V5_NotI_F	CTAGAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGTGAGC
6837	XbaI_V5_NotI_R	GGCCGCTCACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCT
6862	ETV6_NotI_R	ACCGCGGCCGCGCATTCATCTTCTTGGTATATTTGTTC
6885	2xGGAA_XN_1	TCGAGTTATCCGTGGAAGGAAATCTCGC
6886	2xGGAA_XN_2	GGCCGCGAGATTTCCTTCCACGGATAAC
6887	4xGGAA_XN_1	TCGAGTTATCCGTGGAAGGAAGGAAGGAAATCTCGC
6888	4xGGAA_XN_2	GGCCGCGAGATTTCCTTCCTTCCTTCCACGGATAAC
6889	6xGGAA_XN_1	TCGAGTTATCCGTGGAAGGAAGGAAGGAAGGAAGGAAATCTCGC
6890	6xGGAA_XN_2	GGCCGCGAGATTTCCTTCCTTCCTTCCTTCCTTCCACGGATAAC
6891	8xGGAA_XN_1	TCGAGTTATCCGTGGAAGGAAGGAAGGAAGGAAGGAAGGAAGGAAATCTCGC
6892	8xGGAA_XN_2	GGCCGCGAGATTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCACGGATAAC
6893	10xGGAA_XN_1	TCGAGTTATCCGTGGAAGGAAGGAAGGAAGGAAGGAAGGAAGGAAGGAAATCTCGC
6894	10xGGAA_XN_2	GGCCGCGAGATTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCACGGATAAC
6895	20230419_F	CAGGGATCCCTCGAGTTATCCG
6896	2GA_161_R	GTGGCATCGCCCTCGCCCTCG
6897	4GA_191_R	CTTCAGGGTCAGCTTGCCGTAG
6898	6GA_220_R	CTTGCCGGTGGTGCAGATGAAC
6899	8GA_252_R	GAGGGTGGGCCAGGGCAC
6900	10GA_280_R	CCGTAGGTCAGGGTGGTCACGAG
7007	pEASY Blunt F	TAGTCCTGCAGGTTTAAACG

Supplemental Table 4. The list of reagents or resource used in this study

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti V5-Tag mAb	Abclone	AE017
Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L)	AffiniPure	115-035-003
Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L)	AffiniPure	111-035-003
Bacterial and virus strains		
TOP10	Li Lab	N/A
BL21(DE3) pLysis codon+	Li Lab	N/A
Chemicals, peptides, and recombinant proteins		
Glutathione Sepharose 4B	GE	17-0756-01
L-Glutathione Reduced	Sigma	G4251
Ni-NTA beads	QIAGEN	30230
Imidazole	Sigma	I2399
1,6-hexanediol	Sigma	240117
Polyethylene Glycol 8000 (PEG-8000)	Fisher	BP233
Lipofectamine 3000 Transfection Reagent	Invitrogen	CW3007M
TRIzol Reagent	Invitrogen	15596018CN
PrimeScript RT reagent Kit	Takara	RR037A
Paraformaldehyde	Sigma	16005
Oil Red O	Sigma	O0625
Fetal Bovine Serum	Sigma	F8318
3,3',5-Triiodo-L-thyronine	Sigma	T2877
3-Isobutyl-1-methylxanthine	Sigma	I5879
Dexamethasone	Solarbio	D8040
Indomethacin	Sigma	I7378
Insulin	Sigma	I5500
Dual-Luciferase Reporter Assay system	Promega	E1910
2×SYBR Green qPCR Master Mix	EZBioscience	A0001-R2
GST-mCherry	This study	#Prt010
His-EGFP-FUS-ERG	This study	#Prt018
GST-FLI1-Flag	This study	#Prt016
His-EGFP-EWS-FLI1	This study	#Prt032
GST-SYGQ2-FLI1-EGFP	This study	#Prt047
GST-SYGQ2-FLI1-mCherry	This study	#Prt053
GST-FLI1 Ct	This study	#Prt052

GST-SYGQ2-mCherry	This study	#Prt020
GST-SYGQ2-FLI1(3YS)-EGFP	This study	#Prt049
GST-ETV6-V5	This study	#Prt054
GST-ETV6 DBD-V5	This study	#Prt055
Aldolase1 (75KD)	Li Lab	N/A
Ferritin1 (440KD)	Li Lab	N/A
Thyroglobin (669 KD)	Li Lab	N/A

Experimental models: Cell lines

Human: HEK293T	Li Lab	N/A
Mouse: brown adipocyte (BAC)	Xuyun Zhao	N/A