INVENTORY OF SUPPLEMENTAL INFORMATION

Structural Rules and Complex Regulatory Circuitry Constrain Expression of a Notch- and EGFR-Regulated Eye Enhancer

Christina I. Swanson, Nicole C. Evans, and Scott Barolo

SUPPLEMENTAL DATA

Table S1 provides GFP expression data from all transgenic lines examined. These data, requested by reviewers, are relevant to Figures 2 through 6.

Table S2 presents EMSA competition data discussed in the text, relevant to the conclusions drawn from Figure 2 in that they support the notion that the newly mapped regulatory sequences do not merely augment binding of the known regulators to their binding sites.

Figure S1 presents expression data discussed in the text, relevant to the proposal, related to Figure 5, that the Su(H)+Ets+Lz sites in *spa* are “poised” for activation in multiple cell types in the eye.

Table S3 summarizes evolutionary conservation analyses of *spa* and six other developmental enhancers, related to Figure 6 and discussed in the text. These data show that, although overall sequence conservation of *spa* is not unusual, this enhancer has relatively few blocks of contiguous conserved sequence. This is an interesting observation, given that we have shown that *spa* is very intolerant of experimental sequence alterations and rearrangements.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

SUPPLEMENTAL REFERENCES

SUPPLEMENTAL INFORMATION

Structural Rules and Complex Regulatory Circuitry Constrain Expression of a Notch- and EGFR-Regulated Eye Enhancer

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SUPPLEMENTAL DATA

Figure S1, A Synthetic Version of *spa*, Containing Only Su(H)+Lz+Ets Binding Sites, Is Poised for Activation in Multiple Cell Types of the Developing Eye (related to Figure 5)

spa(synth^{NS}), a synthetic enhancer containing the Su(H)+Lz+Ets sites from *spa* in their native arrangement, placed at -846 bp upstream of a promoter, is inactive in all cell types of the eye, in all 8 lines examined (A). When this construct was placed closer to the promoter (-121 bp), no expression was observed in 5 of 7 independent transgenic lines (B). However, 2 of 7 lines show insertion site-dependent activity in the eye: one line is active in cone cells, as shown by co-expression with Cut (C), while another line is active in multiple photoreceptors, as shown by co-expression with Elav (D).

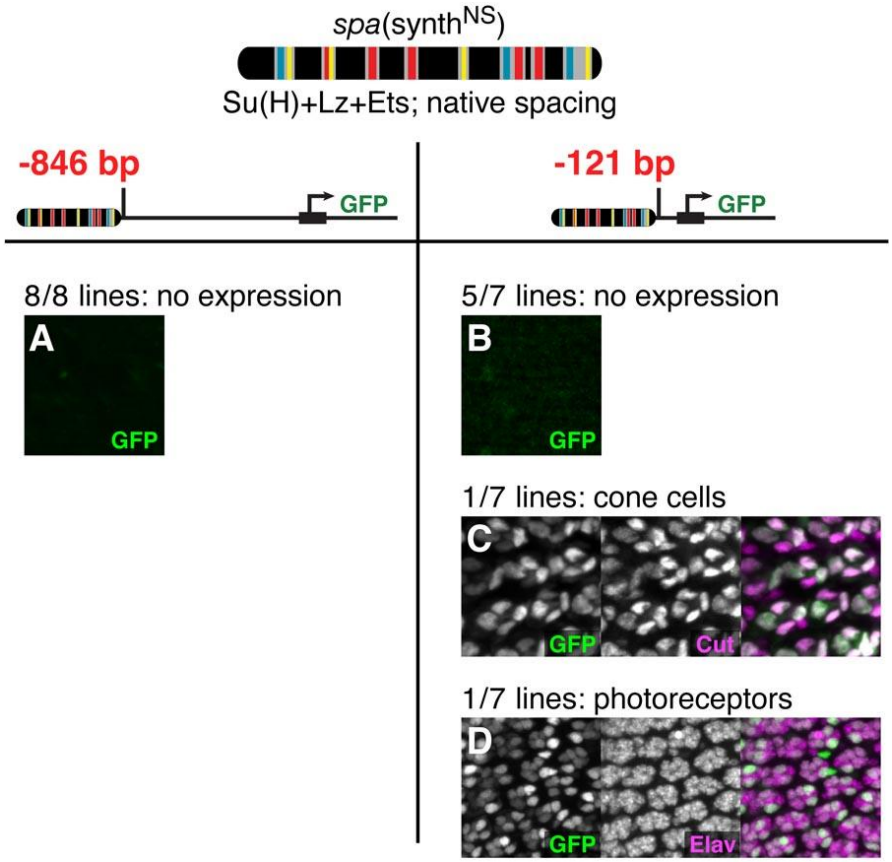

Table S1, Summary of Transgenic Reporter Expression Data (related to Figures 2-5)

Data are presented in a separate Excel file.

All constructs are placed at -846 bp from promoter, except those labeled "-121," which are placed at -121 bp. CC, cone cell expression; PR, photoreceptor 1+6 expression. 3 to 14 independently derived transgenic lines were examined for each construct. See Figure 2 for scoring key. Asterisks on lines of *spa*(synth^{CS}) and 2x*spa*(synth^{CS}) denote lines with weak and incomplete R1+R6 expression, plus weak-to-moderate expression at the posterior margin of the eye disc, which, based on z-position and nuclear size, is likely to include peripodial cells.

Table S2, Novel Regulatory Regions of *spa* Do Not Significantly Contribute to In Vitro Binding of the Known Regulatory TFs (related to Figure 2)

Experimental details and probe sequences are described in Supplemental Experimental Procedures. Probes include novel regulatory regions 1, 4, 5, or 6a, plus any immediately flanking Lz, Su(H), or Ets binding sites (see Figure 6A for annotated sequence). Left, Lz binding to sites flanking regions 1, 5, and 6a; Right, Su(H) binding to sites flanking regions 4 and 6a. Pnt binding was not examined, because the predicted Ets sites flanking these enhancer regions do not bind Pnt in vitro (Flores et al., 2000).

In all cases, mutations to novel regulatory regions of *spa* (all of which reduce enhancer activity in vivo) do not significantly affect binding of Lz or Su(H) to nearby sites. By contrast, binding is reduced in all cases by mutating the Lz or Su(H) sites themselves (red figures).

Note that mutating Su(H) sites has a milder effect on binding than mutating Lz sites, and that a greater excess of competitor is required for Su(H) than for Lz. This apparent difference in relative binding strength is consistent with the fact that the Lz sites perfectly match the optimal binding consensus, while the Su(H) sites deviate from the optimal consensus and are predicted to be lower-affinity sites (Flores et al., 2000; Nellesen et al., 1999).

Lz binding in vitro (given as % competition)				**Su(H) binding in vitro (given as % competition)**		
cold competitor fold excess:	**10x**	**100x**		cold competitor fold excess:	**100x**	**1000x**
Region 1:				**Region 4:**		
1 wt	32.8	74.2		4 wt	33.8	69.8
1 mBS	1.1	1.2		4 mBS	21.0	63.0
m1a	36.8	72.7		m4a	32.3	76.5
m1b	34.2	74.6		m4b	25.3	73.7
m1c	39.6	71.2		m4c	51.1	84.3
Region 5:				**Region 6a:**		
5 wt	37.7	79.4		6a wt	10.3	52.1
5 mBS	5.8	8.9		6a mBS	8.8	36.0
m5a	39.5	85.4		m6a	15.7	55.4
m5b	44.7	81.1				
m5c	39.7	78.4				
Region 6a:						
6a wt	35.1	75.1				
6a mBS	5.6	11.5				
m6a	44.4	72.6				

Table S3, *D. melanogaster* - *D. pseudoobscura* sequence identity and blocks of conservation within *spa* and six other developmental enhancers (related to Figure 6)

Enhancer	Total bp	Conserved bp (%)[a]	10/10 conserved blocks (%)[b]	20/20 conserved blocks (%)[b]
E(spl)m4[c]	279	87.8	74.6	58.1
***Su(H)*ASE**[d]	372	82.8	63.2	40.3
***dpp*VM**[e]	419	70.9	51.3	21.7
***eve*MHE**[f]	311	74.3	45.7	29.3
***eve*st.2**[g]	483	73.1	37.7	19.9
***dpp*D**[h]	372	69.6	36.5	26.1
mean±SEM (excluding *spa*)		**76.4**±3.0	**51.5** ±6.1	**32.6**±5.9
spa[i]	362	**64.6**	**3.9**	**0**
mean±SEM (including *spa*)		**74.7**±3.0	**44.7** ±8.5	**27.9** ±6.8

[a]Derived from BLASTZ alignments of orthologous enhancer sequences. [b]Defined as the total length of contiguous conserved sequences of ≥10 bp or ≥20 bp (counting gaps as mismatched bases) in a BLASTZ alignment, as a percentage of total enhancer length.
[c]Nellesen et al. (1999). [d]Barolo et al. (2000). [e]Sun et al. (1995); Yang et al. (2000); Zaffran et al. (2001); Stultz et al. (2006). [f]Halfon et al. (2000). [g]Small et al. (1992); Arnosti et al. (1996); Andrioli et al. (2002). [h]Müller and Basler (2000). [i]Flores et al. (2000).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Protein Expression and Purification

Lozenge protein was expressed using the TNT T7 Coupled Reticulocyte Lysate System (Promega) from pET3c-Lz (kindly provided by Richard Mann, Columbia University). His-tagged Su(H) was expressed from pRSET-6XHis-Su(H) (kindly provided by Jim Posakony, University of California, San Diego). RosettaBlue (DE3) Competent Cells (Novagen) transformed with pRSET-6XHis-Su(H) were grown overnight in 250 ml of LB plus 30 µg/ml carbenicillin in a 37°C shaking incubator. The next morning, cells were spun down for 10 min at 6000 rpm, and the pellet was resuspended in 10 ml LB. Two 500 ml LB+carb cultures were each inoculated with 2.5 ml of resuspended cells and grown in a 30°C shaking incubator until they reached an OD_{600} of 0.6-0.7. Each 500 ml culture was then induced with 500 µl 1M IPTG and grown at 30°C for an additional 2 hours. Cultures were spun down at 4°C for 10 min at 6000 rpm. Each pellet was resuspended on ice in 10 ml of lysis buffer (0.1 M NaCl, 0.1% Tween-20, 10 mM Tris-Cl (pH 8.0), 5 mM Imidazole, 1 mM DTT, 1 mM PMSF, 1 tablet Complete Mini EDTA-free (Roche)). Cells were lysed at 4°C by sonication (60% power, 5 X 30s, at 30s intervals), then centrifuged for 20 min at 10,000 rpm at 4°C. All following purification steps took place in a 4°C cold room. Two 0.8 X 4 cm Poly-Prep Chromatography Columns (Bio-Rad) were packed with 0.15 ml of Ni-NTA agarose (Qiagen) and cleared with 10 ml Wash Buffer 1 (0.1 M NaCl, 0.1% Tween-20, 10 mM Tris-Cl (pH 8.0), 5 mM Imidazole). Ten ml of supernatant was then applied to each column. Each column was washed with 1 ml Wash Buffer 1, followed by 1 ml Wash Buffer 2 (0.1 M NaCl, 10 mM Tris-Cl (pH 8.0), 15 mM Imidazole). Protein was eluted with 6 X 100 µl elution buffer (0.1 M NaCl, 10 mM Tris-Cl (pH 8.0), 200 mM Imidazole, pH adjusted to 8.0). Elutions containing protein were pooled and glycerol was added to 10% final concentration before being aliquoted, flash frozen with dry ice and ethanol, and stored at -80°C.

In Vitro Binding Assays

Protein-DNA binding was quantitated in vitro with electrophoretic mobility shift assays (EMSAs); relative affinities were determined by competing unlabeled (“cold”) DNA probes against radiolabeled (“hot”) probes for protein binding. DNA probes were made using custom oligos from either Invitrogen or IDT. Gel shift probe labeling reactions contained 37 µl dH_2O, 5 µl 10X PNK Buffer, 1 µl top strand oligo (2 µM), 1 µl bottom strand oligo (2 µM), 5 µl $\gamma^{32}P$, and 1 µl T4 PNK (NEB). Reactions were incubated at 37°C for one hour, boiled at 80°C for 5 min, then allowed to cool slowly to room temperature. Labeled probes were purified twice using Illustra ProbeQuant G-50 Micro Columns (GE). The Lz labeled probe contained the first Lz site from *spa* along with flanking sequence. The Su(H) labeled probe contained the third Su(H) site from *spa* along with flanking sequence. The sequences of labeled probes are below, with Lz sites in blue, Su(H) sites in red, and binding site mutations in bold and underlined:

Lz: aaaatttactatGACCGCAaagctgtttcc
MutLz: aaaatttactatGA**AA**GCAaagctgtttcc
Su(H): tcaagatcttaTTCACATTgaaattgaagc
MutSu(H): tcaagatcttaTT**GGG**ATTgaaattgaagc

Cold competitors were assembled as follows: 10X - 25 µl dH_2O, 5 µl 10X PNK Buffer, 10 µl top strand oligo (2 µM), 10 µl bottom strand oligo (2 µM); 100X - 43 µl dH_2O, 5 µl 10X PNK Buffer, 1 µl top strand oligo (200 µM), 1 µl bottom strand oligo (200 µM); 1000X - 25 µl dH_2O, 5 µl 10X PNK Buffer, 10 µl top strand oligo (200 µM), 10 µl bottom strand oligo (200 µM). Cold competitors were incubated at 37°C for one hour, boiled at 80°C for 5 min, then allowed to cool slowly to room temperature. Sequences of cold competitor probes match the sequences of *in vivo* reporter constructs, and include region of interest along with adjacent binding sites and additional flanking sequence on either side. Sequences of cold competitors are below, with Lz sites in blue, Su(H) sites in red, Ets sites in green, binding site mutations in bold and underlined, and mutations outside binding sites in alternating caps and lower case, where the bases in caps have been mutated by non-complementary transversion:

```
1_wt:   gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtttc
m1a:    gGaGcCaTtCactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtttc
m1b:    gtatcaagtaacGgTgGgAcGaattgaaaaaatttactatGACCGCAaagctgtttc
m1c:    gtatcaagtaactgggtgcctaCtGgCaCaCatttactatGACCGCAaagctgtttc
1_mBS:  gtatcaagtaactgggtgcctaattgaaaaaatttactatGAAAGCAaagctgtttc

4_wt:     caagatcttaTTCACATTgaaattgaagcactattggtgtacgattacaacgctcac-
              attatcaGGATataaaaaaaa
m4abc:    caagatcttaTTCACATTgaaaGtTaCgAaAtCtGgTtTtCcTaGtCcCaAgAtAaA-
              aGtCtcaGGATataaaaaaaa
m4a:      caagatcttaTTCACATTgaaCtGgCaTcCcGaGtggtgtacgattacaacgctcac-
              attatcaGGATataaaaaaaa
m4b:      caagatcttaTTCACATTgaaattgaagcactattTgGgGaAgCtGaAaacgctcac-
              attatcaGGATataaaaaaaa
m4c:      caagatcttaTTCACATTgaaattgaagcactattggtgtacgattacCaAgAtAaA-
              aGtCtcaGGATataaaaaaaa
4_mBS:    caagatcttaTTGACATTgaaattgaagcactattggtgtacgattacaacgctcac-
              attatcaTTATataaaaaaaa

5_wt:   ttatcaGGATataaaaaaaaggtgatagtaattcagcacgactttgtAACCACAaatata
m5abc:  ttatcaGGATataaCaCaCaTgGgCtCgGaCtGcCgAaAgCcAttgtAACCACAaatata
m5a:    ttatcaGGATataCaCaCaCgTtgatagtaattcagcacgactttgtAACCACAaatata
m5b:    ttatcaGGATataaaaaaaaggtTaGaTtCaGtAagcacgactttgtAACCACAaatata
m5c:    ttatcaGGATataaaaaaaaggtgatagtaattcaTcCcTaAtGtgtAACCACAaatata
5mBS:   ttatcaTTATataaaaaaaaggtgatagtaattcagcacgactttgtAAAAACAaatata

6a_wt:   cagattactcCGTGAGTAcaacgtaagtcgggtgaagccagaAACCACAaatcaagttg
m6a:     cagattactcCGTGAGTAcaacTtCaTtAgTgGgCaTcAagaAACCACAaatcaagttg
6a_mBS:  cagattactcCGTGACTAcaacgtaagtcgggtgaagccagaAAAACACaatcaagttg
```

Gel shift reactions were assembled on ice and contained 1 µl 10X Gel Shift Buffer (0.1 M Tris-HCl pH 7.5, 0.5 M NaCl, 10 mM DTT, 10 mM EDTA, 275 µg/ml salmon sperm DNA), 1 µl poly d(I-C) (1 mg/ml), 1 µl DTT (100 µM), 1 µl labeled probe, 1 µl competitor (if included), and protein and dH_2O to a final volume of 10 µl. Lozenge gel shift reactions contained 3 µl of TNT reaction, while Su(H) gel shift reactions contained 5 µl of purified 6XHis-Su(H). Gel shift reactions were incubated on ice for 15 min before being loaded into 5% or 6% acrylamide gels that had been pre-run for 30 min; gels were then run at 120-140V in 0.5X TBE. Gels were dried for one hour at 80°C, exposed overnight to a storage phosphor screen (GE), and scanned using a Typhoon 9400 Variable Mode Imager. Quantification was performed using ImageJ. Calculations of competition were based on measurements of mean signal from which background had been subtracted.

Complete Sequences of Enhancer Constructs

Blue indicates Lz binding sites; red, Su(H) binding sites; and green, Ets (Pnt/Yan) binding sites, as determined by Flores et al. (2000). Wild-type *sparkling* sequences, outside of Lz, Su(H), and Ets binding sites, are in lower case. Where lower case alternates with capitalized sequence, mutated bases (in caps) are subjected to non-complementary transversions.

From Figure 1:

spa(wt)

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtTTCCtgactatgacatagtttttt
ttgctttggtTGTGGGATgtaaatggtcattggaactggacgctgtccctgtcTTCTCACTaagttaatgatcgtaca
acctcaagatcttaTTCACATTgaaattgaagcactattggtgtacgattacaacgctcacattatcaGGATataaaa
aaaaggtgatagtaattcagcacgactttgtAACCACAaataTATGGGAAcacagattactcCGTGAGTAcaacgtaa
gtcgggtgaagccagaAACCACAaatcaagttgttTTCCggtagcttagg

spa(synth^{CS})

tGACCGCAaCgAtTtTTCCtTaAtCtGtTgtTGTGGGATgGaCaGgGcCttggaacGgATCcAtTtcTTCTCACTaCg
TtCaGcGtaTTCACATTgCaCtGtGaGcaGGATaGaCaGAAttCtAACCACAaCtaTATGGGAAcCcCgCtGaAcCGT
GAGTAcCaAgGgAcCgaAACCACAaCtAaCgGtTttTTCCg

spa(synth^{NS})

gGaGcCaTtCaAtTgTtTcAtCaGtTaCaCaCtGtCctatGACCGCAaagAtgtTTCCtgacGaGgCcCtCgGtGtGt
GtTcGtGggtTGTGGGATgtaaCtTgGcattggaactggCcTcGgGcAcGgtcTTCTCACTaagtGaCtTaGcTtCcC
aAcGcCaTaGcttaTTCACATTgaaaGtTaCgAaAtCtGgTtTtCcTaGtCcCaAgAtAaAaGtCtcaGGATataaCa
CaCaTgGgCtCgGaCtGcCgAaAgCcAttgtAACCACAaataTATGGGAAcacaTaGtCctcCGTGAGTAcaacTtCa
TtAgTgGgCaTcAagaAACCACAaatcCaTtGgttTTCCggtaTcGtCgT

From Figure 2:

spa(Δ1)

tGACCGCAaagctgtTTCCtgactatgacatagttttttttgctttggtTGTGGGATgtaaatggtcattggaactgg
acgctgtccctgtcTTCTCACTaagttaatgatcgtacaacctcaagatcttaTTCACATTgaaattgaagcactatt
ggtgtacgattacaacgctcacattatcaGGATataaaaaaaaggtgatagtaattcagcacgactttgtAACCACAa
ataTATGGGAAcacagattactcCGTGAGTAcaacgtaagtcgggtgaagccagaAACCACAaatcaagttgttTTCC
ggtagcttagg

spa(Δ2)

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaCgAtTtTTCCtTaGAattCTgtTGTGGGAT
gGaCaGgGcCttggaactggacgctgtccctgtcTTCTCACTaagttaatgatcgtacaacctcaagatcttaTTCAC
ATTgaaattgaagcactattggtgtacgattacaacgctcacattatcaGGATataaaaaaaaggtgatagtaattca
gcacgactttgtAACCACAaataTATGGGAAcacagattactcCGTGAGTAcaacgtaagtcgggtgaagccagaAAC
CACAaatcaagttgttTTCCggtagcttagg

spa(Δ3)

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtTTCCtgactatgacatagtttttt
ttgctttggtTGTGGGATgtaaatggtcattggaacGTcTCcAtTtcTTCTCACTaCggAaTtcGtaTTCACATTgaa
attgaagcactattggtgtacgattacaacgctcacattatcaGGATataaaaaaaaggtgatagtaattcagcacga
ctttgtAACCACAaataTATGGGAAcacagattactcCGTGAGTAcaacgtaagtcgggtgaagccagaAACCACAaa
tcaagttgttTTCCggtagcttagg

spa(Δ4)

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtTTCCtgactatgacatagtttttt
ttgctttggtTGTGGGATgtaaatggtcattggaactggacgctgtccctgtcTTCTCACTaagttaatgatcgtaca
acctcaagatcttaTTCACATTgCaGAAttCGcaGGATataaaaaaaaggtgatagtaattcagcacgactttgtAAC
CACAaataTATGGGAAcacagattactcCGTGAGTAcaacgtaagtcgggtgaagccagaAACCACAaatcaagttgt
tTTCCggtagcttagg

spa(Δ5)

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtTTCCtgactatgacatagtttttt
ttgctttggtTGTGGGATgtaaatggtcattggaactggacgctgtccctgtcTTCTCACTaagttaatgatcgtaca
acctcaagatcttaTTCACATTgaaattgaagcactattggtgtacgattacaacgctcacattatcaGGATaGaCaG
AAttCtAACCACAaCtaTATGGGAAcCcCgCtGaAtcCGTGAGTAcaacgtaagtcgggtgaagccagaAACCACAaa
tcaagttgttTTCCggtagcttagg

spa(Δ6)

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtTTCCtgactatgacatagtttttt
ttgctttggtTGTGGGATgtaaatggtcattggaactggacgctgtccctgtcTTCTCACTaagttaatgatcgtaca
acctcaagatcttaTTCACATTgaaattgaagcactattggtgtacgattacaacgctcacattatcaGGATataaaa
aaaaggtgatagtaattcagcacgactttgtAACCACAaataTATGGGAAcacagattactcCGTGAGTAcCaAgGgA
cCgaAACCACAaCtAaCgGtTttTTCCgtctaga

spa(m2^{NS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagAtgtTTCCtgacGaGgCcCtCgGtGtGt
GtTcGtGggtTGTGGGATgtaaCtTgGcattggaactggacgctgtccctgtcTTCTCACT…

spa(m4^{NS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtTTCCtgactatgacatagtttttt
ttgctttggtTGTGGGATgtaaatggtcattggaactggacgctgtccctgtcTTCTCACTaagttaatgatcgtaca
acctcaagatcttaTTCACATTgaaaGtTaCgAaAtCtGgTtTtCcTaGtCcCaAgAtAaAaGtCtcaGGAT…

spa(m5^{NS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtTTCCtgactatgacatagtttttt
ttgctttggtTGTGGGATgtaaatggtcattggaactggacgctgtccctgtcTTCTCACTaagttaatgatcgtaca
acctcaagatcttaTTCACATTgaaattgaagcactattggtgtacgattacaacgctcacattatcaGGATataaCa
CaCaTgGgCtCgGaCtGcCgAaAgCcAttgtAACCACAaataTATGGGAAcacaTaGtCctcCGTGAGTA…

spa(m6a^{NS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtTTCCtgactatgacatagtttttt
ttgctttggtTGTGGGATgtaaatggtcattggaactggacgctgtccctgtcTTCTCACTaagttaatgatcgtaca
acctcaagatcttaTTCACATTgaaattgaagcactattggtgtacgattacaacgctcacattatcaGGATataaaa
aaaaggtgatagtaattcagcacgactttgtAACCACAaataTATGGGAAcacagattactcCGTGAGTAcaacTtCa
TtAgTgGgCaTcAagaAACCACAaatcaagttgttTTCCggtagcttagg

From Figure 3:

spa(m1a^{NS})

gGaGcCaTtCactgggtgcctaattgaaaaaatttactatGACCGCA…

spa(m1b^{NS})

gtatcaagtaacGgTgGgAcGaattgaaaaaatttactatGACCGCA…

spa(m1c^{NS})

gtatcaagtaactgggtgcctaCtGgCaCaCatttactatGACCGCA…

spa(m4a^{NS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtTTCCtgactatgacatagtttttt
ttgctttggtTGTGGGATgtaaatggtcattggaactggacgctgtccctgtcTTCTCACTaagttaatgatcgtaca
acctcaagatcttaTTCACATTgaaCtGgCaTcCcGaGtggtgtacgattacaacgctcacattatcaGGAT…

spa(m4b^{NS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtTTCCtgactatgacatagtttttt
ttgctttggtTGTGGGATgtaaatggtcattggaactggacgctgtccctgtcTTCTCACTaagttaatgatcgtaca
acctcaagatcttaTTCACATTgaaattgaagcactattTgGgGaAgCtGaAaacgctcacattatcaGGAT…

spa(m4c^{NS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtTTCCtgactatgacatagttttttttgctttggtTGTGGGATgtaaatggtcattggaactggacgctgtccctgtcTTCTCACTaagttaatgatcgtacaacctcaagatcttaTTCACATTgaaattgaagcactattggtgtacgattacCaAgAtAaAaGtCtcaGGAT…

spa(m5a^{NS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtTTCCtgactatgacatagttttttttgctttggtTGTGGGATgtaaatggtcattggaactggacgctgtccctgtcTTCTCACTaagttaatgatcgtacaacctcaagatcttaTTCACATTgaaattgaagcactattggtgtacgattacaacgctcacattatcaGGATataCaCaCaCgTtgatagtaattcagcacgactttgtAACCACA…

spa(m5b^{NS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtTTCCtgactatgacatagttttttttgctttggtTGTGGGATgtaaatggtcattggaactggacgctgtccctgtcTTCTCACTaagttaatgatcgtacaacctcaagatcttaTTCACATTgaaattgaagcactattggtgtacgattacaacgctcacattatcaGGATataaaaaaaaggtTaGaTtCaGtAagcacgactttgtAACCACA…

spa(m5c^{NS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtTTCCtgactatgacatagttttttttgctttggtTGTGGGATgtaaatggtcattggaactggacgctgtccctgtcTTCTCACTaagttaatgatcgtacaacctcaagatcttaTTCACATTgaaattgaagcactattggtgtacgattacaacgctcacattatcaGGATataaaaaaaaggtgatagtaattcaTcCcTaAtGtgtAACCACA…

spa(m5d^{NS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtTTCCtgactatgacatagttttttttgctttggtTGTGGGATgtaaatggtcattggaactggacgctgtccctgtcTTCTCACTaagttaatgatcgtacaacctcaagatcttaTTCACATTgaaattgaagcactattggtgtacgattacaacgctcacattatcaGGATataaaaaaaaggtgatagtaattcagcacgactttgtAACCACAaataTATGGGAAcacCgCtGactcCGTGAGTA…

spa(m6b^{NS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagctgtTTCCtgactatgacatagttttttttgctttggtTGTGGGATgtaaatggtcattggaactggacgctgtccctgtcTTCTCACTaagttaatgatcgtacaacctcaagatcttaTTCACATTgaaattgaagcactattggtgtacgattacaacgctcacattatcaGGATataaaaaaaaggtgatagtaattcagcacgactttgtAACCACAaataTATGGGAAcacagattactcCGTGAGTAcaacgtaagtcgggtgaagccagaAACCACAaatcCaTtGgttTTCCggtaTcGtagg

From Figure 5:

Mutated bases are bold and underlined.

spa(KO)

gtatcaagtaactgggtgcctaattgaaaaaatttactatGA**AA**GCAaagctgtTT**AA**tgactatgacatagttttttttgctttggtTGTGG**C**ATgtaaatggtcatt**TT**aactggacgctgtccctgtcTT**G**TCACTaagttaatgatcgtacaacctcaagatcttaTT**G**ACATTgaaattgaagcactattggtgtacgattacaacgctcacattatca**TT**ATataaaaaaaaggtgatagtaattcagcacgactttgtAA**AA**ACAaataTATGG**C**AAcacagattactcCGTGA**C**TAcaacgtaagtcgggtgaagccagaAA**AA**ACAaatcaagttg**TT**TTaaggtagcttagg

spa(KO+synth^{CS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatGA**AA**GCAaagctgtTT**AA**tgactatgacatagttttttttgctttggtTGTGG**C**ATgtaaatggtcatt**TT**aactggacgctgtccctgtcTT**G**TCACTaagttaatgatcgtacaacctcaagatcttaTT**G**ACATTgaaattgaagcactattggtgtacgattacaacgctcacattatca**TT**ATataaaaaaaaggtgatagtaattcagcacgactttgtAA**AA**ACAaataTATGG**C**AAcacagattactcCGTGA**C**TAcaacgtaagtcgggtgaagccagaAA**AA**ACAaatcaagttg**TT**TTaaggtagcttaggCTCGAGtGACCGCAaCgAtTtTTCCtTaAtCtGtTgtTGTGGGATgGaCaGgGcCttggaacGgATCcAtTtcTTCTCACTaCgTtCaGcGtaTTCACATTgCaCtGtGaGcaGGATaGaCaGAAttCtAACCACAaCtaTATGGGAAcCcCgCtGaAcCGTGAGTAcCaAgGgAcCgaAACCACAaCtAaCgGtTttTTCCg

spa(KO+synth^{NS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatGA**AA**GCAaagctgtTT**AA**tgactatgacatagttttttttgctttggtTGTGG**C**ATgtaaatggtcatt**TT**aactggacgctgtccctgtcTT**G**TCACTaagttaatgatcgtacaacctcaagatcttaTT**G**ACATTgaaattgaagcactattggtgtacgattacaacgctcacattatca**TT**ATataaaaaaaaggtgatagtaattcagcacgactttgtAA**AA**ACAaataTATGG**C**AAcacagattactcCGTGA**C**TAcaacgtaagtcgggtgaagccagaAA**AA**ACAaatcaagttg**TT**TTaaggtagcttaggCTCGAGgGaGcCaTtCaAtTgTtTcAtC

aGtTaCaCaCtGtCctatGACCGCAaagAtgtTTCCtgacGaGgCcCtCgGtGtGtGtTcGtGggtTGTGGGATgtaa
CtTgGcattggaactggCcTcGgGcAcGgtcTTCTCACTaagtGaCtTaGcTtCcCaAcGcCaTaGcttaTTCACATT
gaaaGtTaCgAaAtCtGgTtTtCcTaGtCcCaAgAtAaAaGtCtcaGGATataaCaCaCaTgGgCtCgGaCtGcCgAa
AgCcAttgtAACCACAaataTATGGGAAcacaTaGtCctcCGTGAGTAcaacTtCaTtAgTgGgCaTcAagaAACCAC
AaatcCaTtGgttTTCCggtaTcGtCgT

spa(1+4+6a+synth^{CS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatCCTAGGAGATCTgaaattgaagcactattggtgtacga
ttacaacgctcacattatcaGCCGGCGGTACCcaacgtaagtcgggtgaagccagaAAaaActcgagtGACCGCAaCg
AtTtTTCCtTaAtCtGtTgtTGTGGGATgGaCaGgGcCttggaacGgATCcAtTtcTTCTCACTaCgTtCaGcGtaTT
CACATTgCaCtGtGaGcaGGATaGaCaGAAttCtAACCACAaCtaTATGGGAAcCcCgCtGaAcCGTGAGTAcCaAgG
gAcCgaAACCACAaCtAaCgGtTttTTCCg

spa(1+4+6a+synth^{CS}, ΔLz)

gtatcaagtaactgggtgcctaattgaaaaaatttactatCCTAGGAGATCTgaaattgaagcactattggtgtacga
ttacaacgctcacattatcaGCCGGCGGTACCcaacgtaagtcgggtgaagccagaAAaaActcgagtGA**AA**GCAaCg
AtTtTTCCtTaAtCtGtTgtTGTGGGATgGaCaGgGcCttggaacGgATCcAtTtcTTCTCACTaCgTtCaGcGtaTT
CACATTgCaCtGtGaGcaGGATaGaCaGAAttCtAA**AA**ACAaCtaTATGGGAAcCcCgCtGaAcCGTGAGTAcCaAgG
gAcCgaAA**AA**ACAaCtAaCgGtTttTTCCg

spa(1+4+6a+synth^{CS}, ΔEts)

gtatcaagtaactgggtgcctaattgaaaaaatttactatCCTAGGAGATCTgaaattgaagcactattggtgtacga
ttacaacgctcacattatcaGCCGGCGGTACCcaacgtaagtcgggtgaagccagaAAaaActcgagtGACCGCAaCg
AtTtTT**AA**tTaAtCtGtTgtTGTGGGATgGaCaGgGcCtt**TT**aacGgATCcAtTtcTTCTCACTaCgTtCaGcGtaTT
CACATTgCaCtGtGaGca**TT**ATaGaCaGAAttCtAACCACAaCtaTATGGGAAcCcCgCtGaAcCGTGAGTAcCaAgG
gAcCgaAACCACAaCtAaCgGtTttTT**AA**g

spa[1+4+6a+synth^{CS}, ΔSu(H)]

gtatcaagtaactgggtgcctaattgaaaaaatttactatCCTAGGAGATCTgaaattgaagcactattggtgtacga
ttacaacgctcacattatcaGCCGGCGGTACCcaacgtaagtcgggtgaagccagaAAaaActcgagtGACCGCAaCg
AtTtTTCCtTaAtCtGtTgtTGTGG**C**ATgGaCaGgGcCttggaacGgATCcAtTtcTT**G**TCACTaCgTtCaGcGtaTT
GACATTgCaCtGtGaGcaGGATaGaCaGAAttCtAACCACAaCtaTATGG**C**AAcCcCgCtGaAcCGTGA**C**TAcCaAgG
gAcCgaAACCACAaCtAaCgGtTttTTCCg

spa(1+6a+synth^{CS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatCCTAGGGGTACCcaacgtaagtcgggtgaagccagaAA
aaACctcgagtGACCGCAaCgAtTtTTCCtTaAtCtGtTgtTGTGGGATgGaCaGgGcCttggaacGgATCcAtTtcT
TCTCACTaCgTtCaGcGtaTTCACATTgCaCtGtGaGcaGGATaGaCaGAAttCtAACCACAaCtaTATGGGAAcCcC
gCtGaAcCGTGAGTAcCaAgGgAcCgaAACCACAaCtAaCgGtTttTTCCg

spa(2Xsynth^{CS})

tGACCGCAaCgAtTtTTCCtTaAtCtGtTgtTGTGGGATgGaCaGgGcCttggaacGgATCcAtTtcTTCTCACTaCg
TtCaGcGtaTTCACATTgCaCtGtGaGcaGGATaGaCaGAAttCtAACCACAaCtaTATGGGAAcCcCgCtGaAcCGT
GAGTAcCaAgGgAcCgaAACCACAaCtAaCgGtTttTTCCgctcgagtGACCGCAaCgAtTtTTCCtTaAtCtGtTgt
TGTGGGATgGaCaGgGcCttGGAAcGgATCcAtTtcTTCTCACTaCgTtCaGcGtaTTCACATTgCaCtGtGaGcaGG
ATaGaCaGAAttCtAACCACAaCtaTATGGGAAcCcCgCtGaAcCGTGAGTAcCaAgGgAcCgaAACCACAaCtAaCg
GtTttTTCCg

spa(2Xsynth^{NS})

gGaGcCaTtCaAtTgTtTcAtCaGtTaCaCaCtGtCctatGACCGCAaagAtgtTTCCtgacGaGgCcCtCgGtGtGt
GtTcGtGggtTGTGGGATgtaaCtTgGcattggaactggCcTcGgGcAcGgtcTTCTCACTaagtGaCtTaGcTtCcC
aAcGcCaTaGcttaTTCACATTgaaaGtTaCgAaAtCtGgTtTtCcTaGtCcCaAgAtAaAaGtCtcaGGATataaCa
CaCaTgGgCtCgGaCtGcCgAaAgCcAttgtAACCACAaataTATGGGAAcacaTaGtCctcCGTGAGTAcaacTtCa
TtAgTgGgCaTcAagaAACCACAaatcCaTtGgttTTCCggtaTcGtCgTGGATCCgGaGcCaTtCaAtTgTtTcAtC
aGtTaCaCaCtGtCctatGACCGCAaagAtgtTTCCtgacGaGgCcCtCgGtGtGtGtTcGtGggtTGTGGGATgtaa
CtTgGcattGGAActggCcTcGgGcAcGgtcTTCTCACTaagtGaCtTaGcTtCcCaAcGcCaTaGcttaTTCACATT
gaaaGtTaCgAaAtCtGgTtTtCcTaGtCcCaAgAtAaAaGtCtcaGGATataaCaCaCaTgGgCtCgGaCtGcCgAa
AgCcAttgtAACCACAaataTATGGGAAcacaTaGtCctcCGTGAGTAcaacTtCaTtAgTgGgCaTcAagaAACCAC
AaatcCaTtGgttTTCCggtaTcGtCgT

spa(Δ1+1)

Repositioned sequence (region 1) is bold and underlined.

tGACCGCAaagctgtTTCCtgactatgacatagttttttttgctttggtTGTGGGATgtaaatggtcattggaactgg
acgctgtccctgtcTTCTCACTaagttaatgatcgtacaacctcaagatcttaTTCACATTgaaattgaagcactatt
ggtgtacgattacaacgctcacattatcaGGATataaaaaaaaggtgatagtaattcagcacgactttgtAACCACAa
ataTATGGGAAcacagattactcCGTGAGTAcaacgtaagtcgggtgaagccagaAACCACAaatcaagttgttTTCC
ggtagcttagg**gtatcaagtaactgggtgcctaattgaaaaaatttactat**

spa(TF scrambled)

Repositioned sequences are bold and underlined.

gtatcaagtaactgggtgcctaattgaaaaaatttact**taTATGGGAAca**gc**tatGACCGCAaa**actatgacatagtt
ttttttgctttg**gaAACCACAaa**aaatggtca**caGGATat**ggacgctgtccctg**gtTTCCtg**gttaatgatcgtacaa
cctcaagatct**ttGGAAct**aattgaagcactattggtgtacgattacaacgctcacattat**taTTCACATTgaa**aaaa
aaaggtgatagtaattcagcacgactttg**tcCGTGAGTAcagtTGTGGGATgt**cagattac**ttTTCCgg**acgtaagtcg
ggtgaagcca**gtAACCACAaa**tcaagttg**tcTTCTCACTaa**tagcttagg

spa(m2,3,6b^{NS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagAtgtTTCCtgacGaGgCcCtCgGtGtGt
GtTcGtGggtTGTGGGATgtaaCtTgGcattggaactggCcTcGgGcAcGgtcTTCTCACTaagtGaCtTaGcTtCcC
aAcGcCaTaGcttaTTCACATTgaaattgaagcactattggtgtacgattacaacgctcacattatcaGGATataaaa
aaaaggtgatagtaattcagcacgactttgtAACCACAaataTATGGGAAcacagattactcCGTGAGTAcaacgtaa
gtcgggtgaagccagaAACCACAaatcCaTtGgttTTCCggtaTcGtCgT

spa(m2,3,5,6b^{NS})

gtatcaagtaactgggtgcctaattgaaaaaatttactatGACCGCAaagAtgtTTCCtgacGaGgCcCtCgGtGtGt
GtTcGtGggtTGTGGGATgtaaCtTgGcattggaactggCcTcGgGcAcGgtcTTCTCACTaagtGaCtTaGcTtCcC
aAcGcCaTaGcttaTTCACATTgaaattgaagcactattggtgtacgattacaacgctcacattatcaGGATataaCa
CaCaTgGgCtCgGaCtGcCgAaAgCcGttgtAACCACAaataTATGGGAAcacaTaGtCctcCGTGAGTAcaacgtaa
gtcgggtgaagccagaAACCACAaatcCaTtGgttTTCCggtaTcGtCgT

From Figure 6:

D. pseudoobscura spa

Predicted Lz/Ets/Su(H) binding sites are underlined and in caps.

gtctcaaataacttcgtgtctaattgaaaaaatgcATCCtGACCGCAaggtgttTTCCtggctctgatggtatttttc
ttttgttgtttttttttatttgtattggtgttgatggtcactggagaggagaagtccactggctctggctgtggctct
gtctctgcatgctaTTCACATGgtgatcaagcaacctaaagctctgaatcagactcagattaagagacaatcagagat
atgatagtatagattacattacaacattcacggtaacGGAAtagacctaataaggtggctgtacagcgcaagtgcaag
tctatAACCACAtaaatacgagcttacgattcagtactatataaatcaggtgaacaccaaagccactaaatctaaaat
cagtTTCCggcTTCCtgtg

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