Supporting Information for:

Caught in the act: covalent crosslinking captures activator-coactivator interactions in vivo

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General Methods

Yeast strain LS41 [JPY9::pZZ41, *Mat*α *his3*Δ*200 leu2*Δ*1 trp1*Δ*63 ura3-52 lys2*Δ*385 gal4* URA::pZZ41] was used for activity and crosslinking experiments and to generate deletion strains (*1*). Strains in which Swi1 and Snf5 are deleted were constructed by gene disruption via PCR in LS41 in accordance with standard protocols (*2*). pBpa was purchased from Chem-Impex International (Wood Dale, IL). All plasmids described below were constructed using standard molecular biology techniques. The sequences of all the isolated plasmids were verified by sequencing at the University of Michigan Core Facility (Ann Arbor, MI).

Table of Plasmids used in this study

Plasmid name	**Function**
pLexAVP16N	Expresses LexA(1-202)+VP16 (413-456)+FLAG tag
pLexAVP16C	Expresses LexA(1-202)+VP16 (446-490)+FLAG tag
pLexAVP16N 439TAG, pLexAVP16N 442TAG, pLexAVP16N 444TAG,	Expresses LexA(1-202)+VP16 (446-490)+FLAG tag with a TAG replacing the codon of the existing amino acid
pLexAVP16C 473TAG, pLexAVP16C 475TAG, pLexAVP16C 479TAG	Expresses LexA(1-202)+VP16 (413-456)+FLAG tag with a TAG replacing the codon of the existing amino acid
pLexAGal4 849TAG, pLexAGal4 867TAG, pLexAGal4 869TAG	Expresses LexA(1-202)+Gal4(840-881)+FLAG tag with a TAG replacing the codon of the existing amino acid
pLexAGcn4 108TAG, pLexAGcn4 110TAG, pLexAGcn4 115TAG, pLexAGcn4 118TAG, pLexAGcn4 120TAG, pLexAGcn4 121TAG, pLexAGcn4 123TAG, pLexAGcn4 124TAG, pLexAGcn4 126TAG, pLexAGcn4 132TAG, pLexAGcn4 140TAG	Expresses LexA(1-202)+Gcn4(107-144)+FLAG tag with a TAG codon replacing the codon of the existing amino acid
pSNRtRNA-pBpaRS	Expresses tRNA under the control of the SNR52 promoter and

	contains synthetase specific for pBpa ptRNA-pBpaRS
pMyc Med15(1-416)	Expresses Med15 (1-416) fused to c-myc tag

Construction of plasmids

1. pLexAVP16 N and pLexAVP16C

A high copy plasmid expressing LexA(1-202)+VP16N (413-456)+FLAG tag and LexA(1-202)+VP16C (446-490))+FLAG tag under the control of the ADH1 promoter was created from pCLexA containing EcoRI and BamHI sites. Primers 5'- catgaattcATGGCCCCCCCGACCGATGTC-3' and 5'-catggatccTTACTTGTCATCGTCGTCCTTGTAGTCTCCCGGCCCCGGGGAATCCC-3' were used to amplify VP16 (413-456) using pMVP16 as a template. The amplified PCR product was digested with EcoRI and BamHI and inserted into pCLexA digested with EcoRI and BamHI and treated with calf intestinal phosphate to create pLexAVP16N.
Primers 5' catgaattcATGTTGGGGGACGGG- 3' and (5'-catggatccTTACTTGTCATCGTCG -3') were used to amplify VP16 (446-490) using pMVP16 as a template. The amplified PCR product was digested with EcoRI and BamHI and inserted into pCLexA digested with EcoRI and BamHI and calf intestinal phosphate treated to create pLexAVP16C.

2. pLexAVP16N 439TAG, pLexAVP16N 442TAG, pLexAVP16N 444TAG, pLexAVP16C 473TAG, pLexAVP16C 475TAG, pLexAVP16C 479TAG

Plasmids containing various amber mutants in the VP16 TAD coding region were derived from pLexAVP16N and pLexAVP16C. To create each plasmid, site-directed mutagenesis was used to replace an existing amino acid codon with TAG codon within the VP16C or VP16N TAD. In general, PCR primers were designed to have ~15 bases of homology on either side of the TAG mutation. QuikChange (Stratagene, La Jolla, CA) was used to incorporate the TAG mutants using manufacturer recommended conditions.

3. pLexAGcn4(107-144)
In a similar fashion to the VP16 plasmid construction, a high copy plasmid expression LexA(1-202)+Gcn4(107-144)+FLAG tag under the control of the ADH1 promoter was created from pCLexA containing EcoRI and BamHI sites.

Primers 5'-GAATTCATGTTTGAGTATGAAAACCTAGAAGACAACTC-3' and 5'-GGATCCGGATTCA ATTGCCTTATCAGCCAATG-3' were used to amplify Gcn4(107-144) from yeast genomic DNA. The amplified product was digested with BamHI and EcoRI and then treated with Calf intestinal phosphatase to create pLexAGcn4.

4. pLexAGal4 (840-881)
pLexA(1-202)+Gal4(840-881) was created as previously described (Majmudar, CY et al, 2009)

5. pMycMed15 (1-416)

A high copy plasmid pMycMed15(1-416) expressing Med15(1-416) under the ADH1 promoter, N-terminally tagged with the c-Myc epitope was constructed by amplifying the DNA sequence encoding Med15(1-415) from yeast genomic DNA using primers (5'-GACAGGATCCATGTCT GCTGCTCCTGTCCAAGAC-3') and (5'- CGATCATATGTCACTGATATAATTTAGAACTTGC-3') and inserted into BamHI and NdeI digested pMyc using standard molecular biology techniques. The pMyc cloning vector was created by inserting an ADH1 driven c-myc epitope tag in pGADT7 (Clontech) followed by restriction sites for gene insertion using site-directed mutagenesis using primers (5'-AGCTATGGAACAAAAGTTGATTTCTGAAGAAGATTTGGGATCCAATGCATATGATCT-3') and (5'-AGCTTGATCATATGCATTGGATCCCAAATCTTCTTCAGAAATCAACTTTTGTTCCAT-3').

Construction of Snf5 and Swi1 deletion strains

Yeast strains in which Snf5 or Swi1 had been deleted were made by gene disruption via PCR using a method described earlier (Longtine, M. S et al, *Yeast* 14, 953–961 (1998)). All the delete strains were derived from LS41 [JPY9::pZZ41, *Matα his3Δ200 leu2Δ1 trp1Δ63 ura3-52 lys2Δ385 gal4* URA::pZZ41]. Plasmid PFa6-TRP1 (generously donated by Karbstein group, University of Michigan) was used as a template to clone out deletion inserts using target-gene-specific primer pairs as designated in Table 2.

Table 2: Primers used for PCR based gene deletion

Primer	**Purpose**	**Primer Sequence 5'-3'**
Snf5- Fwd-1	Round 1 PCR	CATCAAGGGAACATATAGTAAAGAACTACACAAAAGCAACA *CGGATCCCCGGGTTAATTAA*
Snf5- Rev-1	Round 1 PCR	GGTTATTTACATCTCCGGTATATTTTATATATGTGTATATATTTT *GAATTCGAGCTCGTTTAAAC*
Snf5-Fwd-2	Round 2 PCR	**CATAAACACCAAAACAAAGCAT**CATCAAGGGAACATATAGTAAAG
Snf5-Rev-2	Round 2 PCR	**GATAATACAAATTCTTCCAC**GGTTATTTACATCTCCGGTA
Swi1-Fwd	Round 1 PCR	ATGGATTTCTTTAATTTGAATAATAATAATAATAATAATAATAC *CGGATCCCCGGGTTAATTAA*
Swi1-Rev	Round 1 PCR	TCATTCCAAATTGGTTAGGATATCATTTTTTAAATTGTAAAG *GAATTCGAGCTCGTTTAAAC*

The underlined sequences correspond to the sequence on the pFa6-TRP1 plasmid and the sequences in italics are gene specific sequences. The sequences in bold are Snf5 gene specific sequences and are ~ 20 bp upstream and downstream of Snf5 sequence from Round 1 PCR product.

In case of Swi1 deletion, pFa6-TRP1 was used as template and PCR inserts were cloned out using primers Swi1-Fwd and Swi1-Rev. 1-5 ug of the PCR product was transformed into LS41 and spread on plates containing SC media + 2% glucose, lacking uracil and tryptophan. After 3-4 days, the colonies grown were screened for deletion strains by lysing a small amount of the colony using 20 mM NaOH. Briefly, a small amount of the colony (~ 0.25-0.5 μL) was taken into a PCR tube containing 20 μL of 20 mM NaOH. The tube was boiled for 20 min at 95 °C in a PCR machine and spun down. The supernatant (0.5 – 1 μL) was used as a template and using sequencing primers, the deletion was verified by gel electrophoresis and DNA sequencing. In case of the Snf5 deletion, there was no successful deletion with one round of PCR and hence a ~ 60 bp Snf5 specific homologous

sequence was cloned upstream and downstream of the Trp1 sequence by two rounds of PCR using primers described in Table 2. Screening and selection was done as described for Swi1 deletion and verified by DNA sequencing.

Incorporation of pBpa into LexA(1-202)+VP16N and LexA(1-202)+VP16C and expression of myc-Med15 in S. cerevisiae

LS41 yeast was transformed with pLexA+VP16 TAG mutant plasmids and pSNRtRNA-pBpaRS plasmid. Individual colonies were grown to saturation in 5 mL SC media lacking histidine and tryptophan for selection and 2% raffinose at 30 °C with agitation. Starter cultures were then used to inoculate 5 mL SC media lacking histidine and tryptophan and containing 2% raffinose and 2% galactose. For pBpa incorporation, 50 μL of 100 mM pBpa dissolved in 1M NaOH and 50 μL of 1M HCl were added to the above cultures. The cultures were grown overnight at 30 °C, with agitation to an OD_{660} of ~1.0. 3 OD's of cells were harvested and the cell pellets were lysed in 12 μL pellet lysis buffer (50 mM Tris Acetate, pH 7.9, 150 mM KOAc, 20% glycerol, 0.2% Tween-20, 2 mM MgOAc) containing Complete EDTA-free protease inhibitor tablets (Roche), 7 uL of 1 mM DTT, and 7 uL of 4X LDS NuPAGE dye (Invitrogen). Lysates were boiled at 95 °C and analyzed using Western blot with anti-FLAG (M2) antibody (Sigma). To test expression of the myc-Med15(1-416) construct, the same protocol was followed except that LS41 were additionally transformed with the pMyc-Med15(1-416) plasmid and grown in SC media lacking histidine, tryptophan and leucine. Lysates were analyzed using Western blot with anti-myc antibody (Santa Cruz Biotech, sc-40).

β-Galactosidase assays

To evaluate the ability of each LexA+VP16 TAG mutant to activate transcription in the presence or absence of 1 mM pBpa, saturated cultures (SC media + 2% raffinose) of each mutant were used to inoculate 5 mL SC media containing 2% raffinose + 2% galactose but lacking histidine and tryptophan for selection. The cells were grown at 30 °C with agitation to an OD_{660} of ~1.0 and harvested. The activity of each construct was monitored using β-galactosidase assays as previously described (*1*). The reported activity was normalized to total protein concentration in the lysate as measured by Bradford assay (Bio-Rad) using BSA as the standard. Additionally, the reported activity is an average of three independent experiments, each of which tested four individual colonies for all constructs reported.

In vivo cross-linking

To perform in vivo cross-linking, individual colonies of each pLexAVP16 TAG mutant were grown in 5 mL SC media containing 2% raffinose but lacking histidine and tryptophan for selection. The cultures were incubated overnight at 30 °C with agitation. Following incubation, these cultures were used to inoculate 100 mL cultures of SC media containing 2% raffinose and 2% galactose but lacking histidine and tryptophan. For pBpa incorporation, 1 mL of 100 mM pBpa dissolved in 1M NaOH and 1 mL of 1M HCl were added to the above cultures. For control cultures, 1 mL of 1M NaOH and 1 mL of 1M HCl were added. The cultures were incubated overnight at 30 °C with agitation to an OD_{660} of ~1.0. When cultures reached the appropriate OD_{660}, the cells were spun down by centrifuging at 3901 rcf at 4°C for 5 min. Following centrifugation, the cell pellets were washed with SC media lacking histidine and tryptophan. The cell pellets were resuspended in 2mL SC media lacking histidine and tryptophan + 2% raffinose and 2% galactose and then transferred to small cell culture dishes and subjected to UV irradiation at 365 nm light (Eurosolar 15 W UV lamp) with cooling for 0.5 h. The cells were isolated by centrifugation and stored at -80°C until lysis.

For crosslinking studies with myc-Med15(1-416) and the deletion strains, the procedure was identical except that cells were grown in SC media lacking histidine, leucine, and tryptophan and, due to poor growth in raffinose and galactose, deletion cultures were grown in 2% glucose. For lysis, cells were resuspended in 600 µL Lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-Deoxycholate and 2X Complete Mini, EDTA Free Protease Inhibitor (Roche) and lysed using glass beads by vortexing at 4 °C. Subsequently, the lysate was pelleted and the supernatant incubated with 10 µL of LexA antibody (sc-1725, Santa Cruz Biotechnologies) for 2 h at 4 °C for immunoprecipitation. The protein bound to the antibody was isolated by incubation for 1 h with either 50 µL of prewashed protein G magnetic beads (Dynal Corporation, Invitrogen) or 25 uL prewashed protein G agarose beads (Millipore) at 4 ^{o}C. After immunoprecipitation, the beads were washed 6 times with 1 mL Wash Buffer (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.1% Na-Deoxycholate and 1 mM EDTA) and stored dry at -80 ^{o}C until elution. The crosslinked sample was eluted from the beads by heating at 95 ^{o}C for 10 min in NuPAGE 4x LDS Sample buffer (Invitrogen) containing 250 mM DTT and probed by Western Blot analysis using either anti-FLAG (M2) antibody (Sigma) or anti-myc antibody (SC-40, Santa Cruz Biotechnology).

Supplemental Figures

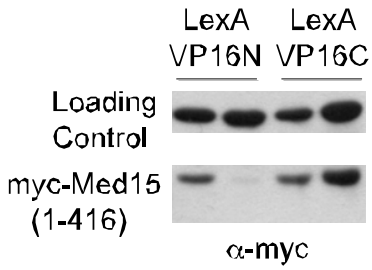

Figure S1. Myc-Med15(1-416) expresses at a moderate level in *S. cerevisiae*. Yeast cells transformed with plasmids encoding two LexA+VP16N and LexA+VP16C constructs, the Bpa specific tRNA/synthetase pair expressed by pSNRtRNA-pBpaRS and myc-Med15(1-416) were grown to mid-log phase OD_{660} in the presence of 1 mM Bpa. 3 ODs of yeast cells were harvested and the cell pellets were lysed and analyzed by Western blot with anti-myc antibody.

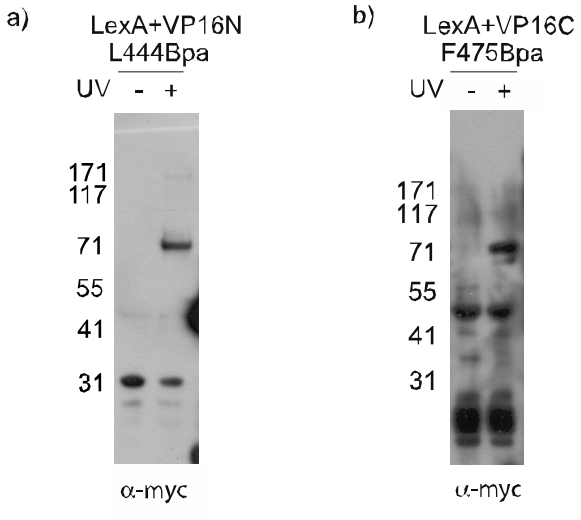

Figure S2. VP16 crosslinks directly to myc-Med15 (1-416) in vivo. a) LexA+VP16N L444Bpa and b) LexA+VP16C F475Bpa were transformed in yeast alongside a plasmid encoding myc-Med15 (1-416) and then irradiated with UV light. Cell lysates were immunoprecipitated with a LexA antibody and subsequent Western blots were probed with a myc antibody. Both constructs display a crosslinked product corresponding to the molecular weight of a LexA+VP16—Med15 crosslinked product (approximately 75 kDa).

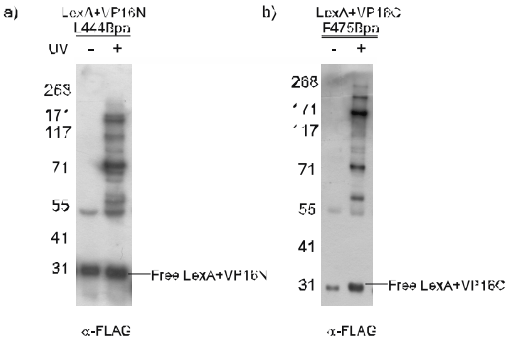

Figure S3. LexA-VP16N L444Bpa and LexA-VP16C F475Bpa crosslink to multiple protein partners in yeast. Live yeast cells bearing plasmids expressing LexA+VP16N L444Bpa or LexA+VP16C F475Bpa fusion proteins were irradiated with UV light (365 nm) for 30 minutes. Subsequently, cell lysates were immunoprecipitated with α-LexA and analyzed by Western blot (α-FLAG). For both constructs, higher molecular weight crosslinked products are formed which roughly correspond to a matching molecular weight of a LexA+VP16-Snf5 (131 kDa), Swi1 (176 kDa), or Snf2 (222 kDa) complex.

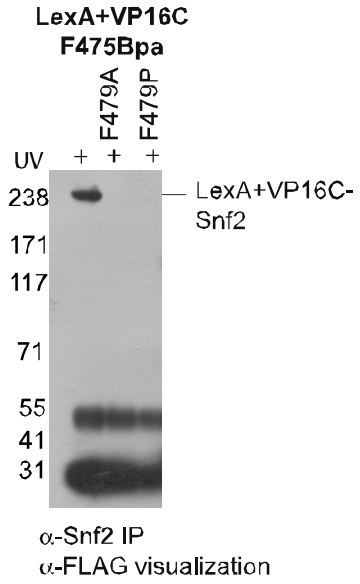

Figure S4. VP16C crosslinks to endogenous Snf2 in vivo. Live yeast cells expressing LexA+VP16C F475Bpa were irradiated with UV light and subsequently the cell lysates were immunoprecipitated with an antibody to Snf2 and resolved by Western blot (α-FLAG), revealing a direct interaction between VP16C and endogenous Snf2. Mutations at position 479 in VP16C known to disrupt VP16-coactivator interactions similarly abrogate crosslinking to Snf2 in vivo.

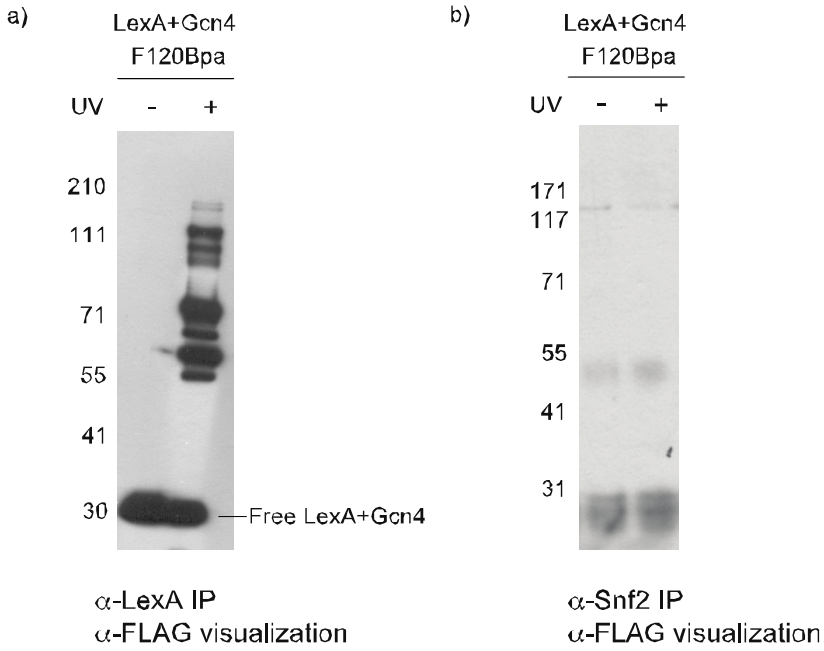

Figure S5. LexA-Gcn4 does not appear to crosslink to Snf2 in vivo. Live yeast cells expressing LexA+Gcn4 W120Bpa were irradiated with 365 nm light for 30 minutes. (a) Cell lysates were immunoprecipitated with an antibody to LexA and resolved by Western blot (α-FLAG), indicating multiple crosslinked products. (b) Cell lysates were

immunoprecipitated with an antibody to Snf2 and resolved by Western blot (α-FLAG). Similar results were obtained with LexA+Gcn4 with Bpa incorporated at positions F108, Y110, D115, K118, T121, L123, F124, N126, T132 or K140.

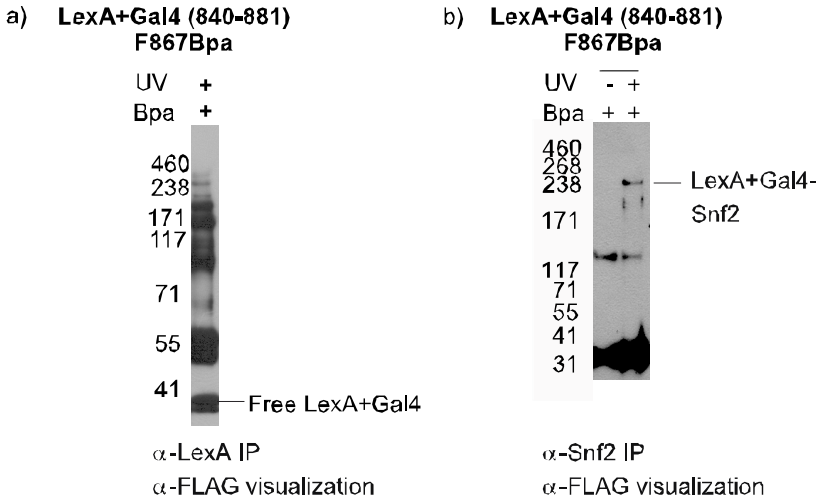

Figure S6. Gal4 crosslinks to Snf2 in live yeast. a) LexA+Gal4 (840-881) F867Bpa was expressed in yeast and subjected to UV irradiation. Cell lysates were immunoprecipitated with LexA antibody and the resulting Western blot was probed with a FLAG antibody, revealing a multi-protein binding profile for the activator. b) After irradiation, lysates were immunoprecipitated with Snf2 antibody and the subsequent Western was probed with a FLAG antibody, revealing a band that corresponds to a LexA+Gal4—Snf2 crosslinked complex (220 kDa).

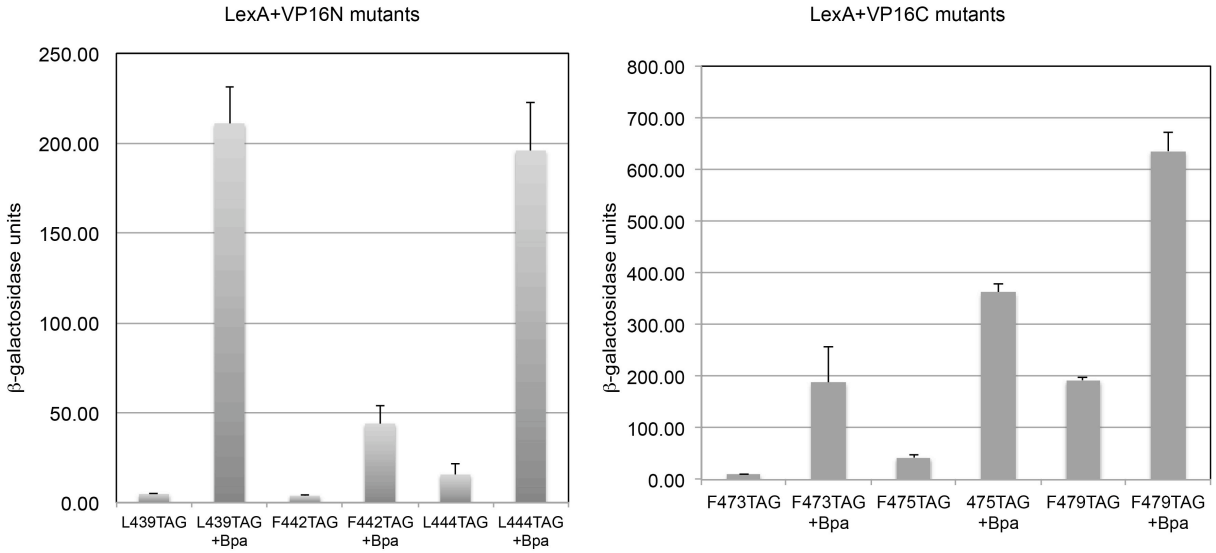

Figure S7. Bpa containing mutants retain transcriptional activity. Six LexA+VP16 TAG constructs were assayed for transcriptional activation potential as measured by a liquid β-galactosidase assay. Each activity is the average of values from at least three independent experiments with the indicated error (SDOM).

References

1. Majmudar, C. Y., Lee, L. W., Lancia, J. K., Nwokoye, A., Wang, Q., Wands, A. M., Wang, L., and Mapp, A. K. (2009) Impact of nonnatural amino acid mutagenesis on the in vivo function and binding modes of a transcriptional activator, *J Am Chem Soc 131*, 14240-14242.
2. Burk, D., Dawson, D., and Stearns, T. (2000) *Methods in Yeast Genetics*, 1 ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor.