Brief Communication

TRIC: capturing the direct cellular targets of promoter-bound transcriptional activators

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Abstract

Transcriptional activators coordinate the dynamic assembly of multi-protein coactivator complexes required for gene expression to occur. Here we combine the power of *in vivo* covalent chemical capture with p-benzoyl-L-phenylalanine (Bpa), a genetically incorporated photocrosslinking amino acid, and chromatin immunoprecipitation (ChIP) to capture the direct protein interactions of the transcriptional activator VP16 with the general transcription factor TBP at the GAL1 promoter in live yeast.

Transcriptional activators are essential in directing the assembly of the RNA polymerase transcriptional machine at individual promoters within the genome. Though it is well understood that this occurs through the formation of protein-protein interactions (PPIs) between DNA-bound activators and individual components of the transcriptional machinery, resolving the network of activator PPIs that underpin this process has been technically challenging.[1] For example, *in vivo* co-localization and chromatin immunoprecipitation (ChIP) studies have identified the complexes that are recruited by activators to the promoter, but distinguishing the individual subunits within these complexes that serve as the activator binding partner(s) *in vivo* has not been possible. Toward this goal, *in vivo* covalent chemical capture with genetically incorporated photo-crosslinking amino acids has emerged as an important tool in the identification of PPIs in cells.[2-5] Here we describe an expansion of this strategy that utilizes the rapid, reversible formaldehyde crosslinking of ChIP together with the site-specific, irreversible crosslinking of the non-natural amino acid p-benzoyl-L-phenylalanine (Bpa) to investigate the direct, in vivo PPIs of DNA-bound activators. We demonstrate that this tandem reversible and irreversible crosslinking (TRIC) approach is able to capture the interaction of the viral activator VP16 and the general transcription factor TBP at the GAL1 promoter in live yeast.

The identities and compositions of the multi-protein complexes that assemble at genes in order to initiate gene expression can vary significantly from promoter to promoter.[6-9] Due to a lack of methods appropriate for determining the individual contacts made by activators at a promoter, these PPIs remain poorly defined. As an example, the recruitment of TATA-binding protein (TBP) by the canonical transcriptional activator VP16 has been studied extensively, yet the mechanism by which TBP arrives at promoters is still contested, even in the case of well-studied promoter contexts such as GAL1. A significant body of *in vitro* data exists to support a direct interaction between VP16 and TBP.[10-13] However, *in vivo* ChIP studies suggest that TBP recruitment to GAL1 is mediated via interactions with the SAGA complex, supporting a model in which the activator is not directly involved in TBP recruitment.[14-17]

To distinguish between the two mechanisms of TBP recruitment, in vivo covalent chemical capture was first used to determine if VP16 directly contacts TBP in living yeast. In this approach, the photo-labile unnatural amino acid Bpa is genetically incorporated into the transcriptional activation domain (TAD) of VP16 using an engineered nonsense suppression system.[18,19] Upon irradiation of live yeast with UV light, Bpa is activated and forms an irreversible covalent bond with any protein partners that are in direct contact with VP16 at the site of incorporation. The covalently captured binding partners can then be isolated and analyzed to determine their identities. As previously described, we used a chimeric activator in which the amino terminal half of the extended VP16 activation domain is fused to the LexA DNA binding motif.[2] We chose to incorporate Bpa at position 444 in the VP16N (413-456) subdomain, as this residue has been shown to be involved in maintaining activator interactions and, consistent with this, our previous work showed a robust and repeatable multi-protein crosslinking profile at this position. Furthermore, work from our group has demonstrated that incorporation at this site does

not impair activator function.[2] LexA+VP16 L444Bpa was expressed in yeast and the cells were irradiated with UV light to activate Bpa and covalently capture any proteins directly contacting VP16. Yeast were then lysed and the lysates immunoprecipitated with an antibody against TBP. After washing away any non-covalently bound proteins, the immunoprecipitated TBP was run on SDS-PAGE and the resulting Western blot was probed with a Flag-HRP antibody to detect the presence of a covalently bound VP16-TBP species. Indeed, a crosslinked band is observed at approximately 60 kDa that corresponds to the additive molecular weight of the LexA+VP16 construct and TBP [Fig. 2(A)]. This data indicates that VP16 directly contacts TBP in live yeast. Consistent with previous reports, introduction of a double point mutation in the VP16 activation domain (L439P and F442P) that abolishes activity also abrogates crosslinking to TBP, indicating that this interaction is specific [Fig. 2(A)].[20,21]

To further substantiate this interaction, covalent chemical capture in which TBP is the captor was carried out. Biochemical data suggest that residues along the concave face of TBP are involved in dynamic exchange with other transcriptional proteins, including activators, which compete for binding at this site.[22-24] We therefore co-expressed LexA+VP16 WT alongside a myc-TBP construct in which the leucine at position 114 was mutated to Bpa (L114Bpa) and carried out covalent chemical capture experiments. We found that incorporation of Bpa at this position in TBP resulted in a crosslinking TBP-VP16 adduct [Fig. 2(B)]. Thus, in support of previous biochemical data, our results indicate that this site on TBP is indeed a contact point for VP16 in vivo.[24]

While demonstrating a direct interaction between VP16 and TBP, the above experiments do not report on any one context. In other words, the crosslinked species likely originate from a variety of different promoter-localized and non-DNA bound VP16 species. We next chose to design a

straightforward method that would allow for the direct examination of the VP16-TBP interaction at the GAL1 promoter present in the yeast strain. The first step of this strategy borrows principles from classic chromatin immunoprecipitation (ChIP) protocols in which formaldehyde is first administered to cells to rapidly stabilize dynamic protein-DNA interactions. The fixed cells are then subjected to our covalent chemical capture workflow in order to activate Bpa and site-specifically crosslink the direct targets of our Bpa-containing activators within the immobilized transcription complexes. The advantage of this tandem crosslinking approach is rooted in the nature of the covalent bonds that are formed by each crosslinking moiety; more specifically, the formaldehyde crosslinks can be readily reversed in an appropriately nucleophilic buffer, whereas the Bpa crosslinks are irreversible under such treatment conditions. Thus, after tandem crosslinking treatment, we could lyse the yeast, isolate the insoluble chromatin-containing pellet, and wash the pellet extensively to remove any non-DNA bound complexes. Similar to ChIP, the DNA could then be solubilized via sonication and immunoprecipitation carried out against the protein of interest, in this case TBP. The formaldehyde crosslinks on the isolated complexes are then reversed, leaving only the irreversible photo-crosslinked activator-coactivator interactions intact and able to be visualized via Western blotting (Fig. 1).

When we executed our tandem reversible and irreversible crosslinking (TRIC) approach, we found that only under conditions where both formaldehyde and UV treatment were used were we able to observe a VP16-TBP crosslink in the DNA fraction, whereas UV alone was sufficient to capture this interaction in yeast lysate [Fig. 2(C)]. When this protocol was repeated to examine the DNA that co-purified during immunoprecipitation, we observed that the GAL1-LacZ gene could only be visualized under conditions in which formaldehyde was present. This indicates that formaldehyde stabilization of promoter-bound proteins is required to observe the interactions of

DNA-bound activators [Fig. 2(D)]. Finally, we executed a straightforward ChIP protocol to show that both LexA+VP16 and TBP co-localize to our reporter gene and confirm that we are indeed observing a direct protein-protein interaction at this specific location in the yeast genome [Fig. 2(E)].

We note that although the protocol is a hybrid of two powerful approaches, optimization efforts in several areas were required in order for this approach to work. The first point of optimization was the duration of formaldehyde crosslinking. We were unable to observe a VP16-TBP interaction using previously reported one-minute rapid mixing protocols.[25] We therefore tested several formaldehyde treatment times and found that five minute treatment with formaldehyde was the shortest interval that could be used to achieve consistent results. As has been noted elsewhere, formaldehyde crosslinking optimization will have to be examined on an individual protein basis in order to achieve sufficient crosslinking results.[26,27] Another key point of optimization for this study involved the extent of yeast lysis prior to sonication of the insoluble chromatin-containing fraction. Yeast have a tough cell wall that is notoriously difficult to break open. We found that under conditions of incomplete lysis, any remaining cells that pelleted with the insoluble fraction would release their cellular contents upon sonication, resulting in contaminating bands in the UV treated lanes of the Western. We thus screened a number of commercially available chemical lysis reagents and mechanical lysis conditions to determine a method that would provide us with the most complete yeast lysis. We evaluated the efficacy of lysis under each condition by measuring the total concentration of protein released in the lysate as well as by visually assessing the extent of lysis by monitoring cells under a microscope. We found that only extended mechanical disruption with glass beads consistently achieved >95% cell lysis and yielded maximal protein release in the cell lysate.

In conclusion, we have described a new in vivo tandem crosslinking approach that is useful in capturing the PPIs of DNA bound activators, as demonstrated by the capture of VP16 and TBP at the GAL1 promoter. The advantage of this strategy over previously described dual crosslinking applications (such as those using formaldehyde and disuccinimidyl suberate, for example) is that the use of a site-specific photo-crosslinking amino acid allows one to distinguish the direct targets of a given protein within a DNA-bound multiprotein complex. This allows a level of resolution that, to the best of our knowledge, has not been achieved prior to this work.

TBP is an essential transcription factor required for gene expression in yeast, yet, as is the case with other requisite transcriptional complexes, the mechanism by which it is recruited to promoters has remained elusive, even in the most well-studied promoter contexts. Using TRIC, we were able to stabilize transcription factor-DNA contacts and then covalently capture proteins that were in direct contact with the Bpa-containing activator VP16 in live yeast. This work suggests that VP16 directly contacts TBP in yeast to recruit the general transcription factor to the GAL1 promoter. While our results thus suggest a direct mechanism of recruitment to GAL1, earlier observations that SAGA is required for TBP localization to GAL1 likely point a role for SAGA in stabilizing TBP residency at this promoter. This work is an important first step toward resolving the PPI map of activators at a single promoter, work that will be useful in building a more complete picture of activator interactions as a whole. Future work will be focused on combining TRIC with DNA microarray technologies to facilitate the identification of the direct interactions of a single activator at individual promoters across the genome.

Methods and Materials

All covalent chemical capture and TRIC experiments were carried out in yeast strain LS41 [JPY9::pZZ41, *Mat his3Δ200 leu2Δ1 trp1Δ63 ura3-52 lys2Δ385 gal4* URA::pZZ41]. Bpa was purchased from Chem-Impex International (Wood Dale, IL). All plasmids used in this study were constructed using standard molecular biology techniques. Sanger sequencing verifying plasmid sequences was performed by the University of Michigan Core Facility (Ann Arbor, MI).

Construction of plasmids

pLexA+VP16N WT

A high copy plasmid expressing LexA(1-202)+VP16N (413-456)+5x FLAG tag under the control of the ADH1 promoter was created via ligation of the fusion gene into a pCLexA-5xflag backbone containing BamHI and SalI sites. Primers 5'-catgaattcATGGCCCCCCCGACCGATGTC-3' and 5'-catGTCGACTTACTTGTCATCGTCGTCCTTGTAGTCTCCCGGCCCCGGGGAATCCC-3' were used to amplify VP16 (413-456) from a pCLexA-VP16-1xflag template. The amplified PCR product was digested with SalI and BamHI and inserted into pCLexA digested with SalI and BamHI and treated with calf intestinal phosphate to create pLexA+VP16N WT-5X Flag.

pLexAVP16N 444TAG

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.

pGADT7 myc-TBP

A high copy plasmid pGADT7 expressing an N-terminally myc-tagged TBP was constructed by amplifying the DNA sequence encoding TBP from yeast genomic DNA using primers (5'-catCATATGATGGCCGATGAGGAACGTTTAAAGG-3') and (5'-atgCTCGAGTCACATTTTTCTAAATTCACTTAGC -3'). The purified PCR product was ligated into a myc-pGADT7 vector digested with NdeI and XhoI using standard molecular biology techniques. The myc-pGADT7 cloning vector was created by inserting a c-myc epitope tag in pGADT7 (Clontech) using site-directed mutagenesis with the following primers: (5'-AGCTATGGAACAAAAGTTGATTTCTGAAGAAGATTTGGGATCCAATGCATATGATCT -3') and (5'-AGCTTGATCATATGCATTGGATCCCAAATCTTCTTCAGAAATCAACTTTTGTTCCAT-3'). Leu114 in TBP was mutagenized to an amber stop codon using site-directed mutagenesis (Qiagen Quikchange Protocol).

In vivo covalent chemical capture

In vivo covalent chemical capture experiments for pLexA+VP16N L444Bpa was carried out as previously described.[2] For crosslinking studies with mycTBP L114Bpa, the procedure was identical except that cells were grown in SC media lacking histidine, leucine, and tryptophan. 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 2 µg 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 50 µL of prewashed protein G magnetic beads slurry (Dynal Corporation, Invitrogen, Carlsbad, CA) and 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, Carlsbad, CA) containing 250 mM DTT and probed using Western Blot analysis using anti-FLAG (M2) antibody (Sigma, St. Louis, MO).

Tandem reversible and irreversible crosslinking

To perform TRIC, 100 mL cultures of yeast were grown in SC media containing 2% Raffinose, 2% Galactose, 1 mL of 100 mM pBpa dissolved in 1M NaOH, and 1 mL 1M HCl. The cultures were incubated overnight at 30°C with agitation and grown to mid-log phase (OD_{660} ~1.0). Cultures receiving UV treatment only were spun down by centrifuging at 3901 rcf at 4^{o}C for 5 min. following which the cell pellets were washed with SC media lacking histidine and tryptophan. These cell pellets were resuspended in 2mL SC media lacking histidine and tryptophan + 2% Raffinose, 2% Galactose and 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.

For cultures receiving only formaldehyde treatment, 3 mL of 37% formaldehyde solution was added directly to the culture and allowed to remain in the incubator for 20 minutes. Repeat of this procedure with a 5 minute formaldehyde incubation yielded the same results as a 20 minute incubation. Cultures were then quenched with 15 mL of 2M Glycine. Cells were then centrifuged and washed with 50 mL SC media lacking histidine and tryptophan. Samples intended to additionally receive UV crosslinking were resuspended in 2 mL SC media (His-,

Trp-) containing 2% Raffinose and 2% Galactose and transferred to a small cell culture dish and subjected to UV irradiation at 365 nm UV light (Eurosolar 15 W UV lamp) with cooling for 0.5h.

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 mechanical disruption at 4°C until >95% lysis was observed. We found in these studies that complete cellular lysis is necessary to eliminate background signal caused by cell lysis during sonication. Subsequent lysates were immunoprecipitated with 8 uL TBP antibody (santa cruz, sc-33736) and incubated for 2 hours at 4°C. The remaining pellet was then washed 4x with "Harsh" ChIP buffer (50 mM HEPES-KOH pH 7.5, 1M NaCl, 1 mM EDTA, 1% Triton X-100, 1% Na-Deoxycholate) followed by 2 washes with standard ChIP 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)). Pellets were resuspended in 600 µL standard ChIP buffer containing protease inhibitor and sonicated at a setting of 10% for 2 minutes with 30 sec pulse on/off (double-step microtip, Fisher Scientific Dismembrator Model 500). Samples were then centrifuged at 4°C for 20 minutes at 14,000 rpm (Eppendorf 5417C). Soluble chromatin (supernatant) was immunoprecipitated with TBP antibody (santa cruz, sc-33736) for 2 hours, 4°C. The protein bound to the antibody was isolated by incubation for 1 h with 50 µL of prewashed protein G magnetic beads (Dynal Corporation, Invitrogen, Carlsbad, CA). 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 and formaldehyde crosslinks reversed by

heating at 95°C for 20 min in SUTEB buffer containing 250 mM DTT and probed using Western Blot analysis using anti-FLAG (M2) antibody (Sigma, St. Louis, MO). For studies examining the DNA IP'd during TRIC, the TRIC protocol was followed with the exception of 50 uL of solubilized chromatin being saved prior to immunoprecipitation. Additionally, lysates were discarded in these experiments.

To examine the size of the sheared chromatin, 50 µL TE/SDS was added to the Input samples and incubated overnight at 65°C to reverse crosslinks. 2.5 µL proteinase K (20 mg/mL stock) was then added and incubated at 50°C for 3 hours to digest proteins, followed by a PCR cleanup. 0.5 µL RNAse A (1 mg/mL stock) was added and then incubated at 37°C for 30 min. Samples were visualized on 1% agarose gel stained with Ethidium bromide. Smears showed between 300-900 bps.

For PCR on TRIC samples, 90 µL TE/SDS was added to 50 uL input and incubated overnight at 65°C followed by PCR Cleanup and elution in 58 µL EB buffer (Qiagen). For immunoprecipitated samples, beads were washed 2x with lysis buffer, 1 time with 500 mM NaCl lysis buffer, 1 time with wash buffer, and two times with TE buffer (10 mM Tris-HCl, 1 mM EDTA, 0.01% SDS (5 g in 500 mL ex). 50 µL elution buffer(50 mM Tris-HCl, 10 mM EDTA, 1% SDS) was then added to the beads and vortexed briefly before incubating at 65°C for 30 minutes, with vortexing every 5 minutes to resuspend the beads. Beads were centrifuged for 30 sec at 3000 rpm and the eluent transferred to a new tube. 120 µL TE/SDS was added and the samples incubated overnight at 65°C. Samples were purified using a PCR cleanup kit (Qiagen) and eluted in 58 µL EB buffer. DNA was quantified and PCR reactions were set up with GAL1-LacZ specific primers (5' CCTTCTCTTTGGAACTTTCAGTAATACGCTTAACTGC 3' and 5'

GGGCGATCGGTGCGGGCCTCTTCGC 3'). Products were visualized on a 1% agarose gel stained with ethidium bromide.

Chromatin Immunoprecipitation at GAL1-LacZ

For chromatin immunoprecipitation, cultures were grown and formaldehyde crosslinked as was done for TRIC experiments. After mechanical glass bead shearing, the lysate and insoluble pellet were resuspended by gentle pipetting. Samples were sonicated and centrifuged as described earlier. Soluble chromatin was separated from the pellet and 10 □L was saved as an input sample. The remaining soluble chromatin was split equally between three 1.75 mL tubes which were then immunoprecipitated with either 2 □g Snf1 antibody (sc-15621, Santa Cruz Biotechnologies), LexA antibody (sc-1725, Santa Cruz Biotechnologies), or control IgG (sc-2027, Santa Cruz Biotechnologies). Immunoprecipitations were allowed to proceed for 2 hours at 4^{o}C followed by incubation for 1 h with ~40 µL of prewashed protein G magnetic Dynabeads slurry (Life Technologies). Beads were washed in the same manner as TRIC DNA samples. Immunoprecipitated complexes were eluted in 50 □L elution buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS) at 65^{o}C for 30 minutes. The eluate was transferred to a new tube and 120 □L of TE-SDS (10 mM Tris-HCl, 1 mM EDTA, 0.1% SDS) buffer was added. To the saved inputs, 90 □L TE-SDS was added. Formaldehyde crosslinks were reversed overnight in a 65°C water bath. Samples were purified using Qiagen PCR Clean Up protocol and eluted in 58 □L Buffer EB. qPCR on all samples and inputs was run using Promega GoTaq qPCR master mix (A6001, Promega) using primers specific for GAL1-LacZ (sequences above). All qPCR runs were carried out on an Applied Biosystems StepOnePlus instrument. At least three independent biological replicates were run for each condition, with each biological replicate run in triplicate for qPCR quantitation. PCR amplification with the designed primers yielded a single band around 450 bps. This band was gel purified and submitted for sequencing. Results returned a sequence for the GAL1-LacZ gene, as expected.

References

1 Mapp AK, Ansari AZ (2007) A TAD further: Exogenous control of gene activation. ACS Chem Biol 2:62-75.

2 Krishnamurthy M, Dugan A, Nwokoye A, Fung Y-H, Lancia JK, Majmudar CY, Mapp AK (2011) Caught in the act: covalent cross-linking captures activator-coactivator interactions in vivo. ACS Chem Biol 6:1321-1326.

3 Mori H, Ito K (2006) Different modes of SecY-SecA interactions revealed by site-directed in vivo photocrosslinking. Proc Natl Acad Sci USA 103:16159-16164.

4 Berg M, Michalowski A, Palzer S, Rupp S, Sohn K (2014) An in vivo photocrosslinking approach reveals a homodimerization domain of Aha1 in S. cerevisiae. PLoS One 9:e89436.

5 Ting S-Y, Schilke BA, Hayashi M, Craig EA (2014) Architecture of the TIM23 inner mitochondrial translocon and interactions with the matrix import motor. J Biol Chem 289:28689-28696.

6 Raab JR, Resnick S, Magnuson T (2015) Genome-wide transcriptional regulation mediated by biochemically distinct SWI/SNF complexes. PLoS Genet 11:e1005748.

7 Wray GA, Hahn MW, Abouheif E, Balhoff JP, Pizer M, Rockman MV, Romano LA (2003) The evolution of transcriptional regulation in eukaryotes. Mol Biol Evol 20:1377-1419.

8 Cheng JX, Floer M, Ononaji P, Bryant GO, Ptashne M (2002) Responses of four yeast genes to changes in the transcriptional machinery are determined by their promoters. Curr Biol 12:1828-2832.

Poss ZC, Ebmeier CC, Taatjes DJ (2013) The mediator complex and transcriptional regulation. Crit Rev Biochem Mol Biol 48:575-608.

0 Klein J, Nolden M, Sanders SL, Kirchner J, Weil PA, Melcher K (2002) Use of a genetically introduced cross-linker to identify interaction sites of acidic activators within native transcription factor IID and SAGA. J Biol Chem 278:6779-6786.

1 Stringer KF, Ingles CJ, Greenblatt J (1990) Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. Nature 345:783-786.

2 Nedialkov YA, Triezenberg SJ (2004) Quantitative assessement of in vitro interactions implicates TATA-binding protein as a target of the VP16C transcriptional activation region. Arch Biochem Biophys 425:77-86.

3 Shen F, Triezenberg SJ, Hensley P, Porter D, Knutson JR (1996) Transcriptional activation domain of the herpesvirus protein VP16 becomes conformationally constrained upon interaction with basal transcription factors. J Biol Chem 271:4827-4837.

4 Mohibullah N, Hahn S (2008) Site-specific cross-linking of TBP in vivo and in vitro reveals a direct functional interaction with the SAGA subunit Spt3. Genes Dev 22:2994-3006.

5 Dudley AM, Rougeulle C, Winston F (1999) The Spt components of SAGA facilitate TBP binding to a promoter at a post-activator-binding step in vivo. Genes Dev 13:2940-2945.

6 Larschan E, Winston F (2001) The S. cerevisiae SAGA complex functions in vivo as a coactivator for transcriptional activation by Gal4. Genes Dev 15:1946-1956.

7 Bhaumik SR, Green MR (2001) SAGA is an essential in vivo target of the yeast acidic activator Gal4p. Genes Dev 15:1935-1945.

8 Chin JW, Cropp TA, Anderson JC, Mukherji M, Zhang Z, Schultz PG (2003) An expanded eukaryotic genetic code. Science 301:964-967.

9 Majmudar CY, Lee LW, Lancia JK, Nwokoye A, Wang Q, Wands AM, Wang L, Mapp AK (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.

0 Ingles CJ, Shales M, Cress DW, Triezenberg SJ, Greenblatt J (1991) Reduced binding of TFIID to transcriptionally compromised mutants of VP16. Nature 351:588-590.

1 Regier JL, Shen F, Triezenberg SJ (1993) Pattern of aromatic and hydrophobic amino acids critical for one of two subdomains of the VP16 transcriptional activator. Proc Natl Acad Sci USA 90:883-887.

2 Nishikawa J, Kokubo T, Horikoshi M, Roeder RG, Nakatani Y (1997) Drosophila TAFII230 and the transcriptional activator VP16 bind competitively to the TATA box-binding domain of the TATA box-binding protein. Proc Natl Acad Sci USA 94:85-90.

3 Mal TK, Takahata S, Ki S, Zheng L, Kokubo T, Ikura M (2007) Functional silencing of TATA-binding protein (TBP) by a covalent linkage of the N-terminal domain of TBP-associated factor 1. J Biol Chem 282:22228-22238.

4 Kim TK, Hashimoto S, Kelleher RJ III, Flanagan PM, Kornbeg RD, Horikoshi M, Roeder RG (1994) Effects of activation-defective TBP mutations on transcription initiation in yeast. Nature 369:252-255.

5 Bryant GO, Ptashne M (2003) Independent recruitment in vivo by Gal4 of two complexes required for transcription. Mol Cell 11:1301-1309.

6 Das PM, Ramachandran K, vanWert J, Singal R (2003) Chromatin immunoprecipitation assay. Biotechniques 37:961-969.

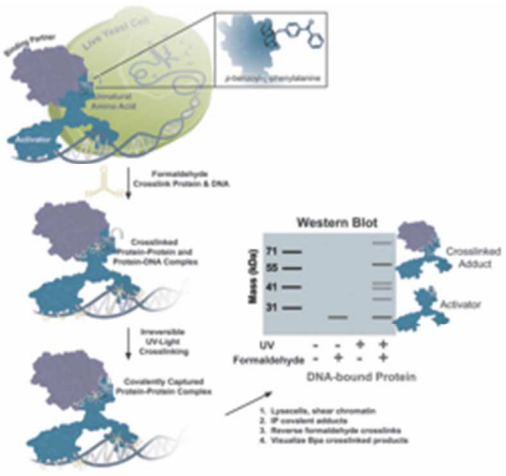
27 Orlando V (2000) Mapping chromosomal proteins in vivo by formaldehyde-crosslinked-chromatin immunoprecipitation. Trends Biochem Sci 25:99-104.

FIGURE LEGENDS

Figure 1. Tandem Reversible and Irreversible Crosslinking (TRIC) allows for the covalent capture of the direct targets of transcriptional activators at a promoter. Yeast cells are treated with formaldehyde to stabilize protein-DNA and protein-protein interactions at the promoter. Treated cells are then gently irradiated with UV light to activate a genetically encoded photocrosslinking amino acid in the transcriptional activation domain (TAD) of the activator coordinating complex assembly. The cells are lysed and the chromatin isolated and sheared via sonication. Following immunoprecipitation of the immobilized complexes, the formaldehyde crosslinks are reversed and the irreversibly covalently linked PPIs are identified by Western blotting.

Figure 2. VP16 directly contacts TBP in yeast. The VP16-TBP interaction was captured in vivo using covalent chemical capture in yeast expressing (a) LexA+VP16N L444Bpa and (b) LexA+VP16N WT and myc-TBP L114Bpa. Yeast were irradiated with UV light and the lysates immunoprecipitated with a TBP antibody. The covalent VP16-TBP products were visualized on Western blot with a □-Flag HRP antibody. (c,d) Tandem reversible and irreversible crosslinking captures the direct targets of DNA bound transcriptional activators. Identical cultures of yeast expressing LexA+VP16N L444Bpa were either crosslinked with UV, formaldehyde, or treated

with a combination of both procedures (formaldehyde followed by UV crosslinking). The chromatin fractions of these cultures were washed to remove non-covalently bound protein and then the chromatin was sheared and solubilized using sonication. Soluble chromatin was then immunoprecipitated with an □-TBP antibody and the formaldehyde crosslinks were reversed. Western blots (c) were probed with an □-Flag antibody and immunoprecipitated DNA was amplified with GAL1-LacZ specific primers and visualized on an agarose gel stained with ethidium bromide (d). (e) Chromatin immunoprecipitation at the GAL1 promoter in yeast. Bars are the mean of at least three biological replicates. Error bars indicate the standard error of the mean.



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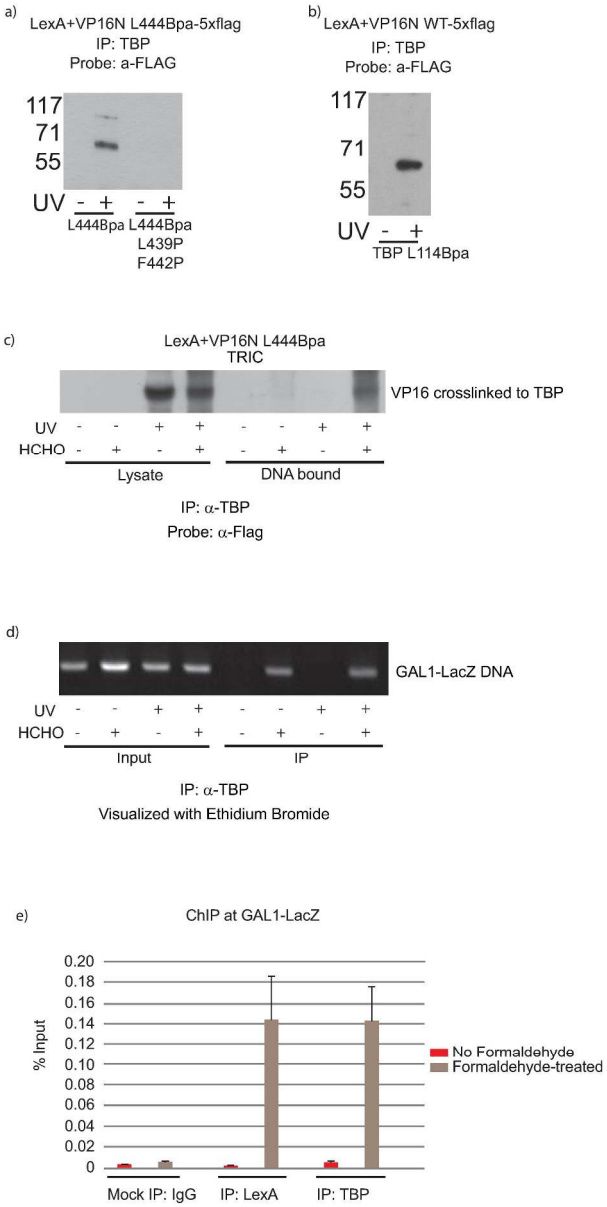

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