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Use of a Genetically Introduced Cross-linker to Identify Interaction Sites of Acidic Activators within Native Transcription Factor IID and SAGA*

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An important goal is to identify the direct activation domain (AD)-interacting components of the transcriptional machinery within the context of native complexes. Toward this end, we first demonstrate that the multisubunit TFIID, SAGA, mediator, and Swi/Snf coactivator complexes from transcriptionally competent whole-cell yeast extracts were all capable of specifically interacting with the prototypic acidic ADs of Gal4 and VP16. We then used hexahistidine tags as genetically introduced activation domain-localized cross-linking receptors. In combination with immunological reagents against all subunits of TFIID and SAGA, we systematically identified the direct AD-interacting subunits within the AD-TFIID and AD-SAGA coactivator complexes enriched from whole-cell extracts and confirmed these results using purified TFIID and partially purified SAGA. Both ADs directly cross-linked to TBP and to a subset of TFIID and SAGA subunits that carry histone-fold motifs.

Most proteins function within the context of large complexes (1). Affinity and specificity of interactions involving large multiprotein complexes often depend on the sum of cooperative interactions, interactions that individually can be rather weak and only moderately specific. For this reason it is desirable to identify directly interacting polypeptides in the context of their native complexes. A particularly clear case for this need is exemplified in the difficulties inherent in identifying the direct targets of transcriptional activation domains (ADs)[1] within transcription complexes.

The ADs of transcriptional activators are thought to recruit large coactivator complexes to promoters by direct protein-protein interactions (2). Two types of coactivator complexes can be distinguished: (i) those that are directly associated with the transcriptional machinery like the RNA polymerase II-associated mediator and the TAF components of the general transcription factor TFIID and (ii) chromatin-associated complexes like the chromatin-remodeling complex Swi/Snf and the histone acetyltransferase (HAT) complex SAGA.

Most attempts to identify the AD-interacting subunits within these coactivator complexes have been based on binding experiments using isolated polypeptides outside of their native holocomplexes. Interactions observed in these analyses were weak, with apparent dissociation constants of $\geq 10^{-7}$ M (3–7). Moreover, many of these AD-target interactions display only moderate specificity. For example, certain nontranscriptional proteins bind ADs with strengths comparable with physiological targets. Furthermore, there is a remarkable correlation between apparent AD "strength" *in vivo* and binding to transcriptional and nontranscriptional proteins *in vitro*, suggesting that "stickiness" is an inherent and important property conferring function to ADs (5). The most critical amino acids in ADs for both *in vivo* function and *in vitro* binding are bulky hydrophobic amino acids, which in the acidic ADs are exposed by their vicinity to negatively charged amino acids (8–13). Hydrophobic surfaces are typically interaction sites between polypeptides within complexes, and it is likely that these surfaces are artificially exposed when these polypeptides are taken out of their native complexes. Based on the common negative-hydrophobic-negative residue distribution pattern of acidic ADs, hydrophobic surfaces exposed in the vicinity of positively charged amino acids are likely to interact, at least to some degree, with acidic ADs. Therefore, *in vitro* binding studies with individual subunits are potentially prone to artifacts. Thus, it is an important challenge to determine the interaction sites of ADs in the context of native complexes, for instance by chemical cross-linking. However, this effort is hampered by the weakness of AD-target interactions relative to the much stronger interactions between the subunits of AD-interacting complexes.

An elegant approach that avoids the formation of large and therefore difficult-to-analyze cross-linking complexes is the use of photoactivatable label transfer cross-linking reagents. This method has been successfully employed to identify several activator targets within intact complexes, including mediator (14), SAGA (15), and Swi/Snf (16) complexes, as well as in bacterial RNA polymerase (17). However, as powerful as this approach is, it does have limitations. First, the label has to be at or immediately abutting the site that directly contacts its target, while at the same time the 15–21 Å photoactivatable

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[1] The abbreviations used are: AD, activation domain; GST, glutathione *S*-transferase; HA, influenza hemagglutinin; HAT, histone acetyl transferase; HF, histone fold; MMPP, magnesium monoperoxyphtaleic acid; SAGA, Spt-Ada-Gcn5-acetyltransferase; TAF, TBP associated factor; TBP, TATA-binding protein; TEV, tobacco etch virus; WCE, whole-cell extract; TFIID, transcription factor IID.

group must not interfere with complex formation. Second, labile, light-sensitive radioactive protein-photoactive variants of ADs must be prepared. Finally, radioactively labeled target proteins are identified by size, a requirement that potentially limits the use of this approach for the analysis of large polypeptides or to analyze subunits with similar sizes relative to each other or to the labeled probe molecule (for instance, see Ref. 14).

Here we describe a variation on protein-protein cross-linking wherein we used immunodetection coupled with electrophoretic mobility changes to score protein-protein interactions. With this approach, we can clearly distinguish all of the subunits of the relevant, multisubunit complexes. We employed a newly developed site-specific cross-linking technique to identify protein-protein interactions termed hexahistidine-mediated cross-linking (18, 19) that uses a common nickel-activated hexahistidine tag as receptor for a nondiffusible cross-linking reagent. With this method, there is no need to chemically modify the activator with photoprobes or radioactivity, and the hexahistidine tag can be genetically introduced at any inert position of the molecule. Finally, the reagent only cross-links residues that are very closely apposed (18, 19).

In this analysis, we concentrated on the interactions of ADs with the TFIID and the SAGA complexes. These complexes partially overlap in subunit composition (20) and function (21), a fact further complicating interpretations based on the analysis of the interactions of ADs with isolated, individual subunits.

TFIID consists of TATA-binding protein (TBP) and 14 TBP-associated factors (TAF_{II}s) (22). Five TAF_{II}s are present in both TFIID and in the 17-subunit SAGA complex (20, 23), and each complex contains a subunit with intrinsic HAT activity (20, 24). Although all but one of the yeast TAF_{II}s are essential for viability, only certain TAF_{II}s, particularly the ones shared by TFIID and SAGA with similarity to histones, are needed for the transcription of a broad set of genes (25). Using the hexahistidine cross-linking method, we have identified AD interacting subunits in the context of intact TFIID and SAGA and also show that although other proposed targets do bind and cross-link to ADs as isolated polypeptides, they are not accessible in the native complexes.

MATERIALS AND METHODS

Yeast Strains and Genomic Tagging—Yeast strains with genomically triple-HA tagged TBP, TAF1, TAF5, TAF6, and TAF10 were described previously (26). All other factors were genomically triple-HA tagged for this work in strain 21R (27) using standard procedures (28).

Antibodies—Immunopurified rabbit polyclonal antibodies directed against TAF1, TAF2, TAF4, TAF5, TAF6, TAF11, TAF12, TAF13, TBP, Ada2, and Gcn5 were described previously (26). The antibody directed against Tra1p was a kind gift from J. L. Workman. Anti-HA mouse monoclonal antibody (clone 12CA5) was purchased from Roche.

Recombinant Proteins—Recombinant GST and GST-His_6 fusion proteins were expressed in *Escherichia coli* from pGexCS (29) variants in which an oligonucleotide encoding His_6 was inserted into the *Nco*I site and from pKM vectors (30). GST-His_6 fusion proteins contain a cleavage site for TEV protease, which increases cross-linking efficiency by providing tyrosine residues as electron donors in the vicinity of the His_6 tag (18). Fusion proteins were bound and purified on glutathione-Sepharose (Amersham Biosciences) using standard protocols (30). TAF9-HA_2 open reading frame was amplified by PCR from the TAF9-HA_3 strain and cloned into pGexCS for expression.

Preparation of WCE—Transcriptionally competent WCE were prepared from 800-ml cultures of each genomically tagged yeast strain. Cells were grown to a density of OD_{600} ≈1, and extracts were prepared according to Woontner *et al.* (31).

Pull-down and Cross-linking—5–10 μg (0.3 μM, or concentrations as indicated in Fig. 1) of GST or GST-activator fusion protein bound to glutathione-Sepharose beads were incubated with 500 μg of WCE protein (~20 μl), each, for 1 h at 4 °C on a tiltboard in 1 ml of transcription buffer (S. Hahn: www.fhcrc.org/science/basic/labs/hahn/): 10 mM Hepes-KOH pH 7.5, 100 mM K-glutamate, 10 mM Mg-acetate, 2.5 mM EGTA, 3.5% glycerol, 0.01% Nonidet P-40). Beads were then washed 3× for 5–10 min in 500 μl of transcription buffer, each, on ice.

Washed beads were resuspended in 500 μl of transcription buffer. 100 μl of the suspension were removed to control GST-fusion protein input (Coomassie stained gel) and 150 μl as control for the pull-down reaction. The remaining beads with bound complexes were pelleted and resuspended in 100 μl of buffer. Hexahistidine tags were complexed with Ni^{2+} by addition of 25 μl of 60 mM Ni-acetate and incubation for 10 min at room temperature. The cross-linking reaction was started by addition of 25 μl of 60 to 120 mM magnesium monoperoxyphtaleic acid (MMPP) and allowed to proceed for 6 min. Reactions were terminated by centrifugation, removal of supernatants, and resuspension of beads in 10 μl of 2× SDS sample buffer. Each cross-linking reaction was repeated at least once with independently prepared extracts. Further information on the His6-mediated cross-linking method can be found at www.uni-frankfurt.de/fb15/mikro/melcher.html.

Tobacco Etch Virus (TEV) Protease Cleavage of Cross-linking Products—Beads from half of the cross-linking reactions were washed 3 times with 500 μl of transcription buffer. Washed beads were resuspended in 10 μl of the same buffer supplemented with 0.1 mM EDTA and 1 mM dithiothrietol plus 1 μl of TEV protease (2.5 μg/μl) and incubated for 30 min at room temperature. Supernatants were made 1× SDS sample buffer, separated by SDS-PAGE, and analyzed by immunoblotting.

SAGA Enrichment and TFIID Purification—To separate SAGA from TFIID, WCE was prepared from 4 liters of culture of yeast cells and loaded onto a nickel-nitrilotriacetic acid agarose column as described (20). Whereas essentially all of TFIID was found in the flow through, SAGA bound to the nickel-nitrilotriacetic acid agarose and was eluted with 300 mM imidazole. TFIID was purified as described (22).

RESULTS

An Acidic Activation Domain Interacts with TFIID, Holopolymerase, Swi/Snf, and SAGA Complexes—To immunologically identify individual subunits of yeast transcription complexes, we genomically tagged and/or raised antibodies against all subunits of TFIID and SAGA as well as against the mediator component Med6 and the Swi/Snf component Swi2 (Fig. 1). Genomically tagged strains were created by introducing a triple influenza virus hemagglutinin (HA)-tag by homologous recombination at the 3′ end of the respective open reading frames. Thus, tagged proteins were expressed from these genes under their native promoters and in their endogenous chromosomal environment. WCEs were prepared from these strains and used for GST-AD pull-down experiments.

We incubated glutathione-Sepharose-bound GST-VP16 AD with transcriptionally competent yeast WCE and determined whether the four coactivator complexes bound to the VP16 AD. As shown in Fig. 2*A*, all four components were retained by saturating amounts of GST-VP16 AD but not by GST alone. To test the specificity of these interactions, transcription extracts were incubated with the wild-type Gal4 and VP16 ADs as well as with transcriptionally inactive variants of the two ADs. Both SAGA (as scored by Ada1p) and TFIID (as scored by TAF4) were retained by the two functional ADs but even under saturating conditions of immobilized ADs, these coactivator complexes did not bind inactive derivatives (Fig. 2*B*). To determine the relative affinities of interactions, we titrated the concentration of immobilized ADs in 3-fold increments down from the saturating concentrations used in *panels A* and *B*. The minimal concentration of Gal4p AD needed to efficiently retain SAGA from WCE was at least 10-fold lower than the concentrations of any of the other coactivator complexes, consistent with SAGA being the predominant complex recruited *in vivo* by Gal4 AD in the absence of a core promoter and other coactivator complexes (32). Surprisingly, TFIID, which is only recruited at sub-stoichiometric levels to the *GAL1* promoter *in vivo* (33), is actually more efficiently retained by Gal4 AD than the mediator and Swi/Snf complexes (Fig. 2*C*), suggesting that the efficient re-

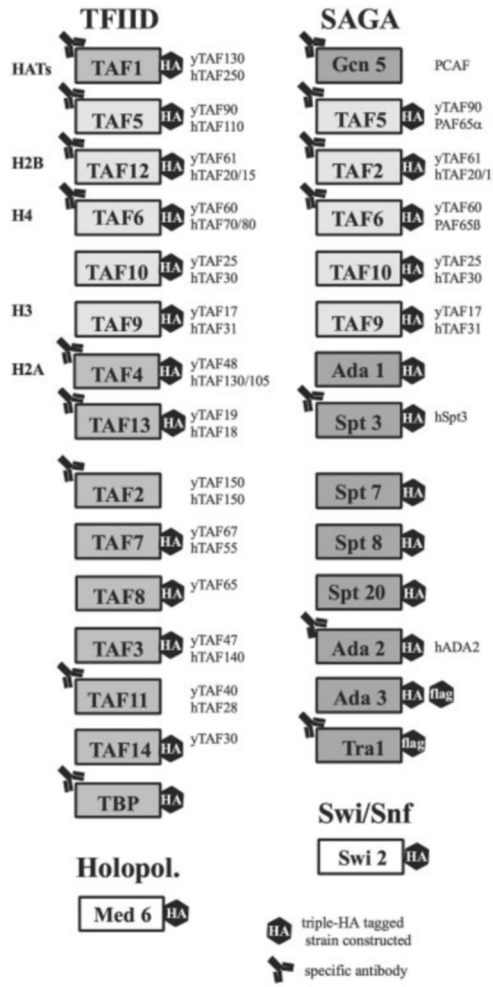

FIG. 1. **Immunological reagents for the detection of specific subunits of transcription complexes.** *Rectangles* represent the indicated 15 subunits of yeast TFIID and 14 subunits of SAGA as well as Med6 and Swi2. Note that after completion of this work, three additional SAGA subunits were identified by a proteomic approach (23). TFIID-specific subunits are shown in *medium gray*, SAGA-specific subunits in *dark gray*, and shared subunits as *light gray rectangles*. *Symbols* indicate antibodies raised and/or genomically triple-HA_3 tagged strains constructed. The antibody against Tra1 was a gift of J. Workman. The old nomenclatures of yeast TAF subunits and their corresponding human homologs are listed to the *right* of yeast TFIID and SAGA subunits. HAT activity and homology to the core histones H2A, H2B, H3, and H4 have been indicated for subunits of both complexes at the *very left*.

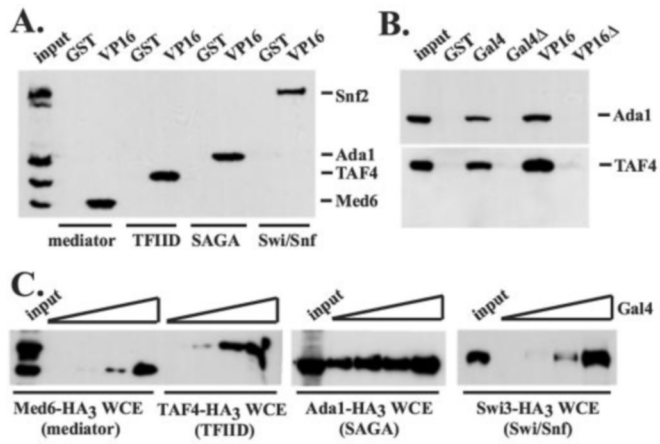

FIG. 2. **Binding of transcription complexes to acidic ADs.** *A*, extracts from four isogenic strains with different factors genomically tagged were incubated with 900 nM GST or GST-VP16AD. HA_3-tagged factors were Med6p (mediator), TAF4 (TFIID), Ada1p (SAGA), and Snf2p (Swi/Snf). *Input*, pooled extracts representing 10% of the binding reactions. Extracts used for pull-downs are indicated on *top*. *B*, TFIID and SAGA do not bind to transcriptionally inactive derivatives of the Gal4p and VP16 ADs. Gal4 AD, Gal4 (841–875); Gal4Δ, Gal4 (841–854); VP16 AD, VP16 (420–490); VP16Δ, VP16(420–456/F442P). The *top panel s*hows pull-downs from the Ada1-HA_3 extract, the *lower panel* from the TAF4-HA_3 extract. *C*, Gal4 AD binds coactivator complexes with variable affinities. Pull-down reactions contained varying concentrations of Gal4 AD (30, 100, 300, 900 nM) to accentuate the relative strength of AD-coactivator interactions. All reactions were separated by SDS-PAGE, blotted to polyvinylidene difluoride membrane, and incubated with anti-HA antibody.

cruitment of mediator (34) likely depends on additional interactions. Importantly, a series of coimmunoprecipitation experiments indicated that TFIID and SAGA remained stable in the WCE during the course of these experiments (data not shown, and see below) indicating that these experiments truly scored the interactions of the coactivator complexes with ADs.

Cross-linking of ADs to TFIID and SAGA Complexes in WCE—We used hexahistidine-mediated cross-linking (19) to probe for direct interactions within the context of intact TFIID and SAGA. Briefly, complexes were collected from WCE by fusion proteins consisting of a GST tag, a hexahistidine tag, and the Gal4 or VP16 AD bound to glutathione-Sepharose beads as described above. The His_6 tag was then complexed with Ni(II) by short incubation with nickel acetate followed by incubation with the peracid MMPP, which activates the complexed Ni(II) to Ni(III). It has been proposed that this Ni(III) extracts an electron from an aromatic side chain, resulting in the formation of highly reactive radicals that lead to fast and efficient 0Å cross-linking (*i.e.* direct cross-linking of side chains, without a linker in between). Importantly, it has been documented that even in crude cellular extracts, only those interacting proteins that are complexed with a hexahistidine-tagged protein become cross-linked (19). Fig. 3*A* shows an outline of the experiment, and Fig. 3*B* shows an example of MMPP-dependent, His_6-mediated cross-linking of the VP16 and Gal4p ADs to TAF12 within TFIID and/or SAGA in WCE.

SAGA and TFIID complexes derived from WCE were bound to the Gal4 and VP16 ADs, cross-linked to the AD with MMPP, and denatured. Cross-linking products were identified by immunoblotting. The *top panel o*f Fig. 3*B* shows the blot incubated with anti-TAF12 antibody. In the lane containing the cross-linking reaction with Gal4 AD, the anti-TAF12 antibody recognized three bands, one band of around 61 kDa corresponding to monomeric TAF12, one band at around 120 kDa corresponding to the 60-kDa GST-His_6-Gal4AD dimer cross-linked to TAF12, and one band that did not enter the gel corresponding to a high molecular weight complex (see below). In the cross-linking reaction with VP16 AD qualitatively the same 61 kDa and high molecular weight bands were visible. However, instead of the 120-kDa band (*i.e.* the TAF12(GST-His_6-$Gal4AD)_2$ adduct), a band of about 130 kDa was recognized by the anti-TAF12 antibody corresponding to the 70-kDa GST-His_6-VP16AD dimer cross-linked to TAF12. The characteristic shift in mobility of the cross-linking complexes, due to the size difference of the Gal4 and VP16 ADs, serves to indicate that the cross-linking complexes recognized by the antibody do indeed contain the two different ADs. Radicals created in the cross-linking reaction can migrate through more than one protein in a complex and can thus cause high molecular weight complexes as well (19). The protein complexes not entering the gel are likely such high molecular weight complexes and were not analyzed further. Only cross-linked complexes consisting solely of the GST-His_6-AD and the subunit detected by the antibody are indicative of direct interaction.

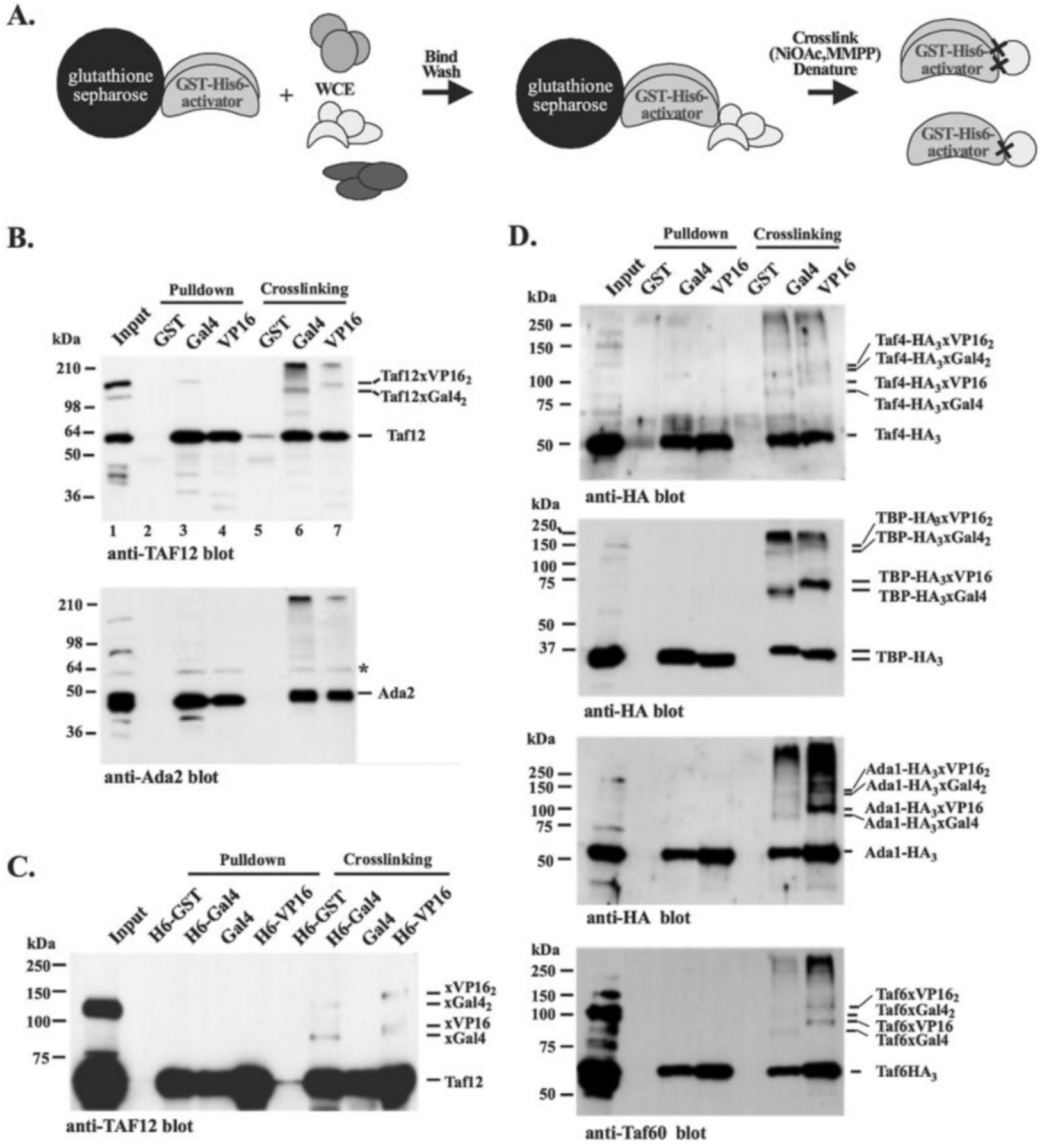

FIG. 3. **The ADs of Gal4 and VP16 cross-link to TBP, TAF12/yTAF$_{II}$61, TAF4/yTAF$_{II}$48, Ada1p, and TAF6/yTAF$_{II}$60 from transcriptionally competent extract (WCE).** *A*, cartoon of binding and cross-linking reaction. The *x* represents cross-links. *B*, specific binding and cross-linking to TAF12. *Top*, immunoblot with anti-TAF12 antibody. *Lane 1*, extract (10% of input). *Lanes 2–4*, pull-down of a TAF12-containing complex with GST-His$_6$, GST-His$_6$-Gal4pAD, and GST-His$_6$-VP16AD. *Lanes 5–7*, His$_6$-mediated cross-linking of bound complexes. TAF12xVP16$_2$ and TAF12xGal4$_2$ represent cross-linking complexes consisting of one TAF12 monomer and one GST-His$_6$-AD dimer. *Below* the anti-TAF12 blot is the same blot stripped and reprobed with anti-Ada2 antibody. *, residual TAF12 signal after stripping. *C*, same as *top panel* of *B*, but including GST-Gal4 AD lacking a His$_6$ tag (Gal4). *D*, detection of TAF4-HA$_3$, TBP-HA$_3$, Ada1-HA$_3$, and TAF6 cross-linking complexes.

The anti-TAF12 blot shown at the *top* of Fig. 2*B* was then stripped and reprobed with anti-Ada2 antibody to determine whether cross-linked AD-Ada2p adducts had also been formed in the same samples and experiment. Like TAF12, Ada2p is also part of the SAGA complex and was retained by the Gal4 and VP16 ADs to the same degree as TAF12 (cf. *lanes 2–4*, *top* and *bottom* blots of Fig. 2*B*). However, no cross-linking product of the correct size was observed between Ada2p and either AD (Fig. 2*B*, *lower panel*), suggesting that the ADs bind the SAGA complex without directly contacting Ada2p.

Untagged SAGA specifically binds to Ni-agarose and can be efficiently separated from TFIID by metal affinity chromatography (20). Hexahistidine-mediated cross-linking might therefore also occur within SAGA in the absence of the His$_6$-tagged AD. To test this possibility, we repeated the above experiment with GST-Gal4AD lacking the His$_6$ tag. As expected, GST-Gal4AD retained TAF12 just as efficiently as GST-His$_6$-Gal4AD, however, no TAF12-AD adducts were observed when the GST-AD-SAGA beads were treated with cross-linker (Fig. 3*C*). We conclude that the SAGA component TAF12 does not form cross-linking products in the absence of an introduced His$_6$ tag. These data provide additional support for the specificity of the observed AD-TAF12 adducts described above.

Having demonstrated the utility of this chemical cross-linking approach, we next performed identical binding and cross-linking reactions with extracts that allowed analysis of each subunit of TFIID and SAGA in turn. Only TAF2 was not examined as we were neither able to genomically tag TAF2 nor reproducibly detect this TAF with our polyclonal anti-TAF2 IgG. Another limitation of our technique applies to the Tra1p subunit of SAGA. The Tra1 monomer has a size of 430 kDa, close to the exclusion limit of the SDS-PAGE gels used. We were therefore not able to separate a potential cross-linked product (*i.e.* Tra1 and the ADs, M_r 430 + 60 kDa) from monomeric Tra1. Each experiment was repeated several times with independently prepared extracts and with different concentrations of cross-linking reagents, with equivalent results. In this extensive series of experiments, in addition to TAF12, we found

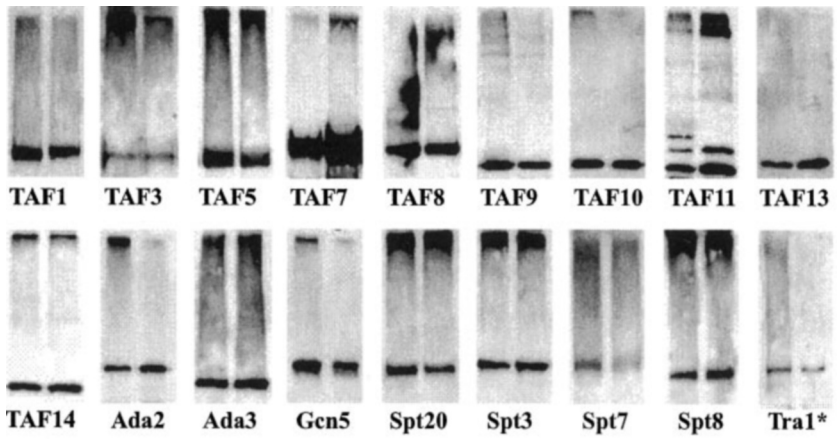

FIG. 4. **Immunological detection of remaining TFIID and SAGA subunits in cross-linking reactions.** Complexes from extracts were bound and cross-linked to His_6-GST-ADs as described in Fig. 3. The *left lane* of each panel contains the cross-linking reaction with Gal4 AD, the *right panel w*ith VP16 AD. The factor immunologically detected is indicated *below* each panel. Each factor was tested in cross-linking reactions from at least two independently prepared extracts. *, Tra1p is shown together with the subunits that do not cross-link to the ADs, although potential Tra1p-AD cross-linking products may not separate from the monomeric 433-kDa Tra1p.

that TBP, TAF4, TAF6, and Ada1p directly cross-linked to both Gal4 and VP16 ADs (Fig. 2*B*). In no case have we observed differential interaction of either AD with the five target proteins. Importantly, although all other subunits were retained specifically and to a very similar degree by GST-AD beads, consistent with TFIID and SAGA being intact, we did not detect any specific cross-linking complexes with any of the other TFIID and SAGA subunits (Fig. 4). With the exception of TBP, the proteins cross-linked contained histone-fold motifs and were the ones with homology to histones H2A (TAF4 and Ada1), H2B (TAF12), and H4 (TAF6) in both complexes.

Confirmation that Cross-linked Complexes Contain ADs Bound to TFIID and SAGA Subunits—The characteristic mobility shift of cross-linking complexes from Gal4 *versus* VP16 reactions argued strongly that these ADs were present in the cross-linked products. To further confirm this conclusion, we engineered a TEV protease cleavage site between the N-terminal tags and the VP16 AD. We reasoned that if the putative AD-protein complexes truly contain the VP16 AD, TEV cleavage should induce a significant mobility shift upon SDS-PAGE fractionation of the digested complexes (see schematic in Fig. 5*A*). We used this TEV-cleavage approach to examine all five of the AD-protein complexes that we had positively scored for direct interactions in our standard cross-linking protocol (*i.e.* TBP, TAF4, TAF6, TAF12, and Ada1).

After pull-down and cross-linking, half of the reactions were incubated with TEV protease before denaturation (Fig. 5*A*). In every case, TEV cleavage induced the predicted dramatic mobility change in these complexes (Fig. 5*B*), demonstrating that the cross-linking complexes did indeed contain the AD. The two major TEV cleavage products correspond to cross-linked adducts in which one or both of the TEV protease sites were cleaved (cf. Fig. 5*A*: TAF4, TAF6, TAF12, Ada1). Note that the cross-linking and cleavage experiment with the TBP-HA_3-tagged strain was performed with a different preparation of AD-fusion protein, which, due to a read-through product, migrates as two distinct monomer bands on SDS-PAGE and consequently gives rise to three different forms of dimers. Like the dimeric fusion protein by itself, both the uncleaved cross-linking product as well as the singly and doubly cleaved complexes migrate as characteristic triplet bands, providing additional proof for the presence of the VP16 AD in the cross-linking complex and in the proteolytic cleavage products.

Ada2p and TAF9 Cross-link to the Gal4 and VP16 ADs When Outside of Their Physiological Context—Ada2p and TAF9 did not cross-link to the Gal4 and VP16 ADs when probed within the context of intact complexes in WCE (Figs. 3*B* and 4). However, it has been reported that recombinant Ada2p (35) and the recombinant *Drosophila* and human TAF9 (d $TAF_{II}40$ and h $TAF_{II}32$) directly bind to the VP16 AD (36, 37). One possible explanation for this apparent discrepancy is that the AD-interacting surfaces of these factors are not accessible in the native complexes but are only (artificially) exposed when in the absence of their normal binding partners. Alternatively, these proteins could also bind ADs in the context of their multiprotein complexes, but our cross-linking method fails to work efficiently with these two proteins. Although the proposed mechanism of hexahistidine-mediated cross-linking does not predict such a selectivity for the target proteins, it was important to experimentally address this possibility. We therefore isolated the *ADA2* and *TAF9-HA_2* genes and expressed and purified the corresponding proteins from *E. coli*. To avoid any potential driving of cross-linking efficiencies by mass action, the concentrations of recombinant Ada2p and TAF9-HA_2 in binding and cross-linking reactions were chosen to be in the same concentration range (about 1 nM) used in the cross-linking experiments with WCE (Figs. 3 and 4). As shown in Fig. 6, both recombinant Ada2p and TAF9-HA_2, as well as TBP as a positive control, bound and cross-linked to the Gal4 and VP16 ADs. We conclude that the inability of Ada2p and TAF9 to cross-link ADs when in the context of SAGA and TFIID is not caused by an inherent incompatibility with the cross-linking reagents, but is most likely due to the inaccessibility of the AD-binding surfaces within Ada2 and TAF9 when these two proteins are present within the context of their native complexes. Of course, we can not rule out the possibility that these surfaces could become accessible upon potential conformational changes when present within TFIID and SAGA.

TAF6 and TAF12 Can Interact with ADs in the Context of Purified TFIID and SAGA—TAF6 and TAF12 are components of both TFIID and SAGA. Thus, the TAF6-and TAF12 AD-cross-linking we observed could have been in the context of TFIID, of SAGA, or of both. Similarly, TBP is also a component of several distinct TBP-TAF complexes, including Pol I- and Pol III-specific TBP-TAF complexes (SL1 and TFIIIB respectively) and due to the fact that WCEs were used in the experiments of Figs. 3–5, we do not know in which context (SL1, TFIID, or TFIIIB) TBP cross-linked with the Gal4 and VP16 ADs. To distinguish between these possibilities and to further support our contention that we are scoring direct AD-target interactions, we examined the cross-linking behavior of ADs to purified TFIID and partially purified SAGA. Separation of TFIID

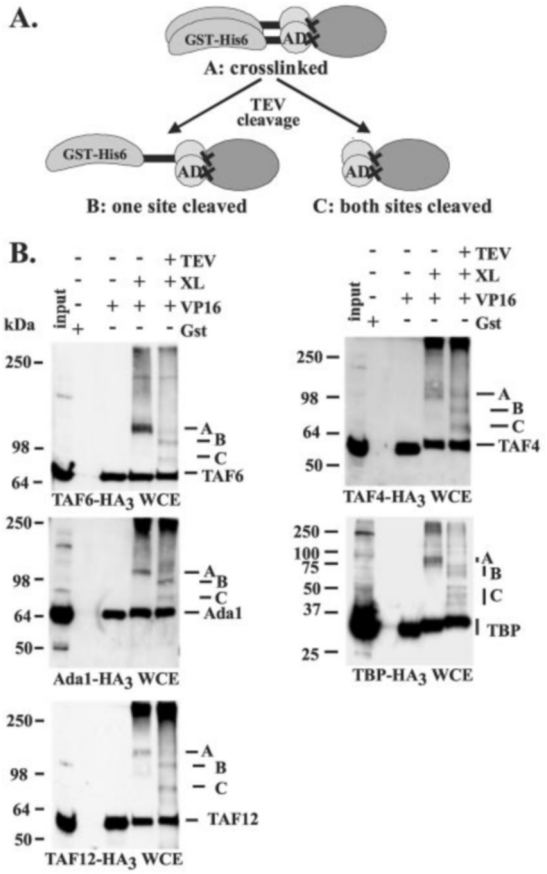

FIG. 5. **Analytical proteolysis of cross-linking products.** *A*, schematic outline of the reaction with the major cross-linking and cleavage products. Immobilized GST-His_6-VP16AD with a cleavage site for the highly specific TEV protease (indicated by the *thick bar*) were bound and cross-linked to complexes from extract. After cross-linking, part of the immobilized fusion proteins with covalently cross-linked TFIID and SAGA subunits were treated with TEV protease before denaturation. *B*, immunoblots of cross-linking and cleavage reactions. All blots were probed with anti-HA antibody. As indicated in *panel A*, *A* represents the uncleaved complex, *B* the complex with one site cleaved, and *C* the doubly cleaved complex. Note that the VP16 preparation used for cross-linking of complexes from the TBP-HA_3 tagged strain differed from the preparation used for the other figures. GST-His_6-VP16AD dimers from this preparation migrate as characteristic triplet bands and serve as additional control.

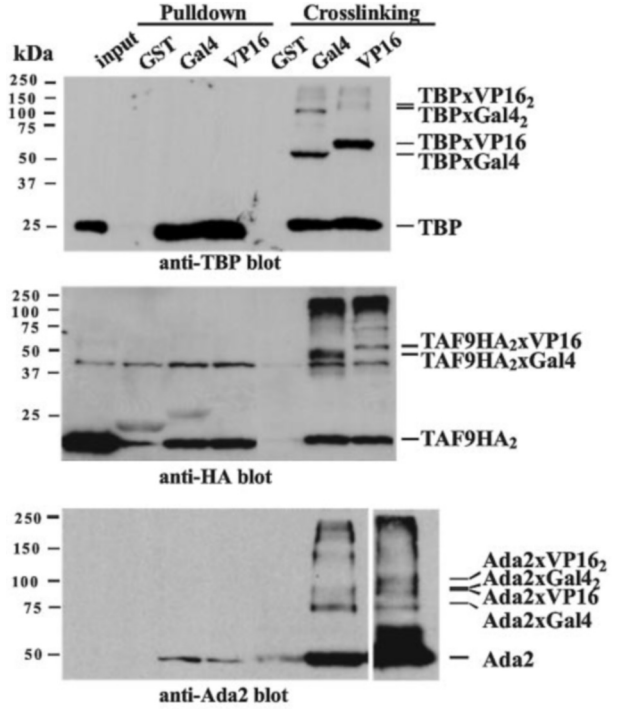

FIG. 6. **Recombinant TAF9 and Ada2p bind and cross-link to ADs.** Recombinant TBP, TAF9-HA_2, and Ada2p were bound and cross-linked to the indicated GST-His_6 fusion proteins. Reactions were performed and analyzed by immunoblotting as described in Fig. 3. *Top*, reactions with TBP as positive control, detected with anti-TBP antibody. *Middle*, reactions with TAF9-HA_2, detected with anti-HA antibody. *Bottom*, reaction with Ada2p, detected with anti-Ada2p antibody.

and SAGA was confirmed by immunoblotting with antibodies against the SAGA-specific Ada2p and the TFIID-specific TAF4 subunits (Fig. 7*A*). As predicted from the data presented above (Figs. 3 and 5) Gal4 AD and VP16 AD cross-linked to TAF6 and TAF12 in the context of both TFIID and SAGA (Fig. 7*B*). For reasons that we do not presently understand, cross-linking to TAF6 varied significantly with different preparations of TFIID and SAGA (data not shown). The ADs also cross-linked to TBP within the purified TFIID complex (Fig. 7*C*), which demonstrates that TBP can interact with ADs in the context of TFIID.

DISCUSSION

AD-target interactions appear to be inherently weak and, because of their hydrophobic nature and limited specificity, direct measurements of these interactions using purified, isolated ADs and (putative) target proteins can be problematic. Here, we describe an approach to analyze AD interactions in the context of native complexes and report the first identification of AD-binding subunits within intact TFIID, as well as the identification of previously unrecognized direct AD contacts within intact SAGA. Our finding that Ada2 and TAF9 can directly interact and cross-link with the Gal4 and VP16 ADs as isolated polypeptides, but apparently not in the context of their native complexes, underscores the utility of our approach.

Our use of HA_3-tagged strains in combination with antibodies raised against specific subunits enabled us to unambiguously test the presence of all subunits except TAF2 and Tra1p in cross-linking products. Because, for most experiments, the same anti-HA antibody was used for detection of differently sized HA_3-tagged proteins, we could exclude immunoreactive signals being due to antibody cross-reactive proteins within the indicated cross-linking products. Three different lines of evidence demonstrate the presence of ADs within cross-linking products. First, the size of cross-linking products is consistent with monomers and dimers of AD fusion proteins cross-linked to the immunologically detected factors. Second, the size difference between the Gal4 and VP16 ADs can be seen in all cross-linking products. Third, incubation with the highly selective TEV protease resulted in the production of the expected specifically cleaved complexes. In addition, the cross-linking products between GST-His_6-VP16AD and TBP-HA_3 as well as their cleavage products migrate as characteristic triplet bands, indicative of the presence of the VP16 AD.

An important feature of His_6-mediated cross-linking is that it functions without a linker between reactive groups. Cross-linking is therefore likely to result from proteins that directly touch each other rather than just being in close proximity. This conclusion is supported by our TEV cleavage analysis (Fig. 5), where in all cases the sizes of cleavage products are consistent with direct cross-linking of the AD rather than cross-linking with other parts of the fusion protein. In contrast, in control experiments using dithiobis(succinimidyl propionate) (DSP), a conventional cross-linker consisting of two reactive groups separated by a 12-Å linker, recombinant TBP cross-linked predominantly to GST, the largest moiety of the His_6-GST-VP16 fusion protein (data not shown). Direct interactions of the ADs with

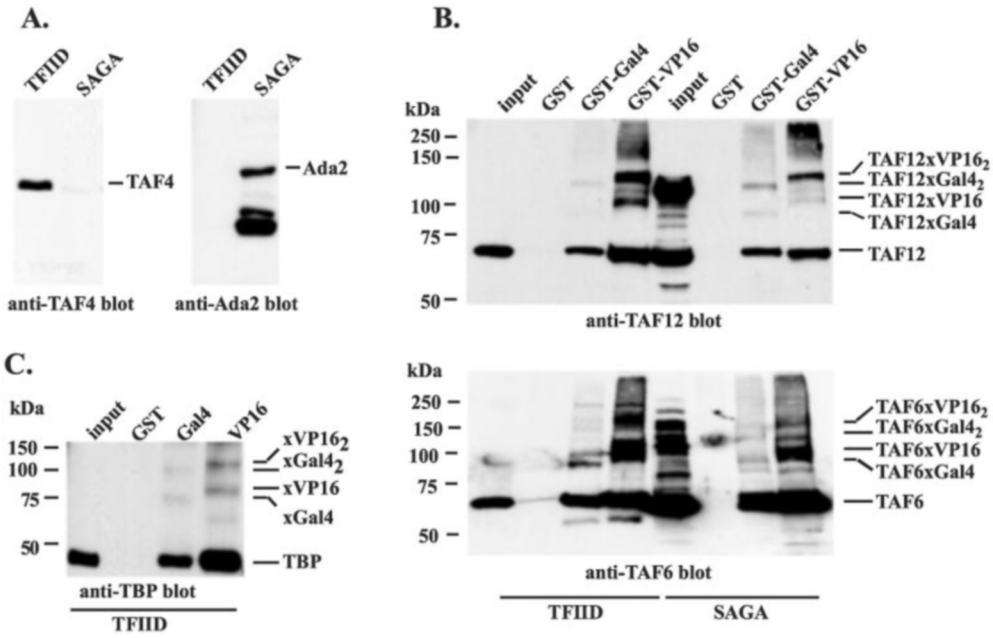

FIG. 7. **Cross-linking reactions with purified TFIID and SAGA.** *A*, purified TFIID and partially purified SAGA were tested for the presence of the TFIID-specific TAF4 and the SAGA-specific Ada2p by immunoblotting. *B*, TAF6 and TAF12 cross-link to ADs in the context of both TFIID and SAGA. TFIID and SAGA preparations used in *A* were bound and cross-linked to the indicated GST fusion proteins. Cross-linked complexes were analyzed by immunoblotting with anti-TAF6 and anti-TAF12 antibodies. *C*, TBP directly binds ADs in the context of purified TFIID. TFIID was bound and cross-linked to the indicated GST fusion proteins. Cross-linked products were analyzed by anti- TBP immunoblotting.

TBP, TAF6, and TAF12 is further supported by binding experiments with recombinant TBP (Fig. 6) and *in vitro*-translated TAF6 and TAF12 (data not shown) and by an *in vivo* interaction between Gal4 and TAF6 (38).

Cross-linking initiates solely at the His_6-tagged protein (19) (see also Fig. 3*C*), which favors the formation of direct cross-linking products between ADs and interacting subunits. All indicated cross-linking complexes with Gal4 and VP16 AD monomers and dimers as well as their corresponding TEV cleavage products migrated with the mobilities expected for direct cross-linking products (Figs. 3, 5, 6, and 7). The only three AD-cross-linking subunits within SAGA (TAF6, TAF12, and Ada1) that we have identified are all of very similar size (54–61 kDa) and consequently produce cross-linking products of similar mobilities. If cross-linking of any of these three proteins would have occurred indirectly via one of the two other subunits, then mobility of the resulting complex would be expected to be significantly retarded relative to the two other complexes (*e.g.* compare the doubly TEV-cleaved cross-linking products of TAF6, TAF12, and Ada1 in Fig. 5, all of which migrate with their calculated sizes between 72 and 79 kDa as opposed to sizes between 132 and 139 kDa expected for any of the combinations of indirect cross-linkings).

The fact that all 14 TFIID subunits detectable and all 14 SAGA subunits were retained by the AD fusion proteins strongly supports the conclusion from co-immunoprecipitation experiments that TFIID and SAGA were intact in our transcriptionally competent WCE preparations. Cross-linking of TBP, TAF6, and TAF12 to the Gal4p and VP16 ADs in reactions with purified TFIID further confirms that interaction occurred in the context of complexes and not with dissociated subunits. Although a lack of a cross-linking signal may not unambiguously exclude a direct interaction, the presence of a cross-linking product consisting solely of the AD and a specific subunit demonstrates a physical AD interaction in the context of native complexes.

The Workman (15) and Struhl (39) labs have described two different approaches to probe for AD targets within coactivator complexes. Brown *et al.* (15) used label transfer cross-linking to identify targets of the VP16 AD within two HAT complexes, SAGA and NuA4. They demonstrated that Tra1p, which is the only common subunit of SAGA and NuA4, directly interacts with acidic ADs in the context of both purified complexes (15). Very recently, Hall and Struhl (39) pioneered a method that combines *in vivo* formaldehyde cross-linking with the immunoprecipitation of activator proteins. Using this approach, they identified TBP, TFIIB, and 9 of the 14 subunits of SAGA, including Tra1, TAF6, TAF12, and Ada1 in Gal4-VP16 immunoprecipitates. Although truly *in vivo*, the formaldehyde-based approach is not site-specific and requires reversal of cross-linking after immunoprecipitation. Thus, this method cannot distinguish which of the 9 subunits of SAGA cross-linked directly to the VP16 AD and which were indirectly cross-linked. However, we note that in this study *in vivo* cross-linking of Ada1 to VP16 AD was not reduced in a Δ*spt3* mutant, a condition in which AD cross-linkings of both Tra1 and TAF12 were strongly reduced (19). This result implies that Ada1 was not indirectly cross-linked to the AD via Tra1 or TAF12, data consistent with our own. Here we have shown that Gal4 and VP16 ADs cross-link with TAF6, TAF12, and Ada1 within native SAGA and with TAF6, TAF12, and TAF4 (homologous to Ada1) subunits of native TFIID. Putative TAF4-TAF12 and TAF6-TAF9 HF heterodimers have been mapped to two different locations each within TFIID. At one of these locations, both heterodimers colocalize and potentially could form a histone octamer-like substructure (40), a structure that can also be generated from recombinant proteins (41).

These identifications of AD-interacting subunits clearly show the limitations of each assay. First, Tra1p is the only SAGA component that, because of its large size, cannot be analyzed by His_6-mediated cross-linking and conversely, Ada1p, TAF6, or TAF12 could not be analyzed by the label-transfer technique because their protein sizes (54–61 kDa) directly overlap with the broad signal from the labeled activator (15). Second, *in vitro*, both NuA4 with its accessible Tra1 subunit and TFIID with its accessible TAF4, TAF6, and TAF12

subunits bind Gal4 and VP16 ADs with specificity and can mediate Gal4-VP16-dependent transcriptional activation *in vitro* (Refs. 42 and 43; Fig. 2*B*). *In vivo*, however, only SAGA, containing both Tra1p and TAF6/12/Ada1, is efficiently recruited by Gal4 AD to Gal4 target genes (32, 44), whereas NuA4 is not detectably recruited at all (45) and TFIID only at substochiometric levels (33) to the same target genes (TFIID is recruited to the *GAL1* promoter at about 20% of the level of TBP and other general transcription factors and inactivation of a TFIID-specific TAF compromises *GAL1* transcription only partially (33, 46, 47)). Moreover, we have shown that TFIID binds Gal4 AD with at least 10-fold lower affinity than SAGA does (Fig. 2*C*). This raises the possibility that Tra1p and TAF6/12/Ada1 as the direct AD-interacting subunits need to cooperate to achieve high affinity binding to acidic ADs and that this high affinity binding is the necessary prerequisite for a stoichiometric recruitment *in vivo*.

What, then, is the physiological role of the interaction between TFIID and acidic activators in yeast? We suggest that (i) TFIID may not be a relevant *in vivo* target for Gal4 at all, (ii) TFIID may be quantitatively recruited to *GAL1* and be essential for its transcription only under certain conditions, comparable with the conditional requirement of Swi/Snf for *GAL1* transcription (48), and/or (iii) the TFIID-AD interaction may not result in recruitment but in the release of an inhibitory interaction between TAF1 and the DNA-binding surface of TBP (49, 50). In support of the last possibility, activators like Gal4 overcome the inhibitory TAF1-TBP interaction, although this interaction poses a severe obstacle *in vivo* to recruitment of the transcriptional machinery by artificial recruitment, *i.e.* in the absence of an AD (51). Given the functional redundancy of TFIID and SAGA (21) and the presence of multiple AD-interacting subunits within these complexes, challenging molecular genetic analyses will be required to determine the contribution of each of the identified interactions for recruitment and transcriptional activation *in vivo*. Analyses involving the shared HF-TAFs is further hampered by the fact that they are necessary for SAGA (52) and TFIID (53, 54) integrity and that changes in the expression level of a histone-fold TAF can dramatically affect the TFIID/SAGA ratio *in vivo* as well as the retention of both TFIID and SAGA by Gal4 and VP16 AD from WCE.[2]

In summary, we have demonstrated that His_6-mediated cross-linking in combination with affinity enrichment and genomic tagging can be used as a powerful technique to study protein-protein interactions. We have applied this technique to identify interaction sites of ADs in native coactivator complexes and have complemented and extended the results obtained by *in vitro* label transfer and *in vivo* formaldehyde cross-linking.

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