Evaluation and Utilization of Photo-Activatable Unnatural Amino Acids for the Study of Protein-Protein Interactions

by

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Table of Contents

List of Figures

List of Tables

Table

Abstract

Protein-protein interactions (PPIs) regulate cellular processes through intricate networks that transfer signals throughout the cell. The interactions possess a range of affinities, lifetimes, and interface areas. In transcriptional regulation networks, activators form strong, concise binding interactions with their masking proteins and transient, moderate affinity interactions with the coactivator complexes and transcriptional machinery. It has been a longstanding goal to fully identify and characterize the protein pairings within transcriptional networks, but the necessarily transient nature of the activator-coactivator interactions essential for the recruitment and assembly of the transcriptional machinery has frustrated efforts to identify the specific pairings using available methods. Due to the limitations in current methods, there is a critical need for additional strategies for capturing transient PPIs in the native environment. Our lab has previously optimized an *in vivo* covalent chemical capture strategy using the photo-activatable unnatural amino acid (UAA), pBpa, to interrogate the interactions of prototypical transcriptional activators, such as Gal4. However, these experiments are technically challenging due to low yields of pBpa crosslinking and difficulties in isolating the resulting adducts and have not yet been applied to interrogate new transcriptional PPI networks. This thesis seeks to expand the applications of covalent chemical capture to new transcriptional systems and optimize the pBpa framework for enhanced reactivity and functionality.

Using the current crosslinking methodology, the metabolic interactions between the novel Hcm1 activator and the heterotrimeric SNF1 signaling complex were examined under nutrient stress in *Saccharomyces cerevisiae*. To this end, a minimal transcriptional activation domain (TAD) sequence of Hcm1 regulated by nutrient conditions was identified and optimized for pBpa incorporation and crosslinking. Additionally, the direct interactions between Hcm1 and the Snf1 kinase and Gal83 scaffolding subunits of the SNF1 complex were captured using two pBpa incorporation

sites within the identified TAD. Thus, demonstrating the modularity of the Hcm1 activator and its ability to regulate the expression of glucose-repressed genes through interactions with dynamic coactivator complexes. Moreover, we identified a novel binding interaction between Hcm1 and Gal83 and demonstrated the strength of *in vivo* covalent chemical capture to interrogate PPIs between activators and dynamic coactivator complexes.

While the exploration of Hcm1 demonstrates the utility of pBpa in novel transcriptional systems, the methods limitations remain. We therefore developed a suite of modified pBpa analogs with enhanced functionality that address the current difficulties faced during the covalent capture of challenging PPIs in cells. We designed a suite of pBpa analogs, substituted with various electron withdrawing groups (F, Cl, CF_3, and Br), that demonstrated up to a three-fold increase in crosslinking yield of *in vitro* activator-coactivator interactions. Furthermore, when incorporated into live yeast, these analogs captured cellular activator-coactivator interactions. Preliminary data demonstrated a visible increase in crosslinking yield for the 4-F Bpa derivative compared to its parent molecule. Upon further optimization, the extent of increase of *in vivo* covalent capture for each analog will be quantifiable. Finally, we created a bifunctional pBpa analogue which possesses a bioorthogonal alkyne handle and facilitates more precise isolation of crosslinked partners for more accurate network analysis. The work presented in this thesis outlines key guidelines for the successful use of *in vivo* covalent chemical capture to identify novel PPIs and extends the utility of the benzophenone crosslinker, thus expanding the current toolbox of chemical probes for mapping PPIs in their native cellular environment.

CHAPTER 1

Introduction to characterizing transcriptional protein-protein interaction networks

1.1 Introduction to protein-protein interaction networks

Protein-protein interactions (PPIs) are essential for all cellular processes; PPIs modulate and mediate protein function through sequential and perfectly timed binding events to direct cellular signals to the proper location in the cell. These interactions connect all aspects of the cell through elaborate PPI networks. Misregulation of both PPIs and their networks is detrimental to cellular health, and has been implicated in a range of diseases, including cancer.[1-8] For example, the transcriptional activator and tumor suppressor p53 participates in hundreds of PPIs required for the regulation of apoptosis, and loss of function mutations within this activator have been shown to be the main contributor to more than 50% of human cancers.[9-14] Due to their fundamental importance in cellular homeostasis, PPIs and their interaction networks are logical targets for extensive characterization and eventual therapeutic intervention.

Within the cell there are a variety of PPIs which are typically defined by their cellular function (i.e. transcriptional regulation, signal transduction, protein folding, etc.).[15-16] However, they can be further categorized based on their binding affinity and the surface area of the binding interface as shown in the quadrant diagram in Figure 1.1A.[17-22] Many of the most well-characterized interactions participate in moderate to high affinity binding events that have small, defined interfaces resembling ligand-protein interactions, such as kinases with their substrate or a transcription factor with its masking protein (upper left quadrant, Figure 1.1A). These interactions are the most well-defined in terms of function and mechanism of action which have led to the development of many small molecule probes which facilitate additional characterization.[23-25] For example, several small molecule classes, such as nutlins, sulfonamides, and benzodiazepinediones, have been developed to target the interaction between p53 and its masking protein MDM2.[26-32]
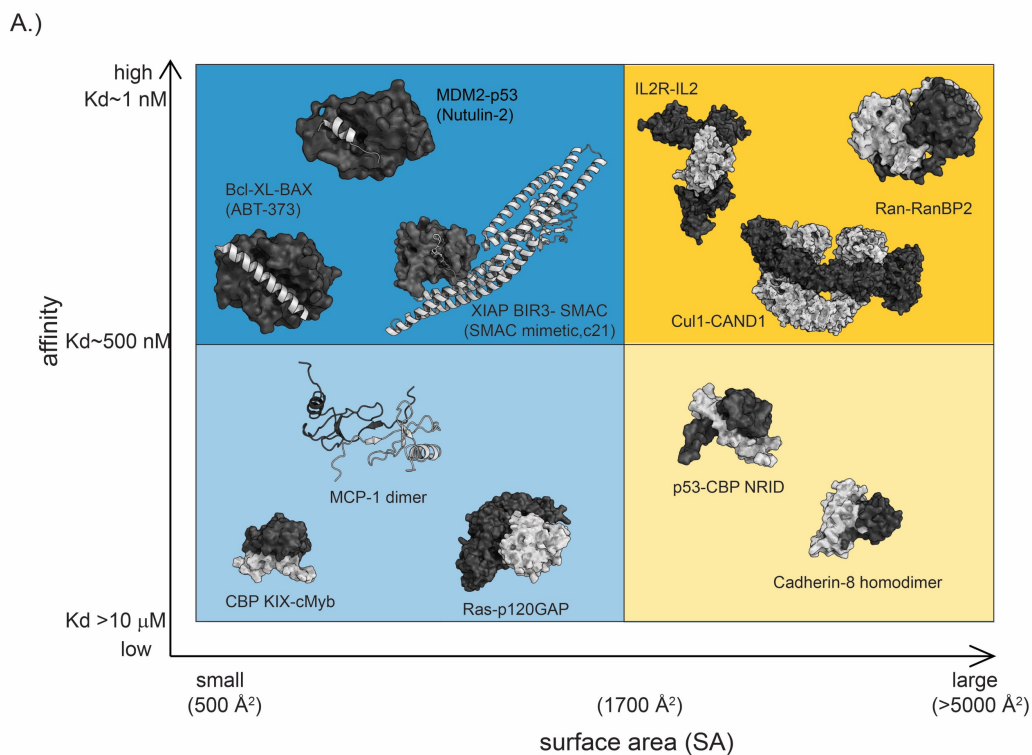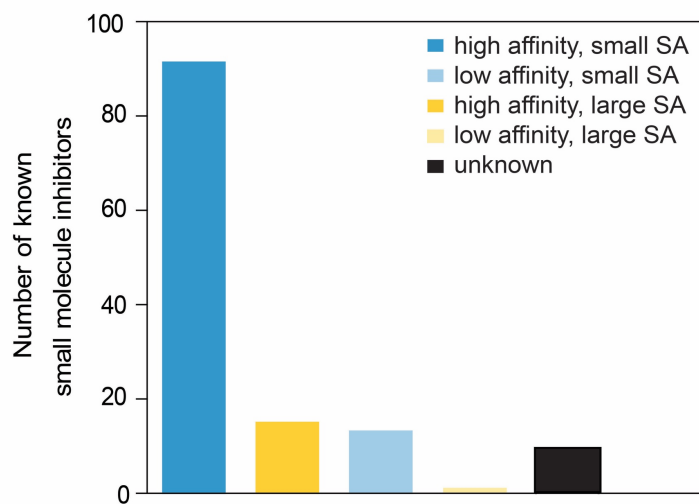
Figure 1.1 PPIs from a variety of cellular processes can be categorized by binding affinity and surface area. A.) Analysis of PPIs using these two variables leads to four main quadrants: strong and concise (dark blue, top left), strong and broad (dark yellow, top right), weak and concise (light blue, bottom left), and weak and broad (light yellow, bottom right). B.) PPIs with known inhibitors were gathered from the 2P2IDB and TIMBAL databases and categorized based on the four quadrants of interactions. The colors on the graph correspond to the matching color quadrant in part A.[17]

Conversely, lower affinity interactions of both small and large surface areas remain poorly understood (see lower quadrants, Figure 1.1A). Due to their weak binding and/or broad undefined binding interface, they have been difficult to capture and characterize with traditional biochemical methods, including affinity purification.[2, 18, 33-36] This lack of basic structural and functional information has made small molecule probe development ineffective. While more than 60% of all reported chemical probes developed to modulate PPIs target strong and concise interactions, less than 10% target the weak affinity interactions seen in the bottom two quadrants (Figure 1.1B).[17, 19-20]

While cellular processes that rely heavily on PPIs within the top two quadrants have been highly characterized, those cellular processes that rely on transient, moderate affinity interactions seen in the lower quadrants, such as transcription, have been an elusive target to this point.[5, 37-39] During transcriptional initiation, there are many moderate to weak affinity, dynamic PPIs ranging in surface areas that must occur to assemble the transcriptional machinery at a specific gene.[40] This complicated network of interactions between transcriptional activators, repressor complexes, coactivators, and the pre-initiation complex required to properly turn on or turn off gene expression has been a long-term target of study. However, a complete map of the interactions required for proper gene expression remains a challenge due to the limitations in current tools.[38, 41]

1.2 Transcriptional PPIs: activators and their elusive binding partners

1.2.1 Transcriptional activators

Transcriptional activators play a key role in orchestrating the initiation of the highly regulated transfer of genetic information from double-stranded DNA to the single stranded mRNA needed for the production of the cellular proteome. These modular proteins minimally consist of a (1) DNA-binding domain (DBD), which localizes the protein to a specific sequence within a gene promoter, and (2) a transcriptional activation domain (TAD), which engages in dynamic PPIs with a variety of coactivator complexes and general transcriptional factors.[40, 42] These interactions lead to the proper assembly of the pre-initiation complex (PIC), including RNA Polymerase II, at the DNA in a signal responsive manner (Figure 1.2). This network of interactions ranges in

binding affinities and surface areas depending on a specific interaction. In the case of the prototypical Gal4 yeast activator, under normal glucose levels its TAD participates in a high-affinity (picomolar K_d) interaction with its masking protein Gal80 which inhibits expression of glucose-repressed genes.[25, 43-44] However, under nutrient stress, Gal4's TAD is liberated allowing several transient, moderate to low affinity interactions with coactivator complexes of widely ranging binding interfaces. These interactions subsequently turn on the expression of glucose-repressed genes, such as GAL1.[43-50]
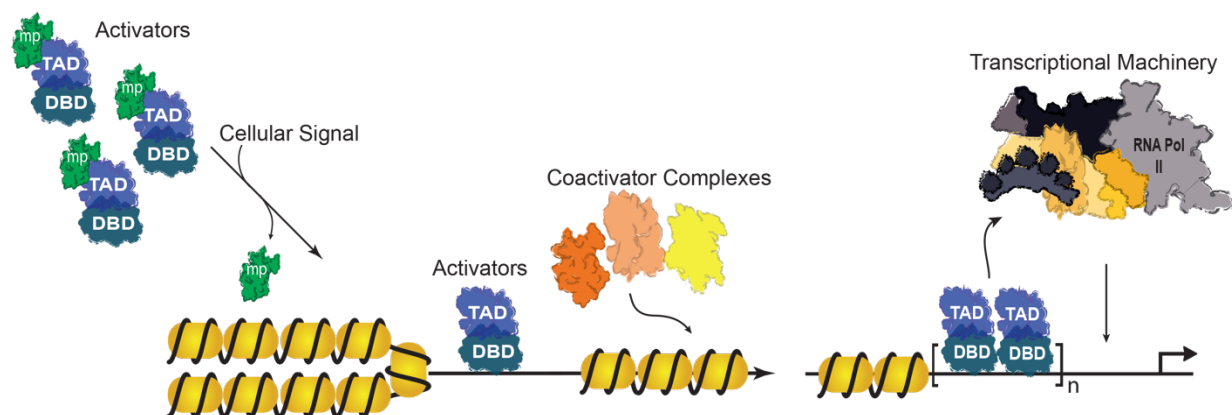
Figure 1.2 Transcriptional initiation scheme. Activators dissociate from their masking protein after a cellular signal and bind to specific DNA sequences through their DNA binding domain (DBD). Upon DNA binding, they recruit several enzymatic coactivator complexes, through a variety of PPIs with their transcriptional activator domain (TAD), to open the DNA for the assembly and binding of the transcriptional machinery, including RNA Polymerase II, for active gene expression.

As noted above, misregulation of activator-associated PPIs has been implicated in several diseases, making activators and their coactivator binding partners desirable targets for therapeutic intervention. However, the transient nature of these interactions makes them difficult to characterize and has limited the development of chemical probes causing them to be termed "undruggable".[5, 38, 51] Additionally, while DBDs have defined structures and biochemical data available[52-53], many activator TADs are intrinsically disordered.[54-59] The most well-studied activators have amphipathic TADs consisting of acidic and polar residues interspersed with hydrophobic residues throughout the amino acid sequence.[60-64] Both the intrinsically disordered nature and hydrophobicity of these activator sequences make them promiscuous *in vitro*.[65] This makes it difficult to determine relevant binding partners *in vivo* based on initial *in vitro*

data. Additionally, while activators such as Gal4 and VP16 often have amphipathic sequences but minimal sequence homology, they still interact with several of the same coactivator complexes at similar binding sites.[66-67] This redundancy and conserved nature of activator-coactivator interactions makes them difficult to selectively target with chemical probes for further characterization.

1.2.2 Transcriptional coactivator complexes: the elusive binding partners of activators

As discussed above, transcriptional activators recruit large, multi-subunit complexes, called coactivators, to prepare the DNA promoter for the assembly of the PIC and RNA Polymerase II for active gene expression (Figure 1.3).[40, 68] During transcriptional initiation, activators bind the specific sequences within the DNA and recruit the chromatin modifying and chromatin remodeling complexes, SAGA and SWI/SNF, respectively, to open and free the DNA from nucleosomes.[46, 69-72] Next, the Mediator coactivator complex bridges activators to the rest of the PIC that consists of the general transcription factors, the TATA-binding protein (TBP), and RNA Polymerase II assembled at genes promoters.[50, 66, 73-81] Each one of these multi-subunit complexes are required for transcriptional initiation and are recruited to the DNA by activators. For example, the amphipathic activators Gal4 and VP16, have been shown to recruit and interact with each of the above-mentioned coactivator complexes in Figure 1.3, demonstrating the redundancy and highly conserved nature of activator-coactivator interactions.[43, 70, 82-83] It is believed that activators have unique subunits within each coactivator complex with which they preferentially interact with. However, the identity of the preferred subunit for individual activators within these complexes remains unclear.
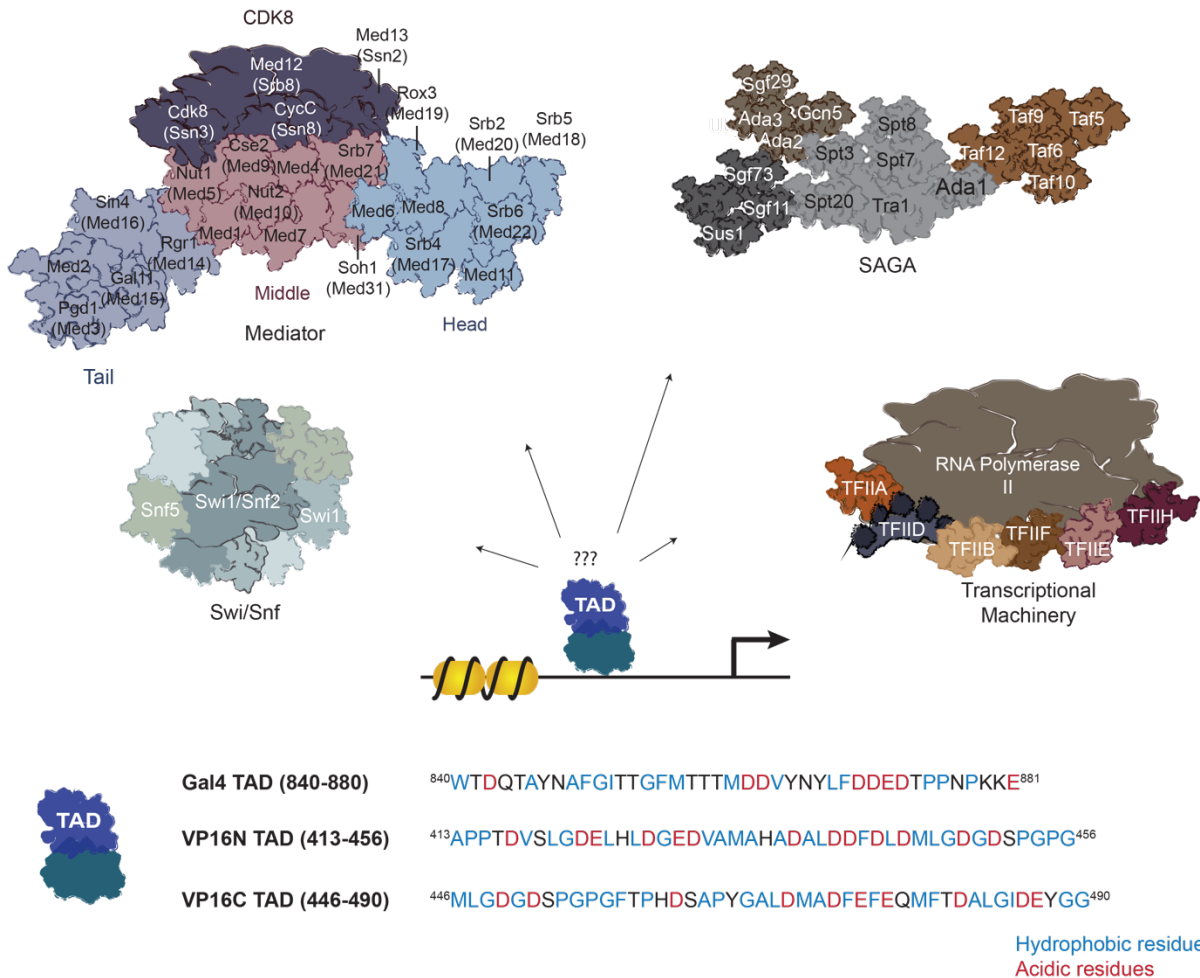
Figure 1.3 Transcriptional activator PPI networks are dynamic and transient in nature. Amphipathic activators, such as Gal4 and VP16, have no sequence homology, but interact with the multisubunit coactivator complexes Swi/Snf, Mediator, SAGA, and the transcriptional machinery to activate gene expression. The exact subunits within these complexes that specifically recognize and bind the diverse amphipathic TAD sequences are still elusive targets of study.

1.3 Challenges faced when characterizing activator-coactivator interactions

During transcriptional initiation, there are more than 65 proteins that must be properly recruited and assembled at the DNA by activators for active gene transcription. This assembly relies on the interactions occur between activators, coactivators, and the transcriptional machinery that vary in stability, binding affinity, and surface area of the interface. The precise timing and order of assembly has been a target of study for several years using different types of detection methods, including genetic and biochemical techniques to decipher the exact binding interactions required for activator specific regulation. However, the mechanistic question of how these coactivator

complexes and activators specifically recognize and bind each other to selectively initiate transcription of activator specific genes persists. A variety of methods developed to interrogate this question are discussed below.[33, 41, 84-85]

1.3.1 Current genetic and ChIP methods applied to transcriptional PPIs

As discussed above, activators recruit several coactivator complexes to the DNA to help prepare the promoter for RNA Polymerase II binding and active transcription. Chromatin immunoprecipitation (ChIP) techniques have helped determine which coactivator complexes are recruited to specific gene promoters by activators, such as Gal4.[45-46, 80, 86-87] This method uses formaldehyde to quickly and non-specifically crosslink DNA-protein and protein-protein interactions in cells (or *in vitro*), immobilizing the assembled PIC to gene promoters at specific times during transcriptional initiation (Figure 1.4).[88] The chromatin is then sheared and the samples are immunoprecipitated for a protein of interest, such as the activator or hypothesized coactivator subunit, to isolate the protein-DNA and protein-protein crosslinked adduct. The protein-DNA adducts are analyzed by reversal of the formaldehyde crosslinked and DNA amplification using promoter specific primers to detect the binding location of the activator and/or coactivator. The protein-protein adducts can be analyzed either by western blotting or mass spectrometry to identify potential binding partners. These results give a detailed picture of the activator-dependent assembly process at specific gene promoters. For example, Ptashne and coworkers used a fast-mixing ChIP method to determine the exact order of Gal4 transcriptional recruitment to the GAL1 promoter under nutrient stress. Upon galactose induction, the SAGA chromatin-modifying complex is recruited to the promoter, followed by the Mediator complex, and finally the general transcription factors, TFIIE, TFIIH, TFIIF, and TBP, together with RNA Polymerase II, which then alone actively transcribes the gene.[82]
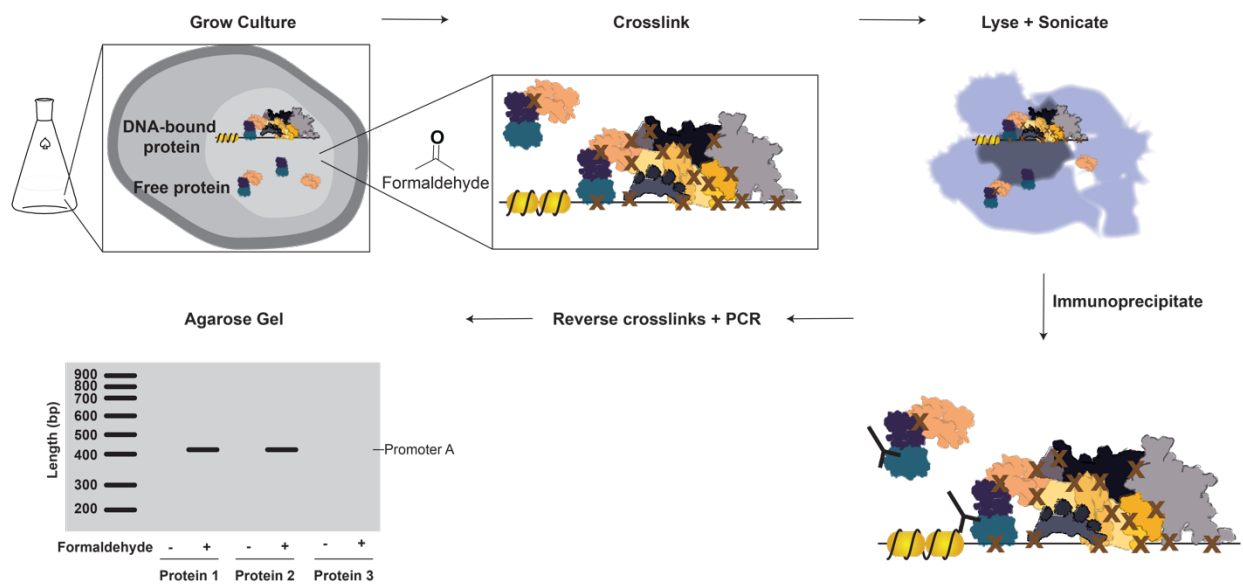
Figure 1.4 Chromatin immunoprecipitation (ChIP) experimental scheme. Cells are grown and formaldehyde crosslinked to freeze all DNA-protein and protein-protein interactions. The cells are lysed and sonicated to shear the chromatin. The lysate is immunoprecipitated with a protein specific antibody to isolate the specific protein-DNA adducts. The formaldehyde crosslinks are reversed and the DNA is amplified using a promoter specific primer and analyzed by agarose gel to determine if the specific protein is bound at that promoter.

Additionally, genetic studies have contributed to understanding the complex assembly through point mutations and deletion of either whole proteins or domains within specific complexes to determine their effect on recruitment, assembly, and function.[89-92] When the Spt20 subunit required for structural stability of the SAGA complex was deleted, Ptashne and coworkers saw complete disruption of transcriptional initiation and loss of RNA Polymerase II binding to the GAL1 promoter, demonstrating the functional importance of this SAGA complex and the Spt20 subunit for functional gene expression. Conversely, when the nonessential Med15 subunit of the Mediator complex was deleted, the SAGA complex and RNA Polymerase II were recruited to the DNA similarly to wild-type cells.[82]

While ChIP has laid the groundwork for identifying the exact coactivator complexes required for transcriptional initiation, the non-specific nature of formaldehyde crosslinking prevents these experiments from differentiating between direct and indirect interactions between these complexes and transcriptional activators. Therefore, the exact subunits within these complexes that act as activator targets *in vivo*, along with the activator binding site within these subunits, remains unclear. Additionally, genetic

studies have been difficult to interpret due to pleiotropic effects seen from knocking out transcriptional genes that are required for several protein interaction networks and regulation of almost all genes.[93] Due to these limitations, these methods have left significant gaps regarding the identities of direct activator binding partners within these complexes.

1.3.2 Current in vitro methods applied to activator-coactivator PPIs

To further characterize and identify activator binding partners *in vitro* biochemical techniques, such as affinity purification, have been used. This technique takes advantage of the selective binding of genetically encoded epitope tags, such as glutathione-S-transferase (GST) or a polyhistidine sequence, that can be appended to a protein of interest.[94-97] The purified epitope-tagged protein (Bait) is bound to an immobilized affinity ligand specific for the epitope tag and incubated with cell lysate to capture any binding partners (Prey). The protein of interest and its binding partners are then eluted from the affinity ligand and analyzed through western blot, probing for a hypothesized binding partner, or by mass spectrometry to identify an unknown interaction (Figure 1.5A). This technique has been extensively used to find coactivator binding partners of the model amphipathic activators Gal4 and VP16.[81, 98-101] Using GST-tethered VP16 and Gal4 TAD peptides, Melcher and colleagues identified TBP as a direct binding partner for both activators.[47, 65] Additionally, using point-mutations with the TADs they found that the strength of the binding interaction was correlated with the activators transcriptional activation; as the binding affinity decreased and becomes tighter, the transcriptional activity increased. Furthermore, several binding studies found several direct binding partners specific for Gal4 and VP16 within each of the transcriptional coactivators discussed above in Figure 1.3.[81, 98-101]

Although these binding studies helped elucidate several activator specific binding partners within these coactivator complexes, this technique is biased towards high affinity interactions with slow kinetics of dissociation.[33] Due to the transient nature and moderate binding affinity seen with many activator-coactivator interactions, these interactions tend to be lost during the wash steps.[95] Additionally, affinity purification studies can be contaminated by high abundance proteins, such as chaperones or

ribosomal proteins, that can drown out low abundance binding partners traditionally seen with transcriptional proteins.[102] Thus, missing some of the weaker interactions and leaving gaps within the activator-coactivator interaction network.
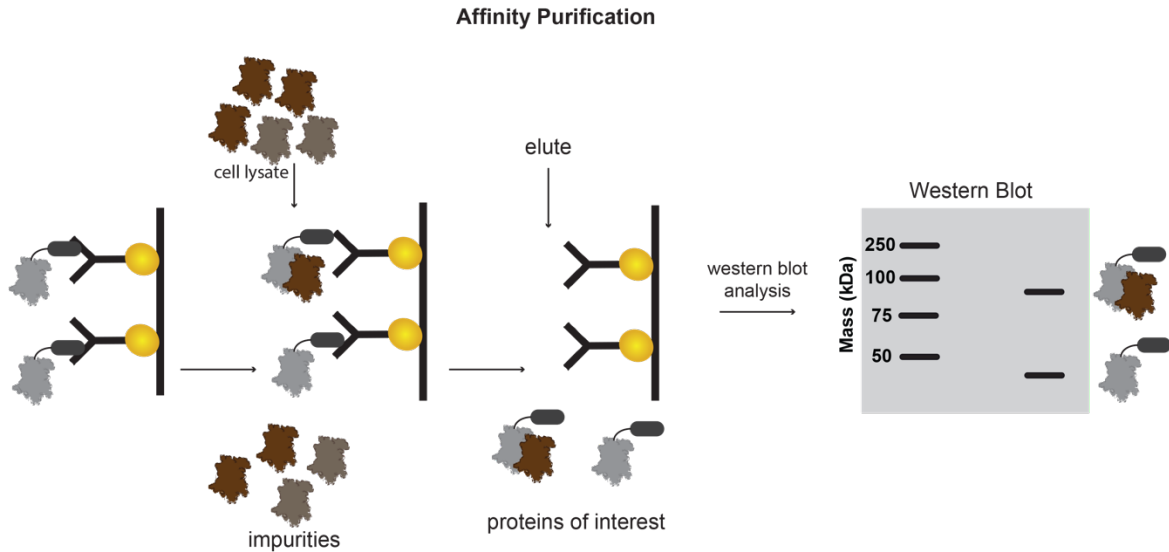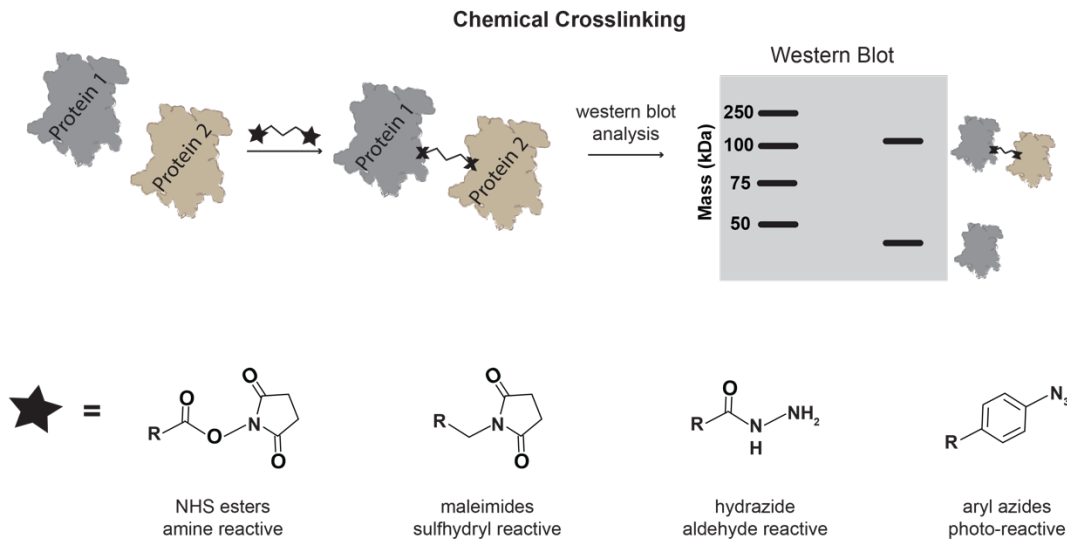
Figure 1.5 Experimental schemes of current *in vitro* methods used to detect and characterize PPIs. A.) Affinity purification of binding partners of the grey immobilized protein of interest. B.) Covalent capture of proteins using chemical crosslinking reagents, such as the N-hydroxysuccinimide, maleimide, hydrazide, and aryl azide reactive groups.

Another approach to capturing activator-coactivator interactions is covalent capture using chemical crosslinking reagents (Figure 1.5B). This technique utilizes chemical crosslinkers containing two reactive groups, such as maleimides or primary amines,

connected by a linker region to capture protein partners.[103-106] These crosslinkers are typically added to cell lysates and chemical attachment between amino acids, such as lysines or cysteines, on two different proteins results in a covalent adduct that stabilizes the PPI so that the interaction can then be easily isolated from the rest of the lysate and analyzed through western blotting or mass spectrometry to identify the binding partners. Homobifunctional chemical reagents, such as di-N-hydroxysuccinimide (NHS) esters, were first used to crosslink proteins through lysines and map large multisubunit complexes, such as the ribosome.[33] Additionally, photo-reactivity crosslinkers have been used to allow more controllable crosslinking. Recently, this strategy has been used to capture several activator-coactivator partners within the SAGA and Mediator complexes.[45, 107] Reeves and Hahn used the ^{125}I-PEAS photo-crosslinker to identify the coactivator subunits, Taf12, Gal11(Med15), and Tra1, as direct binding partners of the Gal4 activator.[45] While this approach enables the covalent capture of moderate affinity PPIs that might be lost in affinity purification studies, the crosslinking reagents used are generally less selective and allow crosslinking independent of the sequence of the binding interface. While these biochemical techniques have helped decipher some of the direct activator-coactivator interactions *in vitro*, many of these interactions are too weak or dynamic to be characterized outside of their native environment. Thus, *in vivo* methods are required to gain a better understanding of the transient activator-coactivator interactions required for regulated transcription.

1.3.3 Current in vivo methods applied to transient PPIs

While *in vitro* biochemical techniques can give initial information about stable, moderate to high affinity interactions, *in vivo* methods allow for transient interactions to be captured and characterized in their native cellular environment. Co-immunoprecipitation is a popular method for identifying physiologically relevant PPIs in the cell and utilize target-specific antibodies to capture proteins that are bound to the specific target protein (Figure 1.6A).[108-112] For a co-immunoprecipitation experiment, whole cell lysate is incubated with an antibody specific for the protein of interest to capture both the protein and its binding partners. The samples are typically immobilized to Protein A or Protein G covalently attached to sepharose or magnetic beads, washed

to get rid of any nonspecific binding partners, and then eluted to obtain the antibody-bound protein of interest and its binding partners. The proteins are then analyzed either by western blot probing with an antibody specific for a hypothesized binding partner or through mass spectrometry to identify novel binding partners. These antibodies can be specific for endogenous proteins or for epitope tags, such as a polyhistidine or Flag tag, that are genetically encoded into a protein of interest. This technique allows for the capture and identification of PPIs in their native cellular environment and enables post-translational modifications that might be essential for the interaction that would not be present in purified proteins used in *in vitro* affinity purification techniques. However, this method tends to have high background from nonspecific binding of the antibody to other proteins within the lysate or from nonspecific binding of proteins to the target protein that were not successfully washed away. These proteins are typically high abundance proteins, such as ribosomal proteins, that can drown out low abundance proteins, such as coactivator complexes. Additionally, to lower nonspecific background binding harsher wash conditions are needed and lead to the loss of transient, weak affinity interactions, such as those between activators and coactivators.[33, 51] Furthermore, this technique relies on protein specific antibodies, that might not be commercially available, or genetically encoded epitope tags that could negatively affect the structure and/or function of the protein being studied.[41, 113]

The yeast-two hybrid (Y2H) system is another common technique used for detection and verification of *in vivo* PPIs, and it has been used to detect more than 50% of all reported interactions in the literature (Figure 1.6B).[85, 114-115] This system relies on the fact that transcription factors have two modular domains, a DBD and TAD, that can be expressed separately from each other.[40] In principle, the DBD can bind to DNA on its own but cannot activate transcription until fused to a TAD. Similarly, the TAD can interact with the rest of the transcriptional machinery required for gene activation, but cannot activate transcription until fused to a DBD. The Y2H system takes advantage of these principles to screen for possible PPIs.
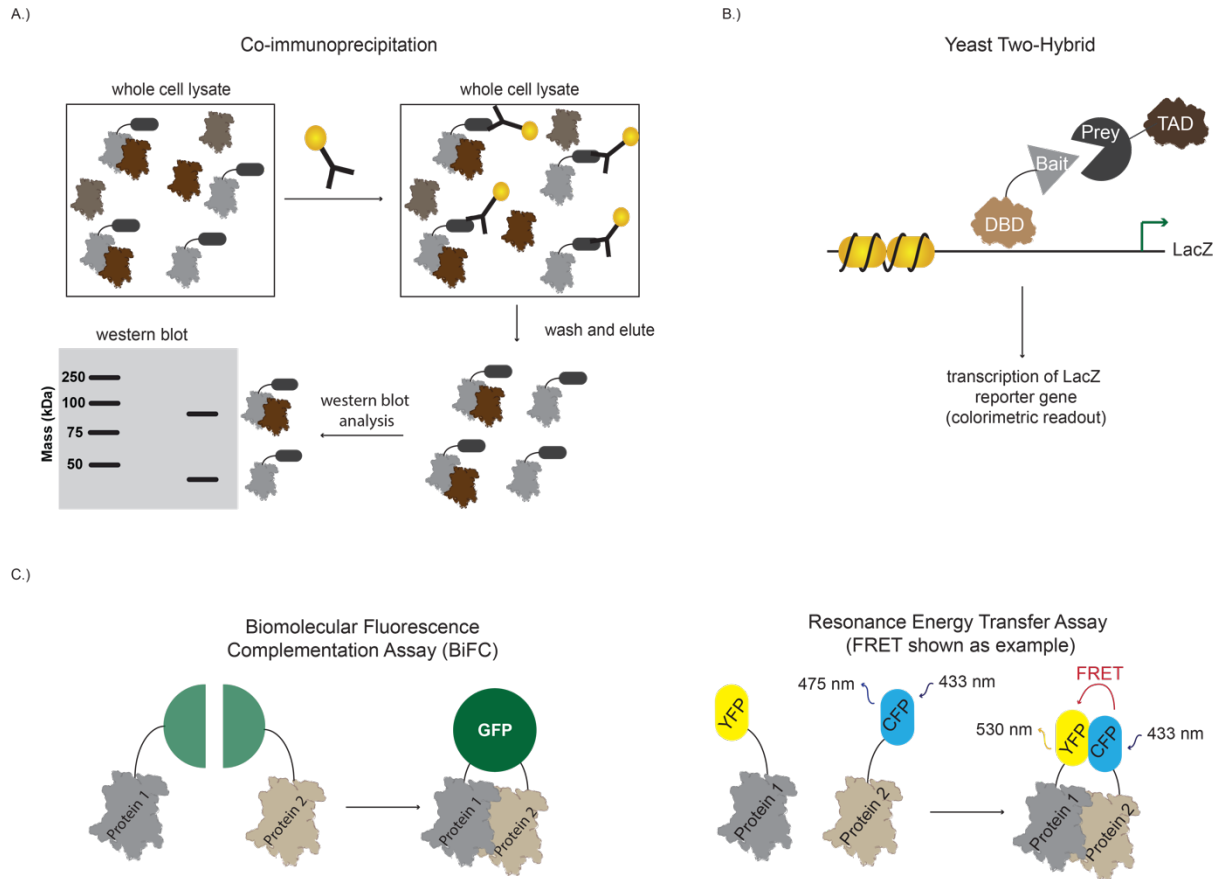
Figure 1.6 Experimental schemes of current *in vivo* methods used to detect and characterize PPIs. A.) Co-immunoprecipitation of binding partners using antibodies and/or genetically incorporated epitope tags. B.) Yeast Two-Hybrid method of screening for binary binding partners. C.) Fluorescent and luminescent visualization of binary *in vivo* interactions.

For these experiments, a plasmid expressing the specific protein of interest (Bait) fused to the DBD of some transcriptional activator, such as Gal4, and a plasmid expressing a possible binding partner (Prey) fused to the activator's TAD are expressed in live yeast. The yeast strain has a genomically encoded reporter gene, such as the β-galactosidase LacZ gene, with DNA binding sites specific for the DBD encoded within the promoter. If the Prey protein is a direct binding partner of the Bait protein, the interaction will bridge the DBD and TAD forming an active transcription factor able to activate transcription of the reporter gene and give a measurable colorimetric readout. Conversely, if the Prey and Bait proteins do not interact, there will be no transcription and no measurable readout. This is an advantageous method for studying transient, moderate-weak affinity interactions *in vivo* since the reporter gene results in signal amplification and the interactions do not need to be isolated from cells. However, only

binary interactions can be studied in this system and there is a significantly high false-positive rate for this method; approximately 50% of identified interactions are not reliable and must be validated through other methods.[116] Additionally, the identification of PPIs required for normal transcriptional regulation has been challenging using the Y2H strategy. Many proteins normally required for transcription, such as coactivator subunits, can recruit the rest of the PIC and RNA Polymerase II when fused to a DBD, thus activating transcription without the need for an activator TAD. For example, Ptashne and coworkers demonstrated approximately identical activated LacZ gene expression to the model activator LexA+Gal4 using a chimeric transcription factor containing the LexA DBD fused to the Gal11 (Med15) Mediator subunit.[117] Additionally, several other chimeric transcription factors using coactivator subunits as the "TAD" showed similar activation of the LacZ gene when fused to the LexA DBD. Thus, illustrating the limitations of using the Y2H system for identifying activator-coactivator interactions *in vivo*.

Other widely used techniques, such as bimolecular fluorescence complementation (BiFC)[118-121], forster resonance energy transfer (FRET)[122-125], and bioluminescence resonance energy transfer (BRET)[126-128] have been used to detect and visualize PPIs *in vivo* (Figure 1.6C). These techniques rely on the association of fluorescent protein fragments or energy transfer giving a measurable readout upon protein binding for BiFC or BRET and FRET, respectively. For example, Bhaumik and coworkers used a FRET screen to determine which subunits within the SAGA chromatin-modifying complex were direct binding partners of the Gal4 activator. Using a cyan fluorescent protein (CFP)-fused Gal4 activator construct and several yellow fluorescent protein (YFP)-fused SAGA subunits, they identified the Tra1 subunit as a direct binding target of Gal4 required for transcriptional activation.[129] While these techniques can identify the transient, weak affinity interactions between activators and coactivators in cells, they required genetic incorporation of large protein complexes to activators and coactivators that could affect their overall function in the cell, potentially changing their binding interactions. Additionally, these techniques require extensive instrumentation that might not be available for high-throughput screens of *in vivo* PPIs.[85]

1.3.4 Difficulties in making functional predictions from PPI studies

While the current PPI detection techniques discussed above have deciphered some cellular interaction networks, many of these interactions are between stable complexes with moderate to high binding affinities (low micromolar to nanomolar binding constants). The more transient, moderate affinity interactions seen between transcriptional activators and coactivators have been harder to characterize using the traditional methods, and this has led to conflicting data within the field regarding the identities of activator specific direct and indirect binding partners. For example, ChIP reports propose that the prototypical VP16 transcriptional activator binds indirectly to TBP through a bridged interaction with the SAGA chromatin-modifying complex, while biochemical *in vitro* binding studies have identified TBP as a direct binding partner of VP16.[65, 98, 130-132] Additionally, while Melcher and coworkers were able to identify a direct binding between the Gal4 and VP16 activators and TBP that positively correlated with transcriptional activity using a GST-pulldown, they also identified lysozyme, a nontranscriptional relevant protein, as a direct binding partner of these TADs with a similar relationship between binding and activity.[65] Due to these controversies and limitations seen in the current methods, there is a dire need for additional tools to capture activator-coactivator interactions in their native cellular environment.

1.4 Covalent chemical capture

Covalent chemical capture utilizing photo-activatable unnatural amino acids (UAA), such as *p*-benzoyl-L-phenylalanine (pBpa), enables the capture of transient, moderate affinity interactions in live cells.[133-136] Using the nonsense suppression method developed by Peter Schultz, pBpa can be site-specifically incorporated into a protein of interest at a residue replaced by the amber stop codon, TAG (Figure 1.7A).[137-141]
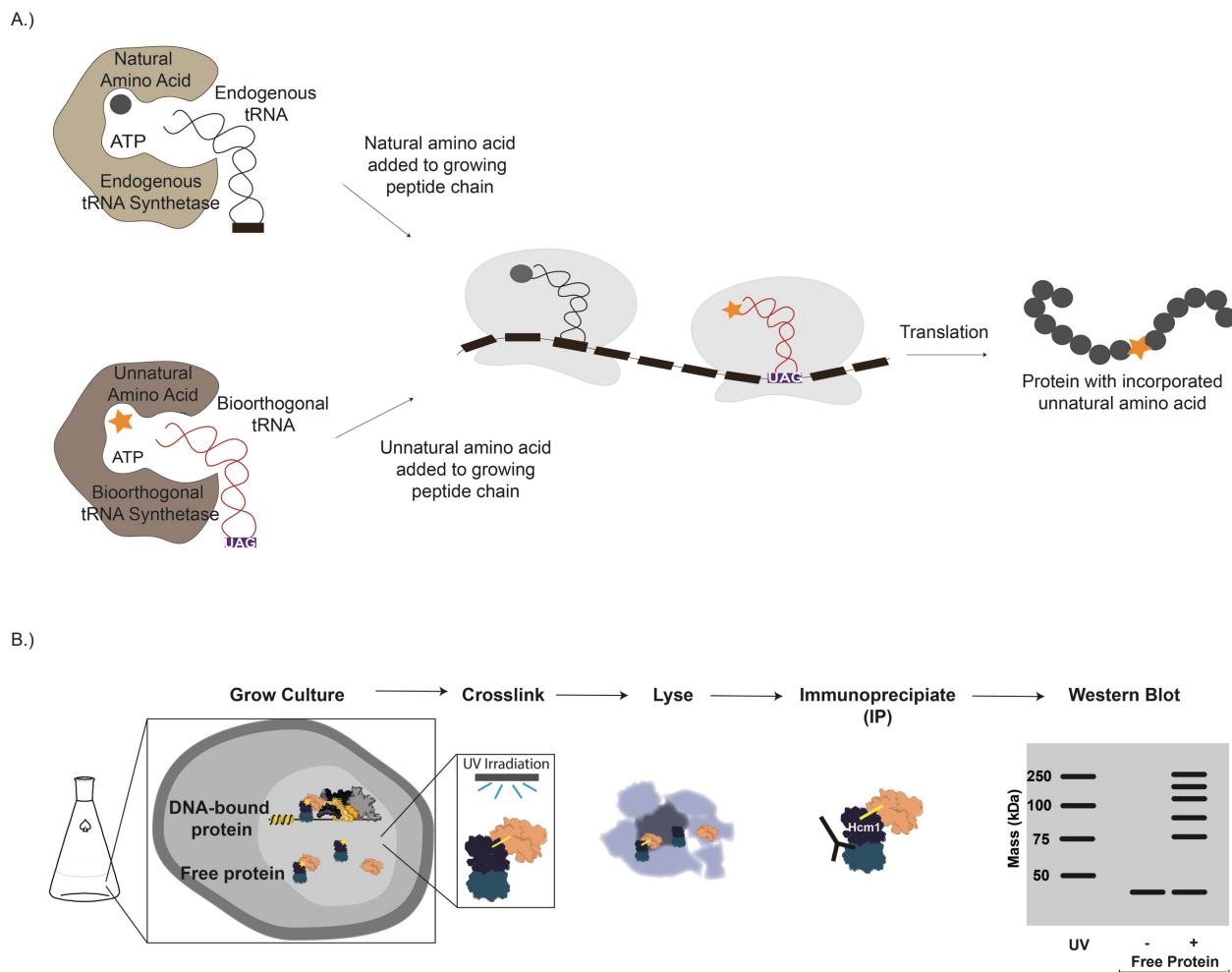
Figure 1.7 A.) Amber nonsense suppression enables the site-specific incorporation of unnatural amino acids (UAAs) into a protein of interest at the mutated amber stop codon (TAG) using a bioorthogonal tRNA/tRNA synthetase pair. B.) *In vivo* covalent chemical capture enables the capture and characterization of the direct binding partners of our UAA-incorporated protein of interest.

Upon irradiation, a covalent adduct is created between the UAA-incorporated protein and it's endogenous binding partner(s), capturing a snapshot of a cellular process at the time of irradiation. These covalently-linked interactions can then be isolated from cells using standard immunological techniques and resolved either through western blot or mass spectrometry analysis to characterize the specific interactions (Figure 1.7B).[141-142]

Currently, there are three photo-crosslinking moieties, benzophenone, aryl-azide, and diazarine, commonly used in the field to capture challenging PPIs. Each of these crosslinkers have very different crosslinking mechanisms that are critical for successful capture of PPIs and must be carefully considered for the system being studied (Figure 1.8).[143] While the fast reactivity of both the aryl-azide and the diazarine moiety is

advantageous for transient interactions, the benzophenone's reversibility and resistance to water enables more selective capture of solvent exposed PPIs that are commonly seen with these transient systems. Due to the large undefined surface area and solvent exposure normally seen between transcriptional activators and their coactivator binding partners, our lab has optimized a covalent chemical capture system using pBpa to create a detailed map of these complex interaction networks.[144-149]

Initially, Dr. Chinmay Majmudar and former Mapp Lab members examined the impact pBpa incorporation had on the function of the Gal4 transcriptional activator and its binding interaction with its masking protein, Gal80.[147] Using ten sites of pBpa incorporation, they found that UAA incorporation had little impact on the proteins function and that the Gal4-Gal80 binding interface extended beyond the residues within the Gal4 TAD initially reported in the literature.[150] Additionally, while bulkier more hydrophobic residues, such as phenylalanine and tryptophan, are ideal for pBpa incorporation, incorporation can also be extended to polar residues, such as aspartic acid and threonine, with minimal impact on protein function. While this initial study demonstrated the utility of *in vivo* covalent chemical capture to examine high affinity interactions between a transcriptional activator and its masking protein (low nanomolar binding affinity), our group has extended this strategy towards transient, moderate affinity interactions between the model activators Gal4 and VP16 and several coactivator complexes, including TBP[149], the SWI/SNF chromatin remodeling complex[144], the Mediator complex[144], and the SNF1 complex[145].

1.5 Dissertation Overview

In the remaining chapters of this thesis, I demonstrate the utility of pBpa to capture the transient, moderate affinity interactions of a novel transcriptional activator in *Saccharomyces cerevisiae* and evaluate the crosslinking capabilities of a suite of pBpa analogs with enhanced functionality to capture PPIs in live yeast. In Chapter 2, the PPI network between the Hcm1 forkhead box transcriptional activator and the SNF1 complex is interrogated under nutrient stress using pBpa photo-crosslinking. During this study, I have identified a minimal TAD sequence for Hcm1 and captured its interaction with two subunits of the SNF1 complex, Snf1 and Gal83, which further demonstrates

the strength of *in vivo* covalent chemical capture to interrogate the PPIs between activators and dynamic, interchangeable coactivators. Additionally, these data have laid the ground work for further interrogation of Hcm1's larger transcriptional PPI network in yeast.
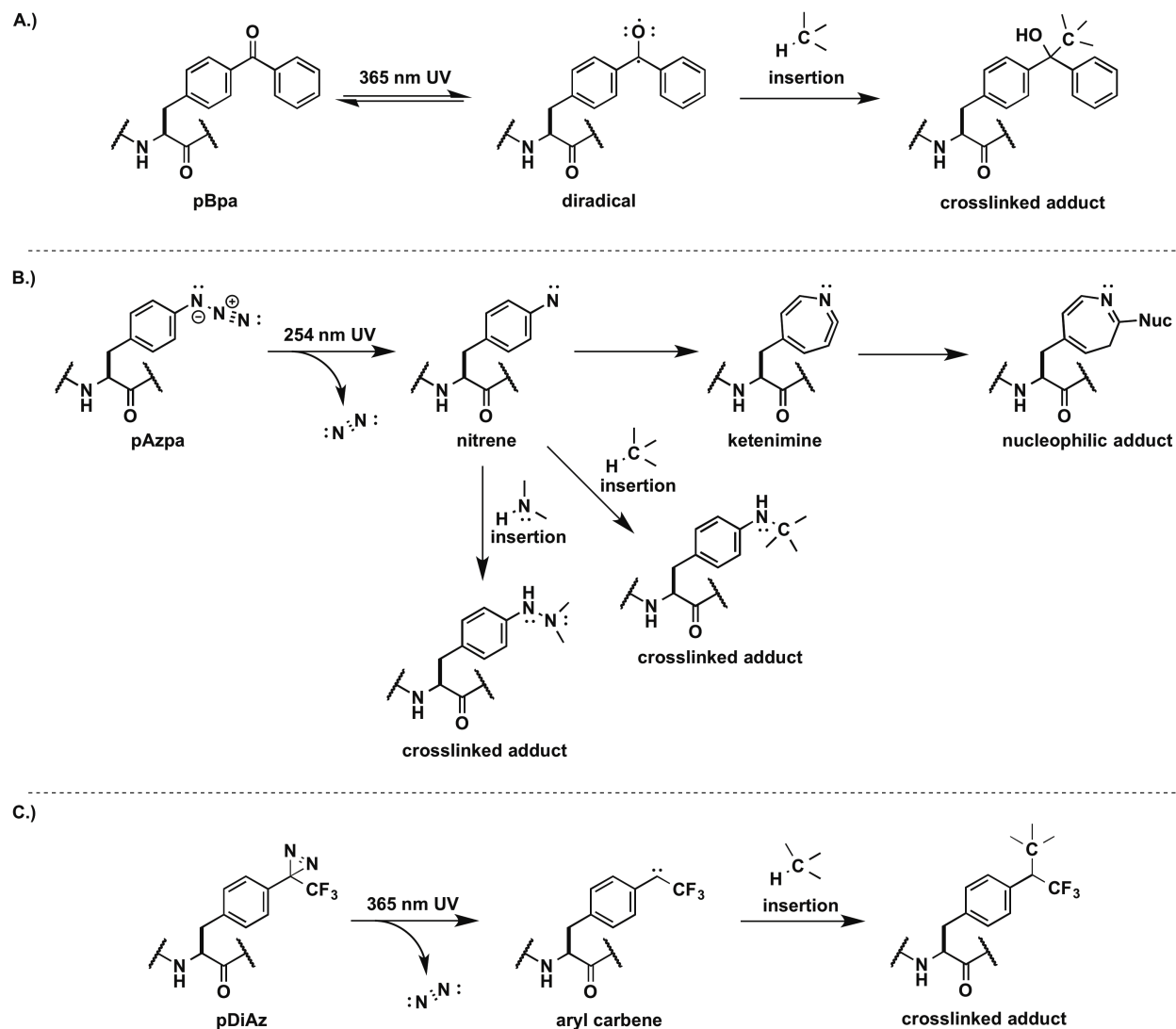
Figure 1.8 Reaction mechanisms for A.) *p*-benzoyl-L-phenylalanine (pBpa), B.) *p*-azido-L-phenylalanine (pAzpa), and C.) *p*-trifluoromethyl-diazo-L-phenylalanine (pDiAz)

While we have shown the strength of pBpa photo-crosslinking for the capture of transient activator-coactivator interactions, there are still limitations with this covalent chemical capture strategy, including the slow reactivity of the molecule and the difficulties seen when isolating crosslinked products. In the second half of my thesis, these limitations are addressed and a suite of pBpa analogs with enhanced

functionalities are described and evaluated for their ability to tackle the current limitations in the crosslinking technology. In Chapter 3, I examine the effects of sequence context and reaction mechanism on the success of covalent chemical capture of *in vivo* PPIs. Additionally, I present a suite of electron-withdrawing group (EWG) containing-pBpa analogs hypothesized to have enhanced reactivity and characterize their overall ability to capture *in vitro* and *in vivo* activator-coactivator interactions. In Chapter 4, I have developed a strategy to isolate *in vivo* crosslinking PPIs using a bifunctional pBpa analog, BPKyne, which contains a bioorthogonal alkyne handle that facilitates isolation of crosslinked adducts upon functionalization with a biotin-azide probe. The pBpa analogs developed in this thesis extend the utility of the benzophenone crosslinker and expands our toolbox of chemical probes for mapping PPIs in their native cellular environment.

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CHAPTER 2[1]

Elucidation of SNF1 recruitment by Hcm1 through *in vivo* covalent chemical capture

2.1 Introduction

Transient, moderate affinity protein-protein interactions (PPIs) play critical roles in all cellular processes, including transcription.[1] During transcriptional initiation, activators bind to specific DNA sequences and recruit several coactivator complexes and the transcriptional machinery, including RNA Polymerase II, to specific gene promoters through several perfectly timed and executed PPIs.[2] These activator-coactivator interactions are tightly choreographed and misregulation has been implemented in several diseases, including cancer, making them a logical target for characterization and eventual therapeutic intervention.[3-9] Possessing a range of binding affinities and surface areas, these interactions have traditionally been difficult to capture and characterize and has led to contradictory and incomplete maps of transcriptional PPI networks.[1, 10-12] For example, reports propose that the prototypical VP16 transcriptional activator binds indirectly to the TATA-binding protein, TBP, through a bridged interaction with the SAGA chromatin-modifying complex, while other studies have identified TBP as a direct binding partner of VP16.[13-17] This lack of consensus demonstrates the limits of the current methods to capture and characterize activator-coactivator interactions. In our hands, *in vivo* covalent chemical capture utilizing photo-activatable unnatural amino acids (UAAs) enables the direct capture and characterization of activator-coactivator interactions in their native cellular environment. For example, using the covalent chemical capture strategy we have captured the VP16-TBP interaction at the GAL1

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[1] The majority of the chapter is not yet published. The individual contributions to the data presented in this chapter is as follows: Cassandra Joiner made and tested the expression and transcriptional activity of all LexA+Hcm1 TAD constructs. Cassandra Joiner made all the LexA+Hcm1 (201-300) TAG constructs and along with Uma Jasty and Samantha DeSalle examined the expression and transcriptional activity of each mutant. Cassandra Joiner ran all crosslinking experiments between Hcm1 and the SNF1 complex, along with Samantha DeSalle.

promoter, indicating a direct interaction between the two proteins during transcriptional initiation.[18] Additionally, our lab has utilized this strategy to further characterize activator-coactivator interactions in *Saccharomyces cerevisiae*, such as the interactions between VP16 and the SWI/SNF chromatin remodeling complex and the interactions between Gal4 and the SNF1 complex.[19-20] Nevertheless, the current activators used in these covalent capture experiments are well characterized with extensive biochemical data that help determine the best sites for UAA incorporation.

In this chapter, the *in vivo* covalent chemical capture method using the photo-activatable UAA *p*-benzoyl-L-phenylalanine (pBpa) is extended to the less characterized yeast forkhead box transcriptional activator, Hcm1. This activator has been reported as a stress regulator during carbon, nitrogen, and oxidation stress.[21-22] Under low glucose conditions, Hcm1 is shuttled into the nucleus where it has been proposed to interact with several coactivator complexes, including the SNF1/AMPK complex, to regulate glucose-repressed genes.[22-24] In an *in vitro* study, Hcm1 was found to be directly phosphorylated by the Snf1 kinase.[22] Thus, we hypothesized that Hcm1 directly interact with the SNF1 complex under glucose limiting conditions and sought to determine the mechanism by which this interaction occurs. In this chapter, we identify a minimal Hcm1 transcriptional activation domain (TAD) that is transcriptionally active and regulated by carbon signaling when fused to the bacterial LexA DNA binding domain (DBD). Using this LexA+Hcm1 chimeric activator we capture its interaction with the SNF1 complex through the Snf1 kinase and Gal83 β-scaffolding subunits. These findings demonstrate the strength of *in vivo* covalent chemical capture to interrogate the PPIs between activators and dynamic coactivator complexes that are regulated by the cellular environment. Additionally, these findings demonstrate Hcm1's ability to function as a modular minimal TAD to regulate nutrient stress response genes.

2.2 Capturing transcriptional activator – coactivator interactions

2.2.1 Transcriptional activator – coactivator interactions

Transcriptional gene activation is an immense multi-step process orchestrated by a vast web of PPIs between transcriptional activators and coactivators required for the proper assembly and recruitment of the pre-initiation complex (PIC), including RNA

Polymerase II, at a specific gene promoter to initiate expression.[2, 9, 25-26] Transcriptional activators are modular proteins that minimally consist of a DNA binding domain (DBD) that binds to specific sequences of DNA within a gene promoter and a transcriptional activation domain (TAD) that participates in various PPIs to recruit the transcriptional machinery to the gene promoter for active transcription (Figure 2.1).
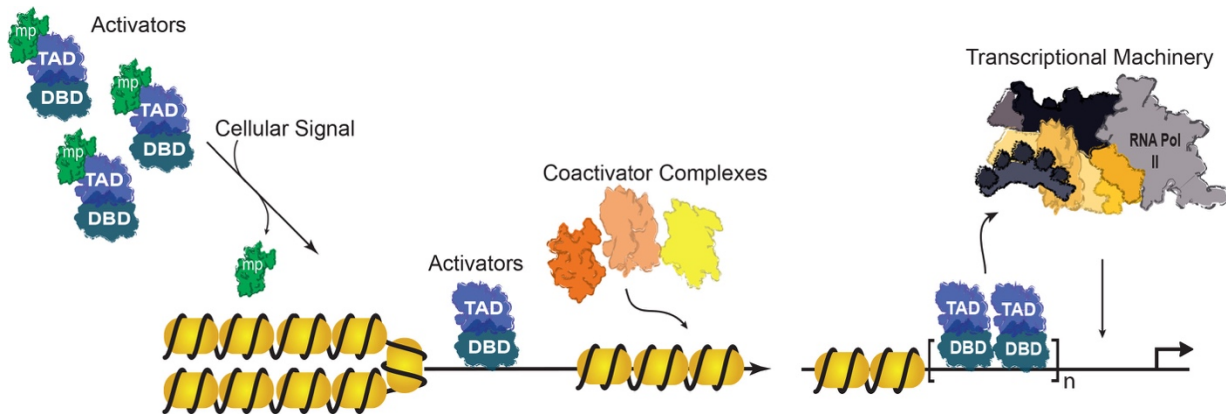
Figure 2.1 Transcriptional initiation schematic. Upon a cellular signal, transcriptional activators dissociate from their masking proteins and bind specific DNA sequences. Through several direct and indirect interactions, they recruit several enzymatic coactivator complexes to prepare the DNA for the assembly of the transcriptional machinery, including RNA Polymerase II, and active transcription.

The interactions between activators and other transcriptional proteins can range drastically in binding affinity and surface area (Figure 2.2). For instance, the interaction between an activator and its masking protein typically has a small surface area and very tight binding (nanomolar dissociate constants) making them the easiest to capture with traditional methods, such as affinity purifcation.[9, 12, 27-28] However, the interactions between an activator and the coactivator complexes are relatively weak affinity and range in binding interfaces from small, defined binding sites to large, poorly defined surfaces. [13, 29-31] Additionally, these interactions are short lived, making them very challenging to characterize using traditional biochemical methods that are more effective for stable binding events.
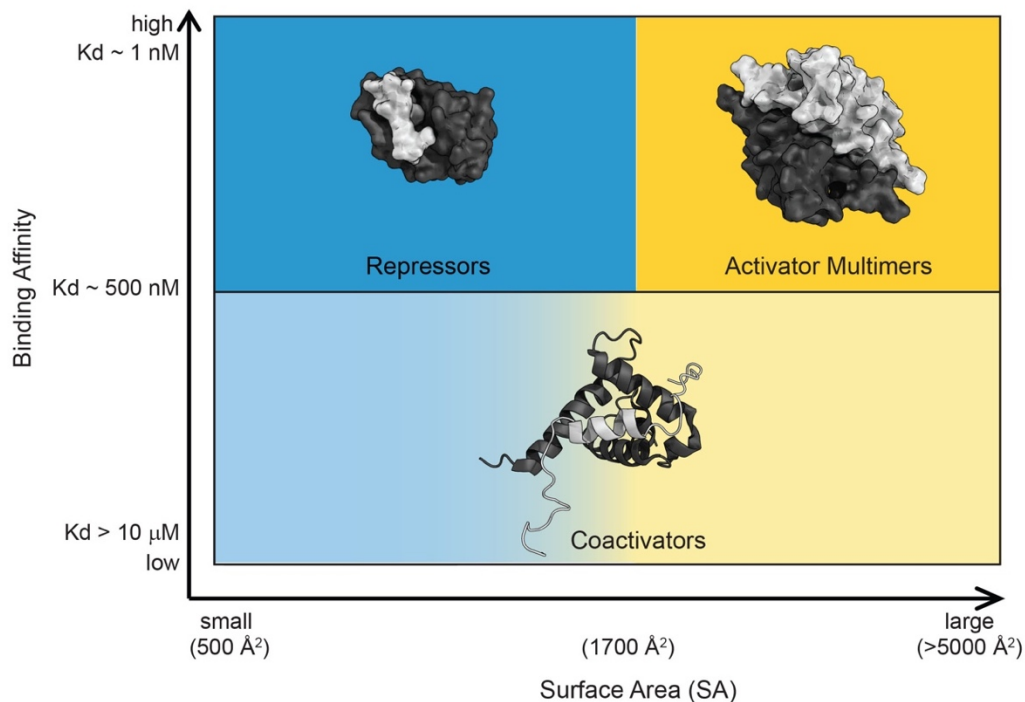
Figure 2.2 Transcriptional PPIs can be categorized based on the binding affinity of the interaction and the surface area of the binding interface. This analysis can lead to roughly four quadrants: strong and concise, strong and broad, weak and concise, and weak and broad. Interactions between activators and their repressor proteins fall under the strong and concise category. Activator dimers or multimers fall under weak and concise interactions and activator-coactivator interactions are weak binding events that range in concise and broad surface areas.[32]

2.2.2 Difficulties capturing activator-coactivator interactions

While *in vivo* transcriptional PPI studies such as chromatin immunoprecipitation (ChIP) have determined the specific coactivator complexes that are recruited by activators to gene promoters during transcriptional initiation, the direct binding partners within those large multi-subunit complexes have not been clearly defined.[33-35] Using ChIP, Ptashne and coworkers have determined the timing by which coactivator complexes, including the SAGA complex, Mediator complex, TBP, and RNA Polymerase II, are recruited to the GAL1 promoter by the prototypical activator Gal4 during transcriptional initiation (Figure 2.3).[33] However, Gal4's direct binding partners within these complexes are still debated within the field.[36-42] Additionally, each of these coactivator complexes are recruited to almost all gene promoters within the cell by multiple activators during transcriptional initiation resulting in redundancies in activator-coactivator interactions.[13, 43-45]
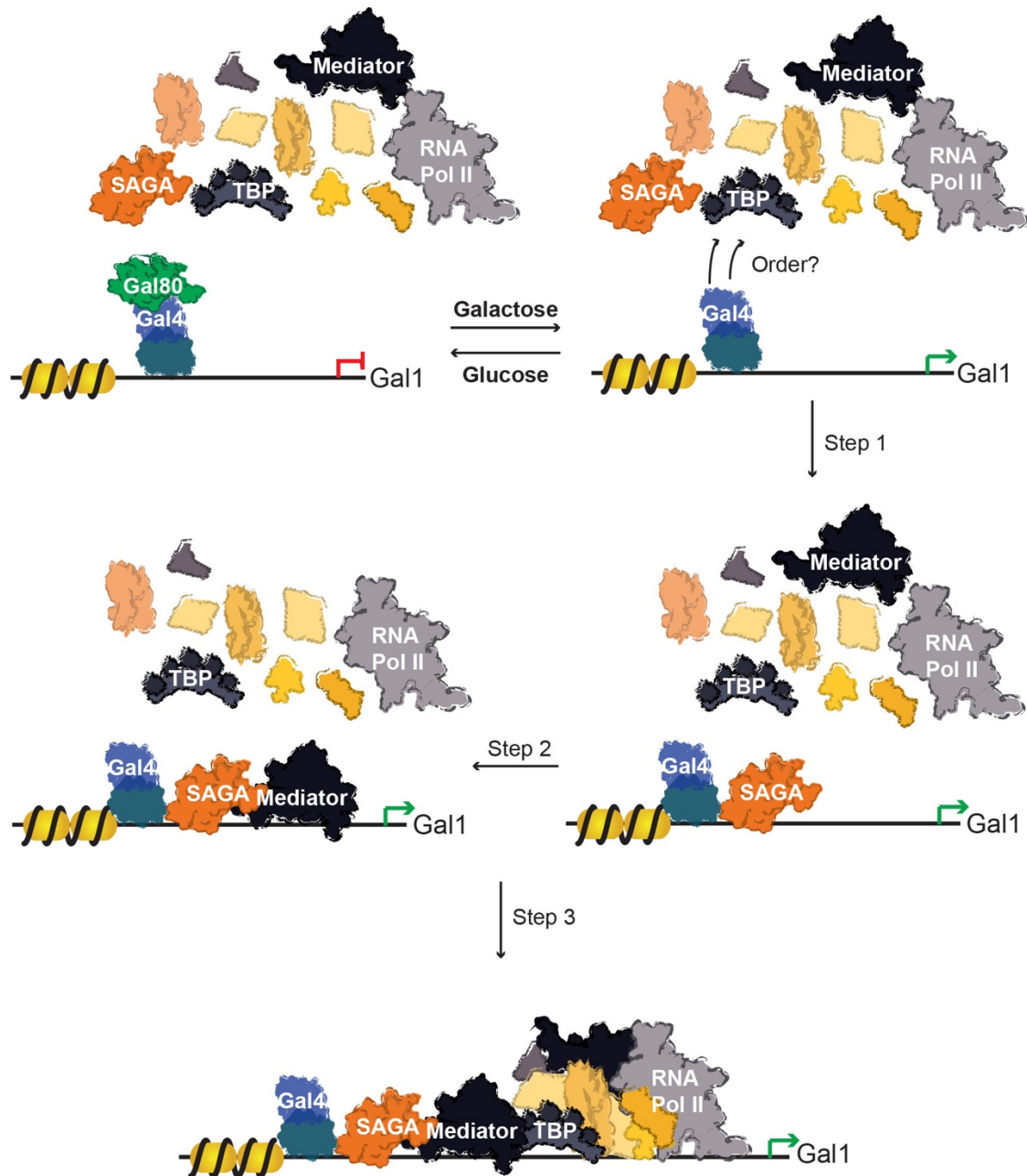
Figure 2.3 Ordered transcriptional recruitment by the model Gal4 activator. The three-step timed recruitment and assembly of the pre-initiation complex (PIC) by the Gal4 activator was determined through fast-mixing ChIP experiments using representative subunits from each complex. Under normal glucose conditions, Gal80 binds to the Gal4 TAD inhibiting interactions with the PIC. However, upon galactose induction, Gal4 recruits the (Step 1) SAGA complex (Spt20) to the promoter, followed by the (Step 2) Mediator complex (Gal11 and Srb4). Finally, TBP, TFIIE(Tfa2), TFIIH (Tfb3), TFIIF (Tfg1), and RNA Polymerase II (Rpb1) arrive at the promoter simultaneously (Step 3). After assembly, RNA Polymerase II moves into the gene along and quickly begins transcription.[33]

Although *in vitro* assays have determined several coactivator binding partners for different activators within these complexes, *in vivo* interaction studies have not yet

confirmed which of those proposed partners are relevant in cells.[15, 29, 46-47] Due to their intrinsically disordered and hydrophobic nature, the TADs of activators have been reported to be promiscuous binders, particularly *in vitro*. In an *in vitro* GST-binding study using several amphipathic TADs, including the prototypical Gal4 and VP16 activators, several direct binding partners within these coactivators and the transcriptional machinery were identified, including TBP.[13] Moreover, there was a correlation seen between *in vitro* binding and *in vivo* transcriptional activity. As the binding affinity decreased between an activator and coactivator, the transcriptional activity increased demonstrating the relevance of each interaction. However, this approach also identified the nontranscriptional and cellularly irrelevant lysozyme protein as a direct binding partner for these TADs. These findings illustrate the limitations of current methods to distinguish and correlate direct interactions to relevant functions within the cell. Therefore, there is a critical need for *in vivo* techniques that can capture the direct activator-coactivator interaction in their cellular environment.

2.2.3 Covalent chemical capture in cells

Covalent chemical capture utilizing photo-activatable UAAs has become a powerful tool for capturing and characterizing PPIs in their native cellular environment.[48-50] With the advancement of amber nonsense suppression by the Schultz group, photo-crosslinking amino acids, such as pBpa, can be site-specifically incorporated into a protein of interest and upon UV irradiation direct binding partners can be captured through radical mechanisms that create a covalent adduct between the two binding partners (Figure 2.4).[48-49, 51-53] The site-specific incorporation of these UAAs is accomplished using a tRNA and tRNA synthetase pair that was engineered to selectively recognize the UAA of interest. This UAA is incorporated at an amber stop codon in the mRNA sequence.
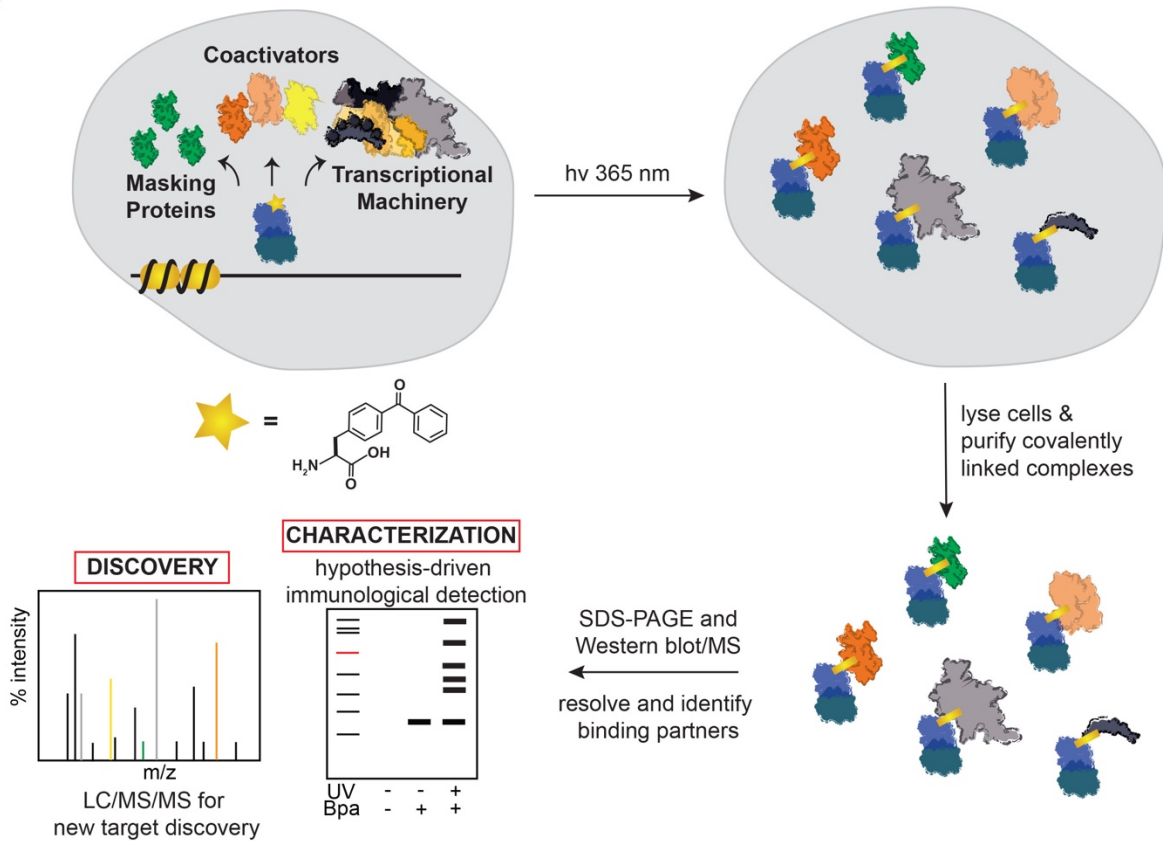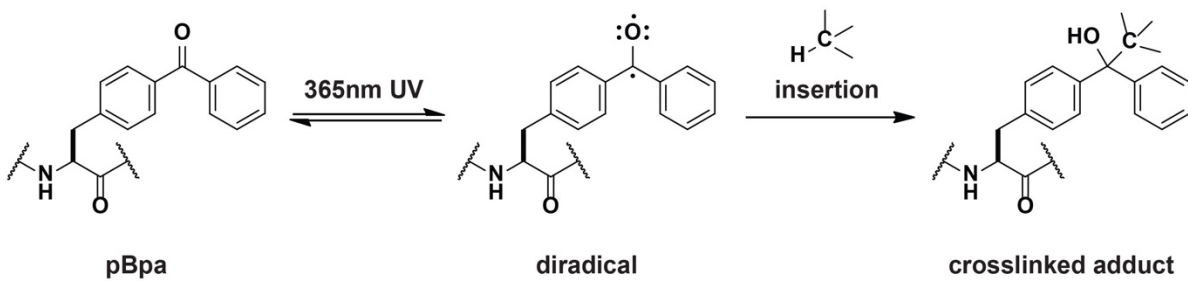
Figure 2.4 *In vivo* covalent chemical capture schematic using pBpa. A.) pBpa is incorporated into the TAD of a transcriptional activator using nonsense suppression. The yeast cells are irradiated with 365 nm UV light to activate pBpa, resulting in the capture of the direct binding partners of the activator. The cells are lysed and the crosslinked products are isolated through a variety of immunological techniques. The crosslinked products can then be resolved either through hypothesis-driven western blot analysis or discovery-driven mass spectrometry analysis to identify the activator's binding partner(s). B.) pBpa reaction mechanism. pBpa is activated by 365nm UV light and upon irradiation a diradical ketyl species is formed. This electrophilic diradical reacts with weak C-H bonds through H-abstraction, followed by a radical recombination with the alkyl radical to form the crosslinked adduct.[54-55]

For example, for the precise incorporation of pBpa into the open reading frame of a protein of interest a pBpa specific tRNA synthetase is used. This synthetase is derived to selectively charge the tyrosyl tRNA variant that contains the anti-codon loop region

that recognizes the amber stop codon (UAG) mutated into the mRNA of the protein. Additionally, the tRNA/tRNA synthetase system is designed to react bioorthogonally in the host organism such that the tRNA synthetase does not recognize any of the endogenous amino acids or charge the host tRNAs with pBpa and vice versa (Figure 2.5).[56]

2.2.4 In vivo photo-crosslinking of activator-coactivator interactions in yeast

After decades of tRNA/tRNA synthetase design and optimization, the incorporation of UAAs through nonsense suppression can be used in several model organisms, including *S. cerevisiae*.[48, 56-60] The first reported incorporation of pBpa in yeast was originally demonstrated by the Schultz group utilizing the *Escherichia coli* tyrosyl tRNA/tRNA synthetase system (tRNA$^{Tyr}_{CUA}$-TyrRS).[53] Using this system, they reported high expression yields for incorporation of pBpa into the highly abundant superoxidase dismutase, a protein that can be highly overexpressed in yeast; however, when this system was applied to the Gal4 transcriptional activator by former members of the Mapp Lab, little to no incorporation or expression of this modestly expressed protein was seen.[25, 61] A collaboration with Lei Wang at the Salk Institute produced a more effective expression system for the tRNA, ultimately leading to levels of pBpa-containing Gal4 expressed at 5-20% levels of the wild-type protein.[58]

Over the past ten years, our group has further optimized this strategy for the capture and characterization of activator-coactivator interactions in live yeast using pBpa.[18-20, 25, 61] pBpa is advantageous for the interrogation of *in vivo* PPIs as it is activated at the longer 365 nm wavelength that in not harmful or damaging to DNA and proteins compared to the shorter wavelengths needed for other crosslinkers.[11] Photo-activation of pBpa leads to the covalent capture of direct binding partners of the UAA-incorporated activator which then can be isolated from live yeast upon lysis and analyzed by traditional immunological techniques, such as western blotting, or by mass spectrometry methods (Figure 2.4).

While the current application of this *in vivo* covalent chemical capture strategy toward understanding activator-coactivator interactions has been used with prototypical activators with extensive biochemical data, in this chapter we sought to extend this

method to examine the interactions between the less characterized Hcm1 yeast nutrient response activator and the SNF1 complex under nutrient stress.
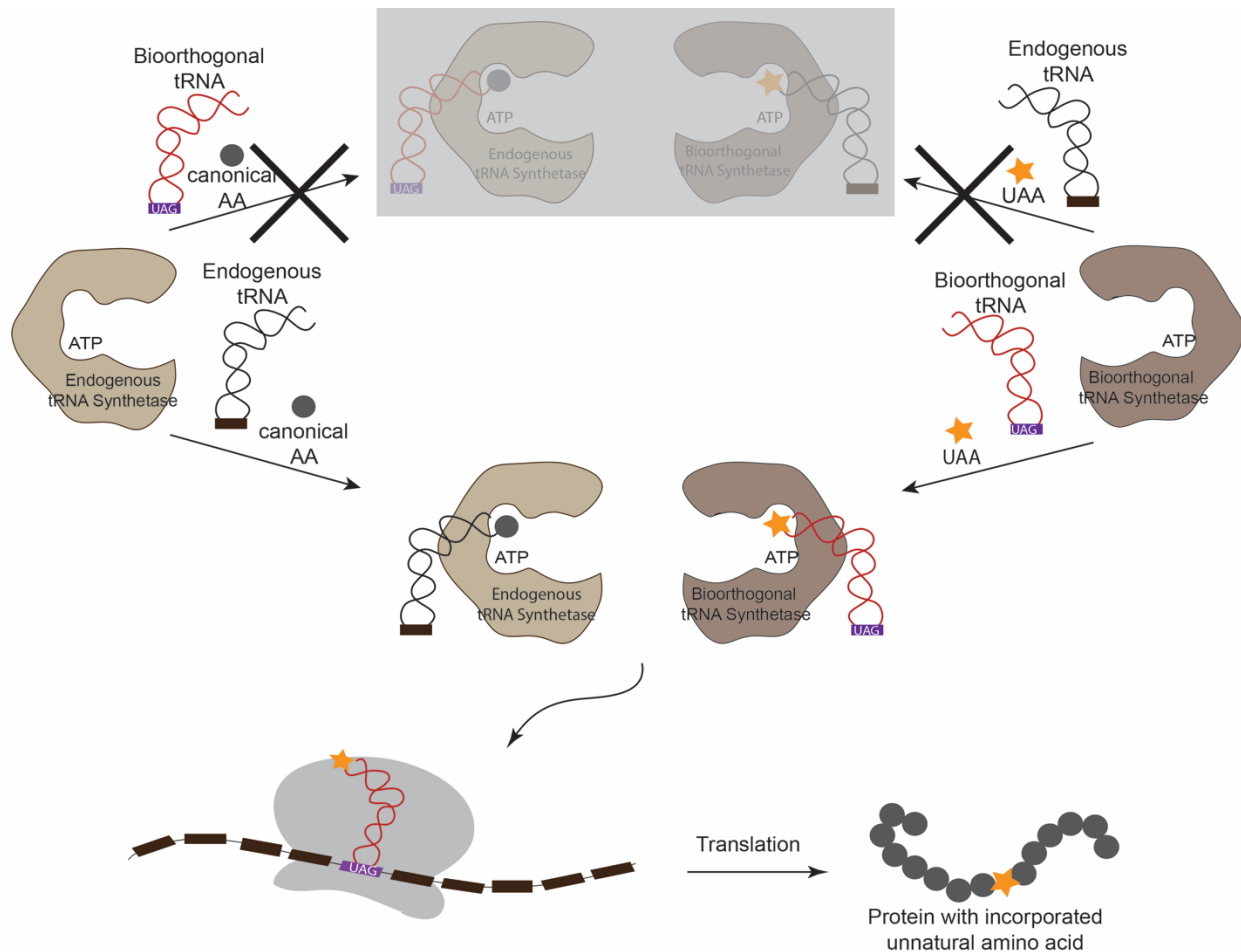
Figure 2.5 Amber nonsense suppression. Nonsense suppression allows the incorporation of an unnatural amino acids (UAAs) into protein or peptide chains. Successful incorporation requires a bioorthogonal tRNA/tRNA synthetase pair (red and dark brown, respectively) that recognizes the purple amber stop codon (UAG) that is site-specifically mutated into the mRNA sequence during translation.

2.3 The yeast SNF1 complex regulates cellular metabolism through activator interactions

2.3.1 The yeast SNF1 complex

The yeast SNF1 protein complex is the founding member of the SNF1/AMPK family of enzymes and is required for the yeast adaptation to alternative carbon sources when glucose is limited. Analogous to the mammalian AMPK complex, SNF1 is a heterotrimeric protein consisting of an enzymatic subunit, Snf1, that phosphorylates

several proteins under nutrient stress, a regulatory subunit, Snf4, that binds and regulates Snf1 activity, and interchangeable β-scaffolding subunits that determines the cellular location and function of the complex (Sip1, Sip2, or Gal83).[62-63] This enzymatic complex is highly regulated by cellular nutrient levels. During normal to high glucose levels, the Snf1 enzymatic subunit is negatively regulated by the Reg1-Glc7 protein phosphatase 1, along with being auto-inhibited by the Snf4 subunit.[64-66]
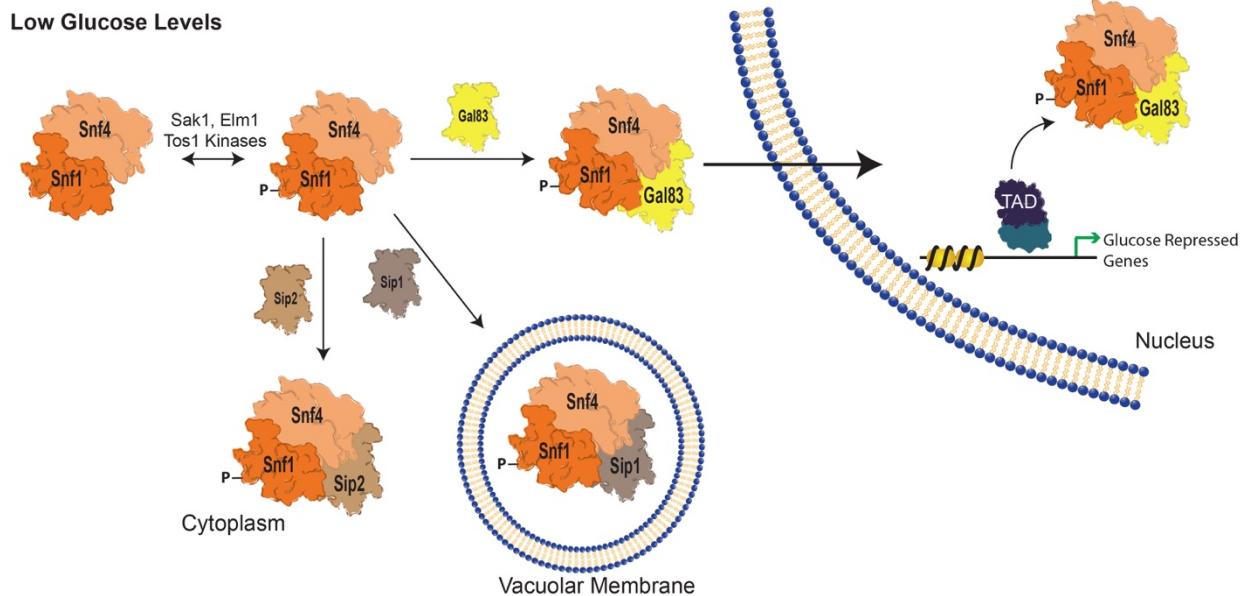
Figure 2.6 SNF1 regulation under nutrient stress. The Snf1 catalytic subunit is activated through phosphorylation by either Sak1, Elm1, or Tos1 kinases under glucose limitation and forms three different complexes with its β-scaffolding subunits, Gal83, Sip2, and Sip1. These scaffolding proteins control the cellular localization and the function of the complex. Under low glucose levels, the Snf1 subunit forms a complex with the Gal83 β-scaffolding subunit, which controls its translocation into the nucleus to activate the gene expression of glucose-repressed genes.[63]

However, under glucose limiting conditions, the Snf1 enzyme is activated through phosphorylation of residue T210 on the activation loop by one of the upstream kinases Sak1, Tos3, or Elm1.[67-69] Upon activation, one of the β-scaffolding subunits bind to the SNF1 complex and directs the complex to its specific subcellular location (Figure 2.6).[62] For instance, Sip1 relocalizes to the vacuolar membrane, while Gal83 localized to the nucleus and Sip2 remains cytoplasmic.[62, 70-71] While all three of these scaffolding subunits are equally active and have overlapping actions, Gal83 has been reported as the major contributor to SNF1's response to glucose limitations and has been shown to mediate the interactions between SNF1 and transcriptional activators and the

transcriptional machinery.[72-76] For example, Gal83 directly interacts with the Sip4 transcriptional activator and facilitates the Snf1-dependent phosphorylation of Sip4 under glucose limiting conditions, while Sip1 and Sip2 show no evidence of interaction with Sip4.[77] Thus, demonstrating Gal83's importance in SNF1's transcriptional regulation and stress response activity during glucose limitation.

2.3.2 The SNF1 complex regulates GAL1 expression under galactose stress

As discussed above, the SNF1 complex has been implicated in the regulation of glucose-repressed stress response genes in the cell. During normal glucose levels, the SNF1 complex is inactive and sequestered in the cytoplasm. Subsequently, the Mig1 transcriptional repressor protein localizes to the nucleus, where it can recruit the Cyc8-Tup1 inhibitor complex to the DNA and inhibit the GAL1 glucose-repressed gene. Conversely, when glucose is limiting and galactose is present, the Snf1 kinase is activated and the SNF1 complex can translocate into the nucleus using the Gal83 nuclear localization signal and phosphorylate the Mig1 protein. Thus, causing Mig1 to be shuttled into the cytoplasm releasing the DNA for RNA Polymerase II recruitment and transcriptional activation of GAL1 (Figure 2.7).[78-82] Additionally, recent reports have shown that the SNF1 complex interacts with several members of the transcriptional PIC, including the Mediator complex, during glucose limiting conditions. [74-76]
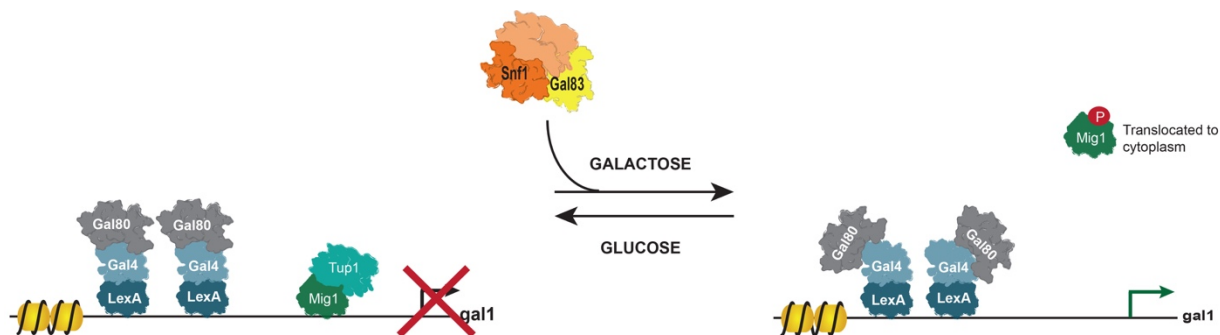
Figure 2.7 SNF1 regulation of the GAL1 promoter under glucose-limiting conditions. Under normal glucose levels Mig binding to the Gal1 promoter and recruits the Tup1 inhibitor complex. However, under galactose induction, the SNF1 complex phosphorylates the Mig1 protein releasing it from the DNA, inducing nuclear translocation and activation of the GAL1 gene.

Furthermore, the Snf1 kinase and Gal83 scaffolding subunit of the SNF1 complex were captured in a recent proteomics study from our lab examining novel enzymatic

targets of the LexA+Gal4 transcriptional activator.[20] In this study, *in vivo* covalent chemical capture using pBpa was combined with shot-gun mass spectrometry proteomics analysis to determine the native binding partners of the LexA+Gal4 activator in an unbiased fashion. Further verification of these experimental hits determined that the Snf1 and Gal83 subunits of the SNF1 complex were direct binding partners of Gal4. Additionally, using tandem reversible formaldehyde crosslinking and irreversible photo-crosslinking (TRIC), they discovered that the interaction between Snf1 and Gal4 occurred at the GAL1 promoter under glucose-limiting conditions, demonstrating the role the SNF1 complex plays in galactose regulation. Concurrently, they also examined the interaction between the LexA+VP16 model activator and the SNF1 complex at the GAL1 promoter. Using identical experimental conditions, they verified that the Snf1 kinase directly interacted with both subdomains of the VP16 TAD, while Gal83 only interacts with the C-terminal subdomain of VP16. Similarly, the Snf1-VP16 interaction was located at the GAL1 promoter when TRIC experiments were used. These findings demonstrate that Snf1 and Gal83 are shared targets of amphipathic activators in yeast.

2.4 Functional interrogation of the Hcm1 activator for UAA incorporation
2.4.1 The Hcm1 forkhead box transcriptional activator

Hcm1, a forkhead box transcriptional activator, has been reported as a yeast homolog for both the mammalian FoxM1 and FoxO3 transcription factors. Similarly to FoxM1, Hcm1 was first reported as a cell cycle regulator of late S-phase genes involved in chromosome segregation, spindle dynamics, and budding.[83-84] This activator is periodically expressed during late G1 and early S phase and is highly regulated by the cyclin-dependent kinase, Cdk1, which maintains genome stability during cell cycle progression.[84] Through post-translational modifications to either the N-terminus or C-terminus of Hcm1, Cdk1 can regulate its degradation or transcriptional activation, respectively.[85-87] This allows for both the precise activation and expression of Hcm1 during a finite window of time which facilitates the productive progression through the cell cycle.

Recently, Hcm1 has recently been shown to regulate environmental stress, such as carbon, nitrogen, and oxidative stress, and longevity in yeast analogous to the

mammalian forkhead box FoxO stress regulators.[21-22, 87] Both forkhead transcriptional activators exhibit subcellular translocation upon cellular stimuli to activate stress response genes.[88-90] For example, under oxidative stress Hcm1, like FoxO, is shuttled into the nucleus following direct interaction with and deacetylation by the histone deacetylase Sir2 (the ortholog of mammalian Sirt1) to turn on genes required for oxidative stress resistance and mitochondrial metabolism.[21, 91-92]

More importantly, under carbon stress, Hcm1 and FoxO3a are shuttled into the nucleus, following post-translational modifications, to turn on glucose-repressed genes.[22, 88, 93-95] During this process, it has been shown that both Hcm1 and FoxO3a are phosphorylated by the SNF1/AMPK complex, making them a substrate for the metabolic regulator.[22, 96-97] Rodriguez-Coleman and coworkers have shown that under glucose-limiting conditions Hcm1 is shuttled into the nucleus, however, in a Snf1 knockout strain, Hcm1 accumulated in the cytoplasm. Additionally, in an *in vitro* study, Hcm1 was directly phosphorylated by the Snf1 catalytic subunit of the SNF1 complex, but phosphorylation was lost when a Snf1 kinase dead mutant was used under identical conditions,[22] thus demonstrating the conserved regulation of Hcm1/FoxO3 under carbon stress by the energy sensing SNF1/AMPK complex.

While Hcm1 has been reported as a substrate *in vitro*, the existence of one or more SNF1/AMPK-Hcm1 PPIs at gene promoters has not been assessed. Therefore, we sought to examine the interaction between Hcm1 and the SNF1 complex in living cells. The SNF1 complex interacts with both prototypical Gal4 and VP16 activators through its Snf1 and Gal83 subunits in live yeast under glucose limiting conditions. Unlike Hcm1, neither of these model activators have been reported as substrates of the Snf1 subunit. We also sought to determine whether the Hcm1-SNF1 interaction mechanism is different from the non-substrate activator proteins, Gal4 and VP16, using *in vivo* covalent chemical capture.
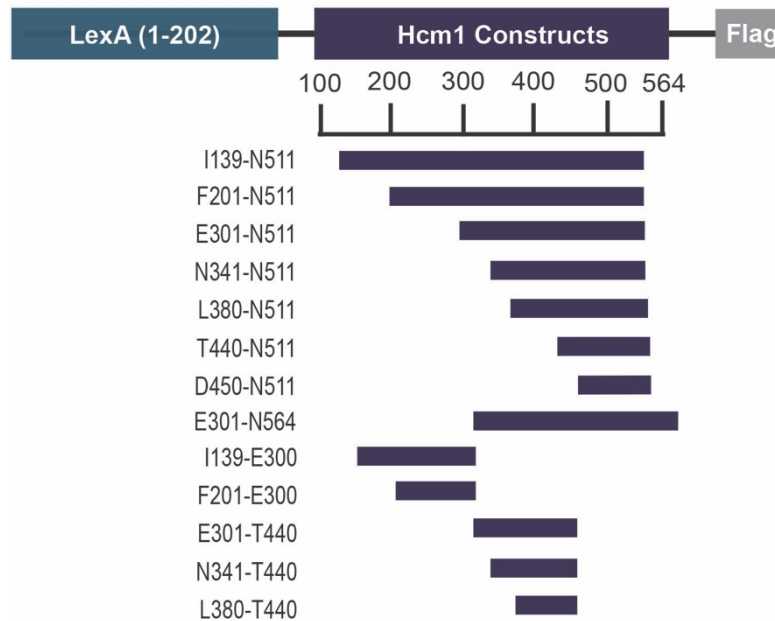
Figure 2.8 LexA+Hcm1 TAD constructs used to determine a minimal TAD sequence. Yeast expression plasmids containing several Hcm1 TAD sequences between residues 139-564 fused to the bacterial LexA DBD and a flag tag for detection were constructed to screen for a minimal TAD sequence that would be regulated by glucose signaling.

2.4.2 Identification of a Hcm1 minimal TAD regulated by nutrient signaling

To extend the covalent chemical capture strategy to Hcm1, we first wanted to define a minimal TAD sequence that would recapitulate both transcriptional activity and glucose regulation. Hcm1 is a 564 amino acid protein with a defined forkhead box DBD domain that resides in the N-terminus of the protein (residues 100-200) which takes on a winged helix-turn-helix fold, but, the C-terminus of the protein is less defined with no clear TAD sequence based on homology models.[98] Zhu and coworkers showed that Hcm1 (139-511) can act as a strong transcriptional activator when fused to the Gal4 DBD.[98] The SNF1 complex is a key member of the nutrient sensing pathway and we hypothesized regions of Hcm1 regulated by changes in nutrient conditions would interact with the SNF1 complex under nutrient stress. So, we sought to identify regions of Hcm1 that were regulated by changes in nutrient conditions within the C-terminal 139-511 region.

Through standard mutagenesis techniques, thirteen Hcm1 TAD constructs fused to the bacterial LexA DBD were made based on known phosphorylation sites (Figure 2.8). Each LexA+Hcm1 TAD construct was expressed in live yeast and their expression and

transcriptional activity under varied nutrient conditions were examined using a genomically incorporated GAL1-LacZ reporter containing two LexA DNA binding sites (Figure 2.9A and B).

A.)

LexA+Hcm1 TADs-1X Flag

LexA+Hcm1

Visualization: α-Flag-HRP

B.)

**LexA+Hcm1-1x Flag TAD Constructs
Transcriptional Activity**
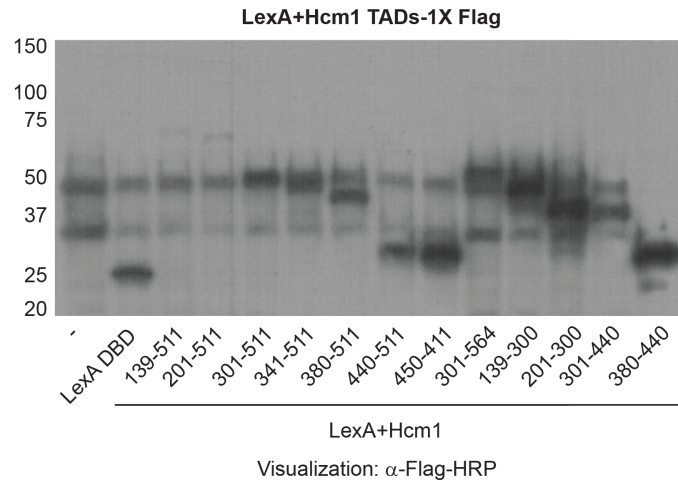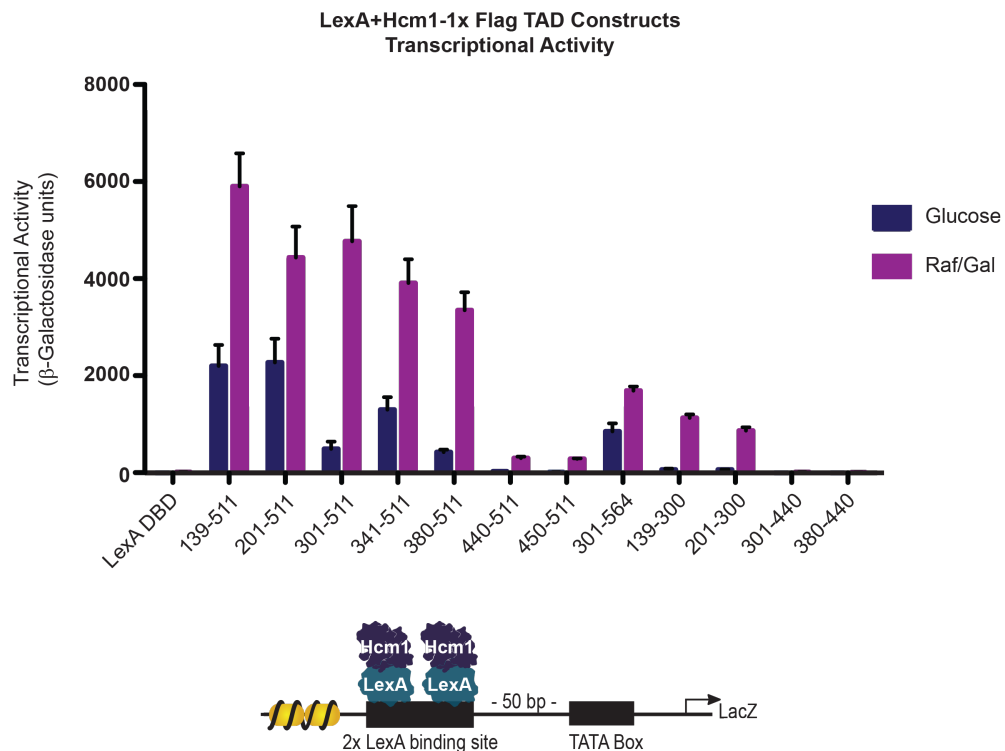
Figure 2.9 *In vivo* characterization of LexA+Hcm1 TAD constructs. A.) Expression of LexA+Hcm1 TADs in live yeast analyzed by western blot with α-Flag. B.) The activation potential of each LexA+Hcm1 TAD construct was measured by liquid β-Galactosidase assay. In the yeast strain, β-galactosidase expression is controlled by a GAL1-LacZ promoter containing two LexA binding sites from LexA+Hcm1 binding.

When expressed in yeast, each activator construct's transcriptional activity varied. As the constructs were shortened, the activity decreased slightly until residues 380-440 were deleted and activity was ablated. While an obvious requirement for a transcriptional activator is that they activate transcription, the nutrient regulation of these Hcm1 regions simulate more of its endogenous function. When analyzing the Hcm1 constructs' transcriptional function there was little difference in absolute transcriptional activity between LexA+Hcm1 (201-511) and LexA+Hcm1 (301-511), however, there was a noticeable difference seen in nutrient regulation between the two constructs, with LexA+Hcm1 (301-511) being inhibited by normal glucose levels more than the (201-511) construct. Thus, we hypothesized that a LexA+Hcm1 (201-300) construct would transcriptionally undergo nutrient signaling similar to the reported wild-type protein. When LexA+Hcm1 (201-300) was expressed in yeast, the chimeric protein was activated under galactose stress conditions, but was inhibited under normal glucose conditions.

While this construct exhibits nutrient regulated transcription like the wild-type protein, there is little biochemical data on the Hcm1 activator, and we sought to determine how its primary and secondary sequence compared to the model amphipathic activators, Gal4 and VP16. Normally, amphipathic activators are intrinsically disordered and take on a random coil structure until binding to a coactivator partner where they form an alpha helical structure.[9, 29, 99-103] After further examination of the Hcm1 (201-300) sequence, there was strong amphipathic characteristics with two coactivator binding motifs ($\phi XX\phi$, where ϕ are hydrophobic residues, usually leucine, and X are nonspecific) at residues 219-221 and 286-289.[104] Furthermore, secondary structure predictions showed three sites of alpha helicity within the sequence around residues 205-211, 218-227 and 283-291 (Figure 2.10). From these characteristics, we decided to use the LexA+Hcm1 (201-300) chimeric transcription factor to examine the Hcm1-SNF1 interaction using *in vivo* covalent chemical capture.

Figure 2.10 LexA+Hcm1 (201-300) sequence analysis. A.) LexA+Hcm1 (201-300) amino acid sequence. Hcm1 (201-300) is amphipathic in nature with the majority of the sequence consisting of hydrophobic and acidic residues (blue and red, respectively) and intrinsically disordered with three predicted alpha helical regions (residues designed with black underline). Additionally, the two coactivator binding motifs are designated by ϕXXϕ, where ϕ are hydrophobic residues, usually leucine, and X are nonspecific B.) Secondary structure prediction of Hcm1 (201-300) by PSIPRED server.[105-106]

2.4.3 pBpa incorporation into the Hcm1 (201-300) TAD

Before the Hcm1-SNF1 interaction could be characterized, the impact of pBpa incorporation on the protein's expression and activity were examined. Using the secondary structure prediction of Hcm1 (201-300) (Figure 2.10), incorporation sites were chosen at residues around the three predicted regions of alpha helicity on the N- and C-terminus of the sequence that have been shown to allow UAA incorporation with minimal impact on protein function.[25] Using site-directed mutagenesis to incorporate the amber stop codon, TAG, in place of the original residue, eight LexA+Hcm1 TAG constructs were made and the expression and transcriptional activity was examined (Figure 2.11). For proper expression, when pBpa is present the full-length protein should be expressed at similar levels to the wild-type protein, but, when pBpa is left out of the culture media, the host translation machinery should recognize the amber stop codon and cleave the protein at that site, resulting in truncated protein. As seen in Figure 2.11B, each full-length LexA+Hcm1 TAG construct is expressed only in the presence of the UAA. However, when the transcriptional activity of each construct was

examined, during glucose-limiting conditions only LexA+Hcm1 T218TAG and Lexa+Hcm1 T272TAG showed activity levels similar to the wild-type LexA+Hcm1 (201-300) protein (Figure 2.11C).
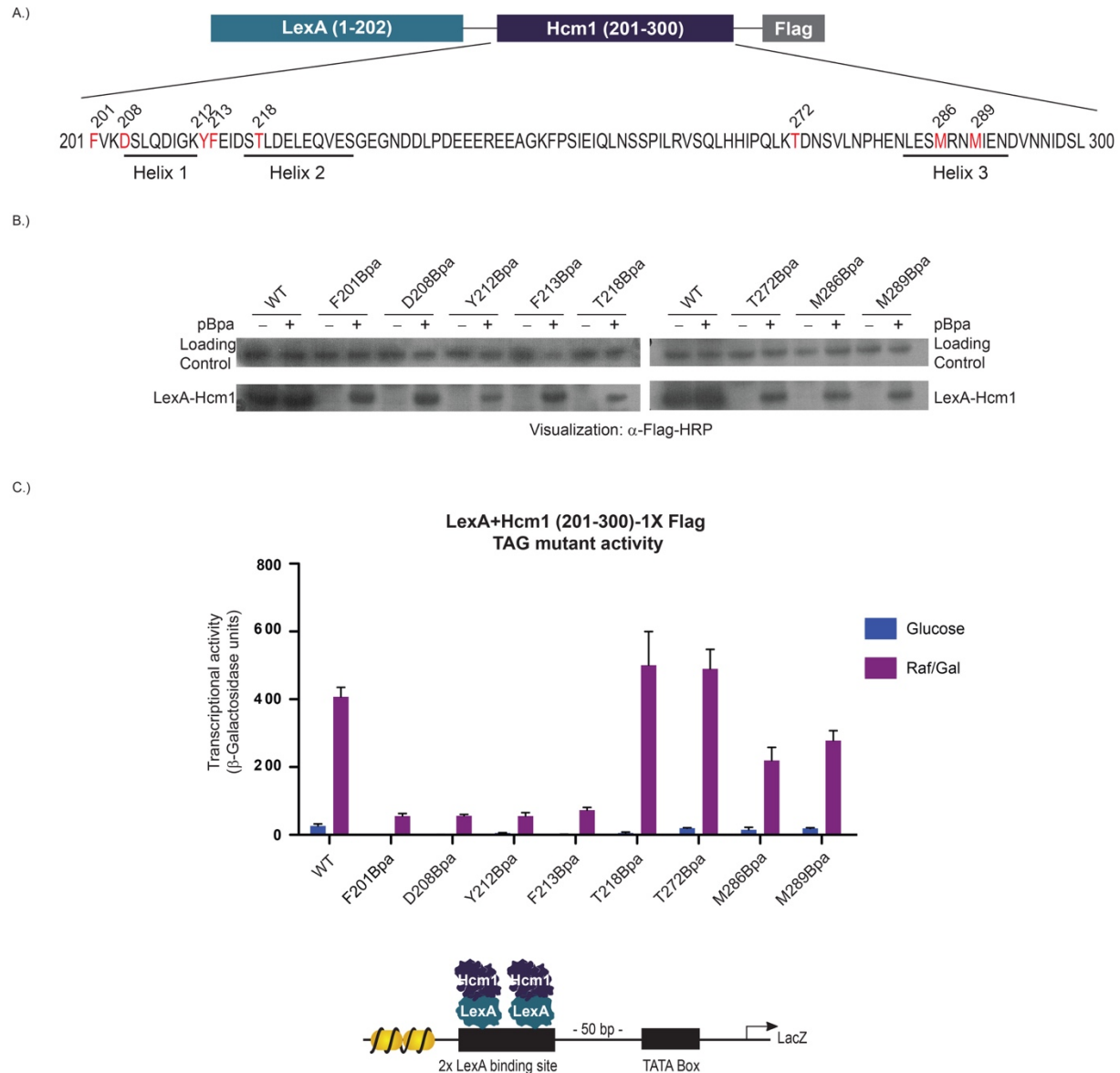
Figure 2.11 *In vivo* incorporation and transcriptional activation of LexA+Hcm1 (201-300) TAG mutants. A.) A plasmid encoding the bacterial LexA DBD fused to Hcm1 (201-300) and a flag tag for detection. Position at which pBpa mutagenesis was carried out are indicated in red. The proposed alpha helical regions are indicated by a black underline. B.) *In vivo* incorporation of pBpa into LexA+Hcm1 (201-300) in live yeast using the pBpa specific *E. coli* tyrosyl tRNA/tRNA synthetase pair in the presence or absence of 1 mM pBpa. C.) β-galactosidase assessment of activation potential of each mutant in LexA+Hcm1 (201-300).

Alongside the activity assays, we examined the crosslinking profiles for each of the TAG mutants and found that each construct had a multi-protein binding profile with

several crosslinked products detected by western blot (Figure 2.12). The decreased crosslinking profile seen for the M286Bpa and M289Bpa constructs are due to the proximal methionine near the pBpa incorporation sites that slightly quench the crosslinking activity. This reactivity phenomenon is discussed in Chapter 3. Nevertheless, based on the expression and activity data, both the LexA+Hcm1 T218Bpa and LexA+Hcm1 T272Bpa chimeric proteins were chosen as the best constructs to move forward with to interrogate the Hcm1-SNF1 interaction under nutrient stress conditions.
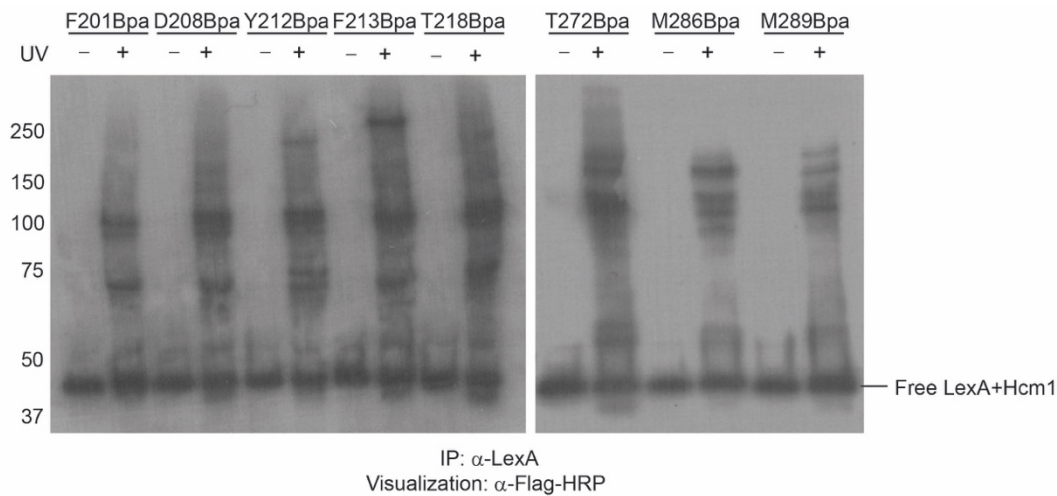
Figure 2.12 *In vivo* photo-crosslinking profiles of LexA+Hcm1 (201-300) TAG mutants. Live yeast expressing the pBpa incorporated LexA+Hcm1 (201-300) TAG mutants were irradiated at 365 nm for 30 minutes on ice to capture Hcm1's endogenous binding partners. The yeast cells were lysed and immunoprecipitated with α-LexA to isolate the activator crosslinked products. The crosslinked products are visualized by western blot probing with α-Flag.

2.5 Examination of the Hcm1-SNF1 interaction network in yeast

While our lab has captured the shared interaction between the Snf1 and Gal83 subunits and the chimeric amphipathic activators LexA+Gal4 and LexA+VP16 using *in vivo* covalent chemical capture, these activators are not known substrates of Snf1.[20] However, Hcm1 is a known substrate of the SNF1 complex and phosphorylation of the wild-type protein by the Snf1 catalytic subunit has been reported under glucose-limiting conditions.[22] We hypothesized that the LexA+Hcm1 (201-300) chimeric protein would directly interact with the SNF1 complex. However, which subunits Hcm1 interacts with and if Hcm1 interacts with the SNF1 complex similarly to the Gal4 and VP16 activators

is still unclear. Thus, we examined Hcm1's interactions with the Snf1 catalytic subunit and the Gal83 β-scaffolding subunit using covalent chemical capture in live yeast.

2.5.1 Capturing a LexA+Hcm1 – Snf1 interaction

First, the interaction between Hcm1 and the Snf1 catalytic subunit was assessed under galactose stress conditions. These conditions should shuttle both the SNF1 complex and Hcm1 proteins into the nucleus and activate transcription of the GAL1 gene. Initially an overexpressed Myc-tagged Snf1 construct was used to examine this interaction. For these experiment, live yeast expressing either the LexA+Hcm1 218Bpa-Flag or LexA+Hcm1 272Bpa-Flag proteins and Myc-tagged Snf1 were irradiated at 365 nm UV light to capture the Hcm1-Snf1 interaction. Upon lysis, immunoprecipitation for the LexA-Hcm1 protein, and western blot analysis probing for the myc-tagged Snf1 subunit, the Hcm1-Snf1 interaction was seen for both Hcm1 218Bpa and Hcm1 272Bpa (Figure 2.13A).

While encouraging, the experiments shown above were run using an overexpressed Snf1 variant which could be forcing the interaction to occur under these conditions. Thus, we next examined the Hcm1 interaction with endogenously expressed Snf1. For these experiments, yeast expressing with LexA+Hcm1 218Bpa-Flag or LexA+Hcm1 272Bpa-Flag were irradiated and lysed. Following immunoprecipitation for the endogenous Snf1 protein, and western blot analysis probing for the Hcm1 protein, the Hcm1 interaction with Snf1 was again seen for both Hcm1 constructs (Figure 2.13 B). These results confirm that under glucose-limiting conditions LexA+Hcm1 (201-300) directly interacts with the Snf1 catalytic subunit.

2.5.2 Capturing a LexA+Hcm1-Gal83 interaction

While we identified the Snf1 catalytic subunit as a binding partner of LexA+Hcm1 (201-300) during nutrient stress conditions, we wanted to determine whether other subunits within the SNF1 complex are Hcm1 binding partners. Under galactose conditions, the Gal83 β-scaffolding subunit binds to the SNF1 complex and shuttles it into the nucleus to regulate transcription of nutrient stress response genes, such as GAL1.[63] As discussed above, Gal83 directly interacts with both Gal4 and VP16 in yeast

at the GAL1 promoter and we sought to determine whether this interaction is shared with Hcm1.
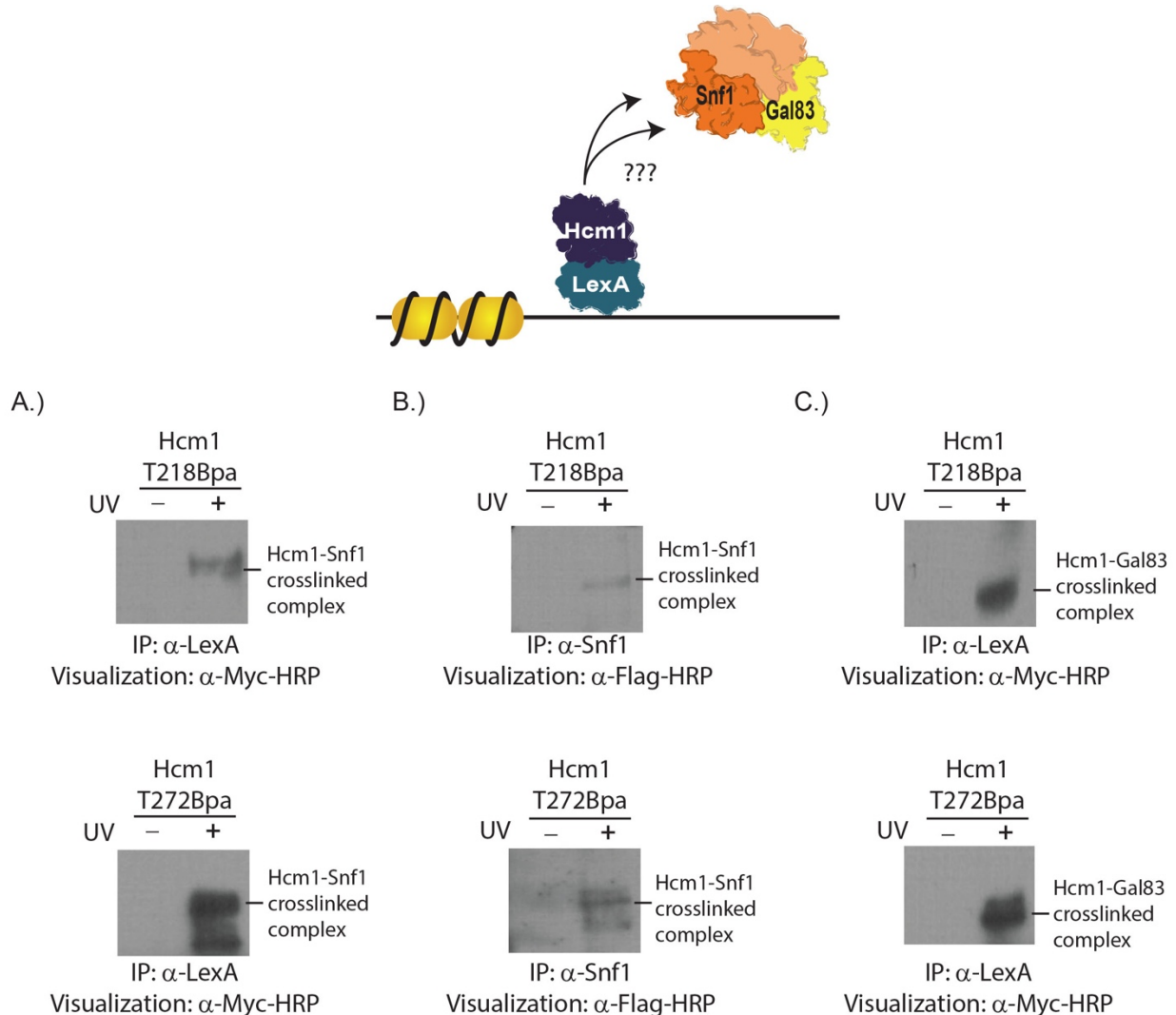
Figure 2.13 Interrogation of Hcm1 interactions with the SNF1 complex. A.) Hcm1 interacts with Myc-tagged Snf1 when pBpa is incorporated at either position 218 or 272. Crosslinking of LexA+Hcm1 and Myc-tagged Snf1 was analyzed by western blot of cell lysates with α-Myc. B.) Hcm1 interacts with endogenous Snf1 when pBpa is incorporated at either position 218 or 272. Crosslinking of LexA+Hcm1 and endogenous Snf1 was analyzed by western blot with α-Flag antibody of cell lysates immunoprecipitated with α-Snf1. C.) Hcm1 interacts with Myc-tagged Gal83 when pBpa is incorporated at either position 218 or 272. Crosslinking of LexA+Hcm1 and Myc-tagged Gal83 was analyzed by western blot of cell lysates with α-Myc.

To examine the interaction between the Gal83 scaffolding subunit and our LexA-Hcm1 (201-300) chimeric protein a Myc-tagged Gal83 construct was used due to Gal83 antibody limitations. Nevertheless, yeast expressing either the LexA+Hcm1 218Bpa-

Flag or LexA+Hcm1 272Bpa-Flag proteins and Myc-tagged Gal83 were irradiated, lysed, and immunoprecipitated for the Hcm1 protein. Following western blot analysis probing for the Myc-tagged Gal83 protein, the Hcm1-Gal83 interaction was captured for both Hcm1 constructs demonstrating a direct interaction between these two proteins and a shared interaction network among diverse amphipathic activators (Figure 2.13C). These data show that the LexA+Hcm1 (201-300) minimal TAD directly interacts with the SNF1 complex through the Snf1 catalytic subunit and the Gal83 β-scaffolding subunit. Thus, demonstrating the conservation of the Hcm1/FOXO3a-SNF1/AMPK interaction and SNF1's recruitment by amphipathic activators. Additionally, this study demonstrates the strength of *in vivo* covalent chemical capture to interrogate PPIs between activators and dynamic interchangeable coactivator complexes.

2.6 Conclusions and Future Directions

In this chapter, we determined a minimal TAD sequence for Hcm1 that is regulated by carbon source in a manner similar to the wild type activator. Furthermore, these data show that the mechanism by which the SNF1 complex interacts with LexA+Hcm1 under nutrient stress is similar to the other yeast stress response activators, such as Gal4. These data also indicate that the Snf1 and Gal83 subunits of the SNF1 complex are shared binding partners of many amphipathic activators in yeast. However, the experiments discussed in this chapter between SNF1 and Hcm1, Gal4, and VP16 are all located at the GAL1 promoter raising the question as to whether these interactions are promoter specific or activator specific. While multi-subunit coactivators, such as the Mediator and SAGA complexes, are required for transcriptional initiation, it has been proposed in the literature that specific gene promoters determine the protein complexes that are required for expression.[107-113] However, it has also been shown that while the interaction between transcriptional activators and coactivators are highly redundant, specific TADs of transcriptional activators either interact with different subunits within these coactivator complexes or in different confirmations with a similar coactivator subunit.[13, 43, 45, 114] For example, while our lab has captured the direct interaction between VP16 and the Snf1 kinase of the SNF1 complex with both subdomains, only one subdomain interacts with the Gal83 scaffolding subunit.[20] Additionally, a study

interrogating VP16's recruitment of the SWI/SNF chromatin remodeling complex showed the subdomains work cooperatively to recruit SWI/SNF with VP16N directly binding to the Snf5 and Snf6 subunits and VP16C binding to the Snf2 within the complex.[19] Thus, demonstrating some sequence specificity within the activator TADs that are required for individual activator-coactivator interactions.

To determine whether the Hcm1-SNF1 interactions are protein specific or promoter specific, a Hcm1 (1-564) full-length construct has been made. Using the 218 and 272 incorporation sites, the Hcm1-SNF1 interactions will be examined using *in vivo* covalent chemical capture under nutrient stress. Additionally, to further assess whether these activator-SNF1 interactions are dependent on the GAL1 promoter, a variety of different DBDs, such as the Gal4 (1-147) or Hcm1 (100-200), could be used to examine the similarities and differences in SNF1 recruitment at different promoters. These studies could help clarify whether these artificial chimeric activators commonly used for interrogating transcriptional PPI networks are capturing activator specific or promoter specific interactions in the cell.

2.7 Materials and Methods

 LS41 [JPY9::pZZ41, Matα his3Δ200 leu2Δ1 trp1Δ63 ura3-52 lys2Δ385 gal4 URA::pZZ41] yeast was used for all experiments. pBpa was purchased from Chem-Impex International (Wood Dale, IL). All plasmids described below were constructed using standard molecular biology techniques and the sequences of all isolated plasmids were validated by sequencing at the University of Michigan Core Facility (Ann Arbor, MI).

Table 2.1 Plasmids used in Chapter 2

Plasmid Name	Function
pLexA-1X Flag	Expresses LexA(1-202)+1X Flag
pLexA+Hcm1(139-511)-1X Flag	Expresses LexA(1-202)+Hcm1(139-511)-1X Flag
pLexA+Hcm1(201-511)-1X Flag	Expresses LexA(1-202)+Hcm1(201-511)-1X Flag

pLexA+Hcm1(301-511)-1X Flag	Expresses LexA(1-202)+Hcm1(301-511)-1X Flag
pLexA+Hcm1(341-511)-1X Flag	Expresses LexA(1-202)+Hcm1(341-511)-1X Flag
pLexA+Hcm1(380-511)-1X Flag	Expresses LexA(1-202)+Hcm1(380-511)-1X Flag
pLexA+Hcm1(440-511)-1X Flag	Expresses LexA(1-202)+Hcm1(440-511)-1X Flag
pLexA+Hcm1(450-511)-1X Flag	Expresses LexA(1-202)+Hcm1(450-511)-1X Flag
pLexA+Hcm1(301-564)-1X Flag	Expresses LexA(1-202)+Hcm1(301-564)-1X Flag
pLexA+Hcm1(139-300)-1X Flag	Expresses LexA(1-202)+Hcm1(139-300)-1X Flag
pLexA+Hcm1(201-300)-1X Flag	Expresses LexA(1-202)+Hcm1(201-300)-1X Flag
pLexA+Hcm1(301-440)-1X Flag	Expresses LexA(1-202)+Hcm1(301-440)-1X Flag
pLexA+Hcm1(380-440)-1X Flag	Expresses LexA(1-202)+Hcm1(380-440)-1X Flag
pLexA+Hcm1 F201TAG-1X Flag	Expresses LexA(1-202)+Hcm1(201-300)+1X Flag tag with a TAG codon replacing the codon of the existing amino acid
pLexA+Hcm1 D208TAG-1X Flag	Expresses LexA(1-202)+Hcm1(201-300)+1X Flag tag with a TAG codon replacing the codon of the existing amino acid
pLexA+Hcm1 Y212TAG-1X Flag	Expresses LexA(1-202)+Hcm1(201-300)+1X Flag tag with a TAG codon replacing the codon of the existing amino acid
pLexA+Hcm1 F213TAG-1X Flag	Expresses LexA(1-202)+Hcm1(201-300)+1X Flag tag with a TAG codon replacing the codon of the existing amino acid
pLexA+Hcm1 T218TAG-1X Flag	Expresses LexA(1-202)+Hcm1(201-300)+1X Flag tag with a TAG codon replacing the codon of the existing amino acid

pLexA+Hcm1 T272TAG-1X Flag	Expresses LexA(1-202)+Hcm1(201-300)+1X Flag tag with a TAG codon replacing the codon of the existing amino acid
pLexA+Hcm1 M286TAG-1X Flag	Expresses LexA(1-202)+Hcm1(201-300)+1X Flag tag with a TAG codon replacing the codon of the existing amino acid
pLexA+Hcm1 M289TAG-1X Flag	Expresses LexA(1-202)+Hcm1(201-300)+1X Flag tag with a TAG codon replacing the codon of the existing amino acid
pLexA+Hcm1 F201TAG-4X Flag	Expresses LexA(1-202)+Hcm1(201-300)+4X Flag tag with a TAG codon replacing the codon of the existing amino acid
pLexA+Hcm1 D208TAG-4X Flag	Expresses LexA(1-202)+Hcm1(201-300)+4X Flag tag with a TAG codon replacing the codon of the existing amino acid
pLexA+Hcm1 Y212TAG-4X Flag	Expresses LexA(1-202)+Hcm1(201-300)+4X Flag tag with a TAG codon replacing the codon of the existing amino acid
pLexA+Hcm1 F213TAG-4X Flag	Expresses LexA(1-202)+Hcm1(201-300)+4X Flag tag with a TAG codon replacing the codon of the existing amino acid
pLexA+Hcm1 T218TAG-4X Flag	Expresses LexA(1-202)+Hcm1(201-300)+4X Flag tag with a TAG codon replacing the codon of the existing amino acid
pLexA+Hcm1 T272TAG-4X Flag	Expresses LexA(1-202)+Hcm1(201-300)+4X Flag tag with a TAG codon replacing the codon of the existing amino acid
pLexA+Hcm1 M286TAG-4X Flag	Expresses LexA(1-202)+Hcm1(201-300)+4X Flag tag with a TAG codon replacing the codon of the existing amino acid
pLexA+Hcm1 M289TAG-4X Flag	Expresses LexA(1-202)+Hcm1(201-300)+4X Flag tag with a TAG codon replacing the codon of the existing amino acid
pSNRtRNA-pBpaRS	Expressed tRNA under the control of the SNR52 promoter and contains synthetase specific for pBpa
pSnf1-6XMyc	Expresses full-length Snf1+6X c-Myc tag on the C-terminus of the protein
pGal83-6XMyc	Expresses full-length Gal83+6X c-Myc tag on the C-terminus of the protein

Table 2.2 Primers used in Chapter 2

Primer ID	Sequence	Description
P1	5' GGCTGGAATTCCCGGAAGGATCCGTCGACGACT ACAAGGACGACGATGACAAGTAACTGCAGCCAAGC TAATTCCGGG 3'	SDM pLexA-Flag For
P2	5' CCCGGAATTAGCTTGGCTGCAGTTACTTGTCAT CGTCGTCCTTGTAGTCGTCGACGGATCCTTCCGGG AATTCCAGCC 3'	SDM pLexA-Flag Rev
P3	5' GGACACGGATCCATCCACGTTCACTTCCCTTATT ACAAGCAG 3'	BamH1-Hcm1 139 For
P4	5' GGACACGGATCCTTTGTAAAGGACTCCTTACAA GACATTGGG 3'	BamH1-Hcm1 201 For
P5	5' GGACACGGATCCGAACCTCCTTATGTCATGAAG AAATATC 3'	BamH1-Hcm1 301 For
P6	5' GGACACGGATCCAATACACTCCCTATAACTAG GCAAAGTCTCC 3'	BamH1-Hcm1 341 For
P7	5' GGACACGGATCCAATACACTCCCTATAACTAGC GCAAAGTCTCC 3'	BamH1-Hcm1 380 For
P8	5' GGACACGGATCCACCCCATCGCGGTTGATAAGC ACACCTAAG 3'	BamH1-Hcm1 440 For
P9	5' GGACACGGATCCGACGGTAACTCGATTTTGAGG AAATGGCAG 3'	BamH1-Hcm1 450 For
P10	5' CCGAGCGTCGACATTCGTTGCGCTTGTGAGGA CATCGGGTGC 3'	Sal1-Hcm1 511 Rev
P11	5' CCGAGCGTCGACCTTTTCATTACCGCTATCGTT GGAAGGGTG 3'	Sal1-Hcm1 564 Rev
P12	5' CCGAGCGTCGACCAAGGAATCTATATTGTTGA CATCGTTTTC 3'	Sal1-Hcm1 300 Rev
P13	5' CCGAGCGTCGACGGTTTTCTCTAAGCCATCGG	Sal1-Hcm1 440

	AATGCCTC 3'	Rev
P14	5'CTGGAATTCCCGGAAGGATCCTAGGTAAAGGACT CCTTACAAGAC 3'	SDM Hcm1 WT F201TAG For
P15	5' GTCTTGTAAGGAGTCCTTTACCTAGGATCCTT CCGGGAATTCCAG 3'	SDM Hcm1 WT F201TAG Rev
P16	5' CCTTTGTAAAGGACTCCTTACAATAGATTGGGA AGTATTTTGAAATAG 3'	SDM Hcm1 WT TAG D208TAG For
P17	5' CTATTTCAAAATACTTCCCAATCTATTGTAAGG AGTCCTTTACAAGG 3'	SDM Hcm1 WT D208TAG Rev
P18	5' CTCCTTACAAGACATTGGGAAGTAGTTTGAAATA GATTCTACACTTG 3'	SDM Hcm1 WT Y212TAG For
P19	5' CAAGTGTAGAATCTATTTCAAACTACTTCCCAA TGTCTTGTAAGGAG 3'	SDM Hcm1 WT Y212TAG Rev
P20	5' CTTACAAGACATTGGGAAGTATTAGGAAATAGA TTCTACACTTGATG 3'	SDM Hcm1 WT F213TAG For
P21	5' CATCAAGTGTAGAATCTATTTCCTAATACTTCCC AATGTCTTGTAAG 3'	SDM Hcm1 WT F213TAG Rev
P22	5' GACATTGGGAAGTATTTTGAAATATAGTCTACA CTTGATGAATTAGAAC 3'	SDM Hcm1 WT T218TAG For
P23	5' GTTCTAATTCATCAAGTGTAGACTATATTTCAAA ATACTTCCCAATGTC 3'	SDM Hcm1 WT T218TAG Rev
P24	5' CATCACATACCGCAATTGAAATAGGACAACAGT GTACTGAACCCTC 3'	SDM Hcm1 WT T272TAG For
P25	5' GAGGGTTCAGTACACTGTTGTCCTATTTCAATT GCGGTATGTGATG 3'	SDM Hcm1 WT T272TAG Rev
P26	5' CCTCACGAAAACCTAGAATCGTAGCGGAACAT GATAGAAAACGATGTC 3'	SDM Hcm1 WT M286TAG For
P27	5' GACATCGTTTTCTATCATGTTCCGCTACGATTC TAGGTTTTCGTGAGG 3'	SDM Hcm1 WT M286TAG Rev

P28	5' GAAAACCTAGAATCGATGCGGAACTAGATAGAA AACGATGTCAACAATATAG 3'	SDM Hcm1 WT M289TAG For
P29	5' CTATATTGTTGACATCGTTTTCTATCTAGTTCCGC ATCGATTCTAGGTTTTC 3'	SDM Hcm1 WT M289TAG Rev
P30	5' CAATATAGATTCCTTGGTCGACGACTACAAGGAC GACGATGACAAGGACTACAAGGACGACGATGAC 3'	SDM 1X Flag addition For
P31	5' GTCATCGTCGTCCTTGTAGTCCTTGTCATCGTC GTCCTTGTAGTCGTCGACCAAGGAATCTATATTG 3'	SDM 1X Flag addition Rev

Construction of plasmids

1. <u>pLexA-1X Flag</u>

   A high copy plasmid expressing LexA (1-202)-1X Flag tag under the control of the ADH1 promoter was derived from pLexA. Using site directed mutagenesis, a Flag tag was added at the carboxy terminus of the pLexA plasmid. PCR primers were designed to have ~15-20 base pairs of homology on either side of the Flag tag sequence. Primers P1 and P2 were used to amplify in the Flag tag sequence using the pLexA parent plasmid as the template. The amplified PCR product was digested with Dpn1 restriction enzyme to produce the new plasmid.

2. <u>pLexA+Hcm1 TAD-1X Flag mutants</u>

   High copy plasmids containing various Hcm1 minimal TAD sequences fused to the LexA DBD under the control of the ADH1 promoter were created from pLexA-1X Flag containing BamH1 and Sal1 restriction sites. Primers for each minimal TAD construct were used to amplify Hcm1 using LS41 genomic yeast DNA as a template. The amplified PCR products were digested with BamH1 and Sal1 restriction enzymes and ligated into BamH1 and Sal1 digested pLexA-1X Flag using T4 DNA ligase to produce the LexA+Hcm1-1X Flag constructs.

3. pLexA+Hcm1 (201-300)-1X Flag TAG mutants (F201TAG, D208TAG, Y212TAG, F213TAG, T218TAG, T272TAG, M286TAG, M289TAG)

Plasmids containing various amber mutations within the Hcm1 minimal TAD were derived from the pLexA+Hcm1 (201-300)-1X flag parent plasmid. Using site directed mutagenesis, single point mutations were made by replacing an existing amino acid in the Hcm1 TAD with the TAG codon. PCR primers were designed to have ~15-20 base pairs of homology on either side of the TAG mutation. Each set of primers were used to amplify the Hcm1 amber mutations using pLexA+Hcm1 (201-300)-1X Flag as the template. The amplified PCR product was digested with Dpn1 restriction enzyme to product the new mutated plasmid.

4. pLexA+Hcm1 (201-300)-4X Flag

A high copy plasmid expressing LexA+Hcm1 (201-300)-4X Flag tag under the control of the ADH1 promoter was derived from pLexA+Hcm1 (201-300)-1X Flag. Using site directed mutagenesis, 3 Flag tags was added at the carboxy terminus of the pLexA+Hcm1 (201-300)-1X Flag plasmid. PCR primers were designed to have ~15-20 base pairs of homology on either side of the Flag tag sequence. Primers P30 and P31 were used in three sequential PCR reactions to amplify in the three Flag tags using the pLexA+Hcm1 (201-300)-1X Flag parent plasmid as the template. The amplified PCR product was digested with Dpn1 restriction enzyme to product the new plasmid.

5. pLexA+Hcm1 (201-300)-4X Flag TAG mutants (F201TAG, D208TAG, Y212TAG, F213TAG, T218TAG, T272TAG, M286TAG, M289TAG)

Plasmids containing various amber mutations within the Hcm1 minimal TAD were derived from the pLexA+Hcm1 (201-300)-4X flag parent plasmid. Using site directed mutagenesis, single point mutations were made by replacing an existing amino acid in the Hcm1 TAD with the TAG codon. PCR primers were designed to have ~15-20 base pairs of homology on either side of the TAG mutation. Each set of primers were used to amplify the Hcm1 amber mutations using pLexA+Hcm1 (201-300)-4X Flag

as the template. The amplified PCR product was digested with Dpn1 restriction enzyme to product the new mutated plasmid.

Expression assay of LexA+Hcm1 TAD+1X Flag constructs

LS41 yeast was transformed with pLexA-1X Flag or one of the pLexA-Hcm1 TAD-1X Flag constructs. Individual colonies were grown in 5 mL SC media containing 2% raffinose, but lacking histidine and uracil for selection. The cultures were incubated at 30 °C with 250 rpm agitation. Following incubation, these cultures were used to inoculate 5 mL cultures of SC media containing 2% raffinose and 2% galactose and the cultures were incubated at 30 °C with agitation to an OD_{660} of 1.0. Three ODs were isolated, washed with sterile water, and stored at -20 °C. The samples were lysed in 10 µL 4X NuPAGE LDS Sample loading buffer (Invitrogen), 10 µL 1X lysis buffer (50 mM tris-acetate pH 7.9, 100 mM potassium acetate, 20% glycerol, 0.2% Tween-20, 2 mM β-mercaptoethanol, and 2 mM magnesium acetate), and 10 µL 1 M DTT by boiling at 95 °C for 10 min. The samples were run on a 3-8% tris-acetate SDS-PAGE gel and analyzed by western blot with the anti-Flag (M2) antibody (Sigma Aldrich).

β-Galactosidase assay

For the LexA+Hcm1 TAD constructs, LS41 yeast was transformed with either pLexA-1X Flag or one of the pLexA+Hcm1 TAD-1X Flag plasmids. Individual colonies were grown in 5mL SC media containing 2% raffinose, but lacking histidine and uracil for selection. The cultures were incubated at 30 °C with 250 rpm agitation. Following incubation, these cultures were used to inoculate 5 mL cultures of SC media containing either 2% glucose or 2% Raffinose and 2% galactose which were subsequently incubated at 30 °C with agitation to an OD_{660} of 1.0 and harvested. The activity of each construct was assessed using β-Galactosidase assay as previously described.[25]

For the LexA+Hcm1 (201-300)-1X Flag TAG mutant constructs, LS41 yeast was transformed with either pLexA-Hcm1 (201-300)-1X Flag or one of the pLexA+Hcm1 TAG-1X Flag plasmids and the PSNRtRNA-pBpaRS plasmid. Individual colonies were grown in 5mL SC media containing 2% raffinose, but lacking histidine, tryptophan, and

uracil for selection. The cultures were incubated at 30 °C with 250 rpm agitation. Following incubation, these cultures were used to inoculate 5 mL cultures of SC media containing either 2% glucose or 2% Raffinose and 2% galactose, with or without 1 mM pBpa (dissolved in 1 M NaOH), and 1 M HCl, which were subsequently incubated at 30 °C with agitation to an OD_{660} of 1.0 and harvested. The activity of each construct was assessed using β-Galactosidase assay as previously described.[25]

UAA incorporation and expression

LS41 yeast was transformed with pLexA+Hcm1WT-1X Flag or one of the pLexA+Hcm1 TAG mutant-1X Flag constructs and pSNRtRNA-pBpaRS plasmids. Individual colonies were grown in 5 mL SC media containing 2% raffinose, but lacking histidine, tryptophan, and uracil for selection. The cultures were incubated at 30 °C with 250 rpm agitation. Following incubation, these cultures were used to inoculate 5 mL cultures of SC media containing 2% raffinose and 2% galactose, with or without 1 mM pBpa (dissolved in 1 M NaOH), and 1 M HCl. The cultures were incubated at 30 °C with agitation to an OD_{660} of 1.0. Three ODs were isolated, washed with sterile water, and stored at -20 °C. The samples were lysed in 10 μL 4X NuPAGE LDS Sample loading buffer (Invitrogen), 10 μL 1X lysis buffer (50 mM tris-acetate pH 7.9, 100 mM potassium acetate, 20% glycerol, 0.2% Tween-20, 2 mM β-mercaptoethanol, and 2 mM magnesium acetate), and 10 μL 1 M DTT by boiling at 95 °C for 10 min. The samples were run on a 3-8% tris-acetate SDS-PAGE gel and analyzed by western blot with the anti-Flag (M2) antibody (Sigma Aldrich).

In vivo photo-crosslinking to capture endogenous Hcm1 binding partners

For *in vivo* photo-crosslinking, a colony of one of the LexA+Hcm1 TAG-4X Flag constructs was grown in 5 mL SC media containing 2% raffinose, but lacking histidine, tryptophan, and uracil for selection. The cultures were incubated at 30 °C with 250 rpm agitation. Following incubation, these cultures were used to inoculate 100 mL cultures of SC media containing 2% raffinose and 2% galactose, with 1 mM pBpa (dissolved in 1 M NaOH), and 1 M HCl. The cultures were incubated at 30 °C with agitation to an OD_{660} of 1.0. For each culture, the cells were isolated by centrifugation and washed with the SC

media lacking histidine, tryptophan, and uracil. The cell pellets were resuspended in 2 mL SC media containing 2% raffinose and galactose and then transferred to small culture dishes and subjected to UV irradiated at 365 nm light (Eurosolar 15W UV lamp) with cooling for 30 minutes. The cells were isolated by centrifugation and stored at -20 °C until lysis. The control samples were washed with 1 mL SC media containing 2% raffinose and 2% galactose, isolated by centrifugation, and stored at -20 °C until lysis.

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 immunoprecipitated with anti-LexA antibody (sc-1725, Santa Cruz Biotechnologies) for 2 hours at 4 °C. The proteins bound to the antibody were isolated by incubation for 1 hour with 40 µL pre-washed protein G Magnetic Dyna beads (Life Technologies) at 4 °C. After incubation, the beads were washed six 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 at -20 °C until elution. The samples were eluted from the beads by heating at 95 °C for 10 minutes in 10 µL NuPAGE 4X LDS Sample Loading Buffer (Invitrogen), 10 µL water, and 10 µL 1M DTT. The samples were run on a 3-8% tris-acetate SDS-PAGE gel and analyzed by western blot using anti-Flag (M2) antibody (Sigma Aldrich).

In vivo photo-crosslinking to Snf1 and Gal83

For *in vivo* photo-crosslinking with Snf1- and Gal83-6xMyc, a colony of either LexA+Hcm1 T218Bpa-4X Flag: Snf1/Gal83-6xMyc or LexA+Hcm1 T272Bpa-4X Flag: Snf1/Gal83-6xMyc constructs was grown in 5 mL SC media containing 2% raffinose, but lacking histidine, leucine, tryptophan, and uracil for selection. The cultures were incubated at 30 °C with 250 rpm agitation. Following incubation, these cultures were used to inoculate 100 mL cultures of SC media containing 2% raffinose and 2% galactose, with 1 mM pBpa (dissolved in 1 M NaOH), and 1 M HCl. The cultures were incubated at 30 °C with agitation to an OD_{660} of 1.0. For each culture, the cells were isolated by centrifugation and washed with the SC media lacking histidine, tryptophan,

and uracil. The cell pellets were resuspended in 2 mL SC media containing 2% raffinose and 2% galactose and then transferred to small culture dishes and subjected to UV irradiated at 365nm light (Eurosolar 15W UV lamp) with cooling for 30 minutes. The cells were isolated by centrifugation and stored at -20 °C until lysis. The control samples were washed with 1 mL SC media containing 2% raffinose and 2% galactose, isolated by centrifugation, and stored at -20 °C until lysis. For crosslinking studies with endogenous Snf1, the procedure was identical except that cells were grown in SC media lacking histidine, tryptophan, and uracil.

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 immunoprecipitated with anti-LexA antibody (sc-1725, Santa Cruz Biotechnologies) for the Myc-tagged constructs or anti-Snf1 (sc-15621, Santa Cruz Biotechnologies) for endogenous Snf1 for 2 hours at 4 °C. The proteins bound to the antibody were isolated by incubation for 1 hour with 40 µL pre-washed protein G magnetic Dyna beads (Life Technologies) at 4 °C. After incubation, the beads were washed six 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 at -20 °C until elution. The samples were eluted from the beads by heating at 95 °C for 10 minutes in 10 µL NuPAGE 4X LDS Sample Loading Buffer (Invitrogen), 10 µL water, and 10 µL 1M DTT. The samples were run on a 3-8% tris-acetate SDS-PAGE gel and analyzed by western blot using either anti-cMyc antibody (sc-40, Santa Cruz Biotechnologies) for the Myc-tagged proteins or anti-Flag (M2) antibody (Sigma Aldrich) for endogenous Snf1 experiments.

2.8 References

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CHAPTER 3[1]

Effects of sequence context and electron-withdrawing groups on pBpa reactivity

3.1 Introduction

Protein-protein interactions (PPIs) between transcriptional activators and coactivators are necessary for the initiation of gene expression, and misregulation of these interactions has been implicated in an array of human diseases.[1-5] Therefore, there is great interest in identifying methods to capture and characterize these interactions. However, PPIs which regulate transcription often are transient in nature and typically have moderate to weak affinities which makes them difficult to study by traditional biochemical techniques.[6-11] Historically, photo-crosslinkers, especially benzophenone, have been used to covalently capture and characterize known PPIs of protein complexes that bind with moderate to high affinity and have small defined binding interfaces.[12-21] Recently, with the advancement of amber nonsense suppression, photo-labile unnatural amino acids (UAAs), such as the benzophenone derivative *p*-benzoyl-L-phenylalanine (pBpa), have been incorporated into proteins of interest and used to capture the more transient PPIs in their native cellular environment.[22-39] For example, Hahn and coworkers have used pBpa to map the transcriptional interactions between the TATA-binding protein (TBP) and several coactivator complexes *in vitro* and *in vivo*, including the SAGA chromatin modifying complex and the TFIIA general transcription factor.[40] Additionally, our lab has previously captured the interaction between TBP and

the Gal4 and VP16 transcriptional activators in *Saccharomyces cerevisiae* using pBpa.[41]

Nevertheless, the successful application of covalent chemical capture remains highly dependent on the affinity and lifetime of the protein complex of interest, the environment of the PPI interface, and the reactivity preference of the photo-crosslinker. With every crosslinking experiment, there are several considerations to be made to insure the effective capture of a specific PPI. These include the reaction mechanism of the photo-crosslinker, the sequence environment surrounding the incorporation site, and the solvent exposure at the PPI interface. Currently, the benzophenone scaffold is widely used in the field for covalent capture of *in vivo* PPIs because of its stability and minimal reactivity with water.[42] However, the benzophenone crosslinking group is the least reactive and requires more irradiation time to capture certain interactions.[32, 43] In the first half of this chapter, we examine the effects by which the sequence environment of the Gal4 transcriptional activation domain (TAD) and the crosslinking mechanism of the photo-crosslinker have on the capture of the Gal4-Gal80 interaction. In the second half of the chapter, we report a suite of pBpa analogs containing electron-withdrawing substituents within the benzophenone scaffold that are hypothesized to have enhanced reactivity. Additionally, conditions for the incorporation and characterization of this suite of mono-substituted pBpa analogs were developed and implemented to fully characterize the change in crosslinking reactivity and yield seen in both *in vitro* and *in vivo* covalent chemical capture experiments.

3.2 Sequence context and crosslinking mechanism are crucial to crosslinking success

While our lab and others have successfully used *in vivo* covalent chemical capture to characterize cellular PPIs, there are considerations that need to made for each photo-crosslinker being used in a specific system, such as the sequence context in which the crosslinker is being incorporated.[28-29, 41, 44]
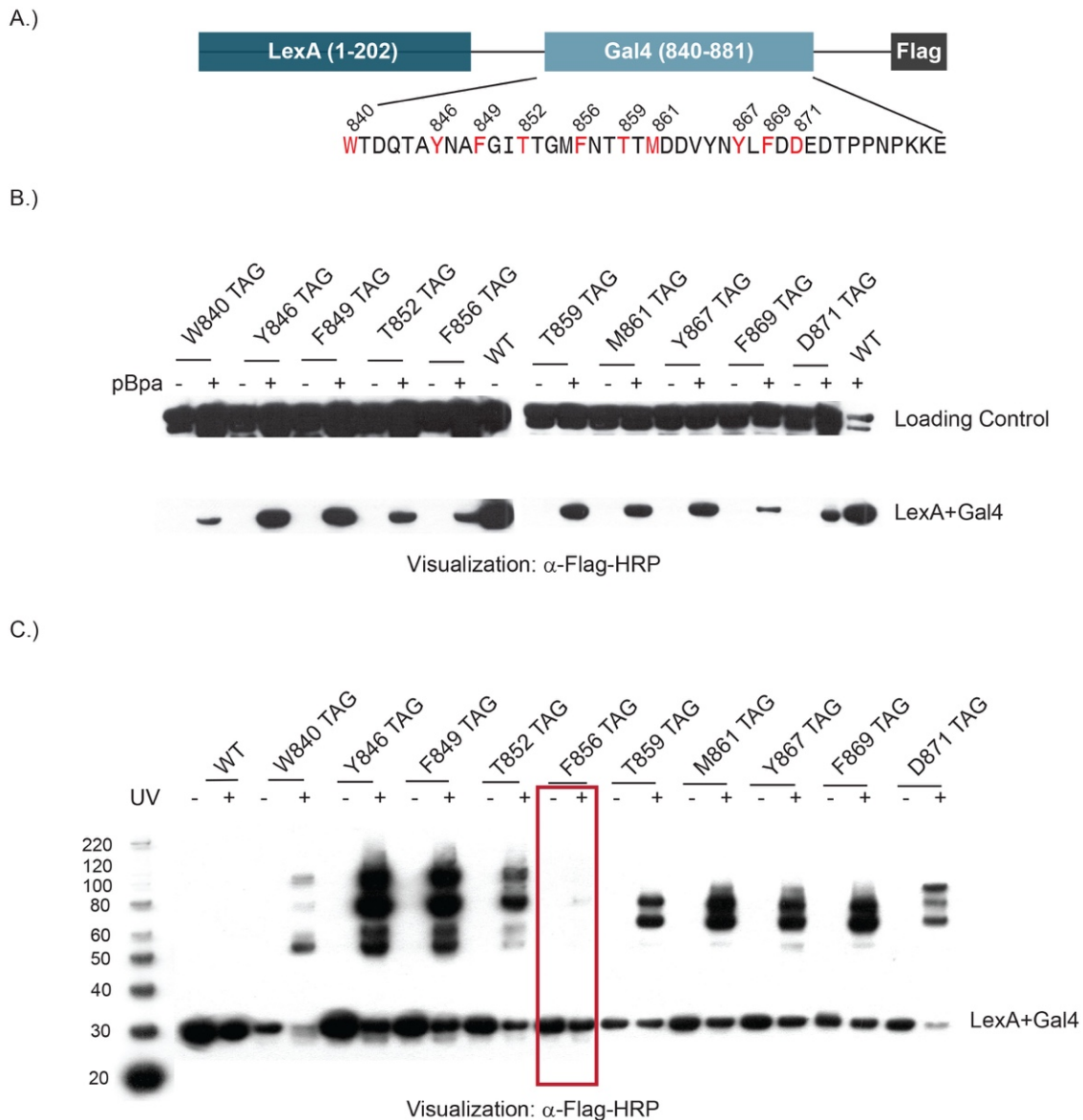
Figure 3.1 *In vivo* incorporation and crosslinking of LexA+Gal4 TAG mutants A.) A plasmid encoding the bacterial LexA DNA binding domain fused to the Gal4 (840-880) transcriptional activation domain (TAD) and a flag tag for detection. Position at which pBpa mutagenesis was carried out are indicated in red. B.) *In vivo* incorporation of pBpa into LexA+Gal4 TAD in live yeast using the pBpa-specific *E. coli* tyrosyl tRNA/tRNA synthetase pair in the presence or absence of 2 mM pBpa. C.) *In vivo* crosslinking of LexA+Gal4 pBpa incorporated protein in live yeast. Yeast expressing LexA+Gal4 mutants in media containing 2 mM pBpa were irradiated at 365 nm UV light, lysed, immunoprecipitated with α-LexA, and analyzed by western blot probing for the Flag-tagged activator. The loss of pBpa crosslinking at position 856 is indicated in the red box. Adapted with permission from [29]. Copyright 2009 American Chemical Society.

For example, in one study where the benzophenone crosslinker was used to interrogate the endogenous interactions of the prototypical Gal4 transcriptional activator and the interaction with its masking protein Gal80 was not captured despite ample evidence that

it was in effect.[29] Under normal glucose levels, the Gal80 protein binds to Gal4's TAD, inhibiting its activity. In this study pBpa was incorporated in several positions across the TAD of Gal4 to examine the expression and crosslinking profile at each position in live yeast (Figure 3.1). When pBpa was incorporated at position F856, there was a reduction in total crosslinking and the Gal4-Gal80 product around 80 kDa was not observed (Figure 3.1C, red box). However, the Gal4 F856pBpa mutant was fully expressed and remained repressed by Gal80 under normal glucose conditions indicating that the functional complex formed but was unable to be successfully captured by pBpa (Figure 3.1B). Thus, it was clear that there was some additional factor preventing effective Gal4-Gal80 crosslinking.

3.2.1 The Gal4-Gal80 interaction

Gal4 regulates genes responsible for galactose catabolism under conditions of limited glucose availability, subsequently acting as a nutrient sensor.[41, 44-48] This activator is highly regulated by its masking protein, Gal80, which binds to Gal4's TAD at low picomolar binding constant under normal glucose environmental conditions. When glucose is limited and galactose is present there is a conformational change at the binding interface which releases Gal4's TAD to recruit coactivator complexes and the transcriptional machinery to the DNA promoter to activate the expression of glucose-repressed genes (Figure 3.2). The Gal4-Gal80 interaction has been comprehensively studied, and several structural and functional studies have mapped the key residues responsible for this interaction.[49-52] Position 856 has been shown to be a key residue required for the direct interaction between Gal4 and Gal80, and point mutations at this position have been shown to be significantly less sensitive to Gal80's inhibition and drastically inhibit the binding of this complex.[48] Therefore, this position was hypothesized to be a strong site for covalent chemical capture. However, when pBpa was incorporated at position 856 within the Gal4 TAD, the interaction between Gal4 and its masking protein Gal80 was not captured.
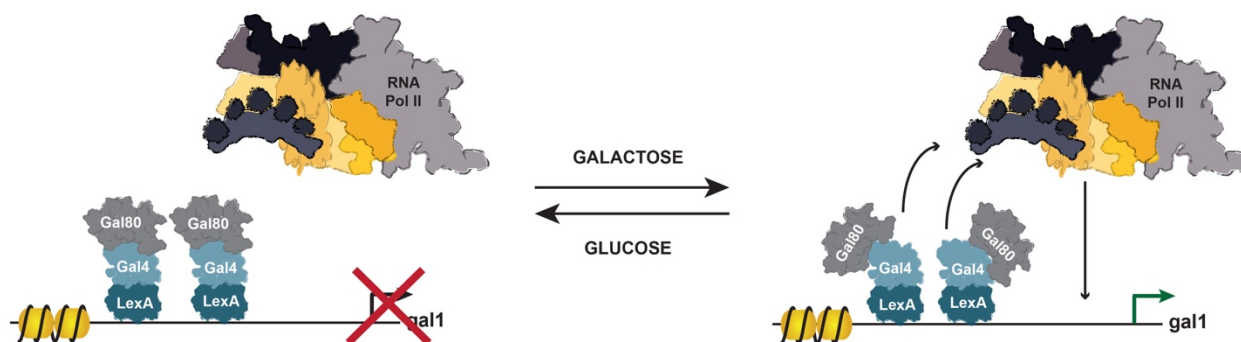
Figure 3.2 Prototypical Gal4 transcriptional model system. Under normal glucose conditions, Gal80 binds to the Gal4 TAD and inhibits GAL1 transcription. However, under glucose limiting conditions and in the presence of galactose, Gal80 changes confirmation releasing Gal4's TAD to recruit the pre-initiation complex (PIC), including RNA Polymerase II, to the GAL1 promoter through a variety of transient PPIs needed to activate transcription.

3.2.2 Different crosslinking mechanisms between pBpa and pAzpa

Several lines of evidence indicate that benzophenones react preferentially with methionines over the other nineteen amino acids.[42, 53-58] After further examination of the F856 incorporation site, it was noticed that there were two methionines nearby at position 855 and 861. Based on the crosslinking mechanism of pBpa, we hypothesized that these nearby methionines (M855 and M861) within the Gal4 TAD could interfere with the crosslinking reactivity of pBpa for Gal80. Upon irradiation with 365 nm light, the benzophenone core in pBpa forms a diradical that reacts with activated C-H bonds via initial H• abstraction by an oxygen diradical and subsequent recombination to yield a carbon-carbon bond between pBpa and either the backbone or side chains of nearby amino acids (Figure 3.3A). When there are no binding partners nearby, then the activated pBpa molecule relaxes back to the ground state and is available for subsequent reactivation.[32, 53] The C-H bonds adjacent to the methionine thioester are particularly reactive towards the pBpa diradical, likely because of the stabilizing influence of the sulfur on the initial radiation following C-H abstraction. It has been experimentally observed that pBpa's crosslinking efficiency and reactivity constraints can be drastically altered by the proximity of methionines. Previous studies have shown that pBpa can react with methionines beyond its usual distance limit of 3.1 A.[54-55, 59] In a recent report, when individual methionines were placed at unique sites within a PPI interface, pBpa was preferentially drawn to the inserted methionine, shifting the site of

crosslinking. This has been termed the "Methionine-magnet effect".[54] Therefore, due to the two methionines in close proximity to the pBpa incorporation site, we hypothesized that these methionines were "quenching" the reactivity of the pBpa molecule. Thus, causing the neighboring methionine residues within the Gal4 TAD to out-compete the residues within the Gal4 binding site on the Gal80 protein. To test this hypothesis, we sought to examine pBpa's ability to capture Gal80 when both the M855 and M861 residues where mutated to alanine.

To complement the pBpa crosslinking results in this study we used the *p*-azido-L-phenylalanine (pAzpa) UAA, which was also site-specifically incorporated into the Gal4 TAD at position 856. Like pBpa, aryl-azides are reactive to C-H and heteroatom-H bonds, but the amino acid reactivity is less defined due to the more complicated reaction mechanism. Upon irradiation with 254 nm light, the aryl-azide loses a dinitrogen group forming a nitrene which reacts with neighboring C-H and heteroatom-H bonds to form a new covalent adduct. However, if there are no X-H atoms available within 10^{-4} seconds of activation, the nitrene will convert to the more stable ketenimine, which only reacts with nucleophiles, including the surrounding solvent (Figure 3.3B). This subsequently reduces the crosslinking yield and increases nonspecific crosslinking reactions.[32, 60] Although the differences in crosslinking mechanisms between the two crosslinkers have been known, a direct comparison of the effects of the different reactivities on crosslinking success has yet to be demonstrated. We further compared the effects of these mechanisms on the ability of the crosslinkers to capture the Gal4-Gal80 interaction when incorporated at position 856 within the Gal4 TAD. Additionally, there have been no reports of amino acid preference for the pAzpa crosslinker compared to "Methionine-magnet effect" seen with pBpa, making it a strong control for the alanine replacement experiments.

3.2.3 LexA+Gal4 856UAA alanine mutants show little impact on protein function

To examine the possibility of M855 and M861 acting as "methionine magnets" and artificially quenching pBpa at the Gal4-Gal80 interface, we made methionine to alanine point mutations at both positions using site-directed mutagenesis and examined the expression levels and transcriptional activity of each mutation. When pBpa was present

each mutant had expression levels similar to the wild-type protein. Additionally, each mutant was properly inhibited by Gal80 under normal glucose levels with only mild deficiencies seen in activated transcription of GAL1 under galactose conditions for the M861A and M855A/M861A constructs (Figure 3.4A and B). Similarly to pBpa, the expression and activity for each mutant when pAzpa was present were minimally perturbed (Figure 3.4C and D).
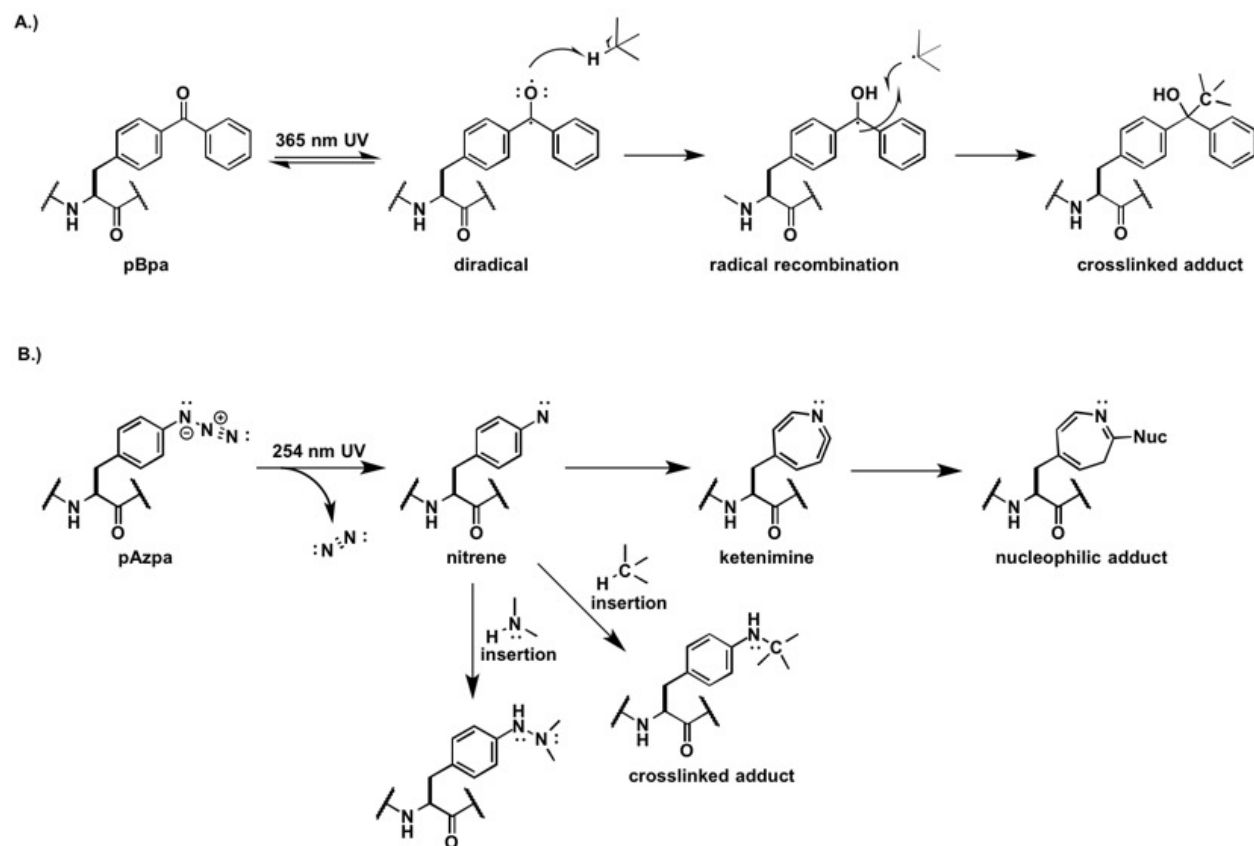
Figure 3.3 Photo-crosslinking reaction mechanisms of A.) *p*-benzoyl-L-phenylalanine (pBpa) and B.) *p*-azido-L-phenylalanine (pAzpa)

3.2.4 Site of incorporation and crosslinking mechanism are key to successful covalent capture

To determine if pBpa's reactivity preference for methionines was the cause of the loss of Gal4-Gal80 covalent capture, we first examined the effect of each alanine mutation on pBpa's crosslinking ability. In these experiments, live yeast expressing LexA+Gal4 856Bpa WT or one of the three alanine mutants and Myc-tagged Gal80 were grown in glucose and irradiated at 365 nm to capture the Gal4-Gal80 interaction. Upon lysis and western blot analysis probing for the Myc-tagged Gal80-Gal4 interaction,

the crosslinked product was only captured when one or both methionines were mutated to an alanine (Figure 3.5A).
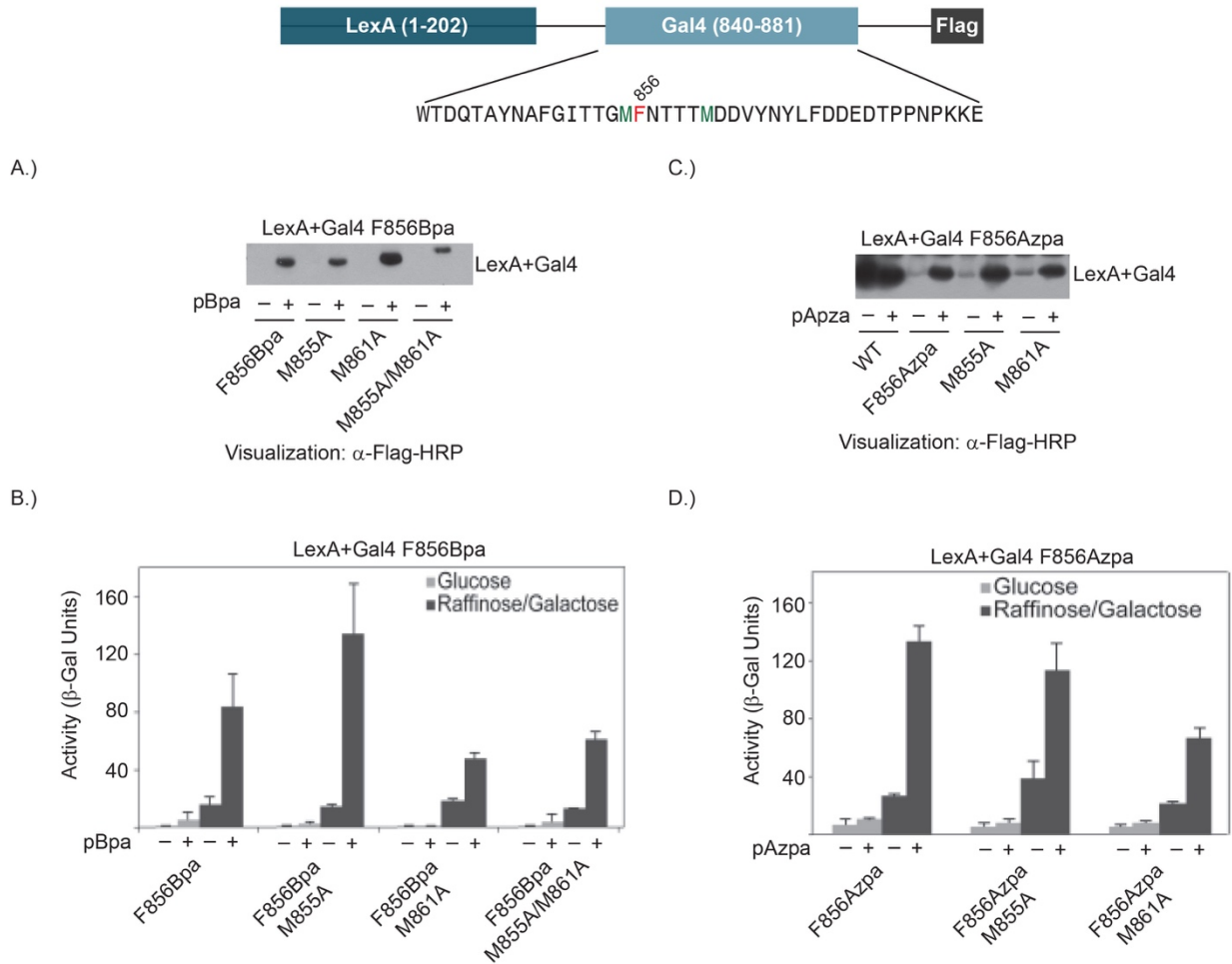
Figure 3.4 The effects of alanine mutants on the expression and activity of LexA+Gal4 F856Bpa and LexA+Gal4 F856Azpa. A.) Expression of LexA+Gal4 F856Bpa analyzed by western blot with α-Flag. B.) The activation potential of each mutant in LexA+Gal4 F856Bpa was measured by liquid β-galactosidase assay. In the yeast strain tested, β-galactosidase expression was controlled by a GAL1 promoter containing two LexA binding sites for LexA+Gal4 binding. C.) Expression of LexA+Gal4 F856Azpa analyzed by western blot with α-Flag. D.) β-galactosidase assessment of activation potential for each mutant in LexA+Gal4 F856Azpa. Adapted with permission from [61]. Copyright 2014 John Wiley and Sons.

As seen in the western blot, less quenching of pBpa was observed when position 855 is mutated to an alanine due to being located directly next to the incorporation site. This demonstrates that the intramolecular interaction between the methionine and pBpa out competes the intermolecular interaction between pBpa and the binding interface of

Gal80. Therefore, careful consideration of UAA incorporation site within a protein of interest is key to the successful application of the covalent chemical capture strategy.

Figure 3.5 The effects of alanine mutants on the crosslinking of LexA+Gal4 F856Bpa and LexA+Gal4 F856Azpa. A.) Crosslinking of LexA+Gal4 F856Bpa to Myc-tagged Gal80 as shown by western blot of cell lysates with α-cMyc antibody. B.) Crosslinking of LexA+Gal4 F856Azpa to Myc-tagged Gal80 as shown by western blot of cell lysates with α-cMyc antibody.

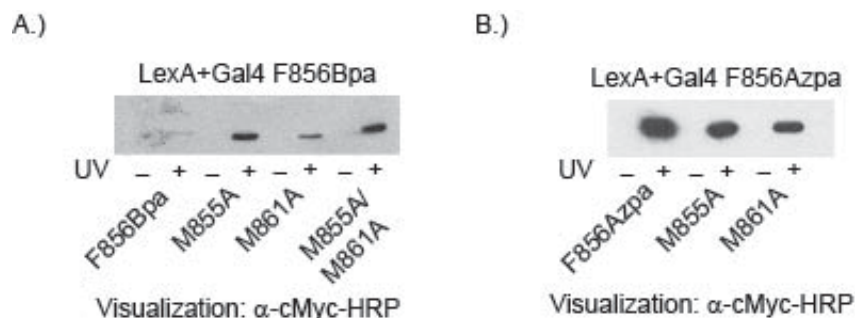To compliment the experiments above and further explain why a negative crosslinking result was seen when pBpa was incorporated at position 856, we used the pAzpa crosslinker, which has a different crosslinking mechanism from pBpa, to capture the Gal4-Gal80 interaction. Using identical conditions, live yeast expressing LexA+Gal4 856pAzpa WT or one of the three alanine mutants and Myc-tagged Gal80 were grown in glucose and irradiated. Upon lysis and western blot analysis, each one of the constructs captured the Myc-tagged Gal80-Gal4 interaction without incident (Figure 3.5B). As expected, introduction of an alanine at positions 855 and 861 yielded no changes in pAzpa crosslinking, consistent with the reactivity profile of this amino acid. These data illustrate that differences in crosslinking mechanism also plays a critical role in the outcome of covalent chemical capture experiments. In other words, relevant binding partners could be missed in an unbiased study due to the presence of methionines in the UAA-containing protein or absence of methionines in potential binding partners. Therefore, it is critical to carry out crosslinking experiment with more than one UAA mutant and more than one UAA to avoid false positives and/or negatives, such as the one discussed above. As illustrated here, a small change in position and crosslinker can have a dramatic effect on successful covalent capture.

3.3 Photo-activatable unnatural amino acids with enhanced reactivity

While sequence context of the PPI interface is important for the success of a crosslinking experiment, the success of covalent chemical capture of an *in vivo* PPI also depends on the innate reactivity of the crosslinker. For example, pBpa reacts through a di-radical mechanism that allows the excited molecule to relax to the ground state and be reactivated if no protein is captured, but this slow reactivity can limit the amount of transient interactions pBpa can capture during a set period of time. Additionally, while pAzpa has a faster crosslinking reactivity than pBpa, once the molecule is activated if no protein is captured, the nitrene will convert to the more stable ketenimine, reducing its crosslinking reactivity to nucleophiles and the surrounding solvent, ultimately increasing nonspecific crosslinking reactions. [32, 53] Our lab has previously used pBpa to characterize the low affinity interaction between the Mediator subunit, Med15, and the transcriptional activator, Gcn4, *in vitro*.[62-63] However, when this study was transitioned to cells this interaction could not be observed under any conditions examined, pointing to a critical limitation of pBpa's reactivity. Therefore, we sought to improve the reaction rates of the benzophenone scaffold. Upon irradiation, the unbound n electrons belonging to the carbonyl oxygen are excited to the π^* orbital, leading to the n-π^* transition. Several studies have looked at the effects substituents had on the reactivity of the benzophenone scaffold.[42, 64-65] While electron withdrawing groups (EWGs) appended around the benzophenone rings increases the energy of the occupied n orbital (HOMO) and decreases the energy of the unoccupied π^* optimal (LUMO) of the carbonyl oxygen, decreasing the transition gap for the oxygen electrons and increasing the reactivity for H-abstraction, electron donating groups (EDGs) do the opposite.[66] Accordingly, we hypothesized that a suite of mono-substituted pBpa analogs containing electron withdrawing substituents (F, Cl, Br, and CF_3) at positions around the benzophenone scaffold would increase the efficiency of H-abstraction, leading to increased reactivity and crosslinking yields. This increase in reactivity would be advantageous in studying the transient PPIs that might be missed by the parent pBpa molecule during the allotted experimental time, such as the Gcn4-Med15 interaction discussed above. Dr. Meghan Breen, a post-doctoral fellow in the Mapp Lab, has synthesized seven mono-substituted pBpa analogs that contain EWGs (Cl, F, Br, and CF_3) at either the meta or para

positions of the benzophenone ring through an air-tolerant carbonylative Suzuki-Miyaura coupling with mono-substituted boronic acids and 4-iodo-L-phenylalanine (Figure 3.6).[67]
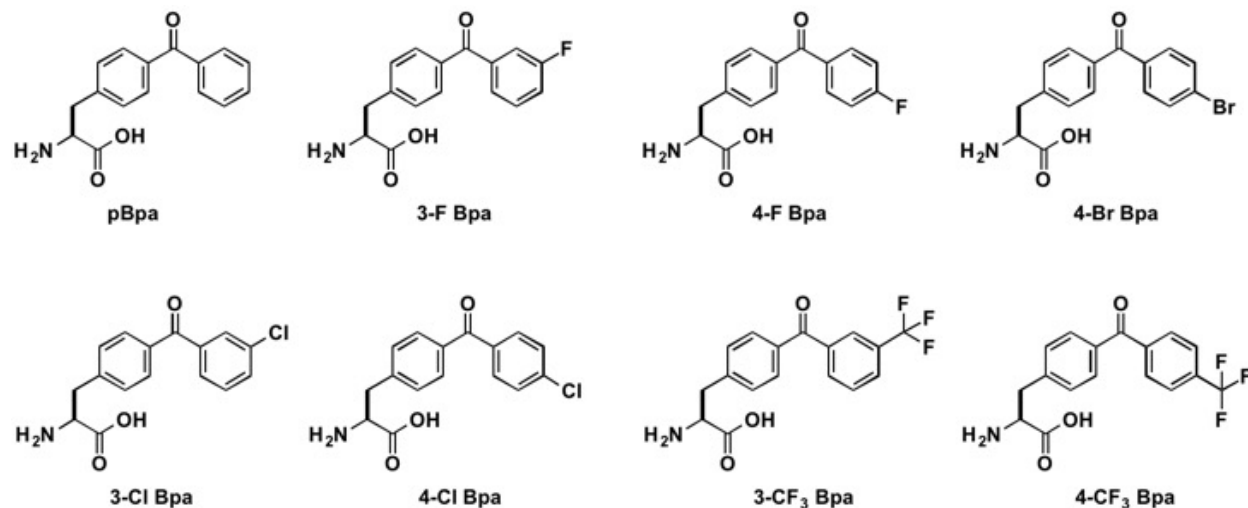
Figure 3.6 Suite of electron withdrawing group (EWG)-containing pBpa analogs

3.3.1 Incorporation of pBpa analogs into live yeast using amber nonsense suppression

With the synthesized analogs in hand, we sought to incorporate them into the model Gal4 activator using amber nonsense suppression and test their crosslinking activity. Recently, it has been shown that bioorthogonal tRNA synthetases developed for specific UAAs can incorporate analogs of the cognate UAA without any further mutagenesis to the active site.[68-72] Using the *Escherichia coli* tyrosyl tRNA/tRNA synthetase system (tRNA$^{Tyr}_{CUA}$-TyrRS), I have identified conditions to test the incorporation of each of the analogs into the Gal4 TAD at position 849 in live yeast. This position has been well characterized for UAA incorporation and was used for further characterization of these pBpa analogs. As shown in Figure 3.7, five of the seven analogs were incorporated into LexA+Gal4 at position 849 without any alterations to the synthetase (3-F Bpa, 4-F Bpa, 4-Cl Bpa, 4-CF$_3$ Bpa, and 4-Br Bpa).

After examination of the crystal structure of the *E. coli* tyrosyl tRNA synthetase, larger EWG groups, such as Cl and CF$_3$, at the meta position would interfere with the backbone of the active site residues and not allow proper docking of the benzophenone core within the synthetase active site. Thus, abolishing synthetase charging of the pBpa

specific tRNA and ultimately inhibiting incorporation of these analogs into the Gal4 TAD by the bioorthogonal tRNA.[73] This observation explains why the levels of 3-Cl Bpa incorporation is approximately 50% of pBpa's and why the 3-CF$_3$ Bpa analog completely failed to be incorporated. Due to the failure of the synthetase to incorporate the 3-CF$_3$ Bpa analog, we did not synthesize the 3-Br Bpa analog, which has a similar van der Waals radius. While the crosslinking reactivity of the seven pBpa analogs will be characterized *in vitro*, only the five that were successfully incorporated into the Gal4 TAD will be further characterized *in vivo* for their ability to increase crosslinking yields of Gal4's endogenous binding partners.
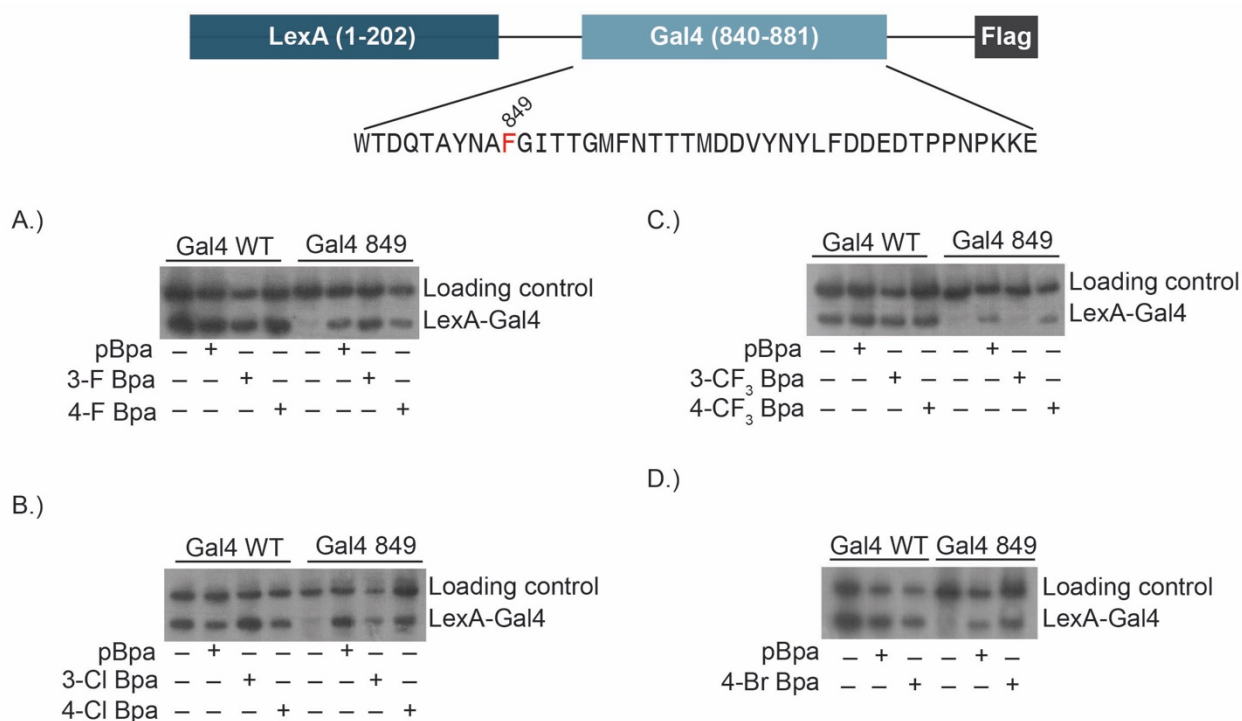
Figure 3.7 *In vivo* incorporation of EWG-containing pBpa analogs into LexA+Gal4 849TAG using the pBpa specific *E. coli* tyrosyl tRNA/tRNA synthetase pair. A.) Incorporation of 3-F and 4-F Bpa into LexA+Gal4 849TAG in the presence or absence of 1 mM UAA. B.) Incorporation of 3-Cl and 4-Cl Bpa into LexA+Gal4 849TAG in the presence or absence of 1 mM UAA. C.) Incorporation of 3-CF$_3$ and 4-CF$_3$ Bpa into LexA+Gal4 849TAG in the presence or absence of 1 mM UAA. D.) Incorporation of 4-Br Bpa into LexA+Gal4 849TAG in the presence or absence of 1 mM UAA.

3.3.2 VP16-Med25 model system

First, we examined each analogs ability to increase crosslinking yields in an *in vitro* setting. For these experiments, the model interaction between the well characterized VP16 activator and the activator interacting domain (AcID, residues 394-543) of the

Med25 coactivator subunit was used. The VP16 transcriptional activator is responsible for the activation of early viral genes during infection, however, its amphipathic TAD (residues 413-490) has been extensively used as a model to examine transcriptional activation.[74-85] VP16 participates in several PPIs between coactivator complexes, such as the Mediator complex, through its TAD.[14, 86-97] The multi-subunit Mediator complex has been reported to be a critical bridge between transcriptional activators and the pre-initiation complex (PIC) during transcriptional initiation. [98-103] Through its Med25 AcID domain, the Mediator complex interacts with several transcriptional activators including VP16.[95, 104-105] Both the Cramer and Wagner groups reported NMR structures of this interaction between Med25 AcID and the VP16 N- and C-terminal TADs.[106-107]

3.3.3 EWGs increase crosslinking yield of the pBpa scaffold in vitro

It has been shown previously that a minimal peptide sequence of eight key residues (DFDLDMLG) within the amino terminal half of VP16 is sufficient for transcriptional activation and participation in interactions with transcriptional coactivator subunits, including Med25.[62, 108-110] Using this minimal TAD, N-terminally biotinylated VP16 peptides were made with position F442 mutated to either pBpa or one of the pBpa analogs in Figure 3.6. Their binding affinities to Med25 were measured using an enzyme-linked immunosorbent assay (ELISA) (Figure 3.8). Position F442 has been shown to be critical for the VP16-Med25 interaction and VP16 transcriptional activation. Thus, larger phenylalanine derivatives still allow binding. However, the bulk added to each derivative would protrude into the binding interface minimally weakening the interaction as seen in our binding assays with the EWG containing pBpa analogs.[94, 107]

Once the binding affinities of each biotinylated VP16 analogs to Med25 AcID were examined, a quantitative *in vitro* plate assay was developed to measure the crosslinking yields of each analog. For each experiment, the VP16 analogs were incubated on Med25 coated plates and irradiated with 365 nm UV light to capture the Med25-VP16 interaction. Following incubation with streptavidin-HRP and tetramethylbenzidine (TMB) substrate, the plates were read at 450 nm and the values of crosslinked and non-crosslinked samples were subtracted to give a crosslinking yield (Figure 3.9A).
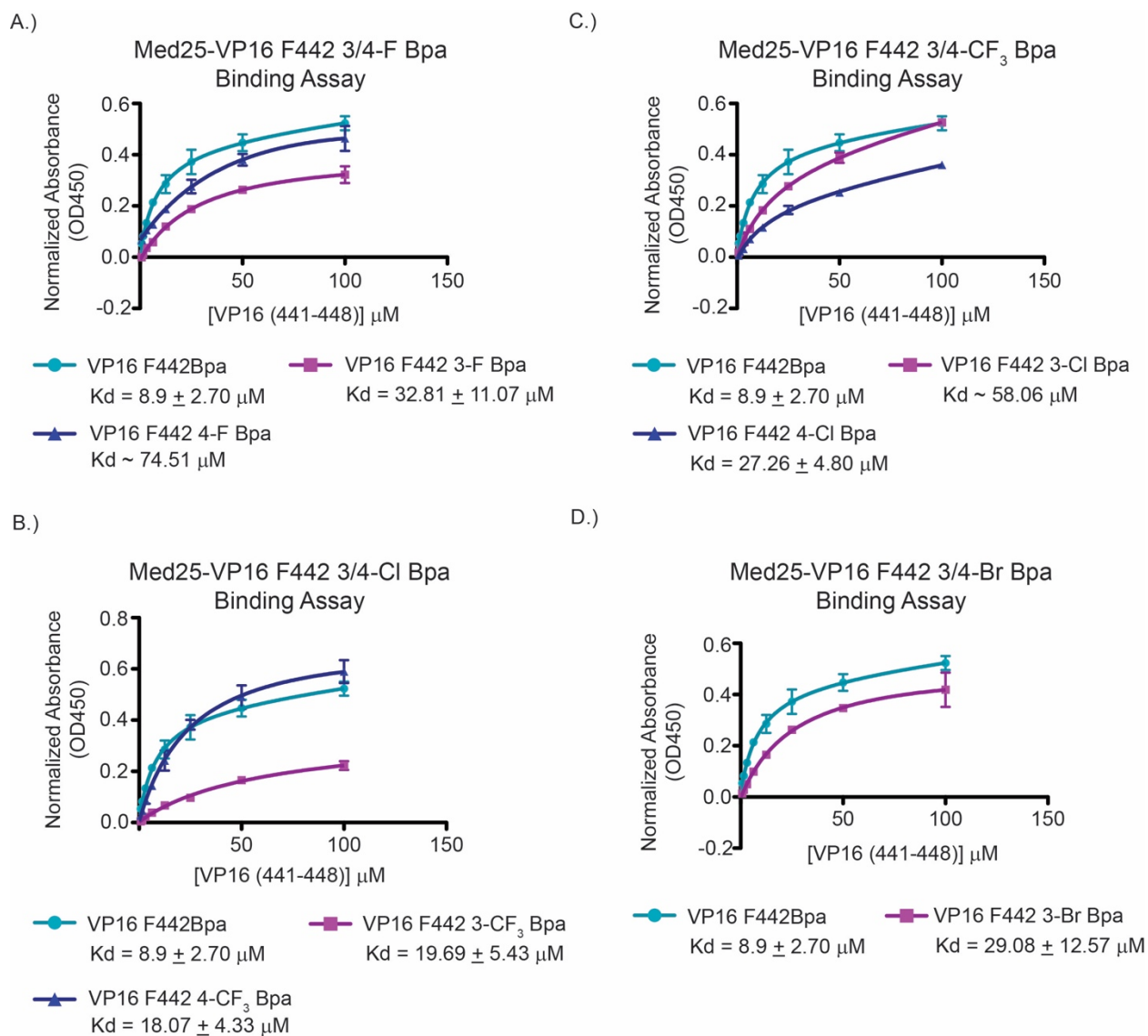
Figure 3.8 *In vitro* Med25-VP16 (441-448) F442UAA binding curves. Med25-coated polystyrene plates are incubated with biotinylated VP16 (441-448) with pBpa or one of the EWG-containing pBpa analogs at position F442 for 2 hours. The plates are incubated with streptavidin-HRP and reacted with TMB substrate to give a visible read-out that can be read at 450 nm absorbance. The binding constants were calculated using the following equation: $Y = \frac{Bmax \times X}{Kd + X} + NS \times X + Background$

As discussed above, EWGs decrease the excitation energy gap for the n-π* transition of the carbonyl oxygen's electrons, making the benzophenone core more reactive and ultimately increasing the crosslinking yield of the molecule. We hypothesized that this suite of EWG-containing pBpa analogs would increase the capture and crosslinking yield of the Med25-VP16 interaction. As seen in Figure 3.9B and C, there is approximately a three-fold increase in crosslinking yield of the Med25

AcID-VP16 interaction for each of the EWG-containing pBpa analogs compared to pBpa. These results support the predicted effect of appending EWGs onto the pBpa framework; through decreasing the energy needed for excitation of the oxygen radical, crosslinking efficacy increased for each analog versus the unaltered pBpa.
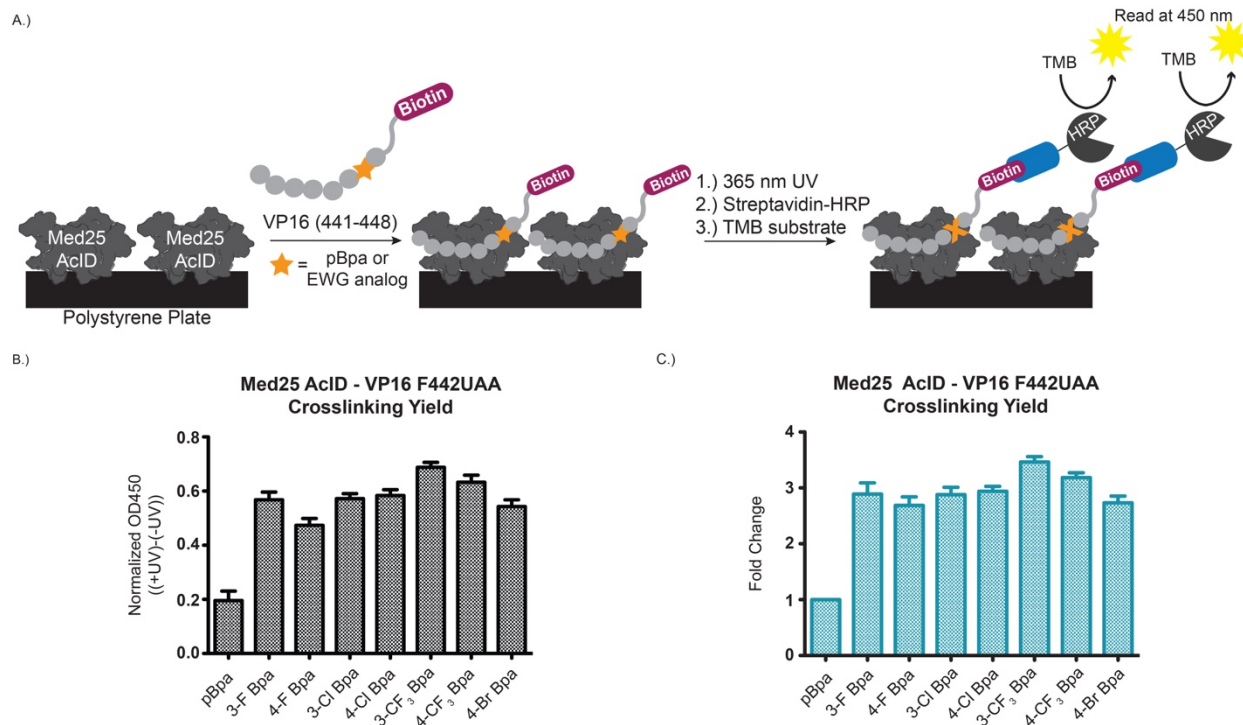
Figure 3.9 Med25-VP16 F442UAA *in vitro* crosslinking. A.) *In vitro* crosslinking experimental scheme. Med25-coated polystyrene plates are incubated with biotinylated VP16 (441-448) with pBpa or one of the EWG-containing pBpa analogs at position F442 for 30 minutes, following irradiation with 365 nm UV light for 10 minutes on ice to capture the Med25-VP16 interaction. The plates are incubated with streptavidin-HRP and reacted with TMB substrate to give a visible read-out that can be read at 450 nm absorbance. B.) Med25-VP16 F442UAA *in vitro* crosslinking yields. The crosslinking yield of each EWG-containing analog was calculated by subtracting the absorbance's from the irradiated (+UV) and non-irradiated (-UV) plates to yield a crosslinking yield. C.) Med25-VP16 F442UAA *in vitro* crosslinking yield fold changes compared to the parent pBpa molecule. All EWG-containing pBpa analogs have approximately 3 fold increase in crosslinking yield of the Med25-VP16 interaction.

3.3.4 EWG Bpa analogs capture the Gal4-Gal80 interaction in live yeast

While the *in vitro* crosslinking data showed a statistically significant increase for each analog using the simplified VP16-Med25 system, we sought to determine the effects of the EWG substituents on the crosslinking when incorporated into the Gal4 activator in live yeast cells. For these experiments, live yeast expressing LexA+Gal4 849TAG: Myc-Gal80 with either pBpa or one of the five EWG analogs (3-F Bpa, 4-F Bpa, 4-Cl Bpa, 4-CF$_3$ Bpa, and 4-Br Bpa) were grown in glucose and irradiated to capture all of

Gal4's binding partners. Upon lysis, the samples were immunoprecipitated for the LexA DNA binding domain to isolate all LexA+Gal4 protein interactions and analyzed by western blot. To fully characterize the crosslinking abilities of the UAAs, I developed a duplex western blotting methods such that both the binding profile of Gal4 and its direct interaction with Gal80 could be seen on the same western blot (Figure 3.10). Briefly, the membrane containing the crosslinked samples is simultaneously probed with a Flag-specific antibody for the LexA+Gal4-5x Flag specific interactions and a Myc-specific antibody for the Gal80-6x Myc specific interactions. Near-IR secondary antibodies are conjugated to their respective primary antibody for visualization and quantification of each analogs crosslinking yield.

As seen in Figure 3.10, the protein bands in red are free LexA+Gal4-5X Flag and its endogenous binding partners, while the protein bands in green are free Gal80-6X myc and its interaction with Gal4. In this duplex western blot, each of the five EWG-containing pBpa analogs capture Gal4's endogenous binding partners (red) in live yeast. While the expression levels of LexA+Gal4 are varied between the different analogs, each analog captured the Gal4-Gal80 interaction in yellow. The decrease in activator expression levels containing 3-F Bpa and 4-CF$_3$ Bpa could be due to low incorporation of the UAAs into the Gal4 TAD, although each UAA used in these experiments previously demonstrated similar expression levels to pBpa in Figure 3.7. While no conclusions can be made without further optimization, this preliminary blot shows that the 4-F Bpa analog has a visible increase in crosslinking yields compared to the parent pBpa molecule for both the Gal4-Gal80 interaction and the other Gal4 crosslinked adducts. These initial findings support our guiding hypothesis that adding electron-withdrawing substituents would increase the reactivity of the benzophenone scaffold, subsequently increasing the crosslinking yields. While this experiment requires further optimization to ensure that the variation in Gal4 expression levels is not due to incorporation issue and to further validate these analogs, the initial data shows the 4-F Bpa analog as a strong candidate for further analysis of weaker binding interactions that might have eluted the parent pBpa molecule, such as the Gcn5-Med15 interaction.
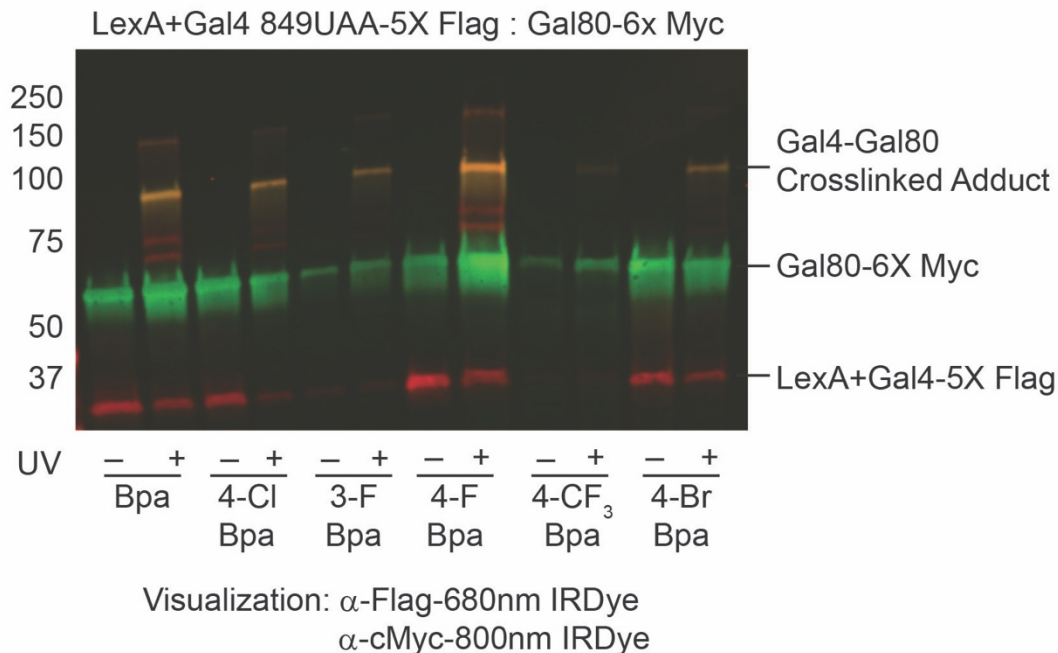
Figure 3.10 LexA+Gal4 849UAA *in vivo* crosslinking. Live yeast cells expressing UAA-containing LexA+Gal4-5X Flag and Gal80-6x Myc are irradiation, lysed and immunoprecipitated with α-LexA to isolate all LexA+Gal4 binding partners. The samples were run on a SDS-PAGE gel and analyzed by western blot simultaneously probing with a Flag-specific antibody for the LexA+Gal4-5x Flag interactions and a Myc-specific antibody for the Gal80-6x Myc interactions. The 680nm (red) and 800nm (green) near-IR fluorescent secondary antibodies were conjugated to the Flag- and Myc-specific antibodies for visualization, respectively. The membrane was visualized using the c600 Azure imager to give the duplex image. Each Bpa analog captured Gal4's endogenous partners (red), including the Gal4-Gal80 interaction (yellow).

3.4 Conclusions and Future Directions

In vivo covalent chemical capture using the photo-activatable UAA pBpa is a powerful technique for capturing well characterized transient *in vivo* PPIs. However, the present and future applications of *in vivo* covalent chemical capture are to discover previously unknown PPIs. The results in the first half of this chapter illustrate that not only is optimization of UAA incorporation a key factor in successful application of the covalent chemical capture strategy, but careful consideration of the innate reactivity of the chosen UAA utilize is also critical. While the longer lifetime and lower reactivity toward solvent makes pBpa an attractive choice for the study of PPIs that occur through shallow, exposed binding sites, seen between activators and coactivators, the marked preference of pBpa for methionines raises some concerns that crosslinking results could be influenced by the presence of methionine in the UAA-containing protein, as shown in the data above, or by a lack of methionines in potential binding partners.[111] This

methionine magnet effect could cause relevant binding partners to be missed in an unbiased study due to either of these factors. To avoid false negatives, it is critical to carry out crosslinking with more than a single UAA mutant and/or with more than one UAA, as seen is the data in the first half of this chapter. In the case of Gal4-Gal80, a small change in position and crosslinking mechanism can have a dramatic effect on crosslinking.

Although pBpa has a marked preference for methionines, its stability and minimal reactivity to solvent makes it a strong tool for the covalent capture of transient *in vivo* PPIs. However, its crosslinking reactivity is rather slow compared to the aryl azides and diazarines used in the field. To overcome this limitation, it has been hypothesized that substituting EWGs around the benzophenone scaffold would increase the reactivity of the oxygen radical of the activated molecule and increase crosslinking yields. In the second half of this chapter, we developed a suite of EWG-containing pBpa analogs that support the predicted effect. Through decreasing the energy needed for excitation of the oxygen radical, crosslinking efficacy increased approximately three-fold for each analog versus the unaltered pBpa when capturing the *in vitro* interaction between VP16 and Med25. Additionally, I have identified conditions for the incorporation of all seven of the Bpa analogs into the yeast Gal4 TAD using amber nonsense suppression. Upon incorporation, I found that five out of seven analogs were incorporated into the Gal4 TAD without requiring alterations to the tRNA synthetase, demonstrating the first reported incorporation of 3-F Bpa, 4-Cl Bpa, 4-CF$_3$ Bpa, and 4-Br Bpa into live cells. Furthermore, upon irradiation of live yeast cells expressing UAA-containing LexA+Gal4, each of the five incorporated analogs captured Gal4's endogenous binding partners, including its masking protein Gal80, with 4-F Bpa illustrating a marked increase in crosslinking yield of the Gal4 crosslinked adducts. These finding further support the predicted effect of appending EWGs onto the pBpa framework and expands our toolbox of chemical probes for capturing challenging PPIs in their native cellular environment. Furthermore, utilizing the pBpa analogs developed and applying the considerations presented in this chapter will facilitate the successful implementation of *in vivo* covalent capture for studying PPIs involved in a variety of biological processes.

3.5 Materials and Methods

LS41 [JPY9::pZZ41, Matα his3Δ200 leu2Δ1 trp1Δ63 ura3-52 lys2Δ385 gal4 URA::pZZ41] yeast was used for all *in vivo* experiments. pBpa was purchased from Chem-Impex International (Wood Dale, IL). pAzpa was purchased from Bachem and Chem-Impex International (Torrance, CA). The EWG pBpa analogs were synthesized by Dr. Meghan E. Breen through an air-tolerant carbonylative Suzuki-Miyaura coupling.[67] All plasmids described below were constructed using standard molecular biology techniques and the sequences of all isolated plasmids were validated by sequencing at the University of Michigan Core Facility (Ann Arbor, MI).

Table 3.1 Plasmids used in Chapter 3

Plasmid Name	Function
pLexA+Gal4 F856TAG-1X Flag	Expresses LexA(1-202)+Gal4(840-881)+1X Flag tag with a TAG codon replacing the codon of the existing amino acid
pLexA+Gal4 F856TAG M855A-1X Flag	Expresses LexA(1-202)+Gal4(840-881)+1X Flag tag with a TAG codon replacing the codon of the existing amino acid and a methionine to alanine mutation at position 855
pLexA+Gal4 F856TAG M861A-1X Flag	Expresses LexA(1-202)+Gal4(840-881)+1X Flag tag with a TAG codon replacing the codon of the existing amino acid and a methionine to alanine mutation at position 861
pLexA+Gal4 F856TAG M855A M861A-1X Flag	Expresses LexA(1-202)+Gal4(840-881)+1X Flag tag with a TAG codon replacing the codon of the existing amino acid and a methionine to alanine mutation at position 855 and 861
pLexA+Gal4WT-5X Flag	Expresses LexA(1-202)+Gal4(840-881)+5X Flag tag
pLexA+Gal4 F849TAG-5X Flag	Expresses LexA(1-202)+Gal4(840-881)+5X Flag tag with a TAG codon replacing the codon of the existing amino acid
pSNRtRNA-pBpaRS	Expressed tRNA under the control of the SNR52 promoter and contains synthetase specific for pBpa
pSNRtRNA-pAzpaRS	Expressed tRNA under the control of the SNR52 promoter and contains synthetase specific for pAzpa

p1XMyc Gal80	Expresses full-length 1X c-Myc tag+Gal80
pGal80-6X myc	Expresses full-length Gal80+6X c-Myc tag
pMed25 AcID-6X Myc	Expresses Med25(394-543)+6X His tag

Table 3.2 Primers used in Chapter 3

Primer ID	Sequence	Description
P1	5' GTTTGGAATCACTACAGGGGCTTAGAATACC ACTACAATGG 3'	SDM pLexA+Gal4 856TAG-1X Flag M855A For
P2	5' CCATTGTAGTGGTATTCTAAGCCCCTGTAGTG ATTCCAAAC 3'	SDM pLexA+Gal4 856TAG-1X Flag M855A Rev
P3	5' GGGATGTAGAATACCACTACAGCTGATGATG TATATAACTATC 3'	SDM pLexA+Gal4 856TAG-1X Flag M861A For
P4	5' GATAGTTATATACATCATCAGCTGTAGTGGTA TTCTACATCCC 3'	SDM pLexA+Gal4 856TAG-1X Flag M861A Rev
P5	5' CGTTGGAATCACTACAGGGCTTAGAATACCA CTACAGCTCATCATCTATATAACTATC 3'	SDM pLexA+Gal4 856TAG-1X Flag M855A M861A For
P6	5' GATAGTTATATACATCATCAGCTGTAGTGGTA TTCTAAGCCCCTGTAGTGATTCAAACG 3'	SDM pLexA+Gal4 856TAG-1X Flag M855A M861A Rev
P7	5' GGACACCTCGAGATGGACTACAACAAGAGAT CTTC 3'	Xho1-Gal80 For
P8	5' CCGAGCCATATGTAAACTATAATGCGAGATAT TGC 3'	Nde1-Gal80 Rev

Table 3.3 Peptides used in Chapter 3

Name	Amino acid sequence
VP16 F442Bpa	Biotin-miniPEG-NH-D**Bpa**DLDMLG-CONH$_2$
VP16 F442 3-Cl-Bpa	Biotin-miniPEG-NH-D**3-Cl-Bpa**DLDMLG-CONH$_2$
VP16 F442 4-Cl-Bpa	Biotin-miniPEG-NH-D**4-Cl-Bpa**DLDMLG-CONH$_2$
VP16 F442 3-F-Bpa	Biotin-miniPEG-NH-D**3-F-Bpa**DLDMLG-CONH$_2$
VP16 F442 4-F-Bpa	Biotin-miniPEG-NH-D**4-F-Bpa**DLDMLG-CONH$_2$
VP16 F442 3-CF$_3$-Bpa	Biotin-miniPEG-NH-D**3-CF$_3$-Bpa**DLDMLG-CONH$_2$
VP16 F442 4-CF$_3$-Bpa	Biotin-miniPEG-NH-D**4-CF$_3$-Bpa**DLDMLG-CONH$_2$
VP16 F442 4-Br-Bpa	Biotin-miniPEG-NH-D**4-Br-Bpa**DLDMLG-CONH$_2$

Construction of plasmids:

1. <u>pLexA+Gal4 856TAG M855A-1X Flag, pLexa+Gal4 856TAG M861A-1X Flag, and pLexA+Gal4 856TAG M855A M861A-1X Flag</u>

   Plasmids containing various alanine mutations within the Gal4 TAD were derived from the pLexA+Gal4 856TAG-1X flag parent plasmid. Using site directed mutagenesis, single point mutations were made by replacing an existing amino acid in the Gal4 TAD with the GCT codon. PCR primers were designed to have ~15-20 base pairs of homology on either side of the GCT mutation. Each set of primers were used to amplify the Gal4 alanine mutations using pLexA+Gal4 856TAG-1X Flag as the template. The amplified PCR product was digested with Dpn1 restriction enzyme to produce the new mutated plasmid.

2. <u>pGal80-6x Myc</u>

   A high copy plasmid expressing full length Gal80-6x Myc under the control of the ADH1 promoter was created from pGADT7 containing Xho1 and Nde1 restriction sites. Primers P7 and P8 were used to amplify Gal80 using LS41 yeast genomic DNA as the template. The amplified PCR product was digested with Xho1 and Nde1 restrictions enzymes and inserted into Xho1 and Nde1 digested pGADT7 using T4 DNA ligase to create pGal80-6X Myc.

Incorporation of pBpa or pAzpa into LexA+Gal4 856TAG-1xFlag

LS41 yeast was transformed with pLexA+Gal4 856TAG-1X Flag or pLexA+Gal4WT-1X Flag and either pSNRtRNA-pBpaRS or pSNRtRNA-pAzpaRS plasmids. Individual colonies were grown in 5 mL SC media containing 2% raffinose, but lacking histidine, tryptophan, and uracil for selection. The cultures were incubated at 30 °C with 250 rpm agitation. Following incubation, these cultures were used to inoculate 5 mL cultures of SC media containing 2% raffinose and 2% galactose, with or without 1 mM pBpa / 1 mM pAzpa (dissolved in 1 M NaOH), and 1 M HCl. The cultures were incubated at 30 °C with agitation to an OD_{660} of 1.0. Three ODs were isolated, washed with sterile water, and stored at -20 °C. The samples were lysed in 10 μL 4X NuPAGE LDS Sample Loading Buffer (Invitrogen), 10 μL 1X lysis buffer (50 mM tris-acetate pH 7.9, 100 mM potassium acetate, 20% glycerol, 0.2% Tween-20, 2 mM β-mercaptoethanol, and 2 mM magnesium acetate), and 10 μL 1 M DTT by boiling at 95 °C for 10 min. The samples were run on a 3-8% tris-acetate SDS-PAGE gel and analyzed by western blot with the anti-Flag (M2) antibody (Sigma Aldrich).

β-Galactosidase assays

LS41 yeast were transformed with pLexA+Gal4 WT-Flag or one of the pLexA+Gal4 856TAG-1X Flag alanine mutant constructs and either pSNRtRNA-pBpaRS or pSNRtRNA-pAzpaRS plasmids. Individual colonies were grown in 5mL SC media containing 2% raffinose, but lacking histidine, tryptophan, and uracil for selection. The cultures were incubated at 30 °C with 250 rpm agitation. Following incubation, these cultures were used to inoculate 5 mL cultures of SC media containing either 2% glucose or 2% Raffinose and 2% galactose with or without 1 mM pBpa / 1 mM pAzpa (dissolved in 1 M NaOH), and 1 M HCl which were subsequently incubated at 30 °C with agitation to an OD660 of 1.0 and harvested. The activity of each construct was assessed using β-Galactosidase assay as previously described.[29]

In vivo crosslinking analysis of LexA+Gal4 856Bpa/Azpa-1X Flag:1x myc-Gal80 alanine mutations

For *in vivo* photo-crosslinking, a colony of either LexA+Gal4 856 TAG-1X Flag:1x Myc Gal80, LexA+Gal4 856 TAG M855A-1X Flag:1x Myc Gal80, LexA+Gal4 856 TAG M861A-1X Flag:1x Myc Gal80, or LexA+Gal4 856 TAG M855A M861A-1X Flag:1x Myc Gal80was grown in 5 mL SC media containing 2% glucose, but lacking histidine, tryptophan, leucine and uracil for selection. The cultures were incubated at 30 °C with 250 rpm agitation. Following incubation, these cultures were used to inoculate 100 mL cultures of SC media containing 2% glucose, with 1 mM pBpa or 1 mM pAzpa analog (dissolved in 1 M NaOH), and 1 M HCl. The cultures were incubated at 30 °C with agitation to an OD_{660} of 1.0. For each culture, the cells were isolated by centrifugation and washed with the SC media lacking histidine, tryptophan, and uracil. The cell pellets were resuspended in 2 mL SC media containing 2% glucose and then transferred to small culture dishes and subjected to UV irradiated at 365nm light (Eurosolar 15W UV lamp) with cooling for 30 minutes. The cells were isolated by centrifugation and stored at -20 °C until lysis. The control samples were washed with 1 mL SC media containing 2% glucose, isolated by centrifugation, and stored at -20 °C until lysis.

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 immunoprecipitated with anti-LexA antibody (sc-1725, Santa Cruz Biotechnologies) for 2 hours at 4 °C. The proteins bound to the antibody were isolated by incubation for 1 hour with 40 μL pre-washed Dynabeads® protein G magnetic beads (ThermoFisher) at 4 °C. After incubation, the beads were washed six 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 at -20 °C until elution.

The samples were eluted from the beads by heating at 95 °C for 10 minutes in 10 μL NuPAGE 4X LDS Sample Loading Buffer (Invitrogen), 10 μL water, and 10 μL 1M DTT.

The samples were run on a 3-8% tris-Acetate SDS-PAGE gel and analyzed by western blot with the anti-Flag (M2) antibody (Sigma Aldrich).

Extinction coefficient calculation of EWG pBpa analogs

Each amino acid analog was dissolved in 1M NaOH to a final concentration of 100 μM. Serial dilutions were made and the absorbance was measured for each concentration on the NanoDrop 1000 Spectrophotometer (ThermoFisher) at 280 nm. Concentration vs. absorbance was plotted and the extinction coefficient was determined following Beer's Law and the following equation: Absorbance = εLc where L is 1 cm and the slope of the line is the extinction coefficient, ε.

Table 3.4 Extinction coefficients for each pBpa analog used in Chapter 3

pBpa analog	Extinction Coefficient	Wavelength
pBpa	12083 $M^{-1}cm^{-1}$	280 nm
3-Cl Bpa	10698 $M^{-1}cm^{-1}$	280 nm
4-Cl Bpa	17559 $M^{-1}cm^{-1}$	280 nm
3-F Bpa	12058 $M^{-1}cm^{-1}$	280 nm
4-F Bpa	13528 $M^{-1}cm^{-1}$	280 nm
3-CF_3 Bpa	12280 $M^{-1}cm^{-1}$	280 nm
4-CF_3 Bpa	14966 $M^{-1}cm^{-1}$	280 nm
4-Br Bpa	14569 $M^{-1}cm^{-1}$	280 nm

Peptide synthesis

All peptides were synthesized using RINK amide AM resin (Acros Organics) using standard HBTU/HOBT/DIEA solid-phase peptide synthesis protocols as previously described.[112] FMOC-Bpa was purchases from Chem-Impex International and the FMOC-Bpa EWG analogs were synthesized by Dr. Meghan Breen. The peptides were cleaved using 92.5% trifluoroacetic acid, 2.5% triisopropylsilane, 2.5% 1,2-ethanedithiol, and 2.5% water and subsequently precipitated with chilled diethyl ether. The products were purified to homogeneity using reverse-phase HPLC on a C18 column with a gradient solvent system (Buffer A: 20 mM ammonium acetate, Buffer B: acetonitrile) and

stored at -20 $^{\circ}$C. Concentrations for each peptide was determined using the extinction coefficients calculated above.

Med25 AcID-6xHis expression and purification

Expression of Med25 AcID-6xHis was carried out in BL21ai *E. coli* cells. 25 mL starter cultures from a single colony were grown overnight at 37 $^{\circ}$C (250 rpm) in LB broth supplemented with ampicillin (100 μg/mL) and streptomycin (100 μg/mL) before addition to 1 L of Terrific Broth (1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 4% (v/v) glycerol, and 1X phosphate buffer (17 mM KH_2PO_4, 72 mM K_2HPO_4)) supplemented with ampicillin (100 μg/mL) and streptomycin (100 μg/mL). After an OD_{600} of 0.7 was reached, the cultures were cooled to 22 $^{\circ}$C for 1 hour, and expression was induced with IPTG (final concentration 0.5 mM) and 0.02% arabinose overnight at 22 $^{\circ}$C. The cells were pelleted by centrifugation at 6000 rpm for 20 minutes and stored at -80 $^{\circ}$C until lysis.

The cell pellet was resuspended in lysis buffer (50 mM sodium phosphate pH 7.2, 300 mM NaCl, 10 mM imidazole, b-mercaptoethanol, and 2X Complete Mini EDTA Free Protease Inhibitor (Roche)) and lysed using sonication. The His-tagged protein was isolated using a Ni-NTA 5 mL HiTrap™ HP column (GE Life Sciences) with a gradient solvent system (Buffer A: 50 mM sodium phosphate, 300 mM NaCl, 30 mM imidazole, pH 7.2, Buffer B: 50 mM sodium phosphate, 300 mM NaCl, 400 mM imidazole, pH 7.2). The fractions were pooled and the Med25 protein was isolated through cation-exchange chromatography using a 5 mL HiTrap SP HP (GE Life Sciences) column with a gradient solvent system (Buffer A: 50 mM sodium phosphate, pH 7.2, Buffer B: 50 mM sodium phosphate, 1 M NaCl, pH 7.2).

The resulting fractions' identity were verified by SDS-PAGE with appropriate molecular weight standards. Fractions containing Med25 AcID-6xHis were combined and placed in dialysis tubing (Pierce) and buffer exchanged to storage buffer (10 mM sodium phosphate pH 6.8, 100 mM NaCl, 10% glycerol, and 1 mM DTT) overnight at 4 $^{\circ}$C. The purified protein was concentrated using a 3K centrifugal filter device (Millapore) and the

concentration was measured using absorbance at 280 nm. The molecular weight was verified by QTOF-MS.

In vitro binding assay

0.4 mg/mL of purified Med25 AcID-6xHis diluted in 50 mM MES buffer, pH 5.2 was bound to a clear, flat bottom, polystyrene, 96-well plate (Fisherbrand) overnight at 37 $^{\circ}$C. The plate was washed three times with PBS-T (10 mM $Na_2HPO_4 \cdot 7H_2O$, 1.7 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, 0.05% Tween-20, pH 7.4) for 5 minutes each. Varying concentrations of the corresponding VP16 peptide diluted in binding buffer (25 mM HEPES, 40 mM KCl, 8 mM $MgCl_2$, 100 mM NaCl, 0.01% Tween-20, pH 7.4) was incubated on Med25-coated plate while rocking for 2 hours at room temperature. The plate was washed three times with PBS-T for 5 minutes each, followed by blocking for 5 minutes with SuperBlock™ (PBS) Blocking Buffer (ThermoFisher). The plate was incubated with 1:2,000 streptavidin-HRP (Abcam, ab7403) in SuperBlock™ (PBS) Blocking Buffer for 1 hour at room temperature on benchtop. The plate was washed three times with PBS-T for 5 minutes each, followed by incubation with TMB substrate (Cell Signaling) for 1 minute until solutions turned blue. The reaction was quenched with 1 M HCl and the plate was read at OD_{450} on the Spectramax M5 microplate reader (Molecular Devices).The binding constant (Kd) was calculated using the following equation: $Y = \frac{Bmax \times X}{Kd + X} + NS \times X + Background$

Bmax - the maximum specific binding in the same units as Y

NS - the slope of nonspecific binding in Y units divded by X in units

Background – the amount of nonspecific binding with no added ligand

In vitro crosslinking assay

0.4 mg/mL of purified Med25 AcID-6xHis diluted in 50 mM MES buffer, pH 5.2 was bound to two clear, flat bottom, polystyrene, 96-well plates (Fisherbrand) overnight at 37 $^{\circ}$C. Both plates were washed three times with PBS-T (10 mM $Na_2HPO_4 \cdot 7H_2O$, 1.7 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, 0.05% Tween-20, pH 7.4) for 5 minutes each. 12.5 μM of the corresponding VP16 peptide diluted in binding buffer (25 mM HEPES, 40 mM KCl, 8 mM $MgCl_2$, 100 mM NaCl, 0.01% Tween-20, pH 7.4) was incubated on the

Med25-coated plates while rocking for 30 minutes at room temperature. One plate (+UV plate) was crosslinked at 365 nm (Eurosolar 15W UV lamp) with cooling for 10 minutes and washed three times with PBS-T for 5 minutes each. The (-UV) plate was also washed three times with PBS-T for 5 minutes each. The plates were blocked for 5 minutes with SuperBlock™ (PBS) Blocking Buffer (ThermoFisher), followed by incubation with 1:2,000 streptavidin-HRP (Abcam, ab7403) in SuperBlock™ (PBS) Blocking Buffer for 1 hour at room temperature on benchtop. The plates were washed three times with PBS-T for 5 minutes each, followed by incubation with TMB substrate (Cell Signaling) for 1 minute until solutions turned blue. The reaction was quenched with 1 M HCl and the plates were read at OD_{450} on the Spectramax M5 microplate reader (Molecular Devices).

In vivo crosslinking analysis of LexA+Gal4 849Bpa/EWG Bpa analogs-5X Flag: Gal80-6x Myc

For *in vivo* photo-crosslinking, a colony of LexA+Gal4 849 TAG-5X Flag: Gal80-6x Myc was grown in 5 mL SC media containing 2% glucose, but lacking histidine, tryptophan, leucine, and uracil for selection. The cultures were incubated at 30 °C with 250 rpm agitation. Following incubation, these cultures were used to inoculate 100 mL cultures of SC media containing 2% glucose, with 1 mM pBpa or 1 mM EWG pBpa analog (dissolved in 1 M NaOH), and 1 M HCl. The cultures were incubated at 30 °C with agitation to an OD660 of 1.0. For each culture, the cells were isolated by centrifugation and washed with the SC media lacking histidine, tryptophan, leucine, and uracil. The cell pellets were resuspended in 2 mL SC media containing 2% glucose and then transferred to small culture dishes and subjected to UV irradiated at 365nm light (Eurosolar 15W UV lamp) with cooling for 30 minutes. The cells were isolated by centrifugation and stored at -20 °C until lysis. The control samples were washed with 1 mL SC media containing 2% glucose, isolated by centrifugation, and stored at -20 °C until lysis.

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 was immunoprecipitated with anti-LexA antibody (sc-1725, Santa Cruz Biotechnologies) for 2 hours at 4 °C. The proteins bound to the antibody were isolated by incubation for 1 hour with 40 µL pre-washed Dynabeads® protein G magnetic beads (ThermoFisher) at 4 °C. After incubation, the beads were washed six 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 at -20 °C until elution.

The samples were eluted from the beads by heating at 95 °C for 10 minutes in 10 µL NuPAGE 4X LDS Sample Loading Buffer (Invitrogen), 10 µL water, and 10 µL 1M DTT. The samples were run on a 4-15% Mini-PROTEAN® TGX™ pre-cast SDS-PAGE gel (Bio-Rad). The gel was transferred to immobilin PDVF membrane (Millapore) and blocked for 1 hour at room temperature using SuperBlock™ (PBS) Blocking Buffer (ThermoFisher). The membrane was incubated with both mouse anti-Flag (M2) (Sigma Aldrich, F1804) and rabbit anti-cMyc (Sigma Aldrich, C3956) primary antibodies for 1 hour at room temperature. The membrane was washed three times for 10 minutes each with PBS-T (10 mM $Na_2HPO_4 \cdot 7H_2O$, 1.7 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, 0.05% Tween-20, pH 7.4), followed by incubation with anti-mouse 680nm (LI-COR, 926-68072) and anti-rabbit 800nm (LI-COR, 926-32211) secondary antibodies for 1 hour at room temperature. The membrane was washed three times for 10 minutes each with PBS-T, 5 minutes with PBS (10 mM $Na_2HPO_4 \cdot 7H_2O$, 1.7 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, pH 7.4), and visualized using the Azure c600 western blot imager (Azure Biosystems). Crosslinking yields were quantified using ImageJ and the relative amount of Gal4-Gal80 crosslinked product for each experiment was expressed as follows ((Gal4 Bpa analog-Gal80 crosslinked product/Gal4 Bpa-Gal80 crosslinked product)*100).

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CHAPTER 4[1]

A bifunctional amino acids to address the challenges of isolating *in vivo* crosslinked products

4.1 Introduction

Transient and moderate affinity protein-protein interactions (PPIs) play crucial roles across cellular processes, but they are often intractable to characterize in their native environments.[1-6] In the case of transcriptional initiation, the complexes formed between transcriptional activators and coactivators are instrumental in the proper assembly of the transcriptional machinery, yet the necessarily transient interactions have frustrated efforts to identify specific protein pairings.[7-12] A breakthrough was realized with genetic code expansion via amber nonsense suppression, enabling the site specific incorporation of photo-activatable amino acids into protein partners in living cells for covalent capture experiments.[13-20] Over the last several years our lab has demonstrated that the photo-activatable unnatural amino acid (UAA) *p*-benzoyl-L-phenylalanine (pBpa) effectively captures even the modest affinity interactions between DNA-bound transcriptional activators and their binding partners *in vivo*, allowing the creation of a detailed map of the key PPIs that define transcriptional activation.[21-24] Nonetheless, these experiments are technically challenging and require several steps of isolation and purification post-crosslinking. This is typically accomplished through immunoprecipitation and/or affinity purification of the protein of interest or its binding

partners and can vary significantly in efficiency, especially for low abundance proteins. Further, immunoprecipitation requires effective antibodies and/or the genetic incorporation of epitope tags into the protein of interest.[11, 25-26] Therefore, there is a real need for additional methods for isolation of *in vivo* crosslinked proteins.
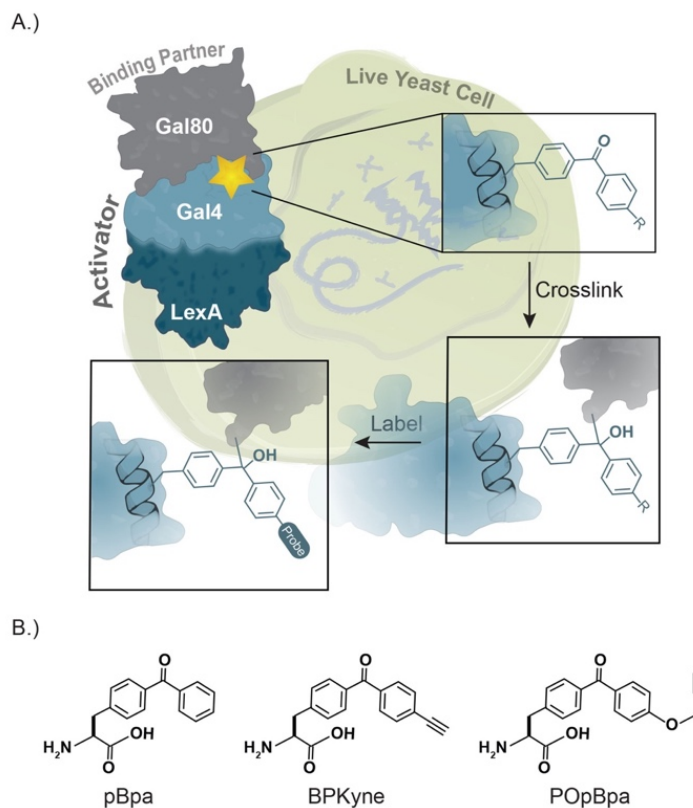
Figure 4.1 A.) Experimental scheme of bifunctional UAA crosslinking and bioconjugation by CuAAC. B.) Photo-labile UAAs. *p*-benzoyl-L-phenylalanine (pBpa), 4-ethynyl-*p*-benzoyl-L-phenylalanine (BPKyne), and 4-propargyloxy-*p*-benzoyl-L-phenylalanine (POpBpa)

To address this, we hypothesized that a bifunctional UAA containing both a photo-activatable group and a moiety that enables post-crosslinking derivatization would facilitate detection, isolation, and/or identification of PPIs in the context of the native cellular environment. To test this, we have developed two bifunctional pBpa derivatives, 4-ethynyl-*p*-benzoyl-L-phenylalanine (BPKyne) and 4-propargyloxy-*p*-benzoyl-L-phenylalanine (POpBpa) which both contain an alkynyl moiety that can be functionalized post-crosslinking using the copper-mediated azide-alkyne Huisgen cycloaddition (CuAAC) (Figure 4.1).[27-31] In this chapter, we sought to incorporate these bifunctional amino acids into proteins in live yeast using nonsense suppression, and

functionalization of the alkyne handle post-crosslinking would enable isolation and visualization of crosslinked products. Here we fully characterize both bifunctional amino acids for their ability to be incorporated into the prototypical yeast Gal4 transcriptional activator and capture the Gal4-Gal80 transcriptional complex discussed in Chapter 3.[21, 32-36] We further demonstrate that alkyne functionalization enables isolation and purification of the *in vivo* crosslinked products from live yeast cells.

## 4.2 Bifunctional UAAs for the isolation of *in vivo* crosslinked products

UAA incorporation has become a strong tool for the integration of new chemistries within proteins in the cell.[20, 37-41] For example, photo-labile UAAs, such as pBpa, allows C-C bond formation between binding partners in the presence of UV irradiation[13, 42-43], while azido-containing UAAs, such as *p*-azido-L-phenylalanine (pAzpa) can either be irradiated by UV light to form C-C bonds or can be coupled with a functional handle to label a protein of interest through the CuAAC or Staudinger ligation reactions.[44-49] However, current methods only allow for one UAA and ultimately one new functionality to be productively incorporated into a protein of interest at a time. There have been a few reports of simultaneous double incorporation of UAAs that allows for different chemical groups and labels to be added to the protein, however, these methods are not trivial and protein expression yields are extremely low.[50-54] Recently, bifunctional UAAs have been developed to allow for two novel functionalities to be integrated into a protein at one site.[18, 55] For example, Yamaguchi and coworkers developed a Z-lysine derivative that contained amino and azido groups that enabled the creation of different protein conjugates in live cells.[18] However, these current bifunctional amino acids contain a nonspecific amino group that can react with the native cellular environment and only one bioorthogonal group.

   Due to the limitation in the current methods, we sought to develop a bifunctional amino acid containing both a photo-reactive group for the capture of *in vivo* protein partners and a bioorthogonal reactive group that would enable derivatization with functional handles, such as biotin, for the isolation of the crosslinked products. This capture and tag strategy has been used in photo-affinity labeling (PAL) for several years. Multiple reports have utilized the benzophenone and alkynyl reactive groups in

small molecule activity-based probes for proteomic screening of drug targets.[56-61] Briefly, the small molecule binds to a protein of interest, and upon UV irradiation the benzophenone crosslinker covalently tethers the small molecule to the protein. The alkynyl group can subsequently be functionalized with a biotinylated azide probe using CuAAC to enable isolation of the small molecule-protein complex. We hypothesized that a bifunctional pBpa analog containing an alkynyl group would similarly enable the capture of PPIs when incorporated into a protein of interest and isolation of the complex following appendage of a biotin handle. To test this, we developed two bifunctional pBpa analogs, BPKyne and POpBpa. BPKyne which has originally been reported for use in synthetic peptides and *in vitro* crosslinking by the Ernst group has a terminal alkyne directly appended to the benzophenone ring, while POpBpa has a more reactive propargyl group that we hypothesized would be more effective during labeling of the crosslinked product.[28, 62] Both molecules were characterized based on their ability to be incorporated into the LexA+Gal4 protein using the *Escherichia coli* tyrosyl tRNA/synthetase pair (tRNA$^{Tyr}_{CUA}$-TyrRS) and its ability to capture Gal4's binding partners and isolate these crosslinked products from yeast cells.

4.2.1 BPKyne can be incorporated into LexA+Gal4 849TAG using amber nonsense suppression

Previously, our lab has used the well-established *E. coli* tyrosyl tRNA/synthetase pair (tRNA$^{Tyr}_{CUA}$-TyrRS) to incorporate pBpa into proteins in their native cellular environment using nonsense suppression.[21-24] As discussed in Chapter 3, it has recently been shown that the bioorthogonal tRNA synthetases can incorporate analogs of the cognate UAA without any further alterations in some cases.[15, 63-67] An examination of the crystal structure of the *E. coli* tyrosyl tRNA synthetase suggested that due to the small van der Waals radius of the alkynyl moiety, BPKyne should fit in the active site and be incorporated without any modifications to the synthetase, however, the propargyl group of POpBpa might be too large for the active site.[15] To test this, we compared the expression levels of the chimeric transcriptional activator LexA+Gal4 when pBpa, BPKyne, or POpBpa was incorporated at position 849 within the Gal4 transcriptional activation domain (TAD) (Figure 4.2A).
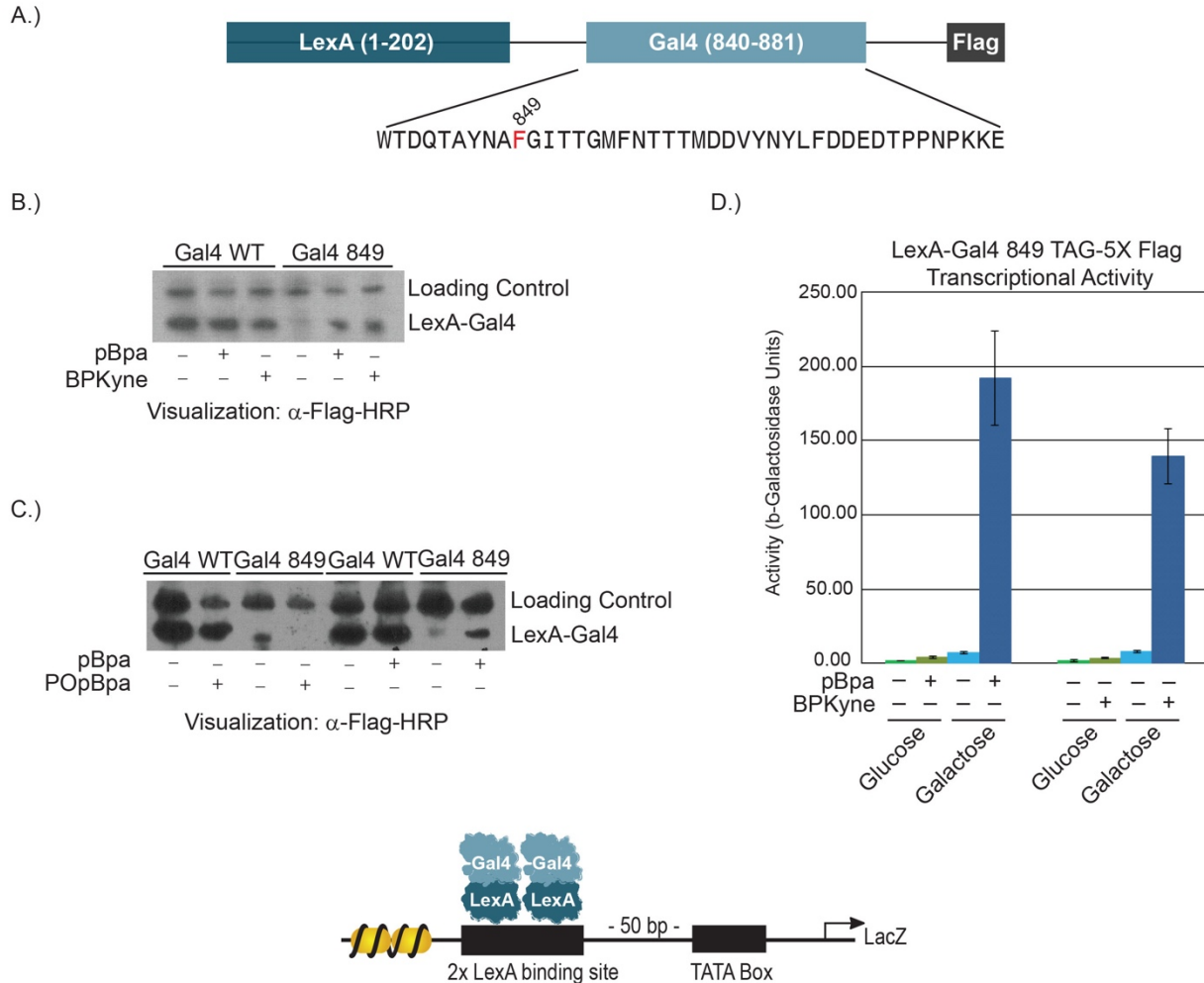
Figure 4.2 Analysis of bifunctional UAAs incorporation in the LexA+Gal4 849TAG-Flag protein. A.) A plasmid encoding the LexA DNA binding domain (DBD) fused to the Gal4 transcriptional activation domain (TAD) and a Flag tag was constructed. Position 849 (in red) was mutated to the amber stop codon for pBpa, BPKyne, and POpBpa incorporation. B.) BPKyne incorporation was compared to that of pBpa at position 849 of Gal4 by using *E. coli* tRNA$^{Tyr}_{CUA}$-TyrRS in the presence or absence of 1 mM pBpa or BPKyne. C.) POpBpa incorporation was compared to that of pBpa at position 849 of Gal4 by using *E. coli* tRNA$^{Tyr}_{CUA}$-TyrRS in the presence or absence of 1 mM pBpa or POpBpa. Expression levels of LexA+Gal4 849UAA mutants relative to LexA+Gal4 WT were quantified by using ImageJ.[68] D.) β-Galactosidase assays were performed using yeast cells expressing the LexA+Gal4 849TAG-5x Flag and pSNRtRNA-pBpaRS plasmids to assess the ability of LexA+Gal4 849Bpa and LexA+Gal4 849BPKyne to upregulate transcription of an integrated pGal1-LacZ reporter gene in *Saccharomyces cerevisiae*. Under identical conditions, there is no significant difference in activity between pBpa or BPKyne incorporated LexA+Gal4 protein when analyzed by student's t-test with p$\leq$0.05.

Using identical conditions, the expression level of BPKyne incorporated LexA+Gal4 was found to be within 10% of that observed with pBpa (Figure 4.2B). Additionally, incorporation of BPKyne did not alter LexA+Gal4-mediated transcriptional activation (Figure 4.2D). However, when POpBpa was introduced to the media, the tRNA/tRNARS

system was unable to incorporate the unnatural into the LexA+Gal4 protein resulting in no production of the full length protein (Figure 4.3C). These data illustrate the substrate flexibility of the tRNA/synthetase pair for small, rigid changes to pBpa seen in BPKyne, but not for more drastic, flexible changes seen in POpBpa. The rest of the chapter will focus on the *in vivo* characterization of BPKyne.

4.2.2 BPKyne captures the Gal4-Gal80 interaction in live yeast

*Next, w*e evaluated the effect of the alkynyl moiety on the photochemical reactivity of the benzophenone through *in vivo* photo-crosslinking experiments using the LexA+Gal4 transcriptional activator (Figure 4.3A). Live yeast expressing LexA+Gal4 with either pBpa or BPKyne incorporated at position 849 were irradiated with 365 nm light to capture Gal4's endogenous binding partners. Upon lysis and western blot analysis probing for the Flag-tagged LexA+Gal4 activator, several crosslinked products were captured by both photo-crosslinkers. Both molecules captured a distinct product around 80 kDa, consistent with the Gal4-Gal80 complex (Figure 4.3B).[21]

To confirm this, a 6x-Myc-tagged Gal80 construct was transformed into live yeast with the UAA-incorporated LexA+Gal4 fusion protein. After irradiation, lysis, and western blot analysis probing for the Myc-tagged Gal80 protein, both pBpa and BPKyne captured a Gal4-Gal80 crosslinked product (Figure 4.3C). When comparing the amount of crosslinked Gal4-Gal80, BPKyne's crosslinking efficiency is approximately 65% of the parent molecule, pBpa, at least in this context. This small decrease was expected due to the addition of an electron-donating moiety, which was predicted to slightly decrease the reactivity of the molecule.[69] However, despite this decrease BPKyne still captures the same biologically relevant interactions as pBpa (Figure 4.3B and C).
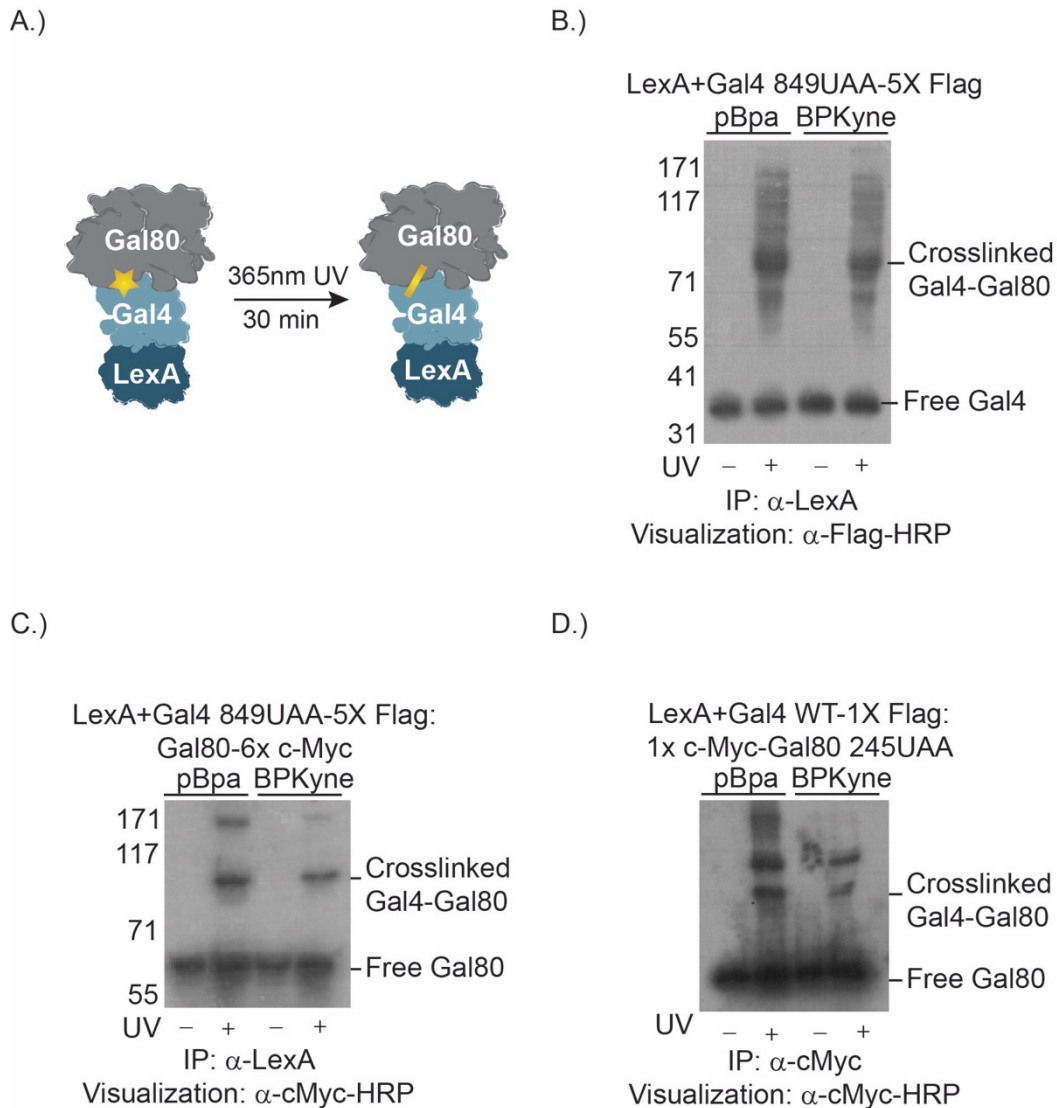
Figure 4.3 Analysis of BPKyne crosslinking in the LexA+Gal4 849TAG-Flag and Gal80 245TAG-Myc tagged proteins. A.) Experimental scheme of LexA+Gal4 849UAA – Gal80 crosslinking. Live yeast expression LexA+Gal4 with either pBpa or BPKyne incorporated at position 849 are irradiated at 365 nm live to capture Gal4's binding partners through a covalent bond. B.) BPKyne captures several of Gal4's endogenous protein partners including Gal80 at approximately 80 kDa. C.) BPKyne captures the Myc-Gal80 interaction with Gal4 to confirm the Gal80 crosslinked band at 80 kDa. D.) BPKyne captures the Gal80-Gal4 interaction when BPKyne is incorporated in Gal80 at position 245. The crosslinking yield of LexA+Gal4 849BPKyne-Gal80 relative to LexA+Gal4 849pBpa-Gal80 was quantified by using ImageJ.[68]

4.2.3 BPKyne incorporated proteins are directly functionalized post-crosslinking

Once incorporation and crosslinking were confirmed, the bioconjugation capability of BPKyne was characterized post-crosslinking and compared to traditional immunological methods for the isolation of crosslinked products. Towards this end, the UAA was incorporated into a Gal80 construct at position 245 mutated through amber nonsense

suppression. This site is located at the outer edge of the Gal4 binding interface. Thus, when this construct was irradiated only the Gal80-Gal4 complex was captured, allowing for a single interaction to be visualized (Figure 4.3D).[21] To demonstrate that the bioorthogonal alkyne handle of BPKyne could be functionalized post-crosslinking, live yeast expressing Flag-tagged LexA+Gal4 and Myc-tagged Gal80 with pBpa or BPKyne incorporated at position 245 were grown under glucose conditions and irradiated to capture the Gal4-Gal80 binding event. After lysis, biotin-PEG$_3$-azide was conjugated to the BPKyne incorporated Gal80 species via a Huisgen cycloaddition in whole cell lysate using copper (II) sulfate, tris-(3-hydroxypropyltriazoylmethyl)amine (THPTA), and sodium ascorbate at 37°C (Figure 4.4A).[27-28]
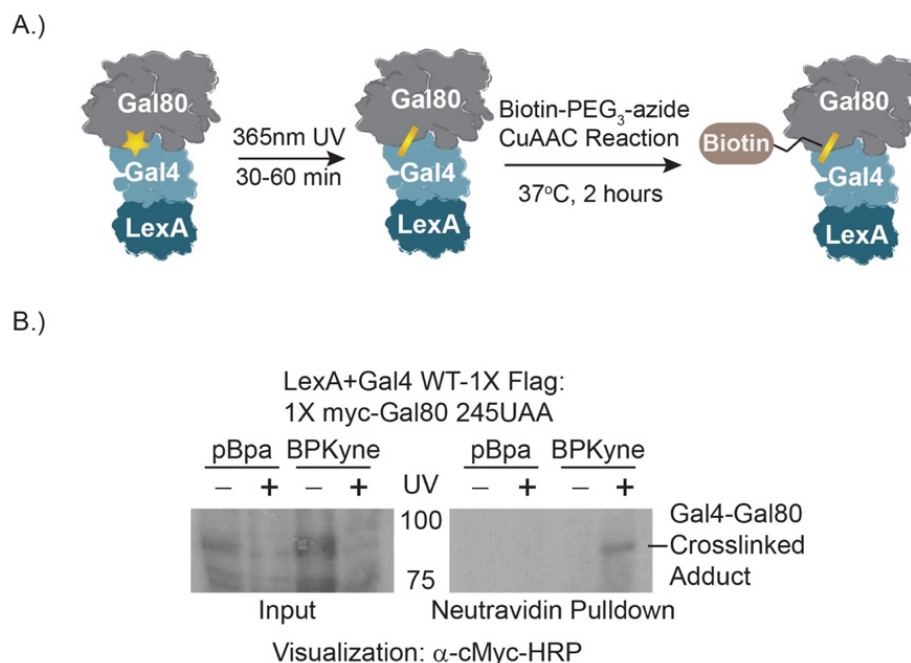
Figure 4.4 Analysis of BPKyne bioconjugation by CuAAC. A.) Experimental workflow for isolation of Gal80 245BPKyne – Gal4 crosslinked products from yeast cells. B.) Biotinylation of Gal4-Gal80 crosslinked product through CuAAC cycloaddition. The BPKyne-incorporated Gal80-Gal4 crosslinked product was isolated from solution using CuAAC and Neutravidin magnetic beads and analyzed by western blot (α-Myc). The Gal4-Gal80 crosslinked product is only isolated in the presence of BPKyne and UV irradiation when conjugated to the biotin probe.

After 2 hours, the biotin-conjugated proteins were isolated using neutravadin magnetic beads and analyzed by western blot probing for Myc-tagged Gal80 species (Figure 4.4B). With this strategy, the LexA+Gal4-Gal80 complex was only observed for BPKyne incorporated proteins that had been irradiated and functionalized with the

biotin-azide probe demonstrating the ability of the bioorthogonal alkyne handle to be specifically labeled. As a comparison, traditional immunological techniques were used to isolate the UAA-containing Gal80-Gal4 crosslinked complex and visualized by western blot analysis (Figure 4.3D). Importantly, when visualized by western blot using the cMyc-HRP antibody, less background was seen when BPKyne containing samples isolated through CuAAC and neutravadin pull-down were compared to UAA containing proteins immunoprecipitated with α-Myc, which results in nonspecific isolation of all proteins containing an endogenous Myc epitope (Figure 4.4B and 4.3D). These experiments illustrate the advantages of the bifunctional BPKyne molecule, which captures specific PPIs upon irradiation and allows for isolation of these interactions from their cellular environment post-functionalization.

4.3 Conclusions and Future Directions

Here we have demonstrated the first incorporation of the bifunctional UAA, BPKyne, into live yeast cells using the *E. coli* tyrosyl tRNA/synthetase system and have illustrated the utility of BPKyne for the isolation of crosslinked products from their native environment. Utilizing the Gal4 and Gal80 yeast proteins we have shown that BPKyne is incorporated with similar expression yields compared to pBpa without requiring further mutagenesis. Along with the similar crosslinking reactivity compared to pBpa, we have illustrated that BPKyne incorporated protein can be isolated from whole cell lysate after functionalization with a biotinylated azide probe. While we used western blotting for visualization in this proof of principle study, mass spectrometry could also be used for characterization of isolated crosslinked adducts. This strategy also enables the capture and isolation of PPIs for which antibodies are not efficient or available and/or when genetically encoded epitope tags, such as Myc or Flag, cannot be appended without impairment of protein structure or function. The bioorthogonal alkyne handle enables the direct labeling of crosslinked PPIs of interest, which will be particularly advantageous in the discovery of novel PPIs.

4.4 Materials and Methods

LS41 [JPY9::pZZ41, Matα his3Δ200 leu2Δ1 trp1Δ63 ura3-52 lys2Δ385 gal4 URA::pZZ41] yeast was used for all experiments. pBpa was purchased from Chem-Impex International (Wood Dale, IL). All plasmids described below were constructed using standard molecular biology techniques and the sequences of all isolated plasmids were validated by sequencing at the University of Michigan Core Facility (Ann Arbor, MI).

Table 4.1 Plasmids used in Chapter 4

Plasmid Name	Function
pLexA+Gal4WT-5X Flag	Expresses LexA(1-202)+Gal4(840-881)+5X Flag tag
pLexA+Gal4849TAG-5X Flag	Expresses LexA(1-202)+Gal4(840-881)+5X Flag tag with a TAG codon replacing the codon of the existing amino acid
pSNRtRNA-pBpaRS	Expressed tRNA under the control of the SNR52 promoter and contains synthetase specific for pBpa
pGal80-6X Myc	Expresses full-length Gal80+6X c-Myc tag
pLexA-Gal4WT-1X Flag	Expresses LexA(1-202)+Gal4(840-881)+1X Flag tag
pMyc Gal80 245TAG	Expresses full-length 1X Myc+Gal80 with a TAG codon replacing the codon of the existing amino acid

UAA incorporation and expression

LS41 yeast was transformed with pLexA+Gal4 849 TAG-5X Flag, pLexA+Gal4WT-5X Flag, or Myc-Gal80 245TAG and pSNRtRNA-pBpaRS plasmids. Individual colonies were grown in 5 mL SC media containing 2% raffinose, but lacking histidine, tryptophan, and uracil for selection of LexA+Gal4 or but lacking histidine, tryptophan, leucine and uracil for selection of Myc-Gal80. The cultures were incubated at 30 °C with 250 rpm agitation. Following incubation, these cultures were used to inoculate 5 mL cultures of SC media containing 2% raffinose and 2% galactose, with or without 1 mM pBpa / 1 mM BPKyne (dissolved in 1 M NaOH), and 1 M HCl. The cultures were incubated at 30 °C with agitation to an OD_{660} of 1.0. Three ODs were isolated, washed with sterile water, and stored at -20 °C. The samples were lysed in 10 μL 4X NuPAGE LDS Sample

Loading Buffer (Invitrogen), 10 μL 1X lysis buffer (50 mM tris-acetate pH 7.9, 100 mM potassium acetate, 20% glycerol, 0.2% Tween-20, 2 mM β-mercaptoethanol, and 2 mM magnesium acetate), and 10 μL 1 M DTT by boiling at 95 °C for 10 min. The samples were run on a 3-8% tris-acetate SDS-PAGE gel and analyzed by western blot with the anti-Flag (M2) antibody (Sigma Aldrich) for the pLexA-Gal4 expression samples and anti-cMyc-HRP (9E10) antibody (Santa Cruz) for the Myc-Gal80 expression samples. Expression levels were quantified using ImageJ and relative levels of LexA-Gal4 protein for each experiment were expressed as follows ((experimental/WT LexA-Gal4) *100).

β-Galactosidase assays

LS41 yeast was transformed with pLexA+Gal4 849 TAG-5X Flag or pLexA+Gal4WT-5X Flag and pSNRtRNA-pBpaRS plasmids. Individual colonies were grown in 5mL SC media containing 2% raffinose, but lacking histidine, tryptophan, and uracil for selection. The cultures were incubated at 30 °C with 250 rpm agitation. Following incubation, these cultures were used to inoculate 5 mL cultures of SC media containing either 2% glucose or 2% Raffinose and 2% galactose with or without 1 mM pBpa / 1 mM BPKyne (dissolved in 1 M NaOH), and 1M HCl which were subsequently incubated at 30 °C with agitation to an OD660 of 1.0 and harvested. The activity of each construct was assessed using B-Galactosidase assay as previously described.[21]

LexA+Gal4 849TAG-5X Flag in vivo photo-crosslinking

For *in vivo* photo-crosslinking, a colony of LexA+Gal4 849 TAG-5X Flag was grown in 5 mL SC media containing 2% raffinose, but lacking histidine, tryptophan, and uracil for selection. The cultures were incubated at 30 °C with 250 rpm agitation. Following incubation, these cultures were used to inoculate 10 mL cultures of SC media containing 2% glucose, with 1 mM pBpa or 1 mM BPKyne (dissolved in 1 M NaOH), and 1 M HCl. The cultures were incubated at 30 °C with agitation to an OD_{660} of 1.0. For each culture, the cells were isolated by centrifugation and washed with the SC media lacking histidine, tryptophan, and uracil. The cell pellets were resuspended in 2 mL SC media containing 2% glucose and then transferred to small culture dishes and subjected to UV irradiated at 365nm light (Eurosolar 15W UV lamp) with cooling for 30 minutes.

The cells were isolated by centrifugation and stored at -20 °C until lysis. The control samples were washed with 1 mL SC media containing 2% glucose, isolated by centrifugation, and stored at -20 °C until lysis.

For crosslinking studies with Myc-Gal80, the procedure was identical except that cells were grown in SC media lacking histidine, leucine, tryptophan, and uracil. For lysis, cells were resuspended in 350 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 was immunoprecipitated with anti-LexA antibody (sc-1725, Santa Cruz Biotechnologies) for 2 hours at 4 °C. The proteins bound to the antibody were isolated by incubation for 1 hour with 8 µL pre-washed protein G magnetic beads (Millapore) at 4 °C. After incubation, the beads were washed six 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 at -20 °C until elution.

The samples were eluted from the beads by heating at 95 °C for 10 minutes in 10 µL NuPAGE 4X LDS Sample Loading Buffer (Invitrogen), 10 µL water, and 10 µL 1M DTT. The samples were run on a 3-8% tris-acetate SDS-PAGE gel and analyzed by western blot using either anti-Flag (M2) antibody (Sigma Aldrich) for endogenous crosslinking profile or anti-cMyc antibody (SC-40, Santa Cruz Biotechnology) for Myc-Gal80 crosslinking. Crosslinking yields were quantified using ImageJ and the relative amount of Gal4-Gal80 crosslinked product for each experiment was expressed as follows ((Gal4 BPKyne-Gal80 crosslinked product/Gal4 Bpa-Gal80 crosslinked product) *100).

LexA+Gal4 WT-1X Flag:1X myc-Gal80 245TAG whole cell lysate preparation
A colony of LexA+Gal4 WT-1X Flag:1X myc-Gal80 245TAG was grown in 5 mL SC media containing 2% raffinose, but lacking histidine, tryptophan, leucine and uracil for selection. The cultures were incubated at 30 °C with 250rpm agitation. Following incubation, these cultures were used to inoculate 100 mL cultures of SC media containing 2% glucose, with 1 mM pBpa or 1 mM BPKyne (dissolved in 1 M NaOH), and

1 M HCl which were subsequently incubated at 30 °C with agitation to an OD_{660} of 2.0. For each culture, the cells were isolated by centrifugation and washed with the SC media lacking histidine, tryptophan, leucine, and uracil. The cell pellets were resuspended in 2 mL SC media containing 2% glucose and then transferred to small culture dishes and subjected to UV irradiated at 365 nm light (Eurosolar 15W UV lamp) with cooling for 45 min. The cells were isolated by centrifugation and stored at -80 °C until lysis. The control samples were washed with 1 mL SC media containing 2% glucose, isolated by centrifugation, and stored at -80 °C until lysis. 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. The supernatant was split into 2 mg protein aliquots and stored at -80 °C until CuAAC labeling.

Traditional immunoprecipitation of LexA+Gal4 WT-1X Flag: 1X myc-Gal80 245pBpa crosslinked products

Whole cell lysate was incubated at 4 °C for 2 hours with either 10 μL anti-LexA (sc-1725, Santa Cruz Biotechnologies) or anti-cMyc (9E10) (SC 40, Santa Cruz Biotechnologies) antibody. The proteins bound to the antibody were isolated by incubation for 1 hour with 40 μL pre-washed Dynabeads® protein G magnetic beads (ThermoFisher) at 4 °C. After incubation, the beads were washed six time 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 at -20 °C until elution. The crosslinked samples were eluted from the beads by heating at 95 °C for 10 minutes in 10 μL NuPAGE 4X LDS Sample Loading Buffer (Invitrogen), 10 μL water, and 10 μL 1M DTT. The samples were run on a 3-8% Tris-acetate SDS-PAGE gel and analyzed by western blot using anti-cMyc-HRP antibody (SC-40, Santa Cruz Biotechnology) for Gal80-containing crosslinked products.

Copper-catalyzed alkyne-azide cycloaddition (CuAAC) labeling of LexA+Gal4 WT-1X Flag: 1X myc-Gal80 245BPKyne crosslinked products

2 mg/mL proteome was combined with 500 µM biotin-PEG3-azide (Sigma-Aldrich), 1% sodium dodecyl sulfate, 5 mM Tris(3-hydroxypropyltriazolylmethyl) amine (THPTA, Sigma-Aldrich), 1 mM $CuSO_4$ in water, and 5 mM sodium ascorbate in water at 37 °C with 250rpm agitation. After 2 hours, the samples were purified through acetone precipitation overnight at -20 °C followed by resuspension in 1X PBS pH 7.4 (ThermoFisher) and 0.5% SDS. Samples were combined with 50 µL GE Sera-Mag™ SpeedBeeds™ Neutravidin Particles and incubated at room temperature with rocking for 1.5 hours. After pulldown, the beads were washed six 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 at -80 °C until elution. The click samples were eluted from the beads by heating at 95 °C for 10 minutes in 10 µL NuPAGE 4X LDS Sample Loading Buffer (Invitrogen), 10 µL water, and 10 µL 1 M DTT. The samples were run on a 3-8% tris-acetate SDS PAGE gel and analyzed by western blot using anti-cMyc-HRP antibody (SC-40, Santa Cruz Biotechnology) for Myc-Gal80 crosslinking.

4.5 References

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CHAPTER 5
Conclusions and Future Directions

5.1 Conclusions

Protein-protein interaction (PPI) networks direct all cellular processes, including cell cycle regulation, protein folding, and transcription.[1] These networks consist of hundreds of protein contacts that range in affinities, surface areas, and lifetimes. For instance, transient, moderate affinity interactions that make up many regulatory processes, including transcription, enable several different protein contacts at a single binding site on a core protein.[2-3] Transcriptional activators act as core proteins recruiting several multisubunit complexes to specific gene promoters through transient interactions with their transcriptional activation domains (TADs).[4] These perfectly timed interactions range in surface areas and affinities to orchestrate the assembly of the transcriptional machinery, including RNA polymerase II, to genes to activate expression. Misregulation of transcriptional PPI networks has been implicated in many diseases, making these networks logical targets for interrogation and modulation by chemical probes and eventually therapeutic interventions.[5-6] However, the transient nature of these interactions makes them challenging to capture with traditional methods, such as affinity purification and/or chromatin immunoprecipitation (ChIP).[7-9] While ChIP studies have determined the timed recruitment of the large coactivator complexes, including SAGA, Mediator, the general transcriptional factors, and RNA Polymerase II by the prototypical Gal4 activator at the GAL1 promoter in yeast, the direct activator binding partners within these complexes are still unclear.[10] While the interactions between transcriptional activators and coactivators have been sought-after targets for small molecule manipulation for years, however, the detailed map of these interactions have been intractable due to the limitations of the current methods available.

Recently, *in vivo* covalent chemical capture using photo-activatable unnatural amino acids (UAAs), such as *p*-benzoyl-L-phenylalanine (pBpa), has been a powerful tool for

characterizing transient PPIs. Our lab has optimized pBpa crosslinking to identify the direct binding partners of the prototypical activators, Gal4 and VP16, within coactivator complexes in *Saccharomyces cerevisiae*.[11-15] However, this strategy has been used with model activators and proteins with ample biochemical data to help determine key incorporation sites and there have been limitations observed with the benzophenone scaffold and characterizing the crosslinked adducts captured. As demonstrated in Chapter 2, this pBpa covalent chemical capture strategy can be extended to novel activator-coactivator interactions with minimal to no biochemical data to help guide a hypothesis-driven investigation. Additionally, the suite of pBpa analogs with enhanced functionality characterized in Chapter 3 and 4 begin to address the limitations seen with the parent benzophenone scaffold, such as slow crosslinking reactivity, and the current crosslinking strategy, such as limited methods for the isolation of the crosslinked adducts, respectively. Ultimately, the work performed in this dissertation not only extends the current pBpa crosslinking technology to a less characterized activator system, but it also expands the toolbox of chemical probes and methods for completing the transcriptional interaction network of activators as well as the PPI networks required for the regulation of cellular homeostasis.

5.1.1 *In vivo covalent capture is a strong tool for the capture of activator-coactivator interactions*

A key finding in this work is that covalent chemical capture, using genetically incorporated pBpa, is a powerful tool to interrogate the challenging PPIs between activators and the dynamic, interchangeable coactivator complexes that regulate transcription. In Chapter 2, the utility of this methodology is extended to interrogate the interactions of the less characterized yeast forkhead box transcriptional activator, Hcm1, under nutrient stress. In this chapter, we identified a minimal TAD region of Hcm1 that is regulated by nutrient signaling and actives transcription when fused to the bacterial LexA DNA binding domain (DBD). Additionally, using two sites for pBpa incorporation, we covalently captured Hcm1's interactions with the SNF1 signaling complex through the Snf1 kinase subunits and the Gal83 interchangeable β-scaffolding subunit under glucose-limiting conditions. These finding demonstrate the modularity of the Hcm1

activator and its ability to regulate the expression of glucose-repressed genes through interactions with dynamic coactivator complexes.

Additionally, while Chapter 2 demonstrates that *in vivo* covalent chemical capture using pBpa enables the interrogation of transient, moderate affinity interactions within the transcriptional PPI network, Chapter 3 demonstrates that a negative result must be interpreted cautiously. The affinity and lifetime of the protein complex, the environment of the PPI interface, and the reactivity of the photo-crosslinker could all play a role in the lack of a crosslinking product. Therefore, the site of UAA incorporation and the photo-crosslinker's innate reactivity is critical to the success of an *in vivo* crosslinking experiment and must be given careful consideration.

5.1.2 *Electron withdrawing groups increase crosslinking reactivity of pBpa*

Although sequence context of the PPI interface is critical for the successful covalent chemical capture, the crosslinking reactivity of the crosslinker is also important for the capture of challenging PPIs. As discussed in Chapter 3, pBpa's reversibility and minimal reactivity to the surrounding solvent is advantageous for the capture of transient PPIs seen between transcriptional activator and their coactivator partners. However, its slow reactivity compared to the diazarine and aryl-azide crosslinkers could miss weaker affinity interactions causing critical interaction gaps within the transcriptional PPI network. To overcome this limitation, a suite of mono-substituted pBpa analogs containing electron withdrawing groups (EWGs - F, Cl, CF_3, Br) appended to the benzophenone scaffold were developed and their crosslinking reactivities were characterized both *in vitro* and *in vivo*. As demonstrated in the *in vitro* studies in Chapter 3, the decrease in the energy needed for excitation of the radical by the EWGs increased the crosslinking efficiency of the Med25-VP16 interaction of each analog by approximately three-fold versus the unaltered pBpa molecule. Additionally, preliminary results show that each analog is able to be incorporated into the LexA+Gal4 TAD using amber nonsense suppression and can capture the Gal4-Gal80 interaction in live yeast. Additionally, the 4-F Bpa analog showed a marked increase in crosslinking yield of the Gal4 crosslinked adducts, making it a strong candidate for further analysis of weaker binding interactions that might have eluted the pBpa molecule. While further

optimization is needed to determine the full effect of these EWGs on pBpa's crosslinking reactivity in live cells, these findings demonstrates the positive effect of appending EWGs onto the pBpa framework and expands our toolbox of chemical probes for capturing transient, weak-affinity PPIs in their native cellular environment.

5.1.3   Bifunctional UAA enables the isolation of crosslinked adducts from live cells

While Chapter 2 has shown that *in vivo* covalent chemical capture holds promise in elucidating many uncharacterized PPIs that might have eluted traditional biochemical techniques, current covalent capture methods often require several steps of isolation and purification post-crosslinking. This is typically accomplished by immunoprecipitation or affinity purification that can vary in purification efficiency depending on the antibody or endogenous tag used. To address this limitation, we hypothesized that a bifunctional UAA that contained a photo-labile group and a bioorthogonal moiety that would enable post-crosslinking functionalization would facilitate detection and isolation of *in vivo* PPIs. The bifunctional pBpa derivative, BPKyne, contains a bioorthogonal alkyne capable of functionalization with a variety of azide-probes using Huisgen cyclization to facilitate the isolation of crosslinked products from live cells. In Chapter 4 using the model LexA+Gal4 activator, I demonstrate the first reported incorporation of BPKyne into live yeast via amber nonsense suppression and isolation of crosslinked partners of the BPKyne incorporated Gal4 protein. This strategy enables the direct labeling of crosslinked interaction using a bioorthogonal alkyne handle, which will be particularly advantageous for the discovery of novel PPIs.

5.2 Future Directions

5.2.1   Interrogation of the full Hcm1-transcriptional PPI network during nutrient stress

In Chapter 2, the interactions between the Hcm1 activator and the SNF1 signaling complex were captured using *in vivo* covalent chemical capture. While this study has laid the groundwork for interrogating Hcm1's full transcriptional PPI network using the covalent capture strategy, it used an artificial chimeric LexA+Hcm1 activator. To fully characterize the Hcm1 interaction network with the SNF1 complex and confirm the interactions between SNF1 and Hcm1's minimal TAD, the full length Hcm1 protein

should be examined. Using the Hcm1 T218 and T272 incorporation sites optimized in Chapter 2, the interactions between Hcm1 and the SNF1 complex will be assessed under normal glucose conditioned and galactose stress conditions. Additionally, using the tandem reversible and irreversible crosslinking (TRIC) technique developed by former lab members, the interaction can be analyzed at Hcm1 specific genes giving insight into the mechanism by which Hcm1 regulates glucose repressed genes.[15]
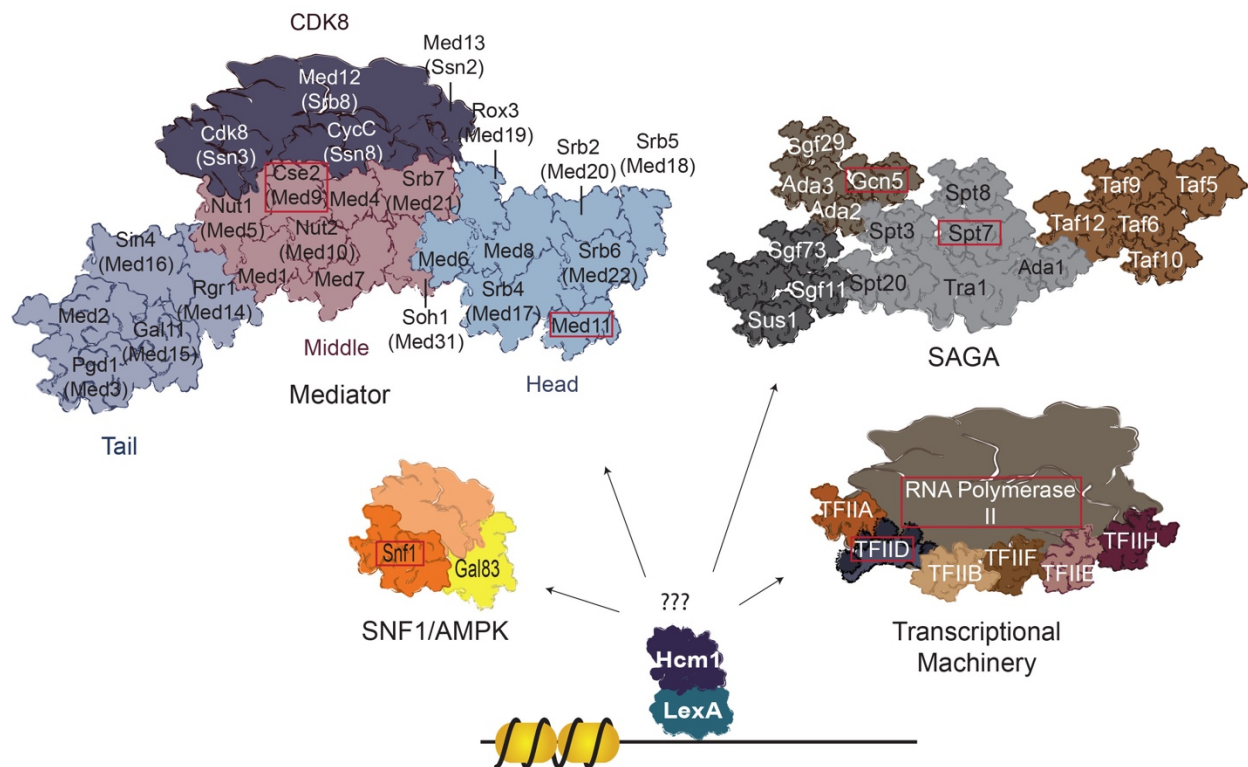
Figure 5.1 Hcm1 recruits several coactivators, such as the SNF1/AMPK, Mediator, and SAGA complexes, and the transcriptional machinery the DNA to express nutrient stress response genes. Several genetic and physical interaction studies proposed binding partners within these multisubunit complex (designated in red boxes). The proposed binding partners are as follows: Mediator complex – Med9[16] and Med11 subunits[17], SAGA – Spt7 and Gcn5[18], SNF1/AMPK – Snf1 kinase[19], RNA Polymerase II – Rpb3[16], and TFIID – Taf1[20].

While Chapter 2 looks at Hcm1's interactions within the SNF1 coactivator complex, reports have proposed several binding partners within the coactivator complexes in Figure 5.1.[16-18, 21] However, there is little known about the mechanism by which Hcm1 recruits these complexes to the promoter or whether these interactions are direct or indirect binding events. To initially verify the proposed binding partners of Hcm1 within these coactivator complexes, *in vivo* covalent chemical capture can be used with pBpa

incorporated into the full-length Hcm1 protein. Once the initial subunits are verified as direct binding partners, a full mass-spectrometry (MS) proteomic study should be run to identify other direct binding partners within these multi-subunit complexes. Our lab has recently used tandem *in vivo* covalent capture MS proteomics to identify cellular targets of the Gal4 activator under nutrient stress.[12] This study captured several novel endogenous binding partners of Gal4 partners, including the SNF1 complex. Using the crosslinking protocol optimized by former lab members, the cellular targets of Hcm1 can be captured and identified through a variety of MS proteomic methods creating a map of Hcm1's transcriptional PPI network during nutrient stress.

5.2.2 *Examination of chimeric transcriptional activator interaction specificity*

Our lab has demonstrated the strength of the *in vivo* covalent chemical strategy for capturing the transient, moderate affinity interactions between transcriptional activators and coactivators. In an effort to create a complete map of specific activator interactions during transcriptional initiation, we have identified several direct coactivator binding partners for the Gal4, VP16, Gcn4, and Hcm1 amphipathic activators in live yeast. While encouraging, these experiments used artificial chimeric LexA DBD+TAD constructs that were localized to the GAL1 promoter which raises the question of whether these activator-coactivator interactions are promoter specific or activator specific. While many of these coactivator complexes, such as the Mediator and SAGA complexes, are required for transcriptional initiation, reports have shown that specific genes determine the exact protein complexes needed for expression.[22-24] Conversely, it has also been shown that while the interactions between transcriptional activators and coactivators are highly redundant, specific activators interact with different subunits within the large coactivator complexes or through a different confirmation and/or binding site within a similar coactivator subunit.[25-27]
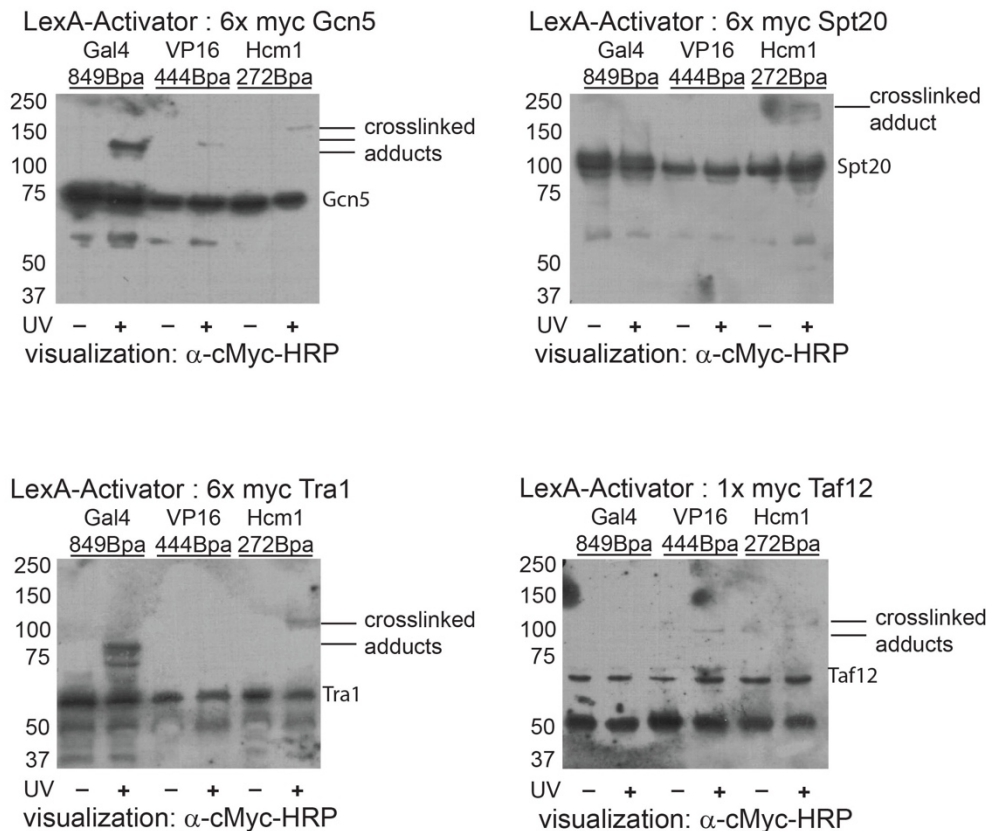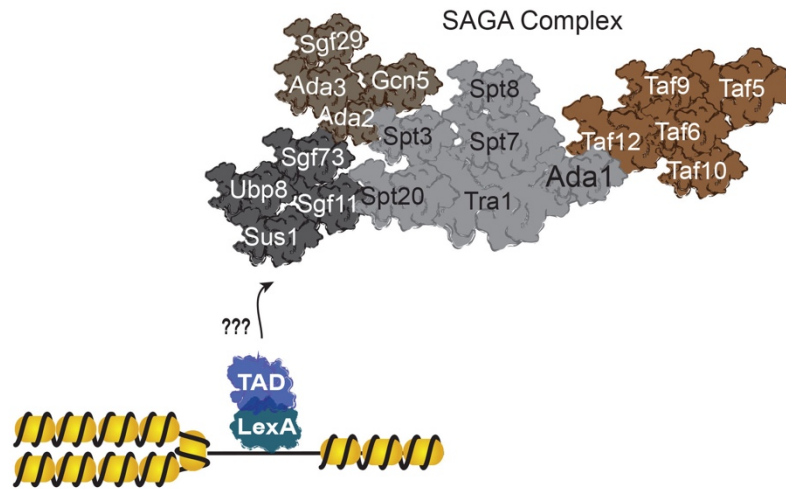
Figure 5.2 Interrogation of SAGA activator interactions. Live yeast expressing pBpa incorporated Flag tagged LexA+TAD activators (LexA+Gal4, LexA+VP16, and LexA+Hcm1) and Myc-tagged SAGA subunits (Spt20, Gcn5, Tra1, and Taf12) were irradiated, lysed, and immunoprecipitated with α-LexA to capture the activator specific interactions. Samples were analyzed by western blot probing for the myc-tagged SAGA subunits. Based on the western blots, the SAGA-activator interactions are as follows: Gcn5 interacts with all three activator TADs, Spt20 only interacts with Hcm1, Tra1 interacts with Gal4 and HCm1, and Taf12 interacts with VP16 and Hcm1.

While using LexA+TAD chimeras at the GAL1 promoter to interrogate the activator-coactivator interaction network, we have seen some specificity between activator TADs

using *in vivo* covalent chemical capture. For example, while Gal4 and both VP16 subdomains interact with the Snf1 kinase domain of the SNF1 signaling complex, Gal4 and only one subdomain of VP16 interact with the Gal83 β-scaffolding subunit.[12] Additionally when examining activator interactions with the SAGA complex, we found that Gal4, VP16 and Hcm1 interact with the Gcn5 acetyltransferase domain, while only Hcm1 and Gal4 interact with the Tra1 activator interacting domain (Figure 5.2). These findings demonstrate some specificity programmed within the activator TAD sequences. However, in these studies only one incorporation site was used for each artificial activator which might affect covalent capture as described in Chapter 3. To fully examine the specificity of each TAD sequence, chimeric activators can be designed with different DNA binding domains changing the promoter localization. For example, Gal4 (1-147) or Hcm1 (100-200) could be used to localize each activator to Gal4 or Hcm1 specific genes to assess whether these interactions are determined by the promoter or the specific TAD sequence. Additionally, full-length activators could be used to fully characterize each TAD's interaction network and determine whether these artificial transcription factors are good models to study activator-coactivator interactions in their cellular environment.

5.2.3 *Mass Spectrometry proteomic screen of activator interaction network using pBpa analogs with enhanced functionality*

While our lab has captured several direct binding partners of amphipathic activators in live yeast, these experiments have been more hypothesis-driven; only examining one activator-coactivator interaction at a time using traditional immunological techniques and western blot analysis. To fully interrogate the interactions between an activator and a whole complex through these methods would be very tedious and time-consuming. However with the advancement in MS proteomic techniques, the endogenous interacting partners of a transcriptional activator can be examined through one experimental screen, and affinity purification coupled with quantitative MS has become a leading method for characterizing *in vivo* PPI networks. Additionally, using *in vivo* covalent chemical capture our lab has captured the endogenous binding partners of the LexA+Gal4 activator and identified several direct enzymatic binding partners through

quantitative MUDPIT mass spectrometry, including the Snf1 and Gal83 subunits of the SNF1 signaling complex.[12]

This proteomic screen demonstrates the strength of coupling crosslinking and quantitative proteomics to quickly identify multiple binding partners of transcriptional activators. However, there are still critical limitations seen with the current protocol that must be optimized to analyze the different activator PPI networks. First, while the *in vivo* covalent chemical capture method is routine, several steps of isolation and purification are required to obtain the crosslinked products for MS analysis. This is typically carried out through immunological techniques that rely on commercially available antibodies that vary in availability and specificity for a given target. Additionally, genetically incorporated epitope tags are required when protein-specific antibodies are not available which could negatively affect the function and structure of the proteins of interest or capture nonspecific proteins containing the epitope tag within their endogenous amino acid sequence. For example, in the Gal4 crosslinking proteomic screen the Snf1 kinase was initially ruled out as a target for Gal4 due to its high spectral count in both the –UV and +UV samples. However, after further examination of the Snf1 amino acid sequence, an endogenous polyhistidine tag was found causing the protein to be pulled out of lysate during the nickel affinity purification step in the –UV samples. To overcome this limitation, the BPKyne bifunctional amino acid developed in Chapter 4 could be used to capture and facilitate the isolation of the crosslinked adducts from cell lysate through its bioorthogonal alkyne handle, decreasing the large amount of nonspecific background proteins pulled out from epitope tags and the need for protein-specific antibodies (Figure 5.3).

While BPKyne could improve the purification and isolation of crosslinked products for MS analysis, the 4-Br Bpa analog developed in Chapter 3 could enable quantitative proteomic analysis without the need for expensive isotopic labeling systems, such as stable isotope labeling using amino acids in cell culture (SILAC) systems.[28-29] Compared to proteogenic elements that only exist in one predominant isotope, bromine exists naturally as two stable isotopes, ^{79}Br and ^{81}Br, that are equally abundant.[30]
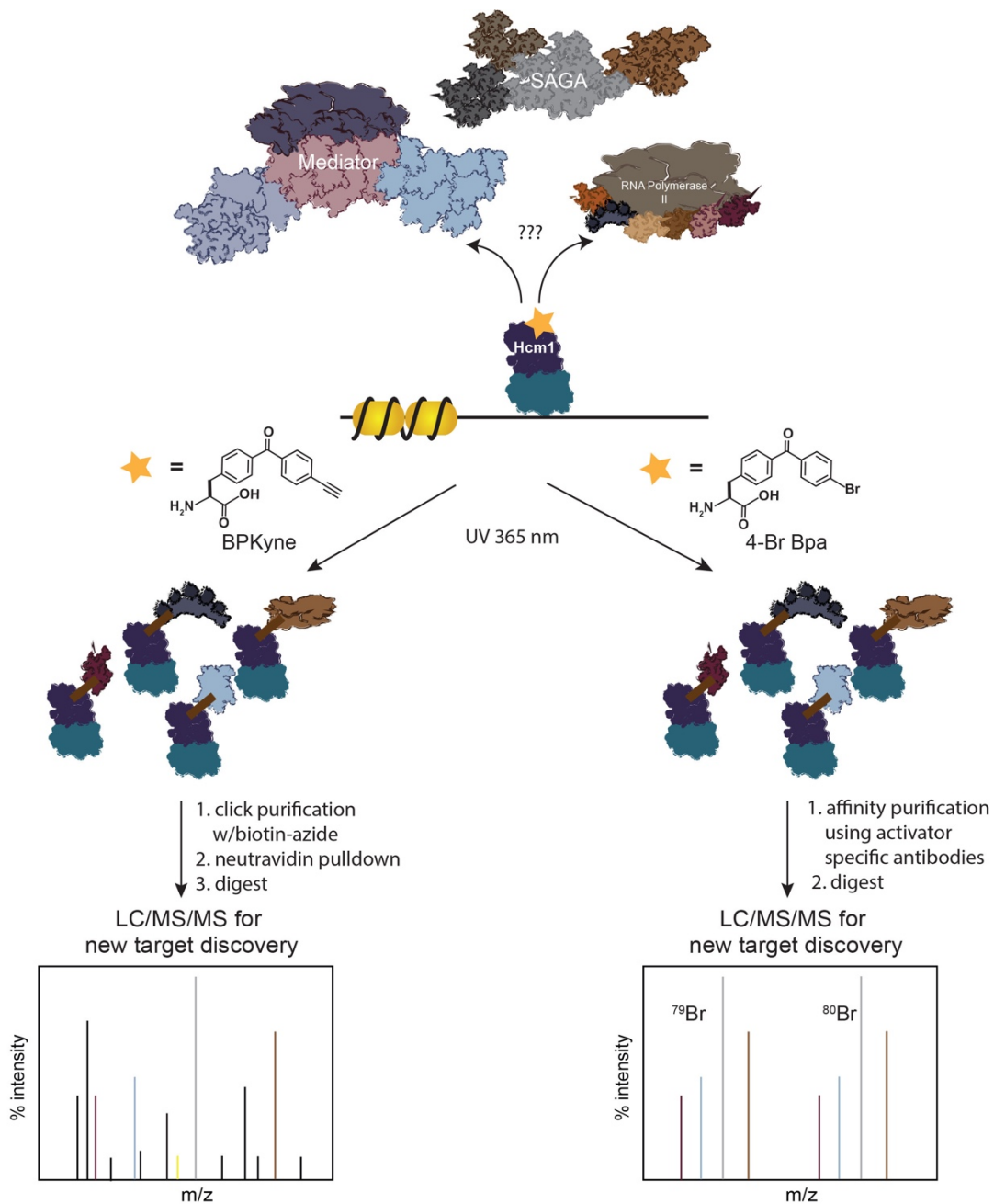
Figure 5.3 Mass Spectrometry screen of crosslinked activator interactions using pBpa analogs. (Left) Using BPKyne, the crosslinked products can be labeled with a biotin-azide through CuAAC and isolated from cell lysate using Neutravidin magnetic beads. Upon mass spectrometry analysis, the activator interacting partners can be identified through elevated spectral counts in +UV samples compared to –UV samples. (Right) Using 4-Br Bpa, the crosslinked products are isolated and purified through affinity purification using antibodies specific to the activator of interest. Upon mass spectrometry analysis, the activator interacting partners can be quantitatively identified by the mass differences in Br isotopes found in the crosslinker.

142

When 4-Br Bpa is incorporated into an activator of interest, the endogenous binding partners can be captured and upon MS analysis, the protein targets can be quantified between samples to accurately identify interaction partners with minimal bias between experimental replicates (Figure 5.3). Due to bromine's distinct isotopic signature, using 4-Br Bpa enables the capture and identification of activator interacting partners that may be drowned out by high abundance proteins.[31] While several novel binding partners of Gal4 were identified in the crosslinking proteomics screen, no transcriptionally relevant complexes were observed, mainly due to their low abundance in the cell (<500 copies). Using 4-Br Bpa or BPKyne, rather than the parent pBpa crosslinker, could possibly enable to capture and identification of the low abundance proteins required for transcriptional initiation that have eluted the current strategy.

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