# Sequence Context and Crosslinking Mechanism Affect the Efficiency of In Vivo Capture of a Protein–Protein Interaction

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## ABSTRACT:

*Protein–protein interactions (PPIs) are essential for implementing cellular processes and thus methods for the discovery and study of PPIs are highly desirable. An emerging method for capturing PPIs in their native cellular environment is in vivo covalent chemical capture, a method that uses nonsense suppression to site specifically incorporate photoactivable unnatural amino acids (UAAs) in living cells. However, in one study we found that this method did not capture a PPI for which there was abundant functional evidence, a complex formed between the transcriptional activator Gal4 and its repressor protein Gal80. Here we describe the factors that influence the success of covalent chemical capture and show that the innate reactivity of the two UAAs utilized, (*p*-benzoylphenylalanine (pBpa) and* p*-azidophenylalanine (pAzpa)), plays a profound role in the capture of Gal80 by Gal4. Based upon these data, guidelines are outlined for the successful use of in vivo photo-crosslinking to capture novel PPIs and to characterize the interfaces. © 2013 Wiley Periodicals, Inc. Biopolymers 101: 391–397, 2014.*

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## INTRODUCTION

Protein–protein interactions (PPIs) underlie virtually all cellular functions and regulate both the location and timing of specific activities.[1–3] In the case of gene transcription, for example, PPIs between transcription factors and histone acetyl transferases such as CBP/p300 localize the enzymatic activity of the HAT to specific promoters, leading to transcriptional upregulation.[4,5] Mis-regulation of PPIs is associated with many human diseases and with the successful development of clinically relevant PPI inhibitors, there is renewed interest in discovering and characterizing PPIs with the goal of identifying new therapeutic targets.[2,6–11] Since many PPIs occur in the context of multi-component complexes, it is particularly valuable to carry out such studies in the native cellular environment.

We recently described the implementation of a powerful approach that enables the study of PPIs *in vivo*, a strategy that uses nonsense suppression to site-specifically incorporate a photoactivatable amino acid into a protein of interest in *Saccharomyces cerevisiae* (Figure 1).[12,13] Photo-crosslinking is then carried out with live cells, enabling the capture of protein binding partners in their native context. Our first example lead to

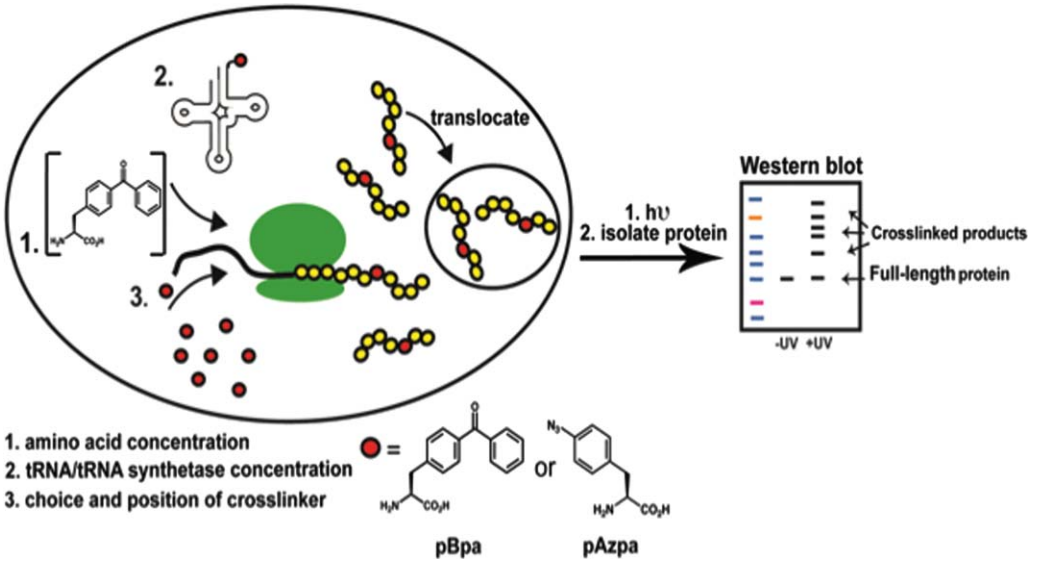


**FIGURE 1** A schematic of *in vivo* crosslinking utilizing UAAs such as pBpa and pAzpa. In this strategy, nonsense suppression is used to incorporate the UAA of choice into the protein. Subsequent UV irradiation leads to activation of the UAA, enabling it to form crosslinks with nearby binding partners. The crosslinked complexes can then be analyzed by Western blot as indicated or by techniques such as mass spectrometry. Factors influencing the success of the overall strategy include efficient incorporation of the UAA (affected by UAA availability and expression of the tRNA/tRNAsynthetase pair) and the identity of the UAA as well as its positioning within the protein.

the *in situ* characterization of the binding interface between the transcriptional activator Gal4 and its masking protein Gal80. For these experiments, the unnatural amino acid *p*-benzoyl-L-phenylalanine (pBpa) was incorporated into 10 different positions within the transcriptional activation domain of Gal4 and photo-crosslinking was carried out in living yeast under conditions in which Gal80 was expected to bind Gal4. While we detected complex formation via *in vivo* crosslinking at most positions, we noted several instances where functional data and existing structural data for the Gal4–Gal80 complex supported a direct interaction yet little to no crosslinking was observed.[12,14–18] For example, Phe856 has been observed in structural studies to be buried within the Gal4•Gal80 binding interface.[17,18] When Phe856 was replaced with pBpa, the resulting Gal4 mutant was repressed in the presence of Gal80, suggesting that the mutation did not negatively impact the Gal80 binding interaction, yet no Gal4–Gal80 covalent complex could be observed after crosslinking.

A negative result in an *in vivo* photo-crosslinking experiment could arise from a variety of factors, including the lack of a binding interaction, low unnatural amino acid (UAA) incorporation yield and/or fidelity, poor positioning of the UAA, and the poor reactivity of the activated UAA with the amino acids in the binding partner. In the case of the Phe856pBpa Gal4 mutant, there was functional evidence excluding the lack of a binding interaction as an explanation. Here we describe an examination of the remaining facets of the *in vivo* crosslinking experiment and, importantly, demonstrate the substantial role that the crosslinking mechanism and the sequence context play in the ability to capture a PPI. This case study of a PPI provides a framework for designing successful *in vivo* crosslinking experiments.

## RESULTS AND DISCUSSION

Gal4 is a well-characterized transcriptional activator that regulates genes responsible for galactose catabolism in yeast and its function is highly regulated by the inhibitory protein, Gal80.[14–16,19] In the presence of glucose, Gal80 binds Gal4 tightly, thus preventing Gal4 from recruiting the necessary transcriptional complexes to upregulate gene expression. Conversely, in the absence of glucose and in the presence of galactose, inhibition of Gal4 by Gal80 is lifted, allowing transcription to occur. We chose to use this well-characterized interaction as a predictable model under which we could evaluate the impact of UAA incorporation on activator binding and function. Furthermore, extensive biochemical and structural studies have provided information on the key residues in Gal4 involved in directly contacting Gal80, thus mapping out

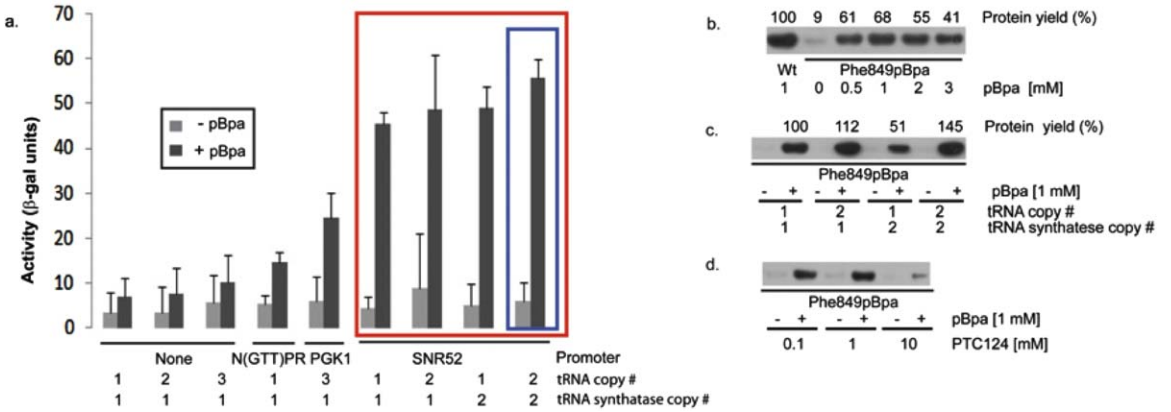


**FIGURE 2** Optimal conditions for expression of pBpa mutants of LexA+Gal4. (a) The efficiency of pBpa incorporation under various tRNA/synthetase expression systems was assessed by $\beta$-galactosidase assays. In this system the amount of activity, which is the average values of three independent experiments with the indicated error (SDOM), is related to the expression of full-length LexA+Gal4 in which Phe849 was replaced with pBpa.[22,24,25] The LS41 yeast strain used for this study bears an integrated $\beta$-galactosidase reporter controlled by two LexA binding sites approximately 50 bp upstream of the transcription start site. Among the combinations evaluated, the pSNR52 tRNA/aaRS system (red box) in which the expression plasmid carried two copies of both the tRNA and the synthetase (blue box) provided the best yield of LexA+Gal4 Phe849pBpa. (b) The effect of pBpa concentration in the growth media on incorporation yield was assessed via quantitated Western blot ($\alpha$-FLAG) of LexA+Gal4 Phe849pBpa bearing a FLAG tag at the C-terminus. The % yield of LexA+Gal4 Phe849pBpa mutant relative to WT LexA+Gal4 is indicated. To account for loading variations, each band was normalized to $\alpha$-tubulin. (c) A Western blot ($\alpha$-FLAG) comparison of LexA+Gal4 Phe849pBpa expressed with different tRNA/tRNAsynthetase expression systems. The % yield of LexA+Gal4 Phe849pBpa mutant is calculated relative to WT LexA+Gal4. To account for loading variations, each band was normalized to $\alpha$-tubulin. (d) A Western blot ($\alpha$-FLAG) comparison of LexA+Gal4 Phe849pBpa expressed in the presence of increasing concentrations of PTC124. Additional details can be found in the Methods section.

positions where pBpa incorporation was most likely to yield crosslinks.[17,18,20,21] As in previous reports, a heterologous construct was utilized in which the transcriptional activation domain of Gal4 (residues 840–881) was fused to the LexA DNA binding domain (LexA+Gal4). The advantage of this construct is a single promoter containing binding sites for LexA exists within the yeast strain used in our studies (LS41), simplifying functional analyses of all mutant proteins.[12] Additionally, a FLAG tag at the c-terminus of the construct facilitates purification and detection.

As described by us and others, the fidelity and efficiency of UAA incorporation can vary significantly with protein and amino acid position.[12,22] Additionally, efficient incorporation of an UAA requires the UAA, a tRNA for that amino acid, and a synthetase (RS) to charge the tRNA. Thus, the expression of these components is an important variable in the successful implementation of the nonsense suppression strategy. In our initial studies, little to no incorporation of pBpa was observed for LexA+Gal4 using the originally reported expression system.[12,23] Upon alteration of the tRNA/RS copy number and the promoter controlling expression of these elements, we found that pBpa incorporation was increased, although this also lead to some loss in incorporation fidelity. However, we also tested an expression system developed by Wang and Wang that utilizes a PolIII promoter (pSNR52) containing consensus A and B box sequences to control tRNA expression.[22] As determined by Western blot and functional experiments, use of the Wang tRNA expression system resulted in the best yield with high fidelity of a LexA+Gal4 construct in which Phe849 has been replaced with pBpa (Figure 2a). Further efforts to optimize this system demonstrated that neither increasing the concentration of pBpa in the growth media beyond 1 mM nor adding a premature stop codon suppressor (PTC124) increased yield significantly (Figures 2b–2d). Thus, for incorporation experiments with transcriptional activators, we find the optimal conditions to include use of a system that employs eukaryotic pol III promoter elements to drive the expression of multi-copy tRNA/RS genes for incorporation of UAAs provided at a concentration of 1 mM in yeast growth media.

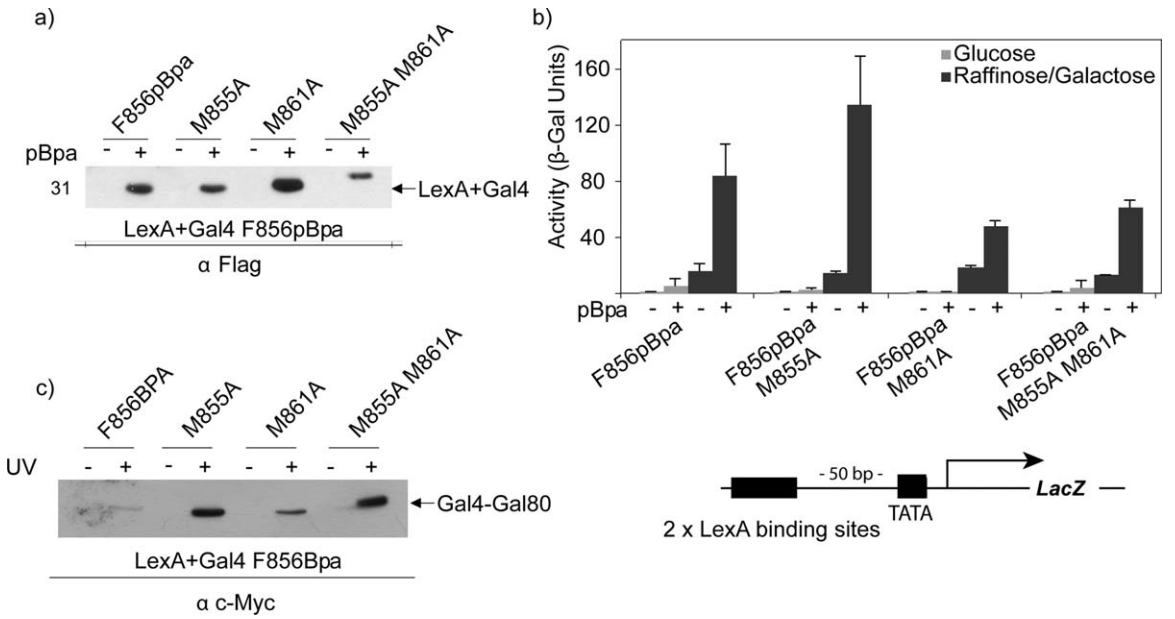


**FIGURE 3** The effect of methionine on LexA+Gal4 F856pBpa crosslinking to Gal80. (a) Expression of LexA+Gal4 F856pBpa as detected by Western blot of cell lysates with α-Flag. (b).The activation potential of each mutant was measured by liquid β-galactosidase assays. In the yeast strain tested, β-galactose expression was controlled by a Gal1 promoter containing two LexA binding sites for LexA+Gal4 binding. (c) Crosslinking of LexA+Gal4 F856pBpa to myc-tagged Gal80 as shown by Western blot of cell lysates with α-Myc antibody.

## Covalent Capture with LexA+Gal4

As described earlier, when *in vivo* crosslinking was carried out with LexA+Gal4 Phe856Bpa no complex between Gal4 and Gal80 was observed despite several lines of experimental and literature evidence supporting an interaction. Believing that this may be due to poor expression of the mutant Gal4, we evaluated pBpa incorporation at position 856 using the optimized incorporation conditions outlined in the previous section. Subsequent Western blot analysis and functional data indicated that LexA+Gal4 Phe856pBpa was not only being expressed, but that it was also fully functional and sensitive to Gal80 inhibition in the presence of glucose (Figure 3a,b). Thus, we examined other parameters that would produce a negative result, focusing on the crosslinking mechanism of pBpa in addition to a second photo-crosslinking UAA, *p*-azidophenylalanine (pAzpa).

pBpa forms a diradical upon UV irradiation at 350–365 nm and then undergoes a C—H insertion reaction with nearby backbones and amino acid side chains.[26]Although pBpa is capable of inserting into most C—H bonds, experimentally pBpa reacts preferentially with methionine (Met) where it will react at distances beyond the 3.1 Å reactivity radius.[27] Specifically, the apparent preference of pBpa for methionine suggests that pBpa efficiency can be altered dramatically when placed in close proximity to methionine's thioether side chain.[27–29] Further examination of position 856 in the Gal4 TAD reveals two methionines in close proximity to the pBpa side chain; thus, we hypothesized that these methionines at positions 855 and 861 are internally "quenching" pBpa, thereby preventing it from crosslinking to Gal80. Consistent with this hypothesis, when Met855 and Met861 are mutated either individually or collectively to alanine, we see that the resulting mutants are functional and, importantly, that pBpa crosslinking to Gal80 is restored (Figure 3a–c). These data are consistent with a model in which an intramolecular crosslink was competing with the intermolecular reaction in the LexA+Gal4 Phe856pBpa mutant and lead to a false negative in our original experiments.

A second photoactivable UAA that can be incorporated into proteins in *S. cerevisiae* using the nonsense suppression method is pAzpa. The amino acid preference for pAzpa crosslinking is less clear because it has a more complex crosslinking mechanism compared to pBpa.[26] During excitation at ~254 nm light it forms a nitrene and it is at this state that insertion into C—H or heteroatom-H bonds occurs. If, however, insertion does not take place during the $\sim 10^{-4}$ s excitation (determined for simple nitrenes in a polystyrene matrix) it will rearrange to a more stable ketenimine.[26,30] Once rearranged, the ketenimine reacts with nucleophiles. While these differences in crosslinking reactivity between pBpa and pAzpa are known, a direct comparison of the effect of these reactivities

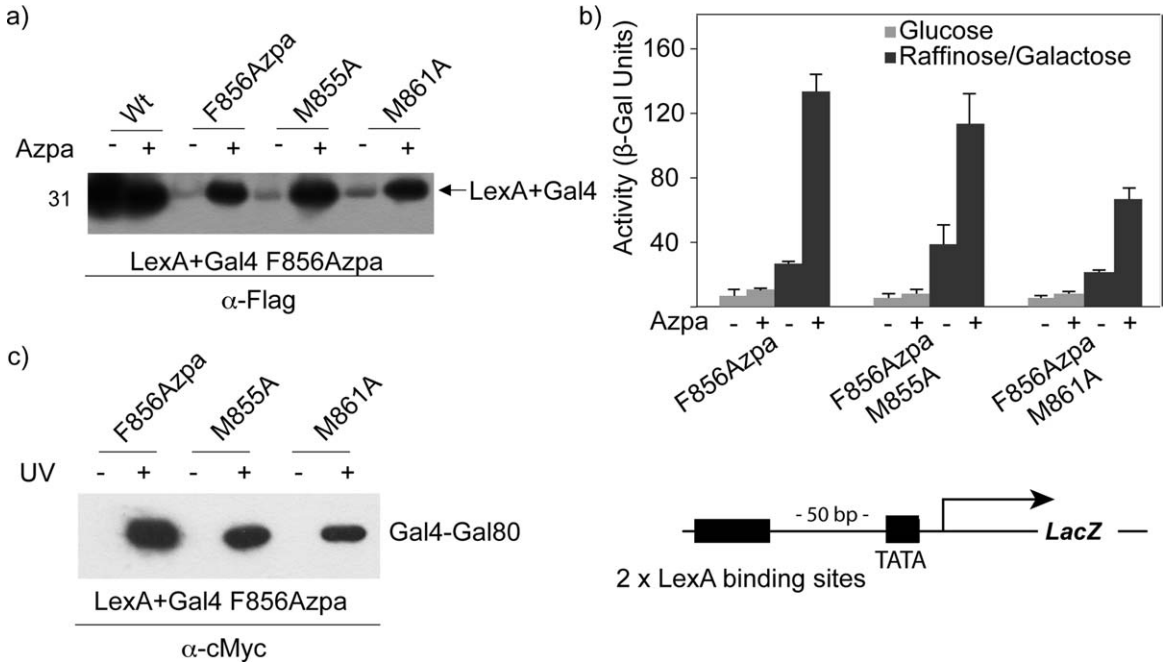


**FIGURE 4** Neighboring methionines have little effect on LexA+Gal4 F856Azpa crosslinking to Gal80. (a) Expression of LexA+Gal4 F856Azpa as detected by Western blot of cell lysates with α-Flag antibody. (b) The activation potential of each mutant was measured by liquid beta-galactosidase assays as in Figure 3. Each value is the average of at least three independent experiments with the indicated error (SDOM). (c) Crosslinking of LexA+Gal4 F856Azpa to myc-Gal80 as shown by Western blot of cell lysates with α-Myc antibody. See Methods for details.

on the experimental outcome of crosslinking studies has yet to be established. Utilization of the expression conditions outlined earlier lead to the incorporation of pAzpa at position 856 and the resulting mutant was fully functional (Figures 4a and 4b). A direct comparison of pAzpa and pBpa crosslinking at position 856 reveals that pAzpa crosslinks readily with Gal80 whereas pBpa does not (Figure 4c). As expected, introduction of alanine at positions 855 and 861 yielded no changes in Azpa crosslinking, consistent with the reactivity profile of this amino acid. These data indicate that the difference in reactive mechanisms of crosslinkers play a critical role in the outcome of crosslinking experiments.

## CONCLUSIONS

Although the Gal4•Gal80 PPI that is the focus of this study is a well-characterized complex, the present and future applications of *in vivo* covalent capture are to discover previously unknown PPIs. The results shown here illustrate that not only is optimization of UAA incorporation a key factor in successful application of the strategy, but careful consideration of the innate reactivity of the UAA utilized is also critical. The longer lifetime and lower reactivity toward solvent makes pBpa an attractive choice, particularly for PPIs that occur through shallow, exposed binding sites. However, the marked preference of pBpa for methionine raises some concern that crosslinking results could be influenced by the presence of methionine in the UAA-containing protein as shown here or by a lack of methionines in potential binding partners.[31] In other words, relevant binding partners could 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, since, as illustrated here and in a previous study, a small change in position can have a dramatic effect on crosslinking. Additionally, it is most useful to use more than one UAA in a study. In the case of Gal4•Gal80, the use of the UAA pAzpa at position 856 within Gal4 restored crosslinking with Gal80. Applying the considerations presented here will facilitate the successful implementation of *in vivo* covalent chemical capture for studying PPIs involved in a variety of biological processes.

## 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). pAzpa was purchased from Bachemand Chem-Impex (Torrance, CA). All plasmids described below were constructed using standard molecular biology techniques and the sequences of all the isolated plasmids were

**Table of Plasmids Used in this Study**

| Plasmid Name | Function |
|---|---|
| pLexAGal4 | Expresses LexA(1–202)+Gal4(840–881)+FLAG tag |
| pLexAGal4 849TAG, pLexAGal4 856TAG, pLexAGal4 F856TAG M855A, pLexAGal4 F856TAG M861A, pLexAGal4 F856TAG M855A M861A | Expresses LexA(1–202)+Gal4(840–881)+FLAG tag with a TAG replacing the codon of the existing amino acid |
| pSNRtRNA-pAzpaRS | Expresses tRNA under the control of the SNR52 promoter and contains synthetase specific for pAzpa |
| pSNRtRNA-pBpaRS | Expresses tRNA under the control of the SNR52 promoter and contains synthetase specific for pBpa |
| c-Myc-Gal80 | Expresses full-length Gal80+c-Myc tag |

verified by sequencing at the University of Michigan Core Facility (Ann Arbor, MI).

## Incorporation of pBpa or pAzpa into LexA(1–202)+Gal4(840–881)

LS41 yeast were transformed with pLexAGal4849 TAG mutants and either pSNRtRNA-pBpaRS or pSNRtRNA-pAzpaRS plasmid. A single colony of transformed LS41 was inoculated in a 4 mL synthetic dropout yeast media lacking histidine, tryptophan, and uracil containing 2% raffinose and grown overnight at 30°C. These starter cultures were used to inoculate a set of expression cultures made of 4 mL synthetic dropout media lacking His, Trp, and Ura plus 2% raffinose, 2% galactose, 10 m*M* HCl and either 10 m*M* NaOH for cultures lacking UAA or 1 m*M* UAA in 10 m*M* NaOH. Cultures were grown to mid-log phase OD660 and 3 OD's of cells were collected, washed with sterile DI water, and snap frozen in liquid nitrogen. Pellets were then lysed with pellet lysis buffer (50 m*M* Tris acetate pH 7.9, 150 m*M* KOAc, 20% glycerol, 0.2% Tween 20, and 2 m*M* MgOAc) including DTT and NuPAGE lithium dodecyl sulfate loading dye and boiled at 95°C for 10 min. Lysates were loaded onto an 8% Tris-acetate gel and run in Tris-acetate running buffer (Invitrogen). Proteins were then transferred to polyvinyl difluoride using a semi-dry transfer apparatus, blocked in 5% milk in phosphate buffered saline and Tween-20 (PBST), and then incubated with a 1:1000 dilution of α-Flag-horseradish peroxidase antibody (Sigma) to detect full length LexA+Gal4. In the case of the PTC124 (MedChem Express) experiments, varying concentrations of PTC124 (0, 0.1, 1, and 10 μ*M*) was first dissolved in Dimethyl sulfoxide and then added to expression cultures.

## Western Blot Quantitation

The quantitation of the LexA-Gal4 protein levels was performed on a representative Western Blot using the Adobe Photoshop method as previously reported by Miller et al.[32] Here, for each lane, the LexA+Gal4 protein, detected by an anti-FLAG antibody, (1:5000, Sigma M2) was normalized to that of α-Tubulin. α-Tubulin was detected using a monoclonal α-Tubulin antibody, YL1/2 (1:5000, Santa Cruz Biotechnologies), the blots were developed using a horseradish peroxidase-conjugated secondary antibody (1:20,000, Santa Cruz Biotechnologies) and then visualized by chemiluminescence with ECL plus (GE Healthcare). The relative amount of LexA-Gal4 protein for each experiment was expressed as follows ((experimental value/WT LexA-Gal4) × 100).

## *β*-Galactosidase Assays

To evaluate the ability of each LexA+Gal4 TAG mutant to activate transcription, in the absence or presence of 1 m*M* or 2 m*M* pBpa, saturated cultures (SC media + 2% raffinose) of each mutant were used to inoculate 5 mL SC media lacking histidine, uracil, and tryptophan supplemented with 2% glucose or 2% raffinose + 2% galactose or 2% raffinose and grown to mid-log phase OD660 before being harvested. The activity of each construct was monitored using a liquid *β*-galactosidaseprotocol, previously described by our group.[12]

## In Vivo Photo-crosslinking

In vivo photo-crosslinking experiments were carried out as previously reported.[12,13]

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