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CLOCKWORK: EXPLORING ACTIVATION MECHANISMS OF THE CIRCADIAN TRANSCRIPTION FACTOR CLOCK:BMAL1

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Abstract

All living organisms experience daily changes in light and temperature that influence mood, metabolism, and overall health. In order to successfully synchronize behavioral and biochemical processes into rhythms that coincide with external light/dark cycles, most organisms have internal molecular clocks. These circadian (*about a day*) clocks regulate biology throughout a 24-hour period. The mammalian molecular circadian oscillator is generated by a transcriptional-translational feedback loop, driven by the heterodimeric transcription factor CLOCK:BMAL1, which binds to DNA and subsequently recruits transcriptional coactivators, such as CBP/p300, to initiate transcription. CBP and its homolog p300 each have multiple modular domains that can make multivalent interactions with transcription factors to achieve ultrasensitive, or cooperative, regulation of their activity. We previously showed that BMAL1 transactivation domain (TAD) interacts with the KIX domain of CBP and p300. My objective was to identify additional domains within CBP that can also interact directly with the BMAL1 TAD to explore the molecular basis for cooperative regulation by CBP. I found that the TAZ1 domain of CBP also interacts with the TAD, using nuclear magnetic resonance (NMR) spectroscopy and other biophysical assays to characterize the nature of their interactions. A comparison of TAD interactions with the KIX and TAZ1 domains of CBP provides insight into the potential cooperative recruitment of CLOCK:BMAL1 by CBP, which will improve our understanding of the mammalian circadian clock and could lead to new pharmaceutical strategies to improve human health by reinforcing clock control of physiology.

Introduction

All living organisms experience daily oscillations in light and temperature that cause predictable changes in susceptibility to environmental stressors such as UV-induced DNA damage, predation, and food availability. These daily and seasonal changes result from earth's rotation about its axis, generating a 24-hour solar day[1]. In order to successfully synchronize biochemical processes into rhythms that coincide with external light/dark cycles, most organisms have developed circadian rhythms. These rhythms control a variety of physiological functions, including sleep-wake cycles, body temperature, hormone secretion, locomotory activity, and feeding behavior[2]. Disruption of circadian rhythms leads to increased risk of diseases throughout the body[3], suggesting that supporting the function of molecular circadian clocks could promote systemic improvements to human health.

Internal molecular clocks are responsible for intrinsic rhythmic changes in physiology. Circadian clocks are self-sustaining and occur autonomously at the cellular level without the need for environmental signals. However, because the period of these intrinsic oscillations are often not exactly 24 hours, environmental cues serve to synchronize the circadian clocks with the solar day[4]. The most predominant environmental signal used to synchronize circadian clocks is light. In mammals, specialized cells in the retina are able to detect light signals and transmit information to the main circadian oscillator or 'master clock' located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus[5]. This 'master clock' is responsible for regulating and coordinating circadian rhythms throughout the body. Functional self-sustaining circadian clocks are located in essentially every peripheral tissue in the body, which are directly controlled by the core oscillator creating a unified circadian rhythm[6].

The circadian oscillator is generated by a set of dedicated 'clock proteins' in a transcriptional-translational feedback loop, which drives circadian rhythms at single cellular level. The core clock proteins CLOCK and BMAL1 contain both basic helix-loop-helix and PER-ARNT-SIM (bHLH-PAS) domains that interact to form a heterodimeric transcription factor. The CLOCK:BMAL1 transcription factor binds to E-box promoter regions on DNA and subsequently recruits transcriptional coactivators that are needed to initiate transcription. CLOCK:BMAL1 activates transcription of the other core clock genes PERIOD (PER) and CRYPTOCHROME (CRY). PER and CRY proteins interact and translocate into the nucleus to eventually bind CLOCK:BMAL1 and inhibit further transcription, thereby closing the transcriptional feedback loop[7] **(Figure 1A)**. The circadian clock is therefore made by going through this feedback loop once each day. In addition to controlling the expression of these core clock proteins, CLOCK:BMAL1 orchestrates the expression of over 40% of the genome on a daily basis to provide temporal control over behavior and physiology[8]. Therefore, learning how CLOCK:BMAL1 is regulated represents an important step in understanding the molecular basis of circadian timing and its control over human health.

The crystal structure of the DNA-binding bHLH and PAS dimerization domains of CLOCK:BMAL1 sheds some light on the mechanism of the molecular clock; however, it lacks the intrinsically disordered C-terminal regions of CLOCK and BMAL1 including the transactivation domain (TAD) of BMAL1[9]. Large, unstructured regions are commonly found in transcriptional regulators, which allows them to interact with many different proteins to integrate a variety of upstream regulatory signals[10]. The BMAL1 TAD (residues 597-626) is necessary for circadian cycling and interactions with transcriptional regulators[11] **(Figure 1B)**. The TAD structure is composed of an N-terminal hydrophobic region, a central alpha helix, and a

C-terminal ‘switch’ region that has two distinct conformations due to *cis*/*trans* proline isomerization at the tryptophan-proline (WP) peptide bond[12] **(Figure 1C)**.

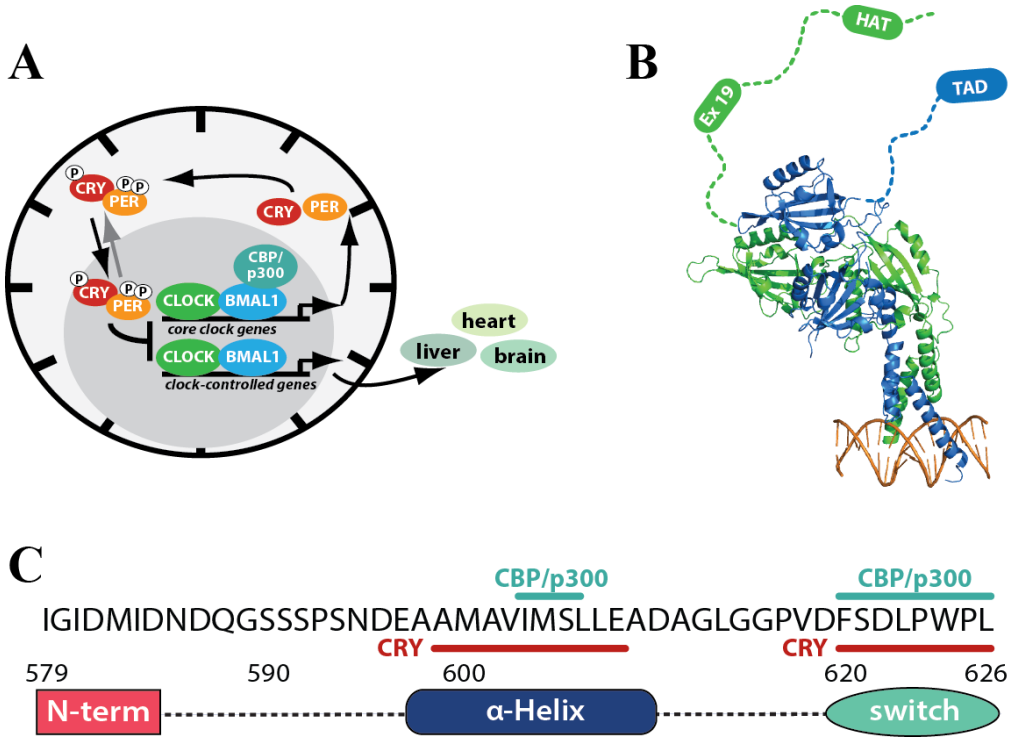


Figure 1. Feedback loops drive the molecular clock. (A) The CLOCK:BMAL1 complex regulates the transcriptional-translational feedback loop through binding of regulatory proteins. A trade-off between activation (CBP/p300) and repression (CRY, PER) sets the internal 24–hour period of the clock. **(B)** The CLOCK:BMAL1 crystal structures (PDB:4F3L and 4H10) provide insight into the function of the core transcription factor, however the structure lacks the unstructured C-terminal regions (dashed lines). The transactivation domain (TAD) of BMAL1, shown in blue, is required for circadian cycling. **(C)** The BMAL1 TAD is composed of an N-terminal hydrophobic region, a central helical region, and a C-terminal proline switch comprising the ‘WP’ peptide bond. Regions of known interactions with regulators CRY and CBP/p300 have been displayed over the sequence.

The molecular circadian clock is broadly understood at the genetic level (**Figure 1A**), but the molecular basis for generation of 24-hour timing and persistent transcriptional oscillations is not well understood. It is known that cooperative regulation leads to ultrasensitive, switch-like responses between ‘off’ and ‘on’ states required to keep the transcription-based clock running day after day[13] **(Figure 2A)**. Currently, the basis for cooperative regulation of CLOCK:BMAL1 and how it might contribute to generation of sustained transcriptional oscillations is not fully

understood. CLOCK:BMAL1 may engage in cooperative interactions with both transcriptional coactivators and tandem binding sites on DNA to generate robust circadian oscillations. CLOCK:BMAL1 has been shown to bind cooperatively to tandem E-box binding sites in the promoters of core clock genes (e.g. *Per2*, *Rev-Erbα*). Two individual CLOCK:BMAL1 complexes bind to these tandem E-box elements with an absolute requirement for only 6 or 7 nucleotides between the two sites—shortening the spacing to 5 nucleotides or extending it to only 8 nucleotides interferes with the cooperative binding of CLOCK:BMAL1 and eliminates circadian rhythms[14,15].

Transactivation of the CLOCK:BMAL1 complex is primarily accomplished through the recruitment of transcriptional coactivators such as CREB-binding protein (CBP) and its homolog p300, both of which facilitate histone acetylation and help to recruit additional transcriptional machinery[16,17]. CBP and p300 are multidomain proteins that often utilize multivalent interactions to achieve cooperative regulation of other transcription factors. For example, p53 binds to DNA as a tetramer and uses its four TADs to simultaneously interact with different domains on CBP/p300[18,19]. We hypothesize that CLOCK:BMAL1 complexes bound to tandem sites on DNA may utilize their TADs to potentially interact with multiple domains of CBP/p300 **(Figure 2B)**. There are four domains on CBP/p300 that could potentially interact with TADs on transcription factors, which allows for the potential of simultaneous interactions between multiple CBP/p300 domains to elicit highly cooperative regulation of transcriptional activation **(Figure 2C)**.

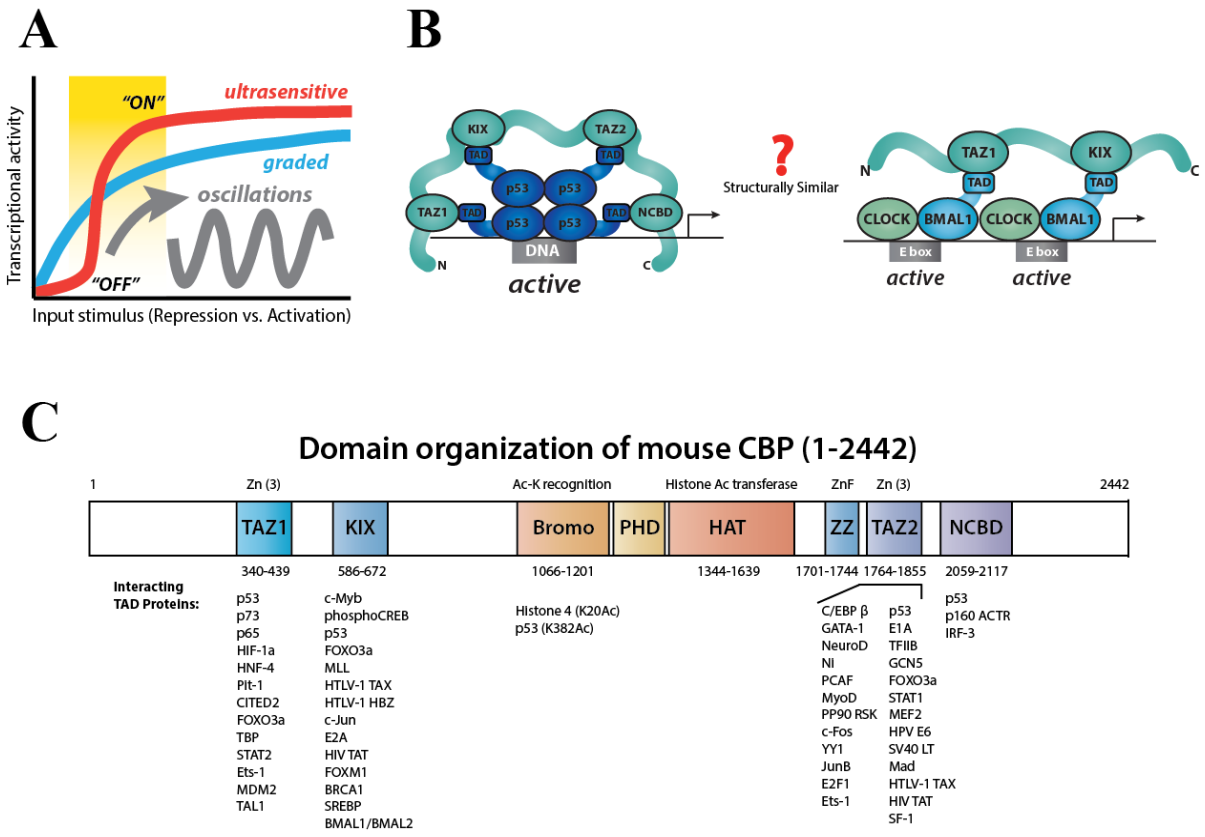


Figure 2. Model of CLOCK:BMAL1 cooperativity with transcriptional activator CBP/p300.
(A) Ultrasensitivity is needed to elicit switch-like responses in biological oscillators. **(B)** Tetrameric p53 can bind simultaneously to four different domains of CBP/p300. We propose that tandem complexes of CLOCK:BMAL1 may also engage in cooperative interactions with multidomain CBP/p300. **(C)** CBP has at least four domains that are known to interact with TADs: TAZ1, KIX, TAZ2, and NCBD domains interact with many transcription factors as shown above.

To determine how the CLOCK:BMAL1 complex might be cooperatively regulated by CBP/p300, we first set out to identify the molecular basis of their interactions. We previously showed that the BMAL1 TAD interacts with the KIX domains of both CBP and p300[11]. Because of the similarity between CBP and p300, we have chosen to focus on CBP to identify additional domains that may also interact with the BMAL1 TAD. In theory, the TAZ1, TAZ2, and NCBD domains of CBP/p300 each have the potential to interact with the BMAL1 TAD. However, most transcription factors only interact with a subset of these domains **(Figure 2C)**, so we expect that the BMAL1 TAD will exhibit some preference in its interactions with CBP domains. Through a

series of biophysical and biochemical assays, I identified that the TAZ1 domain interacts with the BMAL1 TAD at a site that overlaps with the binding site of the repressor CRY. I also showed that TAZ1 preferentially binds to one conformation of the BMAL1 TAD switch that is needed for proper circadian cycling. This differential binding could lead a regulatory role in the interaction between CBP/p300 and BMAL1. Identifying the molecular basis for interactions between the circadian transcription factor CLOCK:BMAL1 and its coactivator CBP represents a large step forward in our understanding of the circadian clock.

Results

Optimizing purification of the TAZ1 domain of CBP

The TAZ1 domain of CBP was initially cloned into various expression plasmids with several different fusion protein tags, including His_6, His_6GB1, or His_6NusA-XL, to enhance the soluble expression and affinity purification of recombinant TAZ1 from *E. coli*. Expression and solubility tests were performed with all three tagged constructs, but each of these affinity tags led to misfolding and aggregation of the solubly expressed TAZ1 protein. We also found that any well-folded TAZ1 we could obtain from soluble preparations copurified with nucleic acids from *E. coli*, so we set out to establish a new purification procedure.

After further testing and optimization, use of a tagless TAZ1 expression construct led to the best recovery of protein after purification. A 4 hour bacterial growth at 37°C led to high expression of the tagless TAZ1 domain; however, the lack of a solubilizing tag drove TAZ1 to the insoluble fraction of the cell lysate. We exploited this to purify the insoluble inclusion bodies from *E. coli* lysates, in which TAZ1 was unfolded but at high (>90%) purity. TAZ1 was resolubilized from inclusion bodies with addition of the chemical denaturant, urea. Solubilized and unfolded TAZ1 protein was then passed over a cation exchange column to bind the protein and remove the urea; purifying the insolubly expressed protein in this manner also had the benefit of removing non-specifically bound nucleic acids. Once eluted from the cation exchange column with high salt, TAZ1 was in a soluble, but unfolded state.

To get meaningful biochemical data, TAZ1 needs to be folded in its native state. The three main alpha helices in TAZ1 coordinate binding to three zinc molecules **(Figure 3A)**. Observation of alpha helical secondary structure indicates that TAZ1 has refolded into its native tertiary structure, because it was previously shown that TAZ1 can take on this helical structure

only after binding to three equivalents of zinc. Therefore, three equivalents of zinc were added to the soluble, unfolded protein to initiate folding of TAZ1 and circular dichroism was utilized to examine the protein secondary structure. Misfolded or unfolded protein would be easily identified by CD spectra, which indicates unfolded (or random coil) protein with a single minimum at 200 nm **(Figure 3B)**. The CD spectrum of purified, zinc-bound TAZ1 confirmed that our purification procedure produced a well-folded alpha helical protein with the characteristic minima at 208 and 222 nm **(Figure 3C)**.

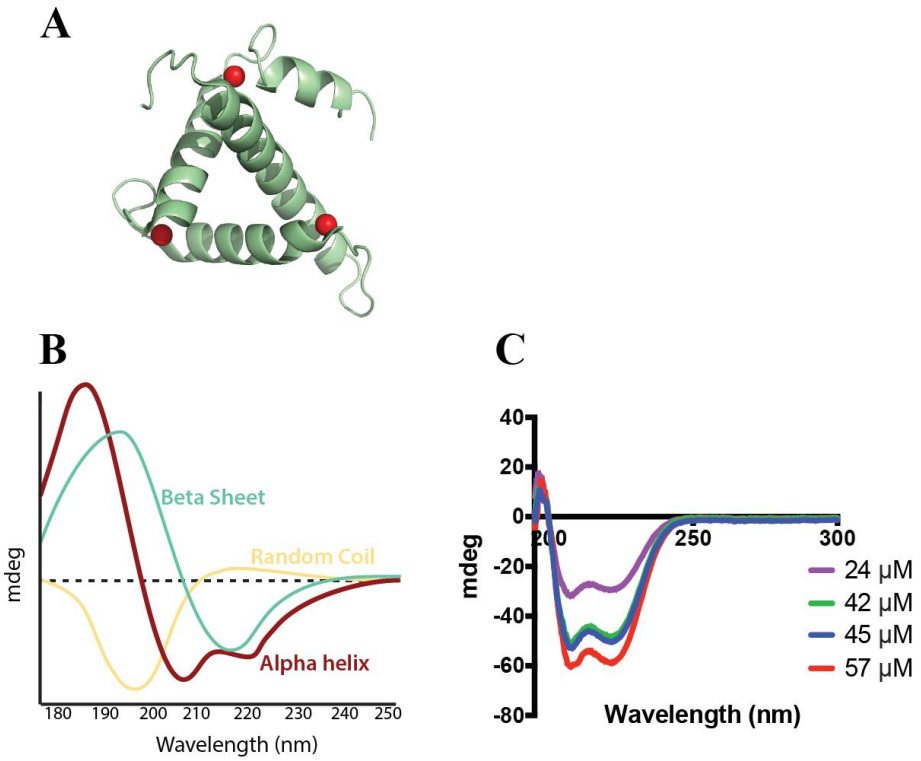


Figure 3. Validating proper refolding of the TAZ1 domain. (A) The TAZ1 (transcriptional adapter zinc binding) domain (PDB:1U2N) of CBP is a zinc-binding motif that has four alpha helices, which are packed against one other to form a hydrophobic core[20]. Three zinc atoms are each coordinated to one histidine and three cysteine residues located at the end of one helix, in the interconnecting loop, and the beginning of the second helix[21]. **(B)** Alpha helix, beta sheet, and random coil secondary structures give rise to a characteristic shape and magnitude in the CD spectrum. The CD spectrum of a given protein represents a linear combination of these features. **(C)** The alpha helical structure of refolded TAZ1 was confirmed at various concentrations by CD.

TAZ1 interacts with the BMAL1 transactivation domain

To determine if the TAZ1 domain can interact with the BMAL1 TAD, I utilized NMR spectroscopy to identify and map protein interaction sites at atomic resolution. 2D ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) NMR experiments allow us to visualize the chemical shift of an NMR-active, spin-½ ^{15}N nucleus that is directly bound to a proton. Every amino acid other than proline contains this amide bond, giving us one backbone peak for each of these amino acids in an HSQC spectrum. The chemical shift of these peaks are highly sensitive to the local chemical environment. Therefore, we can monitor ^{15}N-labeled BMAL1 TAD by NMR in the presence of unlabeled TAZ1; if the proteins interact directly, it will lead to changes in peak location or intensity of the ^{15}N-labeled nuclei at the binding site(s).

I collected a series of HSQC spectra of ^{15}N BMAL1 TAD alone or with increasing amounts of TAZ1 **(Figure 4A)**, and confirmed that a BMAL1 TAD-TAZ1 complex was formed. NMR titration experiments revealed that significant changes in peak intensity occurred throughout the ^{15}N BMAL1 TAD. Here, a loss in peak intensities suggests a change in protein dynamics that is indicative of complex formation at these sites. To identify the residues on the TAD that interact with TAZ1, I measured peak intensities in the absence and presence of increasing concentrations of TAZ1 **(Figure 4B)**. These analyses suggest that TAZ1 interacts with three distinct sites on the TAD: the N-terminal hydrophobic region, α-Helix, and the switch. The three main alpha helices of TAZ1 provide three interfaces for interacting proteins, suggesting that each TAD motif could dock onto one of TAZ1 helices, a common binding motif for other TADs[22]. This is in contrast to the KIX domain, which only binds to the alpha helix and switch regions of BMAL1 TAD. These data suggest that the hydrophobic region at the N-terminus of the TAD may be particularly important for distinguishing binding by the KIX and

TAZ1 domains. These data also show that, like KIX, TAZ1 interacts with the BMAL1 TAD at sites that overlap with the binding site of the repressor CRY[11]. This furthers our understanding of how the CLOCK:BMAL1 complex can be regulated from a repressed to activate state.

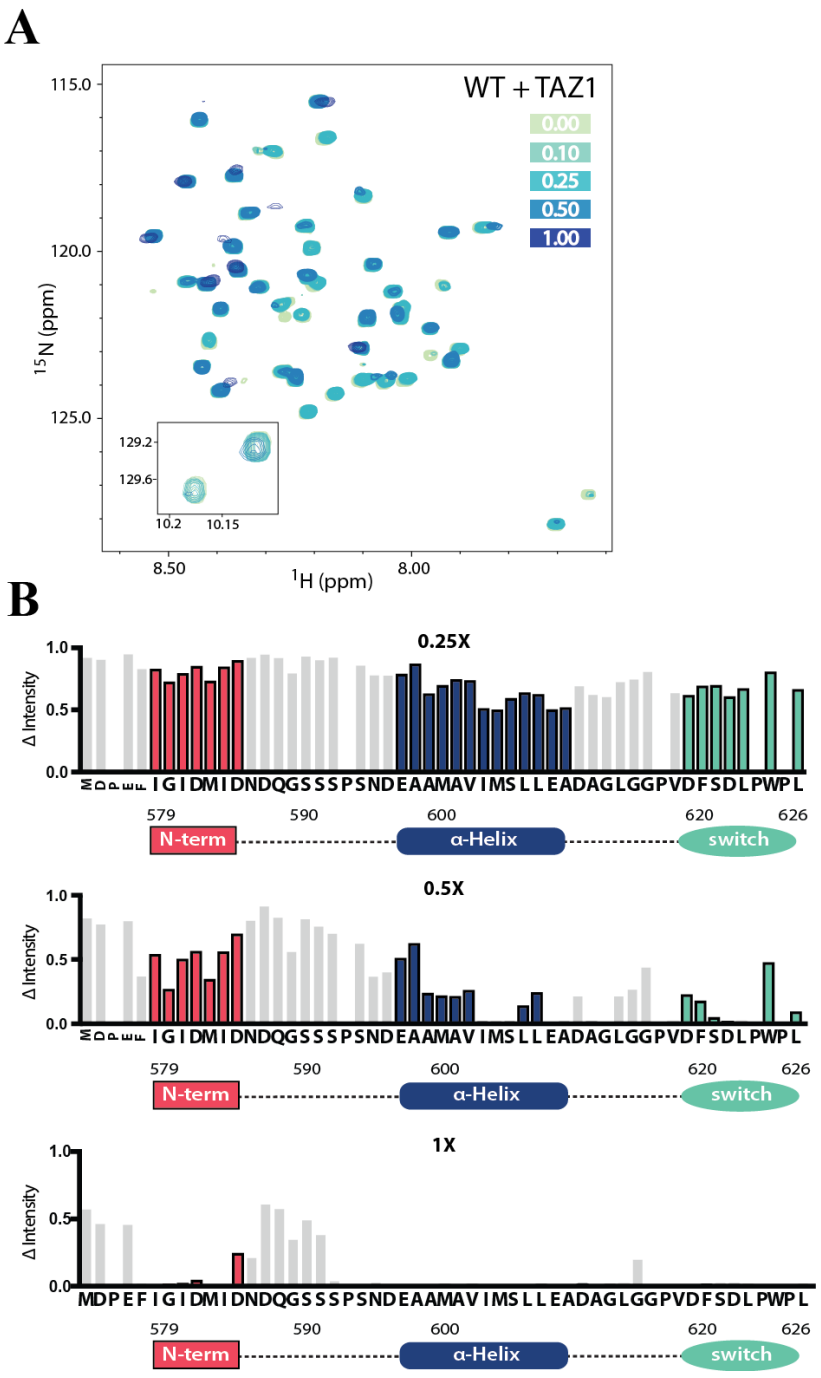


Figure 4. Mapping BMAL1 TAD binding with TAZ1. (A) HSQC spectra of ^{15}N BMAL1 TAD titrated with increasing amounts of TAZ1, represented from green to blue. The TAZ1 protein was added stepwise in 0.10X, 0.25X, 0.50X, and 1.00X molar increments. **(B)** Differential peak intensities were calculated for each amino acid in the BMAL1 TAD by dividing peak intensities of the TAD with increasing concentrations of TAZ1 by the intensity of the BMAL1 TAD alone. A Δ Intensity of 1 indicates no change in peak intensity, representing no interaction. Decreasing Δ Intensity values indicate a change in dynamics that we attribute to protein binding. Because proline gives no HSQC signal due to its lack of backbone amide, the proline residue is represented with a Δ Intensity value = 0.

Probing interactions of TAZ1 with BMAL1 by NMR

To investigate how all three regions of the BMAL1 TAD collectively interact with TAZ1, we utilized a mutated BMAL1 TAD that disrupts binding of key transcriptional regulators by mutating L606A and L607A in the alpha helical region, referred to as the LL/AA mutant[11]. NMR HSQC spectra of BMAL1 TAD LL/AA alone and with increasing amounts of TAZ1 were collected **(Figure 5A)**. In contrast to the wild type protein, we measured only small changes in chemical shift perturbations throughout the BMAL1 TAD protein. Differences in peak intensities were plotted, and identified that mutating the alpha helical region significantly interfered with binding to the BMAL1 TAD **(Figure 5B)**. From the analysis of peak intensities, it is clear that the mutant essentially eliminated interaction of the three regions of the BMAL1 TAD with the TAZ1 domain. The KIX domain also demonstrated decreased affinity for the LL/AA mutant; however, in that case we still observed moderately weak binding at the helix and switch region by NMR[11]. Overall, the dramatic decrease in affinity for TAZ1 suggests that binding of the α-Helix, switch, and N-terminal regions are more dependent on one another for TAZ1 binding than they are for KIX, indicating distinct binding mechanisms.

Using NMR spectroscopy, we recently discovered that there is a slow switch between *trans* and *cis* conformers of the protein backbone at the tryptophan-proline (W624-P625)[12]. Each amino acid in the switch region of the BMAL1 TAD shows two distinct peaks because the WP peptide bond exists in two states: the *cis* and *trans* conformers. Because peptide bonds are almost always in the *trans* configuration due to steric hindrance, this slow switch could act as a regulatory mechanism to control CLOCK:BMAL1 activity. Strikingly, the only residues of BMAL1 TAD LL/AA mutant that retained interactions with TAZ1 were those in the switch region, and specifically, those in the *cis* configuration. These data suggest that TAZ1

preferentially binds to the *cis* conformer of the BMAL1 TAD. Throughout our study of CLOCK:BMAL1 regulatory proteins, the TAZ1 domain is the first to exhibit an apparent differential affinity for the *cis* and *trans* conformers. Therefore, TAZ1 is poised to play a vital and exciting role in regulating the BMAL1 TAD *in vivo* due to its preferential binding properties.

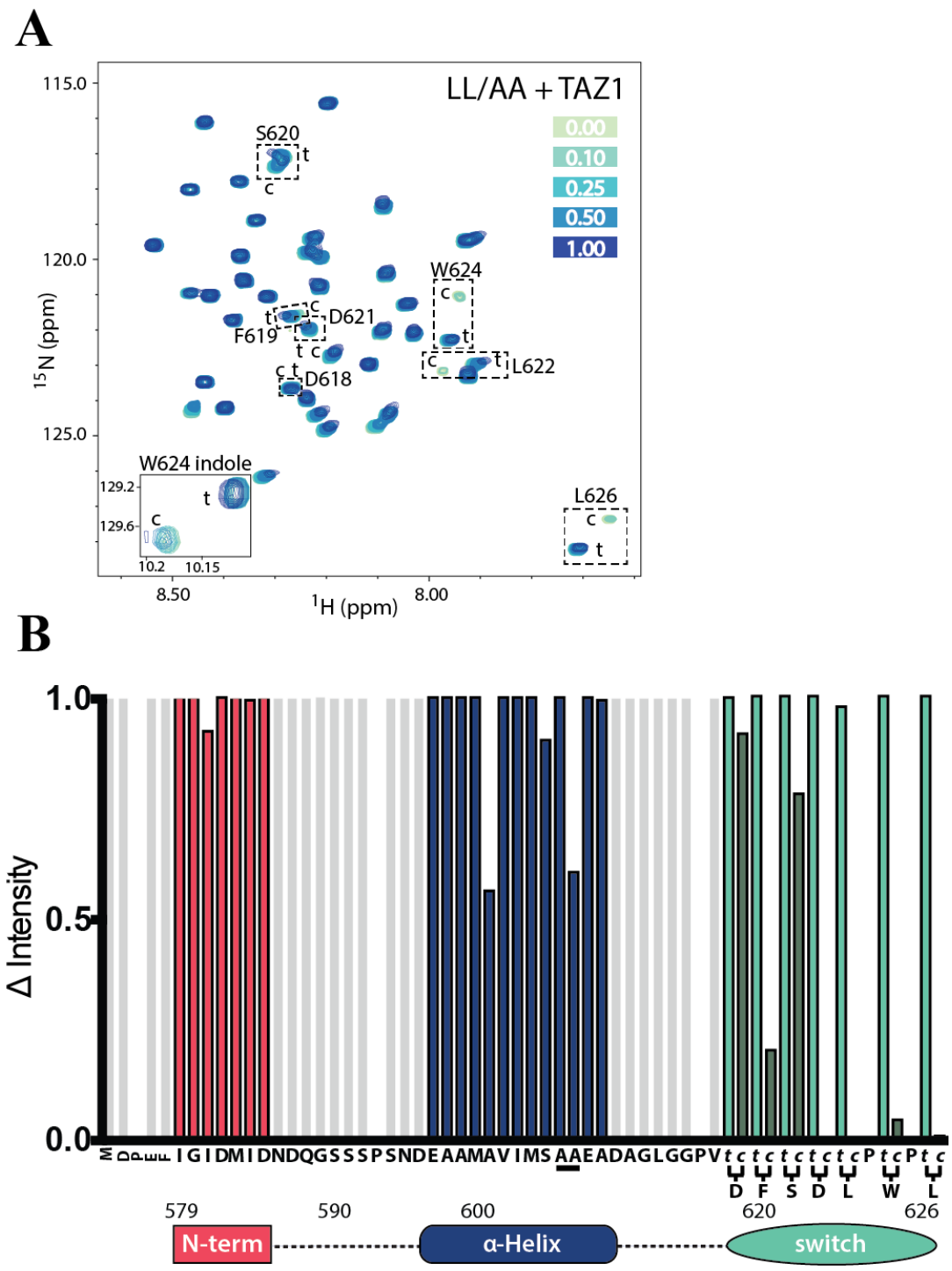


Figure 5. LL/AA mutant decreases overall binding, except cis conformational interactions. (A) HSQC spectra of ^{15}N BMAL1 TAD LL/AA titrated with increasing amounts of TAZ1, represented from green to blue. Many of the change intensity with increasing amounts of TAZ1, indicating little change in the chemical environment. The TAZ1 protein was added stepwise in 0.10X, 0.25X, 0.50X, and 1.00X increments. **(B)** Differential peak intensities for each amino acid in the LL/AA mutant are plotted. This plot includes the changes in peak intensities for amino acids in *cis* configuration. Proline gives rise to no signal in the HSQC and is plotted at an intensity value of 0.

Differential binding between TAZ1 and BMAL1 TAD

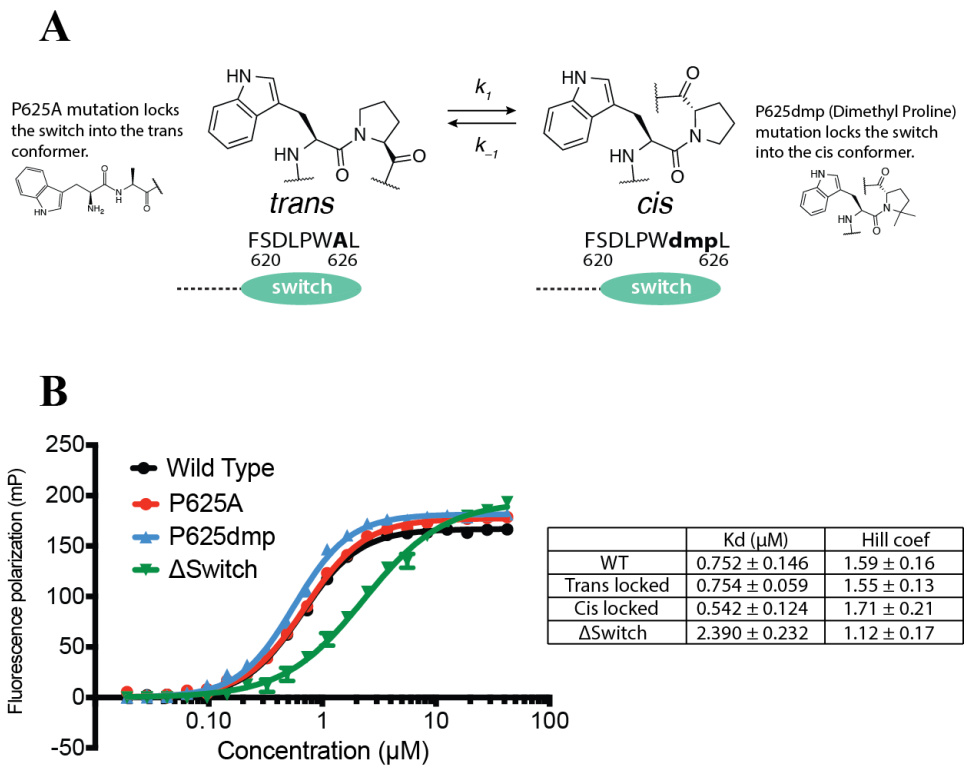
To further explore how proline isomerization may affect the affinity of BMAL1 TAD for the TAZ1 domain, we performed fluorescence polarization (FP) assays. Our lab previously designed truncated fluorescently-labeled BMAL1 TAD constructs that contained only the α-Helix and switch regions. Since TAZ1 showed differential binding in the switch region, mutations of critical residues needed for isomerization were tested for binding to TAZ1: WT, P625A, P625dmp, and ΔSwitch. The P625A mutation locks the peptide bond into the *trans* conformer, while the P625dmp (5,5 dimethyl proline) mutation locks the backbone into the *cis* conformer **(Figure 6A)**. The ΔSwitch mutation completely removes the switch region of BMAL1, leaving only the α-Helix to interact.

Fluorescence polarization allows one to monitor changes in the rotation time of fluorescently-labeled molecules; as TAZ1 binds to the labeled BMAL1 TAD, the rotational rate of the bound molecule decreases due to its increased size. This difference in rotation from the unbound to bound states is used to plot out the concentration-dependence of complex formation, to derive the equilibrium dissociation constant (K_d) and Hill coefficient (h) of the reaction **(Figure 6B**) to report on the affinity and potential cooperativity of binding, respectively.

The FP assay provided evidence of cooperative interactions between the α-Helix and switch regions of the TAD and the TAZ1 domain. The Hill coefficients for the WT, P625A, and P625dmp mutants were all above the value of 1, which indicates positive cooperative binding. We interpret this to mean that once TAZ1 initially binds to the first region of the TAD, a second region of the TAD binds with increased affinity. From our LL/AA mutational data, we have shown the α-Helix region is the primary binding interface for transcriptional regulators[11]. The ΔSwitch mutant recorded a decreased Hill coefficient and much closer to the value of 1,

representing noncooperative binding; this is expected because only the α-Helix region is available to bind in this construct. The positive cooperativity found between the α-Helix and switch regions contribute to the understanding of the TAZ1 binding mechanism.

In exploring differential affinity of TAZ1 for the switch conformers, we only noted slight differences in affinity between the WT, P625A, and P625dmp peptides. The wild type BMAL1 TAD binds to TAZ1 with an affinity of 0.752 μM, the *trans*-locked P625A mutant binds with an affinity of 0.754 μM, and the *cis*-locked P625dmp mutant binds with a slightly higher affinity of 0.542 μM. However, the difference in binding between the *cis* and *trans* states is not large enough at this point to make a conclusion regarding differential binding. It is worth nothing that the fluorescently-labeled BMAL1 TAD peptide used in these assays does not include the N-terminal region; this truncated peptide was originally designed based on our KIX binding data [11], which indicated that only the α-Helix and switch regions were important for TAD binding. Given our NMR data for TAZ1, the N-terminal region lacking from this truncated peptide may be critical for differential binding of the switch region. Overall, the switch region is clearly important for binding to the TAZ1 domain, as the ΔSwitch mutant binds with an affinity of 2.390 μM, several fold weaker than the wild type peptide.



	Kd (μM)	Hill coef
WT	0.752 ± 0.146	1.59 ± 0.16
Trans locked	0.754 ± 0.059	1.55 ± 0.13
Cis locked	0.542 ± 0.124	1.71 ± 0.21
ΔSwitch	2.390 ± 0.232	1.12 ± 0.17

Figure 6. Characterization of cis/trans binding affinities through fluoresce polarization. (A) P625A and P625dmp mutants lock the TAD into a *cis* or *trans* conformational state. **(B)** WT, P625A, P625dmp, and ΔSwitch mutants are plotted with increasing TAZ1 concentration against fluorescence polarization. The corresponding K_d and Hill coefficients are listed as average values with standard deviations from three independent assays.

Discussion

Through biophysical and biochemical assays, I determined that the TAZ1 domain of CBP interacts with three distinct regions of the BMAL1 TAD, demonstrating that multiple domains of CBP interact with BMAL1. Selective binding of multiple CBP domains with the BMAL1 TAD could therefore play a role in eliciting cooperative regulation of CLOCK:BMAL1 heterodimers bound to tandem sites on DNA. Cooperativity gives rise to ultrasensitive responses that play a role in the switch-like activation of CLOCK:BMAL1 complexes from a mostly "off" to a mostly "on" state with small change in coactivator concentration. More work will be needed to see how the coactivator CBP simultaneously interacts with multiple TADs from adjacent BMAL1, but it

is exciting to note that the two TAD-binding modular domains from CBP are located next to one another in the coactivator, simplifying models for recruitment to proximal TADs.

As part of this work, I discovered that CBP TAZ1 appears to exhibit differential binding for the two conformations of the BMAL1 TAD C-terminal switch, which could lead to a major regulatory role in the circadian clock. TAZ1 appears to preferentially bind to the *cis* conformer of the BMAL1 TAD, which does not occur between BMAL1 and other transcriptional regulators examined thus far. Therefore, the TAZ1 domain of CBP is the only regulator studied by our lab that interacts at three distinction regions of the TAD and the first to demonstrate the potential for differential binding affinities at the *cis*/*trans* switch region. Through future research, we hope to define the mechanism by which other domains of CBP/p300 (e.g. TAZ2, NCBD) interact with CLOCK:BMAL1 **(Figure 7)**. By identifying the molecular basis of interactions between the circadian transcription factor CLOCK:BMAL1 and its coactivator CBP, we will provide key insight into important steps in generation of the mammalian circadian clock.

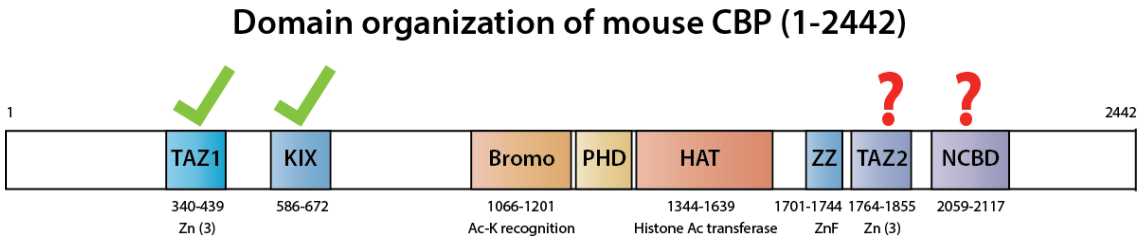


Figure 7. Domain organization of multidomain CBP. The TAZ1 and KIX domains have now been confirmed to interact with the BMAL1 TAD. The interaction of multiple domains with BMAL1 could lead to ultrasensitive responses that regulate the "on" and "off" states of the circadian clock via CLOCK:BMAL1. Further research will explore whether or not the TAZ2 and NCBD domains also interact with BMAL1.

Methods

Cloning the TAZ1 domain

The mammalian expression vector for mouse CBP was kindly provided by Andrew Liu (University of Memphis). The TAZ1 domain was amplified through PCR, treated with restriction digest enzymes BamH1/NotI or NdeI/NotI for ligation into the appropriate expression vectors. DNA encoding the TAZ1 domain was ligated into the parallel series of bacterial expression vectors (based on the pET22b backbone) containing a His_6, His_6GB1, or His_6NusA-XL tags for soluble expression, or the pET22b vector with no affinity tag for insoluble expression. All constructs were validated by sequencing.

Growth and Purification of the TAZ1 domain

Tagless-TAZ1 constructs were transformed into the *Escherichia coli* (*E. coli*) Rosetta (DE3) strain for recombinant protein expression. Large-scale growths were performed in Luria Broth (LB) media containing ampicillin to promote plasmid selectivity and chloramphenicol to maintain expression of rare codons that enhance expression of mammalian proteins. The large-scale LB growths were performed at 37 °C; once the measured optical density of the cells were 0.6-0.8 units of optical density at 600 nm (OD_{600}), expression was induced with 1 mM IPTG and 50 µM $ZnSO_4$ and grown for 4 hours at 37 °C. Bacterial pellets were collected by centrifugation, and the cells were resuspended in Buffer A (50mM Tris pH 7.5, 300mM NaCl, 20mM Imidazole, 20mM DTT, 50µM ZnSO4) and lysed by high pressure on ice. The crude extract was separated by centrifugation at 19,000 rpm at 4 °C. The insoluble pellet containing TAZ1 protein was washed once with Buffer A and repelleted through centrifugation. The final insoluble pellet was resuspended in Buffer B (10 mM Tris pH 7.5, 20 mM NaCl, 6 M Urea, 20 mM DTT, 50 µM

$ZnSO_4$). Full resuspension of the inclusion bodies was achieved through sonication (40% amplitude, 15 sec on, 30 sec off) and vortexing, and the solubilized pellet was centrifuged to remove insoluble particulates. The supernatant from this spin was filtered with a 0.40 micron membrane and passed over a SP sepharose cation exchange column using a fast performance liquid chromatography (FPLC) instrument. Protein was eluted with a gradient from Buffer C (50 mM Tris pH 7.5, 20 mM NaCl, 20 mM DTT, 50 µM $ZnSO_4$) to Buffer D (50 mM Tris pH 7.5, 1 mM NaCl, 20 mM DTT, 5 0µM $ZnSO_4$). After elution, three equivalents of $ZnSO_4$ were added to fractions containing TAZ1, to properly refold the previously denatured protein. Protein concentration was determined by absorbance at 280 nm utilizing a calculated extinction coefficient of 5,500. Protein concentration was additionally verified through Bradford assay. Refolded protein was then run on a preparative Superdex 75 size exclusion column, and eluted in TAZ Buffer (10 mM MES pH 6.5, 50 mM NaCl, 2 mM DTT). Each purification step was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Circular dichroism

CD spectra were acquired on a JASCO J-1500 CD spectrometer. Four independent spectra were obtained with quartz cells of 1 mm path length with protein samples containing 24, 42, 45 and 57 µM TAZ1 protein in TAZ Buffer. In order to correct for the absorbance of the buffer and cuvette, a zero blanking of the spectrophotometer with buffer alone was performed.

Nuclear magnetic resonance spectroscopy

^{15}N-labeled BMAL1 TAD proteins were previously purified and stored at -80 °C in 10 mM MES pH 6.5, 50 mM NaCl[11]. Proteins were thawed at room temperature and DTT was added to a

final concentration of 2 mM. ^{1}H-^{15}N Heteronuclear Single Quantum Coherence (HSQC) spectra were acquired on a triple resonance, cryoprobe equipped Varian Unity Inova 600 MHz spectrometer at 25 °C. HSQC spectra of unbound ^{15}N labled BMAL1 TAD or ^{15}N BMAL1 TAD LL/AA were recorded at 100 µM final concentration; TAZ1 was gradually titrated into solutions with the ^{15}N BMAL1 constructs and spectra were recorded again. The data were processed with using NMRPipe/NMRDraw. Peak intensities were measured with NMRViewJ through integration of NMR signals of assigned chemical shifts as previously done[11].

Fluorescence Polarization

The BMAL1 TAD WT, P625A, P625dmp and ΔSwitch peptide probes were purchased from Bio-Synthesis Inc (Lewisville, TX) with a 5,6-TAMRA fluorescent probe covalently attached to the N-terminus. TAZ1 binding assays were performed in 10 mM MES pH 6.5, with 50 mM NaCl and 0.05% (volume/volume) Tween-20. Binding was monitored by changes in fluorescence polarization with a Perkin Elmer En Vision 2103 Multilabel plate reader with excitation at 531 nm and emission at 595 nm. The Hill coefficient and equilibrium binding dissociation constant (K_d) were calculated by fitting the dose-dependent change in millipolarization (Δmp) to a one-site specific binding model in GraphPad Prism, with averaged Δmp values from duplicate or triplicate assays. Data shown are from one representative experiment of three independent assays.

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