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ANION-DRIVEN CONFORMATIONAL CHANGES IN CK1δ REGULATE CIRCADIAN RHYTHMS

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By
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Abstract

Circadian rhythms are periodic changes in an organism's physiology that align with the 24-hour solar day. In order to conserve energy, organisms generate circadian rhythms via a core molecular clock that oscillates gene transcription of important cellular functions such as the sleep/wake cycle, metabolism, DNA repair, and hormone production/release (Lowrey and Takahashi 2004). The core molecular clock is generated by an interlocking transcription/translation feedback loop of core clock proteins (Reppert and Weaver 2002, Ko and Takahashi 2006). The molecular clock protein, Period2 (PER2), is integral to maintaining a 24-hour period (Chen, Schirmer et al. 2009). PER2 forms a repressive complex with cryptochromes (CRY1/2), and inhibits the transcription of clock genes, including itself (Ko and Takahashi 2006, Partch, Green et al. 2014). A key regulator of PER2 is casein kinase 1δ (CK1δ), which phosphorylates PER2 at two distinct regions, causing either proteasomal degradation via a degron or stabilization by phosphorylation of the Familial Advanced Sleep Phase (FASP) region downstream (Etchegaray, Machida et al. 2009). Mutations in the FASP region of PER2 that inhibit CK1δ phosphorylation or mutations in CK1δ that alter activity on the FASP region cause a short circadian period, leading to an advance in sleep phase (Xu, Toh et al. 2007, Narasimamurthy, Hunt et al. 2018, Philpott, Narasimamurthy et al. 2019). In this thesis, the structure of CK1δΔC and the FASP sequence were investigated to determine crucial residues and regions that enable CK1δ regulation of PER2. CK1δΔC was crystallized in the absence of anions to reveal two conformations of the activation loop that influence substrate selection. This suggests that negatively charged species, such as phospho-serines from the FASP region, could influence the activity of CK1δ by changing the conformation of the activation loop. Adjacently, the FASP region was examined in order to determine important residues for CK1δ activity. The identity of the plus-one residue following the targeted serine was found to be crucial for kinase activity, and therefore PER2 stability. This work has made an important contribution to understanding the molecular determinants underlying CK1δ regulation of the FASP region, which ultimately establishes a ~24-hour circadian period in humans.

Introduction

Circadian rhythms are patterns of behavior and physiology that oscillate with a ~24-hour periodicity in the absence of external stimuli but are synchronized to an exact 24-hour cycle in accordance with the solar day (Turek 1985). Circadian rhythms are found in nearly all eukaryotes and cyanobacteria where they enable organisms to anticipate regular changes in their external environment. Temporal regulation of metabolism and gene transcription allow organisms to conserve energy (Lowrey and Takahashi 2004). In mammals, behavioral and physiological changes are established by a molecular clock that generates temporal oscillations in gene transcription via an interlocking transcription/translation feedback loop of core clock proteins (Bass and Lazar 2016).

Four proteins are crucial for establishing the transcription/translation feedback loop that generate circadian rhythms in animals: the protein subunits CLOCK and BMAL form a heterodimeric transcription factor that drives the transcription of ~3000 unique genes including its repressors, *Per* and *Cry*, which form part of a repressive complex that ultimately interacts directly with CLOCK:BMAL and inhibits gene transcription (Ko and Takahashi 2006, Koike, Yoo et al. 2012, Takahashi 2015). PER and CRY are degraded by proteasome ubiquitin-dependent pathways, allowing for transcriptional activation to restart and establishing a ~24-hour period (**Figure 1**) (Zhou, Kim et al. 2015, Aryal, Kwak et al. 2017).

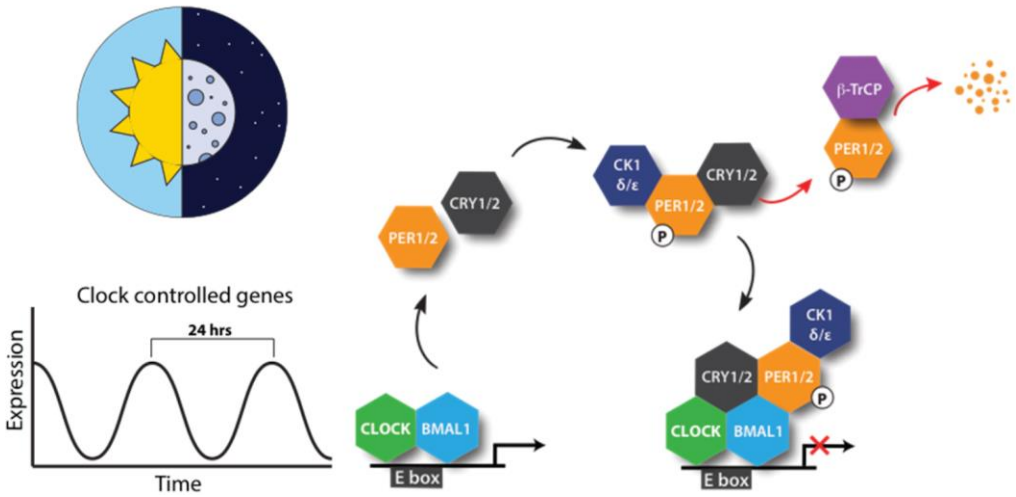

Figure 1 | A transcription/translation feedback loop establishes periodic gene expression. The transcription factor CLOCK:BMAL1 promotes the transcription of core clock genes that encode for the proteins PER1/2 and CRY1/2, which form a complex with the kinases CK1δ or CK1ε. Phosphorylation of PER1/2 by CK1δ or CK1ε can either lead to its degradation or enhance its stability to control clock timing. In the nucleus, complexes of CRY1/2, PER1/2, and CK1δ/ε bind directly to CLOCK:BMAL1, nucleating formation of the repressive complex and causing inhibition of gene transcription.

Although most components of the clock are embedded within the transcription/translation feedback loop, nearly all of them can be expressed constitutively and the clock still functions; however, constitutive expression of PER2 in mice causes complete loss of circadian rhythms, implicating daily changes in PER2 abundance as an important determinant of the molecular clock (Chen, Schirmer et al. 2009). Therefore, PER2 abundance is tightly controlled at the transcriptional, post-transcriptional, and post-translational levels (Aryal, Kwak et al. 2017). At the protein level, PER2 stability is regulated by kinase activity from CK1δ (Etchegaray, Machida et al. 2009). Polymorphisms in PER2 and CK1δ/CK1ε give rise to altered circadian periods and lead to human sleep disorders such as: Familial Advanced Sleep Phase Syndrome (FASPS), which results in a circadian period shorter than 24-hours, and Delayed Sleep Phase Syndrome which results in a period longer than 24-hours (Lowrey, Shimomura et al. 2000, Toh, Jones et al. 2001, Xu, Padiath et al. 2005). Therefore, understanding the basis by which CK1δ regulates PER2 stability could help provide new strategies for controlling circadian rhythms and these sleep disorders in humans.

PER2 stability is regulated by CK1δ kinase activity through a 'phosphoswitch' where two regions of PER2, one causing increased stability and the other causing degradation, compete as potential substrates for CK1δ activity (Zhou, Kim et al. 2015). Phosphorylation of the degron at Serine 478 (S478) recruits the E3 ubiquitin ligase β-TrCP in a CK1-dependent manner to initiate degradation of PER2 (Eide and Virshup 2001, Eide, Woolf et al. 2005). The phosphoswitch model posits that degron phosphorylation is counteracted by phosphorylation of a downstream region, introducing a delay in the degradation of PER2 that is essential for the ~24-hour timing of circadian rhythms. However, the detailed mechanism by which the phosphoswitch works is currently not understood.

The stabilizing region of PER2, the FASP region, is located within the kinase binding domain of PER2 that allows the kinase to remain stably anchored to PER2 throughout the day (Aryal, Kwak et al. 2017). This region is named after a point mutation in the first serine in the sequence (S662G), which gives rise to Familial Advanced Sleep Phase (FASP) syndrome (Toh, Jones et al. 2001). FASP syndrome is characterized by a 4-hour advance in sleep phase (short clock phenotype) and is caused by the loss of phosphorylation of S662 and downstream serines in this region (Toh, Jones et al. 2001, Narasimamurthy, Hunt et al. 2018). The FASP region contains five target serines, four of which closely resemble the established CK1 consensus sequence motif, pSxx**S**, where priming phosphorylation (pS) templates high kinase activity 3 residues downstream (bold) (Flotow, Graves et al. 1990, Goldsmith, Akella et al. 2007). In PER2 a similar motif is found, where the first serine starts at S662 and ends at S674, but the so-called

priming serine at S662 lacks the upstream phosphorylated serine found in the CK1 consensus motif (**Figure 2A**). Despite this deviation from the CK1 consensus motif, our lab has shown that CK1δ phosphorylates the priming serine at S662 in a rate-limiting step for FASP phosphorylation (Narasimamurthy, Hunt et al. 2018). Moreover, we showed that the kinase phosphorylates the five serines in a sequential manner, first acting slowly on the non-consensus priming site, and then acting with high efficiency on the downstream consensus sites. We also showed that mutating the priming site eliminates all downstream phosphorylation in PER2 leaving this stabilizing site inactive (Narasimamurthy, Hunt et al. 2018).

To visualize the phosphorylation state of individual amino acids in the FASP region, we used an NMR-based kinase assay developed by the Selenko lab (Theillet, Rose et al. 2013). My mentor, Sabrina Hunt, showed that mutating the first serine in this motif (S662), to a glycine or alanine results in the loss of kinase activity on all downstream serines in the motif (S665, S668, S671, and S674) (**Figure 2B**) (unpublished data). Conversely, mutating the first serine to an aspartate (S662D), which can chemically resemble a phosphorylated serine, allows for weak but constitutive priming of the downstream phosphorylation of serines in the motif , leading to a long circadian period in mice (**Figure 2C**) (Xu, Toh et al. 2007). Thus, phosphorylation of the non-consensus S662 site of PER2 is crucial for stability of PER2, and therefore important for establishing circadian period.

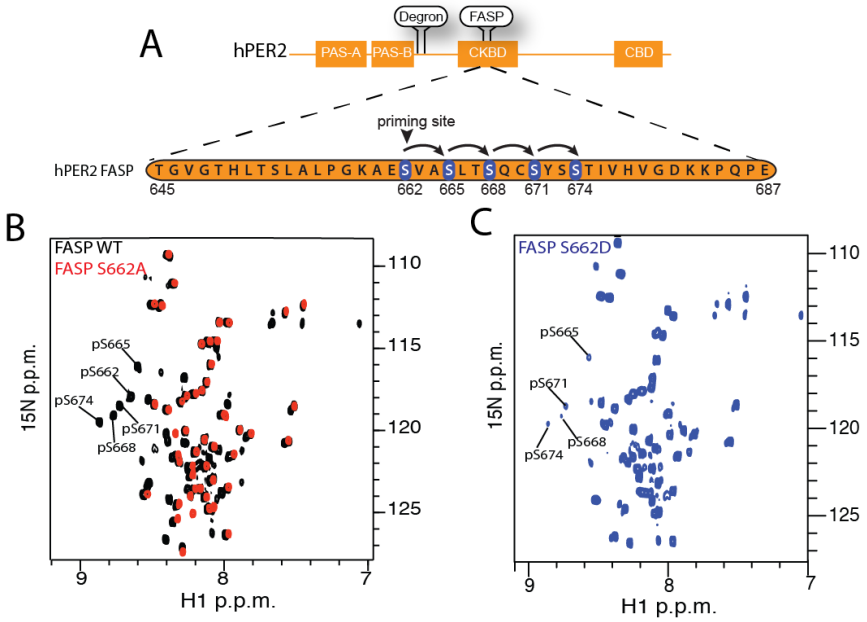

Figure 2 | Mutations in the PER2 FASP region result in altered circadian rhythms. (A) Diagram of human PER2 (hPER2) showing the locations and sequence of the FASP and degron sequences. PAS, PER-ARNT-SIM domains; CKBD, Casein Kinase-Binding Domain; CBD, CRY-Binding Domain. Below, the FASP region is highlighted with CK1 phosphorylation sites shown in blue. **(B)** Overlay of $^{15}N/^{1}H$ HSQC spectra from the NMR kinase assay with 200 µM ^{15}N FASP peptide (black) or FASP S662A (red) incubated with 1 µM CK1δ kinase domain 3 hr at 30°C. **(C)** Overlay of $^{15}N/^{1}H$ HSQC spectra with 200 µM ^{15}N FASP S662D (blue) incubated with 1 µM CK1δ 3 hr at 30°C.

CK1δ is the primary kinase that regulates PER2 stability; therefore, understanding how CK1δ acts upon PER2 is crucial for understanding how circadian period is established (Etchegaray, Machida et al. 2009). CK1δ possesses multiple anion-binding sites that are completely conserved amongst the CK1 family in eukaryotes from humans to green algae (**Figure 3**). Site 1 and site 2 anion binding pockets are located near the catalytic cleft and therefore thought to play a role in substrate binding and catalytic activity (Longenecker, Roach et al. 1996). Site 1 is thought to coordinate phosphorylated serines of the pSxxS recognition motif and position subsequent downstream serines for further phosphorylation (Longenecker, Roach et al. 1996, Zeringo and Bellizzi III 2014). Site 2 is more generally conserved amongst serine/threonine kinases, where it typically coordinates a phosphorylated residue from the activation loop. A number of kinases are regulated in this manner, serving as an on/off switch for catalytic activity in most serine/threonine kinases (Johnson, Noble et al. 1996). However, CK1δ does not require phosphorylation of its activation loop for kinase activity and is considered to be constitutively active (Goldsmith, Akella et al. 2007).

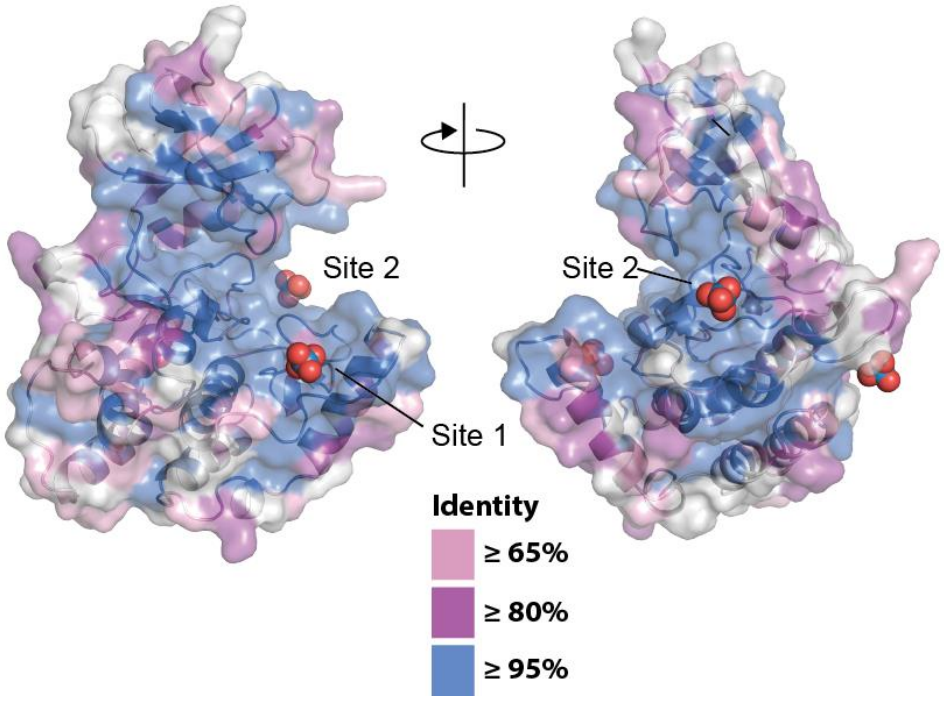

Figure 3 | Conservation of anion binding sites in CK1δΔC. Percent identity of site 1 and site 2 anion binding pockets from the following 20 species: *Danaus plexippus*, *Drosophilia melanogaster*, *Platynereis dumerilii*, *Euydice pluchra*, *Homo sapiens*, *Mus musculus*, *Danio rerio*, *Mesocricetus auratus*, *Nematostella vectensis*, *Cyanidoschyzon tauri*, and *Chlamydomonas* mapped onto CK1δ (1CKJ chain B).

Notably, CK1δ also differs from other serine/threonine kinases at site 2 by a deviation in the conserved 'APE' motif in this region. The 'APE' motif interacts with the +1 residue that follows the targeted serine and typically regulates substrate specificity in most kinases (Goldsmith, Akella et al. 2007). Therefore, aside from knowing that the CK1 family prefers the phosphorylated pSxxS motif, little is known about its substrate selectivity, and nothing is known about how the kinase selects non-consensus sites for activity. CK1δ also has an unstructured auto-inhibitory C-terminal tail that is often removed when doing kinetic assays *in vitro* and crystallography experiments (Graves and Roach 1995, Rivers, Gietzen et al. 1998).

Polymorphisms in CK1δ/ε presumably cause altered circadian periods by changing the structure and chemical environment of CK1δ/ε, and thus alter its activity on the FASP and/or degron sequence of PER2. The first circadian polymorphism observed in any mammal was first isolated from a Syrian hamster. The CK1ε (R178C) mutant was called *tau*, and shown to cause a 4-hour shortening of circadian period (Ralph and Menaker 1988, Lowrey, Shimomura et al. 2000). Because of its reduced activity on the common substrate, casein, the *tau* mutation was thought to cause a general loss of kinase activity, that ultimately resulted in a short period phenotype (Lowrey, Shimomura et al. 2000, Eide and Virshup 2001). However, it was later shown that the *tau* mutant had a surprising gain of function on the PER2 degron (Gallego, Eide et al. 2006). Although these data helped to explain the short period phenotype, as premature degradation of PER2, it wasn't clear how the *tau* mutant could increase kinase activity on the degron.

Over the last year, our lab has extensively studied the *tau* mutant to better understand its ability to dramatically alter circadian timing. Importantly, inserting the *tau* mutation into the truncated CK1δΔC kinase displays the same substrate activity *in vitro* as observed in the CK1ε *tau* mutant (Philpott, Narasimamurthy et al. 2019). Using a variety of kinase assays, from cellular assays using phosphospecific antibodies for S478 in the degron and the priming site S659 (in the mouse FASP, not pictured), to radiolabeled kinase assays *in vitro* (**Figure 4A-B**), our lab made the important discovery that while *tau* does indeed have increased activity on the degron site *in vitro*, it exhibits a loss of activity on the FASP site. These data suggest that the *tau* mutant somehow alters substrate recognition to 'flip' the specificity; where the wild-type (WT) protein has preference for the FASP substrate over the degron, and the *tau* mutant has preference for the reverse.

To better understand the cause of the *tau* mutant phenotype, my mentor Jon Philpott crystallized and solved the structure of the CK1δ kinase domain with the *tau* mutation (Philpott, Narasimamurthy et al. 2019). The crystal structure of the *tau* mutant (6PXN) revealed the loss of

anion binding at site 1 and site 2 (**Figure 4C**). Site 1 anion binding was most likely lost due to the mutation itself (R178C), as this residue coordinates an anion at site 1 in all other CK1δ kinase domain crystal structures (Longenecker, Roach et al. 1996). However, the loss of anion binding in site 2 was unexpected since the *tau* mutation is not part of the activation loop. Instead, we found that the loss of anion binding in site 2 is due to an allosterically-induced conformational change in the activation loop that physically blocks an anion from binding (**Figure 4C)**. Starting with a backbone flip of a conserved glycine, G175, the activation loop takes on a 'loop up' conformation. This particular conformation has been observed in a few other serine/threonine kinases that have atypical regulation of their activation loop (Nolen, Taylor et al. 2004). Therefore, we hypothesized that the cause of the *tau* phenotype resulted from this change in activation loop conformation

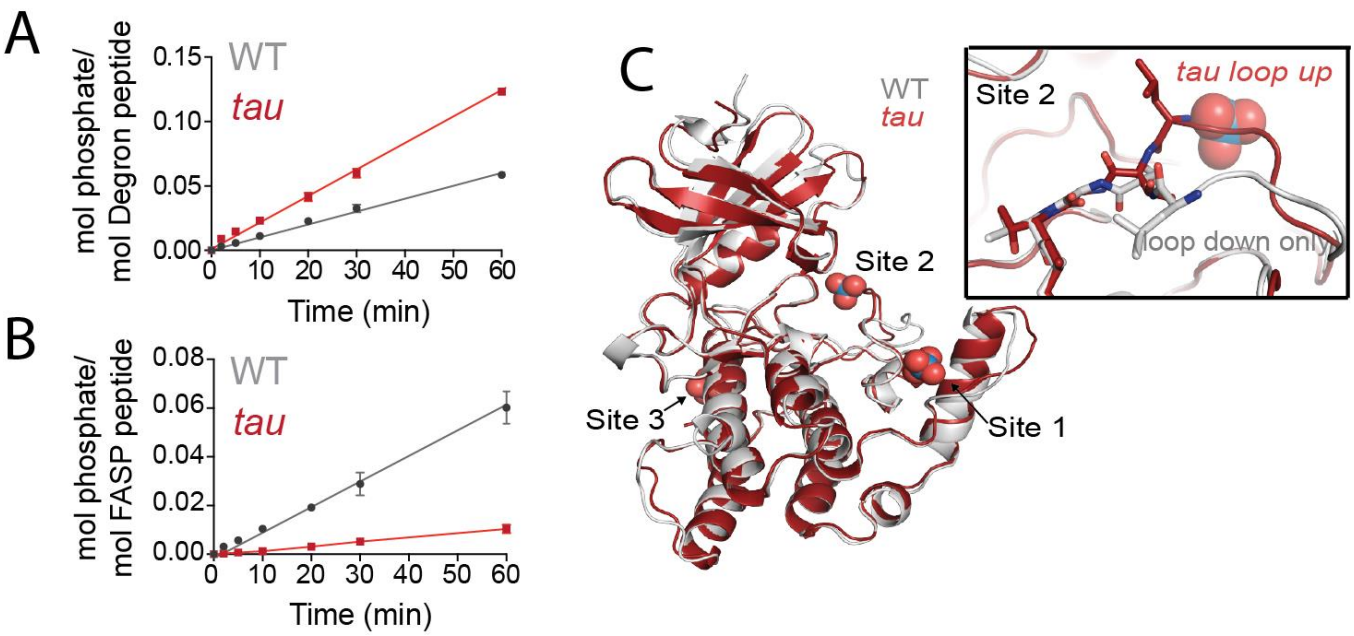

Figure 4 | Structure of the CK1δ kinase domain *tau* mutant. (A) Radiolabeled kinase assay with WT CK1δ kinase domain or *tau* (200 nM) and degron peptide (200 µM), (n = 4 with s.d.). **(B)** Radiolabeled kinase assay with WT CK1δ kinase domain or *tau* (20 µM) and FASP peptide (200 µM). **(C)** *Tau* structure (PDB 6PXN) and zoom-in of site 2 with both loop conformations. **Figures were taken from (Philpott, Narasimamurthy et al. 2019)**

Notably, only one other crystal structure of the CK1δ kinase domain in the RCSB Protein Data Bank (PDB) captured the kinase with the loop up conformation. Unlike all other structures of CK1δ in the PDB, the 1CKJ structure was initially crystallized with a low concentration of anions, and then later soaked in tungstate to map anion-binding sites (Longenecker, Roach et al. 1996). One other CK1δ crystal structure (4JJR) did not use any anions to crystalize CK1δ,

but lacked sufficient electron density to model the activation loop in the loop up conformation (Zeringo and Bellizzi III 2014). These crystal structures led me to hypothesize that the presence of anions would suppress the loop up conformation in WT CK1δ.

Interestingly, the *tau* mutant was crystallized in a high concentration of anions, yet we still observed the activation loop in its loop up conformation. This suggests that the tau mutation somehow stabilizes this alternate conformation of the activation loop. To test this, we collaborated with Clarisse Ricci in the McCammon lab at UCSD. She ran extended molecular dynamics (MD) simulations of both the *tau* and WT CK1δ kinase domain structures, which found that the loop up conformation indeed represents an energy minimum for the *tau* mutant, but not for the WT CK1δ (Philpott, Narasimamurthy et al. 2019). In conjunction with the detailed biochemical and cell-based assays in our current study (Philpott, Narasimamurthy et al. 2019), these molecular dynamics simulations help to explain the *tau* phenotype and support our hypothesis that stabilizing the loop up conformation of CK1δ leads to increase activity on the degron, and decreased activity on the FASP region of PER2.

In this thesis, I sought to determine if crystallizing the WT CK1δ kinase domain in a solution with no anions would allow the activation loop to take on the loop up conformation. To do this, I crystallized CK1δ in the absence of anions and solved the structure to 2.0Å (6PXO). In the absence of anions, this structure revealed that the WT kinase can indeed take on the same two activation loop conformations as the *tau* mutant and prior 1CKJ structure, providing further evidence that the WT CK1δ undergoes the same molecular switch in activation loop conformations and that its conformation is likely influenced by the presence of anions. I also studied the FASP region to identify what substrate residues are required for CK1δ activity on this important regulatory site. I found that the +1 residue of the priming serine is crucial for CK1δ activity on the FASP region. Therefore, the overall circadian period can be altered by mutation to the +1 residue of the priming serine in the FASP region, or by the stabilization of the loop up conformation of the activation loop.

Results

Optimization of recombinant CK1δ expression and purification

I spent a significant amount of time optimizing the purification of CK1δ due to low yields for the recombinant protein. Two versions of the truncated kinase missing its C-terminal tail (CK1δΔC, either residues 1-294 or 1-317) were found to induce and purify better than the full-length kinase. The truncated form of the kinase was used for crystallization and NMR experiments because unlike full length CK1δ, it has been previously crystallized and does not auto-inhibit kinase activity (Graves and Roach 1995, Longenecker, Roach et al. 1996, Rivers, Gietzen et al. 1998). Several different affinity and solubilization tags were used to express and purify CK1δΔC, including His-NusA-XL (HNXL), His-MBP, His-SUMO, and His-GST tags, as well as a variety of buffering solutions during purification to improve the final yield of CK1δΔC. Low yields appear to be mainly due to poor solubility during expression and cleavage of the affinity tags after initial purification. Optimization of purification is still ongoing. The crystals obtained in this thesis were from protein purified with His-MBP and/or His-GST tags, but the highest yields obtained thus far were from His-SUMO-tagged CK1δ construct with an average of ~2 mg per L of bacterial culture.

Anions influence the conformation of the CK1δΔC activation loop

Only one other structure of CK1δΔC in the PDB (1CKJ) captures the kinase with the two loop conformations observed in the *tau* structure (Longenecker, Roach et al. 1996). This structure (from 1996) was crystallized in a low concentration of anions and the authors hinted at the possibility of anions influencing the conformation of the activation loop (Longenecker, Roach et al. 1996). To systematically compare the loop conformations of all the CK1δΔC PDB entries, the exact distances between L173 on the activation loop and either Y225 or L152 was measured in all of the existing structures as a proxy for the activation loop in its up or down conformation and plotted for all existing CK1δΔC structures (**Figure 5A-B**) (Philpott, Narasimamurthy et al. 2019). As mentioned before, one other structure of CK1δΔC (4JJR) was also crystallized in the absence of anions and mentioned the possibility of a distinct loop up conformation, but it lacked sufficient electron density to model the activation loop in (Zeringo and Bellizzi III 2014). I used the crystallization conditions for this structure as the starting point for my screens to optimize crystallization of CK1δΔC without anions.

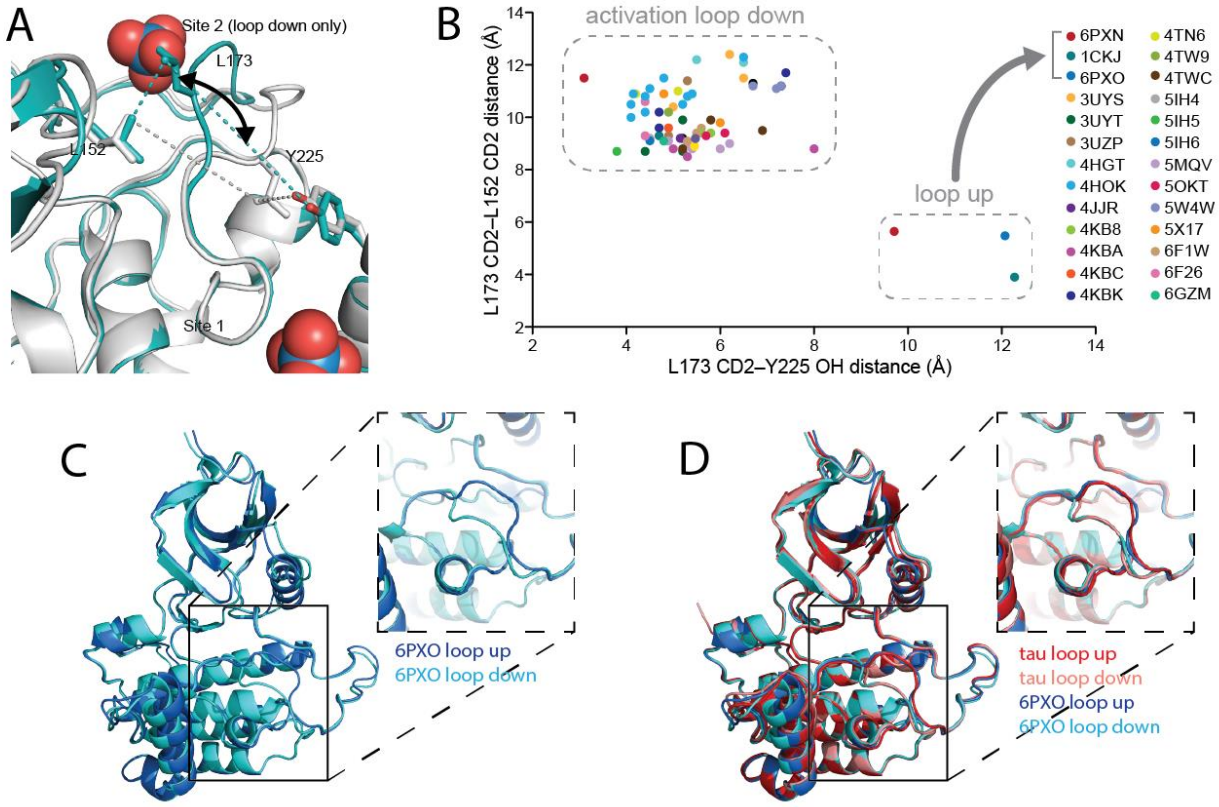

Figure 5 | Anion-free WT CK1δΔC has the same two activation loop conformations as *tau*. (A) The distance between L173 on the loop and either L152 or Y225 reports on the activation loop conformation in WT CK1δΔC(PDB: 1CKJ). **(B)** Using the measurements in **(A)**, activation loop conformation of CK1δΔC structures in the PDB were plotted. **(C)** The crystal structure of the **WT** CK1δΔC under anion-free conditions (6PXO). Two molecules from the asymmetric unit in the crystal structure are overlaid on top of each other, highlighting the two loop conformations. **(D)** Overlay of 6PXO and *tau* structure, highlighting the two conformations of the activation loop. **Panel **(A)** and **(B)** were taken from (Philpott, Narasimamurthy et al. 2019)**

To determine if the WT CK1δΔC could take on the two activation loop conformations in the absence of anions, I crystallized WT CK1δΔC in the absence of anions and solved the structure to 2.0Å resolution (6PXO) (**Figure 5C**). The anion-free WT CK1δΔC structure showed the activation loop in the same two conformations as the *tau* and 1CKJ structure (**Figure 5D**), confirming my initial hypothesis. Importantly, the crystal structure was solved with two CK1δΔC molecules in each asymmetric unit, and just like with the *tau* structure, I found that one molecule of the kinase had an activation loop in the down conformation, while the other was in the up conformation. This structure provides evidence that CK1δ has an intrinsically dynamic activation loop that is influenced by the presence of anions. This ultimately suggests that the overall substrate activity of CK1δ, and therefore the length of circadian period, could be regulated by the presence of anions or possibly by phosphorylated substrate such as the FASP region.

Molecular determinants of CK1δΔC activity on the FASP region

To investigate which residues are required for non-consensus CK1δΔC activity on the FASP substrate, I introduced several different mutations using site-directed mutagenesis, and then expressed and purified these substrates. I visualized kinase activity on them using the NMR kinase assay, which allows atomic resolution analysis of substrate phosphorylation. I first tested FASP priming site mutations (S662A/L666V and S662A/L666V/T667A), which were designed to eliminate priming on the canonical site at S662 and recover non-consensus priming activity on the downstream serine by mimicking key determinants of the canonical priming serine's downstream environment at the next serine (S665). Interestingly, I found that introducing the L666V mutation to the S662A FASP mutant was sufficient to regain kinase activity at the new priming site (S665) and allow for kinase activity on all downstream serines (**Figure 6A**). Another priming mutant that even more closely reconstructs the priming serine's downstream environment (S662A/L666V/T667A) also regained kinase activity (**Figure 6B**).

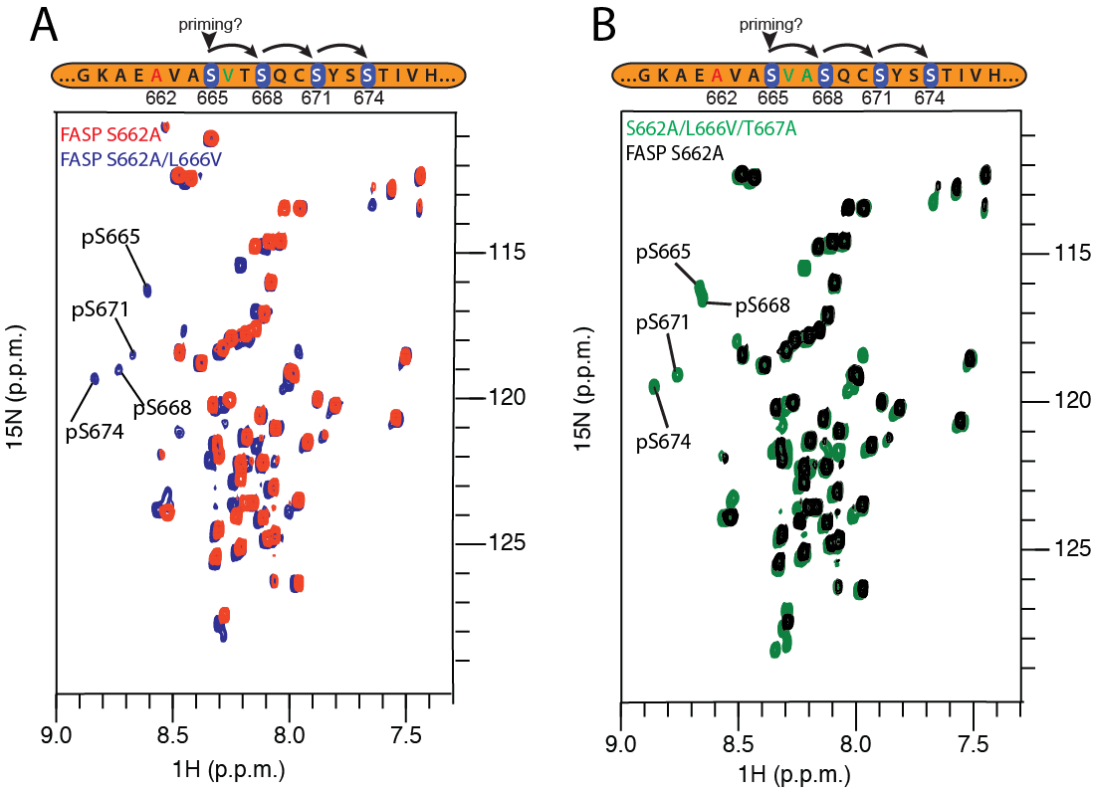

Figure 6 | Mutations to the FASP peptide reveal the importance of the +1 residue of the priming serine. (A) Overlay of $^{15}N/^{1}H$ HSQC spectra with 200 μM ^{15}N FASP S662A (red) or S662A/L666V (purple), incubated with 1 μM CK1δΔC 3 hr at 30°C showing that L666V mutation is sufficient to recapitulate kinase activity. **(B)** Overlay of $^{15}N/^{1}H$ HSQC spectra with 200 μM ^{15}N FASP S662A (black) or S662A/L666V/T667A (green), incubated with 1 μM CK1δΔC 3 hr at 30°C.

I then wanted to see if CK1δΔC vastly prefers the downstream serines from a phospho-serine mimic that resembles a consensus motif and would not phosphorylate the non-consensus serine (S662). To test if non-consensus priming would still occur on the first serine (S662), the mutation S665D was introduced to mimic a phosphoserine and generate what resembles the pSxxS motif starting at S665. The ^{15}N FASP S665D was used in the NMR kinase assay and revealed peaks that likely correspond to phosphorylated S662 (pS662) as well as for the serines downstream of S665D (**Figure 7A**). This data suggests that introducing aspartate, a phosphomimetic residue, downstream of the canonical priming site can still trigger phosphorylation of the pSxxS consensus sites as well as non-consensus phosphorylation. However, to be completely sure of the serines that are getting phosphorylated, chemical shift assignments need to be completed on the phosphorylated S665D mutant peptide. A time-resolved series of NMR spectra would provide kinetic data to help better understand the preference of CK1δΔC on the non-consensus canonical priming site at S662 compared to the aspartate-primed S668 residue.

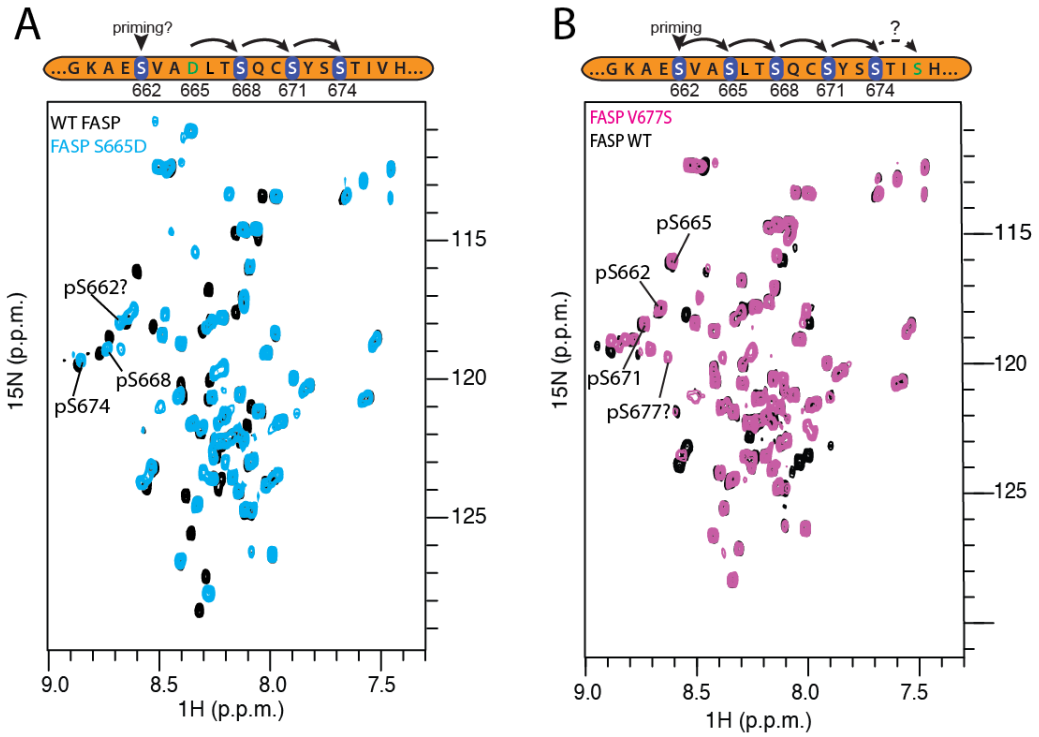

Figure 7 | CK1δΔC phosphorylates consensus and non-consensus FASP substrate. (A) Overlay of ^{15}N/^{1}H HSQC spectra with 200 µM ^{15}N WT FASP peptide (black) or S665D (blue), incubated with 1 µM CK1δΔC 3 hr at 30°C showing that CK1δΔC can phosphorylate non-consensus and consensus FASP substrates on the same peptide. **(B)** Overlay of ^{15}N/^{1}H HSQC spectra with 200 µM ^{15}N WT FASP peptide (black) or V677S (pink), incubated with 1 µM CK1δΔC 3 hr at 30°C showing that CK1δΔC can continue consensus activity further down the FASP substrate.

Very little is known about why the FASP region contains five serines arrayed with consensus spacing. One hypothesis we are exploring is that the phosphorylated serines of the FASP region might bind to anion-binding sites on the kinase to act as a competitive inhibitor blocking activity on the degron region. Therefore, we want to understand as much as possible about kinase activity on the FASP substrate. I made the V677S to see if the addition of another downstream serine that follows the pSxxS consensus motif would get phosphorylated. To keep the SxxS motif intact, V677 was mutated to a serine and the ^{15}N-labeled peptide was purified and visualized with the NMR kinase assay. The appearance of a new peak in the chemical shift range of phosphoserines at a position of ~120 p.p.m. (^{15}N) and ~8.6 p.p.m (^{1}H) is likely from the new serine introduced with the V677S mutant (**Figure 7B**). Once again, chemical shift assignments are required to assign the residues in the FASP V677S mutant, but it is likely that CK1δ will continue to phosphorylate additional downstream serines.

PER2 binding interactions with CK1δ

PER2 and CK1δ form a tight complex that travels from the cytoplasm to the nucleus together (Aryal, Kwak et al. 2017). In an attempt to better understand how PER2 anchors to CK1δ and how the FASP region interacts with CK1δ, I engineered a minimal kinase binding domain of PER2, called SYQ-LT, for two conserved motifs that are required for kinase binding (Eide and Virshup 2001). I deleted flexible, unconserved regions of ~20-40 residues and joined the key motifs with the central FASP region to limit the amount of unstructured regions that might impede crystal formation. However, when I tried to purify the SYQ-LT protein in isolation for biochemical studies, I found that it degraded too quickly to use for binding assays or crystallography with the kinase (**Figure 8A**). I then tried co-purifying CK1δ with SYQ-LT and was successful in obtaining a stable complex with both the full length CK1δ and truncated CK1δΔC that co-eluted on size exclusion chromatography and was used for crystallography experiments (**Figure 8 B-D**). Crystal screens of this complex generated small, non-singular needle crystals that diffracted to 20 Å. I tried extensive optimization of these crystals, including using hanging drop, sitting drop, under oil conditions, seeding with existing crystal stocks, incubating at 4°C and 22°C, and purifying in the presence of ATP/Mg^{2+}. None of these attempts successfully improved the quality of the crystals. My current work is focused on redesigning the SYQ-LT construct to eliminate the FASP region, which could be heterogeneously

phosphorylated, in order to capture the anchoring interactions by the SYQ and LT motifs on the kinase domain.

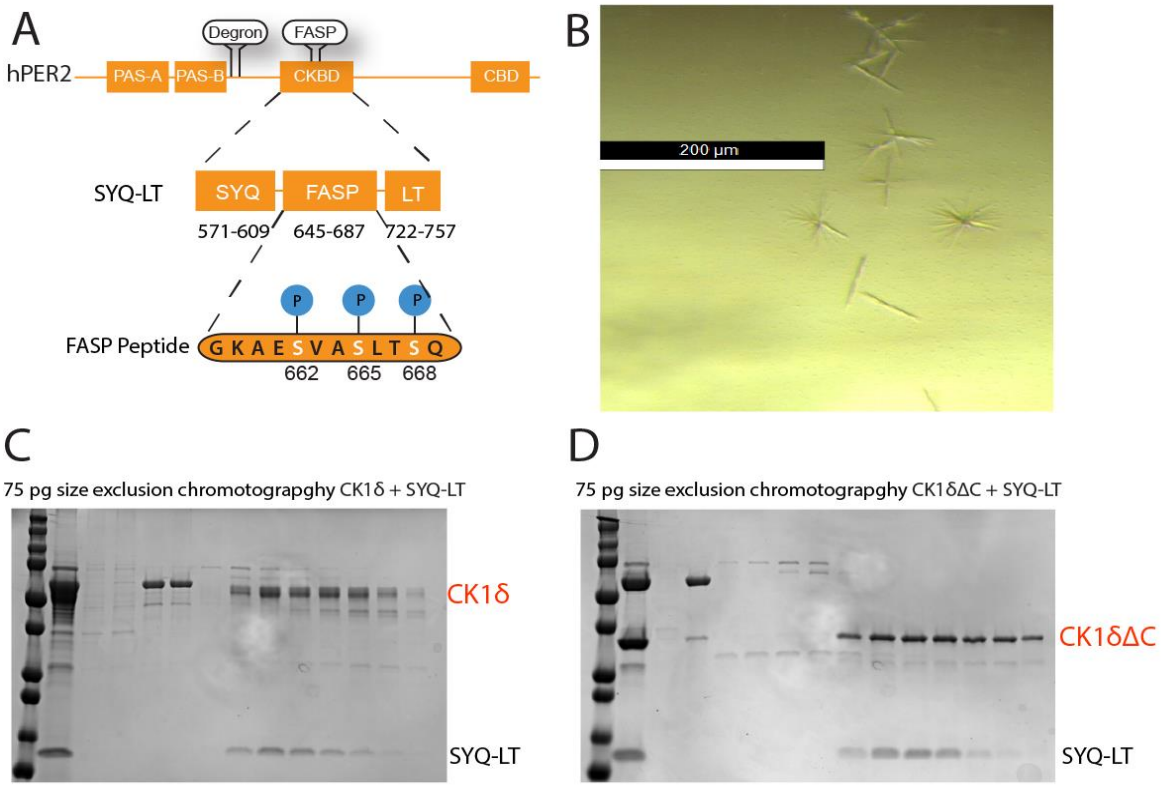

Figure 8 | A minimal, engineered PER2 kinase binding domain (SYQ-LT) co-elutes with CK1δ and CK1δΔC. **(A)** Diagram showing domains and regions of full length human PER2, the minimal kinase binding domain (called SYQ-LT) used in size exclusion and crystallography experiments, and the minimal phosphorylated FASP peptide purchased from Biosynthesis used in co-crystallization experiments. **(B)** Crystals of co-purified CK1δ and SYQ-LT that diffracted to 20 Å. **(C)** SDS-PAGE gel of fractions from Superdex 75 size exclusion chromatography showing that full-length CK1δ co-elutes with SYQ-LT. **(D)** Same as **(C)**, but showing that CK1δΔC co-elutes with SYQ-LT.

Preliminary data from the lab shows that the phosphorylated FASP peptide can act as a competitive inhibitor to regulate kinase activity. Therefore, in addition to capturing the anchoring interactions that keep the kinase bound to PER, it would also be helpful to see how the phosphorylated FASP peptide binds to the kinase domain. I co-crystallized a small phosphorylated FASP peptide containing the first 3 phospho-serines (**Figure 8A**) with CK1δΔC and obtained crystals, but no density was observed for the FASP peptide in the CK1δΔC structure. Future experiments should consider using higher concentrations of this

phosphorylated FASP peptide or a longer peptide containing all 5 phospho-serines because this might further stabilize binding and therefore increase electron density for the FASP peptide in the crystal structure.

Discussion

Conformational changes of CK1δΔC cause a reversal of activity on two crucial regions of PER2, the FASP region and the degron site, that ultimately lead to an altered circadian period. The presence of anions is sufficient to influence the conformation of the activation loop of WT CK1δΔC. Stabilization of the activation loop in the up conformation, as observed with the *tau* mutant, results in decreased activity on the FASP region and increased activity on the degron site of PER2 (Philpott, Narasimamurthy et al. 2019). The FASP region of PER2 is known to be required for PER2 stability and contains a non-consensus SxxS motif that is phosphorylated by CK1δ. The residues directly following the first serine in this motif, are important for CK1δ selection and activity and mutation of this residue is sufficient to regain CK1δΔC activity on all downstream serines in the S662A FASP mutant.

Crystallization of CK1δΔC in the absence of anions confirms that the activation loop conformation of WT CK1δ is influenced by the presence of anions and is in a dynamic equilibrium between the two states. It is therefore likely that the activation loop conformation may be also affected by phosphoserines from biological substrates or its own auto-phosphorylated tail (Kawakami, Suzuki et al. 2008, Fustin, Kojima et al. 2018). Work with the *tau* mutant shows that stabilizing the loop up conformation of the activation loop causes a gain of function on the degron over the FASP sequence, eventually leading to a shortened period. It could therefore be possible to design small molecules that stabilize the loop up or loop down conformation in WT CK1δ, causing an altered circadian period.

It is still unclear if the *tau* phenotype is due primarily to the conformational change of the activation loop, or from the loss of anion binding at site one. Another short period CK1δ mutant, K224D, loses anion coordination at site one that is observed in other CK1δ crystal structures coordinating an anion (Longenecker, Roach et al. 1996, Shinohara, Koyama et al. 2017). K224D also has improved activity on the degron and decreased activity on the FASP region relative to WT CK1δ, but unlike the *tau* mutant, K224D retains a higher degree of non-consensus priming activity on S662 (Philpott, Narasimamurthy et al. 2019). It could be that the activation loop conformation affects non-consensus substrate activity and site 1 is the anchoring point for subsequent pSxxS motif phosphorylation as suggested previously (Longenecker,

Roach et al. 1996, Zeringo and Bellizzi III 2014, Philpott, Narasimamurthy et al. 2019). A crystal structure of K224D would reveal the conformation of the activation loop and its role in retaining non-consensus activity.

To unequivocally know where the anchoring interactions are between CK1δ and PER2, crystallization of CK1δ or CK1δΔC with the PER2 kinase binding domain would be required. We showed that both CK1δ and CK1δΔC co-elute with the SYQ-LT construct on size exclusion chromatography, supporting previous work that suggests that they form a tight complex (Aryal, Kwak et al. 2017). Unfortunately, co-crystallization experiments have been unsuccessful thus far. Future co-purification experiments should use a redesigned the minimal PER2 construct that retains all predicted structured regions of the PER2 kinase binding domain, as well as highly conserved residues. Additionally, seeding and an additive screen should be used on the current construct in an attempt to improve crystal quality.

Looking at the FASP region can also give insight into the mechanism of kinase selection and activity. After eliminating kinase activity on FASP with the S662A mutant, it was found that activity can be recapitulated with the L666V mutation. Leucine and valine are very similar amino acids sharing similar shape, size, and chemical properties, making it somewhat surprising that this mutation is sufficient for regaining kinase activity. Leucine is slightly longer than valine and may be too large for priming activity to occur. This experiment revealed a high degree of CK1δΔC specificity towards the +1 residue of the priming serine. Future experiments should introduce the V663L mutation to see if this is sufficient to lose CK1δΔC activity at the physiologically relevant priming serine. If the size of the +1 residue on the FASP peptide is crucial for kinase activity, the *tau* phenotype could be caused by steric hindrance between the loop up conformation and the +1 residue of the priming serine. Inserting V663L, V663A, and V663G to the FASP region and incubating with *tau* and WT CK1δΔC would test if the size of the plus one amino acid is the cause of the *tau* phenotype.

Materials and methods:

Protein Growth and Purification

His-glutathione S-transferase (His-GST)-tagged CK1δ, His-maltose binding protein (His-MBP)-tagged CK1δΔC (1-294), and His-NusA with flexible linker (His-HNXL)-tagged SYQ-LT and FASP priming mutants were transformed into the *Escherichia coli* Rosetta (DE3) strain for protein expression and purification. His-GST-CK1δ, His-MBP-CK1δΔC, and His-HNXL-SYQ-LT were grown in Luria Broth and induced with 0.5 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG). His-HNXL-FASP mutants were grown in M9 minimal media in the presence of 1 g/L $^{15}NH_4Cl$ and induced with 0.5 mM IPTG. All cell cultures were induced at an OD_{600} of 0.6-0.8 and grown at 18℃ for approximately 16 hr.

His-MBP-CK1δΔC was isolated from the supernatant of the cell lysate via an MBP affinity chromatography column. 6 mg of His-TEV protease was used to cleave the His-MBP tag from CK1δΔC and incubated at 4℃ overnight. CK1δΔC was isolated from the protease reaction via a Ni^{2+} nitrilotriacetic acid (Ni-NTA) affinity chromatography column and then run on a HiLoad 16/600 Superdex 75 pg (GE Healthcare) size exclusion chromatography column. Isolated CK1δΔC was buffer exchanged into 50 mM Tris pH 7.5, 200 mM NaCl, 1 mM EDTA, 5 mM 2-mercaptoehanol (βME), and 0.05% TWEEN buffer, frozen in small aliquots in liquid nitrogen and stored at -80℃.

His-HNXL-FASP priming mutants (S662A/L666V, S662A/L666V/T667A, S665D, V677S) were isolated from the supernatant of the cell lysate via Ni^{2+}-nitrilotriacetic acid (Ni-NTA) affinity chromatography column and then buffer exchanged into a non-imidazole buffer. The tag was cleaved using 4 mg of His-TEV protease incubated at 4℃ overnight. The FASP priming mutants were then separated from the protease reaction using another Ni-NTA affinity chromatography column. The FASP priming mutants were then run on a HiLoad 16/600 Superdex 75 pg (GE Healthcare) size exclusion chromatography column and stored in 17.5 mM MES pH 6, 35 mM NaCl, 0.7 mM EDTA, 1.4 mM TCEP, 7.7 mM $MgCl_2$ buffer.

His-GST-CK1δ and HNXL-SYQ-LT were mixed and co-lysed on ice using 15/45 second on/off blasts for a total run time of 8 minutes. The complex was then isolated from the cell lysate supernatant via a GST affinity chromatography column and the tag was cleaved using 8 mg of His-TEV protease incubated at 18℃ overnight. The complex was then isolated from the protease reaction using a Ni-NTA affinity chromatography column and injected onto a HiLoad 16/600 Superdex 75 pg (GE Healthcare) size exclusion chromatography column. The complex

was stored in 50 mM Tris pH 7.5, 500 mM NaCl, 1 mM EDTA, 2 mM TCEP, and 0.05% TWEEN buffer.

Crystallization (Longenecker, Roach et al. 1996) and data collection

Crystallization conditions were based off of the low anion concentration conditions used in the Zeringo et al. 2013 paper that crystallized CK1δΔC in the absence of anions (PDB 4JJR). The anion-free structure that I solved (6PXO), was crystallized at 8.69 mg/mL in 0.2 M succinic acid pH 5.5, 25% PEG 3350 using a 1:1 ratio hanging drop vapor diffusion method at 22℃. Diffraction data were collected using beamline 8.3.1 at the advanced light source (ALS), Lawrence Berkeley National Laboratory. Data was indexed, integrated, and merged using the CCP4 software suite (Winn, Ballard et al. 2011). Structures were solved by molecular replacement with Phaser MR (McCoy, Grosse-Kunstleve et al. 2007) using the CK1δΔC *tau* structure (PDB: 6PXN). Model building was performed with Coot (Emsley, Lohkamp et al. 2010) and structure refinement was performed with PHENIX (Adams, Afonine et al. 2011). All structural alignments were generated using PyMOL Molecular Graphics System 2.0 (Schrödinger).

Nuclear Magnetic Resonance Spectroscopy

200 µM of ^{15}N-labeled FASP priming mutant (FPM) was incubated with 1 µM of CK1δΔC in a buffer containing 1X protease inhibitors (Pierce), 0.25 mM ATP, 5 mM β-glycerol phosphate, and 0.1 nM Na_3VO_4 for 3 hours at 30℃. ^{15}N-HSQC spectra were collected using a Varian INOVA 600 MHz spectrometer. Peak volumes were calculated and normalized as described in (Narasimamurthy, Hunt et al. 2018).

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