The dramatic impact of the PER-DBT interaction on circadian timekeeping and temperature compensation

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Abstract

Circadian timekeeping depends on protein-protein interactions as well as post-translational modifications, yet the structural details that underlie these events remain largely uncharacterized. We used AlphaFold 2.3 to predict the interaction region between the *Drosophila* PERIOD (PER) protein and the kinase Doubletime (DBT). The structure identified two conserved helical domains of PER, SYQ and LT, as critical for the interaction. We made mutations within these domains and used mutant *period* rescue constructs to assess their effects on circadian period and temperature compensation in vivo. A charge-reversal mutation (E to K) in the SYQ domain caused severe period lengthening and temperature sensitivity, which was rescued by a compensatory charge-reversal mutation in a predicted interacting LT domain residue (K to E); this validated an AlphaFold predicted interaction between two charged residues in SYQ and LT. Three additional mutations in the SYQ domain showed varying effects on period length and temperature compensation, with one uniquely displaying period shortening at higher temperatures. Most strikingly, two mutations in the LT domain (L770A, L775A) resulted in the longest recorded periods for single point mutations in *period*, with free-running periods > 44 hours at 25C. These also showed extreme temperature sensitivity with a magnitude of change >20 hrs. over the physiological temperatures tested. RNA-sequencing analysis of the L775A mutant shows that these behavioral changes are paralleled by altered molecular cycling. Our findings underscore the power of AlphaFold in predicting a crucial protein-protein interaction even with a largely disordered protein partner and reveal that the PER-DBT binding interface plays a crucial role in maintaining proper timekeeping and temperature-compensated circadian rhythms.

Introduction

Circadian rhythms are an evolutionarily conserved process that allows an organism to appropriately time cellular and physiological functions to the cyclical patterns of nature. There are three basic definitions: 1) the rhythms have a free-running period of ~ 24 hrs.; 2) they can entrain to external cues such as light and temperature; 3) the rhythms have a temperature coefficient (Q10) of ~ 1, meaning that the period length changes only marginally over a substantial biological temperature ranges[1–3]. Central to timekeeping is a set of conserved eukaryotic proteins, which engages in a well-characterized transcription-translation feedback loop (TTFL)[4–7]. Nonetheless, detailed mechanistic insight into timekeeping and especially temperature insensitivity remains limited.

What is known about circadian mechanisms have largely relied on mutations within these core clock proteins and how these mutant proteins affect these processes. *Drosophila* has featured prominently in these studies because of its genetic facility and because of its well-characterized circadian processes. Indeed, many of these rate and temperature-sensitive mutants have been identified in two of the conserved negative regulators of the *Drosophila* TTFL, Period (PER) and the kinase Doubletime (DBT); the mammalian ortholog of DBT is casein kinase 1 or CK1. This enzyme contributes to the overall rate of the circadian clock in part through a series of programmed phosphorylation events on PER.

Two well-studied phospho-domains of PER are the degron whose phosphorylation results in PER degradation and resetting of the molecular clock[8–10], and the per-short (in flies) or FASP domain (in mammals) which inhibits degron phosphorylation[11–13]. The interchange between these two regions not only regulates the timing of the clock[14–16], but the preference in phosphorylation for these regions at different temperatures creates a 'phosphoswitch' which is a prevailing mechanistic explanation for circadian temperature independence[11,12,16,17]. Other classical mutations like dbt^S or per^L are known to have defects in both period length and temperature independence, but their underlying mechanisms are unclear[18–23].

AlphaFold's potential contribution to circadian rhythm research lies in its ability to accurately model the structures of key clock proteins and their complexes, offering valuable insights into their binding interfaces and functional relationships[24,25]. AlphaFold has been used to probe intra- and inter-protein interaction domains[24,25] and has even been used to screen novel interactors in a large

protein complex[26–28]. Given the wide availability of this tool, and the lack of circadian complex structures, we sought to determine if it could give insight into interactions in the circadian protein system. In this study we utilized AlphaFold to model the DBT-PER interaction with the aim of discovering which residues are important as the structural basis of their functional relationship. By combining computational predictions with experimental validation in flies, this study seeks to provide a more comprehensive understanding of the molecular mechanisms governing circadian rhythmicity and their ability to maintain temperature independence.

Results

AlphaFold Predictions

To identify regions of the Period protein important for the PER::DBT interface we used AlphaFold 2.3 with full-length sequence for both proteins. Five different seeds were used to predict five structures per seed for a total of 25 predicted PER::DBT dimers (Supp. Fig. 1-1A). These structures were then analyzed for regions with consistently high predicted local distance difference test (pLDDT) scores and low predicted aligned error (PAE) scores to identify regions most likely to be biologically accurate.

Regions with consistent pLDDT scores >70 for PER were the two PAS domains, the F-helix, and two highly conserved regions of PER, namely its SYQ and LT domains (Supp. Fig. 1-2)[29–31]. These two regions lay within the PER-DBT binding domain[29,30], and are predicted to form alpha helices (Fig. 1A)[16,32]. The remaining ~70% of PER is unstructured, consistent with previous predictions of PER[20,33]. In DBT, similar scores were identified for all regions and are consistent with crystal structures of CK1d[13,34]. The exception was the C-terminus, which is predicted to be unstructured (Fig. 1B). Similar scores in precisely the same regions were noted for human, mouse, and *Xenopus* orthologs of PER2 and CK1d (Supp. Fig. 1-1), indicating consistent predictions in these homologous regions. Predicted aligned error (PAE) scores showed high confidence in the relative internal position of the PAS domains of PER as well as the internal position of the N- and C-lobes of DBT (Fig. 1A-B). The remaining disordered portions of each protein were not confidently positioned in relation to these two main globular regions.

PAE scores between PER and DBT identified the PER SYQ and LT domains as high confidence interaction regions with DBT (Fig. 1A-B). These domains were also positioned near DBT, indicating possible detailed regions of interaction between the two proteins (Fig. 1A). While the contribution of the LT domain to PER::DBT/CK1 binding and circadian rhythm are well known[35,36], the SYQ domain has not been directly studied. SYQ has been implicated in kinase binding through large deletions and chimeric proteins in mammalian systems[29,30], but these included the LT domain making the individual contribution of SYQ unclear.

Closer analysis of these regions identified residues throughout the SYQ domain (F626, N622, N618) and at the N-terminal region of the LT domain (L770, L775) that could be important for a PER/DBT binding interface (Fig. 1C-D). Indeed, mutations at an orthologous location to L775 identified it as important for circadian function and PER-DBT binding[37]. An additional intra-PER interaction between the SYQ and LT domains (E621, K789) was also identified (Fig. 1D). Alignments between drosophila PER and evolutionary orthologs showed that these residues and the SYQ and LT sequences more generally have high conservation across the animal kingdom indicating their likely importance for PER function (Fig. 1E). A simple hypothesis is that this interaction is important for DBT-mediated PER phosphorylation.

Testing the AF predictions

To test these predictions in vivo, we created point mutants for each of these predicted interaction sites. Mutations were made in a canonical PER(13.2) rescue plasmid and injected into flies using a PhiC31 site on the third chromosome. Mutant flies were then crossed to virgin Per01 flies and F1 males were collected and assayed for behavior. This breeding scheme allows for the stoichiometry of PER to be maintained (one active allele/fly) since increasing the copy number of PER is known to have circadian behavioral effects[38,39].

A predicted charge cluster between E621 and K789 was predicted to be a critical intra-PER interaction site between the LT and SYQ domains (Fig. 2A). The position of these residues offered a unique opportunity to easily test the effects of single and double mutations at this site. We first decided to test the effect of charge removal and charge reversal with the creation of E621A and E621K mutants respectively. Both mutants showed an increased free-running period with E621A lengthening by ~45 min and E621K lengthening by ~5.5 hours at 25C (Fig. 2B). The proximity of E621 to K789 likely explains this disparity as a charge swap would likely disrupt the position K789, and

likely the whole LT helix, more than the mutation to an alanine. As the LT helix is already known to be important for binding to DBT[35,37], a reasonable working hypothesis is that these mutations decrease the affinity of the DBT for PER and consequently decrease the rate of phosphorylation resulting in a lengthened circadian period.

The E621K mutant also had a significant impact on the temperature independence of the circadian rhythm. We measured a period increase of 5 hours between 18C and 29C with longer periods at higher temperatures (Fig. 2C); this is the largest recorded period change due to temperature we could find. The effect of temperature was most pronounced at higher temperatures. E621A did not have a significant change in period over the same temperature range (Supp. Fig 2-1), suggesting that the positive charge at this position has a bigger effect than the absence of the negative charge.

Remarkably, the double mutant E621K/K789E was able to restore most of the temperature independence of the free-running period. There was only a small increase in the free running period at 29C (Fig. 2D), much reduced compared to the E621K mutant (Fig 2C). The near-complete rescue of a wild-type phenotype supports the predicted structure and specifically the interaction between residues E621 and K789, and it also gave us confidence that mutations in our other predicted interaction residues would prove informative.

Neighboring SYQ Domain Mutations have opposite effects on temperature independence

We next tested amino acids within the PER SYQ domain for their effects on circadian period. SYQ was first recognized as a short sequence in the *C. elegans* heterochronic gene lin-42, which is highly conserved with eukaryotic PER. Although this domain, as well as its nearby LT domain, lies within the PER CK1 binding domain (CK1BD), there have been no studies addressing the role of the SYQ region distinct from the nearby LT region. To test the role of SYQ in circadian rhythms three residues were chosen because their side chains are oriented towards DBT: F626, which is predicted to interact with a small hydrophobic region in the C-lobe of DBT, and N622 and N618, which interact with positively charged regions on the C- and N-lobe of DBT, respectively (Fig. 3A). N618A and N622A both resulted in lengthened periods at 25C like the previous mutations, whereas F626A resulted in total arrhythmicity (Fig. 3B).

We also assayed the two rhythmic strains for their response to temperature. They were both temperature-sensitive but in opposite directions. N622A caused a 1.25 hr. increase in period between 18C and 29C, consistent with the above mutations (Fig. 3D). In contrast, N618A decreased in period by 2.6 hr. between 18C and 27C (Fig. 3C). Interestingly, between 27C and 29C there was a small but statistically significant increase in period, suggesting perhaps a different effect of temperature above 27C.

LT Domain mutations result in dramatic long and temperature sensitive periods

The LT domain has previously been shown to be important for binding to DBT and CK1d in flies and mammals respectively[35–37]. In addition to the K789 residue involved in the charge cluster described above, AlphaFold identified two hydrophobic residues, L770 and L775, which interact with a small hydrophobic pocket in the N-lobe of the kinase (Fig. 4A). A previous study in mice showed that the ortholog of L775 was important for kinase binding, but the effect on the circadian period was complicated[37].

In flies, both mutations, L770A and L775A have very large effects on circadian period at the standard assay temperature of 25C; they had average free-running periods of 44.8 and 47.4 hours, respectively (Fig. 4B). These represent the longest periods from a single *period* point mutant to our knowledge. It is perhaps noteworthy that the standard deviation of period was quite large, which we attribute to either an algorithmic issue in identifying flies with this long of a period or to these mutants being very sensitive to minor differences in temperature, perhaps even within a single incubator.

Consistent with this second hypothesis, we observed that both L770A and L775A flies had very steep temperature response curves (Fig. 4C-D). L770A had a change of 13.4 hrs. from 18C to 29C (Fig. 4C). L775A was arhythmic at 27C and 29, but rhythmic at lower temperatures. Moreover, the average period changed by a remarkable 22.4 hrs. between 18C and 25C (Fig. 4D). Like L770A at 25C, L775A also has a large standard deviation in period length at 22C. These temperatures seem to be close to the inflection point of the period-temperature profiles. Taken together with the high degree of change overall, this is a likely explanation for the high level of variation within groups at these rhythmic temperatures.

Elongated behavior is due to a lengthened molecular clock

Are the extreme phenotypes L770A and L775A a behavioral oddity? To address this possibility, we assayed molecular cycling.

To observe RNA cycling, L775A mutant and WT flies were entrained at 25C for 5 days and then frozen at 4 hr. intervals over the first two days of darkness (DD 1-2). Heads were isolated, RNA was purified, and libraries prepared using xGen. Reads were mapped and transcripts normalized for direct comparison between samples.

Consistent with the behavior, the core clock genes of L775A flies undergo RNA cycling with a ~48 hr. period (Fig 5A). WT fly heads were assayed in parallel. WT clock genes experienced ~ 2 cycles of RNA cycling over the 48hr collection time, whereas L775A flies were only had ~1.

Discussion

We show here not surprisingly that AlphaFold is a powerful tool for the identification of predicted protein-protein interaction sites between the large circadian protein PER and the circadian kinase CK1/DBT. AlphaFold has become a valuable part of hypothesis generation for proteins and systems for which there are few solved structures[24]. Importantly, AlphaFold predicted important interaction domains even in a substantially intrinsically disordered protein like PER. These two small helical domains, SYQ and LT, are in the middle of a large intrinsically disordered C-terminus and thus had little structural context to indicate where or how they would interact with the CK1/DBT kinase (Supp. Fig. 1-1). Although the interaction confirms and extends prior studies[35,35,37], it is our mutational studies in flies that really cement the interaction. They indicate that many detailed features of the predicted interaction are correct and even more importantly that the interaction is essential to maintain the temperature independence of the circadian clock.

Arguably most convincing is the rescue of the E621K SYQ mutation with the addition of a second PER mutation, the K789E LT mutation (Fig. 2). Despite two radical amino acid changes (acidic to basic, and basic to acidic), the near perfect restoration of period and temperature independence strongly indicates that these two residues interact as proposed and supports the notion that the LT and SYQ domains are positioned as indicated by our AlphaFold predictions and that both are required for normal circadian function.

Also convincing are the striking results of the L770A and L775A mutations, residues that sit at the top of the LT domain (Fig. 4A). They are not just a behavioral anomaly because RNA cycling is also dramatically affected by the L775A mutation (Fig. 5). These data fit well with the dramatic effect of a mammalian study of a L/G mutation at the orthologous position to L775, which indicated the importance of this residue for CK1 binding and for maintaining proper circadian period[37]. Together with our results, this data reinforces the importance of the LT region to kinase binding and activity.

These mutants and almost all others tested lengthen period with higher temperature. The simple suggestion is that higher temperature causes weaker binding of these mutant PERs to DBT, which becomes rate-limiting for kinase activity and lengthens circadian period. The prevailing mechanisms of temperature independence largely rely on the opposing phosphorylation kinetics of the degron and FASP domains, but do not consider how direct binding between PER and DBT contributes to this mechanism. Additional biochemical and biophysical experiments are now required to confirm the contribution and magnitude of this binding to the mechanisms of temperature independence.

An exception is the N618A mutation, which shortens period with higher temperature. This suggests that this mutation makes a different biochemical step rate-limiting, perhaps a catalytic event rather than a required prior association between PER and DBT. A simple interpretation is that this event is faster at higher temperature as would be expected for enzyme rate (Q10>1) but is normally not rate-limiting with wild-type sequences.

All these considerations beg the interesting question of how the circadian period of the wild-type system normally achieves temperature independence. An individual step involved in DBT-mediated PER phosphorylation, for example the DBT-PER dissociation constant or the DBT catalytic rate, might be truly rate-limiting and temperature-independent in the full in vivo setting. However, we think it is more likely that two key events, perhaps even these two steps, have approximately the same temperature coefficient and are offsetting in determining overall rate. Both of these steps may be affected in the individual mutants studied here but for each mutant one more potently than the other, giving rise to either an overall increase or decrease in period length with increasing temperature. In addition, the unusual feature of PER phosphorylation, the fact that the protein experiences a large number of events (likely >50) over several hours prior to degradation, may be relevant to solving this enigma. Direct measurement of these biochemical processes will be required to fully address this issue of temperature-independence.

Materials and Methods

Fly Stocks and Rearing

Flies were raised on standard cornmeal medium supplemented with yeast at 25°C under 12h light: 12h dark conditions.

Generation of Fly Lines

To generate the Period mutants, pAttB-Per(13.2)-HA-HIS (gift from Joanna Chiu, UC Davis) was used as the WT control. All other mutants were generated by GenScript Biotech. Wildtype and mutant constructs were verified using whole-plasmid sequencing.

Sequencing-verified plasmids were injected into the third-chromosome VK00027 site by BestGene, Inc (Chino Hills, CA, USA). Successful transformants were screened by eye color.

Circadian Behavior Assay

For behavior analysis of flies, 0-5 day old males were collected and aged until ~ 2 weeks old using rearing method above with food changes every 3-4 days. Male flies were loaded into Drosophila Activity Monitor (DAM) tubes containing sucrose food (4% sucrose and 2% agar). Flies were entrained to at least 3 days of a 12 hour light: 12 hour dark cycle before switching to constant darkness for at least 12 days. Circadian free-running periods were determined using the Rethomics package in R[40].

Generation of Sequencing Libraries

RNA from fly heads (10-15) was extracted using TRIzol reagent (ThermoFisher #15596026). Libraries were generated using the xGen RNA Library Prep Kit (IDT #10009814) using mRNA isolated using the NEBNext Poly(A) mRNA Isolation Kit (NEB #E7490). Libraries were purified using AMPure XP Beads (Beckman Coulter #A63881) and quantified using a High Sensitivity D5000 ScreenTape (Agilent # 5067-5592) on a TapeStation.

These libraries were then sequenced on Illumina NovaSeq6000 at Novogene Corporation Inc. (Sacramento, CA, USA) generating 20-38million total raw paired end reads (2*150bp) per sample.

Sequencing Data Analysis

Pair-end reads were pseudoaligend and quantified using Salmon[41]. Count tables were imported into R using Tximeta[42] and transcript-level counts were summarized to gene-level counts. Gene-level counts were normalized using DESeq2[43].

Statistical Analysis

All behavior was analyzed using the Brown-Forsythe and Welch one-way ANOVA with Dunnett's T3 multiple comparisons test in GraphPad Prism.

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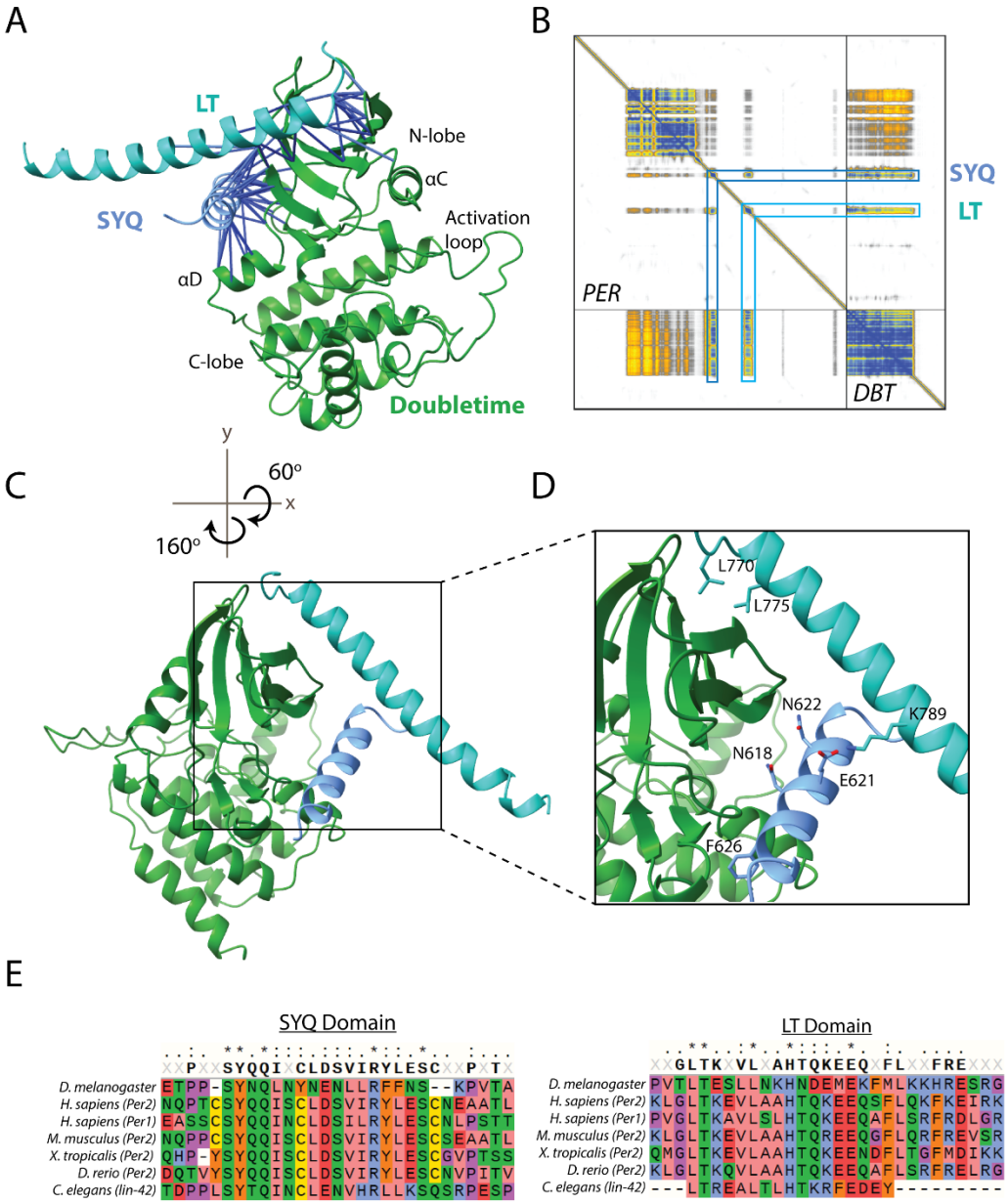

Figure 1. **AlphaFold predicts an interaction between the kinase DBT and Period LT and SYQ domains.** The AlphaFold prediction of the kinase DBT with the PER LT and SYQ domains (A). PAE scores between PER and DBT are mapped onto the structure with a distance of 4 Å and show a high degree of confidence between these domains. A representative PAE plot in (B) shows these regions as the highest degree of confidence between the two structures. Rotation of the complex (C) shows that LT and SYQ sit orthogonal to one another around the N-lobe of the kinase. The location of predicted binding residues are shown in (D). The SYQ and LT domains of PER are highly conserved across species (E).

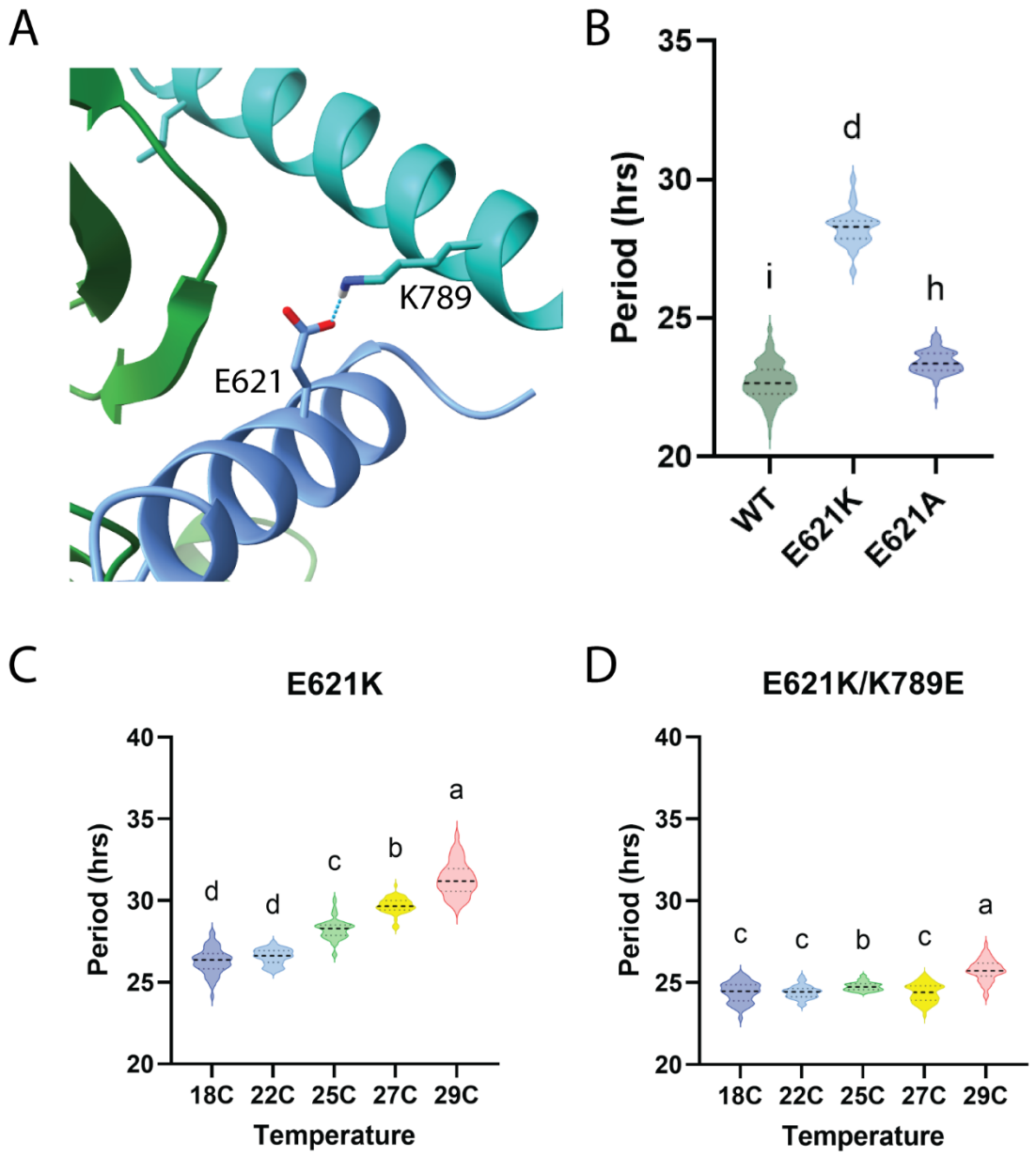

Figure 2. Restoration of temperature independence with double-mutant charge swap between SYQ and LT helices. The putative charge cluster is predicted to be assembled from E621 on the SYQ domain, K789 on the LT domain(A). A predicted H-bond is shown as a blue dashed line between these residues. K621E/A mutants both show lengthened periods at 25C with the charge-swapped K621E having an effect of significantly larger magnitude (B). This mutation also had a large effect on the temperature independence of the circadian period (C) with significantly increased periods at increased temperatures. Significantly, the double mutant K789E/E621K (D) largely rescued both the free-running period and temperature independence of the free-running period.

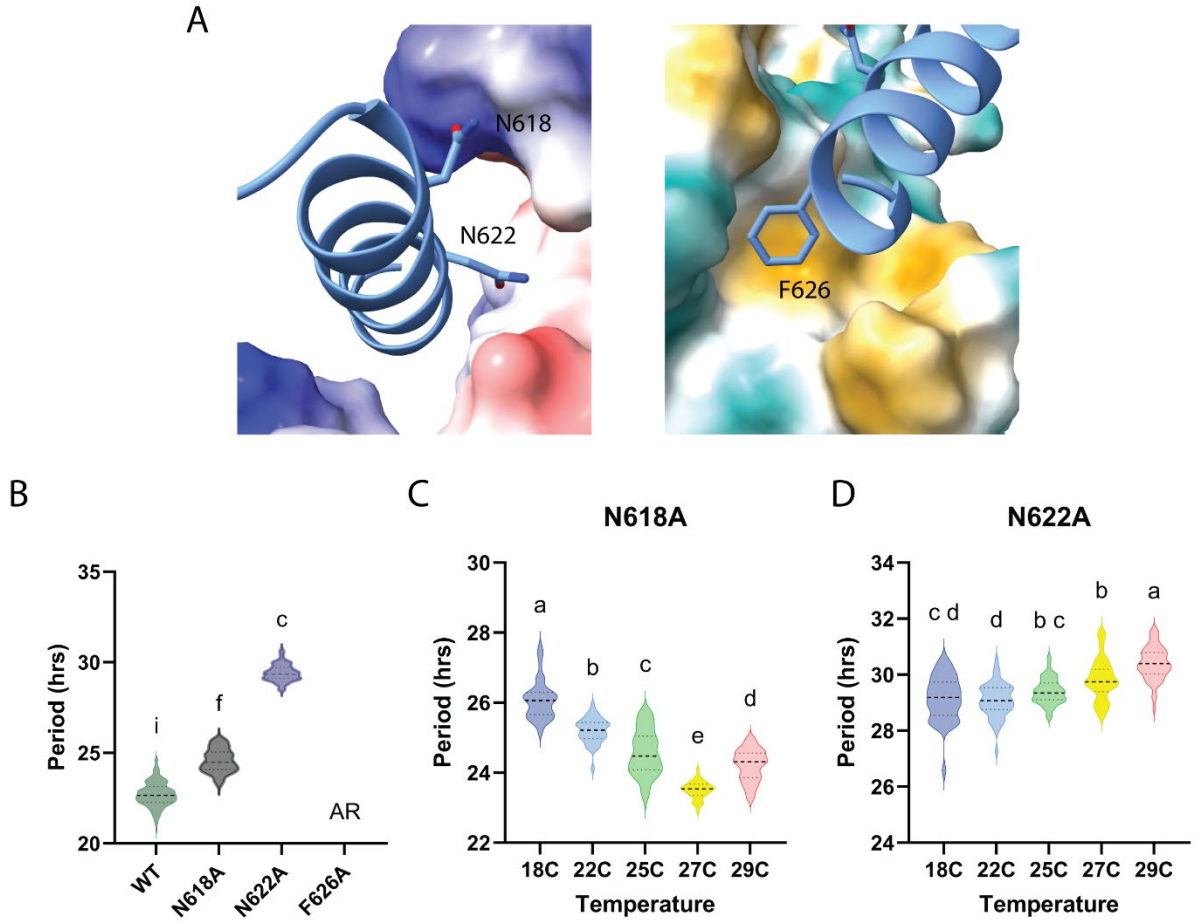

Figure 3. Adjacent mutations in SYQ domain result in opposing temperature-dependent effects on the circadian rhythm. In the SYQ domain, three residues are predicted to interact with DBT. The polar residues N618 and N622 are shown interacting with charged areas adjacent to the catalytic cleft of DBT (A, left, DBT shown as surface model colored by electrostatic potential) while F626 interacts with a small hydrophobic region on the C-lobe of DBT (A, right, DBT shown as surface model colored by hydrophobicity). N to A mutations result in long-period mutants while the F to A mutant is arrhythmic (B) at 25C (B). Interestingly, while both N618A and N622A result in a loss of the temperature independence of the circadian clock, the free running period of N618A decreases with increasing temperature (faster clock at higher temperatures) (C) while N622A follows the trend of the other mutants by having a longer period at higher temperatures (D).

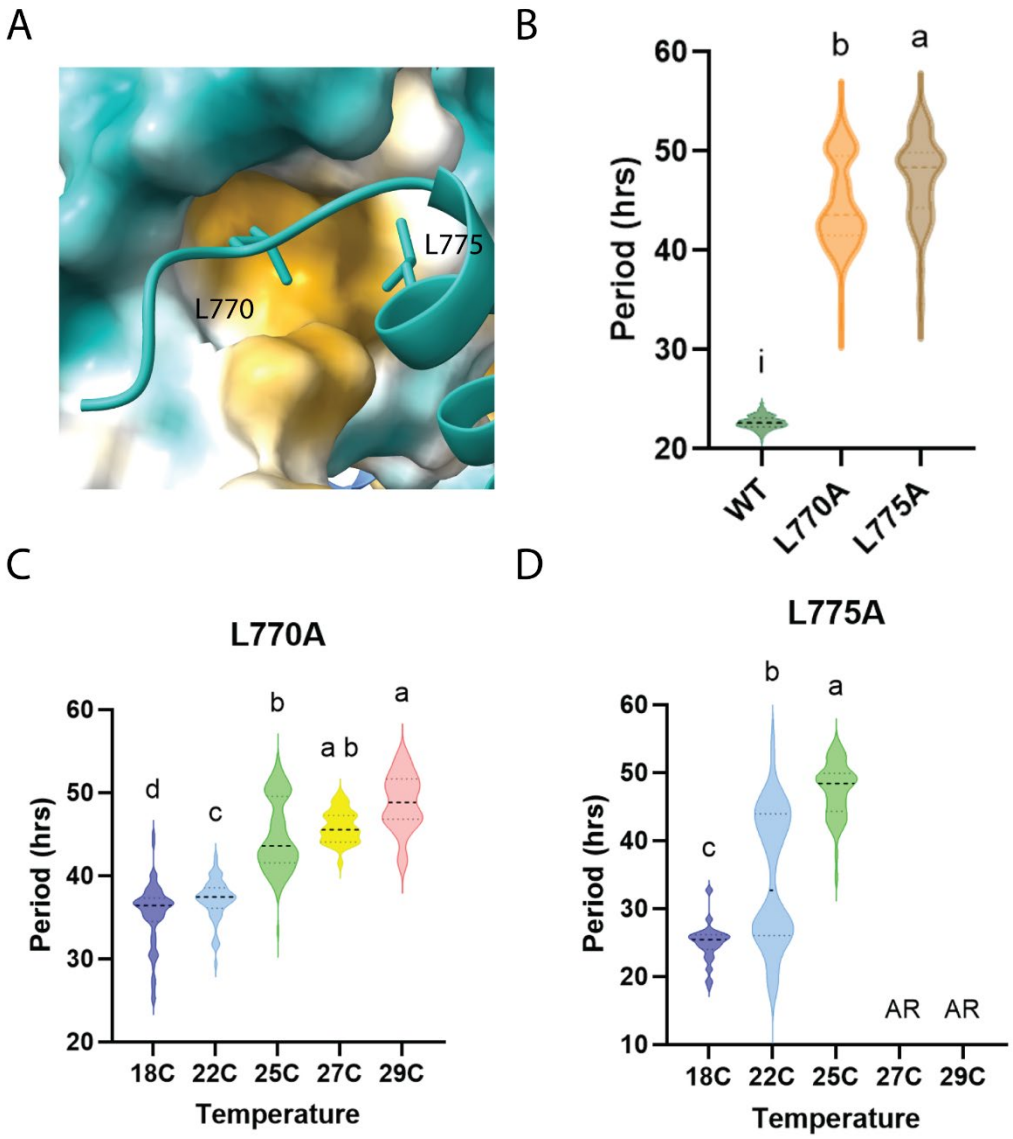

Figure 4. Disruption of hydrophobic anchor between DBT and LT domain results in 48-hour period. Both L770 and L775 are predicted to sit within a small hydrophobic pocket of the N-lobe of DBT (A). DBT is shown as a hydrophobicity surface model. Mutation of L770 or L775 to alanine results in a near doubling of the free running period at 25C (B). Both mutations are also extremely sensitive to temperature (C-D) with both mutants showing an increase in circadian period with increased temperature.

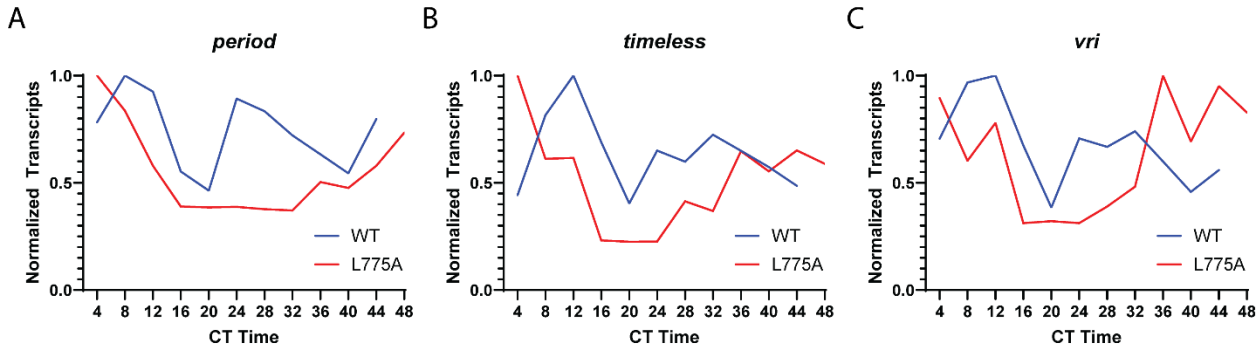

Figure 5 – The molecular clock of the ultra-long period mutants also cycles with an ~48 hour period. Direct observation of *per* (A) *tim* (B), and *vri* (C) transcripts from L775A and WT heads show that the period of transcript cycling matches closely with the period of the behavior. Part of the lengthening can be attributed to an elongated transcriptional trough, possibly indicating a longer than normal inhibition period of these mutants.