Cooperative Interaction between Phosphorylation Sites on PERIOD Maintains Circadian Period in *Drosophila*

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Introduction: Circadian rhythms in Drosophila rely on cyclic regulation of the period (per) and timeless (tim) clock genes. The molecular cycle requires rhythmic phosphorylation of PER and TIM proteins, which is mediated by several kinases and phosphatases such as Protein Phosphatase-2A (PP2A) and Protein Phosphatase-1 (PP1).

Methods: Here we used mass spectrometry to identify 35 "phospho-occupied" serine/threonine residues within PER, 24 of which are specifically regulated by PP1/PP2A. We found that cell culture assays were not good predictors of protein function in flies and so we generated per transgenes carrying phosphorylation site mutations and tested for rescue of the per^{01} arrhythmic phenotype.

Results: Surprisingly, most transgenes restore wild type rhythms despite carrying mutations in several phosphorylation sites. One particular transgene, in which T610 and S613 are mutated to alanine, restores daily rhythmicity, but dramatically lengthens the period to ~30hrs. Interestingly, the single S613A mutation extends period length by 2-3 hours, while the single T610A mutation has a minimal effect, suggesting these phospho-residues can substitute for each other to a large extent. Conservation of S613 from flies to humans suggests that it has a critical role in determining circadian period. Biochemical data indicate defects in overall phosphorylation and altered timely degradation of PER carrying the double or single S613A mutation(s). Immunohistochemical analysis revealed that PER-T610A/S613A exhibits an extended period of protein expression during the middle of the day. Our results also suggest that PER undergoes nuclear-to-cytoplasmic redistribution in some clock cells prior to its daily decline.

Conclusions: Together these data identify specific phosphorylation events that are critical for PER stability and circadian period, demonstrate that cooperativity between phosphorylation sites maintains PER function, and reveal novel features of PER regulation.

Trainee Award Eligible