

Supporting Information

Methods

Plasmids- To generate phospho-mutant *Clock* constructs, the following primers and their complement were used in the PCR reactions: CLOCK(S406A): 5'-gctgacaaagctcaagattct-3', CLOCK(S416A): 5'-aacacagtggctctcaaggaa-3', CLOCK(SS427/429AA): 5'-ggaggcagaaggagctggggcgtgatcaaacct-3', CLOCK(SS431/433): 5'-ccaactcctgctgccgcctctagaagc-3', CLOCK(SS434/436AA): 5'-ccttctgcctccgctagagcctcacgaaagtca-3', CLOCK(S437A): 5'-tctagaagcgcacgaaagtca-3', agctcacgaaaggaagatcacaccgcagtc-3', CLOCK(SS440/441AA): 5'-agctcacgaaaggetgctcacaccgcagtc-3', CLOCK (ST450/451AA): 5'-gaccctccgccgaccgacaaag-3'', CLOCK(ST459/461AA): 5'-ctactgatgctagcgtcctcccag-3', CLOCK(SS434/436DD): 5'-ccttctgcctccgatagagactcacgaaagtca-3', CLOCK(S437E): 5'-tctagaagggaacgaaagtca-3', CLOCK(S440/441ED): 5'-agctcacgaaaggaagatcacaccgcagtc-3', CLOCK(S427A): 5'-ttgatcacgcccccaactcct3', CLOCK(S429A): 5'-cacagcccagctccttctgcc-3', CLOCK(S431A): 5'-ccaactcctgctgctcctct-3', CLOCK(S433A): 5'-ccttctgccgcctctagaagc-3'. CLOCK primer 5'-agtttatgcagaagttag-3' was used to sequence each CLOCK mutant.

To generate GAL4-fused constructs, full-length *mBmal1* was amplified by PCR using 5'-gaaggatccgcgaccagagaatggac-3' and 5'-gaagtcgacctacagcgccatggcaa-3' oligonucleotides as primers. Full-length *mClock* was amplified using 5'-gaaggatccgtgtttaccgtaaggtgt-3' and 5'-gaagtcgacctccagtggtggacctt-3' oligonucleotides as primers. PCR products were digested with *BamHI* and *Sall* restriction endonucleases and cloned in *BamHI/XhoI* sites of pCMV-GAL4.

Supplementary Fig.1

A

NetPhos 2

S-427 0.985

S-431 0.033

S-433 0.986

S-434 0.985

S-436 0.987

S-437 0.998

S-440 0.997

S-441 0.987

S-446 0.709

S-449 0.973

T-451 0.541

T-459 0.905

P

B

NetPhosK 1

S-427 GSK3 0.51

T-429 GSK3 0.51

S-434 PKC 0.79

S-436 PKC 0.79

S-437 PKC 0.91

S-440 RSK 0.53

S-441 PKC 0.57

S-446 PKC 0.74

T-451 P38MAPK 0.52

T-461 CDK5 0.62

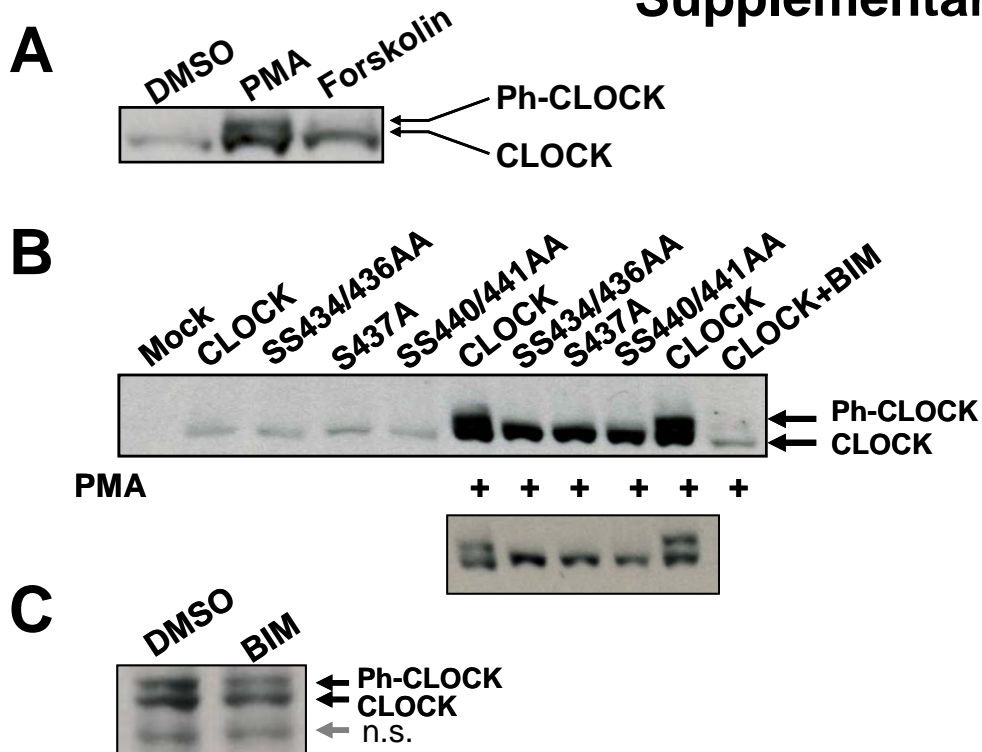
Identification of CLOCK phospho-serines.

A. The Proteomic Server NetPhos 2 predicts a “hot spot” of kinase activity with Ser437 representing the most likely phosphorylated CLOCK residue.

B. The NetPhosK 1 Server predicted that Ser437 is part of a PKC consensus site as well as adjacent Ser436 and 441.

Ser427 and 429 are potential GSK-3 phosphorylation sites.

Supplementary Fig. 2



BMAL induced CLOCK phosphorylation is PKC-independent

- A. Long-term PMA treatment facilitates CLOCK phosphorylation. Cells expressing ectopic CLOCK were treated with DMSO, 100nM PMA, or 10uM forskolin for 16 hours. CLOCK protein profiles were analyzed by Western Blot using anti-HA antibody. Arrows indicate position of phosphorylated and un-phosphorylated forms of CLOCK.
- B. Inhibition of PKC does not prevent PMA-facilitated CLOCK phosphorylation. Cells were transfected with plasmids encoding individual HA-tagged CLOCK proteins and then treated with either PMA or DMSO. After a 16-hrs treatment cells were boiled in SDS sample buffer and analyzed by Western Blot with anti-HA antibody. PMA induced the phosphorylation of wild type CLOCK only. Pretreatment of cells with specific PKC inhibitor BIM negated PMA-mediated increase in CLOCK proteins, but did not prevent CLOCK phosphorylation. The bottom panel shows shorter exposure of the same blot.
- C. BMAL induced CLOCK phosphorylation is PKC-independent. HEK-293T cells co-expressing BMAL1 and CLOCK, were treated with either DMSO or BIM followed by Western Blot for detection of CLOCK. n.s. – non-specific band.