Figure S1

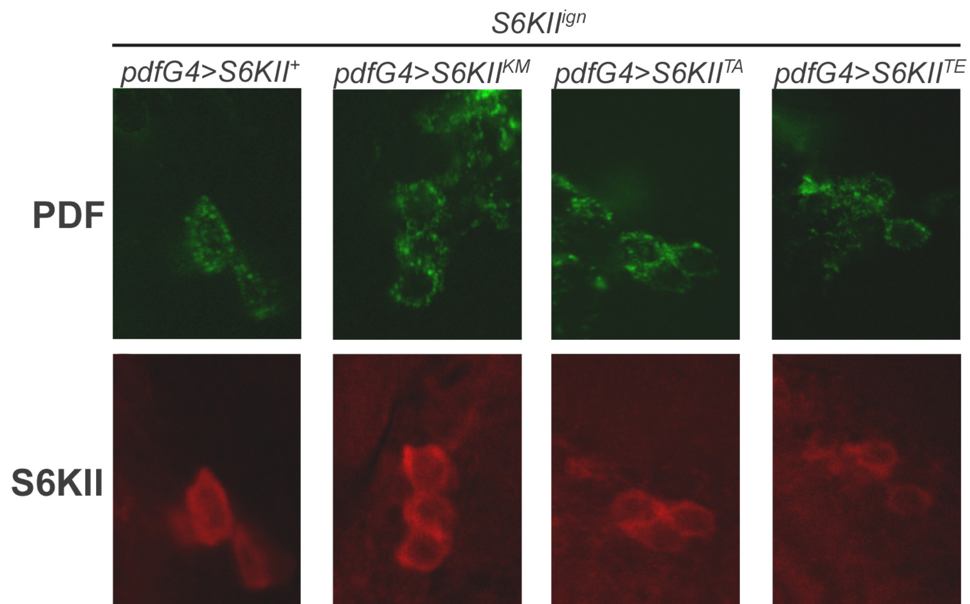
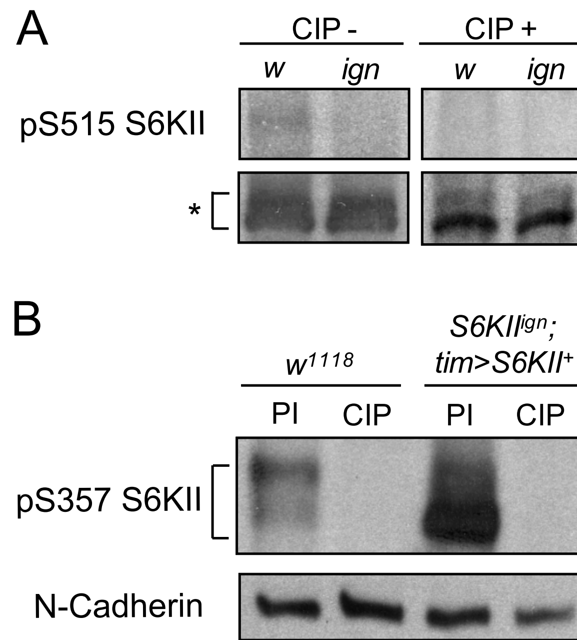


Figure S2



SUPPLEMENTAL TABLE 1. Period estimates and sample sizes for *S6KII* mutants and other genotypes. (**Bold** indicates genotypes with short period phenotypes).

Genotype	(h ± SEM)	N
w^{1118} (control)	23.9±0.05	113
***S6KII*^{ign}/Y**	22.9±0.07	88
***S6KII*^{ign}/*Y;pdf-Gal4/+;* +/+ (control)**	23.0±0.09	32
***S6KII*^{ign}/*Y;tim-UAS-Gal4/+;+/+* (control)**	23.0 ±0.06	44
S6KII^{ign}*/Y;pdf-Gal4/+;UAS-S6KII/+*	23.9±0.10 [1]	90
S6KII^{ign}*/Y;tim-UAS-Gal4/+;UAS-S6KII/+*	23.8±0.04 [1]	90
S6KII*^{ign}*/Y;+/+;UAS-S6KII/+	23.1±0.06	52
S6KII^{ign}*/Y; tim-UAS-Gal4/+; UAS-S6KII*^{KR}*/+*	23.7±0.04 [1]	96
S6KII*^{ign}*/Y; +/+; UAS-S6KII*^{KR}*/+	23.2±0.06	45
S6KII*^{ign}*/Y; tim-UAS-Gal4/+; UAS-S6KII*^{RA}*/+	23.2±0.06 [2]	74
S6KII*^{ign}*/Y; +/+; UAS-S6KII*^{RA}*/+	23.1±0.06	76
S6KII*^{ign}*/Y; tim-UAS-Gal4/+; UAS-S6KII*$^{KR/RA}$*/+	23.0±0.07 [2]	44
S6KII*^{ign}*/Y; +/+; UAS-S6KII*$^{KR/RA}$*/+	23.0±0.06	53
S6KII*^{ign}*/Y; pdf-Gal4/+; UAS-S6KII*^{KM}*/+	23.0±0.06 [2]	63
S6KII*^{ign}*/Y; +/+; UAS-S6KII*^{KM}*/+	22.8±0.07	54
S6KII*^{ign}*/Y; pdf-Gal4/+; UAS-S6KII*^{TA}*/+	23.3±0.05 [2]	45
S6KII*^{ign}*/Y; +/+; UAS-S6KII*^{TA}*/+	23.1±0.07	40
S6KII^{ign}*/Y; pdf-Gal4/+; UAS-S6KII*^{TE}*/+*	23.9±0.05 [1]	57
S6KII*^{ign}*/Y; +/+; UAS-S6KII*^{TE}*/+	23.0±0.10	44
S6KII*^{ign}*/Y; tim-UAS-Gal4/+; UAS-S6KII*^{SA}*/+	23.3±0.05 [2]	56
S6KII*^{ign}*/Y; +/+; UAS-S6KII*^{SA}*/+	23.2±0.06	44
S6KII*^{ign}*/Y; tim-UAS-Gal4/+; UAS-S6KII*^{SD}*/+	23.0±0.06 [2]	49
S6KII*^{ign}*/Y; +/+; UAS-S6KII*^{SD}*/+	22.8±0.10	27

[1] significantly different from *S6KII*^{ign}*;UAS* unrescued control, p<0.001. [2] not significantly different from *S6KII*^{ign}*;UAS* unrescued control.

SUPPLEMENTAL TABLE 2. Summary of the behavioral and molecular analysis of S6KII mutants

Residue	Mutation Abbrevation	Rescues ign short period?	pS515 immunoreactivity	pS357 immunoreactivity	pT732 immunoreactivity
	S6KII+ (wild-type)	yes	high intensity band	high intensity band	high intensity band
K231R	**KR** (N-terminal kinase dead)	yes	NS	NS	NS
R902A	**RA** (ERK docking deficient)	no	*	NS	*
K231R, R902A	**KRRA** (N-terminal kinase dead + ERK docking deficient)	no	*	NS	*
K597M	**KM** (C-terminal kinase dead)	no	*	*	*
T732A	**TA** (unphosphorylatable ERK phos site)	no	NS	NS	Not Detected
T732E	**TE** (pseudo-phosphorylated ERK phos site)	yes	NS	NS	Not Detected
S515A	**SA** (unphosphorylatable C-terminal kinase phos site)	no	Not Detected	*	NS
S515D	**SD** (pseudo-phosphoryated C-terminal kinase phos site)	no	Not Detected	*	NS

***** Significant difference vs S6KII$^+$, $p \leq 0.03$. **NS** = not significant. ANOVA with Dunnett's post test.

SUPPLEMENTAL FIGURE LEGENDS

SUPPLEMENTAL FIGURE S1. Expression of S6KII mutant proteins, using *pdf-Gal4*, can be detected in the PDF neurons. Upper panels show PDF (green) and lower panels show S6KII (red) immunoreactive signals, respectively, in the cytoplasm of PDF cells overexpressing wild-type and mutant forms of S6KII. *S6KII^{ign}* null mutants completely lacked S6KII signal (see our previous publication (1) for example), indicative of antibody specificity. Six brains were examined for each genotype in these experiments, with similar results. Images are 2.0 µm sections from the middle of the small ventral lateral neurons. Comparable expression was seen in the large ventral lateral neurons (data not shown).

SUPPLEMENTAL FIGURE S2. pS515 and pS357 S6KII antibodies specifically detect phosphorylated fly S6KII. A. Identically prepared blots were incubated in buffer with or without calf alkaline phosphatase (CIP) prior to pS515 antibody incubation. The non-specific bands indicated by the * demonstrate that total protein levels were comparable in each lane. CIP incubation eliminated the pS515 signal in the wild-type (*w*) lysate. B. Blot for which lysates were treated with either phosphatase inhibitors (PI) or calf alkaline phosphatase (CIP). CIP eliminated the pS357 signal in lysates from the wild type (*w^{1118}*) or flies overexpressing S6KII^{+} in clock neurons (*S6KII^{ign}; tim>S6KII^{+}*). N-cadherin served as a loading control.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Phospho-antibody specificity - Phosphate specificity of mammalian pSer380-RSK and pSer221-RSK antibodies against fly protein was tested via de-phosphorylation of membranes after transfer or via de-phosphorylation of lysates before SDS-PAGE. To de-phosphorylate membranes after transfer, protein lysates were first prepared, run on a gel and transferred to a PVDF membrane as described above. Membranes were blocked with 5% BSA in TBS with 0.1% Triton X-100 for one hour, room temperature (a milk block may increase background signal since milk contains phospho-proteins). Prior to antibody incubation, half of the blot was incubated overnight at 37ºC in calf alkaline phosphatase (CIP) buffer alone and the other half in buffer containing calf alkaline phosphatase (1 unit/ug protein). CIP buffer, pH 7.9, contains 100mM NaCl, 50mM Tris-HCl, 10mM $MgCl_2$ and 1mM DTT. To de-phosphorylate lysates, protein was extracted in a modified RIPA buffer (50mM KCl, 10mM HEPES, 5mM Tris-HCL, 10% glycerol, 2mM EDTA, 1% TritonX-100) with 1mM DTT, 0.4% NP40, and a 1:100 dilution of Halt protease inhibitor cocktail (Pierce). Each sample was aliquoted into two tubes containing the proper amount of protein for loading, one of which received CIP (10 units/ul) and the other PhosStop (1:10 dilution). Samples were incubated at 37ºC for one hour before proceeding with normal western immunoblotting procedure.

Immunostaining methods - Hand dissected whole mounts of the fly brain were stained with antibodies according to published procedures (2), with the addition of phospho-protein protection. Brains were dissected on ice in PBS with 8% PFA, 0.5% Triton-X and PhosStop phosphatase inhibitors (Roche). All subsequent incubations were on ice and in solutions containing PhosStop. Mouse anti-PDF (pigment dispersing factor), 1:50 (Developmental Studies Hybridoma Bank) and guinea pig anti-RSK, 1:1,000 (3) antisera were used. Brain images were acquired using a Leica TCS SP2 AOBS confocal microscope. Images represent 1.5um optical sections from the middle of the cells acquired with a 40x objective, 3.0x zoom and analyzed using the Leica LCS 'Lite' software.

REFERENCES

1. Akten, B., Tangredi, M. M., Jauch, E., Roberts, M. A., Ng, F., Raabe, T., and Jackson, F. R. (2009) *J. Neurosci.* **29,** 466-475
2. Suh, J. and Jackson, F. R. (2007) *Neuron* **55,** 435-447
3. Kim, M., Lee, J. H., Koh, H., Lee, S. Y., Jang, C., Chung, C. J., Sung, J. H., Blenis, J., and Chung, J. (2006) *EMBO J.* **25,** 3056-3067