Supporting Online Material

Materials and Methods

Fly stocks

Flies were maintained on standard food containing molasses, cornmeal, and yeast at room temperature. In our screen for mutants with reduced sleep, a total of 3473 transposon lines were used. The strains used in the original screen and subsequent experiments (except for the *P2* insertion) were obtained from the Bloomington Stock Center (Bloomington, Indiana) and the *Drosophila* Gene Disruption Project (*1*) (http://flypush.imgen.bcm.tmc.edu/pscreen/). The *P2* insertion (*f01257*) was obtained from the Exelixis collection at the Artavanis-Tsakonas laboratory (Harvard University). eas^{1} and *qvr* mutants were obtained from Dr. C.-F. Wu. Sh^{14} and Hk^{1} flies were obtained from the Bloomington Stock Center. Sh^{mns} was a gift from Dr. C. Cirelli. For the screen, each line was outcrossed to the *iso31* background twice and balanced before testing homozygotes. For subsequent examination of *sss* mutants, sss^{P1}, sss^{P2}, and sss^{qvr} were outcrossed to the *iso31* background 5 times, and balanced mutant and sibling control lines were established for each allele. Transgenic fly lines bearing the genomic *sss* rescue construct (TG1-3) were generated by standard techniques (*2*) in an *iso31* background (Rainbow Transgenics).

Generation of excision lines

Precise and imprecise excision lines were derived from the sss^{P1} line by mobilizing the P element using the Δ2-3 recombinase. By screening 49 excision lines by PCR

amplification and sequencing, we obtained several precise excision lines and one imprecise excision line. The imprecise excision line (*Δ40*) removes 1069 base pairs (from +1756 to +2824 of the *sss* genomic region relative to the translational start site). The cDNA from the *Δ40* line was sequenced between the translational start site and what corresponds to the stop codon in a wild-type strain; the *Δ40* protein is predicted to include the first 35 amino acids of SSS and 24 amino acids unrelated to SSS before encountering a stop codon. As the first 32 amino acids of SSS constitute the signal peptide, only 3 out of 126 amino acids of the mature protein are expected to be intact in the *Δ40* line, and thus the allele is likely to be null. In an initial experiment, three precise excision lines were assayed for sleep, and since they all had sleep amounts similar to wild-type control lines, one of them was selected for further characterization.

Sleep and circadian assays

Flies were entrained to a 12 hr:12 hr light:dark (LD) cycle for at least two days before being assayed for sleep in glass tubes containing 5% sucrose and 2% agarose using the *Drosophila* Activity Monitoring System (Trikinetics) in an incubator at 25^{o}C. For the screen, up to 8 female flies of 5 to 10 days of age were tested per line. In subsequent experiments on *sss* mutants, 4- to 7-day old male and female flies were monitored for sleep behavior. For sleep measurements, activity counts were collected in 30-sec or 1-min bins in LD for 2 days, and a moving window was used to identify sleep as periods of inactivity lasting at least 5 minutes (*3, 4*). Sleep parameters were computed using MATLAB-based (MathWorks) custom software. For analysis of circadian behavior, activity counts were collected in 30-min bins in DD over a 6-day period and analyzed

using ClockLab (Actimetrics) as previously described (*5*). One-way analyses of variance (ANOVAs) with genotype as a between-subject factor (and if there was a significant effect) followed by post-hoc comparisons with the Bonferroni correction were used to compare sleep and circadian parameters of more than two genotypes. For comparisons of two genotypes, unpaired t-tests with unequal variances were used. For analysis of sleep bout duration, which is not normally distributed, Mann-Whitney U test was used.

Sleep deprivation

Mechanical stimulation was applied for 2 seconds at random intervals averaging 20 seconds by a custom-built device to deprive flies of sleep for six hours (ZT 18-24) or 12 hours (ZT 12-24) in the second half of the night. Locomotor activity was monitored during mechanical stimulation, and only data from flies that were deprived of sleep by at least 75% compared with baseline conditions were included. Rebound sleep was calculated as the difference in the amount of sleep between the deprived and undisturbed control animals during the first 6 hours following deprivation. To account for individual differences in baseline sleep, pre-deprivation sleep was subtracted from post-deprivation sleep at ZT0-6 for each fly. Similarly, change in sleep latency due to deprivation was computed as the difference in latency to sleep between the deprived and undisturbed animals. Pre-deprivation sleep latency was subtracted from post-deprivation latency at ZT0 to account for individual differences. Two-way ANOVAs with genotype and deprivation as between-subject variables were performed to assess statistical significance of differences in rebound sleep and latency change between control and mutant strains.

Other behavioral assays

For assessment of general behaviors, 5-10 day old female flies were used (unless noted otherwise). Experimental flies were allowed to recover from CO_2 anesthesia for at least 1 day prior to testing. To measure phototaxis, a modified version of the fast phototaxis assay was used (*6*). In the dark, flies were quickly tapped down into a 17x100 mm tube connected to another similar tube, both of which were then laid in a horizontal position. Flies were exposed to light (15W fluorescent bulb) either proximal or distal to the original tube to assess propensity to run away from or towards the light, respectively. After 30 seconds, the number of flies in the original tube was counted. To assess the ability of flies to distinguish between attractive and aversive tastes, animals were given a modified two-choice preference test (*7*). 2% agarose plus 1 mM or 5 mM sucrose was evenly split across the bottoms of 17x100 mm vials. Each of the two food sources was supplemented with either red or blue food coloring, and in one set of experiments 1 mM quinine (Sigma) was added to the higher concentration of sugar. Flies were starved for 12-16 hrs, then added to vials and allowed to feed for 1 hr in the dark (to avoid influence of food color). After feeding, animals were frozen and examined visually for feeding preference by assessing the color of their abdomens. To assess bang-sensitivity, male flies were vortexed in vials at maximum speed for 10 seconds and examined for paralysis; eas^{1} flies were used as a positive control. For climbing assays, flies were gently tapped down into a vertical 17x100 mm tube, and the number of flies able to climb 9 cm in 5 and 10 seconds was counted. To elicit ether-induced leg shaking, we anesthetized flies using diethyl ether (Sigma) and observed for the characteristic high-frequency leg-shaking phenotype. Sh^{mns} flies were used as a positive control for ether-

induced leg-shaking. Differences in all general behavioral assays were assessed statistically using Chi-square tests.

Longevity assay

Background control and sss^{P1} mutant flies were maintained in a 12 hr:12 hr LD cycle at 25^{o}C. Groups of about 30 flies (males and females mixed) were collected into vials within 24 hr of eclosion. Flies were transferred to fresh vials and the number of dead flies counted every 2 days. Log-rank tests were performed to compare longevity of *sss* flies to that of control flies.

Molecular Biology

mRNA from adult fly heads was isolated using the Ultraspec RNA Isolation System (Biotecx) and reverse transcribed using Superscript III (Invitrogen). *sss* cDNA was amplified by RT-PCR using primers encoded by 5'-GGT TGG CCA GTA GTA ACT GGG AC-3' and 5'-GTC GAC GAG CCT AAC ACT TTC TAT CTG CTG AGC-3'. Three independent clones derived from multiple PCRs were subcloned using the TOPO TA-cloning system (Invitrogen) and sequenced in both directions. The cloned *sss* open reading frame is the same as the predicted sequence CG33472-RB in Flybase except for a few base changes. We did not observe RT-PCR products corresponding to the other predicted sequence CG33472-RA, suggesting that it is either rare or artifactual.

To construct pIZ-*sss*, the cloned *sss* cDNA was PCR amplified using the following primers: 5'-CGG AAT TCC GGC AAG ATG TGG ACG C-3' and 5'-AAC TCG AGC TAT CTG CTG AGC AAT TGA CC-3'. The PCR fragment was then

inserted into the pIZ/V5-His vector (Invitrogen), and the construct was verified by sequencing.

To generate the genomic *sss* rescue construct, ~9.8 kb of genomic sequence containing the entire 5'UTR and 3'UTR was recombined into the P[acman] vector by gap repair as described (*8*). Primers to amplify the left homology arm (LA) were designed ~400 bp upstream of the start of the 5'UTR and were as follows: 5'-CTT GTA CTC TCA TGC GCT C-3' and 5'-CCA CAA CAC TTT AGT GCA TCG C-3'. Primers to amplify the right homology arm (RA) were designed ~300 bp downstream of the end of the 3'UTR and were as follows: 5'-GGT GCT TCC AAC TCG CTT TGC-3' and 5'-CGT GCG AGC TAT CGG AAA CAC TC-3'. LA and RA were cloned into the P[acman] vector and confirmed by sequencing. Recombination was induced between linearized P[acman]-LA/RA and BACR09A11 (Children's Hospital Oakland Research Institute), and the desired recombinant was detected by PCR and then partially sequenced to confirm recombination.

To determine the molecular basis of the *qvr* mutation, we sequenced the coding region and intron-exon boundaries of the genomic DNA of *qvr* mutants and wild-type control flies, and did not find any sequence difference that would cause an amino acid substitution. We observed a few base changes in introns, however, and to determine if splicing is altered, we amplified *sss* cDNA in *qvr* mutants by RT-PCR using primers encoded by 5'-CGG AAT TCC GGC AAG ATG TGG ACG C-3' and 5'-AAC TCG AGC TAT CTG CTG AGC AAT TGA CC-3'. Three distinct bands were observed in *qvr* mutants compared with a single band in wild-type flies. All three *qvr* bands were sequenced, revealing altered splicing of Intron 6. Two of the three transcripts are

predicted to introduce a frame shift, but one of them is predicted to be in frame resulting in a 21 amino acid insertion.

Transient transfection and PI-PLC treatment

Drosophila S2R+ (*9*) cells were transfected with pIZ-*sss* (150 ng) in 24-well plates using Effectene (Qiagen). Cells were maintained at room temperature for two or three days before being processed for Western analysis or immunostaining. For PI-PLC treatment, cells were washed in PBS (10 mM phosphate buffer, pH 7.2, 0.15 M NaCl) once, and were incubated with or without PI-PLC (1 U/mL, Sigma) in PBS for 1 hr at 28^{o}C.

Western analysis and antibody production

Western blot analysis of S2R+ cell lysates and fly head extracts was performed as described (*5, 10*). For comparing SSS expression levels of different genotypes, head extracts from 8 females were loaded per lane. For comparison of different tissues, an equal amount of total protein (~40 ug) was loaded per lane. To assay release of SSS by PI-PLC treatment, protein in the medium was concentrated about 20-fold using a Microcon YM-10 filter (Millipore), and 100% of the concentrated medium or 8% of the cell extract was loaded per lane. The PA0681 rabbit antibody to SSS was raised against a peptide: DSWTDARCKDPFNYTALPR (Open Biosystems). We did not detect specific staining by the antibody in whole-mount brain samples, probably because the antibody poorly recognizes glycosylated SSS. We were able to circumvent this problem in Western analysis by first deglycosylating blots using Peptide N-Glycosidase F (PNGase F, New England Biolabs) before incubating them with the antibody to SSS. Antibodies

to SSS, Sh (DN16, Santa Cruz biotechnology), and MAPK (Sigma) were used at 1:500, 1:1000, and 1:2500, respectively.

Immunostaining

Flies entrained to a 12 hr:12 hr LD cycle were collected at ZT2, 8, 14, and 20, and immunostaining of whole-mount brain samples was performed as described (*10*). Samples were incubated with antibodies to PER (UPR34) at 1:1500 and Pigment Dispensing Factor (PDF, HH74) at 1:1000. PDF staining was used to identify ventral lateral neurons. Four to six fly brains were examined per condition. For immunostaining of S2R+ cells, transfected cells were fixed with 4% formaldehyde in PBS for 30 min at room temperature. After three quick washes with PBS, non-specific binding was blocked with culture medium (10% fetal bovine serum in Schneider's medium, Gibco). Cells were then incubated with antibody to SSS in culture medium at 1:250 for 1 hr, washed with culture medium for 15 min three times, and incubated with Cy3-conjugated anti-rabbit antibody at 1:500 for 1hr, followed by three washes with culture medium. To permeabilize cells, 0.1% Triton X-100 was added to the culture medium during fixing, antibody incubation and washing. Immunostained samples were imaged with a Leica confocal microscope.

Supplementary Table 1. Circadian rhythm parameters of *sss* mutants and controls.

Genotype	Number of flies	% rhythmic	Tau ± SEM (hr)	FFT ± SEM
ctrl	64	96.8	23.4 ± 0.03	0.165 ± 0.006
sss^{P1}	81	30.5	23.3 ± 0.09	0.056 ± 0.008
ctrl/*sss*	76	100	23.3 ± 0.04	0.130 ± 0.005
sss^{P2}/sss^{P1}	65	97.1	23.3 ± 0.04	0.109 ± 0.005

X^2 periodogram analysis was performed for each fly to determine the free-running period, tau. Relative FFT, determined by fast Fourier transform analysis, is a measure of rhythm strength. Pooled data for ctrl/sss^{P1} and ctrl/sss^{P2} (ctrl/*sss*) are presented.

References for Supporting Online Material

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