Identification of SLEEPLESS, a Sleep-Promoting Factor

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Sleep is an essential process conserved from flies to humans. The importance of sleep is underscored by its tight homeostatic control. Through a forward genetic screen, we identified a gene, *sleepless*, required for sleep in *Drosophila*. The *sleepless* gene encodes a brain-enriched, glycosylphosphatidylinositol-anchored protein. Loss of SLEEPLESS protein caused an extreme (>80%) reduction in sleep; a moderate reduction in SLEEPLESS had minimal effects on baseline sleep but markedly reduced the amount of recovery sleep after sleep deprivation. Genetic and molecular analyses revealed that *quiver*, a mutation that impairs *Shaker*-dependent potassium current, is an allele of *sleepless*. Consistent with this finding, Shaker protein levels were reduced in *sleepless* mutants. We propose that SLEEPLESS is a signaling molecule that connects sleep drive to lowered membrane excitability.

Insufficient and poor-quality sleep is an increasing problem in industrialized nations. Chronic sleep problems diminish quality of life, reduce workplace productivity, and contribute to fatal accidents (*1*). Although the biological needs fulfilled by sleep are unclear (*2*), they are likely to be important, because sleep is conserved from flies to humans (*3*–*7*) and prolonged sleep deprivation can lead to lethality (*8*–*10*). Identifying mechanisms that control sleep may lead to novel approaches for improving sleep quality.

Sleep is regulated by two main processes: circadian and homeostatic (*11*, *12*). The circadian clock regulates the timing of sleep, whereas the homeostatic mechanism controls the need for sleep. Homeostatic pressure to sleep increases with time spent awake and decreases with time spent asleep. Homeostatic control is thought to influence sleep under normal (baseline) conditions as well as recovery (rebound) sleep after deprivation. However, the molecular mechanisms underlying homeostatic regulation of sleep have remained unclear.

A powerful approach to unraveling a poorly understood biological process is to conduct unbiased genetic screens to identify molecules required for that process. The *Drosophila* model for sleep is well suited for such an approach, which proved invaluable for elucidation of the molecular basis of the circadian clock. Although several *Drosophila* genes have been implicated in sleep regulation [for example, (*13*–*15*)], only one of these, the gene encoding the Shaker (Sh) K^+ channel, was isolated as a result of a genetic screen (*16*). A mutation in this gene causes one of the shortest-sleeping phenotypes known, validating the use of screens and suggesting that control of membrane excitability is a critical requirement for sleep.

Using a large-scale, unbiased genetic screen, we identified a gene, *sleepless* (*sss*), which is required in *Drosophila* for both normal baseline sleep and rebound sleep after deprivation. We find that *sss* encodes a brain-enriched, glycosylphosphatidylinositol (GPI)–anchored membrane protein. We also show that *quiver* (*qvr*), a mutation causing impaired *Sh*-dependent K^+ current (*17*, *18*), is an allele of *sss*, and that Sh protein levels are reduced in *sss* mutant flies. We propose that the SSS protein signals homeostatic sleep drive by enhancing K^+ channel activity and thus reducing neuronal excitability.

Identification of *sss*. To identify genes involved in sleep regulation, we carried out a forward genetic screen for *Drosophila* mutants with reduced daily sleep. We screened ~3500 mutant lines bearing transposon insertions. A histogram summarizing the daily sleep of these lines is shown in Fig. 1A. We selected for further study the mutant line with the lowest amount of daily sleep, which we named *sleepless* (*sss*). To homogenize the genetic background, we outcrossed this strain five times into an isogenic wild-type strain, *iso31*, a line generated specifically for use in behavioral experiments (*19*). Both daytime and nighttime sleep were severely reduced in both male and female *sss* mutants relative to background controls (Fig. 1B). Indeed, a small percentage of *sss* flies (~9% for both males and females) in our assay did not sleep at all—a phenotype never seen in control flies. To our knowledge, *sss* mutants exhibit the most extreme reduction in daily sleep (>85% for males and >80% for females; Fig. 1C) attributable to a single gene mutation.

Despite this extreme reduction in daily sleep, waking activity (defined as activity counts per minute awake) was not significantly elevated in this mutant (Fig. 1D), which suggests that the mutant is not hyperactive when awake (*20*). The marked decrease in sleep amount was largely due to a sharp reduction in the duration of sleep bouts (Fig. 1E). However, decreased sleep in the *sss* mutant was also attributable in part to a significant reduction in the number of daily sleep bouts (Fig. 1F). These phenotypes are recessive in mutant animals, because flies bearing one copy of the *sss* mutation behaved similarly to background controls (Fig. 1, C to F).

SSS is a brain-enriched, GPI-anchored protein. *sss* mutants bear a P-element insertion (*EY04063*, which we refer to as *P1*) in the open reading frame of a gene designated CG33472 by the *Drosophila* Genome Project. The genomic structure of this gene consists of two noncoding exons and five coding exons, the last of which also contains a 3′ untranslated region (3′UTR) predicted to be ~3.9 kb (Fig. 2A). In addition to the original *P1* insertion line, there is a second line, which we call *P2*, bearing a transposon insertion (*f01257*) in the 3′UTR. The SSS protein is predicted to contain a signal peptide, an N-type glycosylation site, and a potential GPI attachment site (Fig. 2, B and C). SSS is well conserved in other insect species, and there is a potential *Caenorhabditis elegans* homolog (F31F6.8 in Wormbase, 46% similarity for amino acids 51 to 133) but no obvious vertebrate homologs. Nonetheless, there may be functional vertebrate homologs with conserved downstream pathways.

To characterize the SSS protein, we used a peptide antigen to generate an antibody (*21*). This antibody recognized two bands on Western blots of wild-type head extracts that were not detectable in sss^{P1} mutant extracts (Fig. 2D), which suggests that sss^{P1} is a severe hypomorph or null allele. Because SSS contains a consensus site for N-type glycosylation, we deglycosylated proteins from head extracts and examined SSS mobility by Western blotting. Under these conditions, only a single band of a lower apparent molecular weight than the two untreated bands was detectable (Fig. 2D), indicating that SSS is glycosylated in vivo.

Because *sss* also contains a potential GPI attachment site, we next examined subcellular localization of SSS. Transfection of *Drosophila* $S2R^+$ cells with a wild-type *sss* construct and staining with the SSS antibody under nonpermeabilizing conditions revealed a subset of the SSS protein expressed on the cell surface (Fig. 2E). Treatment of the cells with phosphatidylinositol-specific phospholipase C (PI-PLC) resulted in severe reduction of surface expression (Fig. 2F) and release of the SSS protein into the culture medium (Fig. 2G). These results show that the SSS protein is attached to the extracellular surface of the plasma membrane with a GPI anchor and can be released by cleavage with PLC.

Using our SSS antibody, we found that SSS protein levels are enriched in fly brain and head relative to body (Fig. 2H). Consistent with these findings, *sss* mRNA expression is enriched by a factor of 23 to 42 in brain relative to whole fly [Adult *Drosophila* Gene Expression Atlas (*22*)]. SSS protein levels did not cycle in a circadian fashion, nor did they change after sleep deprivation (fig. S1, A and B) (see below).

Genetic analysis of *sleepless*. To determine whether the sleep phenotype maps to the *sss* locus, we crossed sss^{P1} to two deficiencies that remove the locus. As predicted, both deficiencies

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failed to complement the short-sleeping phenotype of sss^{P1} (fig. S2, A and B). To confirm that the sleep phenotype in sss^{P1} mutants is caused by disruption of the *sss* gene, we mobilized the P-element to generate precise and imprecise excision lines. Precise excision of the P-element restored daily sleep amount in *sss* mutants to wild-type levels (Fig. 3A and fig. S2C). We also obtained an imprecise excision allele (*Δ40*) that removes part of the *sss* coding region and is likely to be a null allele (fig. S2D). Consistent with this interpretation, $sss^{\Delta 40}$ mutants produced an undetectable level of the SSS protein (Fig. 3B). Sleep in this mutant is reduced as severely as in the *P1* mutant; the phenotype maps to the *sss* gene, because the *Δ40* allele failed to complement the *P1* allele (Fig. 3A and fig. S2C).

We next tested whether expression of wild-type SSS from a transgene could rescue the sleep phenotype of sss^{P1} mutants. Daily sleep amount was fully rescued to wild-type levels in sss^{P1} mutants carrying a genomic *sss* transgene (Fig. 3C and fig. S2E). Together with the results of the deficiency and excision experiments, the rescue data provide strong evidence that disruption of the *sss* gene is responsible for the marked reduction in sleep in sss^{P1} mutants.

As described above, sss^{P2} mutants harbor an independent transposon insertion in the 3′UTR of the *sss* gene. Homozygous sss^{P2} mutant females had amounts of daily sleep similar to those of controls, whereas mutant males had slightly lower amounts of sleep than controls (Fig. 3D and fig. S2F). In contrast, sss^{P2}/sss^{P1} trans-heterozygous mutants had a ~30% reduction in daily sleep relative to control/sss^{P1} flies. These data suggest that the *P2* insertion is a weaker allele than the original *P1* insertion. To examine the biochemical basis of this possibility, we performed Western analysis on head lysates from mutant and control flies. As noted above, the *P1* insertion severely reduced baseline sleep and rendered SSS undetectable (Figs. 2D and 3B). In contrast, the *P2* insertion, which had a minimal effect on baseline sleep, caused a moderate reduction in the level of SSS protein relative to control flies (Fig. 3E). Finally, trans-heterozygous sss^{P1}/sss^{P2} flies, which exhibit a ~30% reduction in sleep, had a greatly reduced but still detectable level of SSS protein. These data suggest that the amount of daily sleep is correlated with the level of SSS protein and that large reductions of SSS protein are necessary to cause a substantial change in daily sleep.

Reduced homeostatic response in *sss* mutants. We next sought to determine whether *sss* mutants have defects in their homeostatic response to sleep deprivation. We did not observe rebound sleep in sss^{P1} flies, but sss^{P1} flies do not have much sleep to deprive. Thus, we tested sss^{P1}/sss^{P2} trans-heterozygous flies, which still have moderate amounts of sleep, as well as sss^{P2} homozygotes, which have essentially normal amounts of sleep.

Mechanical stimulation resulted in equivalent sleep loss in sss^{P2} homozygous flies and controls; sleep loss was moderately reduced in sss^{P1}/sss^{P2} flies relative to controls (Fig. 4A and fig. S3A). Whereas control flies showed substantial rebound sleep after deprivation, sss^{P1}/sss^{P2} flies had little or none (Fig. 4B and fig. S3B). Unexpectedly, we observed a similar lack of rebound sleep in sss^{P2} homozygous flies. In addition, when lights were turned on, control animals went to sleep faster after deprivation, but this effect was significantly less pronounced or nonexistent in sss^{P2} and sss^{P1}/sss^{P2} mutants (Fig. 4C and fig. S3C).

Although other genes have been suggested to play a role in homeostatic regulation of sleep, assessment of rebound sleep in animals bearing mutations in these genes is often confounded by concomitant reductions in baseline sleep (*13*, *16*, *23*, *24*). The amount of rebound sleep generally increases with sleep lost (*25*, *26*). Thus, when comparing the effects of sleep deprivation in animals with different amounts of baseline sleep (which leads to loss of different amounts of sleep), it is unclear whether rebound sleep should be compared in absolute terms or relative to amount of sleep lost. We have circumvented this problem by using the sss^{P2} mutant to study the contribution of SSS to sleep homeostasis. The finding that sss^{P2} animals exhibit markedly reduced rebound sleep, but minimally affected baseline sleep, provides strong evidence that sleep homeostasis is impaired in these mutants.

Effect of *sss* on other behaviors and longevity. To further characterize *sss* mutants, we examined several other behavioral phenotypes. Because mutations in certain central clock genes cause baseline and rebound sleep phenotypes (*9*, *27*–*30*), we analyzed the circadian rhythm phenotypes of *sss* mutants. Whereas sss^{P1} mutants exhibited weak rhythms, almost all sss^{P1}/sss^{P2} trans-heterozygous mutants, which displayed a ~30% reduction in daily sleep time, were rhythmic (Fig. 5, A and B, and table S1). Furthermore, daily oscillations in the level of PERIOD (PER) protein in the ventral lateral neurons (clock cells) remained intact in sss^{P1} mutants (Fig. 5C), which suggests that the reduced behavioral rhythmicity seen in these mutants is not due to a defect in the central clock.

Several other behaviors that we tested also appear normal. We found that the phototactic

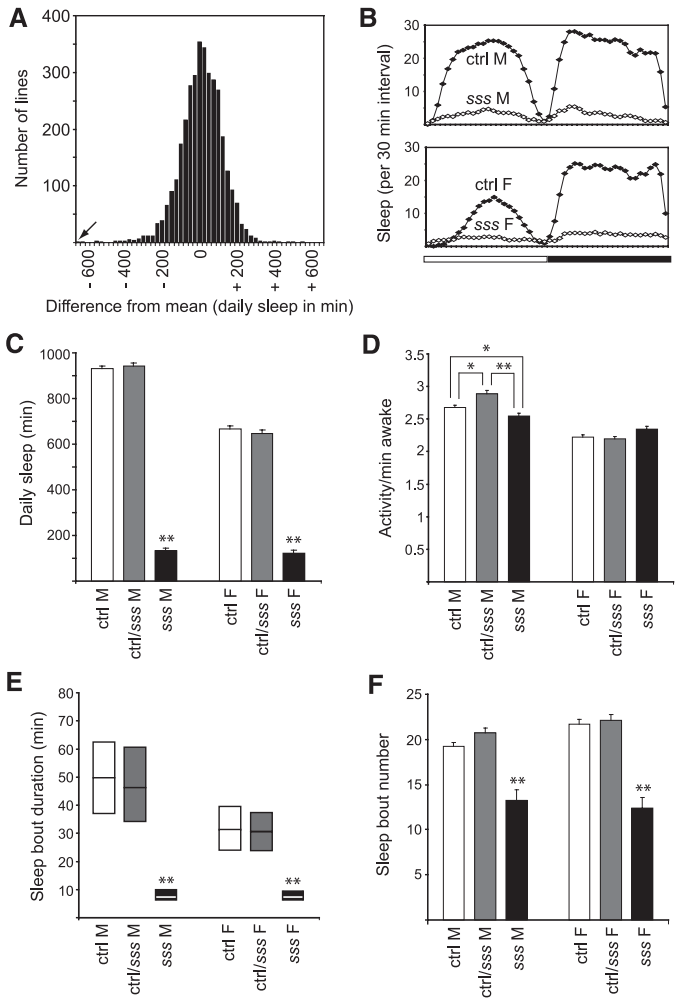

Fig. 1. Sleep phenotype of *sss* mutants. (**A**) Histogram showing the distribution of daily sleep for ~3500 mutant lines (~8 female flies per line). For each line, daily sleep is shown as the difference from the mean of a group of about 100 to 250 lines tested simultaneously. The arrow indicates the *sss* mutant line. (**B**) Sleep profile in 30-min intervals for *sss* flies (open diamonds) versus background controls (ctrl, solid diamonds). Data for male (M) and female (F) flies are shown. The bar below the *x* axis indicates 12-hour light and 12-hour dark periods. (**C**) Daily sleep amount for control (162 males and 148 females), control/*sss* (111 males and 113 females), and *sss* flies (146 males and 148 females). Data from the same flies are shown in (C) to (F). (**D** to **F**) Activity counts per minute awake (D), sleep bout duration (E), and daily number of sleep bouts (F) for male and female control, control/*sss*, and *sss* flies. In this and subsequent figures, error bars represent SEM. $*P < 0.05$, $**P < 0.0001$. For (C), (E), and (F), significance level is shown for *sss* mutants compared to both control and control/*sss* flies. For (D), significance level is shown for pairwise comparisons as indicated by lines. In (E), sleep bout duration, which is not normally distributed, is presented as simplified box plots. The line inside each box indicates the median; the top and bottom represent 75th and 25th percentiles, respectively. About 9% of flies exhibiting zero sleep were excluded from calculation of sleep bout duration.

responses of sss^{P1} mutants are similar to those of controls (fig. S4A) and that sss^{P1} mutants perform as well as controls in a taste discrimination assay (fig. S4B). sss^{P1} flies ($n = 43$) did not exhibit a bang-sensitive paralytic phenotype, whereas 89% ($n = 56$) of *easily shocked* (eas^{1}) flies used as a positive control did exhibit this phenotype. On the other hand, the sss^{P1} mutants appeared somewhat uncoordinated, and fewer mutants were able to climb a specific distance in given amounts of time relative to controls (fig. S4C). However, despite their apparent difficulties with coordination, sss^{P1} mutants spent more time walking than controls and were capable of flying and mating. Consistent with the widely held view that sleep serves essential biological functions, sss^{P1} mutants also exhibited a shortened life span relative to background controls (Fig. 5D and fig. S5).

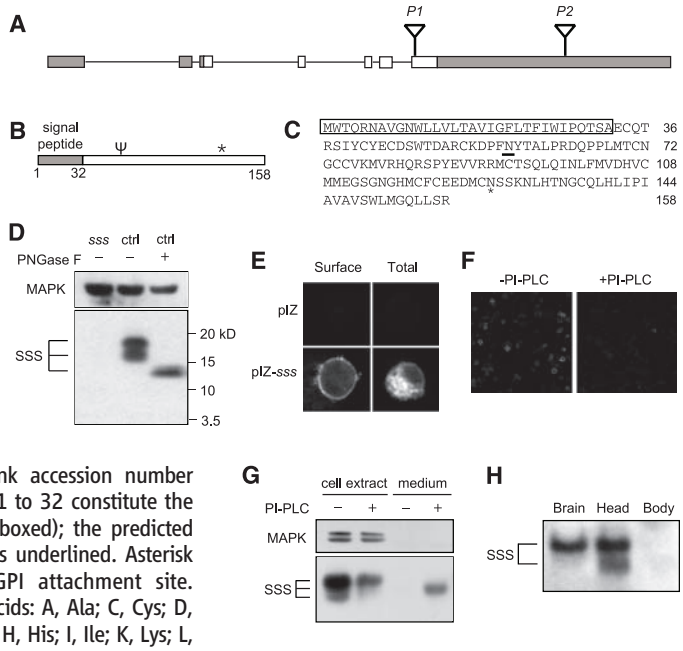

Fig. 2. *sss* encodes a brain-enriched, GPI-anchored protein. (**A**) Schematic of the genomic structure of the *sss* locus. Noncoding regions of the cDNA are shaded; coding regions are shown in white. (**B**) Schematic of structural features of the SSS protein. The primary sequence contains a predicted signal peptide, an N-type glycosylation site (Ψ), and a potential GPI attachment site (*). (**C**) Amino acid sequence of SSS (GenBank accession number EU816195). Amino acids 1 to 32 constitute the predicted signal peptide (boxed); the predicted N-type glycosylation site is underlined. Asterisk denotes the predicted GPI attachment site. Abbreviations for amino acids: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. (**D**) Glycosylation of the SSS protein. Western blot analysis with SSS antibody revealed two bands detected in head extracts from wild-type (ctrl) but not *sss* flies. Deglycosylation of head extracts by treatment with PNGase F resulted in detection of a single band. Because our antibody to SSS does not recognize glycosylated SSS well, Western blots were treated with peptide *N*-glycosidase F (PNGase F) before being probed with antibody to SSS. In this and subsequent Western blots, antibody to mitogen-activated protein kinase (MAPK) was used to control for loading. (**E**) Surface expression of SSS in cultured *Drosophila* cells. S2R^{+} cells were transfected with a pIZ-*sss* construct and stained with or without permeabilization to assay for total or surface expression, respectively. Transfection with the pIZ vector alone shows specificity of our SSS antibody. (**F**) Reduced surface expression of SSS after PI-PLC treatment. S2R^{+} cells transfected with a pIZ-*sss* construct were stained without permeabilization after PI-PLC (+) or mock (–) treatment. (**G**) Release of SSS into the culture medium by PI-PLC. Western blot analysis of S2R^{+} cells transfected with pIZ-*sss* was performed after PI-PLC (+) or mock (–) treatment. (**H**) Enrichment of SSS expression in brain and head versus body. An equal amount of total protein (~40 μg) was loaded per lane. The experiments in (D) through (H) were performed three or four times with similar results.

***sss* is allelic to *qvr* and affects Sh expression.** Because two short-sleeping mutants, *Sh* and *Hyperkinetic* (*Hk*), exhibit ether-induced leg shaking, we assayed *sss* mutants for this phenotype (*16*, *23*). We found that both sss^{P1} and sss^{P2} mutants show ether-induced leg shaking. Notably, *qvr*, a mutant for which the underlying molecular defect is unknown, also has a leg-shaking phenotype, and this phenotype has been mapped close to *sss* (*17*). Because *qvr* mutants exhibit impaired *Sh*-dependent K^{+} current (*18*), identification of *qvr* as an allele of *sss* would implicate *Sh* as an effector of SSS function.

Genetic and molecular analyses confirmed that *qvr* is indeed an allele of *sss*. The *qvr* mutation failed to complement sss^{P1} for the leg-shaking phenotype. Similarly, after being outcrossed five times, *qvr* mutants showed a significant decrease in sleep relative to wild-type controls, and sss^{P1}/qvr trans-heterozygotes showed a further reduction in sleep (Fig. 6A and fig. S6).

We next investigated the molecular basis of the *qvr* mutation. Reverse transcription polymerase chain reaction (RT-PCR) of *sss* transcripts in *qvr* mutants produced three bands, whereas that of wild-type *sss* transcripts produced a single band (Fig. 6B), indicating splicing defects in *qvr* mutants. None of the three *qvr* bands showed the same electrophoretic mobility as the wild-type control band. Sequencing of the RT-PCR products revealed altered splicing of the last intron (intron 6) of *sss* in the *qvr* mutant (Fig. 6C). A single base change found in the intron is likely to be responsible for the defective splicing (Fig. 6D). Only one of the three *qvr* transcripts (*qvr* 2) is predicted to be in frame (resulting in an insertion of 21 amino acids) and thus has the potential to produce functional SSS protein. Western analysis of *qvr* mutants revealed a small amount of SSS with a slightly higher apparent molecular weight than wild-type SSS protein, which may correspond to the product of the in-frame *qvr* 2 transcript (Fig. 6E).

Because *qvr* mutants were shown to have severely reduced *Sh*-dependent K^{+} current (*18*), we examined whether Sh protein levels are affected in *sss* mutants. We found that one form of

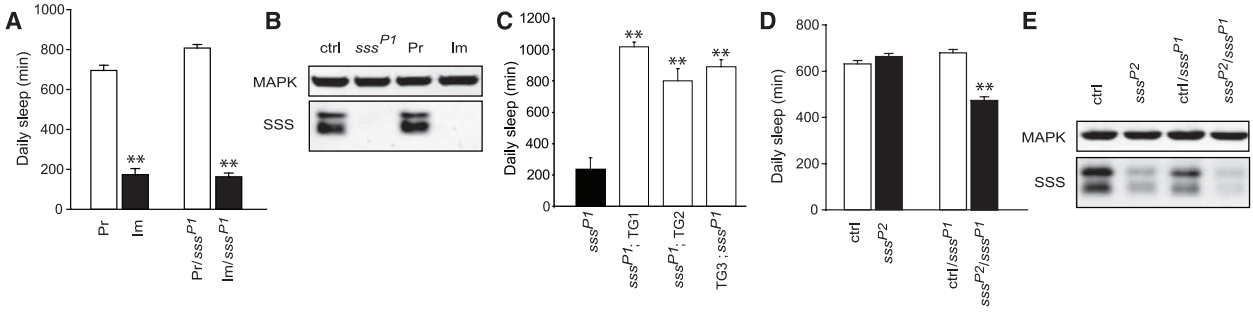

Fig. 3. Genetic analysis of *sss*. (**A**) Daily sleep amount for precise excision (Pr, $n = 26$), $sss^{\Delta 40}$ imprecise excision (Im, $n = 15$), precise/sss^{P1} (Pr/sss^{P1}, $n = 24$), and imprecise/sss^{P1} (Im/sss^{P1}, $n = 35$) female flies. (**B**) Western blot analysis of SSS protein levels. Similar levels of SSS protein are seen in head extracts from background control (ctrl) and precise excision (Pr) flies. SSS protein is undetectable in sss^{P1} and $sss^{\Delta 40}$ imprecise excision (Im) flies. Similar results were obtained in two additional experiments. (**C**) Daily sleep amount for female sss^{P1} mutant flies with (TG1, $n = 15$; TG2, $n = 8$; TG3, $n = 16$) or without ($n = 16$) a genomic *sss* transgene. TG1, 2, and 3 refer to three independent transgene insertions, and one or two copies of the transgene were present in the flies tested. (**D**) Daily sleep amount for sss^{P2} ($n = 110$) versus background control (ctrl, $n = 80$) as well as control/sss^{P1} ($n = 80$) versus sss^{P2}/sss^{P1} ($n = 112$) female flies. (**E**) Reduced levels of SSS protein in sss^{P2} and trans-heterozygous sss^{P2}/sss^{P1} flies. Similar results were obtained in three additional experiments. Data from male flies of the genotypes shown in (A), (C), and (D) are shown in fig. S2. *$P < 0.05$, **$P < 0.0001$.

Sh protein is expressed at a substantially reduced level in sss^{P1} mutants relative to wild-type flies (Fig. 6F), which suggests that SSS affects Sh at least in part through its protein expression. These results establish SSS as an important regulator of the Sh K^+ channel.

Discussion. We have identified a *Drosophila* gene required for homeostatic regulation of sleep under normal conditions and after sleep deprivation. Although genes have been identified that regulate sleep-wake stability and baseline sleep amount, few have been shown to be important for sleep rebound (*13*, *15*, *31*–*35*). Thus, further analysis of SSS function may provide a rare opportunity to gain mechanistic insight into the homeostatic regulation of sleep.

It is worth noting that sss^{P2} animals show a moderate reduction in SSS protein and a minimal reduction in baseline sleep, but have severely reduced sleep rebound. The differential requirement for SSS protein in normal versus rebound sleep may be explained in the context of the two-process model of sleep regulation, where sleep is postulated to be controlled by the opposing influences of circadian waking drive and homeostatic sleep drive (*11*, *36*). In this context, for early-morning rebound sleep to occur, a strong homeostatic signal promoting sleep would be required to counteract a strong circadian input keeping the flies awake. At night, when circadian waking drive is weaker or absent, a relatively low level of homeostatic input may suffice to allow flies to sleep. The moderate level of SSS protein in sss^{P2} mutants may be within the range where sleep is possible when a wake-promoting circadian signal is low (at night), but not when it is high (in the early morning). In contrast, sss^{P1} and $sss^{\Delta 40}$ mutants, which have undetectable levels of SSS expression, display severe reductions in both baseline and rebound sleep. In these mutants, the sleep-promoting signal may be too low to allow flies to sleep even when the circadian waking drive is weak at night.

Clues to the role of SSS at the cellular level come from our biochemical characterization of this molecule. The SSS protein is a GPI-anchored membrane protein enriched in the brain. GPI-anchored proteins can function as ligands or co-receptors and can also act as diffusible signals after cleavage of the GPI anchor (*37*, *38*). Although we were unable to detect circadian or homeostatic regulation of the total levels of SSS protein, such regulation may occur at the level of cleavage of the GPI anchor. Regulation of release is known to be controlled by time of day for other proteins that do not cycle in overall levels, such as pigment-dispersing factor, a molecular output of clock neurons (*39*). Alternatively, SSS may be regulated in a subset of cells that express it, which would be undetectable on our Western blots.

A potential mechanism by which SSS regulates sleep is suggested by our finding that *qvr* is an allele of *sss* and that Sh protein levels are reduced in *sss* mutants. Furthermore, *qvr* mutants exhibit markedly impaired *Sh*-dependent K^+ current at the larval neuromuscular junction (*18*). Thus, we propose that SSS lowers membrane excitability by modulating K^+ channel expression and activity. It is striking that among thousands of mutants screened in *Drosophila*, two with the strongest sleep phenotypes affect the *Sh* K^+ channel (*16*) and its putative regulator, *sss*. Reduced membrane excitability may thus be a central feature of sleep. Collectively, our data suggest that SSS is a signaling molecule that links homeostatic sleep drive to neuronal excitability.

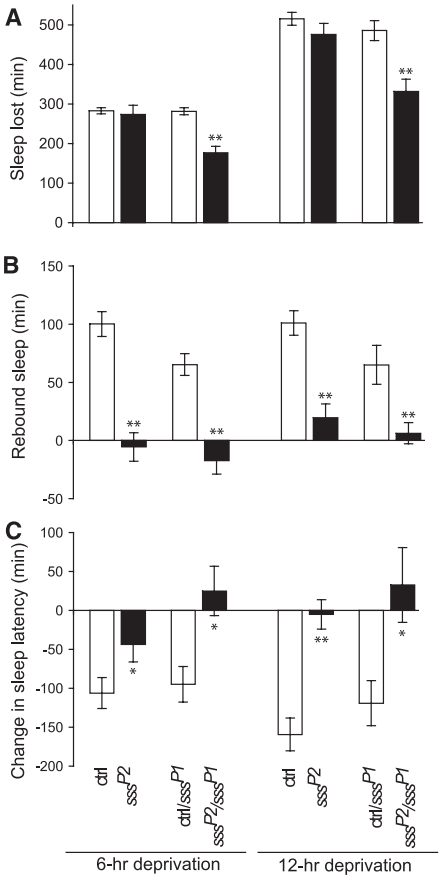

Fig. 4. Reduced homeostatic response to sleep deprivation in female *sss* mutants. (**A**) Amount of sleep lost during 6 or 12 hours of deprivation by the end of the dark period for background control (ctrl), sss^{P2}, control/sss^{P1}, and sss^{P2}/sss^{P1} flies. Data from 13 to 56 female flies are shown. (**B**) Amount of sleep gained during 6 hours of recovery after deprivation as in (A). (**C**) Change in sleep latency after deprivation relative to undisturbed controls as in (A). Sleep latency is defined as the time between the end of deprivation (which coincided with light onset) and the start of a sleep bout. Data from male files are shown in fig. S3. *$P < 0.05$, **$P < 0.001$.

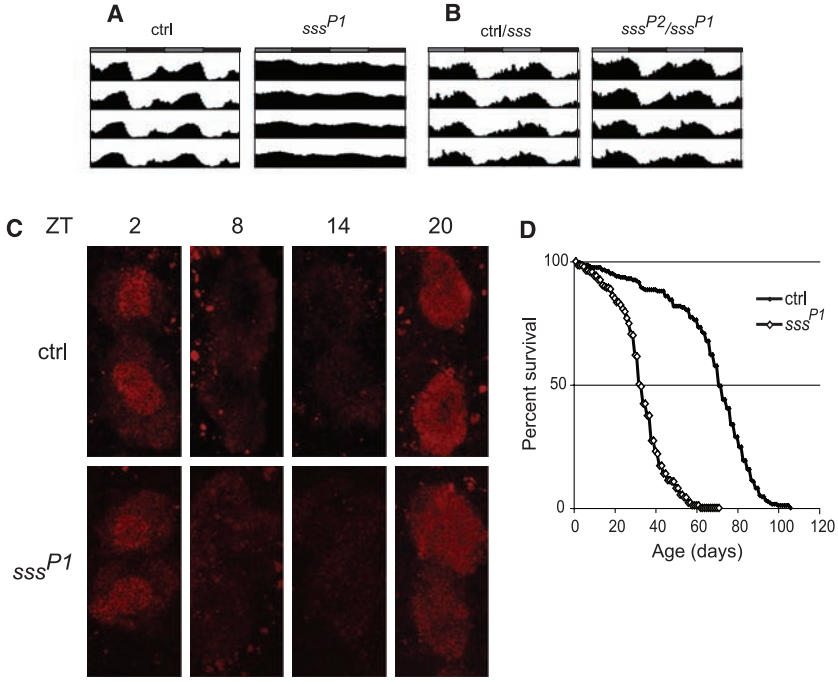

Fig. 5. Circadian rhythm and longevity phenotypes of *sss* mutants. (**A**) Average activity records for background control (ctrl, $n = 64$) and sss^{P1} male flies ($n = 81$) assayed in constant darkness (DD). The activity records are double-plotted so that each horizontal line represents data for 2 days. The gray and black bars above each activity record indicate subjective day and night, respectively. (**B**) Activity records showing average activity in DD for control/sss^{P1} and control/sss^{P2} ($n = 76$) versus sss^{P2}/sss^{P1} ($n = 65$) male flies. Circadian data for control/sss^{P1} and control/sss^{P2} flies were statistically similar and thus were pooled. (**C**) Cycling of PER protein in large ventral lateral neurons in control and sss^{P1} mutants. Ventral lateral neurons for control and sss^{P1} flies were stained for PER at indicated Zeitgeber times (ZT). PER protein levels are elevated at ZT2 and ZT20 and are low at ZT8 and ZT14. (**D**) Survivorship curves of background control (solid diamonds) and sss^{P1} (open diamonds) flies. Female *sss* flies ($n = 187$) show a significantly shorter life span ($P < 0.0001$) than controls ($n = 198$). Data from male flies are shown in fig. S5.

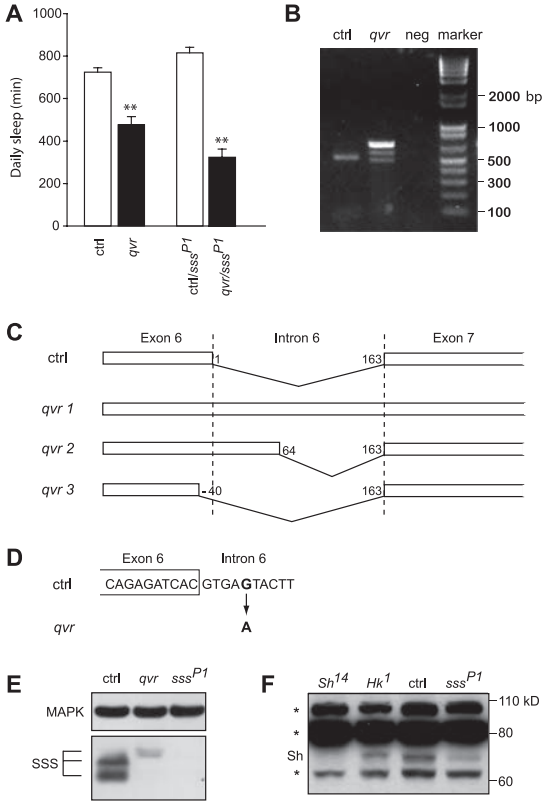

Fig. 6. *sss* is allelic to *qvr* and affects Sh expression. (**A**) Daily sleep amount for *qvr* ($n = 31$), versus background control ($n = 32$) as well as control/sss^{P1} ($n = 30$) versus *qvr*/sss^{P1} ($n = 32$) female flies. **$P < 0.0001$. (**B**) Altered *sss* transcripts in *qvr* mutants. RT-PCR products were obtained with *qvr* and background control (ctrl) RNA and water was used as a negative control (neg). (**C**) Schematic representation of *sss* transcripts in *qvr* mutants. *qvr* 1, 2, and 3 correspond to the top, middle, and bottom bands, respectively. In background control transcripts, 163 nucleotides of intron 6 are spliced out. In contrast, the entire intron is present in *qvr* 1 transcripts. In *qvr* 2 and 3 transcripts, splice donor sites differ from the one used in wild-type control transcripts, as indicated by the nucleotide numbers for splice sites. (**D**) Sequence change in *qvr* genomic DNA in intron 6 of *sss*. The fifth nucleotide in intron 6 has a G → A transition. (**E**) Altered expression of SSS in *qvr* mutants. Fly head extracts from background control, *qvr*, and sss^{P1} flies were analyzed by Western blotting with SSS antibody. (**F**) Reduced expression of Sh in *sss* mutants. Western blot analysis of head extracts with Sh antibody reveals a Sh-specific band that is substantially reduced in sss^{P1} mutants relative to background control flies. Sh^{14} flies were used to identify a Sh-specific band, and Hk^1 flies were used as an additional control. Nonspecific bands (*) may have obscured additional Sh bands. The experiments in (E) and (F) were performed three times with similar results.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/321/5887/372/DC1
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REPORTS

Properties of Gamma-Ray Burst Progenitor Stars

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We determined some basic properties of stars that produce spectacular gamma-ray bursts at the end of their lives. We assumed that accretion of the outer portion of the stellar core by a central black hole fuels the prompt emission and that fall-back and accretion of the stellar envelope later produce the plateau in the x-ray light curve seen in some bursts. Using x-ray data for three bursts, we estimated the radius of the stellar core to be $\sim(1 - 3) \times 10^{10}$ cm and that of the stellar envelope to be $\sim(1 - 2) \times 10^{11}$ cm. The density profile in the envelope is fairly shallow, with $\rho \sim r^{-2}$ (where ρ is density and r is distance from the center of the explosion). The rotation speeds of the core and envelope are ~0.05 and ~0.2 of the local Keplerian speed, respectively.

Observations of gamma-ray bursts (GRBs) suggest that the activity at the center of these explosions lasts for several hours (*1*, *2*). The most compelling evidence is provided by three bursts (*3*)—GRBs 060413, 060607A, and 070110—that show a sudden decline in their x-ray light curves (LCs) a few hours after the prompt burst (Fig. 1). The flux decline is by a factor of 10 or more and is much too sharp for the radiation to originate in an external forward shock (FS) (*4*); the most likely explanation is continued activity at the center of the explosion, at least until the time of the decline. Additional evidence for continued activity of the central engine is provided by the x-ray flares seen in many GRBs (*5*–*7*) and also by those bursts whose x-ray and optical afterglow LCs are mutually incompatible with a common origin (*8*, *9*). In fact, central engine activity is implicated whenever the observed flux variability time scale, δt, is much smaller than the time

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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.

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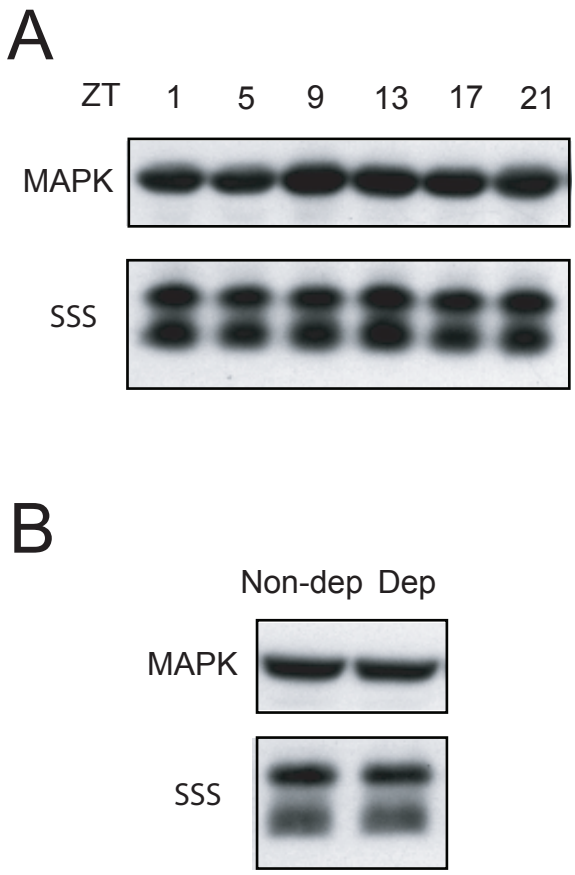

fig. S1. Regulation of SSS protein. (**A**) Circadian profile of SSS protein in head extracts. Wild-type fly heads were collected at indicated Zeitgeber times (ZT), and SSS levels were determined by Western blot analysis. (**B**) SSS protein levels in head extracts do not change in response to sleep deprivation. The SSS protein level of wild-type (*iso31*) flies that were deprived of sleep for 8 hours during ZT 12-20 (Dep) is comparable to that of wild-type flies that were not deprived (Non-dep). Each of these experiments was performed 3 times with similar results.

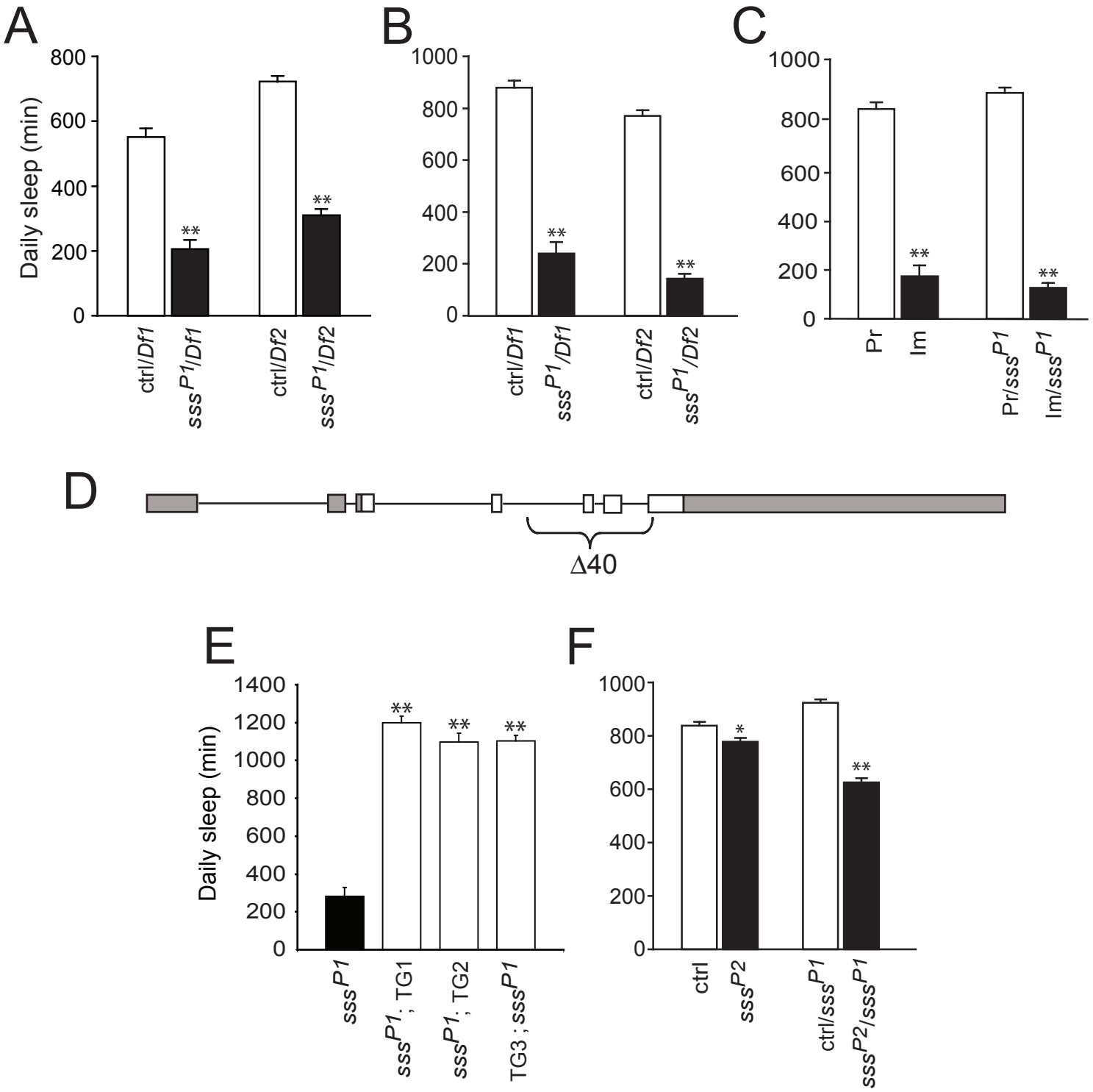

fig. S2. Genetic analysis of *sss* flies. **(A-B)** Deficiency mapping of the *sss* mutation. Daily sleep in minutes is shown for *Df(2R)ED2219* (*Df1*) and *Df(2R)en-B* (*Df2*) flies crossed to either background control (ctrl) or sss^{P1} flies. 21-65 female (A) and 18-42 male (B) flies were tested in each condition. (**C**) Daily sleep amount for precise excision (Pr; n=25), $sss^{\Delta 40}$ imprecise excision (Im, n=18) , precise/sss^{P1} (Pr/ sss^{P1}, n=29), and imprecise/sss^{P1} (Im/sss^{P1}, n=33) male flies. (**D**) Schematic of the *sss* genomic region and the imprecise excision allele, Δ*40*. The bracket indicates bases deleted in the Δ*40* allele. (**E**) Daily sleep amount for male sss^{P1} mutant flies with (sss^{P1};TG1-3, n=16, 8, 15, respectively) or without (sss^{P1}, n=13) a genomic *sss* transgene. TG1-3 refer to three independent transgenic insertions and either 1 or 2 copies of the transgene were present in the flies tested. **(F)** Daily sleep amount for sss^{P2} (n=106) versus background control (ctrl, n=80), as well as ctrl/sss^{P1} (n=79) versus sss^{P2}/ sss^{P1} (n=112) male flies. $^{*}P < 0.05$; $^{**}P < 0.0001$.

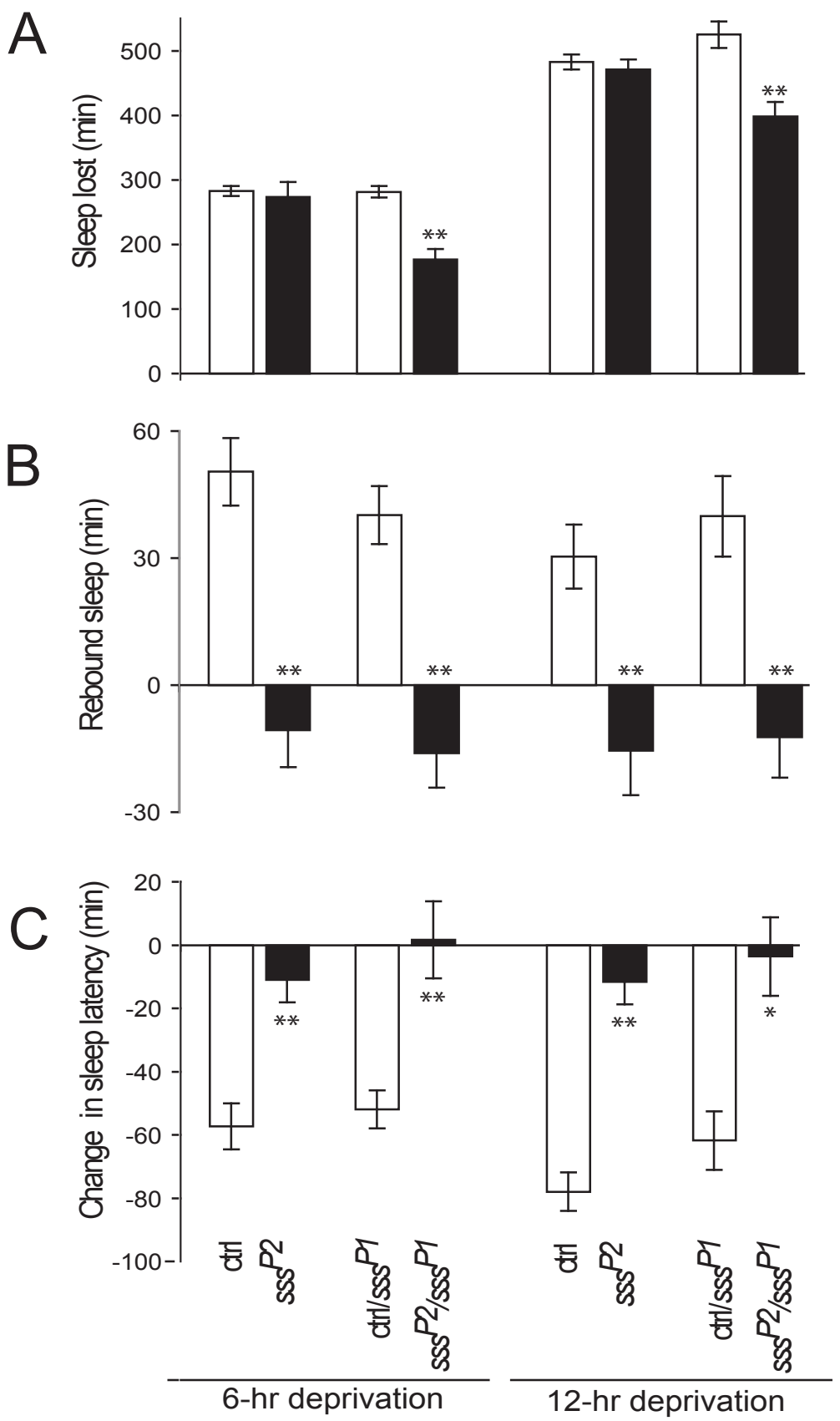

fig. S3. Reduced homeostatic response to sleep deprivation in male *sss* mutants. **(A)** Amount of sleep lost during 6 or 12 hours of deprivation at the end of the dark period for background control (ctrl), sss^{P2}, ctrl/sss^{P1}, and sss^{P2}/sss^{P1} flies. Data from 18-60 male flies are presented. **(B)** Amount of sleep gained during 6 hours of recovery following deprivation as in (A). **(C)** Change in sleep latency following deprivation, compared to undisturbed controls as in (A). $*P < 0.05$; $**P < 0.001$.

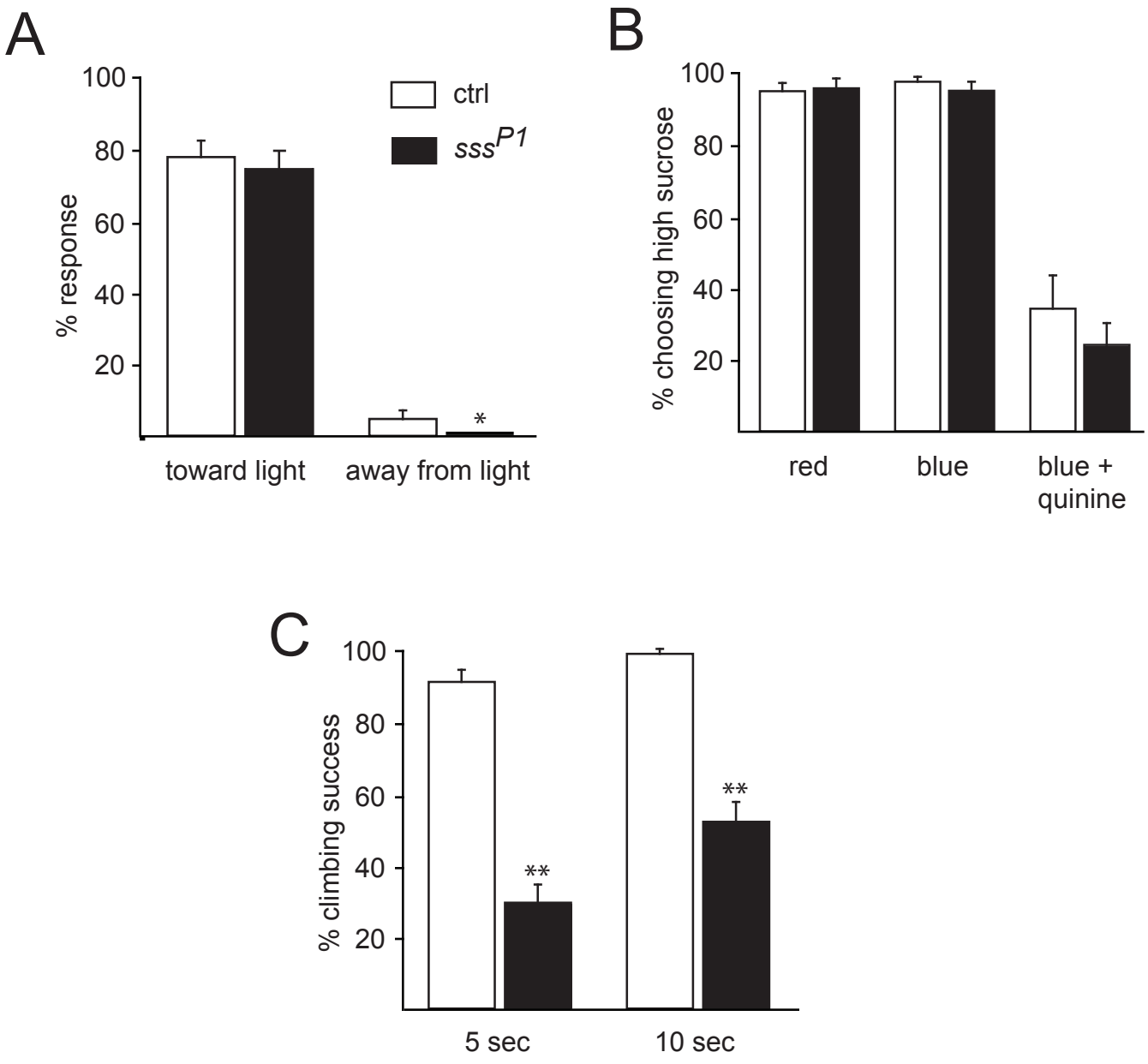

fig. S4. General behavioral assays for *sss* mutants. (**A**) Percent of control and sss^{P1} flies that run toward a distal light source or away from a proximal light source is shown. 79-82 flies were tested in each condition. (**B**) Percent of control and sss^{P1} flies that choose 5 mM over 1 mM sucrose is shown. Addition of food coloring (red or blue) or quinine to the 5 mM sucrose condition is denoted below the results of each of three experiments. No preference is observed for color of food. Control and mutant flies have an equivalent preference for 5 mM over 1 mM sucrose and an equivalent avoidance of 1 mM quinine in the presence of the higher concentration of sugar. 26-95 animals were used in each condition. (**C**) Percent of control and sss^{P1} flies that climb 9 cm in either 5 or 10 seconds is shown. 74-83 animals were tested in each condition. In all three panels, white depicts control and black depicts sss^{P1} animals. $*P < 0.05$; $**P < 0.0001$.

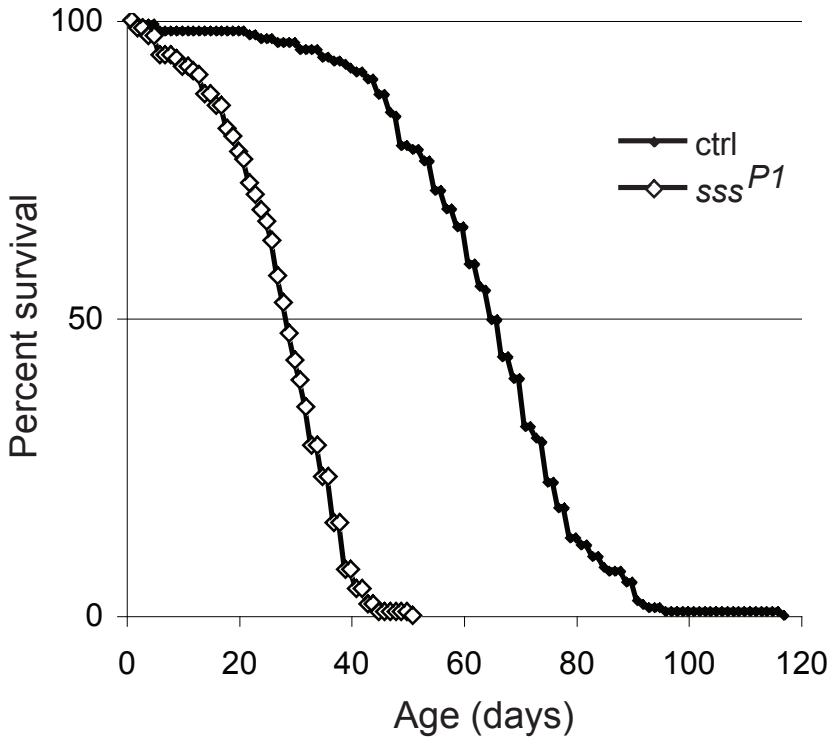

Fig. S5. Survivorship curves of background control (ctrl, closed diamonds) and sss^{P1} (open diamonds) flies. Male sss^{P1} flies (n=154) show a significantly shorter lifespan ($P < 0.0001$) than male control flies (n=161).

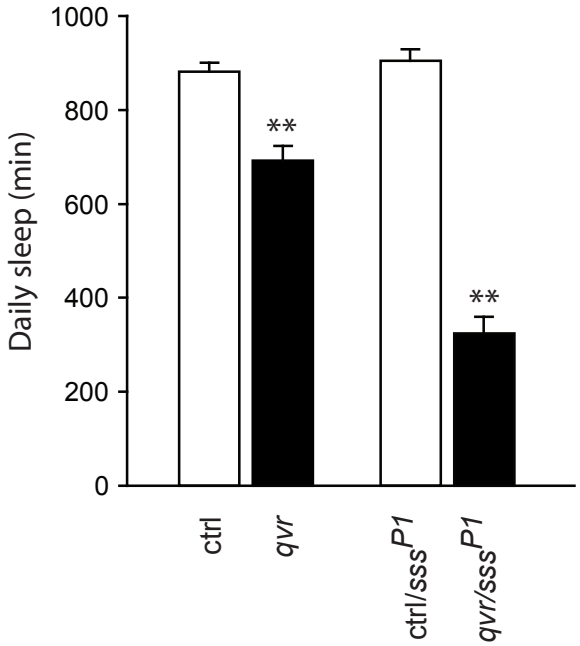

fig. S6. Daily sleep amount for *qvr* (n=32) versus background control (ctrl, n=29), as well as ctrl/sss^{P1} (n=30) versus *qvr*/sss^{P1} (n=31) male flies. $^{**}P < 0.0001$.