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Supplementary Materials for

Evolutionarily conserved regulation of sleep by epidermal growth factor receptor signaling

Daniel A. Lee, Justin Liu, Young Hong, Jacqueline M. Lane, Andrew J. Hill, Sarah L. Hou, Heming Wang, Grigorios Oikonomou, Uyen Pham, Jae Engle, Richa Saxena, David A. Prober*

*Corresponding author. Email: dprober@caltech.edu

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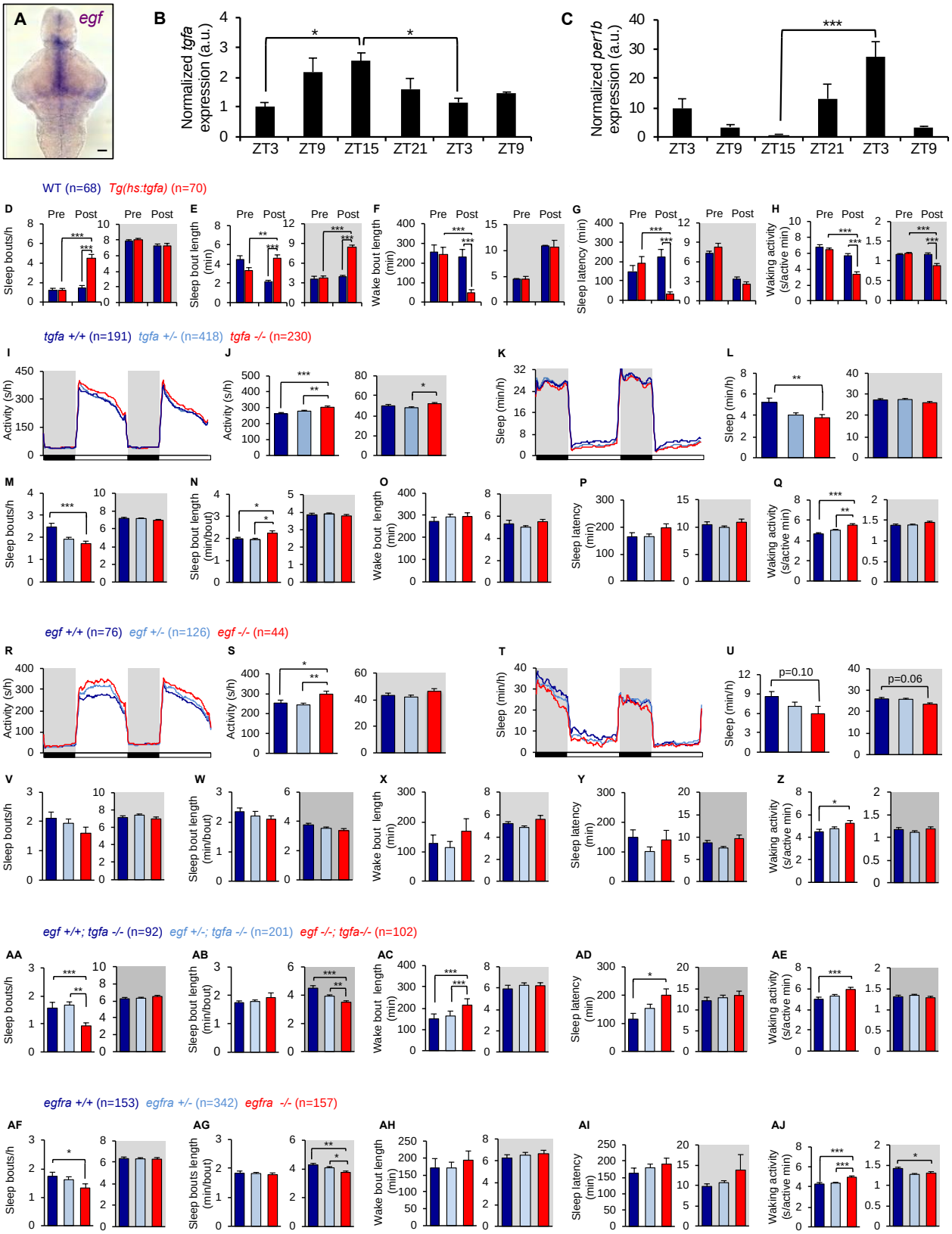
A
egf
B
Normalized tgfa expression (a.u.)
ZT3 ZT9 ZT15 ZT21 ZT3 ZT9
C
Normalized per1b expression (a.u.)
ZT3 ZT9 ZT15 ZT21 ZT3 ZT9
WT (n=68) Tg(hs:tgfa) (n=70)
D Sleep bouts/h
E Sleep bout length (min)
F Wake bout length (min)
G Sleep latency (min)
H Waking activity (s/active min)
Pre Post Pre Post
tgfa +/+ (n=191) tgfa +/- (n=418) tgfa -/- (n=230)
I Activity (s/h)
J Activity (s/h)
K Sleep (min/h)
L Sleep (min/h)
M Sleep bouts/h
N Sleep bout length (min/bout)
O Wake bout length (min)
P Sleep latency (min)
Q Waking activity (s/active min)
egf +/+ (n=76) egf +/- (n=126) egf -/- (n=44)
R Activity (s/h)
S Activity (s/h)
T Sleep (min/h)
U Sleep (min/h)
p=0.10
p=0.06
V Sleep bouts/h
W Sleep bout length (min/bout)
X Wake bout length (min)
Y Sleep latency (min)
Z Waking activity (s/active min)
egf +/+; tgfa -/- (n=92) egf +/-; tgfa -/- (n=201) egf -/-; tgfa-/- (n=102)
AA Sleep bouts/h
AB Sleep bout length (min/bout)
AC Wake bout length (min)
AD Sleep latency (min)
AE Waking activity (s/active min)
egfra +/+ (n=153) egfra +/- (n=342) egfra -/- (n=157)
AF Sleep bouts/h
AG Sleep bouts length (min/bout)
AH Wake bout length (min)
AI Sleep latency (min)
AJ Waking activity (s/active min)

Fig. S1. Effects of gain and loss of EGFR signaling on sleep architecture. (**A**) ISH using an *egf*-specific riboprobe in a 6-dpf zebrafish brain. Scale: 50 μm. (**B,C**) qPCR analysis of *tgfa* (**B**) and *per1b* (**C**) expression in 14:10 h light:dark conditions, each normalized to *ef1a*, over 36 hours beginning at 5-dpf. RNA from twenty pooled animals was assayed at each time point. Pooled data from 3 independent biological replicates shows a significant difference between peak and trough transcript level for *tgfa* and *per1b* (*p<0.05, ***p<0.005, One-way ANOVA, Holm-Sidak test). a.u.= arbitrary units. (**D-H**) In *Tg(hs:tgfa)* animals, heat shock-induced TGFa overexpression increased daytime sleep bout number (**D**) and daytime and nighttime sleep bout length (**E**) compared to WT siblings. TGFa overexpression also decreased daytime wake bout length (**F**) and sleep latency (time to first sleep bout) (**G**), as well as daytime and nighttime waking activity (**H**) compared to WT siblings. (**I-AJ**) Genetic loss of EGFR signaling components increased locomotor activity and decreased sleep compared to sibling controls. (**I-Q**) *tgfa* -/- animals were more active during the day and night, and slept less during the day, than *tgfa* +/+ siblings. (**M-Q**) *tgfa* -/- animals had fewer and longer sleep bouts, and higher waking activity, compared to *tgfa* +/+ siblings during the day. (**R-Z**) *egf* -/- animals exhibited increased daytime activity and waking activity, and showed a trend of less sleep during the day and night, compared to *egf* +/+ siblings. (**AA-AE**) *egf* -/-; *tgfa* -/- animals had fewer sleep bouts, longer wake bouts, longer sleep latency, and higher waking activity during the day, and shorter sleep bouts at night, compared to *egf* +/+; *tgfa* -/- siblings. (**AF-AJ**) *egfra* -/- animals have fewer sleep bouts and higher waking activity during the day, and shorter sleep bouts and lower waking activity at night, compared to *egfra* +/+ siblings. Mean ± SEM from 2 (**D-H**), 11 (**I-Q**), 3 (**R-Z**), 8 (**AA-AE**) and 9 (**AF-AJ**) pooled experiments are shown. n=number of animals. *p<0.05, **p<0.01, ***p<0.005 by Two-way ANOVA with Holm–Sidak test (**D-H**) or One-way ANOVA with Holm–Sidak test (**I-AJ**).


A
TGFA_Hs
TGFA_Dr
TGFA_Dr mut
EGF REPEAT
TMD
B
EGF_Hs
EGF_Dr
EGF_Dr mut
EGF REPEAT
C
EGFR_Hs
EGFRA_Dr
EGFRA_Dr mut
TRANSMEMBRANE DOMAIN

Fig. S2. Amino acid alignment of human and zebrafish TGFa, EGF, and EGFR. Alignment of the amino acid sequence of human (*Hs*), WT zebrafish (*Dr*) and mutant zebrafish (*Dr* mut) TGFa (**A**), EGF (**B**) and EGFR (**C**). Green and black lines above alignments indicate EGF repeat domains and transmembrane domains (TMD), respectively. TGFa *Dr* mut has a 7 bp deletion after amino acid 8, resulting in a translational frame shift that generates a predicted protein that lacks both of these domains. EGF *Dr* mut has a 26 bp insertion after amino acid 142, resulting in a translational frame shift that generates a predicted protein that lacks 5 EGF domains and the TMD. EGFRa *Dr* mut contains an 11 bp deletion and 27 bp insertion after amino acid 264, resulting in a translational frame shift before the TMD and intracellular domains required to interact with downstream effectors. Colors indicate amino acids with similar chemical properties. Grey shading indicates frame shifted sequence in mutant proteins.

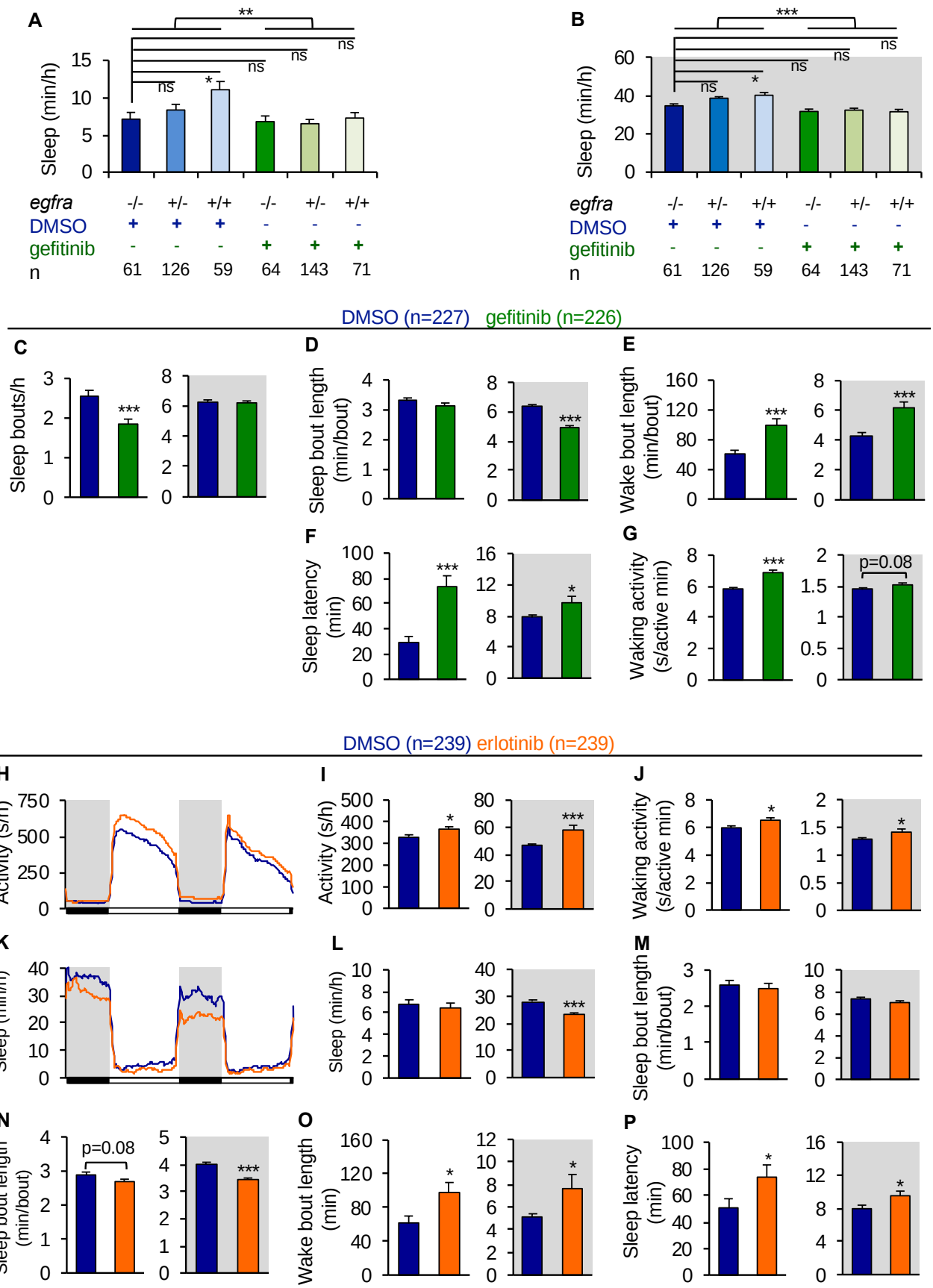
A
B
Sleep (min/h)
egfra -/- +/- +/+ -/- +/- +/+
DMSO + + + - - -
gefitinib - - - + + +
n 61 126 59 64 143 71
DMSO (n=227) gefitinib (n=226)
C
Sleep bouts/h
D
Sleep bout length (min/bout)
E
Wake bout length (min/bout)
F
Sleep latency (min)
G
Waking activity (s/active min)
p=0.08
DMSO (n=239) erlotinib (n=239)
H
Activity (s/h)
I
J
K
Sleep (min/h)
L
M
N
O
Wake bout length (min)
P

Fig. S3. Gefitinib does not enhance *egfra*$^{-/-}$ phenotype and effects of EGFR inhibitors on sleep architecture. (**A,B**) Gefitinib-treated animals sleep less than DMSO vehicle-treated siblings during the day and night, and DMSO-treated *egfra* -/- animals sleep less than DMSO-treated *egfra* +/+ siblings during the day and night, but gefitinib-treated *egfra* -/- animals do not sleep less than DMSO vehicle-treated *egfra* -/- animals. Thus, gefitinib treatment does not enhance the *egfra* -/- phenotype. Data are from night 5 dpf (**B**) and day 6 dpf (**A**) (24 h total). (**C-G**) WT animals treated with gefitinib had fewer sleep bouts, longer wake bouts, increased sleep latency and increased waking activity during the day, and shorter sleep bouts, increased sleep latency and longer wake bouts at night compared to DMSO control-treated siblings. (**H-P**) WT animals treated with erlotinib were more active during the day and night (**H,I**) and slept less at night (**K,L**) compared to DMSO control-treated siblings. (**J,M-P**) Erlotinib-treated animals had shorter sleep bouts at night, and longer wake bouts, increased sleep latency and increased waking activity during the day and night, compared to DMSO-treated siblings. Pooled data from 5 (**A-B**), 6 (**C-G**) and 5 (**H-P**) experiments are shown. Bar graphs show mean ± SEM. n=number of animals. *p<0.05, **p<0.01, ***p<0.005, n.s. p>0.05 by Two-way ANOVA with Dunnett's test (**A,B**) or Student's t-test (**C-G,I,J,L-P**).

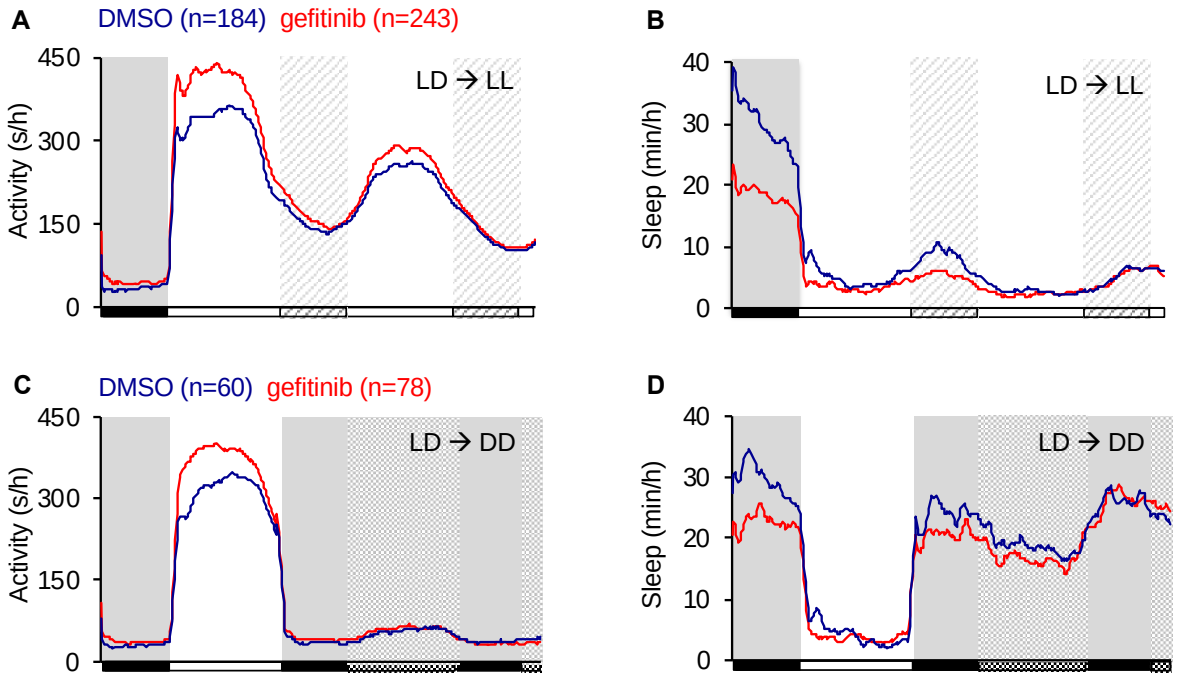

Fig. S4. EGFR signaling is not required for behavioral circadian rhythms. Locomotor activity and sleep behavioral traces of WT animals that were entrained in 14:10 h light:dark conditions until 5-dpf, and then shifted to constant light (**A,B**) or constant dark (**C,D**) free-running conditions. WT animals treated with gefitinib starting on the afternoon of 4-dpf were more active and slept less than DMSO-treated siblings, but showed normal circadian regulation of locomotor activity and sleep, and apparently normal circadian period length and phase. Pooled data from 6 (**A,B**), and 2 (**C,D**) independent experiments are shown. n=number of animals. Black, white, and hatched bars under behavioral traces indicate night (10 h), day (14 h), subjective night (10 h, **A,B**), and subjective day (14 h, **C,D**) respectively.

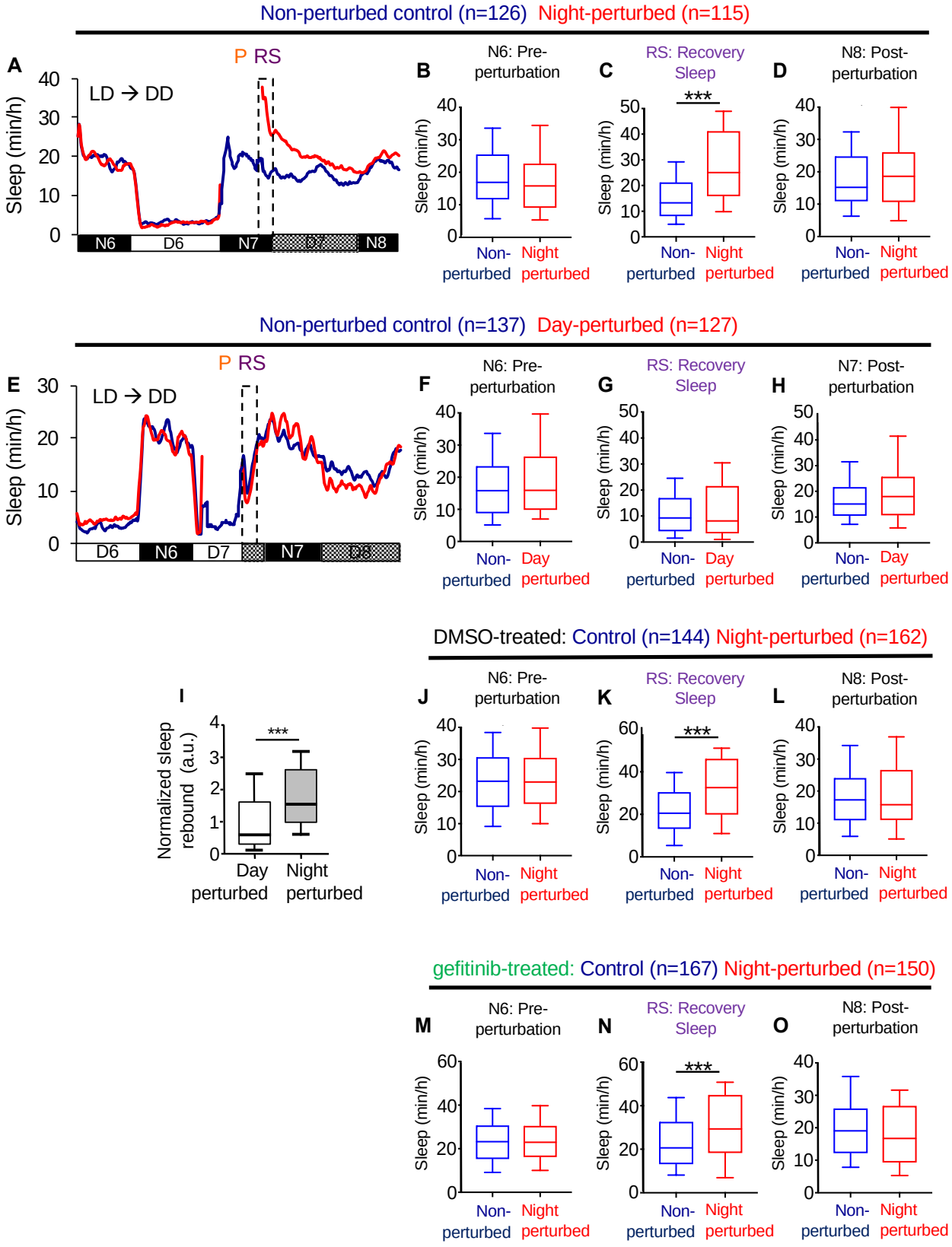
Non-perturbed control (n=126) Night-perturbed (n=115)
A
P RS
LD → DD
Sleep (min/h)
N6
D6
N7
D7
N8
B
N6: Pre-perturbation
C
RS: Recovery Sleep
***
D
N8: Post-perturbation
Non-perturbed
Night perturbed
Non-perturbed control (n=137) Day-perturbed (n=127)
E
F
N6: Pre-perturbation
G
RS: Recovery Sleep
H
N7: Post-perturbation
Day perturbed
DMSO-treated: Control (n=144) Night-perturbed (n=162)
I
Normalized sleep rebound (a.u.)
J
N6: Pre-perturbation
K
RS: Recovery Sleep
L
N8: Post-perturbation
gefitinib-treated: Control (n=167) Night-perturbed (n=150)
M
N6: Pre-perturbation
N
RS: Recovery Sleep
O
N8: Post-perturbation

Fig. S5. Validation of an SD assay, and EGFR signaling is required for normal homeostatic regulation of sleep. (**A**) Sleep behavioral traces for WT animals that were sleep deprived during the first 6 h of night at 7-dpf (P, orange) followed by a period of recovery sleep during the remaining 4 h of night (RS, purple) (red trace), as well as their non-perturbed siblings (blue trace). (**B-D**) Quantification of sleep during the night before the night perturbation (N6: 6-dpf), during the 4 h immediately after the night perturbation (RS: last 4 h of night at 7-dpf), and during the night after the night perturbation (N8: night of 8-dpf). Night perturbed animals showed significantly more sleep than non-perturbed controls only during the SR period (**C**). (**E**) Sleep behavioral traces for WT animals that were perturbed for 6 h during the middle of the day (ZT2-ZT8) at 7-dpf (P, orange) followed by a 4 h period of recovery sleep (RS) immediately thereafter, during which time they were monitored in the dark (red trace), as well as their non-perturbed siblings (blue trace). Animals were maintained in constant dark for the remainder of the experiment. (**F-H**) Quantification of sleep during the night before the day perturbation (N6: 6-dpf), during the 4 h immediately after the day perturbation (RS: 4 hours of subjective day at 7-dpf), and during the night after the day perturbation (N7: night of 7-dpf). There was no significant difference in the amount of sleep between perturbed and non-perturbed animals during any of these time periods. (**I**) Normalized sleep rebound following perturbation during the day or night for WT animals. Normalized sleep rebound is calculated as the amount of sleep of each perturbed animal during the first 4 h of recovery sleep (RS, purple) divided by the average amount of sleep of all non-perturbed control animals during this time period. (**J-O**) Further quantification of gefitinib sleep deprivation experiment (**Fig. 3D-3G**). Quantification of sleep during the night before sleep deprivation (N6: 6-dpf), during the 4 h immediately after sleep deprivation (RS: last 4 h of night at 7-dpf), and during the night after sleep deprivation (N8: night of 8-dpf) in DMSO-treated (J-L) or gefitinib-treated (**M-O**) WT zebrafish. Both perturbed gefitinib- and DMSO vehicle-treated animals slept more than non-perturbed but identically treated controls during the RS period, but not during the nights before or after sleep deprivation. Pooled data from 5 experiments are shown. n=number of animals. a.u. = arbitrary units. Black, white, and hatched bars under behavioral traces indicate night (10 h), day (14 h), and subjective day, respectively. ***$p<0.005$ by Mann-Whitney test.

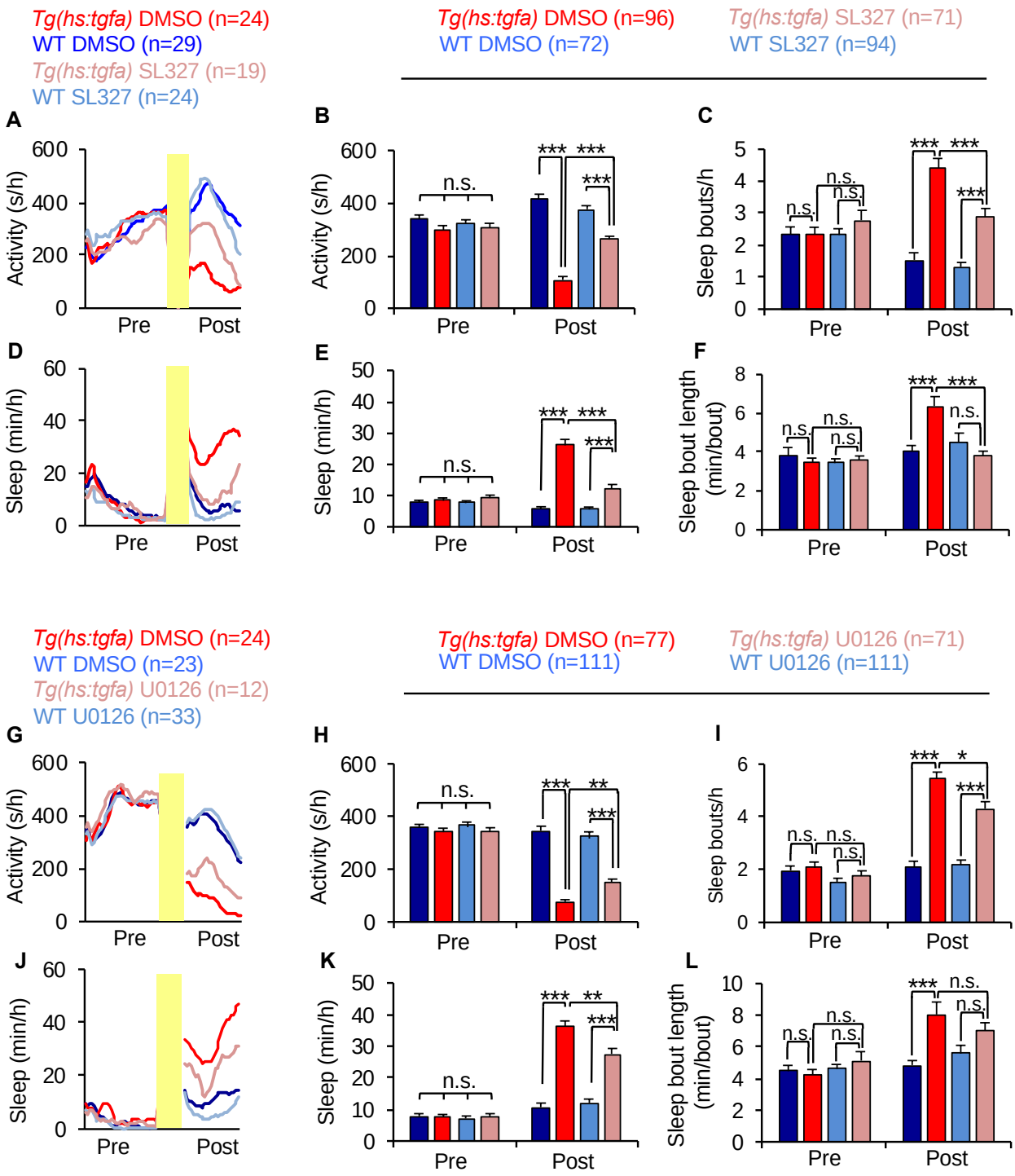
Tg(hs:tgfa) DMSO (n=24)
WT DMSO (n=29)
Tg(hs:tgfa) SL327 (n=19)
WT SL327 (n=24)
Tg(hs:tgfa) DMSO (n=96)
WT DMSO (n=72)
Tg(hs:tgfa) SL327 (n=71)
WT SL327 (n=94)
A
B
C
D
E
F
Activity (s/h)
Sleep bouts/h
Sleep (min/h)
Sleep bout length (min/bout)
Pre
Post
n.s.
***
Tg(hs:tgfa) DMSO (n=24)
WT DMSO (n=23)
Tg(hs:tgfa) U0126 (n=12)
WT U0126 (n=33)
Tg(hs:tgfa) DMSO (n=77)
WT DMSO (n=111)
Tg(hs:tgfa) U0126 (n=71)
WT U0126 (n=111)
G
H
I
J
K
L
**
*

Fig. S6. Inhibition of MAPK/ERK signaling suppresses TGFa overexpression–induced sleep. *Tg(hs:tgfa*) and their WT siblings were treated with the MEK1/2 antagonists SL327 (**A-F**) or U0126 (**G-L**), or DMSO vehicle control, immediately after heat shock (yellow bars). Both MEK1/2 antagonists suppressed TGFa overexpression-induced effects on locomotor activity (**A,B,G,H**), sleep (**D,E,J,K**) and sleep bout number (**C,I**) compared to DMSO-treated siblings. Treatment with SL327, but not U0126, blocked the TGFa overexpression-induced effect on sleep bout length compared to DMSO-treated controls. Pooled data from 5 experiments are shown. Bar graphs show mean ± SEM. Pre- and Post-HS data is calculated for the day of HS. n=number of animals. *p<0.05, **p<0.01, ***p<0.001, n.s. p>0.05 by Two-way ANOVA with Holm–Sidak test.

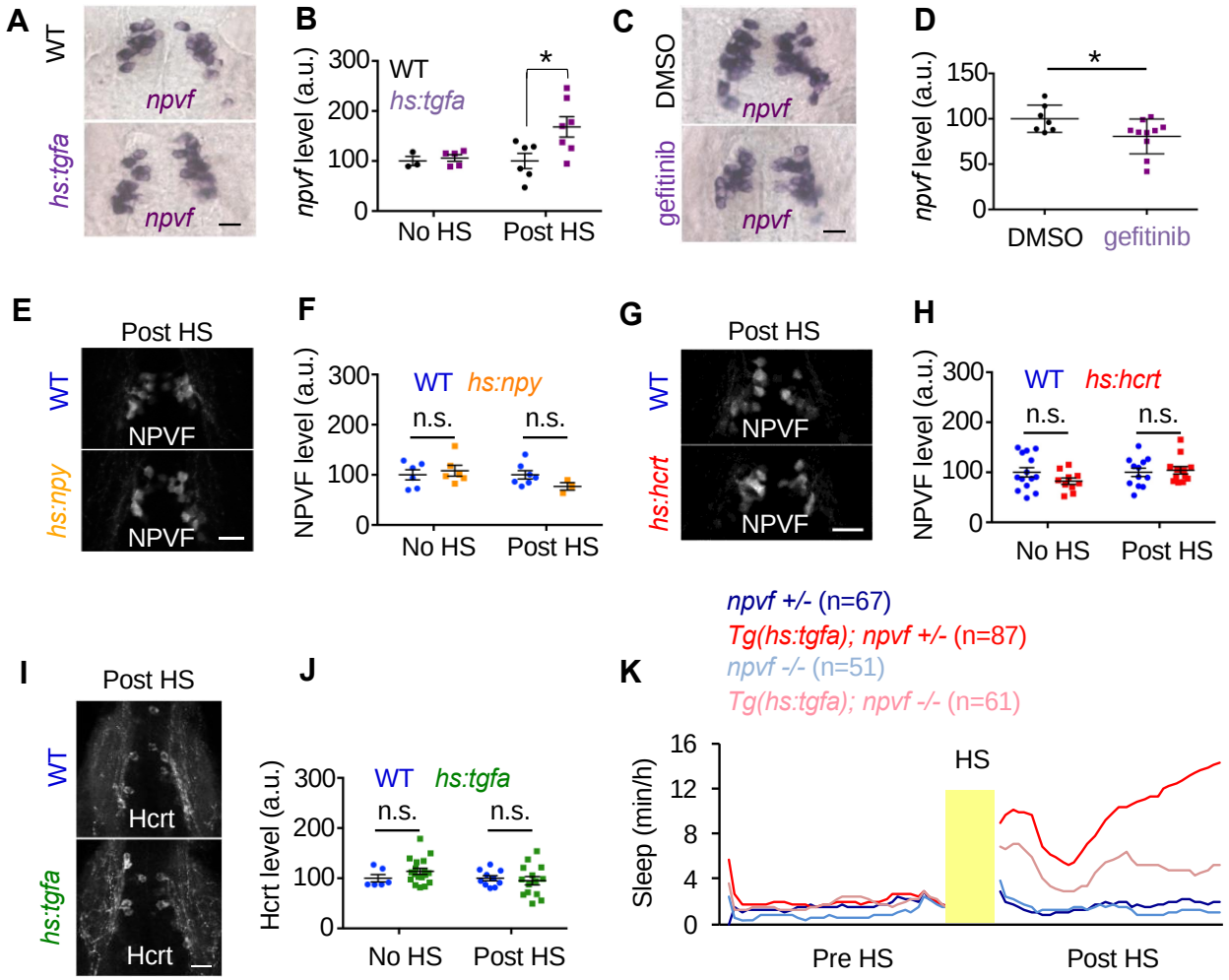

Fig. S7. EGFR signaling regulates *npvf* expression, and TGFa overexpression–induced sleep is suppressed in *npvf* mutant animals. (A,B) Increased *npvf* mRNA was observed using ISH at 2 h after heat shock in *Tg(hs:tgfa)* animals compared to WT siblings. (**C,D**) Decreased *npvf* mRNA was observed at 45 min after treatment of WT animals with gefitinib compared to DMSO. (**E-H**) No significant difference in NPVF protein level was observed using IHC at 2 h after heat shock in *Tg(hs:npy)* or *Tg(hs:hcrt)* animals compared to their WT siblings. (**I,J**) No significant difference in Hcrt protein level was observed using IHC at 2 h after heat shock in *Tg(hs:tgfa)* animals compared to WT siblings. Representative images (**A,C,E,G,I**) and quantification of average pixel intensity (**B,D,F,H,J**) are shown. Graphs show mean ± SEM. Each data point represents one animal. *p<0.05, n.s. p>0.05 by Two-way ANOVA with Holm–Sidak test (**B,F,H,J**) or Student's t-test (**D**). Scale: 20 μm. (**K**) After heat shock, increased sleep in *Tg(hs:tgfa)* animals was partially suppressed in *npvf* -/- animals compared to their *npvf* +/- siblings. Pooled data from 3 experiments is shown. n=number of animals. Data shown in the line graph is quantified using bar graphs in **Fig. 5E**.

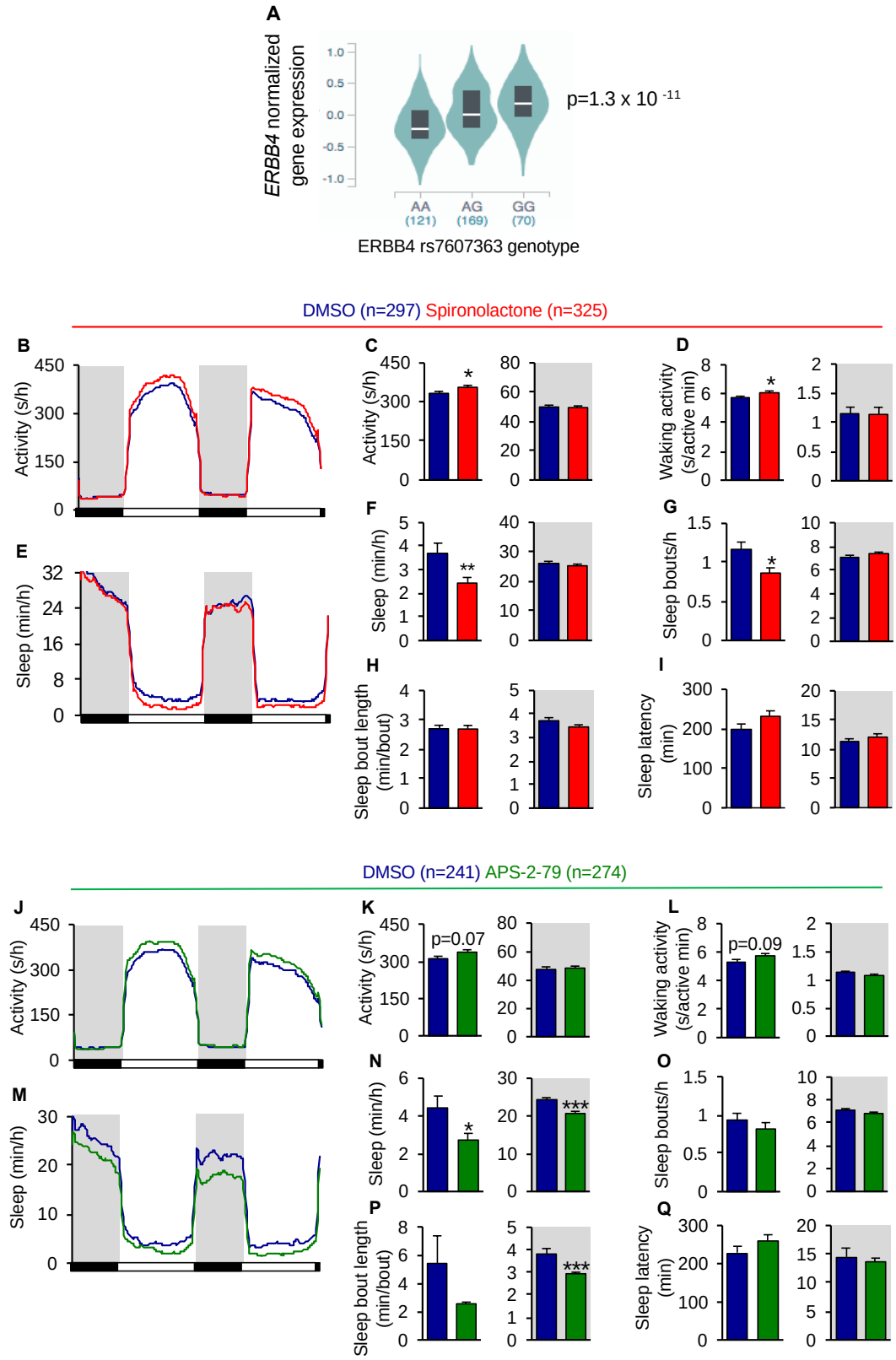
A
ERBB4 normalized gene expression
1.0
0.5
0.0
-0.5
-1.0
AA (121)
AG (169)
GG (70)
p=1.3 x 10 -11
ERBB4 rs7607363 genotype
DMSO (n=297) Spironolactone (n=325)
B
Activity (s/h)
C
Activity (s/h)
D
Waking activity (s/active min)
E
Sleep (min/h)
F
Sleep (min/h)
G
Sleep bouts/h
H
Sleep bout length (min/bout)
I
Sleep latency (min)
DMSO (n=241) APS-2-79 (n=274)
J
Activity (s/h)
K
Activity (s/h)
p=0.07
L
Waking activity (s/active min)
p=0.09
M
Sleep (min/h)
N
Sleep (min/h)
O
Sleep bouts/h
P
Sleep bout length (min/bout)
Q
Sleep latency (min)

Fig. S8. Association of *ERBB4* sleepiness allele with increased *ERBB4* expression in humans and pharmacological inhibition of KSR2 or ERBB4 decrease sleep in zebrafish.
(A) Significant association is observed between *ERBB4* rs7607363 genotypes (G vs A allele) with rank normalized gene expression of *ERBB4* in human Tibial nerve (n=360 samples; normalized effect size of 0.25, $p=1.3 \times 10^{-11}$, linear regression analysis). (**B-I**) Pharmacological inhibition of ERBB4 by treatment of WT zebrafish with spironolactone resulted in less sleep (**E,F**) and more activity (**B,C**) during the day compared to DMSO-treated siblings. These changes were due to increased waking activity (**D**) and fewer sleep bouts (**G**). (**J-Q**) Pharmacological inhibition of KSR2 by treatment of WT zebrafish with APS-2-79 resulted in less daytime and nighttime sleep (**M,N**), shorter nighttime sleep bouts (**P**), and a trend of increased daytime activity (**J,K**) compared to DMSO-treated siblings. Pooled data from 10 (**B-I**) and 8 (**J-Q**) experiments are shown. Bar graphs show mean ± SEM. n=number of animals. *p<0.05, **p<0.01, ***p<0.005 by Student's t-test.

Table S1. Variants at *ERBB4* and *KSR2* associate with self-reported measures of sleep quality and quantity in U.K. Biobank subjects.

Gene	SNP	CHR	BP	Effect Allele	Alt Allele	EAF	INFO	Phenotype	Beta	StdErr	P-value
ERBB4	rs7607363	2	213,402,705	G	A	0.44	1.00	Sleep Duration, hrs	0.004	0.002	0.095
								Daytime Sleepiness, increased frequency	**0.006**	**0.001**	**8.00×10^{-9}**
								Hypersomnolence, log odds	0.041	0.052	0.064
								Difficulty Waking Up, increased difficulty	-0.003	0.002	0.031
								Daytime Napping, increased frequency	0.005	0.001	7.50×10^{-4}
								Frequent Insomnia Symptoms, log odds	0.000	0.001	0.790
								Chronotype, morningness	-0.004	0.003	0.071
KSR2	rs1846644	12	117,938,380	C	T	0.41	1.00	**Sleep Duration, hrs**	**0.013**	**0.002**	**5.30×10^{-9}**
								Daytime Sleepiness, increased frequency	**0.011**	**0.001**	**2.50×10^{-27}**
								Hypersomnolence, log odds	0.074	0.052	8.80×10^{-4}
								Difficulty Waking Up, increased difficulty	0.002	0.002	0.140
								Daytime Napping, increased frequency	**0.018**	**0.001**	**2.00×10^{-41}**
								Frequent Insomnia Symptoms, log odds	-0.003	0.001	0.036
								Chronotype, morningness	0.003	0.003	0.270

CHR=chromosome, BP=base pair position in hg19, EAF=effect allele frequency, INFO=imputation quality metric, Beta=effect size, StdErr=standard error. n=453,964. Traits and P-values in bold indicate genome-wide significant associations (withstand correction for all SNPs tested for that trait).

Results for the following traits were looked up from GWAS summary statistics available at Sleep Disorder Knowledge Portal (http://sleepdisordergenetics.org/): Self-report Sleep duration (*5*), Daytime sleepiness (*4*); Frequent insomnia symptoms (*7*) and Chronotype (*8*).

Table S2. Descriptive characteristics of U.K. Biobank subjects of European ancestry used for sleep trait analysis.

	Chronotype	Difficulty Waking Up	Sleep Duration	Frequent Insomnia Symptoms	Daytime Napping	Excessive Daytime Sleepiness
N	451,963	452,724	446,953	237,627	339,400	451,937
	Definite morning =108,083 (24%)	Not at all easy =17,210 (4%)	≤6 hours =106,388 (24%)	Never/rarely =108,357 (46%)	Never/rarely =203,962 (60%)	Never/rarely =347,213 (77%)
	Somewhat morning =145,323 (32%)	Not very easy =61,959 (14%)	7-8 hours =306,318 (68%)	Usually =129,270 (54%)	Sometimes =121,612 (36%)	Sometimes =92,746 (2%)
	Don't know =46,847 (10%)	Fairly easy =225,867 (50%)	≥9 hours =34,247 (8%)		Usually =13,826 (4%)	Often =11,950 (3%)
	Somewhat evening =115,629 (26%)	Very Easy =147,688 (33%)				All of the time =28 (<1%)
	Definite evening =36,081 (8%)					
Sex, male	206,691 (46%)	207,116 (46%)	205,125 (46%)	112,477 (47%)	149,304 (44%)	206,733 (46%)
Age, years	56.77±8.02	56.78±8.03	56.75±8.03	56.68±8.04	56.71±8.04	56.77±8.02
Body Mass Index (BMI)	27.4±4.76	27.4±4.76	27.39±4.75	27.56±4.87	26.72±4.21	27.39±4.76
Sleep duration, hrs	7.17±1.08	7.17±1.08	7.17±1.08	7.05±1.16	7.20±0.98	7.17±1.08

Mean ± standard deviation or N (%) are shown.