Supporting Information

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SI Materials and Methods

Animals. Mice used in this study (C57BL/6 background) were handled in accordance with The University of Tokyo's guidelines for the care and use of laboratory animals. Mice were reared in the compartments in which the environmental conditions were controlled (room temperature, 23 ± 1 °C). Animals had free access to commercial chow (CLEA Japan) and tap water available ad libitum. *Ask*-TKO mice were generated by crossing *Ask1*-KO (1), *Ask2*-KO (2), and *Ask3*-KO (3) mice. The *Ask*-TKO mice were bred with PER2::LUC knockin mice (4), and *Ask*-TKO/PER2::LUC MEF lines were prepared as described previously (5).

Cell Culture and Real-Time Monitoring of Circadian Rhythms of Cultured Cells. MEFs from PER2::LUC mice (4), NIH 3T3 cells (RIKEN Cell Bank), HEK293A cells (Invitrogen), and U2OS cells (American Type Culture Collection) were maintained at 37 °C under 5% CO_2 and 95% air in DMEM (Sigma-Aldrich or Wako Chemicals) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS. Real-time monitoring of the cellular bioluminescence rhythms was performed as described previously (6) with minor modifications. In brief, PER2::LUC MEFs were plated on 35-mm dishes (1.0×10^6 cells/dish) or 24-well plates (2.5×10^5 cells/well) and then cultured at 37 °C under 5% CO_2. After 24 h, the cells were treated with 0.1 μM (final concentration) dexamethasone for 2 h, after which the media were replaced by recording media [phenol-red free DMEM (Sigma-Aldrich) supplemented with 10% FBS, 3.5 g/L glucose, 25 U/mL penicillin, 25 μg/mL streptomycin, 0.1 mM luciferin, and 10 mM Hepes-NaOH, pH 7.0]. The bioluminescence signals of the cultured cells were continuously recorded for 5–10 d at 37 °C in air with a Kronos AB-2500 or AB-2550 (Atto), LumiCycle (Actimetrics), or CL24A (Churitsu) luminometer. NIH 3T3 cells that stably expressed the *Bmal1*-luc reporter (7) were used in Fig. S1 *A* and *E*. NIH 3T3 cells or U2OS cells plated on 24-well plates were transiently transfected by *Bmal1*-luc/pGL4.12, a firefly luciferase reporter under regulation of the mouse *Bmal1* promoter region (nucleotides from −95 to +168; +1 is the transcription start site) (Fig. 5 *A* and *B* and Fig. S1 *B*, *F*, and *H*). For transient transfection, the cells were plated in 24-well plates at 24 h before the experiments and were then transiently transfected using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's protocol. In Fig. 5 *A* and *B*, Flag-hASK3/pcDNA3 and Flag-hASK3 KM/pcDNA3 were used, as described previously (3).

Calculation of the Circadian Period and Amount of Phase-Shift. The raw data of bioluminescence rhythms were detrended by subtracting their 24-h moving averages and were smoothed by their 2-h moving averages. In Fig. 1*A*, Fig. S1 *A* and *B*, and Fig. S2 *A* and *B*, the period length of each day was calculated as an interval between the trough (or peak) of the day and the next trough (or peak) of the bioluminescence rhythms, as shown in each panel. In the other figures, the circadian period was calculated from the slope of a regression line fitted to time points of consecutive peaks and troughs of cellular rhythms by the least squares method. For the fitting, we selected at least five time points of peaks and troughs of cellular rhythms in which any time point within 12 h after Dex treatment was eliminated. To determine the amount of phase shift induced by hypertonic or oxidative pulse treatment, the regression lines before and after the pulse treatments were extrapolated to time points of the pulse treatments and the phase differences at the time points were calculated.

Osmotic Stress Treatments. The extracellular osmolarity was measured with an OSMOMAT 030 osmometer (Asahi Life Science). For chronic hypertonic stimuli, NaCl (final concentration 100 mM) or sorbitol (final concentration 200 mM) was added to the cultured media. For pulse hypertonic stimuli, the cultured media (2.5 mL) were removed, and 0.88 mL of the used media were rapidly returned back to the original wells after mixing with 0.12 mL of 5 M sorbitol in the fresh media (final concentration 600 mM). After a 30-min incubation, the hypertonic media were replaced by the residual used media that had been kept at 37 °C (Fig. S3*A*). For hypotonic stimuli, the cultured media were diluted by mixing with distilled water.

Extrinsic and Intrinsic Oxidative Stress Treatment. For extrinsic oxidative pulse stress, the cultured media (2.5 mL) were removed, and 0.9 mL of the used media were rapidly returned back to the original wells after mixing with 0.1 mL of 4 mM H_2O_2 (final concentration 0.4 mM) in the fresh media. After a 30-min incubation, the oxidative media were replaced by the residual used media that had been kept at 37 °C, in the same way as pulse hyperosmotic stress (Fig. S3*A*). For intrinsic oxidative stress, ATZ (final concentration 20 mM or 80 mM) was added to the cultured media.

Dual Luciferase Reporter Assay. To quantify CLOCK/BMAL1-dependent transactivation, we carried out a dual luciferase reporter assay as described previously (8) with minor modifications. In brief, we transiently transfected HEK293T cells (American Type Culture Collection) in 12-well plates using polyethylimine with 200 ng of Myc-CLOCK/pSG5 and 50 ng of BMAL1/pcDNA3.1 in combination with 20 ng of a firefly luciferase plasmid harboring the intronic regions of the *Ask1* gene locus as a reporter, and 2 ng of a *Renilla* luciferase plasmid (pRL-SV40) as an internal control. We adjusted the total amount of DNA (822 ng) by adding the empty expression plasmids. The following DNA sequences were inserted to pGL3N, a modified pGL3 promoter vector (9): *Ask1*-WT, CTAGT GATTG TCACA TGCTG GGTGC TGTGC CTGGA GCTCC ACACG TGTGA TCTT; *Ask1*-mut, CTAGT GATTG TACCA GTCTG GGTGC TGTGC CTGGA GCTCC AACCG GTTGA TCTT.

Real-Time qRT-PCR. Total RNA was prepared from the mouse liver or MEFs using TRIzol reagent (Invitrogen) and the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The qRT-PCR analyses were performed as described previously (9). Total RNA was reverse-transcribed using Go Script Reverse-Transcriptase (Promega) with both anchored (dT)15 primer and random oligo primer, and the reaction mixture was treated with RNase H (TaKaRa). The cDNA was subjected to real-time qRT-PCR (StepOnePlus Real-Time PCR System; Applied Biosystems) using GoTaq Master Mix (Promega) with the following gene-specific primers: for *Per1*, 5′-CTGAC AAGCT GCTGA AGTGG-3′ and 5′-GGTAG AGCTG AGAAC ACCTG G-3′; for *Per2*, 5′-GCTCA CTGCC AGAAC TATCT CC-3′ and 5′-CCTCT AGCTG AAGCA GGTTA AG-3′; for *Dec1*, 5′-CTATC TCATC CCACC ATCGG-3′ and 5′-GAATC TTCTC TGTGG GTCTG C-3′; for *Dec2*, 5′-AGGGA GGAAG AGTAA GAGAT GC-3′ and 5′-GGAAG GGAGT GTCAA AGGGT-3′; for *Cry1*, 5′-TCCAG CGACA GAGCA GTAAC-3′ and 5′-AACAC AGACT GTCCA CGCAG-3′; for *Cry2*, 5′-GCATC ATTGG CGTGG ACTAC-3′ and 5′-CCACA GGGTG ACTGA GGTCT-3′; for *Dbp*, 5′-CCAAT

CATGA AGAAG GCAAG G-3′ and 5′-AGGAT TGTGT TGATG GAGGC-3′; for *E4bp4*, 5′-ATCGG AACAC TGGCA TCAC-3′ and 5′-TATCT GACTA CACGC CAGGC-3′; for *Rev-erbα*, 5′-AGAAT GTTCT GCTGG CATGT C-3′ and 5′-TTGAG CTTCT CGCTG AAGTC-3′; for *Rev-erbβ*, 5′-TGAGC AAGTC TCCAT ATGTG G-3′ and 5′-AGGCC TCATT TGGAT GGTT-3′; for *Bmal1*, 5′-TGGTA CCAAC ATGCA ATGC-3′ and 5′-AGTGT CCGAG GAAGA TAGCTG-3′; for *Clock*, 5′-CAAGG TCAGC AACTT GTGAC C-3′ and 5′-AGGAT GAGCT GTGTC GAAGG-3′; for *Rps29*, 5′-TGAAG GCAAG ATGGG TCAC-3′ and 5′-GCACA TGTTC AGCCC GTATT-3′; for *Ask1*, 5′-CGTGC TGGAC CGTTT TTAC-3′ and 5′-TCTCG CACTC CAAGA TGGTA -3′; for *Ask2*, 5′-CTGCT GAGCC CTGAC ATTG-3′ and 5′-ATGAT GGCCG AGTAG TCCTG-3′; for *Ask3*, 5′-GAAAT CCCAG AGAGA GATAT CAGG-3′ and 5′-TGTTT GAGAT ACTTG TGCAG AGC-3′.

Preparation of Nuclear Proteins. The nuclear proteins and cytoplasmic proteins were isolated as described previously (10). In brief, the mouse tissue (1 g wet weight) was washed with ice-cold PBS and homogenized at 4 °C with 9 mL of ice-cold buffer A composed of 10 mM Hepes-NaOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 4 μg/mL aprotinin, 4 μg/mL leupeptin, 50 mM NaF, and 1 mM Na_3VO_4. The homogenate was centrifuged twice (5 min each, 700 × *g*), and the resultant precipitate was resuspended in 2 mL of ice-cold buffer C composed of 20 mM Hepes-NaOH (pH 7.8), 400 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 2% (vol/vol) glycerol, 1 mM DTT, 1 mM PMSF, 4 μg/mL aprotinin, 4 μg/mL leupeptin, 50 mM NaF, and 1 mM Na_3VO_4. After gentle mixing at 4 °C for 30 min, the suspension was centrifuged twice (30 min each, 21,600 × *g*), and the final supernatant was used as "nuclear extract."

Antibodies, Immunoprecipitation, and Immunoblot Analysis. The anti-CLOCK mAb CLNT1 was used for immunoprecipitation as described previously (10). In immunoblot analysis, the following antibodies were used: CLSP3 anti-CLOCK mAb (10), BIBH2 anti-BMAL1 mAb (10), anti-E4BP4 (MBL), anti-PER2 (ADI), anti-REV-ERBα (MBL), anti-DBP (MBL), anti-CRY1 (MBL), anti-TBP (Santa Cruz Biotechnology), anti-ASK1 (ab45178; Abcam), anti–phospho-ASKs (originally generated as phospho-ASK1 pT845) (1), anti-JNK (sc-571; Santa Cruz Biotechnology), anti–phospho-JNK (9251; Cell Signaling Technology), anti-p38 (9228; Cell Signaling Technology) and anti–phospho-p38 (9211; Cell Signaling Technology). The bound primary antibodies were detected by horseradish peroxidase-conjugated anti-rabbit, anti-mouse (Kirkegaard & Perry Laboratories), or anti-guinea pig (Cell Signaling Technology) IgG antibody.

Recording of Mouse Behavioral Rhythms. Behavioral rhythms of mice were recorded as described previously (11), with minor modifications. In brief, 8- to 16-wk-old male mice were housed individually in a polycarbonate cage equipped with a running wheel. The animals were maintained in a light-tight chamber and were entrained to the 12-h LD cycles for at least 2 wk. Responses of the mouse circadian clock to single light pulses were examined after releasing the mice into the DD conditions for 14 d or longer. Single light pulses (30 min, approximately 400 lux) were applied every 2–3 wk at two different CTs in a subjective night phase (CT22 and CT14), where the activity onset was designated as CT12. Responses of the circadian clock to different light intensities in the LL condition were examined for 21 d or longer after monitoring the behavioral rhythms in DD for 17 d or longer. Fluorescent lamps were used in Fig. 4 *B–E* (approximately 400 lux). Light-emitting diodes (NS2W157AR-H3; Nichia) were used to precisely coordinate the light intensity in Fig. S5*D*. The locomotor activity rhythms of mice were measured by wheel revolutions in 5-min bins and analyzed using ClockLab software (Actimetrics). The circadian periods were analyzed by a χ^2 periodogram with $P < 0.001$, based on the locomotor activity in days 4–17 after the start of each condition.

Preparation of Phosphopeptides. Enzymatic digestion of proteins in MEF lysates was performed according to a previously described protocol (12) with modifications. In brief, MEFs plated in a 35-mm dish were harvested with ice-cold PBS, and the cell pellet was lysed in 200 μL of PTS buffer (100 mM NH_4HCO_3, 12 mM sodium deoxycholate, and 12 mM sodium *N*-lauroylsarcosinate) containing 1 mM DTT, 1 mM PMSF, 4 μg/mL aprotinin, 4 μg/mL leupeptin, and Phosphatase Inhibitor Cocktails 2 and 3 (Sigma-Aldrich), followed by extensive sonication in an ultrasonic disruptor (UR-21P; TOMY; output power 2 for 30–45 s). Each sample in the PTS buffer was reduced with 10 mM DTT at 60 °C for 30 min and then alkylated by incubation with 22 mM iodoacetamide at 37 °C for 30 min in the dark. The resultant protein sample was diluted with 100 mM NH_4HCO_3 solution up to 1 mL and digested with trypsin (Sigma-Aldrich) at 1:100 (wt/wt) by incubation at 37 °C for 18 h in the dark. After the digestion, an equal volume of ethyl acetate was added to the sample, and the mixture was acidified with 0.5% TFA and then well mixed to transfer the detergents into an organic phase. After the sample was centrifuged at 15,700 × *g* for 2 min at room temperature, an aqueous phase containing peptides was collected. The sample was concentrated by a centrifugal evaporator (EYELA) and desalted using a MonoSpin C18 column (GL Sciences). After 10% of the eluate was dried by the evaporator, it was analyzed by LC-MS/MS analysis as a sample labeled "total peptides." The remaining eluate was dried and applied to a high-select Fe-NTA phosphopeptide enrichment kit (Thermo Fisher Scientific). The enriched sample, labeled "phosphopeptides," was dried before LC-MS/MS analysis.

LC-MS/MS–Based Proteomic Analysis. The dried and desalted peptides were dissolved in distilled water containing 2% acetonitrile and 0.1% TFA. The LC-MS/MS analyses were performed using a mass spectrometer (Q Exactive Plus, Thermo Fisher Scientific) equipped with a nano ultra-HPLC system (Dionex Ultimate 3000; Thermo Fisher Scientific). The peptides were loaded to the LC-MS/MS system with a trap column (0.3 × 5 mm L-column ODS; Chemicals Evaluation and Research Institute) and a capillary column (0.1 × 150 mm L-column ODS; Chemicals Evaluation and Research Institute) at a flow late of 10 μL/min. The loaded peptides were separated by a gradient using mobile phases A (1% formic acid in distilled water) and B (1% formic acid in acetonitrile) at a flow late of 300 nL/min (2–40% B for 85 min, 40–95% B for 5 min, 95% B for 10 min, 95–2% B for 0.1 min, and 2% B for 5 min). The eluted peptides were electrosprayed (2.0 kV) and introduced into the MS equipment (positive ion mode, data-dependent MS/MS). Each of the most intense precursor ions (up to the top 10) was isolated and fragmented by higher collision energy dissociation (HCD) with the normalized collision energy (27%). For full MS scans, the scan range was set to 350–1,500 *m*/*z* at a resolution of 70,000, and the automatic gain control (AGC) target was set to 3e6 with a maximum injection time of 60 ms. For MS/MS scans, the precursor isolation window was set to 1.6 *m*/*z* at a resolution of 17,500, and the AGC target was set to 5e5 with a maximum injection time of 100 ms. The Orbitrap mass analyzer was operated with the "lock mass" option to perform shotgun detection with high accuracy. The raw spectra were extracted using Proteome Discoverer 2.2 (Thermo Fisher Scientific) and searched against the mouse SwissProt database (TaxID 10,090 and subtaxonomies, v2017-06-07) with following settings. The parameter of the cleavage was set to trypsin, and the missed cleavage was allowed up to 2. The mass tolerances were set to 10 ppm for the precursor ion and 0.02 Da for the fragment ion. As for protein modifications, we set carbamidomethylation (+57.021 Da) at

Cys; oxidation (+15.995 Da) at Met; phosphorylation (+79.966 Da) at Ser, Thr, and Tyr; and GG (+114.043 Da) at Lys as dynamic (non-fixed) modifications for peptide and acetylation (+42.011 Da) at the amino terminus as a dynamic modification for protein terminus. The amount of each peptide was semiquantified using peak area with Precursor Ions Quantifier in Proteome Discoverer 2.2.

1. Tobiume K, et al. (2001) ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO Rep* 2:222–228.
2. Iriyama T, et al. (2009) ASK1 and ASK2 differentially regulate the counteracting roles of apoptosis and inflammation in tumorigenesis. *EMBO J* 28:843–853.
3. Naguro I, et al. (2012) ASK3 responds to osmotic stress and regulates blood pressure by suppressing WNK1-SPAK/OSR1 signaling in the kidney. *Nat Commun* 3:1285–1295.
4. Yoo SH, et al. (2004) PERIOD2:LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci USA* 101:5339–5346.
5. Hirano A, et al. (2013) FBXL21 regulates oscillation of the circadian clock through ubiquitination and stabilization of cryptochromes. *Cell* 152:1106–1118.
6. Kon N, et al. (2008) Activation of TGF-beta/activin signalling resets the circadian clock through rapid induction of Dec1 transcripts. *Nat Cell Biol* 10:1463–1469.
7. Yoshitane H, et al. (2012) JNK regulates the photic response of the mammalian circadian clock. *EMBO Rep* 13:455–461.
8. Pekovic-Vaughan V, et al. (2014) The circadian clock regulates rhythmic activation of the NRF2/glutathione-mediated antioxidant defense pathway to modulate pulmonary fibrosis. *Genes Dev* 28:548–560.
9. Yoshitane H, et al. (2014) CLOCK-controlled polyphonic regulation of circadian rhythms through canonical and noncanonical E-boxes. *Mol Cell Biol* 34:1776–1787.
10. Yoshitane H, et al. (2009) Roles of CLOCK phosphorylation in suppression of E-box-dependent transcription. *Mol Cell Biol* 29:3675–3686.
11. Terajima H, et al. (2017) ADARB1 catalyzes circadian A-to-I editing and regulates RNA rhythm. *Nat Genet* 49:146–151.
12. Masuda T, Tomita M, Ishihama Y (2008) Phase transfer surfactant-aided trypsin digestion for membrane proteome analysis. *J Proteome Res* 7:731–740.

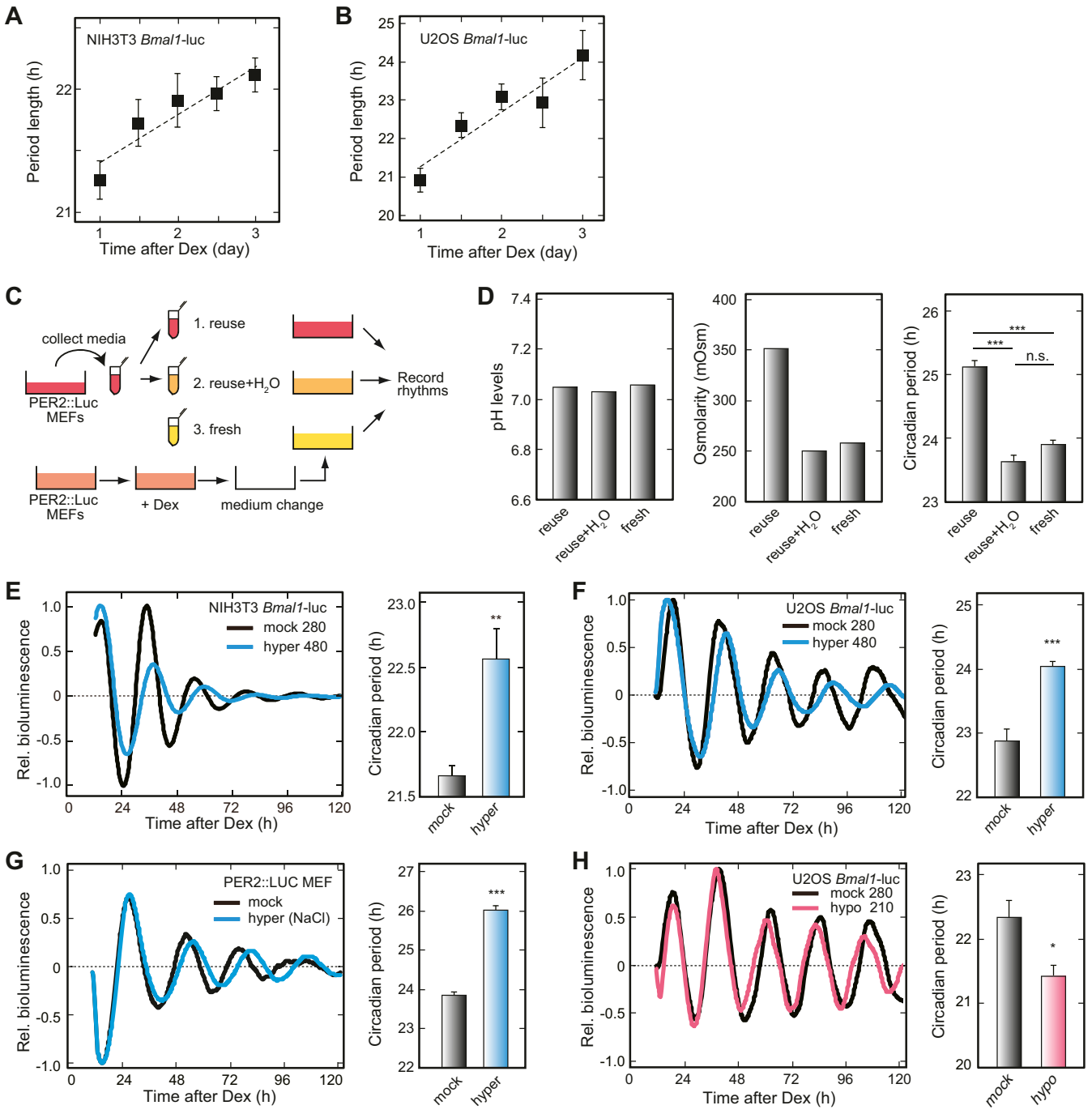

Fig. S1. The osmotic changes of media regulate the circadian period of the cellular rhythms. (*A*) Change in the circadian period length in NIH 3T3 cells (stably expressing *Bmal1*-luc) over several days. The period length was calculated as in Fig. 1*A* ($n = 40$). (*B*) Change in the circadian period length in U2OS cells (transiently expressing *Bmal1*-luc) over several days. The period length was calculated as in Fig. 1*A* ($n = 6$). (*C*) Scheme of the experiment examining the period-lengthening effect of the reused media. The used media were obtained from cultures of PER2::LUC MEFs at day 7 (reuse). The reused media were diluted to isotonicity by adding distilled water (reuse+H_2O). (*D*) Effect of the reused media on the circadian period of the cellular rhythms (*Right*), along with the measured pH (*Left*) and measured osmolarity (*Middle*) of the indicated media. (*E*) Cellular rhythms were recorded from NIH 3T3 cells stably expressing *Bmal1*-luc. For hypertonic treatment, 200 mM sorbitol was added to the recording media (approximately 280 mOsm) to give the final osmolarity of approximately 480 mOsm (blue). (*F*) Cellular rhythms were recorded from U2OS cells transiently expressing *Bmal1*-luc. For hypertonic treatment, 200 mM sorbitol was added to the recording media (approximately 280 mOsm) to give the final osmolarity of approximately 480 mOsm (blue). (*G*) Cellular rhythms were recorded from PER2::LUC MEFs. For hypertonic treatment, 100 mM NaCl was added to the recording media (approximately 280 mOsm) to give the final osmolarity of approximately 480 mOsm (blue). For mock treatment, the same volume of the recording media was added (black in *E*–*G*). (*H*) Cellular rhythms were recorded from U2OS cells transiently expressing *Bmal1*-luc. For hypotonic treatment, the recording media were diluted to give the final osmolarity of approximately 210 mOsm by distilled water (red). For mock treatment, the same volume of 140 mM NaCl solution was added to isotonicity (approximately 280 mOsm; black). In *D*–*H* data are mean ± SEM. $n = 3$. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; not significant (n.s.), $P \geq 0.05$ vs mock treatment, Student's *t* test.

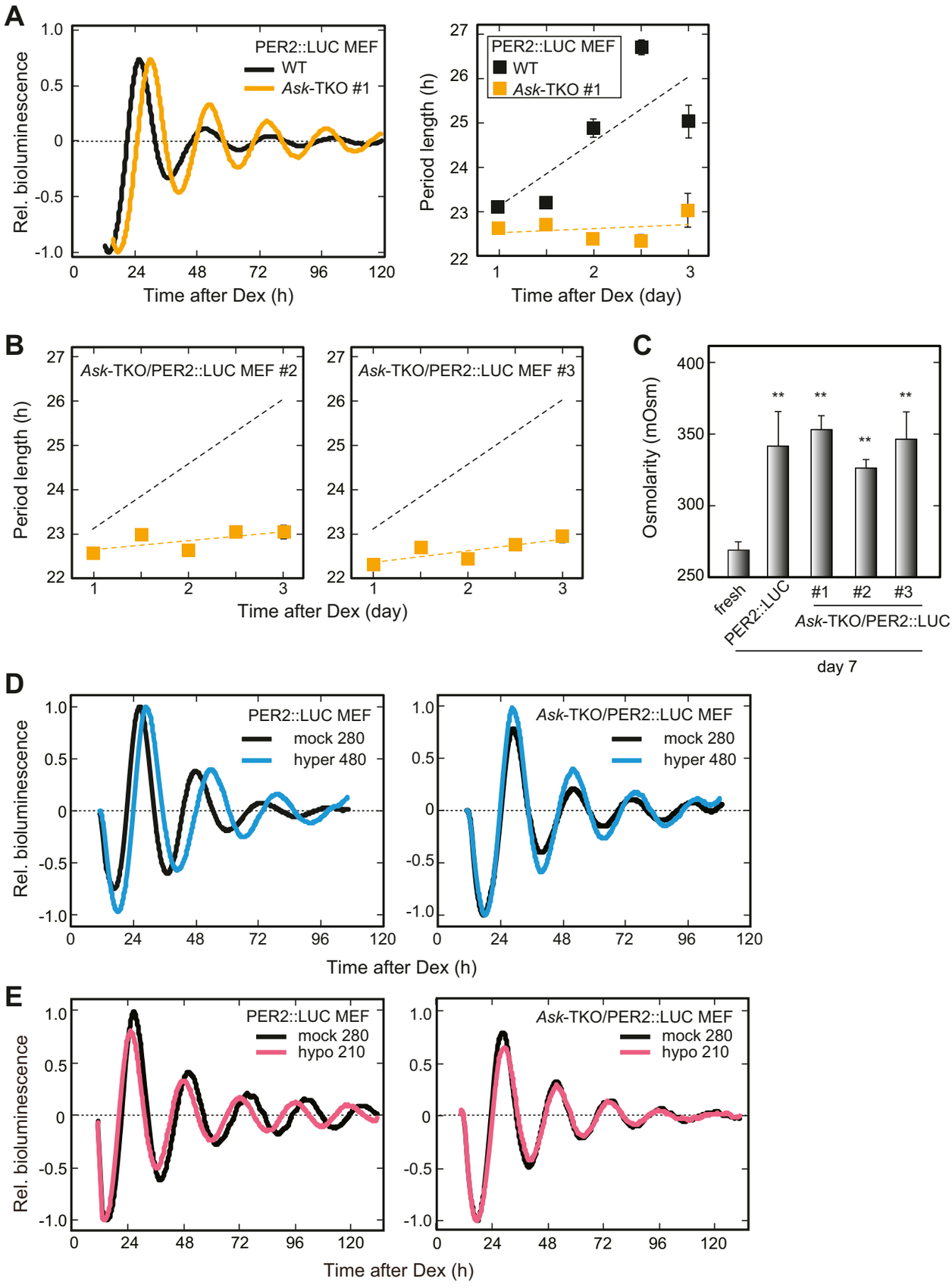

Fig. S2. Chronic osmotic treatment affects the circadian period of the cellular rhythms through ASK signaling. (*A*) Effect of *Ask*-TKO on the circadian period of the cellular rhythms. Cellular rhythms were recorded from PER2::LUC (black) and *Ask*-TKO/PER2::LUC MEFs (orange). The period length was calculated as in Fig. 1*A*. (*B*) The cellular rhythms were recorded from *Ask*-TKO/PER2::LUC MEFs #2 and #3 isolated from independent embryos. *Ask*-TKO/PER2::LUC MEFs #1 were used exclusively in this study, except in the experiments depicted in *B* and *C*. The period length was calculated as in Fig. 1*A*. (*C*) Measured osmolarity of the recording media recovered from the culture of PER2::LUC MEFs or *Ask*-TKO/PER2::LUC MEFs #1–3 at day 7. Data are mean ± SEM. $^{**}P < 0.01$ vs. fresh recording media, Student's *t* test (fresh, $n = 8$). (*D* and *E*) Representative data of the cellular rhythms shown in Fig. 1 *E* and F.

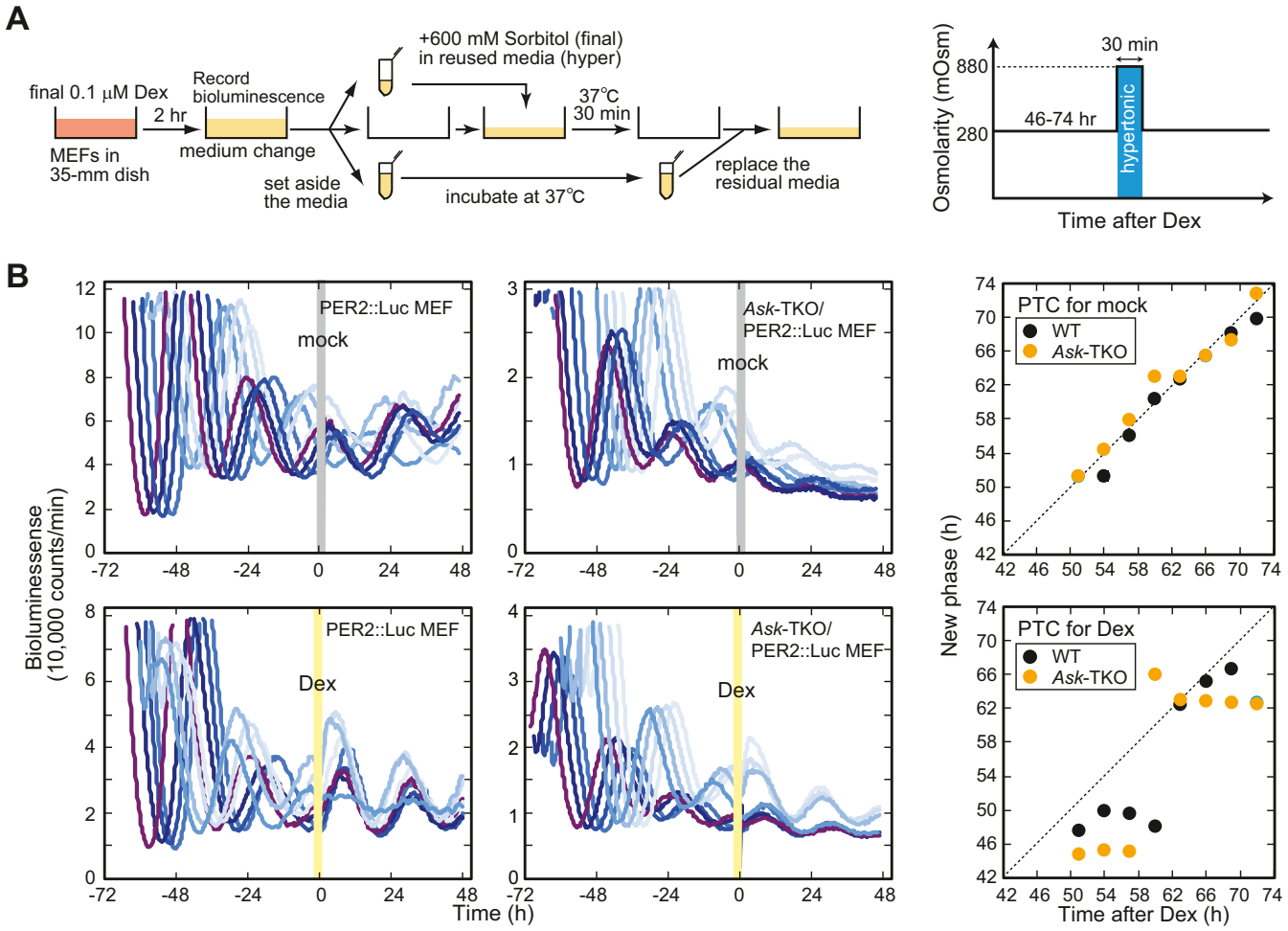

Fig. S3. Experimental scheme for acute hypertonic treatment and Dex-induced resetting in *Ask*-TKO MEFs. (*A*) Experimental scheme for acute hypertonic treatments to investigate the phase-shift of the cellular rhythms. (*Left*) For hypertonic pulses, the media were removed at various circadian phases, and 0.88 mL of the used media were rapidly returned back to the original well after mixing with 0.12 mL of 5 M sorbitol in the fresh media (final concentration 600 mM). For mock treatment, 0.12 mL of the fresh media was added. After a 30-min incubation, the hypertonic media were replaced by the residual used media that had been kept at 37 °C. (*Right*) Time course of the osmolarity changes. (*B*) Phase response of PER2::LUC (*Left*) and *Ask*-TKO/PER2::LUC MEFs (*Middle*) in response to dexamethasone (Dex) treatment. Dex (final concentration 0.1 μM) or the same volume of the fresh media (mock) was added to the recording media at the various CTs, and this timing was set to time 0 in this figure. (*Right*) PTCs for the cellular rhythms in response to the Dex treatment.

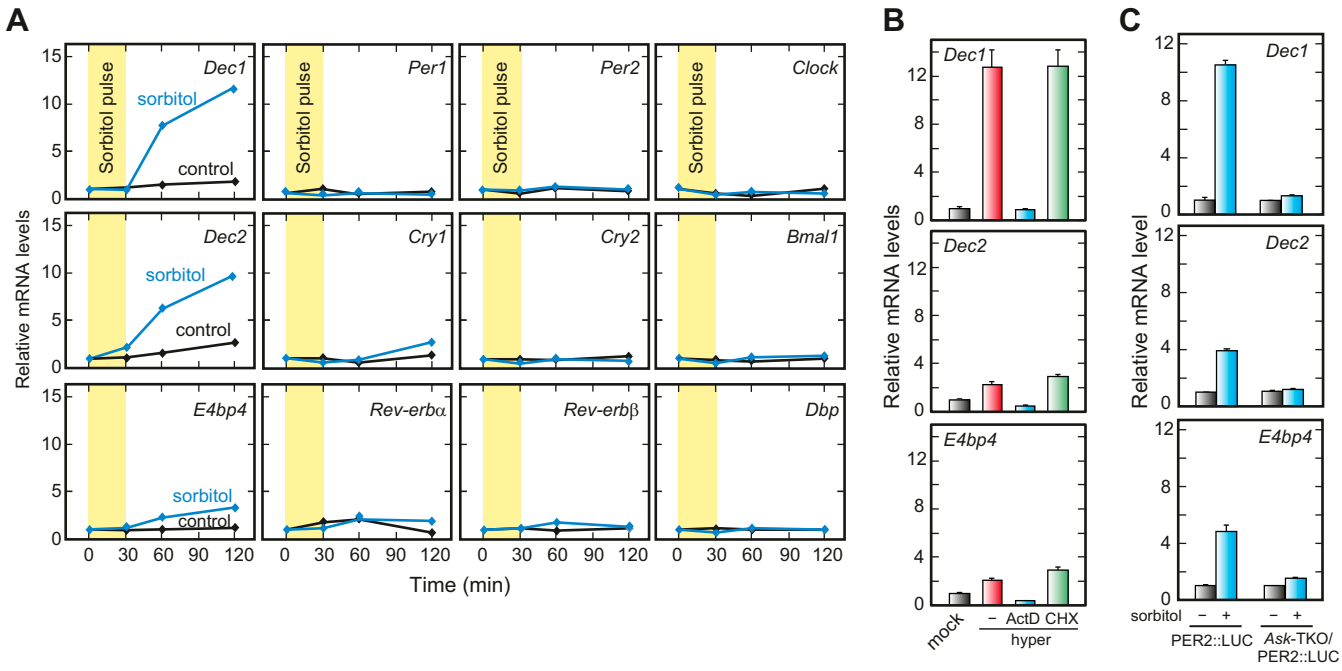

Fig. S4. Early responses of clock genes as entry points of the osmotic effects through ASKs. (*A*) Temporal changes in mRNA levels of clock related genes after hypertonic treatment. PER2::LUC MEFs were cultured in the recording media, and the hypertonic treatment was applied for 30 min (illustrated by the yellow zone in each panel) at TAD 66 h. For mock treatment, the same volume of the recording media was added. The cells were collected at 0 min (starting point of the treatment), 30 min (end point of the treatment), and 60 min and 120 min after the treatment, and the relative mRNA levels at time 0 was set to 1. (*B*) Effect of cycloheximide (CHX) and actinomycin D (ActD) on the osmolarity-dependent inductions of *Dec1*, *Dec2*, and *E4bp4* transcripts. CHX (0.8 μM) or ActD (50 μM) was added 1 h before the hypertonic treatment. (*C*) Changes in *Dec1*, *Dec2*, and *E4bp4* mRNA levels after the hypertonic treatment in *Ask*-TKO/PER2::LUC MEFs. (*B* and *C*) The cells were cultured in the recording media, and the hypertonic treatment was applied for 30 min at TAD 66 h. For mock treatment, the same volume of the recording media was added. The cells were collected at 60 min (30 min after the end point of the treatment). The mean value of each mock was set to 1. Data are mean ± SEM. $n = 3$.

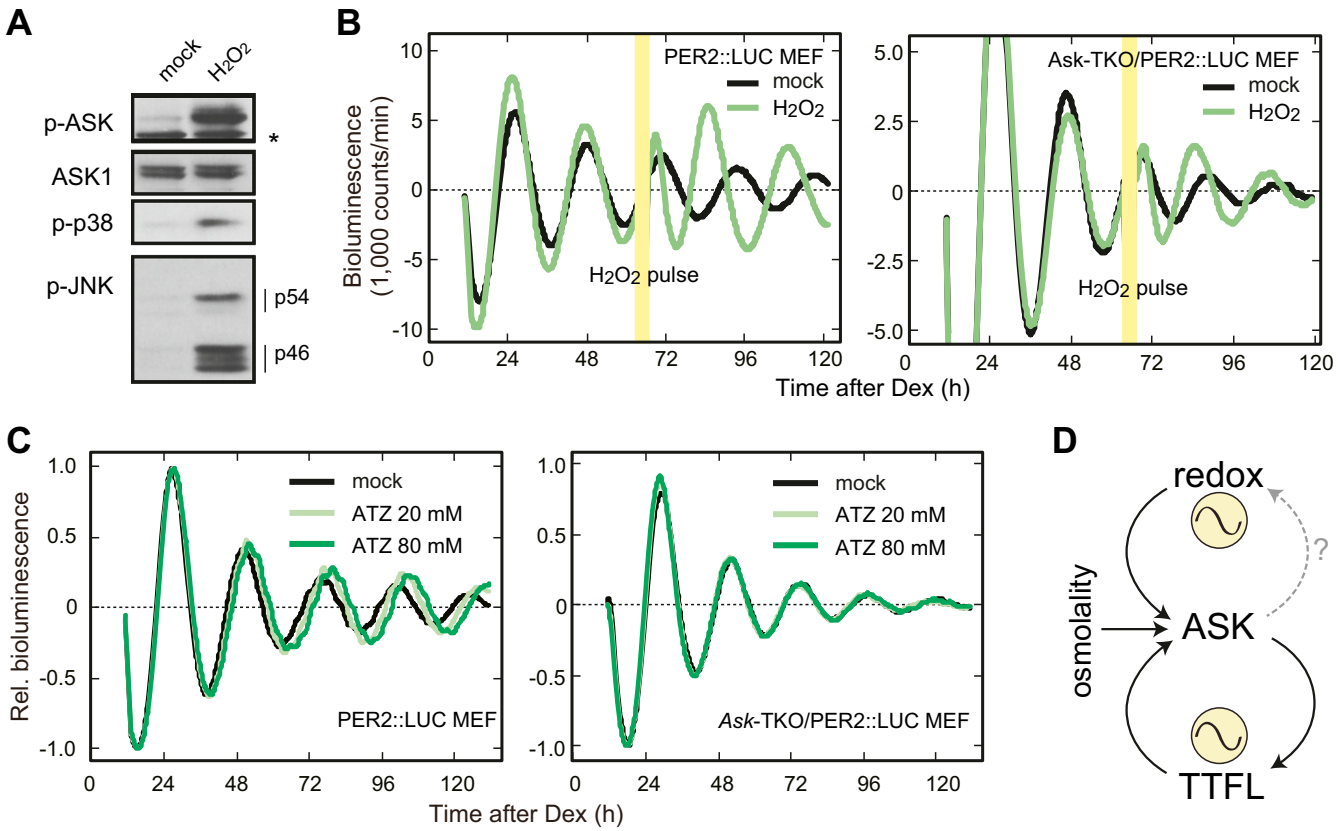

Fig. S5. Oxidative stress inputs to the circadian clock through ASK signaling. (*A*) Immunoblot analysis for the phosphorylation states of endogenous ASKs, p38, and JNK in HEK293A cells. HEK293A cells were collected at 30 min after treatment of the cells with 1 mM H_2O_2 (final concentration). For mock treatment, the same volume of the recording media was added. The asterisk represents a nonspecific band. For JNK immunoreactive bands, p54 and p46 represent two JNK isoforms. (*B*) Representative data of the cellular rhythms shown in Fig. 4*A*. (*C*) Representative data of the cellular rhythms shown in Fig. 4*B*. (*D*) Model for the interlocking interaction between the redox cycle and the TTFLs through ASK signaling. The changes in extracellular osmolarity were also input to the clock.

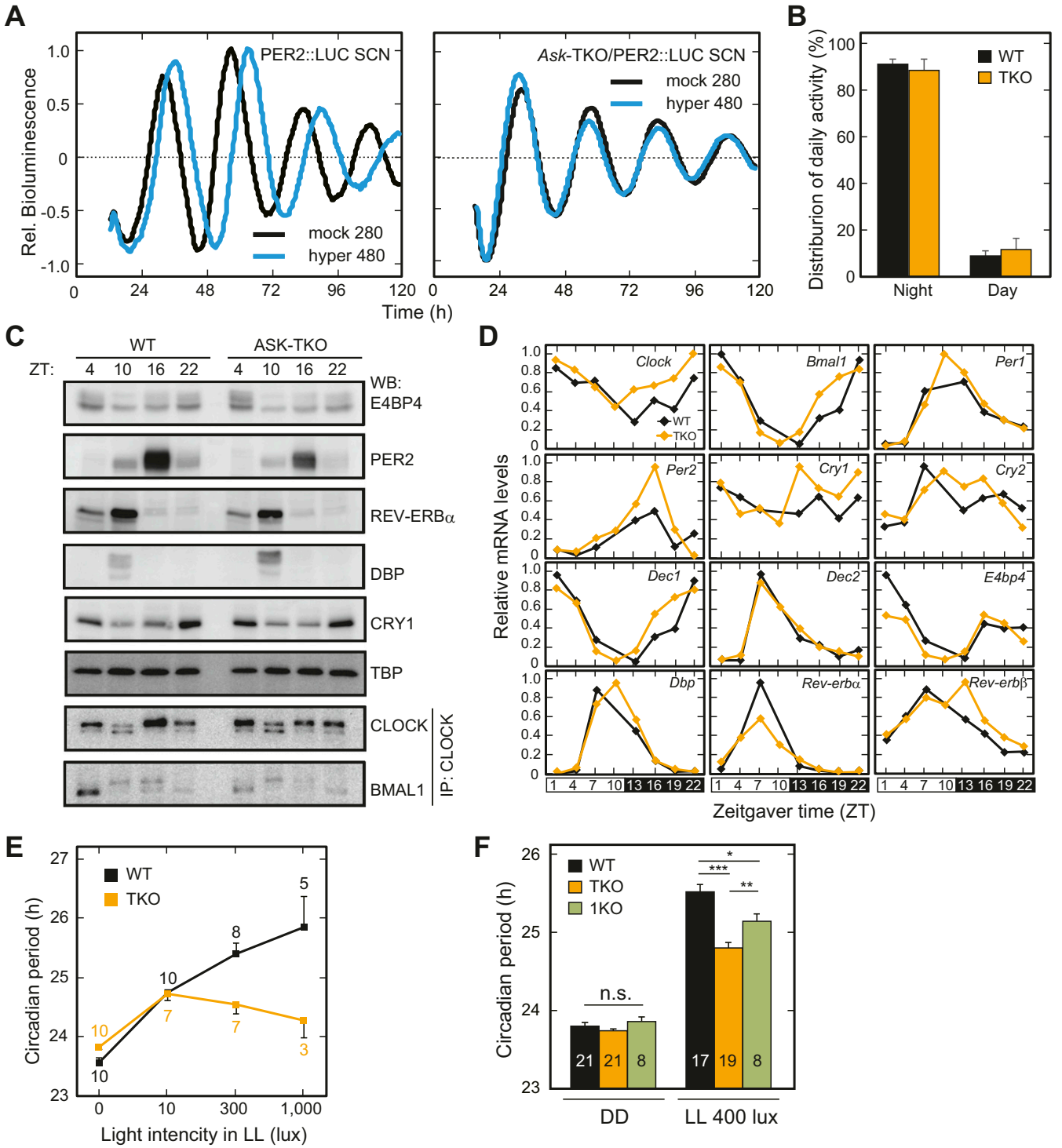

Fig. S6. The effect of *Ask*-TKO at tissue and behavioral levels. (*A*) Representative data of the SCN rhythms shown in Fig. 4*C*. (*B*) Distribution of the activity levels in 12-h light-phase (day) and 12-h dark-phase (night). Data are mean ± SEM. WT, $n = 21$; *Ask*-TKO, $n = 21$. (*C*) Temporal expression profiles of the clock proteins in the liver nucleus. Nuclear extracts prepared at the indicated time points were subjected to immunoblot analysis. TATA-binding protein (TBP) served as a loading control. For immunoblotting of CLOCK and BMAL1, the liver nuclear extracts were immunoprecipitated with anti-CLOCK mAb (IP: CLOCK). (*D*) Temporal expression profiles of clock-related genes in the liver. The relative levels of mRNA were examined by qRT-PCR and normalized by *Rps29*. (*E*) Change in the circadian period of the behavior rhythms under constant light (LL) conditions with various light intensities. Data are mean ± SEM. The numbers near the plots indicate the sample sizes. (*F*) Free-running periods of the behavioral rhythms under DD and LL (approximately 400 lux) conditions in *Ask1* single KO mice. The data for WT and *Ask*-TKO mice are reproduced from Fig. 4*E*. The numbers in the bars indicate the sample sizes. Data are mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; not significant (n.s.), $P \geq 0.05$, Student's *t* test.

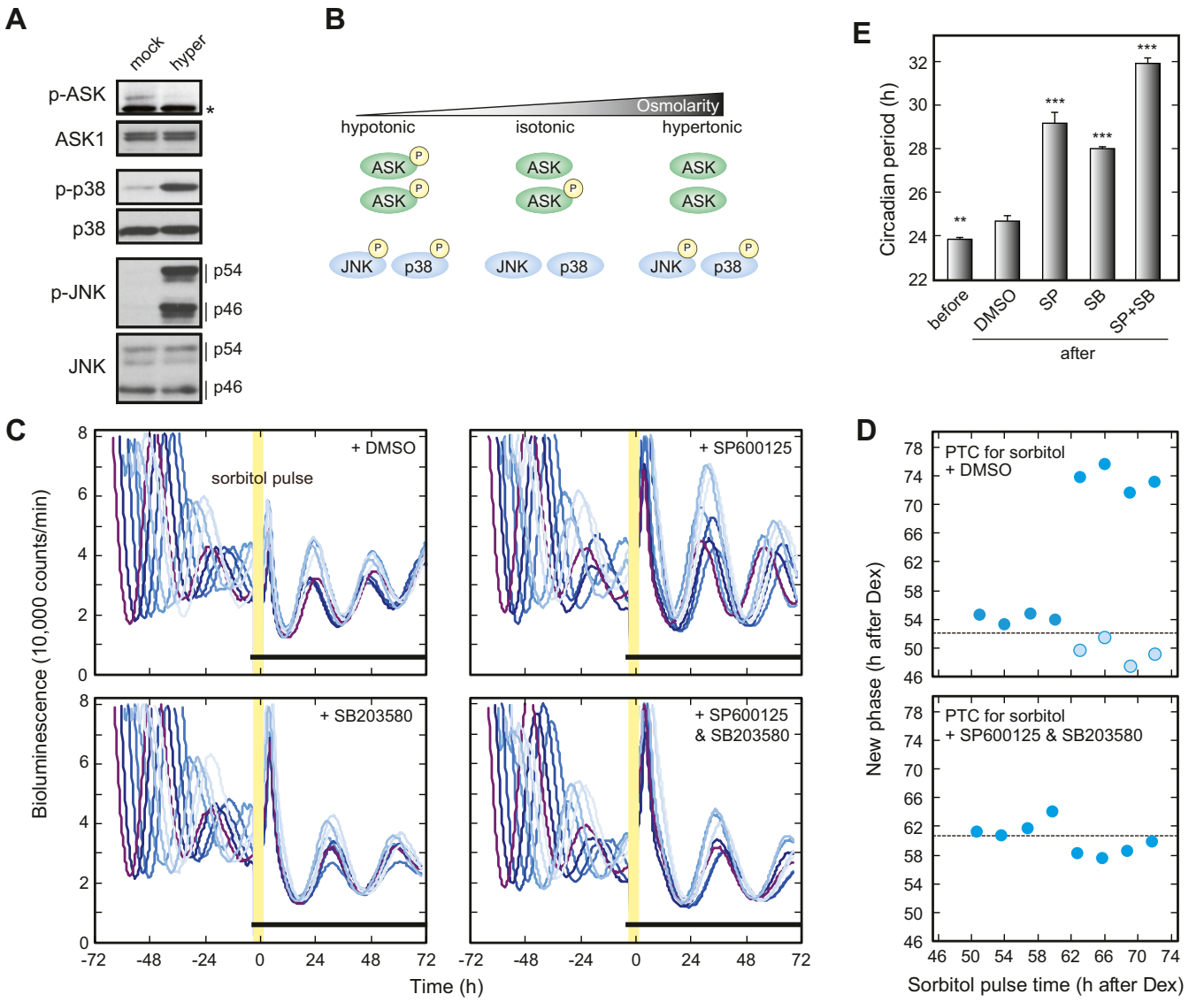

Fig. S7. JNK and p38 make no obvious contribution to the hyperosmolarity-induced resetting. (*A*) Immunoblot analysis for the phosphorylation of ASKs, p38, and JNK in response to hyperosmotic stress. HEK293A cells were collected 30 min after the treatment of the indicated stimuli. For hypertonic treatment, 200 mM sorbitol was added to the media to give a final osmolarity of approximately 480 mOsm. For mock treatment, the same volume of the recording media was added. The asterisk represents a nonspecific band. The JNK immunoreactive bands, p54 and p46, represent two JNK isoforms. (*B*) Regulation of MAPK signaling pathways under hyperosmotic and hypoosmotic stress. In most conditions and cell types, JNK and p38 are phosphorylated in response to hyperosmotic stress and hypoosmotic stress. In contrast, ASKs are dephosphorylated during hyperosmotic stress and phosphorylated during hypoosmotic stress. (*C*) Phase response of PER2::LUC MEFs to the acute hypertonic treatment in the presence of inhibitors for stress-activated MAPKs. SP600125 (20 μM) and/or SB203580 (20 μM) or DMSO as a control were added to the media at 1 h before the hypertonic pulse (indicated by horizontal black lines). The cells were stimulated by a 30-min pulse of 600 mM sorbitol (final concentration) at various circadian phases, and this timing was set to Time 0 in this figure. (*D*) PTC for the cellular rhythms in response to the hypertonic pulse in the presence of SP600125 and SB203580 (*Bottom*). The open circles in PTC indicate the extrapolated new phases by subtracting intrinsic cellular periods from actual new phases. (*E*) Effect of MAPK inhibitors on the circadian period of the PER2::LUC MEFs. The circadian period was calculated from the time points of peaks and troughs of bioluminescence before and after treatment with the indicated inhibitors. Both SP600125 and SB203580 had remarkable period-lengthening effects, as reported previously (1, 2), by which inhibitions of JNK and p38 were verified under the condition. The significant difference between "before" and "DMSO" is consistent with the data shown in Fig. 1*A* and Fig. S1 *A* and B. Data are mean SEM. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. DMSO treatment (before, $n = 32$; DMSO, SP, SB, and SP+SB, $n = 8$), Student's t test.

1. Yoshitane H, et al. (2012) JNK regulates the photic response of the mammalian circadian clock. *EMBO Rep* 13:455–461.
2. Yagita K, Yamanaka I, Koinuma S, Shigeyoshi Y, Uchiyama Y (2009) Mini screening of kinase inhibitors affecting period-length of mammalian cellular circadian clock. *Acta Histochem Cytochem* 42:89–93.

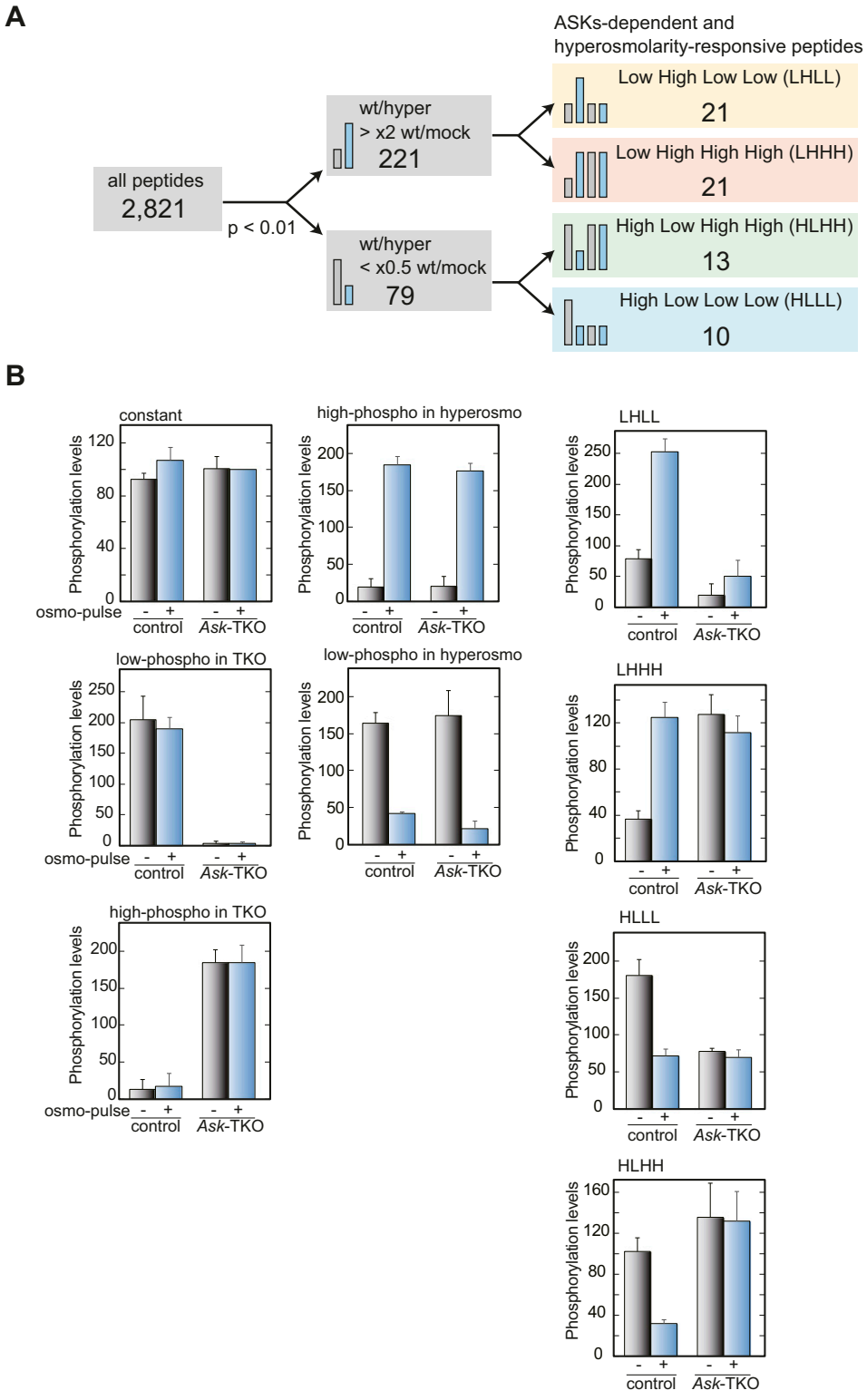

Fig. S8. ASK-dependent and hyperosmotic stress-responsive phosphopeptides. (*A*) Scheme for identifying ASK-dependent and hyperosmotic stress-responsive phosphopeptides from 2,821 phosphopeptides detected in the LC-MS/MS–based proteomic analysis. (*B*) Representative patterns of phosphorylation levels of ASK-dependent and hyperosmotic stress-responsive peptides. Data are mean ± SEM. $n = 3$.

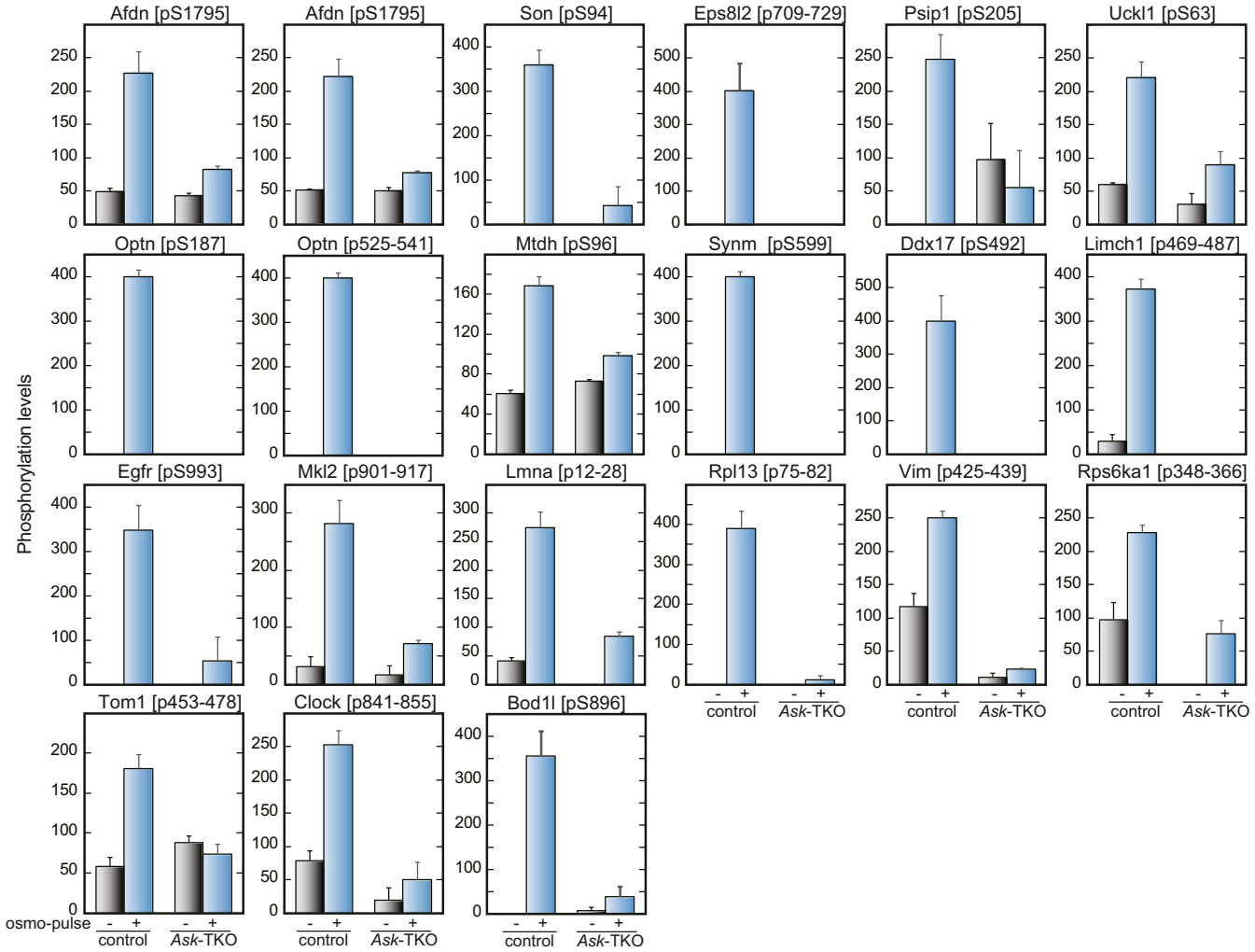

Fig. S9. All of the "LHLL-pattern" phosphopeptides in the LC-MS/MS–based proteomic analysis. A total of 21 phosphopeptides were up-regulated by hyperosmotic treatment in control MEFs but were constantly low in the *Ask*-TKO MEFs. This "LHLL" group included a phosphopeptide derived from CLOCK protein. Data are mean ± SEM. $n = 3$.

Other Supporting Information Files

Dataset S1 (XLSX)
Dataset S2 (XLSX)