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Supplemental Information

Klf15 Orchestrates Circadian Nitrogen Homeostatsis

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Supplemental Experimental Procedures

Neurobehavioral Analysis

Behavioral Assessment - Y-Maze

Spontaneous alternation behavior and exploratory activity, a hippocampal-associated task, is measured using a Y-maze (32 cm (long) X 10 cm (wide) with 26-cm walls). Briefly, each animal is placed in one of three arms of the Y-maze (alternating arms across animals in each group) and each arm entry is recorded for duration of 6 minutes. An alternation is defined as 3 entries in 3 different arms (i.e. 1, 2, 3 or 2, 3, 1 etc). % number of alternations is calculated as (total alternations/total number of entries – 2)*100. The maze is cleaned with ethanol between each animal to minimize odor cues (Casadesus et al., 2006).

Delayed/Trace Fear Conditioning

Pavlovian fear conditioning is a paradigm in which an initially neutral stimulus such as a tone (conditioned stimulus, CS), through the pairing with an aversive unconditioned stimulus (US) such as a mild foot-shock, acquires aversive properties and comes to elicit a fear related freezing response. Contextual fear conditioning is generally thought to be hippocampus-dependent testing for the ability of the animal to associate the US with the context in which it was presented. On the other hand, cued fear conditioning, in which the animal responds to the CS presentation in an altered context is thought to be hippocampus-independent. A two day protocol is designed to first train the animals to associate the US and CS and subsequently test for post-reactivation long-term memory 24 hours after training.

Day 1 – Training: On the first day animals are allowed to habituate in the chamber (Med Associates, Burlington, VT) for 2 min and are then presented with a white noise (80dB) for 30 sec, this stimulus is designated as the conditioned stimulus (CS). After a 2 second interval the animals are administered a 0.5mA shock, this is designated as the unconditioned stimulus (US). This procedure is repeated 5 times.

Day 2 – Contextual/altered context/cued testing: 24 h after training, animals are placed back in the original chamber and freezing bouts are scored during 5 min to determine the associations of the US with the context (contextual). Freezing

measurements are automated using appropriate software (Med Associates, Burlington VT) designed to gather 30 observations in 5 min. After contextual freezing is measured, animals are returned to their home cage for 1 h. The chamber environment is modified (new walls, flooring and odor cues) and the animal is introduced in the “new” chamber for 6 min. Freezing rate is quantified as described in the contextual test for 3 min in the absence of the CS (altered context). For the remaining 3 min the animal is presented with the CS in the altered context and scored for freezing behavior as described previously, to determine the cued fear conditioning score (Greco et al., 2010).

Morris Water Maze

Animals were trained in a black circular pool (112 cm) in a room containing distal visual cues. Pool water was whitened with non-toxic white dye and temperature maintained at 24°C. A white escape platform (10.5 cm in diameter) located approximately 0.5cm beneath the water level was placed in the center of the NE quadrant of the pool. Animals were introduced into the pool facing the wall from different quadrants to control for location bias and were tested on 8 trials per day, subdivided into 2 blocks, 30 minutes apart over 4 days; each trial was 60 seconds long. A pre-training session in which all animals were allowed to swim in the pool, and were gently guided to the platform was also performed. During each trial, if the animal did not find the platform during the allocated task time, it was gently guided towards the platform where it remained for 15 seconds and then immediately placed back into the water from the next start position for the next trial. Swim time, path length, and swim speed was recorded using a video tracking system and software (Ethovision, Noldus Information Technology, Wageningen, The Netherlands). On day 4, trial 8 was designated as probe trial in which the platform was lowered to measure spatial strategy and short-term retention. During probe trials animals were allowed to swim for 60 seconds without the possibility to escape; percent time spent in the quadrant where the platform was previously located, and platform crossings and latency were measured. After the completion of this trial the platform was rendered visible and all animals underwent a session to test for visual acuity (Bryan et al., 2010).

SUPPLEMENTAL REFERENCES

Bryan, K.J., Mudd, J.C., Richardson, S.L., Chang, J., Lee, H.G., Zhu, X., Smith, M.A., and Casadesus, G. (2010) Down-regulation of serum gonadotropins is as effective as estrogen replacement at improving menopause-associated cognitive deficits. J Neurochem *112*, 870-881.

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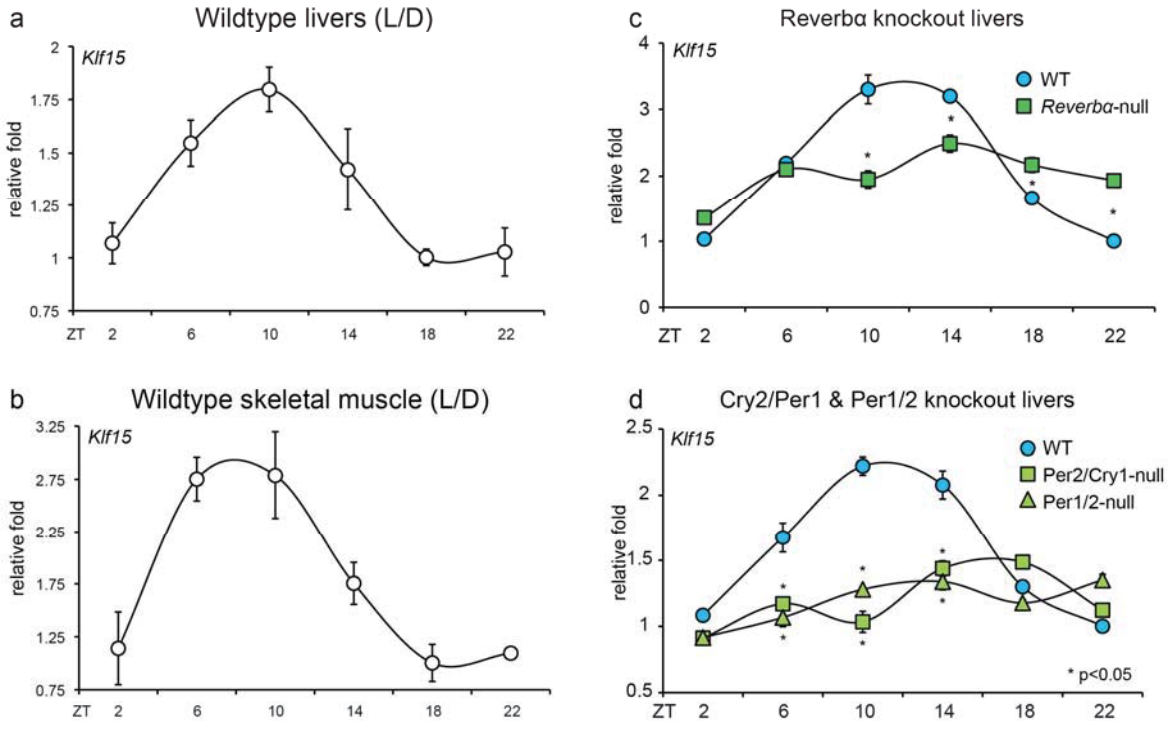
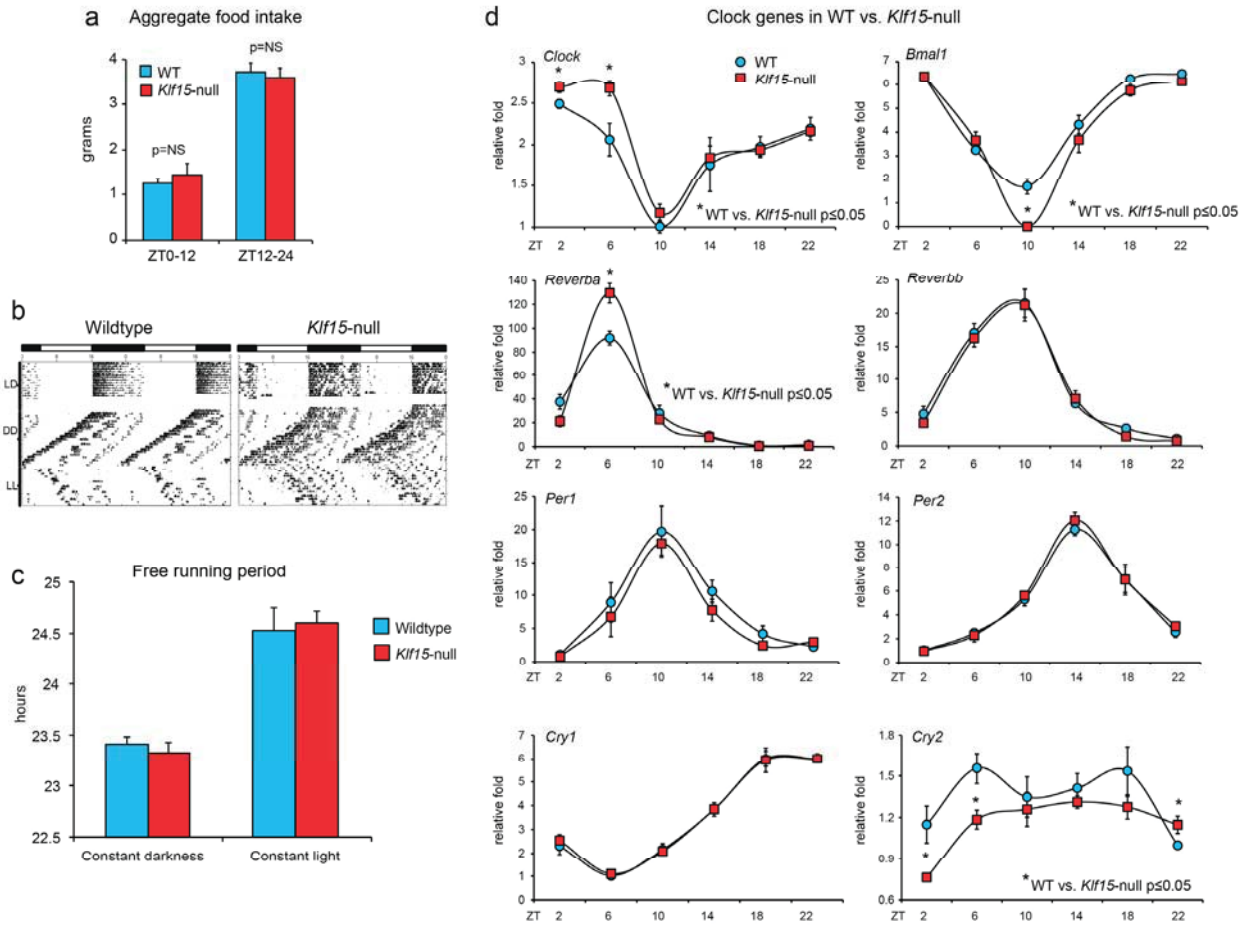
Greco, S.J., Bryan, K.J., Sarkar, S., Zhu, X., Smith, M.A., Ashford, J.W., Johnston, J.M., Tezapsidis, N., and Casadesus, G. (2010) Leptin reduces pathology and improves memory in a transgenic mouse model of Alzheimer's disease. J Alzheimers Dis *19*, 1155-1167.



Supplemental figure 1

Figure S1. Klf15 Expression in Wild-Type and Clock Mutant Livers and Skeletal Muscles, Related to Figure 1

(a and b) *Klf15* mRNA expression in (a) WT liver and (b) skeletal muscle under L/D conditions (n=4 per time point, ANOVA $p<0.05$). *Klf15* mRNA accumulation in (c) Per1/2 KO, Per2/Cry1 KO and (d) Reverba-null livers. Data presented as mean ± SEM.



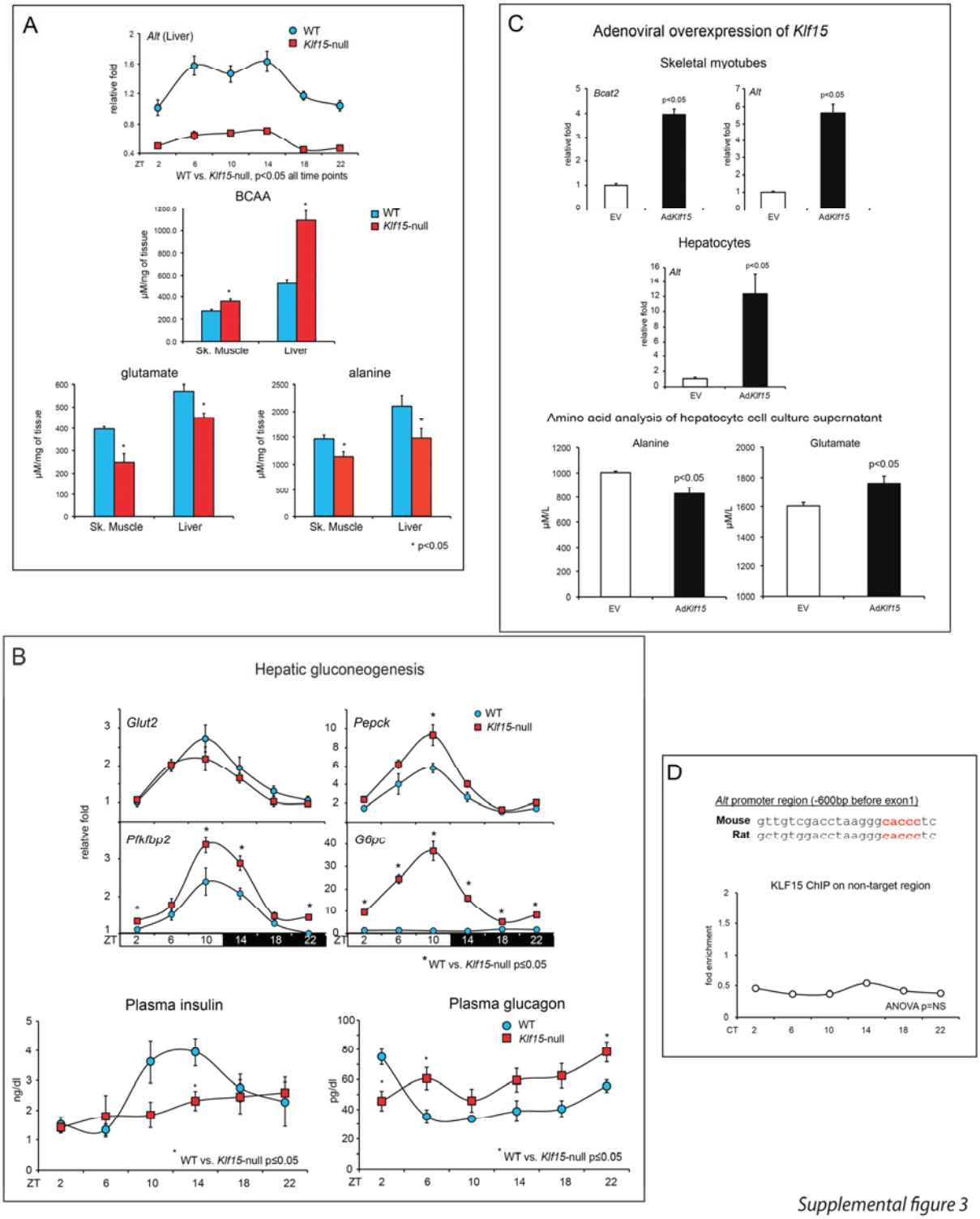
Supplemental figure 2

Figure S2. The Feeding and Activity of *Klf15*-Null Mice Are Similar to Their Controls and They Have Similar Clock Gene Expression, Related to Figure 2

(A) Aggregate food intake in WT and *Klf15*-null mice (n=4 per group

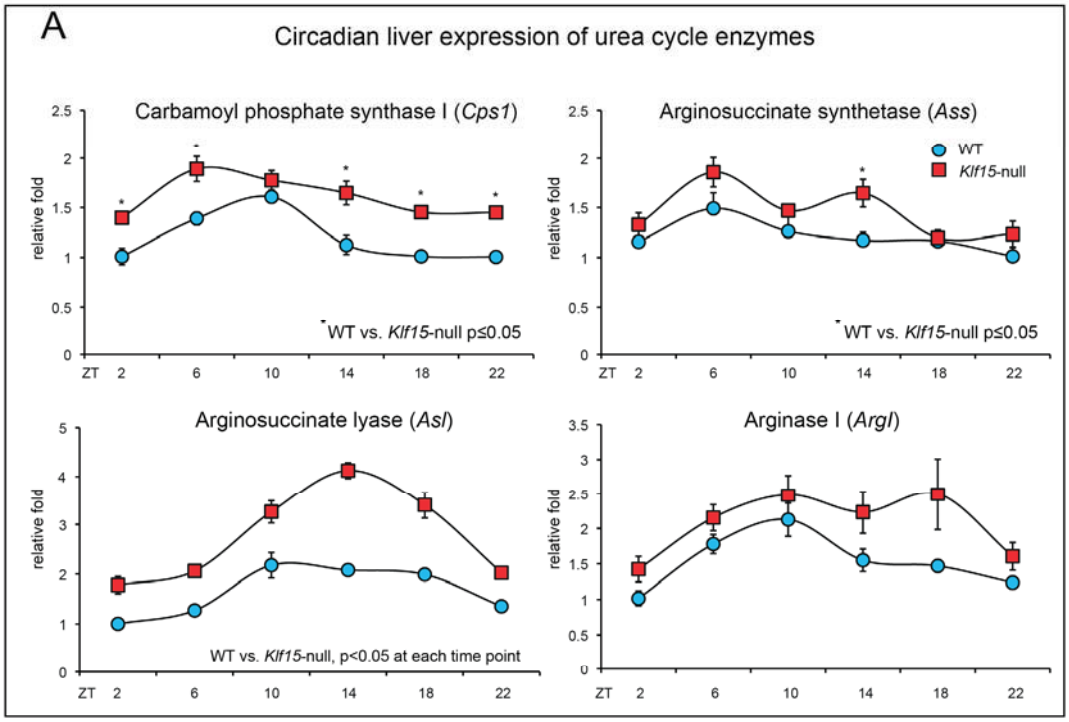
(B and C) WT and *Klf15*-null mice in a light-dark cycle (LD), constant dark (DD), or constant light (LL). The period of lights on and lights off is shown in the bars at the top of the figure. Each line represents a 48-hour period, with the dark bars indicated periods of wheel-running activity (n=14 per group).

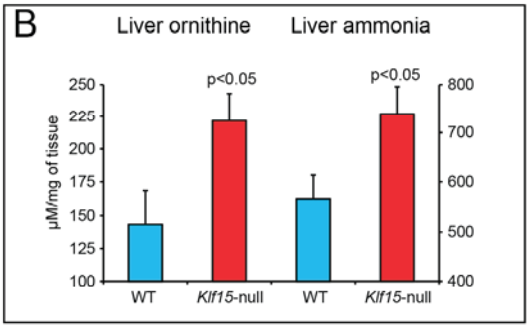
(D) Clock gene expression in WT and *Klf15*-null livers (n=4 per group per group). Data presented as mean ± SEM.

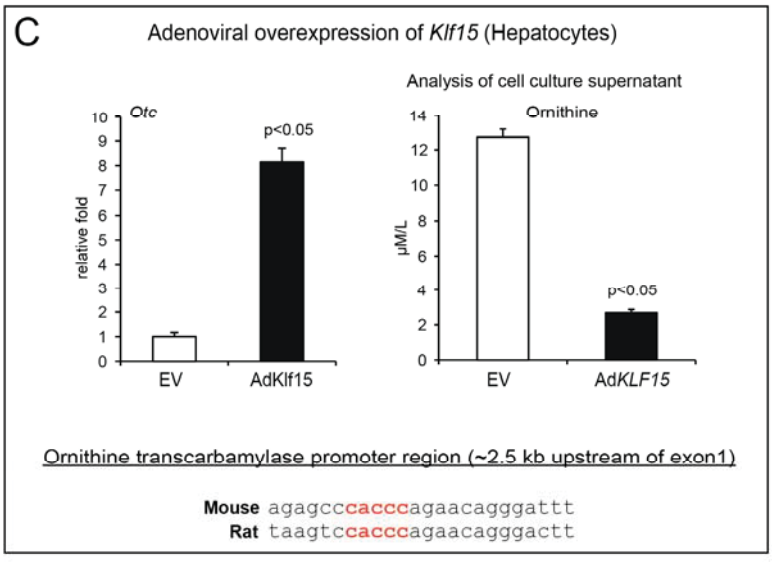


Supplemental figure 3

Figure S3. A Role for *Klf15* in Amino Acid Metabolism, Related to Figure 3
(A) *Alt* expression in WT and *Klf15*-null livers (n=4 per group per time point), and skeletal muscle/liver concentration of BCAA, glutamate and alanine in WT and *Klf15*-null mice measured at ZT7 (n=4 per group).
(B) Liver expression of gluconeogenic enzymes in WT, *Klf15*-null mice (n=4 per group per time point) and plasma insulin/glucagon in WT and *Klf15*-null mice (n=5 per group per time point).
(C) Adenoviral overexpression of *Klf15* compared to empty virus (EV) in skeletal myotubes and hepatocytes for *Alt* and *Bcat2* (n=3 per group) and AA analysis of cell culture supernatant from hepatocytes over expressing *Klf15* (n=3 per group).
(D) Conserved Krüppel-binding region, i.e., C(A/T)CCC on the *Alt* promoter, and ChIP for KLF15 on a non-target region (n=3 per time point). Data presented as mean ± SEM.

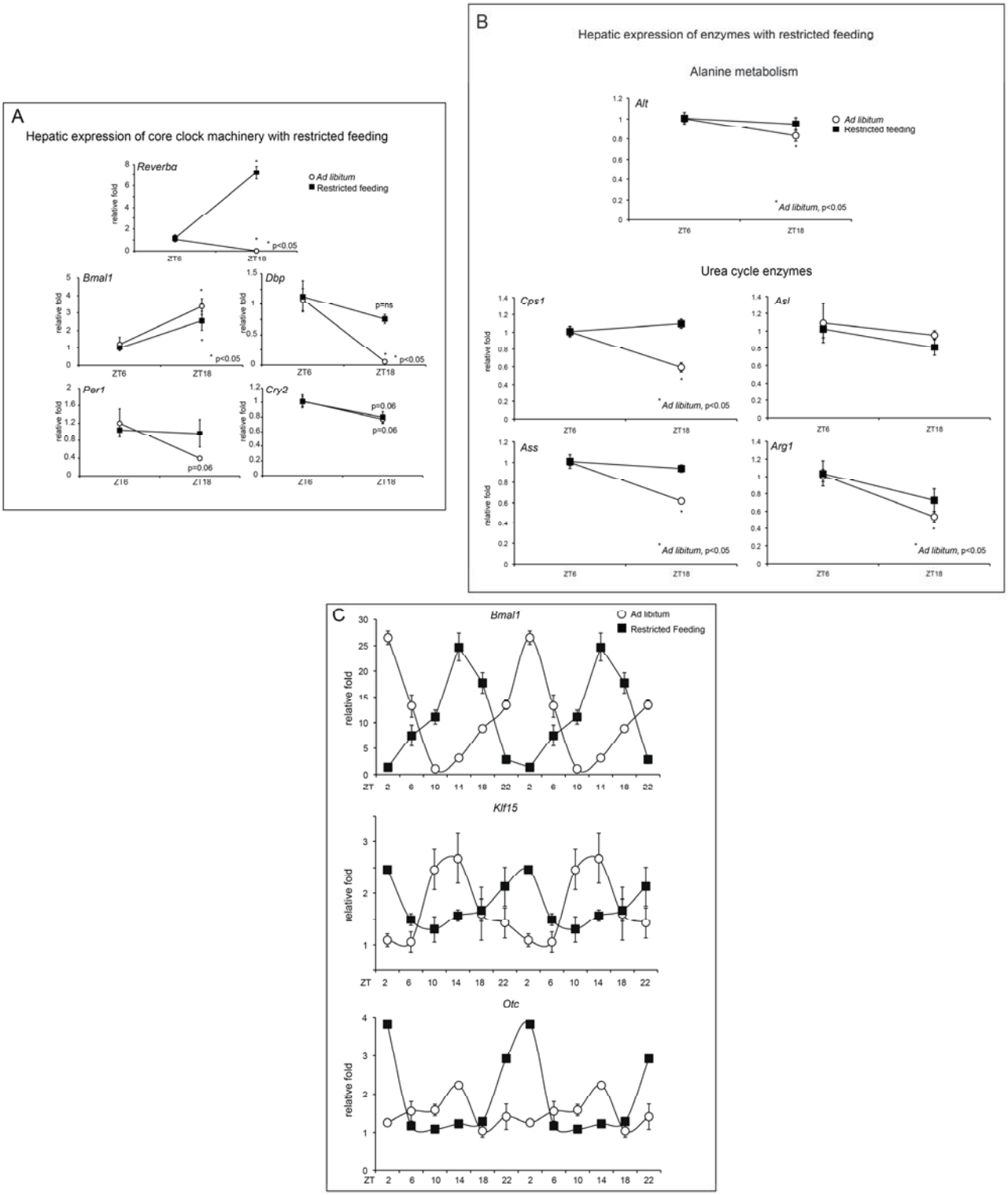






Supplemental figure 4

Figure S4. A Role for *Klf15l* in Ureagenesis, Related to Figure 4
(A) Expression of urea cycle enzymes (*Cps1, Ass, Asl and Arg1*) in WT and *Klf15*-null mice (n=4 per group per time point).
(B) Liver ornithine and ammonia from WT and *Klf15*-null at ZT7 (n=4 per group).
(C) Adenoviral overexpression of *Klf15* in hepatocytes compared to EV, and analysis of cell culture supernatant (n=3 per group) and conserved Krüppel-binding region on the *Otc* promoter. Data presented as mean ± SEM.



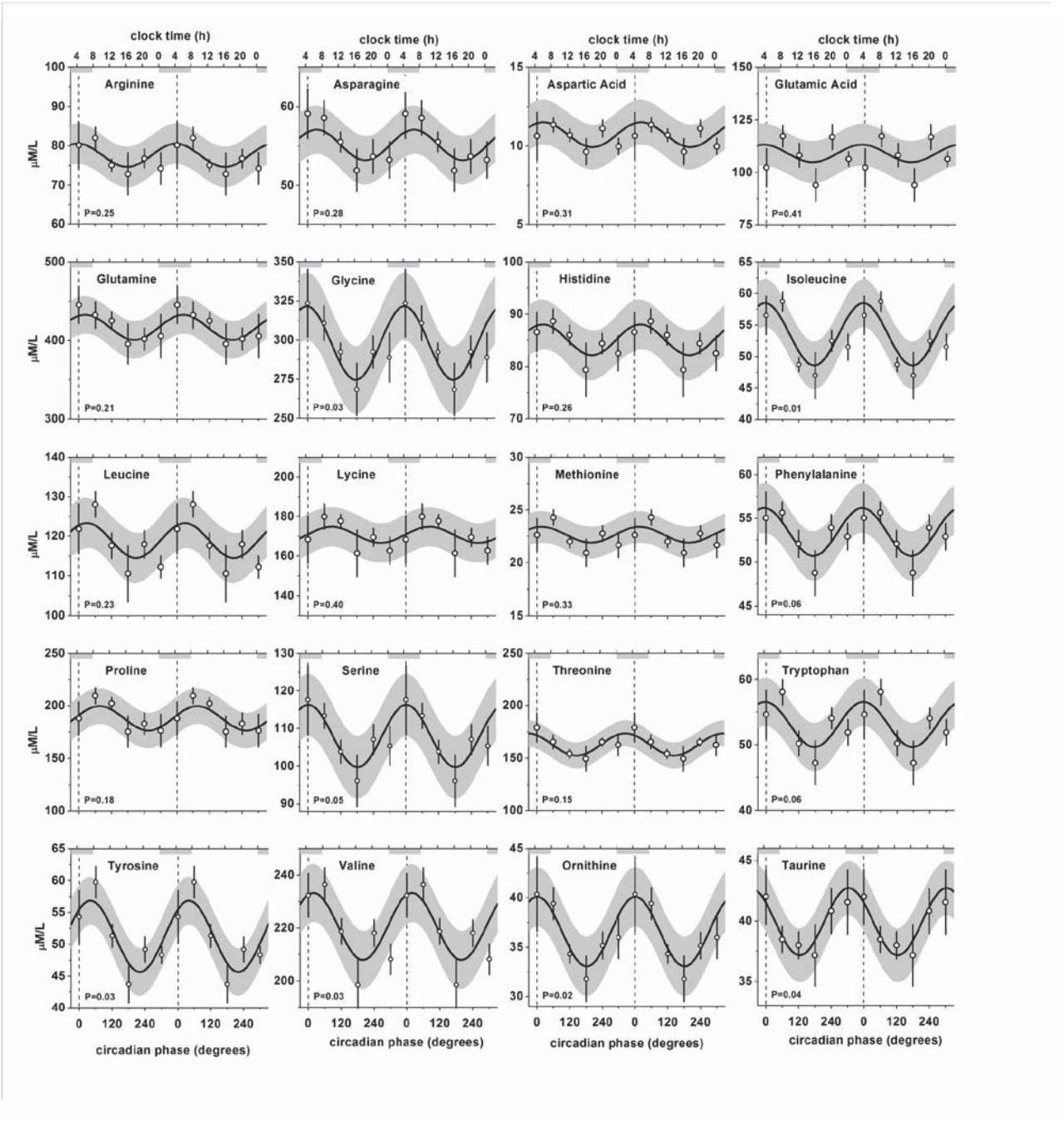
Supplemental figure 5

Figure S5. Restricted Feeding, Related to Figure 5

(A) Liver expression of core clock machinery components following *ad libitum* or restricted feeding (n=5 per group per time point).

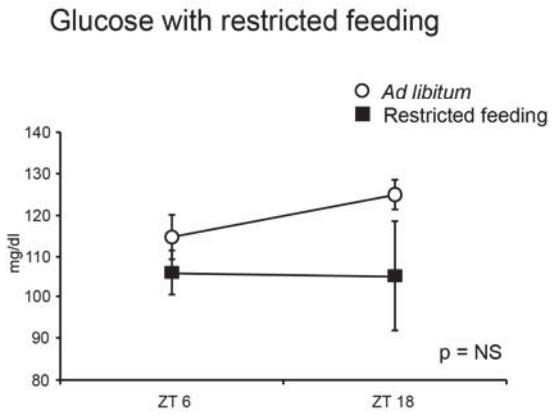
(B) Liver expression of AA metabolic enzymes (*Alt*), and urea cycle enzymes (*Cps1, Asl, Ass* and *Arg1*) following *ad libitum* or restricted feeding (n=5 per group per time point).

(C) Following restricted feeding for one month, liver gene expression for *Bmal1, Klf15* and *Otc* was compared to mice fed *ad libitum* (n=4 per time point per group). Data is double plotted to illustrate rhythmicity. Data presented as mean ± SEM.



Supplemental figure 6

Figure S6. Human Amino Acid and Urea Cycle Intermediates, Related to Figure 6
Summary data of all human amino acids from the forced desynchrony study not included in Figure 9 of the manuscript, and plasma ornithine, taurine. Axes and symbols as in Fig. 7 in main text.



Supplemental figure 7

Figure S7. Glucose with Restricted Feeding
Glucose following *ad libitum* or restricted feeding (n=5 per group per time point). Data presented as mean ± SEM.

Circadian Amino Acid Data

	Amino Acid	WT (D/D) - CT (Hours)				WT (L/D) - ZT (Hours)				*Klf15*-null (L/D) - ZT (Hours)			Changes with feeding
		Zenith	Nadir	Oscillation	ANOVA (p value)	Zenith	Nadir	Oscillation	ANOVA (p value)	Zenith	Nadir	Level	
	Total Amino Acids	22	6	Yes	0.01	18	2	Yes	0.02	No rhythm		Increased*	Yes
Non-essential amino acids	Alanine	2	10	Yes	0.01	22	10	Trend	0.06	No rhythm		Reduced*	Yes
Non-essential amino acids	Arginine	2	14	Yes	<0.0001	22	14	Yes	0.0001	2	14	Reduced*#	Yes
Non-essential amino acids	Asparagine			No	NS			No	NS			Increased*#	
Non-essential amino acids	Aspartate	2	10	Yes	0.01	10	2	Yes	0.0006	22	2	#	Yes
Non-essential amino acids	Cysteine	6	22	Yes	0.007	2	6	Yes	0.04	No rhythm			No
Non-essential amino acids	Glutamate			No	NS			No	NS			Reduced*#	
Non-essential amino acids	Glutamine	10	2	Yes	0.05	10	22	Yes	0.02	10	2	Increased*#	Yes
Non-essential amino acids	Glycine			No	NS	10	2	Trend	0.07	6	2	Reduced*#	No
Non-essential amino acids	Proline	22	6	Yes	0.01	14	6	Yes	0.03	18	2	Increased*#	Yes
Non-essential amino acids	Serine			No	NS			No	NS			Reduced*#	
Non-essential amino acids	Tyrosine	22	14	Yes	<0.0001			No	NS			Increased*#	Yes
Essential amino acids	BCAA	22	14	Yes	0.01	14	6	Yes	0.0002	18	6	Increased*#	Yes
Essential amino acids	Lysine	22	14	Yes	0.004	2	10	Yes	0.02	6	14	Increased*#	Yes
Essential amino acids	Histidine			No	NS			No	NS			Increased*#	Yes
Essential amino acids	Methionine			No	NS	14	6	Yes	0.002	14	2	Increased*#	Yes
Essential amino acids	Phenylalanine	22	6	Yes	0.0002	14	6	Yes	0.05	10	6	Increased*#	Yes
Essential amino acids	Threonine	22	6	Yes	0.03	18	10	Yes	0.03	6	2		Yes
Essential amino acids	Tryptophan	22	6	Yes	<0.0001	14	2	Yes	0.01	No rhythm		Increased	Yes

* WT vs. *Klf15*-null p≤0.05 in at least one time point
# ANOVA p<0.05 in *Klf15*-null mice

Supplemental Table 1

Supplemental Table 1. Summary of Mouse Amino Acid Data.