

# Temporal orchestration of circadian autophagy rhythm by C/EBP $\beta$

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**Temporal organization of tissue metabolism is important for maintaining nutrient and energy homeostasis in mammals. Autophagy is a conserved cellular pathway that is activated in response to nutrient limitation, resulting in the degradation of cytoplasmic components and the release of amino acids and other nutrients. Here, we show that autophagy exhibits robust circadian rhythm in mouse liver, which is accompanied by cyclic induction of genes involved in various steps of autophagy. Functional analyses of transcription factors and cofactors identified C/EBP $\beta$  as a potent activator of autophagy. C/EBP $\beta$  is rhythmically expressed in the liver and is regulated by both circadian and nutritional signals. In cultured primary hepatocytes, C/EBP $\beta$  stimulates the program of autophagy gene expression and is sufficient to activate autophagic protein degradation. Adenoviral-mediated RNAi knock-down of C/EBP $\beta$  *in vivo* abolishes diurnal autophagy rhythm in the liver. Further, circadian regulation of C/EBP $\beta$  and autophagy is disrupted in mice lacking a functional liver clock. We have thus identified C/EBP $\beta$  as a key factor that links autophagy to biological clock and maintains nutrient homeostasis throughout light/dark cycles.**

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## Introduction

Organisms evolve diverse strategies to adapt their nutrient and energy metabolisms to the light/dark cycles on the earth. In mammals, the circadian clocks coordinate diurnal rhythms of behaviour and physiology, including major pathways of nutrient and energy metabolism (Rutter *et al*, 2002; Wijnen and Young, 2006; Green *et al*, 2008). These pacemakers are self-sustained oscillators in the brain and peripheral tissues, which synchronize their downstream transcriptional output

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(Panda *et al*, 2002; Storch *et al*, 2002; Ueda *et al*, 2002). Clocks in different tissues can be entrained by distinct external cues, such as light, temperature and nutritional signals. Genetic disruption of components in mammalian clock results in metabolic disorders, including perturbations in lipid and glucose homeostasis (Rudic *et al*, 2004; Turek *et al*, 2005; Lamia *et al*, 2008). In humans, disruption of circadian rhythm has been associated with increased risk for obesity and cardiovascular disease (Leproult and Van Cauter, 2010).

Autophagy is a cellular process that delivers cytosolic components to lysosomes for degradation (Levine and Kroemer, 2008; Mizushima *et al*, 2008; Yang and Klionsky, 2010). In response to metabolic stress, such as starvation, autophagy is highly induced to degrade glycogen, lipid droplets, and cytosolic components to provide a source of nutrients and metabolic fuel (Rabinowitz and White, 2010). The essential role of autophagy is underscored by neonatal lethality in pups lacking ATG5, an essential factor in the autophagy pathway (Kuma *et al*, 2004). In addition, autophagy plays an important role in removing protein aggregates and damaged organelles (Komatsu *et al*, 2005, 2006, 2007; Ebato *et al*, 2008; Klionsky *et al*, 2008). Genetic disruption of autophagy genes results in the accumulation of ubiquitinated protein aggregates and abnormal organelles in hepatocytes, neurons, and pancreatic  $\beta$ -cells. Recent studies have also implicated autophagy in the control of hepatic lipid metabolism and the development of insulin resistance (Singh *et al*, 2009a; Yang *et al*, 2010). Electron microscopy studies conducted in 1970s have demonstrated that the abundance of autophagic vacuoles varies according to the time of day in several rat tissues (Pfeifer and Scheller, 1975; Reme and Sulser, 1977; Sachdeva and Thompson, 2008). However, whether autophagy is regulated by clock and the molecular mechanisms underlying circadian autophagy rhythm have not been defined. In this study, we show that autophagy displays robust circadian rhythm in mouse liver, which is accompanied by rhythmic induction of autophagy genes. We further identified C/EBP $\beta$  as a key transcription factor that links autophagy to circadian pacemaker and maintain nutrient homeostasis throughout light/dark cycles.

## Results

### **Autophagy undergoes diurnal rhythm during the day**

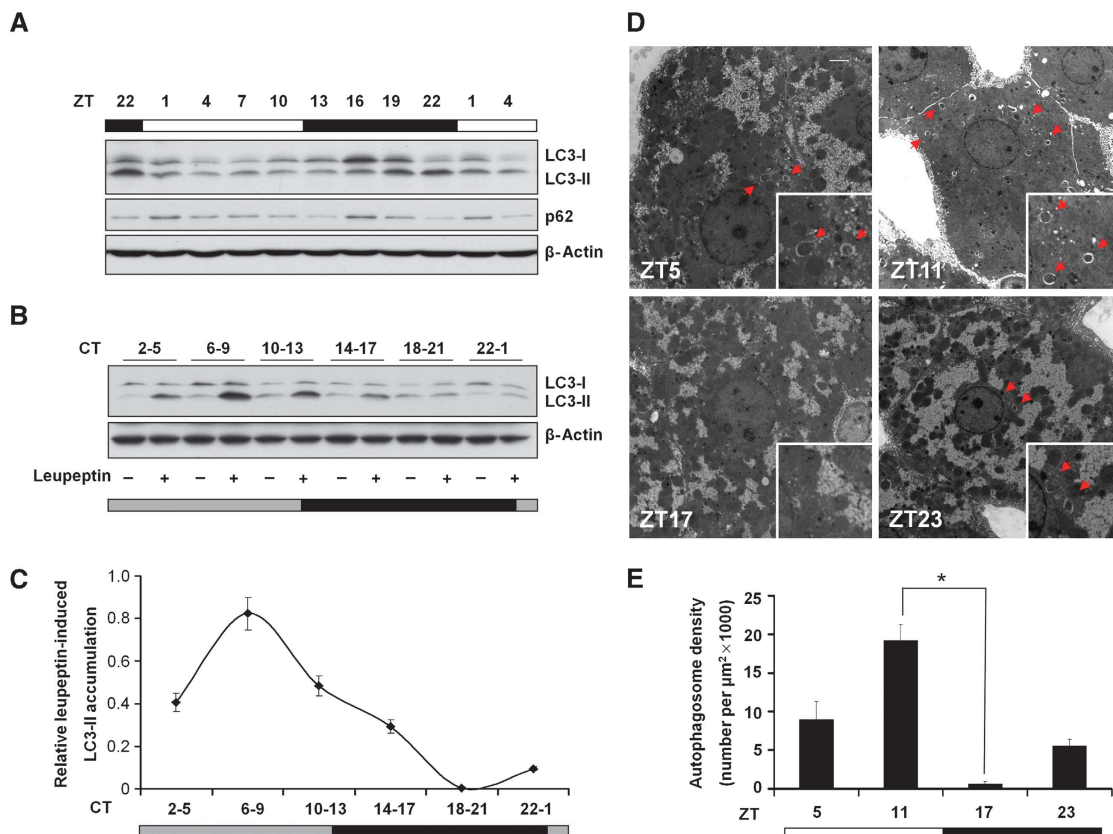
To determine whether autophagy is rhythmically activated during light/dark cycle, we examined molecular markers of autophagy, performed electron microscopy and expression profiling of autophagy-related genes. Microtubule-associated protein 1 light chain 3 (LC3) is the mammalian homologue of yeast Atg8 that undergoes conjugation to phosphatidylethanolamine upon autophagy induction (Mizushima, 2004; Klionsky *et al*, 2008). The unconjugated LC3 (LC3-I) is localized to cytosol whereas lipid-conjugated form (LC3-II) resides on autophagosome membrane. Immunoblotting ana-

lyses of mouse liver lysates harvested at different time points indicate that relative amounts of LC3-I and LC3-II exhibit a strong circadian rhythm in the liver (Figure 1A). We further examined the expression of sequestosome 1 (p62), a protein that interacts with LC3 and delivers autophagy cargos for lysosomal degradation (Komatsu *et al*, 2007). We found that p62 protein levels also undergo cyclic regulation through light/dark cycle (Figure 1A). Circadian regulation of LC3-I/II and p62 was also observed in other tissues, including skeletal muscle, heart, and kidney (Supplementary Figure S1).

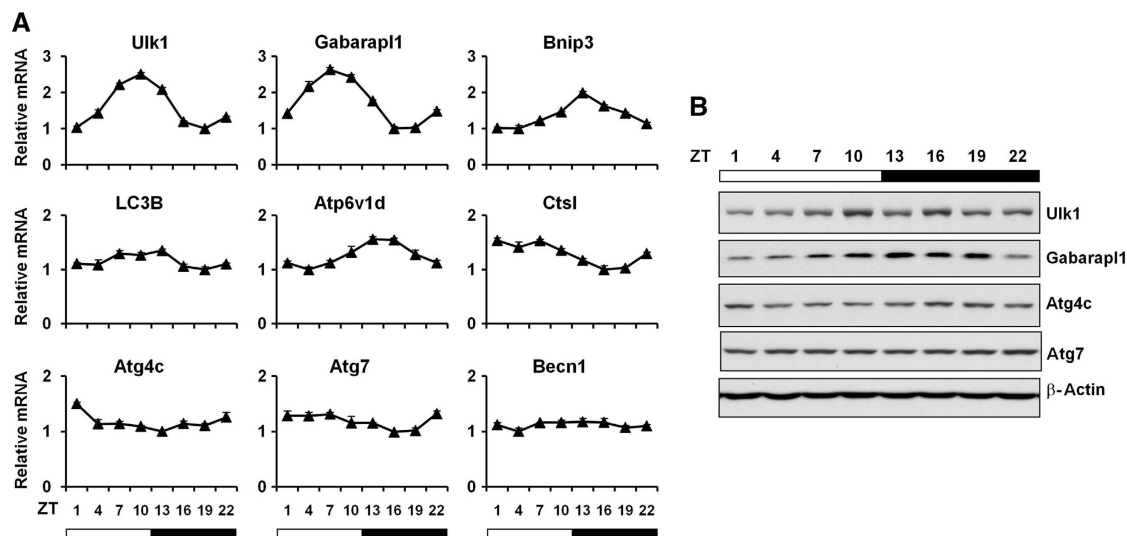
While the relative abundance of LC3-I and LC3-II is a useful marker for autophagy under certain conditions, their steady-state levels do not provide an accurate assessment of autophagy flux. A new method using leupeptin, a lysosomal protease inhibitor, was recently developed to measure autophagy flux in mouse liver (Haspel *et al*, 2011). To further clarify whether autophagy activity is circadianly regulated, we injected a single dose of PBS or leupeptin into mice kept under constant darkness and harvested tissues 3 h following injection. A total of six time points spanning one light/dark cycle was examined. As shown in Figure 1B and C, the rate of LC3-I

to LC3-II conversion peaks at CT6-9 and remains elevated at CT10-13 following leupeptin injection. In contrast, the rate of LC3 conversion was modest in the dark phase. We next performed transmission electron microscopy on liver samples to assess autophagosome formation at different time points. Consistent with the flux studies, we found that autophagosome is most abundant in the afternoon (ZT11), rapidly decreases at night (ZT17), and rise again throughout the light phase (ZT5) (Figure 1D and E). Cyclic appearance of autophagic vacuoles was also observed in previous electron microscopy studies in rat tissues (Pfeifer and Scheller, 1975; Reme and Sulser, 1977; Sachdeva and Thompson, 2008). Together, our results indicate that autophagy activity, as revealed by the rate of LC3-I to LC3-II conversion and autophagosome formation, is highly rhythmic in the liver.

We next examined whether the expression of autophagy genes is regulated by circadian signals. Quantitative PCR (qPCR) analyses indicate that several genes involved in autophagy display robust oscillation throughout the light/dark cycle, including genes involved in the autophagosome formation (Unc-51 like kinase 1 (*Ulk1*), GABA(A) receptor-associated protein like 1 (*Gabarapl1*), *LC3B*), mitophagy



**Figure 1** Rhythmic induction of autophagy in the liver. (A) Immunoblotting of liver lysates using indicated antibodies. Pooled samples from four mice were used for each time point. ZT0 and 12 represent the onset of light and dark cycles, respectively. The figure represents one of three independent sets of circadian samples. (B) Immunoblots showing LC3-II levels in the livers from mice injected with PBS (-) or leupeptin (+) 3 h before tissue harvest. Pooled samples from three mice were used for each lane. (C) Quantitation of *in vivo* autophagy flux. Following normalization to  $\beta$ -actin in (B), relative leupeptin-induced LC3-II accumulation was calculated by subtracting LC3-II levels in PBS-treated from leupeptin-treated mice for each time point. Data represent mean  $\pm$  s.d. of one representative experiment. (D) Transmission electron micrograph of liver sections at ZT5, 11, 17, and 23. The scale bar in the upper right corner of ZT5 figure represents two microns. The right lower corner shows the higher magnification highlighting a cytosolic region. Note the presence of double-membraned autophagosomes (arrow head). (E) Quantitation of autophagosome abundance in (D). Data represent mean  $\pm$  s.e. \* $P < 0.000001$  (ZT11 versus 17,  $n = 10-16$  cells). Student's *t*-test was applied.



**Figure 2** Rhythmic induction of autophagy gene expression in the liver. (A) qPCR analysis of autophagy genes at different time points of mouse livers. Pooled samples from four mice were used for each time point. Data represent mean  $\pm$  s.d. of one of three independent studies. *Becn1* serves as an example gene with modest diurnal oscillation at mRNA level. (B) Immunoblots of autophagy proteins in mouse livers at indicated time points. Pooled samples from four mice were used for each time point.

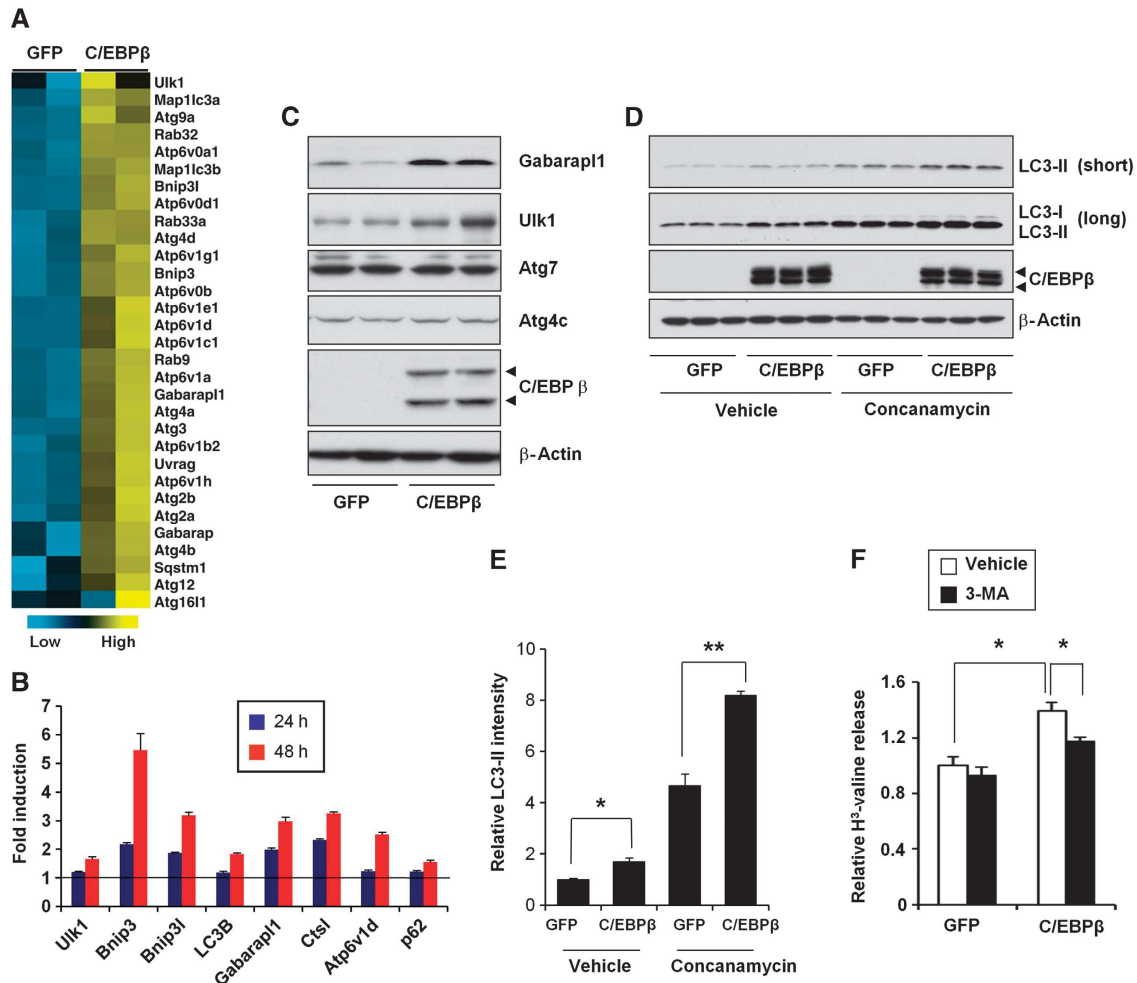
(BCL2/adenovirus E1B interacting protein 3 (*Bnip3*)), and lysosomal degradation (lysosomal protease Cathepsin L (*Ctsl*) and lysosomal ATPase subunit (*Atp6v1d*)) (Figure 2A). Cyclic mRNA expression of these genes was also observed in microarray data set of high-resolution temporal transcriptional profiling of livers from mice kept under constant darkness (Hughes *et al*, 2009; Supplementary Figure S2). Similarly, protein levels of *Ulk1* and *Gabarapl1* are rhythmically regulated (Figure 2B). In contrast, the expression of *Atg7* and beclin 1 (*Becn1*) remains similar at different time points. Pronounced rhythmic autophagy gene expression was also observed in heart, and to a lesser extent, in the skeletal muscle (Supplementary Figures S3 and S4). Recent studies demonstrate that only a small fraction of rhythmic genes is truly clock regulated (Vollmers *et al*, 2009). We next analysed temporal regulation of autophagy genes under starvation conditions to assess the relative contribution of circadian and nutritional signals. While the mRNA levels of several autophagy genes (*LC3B*, *Gabarapl1*, *Ulk1*, *Bnip3*, and *Ctsl*) remain elevated at all time points in the fasted state, they appear to retain circadian regulation, albeit with altered phase characteristics (Supplementary Figure S5).

### C/EBP $\beta$ induces autophagy gene expression and protein degradation

In the liver,  $\sim$ 10% of all transcribed genes are rhythmically expressed (Panda *et al*, 2002; Storch *et al*, 2002; Ueda *et al*, 2002). Cyclic induction of autophagy genes suggests that transcriptional regulation may play an important role in driving the circadian autophagy rhythm. While the molecular components that execute various steps of autophagy have been extensively studied, the regulatory network that governs the program of autophagy gene transcription remains poorly understood. Forkhead box O3 (*Foxo3*) has been recently implicated in the nutritional regulation of skeletal muscle autophagy (Mammucari *et al*, 2007; Zhao *et al*, 2007). However, we failed to observe robust induction of autophagy genes by *Foxo3* in cultured primary hepatocytes, suggesting that tissue-specific regulatory mechanisms are likely involved.

To identify factors that control the program of autophagy gene expression, we examined a set of transcription factors and cofactors known to regulate mammalian clock and/or hepatic starvation response, including *Bmal1*, *ROR $\alpha$* , *ROR $\gamma$* , *Crtc2*, *C/EBP $\alpha$* , *C/EBP $\beta$* , *BAF60a*, *BAF60c*, and *PPAR $\alpha$* . We transduced primary hepatocytes with adenovirus expressing individual factors and analysed autophagy gene expression and activation by qPCR and immunoblotting, respectively. These analyses revealed that *C/EBP $\beta$*  strongly stimulates the expression of autophagy genes and the appearance of LC3-II in transduced hepatocytes. In contrast, other factors have modest effects on this pathway. Microarray profiling of primary hepatocytes transduced with control (GFP) or *C/EBP $\beta$*  adenoviruses indicates that *C/EBP $\beta$*  stimulates the expression of many genes involved in different steps of the autophagy pathway, such as *Ulk1*, *Gabarapl1*, *LC3B*, and *Bnip3* (Figure 3A–C). Notably, *C/EBP $\beta$*  also induces the expression of a number of lysosomal genes, particularly subunits of the vacuolar-type H<sup>+</sup>-ATPase that is responsible for lysosomal acidification. The expression of *Atg4c* and *Atg7* appears largely unaffected by *C/EBP $\beta$* , suggesting that not all autophagy genes are subjected to transcriptional regulation.

We next examined the effects of *C/EBP $\beta$*  on autophagic protein degradation in cultured hepatocytes. Compared with control, adenoviral-mediated expression of *C/EBP $\beta$*  significantly increased LC3-II levels in transduced hepatocytes (Figure 3D and E). The induction of LC3-II is further augmented in the presence of concanamycin, a vacuolar ATPase inhibitor. The induction of LC3-II by *C/EBP $\beta$*  is abolished by 3-methyladenine (3-MA), an autophagy inhibitor, but not by proteasome inhibitor PS341 (Supplementary Figure S6). We do not observe changes in p62 protein levels, likely as a result of concomitant increase in its mRNA expression and turnover when autophagy is stimulated by *C/EBP $\beta$* . Interestingly, *C/EBP $\beta$*  increases LC3-II levels without affecting mTOR activity (Supplementary Figure S7). Further, inhibition of mTOR by *Torin1* leads to autophagy induction but has modest effects on *C/EBP $\beta$*  expression, suggesting that mTOR and *C/EBP $\beta$*  may regulate autophagy through distinct



**Figure 3** Induction of autophagy gene expression and autophagy process by C/EBP $\beta$ . (A) Clustering analysis of autophagy-related genes in primary hepatocytes transduced with GFP or C/EBP $\beta$  adenoviruses for 48 h. Blue and yellow represent low and high mRNA expression, respectively. (B) qPCR analysis of autophagy gene expression. Fold induction by C/EBP $\beta$  in primary hepatocytes 24 h (blue) or 48 h (red) following adenoviral transduction is shown. Data represent mean  $\pm$  s.d. of one representative experiment. (C) Protein expression of autophagy genes in hepatocytes transduced with GFP or C/EBP $\beta$  adenoviruses. Arrowheads point to two C/EBP $\beta$  isoforms generated from alternative translation start sites. (D) Immunoblots of total lysates from transduced primary hepatocytes treated with vehicle or concanamycin in triplicates. Blots with different exposure time were shown to illustrate LC3-I and LC3-II signals. (E) Quantitation of LC3-II protein levels following normalization to  $\beta$ -actin. Data represent mean  $\pm$  s.e. Student's *t*-test was applied. \* $P$  < 0.01; \*\* $P$  < 0.001. (F) Protein degradation assay in transduced hepatocytes in the absence (open) or presence of 3-MA (filled). Note that C/EBP $\beta$ -inducible proteolysis is sensitive to 3-MA treatment. Student's *t*-test was applied. \* $P$  < 0.05.

mechanisms. To determine whether C/EBP $\beta$  is sufficient to stimulate autophagic protein degradation, we transduced hepatocytes with GFP or C/EBP $\beta$  adenoviruses, labelled the cells with [<sup>3</sup>H]-valine for 24 h, and measured the release of radiolabelled amino acid in culture medium to estimate protein. Compared with GFP, protein degradation rate is significantly enhanced in primary hepatocytes transduced with C/EBP $\beta$  adenovirus. Further, this augmentation of protein degradation is sensitive to 3-MA treatments (Figure 3F). Together, we conclude that C/EBP $\beta$  stimulates the program of autophagy gene expression and is sufficient to enhance autophagic protein degradation.

#### C/EBP $\beta$ stimulates the transcription of autophagy genes through direct promoter occupancy

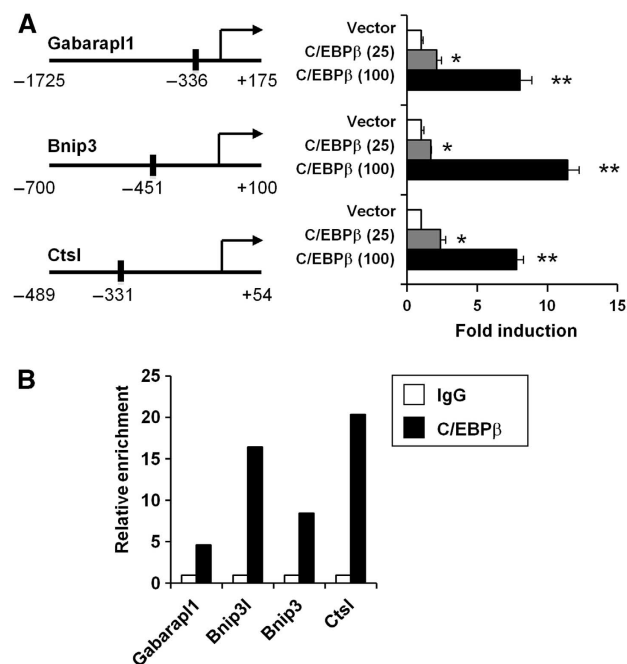
To determine whether C/EBP $\beta$  directly regulates autophagy gene transcription, we constructed luciferase reporter constructs for the proximal promoters of *Gabrarapl1*, *Bnip3*, and *Ctsl*, most highly induced genes by C/EBP $\beta$ , and

examined the effects of C/EBP $\beta$  on their transcriptional activity. Transient co-transfection of C/EBP $\beta$  strongly induces luciferase activity in reporter gene assays (Figure 4A). Motif analyses revealed several putative C/EBP $\beta$  binding sites on these promoter sequences. We next performed chromatin immunoprecipitation (ChIP) analyses to assess direct occupancy of C/EBP $\beta$  in the native chromatin environment using chromatin extracts prepared from mouse livers. Compared with IgG control, we observed robust enrichment of C/EBP $\beta$  in the proximity of predicted binding sites on *Bnip3*, *Ctsl*, and *Gabrarapl1* promoters. In addition, we also found that C/EBP $\beta$  is recruited to a C/EBP $\beta$  binding site residing in the first intron of *Bnip3l* (Figure 4B).

#### C/EBP $\beta$ is regulated by both circadian and nutritional signals

We next explored whether C/EBP $\beta$  itself is under the control of biological clock and nutritional status in mice. qPCR analysis of liver RNA isolated at different time points revealed

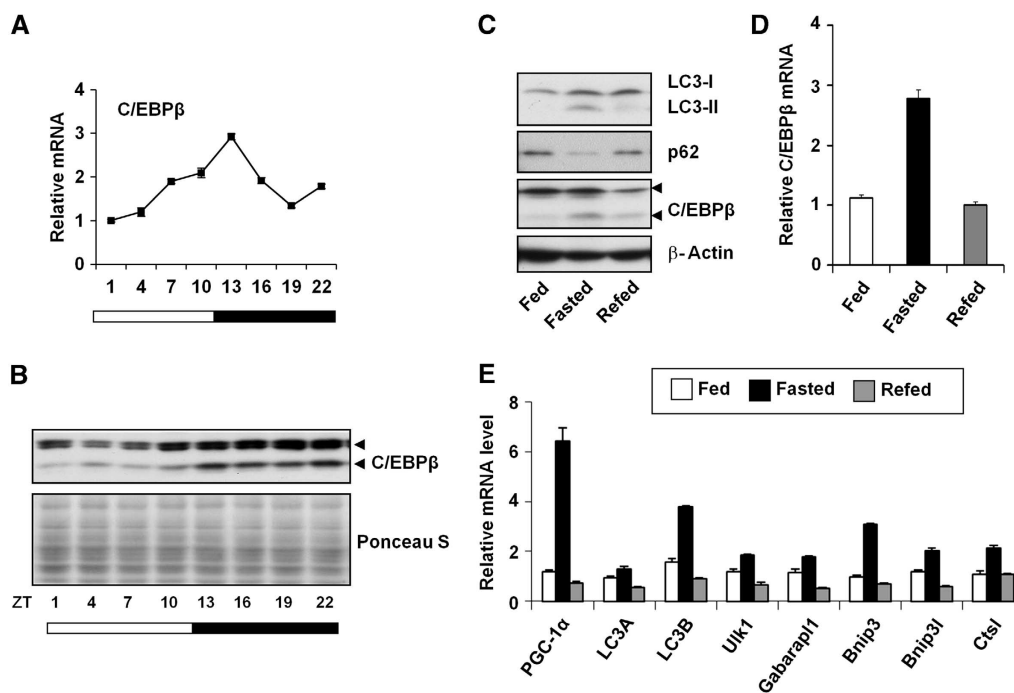
that C/EBP $\beta$  mRNA expression exhibits a robust diurnal rhythm and peaks at ZT13 (Figure 5A). Accordingly, C/EBP $\beta$  protein expression is also cyclic and reaches its



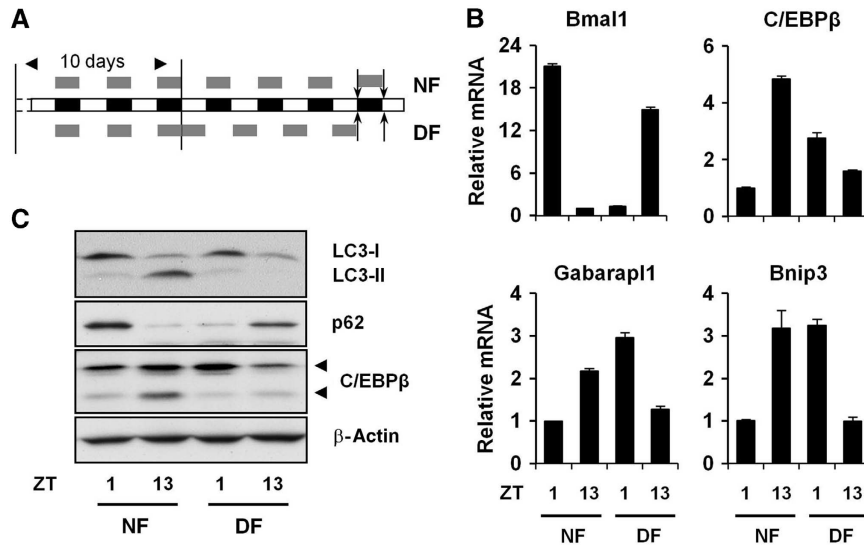
**Figure 4** C/EBP $\beta$  stimulates the transcription of autophagy genes through direct promoter occupancy. (A) Transcriptional assays using indicated promoter luciferase constructs in the presence of vector (open), 25 ng (grey), or 100 ng (filled) of C/EBP $\beta$  expression plasmid. Predicted C/EBP $\beta$  binding sites are indicated with solid bars. Data represent mean  $\pm$  s.e. Student's *t*-test was applied. \**P* < 0.01 (vector versus 25 ng C/EBP $\beta$ ); \*\**P* < 0.001 (vector versus 100 ng C/EBP $\beta$ ). (B) ChIP assay using control IgG (open) or C/EBP $\beta$  (filled) antibodies. Relative enrichment was determined by qPCR.

highest levels during the dark phase (Figure 5B). We note that the induction of several autophagy genes tends to lag C/EBP $\beta$  protein expression, likely reflecting the time needed for the accumulation of these target mRNA. In response to starvation, hepatic C/EBP $\beta$  mRNA and protein expression is significantly elevated (Figure 5C and D), accompanied by increased LC3-II and reduced p62 protein levels. In addition, mRNA expression of autophagy genes, such as *Ulk1*, *Gabarapl1*, *LC3B*, *Bnip3*, *Bnip3l*, and *Ctstl*, is also induced following starvation (Figure 5E).

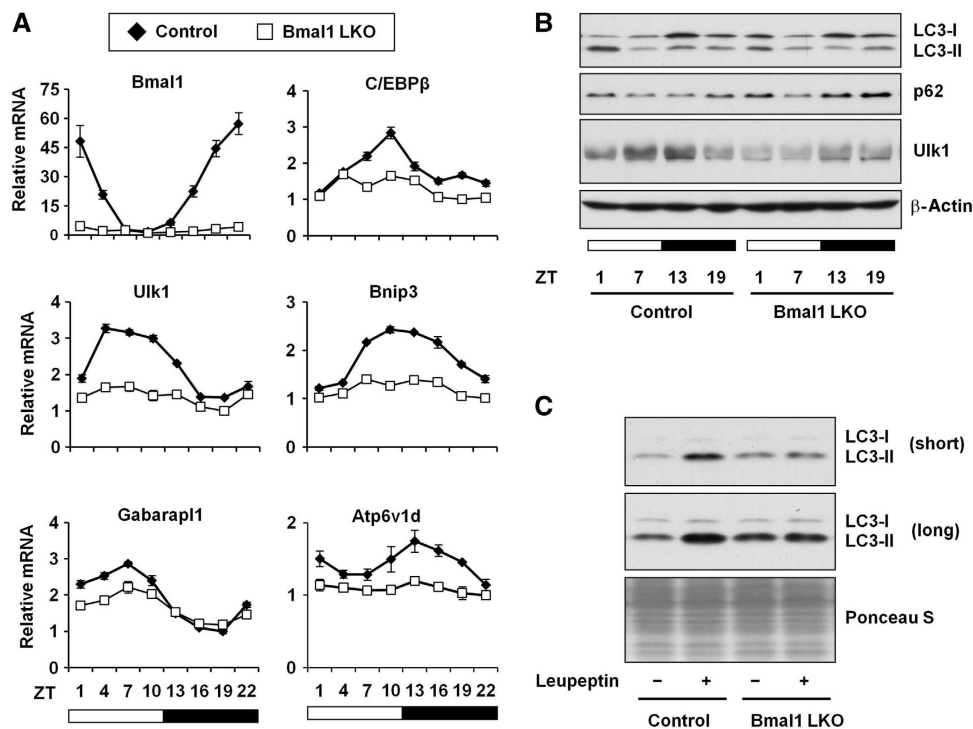
Unlike the central pacemaker residing in the suprachiasmatic nucleus, peripheral clocks are highly sensitive to feeding (Damiola *et al*, 2000). Altered meal timing, or restricted feeding, in mice results in phase resetting of peripheral clocks within several days. To examine whether the phase of C/EBP $\beta$  expression and autophagy is tightly aligned with the phase of liver clock, we subjected C/57Bl6J male mice to night feeding from ZT13 to ZT1 for a period of 10 days and switch half of the group to day feeding (DF) from ZT1 to ZT13 for an additional 4 days (Figure 6A). We harvested the livers at the end of the feeding switch and examined autophagy gene expression and autophagy markers. As shown in Figure 6B, we found that, as expected, feeding switch leads to phase resetting of the expression of *Bmal1*, a core clock gene that is indispensable for oscillator function. The expression of other clock genes also undergoes phase resetting following restricted feeding (Supplementary Figure S8). C/EBP $\beta$  expression is higher at ZT13 before food addition in the night-feeding group and this temporal pattern is completely reversed following the switch to DF (Figure 6B and C). Phase resetting of C/EBP $\beta$  expression is accompanied by a switch of autophagy genes, such as *Gabarapl1* and *Bnip3*, as well as a phase reversal of p62 protein levels. These results suggest that



**Figure 5** C/EBP $\beta$  expression is regulated by circadian and nutritional signals. (A, B) Hepatic C/EBP $\beta$  mRNA and protein expression at different time points. (C) Immunoblotting of liver lysates from fed, 24 h-fasted or 24 h-refed mice (after 24 h fast). Samples were collected at ZT4. (D, E) qPCR analysis of hepatic gene expression from fed, 24 h-fasted or 24 h-refed mice, the same group as in (C). Pooled samples from 3 to 5 mice were used for each data point. Data in (A), (D), and (E) represent mean  $\pm$  s.d.



**Figure 6** Regulation of C/EBP $\beta$  and autophagy by restricted feeding. (A) The diagram indicates restricted feeding schedule and food availability (grey box, arrows indicate tissue harvest times). (B) qPCR analysis of hepatic gene expression in mice fed during dark (NF) or light (DF) phase. (C) Immunoblotting of liver lysates from mice undergoing restricted feeding. Pooled samples from 3 to 5 mice were used for each data point. Data in (B) represent mean  $\pm$  s.d.



**Figure 7** Liver autonomous clock is required for normal autophagy rhythm. (A) qPCR analysis of hepatic genes in control (filled diamond) and liver-specific *Bmal1* knockout mice (open square, *Bmal1* LKO). (B) Immunoblots of total liver lysates in control and *Bmal1* LKO mice using indicated antibodies. (C) *In vivo* autophagy flux assay was performed at ZT6-9. Pooled samples from 3 to 4 mice were used for each group. Data in (A) represent mean  $\pm$  s.d.

C/EBP $\beta$  is downstream of circadian and nutritional signaling pathways.

To test whether diurnal regulation of C/EBP $\beta$  is dependent on a functional tissue clock, we examined hepatic gene expression in liver-specific *Bmal1* knockout mice. Hepatic *Bmal1* deficiency disrupts clock in the liver without affecting the central pacemaker (Storch *et al*, 2007; Lamia *et al*, 2008), allowing us to dissect tissue-autonomous role of the clock

oscillator on C/EBP $\beta$  and autophagy gene expression. Similar to previous studies, rhythmic expression of core clock genes is defective in liver-specific *Bmal1* knockout mice (Figure 7A; Supplementary Figure S9). Interestingly, the amplitude of C/EBP $\beta$  mRNA oscillation is greatly diminished in the *Bmal1*-deficient mouse livers. In addition, diurnal regulation of *Bnip3* and *Ulk1* mRNA expression is nearly completely abolished, whereas the amplitude of *Gabarap1* mRNA

expression is significantly reduced in the absence of Bmal1. Autophagy marker and flux assays indicate that while the steady-state levels of LC3 isoforms and p62 are modestly affected, peak autophagy flux (ZT6-9) is significantly diminished in clock-deficient mouse livers (Figure 7B and C). These results strongly suggest that cyclic expression of C/EBP $\beta$  and autophagy genes is under the control of biological clock in a tissue-autonomous manner.

### C/EBP $\beta$ is essential for physiological regulation of autophagy in the liver

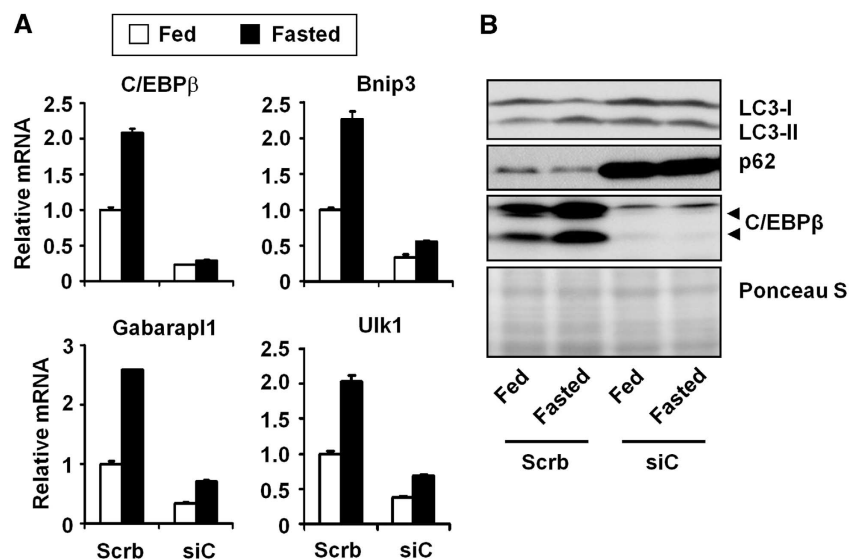
To critically assess whether C/EBP $\beta$  is required for physiological regulation of autophagy, we performed *in vivo* RNAi knockdown of C/EBP $\beta$  in the liver. We transduced mice with adenoviruses that express control shRNA or shRNA directed against C/EBP $\beta$  through tail vein injection (Cui et al, 2005). Recombinant adenoviruses efficiently and selectively transduce hepatocytes and have been widely used to knock down endogenous genes in the liver. We first examined whether C/EBP $\beta$  is required for the induction of autophagy in response to starvation. Compared with control, C/EBP $\beta$  mRNA and protein levels are significantly reduced in livers transduced with siC/EBP $\beta$  adenovirus (Figure 8). Basal and starvation-induced expression of autophagy genes, such as *Gabarapl1*, *Bnip3*, and *Ulk1*, is severely diminished when endogenous C/EBP $\beta$  is depleted. Further, p62 protein levels are drastically elevated in the knockdown liver, consistent with a blockade of autophagy in the liver of C/EBP $\beta$  knockdown mice.

We next examined whether C/EBP $\beta$  is responsible for driving the daily cycles of autophagy gene expression and autophagy process in the liver. We harvested liver from mice transduced with control or siC/EBP $\beta$  adenoviruses at four time points. As expected, rhythmic expression of C/EBP $\beta$  mRNA and protein is significantly diminished in livers transduced with siC/EBP $\beta$  adenovirus (Figure 9A and B). Gene expression analysis revealed that C/EBP $\beta$  knockdown

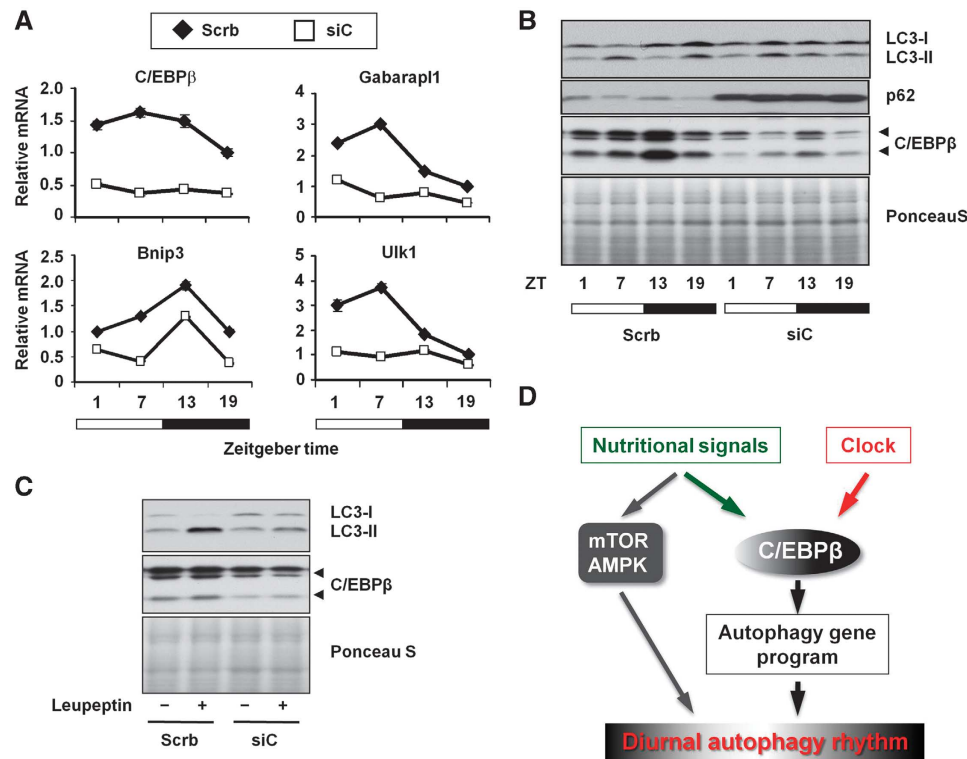
significantly perturbs circadian regulation of autophagy genes, such as *Ulk1* and *Gabarapl1* (Figure 9A). Immunoblotting analyses indicate that while p62 protein is cyclic in control livers, its levels are constitutively elevated in the knockdown livers (Figure 9B). In addition, diurnal regulation of LC3-I and LC3-II protein levels is also altered when hepatic C/EBP $\beta$  is reduced. To ascertain whether autophagy flux is reduced in response to C/EBP $\beta$  knockdown, we examined leupeptin-induced LC3-II accumulation during peak hours of autophagy (ZT6-9) and found that autophagy flux was nearly abolished in mouse livers transduced with siC/EBP $\beta$  (Figure 9C). Together, we proposed that C/EBP $\beta$  serves as a link between nutritional and circadian signals and the autophagy gene program to coordinate rhythmic activation of autophagy (Figure 9D).

## Discussion

In this study, we characterized the temporal organization of autophagy in mouse tissues and identified C/EBP $\beta$  as a key factor that orchestrates circadian autophagy rhythm in the liver. Circadian induction of autophagy was observed in mouse liver, skeletal muscle, heart, and kidney (Figure 1; Supplementary Figure S1). In the liver, autophagy flux is highest in the afternoon, rapidly decreases at night, and rises again throughout the light phase. Interestingly, the periods with low autophagy activity appear to correlate with feeding that occurs after the onset of dark phase in rodents. This correlation is consistent with the fact that autophagy activity is highly sensitive to nutritional status. In mice fasted for 24 h, hepatic expression of several autophagy genes still appears to be cyclic, suggesting that circadian timing cues also impinge on the autophagy gene program. Furthermore, liver-specific Bmal1 deficiency results in altered rhythmic expression of autophagy genes and significantly reduced autophagy flux at peak hours, indicating that the core



**Figure 8** C/EBP $\beta$  is essential for physiological regulation of autophagy in the liver. (A) qPCR analysis of hepatic genes in mice transduced with control (Scrb) or siC/EBP $\beta$  (siC) adenoviruses under fed (open) and 16 h-fasted (filled) conditions. Samples were harvested at ZT19. (B) Immunoblots of liver lysates in mice from (A) under fed and fasted conditions. Pooled samples from 3 to 5 mice were used for each data point. Data in (A) represent mean  $\pm$  s.d.



**Figure 9** C/EBP $\beta$  is essential for circadian autophagy regulation in the liver. (A) qPCR analysis of hepatic genes in mice transduced with control (Scrb, filled diamond) or siC/EBP $\beta$  (siC, open square) adenoviruses harvested at indicated time points. Data represent mean  $\pm$  s.d. (B) Immunoblots of liver lysates in mice from (A) at indicated time points. (C) *In vivo* autophagy flux assay was performed in mice transduced with control (Scrb) or siC/EBP $\beta$  (siC) adenoviruses at ZT6-9. Pooled samples from 3 to 5 mice per group were analysed. (D) Model depicting circadian autophagy regulation through C/EBP $\beta$ . Note that C/EBP $\beta$  receives both circadian and nutritional input and coordinately regulates the program of autophagy gene expression.

molecular clock is required for normal circadian regulation of autophagy.

Interestingly, robust rhythmic autophagy gene expression was only observed in the liver and heart, suggesting that autophagy is likely regulated by distinct mechanisms in peripheral tissues (Supplementary Figures S3 and S4). Previous studies have demonstrated that FoxO3 is nutritionally regulated and induces autophagy in skeletal muscle (Mammucari *et al*, 2007; Zhao *et al*, 2007). However, it only modestly increases autophagy gene expression in hepatocytes, suggesting that distinct transcriptional networks may be responsible for the regulation of autophagy in different tissues. In support of this notion, we identified bZIP transcription factor C/EBP $\beta$  as a potent activator of the autophagy gene program and autophagic protein degradation. C/EBP $\beta$  stimulates the expression of a set of core autophagy genes as well as lysosomal genes and is required for the induction of autophagy in response to starvation. Recently, transcription factor EB (TFEB) was found to regulate lysosomal biogenesis and play an important role in autophagy (Sardiello *et al*, 2009; Settembre *et al*, 2011). While the significance of TFEB in circadian autophagy remains unknown, together these studies underscore an important role of transcriptional control in physiological regulation of autophagy.

C/EBP $\beta$  appears to serve as a target of both nutritional and circadian signals in the liver. Rhythmic expression of C/EBP $\beta$  requires a functional tissue clock, suggesting that the circadian pacemaker may exert direct effects on C/EBP $\beta$  expression. Consistently, we found that rhythmic autophagy

gene expression persists in constant darkness and during starvation. As such, nutritional and circadian signals likely provide distinct cues that are integrated by C/EBP $\beta$  in physiological regulation of autophagy. The molecular mechanism underlying clock regulation of C/EBP $\beta$  remains unknown. The significance of C/EBP $\beta$  in autophagy is supported by the observations that starvation-induced autophagy gene expression and autophagy flux are impaired in response to RNAi knockdown. In addition, knockdown of C/EBP $\beta$  severely impairs autophagy gene expression and disrupts physiological regulation of autophagy throughout light/dark cycles. These studies strongly implicate C/EBP $\beta$  as a key integrator of nutritional and circadian signals that orchestrates cyclic autophagy activation in the liver.

Rhythmic activation of autophagy in mammalian tissues likely provides a steady supply of nutrients throughout the light/dark cycles. The bulk degradation of cellular components provides amino acids and lipids that serve as a critical source of fuel and substrates for biosynthetic pathways in the tissue. In addition, these nutrients also enter systemic circulation and supply metabolites for organismal energy homeostasis, such as blood glucose control. Recent studies have implicated autophagy in the regulation of hepatic lipid metabolism, adipocyte function, and the pathogenesis of insulin resistance (Singh *et al*, 2009a,b; Yang *et al*, 2010). Impaired autophagy reduces triglyceride hydrolysis in lipid droplets and may participate in the pathogenesis of hepatic steatosis. Whether C/EBP $\beta$  is involved in this context remains presently unknown. Given that disruption of circadian clock



increases the risk for metabolic disorders (Copinschi *et al.*, 2000), it is possible that aberrant temporal regulation of autophagy may contribute to altered hepatic lipid metabolism in obesity.

## Materials and methods

### Animals

All animal experiments were performed according to procedures approved by the University Committee on Use and Care of Animals. C57BL/6J male mice were fed *ad lib* and maintained in 12/12 h light/dark cycles. For circadian studies, four mice were dissected every 3 h for a period of 24 h. Tissues were immediately frozen for the preparation of protein lysates and total RNA. Bmal1 flox/flox mice were purchased from Jackson Laboratory (Stock #007668) and crossed with Albumin-Cre transgenic mice (Stock #003574) to obtain the liver-specific Bmal1 knockout mouse. In restricted feeding experiment, C57BL/6J male mice were fed exclusively at night (night feeding from ZT13 to ZT1, NF) for a total of 10 days. On day 11, the animals were divided into two groups that were kept on NF or switched to DF from ZT1 to ZT13. Animals were sacrificed at two time points (ZT1 and 13) 4 days following the feeding switch. For *in vivo* autophagy flux measurements, mice were injected intraperitoneally a single dose of PBS or leupeptin (40 mg/kg). Tissues were harvested 3 h following the injection during which food was restricted. All the procedures were performed in a red dim light for constant darkness condition. Pooled liver lysates from 3 to 5 mice were used for LC3 immunoblotting. Quantitation of LC3-II protein was performed using ImageJ (<http://rsbweb.nih.gov/ij/>). The autophagy flux rate was determined by leupeptin-induced LC3-II accumulation normalized to  $\beta$ -actin.

### In vitro and in vivo adenoviral transduction

Primary hepatocytes were transduced with recombinant adenoviruses expressing GFP or C/EBP $\beta$  with moiety of infection from ~2 to 10. For *in vivo* adenoviral transduction, C57BL/6J male mice (3–5 per group) were transduced with purified adenoviruses through tail vein injection (0.2 OD per mouse), as previously described (Li *et al.*, 2008). The titres of all adenoviruses were determined based on the expression of GFP and adenoviral gene AdE4 before use to ensure similar doses were administered in the studies.

### Protein and RNA analysis

Immunoblotting studies were performed using specific antibodies for LC3 (LC3-5F10, Nanotools), p62 (PW9860, Enzo Life Sciences), Gabarapl1 (11010-1-AP, ProteinTech Group, Inc), Ulk1 (A7481, Sigma), Atg4c (AP1810c, Abgent), Atg7 (AP1813b, Abgent), C/EBP $\beta$  (sc-150, Santa Cruz), and  $\beta$ -actin (A4700, Sigma). For inhibitor treatments, transduced hepatocytes were incubated in the presence of vehicle, 100 nM concanamycin A, 10 mM 3-MA, or 500 nM PS341 for 60 or 90 min before harvest. Hepatic gene expression was analysed by qPCR using specific primers as previously described (Liu *et al.*, 2007) or in Supplementary Table S1. Data represent mean  $\pm$  s.d. Microarray was carried out using total RNA from primary hepatocytes transduced with GFP or C/EBP $\beta$  adenoviruses for 48 h using Affymetrix mouse 430 2.0 chips and analysed using dChip software.

### ChIP assay

ChIP assay was performed essentially as described (Li *et al.*, 2008). To obtain chromatin lysates from mouse livers, liver nuclei were isolated and then crosslinked in 1% formaldehyde for 15 min followed by sonication. After being precleared with protein G agarose beads, chromatin lysates were immunoprecipitated using

antibodies against C/EBP $\beta$  (sc-150X, Santa Cruz) or control mouse IgG in the presence of BSA and salmon sperm DNA. Beads were extensively washed before reverse crosslinking. DNA was purified using a purification kit (QIAGEN) and subsequently analysed by qPCR using primers flanking the predicted binding sites on promoter or intron regions (Supplementary Table S1).

### Reporter gene assays

AD293 cells were transiently transfected with indicated plasmids using polyethylenimine (Polysciences, Inc) in triplicates. Equal amounts of DNA were used for all transfection combinations by adding appropriate vector DNA. Relative luciferase activities were determined 24 h following transfection.

### Transmission electron microscopy

Mice were first perfused with Sorensen's buffer (0.1 M, pH 7.4) and then Karnovsky's fixative buffer. The liver is post-fixed in Karnovsky's fixative buffer for 24–48 h before embedding. The sample preparation for EM was performed by Microscopy and Image Analysis Laboratory at University of Michigan. The images were taken by Philips CM-100 transmission electron microscope. The quantitation of autophagosome density was carried out on 10–16 hepatocytes per time point. The autophagosome number was counted manually, and the cytoplasmic area is measured using software ImageJ.

### Protein degradation assay

Primary hepatocytes transduced with GFP or C/EBP $\beta$  adenovirus were first labelled in valine-free DMEM containing 0.1% BSA plus 1.16  $\mu$ M [ $^3$ H] L-valine (863.0 mCi/mmol, Moravak Biochemicals, Inc) for 24 h, washed, and then chased in DMEM containing additional 10 mM unlabelled valine for 4 h. Following chase, the hepatocytes were washed and incubated in the same chase media in the presence of DMSO or 10 mM 3-MA. The culture media were collected 12 h after the treatments. Radioactive amino-acid content in culture media and total radioactivity in hepatocytes lysates were determined (Mizushima, 2004). Protein degradation rate is calculated by normalization of released [ $^3$ H] L-valine to total radioactivity in the cell.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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*Author contributions:* DM and JDL formulated the concept, designed and performed the studies, and wrote the manuscript. SP provides experimental materials.

## Conflict of interest

The authors declare that they have no conflict of interest.

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