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## Adipose tissue CLK2 promotes energy expenditure during high fat diet intermittent fasting

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### Summary

A promising approach to treating obesity is to increase diet-induced thermogenesis in brown adipose tissue (BAT), but the regulation of this process remains unclear. Here, we find that CDC-like kinase 2 (CLK2) is expressed in BAT and upregulated upon refeeding. Mice lacking CLK2 in adipose tissue exhibit exacerbated obesity and decreased energy expenditure during high fat intermittent fasting. Additionally, tissue oxygen consumption and protein levels of UCP1 are reduced in CLK2-deficient BAT. Phosphorylation of CREB, a transcriptional activator of UCP1, is markedly decreased in BAT cells lacking CLK2 due to enhanced CREB dephosphorylation. Mechanistically, CREB dephosphorylation is rescued by inhibition of PP2A, a phosphatase that targets CREB. Our results suggest that CLK2 is a regulatory component of diet-induced thermogenesis in BAT through increased CREB-dependent expression of UCP1.

### Graphical abstract

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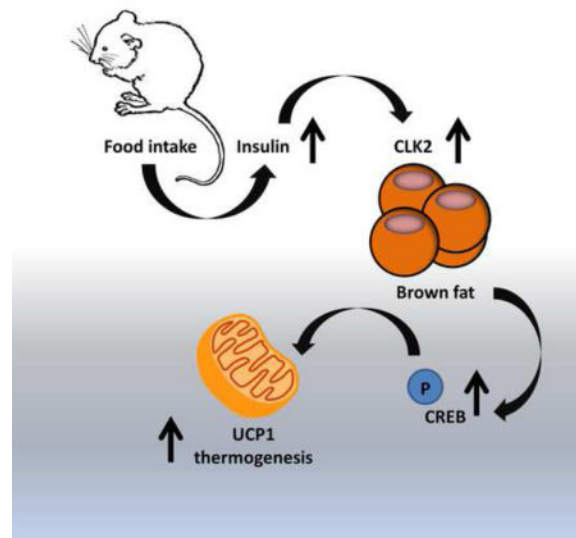
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#### Author Contributions

M.H. designed and performed experiments, analyzed data, and wrote the manuscript. C.L. and F.V. helped with the experiments and revised the manuscript. A.K.R, M.T. and J.A.H. revised the manuscript. C.T. and K.S. helped with the revision and analyzed the data. P.P. designed experiments, analyzed data and wrote the manuscript.



## Keywords

Clk2; intermittent fasting; brown fat; high fat diet; diet-induced thermogenesis; refeeding; UCP1; p-CREB; PP2A

## Introduction

Obesity is a worldwide health problem with numerous associated complications, such as cardiovascular disease, type 2 diabetes and cancer (Haslam and James, 2005). Obesity results from an imbalance between energy intake and energy consumption, leading to excessive storage of spare energy as fat in adipose tissue (Lowell and Spiegelman, 2000). Strategies to tackle obesity include reducing energy intake and increasing energy expenditure, such as through exercise. For many people, neither of these strategies are easy to pursue and relapse rates on many anti-obesity programs are high, emphasizing the need for new approaches to promote sustained weight loss (Nedergaard et al., 2007).

As an alternative to caloric restriction or as a complementary intervention, intermittent fasting (IF) comprises various diets that cycle between a period of fasting and non-fasting. IF protocols have gained increased attention as weight loss strategies, as they may produce similar results as caloric restriction in humans, and are easier to pursue for many individuals (Seimon et al., 2015). However, little is known about the physiological and metabolic changes and processes involved in IF.

Brown adipose tissue (BAT) is a highly metabolically active fat depot characterized by uncoupling protein 1 (UCP1)-positive, multilocular, thermogenic adipocytes. Unlike white fat, which is abundant and designed to store energy, BAT is found in relatively small areas of the neck and perirenal regions in adult humans, and acts to uncouple respiration to consume energy and produce heat (Peirce et al., 2014). Activation of BAT thus increases energy expenditure, and is regulated with age, outdoor temperature, and body mass index (Cypess et al., 2009). The metabolic impact of activated BAT in adult humans has been

highlighted by numerous publications in recent years (Nedergaard et al., 2007; van Marken Lichtenbelt et al., 2009).

The role of BAT activity in diet-induced thermogenesis (DIT) has been studied in rodents and humans (Cannon and Nedergaard, 2010; Lee et al., 2016; Rothwell and Stock, 1979). The purpose and extent of DIT is controversial, though the capacity for DIT is inversely associated with obesity in mice (Bachman et al., 2002), suggesting that BAT activation during DIT may dissipate energy in order to maintain body weight following excess energy intake. Data in IF mice also suggest that an increase in energy expenditure through DIT contributes to the beneficial effects observed during IF, yet the molecular and physiological regulations involved are unknown (Hatori et al., 2012). The function of BAT activation during DIT likely extends beyond simply controlling weight, as additional protective functions for BAT activation include improving glucose tolerance, increasing insulin sensitivity, and enhancing metabolism of lipids from the bloodstream, which can all improve systemic metabolism in the face of increased caloric load (Harms and Seale, 2013).

Mechanistically during DIT, in response to feeding, the sympathetic nervous system is activated, causing stimulation of beta-adrenergic receptors ( $\beta$ ARs) in the plasma membrane of brown adipocytes. Adenyl cyclase is then activated, producing cyclic AMP (cAMP). cAMP promotes the activity of protein kinase A (PKA), also known as cAMP-dependent protein kinase. PKA signaling promotes lipolysis as well as upregulation and activation of UCP1, a mitochondrial proton carrier that increases thermogenesis by uncoupling oxidative phosphorylation (van Marken Lichtenbelt et al., 2009). The contribution of beta-adrenergic signaling to DIT has been shown in mice lacking all three beta adrenergic receptors ( $\beta$ -less mice) (Bachman et al., 2002). Brown adipocytes in these animals are thermogenically compromised and have decreased UCP1 protein, and the mice develop massive obesity on a high calorie diet due to reduced energy expenditure induction. Additional regulatory components of the feeding response in BAT that contribute to diet-induced energy expenditure have not been well-studied.

Cdc2-like kinase 2 (CLK2) is a dual-specificity kinase, which belongs to the evolutionarily conserved Clk-family (Hanes et al., 1994). We recently reported that CLK2 is regulated by the liver fed/fasting response. CLK2 responds to feeding and high fat diet and controls hepatic gluconeogenesis and the ketogenic fasting response (Rodgers et al., 2010; Tabata et al., 2014). In response to feeding, CLK2 is stabilized by Akt activation and directly hyperphosphorylates peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ) at the SR domain to repress its activity toward target genes (Rodgers et al., 2010). CLK2 has also been reported to play a role in SR protein recruitment and pre-mRNA splicing (Corkery et al., 2015). In addition, hepatic CLK2 interacts with regulatory subunits of the protein phosphatase 2A (PP2A) complex. We reported that CLK2 promotes the assembly of this phosphatase enzymatic complex in liver. CLK2 phosphorylates the PP2A regulatory subunit B56 $\beta$  (PPP2R5B, B' $\beta$ ), which is a critical regulatory step in the assembly of the PP2A holoenzyme complex on Akt leading to dephosphorylation and attenuation of Akt activity (Rodgers et al., 2011). Although the contribution of CLK2 to metabolic control in the liver has been addressed, the role of adipose CLK2 in energy balance is unknown.

Here, we investigate the function of adipose CLK2 in energy expenditure and BAT thermogenic activity. Mice lacking CLK2 in adipose tissue have defective DIT in BAT and an increase in body weight when subjected to high fat diet under an IF dietary regimen. CLK2 is activated upon refeeding in BAT and increases energy expenditure through sustained activation of CREB transcriptional activity and increased UCP1 expression. The CLK2 effects on CREB phosphorylation are mediated through attenuation of CREB inhibitory dephosphorylation by PP2A protein phosphatase. These results indicate that BAT CLK2 increases diet-mediated energy expenditure and protects against obesity.

## Results

### CLK2 is enriched in BAT and upregulated upon refeeding

To assess the potential regulatory role of CLK2 in adipose tissue, we first analyzed the expression of CLK2 mRNA transcripts in different fat depots (Figure 1A). CLK2 mRNA levels were enriched in interscapular BAT compared to subcutaneous inguinal white adipose tissue (iWAT) and visceral epididymal white adipose tissue (eWAT) depots (Figure 1A). Similar to our previous findings in the liver, CLK2 mRNA levels in BAT did not change upon fasting or refeeding (Figure 1B), and other members of the CLK family were not regulated on the mRNA level upon fasting and refeeding (Figure S1). However, CLK2 protein levels were increased during refeeding (Figure 1B). Insulin increased CLK2 protein level in differentiated primary brown adipocytes after 8 hours, with attenuation of CLK2 expression at 16 hours (Figure 1C). This effect was abolished upon pretreatment with the PI3K Inhibitor LY294002, suggesting a mechanism downstream of PI3K signaling. In addition, inhibiting protein translation with cycloheximide did not prevent the increase in CLK2 protein upon insulin treatment. Finally, treatment with insulin and the proteasome inhibitor MG132 sustained the increase in CLK2 protein levels after 16 hours, suggesting that proteasomal degradation contributes to CLK2 protein accumulation (Figure 1C). Taken together, these results indicate that CLK2 protein levels are increased upon insulin signaling and degraded via the proteasome, suggesting a mechanism of CLK2 regulation that is similar as previously described (Rodgers et al., 2010). These results demonstrate that CLK2 is regulated at the protein level in BAT in response to feeding, and that it may contribute to metabolic and energetic processes associated with food intake.

### Adipose-specific CLK2 KO mice exhibit decreased energy expenditure upon IF

To determine the role of CLK2 in whole body energy balance, we generated two different strains of CLK2-deficient mice. We used the CLK2 floxed (*Clk2<sup>f/f</sup>*) mice that have been previously described (Tabata et al., 2014) to obtain a CLK2 constitutive total body knockout mouse (*Clk2<sup>Δ</sup>KO*) (Lewandoski et al., 1997). These mice were used to isolate primary cells for adipocyte differentiation *in vitro*. CLK2 mRNA and protein were undetectable in primary differentiated brown adipocytes derived from these animals compared to *Clk2<sup>f/f</sup>* wild type control mice (Figure 2A), and there was no compensatory regulation of other CLK family members (Figure S2A). For *in vivo* phenotyping studies, we generated a second strain of mice by crossing *Clk2<sup>f/f</sup>* mice with adiponectin-driven Cre recombinase (Adiponectin-Cre-EVDR/J) mice to delete CLK2 in adipose tissue (*Clk2<sup>a</sup>KO*). Levels of

CLK2 mRNA were markedly reduced in all adipose depots of these animals (Figure 2B) relative to *Clk2<sup>f/f</sup>* control mice.

The fact that CLK2 is enriched in brown fat suggests that this kinase might contribute to thermogenesis. However, no differences in body weight or core body temperature after short-term cold exposure were observed in *Clk2*KO mice (Figure S2B and C). To further challenge these *Clk2*KO mice, they were fed with high fat diet for a period of three months. *Clk2*KO mice had a modest but consistent increase in body weight gain over time compared to *Clk2<sup>f/f</sup>* mice (Figure 2C). A comprehensive lab animal monitoring system (CLAMS) was used to assess parameters linked to energy balance in these mice. *Clk2*KO mice had a slight reduction in total oxygen consumption that did not reach statistical significance (Figure 2D). However, when subjected to refeeding after a 24 hour fast (Figure 2E), *Clk2*KO mice showed a marked decrease in oxygen consumption during the refeeding period (Figure 2F) without a change in food intake or movement (Figure S2D–G).

Since whole body energy expenditure was decreased in *Clk2*KO mice upon HFD refeeding, we subjected the mice to a dietary regimen of HFD combined with IF (Hatori et al., 2012), allowing access to food only during the active (night) phase for a period of twelve weeks (Figure 3A). The rationale was to use a dietary model of increased food caloric intake that causes elevated rates of energy expenditure. The IF regimen synchronizes and restricts daily eating times and causes an increase in energy expenditure and a decrease in body weight compared to *ad libitum* HFD (Hatori et al., 2012). As expected, the *Clk2<sup>f/f</sup>* mice gained less weight during IF (Figure 3B) than when fed *ad libitum* (Figure 2C). Although the *Clk2*KO mice also gained less weight than when fed *ad libitum*, the IF regimen uncovered an increased propensity for obesity, with the *Clk2*KO mice gaining significantly more weight than the *Clk2<sup>f/f</sup>* mice (Figure 3B). Blood glucose and insulin levels were slightly but non-significantly increased in *Clk2*KO mice compared to *Clk2<sup>f/f</sup>* mice (Figure 3C and D). Consistent with this increase in body weight, whole body oxygen consumption was reduced in *Clk2*KO mice (Figure 3E), without any difference in food intake or movement (Figure S3A–C). Of note, IF lead to slightly decreased food intake in both genotypes compared to *ad libitum* feeding, but with no difference between control and *Clk2*KO mice (Figure S3D). These results indicate that adipose tissue CLK2 is necessary to fully increase energy expenditure in the IF dietary regimen and maintain reduced body weight.

### CLK2 decreases BAT oxygen consumption cell autonomously

BAT thermogenic activity increases in response to high calorie diets to dissipate excess energy and ameliorate body weight gain (Harms and Seale, 2013; Rothwell and Stock, 1979). Since *Clk2*KO mice exhibited reduced energy expenditure during different high fat dietary regimens, we investigated whether oxygen consumption was altered specifically in BAT from WT and *Clk2*KO mice using *ex vivo* tissue analysis. Minced BAT from WT and *Clk2*KO fasted (24h) and refed (18h) mice (Figure 4A) were used to measure oxygen consumption (Figure 4B). Consistent with the CLAMS results (Figure 2F), oxygen consumption rates were significantly lower in CLK2-deficient brown fat tissue (Figure 4B). In contrast, there was no difference in oxygen consumption in iWAT or eWAT, or in BAT after fasting (Figure S4A–C). These results indicate that the decrease in energy expenditure

of *Clk2aKO* mice stems from a cell autonomous defect in brown adipocyte respiration after refeeding.

### Loss of CLK2 in brown adipocytes causes a marked reduction in UCP1 expression

One of the main thermogenic mechanisms in brown adipocytes involves mitochondrial uncoupled respiration that bypasses ATP synthesis and dissipates heat. A key component of this mechanism is uncoupling protein 1 (UCP1), an inner mitochondrial proton carrier that decreases membrane potential and conductance (Lowell and Spiegelman, 2000). To assess whether the decreased energy expenditure in *Clk2aKO* mice or oxygen consumption in CLK2-deficient brown adipocytes was associated with repressed expression of UCP1, we first measured UCP1 mRNA transcripts and protein levels in BAT from mice exposed to different dietary treatments. UCP1 mRNA was reduced in BAT from lean *Clk2aKO* mice after refeeding (Figure 4C) and after 12 weeks of IF compared to controls (Figure 4E). In alignment with this finding, protein levels for UCP1 were also decreased in CLK2-deficient mice (Figure 4D and 4F). However, despite profound changes in UCP1 protein, no changes were observed in mitochondrial mass (Figure S4D–F). These results suggest that BAT CLK2 controls the transcription of UCP1 but has no effect on mitochondrial biogenesis, as markers of mitochondrial mass remain largely unaffected.

Next, to determine if UCP1 mRNA and protein changes in BAT are cell autonomous, we isolated primary brown adipocytes from *Clk2aKO* mice. CLK2-deficient brown adipocytes had decreased UCP1 mRNA and substantially reduced UCP1 protein levels (Figure 5A and B). However, the degree of differentiation analyzed by measurement of specific differentiation markers including aP2, adiponectin, and PPAR $\gamma$  was similar between the two genotypes (Figure 5B). In addition, no differences were observed in markers of mitochondrial mass (Figure 5C). Although basal levels of UCP1 were reduced in CLK2-deficient brown adipocytes, fold induction of its mRNA and protein upon norepinephrine stimulation was similar between control and *Clk2aKO* cells (Figure 5D and E), demonstrating intact adrenergic signaling in the *Clk2aKO* cells.

To further support the effects of CLK2 on UCP1 gene expression, primary brown adipocytes derived from *Clk2<sup>f/f</sup>* or *Clk2aKO* mice were infected with adenoviruses encoding wild type CLK2 or a kinase inactive mutant (K192R), as described previously (Rodgers et al., 2010; Rodgers et al., 2011). CLK2 ectopic expression rescued the levels of UCP1 mRNA levels in CLK2-deficient brown adipocytes, but the K192R mutant did not affect UCP1 transcript levels (Figure 5F). Lastly, using a Seahorse Extracellular Flux Analyzer, the oxygen consumption rate of primary adipocytes lacking CLK2 was reduced (Figure 5G). These results suggest that CLK2 increases UCP1 protein and mRNA levels in brown adipocytes in a cell-autonomous manner.

### Brown adipose CLK2 deficiency causes PP2A-dependent CREB dephosphorylation

One of the major regulatory signals that controls energy expenditure in BAT is the canonical cAMP/PKA pathway, which is activated in response to cold or increased food intake (Bachman et al., 2002). A major effector of this response is UCP1, which is regulated through this pathway partially at the transcriptional level (Bachman et al., 2002; Lowell and

Spiegelman, 2000). To determine whether components of the cAMP/PKA pathway are involved in CLK2-dependent control of UCP1 expression, we first measured intracellular levels of cAMP. Basal or forskolin-induced cAMP levels were similar in *Clk2<sup>f/f</sup>* and *Clk2<sup>ΔKO</sup>* brown adipocytes (Figure 6A). Since adrenergic signaling stimulates lipolysis during energy deficits (Peirce et al., 2014), we also measured lipolysis in primary differentiated adipocytes obtained from subcutaneous fat. Isoproterenol treatment induced similar levels of glycerol release and similar induction of lipolytic genes, suggesting that the lipolytic response is not affected in the absence of CLK2 (Figure S5A and B). Since the UCP1 promoter contains cAMP regulatory elements that bind to CREB (Koo et al., 2005; Lowell and Spiegelman, 2000), we measured levels of phospho-CREB (the activated form) after forskolin treatment in *Clk2<sup>f/f</sup>* and *Clk2<sup>ΔKO</sup>* brown adipocytes. Similar levels of phospho-CREB were observed upon these conditions in the presence or absence of CLK2 (Figure 6B), suggesting that initial increases in CREB phosphorylation are not affected by CLK2.

However, we found a decrease in CREB phosphorylation in BAT of *Clk2<sup>ΔKO</sup>* mice after IF compared to WT controls (Figure 6C), suggesting that the rate of CREB dephosphorylation could be involved in the regulation of energy expenditure by Clk2. CREB dephosphorylation is a mechanism to attenuate the cAMP response. Dephosphorylation occurs, depending on cell types and conditions, between 30 minutes and several hours after the initial stimulation (Koo et al., 2005). Notably, we found that lack of CLK2 in brown adipocytes decreased levels of phospho-CREB during the attenuation phase (Figure 6D, Figure S5C). This result suggests that CLK2 might affect the CREB phosphatase that dephosphorylates phospho-CREB at Ser 177 (Wadzinski et al., 1993). Since we previously found that hepatic CLK2 affects PP2A activity towards Akt, we used the PP2A inhibitor okadaic acid to suppress PP2A catalytic activity in WT and *Clk2<sup>ΔKO</sup>* brown adipocytes. In the absence of okadaic acid, and after 1 hour of forskolin treatment, CLK2-deficient brown adipocytes increased the levels of phospho-CREB similar to WT cells (Figure 6D). However, a strong reduction in phospho-CREB was observed in *Clk2<sup>ΔKO</sup>* cells after 4 hours. The presence of okadaic acid completely suppressed phospho-CREB reduction at 4 hours in CLK2-deficient cells (Figure 6D), suggesting that PP2A inhibition is involved in Clk2-mediated CREB phosphorylation. Treatment with okadaic acid also rescued UCP1 gene expression in *Clk2<sup>ΔKO</sup>* cells (Figure S5D). To test whether increased CREB phosphorylation in the presence of CLK2 leads to increased transcriptional activity, we also performed a luciferase reporter assay using a cAMP-responsive binding element reporter. Consistent with the effects on CREB phosphorylation, CLK2 strongly increased luciferase activity, while the kinase dead CLK2 mutant K192R or a GFP control plasmid had no effect on the activity (Figure 6E). Taken together, these data show that CLK2 in brown adipocytes does not affect cyclic AMP signaling induction, but attenuates the termination of this signal, likely by inhibiting CREB dephosphorylation and maintaining increased CREB transcriptional activity.

## Discussion

In these studies, we show that CLK2 controls the thermogenic process in BAT and regulates energy expenditure. CLK2 expression is enriched in brown compared to white fat, and CLK2 is upregulated upon refeeding at the protein level. Mice lacking CLK2 in adipose

tissue show decreased energy expenditure, mainly through attenuated BAT oxygen consumption. This reduced respiration rate impairs the anti-obesity effects of a time-restricted feeding regimen. Brown adipocytes lacking CLK2 exhibit decreased levels of UCP1, which may account, at least in part, for the decrease in respiration. Furthermore, CLK2-deficient cells show decreased levels of active phospho-CREB, indicating that CLK2 increases CREB transcriptional activity in BAT. Inhibition of PP2A phosphatase, which targets and inactivates CREB, rescues CREB phosphorylation in CLK2-deficient cells. Overall, these results indicate that CLK2 contributes to DIT, particularly in intermittent HFD fasting regimens.

Increased attention has been paid to IF regimens in humans, and studies have shown a similar effect on weight loss compared to caloric restriction (Seimon et al., 2015). A concept widely accepted and accountable for weight loss on time restricted feeding is DIT (Cannon and Nedergaard, 2010; Rothwell and Stock, 1979). Our results propose that CLK2 regulates DIT. Interestingly, CLK2 appears not to be involved in the initial activation of BAT through beta adrenergic signaling, but CLK2 action prolongs the effects on the thermogenic transcriptional program. Our findings showing that CLK2 affects energy expenditure at late time points of refeeding (12–24h) are consistent with a model that BAT CLK2 contributes to DIT. Thus, CLK2 does not play a role in the vital circuits maintaining body core temperature, but permits a sustained activation of brown adipose thermogenic response during nutrient excess. This allows the body to invest in effective thermogenesis in phases of caloric overload, but not during times of low energy intake. This model is supported by our results that CLK2 deficiency has no effect on cold-induced thermogenesis, and that CLK2-deficient cells are able to induce UCP1 protein and mRNA upon stimulation with norepinephrine.

We and others have previously shown that feeding increases CLK2 in insulin-sensitive tissues such as the liver (Nam et al., 2010; Rodgers et al., 2010; Rodgers et al., 2011; Tabata et al., 2014). In hepatocytes, CLK2 is directly phosphorylated by AKT and auto-phosphorylated at several residues, leading to stabilization of the protein (Nam et al., 2010; Rodgers et al., 2010). Our results suggest a similar mechanism of CLK2 activation in BAT. However, we did not detect this regulation in white adipose depots, likely due to low levels of CLK2 protein. Consistent with this, we did not observe any changes related to energetic processes in white adipose depots of CLK2aKO compared to WT mice. However, future studies with BAT-specific Clk2 knockout would be informative to clarify this specificity.

Not much is known about the termination of the CREB Ser133 phosphorylation signal, which leads to lower transcriptional activity. It has been reported that PKA-phosphorylated CREB is a substrate of PP2A (Wadzinski et al., 1993). In hepatocytes, CLK2 affects the PP2A holoenzyme complex assembly by phosphorylating a regulatory subunit, thereby regulating its phosphatase activity on phosphorylated AKT (Rodgers et al., 2011). We have shown that CREB phosphorylation is attenuated over time in CLK2-deficient cells and that the levels of phospho-Creb are lower in BAT of mice deficient in CLK2. As total CREB protein levels and initial CREB phosphorylation are not affected, a role for CLK2 in the termination of CREB activation is likely. CLK2 is known to mediate the activity of PP2A, which can dephosphorylate phospho-CREB (Cohen, 1989), and PP2A inhibition completely



rescued decreased phospho-CREB levels in CLK2-deficient primary adipocytes. We have previously shown in hepatocytes that CLK2 increases PP2A activity toward AKT (Rodgers et al., 2011). However, this effect appears to be tissue-specific, because we did not detect any changes in BAT AKT phosphorylation, suggesting cell type-specific regulation of CLK2 activity toward the PP2A holoenzyme complex. It can be speculated that CLK2 in BAT interferes with the formation of a PP2A complex that preferentially targets phospho-CREB. The precise mechanisms of how CLK2 controls PP2A and CREB phosphorylation are unclear at this point, but it is likely that CLK2 phosphorylates components of the PP2A regulatory subunits providing specificity to the phosphatase catalytic activity.

In summary, our studies indicate that CLK2 mediates energy expenditure through increases in BAT oxygen consumption in postprandial fed states. Notably, these increases in energy expenditure are sufficient to maintain a decreased body weight upon a HFD IF dietary regimen. The fact that CLK2 is regulated by insulin and specifically controls the BAT thermogenic process in response to specific time-restricted food intake dietary patterns opens the possibility of developing drug-targeted therapies using this kinase or components of this pathway. BAT-specific targeting of the Clk pathway has the potential to increase energy expenditure and recapitulate the beneficial weight loss effects of IF, thereby revealing a potential anti-obesity therapeutic strategy.

## Experimental Procedures

### Animal experiments

All experiments and protocols were approved by the Institutional Animal Care and Use Committees of Dana-Farber Cancer Institute or Beth Israel Deaconess Medical Center. *Clk2*-AdipoCre mice (*Clk2aKO*) and *Clk2* Zp3-Cre mice (*Clk2λKO*) were generated by breeding animals harboring a floxed *Clk2* allele with transgenic mice expressing Adiponectin-Cre recombinase or *Zona pellucida*-Cre, respectively (Jeffery et al., 2014; Lewandoski et al., 1997). Mice were maintained on standard chow or 60% high fat diet (Research Diets) with 12h dark/light-cycles. For IF studies, access to food was regulated by transferring mice daily between cages with food and water and cages with water only according to a previously published protocol (Hatori et al., 2012). For cold exposure experiments, mice were placed in 4°C incubators at indicated time points, and body temperature was measured with a rectal probe. For metabolic studies, energy expenditure was analyzed using a Comprehensive Lab Animal Monitoring System (Columbus Instruments). Mice were acclimated for 24h before measurements were taken. For *in vivo* oxygen consumption, mean oxygen consumption per hour of each individual mouse was determined and calculated for a 24h or 12h period, respectively. A representative 24h interval was chosen to calculate oxygen consumption and for fasting or feeding periods, and the last 12 hours of a 24h period was used for the calculation.

### Food intake measurements

Food intake was measured in metabolic cages continuously by feeders connected to a scale and recorded by the system. For long term studies, mice were housed individually and food

intake was measured weekly weighing the food before and after the measurement period. Food intake per day was calculated from the data obtained.

### Oxygen consumption

Direct *ex vivo* tissue respiration was performed using a Clark electrode (Strathkelvin Instruments). Freshly isolated tissue was minced in respiration buffer (1.5 mM Pyruvate, 25 mM Glucose and 2% BSA) and placed in electrode chambers. O<sub>2</sub> consumption rate was normalized to tissue weight.

Oxygen consumption in differentiated adipocytes was measured using the Seahorse XF24 Extracellular Flux Analyzer. Cells were plated and differentiated on Seahorse XF24 V7 plates. Prior to the assay, the cell media was changed to DMEM media lacking NaHCO<sub>3</sub> and containing 25 mM glucose and 1 mM sodium pyruvate, with pH adjusted to 7.4. Oxygen tension was measured and normalized to protein content in each well.

### Primary adipocytes cell culture

The stromal vascular fraction (SVF) of brown adipose tissue of 6 weeks old mice was isolated by collagenase digestion followed by two alternative filtrations steps (using 100 and 40 µM strainers) and centrifugations during 5 min at 500 × (g). Then, cells were plated and differentiated upon confluency with an adipogenic cocktail (0.5 mM IBMX, 1 µM Dexamethasone, 1 µM Rosiglitazone, 0.02 µM Insulin and 1 nM T3) for 48h. The cells were maintained in 0.02 µM Insulin and 1 nM T3 and harvested at day 6–8 post differentiation.

### Treatment of primary adipocytes

Prior to treatment with insulin, cells were preconditioned in medium containing serum, but no insulin overnight. Insulin was added to the medium at indicated concentrations. For PI3K inhibitor studies, LY294002 (Tocris Bioscience) was added to the medium 16 hours before the treatment at 50 µM concentration. Cycloheximide (Cell Signaling) was used at 50 µg/ml and added at the beginning of the assay. Proteasome inhibitor MG132 (Cell Signaling) was added at 10 µM concentration at the beginning of the assay.

Forskolin (Sigma Aldrich) and norepinephrine (Sigma Aldrich) were used at indicated concentrations. Prior to any treatment, fresh medium was added 1 hour in advance.

### Gene expression and Western Blot analysis

Total RNA from cultured cells or tissues was purified using TRIzol (Invitrogen) for cDNA synthesis (ABI High Capacity Reverse Transcription Kit). Relative mRNA expression was quantified by qPCR using SYBR Green dye (ABI) and specific primers (see Table S1 for primer sequences). For Western Blotting, whole cell lysates were prepared with RIPA buffer, separated by SDS-PAGE and transferred to ImmobilonP membranes (Millipore). The following antibodies were used: anti-CLK2 (NeoBioLab), anti-UCP1 (Abcam), pCREB (Cell Signaling), total CREB (Cell Signaling), anti-Tubulin (Millipore), anti-Lamin (Abcam), anti-Actin (Cell Signaling), anti-Porin (Abcam) anti-pAKT S473 (Cell Signaling), and anti-Pan AKT (Cell Signaling).

## Mitochondrial DNA measurement

DNA was isolated from tissues or cells using a kit according to the manufacturers instruction (DNeasy Blood & Tissue Kit, Quiagen). DNA concentration was adjusted and qRT PCR was performed using primers for a genomic locus (18s) and a mitochondrial locus (Cox1). Relative mitochondrial DNA amount was calculated as a ratio of genomic versus mitochondrial DNA.

## Lipolysis assay

Determination of free glycerol was performed as described (Eguchi et al., 2011). In brief, primary adipocytes were washed twice with DMEM and then incubated for 6 h in serum-free DMEM containing 1% fatty acid-free BSA in the presence or absence of 10  $\mu$ M isoproterenol. Conditioned medium was then analyzed for glycerol using the Free Glycerol Determination Kit (Sigma). Adipocytes were then rinsed with PBS and harvested in RIPA buffer. The protein content of cell lysates was determined using the DC Protein Assay kit (BioRad).

## Constructs

Plasmids and adenoviral constructs of CLK2 and the kinase-dead K192R control were used as previously published (Rodgers et al., 2010).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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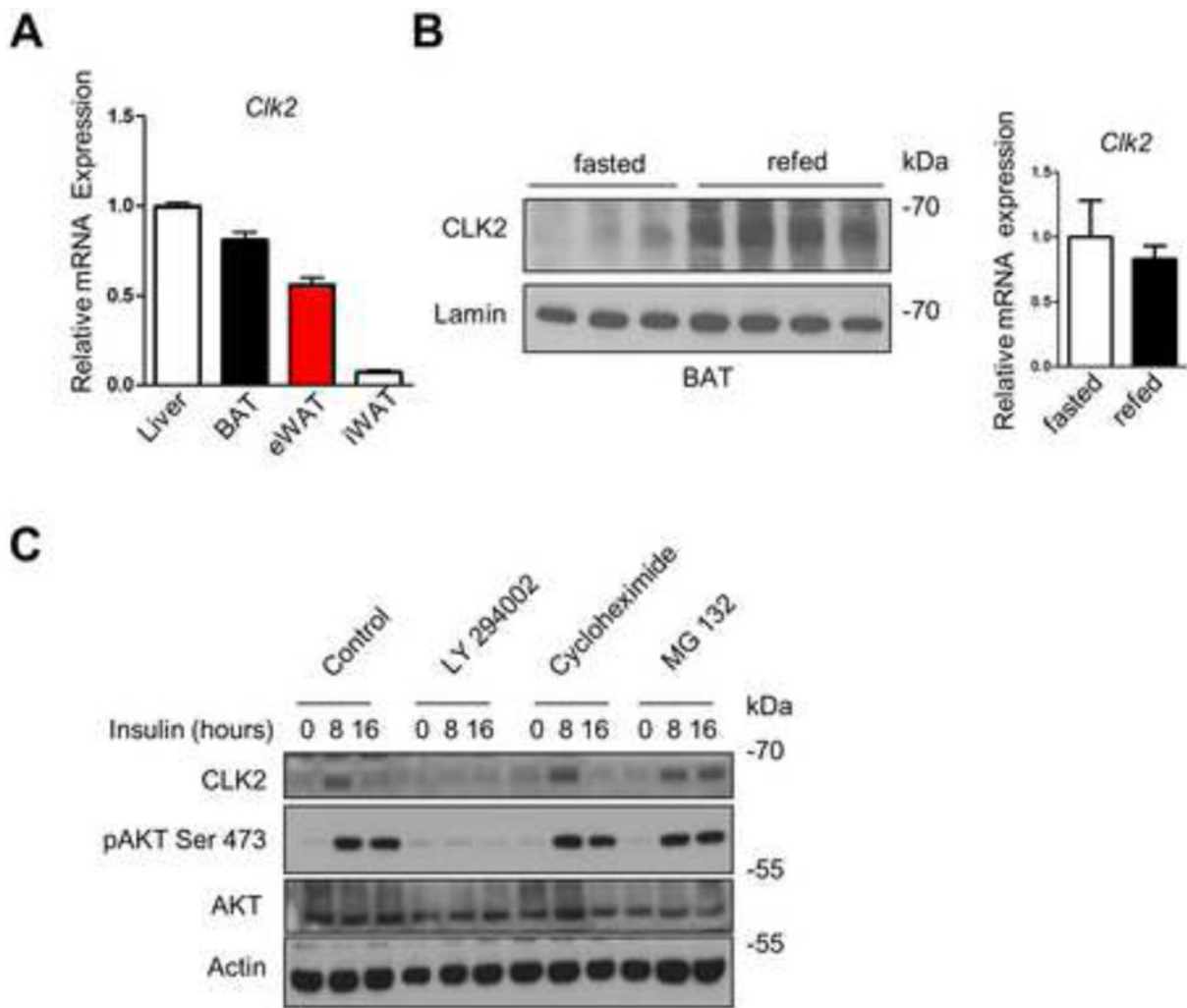
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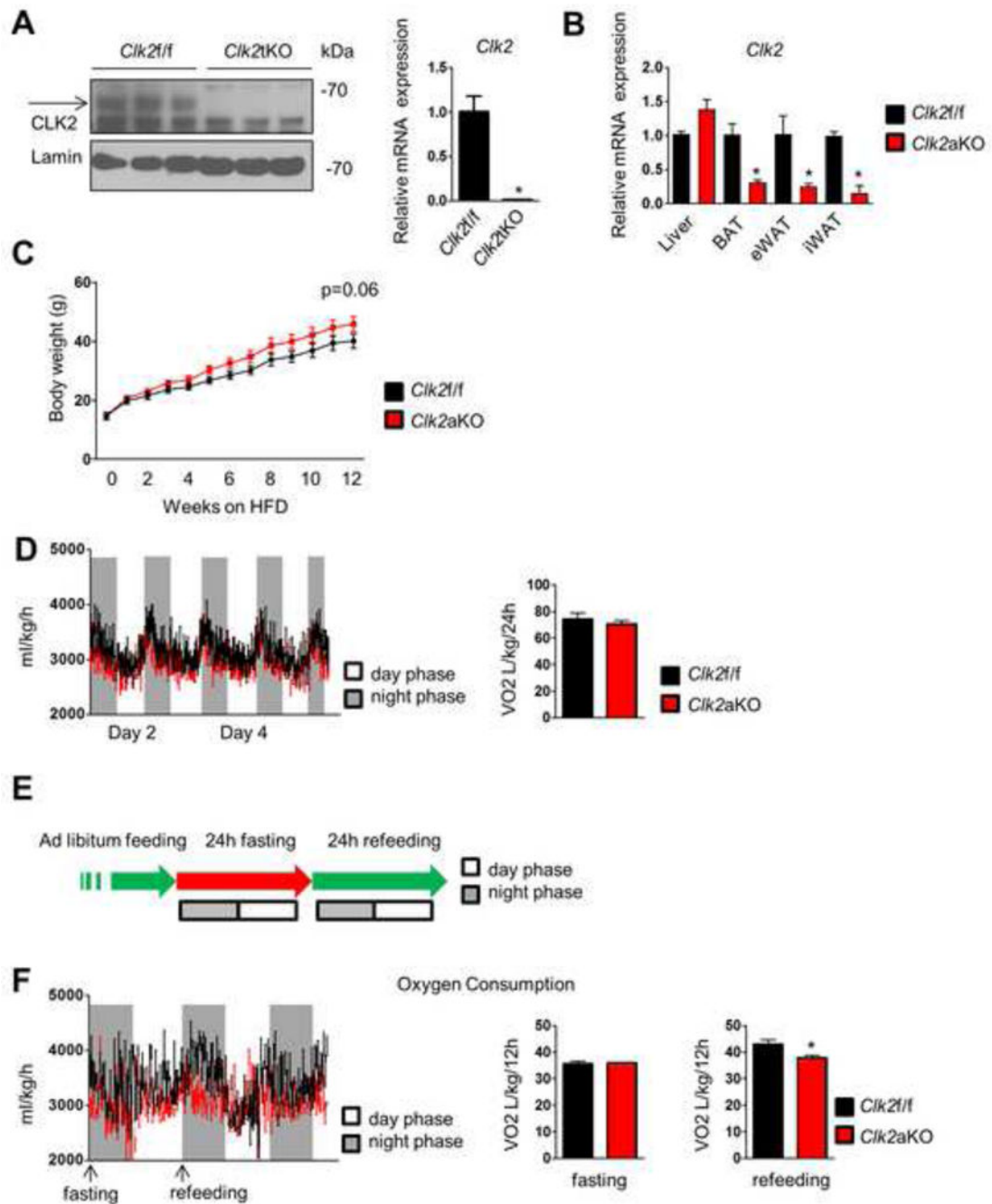
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**Figure 1. CLK2 is enriched in brown adipose tissue and regulated by feeding**

A) qRT-PCR analysis of CLK2 expression in tissue extracts of *Clk2<sup>f/f</sup>* mice as indicated (n=5). B) Western blots of BAT nuclear extracts from *Clk2<sup>f/f</sup>* mice after fasting (24h) or refeeding (12h) (left panel) and qRT-PCR analysis of BAT from *Clk2<sup>f/f</sup>* mice after fasting (24h) or refeeding (12h) (right panel). C) Western blot of primary brown adipocytes treated with 100 nM insulin for 0, 8 or 16 hours and pre-treated as indicated. Data shown as mean  $\pm$  SEM. Student's t-test (2 data sets) or one-way-ANOVA (multiple data sets) were performed and  $p < 0.05$  was considered to be significant and indicated with \*. (See also Figure S1)



**Figure 2. CLK2 deletion in adipose tissue decreases energy expenditure**

A) Western blot from primary adipocytes derived from *Clk2<sup>ΔKO</sup>* mice (left panel) and qRT-PCR analysis from cells of the same experiment (right panel). B) qRT-PCR analysis of *Clk2* levels from *Clk2<sup>f/f</sup>* and *Clk2<sup>ΔKO</sup>* mice as indicated. C) Body weights of *Clk2<sup>f/f</sup>* and *Clk2<sup>ΔKO</sup>* mice chronically fed a HFD (n=5 per group). D) Oxygen consumption corrected by body weight of *Clk2<sup>f/f</sup>* and *Clk2<sup>ΔKO</sup>* mice chronically fed a high fat diet (HFD) (n= 8 per group, 13 weeks). E) Schematic of fasting and refeeding studies used in Figure 2F. F) Oxygen consumption corrected by body weight of *Clk2<sup>f/f</sup>* and *Clk2<sup>ΔKO</sup>* mice chronically

fed a HFD (n= 3 per group and fasted (24h) or refed (24h). Data shown as mean  $\pm$  SEM. Student's t-test (2 data sets) or one-way-ANOVA (multiple data sets) were performed and  $p < 0.05$  was considered to be significant and indicated with \*. (See also Figure S2)

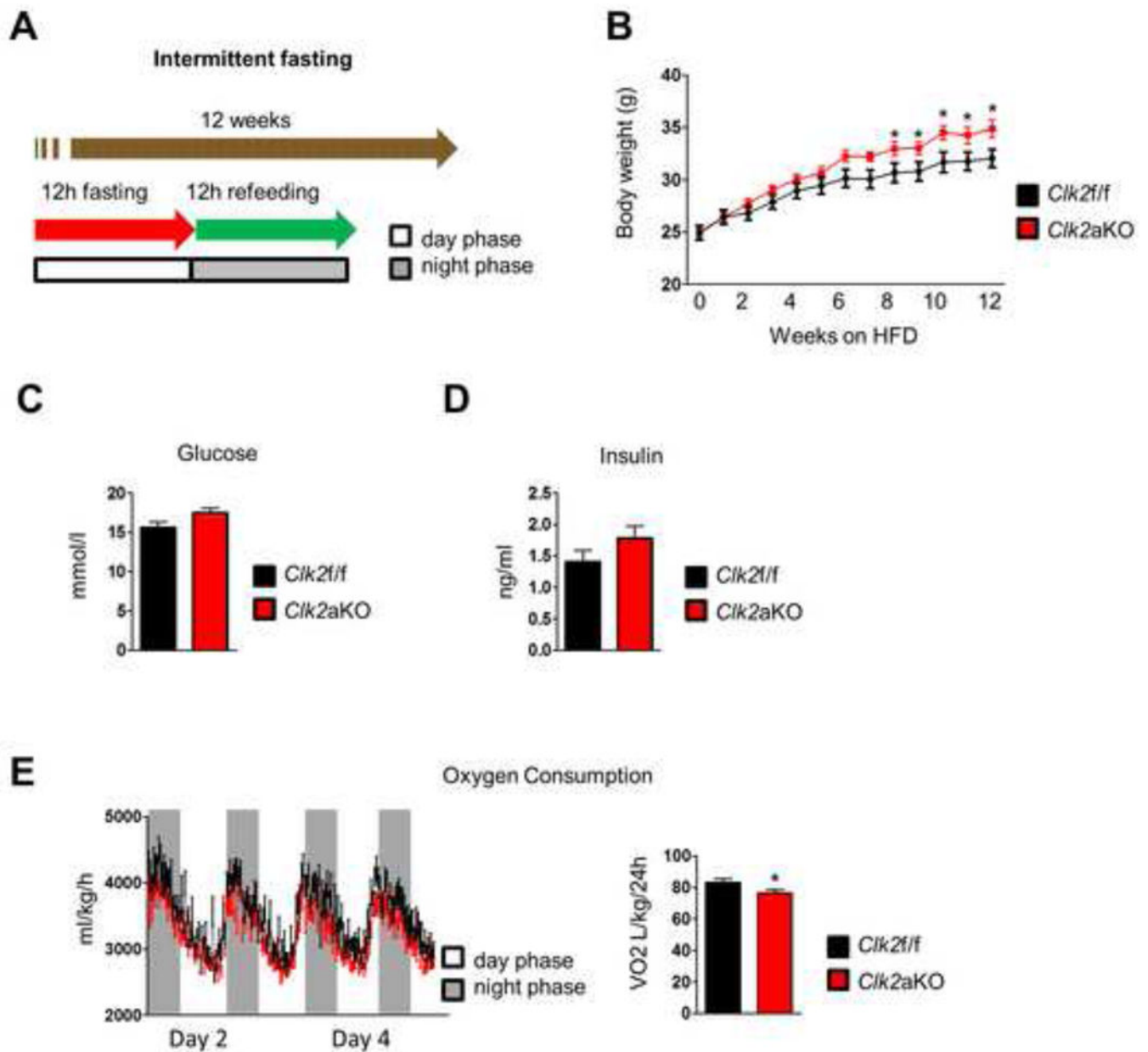
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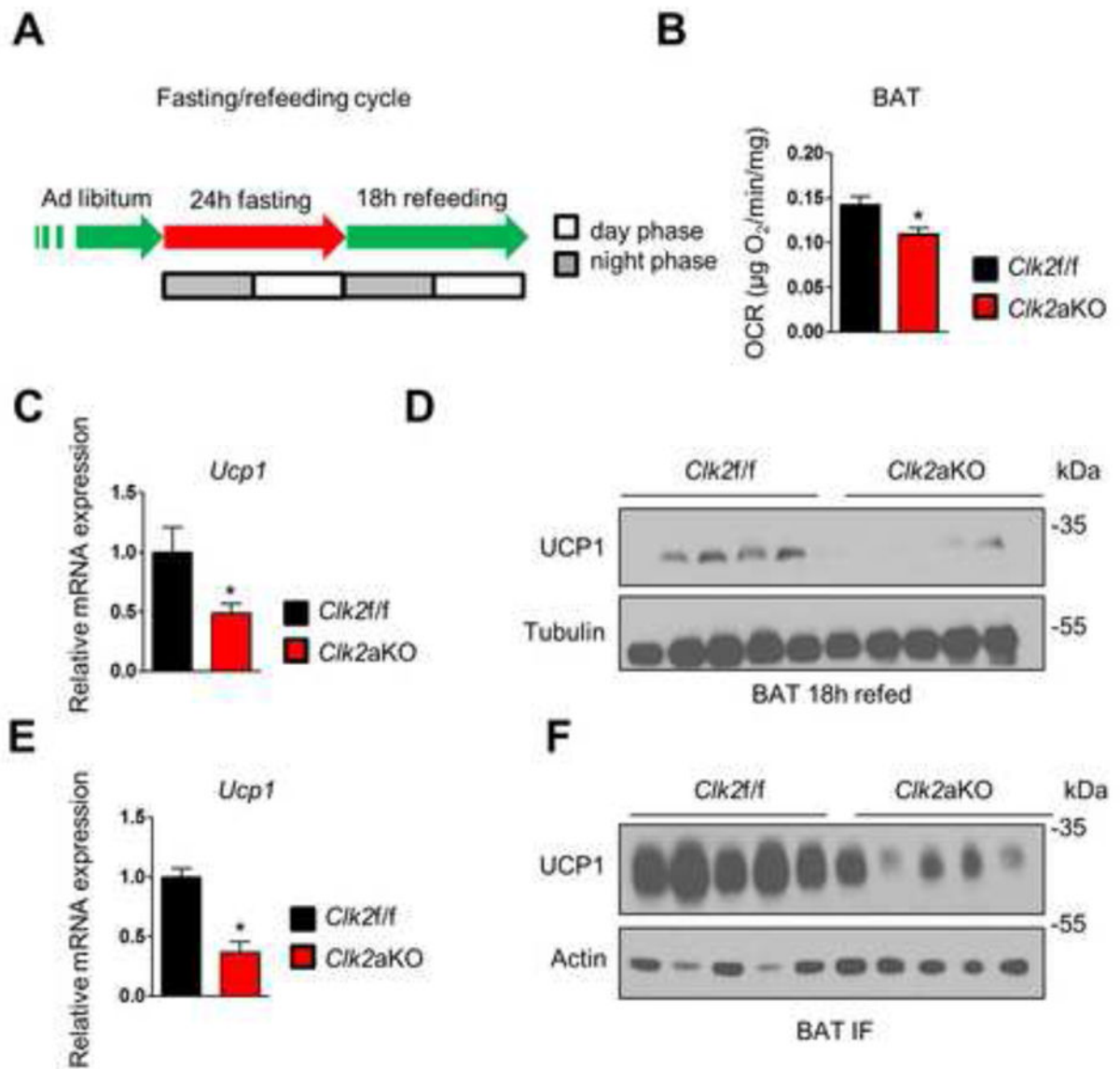
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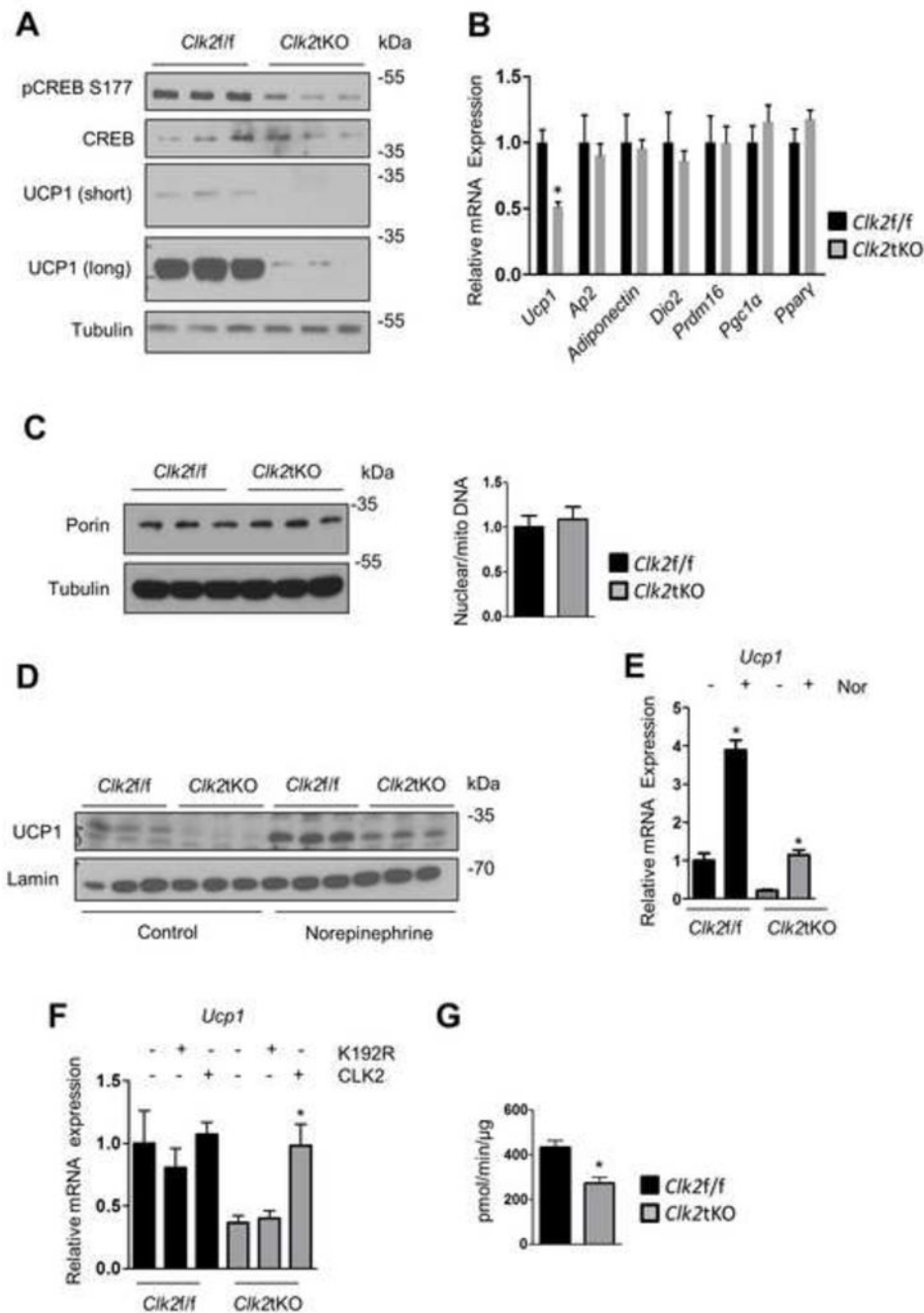
**Figure 3. CLK2 deficiency exacerbates obesity during an IF regimen**

A) Schematic of IF dietary regimen used in Figure 3. B) Body weight of *C1k2f/f* and *C1k2aKO* mice chronically subjected to IF and HFD (n= 10 per group). C) Blood glucose of *C1k2f/f* and *C1k2aKO* mice after 12 weeks of IF and 2h fast before sample collection. D) Serum insulin levels of *C1k2f/f* and *C1k2aKO* mice after 12 weeks of IF and 2h fast before sample collection. E) Oxygen consumption corrected by body weight of *C1k2f/f* and *C1k2aKO* mice chronically subjected to IF and HFD (n= 8 per group). Data shown as mean  $\pm$  SEM. Student's t-test (2 data sets) or one-way-ANOVA (multiple data sets) were performed and  $p < 0.05$  was considered to be significant and indicated with \*. (See also Figure S3)



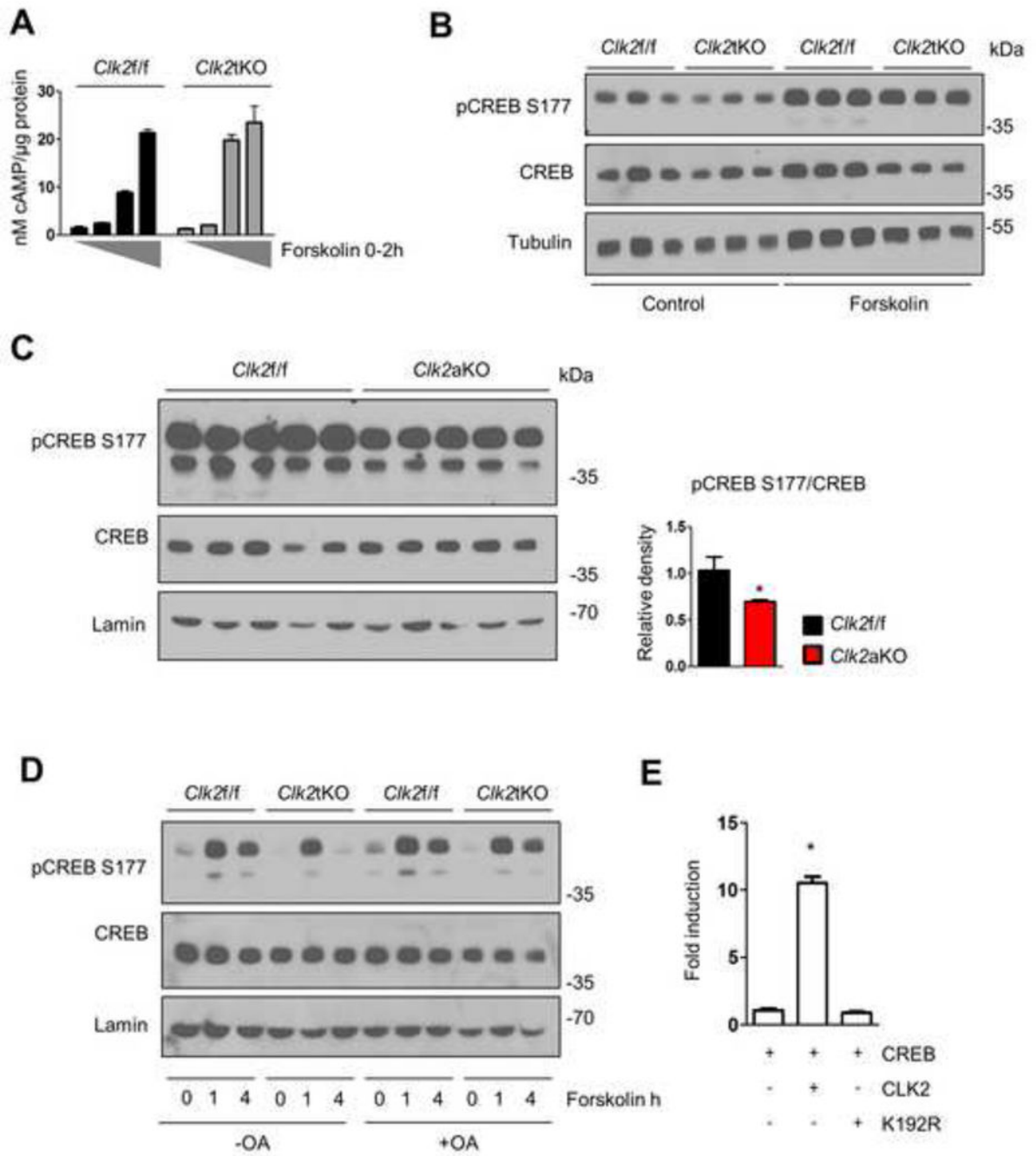
**Figure 4. BAT oxygen consumption and UCP1 expression is reduced with CLK2 deficiency**

A) Schematic of fasting and refeeding studies used in Figure 4. B) Oxygen consumption rates of BAT explants from *C1k2f/f* and *C1k2aKO* mice after 18h refeeding (n=5). C) qRT-PCR analysis of BAT from lean *C1k2f/f* and *C1k2aKO* mice after 18h refeeding (n=5). D) Western blot analysis of BAT from lean *C1k2f/f* and *C1k2aKO* mice after 18h refeeding. E) qRT-PCR analysis of BAT from *C1k2f/f* and *C1k2aKO* mice subjected to IF for 12 weeks (n=5). F) Western blot analysis of BAT from *C1k2f/f* and *C1k2aKO* mice subjected to IF for 12 weeks. Data shown as mean  $\pm$  SEM. Student's t-test (2 data sets) or one-way-ANOVA (multiple data sets) were performed and  $p < 0.05$  was considered to be significant and indicated with \*. (See also Figure S4)



**Figure 5. CLK2-deficient brown adipocytes have a cell-autonomous defect in UCP1 expression**  
 A) Western blots of primary brown adipocytes from *Clk2f/f* and *Clk2tKO* mice. B) qRT-PCR analysis from primary brown adipocytes. C) Western blots from primary brown adipocytes and qRT-PCR analysis from nuclear and mitochondrial DNA of primary brown adipocytes. D) Western blot from primary brown adipocytes treated with 100 nM norepinephrine for 24 hours. E) qRT-PCR analysis from primary brown adipocytes treated for 2 hours with 100 nM norepinephrine as indicated. F) qRT-PCR analysis from primary brown adipocytes infected with adenoviruses as indicated and harvested 36h post infection.

G) Oxygen consumption rate in primary brown adipocytes. Cell culture experiments were done in triplicate and at least 3 independent experiments were performed. Data shown as mean  $\pm$  SEM. Student's t-test (2 data sets) or one-way-ANOVA (multiple data sets) were performed and  $p < 0.05$  was considered to be significant and indicated with \*.



**Figure 6. CLK2 decreases CREB dephosphorylation via a PP2A phosphatase-dependent pathway**

A) cAMP measurements in whole cell lysates after 10  $\mu$ M forskolin treatment (0, 15 min, 1h, 2h). B) Western blot from primary brown adipocytes after 1 hour 10  $\mu$ M forskolin treatment as indicated. C) Western blot of BAT from *CiK21/f* and *CiK2a/KO* mice subjected to IF for 12 weeks. D) Western blot from primary brown adipocytes after 10  $\mu$ M forskolin treatment as indicated in the presence or absence of 5 nM okadaic acid (OA). E) Luciferase reporter assay from U2OS cells transfected with a cyclic AMP response binding element reporter, CREB and CLK2 or control DNA as indicated. Cell culture experiments were done

in triplicate and at least 3 independent experiments were performed. Data shown as mean  $\pm$  SEM. Student's t-test (2 data sets) or one-way-ANOVA (multiple data sets) were performed and  $p < 0.05$  was considered to be significant and indicated with \*. (See also Figure S5)

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