

## Diet-induced obese rats exhibit impaired LKB1–AMPK signaling in hypothalamus and adipose tissue<sup>☆</sup>

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### ABSTRACT

AMPK not only acts as a sensor of cellular energy status but also plays a critical role in the energy balance of the body. In this study, LKB1–AMPK signaling was investigated in diet-induced obese (DIO) and diet resistant (DR) rats. In hypothalamus, DIO rats had lower level of LKB1, AMPK $\alpha$  and pAMPK $\alpha$  than chow-fed or DR rats. Both orexigenic peptide NPY and anorexigenic peptide POMC expression were reduced in hypothalamus of DIO rats. i.c.v. injection of AICAR, an activator of AMPK, increased NPY expression but did not alter POMC expression in DIO rats. In periphery, LKB1 protein content and pAMPK $\alpha$  level were lower in the adipose tissue of DIO rats compared to chow-fed and DR rats. Moreover, pAMPK $\alpha$  and LKB1 protein levels obtained from epididymal fat pad were inversely correlated with epididymal fat mass. LKB1 protein content and pAMPK $\alpha$  in skeletal muscle of DIO rats were not different from those in the muscles of chow-fed and DR rats. In summary, DIO rats, but not DR rats, have impaired LKB1–AMPK signaling in hypothalamus and adipose tissue, suggesting the disturbed energy balance observed in DIO rats is related with abnormalities of AMPK signaling in a tissue specific manner.

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### 1. Introduction

Obesity is the consequence of an imbalance between energy intake and expenditure. The prevalence of obesity all over the world suggests that there is a fundamental weakness in the regulation of appetite and energy homeostasis. Diet-induced obesity (DIO) in rats shares several common features with human obesity [18]. As in many of the human obese cases, the animal model appears to follow a polygenic mode in inheritance. Thus, the physiological changes observed in this animal model should provide a useful insight into the development of obesity in humans. Studies have shown that some animals become obese while others remain lean when fed on a high-fat (HF) diet [18,36]. This phenomenon of phenotype

variation in response to environmental challenges can be found in different strains of rodents [3,34]. However, the underline mechanisms by which these animals response different to environment are largely unknown.

AMP-activated kinase (AMPK) is a serine/threonine kinase consisting of an  $\alpha$  catalytic subunit and two regulatory subunits,  $\beta$  and  $\gamma$  [5]. Once activated, AMPK in the cell switches off energy consuming pathways and switches on energy-producing pathways, acting to reestablish normal cellular energy balance [32]. Liver kinase B1 (LKB1) was identified as a major AMPK kinase [9,29,35,40]. LKB1 is constitutively active and phosphorylates AMPK at site Thr172 of the  $\alpha$  subunit [6,7]. AMPK activation is abolished in cells lacking LKB1 expression or in rodent following deletion of LKB1 [6,27,30]. When activated by LKB1, AMPK elicits its effects by regulating activities of key metabolism enzymes, acetyl CoA carboxylase (ACC). ACC is phosphorylated by AMPK and thus inactivated, thereby decreasing malonyl CoA levels, resulting in disinhibition of carnitine palmitoyl transferase-1 (CPT-1) and an increase of fatty acid oxidation [10].

Recent results suggest that AMPK may have a wider role in regulating whole-body energy metabolism. AMPK was reported to stimulate glucose uptake and fatty acid oxidation in muscle, inhibit fatty acid and cholesterol synthesis in liver and inhibit fatty acid synthesis in adipose tissue [10]. In addition to its role in the periphery, AMPK also regulates energy intake by integrating signals from hormones and nutrients to coordinately regulate the energy

**Abbreviations:** AMPK, AMP-activated protein kinase; LKB1, liver kinase B1; ACC, acetyl CoA carboxylase; DIO, diet-induced obesity; DR, diet resistant; CPT-1, carnitine palmitoyl transferase-1; AICAR, 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside; FFA, free fatty acid; UCP1, uncoupling protein-1; HSL, hormone-sensitizing lipase; VLDL, very low-density lipoprotein;  $\alpha$ -MSH,  $\alpha$ -Melanocyte Stimulating Hormone; NPY, neuropeptide Y; POMC, proopiomelanocortin.

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balance in the hypothalamus [1,12,16,24]. For instance, AMPK activates neuropeptide Y neurons in the hypothalamic arcuate nucleus to increase food intake in rats [14].

Although AMPK plays a critical role in regulating energy metabolism, it has not been extensively studied in animals that were rendered obese by the chronic feeding of a high-fat diet. Thus, an important goal of this study is to determine whether diet-induced obesity is associated with alterations in LKB1–AMPK signaling. Emphasis was put on the possible differences in AMPK cascade between DIO and diet resistant (DR) rats.

## 2. Materials and methods

### 2.1. Animals and experimental protocol

Male Sprague–Dawley rats (160–180 g) were obtained from Vital Company, Beijing. Animals were housed in a facility with ambient temperature ( $22 \pm 2^\circ\text{C}$ ) and maintained in 12/12 h light–dark cycles (light on from 07:00 to 19:00 h). To acclimatize to the new environment, all rats were fed with standard laboratory chow and water available *ad libitum* during the first week of the experiment. All procedures were performed in accordance with institutional guidelines of the Animal Care Committee of the Peking University. Animals were then randomly divided into two groups: (1) chow-fed group ( $n=20$ ), fed with standard laboratory chow (Vital Company Beijing, 3.80 kcal/g), consisting of 5% fat, 55% carbohydrates, 22% protein, 7% ash and 5% fiber; (2) HF group ( $n=100$ ), fed with HF diet (4.76 kcal/g) consisting of 30% fat, 40% carbohydrate, 15.5% protein, 4% ash, and 3% fiber [36]. Body weight was monitored once every week at 9 am.

After feeding for 9 weeks, 44 rats on HF diet gaining weights surpassing the maximal body weights of rats in chow-fed group were designated as DIO group. 27 rats in HF diet group gaining their body weights less than the average of chow-fed group were designated as the DR group. 29 rats in the HF diet group gaining weights between the average and the maximum body weights of the chow-fed group were discarded. DIO, DR and chow-fed group, respectively, were housed individually, and fed with the corresponding diet. Food intake and body weight were measured daily for 3 weeks. Adipose tissue, liver, muscle, hypothalamus and plasma samples were collected at the end of the study.

Twenty rats in DIO group were implanted with permanent stainless steel cannula into the third ventricle of the brain: 1.8 mm caudal to Bregma, 0 mm lateral to the midline, and sunk to a depth of 8 mm below the surface of the skull. Correct cannula placement was tested by intracerebroventricular (i.c.v.) Angiotensin II (50 ng/5  $\mu\text{l}$ ) injection, which induced a dipsogenic response. An increased drinking response of at least 3 ml of water during the 30 min following the Angiotensin II administration confirmed correct placement. Implanted rats were housed in individual cages. 5-Aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR) (Phoenix, Belmont, CA, USA) dissolved in saline was injected i.c.v. in a volume of 5  $\mu\text{l}$  immediately preceding dark onset, food intake measurements were then taken at 1 h, 3 h, and 12 h later.

### 2.2. Determination of circulating metabolites

Blood samples were collected into vials containing ethylenediamine tetraacetic acid (EDTA, 24 mg/ml, pH 7.4) and centrifuged at  $1000 \times g$  for 15 min, the plasma was then stored at  $-20^\circ\text{C}$  until the analysis for glucose, triglycerides and free fatty acid (FFA) was to be performed. Glucose and triglycerides were analyzed by the biochemistry Analysis Center of the third affiliated hospital of the

Peking University Health Science Center (HITACHI 7170, Japan). Plasma FFA were determined according to Miles et al. [23].

### 2.3. Western blot analysis

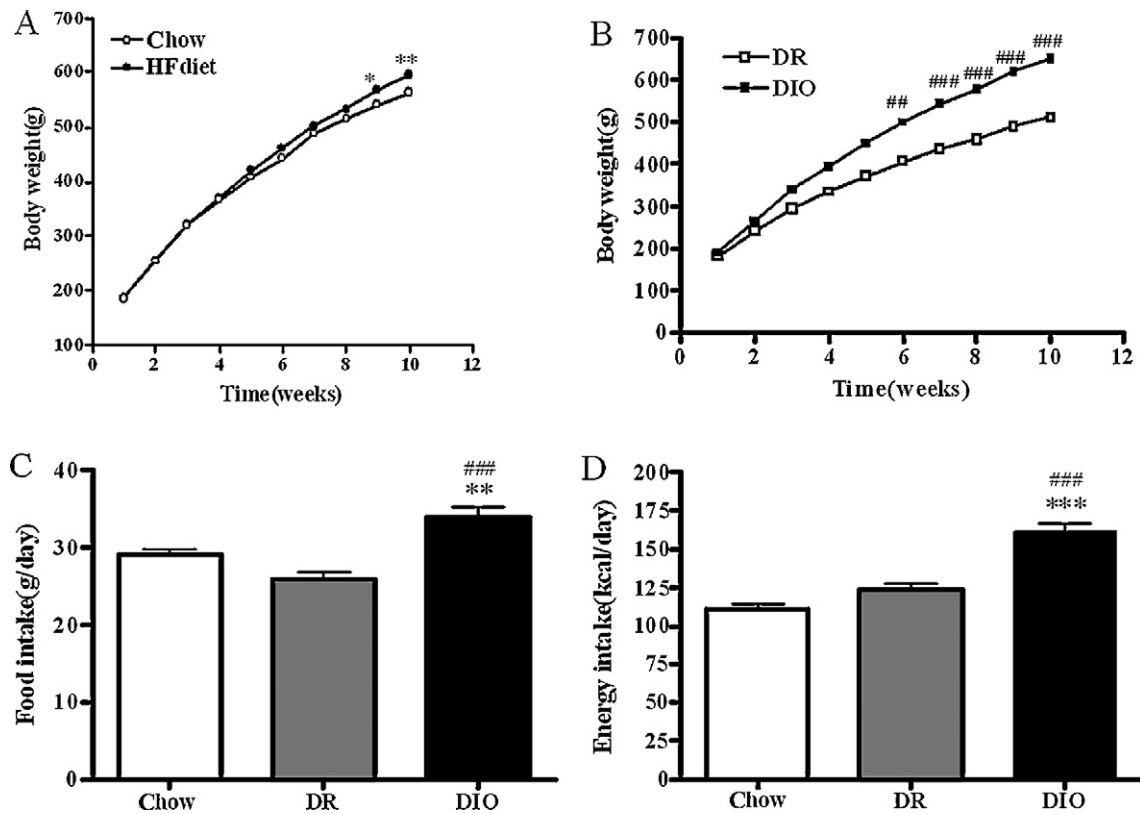
Hypothalamus were dissected using as landing markers the optic chiasma rostrally, and the mammillary bodies caudally to a depth of 2 mm. Dissected hypothalamus, gastrocnemius muscle and adipose tissue were immediately frozen in liquid nitrogen. Tissues were homogenized in 500  $\mu\text{l}$  of lysis buffer (50 mM Tris–HCl, pH 7.5, 250 mM sucrose, 5 mM sodium pyrophosphate, 50 mM NaF, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 50) and then centrifuged at  $10,000 \times g$  for 10 min. After supernatant was collected, protein content was determined by BCA kit (Pierce Biochemicals, USA). AMPK $\alpha$  phosphorylation (pAMPK $\alpha$ ) was determined with 10% SDS acrylamide gels by using the antibody that recognizes phosphoThr 172 of AMPK $\alpha$  (1:1000, cell signaling). Blots were re-probed with antibodies to AMPK $\alpha$  (1:1000, cell signaling) or LKB1 (1:1000, upstate). The results were visualized using chemiluminescent substrate kit (Pierce). Relative band intensities were assessed by densitometric analysis (Scion Image Analysis software program).

### 2.4. Immunohistochemistry and in situ hybridization

Rats were perfused under sodium pentobarbital (50 mg/kg, i.p.) anesthesia with 100 ml of saline followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and kept in the same fixative overnight at  $4^\circ\text{C}$  and then cryoprotected overnight in 30% sucrose solution. Frozen sections were cut at 20  $\mu\text{m}$  and immunohistochemistry was performed. Sections were blocked in phosphate-buffered saline (PBS) containing 5% goat serum, 0.1% bovine serum albumin, 0.3% Triton-X 100, for 1 h at room temperature and incubated, respectively, with antibodies against neuropeptide Y (NPY) (1:4000, Phoenix Pharm, Belmont, CA, USA) or  $\alpha$ -Melanocyte Stimulating Hormone ( $\alpha$ -MSH) (1:4000, Phoenix Pharm, Belmont, CA, USA) over night at  $4^\circ\text{C}$ . Signal was visualized by using a Vectastain ABC kit (Vector). For *in situ* hybridization, digoxigenin-labeled antisense cRNA probe was generated from a plasmid containing the NPY gene (NM.012614, 76–426) or proopiomelanocortin (POMC) gene (NM.139326, 57–438). Sections were washed in PBS, treated with proteinase K, fixed, acetylated, and hybridized overnight at  $58^\circ\text{C}$  using 2 ng/ml cRNA probes. After hybridization, slides were washed in  $4 \times \text{SSC}$  containing 50% formamide for 30 min, incubated in RNase A (20  $\mu\text{g}/\text{ml}$ ) for 30 min at  $37^\circ\text{C}$ , washed in  $2 \times \text{SSC}$ ,  $0.1 \times \text{SSC}$  for 30 min at  $37^\circ\text{C}$ , and then washed in PBS twice for 10 min. After immersed in 1.5% blocking reagent, sections were incubated with anti-Dig-AP antibody (1:1000, Roche) for 4 h at  $37^\circ\text{C}$ , and were then washed in PBS for 10 min twice, buffer-1 (100 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.01% Tween 20) for 10 min and buffer-2 (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM  $\text{MgCl}_2$ ) for 3 min. Finally, sections were stained using NBT/BCIP (400  $\mu\text{g}/\text{ml}$ , 200  $\mu\text{g}/\text{ml}$ ). For control purposes, hybridization was also performed without a probe or in the presence of a sense probe. In control experiments, no staining was performed. Four sections from the arcuate nucleus of each animal were analyzed.

### 2.5. Measurement of cell size and DNA content in adipose tissue

Epididymal adipose tissue was removed from 4% paraformaldehyde perfused rats, kept in the same fixative overnight at  $4^\circ\text{C}$ , dehydrated, and embedded in paraffin for subsequent sectioning. Sections (5  $\mu\text{m}$ ) were stained with hematoxylin and eosin, and cell size was measured by a computerized image analysis program (Q-win, Leica Microsystems, Germany). For DNA analysis, freshly



**Fig. 1.** Body weight and food intake of chow or high fat (HF) fed rats. (A) Body weight gain of HF (4.76 kcal/g,  $n=80$ ) or chow fed rats (3.80 kcal/g,  $n=20$ ). (B) Body weight gain of DR and DIO rats during HF feeding. (C) Food intake (g/day) of chow-fed, DR and DIO rats ( $n=10$ ). (D) Energy intake (kcal/day) of chow-fed, DR and DIO rats ( $n=10$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. chow; # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  vs. DR.

isolated Epididymal adipose tissue was digested with proteinase K, and DNA was extracted with phenol:chloroform. DNA content was measured by spectrophotometry ( $\lambda = 260$  nm).

## 2.6. Statistical analysis

All data are expressed as mean  $\pm$  SEM. Statistical differences among groups were determined by using one-way ANOVA and Fisher's *post hoc* tests. Correlation analysis was performed by the Pearson product-moment method. For all analyses, a  $p < 0.05$  was considered to be statistically significant.

## 3. Results

### 3.1. Body weight and food intake

Fig. 1A shows that rats fed the high fat (HF) diet gained more body weight than those fed a chow diet over a 9-week period. Differences in body weight became noticeable from 6 weeks onwards and became significant after 9 weeks. The rats fed with HF diet were separated into two groups at the end of 9 weeks. 44 of them had body weights surpassing the maximum body weight in chow-fed group, so were described as DIO, 27 of them had body weights less than the average bodyweight of chow-fed group, and were chosen as DR. When backtracking to the beginning of high fat feeding, DIO and DR rats showed similar initial body weights (Fig. 1B).

During the period of 9–11th week, a significantly higher food or energy intake per day was observed in the DIO group compared to the other two groups (Fig. 1C and D).

### 3.2. Body weight distribution and metabolic parameter measurements

Epididymal, circumrenal and epiloonal depots account for the majority of visceral adipose tissue mass. A significantly larger mass was observed for each adipose depot in DIO rats as compared to chow-fed and DR rats (Table 1), while chow-fed and DR rats had no difference in adipose weight. Microscopic analysis of epididymal adipose tissue revealed that DIO rats had larger adipocytes than chow-fed and DR rats (Fig. 2A and B), whereas total DNA contents were comparable in all groups (Fig. 2C). Remarkable augmentation of liver weight was also observed in DIO rats. The increase in liver weight is likely caused by the accumulation of lipid in hepatocytes (Table 1, Fig. 2D). The weights of other organs, including heart, gastrocnemius muscle and testes were similar between DIO and DR or chow-fed rats (Table 1).

In addition to lipid accumulation in adipocytes, DIO rats showed higher concentrations of plasma triglyceride (2.0 mmol/l) and free fatty acid (FFA) (0.068 mmol/l) levels than DR rats (1.2 and 0.042 mmol/l) (Table 1). However, plasma glucose concentrations (9.3, 8.8 and 8.8 mmol/l) were similar among all three groups (Table 1).

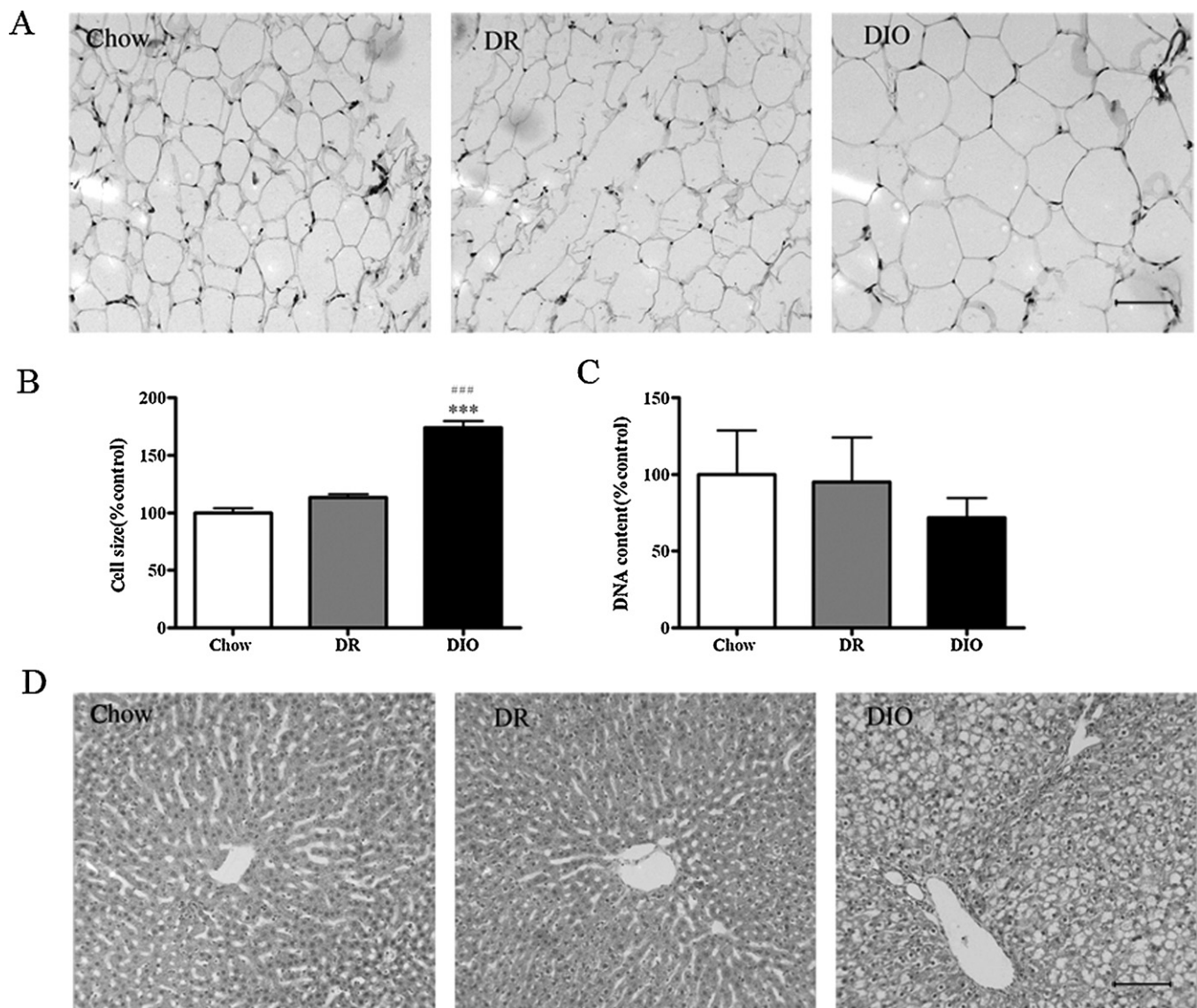
### 3.3. AMPK signaling in hypothalamus

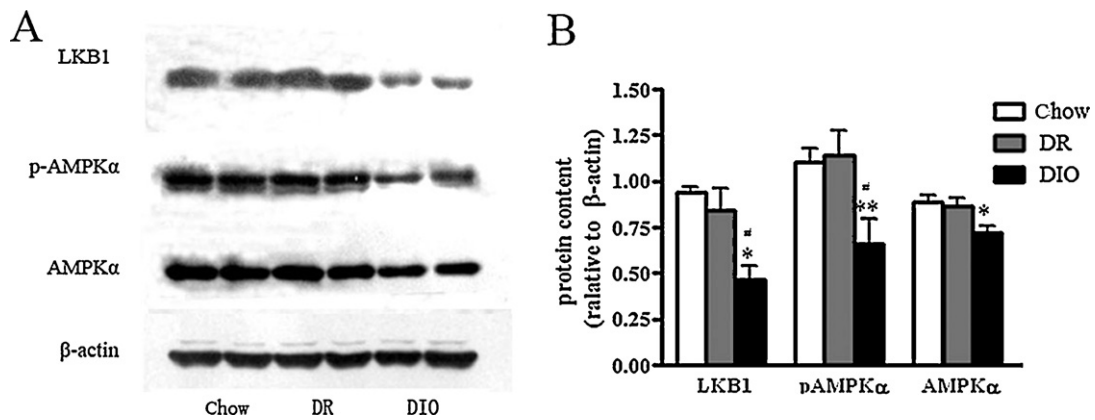
Hypothalamic LKB1 content was lower in DIO rats than that of chow-fed (–50%) or DR (–44%) rats (Fig. 3A and B). pAMPK $\alpha$  levels which represent the activity of AMPK in the hypothalamus of DIO rats were decreased significantly compared to that of chow-fed (–40%) or DR (–42%) rats (Fig. 3A and B). Total protein level of AMPK $\alpha$  was also decreased in hypothalamus of DIO rats compared

**Table 1**  
Terminal tissue weights and plasma metabolic parameters ( $n = 5$ ).

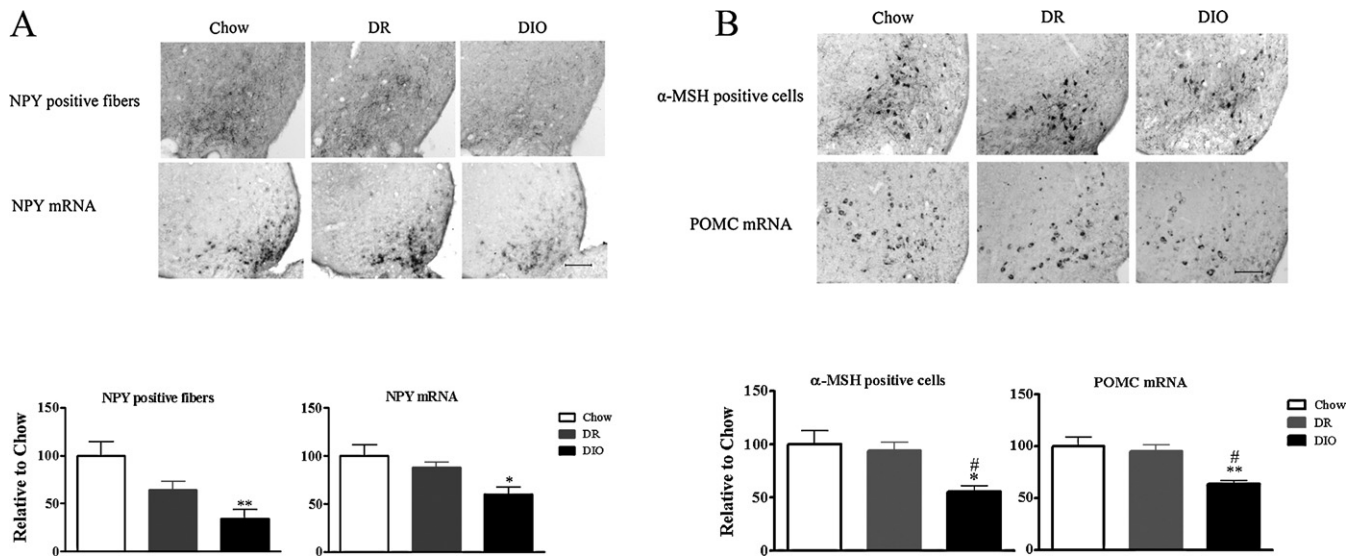
	Chow	DR	DIO
Epididymal fat mass (g)	9.1 ± 0.79	8.9 ± 0.31	21.9 ± 1.42 <sup>***,###</sup>
Circumrenal fat mass (g)	13.2 ± 1.14	17.2 ± 0.98	32.8 ± 1.49 <sup>***,###</sup>
Epiploon fat mass (g)	1.4 ± 0.14	1.2 ± 0.11	2.3 ± 0.28 <sup>*,##</sup>
Fat weight/body weight	0.05 ± 0.002	0.06 ± 0.003	0.11 ± 0.005 <sup>***,###</sup>
Gastrocnemius muscle (g)	3.2 ± 0.36	3.3 ± 0.22	3.1 ± 0.33
Liver (g)	19.3 ± 0.9	16.1 ± 1.08 <sup>**</sup>	24.0 ± 0.64 <sup>***</sup>
Testes (g)	3.7 ± 0.26	3.2 ± 0.11	3.7 ± 0.19
Heart (g)	1.6 ± 0.08	1.5 ± 0.06	1.7 ± 0.04
Plasma glucose (mmol/l)	8.8 ± 0.28	8.8 ± 0.35	9.3 ± 0.34
Plasma triglycerides (mmol/l)	1.4 ± 0.16	1.2 ± 0.19	2.0 ± 0.22 <sup>#</sup>
Plasma free fatty acid (mmol/l)	0.054 ± 0.005	0.042 ± 0.005	0.068 ± 0.004 <sup>##</sup>

Data are means ± S.E.

<sup>\*\*</sup>  $p < 0.01$  vs. chow.<sup>\*\*\*</sup>  $p < 0.001$  vs. chow.<sup>#</sup>  $p < 0.05$  vs. DR.<sup>##</sup>  $p < 0.01$  vs. DR.<sup>###</sup>  $p < 0.001$  vs. DR.**Fig. 2.** Adipocyte DNA content, adipose and liver morphology in chow-fed, DR, and DIO rats. (A) Paraffin-embedded sections of epididymal adipose tissue were stained with hematoxylin and eosin ( $n = 5$ ). (B) Average size of adipocyte. (C) DNA content of epididymal depot ( $n = 4$ ). (D) Paraffin-embedded sections of liver were stained with hematoxylin and eosin ( $n = 5$ ). <sup>\*\*\*</sup> $p < 0.001$  vs. chow; <sup>###</sup> $p < 0.001$  vs. DR.



**Fig. 3.** AMPK signaling in hypothalamus. (A) Representative immunoblots in each group to show LKB1 protein content, pAMPK $\alpha$ , and total AMPK $\alpha$  protein content ( $n=5$ ). (B) Quantization of protein levels in each blot by densitometry shown in (A). \* $p < 0.05$ , \*\* $p < 0.01$  vs. chow; # $p < 0.05$  vs. DR.



**Fig. 4.** (A) NPY positive fibers ( $n=5$ ) and mRNA ( $n=4$ ) were measured in chow-fed, DR and DIO rats. (B)  $\alpha$ -MSH peptide ( $n=5$ ) and POMC mRNA ( $n=4$ ) were measured in chow-fed, DR and DIO rats. \* $p < 0.05$ , \*\* $p < 0.01$  vs. chow; # $p < 0.05$  vs. DR.

with chow-fed rats (Fig. 3A and B). Although the DR rats were fed the same HF diet as DIO rats, AMPK signaling in hypothalamus of DR rats was not different from that of chow-fed group (Fig. 3A and B). Since a number of hypothalamic neuropeptides play important roles in energy homeostasis, we then measured the expression of orexigenic peptide NPY and anorexigenic peptide POMC in arcuate nucleus. mRNA and peptide levels of NPY were decreased in DIO rats compared to the chow group (Fig. 4A). Reduced POMC mRNA and  $\alpha$ -MSH peptide level were detected in DIO rats compared to those in chow-fed and DR rats (Fig. 4B). The reduced expression of NPY in DIO rats was restored by i.c.v. administration of AICAR (Fig. 5A), which is along with increased food intake (Fig. 5C). Yet, neither POMC mRNA nor  $\alpha$ -MSH peptide level was influenced by AICAR administration (Fig. 5B).

### 3.4. AMPK signaling in adipose tissue

As shown in Fig. 6A and B, pAMPK $\alpha$  levels in the adipose tissue of DIO rats were decreased by 28% and 36% compared to chow-fed and DR rats, respectively. LKB1 protein content in the adipose of DIO rats was also reduced by 42% and 41%, when compared to chow-fed and DR rats, respectively. In contrast, there were no differences in AMPK $\alpha$  protein level among all three groups. Although the DR rats

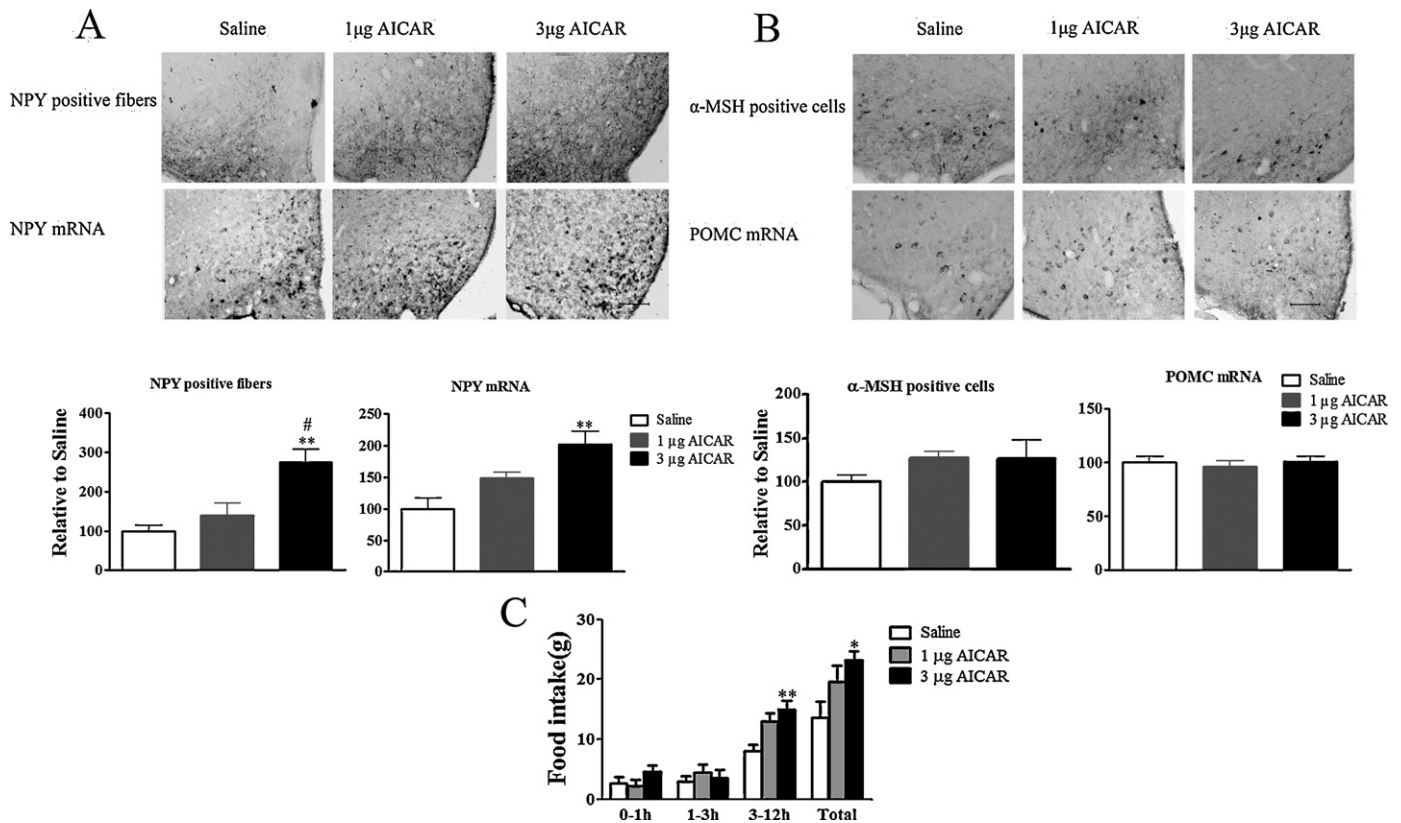
were fed the same HF diet as DIO rats (Fig. 6A and B), AMPK signaling in the adipose tissue of DR rats was not different from that of chow-fed control. Furthermore, pAMPK $\alpha$  ( $r=0.773$ ,  $p < 0.001$ ) and LKB1 ( $r=-0.655$ ,  $p < 0.01$ ) protein levels in all groups were inversely correlated with the weight of epididymal fat depots (Fig. 6C and D).

### 3.5. AMPK signaling in muscle

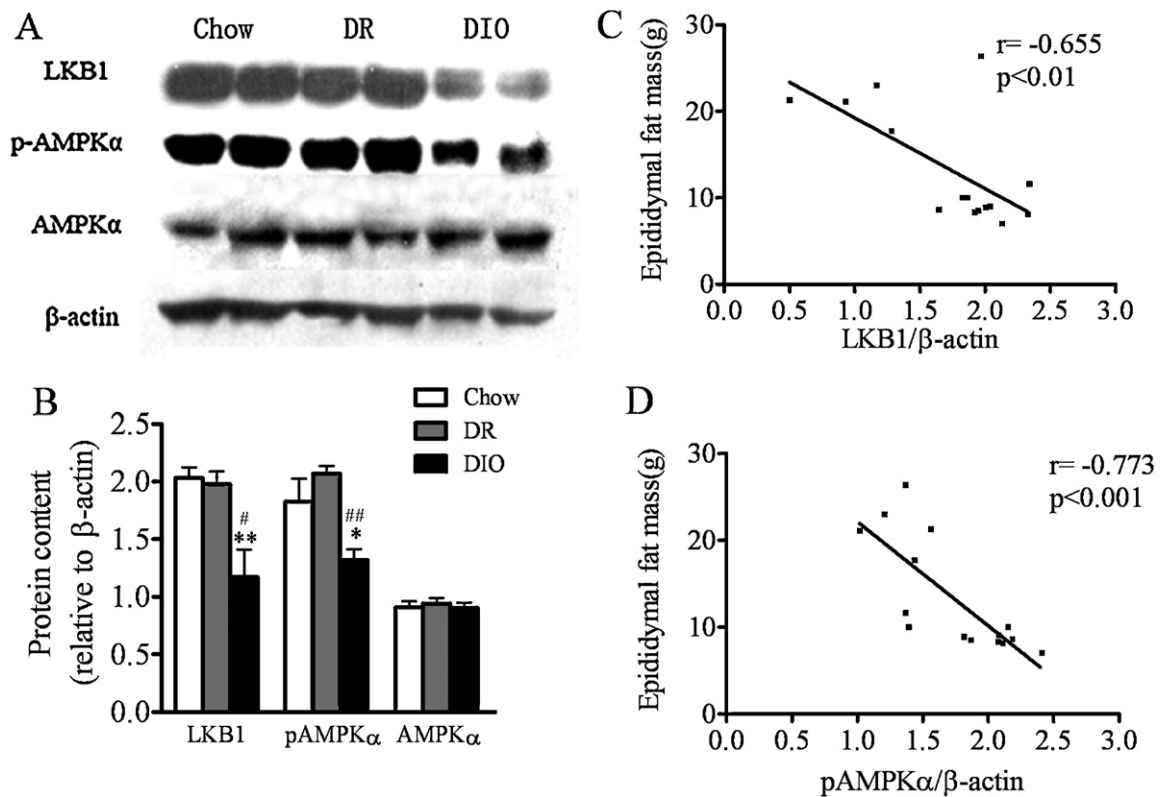
Although AMPK is proposed to play a critical role in glucose and lipid metabolism in muscle, we did not detect any differences of AMPK cascade in the muscle of DIO rats when compared to DR or chow-fed rats (Fig. 7).

## 4. Discussion

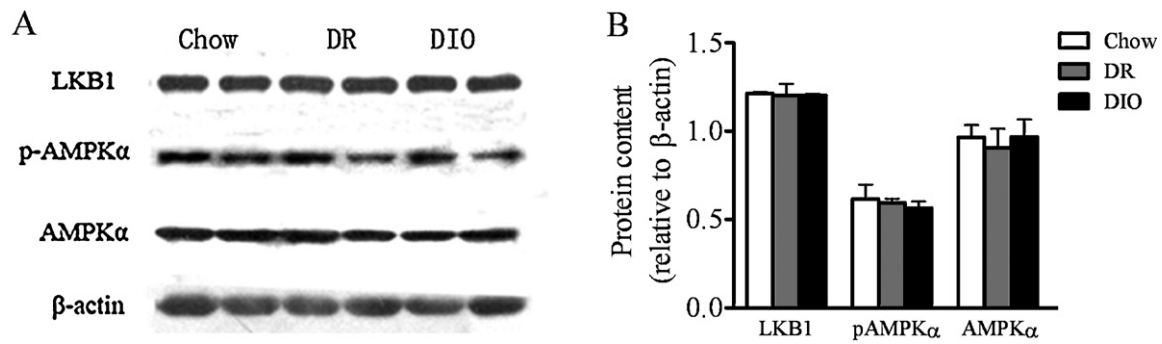
Although the SD rats showed a general trend of increase in energy intake and body weight when fed a HF diet, they diverged into two populations of weight gainers after 9 weeks' time (Fig. 1). About half of the rats on HF diet developed DIO with body weight higher than the highest in the control group fed with standard chow, while the rest of rats fed with HF diet did not gain more weight when compared to the chow-fed group. This result is in line with the previously published studies [18,37]. In fact, 27% of



**Fig. 5.** (A) Positive fibers and mRNA levels of NPY were measured 12 h after AICAR treatment ( $n=5$ ). (B)  $\alpha$ -MSH peptide and POMC mRNA levels were measured 12 h after AICAR treatment ( $n=5$ ). (C) Food intake was measured from DIO rats ( $n=5$ ) at different time points after an i.c.v. injection of saline or AICAR. \* $p < 0.05$ , \*\* $p < 0.01$  vs. saline; # $p < 0.05$  vs. 1  $\mu$ g AICAR.



**Fig. 6.** AMPK signaling in the epididymal depot. (A) Representative immunoblots in each group to show LKB1 protein content, pAMPK $\alpha$ , and total AMPK $\alpha$  protein content ( $n=5$ ). (B) Quantization of protein levels in each blot by densitometry shown in (A). (C) Correlation between relative LKB1 levels and masses of Epididymal depots ( $n=5$ ). (D) Correlation between relative pAMPK levels and masses of epididymal depots ( $n=5$ ). \* $p < 0.05$ , \*\* $p < 0.01$  vs. chow; # $p < 0.05$ , ## $p < 0.01$  vs. DR.



**Fig. 7.** AMPK signaling in the gastrocnemius muscle. (A) Representative immunoblots in each group to show LKB1 protein content, pAMPK $\alpha$ , and total AMPK $\alpha$  protein content ( $n = 5$ ). (B) Quantization of protein levels in each blot by densitometry shown in (A) ( $n = 5$ ).

the rats fed with HF diet gained body weight at a value less than the average of control group and were thus designated as DR rats. When backtracking to the beginning, those who ended up developing obesity had similar initial weights as those who remained lean. This observation suggests that DIO and DR rats response different to high fat diet, DIO rats ate more and accumulated more fat than DR rats.

In hypothalamus, activation of AMPK stimulates food intake, whereas inhibition of AMPK reduces food intake [24]. In the present study, obese rats consumed more food than both DR and chow fed rats. We suspect that hypothalamic AMPK pathway may be activated in DIO rats, and thereafter result in hyperphagia. To our surprise, we observed that DIO rats had lower pAMPK $\alpha$ , AMPK $\alpha$  and LKB1 levels in hypothalamus (Fig. 3). In addition, NPY positive fibers and mRNA level were reduced in DIO rats as well (Fig. 4A). NPY is a potent stimulator of food intake produced in arcuate nuclear of hypothalamus. It was reported that AMPK was co-localized with NPY in a subpopulation of arcuate nuclear neurons [11]. Dominant negative AMPK mutation caused a decrease of NPY expression, but NPY did not change  $\alpha$ 2AMPK activity in any hypothalamic region, suggesting that AMPK acts upstream of NPY [24]. A most recent paper indicated that AMPK activated NPY neurons by Ca<sup>2+</sup> influx thereby causing NPY-stimulated food intake [14]. In our study, although the AMPK–NPY orexigenic system was inhibited, DIO rats were still overeating. This observation could at least partly be explained by the decreased expression of POMC and its producer  $\alpha$ -MSH in hypothalamus (Fig. 4B), consistent with the report that gene knockout of POMC was sufficient to induce hyperphagia and obesity [41,43]. Previous studies reported that POMC was regulated by the signal pathways other than the AMPK cascade [12,14]. Our observation that i.c.v. administration of AICAR did not influence the expression of POMC and  $\alpha$ -MSH (Fig. 5B) agrees with this concept. Therefore, we speculate that the down regulation of POMC is a primary driven of hyperphagia in DIO Rats independent of AMPK cascade. On the other hand, inhibition of AMPK–NPY orexigenic system could be a compensation response to energy overload in obese rats. In support of this idea, when i.c.v. injection of AICAR was used to activate AMPK, NPY expression was increased and DIO rats ate more than saline control (Fig. 5A and C).

Body distribution data showed that extra body weight gain in DIO rats mainly due to fat accumulation. Increase in adipose tissue mass was accompanied by a cellular hypertrophy without changes in the number of cells in the fat depots (Fig. 2). It is evident that body fat content is controlled, at least partially, by the metabolism of adipose tissue itself. Mice with over-expressed UCP1 in adipose tissue were reported to be resistant toward diet-induced obesity [17]. On the other hand, over-expression of  $\alpha$ 2-adrenergic receptors in adipose tissue of  $\beta$ 3-adrenergic receptor-deficient mice promoted diet-induced obesity [2]. In these transgenic models, metabolic changes in white adipose tissue were mostly responsible for the

altered accretion of body fat, highlighting the importance of lipid metabolism in adipose tissue. It was proposed that mice lacking AMPK $\alpha$ 2 subunit had increased fat mass when fed with a high fat diet [38]. In this study, we observed that pAMPK $\alpha$  level in adipose tissue was inversely correlated with adipose tissue mass (Fig. 6D). DIO rats exhibited a reduction of the pAMPK $\alpha$  level in the white adipose tissue, implying an inhibition of AMPK activity (Fig. 6A and B). In contrast, the pAMPK $\alpha$  level in DR rats fed with the same HF diet was not different compared with that of chow-fed rats. This finding suggests that intact AMPK signaling could contribute to the mechanisms by which DR rats maintain normal body fat. In line with this concept, sustained AMPK activation in obese Zucker rats by long-term administration of AICAR also resulted in a reduction of epididymal and retroperitoneal fat pads up to 30–40%, although no difference in total body weights were observed in these rats [4,39]. In the present study, we do not know whether reduced AMPK activity in DIO rats was genetically inherited or induced by high fat challenge, but we speculate that the reduced AMPK activation in adipose could help those rats accumulate more fat mass during the development of obesity.

LKB1 phosphorylates and activates AMPK if AMP concentration rises in the cell and binds to the  $\gamma$  subunit, transforming into a suitable substrate [40]. A critical finding from this study (Fig. 6A) is that obese rats have markedly decreased LKB1 content in the adipose tissue compared to chow-fed and DR rats. Although LKB1 is discovered initially as a tumor suppressor, this finding indicates that LKB1 might directly act as a regulator of energy metabolism. In adipose tissue, several lines of evidence suggest that LKB1 is involved in AMPK activation [19,28]. In transgenic mice over expressing UCP1 in white adipose tissue, the AMP/ATP ratio is increased and AMPK is activated [22]. Intriguingly, most attempts to identify physiological regulators of LKB1 failed and LKB1 was thought to be constitutively active [20,26]. Therefore we suggest that lower protein levels of LKB1 may partly contribute to the diminished AMPK activity in DIO rats. Another upstream kinase, calmodulin kinase kinase  $\beta$  was recently described to activate AMPK in the presence of an increased calcium concentration, independent of an increase in AMP concentration [29]. To our knowledge, a potential role of calmodulin kinase kinase  $\beta$  in AMPK activation has not been demonstrated in adipose tissue.

AMPK has been proposed as a key molecule mediating adaptations in response to exercise training, such as increasing lipid oxidation and glucose uptake [13]. But we did not observe significant changes of LKB1–AMPK pathway in skeletal muscle of DIO rats in the resting state (Fig. 7). In line with our observation, it has been reported that there are no changes in the basal activity of AMPK of the skeletal muscle in obese humans, compared to lean controls [33]. The muscle AMPK protein content and activity are also normal in type 2 diabetic subjects [8,15,25]. However, the regulation of AMPK in muscle remains controversial. Some reports

demonstrate that AMPK protein level and activity have a trend to increase in obese mice [21], and the content of LKB1 and phosphorylated AMPK are lower in the muscle tissue of obese Zucker rats than the lean controls [31]. One possible explanation for this discrepancy is that animal models used in these studies are different. In addition, different isoforms of AMPK are expressed in glycolytic and oxidative muscle fibers [42]. Therefore it is not surprising that different results are obtained since different parts of skeletal muscles are used in those studies. It is still important for us to address whether AMPK signaling exhibits abnormalities in muscle of DIO rats in response to exercise.

In summary, tissue specific dysregulation of LKB1–AMPK signaling was detected in DIO rats. DIO rats were hyperphagic despite the down-regulated LKB1–AMPK signaling and reduced expression of NPY in the hypothalamus. Reduced POMC expression may account for the hyperphagic phenotype of the DIO rats. DIO rats had elevated body weight, particularly in the size of abdominal adipose tissue depots and impaired LKB1–AMPK signaling in adipose tissue. DIO rats did not show significant changes of LKB1–AMPK signaling in skeletal muscle. Although DR rats were fed with the same HF diet as the DIO rats, they kept normal appetite and visceral fat mass without abnormalities in AMPK cascade.

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