Supplemental Information

Grb10 Promotes Lipolysis and Thermogenesis by Phosphorylation-dependent Feedback Inhibition of mTORC1

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Supplemental Experimental Procedures

Materials

Adenoviruses harboring the Grb10 gene were generated as described in our previous study (Langlais et al., 2004). CL316243, L-leucine, isoproterenol, L-carnitine, free glycerol reagent, rapamycin (for cell culture studies) and Tween-80 were from Sigma; α-cyclodextran was from Fisher Scientific; NEFA C Kit was from Wako; ^{14}C-pamitate was from Moravek Biochemicals; AKTi VI and PD98059 were from Calbiochem; A polyclonal antibody to Grb10 was described previously (Wang et al., 2007); Antibodies to UCP1, C/EBPβ and PRDM16 were obtained from Abcam; Antibodies to Akt and tubulin were from Biosource and Sigma, respectively. All other antibodies were from Cell Signaling. PEG-400 was from Hampton Research. Rapamycin for mouse studies was from LC laboratories.

Generation of Grb10-suppressed brown preadipocytes

The brown preadipocyte cell line was a generous gift from Dr. Jiandie Lin (Univ. of Michigan) and was described previously (Uldry et al., 2006). Grb10-suppresed or scramble control brown preadipocytes were generated by using the small RNA interference approach according to a similar protocol as described previously (Ramos et al., 2006). Positive stable cell lines were selected with G418 and confirmed by Western blot with an anti-Grb10 antibody.

Fat cell size and number measurement

Fat cell size measurement was performed according to the procedure as described (Mul et al., 2013). The sections cut from fat tissue pads were stained with hemotoxylin and eosin. Five-sections/per animal were cut at positions distributed equally throughout the pad and 3 pictures

were obtained per section. The average diameter of adipocyte cell was analyzed using NIH Image J software. Total adipocyte numbers were estimated by manual counting cells on H&E slides from at least three 20× fields of three mice per genotype and approximated assuming cubic packing as described (Mancuso et al., 2010).

Lipolysis

Lipolysis was performed according to the procedure as described (Ahmadian et al., 2011). Tissue samples (~40-60 mg) of epididymal white adipose tissue (eWAT), inguinal WAT (iWAT), and interscapular brown fat pads from overnight fasted mice were dissected and incubated in 500µl of KRB buffer (12 mM HEPES, 121 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, and 0.33 mM $CaCl_2$) containing 2% fatty acid free BSA and 0.1% glucose with or without 10 µM isoproterenol at 37°C for 6 hours. The KRB buffer were collected and used for fatty acid and free glycerol analysis using the NEFA C Kit (Wako) and Free Glycerol Reagent (Sigma), respectively. The levels of fatty acid and free glycerol were normalized to the mass of the tissue sample. To measure lipolysis in cultured cells, differentiated primary brown adipocytes seeded onto 12-well plates were electroporated with an empty plasmid or plasmids encoding wild type or the phosphorylation site mutated Grb10. Eighteen hours post-transfection, cells were washed with KRB containing fatty acid-free BSA (4%) and then incubated with 200 µl cell culture medium with or without 10 µM isoproterenol for 16 hours. The medium was collected and used for fatty acid and glycerol measurement. The levels of fatty acid and free glycerol were normalized to total protein levels in the cells.

Glucose and insulin tolerance test

5-week old male $Grb10^{fKO}$ and control mice were fed with normal chow or a HFD (45% kcal from fat, D12451; Research Diets Inc) for 12 weeks. The glucose and insulin tolerance test were performed as described in our previous study (Liu et al., 2012).

Cold stress experiments

Male mice (3 month-old) were individually housed in cages kept at 4^oC for 4 hours every day with free access to food and water continuously for 4 days. iWAT, eWAT, and brown fat were dissected and subjected to gene expression analysis. For cold tolerance studies, core body

temperature was monitored using a telemetry system at various times after the start of cold exposure. The Mini-Mitter implantable bio-telemetric thermo-sensors were surgically implanted into the peritoneal cavity of $Grb10^{fKO}$ and the control mice (n=5-6/group), and all the mice recovered for 1 week. Mice were individually housed in cages with free access to food and water continuously and kept near room temperature condition (~24 ^{o}C) for 24 hours followed with exposure to cold (4 ^{o}C) for 6 hours. Core body temperature was monitored at every 15 minutes at room temperature condition and every 10 minutes after the start of cold exposure using the Mini-Mitter implantable bio-telemetric thermo-sensors. The data were processed with the Vital View software.

Real-time PCR

Tissue samples were homogenized in Trizol (Invitrogen) and total RNA was isolated according to the manufacturer's suggested protocol. 1 μg of RNA was used for cDNA synthesis (Qiagen). Quantitative PCR reactions were performed using the SYBR green mix (Qiagen) and quantitated using Applied Biosystems 7900 HT sequence detection system. Duplicate runs of each sample were normalized to β-actin to determine relative expression levels. The sequences for the primer pairs used in this study are listed in supplementary table 1.

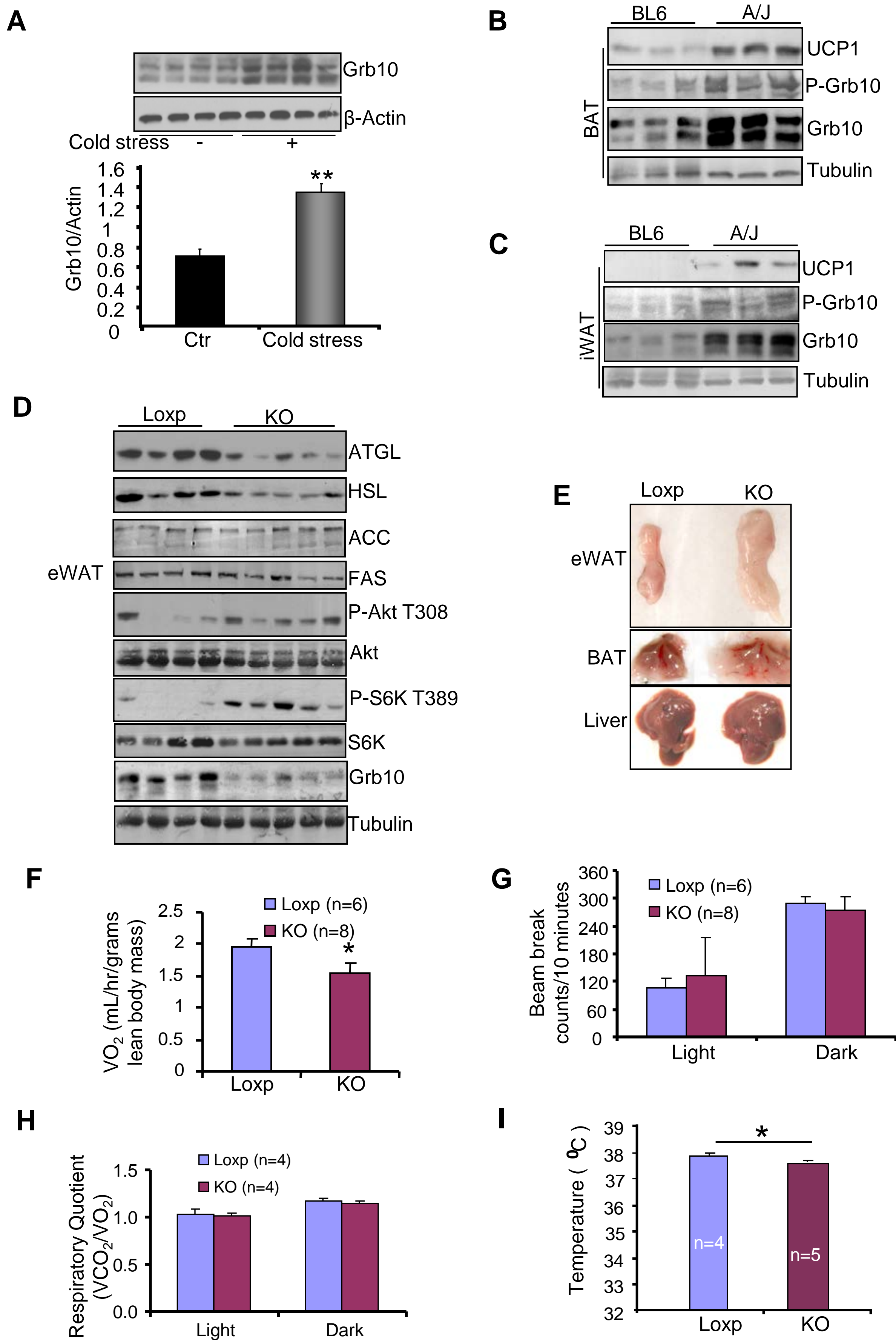
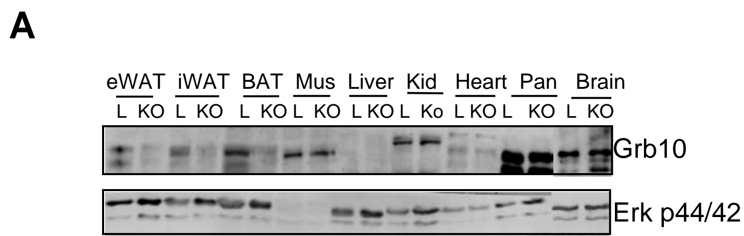
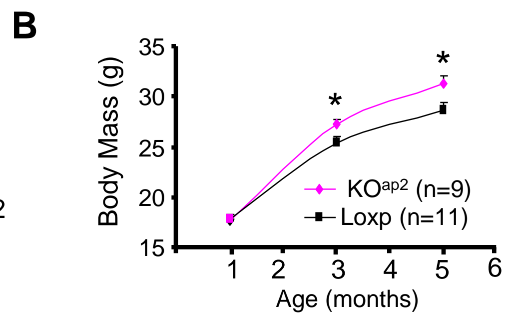
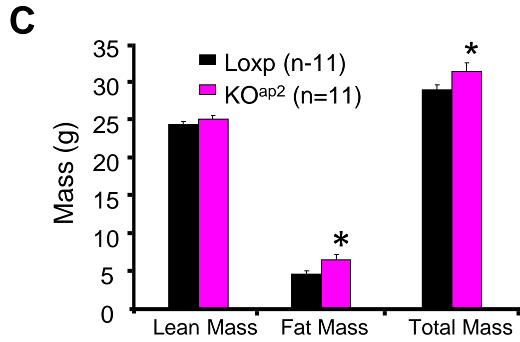


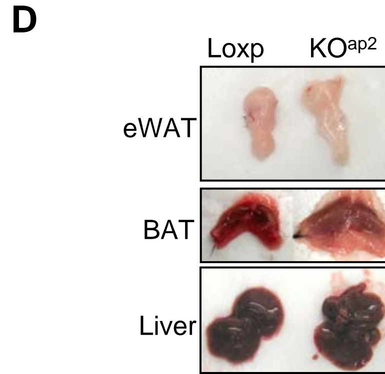
Fig.S1

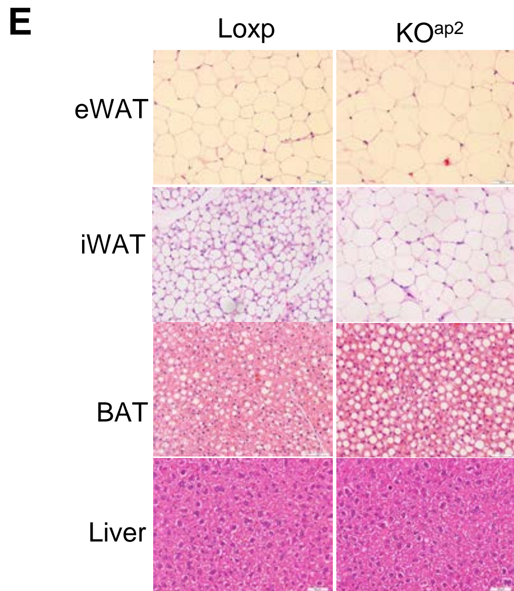
Fig. S1 The expression level of Grb10 is positively associated with lipolysis, thermogenesis, and energy expenditure (Related to Fig. 1, Fig. 2 and Fig. 4). (A) Grb10 expression was induced by cold stress (4 ^{o}C, 4hrs/day, 4 days) in the interscapular BAT of male rats. ** p<0.01 (n=4). The cellular levels of Grb10, phosphor-Grb10 at $Ser^{501/503}$, and UCP1 were upregulated in BAT **(B)** and iWAT **(C)** of A/J mice (n=3) compared to C57BL/6 mice (n=3). **(D)** The expression of key enzymes involved in lipid metabolism and the activities of Akt and mTOR in eWAT of $Grb10^{fKO}$ and control mice. (**E**) A representative photograph of eWAT, interscapular BAT, and liver from 5 month-old $Grb10^{fKO}$ and control mice are shown. 5-month-old $Grb10^{fKO}$ mice and Loxp control mice were housed in individual metabolic cages for 48 h, and the resting metabolic rate (**F**), total activity (**G**), and the respiratory quotient (VCO_2/VO_2) (**H**) of $Grb10^{fKO}$ mice and control mice were measured. Results shown are means ± SEM, monitored during light and dark hours. *P < 0.05. **(I)** The average of core body temperature of $Grb10^{fKO}$ mice and control mice measured with Mini-mitter telemetry system at 24°C within 24 hours. Data are presented as mean ± S.E.M. *P<0.05.

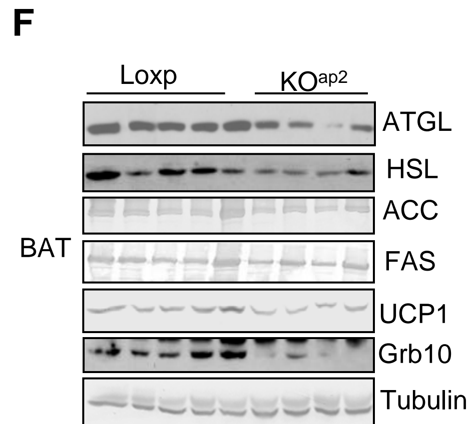


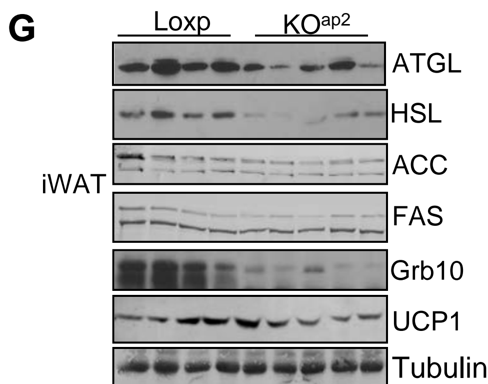


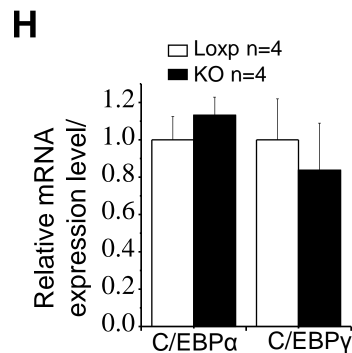












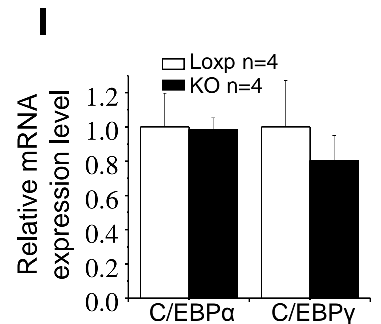


Fig.S2

Fig. S2. Fat-specific disruption or suppression of Grb10 leads to impaired lipolysis and fat expansion (Related to Fig. 2). The $Grb10^{fKO-aP2}$ (KO^{ap2}) mice were generated by crossing the Grb10 floxed mice with the aP2 (Fabp4)-cre mice. Male mice were used in all experiments. **(A)** The expression levels of Grb10 in different tissues in KO^{ap2} and Loxp control mice. KO^{ap2} and control mice at the age of 3 months were euthanized. Various tissues were collected from the mice and homogenized for Western blot analysis. Mus, muscle; Kid, kidney; Pan, Pancreas. **(B)** Body weight gains of KO^{ap2} and Loxp control mice. **(C)** Tissue composition of the KO^{ap2} (n=11) and control littermates (n=11) was analyzed by dual-energy x-ray absorptiometry (DEXA). A representative photograph (**D**) and hemotoxylin and eosin (H&E) staining (**E**) of eWAT, interscapular BAT, and liver tissues from 5 month-old KO^{ap2} and Loxp control mice. The expression levels of ACC, FAS, ATGL, HSL, and UCP1 in BAT **(F)** and iWAT **(G)** of KO^{ap2} and control mice was determined by Western blot using specific antibodies as indicated. The mRNA levels of differentiation markers such as C/EBPα and C/EBPβ in BAT **(H)** and iWAT **(I)** of $Grb10^{fKO}$ and control mice were determined by RT-PCR and normalized to β-actin. All data in 2B, 2C, 2H and 2I are presented as mean ± S.E.M. $*P<0.05$.

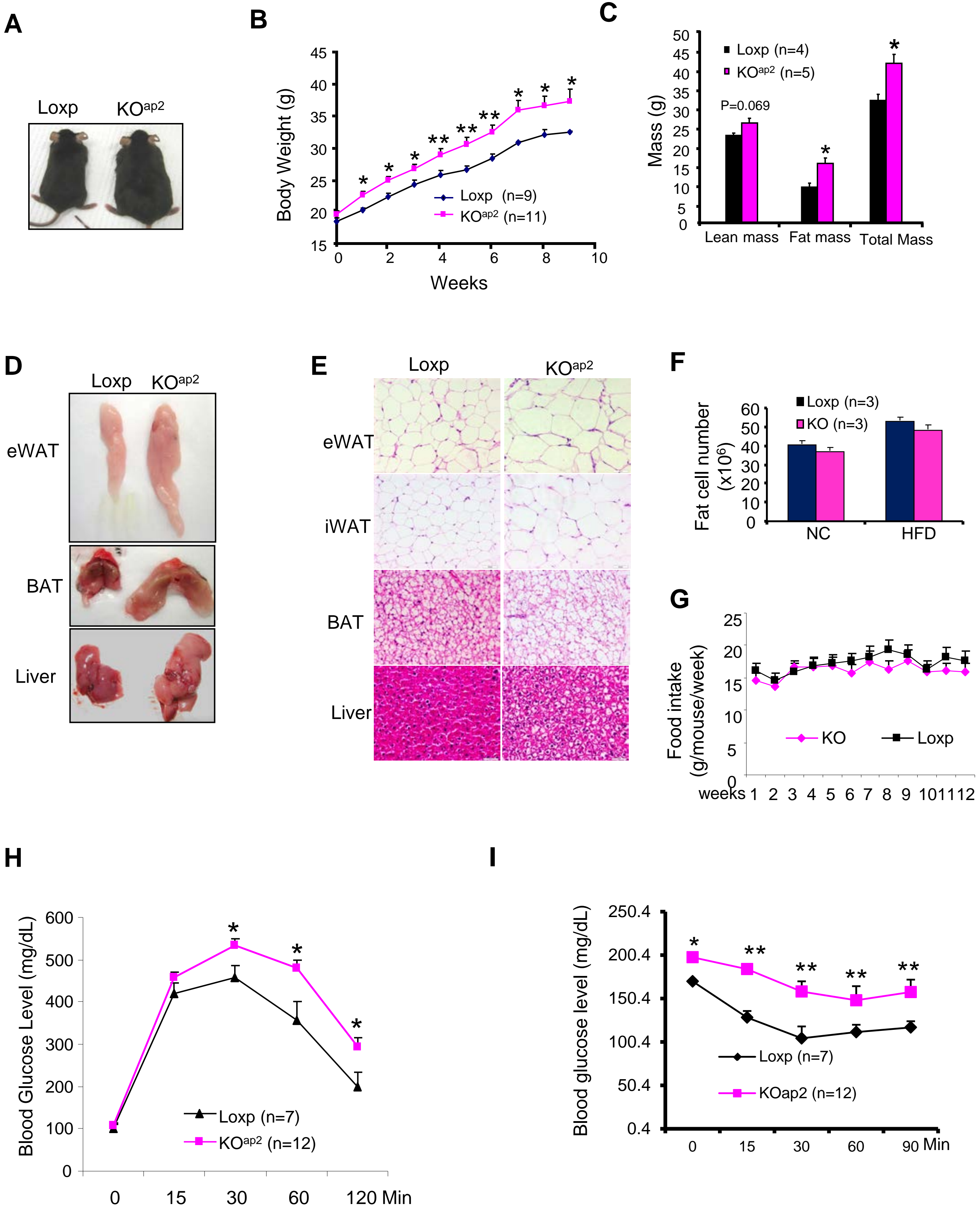


Fig.S3

Fig. S3. Fat tissue-specific disruption of Grb10 exacerbated diet-induced obesity and insulin resistance (Related to Fig.3). (A) A representative photograph of 5 week-old $Grb10^{fKO-aP2}$ (KO^{ap2}) mice and control mice fed with HFD for 12 weeks. **(B)** Body weight gain of KO^{ap2} and control mice during HFD feeding. **(C)** Tissue composition of HFD-fed KO^{ap2} (n=5) and control littermates (n=4). A representative image **(D)** and H&E staining (**E**) of eWAT, BAT, and liver tissues of HFD-fed KO^{ap2} and control mice. **(F)** Fat cell numbers of HFD-fed KO^{ap2} and control mice were determined as described in Experimental Procedures. **(G)** Food intake of KO^{ap2} and control mice under HFD feeding condition. Glucose tolerance test (GTT) **(H**) and insulin tolerance test (ITT) **(I)** were performed on HFD-fed KO^{ap2} and control mice. All data in 3B, 3C, and 3F-3I were presented as mean ± S.E.M. ANOVA was used for statistical analysis in 3H and 3I. The t-test was used for statistical analysis of data in 3B, 3C and 3F-3G. *P<0.05; **P<0.01.

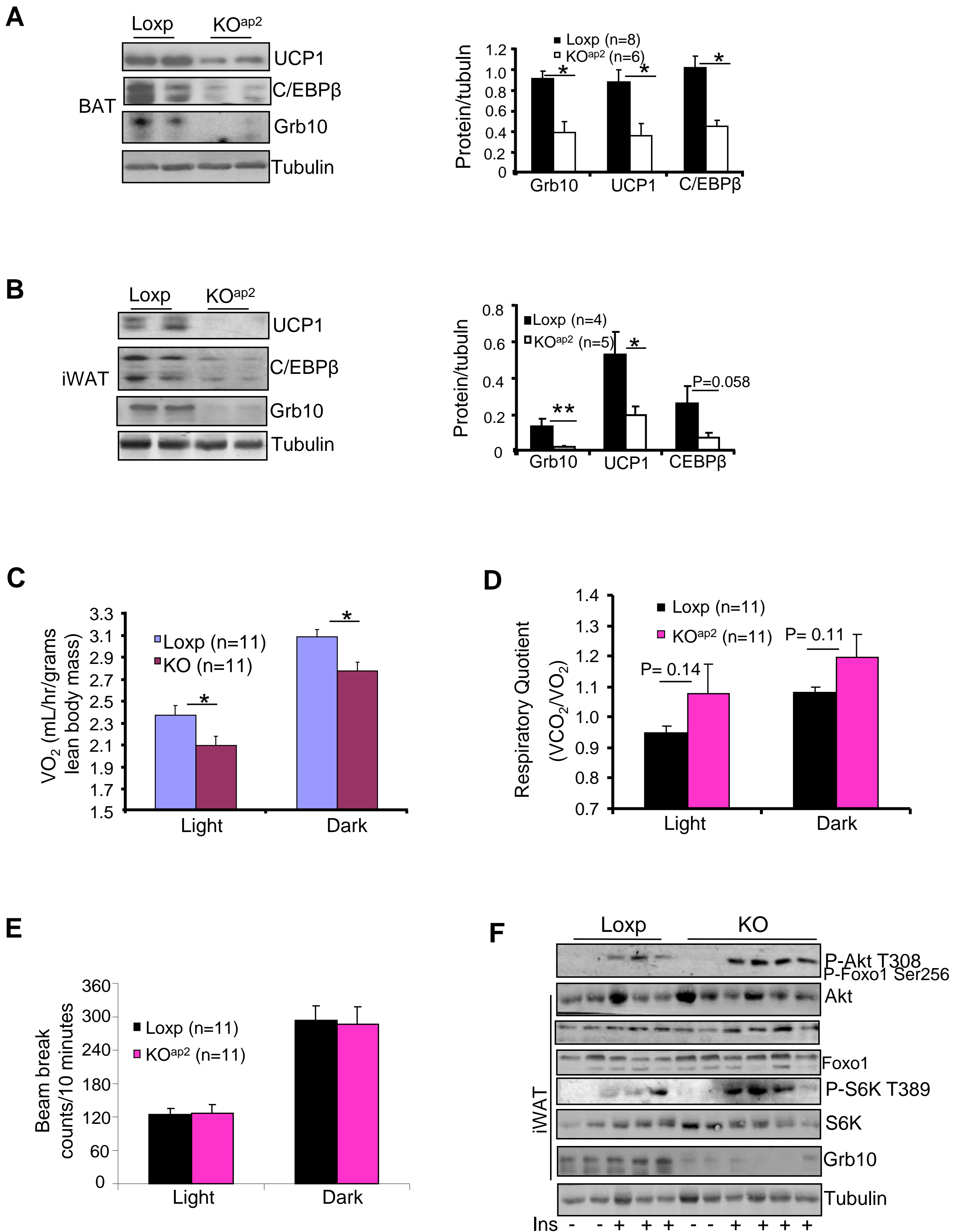


Fig.S4

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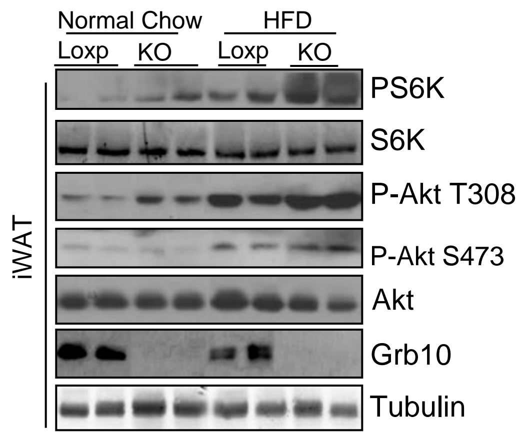

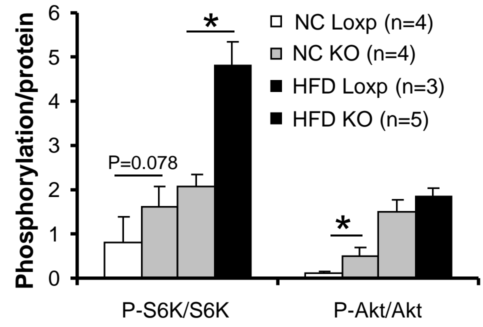

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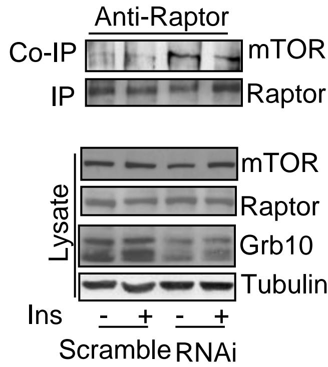

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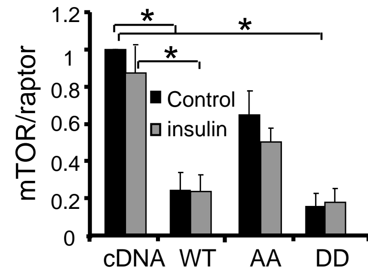

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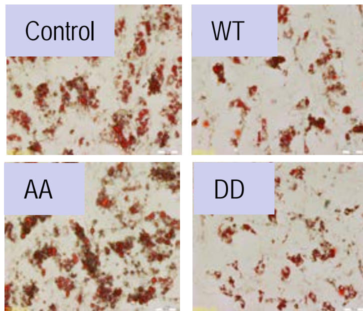
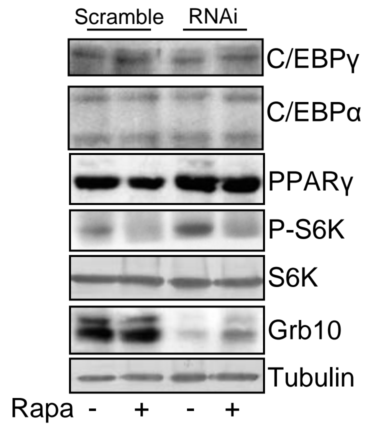
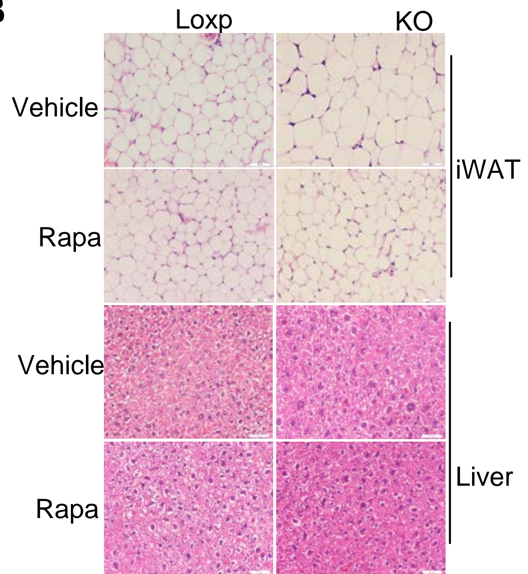
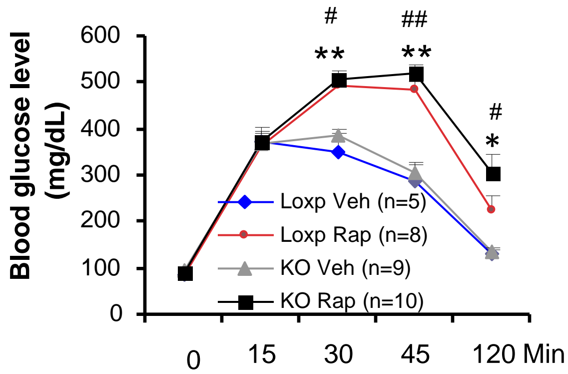


Fig.S4

Fig. S4. Fat-specific disruption of Grb10 leads to super activation of mTORC1 and impaired thermogenic function in BAT and iWAT (Related to Fig. 4, Fig. 5 and Fig. 6). The expression of UCP1 and C/EBPβ in BAT **(A)** and iWAT **(B)** of KO^{ap2} and control mice. Five month-old KO^{ap2} (n=5) and control mice (n=6) were euthanized and various adipose tissues were collected and homogenized for Western blot analysis. Tubulin was used as loading control. 5-month-old $Grb10^{fKO}$ mice and Loxp control mice were housed in individual metabolic cages for 48 hrs, and oxygen consumption, which is normalized to lean body mass (**C**), respiratory quotient (**D**), and total activity (**E**) of $Grb10^{fKO\text{-}ap2}$ mice and control mice were measured. Results shown are means ± SEM, monitored during light and dark hours. *P < 0.05. **(F)** Insulin-stimulated Akt and S6K phosphorylation in iWAT of $Grb10^{fKO}$ (KO) and Loxp control mice. **(G)** Phosphorylation of S6K and Akt in iWAT of $Grb10^{fKO}$ and Loxp control mice (3-5 mice/treatment group) under normal chow and HFD feeding conditions. Western blot images were semi-quantified and data were presented as mean ± S.E.M. *P<0.05. (**H**) Grb10-suppressed brown preadipocytes (RNAi) and scramble control cells were serum starved and treated with or without 10 nM insulin for 20 min. Raptor was immunoprecipitated from cell lysates and co-immunoprecipitated mTOR was detected by Western blot. Data are representative of three independent experiments with a similar result. **(I)** The ratio of co-immunoprecipitated mTOR to immunoprecipitated raptor shown in Fig. 6F was quantified. (**J**) Oil red O staining of Grb10-deficient primary brown adipocytes transfected with vector or vectors encoding wild-type (WT) Grb10, $Grb10^{S501/503A}$ (AA) or $Grb10^{S501/503D}$ (DD).







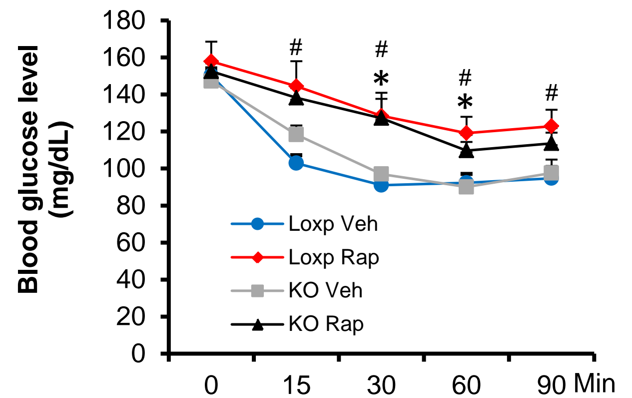


Fig.S5

Fig. S5. Rapamycin administration reduced fat mass gain but induced insulin resistance (Related to Fig. 7). (A) The effect of rapamycin treatment on differentiation markers such as PPARγ, C/EBPα, and C/EBPγ in Grb10-suppressed brown adipocytes. Grb10 scramble and suppressed brown preadipocytes were differentiated into adipocytes. 48 hours post induction of differentiation, the cells were treated with or without 5 nM rapamycin for 72 hours. Data are representative of three independent experiments with a similar result. **(B)** The effect of rapamycin treatment on the fat cell size of iWAT and liver of $Grb10^{fKO}$ mice and Loxp control mice. **(C)** Glucose tolerance test (GTT) and **(D)** insulin tolerance test (ITT) were performed on $Grb10^{fKO}$ (KO) and control mice treated with or without rapamycin for 8 weeks. GTT and ITT data were presented as mean ± S.E.M. ANOVA was used for statistical analysis. *P<0.05 ($Grb10^{fKO}$ mice treated with vehicle compared to $Grb10^{fKO}$ mice treated with rapamycin); #P<0.05 (Loxp control mice treated with vehicle compared with Loxp control mice treated with rapamycin).