Skp2 Controls Adipocyte Proliferation during the Development of Obesity*

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The increase in the mass of adipose tissue during the development of obesity can arise through an increase in cell size, an increase in cell number, or both. Here we show that long term maintenance of C57BL/6 mice on a high fat diet (for ~25 weeks) induces an initial increase in adipocyte size followed by an increase in adipocyte number in white adipose tissue. The latter effect was found to be accompanied by up-regulation of expression of the gene for the F-box protein Skp2 as well as by down-regulation of the cyclin-dependent kinase inhibitor p27Kip1, a principal target of the SCF^Skp2 ubiquitin ligase, in white adipose tissue. Ablation of Skp2 protected mice from the development of obesity induced either by a high fat diet or by the lethal yellow agouti (A^y) mutation, and this protective action was due to inhibition of the increase in adipocyte number without an effect on adipocyte hypertrophy. The reduction in the number of adipocyte caused by Skp2 ablation also inhibited the development of obesity-related insulin resistance in the A^y mutant mice, although the reduced number of β cells and reduced level of insulin secretion in Skp2-deficient mice resulted in glucose intolerance. Our observations thus indicate that Skp2 controls adipocyte proliferation during the development of obesity.

The worldwide epidemic of obesity is a serious threat to public health, in part because the increase in the mass of white adipose tissue (WAT) in obese individuals increases the risk for development of insulin resistance and type 2 diabetes mellitus (1). The expansion of WAT during the development of obesity can occur through increases in cell number (adipocyte hyperplasia) and in cell size (adipocyte hypertrophy) (2, 3). However, the precise contribution of adipocyte number to the pathogenesis of obesity and obesity-related insulin resistance remains unclear.

The number of adipocytes is thought to increase as a result of the proliferation of preadipocytes and their subsequent differentiation into mature adipocytes (4, 5). Both the proliferation and differentiation of preadipocytes are characterized by marked changes in the pattern of gene expression that are achieved by the sequential induction of various transcription factors, including members of the CCAAT/enhancer-binding protein (C/EBP-α, -β, and -δ), peroxisome proliferator-activated receptor (PPAR-γ, -β, and -δ), basic helix-loop-helix (SREBP-1c), and Kruppel-like zinc-finger factor (KLF-5 and -15) families (6, 7). These proteins are thought to act synergistically in the transcriptional activation of a variety of adipocyte-specific genes, with each also reciprocally activating the expression of the others (8, 9).

The cell cycle plays an important role in adipogenesis, given that inhibition of DNA synthesis prevents the differentiation of 3T3-L1 preadipocytes into adipocytes (10). Cell proliferation is regulated at each phase of the cell cycle by the activation and deactivation of various cell cycle-related proteins, including cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs) (11, 12). CDK4 and CDK2 regulate the transition from G1 to S phase of the cell cycle as well as progression of S phase by phosphorylating substrates such as the retinoblastoma protein (Rb) (13, 14). Phosphorylation of Rb induces its dissociation from the E2F1 complex, the latter of which then promotes cell cycle progression (15). Evidence suggests that CDK2 (16), CDK4 (17), Rb (18), and E2F1 (19) are essential not only for cell proliferation but also for differentiation during adipogenesis. CKIs include two families of proteins, the Cip (Kip) family and Ink4 family, and are central players in the exit of cells from the cell cycle (20). The loss of p27Kip1 or p21Cip1 in mice leads to adipocyte hyperplasia as a result of increased proliferation or recruitment of preadipocytes (21), suggesting that

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‡ The abbreviations used are: WAT, white adipose tissue; CDK, cyclin-dependent kinase; Cki, cyclin-dependent kinase inhibitor; Rb, retinoblastoma protein; SCF, Skp1-Cullin-F-box protein; PDK1, phosphoinositide-dependent kinase 1; Erk, extracellular signal-regulated kinase; E3, ubiquitin-protein isopeptide ligase.
these CKIs are important in regulation of adipocyte number. The SCF (Skp1-Cullin-F-box protein) ubiquitin ligase (E3) complex targets CKIs for degradation by the 26 S proteasome and thereby regulates cell cycle progression (22). Skp2, the substrate-binding subunit of the SCF complex, contributes to the degradation of p27kip1, an inhibitor of CDK2 and CDK1 activities that promote entry into S phase and mitosis, respectively. The abundance of Skp2 is increased in various human cancers, in which its expression is inversely related to that of p27kip1 (23).

We have now investigated the role of Skp2 in adipogenesis and show here that the amount of Skp2 mRNA is increased in WAT during the development of obesity in mice. Our genetic analyses further suggest that Skp2 controls adipocyte proliferation during the development of this condition in mice.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Other Reagents**—Antibodies to Skp2 and to Erk1 or Erk2 (Erk1/2) were obtained from Zymed Laboratories Inc. (San Francisco, CA) and Cell Signaling Technologies (Beverly, MA), respectively. Antibodies to p27kip1 and to cyclin E1 were from Santa Cruz Biotechnology (Santa Cruz, CA). An adenovirus encoding Skp2 was generated as described previously (25).

**Animals**—Male C57BL/6 and KK y/TaJcl mice were obtained from CLEA Japan. Skp2 knock-out mice were generated as described previously (24). Parental Skp2+/− mice used to generate Skp2+/+, Skp2−/−, and Skp2+/−/− littermates for the present study were derived by backcrossing the C57BL/6/129SV knock-out strain onto the C57BL/6 background for seven or eight generations. We crossed KKAy/TaJcl mice with Skp2+−/+ mice on the C57BL/6 background and then crossed resulting male A+/−;Skp2+/− and female +/+; Skp2−/− animals to obtain male A+/−;Skp2−/− and A+/−;Skp2+/− littermates on the C57BL/6 and KK hybrid background. Only male mice were used for experiments. For examination of the effects of a high fat diet, mice were fed from 4 weeks of age with chow containing 30% fat by weight as described previously (26). Experiments with mice were performed according to the guidelines of the ethical committee of Kobe University Graduate School of Medicine.

**Determination of Adipocyte Size and Number**—Samples of mouse adipose tissue for determination of cell size were obtained from the epididymal or subcutaneous fat pads. The tissue (∼100 mg) was fixed with osmium tetroxide (Sigma) as described (27), suspended in isotonic saline, and passed through a 250-μm nylon filter to remove fibrous elements. The filtrate was then passed through a 250-μm osmium tetroxide (Sigma) as described (27), suspended in isotonic saline, and passed through a 250-μm nylon filter to remove fibrous elements. The filtrate was then passed through a 250-μm osmium tetroxide (Sigma) as described (27), suspended in isotonic saline, and passed through a 250-μm nylon filter to remove fibrous elements. The filtrate was then passed through a 250-μm osmium tetroxide (Sigma) as described (27), suspended in isotonic saline, and passed through a 250-μm nylon filter to remove fibrous elements. The filtrate was then passed through a 250-μm osmium tetroxide (Sigma) as described (27), suspended in isotonic saline, and passed through a 250-μm nylon filter to remove fibrous elements. The filtrate was then passed through a 250-μm osmium tetroxide (Sigma) as described (27), suspended in isotonic saline, and passed through a 250-μm nylon filter to remove fibrous elements. The filtrate was then passed through a 250-μm osmium tetroxide (Sigma) as described (27), suspended in isotonic saline, and passed through a 250-μm nylon filter to remove fibrous elements. The filtrate was then passed through a 250-μm osmium tetroxide (Sigma) as described (27), suspended in isotonic saline, and passed through a 250-μm nylon filter to remove fibrous elements. The filtrate was then passed through a 250-μm osmium tetroxide (Sigma) as described (27), suspended in isotonic saline, and passed through a 250-μm nylon filter to remove fibrous elements.

The tissue weight of the stromal-vascular fraction, including preadipocytes, is 5% of WAT weight, the total adipocyte volume (milliliters), the latter of which was determined from the average value of adipocyte diameter directly measured with the Coulter counter.

**Immunoblot and Quantitative Reverse Transcription-PCR Analyses**—Immunoblot analysis was performed as described previously (30, 31). Reverse transcription and real-time PCR analysis was also performed as described (32) with a Sequence detector (model 7900, Applied Biosystems) and with 36B4 mRNA as the invariant control. The primers (upstream and
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downstream, respectively) were 5’-AGAAAATCTTCTCGGC-CGGTCAATCA-3’ and 5’-CTCCACCTCTGCGA-CTATCTGC-3’ for p27kip1 mRNA, 5’-AAGCTC-3’ and 5’-GTTC-3’ for p27kip1 mRNA, and 5’-ATGAGCTTGCAATCA-3’ and 5’-AGAAATCTCTTCGGC-3’ for cyclin E1 mRNA. The primers for mouse 36B4 mRNA were as described previously (32).

Histology and Immunostaining—The pancreas and adipose tissue of mice were fixed overnight in buffered 10% formaldehyde, embedded in paraffin, and sectioned at a thickness of ~5 μm. The sections were mounted on Silane-treated slides and either stained with hematoxylin-eosin or subjected to immunostaining with antibodies to insulin and to glucagon (Dako, Kyoto, Japan). Immune complexes were detected with Cy3- or fluorescein isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Images were acquired with a confocal laser-scanning microscope (LSM5 PASCAL, Carl Zeiss). The size of pancreatic islets and that of β cells (islet size/β cell number) were determined by analysis of at least 50 islets per animal with LSM Image Browser software (Carl Zeiss). Islet density (total islet area/pancreas area) was determined with an image analyzer (Image Processor for Analytical Pathology, Sumika Technoservice, Hyogo, Japan). Sections of the pancreas were also stained with antibodies to insulin (Santa Cruz Biotechnology), horseradish peroxidase-conjugated secondary antibodies, and diaminobenzidine (DAB substrate kit, Dako).

Glucose Tolerance Test, Insulin Tolerance Test, and Measurement of Insulin Release—Mice were deprived of food for 16 h before oral loading with glucose (2 g per kilogram of body mass) for a glucose tolerance test, during which blood samples were collected at various times (0–120 min). An insulin tolerance test was performed with mice in the randomly fed state; blood glucose concentration was determined at various times (0–120 min) after intraperitoneal injection of human regular insulin (0.75 units/kg). Insulin release was measured as described previously (33) with slight modifications. Mice that had been deprived of food for 16 h were thus subjected to intraperitoneal injection of glucose (3 g/kg), the plasma concentration of insulin was measured at various times (0–30 min) thereafter, and insulin release was evaluated from the area under the curve between 0 and 15 min.

Analysis of Metabolic Parameters—Blood glucose level was determined with a glucometer (Glutest Pro, Sanwa Kagaku Kenkyusho, Nagoya, Japan), and plasma insulin concentration was measured with an enzyme-linked immunosorbent assay kit and a mouse insulin standard (Shibayagi, Gunma, Japan). Serum leptin was measured with an enzyme-linked immunosorbent assay kit for mouse leptin (Morinaga, Tokyo, Japan).

Statistical Analysis—Data are presented as means ± S.E. Differences between two groups were evaluated by Student’s unpaired two-tailed t test as performed with StatView software. A p value of <0.05 was considered statistically significant.

TABLE 1

| Parameter                  | Skp2+/+ | Skp2+/− | Skp2−/− |
|----------------------------|---------|---------|---------|
| Body weight (g)            | 32.1 ± 0.7 | 34.9 ± 2.4 | 21.6 ± 0.4* |
| WAT/BW (mg/g)              | 20.5 ± 2.5 | 19.1 ± 1.7 | 14.7 ± 1.2b |
| Epididymal WAT             | 13.1 ± 0.9 | 12.8 ± 0.6b | 10.3 ± 0.6a |
| Subcutaneous WAT           | 6.9 ± 1.0 | 7.1 ± 0.7 | 4.1 ± 0.4b |
| Retropitoneal WAT          | 4.6 ± 0.1 | 4.7 ± 0.1 | 4.2 ± 0.1 |
| BAT/BW (mg/g)              | 46.5 ± 0.8 | 45.1 ± 1.0 | 46.7 ± 1.7 |
| Liver/BW (mg/g)            | 10.2 ± 0.5 | 11.1 ± 0.3 | 9.1 ± 1.1 |
| Muscle/BW (mg/g)           | 2.9 ± 0.2 | ND* | 2.9 ± 0.1 |
| Spleen/BW (mg/g)           | 3.0 ± 0.3 | 8.3 ± 0.4 | 6.9 ± 0.4a |

* p < 0.005 versus Skp2+/− mice.
* p < 0.05 versus Skp2+/− mice.
* ND, not determined.

FIGURE 2. Effects of loss of Skp2 on the numbers of adipocytes in WAT and β cells in the pancreas. A–D, hematoxylin-eosin-stained sections of (A) as well as adipocyte size distribution (B), mean diameter of adipocytes (C), and relative number of adipocytes (D) in epididymal WAT of Skp2+/+ and Skp2−/− mice at 20 weeks of age. Scale bar in A, 100 μm. Data in B–D are means (±S.E.) of values from four mice of each genotype. *, p < 0.05. E and F, islet density (E) and mean islet size (F) in Skp2+/+ and Skp2−/− mice at 20 weeks of age. Data are means ± S.E. of values from six mice of each genotype. G, hematoxylin-eosin (H/E) staining and immunofluorescence staining for insulin (red) and glucagon (green) in pancreatic sections of Skp2+/+ and Skp2−/− mice at 20 weeks of age. Scale bars, 20 μm. H and I, size (H) and number (I) of β cells in Skp2+/+ and Skp2−/− mice at 20 weeks of age. Data are means ± S.E. of values from five mice of each genotype. *, p < 0.05; ***, p < 0.005.
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RESULTS

Effect of a High Fat Diet on Adipocyte Number in WAT—We first examined the size and number of adipocytes in WAT of C57BL/6 mice fed a high fat diet or regular chow from 4 weeks of age. Maintenance of mice on the high fat diet increased body weight and the weight of epididymal WAT by 9.0 and 0.45 g, respectively, after 10 weeks and by 19.9 and 1.3 g, respectively, after 26 weeks, compared with mice fed the normal diet (Fig. 1, A and B). The high fat diet also induced a marked increase in the size of adipocytes (Fig. 1C) but had no effect on adipocyte number (Fig. 1D) in epididymal WAT after 10 weeks. In contrast, after 26 weeks, the high fat diet increased both adipocyte size and number (Fig. 1, C and D). The size of adipocytes did not differ significantly, however, between mice fed the high fat diet for 10 or 26 weeks. These results thus showed that an increase in adipocyte size precedes an increase in adipocyte number in C57BL/6 mice fed a high fat diet.

The loss of p27Kip1 in mice results in adipocyte hyperplasia as a result of increased proliferation or recruitment of preadipocytes (21). We therefore examined whether the high fat diet affected p27Kip1 expression in WAT. Maintenance of C57BL/6 mice on the high fat diet for 10 weeks, which had no effect on adipocyte number, did not affect the abundance of p27Kip1 in epididymal WAT, as compared with that apparent in animals fed the normal diet (Fig. 1E). In contrast, feeding with the high fat diet for 26 weeks resulted in a marked down-regulation of the amount of p27Kip1 in WAT without an effect on the amount of the corresponding mRNA (Fig. 1, E and F). Although Skp2 was not detected in epididymal WAT by immunoblot analysis, quantitative reverse transcription-PCR analysis revealed that the amount of Skp2 mRNA in this tissue was increased in mice fed the high fat diet for 26 weeks compared with that in those fed normal chow (Fig. 1, E and F). Similar results with regard to the effects of the high fat diet on p27Kip1 and Skp2 expression were obtained with subcutaneous WAT (data not shown). Feeding of mice with the high fat diet for 26 weeks also induced a slight decrease in the abundance of cyclin E1, another substate of SCFSkp2 (23), in epididymal WAT without a significant effect on the amount of the corresponding mRNA (Fig. 1, E and F). The high fat diet thus both increased the expression of Skp2 and down-regulated that of p27Kip1 and cyclin E1 in WAT, and these effects were accompanied by an increase in adipocyte number.

Adipocyte and Pancreatic β Cell Numbers in Skp2<sup>−/−</sup> Mice—To investigate whether the loss of Skp2 affects the proliferative response of adipocytes to a high fat diet, we first determined WAT weight in Skp2<sup>−/−</sup> mice fed with normal chow. As previously described (25), Skp2<sup>−/−</sup> mice are smaller than their Skp2<sup>+/+</sup> littersmates; their body weight was thus reduced by ~30% compared with that of wild-type animals at 20 weeks of age (Table 1). The Skp2<sup>−/−</sup> mice at 20 weeks of age were also lean, as reflected in marked reductions in the ratios of epididymal, subcutaneous, or retroperitoneal WAT weight to body weight. Neither body weight nor the ratios of WAT weight to body weight differed significantly between Skp2<sup>−/−</sup> and Skp2<sup>+/+</sup> mice. Measurement of adipocyte diameter revealed that adipocytes of epididymal WAT were slightly larger in Skp2<sup>−/−</sup> mice than in Skp2<sup>+/+</sup> mice (Fig. 2, A–C). The number of adipocytes in epididymal WAT was reduced in Skp2<sup>−/−</sup> mice compared with that in Skp2<sup>+/+</sup> mice (Fig. 2D), and this difference thus appeared to be responsible for the difference in WAT mass.

There were no significant differences in the ratios of brown adipose tissue, liver, muscle (soleus plus gastrocnemius), or spleen weight to body weight among Skp2<sup>+/+</sup>,

![Table 2: Blood glucose and plasma insulin concentrations of Skp2<sup>+/+</sup>, Skp2<sup>+/−</sup>, and Skp2<sup>−/−</sup> mice fed high fat or regular diets](image)

| Parameter                  | Regular diet | High fat diet |
|----------------------------|--------------|--------------|
|                            | Skp2<sup>+/+</sup> | Skp2<sup>+/−</sup> | Skp2<sup>−/−</sup> |
| Glucose, fasting (mg/dl)   | 80 ± 4       | 74 ± 2       | 88 ± 7       |
| Glucose, fed (mg/dl)       | 138 ± 3      | 137 ± 2      | 135 ± 2      |
| Insulin, fasting (ng/ml)   | 0.12 ± 0.03  | 0.07 ± 0.03  | 0.24 ± 0.07  |
| Insulin, fed (ng/ml)       | 0.52 ± 0.11  | ND           | 0.49 ± 0.05  |
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Skp2+/− and Skp2−/− mice (Table 1). However, the ratio of the weight of the pancreas to body weight was significantly smaller in Skp2−/− mice than in their wild-type littermates. A detailed histological analysis of pancreatic sections revealed no differences in islet density (Fig. 2E) or islet size (Fig. 2, F and G) between Skp2+/− and Skp2−/− mice. In contrast, β cells were hypertrophic, manifesting abnormalities such as nuclear enlargement, and were reduced in number in Skp2−/− mice (Fig. 2, H and I). These mutant mice thus have reduced numbers of adipocytes in WAT and of β cells in the pancreas compared with Skp2+/− mice.

Glucose Intolerance in Skp2−/− Mice—The blood glucose and plasma insulin concentrations in the fasted or fed state did not differ significantly between Skp2−/− and Skp2+/− mice at 11–12 weeks of age (Table 2). However, an oral glucose tolerance test revealed marked glucose intolerance in Skp2−/− mice (Fig. 3A). An insulin tolerance test showed that Skp2−/− and Skp2+/+ mice were similarly sensitive to the glucose-lowering effect of exogenous insulin (Fig. 3B). To evaluate further the function of pancreatic β cells in Skp2−/− mice, we measured the release of insulin in response to glucose stimulation (33). The insulin secretory response of Skp2−/− mice was reduced compared with that of Skp2+/+ mice as early as 2 min after intraperitoneal injection of glucose, and this difference remained apparent for up to 30 min (Fig. 3C). The area under the curve for the plasma insulin concentration between 0 and 15 min after glucose injection was significantly smaller for Skp2−/− mice than for their wild-type littermates (Fig. 3D). These data indicated that the reduced number of β cells in Skp2−/− mice is reflected in a reduced insulin secretory response to glucose.

Resistance of Skp2−/− Mice to Diet-induced Obesity—Wild-type mice fed the high fat diet gained 11.0 and 9.9 g in body weight between 8 and 16 weeks and between 20 and 28 weeks of age, respectively (Fig. 4, A and B). Similarly, Skp2−/− mice on this diet gained 11.1 and 8.7 g from 8 to 16 weeks and from 20 to 28 weeks, respectively. In contrast, Skp2+/− mice fed the high fat diet gained only 6.8 g from 8 to 16 weeks and 2.5 g from 20 to 28 weeks of age. The gain in the weight of WAT induced by the high fat diet was also significantly smaller for Skp2+/− mice than for their wild-type littermates (Fig. 4C). Whereas the size of adipocytes in epididymal WAT was similar for 28-week-old Skp2+/− and Skp2−/− mice, it had no such effects in that of Skp2−/− mice (Fig. 4D). An insulin tolerance test showed that Skp2+/− and Skp2−/− mice were similarly sensitive to the glucose-lowering effect of exogenous insulin (Fig. 3B). To evaluate further the function of pancreatic β cells in Skp2−/− mice, we measured the release of insulin in response to glucose stimulation (33). The insulin secretory response of Skp2−/− mice was reduced compared with that of Skp2+/+ mice as early as 2 min after intraperitoneal injection of glucose, and this difference remained apparent for up to 30 min (Fig. 3C). The area under the curve for the plasma insulin concentration between 0 and 15 min after glucose injection was significantly smaller for Skp2−/− mice than for their wild-type littermates (Fig. 3D). These data indicated that the reduced number of β cells in Skp2−/− mice is reflected in a reduced insulin secretory response to glucose.
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mice (Fig. 4G). The amounts of p27\(^{kip1}\) and cyclin E1 mRNAs in epididymal WAT did not differ between mice of the two genotypes maintained on the high fat diet (Fig. 4H). These results suggested that Skp2-dependent degradation of p27\(^{kip1}\) and cyclin E1 may be required for adipocyte proliferation in response to feeding a high fat diet.

Metabolic Phenotype of Skp2\(^{-/-}\) Mice Fed a High Fat Diet—We next investigated whether the resistance of Skp2\(^{-/-}\) mice to obesity induced by a high fat diet was accompanied by protection from diet-induced insulin resistance. No significant difference in blood glucose or plasma insulin concentrations in the randomly fed state was apparent between 8- to 16-week-old Skp2\(^{-/-}\) and Skp2\(^{+/+}\) mice maintained on the high fat diet (Fig. 5, A and B, and Table 2). Whereas, both Skp2\(^{+/+}\) (Fig. 5B) and Skp2\(^{+/-}\) (data not shown) mice fed the high fat diet manifested pronounced hyperinsulinemia at 28 weeks of age, the plasma insulin concentration of Skp2\(^{-/-}\) mice maintained on this diet did not differ between 16 and 28 weeks of age (Fig. 5B). Furthermore, in contrast to those of Skp2\(^{+/-}\) mice, pancreatic sections of Skp2\(^{-/-}\) mice showed no compensatory increase in \(\beta\) cell mass in response to the high fat diet (Fig. 5C). Although these findings suggested that the protection from obesity conferred by Skp2 ablation was associated with prevention of the induction of insulin resistance by a high fat diet, an insulin tolerance test revealed that sensitivity to exogenous insulin did not differ between 28-week-old Skp2\(^{-/-}\) and Skp2\(^{+/-}\) mice fed such a diet (Fig. 5D).

Resistance of Skp2\(^{-/-}\) Mice to Obesity Induced by the Lethal Yellow Agouti (\(A^y\)) Mutation—To confirm that loss of Skp2 protects against the development of obesity and obesity-induced insulin resistance, we generated Skp2\(^{-/-}\) mice with the lethal yellow agouti (\(A^y\)) mutation. Mice that contain this mutation are studied as a well characterized model of obesity. We thus first crossed Skp2\(^{+/-}\) mice and KA\(^y\) mice, in which the \(A^y\) mutation is present on the KK background (34). We then obtained male \(A^y^+;Skp2^{-/-}\) and \(A^y^+;Skp2^{+/-}\) littermates on the C57BL/6 and KK hybrid background by crossing \(A^y^+;Skp2^{-/-}\) males and \(+/-;
Skp2+/− females. A, blood glucose (A) and plasma insulin (B) concentrations in A+/−;Skp2−/− and A+/++;Skp2−/− littersmates at the indicated ages. Data are means ± S.E. of values from six to eight mice of each genotype. *, p < 0.05; **, p < 0.005; †, p < 0.001 versus the corresponding value for A+/++;Skp2−/− mice. C, glucose tolerance test during an oral glucose tolerance test in A+/−;Skp2−/− and A+/++;Skp2−/− mice at 12 weeks of age. Data are means ± S.E. of values from 7 to 11 mice of each genotype. *, p < 0.05; **, p < 0.005 versus the corresponding value for A+/++;Skp2−/− mice. D, glucose clearance during an insulin tolerance test in A+/−;Skp2−/− and A+/++;Skp2−/− mice at 16 weeks of age. Blood glucose concentration is expressed as a percentage of the initial value. Data are means ± S.E. of values from six or seven mice of each genotype. *, p < 0.05; †, p < 0.01 versus the corresponding value for A+/++;Skp2−/− mice. E, insulin immunostaining of pancreatic sections of A+/−;Skp2−/− and A+/++;Skp2−/− littersmates at 18 weeks of age. Sections were counterstained with hematoxylin. White scale bars, 500 μm; yellow scale bars, 100 μm.

DISCUSSION

The enlargement of WAT during the development of obesity is mediated by an increase in the size of existing adipocytes and the formation of new adipocytes, the latter of which is referred to herein as adipocyte proliferation. We have shown that maintenance of C57BL/6 mice on a high fat diet for 10 weeks induced adipocyte hypertrophy without an increase in adipocyte number. In contrast, feeding mice such a diet for an additional 16 weeks resulted in a ~2.2-fold increase in adipocyte number without a further increase in adipocyte size. The expansion of WAT mass in mice fed a high fat diet thus appears to be characterized by an initial increase in adipocyte size, until a maximal size is reached, followed by an increase in adipocyte number.

Maintenance of mice on a high fat diet for 26 weeks also resulted in down-regulation of the expression of p27Kip1 in WAT. Regulation of p27Kip1 function is mediated in part by Skp2-dependent ubiquitination of p27Kip1, which promotes its proteasomal degradation (23). Although we were not able to detect induction of Skp2 in WAT by immunoblot analysis, the down-regulation of p27Kip1 in WAT of mice fed the high fat diet was accompanied by an increase in the amount of Skp2 mRNA. The high fat diet-induced down-regulation of p27Kip1 was also not apparent in WAT of C57BL/6 mice. Furthermore, the lack of Skp2 protected mice on such a diet from the development of obesity, and this effect was attributable to inhibition of adipocyte proliferation rather than to prevention of adipocyte hypertrophy. Recent studies have demonstrated a functional role for Skp2 in mediating p27Kip1 degradation during the replicating process of adipocyte differentiation in 3T3-L1 preadipocytes in vitro (35, 36). Taken together, these data suggest that Skp2-dependent degradation of p27Kip1 directly controls adipocyte proliferation during the development of obesity.

In addition to a reduced number of adipocytes in WAT, Skp2−/− mice fed a high fat diet manifested a reduced number of β cells in the pancreas. We have previously shown that overexpression of p27Kip1 in β cells induced hyperglycemia in mice as a result of inhibition of β cell proliferation (37). The transcription factor E2F1, which controls the G1 to S transition of the cell cycle, regulates both the growth of the pancreas and the number of β cells, but the size of β cells in E2F1 knock-out mice was found not to differ from that in wild-type animals (38). The number and size of pancreatic β
cells were recently shown to be reduced in mice that lack receptors for both insulin and insulin-like growth factor-1 in β cells as well as in those lacking phosphoinositide-dependent kinase 1 (PDK1) in these cells (39, 40). Haploinsufficiency of the gene for the transcription factor Foxo1 in these PDK1 mutant mice resulted in a marked increase in the number, but not in the size, of β cells (40). In addition, Foxo1, which is regulated by a PDK1-dependent insulin signaling pathway, functions through Skp2 to regulate tumor cell proliferation (41). These findings suggest that Skp2 may be a target of a PDK1–Foxo1 signaling pathway that regulates β cell number.

Given the role of Skp2 in cell cycle progression, the reduced numbers of adipocytes and β cells that result from Skp2 deficiency in mice fed a high fat diet likely reflect the operation of a cell-autonomous mechanism. However, given that insulin stimulates both preadipocyte replication and adipocyte differentiation in vitro (3, 42, 43), the reduced number of β cells and reduced level of insulin secretion apparent in Skp2−/− mice may contribute to the inhibition of adipocyte proliferation during feeding on a high fat diet. Conversely, it is also possible that the reduced number of adipocytes may play a role in the reduction in β cell number.

To investigate the consequences of a decrease in the number of adipocytes in WAT on whole-body insulin sensitivity, we performed an insulin tolerance test in Skp2+/+ and Skp2−/− mice with the obesity-inducing Aβ mutation. Loss of Skp2 in the Aβ mutant mice resulted in marked potentiation of the glucose-lowering effect of exogenous insulin, suggesting that the reduction in the number of adipocytes caused by Skp2 ablation in these animals inhibited the development of obesity-related insulin resistance. However, given that hyperinsulinemia per se induces insulin resistance (44) and that, in contrast to the Aβ+/+; Skp2+/+ mice, the Aβ+/−;Skp2−/− mice did not develop hyperinsulinemia, generation of adipocyte-specific Skp2 knock-out mice will be required to confirm this conclusion.

In summary, the intake of excess calories associated with feeding on a high fat diet in mice, and likely that associated with overeating in humans, results in storage of the extra energy initially through an increase in adipocyte size. Adipocytes do not have an unlimited capacity for enlargement, however, and long term intake of excess calories eventually results in an increase in adipocyte number to accommodate storage of the surplus energy. We have now shown that this increase in adipocyte number is associated with up-regulation of Skp2 expression in WAT. Furthermore, ablation of Skp2 resulted in inhibition of the increase in adipocyte number and improvement of obesity-related insulin resistance in obesity-prone mice. We therefore propose that Skp2 plays an essential role in adipocyte proliferation during the development of obesity. Identification of the mechanism responsible for the up-regulation of Skp2 expression in WAT during the development of obesity may provide a basis for new therapeutics targeted to obesity and obesity-related metabolic disorders.

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