experiments by manual removal of the vitelline membrane after exposure to a hypertonic solution[12]. Cell-attached patches were obtained with pipettes (resistance of 10–20 MΩ) filled with a solution containing 80 mM Li^+.

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Protection from obesity-induced insulin resistance in mice lacking TNF-α function

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Obesity is highly associated with insulin resistance and is the biggest risk factor for non-insulin-dependent diabetes mellitus[1–3]. The molecular basis of this common syndrome, however, is poorly understood. It has been suggested that tumour necrosis factor (TNF)-α is a candidate mediator of insulin resistance in obesity, as it is overexpressed in the adipose tissues of rodents and humans[4–10] and it blocks the action of insulin in cultured cells and whole animals[10–14]. To investigate the role of TNF-α in obesity and insulin resistance, we have generated obese mice with a targeted null mutation in the gene encoding TNF-α and those encoding the two receptors for TNF-α. The absence of TNF-α resulted in significantly improved insulin sensitivity in both diet-induced obesity and that resulting for the *ob/ob* model of obesity. The TNFα-deficient obese mice had lower levels of circulating free fatty acids, and were protected from the obesity-related reduction in the insulin receptor signalling in muscle and fat tissues. These results indicate that TNF-α is an important mediator of insulin resistance in obesity through its effects on several important sites of insulin action.

It has been demonstrated previously that expression of TNF-α in adipose tissue is elevated in a variety of experimental obesity models[4–6] and in obese humans[7–10], and might represent an important component of the link between obesity and insulin resistance[4,15]. TNF-α blocks the action of insulin through its ability to inhibit insulin receptor tyrosine kinase activity[10–14], although other mechanisms, such as the quantitative regulation of glucose transporters, have also been proposed[16,17]. To investigate the role of TNF-α in obesity-induced insulin resistance, we first generated obese mice that had no functional copy of the gene encoding TNF-α[18]. This was accomplished by placing mice homozygous for a targeted null mutation in the TNF-α gene (TNF-$\alpha^{-/-}$) and their control littermates (TNF-$\alpha^{+/+}$) on a high-fat (50% of the total calories in the form of fat) and high-caloric diet (5,286 kcal kg^{-1}, Bioserve, NJ). On this diet, both TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$ mice developed marked obesity compared with mice kept on a standard rodent diet (Fig. 1a). In both the lean (standard diet) and obese (high-fat diet) groups, the total body weights of TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$ mice were similar throughout the 16-week study. The total weight gain in the obese TNF-$\alpha^{+/+}$ animals on the high-fat diet tended to be slightly larger than that of the obese TNF-$\alpha^{-/-}$ mice (39.99 ± 0.9 versus 37.89 ± 1.12 g; Fig. 1a) but overall this difference was not significant. The average weights of the epididymal fat pad of the lean TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice were also similar (0.41 ± 0.09 versus 0.54 ± 0.06 g; Fig. 1b). In the obese group, fat pads of the TNF-$\alpha^{+/+}$ animals weighed ~22% more than those of the TNF-$\alpha^{-/-}$ mice (2.64 ± 0.15 versus 2.16 ± 0.14 g, $P < 0.05$). These results suggested that the absence of TNF-α did not have a significant effect on the development of dietary obesity, except for a potential small difference in adiposity. Further understanding of this issue requires detailed analysis of the body composition of these animals.

Because the effect of TNF-α in obesity is mainly on the action of insulin, we next investigated glucose homeostasis in TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ animals rendered obese by the high-fat diet. Measurement of fasting blood glucose levels demonstrated that all animals (lean and obese, TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$) remained euglycaemic throughout the study, although the lean TNF-$\alpha^{-/-}$ mice had the lowest fasting blood glucose levels (Fig. 2a). In contrast, fasting hyperinsulinaemia became apparent in obese TNF-$\alpha^{+/+}$ mice 4 weeks after the start of the high-fat diet and continued to increase in the following weeks (1.3 ± 0.3 ng ml^{-1} at 8 weeks and 2.38 ± 0.6 ng ml^{-1} at 12 weeks; Fig. 2b). However, the fasting

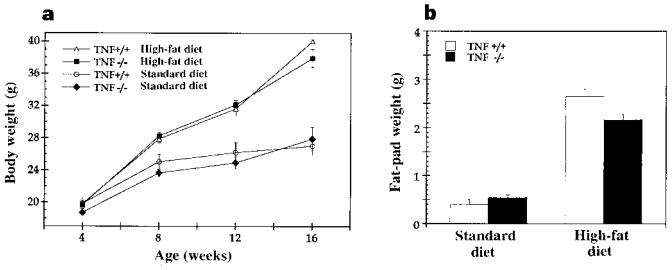

Figure 1 Growth curves and adiposity of TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$ mice. **a**, Development of diet-induced obesity in TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$ mice ($n = 10$ in each group). **b**, Epididymal fat-pad weights of TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$ mice.

insulin concentrations in obese TNF-$\alpha^{-/-}$ mice were significantly lower (0.4 ± 0.07 ng ml^{-1} at 8 weeks and 0.57 ng ml^{-1} at 12 weeks, $P < 0.001$) than those of the obese TNF-$\alpha^{+/+}$ animals (Fig. 2b), and were the same as those of the lean mice (0.41 ± 0.02 ng ml^{-1} at 8 weeks and 0.627 ± 0.2 ng ml^{-1} at 12 weeks) throughout the study. At 12 weeks, the fasting insulin levels in the obese TNF-$\alpha^{+/+}$ group were increased ~fourfold compared with those of the obese TNF-$\alpha^{-/-}$ mice (Fig. 2b). The rise in serum insulin concentrations in the presence of euglycaemia indicates strongly that there is a compensatory response to the development of obesity-induced insulin resistance in the wild-type obese mice. These results indicate that the mice deficient in TNF-α were protected from the development of obesity-induced insulin resistance that was observed in obese wild-type animals.

To investigate this possibility directly, we performed intraperitoneal insulin and glucose tolerance tests on these mice. The hypoglycaemic response to insulin was less in the obese TNF-$\alpha^{+/+}$ mice at 45–90 min than that in obese TNF-$\alpha^{-/-}$ animals (Fig. 2c). The intraperitoneal glucose tolerance tests also revealed a higher degree of hyperglycaemia in the obese TNF-$\alpha^{+/+}$ animals after 30–90 min than that in the obese TNF-$\alpha^{-/-}$ mice (Fig. 2d). Thus both of these tests indicated that there was marked insulin resistance in obese TNF-$\alpha^{+/+}$, but not in obese TNF-$\alpha^{-/-}$ mice. We did not observe any differences in the lean group between the TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice in insulin and glucose tolerance tests. These results demonstrate that the genetic absence of TNF-α can significantly

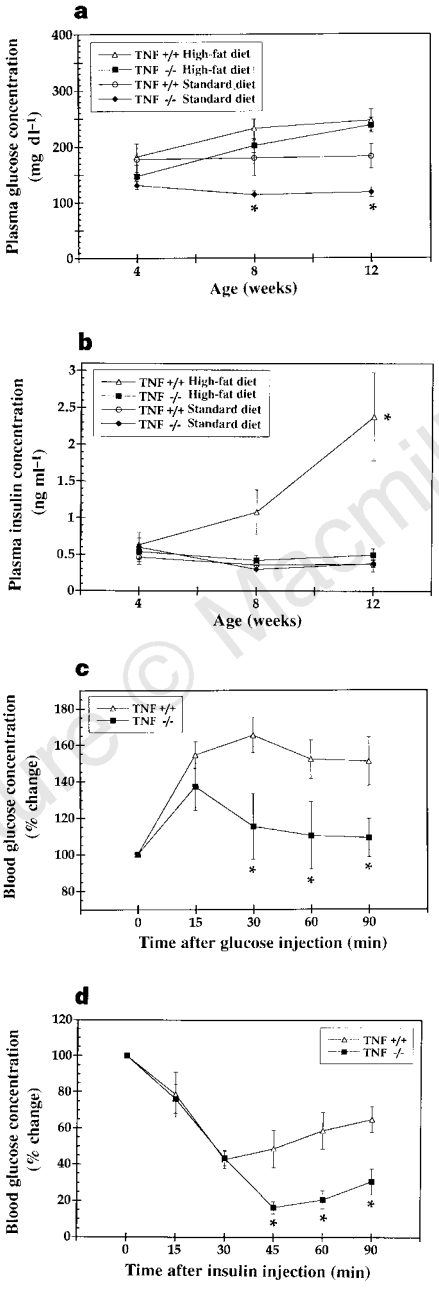

Figure 2 Measures of glucose homeostasis in TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$ mice. **a**, **b**, Fasting glucose (**a**) and insulin (**b**) concentrations. **c**, **d**, Glucose (**c**) and insulin (**d**) tolerance tests. Asterisks indicate $P < 0.05$. Investigations of the dynamics of the responses to the tolerance tests were done by ANOVA repeated measures analysis (Statview 4.01, Abacus Concepts), and demonstrated statistically significant differences between the TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$ mice in both tests ($P < 0.05$).

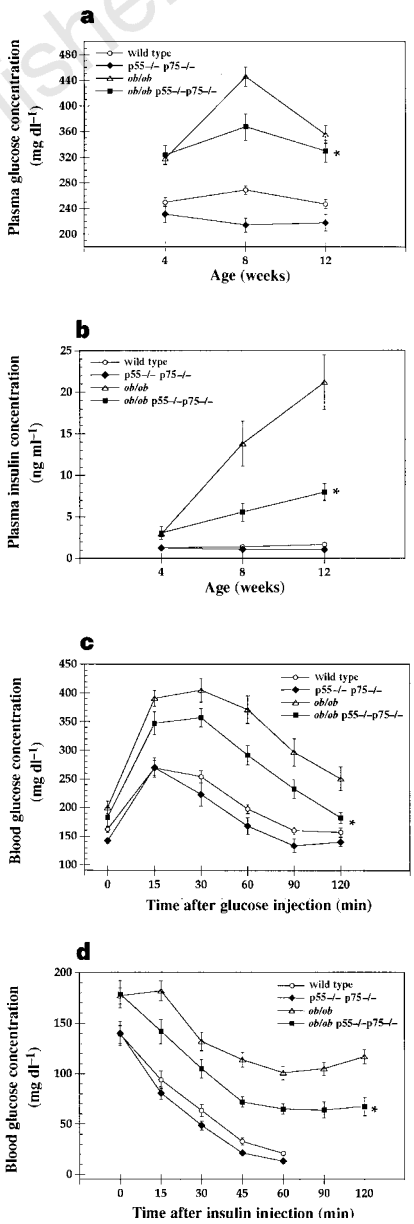

Figure 3 Measures of glucose homeostasis in *ob/ob* and *ob/ob* p55$^{-/-}$p75$^{-/-}$ mice. **a**, **b**, Fasting plasma glucose (**a**) and insulin (**b**) concentrations. **c**, **d**, Glucose (**c**) and insulin (**d**) tolerance tests. Mice studied: *ob/ob* ($n = 51$ in **a** and **b**, and 41 in **c** and **d**); *ob/ob* p55$^{-/-}$p75$^{-/-}$ ($n = 17$). Asterisks indicate $P < 0.05$.

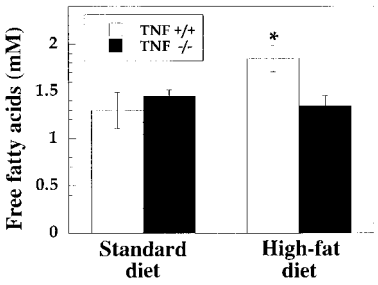

Figure 4 Circulating free fatty-acid levels in TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$ mice. Asterisk indicates $P < 0.05$; two-tailed Student's *t*-test comparing free fatty-acid levels in TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$ mice.

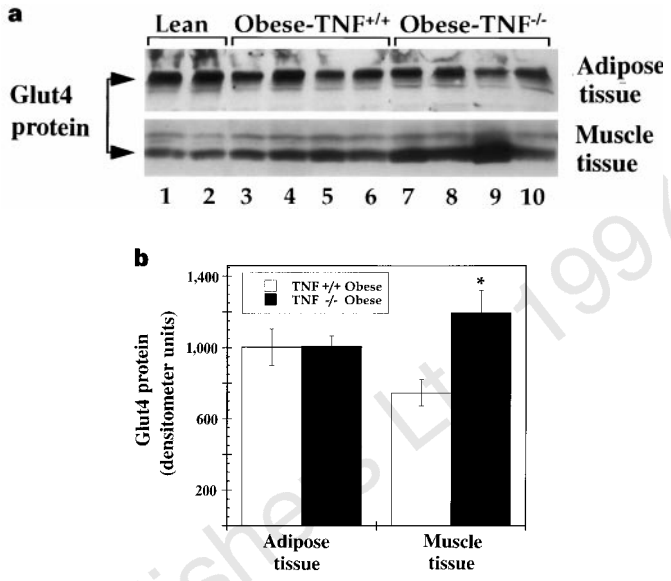

Figure 5 The levels of Glut4 protein in TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$ mice. **a**, A representative immunoblot showing Glut4 protein level in fat and muscle tissues. **b**, Immunoblots are quantified by NIH Image 3.01 image-analysis software and presented as arbitrary units. Six mice were used in each analysis in two independent experiments. Asterisk indicates $P < 0.05$.

reduce development of insulin resistance associated with dietary obesity.

To test the importance of the TNFα-activated pathway of insulin resistance in the most severe, genetic model of obesity, we generated genetically obese (*ob/ob*) mice with targeted mutations in both p55 and p75 TNF receptors, effectively abolishing the signalling and function of TNF-α in these animals[19]. This experiment allowed us both to investigate the action of TNF-α in a different and more severe genetic model of obesity, and through an alternative strategy to block the action of TNF-α. As expected, the *ob/ob* mice developed early onset and severe obesity regardless of the TNF receptor allele they carried[20]. There was no significant difference in body weights or body compositions between the obese animals lacking TNF-α function (*ob/ob p55*$^{-/-}$ *p75*$^{-/-}$) and obese control animals (*ob/ob*) (unpublished data).

To determine the state of insulin sensitivity in these animals, we measured the fasting plasma glucose and insulin concentrations and performed insulin and glucose tolerance tests. As previously observed, *ob/ob* animals (wild-type at TNF receptor loci) developed a moderate and transient fasting hyperglycaemia by eight weeks of age ($445.5 \pm 15.3\,mg\,dl^{-1}$; Fig. 3a) that subsided at 12 weeks[20]. In contrast, the increase in fasting blood glucose levels in the *ob/ob* mice lacking TNF-α function (*ob/ob p55*$^{-/-}$ *p75*$^{-/-}$) was small and the hyperglycaemia was milder ($367.2 \pm 20.7\,mg\,dl^{-1}$ at 8 weeks of age). The *ob/ob* animals also displayed a severe and progressive hyperinsulinaemia during the course of the study (13.8 ± 2.7 and $21.2 \pm 3.3\,ng\,ml^{-1}$ at 8 and 12 weeks of age, respectively; Fig. 3b). However, the *ob/ob* mice lacking TNF-α function (*ob/ob* p55$^{-/-}$ p75$^{-/-}$) displayed significantly lower fasting plasma insulin levels throughout the study (5.6 ± 1.1 and $8.0 \pm 1.0\,ng\,ml^{-1}$ at 8 and 12 weeks of age, respectively; Fig. 3b) than the *ob/ob* animals with functional TNF-α signalling.

We also determined insulin sensitivity in *ob/ob* mice that are wild type or mutant at the loci of both TNF receptors by performing intraperitoneal insulin and glucose tolerance tests (Fig. 3c, d). Both of these tests demonstrated significantly increased insulin sensitivity in the obese mice lacking TNF-α function (*ob/ob* p55$^{-/-}$ p75$^{-/-}$) compared with obese controls (*ob/ob*). However, the *ob/ob* animals that are deficient in TNF receptors were still insulin resistant. This finding shows that interfering with TNF-α signalling through null mutations in both TNF receptors results in a significant but incomplete protection from the insulin resistance associated with the *ob/ob* phenotype. This indicates that several factors combine to result in insulin resistance in obesity, and that TNF-α is not solely responsible for non-insulin-dependent diabetes mellitus in mice.

After confirming the significant improvement in insulin sensitivity in mice lacking TNF-α function in both mild (diet-induced) and severe (genetically induced) forms of obesity, we examined the potential molecular mechanisms that might underlie the protection from insulin resistance in the absence of TNF-α function. For this analysis, we investigated the dietary model of obesity because it more closely resembles the level of obesity and insulin resistance seen in humans. We examined three potential sites of TNF-α action that might mediate insulin resistance: regulation of free fatty-acid levels; numbers of glucose transporters; and insulin receptor activity.

Elevated free fatty-acid levels in obesity are potential contributors to the development of insulin resistance[1]. To investigate whether TNF-α deficiency influenced lipid metabolism, we measured serum free fatty-acid and triglyceride concentrations in TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice. Serum triglyceride levels in the lean TNF-$\alpha^{-/-}$ mice were lower (26%) than in the lean TNF-$\alpha^{+/+}$ animals (16.6 ± 1.4 versus $22.6 \pm 3.3\,mg\,dl^{-1}$). However, no significant differences were observed in triglyceride levels between the obese TNF-$\alpha^{-/-}$ mice and the obese TNF-$\alpha^{+/+}$ animals (22.4 ± 3.8 versus $23.6 \pm 3.4\,mg\,dl^{-1}$). As expected, the obese TNF-$\alpha^{+/+}$ animals had higher levels of circulating free fatty acids than the lean animals (1.84 ± 0.1 versus 1.3 ± 0.1 mM, $P < 0.05$; Fig. 4). In contrast, the free fatty-acid levels in the obese TNF-$\alpha^{-/-}$ mice were indistinguishable from those of the lean animals (1.34 ± 0.1 versus 1.3 ± 0.1 mM; Fig. 4). These results suggest that TNF-α contributes, either directly or indirectly, to the dyslipidaemia of obesity. This effect of TNF-α might be related to the increased insulin sensitivity in the obese TNF-$\alpha^{-/-}$ mice, as free fatty acids are believed to contribute to systemic insulin resistance in obesity.

Multiple sites of insulin signalling are known to be defective in obesity–diabetes syndromes[21]. These include the quantitative changes in insulin-sensitive glucose transporters and reduced signalling capacity of the insulin receptor in insulin-sensitive tissues[21]. It is important to understand the mechanistic basis of increased insulin sensitivity in the absence of TNF-α in obesity. In cultured adipocytes, hepatocytes, fibroblasts, muscle cells and myeloid cells, TNF-α inhibits the insulin-stimulated tyrosine kinase activity of the insulin receptor[11–15]. In adipocytes and L6 myoblasts, TNF-α also downregulates the expression of the insulin-sensitive glucose transporter Glut4 (refs 4, 16, 22). Although pharmacological studies in rats have attributed most of the action of TNF-α to its effects on insulin-receptor signalling[23], the exact molecular basis of the TNFα-induced systemic insulin resistance *in vivo* has remained contro-

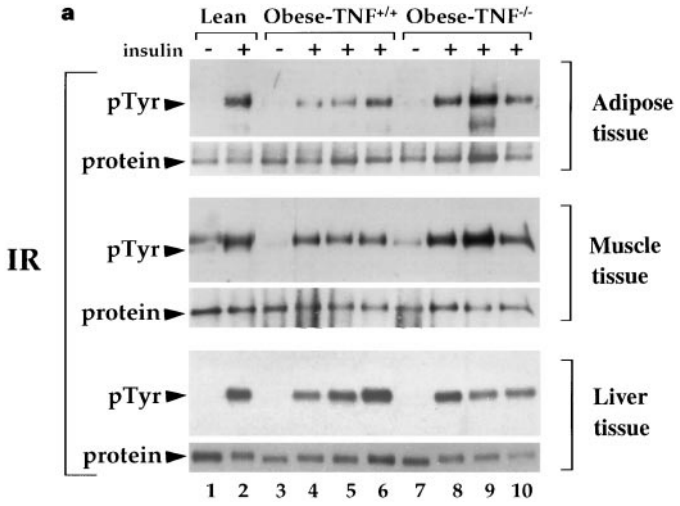

Figure 6 Levels of insulin-stimulated tyrosine phosphorylation of insulin receptor in fat, muscle and liver tissues of TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$ mice. **a**, Representative immunoblot showing insulin-receptor (IR) tyrosine phosphorylation (pTyr) before (–) or after (+) insulin stimulation. **b**, Immunoblots are quantified by NIH Image 3.01 image-analysis software and presented as arbitrary units. Six mice were used in each analysis in three independent experiments. Asterisks indicate $P < 0.05$.

versial. To address these issues definitively, we examined the amount of Glut4 protein in the adipose and muscle tissues, and determined the insulin-stimulated tyrosine phosphorylation of the insulin receptor in fat, muscle and liver tissues of obese TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice.

In adipose tissue, levels of Glut4 protein in the obese mice were slightly lower than the lean controls (27%), but no significant difference was observed between the obese TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ animals (Fig. 5a, b). Because adipose tissue is the primary site of TNF-α expression in obese rodents[4], this observation excludes the possibility that TNF-α is involved in the obesity-related quantitative regulation of Glut4 in adipose tissue. In muscle tissue, we did not observe a significant downregulation of Glut4 protein in TNF-$\alpha^{+/+}$ obese mice compared with the lean animals (Fig. 5a). Glut4 protein levels in muscle tissue were higher in TNF-$\alpha^{-/-}$ obese mice than in TNF-$\alpha^{+/+}$ animals (Fig. 5a, b). Although this result might suggest a role for TNF-α in the quantitative regulation of Glut4 in muscle, it is unlikely to explain the difference in insulin sensitivity seen between obese wild-type and TNFα-deficient animals.

The binding of insulin to its receptor initiates a phosphorylation cascade that starts with the autophosphorylation of the insulin receptor on multiple tyrosine residues[24]. This autophosphorylation is critical for the proper activation of the insulin-receptor tyrosine kinase and signalling events further downstream that mediate the biological actions of insulin[24]. To determine the effects of TNF-α deficiency on the signalling capacity of the insulin receptor *in vivo*, we examined the ability of insulin to stimulate the tyrosine phosphorylation of the insulin-receptor β-chain in adipose, muscle and liver tissues of TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$ animals. Following stimulation with insulin, phosphorylation of the 97k β subunit of the insulin receptor was visible in fat, muscle and liver tissues of all animals (Fig. 6). As expected, the insulin-stimulated autophosphorylation of the insulin receptor in obese TNF-$\alpha^{+/+}$ animals was lower than that of the lean animals in all tissues examined (70% in fat, 35% in muscle and 25% in liver; Fig. 6). There was a significant increase in autophosphorylation of the insulin receptor in the obese TNF-$\alpha^{-/-}$ animals compared with that of the TNF-$\alpha^{+/+}$ obese animals, which had levels approaching those observed in lean mice (Fig. 6). This increased insulin-stimulated phosphorylation of insulin receptor in the obese TNF-$\alpha^{-/-}$ animals was most significant in fat and muscle tissues (50% in fat and 46% in muscle, $P < 0.05$). The differences seen in insulin-receptor phosphorylation in liver were not statistically significant. In the lean group, the level of insulin-receptor phosphorylation was similar between the TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$ mice. These results demonstrate that, in the absence of TNF-α, the signalling capacity of the insulin receptor is significantly protected from obesity-induced downregulation in fat and muscle tissues.

We have provided clear evidence that the action of TNF-α is an important component of the link between obesity and insulin resistance in at least two different models (diet-induced and genetic, *ob/ob*) of murine obesity. As well as demonstrating the *in vivo* role of TNF-α in insulin resistance, our results also show that the action of TNF-α in obesity involves several potential targets that could influence systemic insulin action. First, the obese TNF-$\alpha^{-/-}$ mice have lower free fatty-acid levels than obese wild-type animals. This reduction in free fatty acids despite significant obesity might be the direct result of the loss of the lypolytic effects of TNF-α in adipose tissue, or alternatively might reflect the increased efficiency of insulin to suppress lypolysis in the absence of TNF-α[1]. Second, the obese TNF-$\alpha^{-/-}$ animals had higher levels of Glut4 protein in their muscle tissues. Finally, the obese TNF-$\alpha^{-/-}$ animals were spared from obesity-induced deficiencies in insulin-receptor signalling in fat and muscle tissues. This observation probably represents the most important effect of TNF-α in the generation of obesity-induced insulin resistance, owing to the critical importance of insulin-receptor signalling in generating the biological actions of insulin[24].

The extent of the role of TNF-α in the insulin resistance of dietary obesity appeared to be greater than its involvement in the *ob/ob* genetic model of obesity. This might simply be due to the extreme nature of the *ob/ob* phenotype compared to the dietary obesity. However, it is possible that the role of TNF-α is different as a result of the specific aetiology of obesity and the genetic background. Alternatively, TNF receptor deficiency might be distinct from TNF-α ligand deficiency, although existing data render this highly unlikely. Generation of additional cross-breedings with different obesity models, such as *db*, *tub* or *agouti*[25], should address these questions. □

Methods

Generation of *ob/ob* mice deficient in TNF receptors. Mice deficient in both TNF receptor 1 and TNF receptor 2 (p55$^{-/-}$ p75$^{-/-}$; C57BL/6/129 mix) were back-crossed to C57BL/6 mice for three generations[19], then intercrossed with *OB/ob* mice (Jackson Laboratories, Maine) to produce animals heterozygous at the p55, p75 and *ob* loci (p55$^{+/-}$, p75$^{+/-}$, *OB/ob*), which also involved two additional back-crosses to C57BL/6, the background strain of the *ob/ob* mutation. The resulting triple-heterozygote animals were cross-bred with each other to produce obese littermates with mutations in both TNF receptors (*ob/ob* p55$^{-/-}$ p75$^{-/-}$) and with intact functional TNF receptors as controls (*ob/ob*).

Diet study and metabolic measurements. Male mice homozygous for a targeted null mutation at the TNF-α locus[18] and their wild-type littermates (C57BL/6 × 129 genetic background) were housed in a barrier-free facility and given a high-fat, high-carbohydrate diet *ad libidum* (Diet F3282, Bioserve, NJ) at 4 weeks of age ($n = 10$), and were studied for the next 12 weeks. Identical groups of animals ($n = 5$) were given standard rodent chow to act as controls. Total body weights were measured weekly for 16 weeks, starting at 4 weeks of age. Blood samples were collected after a 6-h fast at 4, 8 and 12 weeks of age. Serum glucose concentrations were measured by using gluco-analyser blood glucose strips (Medisense). Serum insulin was measured with a monoclonal anti-rat insulin radioimmunoassay (Linco). The triglyceride and free fatty-acid levels in serum were determined (GPO Trinder, Sigma and Wako assays, respectively) using 12-week-old animals. Glucose and insulin tolerance tests were performed on conscious mice after a 6-h fast[26]. Glucose tolerance tests were done by intraperitoneal administration of glucose (3 mg per g body weight) and measurement of blood glucose at 15, 30, 60 and 90 min in 18-week-old mice. Insulin tolerance tests were done similarly, except for the injection of human insulin (1 IU per kg; Eli Lilly) and an additional glucose measurement at 45 min. Fasting plasma glucose and insulin concentration and insulin sensitivity were also determined as described above for *ob/ob* and *ob/ob* $p55^{-/-}$ $p75^{-/-}$ mice.

***In vivo* insulin-stimulated insulin-receptor phosphorylation.** After an overnight fast, mice were anaesthetized by intraperitoneal administration of 10 mg per kg xylasine and 100 mg per kg ketamine. The abdominal cavity was opened, and 25 mIU per kg insulin (Eli Lilly) or an equal volume of vehicle were administered through the portal vein. Liver, adipose (inguinal fat pads) and muscle (hindlimb) tissues were collected 120 s after the injection and immediately stored in liquid nitrogen. Protein extracts from the tissue samples were prepared as described[23].

Immunoprecipitation and immunoblotting. Total protein extract (750 μg) was immunoprecipitated for 5 h at 4 °C by adding 1 μg ml^{-1} rabbit anti-insulin receptor antibody (gift from B. Cheatham and C. R. Kahn). Immune complexes were collected, washed and electrophoresed as described[23]. After electrophoresis, the proteins were transferred to nitrocellulose membranes and protein immunoblot analysis was performed using a 1:2,000 dilution of a monoclonal anti-phosphotyrosine (gift from T. Roberts) or a 1:500 dilution of the polyclonal anti-insulin receptor as the primary antibody (gift from B. Cheatham and C. R. Kahn), followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Promega) for detection. For Glut4 immunoblots, 100 μg of protein extract was used and immunoblot analysis was performed by both a rabbit polyclonal (gift from B. Kahn) and a mouse monoclonal (gift from P. Pilch) anti-Glut 4 antibody, as described above.

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Control of compartmental affinity boundaries by Hedgehog

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In *Drosophila*, each segmental primordium is subdivided into two cell populations, the anterior (A) and posterior (P) compartments by the selective activity of the transcription factor Engrailed (En) in P cells[1–4]. Under En control, P cells secrete, but cannot respond to, the signalling protein Hedgehog (Hh)[5–7]. In contrast, and by default, A cells are programmed to respond to Hh by expressing other signalling molecules, such as Decapentaplegic (Dpp) and Wingless (Wg), which organize growth and patterning in both compartments[5,7–9]. Cells of the A and P compartments do not intermix, apparently as a consequence of their having distinct cell affinities that cause them to maximize contact with cells of the same compartment, while minimizing contact with cells from the other compartment[10]. This failure to mix has previously been ascribed to an autonomous and direct role for En in specifying a P, as opposed to an A, cell affinity[3,11–13]. However, an alternative hypothesis is that Hh secreted by P cells induces A cells to acquire a distinct cell affinity, ensuring that a stable 'affinity boundary' forms wherever P and A cells meet. Here we show that the affinity boundary that segregates A and P cells into adjacent but immiscible cell populations is to a large extent a consequence of local Hh signalling, rather than a reflection of an intrinsic affinity difference between A and P cells.

To distinguish between these two hypotheses we used a mutation in the gene *smoothened* (*smo*)[14], which encodes an essential component of the Hh signal-transduction pathway[15,16], to block the ability of A-compartment cells to receive and transduce Hh[17]. If distinct A and P cell affinities are specified autonomously by the state of En expression ('off' for A and 'on' for P), then anterior cells should retain their A-compartment affinity and sort out from P cells, even if their ability to respond to Hh is blocked by the loss of

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