Transgenic rescue of insulin receptor–deficient mice

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The role of different tissues in insulin action and their contribution to the pathogenesis of diabetes remain unclear. To examine this question, we have used genetic reconstitution experiments in mice. Genetic ablation of insulin receptors causes early postnatal death from diabetic ketoacidosis. We show that combined restoration of insulin receptor function in brain, liver, and pancreatic β cells rescues insulin receptor knockout mice from neonatal death, prevents diabetes in a majority of animals, and normalizes adipose tissue content, lifespan, and reproductive function. In contrast, mice with insulin receptor expression limited to brain or liver and pancreatic β cells are rescued from neonatal death, but develop lipoatrophic diabetes and die prematurely. These data indicate, surprisingly, that insulin receptor signaling in noncanonical insulin target tissues is sufficient to maintain fuel homeostasis and prevent diabetes.

Introduction

The mechanism of insulin action is a central question in biology, with important ramifications for human disease (1). Impaired insulin action, or insulin resistance, is commonly observed in type 2 diabetes and predisposes to obesity, arteriosclerosis, and cardiovascular diseases (2). It remains unclear whether insulin resistance is initially restricted to selected tissues and cell types or whether it represents a systemic abnormality of insulin action. To examine the contribution of individual tissues and cell types to the pathogenesis of insulin resistance, we and others have generated constitutive and conditional null alleles of insulin receptor (Insr) to inactivate its function in mice (3). Complete Insr ablation results in early postnatal death from diabetes (4, 5). In contrast, conditional Insr ablation in specific tissues results in mild but complex metabolic abnormalities (6–9). The latter experiments support two conclusions: (a) that insulin resistance in any given tissue can be compensated for through substrate redistribution to other organs (7, 10), and (b) that insulin has direct actions on tissues that have generally not been considered insulin sensitive, such as pancreatic β cells and brain (7, 8, 11). These observations complement work showing that combined functional Insr knockout in muscle and adipose tissue does not lead to diabetes (12).

To study the role of noncanonical insulin target tissues in the pathogenesis of type 2 diabetes and to circumvent the problem of compensation by other tissues, we used a genetic reconstitution approach. We asked whether re-expression of Insr in selected tissues of Insr-deficient mice would confer protection from the metabolic abnormalities that cause diabetes. Our data lend support to the surprising conclusion that insulin action in tissues that are not dependent on insulin for glucose uptake (liver, brain, and pancreatic β cells) is sufficient to restore metabolic homeostasis and prevent diabetes.

Results

In attempting to rescue Insr-deficient mice, we had to consider the peculiar metabolism of the newborn mouse, which is dependent mainly on the liver’s ability to generate ketones as an energy source for the developing brain (13). Although the early postnatal period is characterized by a fall in insulin levels (13), the establishment of insulin-dependent fuel-sensing mechanisms is required to restrain hepatic gluconeogenesis and promote glycogen storage. Indeed, liver failure appears to be the leading cause of death of Insr knockouts (4, 5). Therefore, we considered reconstitution of Insr expression in liver the sine qua non for further metabolic compensation.

Generation of transhthyretin-Insr lines. We generated three lines of transgenic mice expressing human INSR cDNA from the transthyretin (Ttr) promoter (referred to here as L1, L2, and L3). The Ttr promoter has been shown to drive transgene expression in liver and choroid plexus. Moreover, it shows copy number– and integration site–dependent expression in pancreatic β cells and brain (14–16). Southern blot analysis indicated that transgene copy number varied between 20 (L1) and 1 (L3) (data not shown). We used Northern blotting to confirm transgene expression in liver. We detected the highest levels of transgene-encoded mRNA (INSR mRNA) in L1 liver. The levels in L2 and L3 livers were approximately 37% and 29% of those in L1 liver (Figure 1A). RT-PCR revealed that, in addition to liver, pancreatic islets expressed the transgene in all three lines (Figure 1B). Based on previous work with the Ttr promoter in transgenic mice, this expression is likely to arise from pancreatic β cells (14, 17). To determine whether the transgene was also expressed in brain, we dissected different brain parts from the three lines and performed real-time RT-PCR using a primer set that specifically amplifies human INSR. We detected expression of the transgene in all brain parts derived from L1 mice but in none of those derived from L2 and L3 mice (Figure 1C). Widespread brain expression from the Ttr promoter has also been reported (18, 19).

Characterization of Ttr-Insr mice. We intercrossed the three lines with Insr+/- mice to generate Ttr transgenic mice lacking endogenous Insr (Ttr-Insr−/-). Unlike Insr+/- mice, which died within days

Nonstandard abbreviations used: actin (ac); insulin receptor (Insr); internal ribosome entry site (IRES); rat insulin II promoter (Rip); transthyretin (Ttr).

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of birth, *Ttr-Insr−/−* mice from all three lines survived to adulthood. All three lines showed evidence of growth retardation at 1 month. The body weights of male L1 (Figure 2A) and L2 (Figure 2B) mice were about 80% those of control littermates, whereas those of male L3 mice (Figure 2C) were about 60% those of control littermates. L1 mice exhibited limited post-weaning “catch-up” growth, whereas L2 and L3 mice remained growth retarded throughout life. We obtained similar data in female mice (not shown). Necroscopic...
analyses indicated that L2 and L3 mice lacked white adipose tissue while retaining interscapular brown adipose tissue. In addition, both L2 and L3 mice showed considerable hepatomegaly and fatty liver degeneration (Figure 2D), as expected from their lipoatrophic state. In contrast, L1 mice had normal adipose tissue content and distribution (not shown).

We assessed Insr expression by immunoblot and insulin-binding assay. In liver, immunoreactive Insr levels were 57% of WT in L1 mice and 75% of WT in L2 and L3 mice (Figure 3A). Interestingly, L1 mice expressed lower protein levels, despite having higher levels of mRNA (compare Figure 1A with Figure 3A). This discrepancy is likely to arise from integration site effects. In islets, L1 mice expressed Insr at levels about 50% of WT, whereas L2 and L3 mice showed levels about fivefold higher than WT. As seen previously with other receptors of the Insr family expressed in pancreatic β cells (20, 21), the band corresponding to the 190-kDa receptor precursor was more prominent in pancreatic β cells than in other cell types (Figure 3B). Consistent with the results of real-time RT-PCR (Figure 1C), we detected Insr immunoreactivity in all brain parts from L1 Ttr-Insr−/− mice but in none of those from L2 and L3 mice. Expression levels were uniformly lower than in control mice, reaching about 36% of WT in hypothalamus, 26% in thalamus, 51% in cortex, 63% in olfactory lobe, 53% in cerebellum, 59% in hippocampus, and 39% in brainstem (Figure 3C). We did not detect Insr immunoreactivity in muscle, heart, spleen, and adipose tissue. We observed representative blots of 3- to 4-month-old mice. We obtained similar results with specimens from mice of different ages. We could not obtain adipose tissue from L2 and L3 mice because they are lipoatrophic.
Metabolic profile of Ttr-Insr mice. To determine the physiological consequences of limited restoration of Insr expression, we measured glucose and insulin levels in mice of various ages. At 2 months of age, 90% of L1 mice showed normal glucose levels and 10% showed glucose levels in the diabetic range (defined as mean + 2 SD). By the age of 6 months, the percentage of L1 mice that were diabetic rose to 35% (6 of 17), but they all had normal lifespans. In contrast, all L2 and L3 Ttr-Insr–/– mice developed diabetes by 2 months and died between 3 weeks and 8 months of age. Indeed, in L3 mice we observed hyperglycemia as early as 4 weeks of age, and 90% of mice died by 2 months of age (Figure 4A and Table 1). The variability of the phenotype in L2 and L3 mice, which have similar expression levels and tissue patterns of the transgene, is likely to arise from the contribution of modifier genes on the outbred C57BL/6 × FVB × 129/Sv genetic background. All lines of Ttr-Insr–/– mice showed substantial increases in circulating insulin levels (Figure 4B and Table 1). As in other models of insulin resistance (22), we observed a “bell-shaped correlation” between glucose and insulin values (Figure 4C). The variability of the phenotype in L2 and L3 mice, which have similar expression levels and tissue patterns of the transgene, is likely to arise from the contribution of modifier genes on the outbred C57BL/6 × FVB × 129/Sv genetic background. All lines of Ttr-Insr–/– mice showed substantial increases in circulating insulin levels (Figure 4B and Table 1). We express glucose as mg/dl, insulin as ng/ml, and glycogen as mg/g protein. Statistically significant differences are indicated as follows: *P < 0.005; **P < 0.001. N/A, not applicable. 2-mo, 2-month-old mouse.
modifies the transgene effect, as we have shown previously for \( \text{Insr} \) mutations (23). To rule out the possibility that the main effect of the \( Ttr \)-driven transgene is due to genetic background, we intercrossed L1 mice with L2 and L3 mice. In the resulting F1 generation, single hemizygosity for L1 and compound hemizygosity for the L1/L2 or L1/L3 combination of transgenic alleles consistently conferred protection from diabetes and premature death, whereas all single-hemizygous mice carrying the L2 and L3 transgenes, alone or in combination, died of diabetes. These data indicate that the protective effect is tightly associated with the L1 transgene and not with modifier genes.

We next assessed gene expression in livers of 4-week-old \( Ttr-\text{Insr}^{+/–} \) mice using real-time RT-PCR. We detected an increase of about 80% in glucose-6-phosphatase (\( \text{G6pc} \)) exclusively in L2 mice, whereas phosphoenolpyruvate carboxykinase (\( \text{Pck1} \)) levels were unperturbed. In L2 and L3 mice we also detected an increase of about twofold in insulin-like growth factor–binding protein 1 (\( \text{Ifgbp1} \)) and a decrease of 60–80% in glycogen synthase (\( \text{Gys1} \)) levels. As expected, given the reduced \( \text{Gys1} \) expression, we detected a decrease in liver glycogen content in L2 and L3 mice compared with control mice, whereas glycogen content in L1 mice was normal (Table 1). These results are consistent with hepatic insulin resistance in L2 and L3 mice, although some changes, such as the decrease in glycogen levels,
could be secondary to diabetes. Interestingly, L1 mice show mild changes in hepatic gene expression than L2 mice do, although the latter express higher transgene levels.

Pancreatic β cell compensation in Ttr-Insr mice. We measured pancreatic islet morphology and morphometry as well as islet function in Ttr-Insr–/– mice. At 1 month of age, L1 mice showed islets of normal size with normal architecture (Figure 5A) and a moderate reduction in β-cell mass (Figure 5B). L2 and L3 mice exhibited increases of about threefold and twofold, respectively, in β-cell mass (Figure 5, A and B). We also analyzed islet morphology in older animals (4 months old). At this age, L1 mice exhibited normal islet size and architecture, whereas in islets from L2 and L3 mice we found extensive loss of insulin-positive cells, with altered islet architecture (Figure 5C). In contrast, we failed to detect alterations in the expression or subcellular localization of the insulin gene transcription factor Pdx1 (Figure 5C). In contrast, we failed to detect alterations in the expression or subcellular localization of the insulin gene transcription factor Pdx1 (Figure 5C).

Normal reproductive behavior in L1 but not in L2 and L3 Ttr-Insr mice. We also evaluated the fertility of the three Ttr-Insr–/– lines. We set up brother-sister intercrosses and backcrosses with Insr+/– mice (Table 2). Both male and female L1 mice were fertile in intercrosses and backcrosses. L2 and L3 male mice produced offspring when backcrossed onto Insr+/– female mice, whereas L2 and L3 female mice were sterile. The observation that L1 intercrosses yielded offspring and a normal litter size indicates that Insr signaling in the gonads and reproductive system is not required for germ cell maturation and successful completion of pregnancy. Female L2 and L3 mice could be infertile owing to diabetes. However, because these mice do not express Insr in the brain, their reproductive phenotype could also be interpreted as supporting the idea of a role for brain Insr in the regulation of gonadotropin production and ovulation (8).

Generation of single-tissue Insr knock-ins in brain and pancreatic β cells. The surprising difference in metabolic control between mice expressing Insr in brain, liver, and β cells and those with expression limited to liver and β cells raised the question of whether restoring insulin action in the brain alone would suffice to prevent diabetes in Insr knockouts. To address this question, we used a conditional locus knock-in approach (24). We modified the βac locus by homologous recombination in ES cells. The modified
locus contained an internal ribosome entry site (IRES) at the 3′ end of exon 6, followed by a promoterlesslox-neo-lox cassette and by the INSR cDNA (Figure 6, A and B, and Methods). We generated mice heterozygous for the modified βac locus, referred to as βac/INSR in Figure 6. By breeding these mice with transgenic mice expressing cre recombinase under the control of a tissue-specific promoter, we ensured excision of thelox-neo-lox cassette in a tissue-specific fashion and juxtaposition of the INSR cDNA with IRES. Thus, cre-mediated recombination enables transcription of a bicistronic βac/INSR mRNA from the IRES (these mice are referred to as βac/INSR Δlox in Figure 6). We then intercrossed the resulting mice with Insr+/− mice to introduce a null mutation of the endogenous Insr. By intercrossing the resulting F1 progeny, we obtained βac/INSR Δlox mice lacking the endogenous Insr (referred to here as βac/INSR/Δox/Insr−/− mice). We generated four different brain-restricted knock-ins to reactivate Insr expression selectively in neurons using synapsin-cre (25) and calmodulin kinase II-cre (26) transgenic mice, or more broadly in neurons and glia using nestin-cre (Nos-cre) (27) and heat shock protein 70-cre (Hsp6-cre) (28) transgenic mice. In all instances, we observed the expected pattern of expression of the INSR cDNA in different brain sections, without expression outside the brain (Figure 6C). In all four lines, Insr expression was restored to physiological or near-physiological levels. We observed the highest expression in Syn-Insr mice and the lowest, in Hsp6-Insr mice (for nomenclature, see Methods). The metabolic phenotype was similar in all four lines (Table 3). Mice were born in normal mendelian ratios and showed no apparent abnormalities or growth defects but developed diabetes within a week of birth and by 3 weeks of age were frankly diabetic. Most animals died within 6–8 weeks of birth, although few survived up to 3 months. Insulin levels were extremely elevated, indicative of insulin resistance. Mice bearing the intact βac/INSR allele on an Insr−/− background died of diabetic ketoacidosis within few days of birth, similar to Insr−/− mice. These findings provide a necessary negative control indicating that the βac/INSR allele does not allow for INSR expression in the absence of cre. While in principle it would be desirable to recreate the liver/brain pattern of expression using this approach to confirm the transgenic data, we have thus far been unable to obtain robust prenatal expression of Insr in liver using the available transgenic cre lines.

Finally, to address the contribution of Insr in the pancreatic β cell to the observed phenotype, we used the modified the βac/INSR allele to generate mice with β cell–restricted Insr expression via intercrosses with rat insulin II promoter–cre (Rip-cre) transgenic mice (29) (Figure 6D). The phenotype of these mice was identical to that of Insr−/− mice, suggesting that insulin signaling in the β cell is not sufficient to restore even partial metabolic control.

**Discussion**

In this study, we demonstrate that Insr expression in liver and pancreatic β cells is sufficient to rescue Insr-deficient mice from perinatal lethality but that concomitant expression of Insr in brain is required to prevent diabetes throughout life. The marked effect that restoration of hepatic Insr expression has on the survival of Insr-deficient mice can be accounted for by the dominant role of the liver during lactation in rodents (13). However, the effect of brain Insr in protecting L1 mice from diabetes provides a compelling demonstration of the role of insulin action in the brain (30). Interestingly, L1 mice are resistant to diabetes despite the fact that of the three Ttr-Insr lines characterized, they displayed the lowest levels of transgene expression in both liver and β cells. This observation makes it unlikely that the phenotypic differences among the three transgenic lines can be ascribed to their hepatic insulin signaling or β-cell compensatory ability. Indeed, measurements of insulin-induced Akt activity in livers of L1 mice revealed no differences compared with those of WT mice (data not shown).

**Brain and insulin sensitivity.** Three studies have addressed the role of Insr in the brain by gene inactivation. Conditional ablation of Insr in nestin-positive neurons results in obesity and decreased female fertility (8), while inhibition of hypothalamic Insr function results in insulin resistance and impaired inhibition of hepatic glucose output (31, 32). Our data in brain-restricted Insr knock-ins indicate that the effect of the brain requires an insulin-sensitive liver to reverse the main abnormalities of the metabolic syndrome. This is consistent with the brain’s role in modulating hepatic glucose homeostasis (32). Brain control of insulin action could be exerted in a cell-autonomous fashion, for example through release of endocrine mediators to activate insulin-independent pathways of fuel metabolism (33), or could be due to local release of neuromediators through peripheral nerve endings (34). Intriguingly, the longevity (dauer) phenotype caused by mutations in the Insr ortholog daf-2 in Caenorhabditis elegans can be rescued by selective daf-2 re-expression in the brain (35). Moreover, regulation of lifespan in the roundworm has been shown to depend on neural and reproductive inputs (36).

Another intriguing feature of the brain-liver interaction is the reversal of the lipoatrophic phenotype of L2 and L3 mice when Insr expression is reconstituted in the brains of L1 mice. It bears emphasizing that none of the three lines expresses Insr in the adipocyte, indicating that the trophic effect of the brain on adipocytes is cell autonomous, as far as insulin signaling is concerned. Although it may prove difficult to demonstrate whether this effect is due to increased differentiation of pre-adipocytes or decreased lipolysis in existing adipocytes, the findings nevertheless lend support to the idea that the effects of insulin in the central nervous system have profound consequences for peripheral metabolism.

**The metabolic balance of power.** Conditional mutagenesis using the cre/loxP system provides a new approach for understanding the integrated physiology of insulin action. Classically, insulin resistance has been thought to arise from an impairment of

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**Table 3**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT (n = 19)</th>
<th>Syn-Insr (n = 6)</th>
<th>Camk-Insr (n = 10)</th>
<th>Hs6-Insr (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>8.9 ± 0.4</td>
<td>5.8 ± 0.4b</td>
<td>4.7 ± 0.2b</td>
<td>6.0 ± 0.2b</td>
</tr>
<tr>
<td>Glucose</td>
<td>126 ± 6</td>
<td>332 ± 46b</td>
<td>367 ± 16b</td>
<td>353 ± 13b</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.7 ± 0.2</td>
<td>8.9 ± 3.4a</td>
<td>47.6 ± 22.6</td>
<td>45.5 ± 4.5a</td>
</tr>
<tr>
<td>Ketoadicosis</td>
<td>None</td>
<td>5/6</td>
<td>9/10</td>
<td>16/19</td>
</tr>
<tr>
<td>Liver steatosis</td>
<td>None</td>
<td>5/6</td>
<td>10/10</td>
<td>17/19</td>
</tr>
</tbody>
</table>

We measured whole-blood glucose (mg/dl), serum insulin (ng/dl), and urinary ketone levels (Ketoacidosis is defined as acetonacetate greater than 9 mg/dl or acetone greater than 70 mg/dl) in fed mice. We scored for liver steatosis by visual inspection. Statistically significant differences are indicated as follows: bP < 0.05, ap < 0.01.
insulin-dependent glucose uptake and glycogen synthesis in skeletal muscle and adipose tissue. However, the onset of clinical diabetes requires an increase in hepatic glucose output, with attendant pancreatic β-cell failure. This has led to the view that two separate defects, one in insulin action and one in pancreatic β-cell function, are required for the development of type 2 diabetes. Results obtained using mice with targeted mutations in genes required for insulin action have increasingly challenged this view. For example, the demonstration that ablations of Insr (11) and Igf1r (21, 37), as well as their main substrates Irs1 (38) and Irs2 (39), impair this view. For example, the demonstration that ablations of Insr genes required for insulin action have increasingly challenged this view. For example, the demonstration that ablations of Insr (39), impair (11) and Igf1r (21, 37), as well as their main substrates Irs1 (38) and Irs2 (39), suggest that insulin signaling also plays a role in insulin secretion and in β cell compensation to insulin resistance (40). On the other hand, the lack of diabetes or insulin resistance in mice lacking Insr in tissues that possess insulin-dependent mechanisms of glucose uptake has raised the possibility that the contribution of this pathway to the pathogenesis of type 2 diabetes has been overstated (41). The present data provide the firmest evidence to date that insulin signaling in noncanonical insulin target tissues (brain and β cells) as well as liver, an organ at the “crossroads” of direct and indirect mechanisms of insulin action (42), is indeed essential for the maintenance of euglycemia.

The results of our study should not be “over-interpreted” to indicate that muscle and fat are unimportant in insulin signaling. The absence of diabetes in Insr-transgenic knockout mice can be explained by the presence of compensatory pathways based on muscle contraction (43) and IGF1 receptor signaling (44). Indeed, inactivation of the insulin-responsive glucose transporter Glut4 in muscle (45) or fat (46) has a more profound effect than Insr inactivation (6, 9), suggesting that multiple pathways converge to promote glucose uptake. Likewise, the mechanism by which insulin regulates hepatic glucose production remains unclear (42) and appears to require both direct effects on the hepatocyte as well as indirect effects, mainly through the brain (32). Further metabolic studies of L1 mice will allow us to draw a more definitive conclusion as to which effects predominate in mice. In summary, mice with tissue-restricted Insr expression provide insight into the relative contributions of individual organs to the pathophysiology of insulin resistance, and suggest that therapeutic alternatives based on preserving insulin sensitivity in the brain should be pursued.

**Methods**

**Animal production and genotyping.** The Trir-Insr transgene was engineered by introduction of a 4.3-kb DNA XbaI-SpeI fragment encoding INSR cDNA at the Stal site of the Trir exon 3 plasmid, containing 3 kbp of the human Trir promoter and exons 1–3. This promoter fragment has been shown to confer expression in hepatocytes and pancreatic β cells. When the promoter is of high copy number (more than 6 copies), expression in the brain and retinal pigment epithelium has also been demonstrated (17). This purified DNA fragment was microinjected into fertilized C57BL/6 × FVB eggs as described (17). Three founder transgenic mice expressing the Trir-Insr cDNA were intercrossed with Insr+/− mice and the resulting progeny were intercrossed to yield Trir-Insr+/− mice. Insr+/− mice have been described previously (4). The animals were maintained on a mixed background derived from 129/Sv, C57BL/6, and FVB. Genotyping was performed as follows. The WT Insr allele was detected using primers 5′-CTGTGCACCTCCCTGCTCA-3′ and 5′-TCTTTGCGCTGT- GCTCCACTCCT-3′; and the null Insr allele was detected using primers 5′-GATCGGCAATGGAACAGATG-3′ and 5′-CGC- CAAGCTTCTCAGAATAT-3′. The product of the WT allele is approximately 300 bp in length and that of the null allele is approximately 700 bp in length. PCR amplification conditions for the WT Insr allele were as follows: 4 minutes at 94°C, followed by 30 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, and then 72°C for 7 minutes. We used similar conditions for the null Insr allele, except that the annealing temperature was 56°C and the final extension was for 15 minutes. We detected the transgene using primers 5′-TACCCGGAGAGGTTGTGCCC-3′ and 5′-ATGGTCGGCCAAACTCTGGAG-3′. PCR amplification conditions were 4 minutes at 94°C followed by 30 cycles at 94°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute, and then 72°C for 7 minutes. The product was approximately 500 bp in length.

**Targeted mutagenesis in ES cells and generation of βac/INSR knock-in mice.** We cloned an INSR cDNA (isoform B) in a modified βac genomic fragment encompassing exons 4–6 of the βac locus (24). The targeting vector was linearized with PmeI and was electroporated into R1 ES cells. After G418 selection, we isolated genomic DNA from individual clones and analyzed them by Southern blotting to detect homologous recombinants by Pctl digestion. Mice carrying the modified βac locus (βac/INSR) were generated by blastocyst injection as described previously (4). To obtain mice with tissue-restricted Insr expression, we bred βac/INSR mice with Insr+/− mice to obtain βac/INSR+/− mice. Similarly, we obtained Insr+/− mice carrying the various cre transgenen: synapsin-cre (Syn-cre/Insr−/−), calmodulin kinase II-cre (Camk-cre/Insr−/−), nestin-cre (Nes-cre/Insr−/−), heat shock protein 70-cre (Hsp-cre/Insr−/−), and rat insulin II promoter-cre (Rip-cre/ Insr−/−). After cre-mediated recombination, the βac/INSR allele is expected to undergo deletion of the lox-neo-lox cassette, thus yielding a βac/INSR Alox allele. Intercrosses between the double-heterozygous βac/INSR+/− mice and cre+/−/Insr−/− mice yielded mice of the following genotypes, used for further analyses: WT, βac/INSR/Insr−/−, and βac/INSR Alox/Insr−/−. For simplicity, these last five types of mice are referred to as Syn-Insr, Camk-Insr, Nes-Insr, Hs6-Insr, and Rip-Insr, respectively.

**Hybridization studies.** We performed Southern analysis on HindIII-digested DNA obtained from tail tissue using a cloned fragment of the INSR cDNA (nucleotides 2,091–3,068) as a 32P-labeled hybridization probe. We isolated total RNA from liver using TRIzol (Life Technologies, Gaithersburg, Maryland, USA). We size-fractionated isolated samples on a denaturing formaldehyde agarose gel and transferred them to a nylon membrane for Northern hybridization according to standard techniques, using the same probe described for Southern analysis. The probe detects human INSR but not mouse Insr mRNA.

**RT-PCR and real-time RT-PCR analysis.** We isolated mRNA using the Micro-FastTrack 2.0 Kit (Invitrogen, Carlsbad, California, USA) and used this mRNA to synthesize cDNA using a GeneAmp RNA PCR kit (Perkin-Elmer, Boston, Massachusetts, USA). We isolated total RNA using TRIzol (Life Technologies, Gaithersburg, Maryland, USA). We size-fractionated isolated samples on a denaturing formaldehyde agarose gel and transferred them to a nylon membrane for Northern hybridization according to standard techniques, using the same probe described for Southern analysis. The probe detects human INSR but not mouse Insr mRNA.