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Supplemental Information

Irs1 Serine 307 Promotes

Insulin Sensitivity in Mice

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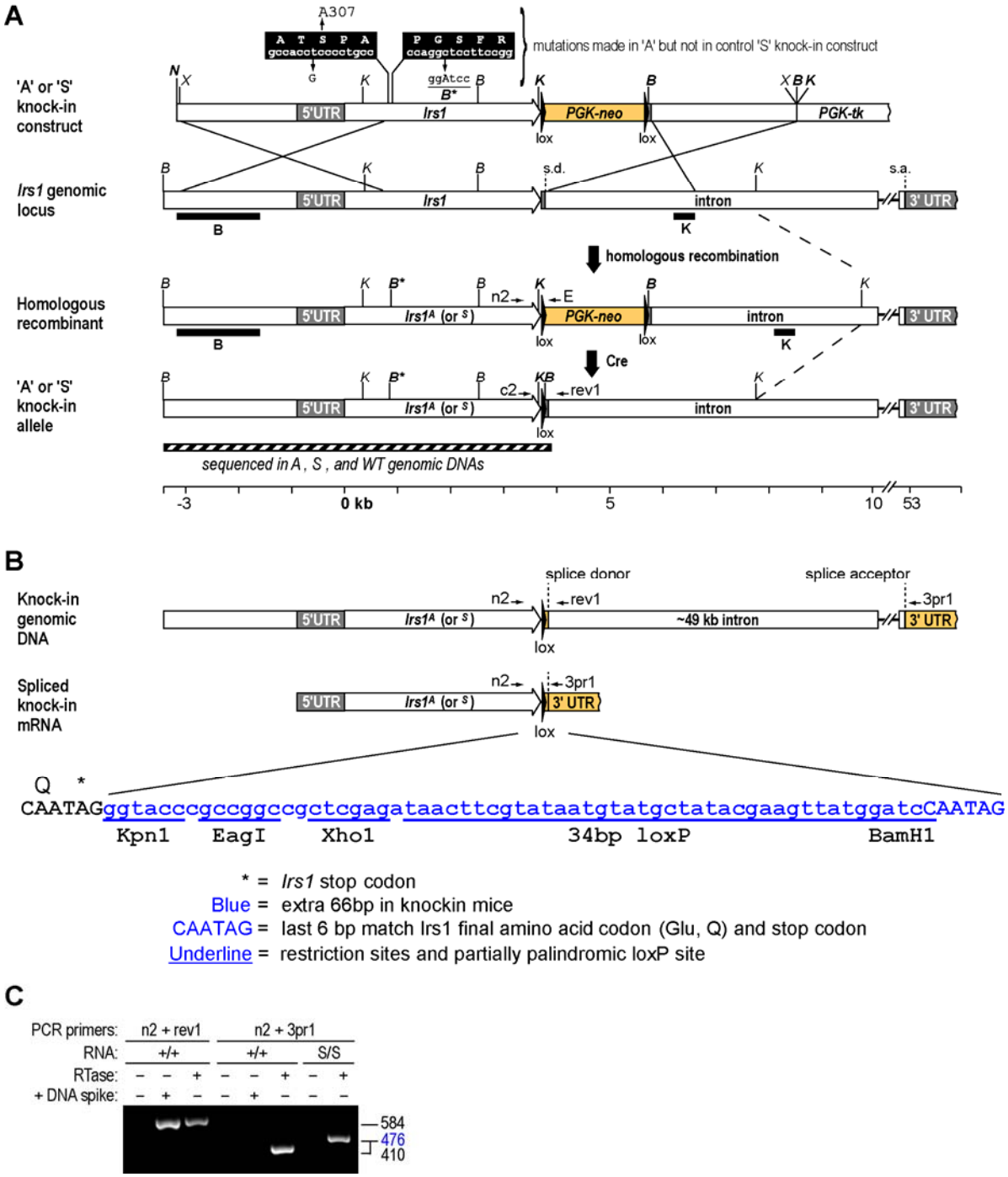

Figure S1. Construction of *Irs1*^{A} (Ser307Ala) and *Irs1*^{s} (non-mutant control) knock-in alleles

(**A**) Homologous recombination of mutant ('A') and control ('S') plasmid constructs with the single-exon *Irs1* gene; restriction sites *N*: *Not*I; *X*: *Xho*I; *K*: *Kpn*I; *B*: *BamH*I (**bold** sites introduced via plasmid construct); black bars: probes 'B' and 'K' for identification of homologous recombinants in *BamH*I- or *Kpn*I-digested DNA; *Cre*: *Cre* recombinase, provided by mating to female EIIa-Cre mice; lox: loxP substrate of Cre recombinase; s.d.: splice donor; s.a.: splice acceptor; UTR: untranslated region; striped bar: sequenced portion of knock-in genomic DNAs; PCR primers: n2, c2, E, rev1.

(**B**) Detail of extra 66 bp in knock-in mice immediately following the *Irs1* stop codon. Length of the 5' UTR in the *Irs1* transcript is imputed from the literature. PCR primers: n2 + rev1 (amplify unspliced mRNA and genomic DNA); n2 + 3pr1 (amplify spliced mRNA).

(**C**) Normal splicing of Irs1 across the extra 66 DNA bp in knock-in mice. Indicated total mRNAs (RNA) were subjected to oligo-dT/random-primed reverse transcription with (+) or without (−) reverse transcriptase (RTase), then spiked (+) or not spiked (−) with wild-type genomic DNA (+ DNA spike) and subjected to PCR with the indicated primers (locations in B).

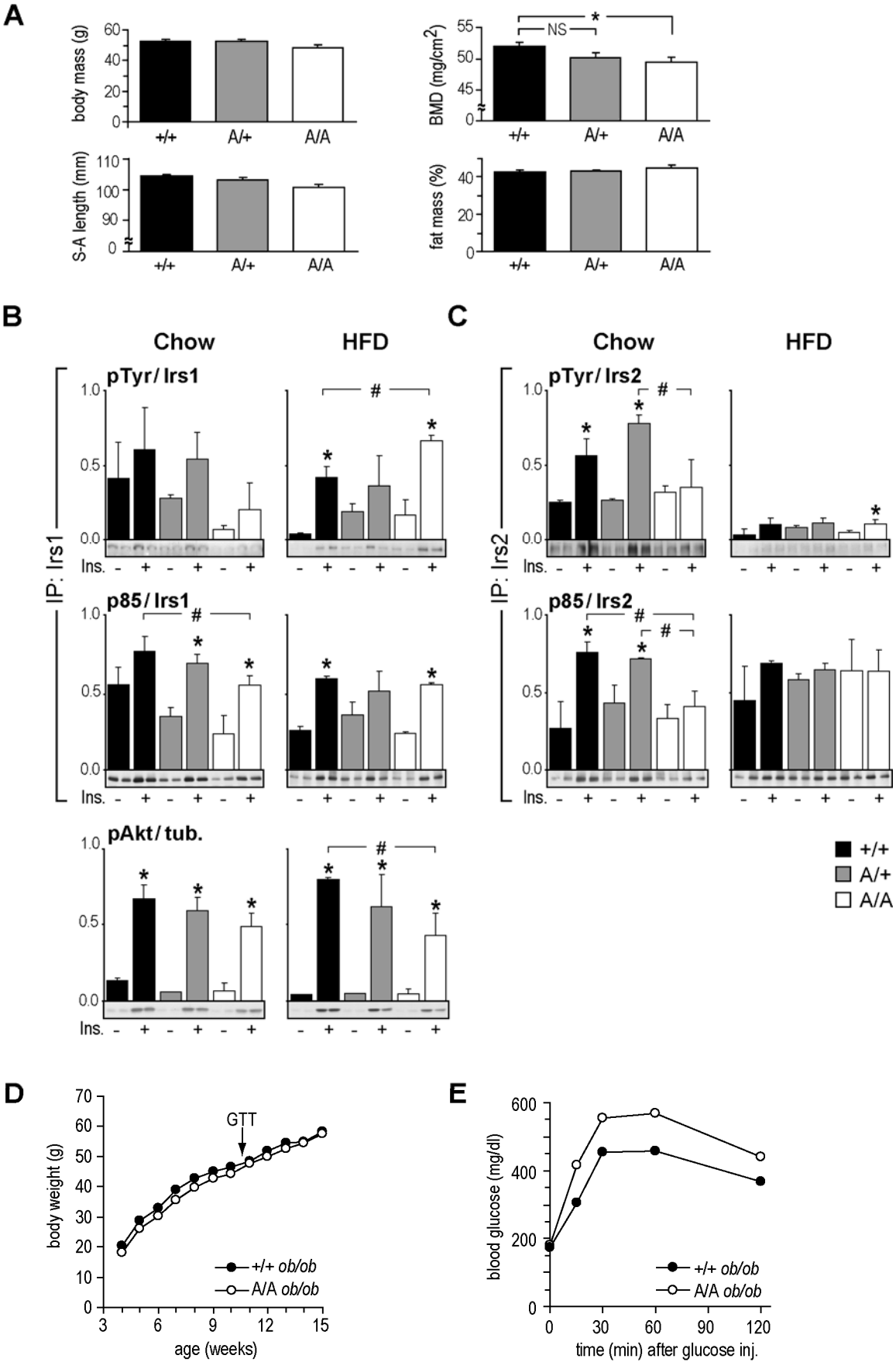
A
body mass (g)
BMD (mg/cm2)
S-A length (mm)
fat mass (%)
+/+
A/+
A/A
NS
B
C
Chow
HFD
pTyr/ Irs1
pTyr/ Irs2
p85/ Irs1
p85/ Irs2
pAkt/ tub.
IP: Irs1
IP: Irs2
Ins.
+/+
A/+
A/A
D
GTT
body weight (g)
age (weeks)
+/+ ob/ob
A/A ob/ob
E
blood glucose (mg/dl)
time (min) after glucose inj.

Figure S2. Additional data from HFD-fed and *ob/ob* mice

(**A**) Body composition of HFD-fed wild-type (+/+), heterozygous (A/+), and homozygous (A/A) mice, assayed by dual X-ray absorptiometry.

(**B-C**) Immunoprecipitation (IP:) and immunoblot analysis of insulin signaling by Irs1 and Akt or (C) Irs2 in liver of chow- and HFD-fed mice; pTyr: phosphotyrosine; pAkt: phospho-$Thr308^{Akt}$. In panels B and C, the estimated means of phosphotyrosine, p85, or phospho-$Thr308^{Akt}$ signals (see methods) were normalized to the average total Irs1, Irs2, or tubulin concentrations; * = significant difference (Bonferroni $p<0.05$) vs. unstimulated; # = significant difference (Bonferroni $p<0.05$) between indicated insulin-stimulated samples.

(**D-E**) Body weight gain and (E) glucose tolerance test for a pair of littermate +/+ and A/A male mice on the (C57BL6) *ob/ob* background.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of Knock-in Mice

The mutant Ser307Ala knock-in allele (*Irs1*^{A}, A) was generated using a targeting construct based on the pPNT vector (Figure S1A) modified to contain two loxP sites flanking the *PGK-neo* selectable marker (pPNT2loxP). The floxed *PGK-neo* gene was immediately preceded by unique *Not*I and *Xho*I sites and immediately followed by a unique *BamH*I site. The right arm of the construct was inserted at the *BamH*I site and consisted of the first 3 kb of (129Sv) mouse genomic DNA immediately following the *Irs1* stop codon (extending 3' to an *Xba*I site) and engineered to have *BamH*I ends. The left arm of the construct was generated in a separate plasmid (pBS8) and consisted of ~6.8 kb of contiguous (129Sv) mouse *Irs* genomic DNA, stretching from an upstream *Xba*I site to the stop codon of the single-exon *Irs1* gene.

Mutagenic primers (mutant nucleotides capitalized) were used to introduce the Ser307Ala mutation in pBS8,

sense: 5'- gcatcactgccaccGcccctgccagta,

anti-sense: 5'- tactggcaggggCggtggcagtgatgc,

followed by a novel *BamH*I site,

sense: 5'- cctgccagtatggtgggtgggaaaccaggAtcc<u></u>ttccgg,

anti-sense: 5'- ccggaaggaTcctggtttcccacccaccatactggcagg.

The novel *BamH*I marked, but did not alter the *Irs1* coding sequence, and was used for genotyping (below). A portion of the doubly-mutated pBS8 was subcloned into fresh pBS8 backbone and then sequenced to verify the absence of any undesired mutations. The completed left arm was ligated into the unique *Not*I of pPNT2loxP as an *Eag*I fragment, regenerating the *Not*I site on the 5' side in the sense of the *Irs1* gene; this site was used to linearize the mature construct for transfection into R1 ES cells. Potential homologous recombinant ES cells emerging from double-selection in G418 and gancyclovir were screened by sequential Southern blotting of KpnI- and BamHI-digested genomic DNA with probes K and B (Figure S1A). Correctly targeted (*Irs1*$^{A/+}$) clones gave 6009 bp recombinant and 7478 bp wild-type bands with probe K, and 4327 bp recombinant and 5854 bp wild-type bands with probe B.

The control Ser307Ser knock-in allele (*Irs1*^{S}, S) was generated by essentially identical means as a byproduct of targeting of the wild-type *Irs1*^{lox} conditional allele (Dong et al., 2008). In this case, 3' homologous recombinants were identified (as above, probe K) that failed to incorporate a loxP site ~1.6 kb upstream of the *Irs1* start codon within the *Irs1*^{lox} targeting construct. These were then verified to have undergone 5' homologous recombination between the lost loxP marker and downsteam *PGK-neo* marker using Southern blotting of BamHI-digested ES cell DNA with a ~1 kb *Bst*BI-*BamH*I probe from near the beginning of the *Irs1* coding sequence. In this assay, positive clones gave only the above 5854 bp *BamH*1 fragment.

Several correctly targeted *Irs1*$^{A/+}$ and *Irs1*$^{S/+}$ ES cell clones were injected separately into C57BL/6 blastocysts to generate highly chimaeric males potentially bearing *Irs1*^{A} or *Irs1*^{S} germ cells. Sperm of these mice were sampled by repeated mating to C57BL/6J females to generate (agouti) *Irs1*$^{A/+}$ and *Irs1*$^{S/+}$ founder mice, which were used to establish a single *Irs1*^{A} line and two *Irs1*^{S} lines. Male *Irs1*$^{A/+}$ and *Irs1*$^{S/+}$ mice were mated to a "deleter" Cre strain (129Sv; EIIa-Cre, Jackson Laboratories) to remove the floxed *PGK-neo* gene. Progeny of this cross were screened using the primers c2 and rev1 (described below, locations in Figure S1A) to identify instances of *PGK-neo* deletion and the EIIa-Cre transgene was bred away during nine subsequent backcrosses to inbred C57BL/6 mice. Deletion of *PGK-neo* yielded 424 bp recombinant and 358 bp wild-type bands, owing to the insertion of 66 bp between the *Irs1* stop codon and the first nucleotide of the *Irs1* 3' UTR in the knock-in alleles. The exact sequence of the 66 bp insertion is shown in

Figure S1B. In the 21 bp following the insertion and preceding the splice site of Irs1 mRNA, two base pairs not conserved in mice, rats, and humans were also changed to match the rat *Irs1* sequence: caatagcTtaactggacGtcacagcag (changes capitalized). Eventual sequencing of the entire 6.8 kb left arm region of the $Irs1^{A}$ and $Irs1^{S}$ alleles from mouse tail DNA showed no differences in *Irs1* 5' control regions or Irs1 protein coding potential other than those described here.

Mouse genotyping

Mice and embryos were genotyped using PCR with primers shown in Figure S1A. The presence of knock-in alleles still containing the *PGK-neo* gene was observed using the three primers:

n2 (5'- gcagtgaggatgtaaaacgccacagctctgcatc),
E (5'- ggggccctcgacataacttcgtatagcatacat), and
rev1 (5'- agagagaagccctttctgtggctgctccaaacaca),

in the ratio 2:1:2, yielding 386 bp knock-in and 584 bp wild-type products.

Following *Cre*-mediated removal of *PGK-neo*, the progeny of $Irs1^{A/+}$ or $Irs1^{S/+}$ pairs were genotyped using primers:

c2 (5'- cagcaatgagggcaactccccaagacgctcca) and
rev1 (above),

yielding 424 bp knock-in and 358 bp wild-type products.

The progeny of $Irs1^{A/S}$ compound heterozygote matings were distinguished using a four-primer allele-specific PCR with nested primers surrounding the Ser307Ala mutation:

S7AF3 (5'- gcagcaaaagccagtcttcatcc),
NotBamR (5'- aggcacgcacccggaaggag),
BamF (5'- agtatggtgggtgggaaaccagga), and
S7AR (5'- ctggggtgacactgcggaagg).

In this assay, PCR of the *BamH*I-marked $Irs1^{A}$ allele (primers BamF + S7AR) gives a 399 bp product, while PCR of the $Irs1^{S}$ allele (primers S7AF3 + NotBamR) gives a 185 bp product. PCR of either allele (outer primers S7AF3 + S7AR) gives a 542 bp product.

RT-PCR of the *Irs1* transcript

Proper splicing of knock-in transcripts (Figure S1C) was verified by reverse-transcriptase PCR (RT-PCR) with the primers:

n2 (above) and
3prev1 (5'- tgaaatagttcgagtctgggtacccatgag),

giving 476 bp knock-in and 410 bp wild-type products.

Generation of LKO2-background mice

To produce mice lacking hepatic Irs2, mixed background (129Sv × C57BL/6) *Alb-*Cre^{+} $Irs1^{lox/lox}Irs2^{lox/lox}$ (LDKO) males {Dong, 2008 23028 /id} were bred with (C57BL/6) $Irs1^{A/+}$ females. The progeny were intercrossed to make the $Irs2^{lox}$ allele homozygous, after which *Alb-*Cre^{+} $Irs1^{A/lox}Irs2^{lox/lox}$ (A/lox::LKO2) and *Alb-*Cre^{+} $Irs1^{+/lox}Irs2^{lox/lox}$ males (+/lox::LKO2) were crossed with Cre-negative females to produce the mice in Figure 4.

Isolation of primary hepatocytes

For isolation of primary hepatocytes, livers of 6-month-old male mice were perfused via the vena cava using a syringe pump for 2 min. at ~3 mL/min. and then for 6 min. at ~7 mL/min., first with PS1 (1X HBSS, 1mM EGTA, 5.5 mM glucose) containing 100 U/mL penicillin/100 μg/mL streptomycin (P/S) to clear, then with PS2 (1X HBSS, 1.5 mM $CaCl_2$, 5.5 mM glucose, plus P/S) containing 0.375 mg/mL (~88 U/mL) of collagenase (Type 1, Worthington). Liver cells were dissociated by agitation with forceps in 5 mL of PS2 in a petri dish, then filtered through a 70

μm nylon filter and placed on ice. Non-parenchymal and dead cells were removed as follows. The filtered cells were spun once at 240 × *g*, resuspended in 8 mL of cold Williams E medium (Gibco) plus P/S, then mixed with 10 mL of a 1:9 (v/v) mix of 10X HBSS:Percoll (Amersham), and spun at 240 × *g* for 8 min. The pelleted cells (mostly live hepatocytes) were then washed twice with 10 mL of Williams E medium plus P/S, resuspended in 10 mL of growth medium (Williams E, P/S, L-glutamine, and 10% FBS), and counted. Cells were plated at a density of $4\times10^4/cm^2$ on plates coated with rat tail collagen (Collaborate Biochemical) and incubated for for ~3 h to allow attachment of hepatocytes. The attached hepatocytes were washed twice with warmed Williams E plus P/S and L-glutamine and incubated for 12 h in this medium before treatment with insulin.

Antibodies

Antibodies to actin, tubulin, and p110α, and phospho-specific antibodies to Akt (T308), Tsc2 (T1462), S6k (T389), Foxo1 (S256), and Irs1 (S302) and were from Cell Signaling Technology. Polyclonal antibodies to Irs1, Irs2, p85 and phospho-S307 of Irs1 were from UBI/Millipore, and anti-phosphotyrosine (4G10) and actinin antibodies were from Sigma. Irs2 was precipitated using protein A-purified polyclonal rabbit serum raised against the full length protein. In primary hepatocyte experiments, Irs1 was precipitated using a phosphorylation-insensitive mouse monoclonal antibody raised against an Irs1 peptide. In Fig 3F, Irs1 Ser/Thr phosphorylation was quantitated using a partial panel of mouse monoclonal antibodies previously determined to be highly site- and phospho- specific by peptide ELISA and/or peptide competition of Western blot immunoreactivity toward phosphorylated Irs1 (lysate of CHO-IR/Irs1 cells stimulated with okadaic acid).