Supplementary Section.

The role that inactivation of GSK3 isoforms resulting from Ser9/Ser21 phosphorylation plays in insulin-signalling and Wnt-mediated β-catenin stabilisation defined by knockin analysis.

By

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Materials. Protein G–Sepharose, ^{14}C-glucose-6-phosphate, 2-deoxy-D-[1-^{3}H]-Glucose, D-[^{14}C]-Mannitol, ^{32}Pγ-ATP were purchased from Amersham Pharmacia Biotech, protease-inhibitor cocktail tablets and Taq Polymerase were purchased from Roche. Restriction enzymes were from New England Biolabs. Precast SDS polyacrylamide Bis-Tris gels and tissue culture reagents were from Invitrogen. Tween-20, glycogen Type III from rabbit liver and glucose 6-phosphate were from Sigma. Human insulin from Novo-Nordisk and Glucose solution from Baxter were obtained from Ninewells Pharmacy, Dundee. PP1γ was expressed in *E. Coli* and purified as previously described (Alessi et al., 1993). Phosphocellulose P81 paper and cellulose 31ETCHR paper were from Whatman. Scintillation fluid was 'Optiscint Hisafe' from Perkin Elmer. Polyethylenimine (PEI) was from Polysciences (Warrington, PA, Cat no 23966). Dual-Luciferase Reporter Assay System (E1910) was from Promega (UK). All peptides were synthesised by Dr Graham Bloomberg at the University of Bristol.

Antibodies. The following antibodies were raised in sheep and affinity purified on the appropriate antigen: Total PKB (isolated PH domain of PKBα), Total GSK3α (residues 471-483 of rat GSK3 α, QAPDATPTLTNSS), used for immunoblotting, Total GSK3α (residues 314-327 of mouse GSK3α, LLGQPIFPGDSGVD), used for immunoprecipitation, Total GSK3β (full length human protein), used for immunoblotting and immunoprecipitation, Glycogen Synthase phospho-641 (residues 635-650 of mouse Glycogen Synthase, RYPRPVpSVPPSPSLSR), Glycogen Synthase phospho-645 (residues 635-650 of mouse Glycogen Synthase, RYPRPVSVPPpSPSLSR) and Glycogen Synthase phospho-641/645 (residues 635-650 of mouse Glycogen Synthase, RYPRPVpSVPPpSPSLSR). The total PKBα antibody used to immunoprecipitate PKBα

was a mouse monoclonal antibody raised against residues 1-149 of human PKB and was purchased from Upstate Inc (#05-591). The following antibodies were purchased from Cell Signalling Technology and the catalogue number indicated: PKB phospho S473-P (#9271), GSK3α/GSK3β phospho S21-P/S9-P (#9336). The Pan-GSK3 isoform antibody used for quantification of GSK3α and GSK3β levels in Figure 4 (#44-610) and the glycogen synthase phospho S641-P/S645-P antibody used to monitor hepatic glycogen synthase phosphorylation (#44-1092G) were from Biosource, and the anti-GSK3α/GSK3β phospho Y279-P/Y216-P (#612312) was from Transduction labs. The total glycogen synthase antibody was a mouse monoclonal antibody and was purchased from Chemicon (#MAB3106). Secondary antibodies coupled to horseradish peroxidase were from Pierce.

General methods and buffers. Restriction enzyme digests, DNA ligations, site directed mutagenesis, PCR, Southern Blotting and other recombinant DNA procedures were performed using standard protocols. All DNA constructs were verified by DNA sequencing which was performed by The Sequencing Service, School of Life Sciences, University of Dundee, Scotland using DYEnamic ET terminator chemistry (Amersham Pharmacia Biotech) on Applied Biosystems automated DNA sequencers. Lysis Buffer: 50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1% (by mass) Triton-X 100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1 μM microcystin-LR, 0.1% (by vol) 2-mercaptoethanol and 'Complete' proteinase inhibitor cocktail (one tablet per 50 ml). Buffer A was 50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 0.1% (by vol) 2-mercaptoethanol.

Construction of the GSK3α 21A knockin targeting vector. Genomic sequence information for GSK3α was obtained from the Celera database. A fragment of the coding region of GSK3α was amplified from EST AW214809 and used to probe a 129Sv mouse BAC library (Incyte genomics screening service). The resultant BAC clone that contained the 5' end of the mouse GSK3α gene was used to make a knockin targeting vector. Sequence analysis revealed that the region of the gene encoding Ser21 was located in exon 1. The targeting vector was constructed to introduce a pMC1-neo-pA cassette flanked by loxP sites (floxed) (Gu et al., 1993) at the 3' end of exon 1 (see supplementary Fig 1A). The 5' homology arm was constructed with a 4242bp *Xba*I fragment and a 2500bp PCR fragment containing exon 1. The 3' arm was generated by PCR of a 2500bp region. The sequence in exon 1 contained within the 5' arm coding Ser21 was mutated to code Ala using standard mutagenesis techniques. The existence of a *Pst*I site within the Neomycin cassette formed the basis for the southern blot screening. The PGK-TK-pA cassette was included at the 3'end of the construct for negative selection (supplementary

Fig 1A). Further details of the sequence of this targeting construct are available by request. The final knockin construct was purified on a cesium chloride gradient and was linearised using *Not*I before electroporation.

Construction of the GSK3β 9A knockin targeting vector. Genomic sequence information for GSK3β was obtained from the Celera database. A 1026bp fragment consisting of the 3' end of exon 1 and the 5' end of intron 1 of the mouse GSK3β gene was generated by PCR of mouse genomic DNA. This was used to probe a 129Sv mouse BAC library (The Centre for Applied Genomics, Toronto). The resultant BAC clone that contained the 5' end of the mouse GSK3β gene was used to make a knockin targeting vector. Sequence analysis revealed that the region of the gene encoding Ser9 was located in exon 1. The targeting vector was constructed to introduce a pMC1-neo-pA cassette flanked by loxP sites (floxed) (Gu et al., 1993) at the 3' end of exon 1 (see supplementary Fig 1D). The 5' homology arm was constructed with a 5395bp *Asu*II fragment and a 2500bp PCR fragment containing exon 1. The 3' arm was generated by PCR of a 2500bp region. The sequence in exon 1 contained within the 5' arm coding Ser9 was mutated to code Ala using standard mutagenesis techniques. The introduction of an *Eco*RI site in the 5' arm and a *Kpn*I site in the 3' allowed screening of recombinant ES cell lines by southern blot. The PGK-TK-pA cassette was included at the 3'end of the construct for negative selection (supplementary Fig 1D). Further details of the sequence of this targeting construct are available by request. The final knockin construct was purified on a cesium chloride gradient and was linearised using *Not*I before electroporation.

ES Cell Targeting. E14 mouse embryonic stem (ES) cells (kindly provided by Dr H. van der Putten) were grown in Dulbecco's modified eagle's medium containing high glucose supplemented with 15% foetal calf serum, 0.1mM non-essential amino acids, antibiotics (100 units penicillin G, 100 μg/ml streptomycin), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol and 25 ng/ml Murine Leukaemia Inhibitory Factor. During the initial selections ES cells were maintained on a feeder layer of mitotically inactivated G418 resistant primary embryonic fibroblasts, derived from MTK-Neo mice (Stewart et al., 1987). Targeting of the GSK3α or GSK3β gene to obtain GSK3$\alpha^{+/21A}$ or GSK3$\beta^{+/9A}$ ES cells (supplementary Fig 1) was performed using standard procedures (Joyner, 1993). Briefly, following electroporation, cells were plated in the absence of G418 for 48 h and then grown in the presence of 0.2 mg/ml G418 and 2 μM Gancyclovir (thymidine kinase negative selection) for 10-14 days. Colonies were picked and cultured in the absence of G418 and Gancyclovir. Southern blotting identified targeted cell lines (GSK3$\alpha^{+/21A}$ and GSK3$\beta^{+/9A}$). ~5% of cell lines screened were correctly targeted. In order to remove the neomycin cassette from GSK3$\alpha^{+/21A}$ ES cells, $1x10^7$ cells were transfected

with 30 μg pMC-CrePuro, which encodes for puromycin resistance and Cre recombinase, by electroporation using a Biorad GENE PULSER II, set at 240 V and 500 μF. As described below, in the case of the GSK3β knockin mice, the neomycin cassette was removed by crossing the mice with Cre expressing mice. The pMC-CrePuro plasmid was prepared by cloning the blunted *Asc*I fragment of pKO SelectPuro (Stratagene) containing the puromycin gene under the PGK promoter, into the blunted *Hind*III site of pMC-Cre (Gu et al., 1993). The cells were plated out onto gelatinised tissue culture plastic and puromycin was added to the medium at a concentration of 2 μg/ml during days 2-4. On day 4 the cells were trypsinised and seeded at low density (1000 cells per 10 cm dish) and colonies were picked in duplicate into 96 well tissue culture plates. One plate was subjected to PCR analysis using Primers P1 (5'- 3' TTGAAGTGGCTGGTACTGGCTCTG) and P2 (5'-3' GTGTGCTCCAGAGTAGTACCTAGC) and the other plate used for expansion of positive ES cells. As depicted in supplementary figure 1C, primers P1 and P2 result in a 271 bp product from the wild type allele and a 317 bp product from the targeted allele after the neomycin cassette had been excised. Approximately 20% of puromycin resistant cell lines had the neomycin cassette fully excised.

Generation of GSK3α$^{+/21A}$ and GSK3β$^{+/9A}$ mice. All animal studies and breeding performed in this study was approved by the University of Dundee ethical committee and performed under a UK Home Office project license. Heterozygous knockin GSK3α$^{+/21A}$ or GSK3α$^{+/9A}$ ES cell clones were microinjected into C57Black6 X Balb/c blastocysts, which were then re-implanted into recipient female mice. Chimeric mice that had a high degree of ES cell contribution were identified by coat colour and were then crossed to C57Black6 mice. Genotyping of GSK3α$^{+/21A}$ mice was carried out by PCR of genomic DNA isolated from tails using primers P1 and P2, originally used to screen cells for the excision of the neomycin cassette were used to genotype the mice (supplementary Fig 1C). GSK3β(+/9A) mice still had the neomycin selection cassette present between exon 1 and exon 2 of the GSK3β gene. In order to remove the selection cassette, these mice were crossed to Bal1 mice, which express the Cre recombinase enzyme ubiquitously. The resulting progeny were subjected to PCR genotyping using Primers P3 (5'- 3' TCACTGGTCTAGGGGTGGTGGAAG) and P4 (5'-3' GGAGTCAGTGACAACACTTAACTT). As depicted in supplementary figure 1F, primers P1 and P2 result in a 233 bp product from the wild type allele and a 352 bp product from the targeted allele after the neomycin cassette had been excised.

Immunoprecipitation and assay of protein kinases. 500 μg of skeletal muscle lysate was used to immunoprecipitate PKBα, 1mg was used for GSK3α and 100μg was used

for GSK3β. The lysates were incubated at 4 ºC for 1 h on a shaking platform with 5 μg of each antibody coupled to 10 μl of protein G-Sepharose (50% beads by volume). In the case of PKBα the immunoprecipitates were washed twice with 1 ml of Lysis Buffer containing 0.5 M NaCl, and once with 1 ml of Buffer A (50 mM Tris pH 7.5, 0.1 mM EGTA, 0.1% (by vol) 2-mercaptoethanol). The standard assay (50 μl) contained: washed Protein G-Sepharose immunoprecipitate, 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (by vol) 2-mercaptoethanol, 2.5 μM PKI (TTYADFIASGRTGRRNAIHD, peptide inhibitor of cyclic-AMP-dependent protein kinase), 10 mM magnesium acetate, 0.1 mM [γ^{32}P]ATP (~200 cpm/pmol) and Crosstide (GRPRTSSFAEG, 30 μM) (Alessi et al., 1996). The assays were carried out for 30 min at 30ºC, the assay tubes being agitated continuously to keep the immunoprecipitate in suspension, then terminated and analysed as described previously (Alessi et al., 1995). 1 mUnit of activity was that amount of enzyme that catalysed the phosphorylation of 1 pmol of substrate in 1 min. In the case of GSK3α and GSK3β the immunoprecipitates were washed once with 1 ml of Lysis Buffer containing 0.5 M NaCl, and twice with 1 ml of Buffer A. The pellets were then dephosphorylated and reactivated by treatment with 12μl 100mU/ml PP1γ for 30 min at 30ºC. The reaction was terminated by the addition of 6 μl 100μM microcystin-LR (MacKintosh et al., 1990). Control incubations were carried out with 12μl 100mU/ml PP1γ preincubated with 6 μl 100μM microcystin-LR for 30 min at 30ºC. The assay (50 μl) contained: washed Protein G-Sepharose immunoprecipitate, 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (by vol) 2-mercaptoethanol, 10 mM magnesium acetate, 0.1 mM [γ^{32}P]ATP (~200 cpm/pmol) and Phospho-GS2 (YRRAAVPPSPSLSRHSSPHQpSEDEEE, 20 μM) and was carried out for 30 min at 30ºC, the assay tubes being agitated continuously to keep the immunoprecipitate in suspension. The reactions were terminated and analysed as for assay of PKB. The activity of GSK3α/β was expressed as the specific activity (Fig 2B) or reactivation ratio (Fig 6C), activity –PP1γ/ activity +PP1γ.

Immunoblotting. Unless indicated otherwise, 20μg of protein lysate in SDS Sample Buffer was subjected to SDS/polyacrylamide gel electrophoresis and transferred to nitrocellulose. For phospho-specific antibody blots the nitrocellulose membranes were immunoblotted at 4ºC for 16h using the indicated antibodies (1-2 μg/ml for the sheep antibodies or 500-1000 fold dilution for commercial antibodies) in the presence of 10 μg/ml of the de-phosphopeptide antigen used to raise the antibody for sheep antibodies. The blots were incubated in 50 mM Tris/HCl pH 7.5, 0.15M NaCl, 0.2% (by vol) Tween containing 5% (by mass) skimmed milk. For immunoblotting of total PKBα, GSK3α, and GSK3β, the nitrocellulose membranes were immunoblotted for 2 h at room

temperature using the antibodies as above. Detection was performed using horse radish peroxidase conjugated secondary antibodies and the enhanced chemiluminescence reagent.

Quantitative immunoblot LI-COR analysis. The immunoblots were incubated with antibodies in 50 mM Tris/HCl pH 7.5, 0.15M NaCl, 0.2% (by vol) Tween containing 5% (by mass) skimmed milk overnight at 4 ºC. The blots were washed and incubated for 1 hour with fluorescently labelled anti-mouse secondary antibody at room temperature. The blots were analysed using a LI-COR Odyssey infrared detection system following the manufactures guidelines. The band intensity was quantified using LI-COR software. Using this approach more quantitative analysis of immunoblots can be achieved than using the standard chemiluminescence techniques (see http://www.licor.com/).

Cloning of human and mouse GSK3α and GSK3β. The cloning of human GSK3β in the pEBG2T vector has been described earlier (Shaw and Cohen, 1999). The coding region of human GSK3α (NCBI accession number, NP_063937) was amplified by PCR using an EST from IMAGE Consortium as a template (IMAGE CLONE 3903896, NCBI accession number, BC027984) with primers: 5-GGATCCAGCGGCGGCGGGCCTTCGGGAGGC-3' and 5'-GCGGCCGCTCAGGAGGAGTTAGTGAGGGTAGGTGT-3'. This fragment was ligated into pCR2.1-TOPO vector (Invitrogen), the sequence verified, then subcloned as a *Bamh1-Not1* fragment into pEBG2T expression vector. The highly GC-rich 0.5 Kb N-terminal region of mouse GSK3α cDNA was amplified from IMAGE clone 6513981 (NCBI accession number, BQ964176) using primers 5'-GGATCCATGAGCGGCGGCGGGCCTTCGGGA-3' and 5'-CCTCACAATATTGCAGTGGTCCAGC-3. The PCR product was cloned and sequenced in pCR2.1-Topo vector, then the 0.5 Kb *Bamh1-Pst1* fragment coding for amino acid 1-160 and the *Pst1-Not1* fragment from EST NCBI Acc. BQ964176 coding for the rest of the cDNA (including 1 Kb non-translated region) was ligated with a three-way ligation into the *Bamh1-Not1* site of pEBGT vector. The full length coding region of mouse GSK3β (NCBI accession number, AF156099) was amplified from IMAGE clone 647729, (NCBI accession number, AW230870) with the following primers: 5'-ACTAGTATGTCGGGGCGACCGAGAACCACCTCC-3' 5'-GCGGCCGCTCAGGTGGAGTTGGAAGCTGATGCAGA-3'. The resulting PCR product was ligated into pCR2.1-TOPO vector, sequenced then subcloned as a *Spe1-Not1* fragment into pEBG2T expression vector.

PEI transfection and purification of GST-GSK3 isoforms. HEK-293 cells cultured on 10-cm-diameter dishes were transfected with 10 μg of the pEBG2T construct encoding

the indicated isoforms of human and mouse GSK3 using the PEI transfection reagent (Durocher et al., 2002). For each dish, 10μg of DNA was transfected using 20μl of 1mg/ml PEI. At 36 h post-transfection, the cells were lysed in 0.5 ml of ice-cold lysis buffer, the lysates pooled, centrifuged at 4 °C for 30 min at 15,000g and the GST fusion proteins were purified by affinity chromatography on glutathione–Sepharose and stored in buffer A containing 0.27 M sucrose, as described previously for GST–PDK1 (Alessi et al., 1997).

Synthesis of AR-AO14418. The GSK3 inhibitor AR-AO14418 was efficiently synthesised from 4-methoxybenzylcyanate and 2-amino-5-nitrothiazole synthesised in 73% yield through modification of the procedure of Bhat and co-workers (Bhat et al., 2003).

Glucose Tolerance Test. Following an overnight fast, mice were weighed and basal blood glucose was measured using the Esprit Blood Glucose Monitoring System (Bayer) following tail incision. The mice were then injected intraperitoneally with 2 mg/g of glucose. Blood glucose was then measured at the indicated times. For the experiment shown in Fig 6E, mice that had been fasted overnight were anaesthetized 13.5 mg/kg of Sagatal (pentobarbitone sodium) 30 min prior to injection of glucose.

***in situ* muscle contraction**. Mice weighing 20-40 g were terminally anaesthetized. The sciatic nerve of both legs was exposed and electrodes were attached to the nerve on one of the legs. This leg was subjected to electrical stimulation using a Grass S88 pulse generator for 10 min (train rate, 1/s; train duration, 500 ms; pulse rate, 100 Hz; duration, 0.1 ms at 2-5 V), and the other leg served as a sham-operated non-contracting control. Immediately after nerve stimulation, mice were sacrificed by cervical dislocation and hind limb muscles (gastronemius complex, tibialis anterior, extensor digitorum longus) were isolated rapidly and frozen in liquid nitrogen.

Glucose transport in isolated muscle. Mice were fasted for 16 hr before study and sacrificed by cervical dislocation and their soleus muscles were removed. The method used to calculate glucose uptake was similar to that described previously (Bruning et al., 1998). Tendons from both ends of each muscle were tied with suture (silk 4-0) and mounted on an incubation apparatus. The muscles were incubated for 50 min at 37°C in 8 ml Krebs-Ringer-Bicarbonate (KRB) buffer (117 mM NaCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24.6 mM $NaHCO_3$) containing 2 mM Pyruvate in the presence or absence of 100 nM insulin. Transport was then measured in 2 ml KRB buffer containing 1 mM 2-deoxy-D-[1-^{3}H]-Glucose (1.5 μCi/ml) and 7 mM D-[^{14}C]-Mannitol (0.3 μCi/ml) at 30°C for 10 min in the presence or absence of 100 nM insulin. Both the KRB and transport buffer were continuously gassed with 95 % O_2-5 % CO_2. To terminate

the transport, muscles were dipped in KRB buffer containing 80 μM cytochalasin B at 4˚C. Muscles were blotted on filter paper, trimmed, and frozen in liquid Nitrogen. Muscles were processed by incubating in 250 μl 1N NaOH at 80˚C for 10 min, neutralized with 250 μl 1N HCl, and particulates were precipitated by centrifugation at 13,000 g for 2 min. ^{3}H and ^{14}C radioactivity present in 350 μl of the supernatant was measured by scintillation counting. Blanks were subtracted from values obtained from muscle derived from the same mouse that had been through the same treatments in the absence of radioactivity. From the ^{14}C specific radioactivity the extracellular water volume present in the muscle was calculated. This enables the extracellular ^{3}H volume to be determined and subtracted from the total ^{3}H radioactivity to calculate the intracellular amount of 2-deoxy-D-[1-^{3}H]-Glucose transported per gram of muscle per hour.

Incubation of isolated muscle with insulin and AR-A014418. Mice weighing 20-40 g were killed by cervical dislocation and the soleus muscle was rapidly extracted. Tendons from both ends of the muscle were tied with suture and mounted on an incubation apparatus to maintain resting length. The muscles were incubated for 1 hr at 37˚C in 8 ml Krebs-Ringer-Bicarbonate (KRB) buffer (117 mM NaCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24.6 mM $NaHCO_3$) containing 2 mM Pyruvate in the presence or absence of 100 nM insulin or 20 μM AR-A014418 (dissolved in DMSO) or DMSO as a control, that had been added to the 8 ml KRB buffer immediately prior to incubation of the muscle. The buffers were continuously gassed with 95 % O_2-5 % CO_2. Immediately after treatments, muscles were quickly isolated and frozen in liquid Nitrogen.

Measurement of glycogen. 30-40 mg quadricep muscle or liver was hydrolyzed in 2 N HCl by heating at 100°C for 2 h. The resulting solution was neutralised, and the resulting free glucosyl units were assayed spectrophotometrically using a hexokinase-dependent assay kit from Amresco (Ohio, USA) as described previously (Bondar and Mead, 1974).

Measurement of plasma insulin levels. Blood was collected from mice following cervical dislocation and incubated on ice for 30 min. The blood was then centrifuged at 3,000 g for 10 min and the plasma supernatant was collected. The plasma insulin level was measured using ultra sensitive Insulin ELISA kit (90060) and mouse insulin standards (90090) both purchased from Crystal Chem Inc. USA. 15 μl of plasma was used for each assay and mouse insulin standards from 0–6 ng/ml were used in order to generate a standard curve.

Generation of homozygous knockin ES cells. Homozygous knockin GSK3α/β$^{21A/21A/9A/9A}$ ES cells were isolated from morulas resulting from a GSK3α/β $^{21A/21A/9A/9A}$ X GSK3α/β$^{21A/21A/9A/9A}$ cross using standard procedures (Joyner, 1993). Briefly, 8 cell compacted morulas were isolated from the oviducts of females 2.5 days after

mating (as determined by plug). The morulas were washed in M2 medium (Sigma) and then transferred to culture drops of M16 medium (Sigma) and kept overnight at 37°C and 5% CO_2. The blastocysts were then transferred to 96 well plates that had been coated with a feeder layer of mitotically inactivated primary embryonic fibroblasts. Cells were then cultured in 25% FCS and 50 ng/ml Murine Leukaemia Inhibitory Factor. After 10 days cell lines were expanded to 24 well plates and subsequently genotyped. Similarly, wild type ES cells were isolated from blastocysts isolated by crossing wild type mice of a similar genetic background to the homozygous knockin GSK3α/β$^{21A/21A/9A/9A}$ mice.

Wnt-signalling studies. Medium containing Wnt3a as well as control medium not containing Wnt, was generated by culturing Wnt3a expressing L-Wnt3a cells and parental L-cells respectively as described previously (Willert et al., 2003). These cells were kindly provided to us by Roel Nusse and colleagues at the University of Stanford. In order to measure transcriptional activity controlled by Wnt3a we employed a β-catenin luciferase reporter construct or a similar construct where the LEF/TCF binding sites had been mutated, kindly provided to us by Freddy Radtke and colleagues at the Ludwig Institute for Cancer Research, Lausanne. Wild type and GSK3α/β$^{21A/21A/9A/9A}$ knockin ES cells were cultured in 12-well plates and were transfected with 2 μg of indicated reporter construct. As a control, the cells were also co-transfected with a Renilla luciferase construct under the control of an SV-40 promoter, to enable differences in confluency or transfection efficiency to be accounted for. 24 h post-transfection, cells were incubated in medium containing Wnt3a or control medium. Luciferase activity was assessed following lysis of the cells in 200 μl of Promega Passive lysis buffer using the Promega dual reporter system according to manufacturers instructions.

Supplementary Section References

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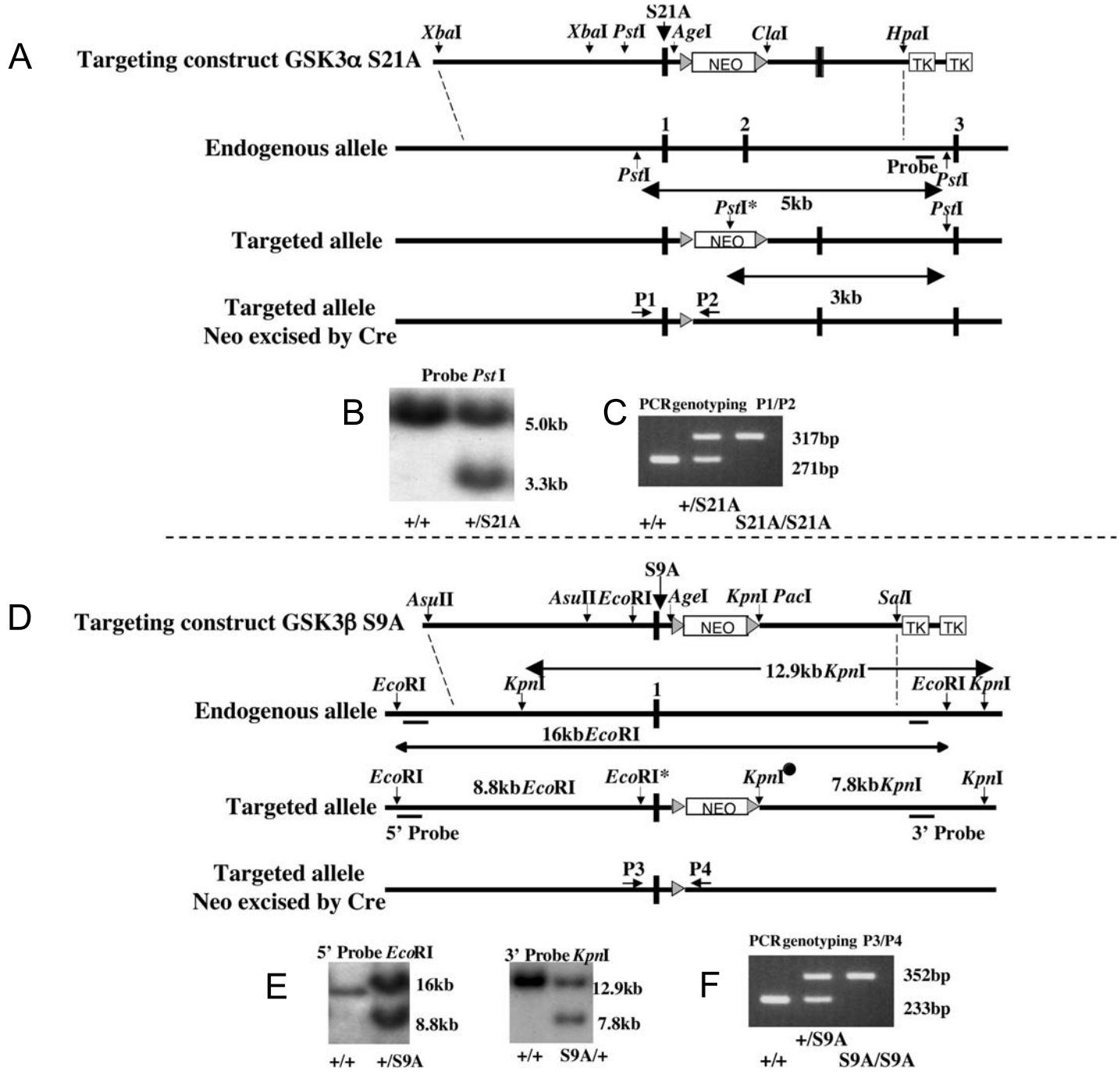
Supplementary Figures

Supplementary Figure 1 – GSK3α and GSK3β knockin strategy: A, Diagram depicting the GSK3αS21A knockin construct, the endogenous GSK3α allele containing exons 1 and 2, the targeted allele with the neomycin selection cassette still present, and the targeted allele with the neomycin cassette removed by Cre recombinase. The black boxes represent exons and the grey triangles represent LoxP sites. The two *Pst*I restriction sites present in the endogenous allele are shown and the extra site, introduced as part of the neomycin cassette is marked with an asterisk. B, After positive and negative selection of cell lines (see Materials and Methods), genomic DNA from the indicated cell lines was purified and digested overnight using *Pst*I, the digested DNA electrophoresed on a 1% agarose gel and transferred to nitrocellulose. The membrane was then incubated with a ^{32}P-labelled 3' probe. The 3' probe detects a fragment of 5kB from the wild type allele and 3.3kB from the targeted knockin allele. C, The indicated mice were genotyped using PCR primers P1 and P2. The wild type allele generates a 271bp product while the knockin allele generates a 317bp product due to the presence of the LoxP site and flanking region, which remains in an intronic region following Cre mediated excision of the neomycin selection cassette. D, As above except that the diagram depicts the GSK3βS9A knockin strategy. The two *Eco*RI restriction sites present in the endogenous allele are shown and the additional site, introduced as part of the targeting construct, is marked with an asterisk. Moreover, 2 *Kpn*I restriction sites present in the endogenous allele are shown. An extra site, introduced as part of the targeting construct is marked with an black circle. E, This allowed screening with a 5'probe, which after *Eco*RI digestion detects a fragment of 16kB from the wild type allele and 8.8kB from the targeted knockin allele. This also allowed screening with a 3'probe, which after *Kpn*I digestion detects a fragment of 12.9kB from the wild type allele and 7.8kB from the targeted knockin allele. F, The indicated mice were genotyped using PCR primers P3 and P4. The wild type allele generates a 233bp product while the knockin allele generates a

352bp product due to the presence of the remaining LoxP site in an intronic region.
Supplementary Figure 2 – Growth and glucose tolerance of knockin mice. A, The indicated male and female wild type (WT) and knockin (KI) mice were weighed once a week between the ages of 4 and 16 weeks. Wild type mice are represented by a solid line and knockin mice by a dotted line. Each point represents the mean ± SEM. n= number of mice analysed in each group. No statistical difference in the mean weights was observed for any group using a Student's T-test. B, Glucose tolerance test of the indicated 20 week old mice. Mice were injected intraperitoneally with 2 mg/g glucose solution and the blood glucose concentration was determined at the indicated times. n indicates the number of mice in each group. The data are presented as the mean ± SEM. Approximate equal numbers of male and female mice were used in B.

Supplementary figure 1



Supplementary figure 2