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Research Article

The Effect of *SH2B1* Variants on Expression of Leptin- and Insulin-Induced Pathways in Murine Hypothalamus

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Supplemental Material

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Sh2b1 Knockdown

For the Sh2b1 knockdown, CLU188 cells (Biozol, Eching, Germany) were co-transfected with mRNA of an SH2B1 specific zinc finger nuclease assay (Sigma Aldrich, St. Louis, MO, USA) and a plasmid containing the selection cassette for neomycin and GFP (obtained from Sigma Aldrich, St. Louis, MO, USA). The zinc finger nuclease assay was designed to cut the DNA in the dimerization domain of Sh2b1, thus even a gene copy which was cut by the zinc finger but did not insert into the plasmid should lead to a truncated and non-functional protein. The plasmid encoded for GFP and the neomycin cassette as selection markers. These genes were surrounded by restriction sites identical to the ones in the dimerization domain of Sh2b1. Hence after insertion of the plasmid only within the restriction sites of the zink finger nucleases, the cells are able to produce GFP and the resistance necessary for cell division in media containing G418 as a selection marker. Cells were plated in 24-well plates 48 hours prior to the experiment with concentrations of 40,000 cells per well. Fresh medium (0.5 ml per well DMEM, Life Technologies, Carlsbad, CA, USA, with 10% FBS, Merck Millipore, Darmstadt, Germany, and Penicillin-Streptomycin, Life Technologies, Carlsbad, CA, USA,) was provided to the cells 1h prior to transfection with calcium phosphate (CaPo) mix (8 µl H2O, 8 µl 2x HEBES and 1.1 µl 2.5 M calcium chloride solution). The appropriate amount of DNA/RNA (0.2ng SH2B1 clone DNA and 0.9 ng empty vector; total DNA/RNA amount 1.1 ng per well) was added to the mix. 1.1 µl calcium chloride solution per well was added. The transfection mix as incubated at room temperature for 20 min.

Cells were controlled for insoluble precipitate produced by the CaPho-bound DNA particles in the media and incubated for 24 h at 37°C / 5% CO_2. Then the media were replaced with G418 (300µg/ml, Life Technologies, Carlsbad, CA, USA) containing media. Cells were analyzed for GFP production by microscopy with a FITC filter (Zeiss, Oberkochen, Germany). Transfection efficiency was calculated by the amount of cells producing GFP divided by the total number of cells. Only wells with a transfection efficiency above 79.6% were used. The knockdown reduced the amount of Sh2b1 protein in the CLU188 by 29.5% as determined by Western blot (SH2B1 Antibody by Abcam, Cambridge, UK).

Transfection of the human SH2B1 clones

To analyze the impact of human SH2B1 mutations on leptin and insulin signaling, the hypothalamic CLU188 cells containing the Sh2b1 knockdown were transfected (calcium phosphate method) with SH2B1 vectors comprising different variants under a CMV promoter (Origene, Rockville, MD, USA): α wild type, Arg67Cys: rs781063312, Lys150Arg: rs141195883, Thr175Ala: rs181294111, Thr343Met: rs139298340, Thr484Ala: rs7498665, Ser616Pro: rs142515048, Pro689Leu. Mutation insertion was provided by a commercial partner (GenScript, Piscataway, NJ, USA).

Cell stimulation

After transfection with the human SH2B1 clones with or without variants, the cells were stimulated with 100 µg murine leptin or insulin (Sigma Aldrich, St. Louis, MO, USA) in DMEM (LifeTechnologies, Carlsbad, CA, USA) containing 10% FBS (Merck Millipore, Darmstadt, Germany) and Penicillin and Streptomycin (LifeTechnologies, Carlsbad, CA, USA). The cells were incubated for 1h at 37°C / 5% CO_2 and were harvested after the medium was exchanged with ice cold PBS (Life Technologies, Carlsbad, CA, USA) using a scraper. The 1h incubation time was derived from previous experiments by Morris et al. (2009) and Chen et al. (2013) for insulin stimulation and our group (Volckmar et al. 2012) for leptin stimulation. For the expression analysis, the cells were immediately frozen and stored at -80°C. Expression of leptin receptor and insulin receptor was confirmed experimentally (TaqMan assay, expression array).

Expression analysis

The stimulated cells were thawed from -80°C on ice and the RNA was extracted using the RNAeasy Mini prep (Qiagen, Hilden, Germany). This kit includes genomic DNA reduction by minimal co-binding of DNA and RNA to the columns. After RNA extraction, the amount of RNA was measured using NanoDrop 2000 (Thermo Fisher Scientific, Oberhausen, Germany).

To further reduce the amount of contaminating DNA, the RT² First Strand Kit (Qiagen, Hilden, Germany) contains a gDNA elimination step which is performed before transcription. The gDNA elimination buffer was incubated with the RNA for 5 minutes at 37°C, then immediately placed on ice. For reverse transcription, the kit supplies all buffers and enzyme. The RT takes 20 minutes at 40°C.

The reversely transcribed cDNA was diluted and used with 2x SYBR green master mix (Qiagen) for insulin signaling pathway and JAK/STAT signaling pathway RT² Profiler PCR arrays (Qiagen, Hilden, GermanyStepOnePlus™ Real-Time PCR System; Life Technologies, Carlsbad, CA, USA). 40 PCR cycles at 60°C elongation temperature according to the manufacturer (Qiagen, Hilden, Germany). The analysis of the runs was performed with the software StepOnePlus™ Real-Time PCR System 2.1 (Life Technologies, Carlsbad, CA, USA).

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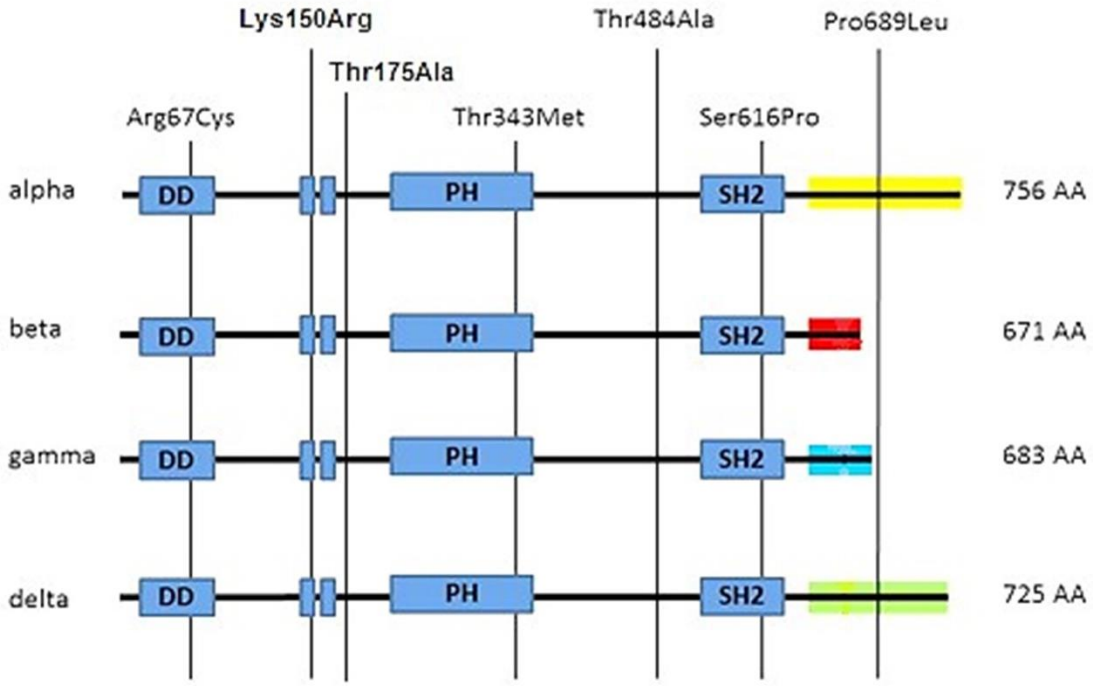
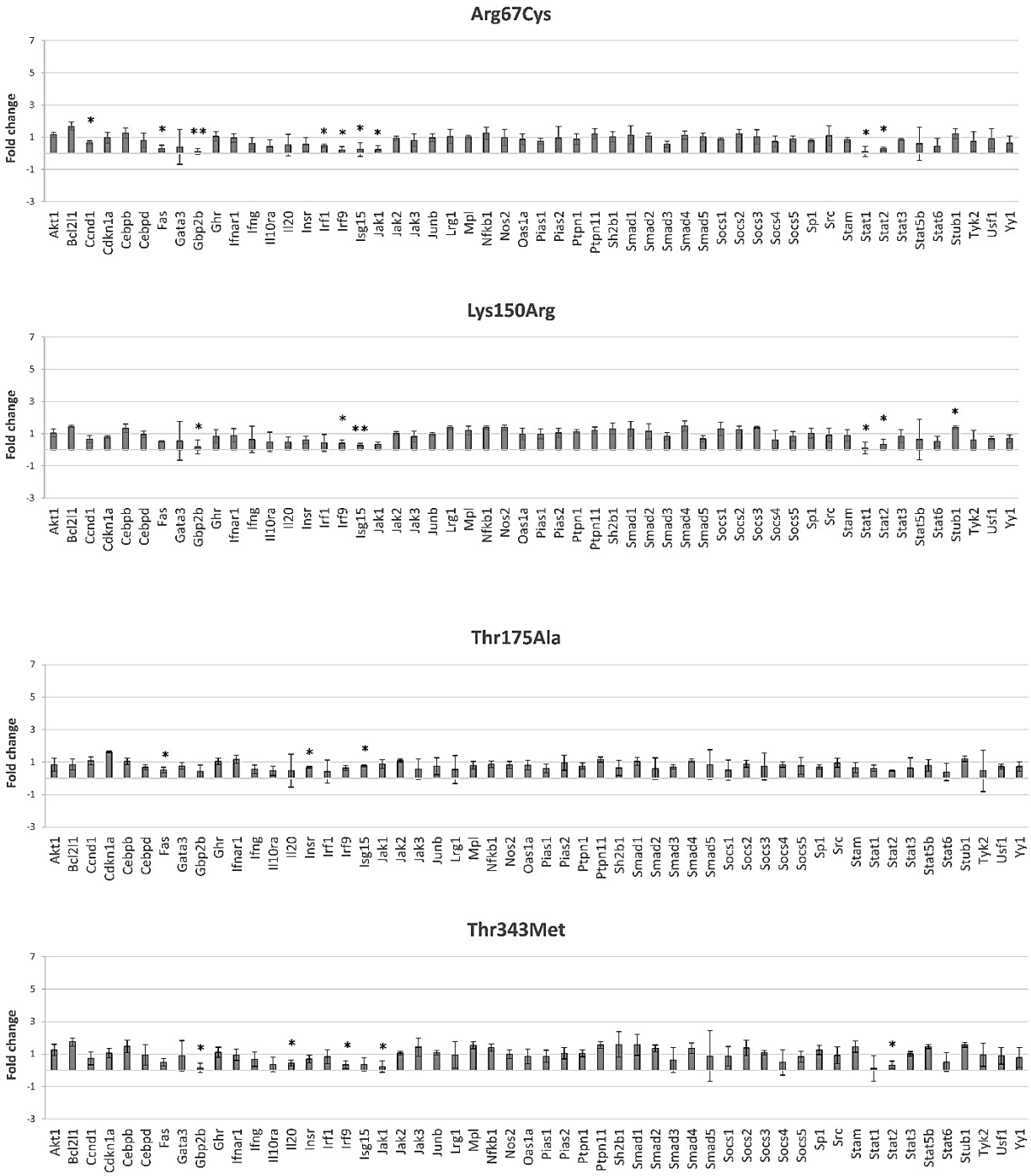
Supplementary figures

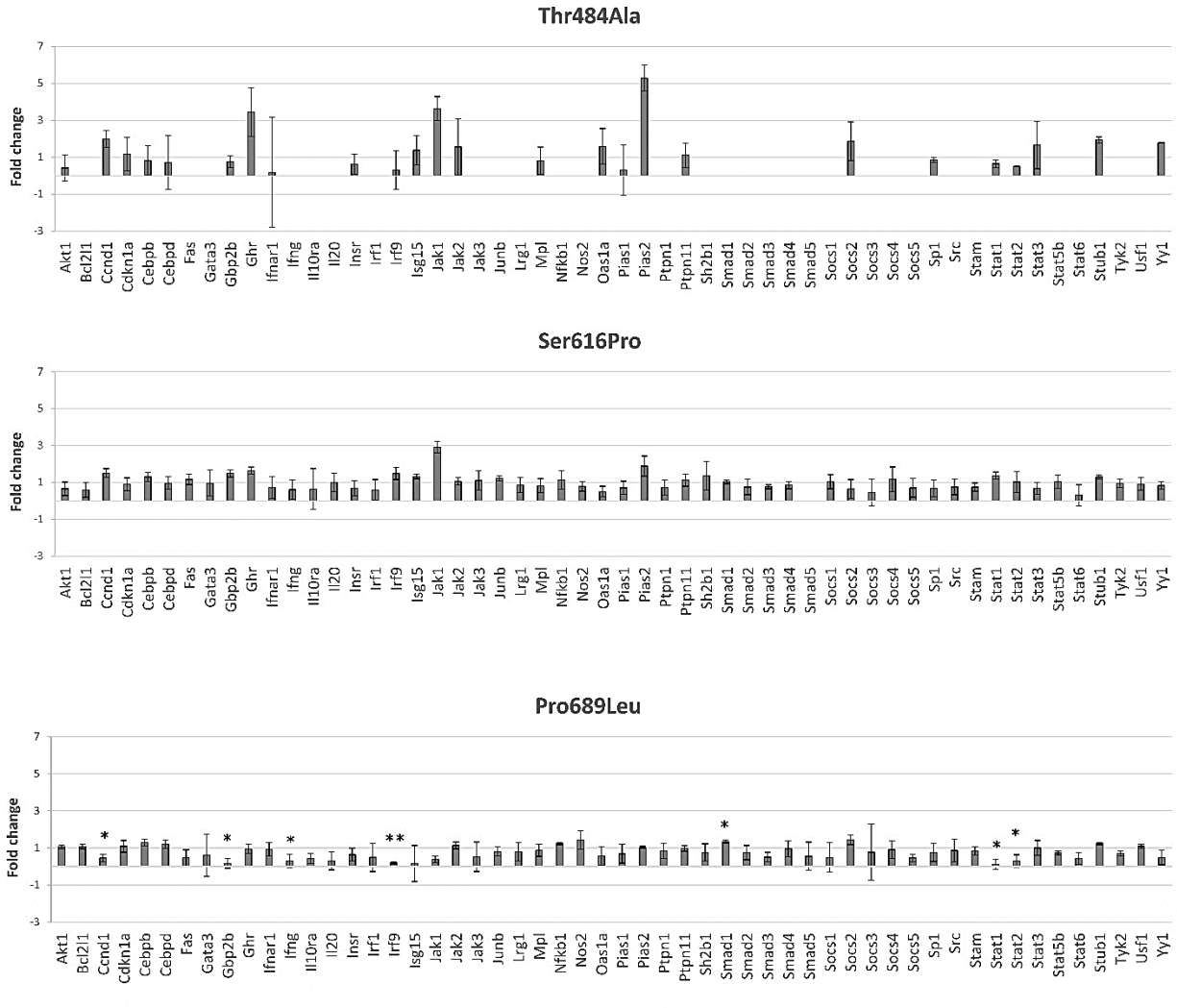
Supplementary Figure 1: All four isoforms (splice variants) of human *SH2B1* with the variants analyzed in this study



The different domains of SH2B1 (Dimerization (DD), Nuclear localization, Pleckstrin Homology (PH) and SH2) are represented as blocks. While the isoforms share the same sequence up to amino acid 632, the C-terminal tails (depicted with different colors) have different amino acid sequences due to different splicing and reading frames. The variant Pro689Leu only affects the alpha and delta splice variant of SH2B1.

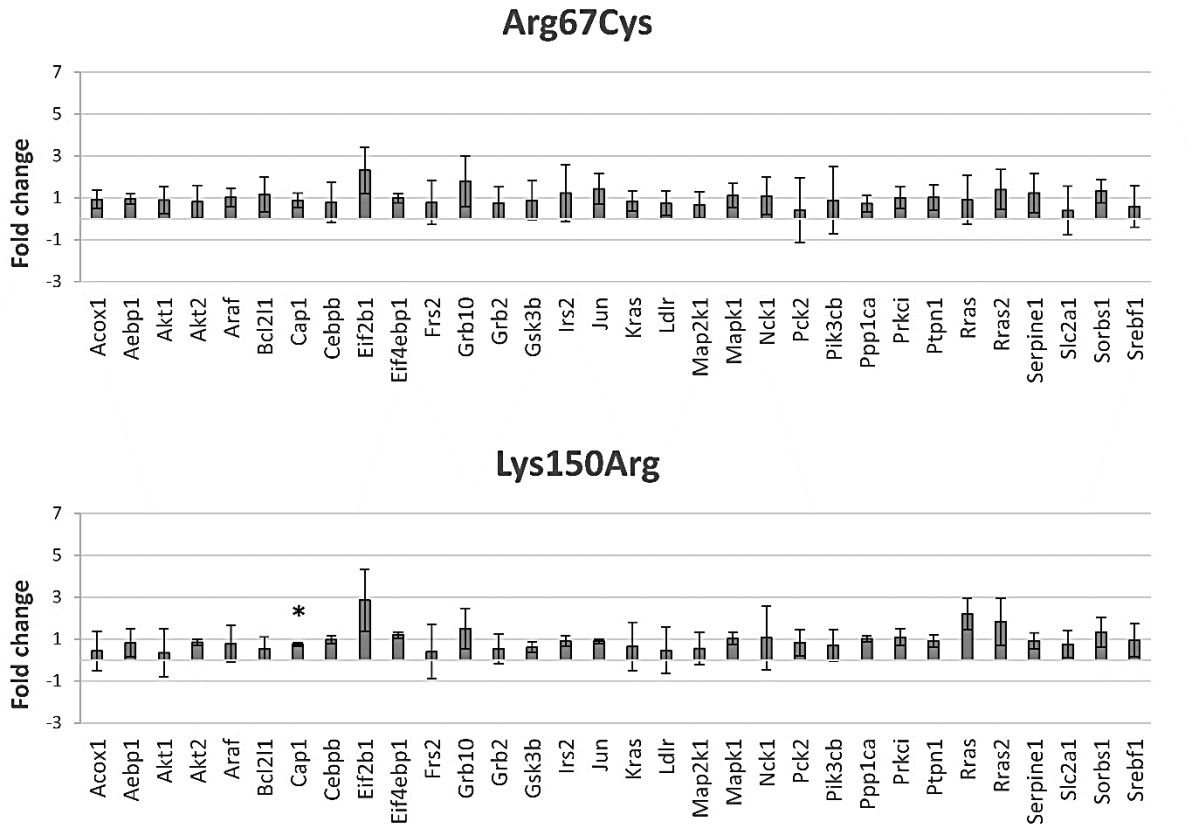
Supplementary Figure 2: Expression patterns for hypothalamic CLU188 cells transfected with different human *SH2B1* variants and stimulated with leptin

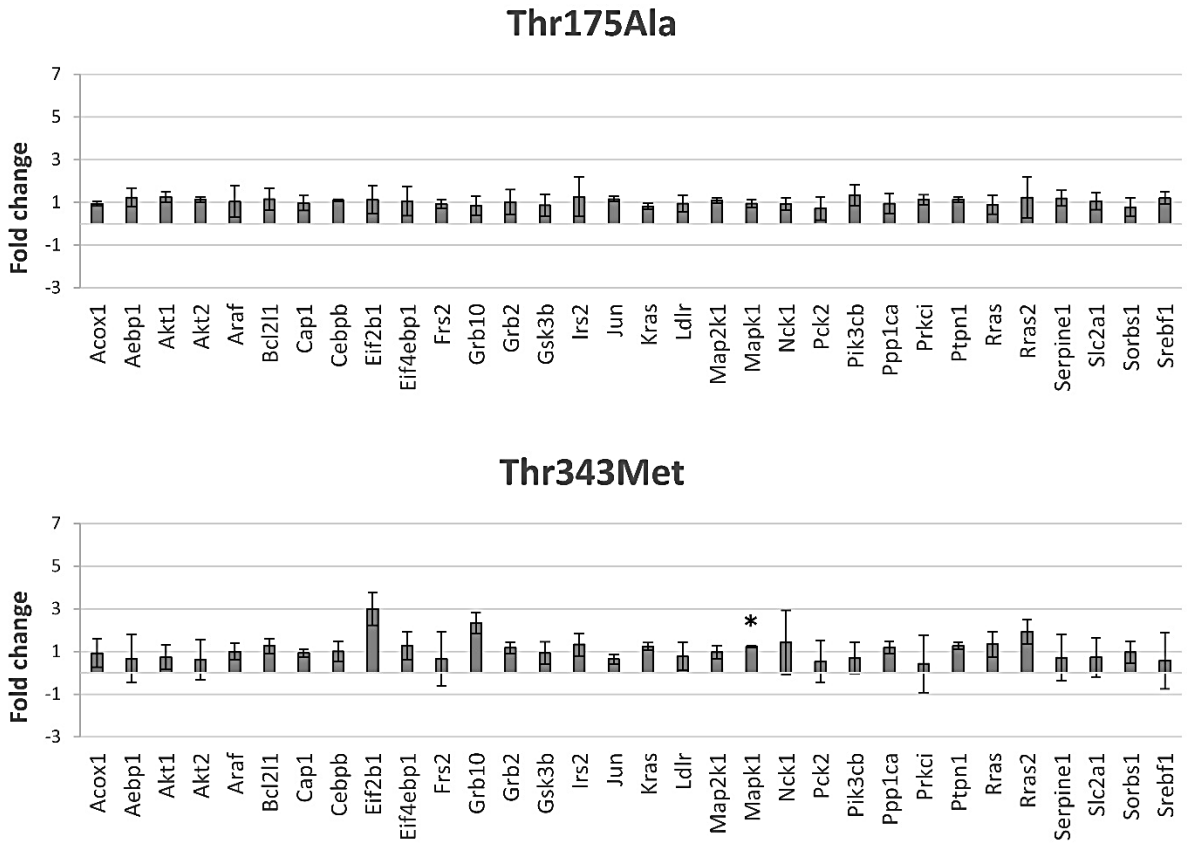




Expression patterns for hypothalamic CLU188 cells transfected with different human *SH2B1* variants and stimulated with leptin using the Qiagen RT² Profiler PCR Array for the murine JAK / STAT Signaling Pathway, showing only the genes expressed more than the recommended threshold of Ct 35; sorted by mutation; * = $p \leq 0.01$, ** = $p \leq 0.001$.

Supplementary Figure 3: Expression patterns for hypothalamic CLU188 cells transfected with different human *SH2B1* variants and stimulated with insulin




Thr175Ala
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Akt2
Araf
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Cap1
Cebpb
Eif2b1
Eif4ebp1
Frs2
Grb10
Grb2
Gsk3b
Irs2
Jun
Kras
Ldlr
Map2k1
Mapk1
Nck1
Pck2
Pik3cb
Ppp1ca
Prkci
Ptpn1
Rras
Rras2
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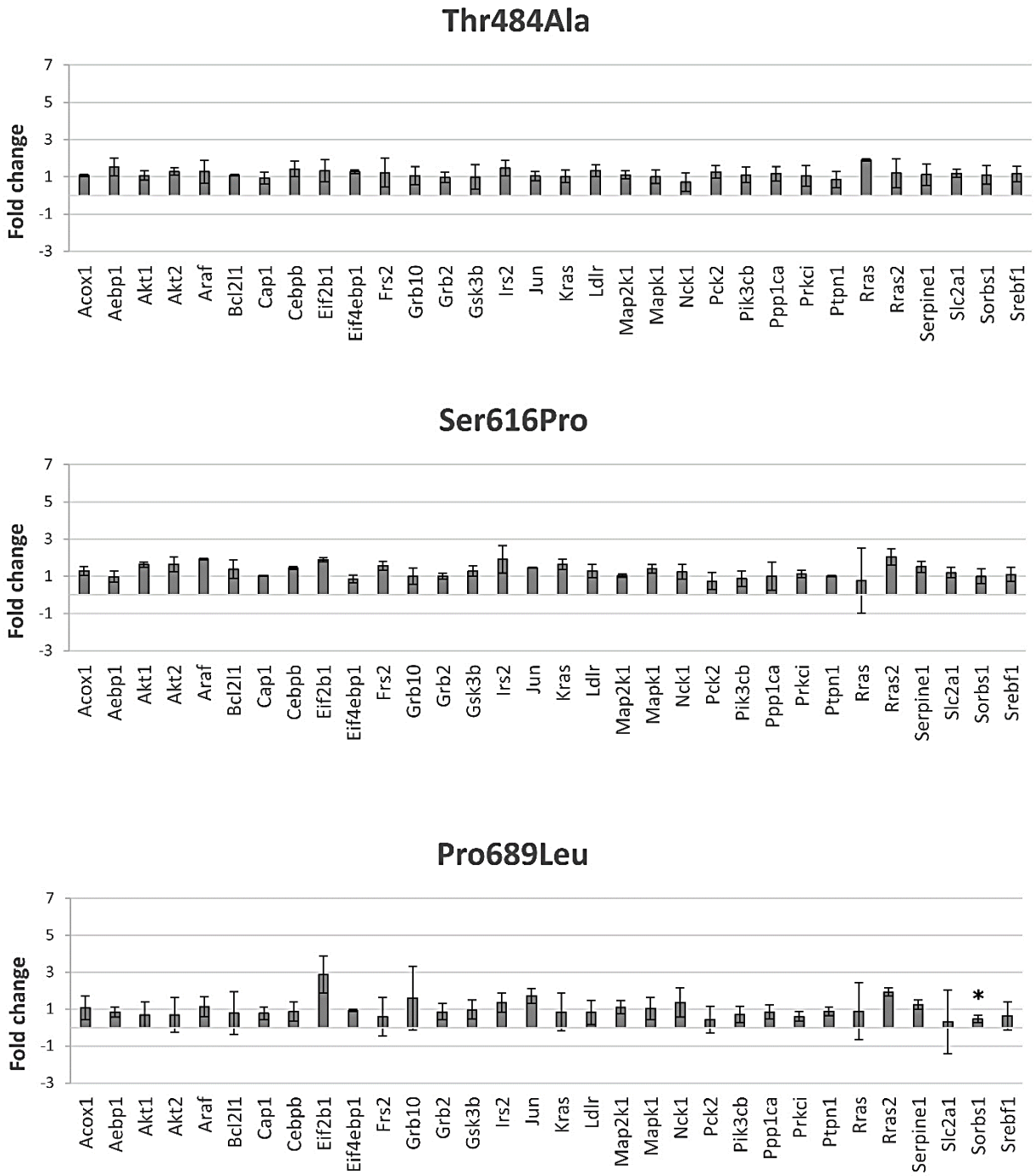
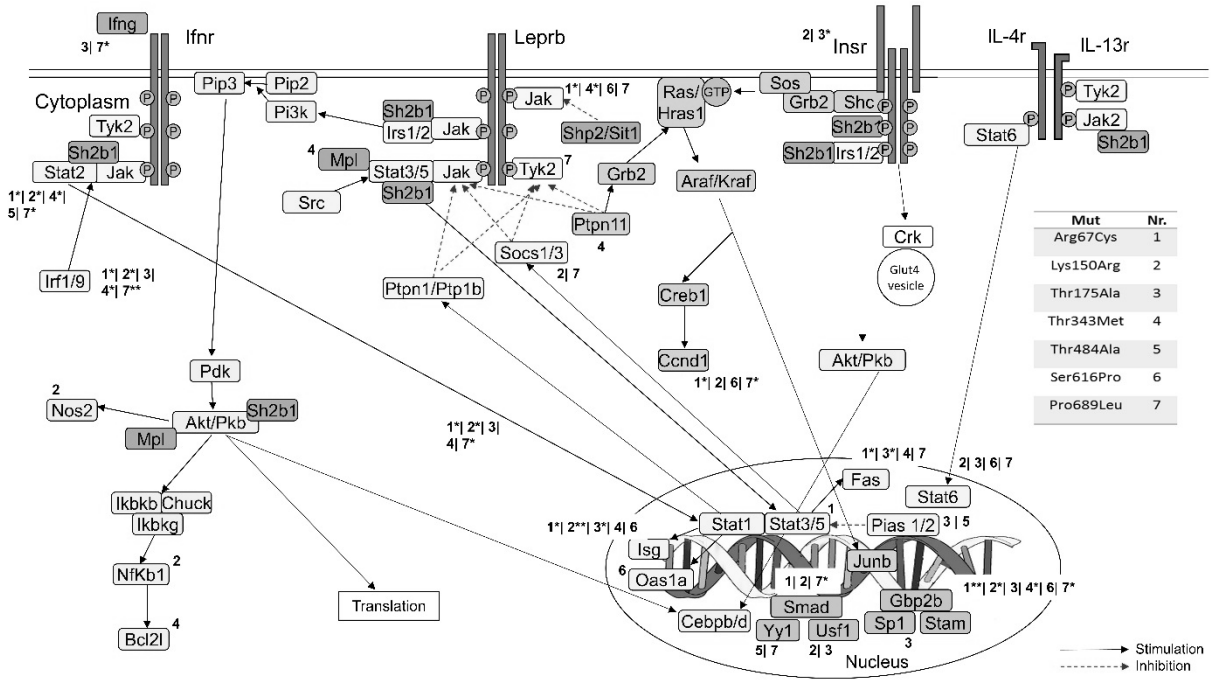


Expression patterns for hypothalamic CLU188 cells transfected with different human *SH2B1* variants and stimulated with insulin using the Qiagen RT² Profiler PCR Array for the murine Insulin Signaling Pathway, showing only the genes expressed more than the recommended threshold of Ct 35; sorted by mutation; * = $p \leq 0.01$.

Supplemental Figure 4: Effect of the *SH2B1* variants on JAK / STAT signaling genes



Overview of some of the analyzed leptin-responsive genes and the effect of *SH2B1* variants on these genes (adapted from [1,2]**)**. After stimulation with the satiety hormone leptin, the long form of the leptin receptor (Leprb) dimerizes and is phosphorylated at positions Tyr-986, Try-1078 and Tyr-1141. It binds Janus kinase 1 and 2 (JAK1 and JAK2) or tyrosine kinase 2 (TYK2) which activate several downstream cascades [3]:

JAK, the insulin receptor substrate (IRS1/2) and SH2B1 form a complex and phosphoinositid-3-kinase (PI3K) transfers the phosphorylation of IRS1/2 and activates thereby phosphatidylinositol-4, 5-bisphosphate (PIP2) to phosphatidylinositol-3, 4, 5-trisphosphate (PIP3). The protein kinase B/ thymoma viral proto-oncogene (PKB/AKT) bind with its PH-domain to PIP3 and is activated via phosphorylation by the phosphoinositide-dependent protein kinase 1 (PDK1) at its amino acids serine and threonine. Activated AKT can phosphorylate other substrates and thereby activate or inhibit several downstream cascades. For example, it can activate nitric oxide synthase 2 (NOS) which influences the NO/ONOO ratio that is associated with atherosclerosis and diabetes [4]. If AKT is activated, then it induces the action of IkappaB (Ikbkb, Ikbkg) kinases (IKKs) which again induces nuclear translocation of NF-kappaB (NFkB1) [5] and hence the transcription of apoptosis regulator Bcl-X (BCL2) [1].

JAK provides binding sites for downstream signalling molecules such as signal transducer and activator of transcription proteins (STAT3 and STAT5) [6]. Activated Stats dimerize and translocate into the nucleus where they induce transcription of genes such as suppressor of cytokine signaling 3 (SOCS3) which inhibits its LEPRB and JAK binding [7]. Protein tyrosine phosphatase, non-receptor type 1 (PTPN1) is another negative regulator of leptin signalling as it dephosphorylates JAK and STAT [8].

Protein tyrosine phosphatase, non-receptor type 11 (PTPN11) has a dual function after phosphorylation by leptin activation. On one hand it dephosphorylates JAK which decreases downstream signalling of LEPRB, on the other hand it activates the RAS/RAF/MEK cascade via growth factor receptor-bound protein 2 (GRB2) [9,10]. These lead to the expression of the anti-apoptosis genes jun-b oncogene (JUNB) and jun-c oncogene (JUN) [11].

Several proteins in leptin signalling exhibit modulatory characteristics, like signaling threshold regulating transmembrane adaptor 1 (SIT1/SHP2), V-Src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (SRC), myeloproliferative leukemia virus oncogene (MP1) and SH2B1. These modulators increase phosphorylation of several protein tyrosine kinases for example Akt, Jak, map kinases and the insulin receptor (INSR) [12–14].

In addition to STATs, other transcriptional regulators are also regulated by leptin signalling. Leptin elicits the "mothers against decapentaplegic homolog" (SMAD) activation by transforming growth factor beta signalling [15]. Trans-acting transcription factor 1 (SP1) binds to the leptin promoter and increases leptin levels [16]. Signal transducing adaptor molecule (STAM) transcription binds directly to JAK and is elicited by IL2 stimulation [17]. IL9 increases the level of upstream transcription factor 1 (USF1) which is a transcription-factor for lipid metabolism [18]. NFkB1 targets yin and yang 1 (YY1) [19] which possesses strong binding affinity for SP1 and increases leptin transcription synergistically [20].

Besides the LEPRB there are other receptors that utilize a similar downstream network. One of these receptors is INSR which shares the AKT pathway and the RAS/RAF/MEK cascade with leptin [21]. V-crk avian sarcoma virus CT10 oncogene homolog (CRK) controls glucose uptake by glucose transporter type 4 (Glut4) mediated by IRS and PI3K [22]. Other receptors are interferon receptors (IFNR, IFNAR1) which are activated for example by interferon gamma (IFNG). Like LEPRB, the receptors dimerize upon activation and bind the JAK-STAT complex which in turn translocates to the nucleus and activates transcription of several target genes [23] such as Interferon (IFN) stimulated gene product 15 (ISG15), an ubiquitin-like protein that interacts with various viral and host downstream effectors either involved in interferon signaling other in type I IFN signaling respectively. This kind of interaction leads to ISGylation of the binding proteins and with it to their modification and suppression [24]. This process is regulated by interferon regulatory factors 1 and 9 (IRF1 and 9) [25].

The type II IL-4Rα/IL-13Rα1 receptor system is part of the Il-4/IL-13/Stat-6 signaling pathway. Both cytokines Il-4 and IL-13 bind to different subunits (IL-4Rα and IL-13Rα1) of the same receptor complex [26]. After binding of IL-13, IL-13Rα1 activates TYK2 and JAK2. The latter induces tyrosine phosphorylation of cytoplasmic IL-4R triggering recruitment of Stat6 SH2 domain and phosphorylation by JAK. Actived STAT6 dimerizes and translocates to the nucleus where it regulates transcription of genes involved in allergy via Stat-specific DNA sequence elements [27]. This signalling pathway is principally involved in allergic airway inflammation [28] and the development of asthma [26].

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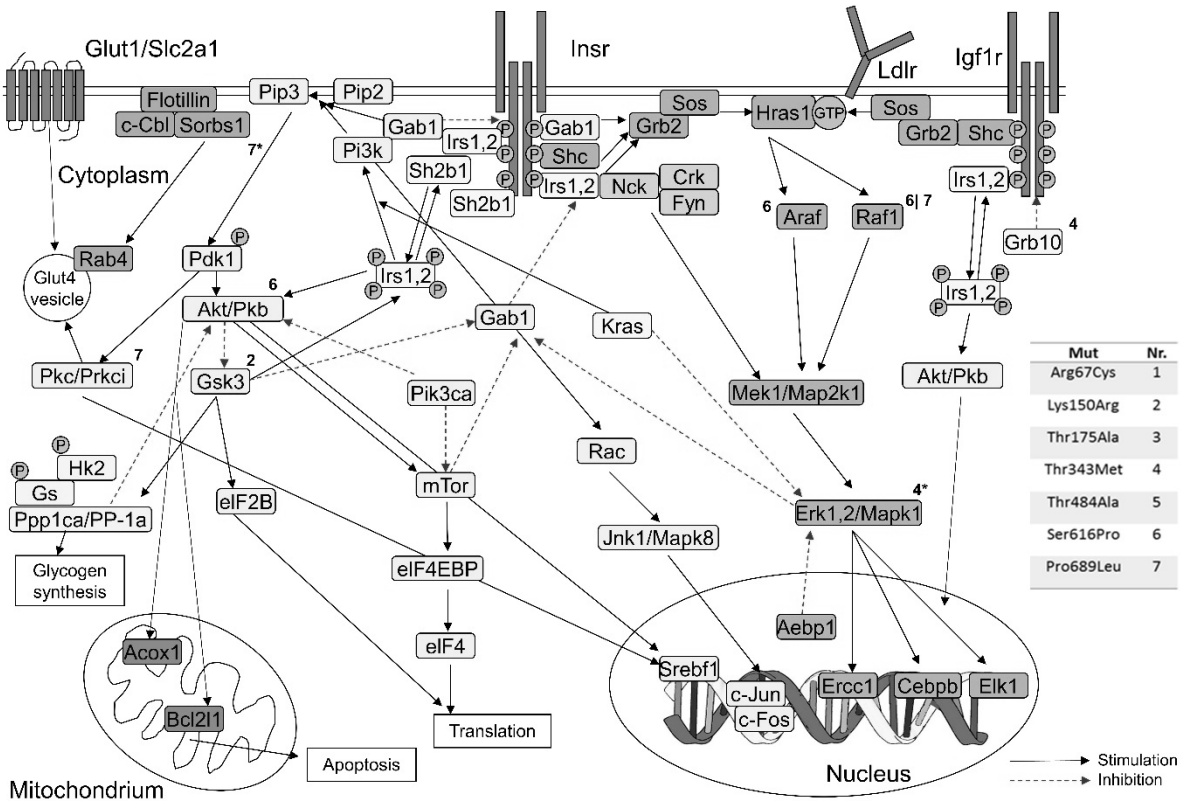
Supplemental Figure 5: Effect of the *SH2B1* variants on Insulin signaling genes



Overview of some of the analyzed insulin-responsive genes and the effect of *SH2B1* variants on these genes (adapted from [1]**)**. The circulating hormone insulin binds to the insulin receptor (INSR) leading to dimerization and tyrosine phosphorylation of the receptor. Therefore, SH2B1 binds directly to phospho-Tyr^{1158} of the insulin receptor via its SH2 domain stimulating the kinase activity of the receptor [2]. This induces binding of insulin receptor substrates 1 and 2 (IRS1 and 2) which are in turn phosphorylated and form a complex with SH2B1 and phosphoinositid-3-kinase (PI3K). PI3K transfers the phosphorylation of IRS1/2 and activates thereby phosphatidylinositol-4,5-Bisphosphate (PIP2) to Phosphatidylinositol-3,4,5-Trisphosphate (PIP3) in the membrane [3]. Subsequently the kinase 3-phosphoinositide-dependent protein kinase 1 (PDK1) interacts via its PH domain with PIP3 and activates thereby protein kinase B or AKT (PKB/AKT; [4]. Activated AKT can activate or inhibit several downstream cascades by phosphorylation of other substrates.

For example, AKT increases the phosphorylation of the mechanistic target of rapamycin (mTOR) which enhances the activity of eukaryotic translation initiation factor 4E binding protein (EIF4EBP). This allows binding of EIF4EBP to eukaryotic translation initiation factor 4E (EIF4E) which increases the rate of translation initiation [5]. Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) is a negative regulator of both mTOR and AKT [6]. Protein phosphatase 1, catalytic subunit, alpha isozyme (PPP1CA) also negatively influences AKT by dephosphorylating at Thr450 [7]. AKT on

the other hand inactivates glycogen synthase kinase-3 (GSK3) which induces via eukaryotic translation initiation factor 2B (EIF2B) the translation of proteins [8]. GSK3 also stimulates the glycogen synthesis via phosphorylation of hexokinase 2 (HK2) [9] and glycogen synthase (GS) [10].

PDK1 phosphorylation also increases protein kinase c (PKC) phosphorylation [11]. Both AKT and PKC increase the insulin-dependent regulation of transcription factor sterol regulatory element binding transcription factor 1 (SREBF1) which is required for lipid homeostasis [12]. The AKT independent mechanism of PKC phosphorylation leads to glucose transporter 4 (Glut4) translocation [13]. The translocation of Glut4 from intracellular pools to the plasma membrane is the main insulin-stimulated glucose transport [14]. Member RAS oncogene family 4 (RAB4) which interacts with microtubule [15] also mediates this process. It can be initiated independent of PI3K by recruitment of the Cbl proto-oncogene, E3 ubiquitin protein ligase (c-CBl) and sorbin and SH3 domain containing 1 (SORBS1) complex to Glut4 vesicle [16] containing flotillin (FLOT1) [17].

Ras-related C3 botulinum toxin substrate 1 (RAC1), a member of the Rho family of small GTPases, activates the transcription factors finkel-biskis-jinkins (FBJ) murine osteosarcoma viral proto-oncogene homolog (c-FOS) and jun proto-oncogene (JUN) by the TNF-alpha induced activation of mitogen-activated protein kinase 8 (MAPK8), also known as c-Jun N-terminal kinase 1 (JNK11); this process is PI3K dependent [18].

Insulin stimulation also affects fatty acid synthesis; for example, PDK phosphorylation can increase Acyl-CoA oxidase 1, palmitoyl (ACOX1) levels in mitochondria, which is the first enzyme of the fatty acid beta-oxidation pathway [19]. Another mitochondrial protein activated by PI3K and AKT is the anti-apoptotic regulator BCL2-like 1 (BCL2L1) [20].

Another arm of the INSR signal transduction is the RAS-pathway which is induced by the association of either phosphorylated IRS1/2 or SHC (Src homology 2 domain containing) transforming protein 1 (SHC) with a complex of growth factor receptor-bound protein 2 (GRB2) and son of sevenless homolog 1 (SOS) [21]. Both steps activate harvey rat sarcoma viral oncogene homolog (HRAS1). HRAS1 forms a complex with GTP and thereby activates proteins of the RAF family for example V--1 murine leukemia viral oncogene homolog 1 (RAF1) or V-Raf murine sarcoma 3611 viral oncogene homolog (ARAF), and mitogen-activated protein kinase kinase 1 (MAP2K1 alias MEK1). Both induce phosphorylation of mitogen-activated protein 1 (MAPK1 alias ERK1 and 2) [22]. This can lead to the accumulation of excision repair cross-complementation group 1 (ERCC1) [23], CCAAT/enhancer binding protein (C/EBP) beta (CEBPB) [24] or ELK1, member of ETS oncogene family (ELK) [25] in the nucleus. ERK phopshorylation also leads to increased transcription of the low-density lipoprotein receptor (LDLR) which regulates the uptake of lipids in an insulin dependent manner [26].

The phosphorylation of MAP2K1 and ERK is negatively regulated by the expression of the transcriptional repressor AE binding protein 1 (AEBP1) [27]. The phosphorylation of MAP2K1 can also be initiated by binding of phosphorylated IRS to NCK adaptor protein (NCK) which forms a complex with either GRB2-SOS or CRK and FYN oncogene related to SRC, FGR, YES [28].

The growth factor receptor-bound protein 10 (GRB10) binds directly to the INSR and the IGF1R and inhibits the autophosphorylation after insulin stimulation, hence it serves as a negative regulator of insulin signalling [29,30].

RAS/ERK and PI3K/AKT pathways are in many ways regulated by GAB1. On the one hand, GAB1 binds to PI3K and increases its activation [31]. It can also influence the RAS-pathway by recruiting GRB2-SOS like IRS [32]. On the other hand, downstream actors of PI3K-AKT like mTOR, GSK3 or ERK provide negative feedback by disabling phosphorylation of GAB1 [31]. Also kirsten rat sarcoma viral oncogene homolog (KRAS) influences negatively the insulin signalling pathway by decreasing ERK phosphorylation and with it increasing the negative regulation of IRS on PI3K [33].

Another regulator of INSR function is the receptor internalisation by endocytosis, which momentarily decreases the cell response to insulin. The receptor then is either returned to the cell membrane or degraded after dephosphorylation [34]. The insulin-like growth factor 1 receptor (IGF1R) is homologous to INSR and activates many pathways in a similar manner. Heterodimers of INSR and IGF1R have been observed [35].

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Supplementary table S1. Overview of genes with significant changes in gene expression after transfection of CLU188 cells with human *SH2B1* containing variants and stimulation with leptin

Gene	Mutation	wt: Mean (SD)	mut: Mean (SD)	MD [95% CI]	T	*p*
Ccnd1	Arg67Cys	3.02 (0.03)	3.60 (0.10)	-0.58 [-0.83, -0.33]	-7.38	0.005
	Pro689Leu	3.02 (0.03)	4.14 (0.21)	-1.12 [-1.62, -0.62]	-7.08	0.006
Fas	Arg67Cys	6.71 (0.17)	8.41 (0.20)	-1.70 [-2.25, -1.15]	-9.89	0.002
	Thr175Ala	6.71 (0.17)	7.68 (0.17)	-0.96 [-1.45, -0.47]	-6.26	0.008
Gbp2b	Arg67Cys	5.80 (0.01)	8.92 (0.18)	-3.12 [-3.53, -2.70]	-23.83	0.0002 **
	Lys150Arg	5.80 (0.01)	8.32 (0.44)	-2.52 [-3.56, -1.48]	-7.70	0.005
	Thr343Met	5.80 (0.01)	8.53 (0.28)	-2.73 [-3.39, -2.06]	-13.07	0.001
	Pro689Leu	5.80 (0.01)	8.40 (0.25)	-2.60 [-3.20, -2.00]	-13.80	0.001
Ifng	Pro689Leu	6.79 (0.10)	8.57 (0.37)	-1.78 [-2.68, -0.88]	-6.29	0.008
Il20	Thr343Met	6.87 (0.11)	8.08 (0.18)	-1.20 [-1.67, -0.73]	-8.16	0.004
Insr	Thr175Ala	4.87 (0.10)	5.42 (0.07)	-0.55 [-0.78, -0.31]	-7.24	0.005
Irf1	Arg67Cys	6.59 (0.06)	7.67 (0.11)	-1.09 [-1.35, -0.82]	-12.99	0.001
Irf9	Arg67Cys	3.95 (0.06)	6.19 (0.20)	-2.24 [-2.73, -1.76]	-14.72	0.001
	Lys150Arg	3.95 (0.06)	5.23 (0.21)	-1.29 [-1.80, -0.77]	-7.96	0.004
	Thr343Met	3.95 (0.06)	5.50 (0.23)	-1.56 [-2.12, -1.00]	-8.83	0.003
	Pro689Leu	3.95 (0.06)	6.36 (0.05)	-2.42 [-2.57, -2.26]	-49.73	0.000018**
Isg15	Arg67Cys	5.63 (0.01)	7.66 (0.43)	-2.03 [-3.05, -1.02]	-6.37	0.008
	Lys150Arg	5.63 (0.01)	7.51 (0.13)	-1.88	-19.32	0.0003 **

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				[-2.19, -1.57]		
	Thr175Ala	5.63 (0.01)	6.00 (0.07)	-0.37 [-0.53, -0.22]	-7.64	0.005
Jak1	Arg67Cys	0.47 (0.40)	2.59 (0.26)	-2.12 [-3.02, -1.21]	-7.41	0.005
	Thr343Met	0.47 (0.40)	2.59 (0.35)	-2.11 [-3.18, -1.05]	-6.30	0.008
Smad1	Pro689Leu	6.70 (0.03)	6.26 (0.08)	0.43 [0.23, 0.64]	6.73	0.007
Stat1	Arg67Cys	4.32 (0.15)	7.44 (0.32)	-3.12 [-3.92, -2.32]	-12.40	0.001
	Lys150Arg	4.32 (0.15)	7.44 (0.36)	-3.12 [-4.01, -2.22]	-11.11	0.002
	Pro689Leu	4.32 (0.15)	7.55 (0.28)	-3.23 [-3.93, -2.53]	-14.67	0.001
Stat2	Arg67Cys	5.30 (0.17)	7.10 (0.10)	-1.80 [-2.18, -1.42]	-15.09	0.001
	Lys150Arg	5.30 (0.17)	6.93 (0.33)	-1.63 [-2.46, -0.79]	-6.18	0.009
	Thr343Met	5.30 (0.17)	6.95 (0.24)	-1.65 [-2.30, -1.01]	-8.17	0.004
Stub1	Lys150Arg	3.70 (0.05)	3.23 (0.09)	0.47 [0.25, 0.69]	6.69	0.007

Detailed description of genes that showed significant changes in gene expression (nominal *p*-value below 0.01; ** nominal *p*-value below 0.001) after applying t-test for equality of means (df = 3). wt: wild type; mut: mutation; MD: mean difference; SD: standard deviation; df: degrees of freedom; T: test statistic; CI: confidence interval.

Obes Facts
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Supplementary table S2. Overview of genes with significant changes in gene expression after transfection of CLU188 cells with human *SH2B1* containing variants and stimulation with insulin

Gene	Mutation	wt: Mean (SD)	mut: Mean (SD)	MD [95% CI]	T	*p*
Cap1	Lys150Arg	4.09 (0.02)	4.52 (0.08)	-0.43 [-0.62, -0.24]	-7.19	0.006
Mapk1	Thr343Met	4.31 (0.03)	4.00 (0.05)	0.31 [0.17, 0.45]	6.92	0.006
Sorbs1	Pro689Leu	7.08 (0.02)	8.20 (0.20)	-1.12 [-1.60, -0.64]	-7.44	0.005

Detailed analysis of genes that showed significant changes in gene expression (nominal *p*-value below 0.01) after applying t-test for equality of means (df = 3). wt: wild type; mut: mutation; MD: mean difference; SD: standard deviation; df: degrees of freedom; T: test statistic; CI: confidence interval.

Obes Facts
DOI: 10.1159/000486962