

Research Article

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

Johanna Giuranna^a Anna-Lena Volckmar^{a, b} Anna Heinen^a
Triinu Peters^a Börge Schmidt^c Anne Spieker^a Helena Straub^a
Harald Grallert^d Timo D. Müller^e Jochen Antel^a Ute Haußmann^f
Hans Klafki^{f, g} Rui Liangyou^h Johannes Hebebrand^a Anke Hinney^a

^aDepartment of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; ^bInstitute of Pathology, Heidelberg University Hospital, Heidelberg, Germany; ^cInstitute for Medical Informatics, Biometry and Epidemiology (IMIBE), University Hospital Essen, University of Duisburg-Essen, Essen, Germany; ^dInstitute of Epidemiology, Helmholtz-Zentrum Munich, Munich, Germany; ^eInstitute of Diabetes and Obesity, Helmholtz-Zentrum Munich, Munich, Germany; ^fDepartment of Psychiatry and Psychotherapy, Faculty of Medicine, University Hospital Essen, Essen, Germany; ^gDepartment of Psychiatry and Psychotherapy, University Medical Center Göttingen (UMG), Georg-August-University Göttingen, Göttingen, Germany; ^hMolecular & Integrative Physiology, University of Michigan Medical School, Ann Arbor, MI, USA

Keywords

GWAS · *SH2B1* · JAK · STAT · IRS · Expression genome-wide · Locus

Abstract

Objective: We aimed to determine the effect of human *SH2B1* variants on leptin and insulin signaling, which are major regulators of energy homeostasis, on the RNA level. **Methods:** We analyzed the expression of infrequent alleles of seven *SH2B1* variants (Arg67Cys, Lys150Arg, Thr175Ala, Thr343Met, Thr484Ala, Ser616Pro, and Pro689Leu) in response to insulin or leptin cell stimulation. Two of these were identified in own mutation screens, the others were predicted to be deleterious or to serve as controls. The variants were analyzed in a homologous system of mouse hypothalamic cells. Changes in expression of downstream genes were measured. Student's t-test for independent samples was applied, and effect sizes using Cohen's d

Johanna Giuranna and Anna-Lena Volckmar contributed equally.

Prof. Dr. Anke Hinney
Department of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy
University Hospital Essen, University of Duisburg-Essen
Wickenburgstraße 21, 45147 Essen, Germany
anke.hinney@uni-due.de

with 95% confidence intervals were therefore calculated. **Results:** In 34 of 54 analyzed genes involved in leptin (JAK/STAT or AKT) signaling, variants nominally changed expression. The expression of three genes was considerably increased (p values ≤ 0.001 : *Gbp2b* (67Cys; $d = 25.11$ (–3.53, –2.70)), *Irf9* (689Leu; $d = 44.65$ (–2.57, –2.26)), and *Isg15* (150Arg; $d = 20.35$ (–2.19, –1.57))). Of 32 analyzed genes in the insulin signaling pathway, the expression of 10 genes nominally changed ($p \leq 0.05$), three resulted in p values ≤ 0.01 (*Cap1* (150Arg; $d = 7.48$ (–0.62, –0.24)), *Mapk1* (343Met; $d = -6.80$ (0.17, 0.45)), and *Sorbs1* (689Leu; $d = 7.82$ (–1.60, –0.64))). **Conclusion:** The increased expression of genes in the leptin (JAK/STAT or AKT) signaling pathway implies that the main mode of action for human *SH2B1* mutations might affect leptin signaling rather than insulin signaling.

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Introduction

The Src homology 2B adaptor protein 1 gene (*SH2B1*) is derived from genome-wide association studies (GWAS) for BMI and obesity [1–4]. The infrequent alleles of single nucleotide polymorphisms (SNPs) at *SH2B1* confer polygenic effects on obesity. A coding SNP in *SH2B1* (rs7498665 Thr484Ala) [1, 2] and variants in linkage disequilibrium are associated with obesity and related traits [3, 4]. The association has been replicated in different ethnicities and age groups [5–11]. In addition, a copy number variation (CNV; 200–500 kb) covering a region on chr16p11.2 that harbors *SH2B1* is associated with weight phenotypes. While a deletion in this region is associated with increased BMI and obesity risk [12, 13], the reciprocal duplication is associated with underweight [14].

Two independently generated *Sh2b1* knockout (KO) models display an altered body weight. *Sh2b1* KO mice are obese, hyperphagic, hyperlipidemic, leptin- and insulin-resistant, hyperglycemic, and glucose-intolerant [15–17]. A genetic rescue of the SH2B1 beta isoform in neurons eliminated both obesity and insulin resistance phenotypes [17]. The *Drosophila melanogaster* KO led to increased fat mass although the overall body size shrank. In contrast to the murine model [17], a neuronal KO of *Sh2b1* was not sufficient to elicit the phenotype in *Drosophila* [18].

Leptin and insulin signaling are the main pathways involved in SH2B1 action on energy homeostasis [19]. SH2B1 promotes leptin signaling by stimulating Janus kinase (JAK1 and JAK2) activity and the assembly of JAK2 / insulin receptor substrate (IRS1 and IRS2) complexes [20–22]. SH2B1 also enhances insulin receptor autophosphorylation after insulin stimulation and protects IRS and JAK from dephosphorylation [23, 24]. Additionally, JAK and IRS are also downstream effectors of growth hormone (GH), insulin like growth factor 1 (IGF-1), (nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), leading to the assumption that the signaling of all these hormones is potentially affected by *SH2B1* mutations [19].

We previously reported a rare variant (β Thr656Ile/ γ Pro674Ser), affecting the beta and gamma splice variants of *SH2B1*. The infrequent variant allele was detected exclusively in 3 out of 11,406 obese individuals but not in 4,568 normal-weight controls [8]. When analyzing the impact of the obesity-associated SNP rs7498665 (Thr484Ala) and the rare variant β Thr656Ile/ γ Pro674Ser on STAT3-mediated leptin signaling, we did not observe functional effects [8].

SH2B1 mutations were detected among 800 obese children and adolescents with insulin resistance (Pro90His, Thr175Asn, Pro322Ser, Phe344Leufs*20, Thr564Ala, Ala663Val, Ala723Val) [25, 26]. All variants impaired GH-induced cell motility when present in the

alpha isoform of SH2B1. Except for the frameshift variant (Phe344Leufs*20) that lacks the SH2 domain, all variants interfered with insulin- or leptin-induced IRS2 phosphorylation [26].

We analyzed the role of *SH2B1* variants in pathways of energy homeostasis by their interaction with insulin receptor and leptin receptor signaling. We chose murine hypothalamic cells, as the hypothalamus is one of the key players in energy balance regulation [2]. Hence, we assayed the functional implications of seven human *SH2B1* variants at the RNA level. The variants were chosen as they were associated with obesity (Thr484Ala), located in functional domains of SH2B1 (dimerization domain: Arg67Cys; nuclear localization domain: Lys150Arg; pleckstrin homology domain: Thr343Met; SH2 domain: Ser616Pro) and predicted (by PolyPhen 2) to have a functional effect (probably damaging) or to serve as potential controls (Thr175Ala, Pro689Leu).

Material and Methods

Mutation Screen

The coding region of *SH2B1*, located at chr16: 28,858,010 – 28,885,533 (hg18/ NCBI 36; accession number: ENSG00000178188, MIM: 608937), was screened for mutations by denaturing high pressure liquid chromatography (dHPLC) using the Transgenomic wave genetic analyzer (Transgenomic, Crewe, UK) and Sanger re-sequencing as described previously [27] in 93 lean adults (26.7% males, age 21.18 ± 6.99 years, BMI 17.98 ± 1.08 kg/m²). This study group was selected in order to identify further variants in *SH2B1* that might rather exert a weight-lowering effect. Detailed information regarding used temperatures and primers for PCR and dHPLC analysis can be obtained from the authors. All PCR amplicons with dHPLC patterns derived from the wild-type pattern were re-sequenced as described previously [8]. At least two experienced individuals independently assigned the genotypes; discrepancies were solved either by reaching consensus or by re-genotyping.

The non-synonymous variants were then genotyped in a sample of 355 trios comprising a child with (extreme) obesity and both parents by MALDI TOF (index patients: 45.37% males, age 13.72 ± 3.12 years, BMI 32.02 kg/m²; BMI Standard Deviation Score (BMI SDS) 4.13 ± 2.06; parents: age: 42.85 ± 6.14, BMI 29.95 ± 6.15 kg/m² [8, 28]), in an independent sample of 453 cases (42.60% males, age 14.37 ± 3.75 years, BMI 30.28 kg/m², BMI SDS 4.51 ± 2.15), and in 342 controls (39.08% males, age 26.08 ± 5.75 years, BMI 18.09 ± 1.14 kg/m² [29]). These studies were approved by the Ethics Committees of the respective Universities and were performed in accordance with the Declaration of Helsinki.

Cells

To provide insight into the hypothalamus as the main weight regulatory area of the brain, we chose a murine hypothalamic cell line, which expresses both insulin receptor and leptin receptor. The hypothalamic CLU188 cells ('Adult mouse hypothalamic cell line mHypoA-2/28 CLU188'; Biozol, Eching, Germany) were cultured in DMEM (Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Merck Millipore, Darmstadt, Germany) and penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA) at 37 °C in humidified atmosphere containing 5% CO₂. After knockdown of *Sh2b1* via co-transfected Zinc finger nuclease (Sigma Aldrich, St. Louis, MO, USA) and a plasmid containing the selection cassette for neomycin and green fluorescent protein (GFP) (obtained from Sigma Aldrich; supplementary data, available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=486962>), G418 (300 µg/ml; Life Technologies) was added to the media to maintain the *Sh2b1* knockout (for details see supplementary data, available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=486962>).

The *Sh2b1* KO cells were transiently transfected (calcium phosphate method, see supplementary data, available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=486962>) with human *SH2B1* alpha splice form clones (wild-type, 67Cys, 150Arg, 175Ala, 343Met, 484Ala, 616Pro, 689Leu) under a CMV promoter (all clones were provided by Origene, mutation induction by Genscript, Piscataway, NJ, USA). The variants were chosen as they were associated with obesity, located in functional domains of SH2B1, and predicted by PolyPhen 2 to have a functional effect, or to serve as potentially 'like wild-type' controls as predicted by PolyPhen 2 (table 1). After 24 h, the cells were stimulated with either leptin or insulin (100 ng/ml each; Sigma Aldrich) for 1 h and immediately harvested and frozen at -80 °C. As a negative control, non-

Table 1. Non-synonymous variants of human SH2B1 chosen for in vitro analysis

Variant: amino acid exchange (rs-number)	MAF in CEU	PolyPhen2 (Class:Score)	Functional domain	Reference
Arg67Cys (rs781063312)	NA	probably damaging (1.000)	dimerization	dbSNP
Lys150Arg (rs141195883)	0.000	possibly damaging (0.952)	nuclear localization domain	dbSNP, lean controls in this study
Thr175Ala (rs181294111)	0.001	benign (0.036)	none	dbSNP
Thr343Met (rs139298340)	0.000	probably damaging (0.998)	Pleckstrin homology	dbSNP
Thr484Ala (rs7498665)	0.331	benign (0.000)	none	[8], lean controls in this study
Ser616Pro (rs142515048)	0.000	possibly damaging (0.876)	Src homology 2	dbSNP
Pro689Leu	NA	benign (0.000)	none	dbSNP

MA = Minor allele frequency; CEU = Northern Europeans from Utah.

dbSNP (www.ncbi.nlm.nih.gov/snp/), PolyPhen 2 (www.genetics.bwh.harvard.edu/pph2/).

transfected cells were also treated and harvested as just described. We also used non-stimulated CLU188 cells as an additional experimental control.

The identity of the mouse cells was confirmed by the DSMZ (www.dsmz.de).

Expression Analysis

The stimulated cells were thawed, and the RNA was extracted using the RNeasy Mini prep (Qiagen, Hilden, Germany). Reverse transcription of 25 ng RNA was performed with RT² First Strand Kit (Qiagen). To gain further insight into the impact of the analyzed *SH2B1* variants on insulin and leptin signaling, insulin signaling pathway and JAK/STAT signaling pathway, RT² Profiler PCR arrays (Qiagen) were used according to the manufacturer's handbook on a StepOnePlus™ Real-Time PCR System (qRT-PCR; Life Technologies) with the respective software. The threshold for analysis was Ct 35 as higher Ct values indicate very low initial mRNA amounts. Here, small pipetting errors would cause strong changes in expression. The experiments were performed in duplicates or triplicates (means, standard deviations, and exact replicate numbers are indicated in the figures).

Statistical Analyses

To analyze the obesity association in the case/control sample, Fisher's exact test (allelic association) was done with PLINK [30]. Additionally, an asymptotic, 2-tailed p value for the transmission disequilibrium test was calculated with PLINK for the family based sample. If not stated otherwise, all p values are asymptotic, two-sided and not corrected for multiple testing.

For the in vitro tests and the quantitative expression analysis, all measured Ct values of the target genes were normalized to the mean of moderately expressed housekeeping genes using the $\Delta\Delta$ Ct method. Then the fold change was calculated in order to compare the final value and the initial value (wild-type) for each gene. Mean differences in amounts of mRNA between variants were tested applying independent samples t-test. To analyze the homogeneity of variances, the Levene's test was used. These analyses were performed using IBM® SPSS® Statistics 24.0.1 for Windows (IBM, Armonk, NY, USA). Given p values are nominal and two-sided unless stated otherwise. A significance level of $p \leq 0.05$ was applied, and for the figures only p values ≤ 0.01

were considered. Effect sizes (Cohen's *d*) of nominally altered gene expression patterns for each *SH2B1* variant were calculated according to the formula:

$$d = X_{\text{var}} - X_{\text{wt}} / SD_p \quad (1),$$

where *X* was the mean of calculated ΔCt values of genes for each *SH2B1* variant (var) or for wild-type respectively, and SD_p is the pooled standard deviation calculated as:

$$SD_p = \sqrt{\frac{SD^2_{\text{var}} + SD^2_{\text{wt}}}{2}} \quad (2),$$

being SD_{var} the standard deviation of genes for each variant and SD_{wt} the standard deviation of genes for the wild-type. Effect sizes were interpreted as small (0.2), medium (0.2–0.5) and large (>0.8) [31].

Results

Mutation Screen

In the 93 lean adults, we detected two non-synonymous variants (rs141195883 – Lys150Arg and rs7498665 – Thr484Ala); both have previously been reported [8, 25]. The infrequent allele of Lys150Arg was not associated with obesity in 355 trios comprising a child with (extreme) obesity and both parents (transmission equilibrium; nominal *p* = 0.32) or an independent sample of 453 children and adolescents with extreme obesity versus 342 lean adults (nominal *p* = 0.51). Minor allele frequency was low (0.14%) so that the association analyses were underpowered. For rs7498665, we previously described an association with obesity in our study groups (nominal *p* = 0.009 in 705 trios with obesity, OR = 1.23) [8].

Expression Analyses

To analyze the effects of the *SH2B1* variants (supplemental fig. 1, available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=486962>) on expression downstream of STAT, AKT and IR/IRS, arrays for gene expression of the JAK/STAT and insulin pathways were used. Although the respective qRT-PCR panels allow a wide range of different expression analyses, we focused on the genes of the pathways that are most relevant for energy homeostasis maintenance in the brain.

After stimulation with leptin, the expression of downstream genes in JAK/STAT, AKT, interferon (IFN), and interleukin signaling were altered by at least one of the *SH2B1* variants compared to wild-type (table 2; supplemental table 1, available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=486962>). Of the 84 genes included in the panel, 54 genes showed expression in the range necessary for a valid analysis (Ct threshold below 35 cycles). Of these, 34 genes were nominally affected by at least one of the variants in *SH2B1* (supplemental fig. 2, available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=486962>). Changes in expression (fold change) of *Gbp2b* (67Cys, 0.12 ± 0.18 ; $p_{\text{nominal}} = 0.0002$), *Irf9* ($\alpha 689\text{Leu}$, 0.19 ± 0.05 ; $p_{\text{nominal}} = 0.000018$), *Isg15* (150Arg, 0.27 ± 0.13 ; $p_{\text{nominal}} = 0.0003$) were nominally significant ($p \leq 0.001$; fig. 1). The differences at the transcription levels were never limited to genes in a specific part of the pathway but rather distributed across all genes within leptin signaling. For SNP rs7498665 (484Ala), we observed for 29 genes (out of 54 genes analyzed for all mutations) that the expression was too low for a proper analysis, despite an expression of four housekeeping genes that was comparable to the other *SH2B1* variants. Hence we did not include these 29 genes in our analysis for this SNP. Among the remaining 25 genes, 484Ala increased expression of *Pias2*, *Stat2*, *Stub1*, and *Yy1* nominally (table 2).

After insulin stimulation, four of the variants altered the expression of at least one gene in the insulin signaling pathway (table 3; supplemental table 2, available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=486962>). Of the 32 genes that were expressed on

Table 2. Changes in gene expression after transfection of CLU188 cells with human *SH2B1* containing variants and stimulation with leptin

	Arg67Cys		Lys150Arg		Thr175Ala		Thr343Met		Thr484Ala		Ser616Pro		Pro689Leu	
	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)
<i>Bcl2l1</i>	-	-	-	-	-	-	0.041	-3.08 (0.06, 1.56)	-	-	-	-	-	-
<i>Ccnd1</i>	0.005*	7.63 (-0.83, -0.33)	0.040	3.64 (-1.14, -0.05)	-	-	-	-	-	-	0.044	-3.54 (0.03, 1.15)	0.006*	7.44 (-1.62, -0.62)
<i>Fas</i>	0.002*	9.21 (-2.25, -1.15)	-	-	0.008*	5.67 (-1.45, -0.47)	0.014	4.95 (-1.64, -0.39)	-	-	-	-	0.048	3.28 (-2.17, -0.02)
<i>Gbp2b</i>	0.0002**	25.11 (-3.53, -2.70)	0.005*	8.11 (-3.56, -1.48)	0.024	4.46 (-2.21, -0.31)	0.001*	13.77 (-3.39, -2.06)	-	-	0.032	-4.01 (0.09, 1.05)	0.001*	14.52 (-3.20, -2.00)
<i>Ghr</i>	-	-	-	-	-	-	-	-	-	-	0.021	-4.37 (0.20, 1.22)	-	-
<i>Ifng</i>	-	-	-	-	0.024	4.36 (-1.46, -0.21)	-	-	-	-	-	-	0.008*	6.52 (-2.68, -0.88)
<i>Il10ra</i>	0.039	3.40 (-2.39, -0.12)	-	-	0.024	3.85 (-1.94, -0.27)	0.029	3.85 (-2.64, -0.28)	-	-	-	-	0.020	4.16 (-2.00, -0.38)
<i>Il20</i>	-	-	0.024	4.33 (-1.86, -0.26)	-	-	0.004*	7.95 (-1.67, -0.73)	-	-	-	-	0.018	4.90 (-2.90, -0.56)
<i>Insr</i>	-	-	0.031	3.90 (-1.26, -0.12)	0.005*	6.19 (-0.78, -0.31)	-	-	-	-	-	-	-	-
<i>Irf1</i>	0.001*	12.93 (-1.35, -0.82)	-	-	-	-	-	-	-	-	-	-	-	-
<i>Irf9</i>	0.001*	15.23 (-2.73, -1.76)	0.004*	8.23 (-1.80, -0.77)	0.01	6.19 (-1.08, -0.26)	0.003*	9.15 (-2.12, -1.00)	-	-	-	-	0.000018**	44.65 (-2.57, -2.26)
<i>Isg15</i>	0.008*	6.72 (-3.05, -1.02)	0.0003**	20.35 (-2.19, -1.57)	0.005*	7.96 (-0.53, -0.22)	0.016	5.21 (-2.37, -0.51)	-	-	0.023	-4.50 (0.10, 0.70)	-	-

Table 2 continued on next page

Table 2. Continued

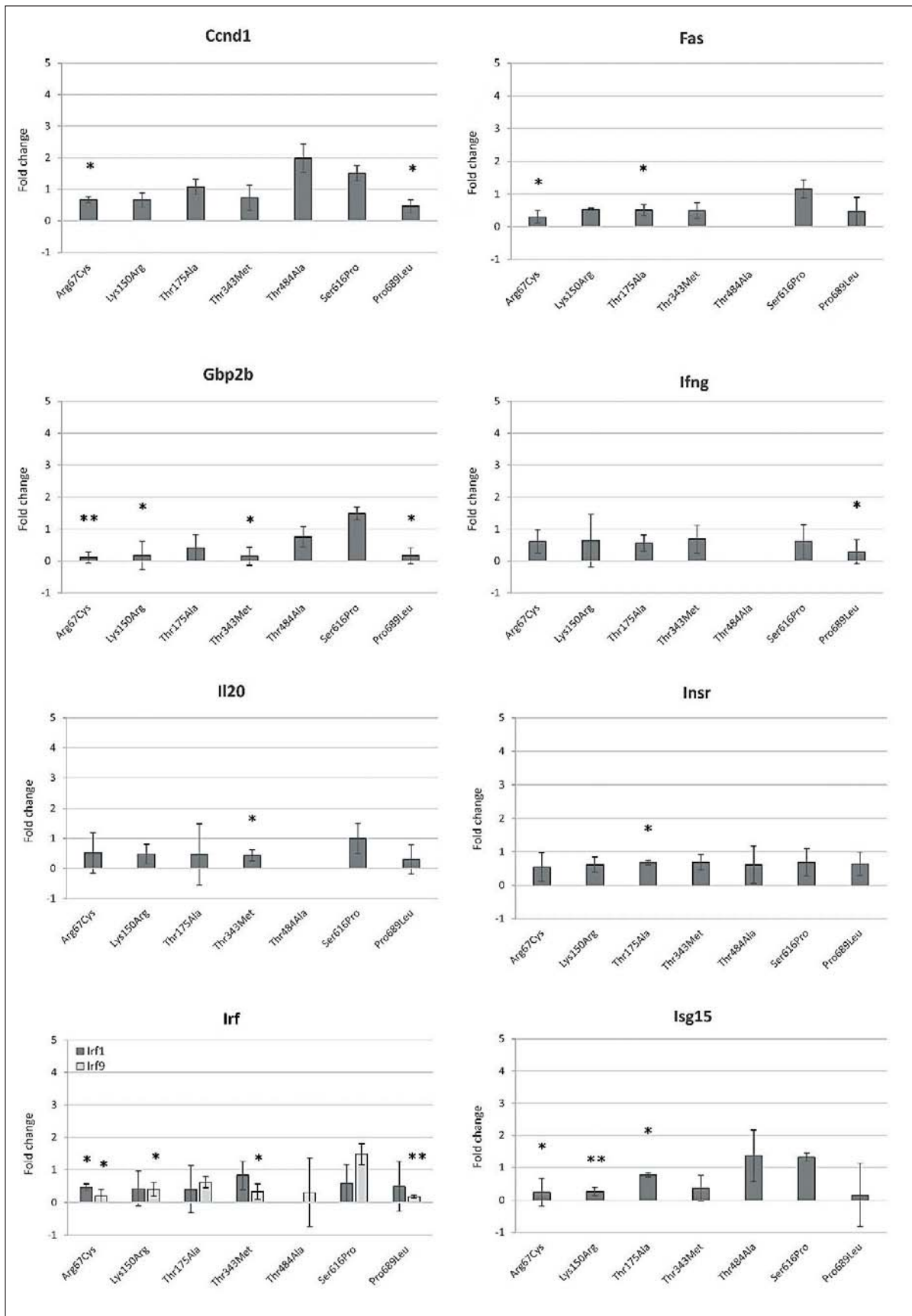
	Arg67Cys		Lys150Arg		Thr175Ala		Thr343Met		Thr484Ala		Ser616Pro		Pro689Leu	
	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)
Jak1	0.005*	6.28 (-3.02, -1.21)	-	-	-	-	0.008*	5.63 (-3.18, -1.05)	-	-	0.016	-4.31 (0.55, 2.53)	0.011	4.52 (-2.19, -0.61)
Mpl	-	-	-	-	-	-	0.040	-3.39 (0.06, 1.19)	-	-	-	-	-	-
Nfkb1	-	-	0.032	-3.24 (0.07, 0.85)	-	-	-	-	-	-	-	-	-	-
Nos2	-	-	0.030	-4.10 (0.09, 0.85)	-	-	-	-	-	-	-	-	-	-
Oas1a	-	-	-	-	-	-	-	-	-	-	0.025	4.07 (-1.75, -0.24)	-	-
Pias1	-	-	-	-	0.041	3.49 (-1.41, -0.05)	-	-	-	-	-	-	-	-
Pias2	-	-	-	-	-	-	-	-	0.045	-4.55 (0.13, 4.68)	-	-	-	-
Ptpn11	-	-	-	-	-	-	0.024	-4.37 (0.16, 1.11)	-	-	-	-	-	-
Smad1	-	-	-	-	-	-	-	-	-	-	-	-	0.007*	-6.97 (0.23, 0.64)
Smad3	0.035	3.09 (-1.54, -0.11)	-	-	-	-	-	-	-	-	-	-	0.032	3.37 (-1.81, -0.16)
Smad5	-	-	0.04	3.37 (-1.06, -0.04)	-	-	-	-	-	-	-	-	-	-
Socs3	-	-	0.01	-7.18 (0.25, 0.66)	-	-	-	-	-	-	-	-	-	-
Socs5	-	-	-	-	-	-	-	-	-	-	-	-	0.011	5.18 (-1.74, -0.50)

Table 2 continued on next page

Table 2. Continued

	Arg67Cys		Lys150Arg		Thr175Ala		Thr343Met		Thr484Ala		Ser616Pro		Pro689Leu	
	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)
Sp1	-	-	-	4.36 (-0.92, -0.17)	-	-	-	-	-	-	-	-	-	-
Stat1	0.001*	12.51 (-3.92, -2.32)	0.002*	11.30 (-4.01, -2.22)	0.026	3.96 (-1.23, -0.16)	0.013	5.55 (-5.02, -1.25)	-	-	-	-	0.001*	14.62 (-3.93, -2.53)
Stat2	0.001*	12.63 (-2.18, -1.42)	0.009*	6.15 (-2.46, -0.79)	-	-	0.004*	7.84 (-2.30, -1.01)	0.015	8.10 (-1.53, -0.47)	-	-	0.007*	6.48 (-2.59, -0.89)
Stat3	0.023	4.54 (-0.37, -0.06)	-	-	-	-	-	-	-	-	-	-	-	-
Stat6	-	-	0.04	3.50 (-1.83, -0.12)	0.045	3.37 (-2.67, -0.06)	-	-	-	-	0.031	3.93 (-3.13, -3.30)	0.021	4.33 (-2.08, -0.36)
Statub1	-	-	0.007*	-6.61 (0.25, 0.69)	-	-	0.010	-5.97 (0.29, 1.00)	0.016	-7.81 (0.43, 1.47)	0.029	-4.03 (0.07, 0.37)	0.011	-5.37 (0.14, 0.49)
Tyk2	-	-	-	-	-	-	-	-	-	-	-	-	0.040	3.04 (-0.98, -0.05)
Usf1	-	-	0.018	4.92 (-0.93, -0.18)	0.032	3.95 (-0.85, -0.08)	-	-	-	-	-	-	-	-
Yy1	-	-	-	-	-	-	-	-	0.014	-8.28 (0.40, 1.26)	-	-	0.041	3.53 (-2.01, -0.08)

For the depicted genes we observed nominal changes (nominal p value < 0.05) in expression (biological triplicate) for at least one of the analyzed *SH2B1* variants (*Nominal p value < 0.01; **Nominal p value < 0.001). Most severe changes in expression were detected for Gbp2b (67Cys, pnominal = 0.0002), Irf9 (6689Leu, pnominal = 0.00018) and Isg15 (150Arg, pnominal = 0.0003).
Gene names: BCL2-like 1 (Bcl2l1); cyclin D1 (Ccnd1); fatty acid synthase (Fas); guanylate binding protein 2, interferon-inducible (Gbp2b); growth hormone receptor (Ghr); interferon, gamma (Ifng); interleukin 10 receptor, alpha (Il10ra); interleukin 20 (Il20); insulin receptor (Insr); interferon regulatory factor 1 and 9 (Irf1, Irf9); interferon-stimulated gene 15 (Isg15); janus kinase 1 (Jak1); myeloproliferative leukemia virus oncogene (Mpl); nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (Nfkb1); nitric oxide synthase 2 (Nos2); 2'-5' oligoadenylate synthetase 1A (Oas1a); protein inhibitor of activated Stat 1 (Pias1); protein tyrosine phosphatase, non-receptor type 11 (Ptpn11); SMAD family member 1, 3 and 5 (Smad1, Smad3, Smad5); suppressor of cytokine signaling 3 and 5 (Socs3, Socs5); trans-acting transcription factor 1 (Stat1); signal transducer and activator of transcription 1, 2, 3 and 6 (Stat1, Stat2, Stat3, Stat6); STIP1 homology and U-Box containing protein 1 (Stub1); tyrosine kinase 2 (Tyk2); upstream transcription factor 1 (Usf1); Yin Yang 1 (Yy1).



(For rest of figure and legend see next page.)

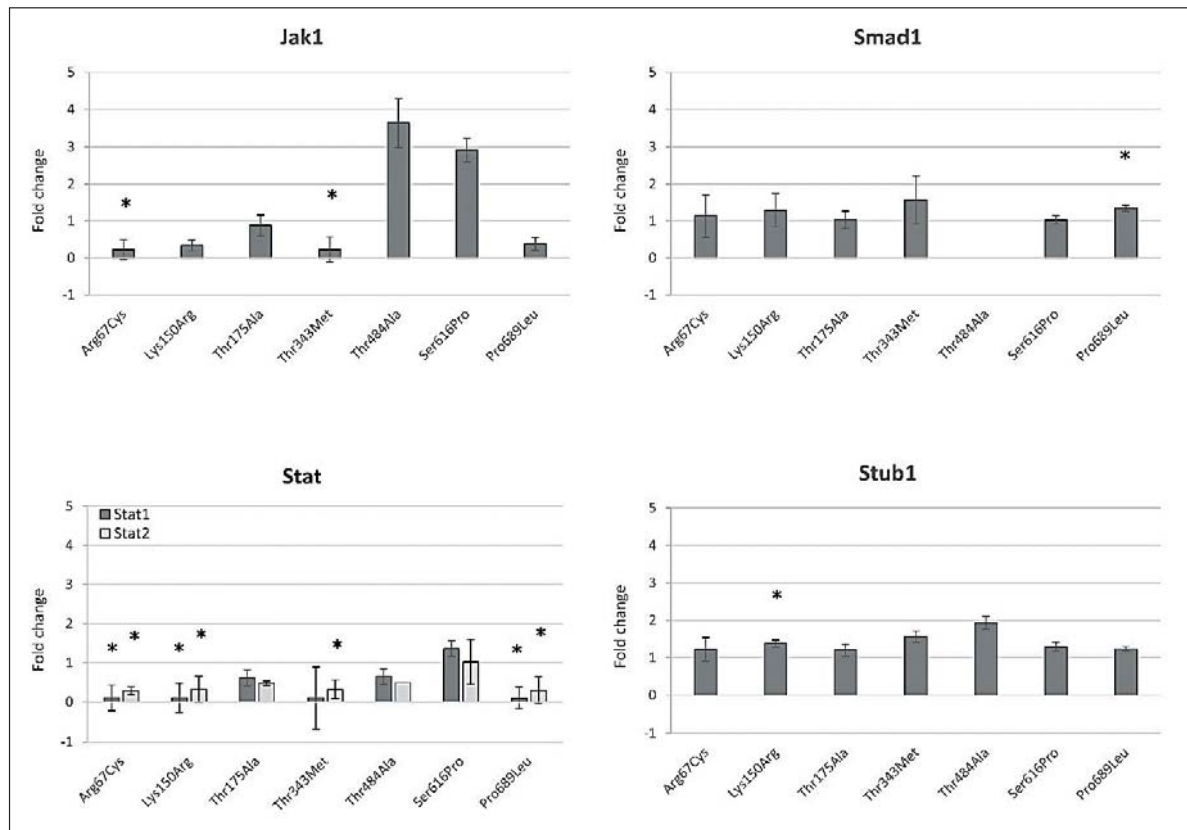


Fig. 1. Genes with expression changes in hypothalamic CLU188 after stimulation with leptin. For the depicted genes, we observed nominal changes in expression (biological triplicate) for at least one of the analyzed *SH2B1* variants ($p \leq 0.05$). Fold change from the alpha wild-type splice form is given. *Nominal p value < 0.01, **Nominal p value < 0.001. Most severe changes in expression were detected for *Gbp2b* (67Cys, $p_{\text{nominal}} = 0.0002$), *Irf9* ($\alpha 689\text{Leu}$, $p_{\text{nominal}} = 0.000018$), *Isg15* (150Arg, $p_{\text{nominal}} = 0.0003$). Gene names: cyclin D1 (*Ccnd1*); Fatty acid synthase (*Fas*); guanylate binding protein 2, interferon-inducible (*Gbp2b*); interleukin 20 (*Il20*); insulin receptor (*Insr*); interferon regulatory factor 1 and 9 (*Irf1*, *Irf9*); interferon-stimulated gene 15 (*Isg15*); Janus kinase 1 (*Jak1*); SMAD family member 1 (*Smad1*); signal transducer and activator of transcription 1 and 2 (*Stat1*, *Stat2*); STIP1 homology and U-Box containing protein 1 (*Stub1*).

a level that could be analyzed (supplemental fig. 3, available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=486962>), 10 showed nominally increased expression ($p \leq 0.05$) for at least one of the variants. Expression differences below 0.01 were detected for *Cap1* (150Arg, 0.74 ± 0.08 , $p_{\text{nominal}} = 0.006$), *Mapk1* (343Met, 1.24 ± 0.05 , $p_{\text{nominal}} = 0.006$), *Sorbs1* (689Leu, 0.46 ± 0.020 , $p_{\text{nominal}} = 0.005$; fig. 2). Here 484Ala did not decrease the expression below the analysis threshold. None of the variants increased the expression of several genes belonging to a specific arm of the signaling pathway downstream of the insulin receptor.

Discussion

Recent GWAS confirmed association of the chromosomal region 16p11.2 with BMI variation and obesity [4]. *SH2B1* most likely contributes to this effect. In a previous mutation screen, we identified seven variants, three of which were non-synonymous [8]. Here, we addi-

Table 3. Changes in gene expression after transfection of CLU188 cells with human *SH2B1* containing variants and stimulation with insulin

	Arg67Cys		Lys150Arg		Thr175Ala		Thr343Met		Thr484Ala		Ser616Pro		Pro689Leu	
	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)	p	Cohen's d (95% CI)
Akt1	-	-	-	-	-	-	-	-	-	-	0.011	-5.10 (0.31, 1.11)	-	-
Araf	-	-	-	-	-	-	-	-	-	-	0.036	-10.61 (0.22, 1.68)	-	-
Cap1	-	0.006*	7.48 (-0.62, -0.24)	-	-	-	-	-	-	-	-	-	-	-
Grb10	-	-	-	-	-	-	0.045	-3.48 (0.05, 2.41)	-	-	-	-	-	-
Gsk3b	-	0.029	4.07 (-1.26, -0.13)	-	-	-	-	-	-	-	-	-	-	-
Mapk1	-	-	-	-	-	-	0.006*	-6.80 (0.17, 0.45)	-	-	-	-	-	-
Prkci	-	-	-	-	-	-	-	-	-	-	-	-	0.031	3.95 (-1.38, -0.13)
Raf1	-	-	-	-	-	-	-	-	-	-	0.041	-3.14 (0.05, 1.14)	0.044	3.01 (-1.08, -0.03)
Rras2	-	-	-	-	-	-	-	-	-	-	-	-	0.012	-5.49 (0.39, 1.49)
Sorbs1	-	-	-	-	-	-	-	-	-	-	-	-	0.005*	7.82 (-1.60, -0.64)

For the depicted genes, we observed nominal changes in expression (biological triplicate) for at least one of the analyzed *SH2B1* variants (*Nominal p value < 0.01.)

Gene names: v-akt murine thymoma viral oncogene homolog 1 (Akt1); V-Raf Murine Sarcoma 3611 Viral Oncogene Homolog (Araf); cyclase-associated protein 1 (Cap1); growth factor receptor-bound protein 10 (Grb10); glycogen synthase kinase 3 beta (Gsk3b); mitogen-activated protein kinase 1 (Mapk1); protein kinase C iota (Prkci); Raf-1 proto-oncogene, serine/threonine kinase (Raf1); related RAS viral (r-ras) oncogene homolog 2 (Rras2); sorbin and SH3 domain containing 1 (Sorbs1).

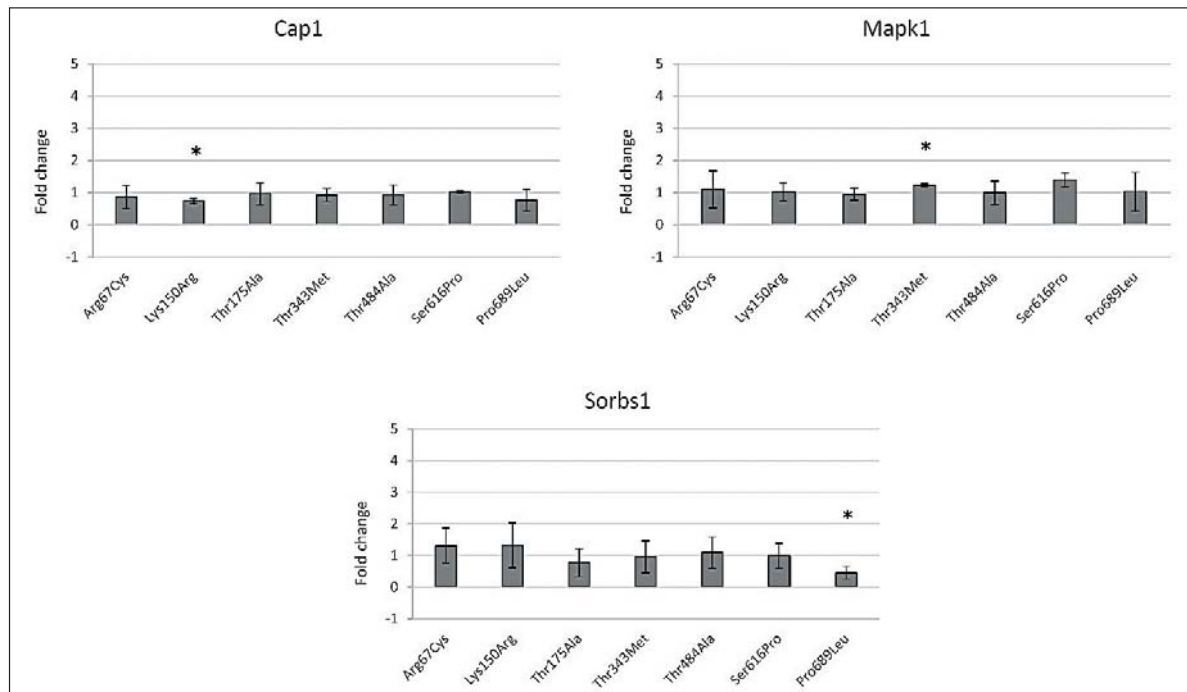


Fig. 2. Genes with expression changes in hypothalamic CLU188 after stimulation with insulin. For the depicted genes, we observed nominal changes in expression (biological triplicate) for at least one of the analyzed *SH2B1* variants. Fold change from the alpha wild-type splice form is given. *Nominal p value < 0.01. Gene names: cyclase-associated protein 1 (*Cap1*); mitogen-activated protein kinase 1 (*Mapk1*); sorbin and SH3 domain containing 1 (*Sorbs1*).

tionally detected a known infrequent variant (*Lys150Arg*) among 93 lean individuals. We included this variant in our in vitro analysis, as the in silico prediction implied a functional effect of the infrequent allele. The mutated amino acid is located at a key position of the nuclear localization domain of *SH2B1*. This domain leads to nuclearization of β *SH2B1* which drives the enhancement of NGF-induced neurite outgrowth [26].

In addition, we also analyzed four variants as they potentially entail an altered function based on the following considerations (table 1): i) Functional domain: *SH2B1* comprises four main functional domains: nuclear localization domain [25], dimerization domain [20], central pleckstrin homology domain (PH) [32], and the highly conserved amino-terminal SH2 domain [21]. Hence we chose at least one variant for each of these domains. ii) In silico prediction: variants with a prediction (PolyPhen2) of ‘possibly damaging’ or ‘probably damaging’ were used. iii) One variant was additionally included; *Thr484Ala* (rs7498665) because of the previously described obesity association [1, 2, 6, 8].

Expression Analyses

Variants nominally affected the expression of genes in the leptin and insulin signaling pathways (supplemental fig. 4 and 5, available at <http://content.karger.com/ProdukteDB/produkte.asp?doi=486962>). Three genes (*Gbp2b*, *Irf9*, and *Isg15*) were expressed at a higher level by *SH2B1* variants (*Gbp2b* – 67Cys, *Irf9* – 689Leu, and *Isg15* – 150Arg) after stimulation with leptin. *STAT1* is activated after leptin stimulation by JAK1 and JAK2 in mice [33]. The transcription factor *Stat1* subsequently nuclearizes and binds to the promoter region of *Gbp2b* [34]. *GBP2B* contributes to immune system functions against intercellular pathogens

[35]. Another downstream effector of STAT1 is ISG15, an ubiquitin-like modifier that binds to cytoplasmic and nuclear proteins ISGylating them. ISG15 has not only an anti-viral role, as assumed at the beginning, but also an oncogenic role [36, 37]. In our study, both direct STAT1 interaction partners showed nominally increased expression for different mutations. The *SH2B1* variant 67Cys, located in the dimerization domain, increased the expression of *Gbp2b*. The C-terminal *SH2B1* variant 689Leu increased the expression of *Irf9*, while 150Arg only affected the expression of *Isg15*.

IRF9 on the other hand mediates, in response to type I IFN (IFN-I), innate immunity [38]. It forms a complex with STAT1 and STAT2 activating IFN-I-stimulated gene factor 3 (ISGF3) and with it the transcription of IFN-stimulated genes (ISG) [39]. In this process, the interaction of STAT1 and IRF9 is indispensable for binding with a specific DNA sequence called IFN-stimulated response element (ISRE) that is located in the promoter of ISG. Furthermore, STAT2 activates ISRE-containing genes transcription by binding through its topologically associating domain to ISRE [40]. The overexpression of IRF9 in both diet-induced and genetically (*ob/ob*) obese mice was found to reduce insulin resistance, hepatic steatosis, and inflammation [41]. Although nuclearization of SH2B1 has been shown for the beta, but not the alpha, splice variant of the gene, the differential expression pattern does not have to be a direct outcome of SH2B1 acting as a transcription factor. Whether SH2B1 has to bind specific partners for nuclear shuttling is still unclear [26], although binding of STATs could well be one of them [42].

The panels used for gene expression analysis compared the expression of 84 target genes in the respective pathway with the expression of four housekeeping genes. The average expression of the genes (i.e., the PCR cycle by which the fluorescence signal was above threshold (Ct)) after conversion to delta Ct (by fitting them to the average Ct of the four housekeeping genes) occurred between the 24th and 30th cycle. Despite this range which allows detecting diversion to lower expression (up to cycle 35, upper threshold for analysis) or higher expression (down to cycle 20), we only observed expression increases. One explanation would be that additional SH2B1, regardless of introduced variants, would serve as additional signal increaser for both insulin and leptin signaling. Hence, it would increase expression of the target genes without or with very subtle effects of the variants. The only exception was the SNP rs7498665 (484Ala) that decreased expression of 29 of 54 genes. These 29 genes could not be analyzed further as the values were below the analysis threshold. Although a meaningful analysis was not possible, we like to point out that we took care that the RNA amount was exactly the same for all samples (see 'Material and Methods'). Additionally, the biological triplicates all replicated the effect with very little standard deviation (below 5%, see 'Results'). As the expression of the other genes in the same panel and all housekeeping genes after transfection with *SH2B1* Thr484Ala were within the normal ranges, our data suggest that the introduction of 484Ala might lead to aberrantly lower *Sh2b1* expression. However, the method we used precluded a definitive analysis of this assumption.

In contrast to leptin signaling, stimulation of 484Ala-transfected CLU188 cells with insulin did not lead to significant changes in expression of genes related to insulin signaling. Expression of half of the genes of the panel (49 out of 84) was too low for meaningful analyses in the CLU188 cells although expression of housekeeping genes was comparable to the other transfected variants. Nonetheless, expression of the genes *Cap1*, *Mapk1*, and *Sorbs1* increased by *SH2B1* variants (*Cap1* – 150Arg, *Mapk1* – 343Met and *Sorbs1* – 689Leu) after insulin stimulation. CAP1 binds directly to resistin, a hormone that induces insulin resistance and type 2 diabetes in obese mice [43]. In humans, resistin, through insulin/IGF-1R, induces the generation of cytokines and interleukins, which act in a pro-inflammatory manner [44]. SORBS1 which is also called c-Cbl-associated protein (CAP) is involved in the insulin-stimulated transport of glucose into target cells in mice. Insulin mediates the phosphorylation of c-Cbl

from the c-Cbl-CAP complex, which dissociates from the insulin receptor and binds to other substrates like flotillin in the plasma membrane. This leads to the migration of vesicles containing glucose transporter 4 from cytoplasm to the plasma membrane [45]. A significant association between a polymorphism of the threonine/alanine amino acid substitution at codon 228 (Thr228Ala) of SORBS1 and insulin resistance, obesity, and type 2 diabetes was already detected in a prior case-control study [46]. Furthermore, a recent study found other genetic variants of *Sorbs1* significantly associated with blood pressure and hypertension, providing also a possible correlation with insulin resistance [47].

MAPK1, also known as ERK2, is part of the insulin-stimulated MAP kinase pathway. For its activation, recruitment and activation of a number of substrates like RAS or MEK are necessary. This leads in the end to the phosphorylation of ERK1. Once in the nucleus, phosphorylated ERK1 acts on transcription factors inducing proliferation and differentiation [48]. The implication of ERK1 in the regulation of obesity was recently assessed by feeding ERK1 knockout (*Erk1*^{-/-}) mice a high-fat diet. *Erk1*^{-/-} mice maintained on this diet were not only more obese but also gained more fat and liver mass than their WT obese counterparts maintained on the same diet. In addition, blood cholesterol, triglyceride, and insulin concentrations were higher in *Erk1*^{-/-} obese mice compared to wild-type obese animals [49].

Limitations of this study were as follows: i) The knock down of the endogenous SH2B1 only reduced 29% of the endogenous protein. ii) The sample size was relatively small ($n \leq 5$). The latter could lead to a lower statistical power and to high false-positive rate [50]. iii) It has to be noted that it cannot be excluded that the *SH2B1* variants produce dominant negative effects, since this phenomenon is already known for SH2B1 in mice. For example, some studies described a murine *Sh2b1* mutant (Arg555Glu) with an insufficient SH2 domain to function as a negative dominant variant that inhibits IRS1 phosphorylation in cultured cells [24, 51] and in transgenic mice [17] causing obesity and insulin resistance. Dominant negative effects in genes have more severe functional impact than other haploinsufficiencies in which only one gene copy remains functional [52, 53]; it would be of interest to analyze whether the *SH2B1* variants here studied have this kind of effect and whether this effect is dose-dependent.

Conclusion

Our findings suggest that *SH2B1* variants robustly affect expression of genes in leptin signaling. We detected that SNP rs7498665 (Thr484Ala) decreases expression of genes involved in leptin signaling below the analysis threshold which is to date the first functional impact described for this variant. Taken together, our results suggest that leptin rather than insulin signaling is relevant for the mode of action of *SH2B1* variants on energy homeostasis.

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Disclosure Statement

The authors declared no conflict of interest.

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Erratum

The article by Giuranna J, Volckmar AL, Heinen A, Peters T, Schmidt B, Spieker A, Straub H, Grallert H, Müller TD, Antel J, Haußmann U, Klafki H, Liangyou R, Hebebrand J, Hinney A: The Effect of SH2B1 Variants on Expression of Leptin- and Insulin-Induced Pathways in Murine Hypothalamus. *Obes Facts* 2018;11:93–108 was published with some serious errors:

- (1) The name of one of the coauthors, Lingyou Rui, was not correctly given: The last name is Rui, and the first name is Liangyou. The correct author names are as follows:

Johanna Giuranna^a Anna-Lena Volckmar^{a, b} Anna Heinen^a Triinu Peters^a Børge Schmidt^c
Anne Spieker^a Helena Straub^a Harald Grallert^d Timo D. Müller^e Jochen Antel^a
Ute Haußmann^f Hans Klafki^{f, g} Liangyou Rui^h Johannes Hebebrand^a Anke Hinney^a

- (2) The data included in our manuscript are correct. However, by mistake the point estimation for mean differences was omitted, thus in the Abstract and tables 2 and 3 the text is misleading. The abstract is corrected as follows:

Objective: We aimed to determine the effect of human *SH2B1* variants on leptin and insulin signaling, major regulators of energy homeostasis, on the RNA level. **Methods:** We analyzed the expression of infrequent alleles of seven *SH2B1* variants (Arg67Cys, Lys150Arg, Thr175Ala, Thr343Met, Thr484Ala, Ser616Pro and Pro689Leu) in response to insulin or leptin cell stimulation. Two of these were identified in own mutation screens, the others were predicted to be deleterious or to serve as controls. The variants were analyzed in a homologous system of mouse hypothalamic cells. Changes in expression of downstream genes were measured. Student's t-test for independent samples was applied and effect sizes using Cohen's d were calculated. **Results:** In 34 of 54 analyzed genes involved in leptin (JAK/STAT or AKT) signaling, variants nominally changed expression. The expression of three genes was considerably increased (p values ≤ 0.001 : *Gbp2b* (67Cys; d = 25.11), *Irf9* (689Leu; d = 44.65) and *Isg15* (150Arg; d = 20.35)). Of 32 analyzed genes in the insulin signaling pathway, the expression of 10 genes nominally changed (p ≤ 0.05), three resulted in p values ≤ 0.01 (*Cap1* (150Arg; d = 7.48), *Mapk1* (343Met; d = -6.80) and *Sorbs1* (689Leu; d = 7.82)). **Conclusion:** The increased expression of genes in leptin (JAK/STAT or AKT) signaling implies that the main mode of action for human *SH2B1* mutations might affect leptin signaling rather than insulin signaling.

- (3) Corrected versions of tables 2 and 3 are also given.

Erratum

Table 2. Changes in gene expression after transfection of CLU188 cells with human *SH2B1* containing variants and stimulation with leptin

	Arg67Cys			Lys150Arg			Thr175Ala			Thr343Met			Thr484Ala			Ser616Pro			Pro689Leu			
	p	MD (95% CI)	d	p	MD (95% CI)	d	p	MD (95% CI)	d	p	MD (95% CI)	d	p	MD (95% CI)	d	p	MD (95% CI)	d	p	MD (95% CI)	d	
<i>Bcl2l1</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Ccnd1</i>	0.005*	-0.58 (-0.83; -0.33)	0.040	-	-0.60 (-1.14; -0.05)	-	-	-	-	-	-	-	-	-	-	0.044	0.59 (0.03; 1.15)	-	0.006*	7.44 (-1.62; -0.62)	-	-
<i>Fas</i>	0.002*	-1.70 (-2.25; -1.15)	3.64	-	-	-	0.008*	-0.96 (-1.45; -0.47)	0.014	-	-	-	-	-	-	-	-	-	0.048	7.44 (-1.62; -0.62)	-	-
<i>Gbp2b</i>	9.21	-3.12 (-3.53; -2.70)	0.005*	-	-2.52 (-3.56; -1.48)	-	5.67	-1.26 (-2.21; -0.31)	4.95	-	-	-	-	-	-	0.032	0.57 (0.09; 1.05)	-	3.28	0.001*	-	-
<i>Ghr</i>	0.0002**	-3.12 (-3.53; -2.70)	8.11	-	-	-	0.024	-1.26 (-2.21; -0.31)	0.001*	-	-	-	-	-	-	-	-	-	14.52	-2.60 (-3.20; -2.00)	-	-
<i>Ghr</i>	25.11	-	-	-	-	-	4.46	-	13.77	-	-	-	-	-	-	0.021	0.71 (0.20; 1.22)	-	-	-	-	-
<i>Irf1</i>	-	-	-	-	-	-	0.024	-0.84 (-1.46; -0.21)	-	-	-	-	-	-	-	-	-	-	0.008*	-1.78 (-2.68; -0.88)	-	-
<i>Irf1</i>	12.93	-1.09 (-1.35; -0.82)	3.90	-	-1.26; -0.12)	-	4.36	-1.11 (-1.94; -0.27)	0.029	-	-	-	-	-	-	-	-	-	6.52	-1.18 (-2.00; -0.38)	-	-
<i>Irf9</i>	0.001*	-2.24 (-2.73; -1.76)	0.031	-	-1.06 (-1.86; -0.26)	-	0.024	-1.11 (-1.94; -0.27)	3.85	-	-	-	-	-	-	-	-	-	0.020	-	-	-
<i>Irf9</i>	15.23	-1.88 (-2.19; -1.57)	3.90	-	-	-	3.85	-	7.95	-	-	-	-	-	-	-	-	-	4.16	-1.73 (-2.90; -0.56)	-	-
<i>Irf9</i>	0.008*	-2.03 (-3.05; -1.02)	0.005*	-	-	-	0.005*	-0.55 (-0.78; -0.31)	0.004*	-	-	-	-	-	-	-	-	-	0.018	-	-	-
<i>Irf9</i>	6.72	-	3.90	-	-	-	6.19	-	7.95	-	-	-	-	-	-	-	-	-	4.90	-	-	-
<i>Irf9</i>	0.001*	-2.24 (-2.73; -1.76)	0.004*	-	-1.29 (-1.80; -0.77)	-	0.01	-0.67 (-1.08; -0.26)	0.003*	-	-	-	-	-	-	-	-	-	0.000018**	-2.42 (-2.57; -2.26)	-	-
<i>Irf9</i>	15.23	-1.88 (-2.19; -1.57)	3.90	-	-	-	5.31	-0.37 (-0.53; -0.22)	9.15	-	-	-	-	-	-	0.023	0.40 (0.10; 0.70)	-	44.65	-	-	-
<i>Irf9</i>	0.008*	-2.03 (-3.05; -1.02)	0.0003**	-	-1.88 (-2.19; -1.57)	-	0.005*	-0.37 (-0.53; -0.22)	0.016	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Irf9</i>	6.72	-	20.35	-	-	-	7.96	-	5.21	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2 continued on next page

Erratum

Table 2. Continued

	Arg67Cys			Lys150Arg			Thr175Ala			Thr343Met			Thr484Ala			Ser616Pro			Pro689Leu		
	p	MD	(95% CI)	p	MD	(95% CI)	p	MD	(95% CI)	p	MD	(95% CI)	p	MD	(95% CI)	p	MD	(95% CI)	p	MD	(95% CI)
<i>Jak1</i>	0.005*	-2.12	(-3.02; -1.21)	-	-	-	-	-	-	0.008*	-2.11	(-3.18; -1.05)	-	-	-	0.016	1.54	(0.55; 2.53)	0.011	-1.40	(-2.19; -0.61)
<i>Mpl</i>	-	-	-	-	-	-	-	-	-	0.040	0.62	(0.06; 1.19)	-	-	-	-4.31	-	-	4.52	-	-
<i>Nfkb1</i>	-	-	-	0.032	0.46	(0.07; 0.85)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Nos2</i>	-	-	-	0.030	0.47	(0.09; 0.85)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Oas1a</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.025	-0.99	(-1.75; -0.24)	-	-	-
<i>Pias1</i>	-	-	-	-	-	-	0.041	-0.73	(-1.41; -0.05)	-	-	-	-	-	-	-	-	-	-	-	-
<i>Pias2</i>	-	-	-	-	-	-	3.49	-	-	-	-	-	-	-	-	0.045	2.40	(0.13; 4.68)	-	-	-
<i>Ptppn11</i>	-	-	-	-	-	-	-	-	-	-	-	-	0.024	0.64	(0.16; 1.11)	-	-	-	-	-	-
<i>Smad1</i>	-	-	-	-	-	-	-	-	-	-	-	-	-4.37	-	-	-	-	-	-	0.007*	0.43
<i>Smad3</i>	0.035	-0.83	(-1.54; -0.11)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-6.97	(0.23; 0.64)	-
<i>Smad5</i>	-	-	-	0.04	-0.55	(-1.06; -0.04)	-	-	-	-	-	-	-	-	-	-	-	-	0.032	-0.99	(-1.81; -0.16)
<i>Sox3</i>	-	-	-	3.37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Sox5</i>	-	-	-	0.01	0.45	(0.25; 0.66)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-7.18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.011	-1.12	(-1.74; -0.50)
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.18	-	-

Table 2 continued on next page

Erratum

Table 2. Continued

	Arg7Cys			Lys150Arg			Thr175Ala			Thr343Met			Thr484Ala			Ser616Pro			Pro689Leu												
	p	MD (95% CI)	d	p	MD (95% CI)	d	p	MD (95% CI)	d	p	MD (95% CI)	d	p	MD (95% CI)	d	p	MD (95% CI)	d	p	MD (95% CI)	d										
<i>Sp1</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-									
<i>Stat1</i>	0.001*	-3.12 (-3.92; -2.32)	0.002*	-3.12 (-4.01; -2.22)	0.026	-0.69 (-1.23; -0.16)	0.013	-3.13 (-5.02; -1.25)	5.55	0.015	-1.00 (-1.53; -0.47)	8.10	0.031	-1.72 (-3.13; -0.30)	4.33	0.001*	-3.23 (-3.93; -2.53)	14.62	0.007*	-1.74 (-2.59; -0.89)	6.48	0.021	-1.22 (-2.08; -0.36)	0.011	0.31 (0.14; 0.49)	0.040	-0.51 (-0.98; -0.05)	3.04	0.041	-1.04 (-2.01; -0.08)	3.53
<i>Stat2</i>	0.001*	-1.80 (-2.18; -1.42)	0.009*	-1.63 (-2.46; -0.79)	-	-	0.004*	-1.65 (-2.30; -1.01)	7.84	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
<i>Stat3</i>	0.023	-0.21 (-0.37; -0.06)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
<i>Stat6</i>	-	-	0.04	-0.98 (-1.83; -0.12)	0.045	-1.36 (-2.67; -0.06)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
<i>Stat1</i>	-	-	0.007*	0.47 (0.25; 0.69)	-	-	0.010	0.65 (0.29; 1.00)	-5.97	0.016	0.95 (0.43; 1.47)	-7.81	0.029	0.37 (0.07; 0.67)	0.011	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
<i>Tyk2</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
<i>Usf1</i>	-	-	0.018	-0.56 (-0.93; -0.18)	0.032	-0.46 (-0.85; -0.08)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
<i>Yy1</i>	-	-	4.92	-	3.95	-	-	-	-	0.014	0.83 (0.40; 1.26)	-8.28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

p = p value; MD = mean difference; d = effect size Cohen's d; 95% CI = 95% confidence intervals.

For the depicted genes we observed nominal changes (nominal p-value below 0.05) in expression (biological triplicate) for at least one of the analyzed *SH2B1* variants (*nominal p value below 0.01; **nominal p value below 0.001). Most severe changes in expression were detected for *Gbp2b* (67Cys; $p_{\text{nominal}} = 0.0002$), *Irf9* (689Leu; $p_{\text{nominal}} = 0.00018$) and *Isg15* (150Arg; $p_{\text{nominal}} = 0.0003$).

Gene names: BCL2-like 1 (*Bcl2l1*); cyclin D1 (*Ccnd1*); fatty acid synthase (*Fas*); guanylate binding protein 2; interferon-inducible (*Gbp2b*); growth hormone receptor (*Ghr*); interferon; gamma (*Irfng*); interleukin 10 receptor; alpha (*Il10ra*); interleukin 20 (*Il20*); insulin receptor (*Insr*); interferon regulatory factor 1 and 9 (*Irf1*; *Irf9*); interferon-stimulated gene 15 (*Isg15*); janus kinase 1 (*Jak1*); myeloproliferative leukemia virus oncogene (*Mpl*); nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*Nfkb1*); nitric oxide synthase 2 (*Nos2*); 2'-5' oligoadenylate synthetase 1A (*Oas1a*); protein inhibitor of activated Stat 1 (*Pias1*); protein tyrosine phosphatase; non-receptor type 11 (*Ptpn11*); SMAD family member 1; 3 and 5 (*Smad1*; *Smad3*; *Smad5*); suppressor of cytokine signaling 3 and 5 (*Socs3*; *Socs5*); trans-acting transcription factor 1 (*Sp1*); signal transducer and activator of transcription 1; 2; 3 and 6 (*Stat1*; *Stat2*; *Stat3*; *Stat6*); STIP1 homology and U-Box containing protein 1 (*Stub1*); tyrosine kinase 2 (*Tyk2*); upstream transcription factor 1 (*Usf1*); Yin Yang 1 (*Yy1*).

Table 3. Changes in gene expression after transfection of CLU188 cells with human *SH2B1* containing variants and stimulation with insulin

	Arg67Cys		Lys150Arg		Thr175Ala		Thr343Met		Thr484Ala		Ser616Pro		Pro689Leu	
	p	MD (95% CI)	p	MD (95% CI)	p	MD (95% CI)	p	MD (95% CI)	p	MD (95% CI)	p	MD (95% CI)	p	MD (95% CI)
<i>Akt1</i>	-	-	-	-	-	-	-	-	-	-	0.011	0.71	-	-
<i>Araf</i>	-	-	-	-	-	-	-	-	-	-	-5.10 (0.31; 1.11)	-	-	-
<i>Cap1</i>	-	-	0.006*	-0.43 (-0.62; -0.24)	-	-	-	-	-	-	0.036	0.95	-	-
<i>Grb10</i>	-	-	7.48	-	-	-	0.045	1.23 (0.05; 2.41)	-	-	-10.61 (0.22; 1.68)	-	-	-
<i>Gsk3b</i>	-	-	0.029	-0.70 (-1.26; -0.13)	-	-	-3.48	-	-	-	-	-	-	-
<i>Mapk1</i>	-	-	4.07	-	-	-	0.006*	0.31 (0.17; 0.45)	-	-	-	-	-	-
<i>Prkci</i>	-	-	-	-	-	-	-6.80	-	-	-	-	-	0.031	-0.75 (-1.38; -0.13)
<i>Raf1</i>	-	-	-	-	-	-	-	-	-	-	0.041	0.59	0.044	-0.55 (-1.08; -0.03)
<i>Rras2</i>	-	-	-	-	-	-	-	-	-	-	-3.14 (0.05; 1.14)	-	3.01	0.94 (0.39; 1.49)
<i>Sorbs1</i>	-	-	-	-	-	-	-	-	-	-	-	-	-5.49	-1.12 (-1.60; -0.64)

p = p value; MD = mean difference; d = effect size Cohen's d; 95% CI = 95% confidence intervals.

For the depicted genes, we observed nominal changes in expression (biological triplicate) for at least one of the analyzed *SH2B1* variants. *Nominal p value below 0.01. *Gene names:* v-akt murine thymoma viral oncogene homolog 1 (*Akt1*); V-Raf Murine Sarcoma 3611 Viral Oncogene Homolog (*Araf*); cyclase-associated protein 1 (*Cap1*); growth factor receptor-bound protein 10 (*Grb10*); glycogen synthase kinase 3 beta (*Gsk3b*); mitogen-activated protein kinase 1 (*Mapk1*); protein kinase C iota (*Prkci*); Raf-1 proto-oncogene, serine/threonine kinase (*Raf1*); related RAS viral (r-ras) oncogene homolog 2 (*Rras2*); sorbin and SH3 domain containing 1 (*Sorbs1*).