Supplementary Methods

Expression plasmids

The PC12 and RAW264.7 cells used to characterize the functional impact of the mutants are derived from rat and mouse respectively, so we used a cDNA encoding a rodent (rat) SH2B1β. Mutant GFP-SH2B1β cDNA was created using QuikChange Site-Directed Mutagenesis Kit (Stratagene). Mouse IRS2 was cloned into pcDNA(3.1-) vector (Flag-tag at N-terminus).

Cell culture and transfection

Q293A cells stably expressing the long form of the mouse leptin receptor (293^{LRb})(S1), HEK 293 cells, and 293T cells were transiently transfected using the polyethylenimine (PEI) method. PC12 cells were cultured in RPMI 1640 (ATCC) containing 10% horse serum (Invitrogen) and 5% fetal bovine serum (FBS) (Atlanta Biologicals) on collagen-coated dishes and transfected as described previously (18). RAW264.7 cells (J. Swanson, University of Michigan,) were cultured in DMEM (Invitrogen) supplemented with 8% heat inactivated FBS (Atlanta Biologicals), 1 mM L-glutamine and 1 mM antibiotic-antimycotic (Invitrogen) at 37°C in 5% CO_2. RAW264.7 cells were transiently transfected using Amaxa nucleofector (Lonza) using solution V and setting D32.

Neuronal differentiation of PC12 cells

Transfected PC12 cells were plated on collagen-coated 60-mm dishes. 24 h post transfection, 25 or 50 ng/ml NGF (BD Bioscience) was added to RPMI 1640 containing 5% horse serum, 1% FBS to induce PC12 cell differentiation. NGF-containing differentiation medium was refreshed two days later. The cells were visualized by fluorescence microscopy (Nikon Eclipse TE200). The percent of GFP+ cells that were differentiated (neurite outgrowths >2 times the length of the cell body) was determined.

Cell Imaging

Transfected 293T cells plated on 35-mm, poly-d-lysine-coated glass-bottom culture dishes (MatTek Corp., Ashland, MA) were incubated for 8h in growth medium supplemented with 20nM leptomycin B (Sigma) to inhibit Crm1-dependent nuclear export. GFP+ cells were visualized with an Olympus FluoView 500 laser scanning confocal microscope using a 60x water-immersion objective and FluoView version 5.0 software. Linescan profiles were obtained and analysed using MetaVue Software (Universal Imaging, Sunnyvale, CA).

GH-induced migration of RAW264.7 macrophages

RAW264.7 cells transiently expressing SH2B1β WT or mutant were incubated in growth medium for 24h, then in serum-free medium (1% BSA) overnight. Transfected cells suspended in serum-free medium were added to the upper chamber of a Transwell (Costar) unit with 5-μm pore size; the lower chamber contained serum-free medium with 500ng/ml recombinant human GH (gift of Eli Lilly & Co.). Transwell units were incubated at 37°C for 18 h, fixed in methanol and air-dried. Cells in the upper chamber were removed with a cotton swab. Filter membranes were stained with hematoxylin and eosin (H&E) (1:10 dilution) for 1 h and then washed with ddH2O. Cells that had migrated were counted (≥3 independent fields per condition) under a light microscope.

SH2B1 dimerization

HEK293 cells were transiently transfected with Flag- and GFP-tagged SH2B1β constructs. 36h post-transfection, cells were washed with PBS, harvested in NP-40 lysis buffer and clarified by centrifugation. Lysate (3 mg protein) was pre-cleared by incubation with Protein A agarose (Sigma) at 4°C for 30 min before incubation with anti-FLAG M2 affinity gel (Sigma) at 4°C overnight. Immunoprecipitates were washed, resuspended in sample buffer and filtered through a Spin-X filter to remove the resin. NuPAGE reducing agent (1X; Invitrogen) was added to the eluted samples, which were subjected to electrophoresis and immunoblot analysis.

Statistics

To test for statistically significant differences in values from the neuronal differentiation assay (n=4-8 experiments, 60-120 cells/condition/experiment), a one-way ANOVA with a Dunnett's multiple comparison post-test was applied. For the values from each day of the assay (days 1-4), the ANOVA yielded *p*-values < 0.0001 and the Dunnett's post-test showed significant differences between GFP-SH2B1β WT-expressing cells and every other condition (alpha = 0.05). For nuclear accumulation of GFP-SH2B1β (n=3-5 experiments, 76-150 cells/condition/experiment) and subcellular localization (n=16-26), statistical significance was determined using an unpaired, two-tailed Student's *t*-test. For the motility assay (n=4), a paired, one-tailed Student's *t*-test was used.

Study subjects

The Genetics of Obesity Study (GOOS) cohort consists of 4300 probands with severe obesity (Body Mass Index Standard Deviation Score (BMI SDS) >3) before 10 years of age. BMI SDS values were calculated using UK reference data (S2). We identified three hundred patients who also exhibited disproportionate insulin resistance as defined by the presence of acanthosis nigricans, the development of type 2 diabetes in early adolescence and/or markedly elevated plasma insulin levels (top decile for age, gender and body mass index). The mean (±SD) BMI SDS for this group of 300 chosen for study was 4.3 ± 1.4. In adults, overweight was defined as BMI 25–29.9 kg/m^2, obesity as BMI > 30 kg/m^2 according to WHO criteria. In children (<18yrs), we used definitions proposed by the International Obesity Task Force: overweight defined as >91st and obesity as >99th percentile for age-adjusted BMI.

Supplementary references

S1. Duan, C., Li, M., and Rui, L. 2004. SH2-B promotes insulin receptor substrate 1 (IRS1)- and IRS2 mediated activation of the phosphatidylinositol 3-kinase pathway in response to leptin. *J Biol Chem* 279:43684-43691.

S2. Cole, T.J., Freeman, J.V., and Preece, M.A. 1995. Body mass index reference curves for the UK, 1990.
Arch Dis Child 73:25-29.

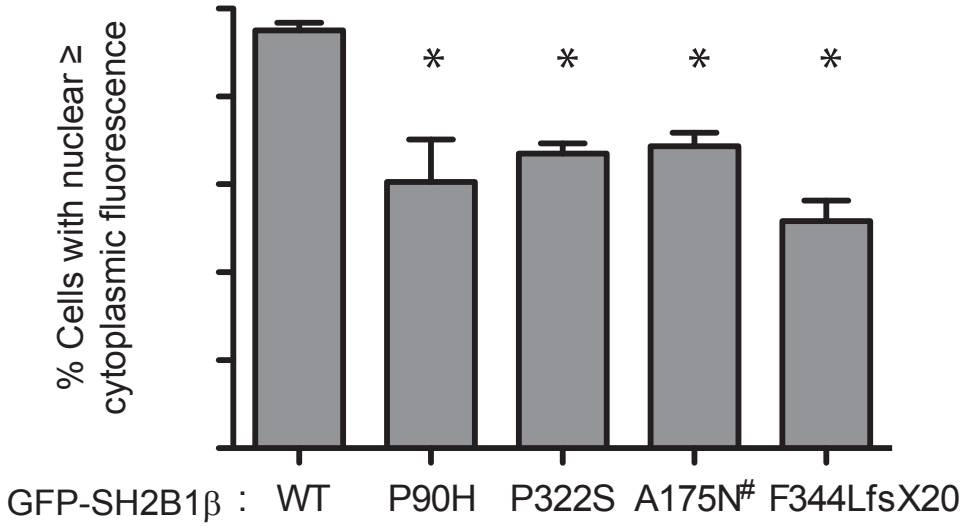

Figure S1. SH2B1β mutants exhibit an impaired ability to accumulate in the nucleus when nuclear export is inhibited. 293T cells transiently expressing GFP-SH2B1β WT or mutant were treated with the nuclear export inhibitor leptomycin B (LMB) (20mM) for 8 h and imaged using fluorescent confocal microscopy as described for Figure 2B. The percentage of GFP-positive cells (76-150 cells/condition/assay) that exhibited nuclear fluorescence signal intensity greater than or equal to the cytoplasmic fluorescence signal intensity was determined. Means ± SEM are shown where n = 5, 4 and 3 independent experiments for WT, point mutants and F344LfsX20, respectively. (*) $p<0.05$ compared to WT using an unpaired, two-tailed Student's t test. $p = 0.0128$, 0.0004, 0.0001 and 0.0062 for P90H, P322S, A175N AND F344LfsX20, respectively. (#) All experiments were performed using rat SH2B1β where Thr175 is Ala175 and therefore the T175N human mutation is referred to as A175N.