

## Functional characterisation of obesity-associated variants involving the alpha and beta isoforms of human SH2B1

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We have previously reported rare variants in Src homology 2 (SH2) B adaptor protein 1 (*SH2B1*) in individuals with obesity, insulin resistance and maladaptive behaviour. Here, we identify four additional *SH2B1* variants by sequencing 500 individuals with severe early-onset obesity. *SH2B1* has four alternatively spliced isoforms. One variant (T546A) lies within the N-terminal region common to all isoforms. As shown for past variants in this region, T546A impairs SH2B1 $\beta$  enhancement of nerve growth factor (NGF)-induced neurite outgrowth and the individual with the T546A variant exhibits mild developmental delay. The other three variants (A663V, V695M and A723V) lie in the C-terminal tail of SH2B1 $\alpha$ . SH2B1 $\alpha$  variant carriers were hyperinsulinemic but did not exhibit the behavioural phenotype observed in individuals with *SH2B1* variants that disrupt all isoforms. In vitro assays, SH2B1 $\alpha$ , like SH2B1 $\beta$ , enhances insulin- and leptin-induced IRS2 phosphorylation and growth hormone (GH)-induced cell motility. None of the variants affect SH2B1 $\alpha$  enhancement of insulin- and leptin-induced IRS2 phosphorylation. However, T546A, A663V and A723V all impair the ability of SH2B1 $\alpha$  to enhance GH-induced cell motility. In contrast to SH2B1 $\beta$ , SH2B1 $\alpha$  does not enhance NGF-induced neurite outgrowth. These studies suggest that genetic variants that disrupt isoforms other than SH2B1 $\beta$  may be functionally significant. Further studies are needed to understand the mechanism by which the individual isoforms regulate energy homeostasis and behaviour.

**S**rc homology 2 (SH2) B adaptor protein 1 (*SH2B1*) is a member of a family of scaffold proteins implicated in signaling downstream of a variety of receptor tyrosine kinases and cytokine receptors that bind to JAKs. These include receptors for leptin, insulin, growth hormone (GH), IGF-1, nerve growth factor (NGF), and brain-de-

rived neurotrophic factor (BDNF) (reviewed in (1)). In mice, targeted deletion of *Sh2b1* results in marked leptin resistance, increased food intake, severe obesity and insulin resistance. An intermediate obesity phenotype is seen in heterozygous null mice fed a high fat diet (2, 3), suggesting that the obesity phenotype is dosage-dependent.

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Abbreviations:

Given the large number of receptor tyrosine kinases and cytokine receptor/JAK complexes that bind to SH2B1 (1), dissecting the molecular mechanisms by which SH2B1 regulates energy balance and glucose homeostasis has proved challenging. SH2B1 is alternatively spliced to yield four isoforms ( $\alpha, \beta, \delta, \gamma$ ) that vary in length from 671 to 756 amino acids. All isoforms share a phenylalanine (Phe) zipper dimerization domain, nuclear localization sequence (NLS), nuclear export sequence (NES), Pleckstrin homology (PH) domain and SH2 domain but exhibit unique C-termini that vary in length from 40 (SH2B1 $\beta$ ) to 125 (SH2B1 $\alpha$ ) amino acids (Figure 1) (4). The human SH2B1 isoforms have distinct expression patterns. While the  $\beta$  and  $\gamma$  isoforms are widely expressed, the  $\alpha$  and  $\delta$  isoforms are restricted to brain regions (5). Although very little is known about the physiological relevance of the different SH2B1 isoforms, neuron-specific restoration of the beta isoform in *Sh2b1* null mice rescues the obese phenotype (6).

We previously reported rare genetic variants in SH2B1 (P90H, T175N, P322S, F344Lfs\*20) that are located in the N-terminal 631 amino acids shared by all four isoforms (1–631 region). Individuals carrying these variants exhibit severe early-onset obesity and insulin resistance, and a neurobehavioural phenotype characterized by delayed speech and language development, and maladaptive behavior (5). These variants disrupted SH2B1 cellular function in *in vitro* assays that measured GH-induced cell motility and NGF-induced neurite outgrowth. An additional SH2B1 variant (g.9483C/T), which affects only the beta (T656I) and gamma (P674S) isoforms, was also recently identified in obese subjects (7). This variant had no functional effect in the one assay tested (SH2B1 enhancement of leptin stimulation of STAT3 activity).

Here we describe four additional SH2B1 variants identified by sequencing a further 500 unrelated severely obese individuals from the Genetics of Obesity Study (GOOS) cohort. We performed a series of functional studies of these new variants and those previously identified by us (P90H, T175N, P322S, F344Lfs\*20) within the context of SH2B1 $\alpha$ . There is evidence to support not only the role of rare variants in SH2B1 in severe obesity but also of common variants with a broader role in the regulation of body mass index (BMI). As such, we also studied a common coding variant (rs7498665; A484T) that has been strongly associated with BMI in genome wide association studies (8, 9).

## Materials and Methods

### SH2B1 variant analysis

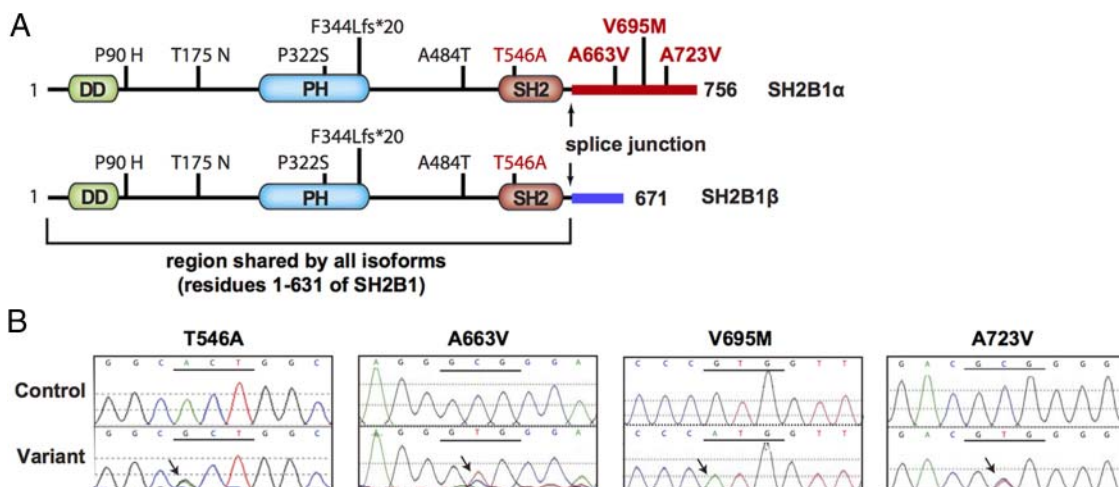
500 individuals with severe early-onset obesity (defined as a BMI sds > 3; onset < 10 years) were randomly selected from the Genetics of Obesity Study (GOOS) cohort study. Primers were designed to cover the coding sequence (NM015503) and splice junctions of SH2B1. Variant screening was performed using PCR, followed by direct sequencing using BigDye terminator chemistry (Applied Biosystems, UK) and analysis on an ABI 3730 automated sequencer (Applied Biosystems, UK).

Methods for functional studies are similar to those described previously (5) and are in the Supplement.

## Results

### Identification of novel SH2B1 variants in severely obese individuals

We previously identified four variants in SH2B1 (P90H, T175N, P322S, F344Lfs\*20) in individuals with



**Figure 1.** Identification of novel variants in SH2B1. A) Schematic showing the location of variants identified in SH2B1 identified in individuals with severe obesity. The novel variants identified in this study are shown in red. Variants reported previously and the common SNP (A484T) are shown in black. DD = dimerization domain, PH = Pleckstrin homology domain, SH2 = SH2 domain. B) Sequence traces of the novel variants in this study.

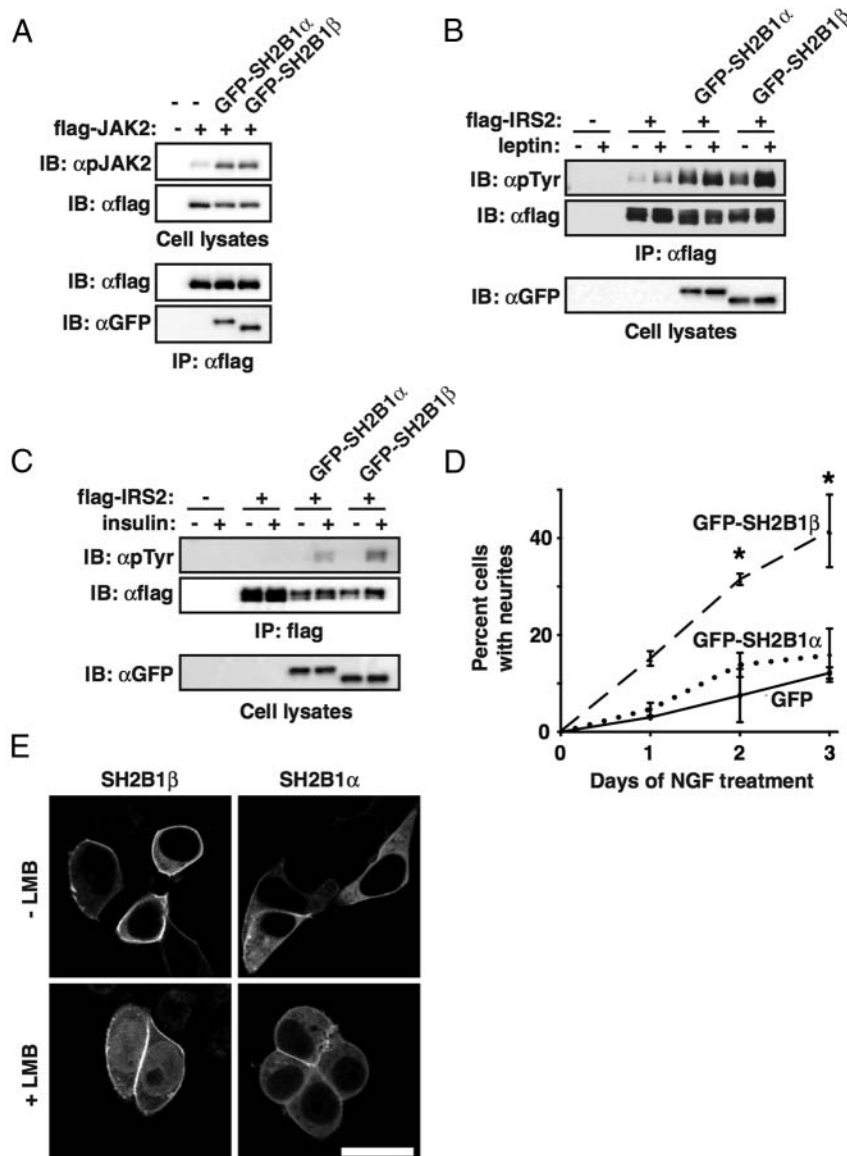
severe early-onset obesity from the Genetics of Obesity (GOOS) cohort (5). In the present study, we sequenced *SH2B1* in 500 additional individuals from this cohort. In addition to another individual carrying the T175N variant, we found three novel heterozygous variants in unre-

lated severely obese individuals: T546A ( $n = 1$ ), A663V ( $n = 14$ ) and A723V ( $n = 1$ ) (Table 1). One individual was homozygous for V695M. As with the previously reported variants, the T546A variant is present in all four *SH2B1* isoforms. However, the three other variants (A663V, V695M and A723V) affect the unique C-terminal tail of *SH2B1* $\alpha$  (Figure 1). We sequenced *SH2B1* in 28 available family members of severely obese probands (Table 1). A663V variants did not cosegregate with obesity in families in a classical Mendelian manner, suggesting that *SH2B1* variants may predispose to obesity against a background of other genetic and environmental factors. There were an equal number of male and female mutation carriers (Table 1).

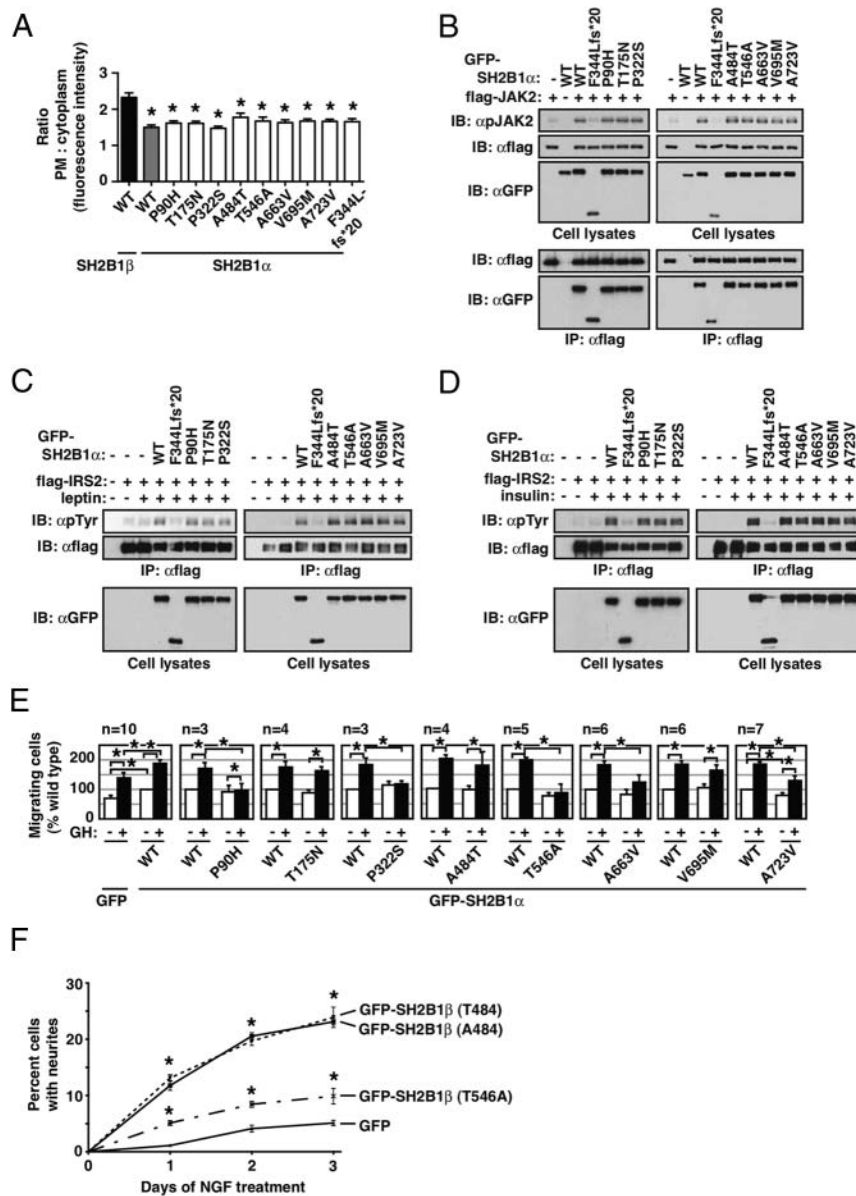
Adult variant carriers were hyperinsulinaemic (mean fasting plasma insulin  $128 \pm 32$  pmol/l; reference range 0–60 pmol/l), but euglycaemic; liver function tests, lipid profiles and final height were in the normal range. The individual with the T546A variant had mild developmental delay (Table 1). However, no neurobehavioral abnormalities were reported in individuals carrying the A663V, V695M or A723V variants.

### Differences in cellular signaling mediated by human *SH2B1* $\alpha$ and $\beta$ isoforms

We next explored the molecular mechanisms by which these variants might disrupt *SH2B1* function. We first studied the ability of human *SH2B1* $\alpha$  to mediate signaling in response to a number of ligands. As a point of reference, we compared these findings to those obtained using human *SH2B1* $\beta$ . Both *SH2B1* $\alpha$  and *SH2B1* $\beta$  bind to JAK2 and enhance JAK2 autophosphorylation to a similar degree (Figure 2A), consistent with results of Nishi et al (10). *SH2B1* $\beta$  is reported to bind to IRS proteins and promote their tyrosyl phosphorylation in response to insulin and leptin (11, 12). Like *SH2B1* $\beta$ ,



**Figure 2.** Comparison of *SH2B1* $\alpha$  and *SH2B1* $\beta$  in vitro. A) 293 cells were transfected with the indicated constructs. Proteins in lysates were immunoprecipitated with anti-flag agarose. Immunoblotting was performed using the indicated antibodies. Results are representative of 3 experiments. B) 293<sup>LRb</sup> cells were transfected as shown, serum-starved, and then treated with 100ng/ml leptin for 5 minutes. Results are representative of 4 experiments. C) 293 cells were transfected with the indicated constructs, serum-starved and then stimulated with 100 nM insulin for 5min. Results are representative of 4 experiments. D) PC12 cells were transiently transfected with the indicated constructs and treated with 25ng/ml NGF to induce neurite outgrowth. Results show the % of GFP+ cells with neurites greater than twice cell body length. Means  $\pm$  range,  $n = 2$  different experiments with 300 cells counted per condition per day of NGF treatment. \* $P < .05$  compared to GFP+ cells at the same time-point. Statistical significance was assessed using two-way ANOVA and Bonferroni's Multiple Comparisons post-test. E) Live 293T cells transiently expressing GFP-tagged human *SH2B1* $\beta$  or *SH2B1* $\alpha$  were incubated  $\pm$  leptomycin-B (LMB, 20 nM) for 4h and imaged using fluorescent confocal microscopy. Each image is representative of 50–60 cells visualized in 2 separate experiments. Scale bar = 20  $\mu$ m.



**Figure 3.** Characterization of novel human variants in SH2B1. A) Live 293T cells transiently expressing GFP-tagged rat SH2B1 $\beta$ , human SH2B1 $\alpha$  WT or human SH2B1 $\alpha$  mutants were stained with the plasma membrane marker wheat germ agglutinin Alexa Fluor 594 and imaged by confocal microscopy. Green and red signal intensity across each cell were determined using line scan analysis (MetaVue). The ratio of the plasma membrane: cytoplasmic signal intensity is shown (mean  $\pm$  SEM). \* =  $P < .0001$  by two-tailed, unpaired Student's  $t$  test compared to the ratio for rat SH2B1 $\beta$  WT,  $n = 13$ –19 cells/condition. B) 293 cells were transfected with the indicated constructs and resulting lysates subjected to immunoprecipitation with anti-flag agarose. Immunoblotting was performed using the indicated antibodies. Results are representative of 3 experiments. C) 293<sup>Rb</sup> cells were transfected as shown, serum-starved, and treated with 100ng/ml leptin for 5 minutes. Results are representative of 4 experiments. D) 293 cells were transfected with the indicated constructs, serum-starved and stimulated with 100 nM insulin for 5min. Results are representative of 4 experiments. E) RAW264.7 cells ( $2 \times 10^5$  cells/well) were transiently transfected as indicated. Migration was analyzed using a transwell migration assay with or without GH (500 ng/ml) in the lower chamber for 18 hours. Values are normalized to the non-hGH treated GFP-SH2B1 $\alpha$  cells. Mean  $\pm$  SEM from 3–10 independent experiments. \* =  $P < .05$  by one-tailed paired Student's  $t$  test. F) PC12 cells transiently expressing GFP, or GFP-tagged human SH2B1 $\beta$  T484, A484, or T546A, were treated with NGF (25 ng/ml) to induce neurite outgrowth. Results show the % of GFP+ cells with neurites greater than twice cell body length were counted. Means  $\pm$  SEM,  $n = 3$  different experiments with 300 cells counted per condition per day of NGF treatment. \*  $P < .05$  compared to GFP cells at the same time-point. GFP-SH2B1 $\beta$ T546A cells exhibited a statistically lower ( $P < .05$ ) number of

SH2B1 $\alpha$  enhances both leptin-stimulated (Figure 2B) and insulin-stimulated (Figure 2C) tyrosyl phosphorylation of IRS2. Next, we sought to determine whether SH2B1 $\alpha$  is involved in mediating the effects of neurotrophins such as NGF. Surprisingly, while SH2B1 $\beta$  enhances NGF-induced neurite outgrowth of PC12 cells (13), SH2B1 $\alpha$  does not (Figure 2D). SH2B1 $\beta$  shuttles between the nucleus and the cytoplasm (14). Shuttling is thought to be necessary for SH2B1 $\beta$  to enhance transcription of NGF-responsive genes such as uPAR, MMP3 and MMP10 (15, 16), which are implicated in neurite outgrowth of PC12 cells (16). When 293T cells expressing either SH2B1 $\alpha$  or SH2B1 $\beta$  are treated with the nuclear export inhibitor, leptomycin B, only the beta isoform is retained in the nucleus (Figure 2E). These results indicate that SH2B1 $\alpha$  and SH2B1 $\beta$  share the ability to mediate signaling downstream of insulin, leptin and GH. However, only the beta isoform translocates to the nucleus and promotes NGF-induced neurite outgrowth.

### Functional characterization of variants affecting SH2B1 $\alpha$

We next investigated the functional consequences of variants when expressed in the SH2B1 $\alpha$  isoform. The distribution of SH2B1 $\alpha$  between the plasma membrane and the cytoplasm is not altered by any of the variants (Figure 3A). However, compared to SH2B1 $\beta$ , the intensity of SH2B1 $\alpha$  in the plasma membrane relative to the cytoplasm is diminished (Figure 2E, 3A). Except for the frameshift mutant F344Lfs\*20 that lacks the SH2 domain, none of the variants affect the ability of SH2B1 $\alpha$  to enhance JAK2 autophosphorylation, or leptin- or insulin-induced tyrosyl phosphorylation of IRS2 (Figures 3B–D). As reported previously for SH2B1 $\beta$  (5), the P90H and

P332S variants reduce the ability of SH2B1 $\alpha$  to stimulate GH-induced cell migration. The T546A, A663V and A723V variants also reduce GH-induced cell migration (Figure 3E). T175N and V695M and the common SNP (A484T) have no impact. Finally, we tested the effect of the variants on SH2B1 enhancement of NGF-induced neurite outgrowth. Like wild-type SH2B1 $\alpha$  (Figure 2D), SH2B1 $\alpha$  A663V, V695M and A723V do not enhance NGF-induced neurite outgrowth (data not shown). However, like the previously described human variants in the 1–631 region, the T546A variant impairs the ability of SH2B1 $\beta$  to enhance NGF-induced neurite outgrowth. The A484T SNP has no effect on SH2B1 $\beta$  enhancement of NGF-induced neurite outgrowth (Figure 3F).

## Discussion

Here we describe the identification of four novel variants in *SH2B1* that are present in individuals with obesity and insulin resistance. Some of the variants we found in severely obese individuals are also found in publicly available exomes (Table 1). However, as BMI and additional phenotypic information for individuals in these datasets are not available, the precise contribution of these variants to obesity remains to be established.

These findings suggest that *SH2B1* contains a spectrum of common and rare alleles which contribute to BMI and obesity predisposition with a broad range of penetrance, from low to more highly penetrant rare alleles. One variant, A663V, was identified in 14 severely obese individuals in the GOOS cohort as well as in many publically available exomes. In cells, A663V affected the ability of SH2B1 to enhance cell motility in response to GH, therefore, it is possible that this variant may contribute to the phenotype of variant carriers. Additional genetic studies will be needed to determine whether this variant is significantly enriched in obese cohorts compared to controls. The nucleotide change that causes the A663V variant in SH2B1 $\alpha$  also causes an amino acid change (R680C) in the delta isoform of SH2B1. Mutation of this residue in SH2B1 $\delta$  impairs the ability of SH2B1 $\delta$  to enhance NGF-induced neurite outgrowth (data not shown), a finding that requires further investigation.

We also studied the common coding SNP, A484T. A484T did not impact upon SH2B1 $\alpha$  or SH2B1 $\beta$  in the functional assays employed here. This is consistent with a previous study (7), which was unable to demonstrate any functional consequence of the A484T SNP. It is possible

that this SNP affects cellular functions of SH2B1 other than those tested or it may have subtle functional consequences that cannot be detected in the cell systems and assays we employed.

We previously showed that individuals carrying heterozygous variants in the 1–631 region of SH2B1 were hyperphagic, with a reduced final height, elevated plasma insulin levels that are disproportionate to the degree of obesity, and surprisingly, maladaptive behavior (5). Consistent with these findings, the individual carrying the T546A variant had a markedly elevated fasting plasma insulin of 123 pmol/l at age 6 years and mild developmental delay. While individuals carrying the A663V, V695M and A723V variants were hyperinsulinaemic, none of them were reported to display any of the behavioral characteristics reported previously. Given the small number of individuals studied, these observations need to be replicated in additional studies. We also need to determine, at the mechanistic level, whether the behavioral phenotype results from disruption of a specific SH2B1 isoform (eg, SH2B1 $\beta$ ) or a function emanating from the 1–631 region shared by all 4 isoforms.

Three of the variants identified in this study are present within the unique C-terminal tail of SH2B1 $\alpha$  but not SH2B1 $\beta$ . A limited number of studies have compared the actions of the various isoforms in vitro. All four SH2B1 isoforms enhanced mitogenesis and cell proliferation in response to IGF-1, insulin and platelet-derived growth factor (PDGF) stimulation (4, 17). Stable expression of each isoform in NIH3T3 fibroblasts led to enhanced insulin receptor autophosphorylation and phosphorylation of IRS1 (17). In 3T3-L1 cells, all four isoforms enhance insulin-stimulated glucose and amino acid transport, glycogen synthesis, lipogenesis, Akt activity, and p70 S6 kinase activity (18). In all of these assays, SH2B1 $\alpha$  was as effective as, or more effective than, SH2B1 $\beta$ . Thus, it was surprising to observe the inability of SH2B1 $\alpha$  to enhance NGF-induced neurite outgrowth. The finding that the gamma and delta isoforms of SH2B1 resemble SH2B1 $\beta$  in their ability to enhance NGF-induced neurite outgrowth (data not shown) suggest that the unique C-terminal tail of SH2B1 $\alpha$  inhibits at least some functions mediated by the region of SH2B1 between amino acids 1–631. In contrast to its inability to promote NGF-induced neurite outgrowth, SH2B1 $\alpha$ , like SH2B1 $\beta$ , was found to enhance GH-induced macrophage motility. Exactly how SH2B1 stimulates motility is not known. However, one of the proline-rich regions present in all isoforms has been shown to bind Rac (23), a protein known to be involved in motility. Ligand-dependent phosphorylation of tyrosines within SH2B1 appears to be critical for SH2B1

neurite outgrowths at days 1–3 compared to GFP-SH2B1 $\beta$ A484 and T484. Statistical significance was assessed using two-way ANOVA and Bonferroni's Multiple Comparisons post-test.

enhancement of GH-dependent cell motility (24) suggesting that these phosphorylated tyrosines may recruit critical proteins to SH2B1 complexes. SH2B1 $\beta$  has also been shown to increase NGF-induced migration of PC12 cells in a wounding assay, perhaps by a protein kinase C-dependent process (25). The finding that most of the human variants impair the ability of SH2B1 $\alpha$  and  $\beta$  to enhance motility raises the possibility that regulation of the actin cytoskeleton and/or motility of cells is an important and vital component of SH2B1 function that plays a critical role in the ability of SH2B1 to regulate energy balance and the response to insulin.

SH2B1 is among a small number of adaptor proteins that undergo nucleocytoplasmic shuttling (14) although its exact role within the nucleus is not yet clear. Our previous studies suggest that neurite outgrowth requires nuclear SH2B1 (16). Human mutations such as P90H, T175N, P322S and F344Lfs\*20 that reside in the 1–631 region of SH2B1 impair both nuclear accumulation in the presence of LMB and enhancement of NGF-induced neurite outgrowth (5). Our finding here that SH2B1 $\alpha$  neither accumulates in the nucleus nor enhances NGF-induced neurite outgrowth is consistent with SH2B1 $\beta$  enhancement of neurite outgrowth requiring nuclear SH2B1 $\beta$ . In contrast to neurite outgrowth, SH2B1 $\beta$  enhancement of GH-induced macrophage motility does not require its nuclear localization (26), a finding consistent with our observation here that SH2B1 $\alpha$  retains the ability to enhance macrophage motility despite its inability to enter the nucleus. It is possible that the unique C-terminal tail of SH2B1 $\alpha$  interferes with the region of SH2B1 that is required for nuclear localization. Masking of the region around the NLS in SH2B1 $\alpha$  might explain why altering amino acid 175, which lies near the NLS, impairs the ability of SH2B1 $\beta$  but not SH2B1 $\alpha$  to enhance GH-induced macrophage migration.

In summary, we have identified additional SH2B1 variants in individuals with obesity and that implicate SH2B1 isoforms besides SH2B1 $\beta$  as important for the regulation of body weight. Further studies will be needed to understand how the distinct C-terminal tails of the alpha, beta, gamma and delta isoforms influence SH2B1 function and their precise roles in vivo.

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