RESEARCH ARTICLE

σ1B adaptin regulates adipogenesis by mediating the sorting of sortilin in adipose tissue

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ABSTRACT

Here, we describe altered sorting of sortilin in adipocytes deficient for the σ1B-containing AP-1 complex, leading to the inhibition of adipogenesis. The AP-1 complex mediates protein sorting between the trans-Golgi network and endosomes. Vertebrates express three AP1 σ1 subunit isoforms – σ1A, σ1B and σ1C (also known as AP1S1, AP1S2 and AP1S3, respectively). σ1B-deficient mice display impaired recycling of synaptic vesicles and lipodystrophy. Here, we show that sortilin is overexpressed in adipose tissue from σ1B−/− mice, and that its overexpression in wild-type cells is sufficient to suppress adipogenesis. σ1B-specific binding of sortilin requires the sortilin DxxD-x12-DSxxxL motif. σ1B deficiency does not lead to a block of sortilin transport out of a specific organelle, but the fraction that reaches lysosomes is reduced. Sortilin binds to the receptor DLK1, an inhibitor of adipocyte differentiation, and the overexpression of sortilin prevents DLK1 downregulation, leading to enhanced inhibition of adipogenesis. DLK1 and sortilin expression are not increased in the brain tissue of σ1B−/− mice, although this is the tissue with the highest expression of σ1B and sortilin. Thus, adipose-tissue-specific and σ1B-dependent routes for the transport of sortilin exist and are involved in the regulation of adipogenesis and adipose-tissue mass.

KEY WORDS: AP-1, Adipogenesis, Clathrin, DLK1, Sortilin

INTRODUCTION

We demonstrate that adaptor protein AP-1-complex-dependent protein sorting in adipocyte precursor cells is involved in the regulation of adipocyte differentiation and, thus, in the regulation of adipose tissue mass during development. AP-1 acts as an adaptor for clathrin during transport by clathrin-coated vesicles (CCVs). It is expressed ubiquitously, mediating protein sorting between the trans-Golgi network (TGN) and endosomes, which is essential for vertebrate development (Meyer et al., 2000; Zizioli et al., 2010; Zizioli et al., 1999). In mammals, there are four members of the family of AP-1 complexes, with tissue-specific expression of subunit isoforms (Boehm and Bonifacino, 2001; Robinson, 2004; Glyvuk et al., 2010). The ubiquitous AP-1A complex is formed by the two large adaptin subunits γ1 and β1 (also known as AP1G1 and AP1B1, respectively), and the subunits μ1A and σ1A (also known as AP1M1 and AP1S1, respectively). The σ1B and σ1C subunit isoforms (also known as AP1S2 and AP1S3, respectively) show an almost complementary tissue-specific expression pattern, with most tissues expressing σ1A and just one of the other two isoforms (Glyvuk et al., 2010). A ‘knockout’ of the X-chromosomal σ1B adaptin in mice (σ1B−/− mice) causes hypoactivity and defects in learning and memory, and impairs synaptic vesicle recycling in the hippocampus. A reduction in the number of synaptic vesicles is accompanied by the appearance of large organelles, indicating σ1B-dependent recycling of synaptic vesicles through endosomes (Glyvuk et al., 2010).

Here, we report a lipodystrophy in σ1B−/− mice. Recently, adipose-tissue-derived vascular stem cells gained much interest because they are a readily accessible source of endogenous stem cells, which can be used for tissue repair and reconstitution (Zuk, 2010). However, little is known about vesicle trafficking and signal transduction pathways that regulate adipocyte differentiation in vivo (Gesta et al., 2007). Most studies in this area are based on the use of 3T3-L1 pre-adipocyte cells, which can be induced to differentiate into adipocytes in conditioned medium by stimulation with glucocorticoid, cAMP and insulin, just like adipose-tissue-derived vascular stem cells (Cristancho and Lazar, 2011; Rosen and MacDougald, 2006). Expression of the transcription factor peroxisome proliferator-activator receptor γ (PPARγ), the master regulator of adipogenesis, is upregulated during differentiation, but the precise signal transduction pathways controlling this are not known. Regulation of adipogenesis is complex and modulated by multiple pathways. For example, hormonal signalling by Wnt and TGFβ family members either induces or inhibits adipogenesis. Also, the cell contacts to the extracellular matrix are involved in the regulation of adipogenesis. Cells that are firmly attached to the extracellular matrix do not differentiate, whereas loosely attached cells do (Cristancho and Lazar, 2011). The delta-like 1 homologue DLK1 (also known as Pref-1) receptor inhibits adipocyte differentiation, thereby regulating the formation of adipose tissue during development (Moon et al., 2002; Wang et al., 2006).

Here, we have found sortilin expression to be increased specifically in adipose tissue from σ1B−/− mice. In adipocytes, the neotrophin-binding receptor sortilin is a major protein in glucose transporter GLUT4 (also known as SLC2A4) storage vesicles (Lin et al., 1997; Morris et al., 1998). However, we found that GLUT4 expression was not altered and serum glucose homeostasis was normal. This indicates the existence of an adipocyte-specific and GLUT4-vesicle-independent sortilin function. Sortilin binds to soluble ligands and forms heterodimeric complexes with other receptors and pro-hormones with transmembrane domains. Complex formation can promote
antegrade as well as retrograde transport of these receptors and, thus, alter hormone signalling, but complex formation can also alter receptor–ligand specificities and, thus, the activity of signalling pathways (Hermey, 2009; Larsen et al., 2010; Willnow et al., 2008). We found that AP-1-dependent sortilin sorting is mediated by two motifs, one of which mediates sorting by AP-1 complexes containing σ1A or σ1B (AP-1–σ1A or AP-1–σ1B complexes) and the second of which binds specifically to σ1B. Importantly, sortilin is not overexpressed in the brains of σ1B−/− mice, although the brain is the tissue of highest sortilin and σ1B expression. This demonstrates the existence of adipocyte-specific σ1B-dependent sortilin trafficking routes that are involved in the regulation of adipogenesis and tissue mass. Sortilin mRNA levels were shown to be altered during adipogenesis and in obese mice and humans, but a direct sortilin function in the regulation of adipogenesis has not been reported previously (Kaddai et al., 2009; Maeda et al., 2002). Here, we show that sortilin binds to the adipogenesis-limiting receptor DLK1, thereby preventing its downregulation. The overexpression of DLK1 inhibits adipogenesis, causing lipodystrophy. We conclude from our data that pre-adipocytes and adipocytes possess tissue-specific AP-1-dependent sortilin trafficking routes, which differ from those in neurons, and that sortilin transport along these pathways by σ1B is involved in the regulation of adipocyte differentiation and, thus, in the regulation of the formation of adipose tissue during development.

RESULTS
Lipodystrophy in σ1B−/− mice
We observed that σ1B-deficient animals appeared to have a hump, but X-ray analysis revealed no abnormalities of spine morphology. Animals were sacrificed and, after skin removal, a severe lipodystrophy of subcutaneous adipose tissues was observed. The complete loss of inter-scapulae adipose tissue that was observed in some individuals caused the hump-like appearance (Fig. 1A). To quantify the lipodystrophy, we determined the mass of epididymal adipose tissue (a visceral tissue in mice) by gravimetry. Epididymal adipose tissue is the preferred tissue for adipocyte analyses, because it is not attached to neighbouring tissues and can be isolated without tissue loss or contamination. The σ1B−/− animals turned out to have a 50% reduction in the amount of epididymal adipose tissue (Fig. 1B).

Reduced mass of the adipose tissue was also observed in females and so is not sex specific. Moreover, adult σ1B−/− animals (5 months) were 15% lighter than the wild-type controls, indicating that the mass of adipose tissues in general (not just the small epididymal and inter-scapula tissues) is reduced (Fig. 1B). A computed tomography (CT) scan of the entire animal was performed to determine the change in the total volume of adipose tissues (Lubura et al., 2012). The CT scan revealed a ~19% reduction in adipose tissue volume in σ1B−/− animals (Fig. 1B), reflecting that, within the body, the degree of adipose tissue volume in σ1B−/− animals (middle) and their total adipose tissue volume (right) were also reduced (n=9 per genotype). Each clone was tested in duplicate. Data show the mean±s.d. The weight of 5-month-old σ1B−/− animals (middle) and their total adipose tissue volume (right) were also reduced (n=9 per genotype).

Fig. 1. Lipodystrophy and impaired adipogenesis. (A) A litter of two wild-type (wt) and two σ1B−/− animals (left panels) and the subcutaneous tissues of one wild-type and one σ1B−/− mouse (right panels) are shown. σ1B−/− animals appear to have a hump due to the loss of inter-scapula (arrows) and other subcutaneous adipose tissues. (B) Left, a reduction in epididymal adipose tissue mass was observed in σ1B−/− animals (n=6 per genotype). Data show the mean±s.d. The weight of 5-month-old σ1B−/− animals (middle) and their total adipose tissue volume (right) were also reduced (n=9 per genotype). (C) Adipogenesis of primary MEFs was determined by Oil Red staining and quantification (wild-type, n=6; σ1B−/−, n=9). Each clone was tested in duplicate. Data show the mean±s.d (D) Stromal stem cell adipogenesis was investigated. For the wild-type group, a total of 6574 cells were counted (6% adipocytes); for the σ1B−/− group, a total 9007 cells were counted (3.6% adipocytes) (wt n=9, σ1B−/− n=10). For box and whisker plots, the boxes show the mean, 25th and 75th percentiles, and whiskers show xxx.
reduction varies between the different types of adipose tissues. Accordingly, previous reports in other mouse models exhibiting altered adipose tissue mass have described that various adipose tissue types differ in terms of hyperplasia and their contribution to energy storage (Roubtsova et al., 2011).

In the present case, the comparatively low adipose tissue mass of \( \sigma 1B^{-/-} \) animals could result from either a reduced storage of lipids in adipocytes (and consequently smaller cells) or from a reduction in the total number of mature cells. Hyperplasia of adipocytes leads to a high variability of cell sizes within a tissue. To compare the size of large numbers of wild-type and \( \sigma 1B^{-/-} \) cells, we subjected isolated epididymal adipocytes to flow cytometric ‘sideward-scatter’ analysis. As the result showed no difference in size between cells from \( \sigma 1B^{-/-} \) mice and those of the isogenic wild-type controls (supplementary material Fig. S1A), the observed decrease in tissue mass must be caused by a reduction in adipocyte numbers and, presumably, by compromised adipogenesis.

**Adipocyte functions and insulin sensitivity**

In adult animals, adipocyte cell numbers are stable and only 10% of the adipocytes are removed and replaced by differentiation of precursor cells. An increase in adipose tissue mass in adults is caused by increased cell size (adipocyte hyperplasia up to 100 \( \mu \)m in diameter) due to the enlarged lipid droplets (Rigamonti et al., 2011). Therefore, we tested for lipid storage by offering animals a lipid-rich diet ad libitum (21% lipids instead of 5%). Growth rates were determined between 4 and 14 weeks of age. The \( \sigma 1B^{-/-} \) animals gained weight like their isogenic wild-type controls and both cohorts gained weight faster than their respective control groups consuming a diet with the regular lipid content (supplementary material Fig. S1B). At this young age, we did not detect significant differences in body weight between the genotypes, irrespective of the diet. However, this is to be expected, because the contribution of adipose tissues to the body mass is minor in young and growing animals and an extremely large cohort would therefore be required to establish the small differences in body weight caused by a lipodystrophy. From these results, we conclude that \( \sigma 1B^{-/-} \) animals do store lipids and that their adipocytes increase in volume like wild-type cells.

\( \sigma 1B \) is overexpressed in many breast tumors, which suggests that the development of this adipose tissue might depend on \( \sigma 1B \) (Sinclair et al., 2003). The normal breeding success of \( \sigma 1B^{-/-} \) animals demonstrated that mammary gland development is not significantly impaired. Also, the histological analysis of milk ducts in 4-week-old animals did not reveal a delayed development. Thus, \( \sigma 1B \) overexpression in these tumor cell lines might be associated with their hyperproliferation. To confirm normal adipocyte function in \( \sigma 1B^{-/-} \) animals, we performed additional experiments addressing this.

Adipose tissues are involved in the reduction of blood glucose levels and, through the secretion of a number of adipokines, in the regulation of food intake and energy metabolism (Ronti et al., 2006). An important contribution to the clearance of excess blood glucose is the insulin-induced redistribution of the glucose transporter GLUT4 from cytoplasmic storage vesicles to the plasma membrane of adipocytes and myocytes, both of which express \( \sigma 1B \) (Glyvuk et al., 2010; Watson and Pessin, 2006). We performed glucose tolerance tests to analyse this tissue function. Basal glucose concentrations did not differ between wild-type and \( \sigma 1B^{-/-} \) animals, and were in the expected range of 50–70 mg/dl blood in both cohorts. After a 16-h starvation period, 2 g of glucose/kg body mass were injected intraperitoneally. Owing to the intra-peritoneal injection of glucose, the absorption of glucose and the increase in blood glucose concentrations varied. One group showed a 2.5–3-fold increase (n=4 wild-type mice; n=5 \( \sigma 1B^{-/-} \) mice) and the second group showed a 4–5-fold increase (n=3 for both) in blood glucose concentrations (supplementary material Fig. S1C). Irrespective of the maximum serum glucose concentrations achieved upon the injection, the concentrations were reduced to steady-state levels equally fast in \( \sigma 1B^{-/-} \) animals and in their isogenic controls. This demonstrates a comparable insulin-sensitivity and a similar capacity for insulin-dependent regulation of blood glucose. Thus, insulin receptor signalling and GLUT4 recycling appear to be unaffected by the \( \sigma 1B \) deficiency.

We also tested for adipose tissue functions by determining the serum concentrations of the adipokines adiponectin, resistin and leptin and, in addition, of the insulin-like growth factor (IGF-1), which, like insulin, is involved in the regulation of adipocyte differentiation. No differences were detected between wild-type and \( \sigma 1B^{-/-} \) mice, which is in line with findings regarding lipid and glucose consumptions and the general lack of alternative phenotypes in other tissues (supplementary material Fig. S1E). A overall reduction in adipose tissue mass, displaying various degrees of reduction in different adipose tissues, might be caused by enhanced heat production by brown and beige adipocytes or by the alternative use of metabolites (Roubtsova et al., 2011; Schulz et al., 2013). We therefore determined the respiratory coefficient, which correlates physical activity with \( O_2 \) consumption and \( CO_2 \) production, and we found this coefficient to be unaltered in \( \sigma 1B^{-/-} \) animals. This confirmed that the reduction in tissue mass is caused by a defect in adipogenesis. Taken together, it follows from the above findings that the major metabolic functions of adipose tissues are not impaired by the \( \sigma 1B \)-deficiency. Also, our data did not provide any indication of adipose-tissue-type-specific defects, but strongly suggested that the reduction in adipose tissues is due to defects in early stages of adipogenesis.

**Adipocyte differentiation**

As the number but not the functions of mature adipocytes appeared to be affected by \( \sigma 1B \) deficiency, we then tested for reduced adipogenesis. Several model systems have been established to study adipocyte differentiation (Gesta et al., 2007; Rosen and MacDougald, 2006). Primary mouse embryonic fibroblasts (MEFs) can be induced to differentiate into adipocytes by the addition of insulin, dexamethasone and 3-isobutyl-1-methylxanthine to the medium. Efficient differentiation of MEFs into adipocytes, whereas \( \sigma 1B \)-deficient clones (n=6) displayed efficient differentiation of MEFs into adipocytes, whereas \( \sigma 1B^{-/-} \) clones (n=9), by comparison, exhibited a 65% reduction in adipocyte differentiation, as determined by the quantification of Oil Red staining after dye extraction (Fig. 1C). Although MEFs are not regular adipocyte precursors, the reduced differentiation rate is within the range of the reduction in adipocyte tissue mass observed in vivo (Fig. 1B). Next, we isolated vascular stromal stem cells from the epididymal adipose tissue and induced cells to differentiate into adipocytes using the conditioned medium. In the \( \sigma 1B^{-/-} \) stem cell cultures, only 3.6% of the cells (323 out of 9007) had differentiated, which was
considerably less than the 6% (395 out of 6583) of cells in cultures from the isogenic controls (Fig. 1D). Unfortunately, dye quantification was not possible, owing to a high background staining and the relatively low number of differentiated cells obtained from tissue isolates. Notably, the *in vitro* differentiation rate of the wild-type precursor cells is not unlike the adipocyte turnover rate of 10% found *in vivo* (Rigamonti et al., 2011).

σ1B−/− vascular stromal stem cells did not detach from the surface and, thus, did not undergo cell death. They detached from each other and rounded up, excluding the possibility that enhanced surface binding could account for the reduction in differentiation. Based on the data described above, we conclude that inhibition of adipogenesis is the primary cause for the lipodystrophy observed in σ1B−/− animals.

**Sortilin expression and adipogenesis**

GLUT4 expression is strongly upregulated during adipogenesis and although the glucose-tolerance assay demonstrated normal glucose serum homeostasis in σ1B−/− mice, GLUT4 levels might nevertheless be reduced. To test this, we measured GLUT4 expression and, in addition, we measured the expression of the Vps10-domain-containing receptor sortilin, which is a major component of GLUT4 storage vesicles and has been proposed to regulate the biogenesis of these vesicles (Hashiramoto and James, 2000; Lin et al., 1997; Shi and Kandror, 2007). Semi-quantitative western blot analysis demonstrated that GLUT4 expression is normal in σ1B−/− adipocytes (Fig. 2A; supplementary material Fig. S2), but also that sortilin expression is increased 4.5-fold (Fig. 2A). The increase in sortilin could even be observed by indirect immunofluorescence microscopy of adipose tissue cryosections (Fig. 2B). Northern blot analysis did not reveal a change in either sortilin or GLUT4 mRNA levels (supplementary material Fig. S2). Thus, the increased sortilin expression has to be caused by altered sortilin trafficking, delaying its lysosomal degradation.

To examine whether the increased expression of sortilin is sufficient to inhibit adipocyte differentiation, we overexpressed sortilin in the adipocyte precursor model cell line 3T3-L1. Eight independent clones with fivefold to eightfold overexpression of sortilin were induced to differentiate, and the differentiation rates were determined by Oil Red staining. These transfected clones exhibited a 70% reduction in differentiation rate compared with that of mock-transfected cells (Fig. 2C). Thus, sortilin overexpression alone seems sufficient to cause a reduction in adipocyte differentiation. Sortilin expression is very low in 3T3-L1 cells, and it does not change during their adipogenesis (Fig. 2D,E); however, in 3T3-L1 transfectants, the expression of sortilin increased even further over time (>24 h). This indicates that sortilin overexpression is self-sustaining – this presumably involves the saturation of sorting steps leading into pathways that are directly linked to degradation pathways.

The insulin-signalling cascade that induces and sustains adipogenesis involves the Akt kinase (also known as PKB) as an essential component. Activation of Akt involves its phosphorylation at S473 (Watson and Pessin, 2006) and leads to expression of the transcription factor PPARγ (Gesta et al., 2007; Rosen and MacDougald, 2006). We tested for Akt expression as well as for S473 phosphorylation, and found neither to be reduced (Fig. 2D). This again indicates a functional insulin-signalling cascade, which should lead to adipogenesis.

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**Fig. 2.** Sortilin overexpression and adipogenesis. (A) Sortilin and GLUT4 expression in wild-type (wt) and σ1B−/− adipocytes was investigated by western blotting (upper panel) and quantified by normalisation to Hsc70 expression (lower panel). Data show the mean±s.d. (B) Immunofluorescence microscopy was used to examine the expression of sortilin (red) in cryosections of epidymal adipocytes. Nuclei were stained with DAPI (blue). The boxed area in the left image is shown at a higher magnification in the image on the right. Scale bar: 20 μm. (C) Sortilin-overexpressing 3T3-L1 cell lines show reduced adipogenesis, as determined by staining with Oil Red. Data show the mean±s.d. (D) Akt expression and Akt activation (p-Akt) during the differentiation of sortilin-overexpressing (Sort) and control (ø) 3T3-L1 cell lines was examined by western blotting. (E) PPARγ expression during adipogenesis of 3T3-L1 cell lines was investigated by using western blotting.
However, in sortilin-overexpressing cell lines, PPARγ expression was found to be reduced, in line with the reduced differentiation rate (Fig. 2E).

**Sortilin sorting by AP-1**

Next, we tested for specific sortilin binding to σ1B. Sortilin contains canonical YxxØ-based (O is a bulky hydrophobic amino acid) and dileucine-based sorting motifs, which can be bound by μ adaptins and σ adaptins respectively (Fig. 3). The dileucine motifs of sortilin and the mannos 6-phosphate (MPR) receptor MPR300 (also known as IGF2R) have been shown to bind to the monomeric clathrin-adapter protein GGA2 (Nielsen et al., 2001). Like AP-1, GGA2 binds to the TGN and to endosomes, and both are essential for development (Govero et al., 2012; Meyer et al., 2000; Zizioli et al., 2010; Zizioli et al., 1999). Both receptors contain canonical YxxØ-type and dileucine motifs, and a chimera of the MPR luminal domain and the sortilin cytoplasmic tail is able to restore lysosomal enzyme sorting in MPR-deficient cells, indicating that the sortilin and MPR itinerary are largely identical (Nielsen et al., 2001). Whereas μA binds to the YxxØ-type motif, γ1–σ1 adapin hemi-complexes target dileucine-based sorting motifs (Doray et al., 2007; Heldwein et al., 2004; Janvier et al., 2003; Kelly et al., 2008; Ohno et al., 1998; Owen et al., 2001; Traub, 2009). We tested for sortilin binding to μA by using the yeast two-hybrid (Y2H) system, and we tested its binding to σ1A, σ1B and σ1C by using the Y3H system. The test included the full-length sortilin cytoplasmic domain (comprising both the proximal YxxØ and the distal dileucine motif) and a truncated cytoplasmic domain of amino acids 30–53 covering the dileucine motif but not the YxxØ motif (Fig. 3). The cytoplasmic domains of MPR300 and MPR46 (also known as M6PR) were used as controls. As demonstrated (Fig. 3), the sortilin YxxØ motif showed weak binding to μA, compared with the binding of MPR300 to μA, whereas the truncated sortilin cytoplasmic domain lacking the YxxØ motif showed no binding activity. The MPR46 tail displayed auto-activation (Fig. 3A). By contrast, both the full-length and the truncated cytoplasmic domain of sortilin (reflecting the activity of the dileucine motif) interacted with γ1–σ1B, weakly with γ1–σ1A and not with γ1–σ1C (Fig. 3B,E). The sortilin dileucine motif differs from canonical motifs that bind to σ1 or the σ2 and σ3 adaptins of the AP-2 and AP-3 complexes. These carry an E at position −4, whereas the sorting motif is SxxxLL (Doray et al., 2007; Sitaram et al., 2012). Moreover, S47 is part of a canonical casein kinase-2 (CK2) phosphorylation motif, providing a mechanism for regulating σ1B-dependent sorting. Converting S47 into E abolished σ1B binding but did not increase affinities towards σ1A or σ1C (Fig. 3C). Exchanging L51 for A also abolished binding, confirming that σ1B-binding is based on the dileucine motif. However, this mutant did exhibit weak binding to σ1C, indicating that residues at this position mediate σ1C specificity. In the sortilin cytoplasmic domain, the residues between S47 and L51 are all acidic. σ1A prefers E over D at −2 in the dileucine motif and, in another motif, it prefers P or F at −1 (Doray et al., 2007; Poirier et al., 2013; Sitaram et al., 2012). Sortilin carries an E at position −2 and, accordingly, we changed the charge at −3 and −1 and replaced D with R. This reduced σ1B binding only slightly, whereas σ1A binding was slightly increased. This did not affect the specificity towards σ1C (Fig. 3C).

We next tested for AP-1 binding in pull-down experiments on a GST-tagged sortilin cytoplasmic domain (GST–sortilin-CD), using western blotting with an antibody against γ1 adaptin to determine AP-1 precipitation. As can be seen (Fig. 3D), cytosolic AP-1 complexes from wild-type cells bound to the full-length GST–sortilin-CD, but bound only weakly to the truncated cytoplasmic domain lacking the YxxØ motif. The trimeric γ1–β1–σ1 complexes isolated from μ1A−/− cells also bound to both tail constructs (Meyer et al., 2000), and binding to the full-length tail was more stable than binding to the truncated cytoplasmic domain lacking the YxxØ motif. Because no dileucine motif is present within the membrane-proximal 30 amino acids, this part of the sortilin cytoplasmic domain must either contain a novel and as-of-yet unidentified σ1-binding motif or the upstream residues allow for a more stable γ1–σ1B binding. As indicated by the Y3H results, AP-1–σ1A complexes isolated from σ1B−/− cells did not bind to the dileucine motif tail sequence. In summary, these experiments confirm the Y2H and Y3H results.

The S47E mutation did not change the sequence into a general σ1-binding motif, indicating that upstream residues prevent binding to σ1A and σ1C. In order to identify additional residues that contribute to the σ1B specificity, we compared the sequence to that of the Niemann-Pick type C disease protein NPC1, the dileucine motif of which binds to all three σ1 adaptins (Poirier et al., 2013). The comparison showed only four identical residues and demonstrated charge differences at three positions (acidic in sortilin, Fig. 3E). D30 and D33 in sortilin are replaced by N and K in NPC1, and D46 is replaced by a Y. As predicted, D30N and D33K mutations reduced σ1B binding, but increased the affinity towards σ1A and σ1C. A D46T substitution did not reduce σ1B binding, but increased affinities towards σ1A and σ1C (Fig. 3E). It follows that all three residues contribute to σ1B-binding specificity, although by different mechanisms. To test for additive effects, we then combined each of these mutations with the S47E mutation, which abolished σ1B binding without enhancing binding to the others (Fig. 3C). In this combination, S47 turned out to be also essential for σ1A and σ1C binding, whereas σ1B binding was no longer sensitive to S47E (Fig. 3E). The upstream residues appear to determine the orientation of the tail on the γ1–σ1 surface, thereby changing the sequence requirements for binding of the E/SxxLL motif. In summary, we can conclude that the D-x2-D-x12-DS-x3-L motif determines the specificity of σ1B binding.

We used adaptin ‘knockout’ MEF cell lines to test for σ1B- and μA-dependent sortilin transport. In μ1A−/− MEFs, MPRs are redistributed from their preferential TGN localisation to endosomes, due to a block in retrograde transport. MPR expression levels and protein half-lives are not changed (Meyer et al., 2000). Also, sortilin is redistributed to adipocytes as determined by density gradient centrifugations to determine AP-1 precipitation. As can be seen (Fig. 3D), cytosolic AP-1 complexes from wild-type cells bound to the full-length GST–sortilin-CD, but bound only weakly to the truncated cytoplasmic domain lacking the YxxØ motif. The trimeric γ1–β1–σ1 complexes isolated from μ1A−/− cells also bound to both tail constructs (Meyer et al., 2000), and binding to the full-length tail was more stable than binding to the truncated cytoplasmic domain lacking the YxxØ motif. Because no dileucine motif is present within the membrane-proximal 30 amino acids, this part of the sortilin cytoplasmic domain must either contain a novel and as-of-yet unidentified σ1-binding motif or the upstream residues allow for a more stable γ1–σ1B binding. As indicated by the Y3H results, AP-1–σ1A complexes isolated from σ1B−/− cells did not bind to the dileucine motif tail sequence. In summary, these experiments confirm the Y2H and Y3H results.

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Fig. 3. Sortilin binding to μ1A and σ1 adaptins. The binding motifs present in the sortilin cytoplasmic domain that are characterised in A–C are boxed. (A) Y2H assay of the interaction of sortilin with μ1A. MPR served as a control. His, histidine; Ade, adenine; d, days at 30 °C. (B) Y3H assay to investigate the interaction of sortilin with γ1 and σ1 isoforms. FL, full-length sortilin cytoplasmic domain; 30-53, truncated cytoplasmic domain. (C) Characterisation of the dileucine-based σ1B-binding motif. In the wild-type sequence, the residues that are mutated in other constructs are shown in blue. In the mutant sequences, the mutated residue is shown in red. (D) Pulldown experiments were performed to investigate the binding of AP-1 (blots were probed for γ1) from wild-type (ct), μ1A /− and σ1B /− cell lines to sortilin sequences. FT, flow-through; E, eluate. (E) Upper panel, sequence comparison of NPC1 and sortilin tail sequences. The sequences are colour coded for polarity (red, acidic; blue, basic; green, polar). Arrowheads, the essential S47 and L51; arrows, residues in sortilin that are replaced by those from the NPC1 tail; asterisk, conserved residues. Lower panel, the results of the Y3H analysis of the contributions of specific sequences to σ1-specific binding are shown.
Therefore, neither sortilin overexpression nor σ1B deficiency blocks sortilin export from a specific organelle. Instead, the sortilin-dependent inhibition of adipogenesis has to be caused by altered sortilin trafficking kinetics, lowering the fraction of sortilin that reaches the lysosomes. Owing to the large volume of the lipid droplet, the cytoplasmic volume is very small and this cannot be tested directly by video microscopy.

The suggested phenotype is in line with the neurological phenotype of the σ1B−/− mice, which have a reduction in the recycling of synaptic vesicles through endosomes, but not a complete block (Glyvuk et al., 2010). Although brain is the tissue with the highest σ1B and sortilin expression, sortilin expression is not altered in the brains of σ1B−/− mice (supplementary material Fig. S4; Glyvuk et al., 2010; Willnow et al., 2008). In conclusion, our data provide strong evidence for an adipose-tissue-specific σ1B-dependent sortilin trafficking route.

Inhibition of adipogenesis by sortilin

Adipocyte differentiation is regulated during development to limit the adipose tissue mass. The receptor DLK1 has been shown to negatively regulate adipocyte differentiation (Sul, 2009). However, its expression increases initially after the induction of adipogenesis and subsequently decreases to allow adipogenesis to proceed once the precursor cell is committed to become an adipocyte. This DLK1 expression profile during differentiation differs from its expression in other tissues (Sul, 2009; Wang and Sul, 2009). We found DLK1 to be overexpressed in σ1B−/− adipocytes, as well as in the sortilin-overexpressing 3T3-L1 cell lines, whereas its expression is reduced in sortilin−/− adipocytes (Fig. 4A,B; supplementary material Fig. S4B,C). Expression of DLK1 is stable during the first 6 h of adipocyte differentiation and starts to decline after 24 h (Kim et al., 2007). In sortilin-overexpressing cell lines, DLK1 expression follows the same pattern, but its expression level is higher than the highest...
expression level in the control cell lines at any given time-point (Fig. 4A). DLK1 does not contain canonical YxxØ- or dileucine-based sorting motifs, indicating that it might interact with sortilin and be transported in the resulting heterodimeric complex. Such an event might prolong the half-life of a protein (Kim and Hempstead, 2009). Neither DLK1 nor sortilin expression is increased in σ1B<sup>-</sup>-brain, supporting the existence of the suggested tissue specificity of this σ1B-sorting pathway (supplementary material Fig. S4B).

We tested the localisation of sortilin and DLK1 in wild-type and sortilin-overexpressing 3T3-L1 cells by confocal microscopy (Fig. 4C), and we found them to colocalise at the perinuclear TGN, where sortilin predominates. There was also a population of sortilin-containing periplasmic organelles, which did not contain detectable amounts of DLK1 and vice versa. Thus, it appears that sortilin and DLK1 traffic through common compartments and that complex formation could change the DLK1 sorting. The plasma membrane pool of sortilin, which accounts for 10% of the total cellular pool, was not detectable, but the plasma membrane was labelled by the anti-DLK1 antibody in both cell lines. Thus, DLK1 also reaches the cell surface during sortilin overexpression and is able to mediate the inhibition of adipogenesis.

**DLK1–sortilin complex formation**

We tested for the formation of a sortilin–DLK1 complex by using surface plasmon resonance. Ectodomains were expressed and purified from culture medium, and experiments were performed using immobilised sortilin. The sortilin ectodomain forms a ten-bladed β-propeller, which binds to a number of different ligands, including the receptor-associated protein (RAP) and the pro-sequence peptide of sortilin itself, both of which compete with all other known ligands for binding (Munck Petersen et al., 1999; Petersen et al., 1996; Quistgaard et al., 2009). DLK1 binding to sortilin was tested in the absence and presence of these ligands. As demonstrated (Fig. 4D), DLK1 bound to sortilin with a K<sub>d</sub> of ~65 nM (Fig. 4D). Moreover, following saturation of sortilin with RAP or sortilin pro-peptide (5 μM), the binding of DLK1 was completely abolished, signifying the specificity of the reaction and supporting the proposed functions of σ1B and sortilin in controlling adipogenesis.

**DISCUSSION**

We describe a novel molecular mechanism regulating adipocyte precursor cell differentiation. Adipose-derived stem cells are of major interest, because they are a readily accessible source of endogenous stem cells, which can be used for tissue repair and regeneration (Zuk, 2010). The new mechanism involves the AP-1–σ1B complex and the Vps10-domain-containing receptor sortilin.

Among the family of heterotetrameric AP complexes, AP-1 forms the highest number of tissue-specific complexes because, of all adaptins, AP-1 has the highest number of tissue-specific isoforms. Our knowledge about the functions of these complexes and the underlying molecular mechanisms is very limited. Although knockouts of the ubiquitously expressed isoforms are embryonic lethal, knockouts of tissue-specific isoforms are viable, but reveal impaired tissue functions (Glyvuk et al., 2010; Meyer et al., 2000; Takahashi et al., 2011; Zizioli et al., 2010; Zizioli et al., 1999). σ1A is ubiquitously expressed at comparable levels in most tissues, whereas its expression in brain is several-fold higher. The expression of σ1B and σ1C is tissue specific. Most tissues express two σ1 adaptins, σ1A plus σ1B or σ1A plus σ1C (Glyvuk et al., 2010). Here, we describe a defect in adipocyte differentiation due to a tissue-specific function of the AP-1–σ1B complex in the sorting of the Vps10-domain-containing neurotrophin receptor sortilin, leading to an increased sortilin expression in the absence of AP-1–σ1B. Sortilin mRNA is upregulated in mesenchymal stem cells after they are induced to differentiate into osteoblasts instead of adipocytes (Maeda et al., 2002), and sortilin mRNA is downregulated in adipose tissues of obese mice and humans (Kaddai et al., 2009). However, a direct function of sortilin in regulating adipogenesis has not been shown previously. Sortilin expression is increased in σ1B<sup>-</sup>-adipose tissue, whereas its expression in brain, the tissue with highest sortilin and AP-1–σ1B expression, is not altered. Therefore, the sortilin-dependent phenotype of the σ1B<sup>-</sup>-mouse cannot be directly compared to the phenotypes of the sortilin<sup>-</sup>-mouse, which lacks sortilin expression in every tissue. Based on our data, sortilin<sup>-</sup>-mice could be obese, but they are not; they are, in fact, smaller than their wild-type controls. This more general developmental defect might prevent enhanced adipogenesis as well (Jansen et al., 2007), because sortilin is expressed in many tissues and has multiple functions, loss of which contribute to the phenotype of the mouse. The data presented here demonstrate that sortilin is directly involved in the regulation of adipocyte-precursor differentiation and thus in the regulation of the mass of adipose tissue. Mesenchymal stem cells of the various adipose tissues are identical and can fully substitute for one another (Cristancho and Lazar, 2011; Nuttall et al., 2014), which explains the fact that all adipose tissues are affected. Sortilin is overexpressed in σ1B<sup>-</sup>-adipose tissue, it is colocalised with the adipogenesis-inhibiting DLK1 receptor (Wang et al., 2010) and it is able to form a complex with DLK1, thus preventing DLK1 downregulation. The resulting DLK1 overexpression leads to enhanced inhibition of adipogenesis.

We identified the residues within the dileucine-based sortilin sorting motif and the residues located further upstream that mediate its specific binding to the σ1B adaptor. The sortilin cytoplasmic domain contains a membrane-proximal YxxØ and a distal dileucine-based sorting motif. The YxxØ motif does bind to μ1A and sortilin is misplaced in μ1A<sup>-</sup>-cells. The sortilin dileucine motif, previously demonstrated to be bound by GGA2 (Nielsen et al., 2001), also binds specifically to a γ–σ1B hemi-complex. Previously characterised dileucine-based motifs binding to σ1A are of the E/DxxLL type (Doray et al., 2007; Janvier et al., 2003; Sitaram et al., 2012). The sortilin motif consists of SxxLL, and the serine is essential for σ1B binding. This serine is part of a CK2 consensus motif, which would allow the regulation of sortilin sorting by AP-1–σ1B. The ExxLL motif binding site, as determined in σ2, is conserved in σ1A, and comparison of the σ1B and σ1C structures with that of σ1A does not reveal differences that could readily explain this sorting motif specificity (not shown) (Heldwein et al., 2004; Kelly et al., 2008). The sortilin tail and the SxxLL peptide has to bind in a different orientation to that of ExxLL-based motifs, because the contribution of S47 to σ1 binding depends on the upstream residues of the D-x2-D-x12-DS-x3-L motif. The identification of additional σ1-isoform-specific sorting motifs will allow a systematic analysis of the contributions of individual residues to the specificities and further elucidation by co-crystallisation. Based on the present knowledge, σ1 isoforms do not contribute to compartment-specific binding of AP-1, and it is therefore difficult to picture how AP-1–σ1B could mediate an adipocyte-specific
controls were obtained from heterozygous matings and are isogenic with the deficient animals. Mice of >6 months of age were used. Cell lines were transfected with magnetic beads (MARTa, Iba, Göttingen, Germany). All animal experiments were approved and performed according to international guidelines. Murine monoclonal anti-sortilin antibody (for microscopy) and rabbit polyclonal anti-sortilin antibody (for western blotting) have been described previously (Munck Petersen et al., 1999; Nielsen et al., 2007). Antibodies against AKT, phosphorylated AKT and PPARγ were from Cell Signaling Technology (Danvers, MA). Antibody against DLK1 was from Abcam (Cambridge, UK), and that against β-actin was from Sigma (Seelze, Germany). Antibodies against ERK1/2, phosphorylated ERK1/2, γ, 1, α and were from BD (Heidelberg, Germany). The following enzyme-linked immunosorbent assay (ELISA) kits were used; Resistin and IGF-1 Quantikine® (R&D Systems, Wiesbaden-Nordenstadt, Germany), Leptin (Mediagnost, Reutlingen, Germany) and Adiponectin (Invitrogen, Karlsruhe, Germany).

**CT analysis**

*In vivo* microCT was performed using a low-dose in vivo microCT (QuantumFX, Perkin Elmer) with the following parameters: field of view, 73×73 mm²; tube voltage, 50 kVp; tube current, 200 μA; 17-s scanning time per one full rotation utilizing 2000 projection images. Reconstructed volumes used a matrix of 256×256×384 with an isotropic voxel size of 295 μm. Expressed in CT numbers, fat has an average value of about −200 to −50 and muscle about 10 to 4 (Lubura et al., 2012).

**Glucose-tolerance test and lipid-rich diet**

Mice of 12–16 weeks of age were starved for 16 h. Glucose (2 g/kg body weight in physiological NaCl solution adjusted to 150 μl/30 g body weight) was injected intra-peritoneally, and 5-μl blood samples were collected from the tail vein. Blood glucose was determined with a glucometer (Ascencia Contour®, Bayer, Leverkusen, Germany). Starting at postnatal day 30, animals were fed a high-fat diet (D12108 R/M (sniff, Soest, Germany) ad libitum), which contains 21% lipids compared with 5% in the regular diet. Animals were weighed at 7-day intervals.

**Flow cytometric analysis**

Tissues were minced (Kreb-Ringer bicarbonate) and incubated for 30 min at 37°C with 10 mg/ml crude collagenase type II (Sigma), and adipocytes were transferred into PBS to a final density of 1×10⁶ cells/ml. A total of 30,000 cells per animal were analysed using a FACScan flow cytometer (BD, Heidelberg, Germany).

**Cell differentiation**

Adipose tissue was incubated in 0.075% collagenase type II (Sigma) for 45 min at 37°C and neutralised with 10% fetal bovine serum (FBS, PAN, Aidenbach, Ger). Cells were centrifuged at 1200 g for 10 min, and the stromal vascular fraction was filtered through a 100-μm nylon strainer and incubated in DMEM plus 10% FBS. Cells were expanded by serial passaging. The differentiation of murine adipose-tissue-derived stem cells and MEFs (embryonic day 12.5) was induced by treatment with insulin (10 μM), dexamethasone (1 μM), IBMX (0.5 mM) and indomethacin (200 μM) (Sigma). On day 14, cells were counted. The average fraction of adipocytes in wild-type stem cell cultures of one experiment was taken as 100% adipogenesis. The fraction of adipocytes identified in individual wells containing wild-type and knockout stem cells cultivated in parallel are expressed relative to the 100%.

**3T3-L1**

3T3-L1 cells were treated with insulin (25 μM), dexamethasone (1 μM) and IBMX (0.5 mM) (Sigma). After day 2, cells were cultured with only insulin. At day 4, adipocytes were exposed to Oil Red (Sigma). Cells were washed with 60% isopropanol and the dye was measured at 510 nm. Cells were lysed at 4°C in a solution containing 1% Triton X-100, 0.15 mM Tris-HCl, 1 mM EDTA pH 8.0, proteinase inhibitors at 1:100 (PIC, Sigma), 10 mM calyculin A and 1 mM Na₃VO₄ (Sigma). Western blot analysis was performed with

**MATERIALS AND METHODS**

**Mice, cell lines, antibodies and ELISA**

The mouse model used here was as described previously (Glyvak et al., 2010). The animals had a mixed SV129/BL6 background and wild-type pathway. One possibility would, however, be the tissue- and compartment-specific phosphorylation of S47 and AP-1, and the sorting of tissue-specific proteins required for cell-type-specific vesicle–endosome fusion reactions.

How does sortilin missorting due to σ1B deficiency lead to sortilin and DLK1 overexpression? Sortilin does not accumulate in a specific compartment and, thus, only its transport kinetics can be altered, which is in line with the reduced rate (but not complete block) of synaptic vesicle recycling in σ1B−/− synapses. Unfortunately, the volume of the adipocyte cytoplasm is too small to analyse this directly by microscopy. The data demonstrate a tissue- and AP-1-σ1B-specific endosomal pathway for sortilin, which is associated with its endolysosomal degradation. σ1B deficiency leads to sortilin sorting primarily by μ1A binding and sorting by the AP-1-σ1A complex into a recycling pathway associated with slow sortilin turnover (Fig. 5). Sortilin sorting by AP-1-σ1B leads to enhanced sortilin transport into endolysosomal pathways. The way in which AP-1-σ1B-dependent protein sorting differs between adipose tissue and the brain (and the identification of the molecular machineries involved) is under investigation.
crude cell extracts (SuperSignal®, Thermo Scientific, Heidelberg, Germany; Fuji BAS1000, Fuji, Japan).

**Y2H and Y3H analysis**
cDNAs encoding the cytoplasmic domains of MPRs and sortilin were cloned into pGBT9, and that encoding µ1A was cloned into pGADT7 (Clontech, Saint-Germain-en-Laye, France). AH109 transformants were grown at 30°C for 3–6 days. For Y3H, cDNAs encoding the sortilin cytoplasmic domain and σ1 subunits were cloned into multiple cloning site (MCS1) and MCS2 of pBridge, respectively (Clontech). The cDNA encoding γ1 adaptin was cloned into pGADT7. Plasmids were transformed into the yeast tester strains CG1945 and HF7c.

**GST-pulldown experiments**
GST-fusion proteins of sortilin cytoplasmic domains were expressed in *Escherichia coli* BL21(DE3) and bound to glutathione–Sepharose 4B beads (GE Healthcare, Freiburg, Germany). MEFs were lysed (PBS pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 5 mM iodoacetamide) and the lysate was centrifuged at 100,000 g. The resulting supernatant was used as the protein sample. 1.5 mg/ml protein was added to 150 µg of GST-fusion proteins or GST coupled to the beads (100 µl) and incubated for 16 h at 4°C. Beads were washed with 0.1% Triton X-100, and elution was performed with 10 mM reduced glutathione in PBS, pH 8.0.

**Immunofluorescence microscopy**
Cells transfected with sortilin were fixed with 3% paraformaldehyde (PFA) at 18 h post-transfection. Antibodies bound to sortilin and DLK1 were detected with Alexa-Fluor-488- and Alexa-Fluor-568-labelled secondary antibodies (Invitrogen) and visualised by using confocal microscopy (Zeiss LSM 510; Carl Zeiss, Oberkochen, Germany). Adipose tissue was perfused with 3% PFA. Frozen tissues were cut (Leica SM2000 R) and mounted (Dako, Glostrup, Denmark), and images were acquired with a Leica SP2 LSM microscope (Leica, Wetzlar, Germany). Antibodies bound to sortilin were detected with anti-rabbit-IgG–Texas-Red (Invitrogen).

**Surface plason resonance**
Surface plasmon resonance analysis (Biacore 3000, Biacore, S) equipped with CM5 sensor chips activated as described previously (Munck Petersen et al., 1999). Human cDNA encoding the sortilin ectodomain [sequence encoding amino acids M33 to S723 in pcDNA3.1/myc-] was expressed in CHO cells and purified using Talon beads (Invitrogen, OR). The resulting supernatant was used as the protein sample. 1.5 mg/ml protein was added to 150 µg of GST-fusion proteins or GST coupled to the beads (100 µl) and incubated for 16 h at 4°C. Beads were washed with 0.1% Triton X-100, and elution was performed with 10 mM reduced glutathione in PBS, pH 8.0.

**Competing interests**
The authors declare no competing interests.

**Author contributions**
J.B., J.V.L., K.R., C.G. and M.K. performed and analysed experiments; C.M.P. and P.S. designed and analysed the experiments and wrote the manuscript.

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**Supplementary material**
Supplementary material available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.146886/-/DC1

**References**
Kim, T. and Hempstead, B. L. (2009). NRH2 is a trafficking switch to regulate sortilin localization and permit prionneurotrope-induced cell death. EMBO J. 28, 1612-1623.


Supplementary Figures: σ1B-Adaptin sorts Sortilin in Adipose tissue regulating Adipogenesis

Fig. S1 A: Comparison of adipocyte cell sizes analysed by FACS ‘sideward-scatter’. A data set of two animals is shown (3 animals/genotype; 30,000 cells/animal). B: Weight gain after feeding a lipid-rich diet. Due to the differences in weight, these were normalised and changes are expressed as % increase. Starting at postnatal day 30, 4 animals/genotype were fed ssniff D12108 R/M ad lipido, (21% lipids compared to 5%) Control: 8 wt, 6 σ1B -/- . Mice were balanced in 7 day intervals. Lipid-rich: solid lines; regular food: dashed lines; wt black, σ1B -/- red. C: Glucose-tolerance tests after intra-peritoneal injection of glucose solution. Transport of the injected glucose into the blood is variable and wt and σ1B -/- animals were grouped according to the maximal glucose serum levels. Blood glucose concentrations expressed as x-fold changes of normal. wt-black, σ1B -/- red, ±s.d.. Irrespective of the increase in blood glucose, σ1B -/- animals normalised their blood glucose concentration as fast as wt animals. Experimental details are described in the results section describing the analysis of adipocyte functions. D: Serum concentrations of three adipokines and of IGF-1 by ELISA (±s.d.; see Mat&Meth). Serum from 18 animals/genotype were determined in duplicates. Leptin was determined with two different ELISA-kits, but data obtained with both showed the same large variabilities between the individuals.
Fig. S2 Analysis of sortilin and GLUT4 expression in adipose tissue by WB and Northern-blot (NB). WB analysis of GLUT4 expression. Sortilin analysis as well as the diagrams are shown in figure 2 of the main manuscript. Sortilin and GLUT4 mRNA expression analysis by NB. Diagram shows the average and standard-deviation of signals obtained from 3 animals per genotype, ±s.d.. Sortilin expression is comparably low in liver whereas GLUT4 is not expressed and thus liver mRNA served as a negative control. Signals were normalised against GAPDH (glycerinaldehyde-3-phosphate dehydrogenase). The amount of RNA loaded does not alter the quantification.
Fig. S3 Sortilin sorting by AP-1 complexes. Upper left panel: In μ1A -/- MEF sortilin is redistributed from the peri-nuclear TGN to peripheral endosomes, as it has been shown for MPR300. In σ1B -/- MEF sortilin and MPR300 are perinuclear like in controls (ct) (picture axis 100 μm). Bottom panel: Endocytosis of anti-sortilin antibodies by control (ct) and μ1A -/- (AP-1-deficient cells).
Fig. S4  

**A:** Sortilin and GLUT4 distribution in primary adipocyte cultures ± insulin addition (10 nM, 30 min). Cellulare sub-fractions were prepared by sucrose-density centrifugations as described (Carvalho E. et al. J Biol Chem 2004, 279 (20), 21598-605). One experiment is shown as an example. Quantification of WBs revealed a 50-100% increase of GLUT4 in the PM fraction upon insulin addition and an increase in sortilin of up to 50%. Sortilin and GLUT4 content of the high-density-membrane (HDM) fraction increase upon insulin addition as it is described for 3T3-L1 and rat adipocyte cultures (reviewed in Holman, G.D. & Sandoval, I. Moving the insulin-regulated glucose-transporter GLUT4 in and out of storage. Trends in Cell Biology 2001 Vol. 11, No. 4. 173-179). We did not detect differences between the genotypes.

The small pool of GLUT4 in the PM fraction of primary adipocytes is hard to detect and in many publications more protein of the PM fraction than of HDM or low-density-membrane (LDM) are added. We loaded the same gradient volume aliquots of each fraction to determine the distribution of sortilin and GLUT4, instead of normalising the load according to the protein content of each fraction and to load different amounts of membrane. That the PM fraction of GLUT4 is not reduced is demonstrated by the glucose tolerance experiments of the animals (Fig. S1C). The in-vivo data thus confirm the in-vitro data. σ1B-deficiency does not lead to a complete block of sortilin transport out of a specific sub-cellular compartment, but that the kinetics of its itinerary are altered. This is in line with the defect in synaptic vesicle recycling of the animals, which is also impaired and not completely abolished.

**B:** Sortilin and DLK1 expression in brain as determined by semi-quantitative WB from 4 animals per genotype (±s.d., representative WB).

**C:** Representative WB showing the reduced DLK1 expression in epididymal adipose tissue of sortilin -/- mice. The quantification of these experiments is shown in figure 4B.