Prosaposin facilitates sortilin-independent lysosomal trafficking of progranulin

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Mutations in the progranulin (PGRN) gene have been linked to two distinct neurodegenerative diseases, frontotemporal lobar degeneration (FTLD) and neuronal ceroid lipofuscinosis (NCL). Accumulating evidence suggests a critical role of PGRN in lysosomes. However, how PGRN is trafficked to lysosomes is still not clear. Here we report a novel pathway for lysosomal delivery of PGRN. We found that prosaposin (PSAP) interacts with PGRN and facilitates its lysosomal targeting in both biosynthetic and endocytic pathways via the cation-independent mannose 6-phosphate receptor and low density lipoprotein receptor-related protein 1. PSAP deficiency in mice leads to severe PGRN trafficking defects and a drastic increase in serum PGRN levels. We further showed that this PSAP pathway is independent of, but complementary to, the previously identified PGRN lysosomal trafficking mediated by sortilin. Collectively, our results provide new understanding on PGRN trafficking and shed light on the molecular mechanisms behind FTLD and NCL caused by PGRN mutations.

Introduction

Proper lysosomal function is essential for cellular health and long-term neuronal survival (Nixon and Cataldo, 2006; Lee and Gao, 2008). Defects in lysosomal function cause a group of metabolic diseases known as lysosomal storage disorders, which are characterized by the accumulation of lysosomal cargoes and autophagic vacuoles and are often accompanied by severe neurodegenerative phenotypes (Bellettato and Scarpa, 2010). More recently, lysosomal dysfunction has also been implicated in adult-onset neurodegenerative diseases, including Alzheimer’s, Parkinson’s, and amyotrophic lateral sclerosis (Nixon et al., 2008; Cook et al., 2012). Thus lysosomal dysfunction is closely linked to neurodegeneration.

Progranulin (PGRN) is an evolutionarily conserved, secreted glycoprotein of 7.5 granulin repeats that was recently implicated in several neurodegenerative diseases (Ahmed et al., 2007; Cenik et al., 2012). PGRN haploinsufficiency resulting from heterozygous mutations in the Granulin (GRN) gene is one of the major causes of frontotemporal lobar degeneration (FTLD) with ubiquitin-positive inclusions (FTLD-U; Baker et al., 2006; Cruts et al., 2006; Gass et al., 2006). It is believed that the neurotrophic and anti-inflammatory functions of PGRN and granulin peptides prevent neurodegeneration in the aging brain (Ahmed et al., 2007; Cenik et al., 2012). However, several recent studies have suggested a surprising role of PGRN in lysosomes (Ahmed et al., 2010; Belcastro et al., 2011; Smith et al., 2012; Götzl et al., 2014). First, homozygous PGRN mutant human patients exhibit neuronal ceroid lipofuscinosis (NCL; Smith et al., 2012), a lysosomal storage disease characterized by the accumulation of autofluorescent storage material (lipofuscin, which is also seen in PGRN knockout mice (Ahmed et al., 2010). Second, PGRN is transcriptionally coregulated with several essential lysosomal genes (Belcastro et al., 2011). Finally, FTLD-U/GRN patients also exhibit typical pathological features of NCL patients (Götzl et al., 2014), suggesting FTLD and NCL caused by GRN mutations are pathologically linked and lysosomal dysfunction caused by PGRN mutations might serve as the common mechanism. Thus PGRN actions in lysosomes are essential for neuronal health. However, how PGRN gets delivered into lysosomes and its functions therein remain to be elucidated.

Previously, we have shown that sortilin, a member of the Vps10 family, mediates the delivery of PGRN into lysosomes (Hu et al., 2010). However, PGRN is still partially localized to lysosomes in sortilin-deficient neurons, suggesting an additional sortilin-independent mechanism for PGRN lysosomal trafficking. To understand these additional pathways and PGRN action in lysosomes, we performed a proteomic screen and

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uncovered a novel interaction between PGRN and prosaposin (PSAP), the precursor of saposin peptides (A, B, C, and D) that serve as activators of lysosomal sphingolipid metabolizing enzymes (O’Brien and Kishimoto, 1991; Qi and Grabowski, 2001; Matsuda et al., 2007). We demonstrate that PSAP helps target PGRN into lysosomes in both biosynthetic and endocytic pathways independent of sortilin, via the cation-independent mannose 6-phosphate receptor (M6PR) and low density lipoprotein receptor-related protein 1 (LRP1).

Results

PGRN physically interacts with PSAP

To understand PGRN regulation and function, we searched for PGRN interactors using a stable isotope labeling by amino acids in cell culture (SILAC) based proteomic screen (Fig. 1 A). In addition to several members of the protein disulfide isomerase (PDI) family, which have been shown to regulate PGRN folding and secretion (Almeida et al., 2011), we identified PSAP as the top hit from the screen (Fig. 1 B and Table S1). PSAP is a secreted glycoprotein and the precursor of saposin peptides that serve as activators of sphingolipid degradation in lysosomes (Matsuda et al., 2007). The PGRN–PSAP interaction was further verified by coimmunoprecipitation with overexpressed PGRN and PSAP in HEK293T cells (Fig. 2 A) and endogenous levels of PGRN and PSAP both in the medium and cell lysates of mouse fibroblasts (Fig. 2, B and C). Both PSAP and saposin peptides are detectable in the fibroblast lysates, but only full-length PSAP is present in the PGRN immunoprecipitate, suggesting saposins do not bind to PGRN under this immunoprecipitation condition (Fig. 2 B). Purified recombinant PGRN and PSAP (Fig. S1) strongly interact with each other at a stoichiometric ratio of 1:1 (Fig. 2 D), indicating a direct physical interaction between PGRN and PSAP.

PSAP facilitates lysosomal delivery of PGRN in the biosynthetic pathway

Because both PGRN and PSAP are lysosomal proteins, we hypothesized that they might regulate each other’s lysosomal trafficking. To test this, we evaluated the effect of PGRN or PSAP ablation on the other’s localization to lysosomes. In the wild-type fibroblast, PGRN and PSAP are colocalized within late endosomes/lysosomes, which are labeled by the lysosomal membrane protein LAMP1 (Fig. 3 A). Loss of PGRN has no obvious effect on PSAP lysosomal localization (Fig. S2). However, PSAP deficiency results in complete disruption of PGRN lysosomal localization in fibroblasts (Fig. 3, A and B). Loss of lysosomal PGRN signal is accompanied by increased secretion of PGRN to the extracellular space (Fig. 3, C and D) and increased colocalization of PGRN with the ER marker PDI (Fig. 3 E). To rule out a general defect of lysosomal targeting in PSAP−/− fibroblasts, we examined the localization of two other lysosomal proteins, cathepsin D and TMEM106B (Chen-Plotkin et al., 2012; Lang et al., 2012; Brady et al., 2013). Both cathepsin D and TMEM106B are lysosomally localized in PSAP−/− fibroblasts (Fig. 4, A and B), confirming that PSAP deficiency does not cause a general defect in lysosomal trafficking. Mislocalization of PGRN is also observed in PSAP-deficient fibroblasts generated by the constitutive expression of shRNAs targeted to PSAP, as well as by clustered regularly interspaced short palindromic repeats (CRISPR)–mediated genome editing (Fig. S3 A; Cong et al., 2013; Mali et al., 2013). To further demonstrate that this defect is a result of direct loss of PSAP function, we infected PSAP−/− fibroblasts with lentivirus expressing mCherry-tagged PSAP. Lysosomal localization of PGRN is fully restored upon the expression of mCherry-PSAP in PSAP−/− fibroblast (Fig. S3 B). However, addition of recombinant PSAP protein to the medium to deliver PSAP through the endocytic pathway failed to rescue endogenous PGRN trafficking defects in PSAP−/− fibroblasts (Fig. S3 C), supporting that PSAP is required for the lysosomal trafficking of PGRN in the biosynthetic pathway in fibroblasts.

PSAP facilitates lysosomal delivery of PGRN from the extracellular space

Receptor-mediated endocytosis from the extracellular space is another mechanism to deliver proteins to lysosomes. Because both PGRN and PSAP are secreted, we examined whether PSAP could also facilitate lysosomal delivery of PGRN from the extracellular space. Recombinant human PSAP and PGRN were purified from transfected HEK293T cells and added alone or in
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Figure 2. Physical interaction between PGRN and PSAP. (A) PGRN and PSAP interact when overexpressed in HEK293T cells. PSAP-V5 and FLAG-PGRN constructs were transfected into HEK293T cells as indicated. Lysates were prepared 2 d later and immunoprecipitated with anti-FLAG antibodies. (B and C) PSAP and PGRN interact with each other at endogenous levels in fibroblasts. Cell lysates (B) and conditioned medium (C) from wild-type (WT), PSAP\(^{-/-}\) (PS\(^{-/-}\)), and PGRN\(^{-/-}\) (GRN\(^{-/-}\)) fibroblasts were immunoprecipitated using rabbit anti–mouse PGRN antibodies. The presence of PGRN and PSAP in the immunoprecipitation was detected using sheep anti–mouse PGRN and rabbit anti–mouse PSAP antibodies, respectively. Asterisk indicates IgG bands. (D) Direct interaction between PGRN and PSAP. Purified recombinant his-PSAP and Flag-PGRN proteins of indicated amounts were mixed in 100 µl PBS + 0.2% Triton X-100 for 1 h before adding anti-FLAG beads. Beads were washed after a 2 h incubation. 0.2 µg of each purified protein was loaded as input. After SDS-PAGE, the gel was stained with Krypton dye to visualize proteins. The binding ratio of PGRN–PSAP was provided based on the densitometric evaluation of PGRN and PSAP band intensities. All the results shown are the representative pictures from at least three independent experiments.

Figure 3. PSAP is required for PGRN lysosomal targeting in fibroblasts. (A) Mislocalization of PGRN in PSAP\(^{-/-}\) fibroblasts. Immunostaining for PGRN, LAMP1, and PSAP in fibroblasts derived from wild-type and PSAP\(^{-/-}\) mice using sheep anti–mouse PGRN, rat anti–mouse LAMP1, and rabbit anti–mouse PSAP antibodies. PGRN mislocalization was observed in all the PSAP\(^{-/-}\) fibroblasts examined. (B) Quantification of PGRN localization within LAMP1-positive vesicles in wild-type and PSAP\(^{-/-}\) fibroblasts using ImageJ. Data are presented as mean ± SEM from three independent experiments. ***, P < 0.001, Student’s t test. (C) Increased PGRN secretion in PSAP\(^{-/-}\) fibroblasts. Fibroblasts were cultured in serum-free medium for 48 h before the lysates and conditioned media (CM) were harvested. Proteins from the conditioned media were precipitated with TCA. (D) Quantification of experiments in C. PGRN levels are normalized to transferrin in the conditioned media and Gapdh in the cell lysates. Data are presented as mean ± SEM from four independent experiments. *, P < 0.05, Student’s t test; N.S., no significance. (E) Immunostaining for PGRN, PDI, and cathepsin D in fibroblasts derived from wild-type and PSAP\(^{-/-}\) mice. Representative images from three replicated experiments are shown. Bars: (main) 20 µm; (inset) 5 µm.
combination to fibroblasts isolated from PGRN−/− mice. Because the recombinant PGRN used in these experiments has a 6x histidine tag at its C terminus and the PGRN C terminus is required for its binding to sortilin (Zheng et al., 2011), these recombinant PGRN proteins are not capable of sortilin binding. After 12 h of incubation, purified PSAP alone is delivered to lysosomes labeled by the lysosomal marker LAMP1, whereas purified PGRN alone is not endocytosed (Fig. 5 A). However, the addition of PSAP with PGRN leads to lysosomal accumulation of both molecules (Fig. 5 A). Similar results were obtained with primary cortical neurons (Fig. S4). Western blot analysis confirmed the immunostaining results and showed that PGRN is only present in the cell lysate in the presence of PSAP (Fig. 5 B). Moreover, endocytosed PSAP is processed into saposin peptides, indicating lysosomal delivery of PSAP (Fig. 5 B). Consistent with this active endocytosis, a significant decrease in the extracellular levels of recombinant PGRN and PSAP proteins was observed (Fig. 5, B and C). Collectively, these experiments clearly demonstrate that PSAP facilitates lysosomal delivery of PGRN from the extracellular space in both fibroblasts and neurons.

**PSAP and sortilin are two independent pathways for PGRN lysosomal trafficking**

Previously, we showed that a transmembrane protein of the VPS10 family, sortilin, interacts with PGRN and mediates its lysosomal delivery (Hu et al., 2010). We found that sortilin protein is barely detectable in fibroblasts compared with the neuronal cell line N2a (Fig. 6 A). Consistent with this expression pattern, sortilin is dispensable for PGRN lysosomal trafficking in fibroblasts because PGRN remains localized to lysosomes in sortilin-deficient fibroblasts (Fig. 6 B). Moreover, PSAP-mediated lysosomal delivery of PGRN from the extracellular space is not affected by sortilin deletion in fibroblasts and neurons (Fig. S5). However, ectopic expression of GFP-tagged sortilin in PSAP-deficient fibroblasts fully restored PGRN lysosomal localization (Fig. 6 C). These results demonstrate that sortilin and PSAP are two independent and complementary pathways for PGRN lysosomal trafficking.

**Figure 4.** PSAP is not required for lysosomal localization of cathepsin D and TMEM106B in fibroblasts. [A] Immunostaining for PGRN, LAMP1, and cathepsin D in fibroblasts derived from wild-type and PSAP−/− mice. (B) Immunostaining for PGRN, LAMP1, and TMEM106B in fibroblasts derived from wild-type and PSAP−/− mice. Representative images from three replicated experiments are shown. Bars: (main) 20 µm; (inset) 5 µm.
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and LRP1 are critical for PSAP-mediated PGRN lysosomal delivery from the extracellular space.

**PSAP regulates PGRN trafficking in vivo**

Lysosomal PGRN and PSAP localization is prominent in neurons in the mouse brain, as shown by colocalization with LAMP1 and cathepsin D (Fig. 9, A and B). To determine the in vivo role of PSAP in PGRN lysosomal localization, we examined PGRN localization in wild-type and PSAP<sup>−/−</sup> mouse brains. Consistent with a previous study, massively enlarged lysosomes were detected in PSAP<sup>−/−</sup> neurons because of defects in sphingolipid degradation (Fujita et al., 1996). In most cases, the entire PSAP<sup>−/−</sup> neuron is packed with enlarged lysosomes (Fig. 9 C). More PGRN signals outside of the LAMP1-positive lumen were observed in PSAP<sup>−/−</sup> neurons compared with wild type (Fig. 9, C and D), although a significant amount of PGRN remains localized in the lysosomal lumen as small puncta in the PSAP<sup>−/−</sup> neurons, probably because of the high level of neuronal sortilin expression (Fig. 9 B). In contrast, another lysosomal resident protein, cathepsin D, remains in lysosomes labeled by LAMP1 in PSAP<sup>−/−</sup> neurons (Fig. 9 C), suggesting PSAP is specifically required for PGRN lysosomal targeting in neurons. Consistent with the critical role of PSAP in PGRN lysosomal targeting, serum PGRN levels were increased approximately twofold in PSAP<sup>+/−</sup> mice and approximately fivefold in PSAP<sup>−/−</sup> mice (Fig. 9 E). These results strongly support that PSAP plays a critical role in regulating PGRN trafficking in vivo.

**Discussion**

In this study, we demonstrate a novel interaction between PSAP and PGRN that facilitates lysosomal trafficking of PGRN. We show that this pathway is independent from the previously identified sortilin-mediated lysosomal trafficking of PGRN (Fig. 10). The PSAP and sortilin-dependent PGRN lysosomal targeting mechanisms seem to compensate for each other. In fibroblasts, which express very low levels of sortilin, the PSAP pathway appears essential for PGRN lysosomal targeting, but ectopic expression of sortilin is capable of rescuing PGRN localization in the absence of PSAP. In adult cortical neurons, both PSAP and sortilin are highly expressed (Fig. 9 B) and are
Figure 6. Sortilin and PSAP comprise two independent and complementary pathways for PGRN lysosomal targeting. (A) Immunoblot for sortilin with lysates prepared from fibroblasts and N2a cells. Gapdh was used as a loading control. (B) Immunostaining for PGRN, LAMP1, and PSAP in fibroblasts derived from wild-type and Sort1−/− mice. (C) Ectopic expression of sortilin in PSAP−/− fibroblasts rescues the PGRN trafficking defect. PSAP−/− fibroblasts were transfected with GFP-sortilin. 48 h after transfection, the cells were fixed and stained with sheep anti–mouse PGRN and rabbit anti–Cathepsin D. Representative images from three replicated experiments are shown. Bars: (main) 20 µm; (inset) 5 µm.

Figure 7. PSAP interacts with M6PR. (A) Volcano plot of the SILAC experiment using recombinant Flag-PSAP protein to identify interactors from cell lysates prepared from fibroblasts grown in SILAC medium. The top hits are highlighted. (B) PSAP interacts with M6PR. Lysates prepared from PSAP−/− fibroblasts were incubated with anti-FLAG antibody-conjugated beads with Flag-PSAP or beads only. After washing, the immunoprecipitation products were analyzed by Western blot using anti-M6PR and anti-Flag antibodies. (C) PSAP bridges the binding between PGRN and M6PR. Lysates prepared from PSAP−/− fibroblasts were incubated with anti-FLAG antibody-conjugated beads with purified FLAG-PGRN or FLAG-PGRN+PSAP or beads only. After washing, the immunoprecipitation products were analyzed by Western blot using anti-M6PR, anti-PSAP, and anti-Flag antibodies. Representative images from three replicated experiments are shown.
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Prosaposin facilitates lysosomal delivery of progranulin (PGRN) through the endolysosomal pathway, which is critical for PGRN trafficking. This is demonstrated by the mislocalization of PGRN in M6PR-deficient fibroblasts (Fig. 8A), where PGRN and PSAP are mislocalized. Quantification of PSAP (Fig. 8B) and PGRN (Fig. 8C) localization within LAMP1-positive vesicles in control and M6PR−/− fibroblasts shows a significant reduction in PGRN–PSAP endocytosis (Fig. 8D and E) mediated by M6PR. Similarly, LRP1 is also critical for PGRN–PSAP uptake in fibroblasts (Fig. 8F and G).

Several receptors have been shown to mediate PSAP trafficking, including the mannose-6-phosphate receptor (Vielhaber et al., 1996; Qian et al., 2008), sortilin (Hu et al., 2010), and LRP1 (Hiesberger et al., 1998). Among these, both M6PR and LRP1 have been reported to mediate PSAP endocytosis in fibroblasts specifically. Deletion of either M6PR or LRP1 causes significant reduction in PGRN–PSAP endocytosis (Fig. 8), supporting the finding that PSAP gets a "piggyback ride" from PSAP mainly through M6PR and LRP1 in fibroblasts. Results from our SILAC screen (Fig. 7A) and coimmunoprecipitation (Fig. 7B and C) clearly demonstrated a direct interaction between PSAP and M6PR. However, we failed to detect an interaction between PSAP and LRP1 in the SILAC screen (Table S2) and in the coimmunoprecipitation experiment (unpublished data). How LRP1 regulates PSAP trafficking remains to be determined. One possibility could be that the interaction between PSAP and LRP1 is very weak and transient. Alternatively, LRP1 might indirectly regulate PSAP trafficking. Nevertheless, consistent with our findings, a recent study has demonstrated an important role of LRP1 in the M6PR-independent lysosomal delivery of multiple lysosomal luminal proteins from the extracellular space (Markmann et al., 2015).

Processing of PSAP in the endolysosomal compartments generates four saposin peptides (A, B, C, and D) that play critical roles in lysosomal sphingolipid degradation. PSAP
and saposin deficiency has been linked to many types of lysosomal storage disorders, including Gaucher disease caused by saposin C deficiency, Krabbe disease caused by saposin A deficiency, and metachromatic leukodystrophy caused by saposin B deficiency (Matsuda et al., 2007). The current study focuses on the role of PSAP in PGRN trafficking. Conversely, PGRN might regulate PSAP processing or saposin function in lysosomes. It is also puzzling that PGRN accumulates in the ER in PSAP−/− fibroblasts. One explanation for the ER localization of PGRN in PSAP−/− cells is that PSAP might facilitate the trafficking of PGRN from the ER to the Golgi. However, the increased secretion of PGRN in PSAP−/− cells fails to support this possibility, although it remains to be determined whether PSAP affects the kinetics of PGRN transport from the ER to the Golgi. Additionally, it is known that both PGRN and PSAP are secreted and have neurotrophic functions (Ahmed et al., 2007; Meyer et al., 2014). Their comparable levels secreted in human serum (Ghidoni et al., 2008; Finch et al., 2009; Koochekpour et al., 2012) and the 1:1 stoichiometric interaction between PGRN and PSAP (Fig. 2 D) support the idea that these two proteins might form a complex extracellularly to promote cell survival.

How PGRN functions in lysosomes is another mystery. One hypothesis is that PGRN is processed into granulin peptides in lysosomes (Cenik et al., 2012). Future proteomic studies to identify PGRN/GRN binding partners in lysosomes will help us understand their critical roles in regulating lysosomal activities. Recently, PGRN has also been shown to regulate the activity of TFEB through mTORC1 signaling in microglia after injury

Figure 9. PSAP regulates PGRN trafficking in vivo. (A) PGRN and PSAP colocalize with LAMP1 in cortical neurons of the adult mouse brain. (B) Both sortilin and PSAP are expressed in cortical neurons of the adult mouse brain. (C) Enlarged lysosomes and PGRN mislocalization in cortical neurons of PSAP−/− mice. PSAP−/− and littermate wild-type mice were sacrificed at postnatal day 21 and 15-μm brain sections were stained as indicated. Bars: (main) 20 μm; (inset) 5 μm. (D) Quantification of PGRN signals inside LAMP1-positive vesicles in C using ImageJ. Data are given as mean ± SEM from three pairs of mice: *, P < 0.05, Student’s t test. (E) Serum PGRN levels in newborn wild-type, PSAP+/−, and PSAP−/− litterate mice before the appearance of any apparent lysosomal phenotype in PSAP−/− mice. Data are given as mean ± SEM from four groups of mice. **, P < 0.01; ***, P < 0.001, one-way analysis of variance.

Figure 10. A model for PGRN lysosomal targeting. PSAP and Sortilin are two independent and complementary pathways for PGRN lysosomal targeting in both biosynthetic and endocytic pathways. M6PR and LRP1 are required for PSAP-mediated PGRN lysosomal trafficking.
Endolysosomal dysfunction has been implicated in many neurodegenerative diseases, including FTLD-U. Correspondingly, VCP/p97 and CHMP2B, two FTLD-U–associated genes, are involved in membrane trafficking and autophagy (Lee and Gao, 2008; Rusten and Simonsen, 2008; Ju et al., 2009). The FTLD-U risk factor TMEM106B regulates lysosomal morphology and function (Brady et al., 2013). The newly identified mutated gene in FTLD-U/ALS, C9Orf72, might also be involved in membrane trafficking (Farg et al., 2014). Furthermore, accumulating evidence suggests a critical role of PGRN in lysosomes. The fact that total loss of PGRN results in NCL and haploinsufficiency of PGRN leads to FTLD-U indicates that these two diseases are linked to each other and lysosomal dysfunction might serve as a common disease mechanism. In line with this, a recent study has found that NCL patients develop pathological TDP-43 phosphorylation, which is typically seen in FTLD patients, and FTLD-U/GRN patients have NCL-like pathology (Götzl et al., 2014). Future studies on PGRN–PSAP interactions and function of PGRN/granulin peptides in lysosomes will reveal novel insights into the cellular processes that might be affected in FTLD-U, NCL, and possibly other neurodegenerative diseases, including Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis.

Materials and methods

DNA and plasmids

Human PSAP cDNA in the pDONR223 vector was a gift from H. Yu (Cornell University, Ithaca, NY). His- and FLAG-tagged PSAP construct was obtained by cloning PSAP into pSectag2B vector (Invitrogen). PSAP-V5 construct was obtained through a gateway reaction (Invitrogen). His- and FLAG-tagged PSAP constructs were previously described (Fujita et al., 1996). His- and FLAG-tagged PSAP constructs were previously described (Fujita et al., 1996).

C57/B16 and PGRN−/− (Yin et al., 2010) mice were obtained from the Jackson Laboratory and housed in the Weill Hall animal facility. Sortilin knockout mice (Nykjaer et al., 2004) were a gift from S. Strittmatter (Yale University, New Haven, CT) and A. Nykjaer (Aarhus University, Aarhus, Denmark). PAP−/− mice were previously described (Fujita et al., 1996).

Cell culture, DNA transfection, and virus infection

HEK293T, T98G, and BV2 cells were maintained in DMEM (Cellgro) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Invitrogen) in a humidified incubator at 37°C and 5% CO2. Cells were transiently transfected with polyethyleneimine as described previously (Vancha et al., 2004). FH14 cells stably expressing FLAG-human-PGRN-6Xhis were a gift from A. Ding (Weill Medical College, New York, NY). FH14 cells were grown in DMEM + 10% FBS + 100 μg/ml hygromycin.

Retroviruses were generated by transfecting pSuper-mPSAP shRNA or control shRNA construct together with pCMV-VSVG in Phoenix 293T cells.

Primary cortical neurons were isolated from P0–P1 pups using a modified protocol (Beaudoin et al., 2012). Cortices were rapidly dissected from the brain in 2 ml HBSS supplemented with B27 (Invitrogen) and 0.5 mM l-glutamine (Invitrogen) at 4°C. Meninges and excess white matter were removed before digestion with papain (2 mg/ml in HBSS; Worthington) and DNase1 (1 mg/ml in HBSS; Sigma-Aldrich) for 12 min at 37°C. Tissues were then dissociated using fire-polished glass pipettes. Cells were spun down and resuspended in Neurobasal medium (Invitrogen) plus B27 and plated onto poly-lysine (Sigma-Aldrich)–coated dishes.

Primary mouse fibroblasts were isolated from wild-type, PGRN−/−, Sort1−/−, and PSAP−/− mouse newborn pups and grown in DMEM plus 10% FBS.

Protein purification

GST-PSAP proteins were purified using Gst beads from Origami B(DE3) bacterial strains (EMD Millipore) after 0.1 mM IPTG induction overnight at 20°C. The proteins were eluted with glutathione.
FLAG-PGRN-his protein was purified from the serum-free culture medium of FH14 cells (HEK293T cells stably expressing FLAG-human-PGRN-6Xhis) using cobalt beads. Protein was eluted with imidazole. His-tagged human PSAP was purified from the culture medium (serum free) of HEK293T cells transfected with his-PSAP construct using cobalt beads. All purified proteins were further concentrated and changed to PBS buffer using the Centricon device (EMD Millipore).

**SILAC and mass spectrometry analysis**

T98G cells and immortalized fibroblasts were grown a minimum of five generations in DMEM with 10% dialyzed FBS (Sigma-Aldrich) supplemented with either light (C12, N14 arginine and lysine) or heavy (C13, N15 arginine and lysine) amino acids. To search for PGRN interactors, the heavy T98G cells were transfected in two 15-cm dishes with GFP-PGRN expression constructs, whereas the light T98G cells were transfected with pEGFP-C1 as a control. 2 d after transfection, cells were lysed in 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, and 0.1% deoxycholic acid with protease inhibitors (Roche). The lysates were subjected to anti-GFP immunoprecipitation using GFP-Trap beads (ChromoTek). The presence of GFP and GFP-PGRN in immunoprecipitated samples was confirmed by SDS-PAGE and silver staining. Samples were then mixed and boiled 5 min with 1% DTT followed by alkylation by treating samples with a final concentration of 28 mM iodoacetamide. Proteins were precipitated on ice for 30 min with a mixture of 50% acetone/49.9% ethanol/0.1% acetic acid. Protein was pelletted and washed with this buffer, reprecipitated on ice, and dissolved in 8 M urea/50 mM Tris (pH 8.0) followed by dilution with three volumes of 50 mM Tris (pH 8.0)/150 mM NaCl. Proteins were digested overnight at 37°C with 1 µg of mass spectrometry grade Trypsin (Promega). The resulting peptide samples were cleaned up for mass spectrometry by treatment with 10% formic acid and 10% trifluoroacetic acid and washed twice with 0.1% acetic acid on precolumn equilibrated Sep-Pak C18 cartridges (Waters). Samples were eluted with 80% acetonitrile/0.1% acetic acid into silanized vials (National Scientific) and evaporated using a SpeedVac. Samples were redissolved in H2O with ~1% formic acid and 70% acetonitrile. Peptides were separated using hydrophilic interaction liquid chromatography on an Ultimate 300 LC (Dionex). Each fraction was evaporated with a SpeedVac and resuspended in 0.1% trifluoroacetic acid with 0.1% DTT.

Immunoprecipitation and protein analysis

Cells were lysed in 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, and 0.1% deoxycholic acid with protease inhibitors (Roche). The lysates were subject to anti-FLAG immunoprecipitation using anti-FLAG antibody–conjugated beads (Sigma-Aldrich) or incubated with anti-PGRN antibodies followed by protein G beads (Genscript). Beads were washed with 50 mM Tris, pH 8.0, 150 mM NaCl, and 1% Triton X-100 after 4 h of incubation. Western blot was visualized using the Licor-Odyssey system as described previously (Brady et al., 2013).

To determine the direct interaction between purified PGRN and PSAP, proteins were mixed in PBS + 0.2% Triton X-100 for 1 h before incubating with anti-FLAG antibody–conjugated beads for another 2 h. Beads were washed three times with PBS + 0.2% Triton X-100. Samples were run on SDS-PAGE and the gel is stained with Krypton dye (Thermo Fisher Scientific) and scanned using the Licor-Odyssey system. The PGRN and PSAP levels in the medium were analyzed after trichloroacetic acid precipitation as described previously (Brady et al., 2013). For brain staining, mice were perfused and fixed with 4% paraformaldehyde. 10-µm-thick brain sections were cut with a cryotome. Tissue sections were blocked and permeabilized with 0.05% saponin in Odyssey blocking buffer before incubating with primary antibodies overnight.

**Immunofluorescence staining**

Cells were fixed, permeabilized with 0.05% saponin, and visualized using immunofluorescence microscopy as previously described (Brady et al., 2013). For brain staining, mice were perfused and fixed with 4% paraformaldehyde. 10-µm-thick brain sections were cut with a cryotome. Tissue sections were blocked and permeabilized with 0.05% saponin in Odyssey blocking buffer before incubating with primary antibodies overnight.

**Microscope image acquisition**

Microscope image acquisition was performed at room temperature (21°C) on a CSU-X spinning disc confocal microscope system (Intelligent Imaging Innovations) using an inverted microscope (DMi600B; Leica), 100x/1.46 NA objective, and the CoolSnap HQ2 camera (Photometrics). Micrographs were acquired in Slidebook (Intelligent Imaging Innovations), which was also used to create single and merged images.

**Quantitative analysis of lysosomal PSAP, PGRN, and endocytosed PGRN**

The ImageJ program was used to process images. To quantify the degree of colocalization between PSAP or PGRN and the lysosomal marker LAMP1, the JACoP plugin was used to generate Manders’ coefficients (Bolte and Cordelières, 2006). To quantify endocytosed PGRN, the entire cell was selected and the fluorescence intensity was measured directly by ImageJ. For each experiment, at least 12 pairs of cells were measured and the data from three or four independent experiments were used for statistical analysis.

**ELISA**

Serum samples were collected from newborn pups and analyzed using mouse PGRN ELISA kit (R&D Systems) according to the manufacturer’s instructions.

**Statistical analysis**

The data were expressed as means ± SEM. Statistical significance between multiple groups was compared by one-way analysis of variance followed by Bonferroni’s multiple comparison test. Two-group analysis was performed using the Student’s t test. P-values <0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism4 software (GraphPad Software).

**Online supplemental material**

Fig. S1 shows coomassie staining of SDS-PAGE with purified Flag-PGRN-his and PSAP proteins used in this study. Fig. S2 shows that PGRN is not required for PSAP lysosomal targeting in fibroblasts. Fig. S3 shows mislocalization of PGRN in fibroblasts with PSAP expression knocked down by shRNA or ablated with CRISPR. Fig. S4 shows that PSAP facilitates PGRN lysosomal targeting from the extracellular space in neurons. Fig. S5 shows that PSAP facilitates PGRN lysosomal targeting from the extracellular space in Sort1−/− fibroblasts and neurons. Table S1 lists SILAC hits identified with GFP-PGRN in T98G cells. Table S2 lists SILAC hits identified with FLAG-PSAP pull-down in fibroblasts.

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Supplemental material

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Figure S1. **Coomassie staining of SDS-PAGE with purified Flag-PGRN-his and PSAP proteins used in this study.** Conditioned media were collected from HEK293T cells expressing Flag-PGRN-his or his-PSAP and incubated with cobalt beads. Proteins were eluted with imidazole and buffers were changed to PBS using the Centricon device.

Figure S2. **PGRN is not required for PSAP lysosomal targeting in fibroblasts.** Immunostaining for PGRN, LAMP1, and PSAP in fibroblasts derived from wild-type and PGRN−/− mice using sheep anti–mouse PGRN, rat anti–mouse LAMP1, and rabbit anti–mouse PSAP antibodies. Representative images from three replicated experiments are shown. Bars: (main) 20 µm; (inset) 5 µm.
Figure S3. **Mislocalization of PGRN in fibroblasts with PSAP expression knocked down by shRNA or ablated with CRISPR.** (A) Immortalized wild-type fibroblasts were infected with retroviruses expressing shRNAs against mPSAP or pLentiCRISPR viruses expressing guide RNAs targeted to mPSAP. Cells were selected with puromycin and stained with sheep anti-PGRN, rat anti-LAMP1, and rabbit anti-PSAP antibodies. In PSAP knockdown samples, two cells with or without visible PSAP expression were shown. (B) Expression of mCherry-PSAP fully rescues the PGRN localization defect in PSAP−/− fibroblasts. PSAP−/− fibroblasts were infected with lentiviruses expressing mCherry-PSAP and stained with sheep anti-PGRN and rat anti-LAMP1 antibodies. (C) PSAP−/− fibroblasts were treated with purified PSAP (5 µg/ml) for 12 h. PSAP and PGRN localization was examined as in B. Representative images from three replicated experiments are shown. Bars: (main) 20 µm; (inset) 5 µm.
PSAP facilitates PGRN lysosomal targeting from the extracellular space in neurons. Recombinant human PSAP and human PGRN were added to primary cortical neurons cultured from newborn PGRN−/− mice at a concentration of 5 µg/ml. After 12 h, cells were washed with PBS and fixed and stained with antibodies specific for human PGRN, mouse LAMP1, and human saposin B (PSAP) proteins. Representative images from three replicated experiments are shown. Bars: (main) 20 µm; (inset) 5 µm.
Figure S5. **PSAP facilitates PGRN lysosomal targeting from the extracellular space in Sort1−/− fibroblasts and neurons.** (A and B) Recombinant human PSAP and human PGRN were added to Sort1−/− fibroblasts (A) and cortical neurons (B) at a concentration of 5 µg/ml in serum-free medium. After 12 h, cells were washed with PBS and fixed and stained with antibodies specific for human PGRN, mouse LAMP1, and human saposin B (PSAP) proteins. Representative images from three replicated experiments are shown. Bars: (main) 20 µm; (inset) 5 µm.

Tables S1 and S2 lists SILAC hits identified with GFP-PGRN in T98G cells and with FLAG-PSAP pull-down in fibroblasts, respectively.