Published in final edited form as:

Matrix Biol. 2017 December ; 64: 27–39. doi:10.1016/j.matbio.2017.04.001.

The perlecan-interacting growth factor progranulin regulates ubiquitination, sorting, and lysosomal degradation of sortilin

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Abstract

Despite extensive clinical and experimental studies over the past decades, the pathogenesis and progression to the castration-resistant stage of prostate cancer remains largely unknown. Progranulin, a secreted growth factor, strongly binds the heparin-sulfate proteoglycan perlecan, and counteracts its biological activity. We established that progranulin acts as an autocrine growth factor and promotes prostate cancer cell motility, invasion, and anchorage-independent growth. Progranulin was overexpressed in prostate cancer tissues vis-á-vis non-neoplastic tissues supporting the hypothesis that progranulin may play a key role in prostate cancer progression. However, progranulin's mode of action is not well understood and proteins regulating progranulin signaling have not been identified. Sortilin, a single-pass type I transmembrane protein of the Vps10 family, binds progranulin in neurons and targets progranulin for lysosomal degradation. Significantly, in DU145 and PC3 cells, we detected very low levels of sortilin associated with high levels of progranulin production and enhanced motility. Restoring sortilin expression decreased progranulin levels, inhibited motility and anchorage-independent growth and destabilized Akt. These results demonstrated a critical role for sortilin in regulating progranulin and suggest that sortilin loss may contribute to prostate cancer progression. Here, we provide the novel observation that progranulin downregulated sortilin protein levels independent of transcription. Progranulin induced sortilin ubiquitination, internalization via clathrin-dependent endocytosis and sorting into early endosomes for lysosomal degradation. Collectively, these results constitute a regulatory feed-back mechanism whereby sortilin downregulation ensures sustained progranulin-mediated oncogenesis.

Keywords

Progranulin; Sortilin; Prostate cancer; Castration-resistant prostate cancer cells

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Introduction

Prostate cancer is one of the most frequently diagnosed cancers in the United States with 161,360 new cases and an estimated 26,730 deaths in 2017 [1]. Despite extensive clinical and experimental studies, the pathogenesis of prostate cancer remains largely unknown. In addition, the molecular mechanisms driving the transition to the castration-resistant stage remain very poorly characterized.

Progranulin is a secreted glycoprotein, which plays an important role as a bona fide growth factor in cell proliferation, angiogenesis, wound healing and transformation [2-4]. In addition, progranulin regulates inflammation and neurodegeneration [5], and has been causatively linked to the development of frontotemporal dementia (FTD). Progranulin binds with high affinity to domain V of perlecan (also known as endorepellin), a heparan-sulfate proteoglycan expressed and localized in nearly all basement membranes and cell surfaces [6-8] and, together, interact within the perivascular space of the tumor blood vessels [9]. The modular architecture of perlecan enables homeostatic regulation for a plethora of cellular processes including cell adhesion [10,11], development of bone and cartilage [12-15], cardiogenesis [16], osteoarthritis [17], endocytic processes [18], inflammation [19], as well as prostate cancer angiogenesis and autophagy [20,21]. Endorepellin is present in the blood and in several body fluids where it is released from perlecan by partial proteolysis of the protein core via matrix metalloproteinases, a large family of metalloendo- or metalloexopeptidases responsible for orchestrating a number of biological processes in development and pathology [22-28].

We have established that progranulin plays a critical role as an autocrine growth factor in bladder cancer by promoting cell motility and invasion [29-32]. Progranulin regulates F-actin remodeling by interacting with the F-actin binding protein drebrin [33]. In addition, progranulin levels are essential for tumorigenesis *in vivo* and confer resistance to cisplatin treatment [34]. Although progranulin was discovered over two decades ago, the functional membrane receptor has remained elusive, precluding a full understanding of progranulin signaling in many cancers where progranulin plays a critical protumorigenic role. Significantly, we recently discovered that EphA2, a member of a large family of receptor tyrosine kinases (RTKs) [35-37], is a functional signaling receptor for progranulin that regulates progranulin-dependent angiogenesis [38].

Our laboratories have demonstrated that progranulin plays an important role in prostate cancer by promoting castration-resistant prostate cancer cell motility [30] and contributes as an autocrine growth factor to the transforming phenotype by modulating invasion and anchorage-independent growth [30]. In addition, progranulin is up-regulated in prostate cancer tissues vis-á-vis non-neoplastic tissue controls [30]. Collectively, these results suggest a possible role of progranulin in driving the transition to the castration-resistant stage of prostate cancer.

A growing body of evidence now supports the important role of progranulin in several pathologies [39]. However, the mechanisms regulating progranulin action are still very

poorly characterized. Furthermore, very few proteins regulating early stages of progranulin signaling have been identified. Sortilin, a single-pass type I transmembrane protein of the Vps (Vacuolar protein sorting) 10 family [40] has been found as a novel progranulin binding protein in neurons where it negatively regulates extracellular progranulin by targeting progranulin for rapid endocytosis and lysosomal degradation [40,41].

We recently reported that sortilin is expressed at very low levels in castration-resistant PC3 and DU145 cells [32,42]. Significantly, enhancing sortilin expression severely reduced progranulin levels and inhibits motility, invasion, proliferation and anchorage-independent growth of these cells [42]. These results were recapitulated by targeting endogenous progranulin [42]. Accordingly, stable ablation of endogenous sortilin enhanced progranulin levels and promoted motility, invasion and anchorage-independent growth [42]. Finally, we characterized the mechanisms of sortilin action and demonstrated that sortilin promoted progranulin endocytosis and clearance from the medium through clathrin-dependent pathways, sorting into early endosomes followed by lysosomal degradation. Collectively, these results indicate a critical role for sortilin in regulating progranulin action in castration-resistant cells suggesting that sortilin loss may contribute to prostate cancer progression.

Here, we demonstrate that chronic progranulin stimulation of PC3 and DU145 downregulated sortilin protein levels but did not affect sortilin transcription. Progranulin promoted sortilin ubiquitination, internalization from the cell membrane via clathrin-dependent endocytosis and eventual sorting into early endosomes for lysosomal degradation. Collectively, these results underscore an oncogenic feed-back mechanism by which progranulin-mediated downregulation of sortilin ensures sustained progranulin-induced prostate tumorigenesis.

Results

Prolonged progranulin stimulation regulates sortilin protein stability

We have previously shown that sortilin negatively regulates progranulin action in castration-resistant prostate cancer cells by mediating progranulin uptake from the media and subsequent sorting for lysosomal degradation [42]. However, sortilin expression is very low in PC3 and DU145 cells [32,42] suggesting that progranulin might modulate sortilin levels to ensure sustained progranulin-induced oncogenesis.

Having recently demonstrated that progranulin stimulation did not affect sortilin mRNA levels [38], we investigated whether prolonged progranulin exposure of PC3 and DU145 cells might affect sortilin stability. As shown (Fig. 1A, B), progranulin stimulation resulted in a significant decrease, up to 40% inhibition from basal conditions, in sortilin protein levels in both PC3 and DU145 cell lysates. Notably, progranulin action in regulating sortilin levels was also confirmed in the presence of the translational elongation inhibitor cycloheximide (CHX) in Fig. 1C, D indicating that progranulin stimulation of PC3 and DU145 cells regulates sortilin protein stability but not sortilin protein synthesis.

Progranulin depletion affects sortilin stability

We recently generated PC3 and DU145 cells with stable progranulin depletion, via shRNA, which was associated with a significant inhibition of cell growth and motility in reduced-serum condition [42]. In addition, progranulin-depleted PC3 and DU145 cells were severely impaired in their ability to grow in anchorage-independency [42]. To assess whether endogenous progranulin may contribute to the regulation of sortilin levels, we repeated the immunoblot analysis of sortilin protein levels in control-and progranulin-depleted PC3 and DU145 cells [42] at different time points of serum-withdrawal. In both cell lines sortilin proteins were stabilized in the absence of endogenous progranulin as compared to scramble-transfected PC3 and DU145 cells (Fig. 2A, B) and the effect on sortilin stability was recapitulated in both cell lines following cycloheximide treatment (Fig. 2C, D). Collectively, these results confirm that endogenously produced progranulin promotes sortilin downregulation in PC3 and DU145 cells.

Progranulin promotes sortilin ubiquitination

The ubiquitin-protein system plays a major role in the turnover and degradation of a vast number of regulatory proteins [43,44]. Previous experiments have demonstrated that palmitoylation and ubiquitination play opposite roles in the stability and turnover of sortilin and serve as a control mechanism that fine tunes the amount of lysosomal sorting and trafficking in cells [45].

To this end, we tested whether progranulin may mediate sortilin ubiquitination after transient transfections with an HA-Ubiquitin (Ub) expression plasmid using a well-established methodology used in our laboratories [46-48]. PC3 and DU145 cells were serum-starved for 24 h then stimulated for 1 h with 40 nM progranulin. Lysates (2 mg) were immunopre-cipitated with anti-sortilin antibodies. Ubiquitination of endogenous sortilin was detected with anti-HA antibodies as a high-molecular-weight smear (Ub-Sortilin) in both PC3 (Fig. 3A) and DU145 cells (Fig. 3B). HA blot on total lysates was performed to assess similar HA-Ubiquitin transfection efficiency in unstimulated and progranulin-stimulated PC3 and DU145 cells (Fig. 3A,B).

As shown in Fig. 2, progranulin depletion in PC3 and DU145 cells stabilized sortilin protein levels suggesting that endogenous progranulin may modulate sortilin ubiquitination. Thus, we repeated the transient ubiquitination assay and transfected a HA-Ubiquitin expression plasmid into control- and progranulin-depleted PC3 cells [42] and sortilin ubiquitination in serum-free media was assessed. After sortilin immunopre-cipitation, we detected robust sortilin ubiquitination in control-transfected PC3 cells, which was considerably reduced in PC3 cells depleted of endogenous progranulin (Fig. 4A). The transfection efficiency of the HA-Ubiquitin plasmid was confirmed by immunoblot with anti-HA antibodies in lysates from control-and progranulin-depleted PC3 cells (Fig. 4A).

To confirm that sortilin ubiquitination in the various PC3 cell lines was detectable at endogenous levels of ubiquitin, we immunoprecipitated sortilin and assessed sortilin ubiquitination by immunoblot with anti-ubiquitin antibodies. The characteristic high molecular-weight smear of ubiquitinated sortilin was detectable in control-transfected PC3

cells but was severely reduced in progranulin-depleted cells (Fig. 4B) despite equal amounts of immunoprecipitated sortilin and similar levels of total ubiquitinated proteins (Fig. 4B). Altogether, these results indicate that progranulin plays an important role in modulating sortilin ubiquitination.

Progranulin induces clathrin-dependent but caveolin-1-independent sortilin endocytosis

Ubiquitination of tyrosine-kinase receptors and membrane proteins plays an important role in modulating endocytosis from cell membranes [44,49,50] into clathrin-dependent or independent pathways [51,52], as shown for the insulin-like growth factor receptor I (IGF-IR) [46,47] and the insulin receptor (IR) [48,53]. To this end, we investigated PC3 cells stably expressing GFP-tagged sortilin [42] to determine the mechanism by which progranulin mediates sortilin internalization from the plasma membrane. Therefore, we evaluated whether sortilin may co-localize with clathrin or caveolin-1, two well-established markers of clathrin-dependent and -independent endocytic pathways [51,52,54], following progranulin stimulation via confocal laser microscopy. Significantly, after 5 min of progranulin stimulation we did not detect any significant co-localization between sortilin and caveolin-1 (Fig. 5A, enlarged insets). On the contrary, sortilin significantly co-localized ($P < 0.05$) with clathrin-enriched vesicles (Fig. 5B, enlarged insets), indicating that progranulin favors clathrin-mediated endocytosis of sortilin.

Progranulin promotes sortilin sorting into early endosomes and lysosomes.

To further characterize progranulin-induced trafficking of sortilin, we assessed whether sortilin co-localized with EEA1 (early endosome antigen 1), a marker of early endosomes, or with LysoTracker, which specifically labels lysosomes [54]. Sortilin co-localized ($P < 0.05$) in EEA1-positive endosomes at 15 min of progranulin stimulation (Fig. 6A, enlarged insets), which was followed by sortilin trafficking into the lysosomal compartment, as demonstrated by significant ($P < 0.01$) co-localization of sortilin with LysoTracker. Co-localization increased at 1 h of progranulin uptake (Fig. 6B, enlarged insets).

Sortilin levels are stabilized by lysosomal inhibition

To indeed confirm that progranulin-induced sortilin degradation occurs in lysosomes we measured, by live cell microscopy, the levels of internalized GFP-Sortilin in the presence or absence of leupeptin, a specific lysosomal inhibitor. Serum-starved PC3/ GFP-Sortilin cells were preincubated on ice to inhibit internalization followed by incubation on ice with progranulin for 1 h. After transferring to 37 °C, cells were imaged continuously for 840 s (14 min) to determine the fluorescence intensity associated with GFP-Sortilin levels. Sortilin levels were considerably reduced ($P < 0.01$) upon progranulin exposure (Fig. 7, SFM + progranulin) compared to serum-starved PC3 cells (Fig. 7, SFM). Importantly, blocking the lysosomal degradative pathway significantly reduced ($P < 0.05$) sortilin degradation in PC3/ GFP-Sortilin cells (Fig. 7, SFM + progranulin + leupeptin) confirming progranulin-dependent sortilin degradation within the lysosomes.

Collectively, these results demonstrate that progranulin evokes sortilin endocytosis preferentially through a clathrin-dependent pathway followed by sorting into early endosomes and subsequent targeting to lysosomes for degradation.

Discussion

Localized prostate cancer can be managed by, surgery or radiation therapy. However, a significant proportion of patients with localized prostate cancer treated with radical prostatectomy will develop bio-chemical recurrence, subsequent local recurrence, and metastases [55-58]. Moreover, there is a lack of biochemical recurrence, subsequent local recurrence, and metastases [55-58]. Moreover, there is a lack of reliable prognostic biomarkers capable of identifying low or intermediate-grade prostate cancers that are likely to progress to metastatic disease.

We have previously demonstrated that progranulin plays an important role in castration-resistant prostate cancer cells as acts as an autocrine growth factor evoking motility, invasion and anchorage-independent growth [30]. We have also shown that the transmembrane protein sortilin negatively regulates progranulin action by promoting progranulin uptake from cell media, internalization and sorting for degradation in the lysosomes [42]. However, sortilin protein levels are very low in PC3 and DU145 prostate cancer cells [42] suggesting possible feedback mechanisms that would ensure the maintenance of sustained progranulin bioavailability and consequent oncogenic effects.

In this study, we discovered that the interaction of progranulin with sortilin has a major effect in regulating sortilin protein levels and we show that: a) Prolonged stimulation of PC3 and DU145 cells with progranulin triggers sortilin downregulation in the absence or presence of cyclohexamide. b) Depletion of endogenous progranulin stabilizes sortilin in the absence or presence of cyclohexamide. c) Exogenous and endogenous progranulin modulates sortilin ubiquitina-tion. d) Progranulin induces sortilin endocytosis via a clathrin-dependent pathway. d) Progranulin mediates sortilin trafficking into early endosomes and sorting into lysosomes. e) Lysosomal inhibitors restore sortilin protein levels.

Sortilin was originally identified as a novel progranulin binding partner in neurons where sortilin acts as a negative regulator of extracellular progranulin levels by targeting progranulin for rapid endocytosis and lysosomal degradation [40,41]. Notably, progranulin levels are significantly increased in *Sort1* null mice [40]. Reduced progranulin levels are associated with frontotemporal dementia similarly to haploinsufficiency associated with progranulin gene mutations [59,60] and targeted manipulation of the sortilin/progranulin axis rescues progranulin haploinsufficiency [61].

Our recent work suggests that the relative expression levels and functional interactions between sortilin and progranulin have a critical role in regulating progranulin-dependent responses in castration-resistant prostate cancer cells as in fact modulating sortilin levels by either overexpression or genetic ablation profoundly affected progranulin-mediated motility, invasion and anchorage-independent growth [42]. Significantly, sortilin overexpression considerably reduced Akt activation in the absence of serum [42]. Notably, sortilin overexpression was also associated with a two-fold decrease of Akt suggesting that sortilin overexpression downregulates Akt and thereby only indirectly affects Akt phosphorylation. These results were recapitulated in progranulin-depleted PC3 cells, which showed a similar reduction of total Akt levels and phosphorylation. Interestingly, PI3K was unaffected by

either sortilin overexpression or progranulin depletion indicating this effect is specific for Akt and not a more general perturbation of the PI3K/Akt pathway [42].

We previously demonstrated that sortilin promotes clathrin-dependent internalization of progranulin, sorting into early endosomes and subsequent lysosomal degradation [42]. Similarly, progranulin-induced sortilin turnover is clathrin-dependent suggesting sortilin may internalize from the cell membrane in complex with progranulin *en route* for lysosomal degradation. Importantly, we did not detect any effect of progranulin in regulating sortilin mRNA levels [38] indicating that progranulin regulation of sortilin levels occurs post-translationally by affecting sortilin turnover.

We have recently identified EphA2 as the functional progranulin receptor [38]; EphA2 is well expressed in castration-resistant prostate cancer cells [38], which inversely express low levels of sortilin [42]. Therefore, these results suggest that the clear majority of progranulin would preferentially bind EphA2 at the cell surface and only sortilin after EphA2 saturation to ensure that only a small pool of progranulin would be internalized and degraded in a sortilin-dependent fashion. The present results support a model whereby progranulin activates a feedback loop by which it promotes additional sortilin degradation for continued progranulin oncogenic function, potentially via EphA2.

A complementary but sortilin-independent mechanism of trafficking progranulin to lysosomes has been recently identified [62] and is mediated by prosaposin (PSAP) via the cation-independent mannose 6- phosphate receptor and low density lipoprotein receptor-related protein 1 [62]. However, whether this alternate pathway plays a role in regulating progranulin levels and action in prostate cancer cells has not been established. Progranulin is likely to internalize in complex with EphA2; however, the mechanisms regulating progranulin/EphA2 endocytosis remain undefined.

Our study provided the first evidence of progranulin-induced sortilin ubiquitination, which is clearly detectable as high molecular-weight smears in both PC3 and DU145 cells. This sortilin ubiquitination pattern may suggest the attachment of topologically complex polyubiquitin chains (polyubiquitination). However, recent data has demonstrated that membrane receptors, such as the EGFR and IGF-IR [47,63] can be not only polyubiquitinated but also monoubiquitinated at multiple sites (multiubiquitinated), which ensure receptor internalization and trafficking for degradation. Experiments are currently under way to elucidate whether progranulin may induce sortilin polyubiquiti-nation or monoubiquitination at multiple sites.

The oncosuppressive small leucine rich proteoglycan (SLRP) decorin [64] regulates key downstream signaling processes indirectly by sequestering growth factors or directly via the antagonism of receptor tyrosine kinases that impacts cell growth, angiogenesis, and autophagy [53,64-69]. In addition, the systemic delivery of an oncolytic adenovirus expressing decorin inhibits bone metastasis in a mouse model of human prostate cancer [70]. Thus, we can speculate that the anti-oncogenic function of decorin in prostate cancer might relate, at least in part, to an inhibitory action on the progranulin/EphA2 axis. However, it is important to notate the complexity and unique functions among the various extracellular

SLRPs insofar as biglycan, the closest relative of decorin, suppresses potent anti-angiogenic effectors, such as endostatin [71].

Very recently published work has shown that glucose deprivation regulates the progranulin-sortilin axis in PC12 neuronal cells [72]. Changes in glucose concentration induced progranulin expression in a p38-dependent but AMPK-independent manner, while simultaneously reducing sortilin expression by modulating AMPK activation [72]. Whether this glucose-dependent regulatory mechanism of the progranulin/ sortilin axis is conserved in prostate cancer cells remains to be elucidated.

In conclusion, we discovered a novel regulatory feedback mechanism. Progranulin destabilizes sortilin via ubiquitination and lysosomal degradation to override the negative modulatory functions of sortilin thereby assuring the sustainable pro-tumorigenic actions of progranulin in castration-resistant prostate cancer.

Experimental procedures

Cell lines

PC3 and DU145 cells were obtained from the ATCC. PC3 cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS). DU145 cells were maintained in EARL-modified MEM medium supplemented with 10% FBS, 1% non-essential amino acid (NEAA) and 1% vitamins. Serum-free medium (SFM) is DMEM containing 0.1% bovine serum albumin (BSA) and 50 μg/ml of bovine transferrin (Sigma-Aldrich).

PC3 and DU145 cells stably depleted of endogenous progranulin [42] were generated by transfecting the pRS-shRNA-control (scrambled shRNA) and pRS/shPGRN plasmids (OriGene Technologies, Inc.) using the TransIT®-Prostate Transfection Kit (Mirus). Cells were selected in medium supplemented with 2 μg/ml of puromycin. After selection, pools of control and progranulin-depleted PC3 and DU145 cells were tested for progranulin expression levels in cell lysates and conditioned media by immunoblot using anti-progranulin polyclonal antibodies (US Biologicals) as previously described [42].

PC3/GFP-Sortilin cells [42] were generated by transfecting a human sortilin cDNA in the pEGFP-C1 vector (a kind gift from Dr. Stephen M. Strittmatter, Yale University School of Medicine), using the TransIT®-Prostate Transfection Kit (Mirus). Cells were selected in medium supplemented with 2 mg/ml of G418, pooled and tested for sortilin expression in cell lysates by immunoblot using anti-sortilin polyclonal antibodies (Abcam).

Human recombinant progranulin

Human progranulin was purified from media conditioned by 293-EBNA/progranulin-overexpressing cells. This cell line expresses a His6-tagged human progranulin. Serum-free conditioned medium was concentrated with polyethylene glycol, dialyzed, purified on Ni-NTA resin and eluted with 250 mM imidazole, as previously described [9,73].

Determination of sortilin stability

Sortilin protein levels were determined by immunoblot with anti-sortilin antibodies after a time course of recombinant progranulin (40 nM) stimulation under serum-free conditions as previously described [42]. Sortilin levels in progranulin-depleted cells and controls were assessed in serum-free media. 100 μM of cycloheximide (CHX) was used in the experiments. Intensities of protein bands were analyzed by densitometry using ImageJ software (National Institutes of Health). Sortilin band intensities for each treatment condition were normalized over β-actin.

Sortilin ubiquitination assays

Ubiquitination assays were performed with the protocol previously established in our laboratories [46-48]. Briefly, PC3 and DU145 cells were plated onto 10 cm plates and transiently transfected with 10 μg of the eight-hemagglutinin (HA)-tagged ubiquitin construct (HA-Ub) (kindly provided by D. Bohmann) using the TransIT 2020 transfection reagent (Mirus). After 24 h, cells were shifted to serum-free medium (SFM) for an additional 24 h and then stimulated for 1 h with 40 nM of recombinant progranulin. Lysates from several plates were pooled, and 2 mg of proteins were immunoprecipitated in HNTG buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.2 mM sodium orthovanadate, protease inhibitor mix [Roche]) supplemented with 20 μM of the proteasome inhibitor MG132 (Calbiochem) and 100 μM of leupeptin as lysosomal inhibitors (Roche) to accumulate the ubiquitinated species. Filters were immunoblotted with anti-HA monoclonal antibodies (P4D1, Covance) to detect ubiquitinated proteins. Ubiquitinated species appear as a high-molecular-weight smear. Filters were then incubated with anti-sortilin polyclonal antibodies (Abcam). Sortilin ubiquitination in PC3/shSCR and PC3/shPGRN was assessed in serum-free media.

To detect sortilin ubiquitination at endogenous levels of ubiquitin, lysates of PC3/shSCR and PC3/shPGRN were immunoprecipitated with anti-sortilin antibodies and sortilin ubiquitination detected by immunoblot with anti-ubiquitin monoclonal antibodies (Covance).

Confocal microscopy

GFP-Sortilin-overexpressing PC3 cells were plated onto cover glass (Corning) in 35 mm plates, serum-starved for 24 h and then incubated with or without progranulin (40 nM) at 37 °C for 5 min (clathrin and caveolin-1), 15 min (EEA1) or 30 min (LysoTracker). Cells were thenwashed with 1 × PBS and fixed with 4% PFA for 20 min at room temperature. Subsequently, slides were subjected to immunofluorescence and confocal microscopy analysis as previously described [74-77]. Primary antibodies were anti-clathrin and anti-caveolin-1 monoclonal antibodies (BD Transduction Laboratories), anti-EEA1 (BD Transduction Laboratories). Lysosomes were detected using LysoTracker deep red (life technologies). Secondary antibodies were goat anti-mouse IgG Alexa Fluor® 594 and goat anti-rabbit IgG Alexa Fluor® 594 antibodies (Invitrogen). Confocal analysis was carried out using a 63×, 1.3 oil-immersion objective of a Zeiss LSM-780 confocal laser-scanning microscope at the Sidney Kimmel Cancer Center Bioimaging Facility. All images were

analyzed using ImageJ (National Institute of Health) and Adobe Photoshop CS6 (Adobe Systems). Colocalization was quantified using ImageJ (National Institute of Health).

For live cell experiments in the presence of lysosomal inhibitors, GFP-sortilin-overexpressing PC3 cells were plated onto 24 well glass-bottom culture dishes (Ibid Corporation). Cells were serum-starved for 24 h, incubated on ice for 1 h to block endocytosis and then incubated with recombinant progranulin (40 nM) with or without 100 M leupeptin for 1 h on ice. Cells were then transferred at 37 °C and imaged every 12 s for 14 min by confocal microscopy (Zeiss LSM 510 UV META) with 40× objective. The background-corrected GFP intensity in 5 independent areas was obtained in each cell and were plotted relative to time = 0 using the Zeiss AIM4.2 SP1 software package.

Statistical analysis

Results of multiple experiments are expressed as mean ± SD. All statistical analyses were carried out with SigmaStat for Windows version 3.10 (Systat Software, Inc., Port Richmond, CA). Results were compared using the two-sided Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

Acknowledgments

This work was supported in part by the Benjamin Perkins Bladder Cancer Fund and The Schorsch Family Fund for Innovative Medical Research and Care (A. M.), National Institutes of Health Grants RO1 CA164462 (A.M., R.V.I.), and CA39481 and CA47282 (R.V.I.), and grant from the Associazione Italiana per la Ricerca sul Cancro (AIRC) (grant n. AIRC IG 19242)(A.B.). The Bioimaging Core Facility of the Sidney Kimmel Cancer Center is supported by NIH/NCI (P30CA056036).

Abbreviations used:

PGRN	progranulin
Ub	ubiquitin
EEA1	Early Endosomal Autoantigen 1
SFM	serum-free medium

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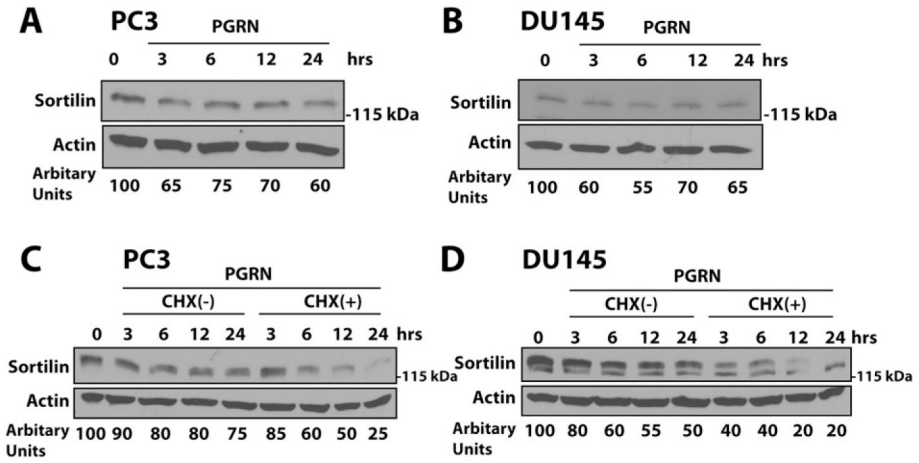

Fig. 1.

Progranulin regulates sortilin stability. (A–B), PC3 and DU145 cells were serum-starved for 24 h and then stimulated for the indicated time points with progranulin (40 nM). Samples were lysed and sortilin levels were assessed by immunoblot with anti-sortilin antibodies. (C–D) Cyclohexamide (CHX, 100 μM) was supplemented with progranulin. Sortilin levels were normalized to β-actin. Densitometric analysis is expressed as arbitrary units and is the average of two independent experiments.

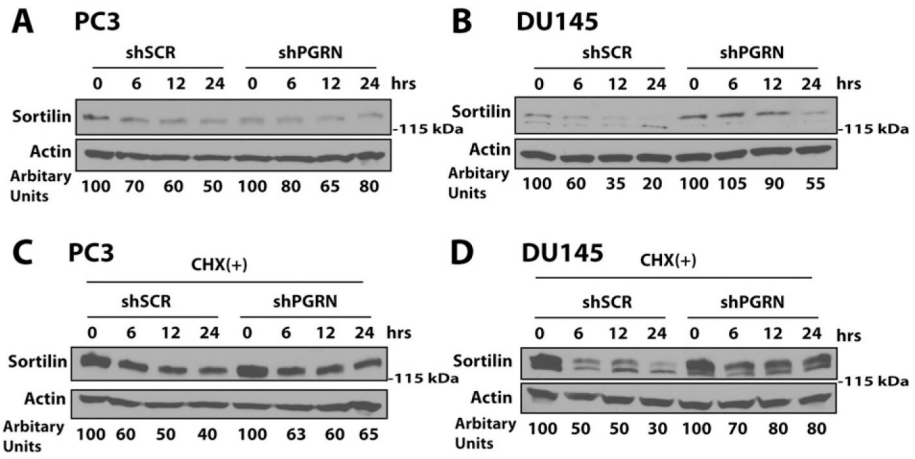

Fig. 2.

Progranulin depletion enhances sortilin stability. (A–B), Controls (shSCR) and progranulin-depleted (shPGRN) PC3 and DU145 cells were plated in serum-rich media overnight and then transferred to serum-free media for the indicated time points. Samples were lysed and sortilin protein levels were assessed by immunoblot with anti-sortilin antibodies. (C–D) Cyclohexamide (CHX, 100 μM) was incubated for the indicated time points. Sortilin levels were quantified over β-actin. Densitometric analysis is expressed as arbitrary units and represents the average of two independent experiments.

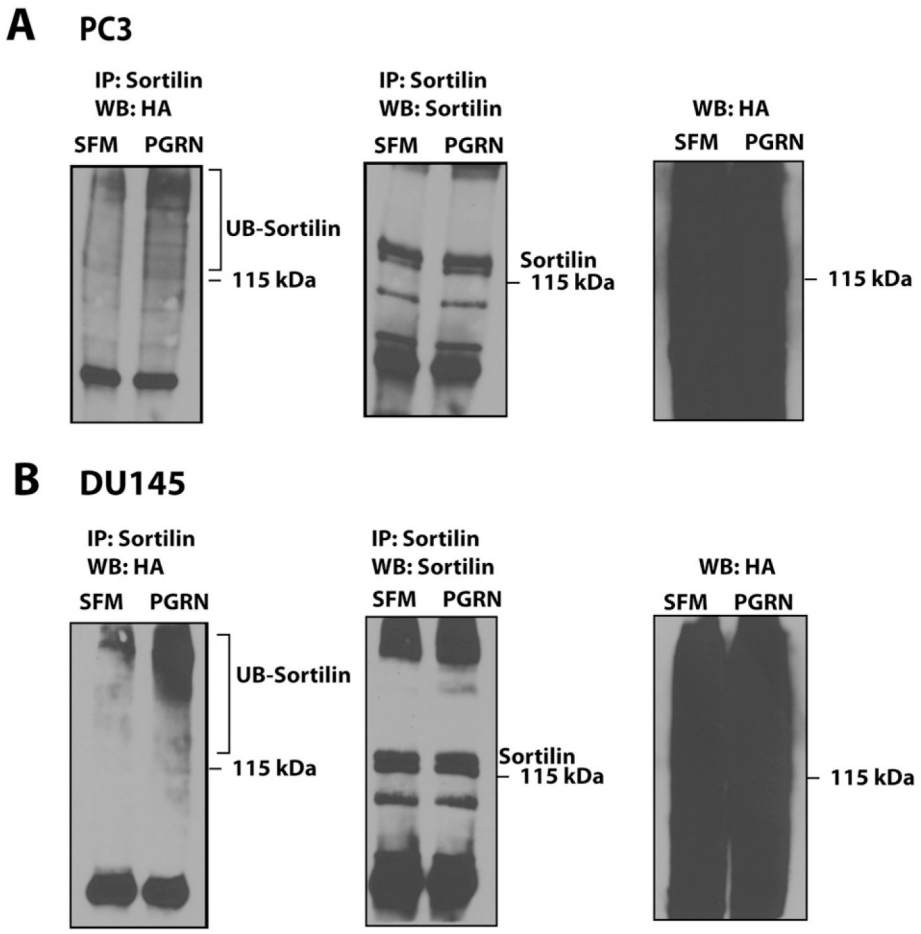

Fig. 3.

Progranulin promotes sortilin ubiquitination. (A) PC3 and (B) DU145 cells were transiently transfected with a HA-Ubiquitin plasmids as described in Experimental procedures. After 24 h, cells were serum-starved for an additional 24 h followed by stimulation with 40 nM progranulin (PGRN) for 1 h. Both samples were supplemented with proteosomal and lysosomal inhibitors to accumulate ubiquitinated species. Lysates were immunoprecipitated with anti-sortilin antibodies, resolved via SDS PAGE and membranes incubated with anti-HA and anti-sortilin antibodies. Experiments shown are representative of two independent experiments from four pooled independent samples.

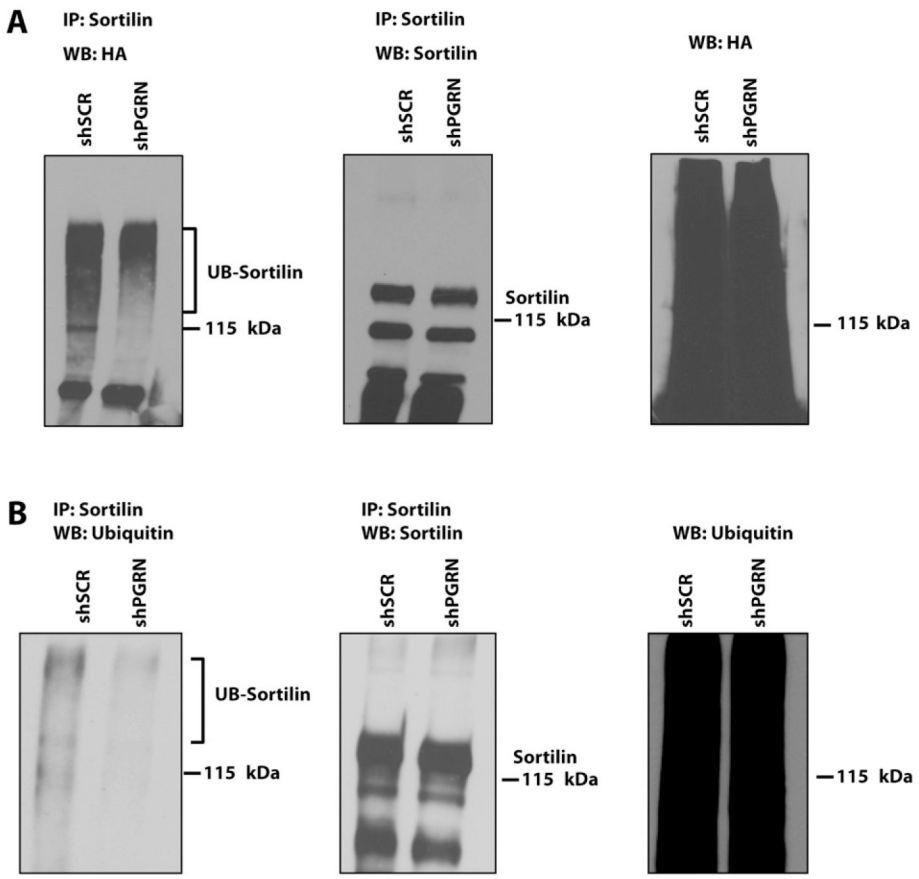

Fig. 4.

Progranulin depletion inhibits sortilin ubiquitination. (A) PC3/shSCR and PC3/shPGRN cells were grown overnight in serum-rich media, transiently transfected with an HA-Ubiquitin plasmid and then transferred after extensive washing to serum-free conditions supplemented with proteosomal and lysosomal inhibitors for 1 h. Lysates were immunoprecipitated with anti-sortilin antibodies, run on SDS PAGE and filters incubated with anti-HA and anti-sortilin antibodies. (B) To detect sortilin ubiquitination at endogenous ubiquitin levels, PC3/shSCR and PC3/shPGRN cells were grown overnight in serum-containing media and then transferred after extensive washing to serum-free condition supplemented with proteosomal and lysosomal inhibitors for 1 h. Lysates were immunoprecipitated with anti-sortilin antibodies, run on SDS PAGE and membranes incubated with anti-ubiquitin and anti-sortilin antibodies. Experiments shown are representative of two independent experiments from four pooled independent samples.

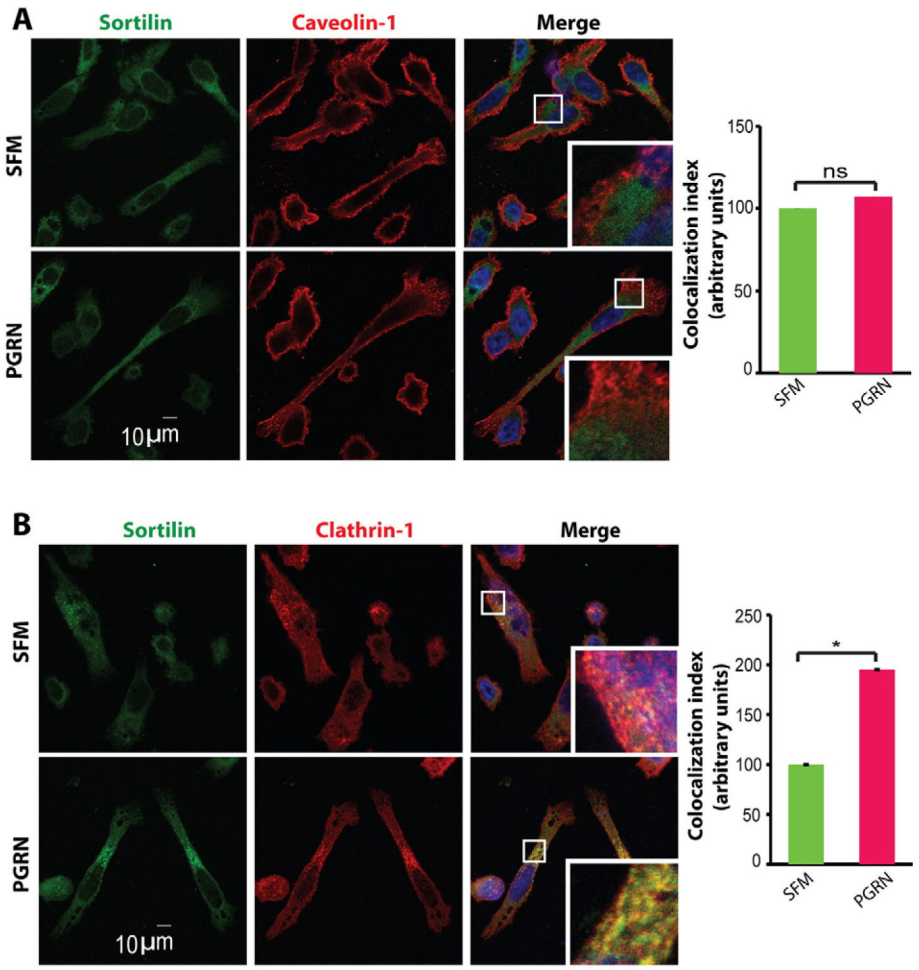

Fig. 5.

Progranulin promotes sortilin internalization via a clathrin-dependent but caveolin-1-independent pathway. (A) Sortilin co-localization (yellow) with caveolin-1 (red) or (B) clathrin (red) was assessed in PC3/GFP-sortilin cells by confocal laser microscopy. Pictures are representative of at least ten fields from three independent experiments. An average of 100 cells was examined for each condition. Scale bar ~10 μm. Nuclei were stained with DAPI. Insets were digitally magnified 3×. Co-localization index is expressed in arbitrary units and was calculated using ImageJ (*$P < 0.05$).

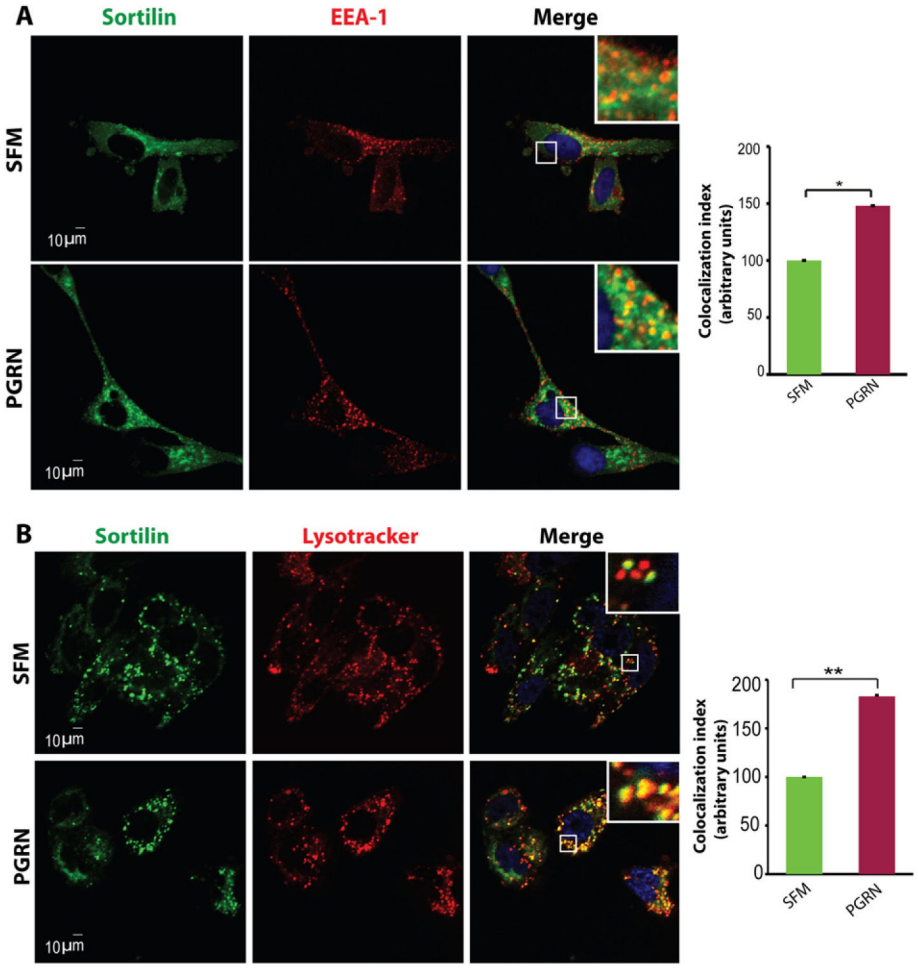

Fig. 6.

Progranulin promotes sortilin trafficking into early endosomes and lysosomes. (A) Sortilin (green) co-localization with EEA1 (red) or (B) lysosomes (red, Lysotracker) was assessed in PC3/GFP-sortilin cells by confocal microscopy. Images are representative of at least ten fields from three independent experiments. An average of 100 cells was examined for each condition. Scale bar ~10 μm. Nuclei were stained with DAPI. Insets were digitally enlarged 3×. The co-localization index is expressed as arbitrary units and was calculated using ImageJ (*$P < 0.05$, **$P < 0.01$).

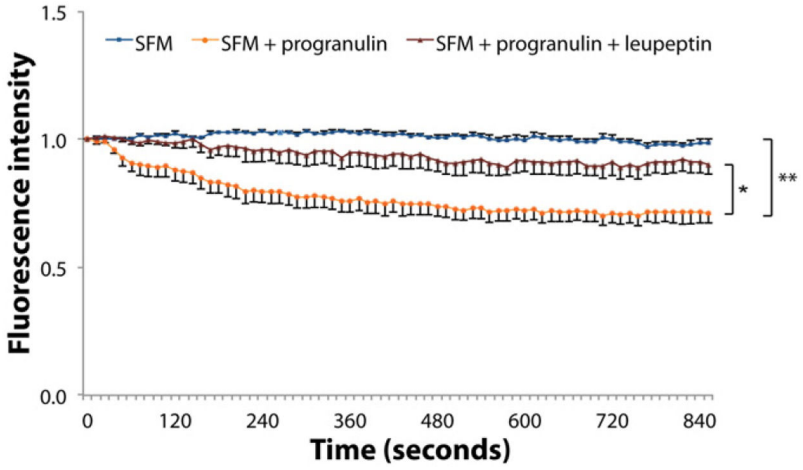

Fig. 7.

Sortilin levels are stabilized by lysosomal inhibition. Serum-starved PC3/GFP-Sortilin cells were incubated with recombinant progranulin (40 nM) with or without leupeptin (100 μM) on ice for 1 h. After washing, cells were shifted to 37 °C and imaged at the indicated time points (seconds). Images were acquired every 12 s for 14 min using live cell confocal microscopy. The background-corrected fluorescence intensity relative to time 0 was quantified and plotted using the software Zeiss AIM 4.2 SP1 and is expressed as a function of time. Error bars represent standard deviation for n = 4 (*$P < 0.05$, **$P < 0.01$).